Strategies for improving *Brassica napus* resistance to *Leptosphaeria maculans*, a causal agent of blackleg disease in canola

by

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ABSTRACT

Rashid M. Harunur, PhD., The University of Manitoba, 2018. Strategies for improving *Brassica napus* resistance to *Leptosphaeria maculans*, a causal agent of blackleg disease in canola. Supervisor: Dr. W. G. Dilantha Fernando

Canola (oilseed rape, *Brassica napus* L.) is a cash crop grown in different countries including Canada, the European Union, China and Australia. The production of this crop is threatened by blackleg disease caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. & De Not. [anamorph: phoma-like (de Gruyter)]. In order to develop a more effective disease management strategy; there is a need to employ crop rotation, introgress new resistance (*R*) genes into elite cultivars, test the effectiveness of existing *R* genes, and predict the emergence of virulent isolates of *L. maculans*.

In this study, sequenced characterised amplified region (SCAR) and derived-cleaved amplified polymorphic sequence (dCAPS) markers linked to the *L. maculans* resistance gene *Rlm6* were developed and their segregation was confirmed by analysis of F_2 and F_3 progeny. In addition, chromosomal segment substitution lines (CSSLs) harbouring *Rlm6* were developed through a combined approach of classical and marker-assisted backcross breeding. The field study suggested a potential effectiveness of a 2-year crop rotation, because of the reduction of disease severity at the end of the 4-year trial. By the end of the study, the *R* genes *LepR1*, *LepR3*, *Rlm2*, and *Rlm4* were effective against *L. maculans*, whereas *Rlm3* was moderately effective. Linear regression analysis suggests an order of effectiveness among the *R* genes tested: *LepR3* > *LepR1*> *Rlm2* > *Rlm4* > *Rlm3*. Moreover, the decreased frequency of effector genes *AvrLm2* and *AvrLm4* in *L. maculans* isolates suggested the gaining of virulence toward *Rlm2* and *Rlm4* within a year. Results from sequencing of the *AvrLm2* gene revealed a shift of *AvrLm2* to *avrLm2* allele due to the accumulation of point mutations.

Overall, CSSLs and DNA markers developed in this study may facilitate breeding *B. napus* varieties resistant to *L. maculans* in the future. It is predicted that rotation of *Rlm2*, *Rlm3*, *Rlm4*, and *Rlm7* could be an opportunity to increase the effectiveness of those *R* genes under a 2-year crop rotation. However, these studies also suggest development of generic epidemiological models for the occurrence of complex molecular interactions in *B. napus-L. maculans* pathosystem, allowing plant breeders to select appropriate *R* genes in the proposed cultivar rotation strategies on the Prairies.

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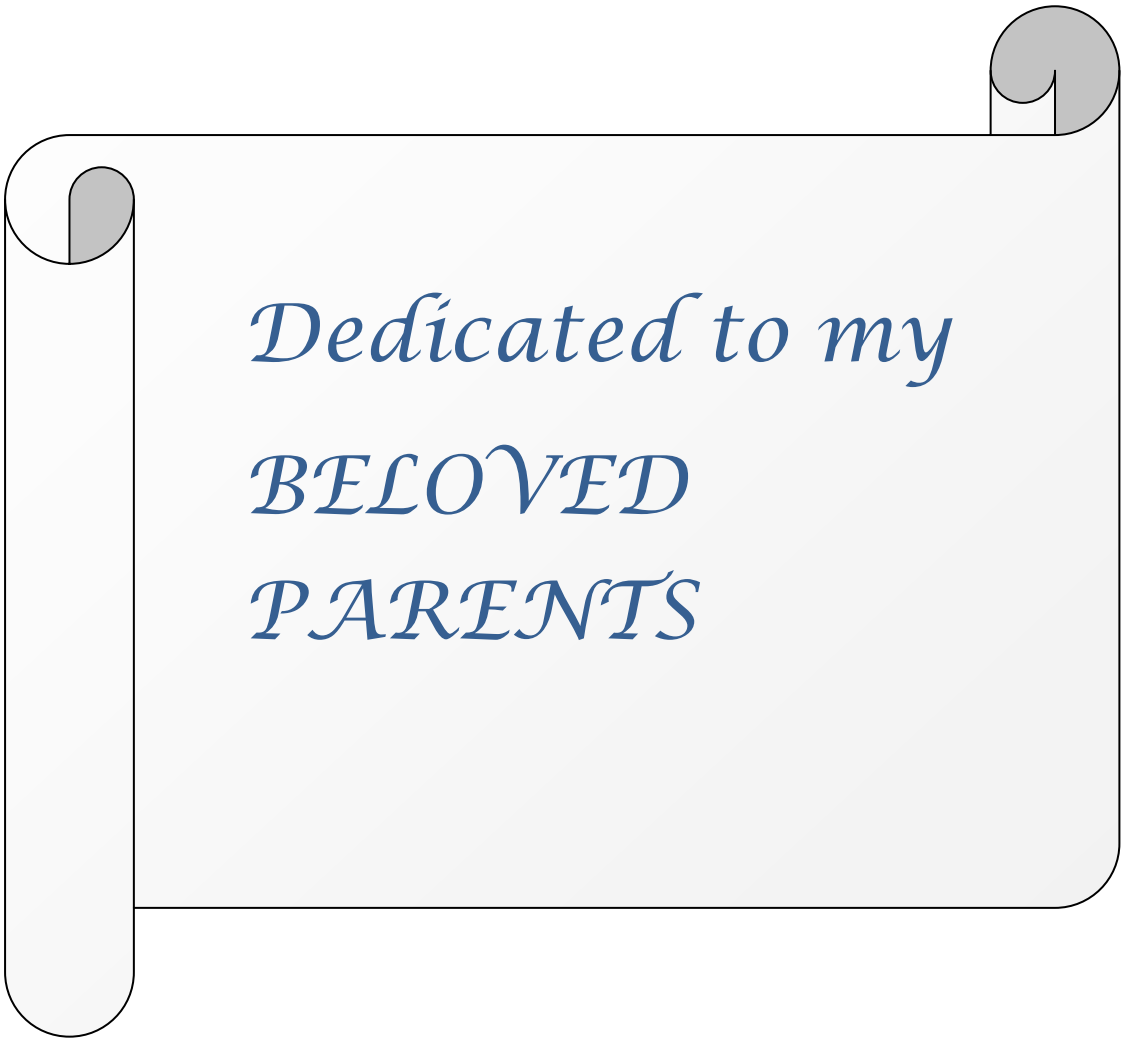
DEDICATION



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FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, Faculty of Graduate Studies at the University of Manitoba. This thesis started with a general introduction, followed by literature review, four manuscripts, general discussion and future directions, and references. The manuscript chapters contain abstract, introduction, materials and methods, results, discussion, and acknowledgments. The first manuscript (Chapter 3) has been accepted in the journal Plant Breeding. The second manuscript (Chapter 4) has been submitted to the journal Euphytica for publication, and the third (Chapter 5) and fourth (Chapter 6) manuscript will be submitted to a journal soon for publication.

1 GENERAL INTRODUCTION

Canola (oilseed rape, *Brassica napus* L.) is a major oilseed crop grown in temperate regions. In Canada, production acreage increased gradually from 6.5 to 22.9 million acres from 1986 to 2015. Concomitantly, total production of canola in Canada also increased from 3.7 to 21.3 million metric tonnes (CCC 2016a; CCC 2017a), making it the world's largest canola producer. Canola oil export by Canada has increased gradually from 11.5 to 28.7 million tonnes during the period from 2007 to 2016 (CCC 2017b). Recently, the Canola Council of Canada proposed to increase production from the current 42.3 to 52 bushels per acre, the equivalent of 26 million metric tonnes, by 2025 (CCC 2016b). However, the production of canola is hampered by a few major diseases such as blackleg, *Sclerotinia* stem rot, and clubroot (Li and McVetty 2013).

Blackleg or stem canker, is a major disease of canola caused by the ascomycete fungal pathogen *Leptosphaeria maculans*. The disease has a great economic impact on the canola industry of Australia, Canada, Europe and parts of Asia with severe yield losses (West et al. 2001; Howlet 2004; Fitt et al. 2006). Genetic studies reported two types of resistance against blackleg pathogen *L. maculans*; qualitative resistance referred to as monogenic / seedling / race-specific / *R* gene / vertical resistance, whereas quantitative resistance is referred to as polygenic / adult plant / race non-specific / horizontal / QTLs resistance (Pongam et al. 1998; Balesdent et al. 2001; Jestin et al 2011; 2015). The *R* gene mediated resistance usually follows the gene-for-gene concept proposed by Flor (1971). In this theory, proteins encoded by *R* genes in the host are able to recognize small secreted proteins encoded by *Avr* genes from the pathogen. As a result, the colonization of *L. maculans* is restricted in a resistant host by programmed cell death which is often induced by a hypersensitive response (Flor 1971; Jones and Dangl 2006). Recently, a very high frequency of *AvrLm6* in *L. maculans* isolates

cultured from Canadian canola stubbles have been reported (Kutcher et al. 2011; Liban et al. 2016), stressing the necessity to develop canola cultivars with the corresponding *R* gene *Rlm6*.

To date, at least 16 *R* genes and many quantitative trait loci (QTLs) linked to blackleg resistance have been identified in different *Brassica* species (Raman et al. 2013). B-genome *Brassica* species *B. juncea*, *B. nigra*, and *B. carinata* display strong resistance to *L. maculans* (Roy 1978, 1984, Sjodin and Glimelius 1988, Rimmer and van den Berg 1992), indicating a valuable source of blackleg resistance. In particular, *B. juncea* is reported to carry the blackleg resistance gene *Rlm6* (Christianson et al. 2006; Balesdent et al. 2002; Chèvre et al. 1997) and thus has the potential to be used in current breeding efforts.

The effective management of host resistance depends on the understanding of the host-pathogen interaction. To date, several investigations have been undertaken worldwide to manage blackleg disease in canola. *L. maculans* has shown the evolutionary ability to lose avirulence genes and gain virulence to *B. napus* as evidenced by the emergence of new races in western Canada (Chen and Fernando 2006; Kutcher et al. 2007). Currently, almost all existing canola cultivars in western Canada carry the single resistance (*R*) gene *Rlm3* (Zhang et al. 2016). Though the *R* gene-mediated resistance is often very effective in disease control, repeated use of a single *R* gene may result in resistance breakdown as was observed in Australia (Sprague et al. 2006), France (Rouxel et al. 2003), and Canada (Zhang et al. 2016); thus, it is imperative that proper management strategies be employed to enhance or maintain host resistance. The management of host resistance requires an integrated approach including the use of resistant cultivars (Kutcher et al. 2013), crop rotation (Kutcher et al. 2013), deployment of resistant cultivars (Marcroft et al. 2012a), a combination of qualitative resistance into quantitative backgrounds (Brun et. al. 2010), stubble management and minimal use of fungicides (Kutcher et al. 2011).

Blackleg has been managed in Canada mostly by the cultivation of canola varieties harbouring *R* genes (West et al. 2001; Kutcher et al. 2010a). To enhance the durability of single *R* genes to *L. maculans*, it is important to continue developing canola varieties with novel resistance genes and new agronomic strategies. Despite the plethora of studies that focused on management *L. maculans* in canola and recent advancements in understanding the genetic constitution of the pathogen, our understanding of this pathosystem is still at its infancy. Therefore, the present study aimed to further the current knowledge of the canola-blackleg pathogen through the following objectives:

1. Development of DNA markers linked to B-genome resistance gene *Rlm6* and characterize their inheritance patterns in interspecific hybrids derived from *Brassica napus* x *B. juncea*.
2. Development of backcross (BC) populations harbouring *Rlm6* which can be employed in variety development programmes.
3. Investigation of effectiveness of *R* gene in a 2-year canola-wheat crop rotation over a 4-year field study.
4. Investigation of impact of *B. napus*-*L. maculans* interaction in the emergence of virulent isolates of *L. maculans* in a 4-year field study.

2 LITERATURE REVIEW

2.1 The host

2.1.1 *Brassica* species

The genus *Brassica* belongs to the family Brassicaceae, comprising 37 different species. Among them six species are available for commercial cultivation worldwide. They are; *B. rapa* L. (n=10, AA genome), *B. nigra* (L.) Koch (n=8, BB genome), *B. oleracea* L. (n=9, CC genome), *B. juncea* (L.) Czern (n=18, AB genomes), *B. carinata* (A.) Braun (n=17, BC genomes), and *B. napus* (n=19, AC genomes). Their genetic relationships have been described by the U triangle (Nagaharu 1935). The *Brassica* species *B. rapa* also known as rapeseed, the ancestor of canola, was grown in about 5000 BC in China (Yan 1990). It is suggested by the ancient literature (Singh 1958) that at the same time *Brassica campestris* cultivar "Yellow Sarson" was grown in Indian subcontinent. *B. napus* (2n=28, AACC) is a relatively new species derived from an interspecific cross between *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC) (Nagaharu 1935; Gupta and Pratap 2007). *B. rapa*, a diploid lineage of *B. napus* has wider distribution among the *Brassica* species and it is thought that the Himalayan regions is its centre of origin (Downey and Robbelen 1989). *Brassica* species have a large number of duplicated regions in the genomes due to duplication and triplication events during their evolution (Jenczewski et al. 2013; Fomeju et al. 2015).

2.1.2 History of canola

Canola (oilseed rape, *Brassica napus* L.) is one of the stable oilseed crops grown today in Canada and other temperate parts of the world. In Canada, Polish immigrants Fred and Olga Solvoniuk grew *B. rapa* rapeseed in Canada for the first time at their home garden in Saskatchewan in the late 1920s (CCC 2017c). Seeds had come from their homeland – hence *B. rapa* then comes to be known as Polish rapeseed in Canada. In 1942 during the Second

World War, the supply of rapeseed oil in Canada coming from Europe and Asia was cut off and the Department of Agriculture's Forage Division took the initiative to grow edible oilseed rape for the first time (Special Crops 1944; CCC 2017c). In 1943, a group of Canadian farmers started growing *B. napus* on a small scale to meet local demands for lubricants for steam engines (CCC 2017c). These seeds came from United States, whose original genotype were from Argentina - thus *B. napus* came to be known as Argentine rapeseed. It seems that both Polish and Argentine rapeseed were grown on the Canadian Prairies and in 1946 the first *B. rapa* cultivar was registered in Canada for commercial production (CCC 2017c), which contained a high level of glucosinolates (Greer 1950). In 1954, a *B. napus* cultivar "Golden" was released by the Saskatoon research centre for improved oil content and lodging resistance (CCC 2017c). High levels of glucosinolates, and erucic acid are not suitable for human, and animal consumption (Gupta and Pratap 2007), so their reduction by breeding high quality rapeseed varieties was essential. In 1957 the first rapeseed breeding program was taken over by Dr. Richard Keith Downey at the Agriculture Canada Research Station in Saskatoon and a year later Dr. Baldur Stefansson from the University of Manitoba also started a breeding program in rapeseed. Both of them had been working on a world collection of rapeseed germplasm. From this core collection Dr. Keith Downey released a *B. napus* cultivar "ORO" with low-erucic acid in 1968, and from the same collection he released a *B. rapa* cultivar "Span" in 1971 (CCC 2017c). A *B. napus* cultivar "Tower" derived by a cross between rapeseed and closely-related forage was released from Dr. Stefansson's breeding program in 1974 (Redekop 2002; CCC 2017c). The variety "Tower" was released in Canada as the first "double-low" (low glucosinolate and low linoleic acid) variety. At the same time the term canola was proposed to brand the combination of oil and meal quality, and finally, in 1978 industry officially adopted the term canola in where 'Can' refer to Canadian and 'Ola' for oil (CCC 2017c). The canola trademark was registered

by the Canadian Oilseed Processors Association (formerly known as Western Canadian Oilseed Crusher's Association), and subsequently in 1980 it became the asset of the Canola Council of Canada. Finally, it was revised by the Trademarks Branch, Department of Consumer and Corporate Affairs Canada in 1986 (CCC 2017c) to fit to the following definition to qualify as a canola, "*canola oil must contain less than 2% erucic acid, and the solid component of the seed must contain less than 30 micromoles per gram of glucosinolates*" (CCC 2017c).

2.1.3 Economic importance of canola

Canola (*B. napus* / oilseed rape) is an economically important crop in the industrial world, particularly in Europe and North America, and countries such as China and Australia. It is ranked third in terms of source of vegetable oil in the world (Sovero 1993), second in North American food industries (CanolaDigest 2012), and it is presently more economical than peanut, cottonseed, and sunflower (Sovero 1993) Canola is not only a source of edible oil but it also has a wider range of industrial purposes (Hayward 2012). *B. napus* cultivation acreage has increased exponentially worldwide in the last 40 years, as its demand and price have increased (Hammoudi et al. 2012). In Canada, production acreage increased gradually from 6.5 to 20.4 million acres during the period from 1986 to 2015. Similarly, total production of canola in Canada also increased from 3.7 to 18.4 million metric tonnes from 1986 to 2015 (CCC 2016a), making it the world's largest canola producer. "Growing the opportunity" is the new motto of Canola Council of Canada in where they propose to increase production to 52 bushels per acre to achieve a new target of 26 million metric tonnes by 2025 (CCC 2016b). Canola acreage has also increased constantly in the United States (Bunting 1986). It seems to be a significant contributor to the Australian economy as well (Jones et al. 2001). The steady increase in its production has led to a steady increase of its export. Canola oil export by Canada has increased from 11.5 to 28.7 million tonnes during

the period from 2007 to 2016 (CCC 2017b). Thus, the canola industry has a huge impact on the Canadian economy, creating 250,000 jobs, resulting in a total economic activity of $26.7 billion per year (LMC International 2016).

2.1.4 Cultivation environment

Canola (*B. napus* / oilseed rape) is a cool temperature loving crop. It requires low night temperatures to recuperate from extreme hot or dry conditions. Soil conditions play an important role for better seed germination and uniform seedling growth. In western Canada, the maximum production of canola is seen in the black and grey soil regions. Loamy soil, which usually does not form a hard layer at the soil surface, is the best suited for canola production, resulting in good seedling emergence. Warm and dry winters with little snow generally allows for early seeding on the Canadian Prairies. Seeding on a well aerated, well-structured seedbed with less stubble plus soil temperatures of $>5^0$C improves seed germination and seedling growth. While annual and perennial weeds are found in canola fields and can be controlled by spring tillage, the use of herbicides either pre-plant or foliar applied and/or both, herbicide resistant genetically modified varieties are grown extensively (Orson 1995).

2.2 The pathogen – Leptosphaeria maculans

2.2.1 Biology

The blackleg pathogen was first identified in cabbage (*Brassica oleracea* var. capitata L.) stem and classified as *Sphaeria lingam* (Tode 1791). Later on it was found in *B. oleracea* and reclassified as *Phoma lingam* (Desmaziere 1849). Subsequently, it was confirmed as *Leptosphaeria maculans* (Desm.) Ces & De Not. in New Zealand through the study of the sexual stage of *Phoma lingam* (Punithalingam and Holliday 1972). The taxonomy and nomenclature associated with *L. maculans* was a bit confusing for a short period of time due to morphological similarity of several species (Howlett et al. 2001). As a result, the

international Blackleg of Crucifers Network (ICBN) was established in 1994 and continues to address the taxonomic problems. Recent studies recognized many genera under the family *Leptosphaeria*, and their asexual morphs considered as phoma-like instead of *Phoma lingam* (Kaczmarek and Jedryczka 2011; de Gruyter et al. 2013; Wijayawardene et al. 2014; Ariyawansa et al. 2015). Today, *L. maculans* is considered as a hemibiotrophic fungal pathogen belonging to the kingdom – Mycota, phylum – Ascomycota, Class – Dothideomycetes, Order – Pleosporales, Family – *Leptosphaeriaceae*, Genus – *Leptosphaeria*, and Species – *maculans*. The pathogen has both sexual and asexual reproduction systems in where the sexual stage is maintained as *L. maculans* (Desm.) Ces & De Not. and the asexual stage considered as phoma-like (de Gruyter et al. 2013; Wijayawardene et al. 2014; Ariyawansa et al. 2015). The ascus of this group, which is enclosed by a double wall consisting of a thin fragile outer shell and a thick flexible inner wall, is bitunicate. These are enclosed in a flask-shape vessel called a pseudothecium. These are the sexual organs known as fungal fruiting bodies, releasing the enclosed ascospores to disperse them. The elongation of the bitunicate asci have been observed in both ascending and descending order within a group of preformed branching pseudoparaphyses. At the matured stage the shell splits open so that the inner wall can absorb water. As a result, the ostiole of the pseudothecia is formed at the tip of the fruiting bodies due to lysis of the thin outer layer (Webster 1993). The ascospores can be discharged successfully in about 5 seconds and the asci tend to be shrunken after releasing ascospores (Hodgetts 1917). On the other hand, the anamorph – phoma-like (de Gruyter et al. 2013) is characterised by a thin-walled, flask-shaped structure called pycnidia, which are known as asexual fruiting bodies. They usually produce abundant rod-shaped pycnidiospores. These are released en mass and ooze out from the pycnidial tip, and spread through the splash of rain.

2.2.2 Pathogenicity group (PG) and avirulence genes of *Leptosphaeria maculans*

The taxonomy and nomenclature associated with *L. maculans* is a bit confusing due to morphological similarity of several species (Howlett et al. 1991; Howlett et al. 2001). The fungus is divided into two groups ("A" and "B") based on their virulence in *Brassica*, SRAP and RFLP analysis, and the production of phytotoxin (Koch et al. 1991; Rouxel et al. 2004; Rouxel and Balesdent 2005). The strains that are able to cause stem canker in *Brassica* and to produce the chemical phytotoxin are classified as group "A". These strains are considered aggressive as they are highly virulent. In contrast, the strains that are unable to cause stem canker in *Brassica* are classified as group "B". These strains are also considered non-aggressive as they are weakly virulent. Group "B" strains are morphologically different from group "A"and are mostly associated with a less damaging pale brown upper stem lesion termed as phoma leaf spot. This phenotype leads to rename group "B" as a new species *Leptosphaeria biglobosa* sp. nov (Shoemaker and Brun 2001).

Isolates cultured from cruciferous weeds (Williams and Fitt 1999) were also classified as *L. maculans* but are genetically different from "A" and "B" groups (Balesdent et al. 1998; Purwantara et al. 2000). The "A" and "B" group isolates also showed cross incompatibility, while pseudothecial reproduction was absent (Somda et al. 1997). However, both species are available, but not equally distributed in all regions where blackleg has been identified (Rouxel et al. 2004). Variability for the virulence of *L. maculans* was reported for the first time in 1927 (Cunningham 1927). North American *L. maculans* populations have a low level of genetic diversity with a low variety of avirulence genes (Balesdent et al. 2005) as compared to European and Australian isolates (Kutcher et al. 1993).

Later on group "A" pathotype was further subdivided into three pathogenicity groups; PG2, PG3 and PG4 based upon their response in the cotyledons of *B. napus* cultivars 'Westar', 'Glacier', and 'Quinta' (Koch et al. 1991; Mengistu et al. 1991; Balesdent et al.

2005). Another aggressive pathotype PGT was detected from the North American *L. maculans* populations (Chen and Fernando 2006; Rimmer 2006). On the other hand, group "B" pathotype was subdivided into three genetically diverse subgroups i.e. NA1, NA2 and NA3 (Koch et al. 1991). A number of studies including isozyme and protein (Balesdent et al. 1992; Gall et al. 1995; Somda et al. 1996), internal transcribed spacers (Balesdent et al. 1998) and AFLP (Purwantara et al. 2000) reported the dissimilarity between the groups and among the subgroups. However, earlier studies on canola-*L. maculans* interaction were mainly based on PG groups while specific avirulence (*Avr*) genes were not identified.

Today most of the studies in the canola-blackleg pathosystem have been done using a differential set of *L. maculans* isolates which carry specific *Avr* genes. Understanding of the *Avr* gene profile in *L. maculans* has a great significance in stewarding *R* genes managing blackleg disease. For example, analysis of *L. maculans Avr* genes from field populations suggested possible effectiveness of *Rlm6* and *Rlm7* in Europe (Stachowiak et al. 2006); and also suggested ineffectiveness of *Rlm3* in Canada (Zhang et al. 2016). The availability of genome sequence of *L. maculans* facilitated the identification of novel *Avr* genes, and validated the interaction between *Avr*- and *R*- genes (Selin et al. 2016). To date fourteen *Avr* genes from *L. maculans* have been identified, and among them seven of the *Avr* loci have been cloned (Table 2.1). Eight of the *L. maculans Avr* genes are found to be in two distinct clusters; *AvrLm1- 2- 6* (Balesdent et al. 2002), and *AvrLm3-4-7-9-AvrLepR1* (Balesdent et al. 2005; Ghanbarnia et al. 2012). This suggests the possible relationship between an *Avr* gene cluster in *L maculans* and the corresponding *R* gene cluster in the host (Delourme et al. 2004). Results of *Avr* gene analysis from *L. maculans* isolates collected from Manitoba indicated a very high frequency of *AvrLm2, 4, 5, 6, 7,* and *11,* whereas very low frequency of *AvrLm3* and *AvrLm9* (Zhang et al. 2016). In Canada, the frequency of both *AvrLm3* and *AvrLm9* in *L. maculans* isolates has decreased over time

(Kutcher et al. 2010b; Liban et al. 2013; Zhang et al. 2016; Fernando et al. 2018). As a result, the increasing of *AvrLm4* and *AvrLm7* frequency in *L. maculans* isolates has been observed over time (Dilmaghani et al. 2009; Liban et al. 2013; Zhang et al. 2016). Moreover, previous studies also showed that very few isolates of *L. maculans* carried *AvrLm3* and *AvrLm7* genes together (Balesdent et al. 2006; Dilmaghani et al. 2009; Kutcher et al. 2010b; Zhang et al. 2016). This phenomenon suggested an unusual co-existence of the genes *AvrLm3* and *AvrLm7* in *L. maculans* isolates. A recent study showed that the *AvrLm3* gene was only expressed if the isolates did not carry *AvrLm4-7* gene, which is claimed as a 'hide-and-seek' relationship between *Avr* genes *AvrLm3* and *AvrLm4-7* (Plissonneau et al. 2016). Another very recent study in Canada also showed similar 'hide-and-seek' relationship between *Avr* genes *AvrLm5-9* and *AvrLm4-7* (Ghanbarnia et al. 2018). However, this kind of relationship could be clarified by the fact that the *Avr* genes *AvrLm3, 4, 7* and *9* are a part of the *AvrLm3-AvrLm4-AvrLm7-AvrLm9-AvrLepR1* gene cluster (Balesdent et al. 2002, 2005; Ghanbarnia et al. 2012). It is noted that the *AvrLm* gene *AvrLmJ1* first cloned and published by van de Wouw et al. (2014) which was renamed as *AvrLm5* (Plissonneau et al. 2018). A very recent study cloned the gene *AvrLm9* and found the sequence similarity with the gene *AvrLm5*, resulting a new name *AvrLm5-9* was proposed (Ghanbarnia et al. 2018).

Table 2. 1 Cloned avirulence genes of *Leptosphaeria maculans*

Avr genes	Cloning strategy	Fragment size	References
AvrLm1	Map-based	1123 bp	Gout et al. 2006
AvrLm2	Comparative genomic	258 bp	Ghanbarnia et al. 2015
AvrLm3	Genetic and genomic	1357	Plissonneau et al. 2016
AvrLm5-9	Map-based	479 bp	Plissonneau et al. 2018; Ghanbarnia et al. 2018
AvrLm4-7	Map-based	1433 bp	Parlange et al. 2009
AvrLm6	Map-based	774 bp	Fudal et al. 2007
AvrLm11	Genetic and genomic	1453	Balesdent et al. 2013

2.2.3 Evolution of virulence of *Leptosphaeria maculans*

The high evolutionary potential of *L. maculans* is characterised by its life cycle and its genome structure. From the life cycle we have seen that the pathogen can reproduce both sexually and asexually. Sexual reproduction of wind borne ascospores permits recombinant virulent isolates to be generated in each cycle, followed by spreading over great distances (Bokor et al. 1975).

The genome sequencing of *L. maculans* has been published in 2011 (Rouxel et al. 2011; http://www.genoscope.cns.fr). The 45-Mb genome *'brassicae'* (Lmb) showed that the *Avr* genes of *L. maculans* are positioned at 'AT-rich' region surrounded by the degenerated transposable elements (TE) (Rouxel et al. 2011). The high percentage of TE (33%) in Lmb genome along with repeat-induced point mutation (RIP) generated large AT-rich regions, known as AT-isochores (Rouxel et al. 2011; Grandaubert et al. 2014). These characteristics are responsible for the rapid evolution of virulent isolates by chromosomal length polymorphism, repeat-induced point mutation (RIP) and gene deletion (Gout et al. 2007; Fudal et al. 2009; Rouxel et al. 2011; Daverdin et al. 2012). Amino acid substitution has also reported to evolve new strains of *L. maculans* in European populations (Balesdent et al. 2006; Stachowiak et al. 2006; Parlange et al. 2009). Moreover, masking or camouflaging of an *Avr* gene led to the appearance of virulence of another *Avr* gene, which has particularly been seen in *AvrLm4-7* and *AvrLm3* interaction (Plissonneau et al. 2016, 2017) as well as *AvrLm5-9* and *AvrLm4-7* interaction (Ghanbarnia et al. 2018).

2.2.4 Life cycle and infection strategies

The pathogen *L. maculans* is known as a stubble-borne pathogen. The fungus can survive as pseudothecia, pycnidia, and mycelia (Hall 1992) and usually overwinters on canola stubble (Fig. 2.1 step-1). *L. maculans* produces both sexually and asexually on host species including cruciferous weeds. The sexual fruiting bodies called pseudothecia are developed on stubble

and release ascospores in early spring as a primary source of inoculum (Fig. 2.1 step-2). Ascospores mostly travel by the wind, up to 8 km as reported by Bokor et al. (1975) and subsequently, land on either cotyledons or true leaves (Fig. 2.1 step-3) followed by germination in wet conditions. As a result, the fungus produces hyphae through stomatal pores or wounds leading to the development of an infection site (Chen and Howlett 1996; West et al. 2001; Hua et al 2004). The high humidity and moderate temperature at vegetative growth stage is believed to be an ideal condition in lesion development (Ghanbarnia et al. 2009). The initial colonization is biotrophic; later on it becomes necrotrophic and produces pycnidiospores, asexual spores at the infection sites of a susceptible seedling (Fig. 2.1 step-4; Hammond et al 1985; Hammond and Lews 1987). These pycnidiospores are considered a secondary inoculum in the disease cycle. They are believed to be spread to other leaves and neighbouring plants by rain splash (Travadon et al. 2007; Fig. 2.1 step-4). It is thought that they can reproduce asexually within a short period of time, in an about 7-days. In western Canada, asexual mode of infection is considered to be predominant over sexual ascospores (Guo et al 2005; Ghanbarnia et al. 2011). At early stages of infection on cotyledons/leaves, the fungus colonizes intercellular spaces between mesophyll cells; afterwards moving towards the stem via leaf veins / petioles. The disease cycle then goes through a long symptomless phase (usually 6-8 weeks) when the fungus grows systematically as a biotroph, mainly in xylem vessels or inbetween xylem parenchyma and the cortex (Hammond et al. 1985; Huang et al. 2014). Finally, before maturation of the pods, the fungus again becomes necrotrophic and kills cells of the stem cortex resulting in the development of a stem canker at the base of the stem (Fig. 2.1 step-5; Hammond et al. 1985; Sprague et al. 2007; Travadon et al. 2009). This may cause a complete blocking of vascular system, which leads the development of black tissues at the stem cortex, hence the name “blackleg” (Fig. 2.1 step-6). Stem cankers are the major cause of yield loss due to lodging and plants can die off without

producing seeds. Blackening at the cross-section of the stem can be seen when the stem is cut off at the base by shears, at the end of the season. After the growing season the pathogen can overwinter as pseudothecia and/or mycelia on the remaining stubble.

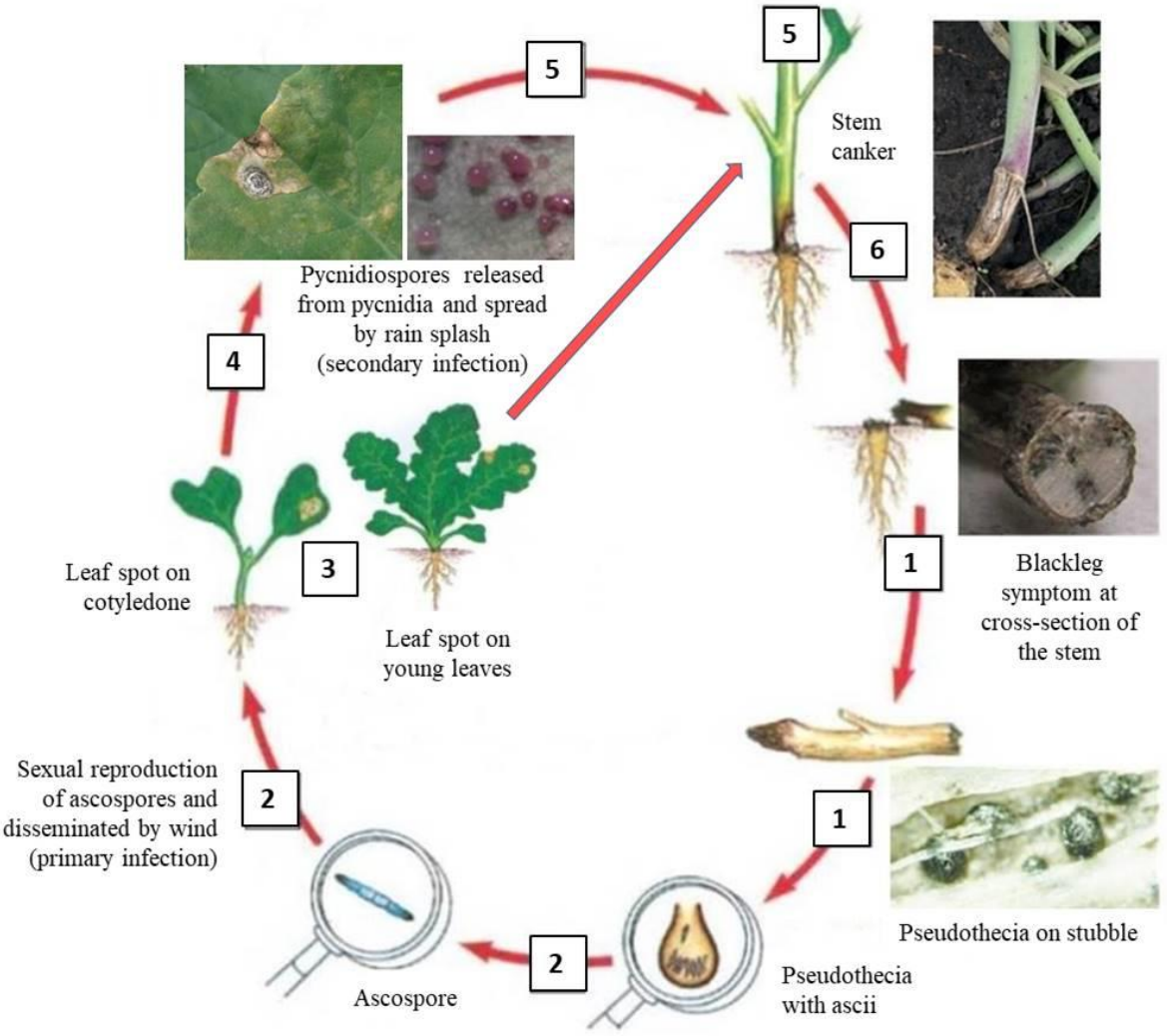

Fig. 2. 1 The life cycle of *Leptosphaeria maculans*, causal agent of blackleg disease in canola. **1**, long-term survival as saprophyte; **2**, producing primary inoculum (ascospores) through sexual reproduction; **3**, short necrotic stage after establishing primary infection; **4**, producing secondary inoculum (pycnidiospore) through asexual cycle; **5**, a long biotrophic stage characterized by the colonization from leaf tissues to stem; and **6**, shift towards necrotrophy. Figure modified from Rouxel and Balesdent (2005) with the kind permission (Appendix I) of the publisher John Wiley & Sons (customercare@copyright.com).

2.2.5 Host range of *Leptosphaeria maculans*

Older literature reported a number of non-crucifers including *Phaseolus* sp., *Humulus* sp., *Swertia perennis*, *Teucrium* sp. or *Artemisia campestris* as hosts of *L. maculans* (Müller 1953; Müller and Tomasevic 1957; Tulasne and Tulasne 1863; Saccardo 1883). Petrie (1969) found about 28 wild crucifer species including weeds to be a host of *L. maculans*. Further

studies on the pathogen species suggested that *L. maculans* could be a pathogen of cruciferous plants and most likely limited to *Brassica* group. Cultivated *Brassica* species; *Brassica napus, B. rapa, B. juncea, B. oleracea, B. carinata, B. nigra* were considered as the main host of *L. maculans*. Recent studies showed that *L. maculans* can be isolated from crucifer species such as radish (*Raphanus sativus*), wild radish (*R. raphanistrum*), white mustard (*Sinapis alba*), or rocket salad (*Eruca vesicaria* ssp. *sativa*) (Johnson and Lewis 1994; Williams and Fitt 1999; Li et al. 2005). However, the recent advancement of molecular techniques facilitated the dissection of the *L. maculans–L. biglobosa* species complex and reported many of the isolates cultured from cruciferous weeds as the subspecies of *L. biglobosa* (Mendès-Pereira et al. 2003). *Arabidopsis thaliana*, another member of Brassicaceae family, was also predicted to be a potential host of *L. maculans*, depending on the genotypes (Bonham et al. 2002; Rouxel and Balesdent 2005).

2.3 The disease – blackleg

2.3.1 Disease occurrence

Blackleg in canola is a devastating disease worldwide, except in China. In Canada, the non-aggressive isolates known as *L. biglobosa* was first detected in the 1960s (Vanterpool 1961, 1963), and at that time blackleg was not an issue in canola production. Thereafter, aggressive isolates known as *L. maculans* were identified in Saskatchewan in the 1970s and further spread to all three provinces of western Canada (McGee and Petrie 1978; Petrie 1979; Petrie et al. 1985; Fernando and Chen 2003; Chen and Fernando 2006) and Ontario (Gugel and Petrie 1992; Rempel and Hall 1993). These two species have coexisted across Europe (Jensen 1994; Kuswinanti et al. 1995; Somda and Brun 1995; Fitt et al. 1997; Szlavik et al. 2006). In Australia, the disease scenario was more epidemic in nature as compared to Canada and Europe from the early 1970s (Bokor et al. 1975), and both species have been reported long since (Plummer et al. 1994; Salisbury et al. 1995). In contrast, the disease occurrence in the

United States has been observed much later as compared to Australia, Europe, and Canada (Lamey 1995; Bradley et al. 2005). China is the only canola growing country where no *L. maculans* isolates have been reported (West et al. 2000; West and Fitt 2005; Fitt et al. 2006; Zhang et al. 2014).

2.3.2 Disease importance

The disease has a great economic impact on the canola industries of Australia, Canada, Europe and parts of Asia with severe yield losses (Fitt et al. 2006; Howlet 2004; West et al. 2001). The severity of blackleg disease differs in canola growing regions due to the differences of climatic conditions, length of growing season, cultivation practices, fungal populations, and cultivars used (West et al. 2001; Fitt et al. 2006). In Canada, 25-50% yield losses had been predicted by Petrie et al. (1985) and Gugel and Petrie (1992), which is much lower than Europe and Australia. Yield losses have varied in different regions; UK – up to 93% (Sansford 1995), Kentucky USA 75-90% (Lamey and Hershman 1993), and Germany as high as 50% (Gugel and Petrie 1992). But in Australia the scenario is more epidemic as 100% yield losses have been reported (Bokor et al. 1975). On the contrary, blackleg disease occurrence in India (*B. juncea* and *B. rapa*) and China (mostly *B. napus*) is very rare. However, a recent study showed an estimated loss of about $1000M per growing season from the canola growing world (Fitt et al. 2008).

2.3.3 Disease epidemiology

Blackleg disease is usually initiated by airborne ascospores in canola growing regions (Fig. 2.1; Hall 1992; Mahuku et al. 1997). Though the infections are initiated by airborne ascospores, but it can also occur by either infected seeds (Kharbanda 1993) or pycnidiospores (Hall 1992). Seed borne inoculum would be a new threat in introducing *L. maculans* where it has not been previously identified, i.e. China (Fitt et al. 2006; Van de Wouw et al. 2015; Fernando et al. 2016). In a dry-wet conditions, the primary inoculum, ascospores, are released

from the pseudothecia originating from crop stubble (Fig 2.1; Mahuku et al. 1997; West et al. 2001). The period of ascospore release varies from season to season and region to region (West et al. 2001). It usually happens in April-August in Australia (Bokor et al. 1975; Khangura et al. 2001), May-August in western Canada (Kharbanda 1993; Rampel and Hall 1993; Guo and Fernando 2005), September-April in Western Europe and September-November in Eastern Europe (West et al. 1999). Showers of sexual spores, ascospores, are believed to be a major inoculum source in Europe (West et al. 2001; Fitt et al. 2006), whereas in western Canada asexual spores, pycnidiospores, are the major inoculum source for the disease development (Guo and Fernando 2005; Ghanbarnia et al. 2011; Dilmaghani et al. 2013). On the other hand, both ascospores and pycnidiospores in combination have proven to be the effective inoculum in Australia (Barbetti 1975; Marcroft et al. 2004a). From the disease cycle (Fig. 2.1) it has been observed both pycnidiospores and ascospores land on cotyledons and/or true leaves followed by germination in a wet condition, to produce hyphae through stomatal pores or wounds (Chen and Howlett 1996). It was also observed that a few ascospores were enough to initiate infection under optimal weather conditions regardless of the port of entry (Wood and Barbetti 1977). In contrast, a few pycnidiospores can infect only wounded cotyledons, leaves, petioles or stems under a controlled environment (Hammond et al. 1985), but can't infect unwounded portions unless applying high conidial concentrations (Vanniasingham and Gilligan 1989). Under laboratory conditions ascospores germinated within 4 hours after landing on leaves at 4-28^{0}C (Hall 1992). It is also reported that four hours was the least wetness period to produce leaf lesions (Buddulph et al. 1999). Nevertheless, the incubation period between infection and development of leaf lesions may vary depending on the cultivar as well as position of the leaves. Leaf spots have been observed throughout the growing season in Australia (Barbetti and Khangura 1999), but it is seen in different time periods in Canada and Europe. It is very common at vegetative to early

flowering stage (June-July) in western Canada, whereas at seedling stage in Ontario (Kharbanda 1993) and Western Europe (Paul and Rawlinson 1992). It actually varies based upon host, pathogen as well as the phase of lesion development.

2.4 Blackleg disease control

2.4.1 Cultural control

Crop rotation is one of the most important strategies in controlling residue-borne plant pathogens, such as *L. maculans* (Curl 1963). In fact, it has a detrimental effect on the growth, survival and reproduction of the causal organism *L. maculans*, thus reducing the pathogen population to a level low so that significant crop damage does not occur. In western Canada, cropping system research indicates that more diverse rotations tend to have fewer pest problems and lower production risk (Bailey et al. 2000; Johnston et al. 2005; Kutcher et al. 2011). Over the last decade, growers have specialized with more and more farmers producing canola in a 4-year rotation, which had been thought to be the standard recommendation (Rimmer et al. 2003). Instead, 3-year rotations of canola with cereals and pulses have been suggested to be sustainable (Catheart et al. 2006; Kutcher et al. 2013). The most severe infections have been observed from 2 -3 years old stubble whereas the fungus can survive in the stubble for more than 5 years (Kharbanda and Tewari 1996). Moreover, there was no difference in disease severity between canola grown in 18-month-old stubble and canola grown in a virgin field (Marcroft et al. 2004b). Another Australian study reported lower disease severity in canola cultivars planted with stubble from different resistance sources as compared cultivars planted in stubble from same resistance sources (Marcroft et al. 2012a) indicating the necessity of rotating oilseed rape with non-host crops for three to four years or longer. In addition, a recent study using resistant cultivars in western Canada showed no significant differences in blackleg disease severity between 2-yrs and 3-yrs crop rotation including canola-wheat and canola-wheat-pea, respectively (Kutcher et al. 2013).

Isolation distance is considered another important strategy in managing blackleg disease. Airborne ascospores released from the stubble of an adjacent field may dampen the efficacy of crop rotation in controlling disease (Kharbanda and Tewari 1996). At one time, 2 km in Western Canada (Petrie 1978) and 5-8 km in Western Australia (Bokor et al. 1975) was recommended for an isolation distance. But a further study from Australia reported that 100 m isolation from 6-month-old canola stubble markedly reduced blackleg disease severity and continuously decreased severity up to distance of 500 m (Marcroft et al. 2004b).

Stubble management is also thought to be an efficient method of disease control. Decomposition of canola stubble by conventional tillage shortens the time that pathogens survive on the stubble and reduces the release of airborne spores (Kharbanda and Tewari 1996). It is suggested that releasing airborne ascospores of *L. maculans* from the stubble can be minimized by either burying stubble during fall or seeding a non-host during spring directly (Kolte 1985). Tillage reduced the release of spores as well as reduced the blackleg disease as compared to zero-till with or without the rotation as seen in Canadian field studies (Guo et al. 2005). Burning or burying of stubble is recommended in Australia along with other residue management approaches (Barbetti and Khangura 1999; Barbetti and Khangura 2000). Deep ploughing is popular in Europe whereas minimum tillage is popular in western Canada (West et al. 2001), in order to achieve conservation of moisture and reduce soil erosion. However, fall tillage along with 3 years crop rotation is considered one of the best strategies in reduction of blackleg severity (Turkington et al. 2000).

Altering seeding date is thought to be an effective method of controlling disease where the crop escapes the conditions / environment conducive to disease development or avoids the high amount of inoculum. In Canada, release of sexual spores occur throughout the growing season, indicating that altering sowing date has no effect on blackleg disease (Kharbanda and Tewari 1996). On the other hand, late sowing is recommended in Australia

(McGee 1977), and early sowing in France (LePage and Penaud 1995) so that seedlings can escape the period of disease development.

2.4.2 Chemical control

Application of fungicides depends upon the disease epidemiology and the level of infection of the crop. Today, fungicides have been used in many different ways such as seed treatments, soil treatments or foliar applications (West et al. 2001). Since seed borne dissemination is believed to be a way of the introduction of the pathogen to a virgin area (Rimmer and Buchwaldt 1995), planting of certified seed that has been treated with a fungicide is recommended in many regions to prevent the introduction of the pathogen. Fungicides are usually used at seed treatment and foliar application in controlling blackleg disease (Kharbanda et al. 1999; West et al. 2001). Kutcher et al. (2013) and Liu (2014) reported that foliar application of fungicides significantly reduced blackleg disease incidence and severity on the Canadian Prairies. It is also important to consider the cost of fungicides. In Europe the higher yield harvested could justify the application of expensive fungicides, but it would be uneconomical to apply them in Canada where seed yield is usually low because of shorter day length and/or early harvesting (West et al. 2001). Moreover, disease forecasting systems, particularly for blackleg, have not been installed in Canada as yet, which could facilitate decisions of fungicide applications at the period of higher disease pressure and protecting the crop from severe yield loss.

2.4.3 Biological control

Biological control refers to a natural phenomenon, involving the use of microorganisms for disease control in a natural setting. Since the biological control agents (BCA) have no detrimental effect on the environment, it seems to be a good alternative to chemical control. Numerous microorganisms including bacteria, fungi, viruses, nematodes, protozoa, viroids, and seed plants have been used as an antagonistic BCA (Cook and Baker 1983; Fravel 1988;

Elad and Kapat 1999; Thanh et al. 2014). The mechanisms employed by BCA are diverse, complicated and differ between pathogen, host plant and the BCA. It is sometimes challenging to determine the potential results of their interactions (Whipps 2001). The core mechanisms of biocontrol are antagonisms such as parasitism, predation, competition, antibiosis, and lysis (Pal and McSpadden Gardener 2006; Athukorala et al. 2009; Ramarathanam et al. 2010; Selin et al. 2010; Thanh et al. 2014).

Bacteria are one of the most popular BCA having multiple mechanisms of disease control including production of siderophores, antifungal antibodies, enzymes, and volatiles (Kloepper et al. 1980; Fravel 1988; Weller et al. 1988; Whipps 1997; Garbeva et al. 2004; Zhang et al. 2006; Athukorala et al. 2009). The ratio of carbon to nitrogen in a nutrient source is a key contributor in the suppression of pathogen by halting or slowing the growth of nearby colonies (Pal and McSpadden Gardener 2006). Fernando et al. (2005, 2007a) have shown that *Pseudomonas spp*. can suppress infection of *Sclerotinia sclerotiorum* in canola via the inhibition of mycelial growth and germination of sclerotia. Different strains of *Pseudomonas spp*. and *Bacillus spp*. were shown to be effective against blackleg pathogen *Leptosphaeria maculans* (Ramarathanam and Fernando 2007b; Ramarathanam et al. 2011). It was reported that bacteria cultured from soil, canola-stubble, and plant-part had a great impact on the suppression of blackleg disease (Ramarathanam and Fernando 2007b; Athukorala et al. 2009). However, blackleg disease was decreased due to the upregulation of induced systemic resistance (ISR) by the inoculations of *Pseudomonas spp.* and *Bacillus spp.* (Ramarathanam et al. 2007b, 2010). Systemic acquired resistance (SAR) also occurs in the entire plant when it has been exposed to a pathogen, resulting in sufficient disease reduction as shown in oilseed rape (Mahuku et al. 1996).

2.4.4 Genetic control – host resistance

2.4.4.1 Qualitative resistance

Qualitative resistance is also referred to as monogenic / seedling / race-specific / *R* gene / vertical resistance in *Brassica*. Qualitative resistance usually responds immediately to the pathogen after their penetration in the host cells. The colonization of *L. maculans* is restricted in a resistant host by program cell death which is often culminated in a hypersensitive response (HR). Until now, eighteen genes resistant to *L. maculans*; *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BMLR1* and *BMLR2* have been identified in different *Brassica* species - *B. rapa*, *B. napus*, *B. juncea* and *B. nigra* (Rimmer and van den Berg 1992; Yu et al. 2005; Delmourme et al. 2006; Rimmer 2006; Yu et al. 2008; Van de Wouw et al. 2008; Long et al. 2011; Balesdent et al. 2002; Balesdent et al. 2013; Raman et al. 2013). Among them, *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9* were identified in *B. napus*. Five *R* genes; *Rlm1*, *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9* were mapped on chromosome A07 (Delourme et al. 2004; Larkan et al. 2016) whereas a single *R* gene *Rlm2* was mapped on chromosome A10 (Delourme et al. 2006). Two genes were identified, each in *B. rapa* (*Rlm8* and *Rlm11*), and *B. juncea* (*Rlm5* and *Rlm6*), and a single gene *Rlm10* in *B. nigra*. Four resistance genes; *LepR1*, *LepR2*, *LepR3*, and *LepR4* were transferred to *B. napus* from *B. rapa* subsp. *sylvestris* (Table 2.2). Several other genes such as *BLMR1, BLMR2*, *LmR1*, *cLmR1*, *cRLM*, *LmFr1*, *LMJR1*, *LMJR2*, *LEM1* have also been identified using uncharacterised *L. maculans* isolates, which could be allelic to the known *R* genes (Table 2.2; Delourme et al. 2004; Delourme et al. 2006; Rimmer 2006).

Table 2. 2 Molecular mapping of major genes resistance to *Leptosphaeria maculans* in *Brassica*

Species	Locus / Gene	Population	Phenotype	Marker genotype	Mapping strategy	Chr.	Reference
B. napus	*Rlm1*	DH	Cotyledon	RAPD	BSA[Z]	A7	Delourme et al. 2004
		F2	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
		DH	Cotyledon / stem canker	SSR, DArT	WGA[Y]	A7	Raman et al. 2012
		Columbus / Westar	Cotyledon / stem canker	SSR	Chromosome specific	A7	Raman et al. 2012
		ILs	Cotyledone	SNP	WGA	A7	Larkan et al. 2016
B. napus	*Rlm2*	F2	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
		BC	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
		DH	Cotyledon / Field	RAPD	BSA	A7	Delourme et al. 2004
B. napus	*Rlm3*	DH	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
		ILs	Cotyledone	SNP	WGA	A7	Larkan et al. 2016
B. napus	*Rlm4*	F2	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
		ILs	Cotyledone	SNP	WGA	A7	Larkan et al. 2016
			Cotyledon / stem canker	SSR	WGA	-	Raman et al. 2012
B. napus	*Rlm7*	F2	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004; Parlange et al. 2009
B. napus	*Rlm9*	DH	Cotyledon	RAPD	BSA	A7	Delourme et al. 2004
B. napus	*BLMR1*	BC2F3	Cotyledon	SRAP, SNP	Selective genotyping	A10	Long et al. 2011
B. napus	*LmFr1*	Cresor / Westar	Field	RFLP	WGA	A7	Dion et al. 1995
B. napus	*LEM1*	Major / Stellar	Cotyledon / Stem	RFLP	WGA	A7	Ferreira et al. 1995
B. napus	*cLmR1*	DH	Cotyledon	RAPD, RFLP	BSA	A7	Mayerhofer et al. 1997
B. napus	*cLmR1*	BC1	Cotyledon	RFLP, SCAR, EST	BSA	A7	Mayerhofer et al. 2005
B. napus	*cRLM*	Maluka / Westar	Cotyledon / Adult	RAPD, AFLP, RFLP	BSA	A7	Rimmer et al. 1999
B. napus	*QRlm*	DH	Cotyledon	SSR	WGA	A10	Raman et al. 2012
B. napus	*Rpg$_3$Dun*	F2	Cotyledon	SRAP	BSA	A7	Dusabenyagasani and Fernando 2005
B. napus	*RlmS*	Surpass400	Cotyledon	-	-	-	Van de Wouw et al. 2008
B. rapa	*LepR1*	DH	Cotyledon / Field	RFLP	WGA	A2	Yu et al. 2005
	LepR2	DH	Cotyledon / Field	RFLP	WGA	A10	Yu et al. 2005
	LepR3	BC	Cotyledon	SSR	CSM[X]	A1 and A10	Yu et al. 2008
		DH	Cotyledon	SSR, SCAR	CSM	A10	Larkan et al. 2013
B. rapa	*LepR4*	BC3S2	Cotyledon / Field	SSR	A-genome specific	A6	Yu et al. 2007
B. rapa	*Rlm8*	156-2-1	Cotyledon	-	-	-	Balesdent et al. 2002
B. rapa	*Rlm11*	02-159-4-1	Cotyledon	-	-	-	Balesdent et al. 2013
B. juncea	*Rlm5*		Cotyledon	-	-	-	Balesdent et al. 2002
B. juncea	*Rlm6*	RILs	Cotyledon / stem canker	RAPD, RFLP	BSA	B8	Chevre et al. 1997
B. juncea	*rjlm2*	ILs	Cotyledon	RAPD, RGA, SCAR	B-genome specific	-	Saal and Struss 2005
B. juncea	*LMJR1*	F2	Cotyledon	RFLP, SSR	WGA	B3 (J13)	Christianson et al. 2006
B. juncea	*LMJR2*	F2	Cotyledon	RFLP, SSR	WGA	B8 (J18)	Christianson et al. 2006
B. nigra	*Rlm10*	Addition	Cotyledon	RAPD,	WGA	B4	Eber et al. 2011;

lines	Isozyme	Delourme et al. 2008

[Z]Bulked segregant analysis, [Y]Whole genome mapping, [X]Chromosome specific mapping

2.4.4.2 Quantitative resistance

Quantitative resistance is also referred to as polygenic / adult plant / race non-specific / horizontal / QTL resistance in *Brassica*. Numerous studies reported quantitative resistance in segregating population derivatives of *B. napus*, *B. juncea* and their hybrids (Cargeeg and Thurling 1980; Ferreira et al. 1995; Saal and Struss 2005; Kaur et al. 2009; Raman et al. 2012). This type of resistance is believed to be more durable as it slows down the *L. maculans* movement from leaf to stem (Fitt et al. 2006) resulting in less severe blackleg at the harvesting period. But the resistance can be overcome when *L. maculans* inoculum pressure is extremely high (Xu et al. 2005). The term 'QTL' has been used to report the quantitative locus in the literature even though a large portion of genotypic variation is defined by the unknown major genes. In conventional genetics, QTL refers to the regions of DNA associated with phenotypic traits that usually have low heritability, low Mendelian segregation and polygenic accumulative effects in the genotypes. Quantitative resistance is thought to be effective in combination with the environmental effect (Xu et al. 2005; Xu et al. 2013). Some of the QTLs identified in different studies are given in Table 2.3.

Table 2. 3 QTLs associated with blackleg resistance identified from mapping populations[z]

Population	Chromosome	LOD# score	%Genetic variation (R^2)[Y]	Additive effect	Reference
Av-Sapphire/Westar 10	A1	2.5-5.6	14-16	-	Kaur et al. 2009
	A2	1.3-3.8	4-26	-	
	C1	0.8-2.9	3-8	-	
	LG1	1.0-1.6	4-10	-	
Caiman$_3$/Westar	A1	2.9-3.5	20.22.7	-	Kaur et al. 2009
	A10	1.4-3.0	5-34	-	
	C5	4.4-5.6	19-23	-	
Camberra$_4$/Westar	A5	0.5-2.6	1.5-33	-	Kaur et al. 2009
	A1/C1	1.9-5.1	17-18	-	
	A10	0.3-2.7	2-31	-	
	C7-2	2.7-3.7	13-24	-	
	LG2	2.1-2.8	14-28	-	
Darmor/Samourai	A1	2.3	6.7	Samourai	Delourme et al. 2008
	A2	2.3-3.02	8.1-14.6	Darmor	
	A6	1.9-2.8	6.2-10.0	Samourai	
	A10	2.7	11.0	Samourai	
	C2	2.0-2.2	8.0-8.4	Darmor	
	C4	2.4-3.2	6.7-12.2	Darmor	
	C8	1.9	-	Samourai	
Darmor/Yudal	A2	2.4-5.5	3.8-8.5	Darmor	Pilet et al. 1998; Delourme et al. 2008
	A4	3.3	4.8	Darmor	
	A6	5.0-12.2	7.2-20.0	Darmor	
	A7	4.5	6.9	Darmor	
	A8	7.2	13.0	Darmor	
	A9	3.3	4.8	Darmor	
	C2	5.5-6.6	8.3-13.3	Yudal	
	C4	4.7-9.5	6.7-15.2	Darmor	
	C8	4.2	6.2	Darmor	
Skipton/Ag-Spectrum	A2	7.0	11.5	Ag-Spectrum	Raman et al. 2012
	A9	2.9	5.0	Skipton	
	A10b	2.2	6.2	Skipton	
	C1	4.2	11.5	Ag-Spectrum	
	C2a	6.8	16.6	Skipton	
	C3	4.2	24.5	Skipton	
	C6	6.1	14.5	Skipton	
	A1a	6.1	26.1	Ag-Spectrum	

[z]scored as internal infection due to canker development at adult plant stage; [Y]range of LOD and R^2 varied with method of regression analysis (simple and composite interval mapping).

2.5 Effectiveness of qualitative resistance

Stubble borne pathogens i.e. *L. maculans* that have both sexual and asexual cycles of reproduction, have been considered a great threat to the durability of qualitative resistance (*R* genes resistance) in canola. Early genetic studies showed a gene-for-gene relationship

between an *R* gene and the corresponding *Avr* gene (Ansan-Melayah et al. 1998; Balesdent et al. 2001). Frequency of the *Avr* genes in *L. maculans* population is the key contributor to the effectiveness of the corresponding *R* genes (Daverdin et al. 2012), which differs in canola growing regions / countries (Balesdent et al. 2005; Balesdent et al. 2006). Moreover, the frequency of specific *Avr* genes in *L. maculans* population is also a vital factor in the rapid evolution of virulent isolates in multiple ways (Gout et al. 2007; Fudal et al. 2009; Daverdin et al. 2012; Balesdent et al. 2006; Stachowiak et al. 2006; Parlange et al. 2009; Plissonneau et al. 2016, 2017). There is a possible fitness cost associated with the pathogen shifting from avirulence to virulence resulting in the breakdown of the host resistance (Vera Cruz et al. 2000; Marcroft et al. 2012b). However, host resistance can be strengthened by utilizing one or more single dominant *R* genes (Johnson and Law 1973) but single *R* genes may not always offer a durable resistance. Because intensive use of a single *R* gene exerts strong selection pressure on the fungal populations, it results in pathogen adaptation to the *R* gene in canola varieties. Previous studies indicated that the pathogen can be adapted to a single *R* gene due to selection pressure within a few years in both experimental conditions and agricultural systems. Due to the adaptation of the pathogen, the breakdown of a single *R* gene has been reported. Studies have shown the breakdown of a single *R* gene *Rlm6* (Brun et al. 2010) and *Rlm1* (Rouxel et al. 2003) in France after three growing seasons. Similarly, *LepR3* resistance in Australia was found ineffective just after three years of releasing a commercial *LepR3*-carrying variety Surpass 400 (Li et al. 2006; Sprague et al. 2006; Van de Wouw et al. 2010a; Van de Wouw et al. 2014). Recently, the breakdown of *Rlm3* resistance was reported in the Canadian Prairies due to the intensive use of *Rlm3*-carrying canola cultivars (Zhang et al. 2016). To cope with this situation, breeders have to develop new cultivars to replace old ones so that the selection pressure of the *R* genes can be avoided. But it requires knowing the pathogen race structure and *Avr* genes distribution in the field population. Today, monitoring

of pathogen population and race structure is feasible and has been used in the last couple of years (Van de Wouw et al. 2010b; Liban et al. 2016). Stacking of several *R* genes and/or deployment of quantitative resistance has been practiced in order to avoid selection pressure to a particular *Avr* gene in the pathogen population. Unfortunately, these strategies have not resulted in a greater durability of resistance (Kolmer et al. 1991; Crute and Pink 1996). In contrast, a recent study (Brun et al. 2010) demonstrated that an *R* gene (*Rlm6*) is more durable when combined in a quantitative genetic background. It is thought that this kind of strategy is more effective in managing blackleg disease when low disease pressure and low variability of *L. maculans* population exists. Since Canada has less pressure from the pathogen compared to Europe and Australia (Balesdent et al. 2005), it may be effective to identify novel *R* genes as well as introgress them into quantitative background. However, a single approach may not offer the durable resistance, indicating an integrated strategy based upon existing farm practices including use of resistant cultivars (Kutcher et al. 2013), rearrangement of resistant cultivars (Marcroft et al. 2012a), crop rotation (Kutcher et al. 2013), stubble management and minimal use of fungicides (Kutcher et al. 2011) need to be implemented for sustainable canola production.

2.6 Introgression of blackleg resistance

The utilization of genetic resistance particularly qualitative resistance has been considered as the most cost-effective and environment friendly action in controlling disease in various crops since the first *R* genes were identified (Jackson and Taylor 1996). Intra- and inter-specific hybridization via conventional plant breeding has played a vital role in gene introgression for disease control. As a consequence, several varieties labelled resistant to blackleg disease are dominating today's commercial cultivation worldwide. Due to the 'breakdown' of several *R* genes by shifting *L. maculans* population (Rouxel et al. 2003a; Sprague et al. 2006; Brun et al. 2010; Van de Wouw et al. 2010a; Van de Wouw et al. 2014;

Zhang et al. 2016), novel sources of resistance are always explored. Interspecific and intergeneric resistant resources have been utilized in order to expand the genetic variability for the resistance to *L. maculans*. *B. napus* germplasm has been largely used in creating genetic variation for resistance to *L. maculans* as reported in previous studies (Rimmer and van den Berg 1992; Rouxel et al. 2003b; Marcroft et al. 2012b). Other *Brassica* species including *B. rapa, B. juncea, B. nigra* and *B. carinata*, as well as another crucifer *Sinapis arvensis,* have been reported to carry blackleg resistance (Roy 1978, 1984; Mithen et al. 1987; Sjodin and Glimelius 1988; Pang and Halloran 1996; Chevre et al. 1997; Snowden et al. 2000; Bohman et al. 2002; Marcroft et al. 2002; Chtistianson et al. 2006; Delourme et al. 2006; Leflon et al. 2007). Some of these sources were utilized in introgression of resistant loci into *B. napus* by conventional backcross breeding (Roy 1978, 1984; Crouch et al. 1994; Howell et al. 2003) or via laboratory techniques including somatic hybridization (Sjodin and Glimelius 1989), and embryo culture (Waara and Glimelius 1995; Gerdemann-Kncorck et al. 1994). Blackleg resistance has been transferred to *B. napus* from *B. rapa* subsp. *sylvestries* using a resynthesized amphidioploid (Crouch et al. 1994) resulting in several high yielding cultivars including Surpass 400, Surpass 404CL, Surpass 501TT, Surpass 603CL, Hyola 43, and Hyola 60 which have been released in Australia. Conventional interspecific hybridization technique has also been used to transfer *R* genes *LepR1-LepR4* into *B. napus* from *B. rapa* subsp. *sylvestries* (Crouch et al. 1994; Yu et al. 2012). Effort has been given to transfer blackleg resistance into *B. napus* from *B. juncea* (Roy 1978, 1984; Prakash and Chopra 1988; Dixelius and Wahlberg 1999; Roussel et al. 1999), resulting few genes have been transferred into *B. napus* from *B. juncea* which showed on linkage group DY717 (Saal et al. 2004). Recently, a successful introgression from *B. juncea* to *B. napus* was reported where developed a cultivar Darmor-MX carrying *L. maculans* resistant gene *Rlm6* (Brun et al.

2010). As well, researchers at the University of Alberta were also able to introgress blackleg resistance genes into *B. napus* from *B. carinata* (Navabi et al. 2010).

2.7 Molecular marker linked to blackleg resistance

Molecular markers have been employed to identify loci linked to blackleg resistance. A number of genotyping techniques based on DNA hybridization including restriction fragment length polymorphism (RFLP), and diversity arrays technology (DArT) have been used for a long time. The most popular PCR-based techniques including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), sequence characterized amplified region (SCAR), amplified fragment length polymorphism (AFLP), and sequence-related amplified polymorphism (SRAP) are currently being used. Very recently, sequence-based techniques such as single nucleotide polymorphism (SNP), restriction-site associated DNA (RAD), and genotyping-by-sequencing (GBS) have become popular for molecular analysis (Li et al. 2001; Lombard et al. 2001; Suwabe et al. 2006; Miller et al. 2007; Sun et al. 2007; Baird et al. 2008; Dusabenyagasani and Fernando 2008; Trick et al. 2009; Durstewitz et al. 2010; Raman et al. 2013b). Among them RFLP, AFLP, SSR, SRAP, RAPD, and SCAR markers have already been used to map genes linked to blackleg resistance (Table 2.2). The latest in molecular tools such as DArT, 60K SNP infinium array, RAD, and GBS are currently being used to map blackleg resistance genes in commercial breeding programs. The loci linked to blackleg resistance have already been mapped via either linkage/QTL mapping or association mapping (Delourme et al. 2004; Jestin et al. 2011; Raman et al. 2012a; Raman et al. 2012b) using structured such as F_2, DH, or BC and unstructured such as breeding lines and diversity panels populations (Table 2.2 and 2.3). Bulked segregant analysis approach was also used (Huang et al. 2006) when extensive genotyping and resources (money and time) are limited (Dusabenyagasani and Fernando 2008). Currently, whole genome analysis (genomic selection) has been used extensively to identify both qualitative and quantitative loci linked to

blackleg resistance (Kaur et al. 2009; Raman et al. 2012b). In addition, several molecular markers particularly linked to the B-genome chromosome carrying blackleg resistance have already been analysed in interspecific hybrids (Chèvre et al. 1997; Barret et al. 1998; Plieske et al. 1998; Plieske and Struss 2001; Saal and Struss 2005; Chèvre et al. 2008).

2.8 Major objectives of the study

As we know blackleg or stem canker, caused by the fungus *L. maculans* has a great economic impact in the canola industry worldwide (Fitt et al. 2008), stressing a need to manage the disease. By this time, a number of investigations have been done to manage blackleg disease in canola, but effective management depends on the understanding of the interaction between host and pathogen (Kutcher et al. 2010a). *L. maculans* has shown the evolutionary ability to loose *Avr* genes and gaining virulence to *B. napus* as was seen with the emergence of new races in western Canada (Chen and Fernando 2006; Kutcher et al. 2007). Currently, almost all existing cultivars in western Canada carry the single *R* gene *Rlm3* (Zhang et al. 2016), but single dominant *R* genes may not offer a durable resistance. The breakdown of single *R* genes has been seen in Australia (Sprague et al. 2006), Canada (Zhang et al. 2016), and Europe (Rouxel et al. 2003a), indicating the need for proper management strategies of host resistance. However, management of host resistance requires an integrated approach such as the use of resistant cultivars (Marcroft et al. 2004a; Kutcher et al. 2013), crop rotation (Kutcher et al. 2013), rearrangement of resistant cultivars (Marcroft et al. 2012a), gene pyramiding (Djian-Caporalino et al. 2014), combination of qualitative resistance into quantitative background (Brun et. al. 2010), stubble management and minimal use of fungicides (Kutcher et al. 2011). Therefore, this study has been undertaken to explore the strategies of improving *Brassica napus* resistance to *L. maculans*, a causal agent of blackleg disease with the following objectives.

- Development of molecular markers SCAR and CAPS linked to the blackleg resistance gene *Rlm6* and characterize their inheritance pattern in F_2 and F_3 generations derived from *B. napus* x *B. juncea*.
- Development of backcross (BC) populations harbouring *Rlm6* which can be employed in variety development programmes.
- Investigation of effectiveness of single *R* gene in a 2-year canola-wheat crop rotation over 4-year field study.
- Investigation of impact of *B. napus-L. maculans* interaction in the emergence of virulent isolates of *L. maculans* in a canola-wheat 2-year crop rotation over 4-year field study.

DEVELOPMENT OF MOLECULAR MARKERS LINKED TO THE *LEPTOSPHAERIA MACULANS* RESISTANCE GENE *RLM6* AND INHERITANCE OF SCAR AND CAPS MARKERS IN *B. NAPUS* X *B. JUNCEA* INTERSPECIFIC HYBRIDS

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3 DEVELOPMENT OF MOLECULAR MARKERS LINKED TO THE *LEPTOSPHAERIA MACULANS* RESISTANCE GENE RLM6 AND INHERITANCE OF SCAR AND CAPS MARKERS IN *B. NAPUS* X *B. JUNCEA* INTERSPECIFIC HYBRIDS

3.1 Abstract

Leptosphaeria maculans causes blackleg disease on *Brassica napus* an economically important oilseed crop. *B. juncea*, has high resistance to blackleg and is a source for the development of resistant *B. napus* varieties. To transfer the *Rlm6* resistance gene from *B. juncea* into *B. napus*, an interspecific cross between *B. napus* 'Topas DH16516' and *B. juncea* 'Forge' was produced, followed by the development of F_2 and F_3 generations. SCAR and CAPS markers linked to the *L. maculans* resistance gene *Rlm6* were developed. Segregation of SCAR and CAPS markers linked to *Rlm6* were confirmed by genotyping of F_2 and F_3 progeny. Segregation of CAPS markers and phenotypes for blackleg disease severity in F_2 plants had a Mendelian ratio of 3:1 in resistant versus susceptible plants, respectively, supporting the assumption that genetic control of resistance was by a single dominant gene. The molecular markers developed in this study, which show linkage with the *L. maculans* resistance gene *Rlm6*, would facilitate marker-assisted backcross breeding in a variety development programmme.

Key words: *Brassica species*, blackleg, *R* gene *Rlm6*, *Leptosphaeria maculans*, CAPS marker, Inheritance

3.2 Introduction

Canola (oilseed rape, *Brassica napus*) is grown extensively in Australia, Europe, North America and Asia. The crop is susceptible to several major pathogens including *Leptosphaeria maculans*, a causal agent of blackleg disease, which is responsible for severe yield losses worldwide (Fitt et al. 2006). Blackleg disease has mainly been controlled by the utilisation of host resistance, crop rotations and fungicide applications (Marcroft et al. 2012a;

Marcroft et al. 2012b; Kutcher et al. 2013). A gene-for-gene interaction between the avirulence (*Avr*) genes in *L. maculans* and the corresponding resistance (*R*) genes in *B. napus*, which determines resistance to the disease, has been reported (Rouxel et al. 2003b; Marcroft et al. 2012a). *R* gene-mediated resistance is often very effective in disease control, while the fungal populations carry a high frequency of the corresponding *Avr* gene (Daverdin et al. 2012). Eighteen *R* genes for resistance to *L. maculans*, *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2*, have been identified in *Brassica* species (Raman et al. 2013). Among them, *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* were identified in *B. napus* (Ansan-Melayah et al. 1998; Balesdent et al. 2001, 2002; Delourme et al. 2004; Van de Wouw et al. 2008); *Rlm5* and *Rlm6* in *B. juncea* (Rimmer and van den Berg 1992; Chèvre et al. 1997, 2008; Balesdent et al. 2002; Christianson et al. 2006); *Rlm8* and *Rlm11* in *B. rapa* (Balesdent et al. 2002, 2013); *Rlm10* in *B. nigra* (Chèvre et al. 1996; Delourme et al. 2011); and five resistance genes, *LepR1*, *LepR2*, *LepR3*, *LepR4*, and *RlmS*, were introgressed from *B. rapa* subsp. *sylvestris* (Yu et al. 2005, 2008, 2013; Van de Wouw et al. 2009; Larkan et al. 2013) and the remaining two genes *BLMR1* and *BLMR2* were identified from *B. napus* cultivar Surpass400 (Long et al. 2011). Most resistance genes have been derived from A-genome species – either from *B. napus* or *B. rapa* – in a cultivar development program, whereas B-genome species are little exploited in the crop improvement programmes.

B-genome species *B. juncea* (AABB) derived from an inter-species hybridization, *B. nigra* (BB) x *B. rapa* (AA), whereas *B. napus* (AACC) developed from *B. oleracea* (CC) x *B. rapa* (AA). The A-genome species *B. rapa* is the common ancestor of *B. napus* and *B. juncea*, which makes an indirect genomic relationship with these two tetraploids revealed by the U triangle (Nagaharu 1935). Earlier studies reported unsuccessful introgression of blackleg resistance to *B. napus* from *B. juncea* (Prakash and Chopra 1988; Dixelius and Wahlberg 1999). Since *Brassica* species with the B-genome, i.e. *B. nigra*, *B. carinata* and *B.*

juncea, display complete resistance to *L. maculans* (Roy 1978, 1984; Sjodin and Glimelius 1988; Rimmer and van den Berg 1992), effort has continued and, recently, *B. napus* lines have been successfully developed with B-genome introgression from *B. juncea* (Brun et al. 2010) and *B. carinata* (Navabi et al. 2010). A recent study has shown a very high frequency of *AvrLm6* in *L. maculans* isolates from Canadian canola fields (Liban et al. 2016), emphasising the need to develop a canola cultivar with the corresponding *R* gene *Rlm6*. Previously developed *Rlm6* harbouring lines are unavailable for commercial breeding. Therefore, investigation into the introgression of B-genome resistance into *B. napus* is essential.

Inheritance of blackleg resistance derived from the B-genome species by a single dominant gene has been reported in earlier studies (Chèvre et al. 1997, 2008; Plieske et al. 1998), suggesting the potential for developing cultivars from interspecific crosses. Cultivar development can be accelerated by marker-assisted selection in backcross populations. Several molecular markers for blackleg resistance have already been reported from several B-genome resistance sources (Chèvre et al. 1997; Barret et al. 1998; Plieske et al. 1998; Plieske and Struss 2001; Saal and Struss 2005). Besides, inheritance studies have also been reported in *Brassica napus* resistance using SCAR markers linked to the locus *cLmR1* (Mayerhofer et al. 2005), and 'indel' markers linked to the locus *LepR3* (Larkan et al. 2013). Since there is no commercial *B. napus* cultivar carrying *Rlm6*, this study has been undertaken as part of an introgression study with the aim of understanding the pattern of inheritance of *Rlm6* in segregating populations derived from a cross between *B. napus* and *B. juncea*. Additionally, the development of SCAR and CAPS markers linked to the locus *Rlm6* (Balesdent et al. 2002; Chèvre et al. 2008) would facilitate marker-assisted backcross breeding in a cultivar breeding program.

3.3 Materials and Methods

3.3.1 Plant materials

The *B. juncea* cultivar 'Forge' was used as pollen donor, and was crossed with the susceptible *B. napus* cultivar 'Topas DH16516'. Resistant progenies were identified in each generation via phenotyping of *L. maculans* isolates carrying the avirulence gene *AvrLm6*, as well as marker genotyping. Only resistant seedlings (those showed hypersensitive responses to the pathogen) were transferred to plastic pot and grown under greenhouse conditions (22ºC and 12-h photoperiod). A SCAR marker linked to *Rlm6* was used to confirm introgression in interspecific hybrids followed by selfing towards next generation.

3.3.2 *Leptosphaeria maculans* isolates

A set of differential isolates of *L. maculans* previously characterised for avirulence genes were used to characterise the *B. juncea* cultivar 'Forge'. A total of 11 isolates (D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, Jn3 and J3), characterised by Leflon et al. (2007), Balesdent et al. (2005, 2013), Marcroft et al. (2012a), and Zhang et al. (2016), were collected from the lab. A single spore isolate, ICBN14 (*AvrLm5, AvrLm6, AvrLepR1*), was chosen for phenotyping interspecific hybrids derived from the *B. napus* x *B. juncea* cross.

3.3.3. Inoculum preparation and DNA extraction from *Leptosphaeria maculans*

Isolates were cultured, and the inoculum was prepared as described by Chen and Fernando (2006) with minor modifications. A paper disc infected with fungus was put on V8 agar medium, which was amended with 0.35% (*w/v*) streptomycine sulphate, and fungus was grown for two weeks at 20ºC. Harvested pycnidiospores were stored at -20ºC for further use. The concentration of spores was diluted to 2 x 10^7 spores mL^{-1} in each inoculation of the study. The CTAB method was followed to extract DNA from fungal pycnidiospores, according to Liban et al. (2016).

3.3.4 Characterization of *Rlm6* in the cultivar 'Forge'

A set of 11 differential isolates (D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, Jn3 and J3), in which 12 *R*-genes can be detected (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, RlmS, LepR1* and *LepR2*), were used to confirm *Rlm6* presence in the cultivar 'Forge'. Seeds were sown in a plastic cell tray using standard growth media and kept in a growth chamber at 21ºC/16ºC (day/night) with a 16-h photoperiod. The fully developed cotyledons of 7-day old seedlings were punctured with tweezers and inoculated with a 10-µL droplet (2×10^7 spores mL^{-1}) of inoculum (two inoculation sites per plant). Inoculated cotyledons were air dried before being placed back in the growth chamber and subsequently watered. Seedling infection was rated 14 days after inoculation using the rating scale of 0–9 (Williams and Delwiche 1979; Appendix III). The average rating score (ARS) was calculated from 12 seedlings (~24 inoculation sites), where ARS ≤4.5 was considered resistant (R) and ARS 4.6–9.0 susceptible (S).

3.3.5 DNA extraction from plant samples

Genomic DNA was extracted from fully developed cotyledons of 7–8-day old seedlings. DNA was extracted following the CTAB method as described by Doyle and Doyle (1990) with slight modifications. In brief, small leaf samples were ground for 2 min in 600 µL of CTAB buffer using a milling apparatus TissueLyser II (Qiagen, Toronto, Canada). The ground sample was incubated at 65ºC for 60 min, followed by adding 600 µL of chloroform and centrifuging for 10 min at 12,000 rpm. Approximately 500 µL of supernatant was pipetted into a fresh 1.5 mL Eppendorf tube. DNA was precipitated by adding isopropanol and washed with ice-cold 70% ethanol. DNA was diluted with milli-Q water and stored at -20ºC.

3.3.6 Marker development

All markers used in this study were PCR-based, taken either from the available literature (Saal and Struss 2005) or designed for this study. Resistant and susceptible parents were selected to make the pooled DNA. Two DNA pools were made containing DNA of either 10 resistant (R) or 10 susceptible (S) parents. Primarily, these pools (R-pool and S-pool) were used for marker development. Suitable restriction enzymes were used to detect the polymorphisms of cleaved amplified polymorphic sequence (CAPS) markers. Finally, the F_2 generation derived from a *B. napus* x *B. juncea* cross was used to confirm the marker efficacy in discriminating resistant and susceptible alleles.

3.3.7 PCR analysis

The PCR amplification reaction contained 50 ng template DNA, 2.5 µL dreamTaq buffer (10x), 2.5 µL of dNTPs (0.2 µM of each dNTP), 0.25 µL of each of the forward and reverse primers (10x), 0.1 µL of dreamTaq DNA polymerase (5 U/µL) and rest of the milli-Q water in a total reaction volume of 20 µL. All PCR amplification was performed according to standard protocols in either an Applied Biosystems GeneAmp 2700 system or an Eppendorf Mastercycler®pro for 35 cycles, each consisting of 30 s at denaturing temperature 94ºC, 30 s at annealing temperature 53ºC, and 1 min at extension temperature 72ºC. PCR products were stained with loading dye, separated on either 1% or 1.5% agarose electrophoretic gels in 1x TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and clear bands were visualised under ultraviolet light. For CAPS markers, 10 µL of PCR product was digested with 10 µL of the appropriate restriction enzyme mixture: 7 µL milli-Q or 5 µL milli-Q plus 2 µL SAM, 2 µL cut smart buffer and 1 µL specific enzyme either *Bsg*I (5 u/µL) or *Hae*III (10 u/µL) (New England *BioLabs* Inc. Ipswich, Massachusetts USA) in a total volume of 20 µL for 3 h at 37ºC. Digested DNA fragments were separated on a 1.5% agarose gel and visualised under ultraviolet light.

For multiplex PCR, DNA from eleven *L. maculans* isolates; D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, JN3, and J3 were used to amplify avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* (Van de Wouw et al. 2014; Fudal et al. 2007). The PCR amplification reaction contained 50 ng template DNA, 2.5 µL GeneDireX buffer (10x), 2.0 µL of dNTPs (0.2 µM of each dNTP), 0.25 µL of each of the forward and reverse primers (10x) link to the genes *AvrLm5/AvrLmJ1* and *AvrLm6*, 0.25 µL of GeneDireX Taq DNA polymerase (5 U/µL) and rest of the milli-Q water in a total reaction volume of 20 µL. Protocol for the PCR amplification, and gel documentation same as described above.

3.3.8 Phenotyping for blackleg resistance

A pot experiment was conducted, using the F_2 generation, at the Department of Plant Science University of Manitoba, under greenhouse conditions (22ºC and 12-h photoperiod). Seeds were directly sown into plastic pots, and two seedlings per pot were allowed to grow until maturity. Cotyledons of 7–8-day old seedlings were inoculated with the *L. maculans* isolate ICBN14 as previously described. Infected plants were grown to maturity and evaluated for their blackleg resistance by estimating the percentage of a blackening area from a cross-sectional stem using a scale of 0–5 (West et al. 2002; Appendix IV):

0 – no visible infection,

1 – disease tissue occupies ≤25% of the cross section,

2 - disease tissue occupies 25–50% of the cross section,

3 – 50–75% of the cross section infected,

4 – more than 75% of the cross section infected, but plant alive, and

5 – 100% of cross section infected and plant dead.

3.3.9 Statistical analysis

A χ^2 goodness-of-fit test was performed to assess the segregation ratios of the CAPS marker for conformity with the expected ratio of 1:2:1. This was also conducted for the segregation

ratio of the phenotypes, resistant versus susceptible, of the F_2 progeny for conformity with the expected ratio of 3:1. The marker genotypes were tested for significant associations with disease severity using SAS version 19 (SAS Institute, Cary NC USA). A *t*-test was performed to compare the mean disease severity of resistant, susceptible and heterozygous groups of the F_2 progeny.

3.4 Results

3.4.1 Confirmation of *AvrLm5/AvrLmJ1* and *AvrLm6* in *Leptosphaeria maculans* isolates

The avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* were amplified via a multiplex PCR from fungal DNA of *L. maculans* isolates including D4, D7, D10, ICBN14, PHW1223, JN3, and J3 (Fig. 3.1; Fudal et al. 2007; Van de Wouw et al. 2014). The avirulence gene *AvrLm5/AvrLmJ1* was amplified in the isolates D3 and R2, whereas the avirulence gene *AvrLm6* was amplified for a single isolate AD746, and neither of these genes were amplified in the isolate D5 (Fig. 3.1). This result confirmed that the isolates D4, D7, D10, ICBN14, PHW1223, JN3, and J3 carried both avirulent genes *AvrLm5/AvrLmJ1* and *AvrLm6*. On the other hand, isolates D3 and R2 carried avirulence gene *AvrLm5* but no *AvrLm6*. A single isolate AD746 carried avirulence gene *AvrLm6* but no *AvrLm5*, and both genes were absent in the isolate D5 (Fig. 3.1).

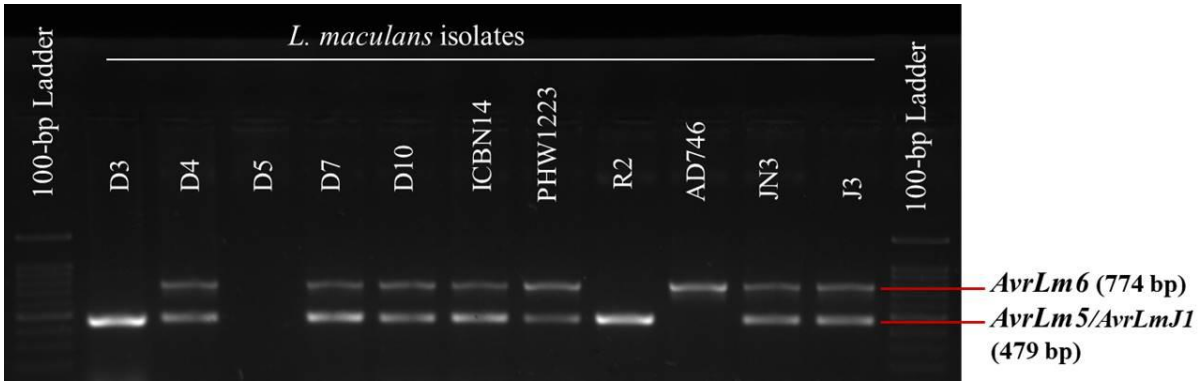

Fig. 3. 1 Amplification of avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* from *Leptosphaeria maculans* isolates used for the phenotype of *Brassica juncea* cultivar 'Forge'.

3.4.2 Confirmation of *Rlm6* in the cultivar 'Forge'

The *Brassica juncea* cultivar 'Forge', which has been predicted to carry a single dominant gene, *Rlm6,* was inoculated by the previously genotyped set of differential isolates of *L. maculans* (Zhang et al. 2016; Fig. 3.1; Table 3.1). The *B. napus* cultivar 'Topas DH16516' was also included in the inoculation study as a control. *B. napus* 'Topas DH16516' was susceptible to all *L. maculans* isolates tested. As a result, no *R*-gene was detected in the cultivar 'Topas DH16516' (Table 3.1). The *B. juncea* cultivar 'Forge' was susceptible to the *L. maculans* isolates D3 (*AvrLm5, AvrLepR1*), D5 (*AvrLm1, 2, 4, 7, S, AvrLepR1, AvrLepR2*), and R2 (*AvrLm5, 7, AvrLepR1*), which indicated that the cultivar 'Forge' did not carry the genes *Rlm1, Rlm2, Rlm4, Rlm5, Rlm7, RlmS, AvrLepR1,* and *AvrLepR2*. On the other hand, the *B. juncea* cultivar 'Forge' was resistant to the *L. maculans* isolates D4 (*AvrLm4, 5, 6, 7, 8, AvrLepR1, AvrLepR2*), D7 (*AvrLm1, 3, 5, 6, 8, AvrLepR1*), D10 (*AvrLm5, 6, 8, 9, S*), ICBN14 (*AvrLm5, 6, AvrLepR1*), PHW1223 (*AvrLm5, 6, 8, 9*), AD746 (*AvrLm3, 6, AvrLepR1*), JN3 (*AvrLm1, 4, 5, 6, 7, 8*), and J3 (*AvrLm2, 3, 5, 6, S*), which indicated that the genes *Rlm3, Rlm6, Rlm8,* and *Rlm9* could be present in the cultivar. Unfortunately, we did not have an appropriate isolate to determine whether *Rlm3, Rlm8,* and *Rlm9* were in fact present in the cultivar 'Forge', but these have been identified previously: the genes *Rlm3* and *Rlm9* were identified in *B. napus* (Balesdent et al. 2002; Delourme et al. 2004) and *Rlm8* was identified

in *B. rapa* (Balesdent et al. 2002). Therefore, based on the available literature we made a scientific assessment that the genes *Rlm3, Rlm8,* and *Rlm9* were not carried by the *B. juncea* cultivar 'Forge'. However, the *B. juncea* cultivar 'Forge' was inoculated again by the *L. maculans* isolates D5, R2, and ICBN14 (Fig. 3.2) those were checked via multiplex PCR for the confirmation of *AvrLm5,* and *AvrLm6* in the pathogen profile (Fig. 3.1). The cotyledon inoculation test showed that the cultivar 'Forge' was susceptible to the isolates D5 (*AvrLm1, 2, 4, 7, S, AvrLepR1, AvrLepR2*) and R2 (*AvrLm5, 7, AvrLepR1*), in which there was an absence of *AvrLm5, 6* and presence of *AvrLm5,* respectively (Fig. 3.2 A and B). The compatible interaction between 'Forge' and the *L. maculans* isolates D5 and R2 indicated that the 'Forge' cultivar did not carry the gene *Rlm5*. On the other hand, the 'Forge' cultivar showed a small dark lesion, typical of a hypersensitive response (HR), to the isolate ICBN14 (*AvrLm5, 6, AvrLepR1*) (Fig. 3.2C) as a result of the gene-for-gene interaction (Flor 1971), confirming that the cultivar 'Forge' must carry a single dominant gene, *Rlm6*. Based on the results from differential testing (Table 3.1), and information from available literature, we could deduce that *Rlm6* was present in the cultivar 'Forge'.

Table 3. 1 Reaction of *Brassica juncea* cultivar 'Forge' to a set of differential isolates of *Leptosphaeria maculans*

Isolates	*Avr* genotypes	*B. napus* (Topas DH16516)	*B. juncea* (Forge)
D3	*AvrLm5, AvrLepR1*	S	S
D4	*AvrLm4, 5, 6, 7, 8, AvrLepR1, AvrLepR2*	S	R
D5	*AvrLm1, 2, 4, 7, S, AvrLepR1, AvrLepR2*	S	S
D7	*AvrLm1, 3, 5, 6, 8, AvrLepR1*	S	R
D10	*AvrLm5, 6, 8, 9, S*	S	R
ICBN14	*AvrLm5, 6, AvrLepR1*	S	R
PHW1223	*AvrLm5, 6, 8, 9*	S	R
R2	*AvrLm5, 7, AvrLepR1*	S	S
AD746	*AvrLm3, 6, AvrLepR1*	S	R
JN3	*AvrLm1, 4, 5, 6, 7, 8*	S	R
J3	*AvrLm2, 3, 5, 6, S*	S	R
Predicted *Rlm* gene		-	*Rlm6*

R and S indicate resistance (~24 cotyledon lesion score) and susceptible (~24 cotyledon lesion score) reaction.



Fig. 3. 2 The gene-for-gene interaction between *Brassica* species and *Leptosphaeria maculans*. The *B. juncea* cultivar 'Forge' showed susceptibility to the *L. maculans* isolates D5 (*AvrLm1*, *AvrLm2*, *AvrLm4*, *AvrLm7*, *AvrLmS*, *AvrLepR1*, *AvrLepR2*) and R2 (*AvrLm5*, *AvrLm7*, AvrLepR1) at 14 days post inoculation (A & B), whereas hypersensitive response to isolates ICBN14 (*AvrLm5*, *AvrLm6*, *AvrLepR1*) (C).

3.4.3 Marker development

The primer sequences listed in Table 3.2 were either collected from available literature (Saal and Struss 2005) or developed for this study. B5-1520 is a SCAR marker, which has a dominant pattern of inheritance, and was developed by Saal and Struss (2005). The primer B5-1520 was originally converted from the amplicon region present in the B-genome species. PCR amplification of the SCAR marker (B5-1520) in the DNA pool, as well as in the F_2 (derived from a cross between *B. napus* and *B. juncea*) generation showed that the band was only present in the resistance genotypes, as described by Saal and Struss (2005) (Fig. 3.3). Variations were identified from the sequences of PCR product between the resistant parental line and the F_1 plant, and new primers were then designed from the sequence of F_1 progeny. The PCR amplification of a new primer B5Rlm6_1 showed a polymorphism in the resistance allele, producing fragments of approximately 650 bp (Fig. 3.3). The sequences of the PCR product of the resistant allele, amplified by the primer B5Rlm6_1, were blasted and resulted in hits in scaffolds ChrUn_random: 36607925-36608309 in *B. napus* and ChrB01: 17530537-17531568 in *B. juncea*. Two primers BnHZ_2 and BjHZ_1 were designed from the blasted

sequence in the B5Rlm6_1 amplicon of gibberellin 2-oxidase gene region. The primer pairs for amplicon BnHZ_2 and BjHZ_1 were yielded to the fragments of 600 and 550 bp, respectively, from both resistant and susceptible individuals. The PCR product amplified by the primers BnHZ_2 and BjHZ_1 were sequenced (Table S3.1) to select specific restriction enzymes (RE) *Bsg*I and *Hae*III to derive codominant CAPS markers (Table 3.2). Both marker assays provided resistance-linked fragments as expected; around 450–550 bp for BjHZ_1 and 300–450 bp for BnHZ_2 (Fig. 3.3; Table 3.2).

Table 3. 2 Sequence characterize amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers used in this study

Primer ID	Primer Sequence (5'–3')[a]	Type	RE[b]	Fragment size (bp[c])	Annealing Temp. (ºC)	Sources
B5-1520	F-TGCCTTTCTCACTTCTTCTCTC R-AGCGTCTATGTCGGTCTTTCAA	Dominant	-	1520	53	Saal and Struss (2005)
B5Rlm6_1	F-GTTACAGAGGGTTGTATCTCATTC R-ACCAGGAGTGGTTAGAAGCTAAT	Dominant	-	650	53	This study
BjHZ_1	F-CCAACCCTTCGAGGTCAATA R-CCAGAGACCCCAGTTAAGCA	Codominant	*Hae*III	540, 450	55	This study
BnHZ_2	F-TCCATGATGTGATAACTATAGACG R-TTAAAGTTTGTGAATTTCTTCCTT	Codominant	*Bsg*I	450, 300	55	This study

[a]F, Forward; R, reverse; [b]RE, restriction enzyme; [c]bp, base pair

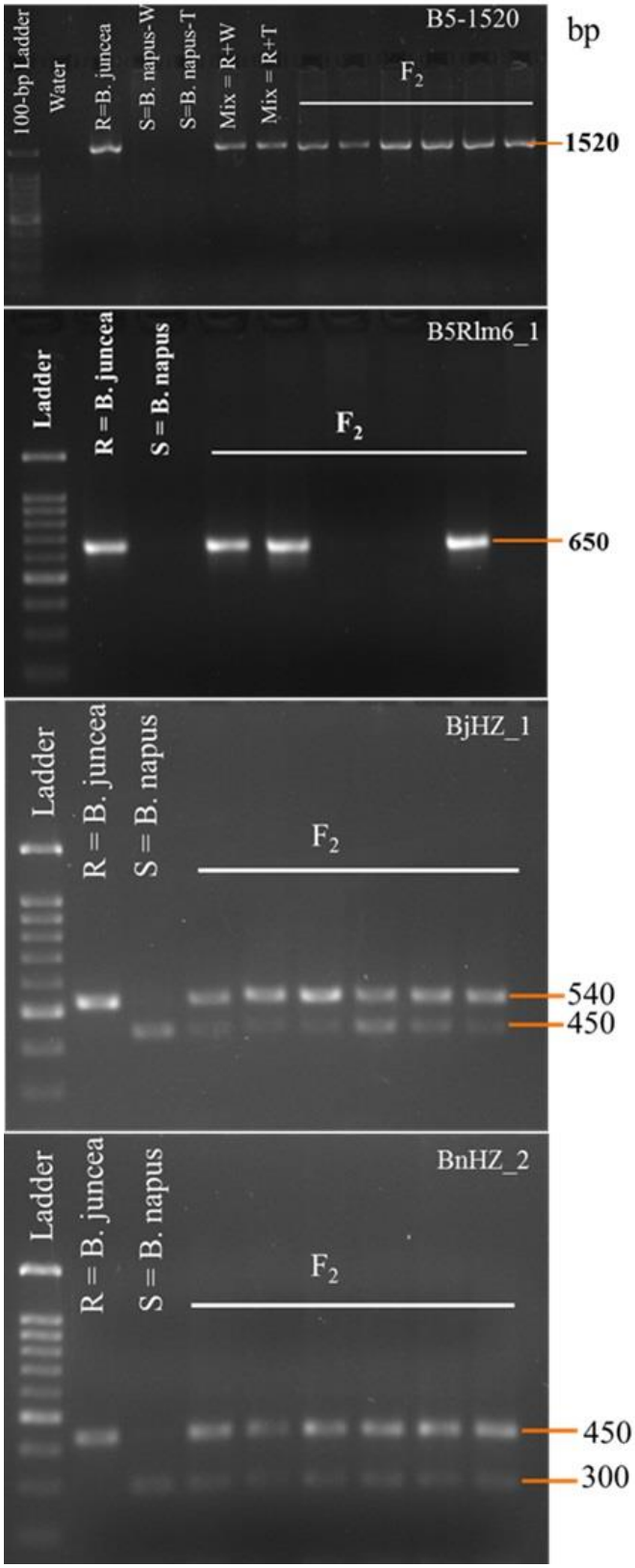

Fig. 3. 3 DNA markers linked to Rlm6 showed polymorphism in the F_2 generation. The SCAR markers B5-1520 and B5Rlm6_1 showed a dominant pattern of polymorphism, whereas CAPS markers BjHZ_1 and BnHZ_2 showed codominant polymorphism. R and S refer to resistance and susceptible, respectively.

3.4.4 Interspecific hybridization, introgression and generation development

Approximately 12 seedlings from each generation were inoculated with the *L. maculans* isolate ICBN14 (*AvrLm5, AvrLm6, AvrLepR1*) for their resistance reaction. Seedlings that showed a hypersensitive reaction to the pathogen were transplanted to plastic pots and grown under greenhouse conditions (22ºC and 12-h photoperiod). In addition, DNA was extracted from the seedlings and was genotyped for the SCAR marker B5-1520. A dominant pattern of inheritance of this marker was yielded, indicating the presence of B-genome region containing the marker fragment in F_1 (Fig. 3.4A). Two seedlings (2 and 3) of 10 F_1 seedlings did not show amplification of the B5-1520 marker, but the remaining seedlings had identical amplification to that described by Saal and Struss (2005), confirming the presence of the B-genome. Introgressed eight F_1 seedlings were selfed to develop the F_2 generation. Similarly, the F_3 generation was also developed from the introgressed F_2 plants (Fig. 3.4B). Three plants (1, 5 and 9) of 9 F_2 showed PCR amplification of B5-1520, indicating that B-genome region containing the marker fragment exist in F_2, but indicating marker distortion, as it did not support the Mendelian segregation ratio of 3:1.

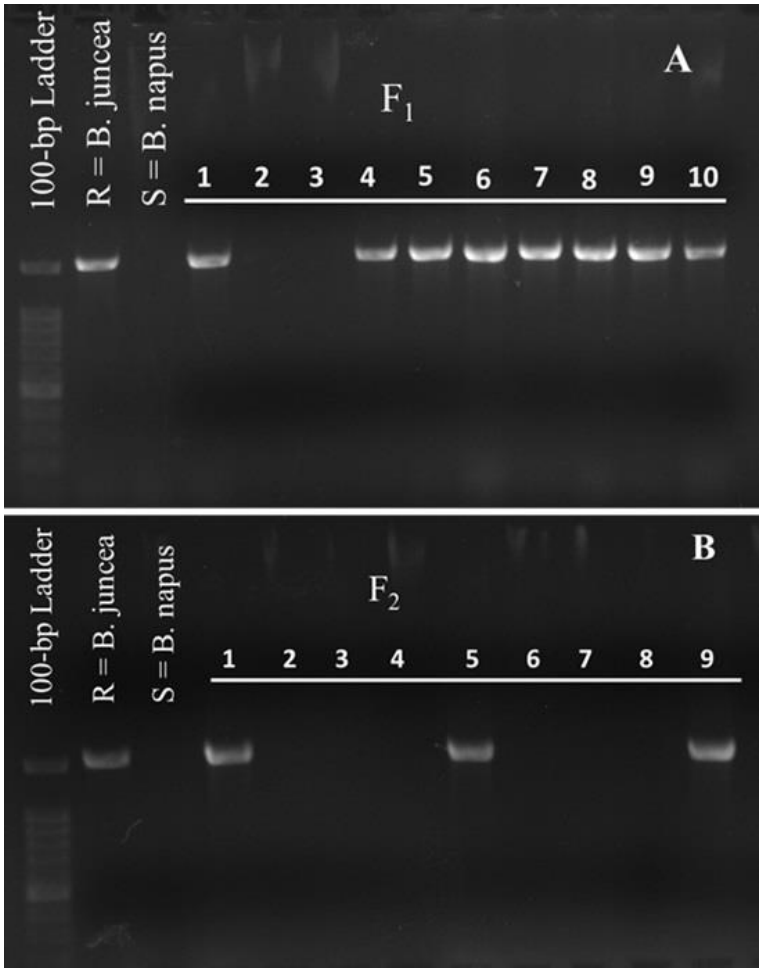

Fig. 3. 4 Gel image showed the confirmation of *Rlm6* introgression in *Brassica napus* x *B. juncea* interspecific hybrids. The SCAR marker B5-1520 yielded a dominant pattern of polymorphism in the F_1 (A), and F_2 (B) generations derived from *Brassica napus* x *Brassica juncea*.

3.4.5 Inheritance of the blackleg resistance gene *Rlm6*

The F_2 and F_3 progeny segregated for the SCAR marker B5Rlm6_1, linked to the *L. maculans* resistance gene *Rlm6*. This was further confirmed by PCR genotyping. Genotype data showed that 52% of F_2 and 28% of the F_3 progeny carried *Rlm6* (Table 3.3). The χ^2 test for a Mendelian segregation of the SCAR marker in the F_2 and F_3 generations showed a significant discrepancy between the observed and expected values ($p < 0.0001$; Table 3.3). However, blackleg disease severity for 142 F_2 plants fit to a Mendelian ratio of 3:1 in resistant versus susceptible plants as expected for the segregation of a single dominant gene (Table 3.4). All susceptible plants (n=36) showed severe disease symptoms, including stem

blackening, the presence of basal cankers and death (Table 3.4). No symptoms were observed on 32 plants of 142 with disease severity 0 (Table 3.4). Seventy two plants showed mild symptoms with disease severity ratings of 1 or 2, not as healthy as control plants (Table 3.4). CAPS marker analysis revealed that 41 plants of 142 were homozygous for the introgression of *Rlm6* from *B. juncea*, 36 were homozygous for the susceptible allele, and the remaining plants (65) were heterozygous for both alleles (Table 3.5), supporting an acceptable fit to a 1:2:1 ratio of resistant : heterozygous : susceptible. Exceptionally, two plants from the heterozygous group (heterozygote for CAPS marker BjHZ_1) were susceptible, as their disease severity was 3 (Table 3.4). Overall, in this study, the χ^2 independence test ($p \leq 0.5014$) for segregation of the phenotypes showed a significant association between the genotypes and phenotypes (Table 3.4).

Table 3. 3 Segregation of the sequence characterize amplified region (SCAR) marker B5Rlm6_1 in F_2 and F_3 generations derived from *Brassica napus* x *Brassica juncea*

Generations	Number of plants genotyped	Resistance		Susceptible		χ^2	*P*
		Observed	Expected	Observed	Expected		
F_2	192	99	144	93	48	56.250	0.0001
F_3	192	53	128	139	64	58.786	0.0001

Table 3. 4 A $\chi 2$ test for the segregation of blackleg resistance gene *Rlm6* in the F_2 progeny derived from *Brassica napus* x *Brassica juncea*

Plant Categories		Disease rating[Z]						Total	χ^2 (2)	*P*
		0	1	2	3	4	5			
Genotype of BjHZ_1[Y]	S	0	0	0	15	11	10	36		
	R	11	22	8	0	0	0	41		
	H	21	22	20	2	0	0	65		
Total		32	44	28	17	11	10	142	1.3806	0.5014

[z]Plants noted as 0 to 2 were considered resistant, whereas plants greater than 2 were susceptible. [Y]S = homozygous for the allele *B. napus*; R = homozygous for the allele *B. juncea*; and H = heterozygous. Noted: χ^2 value was adapted from Likelihood Ratio.

Plants that were either heterozygous or homozygous for the *B. juncea* introgression carrying the gene *Rlm6* showed a high level of resistance to *L. maculans*, ranging from 0 to 2 on the 0–5 scale. The disease severity for the heterozygotes with a mean disease severity of 1.05 was insignificantly higher than that of the homozygotes, with a mean disease severity of 0.93 (Table 3.5). Conversely, homozygous plants for the *B. napus* genome showed severe blackleg disease symptoms; ranging from 3 to 5 with a mean disease severity of 3.89 (Table 3.5), which was significantly higher than the *Rlm6* introgressed plants. Therefore, the results found in this study agreed with the interpretation of resistance, i.e. an acceptable fit to a Mendelian ratio of 3:1 in resistant versus susceptible plants.

Table 3. 5 Blackleg disease severity of the F_2 plants as grouped by their genotypes for a cleaved amplified polymorphic sequence (CAPS) marker linked to *Rlm6* gene

Marker- BjHZ_1[Z]	Number observed	Disease rating[y]	
		Mean	Range
Susceptible (S)	36	3.89 b[X]	3-5
Resistant (R)	41	0.93 a	0-2
Heterozygous (H)	65	1.05 a	0-2
Total	142	1.95	0-5

[y]Disease rating was conducted at maturity stage immediately prior to harvesting. [z]S = homozygous for the allele *B. napus*; R = homozygous for the allele *B. juncea*; and H = heterozygous. [x]Means in column with the same letter are not significantly different ($p \leq 0.0001$, *t*-test).

3.5 Discussion

In this study, we showed that major gene resistance was conferred by a single gene in the *L. maculans–B. juncea* interaction, which appeared to be expressed in cotyledons. The cultivar 'Forge', which carries monogenic resistance conferred by a single resistance gene, was characterised here. In this case, all plants were resistant to the isolate ICBN14 (*AvrLm5, 6, AvrLepR1)* at the cotyledon stage (Table 3.1), indicating seedling resistance as a result of a race-specific 'gene-for-gene' interaction (Flor 1971; Balesdent et al. 2005). Although the *L. maculans* resistance genes *Rlm5* and *Rlm6* have been identified in *B. juncea* in previous

studies (Rimmer and van den Berg 1992; Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006), our data (Table 3.1 and Fig. 3.3) revealed that a single gene, *Rlm6*, is involved in controlling blackleg resistance in the *B. juncea* cultivar 'Forge'.

Resistance, explained by previous studies in *Brassica*, is either monogenic or polygenic, indicating a complexity regarding the expression of resistance genes (Ferreira et al. 1995; Ballinger and Salisbury 1996; Pang and Halloran 1996; Chèvre et al. 1997; Pilet et al. 1998; Elliott et al. 2016). In this study, almost all the F_1 plants showed hypersensitive responses to the *L. maculans* isolate ICBN14 at the cotyledon stage, indicating a race-specific interaction (Flor 1971; Balesdent et al. 2005). In addition, genotyping of the SCAR marker B5-1520 supported that *Rlm6* was likley prsent in the F_1 generation (Fig. 3.4A). Segregation of the SCAR marker B5Rlm6_1 in the F_2 and F_3 generations did not fit to a 3:1 or 2:1 ratio of resistant versus susceptible alleles, respectively, indicating marker skewedness in the F_2 and F_3 generations (Table 3.3). Since the SCAR markers used in this study were dominant, it is unknown whether the frequency of homozygotes or heterozygotes was reduced. Interestingly, the SCAR markers used here were strongly associated with each other and to the locus *Rlm6*. Saal and Struss (2005) conducted a study on the introgression of blackleg resistance from *B. nigra*, *B. juncea* and *B. carinata* to *B. napus*, in which they observed a similar pattern of dominant marker distortion. Plieske and Struss (2001) also found segregation distortion for restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) markers linked to a blackleg resistance gene derived from the B-genome species. Importantly, in this study, segregation of the derived CAPS marker in the F_2 generation had an acceptable fit to a 1:2:1 ratio of resistant versus heterozygous versus susceptible alleles, supporting the genetic control of resistance by a single dominant gene (Table 3.5). A similar pattern of segregation (1:2:1) was observed in a previous study, where a codominant marker derived from a resistant gene analog (RGA) linked to *L. maculans* in an

interspecific hybrid was genotyped (Saal and Struss 2005). Exceptionally, two plants from the heterozygous group displayed moderate symptoms with disease severity ratings of 3 (Table 3.4). This disease severity rating (3) was higher than expected (>2). This could be due to an environmental effect and/or plant characteristics, because these plants grew somewhat slower compared to disease progression in the plant, and leaves were also infected by powdery mildew. Moreover, the F_2 generation was also genotyped for another CAPS marker BnHZ_2 (data not shown), and there was no recombinant identified from 142 individuals for the introgressed region. The CAPS markers developed and genotyped here could be located close to each other, or be in the same locus, which usually limits recombination. Chromosomal rearrangement during introgression could be another plausible explanation of recombination suppression (Chèvre et al. 1997; Yang et al. 2014). However, our study revealed monogenic inheritance of CAPS markers linked to the *L. maculans* resistance gene *Rlm6* in the interspecific hybrids derived from *B. napus* x *B. juncea*. A similar pattern of inheritance was also reported in many *B. napus* cultivars by previous studies (Dion et al. 1995; Ferreira et al. 1995; Mayerhofer et al. 1997; Rimmer et al. 1999; Raman et al. 2012b).

In order to clarify the reason for segregation distortion, both the trait locus and SCAR markers could be analysed further in different populations, such as double haploid, backcross or near isogenic lines, to see whether the similar trend of marker skewedness would occur. This will be the focus for future study in addition to development of more codominant markers in the introgression region. Moreover, study towards fine mapping could also be possible by screening different mapping populations with the molecular markers developed here. It remains unclear whether the recombination is originating from the homeologous position of the *B. napus* genome or from the loss of introgression segments. We believe the latter case did not occur here, as recombinant individuals did not lose their vigor. In summary, based on the study of phenotype and genotype in *B. napus* x *B. juncea* interspecific

hybrids, we demonstrated that the B-genome species *B. juncea* carries a dominant gene *Rlm6*, conferring resistance to the blackleg pathogen *L. maculans*. Additionally, the molecular markers developed here linked to *L. maculans* resistance would accelerate marker-assisted backcross breeding in the future.

3.6 Acknowledgements

We thank Paula Parks, Rob Visser, and Cathy Bay for their help in the greenhouse study and to SaskCanola and AAFC-Growing Forward 2 programs and the NSERC for funding. We also acknowledge Hossein Borhan (Agriculture and Agri-Food Canada, Saskatoon) for providing seed of *B. napus* cultivar 'Topas DH16516'.

Table S3.1 DNA sequence of PCR product amplified by the primers BjHZ_1 and BnHZ_2.

Primer ID	DNA sequence
BjHZ_1	TCCAACCCCNNTCGAGGTCAATATGAGAATATTAAGTTGGAACTGTCAGG GGTTGGGGAATACCCCGACAGTTCGACATCTACAGGGAATACATGGTCAG TATTTCCCTGAGATAATATTCCTCAGTGAGACCAAGAGTAGAAGGTGTTAT TTGGAGTCTATAGTGGAGAAGATGGGGTTCCATGACGTGATAACTATAGA CGCCAGAGGAAAGAGTGGAGGTTTGGCAGTTATGTGGAAGAGTTCATGTA CGGTGGAGGTTTTGCAGGCAAATAAGAGGGTGATTGATTTGAAGGTACAG TGGCAGGATCAAATCTTTTTCCTTACATGCGTATATGGAGATCCTGTCAAG AGTAGAAGGGGAGAGGTATGGGAACGGATTTCACGGATTGGAGCAAATA GGAGGGGGGCTTGGATGCTGATAGGAGACTTTAATGAGCTGATTGATCAA TCTGAAAAGTCAGGAGGAGCAGTAAGGGAAGATCGAGAAGGACAAAAAT TCAAGCAGCTACTGCTTAACTGGGGNNCTCTGGA
BnHZ_2	NNTTTCCCAANAANGTGATAACTATAGACNCCATAGGAAAGAGTGGAGGT TTNGCAGTTATGTGGAAGAGTTCATGTACGGTGGAGGTTTTGCAGGCAAA TAAGAGGGTGATTGATTTGAAGGTGCAGTGGCAGGATCAAATTTTTTTCCT TACCTGTGTATATGGCGATCCGGTCAAGAGTAGAAGGGGAGAAATAGGGA ACGGATTTCACGGATTGGAGCAAATAGAAAGGGGGCTTGGATGCTGATAG GAGACTTTAATGAGCTGATTGATCAATCCGAAAAGTCAGGAGGAGCAGTA AGGGAAGATCGAGAAGGGCAAGAATTCAAGCAGCTACTGCTTAACTGGG GTCTCTGGGACATTAAATACAAGGGGAACCCGCTATCGTGGGCAGGTAAA AGAAACAACATGTTGTGGAAGTGCAGACTGGATAGAGCAGTAGCAAACC AGGAATGGGTGGAGGCTCACCCACAAGCAGCTACCTTCTACCTCCCAAGA GTTCAGTCGGATCACAATCCGATCATTACCTCCTTGGATGGGCATCAAAGG AAGANNNNNNNAATTTTTAAAAA

MOLECULAR AND PHENOTYPIC IDENTIFICATION OF B-GENOME INTROGRESSION LINKED TO *L. MACULANS* RESISTANT GENE *RLM6* IN *BRASSICA NAPUS* X *B. JUNCEA* INTERSPECIFIC HYBRIDS

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Author contributions: M. Harunur Rashid conducted this work as part of his PhD thesis. M. Harunur Rashid developed the materials, designed the experiment, carried out the experiment, analysed the data and wrote the manuscript. Georg Hausner edited the manuscript. Dr. Dilantha Fernando supervised the experiment and edited the manuscript. All authors read and approved the final manuscript before publication.

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4 MOLECULAR AND PHENOTYPIC IDENTIFICATION OF B-GENOME INTROGRESSION LINKED TO *LEPTOSPHAERIA MACULANS* RESISTANT GENE RLM6 IN *BRASSICA NAPUS* X *B. JUNCEA* INTERSPECIFIC HYBRIDS

4.1 Abstract

Blackleg is a devastating disease in canola worldwide, except in China, caused by the fungal pathogen *Leptosphaeria maculans*. The B-genome *Brassica* species were reported to have strong resistance to the blackleg pathogen *L. maculans*. Backcross (BC) generations, BC_1F_1 to BC_4F_1, were derived from a cross *B. napus* x *B. juncea*, where *B. napus* was recurrent parent. Phenotype of *L. maculans* isolate J20 showed that 49% of BC_1F_1, 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 plants were resistant to the isolate J20. Offspring from the interspecific hybridization were also analysed for the presence of dominant type SCAR markers detecting loci linked on the *B. juncea* genome. The plants with *B. juncea* introgression had a decrease in the presence of SCAR marker B5Rlm6_1 ranging from 47% in BC_1F_1 to 30% in BC_2F_1 and further down to 18% in BC_3F_1 and 11% in BC_4F_1 . A similar trend of loci reduction was also observed for the marker B5-1520. However, segregation of SCAR markers and phenotypes for the blackleg resistance in BC_1F_1 plants had an acceptable fit to a Mendelian ratio of resistant versus susceptible, supporting the assumption that the genetic control of resistance is governed by a single dominant gene. The BC generations developed in this study, which shows introgression of *B. juncea* genome linked to *L. maculans* resistance gene *Rlm6*, would facilitate breeding a *B. napus* variety resistant to blackleg in the future.

Key words: *Brassica* species, B-genome, Inheritance, *Leptosphaeria maculans*, *R*-gene *Rlm6*, SCAR markers

4.2 Introduction

Canola (rapeseed / *Brassica napus* L.) is an economically important oilseed crop in many countries including Australia, Canada, the European Union and China. It interacts with a few major pathogens including *Leptosphaeria maculans*, which is the causal agent of blackleg disease resulting in significant yield loss (Fitt et al. 2006). Genetic resistance is one of the most cost-effective and environment friendly strategies to control disease. In canola qualitative resistance often refer to *R* genes which are very effective in controlling blackleg disease, while the fungal pathogen *L. maculans* carries a high frequency of the corresponding *Avr* genes (Daverdin et al. 2012). A recent study showed a very high frequency of *AvrLm6* in *L. maculans* isolates from Canadian canola fields (Liban et al. 2016), indicating the necessity to develop canola cultivar with the corresponding *R* gene *Rlm6*. In addition, one of the goals of canola research programs is the stable introgression of novel resistance from wild or closely related species into elite cultivars through inter- and intra-specific crosses (Ky et al. 2000). So far *L. maculans* resistance (*R*) genes *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2*, have been identified in different *Brassica* species (Raman et al. 2013a). Among *Brassica* species; the B-genome species *B. juncea*, *B. carinata*, and *B. nigra* carry many valuable traits including blackleg resistance (Schelfhout et al. 2006). B-genome species *B. juncea* is reported to carry the blackleg resistance gene *Rlm6* (Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006).

However, *B. juncea* (2n=36, AABB) is derived from an inter-species hybridization between *B. nigra* (2n=16, BB) and *B. rapa* (2n=20, AA), whereas *B. napus* (2n=38, AACC) is derived from an inter-species hybridization between *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC) (Nagaharu 1935). The relationship among the *Brassica* species was revealed by an early cytogenetic study known as the U triangle. This study predicts an indirect genomic relationship between *B. napus* and *B. juncea* as revealed by the U triangle (Nagaharu 1935;

Fig. 4.1). Earlier studies attempted the introgression of blackleg resistance from *B. juncea* to *B. napus* but so far the trait has not been successfully introduced into commercial cultivars (Roy 1984; Prakash and Chopra 1988; Dixelius and Wahlberg 1999; Barret et al. 1998; Chèvre et al. 1997; Saal et al. 2004). Plausible explanation for the failure of introgression could be due to genome incompatibility between B- and A- or C-genome (Leflon et al. 2007). Recent studies reported successful introgression of B-genome into *B. napus* from *B. juncea* (Chèvre et al. 2008; Brun et al. 2010) and *B. carinata* (Navabi et al. 2010). Unfortunately, the introgressed lines are kept as a research tool indicating unavailability of the materials for commercial breeding. Therefore, further efforts for the introgression of the *B. juncea* genome link to blackleg resistant gene *Rlm6* to *B. napus* is essential.

In this study we successfully introgressed a part of the B-genome linked to *Rlm6*, a blackleg resistance gene from *B. juncea* to *B. napus* using an advanced backcross approach (Tanksley and Nelson 1996). More specifically, an interspecific cross *B. napus* x *B. juncea* was made to generate an F_1 hybrid. Later on, four rounds of backcrossing to *B. napus* were performed to develop B-genome-containing substitution lines. This resulted in the production of interspecific hybrids and BC_1F_1 to BC_4F_1 were phenotyped using the *L. maculans* isolate J20 which carries the corresponding avirulence gene *AvrLm6*. Further BC_1F_1 to BC_4F_1 were genotyped with the molecular markers B5Rlm6_1 and B5-1520 linked to the B-genome particularly to the blackleg resistant gene *Rlm6* (Rashid et al. 2018). We are able to demonstrate that the B-genome segment is introgressed into *B. napus*.

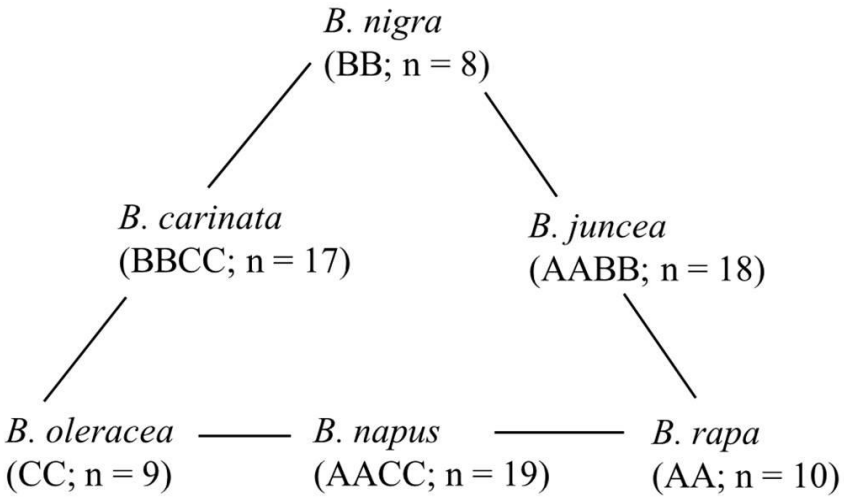
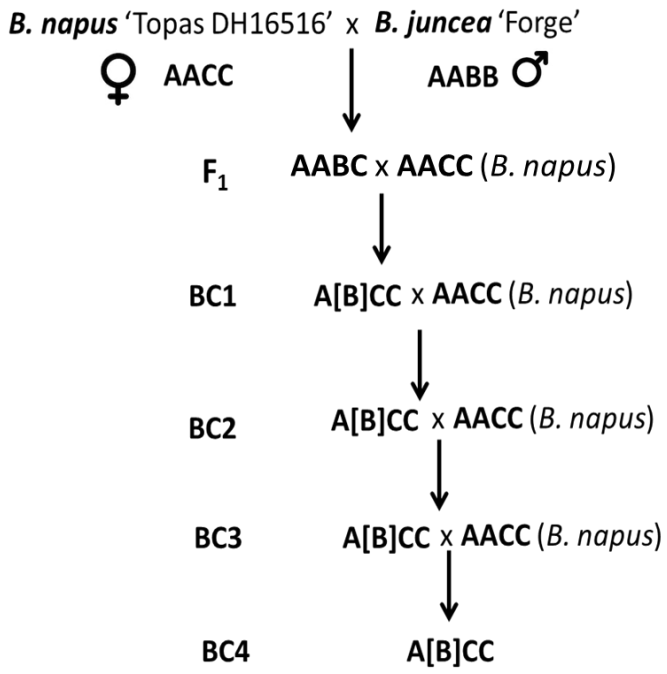


Fig. 4. 1 Relationship among the major *Brassica* species. Idea adapted from cytogenetic studies (Nagaharu 1935).

4.3 Materials and Methods

4.3.1 Plant materials

B. juncea cultivar 'Forge' was used as pollen donor, and crossed with the susceptible *B. napus* line 'Topas DH16516'. F_1's derived from the interspecific hybridization were backcrossed with the recurrent susceptible parent to develop backcross (BC) generations, including BC_1F_1, BC_2F_1, BC_3F_1, and BC_4F_1 (Fig. 4.2). All generations were grown under greenhouse condition at 22°C and 12-h photoperiod throughout the study period.



Pedigree and crosses used in the development of the backcross (BC) generations

Fig. 4. 2 Pedigree and crosses used in the development of the backcross (BC) generations for this study. Plant selection was performed in each generation for disease resistance based on the phenotype of *Leptosphaeria maculans* isolate J20, and the genotype of the SCAR marker B5Rlm6_1.

4.3.2 *Leptosphaeria maculans* isolates

The single spore derived isolate J20 (carried *AvrLm2, AvrLm3, AvrLm6, AvrLepR1*) was chosen for phenotyping interspecific hybrids derived from the cross *B. napus* line 'Topas DH16516' x *B. juncea* cultivar 'Forge'. The isolate J20 was previously characterized for the avirulence genes profile by Zhang et al. (2016).

4.3.3 Inoculum preparations from *Leptosphaeria maculans* isolate J20

Isolate was cultured and inoculum was prepared as described by Chen and Fernando (2006) with minor modifications. In brief, an infected paper disc was put on V8® agar medium which was amended with 0.35% (*w/v*) streptomycin sulfate, and the fungus was grown for 2

weeks at 20°C. Harvested pycnidiospores were stored at -20°C for further use. The spore concentrations were diluted to 2 x 10^7 spores' mL^{-1} each time to serve as inoculation.

4.3.4 Phenotype

Seeds were sown in a plastic cell tray using standard growing media, and kept in a growth chamber at 21°C/16°C (day/night) with a 16-h photoperiod. The fully developed cotyledons of 7-days old seedlings were punctured with a tweezer, and inoculated with a prepared 10-µL droplet (2 x 10^7 spores' mL^{-1}) of *L. maculans* inoculum (two inoculation sites per plant). Inoculated cotyledons were air dried before being returned to the growth chamber and watering. Seedlings were fertilized with water soluble fertilizer 20-20-20 (N-P_2O_5-K_2O) one day after inoculation. Infected cotyledons were rated 13-days after inoculation using a rating scale of 0-9 (Williams and Delwiche 1979; Appendix III). The average rating score (ARS) was calculated, where ARS ≤4.5 was considered resistant (R), and ARS 4.6-9.0 was interpreted as susceptible (S).

4.3.5 DNA extraction from plant samples

Genomic DNA was extracted from the first 1-2 true leaves of seedlings following the CTAB method as described by Doyle and Doyle (1990) with slight modifications. In brief, small leaf samples were ground for 2 min in 600 µL of CTAB buffer using a milling apparatus TissueLyser II (Qiagen, Toronto, Canada). The ground up sample was incubated at 65°C for 60 min followed by adding 600 µL of chloroform, and centrifuged for 12 min at 12000 rpm. Approximately 500 µL of supernatant was recovered and added to a 1.5 ml Eppendorf tube. DNA was precipitated by adding isopropanol, and the DNA pellet was washed with ice-cold 70% ethanol. The DNA pellet was dissolved in 150 µL milli-Q water, and stored at -20°C.

4.3.6 Markers used in genotype determination

PCR-based dominant type SCAR markers B5Rlm6_1 (F-GTTACAGAGGGTTGTATCTCATTC / R-ACCAGGAGTGGTTAGAAGCTAAT) and B5-

1520 (F-TGCCTTTCTCACTTCTTCTCTC / R-AGCGTCTATGTCGGTCTTTCAA) linked to blackleg resistance gene *Rlm6* were used to genotype the parents (Rashid et al. 2018), and all interspecific hybrids including F_1, BC_1F_1, BC_2F_1, BC_3F_1 and BC_4F_1.

4.3.7 PCR analysis

PCR amplification reaction contained ~50 ng template DNA, 2.5 µL of 10x reaction buffer, 2.5 µL of dNTPs (0.2 µM of each dNTP), 0.25 µL of each forward and reverse primer (1.0 µM of each primer), 0.1 µL of dreamTaq polymerase (5 u/µL) and 12.4 µL milli-Q H_20 in a total reaction volume of 20 µL. All PCR amplifications were performed according to standard protocols in either an Applied Biosystems GeneAmp 2700 system (Applied Biosystems, Thermo Fisher Scientific Inc. Canada) or an Eppendorf Mastercycler®pro (Eppendorf, Mississauga, Canada) for 35 cycles, each consisting of 30s at 94°C, 30s at annealing temperatures 53°C and 1 min at 72°C. PCR products were separated on 1.5% agarose gels resolved by electrophoresis in 1x TBE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0). Agarose gels were stained with loading dye (containing ethidium bromide 0.5 µg/ml), and visualized under ultraviolet light.

4.3.8 Statistical analysis

A $\chi 2$ goodness-of-fit test was performed to assess the segregation ratio of the phenotypes, resistant versus susceptible in BC progenies for the conformity of the expected ratio. Similar test was also performed to assess genotypic segregation ratios of the SCAR markers for the conformity of the expected ratio. For each backcross generation the percentage of retained SCAR marker loci was calculated using Microsoft Excel version 10.0. A simple t-test was performed to determine significance levels regarding differences among the generations. The $\chi 2$ goodness-of-fit test was performed using free online software accessed on November 10th 2017 (http://www.socscistatistics.com/tests/goodnessoffit/Default2.aspx).

4.4 Results

4.4.1 Confirmations of the presence of *AvrLm6* in *Leptosphaeria maculans* isolate J20

DNA from three *L. maculans* isolates; ICBN14 (*AvrLm5-9, 6, AvrLepR1*), R2 (*AvrLm5-9, 7, AvrLepR1*), and J20 (*AvrLm2, AvrLm3, AvrLm6, AvrLepR1*) were used as templates to amplify avirulence genes *AvrLm5-9* and *AvrLm6* via a multiplex PCR (Fig. 4.3). The primers for this PCR and amplification protocols were collected from the corresponding publication (Van de Wouw et al. 2014a; Fudal et al. 2007). Both avirulence genes, *AvrLm5-9* and *AvrLm6*, were successfully amplified for the isolate ICBN14. On the other hand, isolates R2 and J20 yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, respectively. This result confirmed that isolate J20 carried the avirulence gene *AvrLm6*, but not *AvrLm5-9* (Fig. 4.3).

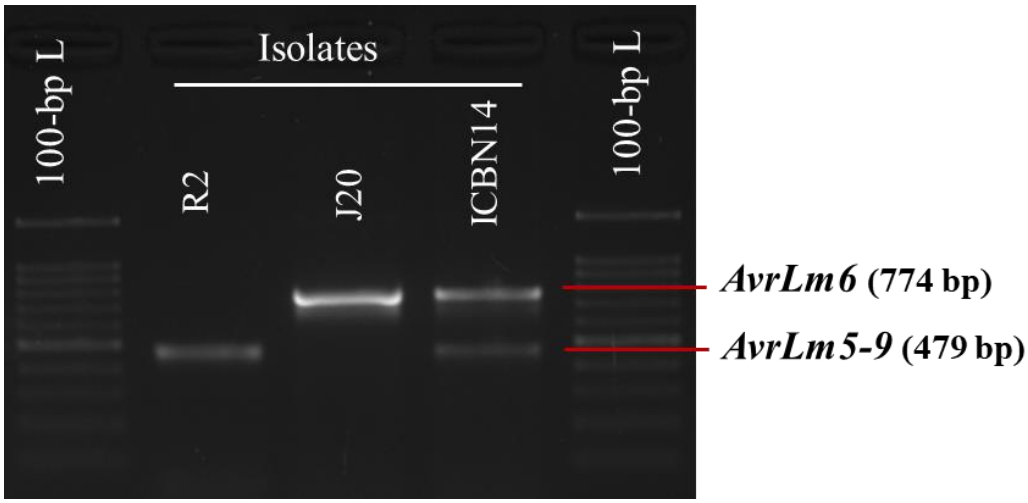


Fig. 4. 3 Confirmation of the *Leptosphaeria maculans* isolate used in the study. Isolate ICBN14 (*AvrLm5-9*, 6, *AvrLepR1*) yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, whereas isolates R2 (*AvrLm5-9*, *AvrLm7*, *AvrLepR1*) and J20 (*AvrLm2*, *AvrLm3*, *AvrLm6*, *AvrLepR1*) yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, respectively.

4.4.2 Interspecific hybridization

Fully expanded 7-days old cotyledons of 'Topas DH16516' (*B. napus* line - no *R* gene) and 'Forge' (*B. juncea* cultivar carries *R* gene *Rlm6*) were inoculated with *L. maculans* isolate J20 (carries avirulence gene *AvrLm6*). Inoculated 'Topas DH16516' showed susceptibility to the

isolate J20 indicating no corresponding *R* gene *Rlm6* was carried by the wild-type susceptible parent (Fig. 4.4A). On the other hand, *B. juncea* cultivar 'Forge' showed small dark chlorotic lesion a sign of a typical hypersensitive response (HR) indicating presence of the corresponding *R* gene *Rlm6* (Fig. 4.4B). 'Topas DH16516' and resistant seedlings of 'Forge' were transplanted to the plastic pot, and grown under greenhouse condition (22ºC and 12-h photoperiod). Interspecific crosses were performed between *B. napus* line 'Topas DH16516' and *B. juncea* cultivar 'Forge'. Harvested F_1 seeds were poorly developed, and a few seeds were germinated on a Petri dish and transplanted to a plastic pot in a growth chamber at 21ºC/16ºC (day/night) with a 16-h photoperiod. Resistant progenies were identified in each generation via phenotyping with *L. maculans* isolate J20. Only resistant F_1 seedlings (those that showed HR to the isolate J20; Fig. 4.4C) were transferred to the plastic pot, and grown under greenhouse conditions (22°C and 12-h photoperiod). In addition a PCR-based SCAR marker B5Rlm6_1 linked to *Rlm6* was also applied to confirm the introgression in backcross progenies (Fig. 4.5).

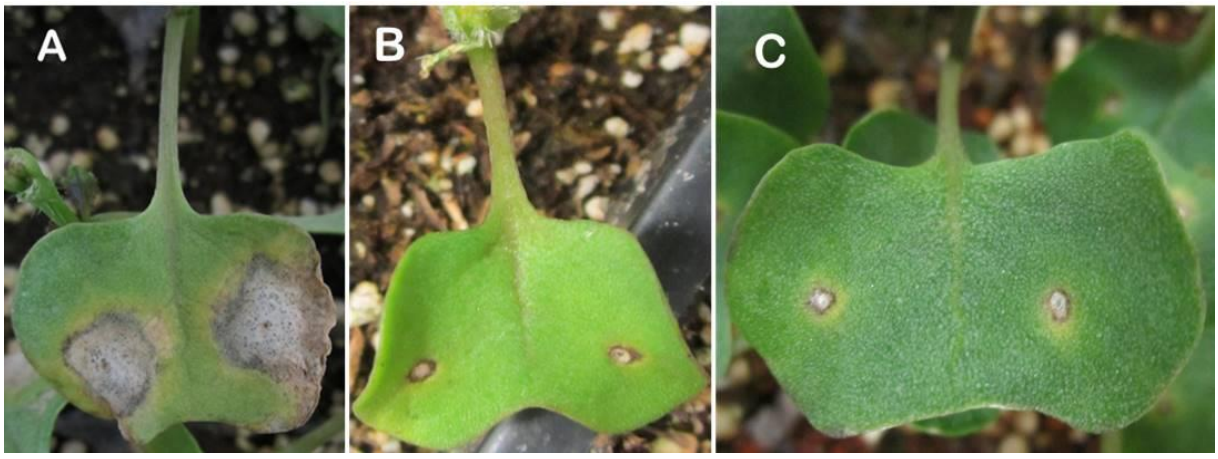


Fig. 4. 4 Phenotype of gene-for-gene interaction between host and *Leptosphaeria maculans* isolate J20. (A) Susceptible parent *Brassica napus* line 'Topas DH16516' showed compatible interaction to J20, (B) Resistant parent *Brassica juncea* cultivar 'Forge' showed incompatible interaction to J20, and (C) an interspecific hybrid also showed incompatible interaction to J20.

4.4.3 Development of backcross generations

For all BC generations seeds were sown in a plastic cell tray using a professional growing media sunshine mix, and kept in a growth chamber at 21°C/16°C (day/night) with a 16-h photoperiod. Fully expanded 7-8 days old cotyledons were inoculated with the *L. maculans* isolates J20 (carry *AvrLm2, AvrLm3, AvrLm6, and AvrLepR1*). The seedlings that showed HR to the pathogen reaction were transplanted to individual plastic pot, and grown under greenhouse condition (22°C and 12-h photoperiod). Moreover, genotyping was also done with the dominant type SCAR marker to confirm the introgression of components of the *B. juncea* genome. Six F_1's were genotyped by the PCR based SCAR marker B5Rlm6_1, which showed polymorphism as expected (Fig. 4.5A). These F_1's were backcrossed with the susceptible recurrent parent towards developing BC_1F_1. Similarly, BC_1F_1 plants were phenotyped using the isolate J20, and genotyped using the marker B5Rlm6_1. Among eleven BC_1F_1 plants, five showed evidence for the introgression of a segment of B-genome from *B. juncea* linked to blackleg resistance gene *Rlm6* (Fig. 4.5B), and those plants were backcrossed with the susceptible recurrent parent to develop BC_2F_1. Subsequently, BC_3F_1 and BC_4F_1 generations were developed from the confirmed BC_2F_1, and BC_3F_1 seedlings, respectively (Fig. 4.5C and 4.5D).

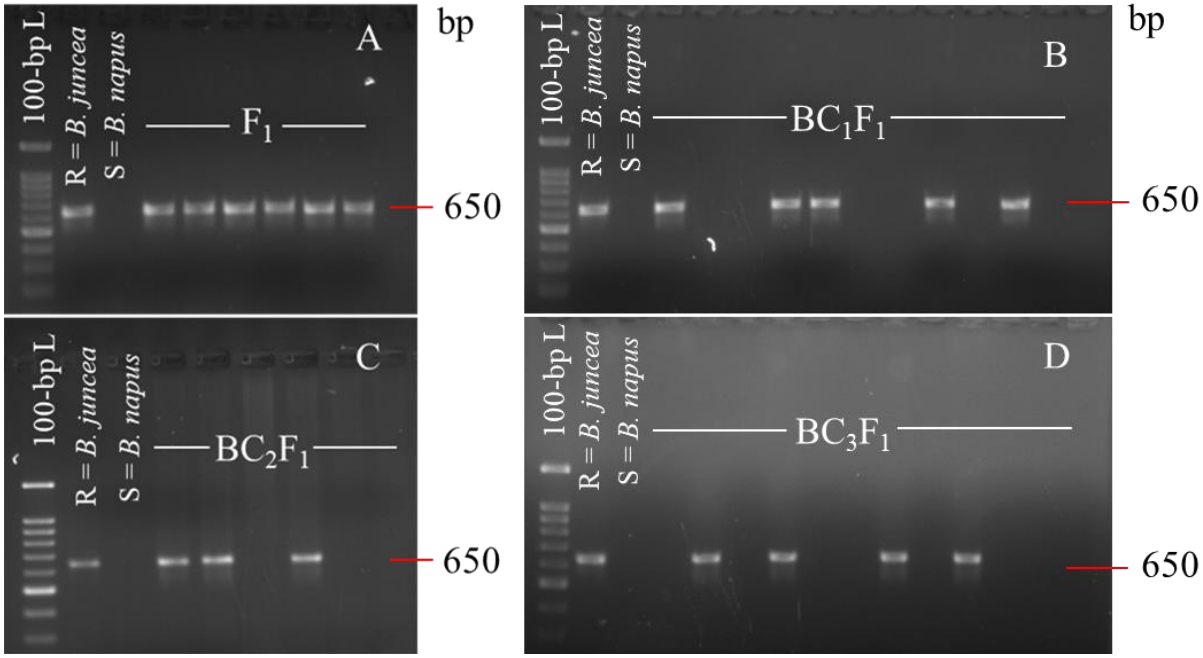


Fig. 4. 5 Confirmation of the introgression of a part of B-genome in the interspecific hybrids such as F_1 (A), BC_1F_1 (B), BC_2F_1 (C), and BC_3F_1 (D) by a SCAR marker B5Rlm6_1. The interspecific hybrids derived from a cross between a *Brassica napus* cultivar 'Topas 'DH16516' and *Brassica juncea* cultivar 'Forge'.

4.4.4 Segregation of blackleg resistance gene *Rlm6* in backcross generations

The BC_1F_1 to BC_4F_1 progenies showed segregation with regards to resistance versus susceptible phenotypes in response to inoculation with *L. maculans* isolate J20 (carries avirulence gene *AvrLm6*). The χ^2 test for the phenotypic segregation of BC_1F_1 generation showed insignificant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.64800$; Table 4.1), which indicates an acceptable fit to a Mendelian segregation in the BC_1F_1 generation. Phenotyping data showed 49% of BC_1F_1 progeny resistant to *L. maculans* isolate J20 (Table 4.1), indicated an acceptable fit to a Mendelian ratio of 1:1 resistance versus susceptible. The χ^2 test for the phenotypic segregation from BC_2F_1 to BC_4F_1 generations showed significant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.00001$; Table 4.1). Phenotyping data showed 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 progeny resistant to *L. maculans* isolate J20 (Table 4.1). As a result, BC_2F_1, BC_3F_1, and

BC_4F_1 generations had an unacceptable fit to a Mendelian ratio of 1:1 with regards to resistant versus susceptible phenotypes.

On the other hand, BC_1F_1 progeny were also segregating for a SCAR marker B5Rlm6_1, which is linked to B-genome of *B. juncea*. The χ^2 test for the segregation of the SCAR marker of BC_1F_1 progeny showed insignificant discrepancy between the observed and expected values for the ratio of presence versus absence of the gene *Rlm6* ($P \leq 0.52607$; Table 4.2), which supports an acceptable fit to a Mendelian segregation in BC_1F_1 generation. Genotyping data showed 47% of the BC_1F_1 progeny carried a single dominant gene *Rlm6* linked to the B-genome of *B. juncea* (Table 4.2) indicated an acceptable fit to a 1:1 ratio of presence versus absence of B-genome fragment in the BC_1F_1 progeny. The χ^2 test for the segregation of the SCAR marker from BC_2F_1 to BC_4F_1 progeny showed significant discrepancy between the observed and expected values for the ratio of presence versus absence of the gene *Rlm6* ($P \leq 0.00006$; Table 4.2), which indicated an unacceptable fit to a Mendelian segregation in BC_2F_1 to BC_4F_1 generations. Again genotyping with the B5Rlm6_1 marker showed that 30% of BC_2F_1, 18% of BC_3F_1, and 11% of BC_4F_1 progeny retained the gene *Rlm6* linked to the B-genome of *B. juncea* (Table 4.2). As a result, BC_2F_1, BC_3F_1, and BC_4F_1 generations had an unacceptable fit to a Mendelian segregation for the gene *Rlm6*. Thus the genotypic segregation of a SCAR marker B5Rlm6_1 is supporting an acceptable fit to a Mendelian ratio of presence versus absence of the B-genome fragment for BC_1F_1 but not for BC_2F_1 to BC_4F_1 generations. Similar segregating patterns were also observed for the marker B5-1520 in all BC generations (Table 4.2). Overall, in this study the χ^2 goodness-of-fit test for the segregation of *B. juncea* B-genome linked to *Rlm6* showed a significant association between the phenotypes (Table 4.1) and the genotypes (Table 4.2) in BC_1F_1 generation, supporting the assumption that the genetic control of resistance is by a single dominant gene.

Table 4. 1 Number of resistant and susceptible plants on the basis of phenotyping with the *Leptosphaeria maculans* isolates J20 among the interspecific hybrids derived from *Brassica napus* x *B. juncea*

Generations	Total # of seedlings	Resistant (R)	Susceptible (S)	Ratio (R:S)		χ^2	*P*(≤0.05)
				Observed	Expected		
BC_1F_1	480	235	245	1:1.0	1:1	0.20800	0.64800
BC_2F_1	288	79	209	1:2.6	1:1	30.9150	0.00001
BC_3F_1	384	57	327	1:5.7	1:1	108.3084	0.00001
BC_4F_1	284	27	257	1:9.5	1:1	111.3998	0.00001

Table 4. 2 Segregation of the dominant type sequence characterize amplified region (SCAR) marker among the different generations derived from *Brassica napus* x *B. juncea*

Markers	Generations	Total # of seedlings	Resistant (R)	Susceptible (S)	Ratio (R:S)		χ^2	*P*(≤0.05)
					Observed	Expected		
B5Rlm6_1	BC_1F_1	244	115	129	1:1.1	1:1	0.4020	0.52607
	BC_2F_1	190	57	133	1:2.3	1:1	15.833	0.00006
	BC_3F_1	192	34	158	1:4.6	1:1	44.703	0.00001
	BC_4F_1	192	21	171	1:8.1	1:1	69.144	0.00001
B5_1520	BC_1F_1	192	91	101	1:1.1	1:1	0.2600	0.60971
	BC_2F_1	190	55	135	1:2.4	1:1	17.623	0.00002
	BC_3F_1	192	34	158	1:4.6	1:1	44.703	0.00001
	BC_4F_1	192	20	172	1:8.6	1:1	71.345	0.00001

4.4.5 Genome configurations

A significant decrease of SCAR markers linked to the *Brassica juncea* genome was found from BC_2F_1 to BC_4F_1 generations. Plants with the additional *B. juncea* genome components showed a decrease from 47.13% in BC_1F_1 to 30% in BC_2F_1, and this was further reduced to 17.71% in BC_3F_1 and 10.94% in BC_4F_1 with respect to the SCAR marker B5Rlm6_1 (Fig. 4.6). The situation was similar for the SCAR marker B5-1520, where 47.40% of BC_1F_1 showed the presence of this SCAR marker but only 10.42% yielded positive results among members of BC_4F_1 (Fig. 4.6). However, in the plants where a fragment of B-genome of *B. juncea* was integrated into the *B. napus* background, the presence of SCAR markers was significantly lower in the more advanced generations (P<0.00001). In contrast, plants with the *B. napus* genome had an increase from 52.87% in BC_1F_1 to 89.06% in BC_4F_1 of *B. napus* genome as the B-genome linked dominant type SCAR marker B5Rlm6_1 appeared to be lost

(Fig. 4.6). Similarly, the retention of the *B. napus* genome was also observed in advanced generation for the marker B5-1520 as well i.e. 52.60% in BC_1F_1 to 89.58% in BC_4F_1 (Fig. 4.6).

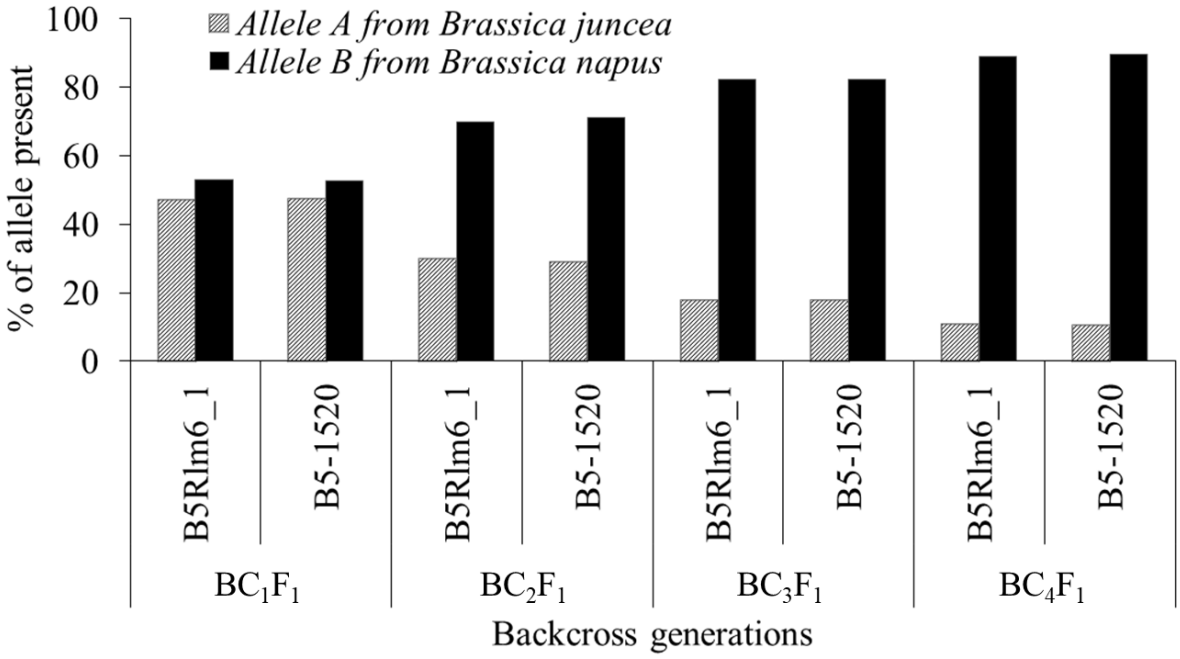


Fig. 4. 6 Bar graph showing the pattern of presence or absence of SCAR markers B5Rlm6_1 and B5-1520 in backcross generations from BC_1F_1 to BC_4F_1. The bars represent the mean frequency of SCAR markers B5Rlm6_1 and B5-1520 linked to the B-genome of *Brassica juncea* as presence (white pattern fill) or absence (black solid fill) in backcross generations derived from *Brassica napus* x *Brassica juncea*. The mean frequency of the SCAR markers, either B5Rlm6_1 or B5-1520, linked to the B-genome fragment is significant among the generations ($P \leq 0.00001$; *t*-test).

4.5 Discussion

In this study we were able to develop a set of BC populations (BC_1F_1 to BC_4F_1) from an interspecific cross *B. napus* x *B. juncea*. We also demonstrated successful introgression of a gene from the B-genome into *B. napus* background. In addition we integrated phenotype and genotype data from the interspecific hybrids in order to analyze the inheritance of a single gene *Rlm6* linked to the B-genome resistance .

Considering the gene-for-gene hypothesis (Flor 1971), an incompatible interaction between *L. maculans* races and the corresponding resistance genes is possible. This kind of

study has been done routinely in canola-blackleg pathosystem since first observed in 1995 (Kuswinanti et al. 1995). Here we observed the hypersensitive reaction between *L. maculans* isolate J20 (carries avirulence gene *AvrLm6*), and *B. juncea* parent Forge or the BC populations (carried *R* gene *Rlm6*) as a result of the gene-for-gene interaction (Fig. 4.4), indicating the study materials retain high level of resistance to *L. maculans*. Earlier studies also showed B-genome *Brassica* species; *B. nigra*, *B. carinata*, and *B. juncea* retain high level of resistance based on the hypersensitive response of the cotyledons upon infection by *L. maculans* (Roy 1978; Sacristan and Gerdemann 1986; Sjodin and Glimelius 1988; Rimmer and van den Berg 1992; Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006). There have been reports on the predicted breakdown of *R* genes in canola including *Rlm3* in Canada (Zhang et al 2016), *Rlm1* in France (Rouxel et al. 2003a), and *LepR3* in Australia (Van de Wouw et al. 2014; Sprague et al. 2006). It could be due to the widespread use of resistant *B. napus* cultivars which may have led to an increase of virulent races of *L. maculans*. Therefore, the characterizations of new sources of resistance against *L. maculans* are essential in controlling blackleg disease effectively. In this case, B-genome based resistance could be an alternative source of controlling blackleg disease. Here we used *B. juncea* cultivar 'Forge' to introduce blackleg resistance into *B. napus* (Fig. 4.5), which carried *R* gene *Rlm6* (Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006; Rashid et al. 2018). This resistance gene was successfully introgressed into *B. napus* confirmed by the polymorphism of a SCAR marker B5Rlm6_1 linked to the B-genome (Fig. 4.5). This line may offer a strategy to breed for blackleg resistant *B. napus* varieties in the future. Previous studies also reported successful introduction of the B-genome into *B. napus* *via* interspecific hybridization (Struss et al. 1991; Zhu et al. 1993; Delourme et al. 1995; Frello et al. 1995; Chèvre et al. 1996; Plieske et al. 1998; Saal and Struss 2005; Chèvre et al.

2008; Brun et al. 2010; Navabi et al. 2010), but the materials are not available for commercial breeding.

In the present study, our phenotype data showed 49% of BC_1F_1, 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 progeny were resistant to *L. maculans* isolate J20, indicating an incompatible interaction between isolate J20 and interspecific hybrids (Table 4.1). The χ^2 test for the phenotypic segregation in BC_1F_1 generation showed insignificant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.64800$; Table 4.1), which indicates an acceptable fit to a Mendelian segregation in the BC_1F_1 generation. Thus the data is supporting a monogenic dominant inheritance for blackleg resistance in the BC_1F_1 population, an important feature which increases the value of the materials for commercial breeding particularly in a variety development programme. A single gene inheritance linked to the B-genome resistance was also reported in previous studies (Struss et al. 1996; Chèvre et al. 1997; Saal et al. 2004; Chèvre et al. 2008; Wang 2016). Wang (2016) investigated the interaction phenotype between blackleg isolates and interspecific hybrids derived from *B. napus* (AACC) x *B. carinata* (BBCC) and *B. napus* x hexaploid *Brassica* species (Meng, AABBCC). She observed a Mendelian segregation in the BC_1F_1, BC_3F_1 and BC_4F_1 generations derived from *B. napus* x *B. carinata*, but no Mendelian segregation in the BC_2F_1. She also could not observe a Mendelian segregation in BC_1F_1 and BC_2F_1 derived from *B. napus* x hexaploid *Brassica* species. Chèvre et al. (2008) also observed different segregation pattern of blackleg rersistance linked to the *R* gene *Rlm6* in the interspecific hybrids derived from *B. napus* cv. Dunkled x KEM lines (*B. napus* x *B. juncea*). A monogenic inheritance of blackleg resistance was also observed in the recombinant inbred lines (Chèvre et al. 1997) and in the F_2 progeny (Pang and Halloran 1996) derived from *B. napus* x *B. juncea*. Struss et al. (1996) investigated three different BC populations derived from different interspecific crosses; *B. napus* x *B. nigra*, *B. napus* x *B. juncea*, and *B. napus* x

B. carinata and observed similar level of resistance among the BC lines as seen in the B-genome donor parents. The present study with the BC generations showed a high similarity in the resistance mechanisms against blackleg. No significant differences in phenotype or levels of resistance were observed in the study materials. However, results of the earlier, and the present study suggest that the resistance genes identified in three B-genome species are identical, indicating the B-genome resistance is due to a single resistance gene in *B. juncea* species.

We were able to demonstrate successful introgression of B-genome blackleg resistance linked to the *R* gene *Rlm6* into *B. napus* via a backcross approach (Fig. 4.5). In this study, a single gene or a gene complex of *B. juncea* was introduced into backcross generations. Our genotype data for the SCAR marker B5Rlm6_1 showed the presence of B-genome introgression rate decreasing towards advanced generations, which was 47% in BC_1F_1, decreased to 27% in BC_2F_1, further decreased to 18% in BC_3F_1, and 11% in BC_4F_1 (Table 4.2). A similar trend was also observed for the marker B5-1520 in all BC generations. Thus the genotypic segregation of SCAR markers linked to the B-genome resistance gene *Rlm6* had an acceptable fit to a Mendelian ratio of presence versus absence of B-genome fragment for BC_1F_1 generation, but not for BC_2F_1 to BC_4F_1. Similar kind of marker skewedness in advanced generations was also observed in previous studies (Dion et al. 1995; Frello et al. 1995; Chèvre et al. 1997; Plieske et al. 1998; Dixelius and Wahlberg 1999; Saal and Struss 2005; Schelfhout et al. 2006; Navabi et al. 2009; Fredua-Agyeman et al. 2014). The present study was supported by Saal and Struss (2005), where they genotyped two segregating populations derived from *B. napus* x *B. juncea* using a dominant type SCAR marker which showed significant deviation from a monogenic inheritance. This study also supported by Dixelius and Wahlberg (1999), where they recorded segregation of RFLP markers, which is linked to the donor genome B, 60% in BC_1F_1, 33% in BC_2F_1, and 10% in

BC_3F_1 from *B. juncea*-derived hybrids whereas *B. carinata*-derived hybrids had segregation from 59% in BC_1F_1 to 36% in BC_2F_1 and further down to 11% in BC_3F_1. *B. nigra*-derived hybrids showed lower segregation of B-genome compared to *B. carinata*- and *B. juncea*-derived hybrids, which was 46% in BC_1F_1 to 25% in BC_2F_1 and further decreased to 8% in BC_3F_1. On the other hand, segregation of RFLP marker linked to blackleg resistance was observed in DH lines and predicted to control genetic resistance by a single major gene *LmFr1* (Dion et al. 1995). Frello et al. (1995) also reported inheritance of 52% RAPD marker linked to a transgene in BC_1 population derived from *B. juncea* x (*B. juncea* x *B. napus*). Similar observations were also reported by Plieske et al. (1998), when they analysed RFLP markers linked to B-genome based blackleg resistance. However, our genotype of SCAR markers B5Rlm6_1 and B5-1520 in BC_1F_1 generation, indicating a monogenic inheritance of *Rlm6* linked to B-genome resistance, as was seen in the earlier studies.We observed that the frequency of SCAR markers was significantly reduced from BC_1F_1 (47.13%) to BC_4F_1 (10.94%) where the B-genome of *B. juncea* was integrated (Fig 4.6), and at the same time the *B. napus* genome contribution was significantly increased from BC_1F_1 (52.87%) to BC_4F_1 (89.06%), which is the result of a possible reduction of the B-genome by the homoeologous recombination. A similar mechanism was predicted for the gene transfer from B-genome species to *B. napus* (Sacristan and Gerdemann 1986; Plieske et al. 1998; Delourme et al. 1995), and from C-genome species to A-genome (Leflon et al. 2006). Introgression of B-genome chromosomes into *B. napus* from *B. juncea* and *B. carinata* was also confirmed by Schelfhout et al. (2006) and Fredua-Agyeman (2014), respectively. We also did not record any significant differences in genotype between marker B5Rlm6_1 and B5-1520 for all four BC generations, indicating there is no segregation deviation from the B-genome. The co-segregation of DNA-markers suggests that both of the SCAR markers are likely located on the introgressed B-genome, and not in the *B. napus* genome. Thus this study suggests a

possible introgression by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with the A- or C-genomes (Plieske et al. 1998). Similar phenomena were found in rice during transfer of bacterial resistance from a wild genotype to cultivated rice (Amante-Bordeos et al. 1992). It is reported that when an amphidiploid such as oilseed rape, is used in interspecific crosses, it is usually challenging to return to the original ploidy level by backcrossing (Bing et al. 1991; Chèvre et al. 1996), because of the tendency of spontaneous genome fixation by amphiploidy (Song et al. 1993). This is in agreement with our outcomes, which indicated a high probability of amphidiploid conservation due to meiotic stability, and sound seed set under normal conditions.

However, it still remains in doubt whether the recombination events are originating from the homoeologous position of the *B. napus* genome or from the loss of introgressed segments or a combination of both types of events. This question could be addressed by Southern blotting or fluorescent *in situ* hybridization (FISH). We developed a custom FISH probe with the specific primer sequences (molecular weight 1.5 kb); unfortunately, there was no FISH signal observed either in the resistant parent or the BC populations. This could be due to the short fragment size of probe, as compared to the larger repetitive fragment requires in a typical FISH study (Jiang and Gill 2006). This is also supported by Schelfhout et al. (2006) where no FISH signals were observed for B-genome. In order to confirm the stabilization of B-genome introgression into *B. napus*, both the trait locus and SCAR markers could be analysed further in double haploid or near isogenic lines, to see if the same direction of marker segregation would occur. This will be the focus for future studies; in addition to the development of more markers in the introgression region. Moreover, a study towards mapping or map-based cloning of the resistance locus could also be possible by screening different mapping populations, and/or by bulked segregant RNA-seq analyses (Liu et al. 2012). This is currently under investigation in our lab. In summary, we demonstrated

successful introgression of B-genome blackleg resistance from *B. juncea* to *B. napus*. The study of phenotypes and genotypes among the interspecific hybrids showed that the material we developed carries a single dominant gene *Rlm6*, conferring resistance to the blackleg pathogen *L. maculans*. Notably, the BC populations developed in this study retained blackleg resistance and therefore can accelerate a variety development program in the future.

4.6 Acknowledgements

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5 CANOLA-WHEAT A 2-YEAR CROP ROTATION INCREASED THE EFFECTIVENESS OF R GENES TO *LEPTOSPHAERIA MACULANS*, A CAUSAL AGENT OF BLACKLEG DISEASE IN *BRASSICA* SPECIES

5.1 Abstract

Blackleg, caused by the ascomycete fungus *Leptosphaeria maculans*, is a major disease of canola in Canada and worldwide. In Canada, canola is usually grown in rotation once every 3-4 years to limit yield losses. Recently, growers have begun to grow canola more intensively because of market demand and cultivars development. A 4-year study from 2014 to 2017 at the Ian N. Morrison Research Station, Carman, Manitoba was conducted to investigate the durability of five single dominant resistant genes under field conditions in a canola-wheat-canola over 2-year rotation. Blackleg incidence was reduced to a maximum of 16% in 2017 compared to 2014 for all *R* genes tested except *Rlm4*. Disease severity was 31-44% higher in 2014 where canola seeded on same stubble compared to 2017 regardless of *R* genes tested except *Rlm2*. Severity rating based on the susceptible cultivar Westar showed that the *R* genes *LepR1*, *LepR3*, *Rlm2*, and *Rlm4* were effective to *L. maculans* in 2017, whereas *Rlm3* was moderately effective. Seed yield was 11-145% higher in 2017 compared to 2014 produced by the introgression lines (ILs) except IL harbouring *Rlm2*. These results suggest that canola cultivars harbouring a resistant gene can be grown more intensively than once every 3-4 years in particular canola-wheat-canola over 2-year rotation. The regression model showed that seed yield decreased linearly as blackleg severity increased. However, linear regression analysis suggests an order of effectiveness among the *R* genes tested: *LepR3* > *LepR1*> *Rlm2* > *Rlm4* > *Rlm3*, which can be included in the proposed cultivar rotation strategies on the Prairies.

Key words: *Brassica napus*, blackleg disease, *Leptosphaeria maculans*, *R* genes effectiveness, crop rotation, wheat

5.2 Introduction

Canola (oilseed rape, *Brassica napus* L.) is the major cash crop in Canada. The crop has a few diseases including blackleg caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph phoma-like (de Gruyter et al. 2013)). It has been identified a major issue of canola production in many growing regions over the world (West et al. 2001; Fitt et al. 2006; Dilmaghani et al. 2009; Marcroft et al. 2012b). In western Canada, blackleg continues to be one of the most serious diseases in canola (Dokken-Bouchard et al. 2011; Lange et al. 2011; McLaren et al. 2011; Zhang and Fernando 2017). The disease can be controlled by an integrated approach including genetic resistance, cultural management and fungicide application (West et al. 2001; Fitt et al. 2006; Marcroft et al. 2012a; Kutcher et al. 2013). A number of single dominant resistance (*R*) genes to *L. maculans* have been identified in different *Brassica* species (Raman et al. 2013a). But lack of durability of *R* genes in canola is especially a big problem against air/stubble borne pathogens (e.g. *L. maculans*) which have both sexual and asexual cycles of reproduction (Ghanbarnia et al. 2011). Early genetic studies showed a gene-for-gene relationship between an *R* gene and the corresponding avirulence (*Avr*) gene (Ansan-Melayah et al. 1998; Balesdent et al. 2001). *R* gene-mediated resistance is often very effective in disease management when the fungal populations harbour a high frequency of the corresponding *Avr* gene (Daverdin et al. 2012). However, intensive use of a single *R* gene exerts strong selection pressure on the fungal populations, resulting pathogen adaptation to the *R* gene in the canola varieties. Due to the adaptation of the pathogen, breakdown of a single *R* gene have been reported in many studies. Studies have shown the breakdown of a single *R* gene *Rlm6* (Brun et al. 2010) and *Rlm1* (Rouxel et al. 2003a) in France after three growing seasons. Similarly, *LepR3* resistance in Australia was not effective in a few years after the release of a commercial *LepR3*-carrying variety Surpass 400 (Sprague et al. 2006; Van de Wouw et al. 2010a; Van de Wouw et al. 2014). Recently, the breakdown

of *Rlm3* resistance was reported in western Canada due to intensive use of *Rlm3*-carrying canola varieties (Zhang et al. 2016), stressing a need of developing proper management strategies of *R* genes for their durability in the canola cultivars.

The crop rotation is one of the most important strategies either controlling residue-borne plant pathogens or increasing the durability of *R* genes (Curl 1963). In fact, it has detrimental effect on the development, persistence and reproduction of the causal agent *L. maculans*, thus decreasing the pathogen population to a level low so that significant yield loss does not occur. Generally productivity and durability of the single *R* genes have been based on complexity or diversity generated by the crop rotations with various crop species (Cook 2006). The trend in crop production worldwide is to be specialized in the production of a limited number of crops to maximize net returns despite the evidence that crop monocultures are detrimental in the long term (Cook 2006; Kutcher et al. 2013). In western Canada, cropping system research indicates that more diverse rotations tend to have fewer pest problems, and lower production risk (Bailey et al. 2000; Johnston et al. 2005; Kutcher et al. 2013). Over the last decade, however, growers have again specialized with more and more farmers producing canola in a 4-year rotation, which has been thought the standard recommendation (Rimmer et al. 2003). Instead, 3-year rotations of canola with cereals and pulses have been suggested to be sustainable (Catheart et al. 2006; Kutcher et al. 2013). These days' 2-year rotation of canola with cereals, and even canola monoculture are becoming common across western Canada due to its market demand and cultivar development with *R* genes. Recently, the Canola Council of Canada projects production of 26 million MT of Canadian canola by 2025 (CCC 2016a), which is actually emphasized an increment of canola yield from the same land area to accommodate the higher demand for canola oil and other canola products. Furthermore, canola is likely to be grown in rotations at higher frequencies than ever before. Studies suggest that the agronomic risks of growing

canola in short crop rotations can be considered with a limited yield reduction, and pest infestation (Johnston et al. 2005; Dosdall et al. 2012; Harker et al. 2012; O'Donovan et al. 2014). In addition to that the recent study using resistant cultivars in western Canada showed no significant difference in blackleg disease severity between 2-yr and 3-yr crop rotation including canola-wheat, and canola-wheat-pea, respectively (Kutcher et al. 2013). However, efficiency of the 2-year canola-wheat rotation in terms of disease reduction is still questionable because it is highly dependent on both blackleg resistance, and herbicide tolerance. At the same time researchers, producers as well as industry need to understand the consequences of intensive canola rotations, and possible unwanted outcomes; i.e. an increase of races virulent to the corresponding *R* genes, which is again an important factor of effectiveness of *R* genes in the canola cultivars.

Blackleg disease has been managed in Canada mostly by the growing of hybrid varieties harbouring *R* genes (West et al. 2001; Kutcher et al. 2010a). In contrast, the virulence of *L. maculans* populations has changed in recent years as reported by Kutcher et al. (2007) and Fernando et al. (2018). As a result, the erosion of genetic resistance is now happening in grower fields in Canada (Zhang et al. 2016), and supported by the moderate to heavy infection of resistant and moderately resistant cultivars in the recent years. Though 3-4 years crop rotations have been suggested to decrease blackleg disease and extended the efficiency of host resistance (Kutcher et al. 2007; 2010a), market demand often lead farmers to grow canola in either short rotations or monoculture. At this time, understanding of disease-yield relationships is a vital factor for estimating the efficiency of *R* genes and the commercial benefits of genetic control (Schoeny et al. 2001), indicating a necessity to establish a relationship between seed yield and blackleg severity, which will ultimately assist growers to predict the possible yield loss at different levels of disease. Our objective of the study was to investigate the effectiveness of canola-wheat over 2-yr rotation on the efficiency

of single *R* genes in a 4-year study. Additionally, we also established a linear relationship between seed yield and blackleg severity of canola, and finally, identified effective *R* genes under disease pressure which can be included in the proposed cultivar rotation strategies on the Prairies.

5.3 Materials and Methods

5.3.1 Plant materials

Five single dominant genes i.e. *Rlm2, Rlm3, Rlm4, LepR1,* and *LepR3* resistance (*R*) to *L. maculans* introgressed into *B. napus* line Topas DH16516 background were used in the study along with a susceptible check Westar. The introgression lines (ILs) harbouring single *R* genes *Rlm2*, *Rlm3*, *Rlm4*, *LepR1* and *LepR3* were developed at AAFC Saskatoon and published by Larkan et al. (2016).

5.3.2 Design of the field experiment

The experiment was established at Ian N. Morrison Research Station at Carman, Manitoba for five consecutive cropping seasons from 2013 to 2017. Plots were located at the south-west end of the station (Appendix V), and where there was no *Brassica* cultivation in the last couple of years. We assumed the plots were completely virgin and there was no *Brassica* grown within ~500 m of surrounding the plots. The experiment started with an initial trial inoculated with a local pathogen population collected from Manitoba which carried specific *Avr* genes (Table 5.1), which simulated the establishment year of the cultivation of ILs each harbouring a single *R* gene. It was followed by five separate 4-year trials (Fig. 5.1), each consistent to recurrent selection of the *L. maculans Avr* genes by one of the five ILs. Experimental plot were set up for canola-wheat-canola over a 2-year crop rotation. All five ILs were sown in a stripe plot design with three replications. Individual plots were measured 8 x 6 m^2, accommodated ten rows of plants, and spaced 0.20-m apart. There was a two meter guard rows seeded with canola cultivar Westar in between *B. napus* IL and wheat (Fig. 5.1).

Temperature and rainfall data were recorded at the research station throughout the study period (Table 5.2).

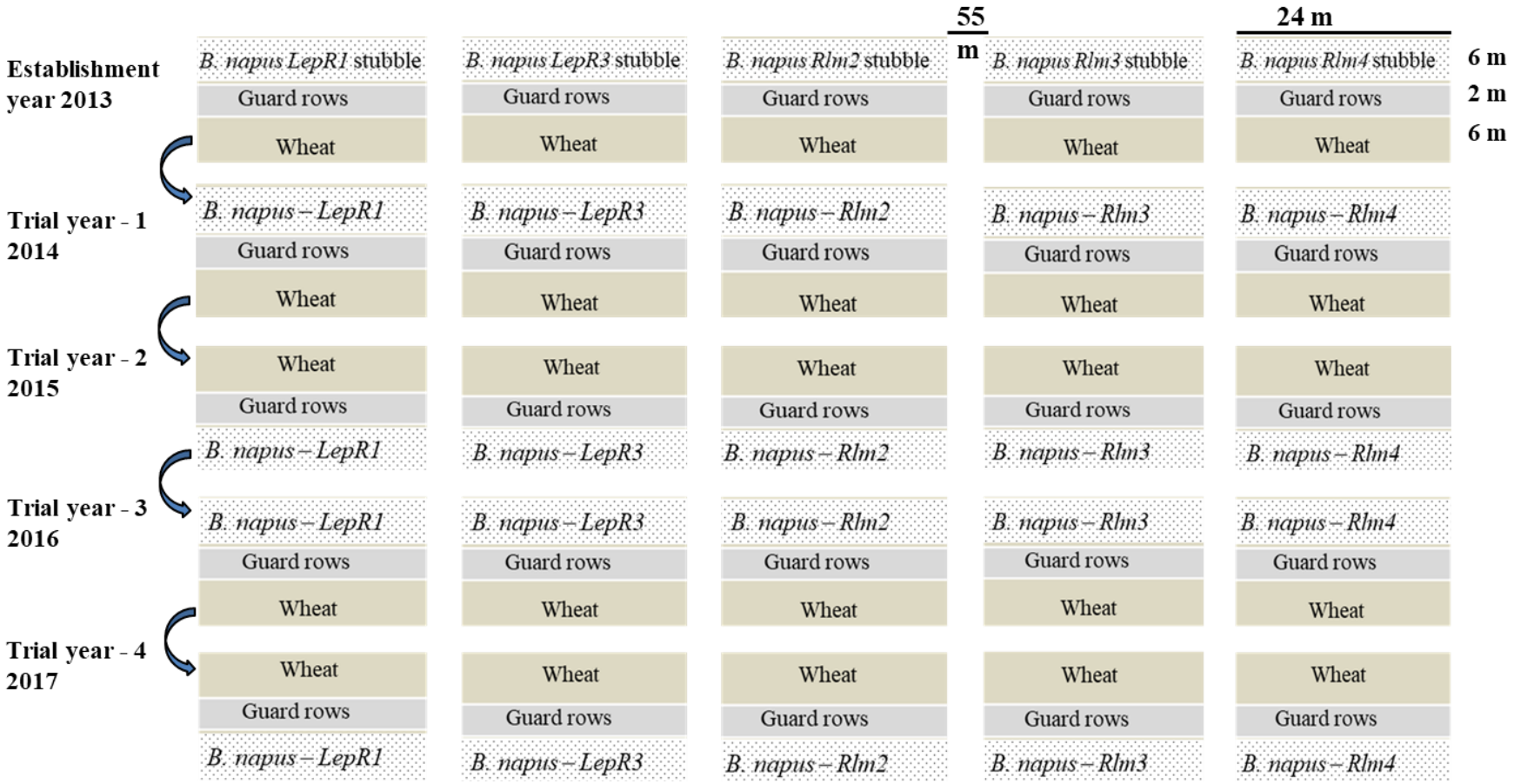

Fig. 5. 1 Field plot layout for a canola-wheat 2-year crop rotation in a 4-year field study at the Ian N. Morrison Research Station, Carman, Manitoba. Susceptible cultivar Westar was seeded as guard rows in between canola and wheat. Note: plot size 24 x 14 m^2 accommodating canola and wheat patches 6 m each, and 2 m guard rows in between. Plot to plot distance 55 m.

5.3.3 Inoculum production and field inoculation

Ten *L. maculans* isolates previously characterized for their *Avr* genes were used to prepare the inoculum (Table 5.1). A mixture of the ten isolates was applied twice at cotyledon, and at two-leaf stage. Each plot was sprayed with 90% avirulent plus 10% virulent isolates of the corresponding *R* gene in the establishment year (2013) and the trial year -1 (2014) to ensure sufficient disease pressure (**Table** S5.1).

Table 5. 1 The avirulence genes profile of the *Leptosphaeria maculans* isolates used in the study.

Isolates	Avirulence genes									
	AvrLm2	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm9*	*AvrLm11*	*AvrLepR1*	*AvrLepR3*
X44	a[1]	a	A	A	A	A	a	A	a	A
X65	A[2]	a	A	A	A	A	a	A	a	A
X108	a	a	A	A	A	A	a	A	A	A
X159	A	a	A	A	A	A	a	A	A	a
RL3	A	a	A	A	A	A	a	A	a	a
RL4	a	a	A	A	A	A	a	A	A	A
DM88	A	a	A	A	A	A	a	A	A	A
MB11-31-2	A	a	A	A	A	A	a	A	A	a
DM96	A	A	a	A	A	a	a	A	a	a
DM118	A	A	a	A	A	a	a	A	a	a

[1]Virulence and [2]Avirulence for the phenotype

5.3.4 Field preparation

A single shallow tillage was performed each year to crust soil surface as the farm practice in western Canada. The systemic insecticide Helix Xtra (Syngenta, Canada) @ 1.5 mL per 100 g was used to treat the seeds before seeding each year. The non-selective herbicide Glyphosate (Roundup ready) was applied pre-emergence of canola and post emergence herbicides including Muster, Eclipse, and Select were applied with its adjuvant Amigo. The plots were hand-weeded as required. *Lepidopteran* insects particularly flea beetles were controlled by using contact insecticide Decis® EC 50.

5.3.5 Assessment of disease incidence

Disease incidence was calculated as percentage of plants affected with at least one leaf lesion in each trial at 30 days after seeding (DAS), 36 DAS and 42 DAS each year from 2014 to

2017. From each plot 50 plants were collected randomly from each replication spread across the plot, making 150 plants in total from each IL.

5.3.6 Assessment of disease severity

Disease severity was evaluated by visual assessment of blackening at the base taking cross sections of the stem a week before harvest usually at the end of August when 30% seeds colour change from green to brown as previously described by Marcroft et al. (2012a). In brief, 50 plants per replication per IL were uprooted and cut at the crown and estimated percentage of internal infection (blackened area) using a scale of 0-5 (West et al. 2002), where: 0 - no visible infection; 1 - disease tissue occupies ≤25% of the cross section; 2 - disease tissue occupies 26-50% of the cross section; 3 - between 51 and 75% of the cross section infected; 4 - more than 75% of the cross section infected but plant alive and 5 - 100% of cross section infected and plant dead (Appendix IV).

5.3.7 Yield assessment

For the assessment of impact of blackleg disease on canola yield, a 1-m^2 area of each replication (8 x 6 m^2) was harvested by sickle and bagged one week after rating of disease severity. The harvested bags were dried under shade before threshing. Mechanical threshing was performed and weighed them to determine overall yield.

5.3.8 Isolation and culture of *Leptosphaeria maculans* isolates from stubble

Blackleg infected canola stems were collected from each plot for all the years. Canola stubble was collected in September just after harvest, when disease symptoms of blackleg were visible at the cross section of the stems. At least 7-8 *L. maculans* isolates were isolated from the stems collected from five different plots separately. Fungal isolation, purification, inoculum preparation and maintenance were performed according to Liban et al. (2016) with some modifications. Briefly, infected stems were surface-sterilized in 50% (v/v) bleach for 1 min and cut to a small pieces. These stem pieces were placed on V8 agar juice medium

amended with 0.0035% (w/v) streptomycin sulphate and incubated under continuous florescent light at room temperature (~25°C) for 7 days. A small portion of pure cultures of *L. maculans* were transferred into a new Petri plate. The full plate culture was flooded with 3 mL of sterile distilled water, and the spores were harvested for the pathogenicity test.

5.3.9 Pathogenicity test

A set of canola cultivar differentials was used in the pathogenicity test. Seeds were sown in a plastic cell tray using standard growth media, and kept in a growth chamber at 21ºC/16ºC (day/night) with a 16-h photoperiod. Four lobes of fully developed cotyledons of 7-days old seedlings were point wounded with a modified tweezer. Each wounded site was inoculated with a droplet of 10 µL pycnidiospores suspension (2×10^7 spores per mL). Inoculated cotyledons were air dried before placing back in the growth chamber, and subsequently watered. Disease symptoms on inoculated cotyledons were evaluated at 13-days post inoculation (dpi) using a rating scale of 0-9 (Williams and Delwiche 1979; Appendix III). At least six plants of each differential variety were rated for the pathogenicity test of an isolate. A total of 24 rating scores were obtained for each isolate-host interaction, and the average rating score was used to determine the interaction phenotype (IP), where IP = 5-9 was considered virulent and IP = 0-4.9 was susceptible (Liban et al. 2016).

5.3.10 Analysis of avirulent genes of *Leptosphaeria maculans* isolates

Avr gene profiles of blackleg isolates were determined by inoculating them onto a set of canola differentials as described in the previous section. Differentials were Topas DH16516 ILs harbouring single *R* gene *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *LepR1*, and *LepR3* developed at AAFC Saskatoon and published by Larkan et al. (2016). *B. rapa* line 01–23-2-1 carried *Rlm7* (Dilmaghani et al. 2009) and cultivar Goéland carrying *Rlm9* (Balesdent et al. 2006) was also included in the differentials. The differential set allowed the identification of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, and *AvrLep3*. Since we do not have

appropriate *Brassica* differential lines to identify the presence or absence of *AvrLm5-9* (Van de Wouw et al. 2014; Ghanbarnia et al. 2018), *AvrLm6* (Fudal et al. 2009), and *AvrLm11* (Balesdent et al. 2013), only PCR was performed to identify these three *Avr* alleles for the isolates collected from the last four years.

5.3.11 DNA extraction and PCR assay

Genomic DNA was extracted from pycnidiospore suspensions of purified *L. maculans* isolates using the method described by Liban et al. (2016). Identification of three avirulence genes *AvrLm5-9* (Van de Wouw et al. 2014; Ghanbarnia et al. 2018), *AvrLm6* (Fudal et al. 2009) and *AvrLm11* (Balesdent et al. 2013) were performed via PCR assay. Primers used for the PCR assay were collected from the corresponding publications (Fudal et al. 2009; Balesdent et al. 2013; Van de Wouw et al. 2014). PCR identification of the above mentioned *Avr* genes were performed for isolates collected from 2014 to 2017.

5.3.12 Statistical analysis

Excel 2010 version was used for data recording, preliminary analysis, and preparing graphs. Statistical analysis was performed using analysis of variance (ANOVA) in SAS version 9.1. The arcsine root square transformation was done for disease incidence (DI) data before running ANOVA. The data for DI, disease severity (DS) and seed yield were submitted to ANOVA where the effect of the ILs harbouring *R* genes, and years taking into account. The means of the genotypes were compared among the years using the least significant difference (LSD) at the 5% level of significance. In addition, a regression analysis was performed using Excel 2010 version to determine the relationships between seed yield and disease severity. Moreover, yield loss for a unit increase of disease severity was calculated using the respective regression model.

5.4 Results

5.4.1 Climatic conditions

Mean temperature during the growing season were normal at the experiment site from 2014 to 2017 (Table 5.2). Growing season temperatures in May were near normal for the year 2015, whereas above the long-term average for the year 2014, 2016, and 2017. Mean temperature in June was normal for 2014 but higher than the long-term average in 2015, 2016, and 2017. In July, mean temperature were normal from 2015 to 2017 but lower than the long-term average for 2014. On the other hand, August temperature was close to normal for the year 2014-2016, but slightly lower than the long-term average for the year 2017 (Table 5.2). Slow melting of snow in May from 2014 to 2017 had an initial impact on the experiments. However, with good moisture, crops recovered reasonably well and at harvest it was easy to determine the extent of yield loss except the year 2016.

Precipitation amounts during the course of this study were near normal for the year 2014, whereas heavier than normal for the year 2015-2016 and considerably drier than normal for the year 2017 (Table 5.2). Early growing season particularly May 2015 and 2016 received precipitation >124-135% of the long-term average, while May 2014 and 2017 was characterized by moisture shortages as experimental site received less precipitation than the normal. The excessive rain in late May 2016 was the cause of late seeding. As a result, there was no harvest of representative yield for the year 2016. The amount of precipitation returned to near normal in June for the year 2015, 2016 and 2017, but June 2014 had received >144% of the long-term average (Table 5.2). The month of July was extremely dry for the year 2014 and 2017 compared to the long-term normal. As a result, crops were drought-damaged so badly that they were unable to respond to moisture received in August and thereafter. Besides, August 2017 was also received precipitation <140% of the long-term average that

could be led to the early senescence, and early harvest. On the other hand, August 2014 received very high precipitation, >140% of the long-term normal (Table 5.2).

Table 5. 2 Mean temperature and total precipitation during the growing seasons of canola May - August from 2014 to 2017 at Carman Manitoba, and long-term average (1996-2013).

Growing period	Temperature (°C)						Precipitation (mm)					
	2014	2015	2016	2017	GSA[1]	LTA[2]	2014	2015	2016	2017	GSA	LTA
May	11.3	10.7	13.6	12.1	11.93	10.62	30.69	98.58	107.88	25.11	65.57	79.42
June	16.6	17.5	17.1	17.1	17.08	16.64	116.70	75.30	95.40	64.2	87.90	81.23
July	17.8	19.9	19.4	19.4	19.13	19.32	27.59	109.12	78.43	23.25	59.60	62.65
August	18.7	18.3	18.4	17.7	18.28	18.42	122.14	47.12	57.66	22.63	62.39	57.16
Mean	16.08	16.45	17.12	16.62	16.57	16.25	-	-	-	-	-	-
GST[3]	-	-	-	-	-	-	297.12	330.12	339.37	135.19	275.45	280.47

[1]Growing season average; Long-term average from 1996 to 2013; [3]Growing season total

5.4.2 Disease incidence

Blackleg incidence was significantly different ($p<0.0001$; Table 5.3) including 30 days after seeding (DAS), 36 DAS, and 42 DAS for all *R* genes tested in the study (Table S5.2). In 2014, disease incidence varied 35-55% among the *R* genes at 30 DAS whereas 45-75% at both 36 DAS, and 42 DAS (Fig. 5.2A). Incidence was slightly lower in 2015 ranging from 30–50% at 30 DAS, and 35-55% at 42 DAS (Fig. 5.2A). Similar trend of the reduction of DI was observed from 3rd and 4th year trial (Fig. 5.2C-D). DI at 42 DAS was similar or higher than the 36 DAS throughout the study period. It could be due to the fact that the pycnidia were produced over the growing season as asexual cycle is known to be predominant in Canada (Guo et al. 2005; Ghanbarnia et al. 2011). Statistically significant differences were observed in DI among the years (Table 5.3). Blackleg incidence was considered to be moderate to high in 2014 for all *R* genes tested, ranging 48–75% of plants infected. In contrast, moderate DI recorded from 2015 to 2017 year for all *R* genes except *Rlm4* while *R* genes rotated with wheat, indicating an impact of crop rotation on blackleg disease symptoms. Significantly higher numbers of plant were infected in 2017 from the plot seeded by the *Rlm4*-harbouring IL (Fig. 5.2E). The interaction between ILs harbouring *R* genes and year was also significant for disease incidence (Table 5.3). Canola production from 2015 and

onward resulted in significantly lower plant infection for all *R* genes tested except *Rlm4* (Fig. 5.2E) indicating a possible role of canola rotation with wheat.

Table 5. 3 Analysis of variance[1] for disease incidence, disease severity, and seed yield of *Brassica napus* Topas-introgression lines in a 4-year study from 2014 to 2017 at Carman Manitoba.

Variables	Source of variation	Degrees of freedom	Type III sum of square	Mean square	F value	P ≤0.05
Disease incidence	Genotypes (IL[2])	4	672.4917	168.1229	21.08	<0.0001
	Year	3	2295.6287	765.2095	95.95	<0.0001
	Rep (Year)	8	94.8000	11.8500	1.49	0.20120
	Genotype (IL)×Year	12	2855.0402	237.9200	29.83	<0.0001
Disease severity	Genotypes (IL)	4	1.8458	0.4614	151.20	<0.0001
	Year	3	0.8371	0.2790	91.44	<0.0001
	Rep (Year)	8	0.0229	0.0028	0.94	0.49770
	Genotype (IL)×Year	12	1.5514	0.1292	42.36	<0.0001
Seed yield	Genotypes (IL)	4	51410.7975	12852.6994	21.36	<0.0001
	Year	2	79269.8395	39634.9197	65.88	<0.0001
	Rep (Year)	6	20092.3407	3348.7235	5.57	0.00100
	Genotype (IL)×Year	8	166833.0609	20854.1326	34.66	<0.0001

[1]Type III tests of fixed effects; [2]Introgression lines

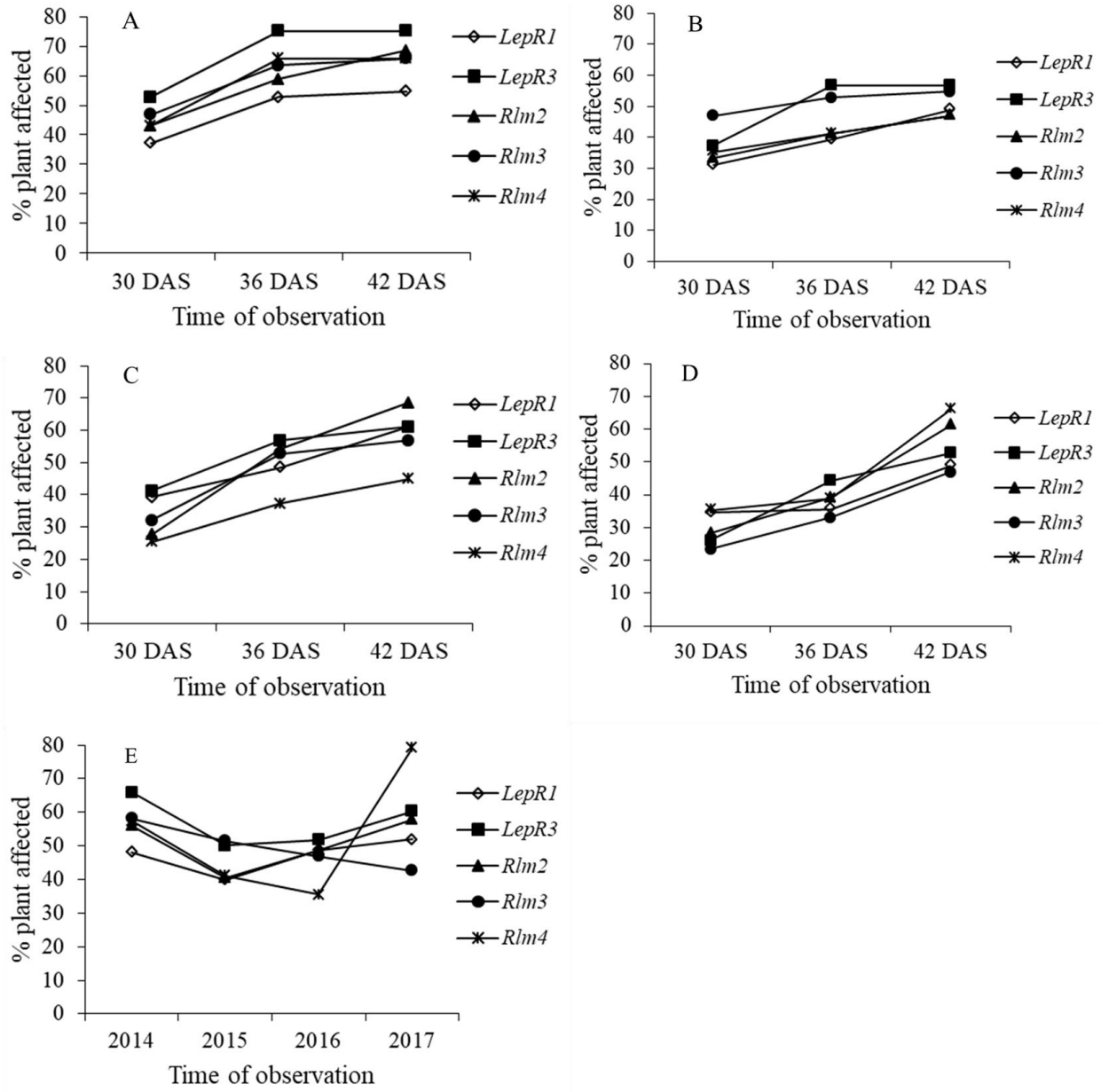

Fig. 5. 2 Disease incidence in the Topas-introgression lines (ILs) carrying a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4*. Disease incidence was calculated as % plants affected from each plot thrice at 30 days after seeding (DAS), 36 DAS and 42 DAS in each cropping season from trial year 2014 (A), 2015 (B), 2016 (C), 2017 (D). (E) Each data point is calculated from the sum of three observations from 2014 to 2017.

5.4.3 Disease severity

Statistically significant differences were found in disease severity ($p<0.0001$) among the *R* genes tested as well as the years (Table 5.3). The higher disease severity of ~1.0 out of a scale 0-5 was revealed by the IL harbouring single *R* gene *LepR3* in the year 2014 whereas the same *R* gene produced much lower disease severity (score 0.2 out of a scale 0-5) in the year

2016 and a little bit higher in 2017 (Fig. 5.3A). Similarly, disease rating of ~1.0 was observed from *R* gene *Rlm3* in the years 2015 and 2016 but lower disease severity (0.52 on a scale 0-5) was observed in 2017 (Fig. 5.3A). On the contrary, the IL carrying *R* gene *Rlm2* showed a disease severity rating of ~1.0 in the year 2017 but lower rating (0.42 out of a scale 0-5) in 2016. The rest of the ILs carrying other *R* genes showed disease severity < 0.70 irrespective of the year (Fig. 5.3A). This indicated an impact of crop rotation on disease severity. The interaction between ILs-harbouring *R* genes and year was also significant for disease severity ($p<0.0001$; Table 5.3). Overall, canola production from 2015 and onward resulted in significantly lower disease severity for all *R* genes tested except *Rlm2* (Fig. 5.3A) indicating a possible role of canola-wheat rotation.

Canola Council of Canada's Western Canada Canola and Rapeseed Recommending Committee (WCC/RRC) usually rated resistant cultivars based on the relative disease severity over the susceptible control in where disease severity of up to 30% is considered as resistant, 31-50% considered moderately resistant, 51-75% considered moderately susceptible, and >75% considered as susceptible to the blackleg pathogen. In our study, statistically significant differences were found in relative disease severity among the ILs-harbouring different *R* genes (Fig. 5.3B). The highest disease severity was 65% of Westar produced by the *Rlm3*-harbouring IL in the year 2015, which was reduced to 34% in the year 2017 (Fig. 5.3B). The *LepR3*-harbouring IL produced relative disease severity of 43% in 2014 which was reduced to 12% in 2017 (Fig. 5.3B). The rest of the *R* genes showed disease severity <30% of Westar irrespective of the year (Fig. 5.3B). Thus the results indicated an effectiveness of crop rotation in reducing disease severity as well as increasing the efficiency of single *R* genes.

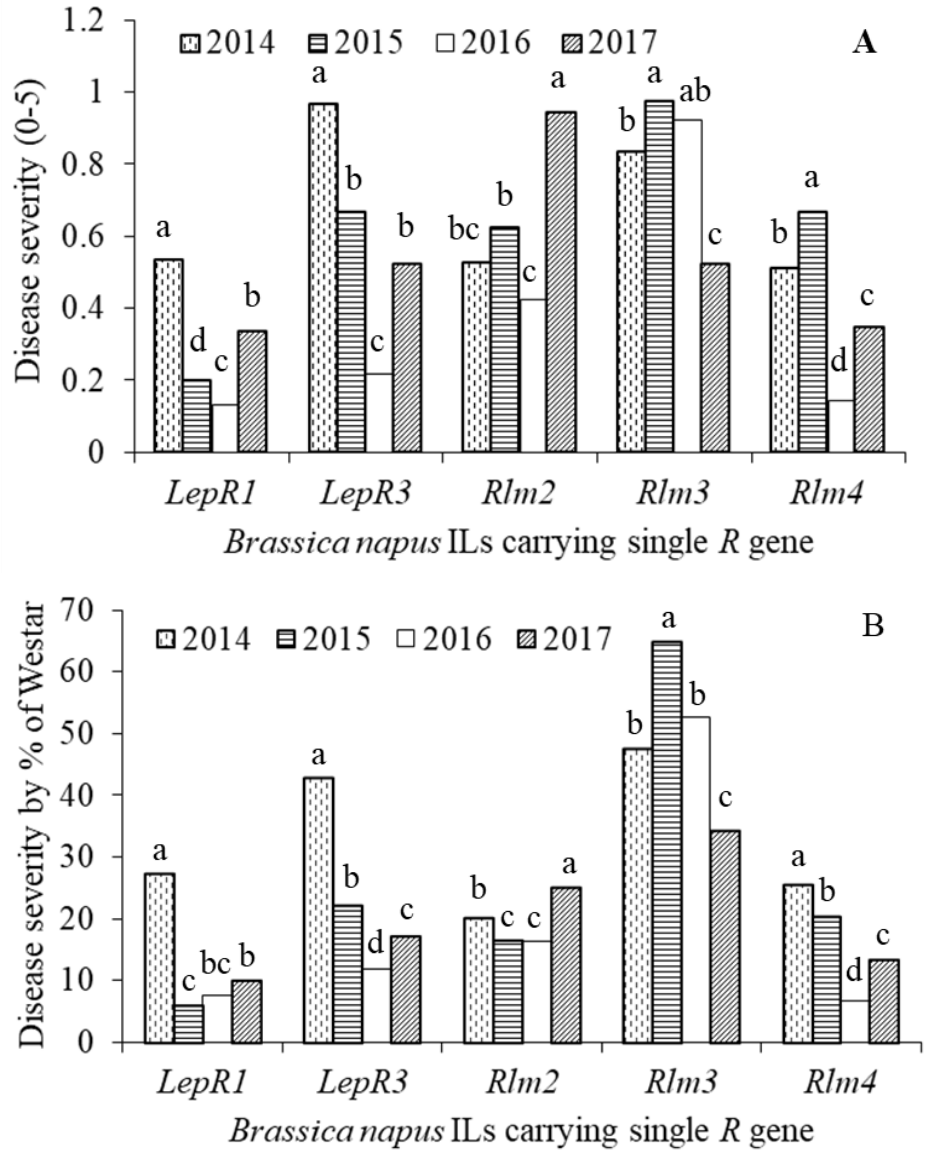

Fig. 5. 3 Disease severity in the Topas-introgression lines (ILs) carrying a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4*. Disease severity rated using a scale of 0-5 (**A**), and relative disease severity by a susceptible cultivar Westar (**B**). Rating scale was followed as West et al. (2002) where: 0 = no visible infection; 1= disease tissue occupies ≤25% of the cross section; 2 = disease tissue occupies 26-50% of the cross section; 3 = between 51 and 75% of the cross section infected; 4 = >75% of the cross section infected but plant alive and 5 = 100% of cross section infected and plant dead. Bars with the same letter are not significantly different at $P \leq 0.0001$ according to Fisher's LSD test.

5.4.4 Analysis of avirulence genes in *Leptosphaeria maculans* isolates

L. maculans isolates cultured from the infected stubble collected from *Rlm3*-stubble in 2014 showed 30% of the isolates carried *AvrLm3*, which could not be isolated from 2015- and 2016-stubble. The frequency of *AvrLm3* carrying isolates was again increased to 30% in the *Rlm3*-stubble in 2017 (Fig. 5.4). As a result, the change of *AvrLm3* to *avrLm3* is postulated since *AvrLm3* carrying isolates could not be isolated from the *Rlm3*-stubble in 2015 and 2016 (Fig. 5.4) and is predicted to be the reason of higher disease severity by the IL carrying *Rlm3* in 2015 and 2016 (Fig. 5.3A). Similarly, isolates cultured from *Rlm2*-stubble showed 50% isolates carried *AvrLm2* in 2014, which was reduced to 25% isolates cultured from the *Rlm2*-stubble in 2015. The frequency of *AvrLm2* carrying isolates was gained back to 45% in the *Rlm2*-stubble in 2016, but completely disappeared from the *Rlm2*-stubble in 2017 (Fig. 5.4). This could be the reason of higher disease severity in 2017 (Fig. 5.3A). The avirulence gene *AvrLm4* was found in 67% isolates cultured from the *Rlm4*-stubble in 2014, which was reduced to 50% isolates the *Rlm4*-stubble in 2015. The avirulence gene *AvrLm4* was returned to 100% isolates cultured from the *Rlm4*-stubble in 2016 but it was again decreased to 57% isolates in 2017 (Fig. 5.4). Isolates cultured from the *LepR1*-stubble showed only 25% isolates carried *AvrLepR1* in 2014, which was increased to 80% isolates in the *LepR1*-stubble collected from 2016, and consistently showed the higher frequency for the following years (Fig. 5.4). The avirulence gene *AvrLepR3* was found in only 15% isolates cultured from the *LepR3*-stubble in 2014, which was increased to 40% isolates from the *LepR3*-stubble in 2015. It was further increased to 90% isolates in the *LepR3*-stubble in 2016 but again decreased to 40% isolates in 2017 (Fig. 5.4). The avirulence genes *AvrLm5*, *AvrLm6*, *AvrLm7* and *AvrLm11* were found at consistently higher frequency in the isolates cultured irrespective of the stubble (Fig. 5.4), and it is expected to be the reason that no canola cultivars carried single *R* genes *Rlm5, Rlm6, Rlm7,* or *Rlm11* in our rotation. The frequency of *AvrLm9* in blackleg

isolates cultured from 2014- to 2017-stubble was consistent with the frequency of *AvrLm3* (Fig. 5.4), which could be due to clustered together (Balesdent et al. 2002, 2005; Ghanbarnia et al. 2012; Zhang et al. 2016). The avirulence gene *AvrLm1* was found to be very diverse in range from low to high frequency irrespective of stubbles and years. Importantly, frequency of *AvrLm1* carrying isolates cultured from the *LepR3*-stubble was increased from 25% in 2014 to 85% in 2017 (Fig. 5.4). However, the return of *AvrLm3*, *AvrLm4*, *AvrLepR1*, and *AvrLepR3* in the stubble of the corresponding *R* genes might be due to the effectiveness of canola-wheat rotation and pathogen adaptation.

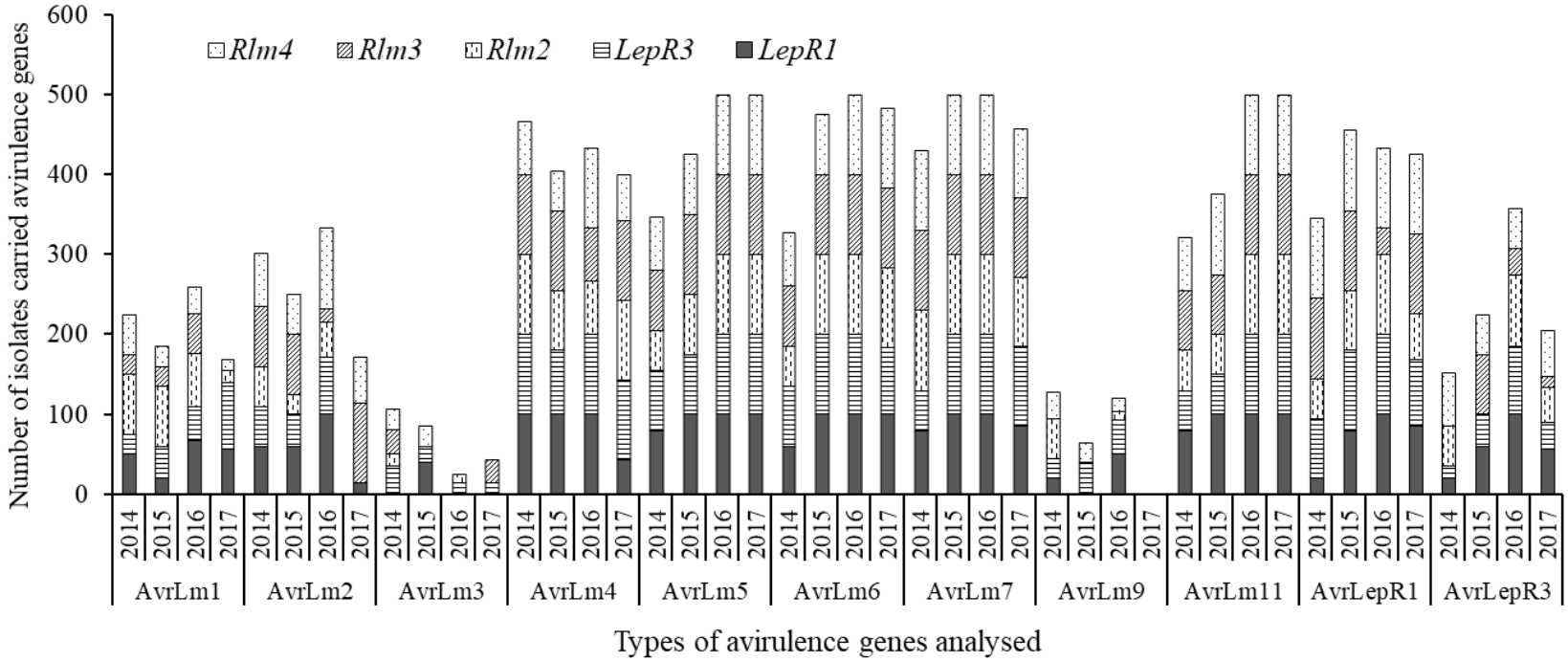

Fig. 5. 4 Analysis of avirulence genes profile in *Leptosphaeria maculans* isolates cultured from infected stubble. Stubble collected from each plot throughout the study period from 2014 to 2017. Bar indicating stubble from different introgression lines with a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4*.

5.4.5 Seed yield

Seed yield significantly varied among the *R* genes-harbouring ILs ($p<0.0001$; Table 5.3). The ILs carrying specific *R* gene *LepR1, LepR3, Rlm2, Rlm3,* and *Rlm4* produced 353-410 g, 233-570 g, 319-402 g, 322-473 g, and 420-525 g seed yield per m^2 area, respectively, (Fig. 5.5A). Seed yield also significantly varied throughout the study period ($p<0.0001$; Table 5.3). Seeding year had no significant effect in seed yield produced by the IL harbouring *LepR1* gene. Canola yield was lowest in 1st year (2014) of the study compared to 4th year (2017) for all ILs tested except *Rlm2*-harbouring IL (Fig. 5.5A). Seed yield per m^2 decreased by 55 g for *Rlm2*-harbouring IL, corresponding to a decline in yield of 15% in 2017 compared to 2014. It may be an effect of drier condition as we have seen less precipitation in 2017 (Table 5.2), which may influence pathogen to be changed from avirulent to virulent and ultimately produced higher disease (Fig. 5.3A; Fig. 5.4). Due to the reduction of blackleg severity in 2017, there was a yield benefit from the ILs harbouring *R* genes *LepR1* (14%), *Rlm3* (20%), and *Rlm4* (11%) (Fig. 5.5 A). A remarkable yield increment of 145% in 2017 compared to 2014 from the *LepR3*-harbouring IL was observed (Fig. 5.5A) indicating a yield potentiality of the gene *LepR3* under disease pressure.

The regression analysis revealed that the seed yield increased for all ILs harbouring different *R* genes as disease severity decreased and vice versa (Fig. 5.5B). The coefficients of the determination for all *R* genes varied from 71% to 99% indicating the fitness of data to the model also varied slightly for the *R* genes tested. For example, the regression model of *LepR1* for seed yield versus disease severity was: $y = -176.758x + 451.61$ where the coefficient of determination ($R^2= 0.9224$) for the model explained 92% fit to the variation of data point. From this model 274.85 g of seed yield per m^2 reduction from *LepR1*-harbouring IL was calculated for every unit increase of disease severity. Similarly, the model for *LepR3*-, *Rlm2*-, *Rlm3*-, and *Rlm4*-harbouring IL has explained the yield reduction of 179.77 g, 314.41 g,

324.53 g, and 317.78 g, respectively for every unit increase of disease severity. Yield reduction of *LepR3* and *LepR1* were less sensitive to disease severity and therefore considered to be more stable compared to the other *R* genes. From the yield reduction point of view, we can predict the effectiveness of *R* genes against *L. maculans* included in the study as *LepR3 > LepR1> Rlm2 > Rlm4 > Rlm3*.

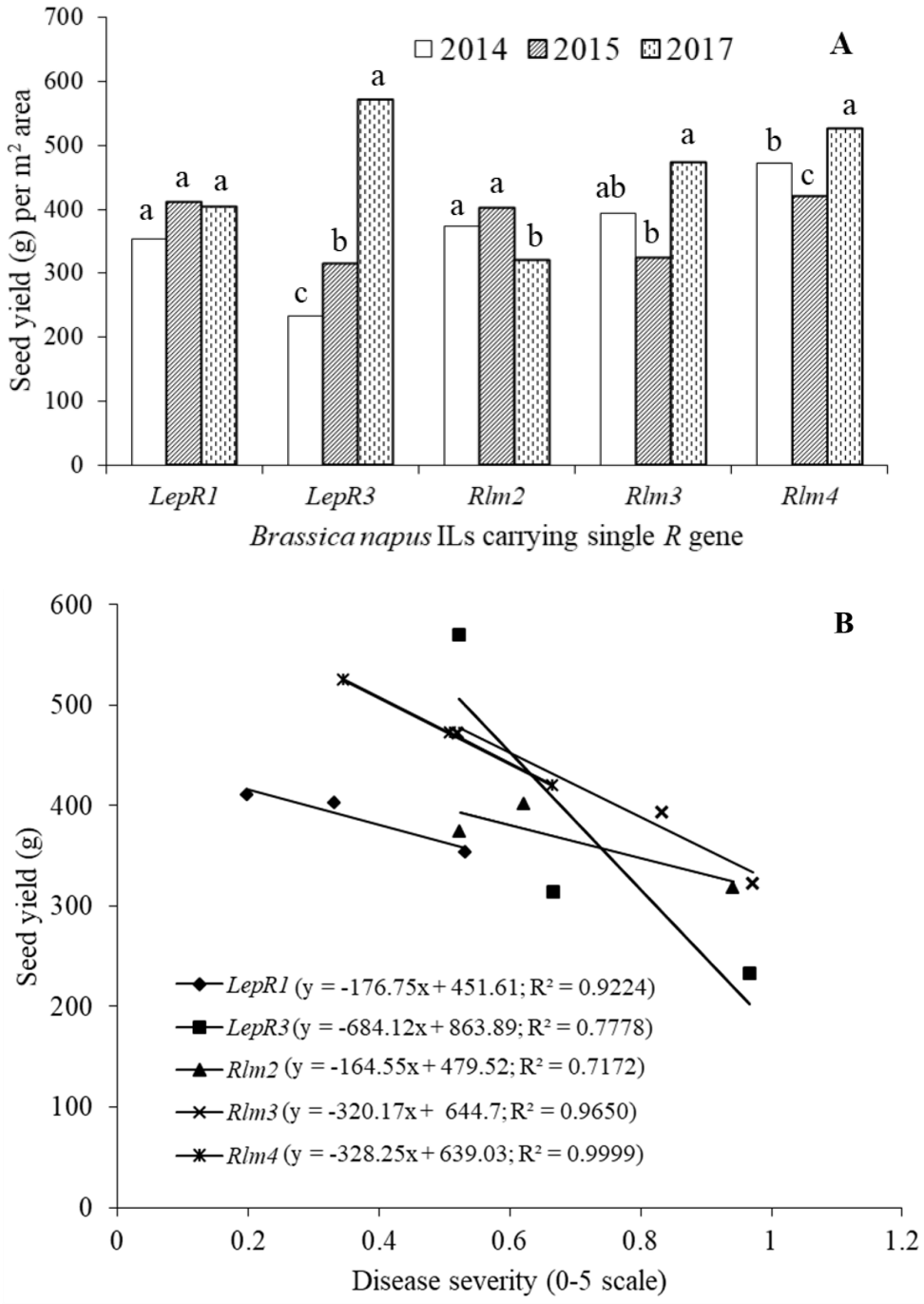

Fig. 5. 5 The representative seed yield from the Topas-introgression lines (ILs) carrying a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4* (A), and the relationship between blackleg disease severity and seed yield (B). Data were collected over three years at Ian Morrison Research Station, Manitoba. Each point represents the mean of three replications × three-years. Blackleg severity was assessed on a 0–5 scale, where: 0 = no disease and 5 = 100% diseased with death of the plant (see text). Bars with the same letter are not significantly different at $P \leq 0.0001$ according to Fisher's LSD test (A).

5.5 Discussion

Based on current literature, this is the first extensive report on the *R* genes performance in *B. napus* assessed in a canola-wheat over 2-year crop rotation. The connection between seeding year and blackleg severity in *B. napus* ILs-harbouring *R* genes over 4-years study clearly showed the effectiveness of canola rotation with wheat on the *R* genes efficiency against *L. maculans*. Five known *R* genes; *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4* were assessed from 2014 to 2017, and all tested *R* genes produced significantly lower disease from 2015 through 2017 as compared to 2014. The strong linear relationship between seed yield and disease severity clearly indicated an order of *R* genes effectiveness; *LepR3* > *LepR1* > *Rlm2* > *Rlm4* > *Rlm3* in a western Canadian field environment.

In this study, early seedling stage (30 DAS) had lower disease incidence as compared to either vegetative or flowering stage, and throughout the growing season had moderate to high disease incidence for all *R* genes tested (Fig. 5.2A-D). It indicated the fact that the pycnidia were produced over the growing season through asexual life cycle of *L. maculans* predominant in western Canada (Guo et al. 2005; Ghanbarnia et al. 2011). Moreover, environmental factors such as temperature, rainfall, and inoculum density could also affect the disease incidence (Hall 1992). Significant differences in the incidence and severity of the disease were observed among the ILs tested, indicated the effectiveness of genetic resistance in combating the disease (Table 5.3; Fig. 5.2, 5.3). Blackleg severity was significantly decreased in 4th year compared to 1st year of the study in *B. napus* ILs except IL harbouring *Rlm2* (Fig. 5.3A). *B. napus* IL harbouring *R* gene *Rlm2* produced unexpectedly higher disease in the 4th year of the study. It could be due to the shifting of pathogen virulence toward *Rlm2*. As we have seen much less precipitation in 2017 compared to the long-term average (Table 5.2), which might trigger pathogen virulence toward *Rlm2*, suggesting compatible interaction between *R*- and *Avr*-gene. *B. napus* ILs carrying *R* genes *LepR1* and *Rlm4* was much less

affected by the disease throughout the study period which is an indication of the stability of these genes at least in the environment of Manitoba. Recent study in western Canada has shown disease severity <0.5 for canola-wheat rotation in a farmer's field (Kutcher et al. 2013), whereas our study found disease severity <1.0. This is a bit higher than the previous study which might be due to the higher disease pressure as two years inoculation had been done here for the 4-year study. Marcroft et al. (2004b) from Australia reported that distance between canola crops is more important than the frequency of canola in the rotation to manage blackleg disease. However, this finding may be true for Australia, but may not be accurate for Canada.

Leptosphaeria maculans is a stubble-borne pathogen of canola (Rimmer et al. 2003). The benefit of crop rotation to control blackleg, similar to other residue-borne diseases, depends on the length of time the infected residue requires to degrade. Continuous canola cultivation does not allow infected stubble to fully decompose; *L. maculans* can complete and repeat its lifecycle, resulting in its multiplication and dissemination. This indicates that blackleg disease management must be improved further with the rotation of canola instead of monoculture. However, the rate of stubble breakdown depends on environmental conditions, i.e. under dry conditions residue breakdown is slower than under wet conditions (Sosnowski et al. 2006). Hot-dry summers in western Canada may increase the amounts of infected canola stubble, which may contribute to more inoculum and a larger pathogen population in the successive years and therefore to greater potential infections of future crops in monoculture. For these reasons, it is of concern that the amount of blackleg-infested stubble in this study was noted to increase dramatically in the patches of guard rows as it followed continuous canola seeding. In addition, larger pathogen populations may increase selection pressure on the pathogen to overcome the genetic resistance in existing canola cultivars (Rouxel et al. 2003a; Li et al. 2005). Despite cultivation of blackleg resistant ILs, the number

of diseased plants was 10-20% lower in 2nd - 4th year of the study as compared to 1st year (Fig**.** 5.2E). Similarly, relative disease severity of 20-30% was also low for ILs carrying *LepR1*, *LepR3*, *Rlm3*, and *Rlm4* from 2015 to 2017 as compared to 2014 (Fig. 5.3B). Our results supported by the previous research where they found crop rotation reduced the incidence and severity of blackleg in canola as compared to monoculture (Guo et al. 2005; Johnston et al. 2005; Kutcher et al. 2013). Results of this study were also supported by the conclusions of Marcroft et al. (2012a), who found the reduction of diseases when a resistant canola cultivar was seeded on stubble from different resistant varieties compared to on its own stubble. In a way "logical" but an important message to farmers to keep rotating varieties with differences sources for resistance.

Variability for the virulence of *L. maculans* has been reported in western Canadian isolates (Kutcher et al. 2007; 2010; Fernando et al. 2018). Knowing of virulence / avirulenec genes in *L. maculans* isolates has an important implication while deploying *R* genes in the management tactics of blackleg disease. For instance, studies on *Avr*-genes in *L. maculans* isolates collected in 2010 and 2011 from canola field showed potential effectiveness of *Rlm2*, *Rlm4*, *Rlm6* and *Rlm7* in Canada (Liban et al. 2016). Similarly, analysis of European isolates of *L. maculans* collected in 2002 and 2003 indicated possible efficiency of *Rlm6* and *Rlm7* in Europe (Stachowiak et al. 2006). This study indicated high frequency of *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLepR1* while very low frequency of *AvrLm3*, and *AvrLm9* in the *L. maculans* isolates cultured from the stubble carried corresponding *R* genes. On the other hand, *Avr*-genes *AvrLm1*, *AvrLm2*, and *AvrLepR3* in the *L. maculans* isolates varied from low to high frequency by the years (Fig. 5.4). Importantly, *Avr*-genes *AvrLm3*, *AvrLm4*, *AvrLepR1*, and *AvrLepR3* were gained back in the *L. maculans* isolates cultured from the stubble of corresponding *R* genes (Fig. 5.4), indicated the effectiveness of canola-wheat rotation and pathogen fitness. Based on knowledge about *Avr*-genes in *L. maculans*

isolates and *R* genes tested in the study *LepR1*, *LepR3*, and *Rlm4* are undoubtedly very effective in present Canadian *R* genes deployment strategies with at least 2-years crop rotation. Additionally, other effective *R* genes such as *Rlm5*, *Rlm6*, *Rlm7*, and *Rlm11* can be introduced into Canadian canola cultivars. On the contrary, based on phenotype *Rlm3* and *Rlm9* were overcome and *Rlm1* and *Rlm2* are predicted to be overcome. Previous work done in our lab showed extensive use of *Rlm3* in Canada, but no evidence for wide use of *Rlm1*, *Rlm2* and *Rlm9* in Canada (Zhang et al. 2016). In addition to the changes of frequency of *Avr*-genes, blackleg disease on canola has varied as shown in Canadian disease survey (http://phytopath.ca/publication/cpds). In this study, the increment of disease was correlated with the decrease of *AvrLm2* frequency from 2017 data (Fig. 5.3A, 5.4). This result strongly supports the reflection of increased disease due to shifting of *Avr*-gene from *AvrLm2* to *avrLm2*.

Variation in disease incidence and blackleg severity was observed among the *R* genes tested, which reflected genetic efficiency against the disease. This was definitely a factor, although not the only factor responsible for the yield difference among the ILs over the years. The decrease of blackleg incidence in 2nd, 3rd, or 4th year among the *R* genes tested except *Rlm4*, indicated that this practice can be expected to result in a decrease in the pathogen population. Since *Rlm4*-carrying IL exhibited higher disease incidence in 4th year unexpectedly, farmers have to be cautious during selection of the cultivars. They may not select the cultivars which carry only *Rlm4*; rather select cultivars which carry *Rlm4* plus another *R* gene or quantitative resistance. On the other hand, blackleg disease severity was decreased in 4th year trial from ILs harbouring *LepR1*, *LepR3*, *Rlm3*, and *Rlm4* but increased in *Rlm2* indicated a possible increase in the pathogen population, even on resistant cultivars (Fig. 5.3A). This unexpected disease increment in 2017 by the *Rlm2*-harbouring IL could be due to shifting of *AvrLm2* to *avrLm2*, which is reflected by the *Avr* genes analysis study (Fig.

5.4), and this kind of sudden shift is believed to be due to the deletion, point mutation and sexual recombination of the pathogen (Gout et al. 2007; Fudal et al. 2009; Daverdin et al. 2012).

It is believed that the effectiveness of *R* genes is the most important factor in controlling blackleg worldwide. Development of canola varieties in combination of qualitative resistance (*R* gene) into quantitative background is one of the best strategies to improve the effectiveness of a single *R* gene (Pietravalle et al. 2006; Brun et al. 2010; Delourme et al. 2014). The alternative approaches of the improvement of the effectiveness of existing *R* genes are cultural management including crop rotation (Kutcher et al. 2013), *R* genes rotation (Marcroft et al. 2012a), and fungicides application (West et al. 2001; Fitt et al. 2006). In this study, canola-wheat a 2-year rotation showed the increment of blackleg resistance among the *R* genes tested (Fig. 5.3B), where *LepR1*, *LepR3, Rlm2*, *Rlm4* showed resistance to blackleg disease, and *Rlm3* showed moderate resistance. However, canola yield was compromised by the impact of blackleg on the crop. The degree of impact was demonstrated statistically by the analysis of linear regression: as blackleg severity increased, seed yield of canola ILs was decreased and vice versa (Fig. 5.5B). Hall (1992) reviewed several studies where he reported a linear relationship between seed yield and disease severity in rapeseed. For example, McGee et al. (1977) categorized plants into two groups such as severely diseased, and healthy or moderately diseased, in where they were able to show a relationship between crop loss and severely diseased plants. In the UK, winter oilseed rape yield-loss model for stem canker was estimated based on disease incidence on stems before harvest (Church and Fitt 1995; Sansford et al. 1996) and yield-loss model for leaf spot was estimated in relation to the area under disease progress curve (AUDPC) at seedling stage (Sutherland et al. 1995). In a recent study in Canada rated disease severity based on stem necrosis on a 0–5 scale and reported a negative linear relationship between seed yield and

disease severity, where they showed 18% reduction of seed yield for each unit increase of disease severity (Hwang et al. 2016). In this study, similar to Hwang et al. (2016) plants were rated for disease severity at maturity using a scale of 0-5 (West et al. 2002). The regression analysis showed a negative linear relationship between seed yield and blackleg severity, as we have seen linear reduction of yield with increase of blackleg severity (Fig. 5.5B). Calculation from the regression model for *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4* has explained the yield reduction of 2748.5 kg, 1797.7 kg, 3144.1 kg, 3245.3 kg, and 3177.8 kg per hectare, respectively, for every unit increase of disease severity (Fig. 5.5B). Our study showed a smaller variation in the coefficients of the determination (R^2), ranging from 71% to 99% among the model tested. Considerable variation in the coefficient of determination also has shown in previous studies (McGee et al. 1977; Hwang et al. 2016). The variation of yield-loss coefficients could be due to the wide range of climatic conditions and production practices under which blackleg occurs (West et al. 2001). The data of the present study were reflected from Manitoba climates and western Canadian production practices. These results will assist industry as well as growers developing appropriate strategies which will reduce blackleg infestation and minimize yield losses. Marcroft et al. (2012a) demonstrated the efficiency of *R*-gene rotation in reduction of disease pressure by manipulating avirulent allele frequency in pathogen populations. However, *R* genes rotation in Canada currently is a bit challenging due to the limited number of *R* genes in existing canola germplasm other than *Rlm3* (Zhang et al. 2016), and the lack of knowledge of *R* genes in commercial canola cultivars as well. The good part is canola breeding company such as Monsanto, and DL seeds started labelling cultivars with *R*-genes, and suggested potential rotation among their cultivars. As the *R*-genes become known and available, and with the advent of *R*-genes on cultivar labels, the knowledge gained from this research will become more useful.

Here we investigated the effectiveness of five *R* genes based on their corresponding *Avr*-gene frequency under field conditions, which recommended an order of efficiency among the *R* genes tested; *LepR3* > *LepR1*> *Rlm2* > *Rlm4* > *Rlm3*. This work will be a potential foundation for breeding programs in the canola industry in Canada which will develop cultivars with effective *R* genes against the blackleg pathogen, and finally, will be included in the proposed cultivar rotation strategies on the Prairies in the near future.

5.6 Acknowledgements

The work was funded by SaskCanola, and AAFC-Growing Forward 2 program. Dr. Hossein Borhan's lab, AAFC Saskatoon Research Station, Saskatchewan is highly acknowledged for providing seeds of *R* genes introgression lines utilized in this study. We would like to thank Paula Parks and Alvin Iverson for help in the field study at Carman Research Station University of Manitoba. Xuehua Zhang and Sakaria Liban are acknowledged for the initial set up of the experiment in the field in 2013, characterizing and selecting the appropriate isolates and their alleles for the experiment.

Table S5.1 Calculation of 90% avirulence plus 10% virulence isolates of *Leptosphaeria maculans* to the corresponding *R* genes.

AvrLepR3	%	Plates	*AvrLm2*	%	Plates	*AvrLepR1*	%	Plates	*AvrLm3*	%	Plates	*AvrLm4*	%	Plates
RL4	18.00%	8.064	RL3	12.86%	5.76	RL4	18.00%	8.064	DM96	45.00%	20.16	RL4	11.25%	5.040
X44	18.00%	8.064	X65	12.86%	5.76	X108	18.00%	8.064	DM118	45.00%	20.16	X108	11.25%	5.040
X65	18.00%	8.064	X159	12.86%	5.76	X159	18.00%	8.064				X159	11.25%	5.040
X108	18.00%	8.064	DM88	12.86%	5.76	DM88	18.00%	8.064				DM88	11.25%	5.040
DM88	18.00%	8.064	MB11-31-2	12.86%	5.76	MB11-31-2	18.00%	8.064				MB11-31-2	11.25%	5.040
			DM96	12.86%	5.76							RL3	11.25%	5.040
			DM118	12.86%	5.76							X44	11.25%	5.040
												X65	11.25%	5.040
avrLepR3	%	Plates	*avrLm2*	%	Plates	*avrLepR1*	%	Plates	*avrLm3*	%	Plates	*avrLm4*	%	Plates
RL3	2.00%	0.896	RL4	3.33%	1.49	RL3	2.00%	0.896	RL4	1.25%	0.56	DM96	5.00%	2.240
X159	2.00%	0.896	X44	3.33%	1.49	X44	2.00%	0.896	X108	1.25%	0.56	DM118	5.00%	2.240
MB11-31-2	2.00%	0.896	X108	3.33%	1.49	X65	2.00%	0.896	X159	1.25%	0.56			
DM96	2.00%	0.896				DM96	2.00%	0.896	DM88	1.25%	0.56			
DM118	2.00%	0.896				DM118	2.00%	0.896	MB11-31-2	1.25%	0.56			
									RL3	1.25%	0.56			
									X44	1.25%	0.56			
									X65	1.25%	0.56			
Total Plates		44.80	Total Plates		44.79	Total Plates		44.80	Total Plates		44.80	Total Plates		44.80
Pooled Total														224.00

Table S5.2 Analysis of variance[1] for disease incidence at 30 days after seeding (DAS), 36 DAS, and 42 DAS of *Brassica napus* introgression lines (IL) in a 4-years study from 2014 to 2017 at the Ian Morrison Research Station, Carman Manitoba

Source of variation	Degrees of freedom	Type III sum of square	Mean square	F value	P value
Genotype (IL[2])	4	1574.46254	393.61564	37.07	<0.0001
DAS	2	14336.71915	7168.35957	675.12	<0.0001
Year	3	7297.13571	2432.37857	229.08	<0.0001
Rep (Year)	8	14.13333	1.76667	0.17	0.99480
Genotype (IL)×Year	12	3577.96883	298.16407	28.08	<0.0001
Genotype (IL)×DAS	8	760.25788	95.03224	8.95	<0.0001
Genotype (IL)×DAS×Year	30	2260.84447	75.36148	7.10	<0.0001

[1]Type III tests of fixed effects; [2]Introgression lines

6 IMPACT OF *BRASSICA NAPUS-LEPTOSPHAERIA MACULANS* INTERACTION IN THE EMERGENCE OF VIRULENT ISOLATES OF *L. MACULANS*, A CAUSAL AGENT OF BLACKLEG DISEASE IN CANOLA

6.1 Abstract

Canola (oilseed rape, *Brassica napus* L.) is one of the most stable oilseed crops grown today in Canada and other temperate parts of the world. The crop is prone to a few major diseases including blackleg, caused by the fungus *Leptosphaeria maculans*. In Canada, growers have recently begun to grow canola more intensively because of the market demand and the development of cultivars harbouring single *R* genes. A 4-year study from 2014 to 2017 at the Ian N. Morrison Research Station Carman Manitoba was conducted to investigate the effect of *B. napus-L.maculans* interaction in the emergence of virulent isolates toward specific *R* genes under field conditions over a 2-year rotation. Blackleg incidence was reduced by a maximum of 40% in 2017 compared to 2014 for all *R* genes tested, except for *Rlm4*. Disease severity was reduced by 52–21% in the 4th year compared to the 1st year, regardless of the *R* genes tested, except for *Rlm2*. Severity rating based on the susceptible cultivar Westar showed that the *R* genes tested here all were resistant to blackleg in 2017. There were no *AvrLm2*- and *AvrLm4*-carrying isolates in 2017, which led to the generation of virulence towards *Rlm2* and *Rlm4* within a year. Sequencing of the *AvrLm2* gene from 2014- and 2015-isolates revealed a shift in the *AvrLm2* to the *avrLm2* allele due to the accumulation of point mutations. In addition to that, masking of the *AvrLm3* phenotype by the presence of the *AvrLm4-7* allele was also confirmed by analysing the phenotypes and genotypes of the isolates collected from 2014 to 2017. Based on previous and current studies, we predict that alternating *Rlm3*, *Rlm4*, and *Rlm7* could provide an opportunity to increase the durability of those *R* genes in a 2-year crop rotation in Canada. Additionally, this study proposed the development of a generic epidemiological model that takes into account complex molecular

mechanisms, allowing plant breeders to select appropriate *R* genes in the proposed cultivar rotation strategies in the Prairies.

Key words: avirulence, blackleg, *Brassica napus*, evolution, gene-for-gene interaction, *Leptosphaeria maculans*, *R* gene

6.2 Introduction

Canola (oilseed rape/*Brassica napus* L.) is an extensively grown crop in Canada and worldwide. The crop is susceptible to a few major diseases, including blackleg or stem canker, which is caused by the fungal pathogen *Leptosphaeria maculans*. The disease can be controlled by the use of resistant varieties, crop rotation, and the application of fungicide (Kutcher et al. 2013; Marcroft et al. 2012a; Fitt et al. 2006; West et al. 2001). The use of genetic resistance is generally a very effective strategy in disease control. Two types of resistance to *L. maculans*, qualitative control by single dominant resistance (*R*) genes and quantitative control by minor genes, have been identified in *Brassica* species (Jestin et al. 2015; 2011; Raman et al. 2013; Balesdent et al. 2001; Pilet et al. 2001; 1998; Pongam et al. 1998). In Canada, most of the germplasm and commercial cultivars of *B. napus* carry single dominant *R* genes (Zhang et al., 2016). *R* genes usually confer race-specific resistance, and follow the typical gene-for-gene interaction proposed by Flor (1971). The wider use of single *R* genes in commercial varieties usually exerts a strong selection pressure for *L. maculans*, which leads to the reduction of corresponding avirulence (*Avr*) genes in populations. For example, the large-scale use of a single *R* gene *Rlm1* in France resulted in a rapid decline of *AvrLm1* in the *L. maculans* isolates (Rouxel et al. 2003a). Similarly, *RlmS*- and *LepR3*-mediated resistance was overcome within three years after their deployment in Australia, causing dramatic yield losses (Sprague et al. 2006; Van de Wouw et al. 2010a). Additionally, the short durability of *Rlm6* and *Rlm7* was also reported from a controlled field experiment in France (Brun et al. 2010; Daverdin et al. 2012; Balesdent et al. 2015). A more recent study in

western Canada reported the loss of *Rlm3*-mediated resistance as the number of *AvrLm3*-carrying isolates decreased in a pathogen population (Zhang et al. 2016); this is believed to be the result of the intensive use of *Rlm3*-harbouring canola varieties. Moreover, the race structure of *L. maculans* in western Canada has also changed over time (Kutcher et al. 2010; Liban et al. 2013); this is believed to be the result of the use of specific *R* gene(s). Fernando et al. (2018) provided further evidence for this when their study revealed a shift in the avirulence allele in Canadian isolates collected from 2010 to 2015. All of those studies indicated that the pathogen can adapt to a single *R* gene due to the selection pressure within a short period of time in both experimental conditions and in agricultural systems. As a result, the pathogenicity of *L. maculans* populations has changed over time in Canadian Prairies (Kutcher et al. 1993; 2007; Keri et al. 2001; Chen and Fernando 2006). This shows the necessity of determining the timeline for the shift of *L. maculans* genes from avirulent to virulent in Canadian field conditions.

The evolutionary potential of *L. maculans* is characterised by its genome structure and its complex life cycle. Sexual reproduction generating wind-borne ascospores permits the generation of recombinant virulent isolates and this can spread over great distances. Furthermore, genome sequencing of *L. maculans* isolate JN3 indicated that avirulence genes usually are distributed in gene-poor and AT-rich regions (Rouxel et al. 2011). These characters are responsible for the rapid evolution of virulent isolates via deletions and repeat-induced point mutations (RIPs) (Gout et al. 2007; Fudal et al. 2009; Daverdin et al. 2012). To date, a total of 16 *Avr* genes have been identified in *L. maculans*, and seven of these (*AvrLm1*, *AvrLm6*, *AvrLm5/J1*, *AvrLm2*, *AvrLm4-7*, and *AvrLm3*) have been cloned (Gout et al. 2006; Fudal et al. 2007; Van de Wouw et al. 2014; Ghanbarnia et al. 2015; Parlange et al. 2009; Balesdent et al. 2013; Plissonneau et al. 2015). Cloning and protein structure analysis reported that *Rlm4*- and *Rlm7*-mediated resistance is triggered by a functional allele of

AvrLm4-7, but *Rlm4*-mediated resistance has been avoided mainly by a single base mutation in the *AvrLm4-7* allele, as seen in European isolates of *L. maculans* (Balesdent et al. 2006; Stachowiak et al. 2006; Parlange et al. 2009). Additional evidence of this was found from a recent study (Fernando et al. 2018), where they found only the *avrLm4AvrLm7* and *AvrLm4AvrLm7* phenotypes; none of the isolates had the *AvrLm4avrLm7* phenotype. It has been reported that a single base mutation C^{358} to G^{358} in the *AvrLm4-7* gene, resulting in an amino acid change from arginine (R) to glycine (G), is sufficient to complement the loss of *Rlm4*-mediated resistance (Parlange et al. 2009). In addition to that, an epistatic interaction has been observed between *AvrLm3* and *AvrLm4-7*, where the *AvrLm3* allele is present but virulent towards *Rlm3*, which is believed to be the reason of masking of the *AvrLm3*-effect in the presence of *AvrLm4-7* allele (Plissonneau et al. 2016, 2017). Earlier phenotypic studies showed that 99% of isolates were avirulent towards *Rlm7* but virulent towards *Rlm3* (Balesdent et al. 2006; Stachowiak et al. 2006). In contrast, recent studies have shown a shift in virulent isolates towards *Rlm7,* with an associated gain of avirulence towards *Rlm3* (Plissonneau et al. 2016). Importantly, the deletion or inactivation of the *AvrLm4-7* allele led to the reappearance of the *AvrLm3* phenotype (Plissonneau et al. 2016). These observations suggested that *AvrLm3* is present in most of the isolates of *L. maculans*, but 'camouflaged' by the *AvrLm4-7* allele (Rouxel and Balesdent 2016). Therefore, we need to understand the complex interaction between avirulence genes and the shift in genes from avirulent to virulent in the Canadian agricultural system.

Crop rotation is thought to be an important strategy controlling residue-borne plant pathogens (Curl 1963). It has an effect on reducing the pathogen population so that significant crop loss does not occur. Generally, the durability of a single *R* gene can be strengthened by longer crop rotations, i.e. 3 or more years (Cook 2006; Kutcher et al. 2013). Due to high market demand for canola, two-year rotations of canola with cereals, or even

canola monoculture are becoming more popular in the Canadian Prairies. The effectiveness of the 2-year canola-cereals-canola rotation in terms of disease suppression or *R* gene durability is still questionable. However, researchers, producers and industry need to understand the possible unwanted outcomes, i.e. an increase in virulence towards the existing *R* genes. Therefore, the aim of this study was to investigate the effect of *B. napus-L. maculans* interactions in the emergence of virulent isolates over a 2-year crop rotation in a 4-year field study to address the question of the sustainability of the short crop rotation.

6.3 Materials and Methods

6.3.1 Plant materials

Lines with single dominant *R* genes with resistance to *L. maculans* (*Rlm*) were used in this study. *Brassica napus* introgressed lines (ILs) harbouring *Rlm2, Rlm3, Rlm4, LepR1,* and *LepR3* were used in the study along with Westar as a susceptible control. The single *R* genes *Rlm2, Rlm3, Rlm4, LepR1* and *LepR3* were introgressed into the Topas DH16516 background by AAFC Saskatoon and published by Larkan et al. (2016).

6.3.2 Design of the field experiment

The field experiments were established at the Ian N. Morrison Research Station (Carman, Manitoba) for five consecutive cropping seasons from 2013 to 2017. Plots were located at the south-west end of the station (Appendix V), and there was no *Brassica* cultivation for the last couple of years, as reported in the brochure published by the station. There had been no canola grown within an area of ~500 m surrounding the plots. Therefore, we assumed that the plots were completely virgin with regards to canola cultivation. The experiment began with an initial trial where plants were inoculated with a pathogen population that carried specific *Avr* genes (Table 6.1), which was considered the establishment year of the trial. This was followed by five separate 4-year trials (Fig. 5.1), each corresponding to the recurrent selection of *L. maculans* populations by one of the five ILs. The trial plots were set up for

canola-wheat-canola over a 2-year crop rotation. Five ILs were sown in a stripe plot design with three replications. Individual plots measured 8 x 6 m^2, accommodating ten rows of plants, which were spaced 0.20 m apart. Two metre gourd rows were seeded with Westar as a control between *B. napus* IL and wheat (Fig. 5.1). Temperature and rainfall data were recorded throughout the study period at the research station (Table 5.2).

6.3.3 Inoculum production and field inoculation

Ten fungal isolates previously characterised for the *Avr* genes of *L. maculans* were used to prepare the inoculum (Table 6.1). Individual plots were inoculated with a mixture of isolates that carried 100% avirulence gene for the corresponding *R* gene in the establishment year (2013) and trial year-1 (2014) to ensure sufficient disease pressure. The inoculum was applied twice, at the cotyledon and at the two leaf stage of canola. Detail calculation of the inoculum preparation for each *R* gene is shown in Table S6.1.

Table 6. 1 Avirulence gene profiles of the *Leptosphaeria maculans* isolates used in the study.

Isolates	Avirulence genes									
	AvrLm2	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm9*	*AvrLm11*	*AvrLepR1*	*AvrLepR3*
X44	a[1]	a	A	A	A	A	a	A	a	A
X65	A[2]	a	A	A	A	A	a	A	a	A
X108	a	a	A	A	A	A	a	A	A	A
X159	A	a	A	A	A	A	a	A	A	a
RL3	A	a	A	A	A	A	a	A	a	a
RL4	a	a	A	A	A	A	a	A	A	A
DM88	A	a	A	A	A	A	a	A	A	A
MB11-31-2	A	a	A	A	A	A	a	A	A	a
DM96	A	A	a	A	A	a	a	A	a	a
DM118	A	A	a	A	A	a	a	A	a	a

[1]Virulence and [2]Avirulence for the phenotype

6.3.4 Intercultural operation

Based on the farm practice in western Canada, a single shallow tillage was performed each year to crust the soil surface. The seeds were treated before seeding each year with the systemic insecticide Helix Xtra (Syngenta, Canada) @ 1.5 mL per 100g. The non-selective herbicide Glyphosate (Roundup ready) was applied pre-emergence of canola and post-

emergence herbicides including Muster, Eclipse, and Select were applied with its adjuvant Amigo. The plots were hand-weeded as required. Contact insecticide Decis® EC50 was used to control insects, particularly flea beetle, as needed.

6.3.5 Assessment of disease incidence

Disease incidence was calculated as the percentage of plants with at least one leaf lesion. Incidence was recorded for each trial three times: at 30 days after seeding (DAS), 36 DAS and 42 DAS in each cropping season. Samples of 50 plants were collected randomly each time from each replicate spread across the plot, making a total of 150 samples from each replication.

6.3.6 Assessment of disease severity

Disease severity was rated by the visual assessment of internal infection at the cross-section of the stem a week before harvest. In brief, 50 canola plants were uprooted randomly from each replicate spread across the plot. Stems were cut off at the crown and an estimated percentage of blackening area at the cross-section of the stem was rated using a scale of 0-5 (West et al. 2002), where: 0 = no visible infection; 1 = disease tissue occupies ≤25% of the cross-section; 2 = disease tissue occupies 26–50% of the cross-section; 3 = between 51 and 75% of the cross-section infected; 4 = more than 75% of the cross-section infected but plant alive and 5 = 100% of the cross-section infected and plant dead (Appendix IV).

6.3.7 Culture of *Leptosphaeria maculans* isolates from stubble

Blackleg infected stubbles were collected from each plot for the entire study period. Canola stubbles were collected right after harvest, when disease symptoms were visible at the cross-section of the stems. At least 7–8 *L. maculans* isolates were isolated from the stems collected from five different plots separately. Fungal isolation, purification, inoculum preparation and maintenance were performed according to Liban et al. (2016) with some modifications. Briefly, infected stems were cut into small pieces and surface-sterilised in 50% (v/v) bleach

for 1 min. These stem pieces were dried under a fume hood before placing them onto V8 agar juice medium treated with 0.0035% (w/v) streptomycin sulphate. An agar plate with stem pieces was incubated under continuous florescent light at room temperature (25°C) for 7–8 days. From these agar plates mycelium was transferred to new agar plates. A small segment of mycelium was obtained from pure culture of *L. maculans* and transferred into a new petri plate. The culture was allowed to cover entire plate. The full plate culture was flooded with 3 mL of sterile distilled water, and the pycnidiospores were harvested for the pathogenicity test.

6.3.8 Pathogenicity test

A set of canola differentials was used in the pathogenicity test (Table 6.2). Seeds were sown in a plastic cell tray using standard growth media, and kept in a growth chamber at 21ºC/16ºC (day/night) with a 16-h photoperiod. Four lobes of fully developed cotyledons of 7-day old seedlings were punctured with modified tweezers. Each punctured site was inoculated with a droplet of 10 µL pycnidiospore suspension (2×10^7 spores per mL). Inoculated cotyledons were air-dried before being placed back in the growth chamber, and subsequently watered. The seedlings were fertilised with all-purpose water soluble fertiliser 20:20:20 ($N:P_2O_5:K_2O$) on the following day of inoculation. Disease symptoms on inoculated cotyledons were evaluated at 13-days post-inoculation (dpi) using a rating scale of 0-9 (Williams and Delwiche 1979; Appendix III). At least six plants for each differential were rated for the pathogenicity test of an isolate. A total of 24 rating scores were obtained for each isolate-host interaction, and the average rating score was used to determine the interaction phenotype (IP), where IP = 5–9 was considered virulent and IP = 0–4.9 was avirulent, as described by Liban et al. (2016).

6.3.9 Development of primers linked to *AvrLm3, AvrLm2* and *AvrLm4AvrLm7*

Sequences for the *AvrLm2* and *AvrLm3* genes were collected from the corresponding publications (Ghanbarnia et al. 2015; Plissonneau et al. 2016). Primer3Plus, a free online

tool, was used to design the primers, which are shown in Table S6.2 (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The original isolates used in inoculum preparation (Table 6.1) were included in the PCR screen to amplify the *AvrLm2*, *AvrLm3*, and *AvrLm4AvrLm7* genes. Control isolates D5, J3, AD746, and R2 were used for showing the polymorphism of *AvrLm2*, *AvrLm3*, *avrLm4avrLm7*, and *avrLm4AvrLm7*, respectively (Zhang et al. 2016). The primers linked to *AvrLm3* generated a 600 bp PCR product from the isolates J3, X44, X65, X108, X159, RL3, RL4, DM88, DM96, DM118 and MB11-31-2 (Fig. 6.1A). Similarly, the primers linked to *AvrLm2* generated a 800 bp PCR product from the isolates D5, X65, X159, RL3, DM88, DM96, DM118, and MB11-31-2, as expected, whereas no amplification product was seen for the isolates X44, X108, and RL4 (Fig. 6.1B). Additionally, a tetra-primer amplification refractory mutation system-PCR (ARMS-PCR) assay (Zou et al. 2018) was employed to differentiate between the *AvrLm4AvrLm7* and *avrLm4AvrLm7* genes in *L. maculans* isolates, which differ by a single point mutation (Parlange et al. 2009). As expected, the *AvrLm4AvrLm7* gene in the *L. maculans* isolates X44, X65, X108, X159, RL3, RL4, DM88, and MB11-31-2 was amplified yielding 235 and 356 bp PCR products, while isolates DM96, and DM118 showed no polymorphism for either the *AvrLm4AvrLm7* or *avrLm4AvrLm7* allele (Fig. 6.1C), and only yielding a 356 bp common PCR product with these primers. Control isolates J3 and AD746 were also not polymorphic for either *AvrLm4AvrLm7* or *avrLm4AvrLm7* alleles, whereas the control isolate R2 yielded 167 and 356 bp PCR products corresponding to the *avrLm4AvrLm7* allele (Fig. 6.1C).

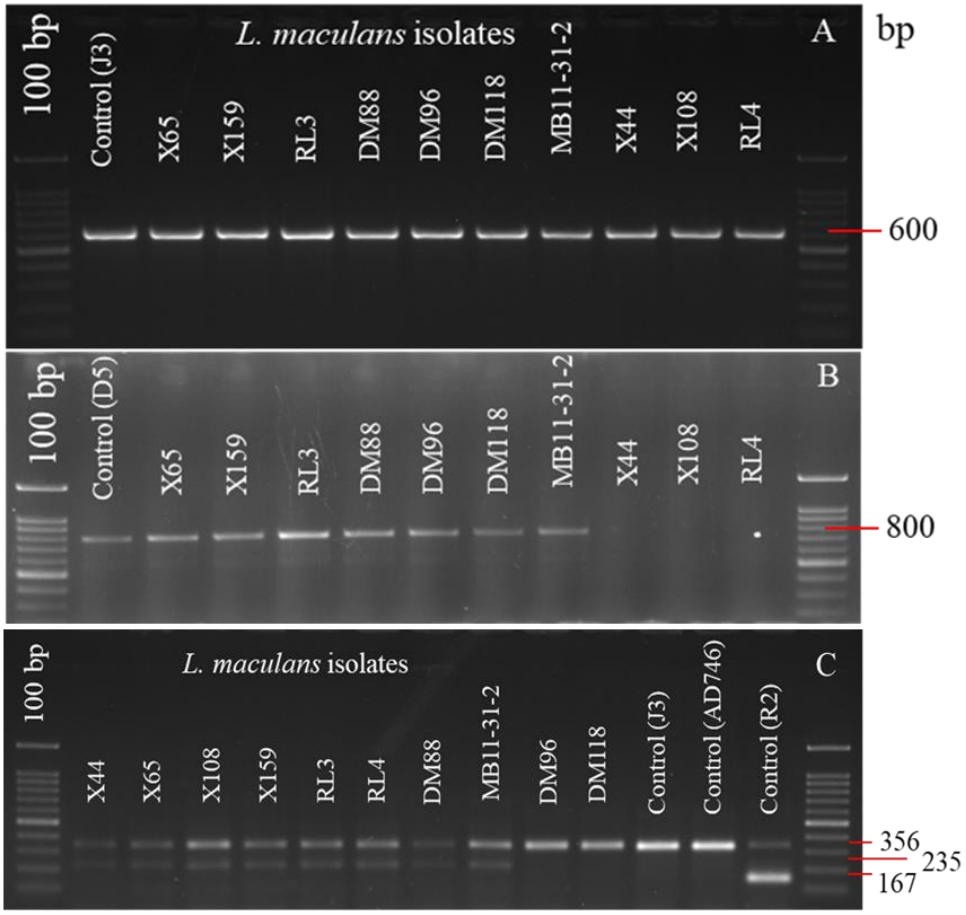

Fig. 6. 1 Amplification of avirulence genes *AvrLm3* (A), *AvrLm2* (B), and *AvrLm4AvrLm7* (C) in the *Leptosphaeria maculans* isolates using newly designed primers linked to the genes. Note that the PCR product sizes 600 bp, 800 bp, 235 bp, and 167 bp are corresponding to *AvrLm3*, *AvrLm2*, *AvrLm4AvrLm7*, and *avrLm4AvrLm7*, respectively; whereas 356 bp is a common amplicon generated by primers linked to *AvrLm4AvrLm7*.

6.3.10 Analysis of avirulence genes of *Leptosphaeria maculans* isolates

The *Avr* gene profile of *L. maculans* isolates was determined by phenotyping a set of canola differentials, as described in Table 6.2. The differential set allowed the identification of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, and *AvrLep3*. Since we do not have access to the canola differential to confirm the presence or absence of *AvrLmJ1/AvrLm5* (Van de Wouw et al. 2014), *AvrLm6* (Fudal et al. 2009) and *AvrLm11* (Balesdent et al. 2013), PCR was performed to identify these three *Avr* genes using isolates collected for the last four years.

Table 6. 2 A set of canola differentials (*Brassica napus* varieties / lines) used to identify avirulence genotypes of *Leptosphaeria maculans* isolates.

Variety/line	Resistance genes	Reference
Topas IL[1]	*Rlm1*	Larkan et al. 2016
Topas IL	*Rlm2*	Larkan et al. 2016
Topas IL	*Rlm3*	Larkan et al. 2016
Topas IL	*Rlm4*	Larkan et al. 2016
Topas IL	*LepR1*	Larkan et al. 2016
Topas IL	*LepR3*	Larkan et al. 2016
01–23-2-1	*Rlm7*	Dilmaghani et al. 2009
Goéland	*Rlm9*	Balesdent et al. 2006
Topas DH16516	No resistance gene	Larkan et al. 2016

[1]Introgression line

6.3.11 DNA extraction and PCR assay

Fungal genomic DNA was extracted from pycnidiospore suspensions of purified *L. maculans* isolates using the method described by Liban et al. (2016). A PCR assay was performed to detect five *Avr* genes: *AvrLm2* (Ghanbarnia et al. 2015), *AvrLm3* (Plissonneau et al. 2016), *AvrLmJ1/AvrLm5* (Van de Wouw et al. 2014), *AvrLm6* (Fudal et al. 2009) and *AvrLm11* (Balesdent et al. 2013). The primers used in the PCR assay were collected from the corresponding literature or designed for this study and these are shown in Table S6.2. Additionally, a tetra-primer amplification refractory mutation system-PCR (tetra-primer ARMS-PCR) assay was employed to differentiate the *AvrLm4AvrLm7* and *avrLm4AvrLm7* genes of *L. maculans* isolates, which differ by a single point mutation. Tetra-primer ARMS-PCR consists of two primer pairs, including an outer primer pair to amplify a common template/target, and an inner primer pair which is allele-specific. The tetra-primer ARMS-PCR protocol was used as described by Zou et al. 2018).

6.3.12 PCR and Sanger sequencing

PCR was performed via the standard protocols in an Eppendorf Mastercycler®pro for 35 cycles, each consisting of 30s at a denaturing temperature of 95ºC, 30s at an annealing temperature of 56ºC, and 1 min at an extension temperature of 72ºC (Eppendorf, Mississauga, Canada). PCR amplifications were performed using the primers linked to *AvrLm2* as

described in the primer development section (Fig. 6.1; Table S6.2). Sequencing of the PCR products was performed by Macrogen USA (https://www.macrogenusa.com). The nucleotide sequences of *AvrLm2* gene of *L. maculans* from original isolates (X65 and DM96) and field-experimental isolates virulent toward *Rlm2* (2014 and 2015) were aligned with a published sequence from NCBI (accession: KM073975.1) by the multiple sequence alignment software MAFFT (Katoh and Standley 2013). Furthermore, the nucleotide sequences were translated into amino acid sequences and aligned with MAFFT to determine the impact of the mutation(s) at the nucleotide level into the gene product (i.e. amino acid sequences).

6.3.13 Statistical analysis

Excel 2010 version was employed for data recording, preliminary analysis and preparing graphs. Statistical analysis was carried out using the PROC MIXED procedure in SAS Studio (Littel et al. 2006). The arcsine root square transformation was performed for disease incidence (DI), whereas log transformation was performed for disease severity (DS) for normality. The data for DI and DS were submitted to ANOVA, including the effect of the genotypes and years as fixed effects. The means of the genotypes for all variables were compared using the Fisher's protected least significant difference (LSD) at the 5% level of significance.

6.4 Results

6.4.1 Climatic conditions

Climate data particularly mean temperature and total precipitation were same as explained in the previous chapter (Section 5.4.1, Table 5.2).

6.4.2 Disease incidence

Blackleg incidence was significantly different for the ILs harbouring a single *R* gene among the years of the study, and for the interaction between them ($p<0.0001$; Table 6.3). Disease incidence (DI) was considered to be moderate to high in 2014 for all of the *R* genes tested,

ranging from 48–75% of plants infected, whereas moderate DI was recorded from 2015 to 2017 for all *R* genes except *Rlm4* (Fig. 6.2), indicating an impact of crop rotation on blackleg disease symptoms. Plot seeded with the IL harbouring *R* gene *Rlm4* infected a significantly higher number of plants in 2017 (Fig. 6.2). A similar level of plant infection was observed throughout the study period. This could be due to the fact that the pycnidia were produced over the growing season as the asexual cycle is predominant in Canada (Ghanbarnia et al. 2011; Guo et al. 2005). Canola production from the 2nd year onwards resulted in significantly lower plant infection for all *R* genes tested, except *Rlm4* (Fig. 6.2), indicating a possible role for canola rotation with wheat.

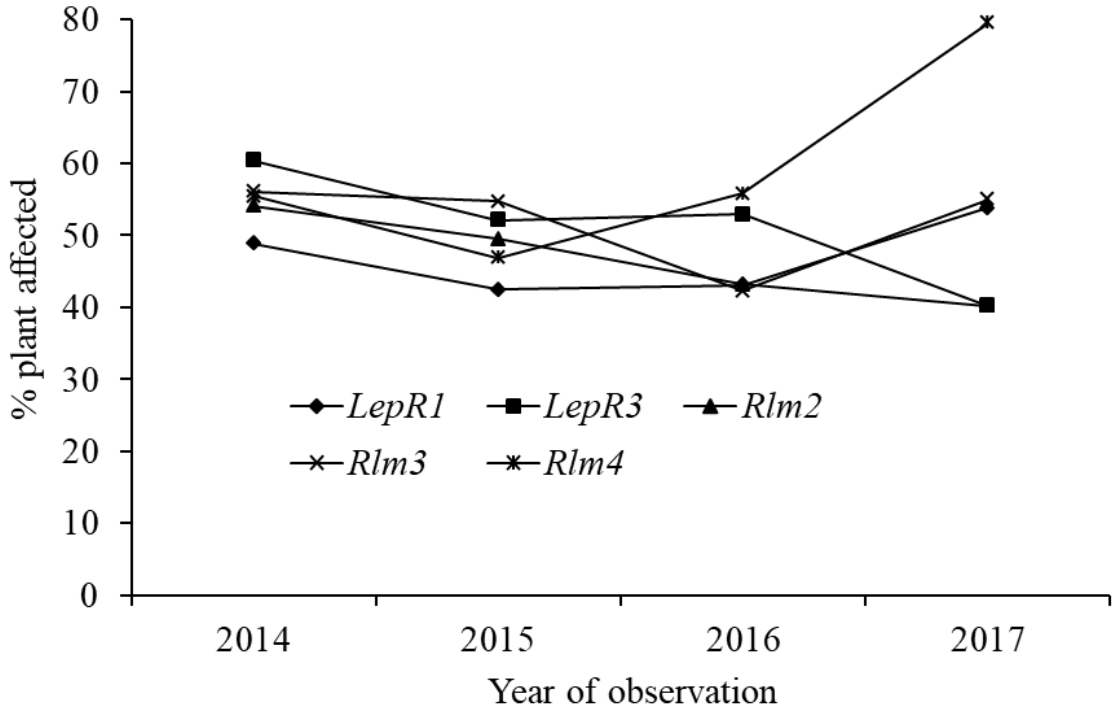

Fig. 6. 2 Disease incidence in the Topas-introgression lines (ILs) carrying a specific resistance gene. Disease incidence was calculated as percentage of plants affected from each plot thrice at 30 days after seeding (DAS), 36 DAS and 42 DAS in each cropping season. Each data point is calculated from the sum of three observations in each year.

6.4.3 Disease severity

Significant differences were found for disease severity among the Topas-ILs tested, among the years of the trial conducted, and in the interaction between them ($p<0.0001$; Table 6.3).

The higher disease severity of 2.5 out of a scale 0–5 was revealed by the *Rlm3*-harbouring IL in the year 2014, whereas the same IL produced a disease severity of 1.5 in 2015, which further decreased to 0.8 in 2017 (Fig. 6.3A). This is an indication of the possible role of crop rotation since; from the 2nd year *B. napus* ILs were seeded on wheat residues. The rest of the ILs carrying other *R* genes showed a disease severity ≤ 1.0 irrespective of the year (Fig. 6.3A). The ILs harbouring the *R* genes *Rlm2* and *Rlm4* had an unexpected increase in disease severity in 2017 (Fig. 6.3A). This might be due to the drier conditions of the year, as less precipitation was observed in 2017 (Table 5.2). Overall, canola production from the 2nd year onwards resulted in significantly lower disease severity for all *R* genes tested, except *Rlm2* (Fig. 6.3A), indicating a possible role for canola-wheat-canola rotation.

The rapeseed recommendation committee (RRC) of the Canola Council of Canada usually rates cultivars based on the relative disease severity by the susceptible control Westar. According to the RRC, disease severity <30% is considered resistant, 30–49% is considered moderately resistant, 50–70% is considered moderately susceptible, and >70% is considered susceptible to blackleg disease. In our study, statistically significant differences were found in relative disease severity among the ILs-harbouring different *R* genes (Fig. 6.3B). The highest disease severity was 84% of Westar produced by the *Rlm3*-harbouring IL in 2014, which was reduced to 47% in 2015, and further reduced to 24% in 2017 (Fig. 6.3B). The *Rlm4*-harbouring IL produced a relative disease severity of 38% of Westar in 2014, which was reduced to 27% in 2017 (Fig. 6.3B). The rest of the ILs harbouring *R* genes *LepR1*, *LepR3*, and *Rlm2* showed disease severity <30% of Westar irrespective of the year (Fig. 6.3B). Thus the results indicate an effectiveness of crop rotation in reducing disease severity as well as increasing the durability of single *R* genes.

Table 6. 3 Analysis of variance[1] for disease incidence, and severity of *Brassica napus* introgression lines in a 4-years study from 2014 to 2017 at Carman Manitoba.

Variables	Source of variation	Degrees of freedom	F value	P ≤0.05
Disease incidence	Genotypes (IL[2])	4	20.27	<0.0001
	Year	3	11.89	<0.0001
	Genotype (IL)×Year	12	17.82	<0.0001
Disease severity	Genotypes (IL)	4	171.36	<0.0001
	Year	3	24.87	<0.0001
	Genotype (IL)×Year	12	15.64	<0.0001

[1]Type III tests of fixed effects; [2]Introgression line

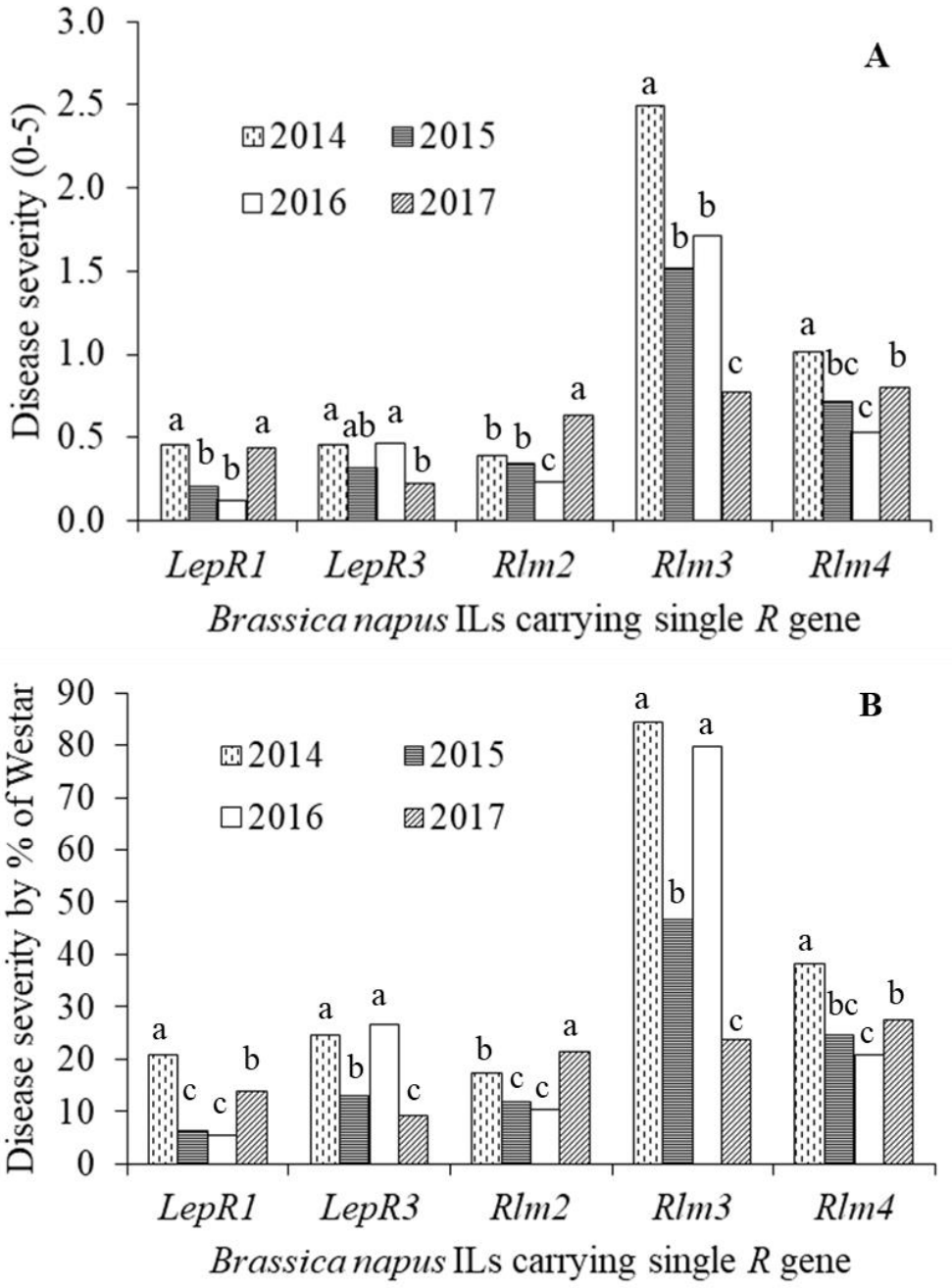

Fig. 6. 3 Disease severity rated from the *Brassica napus* Topas-introgression lines (ILs) harbouring a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4*. Disease severity rated using a scale of 0-5 (**A**), and relative disease severity calculated by a susceptible cultivar Westar (**B**). Rating scale was followed as West et al. (2002) where: 0 = no visible infection; 1 = disease tissue occupies ≤25% of the cross section; 2 = disease tissue occupies 26-50% of the cross section; 3 = between 51 and 75% of the cross section infected; 4 = >75% of the cross section infected but plant alive and 5 = 100% of cross section infected and plant dead. Bars with the same letter are not significantly different at $P \leq 0.0001$ according to Fisher's LSD test.

6.4.4 Frequency of avirulence genes in *Leptosphaeria maculans* isolates

Leptosphaeria maculans isolates cultured from the infected stubbles showed that the frequency of *AvrLm1* was very diverse, from 0 to 100% of isolates tested among the year of study. Importantly, the frequency of *AvrLm1*-carrying isolates cultured from *LepR3*-stubble was high and consistent throughout the study period (Fig. 6.4). Isolates cultured from *Rlm2*-stubble from the 2014 trial showed that 75% of isolates carried *AvrLm2*, which was reduced to 13% in the *Rlm2*-stubble in 2015. The frequency of *AvrLm2* carrying isolates was again increased to 63% in the *Rlm2*-stubble in 2016, but it had completely disappeared in 2017 (Fig. 6.4). As a result, the shift from *AvrLm2* to *avrLm2* is suggested since *AvrLm2*-carrying isolates completely disappeared from the *Rlm2*-stubble in 2017 (Fig. 6.4); this is anticipated to be the reason for the increased disease severity in the *Rlm2*-harbouring IL in 2017 (Fig. 6.4). *L. maculans* isolates cultured from the *Rlm3*-stubble indicated that the *AvrLm3*-carrying isolates increased from 14% in 2014 to 50% in 2015, but could not be isolated from 2016 stubble and again increased to 29% of isolates in 2017 stubble (Fig. 6.4). The shift from *AvrLm3* to *avrLm3* is postulated since *AvrLm3*-carrying isolates could not be isolated from the *Rlm3*-stubble in 2016 (Fig. 6.4) and this is predicted to be the reason for the higher disease severity in the *Rlm3*-harbouring IL in 2016 (Fig. 6.3). In addition to that, an increase in *AvrLm3* from the *Rlm3*-stubble was observed in 2015 and 2017 (Fig. 6.4) and is predicted to be the reason for the lower disease severity in the *Rlm3*-harbouring IL in 2015 and 2017 (Fig. 6.3). *AvrLm4* was found in 86% of isolates cultured from *Rlm4*-stubble in 2014, which was reduced to 29% in 2015 stubble, increased to 43% of isolates in 2016 stubble but could not be isolated from 2017 stubble (Fig. 6.4); this indicates a shift from of *AvrLm4* to *avrLm4*. Isolates cultured from *LepR1*-stubble consistently showed a higher frequency of *AvrLepR1* from 71–100% throughout the study period. *AvrLepR3* was found in only 29% of isolates cultured from *LepR3*-stubble in 2014, which consistently showed a higher frequency for

consecutive years (Fig. 6.4). The avirulence genes *AvrLm5*, *AvrLm6*, *AvrLm7* and *AvrLm11* were found at a consistently higher frequency in the isolates cultured irrespective of the stubble (Fig. 6.4), which is expected because no canola cultivars carry the single *R* genes *Rlm5*, *Rlm6*, *Rlm7*, or *Rlm11* in our rotation. The frequency of *AvrLm9* in blackleg isolates cultured from 2014 to 2017 stubble was consistent with the frequency of *AvrLm3* (Fig. 6.4), which could be due to the linkage between the two genes (Balesdent et al. 2002; 2005; Ghanbarnia et al. 2012; Zhang et al. 2016). However, the return of *AvrLm3*, *AvrLm4*, *AvrLepR1*, and *AvrLepR3* in the stubble of the corresponding *R* genes might be due to the effectiveness of canola-wheat-canola rotation and pathogen adaptation.

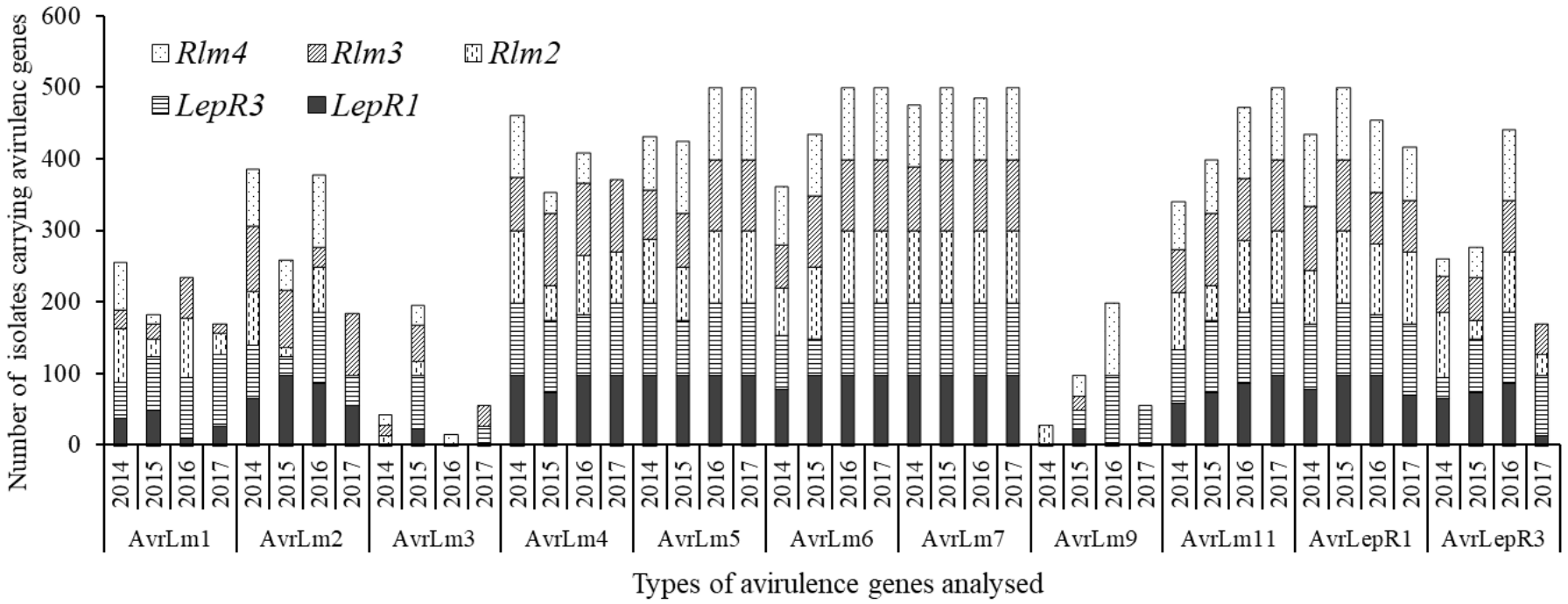

Fig. 6. 4 Frequency of avirulence genes in *Leptosphaeria maculans* isolates cultured from *Brassica napus* infected stubbles. Stubble collected from each plot throughout the study period from 2014 to 2017. Bar indicating stubble from different *B. napus* introgression lines with a specific resistance gene *LepR1*, *LepR3*, *Rlm2*, *Rlm3*, and *Rlm4*. Note that the data based on the phenotype of a set of differentials (**Table 6.1**), and genotype of *AvrLm5/AvrLmJ1*, *AvrLm6*, and *AvrLm11* via PCR as described in the methodology section.

6.4.5 Genotype of *AvrLm2, AvrLm3* and *AvrLm4AvrLm7* in the *Leptosphaeria maculans* isolates

A PCR assay was employed to differentiate between the *AvrLm2* and *avrLm2* genotypes in *L. maculans* isolates. A total of 30 *L. maculans* isolates derived from *Rlm2*-harbouring infected stubbles from 2014 to 2017 were used for examining the genotyping of *AvrLm2* (Table 6.4). As expected (see Fig. 2B), the *AvrLm2* gene in the *L. maculans* isolates was amplified as an 800 bp PCR product (Fig. S6.1). Of the 7 isolates tested from 2014-stubbles, 5 were positive for the allele *AvrLm2*, whereas only a single isolate cultured from 2015-stubble showed any polymorphism (Table 6.4; Fig. S6.1). Similarly 3 of 7 isolates from 2016-stubbles produced the expected PCR products for the allele *AvrLm2*, but none of the isolates from 2017-stubble produced the expected amplicons (Table 6.4; Fig. S6.1), indicating a complete absence of the *AvrLm2* allele in 2017 derived isolates and as predicted indicates a shift from *AvrLm2* to *avrLm2* in the fungal population. However, these results suggest a complete shift from *AvrLm2* to *avrLm2* during the period from 2016 to 2017, which is a reflection of the emergence of new isolates of *L. maculans* in a single cropping season. PCR was also performed to differentiate between *AvrLm3* and *avrLm3* genotypes in *L. maculans* isolates cultured from *Rlm3*-harbouring stubbles from 2014 to 2017 in a field trial. A total of 29 *L. maculans* isolates were used to genotype *AvrLm3;* among them, 21 isolates were polymorphic for the *AvrLm3* genotype (Table 6.4). The *AvrLm3* gene in *L. maculans* isolates was amplified as a 600 bp PCR product (Fig. S6.1; also see Fig. 6.1A). All isolates tested from 2014-stubble were polymorphic for the allele *AvrLm3*, whereas 4 isolates from 2015-stubble were polymorphic (Table 6.4; Fig. S6.1). Similarly, 6 of the 7 isolates from 2016-stubbles upon the PCR assay generated 600 bp PCR products for the allele *AvrLm3*, but only 4 isolates from 2017-stubble produced the expected amplicons (Table 6.4; Fig. S6.1). These results indicate an increase and decrease of the *AvrLm3* allele in the *L. maculans* isolates

from alternative years and are predicted to indicate the effectiveness of crop rotation. Moreover, these results also indicated a partial shift from the *AvrLm3* allele to the *avrLm3* allele in the isolates derived from alternative years.

A tetra-primer ARMS-PCR (Zou et al 2018)) assay was employed to differentiate the *AvrLm4AvrLm7* and *avrLm4AvrLm7* alleles of *L. maculans* isolates, which differ by a single point mutation (Parlange et al. 2009). Using this approach, we were able to amplify distinct PCR products to infer the specific allele of the tested isolates. A total of 29 *L. maculans* isolates derived from *Rlm4*-harbouring infected stubbles from 2014 to 2017 were employed to differentiate between the *AvrLm4AvrLm7* and *avrLm4AvrLm7* alleles. As expected, the *AvrLm4-7* allele in the *L. maculans* isolates was polymorphic yielding 235 and 356 bp PCR products, while the presence of *avrLm4AvrLm7* allele yielded 167 and 356 bp PCR products (Fig. 6.1C; Fig. S6.1). The DNA of all 7 isolates from 2014-stubble amplified for the allele *AvrLm4-7* (235 and 356 bp amplicons). In contrast, 6 of the 7 isolates from 2015-stubble amplified for the allele *AvrLm7* (167 and 356 bp products), and only one isolate amplified for the allele *AvrLm4-7* (Table 6.4; Fig. S6.1), indicating a shift from *AvrLm4AvrLm7* to *avrLm4AvrLm7*. Similarly, 4 of the 7 tested isolates from the 2016-stubble were polymorphic for the allele *AvrLm7* (167 and 356 bp amplicons), and the remaining 3 isolates were polymorphic for the allele *AvrLm4-7* (Table 6.4; Fig. S6.1), indicating that the *AvrLm4-7* allele had returned to these isolates. On the other hand, 8 isolates tested from 2017-stubble only amplified the allele *avrLm4AvrLm7*, generating 167 and 356 bp amplicons (Table 6.4; Fig. S6.1), suggesting a complete absence of the *AvrLm4* allele in these isolates; this is predicted to be due to the change from *AvrLm4* to *avrLm4*. However, these results reflect a complete shift from *AvrLm4* to *avrLm4* within the period from 2016 to 2017, which might indicate the emergence of new isolates of *L. maculans* in a single cropping season.

Table 6. 4 Genotype of the avirulence genes *AvrLm2*, *AvrLm3* and *AvrLm4AvrLm7* in *Leptosphaeria maculans* isolates cultured from *Brassica napus* stubble harbouring genes *Rlm2*, *Rlm3*, and *Rlm4*, respectively.

Year of isolates used	*AvrLm2*		*AvrLm3*		*AvrLm4-7*	
	Presence	Absence	Presence	Absence	Presence	Absence
2014	5	2	7	0	7	0
2015	1	7	4	4	1	6
2016	3	4	6	1	3	4
2017	0	8	4	3	0	8
# isolates	9	21	21	8	11	18
Total isolates	30		29		29	

6.4.6 *AvrLm3* phenotype camouflaged by the *AvrLm4AvrLm7* phenotype

A total of 29 fungal isolates cultured from *Rlm3*-harbouring stubble were phenotyped for their virulence towards *Rlm3*. After 4 years of *Rlm3* selection pressure with a canola-wheat-canola rotation, the frequency of isolates with virulence towards *Rlm3* had reached 75.86% (calculated from Fig. 6.4). In contrast, genotypic data showed that only 27.59% of isolates carried the genotype *avrLm3* (Table 6.4; Fig. S6.1). Phenotypic data from the 2014-stubble revealed that 14.29% of isolates displayed an *AvrLm3* phenotype (Fig. 6.4) whereas all of the isolates cultured from the 2014-stubble displayed an *AvrLm3* genotype (Table 6.4; Fig. S6.1). Isolates cultured from 2015-stubbles showed similar frequencies for the phenotype and genotype for *AvrLm3*. All of the isolates cultured from 2016-stubble were virulent towards *Rlm3* (Fig. 6.4), whereas 85.71% of isolates displayed an *AvrLm3* genotype (Table 6.4; Fig. S6.1). Frequency of isolates cultured from 2017-stubble with virulence towards *Rlm3* was 28.57%, but 57.14% of isolates showed an *AvrLm3* genotype (Table 6.4; Fig. S6.1). These results revealed that the *AvrLm3* allele was absent from 2016-stubble and appeared to be absent from 2014- and 2017-stubble, but actually it is present in most of the isolates (Table 6.4; Fig. S6.1). In fact it appears that the *AvrLm3* phenotype is 'hidden' by the presence of a functional allele of *AvrLm4-7*. While *AvrLm4-7* is present at a high frequency, the interaction between *AvrLm3* and *Rlm3* is compatible, as seen in the 2014 and 2016 phenotypes (Fig. 6.4). Isolates cultured from 2014- and 2016-stubble displayed a high frequency of the *AvrLm3*, and

AvrLm4-7 genotypes (Table 6.4; Fig. S6.1), and at the same time a high frequency of isolates were virulent towards *Rlm3* (Fig. 6.4). In contrast, isolates cultured from 2015- and 2017-stubble displayed a zero to low frequency of the *AvrLm4-7* genotype (Table 6.4; Fig. S6.1), and at the same time a small number of isolates were avirulent towards *Rlm3* (Fig. S6.1), confirming the hypothesis that the *AvrLm3* phenotype is masked by the presence of a functional allele of *AvrLm4-7*.

6.4.7 Polymorphism of *AvrLm2* in isolates virulent towards *Rlm2*

The field experiment was conducted in Manitoba Canada from 2014 to 2017 to monitor the shift in virulent isolates towards the *R* genes when submitted to an increased and constant selection pressure. A total of 30 isolates cultured from *Rlm2*-harbouring stubble from 2014 to 2017 were analysed for their phenotype and genotype. Analysis of the phenotype and genotype data showed that a shift from the *AvrLm2* allele to the *avrLm2* allele in the isolates occurred in a single year (Fig. 6.4; Table 6.4; Fig. S6.1). To confirm this shift, the PCR products linked to the *AvrLm2* gene were sequenced from 2014 and 2015 isolates, along with the two original isolates used in the study (Fig. 6.5). Eighteen (18) nucleotide polymorphisms were detected throughout the coding sequence. These polymorphisms defined 18 different alleles, corresponding to 12 amino acid positions impacted by non-synonymous point mutations and 6 amino acid positions of where the codons acquired synonymous point mutations, leading to a maximum of 12 isoforms of the protein, termed *AvrLm2*-E to *AvrLm2*-V (Table 6.5; Fig. 6.5).

Table 6. 5 Polymorphic sites identified in the *AvrLm2* nucleotide sequence and corresponding amino acids

Polymorphic nucleotide sites																		
	253	259	295	296	334	364	377	402	418	435	437	438	441	445	447	448	449	552
AvrLm2-original	G	C	G	G	G	A	A	G	G	C	C	T	T	A	T	C	A	T
AvrLm2-2014	G	C	C	A	C	G	G	A	A	A	G	A	G	G	G	G	T	G
AvrLm2-2015	A	A	A	A	C	A	A	G	G	C	C	T	T	A	T	C	A	T
Polymorphic amino acid sites and mature protein																		
	84	86	98	-	111	121	125	-	139	145	146		-	-	149	150		151
AvrLm2-original	E	Q	G		E	K	Q		E	N	T				S	H		V
AvrLm2-2014	E	Q	H		Q	R	R		K	K	R				G	V		G
AvrLm2-2015	K	K	N		Q	K	Q		E	N	T				S	H		V



Fig. 6. 5 *AvrLm2* sequence and polymorphism. (A) Alignment of *AvrLm2* nucleotide sequences. Note that the isolates S1_2014 and S2_2015 were the experimental isolates virulent toward *Rlm2*. Isolates S3_original_X65 and S3_original_DM96 were original isolates avirulent toward *Rlm2*, and *AvrLm2*_ref_gene is a reference gene sequence of *AvrLm2*. (B) Alignment of *AvrLm2* protein sequences. DNA from all isolates amplified via PCR using AvrLm2 linked primer showed in **Fig. 6.1** and **Table S6.2.** The specific change in nucleotides and amino acids are shaded.

6.5 Discussion

In this study, five known *R* genes; *LepR1*, *LepR3, Rlm2, Rlm3,* and *Rlm4* were included in a 4-year field study over canola-wheat-canola 2-year crop rotation. Based on *B. napus-L. maculans* interactions, we report the loss of avirulence and gaining of virulence by isolates of *L. maculans* in a single year. A shift from *AvrLm2* to *avrLm2* allele has been observed and is due to the accumulation of point mutations. In addition to that, masking of the *AvrLm3* phenotype by the predominant allele of *AvrLm4-7* was also confirmed by analysing the phenotype and the genotype of isolates. Moreover, gaining back avirulence activity in *L. maculans* isolates was also detected in the alternative years, which is predicted to reflect the effectiveness of canola-wheat-canola rotation.

Disease incidence in *B. napus* ILs decreased from the 2nd year of the study, except for ILs harbouring *Rlm4* (Fig. 6.2). *B. napus* ILs harbouring the *R* genes *Rlm2* and *Rlm4* produced greater disease severity in 2017 (Fig. 6.3). This could be due to the drier conditions seen in 2017. As there was much less precipitation in 2017 compared to the long-term average (Table 5.2), which might trigger pathogens with virulence toward corresponding *R* genes, this suggests a compatible interaction between *R*- and *Avr*-genes. As a result, higher disease incidence was noted by the ILs harbouring the *R* genes *Rlm2* and *Rlm4* in 2017 (Fig. 6.3). On the other hand, disease severity decreased in the alternative years, as shown by the ILs harbouring *LepR3* and *Rlm3* (Fig. 6.3), which could indicate the effectiveness of crop rotation in gaining back avirulence activities of the isolates, and suggests an incompatible interaction between *R*- and *Avr*-genes. *B. napus* ILs carrying the *R* genes *LepR1*, *LepR3*, *Rlm2,* and *Rlm4* were much less affected by the disease throughout the study period, indicating the strong stability of these genes at least in the Manitoban environment. A recent study conducted in western Canada has shown disease severity <0.5 from a canola-wheat-canola rotation under natural conditions (Kutcher et al. 2013). Our study reported a disease

severity <1.0, which is slightly higher than that reported in the previous study. This might be due to the high disease pressure, as we applied extra inoculum in the establishment (2013) as well as in the first (2014) year of this study.

Variability for the virulence of *L. maculans* has been reported in western Canadian isolates (Kutcher et al. 2007, 2010; Fernando et al. 2018). Knowledge of the *Avr* gene profile in *L. maculans* isolates has had a great impact when deploying *R* genes in the management of blackleg disease. For instance, an analysis of *L. maculans* isolates collected in 2010 and 2011 from western Canadian canola fields showed the possible effectiveness of *Rlm2*, *Rlm4*, *Rlm6* and *Rlm7* in Canada (Liban et al. 2016). Similarly, an analysis of *L. maculans* isolates collected in 2002 and 2003 from European canola fields indicated the potential effectiveness of *Rlm6* and *Rlm7* in a European environment (Stachowiak et al. 2006). Our study indicated the high frequency of *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLepR1* alleles, along with the very low frequency of *AvrLm1*, *AvrLm3*, and *AvrLm9* alleles in the *L. maculans* isolates cultured from the stubble carrying the corresponding *R* genes. On the other hand, *AvrLm2*, *AvrLm4*, and *AvrLepR3* varied from low to moderate frequency among the *L. maculans* isolates collected over the years of this study (Fig. 6.4). Importantly, *AvrLm2*, *AvrLm3*, and *AvrLepR3* were gained back among the isolates cultured from the stubble harbouring the corresponding *R* genes (Fig**.** 6.4), indicating the effectiveness of canola-wheat-canola rotation and pathogen fitness. Based on the knowledge of *Avr* genes present among *L. maculans* isolates and *R* genes tested in the study, *LepR1*, *LepR3*, and *Rlm4* are undoubtedly very useful in the current deployment of Canadian *R* genes within the context of at least a 2-year crop rotation. Additionally, other useful *R* genes including *Rlm5*, *Rlm6*, *Rlm7*, and *Rlm11* can be introduced into Canadian canola cultivars. However, some *R* genes should be avoided based on the phenotypes observed for *Rlm3* and *Rlm9* that were overcome and *Rlm1* and *Rlm2* are at risk of being overcome. Surprisingly, there are no reports suggesting the wide use of *Rlm1*,

and *Rlm2* in Canada, instead reports suggest the extensive use of *Rlm3* in Canada (Zhang et al. 2016). A very recent study in Canada claimed a hide-and-seek relationship between *AvrLm9* and *AvrLm4-7* (Ghanbarnia et al, 2018), which is believed to be the reason of disappearing *AvrLm9* in this study. In addition to changes in the frequency of *Avr* genes, blackleg disease in canola has fluctuated, as shown in a recent Canadian disease survey (http://phytopath.ca/publication/cpds). The occurrence of the disease correlated with the declining frequency of *AvrLm2* and *AvrLm4* alleles from the 2017-isolates (Table 6.5; Fig. 6.3). These results strongly support the observation of increase of this disease due to a shift from *AvrLm2* and *AvrLm4* to *avrLm2* and *avrLm4*, respectively (Table 6.5; Fig. S6.1).

As already known, the application of *R* genes in *B. napus* varieties exerts a strong selection pressure on the evolution of virulence towards these *R* genes; therefore, it is important to know the timeframe and the mechanisms of evolution of new *Avr* alleles so that effective strategies can be developed to manage *R* genes and improve their potential durability. The first constraint of this kind of study is the limited number of avirulence genes that have been identified and characterized (cloned and sequenced) to date. The second limitation is the scarcity of historical population surveys after releasing a resistance gene into commercial cultivation. Moreover, models used to predict the stability of *R* genes are often established on the gene-for-gene concept, which predicts short-lived resistance (Brown 2015). More complex types of interactions are now being described in different fungal and oomycetes species, such as *Fusarium oxysporum* (Houterman et al. 2008), *Blumeria graminis* (Bourras et al. 2016), *Phythophtora infestans* (Chen et al. 2012), *Pyrenophora tritici-repentis* (Manning and Ciuffetti 2015) and *Cladosporium fulvum* (Van den Burg et al. 2003; Iida et al. 2015). Similarly, the blackleg pathogen *L. maculans* is also predicted to result in complex interactions with the host (Plissonneau et al. 2016; Plissonneau et al. 2016; Ghanbarnia et al.

2018). Here, we also report a hide and seek phenomenon between the *AvrLm3* and *AvrLm4AvrLm7* alleles of *L. maculans* with regards to agricultural systems.

AvrLm3 was previously considered to be absent in Canadian and European isolates until recently because of the predominant nature of an avirulent allele of *AvrLm4-7* in *L. maculans* isolates (Balesdent et al. 2006; Stachowiak et al. 2006; Liban et al. 2016; Zhang et al. 2016). In this study, we detected an unusual interaction between the avirulence gene *AvrLm3* and *AvrLm4AvrLm7*, because *Rlm3*-mediated resistance is completely lost when an isolate carries an avirulent allele of *AvrLm4AvrLm7* (Fig. 6.4; Table 6.5; Fig. S6.1); this is supported by the previous studies (Plissonneau et al. 2016; 2017) which found that most of the isolates carried a functional allele of *AvrLm3*, but the presence of a functional allele of *AvrLm4-7* 'hides' the *Rlm3*-mediated recognition. In contrast, an inactivated (or absent) *AvrLm4-7* allele leads to the re-emergence of the *AvrLm3* phenotype (Plissonneau et al. 2016). Here, we evaluated the role of *AvrLm3* on pathogenicity and interpreted how isolates could overcome *Rlm3*-mediated resistance. Evaluation of the frequency of *AvrLm3*, *AvrLm4* and *AvrLm7* in the *L. maculans* isolates cultured from 2014- to 2017-stubbles (field trial in Carman Manitoba) harbouring the corresponding *R* genes showed the ongoing breakdown of *Rlm3*-mediated resistance, as 100% of the isolates showed virulence towards *Rlm3* in 2016 (Fig. 6.4). It also confirmed the presence of the *AvrLm4*, and *AvrLm7* phenotypes in *L. maculans* isolates cultured from *Rlm3*-harbouring stubble, with more than 90% of the isolates being avirulent towards *Rlm4* and *Rlm7* (Fig. 6.4), confirming the masking effect of *AvrLm4AvrLm7* allele on the *Rlm3*-mediated resistance in an agricultural system. To test this hypothesis, we assessed the presence of the *AvrLm3*, *AvrLm4*, and *AvrLm7* alleles via PCR in a collection of isolates displaying virulent or avirulent phenotypes towards *Rlm3*, *Rlm4*, and *Rlm7*. We found that the *AvrLm4AvrLm7* allele was appeared to have shifted towards *avrLm4AvrLm7* from the isolates that are virulent towards *Rlm4*, and a copy of *AvrLm3* was

present in most of the isolates regardless of the phenotype displayed (Fig. 6.4; Table 6.5; Fig. S6.1). Similar genotypes were detected by the previous studies in European (Plissonneau et al. 2016) and Canadian isolates. Thus, this study highlights the complexity of the molecular mechanisms shaped by the arms race between *B. napus* and *L. maculans* in an agricultural system. Such complex interactions may require new approaches for disease management. Until now, the rapid breakdown of *R*-gene-mediated resistance has been reported and predicted to be the result of using single *R* genes controlling *L. maculans* (Rouxel et al. 2003; Sprague et al. 2006; Van de Wouw et al. 2010a). The fact that the breakdown of *Rlm7* produced *AvrLm3* isolates (Plissonneau et al. 2016) provides an unparalleled pattern to employ the *R* gene rotation strategy to increase the durability of a single *R* gene in these cases of complex gene-for-gene interactions.

The genome sequence of *L. maculans* showed that the *Avr* genes are positioned near various transposable elements (TEs) and the *Avr* genes encodes cysteine-rich small secreted proteins (Gout et al. 2007; Fudal et al. 2009; Rouxel et al. 2011; Daverdin et al. 2012; Ghanbarnia et al. 2015). The identification and cloning of *AvrLm2* (Ghanbarnia et al. 2015) showed it to be similar to that of other avirulence genes cloned in *L. maculans* (Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Balesdent et al., 2013; Van de Wouw et al. 2014; Plissonneau et al., 2015). Sequencing of *AvrLm2* from our isolates which are virulent towards *Rlm2* revealed an unusual situation for fungal avirulence genes. First, there was no deletion event noted in our data (Fig. 6.6). Deletions in *AvrLm1* are thought to be the main mechanism for *L. maculans* isolate virulence towards *Rlm1* (Gout et al. 2007; Van de Wouw et al. 2010b). Deletions and RIP mutations also occurred in isolates showing virulence towards *Rm6* and *Rlm7* (Fudal et al. 2009; Van de Wouw et al. 2010b; Daverdin et al. 2012). Moreover, deletions were also identified as the predominant mechanism in the rice blast pathogen *Magnaporthe oryzae* (Huang et al. 2014a). In contrast, deletions were absent from

AvrLm2, as shown by Ghanbarnia et al. (2015), which is an agreement with the current study. In the *AvrLm2* allele, point mutations were observed (Table 6.6), which was previously also observed, in the *AvrLm4-7* allele (Daverdin et al. 2012). Second, a high level of allelic polymorphism was found for *AvrLm2* in our isolates, with 19 distinct alleles encoding for 12 different isoforms of the protein (Fig. 6.5), whereas only four isoforms of the protein were detected in *AvrLm2* by a previous study (Ghanbarnia et al. 2015). In *AvrLm1*, two amino acid substitutions leading to three isoforms of the protein were detected in previous studies (Gout et al. 2006; Van de Wouw et al. 2010b). In contrast, a high level of allelic polymorphism was found for *AvrLm3*, with 22 distinct alleles encoding for 11 different isoforms of the protein (Plissonneau et al. 2017). The high levels of polymorphism were also reported for *L. maculans* avirulence genes *AvrLm6* (Fudal et al. 2009; Van de Wouw et al. 2010b) and *AvrLm4-7* (Daverdin et al. 2012), which are in strong agreement with this study. In the current study, only point mutations generating apparently functional versions of the protein were identified for *AvrLm2*, and are predicted to be the reason why *Rlm2*-mediated resistance was avoided (Fig. 6.4). Previous studies also reported that *AvrLm2* and *AvrLm4* circumvent the *Rlm2*- and *Rlm4*-mediated resistance by the development of point mutations (Parlange et al. 2009; Ghanbarnia et al. 2014). Only amino acid alterations in the *Avr2* gene of *F. oxysporum* led to isolates being virulent towards both *I1* and *I2* resistance (Rep et al. 2005; Houterman et al. 2009). Finally, the high level of polymorphism via transitions and transversions in *AvrLm2* is likely to be the result of positive selection. Currently, positive selection is considered to be an evolutionary force on effectors in many species (Aguileta et al. 2009; Stukenbrock and McDonald 2009; Stergiopoulos et al. 2014), and evidence of positive selection is now considered to be one of the criteria by which to detect candidate effector genes (Chen et al. 2013; Sperschneider et al. 2014).

Our genotypic data from 2015- and 2017-isolates also suggested a reduction of *AvrLm3* in an isolate already lacking *AvrLm4AvrLm7* (Table 6.5; Fig S6.1), which may have detrimental effects on the life cycle of *L. maculans*. It has been speculated that the avirulence gene *AvrLm3* may have a role in fungal fitness, along with *AvrLm4AvrLm7*. A similar prediction was also reported while epistatic interactions were observed between *AvrLm3* and *AvrLm4-7* (Huang et al. 2010; Novakova et al. 2016). These results also speculated that the role of *AvrLm3* in *L. maculans* fitness could have favoured the evolution of this complex mechanism, permitting *L. maculans* to avoid selection pressure while conserving *AvrLm3* integrity. A similar association has been reported between *Avr1*, *Avr2* and *Avr3* in *F. oxysporum* (Houterman et al. 2009). The presence of a functional allele of *Avr1* accounts for the suppression of *I2*- and *I3*-mediated resistance in tomato (Houterman et al. 2008). Although a few examples of the suppression of effector-triggered immunity (ETI) by a fungal effector have been shown to date, this may be more common than previously hypothesized in the arms race between plant and pathogenic fungi. In this case, it may allow indispensable effectors to escape the selection pressure employed by the *R* genes under the field conditions.

This study also showed that an increase in the *avrLm4AvrLm7* allele is accompanied by an increase in the frequency of isolates with virulence towards *Rlm4* and vice versa (Table 6.5; Fig. 6.4), which suggests that alternating *Rlm4* and *Rlm7* could have an effect on the *Avr* gene profile in *L. maculans* populations. It has been shown in Canada that the large-scale use of the *Rlm3* gene has led to an increase in the frequency of *avrLm3AvrLm7* isolates (Zhang et al. 2015), which currently would be rendering *Rlm7* as a potential new source of resistance. In France, the reverse situation is currently occurring, with a shift from *avrLm3AvrLm7* to *AvrLm3avrLm7* isolates due to the commercial use of *Rlm7* (Plissonneau et al. 2017). On the other hand, the model of evolution proposed by Plissonneau et al. (2017) suggests that alternating *Rlm3* and *Rlm7* could have an effect by changing the race structure. Based on

previous and current studies, we can predict that alternating *Rlm3*, *Rlm4*, and *Rlm7* could provide an opportunity to increase the durability of those *R* genes in Canada. In fact, the pyramiding of *R* genes in cultivated plant species is believed to increase the durability of such resistance (Mundt 2014). However, the capacity of *L. maculans*, a sexually reproductive pathogen, to overcome *Rlm3* (Zhang et al. 2016), *Rlm4* (this study), and *Rlm7* (Plissonneau et al. 2017) resistance must be taken into consideration for the field deployment of such genetic strategies. In contrast, alternating *Rlm3*, *Rlm4*, and *Rlm7* is likely to limit the risk of the emergence of corresponding virulent isolates. To date, a number of reports have described non-conventional gene-for-gene interactions, including fungal and oomycete pathogens (Houterman et al. 2008; Chen et al. 2012; Manning and Ciuffetti 2015; Bourras et al. 2016; Plissonneau et al. 2016). Such complex interactions between *Avr* genes may also offer new perspectives for the rationale deployment of corresponding *R* genes. Therefore, this study suggest to develop generic epidemiological models that take into account such complex molecular mechanisms, allowing plant breeders to select appropriate *R* genes in the rotation strategy.

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Table S6.1 Calculation of avirulence genes (100%) for the corresponding resistance (*R*) genes used in the study.

AvrLepR3	%	Plates	*AvrLm2*	%	Plates	*AvrLepR1*	%	Plates	*AvrLm3*	%	Plates	*AvrLm4*	%	Plates
RL4	20.00%	8.96	RL3	14.29%	6.40	RL4	20.00%	8.96	DM96	50.00%	22.4	RL4	12.50%	5.60
X44	20.00%	8.96	X65	14.29%	6.40	X108	20.00%	8.96	DM118	50.00%	22.4	X108	12.50%	5.60
X65	20.00%	8.96	X159	14.29%	6.40	X159	20.00%	8.96				X159	12.50%	5.60
X108	20.00%	8.96	DM88	14.29%	6.40	DM88	20.00%	8.96				DM88	12.50%	5.60
DM88	20.00%	8.96	MB11-31-2	14.29%	6.40	MB11-31-2	20.00%	8.96				MB11-31-2	12.50%	5.60
			DM96	14.29%	6.40							RL3	12.50%	5.60
			DM118	14.29%	6.40							X44	12.50%	5.60
												X65	12.50%	5.60
Total Plates		44.80	Total Plates		44.80	Total Plates		44.80	Total Plates		44.80	Total Plates		44.80
Pooled Total														224.00

Table S6.2 Primer used in PCR amplification.

Primer ID	Sequence		Linked gene	References
AvrLm2_3	F-CATGCGGCTAGCCAATTTTC	R-CAGGTTTTCGTGGGGTCTTG	*AvrLm2*	Ghanbarnia et al. 2015
AvrLm3_1	F-AATGCCACGCTGTTGCTTTC	R-GCTGCCTAACGTTCGATCCT	*AvrLm3*	Plissonneau et al. 2015
LemaT070880	F-ACAACCACTCTTCTTCACAGT	R-TGGTTTGGGTAAAGTTGTCCT	*AvrLmJ1 /AvrLm5*	Van de Wouw et al. 2014
AvrLm6ext	TCAATTTGTCTGTTCAAGTTATGGA	CCAGTTTTGAACCGTAGTGGTAGCA	*AvrLm6*	Fudal et al. 2009
uP119060	F-TGCGTTTCTTGCTTCCTATATTT	R-CAAGTTGGATCTTTCTCATTCG	*AvrLm11*	Balesdent et al. 2013
Tetra-primers	Outer-GTAACAAAGTAACGAAGGGCTTAATT Inner for G-TCTAAACCAGTCTCCTGCCC	Outer-GAAAACTCACCTCCGTATCTTTAGTC Inner for C-TAGCTCAGCACCTGGAGTTATATATC	*AvrLm4-7*	Zou et al. 2018

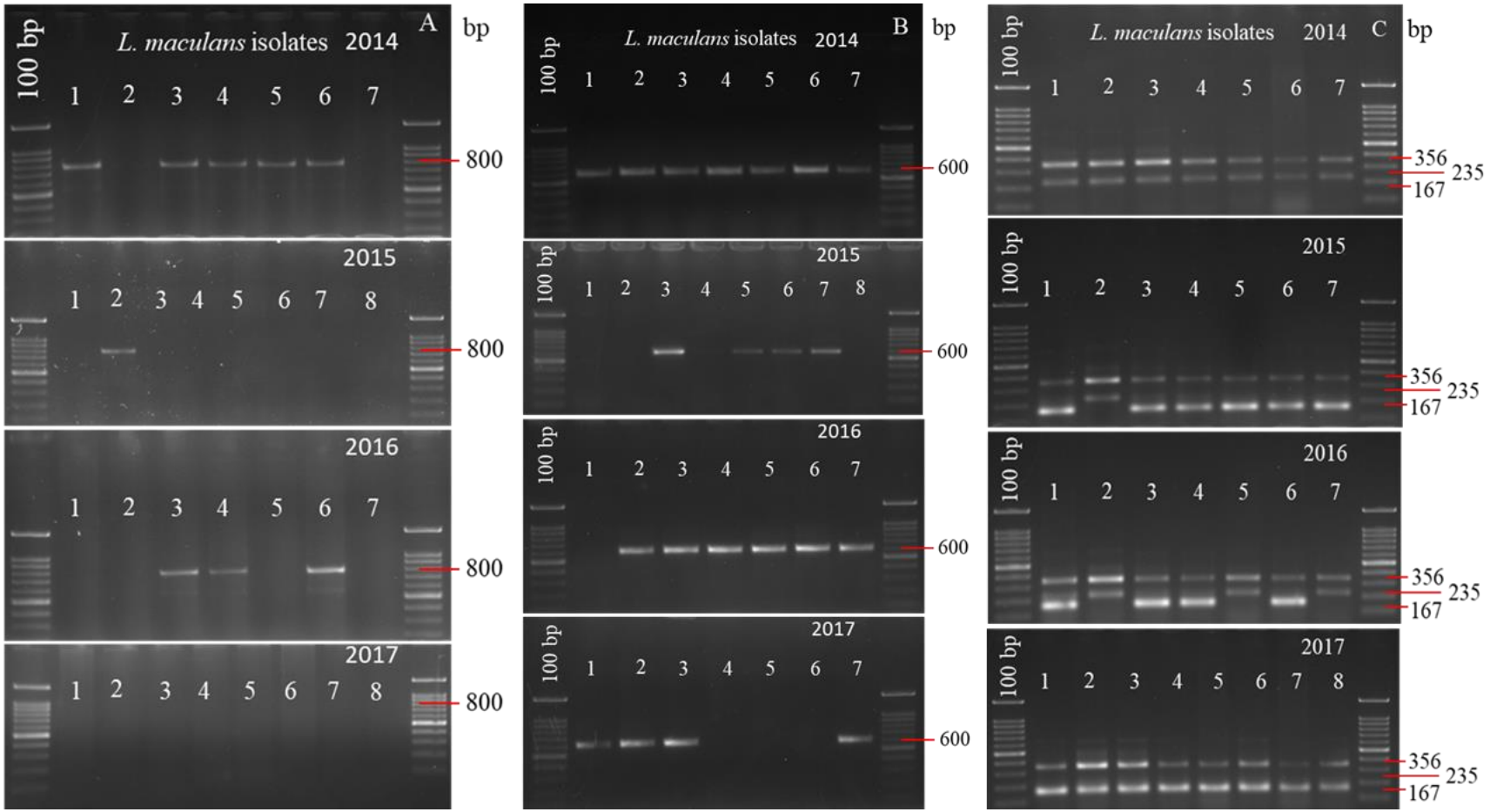

Fig. S6.1 Genotype of *AvrLm2* (A), *AvrLm3* (B) and *AvrLm4AvrLm7* (C) in *Leptosphaeria maculans* isolates cultured from *Brassica napus* stubbles harbouring corresponding *R* genes *Rlm2*, *Rlm3*, and *Rlm4*, respectively. Note that the fragment size 800 bp, 600 bp, 235 bp, and 167 bp are corresponding to *AvrLm2*, *AvrLm3*, *AvrLm4AvrLm7*, and *avrLm4AvrLm7*, respectively; whereas the 356 bp PCR product is a common band noted for for the primers linked to *AvrLm4-7*.

7 GENERAL DISCUSSION AND FUTURE DIRECTIONS

Blackleg disease and its increased occurrence in all three Prairie Provinces are a major issue and constraint faced by both the canola seed industry and the growers. This doctoral study generated research tools that can be utilized by growers and industry. In addition, the seed industry can benefit by utilizing the outcomes from this research in their breeding programs, and developing blackleg resistant lines and/or cultivars that could eventually be effective over a longer period of time and ultimately increase total production allowing greater access to the canola market around the world. Results from this study could assist in increasing canola production from 42.3 to 52 bushels/acre or more and achieve the new target of Canola Council - 26 million metric tonnes of sustained market demand by 2025.

7.1 Development of DNA markers and CSSLs linked to the blackleg resistance gene *Rlm6*

Breeding of canola cultivars can be accelerated by marker assisted selection (MAS). Several marker systems including SCAR, RFLP and AFLP linked to B-genome resistance have been used in *Brassica* breeding (Plieske and Struss 2001; Saal and Struss 2005). SCAR markers linked to the locus *cLmR1* (Mayerhofer et al. 2005), and 'indel' markers linked to the locus *LepR3* (Larkan et al. 2013) have also been used in *B. napus* disease resistance breeding. In this study, SCAR and dCAPS markers linked to the B-genome resistance gene *Rlm6* were developed and studied for their segregation. Segregation of the SCAR marker in the interspecific hybrids did not have an acceptable fit to a Mendelian segregation, indicating marker skewedness. Since the SCAR markers used in this study were dominant, it is unknown whether the frequency of homozygotes or heterozygotes was reduced. In this study, segregation of the dCAPS marker in the interspecific hybrids had an acceptable fit to a Mendelian segregation, supporting the genetic control by a single dominant gene as shown in previous studies (Plieske and Struss 2001; Saal and Struss 2005; Raman et al. 2012a). This

study revealed that a single gene, *Rlm6*, is involved in controlling blackleg resistance in the *B. juncea* cultivar 'Forge', which is introgressed into *B. napus* background. The interspecific hybrids derived from *B. napus* x *B. juncea* were resistant to the *L. maculans* isolate at the cotyledon stage, indicating seedling resistance as a result of a race-specific 'gene-for-gene' interaction (Flor 1971). The interspecific hybrids showed the stable introgression of *B. juncea* resistance into *B. napus* background and was confirmed by the polymorphism of SCAR markers linked to the blackleg resistance gene *Rlm6*. The co-segregation of DNA-markers suggests a possible mechanism of introgression by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with the A- or C-genomes. Additionally, the phenotype of B-genome resistance in the interspecific hybrids followed an acceptable fit to a Mendelian ratio of resistant versus susceptible, supporting a monogenic dominant inheritance for blackleg resistance in the BC4 generation which is considered as chromosomal segment substitution lines (CSSLs; Fukuoka et al. 2010). However, the markers and CSSLs developed here may offer an opportunity towards marker-assisted backcross breeding in a cultivar development program.

7.2 Effectiveness of *R* genes in a canola-wheat 2-year crop rotation

In Canada, blackleg disease has been managed mostly by growing resistant canola varieties along with crop rotation (Kutcher et al. 2010a; Kutcher et al. 2013). Though commercial cultivars labelled as resistant to blackleg have been used for the past 30 years, disease incidence of blackleg has increased in recent years (Canadian plant disease survey; http://phytopath.ca/publication/cpds). The findings from the *R* gene durability study highlighted the effectiveness of a 2-year crop rotation in the reduction of blackleg severity. While the predominant *Rlm3* is not effective in grower fields at present (Zhang et al. 2016), some other genes such as *LepR1*, *LepR3*, *Rlm2* and *Rlm4* will be very effective according to the avirulence genes present in the *L. maculans* isolates (Liban et al. 2016; Fernando et al.

2018). Additionally, other effective *R* genes such as *Rlm5*, *Rlm6*, *Rlm7* and *Rlm11* can be introduced into Canadian canola cultivars. Although the diversity of *R* genes in current canola varieties is quite limited, *R* gene rotation strategies can still be a good choice for the Canadian canola industry. The rotation of *R* genes can minimize disease pressure by manipulating fungal populations as was seen in Australia (Marcroft et al. 2012a). This strategy can be used according to the results from this study and other related studies. For instance, *R* genes can be categorized into two different classes; group-1 (*Rlm1*, *Rlm2*, *Rlm6* and *LepR3*) and group-2 (*Rlm3*, *Rlm4*, *Rlm7*, *Rlm9* and *LepR1*), which can be included alternatively in a 2-year crop rotation to reduce the selection pressure on the fungal populations. The regression analysis in this study showed a straight-forward relationship between seed yield and blackleg severity, which suggested an order of effectiveness of *R* genes tested; *LepR3* > *LepR1* > *Rlm2* > *Rlm4* > *Rlm3* in western Canadian field conditions. This work will be the foundation of breeding programs for the canola industry in Canada which will develop cultivars with effective *R* genes against the blackleg pathogen, and finally, will be included in the proposed cultivar rotation strategies on the Prairies.

7.3 Emergence of virulent isolates in *Brassica napus*-*Leptosphaeria maculans* pathosystem

Although considerable advances in genetic resistance to blackleg disease, the causal agent *L. maculans* still poses a major challenge for canola growers in Australia, Canada and Europe due to the ability of the pathogen to evolve into new avirulence pathotypes. The evolution of new avirulence genes in fungal populations exert a strong selection pressure to the resistance genes, which is believed to be a major challenge in developing appropriate strategies managing resistance genes and improving their potential durability. That's why understanding the molecular mechanisms and timeframe of the emergence of new races is vital. This study strongly supports the observation of an increase in disease due to a shift

from *AvrLm2* and *AvrLm4* to *avrLm2* and *avrLm4*, respectively. A shift from *AvrLm2* to *avrLm2* allele has been observed by the Sanger sequencing and is postulated due to accumulation of point mutations. Prior to the cloning of *AvrLm2* and what is presented here for *AvrLm2* sequence, point mutations and deletions were considered as the main drivers for generating new races of *L. maculans* and many other fungi with highly repetitive genomes. *AvrLm4AvrLm7*-mediated recognition of *AvrLm3* (this study; Plissonneau et al. 2017) and *AvrLm5-9* (Ghanbarnia et al. 2018) reveals an epistatic interaction between pathogen and host. It has also been speculated that the avirulence gene *AvrLm3* may have a role in fungal life-cycle along with *AvrLm4AvrLm7*, improving *L. maculans* fitness while conserving *AvrLm3* integrity. Those are the evidence for the presence of highly dynamic and complex mechanisms in *L. maculans* for the avoidance of host recognition. The emergence of virulent isolates due to epistatic effects, and epigenetic regulation, creates additional challenges to those breeding for disease resistance. However, this does suggest developing generic epidemiological models that take into account the complex molecular mechanisms allowing plant breeders to select appropriate *R* genes in the rotation strategy.

7.4 Contribution to the knowledge and/or industry

This is the first time in Canada that for *B. napus* CSSLs and DNA markers linked to the blackleg resistance gene *Rlm6* were developed, that can be used immediately to develop *B. napus* varieties resistant to *L. maculans*. To the best of our knowledge, this is also the first extensive report on the effectiveness of *R* gene assessed in a 2-year canola-wheat crop rotation. The key findings on the efficiency of *R* genes in Canada can benefit the canola industry as a whole. Primarily, this finding can guide canola breeders to make appropriate plans in breeding for blackleg resistance and furthermore, this finding can also guide canola growers to grow canola in a better way to reduce blackleg severity in canola fields. Results obtained from this study will not only provide us with a better understanding of shifting *L.*

maculans virulence toward *R* genes, but also provide us guidance in order to make proper decisions in blackleg resistance breeding programs in Canada. The outcome of this study provided a recommendation with regards to cultivar rotation based on *R* genes efficiency. This study recommended an order of *R* genes based on their effectivess against *L. maculans*, which can be implemented in the *R* genes deployment strategies on the Prairies.

7.5 Conclusions and future directions

In order to accelerate a breeding program, it is important to develop a variety of research tools such as DNA markers and plant materials which can speed up the breeding programs. The molecular marker SCAR and dCAPS developed in this study, which show linkage to the blackleg resistance gene *Rlm6*, would facilitate marker-assisted backcross breeding in the future. Moreover, the CSSLs derived from *B. napus* x *B. juncea* harbouring blackleg resistance gene *Rlm6* can accelerate a variety development program in the future. The inheritance study of the molecular markers here showed the segregation skewedness. To clarify the reason for segregation distortion, both the trait locus and SCAR markers could be analysed further in different populations, such as double haploid (DH), or near isogenic lines (NILs), to see if the same direction of marker skewedness would occur. Studies speculate that the segregation of molecular markers in DH or NILs could follow the Mendelian genetics. Moreover, studies towards fine mapping or map-based cloning of the resistance locus *Rlm6*, could also be possible by screening different mapping populations and/or by bulked segregant RNA-seq analyses (Liu et al. 2012). To facilitate a proper and effective rotation of *R* genes in disease control, it is important to develop *B. napus* varieties harbouring different *R* genes that confer disease resistance. The CSSLs and molecular markers developed in this study will be the effective tools to develop a *B. napus* variety harbouring *Rlm6*, which will be an excellent addition to the *R* gene deployment strategy on the Prairies. The results of 4-year field studies suggested an order of effectiveness among the *R* genes tested; *LepR3 > LepR1> Rlm2 > Rlm4*

> *Rlm3*. This sequence of efficiency could be the foundation of breeding programs for the canola industry in Canada which will develop cultivars with effective *R* genes against blackleg pathogen. Based on *B. napus*-*L. maculans* interaction, this study reports the loss of avirulence and gaining of virulence by the isolates of *L. maculans* in a single year. A shift from *AvrLm2* to the *avrLm2* allele has been observed and is due to the accumulation of point mutations. In addition, masking of *AvrLm3* phenotype by the predominant allele of *AvrLm4-7* is also confirmed by analysing the phenotype and the genotype of the isolates. These kinds of complex interactions between avirulence genes may also offer new perspectives for the deployment of the corresponding *R* genes. Therefore, this study suggests developing generic epidemiological models that take into account such complex molecular mechanisms allowing plant breeders to select appropriate *R* genes during their deployment. Moreover, reacquisition of avirulence activity in the *L. maculans* isolates was also detected in the alternative year which is predicted to reflect the effectiveness of canola-wheat rotation.

Overall, this study suggests to canola breeders to develop *B. napus* varieties with more effective *R* genes such as *Rlm5, Rlm6, Rlm7* and *Rlm11*. This study also recommend to the canola industry and/or growers to follow appropriate agronomic strategies including deployment of diverse *R* genes with at least a 2-years crop rotation, which will increase the *R* genes efficiency and maximize seed yield.

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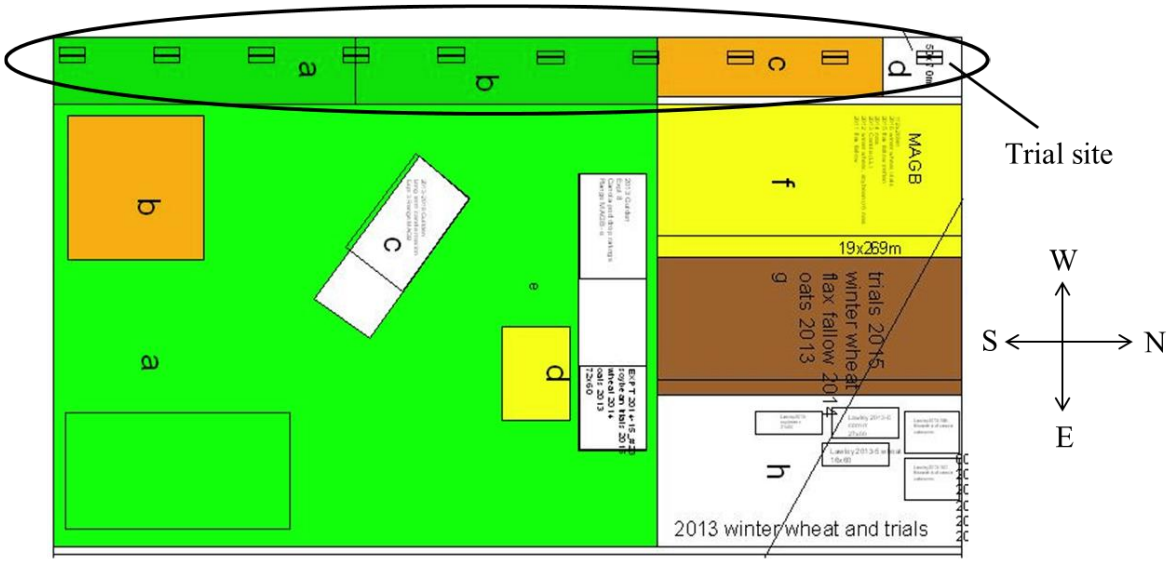
Appendix III. Blackleg disease rating at seedling stage using a scale of 0-9 (Williams and Delwiche 1979). Interaction phenotypes (IP) on 13-days post inoculation (dpi) cotyledons were rated where; 0 - no disease, 1 - dark necrotic lesions with 0.5–1.5 mm diameter and no sporulation, 3 - dark necrotic lesions 1.5–3.0 mm diameter and no sporulation, 5 - dark necrotic lesions 3.0–6.0 mm diameter and no sporulation but lesion size may increase during later infection stage, 7 - tissue collapse and lesions with diffuse margins and sporulation 9 - rapid tissue collapse, lesions with diffuse margins and profuse sporulation. Image adapted from Zhang 2016b.



Appendix IV. A rating scale of 0-5 used to assess blackleg disease severity on canola, showing stem cross sections (West et al. 2002). Where: 0 = no visible infection; 1= disease tissue occupies ≤25% of the cross section; 2 = disease tissue occupies 26-50% of the cross section; 3 = between 51 and 75% of the cross section infected; 4 = >75% of the cross section infected but plant alive and 5 = 100% of cross section infected and plant dead.



Appendix V. Experimental site at Ian N. Morrison Research Station (University of Manitoba) Carman Manitoba.

Appendix VI. List of abbreviations

Abbreviation	**Elaboration**
ANOVA	Analysis of variance
APR	Adult plant resistance
ARS	Average rating score
Avr	Avirulence
DPI	Days pot inoculation
CSSLs	Chromosomal segement substitution lines
H	Heterozygous
HR	Hypersensitive response
ICBN	International blackleg of crucifers network
IR	Intermediate resistant
LTR	Long terminal repeat
MR	Moderately resistant
MS	Moderately susceptible
PCD	Programed cell death
PCR	Polymerase chain reaction
PG	Pathogenicity group
QTL	Quantitative trait loci
R	Resistance
RDS	Relative disease severity
RIP	Repeat-induced point mutation
S	Susceptible
TE	Transposable elements