Eur J Plant Pathol
DOI 10.1007/s10658-015-0819-0

Breakdown of *Rlm3* resistance in the *Brassica napus–Leptosphaeria maculans* pathosystem in western Canada

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Accepted: 17 November 2015

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

Keywords *Brassica napus* · *Leptosphaeria maculans* · *R* genes · Adult plant resistance · Avirulence genes · Canola · Blackleg · Rlm · Avr · Canada

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Introduction

Canola (oilseed rape, *Brassica napus*) is one of the major oilseed crops of the world. Blackleg, caused by the fungal pathogen *Leptosphaeria maculans*, is a serious disease of canola in North America, Australia, Europe and many other regions around the world (Fitt et al. 2006). The disease can be controlled by crop

Published online: 27 November 2015

rotation, fungicide application as well as the use of resistant varieties (West et al. 2001; Fitt et al. 2006). As an environmentally friendly strategy, genetic resistance is generally very effective in disease control. Both seedling resistance controlled by major or seedling *R* genes and adult plant resistance (APR) mediated by quantitative resistance (minor) genes to *L. maculans* have been identified in *B. napus* varieties (Pongam et al. 1998; Balesdent et al. 2001; Pilet et al. 1998, 2001; Jestin et al. 2011, 2015). *R* genes confer race-specific resistance and follow the gene-for-gene concept proposed by Flor (1971). To date, at least 18 major *R* genes against *L. maculans* have been identified in *Brassica* species: *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* from *B. napus*, which have been mapped to two *B. napus* linkage groups, N7 and N10 (Ferreira et al. 1995; Mayerhofer et al. 1997; Ansan-Melayah et al. 1998; Zhu and Rimmer 2003; Rimmer 2006; Delourme et al. 2006); *Rlm8* and *Rlm11* from *B. rapa* (Balesdent et al. 2002, 2013); *Rlm5* and *Rlm6* from *B. juncea* (Chèvre et al. 1997; Balesdent et al. 2002); *Rlm10* from *B. nigra* (Chèvre et al. 1996; Eber et al. 2011); *LepR1*, *LepR2*, *LepR3*, *LepR4* and *RlmS* from re-synthesized *B. rapa subsp. Sylvestris* (Yu et al. 2005, 2007, 2008; Van de Wouw et al. 2009); and *BLMR1* and *BLMR2* from Surpass 400 (Long et al. 2011). To date, two *R* genes, *LepR3* (that interacts with *AvrLm1*) and *Rlm2*, have been cloned (Larkan et al. 2013, 2015). By contrast, at least seven of the corresponding avirulence (Avr) genes have been cloned: *AvrLm1* (Gout et al. 2006b), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLm3* (Plissonneau et al., 2015), *AvrLm5*/*AvrLmJ1* (Van de Wouw et al. 2014a; Balesdent & Howlett unpublished data), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013).

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

Both seedling and adult plant resistance play important roles in blackleg control. It has been shown that a combination of major gene resistance and adult plant resistance can provide effective and durable resistance against blackleg (Kiyosawa 1982; Brun et al. 2010). Selection of blackleg resistant breeding materials is usually based on field evaluations without genetic characterization of *R* genes (Rouxel et al. 2003b). Moreover, the interaction between specific *R* genes and their corresponding avirulence genes in the seedling stage normally results in very low disease severity at the adult plant stage. Therefore, it is difficult to dissect blackleg resistance evaluated under field conditions into major gene resistance and/or adult plant resistance. To develop varieties with a combination of seedling resistance and adult plant resistance, it is necessary to characterize *R* genes in breeding lines and then evaluate APR by reducing the interference of *R* genes.

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

reported changes in the population structure of *L. maculans* in western Canada, which was believed to be the result of the use of specific *R* gene(s). Liban et al. (2013) provided further evidence of this when they reported a shift in avirulence allele frequency in isolates collected in 2010 and 2011. The association between the specific *R* gene(s) in canola varieties as mentioned by Kutcher et al. (2010) and the corresponding avirulence gene(s) in *L. maculans* populations can be revealed by investigating *R* genes in canola varieties and avirulence allele frequencies in field fungal populations.

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

Materials and methods

Brassica napus varieties/lines and seed sample collection

B. napus varieties/lines with known *R* genes were considered as differentials and used to characterize avirulence genes in *L. maculans* isolates. The *B. napus* differentials used in this study are listed in Table 1. A total of 104 *B. napus* varieties/lines, which will be referred to as *B. napus* accessions, included commercial varieties released since 1980s and advanced breeding lines, were kindly provided by commercial seed companies and research institutions. These 104 *B. napus* accessions were used to investigate *R* genes in Canadian *B. napus* germplasm. A collection of 102 *B. napus* seed samples were directly collected from different growers' fields across Manitoba in 2012. These 102 seed samples were used to determine the proportion of *R* genes used in the fields. Of 102 seed samples, 35 were from fields where canola stems were collected and *L. maculans* isolates were identified in this study. These 35 samples were considered a subset of field seed samples.

Table 1 *Brassica napus* varieties/lines used as differentials to identify avirulence genotypes of *Leptosphaeria maculans* isolates

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

Leptosphaeria maculans isolates and canola stem collection

Isolates of *L. maculans* previously characterized for avirulence genes were used to identify *R* genes in canola varieties/lines; these were referred to as differential isolates. A total of 12 isolates (D1-D10, D13, and D14) were provided and previously characterized by scientists at the University of Melbourne to identify 10 avirulence genes (*AvrLm1-AvrLm9* and *AvrLmS*). The avirulence genotypes of *AvrLm1-9* and *AvrLmS* in isolates D8, D9, D13 and D14 were described in Marcroft et al. (2012a). In addition, seven *L. maculans* isolates (ICBN14, PHW1223, JN2/v23.1.2, JN3/v23.1.3, S7, R2, and AD746) were characterized previously. The genotypes of *AvrLm1-AvrLm9* in four isolates (ICBN14, PHW1223, and JN2/v23.1.2) were described in Balesdent et al. (2005); in isolates R2 and S7 were described in Leflon et al. (2007); and in isolate JN3/v23.1.3 was described in Balesdent et al. (2013). Genotypes of *AvrLepR1* and *AvrLepR2* in these 19 differential isolates were characterized in this

study. Genotypes of some *Avr* genes in these 19 differential isolates were further confirmed in this study, using differential varieties/lines listed in Table 1. Additional isolates were collected from western Canada and characterized in this study. The *L. maculans* differentials used in this study are described in Table 2. Canola stems collected after harvest were randomly sampled from 37 growers' canola fields across Manitoba in 2012. Seed samples of 35 of these fields were collected

Table 2 Avirulence genotypes of *Leptosphaeria maculans* differential isolates

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

[a] +/− indicates the presence/absence of a specific avirulence gene

[b] nd indicates the genotype was not determined

and included in 102 seed samples that were used to determine the proportion of *R* genes used in the fields, whereas seed samples were not available for two fields.

Leptosphaeria maculans isolation, inoculum preparation and DNA extraction

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

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

Characterization of avirulence genotypes of *Leptosphaeria maculans* isolates

Cotyledon inoculation and gene-specific PCR assays were used to identify avirulence genotypes of the *L. maculans* isolates. In the cotyledon inoculation assay, *B. napus* differentials used to confirm/characterize the *L. maculans* differential isolates were Westar (no known resistance gene, susceptible check), Darmor, MT29, Falcon, Cooper, Samourai, 01–23-2-1, Quinta, Surpass 400, 1065, Verona, 1135, Columbus, Jet Neuf, Goéland, Bristol, and 02–22-2-1 (Table 1). Avirulence genotyping of 300 *L. maculans* isolates collected from the field was performed with 11 *B. napus* differential varieties/lines: Westar, Quinta, Bristol, Jet Neuf, 01–23-2-1, Goéland, 1065, 1135, 02–22-2-1, Surpass 400, and MT29 (Table 1).

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

Polymerase chain reaction (PCR) characterization of six cloned avirulence (Avr) genes in *L. maculans* isolates collected from growers' field (2012) was performed: *AvrLm1* (Gout et al. 2006b), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLmJ1*/*AvrLm5* (Van de Wouw et al. 2014a, Balesdent & Howlett unpublished data), *AvrLm4-7* (Parlange et al. 2009), and *AvrLm6* (Fudal et al. 2009) and *AvrLm11* (Balesdent et al. 2013). *HaeIII* enzyme (GG^CC) was used to digest the PCR product of *AvrLm4*-7 to detect the SNP mutation of C^{358} to G^{358} that leads to virulence against *Rlm4*. The avirulence/virulence of *AvrLm1*, *AvrLm2*, *AvrLmJ1*, *AvrLm6*, and *AvrLm11* were decided by presence/absence of the corresponding PCR products. The avirulence genotypes of *AvrLm1*, *AvrLm2*, and *AvrLm4* in 300 isolates were a combination of PCR assay and differential test results. The genotypes of *AvrLmJ1*/*AvrLm5*, *AvrLm6*, and *AvrLm11* were only determined by gene-specific PCR assay as we do not have access to any differential varieties that can identify the presence/absence of these three genes.

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

A total of 206 *B. napus* accessions/seed samples were collected for *R* gene characterization, and two trials were performed. Trial I included 104 Canadian *B. napus* accessions. A set of 22 ((D1-D10, D13, D14, S7, ICBN14, PHW1223, R2, AD746, JN2, JN3, J3, J20 and Q12); Table 2) differential isolates, which were able to detect 12 *R* genes (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7*, *Rlm8*, *Rlm9*, *RlmS*, *LepR1*, *LepR2*), were used to characterize *R* genes in these accessions. Three canola accessions (DF78, DF79 and DF80) were resistant to 21 differential isolates and as a

result the *R* genes they carried were difficult to postulate. Thus, twelve more *L. maculans* isolates from Canada (L-MD7-14, L-PC4-1, L-MP1-8, L-Sb1, L-MP1-6, L-Sb7-6, L-Br17-1, L-Mo5-1, L-Br1-16, L-RL25, L-DS103 and L-CV8-7) were used to further detect *R* genes in these three accessions (Table 2). Trial II included 102 canola seed samples collected from different growers' fields in Manitoba in 2012. This analysis identified the *R* genes present in the canola varieties grown by Manitoba growers in 2012. For this trial, a set of 11 *L. maculans* differentials (D3, D4, D5, D7, D10, AD746, JN3, J3, ICBN14, PHW1223 and R2) were used (Table 2). In trial II, 35 seed samples were collected from fields where blackleg infected stems were collected and analyzed in this study.

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

Adult plant resistance evaluation

Adult plant resistance of 104 Canadian *B. napus* accessions provided by companies and research institutions were evaluated under controlled conditions, where cv. Westar was used as a susceptible check. Among differential isolates, D3 infected 101 accessions and caused lesions on the cotyledons as early as 12 dpi. Three accessions, DF78, DF79 and DF80 were resistant to isolate D3 but susceptible to isolate D13. To reduce the interference of seedling resistance during adult plant resistance evaluation, isolate D13 was used to inoculate accessions DF78, DF79 and DF80, and isolate D3 was used to inoculate the other 101 accessions. Seeds were directly seeded into plastic pots (18 cm in diameter), and inoculated with a single *L. maculans* isolate. The experiment was a completely randomized design of three replicates, each with nine plants. The inoculation methods and spore concentration were the same as for the *R* gene identification. Seedling infection was observed 14 days after inoculation and plants without visible symptoms were removed. Infected plants were grown to maturity and evaluated for their blackleg resistance by inspecting internal infection on the cross-section of the crown. Disease severity of basal stems was scored on a 0–5 rating scale (WCC/RRC): 0 - no noticeable infection, 1 - diseased tissue occupies ≤25 % of the cross-section, 2 - diseased tissue occupies 25–50 % of cross-section, 3 - between 50 and 75 % of the cross-section infected, 4 - more than 75 % of the cross-section infected, 5–100 % of cross-section were diseased, plant dead.

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

Data analysis

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

Results

Prevalence of *Rlm3* in Canadian canola varieties/lines

In Experiment I, 85 % of the accessions showed seedling resistance. A total of eight known *R* genes (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm9*, *RlmS*, *LepR1 and LepR2*) were detected (Fig. 1, Table 4). However, 16 accessions were susceptible to all *L. maculans* differential isolates and therefore no *R* gene was detected for these accessions. Some of the accessions carried uncharacterized

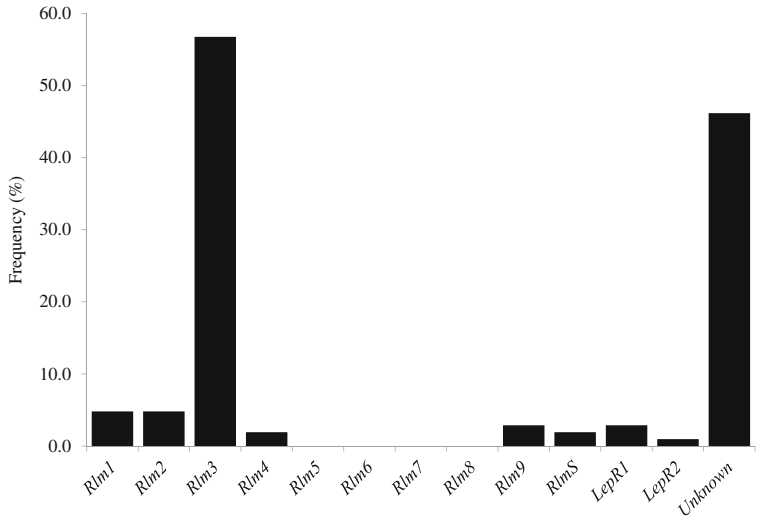

Fig. 1 Percentage of *R* genes in 104 Canadian *Brassica napus* accessions. Unknown means the resistance genotype could not be determined in this study, accessions that carried unknown *R* genes might be due to the presence of a new *R* gene, other known *R* genes that were not tested in this study, or combinations of several *R* genes. *Rlm5* and *Rlm8* might be present in some accessions, but further confirmation is required. *Rlm6* and *Rlm7* were not detected in the accessions tested

resistance genes that could not be deduced using the 22 differential isolates (Fig. 1, Table 4). This type of resistance was considered as unknown resistance in this study. The presence of unknown *R* gene resistance might be due to the effect of a novel *R* gene, other known *R* genes that were not tested in this study, or a combination of a few *R* genes. Among the *R* genes detected, *Rlm3* was present in 59 accessions, followed by *Rlm1* in 5 accessions, and *Rlm2* in 5 accessions. Both *Rlm9* and *LepR1* were detected in three accessions, while *Rlm4* was present in two accessions, and *RlmS* in two accessions. *LepR2* appeared to be present in only one accession. In addition, some of the other *R* genes such as *Rlm5* and *Rlm8* might be present in some accessions, however further confirmation is required. Although we do not have access to variety names of the majority of the 104 accessions tested in this study, variety names of six accessions developed and provided by the University of Alberta, and 11 accessions developed by the University of Manitoba were available and were described in Table 3. Although *R* genes in Q2, Quantum, Conquest, and Hi-Q have been previously characterized (Kutcher, Personal communication), this study confirmed their *R* genes and the results were consistent with the previous study. All six varieties (Conquest, Hi-Q, Q2, Quantum, Cougar CL, Peace) developed by the University of Alberta were released during 1995–2001 and carried *Rlm3*. Of 11 varieties developed by the University of Manitoba, one released in 1995, two released in 2008 and 2012, respectively, carried *Rlm3*, four carried unknown resistance, and four did not carry any *R* gene. The results indicated that *Rlm3* was available in commercial varieties in the early 1990s.

Among 104 accessions, a total of 35 accessions carried a single *R* gene, including *Rlm1* in one accession, *Rlm3* in 32 accessions, *Rlm4* in one accession, and *LepR2* in one accession. A total of 19 accessions carried two resistance genes, and/or a combination of a known resistance gene and an unknown resistance gene or genes, such as *Rlm3* and *LepR1* or *Rlm3* and an unknown *R* gene. Eight *B. napus* accessions carried three or more *R* genes. A total of 26 *B. napus* accessions carried only unknown *R* genes. The rest 16 accessions were susceptible to all 22 differential isolates and did not carry any *R* gene (Table 4).

In Experiment II, *R* gene was present in 58 % of seed samples. Only three *R* genes (*Rlm1*, *Rlm2*, and *Rlm3*) were detected. Among the 102 seed samples, 50 carried *Rlm3*, three carried *Rlm2*, and two carried *Rlm1*. An unknown *R* gene or genes were detected in nine seed samples. Most seed samples showing seedling resistance carried single *Rlm3*. Seed samples from only three fields carried more than one *R* gene: BR1: *Rlm2*, *Rlm3*; BR5: *Rlm2*, *Rlm3*; BR21: *Rlm1*, *Rlm2*, *Rlm3*. Surprisingly, 43 seed samples carried none of the 12 *R* genes that could be detected using the *L. maculans* differential isolates in this study.

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

Table 3 *R* genes and adult plant resistance of 17 Canadian *B. napus* accessions with known variety names

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

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

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

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

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

Adult plant resistance evaluation under controlled environment

In this study, the resistance observed in the APR evaluation of 104 *B. napus* accessions was assumed to be mediated by adult plant resistance genes since none of the varieties/lines tested showed seedling resistance after infection. Disease severity of 104 accessions ranged from 0 to 4.8 (0–5 scale). More than 50 % of *B. napus* accessions showed disease severity of lower than 2. Five accessions showed disease severity of higher than 4 (Fig. 2). Significant differences of the relative disease severity among accessions were observed ($F = 8.30$, $p < 0.0001$). APR evaluation indicated that 58 accessions were either resistant or moderately resistant to blackleg, while the rest accessions were susceptible or moderately susceptible at the adult plant stage (Table 4). Among 58 accessions that exhibited APR, 50 also had *R* gene resistance at seedling stage, and eight exhibited only APR. Of 46 susceptible or moderately susceptible accessions, 8 were susceptible to all differential isolates at the seedling stage, and all others carried seedling resistance. In summary, a large proportion of *B. napus* accessions had both adult plant resistance and seedling resistance due to an *R* gene (most commonly *Rlm3*).

Table 4 Summary of *R* genes and adult plant resistance in 104 Canadian *B. napus* accessions

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

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

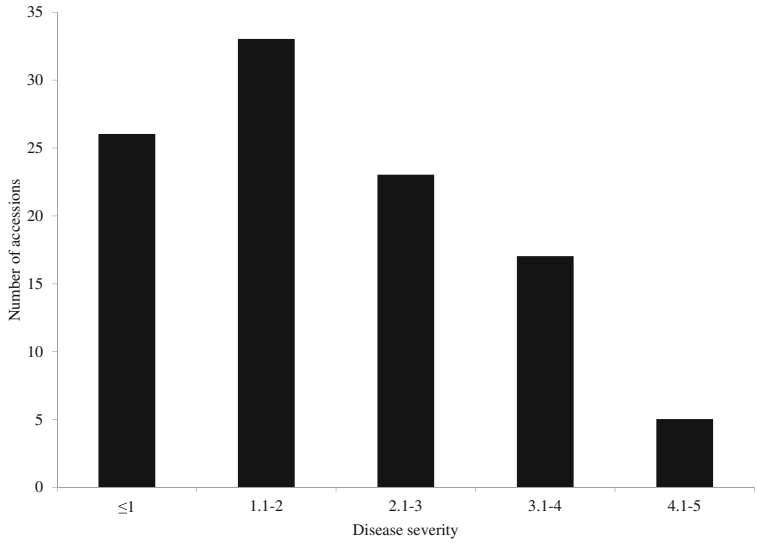

Fig. 2 Frequency distribution of disease severity for adult plant resistance of 104 *B. napus* accessions evaluated under controlled conditions

Avirulence genotype characterization of *Leptosphaeria maculans* isolates

The avirulence genes of all isolates used as differentials in this study are listed in Table 2. The frequency of 12 avirulence alleles in 300 *L. maculans* isolates collected from fields were identified (Fig. 3). Among the 12 avirulence alleles, the frequency of *AvrLm3* was the lowest (2.7 %) and was detected in only eight isolates. Five of the *AvrLm3*-carrying isolates were from Brandon, two were from Morden, and one from Morris. The avirulence alleles of *AvrLm9* and *AvrLepR2* were detected in 3.3 % and 10.7 % of the isolates collection, respectively. A few avirulence genes were detected in higher frequency: *AvrLm1*, 22.0 %; *AvrLepR1*, 39.1 %; *AvrLm2*, 64.3 %; *AvrLm11*, 65.3 % and *AvrLm6*, 66.0 %. The proportion of avirulence alleles *AvrLm4*, *AvrLm5* and *AvrLm7* were the highest, which accounted for 77.1 %, 80.7 %, and 89.2 % of the isolates collection, respectively. Only one differential variety, Surpass 400 (*RlmS*, *LepR3*) could be used to identify *AvrLmS*, and *LepR3* in Surpass 400 interacts with *AvrLm1*. Therefore, we were not able to identify *AvrLmS* in 73 isolates due to the presence of *AvrLm1*. The frequency of *AvrLmS* accounted for 34.4 % of 227 isolates. The number of avirulence genes per isolate ranged from 2 to 9. The majority of the isolates (226) carried five or more avirulence alleles..

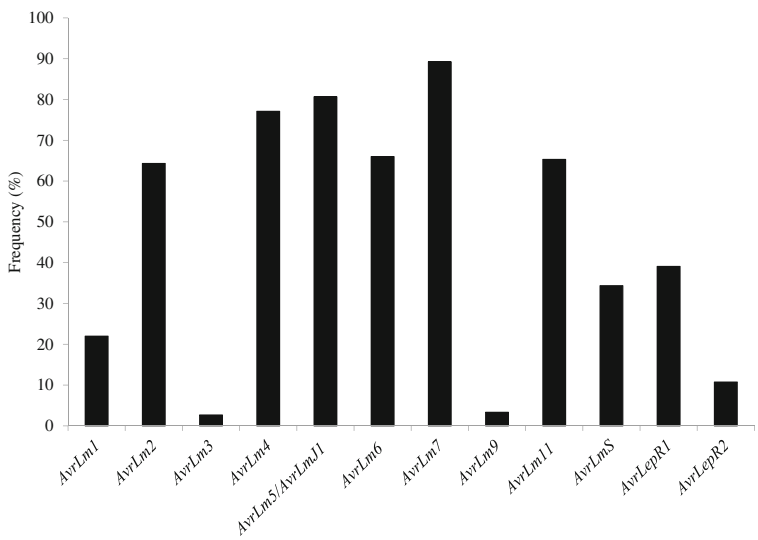

Fig. 3 Frequency of avirulence alleles in a collection of 300 *Leptosphaeria maculans* isolates collected in Manitoba in 2012. *AvrLmS* was assessed in 227 *L. maculans* isolates

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

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

Frequency of Avr alleles in *L. maculans* isolates from 37 canola fields were compared (Table 5). Variations between fields were observed for some avirulence alleles. The frequency of *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, and *AvrLm11* were high in the majority of fields, whereas variations between fields were observed in the frequency of *AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLmS*, *AvrLepR1*, and *AvrLepR2*. *AvrLm3* was only detected in five fields. Among these 5 fields, seed samples from three fields did not carry any *R* gene, seed sample from one field carried unknown resistance, whereas seed samples from one field carried *AvrLm3*.

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

Discussion

To the best of our knowledge, this is the first thorough report on the *R* genes present in Canadian *B. napus* germplasm. A total of eight known *R* genes were detected in 104 *B. napus* accessions with *Rlm3* being clearly the predominent *R* gene identified within the collection. The presence of *Rlm3* within Canadian *B. napus* accessions, and the deficiency of the

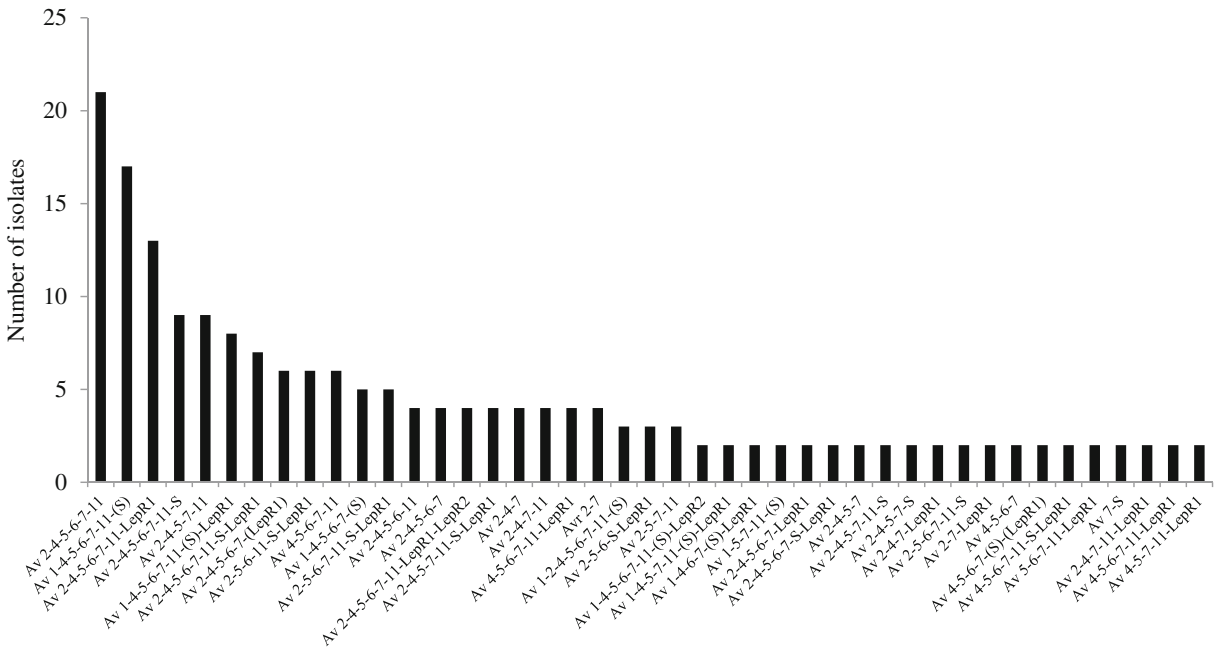

Fig. 4 Major races of *L. maculans* identified from 300 isolates collected in Manitoba in 2012. Race structures were identified based on 12 avirulence alleles

Table 5 *R* genes in seed samples and frequency of avirulence alleles in *L. maculans* isolates collected from 37 canola fields in Manitoba

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

Frequency was calculated as the percentage of isolates carrying a given avirulence allele

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

[b] Frequency of avirulence alleles in isolates collected from the field was not calculated due to small sample size

corresponding *AvrLm3* in the *L. maculans* population clearly indicated the breakdown of *Rlm3* resistance in western Canada. The high frequency of *Rlm3* in Canadian canola accessions is likely due to the use of a single source of resistance in breeding programs since the first report of this disease in Canada in the 1970s (Gugel and Petrie 1992). When pathogenicity groups (PGs) were used to describe *L. maculans* populations, the predominant PG in western Canada was PG2 (Chen and Fernando 2006; Kutcher et al. 2007, 2010). It can be hypothesized that breeding for blackleg resistance was conducted against a pathogen population of limited variability, only PG2 isolates (virulent on *B. napus* cv. Westar, avirulent on Glacier and Quinta). The corresponding *R* genes in differential varieties to PG2 isolates, are *Rlm2* and *Rlm3* in Glacier (Balesdent et al. 2002), *Rlm1* and *Rlm3* in Quinta (Kutcher et al. 2010). Due to the presence of *Rlm3* in both Glacier and Quinta, the probability of introducing *Rlm3* into canola varieties was extremely high. Our findings support this theory as *Rlm1*, *Rlm2*, and *Rlm3* were the top three *R* genes present in the *B. napus* accessions. Furthermore, Canadian blackleg resistant canola varieties were released in the early 1990s (Kutcher et al. 2011), and this study clearly indicated that *Rlm3* was present in Canadian canola varieties released in the 1990s, such as Quantum (1995), Allons (1995), Q2 (1998), and Hi-Q (1999).

The breakdown of *Rlm3* resistance demonstrates the high evolutionary potential of *L. maculans* populations in western Canada where PG2 isolates were dominant for a very long time (Chen and Fernando 2006). More specifically, between 1984 and 1998, only PG1 (*Leptosphaeria biglobosa*) and PG2 isolates were observed, but other PGs (PG3, PG4, PGT) were identified by 1998 (Keri et al. 2001; Chen and Fernando 2006). Balesdent et al. (2005) reported that *AvrLm3* was present in 69.2 % of Canadian *L. maculans* isolates collected during 1985–1992. The frequency of *AvrLm3* in *L. maculans* isolates collected between 1997 and 2005 in western Canada was 17.7 %, much lower than the frequency of other avirulence alleles (Kutcher et al. 2010). Dilmaghani et al. (2009) reported the *AvrLm3* allele was present in about 60 % of *L. maculans* isolates collected in western Canada between 2005 and 2006, but variations between locations were observed. In 2010 and 2011, 8.7 % of *L. maculans* isolates collected in western Canada carried the *AvrLm3* allele (Liban et al. 2013). By 2012, our results demonstrate that the frequency of *AvrLm3* in *L. maculans* isolates collected in Manitoba had dropped to 2.7 %. The frequency of *AvrLm3* isolates varied between locations and years in Canada, which could be indicative of a transitory situation towards *Rlm3*. In addition to changes in the frequency of Avr alleles, disease incidence and severity of blackleg on canola has fluctuated (Canadian disease survey, http://phytopath.ca/publication/cpds). The increase in disease severity correlates with the declining frequency of *AvrLm3* observed from 2005 to 2012, and the increasing use of resistant varieties with *Rlm3* resistance over the years. In addition, the effect of intensive production of canola from the common practice of one canola crop every four years on a field to the very common one canola crop every two years has likely played a role in increased frequency and severity of blackleg (Kutcher et al. 2013). This finding strongly supports the observation of increased disease incidence as a result of the shift from *AvrLm3* to *avrLm3*, mainly due to the repeated use of *Rlm3* (Kutcher et al. 2010; Liban et al. 2013).

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

Knowledge on Avr alleles of *L. maculans* isolates has major implications in deploying *R* genes in management of diseases. For example, studies on avirulence alleles in field populations (2002, 2003) of *L. maculans* suggested potential effectiveness of *Rlm6* and *Rlm7* in Europe (Stachowiak et al. 2006). Results from our study indicated very high frequency of *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, and *AvrLm11*, while very low frequency of *AvrLm3* and *AvrLm9* in *L. maculans* populations in Manitoba (2012 collection). According to our knowledge on Avr alleles in *L. maculans* populations and *R* genes in *B. napus* germplasm, *Rlm2* and *Rlm4* are probably very useful in current Canadian *R* gene

deployment. In addition, other useful *R* genes such as *Rlm5*, *Rlm6*, *Rlm7*, and *Rlm11* can be introduced into Canadian canola varieties. In contrast, *Rlm3* and *Rlm9* were overcome and *Rlm1*, *LepR1*, and *LepR2* are in the process of being overcome. However, except for *Rlm3*, there is no evidence that *Rlm1*, *LepR1*, and *LepR2* are widely used in Canada. We also do not know whether *Rlm9* was used in blackleg control in Canada as *Rlm9* was not detected in this study. In Canada, the frequency of both *AvrLm3* and *AvrLm9* in blackleg populations decreased with time (Kutcher et al. 2010; Liban et al. 2013; Fernando et al. unpublished). This phenomenon suggested intensive use of *Rlm3* in Canada may have resulted in decrease of both *AvrLm3* and *AvrLm9* in isolates as they are in the same gene cluster. In contrast, with the decrease of *AvrLm3*, the frequency of *AvrLm7* in Canadian *L. maculans* populations increased with time (Dilmaghani et al. 2009; Liban et al. 2013). Moreover, only a small number of *L. maculans* isolates have been found to carry both *AvrLm3* and *AvrLm7* in previous studies (Balesdent et al. 2006; Kutcher et al. 2010; Dilmaghani et al. 2009). Our study's findings corroborate this as further evidence of this phenomenon suggesting the co-existence of these two genes in *L. maculans* to be uncommon. Recently, Plissonneau et al. (2015) found *AvrLm3* was only expressed if the isolate did not carry *AvrLm7*, and illustrated the 'hide-and-seek' relationship between *AvrLm3* and *AvrLm7*. These phenomena can be at least partially explained by the fact that *AvrLm3*, *AvrLm7* and *AvrLm9* are part of the *AvrLm3-4-7-9-LepR1* genetic cluster (Balesdent et al. 2002, 2005; Ghanbarnia et al. 2012), and can further provide guidance to blackleg management through appropriate *R*-gene rotations.

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

Durability of resistance is particularly important in blackleg control (Pietravalle et al. 2006). One effective strategy to improve the durability of blackleg resistance is through the development of canola varieties with a combination of *R* genes and APR (Kiyosawa 1982; Pietravalle et al. 2006; Brun et al. 2010; Delourme et al. 2014). Canola accessions that have both APR and *R* gene resistance can increase the durability of *R* gene resistance (Brun et al. 2010; Marcroft et al. 2012b). In our study, about half of the Canadian canola accessions had both APR and *R* gene resistance. Although APR was evaluated under controlled conditions in this study, APR identified under controlled conditions can improve the process of blackleg resistance breeding (Huang et al. 2014), and it may be considered a preliminary evaluation that can be further confirmed by field evaluations. Although APR can be isolate-specific in some cases, as described by Marcroft et al. (2012a), APR is usually considered as race non-specific (Delourme et al. 2006).

Unlike the rapid breakdown of 'sylvestris' resistance in Australia and *Rlm1* in Europe (Rouxel et al. 2003a; Sprague et al. 2006; Van de Wouw et al. 2010) that occurred within a few years, the breakdown of *Rlm3* resistance in western Canada appears to have been much slower. Although *Rlm3*-carrying varieties were released in 1990s, *Rlm3* appeared to be still very effective in 2005 because the *AvrLm3* allele was present in about 60 % of *L. maculans* isolates collected in western Canada between 2005 and 2006 (Dilmaghani et al. 2009). This could be due to the fact that *Rlm3* resistance was deployed in different canola varieties with different genetic backgrounds (with different levels of APR). Although other *R* genes were rarely detected in Canadian canola accessions, they still have a role to play in blackleg control. In addition, a combination of *Rlm3* and APR, or the combination of *Rlm3* with other *R* genes in some commercial varieties may have reduced the speed of the breakdown of *Rlm3* observed. Although both ascospores

(sexual) and pycnidiospores (asexual) can infect oilseed rape, ascospores have many advantages over pycnidiospores during disease epidemiology and therefore contributed more in generating variations at avirulence loci to overcome *R* genes (Dilmaghani et al. 2013). In western Canada, pycnidiospores are a major source of primary inoculum (Ghanbarnia et al. 2011; Guo et al. 2005) and this is another reason that may explain the slowdown of breakdown as it limits genetic variability of the pathogen, along with a very limited spread in space of pycnidiosopres compared to ascospores. In addition, high clonal fractions in *L. maculans* populations in western Canada further confirmed a lack of ascospore-mediated infection and of sexual reproduction (Dilmaghani et al. 2009, 2013). Furthermore, crop rotation, and short cultivation season (3 months) of canola in Canada are not in favour of sexual mating. With this in mind, canola breeders might use the less common *R* genes to develop new varieties, to increase the number of varieties to facilitate *R* gene rotation.

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

Acknowledgments This work was funded by Canola Agronomic Research Program (CARP) of the Canola Council of Canada. Xuehua Zhang is supported by China Scholarship Council (CSC). The authors wish to thank Dr. Angela Van de Wouw at the University of Melbourne for providing differential isolates for *R* gene characterization. The authors thank Paula Parks, Sakaria Liban, Besrat Demoz and Jaqueline Huzar Novakowiski for technical assistance. We are grateful to Drs. Carrie Selin and Shuanglong Huang for internal review of the manuscript. We acknowledge several seed companies and research institutions for providing canola seeds for this study.

References

Ansan-Melayah, D., Balesden, M. H., Delourme, R., Pilet, M. L., Renard, M., Tanguy, X., et al. (1998). Genes for race specific resistance against blackleg disease in *Brassica napus* L. *Plant Breeding*, *117*, 373–378.

Balesdent, M. H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M., & Rouxel, T. (2001). Genetic control and host range of avirulence towards *Brassica napus* cvs. Quinta and Jet Neuf in Leptosphaeria maculans. *Phytopathology,*, *91*, 70–76.

Balesdent, M. H., Attard, A., Kuhn, M. L., & Rouxel, T. (2002). New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology*, *92*, 1122–1133.

Balesdent, M. H., Barbetti, M. J., Hua, L., Sivasithamparam, K., Gout, L., & Rouxel, T. (2005). Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology*, *95*, 1061–1071.

Balesdent, M. H., Louvard, K., Pinochet, X., & Rouxel, T. (2006). A large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. *European Journal of Plant Pathology*, *114*, 53–65.

Balesdent, M. H., Fudal, I., Ollivier, B., Bally, P., Grandaubert, J., Eber, F., et al. (2013). The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *brassica Rapa*. *New Phytologist*, *198*, 887–898.

Brun, H., Chevre, A. M., Fitt, B. D. L., Powers, S., Besnard, A. L., Ermel, M., et al. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist*, *185*, 285–299.

Calderon, C., Ward, E., Freeman, J., Foster, S. J., & Mccartney, H. A. (2002). Detection of airborne inoculum of *Leptosphaeria maculans* and *pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. *Plant Pathology*, *51*, 303–310.

Chen, Y., & Fernando, W. G. D. (2006). Prevalence of pathogenicity groups of *Leptosphaeria maculans* in western Canada and North Dakota, USA. *Canadian Journal of Plant Pathology*, *1*, 533–539.

Chèvre, A. M., Eber, F., This, P., Barret, P., Tanguy, X., Brun, H., et al. (1996). Characterization of *brassica nigra* chromosomes and of blackleg resistance in *B. napus–B. nigra* addition lines. *Plant Breeding*, *115*, 113–118.

Chèvre, A. M., Barret, P., Eber, F., Dupuy, P., Brun, H., Tanguy, X., et al. (1997). Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). *Theoretical and Applied Genetics*, *95*, 1104–1111.

Delourme, R., Pilet-Nayel, M. L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., et al. (2004). A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Phytopathology*, *94*, 578–583.

Delourme, R., Chèvre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias, J. S., et al. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *114*, 41–52.

Delourme, R., Brun, H., Ermel, M., Lucas, M. O., Vallee, P., Domin, C., et al. (2008). Expression of resistance to *Leptosphaeria maculans* in *Brassica napus* double haploid lines in France and Australia is influenced by location. *Annals of Applied Biology, 153*, 259–269.

Delourme, R., Bousset, L., Ermel, M., Duffé, P., Besnard, A. L., Marquer, B., et al. (2014). Quantitative resistance affects the speed of frequency increase but not the diversity of the virulence alleles overcoming a major resistance gene to

Leptosphaeria maculans in oilseed rape. *Infection, Genetics and Evolution*, *27*, 490–499.

Dilmaghani, A., Balesdent, M. H., Didier, J. P., Wu, C., Davey, J., Barbetti, M. J., et al. (2009). The *Leptosphaeria maculans* - *leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology*, *58*, 1044–1058.

Dilmaghani, A., Gout, L., Moreno-Rico, O., Dias, J. S., Coudard, L., Castillo-Torres, N., et al. (2013). Clonal populations of *Leptosphaeria maculans* contaminating cabbage in Mexico. *Plant Pathology*, *62*, 520–532.

Eber, F., Lourgant, K., Brun, H., Lode, M., Huteau, V., Coriton, O., et al. (2011). Analysis of *Brassica nigra* chromosomes allows identification of a new effective *Leptosphaeria maculans* resistance gene introgressed in *Brassica napus*. *Proceeding of the 13th International rapeseed congress*, Prague 5–9 June.

Ferreira, M. E., Rimmer, S. R., Williams, P. H., & Osborn, T. C. (1995). Mapping loci controlling *brassica resistance* to *L. maculans* under different screening conditions. *Phytopathology*, *85*, 213–217.

Fitt, B. D. L., Brun, H., Barbetti, M. J., & Rimmer, S. R. (2006). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, *114*, 3–15.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology*, *9*, 275–296.

Fudal, I., Ross, S., Gout, L., Blaise, F., Kuhn, M. L., Eckert, M. R., et al. (2007). Heterochromatin-like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome: map-based cloning of *AvrLm6*. *Molecular Plant-Microbe Interactions*, *20*, 459–470.

Fudal, I., Ross, S., Brun, H., Besnard, A. L., Ermel, M., Kuhn, M. L., et al. (2009). Repeat-induced point mutation (RIP) as an alternative mechanism of evolution towards virulence in *Leptosphaeria maculans*. *Molecular Plant-Microbe Interactions*, *22*, 932–941.

Ghanbarnia, K., Fernando, W. G. D., & Crow, G. (2011). Comparison of disease severity and incidence at different growth stages of naturally infected canola plants under field conditions by pycnidiospores of *phoma lingam* as a main source of inoculum. *Canadian Journal of Plant Pathology*, *33*(3), 355–363.

Ghanbarnia, K., Lydiate, J. L., Rimmer, S. R., Li, G., Kutcher, H. R., & Larkan, N. J. (2012). Genetic mapping of the *Leptosphaeria maculans* avirulance gene corresponding to the *LepR1* resistance gene of *Brassica napus*. *Theoretical Applied Genetics*, *124*, 505–513.

Ghanbarnia, K., Fudal, I., Larkan, N. J., Links, M. G., Balesdent, M. H., Profotova, B., et al. (2015). Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2*, using an intraspecific comparative genomics approach. *Molecular Plant Pathology*, *6*, 1–11.

Gout, L., Eckert, M., Rouxel, T., & Balesdent, M. H. (2006a). Genetic variability and distribution of mating-type alleles in field populations of *Leptosphaeria maculans* from France. *Applied and Environmental Microbiology*, *72*, 185–191.

Gout, L., Fudal, I., Kuhn, M. L., Blaise, F., Eckert, M., Cattolico, L., et al. (2006b). Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the dothideomycete *Leptosphaeria maculans*. *Molecular Microbiology*, *60*, 67–80.

Gugel, P. K., & Petrie, G. A. (1992). History, occurrence, impact and control of blackleg of rapeseed. *Canadian Journal of Plant Pathology*, *14*, 36–45.

Guo, X. W., Fernando, W. G. D., & Entz, M. (2005). Effects of crop rotation and tillage on blackleg disease of canola. *Canadian Journal of Plant Pathology*, *27*, 53–57.

Huang, Y. J., Qi, A., King, G. J., & Fitt, B. D. L. (2014). Assessing quantitative resistance against *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. *PloS One*, *9*(1), e84924.

Jestin, C., Lodé, M., Vallée, P., Domin, C., Falentin, C., Horvais, R., et al. (2011). Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). *Molecular Breeding*, *27*, 271–287.

Jestin, C., Bardol, N., Lodé, M., Duffé, P., Domin, C., Vallée, P., et al. (2015). Connected populations for detecting quantitative resistance factors to Phoma stem canker in oilseed rape (*Brassica napus* L.). *Molecular Breeding*, *35*, 167.

Keri, M., Kutcher, H. R., & Rimmer, S. R. (2001). Virulence of isolates of *Leptosphaeria maculans* from western Canada on *Brassica napus* differentials. *Canadian Journal of Plant Pathology*, *23*, 199.

Kiyosawa, S. (1982). Genetics and epidemiological modeling of breakdown of plant disease resistance. *Annual Review of Phytopathology*, *20*, 93–117.

Kutcher, H. R., van den Berg, C. G. J., & Rimmer, S. R. (1993). Variation in pathogenicity of *Leptosphaeria maculans* on brassica spp. based on cotyledon and stem reactions. *Canadian Journal of Plant Pathology*, *15*, 253–258.

Kutcher, H. R., Keri, H., Mclaren, D. L., & Rimmer, S. R. (2007). Pathogenic variability of *Leptosphaeria maculans* in western Canada. *Canadian Journal of Plant Pathology*, *393*, 388–393.

Kutcher, H. R., Balesdent, M. H., Rimmer, S. R., Rouxel, T., Chèvre, A. M., Delourme, R., et al. (2010). Frequency of avirulence genes in *Leptosphaeria maculans* in western Canada. *Canadian Journal of Plant Pathology*, *32*, 77–85.

Kutcher, H. R., Fernando, W. G. D., Turkington, T. K., & McLaren, D. L. (2011). Best management practices for blackleg disease of canola. *Prairie Soils & Crops Journal*, *4*, 122–134.

Kutcher, H. R., Brandt, S. A., Smith, E. G., Ulrich, D., Malhi, S. S., & Johnston, A. M. (2013). Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. *Canadian Journal of Plant Pathology*, *35*, 209–221.

Larkan, N. J., Lydiate, D. J., Parkin, I. A., Nelson, M. N., Epp, D. J., Cowling, W. A., et al. (2013). The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *AVRLM1*. *New Phytologist*, *197*, 595–605.

Larkan, N. J., Ma, L., & Borhan, M. H. (2015). The *Brassica napus* receptor-like protein RLM2 is encoded by a second allele of the *LepR3*/*Rlm2* blackleg resistance locus. *Plant Biotechnology Journal*, *13*, 983–992.

Leflon, M., Brun, H., Eber, F., Delourme, R., Lucas, M. O., Vallée, P., et al. (2007). Detection, introgression and localization of genes conferring specific resistance to *Leptosphaeria maculans* from *brassica Rapa* into *B. napus*. *Theoretical and Applied Genetics*, *115*, 897–906.

Liban, S. H., Cross, D. J., Fernando, W. G. D., Peng, G., & Kutcher, H. R. (2013). Practical genomics: geographic mapping of the race structure in the Canadian *Leptosphaeria*

maculans pathogen population. *Acta Phytopathologica Sinica*, *43*, S282.

Long, Y., Wang, Z., Sun, Z., Fernando, W. G. D., McVetty, B. E. P., & Li, G. (2011). Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola variety 'surpass 400'. *Theoretical and Applied Genetics*, *122*, 1223–1231.

Marcroft, S. J., Elliott, V. L., Cozijnsen, A. J., Salisbury, P. A., Howlett, B. J., & Van de Wouw, P. (2012a). Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* varieties based on reactions to isolates with known avirulence genotypes. *Crop Pasture Science*, *63*, 338–350.

Marcroft, S. J., Van de Wouw, A. P., Salisbury, P. A., Potter, T. D., & Howlett, B. J. (2012b). Effect of rotation of canola (*Brassica napus*) varieties with different complements of blackleg resistance genes on disease severity. *Plant Pathology*, *61*, 934–944.

Mayerhofer, R., Bansal, V. K., Thiagarajah, G. R., Stringam, G. R., & Good, A. G. (1997). Molecular mapping of resistance to *Leptosphaeria maculans in* Australian varieties of *Brassica napus*. *Genome*, *40*, 294–301.

Parlange, F., Daverdin, G., Fudal, I., Kuhn, M. L., Balesdent, M. H., Blaise, F., et al. (2009). *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of oilseed rape, and circumvents *Rlm4*-mediated recognition through a single amino acid change. *Molecular Microbiology*, *71*, 851–863.

Pietravalle, S., Lemarié, S., & van den Bosch, F. (2006). Durability of resistance and cost of virulence. *European Journal of Plant Pathology*, *114*(1), 107–116.

Pilet, M. L., Delourme, R., Foisset, N., & Renard, M. (1998). Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (desm.) Ces. et de not., in winter rapeseed (*Brassica napus* L.). *Theoretical and Applied Genetics*, *96*, 23–30.

Pilet, M. L., Duplan, G., Archipiano, M., Barret, P., Baron, C., Horvais, R., et al. (2001). Stability of QTL for field resistance to blackleg across two genetic backgrounds in oilseed rape. *Crop Science*, *41*, 197–205.

Plissonneau, C., Daverdin, G., Ollivier, B., Blaise, B., Degrave, A., Fudal, I., et al. (2015) A game of hide and seek between avirulence genes *AvrLm4-7* and *AvrLm3* in *Leptosphaeria maculans*. *New Phytologist.*

Pongam, P., Osborn, T. C., & Williams, P. H. (1998). Genetic analysis and identification of amplified fragment length polymorphism markers linked to the *alm1* avirulence gene of *Leptosphaeria maculans*. *Phytopathology*, *88*, 1068–1072.

Rimmer, S. R. (2006). Resistance genes *to Leptosphaeria maculans* in *Brassica napus*. *Canadian Journal of Plant Pathology*, *28*, S288–S297.

Rimmer, S. R., & van den Berg, C. G. J. (1992). Resistance of oilseed brassica spp. to blackleg caused by *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology*, *14*, 56–66.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., & Gout, L. (2003a). A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. *European Journal of Plant Pathology*, *109*, 871–881.

Rouxel, T., Willner, E., Coudard, L., & Balesdent, M. H. (2003b). Screening and identification of resistance to *Leptosphaeria maculans* (stem canker) in *Brassica napus* accessions. *Euphytica*, *133*, 219–231.

Sprague, S. J., Balesdent, M. H., Brun, H., Hayden, H. L., Marcroft, S. J., Pinocher, X., et al. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *European Journal of Plant Pathology*, *114*, 33–44.

Stachowiak, A., Olechnowicz, J., Jedryczka, M., Rouxel, T., Balesdent, M. H., Happstadius, I., et al. (2006). Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *European Journal of Plant Pathology*, *114*(1), 67–75.

Van de Wouw, A. P., Marcroft, S. J., Barbetti, M. J., Hua, L., Salisbury, P. A., Gout, L., et al. (2009). Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* variety with 'sylvestris-derived' resistance suggests involvement of two resistance genes. *Plant Pathology*, *58*, 305–313.

Van de Wouw, A. P., Cozijnsen, A. J., Hane, J. K., Brunner, P. C., McDonald, B. A., Oliver, R. P., et al. (2010). Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathogens*, *6*, 1–15.

Van de Wouw, A. P., Lowe, R. G. T., Elliott, C. E., Dubois, D. J., & Howlett, B. J. (2014a). An avirulence gene, *AvrLmJ1*, from the blackleg fungus, *Leptosphaeria maculans*, confers avirulence to *Brassica juncea* varieties. *Molecular Plant Pathology*, *15*, 523–530.

Van de Wouw, A. P., Marcroft, S. J., Ware, A., Lindbeck, K., Khangura, R., & Howlett, B. J. (2014b). Breakdown of resistance to the fungal disease, blackleg, is averted in commercial canola (*Brassica napus*) crops in Australia. *Field Crops Research*, *166*, 144–151.

West, J. S., Kharbanda, P. D., Barbetti, M. J., & Fitt, B. D. L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology*, *50*, 10–27.

Williams, P. H., & Delwiche, P. A. (1979). Screening for resistance to blackleg of crucifers in the seedling stage. In *In: Proc eucarpia conference on the breeding of cruciferous crops, Wageningen* (pp. 164–170.). The Netherlands pp.

Yu, F., Lydiate, D. J., & Rimmer, S. R. (2005). Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theoretical and Applied Genetics*, *110*, 969–979.

Yu, F., Lydiate, D. J., Hahn, K., Kuzmicz, S., Hammond, C., & Rimmer, S. R. (2007). Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* × *B. rapa* subsp. sylvestris. *Proceedings of the 12th IRC*, *Wuhan*, *PRC.*

Yu, F., Lydiate, D. J., & Rimmer, S. R. (2008). Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa* subsp. Sylvestris. *Genome*, *51*, 64–72.

Zhu, B., & Rimmer, S. R. (2003). Inheritance of resistance to *L. maculans* in two accessions of *B. napus*. *Canadian Journal of Plant Pathology*, *3*, 98–103.