Plant Pathology (2016) **65**, 1161–1169 Doi: 10.1111/ppa.12489

Race structure and frequency of avirulence genes in the western Canadian *Leptosphaeria maculans* pathogen population, the causal agent of blackleg in brassica species

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Leptosphaeria maculans is the causal agent of blackleg, a serious disease on canola/rapeseed in western Canada, Australia and Europe. Genetic resistance and extended crop rotation provided effective disease control in western Canada for years but the emergence of new pathogen races has reduced the effectiveness of current management strategies. The objective of this study was to analyse *L. maculans* isolates derived from canola stubble in commercial fields collected in 2010 and 2011 across western Canada for the presence and frequency of avirulence (*Avr*) genes. A total of 674 isolates were examined for the presence of *Avr* alleles *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS* using a set of differential host genotypes carrying known resistance genes or PCR amplification of *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7*. Certain alleles were more prevalent in the pathogen population, with *AvrLm6* and *AvrLm7* present in >85% of isolates, while *AvrLm3*, *AvrLm9* and *AvrLepR2* were present in <10% of isolates. A total of 55 races (different combinations of *Avr* alleles) were detected, with the two most common ones being *AvrLm2–Lm4–Lm6–Lm7* and *AvrLm2–Lm4–Lm6–Lm7–LmS*. Races carrying as many as seven and as few as one known *Avr* allele were detected. Selection pressure from the race-specific resistance genes carried in canola cultivars has probably played a significant role in the current *Avr* profile, which may have also contributed to the recent increase in blackleg observed in western Canada.

Keywords: avirulence genes, blackleg races, canola, western Canada

Introduction

Leptosphaeria maculans causes blackleg on canola (*Brassica napus*) in Canada and in several other countries. The disease was first reported in the Canadian province of Saskatchewan and yield losses up to 50% were observed in individual fields (Gugel & Petrie, 1992). In the early 1990s, commercial cultivars with genetic resistance to blackleg were released, and in combination with 3- to 4-year crop rotations, the disease was successfully controlled for many years. Changes in pathogen race structure were observed in the early 2000s (Chen & Fernando, 2006), probably attributable to the pathogen response to the resistance (*R*) genes in canola cultivars. In recent years producers and agronomists in western Canada have reported severe disease symptoms in cultivars that were registered as resistant, and the incidence and severity of the disease has increased steadily (Morrall, 2014). Changes in virulence may be due to pathogen adaptation to resistance genes in canola cultivars. The lower disease severity in Canada can in part be attributed to a colder climate and shorter growing season compared to Australia and parts of Europe, which limits the growth of the pathogen to only 6 months per year, and possibly because the asexual pycnidia are the dominant inoculum source (Ghanbarnia *et al.*, 2011).

In Canada the vast majority of canola cultivars carry the *R* gene *Rlm3*, while other *R* genes are rarely detected in commercial cultivars (Zhang *et al.*, 2013). This low diversity of *R* genes makes the crop vulnerable to pathogen adaptation. Some *Avr* genes may have a fitness cost (Huang *et al.*, 2006) and will therefore naturally re-emerge in the absence of selection pressure, while others can simply be lost as a result of selection pressure due to host resistance or through genetic drift. In addition, pathogen *Avr* genes have been shown to be subject to increased mutational pressure due to their proximity to transposable elements and localization in gene-poor AC isochores (Rouxel *et al.*, 2011). The dual sexual and asexual life cycles of *L. maculans* in combination with the underlying genetic plasticity of virulence factors provides the pathogen with an exceptional ability to rapidly overcome new resistance sources, as demonstrated in rapid breakdown of cultivars carrying *Rlm1* in France and *LepR3* in Australia (Li *et al.*, 2003; Rouxel *et al.*, 2003).

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Published online 10 January 2016

Resistance to blackleg relies on recognition of a pathogen *Avr* gene product by the corresponding *R* gene product in the host, which triggers cellular processes resulting in the hypersensitive reaction (Hayward *et al.*, 2012).

Fungal *Avr* genes are studied to better understand the risks posed by different pathogen races and to assist in breeding efforts. To date, 16 *Avr* genes have been identified in *L. maculans*: *AvrLm1–Lm11*, *AvrLepR1–LepR3*, *AvrLmS* and *AvrLmJ1* (Hayward *et al.*, 2012; Van de Wouw *et al.*, 2013). Genetic studies have mapped *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLm11*, *AvrLepR1*, *AvrLepR2*, *AvrLepR3*, *AvrLepR4* and *AvrLmJ1*, with most of them being found in two clusters: *AvrLm1–Lm2–Lm6* (Balesdent *et al.*, 2002) and *AvrLm3–Lm4–Lm7–Lm9–LepR1* (Balesdent *et al.*, 2005). Additionally, six *Avr* genes from *L. maculans* have been cloned: *AvrLm1* (Gout *et al.*, 2006), *AvrLm2* (Ghanbarnia *et al.*, 2015), *AvrLm6* (Fudal *et al.*, 2007), *AvrLm4–Lm7* (Parlange *et al.*, 2009), *AvrLm11* (Balesdent *et al.*, 2013) and *AvrLmJ1* (Van de Wouw *et al.*, 2013). Most of the published *Avr* genes have been located through linkage mapping (Gout *et al.*, 2006; Fudal *et al.*, 2007; Parlange *et al.*, 2009), although RNA sequencing (RNA-seq) and comparative DNA sequencing have also been used (Van de Wouw *et al.*, 2013; Ghanbarnia *et al.*, 2015).

Strategies for managing blackleg include clean seed, seed treatment, crop rotation, stubble management, foliar fungicides and genetic resistance (Kutcher *et al.*, 2013). In *B. napus*, both qualitative single gene resistance and quantitative QTL-based resistance have been reported and have been shown to complement one another (Brun *et al.*, 2010). Quantitative or adult-plant resistance (APR) reduces disease severity in the stem and is postulated to be race-nonspecific, while qualitative or major gene resistance is effective only against races carrying the corresponding *Avr* genes. Major resistance genes corresponding to pathogen avirulence genes identified to date include *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm8* (Balesdent *et al.*, 2002), *Rlm5* and *Rlm6* (Chèvre *et al.*, 1997; Balesdent *et al.*, 2002), *Rlm7* and *Rlm9* (Delourme *et al.*, 2004), *Rlm10* (Chèvre *et al.*, 1996), *BLMR1* and *BLMR2* (Long *et al.*, 2011), *LepR1*, *LepR2* and *LepR3* (Yu *et al.*, 2005). Some of these *R* genes have been used widely in canola breeding programmes and in blackleg management. Due to their effectiveness, cultivars carrying major *R* genes are often sown over large areas, resulting in strong selection pressure for virulent pathogen races. The occurrence of sexual reproduction and airborne ascospores of *L. maculans* facilitates the mutation, recombination and long-range dispersal of the pathogen inoculum, while asexual reproduction (pycnidiospores) allows for rapid increase of virulent races (Hayward *et al.*, 2012).

An understanding of the pathogen *Avr* gene profile is essential to selecting *R* genes effective against the pathogen population in a region. The objective of this study was to assess the occurrence and frequency of *Avr* genes in the *L. maculans* population in western Canada. This information can be used by canola breeders to select effective *R* genes to introgress into new cultivars and by producers to choose cultivars effective against the current pathogen population in each region.

Materials and methods

Sample collection

Canola stubble with blackleg symptoms in the basal stem was collected from commercial farmers' fields during disease surveys. These fields were located in the main canola production regions on the Canadian prairies; the cultivar was unknown for most of the fields. In total, 674 isolates were retrieved from stubble samples. They consist of 70 from Alberta 2010, 139 from Saskatchewan 2010, 170 from Manitoba 2010, 64 from Alberta 2011, 108 from Saskatchewan 2011, and 123 from Manitoba 2011. In total 293, 247 and 134 *L. maculans* isolates were isolated from the Canadian provinces of Manitoba, Saskatchewan and Alberta, respectively. Of these, 379 isolates were collected from 2010 stubble and 295 were collected from 2011 stubble.

Fungal isolation and inoculum production

Blackleg was isolated from infected inner basal stem tissue. The diseased stubble pieces, cut down to *c.* 5 mm, were surface-sterilized with 70% ethanol for 5 s, in 10% bleach for 2 min and then rinsed in sterile water prior to incubation on V8 juice agar (V8A; 200 mL V8 juice, 800 mL distilled water, 15 g agar, 0·75 g calcium carbonate, 0·1 g streptomycin sulphate) amended with 0·04 g L^{-1} Rose Bengal in Petri dishes at 22°C. After 4–7 days, when masses of conidia oozed from pycnidia on V8A plates, the conidial ooze from a single pycnidium was transferred with a fine wire under a dissecting microscope and suspended in 250 μL sterile water. The spore suspension was streaked on V8A in Petri dishes and placed on a light bench under cool white fluorescent light (100–150 μmol m^{-2} s^{-1}) at 22–24°C for 4–7 days. One culture from each plate (one sample from each field of collection) derived from a single-spore colony was transferred to a fresh V8A plate. Both *L. maculans* and *L. biglobosa* were isolated and were distinguished by inoculating cotyledons of canola cultivar Westar, which is resistant to *L. biglobosa* (Kutcher *et al.*, 2010). Pathogen inoculum was increased on V8A in Petri dishes under cool white fluorescent light as above for 5–12 days.

Preparation of fungal inoculum and DNA samples

Pycnidiospores were harvested by flooding *L. maculans* cultures with sterile distilled water, and scraping with a bent glass rod to dislodge spores. Spore suspensions were filtered through Miracloth into 50 mL sterile centrifuge tubes. The concentration was estimated using a haemocytometer, adjusted to 10^7 spores mL^{-1} and stored in sterile microcentrifuge tubes at −20°C until use. The remaining mixture of hyphae, pycnidia and spores on the agar plates were scraped with a spatula and placed in sterile 1·5 mL microcentrifuge tubes for DNA extraction.

Host differentials

Host differentials consisted of a set of *B. napus* lines or cultivars and *B. juncea* 'Cutlass', each carrying different single or multiple *R* genes to identify *Avr* alleles in *L. maculans* isolates. The set comprised: Westar (no *R* genes; Delourme *et al.*, 2004),

MT29 (*Rlm1*, *Rlm9*; Delourme *et al.*, 2008), Samouraï (*Rlm2*, *Rlm9*; Rouxel *et al.*, 2003), Cooper (*Rlm1*, *Rlm4*; Balesdent *et al.*, 2002), Glacier (*Rlm2*, *Rlm3*; Balesdent *et al.*, 2002), Verona (*Rlm2*, *Rlm4*; Balesdent *et al.*, 2002), Falcon (*Rlm4*; Rouxel *et al.*, 2003), Cutlass (*Rlm5*, *Rlm6*; this study), 23-2-1 (*Rlm7*; Delourme *et al.*, 2004), Darmor (*Rlm9*; Delourme *et al.*, 2004), 1035 (*LepR1*; Yu *et al.*, 2012), 1065 (*LepR2*; Yu *et al.*, 2012) and Surpass 400 (*LepR3*, *RlmS*; Larkan *et al.*, 2012).

The differential set allowed the deduction of the following avirulence genes in *L. maculans* isolates: *AvrLm1* to *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS*. The *Avr* genes *Avrlm5*, *AvrLm8*, *AvrLm10* and *AvrLepR4* could not be determined due to a lack of differential lines carrying the corresponding *R* genes. The *Avr* gene *AvrLmS* could be deduced from Surpass 400 (*LepR3*, *RlmS*) only for isolates lacking *AvrLm1* because *LepR3* is also able to interact with *AvrLm1* (Larkan *et al.*, 2012). Several cultivars/lines carry more than one *R* gene, requiring multiple cultivars/lines in some cases to deduce the *Avr* genes carried by some isolates. Cutlass was deduced to carry *Rlm5* and *Rlm6* in addition to an undetermined resistance gene based on screening with differential isolates D1 to D14 (Marcroft *et al.*, 2012a).

Pathogenicity test

Differentials were seeded into 96-cell seeding flats filled with Pro-Mix BX w/Mycorrhizae (Premier Tech). Flats were watered daily and maintained in growth chambers (22/14°C, day/night with a 16 h daily photoperiod). After 6–7 days, six seedlings of each differential line were inoculated with a suspension of a single *L. maculans* isolate. Each lobe of the cotyledons was wounded using a pair of modified forceps. A 10 μL droplet of inoculum was pipetted onto each of the two wounds on a cotyledon (four inoculated lobes per plant). Flats were returned to growth chambers once the inoculum droplets were dry. The day following inoculation, the plants were fertilized using 20:20:20 (N:P:K), and emerging true leaves were removed to delay the senescence of cotyledons. Cotyledons were evaluated for interaction phenotype (IP) 12–14 days after inoculation on a rating scale of 0 to 9 based on lesion size, chlorosis or necrosis, and presence of pycnidia as described by Kutcher *et al.* (2010). The mean score from the 24 inoculated lobes was used to determine virulence: avirulent (IP = 0–4·9); virulent (IP = 5–9). The results were analysed and interactions of each isolate–host genotype combination considered to deduce the *Avr* genes carried by each isolate.

The diversity and evenness of the *L. maculans* population

Simpson's index of diversity (IOD) was used to measure diversity of the *L. maculans* population, and Simpson's index of evenness (IOE) to measure evenness of the population (Simpson, 1949). The IOD was calculated by weighing the number of races relative to the total number of *L. maculans* isolates tested; an index of 1 is considered a completely diverse or random population, whereas an index of 0 would represent a single race. The IOE measured the relative abundance of different races in the population, with 1 indicating a perfectly even representation of all races.

DNA extraction, PCR and sequencing

DNA was extracted from a mixture of *L. maculans* pycnidia, conidia and hyphae harvested from 8–12-day-old single-spore cultures using a modification of the procedure developed by Lee & Taylor (1990). The samples were mixed with a lysis buffer (Tris, EDTA, SDS and NaCl), lysed with mechanical beads at 5000 rpm for 30 s, incubated at 65°C for 30 min, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated with 5 M NaCl. Following the final centrifugation, the DNA pellet was dissolved in 100 μL sterile distilled water.

DNA samples from *L. maculans* isolates were tested for *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7* using the appropriate primers (Table S1). The PCR product for *AvrLm4–Lm7* was digested with the *Hae*III enzyme (GG^CC) to detect the SNP mutation C^{358} to G^{358} that leads to virulence against *Rlm4* (Parlange *et al.*, 2009). PCR was performed in a T100 thermal cycler (Bio-Rad) with the following conditions: 3 min at 95°C; 30 cycles of 45 s at 95°C, 30 s at 61°C, 1 min at 72°C; and finally 5 min at 72°C.

The PCR products of three published *Avr* genes were sequenced at Macrogen Inc., with 28 to 33 isolates subjected to single-pass sequencing for *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7*. The sequences were aligned with the CLUSTALW algorithm (Higgins, 1994) conducted within MEGA v. 6 (Tamura *et al.*, 2013). Pairwise alignment was set with a gap penalty of 15 and gap extension penalty of 6·66. The data were exported to the BIOEDIT software where conserved regions were identified with a minimum of 15 residue length and a gap limit of 2 per segment. BIOEDIT was also used to provide nucleotide positional summary and identify SNPs.

Results

Frequency of *Avr* genes and race structure of *L. maculans* in western Canada

A total of 674 *L. maculans* isolates were characterized for 10 *Avr* genes: *AvrLm1*, *AvLm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS*. The presence or absence of *AvrLmS* could only be confirmed for 582 isolates due to the presence of *AvrLm1* in 92 isolates. There was noticeable variation in the pathogen population, depending on the specific *Avr* gene and geographic origin of the isolates. Five *Avr* genes were detected at relatively low frequency: *AvrLm1* (13·7%), *AvrLm3* (8·0%), *AvrLm9* (1·5%), *AvrLepR1* (16·0%) and *AvrLepR2* (0·2%; Fig. 1). Five *Avr* genes were detected in more than half the population: *AvrLm2* (80·6%), *AvrLm4* (71·8%), *AvrLm6* (89·3%), *AvrLm7* (89·8%) and *AvrLmS* (54·8%).

Based on the combinations of various *Avr* genes in the 674 isolates examined, 55 races were identified with frequencies ranging from 0·2 to 22·7% (Fig. 2). Two races, *AvrLm2–Lm4–Lm6–Lm7* at 22·7% and *AvrLm2–Lm4–Lm6–Lm7–LmS* at 22·5%, differing only for *AvrLmS*, accounted for almost half the population, with the third most common race detected at 5·9%. More than 75% of the isolates carried four to five *Avr* genes (Fig. 3). Only one isolate carried seven *Avr* genes (*AvrLm2–Lm3–Lm6–Lm7–Lm9–LmS–LepR1*) and another carried a single *Avr* gene (*AvrLm7*). The average complexity increased slightly in 2011 when compared to 2010 (Fig. 4).

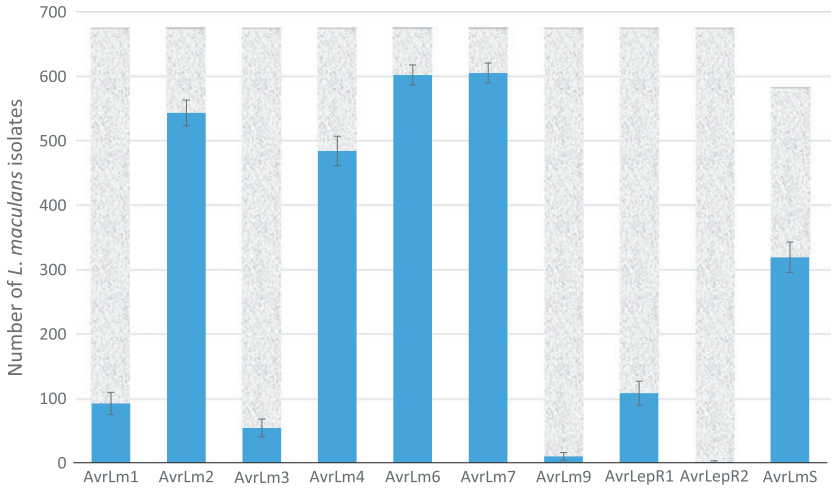

Figure 1 Frequency of *Avr* genes in the population of *Leptosphaeria maculans* collected from commercial canola fields in western Canada in 2010 and 2011 based on analysis using a host differential set and PCR. A total of 674 isolates were examined for potentially carrying 10 known *Avr* genes, with the exception of *AvrLmS* for which 582 isolates were tested (total shown by light grey bars).

Variation in the *L. maculans* population

The *L. maculans* population was genetically diverse, with Simpson's IOD at 0·89 (Table 1). Simpson's IOE was 0·71, indicating a somewhat even population. The two dominant races probably reduced the evenness score significantly. Simpson's IOD and IOE were variable among provinces: Manitoba (293 isolates) had the most diverse and even *L. maculans* population, with IOD and IOE at 0·91 and 0·68, respectively. The respective scores were 0·87 and 0·61 for Alberta (134 isolates) and 0·77 and 0·39 for Saskatchewan (247 isolates). However, IOD did not change significantly between the years, whereas IOE was reduced slightly in 2011 from 2010 (Table 1).

The frequency of *Avr* genes in the pathogen population increased by an average of 3·8% from 2010 to 2011 (Fig. S3). *AvrLepR1*, *AvrLm6*, *AvrLm4*, *AvrLmS* and *AvrLm1* increased by 12·4, 11·8, 7·3, 6·9 and 5·9% respectively, while *AvrLm2* declined by 6·4%. The other avirulence genes surveyed shifted less than 2% between 2010 and 2011. The *Avr* gene frequency also varied somewhat among provinces (Fig. S4), with *AvrLm2* showing the greatest regional variation with 31·8% more isolates carrying *AvrLm2* in Saskatchewan relative to those in Manitoba. The frequency of *AvrLm1*, *AvrLm2*, *AvrLepR1* and *AvrLmS* varied by more than 20% among provinces, while *AvrLm3*, *AvrLm7*, *AvrLm9* and *AvrLepR2* varied by less than 10%. Despite the variation, *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7* were at high frequencies, while *AvrLm1*, *AvrLm3*, *AvrLm9* and *AvrLepR2* were low in the pathogen population in all provinces. Greater variation in the frequency of *Avr* genes was observed among different field sites. Figure 5 compares *L. maculans* isolates from five locations. *AvrLm2* was the most variable locus, displaying large differences between sampling sites such as between Melfort, Saskatchewan and Roland, Manitoba (difference of 78%). *AvrLm1* was observed at a frequency of 63% in Roland and was absent in Melfort. *AvrLm7* was the least variant locus and was consistently above 90% at all five sites.

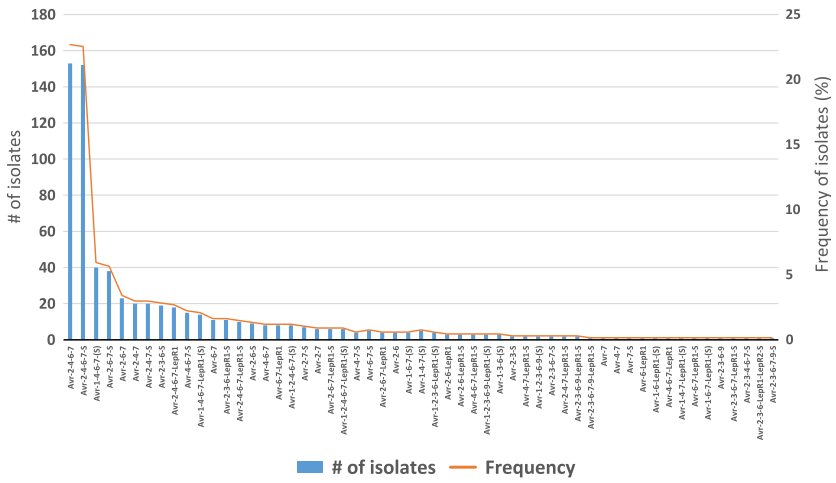

Figure 2 Frequency of 55 *Leptosphaeria maculans* races based on the 10 known avirulence genes carried. A total of 674 isolates were examined for all the avirulence genes with the exception of *AvrLmS* for which 582 isolates were tested.

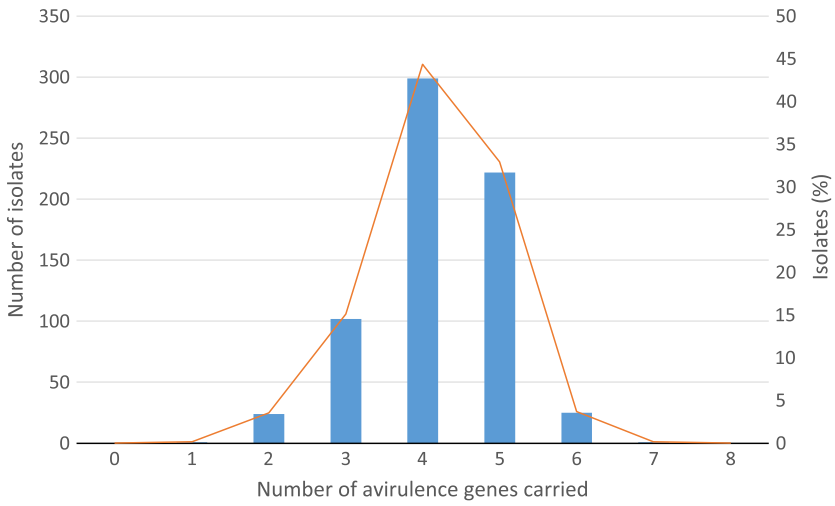

Figure 3 Race complexity for the *Leptosphaeria maculans* population in western Canada: average number of avirulence genes carried by each isolate based on a total of 674 isolates assessed at 10 avirulence gene loci.

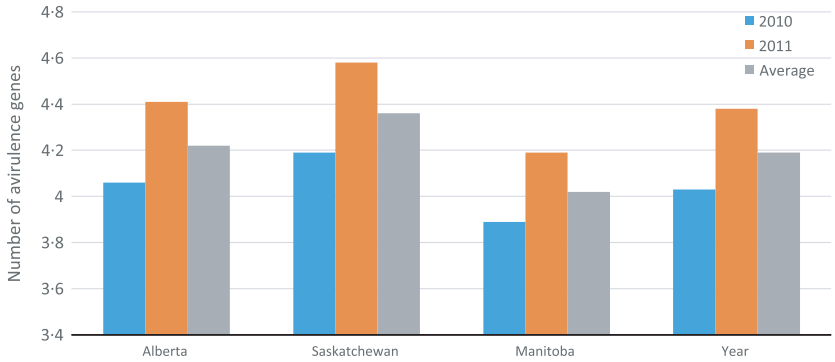

Figure 4 Average number of avirulence genes located in each province and year, based on a total of 674 isolates from western Canada assessed at 10 avirulence gene loci.

Table 1 Simpson's index of diversity (IOD) and index of evenness (IOE) for 674 western Canadian *Leptosphaeria maculans* populations surveyed in 2010 and 2011, in terms of races designated based on the 10 avirulence genes characterized in this study

		Province			
Index	Population	MB	AB	SK	Year
IOD[a]	2010	0·922	0·899	0·781	0·868
	2011	0·891	0·832	0·755	0·826
	Province	0·906	0·865	0·768	0·885
IOE[b]	2010	0·700	0·697	0·460	0·619
	2011	0·667	0·519	0·317	0·501
	Province	0·684	0·608	0·389	0·712

[a]$S = 1 - \Sigma\,(n_i^2 - n_i)/(N^2 - N)$.
[b]$EH = H/\ln R$, with $H = -\Sigma P_i \times \ln P_i$.

Genetic variation of *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7* alleles in the *L. maculans* population

A total of 96 samples were amplified and sequenced for each of the *Avr* gene loci: *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7*. The alignment of the three *Avr* genes resulted in variable numbers of SNPs and alleles, while the size of the conserved region relative to respective PCR products was relatively consistent among the three *Avr* genes (Table 2).

The *AvrLm1* gene carried by 33 *L. maculans* isolates was aligned to the French reference *L. maculans* isolate JN3, with a single conserved region of 438 bp. A total of 58 SNPs were identified among the 33 isolates comprising 13·2% of the conserved region. The 58 SNPs arose from three alleles, with 31 isolates sharing a common allele and two isolates from Manitoba 2010 samples displaying unique haplotypes with many nucleotide substitutions (Table 3). Alignment of the *AvrLm6* gene among 35 isolates with the reference genome identified a single conserved region of 239 bp with a total of 11 SNPs at four alleles. Thirty-two isolates shared a common allele and two isolates revealed unique SNPs. One isolate shared an allele with the reference isolate JN3. The fewest SNPs (4·6%) were identified for *AvrLm6*, relative to the size of the conserved region. Similar alignment of 28 isolates carrying the *AvrLm4–Lm7* gene with the reference genome revealed nine conserved regions separated by deletions involving a combined size of 562 bp. The alignment detected SNPs at the highest frequency (21%) relative to the size of conserved regions, with 110 SNPs identified at nine alleles. Fifteen isolates shared the most common allele, followed by five and two isolates sharing the next two most common alleles, respectively. The remaining six alleles were represented by six different isolates.

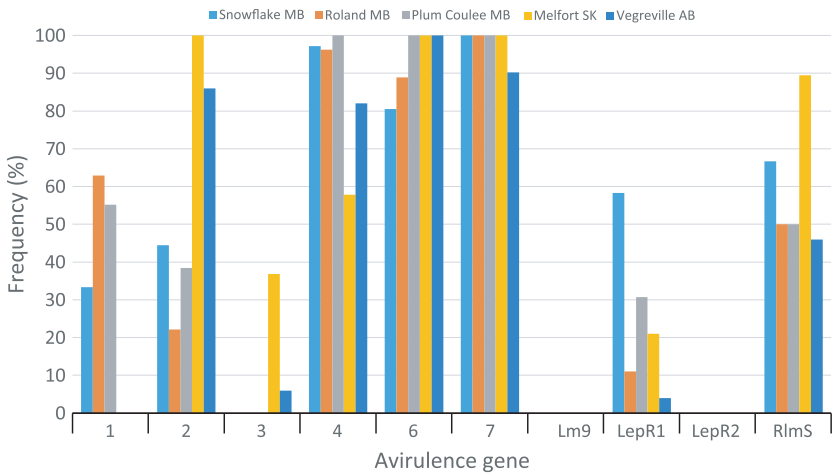

Figure 5 Regional avirulence gene variation in the western Canadian *Leptosphaeria maculans* pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS* for which 584 isolates were characterized.

Table 2 Summary of sequence variation among three avirulence gene loci sequenced in 96 isolates from the western Canadian *Leptosphaeria maculans* pathogen population

	Avirulence gene locus		
Feature	*AvrLm1*	*AvrLm6*	*AvrLm4–Lm7*
No. of isolates	33	35	28
PCR product size (bp)	1123	774	1433
Conserved regions	1	1	9
Conserved regions size (bp)	438	239	562
Conserved region/PCR product (%)	39	31	39
No. of SNPs	58	11	118
SNPs/conserved region (%)	13·24	4·60	21·00
% virulent	86·52	10·81	28·15
% avirulent	13·48	89·19	71·85

Table 3 Allele frequency of *AvrLm1*, *AvrLm6* and *AvrLm4–Lm7* among western Canadian *Leptosphaeria maculans* isolates

Gene	Allele	No. of isolates	Frequency (%)
AvrLm1	0	31	93·9
	1	1	3·0
	2	1	3·0
AvrLm6	0	32	91·4
	1	1	2·9
	2	1	2·9
	3	1	2·9
AvrLm4–Lm7	0	15	53·6
	1	5	17·9
	2	2	7·1
	3	1	3·6
	4	1	3·6
	5	1	3·6
	6	1	3·6
	7	1	3·6
	8	1	3·6

Discussion

The current study determined the frequency of 10 *Avr* genes in 674 *L. maculans* isolates from western Canada based on a set of host differentials and PCR-based markers. A total of 55 races were identified, and among them, two races appeared clearly dominant, accounting for almost half of the population. An earlier study based on 96 isolates reported 16 races of *L. maculans* in western Canada (Kutcher *et al.*, 2010). In this study, 15 races were represented by single isolates. *Leptosphaeria maculans* was isolated from stubble in farmers' fields at the stem canker stage prior to harvest. Isolation of the pathogen directly from stubble of commercial cultivars introduced positive selection pressure towards races virulent on the cultivars grown, all of which are registered with resistance to blackleg. However, this selection pressure would have also occurred if isolation were made from a cultivar with no *R* genes seeded into the commercial stubble, such as Westar, because the isolates collected would be a reflection of selection pressure from the *R* genes in the previous commercial cultivar grown. Because isolates were retrieved directly from commercial stubble, the 2010–11 isolates obtained in this study should be considered to reflect more current *L. maculans* populations. This approach is limited by the inability to detect the low frequency of new races that re-emerge with avirulence genes after selection by commercial stubble due to natural recombination and mutation.

The dominance of two races (*AvrLm2–Lm4–Lm6–Lm7* and *AvrLm2–Lm4–Lm6–Lm7–LmS*) indicates an uneven pathogen population, while the 55 races detected highlights the diversity. The pathogen's race diversity indicates the potential for blackleg to affect canola production in western Canada because each resistance gene examined in the study can be overcome by at least one of the pathogen races detected. Simpson's evenness index, which measures relative frequency of races, was generally lower in Saskatchewan and Alberta than in Manitoba. The higher diversity and evenness indices observed among Manitoba *L. maculans* isolates may point to a larger risk of resistance breakdown in Manitoba when compared to Alberta or Saskatchewan, where the indices were lower. It is surprising to find that Sas-

katchewan had both the fewest races and an uneven distribution of races given that *L. maculans* was first reported in the province in 1975 (McGee & Petrie, 1978) and Saskatchewan's canola acreage is currently the largest in western Canada. However, agricultural practices such as crop rotation, tillage and nitrogen fertilizer application rates all play a role in pathogen reproduction, which may influence race structure and disease pressure (Kutcher *et al.*, 2013). This assessment of disease risk based on the race structure is supported by field survey data that documents increased incidence of stem canker in Manitoba and Alberta compared with Saskatchewan (Figs S1 & S2). Indeed, the first evidence of new pathogen races (PG3 and PG4) was from southern Manitoba (Chen & Fernando, 2006).

Most *L. maculans Avr* genes reside in two major gene clusters, the first of which is the *AvrLm1–Lm2–Lm6* cluster (Balesdent *et al.*, 2002). This study found the frequency of *AvrLm1*, *AvrLm2* and *AvrLm6* to be 13·7, 80·6 and 89·3%, respectively. The decrease in *AvrLm1* has been significant in western Canada, falling from 46% in 1997–2005 samples (Kutcher *et al.*, 2010) to 12·6% in 2005/6 samples (Dilmaghani *et al.*, 2009), to 13·7% in the 2010–11 isolates of this study. *AvrLm2* declined moderately from 96·6% in 1997–2005 samples (Kutcher *et al.*, 2010), to 87·02% in 2005/6 samples (Dilmaghani *et al.*, 2009), and to 80·56% in the 2010–11 isolates of this study. *AvrLm6* declined only slightly from 100% in 1997–2005 samples (Kutcher *et al.*, 2010), to 94·8% in 2005/6 samples (Dilmaghani *et al.*, 2009), and to 89·3% in the 2010–11 isolates of this study. The host resistance genes *Rlm1* and *Rlm2* are present in 10 and 1% of Canadian commercial canola cultivars/breeding lines, respectively (Zhang *et al.*, 2013), but the actual acreage of cultivars carrying these *R* genes is unknown. *Rlm6* resistance is derived from *B. juncea* and has only been introgressed into experimental lines (Chèvre *et al.*, 1997). *AvrLm6* was at consistently high frequency in western Canada several years ago (Kutcher *et al.*, 2010), and is one of the most frequently encountered *Avr* genes in the current study. Stachowiak *et al.* (2006) found *AvrLm6* to be fixed in the *L. maculans* population in Europe. However, in experimental trials, increased virulence at *AvrLm6* was observed after repeated uses of *Rlm6* cultivars in the same research plots in France (Brun *et al.*, 2010). Dilmaghani *et al.* (2009) found *AvrLm6* to be at 99 and 96% in Chile and Western Australia, respectively. No Canadian cultivars with *Rlm6* were detected during germplasm screening (Zhang *et al.*, 2013). In *L. maculans*, a 'hitch-hiking' effect was demonstrated when selection pressure on *AvrLm1* led to increased virulence at *AvrLm6* in Australia despite the absence of any cultivars carrying *Rlm6* (Van de Wouw *et al.*, 2010) and this may also explain the virulence observed at *AvrLm6* in Canada.

The linkage of *AvrLm1–Lm2–Lm6* presents an interesting trend in terms of race structure; 90% of *L. maculans* isolates in western Canada that carried *AvrLm1* lacked *AvrLm2* and vice versa. Only 3·4% of isolates carried both *Avr* genes, while 6·5% lacked both *AvrLm1* and *AvrLm2*. The low frequency of *AvrLm1* at 13·7% in western Canada may have contributed to the relatively high frequency of *AvrLm2* (87·0%). A similar, but opposite, inverse relationship between *AvrLm1* and *AvrLm2* was also reported in Western Australia (Dilmaghani *et al.*, 2009) with *AvrLm1* at 91% and *AvrLm2* at 1%. In contrast, *AvrLm6* did not significantly correlate with the presence or absence of either *AvrLm1* or *AvrLm2* in the current study. While fitness cost may be the first assumption, Dilmaghani *et al.* (2009) also found that almost all Chilean isolates lacked both *AvrLm1* and *AvrLm2*; both *Avr* genes were at <1% in 128 *L. maculans* isolates. This indicates that the frequency of both *AvrLm1* and *AvrLm2* can be suppressed simultaneously given the selection pressure at both loci. Both *AvrLm1* and *AvrLm2* have been cloned and it is clear that they are unique genes and not two variant alleles of the same gene (Gout *et al.*, 2006; Ghanbarnia *et al.*, 2015). *AvrLm4–Lm7* is a single *Avr* gene that interacts with both *Rlm4* and *Rlm7*. Virulence to *Rlm4* is gained through a SNP mutation while virulence to *Rlm7* is gained through a deletion of *AvrLm4–Lm7* (Parlange *et al.*, 2009). The alignment of 28 western Canadian *L. maculans* isolates at the *AvrLm4–Lm7* locus identified 118 SNPs in the conserved region and eight alleles among the isolates. Similar alignments led to 58 SNPs and three haplotypes for *AvrLm1* and 11 SNPs and four haplotypes for *AvrLm6*. This demonstrated that *AvrLm4–Lm7* was the most variable of the three *Avr* genes sequenced despite no *Rlm4* or *Rlm7* cultivars having been identified in Canada (Zhang *et al.*, 2013). *Rlm4* is the only resistance gene overcome primarily through a SNP mutation, and the increased frequency of SNPs in the *AvrLm4–Lm7* gene, in comparison to *AvrLm1* and *AvrLm6*, may explain this observation.

The frequency of *AvrLm3* in western Canada was reduced from 52·8% in 2005/6 (Dilmaghani *et al.*, 2009) to 13·7% in this study. Selection pressure is the most probable factor for this change because *Rlm3* is the most common resistance gene carried in more than half of Canadian cultivars (Zhang *et al.*, 2013). The frequency of the *AvrLm4–Lm7* avirulence gene increased from 25% in 1997–2005 (Kutcher *et al.*, 2010) to 47·2% in 2005/6 (Dilmaghani *et al.*, 2009), and to 89·7% in this study. The increase in *AvrLm4–Lm7* was too rapid to be due to random drift and may be explained by positive selection pressure. When examining the race structure of *AvrLm3* and *AvrLm4–Lm7*, it was observed that, as with *AvrLm1* and *AvrLm2*, approximately 96% of isolates carried either *AvrLm3* or *AvrLm4–Lm7*, only 0·1% carried both, and 3% carry neither. This observation, along with the rapid increase of *AvrLm4–Lm7*, supports the notion that the decline in *AvrLm3* led to an increase in *AvrLm4–Lm7*.

There was a relatively rapid decline in *AvrLm9*, from 60·4% in 1997–2005 (Kutcher *et al.*, 2010) to 56·4% in 2005/6 (Dilmaghani *et al.*, 2009), and to 1·5% in this study. All isolates with *AvrLm9* in this study also carried *AvrLm3*. Thus the decline of *AvrLm9* may also be due

to negative selection at *AvrLm3*, and the potential linkage of *AvrLm3* and *AvrLm9*. *AvrLm9* was reported as rare outside of western Canada (Hayward *et al.*, 2012), and the consistently low frequency among regions, as opposed to variation in other *Avr* genes, suggests there is no fitness cost associated with the loss of this *Avr* gene. *AvrLepR1* is the last *Avr* gene in the *AvrLm3–Lm4–Lm7–Lm9–LepR1* gene cluster and was observed at a relatively low frequency of 16·0%. The corresponding resistance gene, along with the other '*LepR*' resistance genes, were first identified in resynthesized *B. napus* lines that arose from a *B. rapa* subsp. *sylvestris* × *B. oleracea* var. *alboglabra* interspecific cross (Crouch *et al.*, 1994). *LepR2* was also characterized in this study but only a single isolate was found to carry this *Avr* gene. Several 2012 isolates were observed to carry *AvrLepR2* (data not shown) and all isolates were found in Manitoba. *AvrLepR1* and *AvrLepR2* have not been examined in the western Canadian *L. maculans* population previously. *AvrLmS* was detected in 54·8% of the *L. maculans* isolates. Kutcher *et al.* (2010) estimated *AvrLmS* at 97% frequency among 47 isolates collected from 1997–2005 compared to just 54·8% in this study.

The differences in avirulence gene frequencies between different sites shown in Figure 5 are significant and may be due to local selection pressure. The regional and provincial variation demonstrates that a single specific resistance gene would not provide effective control of blackleg at all sites across the Prairie provinces except perhaps *Rlm6* and *Rlm7*, which consistently occurred at high frequencies in this study. The number of *L. maculans* races detected and the geographic variation in *Avr* gene frequency in this study indicates that the *L. maculans* population in western Canada is quite diverse. The prevalence of certain avirulence gene combinations is probably due to avirulence gene clusters, which reduce recombination between proximal genes. The frequency of particular *Avr* genes may be affected by fitness cost (Huang *et al.*, 2006), or the result of initial founder race combinations, and in Canada possibly by lower rates of sexual recombination (Ghanbarnia *et al.*, 2011). The presence of isolates virulent to *Rlm6* and *Rlm7*, which are not widely used in commercial cultivars, points to naturally existing virulent races in the absence of selection pressure. Given that *L. maculans* can lose *Avr* genes to gain virulence, often with no fitness cost, sustained use of any single resistance gene will result in loss of effectiveness. Resistance genes may be stacked into a single cultivar to provide protection against multiple races of the pathogen, and this strategy would be most effective if there is a fitness cost associated with races carrying multiple virulence genes or if virulent races are absent (i.e. pathogen diversity is low) at the time of multi-*R*-gene cultivar deployment. The presence of low frequency races virulent on stacked resistance risks the loss of multiple *R* genes simultaneously when selection pressure degrades resistance. Based on this study, stacking *Rlm6* and *Rlm7* would probably be effective against all races examined in western Canada. Similarly, stacking *Rlm2–Rlm7–LepR1* would provide resistance against all isolates surveyed. However, in the present analysis, an alternate strategy of rotating *R* genes similar to rotating fungicides or herbicides would more likely increase the longevity of *Rlm1* and *Rlm2*. Rotating crops with different blackleg *R* genes has been shown to be effective in field experiments (Marcroft *et al.*, 2012b). Most avirulence genes have no observable correlation in terms of race structure and either stacking or rotation may work equally well. In some cases, a single *L. maculans Avr* gene product interacts with multiple *R* gene products in *B. napus*, such as *AvrLm1* that can be detected by both *Rlm1* and *LepR3*, and *AvrLm4–Lm7*, which is detected by both *Rlm4* and *Rlm7*. In these cases, stacking *Rlm1* and *LepR3* or *Rlm4* and *Rlm7* would not be advisable because a single gene deletion in the pathogen would render two *R* genes ineffective. The linkage of *Avr* genes in the pathogen and the interplay between races and different resistance genes under selection pressure are two important factors to consider when breeding for blackleg resistance in Canada.

Acknowledgements

The authors would like to thank Canola Council of Canada, CARP Program for funding this research. They would also like to thank Paula Parks and Xuehua Zhang for technical assistance. They thank Regine Delourme (INRA), France for providing the differential lines for the study.

References

Balesdent MH, Attard A, Kühn ML, Rouxel T, 2002. New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology* **92**, 1122–33.

Balesdent MH, Barbetti MJ, Hua L, Sivasithamparam K, Gout L, Rouxel T, 2005. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology* **95**, 1061–71.

Balesdent MH, Fudal I, Ollivier B *et al.*, 2013. The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytologist* **198**, 887–98.

Brun H, Chèvre AM, Fitt BD *et al.*, 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist* **185**, 285–99.

Chen Y, Fernando WGD, 2006. Prevalence of pathogenicity groups of *Leptosphaeria maculans* in western Canada and North Dakota, USA. *Canadian Journal of Plant Pathology* **28**, 533–9.

Chèvre AM, Eber F, This P *et al.*, 1996. Characterization of *Brassica nigra* chromosomes and of blackleg resistance in *B. napus–B. nigra* addition lines. *Plant Breeding* **115**, 113–8.

Chèvre AM, Barret P, Eber F *et al.*, 1997. Selection of stable *Brassica napus–B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 1: Identification of molecular markers, chromosomal and genomic origin of the introgression. *Theoretical and Applied Genetics* **95**, 1104–11.

Crouch JH, Lewis BG, Mithen RF, 1994. The effect of a genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans*. *Plant Breeding* **112**, 265–78.

Delourme R, Pilet-Nayel ML, Archipiano M *et al.*, 2004. A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Phytopathology* **94**, 578–83.

Delourme R, Piel N, Horvais R *et al.*, 2008. Molecular and phenotypic characterization of near isogenic lines at QTL for quantitative

resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). *Theoretical and Applied Genetics* **11**, 1055–67.
Dilmaghani A, Balesdent MH, Didier JP *et al.*, 2009. The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology* **58**, 1044–58.
Fudal I, Ross S, Gout L *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* **2**, 459–70.
Ghanbarnia K, Fernando WGD, Crow G, 2011. Role of pycnidiospores of *Leptosphaeria maculans* on infection of different growth stages of canola plants under field conditions. *Canadian Journal of Plant Pathology* **33**, 355–63.
Ghanbarnia K, Fudal I, Larkan NJ *et al.*, 2015. Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2*, using an intraspecific comparative genomics approach. *Molecular Plant Pathology* **16**, 699–709.
Gout L, Fudal I, Kuhn ML *et al.*, 2006. Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the Dothideomycete *Leptosphaeria maculans*. *Molecular Microbiology* **60**, 67–80.
Gugel RK, Petrie GA, 1992. History, occurrence, impact and control of blackleg of rapeseed. *Canadian Journal of Plant Pathology* **14**, 36–45.
Hayward A, McLanders J, Campbell E, Edwards D, Batley J, 2012. Genomic advances will herald new insights into the *Brassica*: *Leptosphaeria maculans* pathosystem. *Plant Biology* **14**, S1–10.
Higgins DG, 1994. CLUSTALV: multiple alignment of DNA and protein sequences. *Methods in Molecular Biology* **25**, 307–18.
Huang YJ, Li Z, Evans N, Rouxel T, Fitt BDL, Balesdent MH, 2006. Fitness cost associated with loss of the *AvrLm4* function in *Leptophaeria maculans* (phoma stem canker of oilseed rape). *European Journal of Plant Pathology* **114**, 77–89.
Kutcher HR, Balesdent MH, Rimmer SR *et al.*, 2010. Frequency of avirulence genes in *Leptosphaeria maculans* in western Canada. *Canadian Journal of Plant Pathology* **32**, 77–85.
Kutcher HR, Brandt SA, Smith EG, Ulrich D, Malhi SS, Johnston AM, 2013. Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. *Canadian Journal of Plant Pathology* **35**, 209–21.
Larkan NJ, Lydiate DJ, Parkin IAP *et al.*, 2012. The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector AVRLM1. *New Phytologist* **197**, 595–605.
Lee SB, Taylor JW, 1990. Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols. A Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 282–7.
Li H, Sivasithamparam K, Barbetti MJ, 2003. Breakdown of a *Brassica rapa* subsp. *sylvestris* single dominant blackleg resistance gene in *B. napus* rapeseed by *Leptosphaeria maculans* field isolates in Australia. *Plant Disease* **87**, 752.
Long Y, Wang Z, Sun Z, Fernando DWG, McVetty PBE, Li G, 2011. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass 400'. *Theoretical and Applied Genetics* **122**, 1223–31.
Marcroft SJ, Elliot VL, Cozinjsen AJ, Salisbury PA, Howlett BJ, Van de Wouw AP, 2012a. Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. *Crop & Pasture Science* **63**, 338–50.
Marcroft SJ, Van de Wouw AP, Salisbury PA, Pottere TD, Howlett BJ, 2012b. Effect of rotation of canola (*Brassica napus*) cultivars with different complements of blackleg resistance genes on disease severity. *Plant Pathology* **6**, 934–44.
McGee DC, Petrie GA, 1978. Variability of *Leptosphaeria maculans* in relation to blackleg of rapeseed. *Phytopathology* **68**, 625–30.
Morrall RAA, 2014. Canadian plant disease survey volume 94 (2014). [http://phytopath.ca/wp-content/uploads/2014/10/cpds-archive/vol94/cpds_vol_94_2014.pdf]. Accessed 26 November 2015.
Parlange F, Daverdin G, Fudal I *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**, 851–63.
Rouxel T, Penaud A, Pinochet X *et al.*, 2003. 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**, 871–81.
Rouxel T, Grandaubert J, Hane JK *et al.*, 2011. Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. *Nature Communications* **15**, 202.
Simpson EH, 1949. Measurement of diversity. *Nature* **163**, 688.
Stachowiak A, Olechnowicz J, Jedryczka M *et al.*, 2006. Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *European Journal of Plant Pathology* **114**, 67–75.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–9.
Van de Wouw AP, Cozijnsen AJ, Hane JK *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**, e1001180.
Van de Wouw AP, Lowe RGT, Elliott CE, Dubois DJ, Howlett BJ, 2013. An avirulence gene, *AvrLmJ1*, from the blackleg fungus, *Leptosphaeria maculans*, confers avirulence to *Brassica juncea* cultivars. *Molecular Plant Pathology* **15**, 523–30.
Yu F, Lydiate DJ, Rimmer SR, 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theoretical and Applied Genetics* **110**, 969–79.
Yu F, Lydiate DJ, Gugel RK, Sharpe AG, Rimmer SR, 2012. Introgression of *Brassica rapa* subsp. *sylvestris* blackleg resistance into *B. napus*. *Molecular Breeding* **30**, 1495–506.
Zhang X, Fernando WGD, Peng G, Kutcher HR, 2013. Characterization of major resistance genes to *Leptosphaeria maculans* in Canadian *Brassica napus* accessions. In: Peng YL, Guo Z, eds. *Proceedings of the 10th International Congress of Plant Pathology. Acta Phytopathologica Sinica* **Vol 43** (Suppl), 404. [http://www.icppbj2013.org/xiazai/Abstract%20of%20ICPP%202013-final.pdf]. Accessed 20 November 2015.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. 2010 Blackleg disease incidence survey. Percentage basal stem canker infection rates in the Canadian Provinces of Alberta, Saskatchewan and Manitoba. Saskatchewan Ministry of Agriculture.

Figure S2. 2011 Blackleg disease incidence survey. Percentage basal stem canker infection rates in the Canadian Provinces of Alberta, Saskatchewan and Manitoba. Saskatchewan Ministry of Agriculture.

Figure S3. Comparison of *Leptosphaeria maculans* avirulence gene frequency in 2010 and 2011 in the western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS*/*AvrLepR5*, for which 582 isolates were characterized.

Figure S4. Comparison of *Leptosphaeria maculans* avirulence gene frequency between provinces in the western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS*, for which 582 isolates were characterized.

Table S1. Primers used to amplify *Leptosphaeria maculans* avirulence genes.

Table S2. Listing of *Leptosphaeria maculans* races among 674 characterized western Canadian isolates sampled in 2010 and 2011. Results are from a combination of interaction phenotypes using a set of differential isolates to characterize *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2*, *AvrLmS* and PCR amplification of published avirulence genes *AvrLm1*, *AvrLm6*, *AvrLm4* and *AvrLm7*. *AvrLmS* could not be determined in 92 isolates and a bracket signifies that the presence or absence of the avirulence gene is unknown.