Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2015) **16**(7), 699–709 DOI: 10.1111/mpp.12228

Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2* using an intraspecific comparative genomics approach

KAVEH GHANBARNIA[1,2], ISABELLE FUDAL[3], NICHOLAS J. LARKAN[1], MATTHEW G. LINKS[1,4], MARIE-HÉLÈNE BALESDENT[3], BRONISLAVA PROFOTOVA[3], W. G. DILANTHA FERNANDO[2], THIERRY ROUXEL[3] AND M. HOSSEIN BORHAN[1,*]

[1]*Saskatoon Research Centre, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada, S7N 0X2*
[2]*Department of Plant Science, University of Manitoba, Winnipeg, MB, Canada, R3T 2N2*
[3]*INRA-Bioger, 78850 Thiverval-Grignon, France*
[4]*Department of Computer Science, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5C9*

SUMMARY

Five avirulence genes from *Leptosphaeria maculans*, the causal agent of blackleg of canola (*Brassica napus*), have been identified previously through map-based cloning. In this study, a comparative genomic approach was used to clone the previously mapped *AvrLm2*. Given the lack of a presence–absence gene polymorphism coincident with the *AvrLm2* phenotype, 36 *L. maculans* isolates were resequenced and analysed for single-nucleotide polymorphisms (SNPs) in predicted small secreted protein-encoding genes present within the map interval. Three SNPs coincident with the *AvrLm2* phenotype were identified within *LmCys1*, previously identified as a putative effector-coding gene. Complementation of a virulent isolate with *LmCys1*, as the candidate *AvrLm2* allele, restored the avirulent phenotype on *Rlm2*-containing *B. napus* lines. *AvrLm2* encodes a small cysteine-rich protein with low similarity to other proteins in the public databases. Unlike other avirulence genes, *AvrLm2* resides in a small GC island within an AT-rich isochore of the genome, and was never found to be deleted completely in virulent isolates.

Keywords: avirulence gene, *Brassica napus*, comparative genomics, *Leptosphaeria maculans*.

INTRODUCTION

Blackleg is a major disease of the oilseed crop *Brassica napus* (canola/oilseed rape) and other *Brassica* crops worldwide. The disease is caused by the ascomycete pathogen *Leptosphaeria maculans* (Fitt *et al*., 2006). Infection starts on the cotyledons and leaves of plants and progresses systemically into the stem. Infection of the stem eventually leads to the formation of lesions at the base of the stem (stem canker) of adult plants, resulting in lodging of the crop and yield loss. Two types of genetic resistance to *L. maculans* have been described in *Brassica* species: qualitative resistance (race-specific) and quantitative resistance (effective at the adult plant stage) (Ansan-Melayah *et al*., 1998; Dion *et al*., 1995; Ferreira *et al*., 1995; Pilet *et al*., 1998; Rimmer, 2006). Qualitative resistance to *L. maculans* mostly follows the gene-for-gene model for plant–pathogen interactions described by Flor (1971) (Ansan-Melayah *et al*., 1998), although some redundancy is also present within the system (Larkan *et al*., 2013; Parlange *et al*., 2009). Major *resistance* (*R*) genes against blackleg reported to date include *Rlm1*, *Rlm2* and *Rlm4* (Ansan-Melayah *et al*., 1998), *Rlm3*, *Rlm5*, *Rlm6* and *Rlm8* (Balesdent *et al*., 2002), *Rlm7* and *Rlm9* (Delourme *et al*., 2004), *LepR1*, *LepR2* and *LepR3* (Larkan *et al*., 2013; Yu *et al*., 2005), and *BLMR1* and *BLMR2* (Long *et al*., 2011), although some redundancy amongst the reported *R* genes is likely (Larkan *et al*., 2013; Raman *et al*., 2013).

Leptosphaeria maculans avirulence genes, capable of triggering their cognate *R* genes to induce resistance, have been named '*AvrLm*' or '*AvrLepR*' to reflect their interaction with the corresponding *R* genes in *Brassica*. Genetic studies in *L. maculans* have identified the genomic location of *AvrLm1, 2, 3, 4, 5, 6, 7, 9, 11* and *AvrLepR1*. Some of these genes are located within two genetic clusters, the *AvrLm1-2-6* cluster (Balesdent *et al*., 2002) and the *AvrLm3-4-7-9-AvrLepR1* cluster (Balesdent *et al*., 2005; Ghanbarnia *et al*., 2012), reflecting contrasting genomic situations. *AvrLm1* and *AvrLm6* are located in a region within the genome in which recombination is suppressed, and are separated by hundreds of kilobases (Fudal *et al*., 2007), whereas the *AvrLm4* and *AvrLm7* specificities are a result of two different alleles of the same gene, renamed *AvrLm4-7* (Parlange *et al*., 2009). Five avirulence genes, *AvrLm1* (Gout *et al*., 2006), *AvrLm4-7* (Parlange *et al*., 2009), *AvrLm6* (Fudal *et al*., 2007), *AvrLm11* (Balesdent *et al*., 2013) and *AvrLmJ1* (Van de Wouw *et al*., 2014), have been cloned. All encode small secreted proteins (SSPs) and, with the exception of *AvrLm1*, are cysteine rich. *AvrLm11* is located on the smallest chromosome in *L. maculans*, which is a conditionally

*Correspondence: Email: hossein.borhan@agr.gc.ca

dispensable chromosome (CDC) extremely enriched in transposable elements (TEs) (Balesdent *et al*., 2013).

All five cloned avirulence genes identified to date from *L. maculans* reside in heterochromatin-like regions with low GC, comprising mosaics of TEs intermingled, truncated and degenerated by repeat-induced point (RIP) mutations (Balesdent *et al*., 2013; Fudal *et al*., 2007; Gout *et al*., 2006; Parlange *et al*., 2009; Van de Wouw *et al*., 2014). The TEs are a site of epigenetic control in which chromatin regulation allows the concerted expression of avirulence effectors at the onset of plant infection (Soyer *et al*., 2014). The transition of *L. maculans* races from avirulence to virulence is a result of multiple molecular events, with the most common being a complete deletion of the gene (Balesdent *et al*., 2013; Daverdin *et al*., 2012; Fudal *et al*., 2009; Gout *et al*., 2007). Other molecular events, such as truncation, RIP mutation or non-RIP mutation, have been reported, resulting in either premature truncations or altered forms of the Avr protein, neither of which are capable of triggering a defence response in the host plant (Daverdin *et al*., 2012; Fudal *et al*., 2009; Parlange *et al*., 2009; Van de Wouw *et al*., 2009). One particular case is that of *AvrLm4-7* in which loss of recognition by the cognate *Rlm4* is a result of a single non-synonymous base mutation that maintains the integrity of the protein and the recognition by *Rlm7* (Parlange *et al*., 2009).

Multiple approaches, such as linkage mapping (Fudal *et al*., 2007; Gout *et al*., 2006; Linning *et al*., 2004; Orbach *et al*., 2000; Parlange *et al*., 2009), reverse genetics (Rep *et al*., 2004; Rivas and Thomas, 2005), cDNA screening (Catanzariti *et al*., 2006; van Kan *et al*., 1991) or the combination of map-based cloning and cDNA screening (Böhnert *et al*., 2004), have been applied to characterize *Avr* genes from plant-pathogenic fungi. Map-based cloning was the major approach utilized to clone five *L. maculans* avirulence genes (Balesdent *et al*., 2013; Fudal *et al*., 2007; Gout *et al*., 2006; Parlange *et al*., 2009; Van de Wouw *et al*., 2014). However, this approach is time consuming and has limitations, such as incompatibility of desired parental isolates for crossing. With the advent of next-generation sequencing and the availability of whole genome sequences of pathogenic fungi (Dean *et al*., 2005; Rouxel *et al*., 2011), a combination of genetic mapping, high-throughput phenotyping and intraspecies comparative genomics can facilitate the identification of avirulence genes. Here, we present the use of parallel genome resequencing as an alternative or complementary approach to map-based cloning of effectors in *L. maculans* and, in particular, the cloning and characterization of the *L. maculans AvrLm2* gene.

RESULTS

Pathotyping of *L. maculans* isolates

Initially, 36 isolates were phenotyped for the occurrence of *AvrLm1*, *AvrLm6* and *AvrLm2* on differential lines harbouring the corresponding resistance genes at Agriculture and Agri-Food Canada (AAFC) Saskatoon (Table 1, isolates 3R11 to 03-02). We also added phenotypic data for another 120 isolates from the INRA-Bioger collection (Table 1, isolates IBCN 18 to NzT4; Table S1, see Supporting Information), with 81 of these isolates being evaluated during the course of this study. The overall data were in accordance with what is currently known about the occurrence of *AvrLm2* and other avirulence genes that are linked to *AvrLm2*. Most of the isolates were avirulent towards *Rlm6* (or showed the presence of the unaltered sequence of *AvrLm6*). Only 18% of the isolates were avirulent towards *Rlm2* genotypes, and they mostly originated from Canada.

Identification of *AvrLm2*

A genetic map of the *AvrLm1-AvrLm6-AvrLm2* region was built previously using an F_1 population consisting of 249 progeny derived from a cross between isolates v23.1.3 and v29.3.1 (Fudal *et al*., 2007; Gout *et al*., 2006; Table S1). These studies placed the *AvrLm2* locus 0.8 cM distant from *AvrLm6* in a genomic region which mostly corresponded to a large AT-rich isochore in which very few genes were present. In this study, the genomes of 36 *L. maculans* isolates from the AAFC collection (14 *avrLm2* and 22 *AvrLm2* isolates) were resequenced, and the resulting sequence reads were mapped to the reference genome v23.1.3. Our analysis focused on the *AvrLm2* genomic interval, corresponding to a 270-kb region containing *AvrLm6*, together with two predicted effector genes *LmCys1* and *LmCys2*, and three other genes (*LmTrans*, *LmGT* and *LmMFS*) (Fig. 1A). As *L. maculans* virulence is often linked to the deletion of the SSP-encoding avirulence gene, we first evaluated the presence–absence polymorphism for two possible candidates, *LmCys1* and *LmCys2*, and examined the correlation with the *AvrLm2* phenotype. *LmCys1* was found to be present in all isolates, whereas *LmCys2* was ruled out as *AvrLm2* based on its absence in all 22 avirulent isolates. After determining that the presence–absence polymorphism in the candidate genes could not account for the observed variation in the *AvrLm2* phenotype, we examined single nucleotide polymorphism (SNP) events within the target genomic region. We found that several SNPs within the *LmCys1* locus perfectly correlated with the phenotypic variation of *AvrLm2* within the first 36 sequenced isolates. Three SNPs were identified that differentiated the *AvrLm2* alleles from the virulent (*avrLm2*) isolates, including the reference isolate v23.1.3. These mutations were SNP^{397} (G in avirulent isolates versus A or C in virulent isolates), SNP^{398} (G in avirulent isolates versus A in virulent isolates) and SNP^{436} (G in avirulent isolates versus C in virulent isolates) (Fig. 1C). We also investigated these SNP polymorphisms in 15 additional isolates by sequencing the polymerase chain reaction (PCR)-amplified allele of *LmCys1*. Nine haplotypes were found in the total of 51 isolates analysed (Table 1). All avirulent isolates contained an invariant

Table 1 Allelic variation at the *LmCys1* locus in a collection of 51 isolates and v23.1.3 (reference isolate), virulent or avirulent towards *Rlm2* and the polymorphic site in its protein.

Isolate	Country*	Year†	*Rlm2*‡	*LmCys1*	Amino acid position§ 133	146	Comments
v23.1.3	France	n/a	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
3R11	Australia	n/a	V	C^{397}, A^{398}, C^{436}	His	Gln	
99-56	Canada, MB	1999	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
98-15	Canada, SK	1998	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
290	France	1985	V	C^{397}, A^{398}, C^{436}	His	Gln	
2354	Canada, ON	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
99-79	Canada, SK	1999	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
7.1	Canada, AB	2005	V	C^{397}, A^{398}, C^{436}	His	Gln	
98-16	Canada, SK	1998	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
00-100	Canada, MB	2000	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
89-13	Canada	1989	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
87-41	USA	1987	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
WA51	Australia	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
WA30	Australia	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
Lifolle5	France	Unknown	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
Lifolle6	France	Unknown	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
SC07-59	Canada, SK	2007	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
IH08-10	Canada, SK	2008	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
CB07-37	Canada, MB	2007	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
SC07-69	Canada, SK	2007	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
VR08-01	Canada, AB	2008	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
VR08-29	Canada, AB	2008	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
IH08-85	Canada, SK	2008	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
PC07-12	Canada, MB	2007	V	C^{397}, A^{398}, C^{436}	His	Gln	
PC07-45	Canada, MB	2007	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
2367	Canada, ON	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
99-22	Canada, SK	1999	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
A94	Canada	n/a	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
86-12	Canada, MB	1986	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
CR07-15	Canada, AB	2007	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
165	Canada	Unknown	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
05-29	Canada, AB	2005	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
04-49	Canada, MB	2004	V	C^{397}, A^{398}, C^{436}	His	Gln	
05-08	Canada, AB	2005	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
89-21	Australia	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
166	Canada	Unknown	V	C^{397}, A^{398}, C^{436}	His	Gln	
03-02	Canada, MB	2003	V	C^{397}, A^{398}, C^{436}	His	Gln	
IBCN 18	Australia	1988	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
IBCN 74 (PHW1245)	France	n/a	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
IBCN 80	Canada	1995	A	G^{397}, G^{398}, G^{436}	Gly	Glu	Contains additional $G^{439}C$ and $T^{589}C$ changes
OMR19	Mexico	2005	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
BBA 62908	Germany	1966	A	G^{397}, G^{398}, G^{436}	Gly	Glu	
IBCN 05	France	1992	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
IBCN 14	Australia	1988	V	A^{397}, A^{398}, C^{436}	Asn	Gln	Contains one additional $G^{541}A$ change
IBCN 15	Australia	1988	V	C^{397}, A^{398}, C^{436}	His	Gln	
IBCN 44	France	1990	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
IBCN 85	Canada	1989	V	C^{397}, A^{398}, C^{436}	His	Gln	
IBCN 88	Canada	1990	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
IBCN 17	Australia	1988	V	A^{397}, A^{398}, C^{436}	Asn	Gln	
WAC 4028	Australia	1984	V	A^{397}, G^{398}, C^{438}	Ser	Gln	Contains three deletions
WAC 7803	Australia	1973	V	A^{397}, A^{398}, C^{436}	Asn	Gln	Contains one additional $C^{490}A$ change
NzT4	New Zealand	n/a	V	A^{397}, A^{398}, C^{436}	Asn	Gln	

*Country/province in which the isolate was collected; AB, Alberta; MB, Manitoba; ON, Ontario; SK, Saskatchewan.

†Year isolate was collected.

‡Interaction phenotype determined following cotyledon inoculation tests on a differential set in which the genotype harbouring *Rlm2* is either Topas-*Rlm2* or Bristol (V, virulent isolate; A, avirulent isolate).

§Asn, asparagine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine.

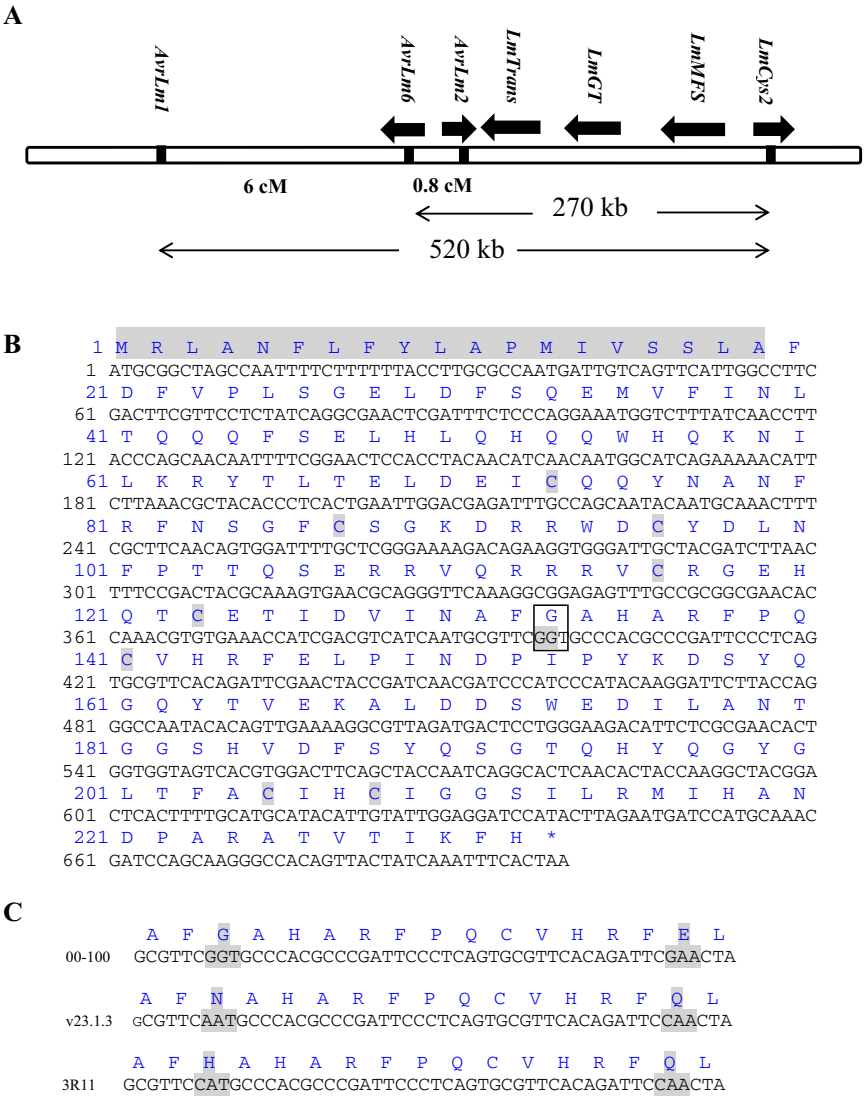

Fig. 1 (A) Schematic representation of genetic and physical distance bordered by *AvrLm1-LmCys2* and putative candidate genes for *AvrLm2*. It should be noted that the gene size and intervals are not to scale. It should also be noted that large AT-rich regions separating *AvrLm1* from *AvrLm2* (*LmCys1*)-*LmTrans-LmGT*, the latter from *LmMSF* and the latter from *LmCys2* are not represented. (B) Nucleotide sequence of the 699 nucleotide region encoding *AvrLm2* and its predicted amino acid sequence. The predicted signal peptide (19 amino acids) and eight cysteine residues are illustrated by grey shading. The mutation of codons $G^{397}G^{398} \rightarrow A/C^{397}A^{398}$, which results in a $G^{133} \rightarrow N^{133}/H^{133}$ change in the amino acids in the protein, leading to the loss of *Rlm2*-mediated recognition specificity, is indicated in a box. (C) Comparison of different haplotypes at the *AvrLm2* locus. Non-synonymous base substitutions and their corresponding amino acid changes are indicated by grey shading.

LmCys1 allele specific to avirulent isolates, except for isolate IBCN80, which also contained additional mutations. Further examination of the sequence variation in virulent isolates showed that A^{398} and C^{436} were invariant in all virulent isolates, except for isolate WAC4028 (see below) (Table 1). The G^{397} polymorphism was more variable and resulted in either an A or C at that position (Table 1). Other mutations were found in the *LmCys1* coding sequence of virulent isolates, but were restricted to one isolate each. All of these mutations led to predicted changes in amino acids (Table 1). The non-synonymous point mutations at bases A^{397}/C^{397} and A^{398} corresponded to either amino acid changes $Gly^{133} \rightarrow Asn^{133}$ or $Gly^{133} \rightarrow His^{133}$, whereas C^{436} corresponded to amino acid changes $Glu^{146} \rightarrow Gln^{146}$ (Fig. 1C; Table 1). Two cases diverging from this simple scheme were found among the sequenced isolates: the *AvrLm2* isolate IBCN80 had two additional point mutations, one of which introduced a stop codon at amino acid 197, producing a truncated predicted protein missing the last 35 amino acids (Table 1). The *avrLm2* isolate WAC 4028 had point mutations leading to a $Gly^{133} \rightarrow Ser^{133}$ change. However, the *LmCys1* sequence in this isolate showed the deletion of a dinucleotide at bases 399–400, followed by a single base mutation at base 454. This introduces multiple mutations between amino acids 133 and 151, including the loss of a cysteine residue.

Functional complementation assay

A genomic *LmCys1* amplicon, including the native promoter region (1109 bp upstream of the ATG start codon) and 42 bp downstream of the predicted open reading frame (ORF) (total length, 1850 bp), from the *AvrLm2* isolate '00-100' was transferred into the fungal transformation vector pNL11. After transforming the virulent isolate v23.1.3 with the *LmCys1* construct, the restoration of the avirulence phenotype was evaluated by the inoculation of transgenic isolates on Topas-*Rlm2* and other *Rlm2*

Table 2 Complementation assays with *AvrLm2* candidate gene. Phenotypic interaction of wild-type and *AvrLm2*-transgenic isolates on different *Brassica* lines harbouring *Rlm2*.

	B. napus lines/cultivars*						
Isolates/transformants†	Westar *Control*	Topas *Control*	T-Rlm2 *Rlm2*	Glacier *Rlm2,Rlm3*	Bristol *Rlm2,Rlm9*	Samourai *RLm2,Rlm9*	Tapidor *Rlm2*
v23.1.3 (*A1a2a3a9*)	V	V	V	V	V	V	V
00-100 (*a1A2A3A9*)	V	V	A	A	A	A	A
v23.1.3: *LmCys1*(AW1)	V	V	A	A	A	A	A
v23.1.3: *LmCys1*(AW2)	V	V	A	A	A	A	A
v23.1.3: *LmCys1*(SNP1)‡	V	V	V	V	V	V	V
v23.1.3: *LmCys1*(SNP2)	V	V	V	V	V	V	V
v23.1.3: *LmCys1*(SNP3)	V	V	V	V	V	V	V
v23.1.3: *LmCys1*(SNP4)	V	V	A	A	A	A	A
v23.1.3: *LmCys1*(Control)	V	V	A	A	A	A	A

*Pathogenicity test on differential lines/cultivar carrying different resistance genes. Each *Leptosphaeria maculans* isolate was tested on 12 seedlings of the differential lines and 12 seedlings of 'Westar' and Topas as highly susceptible controls. The disease reactions were scored 14 days after inoculation and rated using the 0–9 scale described by Williams (1985). T-Rlm2 stands for Topas-Rlm2.

†Isolate v23.1.3 (*A1a2a3a9*) is avirulent on lines harbouring *Rlm1*, but virulent on lines harbouring *Rlm2*, *Rlm3* and *Rlm9*, and isolate 00-100 (*a1A2A3A9*) is virulent on the line harbouring *Rlm1*, but avirulent on lines harbouring *Rlm2*, *Rlm3* and *Rlm9*. v23.1.3: *LmCys1*(AW1) is a transformant with the pNL11-*LmCys1* construct (*LmCys1* avirulent allele was amplified from isolate 00-100). V23.1.3: *LmCys1*(AW2) is a transformant with the pLM4-*LmCys1* construct (*LmCys1* avirulent allele coding region was amplified from isolate 00-100). All constructs were introduced into isolate v23.1.3 by *Agrobacterium*-mediated transformation.

‡The four various alleles of *AvrLm2* (single nucleotide polymorphisms, SNPs) were synthesized and cloned in a Gateway vector (pLM4) downstream of the promoter of the *AvrLm1* effector. These SNPs are: SNP1, $G^{397} \rightarrow A^{397}$ (amino acid change $Gly^{133} \rightarrow Ser^{133}$); SNP2, $G^{397} \rightarrow C^{397}$ (amino acid change $Gly^{133} \rightarrow Arg^{133}$); SNP3, $G^{398} \rightarrow A^{398}$ (amino acid change $Gly^{133} \rightarrow Asp^{133}$); SNP4, $G^{436} \rightarrow C^{436}$ (amino acid change $Glu^{146} \rightarrow Gln^{146}$). The *AvrLm2* open reading frame (ORF) from isolate 00-100 was cloned in the same vector, as a positive control.

cultivars (Glacier DH24287, Bristol, Tapidor and Samourai, Table 2). Twelve transformant selections were tested on the *B. napus* differential lines. Eight of the 12 transformants showed avirulence on cotyledons of *Rlm2* plants, but remained virulent on the susceptible Topas DH16516 and Westar control lines (Fig. 2). Positive transformants showed a wild-type interaction phenotype with the differential lines harbouring other resistance genes (Table 3). This confirmed the identity of *LmCys1* as *AvrLm2*.

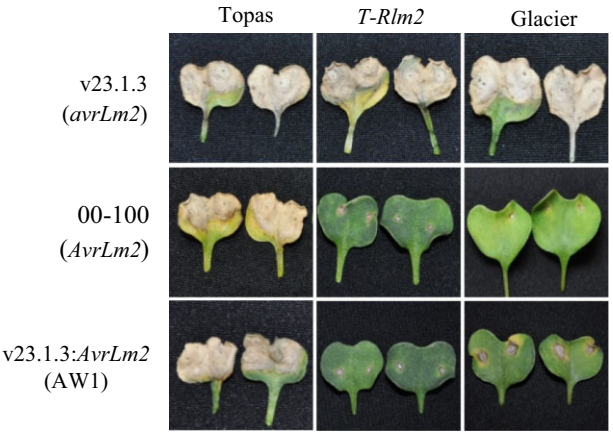

Fig. 2 Phenotypic interaction of wild-type and complemented *Leptosphaeria maculans* isolates on the cotyledons of control (Topas), Topas-*Rlm2* (T-*Rlm2*) and Glacier DH24287 harbouring *Rlm2* and *Rlm3*. Photographs of the infected cotyledons were taken at 14 days post-inoculation. v23.1.3: *AvrLm2* (AW1) contains an avirulent allele from isolate 00-100.

Gene annotation and sequence analysis

The *AvrLm2* (accession: KM073975) ORF is 699 bp with 46% GC content and comprises a single exon. *AvrLm2* encodes a predicted 232-amino-acid protein of 27.046 kDa and contains a predicted N-terminal signal peptide of 19 amino acids. Eight cysteine residues were present in *AvrLm2* and three to four disulfide bonds were predicted, depending on the software used: SCRATCH (Cheng *et al*., 2005), amino acid positions 87–96, 123–141 and 73–116; DISULFIND (Ceroni *et al*., 2006), amino acid positions 73–116, 87–96, 123–208 and 141–205; DIANA (Ferre and Clote, 2005), amino acid positions 73–96, 87–116, 123–205 and 141–208. A search of the National Center for Biotechnology Information (NCBI) non-redundant protein database with AvrLm2 identified distantly related hypothetical proteins in genomes of several *Fusarium oxysporum forma speciales* GenBank accessions (EXK23710.1, EXL90101.1, EXA28588.1 and EXA28676.1). *E*-values ranged between 3×10^{-4} and 1×10^{-6} with 28%–30% identities. However, in all cases, the number and spacing of cysteine residues were identical between all of the proteins. These *F. oxysporum* genes encode for potential secreted proteins based on the presence of the N-terminal signal peptide, lack of a transmembrane domain and subcellular targeting signals. BLASTP of the NCBI database was performed on the *L. maculans* isolate v23.1.3 genome sequence. (*E*-value, 8×10^{-12}; locus tag, LEMA_P09120.1; accession, XP_003845604). LEMA_P09120.1 encodes for a predicted secreted protein with 29% amino acid identity to AvrLm2, and the same number and position of cysteine

Table 3 Pathogenicity test on wild-type isolates and positive transformants on *Brassica napus* lines/cultivars carrying diverse resistance genes.

	B. napus or *B. juncea* lines/cultivars*							
Isolates/ transformants†	Westar *Control*	Topas *Control*	T-Rlm2 *Rlm2*	Quantum *Rlm3*	JetNeuf *Rlm4*	Vulcan-1S *Rlm6*	Roxet *Rlm7*	Goeland *Rlm9*
v23.1.3	V	V	V	V	A	A	A	V
00-100	V	V	A	A	V	A	A	A
v23.1.3: *LmCys1*(AW1)	V	V	A	V	A	A	A	V
v23.1.3: *LmCys1*(AW2)	V	V	A	V	A	A	A	V

*Pathogenicity test on differential lines/cultivar carrying different resistance genes. Each *Leptosphaeria maculans* isolate was tested on 12 seedlings of the differential lines and 'Westar' and Topas as susceptible controls. The disease reactions were scored 14 days after inoculation and rated using the 0–9 scale described by Williams (1985). T-Rlm2 stands for Topas-Rlm2.

†v23.1.3: *LmCys1*(AW1) is a transformant with the pNL11-*LmCys1* construct (*LmCys1* avirulent allele was amplified from isolate 00-100). v23.1.3: *LmCys1*(AW2) is a transformant with the pLM4-*LmCys1* construct (*LmCys1* avirulent allele coding region was amplified from isolate 00-100, driven by the *AvrLm1* promoter). All constructs were introduced into isolate v23.1.3 by *Agrobacterium*-mediated transformation.

residues. *AvrLm2* was absent from all other species of the *L. maculans–L. biglobosa* species complex, except *L. biglobosa* 'thlaspii' (Grandaubert *et al*., 2014b). Compared with this latter species, however, the homologue of *LmCys1* has been translocated to another genome location, as observed previously for other avirulence genes of *L. maculans* (Grandaubert *et al*., 2014a, b).

Functional validation of SNPs associated with *AvrLm2* specificity

Comparison of *AvrLm2* alleles among 51 reference isolates identified SNPs in three locations. These SNPs gave rise to four potential combinations: SNP1, $G^{397} \rightarrow A^{397}$ (amino acid change $Gly^{133} \rightarrow Ser^{133}$); SNP2, $G^{397} \rightarrow C^{397}$ (amino acid change $Gly^{133} \rightarrow Arg^{133}$); SNP3, $G^{398} \rightarrow A^{398}$ (amino acid change $Gly^{133} \rightarrow Asp^{133}$); SNP4, $G^{436} \rightarrow C^{436}$ (amino acid change $Glu^{146} \rightarrow Gln^{146}$). These potential allelic variants of *AvrLm2* were synthesized and transferred to the fungal transformation vector pLM4 under the control of the *AvrLm1* promoter. The *AvrLm2* ORF from isolate 00-100 was used as a positive control. Transgenic v23.1.3 isolates harbouring individual SNP constructs or the *AvrLm2* ORF were tested for the function of the transgene on the *B. napus* differential lines described above. The results showed that only the non-synonymous changes of $G^{397} \rightarrow A/C^{397}$ (SNP1 and SNP2) or $G^{398} \rightarrow A^{398}$ (SNP3), both of which lead to a change in amino acid at Gly^{133}, were responsible for the loss of *Rlm2*-mediated recognition specificity (Table 2).

Expression of *AvrLm2*

AvrLm2 expression was investigated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) during infection and *in vitro* growth with the isolate 00-100. The gene expression level was measured relative to that of actin (Fig. 3). *AvrLm2* expression was very low in germinating conidia compared with other stages. It was expressed at a higher level in infected plants at 3 days post-inoculation (dpi) and peaked at 5 dpi. It then decreased after 5 dpi, but remained at a higher level than during *in vitro* mycelial growth (Fig. 3). The level of expression was higher in mycelium grown axenically than in germinated pycnidiospores, and was comparable with the expression exhibited at 12 dpi (Fig. 3). The virulent allele, *avrLm2*, showed a similar expression pattern (Fig. S1, see Supporting Information).

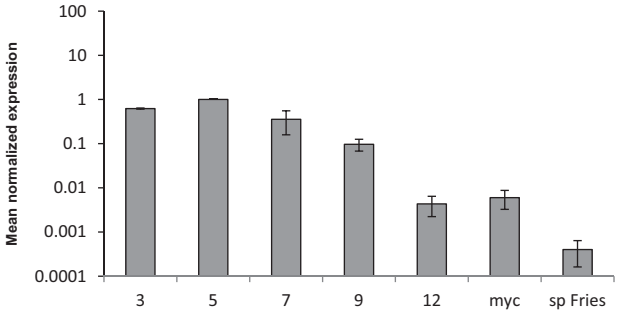

Fig. 3 Expression of representative *AvrLm2* (avirulent) alleles during *in vitro* growth of *Leptosphaeria maculans* and oilseed rape infection. Expression of the *AvrLm2* alleles was analysed in the avirulent isolate 00-100 by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). 3–12, RT-PCR products obtained from RNA isolated from oilseed rape cotyledons (Westar) at 3–12 days post-infection; myc, RT-PCR product obtained from RNA isolated from mycelial culture; sp Fries, RT-PCR product obtained from RNA isolated from conidia germinating in rich medium (Fries). RNA extracted from uninfected cotyledons and water were used as negative controls. Gene expression levels are relative to actin. Each data point is the average of three biological repeats (extractions from different biological material). The standard error of the mean normalized expression level is indicated by error bars.

Genome environment and heterochromatin-based regulation of expression of *AvrLm2*

Recently, Soyer *et al*. (2014) related the heterochromatin-like genome environment of the avirulence gene with an efficient repression of expression during axenic growth, and showed that the repression was relieved during *in planta* infection or in mutants silenced in the expression of two key players in heterochromatin assembly and maintenance, *LmHP1* and

Table 4 Influence of the silencing of *LmHP1* and *LmDIM5* on the expression of selected avirulence genes in axenic culture.

Avirulence gene	Location†	Fold change (*P* value) in:‡	
		Silenced *mHP1*	Silenced *LmDIM5*
AvrLm2	GC island	−5.7*	−9.6*
AvrLm1§	AT-HB	5.6*	16.9*
AvrLm4-7§	AT-HB	8.8*	16.8*
AvrLmJ1	AT-HB	4.2*	4.2
AvrLm6	AT-HB	1.1	1.15
AvrLm11	AT-HB	4.1*	2.9

†AT-HB refers to AT isochores; GC islands refer to regions of more than 1 kb within AT isochores with a GC content of more than 50%.

‡Genes with fold change of less than −1.5 or more than 1.5 in transcript level and an associated *P* value of less than 0.05 (indicated by *) were considered to be significantly down- or up-regulated in the silenced *LmHP1* or silenced *LmDIM5* transformants compared with the wild-type v23.1.3 isolate in axenic culture.

§*AvrLm1* and *AvrLm4-7* data are from Soyer *et al*. (2014).

LmDIM-5. Reassessment of the data including other avirulence genes, such as the newly cloned *AvrLmJ1*, confirmed that the silencing of *LmHP1* or *LmDIM-5* results in increased expression of most of the avirulence genes located in AT isochores, but has little or no effect on *AvrLm6* (Table 4). Surprisingly, a reverse effect was observed for *AvrLm2* with an increased repression of expression in s.*LmHP1* or s.*LmDIM-5* backgrounds (Table 4).

DISCUSSION

In this article, we report the identification of the *L. maculans AvrLm2* gene from the *AvrLm1-AvrLm2-AvrLm6* genetic cluster (Balesdent *et al*., 2002; Fudal *et al*., 2007; Gout *et al*., 2006). AvrLm2, like the majority of effector proteins identified from plant-pathogenic fungi, is a small cysteine-rich secreted protein with limited homology to other proteins in the public protein databases.

Taking an intraspecific comparative genomics approach, as an alternative or complementary approach to map-based cloning, we were able to rapidly identify *AvrLm2* by focusing on a gene that did not show the typical presence–absence polymorphism found for *AvrLm1*, *AvrLm6, AvrLm7* and *AvrLm11*. Thus, we could associate SNPs within the predicted effector *LmCys1* with the phenotypic responses of *L. maculans* isolates on *B. napus* lines containing the *Rlm2* resistance gene. The cloning of *AvrLm2* described here provides an example of the rapid cloning of effector genes through comparative genomics as an alternative or complementary approach to map-based cloning. Map-based cloning has been used successfully to clone fungal Avr genes; however, it is a time-consuming approach, limited by the compatibility of parental isolates for crossing and, in the case of fungal and oomycete plant pathogens, has been applied mainly to species that can be grown axenically. More importantly, the majority of the effectors of *L. maculans* and many other fungal and oomycete plant pathogens sequenced to date are located within a repeat-rich part of the genome that may impede fine mapping or even correct positioning of the target gene. Although a knowledge of the map position of *AvrLm2* facilitated our genome comparison approach to target *AvrLm2*, at the same time the identification of *LmCys1* as *AvrLm2* pointed us to the error in the previously published mapping data (Fudal *et al*., 2007), which excluded *LmCys1* as a candidate.

In the case of *L. maculans*, genome-assisted map-based cloning has been facilitated recently by the availability of a complete repertoire of putative effector-encoding genes (Rouxel *et al*., 2011), which helped in the cloning process of *AvrLm11* and *AvrLmJ1* (Balesdent *et al*., 2013; Van de Wouw *et al*., 2014).

The identification of effectors by comparative genomics requires a high-quality reference genome that has been correctly annotated and contains the gene of interest. In the case in which a reference isolate is virulent because of total deletion of the Avr gene, we would be unable to use this method. The production of an alternative reference genome with a complementary Avr profile to the current reference (v23.1.3) and the mapping of the unknown target genes can rectify these shortcomings. Rapid advances in DNA/RNA sequencing technologies, the expansion of genome databases and the improvement of bioinformatics software promise significant improvement and more common use of such tools in the near future.

Another advantage of the intraspecific comparison approach described here is that it provides detailed information on the allelic variation of all candidate effector genes unearthed in the reference genome, eliminating the need for the time-consuming and repetitive amplification and sequencing of individual candidate genes. Here, we were able to rapidly assign sequence polymorphism to the virulence/avirulence phenotype and showed that only two adjacent nucleotide changes were sufficient for the loss of recognition by *Rlm2*.

Other rare allelic variants of characterized avirulence genes, such as the C-terminal truncation predicted in the *AvrLm2* isolate IBCN80 described above, could help to define functional effector domains. The allelic information collected could be applied to the design of SNP markers for the rapid genotyping of new *L. maculans* isolates with practical application for the management of blackleg disease. For example, Carpezat *et al*. (2014) have recently used a very limited nucleotide polymorphism observed for avirulent versus virulent alleles of the *AvrLm7* gene to develop a diagnostic method based on high-resolution melting (HRM) analysis.

AvrLm2 genotypes are: (i) currently absent in Europe and found only for two isolates obtained before 1975 (Balesdent *et al*., 2006; Stachowiak *et al*., 2006; this study); (ii) rare in Australia with only one isolate of 65 showing the *AvrLm2* phenotype (Dilmaghani *et al*., 2009; this study); (iii) common in current and older popu-

lations from western and eastern Canada, but not in Ontario (Dilmaghani *et al*., 2009; Kutcher *et al*., 2007, 2010), and becoming increasingly less common through southern Manitoba and North Dakota (Chen and Fernando, 2006; Nepal *et al*., 2014); and (iv) prevalent in Mexican populations (Dilmaghani *et al*., 2012) (Table S1). Adaptive mechanisms used by fungi to escape *R* gene recognition have been analysed in detail for those Avr genes which have been submitted to cognate *R* gene selection in either agronomic practice or experimental fields, namely *AvrLm1* (Gout *et al*., 2007), *AvrLm2* (this study), alleles *AvrLm4* (Parlange *et al*., 2009) and *AvrLm7* (Daverdin *et al*., 2012) of *AvrLm4-7* and *AvrLm6* (Fudal *et al*., 2009). At present, such data are not available for the recently cloned *AvrLm11* (Balesdent *et al*., 2013) and *AvrLmJ1* (Van de Wouw *et al*., 2014), whose cognate *R* genes are not used commercially. In Europe, *Rlm2* is likely to have been the oldest resistance gene to be used against *L. maculans* at the time of the re-introduction of winter oilseed rape in the 1960s and 1970s. Thus, cv. Ramses that harboured *Rlm2* was described as being resistant to French populations in 1968–1970 (Rouxel *et al*., 2003b). The selection pressure exerted on populations of *L. maculans* at this time is consistent with the fact that only the two oldest French isolates in collections harbour *AvrLm2* and that current European populations are 100% virulent towards *Rlm2* (Balesdent *et al*., 2006). A similar situation was found later for *Rlm4* with the release of the market-leader cultivar Major in 1971, followed by cv. Jet Neuf in 1977, which was grown on more than 80% of the French acreages (Clement, 1981; Rouxel *et al*., 2003a). Again, this led to a strong selection and to a drastic impoverishment of isolates avirulent towards *AvrLm4* in France, and more generally in Europe (Balesdent *et al*., 2006; Stachowiak *et al*., 2006). This is paralleled by the observation that both *AvrLm2* and the *AvrLm4* allele of *AvrLm4-7* escape from recognition because of point mutations (Parlange *et al*., 2009; this study). Although the intrinsic effector function of *AvrLm4-7* is still unknown, the importance of the effector protein in fungal fitness has been established previously (Huang *et al*., 2010). Here, we show that escape from recognition by *Rlm2* also depends on a few point mutations that all contribute to change the single Gly^{133} residue, and that *AvrLm2* deletion is never observed. The importance of non-synonymous point mutations in these two genes (*AvrLm2* and *AvrLm4-7*) contrasts with that observed by examining the evolution of populations submitted to a new selection exerted by *Rlm1*, *Rlm6* or *Rlm7*. In these cases, the immediate response to the novel pressure was multiple inactivating RIP mutations or complete deletion, with only very few cases of non-synonymous mutations (Daverdin *et al*., 2012; Fudal *et al*., 2007; Gout *et al*., 2006). This suggests the following sequence of events when an avirulence gene is submitted to a novel selection: (i) immediate gene inactivation as a result of multiple RIP mutations of the gene, permitted by the obligate sexual cycle of the fungus and linked with the genome location of *Avr* genes; (ii) simultaneous or secondary deletion of the inactivated gene and its surrounding genomic region comprising only the inactivated gene and its inactivated TE surroundings; and (iii) at a later stage, surge and dissemination of a less detrimental allelic version of the gene, maintaining the effector function whilst preventing recognition by the cognate resistance gene (Daverdin *et al*., 2012). Such a compensatory mechanism would only be set up for genes of importance for fungal pathogenicity, such as *AvrLm2* and *AvrLm4-7*, in contrast with genes, such as *AvrLm1*, whose loss has only a limited effect on fungal fitness (Huang *et al*., 2010).

In *L. maculans*, all currently known avirulence genes occur isolated as single genes within large (typically hundreds of kilobases) AT-rich isochores, genomic regions made up of mosaics of RIP-degenerated and truncated TEs (Fudal *et al*., 2007; Gout *et al*., 2006). This has strong consequences on their mode of evolution under selection, mechanisms of allelic diversification and concerted overexpression at the onset of plant infection (Rouxel *et al*., 2011; Soyer *et al*., 2014). Compared with this common scheme, *AvrLm2* has the unique feature of being accompanied by a second head-to-head gene in the AT isochore, making up a small GC isochore. This single-copy gene, *LmTrans*, has been described to contain a DDE superfamily endonuclease domain predicted to be involved in efficient DNA transposition, and its best match is a putative transposase from *Stagonospora nodorum* (Van de Wouw *et al*., 2010). However, this gene also has strong similarity with the Fot5 transposase of *F. oxysporum* f. sp. *lycopersici* (accession, CAE55867.1; E-value, $8e^{-67}$; identity, 60%). Interestingly, in the genome of *F. oxysporum* f. sp. *lycopersici*, the transposase is located two genes away from the gene *FoSIX1* (accession CAE55870.1) encoding an effector protein which shows limited identity to AvrLm2 (E-value, $6e^{-05}$; identity, 25%), but a good conservation of the cysteine residues. It is unknown whether this may indicate an ancient horizontal gene transfer event.

The fact that *AvrLm2* is not a typical 'lost in the middle of nowhere' Avr gene seems to have consequences on its regulation of expression. Repression of *L. maculans* effector expression in axenic culture has been shown to be a result of the AT-rich isochore environment behaving like heterochromatin (Soyer *et al*., 2014). In addition, the heterochromatin regulation of expression was relieved during plant infection and favoured a concerted and very high overexpression of the avirulence gene in the first stages of plant infection (Balesdent *et al*., 2013; Fudal *et al*., 2007; Gout *et al*., 2006; Parlange *et al*., 2009; Soyer *et al*., 2014; Van de Wouw *et al*., 2014). Here, we show that *AvrLm2* expression is not subject to heterochromatin-based epigenetic control, being adversely affected in a silenced *LmHP1* and *LmDIM5* genetic background compared with most other avirulence genes. Finally, as discussed previously, specific adaptive features (lack of RIP mutations, lack of deletions) may also be linked to the occurrence of *AvrLm2* within a GC island in the middle of an AT isochore. Additional investigations are now needed to confirm that the

characteristics of *AvrLm2* (regulation and adaptive features) are linked to this peculiar genome environment, and/or are strongly interlinked and interdependent on the importance of the gene for fungal fitness.

EXPERIMENTAL PROCEDURE

Fungal material and inoculum preparation

Leptospaeria maculans isolates in this study were sourced from the Rimmer Collection, AAFC Saskatoon, representing isolates collected over 30 years from different geographical regions in the world and two progenies of *in vitro* crosses (Table 1; isolates 3R11 to 03-02). Some of the Canadian isolates in the collection were kindly provided by R. Kutcher, AAFC Melfort, Canada. Isolate '3R11' was kindly provided by Drs A. Van de Wouw and B. Howlett, University of Melbourne, Australia. All isolates were purified as single-spore cultures. Pycnidiospores were harvested from the single-spore cultures after 7–10 days of incubation at 22 °C on V8 juice agar containing 100 μg/mL streptomycin sulfate, as described by Chen and Fernando (2006). All other isolates were from the INRA-Bioger collection and included a series of reference isolates, such as isolates from the International Blackleg of Crucifers Network (IBCN) collection (described in Balesdent *et al*., 2005), one isolate obtained from cabbage in Mexico (OMR19; Dilmaghani *et al*., 2012), a collection of isolates from Western Australia [WTxx isolates; described in Vincenot *et al*. (2008) and Dilmaghani *et al*. (2009)] and a series of collections obtained from samplings performed in France between 1994 (SAM xxxxx collection) and 2003 (V03-Mx-xxx from Versailles and CB-X-x.xx from four locations in France) (Table 1; isolates IBCN 18 to NzT4; Table S1). The fungal culture conditions for growth and sporulation were as reported by Ansan-Melayah *et al*. (1995). For long-term conservation, isolates were stored at 4 °C in 1% malt-agar slant tubes.

Plant material and virulence phenotyping

AAFC isolates were first phenotyped using the *B. napus* differential line Topas-*Rlm2*, harbouring the *Rlm2* resistance gene from the *B. napus* line Glacier DH24287. Topas-*Rlm2* is one of several introgression lines containing individual blackleg *R* genes in the completely susceptible (no *R* gene against *L. maculans*) doubled haploid *B. napus* line Topas DH16516 (N. J. Larkan, unpublished data). This material allowed us to accurately determine the phenotypic response of the isolates to *Rlm2* (Tables 1 and 2). Transgenic isolates were phenotyped on Topas-*Rlm2* (*Rlm2*), Glacier DH24287 (*Rlm2*, *Rlm3*) and Bristol (*Rlm2*, *Rlm9*) (Table 2). Positive transformants (showing restored phenotypic reaction on Topas-*Rlm2*, Glacier and Bristol) were also tested on additional differential cultivars to confirm the interaction of *AvrLm2* only with lines harbouring *Rlm2* (Table 3). Westar and Topas DH16516 (no *R* genes) were used as positive controls for infection by *L. maculans*. The inoculation of *B. napus* cotyledons was performed as described previously (Chen and Fernando, 2006). Each *L. maculans* isolate was tested on 12 seedlings of the differential lines and 12 seedlings of 'Westar' and Topas as highly susceptible controls. The disease reactions were scored 14 days after inoculation and rated using the 0–9 scale described by Williams (1985). Isolates from the INRA-Bioger collection were phenotyped for the presence of *AvrLm1*, *AvrLm6* and *AvrLm2* using a cotyledon inoculation test on the set of differential cultivars as described by Dilmaghani *et al*. (2009) (Table 1, isolates IBCN 18 to NzT4; Table S1).

DNA and RNA manipulation

For PCR and sequencing, DNA was extracted following the cetyltrimethylammonium bromide (CTAB) method of Rogers and Bendich (1985) as modified by O'Gorman *et al*. (1994). Primer sequences were designed using Vector NTI software (Table S2, see Supporting Information). Candidate DNA fragments were sent for synthesis (Eurofins, Huntsville, AL, USA) or amplified through PCR. PCR amplifications were performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) or Q5 DNA Polymerase Master Mix (New England Biolabs, Ipswich, MA, USA) on a C1000 Touch Thermal Cycler (BIO-RAD, Berkeley, CA, USA).

Total RNA was extracted from mycelium grown for 1 week in Fries liquid medium, from germinating conidia grown for 36 h in Fries or from infected leaf tissue, using a PureLink RNA Mini kit (Ambion, Carlsbad, CA, USA) or TRIzol reagent (Invitrogen, CergyPontoise, France) according to the manufacturer's protocol. Total RNA was treated with DNase I RNase-Free (Invitrogen). The RNA concentration was adjusted to 4 μg, and single-strand cDNA was generated using oligo-dT-primed reverse transcription with PowerScript Reverse Transcriptase (Clontech, Palo Alto, CA, USA) or a ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's protocol.

Identification, cloning and transformation of the candidate gene

Leptospaeria maculans isolates were sequenced under contract at the National Research Council of Canada (Saskatoon) by running a paired-end multiplex of 36 isolates across two lanes of Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The depth of sequencing among the 36 isolates provided between nine- and 59-fold coverage of the *L. maculans* genome (Table S3, see Supporting Information). Sequence reads for each isolate were mapped to the reference genome v23.1.3 using Bowtie2 (Langmead and Salzberg, 2012) and visualized using GBrowse 2.0 (Stein, 2013). Candidate gene sequences were either synthesized (Eurofins) or amplified via PCR with Gateway *attB*-tagged primers. These fragments were cloned using the TOPO TA cloning kit (Invitrogen), confirmed by sequencing and transferred to the Gateway entry vector pDONR-Zeo (Invitrogen). Inserts, either including the native promoter or containing only the coding region of the gene, were transferred to the Gateway-compatible fungal expression vectors pNL11 (Larkan *et al*., 2013) and pLM4 (a Gateway vector with the *AvrLm1* promoter; Ma, L., AAFC, Saskatoon, unpublished data), respectively (Tables 2 and 3). Cloning of each insert was confirmed by sequencing. Confirmed pLM4-*AvrLm2* or pNL11-*AvrLm2* constructs were transferred to *Agrobacterium tumefaciens* strain 'AGL1 pTiBo542'. Transformation of pycnidiospores from the *L. maculans* isolate v23.1.3 was performed as described by Utermark and Karlovsky (2008). Transformed colonies were selected after 5–10 days of incubation on Czapek Dox medium enriched with 200 μM cefotaxime and 150 μg/mL hygromycin B, and transferred to V8 medium supplemented with the above antibiotics. For the functional

validation of SNP involvement in *AvrLm2* specificity, different mutagenized allelic forms of *LmCys1* were synthesized (Eurofins) and transferred to pLM4 or pNL11 transformation destination vectors (Table 2).

qRT-PCR

qRT-PCR was performed using a 7700 real-time PCR machine (Applied Biosystems) and SsoFast EvaGreen Supermix (BIO-RAD). RT-PCR was performed for the *AvrLm2* allele with three biological samples. The primers used for qRT-PCR are described in Table S2. *Ct* values were analysed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The *L. maculans* actin was targeted as a reference gene.

ACKNOWLEDGEMENTS

We thank Dr Angela Van de Wouw, Dr Barbara Howlett and Dr Randy Kutcher for kindly providing some of the blackleg isolates. Funding for this research at Agriculture and Agri-Food Canada (AAFC) was provided by the Government of Saskatchewan Agricultural Development Fund (ADF) and Growing Forward 2 (SaskCanola and AAFC). The work at INRA was funded by the Marie Curie Training Site FunGene HPMT-CT-2001-00395 to M. H. Balesdent. Bronislava Profotova was funded by the FunGene project. Thanks are due to Françoise Blaise, Martin Willisecker, Laurent Coudard (INRA-Bioger) and Elena Beynon (AAFC) for technical assistance. The authors declare that they have no conflict of interest.

REFERENCES

Ansan-Melayah, D., Balesdent, M.H., Buèe, M. and Rouxel, T. (1995) Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. *Phytopathology*, **85**, 1525–1529.

Ansan-Melayah, D., Balesdent, M.H., Delorume, R., Pilet, M.L., Tanguy, X., Renard, M. and Rouxel, T. (1998) Genes for race-specific resistance against blackleg disease in *Brassica napus* L. *Plant Breed.* **117**, 373–378.

Balesdent, M.H., Attard, A., Kuhn, M.L. and 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. and 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. and Rouxel, T. (2006) A large scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. *Eur. J. Plant Pathol.* **114**, 53–65.

Balesdent, M.H., Fudal, I., Ollivier, B., Bally, P., Grandaubert, J., Eber, F., Chèvre, A.M., Leflon, M. and Rouxel, T. (2013) The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytol.* **198**, 887–898.

Böhnert, H.U., Fudal, I., Dioh, W., Tharreau, D., Notteghem, J.L. and Lebrun, M.H. (2004) A putative polyketide synthase peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. *Plant Cell*, **16**, 2499–2513.

Carpezat, J., Bothorel, S., Daverdin, G., Balesdent, M.H. and Leflon, M. (2014) Use of high resolution melting analysis to genotype the avirulence *AvrLm4-7* gene of *Leptosphaeria maculans*, a fungal pathogen of *Brassica napus*. *Ann. Appl. Biol.* **3**, 430–440.

Catanzariti, A.M., Dodds, P.N., Lawrence, G.J., Ayliffe, M.A. and Ellis, J.G. (2006) Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell*, **18**, 243–256.

Ceroni, A., Passerini, A., Vullo, A. and Frasconi, P. (2006) DISULFIND, a disulfide bonding state and cysteine connectivity prediction server. *Nucleic Acids Res.* **34** (Suppl. 2), W177–W181.

Chen, Y. and Fernando, W.G.D. (2006) Prevalence of pathogenicity groups of *Leptosphaeria maculans* in Western Canada and North Dakota, USA. *Can. J. Plant Pathol.* **28**, 533–539.

Cheng, J., Randall, A.Z., Sweredoski, M.J. and Baldi, P. (2005) SCRATCH, a protein structure and structural feature prediction server. *Nucleic Acids Res.* **33** (Suppl. 2), W72–W76.

Clement, J.M. (1981) *Larousse Agricole*. Paris: Librairie Larousse.

Daverdin, G., Rouxel, T., Gout, L., Aubertot, J.N., Fudal, I., Meyer, M., Parlange, F., Carpezat, J. and Balesdent, M.H. (2012) Genome structure and reproductive behaviour influence the evolutionary potential of a fungal phytopathogen. *PLoS Pathog.* **8**, e1003020.

Dean, R.A., Talbot, N.J., Ebbole, D.J., Farman, M.L., Mitchell, T.K., Orbach, M.J., Thon, M., Kulkarni, R., Xu, J.R., Pan, H., Read, N.D., Lee, Y.H., Carbone, I., Brown, D., Oh, Y.Y., Donofrio, N., Jeong, J.S., Soanes, D.M., Djonovic, S., Kolomiets, E., Rehmeyer, C., Li, W., Harding, M., Kim, S., Lebrun, M.H., Bohnert, H., Coughlan, S., Butler, J., Calvo, S., Ma, L.J., Nicol, R., Purcell, S., Nusbaum, C., Galagan, J.E. and Birren, B.W. (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature*, **434**, 980–986.

Delourme, R., Pilet-Nayel, M.L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., Brun, H., Renard, M. and Balesdent, M.H. (2004) A cluster of major specific resistance to the blackleg disease [causal agent, *Leptosphaeria maculans* (Des.) Ces. et de Not.] in canola (*Brassica napus* L.). *Theor. Appl. Genet.* **91**, 1190–1194.

Dilmaghani, A., Balesdent, M.H., Didier, J.P., Wu, C., Davey, J., Barbetti, M.J., Li, H., Moreno-Rico, O., Philips, D., Despeghel, J.P., Gout, L. and Rouxel, T. (2009) The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathol.* **58**, 1044–1058.

Dilmaghani, A., Gout, L., Moreno-Rico, O., Dias, J.S., Coudard, L., Castillo-Torres, N., Balesdent, M.H. and Rouxel, T. (2012) Clonal populations of *Leptosphaeria maculans* contaminating cabbage in Mexico. *Plant Pathol.* **62**, 520–532.

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

Ferre, F. and Clote, P. (2005) DIANNA, a web server for disulfide connectivity prediction. *Nucleic Acids Res.* **33** (Suppl. 2), W230–W232.

Ferreira, M., Rimmer, S.R., Williams, P. and Osborn, T. (1995) Mapping loci controlling *Brassica napus* resistance to *Leptosphaeria maculans* under different screening conditions. *Phytopathology*, **85**, 213–217.

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

Flor, H.H. (1971) Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9**, 275–296.

Fudal, I., Ross, S., Gout, L., Blaise, F., Kuhn, M.L., Eckert, M.R., Cattolico, L., Balesdent, M.H. and Rouxel, T. (2007) Heterochromatin-like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome, map-based cloning of *AvrLm6*. *Mol. Plant–Microbe Interact.* **20**, 459–470.

Fudal, I., Ross, S., Brun, H., Besnard, A.L., Ermel, M., Kuhn, M.L., Balesdent, M.H. and Rouxel, T. (2009) Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in *Leptosphaeria maculans*. *Mol. Plant–Microbe Interact.* **22**, 932–941.

Ghanbarnia, K., Lydiate, J.L., Rimmer, S.R., Li, G., Kutcher, H.R., Larkan, N.J., McVetty, P.B.E. and Fernando, W.G.D. (2012) Genetic mapping of the *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*. *Theor. Appl. Genet.* **124**, 505–513.

Gout, L., Fudal, I., Kuhn, M.L., Blaise, F., Eckert, M., Cattolico, L., Balesdent, M.H. and Rouxel, T. (2006) Lost in the middle of nowhere, the *AvrLm1* avirulence gene of the Dothideomycetes *Leptosphaeria maculans*. *Mol. Microbiol.* **60**, 67–80.

Gout, L., Kuhn, M.L., Vincenot, L., Bernard-Samain, S., Cattolico, L., Barbetti, M., Moreno-Rico, O., Balesdent, M.H. and Rouxel, T. (2007) Genome structure impacts molecular evolution at the *AvrLm1* avirulence locus of the plant pathogen *Leptosphaeria maculans*. *Environ. Microbiol.* **9**, 2978–2992.

Grandaubert, J., Balesdent, M.H. and Rouxel, T. (2014a) Evolutionary and adaptive role of transposable elements in fungal genomes. *Ann. Bot. Res.* **70**, 79–107.

Grandaubert, J., Lowe, R.G.T., Soyer, J.L., Schoch, C.L., Van deWouw, A.P., Fudal, I., Robbertse, B., Lapalu, N., Links, M.G., Ollivier, B., Linglin, J., Barbe, V., Mangenot, S., Cruaud, C., Borhan, H., Howlett, B.J., Balesdent, M.H. and Rouxel, T. (2014b) Transposable element-assisted evolution and adaptation to host plant within the *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex of fungal pathogens. *BMC Genomics*, **15**, 891–918.

Huang, Y.J., Balesdent, M.H., Li, Z.Q., Evans, N., Rouxel, T. and Fitt, B.D.L. (2010) Fitness cost of virulence differs between the *AvrLm1* and *AvrLm4* loci in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *Eur. J. Plant Pathol.* **126**, 279–291.

van Kan, J.A.L., van den Ackerveken, F.J.M. and de Wit, P.J.G.M. (1991) Cloning and characterization of cDNA of avirulence gene avr9 of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. *Mol. Plant–Microbe Interact.* **4**, 52–59.

Kutcher, H.R., Keri, M., McLaren, D.L. and Rimmer, S.R. (2007) Pathogenic variability of *Leptosphaeria maculans* in Western Canada. *Can. J. Plant Pathol.* **29**, 388–393.

Kutcher, H.R., Balesdent, M.H., Rimmer, S.R., Rouxel, T., Chèvre, A.M., Delourme, R. and Brun, H. (2010) Frequency of avirulence genes in *Leptosphaeria maculans* in Western Canada. *Can. J. Plant Pathol.* **32**, 77–85.

Langmead, B. and Salzberg, S. (2012) Fast gapped-read alignment with Bowtie2. *Nat. Methods*, **9**, 357–359.

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

Linning, R., Lin, D., Lee, N., Abdennadher, M., Gaudet, D., Thomas, P., Mills, D., Kronstad, J.W. and Bakkeren, G. (2004) Marker-based cloning of the region containing the *UhAvr1* avirulence gene from the basidiomycete barley pathogen *Ustilago hordei*. *Genetics*, **166**, 99–111.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$Ct method. *Methods*, **25**, 402–408.

Long, Y., Wang, Z., Sun, Z., Fernando, D.W.G., McVetty, P.B.E. and Li, G. (2011) Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass 400'. *Theor. Appl. Genet.* **122**, 1223–1231.

Nepal, A., Markell, S., Knodel, J., Bradley, C.A. and del Río Mendoza, L.E. (2014) Prevalence of blackleg and pathogenicity groups of *Leptosphaeria maculans* in North Dakota. *Plant Dis.* **98**, 328–335.

O'Gorman, D., Xue, B., Hsiang, T. and Goodwin, P.H. (1994) Detection of *Leptosphaeria korrae* with the polymerase chain reaction and primers from ribosomal internal transcribed spacers. *Can. J. Bot.* **72**, 342–346.

Orbach, M.J., Farrall, L., Sweigard, J.A., Chumley, F.G. and Valent, B. (2000) A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. *Plant Cell*, **12**, 2019–2032.

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

Pilet, M.L., Delourme, R., Foisset, N. and Renard, M. (1998) Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). *Theor. Appl. Genet.* **97**, 398–406.

Raman, H., Raman, R. and Larkan, N. (2013) Genetic dissection of blackleg resistance loci in rapeseed (*Brassica napus* L.). In: *Plant Breeding from Laboratories to Fields* (Andersen, S.B., ed.), pp. 85–120. Rijeka, Croatia: InTech.

Rep, M., Van Der Does, H.C., Meijer, M., Van Wijk, R., Houterman, P.M., Dekker, H.L., de Koster, C.G. and Cornelissen, B.J. (2004) A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Mol. Microbiol.* **53**, 1373–1383.

Rimmer, S.R. (2006) Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Can. J. Plant Pathol.* **28**, 288–297.

Rivas, S. and Thomas, C.M. (2005) Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*. *Annu. Rev. Phytopathol.* **43**, 395–436.

Rogers, S.O. and Bendich, A.J. (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissue. *Plant Mol. Biol.* **5**, 69–76.

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

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

Rouxel, T., Grandaubert, J., Hane, J.K., Hoede, C., Van de Wouw, A., Couloux, A., Dominguez, V., Anthouard, V., Bally, P., Bourras, S., Cozijnsen, A.J., Ciuffetti, L.M., Degrave, A., Dilmaghani, A., Duret, L., Fudal, I., Goodwin, S.B., Gout, L., Glaser, N., Liglin, J., Kema, G.H., Lapalu, N., Lawrence, C.B., May, K., Meyer, M., Ollivier, B., Poulian, J., Schoch, C.L., Simon, A., Spatafora, J.W., Stachowiak, A., Turgeon, B.G., Tyler, B.M., Vincent, D., Weissenbach, J., Amselem, J., Quesneville, H., Oliver, R.P., Wincker, P., Balesdent, M.H. and Howlett, B.J. (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. *Nat. Commun.* **2**, 202.

Soyer, J.L., El Ghalid, M., Glaser, N., Ollivier, B., Linglin, J., Grandaubert, J., Balesdent, M.H., Connolly, L.R., Freitag, M., Rouxel, T. and Fudal, I. (2014) Epigenetic control of effector gene expression in the plant pathogenic fungus *Leptosphaeria maculans*. *PLoS Genet.* **10**, e1004227.

Stachowiak, A., Olechowicz, J., Jedryczka, M., Rouxel, T., Balesdent, M.H., Happstadius, I., Gladders, P., Latunde-Dada, A. and Evans, N. (2006) Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *Eur. J. Plant Pathol.* **114**, 67–75.

Stein, L.D. (2013) Using GBrowse 2.0 to visualize and share next-generation sequence data. *Brief. Bioinform.* **14**, 162–171.

Utermark, J. and Karlovsky, P. (2008) Genetic transformation of filamentous fungi by *Agrobacterium tumefaciens*. *Nat. Protoc.* http://dx.doi.org/10.1038/nprot.2008.83.

Van de Wouw, A.P., Marcroft, S.J., Barbetti, M.J., Li, H., Salisbury, P.A., Gout, L., Rouxel, T., Howlett, B.J. and Balesdent, M.H. (2009) Dual control of avirulence effectors in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with sylvestris-derived resistance suggests involvement on two resistance genes. *Plant Pathol.* **58**, 305–313.

Van de Wouw, A.P., Cozijnsen, A.J., Hane, J.K., Bruner, P.C., McDonald, B.A., Oliver, R.P. and Howlett, B.J. (2010) Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathog.* **6**, e1001180.

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

Vincenot, L., Balesdent, M.H., Li, H., Barbetti, M.J., Sivasithamparam, K., Gout, L. and Rouxel, T. (2008) Occurrence of a new subclade of *Leptosphaeria biglobosa* in Western Australia. *Phytopathology*, **98**, 321–329.

Williams, P.H. (1985) Crucifer genetics cooperative. *Plant Mol. Boil. Rep.* **3**, 129–144.

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

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Expression of representative *AvrLm2* (virulent) alleles during the *in vitro* growth of *Leptosphaeria maculans* and oilseed rape infection.

Table S1 List and characteristics of the isolates used here for the identification of *AvrLm2*.

Table S2 List of polymerase chain reaction (PCR) primers used in this study.

Table S3 The genome coverage for each of the *Leptosphaeria maculans* isolates used in this study.