Eur J Plant Pathol (2010) 126:279–291
DOI 10.1007/s10658-009-9539-7

Fitness cost of virulence differs between the *AvrLm1* and *AvrLm4* loci in *Leptosphaeria maculans* (phoma stem canker of oilseed rape)

Yong-Ju Huang · Marie-Hélène Balesdent · Zi-Qin Li · Neal Evans · Thierry Rouxel · Bruce D. L. Fitt

Received: 15 April 2009 / Accepted: 28 September 2009 / Published online: 12 October 2009
© KNPV 2009

Abstract To investigate whether the reported fitness cost of virulence at the *AvrLm4* locus in *Leptosphaeria maculans* is common to other loci, near-isogenic (NI) isolates differing at *AvrLm1* locus were produced *in vitro*. Fitness of virulent (*avrLm1*) or avirulent (*AvrLm1*) isolates on *Brassica napus* without the corresponding *R* (resistance) gene *Rlm1* was investigated in controlled environment (CE) and field experiments. Results indicate that there is a measurable fitness cost for *avrLm1* compared to *AvrLm1* isolates in terms of number of lesions, size of lesions, distance grown through leaf tissue towards the petiole in CE experiments and systemic growth from leaf lesions to stems in field experiments. There were differences in fitness cost between the *AvrLm1* and *AvrLm4* loci. There was a cultivar effect on fitness cost of virulence at the *AvrLm1* locus but not at the *AvrLm4* locus. In CE experiments, the optimal temperature for leaf infection was greater for *AvrLm4* isolates than for *AvrLm1* isolates. Field experiment results suggest that on the same host *AvrLm4* isolates are more fit than *AvrLm1* isolates in warmer seasons. The fitness cost at the *AvrLm4* locus was generally greater than at the *AvrLm1* locus, suggesting that the corresponding *R* gene *Rlm4* may be more suitable than *Rlm1* for redeployment in commercial cultivars after an interval of a few years.

Keywords Blackleg · *Brassica napus* (canola) · Durable host resistance · Genotype-environment interactions · Pathogen fitness · *R* gene-mediated resistance

Y.-J. Huang (✉) · Z.-Q. Li · N. Evans · B. D. L. Fitt
Rothamsted Research,
Harpenden, Herts AL5 2JQ, UK
e-mail: yong-ju.huang@bbsrc.ac.uk

M.-H. Balesdent · T. Rouxel
INRA-UMR1290, BIOGER-CPP,
Versailles Cedex 78026, France

Present Address:
Z.-Q. Li
Plant Protection Institute,
Inner Mongolia Academy of Agricultural Sciences,
Huhhot 010031, China

Introduction

Phoma stem canker (blackleg), caused by *Leptosphaeria maculans*, is an economically important disease of *Brassica napus* (oilseed rape, canola, colza) world-wide and causes yield losses worth >$1,000M each growing season (West et al. 2001; Fitt et al. 2008). Host resistance is an economic, effective method for control of the disease; new sources of resistance to *L. maculans* with qualitative resistance (*R*) genes are very effective when first introduced into commercial cultivars (Delourme et al. 2006). Such *R* genes each code for a protein that recognises one of the pathogen effectors, produced by the pathogen to overcome the basal host defence

mechanism (Jones and Dangl 2006). Since pathogen isolates producing an effector recognised by a host *R* gene product are unable to infect the host, historically the genes coding for these effectors have been termed avirulence (*Avr*) genes (Stergiopoulos et al. 2007).

New *R* genes introduced into commercial cultivars are often not durable; for example, *L. maculans* populations can rapidly evolve from avirulence to virulence to render such *R*-gene mediated resistance ineffective after two to three growing seasons (Rouxel et al. 2003; Sprague et al. 2006). This evolution of *L. maculans* populations involves selection of isolates with mutations ranging from a point mutation to complete deletion at the locus of the *Avr* gene (Gout et al. 2007; Fudal et al. 2009; Parlange et al. 2009). Thus, the pathogen *Avr* gene product is no longer 'recognised' by the host *R* gene or is no longer able to modify the plant guard protein (or cannot bind to the guard protein), therefore rendering the host resistance ineffective (Jones and Dangl 2006). For a cost-effective use of *R* genes in agriculture, there is a need to predict the durability (how long they will remain effective) of new *R* genes.

It has been hypothesised that the durability of an *R* gene can be predicted from knowledge of the fitness cost of pathogen virulence at the corresponding locus (Leach et al. 2001). Fitness of an organism is defined as the combined ability for it to survive and reproduce (Pringle and Taylor 2002). Since pathogen effectors play an important role in the fitness of a pathogen, their ability to change to avoid recognition by the host influences the durability of the corresponding *R* gene (Bent and Mackey 2007) because such changes in the effector (*Avr*) gene may damage the pathogen so that it is less able to complete one of the stages in its life-cycle (i.e. have a fitness cost). Evidence in support of this hypothesis comes from theoretical modelling (Pietravalle et al. 2006) and from experimental work with bacterial pathogens (Leach et al. 2001) but there has been little experimental work done with fungal pathogens.

To test experimentally if there are fitness costs of virulence, it is appropriate to study key stages in pathogen life-cycles (Leach et al. 2001; Pringle and Taylor 2002). For *L. maculans*, phoma stem canker epidemics are initiated by ascospores released from crop stem debris (Fig. 1; Huang et al. 2005). Leaf lesions appear after infection through stomata by hyphae from the germinated ascospores. *R* genes operate after infection to prevent the formation of leaf spot lesions (Huang et al. 2006a). In susceptible interactions, the pathogen then grows systemically along the leaf petiole to the stem, where it causes damaging stem base cankers (Fitt et al. 2006). The rate of pathogen growth in petiole and stem tissues is influenced by the operation of quantitative (background, adult plant) resistance (Huang et al. 2009). Therefore, the infection of leaves, growth in stem tissues and the production of ascospores on debris are important stages for studying the fitness of *L. maculans*. Such work with the *L. maculans*/*B. napus* pathosystem has confirmed a fitness cost of virulence at the *AvrLm4* locus in *L. maculans* on hosts without the corresponding *R* gene *Rlm4* (Huang et al. 2006b). It is not clear whether the fitness cost of virulence at the *AvrLm4* locus is specific to that locus or a more general feature of the *Avr* genes in *L. maculans*.

Surveys of populations of *L. maculans* in Europe provide indirect evidence that there may be differences between different loci in the fitness cost associated with virulence. At least eleven *R* genes conferring resistance to *L. maculans* have been identified in *Brassicas* (Delourme et al. 2006; Yu et al. 2008; Van de Wouw et al. 2009). At least nine corresponding *L. maculans Avr* genes have been genetically characterised (*AvrLm1—AvrLm9*) (Balesdent et al. 2002) and *AvrLm1, AvrLm4, AvrLm6* and *AvrLm7* have now been cloned (Gout et al. 2006; Fudal et al. 2007; Parlange et al. 2009). The surveys suggest that there is no fitness cost of virulence at the *AvrLm2* and *AvrLm3* loci since avirulent alleles *AvrLm2* or *AvrLm3* are very rare or completely absent in European populations (Rouxel et al. 2003; Balesdent et al. 2006; Stachowiak et al. 2006). By contrast, they suggest that there may be a fitness cost at *AvrLm1* and *AvrLm4* loci since *AvrLm1* and *AvrLm4* are still present in *L. maculans* populations, although cultivars with *R* genes *Rlm1* and *Rlm4* have been used commercially for >10 years. However, it has been difficult to investigate the fitness costs at *AvrLm2* or *AvrLm3* loci because pairs of near-isogenic (NI) *L. maculans* isolates differing only at the corresponding locus are not available and these *Avr* genes have not been cloned. Recently, pairs of NI *L. maculans* isolates differing only at the *AvrLm1* locus have been developed; it is now possible to experimentally examine the fitness cost at the *AvrLm1* locus to compare with the *AvrLm4* locus.

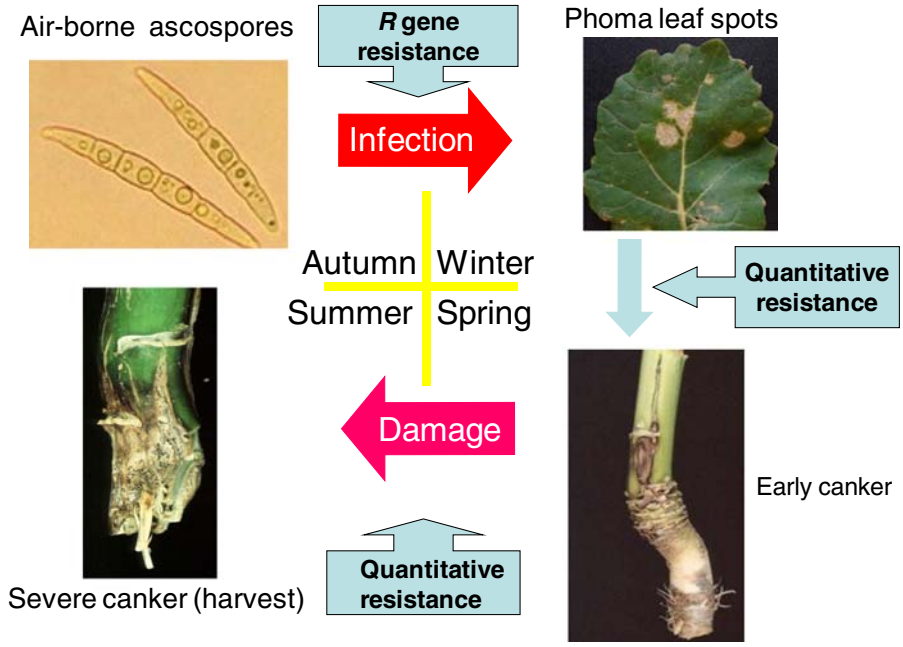

Fig. 1 Seasonal cycle of phoma stem canker epidemics in Europe in relation to components of oilseed rape (*Brassica napus*) resistance to *Leptosphaeria maculans*. Epidemics of this disease are initiated in autumn (September/October) by air-borne ascospores which infect to start development of phoma leaf spots; the pathogen spreads symptomlessly along the leaf petioles in autumn/winter to reach the stem, where stem base cankers or upper stem lesions start to develop in spring (March/April) and may become severe by harvest in summer (July). A gene-for-gene specific host-pathogen interaction operates at the leaf infection stage between host resistance (*R*) genes and pathogen effector (*Avr*) genes. The basis for the quantitative (background, adult plant) resistance which operates in the leaf petiole and stem is less clear. Modified from Fig. 4 in Fitt et al. (2006)

This paper investigates the fitness cost associated with evolution to virulence at the *AvrLm1* locus in *L. maculans* and compares it with the fitness cost at the *AvrLm4* locus. The fitness of avirulent and virulent isolates was investigated on hosts without the corresponding resistance gene *Rlm1* in controlled environments (CEs) using NI *L. maculans* isolates carrying either the avirulent (*AvrLm1*) or virulent (*avrLm1*) alleles, and in natural *L. maculans* populations under field conditions.

Materials and methods

Production of near-isogenic *L. maculans* (*AvrLm1, avrLm1*)

To produce NI isolates of *L. maculans*, differing in alleles only at the *AvrLm1* locus (i.e. *AvrLm1*, *avrLm1*), a back-crossing (BC) scheme, described for production of NI isolates of *L. maculans* at the *AvrLm4* locus (Huang et al. 2006b), was used. Six *AvrLm1* (three *MAT1-1* and three *MAT1-2*) and six *avrLm1* (three *MAT1-1* and three *MAT1-2*) isolates (Table 1) from the BC_5 progeny were randomly selected for producing NI ascospores by *in vitro* crossing. The mycelial growth rates of the six *AvrLm1* and six *avrLm1* isolates on V8 juice agar at 22°C were compared using the method of Huang et al. (2006b).

Ascospores of *L. maculans* with either *AvrLm1* or *avrLm1* alleles were produced by *in vitro* crossing using the six *AvrLm1* isolates or the six *avrLm1* isolates. To compare the germination patterns of ascospores, mature pseudothecia were excised from the colony on agar under a dissection microscope (Olympus, Optical Co., London, UK) and attached to the under-side of the lid of a Petri dish (9 cm diam). Then the lid was placed over a Petri dish containing 1.5% distilled water agar (Oxoid, Basingstoke, UK). When ascospores were released onto the agar, a piece of the agar (2 cm × 1 cm) was excised and placed on a glass slide (7.5 cm × 2.5 cm), then the slide was placed in a Petri dish lined with moistened filter paper. The

Table 1 *In vitro* growth on V8 juice agar at 22°C in darkness of the *Leptosphaeria maculans* near-isogenic isolates *AvrLm1* and *avrLm1* from the progeny produced after five back-crosses (BC_5)

Isolate[a]	*AvrLm1* allele[b]	*MAT* allele[c]	Colony diam (mm)[d]		Growth rate (mm day^{-1})
			6 days	10 days	
V41.1.2	*AvrLm1*	*MAT1-1*	31.2	54.8	5.9
V41.2.1	*AvrLm1*	*MAT1-1*	30.7	56.1	6.3
V41.5.3	*AvrLm1*	*MAT1-1*	31.7	55.9	6.1
V41.2.4	*AvrLm1*	*MAT1-2*	29.7	54.3	6.1
V41.2.5	*AvrLm1*	*MAT1-2*	29.0	49.7	5.2
V41.3.9	*AvrLm1*	*MAT1-2*	29.3	52.2	5.7
V41.2.7	*avrLm1*	*MAT1-1*	29.9	56.9	6.8
V41.4.6	*avrLm1*	*MAT1-1*	30.1	52.5	5.6
V41.5.7	*avrLm1*	*MAT1-1*	19.8	38.3	4.6
V41.1.3	*avrLm1*	*MAT1-2*	27.1	50.9	5.9
V41.2.14	*avrLm1*	*MAT1-2*	18.0	36.4	4.6
V41.3.5	*avrLm1*	*MAT1-2*	28.9	55.3	6.6
SED			0.69	1.39	0.28
(df)			(70)	(70)	(70)

[a] The isolates v23.1.3 and v23.1.2 were crossed to produce the F_1 generation, followed by five back-crosses (BC_5) to v23.1.3. The near-isogenic isolates were selected from the progeny of BC_5. The isolates v23.1.3 and v23.1.2 are sister isolates from an *in vitro* cross (Attard et al. 2002)

[b] Isolates were characterised at the *AvrLm1* locus by inoculation onto cotyledons of oilseed rape cv. Columbus (with resistance gene *Rlm1-Rlm3*) and susceptible controls Westar (with no resistance gene) and 00.22.1.1 (*Rlm3* only)

[c] The mating type (*MAT1-1* or *MAT1-2*) of isolates was determined by PCR

[d] The colony diameter was measured for two perpendicular diameters (mm) per plate 6 and 10 dpi. Data are means of four replicate plates per isolate from two replicate experiments

Petri dish was then incubated at 20°C in darkness for 18 h. The slides were then stained with trypan blue (0.1% w/v in lactophenol) and germination pattern were assessed under a microscope (Huang et al. 2001).

Fitness of near-isogenic *L. maculans* (*AvrLm1, avrLm1*) isolates during leaf infection in CEs

The fitness cost of virulence at the *AvrLm1* locus was studied with oilseed rape cvs Eurol and Darmor (without both *Rlm1* and *Rlm4*, corresponding to *AvrLm1* and *AvrLm4*, also used to investigate fitness of *AvrLm4* and *avrLm4* isolates, Huang et al. 2006b). Darmor has quantitative background resistance against *L. maculans* whilst Eurol does not (Huang et al. 2009). Plants were grown in pots (5 cm diam) containing peat-based compost and a soluble fertiliser. Pots (one plant per pot) were put in trays in four rows with seven pots in each row. The two cultivars were arranged in alternate rows. Plants were grown in a glasshouse, then transferred to a 15°C controlled-environment cabinet (12 h light/12 h darkness, light intensity 210 µmol $m^{-2}s^{-1}$) until they had three fully expanded leaves and were ready for inoculation.

Plants were inoculated using the 'ascospore shower' method (Huang et al. 2006b) with ascospores of either *AvrLm1* isolates or *avrLm1* isolates that had been produced *in vitro*. After inoculation, plants were sprayed with distilled water and covered with tray lids. Two trays (one inoculated with *AvrLm1* isolates, one inoculated with *avrLm1* isolates) were selected at random and transferred to each of the 5, 10, 15, 20 or 25°C growth cabinets. The tray lids were kept on for 48 h to maintain leaf wetness. The experiment was repeated four times, with each experiment inoculated with fresh ascospores produced by a new set of crosses.

To estimate the number of ascospores deposited per unit leaf area, three glass microscope slides (7.5 cm×2.5 cm) were placed in between the plants

in each tray at approximately the same height as the leaves. The number of ascospores deposited on each slide was counted to estimate number of spores deposited cm^{-2}. The maximum length and width of each leaf on ten plants of each cultivar were measured just before inoculation to estimate the leaf area per plant, then the number of ascospores deposited per plant was calculated [total leaf area (cm^2) per plant × number of spores cm^{-2}]. The infection efficiency was then estimated as number of lesions resulting from inoculation with 100 ascospores [(total number of lesions per plant) ÷ (total number of spores deposited per plant) × 100].

Plants were assessed daily by counting the numbers of new phoma leaf spots on each plant, until no new leaf spots appeared. The incubation period (time from inoculation to the appearance of the first lesion) was recorded. The diameters of 10 lesions on plants grown at 15, 20 and 25°C were measured 14–15 days post-inoculation (dpi) and 18–20 dpi for plants grown at 10°C. To compare the distance grown by *AvrLm1* and *avrLm1* isolates after initial infection, affected leaves were detached 18–19 dpi from plants grown at 15, 20 and 25°C, and samples were taken at 1 and 2 cm from the lesion in the direction of the petiole for isolation of *L. maculans* (West et al. 2002).

Analyses of variance (ANOVA) were done to compare components of the fitness (e.g. incubation period, number of lesions, infection efficiency, size of lesions, growth towards the leaf petiole) of *AvrLm1* and *avrLm1* isolates during leaf infection, using the GENSTAT statistical software (Payne et al. 2007). Linear regressions of incubation period against temperature were calculated separately for each inoculum (*AvrLm1* or *avrLm1*) treatment in each replicate experiment. Analyses of position and parallelism were done to assess whether the data were best fitted by a single line or series of parallel lines or series of non-parallel lines for *AvrLm1* and *avrLm1* isolates.

Comparison of fitness of *AvrLm1/avrLm1* and *AvrLm4/avrLm4* isolates in natural populations of *L. maculans*

To compare the fitness of *AvrLm1/avrLm1* and *AvrLm4/ avrLm4* isolates in natural populations of *L. maculans*, isolates recovered from individual phoma leaf spot lesions in autumn, from stem cankers before harvest in summer and from ascospores produced on the stem debris in the following autumn in the 2002/03 and 2003/04 growing seasons (Huang et al. 2006b), were used to assess fitness cost at the *AvrLm1* locus. The cultivars used in these field experiments (Pactol, Darmor and Mohican) did not carry *R* genes *Rlm1* and *Rlm4*. In total, 501 isolates from the 2002/03 growing season and 690 isolates from the 2003/04 growing season were characterised at the *AvrLm1* locus (as *AvrLm1* or *avrLm1*) using cotyledon inoculation tests on cv. Columbus (*Rlm1- Rlm3*), line 00.22.1.1 (*Rlm3* only) and the susceptible control Westar (no resistance genes) (Balesdent et al. 2002). During the two growing seasons, the daily rainfall and temperature were recorded from October (the usual start of phoma leaf spotting) to July (harvest) by an on-site weather station.

Results

Fitness of near-isogenic *L. maculans* (*AvrLm1/avrLm1*) isolates during leaf infection in CEs

For the six avirulent (*AvrLm1*) and six virulent (*avrLm1*) NI isolates selected for production of NI ascospores, there was no difference in growth rate on V8 juice agar between *AvrLm1* (5.9 mm day^{-1}) and *avrLm1* (5.7 mm day^{-1}) isolates at 22°C (Table 1). There was also no difference in growth rate between the two *L. maculans* mating types *MAT1-1* and *MAT1-2*. On water agar at 20°C, ascospores of *AvrLm1* and *avrLm1* isolates produced similar, long germ tubes with a few branches. There was thus no difference in germination patterns between *AvrLm1* and *avrLm1* isolates (data not presented).

More lesions developed on plants inoculated with ascospores of *AvrLm1* isolates than on plants inoculated with ascospores of *avrLm1* isolates ($P<0.01$, 41 df, SED=0.2) (Fig. 2a). More lesions developed at 15–25°C than at 5–10°C ($P<0.05$, 11 df, SED=0.3). On average, more lesions developed on Darmor than on Eurol ($P<0.01$, 41 df, SED=0.2). The infection efficiency was greater at 15–25°C than at 5–10°C ($P<0.05$, 11 df, SED=3.4). The infection efficiency of *AvrLm1* isolates was greater than that of *avrLm1* isolates ($P<0.001$, 42 df, SED=1.4; Table 2). The infection efficiency was greater on Darmor than on Eurol ($P<0.05$, 42 df, SED=1.4). There was no

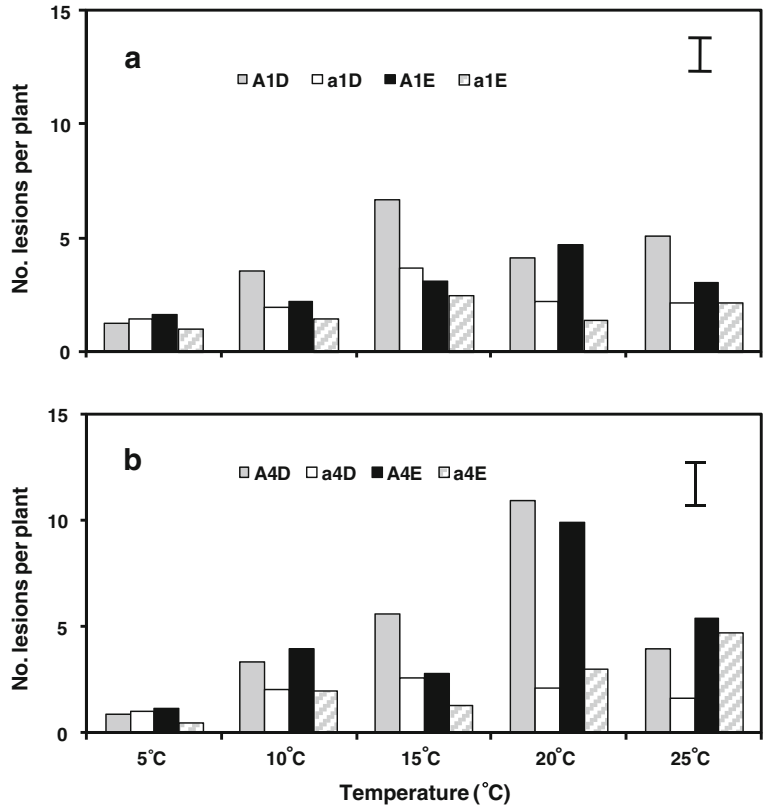

Fig. 2 Numbers of phoma leaf spot lesions which developed at different temperatures on leaves of oilseed rape (*Brassica napus*) cvs Darmor and Eurol (without the resistance genes *Rlm1* and *Rlm4*), inoculated with ascospores of avirulent (*AvrLm1* or *AvrLm4*) or virulent (*avrLm1 or avrLm4*) isolates of *Leptosphaeria maculans* produced *in vitro*. Data are means from four replicate experiments for *AvrLm1*/*avrLm1* **a** or three replicate experiments for *AvrLm4*/*avrLm4* **b**. Data for *AvrLm4* are from experiments described in Huang et al. (2006b). AD and AE represent Darmor and Eurol inoculated with avirulent isolates; aD and aE represent Darmor and Eurol inoculated with virulent isolates. Error bars represent the standard error of differences (a, 43 df; b, 30 df)

significant cultivar-isolate interaction. As temperature increased, the incubation period of both *AvrLm1* and *avrLm1* isolates decreased (Fig. 3). However, there was no difference in incubation period between *AvrLm1* and *avrLm1* isolates or between cvs Darmor and Eurol. The relationship between incubation period (f) and temperature (T) was fitted best by a single line for *AvrLm1* and a*vrLm1* isolates: $f=25.3–0.74T$ ($R^2=0.83$).

The diameters of lesions on plants inoculated with *AvrLm1* isolates were greater than those on plants inoculated with *avrLm1* isolates ($P<0.001$; 22 df; SED=0.3) (Table 2). There was no difference between cvs Darmor and Eurol in lesion diameter. For both *AvrLm1* and *avrLm1* isolates, the diameters of lesions increased with increase in temperature ($P<0.05$; 7 df; SED=0.8). The percentage of lesions which produced hyphae at 1 or 2 cm away from the lesion was greater on plants inoculated with *AvrLm1* isolates than on plants inoculated with *avrLm1* isolates (Table 2). For example, 78% of *AvrLm1* lesions produced hyphae, while only 37% of *avrLm1* lesions produced hyphae at 1 cm away from the lesion at 15°C.

Comparison of fitness of *AvrLm1/avrLm1* and *AvrLm4/avrLm4* isolates during leaf infection in CEs

The pairs of isolates *AvrLm1/avrLm1* and *AvLm4/avrLm4* differed in their optimal temperature for leaf infection. The greatest numbers of lesions developed at 15°C for *AvrLm1/avrLm1* isolates (Fig. 2a) and at 20°C for *AvLm4/avrLm4* isolates (Fig. 2b). The largest differences in infection efficiency were at

Table 2 Fitness of *Leptosphaeria maculans* near-isogenic isolates *AvrLm1* (avirulent) or *avrLm1* (virulent) in terms of infection efficiency, lesion diameter or growth from phoma leaf spot lesions towards the petiole on leaves of oilseed rape (*Brassica napus*) cultivars Darmor and Eurol without the corresponding resistance gene *Rlm1*[a]

Temp. (°C)	*AvrLm1* allele	Infection efficiency[b]		Lesion diam (mm)[c]		% lesions producing hyphae at 1 cm[d]	% lesions producing hyphae at 2 cm
		Darmor	Eurol	Darmor	Eurol		
5	*AvrLm1*	2.4	3.5	-[e]		-	-
	avrLm1	6.0	1.5	-		-	-
10	*AvrLm1*	20.2	14.0	4.2	4.6	-	-
	avrLm1	9.0	4.7	3.8	3.2	-	-
15	*AvrLm1*	28.8	14.1	4.9	4.9	78.2	43.8
	avrLm1	8.7	10.6	3.9	4.1	37.2	7.2
20	*AvrLm1*	14.2	20.2	6.8	7.9	66.4	29.5
	avrLm1	8.5	3.7	5.2	5.1	30.7	5.7
25	*AvrLm1*	15.0	10.9	6.9	6.9	22.0	1.6
	avrLm1	5.9	7.1	5.0	4.3	24.3	0
SED		5.1		1.0		5.4	2.9
(df)		(40)		(17)		(6)	(6)

[a] Data are the mean values from four experiments on cvs Darmor and Eurol. In each experiment, plants were inoculated with fresh ascospores of *AvrLm1* or *avrLm1* produced *in vitro* in a new set of crosses

[b] Infection efficiency was estimated as number of lesions caused by inoculation with 100 ascospores

[c] Phoma leaf spot lesion diameters were measured 18–20 (10°C) or 14–15 (15–25°C) dpi.

[d] Presence of *L. maculans* hyphae shown by isolation from samples taken at 1 or 2 cm away from the lesion towards the petiole, from affected leaves 18–19 dpi.

[e] Not measured

10–20°C for *AvrLm1* and *avrLm1* isolates and at 15–25°C for *AvrLm4* and *avrLm4* isolates (Table 3). Furthermore, there was a cultivar effect on *AvrLm1/avrLm1* isolates but not on *AvrLm4/avrLm4* isolates; more *AvrLm1* or *avrLm1* lesions developed on Darmor than on Eurol (Fig. 2a) but there was no difference in number of *AvrLm4* or *avrLm4* lesions between Darmor and Eurol (Fig. 2b).

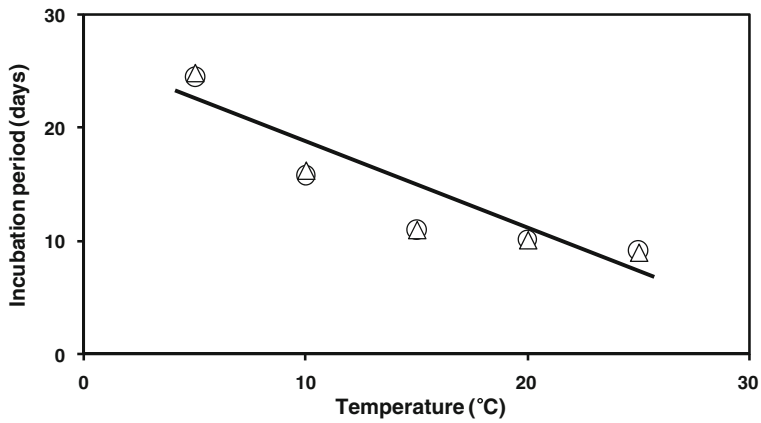

Fig. 3 Effects of temperature on the incubation period (time from inoculation to the appearance of the first lesion) of *AvrLm1* (Δ) and *avrLm1* (○) isolates of *Leptosphaeria maculans* on leaves of oilseed rape cvs Darmor or Eurol (without the resistance gene *Rlm1*). The relationships between incubation period (*f*) and temperature (*T*) were fitted best by a single line $f=25.3-0.74T$ ($R^2=0.83$). Data points are means for the two cultivars in four replicate experiments

There was no difference between *AvrLm1/avrLm1* and *AvrLm4/avrLm4* isolates in the length of the incubation period. The relationship between incubation period (*f*) and temperature (*T*) was similar for *AvrLm1*/*avrLm1* isolates (f=25.3–0.74T) to that for *AvrLm4*/*avrLm4* isolates (f=24.3–0.74T) (Huang et al. 2006b). The largest differences in systemic growth in leaf tissue towards the petiole after initial infection were at 15°C for *AvrLm1* and *avrLm1* isolates and at 25°C for *AvrLm4* and *avrLm4* isolates (Table 3). At 25°C at 2 cm from the leaf lesion, the difference in systemic growth between *AvrLm1* and *avrLm1* isolates (1.6%) was much smaller than that between *AvrLm4* and *avrLm4* isolates (30%).

Comparison of fitness of *AvrLm1/avrLm1* and *AvrLm4/avrLm4* isolates in natural populations of *L. maculans*

During the field experiment in the 2002/03 growing season, the frequency of the *AvrLm1* allele in the *L. maculans* population did not change greatly (Fig. 4a). By contrast, the frequency of the *AvrLm4* allele increased from 6% at the phoma leaf spot stage in autumn to 20% at the stem canker stage before harvest and 15% on stem debris after harvest (Huang et al. 2006b). In the 2003/04 growing season, the frequency of the *AvrLm1* allele increased from 7% at the phoma leaf spot stage to 12% at the stem canker stage before harvest and 10% on debris after harvest (Fig. 4a), while the frequency of the *AvrLm4* allele increased from 8% at the phoma leaf spot lesion stage to 11% at the stem canker stage before harvest and to 12% on debris after harvest. On average, over the two growing seasons, there was no difference between the *AvrLm1* and *AvrLm4* loci in the frequency of avirulent alleles at the leaf lesion stage (initial leaf infection), but at the stem canker stage (growth from leaf lesion to stem) and on debris after harvest (sexual spore reproduction) the frequencies of the *AvrLm4* allele were greater than those of the *AvrLm1* allele (Fig. 4b).

The 2002/03 season was generally hotter than the 2003/04 season. From October to July, average temperature in 2002/03 was 1.1°C greater than in 2003/04 and total rainfall was 3.4 mm greater than in 2003/04. In autumn, mean monthly temperatures in October and December were 2.4 and 1.9°C greater, respectively, in 2002 (11.5, 6.6°C) than 2003 (9.1, 4.7°C) (Fig. 5a), while mean monthly total rainfall values were 0.4 and 19.6 mm greater, respectively, in 2002 (74.2, 67 mm) than 2003 (73.8, 47.4 mm) (Fig. 5b). In spring/summer, the mean temperatures in May and June were 1.3 and 3.0°C greater, respectively, in 2003 (14.0, 20.0°C) than 2004 (12.7, 17.0°C) (Fig. 5a), while the mean monthly total rainfall values were 22.8 and

Table 3 Comparative fitness of two pairs of *Leptosphaeria maculans* near-isogenic isolates (*AvrLm1/avrLm1*, *AvrLm4/avrLm4*[a]) in terms of infection efficiency and systemic growth from phoma leaf spot lesions towards the petiole on leaves of oilseed rape (*Brassica napus*) cvs Darmor and Eurol without the corresponding resistance genes *Rlm1* and *Rlm4*

Temperature (°C)	Infection efficiency (difference between avirulent and virulent isolates)[b]		% lesions producing hyphae at 2 cm (difference between avirulent and virulent isolates)[c]	
	AvrLm1 – avrLm1	*AvrLm4 – avrLm4*	*AvrLm1 – avrLm1*	*AvrLm4 – avrLm4*
5	-0.8	+1.2	[d]	-
10	+10.3	+8.8	+0.7	-
15	+11.8	+14.5	+36.6	-4.8
20	+11.1	+14.6	+23.8	+27.0
25	+6.4	+15.0	+1.6	+30.1

[a] The experiments for *AvrLm4* and *avrLm4* isolates are described in Huang et al. (2006b). Data presented are differences between means from four experiments for *AvrLm1/avrLm1* or three experiments for *AvrLm4/avrLm4*

[b] Infection efficiency was estimated as number of lesions caused by inoculation with 100 ascospores

[c] Presence of *L. maculans* hyphae shown by isolation from samples taken at 2 cm away from the leaf lesion towards the leaf petiole, from affected leaves 18–19 dpi.

[d] Isolation was not done

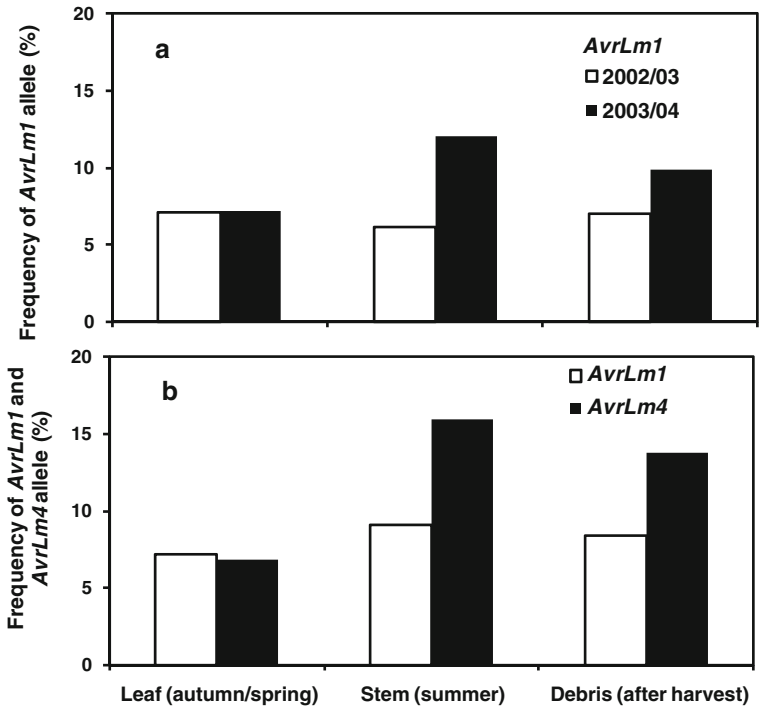

Fig. 4 Frequency (%) of the avirulent alleles *AvrLm1* and *AvrLm4* in natural populations of *Leptosphaeria maculans*. Isolates were obtained from phoma leaf spots (sampled in autumn/spring), stem cankers (sampled in summer before harvest) and stem debris (sampled 3 months after harvest) of oilseed rape cvs Pactol, Darmor and Mohican lacking the resistance genes *Rlm1* and *Rlm4* in 2002/03 and 2003/04 field experiments at Versailles, France. **a** frequency of *AvrLm1* alleles during 2002/03 and 2003/04 growing seasons; **b** mean frequencies for *AvrLm1* and *AvrLm4* alleles over the 2002/03 and 2003/04 growing seasons (501 isolates from 2002/03 and 690 isolates from 2003/04 were classified as *AvrLm1* or *AvrLm4* using a cotyledon inoculation test). Data for *AvrLm4* are presented separately for the two growing seasons in Huang et al. (2006b)

9.8 mm greater, respectively, in 2003 (60.8, 39.2 mm) than 2004 (38.0, 29.4 mm) (Fig. 5b).

Discussion

These results suggest that there are fitness costs of virulence at both the *AvrLm1* and *AvrLm4* loci in *L. maculans* but that there are differences in fitness cost of virulence between the two loci. Firstly, there was a cultivar effect on fitness of *AvrLm1* isolates but not on fitness of *AvrLm4* isolates, for the two cultivars tested. That there were no differences in number of lesions or infection efficiency of *AvrLm4* isolates between cvs Darmor and Eurol suggests that quantitative (background) resistance does not affect fitness at the *AvrLm4* locus (Huang et al. 2006b). In contrast, more *AvrLm1* leaf lesions developed on Darmor than Eurol, suggesting a cultivar effect on fitness at the *AvrLm1* locus. These data show that there is a need to evaluate a range of plant genotypes to detect pathogen fitness costs that may be rare or even absent on some genotypes.

Secondly, these results suggest that there are differences between the *AvrLm1* and *AvrLm4* loci in effects of temperature on fitness cost. Controlled environment experiments suggested that there were differences in the optimal temperature range for leaf infection between *AvrLm1* and *AvrLm4* isolates, with the optimal temperature range for leaf infection 15–25°C for *AvrLm4* isolates and 10–20°C for *AvrLm1* isolates. These results are supported by those of field experiments. The frequency of *AvrLm4* isolates increased more between the leaf infection stage in autumn and the stem canker stage before harvest in the hotter (2002/03) than the cooler growing season (2003/04), suggesting that *AvrLm4* isolates may be

more fit than *avrLm4* isolates at higher temperatures. This suggests that global warming effects on severity of phoma stem canker epidemics (Evans et al. 2008) may benefit *AvrLm4* isolates.

Thirdly, the results suggest that there is a greater fitness cost of virulence at the *AvrLm4* locus than at the *AvrLm1* locus. Evidence is provided by the observation that the difference in infection efficiency at temperatures of 15–25°C in controlled environments (Table 3) is larger between *AvrLm4* and *avrLm4* isolates than between *AvrLm1* and *avrLm1* isolates. This is supported by results from field experiments suggesting that, on the same host, frequencies of the *AvrLm4* allele were greater than those of the *AvrLm1* allele at the stem canker stage (reflecting growth from leaf lesion to stem) and on debris after harvest (sexual spore production) during the hotter season. Investigation of the molecular evolution of virulence has shown that virulence at the *AvrLm1* locus is usually due to a complete deletion of the gene (Gout et al. 2007), whilst the virulence at the *AvrLm4* locus is due to a single-base mutation without deletion of the gene (Parlange et al. 2009). This suggests that the *AvrLm4* gene may be more important than the *AvrLm1* gene for the overall fitness of *L. maculans*. The transition from avirulence to virulence can involve gene deletion, point mutation or insertion of transposon-like elements (Stergiopoulos et al. 2007). Recent work on evolution from avirulence to virulence at the *AvrLm6* locus in *L. maculans* suggests that repeat-induced point mutation is another mechanism leading to virulence in *L. maculans* (Fudal et al. 2009). Differences in fitness costs of virulence between the different *Avr* genes suggest that selection may favour different types of adaptation, which may reflect differences in direct or indirect interactions with *R*-gene encoded proteins (Jones and Dangl 2006).

Results of these experiments with the *L. maculans*-*B. napus* pathosystem provide experimental evidence for a fungal pathogen that it may be possible to predict durability of *R* genes introduced into new cultivars by assessing the fitness cost of virulence. This supports theoretical evidence and experimental evidence with bacterial pathogens (Leach et al. 2001; Pringle and Taylor 2002; Pietravalle et al. 2006; Bent and Mackey 2007). The evidence that fitness cost of virulence at the *AvrLm4* locus is generally greater than that at the *AvrLm1* locus suggests that the corresponding resistance gene *Rlm4* may be more

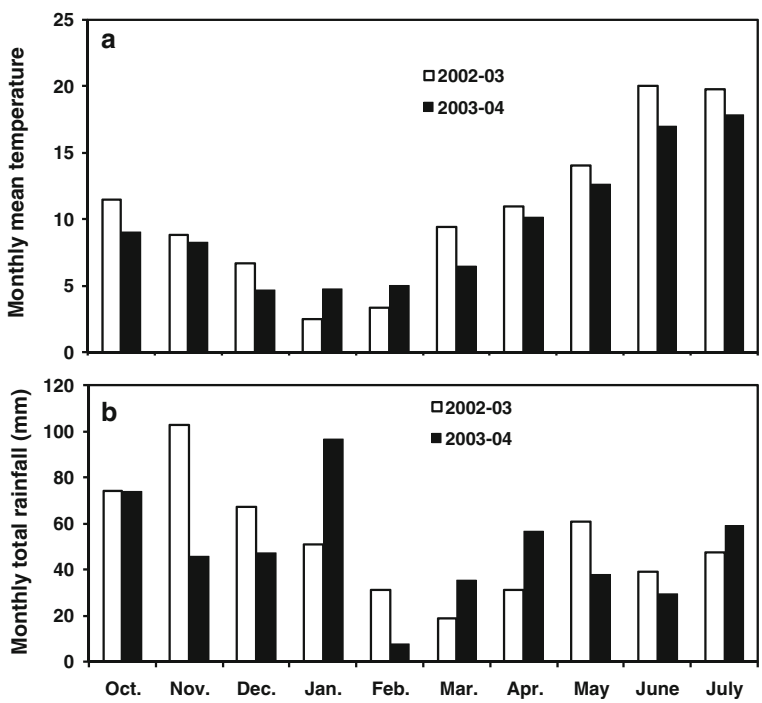

Fig. 5 Monthly mean temperature **a** and total rainfall **b** during 2002/03 and 2003/04 growing seasons for field experiments at Versailles, France

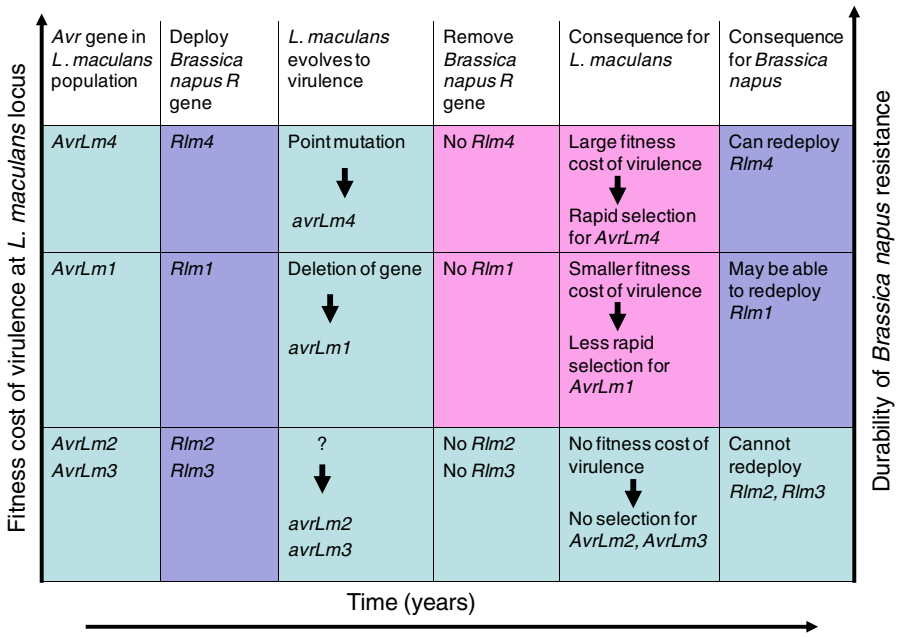

Fig. 6 Relationship between fitness cost of virulence at *Leptosphaeria maculans Avr* loci and potential durability of resistance of the corresponding *R* genes in *Brassica napus* over time (successive winter oilseed rape growing seasons over several years). Differences between *AvrLm4*/*Rlm4*, *AvrLm1*/*Rlm1* and *AvrLm2*/*Rlm2* and *AvrLm3*/*Rlm3* interactions are shown. Shading indicates the phenotype of winter oilseed rape plants associated with each interaction. Initially, there are susceptible (*light blue*) responses (leaf spots and stem cankers develop) on the host since there are no *R* gene products recognising the *Avr* effectors. Then when the corresponding *R* genes (e.g. *Rlm1* and *Rlm4*) are introduced into the host, *R* gene products recognise the effectors and there are resistant responses (*dark blue*). The pathogen then evolves to virulent alleles to avoid the recognition so there are susceptible responses on the host with the *R* genes. Since there are fitness costs of virulence (e.g. *avrLm1* and *avrLm4*), when the *R* genes are withdrawn from host cultivars there is selection for avirulent alleles (e.g. *AvrLm1* and *AvrLm4*). However, the epidemics are less severe (*pink*) since the virulent and avirulent alleles are co-existing and severity of epidemics depends on the proportion of virulent and avirulent alleles in the population. When avirulent alleles are again predominant in the population, the corresponding *R* gene can be redeployed (resistant response). By contrast, when there are no fitness costs of virulence (e.g. *avrLm2* and *avrLm3*), there is no selection for avirulent alleles and the corresponding *R* genes cannot be redeployed

durable than *Rlm1* when it is deployed in commercial cultivars (Fig. 6). This is supported by the evidence that the resistance gene *Rlm4* has been successfully used as a source of resistance for >30 years and is still present in >35% of current commercial French cultivars, and avirulent *AvrLm4* isolates are still present in the French *L. maculans* population (Balesdent et al. 2006; X Pinochet, CETIOM, France and MH Balesdent, unpublished data). However, the differences in the fitness cost of virulence between the *AvrLm1* and *AvrLm4* loci suggest there is a need to investigate fitness cost of virulence for each individual locus. The evidence of fitness costs at the *AvrLm1* and *AvrLm4* loci suggests that, when there is no selection pressure, the frequency of avirulent alleles will increase, probably more rapidly for the *AvrLm4* than for the *AvrLm1* locus. This suggests that, by contrast with *Rlm2* and *Rlm3,* the *R* genes *Rlm1* and *Rlm4* can be re-used when the corresponding avirulent isolates again become predominant in local *L. maculans* populations (Fig. 6). Therefore, information on pathogen fitness cost can be applied when developing strategies for deployment of *R* genes to increase their durability.

Acknowledgements We thank the European Union (SECURE, QLK5-CT-2002-01813), CETIOM, the UK Biotechnology and Biological Sciences Research Council (BBSRC, IPA project, BB/E001610/1), the Department for Environment, Food and Rural Affairs (Defra), the Chadacre Agricultural Trust, the British Council and the Royal Society for supporting the work. We thank Alan Todd and Rodger White for statistical analyses of the data, Michel Renard for supplying seed of Darmor and Eurol, M.

Chabirand for technical assistance, C. Montagnier for field experiment management at Versailles and J. N. Aubertot for weather data. We thank John Lucas for critical reading of the manuscript.

References

Attard, A., Gout, L., Gourgues, M., et al. (2002). Analysis of molecular markers genetically linked to the *Leptosphaeria maculans* avirulence gene *AvrLm1* in field populations indicates a highly conserved event leading to virulence on *Rlm1* genotypes. *Molecular Plant-Microbe Interactions, 15*, 672–682.

Bent, A. F., & Mackey, D. (2007). Elicitors, effectors, and *R* genes: the new paradigm and a lifetime supply of questions. *Annual Review of Phytopathology, 45*, 399–436.

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

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

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

Evans, N., Baierl, A., Semenov, M. A., Gladders, P., & Fitt, B. D. L. (2008). Range and severity of a plant disease increased by global warming. *Journal of the Royal Society Interface, 5*, 525–531.

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

Fitt, B. D. L., Hu, B. C., Li, Z. Q., et al. (2008). Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. *Plant Pathology, 57*, 652–664.

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

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

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

Gout, L., Kuhn, M. L., Vincenot, L., et al. (2007). Genome structure impacts molecular evolution at the *AvrLm1* avirulence locus of the plant pathogen *Leptosphaeria maculans*. *Environmental Microbiology, 9*, 2978–2992.

Huang, Y. J., Toscano-Underwood, C., Fitt, B. D. L., et al. (2001). Effects of temperature on germination and hyphal growth from ascospores of A-group and B-group *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *Annals of Applied Biology, 139*, 193–207.

Huang, Y. J., Fitt, B. D. L., Jedryczka, M., et al. (2005). Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans*) and Poland (*L. biglobosa*). *European Journal of Plant Pathology, 111*, 263–277.

Huang, Y. J., Evans, E., Li, Z. Q., et al. (2006a). Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist, 170*, 129–141.

Huang, Y. J., Li, Z. Q., Evans, N., Rouxel, T., Fitt, B. D. L., & Balesdent, M. H. (2006b). Fitness cost associated with loss of the *AvrLm4* avirulence function in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *European Journal of Plant Pathology, 114*, 77–89.

Huang, Y. J., Pirie, E. J., Evans, N., Delourme, R., King, G. J., & Fitt, B. D. L. (2009). Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). *Plant Pathology, 58*, 314–323.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature, 44*, 323–329.

Leach, J. E., Vera Cruz, C. M., Bai, J., & Leung, H. (2001). Pathogen fitness penalty as a predictor of durability of disease resistance genes. *Annual Review of Phytopathology, 39*, 187–224.

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*, 851–863.

Payne, R. W., Harding, S. A., Murray, D. A., et al. (2007). *The guide to GenStat release 10, part 2: statistics*. Oxford: VSN International. 1096 pages.

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

Pringle, A., & Taylor, J. W. (2002). The fitness of filamentous fungi. *Trends in Microbiology, 10*, 475–481.

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*, 871–881.

Sprague, S. J., Balesdent, M. H., Brun, H., et al. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence population of *Leptosphaeria maculans* in France and Australia. *European Journal of Plant Pathology, 114*, 33–40.

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.

Stergiopoulos, I., De Kock, M. J. D., Lindhout, P., & De Wit, P. J. G. M. (2007). Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals differ-

ent modes of adaptive evolution. *Molecular Plant-Microbe Interactions, 20*, 1271–1283.

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

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

West, J. S., Balesdent, M. H., Rouxel, T., et al. (2002). Colonisation of winter oilseed rape tissues by A/Tox^{+} and B/Tox^{0} *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology, 51*, 311–321.

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