Research Article

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Leptosphaeria maculans isolates with variations in *AvrLm1* and *AvrLm4* effector genes induce differences in defence responses but not in resistance phenotypes in cultivars carrying the *Rlm7* gene

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Abstract

BACKGROUND: The phoma stem canker pathogen *Leptosphaeria maculans* is one of the most widespread and devastating pathogens of oilseed rape (*Brassica napus*) in the world. Pathogen colonization is stopped by an interaction of a pathogen *Avr* effector gene with the corresponding host resistance (*R*) gene. While molecular mechanisms of this gene-for-gene interaction are being elucidated, understanding of effector function remains limited. The purpose of this study was to determine the action of *L. maculans* effector (*AvrLm*) genes on incompatible interactions triggered by *B. napus* noncorresponding *R* (*Rlm*) genes. Specifically, effects of *AvrLm4-7* and *AvrLm1* on *Rlm7*-mediated resistance were studied.

RESULTS: Although there was no major effect on symptom expression, induction of defence genes (e.g. *PR1*) and accumulation of reactive oxygen species was reduced when *B. napus* cv. Excel carrying *Rlm7* was challenged with a *L. maculans* isolate containing *AvrLm1* and a point mutation in *AvrLm4-7* (*AvrLm1*, *avrLm4-AvrLm7*) compared to an isolate lacking *AvrLm1* (*avrLm1*, *AvrLm4-AvrLm7*). *AvrLm7*-containing isolates, isogenic for presence or absence of *AvrLm1*, elicited similar symptoms on hosts with or without *Rlm7*, confirming results obtained with more genetically diverse isolates.

CONCLUSION: Careful phenotypic examination of isogenic *L. maculans* isolates and *B. napus* introgression lines demonstrated a lack of effect of *AvrLm1* on *Rlm7*-mediated resistance despite an apparent alteration of the *Rlm7*-dependent defence response using more diverse fungal isolates with differences in *AvrLm1* and *AvrLm4*. As deployment of *Rlm7* resistance in crop cultivars increases, other effectors need to be monitored because they may alter the predominance of *AvrLm7*.

Supporting information may be found in the online version of this article.

Keywords: apoplast; effector; gene expression; reactive oxygen species; *R* gene-mediated resistance; symptoms

1 INTRODUCTION

Leptosphaeria maculans (syn. *Plenodomus lingam*, anamorph: *Phoma lingam*) causes phoma stem canker of oilseed rape (*Brassica napus*) and annual yield losses of approximately US$1 billion globally.[1] The species name *L. maculans* is preferred here because the alternative species name has not been universally adopted.[2,3] This apoplastic fungal pathogen spreads disease through release of air-borne ascospores.[4,5] Once ascospores land on the surface of *B. napus* cotyledons or leaves, they germinate and subsequently penetrate the surface through stomatal pores.[6,7] Upon entry into the substomatal cavity, the pathogen colonizes the apoplastic space of the foliar mesophyll layer. Following a period of

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symptomless growth for more than a week, lesions with pycnidia, capable of releasing asexual spores, develop within 2 weeks after infection.[6] During incompatible interactions, single dominant *R* genes control foliar resistance by recognizing corresponding pathogen *Avr* genes (that encode effectors) to prevent pathogen growth from the leaf to the stem.[8,9] During compatible interactions, the pathogen continues asymptomatic colonization of the leaf petiole and subsequently the stem for months, before finally causing severe stem canker and yield loss.[10,11]

More than 19 *R* genes against *L. maculans* have been identified in *B. napus* and five of them have been cloned.[12–16] Conversely, the *L. maculans* genome contains >14 genes encoding effectors, 12 of which have been cloned, including *AvrLm1* and *AvrLm4-7*.[17–21] The number of putative effectors is far greater.[22] *Rlm1* and *LepR3* resistance genes, operating against *AvrLm1*, have been rendered ineffective in France and Australia, respectively;[23,24] this breakdown was caused by a decrease in frequencies of avirulent *AvrLm1* in *L. maculans* populations in both France and Australia.[23,25] Conversely, discontinuation of use of *Rlm1* or *LepR3* has resulted in an increase in *AvrLm1* frequencies, suggesting that there is a fitness penalty for the loss of this *AvrLm1* function, confirming previous studies.[25,26] Whereas *AvrLm4-7* and most other effector genes code for cysteine-rich peptides, *AvrLm1* encodes only a single Cys residue.[17] Recognition of AvrLm1 by LepR3 occurs outside the plant cell, not requiring the first 40 amino acids following the signal peptide.[27]

AvrLm1 triggers defence responses in *B. napus* cv. Columbus (carrying *Rlm1*), including salicylic acid (SA) and hydrogen peroxide (H_2O_2) production as well as induction of *ICS1*, a SA biosynthetic gene, *WRKY70* and *PR-1* expression.[28] Pathogen effectors are known to suppress innate immune responses, such as pattern-triggered immunity (PTI).[29] This may explain that *AvrLm4-7* transiently reduces SA production, *PR-1* expression and H_2O_2 accumulation during compatible interactions with susceptible *B. napus* cvs Eurol and ES Astrid.[30] While the AvrLm1 effector is recognized by the receptor-like protein (RLP) LepR3 outside the plant cell,[8] AvrLm1 interacts with the cytosolic signal transducer mitogen-activated protein kinase 9 (MPK9) of *B. napus* to increase host susceptibility.[31] Conversely, documented evidence for suppression of *R* gene-mediated resistance by noncorresponding pathogen effectors is less frequent.[32] In the case of *AvrLm5-9*, *AvrLm3* and *AvrLm4-7*, however, evidence for interference with effector recognition has been obtained; *AvrLm4-7* suppresses both *Rlm3*- and *Rlm9*-mediated resistance.[33,34] These types of epistatic interactions have altered crop protection and rotation strategies to adjust to *L. maculans* isolates carrying multiple *Avr* genes because a simple gene-for-gene model does not always determine the outcome of a particular host–pathogen interaction.[13]

The purpose of this study was to determine the action of *L. maculans* effectors on incompatible interactions triggered by noncorresponding *R* genes. Of particular interest were the effects of *AvrLm1* and *AvrLm4* on the defence responses mediated by the noncorresponding *R* gene *Rlm7*. Hence, the effects of *AvrLm4-7* and presence or absence of *AvrLm1* on *Rlm7*-mediated resistance were tested. Attention was given to effector-triggered defence (ETD) responses – defence-related gene expression and reactive oxygen species (ROS) production.[1] While *AvrLm1* had no effect on *Rlm7*-mediated resistance, *AvrLm4* had a subtle effect in enhancing ETD, although background effects of *L. maculans* isolates could not be excluded.

2 MATERIALS AND METHODS

2.1 Plant, pathogen growth and inoculation

Spring (Topas DH16516, Topas-*LepR3*, Topas-*Rlm4*, Topas-*Rlm7*) and winter (Capitol with *Rlm1* and Excel with *Rlm7*) oilseed rape cultivars/lines were used (Table 1). Seedlings were pre-germinated on filter paper and grown using a mixture of general-purpose compost (Miracle-Gro; Evergreen Garden Care, Cardiff, UK) and John Innes No 3 compost (J Arthur Bowers; Westland Horticulture Ltd, Huntingdon, UK) as described.[35] Controlled environment (CE) chambers (Fitoclima 1200; Aralab, Rio de Mouro, Portugal) were set to a 12 h:12 h, light: dark photoperiod with a light intensity of 250 µmol m^{-2} s^{-1}. Seedlings were grown for 10 days before inoculation.

Leptosphaeria maculans isolates were grown from conidial glycerol stocks stored at −80 °C. The isolates used were v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*) and v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*), derived from a single cross,[36] v29.3.1 (*avrLm1*, *avrLm4-AvrLm7*) and its transformed isogenic strains v29.3.1-T2 and v29.3.1-T3 (*AvrLm1*, *avrLm4-AvrLm7*),[17] and isolate 99–79 (*avrLm1*, *AvrLm2*, *AvrLm4-AvrLm7*)[11] (Table 1). Conidial suspensions were prepared from sporulating cultures on V8 agar.[37] A conidial suspension (10^5 mL^{-1}) was used to infiltrate the abaxial surface of cotyledons with a volume of 10 µL on both sides of the midrib. Inoculated seedlings were kept for 24 h in darkness at 100% humidity in CE chambers (Conviron, Isleham, USA) before re-establishing standard growth conditions.[35] To investigate gene expression, cotyledons were sampled and immediately frozen in liquid nitrogen (N_2) at the same time of the day as the inoculation occurred to avoid diurnal fluctuations.

Table 1. *Brassica napus* cultivars and *Leptosphaeria maculans* isolates used in this study

Cultivar/line or isolate	Description (*Rlm*/*AvrLm* genes)	Reference
B. napus		
Capitol	Winter oilseed rape; *Rlm1*	Gout *et al.* (2006)
Excel	Winter oilseed rape; *Rlm7*	Mitrousia *et al.* (2018)
Topas DH16516	Spring oilseed rape	Larkan *et al.* (2016)
Topas-*LepR3*	Introgression line with *LepR3*	Larkan *et al.* (2016)
Topas-*Rlm4*	Introgression line with *Rlm4*	Larkan *et al.* (2016)
Topas-*Rlm7*	Introgression line with *Rlm7*	Haddadi *et al.* (2022)
L. maculans		
v23.2.1	*avrLm1*, *avrLm2*, *AvrLm4-AvrLm7*, *AvrLm5*, *AvrLm6*, *AvrLm8*	Rouxel *et al.* (2003)
v23.11.9	*AvrLm1*, *avrLm2*, *avrLm4-AvrLm7*, *AvrLm5*, *AvrLm6*, *AvrLm8*	Balesdent *et al.* (2001)
v29.3.1	*avrLm1*, *AvrLm2*, *avrLm4-AvrLm7*	Gout *et al.* (2006)
v29.3.1-T2	*AvrLm1*, *AvrLm2*, *avrLm4-AvrLm7*	Gout *et al.* (2006)
v29.3.1-T3	*AvrLm1*, *AvrLm2*, *avrLm4-AvrLm7*	Gout *et al.* (2006)
99–79	*avrLm1*, *AvrLm2*, *AvrLm4-AvrLm7*	Mitrousia *et al.* (2018)

Seedlings also were wound-inoculated, following standard procedures.[7] For this purpose, plants were grown in a CE chamber (Conviron A1000) with a 16 h:8 h, light:dark photoperiod. Each cotyledon was marked, removing a small plug of leaf tissue with a sterile Pasteur pipet. Cotyledons then were wounded to the left and right of the midrib and inoculated with a droplet of conidial suspension (10^7 mL^{-1}). Incubation and further plant growth were as described. Disease was scored on a scale of 1 to 9 as reported previously.[11]

2.2 Phenotypic assessment and image analysis

A GXM XTC3A1 stereo microscope (GT Vision, Stansfield, UK) was used to obtain images of cotyledons with pycnidia. Numbers of pycnidia were determined using the Cell Counter Plugin for ImageJ;[38] two individuals counted pycnidia independently and a consensus was made. Lesions observed on the abaxial surface of cotyledons were photographed and analyzed using ImageJ.[38] All images were processed equally after conversion to 8-bit. A ruler was used to set a scale of 1 cm. A light background of 20 was used for background subtraction. Threshold was set from 0 to 225. Particles were analyzed from 100 to infinity; circularity was 0.4 to 1.0. Numbers of lesions per cotyledon were determined as was total lesion area per cotyledon; *F*-tests and Student's *t*-tests were used to determine statistically significant differences.

2.3 qPCR experiments for gene expression

Plant tissue was ground to a powder in liquid N_2 and total RNA was extracted using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). RNA was quantified using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was assessed using agarose gel electrophoresis.[39] RNA was treated with RNase-Free DNase (Promega, Madison, WI, USA) and reverse-transcribed using M-MLV RT (H-) Point Mutant (Promega) using oligo-dT_{16}-VN. Sequences of the primers to amplify *PR-1*, *PDF1.2*, *WRKY70* and actin transcripts are listed in Supporting Information, Table S1.

Calibration standards were generated by amplifying cDNA and purifying products. Yeast tRNA (Sigma-Aldrich) was used as a carrier to generate dilution series of PCR products. Each 96-well plate contained calibration standards and samples analysed for a specific experiment. A Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA, USA) was used for PCR amplification and analysis. Two-step PCR parameters were: 95 °C for 15 s, 60 °C for 1 min for 40 cycles. The Relative Standard Curve Method was used to quantify gene expression because gene-specific differences in amplification efficiencies were observed. A minimum of three biological and two technical replicates was used. For all analyses, the same gene-specific thresholds were used for separate experiments. Relative differences in expression were Log_2-transformed for statistical analysis; the Shapiro–Wilk test for normality and the Bartlett's test for homogeneity of variances were used before ANOVA; alternatively, Student's *t*-tests were used. Nonparametric tests also were done using the Wilcoxon rank sum exact test and the Kruskal–Wallis rank sum test.

2.4 Histochemistry

3,3'-Diaminobenzidine (DAB)- and *p*-nitroblue tetrazolium (NBT)-staining were used for detection of H_2O_2 and superoxide radicals, respectively, following published procedures[40] with some modifications. Following DAB staining, tissues were cleared with lactic acid/glycerol/ethanol (1/1/1, v/v/v). ImageJ was used to convert images to 8-bit. Background was subtracted using a rolling ball radius of 30 pixels. The threshold was adjusted from 0 to 220. Particles were analyzed with circularity from 0.2 to 1.0. Statistical analysis was done using ANOVA in Spss.

NBT-stained cotyledons were analyzed using ImageJ. Background was removed by adjusting the colour threshold to determine the total area of the cotyledons. Particles were analyzed with circularity values of 0.2 to 1.0. A colour threshold of 120 to 180, corresponding to blue colour, was applied to quantify the total area stained and subtract the green colour. Spss was used for statistical analysis using ANOVA.

3 RESULTS

3.1 Phenotypic analyses of resistance to *AvrLm1* isolates in *B. napus*

Leptosphaeria maculans isolates were used to differentiate the effects of *AvrLm1* and *AvrLm4-7* during incompatible interactions between *B. napus* genotypes and specific races of the pathogen (Table 1). The *L. maculans* isolates used were v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) and v29.3.1 (*avrLm1*, *avrLm4-AvrLm7*) without *AvrLm1* and v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*) and transformed v29.3.1-T2 and v29.3.1-T3 with *AvrLm1*. The Topas DH16516 line (simply referred to as Topas), with no known *R* genes against *L. maculans*, was compared to resistant cv. Capitol, carrying *Rlm1*, cv. Excel, carrying *Rlm7*, and introgression lines Topas-

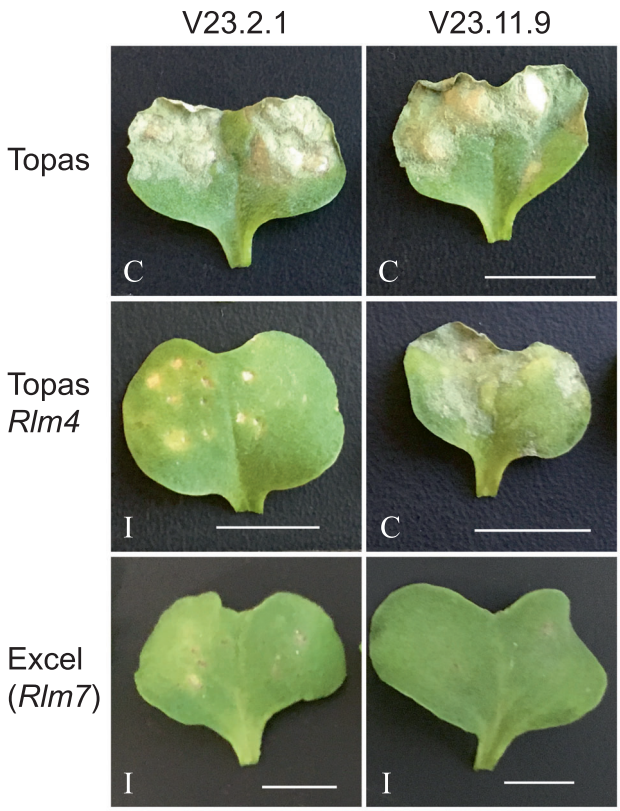

Figure 1. Phenotypic assessment of *Brassica napus* seedlings challenged with *Leptosphaeria maculans*. *L. maculans* isolates v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) and v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*) and host genotypes Topas, Topas-*Rlm4* and cv. Excel (with *Rlm7*) were used. Both cotyledons of each seedling were infiltrated on both sides of the midrib. For illustration, cotyledons with average phenotypes were selected from a total of 10 cotyledons. Compatible (C) and incompatible (I) interactions are indicated. Scale bars are 1 cm; the size of the upper left cotyledon was not determined.

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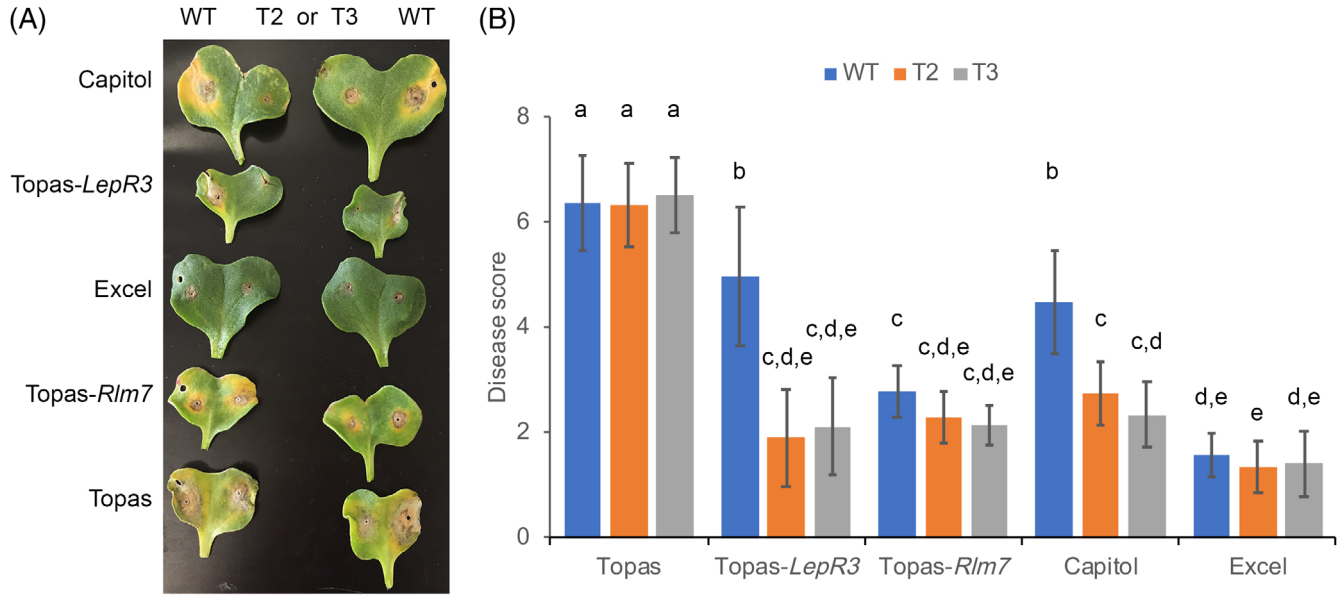

Figure 2. Phenotypic assessment of *Brassica napus* seedlings challenged with *Leptosphaeria maculans*. Isogenic *L. maculans* isolates used were v29.3.1 (*avrLm1*, *avrLm4-AvrLm7*) wild-type (WT) and isolates transformed with *AvrLm1*, v29.3.1-T2 (T2) and v29.3.1-T3 (T3); host genotypes used were Topas, Topas-*LepR3*, Topas-*Rlm7*, cv. Capitol (*Rlm1*) and cv. Excel (*Rlm7*). (A) Cotyledons of each seedling were wounded and inoculated with a droplet of conidia of WT, T2, T3 or WT at four sites. (B) Quantitative analysis of disease scores on a scale of 1 to 9. Means and standard deviations ($n > 7$) are indicated. Differences between means are based on Tukey's honestly significant difference with $\alpha < 0.05$; different letters indicate significant differences.

LepR3, Topas-*Rlm4* and Topas-*Rlm7*. Inoculation of susceptible Topas with isolates v23.11.9 or v23.2.1 resulted in spreading lesions (Fig. 1) and production of many pycnidia (Fig. S1). As predicted, Topas-*Rlm4* was resistant against isolate v23.2.1 but susceptible to v23.11.9 (Fig. 1). The incompatible interaction in Topas-*Rlm4* expressed itself on the adaxial leaf surface as discrete lesions (Fig. 1). The incompatible reaction of cv. Excel was less dramatic, resulting in resistance against both isolates v23.11.9 and v23.2.1 (Fig. 1). Despite incompatibility of cv. Excel against both v23.11.9 and v23.2.1, isolate-specific differences in size and frequencies of discrete lesions were observed (Fig. S2). Both frequency and size of discrete lesions on the abaxial leaf surface of cv. Excel were increased after infection with isolate v23.2.1 compared to isolate v23.11.9 (Fig. S2), but these isolate-specific effects were not statistically significant. Cotyledons subsequently were incubated under high humidity to determine the frequency of asexual sporulation. No significant differences between the incompatible interactions were observed regarding pycnidial numbers per inoculation or the frequency of asexual sporulation as determined by the production of cirrhi (Fig. S1). Thus, only subtle and not statistically significant phenotypic differences were observed between inoculations with isolates v23.2.1 and v23.11.9.

In order to determine whether these phenotypic findings were a result of using isolates with several genetic differences, isogenic isolates v29.3.1, v29.3.1-T2 and v29.3.1-T3 that differed only in a single effector gene as a result of transformation with *AvrLm1*[17] were used. The expression of symptoms [Fig. 2(A)] was consistent with that observed with genetically more diverse isolates (Fig. 1); both cv. Excel (with *Rlm7*) and Topas-*Rlm7* were resistant against isolates v29.3.1 (without *AvrLm1*) and v29.3.1-T2 and v29.3.1-T3 (with *AvrLm1*) [Fig. 2(B)]. Statistical analysis of all different host genotypes clearly showed that significant differences in effector-dependent resistance were observed only when Topas-*LepR3* and Capitol (*Rlm1*) were inoculated with isolates v29.3.1-T2 and v29.3.1-T3 that contained the corresponding *AvrLm1* effector (Fig. 2).

3.2 Isolate-dependent modulation of *Rlm7*-mediated defence gene expression

Induction of plant defence genes was studied 3, 5 and 7 days postinfiltration with pathogen (dpi) in Topas, introgression line Topas-*Rlm4* and cv. Excel (with *Rlm7*). Three separate experiments to test defence gene expression were done (Table S2).

Expression of neither *PR-1* nor *WRKY70* was elevated at 3 dpi in Topas and cv. Excel (Fig. S3). Although a significant increase in *PR-1* expression was observed at 5 dpi, expression of this defence gene increased still further at 7 dpi, particularly when Topas-*Rlm4* was challenged with *AvrLm4-AvrLm7*-containing isolate 99–79 (Fig. S4). *WRKY70* expression was significantly induced at 7 dpi when resistant cv. Excel was inoculated with isolate v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) but not with v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*) (Fig. S3). In separate experiments, *PR-1* expression was significantly increased in resistant cultivars at 7 dpi [Figs 3(A) and S3]. *PR-1* expression was particularly strongly induced in Topas-*Rlm4* when it was challenged with isolates containing the corresponding *AvrLm4-AvrLm7* gene [Figs 3(A) and S4]. Reproducibly, induction of *PR-1* expression was less when cv. Excel was challenged with v23.11.9 compared to v23.2.1, suggesting isolate-specific effects on the *Rlm7*-mediated defence response [Figs 3(a) and S3]. However, whereas induction of *PR-1* expression was less for v23.11.9 than for v23.2.1 when Topas-*Rlm4* was inoculated [Fig. 3(A)], *PR-1* expression was not significantly different when cotyledons of cv. Excel were inoculated with either isolate (Student's *t*-test, $P = 0.194$; Wilcoxon, $P = 0.229$). Analysis of actin and *PR-1* expression revealed cultivar- ($F_{2,31} = 22.608$, $P < 0.001$) and treatment-specific differences ($F_{3,31} = 9.423$, $P < 0.001$), respectively. No cultivar-dependent differences in *PR-1* expression were observed ($F_{2,31} = 1.510$, $P = 0.237$). Conversely, no treatment effects on actin expression were observed ($F_{2,31} = 0.722$, $P = 0.546$). Of note, although pathogen inoculation with either v23.11.9 or v23.2.1 did not induce *PDF1.2* expression at 7 dpi in Topas, Topas-*Rlm4* or cv. Excel, the expression of this gene was reduced when cv. Excel was challenged with v23.11.9 as compared to v23.2.1 [Fig. 3(B)].

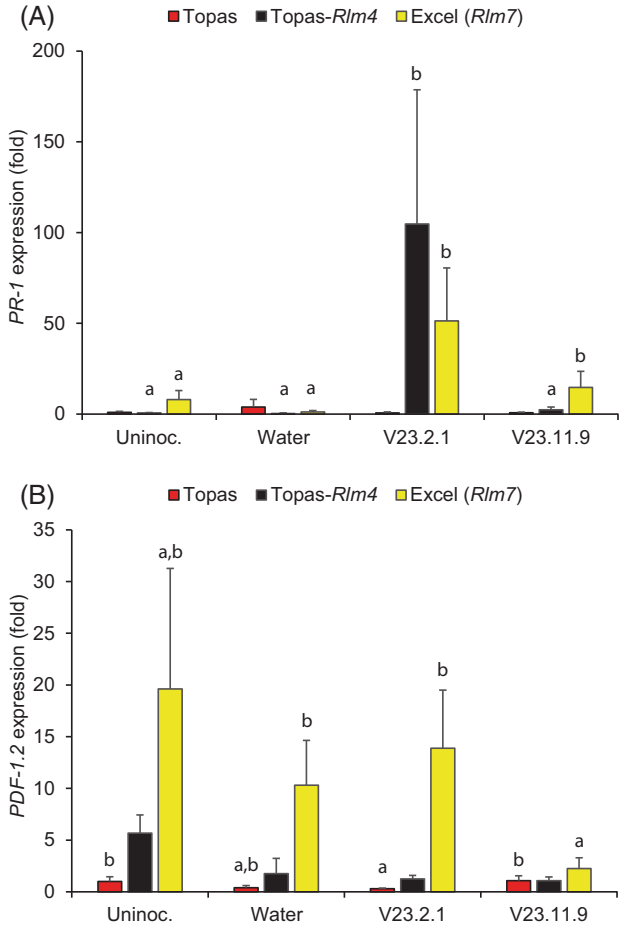

Figure 3. Defence-related gene expression in *Brassica napus* cotyledons challenged with *Leptosphaeria maculans*. RNA was extracted from cotyledons 7 days postinoculation for qPCR analysis. Topas, Topas-*Rlm4* and cv. Excel (*Rlm7*) were infiltrated with isolate v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) or v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*), mock-infiltrated with water or not inoculated (Uninoc.). Relative quantification was used to determine (A) *PR-1* or (B) *PDF1.2* expression relative to actin as a control. Means and standard errors are shown (n = 3 to 4). Significant differences in *PR-1* expression between pathogen- and water-inoculation within cultivars were detected after $\log_2$-transformation of relative expression values. A Student's t-test of $\log_2$-transformed data was used to determine significant differences in *PDF1.2* expression. Different letters within genotype comparisons illustrate statistically significant differences ($P < 0.05$).

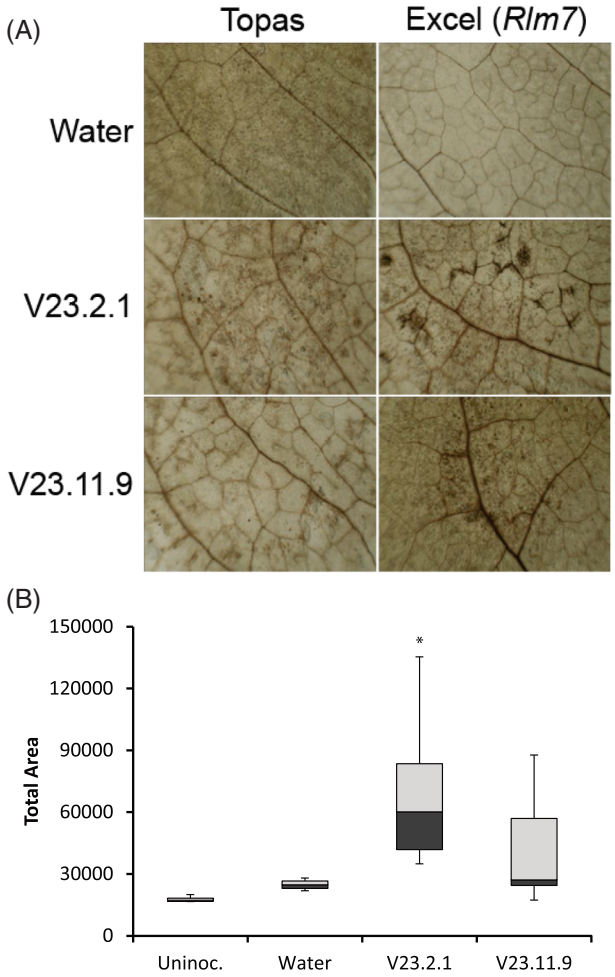

Figure 4. Diaminobenzidine staining of *Brassica napus* cotyledons challenged with *Leptosphaeria maculans*. (A) Micrographs of stained cotyledons at 7 days postinfiltration with water or *L. maculans* isolates v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) or v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*). (B) Box plot of the staining intensity in uninoculated, water- or *L. maculans*-challenged cotyledons of cv. Excel (*Rlm7*). ImageJ was used measure total area (pixels). Sample sizes for controls (n = 6) and pathogen treatments (n = 12) are given. Asterisk indicates statistical significance ($P < 0.05$).

Collectively, data from independent experiments provide evidence that isolate-specific differences in effector composition modulate the defence-related gene expression; *PDF1.2* expression differs between those isolates in cv. Excel carrying the *Rlm7* gene, whereas *PR-1* expression significantly differs only in Topas-*Rlm4* (Fig. 3).

3.3 Isolate-specific modulation of *Rlm7*-mediated reactive oxygen species production

Dyes were used to monitor the production of ROS after inoculation of *B. napus* cultivars with *L. maculans* isolates v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) or v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*). Little change in DAB staining, indicative of H_2O_2 production, occurred at 3 dpi in cotyledons of Topas or cv. Excel inoculated with either of these *L. maculans* isolates (Fig. S5). Likewise, only a minor increase in DAB staining was evident at 7 dpi of susceptible Topas with either *L. maculans* isolate [Figs 4(A) and S5]. A clearer indication of elevated H_2O_2 production was evident after inoculation with either isolate in stained cotyledon tissues of resistant cv. Excel [Fig. 4(A) and S5]. In the case of inoculation with *L. maculans* isolate v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*), a quantitatively significant increase in DAB staining was observed in cotyledons of cv. Excel at 7 dpi [Fig. 4(B)].

Infected cotyledons also were stained using NBT staining, indicative of superoxide formation. No changes in NBT staining were observed at 3 dpi with *L. maculans* but increases in NBT staining occurred in resistant cv. Excel at 7 dpi (Fig. S6). The largest increase in NBT staining was observed when cv. Excel was challenged with *L. maculans* isolate v23.2.1, but this suggestive increase in superoxide formation was not statistically significant. Together, however, these data imply that an oxidative burst occurs in response to *L. maculans* infection, especially when cv. Excel is challenged with *L. maculans* isolate v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*).

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Table 2. Interactions between pathogen isolates and host genotypes

Pathogen isolate	Avr genes	Host genotype	R genes	Interaction
v23.2.1	*avrLm1*, *AvrLm4-AvrLm7*	Topas	None	Compatible
v23.2.1	*avrLm1*, *AvrLm4-AvrLm7*	Topas-*Rlm4*	*Rlm4*	Incompatible
v23.2.1	*avrLm1*, *AvrLm4-AvrLm7*	Excel	*Rlm7*	Incompatible
v23.11.9	*AvrLm1*, *avrLm4-AvrLm7*	Topas	None	Compatible
v23.11.9	*AvrLm1*, *avrLm4-AvrLm7*	Topas-*Rlm4*	*Rlm4*	Compatible
v23.11.9	*AvrLm1*, *avrLm4-AvrLm7*	Excel	*Rlm7*	Incompatible
v29.3.1	*avrLm1*, *avrLm4-AvrLm7*	Topas	None	Compatible
v29.3.1	*avrLm1*, *avrLm4-AvrLm7*	Topas-*LepR3*	*LepR3*	Compatible
v29.3.1	*avrLm1*, *avrLm4-AvrLm7*	Topas-*Rlm7*	*Rlm7*	Incompatible
v29.3.1	*avrLm1*, *avrLm4-AvrLm7*	Capitol	*Rlm1*	Compatible
v29.3.1-T2 or T3	*AvrLm1*, *avrLm4-AvrLm7*	Topas	None	Compatible
v29.3.1-T2 or T3	*AvrLm1*, *avrLm4-AvrLm7*	Topas-*LepR3*	*LepR3*	Incompatible
v29.3.1-T2 or T3	*AvrLm1*, *avrLm4-AvrLm7*	Topas-*Rlm7*	*Rlm7*	Incompatible
v29.3.1-T2 or T3	*AvrLm1*, *avrLm4-AvrLm7*	Capitol	*Rlm1*	Incompatible
99–79	*avrLm1*, *AvrLm2*, *AvrLm4-AvrLm7*	Topas	None	Compatible
99–79	*avrLm1*, *AvrLm2*, *AvrLm4-AvrLm7*	Topas-*Rlm4*	*Rlm4*	Incompatible

4 DISCUSSION

Our results show that irrespective of use of the diverse isolates v23.2.1 (*avrLm1*, *AvrLm4-AvrLm7*) and v23.11.9 (*AvrLm1*, *avrLm4-AvrLm7*) or transgenic isolates expressing *AvrLm1* in the background of v29.3.1 (*avrLm1*, *avrLm4-AvrLm7*), no significant phenotypic differences in recognition by the noncorresponding receptor *Rlm7* were observed (Figs 1 and 2; Table 2). However, defence gene expression (e.g. *PR1*) and production of ROS were greater when cv. Excel was challenged with v23.2.1 rather than v23.11.9 (Figs 3 and 4); this could have been caused by differences in effector gene composition or genetic background effects (Table 3).

It is unlikely that *AvrLm1* would have been responsible for these differences in defence responses because isogenic isolates with or without this effector gene did not influence resistance phenotypes in cv. Excel or Topas-*Rlm7*. Nevertheless, it should be mentioned that transformation of *Arabidopsis thaliana* with *AvrLm1* reduced *PR1* expression *in planta*.[31] Consequently, *AvrLm1*-transformed *A. thaliana* plants were more susceptible to the virulent *Pseudomonas syringae* strain Pst DC3000.[31]

It is more likely that *AvrLm4* triggered a stronger defence response in cv. Excel to isolate v23.2.1 than to isolate v23.11.9. In this context, near-isogenic isolates with *AvrLm4* were more aggressive than *avrLm4* mutants on susceptible host cultivars lacking *Rlm4*, suggesting a fitness penalty for virulent strains.[41] However, a single amino acid change resulting in loss of *AvrLm4-7* recognition by *Rlm4*[18] maintained effector function of *avrLm4-AvrLm7* isolates; *avrLm4-AvrLm7* isolates also containing *AvrLm3* or *AvrLm5-9* were virulent on *B. napus* lines with *Rlm3* or *Rlm9*, respectively.[34,42] The subtle differences in defence gene expression and ROS production reported here also could be the result of a combination of effects based on differences in *AvrLm1* and *AvrLm4* effector composition. Alternatively, different genetic backgrounds of v23.2.1 and v23.11.9 could have been responsible for the observed differences in defence responses.

A reduction in *PDF1.2* expression was observed when the v23.11.9 isolate was used for infection of cv. Excel relative to isolate v23.2.1 [Fig. 3(B)]. The combination of jasmonic acid (JA) and ethylene regulates *PDF1.2* expression.[43,44] JA signalling was suppressed when *AvrLm1* was expressed in *A. thaliana*, although upregulation of JA signalling was observed when MPK9 was overexpressed in *B. napus*.[31]

Our results also show that ROS production is increased postinoculation with isolate v23.2.1 relative to v23.11.9 (Fig. 4). *AvrLm1* activates *PR1* expression, salicylic acid (SA) production and ROS accumulation during the incompatible interaction of *L. maculans* with cv. Columbus carrying the *Rlm1* gene.[30] Contrary to suppression of *PR1* expression by *AvrLm1*, activation of MPK9 leads to increased ROS production, cell death and disease susceptibility.[31] Our finding of less ROS production after inoculation with isolate v23.11.9 is therefore not in conflict with the finding that MPK9 increases ROS concentrations.[31] Although analysis of *Rlm7*-dependent defence responses with isogenic isolates differing in *AvrLm1* composition would be desirable, this was not justified because no phenotypic differences in disease resistance were observed (Fig. 2).

Alteration of host plant defence responses by noncorresponding effectors, such as *AvrLm1* on *Rlm7*-mediated resistance, may be of concern to the plant-breeding industry, considering the increased use of oilseed rape cultivars containing the *Rlm7* gene. In addition to *AvrLm7*, it is recommended to monitor other effector genes in *L. maculans* populations to avoid breakdown of *Rlm7* resistance. We recently noted that a reduction of the *AvrLm7* allele in *L. maculans* populations was correlated with a corresponding increase of the *AvrLm1* allele in *L. maculans* populations.[45,46] Disease management may therefore require development of *LepR3* cultivars to compensate for the decline in *Rlm7*-mediated resistance or use of different *R* genes for crop rotation.[13] Increases in *AvrLm1* have been observed previously after deployment of corresponding *LepR3*-mediated resistance was suspended in Australian cultivars[25]; both *AvrLm1* and *AvrLm4* incur fitness costs.[26,41] As a note of caution, because the correlations to previous studies were not strong (Table 3), different genetic backgrounds used for analysis of defence responses may have contributed to

Table 3. Comparison of different studies on the effects of *AvrLm1* and *AvrLm4* in enhancing susceptibility to or triggering resistance against *Leptosphaeria maculans* in susceptible and resistant hosts

	SA pathway			ET/JA pathway		JA pathway						
	PR1	*WRKY70*	SA	*PDF1.2*	*HEL, CHI*	JA	*LOX2*	*AOS*	H2O2	Susceptibility	Interaction	Reference
avrLm1,AvrLm4	Increase	Increase		Increase	N/A*	N/A	N/A	N/A	Increase	Level	Incompatible	This study
AvrLm1,avrLm4	Decrease	N/A	N/A	Decrease	N/A	N/A	N/A	N/A	Decrease	Level	Incompatible	This study
AvrLm1	Increase	Increase	Increase	N/A	Increase	Level	N/A	N/A	Increase	Decrease	Incompatible	Sasek *et al.*, 2012
AvrLm1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Compatible	Sasek *et al.*, 2012
AvrLm1 OE At†	Decrease	N/A	N/A	N/A	N/A	N/A	Decrease	Decrease	N/A	Increase	Heterologous system	Ma *et al.*, 2018
AvrLm1 transient Nb†	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Heterologous system	Ma *et al.*, 2018
BnMPK9 OE Bn†	Decrease	Decrease	N/A	N/A	N/A	N/A	N/A	Increase	Increase	Increase	Compatible	Ma *et al.*, 2018
AvrLm4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Increase	Compatible	Huang *et al.*, 2006
AvrLm4	Decrease	N/A	Decrease	N/A	Decrease	Level	N/A	N/A	Decrease	Increase	Compatible	Novakova *et al.*, 2016

*Not available.
† *Arabidopsis thaliana* (At), *Nicotiana benthamiana* (Nb), *Brassica napus* (Bn), overexpression (OE), transient expression (transient).

the observed molecular changes in isolates varying in *AvrLm1* and *AvrLm4*.

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HUS, AMA, LRL, MSR, YJH and GKM contributed to experimental work. HUS statistically analysed the data and drafted the manuscript. YJH assisted with experimental design and manuscript revision. BDLF obtained some of the funding and assisted with writing the manuscript. The authors consent to the data policy of the journal.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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