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A new avirulence gene of *Leptosphaeria maculans*, AvrLm14, identifies a resistance source in American broccoli (*Brassica oleracea*) genotypes

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Abstract

In many cultivated crops, sources of resistance to diseases are sparse and rely on introgression from wild relatives. Agricultural crops often are allopolyploids resulting from interspecific crosses between related species, which are sources of diversity for resistance genes. This is the case for *Brassica napus* (oilseed rape, canola), an interspecific hybrid between *Brassica rapa* (turnip) and *Brassica oleracea* (cabbage). *B. napus* has a narrow genetic basis and few effective resistance genes against stem canker (blackleg) disease, caused by the fungus *Leptosphaeria maculans*, are currently available. *B. rapa* diversity has proven to be a valuable source of resistance (*Rlm*, *LepR*) genes, while *B. oleracea* genotypes were mostly considered susceptible. Here we identified a new resistance source in *B. oleracea* genotypes from America, potentially effective against French *L. maculans* isolates under both controlled and field conditions. Genetic analysis of fungal avirulence and subsequent cloning and validation identified a new avirulence gene termed *AvrLm14* and suggested a typical gene-for-gene interaction between *AvrLm14* and the postulated *Rlm14* gene. *AvrLm14* shares all the usual characteristics of *L. maculans* avirulence genes: it is hosted in a genomic region enriched in transposable elements and heterochromatin marks H3K9me3, its expression is repressed during vegetative growth but shows a strong overexpression 5–9 days following cotyledon infection, and it encodes a small secreted protein enriched in cysteine residues with few matches in databases. Similar to the previously cloned *AvrLm10-A*, *AvrLm14* contributes to reduce lesion size on susceptible cotyledons, pointing to a complex interplay between effectors promoting or reducing lesion development.

KEYWORDS

Brassica napus, gene-for-gene, resistance introgression, stem canker

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1 | INTRODUCTION

Crops of agronomic importance are submitted to multiple pathogen challenges, threatening global food security. Whenever enough genetic variation is available, genetic improvement of crops is the most reliable, least expensive and most environment-friendly disease management strategy. However, the use of major gene resistance obeying the gene-for-gene postulate often exerts a strong selection pressure on pathogen populations whenever the corresponding avirulence gene is prevalent in populations, leading to possible “breakdown” of the new resistance source (Lo Presti et al., 2015; Rouxel & Balesdent, 2017). Moreover, it is now thought that a single mutation in one avirulence gene may allow the pathogen to defeat several resistance genes simultaneously, and some complex gene-for-gene systems rely on recognition specificities that can be masked by other avirulence genes (Plissonneau et al., 2017). This indicates that one strategy to hamper resistance gene breakdown is a temporal or geographic alternating of resistance sources exerting sequential nonredundant pressure on populations. The counterpart of the deployment of such strategies is the need for a given crop to have a diversified portfolio of major *R* genes to develop informed strategies to use in a perspective of durability (Brown, 2015).

During the 20th century, breeding for resistance in nearly every crop species resulted in deployment of major disease-resistance genes. Interspecific and intergeneric hybridizations are performed between cultivated species and between cultivated species and their wild relatives, allowing introgression of resistance traits from sexually compatible wild relatives (Katche et al., 2019; Stam & McDonald, 2018). Each parent species can harbour different resistance genes and resynthesizing interspecific hybrids is thus a way to diversify resistance sources. Agricultural crops often are allopolyploids resulting from interspecific crosses between related species (e.g., wheat, oilseed rape, brown mustard, tobacco, triticale) (Mason & Batley, 2015). *Brassica napus* (oilseed rape, OSR, canola) is a natural interspecific hybrid between two diploid *Brassica* species, *B. rapa* (turnip, Chinese cabbage, etc.) and *B. oleracea* (cabbages) (U, 1935). Being of recent origin, *B. napus* is known to have a narrow genetic basis and low diversity of resistance genes, while both parents show a remarkable diversity of morphotypes and genetic diversity (Girke et al., 2012). In Brassicaceae crop breeding programmes, the resistance gene diversity in parent diploid species can be exploited to develop new cultivars with biotic and abiotic stress resistance (Katche et al., 2019).

One of the main diseases of brassica crops is stem canker (blackleg), caused by the ascomycete *Leptosphaeria maculans* (Rouxel & Balesdent, 2005). The fungus develops typical gene-for-gene interaction with its host and most sources of resistance available (*Rlm* genes) originate from *B. rapa* or from other related brassicas such as brown mustard (*B. juncea*) or black mustard (*B. nigra*). In contrast, *B. oleracea* is known to be highly susceptible to the disease, with few resistance sources from this gene pool identified to date. For example, an analysis of 392 accessions of *B. oleracea* from diverse geographic origins and with different morphotypes showed most

of these were extremely susceptible to four *L. maculans* isolates (Dilmaghani et al., 2012). Only six accessions showed moderate resistance against two or three of the isolates. More recently, a survey of 32 Korean cabbage germplasms identified two resistant lines against two *L. maculans* isolates without investigating the genetic control of resistance (Robin et al., 2017).

Here we analyse a small group of genotypes of *B. oleracea* grown in Mexico and identify a novel resistance source both in controlled conditions when inoculated with isolates obtained from *B. napus* in Europe and when the cultivars are grown in the field. Genetic analyses on the fungal side show avirulence is due to a single gene genetically independent from all currently identified *AvrLm* genes and termed *AvrLm14*. Candidate-based and map-based cloning of *AvrLm14* point to a typical avirulence effector gene of *L. maculans* in terms of gene structure, regulation, and genome environment. This first identification and characterization of an avirulence gene towards *B. oleracea* in *L. maculans* may be indicative of the occurrence of a single-gene matching major resistance in broccoli and shows that *B. oleracea* diversity can also be a resource to consider to diversify the resistance genes portfolio used in *B. napus*.

2 | RESULTS

2.1 | A new resistance found in American broccoli genotypes

Six American broccoli cultivars, Domador, Ironman, Marathon, Monaco, Neptuno, and Tlaloc, were found in a preliminary screening to show resistance responses when inoculated with two isolates of *L. maculans* obtained from oilseed rape, v23.1.2 and PHW1223 (data not shown; see Table 1 for a detailed description of the isolates). All these cultivars were previously shown to be highly susceptible to Mexican isolates of *L. maculans*, typically belonging to race Av1-2-4-5-6-7-(8), suggesting these cultivars are devoid of the cognate resistance genes *Rlm1/LepR3*, *Rlm2*, *Rlm4*, *Rlm5*, *Rlm6*, and *Rlm7* (Dilmaghani et al., 2012). To refine these data by including identification of *Rlm3* and *Rlm9*, an additional round of screening was done with a set of eight differential isolates developed at INRAE (Table 1). The expected differential responses were expressed when using control oilseed rape genotypes (e.g., expression of the *Rlm4* resistance towards isolate v23.2.1 only by cv. Jet Neuf) (Figure 1), while, with the main exception of cv. Ironman, all broccoli cvs expressed a moderate but nondifferential resistance response to all eight isolates (Figure 1). This was expressed as a larger than usual symptom with patches of dark necrotic responses and no or sparse sporulation (scoring around “3” in Figure 1) similar to what was described for the *AvrLm1-Rlm1* interaction (Ansan-Melayah et al., 1995) but differing from the <1 mm hypersensitive response (HR) observed in typical gene-for-gene interactions such as the *AvrLm4-Rlm4* or the *AvrLm7-Rlm7* interactions (typical scoring “1” in Figure 1). The highest level of resistance was observed for cv. Monaco, while it was much less effective for cv. Ironman, showing complete susceptibility to isolate

TABLE 1 List and characteristics of *Leptosphaeria maculans* field or laboratory isolates used in this study

Isolate ID	Country of origin	Origin ^a	Year of isolation	Host ^b	Other name	Race ^c	Interaction phenotype in Table/Figure	Reference ^d
BBA62908	Germany	?	1966	<i>Beta vulgaris</i> ?		Av1-2-4-5-6-7-8	Figure S1	[1]
IBCN74	France	F	1974 ?	Cabbage	PHW1245	Av1-2-4-5-6-7-8	Figure S1, Table 4	[1]
a.2	France	F	1990	OSR		Av1-5-6-7-8	Table 4	[2]
H5	France	F	1990	OSR		Av5-6-7-8	Table 4	[1]
Nz-T4* ^e	New Zealand	F	?	Swede	T	Av5-6-8	Table 4	[1]
PHW1223*	Australia	F	?	OSR		Av5-6-8-9	Figure 1, Table 4	[1]
IBCN14	Australia	F	1988	OSR	MD2	Av5-6	Figure S1, Table 4	[1]
IBCN17	Australia	F	1988	OSR	C13	Av4-5-6-7-8	Table 4	[1]
IBCN18	Australia	F	1988	OSR	M1	Av1-2-4-7-(8)	Figure S1, Table 4	[1]
IBCN56*	Canada	F	1992	OSR	BJ-121	Av1-2-3-5-6-9	Figure 1	[1]
IBCN80	Canada	F	1985	OSR	Leroy	Av1-2-3-5-6-9	Figure S1	[1]
WT50	Australia	F	2004	OSR		Av5-7-(8)	Figure S1	[4]
OMR13	Mexico	F	2008	Cabbage		Av1-2-4-(5)-6-7-(8)	Figure S1	[3]
OMR19	Mexico	F	2008	Cabbage		Av1-2-4-5-6-7-(8)	Figure 2, Table 4	[3]
OMR21	Mexico	F	2008	Cabbage		Av1-2-4-(5)-6-7-(8)	Table 3, Table 4	[3]
19.4.24*		L				Av3-5-6-8	Figure 1	[5]
19.2.1		L				Av3-5-6-8	Table 4	[5]
v23.2.1*		L				Av4-5-6-7-8	Figure 1	[6]
v23.1.2*		L			JN2	Av5-6-7-8	Figure 1, Figure 2, Figure S1, Table 3, Table 4	[5, 6]
v23.1.3		L			JN3	Av1-4-5-6-7-8	Table 4	[5, 6]
v23.1.11		L			JN11	Av1-4-5-6-7-8	Table 4	[5, 6]
v23.11.9*		L				Av1-5-6-7-8	Figure 1	[5,6]
V34.18.12*		L				Av2-(5)-7-(8)	Figure 1	[5]
m1-401 – m3-409	France	F	2003	OSR (Mohican)		Single-conidium field population (9 isolates)	Figure S1	[7]
m2-6xx – m10-6xx	France	F	2004	OSR (Mohican)		Single-ascospore field population (78 isolates)	Figure S1	[7]

^aF, Field single-ascospore isolate (single conidium isolates for the m1-401-m3-409 population); L, laboratory isolate originating from in vitro crosses.

^bHost from which the isolate was obtained; swede, *Brassica napus rapifera*; cabbage, *Brassica oleracea*; OSR, oilseed rape (*Brassica napus oleifera*).

^cRaces are defined as the combination of avirulent alleles at the *AvrLm1* to *AvrLm9* loci. Avx-y-z indicates avirulence towards resistance genes *Rlmx*, *Rlmy*, and *Rlmz*. Figures in parentheses indicate missing data at a given avirulence locus.

^d[1] Balesdent et al. (2005); [2] Gall et al. (1994); [3] Dilmaghani et al. (2012); [4] Dilmaghani et al. (2009); [5] Balesdent et al. (2002); [6] Balesdent et al. (2001); [7] Huang et al. (2006).

^eIsolates marked with an asterisk are those used in Figure 1 to search for known resistance gene in broccoli genotypes.

v34.18.12 and moderate but variable susceptibility to four other isolates (Figure 1). Lastly, focusing only on cvs Monaco and Ironman we investigated whether this new resistance could operate against an 87-isolate field population collected in France in the growing season 2003/2004 (Huang et al., 2006). Using the cotyledon-inoculation test, control isolates behaved as expected: European or Australian isolates v23.1.2 (JN2), IBCN18, and WT50 (Table 1) were avirulent while the Mexican isolate obtained from *B. oleracea*, OMR13, was

highly virulent towards the two broccoli genotypes (Figure S1). Four other isolates, IBCN14, IBCN80 and the oldest available isolates in collections, BBA62908 and IBCN74 (both Av1-2-4-5-6-7-8), were also fully or moderately virulent towards the two broccoli genotypes (Figure S1). In the field population, nearly all isolates were avirulent towards the two broccoli genotypes and mostly induced HRs on cv. Monaco. However, two field isolates were moderately or highly virulent towards broccolis, with one showing virulence similar to that

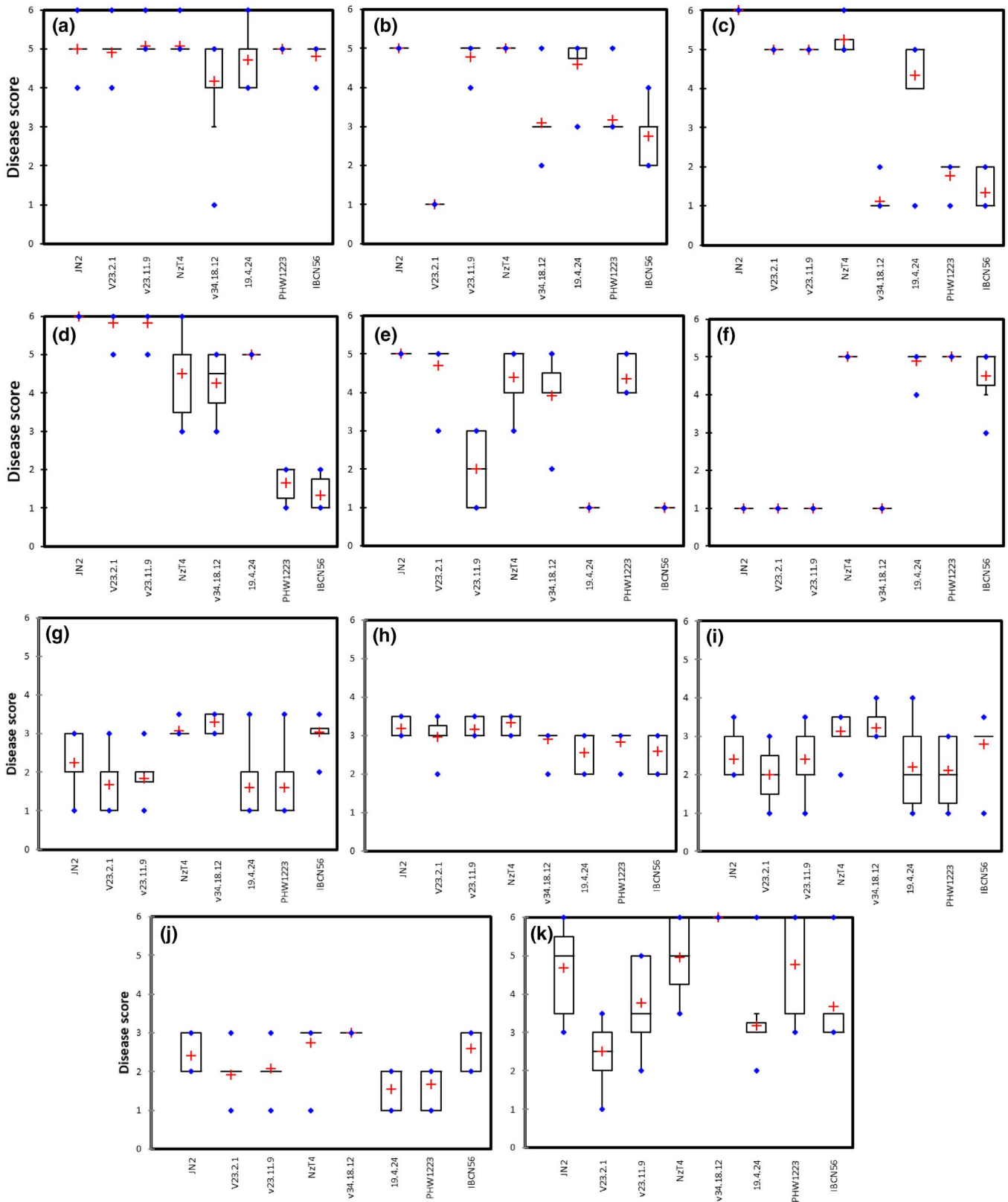


FIGURE 1 Box plot representation of interaction phenotypes of a set of differential isolates with known avirulence genes with a series of oilseed rape (OSR) cultivars and five Mexican broccoli genotypes. The differential OSR set consists of Westar (susceptible control, no *Rlm* gene) (a), Jet Neuf (*Rlm4*) (b), Bristol (*Rlm2* & *Rlm9*) (c), Goéland (*Rlm9*) (d), Columbus (*Rlm1* & *Rlm3*) (e), and O1.23.2.1 (*Rlm7*) (f). The five Mexican cultivars of *B. oleracea* are Neptuno (g), Tlaloc (h), Marathon (i), Monaco (j), and Ironman (k). Spore suspensions were deposited on wounded cotyledons, and symptoms were scored at 14 days postinoculation (dpi) and 18 dpi. Symptom expression at 18 dpi is displayed (except for O1.23.2.1, 14 dpi) using a semiquantitative 1 (avirulent) to 6 (virulent) rating scale in which scores 1–3 represent different levels of resistance (from typical hypersensitive response [HR] to delayed resistance) and 4–6 represent different levels of susceptibility. For each box, the red cross indicates the average scoring (10–12 plants per inoculation point, repeated twice) while the black horizontal line in the rectangle indicates the median scoring; the rectangles comprise 75% of the rating scores

of the most virulent isolates available in the collection and isolated from *B. oleracea*, OMR13 and IBCN74 (Figure S1).

2.2 | The new resistance is effective in the field in France

To assess the effectiveness of this new resistance source under French field conditions, both Monaco and Ironman were included in a field experimental setting alongside a susceptible OSR control, Goéland and new putative sources of resistance introgressed in OSR, such as *Rlm10* originating from black mustard, *B. nigra* (Petit-Houdenot et al., 2019). Field experiments were established for two consecutive years in up to eight locations in France and leaves with symptoms were collected in autumn for isolation and analysis of fungal isolates (Table 2). Mostly, typical greyish leaf symptoms were recovered from Goéland, leading to the isolation of a vast majority of *L. maculans* isolates. In contrast, fewer and less typical symptoms were recovered from leaves of the FU-Eurol-*Rlm10*, Ironman, and Monaco genotypes, leading to the isolation of a majority of isolates of another *Leptosphaeria* species, *L. biglobosa*, unaffected by *Rlm* genes from *Brassica* species (Table 2). Moreover, the ratio of *L. biglobosa*:*L. maculans* was much higher when isolated from the broccoli genotypes compared to FU-Eurol-*Rlm10* (Table 2), substantiating the scarcity of typical symptoms that can be obtained from broccoli genotypes. While more than half of the *L. maculans* isolates recovered from FU-Eurol-*Rlm10* were virulent towards the never used before *Rlm10* resistance, only one isolate fully virulent towards the novel *B. oleracea* resistance could be recovered (Table 2).

Overall, these data indicate that none of the known resistance genes (*Rlm1*–*Rlm9*) are present in American genotypes of broccoli included in this study and that a new resistance source is present in these genotypes. This resistance is effective when faced with French field populations of *L. maculans* but virulence is present in a small ratio of field isolates. The fact that isolates virulent towards the new resistance source are already present in French populations isolated from OSR could be indicative of the occurrence of a typical gene-for-gene interaction and thus that the *B. oleracea* genotypes harbour a single-gene major resistance, tentatively named *Rlm14*.

2.3 | A single-gene control and genetic independence from known avirulence genes

Genetic control of resistance can easily be approximated by characterization of the genetic control of avirulence on the fungal side. Two crosses were undertaken between Mexican virulent isolates (OMR19, OMR21) and European isolates showing avirulence towards broccoli genotypes (Figure 2), cross 66 (OMR21 × v23.1.2; 87 progeny) and cross 67 (OMR19 × v23.1.3; 79 progeny). Cross 66 involved two isolates differing at loci *AvrLm1*, *AvrLm2*, and *AvrLm4*, and was primarily used to evaluate genetic linkage between these loci and the putative

TABLE 2 Characterization of *Leptosphaeria maculans* isolates obtained from field experiments containing new resistant material (*Rlm10* from *Brassica nigra* in a *B. napus* background and the new resistance source from *B. oleracea* described in this study)

Growing season	2008/2009					2009/2010					Total 2008–2010				
	Number (%) of isolates recovered					Number (%) of isolates recovered					Number (%) of isolates recovered				
	Total	Lm ^e	Lb ^e	nd ^e	vir ^a	Total	Lm	Lb	nd	vir	Total	Lm	Lb	nd	vir
Ironman (2008/2009) Monaco (2009/2010)	74	45 (61%)	29 (39%)	0	1 (2%)	28	2 (7%)	25 (89%)	1 (4%)	1 ^b (50%)	102	47 (46%)	54 (53%)	1	2 (4%)
FU-Eurol- <i>Rlm10</i> (<i>Rlm10</i>)	57	51 (89.5%)	4 (7%)	2 (3.5%)	36 (70.6%)	49	39 (80%)	10 (20%)	0	10 (26%)	106	90 (85%)	14 (13%)	2	46 (51%)
Goéland (<i>Rlm9</i>)	101	99 (98%)	2 (2%)	0	nd	66	66 (100%)	0	0	nd	167	165 (99%)	2 (1%)	0	nd

^aThe isolates recovered from leaf symptoms were classified as *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) following PCR amplification of the rDNA internal transcribed spacer (ITS) and Mat loci; nd, the isolate did not belong to any of the species using this criterion or was a mix of both species.

^bNumber (%) of *L. maculans* isolates showing virulence when inoculated on cotyledons of genotypes with novel resistance sources (*Rlm10* or the resistant *B. oleracea*).

^cThe isolate showed an intermediate behaviour when inoculated on cotyledons of Monaco.

avirulence gene (*AvrLm14*) towards broccolis. The progeny was thus assessed on control differential OSR genotypes (Westar: no *R* gene, Columbus: *Rlm1*, Bristol: *Rlm2*, and Jet Neuf: *Rlm4*) and on broccoli cv. Monaco. The progeny was assessed for segregation of avirulence and recombination of avirulence phenotypes between pairs of avirulence genes. All four avirulence genes, including the putative *AvrLm14*, showed a segregation ratio consistent with a single-gene control [*AvrLm1*: 45:42 A:V, $p(\chi^2) = .748$; *AvrLm2*: 40:47 A:V, $p(\chi^2) = .453$; *AvrLm4*: 41:45 A:V, $p(\chi^2) = .670$; avirulence towards Monaco: 50:37 A:V, $p(\chi^2) = .163$; $p(\chi^2) < .001$ if considering a two-gene control]. The high number of recombinant genotypes in the progeny (Table 3) indicated genetic independence between *AvrLm14* and all three other avirulence genes. Cross 67, involving the reference sequenced isolate v23.1.3, was then used for genetic mapping of the new avirulence locus.

2.4 | Identification and characterization of *AvrLm14* points to a typical *AvrLm* gene

To identify the candidate effector gene encoding *AvrLm14*, we firstly undertook an a priori strategy involving systematic PCR amplification of 43 of the genes encoding putative effectors whose repertoire has been established in Rouxel et al. (2011) on a series of isolates showing avirulence or virulence towards broccoli genotypes. Only five of the genes of the repertoire showed complete lack of amplification in the two parental isolates virulent towards broccolis, OMR19 and OMR21 (Table 4). Of these, four genes were also lacking in isolate IBCN74, found here to be virulent towards Monaco and Ironman (Table 4 and Figure S1). In addition, one of the candidates, Lema_T200070, was not predicted in the new version of the genome annotation (Dutreux et al., 2018) due to lack of transcriptomic support. Among the three remaining candidates, only the

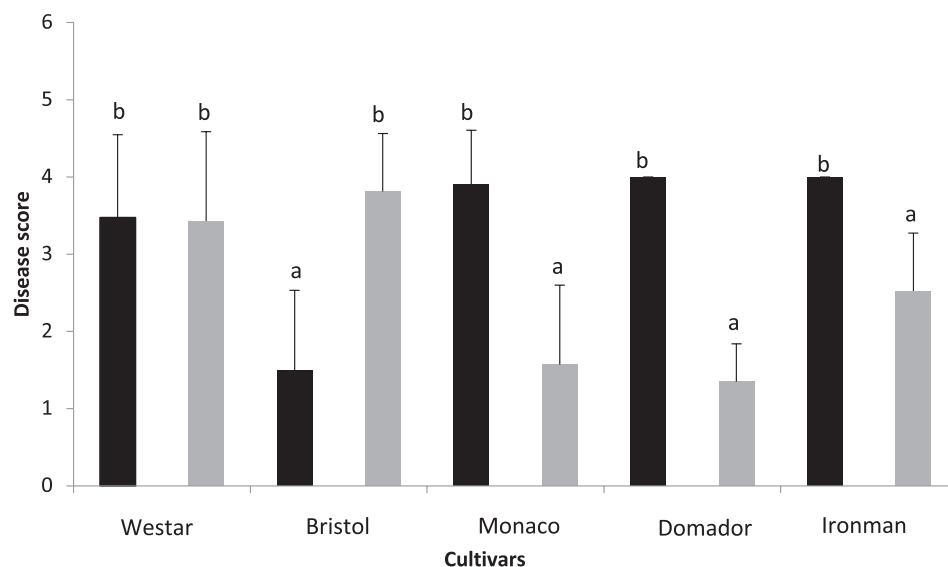


FIGURE 2 Interaction phenotypes of parent *Leptosphaeria maculans* isolates OMR19 (black bars) and JN2 (grey bars) on cotyledons of control oilseed rape genotypes (Westar [no *Rlm* gene] and Bristol [*Rlm2*, *Rlm9*]), and three broccoli cultivars, Monaco, Domador, and Ironman. Symptoms were scored at 13 days postinoculation on a 1–6 scale (Balesdent et al., 2005), with scores 1–3 and 4–6 corresponding to avirulent and virulent phenotypes, respectively. Each bar represents the average (+ SE) of 14–22 inoculation points per interaction and data with different letters are significantly different (Kruskal–Wallis test: $p < .0001$)

TABLE 3 Segregation analysis of interaction phenotypes in progeny of cross #66 shows genetic independence between *AvrLm14* and *AvrLm1*, *AvrLm2*, or *AvrLm4*

	Parental phenotype 1 ^c	Parental phenotype 2 ^a	Recombinant phenotype 1	Recombinant phenotype 2	$p(\chi^2)^b$
<i>AvrLm14</i> vs. <i>AvrLm1</i>	21 (<i>AvrLm14</i> - <i>avrLm1</i>) ^c	18 (<i>avrLm14</i> - <i>AvrLm1</i>)	28 (<i>AvrLm14</i> - <i>AvrLm1</i>)	20 (<i>avrLm14</i> - <i>avrLm1</i>)	.456 ns
<i>AvrLm14</i> vs. <i>AvrLm2</i>	21 (<i>AvrLm14</i> - <i>avrLm2</i>)	19 (<i>avrLm14</i> - <i>AvrLm2</i>)	26 (<i>AvrLm14</i> - <i>AvrLm2</i>)	21 (<i>avrLm14</i> - <i>avrLm2</i>)	.746 ns
<i>AvrLm14</i> vs. <i>AvrLm4</i>	28 (<i>AvrLm14</i> - <i>avrLm4</i>)	18 (<i>avrLm14</i> - <i>AvrLm4</i>)	22 (<i>AvrLm14</i> - <i>AvrLm4</i>)	18 (<i>avrLm14</i> - <i>avrLm4</i>)	.374 ns

^aIsolate v23.1.2, showing virulence at loci *AvrLm1*, *AvrLm2*, and *AvrLm4*, and avirulence at locus *AvrLm14*.

^bIsolate OMR21, showing avirulence at loci *AvrLm1*, *AvrLm2*, and *AvrLm4*, and virulence at locus *AvrLm14*.

^cProbability associated to the χ^2 test for independent segregation (25% of the progeny in each phenotypic class); ns, not significant.

^d*AvrLm*, avirulent phenotype; *avrLm*, virulent phenotype.

lack of amplification of *Lm_JN3_05547* (formerly *Lema_P004720*) fully cosegregated with the virulence phenotype on Monaco in cross 67. Additional genetic mapping based on minisatellite markers generated from the genomic sequence of the supercontig hosting the candidate gene (Bally et al., 2010) delineated a 11.3 cM genetic interval bordered by markers SSR209 and mpb0.69 (Table S1) that covered 547 kb and encompassed 54 predicted genes. This region mostly corresponded to a 399 kb AT-rich isochore (Figure S2). Six genes predicted as encoding putative secreted proteins were identified among the 54 genes, of which only one, the candidate gene *Lmb_JN3_05547*, showed a 0.984 probability to be an effector according to the EffectorP prediction tool (Sperschneider et al., 2018), and was located as a solo-gene in the middle of the AT-rich isochore. Exploitation of RNA-Seq data (Dutreux et al., 2018) pointed to expression kinetics typical of known avirulence genes for this candidate, with a low level of expression in axenic conditions and a strong overexpression upon cotyledon infection 5–9 days postinoculation (dpi) followed by a drastically reduced expression level at later time points (Figure 3). In addition to its location within a large transposable element (TE)-rich genome region, examination of data generated by Soyer et al. (2021) also showed that *Lmb_JN3_05547* was strongly enriched in the heterochromatin mark H3K9me3. RACE-PCR was used to confirm the improved gene annotation obtained in Dutreux et al. (2018) and refine it. It showed that *Lmb_JN3_05547* is a mono-exonic 405-bp gene, with a 131-bp 5' untranslated region (UTR) encompassing a 50 bp intron and a 30 or 107-bp 3' UTR. The gene encoded a 134 amino acid protein encompassing a 17 amino acid signal peptide. The mature protein contained four cysteine residues (Figure S2). The predicted protein had no Blast hit with sequences available in public databases, including

in closely related species such as *L. biglobosa* or other closely related Dothideomycetes.

Complementation of an *avrLm14* isolate with *Lmb_JN3_05547* indicated that 13 out of the 15 complemented isolates had a restored *AvrLm14* phenotype and that the two isolates that were still virulent had no expression of the transgene (data not shown).

As a complementary approach, 14 transformants were recovered following RNAi silencing of the candidate *AvrLm14* in the reference isolate v23.1.3. All of these transformants remained fully virulent on the control oilseed rape cv. Westar (data not shown). On Monaco, isolates showing a low level of silencing of *Lmb_JN3_05547* (residual expression of 60% or higher compared to the avirulent control) still displayed an avirulent phenotype (more than 90% of the inoculation sites), while transformants with a residual expression of *Lmb_JN3_05547* lower than 52% of that of the control displayed virulent phenotypes on a majority of inoculation sites (Figure 4a). Moreover, the two field isolates found to be virulent on Ironman and Monaco (Figure S1) displayed no expression of *Lmb_JN3_05547*, although they contained the corresponding gene (data not shown). All these data agree to confirm that *Lmb_JN3_05547* is *AvrLm14*.

2.5 | Fitness analyses indicate *AvrLm14* restricts expansion of lesion size on cotyledons

Based on the residual expression level of *AvrLm14* in RNAi transformants and their pathogenicity on Monaco, these transformants were classified into two groups, that is, silenced or not (Figure 4). Each of these 14 RNAi transformant was inoculated onto two susceptible *B. napus* varieties, Aviso and ES Astrid, and cotyledon lesion

FIGURE 3 Kinetics of expression of the candidate *AvrLm14*, *Lmb_JN3_05547* (red line) following inoculation of cotyledons of a susceptible oilseed rape cultivar, Darmor-Bzh, at a range of days postinoculation (dpi). This includes the RNA-Seq data described in Dutreux et al. (2018) and Gay et al. (2021), and encompasses the kinetics of three other avirulence genes, *AvrLm2* (green), *AvrLm4-7* (black), and *AvrLm6* (blue) for comparison. Data are $\log_2(\text{RPKM} + 1)$ transformed expression data, scaled to expression of all genes in the genome. Germinating conidia in axenic medium were used as a reference

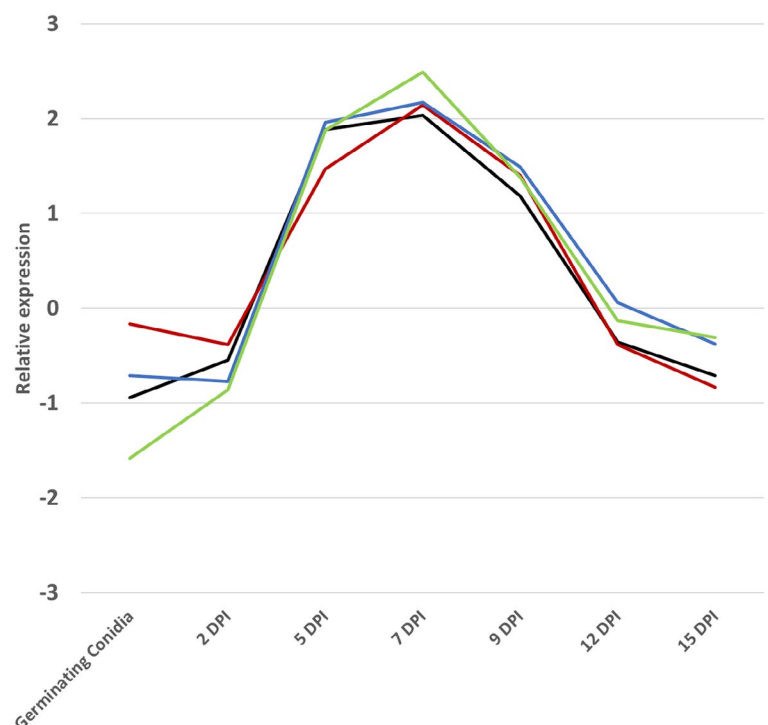


TABLE 4 PCR amplification of the repertoire of effector genes (encoding small secreted proteins, SSPs) identifies five candidates with lack of amplification in the two isolates virulent towards *Brassica oleracea* genotypes OMR19 and OMR 21

	v1 genome ^d annotation	v2 genome annotation	a.2 ^a	H5	v23.1.11	v23.1.2 (JN2)	v23.1.3 (JN3)	OMR19	OMR21	Nz-T4	PHW1223	19.2.1	IBC14	IBC17	IBC18	IBC174
SSP00_4	Lema_T200070	Missing	- ^b	+	+	+	+	-	+	+	+	+	+	+	+/-	-
SSP00_5	Lema_P004720	Lmb_JN3_05547	+	+	+	+	+	-	+	+	+	+	+	+	+	-
SSP05_7	Lema_T200330	Lmb_JN3_09746	-	+	+	+	+	-	-	-	+	+	+	+	+	-
SSP13_1	Lema_T200460	Lmb_JN3_01018	+	+	+	+	+	-	+	+	+	+	-	+	+	-
SSP18_1	Lema_T200500	Lmb_JN3_12622	+	+	+	+	+	-	+	+	+	+	+	+	+	+

^av1 genome annotation according to Rouxel et al. (2011) has been moved to v2 following Nanopore resequencing and RNA-Seq-based annotation of isolate JN3 (Dutreux et al., 2018).

^bDescriptions of isolates are in Table 1. Only isolates OMR19, OMR21, and IBCN74 are virulent towards *B. oleracea* genotypes Ironman and Monaco.

^c+, presence of the expected PCR product; -, lack of PCR amplification.

areas were measured at 12 dpi. Analysis of variance (ANOVA) of mean size of cotyledon lesion indicated no effect of the cultivar on the lesion size ($p = .802$), while the silencing had a significant effect ($p = .015$); the inactivation of *AvrLm14* not only suppressed the avirulence on Monaco (Figure 4a), but led to an increase of cotyledon lesion size on susceptible cultivars (Figure 4b). In addition, this effect functioned in a quantitative manner because the cotyledon lesion size was negatively correlated to the level of residual expression of *AvrLm14* (Spearman test, $p = .017$; Figure 4b).

3 | DISCUSSION

Control of the stem canker disease of *Brassica* strongly depends on the use of resistance genes in the host, at least in part of continental Europe, where no fungicides are used against *L. maculans*. Due to limited genetic resources in *B. napus*, this led in the past to a series of boom-and-bust cycles during which one new effective resistance gene was introduced and widely used on large acreages, exerting a very high selection pressure on fungal populations and eventually leading to a shift from avirulence to virulence in populations and ineffectiveness of the resistance (Rouxel & Balesdent, 2017). Here, we identified a novel resistance source in *B. oleracea* and cloned the corresponding avirulence gene, *AvrLm14*. Without having performed genetic analyses on the plant side, the identification of a single avirulence gene in the fungus strongly suggests the plant-pathogen interaction obeys a canonical gene-for-gene relationship involving one resistance gene in the plant, responsible for resistance to avirulent populations of the fungus. Similar postulates have been done multiple times ever since we cloned the first avirulence gene in *L. maculans* and have always proven to be the rule (e.g., Ansan-Melayah et al., 1998). In this respect the relatively easy cloning of fungal avirulence genes provides us with an efficient tool to identify corresponding single-gene resistance sources in the host.

The *Rlm14* resistance speculated here seems to be effective against a large proportion of *L. maculans* French field populations both when using a collection of isolates in controlled conditions and in field settings under diverse pedoclimatic conditions. However, virulent isolates preexist at a low level in collections (one fully virulent isolate out of 87 field isolate collections) and in field populations (one fully virulent isolate obtained from leaf symptoms). Preexistence of virulent isolates towards a new resistance source has been observed for nearly all *Rlm* genes introduced in France when data were available on population structure prior to the use of the resistance gene (e.g., estimated frequencies of *avrLm7* isolates ranging from 0.006% to 1.3% at Versailles prior to the use of *Rlm7*, or 3.2% of virulent field isolates towards the never used *Rlm11*; Balesdent et al., 2013; Daverdin et al., 2012). This is attributable to the conjunction of large effective pathogen population size, hosting of *AvrLm* genes in dispensable regions of the genome along with the importance of sexual reproduction in the fungal life cycle that maximizes the ability to continuously and locally generate nonconventional inactivating

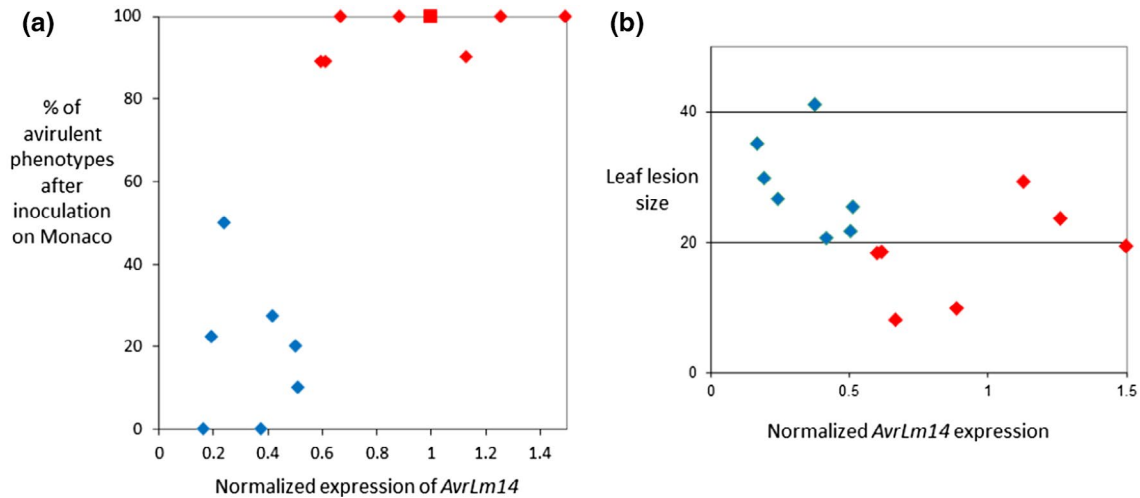


FIGURE 4 RNAi silencing of the expression of *Lmb_JN3_05547* abolishes avirulence towards the *Brassica oleracea* cultivar Monaco but increases lesion size on susceptible *B. napus* varieties Aviso and ES Astrid. (a) Expression of avirulence towards cotyledons of Monaco, when inoculated with the silenced transformants. Data were obtained 15 days postinoculation (dpi) as percentage of resistance responses induced by the isolate and correspond to two independent inoculation tests with 10–12 plant/isolate combination per replicate. The wild-type (WT) avirulent control, JN3, is shown as a red square while JN3 isolates transformed with the silencing construct are represented as diamonds, with blue diamonds corresponding to virulent transformants. (b) The relationship between the level of RNAi silencing of *AvrLm14* and the lesion size on cotyledons of *B. napus* 'Aviso' and 'ES Astrid'. Each dot corresponds to one isolate inoculated on the two susceptible varieties Aviso and ES-Astrid using 10–12 inoculation points per isolate per plant variety, and two independent experiments. At 12 dpi, cotyledons were scanned to measure lesion areas. The transformed isolates are represented as diamonds, with blue diamonds corresponding to isolates showing less than 50% residual expression of *AvrLm14*. For both (a) and (b), the relative expression level was estimated following quantitative reverse transcription PCR (RT-qPCR) from RNA extracted from infected cotyledons, 7 dpi (the time of peak expression of the *L. maculans* effector genes). Expression of *Lmb_JN3_05547* in the silenced isolates is relative to *Actin*. The data correspond to two biological and two technical replicates

mutations in *AvrLm* genes (Daverdin et al., 2012; Rouxel & Balesdent, 2017).

In our collections, one of the two oldest isolates was obtained in Europe from cabbage while the other was obtained from an uncertain host species. Both have the same *AvrLm* gene complement as modern Mexican isolates obtained from *B. oleracea* and are virulent towards *Rlm14*. We previously postulated a North American origin of *L. maculans*, partly because the first recorded damaging epidemics of the disease at the beginning of the 20th century were on cabbage in North America (Dilmaghani et al., 2012). *B. napus* being a crop of recent origin (and being widely used since beginning of the 1960s in France), our data support at least a host shift of *L. maculans* populations originating from *B. oleracea* to get adapted to oilseed rape, which probably necessitated the loss of many avirulence effectors detrimental to be a pathogen of oilseed rape and in contrast the gain of new effectors such as *AvrLm14*, which are detrimental to infect *B. oleracea* but may help infecting oilseed rape.

The relationships between cultivated *Brassica* species have been elucidated in the 1930s, in a relationship known as U's triangle (U, 1935). The diploid species *B. rapa* (turnip, AA), *B. nigra* (black mustard, BB), and *B. oleracea* (cabbages, CC) are natural progenitors of the allopolyploid species *B. juncea* (Indian mustard, AABB), *B. napus* (oilseed rape, AACC), and *B. carinata* (Abyssinian mustard, BBCC). *B. napus* is a species with very low diversity due to its recent origin (7,500 years or less) and to a bottleneck effect associated with

intensive selection for low erucic acid and low glucosinolate content traits (Katche et al., 2019). In addition, there is no natural source of variation because *B. napus* is not found in nature as a wild type. All resistance genes of *B. napus* currently used in commercial varieties (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, *Rlm9*) are located on the A genome of *B. napus* (Lv et al., 2020) or have been introgressed from *B. rapa* into *B. napus* (*LepR1*, *LepR2*, *LepR3*, Yu et al., 2005, 2008; *Rlm11*, Balesdent et al., 2013; *RlmS*, Neik et al., 2020). Most of these, except *Rlm3* to date, have also been found in genotypes of *B. rapa* (Leflon et al., 2007), as well as other resistance genes, *Rlm8* (Balesdent et al., 2002) and *LepR1* (Yu et al., 2005). This suggests that *B. rapa* was the main provider of resistance genes in *B. napus*. In contrast, and while the *B. oleracea* genome (CC) shares a high ancestral synteny with the A genome of *B. rapa* (Chalhoub et al., 2014), suggesting that functional homologues of A genome resistance genes could be found in the C genome, no currently known resistance gene against *L. maculans* is located on the C genome of *B. napus* (Lv et al., 2020). In this respect, the few studies having screened *B. oleracea* genetic resources, and having identified lines showing moderate to high resistance did not further investigate the genetic control of the resistance to conclude on whether major genes for resistance may be present in *B. oleracea* (Dilmaghani et al., 2012; Robin et al., 2017). Without additional information at this stage, we can only speculate on why the A genome harbours so many resistances to *L. maculans* compared to the C genome. The first reason could be a biased interpretation due

to difficulties in correctly interpreting incompatibility phenotypes in the C versus the A genome; actually, many of the common *Rlm* genes induce HR-type, easy to score resistance responses, while intermediate responses are found for *B. oleracea* genotypes. In this respect, the moderately resistant lines of *B. oleracea* identified by Dilmaghani et al. (2012) have not been investigated further. Bayer et al. (2019) suggested that domestication was accompanied by impoverishment in resistance gene analogs (RGAs) in *B. oleracea* compared to the ancestral species *B. macrocarpa*, which displays resistance to pathogens such as *Sclerotinia sclerotiorum* lost in domesticated *Brassica* species. One last speculation could be linked to diversification of genes in the A and C genomes of *B. napus* compared to those of its progenitors. Fu et al. (2019) showed a high 87.1% level of conservation of RGAs of the nucleotide binding site-leucine rich repeat (NBS-LRR) class in the A genome between *B. napus* and *B. rapa* while the ratio was much lower (66.4%) for the C genome between *B. napus* and *B. oleracea* due to expansion of genes of this family in the C genome of *B. napus*. One important way to increase the gene pool of diversity in terms of resistance sources is to exploit the diversity of related mustard species *B. nigra*, *B. juncea*, and *B. carinata*, all sharing the B genome, absent from *B. napus*, and being famed to harbour complete resistance or nonhost resistance to *L. maculans*. Roy (1984) introgressed resistance gene *Rlm6* from *B. juncea* into *B. napus*. Similarly, Chevre et al. (1996) generated a resistant *B. napus* line harbouring one chromosome of *B. nigra* that was later shown to contain the new *Rlm10* gene (Petit-Houdenot et al., 2019). More recently, similar works were performed to introduce one chromosome of *B. carinata* into *B. napus*, allowing the generation of plants resistant to *L. maculans* (Navabi et al., 2010). To date, none of these works resulted in commercial *B. napus* varieties harbouring the new effective resistance. This illustrates that, while the mustards are promising sources of new resistance to exploit, there still are difficulties in performing interspecific hybridization between *B. napus* and the different mustard species to make this the easiest way to generate *B. napus* varieties with improved resistance (Mason & Batley, 2015). In contrast, multiple successes were found to resynthesize allopolyploid *B. napus* by crossing *B. rapa* and *B. oleracea* to increase the pool of available diversity (Katche et al., 2019). Our finding that not only *B. rapa*, but also *B. oleracea* may harbour diversified sources of resistance justifies a continuous genebank screening effort to identify and exploit both *B. rapa* and *B. oleracea* resources. This approach via resynthesized *B. napus* should provide new *B. napus* resources with new *Rlm* genes while interspecific hybridization with mustards should in the long term allow introgression of additional *Rlm* genes. Both these approaches are needed to obtain the diversified pool of *Rlm* genes introgressed into *B. napus* to design adequate strategies for durable management of resistance sources.

The cloning of the 10th avirulence gene (*AvrLm14*) in *L. maculans* illustrates a remarkable identity of traits among all currently cloned *AvrLm* genes, even though they share no sequence identity and are scattered throughout the genome, often on distinct chromosomes. As noticed when having cloned the first avirulence gene of *L. maculans* more than a decade ago (Gout et al., 2006), all of them are

“lost in the middle of nowhere” genes isolated in large AT-rich blocks of degenerated transposable elements (for a review, see Rouxel & Balesdent, 2017). In relation to this genome location we also found recently that all *AvrLm* genes are enriched in H3K9me3 (trimethylation of lysine 9 of histone H3) marks, usually regarded as characteristic of constitutive heterochromatin in other organisms (Soyer et al., 2021). Being associated with this repressive mark, none of the *AvrLm* genes are expressed during vegetative growth (Soyer et al., 2021), but all of them show the same kinetics of expression when the fungus is inoculated onto cotyledons of *B. napus*, with a strong peak of overexpression 5–9 days postinoculation before symptoms are expressed (Figure 3) (Gay et al., 2021). Additionally, all of them encode small secreted proteins, often but not always enriched in cysteine residues, and showing no or few matches in databases, no recognizable domain, and no postulated function. The 10 known *AvrLm* genes are expressed together with tens of other putative effectors (Gay et al., 2021; Gervais et al., 2017; Haddadi et al., 2016). Generally, the quantity of effectors produced at the same time in the same place raises the question of why so many effectors are produced together. This led to the postulate of functional redundancy substantiating the dispensability of avirulence effectors under resistance gene selection (Lo Presti et al., 2015). The data obtained on *AvrLm14*, along with those previously obtained for *AvrLm1*, *AvrLm3*, *AvrLm4-7*, or *AvrLm10A*, showed that functional inactivation or silencing of *AvrLm* genes often results in an observable phenotype, suggesting additive effects rather than redundancy. In many cases, and irrespective of the approach used, *L. maculans AvrLm* genes were first postulated to promote development of leaf lesions. This was shown when comparing near-isogenic lines of the fungus for *AvrLm1* and *AvrLm4-7*, by functional complementation for *AvrLm4-7*, and following RNAi silencing for *AvrLm3* (Huang et al., 2006, 2010; Novakova et al., 2016; Plissonneau et al., 2017). In contrast to *AvrLm1*, *AvrLm3*, or *AvrLm4-7*, two other *AvrLm* genes, *AvrLm10A* (Petit-Houdenot et al., 2019) and *AvrLm14* (this study) have been found, following RNAi silencing experiments, to limit the expansion of the lesions. This suggests the existence of a trade-off between a series of effectors that are produced at the same time with antagonistic effects on lesion size to eventually control the expansion of the leaf lesion. Such fine tuning is likely to be important for infection of oilseed rape, because it is known that leaf lesions supporting asexual sporulation play a minor role in the fungal life cycle due to the low infectivity of pynidiospores and the low importance of secondary infection compared to continuous infection by ascospores under European field conditions (Rouxel & Balesdent, 2005). As postulated by Petit-Houdenot et al. (2019), it seems to make sense to restrict leaf lesion development to prevent early leaf senescence that would compromise the ability of *L. maculans* to reach the leaf vascular tissue in time and eventually the stem, where sexual reproduction takes place. Interestingly, the ability of *AvrLm14* to contribute to restriction of lesion expansion is consistent with its absence in isolates obtained from *B. oleracea*, either in Mexico or in the oldest isolates of the collection, because the pathogenic cycle of *L. maculans* on cabbages is different from that on OSR and mostly relies on asexual sporulation on leaf lesions

with little evidence of systemic colonization of stems to cause stem canker (Dilmaghani et al., 2013). Thus it appears that the production of a mix of effectors promoting or restricting lesion size development provides the fungus with a highly flexible template to adapt to different host species and different pathogenic strategies depending on whether it relies on asexual sporulation for short-cycle cultivation practices such as those used for *B. oleracea* in Mexico or canola in western Canada (Dilmaghani et al., 2012) or rather on sexual reproduction necessitating a lengthy colonization of stem tissues for long-cycle hosts such as winter OSR in Europe.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal material

Isolates used in this study are described in Table 1. Briefly, a series of reference isolates included in the International Blackleg of Crucifer Network collection (IBCN), collected between 1966 and 2004, and for which the spectrum of avirulence gene they contain (mostly described in Balesdent et al., 2005) were used for characterization of genotypes using cotyledon-inoculation tests. OMRx isolates are reference virulent isolates towards Mexican broccoli genotypes. Isolates 19.x.xx, v23.xx.x, and v34.xx.x are genetically bred isolates to generate combinations of avirulence genes unavailable in field isolates. They encompass isolate v23.1.3 (JN3), whose genome sequence is the reference genome sequence for *L. maculans* (Dutreux et al., 2018) and its sister isolate, v23.1.2 (JN2). Fungal cultures were maintained and conidia were produced as previously described (Ansan-Melayah et al., 1995). To examine genetic linkage between *AvrLm1*, *AvrLm2*, *AvrLm4-7*, and *AvrLm14*, cross #66 was performed between isolate OMR21 and isolate v23.1.2. To map *AvrLm14*, a segregating population was built following an in vitro cross (#67) between isolates OMR19 and the reference v23.1.3. In vitro crosses and recovery of random ascospore progeny were done as previously established (Plissonneau et al., 2016).

4.2 | Plant genotypes and inoculation tests

B. napus cultivars and lines used for cotyledon-inoculation tests or field experiments included Westar (control genotype with no *Rlm* gene), Columbus (*Rlm1*, *Rlm3*), Bristol (*Rlm2*, *Rlm9*), Jet Neuf (*Rlm4*), Goéland (*Rlm9*), 01.23.2.1 (*Rlm7*), 00.22.1.1 (*Rlm3*) (Balesdent et al., 2005), Aviso (*Rlm9*), ES Astrid (*Rlm9*), and FU-Eurol-74 (*Rlm10*) (Petit-Houdenot et al., 2019). American broccoli cultivars used were Domador, Ironman, Marathon, Monaco, Neptuno, and Tlaloc. All isolates were phenotyped for their virulence spectrum using a cotyledon-inoculation test. Cotyledons of 10- to 12-day-old seedlings were puncture-inoculated with 10 μ l of inoculum (10^7 pycnidiospores/ml [Balesdent et al., 2001]). Spore suspensions of each isolate were inoculated on 10–12 plants of each of the *B. napus* differentials

and *B. oleracea* cultivars. Symptoms were scored 12–18 dpi using a semiquantitative 1 (avirulent) to 6 (virulent) rating scale in which scores 1–3 represent different levels of resistance (from typical HR to delayed resistance) and 4–6 represent different levels of susceptibility (mainly evaluated by the intensity of sporulation on lesions; Balesdent et al., 2005).

For RNA-Seq analyses, inoculations were done on cv. Darmor-*bzh* as described previously in Dutreux et al. (2018) and samples were recovered at days 2, 5, 7, 9, 12, and 15 dpi. At each time point, eight cotyledons from eight different plants were randomly selected. The plant tissues around the inoculation site were cut with a 10 mm diameter disposable punch and the 16 corresponding samples were pooled together in a sterile Falcon tube, immediately frozen in liquid nitrogen and stored at -80°C until extraction. At each time point, two replicates were recovered and the whole experiment was repeated once.

For aggressiveness studies, isolates were inoculated onto the two susceptible varieties Aviso and ES-Astrid using 10–12 inoculation points per isolate per plant variety. At 12 dpi, cotyledons were scanned to measure lesion areas using DPlan4Lab software (<http://4lab.smyslzivota.cz/DPlan/>). Experiments were repeated twice. Median lesion areas were calculated and the data were subjected to ANOVA and to nonparametric correlation tests (Spearman correlation) using XLSTAT 2013.4.03 (Addinsoft).

4.3 | Field experiments

For evaluation of the effectiveness of the resistance source found in broccoli genotypes, field experiments were set up for two growing seasons in eight locations with contrasting agroclimatic environments: En Crambade and Mondonville (south France), Rennes (Brittany, west France), Versailles, Grignon and Verneuil l'Etang (Ile-de-France region), Rozières and Blois (central France, a region with high acreages of OSR). In France, OSR is usually sown late August to spend winter under a rosette stage. Ascospore infection mostly takes place in autumn (October–December) and leaf symptoms are mostly expressed during this period. To synchronize growth stages between OSR and broccoli genotypes, and to maximize the chances of submission to a wide ascospore shower at the time when plants are the most susceptible to the disease (i.e., before the 6-leaf stage), seeds were presown in individual pots in the greenhouse on 15 September and 15 October, and then transplanted in the field at the 2-leaf stage on 15 October and 15 November, respectively. Development of symptoms was assessed weekly and symptomatic leaves were collected to obtain single pycnidium isolates from leaf spots as described in Balesdent et al. (2006).

4.4 | Genetic mapping

Generation of minisatellite markers and primers for mapping and PCR amplification were done as reported in Bally et al. (2010). On cross #67, linkage analysis among loci was performed using MapMaker/

EXP v. 3.0b software (available online; Whitehead/MIT Center for Genome Research, Cambridge, MA, USA) with a logarithm of odds (LOD) score of 3.0 and a maximum recombination frequency of 20 cM.

4.5 | DNA and RNA manipulations

For PCR, genomic DNA was extracted from conidia with the DNeasy 96 Plant Kit (Qiagen) as already described (Daverdin et al., 2012). Standard PCRs were performed in an Mastercycler EP gradient thermocycler (Eppendorf) as described in Daverdin et al. (2012). Sequencing was performed using a CEQ 8000 automated sequencer (Beckman Coulter) according to the manufacturer's instructions.

Total RNA was extracted from infected cotyledons of *B. napus* or *B. oleracea* genotypes using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Total RNA was treated with RNase-free DNase I (New England Biolabs).

4.6 | Gene annotation

Gene annotation was firstly based on the RNA-Seq data from Dutreux et al. (2018). Annotation of 5' and 3' UTRs, transcription start and stop sites, and intron positions were then confirmed following PCR amplification and sequencing of 3'- and 5'-ends of cDNA using the SMARTer RACE cDNA Amplification kit (Clontech) according to the manufacturer's recommendation and using primers AvrLm14-F, AvrLm14-R, and AvrLm14-int1F (Table S1).

4.7 | Expression analyses using RNA-Seq and RT-qPCR

For RNA-Seq, protocols are described in Dutreux et al. (2018). For the RT-qPCR experiments, all RNA samples were adjusted to 3 µg of RNA and single-strand cDNA was generated using oligo(dT)-primed reverse transcription with PrimerScript reverse transcriptase (TaKaRa) according to the manufacturer's protocol. Samples were analysed from a 10-fold dilution of the original RT products as described by Fudal et al. (2007). Quantitative PCR was performed using a 7900 fast real-time PCR system (Applied Biosystems) and ABsolute SYBR Green ROX dUTP mix (ThermoScientific). To evaluate the expression profile of *AvrLm14* in complemented isolates, C_t values were analysed according to the method described by Muller et al. (2002). In silencing assays, the residual expression of *AvrLm14*, compared to those of the wild-type isolate, were analysed according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Fungal *Actin* or β -*tubulin* was used as a constitutive reference gene and *L. maculans* *EF1-α* relative expression to *Actin* was used as a supplementary control to verify that there was no variation in the level of expression of *Actin* between conditions and between biological

replicates. For each value measured, two to three technical replicates from two biological replicates were performed. Water and uninfected plants were used as negative controls. The RT-qPCR primers used are indicated in Table S1.

4.8 | Functional validation

For functional complementation experiments, a fragment was amplified using primers AvrLm14-XhoIF, which contains a *XhoI* site, and AvrLm14-EcoRIR, which contains an *EcoRI* site. The resulting PCR product was inserted into pGEM-T Easy vector (Promega), generating vector pGEM-AvrLm14. The 1.3 kb *XhoI*/*EcoRI* fragment was inserted into the binary vector pPZPnat1 linearized with the corresponding restriction enzymes, yielding plasmid pPZPnat1-AvrLm14.

The silencing vectors were constructed by inserting two inversely oriented fragments into pJK11. These fragments were PCR amplified with primers SilEcoRAvrLm14GF1/SilSaclAvrLm14GR1 and primers SilBamHIAvrLm14pF2/SilSaclAvrLm14pR2 using as a template cDNA from inoculated cotyledons 7 dpi. The resulting PCR products were digested by *EcoRI*/*SacI* and *BamHI*/*SacI*, respectively, and ligated into the *EcoRI*/*BamHI*-linearized pJK11 vector. The resulting plasmids place the hairpin-inverted repeat fragments under the control of the promoter of *Glomerella cingulata* *pgdA* and the terminator of *Aspergillus nidulans* *trpC*. These expression cassettes were excised by *XhoI*/*SpeI* and subcloned into the corresponding sites of pPZPnat1.

The different constructs were then introduced into *Agrobacterium tumefaciens* C58 (pGV2260) by electroporation at 1.5 kV, 200 Ω, and 25 µF. Transformants were selected on minimal medium supplemented with 50 µg/ml nourseothricin (Werner, BioAgents), purified by single conidium isolation and maintained on selective medium.

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AUTHOR CONTRIBUTIONS

M.H.B. designed the research and acquired the funding. A.D., M.W., P.G., L.C., I.F., and M.E. performed the experiments. A.D., M.W., E.J.G., M.H.B., and T.R. analysed the data. X.P., O.M.R., T.R., and M.H.B. supervised the project. T.R. and M.H.B. wrote the draft manuscript. All authors revised the manuscript.

DATA AVAILABILITY STATEMENT

All genome and transcriptome data are described in Rouxel et al. (2011), Dutreux et al. (2018), and Gay et al. (2020). All other relevant data are provided in the main text or supporting information.

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REFERENCES

- Ansan-Melayah, D., Balesdent, M.H., Buée, M. & Rouxel, T. (1995) Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. *Phytopathology*, 85, 1525–1529.
- Ansan-Melayah, D., Balesdent, M.H., Delourme, R., Pilet, M.L., Tanguy, X., Renard, M. et al. (1998) Genes for race-specific resistance against blackleg disease in *Brassica napus* L. *Plant Breeding*, 117, 373–378.
- Balesdent, M.H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M. & Rouxel, T. (2001) Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. *Phytopathology*, 91, 70–76.
- Balesdent, M.H., Attard, A., Kühn, M.L. & Rouxel, T. (2002) New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology*, 92, 1122–1133.
- Balesdent, M.H., Barbetti, M.J., Li, H., Sivasithamparam, K., Gout, L. & Rouxel, T. (2005) Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology*, 95, 1061–1071.
- Balesdent, M.H., Fudal, I., Ollivier, B., Bally, P., Grandaubert, J., Eber, F. et al. (2013) The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytologist*, 198, 887–898.
- 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.
- Bally, P., Grandaubert, J., Rouxel, T. & Balesdent, M.H. (2010) FONZIE: an optimized pipeline for minisatellite marker discovery and primer design from large sequence data sets. *BMC Research Notes*, 3, 322.
- Bayer, P.E., Golicz, A.A., Tirnaz, S., Chan, C.-K.-K., Edwards, D. & Batley, J. (2019) Variation in abundance of predicted resistance genes in the *Brassica oleracea* pangenome. *Plant Biotechnology Journal*, 17, 789–800.
- Brown, J.K.M. (2015) Durable resistance of crops to disease: a Darwinian perspective. *Annual Review of Phytopathology*, 53, 513–539.
- Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X. et al. (2014) Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science*, 345, 950–953.
- Chevre, A.M., Eber, F., This, P., Barret, P., Tanguy, X., Brun, H. et al. (1996) Characterization of *Brassica nigra* chromosomes and of blackleg resistance in *B. napus*-*B. nigra* addition lines. *Plant Breeding*, 115, 113–118.
- Daverdin, G., Rouxel, T., Gout, L., Aubertot, J.-N., Fudal, I., Meyer, M. et al. (2012) Genome structure and reproductive behaviour influence the evolutionary potential of a fungal phytopathogen. *PLoS Pathogens*, 8, e1003020.
- Dilmaghani, A., Balesdent, M.H., Didier, J.P., Wu, C., Davey, J., Barbetti, M.J. et al. (2009) The *Leptosphaeria maculans*-*Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology*, 58, 1044–1058.
- Dilmaghani, A., Gladieux, P., Gout, L., Giraud, T., Brunner, P., Stachowiak, A. et al. (2012) Migration patterns and changes in population biology associated with the worldwide spread of the oilseed rape pathogen *Leptosphaeria maculans*. *Molecular Ecology*, 21, 2519–2533.
- Dilmaghani, A., Gout, L., Moreno-Rico, O., Dias, J.S., Coudard, L., Castillo-Torres, N. et al. (2013) Clonal populations of *Leptosphaeria maculans* contaminating cabbage in Mexico. *Plant Pathology*, 62, 520–532.
- Dutreux, F., Da Silva, C., d'Agata, L., Couloux, A., Gay, E.J., Istace, B. et al. (2018) *De novo* assembly and annotation of three *Leptosphaeria* genomes using Oxford Nanopore MinION sequencing. *Scientific Data*, 5, 180235.
- Fu, Y., Zhang, Y., Mason, A.S., Lin, B., Zhang, D., Yu, H. et al. (2019) NBS-encoding genes in *Brassica napus* evolved rapidly after allopolyploidization and co-localize with known disease resistance loci. *Frontiers in Plant Science*, 10, 26.
- Fudal, I., Ross, S., Gout, L., Blaise, F., Kuhn, M.L., Eckert, M.R. et al. (2007) Heterochromatin-like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome: map-based cloning of *AvrLm6*. *Molecular Plant-Microbe Interactions*, 20, 459–470.
- Gall, C., Balesdent, M.H., Robin, P. & Rouxel, T. (1994) Tetrad analysis of acid phosphatase, soluble protein patterns and mating type in *Leptosphaeria maculans*. *Phytopathology*, 84, 1299–1305.
- Gay, E.J., Soyer, J.L., Lapalu, N., Linglin, J., Fudal, I., Da Silva, C. et al. (2021) Large-scale transcriptomics to dissect two years of the life of a fungal phytopathogen interacting with its host plant. *BMC Biology*, 19, 55.
- Gervais, J., Plissonneau, C., Linglin, J., Meyer, M., Labadie, K., Cruaud, C. et al. (2017) Different waves of effector genes with contrasted genomic location are expressed by *Leptosphaeria maculans* during cotyledon and stem colonization of oilseed rape. *Molecular Plant Pathology*, 18, 1113–1126.
- Girke, A., Schierholt, A. & Becker, H.C. (2012) Extending the rapeseed gene pool with resynthesized *Brassica napus* L. I: Genetic diversity. *Genetic Resources and Crop Evolution*, 59, 1441–1447.
- Gout, L., Fudal, I., Kuhn, M.L., Blaise, F., Eckert, M., Cattolico, L. et al. (2006) Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the Dothideomycete *Leptosphaeria maculans*. *Molecular Microbiology*, 60, 67–80.
- Haddadi, P., Ma, L., Wang, H. & Borhan, M.H. (2016) Genome-wide transcriptome analyses provides insights into the lifestyle transition and effector repertoire of *Leptosphaeria maculans* during colonization of *B. napus* seedlings. *Molecular Plant Pathology*, 17, 1196–1210.
- Huang, Y.J., Balesdent, M.H., Li, Z.Q., Evans, N., Rouxel, T. & 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). *European Journal of Plant Pathology*, 126, 279–291.
- Huang, Y.J., Li, Z.Q., Evans, N., Rouxel, T., Fitt, B.D.L. & Balesdent, M.H. (2006) 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.
- Katche, E., Quezada-Martinez, D., Katche, E.I., Vasquez-Teuber, P. & Mason, A.S. (2019) Interspecific hybridization for *Brassica* crop improvement. *Crop Breeding, Genetics and Genomics*, 1, e190007.
- Leflon, M., Brun, H., Eber, F., Delourme, R., Lucas, M.O., Vallée, P. et al. (2007) Detection, introgression and localization of genes conferring specific resistance to *Leptosphaeria maculans* from *Brassica rapa* into *B. napus*. *Theoretical and Applied Genetics*, 115, 897–906.
- Livak, K.J. & 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.
- Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M. et al. (2015) Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, 66, 513–545.
- Lv, H., Fang, Z., Yang, L., Zhang, Y. & Wang, Y. (2020) An update on the arsenal: mining resistance genes for disease management of *Brassica* crops in the genomic era. *Horticulture Research*, 7, 34.
- Mason, A.S. & Batley, J. (2015) Creating new interspecific hybrid and polyploid crops. *Trends in Biotechnology*, 33, 436–441.
- Muller, P.Y., Janovjak, H., Miserez, A.R. & Dobbie, Z. (2002) Processing of gene expression data generated by quantitative real-time RTPCR. *BioTechniques*, 32, 1372–1378.

- Navabi, Z.K., Parkin, I.A.P., Pires, J.C., Xiong, Z., Thiagarajah, M.R., Good, A.G. et al. (2010) Introgression of B-genome chromosomes in a doubled haploid population of *Brassica napus* × *B. carinata*. *Genome*, 53, 619–629.
- Neik, T.X., Ghanbarnia, K., Ollivier, B., Scheben, A., Severn-Ellis, A., Larkan, N.J. et al. (2020) Two independent approaches converge to the cloning of a new *Leptosphaeria maculans* avirulence effector gene, AvrLmS-Lep2. *bioRxiv*. <https://doi.org/10.1101/2020.10.02.322479>
- Nováková, M., Šásek, V., Trdá, L., Krutínová, H., Mongin, T., Valentová, O. et al. (2016) *Leptosphaeria maculans* effector AvrLm4-7 affects SA- and ET-signalling and H₂O₂ accumulation in *Brassica napus*. *Molecular Plant Pathology*, 17, 818–831.
- Petit-Houdonot, Y., Degraeve, A., Meyer, M., Blaise, F., Ollivier, B., Marais, C. et al. (2019) A two genes-for-one gene interaction between *Leptosphaeria maculans* and *Brassica napus*. *New Phytologist*, 223, 397–411.
- Plissonneau, C., Blaise, F., Ollivier, B., Leflon, M., Carpezat, J., Rouxel, T. et al. (2017) Unusual evolutionary mechanisms to escape effector-triggered-immunity in the fungal phytopathogen *Leptosphaeria maculans*. *Molecular Ecology*, 26, 2183–2198.
- Plissonneau, C., Daverdin, G., Ollivier, B., Blaise, F., Degraeve, A., Fudal, I. et al. (2016) A game of hide and seek between avirulence genes AvrLm4-7 and AvrLm3 in *Leptosphaeria maculans*. *New Phytologist*, 209, 1613–1624.
- Robin, A.H.K., Larkan, N.J., Laila, R., Park, J.-I., Ahmed, N.U., Borhan, H. et al. (2017) Korean *Brassica oleracea* germplasm offers a novel source of qualitative resistance to blackleg disease. *European Journal of Plant Pathology*, 149, 611–623.
- Rouxel, T. & Balesdent, M.H. (2005) The stem canker (blackleg) fungus, *Leptosphaeria maculans*, enters the genomic era. *Molecular Plant Pathology*, 6, 225–241.
- Rouxel, T. & Balesdent, M.H. (2017) Life, death and rebirth of avirulence effectors in a fungal pathogen of *Brassica* crops, *Leptosphaeria maculans*. *New Phytologist*, 214, 526–532.
- Rouxel, T., Grandaubert, J., Hane, J.K., Hoede, C., van de Wouw, A.P., Couloux, A. et al. (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. *Nature Communications*, 2, 202.
- Roy, N.N. (1984) Interspecific transfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus*. *Euphytica*, 33, 295–303.
- Soyer, J.L., Clairet, C., Gay, E.J., Lapalu, N., Rouxel, T., Stukenbrock, E.H. et al. (2021) Genome-wide mapping of histone modifications in two species of *Leptosphaeria maculans* showing contrasted genomic organization and different host specialization. *Chromosome Research*, 29, 219–236.
- Sperschneider, J., Dodds, P.N., Gardiner, D.M., Singh, K.B. & Taylor, J.M. (2018) Improved prediction of fungal effector proteins from secretomes with EffectorP 2.0. *Molecular Plant Pathology*, 19, 2094–2110.
- Stam, R. & McDonald, B.A. (2018) When resistance gene pyramids are not durable – the role of pathogen diversity. *Molecular Plant Pathology*, 19, 521–524.
- U, N. (1935) Genome-analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany*, 7, 389–452.
- Yu, F., Lydiate, D.J. & Rimmer, S.R. (2005) Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theoretical and Applied Genetics*, 110, 969–979.
- Yu, F., Lydiate, D.J. & 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.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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