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ORIGINAL ARTICLE

Two independent approaches converge to the cloning of a new *Leptosphaeria maculans* avirulence effector gene, *AvrLmS-Lep2*

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Abstract

Brassica napus (oilseed rape, canola) seedling resistance to *Leptosphaeria maculans*, the causal agent of blackleg (stem canker) disease, follows a gene-for-gene relationship. The avirulence genes *AvrLmS* and *AvrLep2* were described to be perceived by the resistance genes *RlmS* and *LepR2*, respectively, present in *B. napus* 'Surpass 400'. Here we report cloning of *AvrLmS* and *AvrLep2* using two independent methods. *AvrLmS* was cloned using combined in vitro crossing between avirulent and virulent isolates with sequencing of DNA bulks from avirulent or virulent progeny (bulked segregant sequencing). *AvrLep2* was cloned using a biparental cross of avirulent and virulent *L. maculans* isolates and a classical map-based cloning approach. Taking these two approaches independently, we found that *AvrLmS* and *AvrLep2* are the same gene. Complementation of virulent isolates with this gene confirmed its role in inducing resistance on Surpass 400, Topas-*LepR2*, and an *RlmS*-line. The gene, renamed *AvrLmS-Lep2*, encodes a small cysteine-rich protein of unknown function with an N-terminal secretory signal peptide, which is a common feature of the majority of effectors from extracellular fungal plant pathogens. The *AvrLmS-Lep2/LepR2* interaction phenotype was found to vary from a typical hypersensitive response through intermediate resistance sometimes towards susceptibility, depending on the inoculation conditions. *AvrLmS-Lep2* was nevertheless sufficient to significantly slow the systemic growth of the pathogen and reduce the stem lesion size on plant genotypes with *LepR2*, indicating the potential efficiency of this resistance to control the disease in the field.

KEYWORDS

avirulence, *AvrLep2*, *AvrLmS*, *Brassica napus*, canola, oilseed rape, resistance

Ting Xiang Neik and Kaveh Ghanbarnia made equal contributions.

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1 | INTRODUCTION

Diseases of field crops are best controlled by the use of resistant varieties. Major race-specific resistance (R) genes, matching avirulence (AVR) genes in the pathogen, are extensively used in crop breeding programmes. However, the widespread and repeated use of cultivars with a single R gene exerts strong selection pressure on the pathogen populations, leading to the emergence of virulent isolates and breakdown of the resistance.

An example of such an arms race between the host crop and its pathogen is the breakdown of *Brassica napus* (oilseed rape, canola) resistance against *Leptosphaeria maculans*, the ascomycete causing stem canker (blackleg), a major disease of oilseed rape (Fitt et al., 2006; Sprague et al., 2006). The *L. maculans* infectious cycle starts with ascospores or conidia germination on cotyledon and leaves. The fungus then asymptomatically colonizes petioles and stems, and finally develops a dry necrosis at the stem base responsible for the main yield losses. Both quantitative, also known as polygenic or adult plant resistance, and single, major gene resistance have been reported in *B. napus* (Delourme et al., 2006). To date, 18 R genes to *L. maculans* have been described, with only three cloned to date: *LepR3*, *Rlm2*, and *Rlm9* (Delourme et al., 2004, 2006; Larkan et al., 2013, 2015, 2020; Long et al., 2011; Rimmer, 2006; Van de Wouw et al., 2009; Yu et al., 2005, 2008). Meanwhile, nine *L. maculans* avirulence genes have already been cloned: *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4-7*, *AvrLm5-9*, *AvrLm6*, *AvrLm10*, *AvrLm11*, and *AvrLm14* (Balesdent et al., 2013; Degrave et al., 2021; Fudal et al., 2007; Ghanbarnia et al., 2015, 2018; Gout et al., 2006; Parlange et al., 2009; Petit-Houdenot et al., 2019; Plissonneau et al., 2016; Van de Wouw et al., 2014). Map-based cloning of the first *AvrLm* genes (*AvrLm1*, *AvrLm6*, and *AvrLm4-7*; Fudal et al., 2007; Gout et al., 2006; Parlange et al., 2009) took many years due to the lack of a *L. maculans* genome sequence and their location in repeat-rich, gene-poor regions of the genome (Rouxel et al., 2011). The availability of a reference genome and a repertoire of effector genes facilitated rapid identification of several further AVR genes, including *AvrLm11*, *AvrLm2*, *AvrLm5-9*, and *AvrLm14* (Balesdent et al., 2013; Degrave et al., 2021; Ghanbarnia et al., 2015, 2018).

B. napus is an allotetraploid (AACC, 2n = 38) species derived from the hybridization of the diploid parents, *Brassica oleracea* (CC, 2n = 18) and *Brassica rapa* (AA, 2n = 20). These related species have been explored as resistance sources to increase the R gene pool in oilseed rape. Four R genes to *L. maculans* (*LepR1*, *LepR2*, *LepR3*, and *LepR4*) have been genetically characterized in *B. rapa* subsp. *sylvestris* (Yu et al., 2005, 2008, 2013), which was used as a source of resistance in the 1990s (Crouch et al., 1994), leading to the release of *B. napus* varieties with "*sylvestris*-derived resistance". From 2000 to 2003, the *B. napus* cultivar Surpass 400 and related cultivars containing *sylvestris*-derived resistance were grown on large acreages across Australia before the resistance was overcome in the Eyre Peninsula region of South Australia (Li et al., 2004; Sprague et al., 2006). Based on the genetic analysis of virulent isolates on Surpass 400, Van de Wouw et al. (2009) reported that at least two avirulence genes, *AvrLm1* and *AvrLmS*, conveyed avirulence towards Surpass 400, supporting the idea of an *RlmS–AvrLmS* interaction in the host plant. On the plant side, genetic analyses described either one or two resistance loci in Surpass 400 (Li & Cowling, 2003; Long et al., 2011; Yu et al., 2008), termed *LepR3* (Yu et al., 2008), *BLMR1* and *BLMR2* (Long et al., 2011), or *LepR3* and *RlmS* (Larkan et al., 2013). Larkan et al. (2013) mapped and cloned *LepR3* from Surpass 400 and showed that *LepR3* recognizes the AvrLm1 protein. *LepR3* can therefore be considered a functional homologue of *Rlm1*, although *LepR3* and *Rlm1* reside on different chromosomes (A10 and A07, respectively). It was also demonstrated that a second resistance gene was present in Surpass 400, probably corresponding to the *AvrLmS* avirulence gene, that is, *RlmS* (Larkan et al., 2013). Yu et al. (2008) noted the possible presence of *LepR2* or a similar gene in Surpass 400 along with *LepR3*, suggesting the possible identity of *RlmS* and *LepR2*.

Identification of R gene content in a cultivar and conclusions on the genetic control of the resistance has traditionally relied on phenotypic evaluation of the interaction based on inoculation tests with *L. maculans* isolates, for which the avirulence gene content may differ between studies. Due to epistatic effects between AVR genes, a single-gene control of a resistant phenotype toward a given isolate may hide a more complex R gene control due to the lack of adequate differential isolates. Cloning of the corresponding AVR genes can thus help us understand the relationships between R genes with different names. In the current study, we report on the cloning of the AVR gene matching *RlmS* and *LepR2* using two independent approaches. The AVR gene recognized by *LepR2*, *AvrLep2*, was cloned following a standard map-based cloning approach, whilst the gene interacting with *RlmS*, *AvrLmS*, was cloned using a bulked-segregant sequencing (BSS) strategy. The two approaches identified the same gene, now referred to as *AvrLmS-Lep2*, which shares all characteristics of *L. maculans* avirulence effector genes. Noticeably, the two strategies converged although the interaction phenotypes differed between laboratories, probably due to the environmental conditions.

2 | RESULTS

2.1 | Approach 1: BSS

2.1.1 | Phenotypic characterization of X82 progeny for BSS

A cross (#82) between isolates WT50 and INV13.269, virulent and avirulent on Surpass 400, respectively (Figure 1) was used to generate a progeny for BSS. Phenotyping of the parental and progeny isolates on an extended *B. napus* differential set revealed that all isolates lacked *AvrLm1* (data not shown) and that *AvrLm6*, *AvrLm4-7*, and *AvrLmS* segregated with the expected 1:1 ratio (Table 1). In addition, eight phenotypic classes were recovered in the progeny, with ratios fitting the hypothesis of independence

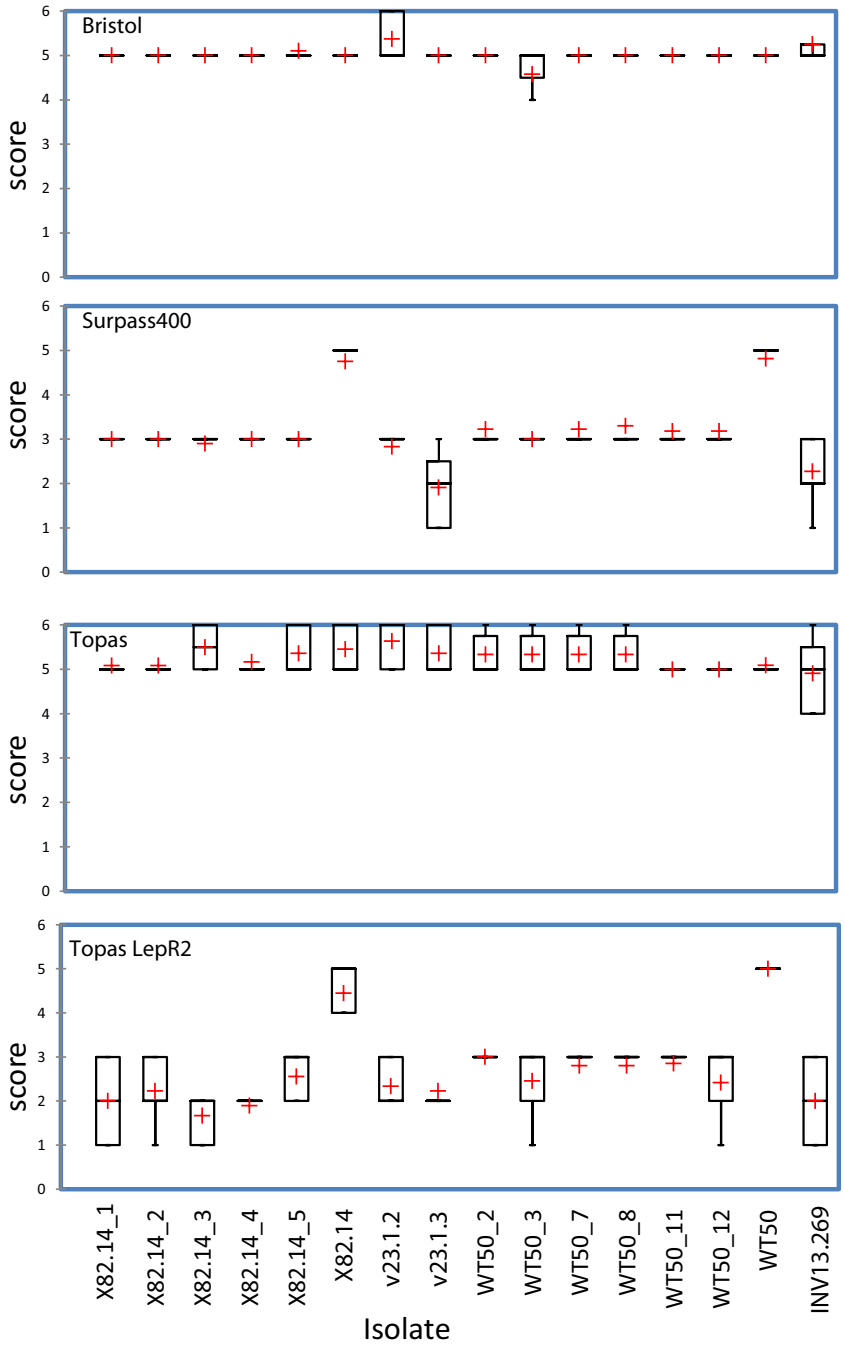

FIGURE 1 Box plot of rating scores of interaction phenotypes between wild-type or transformed isolates of *Leptosphaeria maculans* and four *Brassica napus* genotypes. From top to bottom: susceptible check Bristol (*Rlm2-Rlm9*), Surpass 400 (*LepR3-RlmS*), Topas DH16516 (no R gene), Topas-LepR2 (*LepR2*). For each box, the red cross indicates the score mean and the black horizontal line indicates the score median. The rectangles comprise 75% (Q1–Q3) of the rating scores. Wild-type isolates are in bold; X82.14-i and WT50-j are five and six independent transformed isolates of X82.14 and WT50, respectively, with the candidate gene Lmb_jn3_08343

between *AvrLm6*, *AvrLm4-7*, and *AvrLmS* (χ^2 = 8.25, p = 0.689; Table 1) as previously established (Van de Wouw et al., 2009). Based on phenotyping results, 47 isolates were selected for the preparation of six DNA bulks. Bulk 1 (AS) comprised 25 progeny isolates with an avirulent phenotype on Surpass 400 but either virulent or avirulent on *Rlm6* or *Rlm7*; Bulk 2 (aS) comprised DNA from 22 progeny isolates virulent on Surpass 400; Bulk 3 (A7) contained DNA from 24 avirulent isolates on *Rlm7*, but either virulent or avirulent on *Rlm6* or *RlmS*; and Bulk 4 (a7) contained DNA from 23 isolates virulent on *Rlm7*. Bulks 5 (AS7) and 6 (aS7) contained isolates being avirulent on *RlmS* and *Rlm7*, or virulent on both genes, respectively (Table 2).

2.1.2 | BSS statistics and validation of the BSS strategy

The Illumina whole-genome sequences (2 × 150 bp) were generated from the six bulks and the two parental isolates. After quality trimming, the average number of reads generated was in the range of 47–75 million and the average genome coverage depth ranged between 164× and 272× (Table S1). In total, 65,727 single nucleotide polymorphisms (SNPs) were identified between the parental isolates, excluding 19,650 SNPs in repetitive regions. The average number of SNPs found in the bulked progeny was 64,473 (Table S2). After quality filtering for quantitative trait locus (QTL) mapping in the bulked

TABLE 1 Segregation for virulence on *Rlm6*, *Rlm7*, and *RlmS* (Surpass 400) in the *Leptosphaeria maculans* cross X82 (WT50 x INV13.269)

	Interaction phenotype on			
	Darmor-MX (*Rlm6-Rlm9*)	15.23.4.1 (*Rlm7*)	Surpass 400 (*LepR3-RlmS*)	Number of isolates (%)
Parental isolates				
WT50	V	A	V	na
INV13.269	A	V	A	na
Phenotypic classes in progeny isolates	A	A	A	7 (8.3%)
	A	A	V	11 (13.1%)
	A	V	A	14 (16.7%)
	A	V	V	6 (7.1%)
	V	A	A	12 (14.3%)
	V	A	V	12 (14.3%)
	V	V	A	11 (13.1%)
	V	V	V	11 (13.1%)
A:V ratio (*p* value[b])	38:46 (0.383)	42:42 (1)	44:40 (0.663)	na

Abbreviations: A, avirulent phenotype; na, not applicable; V, virulent phenotype.
[a]*p* value of the X^2 test for a 50:50 segregation ratio.

TABLE 2 Characteristics of DNA bulks and sequence mapping results

Bulk or sample no.	Bulk name (abbreviated name)	Type of isolate	No. of contributing isolates in the bulk	Nb of reads mapped to Lmb_jn3_08343	Fraction of Lmb_jn3_08343 bases covered by at least one read
1	*AvrLmS* (AS)	Progeny, avirulent on Surpass 400	25	626	1.00
2	*avrLmS* (aS)	Progeny, virulent on Surpass 400	22	2	0.37
3	*AvrLm7* (A7)	Progeny, avirulent on *Rlm7*	24	nd	nd
4	*avrLm7* (a7)	Progeny, virulent on *Rlm7*	23	nd	nd
5	*AvrLmS* + *AvrLm7* (AS7)	Progeny, avirulent on Surpass 400 and *Rlm7*	11	809	1.00
6	*avrLmS* + *avrLm7* (aS7)	Progeny, virulent on Surpass 400 and *Rlm7*	9	0	0.00
7	INV13.269 (a7AS)	Parental isolate	1	1056	1.00
8	WT50 (A7aS)	Parental isolate	1	1	0.03

pairs, the total number of SNPs retained was 27,532 (Bulks 3/4), 27,128 (Bulks 1/2), and 26,010 (Bulks 5/6).

To validate the BSS strategy for AVR gene cloning, a QTL-Seq analysis was carried out using Bulk 3 vs. Bulk 4, differing for the gene *AvrLm4-7*. A QTL was found on scaffold JN3_SC03 (v23.1.3 genome; Dutreux et al., 2018) at positions 62,390 to 345,050 (Figure S1, Tables S3 and S4). This region contains 50 predicted genes (Lmb_jn3_03239 to Lmb_jn3_03288) including *AvrLm4-7* (GenBank nucleotide sequence AM998638.1, Lmb_jn3_03262). This result validates the BSS strategy for identification of a genomic region containing a gene of interest and suggests that the size of the bulks (23 and 24 isolates) is adequate for this purpose.

2.1.3 | Identification of a candidate gene for *AvrLmS* using BSS

QTL-Seq results for Bulk 1 (AS) vs. Bulk 2 (aS) and Bulk 5 (AS7) vs. Bulk 6 (aS7) were compared to identify the *AvrLmS* genomic interval. The analysis revealed a QTL for *AvrLmS* within a 335 kb (Bulks 5/6) or 816 kb (Bulks 1/2) segment on JN3_SC09 (Figures 2 and S2, Tables S4–S6). The QTL for both bulked pairs overlapped, with the major QTL supported by the most SNPs and the highest ΔSNP value showing peaks at position 1,481,733 in Bulks 1/2 and Bulks 5/6. Candidate SNP analysis for *AvrLmS* using Bulk 1/2, Bulk 5/6, and both parents (Samples 7/8) confirmed the QTL-Seq results, identifying a total of 437 genome-wide SNPs that segregated with the avirulence trait. Of these, 410 were found on JN3_SC09, with all SNPs found in a 398 kb region overlapping with the QTL region (position 1,477,092–1,874,868 bp). This interval contains 28 genes (Lmb_jn3_08331 to Lmb_jn3_08358). The candidate region also contains a 285 kb AT-rich region (JN3_SC09: 1,533,065–1,818,564), enriched in repeats (Figures S3 and S4, Table S7), typical for genomic regions encompassing AVR genes in *L. maculans* (Rouxel et al., 2011). Only one gene, Lmb_jn3_08343, was located in this AT-rich region. The number of reads mapped to Lmb_jn3_08343 was over 600 for each of the *AvrLmS* bulks and the *AvrLmS* parent INV13.269, with every single base of the coding sequence covered

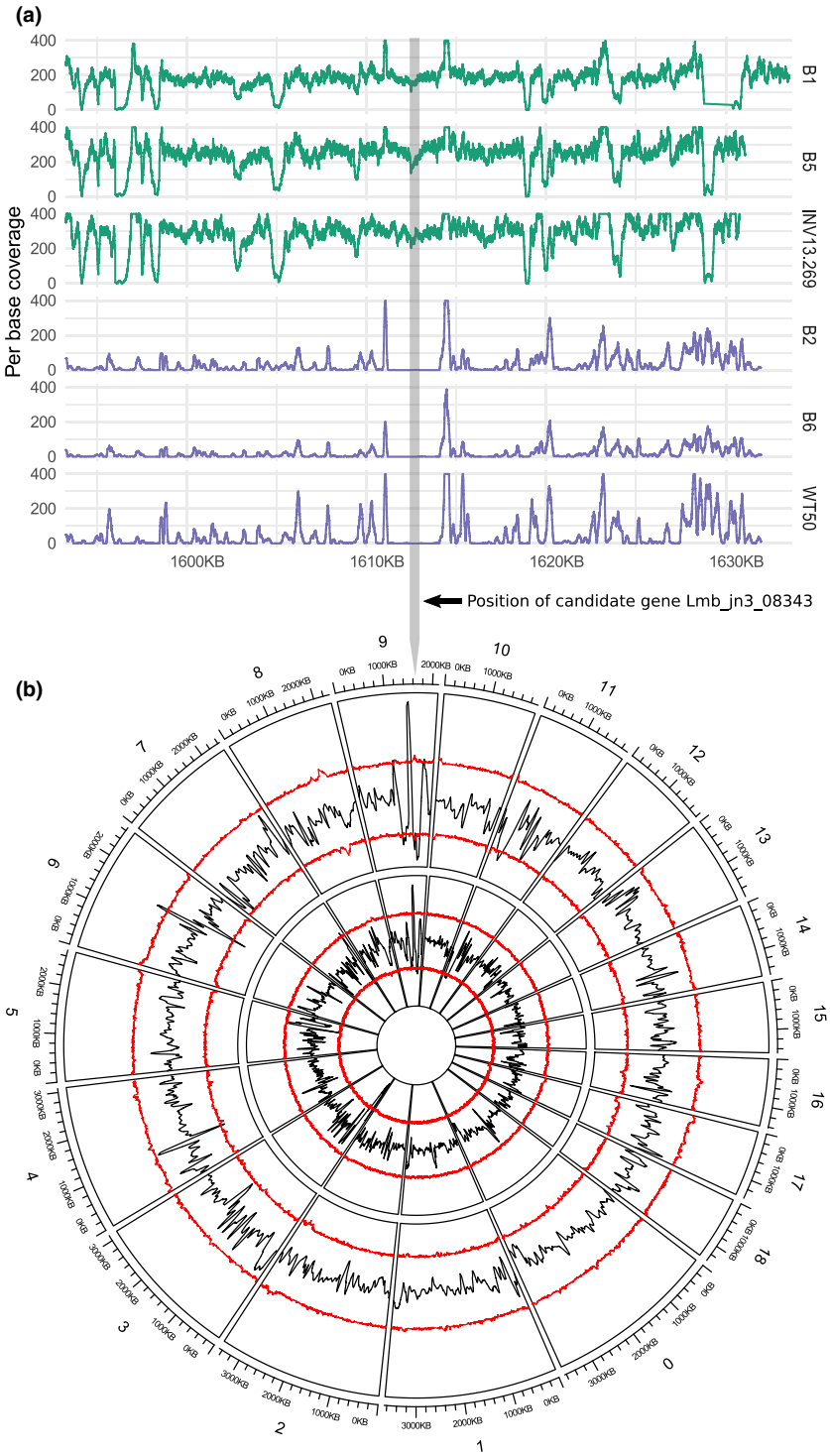

FIGURE 2 Identification of the candidate region for *AvrLmS* by bulked segregant sequencing. (a) Per base coverage for all samples (not including Bulk 3 and 4, *AvrLm7* and *avrLm7*, respectively) for 20 kb upstream and downstream of the gene Lmb_jn3_08343 (coding sequence demarcated with grey vertical bar) on scaffold 9 (JN3_SC09). Samples with *AvrLmS* are shown in green and those with *avrLmS* in purple. The *y* axis limit was set to 400. (b) Circos plot of Δ(SNP-index) between Bulk 1/2 with *AvrLmS* (outer circle) and Bulk 5/6 with *AvrLmS*+*AvrLm7* (inner circle) for the 19 scaffolds larger than 1 Mb. Confidence intervals of 99% (red) are shown. The *y* axis is bounded from −0.5 to 0.8 for the outer plots and from −0.9 to 0.9 for the inner plots. The shared quantitative trait locus (QTL) for *AvrLmS* is located on scaffold 9. Coordinates for QTLs are provided in Tables S5 and S6

by reads (Table 2). Comparatively, only three reads mapped to Lmb_jn3_08343 in the *avrLmS* bulk sample and the virulent parent WT50 (Table 2). Read coverage analysis revealed a c.3 kb region (1,611,953–1,614,969 bp), with zero or close to zero base coverage in all the *avrLmS* bulk samples (Bulk 2, Bulk 6, and parent WT50) whereas all the *AvrLmS* bulk samples (Bulk 1, Bulk 5, and parent INV13.269) had per base coverage between 100 and 400 within this region.

The c.3 kb putative deletion contains the v23.1.3 candidate gene Lmb_jn3_08343 (Figures S2 and S3). This gene fulfils all criteria for a *L. maculans* AVR gene candidate: (a) sequence variation, either in terms of SNPs or presence/absence variation; (b) genomic location in a gene-poor, AT-rich region; and (c) lack of sequence homology with other AVR genes or any protein in the database, except a weak homology with another candidate effector gene in *L. maculans* (Lmb_jn3_03815; 39.50% identity, e-value = 1e−20).

2.2 | Approach 2: Map-based identification of a candidate gene for *AvrLep2*

Contrasting with data obtained at BIOGER, where isolate v23.1.3 (JN3) induced an intermediate resistance reaction of Topas-*LepR2* (Figure 1), the conditions used for pathotyping at AAFC Saskatoon induced for v23.1.3 a susceptibility phenotype on the cotyledons of plants carrying *LepR2* and was thus deemed virulent. The F_1 progeny produced from crossing v23.1.3 (*avrLep2*) and 00-100 (*AvrLep2*) segregated for this interaction phenotype on *LepR2*, with 41 virulent isolates and 57 avirulent isolates, a segregation approximating a 1:1 ratio ($\chi^2 = 2.61$, $p = 0.11$), as expected for genetic control of the phenotype by a single AVR gene. All 98 progeny were virulent on the susceptible line Topas DH16516. One hundred and fifty-five Kompetitive allele-specific PCR (KASP) markers were developed based on the whole-genome sequence and predicted effector genes of *L. maculans* v23.1.3, and applied to the progeny of the v23.1.3 × 00-100 cross. Two markers, K16-S3-1675 and K-S3-2160, closely segregated with the *AvrLep2* locus and spanned a physical interval of approximately 485 kb of the *L. maculans* genome. To more precisely map the *AvrLep2* locus, an additional 11 KASP markers were designed within the *AvrLep2* interval. The resulting map showed that *AvrLep2* resided within an interval of 319 kb between two markers, K-S3-1761 and K-S3-2080 (Figure 3). To improve the predicted gene annotation within the *AvrLep2* interval, previously generated RNA-Seq data produced from *L. maculans*-infected *B. napus* seedlings (Haddadi et al., 2016, 2019) were mapped to the *L. maculans* genome. Genes within the *AvrLep2* interval were manually annotated and a predicted secreted protein was identified as the AvrLep2 candidate.

2.3 | Two approaches, one "typical" avirulence effector gene candidate

The two cloning strategies identified the same candidate gene, Lmb_jn3_08343, which is 426 bp and contains one exon. In isolate v23.1.3, it is located in a typical AT-rich region of 285 kb containing one single gene (Figures 3 and S4). Lmb_jn3_08343 encodes a small (141 amino acid) putative secreted (SignalP v. 4.1; Petersen et al., 2011) protein enriched in cysteines (eight cysteine residues in the mature protein). PCR amplification confirmed the gene was absent in the virulent isolate WT50 and in all virulent isolates in X82 progeny, while sequencing of the gene in the avirulent isolate INV13.269 indicated it is 100% identical to that of v23.1.3.

We examined SNP events within the candidate gene in the previously resequenced genomes of 37 *L. maculans* isolates (including

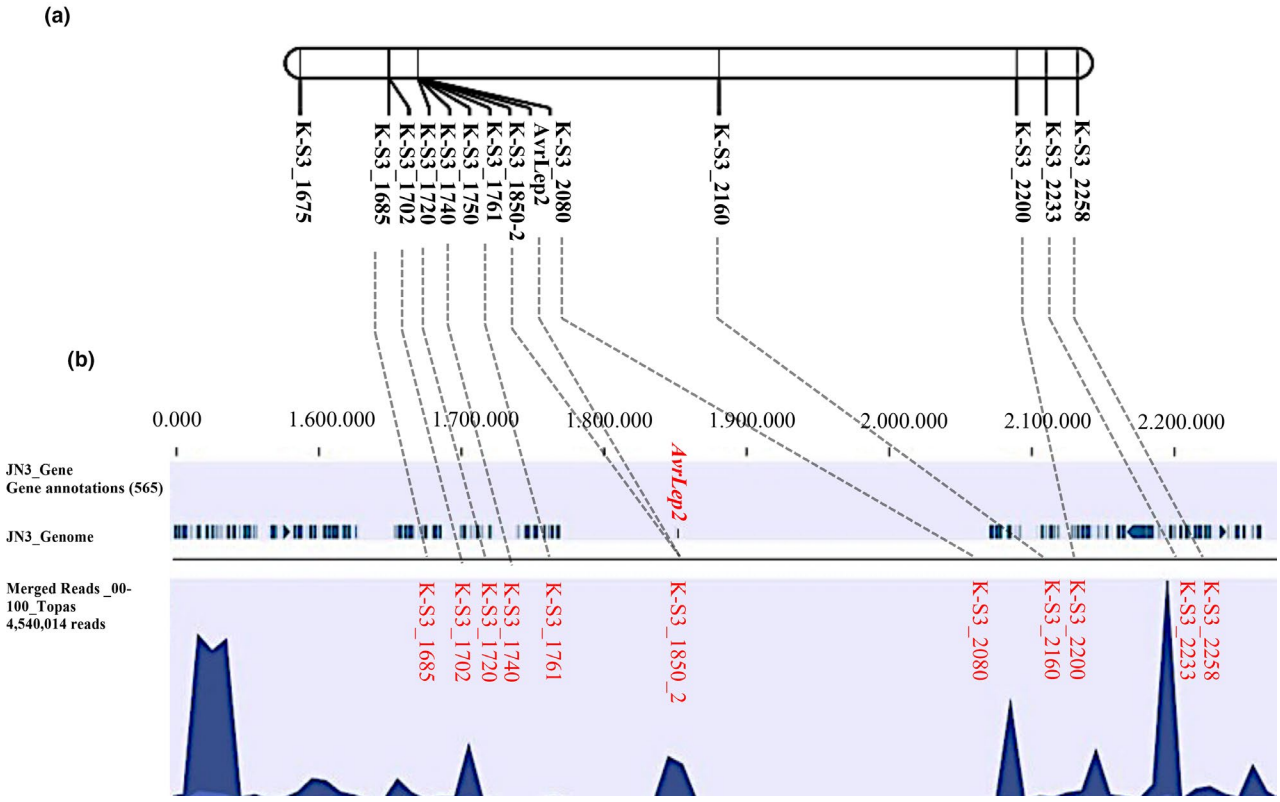

FIGURE 3 Genetic and physical maps of the *AvrLep2* genomic region in *Leptosphaeria maculans* isolate v23.1.3. (a) Position of *AvrLep2* relative to KASP markers on 00-100 × v23.1.3 (SuperContig 3 from v23.1.3 V1 assembly GCF_000230375.1) map. (b) Physical region spanning the *AvrLep2* locus in the isolate v23.1.3. The top lane denotes predicted genes and the bottom lane shows cumulative gene expression level for predicted *L. maculans* genes during infection time course (2–8 days postinoculation)

v23.1.3) from the AAFC collection (3 *avrLep2* and 34 *AvrLep2* isolates; Ghanbarnia et al., 2015). The candidate gene was present in all isolates. In total, eight nucleotide changes were observed in the candidate gene, of which four resulted in nonsynonymous amino acid substitutions at three different positions in the protein (Table S8 and Figure S5). There was no clear relationship between the SNPs found in Lmb_jn3_08343 and the virulence phenotype toward *LepR2*. 00-100 and the majority of Canadian isolates displayed two amino acid substitutions compared to v23.1.3, at positions 93 (Gly→Asp) and 95 (Arg→Gln).

2.4 | Validation of the candidate gene

Two independent validation experiments were performed. First, WT50 (virulent on *RlmS* and *Rlm6*) and its progeny isolate X82.14 (virulent on *Rlm6*, *Rlm7*, and *RlmS*), both of which had the candidate gene deleted, were complemented with the v23.1.3 allele of the candidate gene. All complemented isolates remained fully virulent toward *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm6*, and *Rlm9* (Figure 1). In addition, X82.14 complemented isolates remained virulent towards *Rlm7*. All complemented isolates induced the typical intermediate resistance observed for isolate INV13.269 on Surpass 400. Due to the presence of *AvrLm1* in v23.1.3, which was interacting with *LepR3* present in Surpass 400 (Larkan et al., 2013), the characteristics of the phenotype induced on Surpass 400 by the v23.1.3 Lmb_jn3_08343 allele could not be determined following inoculation with v23.1.3. However, the interaction phenotype of INV13.269 and isolates complemented with the v23.1.3 allele confirmed this allele confers an intermediate resistance phenotype on Surpass 400, which could evolve toward susceptibility with time in some plants, as initially described (Van de Wouw et al., 2009). Finally, all complemented isolates were virulent on Topas DH16516 but displayed a clear resistant phenotype on Topas-*LepR2* (Figures 1, 4, and S6) and on the *RlmS* line 72.1 (Figure 4). Therefore, Lmb_jn3_08343 encodes the avirulence effector protein corresponding to *RlmS* and is also able to elicit the *LepR2* resistance response.

Second, two types of construct for transformation using the *AvrLep2* candidate allele from *L. maculans* isolate 00-100 were produced, either with its native promoter or with the promoter of the avirulence gene *AvrLm1*. After transforming the virulent isolate v23.1.3 with the candidate gene constructs, restoration of avirulence phenotype was evaluated by inoculation of transgenic isolates on Topas-*LepR2* (Table 3). Transformant selections for each of the constructs tested on the *B. napus* differential lines showed avirulence on cotyledons of Topas-*LepR2* plants but remained virulent on the susceptible Topas DH16516 and Westar control lines (Figure 5). Positive transformants also showed wild-type interaction

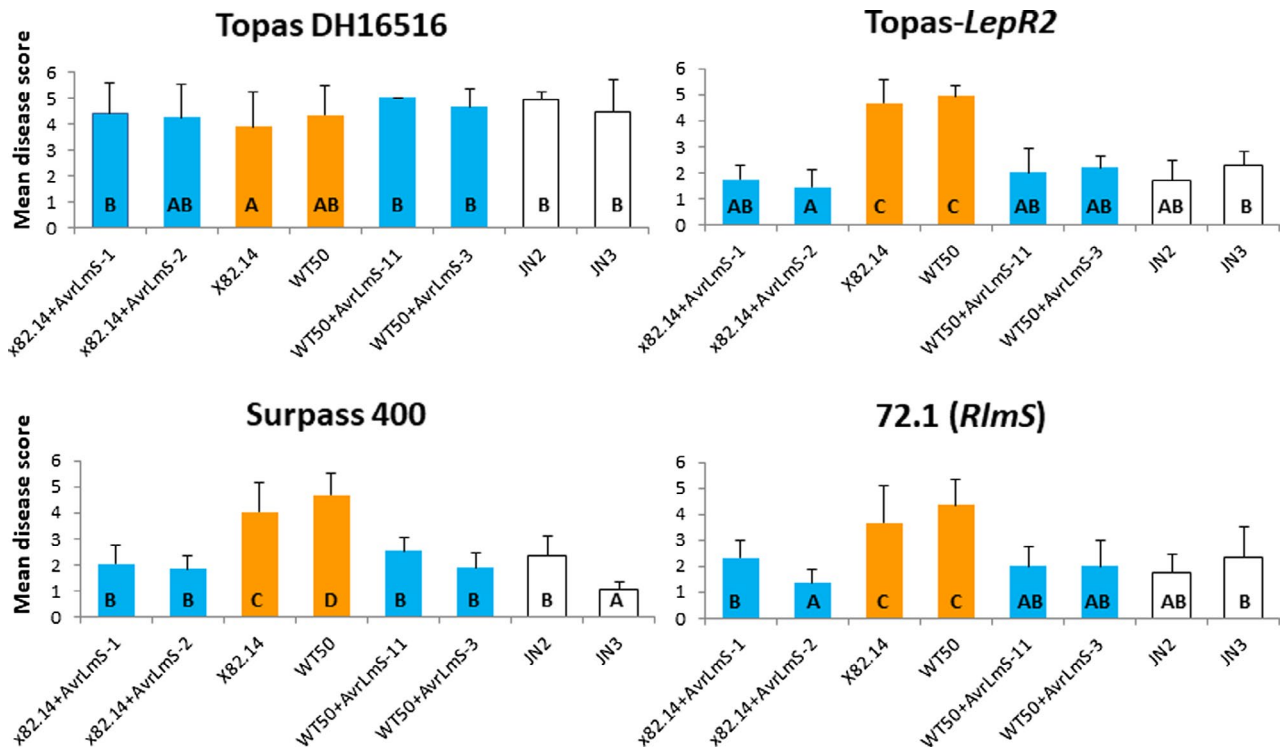

FIGURE 4 Phenotypic confirmation of the elicitation of both *LepR2* and *RlmS* resistance responses by Lmb_jn3_08343. Reference isolates (white, avirulent on *RlmS*), wild-type virulent isolates (orange), and a selection of transformants with the *AvrLmS* candidate gene Lmb_jn3_08343 (blue) were inoculated on Topas DH16156 (devoid of *Rlm* gene), Topas-*LepR2* (*LepR2*), 72.1 (*RlmS*), or Surpass 400 (*RlmS*+*LepR3*). Data are means of disease scores on a 1–6 scale, at 17 days postinoculation including two biological replicates with 6 to 24 plants per each isolate/plant genotype/replicate. For each plant genotype, isolates with the different letters are significantly different ($p < 0.05$) according to the nonparametric Kruskal–Wallis test

TABLE 3 Pathogenicity test for *Leptosphaeria maculans* isolates (wild-type and transformants) on *Brassica napus* lines carrying diverse blackleg resistance (R) genes

Isolates/transformants[a]	*B. napus* lines/cultivars and R gene content										
	Topas (T)	T-*Rlm1*	T-*Rlm2*	T-*Rlm3*	T-*Rlm4*	Vulcan	Roxet	Goéland	T-*LepR1*	T-*LepR2*	T-*LepR3*
	Control	*Rlm1*	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm6*	*Rlm7*	*Rlm9*	*LepR1*	*LepR2*	*LepR3*
v23.1.3	V[b]	A	V	V	A	A	A	V	A	V	A
00-100	V	V	A	A	V	A	V	A	A	A	V
v23.1.3: *AvrLep2* (AW1)	V	A	V	V	A	A	A	V	A	A	A
v23.1.3: *AvrLep2* (AW2)	V	A	V	V	A	A	A	V	A	A	A

[a]v23.1.3: *AvrLep2* (AW1) is a transformant with pNL11-*AvrLep2* construct (*AvrLep2* avirulent allele was amplified from isolate 00-100 with native promoter); v23.1.3: *AvrLep2* (AW2) is a transformant with pLM4-*AvrLep2* construct (*AvrLep2* avirulent allele coding region was amplified from isolate 00-100, driven by *AvrLm1* promoter).

[b]Interactions classified as either virulent (V) or avirulent (A).

phenotypes with the differential lines harbouring other resistance genes (Table 3), confirming the identity and the specificity of the candidate gene as *AvrLep2*.

2.5 | Expression analysis

Previously generated RNA-Seq data from isolate v23.1.2, avirulent (intermediate resistance phenotype) on Surpass 400 (Van de Wouw et al., 2009; Figure S6), were used to compare the expression kinetics of Lmb_jn3_08343 with that of all previously cloned *L. maculans* avirulence genes, following inoculation of cotyledons from a susceptible cultivar (Gay et al., 2021). Lmb_jn3_08343 is highly expressed during cotyledon infection, with a peak of expression at 7 days after infection (dai) in BIOGER's controlled conditions, that is, before symptoms develop. Lmb_jn3_08343 is fully co-regulated with previously cloned avirulence genes, particularly *AvrLm4-7*, *AvrLm5-9*, and *AvrLm3* (Figure 6). Previously generated RNA-Seq data for the infection of the susceptible *B. napus* 'Topas DH16516' by both v23.1.3 and 00-100 (Haddadi et al., 2016) was also examined to determine the expression patterns for both alleles of *AvrLep2*. Peak expression, measured as reads per kilobase of transcript per million mapped reads (RPKM), was observed at 4 dai for both v23.1.3 and 00-100, with *AvrLep2* having a similar expression to *AvrLm5-9* in both isolates (Figure S7).

2.6 | Adult plant tests

After leaf infection and leaf spot development in the field, *L. maculans* grows systemically into the petioles and the stems before switching to necrotrophy and developing the stem canker symptom. To test the functionality of the *LepR2–AvrLep2* interaction during these later stages of plant infection, seedlings of Topas DH16516 and Topas-*LepR2* were inoculated with the control isolates v23.1.3 and 00-100, as well as eight additional native *L. maculans* isolates that had previously been classified as being virulent towards both *LepR2* and *RlmS* based on cotyledon pathotyping. Three of the isolates had deletions of *AvrLep2*, while the remaining five all contained intact *AvrLep2* alleles of either the v23.1.3 (A^{278}) or 00-100 (G^{278}) haplotype, based on whole-genome resequencing data (Table S9). After allowing the infections to proceed from the cotyledon into the stem, there was a visible difference amongst the isolates in internal stem infection of Topas-*LepR2* 12 weeks postinoculation, despite all of them (except the avirulent control 00-100) producing virulent cotyledon interactions. All seven isolates carrying an intact *AvrLep2* allele produced significantly less internal infection in the Topas-*LepR2* plants than in the susceptible Topas DH16516 control plants (Mann–Whitney test, p values ranging from 0.028 to <0.0001) (Figure 7, Table S9). Only one "*AvrLep2*" isolate (AI397) was able to produce relatively high infection in Topas-*LepR2*. In contrast, all three isolates that carried a deletion at the *AvrLep2* locus (B16-13, B18-10, and

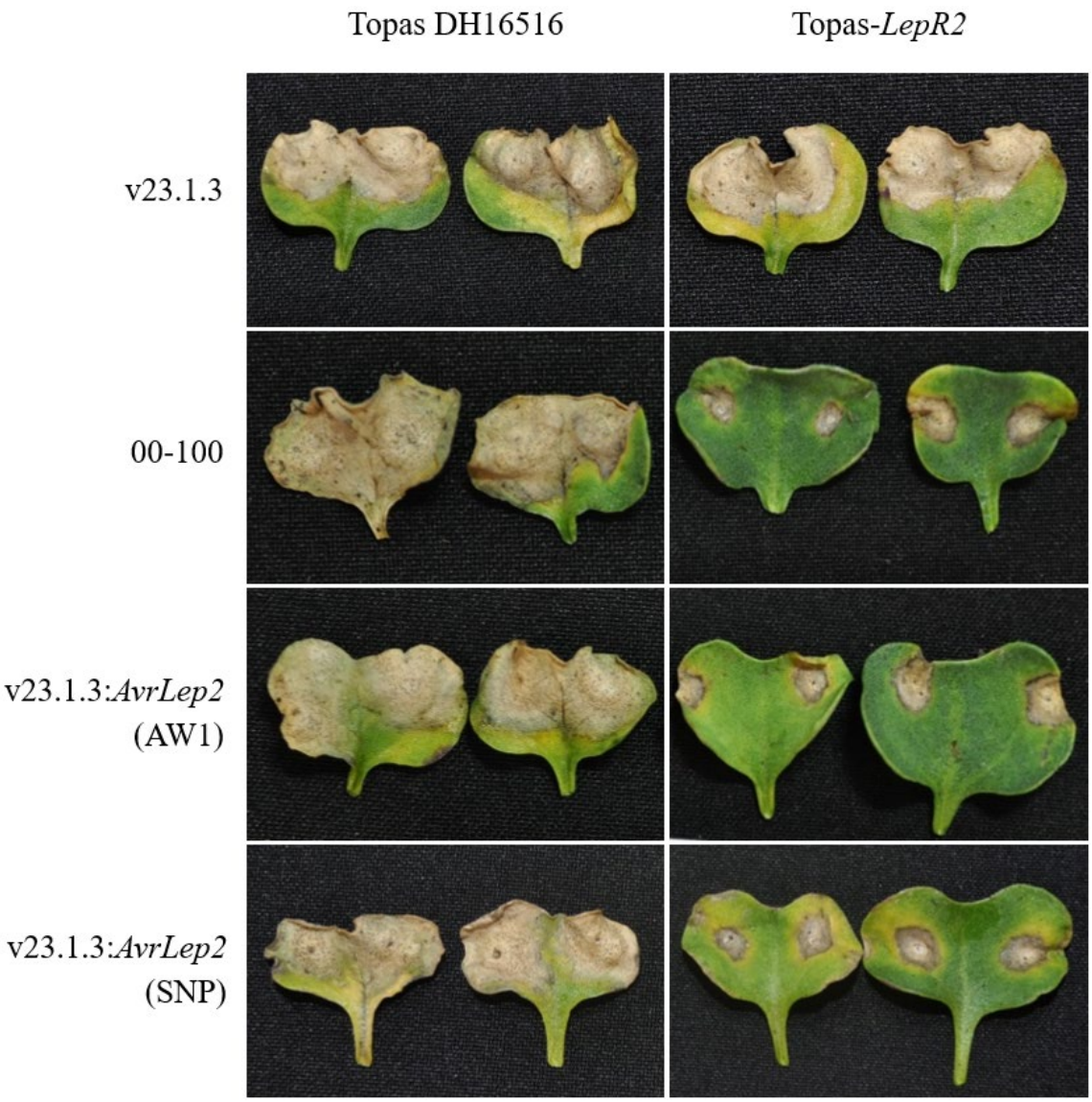

FIGURE 5 Phenotypic interaction of wild-type and complemented *Leptosphaeria maculans* isolates on the cotyledons of control (Topas DH16516) and Topas-*LepR2* lines. Photographs of the infected cotyledons were taken 14 days postinoculation. v23.1.3:*AvrLep2* (AW1) is a transformant with pNL11-*AvrLep2* construct (*AvrLep2* avirulent allele coding region was amplified from isolate 00-100, driven by its native promoter)

B18-11) produced severe and identical levels of infection in both the control Topas DH16516 and Topas-*LepR2* lines.

2.7 | A posteriori control of the consistency of phenotypic data

The sequenced reference isolate v23.1.3 had been previously shared between laboratories but here the interaction phenotypes of v23.1.3 inoculated on Topas-*LepR2* clearly differed between the AAFC (virulent phenotype) and BIOGER experiments (avirulent phenotype) despite the use of the same Topas-*LepR2* seed lot. To resolve this difference, the two v23.1.3 lines maintained for years in parallel at AAFC and BIOGER were shared again and phenotyped on differential plant genotypes, including Topas-*LepR2* at BIOGER. The interaction phenotypes of the two clonal isolates on Topas-*LepR2* were identical, with a clear resistance response compared to virulent control isolates (Figure S8). In addition, the two isolates behaved similarly on all other plant genotypes, including those containing resistance genes *Rlm1* or *Rlm4*, matching *AvrLm1* and *AvrLm4-7* present in v23.1.3. Finally, the sequence of *AvrLmS-Lep2* was identical in the two clonal isolates (data not shown). This suggests that environmental conditions or experimental parameters, not genetic drift after independent subculturing of the isolate in the two laboratories, explain the difference in the phenotypic expression of the *LepR2/AvrLmS-AvrLep2* interaction.

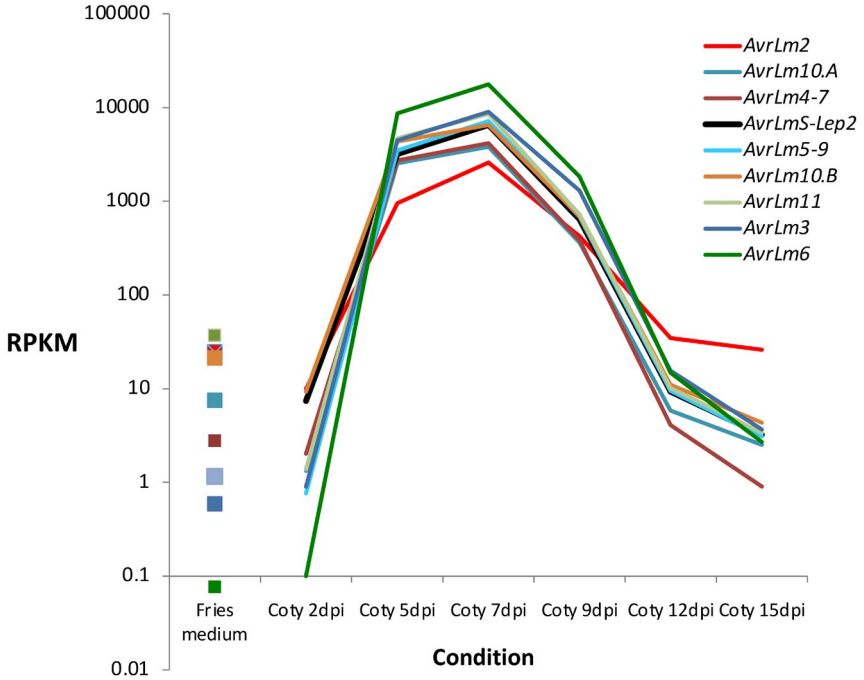

FIGURE 6 Expression of Lmb_jn3_08343 upon infection of oilseed rape cotyledons. Expression kinetics of Lmb_jn3_08343 was compared to that of previously cloned *AvrLm* genes. RNA-Seq data were obtained for isolate v23.1.2 in vitro (Fries medium condition) and following infection of cotyledons of cv. Darmor-*bzh* at 2, 5, 7, 8, 12 and 15 days postinfection (dpi). Values are reads per kilobase of transcript per million mapped reads (RPKM)

3 | DISCUSSION

In search of the avirulence genes matching *RlmS* in Surpass 400 and *LepR2* in Topas-*LepR2* (i.e., *AvrLmS* and *AvrLep2*, respectively), we report here on the independent cloning by two teams of the same avirulence gene, despite clear divergence between interaction phenotypes observed on resistant plant genotypes between the two laboratories. Cloning *AvrLmS* is the first example of applying the BSS strategy to clone a gene of interest from *L. maculans*. BSS is a powerful approach to rapidly identify candidate genes not only in plant species (Dong et al., 2018; Klein et al., 2018) but also in fungi (Hu et al., 2015; Lenhart et al., 2019). Here, we validated BSS on the previously cloned *AvrLm4-7* gene and found that bulks containing only about 10 isolates are sufficient to identify the genomic region containing the candidate gene. On the other hand, *AvrLep2* was cloned using the conventional biparental mapping approach. The gene was renamed here as *AvrLmS-Lep2*. Similar to all other *AvrLm* genes cloned to date, *AvrLmS-Lep2* is located in an AT-rich genomic environment, encodes for a small, secreted protein rich in cysteines, and the gene is highly overexpressed at early stages of cotyledon infections.

The phenotypic interaction of the reference isolate v23.1.3 on Topas-*LepR2* was interpreted as either an intermediate resistance phenotype or a virulent (susceptibility) phenotype. Such intermediate phenotypes have been reported for this interaction in many studies. The phenotypic resistance response of *B. napus* 'Surpass 400' inoculated with avirulent *AvrLmS* isolates was described as intermediate, that is, producing larger lesions than typical hypersensitive response (HR), and sometimes at the edge of virulence, depending on the environmental conditions or time (Van de Wouw et al., 2009). The resistance in Surpass 400 was initially considered to be monogenic, based on field assays in Australia (Li & Cowling, 2003) and genetic mapping (Yu et al., 2008), though later mapping with defined isolates under controlled conditions suggested the occurrence of two genes in Surpass 400 (named as *BLMR1* and *BLMR2*, Long et al., 2011; or *LepR3* and *RlmS*, Larkan et al., 2013). The resistance gene *LepR2* in the doubled-haploid line AD49 was described as limiting, but not preventing, hyphal growth of avirulent isolates, along with restricting sporulation on the infected plant tissues (Yu et al., 2005). In the same study, most field isolates inoculated (22 out of 32) were found to display large, nonsporulating lesions (scores between 3 and 6 on a 0–9 scale), while only eight isolates displayed typical HR (score <3) (Yu et al., 2005). Furthermore, in the study by Yu et al. (2005) the two most virulent isolates on *LepR2* displayed scores between 6 and 6.5, but never reached the scores observed on the susceptible genotypes (scores >7.5). This intermediate resistance phenotype was nevertheless correlated with the stem canker resistance (Yu et al., 2005), suggesting it is sufficient to prevent systemic growth of *L. maculans* in the leaves and stems. Similarly, *B. napus* plants harbouring the resistance gene *BLMR2* derived from the Surpass 400 parent also showed an intermediate resistance response at the cotyledon stage (Long et al., 2011), which correlated with the partial resistance response at the adult plant stage (Dandena et al., 2019), while *BLMR1*, corresponding to *LepR3* (Dandena et al., 2019), gave a strong and typical HR.

Consistent with these published data, such an intermediate resistant phenotype on Surpass 400 was also described here for either the avirulent parental isolate INV13.269 or the transgenic isolates complemented with the v23.1.3 allele of *AvrLmS*. The fluctuating intermediate phenotype resulting from the *AvrLmS–RlmS* or *AvrLep2–LepR2* interaction could be attributed to sequence variation in the gene, with only deleted *AvrLmS-Lep2* alleles, like those found in isolate WT50 or all virulent progeny of cross #82, responsible for clear susceptibility symptoms on Surpass 400 or Topas-*LepR2*, while variations in the nucleotide sequence of the

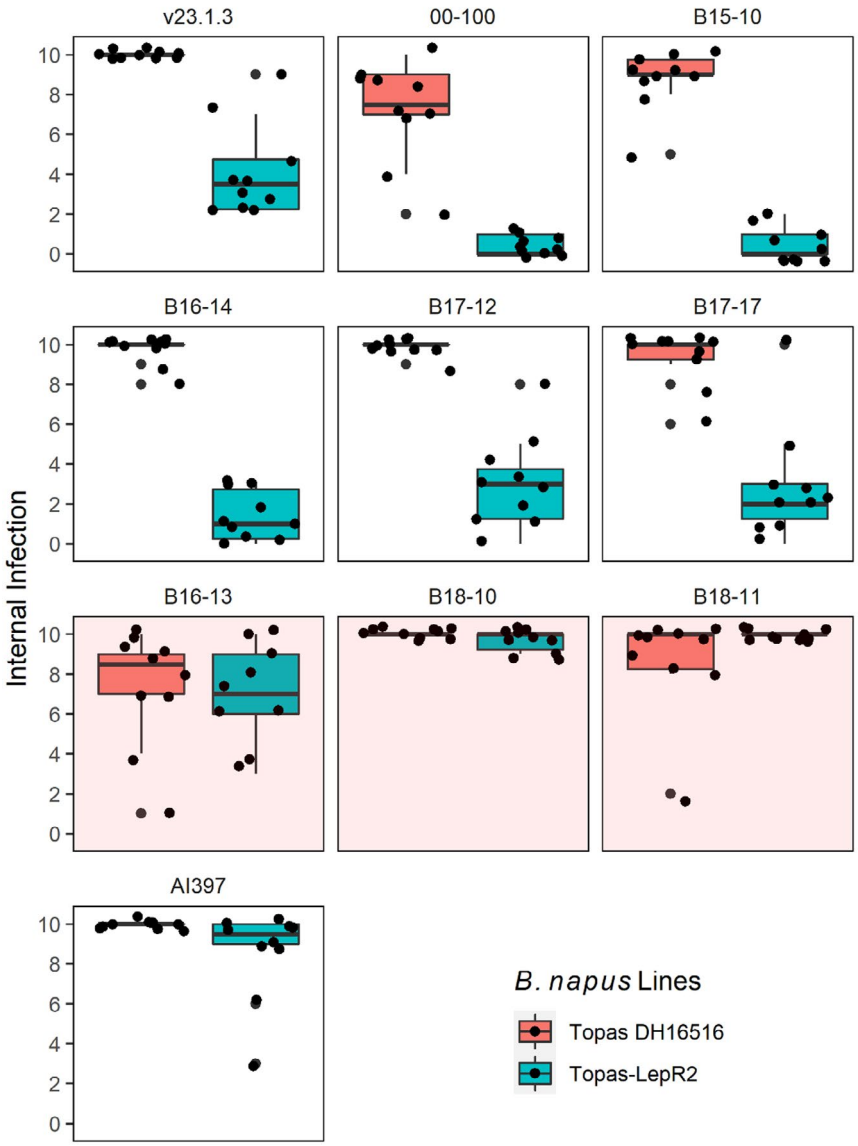

FIGURE 7 Box–whisker plot for internal infection of Topas DH16516 (red) and Topas-*LepR2* (blue) of adult plants by 10 *Leptosphaeria maculans* isolates. Data points (black dots) indicate internal infection (0–10 scale) of individual plants (10 per test). Boxes denote interquartile range (Q1 to Q3), black bars show median score, and whiskers denote range of distribution. Data points outside the whiskers (<1.5 Q3 or >1.5 Q1) are considered outliers. Red-shaded panels indicate isolates for which *AvrLep2* is deleted

gene could correspond to variable degrees of avirulence. However, no relationship between sequence variants and phenotypes was observed amongst sequenced isolates. Alternatively, the virulence phenotype observed in AAFC tests may be due to the expression level of the gene in v23.1.3 and its progeny. Expression data from AAFC indicated that *AvrLmS-Lep2* is expressed at a lower level than some other AVR genes, and at a similar level to *AvrLm5-9*, another AVR gene that generally also elicits an intermediate resistance response (Balesdent et al., 2002; Ghanbarnia et al., 2015; Plissonneau et al., 2018) that can be challenging to identify through cotyledon phenotyping. Another hypothesis to explain the variable expression of symptoms and contrasting interpretation of the interaction could be a strong influence of environmental conditions on the phenotypic outcome (or on expression level), resulting in an intermediate virulence phenotype in AAFC environmental conditions. Previous studies have shown the impact of temperature or humidity on the expression of some *AvrLm–Rlm* interactions (e.g., Huang et al., 2006). Consistent with that, the avirulent phenotype of v23.1.3 on Topas-*LepR2* observed at BIOGER was reproduced here under BIOGER's conditions using seed lots and an isolate used at AAFC. Importantly, regardless of the cotyledon phenotype, it was clearly shown that the presence of an *AvrLep2* allele in any isolate was sufficient to induce *LepR2*-dependent resistance in the adult plant assay, with variable but significant reduction of stem necrosis, while a deletion of the *AvrLmS-Lep2* gene always resulted in similar internal infection and stem lesions in the *LepR2* line as in the susceptible control (Figure 7).

Despite the divergent interpretation of the phenotypes, the use of crosses involving isolates with contrasting phenotypes on the resistant plant genotype was sufficient to allow us to identify and validate Lmb_jn3_08343 as the matching avirulence gene. Both approaches involved crosses between isolates displaying differential phenotypes on Surpass 400 and/or Topas-*LepR2*, with either a highly susceptible × intermediate resistance combination (WT50 × INV13.269) or a virulence × avirulence combination (v23.1.3 × 00-100) that finally targeted the same AVR gene.

Understanding the relationship between allelic variation and interaction phenotypes, and how environmental or experimental conditions, along with the effect of the plant genetic background, can modulate the outcome of the interaction should be further analysed in future work. Supported by our phenotypic data, we have shown that the genes *RlmS* and *LepR2*, both introgressed from *B. rapa* subsp. *sylvestris* sources (Crouch et al., 1994; Van de Wouw et al., 2009) recognize the same effector protein and are thus potentially the same resistance gene. The *AvrLmS-Lep2* transgenic isolates generated here could be further used to determine whether *BLMR2*, also obtained from Surpass 400, also recognizes the same avirulence gene using plant material (near-isogenic lines with or without *BLMR2*) recently described (Zhang et al., 2021). This work illustrates a first step toward the standardization of the complex and divergent terminologies used to describe *L. maculans*–*Brassica* sp. interactions.

4 | EXPERIMENTAL PROCEDURES

4.1 | Approach 1: Bulk segregant analysis

4.1.1 | *L. maculans* isolates and crosses

To map *AvrLmS*, a segregating progeny population was built following an in vitro cross between isolate WT50, isolated in Australia in 2005 (Van de Wouw et al., 2009), and INV13.269, recovered in 2013 in France. In vitro crosses and random ascospore progeny recovery were performed as previously established (Plissonneau et al., 2016).

4.1.2 | Plant genotypes and inoculation tests

Isolates were grown on 20% V8-agar medium to produce conidia. Conidia (10 μl, 10^7 spores/ml) were inoculated onto wounded cotyledons of 10 to 12 10-day-old seedlings per plant genotype. The following *B. napus* plant genotypes were used: Westar or Topas DH161516 (no *R* gene), 15-23-4-1 (*Rlm7*), Pixel (*Rlm4*), Columbus (*Rlm1*, *Rlm3*), Darmor (*Rlm9*), Bristol (*Rlm2-Rlm9*), Darmor-MX (*Rlm6-Rlm9*), 15.22.5.1 (*Rlm3*) (Balesdent et al., 2005), Topas-*LepR2* (*LepR2*; Larkan et al., 2016) Surpass 400 (*LepR3-RlmS*; Larkan et al., 2013), and the *RlmS*-line 72.1, an F_3 selection from Topas × Surpass 400 that retains *RlmS* resistance but lacks *LepR3* (Larkan et al., 2013). Four different isolates were inoculated on each plant. Symptoms were scored two or three times at 12–21 dai using a 1–6 scale, with scores 1–3 and 4–6 corresponding to avirulent and virulent phenotypes, respectively (Balesdent et al., 2005). To compare the interaction phenotypes of progeny isolates with those of parental isolates, the nonparametric Kruskall–Wallis test was applied, with a *p* value threshold set up at 0.05, using XLSTAT v. 2013.4.03. The phenotypes of the progeny selected for BSS were confirmed in an independent inoculation test.

4.1.3 | DNA extraction and bulk preparation

Isolates were grown on Fries liquid medium for 7 days as previously established (Fudal et al., 2007). Mycelium was harvested by vacuum filtration, rinsed with sterile deionized water, and freeze-dried. DNA was then extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA concentration was quantified using a Qubit dsDNA BR Assay Kit (Invitrogen). For library preparation, each isolate sample was adjusted to 20 ng DNA and pooled into six different bulks (Table 2). The final DNA concentration in the bulks was controlled similarly.

4.1.4 | Whole-genome sequencing

Between 74 and 200 ng of DNA was taken from each sample to prepare the DNA library. The DNA library was prepared using the Illumina Nextera DNA Flex Library Prep Kit (Illumina) according to the manufacturer's protocol. Whole-genome sequencing was performed on each of the eight bulks using Illumina Hi-Seq technology with 150 bp PE at the Kinghorn Centre for Clinical Genomics (KCCG) Core Facility at the Garvan Institute of Medical Research (Darlinghurst, Australia).

4.1.5 | Read mapping and variant calling

Quality trimming of reads was carried out using Trimmomatic v. 0.36 (Bolger et al., 2014) with default parameters and the Nextera paired-end adapters provided with the software. Reads were mapped to the reference isolate v23.1.3 (GenBank BioProject: PRJEB24468, Assembly GCA_900538235), using BWA 0.7.17 with the BWA-MEM algorithm (Li, 2013) and default parameters. Duplicates were removed using the Picard MarkDuplicates v. 2.8.1 (Picard Toolkit, 2019). Reads with mapping quality <20 were filtered using SAMtools v. 1.8 (Li et al., 2009). Variants were called using GATK HaplotypeCaller v. 3.6-0-g89b7209 (McKenna et al., 2010) with default parameters. Paired bulked samples were extracted (Bulk 1/2, Bulk 3/4, Bulk 5/6) using VCFtools v. 0.1.15 (Danecek et al., 2011) and variants with a phred-scaled quality score <30 were excluded. Indels were removed using GATK SelectVariants. The public reference genome repeat annotation was used to exclude SNPs occurring within repeats. SNPs that were heterozygous or monomorphic in parental isolates were also excluded. Finally, VCF files were converted to tabular format with GATK VariantsToTable.

4.1.6 | QTL-Seq and candidate SNP analysis

QTL-Seq of paired bulked segregants was carried out using the R package QTLseqr v. 0.7.3 (Mansfeld & Grumet, 2018; Takagi et al., 2013) with the QTL-Seq approach (Takagi et al., 2013). For SNP filtering settings, we used minTotalDepth = 100, maxTotalDepth = 800,

and a minimum genotype quality of 99. Reference allele frequency was required to be ≥0.2 and ≤0.8. Window size was set to $5e^4$. To complement this analysis, SNPs that segregated perfectly between *AvrLmS* (Bulk 1 and 2 including the parent INV13.269) and *avrLmS* (Bulk 5 and 6 including the parent WT50) were identified as candidate SNPs. We considered an SNP as perfectly segregating if it was called homozygous by GATK in all samples and the alleles differed between samples with *AvrLmS* and those with *avrLmS*. The candidate SNP positions were intersected with the gene annotation to identify candidate genes based on the presence of a candidate SNP in the gene sequence or in the 5 kb upstream/downstream region. The candidate region was also queried for long terminal repeats (LTR) using RepeatMasker. The GC content in the candidate QTL region was analysed using seqinr v. 3.4 (Charif & Lobry, 2007) and AT-rich regions were identified with OcculterCut v. 1.1 (Testa et al., 2016).

4.1.7 | Candidate gene analysis

Gene presence/absence variation (PAV) analysis was performed on the *AvrLmS* candidate gene using the SAMtools view utility (Li et al., 2009). Per base coverage of the candidate gene and upstream and downstream regions was calculated using BEDTools v. 2.26.0 (Quinlan & Hall, 2010) and plotted with ggplot2 in R. To search for gene homology of the *AvrLmS* candidate gene, the full nucleotide sequence of the candidate gene was queried using BLAST against the InterProScan database website. The genomic region surrounding the candidate gene, including 10 kb upstream and downstream regions, was also queried using BLAST on the NCBI database website. Expression of *AvrLmS* was examined from infection time course data previously generated following inoculation of isolate v23.1.2 on the susceptible cv. Darmor-*bzh*, or from in vitro culture conditions (Dutreux et al., 2018; Gay et al., 2021).

4.1.8 | Functional validation of the candidate gene

The *AvrLmS* candidate gene was amplified from genomic DNA of v23.1.3 (2537 bp fragment: 1049 bp upstream and 1062 bp downstream of the coding sequence) and cloned into the binary vector pPZPNat1 using Gibson assembly (New England Biolabs). The plasmid was amplified in *Escherichia coli* TOP10 cells, re-extracted, and checked by sequencing (Eurofins Genomics). The construct was introduced into *Agrobacterium tumefaciens* C58 by electroporation at 1.5 kV, 200 Ω, and 25 IF and used for transformation of two virulent isolates, WT50 and X82.14, as described by Gout et al. (2006). Fungal transformants were selected on 50 μg/ml nourseothricin (WERNER BioAgents), purified by single pycnidium isolation, and maintained on selective medium. Twenty-one and seven independent transformants were recovered for WT50 and X82.14, respectively. To control the deletion of the candidate gene in WT50 and in virulent progeny, the primers AvrLms-up (5′-GACTGCAACACCTCTTTTCCA-3′) and AvrLms-low (5′-CGCTCGATCCGTCCCTTATA-3′) were used on genomic DNA using standard PCR procedures and an annealing temperature of 60°C.

4.2 | Approach 2: Map-based cloning

4.2.1 | Phenotyping of mapping population

For mapping *AvrLep2*, an F_1 population produced from the parental isolates v23.1.3 and 00-100, previously used to map the *AvrLm5-9* locus (Ghanbarnia et al., 2018), was shown to also be segregating for the *AvrLep2* phenotype under the controlled growth chamber conditions used at AAFC Saskatoon (Larkan et al., 2013). The *B. napus* line Topas-*LepR2* (Larkan et al., 2016) and the *LepR2* line 1135 (Yu et al., 2012) were used to determine the phenotypic response of the parental isolates and progeny to *LepR2*. *B. napus* cotyledons were inoculated as described previously (Chen & Fernando, 2006). Each *L. maculans* isolate was tested on 12 seedlings of the differential lines and 12 seedlings of Topas as susceptible control. The disease reactions were scored 14 dai and rated using the 0–9 scale described by Williams (1985). Paired-end Illumina sequencing and assembly of parental isolates was previously described by Ghanbarnia et al. (2015).

4.2.2 | Expression analysis by RNA-Seq

Expression of *AvrLep2* was examined from infection time course data previously generated (Haddadi et al., 2016). Briefly, cotyledons of 7-day-old Topas DH16516 seedlings were inoculated with the parental isolates 00-100 and v23.1.3. Mock inoculation with water served as a negative control. Cotyledon discs 6 mm in diameter were excised from the infected cotyledons (four biological replicates) at 2, 4, 6, and 8 dai. RNA was extracted and sequence reads (100 bp paired-end) were generated with Illumina TruSeq high output version 3 chemistry on a HiSeq 2500 (Illumina) at NRC-Plant Biotechnology Institute (NRC-PBI), Saskatoon, Canada.

4.2.3 | Mapping, cloning, and transformation of the candidate gene

SNPs for primer development were selected based on whole-genomic comparison of parental isolates or based on predicted polymorphic effectors from isolate 00-100 and v23.1.3 using CLC Genomic Workbench (v. 8.1.1; CLC Bio). Then the target SNP(s) were used to design the KASP primers using the PrimerPicker software provided by KBioscience (https://www.kbioscience.co.uk/). KASP reactions were performed as per the manufacturer's instructions (LGC Biosearch; https://www.biosearchtech.com). One hundred F_1 progeny were selected to screen KASP markers spanning the whole *L. maculans* genome (Rouxel et al., 2011). A linkage map of *AvrLep2* was constructed using the MAP function of QTL IciMapping v. 3.2

software (Li et al., 2008). Minimum LOD (logs of the odds ratios of linkage vs. no linkage) scores of 6.0 (maximum recombination fraction of 0.6) were used to group loci. After initial linkage between markers and the *AvrLep2* locus was established, additional KASP markers targeted to the *AvrLep2* interval were designed based on genomic polymorphisms to enrich the map. Cloning, transformation, and functional validation of *AvrLep2* was performed as described previously (Ghanbarnia et al., 2015). For functional validation two constructs were produced. First, the open reading frame for the *AvrLep2* candidate gene (426 bp) was amplified and transferred to the fungal transformation vector pLM4 (Ghanbarnia et al., 2015) under the control of the *AvrLm1* promoter. In addition, an *AvrLep2* candidate gene amplicon, including the native promoter region (starting from 1996 bp upstream of the ATG start codon based on the v23.1.3 reference sequence) and 186 bp downstream of the predicted open reading frame (total length 2609 bp), from the *AvrLep2* parental isolate 00-100 was transferred into the fungal transformation vector pNL11 (Larkan et al., 2013). To confirm the *AvrLep2* specificity, the phenotypic response of the parental isolates and positive transformants (showing restored phenotypic reaction on Topas-*LepR2*) was tested on the following *B. napus* differential lines: Topas-*Rlm1*, Topas-*Rlm2*, Topas-*Rlm4*, Topas-*LepR1*, Quantum (*Rlm3*), Roxet (*Rlm7*), Goéland (*Rlm9*), and the *B. juncea* line Vulcan-1S (*Rlm6*) (Larkan et al., 2016). Topas DH16516 (no R genes) was used as a positive control for infection by *L. maculans*.

4.2.4 | Adult plant tests

Isolates carrying different alleles of *AvrLep2* (v23.1.3-type, 00-100-type or deletion, as well as one unique mutation) and classified as virulent towards both *LepR2* (Topas-*LepR2* line) and *RlmS* (72-1) were used to infect Topas DH16516 and Topas-*LepR2* seedlings via a standard cotyledon wounding method. The plants were maintained under controlled conditions (Haddadi et al., 2019) and infection was allowed to progress into the stem (cotyledons were not removed). The resistance phenotype was scored in the adult plants by assessment of internal infection in the stem at 8–12 weeks postinfection. Stem infection was rated using a 0–10 scale, where each graduation corresponds to 10% of the internal cross-section showing infection damage. Results were plotted using the ggplot2 (Wickham, 2016) and reshape2 (Wickham, 2007) packages in R v. 4.0.0 (R Core Team, 2020), run in RStudio v1.3.959.

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AUTHOR'S CONTRIBUTION

T.X.N., K.G., N.J.L., B.O., and A.S.E. conducted the experiments. A.S. and P.H. performed bioinformatics analysis. T.X.N., A.S., B.O., K.G., M.H.Bo., N.J.L., M.H.Ba., and T.R. analysed the data. J.B., T.R., K.G., N.J.L., M.H.Ba., M.H.Bo., and T.X.N. conceived the idea. J.B., T.R., and M.H.Ba. supervised the *AvrLmS* project. M.H.Bo. and W.G.D.F. supervised the *AvrLep2* project. M.H.Ba. coordinated the writing of the publication.

DATA AVAILABILITY STATEMENT

Data is available on request from the authors.

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