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
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 symptomatically 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 major race-specific resistance (R) genes, matching avirulence genes have already been cloned: *AvrLm4-7, AvrLm5-9, AvrLm6, AvrLm10, AvrLm11*, and *AvrLm1*.Mutations in the corresponding AVR genes can thus help us understand the relationships a given isolate may hide a more complex R gene control due to epistatic effects between genes.

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.

## 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.
between AvrLm6, AvrLm4-7, and AvrLmS ($\chi^2 = 8.25$, $p = 0.689$; 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 (A7S) and 6 (aS7) contained isolates being avirulent on RlmS and Rlm7, or virulent on both genes, respectively (Table 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
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.

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

| Parental isolates | Number of isolates (%) |
|-------------------|------------------------|
| WT50              | V A V 15 (23.4%)       |
| INV13.269         | A V A 11 (13.1%)       |

### 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 | 11                                       | 809                                 | 1.00                                                          |
| 6                  | avrLmS + avrLm7 (a7S)        | Progeny, virulent on Surpass 400  | 9                                        | 0                                   | 0.00                                                          |
| 7                  | INV13.269 (a7AS)             | Parental isolate                | 1                                        | 1056                                | 1.00                                                          |
| 8                  | WT50 (A7aS)                  | Parental isolate                | 1                                        | 1                                   | 0.03                                                          |

Abbreviations: A, avirulent phenotype; na, not applicable; V, virulent phenotype.

*p value of the X² test for a 50:50 segregation ratio.
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 F1 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 Rlm5), 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 DH16156 but displayed a clear resistant phenotype on Topas-LepR2 (Figures 1, 4, and S6) and on the Rlm5 line 72.1 (Figure 4). Therefore, Lmb_jn3_08343 encodes the avirulence effector protein corresponding to Rlm5 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](image-url)  
**Figure 4**  
Phenotypic confirmation of the elicitation of both LepR2 and Rlm5 resistance responses by Lmb_jn3_08343. Reference isolates (white, avirulent on Rlm5), wild-type virulent isolates (orange), and a selection of transformants with the AvrLm5 candidate gene Lmb_jn3_08343 (blue) were inoculated on Topas DH16156 (devoid of Rlm gene), Topas-LepR2 (LepR2), 72.1 (Rlm5), or Surpass 400 (Rlm5+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.
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 (A278) or 00-100 (G278) haplotype, based on whole-genome sequencing 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
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 AvrLm5-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/AvrLm5-AvrLep2 interaction.
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., 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 INVI3.269 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
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 BIOCER was reproduced here under BIOCER’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 x intermediate resistance combination (WT50 x INV13.269) or a virulence x avirulence combination (v23.1.3 x 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 analyzed 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.

### 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). 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 QTLseq 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. 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 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).

4.1.7 | 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'-CGCTCGATCCGCTCCCTTATA-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 F1 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 F1 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
under the control of the fungal transformation vector pLM4 (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 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 Rlm5 (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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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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