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Rapid detection of *Leptosphaeria maculans* avirulence gene *AvrLm4-7* conferring the avirulence/virulence specificity on *Brassica napus* using a tetra-primer ARMS-PCR

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Abstract *Leptosphaeria maculans* is the causal agent of blackleg disease in canola (*Brassica napus*), resulting in significant yield loss in canola fields worldwide. *AvrLm4-7* is an avirulence effector gene in *L. maculans*, and a single nucleotide mutation at codon 358 is responsible for the absence of the *AvrLm4* allele. A tetra-primer amplification refractory mutation system-PCR assay (ARMS-PCR) was developed to rapidly differentiate the *AvrLm4AvrLm7* and *avrLm4AvrLm7* genes of *L. maculans* isolates, which differ by a single point mutation. By this approach, we were able to amplify distinct PCR products to infer the gene of the tested isolates. These results were also confirmed through phenotyping, using the cotyledon inoculation test and two canola genotypes with the corresponding resistance genes. The tetra-primer ARMS-PCR assay developed in this study is a simple, rapid, and useful protocol to identify the *AvrLm4-7* alleles in *L. maculans* isolates. This assay has potential applications in the selection of resistant canola cultivars as part of broader antifungal strategies.

Keywords *Leptosphaeria maculans* · *Tetra-primer ARMS-PCR* · *AvrLm4-7* · *Canola*

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Blackleg caused by the fungal pathogen *Leptosphaeria maculans* is one of the most important diseases affecting canola (*Brassica napus*). *L. maculans* is a hemibiotrophic fungal pathogen responsible for the stem canker of *B. napus* and other *Brassica* species. Blackleg disease has been mainly controlled by using resistant cultivars and crop rotations (Fernando et al. 2016). Gene-for-gene interactions between a resistance gene (*R*) in *B. napus* and its corresponding avirulence (*Avr*) gene in *L. maculans* have been extensively studied (Ansan-Melayah et al. 1998; Balesdent et al. 2001). Genome sequencing of one *L. maculans* isolate (JN3) indicated that avirulence genes were usually distributed in gene-poor and AT-rich regions (Rouxel et al. 2011). To date, a total of 16 *Avr* genes in *L. maculans* have been identified, and seven of these (*AvrLm1*, *AvrLm6*, *AvrLm5/J1*, *AvrLm2*, *AvrLm4-7*, *AvrLm3* and *AvrLm1*) have been cloned (Gout et al. 2006; Fudal et al. 2007; Van de Wouw et al. 2014; Ghanbarnia et al. 2015; Parlange et al. 2009; Balesdent et al. 2013; Plissonneau et al. 2015). In a six-year canola disease survey of the avirulence allele profiles of *L. maculans* in Manitoba, Canada, *AvrLm7*and *AvrLm4-7* were found to be carried by more than 90% of the isolates (Fernando et al. 2017). Using a map cloning strategy and structure analysis, it is found that *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of *B. napus*, as well as that a single nucleotide change, which leads to a single amino acid mutation, is responsible for the loss of *AvrLm4* specificity (Parlange et al. 2009). The single base mutation, C^{358} to G^{358} of the *AvrLm4-7* gene, resulting in an amino acid change

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from arginine (R) to glycine (G), was sufficient to complement the lost *Rlm4*-mediated recognition specificity (Parlange et al. 2009). In an investigation of 300 isolates for the occurrence of alleles of *AvrLm4* and *AvrLm7*, only *avrLm4AvrLm7* and *AvrLm4AvrLm7* were present in the collection. No isolate had *AvrLm4avrLm7* and avirulence towards a *Rlm4 B. napus* genotype (Parlange et al. 2009). Also, in our six-year collection from a canola blackleg disease survey, we did not detect the *AvrLm4avrLm7* avirulence allele (Fernando et al. 2017), which was consistent with previous observations by Parlange et al. (2009).

Currently, methods used for the genetic identification of *AvrLm4-7* and *AvrLm7* rely on the polymerase chain reaction (PCR) of the *AvrLm4-7* gene and cotyledon inoculation test on *B. napus* genotypes harbouring *Rlm4* and *Rlm7* genes (Zhang et al. 2016). A single nucleotide mutation provides the possibility to rapidly identify the *AvrLm4-7* and *AvrLm7* gene specificities in *L. maculans* isolates towards the canola resistance by *Rlm4* and *Rlm7* gene. Genotyping of single nucleotide polymorphisms (SNPs) has been widely used to study the genetic determinants of complex diseases. For example, SNP genotyping can be applied to detect the resistance of *Fusarium graminearum* to benzimidazole fungicides caused by a single nucleotide mutation (Hou et al. 2013). Multiple methods for SNP genotyping have been developed, including DNA sequencing, allele-specific PCR, single strand conformation polymorphism PCR, the mismatch amplification mutation assay, PCR-restriction fragment length polymorphism (PCR-RFLP) and TaqMan-MGB (Minor Groove Binder). Each of these strategies has disadvantages [e.g., costly (DNA sequencing), extensive optimization (allele-specific PCR) and lacking restriction enzyme recognition sequence (PCR-RFLP)]. Currently, Kompetitive Allele Specific PCR (KASP) has been widely used and is cost-effective for SNP genotyping. However, it relies on a fluorescent- labelled primer for PCR amplification, the reading of the fluorescence signal by specific platform (e.g., Omega F plate reader, or a real time PCR machine) is needed. The high expense of tools and reagents is another issue to be taken into consideration. A relatively inexpensive and equipment friendly method for SNP genotyping is the tetra-primer amplification refractory mutation system-PCR (Tetra-primer ARMS-PCR). This is a sensitive, reliable, and cost-effective method for detecting single nucleotide mutations (Ye et al. 2001; Hou et al. 2013; Baris et al. 2010). Tetra-primer ARMS-PCR employs two primer pairs, including an outer primer pair for amplifying the common template for the subsequent allele-specific amplification, while the inner primer pair is designed as allele-specific with its 3′ end located at the SNP, respectively, in a single PCR amplification.

In this study, we developed a tetra-primer ARMS-PCR assay to rapidly differentiate the *AvrLm4AvrLm7* and *avrLm4AvrLm7* genes in *L. maculans* isolates based on the determinant SNP (Parlange et al. 2009). A total of 29 *L. maculans* candidate isolates (Can1 to 29) derived from infected stubble in our disease survey collection from Manitoba canola growing fields were employed for DNA extraction and avirulence gene profiling. A whole genome sequenced *L. maculans* isolate, JN3, was also included as a control (Rouxel et al. 2011). All of the isolates were cultured and maintained on V8 juice medium. Two *B. napus* genotypes, Jet Neuf and 02-23-2-1, carrying resistance genes *Rlm4* and *Rlm7* respectively, were used to characterize the avirulence genes in the *B. napus* cotyledon inoculation test. The inoculum preparation, canola cotyledon inoculation test, and rating system were conducted according to Zhang et al. (2016). Here we aimed to distinguish the *AvrLm4AvrLm7* and *avrLm4AvrLm7* genes of *L. maculans* from isolates using a rapid PCR identification, without the *B. napus* cotyledon inoculation test.

According to the SNP at codon 358 (G-C) of the *AvrLm4-7* gene, the tetra-primers of ARMS-PCR were designed using a web-based tool (http://primer1.soton.ac.uk/primer1.html). To increase the specificities of the primers, a mismatch base was introduced at the 3′ end of the two inner allele-specific primers. The primers used in this study were as follows: two common outer primers, 5′–GTA ACA AAG TAA CGA AGG GCT TA ATT–3′ and 5′–GAA AAC TCA CCT CCG TAT CTT TAG TC–3′; an inner primer for the ‘G’ allele, 5′– TCT AAA CCA GTC TCC TGC CC–3′; and an inner primer for the ‘C’ allele, 5′–TAG CTC AGC ACC TGG AGT TAT ATA TC–3′. The PCR amplicons were expected to be 356 bp (common outer primer), 167 bp with specific ‘C’ allele and 235 bp with specific ‘G’ allele. The annealing temperature and concentrations of the outer and inner specific primers were optimized by conventional PCR. Finally, the tetra-primer ARMS-PCR assay was carried out in a total volume of 25 μL, including approximately 50 ng fungal DNA, 2.5 μL of 10× PCR buffer within $MgCl_2$, 2 μL of dNTP (2.5 mM each), 0.4 μL of each outer primer (10 pmol), 1 μL of each inner specific allele primer (10 pmol), and 1.5 U Taq polymerase (Econo Taq DNA polymerase,

Lucigen, Middleton, WI). The PCR cycling was optimized as: 94 °C for 3 min of denaturation; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. The PCR product (8 μL) was then separated and visualised by electrophoresis on a 2.0% agarose gel at 100 V for 45 min and stained with Redsafe (iNtRON BIOTECHNOLOGY, Seongnam-Si, Gyeonggi-do, Korea).

As expected, the *AvrLm4-7* gene in the *L. maculans* isolate at codon 358 harbouring the 'G' allele was amplified with 235 bp and 356 bp bands, while the isolate harbouring the 'C' allele was amplified with 167 bp and 356 bp bands (Fig. 1 and Table 1). Of the 30 tested *L. maculans* isolates, 17 amplified 235 and 356 bp bands for the 'G' allele. These isolates carry the *AvrLm4AvrLm7* gene, according to the result obtained by Parlange et al. (2009). The remaining 13 isolates, producing 167 and 356 bp PCR products ('C' allele), carry the *avrLm4AvrLm7* gene. To confirm the allele specificity of these *L. maculans* isolates, we amplified the *AvrLm4-7* gene and sequenced the PCR product using primers 5′–TAT CGC ATA CCA AAC ATT AGG C–3′ (forward) and 5′–GAT GGA TCA ACC GCT AAC A–3′ (reverse). The sequencing data were consistent with the tetra-primer ARMS-PCR results (Fig. S1). Based on these findings, we conclude that the tetra-primer ARMS-PCR assay is a useful method in the discrimination of specific alleles of the *AvrLm4-7* gene, used to identify the *Avr* genes and races of these isolates.

To further validate the *Avr4-7* gene profile, we inoculated these 30 *L. maculans* isolates onto cotyledons of two canola genotypes, Jet Neuf and 02-23-2-1, harbouring *Rlm4* and *Rlm7* genes respectively. At 14 days post-inoculation (dpi), the infected cotyledons were tested to establish the disease severity. The *L. maculans* isolates ($n = 17$) carried *AvrLm4* and *AvrLm7* when both genotypes (Jet Neuf and 02-23-2-1) showed resistance to these isolates. The remaining 13 isolates carried only *AvrLm7*, since the canola genotype 02-23-2-1 showed resistance to those isolates, whereas Jet Neuf showed susceptibility (Table 1). The avirulence genes characterized by cotyledon inoculation test were consistent with our tetra-primer ASMR-PCR findings. To apply the tetra-primer ARMS-PCR assay in our canola blackleg diseases survey, the assay was used to test 288 *L. maculans* isolates. We found that their tetra-primer ARMS-

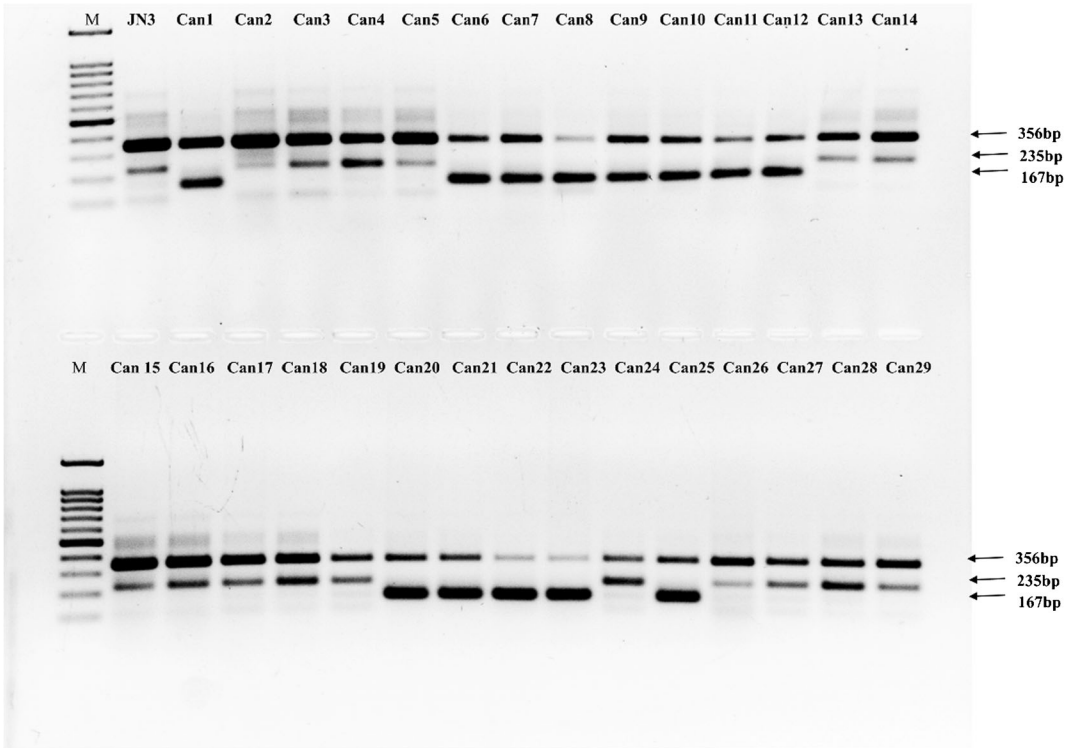

Fig. 1 Tetra-primer ARMS-PCR for detection the genotype of single nucleotide mutation at codon 358 in the *AvrLm4-7* gene of *L. maculans* isolates. M is a DNA ladder (100 bp)

Table 1 Summary of avirulence gene profiles of 30 *L. maculans* isolates determined by tetra-primer ARMS-PCR and canola cotyledon inoculation test

			ARMS-PCR		Plant differential		
Isolate ID	Original source	SNP by sequencing	Amplicons (bp)	*Avr* gene/Genotype	Jet Neuf (*Rlm4*)	02-23-2-1 (*Rlm7*)	*Avr* gene/Phenotype
JN3	JN3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can1	CDS15-18-3	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can2	CDS15-16-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can3	CDS15-107-2	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can4	CDS15-140-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can5	CDS15-50-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can6	DS103	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can7	CDS15-10-1	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can8	CDS15-10-3	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can9	CDS15-11-1	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can10	CDS15-11-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can11	CDS15-79-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can12	CDS15-60-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can13	CDS15-140-1	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can14	CDS15-35-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can15	CDS15-35-2	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can16	CDS15-1-2	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can17	CDS16-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can18	CDS16-5	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can19	CDS16-20-2	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can20	CDS16-7-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can21	CDS16-25-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can22	CDS16-29-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can23	CDS16-33-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can24	CDS16-46-2	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can25	CDS16-40-2	C	167&356	*avrLm4AvrLm7*	*S*	*R*	*AvrLm7*
Can26	CDS16-25-1	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can27	CDS16-26	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can28	CDS16-29-1	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*
Can29	CDS16-33-3	G	235&356	*AvrLm4AvrLm7*	*R*	*R*	*AvrLm4-7*

a: R, S = *Brassica* genotypes that displayed resistance (rating score < 4.5) and susceptibility (rating score 6.1 to 9.0), to *Leptosphaeria maculans* isolates (Zhang et al. 2016). The scores were averaged from at least 6 plants

PCR-determined *Avr* genes were the same as the *Avr* gene profiles characterized by cotyledon inoculation test. The tetra-primer ARMS-PCR assay developed in this study was successfully applied to the detection of the single nucleotide mutation in the *AvrLm4-7* gene that is responsible for the absence of the *AvrLm4* allele. The *Avr* gene profile inferred from the tetra-primer ARMS-PCR assay was validated by DNA sequencing technology and the canola cotyledon inoculation test.

The success of tetra-primer ARMS-PCR assays depends on the specific primer design and a mismatched nucleotide in the primer's 3′ end to bind the mutation codon. Primer specificity and the annealing temperature, as well as the concentration of primers, Mg^{2+} and Taq DNA polymerase, can also influence the sensitivity of

the tetra-primer ARMS-PCR assay. For example, the ratio between the inner primer and outer primer has been reported as 10:1 (Ye et al. 2001), 3:2 (Bu et al. 2004), 5:1 (Guan et al. 2005) and 2.5:1 (Hou et al. 2013). In this study, we optimized the ratio between the inner primer and out primer as 2.5:1, which could amplify with best-visualized fragments to distinguish the avirulence gene in *L. maculans* isolates. The annealing temperature was optimized as 55 °C from 50 °C to 60 °C, and the touchdown PCR strategy was also found to affect the sensitivity of the tetra-primer ARMS-PCR assay. Therefore, optimization of the PCR reagents, conditions and strategies is important for successfully performing the tetra-primer ARMS-PCR assay.

The canola blackleg disease survey along with the profiling of genes of *L. maculans*, and their races is done in order to assist canola growers to select resistant cultivar with a knowledge of the population dynamics of the blackleg pathogen in western Canada. However, to obtain more accurate avirulence gene profiles, researchers would need to conduct both PCR and plant differential tests to determine the *Avr* genes on thousands of *L. maculans* isolates, which is laborious, time-consuming and costly. The absence of *AvrLm4* by a single nucleotide mutation in the *AvrLm4-7* gene allowed us to develop a rapid tetra-primer ARMS-PCR assay to detect the *AvrLm4* and *AvrLm7* allele of *L. maculans* isolates, without the cotyledon inoculation test. In summary, the tetra-primer ARMS-PCR assay developed in this study is a simple, rapid, and economical method to identify the *AvrLm4-7* gene in *L. maculans* isolates and could potentially be used in the identification of other *Avr* genes. This assay will help researchers and growers to select the most resistant canola cultivars as part of broader disease management strategies.

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1) the manuscript has not been published in whole or in part elsewhere;
2) the manuscript is not being considered to submit in another journal currently;
3) the manuscript is not split up to several parts to increase the quantity of submissions.

Conflict of interest The authors declare no conflict of interests.

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