

ORIGINAL ARTICLE

Independent breakdown events of the *Brassica napus* *Rlm7* resistance gene including via the off-target impact of a dual-specificity avirulence interaction

Angela P. Van de Wouw¹  | Elizabeth M. Sheedy² | Andrew H. Ware³ |
Stephen J. Marcroft² | Alexander Idnurm¹

¹School of BioSciences, University of Melbourne, Parkville, Victoria, Australia

²Marcroft Grains Pathology, Horsham, Victoria, Australia

³EP Ag Research, Port Lincoln, South Australia, Australia

Correspondence

Angela P. Van de Wouw, School of BioSciences, University of Melbourne, Parkville, Victoria 3010, Australia.
Email: apvdw2@unimelb.edu.au

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Abstract

Protection of many crops is achieved through the use of genetic resistance. *Leptosphaeria maculans*, the causal agent of blackleg disease of *Brassica napus*, has emerged as a model for understanding gene-for-gene interactions that occur between plants and pathogens. Whilst many of the characterized avirulence effector genes interact with a single resistance gene in the host, the *AvrLm4-7* avirulence gene is recognized by two resistance genes, *Rlm4* and *Rlm7*. Here, we report the “breakdown” of the *Rlm7* resistance gene in Australia, under two different field conditions. The first, and more typical, breakdown probably resulted from widescale use of *Rlm7*-containing cultivars whereby selection has led to an increase of individuals in the *L. maculans* population that have undergone repeat-induced point (RIP) mutations at the *AvrLm4-7* locus. This has rendered the *AvrLm4-7* gene ineffective and therefore these isolates have become virulent towards both *Rlm4* and *Rlm7*. The second, more atypical, situation was the widescale use of *Rlm4* cultivars. Whilst a single-nucleotide polymorphism is the more common mechanism of virulence towards *Rlm4*, in this field situation, RIP mutations have been selected leading to the breakdown of resistance for both *Rlm4* and *Rlm7*. This is an example of a resistance gene being rendered ineffective without having grown cultivars with the corresponding resistance gene due to the dual specificity of the avirulence gene. These findings highlight the value of pathogen surveillance in the context of expanded knowledge about potential complexities for Avr-R interactions for the deployment of appropriate resistance gene strategies.

KEYWORDS

avirulence genes, dual specificity, repeat induced point mutations, resistance breakdown

1 | INTRODUCTION

Brassica napus (canola, oilseed rape) is the second most important oilseed crop worldwide, with 72 million tonnes of seed harvested in

2019 (Zheng et al., 2020). One of the major production constraints of canola is blackleg disease, caused by the fungus *Leptosphaeria maculans*. This disease is found worldwide, with the exception of China, and causes annual yield losses of 5%–20% in Europe, Canada, and

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Australia, with localized epidemics resulting in up to 90% yield loss (Fitt et al., 2006; Hwang et al., 2011; Sprague et al., 2006a; Zheng et al., 2020). *L. maculans* is a sexually reproducing pathogen that releases ascospores from the colonized crop debris (stubble) with each rainfall event during the growing season. These ascospores land on the leaves, flowers, upper stems or branches and can colonize the tissue and grow within the vascular tissue, stopping nutrient flow throughout the plant (Hammond et al., 1985; Sprague et al., 2018).

The impact of blackleg disease can be minimized through the use of cultural, chemical, and genetic approaches. Cultural practices such as rotation of crops and isolation from the previous year's stubble can significantly reduce disease (Marcroft et al., 2003, 2004); however, in regions such as Australia, these approaches are becoming difficult to achieve due to increases in canola production (Van de Wouw et al., 2021). Fungicides can minimize blackleg disease with applications possible at seeding, four- to six-leaf, and 30% bloom growth stages (Peng et al., 2020; Van de Wouw et al., 2016, 2021). Fungicides are available worldwide for control of blackleg disease, with growers in Australia using at least one fungicide on approximately 90% of crops (Van de Wouw et al., 2021).

Whilst the type and dependency of cultural and chemical practices vary across growing regions, the use of genetic resistance is a universal approach to minimizing blackleg disease. There are two types of resistance involved in the *L. maculans*-*B. napus* interaction: quantitative (minor) and qualitative (major) (Delourme et al., 2006). Quantitative or minor gene resistance, whilst poorly understood, is thought to involve the small contribution of a number of minor genes to give an overall resistant phenotype (Stuthmann et al., 2007). Quantitative resistance is generally thought to be expressed in the adult plants, be extremely durable, and provide equal protection against all isolates (Stuthmann et al., 2007). However, in the *L. maculans*-*B. napus* interaction quantitative resistance appears to be isolate-specific and expressed throughout the life of the plant (Jiquel et al., 2021; Marcroft et al., 2012a; Schnippenkoetter et al., 2021).

Qualitative or major gene resistance is well understood in the *L. maculans*-*B. napus* interaction and occurs via a gene-for-gene interaction whereby for each *Brassica* resistance gene there is a corresponding avirulence (Avr) effector gene in *L. maculans* (Balesdent et al., 2005; Flor, 1955). These resistance genes provide protection throughout the life of the plant and have been shown to be effective in all plant parts in the *B. napus*-*L. maculans* interaction (Balesdent et al., 2005; Elliott et al., 2016; Marcroft et al., 2012a; Sprague et al., 2018). To date, 19 resistance genes or alleles of genes have been identified from *Brassica* species (*Rlm1*-*Rlm11*, *Rlm13*, *Rlm14*, *Rlm5*, and *LepR1*-*LepR4*) and five of these genes, *LepR3*, *Rlm2*, *Rlm4*, *Rlm7* and *Rlm9*, have been cloned (Degrave et al., 2021; Delourme et al., 2006; Haddadi et al., 2021; Larkan et al., 2013, 2015, 2020; Long et al., 2011; Raman et al., 2021; Van de Wouw et al., 2009; Yu et al., 2005, 2008, 2013). In comparison, 11 of the corresponding Avr effector genes have been cloned from *L. maculans* (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-Houdent

et al., 2019; Plissonneau et al., 2016, 2018; Van de Wouw et al., 2014b).

Following the identification of many of the Avr effector genes from *L. maculans*, the gene-for-gene interaction was found not to be as simple as first thought. To date, three of the *L. maculans* Avr genes have been shown to have dual specificity (*AvrLm1-R3*, *AvrLm4-7* and *AvrLm5-9*), whereby a single avirulence gene is recognized by two separate resistance genes (for review see Van de Wouw & Howlett, 2020). The reverse has also been shown whereby two avirulence genes, *AvrLm10A* and *AvrLm10B*, are recognized by a single resistance gene (*Rlm10*) (Petit-Houdent et al., 2019). To complicate the interaction even further, *AvrLm4-7* is epistatic over *AvrLm3* and *AvrLm9* whereby when an isolate is avirulent towards *Rlm7*, the *AvrLm3-Rlm3* and *AvrLm9-Rlm9* interactions are masked, and the isolates appear virulent towards both *Rlm3* and *Rlm9* regardless of their genotype (Ghanbarnia et al., 2018; Plissonneau et al., 2016).

The mechanisms of virulence also vary dramatically depending on the avirulence gene (for review see Van de Wouw & Howlett, 2020). Virulence towards *Rlm1* and *Rlm6*, for example, is usually conferred through deletion of the entire corresponding avirulence genes (Fudal et al., 2009; Gout et al., 2007). Virulence towards *Rlm2* and *Rlm4* is usually conferred through single-nucleotide polymorphisms (SNPs) that result in a single amino acid substitution (Ghanbarnia et al., 2015; Parlange et al., 2009). Virulence towards *Rlm7* is conferred by either deletion of the gene or through repeat-induced point (RIP) mutations (Mitrousia et al., 2018; Parlange et al., 2009), which is a genome defence mechanism that occurs during sexual crossing and generates G to A and C to T transitions within repetitive sequences (Selker, 1990).

Whilst genetic resistance provides protection to blackleg, if the pathogen population changes then resistance can be overcome and rendered ineffective. When cultivars harbouring major resistance genes are grown continuously, this creates strong selection pressure towards any isolates in the population that are virulent towards that corresponding resistance gene. These isolates then increase in number, eventually reaching a frequency whereby they render the resistance gene ineffective. This scenario has occurred many times for the *B. napus*-*L. maculans* interaction, such as the breakdown of *LepR3* and *LepR1* in Australia, *Rlm3* in Canada, *Rlm1* in France, *Rlm7* in the UK, and *Rlm6* in trial experiments in France (Brun et al., 2010; Mitrousia et al., 2018; Rouxel et al., 2003; Sprague et al., 2006a; Van de Wouw et al., 2014c; Zhang et al., 2016). This boom-and-bust nature of major gene resistance has led to the development of resistance gene management strategies, initially in Australia and then in Canada, whereby resistance genes are rotated in space and time to minimize the risk of resistance being overcome (Marcroft et al., 2012b; Van de Wouw et al., 2016; Zhang & Fernando, 2018). All commercial cultivars are released to industry with associated resistance groups, which are letters given to represent different resistance genes. Growers can then rotate cultivars with different resistance genes to minimize the risk of resistance being overcome. In Australia, this management strategy

is then supported through the monitoring of disease severity in these resistance groups in field sites across canola-growing regions of Australia, so that warnings can be provided to industry when the effectiveness of specific resistance genes is in jeopardy (Van de Wouw et al., 2016). This monitoring has previously led to issuing warnings on the Eyre Peninsula, South Australia, which saved growers over A\$13 million in potential yield losses (Van de Wouw et al., 2014c). In the current study, we report on the breakdown of *Rlm7* resistance in two separate locations in Australia, whereby the different selection regimes resulted in different allele diversity patterns.

2 | RESULTS

Blackleg disease is monitored in canola cultivars representing different resistance groups at field sites across Australia every year (Table S1). This monitoring allows detection of potential resistance breakdown at regional levels and has previously been used to provide warnings to growers that significant yield losses are imminent due to resistance being overcome (Van de Wouw et al., 2014c). In the current study, this regional monitoring identifies breakdown of *Rlm7* resistance.

2.1 | Breakdown of *Rlm7* resistance due to extensive use of *Rlm7*-containing cultivars

Disease data (leaf lesion severity and percentage internal infection at the crown) were collected from the Hamilton, Victoria site in 2020 (Figure 1). Leaf lesion data collected at the four-leaf growth stage showed that the disease severity for the *Rlm7* (Group H) cultivar was not significantly different from the *Rlm1* (Group A), *Rlm4* (Group B), *Rlm3* (Group C), and *Rlm4*, *Rlm6* (Group BF) cultivars, which harbour resistance genes that are already reported as overcome in Australia (Van de Wouw et al., 2018). In comparison, the *LepR1*, *Rlm1* (Group AD), *Rlm1*, *Rlm4*, *LepR1* (Group ABD), and *Rlm1*, *Rlm4*, *LepR1*, *Rlm6* (Group ABDF) cultivars had significantly lower levels of disease than the *Rlm7* cultivar (Figure 1c). Lastly, the *Rlm7* cultivar had five times more disease at the Hamilton site compared to the national average (Figure 1c).

Similarly, at plant maturity at the end of the growing season, the *Rlm7* cultivar displayed similar levels of crown canker disease to the *Rlm1* (Group A), *Rlm4* (Group B), and *Rlm3* (Group C) cultivars (Figure 1d). Significantly lower levels of disease were observed in the *LepR1*, *Rlm1* (Group AD), *Rlm1*, *Rlm4*, *LepR1* (Group ABD), *Rlm4*, *Rlm6* (Group BF), and *Rlm1*, *Rlm4*, *LepR1*, *Rlm6* (Group ABDF) cultivars compared to the *Rlm7*. Lastly, the *Rlm7* cultivar had almost three times higher disease at the Hamilton site compared to the national average (Figure 1d). These combined results of high levels of disease severity early and late in the growing season and at different stages of plant development suggest that the *Rlm7* resistance may be overcome in the Hamilton, Victoria region.

Four isolates were cultured from lesions following surface sterilization of infected leaves collected at the Hamilton site in 2020. The *AvrLm4-7* gene was PCR amplified and sequenced from these four isolates to determine the genotype at this locus. Each isolate harboured an allele of the *AvrLm4-7* gene that had undergone RIP mutation, resulting in 38 or 40 C-T or G-A transitions throughout the coding region. Three of these four isolates were screened for virulence towards both *Rlm4* and *Rlm7* using in planta phenotyping assays to confirm that the RIP mutations inactivate *AvrLm4-7* and therefore result in virulence. All three isolates (20BL001–20BL003) were virulent towards both *Rlm4* and *Rlm7* (Figure 2 and Table S2). One of these isolates, 20BL001, was transformed with the wild-type copy of the *AvrLm4-7* gene, which restored the avirulence phenotype towards both *Rlm4* and *Rlm7* (Figure S1). Stubble (crop debris) from the *Rlm7* cultivar was collected from the Hamilton site at the end of the 2020 growing season and a further 19 isolates were obtained from the mature stubble in 2021. All 19 isolates (21BL001–21BL019; Table S2) harboured *avrLm4-7* alleles that had undergone RIP mutation, resulting in 35 to 41 C-T or G-A transitions throughout the gene.

Information on farming practices was collected from the grower on whose property the disease monitoring site was located. This grower had been using Grain 'n' Graze farming practices for the past three of four years, whereby cultivars with vernalization requirements are sown in March and then grazed with sheep for a number of months before being allowed to grow through to maturity and harvested for yield (Kirkegaard et al., 2008). The grower had predominately been using the Grain 'n' Graze cultivar Hyola 970CL during this time (G. Kreeck, Southern Farming Systems, personal communication), which harbours resistance gene *Rlm7* (<https://grdc.com.au/GRDC-FS-BlacklegManagementGuide>). This use of an *Rlm7* cultivar over multiple and successive years has presumably led to selection of the *avrLm7* isolates within the *L. maculans* population, resulting in the *Rlm7* resistance gene being overcome.

2.2 | Breakdown of *Rlm7* resistance due to dual specificity of *AvrLm4-7*

Disease severity data were collected at crop maturity from the Yeelanna, South Australia, site on the Eyre Peninsula in 2019 (Figure 3). The *Rlm7* cultivar had similar levels of disease as the *Rlm1* (Group A) and *Rlm4* (Group B) cultivars at this site, although these were significantly lower than the *Rlm3* (Group C) and *Rlm4*, *Rlm6* (Group BF) cultivars (Figure 3). The *Rlm1*, *LepR1* (Group AD), *Rlm1*, *Rlm4*, *LepR1* (Group ABD), and *Rlm1*, *Rlm4*, *LepR1*, *Rlm6* (Group ABDF) cultivars had the lowest level of disease. The *Rlm7* cultivar at the Yeelanna site had five times more disease than the national average.

Stubble was collected from the *Rlm7* cultivar at the Yeelanna site at the end of 2019 and 17 isolates were obtained from the mature stubble in 2020 (Table S2). The *AvrLm4-7* allele was PCR amplified and sequenced from each of these, with eight isolates harbouring

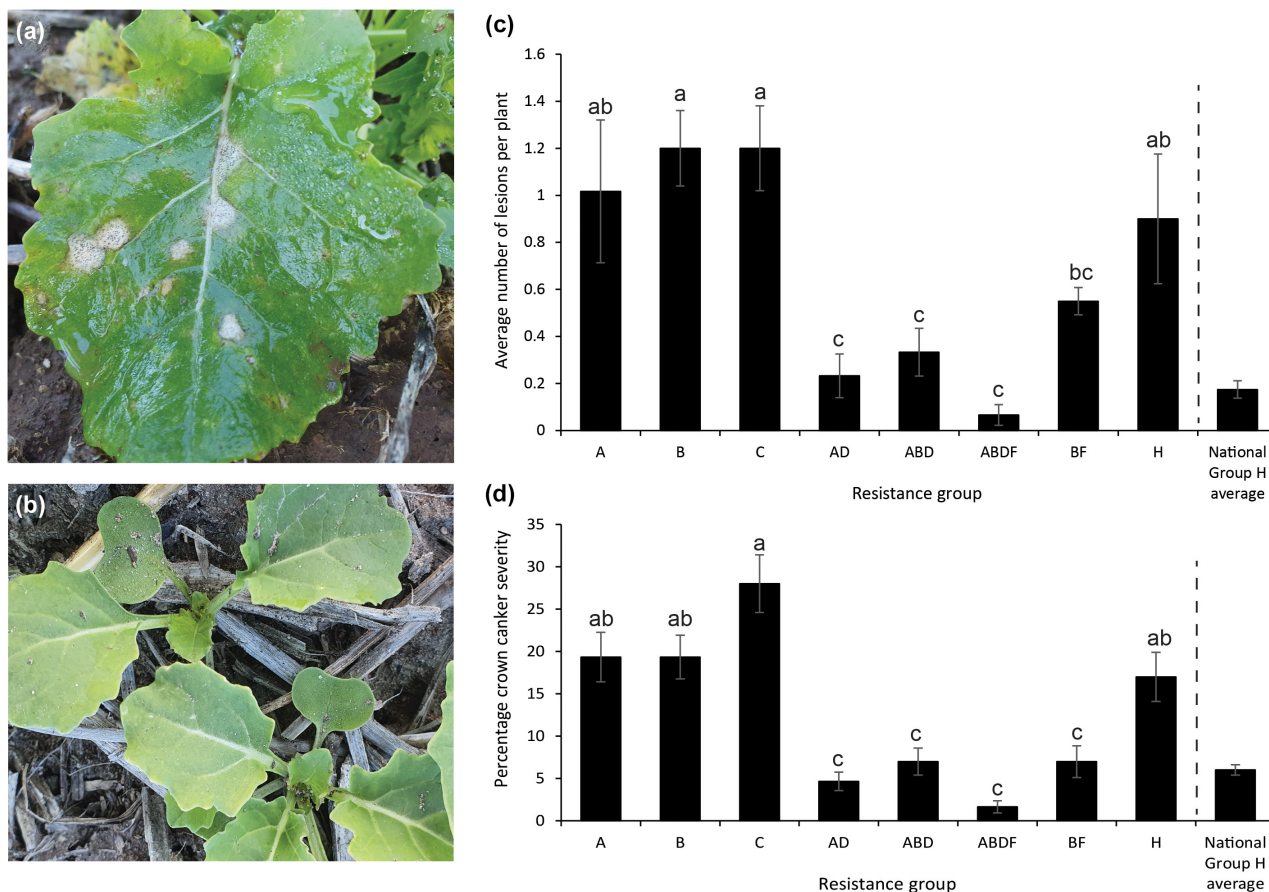


FIGURE 1 Disease severity in the Group H (*Rlm7*) cultivar at the Hamilton, Victoria, monitoring site. (a, b) Leaf lesions were detected on the *Rlm7* (Group H) cultivar at the Hamilton (a) site but not at other sites such as Kaniva, Victoria (b). (c) At the Hamilton site, the average numbers of leaf lesions per plant were recorded for cultivars with different resistance genes (indicated by resistance group on the x axis), including the Group H representative cultivar at the four-leaf stage. The level of leaf lesion infection was much higher in the Group H cultivar at the Hamilton site compared to the national average (last bar on graph, determined from 14 sites). (d) The percentage of crown canker severity was also determined for all cultivars at the Hamilton site at the end of the growing season. Again, the level of crown canker infection was much higher in the Group H cultivar at the Hamilton site compared to the national average. Data are the average of three replicate plots. Bars with the same letter indicate no significant difference ($p < 0.01$)

the *AvrLm47_02* allele, which results in virulence towards *Rlm4* but not *Rlm7* (Table 1). The remaining nine isolates all harboured either the *AvrLm47_09* or *AvrLm47_10* alleles, which both contained RIP mutations throughout the coding region. A subset of six of the isolates harbouring these RIP alleles (20BL183, 20BL184, 20BL186, 20BL187, 20BL188, 20BL193; Table S2) was screened for virulence towards *Rlm7*. As expected, all six isolates were virulent towards both *Rlm4* and *Rlm7* (Figure 2). Five of these isolates (20BL183, 20BL184, 20BL186, 20BL187, 20BL188) were complemented with a wild-type copy of the *AvrLm4-7* gene and shown to restore the avirulence phenotype towards both *Rlm4* and *Rlm7* (Figure S1).

Unlike the situation in Hamilton, Victoria, *Rlm7* cultivars have never been grown on the Eyre Peninsula, as there are no cultivars harbouring *Rlm7* with suitable phenology for the region. However, there has been a high dependency on *Rlm4* cultivars in this region for the past 3 years (Figure 4). Since 2018, over 80% of seed sold on the Eyre Peninsula has been cultivars containing the *Rlm4* resistance gene, with 2019 and 2020 seeing 95% of total seed sold harbouring

Rlm4 (Nutrien Cummins Ag Supplies, personal communication). This suggests that the breakdown of *Rlm7* on the Eyre Peninsula is associated with the high use of *Rlm4* cultivars and the dual specificity of the corresponding avirulence gene, *AvrLm4-7*.

2.3 | Diversity of RIP alleles differs under contrasting selection regimes

The allelic variation for the RIP isolates varied across different selection regimes (Table S2). Of the nine *avrLm7* isolates collected from Yeelanna, only two different RIP alleles were detected, *AvrLm47_09* and *AvrLm47_10*. These two alleles contained 37 (*AvrLm47_09*) and 36 (*AvrLm47_10*) C-T or G-A transitions, each generating 19 amino acid substitutions and nine stop codons (Table 1). Of the eight *AvrLm7* isolates, only a single allele, *AvrLm47_02*, was detected. Conversely, of the 23 *avrLm7* isolates collected from Hamilton, Victoria, 11 different RIP alleles were identified (*AvrLm47_9*, *AvrLm47_11*, *AvrLm47_12*,

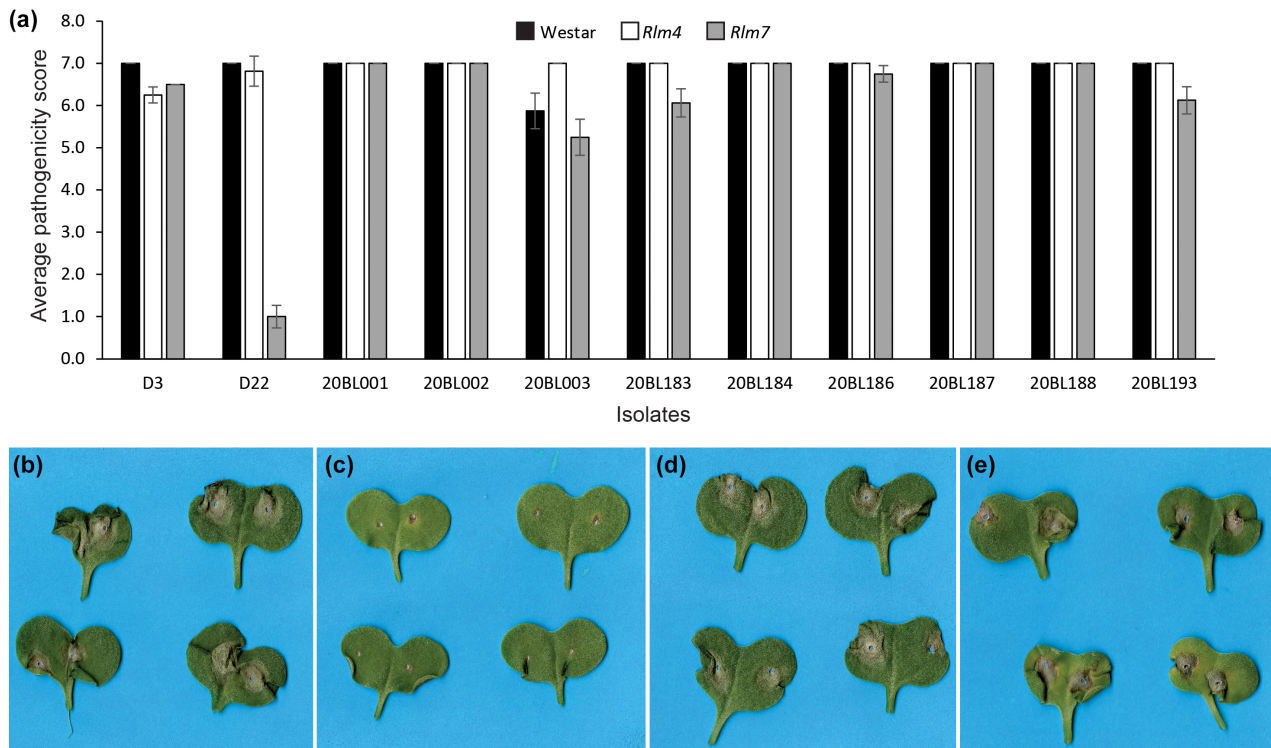


FIGURE 2 Repeat-induced point (RIP) mutations within the *AvrLm4-7* gene of *Leptosphaeria maculans* confers virulence towards *Rlm7*. (a) Eleven isolates (two controls, D3 and D22, and nine representative isolates from 2020, harbouring RIP alleles at *AvrLm4-7*), were screened for virulence towards Westar (no R genes, black bars), *Rlm4* (white bars), and *Rlm7* (grey bars) lines. Lesions developed on Westar (b) following inoculation with isolate D22, with a functioning copy of *AvrLm4-7*, but not Topas-*Rlm7* (c), as expected. Lesions on Westar (d) and Topas-*Rlm7* (e) formed following inoculation with isolate 20BL193, which harbours RIP mutations throughout the *AvrLm4-7* gene. Bars represent the average pathogenicity score from eight plants. Error bars represent standard errors

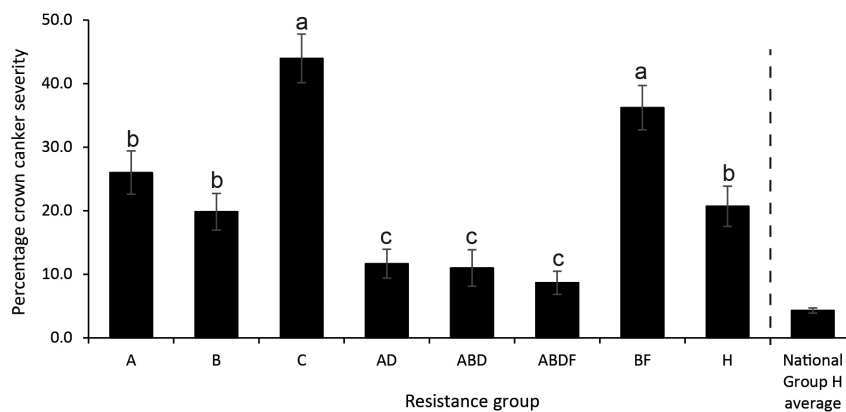


FIGURE 3 Disease severity in the Group H (*Rlm7*) cultivar at the Yeelanna, South Australia, monitoring site. The percentage of crown canker severity was determined for all cultivars at the end of the growing season. The level of disease observed in the Group H cultivar was much higher at the Yeelanna site compared to the national average (last bar on graph). Data are the average of three replicate plots. Bars with the same letter indicate no significant difference ($p < 0.01$)

AvrLm47_13, *AvrLm47_18*, *AvrLm47_19*, *AvrLm47_20*, *AvrLm47_21*, *AvrLm47_22*, *AvrLm47_23*, *AvrLm47_24*, suggesting that the diversity of RIP alleles was much higher in the isolates collected from *Rlm7* cultivars at Hamilton, Victoria, compared to Yeelanna, South Australia.

As a comparison, the *AvrLm4-7* alleles were sequenced from additional isolate populations from Hamilton and Yeelanna that

were collected from alternative resistance sources, not *Rlm7*. No RIP alleles were identified in any of these isolates. For the isolates collected from the *Rlm1*, *LepR1* (Group AD) cultivar at the Yeelanna site, two non-RIP alleles were detected, *AvrLm47_01* and *AvrLm47_02*, both phenotypically *avrLm4* (Table 1). For the isolates from the *Rlm3* (Group C) cultivar at the Hamilton site, three non-RIP alleles were detected: *AvrLm47_00*, *AvrLm47_01*,

TABLE 1 Alleles of the *AvrLm4-7* gene of *Leptosphaeria maculans*

Allele ^a	Frequency of allele (number)	Populations identified in ^b	Mutations ^c		Translational consequences of mutations			Phenotypic consequences ^d	
			Non-RIP	RIP	Syn. subs.	Non-syn. subs.	Stop codons	<i>Rlm4</i>	<i>Rlm7</i>
<i>AvrLm47-0*</i>	1% (1)	2	0	0	0	0	0	Avirulent	Avirulent
<i>AvrLm47-1*</i>	7% (7)	2, 4	2	0	0	2	0	Virulent	Avirulent
<i>AvrLm47-2*</i>	40% (40)	2-5	2	0	0	2	0	Virulent	Avirulent
<i>AvrLm47-4*</i>	3% (3)	5, 6	3	0	0	3	0	Virulent	Avirulent
<i>AvrLm47-5*</i>	1% (1)	7	0	39	10	19	9	Virulent	Virulent
<i>AvrLm47-6*</i>	1% (1)	7	0	42	10	22	9	Virulent	Virulent
<i>AvrLm47-7*</i>	1% (1)	7	0	37	8	19	9	Virulent	Virulent
<i>AvrLm47-8*</i>	1% (1)	7	0	39	9	20	9	Virulent	Virulent
<i>AvrLm47-9</i>	15% (15)	1, 3, 5, 6	0	37	8	19	9	Virulent	Virulent
<i>AvrLm47-10</i>	1% (1)	3	0	36	7	19	9	NT	NT
<i>AvrLm47-11</i>	6% (6)	1, 5, 6	0	38	9	20	8	Virulent	Virulent
<i>AvrLm47-12</i>	1% (1)	1	0	40	11	19	9	Virulent	Virulent
<i>AvrLm47-13</i>	1% (1)	1	0	38	9	19	9	NT	NT
<i>AvrLm47-14</i>	1% (1)	5	0	39	9	20	9	NT	NT
<i>AvrLm47-15</i>	2% (2)	5, 6	0	39	10	19	9	NT	NT
<i>AvrLm47-16</i>	2% (2)	6	0	38	8	20	9	NT	NT
<i>AvrLm47-17</i>	1% (1)	6	0	42	11	21	9	NT	NT
<i>AvrLm47-18</i>	1% (1)	1	0	38	10	18	9	NT	NT
<i>AvrLm47-19</i>	1% (1)	1	0	38	9	19	9	NT	NT
<i>AvrLm47-20</i>	1% (1)	1	0	39	8	21	8	NT	NT
<i>AvrLm47-21</i>	3% (3)	1	0	41	8	20	10	NT	NT
<i>AvrLm47-22</i>	6% (6)	1	0	39	9	21	8	NT	NT
<i>AvrLm47-23</i>	1% (1)	1	0	35	6	18	8	NT	NT
<i>AvrLm47-24</i>	2% (2)	1	0	40	8	21	8	NT	NT

^aAllele nomenclature based on Van de Wouw and Howlett (2012). **AvrLm47-0* through *AvrLm47-8* previously published.

^bPopulation details: 1, Hamilton, Victoria, *Rlm7*; 2, Hamilton, Victoria, *Rlm3*; 3, Yeelanna, South Australia, *Rlm7*; 4, Yeelanna, South Australia, *Rlm1*, *LepR1*; 5, Canberra, Australian Capital Territory, *Rlm7*; 6, Springfield, New South Wales, *Rlm7*; 7, historic isolates, unknown.

^cRepeat-induced point (RIP) mutations were identified as CpA to TpA and TpG to TpA mutations as previously reported (Selker & Garrett, 1988). Non-RIP mutations were identified as other classes of transitions and transversions.

^dPhenotypes are based on either previously published findings (Van Parlange et al., 2009; Van de Wouw & Howlett, 2012) or this study (see Figure 2). NT refers to not tested.

and *AvrLm47_02*. The *AvrLm47_00* isolate is avirulent towards both *Rlm4* and *Rlm7* (Table 1).

Lastly, isolates were also collected from *Rlm7* cultivars from experimental plots at Springfield (New South Wales) and Canberra (Australian Capital Territory) whereby recurrent *Rlm7* selection had also occurred (Table S2). From eight Springfield isolates, six isolates had five different RIP alleles: *AvrLm47_09*, *AvrLm47_11*, *AvrLm47_15*, *AvrLm47_16*, and *AvrLm47_17*. These five RIP alleles harboured up to 42 RIP mutations resulting in 19–21 amino acid substitutions and eight or nine stop codons (Table 1). The remaining two isolates both contained the *AvrLm47_04* allele, which harbours three non-RIP-like mutations resulting in virulence towards *Rlm4* but not *Rlm7* (Table 1). From 10 Canberra isolates, seven had four different RIP alleles: *AvrLm47_09*, *AvrLm47_11*, *AvrLm47_14*, and *AvrLm47_15*

(Table 1). The remaining three isolates harboured two different alleles, *AvrLm47_02* and *AvrLm47_04*.

Molecular variation was analysed for the *AvrLm4-7* locus in the isolates using analysis of molecular variance (AMOVA), taking into consideration geographical location (region) and the cultivar as the source of resistance from where the isolates originated (population). For all 100 isolates significant differences in genetic diversity among regions and populations were detected as well as within populations (Table 2). The largest source of variation was within populations (69%), followed by among populations (22%). The genetic diversity of each population was calculated using Shannon indices, whereby the Hamilton (Vic) population collected from a *Rlm7* cultivar showed the highest level of diversity (Shannon index, $I = 2.08$; genetic diversity, $h = 0.85$) and the Yeelanna (South Australia) population collected

from a *LepR1*, *Rlm1* cultivar showed the lowest ($I = 0.349$, $h = 0.198$) (Figure 5a). The Yeelanna population collected from a *Rlm7* cultivar showed similar levels of diversity ($I = 1.053$, $h = 0.602$) as the Hamilton population from a *Rlm3* cultivar ($I = 1.129$, $h = 0.560$) but much lower than each of the populations collected from *Rlm7* cultivars ($I = 1.696$ – 2.080 , $h = 0.813$ – 0.851). The lower diversity of alleles for the Yeelanna-*Rlm7* population was further exemplified when the genetic diversity indices were calculated only on the 49 isolates harbouring RIP alleles (Figure 5b), whereby this population obtained I index of 0.349 and h index of 0.198. This compares to a range of I indices of 1.386–2.163 and h indices of 0.694–0.858 for each of the other RIP populations. These data suggest that the diversity in RIP alleles is much lower for the Yeelanna *Rlm7* populations compared to all other *Rlm7* populations.

Across the 20 different RIP alleles identified, 33 mutations are common between all alleles (Figures 6a and S2), with a further three to nine mutations responsible for the variation between the different RIP alleles. When compared to a single published RIP allele from isolate Nz-T4 (GenBank: KT804641.1), the same 33 RIP mutations are also in common with this allele (data not shown).

The *avrLm7* RIP alleles (*AvrLm47_05* to *AvrLm47_24*) are phylogenetically distinct from the *AvrLm7* alleles (*AvrLm47_00* to *AvrLm47_4*), forming a separate clade on the phylogenetic tree, which is supported with bootstrap values (Figure 6b). However, no clear pattern of divergence can be seen within the RIP alleles with limited bootstrap support (Figure 6).

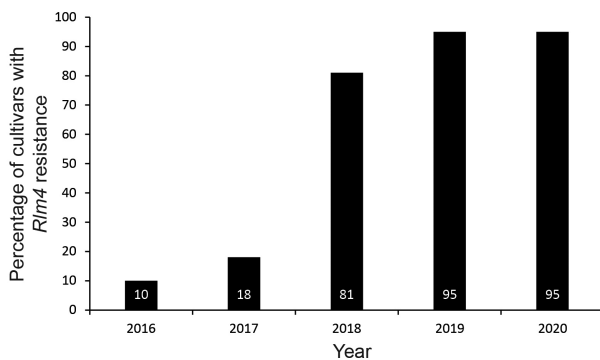


FIGURE 4 Percentage of *Brassica napus* cultivars sown on the Eyre Peninsula, South Australia, between 2016 and 2020 that harbour the *Rlm4* resistance gene

TABLE 2 Analysis of molecular variance results for the *AvrLm4-7* alleles identified across 100 isolates

Source of variation ^a	df	SS	MS	Est. var.	%	p value
Among regions	4	7.851	1.963	0.036	8%	0.001
Among populations	2	4.332	2.166	0.096	22%	0.001
Within populations	93	27.866	0.300	0.300	69%	0.001

Note: Analysis of molecular variance was performed with 999 random permutations. SS, sum of squares; MS, mean of square value.

^aRegions refer to different geographical locations. Populations refer to different cultivars, and therefore sources of resistance, that the isolates were cultured from.

3 | DISCUSSION

The use of major gene resistance is an essential tool in the fight to minimize the impact of plant diseases like blackleg of canola. However, as seen on numerous occasions in Europe, Canada, and Australia, *L. maculans* populations have the ability to evolve rapidly and overcome these resistance genes (Mitrousia et al., 2018; Sprague et al., 2006a; Van de Wouw et al., 2014c; Zhang et al., 2016). Previous resistance breakdowns have involved either the widescale use of the resistance gene on farms or recurring selection in trial settings (e.g., Delourme et al., 2014; Van de Wouw et al., 2014c). The selection pressure from the continual use of the resistance gene has selected for isolates in the population that are virulent, which then increase in frequency, resulting in the corresponding resistance gene being overcome. In the current study, we have identified the breakdown of *Rlm7* associated with two different selection regimes.

The first involves continual use of *Rlm7* cultivars on a specific farm, resulting in the selection of *avrLm7* isolates and therefore the breakdown of *Rlm7* resistance. This is similar to situations in Europe whereby breakdown of *Rlm7* resistance occurred in response to widespread sowing of cultivars carrying this resistance gene (Daverdin et al., 2012; Mitrousia et al., 2018). In the scenario in Australia, RIP mutation in the *avrLm4-7* gene was identified as the only mechanism conferring virulence towards *Rlm7*, whilst in the UK and France gene deletion was identified as the major mechanisms of virulence (Daverdin et al., 2012; Mitrousia et al., 2018). However, in French surveys it was found that in the first year, RIP mutations were the most frequent event giving rise to virulence and these decreased in frequency in the second year (Daverdin et al., 2012). Continual monitoring in Australia might identify a similar situation in which RIP mutations decrease in frequency and deletion alleles increase. Because this was the first time monitoring had been done at this location, it is unknown when the frequency of virulent *avrLm7* isolates began to increase; however, it is likely that it has occurred within a 3- or 4-year time frame that would coincide with the use of the *Rlm7* cultivar Hyola 970 on the farm. In comparison, the breakdown of *Rlm7* resistance was reported over a 4-year window in specific trials in France and was much slower under UK conditions (Daverdin et al., 2012; Mitrousia et al., 2018). It is unclear why the breakdown of resistance occurred much faster in Australia, but this finding is consistent with other resistance genes such as *Rlm1*, which was overcome in

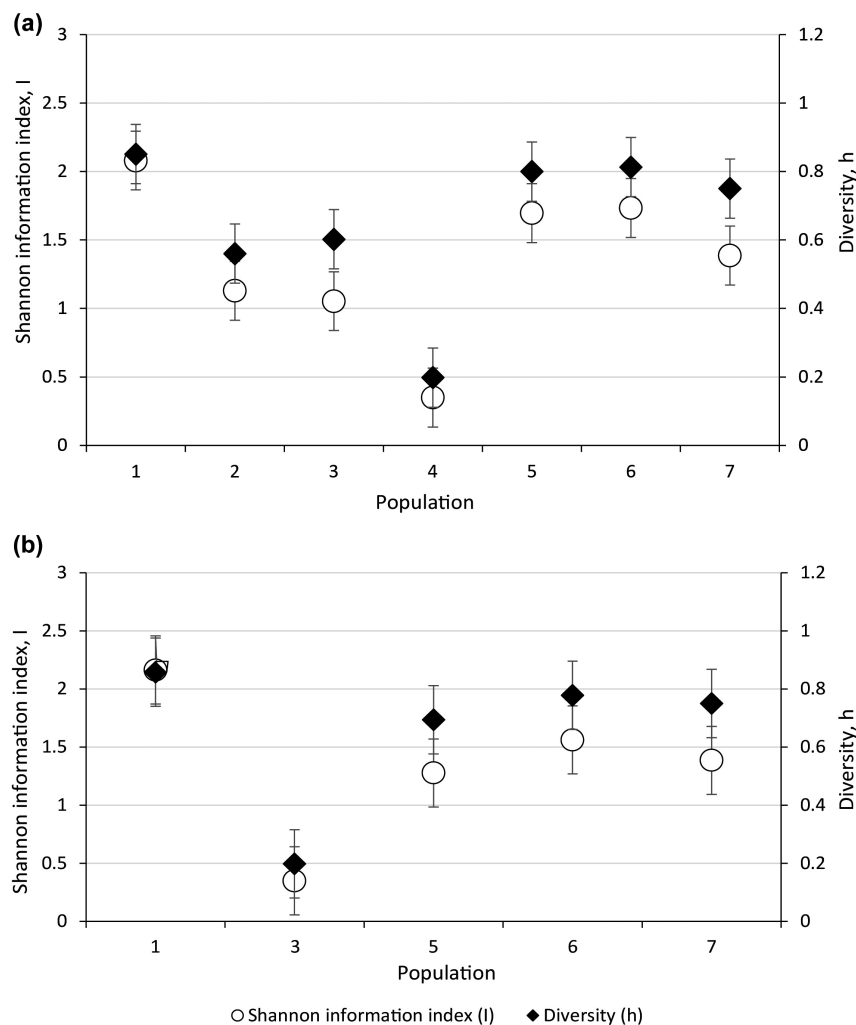


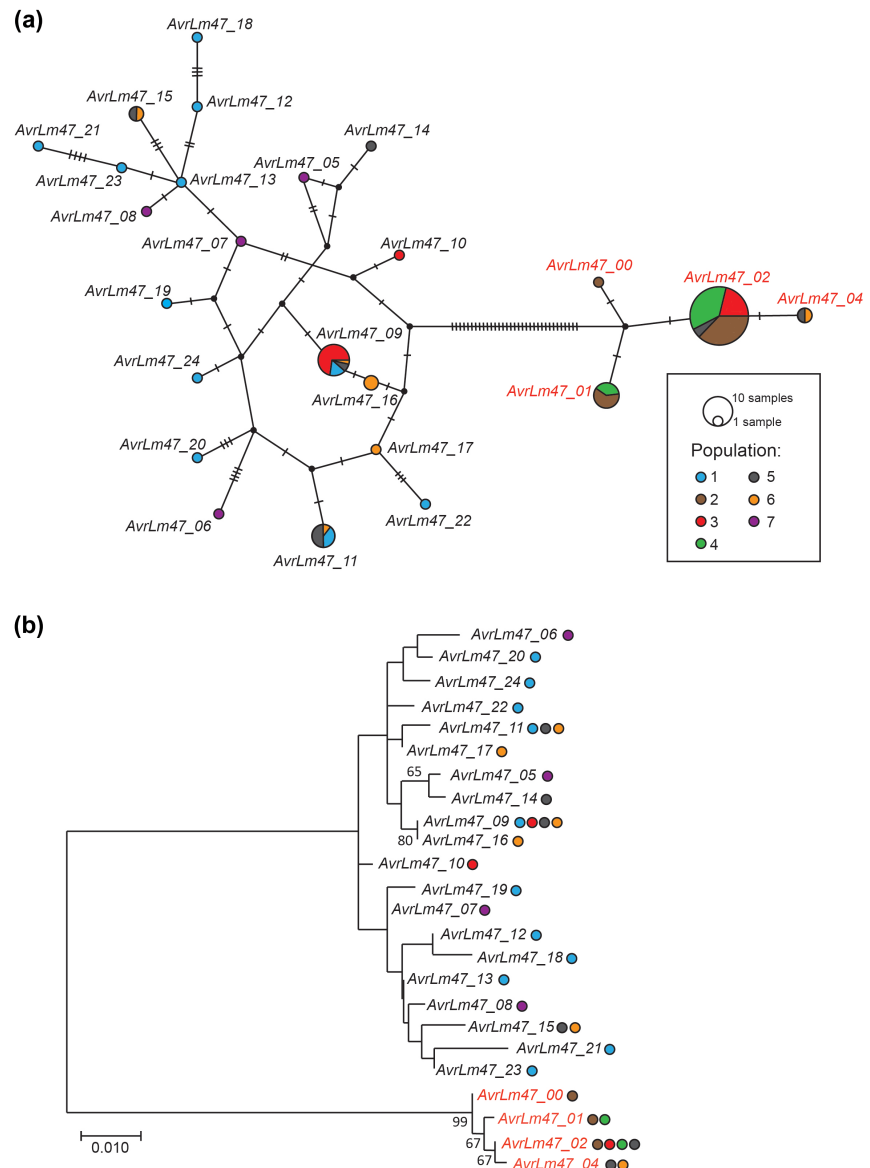
FIGURE 5 Differences in genetic diversity were calculated using Shannon indices for the *AvrLm4-7* allele of *Leptosphaeria maculans* populations. (a) When all seven populations representing 100 isolates were analysed, population 4 showed the lowest diversity and population 1 showed the highest. (b) When the 49 isolates harbouring repeat-induced point mutation alleles were analysed, large differences in diversity were observed for population 3 compared to all other *Rlm7* populations. Population details: 1, Hamilton, Victoria, *Rlm7*; 2, Hamilton, Victoria, *Rlm3*; 3, Yeelanna, South Australia, *Rlm7*; 4, Yeelanna, South Australia, *Rlm1*, *LepR1*; 5, Canberra, Australian Capital Territory, *Rlm7*; 6, Springfield, New South Wales, *Rlm7*; 7, historic isolates, unknown. Error bars represent the standard errors for the Shannon index (*I*) and genetic diversity (*h*) as calculated using GenAlEx v. 6.503

3 years in Australia compared to 10+ years in France, and may be a reflection of the fungal life cycle in Australia, whereby the sexual cycle during which RIP mutation occurs is essential to produce the ascospores to initiate disease (Rouxel et al., 2003; Sprague et al., 2006b). Previously, *Rlm7* has only been available in winter cultivars, and therefore predominantly only used in Grain 'n' Graze farming systems. In 2020 the *Rlm7* cultivar Hyola 970CL set an Australian harvest record of 7.16 t/ha (Thyer, 2021), and in 2021 spring-type *Rlm7* cultivars were released for the first time, which will expand the potential growing area for this resistance gene. Therefore, close monitoring of disease levels will be required to prevent yield losses from resistance breakdown in *Rlm7* cultivars.

The second scenario presented in this study was the breakdown of *Rlm7* on the Eyre Peninsula, South Australia, where no *Rlm7* cultivars have ever been sown. Instead, this region has predominately been sown with *Rlm4* cultivars, suggesting that the breakdown of the *Rlm7* resistance is due to the dual specificity of *AvrLm4-7*, a single avirulence gene recognized by two different resistance genes, *Rlm4* and *Rlm7*. A SNP is the most common mechanism conferring virulence towards *Rlm4* (Parlange et al., 2009) and has been reported in populations in Europe, Canada, and Australia (Daverdin et al., 2012; Fernando et al., 2018; Van de Wouw et al., 2018). Conversely, the

most common mechanism for virulence towards *Rlm7* is either deletion or RIP mutation of the *AvrLm4-7* gene that results in virulence towards both *Rlm7* and *Rlm4* (Daverdin et al., 2012). Previously, the identification of RIP alleles has only been reported from populations that have undergone *Rlm7* selection (either field or trials) or historic populations where the selection regime is unknown (Daverdin et al., 2012; Van de Wouw & Howlett, 2012). In the current study, RIP alleles were detected following multiple years of *Rlm4* cultivation, suggesting that strong selection pressure due to *Rlm4* can lead to selection of both SNPs and RIP alleles conferring virulence towards *Rlm4* and, consequently, virulence towards *Rlm7*. However, under *Rlm4* selection the diversity of RIP alleles was significantly lower than in populations that had been selected through *Rlm7* cultivation (e.g., Figure 7). These data suggest that RIP mutation is potentially triggered at a lower frequency under *Rlm4* selection compared to *Rlm7* selection. Therefore, only a small number of RIP alleles are present in the population during *Rlm4* selection, and then when *Rlm7* cultivars are grown the small number of RIP alleles present are selected for and increase in frequency with low genetic diversity (Figure 7a). However, under *Rlm7* selection, RIP mutation is being triggered more readily and therefore there is a larger pool of *avrLm4-7* alleles being generated and selected (Figure 7b).

FIGURE 6 Haplotype network (a) and phylogenetic tree (b) of the 24 different alleles identified for the *AvrLm4-7* locus of *Leptosphaeria maculans*. *AvrLm47_00* confers an avirulent phenotype towards both *Rlm4* and *Rlm7*; *AvrLm47_01*, *AvrLm47_02*, and *AvrLm47_04* confer a virulent phenotype towards *Rlm4* and an avirulent phenotype towards *Rlm7* (represented in red text). Alleles *AvrLm47_05* to *AvrLm47_24* confer virulence towards both *Rlm4* and *Rlm7*, and were generated through repeat-induced point (RIP) mutations (represented in black text). Population details: 1, Hamilton, Victoria, *Rlm7*; 2, Hamilton, Victoria, *Rlm3*; 3, Yeelanna, South Australia, *Rlm7*; 4, Yeelanna, South Australia, *Rlm1*, *LepR1*; 5, Canberra, Australian Capital Territory, *Rlm7*; 6, Springfield, New South Wales, *Rlm7*; 7, historic isolates, unknown. The size of the circle represents the number of samples detected for each allele (see key for details). The phylogenetic tree features bootstrap values (from 1000 replications) greater than 65%



The mechanisms of RIP mutation are still poorly understood in non-model organisms although the process has been proposed to exist in many fungal species based on genome analyses, especially within the ascomycetes (van Wyk et al., 2021). With the exception of *L. maculans* and some model fungal species, there is limited experimental evidence of RIP mutation existing in other species. Furthermore, there is no report of RIP mutation conferring virulence in other plant pathogen species. Of the 20 RIP alleles identified in this study, 33 mutations were common between them. Do these findings suggest that there is a precursor allele that is predominant in the population that is selected for under *Rlm4/Rlm7* selection? In line with this possibility, the 33 mutations were in common in four historical isolates that were collected in the 1980s. However, these 33 mutations were also shared with an allele sequenced from isolate Nz-T4 from New Zealand. An alternative possibility is that multiple rounds of RIP mutation occur during meiosis, which results in a first-round of mutations that target specific bases, and therefore common in all RIP alleles, and this is then followed by a second round

of mutations that randomly targets remaining potential sites. This is consistent with recent findings in *L. maculans* whereby at least two rounds of RIP mutation occur during meiosis (Van de Wouw et al., 2019). In experiments characterizing RIP mutation using specific constructs carrying DNA duplication, multiple independent alleles are created after crossing. However, the trigger for mutations at the *AvrLm4-7* locus is probably the large regions of repetitive DNA sequence on either side, which may impose a different intensity of the mutation process and hence those 33 sites may always be targeted. Counter to this hypothesis is the range of different mutations found in RIP-derived alleles of *AvrLm6*, which is also found embedded in repetitive DNA (Van de Wouw et al., 2010).

Hence, it is unclear whether the RIP alleles arise independently each time there is selection pressure being imposed by *Rlm4* or *Rlm7* cultivation or if these alleles are always being maintained at low frequency in the population. Fitness penalties in strains that are virulent towards *Rlm4* have been reported from field- and laboratory-based experiments in the UK (Huang et al., 2006, 2010).

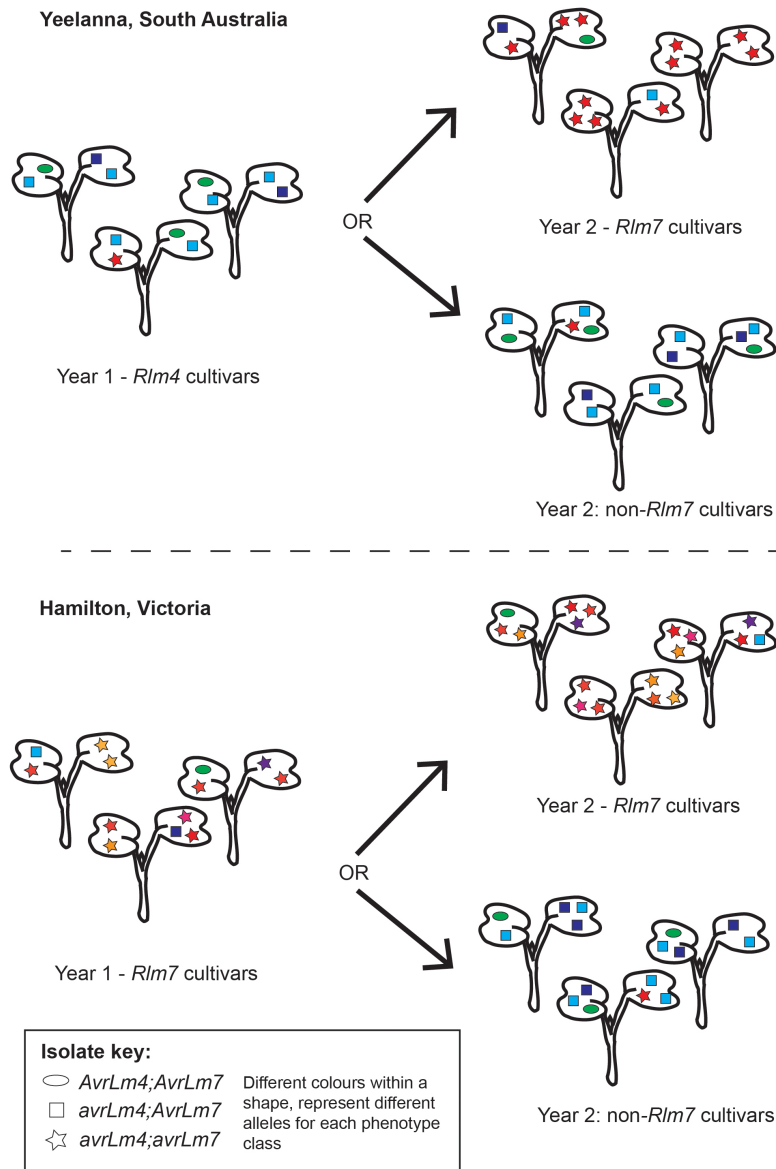


FIGURE 7 Representation of different selection regimes and the impact on genetic diversity within the *AvrLm4-7* locus of *Leptosphaeria maculans*. Different colours within a shape represent different alleles for each phenotype class

No such experiments have been done for strains associated with selection with *Rlm7*; however, it may be reasonable to assume there would be considering the differences associated between avirulent and virulent alleles.

The dual specificity of *Avr* genes has implications for management strategies that are being implemented for growers. In both Australia and Canada, growers are advised to rotate resistance genes in the field to minimize the risk of resistance being overcome (Marcroft et al., 2012b; Van de Wouw & Howlett, 2020; Zhang & Fernando, 2018). When a single avirulence protein is recognized by two different resistance proteins then these resistance genes potentially need to be treated as a single *R* gene when deploying management strategies, depending on the mechanisms conferring virulence. Resistance genes *LepR3* and *Rlm1* are another blackleg disease example whereby two separate *R* genes in *B. napus* recognize a single *Avr* gene in *L. maculans*, *AvrLm1-R3*. Similar to the situation described in the current study, the wide-scale cultivation of *sylvestris* cultivars harbouring *LepR3* resistance

genes led to the selection of virulence at the *AvrLm1-R3* locus. In this situation, gene deletion is the most common mechanism for loss of avirulence and therefore will always result in virulence towards both genes. In Australia, *LepR3* and *Rlm1* are treated as a single resistance group due to the fact that virulence towards one of the genes results in virulence towards both. Due to the previous findings that the emergence of virulence under *Rlm4* selection was due to a single SNP, and therefore only virulence towards *Rlm4* and not *Rlm7*, these two resistance genes have been treated as separate resistance groups in Australia. Rotation to *Rlm7* following *Rlm4* cultivation has therefore been recommended as isolates would only be virulent towards *Rlm4*. However, rotation to *Rlm4* following *Rlm7* selection was not recommended as virulence towards *Rlm7* inactivates the gene and always also leads to virulence towards *Rlm4*. The findings presented in this current study suggest that *Rlm4* and *Rlm7* should not be rotated in either manner because *Rlm4* cultivation can lead to generation of RIP alleles, albeit at a lower frequency.

As more R-Avr interactions are discovered and characterized in plant-microbe interactions, and that information moved into crop protection, establishing situations in which multigene interactions occur can be useful for breeding prioritization strategies and the deployment of new resistance genes.

4 | EXPERIMENTAL PROCEDURES

4.1 | Disease monitoring sites

Blackleg severity was monitored in 14 field sites across Australia in 2019 and 2020 (Figure S3). These disease monitoring sites were located directly adjacent to National Variety Trial yield evaluation sites where cultivars/breeding lines are sown. Two of these sites, Hamilton in Victoria and Yeelanna in South Australia, displayed unusual levels of disease for the *Rlm7* (Group H) cultivar in 2020 and 2019, respectively. For the full list of disease monitoring sites, see Table S1.

At each of these disease monitoring sites, eight cultivars representing different resistance groups were sown. The cultivars grown at each site were ATR-Bonito^{PBR} (*Rlm1*; Group A), SFR650917TT (*Rlm4*; Group B), ATR-Stingray^{PBR} (*Rlm3*; Group C), HyT Tec® Trophy (*Rlm1*, *LepR1*; Group AD), Hyola® 559TT (*Rlm1*, *Rlm4*, *LepR1*; Group ABD), SF Turbine TT (*Rlm4*, *Rlm6*; Group BF), Hyola® 350TT (*Rlm1*, *Rlm4*, *LepR1*, *Rlm6*; Group ABDF), and DG1901TT (*Rlm7*; Group H). Cultivars were sown in three replicate plots (10 × 1 m) with complete randomization, designed using DiGger software (www.austatgen.org/files/software).

In 2019, disease severity was assessed at maturity as described previously (Van de Wouw et al., 2014c). In brief, for each replicate plot 20 plants were chosen at random, uprooted, and then cut at the crown. The cross-section was visually assessed for the percentage of internal infection (discolouration). The average crown canker severity was determined from the 60 plants (20 per replicate) for each cultivar at each site. The national average was determined by averaging the crown canker severity for the *Rlm7* cultivar from all 14 disease monitoring sites.

In 2020, in addition to the maturity assessments described above, the severity of leaf lesions was also determined. At the four-leaf growth stage, the total number of lesions was counted for each of 20 consecutive plants within each plot. For consistency, the 20 plants were always selected from the second row, starting 1 m in from the front of the plot. The average number of lesions per plant was determined for each replicate plot for each cultivar at each site. The national average was determined for the leaf lesions per plant for the *Rlm7* cultivar from all 14 disease monitoring sites.

Differences in disease severity were determined through analysis of variance of the log-transformed mean for each plot. Data were analysed using GenStat v. 16.1.0.

Farm practice information was collected. For the Hamilton (Victoria) site, direct information regarding cultivar use was obtained from the farmer where the site was located by G. Kreeck, the trial

manager for Southern Farming Systems who ran the trial. For the Yeelanna (South Australia) site, cultivar use in the Eyre Peninsula region was collected from Nutrien Ag Supplies, Cummins. This company is the largest distributor of seed for the Eyre Peninsula region and therefore a relevant representative of farming practices.

4.2 | Isolate culturing and pathogenicity screening

All isolates used in this study are listed in Table S2 and the location of their collection sites represented in Figure S3. Isolates were collected following one of two protocols. For the isolates collected from Hamilton, Victoria, in 2020, leaf lesions were collected from the *Rlm7* cultivar from the disease monitoring site. Lesions were excised and isolates cultured using the surface sterilization technique as previously described (Van de Wouw et al., 2017). For the remaining isolates used in this study, stubble (crop debris) was collected from disease monitoring sites, matured, and then individual ascospores discharged as previously described (Van de Wouw et al., 2018). All isolates were maintained on 10% Campbell's V8 juice agar. Four historic isolates were included in which alleles harbouring RIP mutations had been previously described (Van de Wouw & Howlett, 2012).

To complement the *AvrLm4-7* mutations, a wild-type copy of the gene was transformed into six representative isolates by *Agrobacterium tumefaciens*-mediated delivery, as described previously (Van de Wouw et al., 2014a). Transformants were selected on medium containing hygromycin (50 µg/ml) and cefotaxime (100 µg/ml), then isolates obtained from colonies derived from single spores.

The virulence phenotype of each of the isolates was determined by inoculating pycnidiospores onto cotyledons of *B. napus* seedlings of lines Westar (harbouring no major gene resistance), Topas-*Rlm4* (harbouring *Rlm4* resistance gene), and Topas-*Rlm7* (harbouring *Rlm7* resistance gene) (Larkan et al., 2016; Van de Wouw et al., 2018). For each isolate × host interaction, eight plants were inoculated and symptoms determined 14 days postinoculation on a 0 (no darkening around wounds) to 9 (large lesions with prolific sporulation) scale (Koch et al., 1991). Average pathogenicity scores of >5.0 were considered virulent and average pathogenicity scores of <3.0 were considered avirulent (Marcroft et al., 2012a). Isolates D2 and D22 were used as controls, with isolate D2 being virulent towards both *Rlm4* and *Rlm7*, whilst isolate D22 is virulent towards *Rlm4* and avirulent towards *Rlm7*.

4.3 | *AvrLm4-7* allele diversity analysis

Alleles of *AvrLm4-7* were identified by PCR amplification and Sanger sequencing. Genomic DNA was extracted from mycelia of each of the isolates as previously reported (Van de Wouw et al., 2010). The *AvrLm4-7* gene was amplified using primers (5'-AGAAGGGTAAGGGGCAAGTC-3' and 5'-GAAGAACCCTGC TAGATAGGTAAGC-3') and PCR conditions previously reported

(Van de Wouw & Howlett, 2012). Amplicons were purified using a PCR purification kit as per manufacturer's specifications (Qiagen) and then sequenced using BigDye terminator cycling at the Australian Genome Research Facility (Melbourne, Australia). Sequences were analysed using Geneious R9.1.8. All *AvrLm4-7* sequences were aligned to the avirulent *AvrLm4-7* reference sequence of *L. maculans* isolate v23.1.3 (GenBank accession no. AM998638; Parlange et al., 2009) as well as the RIP allele of isolate Nz-T4 (GenBank accession no. KT804641). Allele nomenclature was based on alleles previously published in Van de Wouw and Howlett (2012). RIP mutations were identified as CpA to TpA and TpG to TpA mutations as previously reported (Selker & Garrett, 1988). All allele sequences were uploaded to GenBank (accession numbers OM470482–OM470505).

Differences in genetic diversity and genetic indices were analysed using AMOVA, Nei's genetic distance calculations, Shannon's information index, and diversity index in GenAEx v. 6.503 (Peakall & Smouse, 2012). AMOVAs were performed with 999 random permutations. Haplotype maps of the various *AvrLm4-7* alleles were generated using PopART (<http://popart.otago.ac.nz>) using median-joining and minimum-spanning networks (Bandelt et al., 1999). Maximum-likelihood phylogenetic analyses were conducted using the bootstrap method (1000 replications) using the software MEGA v. 10.2.6 (Kumar et al., 2018).

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DATA AVAILABILITY STATEMENT

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

ORCID

Angela P. Van de Wouw  <https://orcid.org/0000-0001-5147-0393>

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