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Molecular Markers for Identifying Resistance Genes in *Brassica napus*

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Abstract: Blackleg disease, caused by the fungal pathogen *Leptosphaeria maculans*, is the most devastating disease of canola (*Brassica napus*, oilseed rape) worldwide. Breeding for genetic resistance is the most widely used tool for controlling this disease and minimizing the impact on yield. To date, five resistance genes (*Rlm2, LepR3, Rlm4, Rlm7, Rlm9*) have been cloned from *B. napus*, representing alleles of two different gene loci, *Rlm2-LepR3* and *Rlm4-7-9*. We report on the development and validation of Kompetitive Allele-Specific PCR (KASP) markers that can discriminate between the resistant and susceptible alleles of each resistance gene. These markers will provide valuable tools for both researchers and industry through the ability to characterize resistance genes without phenotyping.

Keywords: Kompetitive Allele-Specific PCR (KASP); blackleg; *Leptosphaeria maculans*; canola; molecular breeding

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Citation: Van de Wouw, A.P.; Zhang, Y.; Mohd Saad, N.S.; Yang, H.; Sheedy, E.; Elliott, C.E.; Batley, J. Molecular Markers for Identifying Resistance Genes in *Brassica napus*. *Agronomy* **2022**, *12*, 985. https://doi.org/10.3390/agronomy12050985

Academic Editor: Yong-Bao Pan

Received: 22 March 2022
Accepted: 14 April 2022
Published: 20 April 2022

1. Introduction

Fungal pathogens are estimated to cause yield losses of around 15% in agricultural crops across the world [1]. While cultural and chemical practices are often employed to minimize disease, breeding for resistance is the major strategy used for reducing these losses [2], and blackleg disease of canola (oilseed rape, *Brassica napus*), is no exception. Blackleg disease, caused by the ascomycete fungus *Leptosphaeria maculans*, is estimated to cause global losses to canola growers of approximately USD 1 billion per year [1]. The control of blackleg disease generally involves a three-pronged approach consisting of cultural, chemical, and genetic practices. However, the reliance on cultural and chemical practices changes dramatically in each global region, for example, in Australia 95% of growers apply fungicides, whilst this is much lower in other regions [3,4]. However, breeding for genetic resistance remains a universal approach to minimizing this disease across all canola-growing regions [2].

The *B. napus*–*L. maculans* interaction involves two types of resistance: quantitative (minor gene) and qualitative (major gene) resistance [5]. Quantitative resistance is thought to be conferred by the contribution of a number of minor genes and minimizes the damage caused by the disease [6]. Whilst quantitative resistance remains poorly understood, recent work has shown that for the *B. napus*–*L. maculans* interaction it is expressed throughout the plant and appears to be isolate-specific [7,8]. In comparison, qualitative resistance is well understood for the *B. napus*–*L. maculans* interaction. Qualitative resistance occurs in a gene-for-gene manner whereby for each resistance gene in the host, there is an a virulence effector gene in the pathogen [9]. A total of 18 resistance genes have been genetically mapped from *Brassica* species that confer resistance to blackleg, with five of these genes, *Rlm2*, *Rlm4*, *Rlm7*, *Rlm9* and *LepR3*, having been cloned [5,10–17]. The *Rlm2* and *LepR3* genes are alleles of the same locus (*Rlm2-LepR3*), with each allele encoding a receptor-like protein [16,17]. Similarly, *Rlm4*, *Rlm7* and *Rlm9* are alleles of the same locus (*Rlm4-7-9*), with each allele encoding a wall-associated kinase-like protein [14,15].

With the increase in genomic resources, and therefore the identification of resistance genes, the development of molecular markers associated with resistance genes could dramatically improve genetic gains for controlling disease, through accelerated breeding [18]. Molecular markers for resistance genes reduce the reliance on phenotyping, which can be laborious and cannot always resolve the full genotype. Currently, the characterization of resistance genes in *B. napus* cultivars and advanced breeding lines is reliant on the screening of material with sets of well-characterized differential isolates [7,9,19]. While phenotyping can infer which resistance genes are present through the patterns of resistance and susceptibility displayed by the set of differential isolates, some genes can be masked by the presence of others. For example, when a differential set of isolates is exposed to novel resistance genes, whereby the pattern of resistance and susceptibility is unknown, the presence of known resistance genes in the background may not be detectable. In these situations, additional isolates that have been transformed with specific avirulence genes, known as differential addition isolates (DAI), can be used to detect known resistance genes through screening of progenitor and complemented isolates and looking for changes from virulent to avirulent reactions [20]. However, these phenotyping screens are limited as they require the progenitor isolates to be virulent.

Phenotyping to identify resistance genes is complicated further in the *B. napus*–*L. maculans* interaction due to both dual-specificity and epistasis of avirulence genes. In *L. maculans*, some avirulence genes have dual specificity and are recognised by more than one resistance gene. For example, *AvrLm1-R3* is a single avirulence gene that is recognised by both the *Rlm1* and *LepR3* resistance genes [16,21]. Therefore, when using isolates for phenotyping that are *AvrLm1*, it cannot be determined whether *Rlm1* or *LepR3* is responsible for the resistant reaction. Similarly, the *AvrLm4-7* gene is recognised by both the *Rlm4* and *Rlm7* resistance genes [22]. When an isolate is virulent towards *Rlm7*, the isolate is also automatically virulent towards *Rlm4*, therefore if the host is heterozygous for *Rlm4* and *Rlm7*, this cannot be determined. Lastly, the *AvrLm4-7* gene is epistatic over both the *AvrLm3-Rlm3* and *AvrLm9-Rlm9* interactions and makes mapping and identification of both avirulence and resistance genes complicated [14,23,24]. It is currently unknown whether other epistatic interactions exist in the *B. napus*–*L. maculans* interaction.

The development of molecular markers for the cloned resistance genes of *B. napus* should resolve these phenotyping issues, thus allowing accurate tracking of resistance genes in both commercial cultivars and breeding programs. Since the resistance genes that have been identified are alleles at the same locus, allele-specific markers are required that can discriminate between susceptible and resistant alleles at each of the loci. The Kompetitive Allele-Specific PCR (KASP) genotyping system allows detection of single nucleotide polymorphisms, as well as insertions/deletions, at the specific region of interest (https://www.biosearchtech.com/products/pcr-kits-and-reagents/genotyping-assays/kasp-genotyping-chemistry (accessed on 19 March 2022)). KASP genotyping requires an assay mix that contains two allele-specific primers, which are each fluorescently tagged with a different dye, and one common reverse primer. A competitive allele-specific PCR then allows amplification of each of the relevant amplicons. If the genotype of the sample being screened is homozygous, only one of the two possible fluorescent signals will be generated. If the genotype of the sample being screened is heterozygous, a mixed florescent signal will be generated. Here, we describe the development and validation of KASP markers for discriminating *Rlm2*, *LepR3*, *Rlm9*, *Rlm4* and *Rlm7* from the susceptible alleles at the corresponding loci.

2. Materials and Methods

2.1. Genotypic Characterization of Resistance Genes

The alleles of the *Rlm2-LepR3* and *Rlm4-Rlm7-Rlm9* loci were obtained from 128 *B. napus* cultivars and advanced breeding lines using either PCR amplification of the gene followed by Sanger or MiSeq sequencing, or complete genome sequencing (Table S1). The MiSeq instrument is a benchtop sequencer that allows for both single- or paired-end sequencing of between 36 to 300 base pairs [25]. DNA was extracted from leaf tissues of each *B. napus* cultivar/line using the DNeasy Plant Mini Kit (Qiagen) as per the manufacturer's instructions. For each cultivar/line, leaf tissue was collected from 8 different plants and combined within a single sample.

LepR3/*Rlm2* alleles were amplified using three pairs of specific primers (Supplementary Materials, Table S2) using high-fidelity polymerase (Phusion Hot Start II High-Fidelity DNA Polymerase, Thermo Scientific, Waltham, MA, USA). Each PCR reaction mixture contained 2 µL of sample DNA (50–100 ng/µL), 20 µM of each primer, 25 µL of high-fidelity polymerase reaction mix (1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.02 U/µL Phusion enzyme in final reaction concentration), 1.5 µL DMSO and dH_2O to a final volume of 50 µL. The PCR reaction conditions were: 98 °C, 30 s; (98 °C, 5 s; 60 °C, 10 or 20 s; 72 °C, 2 min 30 s) × 35; 72 °C, 10 min; 4 °C hold. *Rlm4-7-9* alleles were amplified using the PCR primers listed in the Supplementary Materials, Table S1. The long-range PCR (LR-PCR) reaction consisted of 2× Platinum Superfi PCR Master Mix (Thermo Fisher Scientific, United States), 10 µM each of forward and reverse primer and 50 ng of gDNA and followed the LR-PCR (>10 Kbp) thermocycling conditions from the manufacturer's protocol. The resulting products were electrophoresed on a 1% agarose gel before being excised from the gel and purified using the Wizard Plus SV Minipreps DNA Purification System following the manufacturer's protocol (Promega Corporation, Madison, WI, USA). The purified PCR amplicons were sequenced on an Illumina MiSeq platform at the Australian Genome Research Facility (AGRF), Perth, Australia. MiSeq reads were de novo assembled and mapped to the reference Darmor v10 in Geneious Prime 2020 v2.1 [26]. Purified DNA fragments for Sanger sequencing were prepared according to the AGRF Sanger sequencing preparation guide (https://static1.squarespace.com/static/5c6a2bfa11f7845bc7a99405/t/5e1406a73f8ed65e20760957/1578370730168/Sanger+Sequencing+Sample+Preparation+Guide.pdf (accessed on 19 March 2022)), where each 12 µL reaction contained 60 to 90 ng of purified DNA, depending on the amplicon size, and 0.8 pmol/µL of sequencing primer. The list of primers used for Sanger sequencing are summarized in Supplementary Materials, Table S2. Low quality reads at the beginning and the end of the Sanger raw reads were trimmed before mapping to the reference sequences in Geneious Prime 2020 v2.1.

2.2. Phenotypic Characterization of Resistance Genes

The presence and absence of resistance genes *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm6*, *Rlm7*, *Rlm9*, *LepR1*, *LepR2*, *LepR3* and *RlmS* was determined in *B. napus* commercial and advanced breeding lines through phenotyping using 16–20 differential *L. maculans* isolates (Supplementary Materials, Table S3). A set of 16 control lines with known resistance genotypes, were used as controls for all phenotyping (Table 1). Isolates were inoculated onto wounded seedlings and disease development was allowed to progress for 14 days before lesions were scored on the 0–9 scale as previously described [7]. The presence and absence of resistance genes were inferred through patterns of virulence and avirulence for the well-characterized differential isolates, as previously described [7].

Table 1. Control *B. napus* lines/cultivars used for testing molecular markers and phenotyping.

Cultivar/Line	Resistance Gene(s)	Reference
Westar	None	[27]
Topas-DH16516	None	[28]
Topas-Rlm1	Rlm1	[28]
Topas-Rlm2	Rlm2	[28]
Topas-Rlm3	Rlm3	[28]
Topas-Rlm4	Rlm4	[28]
Topas-Rlm7	Rlm7	[28]
Topas-Rlm9	Rlm9	[28]
Topas-LepR1	LepR1	[28]
Topas-LepR2	LepR2	[28]
Topas-LepR3	LepR3	[28]
Express	Rlm2	[27]
Surpass501TT	LepR3, RlmS	[16,21]
BASF3000TR	Rlm4	[7]
Caiman	Rlm7	[7]
ATR-Gem	Rlm1, Rlm9	[7]

Due to discrepancies between the phenotype and genotype data, additional phenotypic characterization of *Rlm9*-harbouring lines was required. A subset of lines was screened using differential addition isolates (DAI) that had been transformed with a functional copy of the *AvrLm9* allele. Isolate D3, virulent towards both *Rlm7* and *Rlm9* (and therefore no epistatic effect of *AvrLm7*), was used as a progenitor strain for transformation. A complementation construct for *AvrLm9* was generated by amplification of an 1810 bp fragment, containing the complete *AvrLm9* ORF and up and downstream regions, from genomic DNA of *L. maculans* isolates D10 using primers AvrLm9_cloningF and AvrLm9_cloningR (Supplementary Materials, Table S2). This fragment was cloned into plasmid pPZP-Hyg*Hind*X as previously described [20,29] and the resulting plasmid transformed into isolate D3 using *Agrobacterium*-mediated transformation as previously described [20,30]. The resulting transformants, D3+AvrLm9#1 and D3+AvrLm9#2 were inoculated onto a subset of control and test lines and compared to the progenitor isolates, D3. Inoculations were carried out as described above.

2.3. *KASP Marker Development and Protocols*

KASP assays were designed through Geneworks Australia (www.geneworks.com.au/ (accessed on 19 March 2022)) following submission of sequences flanking SNPs of interest. Regions were chosen with a SNP that discriminates between the resistant and susceptible alleles and with minimal other variation to allow primer design.

For each gene of interest, allele-specific primers with fluorescent tags (FAM or HEX), and a common primer were designed to allow detection of the two alternative SNPs being targeted (Table 2, Figure 1). For each assay, a primer assay mix was made which consisted of 12 µL of primer X (100 mM), 12 µL of primer Y (100 mM), 30 µL of primer Common (100 mM) and 46 µL of 10 mM Tris-HCL (pH 8.3). The KASP reactions were run in a total volume of 10 µL consisting of 5.0 µL KASP 2x Master Mix (KASP-TF V4.0 Low ROX), 0.14 µL Primer assay mix, 3.86 µL sterile H_2O, and 1.0 µL DNA (1–2 ng/mL). All KASP assays were carried out in a Quantstudio 5 machine using the conditions provided in Table 3. Two replicates were included for every sample in each assay run. Resistant, susceptible, heterozygote (where applicable) and no template control samples were included in each run, and labelled accordingly within the Quantstudio 5 software, for allele discrimination following acquisition of the data. All data were exported from Quantstudio 5 and analyzed using Microsoft Excel. Allele discrimination was performed through the Quantstudio 5 software but manually checked within Microsoft Excel through the analysis of clustering with the known control samples.

Table 2. Primer sequences for Kompetitive Allele-Specific PCR (KASP) markers for the *Brassica napus* resistance genes *Rlm2*, *LepR3*, *Rlm9*, *Rlm4* and *Rlm7*.

Gene	Primer Name	Primer Sequence	SNP	Allele Detected	Fluorescence
Rlm2	Rlm2_AlleleX	GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA	A	*Rlm2*	FAM
	Rlm2_AlleleY	GAAGGTCGGAGTCAACGGATTGATAAGTTGGATAGCAGCTGCAATTG	G	*rlm2*	HEX
	Rlm2_Common	AATCCAAATRCAATACCAGGTATGAAA			
LepR3	LepR3_AlleleX	GAAGGTGACCAAGTTCATGCTATGGTTGCCGGAGTTTWTTCGGAT	A	*LepR3*	FAM
	LepR3_AlleleY	GAAGGTCGGAGTCAACGGATTGGTTGCCGGAGTTTWTTCGGAC	G	*lepR3*	HEX
	LepR3_Common	CCAACAACACTTTCACCAGYTTCGAAA			
Rlm9	Rlm9_AlleleX	GAAGGTGACCAAGTTCATGCTCGTAGAAAGGGCTCCCCGTC	G	*rlm9*	FAM
	Rlm9_AlleleY	GAAGGTCGGAGTCAACGGATTAACGTAGAAAGGGCTCCCCGTA	T	*Rlm9*	HEX
	Rlm9_Common	AACGAACAAGAGTCTACATCACTTCTGAA			
Rlm4 and Rlm7 [1]	Rlm47_AlleleX	GAAGGTGACCAAGTTCATGCTATTTATGTCTCCCGTCCTTTTCCTAT	A	*Rlm7*	FAM
	Rlm47_AlleleY	GAAGGTCGGAGTCAACGGATTTATGTCTCCCGTCCTTTTCCTAC	G	*Rlm4*	HEX
	Rlm47_Common	CACATATCATTTGATCAGAACAAATTAAAT			

[1] No amplification indicates susceptible alleles.

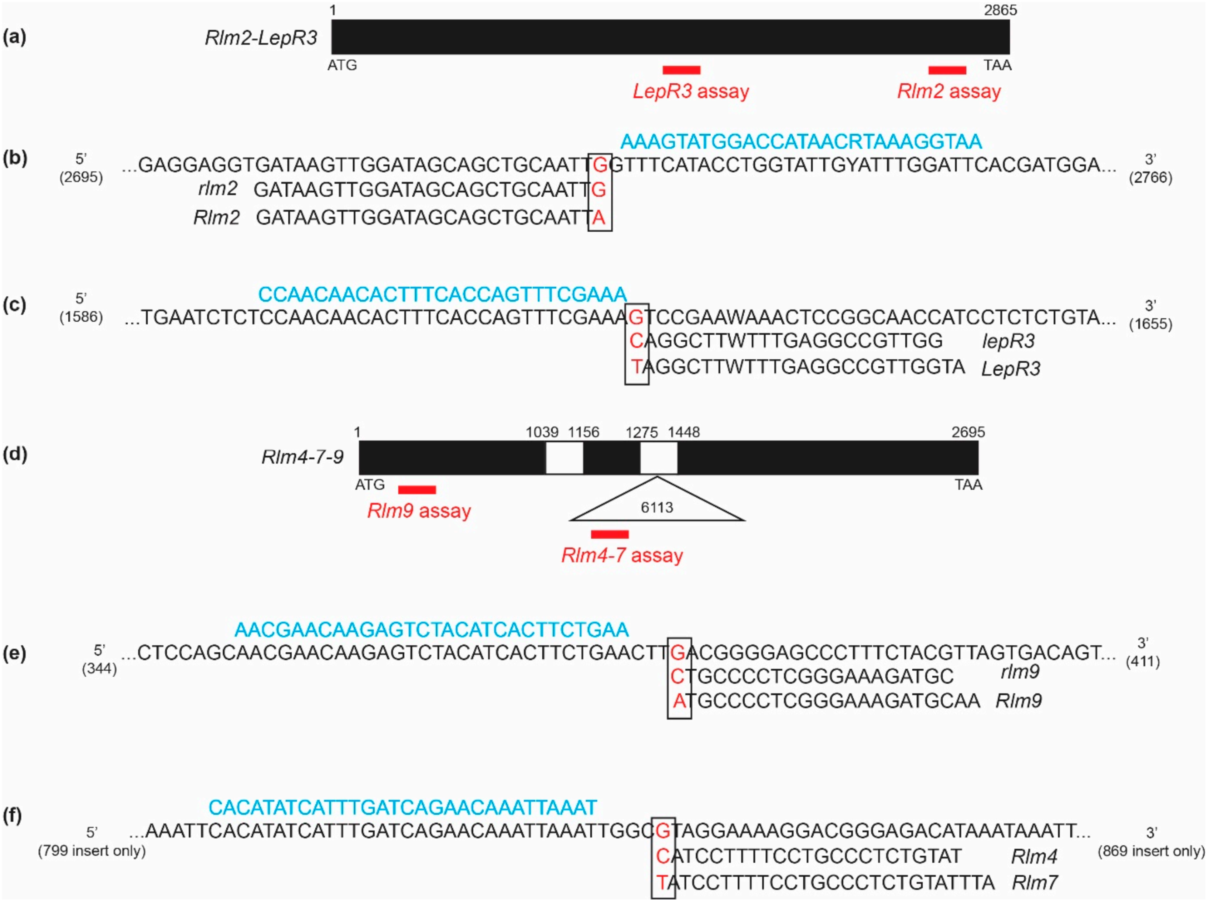

Figure 1. Location and flanking sequences of the Kompetitive Allele-Specific PCR (KASP) markers for *B. napus* resistance genes, *Rlm2*, *LepR3*, *Rlm4*, *Rlm7* and *Rlm9*. Location of the *LepR3* and *Rlm2* assays within the *Rlm2-LepR3* gene (**a**) and the *Rlm9*, *Rlm4* and *Rlm7* assays within the *Rlm4-7-9* gene region (**d**). Exons are indicated in black, introns in white and the 6113 bp insertion characteristic of *Rlm4* and *Rlm7* alleles are indicated by the triangle. The primer binding sites, flanking sequences and targeted SNP for the *Rlm2* (**b**), *LepR3* (**c**), *Rlm9* (**e**) and *Rlm4*/*Rlm7* (**f**) KASP assays are provided. The common primer for each assay is indicated in blue, whilst the allele-specific primers for each gene are highlighted in black with the targeted SNP indicated in red. Nucleotide positions of the flanking sequences within the genes are provided.

Table 3. Thermal cycling conditions for KASP assays using the Quantstudio 5 machine.

Cycles	Temp	Time	Type of Cycling
1×	94 °C	15 min	Denature
10×	94 °C	20 s	PCR
	61–55 °C (decreasing temp each cycle)	60 s	
26×	94 °C	20 s	PCR
	55 °C	60 s	
1×	25 °C	1 min	Cooling
1×	25 °C	30 s	Acquire data
3×	94 °C	20 s	Recycling (if required) [1]
	57 °C	60 s	
1×	25 °C	1 min	
	25 °C	30 s	Second data acquisition

[1] Results were observed after the first data acquisition. If appropriate separation between clusters was not clear, recycling was repeated until clear clusters were detected (maximum of three recycling).

Testing of KASP markers was carried out using the 16 previously characterized control lines listed in Table 1. Mock *rlm2/Rlm2*, *lepR3/LepR3*, *rlm9/Rlm9*, *rlm4/Rlm4*, *rlm7/Rlm7* heterozygote samples were made by mixing equal concentrations of DNA from the Topas-DH16516 susceptible line and the appropriate Topas resistant lines. Mock *Rlm2/LepR3*, *Rlm4/Rlm9*, *Rlm7/Rlm9* and *Rlm4/Rlm7* heterozygotes were also made by mixing equal concentrations of DNA from the appropriate individual lines. Validation of the markers was carried out on up to 595 commercial cultivars and advanced breeding lines that were phenotyped using the differential isolates, as described earlier.

3. Results

3.1. Identification of Resistance and Susceptible Alleles at the LepR3-Rlm2 and Rlm4-7-9 Gene Loci

The *LepR3-Rlm2* and *Rlm4-7-9* loci were sequenced from 128 *Brassica* lines/cultivars to detect all resistant and susceptible alleles at those loci. For the *LepR3-Rlm2* loci, a single *LepR3* and *Rlm2* allele were detected in addition to 10 different susceptible alleles (Supplementary Materials, Figure S1). For the *Rlm4-7-9* loci, two different *Rlm4* alleles, two different *Rlm7* alleles, a single *Rlm9* allele and five susceptible alleles were detected (Supplementary Materials, Figure S2) [15]. All material was also screened with a set of 16–20 differential isolates to confirm the phenotype of each line (data not shown). All phenotype and genotype data correlated, except for three lines, all from the same breeding company, that were identified with the *Rlm9* allele but phenotypically appeared as *rlm9*.

The genomes of these three lines were sequenced and confirmed to have the *Rlm9* allele and no sequence variation within the coding region or up or downstream of the *Rlm9* locus was observed (data not shown). These lines were screened with differential addition isolates (DAI) that were transformed with the *AvrLm9* allele to confirm the *rlm9* phenotype. All isolates were virulent towards the susceptible control, Westar, as well as Topas-*Rlm1* and Topas-*Rlm2*, which do not harbor *Rlm9* (Figure 2). As expected, the D3+*AvrLm9* isolates were all avirulent towards Topas-*Rlm9*, ATR-Gem, ATR-Bonito and ATR-Mako, which all harbor the *Rlm9* gene whilst the progenitor isolate, D3, was virulent. The D3+*AvrLm9* isolates remained virulent towards the Company lines 1–3 suggesting that although the *Rlm9* allele is present, it is not conferring the *Rlm9-AvrLm9* interaction.

3.2. Rlm2 Molecular Marker

The *Rlm2* KASP assay was designed based on a discriminative SNP at position 2728 bp of the CDS of the Westar *rlm2-lepr3* allele (Figure 1, Table 2). Testing of the *Rlm2* KASP marker across the set of 16 control lines showed that the marker could distinguish between homozygous susceptible (*rlm2/rlm2*), homozygous resistant (*Rlm2/Rlm2*) and heterozygous (*Rlm2/rlm2* or *Rlm2/LepR3*) lines/samples (Figure 3a).

The *Rlm2* KASP marker was then validated across a further 479 commercial and advanced breeding lines that had been phenotyped for *Rlm2* (data not shown). Of the 479 lines, four were phenotyped as *Rlm2* and the remaining 475 lines as *rlm2*. When these 479 lines were tested with the *Rlm2* KASP marker, three of the lines were identified as being homozygous for *Rlm2* and one heterozygous for *Rlm2* correlating 100% with the phenotype data.

3.3. LepR3 Molecular Marker

The *LepR3* KASP assay was designed based on a discriminative SNP at position 1622 bp of the CDS of the Westar *rlm2-lepr3* allele (Figure 1, Table 2). Testing of the *LepR3* KASP marker across the set of 16 control lines showed that the marker could distinguish between homozygous susceptible (*lepR3/lepR3*), homozygous resistant (*LepR3/LepR3*) and heterozygous (*LepR3/lepR3* or *Rlm2/LepR3*) lines/samples (Figure 3b).

The *LepR3* KASP marker was then validated across a further 481 commercial and advanced breeding lines that had been phenotyped for *LepR3/Rlm1* (data not shown). Of the 481 lines, 217 were phenotyped as either *Rlm1* or *LepR3*, which are indistinguishable through phenotyping. The remaining 264 lines were phenotyped as *rlm1/lepR3*. Using the *LepR3* KASP marker, 33 of the 217 lines that were phenotypically characterized as having either *Rlm1* or *LepR3* were identified as having *LepR3*, suggesting the remaining 184 were *Rlm1*. Of the 33 lines identified as having *LepR3*, nine of these were heterozygous and 24 were homozygous.

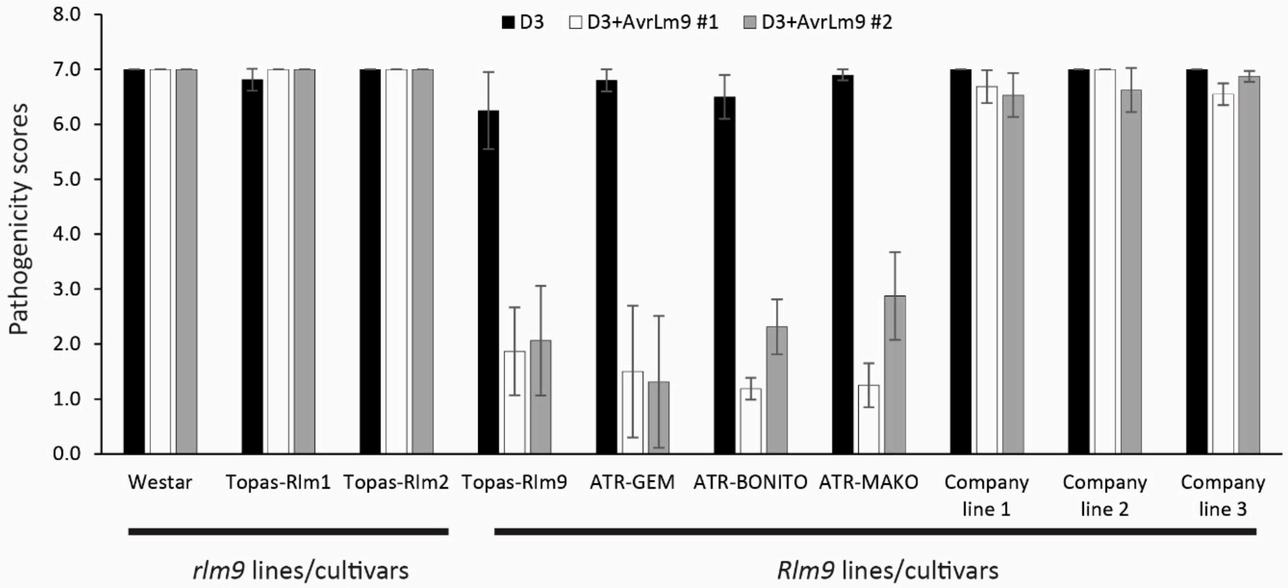

Figure 2. Confirmation of the *Rlm9-AvrLm9* interaction in *B. napus* lines/cultivars with differential addition isolates (DAI). Isolates D3+AvrLm9 #1 and D3+AvrLm9 #2 are avirulent towards *Rlm9* due to transformation of the progenitor isolate, D3, with *AvrLm9*. The *rlm9* cultivars/lines were susceptible to all isolates as expected. The Topas-*Rlm9*, ATR-Gem, ATR-Bonito and ATR-Mako lines were all resistant to the DAI isolates as expected, due to the presence of the *Rlm9* allele. Company line 1–3 unexpectedly showed a susceptible reaction to all isolates despite genotypically harboring the *Rlm9* allele. Error bars represent the standard error from eight replicate plants.

3.4. *Rlm9 Molecular Marker*

The *Rlm9* KASP assay was designed based on a discriminative SNP at position 381 bp within the first exon of the CDS of the Westar *rlm4-7-9* allele (Figure 1, Table 2). Testing of the *Rlm9* KASP marker across the set of 16 control lines showed that the marker could distinguish between homozygous susceptible (*rlm9*/*rlm9*), homozygous resistant (*Rlm9*/*Rlm9*) and heterozygous (*Rlm9*/*rlm9*, *Rlm9*/*Rlm4* or *Rlm9*/*Rlm7*) lines/samples (Figure 3c).

The *Rlm9* KASP marker was then validated across a further 132 commercial and advanced breeding lines that had been phenotyped for *Rlm9* (data not shown). Of the 132 lines, 42 lines were phenotypically detected as having *Rlm9*. Using the *Rlm9* KASP marker, all of these 42 lines were identified as having *Rlm9*, eight of which were heterozygous for *Rlm9* and the remaining 33 were homozygous.

3.5. *Rlm4 and Rlm7 Molecular Marker*

The *Rlm4-7* KASP assay was designed based on a discriminative SNP 853 bp in the insert, present in only *Rlm4* or *Rlm7* alleles at the *Rlm4-7-9* locus (Figure 1, Table 2). This SNP discriminates between *Rlm4* and *Rlm7*, with both *Rlm7* alleles harboring this SNP and therefore detectable. Testing of the *Rlm4-7* KASP marker across the set of 16 control lines showed that the marker could distinguish between homozygous *Rlm4* (*Rlm4*/*Rlm4*), homozygous *Rlm7* (*Rlm7*/*Rlm7*), heterozygous (*Rlm4/Rlm7*) and homozygous susceptible (*rlm4-7*/*rlm4-7*) lines/samples (Figure 3d). Since the KASP marker amplifies within the insertion that is unique to the *Rlm4* and *Rlm7* alleles, no amplification will be detected for susceptible alleles. Therefore, *Rlm4*/*rlm4* or *Rlm7*/*rlm7* heterozygotes will not be distinguishable from the *Rlm4*/*Rlm4* or *Rlm7*/*Rlm7* homozygotes, respectively, making the markers appear dominant rather than co-dominant (Figure 3d).

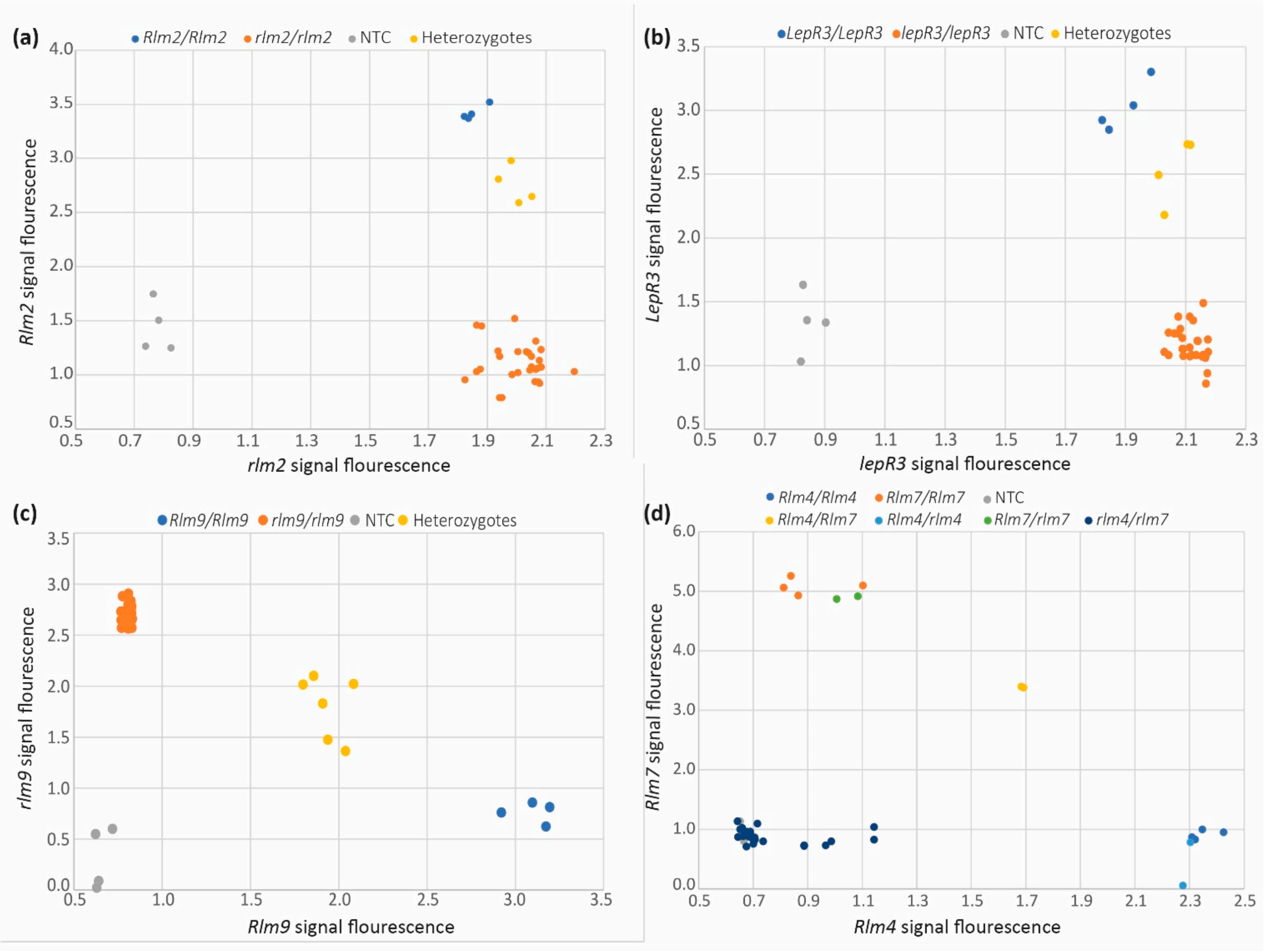

Figure 3. Cluster plot diagrams of Kompetitive Allele-Specific PCR (KASP) assays for *Rlm2* (**a**), *LepR3* (**b**), *Rlm9* (**c**) and *Rlm4* and *Rlm7* (**d**). Each data point represents the fluorescence signal of a single DNA sample. DNA samples from 16 *B. npaus.* (**a**–**c**) The KASP markers for *Rlm2*, *LepR3* and *Rlm9* allow heterozygotes to be distinguished from the homozygote resistant and susceptible lines. (**d**) However, for the KASP marker for *Rlm4* and *Rlm7*, only *Rlm4*/*Rlm7* heterozygotes can be distinguished from the homozygote resistant and susceptible lines as the primer binds within the insertion that is specific to the *Rlm4* and *Rlm7* alleles, and therefore susceptible alleles do not amplify. Two replicates are included for all samples. NTC = no template control.

The *Rlm4-7* KASP marker was then validated across a further 595 commercial and advanced breeding lines that had been phenotyped for *Rlm4* and *Rlm7* (data not shown). The phenotype screening revealed that of the 595 lines, 263 had *Rlm4* whilst 48 had *Rlm7*. Application of the KASP marker across all lines detected *Rlm7* in the 48 lines whilst *Rlm4* was detected in the 263 lines, as well as 18 of the *Rlm7* lines, showing that these 18 lines were heterozygous for *Rlm4*/*Rlm7*, something that phenotyping cannot distinguish as isolates that are virulent towards *Rlm7* are also virulent towards *Rlm4*.

4. Discussion

The use of molecular breeding is rapidly increasing in agricultural crops as the need for improved yields increases with population growth and food-production demands [31]. Molecular markers for resistance genes can aid in the breeding of cultivars by reducing the need for laborious phenotyping and providing unequivocal information about the genotype of the cultivar/line. The KASP markers developed here are allele-specific markers that can discriminate homozygous resistant and susceptible lines from heterozygotes in a high-throughput, low-cost method, and are already being used by commercial breeding companies and private research groups in Australia (Van de Wouw; pers. comm).

In many host–pathogen systems, the longevity of resistance genes requires disease deployment strategies such as rotation of resistance genes in both space and time [32,33]. However, for these types of strategies to work, thorough knowledge of the genotype of the host is required. For the *B. napus–L. maculans* system, there are a number of examples whereby cultivars were commercially released that were considered to have single novel R genes, but upon further characterization were identified as containing up to three resistance genes, one being a novel resistance gene and the others being already deployed resistance genes [21,34,35]. These previously deployed resistance genes were masked due to the presence of novel sources of resistance; the use of molecular markers would prevent these types of scenarios occurring. Furthermore, for pathosystems such as *B. napus*/*L. maculans*, the rotation of single resistance genes is recommended [32]. However, in Australia and Canada, many of the commercial cultivars contain stacks of multiple resistance genes [3,19,35]. The removal of resistance genes to allow the development of single R gene cultivars is extremely laborious using phenotyping alone, and is therefore something in which canola breeders have shown little interest. However, the use of molecular markers for tracking specific resistance genes provides a more feasible opportunity for such strategies. Lastly, the development of molecular markers for the *B. napus* resistance genes will aid in the cloning of additional resistance genes in situations such as the *LepR3/Rlm1* scenario, whereby phenotyping cannot discriminate these genes, thus potentially complicating the mapping.

The development of allele-specific PCR markers requires confidence that the SNP being targeted is specific to the alleles of interest. To achieve this, validation is required from diverse germplasm, representing different genetic lineages such that any sequence variation will be detected and can be accounted for. The identification of two different *Rlm7* alleles was only possible thanks to material being included from Australia, Europe, and America [15]. The whole genome sequencing of different accessions of different species will aid in the development of markers moving forward. Continual sequencing of new germplasm will be required to ensure that the markers are still accurate and that new susceptible or resistant alleles that may be identified are still distinguishable using the markers.

The validation of markers also requires comparisons with sound phenotypic data. The identification of three lines, from a single breeding company, that harbored the *Rlm9* allele but did not display the *Rlm9-AvrLm9* resistant reaction, requires further investigation. Genome sequencing of the entire *Rlm9* gene region showed no polymorphisms in these company lines compared to the *Rlm9* resistant allele. Whilst these lines are not phenotypically *Rlm9*, the KASP marker will detect the *Rlm9* allele in them, resulting in a false positive phenotypically, i.e., the line is genotypically *Rlm9* but phenotypically *rlm9*. The reason for this unusual discrepancy remains unknown; however, one possible explanation is that a second gene is involved that suppresses the expression of *Rlm9* in these lines and therefore prevents recognition of the *AvrLm9* protein. Inoculation experiments using avirulent and virulent isolates could be conducted whereby the expression of *Rlm9* is determined in these lines compared to lines correctly expressing the *Rlm9* and *rlm9* phenotypes. Alternatively, mapping populations could be generated between these unusual *Rlm9* lines and segregation could be looked at to determine whether a second gene was masking the *Rlm9* phenotype.

Through the validation of the molecular markers, it was found that 47% of cultivars/lines harbored the *Rlm4* gene, similar to previous reports that suggested *Rlm4* was present in over half of the Australian cultivars [3,35]. The *Rlm9* resistance gene was present in 32% of cultivars, whilst *LepR3* and *Rlm7* was present in 7% and 8%, respectively. Interestingly, *Rlm2* was only present in four cultivars/lines (0.8%). However, the Australian *L. maculans* population is almost fixed for the virulent *avrLm2* allele [36]. If almost no cultivars harbor the *Rlm2* allele, why is the population remaining virulent? One possibility is that the avirulent allele, *AvrLm2*, has a fitness cost rather than the virulent allele; however, biologically this would be counterintuitive. Alternatively, there may be a second resistance gene that recognizes the AvrLm2 protein, and that second gene is more common in the host and therefore responsible for maintaining selection towards the *avrLm2* isolates.

5. Conclusions

Molecular markers for discriminating resistance and susceptible alleles of resistance genes are valuable tools for research, breeding and management strategies for resistance gene deployment. The cloning of resistance genes, whilst providing insightful biological information, needs to be extended to practical outputs such as molecular markers for breeding and screening of germplasm. The development and deployment of KASP markers for the cloned *B. napus* resistance genes provide such tools for industry and will aid in the identification and breeding of future germplasm.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12050985/s1, Table S1: List of alleles identified at the *Rlm2-LepR3* and *Rlm4-7-9* gene loci from *B. napus* commercial cultivars and advanced breeding lines and the methods for obtaining sequences; Table S2. Primers used for PCR amplification and sanger sequencing of the *Rlm2-LepR3* and *Rlm4-7-9* gene loci from *B. napus* commercial cultivars and advanced breeding lines; Table S3. List of differential *L. maculans* isolates used for determining the presence and absence of resistance genes in *B. napus* cultivars and advanced breeding lines. Figure S1. Alignment of the resistant and susceptible alleles at the Rlm2-LepR3 locus. The SNP used for detecting LepR3 is highlighted in blue, whilst the SNP used for detecting the Rlm2 allele is highlighted in red. Figure S2. Alignment of the resistant and susceptible alleles at the Rlm4-Rlm7-Rlm9 locus. The SNP used for discriminating Rlm4 and Rlm7 is highlighted in blue, whilst the SNP used for detecting the Rlm9 allele is highlighted in red.

Author Contributions: Conceptualization, A.P.V.d.W. and J.B.; Methodology, A.P.V.d.W., J.B., C.E.E. and E.S. Validation, C.E.E., A.P.V.d.W., E.S., Y.Z., H.Y. and N.S.M.S.; Investigation, A.P.V.d.W., C.E.E., E.S., H.Y., Y.Z. and N.S.M.S.; Writing—Original Draft Preparation, A.P.V.d.W.; Writing—Review and Editing, A.P.V.d.W., J.B., C.E.E. and E.S.; Supervision, J.B. and A.P.V.d.W.; Funding Acquisition, A.P.V.d.W. and J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Grains Research and Development Corporation (UWA1905-006RTX).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are either openly available in GenBank or can be made available on request from the corresponding author.

Acknowledgments: The authors would like to thank the Australian breeding companies for access to commercial cultivars and advanced breeding lines for genotypic and phenotypic screening. We thank the Grains Research and Development Corporation for funding.

Conflicts of Interest: The authors declare no conflict of interest.

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