Molecular Markers for Identifying Resistance Genes in Brassica napus

Angela P. Van de Wouw 1,* , Yueqi Zhang 2 , Nur Shuhadah Mohd Saad 2 , Hua Yang 2 , Elizabeth Sheedy 3 , Candace E. Elliott 1 and Jacqueline Batley 2

1 School of BioSciences, University of Melbourne, Parkville, Melbourne, VIC 3010, Australia; candace.elliott@awe.gov.au
2 School of Biological Sciences, The University of Western Australia, Crawley, WA 6009, Australia; 22078946@student.uwa.edu.au (Y.Z.); nur.mohdsaad@research.uwa.edu.au (N.S.M.S.); hua.yang1@uq.net.au (H.Y.); jacqueline.batley@uwa.edu.au (J.B.)
3 Marcroft Grains Pathology, Grains Innovation Park, Horsham, VIC 3400, Australia; biz.sheedy@gmail.com
* Correspondence: apvdw2@unimelb.edu.au

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

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 avirulence 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 fluorescent 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 \textit{Rlm2-LepR3} and \textit{Rlm4-Rlm7-Rlm9} loci were obtained from 128 \textit{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 \textit{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.

\textit{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 MgCl₂, 200 µM of each dNTP and 0.02 U/µL Phusion enzyme in final reaction concentration), 1.5 µL DMSO and dH₂O 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.

\textit{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 \textit{Rlm1}, \textit{Rlm2}, \textit{Rlm3}, \textit{Rlm4}, \textit{Rlm6}, \textit{Rlm7}, \textit{Rlm9}, \textit{LepR1}, \textit{LepR2}, \textit{LepR3} and \textit{RlmS} was determined in \textit{B. napus} commercial and advanced breeding lines through phenotyping using 16–20 differential \textit{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       |                    |           |
| 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-HygHindX 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 H₂O, 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     | Rlm2_AlleleY  | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | G   | rlmt            | HEX          |
|          | Rlm2_Common   | ATCCAAATRCAATACCAGGTATGAA                 |     |                 |              |
| LepR3    | LepR3_AlleleX | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | A   | LepR3           | FAM          |
| LepR3    | LepR3_AlleleY | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | G   | lepr            | HEX          |
|          | LepR3_Common  | CAAACAACACTTTCCACCAGYTTTCAAA             |     |                 |              |
| Rlm9     | Rlm9_AlleleX  | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | G   | rlmt            | FAM          |
| Rlm9     | Rlm9_AlleleY  | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | T   | Rlm9            | HEX          |
|          | Rlm9_Common   | ACGAAAAGAGTGCTACATTCACATCTTCAGAA         |     |                 |              |
| Rlm4 and Rlm7 1 | Rlm47_AlleleX | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | A   | Rlm7            | FAM          |
| Rlm4     | Rlm47_AlleleY | GAAGGTGACCAAGTTCATGCTGATAAGTTGGATAGCAGCTGCAATTA | G   | Rlm4            | HEX          |
|          | Rlm47_Common  | CACATATCATTGATCAGAAACAAATTAAT            |     |                 |              |

1 No amplification indicates susceptible alleles.
Results were observed after the first data acquisition. If appropriate separation between clusters was not clear, thermal cycling conditions for KASP assays using the Quantstudio 5 machine.

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             |
| 26×    | 94 °C| 20 s  | PCR             |
| 1×     | 55 °C| 60 s  | PCR             |
| 1×     | 25 °C| 60 s  | Recycling (if required) |<sup>1</sup> |
| 1×     | 94 °C| 30 s  | Acquire data    |
| 3×     | 94 °C| 20 s  | Recycling (if required) |<sup>1</sup> |
| 1×     | 57 °C| 60 s  | Recycling (if required) |<sup>1</sup> |

<sup>1</sup> 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-Avrlm9 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.

![Graph](image-url)

**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).
were heterozygous for \( \text{Rlm4} \) was detected in the 263 lines, as well as 18 of the \( \text{Rlm7} \)

Application of the KASP marker across all lines detected \( \text{Rlm4} \)
The phenotype screening revealed that of the 595 lines, 263 had

Two replicates are included for all samples. NTC = no template control.

\( \text{LepR3} \)

LepR3 (homozygous for \( \text{Rlm2} \)) advanced breeding lines that had been phenotyped for

3.3. LepR3 Molecular Marker

isolates that are virulent towards \( \text{Rlm7} \)

advanced breeding lines that had been phenotyped for

The use of molecular breeding is rapidly increasing in agricultural crops as 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

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

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

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/10.390/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.

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