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Development of molecular markers linked to the *Leptosphaeria maculans* resistance gene *Rlm6* and inheritance of SCAR and CAPS markers in *Brassica napus* × *Brassica juncea* interspecific hybrids

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Abstract

Leptosphaeria maculans causes blackleg disease on *Brassica napus*, an economically important oilseed crop. *Brassica juncea* has high resistance to blackleg and is a source for the development of resistant *B. napus* varieties. To transfer the *Rlm6* resistance gene from *B. juncea* into *B. napus*, an interspecific cross between *B. napus* "Topas DH16516" and *B. juncea* "Forge" was produced, followed by the development of F_2 and F_3 generations. Sequence characterized amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers linked to the *L. maculans* resistance gene *Rlm6* were developed. Segregation of SCAR and CAPS markers linked to *Rlm6* were confirmed by genotyping of F_2 and F_3 progeny. Segregation of CAPS markers and phenotypes for blackleg disease severity in F_2 plants had a Mendelian ratio of 3:1 in resistant vs. susceptible plants, respectively, supporting the assumption that genetic control of resistance was by a single dominant gene. The molecular markers developed in this study, which show linkage with the *L. maculans* resistance gene *Rlm6*, would facilitate marker-assisted backcross breeding in a variety development programme.

KEYWORDS
blackleg, *Brassica species*, CAPS marker, inheritance, *Leptosphaeria maculans*, *R*-gene *Rlm6*

1 | INTRODUCTION

Canola (oilseed rape, *Brassica napus* L.) is grown extensively in Australia, Europe, North America and Asia. The crop is susceptible to several major pathogens including *Leptosphaeria maculans*, a causal agent of blackleg disease, which is responsible for severe yield losses worldwide (Fitt, Brun, Barbetti, & Rimmer, 2006). Blackleg disease has mainly been controlled by the utilization of host resistance, crop rotations and fungicide applications (Kutcher et al., 2013; Marcroft, Van de Wouw, Salisbury, Potter, & Howlett, 2012). A gene-for-gene interaction between the avirulence (*Avr*) genes in *L. maculans* and the corresponding resistance (*R*) genes in *B. napus*, which determines resistance to the disease, has been reported (Marcroft, Elliott, et al., 2012; Rouxel, Willner, Coudard, and Balesdent, 2003). *R*-gene-mediated resistance is often very effective in disease control, while the fungal populations carry a high frequency of the corresponding *Avr* gene (Daverdin et al., 2012). Eighteen *R*-genes for resistance to *L. maculans*, *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2*, have been identified in *Brassica* species (Raman, Raman, & Larkan, 2013). Among them, *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* were identified in *B. napus* (Ansan-Melayah et al., 1998; Balesdent, Attard, Kuhn, & Rouxel, 2002; Balesdent et al., 2001; Delourme et al., 2004; Van de Wouw et al., 2009); *Rlm5* and *Rlm6* in *B. juncea* (Balesdent et al., 2002; Chèvre et al., 1997, 2008; Christianson, Rimmer, Good, & Lydiate, 2006; Rimmer & van den Berg, 1992); *Rlm8* and *Rlm11* in *B. rapa* (Balesdent et al., 2002, 2013); *Rlm10* in *B. nigra* (Chèvre et al., 1996; Eber et al. 2011); and five resistance genes, *LepR1*, *LepR2*, *LepR3*, *LepR4* and *RlmS*, were introgressed from *B. rapa* subsp. *sylvestris* (Larkan et al., 2013; Van de Wouw et al., 2009; Yu, Gugel, Kutcher, Peng, & Rimmer, 2013; Yu, Lydiate, &

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Rimmer, 2005, 2008) and the remaining two genes *BLMR1* and *BLMR2* were identified from *B. napus* cultivar 'Surpass 400' (Long et al., 2011). Most resistance genes have been derived from A-genome species—either from *B. napus* or *B. rapa*—in a cultivar development programme, whereas B-genome species are little exploited in the crop improvement programme.

B-genome species *B. juncea* (AABB) derived from an inter-species hybridization, *B. nigra* (BB) × *B. rapa* (AA), whereas *B. napus* (AACC) developed from *B. oleracea* (CC) × *B. rapa* (AA). The A-genome species *B. rapa* is the common ancestor of *B. napus* and *B. juncea*, which makes an indirect genomic relationship with these two tetraploids revealed by the U triangle (Nagaharu, 1935). Earlier studies reported unsuccessful introgression of blackleg resistance to *B. napus* from *B. juncea* (Dixelius & Wahlberg, 1999; Prakash & Chopra, 1988). As *Brassica* species with the B-genome, that is, *B. nigra*, *B. carinata* and *B. juncea*, display complete resistance to *L. maculans* (Rimmer & van den Berg, 1992; Roy, 1978, 1984; Sjödin & Glimelius, 1988), effort has continued and, recently, *B. napus* lines have been successfully developed with B-genome introgression from *B. juncea* (Brun et al., 2010) and *B. carinata* (Navabi et al., 2010). A recent study has shown a very high frequency of *AvrLm6* in *L. maculans* isolates from Canadian canola fields (Liban, Cross, Kutcher, Peng, & Fernando, 2016), emphasizing the need to develop a canola cultivar with the corresponding *R*-gene *Rlm6*. Previously developed *Rlm6* harbouring lines are unavailable for commercial breeding. Therefore, investigation into the introgression of B-genome resistance into *B. napus* is essential.

Inheritance of blackleg resistance derived from the B-genome species by a single dominant gene has been reported in earlier studies (Chèvre et al., 1997, 2008; Plieske, Struss, & Röbbelen, 1998), suggesting the potential for developing cultivars from interspecific crosses. Cultivar development can be accelerated by marker-assisted selection in backcross populations. Several molecular markers for blackleg resistance have already been reported from several B-genome resistance sources (Barret et al., 1998; Chèvre et al., 1997; Plieske & Struss, 2001; Plieske et al., 1998; Saal & Struss, 2005). Besides, inheritance studies have also been reported in *B. napus* resistance using SCAR markers linked to the locus *cLmR1* (Mayerhofer et al., 2005), and "indel" markers linked to the locus *LepR3* (Larkan et al., 2013). As there is no commercial *B. napus* cultivar carrying *Rlm6*, this study has been undertaken as part of an introgression study with the aim of understanding the pattern of inheritance of *Rlm6* in segregating populations derived from a cross between *B. napus* and *B. juncea*. Additionally, the development of SCAR and CAPS markers linked to the locus *Rlm6* (Balesdent et al., 2002; Chèvre et al., 2008) would facilitate marker-assisted backcross breeding in a cultivar breeding programme.

2 | MATERIALS AND METHODS

2.1 | Plant materials

The *B. juncea* cultivar 'Forge' was used as pollen donor and was crossed with the susceptible *B. napus* cultivar 'Topas DH16516,' Resistant progenies were identified in each generation via phenotyping of *L. maculans* isolates carrying the avirulence gene *AvrLm6*, as well as marker genotyping. Only resistant seedlings (those showed hypersensitive responses [HRs] to the pathogen) were transferred to the plastic pot and grown under glasshouse conditions (22°C and 12-hr photoperiod). A SCAR marker linked to *Rlm6* was used to confirm introgression in interspecific hybrids followed by selfing towards next generation.

2.2 | *Leptosphaeria maculans* isolates

A set of differential isolates of *L. maculans* previously characterized for avirulence genes were used to characterize the *B. juncea* cultivar 'Forge.' A total of 11 isolates (D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, JN3 and J3), characterized by Leflon et al. (2007), Balesdent et al. (2005, 2013), Marcroft, Elliott, et al. (2012), and Zhang et al. (2016), were collected from the laboratory. A single spore isolate, ICBN14 (*AvrLm5*, *AvrLm6*, *AvrLepR1*), was chosen for phenotyping interspecific hybrids derived from the *B. napus* × *B. juncea* cross.

2.3 | Inoculum preparation and DNA extraction from *L. maculans*

Isolates were cultured, and the inoculum was prepared as described by Chen and Fernando (2006) with minor modifications. An infected paper disc with fungus was put on V8 agar medium, which was amended with 0.35% (*w/v*) streptomycine sulphate, and fungus was grown for 2 weeks at 20°C. Harvested pycnidiospores were stored at −20°C for further use. The concentration of spores was diluted to 2×10^7 spores/ml in each inoculation of the study. The CTAB method was followed to extract DNA from fungal pycnidiospores, according to Liban et al. (2016).

2.4 | Characterization of *Rlm6* in the cultivar 'Forge'

A set of 11 differential isolates (D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, JN3 and J3), in which 12 *R*-genes can be detected (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7*, *Rlm8*, *Rlm9*, *RlmS*, *LepR1* and *LepR2*), were used to confirm *Rlm6* presence in the cultivar 'Forge.' Seeds were sown in a plastic cell tray using standard growth media and kept in a growth chamber at 21°C/16°C (day/night) with a 16-hr photoperiod. The fully developed cotyledons of 7-day-old seedlings were punctured with tweezers and inoculated with a 10-μl droplet (2×10^7 spores/ml) of inoculum (two inoculation sites per plant). Inoculated cotyledons were air-dried before being placed back in the growth chamber and subsequently watered. Seedling infection was rated 14 days after inoculation using the rating scale of 0–9 (Williams & Delwiche, 1979). The average rating score (ARS) was calculated from 12 seedlings (~24 inoculation sites), where ARS ≤4.5 was considered resistant (R) and ARS 4.6–9.0 susceptible (S).

2.5 | DNA extraction from plant samples

Genomic DNA was extracted from fully developed cotyledons of 7- to 8-day-old seedlings. DNA was extracted following the CTAB method as described by Doyle and Doyle (1990) with slight modifications. In brief, small leaf samples were ground for 2 min in 600 μl of CTAB buffer using a milling apparatus TissueLyser II (Qiagen, Toronto, Canada). The ground sample was incubated at 65°C for 60 min, followed by adding 600 μl of chloroform and centrifuging for 10 min at 11,269 × g. Approximately 500 μl of supernatant was pipetted into a fresh 1.5-ml Eppendorf tube. DNA was precipitated by adding isopropanol and washed with ice-cold 70% ethanol. DNA was diluted with milli-Q water and stored at −20°C.

2.6 | Marker development

All markers used in this study were PCR-based, taken either from the available literature or designed for this study. Resistant and susceptible parents were selected to make the pooled DNA. Two DNA pools were made containing DNA of either 10 resistant (R) or 10 susceptible (S) parents. Primarily, these pools (R-pool and S-pool) were used for marker development. Suitable restriction enzymes (RE) were used to detect the polymorphisms of cleaved amplified polymorphic sequence (CAPS) markers. Finally, the F_2 generation derived from a *B. napus* × *B. juncea* cross was used to confirm the marker efficacy in discriminating resistant and susceptible alleles.

2.7 | PCR analysis

The PCR amplification reaction contained 50 ng template DNA, 2.5 μl Dream*Taq* buffer (10×), 2.5 μl of dNTPs (0.2 μM of each dNTP), 0.25 μl of each of the forward and reverse primers (10×), 0.1 μl of Dream*Taq* DNA polymerase (5 U/μl) and rest of the milli-Q water in a total reaction volume of 20 μl. All PCR amplification was performed according to standard protocols in either an Applied Biosystems GeneAmp 2700 system or an Eppendorf Mastercycler®pro for 35 cycles, each consisting of 30 s at denaturing temperature 94°C, 30 s at annealing temperature 53°C, and 1 min at extension temperature 72°C. PCR products were stained with loading dye, separated on either 1% or 1.5% agarose electrophoretic gels in 1× TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and clear bands were visualized under ultraviolet light. For CAPS markers, 10 μl of PCR product was digested with 10 μl of the appropriate RE mixture: 7 μl milli-Q or 5 μl milli-Q plus 2 μl SAM, 2 μl cut smart buffer and 1 μl specific enzyme either *Bsg*I (5 U/μl) or *Hae*III (10 U/μl) (New England *BioLabs* Inc. Ipswich, Massachusetts, USA) in a total volume of 20 μl for 3 hr at 37°C. Digested DNA fragments were separated on a 1.5% agarose gel and visualized under ultraviolet light.

For multiplex PCR, DNA from eleven *L. maculans* isolates: D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, JN3 and J3 were used to amplify avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* (Fudal et al., 2007; Van de Wouw, Lowe, Elliott, Dubois, & Howlett, 2014). The PCR amplification reaction contained 50 ng template DNA, 2.5 μl GeneDireX buffer (10×), 2.0 μl of dNTPs (0.2 μM of each dNTP), 0.25 μl of each of the forward and reverse primers (10×) link to the genes *AvrLm5/AvrLmJ1* and *AvrLm6*, 0.25 μl of GeneDireX *Taq* DNA polymerase (5 U/μl) and rest of the milli-Q water in a total reaction volume of 20 μl. Protocol for the PCR amplification, and gel documentation same as described above.

2.8 | Phenotyping for blackleg resistance

A pot experiment was conducted, using the F_2 generation, at the Department of Plant Science University of Manitoba, under glasshouse conditions (22°C and 12-hr photoperiod). Seeds were directly sown into plastic pots, and two seedlings per pot were allowed to grow until maturity. Cotyledons of 7- to 8-day-old seedlings were inoculated with the *L. maculans* isolate ICBN14 as previously described. Infected plants were grown to maturity and evaluated for their blackleg resistance by estimating the percentage of a blackening area from a cross-sectional stem using a scale of 0–5 (West et al., 2002):

0—no visible infection,
1—disease tissue occupies ≤25% of the cross section,
2—disease tissue occupies 25%–50% of the cross section,
3—50%–75% of the cross section infected,
4—more than 75% of the cross section infected, but plant alive, and
5—100% of cross section infected and plant dead.

2.9 | Statistical analysis

A chi-square goodness-of-fit test was performed to assess the segregation ratios of the CAPS marker for conformity with the expected ratio of 1:2:1. This was also conducted for the segregation ratio of the phenotypes, resistant vs. susceptible, of the F_2 progeny for conformity with the expected ratio of 3:1. The marker genotypes were tested for significant associations with disease severity using SAS version 19 (SAS Institute, Cary, NC, USA). A *t* test was performed to compare the mean disease severity of resistant, susceptible and heterozygous groups of the F_2 progeny.

3 | RESULTS

3.1 | Confirmation of *AvrLm5/AvrLmJ1* and *AvrLm6* in *L. maculans* isolates

The avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* were amplified via a multiplex PCR from fungal DNA of *L. maculans* isolates including D4, D7, D10, ICBN14, PHW1223, JN3 and J3 (Figure 1, Fudal et al., 2007; Van de Wouw et al., 2014). The avirulence gene *AvrLm5/AvrLmJ1* was amplified in the isolates D3 and R2, whereas the avirulence gene *AvrLm6* was amplified for a single isolate AD746, and neither of these genes were amplified in the isolate D5

(Figure 1). This result confirmed that the isolates D4, D7, D10, ICBN14, PHW1223, JN3 and J3 carried both avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6*. On the other hand, isolates D3 and R2 carried avirulence gene *AvrLm5* but no *AvrLm6*. A single isolate AD746 carried avirulence gene *AvrLm6* but no *AvrLm5*, and both genes were absent in the isolate D5 (Figure 1).

3.2 | Confirmation of *Rlm6* in the cultivar 'Forge'

The *B. juncea* cultivar 'Forge,' which has been predicted to carry a single dominant gene, *Rlm6*, was inoculated by the previously genotyped set of differential isolates of *L. maculans* (Zhang et al. 2016, Figure 1 and Table 1). The *B. napus* cultivar 'Topas DH16516' was also included in the inoculation study as a control. *Brassica napus* cultivar 'Topas DH16516' was susceptible to all *L. maculans* isolates tested. As a result, no *R*-gene was detected in the cultivar 'Topas DH16516' (Table 1). The *B. juncea* cultivar 'Forge' was susceptible to the *L. maculans* isolates D3 (*AvrLm5*, *AvrLepR1*), D5 (*AvrLm1*, *2*, *4*, *7*, *S*, *AvrLepR1*, *AvrLepR2*) and R2 (*AvrLm5*, *7*, *AvrLepR1*), which indicated that the cultivar 'Forge' did not carry the genes *Rlm1*, *Rlm2*, *Rlm4*, *Rlm5*, *Rlm7*, *RlmS*, *AvrLepR1* and *AvrLepR2*. On the other hand, the *B. juncea* cultivar 'Forge' was resistant to the *L. maculans* isolates D4 (*AvrLm4*, *5*, *6*, *7*, *8*, *AvrLepR1*, *AvrLepR2*), D7 (*AvrLm1*, *3*, *5*, *6*, *8*, *AvrLepR1*), D10 (*AvrLm5*, *6*, *8*, *9*, *S*), ICBN14 (*AvrLm5*, *6*, *AvrLepR1*), PHW1223 (*AvrLm5*, *6*, *8*, *9*), AD746 (*AvrLm3*, *6*, *AvrLepR1*), JN3 (*AvrLm1*, *4*, *5*, *6*, *7*, *8*) and J3 (*AvrLm2*, *3*, *5*, *6*, *S*), which indicated that the genes *Rlm3*, *Rlm6*, *Rlm8* and *Rlm9* could be present in the cultivar. Unfortunately, we did not have an appropriate isolate to determine whether *Rlm3*, *Rlm8* and *Rlm9* were in fact present in the cultivar 'Forge,' but these have been identified previously: the genes *Rlm3* and *Rlm9* were identified in *B. napus* (Balesdent et al., 2002; Delourme et al., 2004), and *Rlm8* was identified in *B. rapa* (Balesdent et al., 2002). Therefore, based on the available literature, we made a scientific assessment that the genes *Rlm3*, *Rlm8* and *Rlm9* were not carried by the *B. juncea* cultivar 'Forge.' However, the *B. juncea* cultivar 'Forge' was inoculated again by the *L. maculans* isolates D5, R2 and ICBN14 those were checked via multiplex PCR for the confirmation of *AvrLm5/AvrLmJ1*, and *AvrLm6* in the pathogen profile (Figure 1). The cotyledon inoculation test showed that the cultivar 'Forge' was susceptible to the isolates D5 (*AvrLm1*, *2*, *4*, *7*, *S*, *AvrLepR1*, *AvrLepR2*) and R2 (*AvrLm5*, *7*, *AvrLepR1*), in which there was an absence of *AvrLm5/AvrLmJ1 and AvrLm6* and the presence of *AvrLm5/AvrLmJ1*, respectively (Figure 2a and b). The compatible interaction between 'Forge' and the *L. maculans* isolates D5 and R2 indicated that the 'Forge' cultivar did not carry the gene *Rlm5*. On the other hand, the 'Forge' cultivar showed a small dark lesion, typical of a HR, to the isolate ICBN14 (*AvrLm5*, *6*, *AvrLepR1*) (Figure 2c) as a result of the gene-for-gene interaction (Flor, 1971), confirming that the cultivar 'Forge' must carry a single dominant gene, *Rlm6*. Based on the results from differential testing (Table 1), and information from available literature, we could deduce that *Rlm6* was present in the cultivar 'Forge.'

3.3 | Marker development

The primer sequences listed in Table 2 were either collected from available literature or developed for this study. B5-1520 is a SCAR marker, which has a dominant pattern of inheritance and was developed by Saal and Struss (2005). The primer B5-1520 was originally converted from the amplicon region present in the B-genome species. PCR amplification of the SCAR marker (B5-1520) in the DNA pool, as well as in the F_2 (derived from a cross between *B. napus* and *B. juncea*) generation, showed that the band was only present in the resistance genotypes, as described by Saal and Struss (2005) (Figure 3). Variations were identified from the sequences of PCR product between the resistant parental line and the F_1 plant, and new primers were then designed from the sequence of F_1 progeny. The PCR amplification of a new primer B5Rlm6_1 showed a polymorphism in the resistance allele, producing fragments of approximately 650 bp (Figure 3). The sequences of the PCR product of the resistant allele, amplified by the primer B5Rlm6_1, were blasted and resulted in hits in scaffolds ChrUn_random: 36607925–36608309 in *B. napus* and ChrB01: 17530537–17531568 in *B. juncea*. Two primers BnHZ_2 and BjHZ_1 were designed from the blasted sequence in the B5Rlm6_1 amplicon of gibberellin 2-oxidase gene region. The primer pairs for amplicon BnHZ_2 and BjHZ_1 were amplified fragments of 600 and 550 bp, respectively, from both resistant and susceptible individuals. The PCR product amplified by the primers BnHZ_2 and BjHZ_1 was sequenced (Table S1) to select specific RE *Bsg*I and *Hae*III to derive codominant CAPS markers (Table 2). Both marker assays provided resistance-linked fragments as expected; around 450–550 bp for BjHZ_1 and 300–450 bp for BnHZ_2 (Figure 3 and Table 2).

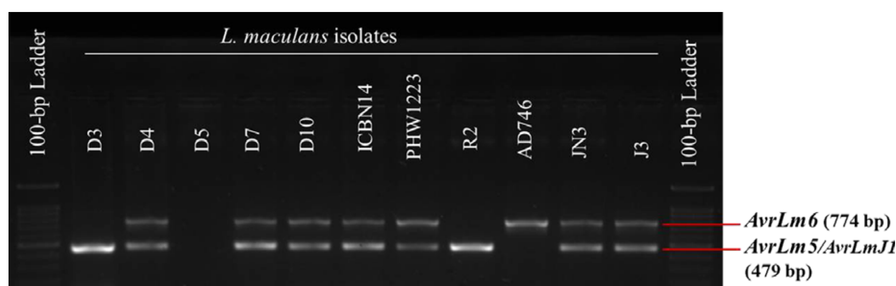

FIGURE 1 Amplification of avirulence genes *AvrLm5/AvrLmJ1* and *AvrLm6* from *Leptosphaeria maculans* isolates used in the study

TABLE 1 Reaction of *Brassica juncea* cultivar 'Forge' to a set of differential isolates of *Leptosphaeria maculans*

Isolates	Avr genotypes	*B. napus* (Topas DH16516)	*B. juncea* (Forge)
D3	*AvrLm5*, *AvrLepR1*	S	S
D4	*AvrLm4*, *5*, *6*, *7*, *8*, *AvrLepR1*, *AvrLepR2*	S	R
D5	*AvrLm1*, *2*, *4*, *7*, *S*, *AvrLepR1*, *AvrLepR2*	S	S
D7	*AvrLm1*, *3*, *5*, *6*, *8*, *AvrLepR1*	S	R
D10	*AvrLm5*, *6*, *8*, *9*, *S*	S	R
ICBN14	*AvrLm5*, *6*, *AvrLepR1*	S	R
PHW1223	*AvrLm5*, *6*, *8*, *9*	S	R
R2	*AvrLm5*, *7*, *AvrLepR1*	S	S
AD746	*AvrLm3*, *6*, *AvrLepR1*	S	R
JN3	*AvrLm1*, *4*, *5*, *6*, *7*, *8*	S	R
J3	*AvrLm2*, *3*, *5*, *6*, *S*	S	R
Predicted *Rlm* gene		—	*Rlm6*

R and S indicate resistance (~24 cotyledon lesion score) and susceptible (~24 cotyledon lesion score) reaction.

3.4 | Interspecific hybridization, introgression and generation development

Approximately 12 seedlings from each generation were inoculated with the *L. maculans* isolate ICBN14 (*AvrLm5*, *AvrLm6*, *AvrLepR1*) for their resistance reaction. Seedlings that showed a hypersensitive reaction to the pathogen were transplanted to plastic pots and grown under glasshouse conditions (22°C and 12-hr photoperiod). In addition, DNA was extracted from the seedlings and was genotyped for the SCAR marker B5-1520. A dominant pattern of inheritance of this marker was yielded, as expected, confirming the introgression of B-genome region containing the marker fragment in F_1 (Figure 4a). Two seedlings (2 and 3) of 10 F_1 seedlings did not show amplification of the B5-1520 marker, but the remaining seedlings had identical amplification to that described by Saal and Struss (2005), confirming the introgression of the B-genome. Introgressed F_1 seedlings were selfed to develop the F_2 generation. Similarly, the F_3 generation was also developed from the introgressed F_2 plants (Figure 4b). Three plants (1, 5 and 9) of nine F_2 showed PCR amplification of B5-1520, confirming the introgression of B-genome region

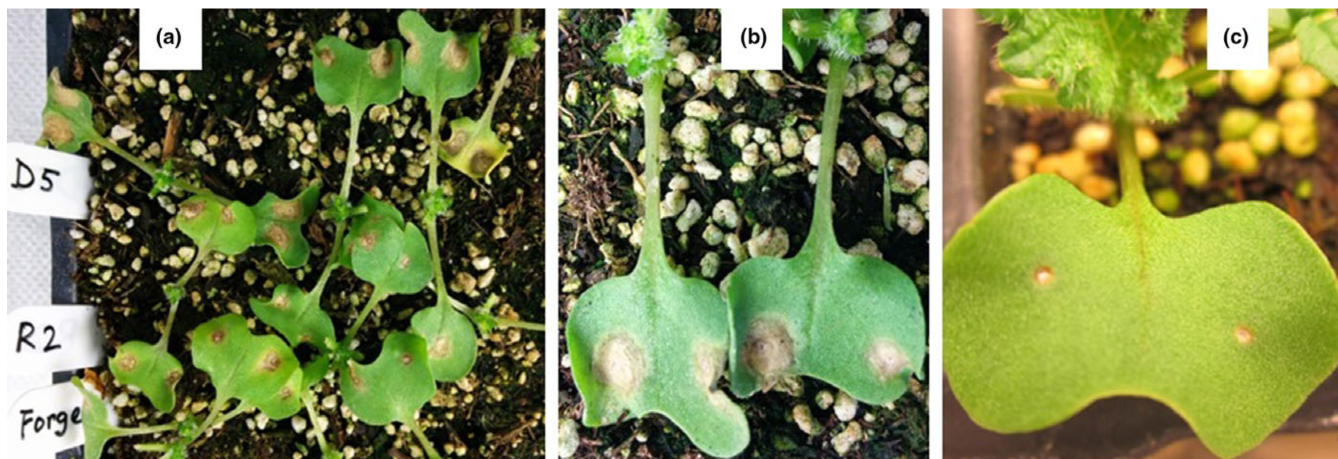

FIGURE 2 The *Brassica juncea* cultivar 'Forge' showed susceptibility to the *Leptosphaeria maculans* isolates D5 (*AvrLm1*, *AvrLm2*, *AvrLm4*, *AvrLm7*, *AvrLmS*, *AvrLepR1*, *AvrLepR2*) and R2 (*AvrLm5*, *AvrLm7*, *AvrLepR1*) at 14 days postinoculation (a and b), whereas hypersensitive response to the isolate ICBN14 (*AvrLm5*, *AvrLm6*, *AvrLepR1*) (c)

TABLE 2 SCAR and CAPS markers used in this study

Primer ID	Primer sequence (5′–3′)	Type	RE	Fragment size (bp)	Annealing Temp. (°C)	Sources
B5-1520	F-TGCCTTTCTCACTTCTTCTCTC	Dominant	—	1,520	53	Saal and Struss (2005)
	R-AGCGTCTATGTCGGTCTTTCAA			650		
B5Rlm6_1	F-GTTACAGAGGGTTGTATCTCATTC	Dominant	—		53	This study
	R-ACCAGGAGTGGTTAGAAGCTAAT					
BjHZ_1	F-CCAACCCTTCGAGGTCAATA	Codominant	*Hae*III	540, 450	55	This study
	R-CCAGAGACCCCAGTTAAGCA					
BnHZ_2	F-TCCATGATGTGATAACTATAGACG	Codominant	*Bsg*I	450, 300	55	This study
	R-TTAAAGTTTGTGAATTTCTTCCTT					

F, forward; R, reverse; RE, restriction enzyme; bp, base pair.

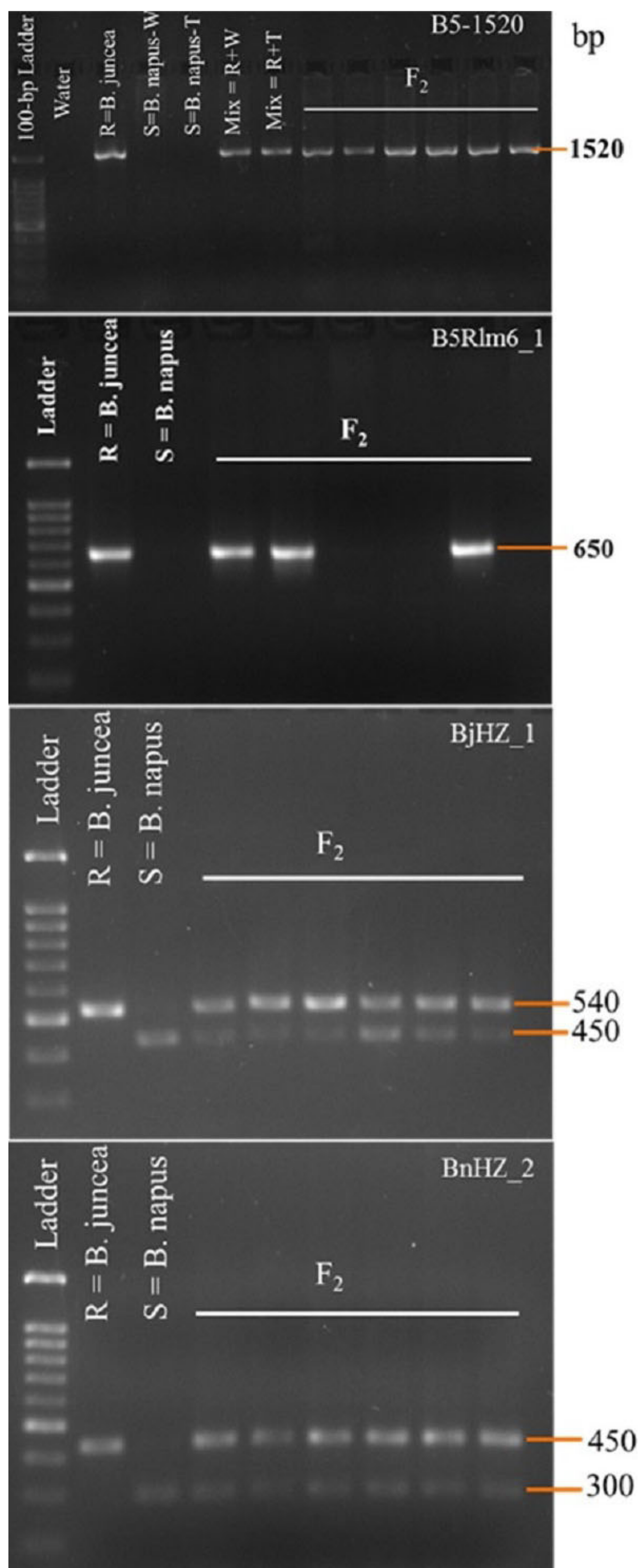

FIGURE 3 DNA markers linked to *Rlm6* showed polymorphism in the F_2 generation. The SCAR markers B5-1520 and B5Rlm6_1 showed a dominant pattern of polymorphism, whereas CAPS markers BjHZ_1 and BnHZ_2 showed codominant polymorphism. R and S refer to resistance and susceptible, respectively

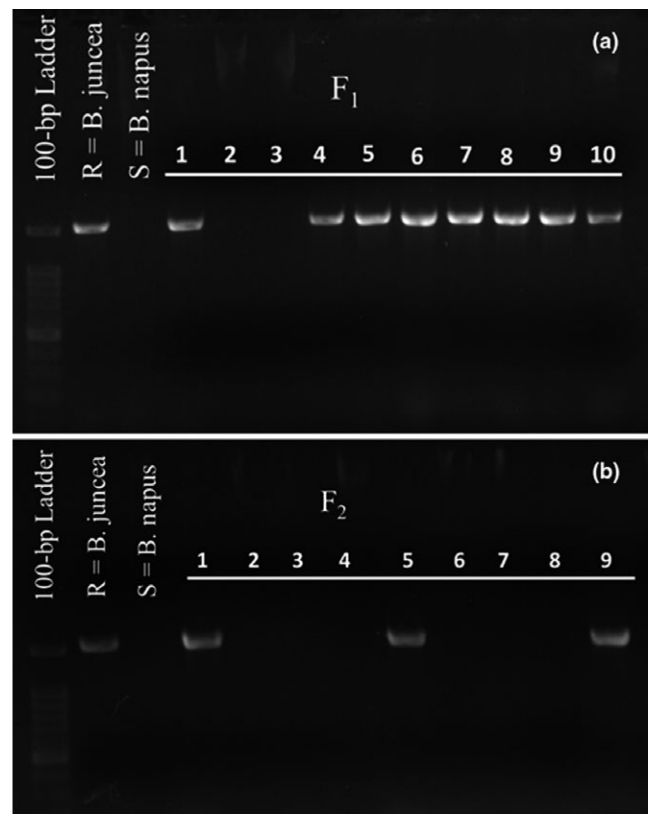

FIGURE 4 Gel image showed the confirmation of *Rlm6* introgression in *B. napus* × *B. juncea* interspecific hybrids. The SCAR marker B5-1520 yielded a dominant pattern of polymorphism in the F_1 (a), and F_2 (b) generations

containing the marker fragment in F_2, but indicating marker distortion, as it did not support the Mendelian segregation ratio of 3:1.

3.5 | Inheritance of the blackleg resistance gene *Rlm6*

The F_2 and F_3 progeny segregated at the SCAR marker B5Rlm6_1, linked to the *L. maculans* resistance gene *Rlm6*. This was further confirmed by PCR genotyping. Genotype data showed that 52% of F_2 and 28% of the F_3 progeny carried *Rlm6* (Table 3). The chi-square test for a Mendelian segregation of the SCAR marker in the F_2 and F_3 generations showed a significant discrepancy between the observed and expected values ($p < .0001$, Table 3).

Evaluation of blackleg disease severity for 142 F_2 plants resulted in an acceptable fit to a Mendelian ratio of 3:1 in resistant vs. susceptible plants, supporting the hypothesis of the genetic control of resistance by a single dominant gene (Table 4). All susceptible plants ($n = 36$) showed severe disease symptoms, including stem blackening, the presence of basal cankers and death (Table 4). No symptoms were observed on 32 plants of 142 with disease severity 0 (Table 4). Seventy-two plants showed mild symptoms with disease severity ratings of 1 or 2, not as healthy as control plants (Table 4). CAPS

TABLE 3 Segregation of the SCAR marker B5Rlm6_1 in F_2 and F_3 generations

Generations	Number of plants genotyped	Resistance		Susceptible		χ^2	*p*
		Observed	Expected	Observed	Expected		
F_2	192	99	144	93	48	56.250	.0001
F_3	192	53	96	139	96	38.521	.0001

TABLE 4 A chi-square test for the segregation of blackleg resistance gene *Rlm6* in the F_2 progeny

Plant categories	Disease rating[a] 0	1	2	3	4	5	Total	χ^2 (2)	*p*
Genotype of BjHZ_1[b]									
S	0	0	0	15	11	10	36		
R	11	22	8	0	0	0	41		
H	21	22	20	2	0	0	65		
Total	32	44	28	17	11	10	142	1.3806	.5014

[a]Plants noted as 0–2 were considered resistant, whereas plants greater than 2 were susceptible.
[b]S = homozygous for the allele *Brassica napus*; R = homozygous for the allele *B. juncea*; and H = heterozygous. Noted: chi-square value was adapted from likelihood ratio.

TABLE 5 Blackleg disease severity of the F_2 plants as grouped by their genotypes for a CAPS marker linked to *Rlm6* gene

Marker-BjHZ_1[†]	Number observed	Disease rating* Mean	Range
Susceptible (S)	36	3.89 b[‡]	3–5
Resistant (R)	41	0.93 a	0–2
Heterozygous (H)	65	1.05 a	0–2
Total	142	1.95	0–5

*Disease rating was conducted at maturity stage immediately prior to harvesting.
[†]S = homozygous for the allele *Brassica napus*; R = homozygous for the allele *B. juncea*; and H = heterozygous.
[‡]Means in column with the same letter are not significantly different ($p \leq .0001$, *t* test).

marker analysis revealed that 41 plants of 142 were homozygous for the introgression of *Rlm6* from *B. juncea*, 36 were homozygous for the susceptible allele, and the remaining plants (65) were heterozygous for both alleles (Table 5), supporting an acceptable fit to a 1:2:1 ratio of resistant: heterozygous: susceptible. Exceptionally, two plants from the heterozygous group (heterozygote for CAPS marker BjHZ_1) were susceptible, as their disease severity was 3 (Table 4). Overall, in this study, the chi-square independence test ($p \leq .5014$) for segregation of the phenotypes showed a significant association between the genotypes and phenotypes (Table 4).

Plants that were either heterozygous or homozygous for the *B. juncea* introgression carrying the gene *Rlm6* showed a high level of resistance to *L. maculans*, ranging from 0 to 2 on the 0–5 scale. The disease severity for the heterozygotes with a mean disease severity of 1.05 was insignificantly higher than that of the homozygotes, with a mean disease severity of 0.93 (Table 5). Conversely, homozygous plants for the *B. napus* introgression showed severe blackleg disease symptoms; ranging from 3 to 5 with a mean disease severity of 3.89 (Table 5), which was significantly higher than the *Rlm6* introgressed plants. Therefore, the results found in this study agreed with the interpretation of resistance, that is, an acceptable fit to a Mendelian ratio of 3:1 in resistant vs. susceptible plants.

4 | DISCUSSION

In this study, we showed that major gene resistance was conferred by a single gene in the *L. maculans*–*B. juncea* interaction, which appeared to be expressed in cotyledons. The cultivar 'Forge,' which carries monogenic resistance conferred by a single resistance gene, was characterized here. In this case, all plants were resistant to the isolate ICBN14 (*AvrLm5*, *6*, *AvrLepR1*) at the cotyledon stage (Table 1), indicating seedling resistance as a result of a race-specific "gene-for-gene" interaction (Balesdent et al., 2005; Flor, 1971). Although the *L. maculans* resistance genes *Rlm5* and *Rlm6* have been identified in *B. juncea* in previous studies (Balesdent et al., 2002; Chèvre et al., 1997; Christianson et al., 2006; Rimmer & van den Berg, 1992), our data (Table 1 and Figure 3) revealed that a single gene, *Rlm6*, is involved in controlling blackleg resistance in the *B. juncea* cultivar 'Forge.'

Resistance, explained by previous studies in *Brassica*, is either monogenic or polygenic, indicating a complexity regarding the expression of resistance genes (Ballinger & Salisbury, 1996, Chèvre et al., 1997; Elliott, Marcroft, Howlett, & van de Wouw, 2016; Ferreira et al. 1995; Pang & Halloran, 1996; Pilet, Delourme, Foisset, & Renard, 1998). In this study, almost all the F_1 plants showed HRs to the *L. maculans* isolate ICBN14 at the cotyledon stage, indicating a race-specific interaction (Balesdent et al., 2005; Flor, 1971). In addition, genotyping of the SCAR marker B5-1520 confirmed the introgression of *Rlm6* in the F_1 generation (Figure 4a). Segregation of the SCAR marker B5Rlm6_1 in the F_2 and F_3 generations did not have an acceptable fit to a 3:1 or 1:1 ratio of resistant vs. susceptible alleles, respectively, indicating marker skewedness in the F_2 and F_3 generations (Table 3). As the SCAR markers used in this study were dominant, it is unknown whether the frequency of homozygotes or heterozygotes was reduced. Interestingly, the SCAR markers used here were strongly associated with each other and to the locus *Rlm6*. Saal and Struss (2005) conducted a study on the introgression of blackleg

resistance from *B. nigra*, *B. juncea* and *B. carinata* to *B. napus*, in which they observed a similar pattern of dominant marker distortion. Plieske and Struss (2001) also found segregation distortion for restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) markers linked to a blackleg resistance gene derived from the B-genome species. Importantly, in this study, segregation of the derived CAPS marker in the F_2 generation had an acceptable fit to a 1:2:1 ratio of resistant vs. heterozygous vs. susceptible alleles, supporting the genetic control of resistance by a single dominant gene (Table 5). A similar pattern of segregation (1:2:1) was observed in a previous study, where a codominant marker derived from a resistant gene analogue linked to *L. maculans* in an interspecific hybrid was genotyped (Saal & Struss, 2005). Exceptionally, two plants from the heterozygous group displayed moderate symptoms with disease severity ratings of 3 (Table 4). This disease severity rating (3) was higher than expectation (>2). This could be due to an environmental effect and/or plant characteristics, because these plants grew somewhat slower compared to disease progression in the plant, and leaves were also infected by powdery mildew. Moreover, the F_2 generation was also genotyped for another CAPS marker BnHZ_2 (data not shown), and there was no recombinant identified from 142 individuals for the introgressed region. The CAPS markers developed and genotyped here could be located close to each other, or be in the same locus, which usually limits recombination. Chromosomal rearrangement during introgression could be another plausible explanation of recombination suppression (Chèvre et al., 1997; Yang et al., 2014). However, our study revealed monogenic inheritance of CAPS markers linked to the *L. maculans* resistance gene *Rlm6* in the interspecific hybrids derived from *B. napus* × *B. juncea*. A similar pattern of inheritance was also reported in many *B. napus* cultivars by previous studies (Dion, Gugel, Rakow, Seguin-Swartz, & Landry, 1995; Ferreira et al., 1995; Mayerhofer, Bansal, Thiagarajah, Stringam, & Good, 1997; Raman et al., 2012).

To clarify the reason for segregation distortion, both the trait locus and SCAR markers could be analysed further in different populations, such as double haploid, backcross or near isogenic lines, to see whether the similar trend of marker skewedness would occur. This will be the focus for future study in addition to development of more codominant markers in the introgression region. Moreover, study towards fine mapping could also be possible by screening different mapping populations with the molecular markers developed here. It remains unclear whether the recombination is originating from the homoeologous position of the *B. napus* genome or from the loss of introgression segments. We believe the latter case did not occur here, as recombinant individuals did not lose their vigour. In summary, based on the study of phenotype and genotype in *B. napus* × *B. juncea* interspecific hybrids, we demonstrated that the B-genome species *B. juncea* carries a dominant gene *Rlm6*, conferring resistance to the blackleg pathogen *L. maculans*. Additionally, the molecular markers developed here linked to *L. maculans* resistance would accelerate marker-assisted backcross breeding in the future.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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