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Molecular and phenotypic identification of B-genome introgression linked to *Leptosphaeria maculans* resistant gene *Rlm6* in *Brassica napus* × *B. juncea* interspecific hybrids

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Abstract Blackleg is a devastating disease in canola worldwide, except in China, caused by the fungal pathogen *Leptosphaeria maculans*. The B-genome *Brassica* species were reported to have a strong resistance to the blackleg pathogen *L. maculans*. Backcross (BC) generations, BC_1F_1 to BC_4F_1, were derived from a cross *B. napus* × *B. juncea*. Phenotype of *L. maculans* isolate J20 showed that 49% of BC_1F_1, 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 plants were resistant to the isolate J20. Offspring from the interspecific hybridization were also analysed for the presence of dominant type SCAR markers detecting loci linked to the *B. juncea* genome. The plants with *B. juncea* introgression had a decrease in the presence of SCAR markers ranging from 47% in BC_1F_1 to 30% in BC_2F_1 and further down to 18% in BC_3F_1 and 11% in BC_4F_1 with respect to the marker B5Rlm6_1. A similar trend of loci reduction was also observed for the marker B5-1520. In contrast, the progression of the *B. napus* genome correlated with an incremental increase in the presence of the two markers with the advancement of the generations. However, segregation of SCAR markers and phenotypes for the blackleg resistance in BC_1F_1 plants had an acceptable fit to a Mendelian ratio of resistant versus susceptible, supporting the assumption that the genetic control of resistance is governed by a single dominant gene. The BC generations developed in this study, which show introgression of the *B. juncea* genome linked to the *L. maculans* resistance gene *Rlm6*, would facilitate breeding a *B. napus* variety resistant to blackleg in the future.

Keywords *Brassica* species · B-genome · Introgression · *Leptosphaeria maculans* · *R*-gene *Rlm6* · SCAR markers

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Introduction

Canola (rapeseed/*Brassica napus* L.) is an economically important oilseed crop in Australia, China, Europe and Canada. It interacts with a few major pathogens including *Leptosphaeria maculans*, which is the causal agent of blackleg disease resulting in significant yield loss (Fitt et al. 2006). Genetic resistance is one of the most cost-effective and environment friendly strategies to control disease. In canola qualitative resistance often refers to *R* genes which are very effective in controlling blackleg disease, while the fungal pathogen *L. maculans* carries a high frequency of the corresponding *Avr* genes (Daverdin et al. 2012). A recent study showed a very

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high frequency of *AvrLm6* in *L. maculans* isolates from Canadian canola fields (Liban et al. 2016), indicating the necessity to develop canola cultivar with the corresponding *R* gene *Rlm6*. In addition, one of the goals of canola research programs is the stable introgression of novel resistance from wild or closely related species into elite cultivars through inter- and intra-specific crosses (Ky et al. 2000). So far *L. maculans* resistance (*R*) genes *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2*, have been identified in different *Brassica* species (Raman et al. 2013). Among *Brassica* species; the B-genome species *B. juncea*, *B. carinata*, and *B. nigra* carry many valuable traits including blackleg resistance (Schelfhout et al. 2006). *B. juncea* is reported to carry the blackleg resistance gene *Rlm6* (Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006;).

However, *B. juncea* (2n = 36, AABB) is derived from a cross between *B. nigra* (2n = 16, BB) and *B. rapa* (2n = 20, AA), whereas *B. napus* (2n = 38, AACC) is derived from a cross between *B. rapa* (2n = 20, AA) and *B. oleracea* (2n = 18, CC) (Nagaharu 1935). The relationship among the *Brassica* species was revealed by an early cytogenetic study known as the U triangle. This study predicts an indirect genomic relationship between *B. napus* and *B. juncea* as revealed by the U triangle (Nagaharu 1935). Earlier studies attempted the introgression of blackleg resistance from *B. juncea* to *B. napus* but so far the trait has not been successfully introduced into commercial cultivars (Roy 1984; Prakash and Chopra 1988; Chèvre et al. 1997; Barret et al. 1998; Dixelius and Wahlberg 1999; Saal et al. 2004). Plausible explanation for the failure of introgression could be due to genome incompatibility between B- and A- or C-genome (Leflon et al. 2007). Recent studies reported successful introgression of B-genome into *B. napus* from *B. juncea* (Chèvre et al. 2008; Brun et al. 2010) and *B. carinata* (Navabi et al. 2010). However, the introgression was unstable as reported by Brun et al. (2010). This could be due to either the source of resistant or susceptible parent or both. Therefore, further efforts for the introgression of the *B. juncea* (resistant cultivar 'Forge') resistance link to blackleg resistant gene *Rlm6* to *B. napus* (susceptible cultivar 'Topas DH16516') is essential.

In this study we successfully introgressed a part of the B-genome linked to *Rlm6*, the blackleg resistance gene from *B. juncea* to *B. napus* using an advanced backcross approach (Tanksley and Nelson 1996). More specifically, an interspecific cross *B. napus* × *B. juncea* was made to generate an F_1 hybrid. Later on, four rounds of backcrossing were performed to develop B-genome-containing substitution lines. This resulted in the production of interspecific hybrids and BC_1F_1 to BC_4F_1 were phenotyped by the *L. maculans* isolate J20 which carries the corresponding avirulence gene *AvrLm6*. Further BC_1F_1 to BC_4F_1 were genotyped with the molecular markers B5Rlm6_1 and B5-1520 linked to the B-genome particularly to the blackleg resistant gene *Rlm6* (Rashid et al. 2018). We are able to demonstrate that the B-genome segment is introgressed into *B. napus* and it is inherited among the interspecific hybrids.

Materials and methods

Plant materials

Brassica juncea cultivar 'Forge' was used as pollen donor, and crossed with the susceptible *B. napus* line 'Topas DH16516'. *B. napus* susceptible line Topas DH16516 was originally produced by G. Séguin-Swartz, Agriculture and Agri-Food Canada (AAFC) Saskatoon, Saskatchewan. F_1 derived from the interspecific cross was backcrossed with the recurrent susceptible parent to develop backcross (BC) generations, including BC_1F_1, BC_2F_1, BC_3F_1, and BC_4F_1 (Fig. 1). All generations were grown under greenhouse condition at 22 °C and 12-h photoperiod throughout the study period.

Leptosphaeria maculans isolates

The single spore derived isolate J20 (carrying avirulence genes *AvrLm2*, *AvrLm3*, *AvrLm6*, *AvrLepR1*) was chosen for phenotyping interspecific hybrids derived from the cross *B. napus* line 'Topas DH16516' × *B. juncea* cultivar 'Forge'. The isolate J20 was previously characterized for the avirulence genes profiled by Zhang et al. (2016).

Inoculum preparations from *L. maculans* isolate J20

Isolates were cultured and inoculum was prepared as described by Chen and Fernando (2006) with minor

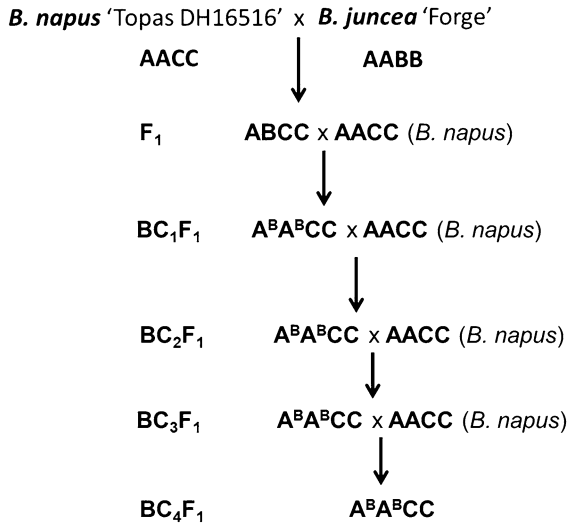

Fig. 1 Pedigree and crosses used in the development of the backcross (BC) generations for this study. Plant selection was performed in each generation for disease resistance based on the phenotype of *Leptosphaeria maculans* isolate J20, and the genotype of the SCAR marker B5Rlm6_1

modifications. In brief, a paper disc was put on V8® agar medium which was amended with 0.35% (w/v) streptomycin sulfate, and the fungus was grown for 2 weeks at 20 °C. Harvested pycnidiospores were stored at − 20 °C for further use. The spore concentrations were diluted to 2×10^7 spores' mL^{-1} each time to serve as inoculation.

Inoculation and infection evaluation

Seeds were sown in a plastic cell tray using standard growing media, and kept in a growth chamber at 21 °C/16 °C (day/night) with a 16-h photoperiod. The fully developed cotyledons of 7-day old seedlings were punctured with tweezers, and inoculated with a prepared 10-µL droplet (2×10^7 spores' mL^{-1}) of *L. maculans* inoculum (two inoculation sites per plant). Inoculated cotyledons were air dried before being returned to the growth chamber and watering. Seedlings were fertilized with water soluble fertilizer 20–20–20 ($N–P_2O_5–K_2O$) 1 day after inoculation. Infected cotyledons were rated 13-days after inoculation using a rating scale of 0–9 (Williams and Delwiche 1979). The average rating score (ARS) was calculated, where ARS $\leq$ 4.5 was considered resistant (R), and ARS 4.6–9.0 was interpreted as susceptible (S).

DNA extraction from plant samples

Genomic DNA was extracted from the first 1–2 true leaves of seedlings 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 up sample was incubated at 65 °C for 60 min followed by adding 600 µL of chloroform, and centrifuged for 12 min at 12,000 rpm. Approximately 500 µL of supernatant was recovered and added to a 1.5 mL Eppendorf tube. DNA was precipitated by adding isopropanol, and the DNA pellet was washed with ice-cold 70% ethanol. The DNA pellet was dissolved in 150 µL milli-Q water, and stored at − 20 °C.

Markers used for *Rlm6* selection

PCR-based dominant type SCAR markers B5Rlm6_1 (F-GTTACAGAGGGTTGTATCTCATTC/R-ACCAGGAGTGGTTAGAAGCTAAT) and B5-1520 (F-TGCCTTTCTCACTTCTTCTCTC/R-AGCGTCTATGTCGGTCTTTCAA) linked to blackleg resistance gene *Rlm6* (Rashid et al. 2018) were used to genotype the parents, and all interspecific hybrids including F_1, BC_1F_1, BC_2F_1, BC_3F_1 and BC_4F_1.

PCR analysis

PCR amplification reaction contained ~ 50 ng template DNA, 2.5 µL of 10× reaction buffer, 2.5 µL of dNTPs (0.2 µM of each dNTP), 0.25 µL of each forward and reverse primer (1.0 µM of each primer), 0.1 µL of dreamTaq polymerase (5 u/µL) and 12.4 µL milli-Q H_20 in a total reaction volume of 20 µL. All PCR amplifications were performed according to standard protocols in either an Applied Biosystems GeneAmp 2700 system (Applied Biosystems, Thermo Fisher Scientific Inc. Canada) or an Eppendorf Mastercycler®pro (Eppendorf, Mississauga, Canada) for 35 cycles, each consisting of 30 s at 94 °C, 30 s at annealing temperatures 53 °C and 1 min at 72 °C. PCR products were separated on 1.5% agarose gels resolved by electrophoresis in 1× TBE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0). Agarose gels were stained with loading dye (containing ethidium

bromide 0.5 μg/mL), and visualized under ultraviolet light.

Statistical analysis

A χ^2 goodness-of-fit test was performed to assess the segregation ratio of the phenotypes, resistant versus susceptible in BC progenies for the conformity of the expected ratio. Similar test was also performed to assess genotypic segregation ratios of the SCAR markers for the conformity of the expected ratio. For each backcross generation the percentage of retained SCAR marker loci was calculated using Microsoft Excel version 10.0. A simple *t* test was performed to determine significance levels regarding differences among the generations. The χ^2 goodness-of-fit test was performed using free online software accessed on November 10th 2017 (http://www.socscistatistics.com/tests/goodnessoffit/Default2.aspx).

Results

Confirmations of the presence of *AvrLm6* in *L. maculans* isolate J20

DNA from three *L. maculans* isolates; ICBN14 (*AvrLm5-9*, *6*, *AvrLepR1*), R2 (*AvrLm5-9*, *7*, *AvrLepR1*), and J20 (*AvrLm2*, *AvrLm3*, *AvrLm6*, *AvrLepR1*) were used as templates to amplify avirulence genes *AvrLm5-9* and *AvrLm6* via a multiplex PCR (Fig. 2). The primers for this PCR and amplification protocols were collected from the corresponding publication (Fudal et al. 2007; Van de Wouw et al. 2014). Both avirulence genes, *AvrLm5-9* and *AvrLm6*, were successfully amplified for the isolate ICBN14. On the other hand, isolates R2 and J20 yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, respectively. This result confirmed that isolate J20 carried the avirulence gene *AvrLm6*, but not *AvrLm5-9* (Fig. 2).

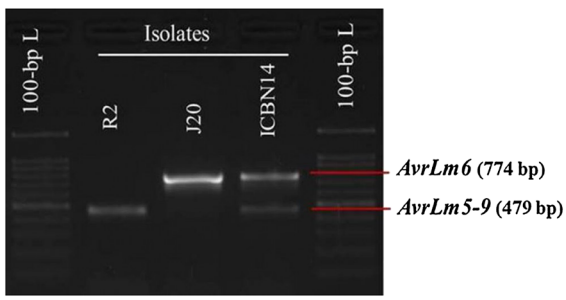

Fig. 2 Confirmation of the *Leptosphaeria maculans* isolate used in the study. Isolate ICBN14 (*AvrLm5-9*, *6*, *AvrLepR1*) yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, whereas isolates R2 (*AvrLm5-9*, *7*, *AvrLepR1*) and J20 (*AvrLm2*, *AvrLm3*, *AvrLm6*, *AvrLepR1*) yielded PCR products for the avirulence genes *AvrLm5-9* and *AvrLm6*, respectively

Interspecific hybridization

Fully expanded 7-days old cotyledons of 'Topas DH16516' (*B. napus* line—no *R* gene) and 'Forge' (*B. juncea* cultivar carries *R* gene *Rlm6*) were inoculated with *L. maculans* isolate J20 (carries avirulence gene *AvrLm6*). Inoculated 'Topas DH16516' showed susceptibility to the isolate J20 indicating no corresponding *R* gene *Rlm6* was carried by the wild-type susceptible parent (Fig. 3a). On the other hand, *B. juncea* cultivar 'Forge' showed small dark chlorotic lesion a sign of a typical hypersensitive response (HR) indicating presence of the corresponding *R* gene *Rlm6* (Fig. 3b). 'Topas DH16516' and resistant seedlings of 'Forge' were transplanted to the plastic pot, and grown under greenhouse condition (22 °C and 12-h photoperiod). Interspecific crosses were performed between *B. napus* line 'Topas DH16516' and *B. juncea* cultivar 'Forge'. Harvested F_1 seeds were poorly developed, and a few seeds were germinated on a Petri dish and transplanted to a plastic pot in a growth chamber at 21 °C/16 °C (day/night) with a 16-h photoperiod. Resistant progenies were identified in each generation via phenotyping with *L. maculans* isolate J20. Only resistant F_1 seedlings (those that showed HR to the isolate J20; Fig. 3c) were transferred to the plastic pot, and grown under greenhouse conditions (22 °C and 12-h photoperiod). In addition a PCR-based SCAR marker B5Rlm6_1 linked to *Rlm6* was also applied to confirm the introgression in backcross progenies (Fig. 4).

Development of backcross generations

For all BC generations seeds were sown in a plastic cell tray using standard growing media, and kept in a growth chamber at 21 °C/16 °C (day/night) with a 16-h photoperiod. Fully expanded 7–8 days old cotyledons were inoculated with the *L. maculans* isolates J20 (carried avirulence genes *AvrLm2*,

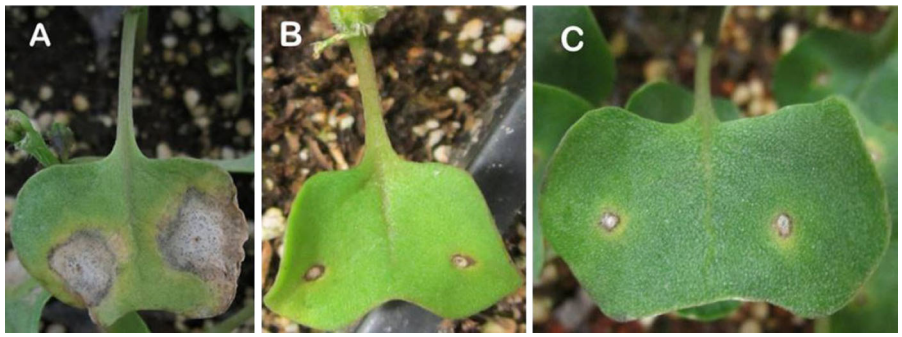

Fig. 3 Phenotype of gene-for-gene interaction between host and *Leptosphaeria maculans* isolate J20. **a** Susceptible parent *Brassica napus* line 'Topas DH16516' showed compatible interaction to J20, **b** resistant parent *Brassica juncea* cultivar 'Forge' showed incompatible interaction to J20, and **c** an interspecific hybrid also showed incompatible interaction to J20

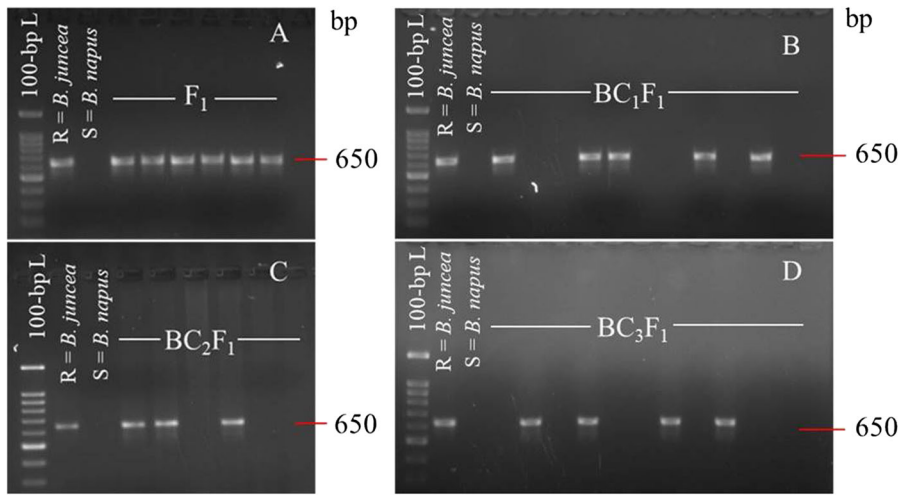

Fig. 4 Confirmation of the introgression of a part of B-genome in the interspecific hybrids such as F_1 (**a**), BC_1F_1 (**b**), BC_2F_1 (**c**), and BC_3F_1 (**d**) by a SCAR marker B5Rlm6_1. The interspecific hybrids derived from a cross between a *Brassica napus* cultivar 'Topas 'DH16516' and *Brassica juncea* cultivar 'Forge'

AvrLm3, *AvrLm6*, *and AvrLepR1*). The seedlings that showed HR to the pathogen reaction were transplanted to the individual plastic pot, and grown under greenhouse condition (22 °C and 12-h photoperiod). Moreover, genotyping was also done with the dominant type SCAR marker to confirm the introgression of components of the *B. juncea* genome. Six F_1's were genotyped by the PCR based SCAR marker B5Rlm6_1, which showed polymorphism as expected (Fig. 4a). These F_1's were backcrossed with the susceptible recurrent parent towards developing BC_1F_1. Similarly, BC_1F_1 plants were phenotyped by the isolate J20, and genotyped by the marker B5Rlm6_1. Among eleven BC_1F_1 plants, five showed evidence for the introgression of a segment of B-genome from *B. juncea* linked to blackleg resistance gene *Rlm6* (Fig. 4b), and those plants were backcrossed with the susceptible recurrent parent to develop BC_2F_1. Subsequently, BC_3F_1 and BC_4F_1 generations were developed from the confirmed BC_2F_1, and BC_3F_1 seedlings, respectively (Fig. 4c, d).

Segregation of blackleg resistance gene *Rlm6* in backcross generations

The BC_1F_1 to BC_4F_1 progenies showed segregation with regards to resistance versus susceptible phenotypes in response to inoculation with *L. maculans* isolate J20 (carried avirulence gene *AvrLm6*). The χ^2 test for the phenotypic segregation of BC_1F_1 generation showed insignificant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.64800$; Table 1), which indicates an acceptable fit to a Mendelian segregation in the BC_1F_1 generation. Phenotyping data showed 49% of BC_1F_1 progeny resistant to *L.*

Table 1 Number of resistant and susceptible plants on the basis of phenotyping with the *L. maculans* isolates J20 among the interspecific hybrids derived from *Brassica napus* × *B. juncea*

Generations	Total # of seedlings	Resistant (R)	Susceptible (S)	Ratio (R:S)		χ^2	$P \leq 0.05$
				Observed	Expected		
BC_1F_1	480	235	245	1:1.0	1:1	0.20800	0.64800
BC_2F_1	288	79	209	1:2.6	1:1	30.9150	0.00001
BC_3F_1	384	57	327	1:5.7	1:1	108.3084	0.00001
BC_4F_1	284	27	257	1:9.5	1:1	111.3998	0.00001

maculans isolate J20 (Table 1), indicated an acceptable fit to a Mendelian ratio of 1:1 resistance versus susceptible. The χ^2 test for the phenotypic segregation from BC_2F_1 to BC_4F_1 generations showed significant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.00001$; Table 1). Phenotyping data showed 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 progeny resistant to *L. maculans* isolate J20 (Table 1). As a result, BC_2F_1, BC_3F_1, and BC_4F_1 generations had an unacceptable fit to a Mendelian ratio of 1:1 with regards to resistant versus susceptible phenotypes.

On the other hand, BC_1F_1 progeny were also segregating to a SCAR marker B5Rlm6_1, which is linked to B-genome of *B. juncea*. The χ^2 test for the segregation of the SCAR marker of BC_1F_1 progeny showed insignificant discrepancy between the observed and expected values for the ratio of *B. juncea* versus *B. napus* genome ($P \leq 0.52607$; Table 2), which supports an acceptable fit to a Mendelian segregation in BC_1F_1 generation. Genotyping data showed 47% of the BC_1F_1 progeny carried a single dominant gene *Rlm6* linked to the B-genome of *B. juncea* (Table 2) indicated an acceptable fit to a 1:1 ratio of *B. juncea* versus *B. napus* genome in BC_1F_1 progeny. The χ^2 test for the segregation of the SCAR marker from BC_2F_1 to BC_4F_1 progeny showed significant discrepancy between the observed and expected values for the ratio of *B. juncea* versus *B. napus* genome ($P \leq 0.00006$; Table 2), which indicated an unacceptable fit to a Mendelian segregation in BC_2F_1 to BC_4F_1 generations. Again genotyping with the B5Rlm6_1 marker showed that 30% of BC_2F_1, 18% of BC_3F_1, and 11% of BC_4F_1 progeny retained the gene *Rlm6* linked to the B-genome of *B. juncea* (Table 2). As a result, BC_2F_1, BC_3F_1, and BC_4F_1 generations had an unacceptable fit to a Mendelian segregation for the gene *Rlm6*. Thus the genotypic segregation of a SCAR marker B5Rlm6_1 is supporting an acceptable fit to a Mendelian ratio of *B. juncea* versus *B. napus* genome for BC_1F_1 but not for BC_2F_1 to BC_4F_1 generations.

Table 2 Segregation of the dominant type sequence characterize amplified region (SCAR) marker among the different generations derived from *Brassica napus* × *B. juncea*

Markers	Generations	Total # of seedlings	Resistant (R)	Susceptible (S)	Ratio (R:S)		χ^2	$P \leq 0.05$
					Observed	Expected		
B5Rlm6_1	BC_1F_1	244	115	129	1:1.1	1:1	0.4020	0.52607
	BC_2F_1	190	57	133	1:2.3	1:1	15.833	0.00006
	BC_3F_1	192	34	158	1:4.6	1:1	44.703	0.00001
	BC_4F_1	192	21	171	1:8.1	1:1	69.144	0.00001
B5-1520	BC_1F_1	192	91	101	1:1.1	1:1	0.2600	0.60971
	BC_2F_1	190	55	135	1:2.4	1:1	17.623	0.00002
	BC_3F_1	192	34	158	1:4.6	1:1	44.703	0.00001
	BC_4F_1	192	20	172	1:8.6	1:1	71.345	0.00001

Similar segregating patterns were also observed for the marker B5-1520 in all BC generations (Table 2). Overall, in this study the χ^2 goodness-of-fit test for the segregation of *Rlm6* linked to B-genome of *B. juncea* showed a significant association between the phenotypes (Table 1) and the genotypes (Table 2) in BC_1F_1 generation, supporting the assumption that the genetic control of resistance is by a single dominant gene.

Genome configurations

A significant decrease of SCAR markers linked to the *Brassica juncea* genome was found from BC_2F_1 to BC_4F_1 generations. Plants with the additional *B. juncea* genome components showed a decrease from 47.13% in BC_1F_1 to 30% in BC_2F_1, and this was further reduced to 17.71% in BC_3F_1 and 10.94% in BC_4F_1 with respect to the SCAR marker B5Rlm6_1 (Fig. 5). The situation was similar for the marker B5-1520, where 47.40% of BC_1F_1 showed the presence of this SCAR marker but only 10.42% yielded positive results among members of BC_4F_1 (Fig. 5). However, in the plants where *B. juncea* B-genome was integrated into the *B. napus* background, the presence of SCAR markers was significantly lower in the more advanced generations ($P < 0.0001$). In contrast, plants with the *B. napus* genome had an increase from 52.87% in BC_1F_1 to 89.06% in BC_4F_1 of *B. napus* genome as the B-genome linked dominant type SCAR marker B5Rlm6_1 appeared to be lost (Fig. 5). Similarly, the retention of the *B. napus* genome was also observed in advanced generation for the marker B5-1520 as well i.e. 52.60% in BC_1F_1 to 89.58% in BC_4F_1 (Fig. 5).

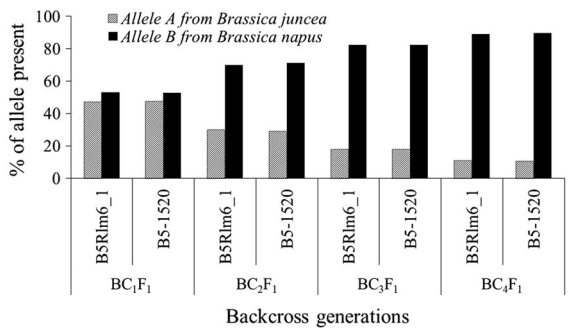

Fig. 5 Bar graph showing the pattern of presence or absence of SCAR markers B5Rlm6_1 and B5-1520 in backcross generations from BC_1F_1 to BC_4F_1. The bars represent the mean frequency of SCAR markers B5Rlm6_1 and B5-1520 linked to the B-genome of *Brassica juncea* as presence (white pattern fill) or absence (black solid fill) in backcross generations derived from *Brassica napus* × *Brassica juncea*. The mean frequency of the SCAR markers, either B5Rlm6_1 or B5-1520, linked to the B-genome fragment is significant among the generations ($P \leq 0.0001$; *t* test)

Discussion

In this study we were able to develop a set of BC populations (BC_1F_1 to BC_4F_1) from an interspecific cross *B. napus* × *B. juncea*. We also demonstrated successful introgression of a part of the B-genome into *B. napus* background. In addition, we integrated phenotype and genotype data to analyze the inheritance of a single gene *Rlm6* linked to the B-genome resistance.

Considering the gene-for-gene hypothesis (Flor 1971), an incompatible interaction between *L. maculans* races and the corresponding resistance genes is possible. This kind of study has been done routinely in canola-blackleg pathosystems since first observed in 1995 (Kuswinanti et al. 1995). Here we observed the hypersensitive reaction between *L. maculans* isolate J20 (carried avirulence gene *AvrLm6*), and *B. juncea* parent and the BC populations (carried *R* gene *Rlm6*) as a result of the gene-for-gene interaction (Fig. 3), indicating the study materials retain high level of resistance to *L. maculans*. Earlier studies also showed B-genome *Brassica* species; *B. nigra*, *B. carinata*, and *B. juncea* retain high level of resistance based on the hypersensitive response of the cotyledons upon infection by *L. maculans* (Roy 1978; Sacristán and Gerdemann 1986; Sjodin and Glimelius 1988; Rimmer and van den Berg 1992; Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006). There have been reports on the predicted breakdown of *R* genes in canola including *Rlm3* in Canada (Zhang et al. 2016), *Rlm1* in France (Rouxel et al. 2003), and *LepR3* in Australia (Sprague et al. 2006; Van de Wouw et al. 2010, 2014). It could be due to the widespread use of resistant *B. napus* cultivars which may have led to an increase of virulent races of *L. maculans.* Therefore, the characterizations of new sources of resistance against *L. maculans* are essential in controlling blackleg disease effectively. In this case, B-genome based resistance could be an alternative source of controlling blackleg disease. Here we used *B. juncea* cultivar 'Forge' to introduce blackleg

resistance into *B. napus* (Fig. 4), which carried *R* gene *Rlm6* (Chèvre et al. 1997; Balesdent et al. 2002; Christianson et al. 2006; Rashid et al. 2018). This resistance gene was successfully introgressed into *B. napus* confirmed by the polymorphism of a SCAR marker B5Rlm6_1 linked to the B-genome (Fig. 4). These lines may offer a strategy for breeding blackleg resistant *B. napus* varieties in the future. Previous studies also reported successful introduction of the B-genome into *B. napus* via interspecific hybridization (Struss et al. 1991; Zhu et al. 1993; Delourme et al. 1995; Frello et al. 1995; Chèvre et al. 1996, 2008; Plieske et al. 1998; Brun et al. 2010; Navabi et al. 2010).

In the present study, our phenotype data showed 49% of BC_1F_1, 27% of BC_2F_1, 15% of BC_3F_1, and 10% of BC_4F_1 progeny are resistant to *L. maculans* isolate J20, indicating an incompatible interaction between isolate J20 and interspecific hybrids (Table 1). The χ^2 test for the phenotypic segregation in BC_1F_1 generation showed insignificant differences between the observed and the expected number of plants for resistant versus susceptible ($P \leq 0.64800$; Table 1), which indicates an acceptable fit to a Mendelian segregation in the BC_1F_1 generation. Thus the data is supporting a monogenic dominant inheritance for blackleg resistance in the BC_1F_1 population, an important feature which increases the value of the materials for commercial breeding particularly in a variety development program. A single gene inheritance linked to the B-genome resistance was also reported in previous studies (Struss et al. 1996; Chèvre et al. 1997; Saal et al. 2004; Chèvre et al. 2008; Wang 2016). Wang (2016) investigated the interaction phenotype between blackleg isolates and interspecific hybrids derived from *B. napus* (AACC) × *B. carinata* (BBCC) and *B. napus* ×hexaploid *Brassica* species (Meng, AABBCC). She observed a Mendelian segregation in the BC_1F_1, BC_3F_1 and BC_4F_1 generations derived from *B. napus* × *B. carinata*, but no Mendelian segregation in the BC_2F_1. She also could not observe a Mendelian segregation in BC_1F_1 and BC_2F_1 derived from *B. napus* ×hexaploid *Brassica* species. Chèvre et al. (2008) also observed different segregation pattern of blackleg rersistance linked to the *R* gene *Rlm6* in the interspecific hybrids derived from *B. napus* cv. Dunkled ×KEM lines (*B. napus* × *B. juncea*). A monogenic inheritance of blackleg resistance was also observed in the recombinant inbred lines (Chèvre et al. 1997) and in the F_2 progeny (Pang and Halloran 1996) derived from *B. napus* × *B. juncea*. Struss et al. (1996) investigated three different BC populations derived from different interspecific crosses; *B. napus* × *B. nigra*, *B. napus* × *B. juncea*, and *B. napus* × *B. carinata* and observed similar level of resistance among the BC lines as seen in the B-genome donor parents. The present study with the BC generations showed a high similarity in the resistance mechanisms against blackleg. No significant differences in phenotype or levels of resistance were observed in the study materials. However, results of the earlier and the present study suggest that the resistance genes identified in the three B-genome species are identical, indicating the B-genome resistance is due to a single resistance gene in *B. juncea* species.

We were able to demonstrate successful introgression of B-genome blackleg resistance linked to the *R* gene *Rlm6* into *B. napus* via a backcross approach (Fig. 4). In this study, a single gene or a gene complex of *B. juncea* was introduced into backcross generations. Our genotype data for the SCAR marker B5Rlm6_1 showed the transmission rate of introgressed segment decreasing towards advanced generations, which was 47% in BC_1F_1, decreased to 27% in BC_2F_1, further decreased to 18% in BC_3F_1, and 11% in BC_4F_1 (Table 2). A similar trend was also observed for the marker B5-1520 in all BC generations. Thus the genotypic segregation of SCAR markers linked to the B-genome resistance gene *Rlm6* had an acceptable fit to a Mendelian ratio of presence versus absence of B-genome fragment for BC_1F_1 generation, but not for BC_2F_1 to BC_4F_1. Similar kind of marker skewedness in advanced generations was also observed in previous studies (Dion et al. 1995; Frello et al. 1995; Chèvre et al. 1997; Plieske et al. 1998; Dixelius and Wahlberg 1999; Saal and Struss 2005; Schelfhout et al. 2006; Navabi et al. 2010; Fredua-Agyeman et al. 2014). The present study was supported by Saal and Struss (2005), where they genotyped two segregating populations derived from *B. napus* × *B. juncea* using a dominant type SCAR marker which showed significant deviation from a monogenic inheritance. This study also supported by Dixelius and Wahlberg (1999), where they recorded segregation of RFLP-based markers, which is linked to the donor genome B, 60% in BC_1F_1, 33% in BC_2F_1, and 10% in BC_3F_1 from *B. juncea*-derived hybrids whereas *B. carinata*-

derived hybrids had segregation from 59% in BC_1F_1 to 36% in BC_2F_1 and further down to 11% in BC_3F_1. *B. nigra*-derived hybrids showed lower segregation of B-genome compared to *B. carinata*- and *B. juncea*-derived hybrids, which was 46% in BC_1F_1 to 25% in BC_2F_1 and further decreased to 8% in BC_3F_1. On the other hand, segregation of RFLP marker linked to blackleg resistance was also observed in DH lines and predicted to control genetic resistance by a single major gene *LmFr1* (Dion et al. 1995). Frello et al. (1995) reported inheritance of 52% RAPD linked marker to a transgene in BC_1F_1 population derived from *B. juncea* ×(*B. juncea* × *B. napus*). Similar observations were also reported by Plieske et al. (1998), when they analysed RFLP markers linked to B-genome based blackleg resistance. However, our genotype of SCAR markers B5Rlm6_1 and B5-1520 in BC_1F_1 generation, indicating a monogenic inheritance of *Rlm6* linked to B-genome resistance, as was seen in the earlier studies. We observed that the frequency of SCAR markers was significantly reduced from BC_1F_1 (47.13%) to BC_4F_1 (10.94%) where the B-genome of *B. juncea* was integrated (Fig. 5), and at the same time the *B. napus* genome contribution was significantly increased from BC_1F_1 (52.87%) to BC_4F_1 (89.06%), which is the result of a possible reduction of the B-genome by the homoeologous recombination. A similar mechanism was predicted for the gene transfer from B-genome species to *B. napus* (Sacristán and Gerdemann 1986; Delourme et al. 1995; Plieske et al. 1998), and from C-genome species to A-genome (Leflon et al. 2006). Introgression of B-genome chromosomes into *B. napus* from *B. juncea* and *B. carinata* was also confirmed by Schelfhout et al. (2006) and Fredua-Agyeman et al. (2014), respectively. We also did not record any significant differences in genotype between marker B5Rlm6_1 and B5-1520 for all four BC generations, indicating there is no segregation deviation from the B-genome. The co-segregation of DNA-markers suggests that both of the SCAR markers are likely located on the introgressed B-genome, and not in the *B. napus* genome. Thus this study suggests a possible introgression by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with the A- or C-genomes (Plieske et al. 1998). Similar phenomena were found in rice during transfer of bacterial resistance from a wild genotype to cultivated rice (Amante-Bordeos et al. 1992). It is reported that when an amphidiploid such as oilseed rape, is used in interspecific crosses, it is usually challenging to return to the original ploidy level by backcrossing (Bing et al. 1991; Chèvre et al. 1996), because of the tendency of spontaneous genome fixation by amphiploidy (Song et al. 1993). This is in agreement with our outcomes, which indicated a high probability of amphidiploid conservation due to meiotic stability, and sound seed set under normal conditions. Moreover, the BC populations were genotyped using dominant type SCAR markers B5Rlm6_1 and B5_1520 (Table 2), which was unable to discover recombinant event for the introgression region between them.

However, it still remains in doubt whether the recombination events are originating from the homoeologous position of the *B. napus* genome or from the loss of introgressed segments or a combination of both types of events. This question could be addressed by Southern blotting or fluorescent in situ hybridization (FISH). We developed a custom FISH probe with the specific primer sequences (molecular weight 1.5 kb), unfortunately, there was no FISH signal observed either in the resistant parent or the BC populations. This could be due to the short fragment size of the probe, as compared to the larger repetitive fragment required in a typical FISH study (Jiang and Gill 2006). This is also supported by Schelfhout et al. (2006) where no FISH signals were observed for B-genome. In order to confirm the stabilization of B-genome introgression into *B. napus*, both the trait locus and SCAR markers could be analysed further in doubled haploid or near isogenic lines, to see if the same direction of marker segregation would occur. This will be the focus for future studies; in addition to the development of more markers in the introgression region. Moreover, a study towards mapping or map-based cloning of the resistance locus could also be possible by screening different mapping populations, and/or by bulked segregant RNA-seq analyses (Liu et al. 2012). This is currently under investigation in our lab.

In summary, we demonstrated successful introgression of B-genome blackleg resistance from *B. juncea* to *B. napus*. The study of phenotypes and genotypes among the interspecific hybrids showed that the material we developed carries a single dominant gene *Rlm6*, conferring resistance to the blackleg pathogen *L. maculans*. Notably, the BC populations developed in this study retained blackleg resistance are available

for commercial breeding and therefore can accelerate a variety development program in the future.

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