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Molecular cytogenetic identification of B genome chromosomes linked to blackleg disease resistance in *Brassica napus* × *B. carinata* interspecific hybrids

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Abstract

Key message **Provide evidence that the *Brassica* B genome chromosome B3 carries blackleg resistance gene, and also the B genome chromosomes were inherited several generations along with *B. napus* chromosomes.**

Abstract Blackleg disease caused by fungus *Leptosphaeria maculans* causes significant yield losses in *Brassica napus*. *Brassica carinata* possesses excellent resistance to this disease. To introgress blackleg resistance, crosses between *B. napus* cv. Westar and *B. carinata* were done. The interspecific-hybrids were backcrossed twice to Westar and self-pollinated three times to produce BC_2S_3 families. Doubled haploid lines (DH1) were produced from one blackleg resistant family. SSR markers were used to study the association between B genome chromosome(s) and blackleg resistance. The entire B3 chromosome of *B. carinata* was associated with blackleg resistance in DH1. A second DH population (DH2) was produced from F_1s

of resistant DH1 lines crossed to blackleg susceptible *B. napus* cv. Polo where resistance was found to be associated with SSR markers from the middle to bottom of the B3 and top of the B8 chromosomes. The results demonstrated that the B3 chromosome carried gene(s) for blackleg resistance. Genomic in situ hybridization (GISH) and GISH-like analysis of the DH2 lines revealed that susceptible lines, in addition to *B. napus* chromosomes, possessed one pair of B genome chromosomes ($2n = 40$), while resistant lines had either one ($2n = 40$) or two pairs ($2n = 42$) of B chromosomes. The molecular and GISH data suggested that the B chromosome in the susceptible lines was B7, while it was difficult to confirm the identity of the B chromosomes in the resistant lines. Also, B chromosomes were found to be inherited over several generations along with *B. napus* chromosomes.

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Introduction

Oilseed rape or canola (*Brassica napus*) contributes about 15 % to the world's total supply of vegetable oils (Rahman et al. 2013). It is cultivated extensively in North America, Europe, China and Australia. In 2012, Canada was the top producing country (15.4 MMT), followed by China (14.0 MMT), India (6.8 MMT), France (5.5 MMT) and Germany (4.8 MMT) (FAOSTAT). Revenue to the major producing countries from canola is projected to increase even further in the coming years due to soaring demands for canola on the world market. However, *B. napus* is affected by many diseases among which blackleg or stem canker caused by the fungus *Leptosphaeria maculans* Desm. (Ces. & de Not) is of major concern mainly because of the significant yield losses associated with the disease worldwide (for reviews, see Gugel and Petrie 1992; West et al. 2001; Howlett

2004; Fitt et al. 2006). Yield loses of up to 50 % have been reported in infected *B. napus* fields (Gugel and Petrie 1992) while total crop failure have also been reported in areas where highly susceptible cultivars are grown or when there is breakdown of a major resistance gene in a susceptible background (Li et al. 2003; Rouxel et al. 2003; Sprague et al. 2006). Breeding for resistance to blackleg offers by far the least expensive, the most environmentally friendly and the best practical means of controlling the disease (Rimmer and Buchwaldt 1995; for reviews, see Fernando et al. 2007; Kutcher et al. 2011).

Genetic mapping studies on the resistance of *B. napus* to *L. maculans* have identified six endogenous race-specific blackleg resistance (*Rlm*) genes in this crop. The loci controlling these genes have been mapped onto the A7 (≈N7) (*Rlm1*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*) and A10 (≈N10) (*Rlm2*) linkage groups of the A-genome (Ansan-Melayah et al. 1998; Balesdent et al. 2001, 2002; Delourme et al. 2004, 2006; Rimmer 2006). Other blackleg resistance genes identified in *B. napus* cultivars and thought to be allelic forms of the genes located on the A7 linkage group or members of the previously mapped gene cluster include *LEM1* in *B. napus* cv. 'Major' (Ferreira et al. 1995), *LmFr1* (Dion et al. 1995) and *CLmR1* (Mayerhofer et al. 2005) in cv. 'Cresor', *LmR1* in cv. 'Shiralee' (Mayerhofer et al. 1997), *cRLMm* in cv. 'Maluka' (Rimmer et al. 1999) and *cRLMrb/aRLMrb* in line 'RB87-62' (Rimmer et al. 1999). Four other disease resistant genes *LepR1* on A2 (≈N2), *LepR2/LepR3* on A10 and *LepR4* on A6 (≈N6) thought to be different from any of the aforementioned genes (Yu et al. 2005, 2008, 2013) as well as *Rlm8* and *Rlm11* which have not been genetically mapped (Balesdent et al. 2002, 2013) have been introgressed into *B. napus* cultivars from *B. rapa*. Thus, to date a total of 12 race-specific resistance genes to *L. maculans* that originate from the A genome of *B. napus* and *B. rapa* have been identified.

Canadian canola breeders, including the University of Alberta, have been utilizing *LmR1* and *cRLMm* resistance present on the A7 linkage group of the Australian canola (*B. napus*) cvs. 'Shiralee' and 'Maluka', respectively, to develop *L. maculans* resistant canola cultivars for the Prairie Provinces where pathogenicity group 2 (PG2) is most prevalent. The two resistant genes were initially thought to be *Rlm4* (see review by Delourme et al. 2006; Hayward et al. 2012), but recent data indicate they could be *Rlm3* since the two are thought to be allelic versions of the same gene and are not present together in the same cultivar. However, more virulent pathotypes of the fungus, e.g. pathogenicity groups PG3, PG4 and PGT, have emerged in the Canadian Praires rendering the resistance ineffective (Fernando and Chen 2003; Kutcher et al. 2007). There is, therefore, the threat of single gene resistance breakdown in *B. napus* cultivars in Canada as was reported in Australia

(Li et al. 2003; Sprague et al. 2006) and France (Brun et al. 2000; Rouxel et al. 2003).

Brassica species that contain the B genome, namely *B. nigra* (BB, $2n = 16$), *B. carinata* (BBCC, $2n = 34$) and *B. juncea* (AABB, $2n = 36$), are known to carry excellent resistance to blackleg that is effective throughout the life of the plant (Roy 1984; Sjödin and Glimelius 1988; Rimmer and van den Berg 1992). Therefore, to produce *B. napus* cultivars that are resistant to blackleg disease many workers have attempted to use these B genome containing *Brassica* species as donors in either sexual crosses (Roy 1978, 1984; Sacristan and Gerdemann 1986; Zhu et al. 1993; Struss et al. 1996; Chèvre et al. 1996, 1997; Plieske et al. 1998; Dixelius 1999; Dixelius and Wahlberg 1999; Navabi et al. 2010, 2011) or somatic-hybridization (Sjodin and Glimelius 1989a, b; Dixelius and Glimelius 1995, Waara and Glimelius 1995; Glimelius 1999).

For the introgression of B genome resistance into *B. napus* several pre- and post- fertilization barriers need to be overcome. In spite of these challenges, Chèvre et al. (1997) successfully introgressed a B genome resistance gene, *Rlm6*, from *B. juncea* into *B. napus*. It is important to incorporate additional resistance genes from the *Brassica* B genome not only to provide resistance to specific pathotypes but also for resistance against emerging new forms of the pathogen as well as to be used in combination with quantitative resistance in this crop. For this, knowledge of the location of the B genome resistance genes is important. A recent paper (Brun et al. 2010) showed that one of the best strategies to minimize the devastating effects of blackleg disease on *B. napus* production is to combine dominant genes and polygenic resistance to prevent breakdown of major genes. Therefore, the introduction of new major resistance genes from *B. carinata* into *B. napus* is important for the diversification of resistance genes for the optimal management of blackleg disease in this crop.

In this work, we used different generations from a *B. napus* × *B. carinata* cross progenies and simple sequence repeat (SSR) markers to show the association of a B genome chromosome of *B. carinata* and blackleg disease resistance in the *B. napus* background. In addition, we studied the behaviour of different B genome chromosomes through genomic in situ hybridization (GISH) and GISH-like techniques.

Materials and methods

Plant materials

The parental materials used for this interspecific cross were *Brassica napus* cv. 'Westar' and *B. carinata* accession #98-14513. Westar is a registered cultivar in Canada and is highly susceptible to blackleg while *B. carinata* line

#98-14513 is highly resistant to this disease. Seed of this line was obtained from the germplasm collection of the Canola Breeding and Research Program at the University of Alberta (U of A). Crosses between Westar × *B. carinata* were done using Westar as female, and the interspecific hybrids were backcrossed twice to Westar and subsequently were self-pollinated three times to produce BC_2S_3 families (Fig. 1). In each generation selection was made for cotyledon resistance to *L. maculans* PG2 isolate #UA3356. Thirty BC_2S_3 families were produced from the *B. napus* cv. Westar × *B. carinata* interspecific cross. Of these, four plants from the family #02-17006 which exhibited resistance to *L. maculans* PG2 isolate #UA3356 at the cotyledon stage were used for the production of DH lines through bulk culture of microspore and the DH population was designated Popl#3 which for simplicity, hereafter will be referred to as DH1 (Fig. 1). The microspore culture technique applied for the production of DH lines was as described by Kebede et al. (2010). To study the inheritance of blackleg resistance and transmission of B genome chromosomes in a different genetic background, we crossed two resistant DH1 lines carrying B genome chromosomes to the oil-rich blackleg susceptible *B. napus* cv. 'Polo' and produced DH lines from the F_1 plants of each of these two crosses (Fig. 1). These two DH populations were designated as Polo-Popl#3[1151] and Polo-Popl#3[1152], and collectively as Polo-Popl#3, which for simplicity, hereafter will be referred to as DH2 population (Fig. 1).
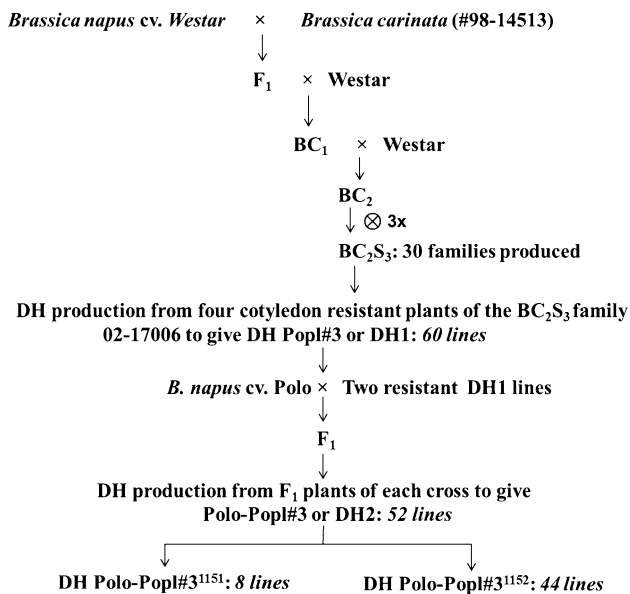
Fig. 1 Interspecific crossing for the introgression of *Leptosphaeria maculans* resistance from *B. carinata* into *B. napus*. The DH1 lines were produced from four cotyledon resistant plants of the BC_2S_3 family #02-17006 while the DH2 lines were produced from F_1 plants obtained by crossing two resistant DH1 lines with Polo

Leptosphaeria maculans culture and inoculum preparation

Leptosphaeria maculans PG2 isolate #UA3356 originally collected from a canola field in Alberta (Canada) and maintained by the Canola Breeding and Research Program at the U of A was used for all inoculation experiments. The isolate was cultured on V8 vegetable juice agar plates containing 40 mg/L Rose Bengal and 100 mg/L streptomycin sulphate (Dhingra and Sinclair 1985). The culture was maintained under 12-h photoperiod at 21 °C in a NuAire™ incubator (Fernbrrok Lane, MN, USA). After 16 days, each plate was flooded with 5 mL sterile distilled water and the culture surface was gently scraped with a sterile metal rod to manually dislodge the pycnidiospores. The pycnidiospore suspensions were then filtered through sterile cheesecloth. The concentration of the spore suspensions were determined using a haemocytometer and adjusted to 1×10^7 pycnidiospores/mL. The prepared inoculum was used immediately or stored at +4 °C for not more than 24 h until use.

Inoculation tests for blackleg resistance

Screening for reaction to *L. maculans* isolate #UA3356 was carried out by inoculating four to eight seedlings of each DH line and each inoculation experiment was replicated two to four times. To do this, 7-day-old seedlings with fully expanded cotyledons were wounded on each lobe and inoculated with *L. maculans* spore suspension as described by Sjödin and Glimelius (1988). Disease symptoms were rated 10 to 13 days after inoculation for cotyledon reaction. The plants were left to grow without any further inoculation and evaluated for stem canker at maturity by cutting stems of the inoculated plants. The severity of the disease symptoms at the cotyledon stage was scored based on the 0–9 scale devised by Delwiche (1980), while the severity of the symptoms at the adult stage was determined based on the 0–5 scale proposed by Western Canada Canola/Rapeseed Recommending Committee (WCC/RRC). Based on mean scores for cotyledon infection, the plants were grouped as resistant (score ≤1), intermediate (score 1 to ≤4) and susceptible (score >4); while based on mean scores for internal necrosis at adult stage, the plants were grouped as resistant (score ≤1), intermediate (score 1 to ≤2) and susceptible (score >2). In these tests, *B. carinata* was used as the resistant control while *B. napus* cultivars Westar and Polo were used as the susceptible controls.

DNA extraction for molecular marker analysis

DNA was extracted from the parental materials (*B. carinata* and the *B. napus* cultivars Westar and Polo) and from the DH lines using the GENELUTE Plant Genomic DNA Kit (Sigma-Aldrich, St. Louis, CA, USA) according to the

manufacturer's instructions. The quality of the DNA was evaluated by spectrometry using the 260/280 nm absorbance ratio method and the DNA concentration estimated at 260 nm with an ND-2000c Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA). DNA was stored at −80 °C until use.

Identification of SSR marker(s) linked to blackleg disease resistance

Bulk segregant analysis as described by Michelmore et al. (1991) was performed using DNA from the resistant parent (*B. carinata*), the susceptible parents (*B. napus* cvs. Westar and Polo), as well as resistant and susceptible bulks of DH1 and by the use of 465 SSR markers. The SSR markers comprised 87, 70 and 273 markers from the *Brassica* A, B and C genomes, respectively, obtained from Dr. Isobel Parkin [Agriculture and Agri-Food Canada (AAFC), Saskatoon Research Centre, Saskatoon, Canada] through a material transfer agreement. In addition, 35 B genome SSR markers obtained from Ramchiary et al. (2007) were also used in the bulk segregant analysis. Putative polymorphic markers were evaluated on the parents as well as on five resistant and five susceptible DH1 lines. Once the linkage association of a marker and disease resistance had been established, fine-scale mapping was done using all DH1 and DH2 lines. Linkage group designation of the B genome chromosomes was done using B1–B8 notation proposed by Lagercrantz and Lydiate (1996) and accepted by the Multinational Brassica Genome Project (MBGP) Steering Committee Meeting of January 2010, while linkage group assignments of the A and C genome chromosomes were done using A1–A10 and C1–C9, respectively.

PCR amplification

Each PCR reaction was performed in a total volume of 12 μL containing 1 × Taq buffer, 2.0 mM MgCl$_2$, 200 μM dNTPs, 0.4 μM forward primer modified at the 5′-end with an M13 tail, 0.4 μM reverse primer, 0.2 μM fluorescently labelled M13 primer, 1.25 U Taq polymerase (Promega Corp., Madison, WI, USA) and 3 to 10 ng of genomic DNA. Amplifications were carried out in an MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad laboratories, Hercules, CA, USA) with initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, optimum annealing temperature of primer for 1 min, and at 72 °C for 1.5 min and a final extension at 72 °C for 30 min. An aliquot of the PCR products was separated by capillary electrophoresis on an ABI PRISM 3730 × 1 DNA analyzer (Applied Biosystems). Amplified PCR products from promising markers

were also separated on 6 % denaturing polyacrylamide gels (Bio-Rad laboratories, Hercules, CA, USA). To confirm the reproducibility of the molecular data we repeated PCR, genotyping and gel electrophoresis in about 10 % of the individual samples.

Meiotic observation in the Polo-Popl#3-DH2 lines

For the study of meiotic behaviour in the interspecific hybrids, immature flower buds were collected from two susceptible and seven resistant DH2 lines. Meiotic chromosome preparations were carried out as described by Leflon et al. (2006) and Mason et al. (2010), that is, the buds were fixed in Carnoy's solution (ethanol: chloroform: acetic acid, 6: 3: 1) for 24 h at room temperature and stored in 50 % ethanol at 4 °C. Anthers were squashed and stained in a drop of 1 % acetocarmine solution. The slides were examined under a phase contrast microscope and chromosome pairing was observed in 10–24 pollen mother cells (PMCs) per plant at metaphase I (MI).

GISH and GISH-like at mitosis with genome-specific probes

Total genomic DNA used for genomic in situ hybridization (GISH) analyses were extracted from young leaves of *B. napus* using the CTAB method (Rogers and Bendich 1988). The genomic DNA samples were autoclaved to give fragments of 100–300 bp and used as a blocking agent. The bacterial artificial chromosome (BAC) clone BoB014O06 from *B. oleracea* (Howell et al. 2002) was used as a probe to identify all C genome chromosomes in the interspecific hybrids using GISH-like technique, while *B. nigra* genomic DNA was used to probe the *Brassica* B genome of *B. carinata* in the interspecific hybrids using GISH (Mason et al. 2010) techniques. Bacterial artificial chromosome BoB014O06 clone and the genomic DNA of *B. nigra* were labelled by random priming with Alexa488-5-dUTP (Molecular probes) and by nick translation with biotin-14-dUTP (Invitrogen), respectively. A 1:50 ratio of *B. nigra* probe to *B. napus* blocking DNA was used to prepare the hybridization mixture. Preparation of slides was as described by Leflon et al. (2006) and Mason et al. (2010). The biotinylated probes were immunodetected by Texas Red avidin DCS (Vector Laboratories).

Statistical analysis

Fisher's exact test was done to investigate independence for resistance and susceptibility among the crosses and Chi-square test for goodness of fit was done to determine segregation pattern for the phenotypic classes.

Results

Evaluation of DH lines for resistance to blackleg PG2 isolate #UA3356

Sixty DH1 lines, obtained from the BC_2S_3 family #02-17006, were evaluated for resistance to *L. maculans* PG2 isolate #UA3356. At the cotyledon stage, 48 % of the lines were found to be resistant, 7 % intermediate and 45 % susceptible (Table 1). The resistant parent *B. carinata* line always had disease score ≤1; therefore, a Chi-square test was done by grouping the DH lines with disease score ≤1 into one class (resistant) and all other DH lines into a separate class (intermediate and susceptible). The distribution of the DH1 lines for cotyledon resistance followed a 1:1 segregation ratio for resistance to susceptibility ($\chi^2 = 0.067$, $P = 0.7957$). Assessment of adult plant resistance could not be carried out in 8 % of the DH1 lines because the plants died before maturity while among the remaining DH1 lines 33 % were found to be resistant, 20 % intermediate and 47 % susceptible (Table 1). Thirty-one per cent of the cotyledon resistant DH1 lines became intermediate resistant at the adult stage while 33 % of the cotyledon intermediate resistant lines became susceptible and 33 % became resistant. This indicates that in spite of the specific resistance gene the fungus was able to continue growth in some plants. On the other hand, almost all (96 %) of the cotyledon susceptible DH1 lines remained susceptible at the adult stage while about 4 % of the susceptible DH1 lines became intermediate resistant at the adult stage).

In the case of DH2, 8 DH lines of Polo-Popl#3[1151] and 44 DH lines of Polo-Popl#3[1152] were evaluated for resistance to *L. maculans* PG2 isolate #UA3356. Cotyledon stage phenotypic evaluation of DH2 lines from the Polo-Popl#3[1151] and Polo-Popl#3[1152] showed that a total of 48 % of the lines were resistant, 6 % intermediate and 46 % susceptible to the isolate (Table 1). At the adult stage, 40 and 28 % of the cotyledon resistant DH2 lines became susceptible and intermediate resistant, respectively, while 32 % of the lines were found to remain resistant. In addition, all

cotyledon intermediate DH2 lines became susceptible at the adult stage. Thus, the fungus was able to grow in some cotyledon resistant and intermediate resistant DH2 lines. Fisher's exact test showed that segregation pattern for cotyledon or adult resistance was not different in the DH2 lines derived from the two crosses. As a result, Chi-square test for fit to 1:1 segregation for resistant to susceptible phenotype was done on pooled data of the DH2 lines of the two crosses. The results fitted well with 1:1 segregation for cotyledon resistance ($\chi^2 = 0.077$, $P = 0.781$) but deviated significantly for adult resistance ($\chi^2 = 26.741$, $P \leq 0.0001$) (Table 1). Also, pooled data of DH1 and DH2 also fitted well to a 1:1 segregation for resistance and susceptibility at the cotyledon stage ($\chi^2 = 0.143$, $P = 0.705$), but not at the adult stage ($\chi^2 = 28.271$, $P \leq 0.0001$) (Table 1). This suggests that cotyledon resistance to *L. maculans* PG2 isolate #UA3356 in DH1 and DH2 populations is under monogenic control. Also, the fact that none of the cotyledon susceptible plants became resistant at the adult stage suggests that the same gene conferring cotyledon resistance is also involved in the control of adult plant resistance.

Genotypic analysis of SSR markers used in the bulk segregant analysis

Analysis of the PCR amplified products of the 87 A genome SSR markers screened showed that 28 produced polymorphic bands, 54 monomorphic bands while 5 failed to amplify genomic DNA in the resistant (*B. carinata*) and susceptible (*B. napus* cvs. Westar and Polo) parents. Among the 273 C genome SSR markers screened, 186 produced polymorphic bands, 61 monomorphic bands while 26 did not amplify genomic DNA in the samples. Comparison of the electrophoretic pattern of PCR-amplified products of the polymorphic SSR markers from the A and C genome ($28 + 186 = 214$) used in the bulk segregant analysis showed that alleles of about 90 % of the markers detected in the *B. napus* parents (but not in *B. carinata*) were also detected in the resistant and susceptible bulks of DH1 and DH2. The remaining 10 % of the polymorphic

Table 1 Cotyledon and adult plant responses to *Leptosphaeria maculans* PG2 isolate #UA3356 in the doubled haploid (DH) population Popl#3 (DH1) derived from (*Brassica napus* cv. Westar × *B. carinata*) × *B. napus* cv. Westar and the DH lines of Polo-Popl#3 (DH2) derived from crossing Polo with two resistant DH lines of Popl#3 (DH1)

DH	Population	Total DH	Cotyledon resistance			Adult resistance		
			Resistant (R)	Intermediate (I)	Susceptible (S)	Resistant (R)	Intermediate (I)	Susceptible (S)
DH1	Popl#3	60	29	4	27	18	11	26
DH2	Polo-Popl#3[1151]	8	4	0	4	1	1	6
	Polo-Popl#3[1152]	44	21	3	20	7	6	31
	Pooled	112	54	7	51	26	18	63

SSR markers from the A and C genomes detected alleles which were shared by both *B. carinata* and the *B. napus* parents, and these alleles were also detected in the DH1 and DH2 bulks. In addition, these markers also amplified alleles from other loci which were unique to *B. carinata*; however, these alleles were not detected in the DH1 and DH2 bulks. Thus, about 10 % of the polymorphic A and C markers may also have amplified the B genome. Based on the molecular marker data, all ten A (A1–A10) and nine C (C1–C9) genome chromosomes were inherited in the interspecific hybrids.

Of the 105 B genome SSR markers screened, 37 markers detected polymorphism between the parents, 62 gave monomorphic bands, while six failed to amplify genomic DNA in any of the samples. Fourteen out of the 37 polymorphic markers, comprising four, one, two, three and four markers from the B1, B2, B4, B5 and B6 chromosomes, respectively, did not detect any alleles in DH1 and DH2. This indicates that not all B genome chromosomes were inherited in the progenies of the *B. napus* × *B. carinata* interspecific cross. However, one marker (SSR #sJ4633) from the terminal region of the B1 chromosome amplified genomic DNA in about 13 and 56 % of both the resistant and susceptible DH1 and DH2 lines, respectively—apparently due to retention of a very small segment of the B1 chromosome in some of the lines (Supplementary Material Table S1). On the other hand, 22 out of the 37 polymorphic SSR markers comprising nine, six and seven from the B3, B7 and B8 chromosomes, respectively, were found to amplify *B. carinata* alleles in the DH1 and DH2 populations. This suggests that the B3, B7 and B8 chromosomes and a small fragment of the B1 were retained in the interspecific progenies. It should be noted that selection for cotyledon resistance was carried out in each generation during the development of the BC_2S_3 family #02-17006; this might have contributed to the retention of the extra B chromosome(s) in the DH1 lines.

Linkage association between B genome chromosomes and blackleg resistance

None of the 28 and 186 polymorphic SSR markers from the A and C genomes identified in the bulk segregant analysis was found to be associated with blackleg resistance. Also, the single SSR marker (sJ4633) from the tip of the B1 chromosome as well as the four (sB1822, sJ7046, sB1752 and sJ8469a) B7-specific SSR markers covering about 90 % of the chromosome did not show any linkage association with blackleg resistance in both DH1 and DH2 (Supplementary Table S1 and Figure S2). On the other hand, some of the polymorphic B3 and B8 SSR markers (see below) showed linkage association with cotyledon resistance and hence were selected for mapping of the two DH populations.

Sixty DH1 lines were genotyped with one, nine, six and seven markers from the B1, B3, B7 and B8 chromosomes (Supplementary Material Table S1). In the case of the DH2 population, 8 and 46 lines from the two crosses, Polo-Popl#3[1151] and Polo-Popl#3[1152], respectively, were genotyped with the same B genome markers used to screen the DH1 population (Supplementary Material Table S1).

In the case of the B3 chromosome, five (sB0860A, sJ34121, sB3751, sA0343a and sB2596) of the nine SSR markers were B-genome-specific and did not amplify Westar or Polo, while the remaining four markers (sJ0954, sJ7166, sB1728 and sB5162) were not specific to the B genome, but produced a clear band in *B. carinata* and the resistant DH1 lines. Eight of the nine SSR markers from the top (sJ0954, sJ7166 and sB0860A), middle (sB1728, sB3751 and sB5162) and bottom (sA0343a and sB2596) of the B3 chromosome were found to co-segregate with cotyledon resistance in DH1 population (Supplementary Material Figure S1). The eight markers included the telomeric markers (sJ0954 and sB2596) from both ends of the chromosome and span a region of about 222 cM based on the B3 linkage map of Navabi et al. (2010). The remaining marker sJ34121, which is specific to the B genome, amplified genomic DNA in only 22 % of the resistant DH1 lines. Therefore, the SSR marker sJ34121, unlike the other eight B3 chromosome markers, did not co-segregate with cotyledon resistance. This may be due to undetected recombination event(s) that occurred during the generation of interspecific hybrid progenies. Based on these eight SSR markers, it can be inferred that the entire B3 chromosome was present in the resistant DH1 lines and was associated with cotyledon resistance. The co-segregation of three of the eight B3 SSR markers with cotyledon resistance in DH1 population is shown in Fig. 2, while the other five markers are shown in Supplementary Material Figure S1. In the DH2 lines, five (sB1728, sB3751, sB5162, sA0343a and sB2596) of the nine markers from middle to bottom of B3 were found to co-segregate with cotyledon resistance. On the other hand, three markers (sJ0954, sJ7166 and sB0860A) from the top of the B3 chromosome failed to detect the B-genome-specific alleles of *B. carinata* in the resistant DH2 lines, while the adjacent marker sJ34121 gave monomorphic bands in both resistant and susceptible DH2 (Supplementary Material Figure S1). Thus, based on the marker data, it can be inferred that the top of the B3 chromosome was lost in the resistant DH2 lines, and the presence of the middle to lower segment of this chromosome was enough to confer cotyledon resistance (Supplementary Material Table S1 and Figure S1).

In the case of the B8 chromosome, four (sJ8370a, sJ6846, sJ3302R and sJ03104) of the seven SSR markers from the middle of the chromosome were found to be B-genome-specific. The telomeric marker (sJ7434) from

Fig. 2 Polyacrylamide gel electrophoretic profiles showing amplified PCR products of resistant and susceptible DH2 lines and the parents *B. carinata* (*C*), Polo (*P*), and Westar (*W*) with three of the eight B3 chromosome SSR markers (sB1728 (*top gel*), sB3751 (*middle gel*), and sB5162 (*bottom gel*)) that co-segregated with blackleg disease resistance. In all gels the resistant bands (indicated by *arrows*) are present in the resistant DH lines as well as in *B. carinata*, but absent in the susceptible DH lines and the susceptible parents, *B. napus* cv. Westar and Polo. See Supplementary Material Figure S3 for gel images showing co-segregation of the other B3 markers with blackleg resistance in DH1 and DH2 lines
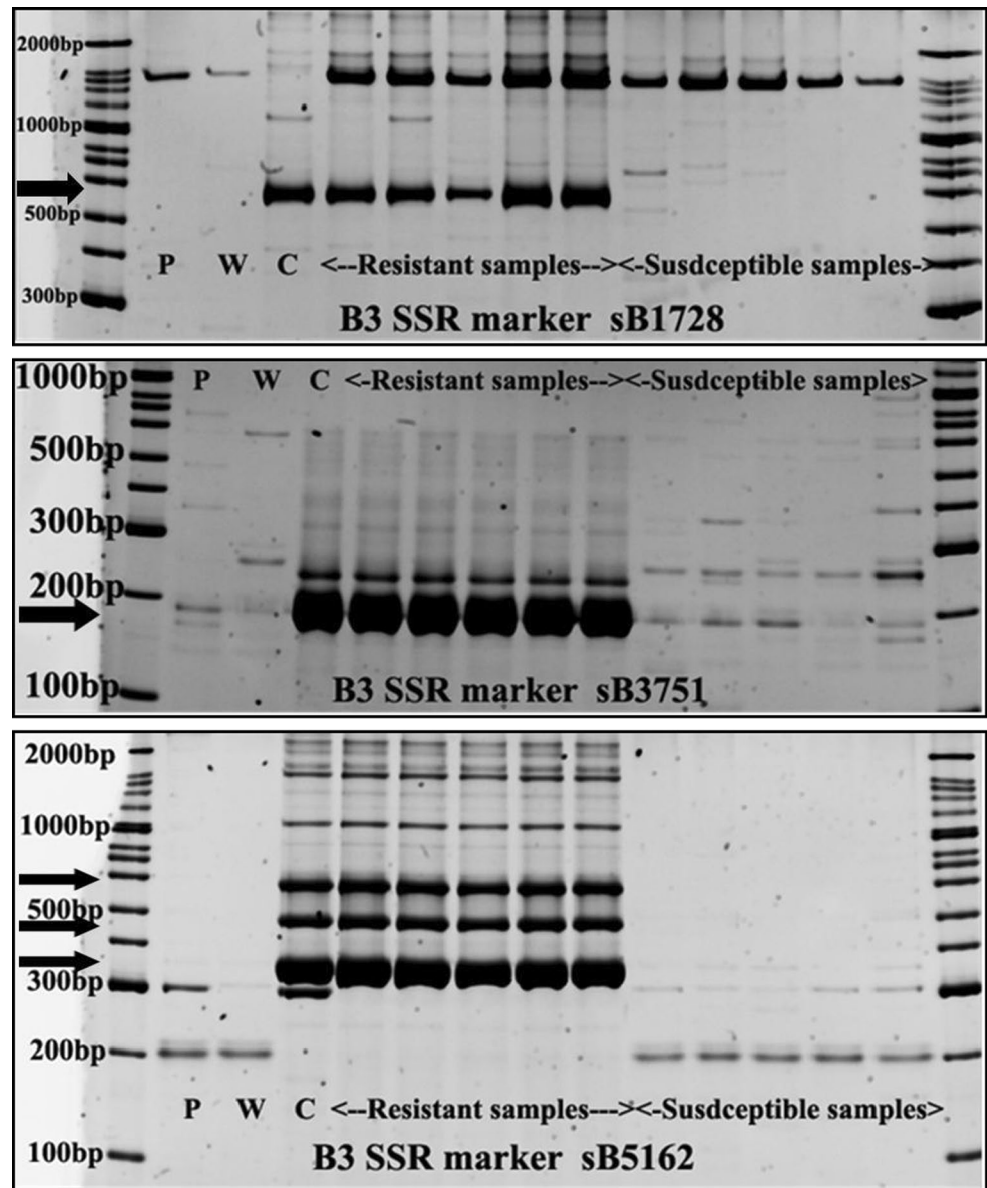
the top of the chromosome was not B-genome-specific but produced a clear band in *B. carinata*. The two markers from the bottom (sA0499F and the telomeric marker sJ1827a) amplified both *B. carinata* and *B napus* giving a similar profile and thus were also not B-genome-specific (Supplementary Material Figure S3). Thus, based on the five B-genome-specific markers (from top to middle) that amplified genomic DNA in the some or all DH1 lines as well as the two markers from the bottom (sA0499F and sJ1827a) that amplified both *B. carinata* and *B. napus*, it can be inferred that the whole B8 chromosome (ca. 453 cM, Navabi et al. 2010) was present in both resistant and susceptible lines, and none of these SSR markers were found to be linked with blackleg resistance. Unlike the DH1 lines, cotyledon resistance in the DH2 lines was found

to co-segregate with three SSR markers (sJ7434, sJ8370a and sJ6846) from the top of the B8 chromosome. The two remaining B-genome-specific B8 markers (sJ3302R and sJ03104) were present in resistant and susceptible plants and so were not associated with blackleg resistance. Thus, in DH2, the top segment of the B8 chromosome was found to co-segregate with cotyledon resistance while the middle to bottom segment of the chromosome did not co-segregate with the resistance (Supplementary Material Table S1 and Figure S3).

Linkage analysis suggested that SSR markers from the top of the B8 and middle to bottom of the B3 chromosome co-segregated in the DH2 population, and these two chromosome segments jointly co-segregated with cotyledon resistance.

Chromosome behaviour and identification of B
chromosomes using GISH and GISH-like technique

Cytogenetic analysis was carried out on two suscepti-
ble DH2 lines and seven resistant DH2 lines. Chromo-
some behaviour at meiosis in pollen mother cells (PMCs)
of the DH2 lines showed that the two susceptible lines
had 40 chromosomes while four and three of the resistant
lines had 40 and 42 chromosomes, respectively (Fig. 4).
In PMCs, we observed mostly bivalents (19.5–20.0 per
cell in $2n = 40$ plants; 20.9 per cell in a $2n = 42$ plant),
and very few univalent (0.21–0.70 per cell) and multiva-
lent (0.04–0.10 per cell). Investigation of mitotic chromo-
somes using GISH and GISH-like techniques showed that
the 40 chromosomes in the susceptible DH2 lines consisted
of two B genome chromosomes (red signals identified by
the *B. nigra* probe fluorescently detected with Texas red
avidin), 18 C genome chromosomes (green signals identi-
fied by the BoB0104O06 BAC clone fluorescently labelled
with Alexa-488) and 20 A genome chromosomes (counter
stained blue with DAPI) (Fig. 5a–c; see also Supplemen-
tary Material Figure S4). On the other hand, GISH/GISH-
like analysis on the seven resistant DH2 lines showed two
different patterns: four lines with a pair of B chromosomes
($2n = 40$) (Fig. 5d–f; see also Supplementary Material Fig-
ure S5) and three lines with two pairs of B chromosomes
($2n = 42$) (Fig. 5g–i; see also Supplementary Material Fig-
ure S6). The A and C genome chromosome content in these
lines were 20 and 18, respectively, the same as the suscepti-
ble lines. Thus, GISH/GISH-like analysis detected one pair
of B genome chromosomes in the susceptible DH2 lines,
while the resistant DH2 lines possessed either one or two
pairs of B chromosomes.

Discussion

In the present study, based on *B. napus* × *B. carinata* inter-
specific hybridization, we provided conclusive evidence
that the B genome chromosome B3 of *B. carinata* carries a
blackleg resistance gene. SSR markers spanning the entire
B3 chromosome were found to co-segregate with cotyledon
resistance in DH1 while five markers covering the middle
to bottom segments of the B3 chromosome were found
to co-segregate with the resistance in DH2. On the other
hand, none of the 360 SSR markers (87 and 273 markers,
respectively) from the *Brassica* A and C genomes used in
this study was found to co-segregate with blackleg disease
resistance and hence blackleg resistance in our interspecific
hybrids progenies is not from endogenous resistance genes
in *B. napus*.

The *Brassica* B genome is known to carry multi-
ple resistance genes (Zhu et al. 1993). Identification and

mapping of these genes has been of interest to researchers
and breeders for stable introgression into *B. napus*. The *Rlm*
genes so far identified to be responsible for blackleg resist-
ance in the B genome include *Rlm5* and *Rlm6* of *B. juncea*
(Balesdent et al. 2002) and *Rlm10* of *B. nigra* (Chèvre et al.
1996, 1997). Chèvre et al. (1996, 1997) used RAPD mark-
ers to demonstrate that cotyledon resistance in *B. nigra*
was located on the B4 chromosome while cotyledon resist-
ance in *B. juncea* is located on the B8 chromosome of their
genetic linkage map. In an RFLP marker study of offspring
from asymmetric hybrids between *B. napus* and the three B
genome species *B. nigra*, *B. juncea* and *B. carinata*, Dix-
elius and Wahlberg (1999) identified four markers co-seg-
regating with adult plant resistance and these were mapped
to three different linkage groups of the B genome. A domi-
nant resistant gene (*LMJR1*) and a recessive resistance gene
(*LMJR2*) associated with blackleg resistance was mapped
to the B7 and B3 chromosomes of *B. juncea* (Christianson
et al. 2006) which corresponds to linkage group G_3 and G_8
chromosomes of *B. nigra*, respectively, based on the con-
sensus linkage map of Lagercrantz and Lydiate (1995) and
Axelsson et al. (2000). It is not possible to compare the
chromosome location of the resistant genes reported in
most of these studies with the present study due to the lack
of common markers or linkage map. The F_1 plants from
our crosses showed no segregation and were all completely
resistant to *L. maculans* isolate #UA3356. Therefore, the
gene on the B3 (middle to bottom) segment that we identi-
fied to be linked to *L. maculans* resistance was dominant
and hence could be different from the recessive gene on the
B3 chromosome identified by Christianson et al. (2006).

Molecular evidence suggests that the three *Brassica*
genomes evolved from a smaller ancestral genome through
extensive chromosome duplication and rearrangements
(Song et al. 1988; Warwick and Black 1991; Lagercrantz
and Lydiate 1996; Lagercrantz 1998; Lysak et al. 2005).
Among the three genomes, the B genome apparently
diverged greatly from the A and C genomes resulting in
little homoeologous pairing between B genome chromo-
somes and either of the related genomes (Attia and Röb-
belen 1986a, b; Attia et al. 1987; Busso et al. 1987; Mason
et al. 2010, 2011). Therefore, the transfer of blackleg dis-
ease resistant gene(s) from the B genome chromosomes
of *B. carinata* (BBCC, $2n = 34$) into *B. napus* genome
(AACC, $2n = 38$) is a difficult task. However, Panjabi et al.
(2008) based on consensus block arrangements of the *Bras-
sica* A (A1–A10), B (B1–B8) and C (C1–C9) genomes
demonstrated that some degree of homoeology between B
genome and A/C genomes exists. Therefore, intergenomic
transfer of genes from the B to the A or C genome can also
occur. Indeed, Chèvre et al. (1997) reported stable intro-
gression of blackleg resistance genes from the B genome of
*B. juncea* into *B. napus*. Using RFLP and RAPD markers,

Plieske et al. (1998) demonstrated that the B genome resistance genes of *B. nigra*, *B. juncea* and *B. carinata* were introgressed at the same location of the *B. napus* genome—apparently due to these three species harbouring the same copy of the resistance gene and sufficient colinearity of the genomic region containing the resistance gene between the B genome and *B. napus*. However, in the present study

two cycles of DH production failed to introgress B genome resistance against *L. maculans* PG2 isolate #UA3356 from *B. carinata* into the *B. napus* genome. Conventional breeding techniques such as backcrossing and pedigree breeding may be applied for introgression of the resistance gene into the *B. napus* genome. These techniques have proved to be effective for introgression of genes from allied species
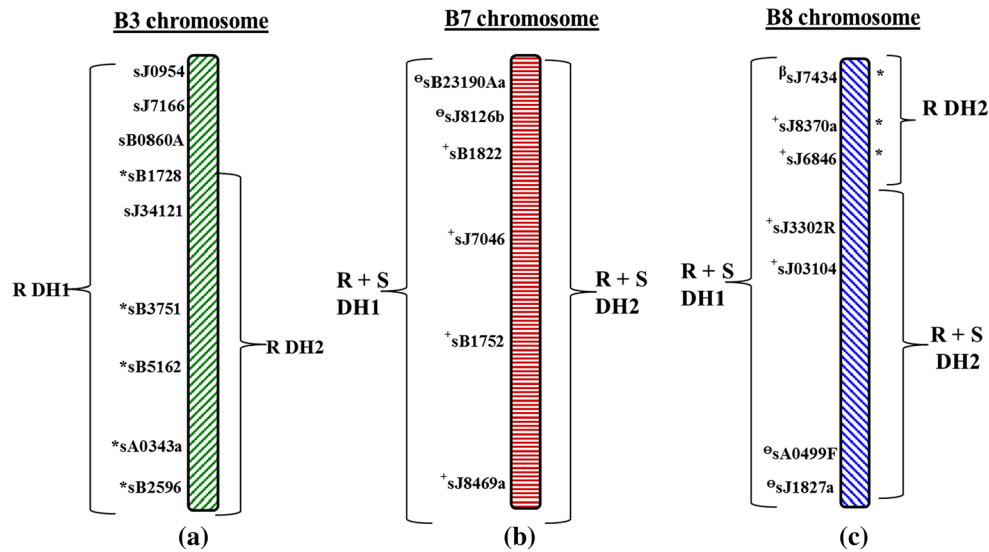

Fig. 3 Summary of genotypic analysis of B genome SSR markers used to study the inheritance of *B. carinata* derived resistance to *L maculans* PG2 isolate #UA3356 in DH1 and DH2 lines derived from *B. napus* × *B. carinata* interspecific cross. **a** The SSR markers from the entire B3 chromosome were present in the resistant (*R*) DH1 lines while markers from only the middle to lower segment were found in resistant DH2 lines. The SSR markers from the B3 chromosome were absent in susceptible (*S*) DH1 and DH2 lines. **b** The SSR markers from the whole B7 chromosome were found in both resistant and susceptible DH1 and DH2 lines. **c** The SSR markers from the entire B8 chromosome were found in the resistant and susceptible DH1 lines as well as resistant DH2 lines, but markers from only middle to bottom were found in the susceptible DH2 lines. *Represents the B genome markers that co-segregate with cotyledon resistance to *L maculans* PG2 isolate #UA3356 in DH2, β represents the markers that amplify both *B. napus* and *B. carinata* but possess alleles that are distinct to the B-genome, + represents the markers that amplify only *B. carinata* but are monomorphic among the resistant and susceptible lines and Θ represents the markers that amplify both *B. napus* and *B. carinata* to give monomorphic bands
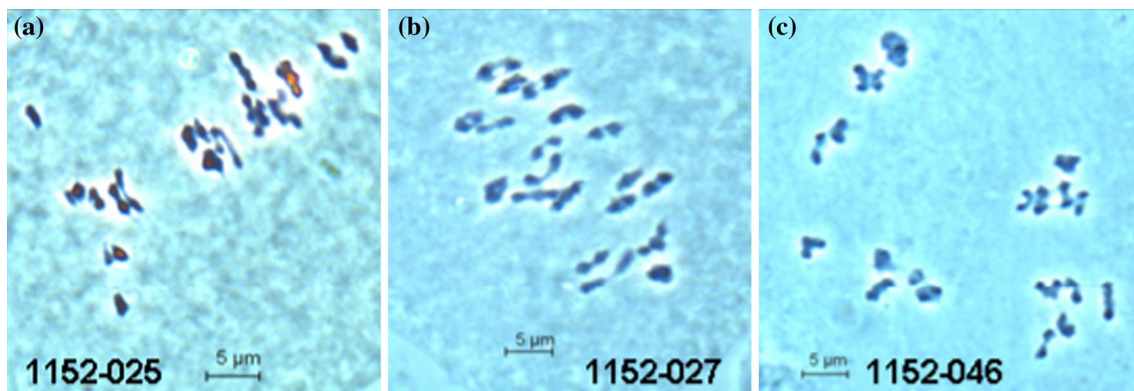
Fig. 4 Chromosome associations at metaphase I of meiosis in pollen mother cells (PMCs). The susceptible lines were observed to have 20 bivalents **a** susceptible DH2 line 1152-025 while resistant DH2 plants had either **b** 20 bivalents (resistant DH2 line 1152-027) or **c** 21 bivalents (resistant DH2 line 1152-046)
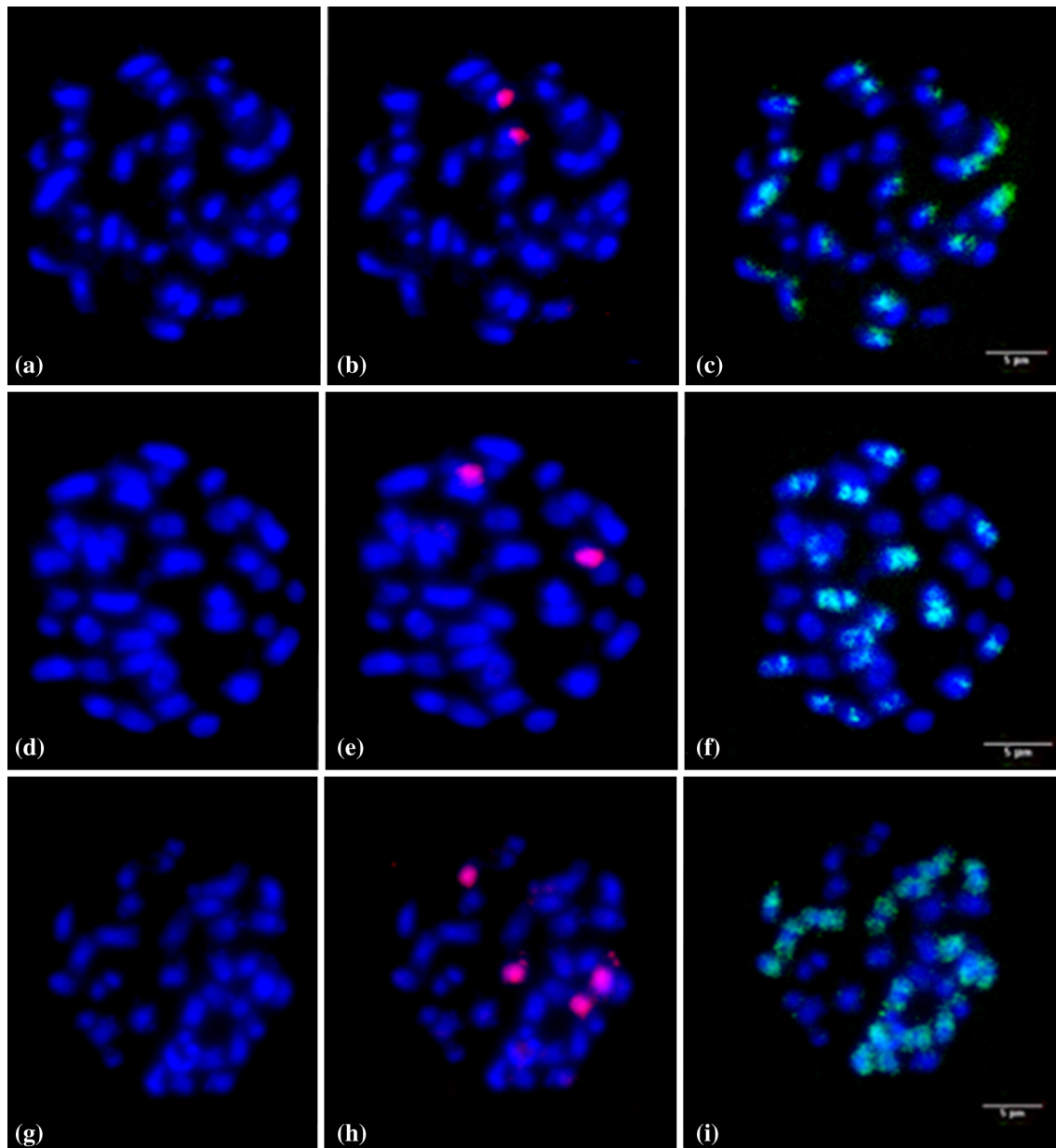
Fig. 5 **a–f** GISH/GISH-like labelling of mitotic chromosome spreads in three DH2 hybrids. Chromosomes painted *red* are from the B genome of *B. carinata*. **a–c** susceptible DH2 line (2*n* = 40), **d–f** resistant DH2 line (2*n* = 40) and **g–i** resistant DH2 line (2*n* = 42). *Bars* 5 μm

such as *Rfo* gene from radish into *B. napus* (Delourme et al. 1991). Also, we are unable to determine if the B genome resistance gene reported in the present study is the same as those identified by Plieske et al. (1998) due to lack of common markers.

Through working with interspecific progenies derived from *B. nigra* × *B. napus*, *B. rapa* × *B. carinata* and *B. oleracea* × *B. juncea* crosses, Struss et al. (1991) found that B genome chromosomes transmit into progeny predominantly through the ovule. In contrast, Chèvre et al. (1991) developed *B. napus*-*B. nigra* disomic chromosome

addition lines for six of the eight *B. nigra* chromosomes. Transmission rate of these alien chromosomes in self-pollinated progeny varied from 50 % to 100 %. This suggests that B genome chromosomes in *B. napus* background can transmit to the progeny through both ovule and pollen, and the rate of transmission can vary depending on the chromosome. In the present study we also found that the B3 chromosome carrying blackleg disease resistance gene was inherited over several generations through both male and female gametes. In addition, almost normal transmission of the B3 chromosome through microspores was found as

evidenced from 1:1 segregation for this chromosome and blackleg disease resistance in the DH population.

In the present study, while applying selection pressure for blackleg resistance at each generation, we found that four chromosomes B1, B3 B7 and B8, were retained as intact or broken chromosomes while the other four B genome chromosomes B2, B4, B5 and B6 were eliminated in the interspecific progenies. Retention of the chromosome B3 may be the consequence of selection for cotyledon resistance as this chromosome is associated with resistance phenotype. Navabi et al. (2010) working with the same *B. napus* × *B. carinata* interspecific progeny and a population derived from another *B. napus* × *B. carinata* interspecific cross (Navabi et al. 2011) also reported that many of the B genome chromosomes (B1, B3, B6, B7 and B8) were retained as either intact or broken chromosomes in interspecific progeny. This is contrary to the general consensus that the B genome chromosomes are very distinct from the A and C chromosomes of *B. napus* and are lost in early generations of interspecific hybrids (Dixelius 1999; Li et al. 2004; Schelfhout et al. 2006).

Cytogenetic analysis was carried out in DH2 and not DH1 lines to investigate the intriguing relationship between the B3, B7 and B8 chromosomes as revealed by the molecular marker work. In the case of the DH1 lines the resistance was clearly attributable to the presence of only the B3 chromosome since all the markers that co-segregated with the resistance were on this chromosome; while only the middle to bottom of this chromosome was present in the resistant DH2 lines (Fig. 3; Supplementary Figure S1). Also, the top of the B8 chromosome was found only in the resistant DH2 lines, and this chromosome segment showed linkage association with the middle to bottom of the B3 chromosome. This suggests that a translocation between the top of the B8 and middle to bottom of the B3 chromosomes might have occurred in the DH2 population.

The meiotic analysis of PMCs from DH2 lines showed that both susceptible and resistant lines possessed more than 38 chromosomes (Figs. 4, 5). The 40 chromosomes observed in susceptible DH2 lines suggest the presence of 20 A^n chromosomes from *B. napus*, 18 $C^{n/c}$ chromosomes from *B. napus* or *B. carinata* and two B^c chromosomes from *B. carinata*. Our molecular marker data suggested that the susceptible lines carried the whole B7 chromosome and a fragment from the middle to bottom of the B8 chromosome (Fig. 3; Supplementary Material Table S1). GISH/GISH-like analysis might have detected only the B7 chromosome, but not the B8 fragment which could be too short to be visualized. In the case of the resistant DH2 lines our molecular marker data suggested that all resistant lines carried a large segment (middle to bottom) of B3, the whole of B7 and the middle to bottom of the B8 chromosome. However, the GISH/GISH-like analysis of the resistant

DH2 lines revealed the presence of one or two pairs of B chromosomes. It is difficult to ascertain the identity of the chromosome(s) detected using GISH/GISH-like techniques in the resistant lines. One of the B chromosomes was apparently B7, the same chromosome detected in the susceptible DH2 lines. The other chromosome detected in the resistant lines with $2n = 42$ could reflect either the B3 or B8. However, because the susceptible lines lacked the B3 chromosome and this chromosome co-segregated with cotyledon resistance, it can be inferred that GISH detected B3 in these lines. For the resistant lines with $2n = 40$ chromosomes, the chromosome detected by GISH could be either B3 or B7, or even B8. The reason why cytological analysis detected only 40 or 42 chromosomes in the resistant DH2 lines when our molecular marker data suggested that these lines carry the B3, B7 and B8 chromosomes, as whole or broken chromosomes, as well as the $2n = 40$ chromosomes detected in the susceptible lines, when our marker data suggested the presence of the B7 and B8 chromosomes is difficult to explain—despite the GISH technique generally being able to detect the A, B and C chromosomes (Navabi et al. 2010, 2011; Mason et al. 2010, 2011). Some chromosomal translocation between the B genome chromosomes or between the B and A/C genome chromosomes might have occurred in these lines. In the latter case, the detection by GISH is size dependent and only experiments with B specific BAC probes of each genomic region could efficiently be used to detect the rearrangements occurring in the plants.

In summary, based on molecular cytogenetic analysis of *B. napus* × *B. carinata* interspecific hybrid progenies through the use of SSR markers and GISH/GISH-like chromosome painting techniques, we demonstrated that the B genome chromosome B3 of *B. carinata* carries a gene conferring resistance at cotyledon stage to *L. maculans* isolate #UA3356. The fact that the B7 and B8 chromosomes were retained in the resistant lines makes it impossible for us to determine whether the inheritance of the B3 chromosome alone is sufficient to confer blackleg disease resistance. The development of DH lines that contain individual B genome chromosomes is needed to understand the mechanism of the resistance. We also demonstrated that a few B genome chromosomes were inherited along with *B. napus* chromosomes for several generations of the interspecific hybrid progenies, as well as transmitted normally through microspores during the production of DH lines. This is contrary to the general consensus that B genome chromosomes are very distinct from the A and C chromosomes of *B. napus* and are easily lost in early generations of interspecific hybrids.

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