Introgression of *Brassica rapa* subsp. *sylvestris* blackleg resistance into *B. napus*

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Abstract

To transfer the blackleg resistance genes, *LepR1* and *LepR2,* from *B. rapa* subsp. *sylvestris* (BRS) into *B. napus*, an inter-specific hybridization between the two species to form allotriploids was made followed by backcrossing to *B. napus*. Analysis of molecular markers in two BC_1 populations, $WT3BC_1$ and $WT4BC_1$ indicated that segregation for BRS and non-BRS alleles on linkage groups N2 and N10, where *LepR1* and *LepR2* respectively are located, fit the ratio 1:1 and recombination frequencies in allotriploid plants were 2-4 times those in the amphidiploid. The number of C-genome chromosomes in the BC_1 plants was determined through marker analysis. Two *L. maculans* isolates, WA51 and pl87-41, which differentiate plants carrying resistance genes, *LepR1* and *LepR2*, were used for resistance evaluation. Surprisingly, only 16.6% of plants were resistant to isolate pl87-41 and 4.0% of plants to WA51 in the $WT3BC_1$, while 8.3% and 9.5% of plants were resistant to the isolates in $WT4BC_1$. A BC_1 plant, WT4-4, which carried *LepR1*, and a BC_2S_2 family WT3-21-25-9, which carried *LepR2*, were identified through analysis of molecular markers and pathology tests. These plants were successively backcrossed with *B. napus* and marker assisted selection was employed in each generation to eliminate non-resistance locus associated BRS genome and to recover the full complement of C-genome chromosomes.

Key words: *Brassica napus*, *Brassica rapa*, *Leptosphaeria maculans*, allotriploid

Introduction

An accession of *B. rapa* subsp. *sylvestris* (BRS) from Sicily was identified with high levels of resistance to *Leptosphaeria maculans* (Mithen et al. 1987). This resistance was transferred into *B. napus* germplasm via development of a synthetic amphidiploid derived from inter-specific hybridization (Crouch et al. 1994). Three resistance genes, *LepR1*, *LepR2* and *LepR3*, which originated from BRS in the Crouch amphidiploid material have been identified in *B. napus* lines. *LepR1* was mapped on *B. napus* linkage group N2 (Yu et al. 2005), *LepR2* and *LepR3* were mapped on N10 (Yu et al. 2004 & 2005). *LepR3* was identified from the *B. napus* cultivars "Surpass 400" and "Hyola 60", while *LepR1* and *LepR2* were found in proprietary *B. napus* breeding lines and are not publicly accessible. To develop AAFC owned *B. napus* lines with the resistance genes, *LepR1* and *LepR2,* we introgressed the R genes from BRS into *B. napus*. Instead of making a re-synthesized amphidiploid as described by Crouch et al. (1994), our approach was to develop materials through inter-specific hybridization between *B. napus* and BRS to produce allotriploid plants and use successive backcrosses with *B. napus* with molecular marker-assisted selection (MAS) and pathology testing.

Information on the transmission of chromosomes in allotriploid derived materials, MAS for progenies from the inter-specific hybridization, disease phenotypic observations and the development of *B. napus* germplasm with resistance genes, *LepR1* and *LepR2,* are presented here**.**

Materials and methods

Plant materials

Seed of the F_1 allotriploid from the cross between a *B. napus* breeding line DH12075 and BRS was kindly provided by Dr. Don Woods at AAFC Crop & Soil Research Section, Alberta. DH12075 is a doubled haploid (DH) line derived from a cross between *B. napus* cultivars Crésor and Westar, (G. Séguin-Schwartz and G. Rakow, AAFC Saskatoon Research Centre). It contains a blackleg resistance gene, *LmR1*, on *B. napus* linkage group N7, originating from "Crésor" (Dion *et al*. 1995; Mayerhofer et al. 2005). N-o-1, a DH line from the *B. napus* cultivar Westar (Sharpe et al. 1995) was pollinated with two allotriploid plants, WT3 and WT4, to produce the first 'backcross' with *B. napus*, namely $WT3BC_1$ and $WT4BC_1$, which consisted of 32 and 66 individuals, respectively. Successive backcrosses with N-o-1 as recurrent parent (female) were performed. Segregation for resistance (R) and susceptibility (S) in each of segregating populations was analyzed with Chi-square tests for goodness of fit (Sokal and Rohlf 1981). Test cross populations, which were made through crosses between a resynthesized *B. napus* line PSA12 (M. Beschorner and D. Lydiate, AAFC Saskatoon Research Centre) with progenies from the allotriploid plants, were used for marker analysis for determining the number of C-genome chromosomes.

The *B. napus* DH lines, AD9 and AD49, which carry *LepR1* and *LepR2* respectively, were kindly provided by Advanta Canada Inc. (Winnipeg, Canada) and used for resistant controls.

Growth conditions, preparation of L. maculans isolates and plant inoculations

Plant growth conditions, preparation of *L. maculans* isolates and plant inoculations were as described previously by Yu et al. (2005). Disease reaction was rated 10-15 days after inoculation (dai) using the 0 to 9 scale described in Williams (1985). Disease ratings of 0-6 were considered as resistant interactions while ratings of 7–9 were considered as susceptible interactions.

Microsatellite analysis

B. napus microsatellite markers with defined loci in the *Brassica napus* A and C genomes have been developed and an AAFC reference microsatellite map has been constructed (D. J. Lydiate and A.G. Sharpe; AAFC Saskatoon Research Centre, Saskatoon). DNA was extracted from young leaves following DNeasy Plant Mini Handbook (QIAGEN). Microsatellite analysis was carried out as described by Naom et al. (1995). Microsatellite alleles were scored as "+" (BRS) and "-" (non-BRS) in the progenies from N-o-1 × allotriploid plants. Genetic distance between marker loci in centi-Morgan (cM) was determined with Mapmaker, version 3.0 (Lander et al. 1987).

Results

Analysis of molecular markers on linkage groups N2 and N10

To identify *LepR1* and *LepR2* in the populations $WT3BC_1$ and $WT4BC_1$, we analyzed plants of N-o-1, DH12075, allotriploids WT3 and WT4 with microsatellite markers on linkage groups N2 and N10 for screening polymorphism. The two BC_1 populations were analyzed with polymorphic markers as shown in Fig 1. Both BC_1 populations segregated for BRS and non-BRS alleles. X^2 analyses indicated that the segregation ratios in all of the markers fit 1:1 in both populations, demonstrating that transmission of the A-genome chromosomes was normal and no distortion in allele segregation occurred.

Genetic distances between markers determined in the two allotriploid BC_1 populations were compared with those in the AAFC reference map that was constructed from an amphidiploid DH population (DH12075 × PAS12). Results (Fig 1) show that recombination events were much more frequent in the allotriploid populations. A region on linkage group N2 from marker CB10172c to CB10416b was 25 cM when mapped in the reference amphidiploid *B. napus* population (Fig 1a). However, the same marker interval was 108 cM when mapped in the $WT3BC_1$ population (Fig 1b) and 92 cM in $WT4BC_1$ population (Fig. 1c), approximately 4 times higher than those in the amphidiploid. Similarly, a 45 cM interval in the amphidipoid reference *B. napus* population, from marker CB10124a to CB10172a on linkage group N10 (Fig. 1d) was 97 cM in both allotriploid populations (Fig. 1e & 1f), 2 times higher than those in the amphidiploid population.

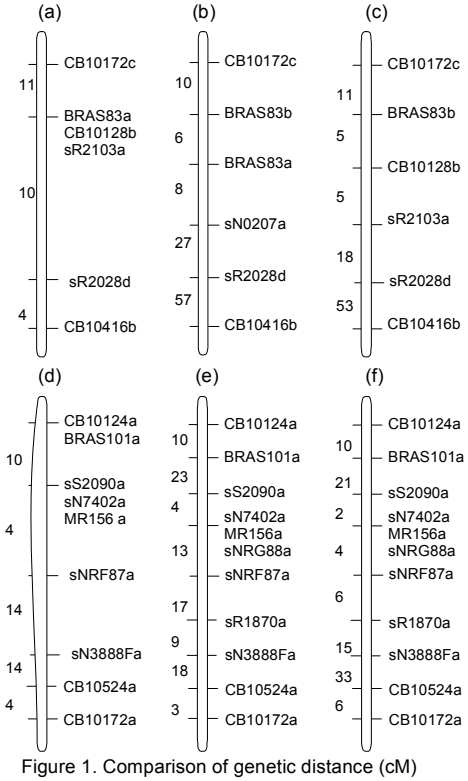

Figure 1. Comparison of genetic distance (cM) between marker loci determined in allotriploid populations with the AAFC reference map of the amphidiploid mapping DH population (DH12075 x PSA12) (a). N2 in amphidiploid, (b).N2 in WT3BC1, (c). N2 in WT4BC1, (d). N10 in amphidiploid, (e). N10 in WT3BC1, (f). N10 in WT4BC1.

Analysis of molecular markers on C-genome chromosomes

C-genome chromosomes in the two BC_1 populations were derived from *B. napus* lines N-o-1 and DH12075. BC_1 plants contained one full set of C-genome chromosomes, which consisted of 9 chromosomes from N-o-1, and a partial set from DH12075 that varied from 0 to 9 chromosomes among individual plants. To estimate the number of C-genome chromosomes donated by DH12075, we analyzed a total of 814 microsatellite markers selected from C- genome linkage groups, N11-N19, of *B. napus* for polymorphism between N-o-1 and DH12075. Only 19 markers, about 2% of the microsatellite loci, 1-3 on each of linkage groups were polymorphic. The low polymorphism is due to DH12075 sharing 50% of its genome with N-o-1. These markers were used to determine the presence of C-genome alleles from DH12075 in each of the BC1 plants. The average number of C-genome chromosomes was 5.9 in $WT3BC_1$ and 5.0 in $WT4BC_1$. These values are higher than the expected value of 4.5, indicating that a high frequency of C-genome transmission from the allotriploid plants to the BC_1 progenies occurred.

Screening for differential isolates of L. maculans *and evaluation of blackleg resistance in cotyledons*

To identify *LepR1* and *LepR2* efficiently in the populations, we screened for isolates that differentiated between disease reaction phenotypes conferred by *LepR1* and *LepR2*, and also eliminate the effect of *LmR1*. Two isolates, pl87-41 and WA51, which give differential reactions on these genes, were identified. Lines with *LepR1* were highly resistant to isolate WA51, but susceptible to isolate pl87-41. Lines with *LepR2* were resistant lines to isolate pl87-41 but were susceptible to isolate WA51. DH12075 carrying *LmR1* was susceptible to both WA51 and pl87-41.

Segregation for R : S to the respective isolates in both populations was expected to be 1:1. However, only 16.6% and 4.0% of 175 plants were resistant to pl87-41 and WA51 respectively in the $WT3BC_1$ population while

8.3% and 9.5% of 85 plants were resistant to the isolates in WT4BC_1 population. X^2 analysis indicated that the segregation ratio did not fit 1:1.

Plants with resistance genes similar to LepR1 *or* LepR2 *were identified*

Five BC_1 plants (Table 1), which exhibited cotyledon resistance to either isolate WA51, pl87-41 or both, and carried BRS molecular markers known to flank *LepR1* or *LepR2* (Fig 1), were chosen to test for cotyledon resistance in advanced generations. A high proportion of susceptible plants was usually observed in BC_2 and BC_1S_1. Relatively higher rates of resistant plants were observed in the BC_1 plants from WT3-21 and WT-4 and these were chosen for further research.

Table 1. Seedling resistance to two isolates of *Leptosphaeria maculans* in backcross and selfed populations from resistant plants identified in BC1 (N-o-1 x allotriploid)

BC1				BC2[4]					BC1S1				
Plant	W/87[1]	Marker[2]	C[3]	%W	%87	%RR	%SS	total	%W	%87	%RR	%SS	total
WT3-18	7/2	R2	3	8.3	8.3	0.0	83.8	12	16.7	0.0	16.7	66.7	12
WT3-21	**3/3**	**R1/R2**	**6**	**5.8**	**19.0**	**8.3**	**66.9**	**121**	**18.5**	**3.2**	**26.6**	**51.6**	**124**
WT4-4	**3/7**	**R1**	**6**	**33.7**	**4.5**	**1.1**	**60.7**	**108**	**70.3**	**3.8**	**0.0**	**25.9**	**41**
WT4-7	9/1	R1	3	4.0	4.0	0.0	92.0	25	18.2	9.1	9.1	63.6	7
WT4-9	7/2	R1/R2	4	0.0	0.0	0.0	100.0	37	0.0	0.0	8.3	91.7	12

[1] Disease ratings, WA51 (W) and pl87-41 (87).
[2] Plants with molecular markers flanking *LepR1* (R1), *LepR2* (R2) or both *LepR1* and *LepR2* (R1/R2).
[3] The number of C-genome chromosomes determined by molecular markers on N11-N19.
[4] The percentage of plants with cotyledon resistance to isolate WA51 (%W), pl87-41(%87), both (%RR), susceptible to both (%SS) and total number of plants tested (Total).

The BC_2 population from N-o-1 × WT4-4, which consisted of 33 R and 12 S plants, was analyzed with polymorphic markers for *B. napus* linkage group N2. Results showed that a resistance gene controlling cotyledon resistance to WA51 was associated with the N2 linkage group and was flanked by microsatellite markers sR2028d and sR12043a (Fig 2a) in the same location as that of *LepR1* on N2 (Yu et al. 2005), strongly suggesting the presence of *LepR1* in this population.

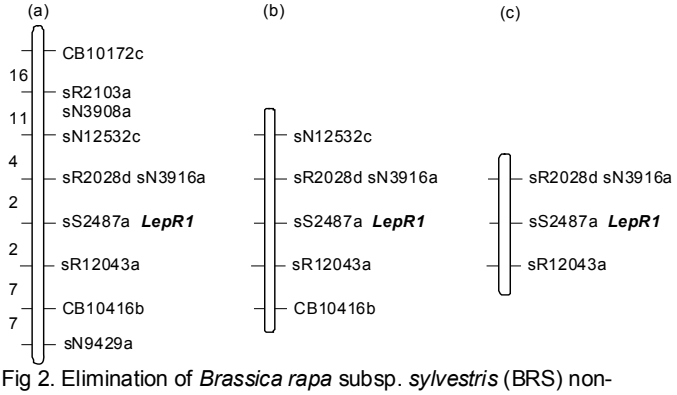

Fig 2. Elimination of *Brassica rapa* subsp. *sylvestris* (BRS) non-resistance gene related genomic background on N2 in plants with *LepR1*. (a). about 50-cM of fragment from BRS in BC_2. (b). 15-cM of fragment from BRS in BC_3, (c). 4-cM of fragment from BRS in BC_4.

The BC_1 plant WT3-21 showed resistance to both isolates and flanking markers for both *LepR1* and *LepR2*. This could indicate the presence of both *LepR1* and *LepR2* or a novel resistance gene(s) that recognised both isolates. To attempt to isolate plants carrying *LepR2* gene only, three BC_2 plants from WT3-21 were selected and self pollinated to produce a BC_2S_1 generation. These plants carried molecular markers associated *LepR2* but not with *LepR1* and were resistant to isolate pl87-41 and susceptible to isolate WA51. Pathology testing with these isolates was continued in the three BC_2S_2 families. One BC_2S_2 population from the BC_2S_1 plant, WT3-21-25-9, consisted of 21 plants and showed similar disease reactions to those in the DH line, AD49, which carries *LepR2*. To confirm the presence of *LepR2* in WT3-21-25-9, a new mapping population was made from BC_2S_2 plants crossed to the susceptible DH line, N-o-1, to produce a F_1 and back crossed with N-o-1 to produce a new back cross population (equivalent to a BC_4 with the allotriploid WT3 using N-o-1 as recurrent parent). Cotyledon resistance to isolate pl87-41 in this population segregated 24 R : 22 S plants and was associated with *B. napus* linkage group N10, flanked by markers sR8548a and sN2551b (Fig 3a). This is the same location as *LepR2* (Yu et al. 2005). These results strongly indicate the presence of *LepR2* in the population.

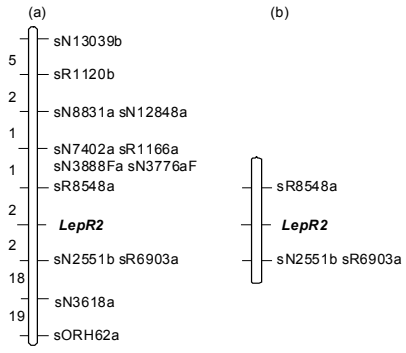

Fig 3. Elimination of *Brassica rapa* subsp. *sylvestris* (BRS) non-resistance gene related genomic background on N10 in plants with *LepR2*. (a). 50-cM of fragment from BRS in BC_4. (b). 4-cM of fragment from BRS in BC_4S_1.

Eliminating the BRS background and recovering C-genome chromosomes

To reduce the BRS genetic background linked to *LepR1* and recover C-genome chromosomes, further back crosses were made with selected breeding lines with *LepR1* to the BC_4 generation. Evaluation for cotyledon resistance to isolate WA51 and analysis of molecular markers on linkage group N2 were carried out in each generation. Two plants that were resistant to isolate WA51 and carried molecular markers either from sR2103a to sN9429a or from CB10172c to CB10416b, were selected for crossing to make the BC_3 generation. Of the 104 resistant plants, ninety-two screened with the molecular markers shown in Fig 2a, and four recombinants, which contained approximately a 15-cM fragment from the BRS on N2 (Fig 2b) were selected for crossing to make BC_4 populations. These four plants were also crossed with a re-synthesized *B. napus* line PSA12 to produce four test cross populations A, B, C and D to determine the number of C-genome chromosomes in the progenies. Three to five almost evenly distributed microsatellite markers on each of the C-genome linkage groups were analyzed. All of the C-genome markers from N-o-1 could be detected in the population A and D, indicating that a full diploid set of C-genome chromosomes were recovered in these populations. Two BC_4 populations derived from the BC_3 plants that were parents of population A and D were selected for further development. To further reduce the BRS genetic background, 184 resistant plants from BC_4 were analyzed with molecular markers shown in Fig 2b. Two recombinant plants were identified with minimal BRS genetic background (about 4-cM of fragment flanking *LepR1* as shown in Fig 2c) and with a full set of C-genome chromosomes.

Molecular markers covering a 50 cM of region from BRS were analyzed in BC_4 plants that carried *LepR2* (Fig 3a). Two plants that only contained the BRS interval from markers sR1120b to sR6903a were crossed with the re-synthesized *B. napus* line PSA12 to produce test cross populations E and F to determine the number of C-genome chromosomes. They were analyzed with C-genome markers. All of the markers from N-o-1 were detected in progenies, indicating that a full diploid set of C-genome chromosomes were recovered in these plants. To further reduce unnecessary BRS background from N10, we screened a BC_4S_1 population, which included 246 homozygous resistant individuals with molecular markers flanking *LepR2*. Of these 246 plants, six plants with minimal BRS genetic background on N10 were obtained (Fig 3b).

Discussion

A lower proportion than expected of plants with resistance to *L. maculans* was found in this study. It is possible that *LepR1* and *LepR2*, which were derived from BRS, may require the presence of some genes or chromosomes from C-genome for complete expression and function in *B. napus*. Although the transmission of C-chromosome was higher than that expected, the majority of the backcross offspring were aneuploid with 0-8 chromosomes from the allotripolid C-genome. Some plants may not have shown resistance associated *LepR1* or *LepR2* due to the absence of the genes or chromosomes required even though the plants carried the R genes.

In this study, allotriploid plants WT3 and WT4 contained unnecessary genetic from *B. rapa* subsp. *sylvestris* and only one set of C-genome chromosomes. BC_1 populations $WT3BC_1$ and $WT4BC_1$ from the *B. napus* × allotriploid showed unexpectedly low proportion of resistant individuals. MAS in combination with traditional breeding methods enabled selection of plants with *LepR1* or *LepR2,* with limited BRS background and a full set of C-genome chromosomes and demonstrated MAS to be efficient for development of lines with desirable genes in plant breeding.

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