Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* × *B. rapa* subsp. *sylvestris*

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Abstract

Two blackleg resistant lines, 16S and 61446, were developed though inter-specific hybridization between *Brassica napus* and *B. rapa* subsp. *sylvestris* and backcrossing to *B. napus*. Classical genetic analysis showed that a single recessive gene in each line contributed to resistance to *L. maculans* and the resistance genes were either allelic or the same. This locus was mapped to *B. napus* linkage group N6, flanked by microsatellite markers sN2189b and sR9571a using a total of 147 plants in BC_1 populations from crosses 61446 × (61446 × PSA12) and 16S × (16S × PSA12). This new resistance gene locus was designated *LepR4*. The two lines were tested for cotyledon resistance to a wide range of isolates and evaluated for stem canker resistance in blackleg nurseries. Results indicated that 16S was highly resistant to most isolates tested and showed a high level of stem canker resistance in the blackleg nurseries. The line 61446 was only resistant to a few isolates and was susceptible in the blackleg nurseries, indicating that these two lines probably carry different resistance alleles at the *LepR4* locus.

Keywords: Brassica napus, Leptosphaeria maculans, *Molecular markers*

Introduction

Blackleg, caused by the dothidiomycete, Leptosphaeria maculans, is one of the most economically important diseases of Brassica napus in Canada, Europe and Australia. Various sources of resistance to L. maculans have been identified and introduced into B. napus. At least 10 race-specific resistance genes including LepR1, LepR2 and LepR3 to L. maculans have been genetically mapped in B. napus (Rimmer, 2006). The three LepR resistance genes, which originated from a wild accession, B. rapa subsp. sylvestris (BRS), have been identified in B. napus materials (Yu et al. 2004 & 2005). LepR1 was mapped on B. napus linkage group N2, and LepR2 and LepR3 map to N10.

To identify new blackleg resistance genes and to transfer them from BRS into *B. napus*, an inter-specific hybridization was made between *B. napus* and the BRS followed by successive backcrossing with *B. napus* followed by inbreeding. Here, we report that a novel blackleg resistance locus *LepR4* was identified from the progenies of *B. napus* × BRS.

Materials and methods

Plant materials

The materials, $WT3BC_1$ and $WT4BC_1$, derived from blackleg susceptible, *B. napus* line N-o-1 × allotriploid plants WT3 and WT4 respectively, were described previously (Yu et al. 2007). Selected plants derived from two BC_1 plants, WT3-21 (from the population $WT3BC_1$) and WT4-16 (from the population $WT4BC_1$), and selected progeny from backcrossing to N-o-1 or selfing were used to identify novel blackleg resistance genes. This resulted in the development of two resistant lines 61146 and 16S.

A resynthesized *B. napus* line, PSA12, (M. Beschorner and D. Lydiate, AAFC Saskatoon Research Centre), was crossed to the resistant lines, 61446 and 16S, and the resulting F_1 plants were backcrossed to the respective resistant parent, either 61446 or 16S, to produce the first backcross (BC_1). Test crosses between 61446 and 16S were also made. The parents, F_1, F_2 and BC_1 or test cross plant populations were inoculated with isolates of *L.* maculans to determine cotyledon disease reaction phenotypes. Segregation for resistance (R) and susceptibility (S) in the F_2 and BC_1 or test cross generations was analyzed with Chi-square tests for goodness of fit (Sokal and Rohlf 1981).

Two *B. napus* doubled haploid lines, AD9 and AD49, previously shown to carry *LepR1* and *LepR2* respectively (Yu *et al*. 2005), 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 are considered resistant interactions while ratings of 7–9 were considered as susceptible interactions.

Evaluation of blackleg resistance under field conditions

Blackleg resistance was evaluated in disease nurseries in Wagga Wagga, New South Wales and Horsham, Victoria, Australia in 2003. A randomized design with three replicates was used. Single rows were sown into blackleg nurseries with infected canola stubble to determine the degree of resistance to blackleg. Plant numbers were counted soon after plant

emergence and again at maturity to determine the percentage plant survival.

Microsatellite analysis

B. napus microsatellite primer pairs with defined loci in the *Brassica napus* A and C genomes (developed by D. J. Lydiate and A.G. Sharpe; AAFC Saskatoon Research Centre, Saskatoon) were used for mapping. DNA was extracted from young leaves following procedures in the DNeasy Plant Mini Handbook (QIAGEN). Microsatellite marker analysis was carried out as described by Naom et al. (1995). Microsatellite alleles were scored as "+" (BRS) and "-" (non-BRS). Genetic distances between marker loci in centi-Morgan (cM) were determined with Mapmaker, version 3.0 using Kosambi's mapping function (Lander et al. 1987).

Results

Resistant plants were identified in the progenies of Brassica napus × BRS

The BC_1 plant WT3-21 was resistant to isolates WA51 and pl87-41, which differentiate between breeding lines carrying LepR1 and LepR2 (Yu et al. 2007). Successive backcrosses with N-o-1 were performed and plants that were resistance to these two isolates were selected in each generation. Seven resistant BC_2 plants were back crossed again with N-o-1 to produce BC_3 families. Four phenotypes, resistant to WA51, resistant to pl87-41, resistant to both isolates, and susceptible to both isolates were observed in BC_3 families. No resistant plants were found in the families 6-01, 6-02 and 6-11 in BC3 as shown in Table 1. Plants from family 6-14 showed that 7.1% of plants were resistant to both isolates (Table 1). Two resistant plants were self-pollinated to produce BC_3S_1 families 6-14-4 and 6-14-5. Ten and three plants were resistant to both isolates out of 44 and 36 plants in 6-14-4 and 6-14-5 populations respectively. Six resistant plants from the family 6-14-4 were self-pollinated to produce BC_3S_2. Cotyledon resistance was further evaluated in BC_3S_2. One BC_3S_2 family, 6-14-4-6, herein designated as 61446 showed 100% of plants resistant to both isolates (Table 1).

Table 1. Cotyledon resistance to *L. maculans* in the progenies of BC1 plants WT3-21 and WT4-16

Family	No. of plants[1]					Generation
	WA	87	RR	SS	%RR	
6-01	0	0	0	39	0.0	BC3
6-02	0	0	0	45	0.0	BC3
6-11	0	0	0	48	0.0	BC3
6-12	1	1	0	26	0.0	BC3
6-14	**7**	**6**	**3**	**26**	**7.1**	**BC3**
6-15	3	1	0	26	0.0	BC3
6-16	3	1	1	28	3.0	BC3
6-14-4	**1**	**0**	**10**	**33**	**30.0**	**BC3S1**
6-14-5	3	4	3	26	11.5	BC3S1
6-14-4-1	2	1	19	1	82.6	BC3S2
6-14-4-2	2	0	14	0	87.5	BC3S2
6-14-4-3	5	0	5	2	41.7	BC3S2
6-14-4-4	4	0	12	2	66.7	BC3S2
6-14-4-6	**0**	**0**	**20**	**0**	**100.0**	**BC3S2**
6-14-4-7	4	0	11	0	73.3	BC3S2
WT4-16	4	6	4	17	12.9	BC1S1
16S-2	**0**	**0**	**20**	**0**	**100.0**	**BC1S2**
16S-3	0	0	8	4	66.7	BC1S2

[1]WA: resistant to WA51; 87: resistant to pl87-41
RR: resistant to both isolates; SS: susceptible to both isolates

Similar to WT3-21, a BC1 plant WT4-16 was resistant to both isolates. Four out of 31 plants in the BC_1S_1 were resistant to the both isolates. Two resistant plants produced sufficient seed to evaluate resistance in BC_1S_2. All of the plants tested in BC_2S_2 family, 16s-2 (herein designated as 16S) were resistant to both isolates (Table 1).

The lines 61446 (BC_3S_2) and 16S (BC_1S_2) were non-segregating for resistance to L. maculans. However, their parental lines, 6-14-4 and WT4-16, did segregate for resistance and susceptibility, with more than 60% of plants susceptible to the both isolates (Table 1). This suggested that the gene(s) responsible for resistance in the lines were recessive.

A single locus control resistance in 61446 and 16S

Resistant lines 61446 and 16S were used to develop new populations to study the inheritance of cotyledon resistance. Crosses were made between the susceptible line PSA12 and the resistant lines, and between 61446 and 16S. Cotyledon disease reaction phenotype was then determined in F_1, F_2, backcross (BC_1) populations or in testcross progenies after inoculation with isolate WA51.

The average disease ratings of susceptible (PSA12) and resistant (61446 and 16S) lines were 8.5, 2.3 and 1.5 respectively. F_1 plants from the crosses PSA12 × 61446 and PSA12 × 16S were all susceptible with mean disease ratings of 7.2 and 8.0 respectively, similar to the susceptible parent PSA12. Analysis of segregation for resistance and susceptibility in the F_2 and BC_1 populations fitted ratios of 1:3 in F_2 and 1:1 in BC_1. This indicated that a single recessive allele was associated with cotyledon resistance in both resistant lines 61446 and 16S.

For the test cross between 61446 and 16S, all F_1 progeny were resistant, indicating that the recessive resistant alleles in the two lines are either allelic or the same, and the resistance gene locus was designated as *LepR4*.

Resistance specificity

Cotyledon resistance in 61446 and 16S was characterized further with L. maculans isolates 2354, pl86-12, OMR1:1, WA74, WA30, Lifolle5, 99-43, 99-56 and 99-79, originating from various countries and isolated from a range of different Brassica species. 16S was resistant to all of the isolates tested with disease ratings ranging from 1.7 to 3.2 at 15 days after inoculation. However, the line 61446 was only highly resistant to isolates 2354, pl86-12 and WA30 with disease ratings of 2.6-3.3. 61446 gave an intermediate level of resistance to isolates WA74, Lifolle5 and 99-43 with disease ratings of 4.3-6.5 and it was susceptible to isolates OMR1:1, 99-56 and 99-79 with disease ratings of 7.0-8.9.

The two lines were evaluated for stem canker resistance in blackleg nurseries in Australia. In Wagga Wagga, New South Wales, 16S showed strong blackleg resistance. All plants survived until the end of the growing season. In contrast, 97% of Westar plants were dead. 61446 showed much weaker resistance than 16S and only 20% of plants survived. There was a higher disease pressure in the blackleg nursery in Horsham, Victoria. More than 40% of plants in 16S survived, which was comparable to most of the Australian resistant cultivars in the trial. However, only 7% of plants in 61446 survived.

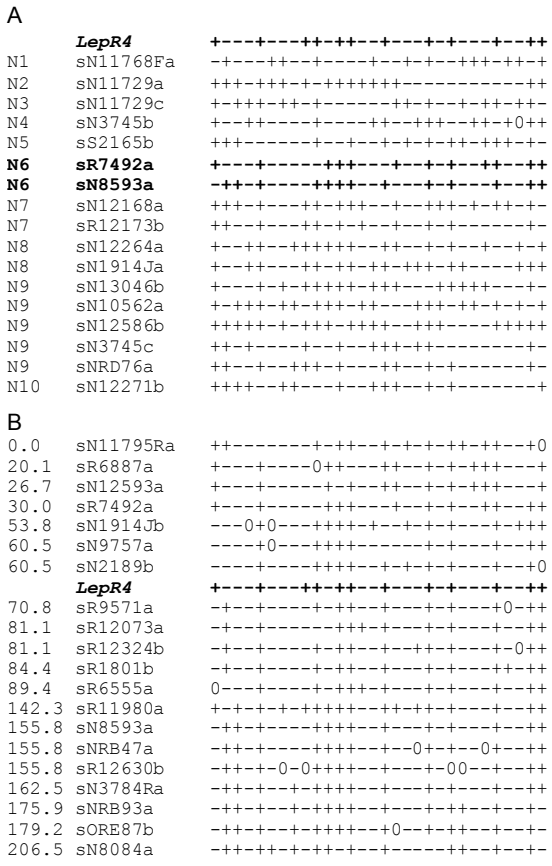

Fig 1. The population 61446 x (PSA12 x 61446) analyzed with microsatellite markers on B. napus linkage group N1-N10. "+": allele from 61446; "-": allele from PSA12. Plants tested with isolate WA51. A. 1-5 polymorphic markers on each of A-genome linkage groups. B. polymorphic markers on linkage group N6. Numbers indicate genetic distance in centi-Morgan (cM) from a reference map (D. J. Lydiate and A.G. Sharpe, unpublished data)

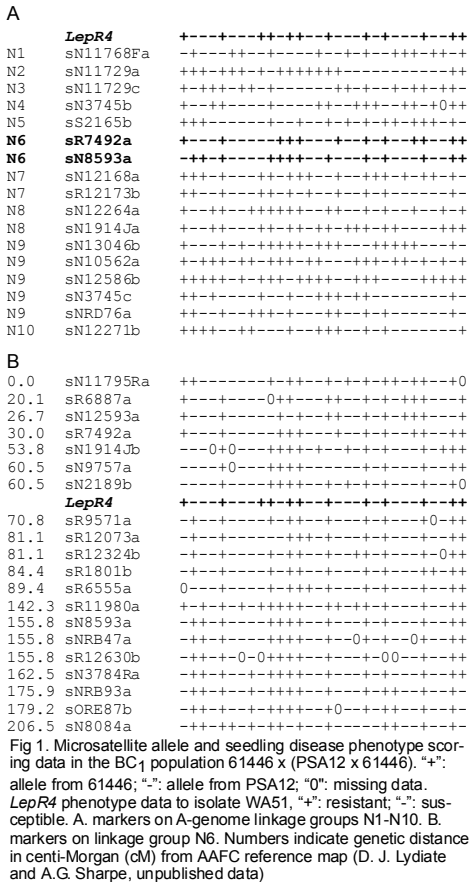

Fig 1. Microsatellite allele and seedling disease phenotype scoring data in the BC_1 population 61446 x (PSA12 x 61446). "+": allele from 61446; "-": allele from PSA12; "0": missing data. *LepR4* phenotype data to isolate WA51, "+": resistant; "-": susceptible. A. markers on A-genome linkage groups N1-N10. B. markers on linkage group N6. Numbers indicate genetic distance in centi-Morgan (cM) from AAFC reference map (D. J. Lydiate and A.G. Sharpe, unpublished data)

Mapping of the resistance locus

Mapping of the resistance gene locus *LepR4* was initiated using microsatellite markers by assaying 30 individuals in a BC_1 population derived from 61446 × (PSA12 × 61446). Plants were tested for cotyledon reaction phenotype to isolate WA51 in the BC_1 and the phenotype confirmed in BC_1S_1. Primer pairs were chosen from the A-genome linkage groups N1-N10. Of 293 markers, 124 were polymorphic between PSA12 and 61446. The BC_1 population was analyzed with 1-5 robust polymorphic markers on each of A-genome linkage groups. Results (Fig 1a) indicated that cotyledon resistance to WA51 in this population was not associated with the markers on linkage groups N1-N5 and N7-N10, but was associated with markers sR7492a and sN8593a on linkage group N6. Polymorphic markers covering the whole of linkage group N6 were further analyzed in the population. The most probable position of *LepR4* on N6 is between microsatellite markers sN2189b and sR9571a (Fig 1b).

To confirm the LepR4 location on linkage group N6, a BC_1 population, which consisted of 117 individuals from 16S × (PSA12 × 16S), was analyzed with microsatellite markers. As previous results had indicated that the resistance alleles in lines 61446 and 16S were allelic, microsatellite markers on linkage group N6 were screened for polymorphism between the resistant parental line 16S and the susceptible parent PSA12. Twenty out of 52 markers showed polymorphism between the parents. The resistance allele in LepR4 locus from resistant line16S was mapped to the same location as that in 61446, flanked by markers sN2189b and sR9571a, in an interval of 17.1 cM, corresponding to a 10.3 cM interval in the AAFC reference map (Fig 2).

Segregation for resistance and susceptibility occurred in BC_1S_1 families that were resistant in BC_1

As the LepR4 resistant alleles are recessive, self-pollinated progenies in

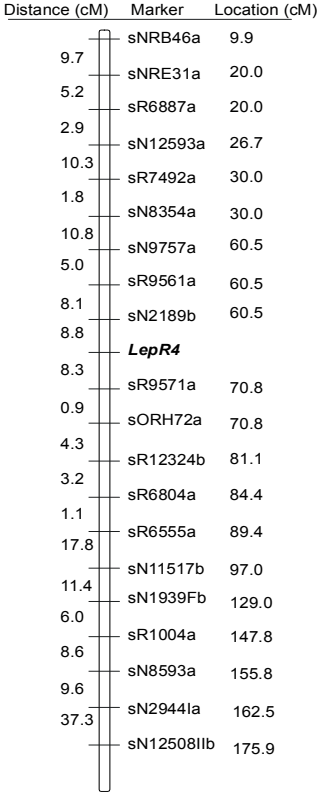

Fig 2. *LepR4* location on *B. napus* linkage group N6. The numbers on the left are genetic distance (cM) determined in the population 16S x (PAS12 x 16S). The numbers on the right indicate marker location (cM) in the reference map (D. J. Lydiate and A.G. Sharpe, unpublished data)

BC_1S_1 from resistant BC_1 plants were expected to be fixed for resistance and non-segregating. However, 7-38% of plants were found to be susceptible to isolate WA51 in about 50% of the BC_1S_1 families that were resistant in BC_1.

Discussion

A small proportion of resistant plants to isolates of *L. maculans* were usually found in the progenies of *B. napus* × BRS (Yu, et al 2007). Two selected lines,

61446 and 16S, showed 100% of plants resistant to two test isolates of *L. maculans*. Genetic analysis, molecular mapping, and pathology testing provided evidence that resistance in these two lines was recessive and perhaps was conditioned by different alleles at the *LepR4* locus. The line 16S (*LepR4b*) is more effective against a range of isolates of *L. maculans* than that in the line 61446 (*LepR4a*). The resistance gene locus, *LepR4*, was mapped on *B. napus* linkage group N6.

Resistance genes *LepR1*, *LepR2* and *LepR3*, originating from *B. rapa subsp. sylvestris* that were identified in previous studies all exert dominate or incomplete dominate resistance. They were mapped on linkage group N2 or N10 (Yu et al. 2004, 2005). Delourme et al. (2004) reported that five blackleg resistance genes *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* cluster on *B. napus* linkage group N7 within a 35 cM interval and the gene *Rlm2* is located on N10. Gene-for-gene interactions between the *Rlm* genes in *B. napus* and avirulence genes *in L. maculans* have been characterized (Ansan-Melayah et al. 1998; Balesdent et al. 2002), implying that these *Rlm* genes are dominant. However, the resistance alleles at the *LepR4* locus identified here is recessive. Recessive blackleg resistance genes *rjlm2* and *LMJR2* from the B-genome were reported recently (Christianson et al. 2006; Saal et al. 2004).

Segregation for resistance and susceptibility was observed in about 50% of the BC_1S_1 families derived from BC_1 plants that showed a resistance phenotype in both 61446 × (PSA12 × 61446) and 16S × (PAS12 × 61446) populations. This suggests that, for these BC_1S_1 families, resistance to L. maculans associated with resistance at the LepR4 locus was not fixed and probably requires interaction with another allele at a different locus that was heterozygous in their parental BC_1 plants. This hypothesis is currently under investigation.

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