Development of a SCAR Marker to Track Canola Resistance Against Blackleg Caused by *Leptosphaeria maculans* Pathogenicity Group 3

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ABSTRACT

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Blackleg of rapeseed and canola (*Brassica napus*) is caused by various pathogenicity groups (PG) of *Leptosphaeria maculans*. The disease occurring in the Canadian prairies for the last two decades was caused by PG2 and was controlled by host resistance. PG3 and PG4 isolates have been found recently in Canada, but there is no resistance available against these pathogenicity groups in commercial Canadian varieties. This study sought to identify canola cultivars that could be used as sources of resistance to PG3 and to develop molecular markers for marker-assisted selection. Resistance to PG3 specifically was found in *B. napus* 'Dunkeld' and 'Quinta', while *B. juncea* 'Cutlass' and 'Domo' proved to be resistant to PG2, PG3, and PG4. A set of F_2 progeny of 'Westar' (susceptible) × 'Dunkeld' was used to identify genetic markers linked to PG3 resistance. These markers were physically located on a BAC clone from *B. rapa* subsp. *pekinensis* containing a homolog to a serine threonine 20 (ste20)-like kinase in *Arabidopsis thaliana*. Thus, we have developed a sequence characterized amplified region (SCAR) marker available for marker-assisted selection in breeding canola for resistance against blackleg caused by *L. maculans* PG3. This work has received a provisional patent (serial # 60/977,933 – Oct. 5, 2007).

Additional keyword: SRAP

Blackleg (Phoma stem canker) of rapeseed and canola (*Brassica napus* L.) is a disease with a worldwide distribution caused by a complex of fungal plant pathogens belonging to *Leptosphaeria maculans* (Desm.) Ces. & Not., anamorph *Phoma lingam* (Tode ex Fr.) Desm. Members of this species historically were divided into two pathotype groups, A and B (also A and NA groups, or Tox^+ and Tox^0 groups), based on characters such as virulence, restriction fragment length polymorphism (RFLP) markers, and secretion of the phytotoxin sirodesmin into culture media (48,49). Group A isolates are highly virulent and cause the damaging stem canker, while B group isolates are weakly virulent (59). Since group B isolates are morphologically different from and cannot sexually cross with group A isolates, they have been recently classified as a new species, *L. biglobosa* R.A. Shoemaker & H. Brun (53). Group A isolates are distributed worldwide and cause significant losses in Australia, North America, and Europe (56). They have been subdivided into pathogenicity groups (PG) 2, 3, 4, and T according to differential disease reactions on a set of *B. napus* cultivars with different resistance genes (23,24,36,45). The subspecies structure of *L. maculans* is made even more complex by the fact that each of the four pathogenicity groups also comprises different races as revealed by analysis conducted with two differential sets (7).

Although restricted to the crucifers, *L. maculans* can infect various species under field and laboratory conditions (10,27). The host and the pathogen establish typical gene-for-gene interactions (20,44,57). The outcome of the host infection is dependent on the presence of a major resistance gene in the host and a corresponding avirulence gene in the pathogen (4–6). Resistance genes have been traced in various *Brassica* species such as *B. juncea*, *B. napus*, *B. rapa*, and *B. nigra* (15,16,42,51,52,54,60). Selection for blackleg resistance relies on cotyledon and adult stage resistance (47). Several authors have reported a significant correlation between resistance in cotyledons and adult plants (8,24,26,35,39). Loci controlling cotyledon resistance as well as field resistance against *L. maculans* have been mapped to the N7 linkage group of the A genome in various *B. napus* genotypes (18,20,33,34,43,46). Molecular markers associated with *Brassica* resistance against blackleg caused by PG2 and useful for maker-assisted selection have also been reported (3).

Blackleg of canola, which had been prevalent in the Canadian prairies for the last two decades, was caused by *L. maculans* PG2 and is well controlled by resistance. In 2002 and 2004, PG3 and PG4 isolates of *L. maculans* were discovered in Manitoba (13,19). Since then, PG3 and PG4 also have been found in Alberta, North Dakota (USA), and more regions in Manitoba (11,14). These new strains of *L. maculans* pose a serious threat to the Prairie canola industry because varieties currently grown are not resistant against them. The objectives of this study were to search for sources of resistance against blackleg of canola caused by *L. maculans* PG3 and to develop molecular markers for resistance-assisted selection.

MATERIALS AND METHODS

Search for sources of resistance. Ten entries from the University of Manitoba canola and *Brassica* germplasm bank were screened for resistance against isolates of *L. maculans* PG2, PG3, and PG4 from our laboratory fungal collection. They were preselected based on their descriptions in terms of resistance against blackleg with a bias toward *B. napus* genotypes from Europe and Australia where PG3 and PG4 had been previously reported (58). Differential canola cultivars Westar, Glacier, and Quinta were used as references (23,36). Cultivars screened for resistance included *B. napus* 'Crésor', 'Dunkeld', 'Oscar', 'Rainbow', and 'Surpass 400', as well as *B. juncea* 'Cutlass' and 'Domo'. The cultivars were evaluated in a completely randomized experiment in the growth chamber with three replicate flats of each genotype per pathogen isolate. Genotypes were grown from seed (one per 6 × 6 × 7 cm flat cell) in a soilless mix (Metromix, W.R. Grace and Co. Ltd., Ajax, Ont.). Seedlings were watered daily and kept under day and night temperatures of 21°C and 18°C, respectively, and a 16-h photoperiod. Seven days after seeding, eight fully expanding cotyledons were wounded on each lobe with a needle and inoculated with 10 µl of spore suspension onto the wound. The inoculated cotyledons were allowed to dry at room temperature for at least 12 h before the next watering. Disease symptoms were rated 12 days after inoculation using a scale of 0 to 9 (14,57): 0, no darkening around the wounds; 1, limited blackening around the wound with a lesion diameter of 0.5 to 1.5 mm, a faint

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chlorotic halo may be present, sporulation absent; 3, dark necrotic lesions, a chlorotic halo of 1.5 to 3.0 mm in diameter may be present, sporulation absent; 5, lesions 3 to 5 mm in diameter and sharply delimited by a dark necrotic margin, may show gray-green tissue collapse, sporulation absent; 7, gray-green tissue collapse, lesion 3 to 5 mm in diameter, sharply delimited by a nondarkened margin; 9, rapid tissue collapse at about 10 days accompanied by profuse sporulation in large lesions, more than 5 mm diameter, with a diffuse margin.

Mapping populations. Based on results from the resistance screening experiment, *B. napus* cultivar Dunkeld was used as a resistant parent in reciprocal crosses with the susceptible cultivar Westar. F_1 progeny from a 'Westar' female parent were evaluated for cotyledon resistance and self-pollinated for the production of F_2 progeny. Segregation of resistance against blackleg caused by *L. maculans* PG3 in the F_2 generation was analyzed at the cotyledon stage, with 'Westar', 'Glacier', and 'Quinta' being included as references. Progeny that exhibited a severity rating of 5 or less on the 0 to 9 rating scale were considered resistant, while those with a higher score were considered susceptible.

DNA isolation and SRAP assays. Leaf and stem tissues from 340 F_2 progeny were collected, freeze dried, ground, and used for genomic DNA isolation with a salt buffer based extraction protocol (1). Bulked segregant analysis (BSA, 37) was used together with sequence-related amplified polymorphisms (SRAP, 28) to search for markers polymorphic among the progeny and to identify those linked to canola resistance against *L. maculans* PG3 from 'Dunkeld'. A 22 sample set of genomic DNA from 'Westar' and 'Dunkeld' parents, 10 susceptible progeny, and 10 resistant progeny, along with two bulk samples, one from resistant and the other from susceptible progeny, were used for screening 180 primer combinations. Ten primer sets that had amplified markers that were polymorphic between parents and among the progeny samples were used to analyze marker segregation within a 92-progeny mapping population. These primer sets are: BG_{16}/ODD_3, PM_{111}/ODD_3, BG_{18}/ODD_{20}, BG_{20}/SA_{12}, EM_2/ME_2, ME_2/DC_1, $Na_{12}A_{02}F$/$Na_{12}A_{02}R$, $NA_{12}D_{04}F$/$NA_{12}D_{04}R$, $Na_{12}F_{12}F$/$Na_{12}F_{12}R$, and $NGA_{111}F$/$NGA_{111}R$ (Table 1). The segregation of two SRAP markers amplified by $Na_{12}A_{02}F$/$Na_{12}A_{02}R$ primer set that were linked to resistance from 'Dunkeld' was confirmed in an additional 248 progeny sample set. Polymerase chain reaction (PCR) amplification reactions were performed in a 13-µl reaction volume containing 15 ng of template DNA, 0.4 µM for each of two primers, 0.75 units of *Taq* polymerase (Fisher brand), 100 mM Tris-HCl (pH 8.0), 500 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM each of dNTPs. PCR amplification was run in a programmable thermal controller (Eppendorf MasterCycler Gradient, Eppendorf Canada Ltd., Mississauga, ON). The first five cycles were run at 94°C for 1 min, 35°C for 50 s, and 72°C for 1 min for denaturing, annealing, and extension, respectively. Then the remainder of the amplification was 36 cycles at 94°C for 50 s, 50°C for 50 s, and 72°C for 1 min. PCR products were separated by electrophoresis using a denaturing 5% polyacrylamide gel containing 7.5 M urea. Gels were then silver stained with the Promega kit (Promega, Madison, WI, USA) according to the manufacturer's specifications. PCR assays were replicated once in order to confirm observed markers. The presence and absence of all fragments between molecular sizes of 50 and 500 base pairs (bp) were scored for each sample. Bands larger than 500 bp or less than 50 bp were not scored because of insufficient resolution.

Table 1. Characteristics of primers used in this study

Primer name	Primer sequence (5′-3′)	Source
BG_{16}	TGATACCACTTGCGATACCA	G. Li[a]
BG_{18}	GCAAGTCTCTCAGGTTATTC	G. Li
BG_{20}	TCCTCTCCACTTTTGTCTTC	G. Li
DC_1	TAAACAATGGCTACTCAAG	G. Li
EM_2	GACTGCGTACGAATTCTG C	G. Li
ME_2	TGAGTCCAAACCGGAGC	G. Li
$Na_{12}A_{02}F$	AGCCTTGTTGCTTTTCAACG	M. Trick[b]
$Na_{12}A_{02}R$	AGTGAATCGATGATCTCGCC	M. Trick
$Na_{12}D_{04}F$	ACGGAGTGATGATGGGTCTC	M. Trick
$Na_{12}D_{04}R$	CCTCAATGAAACTGAAATATGTGTG	M. Trick
$Na_{12}F_{12}F$	CGTTCTCACCTCCGATAAGC	M. Trick
$Na_{12}F_{12}R$	TCCGATGTAGAATCAGCAGC	M. Trick
$NGA_{111}F$	TGTTTTTTAGGACAAATGGCG	M. Trick
$NGA_{111}R$	CTCCAGTTGGAAGCTAAAGGG	M. Trick
ODD_3	CCAAAACCTAAAACCAGGA	G. Li
PM_{111}	CTTTAGCAGCATTGTTACCA	G. Li
SA_{12}	TTCTAGGTAATCCAACAACA	G. Li
BN204F	GGTGCAAACGATGTATTCAAGA	This study
BN204R	CGTTTGTAAAACCGACCTTCA	This study

[a] G. Li, University of Manitoba, Winnipeg, Manitoba, Canada.
[b] M. Trick, BrassicaDB, John Innes Centre, Norwich, UK.

DNA sequencing and SCAR development. DNA bands of SRAP markers linked to resistance were collected from polyacrylamide gels, ground in 50 µl of Tris EDTA, and incubated overnight at 4°C in order to isolate DNA fragments. These fragments were used as template for PCR assays with the corresponding original SRAP primers. The PCR mixture composition used was similar to the one used in the SRAP assays except that cycling protocol was as follows: initial denaturation at 95°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 20 s, and 72°C for 1 min; and a final DNA extension at 72°C for 6 min. PCR products were purified using the Qiaquick PCR purification kit according to the producer specifications (Qiagen Inc., Mississauga, ON, Canada). Purified PCR products were sent for sequencing at Macrogen DNA Sequencing Service (Macrogen USA, Rockville, MD). The software ClustalX (55) was used for sequence alignment, and sequence alignments were edited with the Genedoc software (40). Similarity search with public library sequences was performed with BLASTn and BLASTx (2). A GenBank library sequence with a genomic region similar to our query was used to design new primers in order to develop sequence characterized amplified region (SCAR) markers linked to resistance in 'Dunkeld' against *L. maculans* PG3. These new primers were used to amplify genomic DNA from 'Westar' and 'Dunkeld'. The segregation of polymorphic markers was then analyzed with the 22-progeny sample set used for screening of SRAP primer combinations. Primer sets with markers that segregated as expected were used in analyzing the 92-progeny sample set for linkage mapping. The markers were later confirmed using the additional 248-progeny sample set.

Data analysis. Analysis of variance was performed on data from the search for sources of resistance experiment, and disease severity means were compared using Tukey's test. In the analysis of F_2 progeny from crossing 'Westar' with 'Dunkeld', chi-square test was run with MS Excel software to verify if segregation ratios of the resistance trait and molecular markers fit Mendelian ratios. Linkage analysis was performed with MapMaker Exp 3.0 computer program (25,29) in order to identify molecular markers linked to the 'Dunkeld' resistance gene against blackleg caused by *L. maculans* PG3.

RESULTS

Sources of resistance. Statistical analysis revealed significant differences between *Brassica* genotypes as to their response to blackleg caused by *L. maculans* PG2, PG3, and PG4 (Fig. 1). Genotypes with a disease rating of 5 or less on a 0 to 9 scale were considered resistant, and those with a higher disease rating were susceptible (57). We found resistance against PG3

in the Australian spring canola cultivar Dunkeld comparable to that of the winter cultivar Quinta, which is used as a differential in *L. maculans* pathogenicity group testing and known to carry cotyledon stage resistance genes against *L. maculans* PG2 and PG3. *B. juncea* cultivars Cutlass and Domo exhibited resistance against PG2, PG3, and PG4.

Segregation of resistance. Tests of 340 F_2 'Westar' × 'Dunkeld' progeny for response to PG3 revealed that 94 progeny were susceptible and 246 were resistant. Disease symptom ratings, on a 0 to 9 scale, ranged from 1 to 5 for resistant progeny, with an overall average rating of 2, while the rating for all susceptible progeny was 9 (Fig. 2). According to chi-square analysis (χ^2 = 0.96), the segregation ratio of the resistance against *L. maculans* PG3 within F_2 'Westar' × 'Dunkeld' progeny was not significantly different from 3:1, which was consistent with a single dominant gene model.

Molecular markers. Bulked segregant analysis (BSA) used together with the SRAP methods allowed selection of 10 primer pairs (Table 1) from 180 primer combinations that produced polymorphic markers between parents and within progeny. Fifty-two polymorphic markers within 92 F_2 'Westar' × 'Dunkeld' progeny were obtained using these 10 primer combinations. The segregation of four SRAP markers, including two from each of the primer combinations $Na_{12}A_{02}F/Na_{12}A_{02}R$ and BG_{20}/SA_{12}, was consistent with the resistance segregation within the 92 tested progeny. The two markers from the first primer combination were 200 and 190 bp, and the markers from the last primer pair were 480 and 475 bp (Fig. 3).

Sequence results from the two $NA_{12}A_{02}$ markers showed that they are from CT simple sequence repeat (SSR) regions and that two CT units differentiate 'Westar' from 'Dunkeld' with 17 and 19 repeats, respectively. BLASTn analysis revealed that this sequence has 96% homology (e-value = $1e^{-27}$) with a region of the *Brassica rapa* subsp. *pekinensis* BAC clone KBr0334I14 (GenBank accession number AC189311.1). BLASTx analysis of a 10 kb sequence flanking the *B. rapa* subsp. *pekinensis* homolog to the $NA_{12}A_{02\text{-}200}$ marker identified a region with 92% amino acid similarity with an *Arabidopsis thaliana* serine threonine 20 (ste-20) protein kinase (e-value = $4e^{-173}$). Primers BN204F and BN204R (Table 1) were designed from that clone sequence and provided the SCAR marker BN204 that is also linked to the cultivar Dunkeld resistance against *L. maculans*. The segregation ratio of the BN204 marker within the F_2 generation of 'Westar' × 'Dunkeld' was consistent with a single dominant gene model and also allowed differentiation of homozygotic from heterozygotic resistant progeny (Fig. 4). Linkage analysis identified a linkage group on which the 'Dunkeld' resistance gene against *L. maculans* PG3 (*Rpg_3Dun*) was closely linked to the BN204 SCAR marker and four SRAP

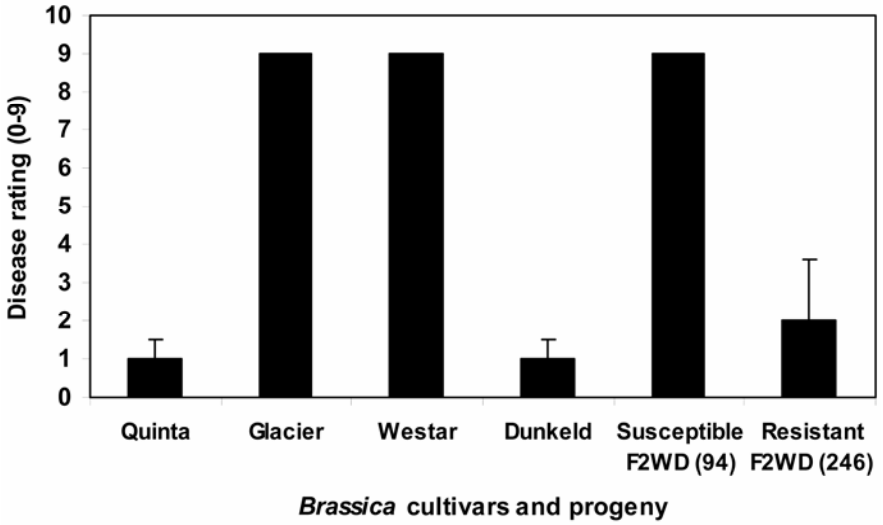

Fig. 2. Segregation of resistance against *Leptosphaeria maculans* PG3 in F_2 'Westar' × 'Dunkeld' progeny. Cultivars Glacier and Quinta were used as references. The disease ratings for 'Glacier', 'Quinta', and the parents 'Westar' and 'Dunkeld' are mean values from 12 seedlings. Disease ratings for F_2 'Westar' × 'Dunkeld' progeny (F_2WD) are average values from 94 susceptible progeny and 246 resistant ones.

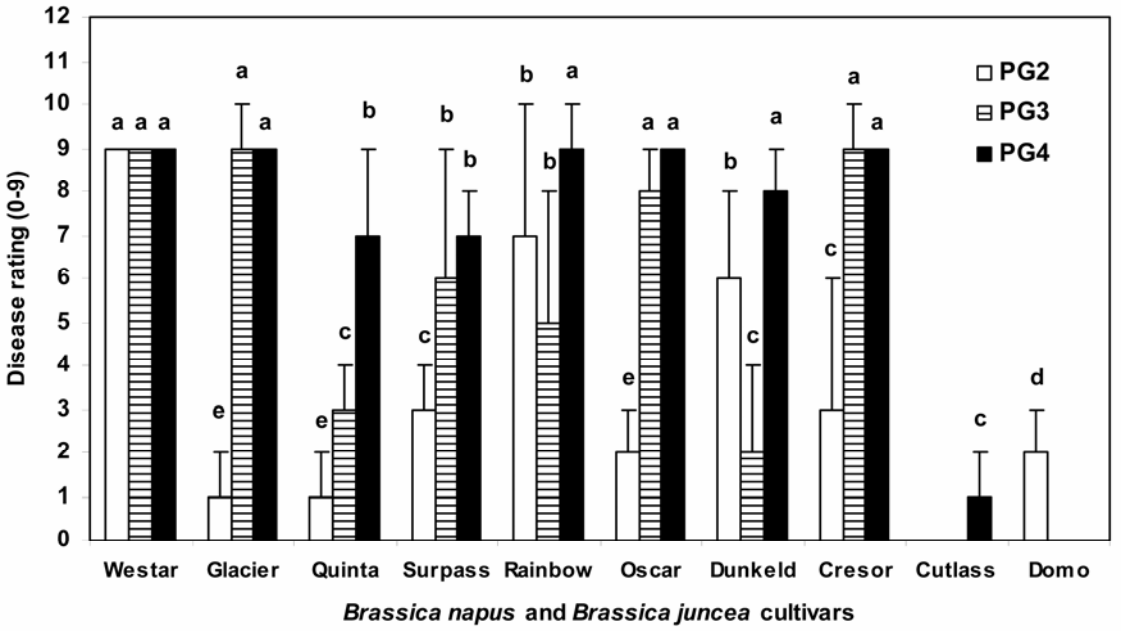

Fig. 1. Response of *Brassica napus* cultivars (Westar, Glacier, Quinta, Surpass 400, Rainbow, Oscar, Dunkeld, and Crésor) and *B. juncea* cultivars (Cutlass, Domo) to blackleg caused by *Leptosphaeria maculans* PG2, PG3, and PG4. Disease ratings for each cultivar are mean values from three replicate pots. Error bars on histograms represent standard deviation values. Letters above bars indicate statistical differences (P = 0.05) according to Tukey's test.

markers (Fig. 5). Three additional SRAP markers including two from BG_{20}/SA_{12} and one from BG_{18}/ODD_{20} were located at 11.3 and 16.4 cM, respectively, from each side of the core region with the resistance gene. Twelve progeny were recombinant for resistance and the molecular markers analyzed in 340 progeny, the SRAP marker $NA_{12}A_{02\text{-}200}$ and the SCAR marker BN204 (data not shown). Such a number was not significant because the calculated χ^2 values of 1.56, 0.06, and 0.25 for the segregation of resistance, NA12A02-200, and BN204, respectively, were lower than the critical value ($\chi^2_{(1, 0.05)} = 3.84$).

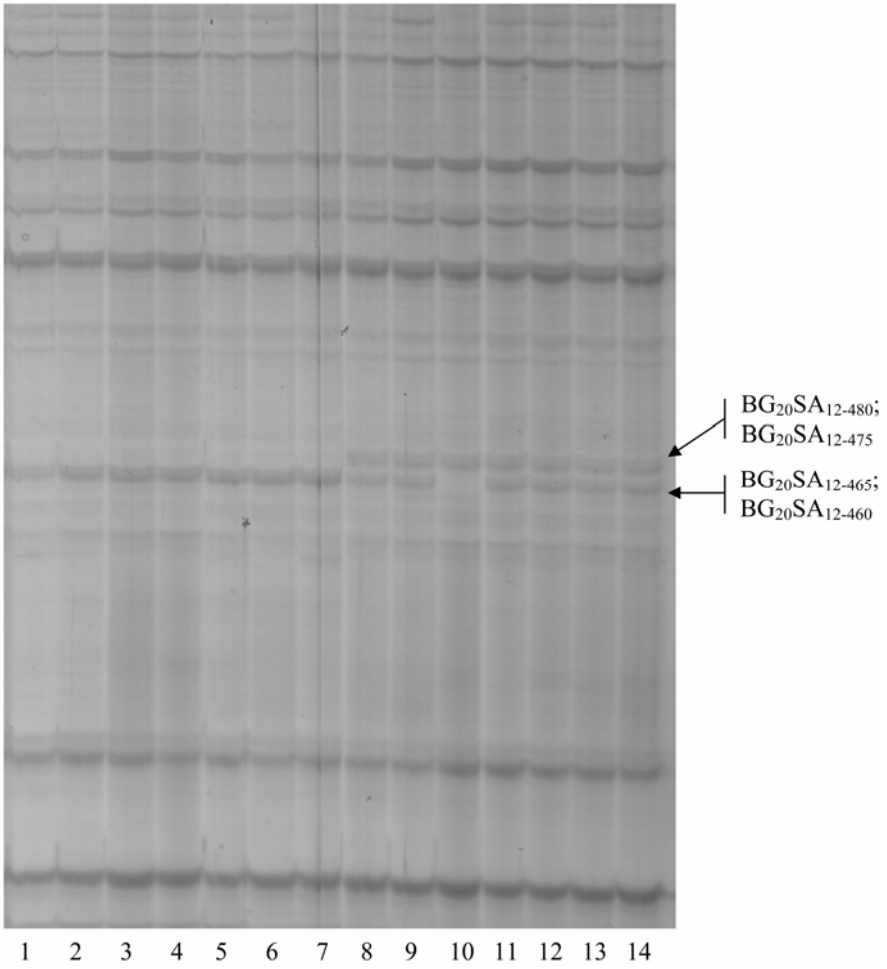

Fig. 3. Segregation of sequence-related amplified polymorphism (SRAP) markers linked to resistance against *Leptosphaeria maculans* in F_2 'Westar' × 'Dunkeld' progeny. Lanes 1 to 7: susceptible progeny, 8 to 14: resistant progeny. The four markers shown on the picture segregated within 92 F_2 'Westar' × 'Dunkeld' progeny in a ratio not significantly different from 3:1. Markers $BG_{20}SA_{12\text{-}480}$ and $BG_{20}SA_{12\text{-}475}$ were absent in all susceptible progeny (lanes 1 to 7) and present in all resistant ones (lanes 8 to 14). Markers $BG_{20}SA_{12\text{-}465}$ and $BG_{20}SA_{12\text{-}460}$ were present in 67 progeny, including all the 23 susceptible ones, and absent in the remaining 25 resistant ones, such as in lane 10.

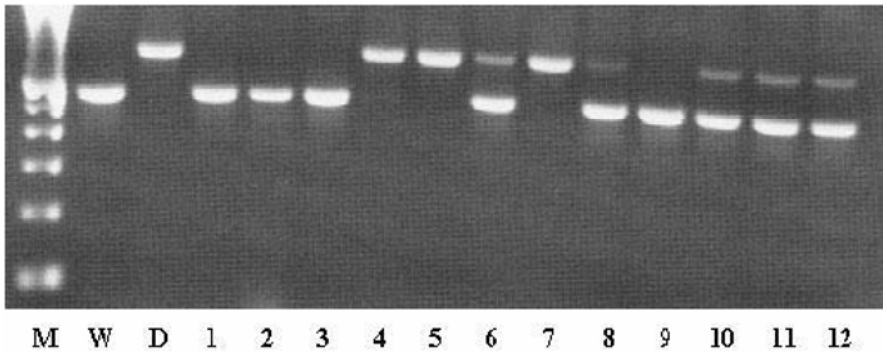

Fig. 4. Segregation of the sequence characterized amplified region (SCAR) marker BN204 linked to resistance against *Leptosphaeria maculans* PG3 in F_2 'Westar' × 'Dunkeld' progeny. M: 100-bp ladder, W: 'Westar', D: 'Dunkeld', and 1 to 12: F_2 'Westar' × 'Dunkeld' progeny. 1 to 3 and 9 represent homozygotic susceptible progeny; 4, 5, and 7 are homozygotic resistant progeny; and 6, 8, and 10 to 12 are heterozygotic resistant progeny.

DISCUSSION

The screening of the University of Manitoba *Brassica* germplasm revealed that sources of canola resistance against blackleg caused by *L. maculans* PG3 were available in two *B. napus* and two *B. juncea* cultivars. We selected 'Dunkeld' for further analysis because, being a spring type, it is easier to manipulate than the winter cultivar Quinta and *B. juncea* 'Cutlass' and 'Domo' in crossing with *B. napus* spring cultivars, such as 'Westar', and with commercial varieties for genetic studies and gene pyramiding. The resistance in 'Dunkeld' against blackleg is thought to be polygenic in part and can be traced back to possible sources present in its pedigree such as *B. napus* 'Chikuzen' and 'Norin' and *B. juncea* BJ168 (21,32). Our resistance screening experiment revealed that 'Dunkeld' was resistant against *L. maculans* PG3 but was susceptible to PG2 and PG4. The results suggest the presence of at least one major resistance gene in addition to polygenic resistance. These results also support the supposition that the decline of 'Dunkeld' blackleg resistance and reduction in grain yields recorded in Australia from 1999 to 2001 were due to a change in virulence in the blackleg pathogen (21). As the three pathogenicity groups coexist in Australia (7), the change in virulence may have resulted from the pathogen population composition shifting to higher proportions of PG2 and PG4 after the release of 'Dunkeld'. The conclusion that a major

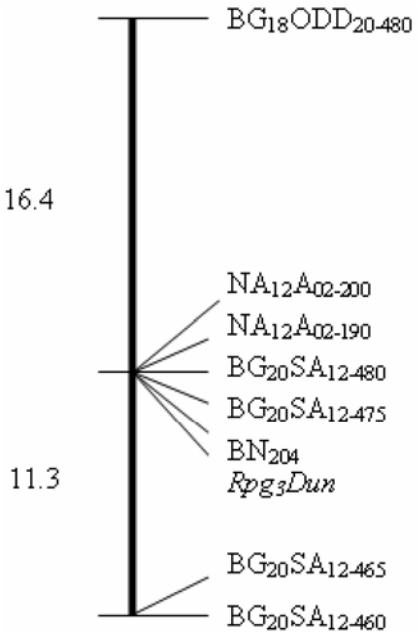

Fig. 5. Linkage group of 'Westar' × 'Dunkeld' population with marker loci linked to resistance gene against *Leptosphaeria maculans* PG3 from 'Dunkeld' (*Rpg_3Dun*). Genetic distance values are expressed in cM units.

gene (*Rpg$_3$Dun*) is responsible for resistance in 'Dunkeld' against PG3 was supported by the resistance segregation ratio of 3:1 in F_2 'Westar' × 'Dunkeld' progeny. *Rpg$_3$Dun* is likely similar to the *Rlm4* gene, which was previously suggested to be the major resistance gene in 'Dunkeld' (50). *Rlm4* has also been located in a cluster of four other tightly linked genes including *Rlm3*, *Rlm7,* and *Rlm9* (17).

Molecular markers linked to the 'Dunkeld' resistance gene against *L. maculans* PG3 were identified with a combination of BSA and SRAP procedures. A linkage group with eight molecular markers linked to this resistance trait was identified. DNA sequencing and BLAST analysis of a sequence from one of these markers allowed us to physically locate this resistance gene on a BAC clone from *B. rapa* subsp. *pekinensis.* Analysis of the *B. rapa* subsp. *pekinensis* genomic region close to the homologs of markers in 'Dunkeld' linked to *Rpg$_3$Dun* revealed that it contains open reading frames producing different putative transcripts that could be alternatively expressed in different stress conditions (9,38). Results from linkage mapping and sequence analysis also suggested that the 'Dunkeld' resistance trait against *L. maculans* PG3 might be associated with a gene encoding protein kinase. Protein kinases have several functions including defense responses (22). Plant serine/threonine kinases and kinase receptors involved in disease resistance have been reported in crops such as barley, tomato, and rice (12,30,31,41). Further studies will help us understand the functions and expression mechanisms of the serine/threonine ste-20 kinase encoding genes in *B. napus* as well as their role in compatible and incompatible interactions between canola and *L. maculans*. The SCAR marker BN204 linked to *Rpg$_3$Dun* resistance gene can be used in marker-assisted selection of canola resistance against blackleg caused by *L. maculans* PG3.

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