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ORIGINAL PAPER

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Identification of two novel genes for blackleg resistance in *Brassica napus*

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Abstract Blackleg, caused by *Leptosphaeria maculans*, is a major disease of *Brassica napus*. Two populations of *B. napus* DH lines, DHP95 and DHP96, with resistance introgressed from *B. rapa* subsp. *sylvestris*, were genetically mapped for resistance to blackleg disease with restriction fragment length polymorphism markers. Examination of the DHP95 population indicated that a locus on linkage group N2, named *LepR1*, was associated with blackleg resistance. In the DHP96 population, a second locus on linkage group N10, designated *LepR2*, was associated with resistance. We developed BC_1 and F_2 populations, to study the inheritance of resistance controlled by the genes. Genetic analysis indicated that *LepR1* was a dominant nuclear allele, while *LepR2* was an incompletely dominant nuclear resistance allele. *LepR1* and *LepR2* cotyledon resistance was further evaluated by testing 30 isolates from Canada, Australia, Europe, and Mexico. The isolates were from *B. napus*, *B. juncea*, and *B. oleracea* and represented different pathogenicity groups of *L. maculans*. Results indicated that *LepR1* generally conferred a higher level of cotyledon resistance than *LepR2*. Both genes exhibited race-specific interactions with pathogen isolates; virulence on *LepR1* was observed with one isolate, pl87-41, and two isolates, Lifolle 5, and Lifolle 6, were virulent on *LepR2*. *LepR1* prevented hyphal penetration, while *LepR2* reduced hyphal growth and inhibited sporulation. Callose deposition was associated with resistance for both loci.

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Introduction

Brassica species provide a range of crops that are of considerable horticultural and agricultural importance. Based on early work by Morinaga (1934) and UN (1935), the genomic relationships among *Brassica* crop species are usually represented by the triangle of U: *B. nigra* (genome BB), *B. oleracea* (CC), and *B. rapa* (AA) being primary diploid species. *B. carinata* (BBCC), *B. juncea* (AABB) and *B. napus* (AACC) are amphidiploids resulting from hybridization between corresponding pairs of the diploid species. *B. napus* (oilseed rape/canola) is important for edible oil production. However, *B. napus*, together with *B. rapa* and *B. oleracea*, are susceptible to infection by the ascomycete, *Leptosphaeria maculans*, which causes blackleg (phoma stem canker), one of the most destructive diseases of oilseed rape/canola crops in North America, Australia, and Europe.

Isolates of *L. maculans* are usually classified into two groups, referred to as the aggressive or highly virulent group and the non-aggressive or weakly virulent group (Koch et al. 1989). *L. maculans* isolates have also been classified into four pathogenicity groups (PGs), based on differential virulence on cotyledons of the *B. napus* cultivars 'Westar', 'Quinta', and 'Glacier' (Koch et al. 1991; Mengistu et al. 1991). Non-aggressive isolates were assigned to PG1 and aggressive isolates can be separated into PG2, PG3, and PG4. Isolates, which are compatible (virulent/susceptible) with 'Westar', and which give an incompatible (avirulent/resistant) interaction phenotype on 'Quinta' and 'Glacier', are classed as PG2. PG3 isolates are virulent on both 'Westar' and 'Glacier' but avirulent on 'Quinta'. PG4 isolates are virulent on all three cultivars. A gene-for-gene relationship has been established by genetic dissection of avirulence genes in *L. maculans* and their corresponding race-specific resistance genes. 'Quinta' contains the race-specific resistance gene, *Rlm1*, matched by the avirulence gene, *AvrRlm1*, in the pathogen. 'Glacier' contains *Rlm2*, matched by *AvrRlm2* (Ansan-Melayah et al. 1998). Subsequently, six

Avr genes have been identified (Balesdent et al. 2001). The corresponding resistance genes have been mapped and shown to cluster on linkage groups YD10 and YD16 (Delourme et al. 2004).

Since chemical protection is difficult and seldom economically advantageous, genetic resistance is the preferred method of control for blackleg in *B. napus* (Rimmer and Buchwaldt 1995). Resistance to blackleg has been identified in *Brassica* species: in *B. napus* (Rimmer and van den Berg 1992), in B-genome species (Chèvre et al. 1996, 1997; Plieske et al. 1998), in *B. rapa* subsp. *sylvestris* (Mithen et al. 1987), and in C-genome species (Mithen and Lewis 1988).

Resistance to blackleg has been identified in several *B. napus* accessions including French and Japanese accessions (Rimmer and van den Berg 1992). Several researchers have described genetic mapping experiments to identify loci in the *B. napus* genome controlling resistance to blackleg. A resistance gene (*LEM1*) from the *B. napus* cultivar 'Major' was mapped on linkage group 6 by Ferreira et al. (1995). Several restriction fragment length polymorphism (RFLP) markers linked to a gene, *LmFr1*, from the cultivar 'Crésor', were identified by Dion et al. (1995). This gene is located on linkage group N7 (Rimmer et al. 1999), which is equivalent to linkage group 6 of Ferreira et al. (1995). A resistance gene from the Australian cultivar 'Shiralee' (designated *LmR1*) has also been mapped to linkage group N7 of *B. napus* (Mayerhofer et al. 1997). Furthermore, resistance genes in other *B. napus* cultivars, including 'Maluka', also map to the linkage group N7 (Rimmer et al. 1999), suggesting that a single gene (or gene cluster) controlling resistance to blackleg in *B. napus* has been used very widely as a component of the resistance used to control blackleg in oilseed rape/canola varieties. It is likely that this gene is the same as *Rlm4*, which has been shown to be present in the French cultivar, 'Major' [previously mapped to linkage group 6 by Ferreira et al. (1995)], and a number of Australian cultivars, including 'Maluka' (Rouxel et al. 2003).

Resistance to blackleg has also been identified in *Brassica* species with the B genome (*B. nigra*, *B. juncea*, and *B. carinata*). These species are highly resistant to blackleg even at the seedling stage, and consequently numerous attempts have been made to transfer B-genome resistance into *B. napus* through interspecific hybridization (Chèvre et al. 1997; Pang and Halloran 1996; Roy 1984; Sacristan and Gerdemann 1986) and somatic hybridization (Sjödin and Glimelius 1989). Keri et al. (1997), using classical genetic methods, identified two genes controlling resistance in *B. juncea*. Resistance genes from *B. nigra* and *B. juncea* were assigned to linkage groups B4 and B8, respectively (Chèvre et al. 1996, 1997). The phenotype of the resistance response associated with the B-genome resistance in *B. nigra*, *B. juncea*, and *B. carinata* is very similar (Plieske et al. 1998).

Resistance to blackleg has been identified in wild accessions of *B. rapa* subsp. *sylvestris* (Mithen et al. 1987) and in *B. insularis*, a species in the *B. oleracea* coenospecies complex (Mithen and Lewis 1988). The *B. rapa* subsp. *sylvestris* resistance to blackleg was transferred into *B. napus*, using a resynthesised amphidiploid formed by interspecific hybridization between *B. rapa* subsp. *sylvestris* and *B. oleracea* subsp. *alboglabra* (Crouch et al. 1994).

The *B. rapa* subsp. *sylvestris* resistance has been introduced into *B. napus* cultivars and breeding lines through conventional breeding based on the original resynthesised *B. napus* plant. The phenotypic behaviour and genetic control of this resistance in *B. napus* is the subject of the current investigation.

Materials and methods

Plant material

DHP95 and DHP96 were populations of doubled haploid (DH) *B. napus* lines derived from distinct F_1 genotypes via microspore culture (Coventry et al. 1988) and developed by Advanta Canada (Winnipeg, Man., Canada). DHP95 consisted of 150 DH lines and was produced from F_1 plants from a cross between a resistant line, 6270, and a susceptible *B. napus* cultivar, 'Springfield'. DHP96 consisted of 300 DH lines and was produced from F_1 plants from a cross between a resistant line, 6279, and a susceptible breeding line, 3027. In both cases, individual DH lines could not be referenced back to a single F_1 plant. The two resistant lines, 6270 and 6279, were derived from the same cross but from different F_1 plants between the resynthesised *B. napus* plant carrying the *B. rapa* subsp. *sylvestris* resistance and a *B. napus* cultivar, 'Polo'. Line 6270 was a BC_2F_5 derived from that cross, while line 6279 was a BC_2F_6. In both lineages, 'Polo' was used as the recurrent parent. The two populations were evaluated for stem canker resistance in blackleg nurseries in Cowra, N.S.W., Australia, in 1998. Segregation for resistance and susceptibility within both populations in Australia was approximately 1:7, and the resistant lines in both populations showed complete canker resistance in the Australian nurseries (G. Buzza and S. Kelly, personal communication). On the basis of the disease nursery data, subsets of the most resistant (R) and most susceptible (S) lines were chosen. These consisted of 16 R lines (AD1-AD16) and 15 S lines (AD17-AD31) from DHP95, and 15 R lines (AD41-AD55) and 15 S lines (AD56-AD70) from DHP96. These lines were used for further genetic analysis.

The DH line, N-0-1, derived from the *B. napus* cultivar 'Westar' (Sharpe et al. 1995) was used as a standard blackleg susceptible genotype in genetic crosses and as a susceptible check for pathology experiments. The *B. napus* cultivars 'Quinta' and 'Glacier' (Mengistu et al. 1991) were used as differentials and resistance checks in pathology tests with *L. maculans* isolates.

Line N-0-1 was used to pollinate the R lines AD9 and AD49. Reciprocal crosses and crosses between AD9 and AD49 were also made. The resulting F_1 plants were

pollinated with line N-0-1 to produce the first backcross (BC_1) or first test cross (T_1) populations. The parents, F_1, F_2, and BC_1 or test cross plants were assayed for cotyledon disease reaction. 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).

An F_1 plant derived from AD9 × AD49 was subjected to microspore culture as described by Coventry et al. (1988), except that microspores instead of plantlets were treated with colchicine to induce chromosome doubling. The line MC1-8 was one of the resulting 78 DH lines.

Growth conditions

Seeds of *B. napus* were sown in 96-well flats (K1020P, Kord Products, Bramalea, Ont., Canada) into a soil-less mix (a mixture of sphagnum peat moss, sand, medium grade vermiculite, fine ground calcium carbonate, fertilizer, and trace elements) and placed in a growth chamber for 3 weeks. Seedlings were transplanted to pots and moved to a greenhouse. Plants were watered daily and kept at 20/18°C day/night temperature with a 16-h photoperiod.

Preparation of *L. maculans* isolates and plant inoculations

All isolates of *L. maculans* were from the collection at the AAFC Saskatoon Research Centre. Inoculum was prepared by culturing 'Westar' cotyledons infected with individual isolates of *L. maculans* on V-8 agar plates containing 100 μg ml^{-1} streptomycin. Detached cotyledons were sterilised in 12% sodium hypochlorite for 2 min, and then washed in sterile water before plating. Plates were incubated at 22°C under continuous fluorescent light. Approximately 2 weeks later, spores were collected by applying 10 ml sterile water to the plate surface and scraping off the submerged pycnidia. The resulting spore suspension was filtered through a double layer of cheesecloth to remove pycnidial remnants, and the spore concentration was adjusted to 2×10^7 spores ml^{-1}, using a hemacytometer. Spore suspensions were stored at −20°C and thawed just before inoculation.

Seven days after sowing, cotyledons of seedlings were wounded with a pair of tissue teeth forceps (Allegiance, VWR Canlab cat. no. 25601-080) and a 10-μl droplet of the pycnidiospore suspension was pipetted on the wound. Inoculated cotyledons were allowed to dry for at least 12 h before the inoculated seedlings were watered. Disease reaction was rated 10 days after inoculation (dai), using the 0–9 scale described in Williams (1985). Disease ratings of 0–6 described R interactions, while ratings of 7–9 described S interactions.

Genetic marker and linkage analyses

DNA extraction, gel electrophoresis, Southern transfer, and Southern hybridization and *Brassica* RFLP probes were as described previously by Sharpe et al. (1995). The linkage of marker loci and loci controlling blackleg resistance was tested with Mapmaker, version 3.0 (Lander et al. 1987), using a log-likelihood threshold of 4.0. Recombination frequencies were converted to map distance, using Kosambi's function (Kosambi 1944).

Light microscopy

Cotyledon tissue around the point of inoculation was excised at 2, 4, 6, and 10 dai and fixed in a solution of 95% ethanol, acetic acid (3:1 v/v) for 2 h. Samples were decolourised with 95% ethanol before clearing in saturated chloral hydrate for 5 days. To visualize fungal hyphae, the cleared cotyledons were stained with 250 μg ml^{-1} trypan blue in a solution of lactic acid, glycerol, and water (1:1:1) for 5 min, rinsed with the same solution, and mounted in 70% glycerol. Callose deposition was detected by staining with 0.1% aniline blue in 0.15 M K_2HPO_4. Cytological events were observed with a Zeiss Photomicroscope III (Carl Zeiss Canada, Mississauga, Ont., Canada) equipped with differential interference contrast and epifluorescence optics.

Electron microscopy

Small pieces of inoculated cotyledon tissue (approximately 2 mm in diameter) around the inoculated wound sites were excised 2 dai and 4 dai. The pieces were prefixed in 2.5% glutaraldehyde in a buffer of 0.05 M sodium phosphate (pH 6.8), washed with the buffer 3 times, post-fixed in 1% osmium tetroxide in the buffer, and dehydrated in a series of washes with increasing ethanol concentrations. After critical point drying, the samples were mounted, sputter-coated with gold, and examined using a Philips 505 scanning electron microscope.

Results

Phenotyping DH populations for cotyledon resistance to isolates of *L. maculans*

Cotyledon assays conducted under controlled environmental conditions, using *L. maculans* isolates, have been employed to accelerate the development of blackleg R varieties (Rimmer and van den Berg 1992). To investigate the correlation between the field-evaluated adult stem canker resistance and cotyledon resistance, the subsets of DHP95 and DHP96 were evaluated in a growth chamber for cotyledon resistance

to a Canadian isolate of *L. maculans*, pl86-12 (PG2), and an Australian isolate, WA30 (PG3). For both DHP95 and DHP96, cotyledon resistance to both isolates completely co-segregated with the stem canker resistance determined in Australia by Advanta (Fig. 1). All DH lines with canker resistance also exhibited cotyledon resistance to isolates pl86-12 (Fig. 1a) and WA30 (Fig. 1b), whereas all lines with stem susceptibility showed cotyledon susceptibility to the isolates. This result provided evidence that cotyledon resistance in the populations was highly correlated to stem resistance, and that the gene(s) controlling resistance in the field and in cotyledons to the two isolates were either the same or tightly linked. Also, it was observed that the R lines from the DHP95, AD1 to AD16, showed higher levels of cotyledon resistance than AD41 to AD55, the R lines from the DHP96 (Fig. 1).

Mapping resistance genes

A RFLP map of *B. napus*, with 19 linkage groups, has been developed (Sharpe et al. 1995). This map includes homology of linkage groups N1–N10 of *B. napus* with those of *B. rapa* (A genome) and N11–N19 with those from *B. oleracea* (C genome). Mapping of the resistance genes was initiated using RFLP markers by assaying the DHP95 and DHP96 subset populations. RFLP probes were chosen from the A-genome linkage groups, because the resistance in these populations was originally derived from the A-genome species, *B. rapa* subsp. *sylvestris*. A set of 24 probes was employed for RFLP analysis (Table 1). Thirty-five polymorphic loci were detected in DHP95, and 34 in DHP96. Since the R parents 6270 (for the DHP95) and 6279 (for the DHP96) were developed from the same original cross with the resynthesised *B. napus* plant, we presumed that similar mapping results would be obtained from the two populations. Surprisingly, RFLP analysis showed that one resistance gene (designated *LepR1*) was mapped in the DHP95 population to linkage group N2, and a second gene was mapped in the DHP96 population to linkage group N10 and designated *LepR2* (Fig. 2). There were seven polymorphic markers on N2 in the DHP95 population and six polymorphic markers on N10 in the DHP96 population (Table 2). *LepR1* co-segregated with pR4b, pO85h, pW180b, pN181a, and pW207a, and was 3 cM and 10 cM from pO3b and pW154a, respectively (Fig. 2a). *LepR2* was located in an interval between pW101a and pR115b, 3 cM from either marker, 11 cM from pN91a, and co-segregated with pN21b, pR34b, and pN53b (Fig. 2b). The genetic distances estimated from the recombination frequencies among linked markers on N2 in the DHP95 and N10 in the DHP96 are consistent with the Sharpe et al. (1995) map. We therefore excluded the possibility of other linkage groups carrying resistance genes in the DHP95 or DHP96, and concluded that a single locus was involved in resistance for each population. This result, that the two populations carried different resistance loci, was confirmed by comparison of polymorphic DNA markers on N2 and N10 (Table 2). Marker pO85h on N2 was linked to *LepR1*, but not to *LepR2*. On the other hand, markers pN53b and pN91a on N10 were associated with *LepR2*, but not with *LepR1*. Our experiments provide support for the view that at least two loci derived from *B. rapa* subsp. *sylvestris* were associated with blackleg resistance in the original amphidiploid line derived from *B. rapa* subsp *sylvestris*.

Fig. 1 Cotyledon disease rating. Stem resistance of doubled haploid (DH) lines was tested in Australian nurseries. AD1 to AD16 were resistant (R) and AD17 to AD31 were susceptible (S) in the DHP95. AD41 to AD55 were R, and AD56 to AD70 were S in the DHP96. W, G, and Q represent differential cultivars, N-0-1 ('Westar'), 'Glacier' and 'Quinta', respectively. Cotyledon disease was rated 10 days after inoculation (dai) with a pycnidiospore suspension (2×10^7 spores ml^{-1}), using a scale of 0–9. Disease ratings of 0–6 were defined as R, 7–9 as S. The values represent the average score of 12 plants ± SE. The experiments were replicated three times with similar results. **a** Inoculated with a Canadian isolate, pl86-12, **b** with an Australian isolate, WA30

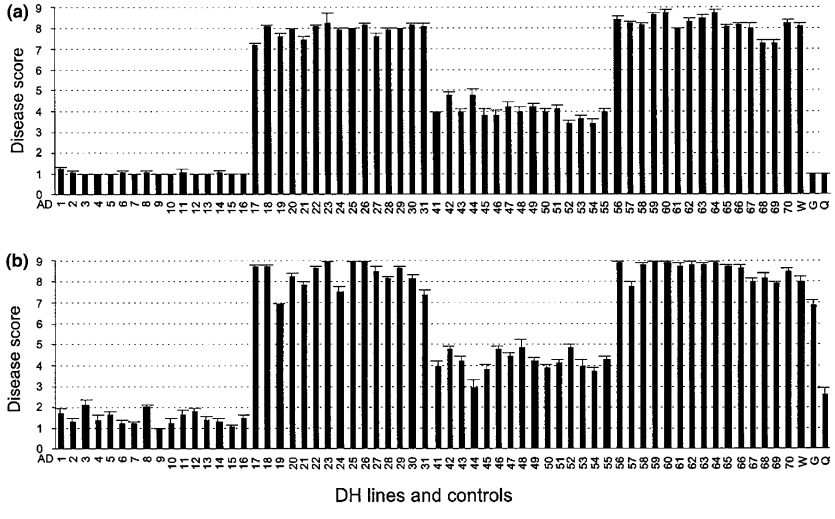

Table 1 Restriction fragment length polymorphism (RFLP) probes used for genetic mapping in DHP95 and DHP96 populations of *Brassica napus* doubled haploid (DH) lines

Probes	Linkage groups[a]	Number of loci detected[b]	
		DHP95	DHP96
pN170	N8, N10	1	1
pN180	N2, N3, N10	3	1
pN199	N6, N10	3	1
pN21	N9, N10	Mono	2
pN53	N3, N5, N10	3	3
pN91	N10	2	3
pO155	N3, N10	1	Mono
pO3	N2, N7	1	2
pO79	N3, N7	1	1
pO85	N2	2	2
pR115	N2, N10	1	3
pR29	N2	Mono	2
pR34	N10	2	1
pR4	N2	3	1
pR72	N2	1	2
pR94	N7	1	3
pW101	N10	1	1
pW148	N3	1	2
pW154	N2	2	Mono
pW161	N2	1	Mono
pW176	N2	Mono	1
pW180	N2	2	Mono
pW228	N7	Mono	1
Total polymorphic loci detected		35	34

[a]A-genome linkage groups of *B. napus* were detected by the probes according to the maps developed by Sharpe et al. (1995)
[b]Polymorphic loci detected, *mono* monomorphic locus

Inheritance of *LepR1* and *LepR2*

One R line from each of the DHP95 and DHP96 populations was used to develop new populations to study the inheritance of cotyledon resistance. Crosses were made between the S line N-0-1 and the R line AD9 (*LepR1*) or AD49 (*LepR2*), and between AD9 and AD49. Cotyledon resistance was then analyzed in F_1, F_2, and backcross (BC_1) populations or testcross progenies after inoculation with the PG2 isolate pl86-12 (Table 3).

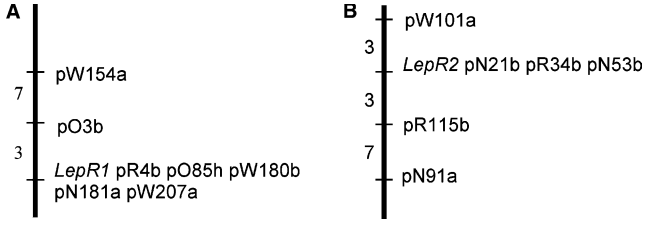

Fig. 2 Genetic map position of *Leptosphaeria maculans* resistance genes *LepR1* and *LepR2* from *Brassica napus*. *LepR1* (**a**) was mapped using 31 DH lines in DHP95, and *LepR2* (**b**) was mapped using 30 DH lines of the DHP96. Numbers are centiMorgans. DNA for RFLP analysis was digested with *Eco*RI, fractionated in 0.8% agarose gels, blotted on Hybond-N^+ nylon membrane, and hybridized with probes. Phenotypic disease evaluation was carried out in the field in Australia and in the greenhouse in Canada

Table 2 RFLP genetic markers mapping to linkage groups N2 or N10 of *B. napus*

Markers	Linkage groups	Linkage with resistance[a]	
		DHP95	DHP96
pO3b	N2	+	Mono
pR4b	N2	+	Mono
pO85h	N2	+	–
pW154a	N2	+	Mono
pW180b	N2	+	Mono
pN181a	N2	+	Mono
pW207a	N2	+	Mono
pN21b	N10	Mono	+
pR34b	N10	Mono	+
pN53b	N10	–	+
pN91a	N10	–	+
pW101a	N10	Mono	+
pR115b	N10	Mono	+

[a]+ Linked to resistance phenotype, – unlinked to resistance phenotype, *mono* monomorphic marker

The average disease ratings of S (N-0-1) and R (AD9) parents were 8.5 and 1.1 respectively. F_1 plants from the cross N-0-1 × AD9 were R, with a mean disease score of 1.4, and AD9 × N-0-1 with 1.0, similar to the R parent AD9. Analysis of the F_2 and BC_1 individuals indicated that segregation for R and S fitted ratios of 3:1 in F_2 and 1:1 in BC_1. This indicates that a single dominant allele was associated with cotyledon resistance and due to a nuclear gene.

We examined plants of the S (N-0-1) and R (AD49) parents. The mean disease rating for N-0-1 was 8.5 and for AD49 was 4.0. Reciprocal crosses of N-0-1 with AD49 produced two F_1 generations. The mean cotyledon disease rating for each of the two F_1 generations was 7.5, which was intermediate between the corresponding homozygous parents, but more similar to the S parent N-0-1. The interaction phenotype of the F_1 plants from the crosses was considered to be S. Examination of segregation in F_2 and BC_1 generations revealed that R:S segregated 1:3 and 0:1 respectively. These results demonstrate that a single allele was responsible for the resistance, and that it was an incompletely dominant nuclear gene.

Further genetic analysis confirmed the inheritance of the two resistance genes. For crosses between AD9 and AD49, all F_1 progeny were R (mean disease rating of 1), resembling the R parent AD9 in their interaction phenotype. In the F_2 population, segregation for R and S fit a 13:3 ratio, and a population derived from the test cross of the F_1 (AD9 × AD49) × N-0-1 fit a 1:1 segregation ratio.

Plants in the F_2 and BC_1 from N-0-1 × AD9 or N-0-1 × AD49 were also tested for segregation for resistance to the PG3 isolate, WA30. Results were consistent with those of isolate pl86-12 (data not shown).

Interaction between *LepR1* and *LepR2*

Cotyledon resistance in AD9 (*LepR1*) and AD49 (*LepR2*) was further characterized with a broad range of isolates of *L. maculans* from Australia, Europe, Mexico,

Table 3 Genetic analysis of blackleg resistance in crosses of the *B. napus* DH lines, AD9 and AD49, with a DH line N-0-1 derived from the cultivar 'Westar'. *R* Resistant, *S* susceptible

Parents and crosses[a]	Type	Disease response[b]			Expected ratio	
		Total	R	S	R:S	X^2
AD9	R parent	24	24	0		
'Westar'	S parent	24	0	24		
AD9 × 'Westar'	F_1	24	24	0		
'Westar' × AD9	F_1	24	24	0		
'Westar' × AD9	F_2	186	132	54	3:1	1.61, $P > 0.10$
'Westar' × ('Westar' × AD9)	BC_1	147	66	81	1:1	1.53, $P > 0.10$
AD49	R parent	24	24	0		
'Westar'	S parent	24	0	24		
AD49 × 'Westar'	F_1	24	0	24		
'Westar' × AD49	F_1	24	0	24		
'Westar' × AD49	F_2	174	40	134	1:3	0.12, $P > 0.50$
'Westar' × ('Westar' × AD49)	BC_1	106	0	106	0:1	
AD9	R parent	24	24	0		
AD49	R parent	24	24	0		
'Westar'	S parent	24	0	24		
AD9 × AD49	F_1	24	24	0		
AD49 × AD9	F_1	24	24	0		
AD9 × AD49	F_2	189	158	31	13:3	0.68, $P > 0.25$
'Westar' × (AD9 × AD49)	Testcross	113	54	59	1:1	0.22, $P > 0.50$

[a] *AD9* R DH line with *LepR1, AD49* R DH line with *LepR2*, *'Westar'* S cultivar
[b] Cotyledons were inoculated with *Leptosphaeria maculans* isolate pl86-12. The interaction phenotype on cotyledons was rated using a 0–9 scale. Disease ratings of 0–6 were defined as R, 7–9 as S

and North America, isolated from different *Brassica* species, and including different pathogenicity groups (Table 4). Generally, AD9 showed a higher level of resistance than AD49. On AD9, lesions were small, necrotic and blackened, and confined to the site of inoculation (disease rating of 0–2.9) for 28 isolates, and one isolate resulted in large, non-sporulating lesions (disease rating of 3.7). Only one isolate, pl87-41, was virulent on AD9, resulting in a large sporulating lesion (disease rating of 8.1). In contrast, AD49 displayed small necrotic blackened lesions to eight isolates and large non-sporulating lesions to 22 isolates, two isolates resulted in disease ratings greater than 6. This suggests that both genes are race-specific and that *LepR1* confers stronger cotyledon resistance than *LepR2* to avirulent isolates.

We have demonstrated that *LepR1* and *LepR2* independently confer resistance to isolates of *L. maculans*. To study the interaction between *LepR1* and *LepR2*, a DH line, MC1-8, carrying both *LepR1* and *LepR2* was developed using pathology screening and flanking markers. MC1-8 showed a similar level of cotyledon resistance to most isolates, as did AD9 (*LepR1*, Table 4). This was expected, because AD9 was highly resistant to the isolates, except pl87-41. Isolates 99-22, M1, WA74, Lifolle6, and 99-43 were more aggressive on AD9 than the other isolates. MC1-8 showed higher levels of resistance to these isolates than AD9, suggesting an additive effect of *LepR1* and *LepR2* when in combination.

Resistance responses

To investigate the resistance response controlled by *LepR1* or *LepR2* to *L. maculans*, we observed cotyledon disease reactions, using AD9 and AD49 after challenging with p186-12. The disease symptoms at 10 dai are shown in Fig. 3. Infection on the S line N-0-1 resulted in extensive tissue collapse, accompanied by profuse sporulation (Fig. 3a). In contrast, infection on AD9 was restricted to a very limited blackening around the inoculation sites (Fig. 3c). Infection on AD49 resulted in 2–3 mm of brown necrotic lesions (Fig. 3b).

Further evaluation of infection was studied on the R lines. At 2 dai, callose deposition in the AD9 and AD49 was observed in the mesophyll cells around the inoculation sites. No hyphae in AD9 and few hyphae in AD49 were observed at the inoculation sites at 4 dai by light microscopy. However, infection on N-0-1 occurred with no callose deposition, and hyphae were extensively ramified among the mesophyll cells at 4 dai (data not shown). Cytological observations among the lines at 10 dai are shown in Fig. 3. Fungal growth in N-0-1 was abundant in the mesophyll cells (Fig. 3j), and pycnidia were formed in the collapsed tissues (Fig. 3g). There was no appreciable callose deposition in mesophyll cells (Fig. 3d). In contrast, hyphae were found among mesophyll cells in AD49 (Fig. 3k), but much less so than in N-0-1. No pycnidia were observed (Fig. 3h). Callose deposition was apparently present (Fig. 3e). This suggests that the resistance response controlled by *LepR2* limited fungal growth and restricted sporulation of *L. maculans* in cotyledons and that resistance was associated with callose deposition. A very intense callose deposition developed around the inoculation site in AD9 (Fig. 3f), and almost no hyphae were observed in the mesophyll cells (Fig. 3i). To investigate whether the pathogen could invade AD9 cotyledons, we examined mesophyll tissues from ten inoculation sites and found

Table 4 Mean cotyledon interaction phenotypes conferred by *B. napus* lines containing *LepR1*, *LepR2*, or both after inoculation with a range of isolates of *L. maculans*

Isolates of *L. maculans*		Plant genotypes						Source of isolates[c]
		AD9 *LepR1*	AD49 *LepR2*	MC1-8 *LepR1*/*LepR2*	'Westar'	'Glacier'	'Quinta'	
778	2, C[b]	1.2	2.8	1.0	8.8	1.0	1.5	*B. napus*
831	2, C	1.4	3.4	1.1	9.0	1.0	1.6	*B. napus*
pl86-12	2, C	1.5	3.6	1.1	8.3	1.3	1.6	*B. napus*
pl86-14	2, C	1.3	3.4	1.0	9.0	1.0	2.0	*B. napus*
99-22	2, C	2.0	4.3	1.0	8.3	0.5	1.4	*B. napus*
99-R1	2, C	1.4	3.8	1.1	8.0	1.1	2.1	*B. napus*
AB97-15	2, C	1.0	4.6	1.0	8.9	1.0	1.0	*B. napus*
AB97-19	2, C	1.7	3.8	1.1	9.0	1.0	1.5	*B. napus*
AC98/11	2, C	0.0	1.1	0.0	7.0	0.0	0.0	*B. napus*
AC98/16	2, C	1.0	2.3	1.0	8.4	0.8	1.2	*B. napus*
AC98/8	2, C	0.1	1.5	0.1	7.0	0.4	0.5	*B. napus*
SA10G	2, C	0.4	3.2	0.2	7.1	0.1	0.6	*B. napus*
M1	2, A	1.8	5.0	1.0	7.0	2.6	1.9	*Brassica juncea*
WA52	2, A	1.6	4.8	1.1	7.6	2.8	1.1	*B. napus*
WA74	2, A	2.9	4.9	1.3	8.2	2.4	1.0	*B. napus*
OMR1:1	2, M	1.4	2.2	1.1	8.4	1.4	2.0	*B. juncea*
OMR11:2	2, M	1.6	3.7	1.2	9.0	2.4	4.3	*Brassica oleracea*
OMR8:1	2, M	2.0	2.2	1.4	7.3	1.8	1.6	*B. oleracea*
OMR9:1	2, M	1.7	2.2	1.5	7.3	1.8	3.6	*B. oleracea*
OMR2:3	2, M	0.8	2.2	0.6	8.0	2.8	2.6	*B. oleracea*
pl87-41	2, U	8.1	1.5	1.1	7.7	1.2	1.7	*B. napus*
2354	3, C	1.2	3.6	1.0	7.8	7.1	2.5	*B. napus*
WA30	3, A	1.2	3.2	0.2	7.8	7.3	4.6	*B. napus*
Lifolle6	3, E	2.6	6.1	1.6	7.6	7.3	4.0	*B. napus*
2367	4, C	1.3	5.8	1.1	7.8	7.9	7.5	*B. napus*
Lifolle5	4, E	1.5	6.5	1.0	7.4	8.1	8.1	*B. napus*
WA51	4, A	1.0	5.9	0.1	7.6	8.1	7.4	*B. napus*
99-79	n, C	1.2	5.0	1.0	8.8	2.4	7.8	*B. napus*
99-43	n, C	3.7	5.5	1.9	8.3	3.1	8.7	*B. napus*
99-56	n, C	1.5	3.7	1.2	8.9	2.3	8.4	*B. napus*

[a]Cotyledon interaction phenotype was rated 10 days after inoculation, using a scale of 0–9. The values represent the average score of 12–24 plants. The experiments were replicated twice with similar results

[b]Pathogenicity group, place where isolates were collected: *2* PG2, *3* PG3, *4* PG4, *n* new PG, *C* Canada, *A* Australia, *E* Europe, *M* Mexico, *U* USA

[c]*Brassica* species from which isolate was obtained

only one with slightly branched mycelium occurring among the mesophyll cells (Fig. 3l). With electron microscopy, we observed that spores germinated on the surface of AD9 cotyledon at 2 dai (Fig. 4a), and abundant mycelia proliferated within the wound site at 4 dai (Fig. 4b). This implies that AD9 (*LepR1*) permitted spore germination on the surface of the cotyledon and hyphal growth in the wound area, but development of the pathogen beyond the wound area was severely restricted.

Discussion

We have shown that resistance in *B. napus* derived from *B. rapa* subsp. *sylvestris* to *L. maculans* is conferred by two loci, *LepR1* located on linkage group N2 and *LepR2* on linkage group N10. The two loci were mapped using two distinct populations. However, R parents of these populations, 6270 in the DHP95, and 6279 in the DHP96, were developed from BC_2F_5 and BC_2F_6, respectively, of the same original cross between a resynthesised *B. napus* line (derived from *B. rapa* subsp *sylvestris* and *B. oleracea*) × S cultivar. The original genetic hypothesis based on the approximate 7:1 S to R segregation ratio observed in the blackleg field nurseries was not substantiated by our results. The cause for this discrepancy is most likely due to heterozygosity in the R parental lines used to make the DH populations, since both DH populations were derived from a number of F_1 plants (G. Buzza, personal communication). If the R parent line was heterozygous, then the F_1 plants used for microspore cultures would be either heterozygous for resistance or homozygous for susceptibility. Alternatively, but probably less likely, is that this ratio may have resulted from segregation distortion. Segregation distortion has been reported in *B. napus* DH populations (Cloutier et al. 1995).

Our observations indicate that the resynthesised *B. napus* line contained at least two unlinked loci associated with blackleg resistance derived from *B. rapa* subsp. *sylvestris*. It seems most likely that two resistance alleles from the resynthesised *B. napus* line were introduced, one into 6270 and the other into 6279, through

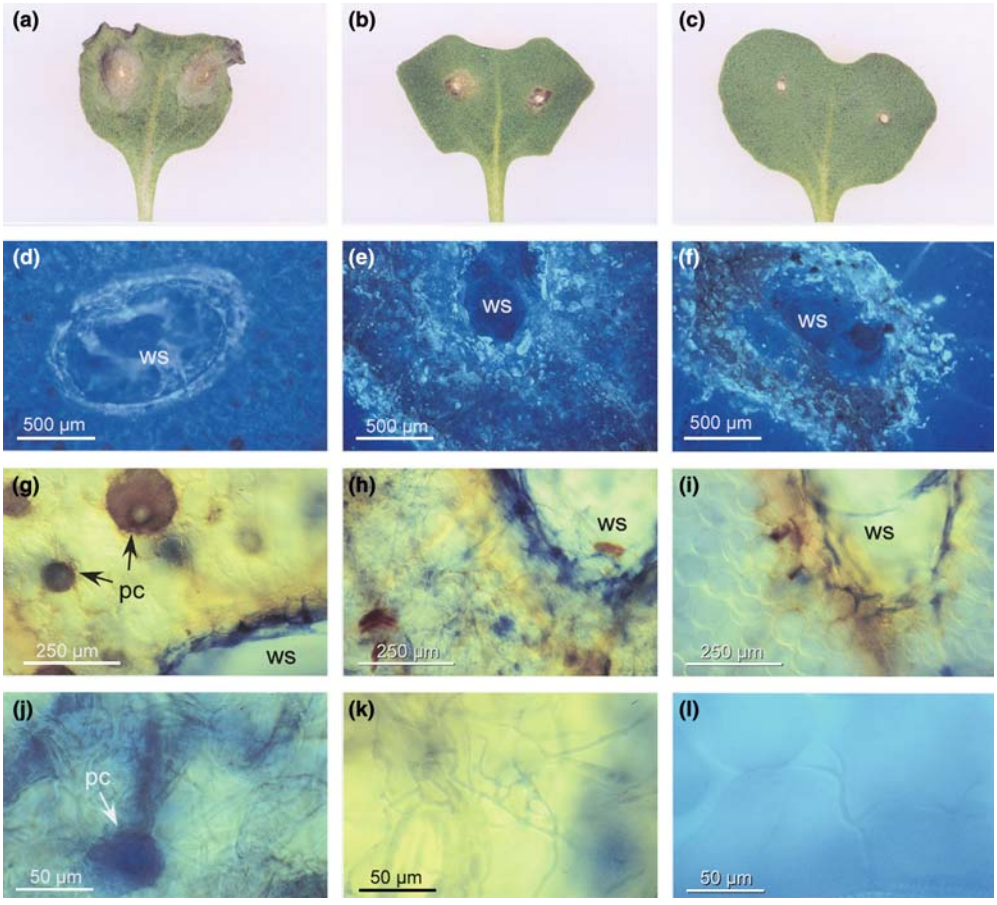

Fig. 3 Cotyledon disease and resistance responses. Photos were taken at 10 dai. Wound site (*ws*), pycnidia (*pc*), and magnification bars are shown. The *left*, *middle*, and *right columns* show cotyledons or cotyledon tissue around inoculation sites obtained from N-0-1, AD49, and AD9 respectively. **a–c** Macroscopic resistance responses. **d–f** Tissues stained with 0.1% aniline blue, indicating callose deposition. **g–l** Differences of mycelial growth and pycnidial formation among the R lines and the S cultivar stained with 250 µg/ml trypan blue

the usual plant breeding procedures of crossing, selfing, and selection. This could be determined with certainty if we were to test the original resynthesised line, the R parents used for DH development, 6270 and 6279, and the S parents with molecular markers and disease phenotyping. Unfortunately, these lines are not available. However, analysis of the pedigrees of 6270 and 6279 showed that these two R parents were derived from different F_1 selections of the resynthesised *B. napus* line × S cultivar (G. Buzza, personal communication). Thus, 6279 (BC_2F_6) is not derived by selfing of 6270 (BC_2F_5). The phenotypic resistance response in the DHP95 and the DHP96 is very different (Fig. 1). Finally, there is segregation for resistance and susceptibility from the progenies of F_2 and testcrosses derived from a cross between R lines AD9 and AD49, chosen from the two populations (Table 3). Therefore, we conclude that the two R parents, 6270 and 6279, carried different and independent resistance alleles. Some resistance genes previously identified in *B. napus* such as *LEM1* ('Major'), *LmFr1* ('Crésor'), *LmR1* ('Shiralee'), *cRLMm* ('Maluka'), and *aRLMrb* (RB87-62) have been mapped genetically (Rimmer et al. 1999). It is interesting that all of these genes are located on linkage group 6 (Ferreira et al. 1995), which corresponds to linkage group N7 in the Sharpe et al. (1995) map (A.G. Sharpe, personal communication). At present, it is still unclear whether the N7 genes present in these cultivars and breeding lines are allelic or not, but it is clear that *LepR1* and *LepR2* described in this study are different from those genes. Delourme et al. (2004) show that five independent blackleg resistance genes (*Rlm1*, *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9)* are located in a 35-cM interval on linkage group DY.10 or equivalent. Rouxel et al. (2003) have shown that 'Maluka' contains *Rlm4*. Consequently, DY.10 must be equivalent to N7.

Recently, the genetic control of a few specific interactions between *B. napus* and *L. maculans* was elucidated with both the pathogen and the host plant (Ansan-Melayah et al. 1995, 1998; Balesdent et al. 2001 and Pongam et al. 1998). Their observations provide evidence for a gene-for-gene relationship in *L. maculans*–*B. napus* interaction. We present evidence that *LepR1* and *LepR2* confer a high or intermediate level of cotyledon resistance to most isolates from different pathogenicity groups. Nonetheless, evidence of race specificity was obtained for both genes. It is likely that both *LepR1* and *LepR2* recognize most races of *L. maculans* that are present in Australia, potentially providing new sources for blackleg disease control.

We have presented evidence that spore germination on cotyledons and hyphal growth in the inoculation sites can occur on line AD9 (*LepR1*), where cotyledon tissue was mechanically wounded. Little hyphal growth was observed beyond the wound site, and deposition of callose (which is a major component of cell wall

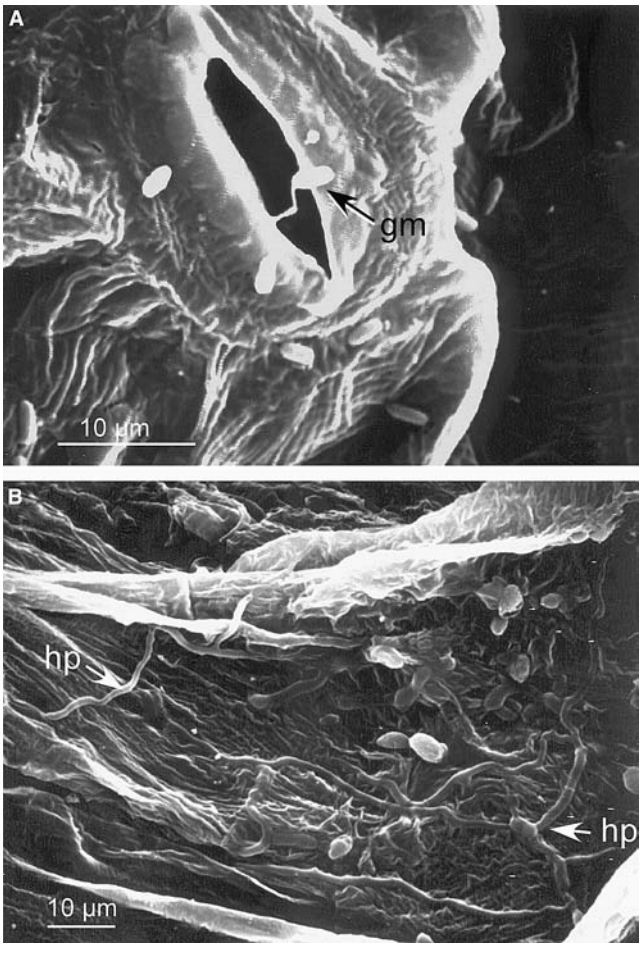

Fig. 4 Scanning electron micrographs. AD9 cotyledons were inoculated with PG2 isolate pl86-12 of *L. maculans*. Magnification bars are presented. **a** Spore germination (*gm*) on the surface of the cotyledon 2 dai. **b** Hyphal growth (*hp*) in the wound site of the cotyledon 4 dai

reinforcement) occurred in *LepR1* tissue but not in the S N-0-1 wound site. This indicates resistance mediated by *LepR1* results in the rapid induction of plant defence mechanisms that inhibit growth of the pathogen. *LepR2* also displayed callose deposition. However, in this case, hyphal growth was reduced rather than completely suppressed, and sporulation was inhibited, resulting in limitation of multiplication and spread of the pathogen within the plant. Enhanced callose formation is correlated with the resistance of some barley cultivars and is proposed to be a mechanism for resistance (Giese et al. 1997).

Stem canker resistance to *L. maculans* is the most important component of resistance required to protect oilseed rape against blackleg, although the pathogen can infect various parts of the plant at different plant growth stages. The resistance described here confers canker resistance under severe blackleg disease situations as frequently occurs in canola production areas in Australia and is thus significantly better than any resistance previously employed. 'Surpass 400' and other related *B. napus* cultivars derived from the *B. rapa* subsp. *sylvestris* accession were regarded as the most resistant Australian cultivars. This resistance has recently been overcome in some regions of Australia. Li and Cowling (2003) reported on the inheritance of resistance in 'Surpass 400' and indicated that the resistance was due to a single dominant gene. Our data indicate that the resistance in 'Surpass 400' is not due to *LepR*1 or *LepR2* but is controlled by another gene that also maps to linkage group N10, about 20 cM distant from *LepR2*. The resistance due to the 'Surpass 400' gene (named as *LepR3*) and *LepR2* can also be distinguished by cotyledon assays, using an isolate of *L. maculans*. This isolate is virulent on lines containing only *LepR2* but avirulent on the lines with the *LepR3* gene (Yu et al. 2004).

In most plant breeding programs, disease assessment of breeding material can be conducted only once a year during the growing season, and the selection efficiency depends on disease development, which varies greatly with environment (Rimmer and van den Berg 1992). Therefore, a cotyledon assay at the seedling stage conducted under controlled environmental conditions, using *L. maculans* isolates, has been used to minimize environmental variation as well as to accelerate the development of cultivars (McNabb et al. 1993). For this to be successful, a close association between seedling and adult stem canker resistance is necessary. In addition, virulence to the seedling resistance in the pathogen population where the resistance will be deployed must be absent or rare. However, a lack of correlation between cotyledon (seedlings) resistance and stem (adult plants) has been reported in resistance sources from *B. napus* and from B-genome species (Salisbury et al. 1995). In this case, cotyledon resistance is an unreliable marker for identifying plants with stem canker resistance derived from these sources. However, in the case of *LepR1* and *LepR2*, the same sets of lines from two DH populations were evaluated for stem resistance in blackleg disease nurseries in Australia, where isolates of *L. maculans* are considered to be generally more aggressive than in Canada (Kutcher et al. 1993), and also for cotyledon resistance to different isolates of *L. maculans* under greenhouse condition. Our results show that cotyledon resistance was consistently associated with stem canker resistance, albeit with a small population, and suggests that the seedling and field resistance phenotypes are controlled by the same gene or by two tightly linked loci. This indicates that cotyledon assays (for phenotyping resistance conferred by *LepR1* and *LepR2*) should be valuable for development of stem canker resistance breeding lines.

To most isolates, *LepR1* conferred greater resistance in the seedling stage than *LepR2*. However, lines containing *LepR2* exhibited a similar level of stem canker resistance as *LepR1* under field conditions, both showing complete stem canker resistance in Australia (G. Buzza, personal communication). Systemic growth of *L. maculans* from leaf infection, via the petiole to the stem is the main pathway for stem canker development (Hammond et al. 1985). Since *LepR1* confers high levels

of resistance in the seedling stage, and very few hyphae grew within the leaves of seedlings, presumably, no or very few hyphae reach the stem. *LepR2* confers partial seedling resistance to most of the isolates, characterized by reduced fungal growth. It is possible that a few hyphae develop in leaves, but that very few reach the vascular bundles and spread down petioles. We presume that very few or no hyphae colonize the stem, again resulting in complete canker resistance. This still needs to be confirmed.

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