Mapping Loci Controlling *Brassica napus* Resistance to *Leptosphaeria maculans* under Different Screening Conditions

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ABSTRACT

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Leptosphaeria maculans, the causal agent of blackleg of crucifers, is a major threat to rapeseed (*Brassica napus*) production throughout the world. Genes controlling blackleg resistance in *B. napus* were mapped using an F_1-derived doubled haploid (DH) population of 105 lines and 138 restriction fragment length polymorphism (RFLP) markers. The host-pathogen interaction phenotype was assessed qualitatively and by several quantitative measurements using different environments and plant developmental stages. A single major locus controlling blackleg resistance (LEM1) was mapped to linkage group 6 based on qualitative scores of the interaction phenotype on inoculated cotyledons. This resistance locus was also identified by interval mapping using quantitative measurements of the interaction phenotype on cotyledon- and stem-inoculated plants. Four other genomic regions were significantly associated with quantitative measurements of resistance on cotyledon and stem, among them a marker locus interval in linkage group 17 that included a pathogenesis related gene (PR2). Two genomic regions associated with resistance in field-evaluated plants were different from those identified in cotyledon- and stem-evaluated plants. The use of different environments and plant developmental stages for mapping disease resistance loci is discussed.

Additional keywords: mapping disease resistance genes, RFLP.

Blackleg of crucifers, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.), is one of the most important diseases of *Brassica* species. The pathogen is a constant threat to crop production, especially to oilseed *Brassica* species (*B. napus* L. and *B. rapa* L. (syn. *B. campestris*)) (7). Despite the importance of this disease and the possibility of manipulating both organisms genetically (25), little research has been devoted to genetic studies of host resistance to the pathogen (19). Resistant rapeseed cultivars of *Brassica napus* (e.g., Jet Neuf, Major, and Crésor) have been developed through breeding. Studies examining the numbers, effects, allelic relationships, and location of the genes controlling resistance are limited. Also, very little information is available about the control of disease resistance at different stages of plant development (1,2,6), and the use of different plant materials, pathogen cultures, and environments has made comparisons among experiments difficult (3,23).

Many host-pathogen relationships are genetically complex and difficult to access experimentally. The use of homozygous plant lines and pathogen isolates, whenever available, can simplify and facilitate the understanding of the genetic mechanisms involved in these relationships. Single ascospore-derived isolates can be obtained from *L. maculans* pseudothecia to reduce genetic variation in the fungus (17), and techniques such as microspore culture of F_1 plants can be used to develop a segregating population of completely homozygous host lines (8,2). Homozygous plant materials can be used to assess the host-pathogen interaction phenotype under different screening conditions and plant developmental stages. Several different measurements of the interaction phenotype can be obtained and used in conjunction with restriction fragment length polymorphism (RFLP) linkage maps to locate important genes related to plant-pathogen interaction. This approach may provide insight on the genetic mechanisms involved in resistance and supply information that can be used in designing breeding strategies.

The *B. napus–L. maculans* association is an interesting biological system to test such an approach. In this study, a *B. napus* F_1-derived doubled haploid (DH) population and RFLP linkage map (5) were used to identify loci controlling blackleg resistance that are expressed under different screening conditions, and to compare the number, location, effects, and parental allelic contributions of loci associated with resistance.

MATERIALS AND METHODS

Plant population development. The *B. napus* biennial cultivar Major, resistant to *L. maculans* isolate PHW 1245, and the susceptible annual cultivar Stellar were crossed and a single hybrid plant was used to develop an F_1-derived DH population (4,5). Three experiments, as described below, were conducted with 105 DH lines, self-pollinated progeny of the two parents, the F_1 hybrid, and controls. The controls were three *B. napus* cultivars (Glacier, Quinta, and Westar) that have differential responses to *L. maculans* isolates (10,18).

Cotyledon interaction. *B. napus* seedlings were grown in a controlled environment chamber at 24 C with continuous cool-white irradiance of 220 $\mu E \cdot m^{-2} \cdot s^{-1}$. Seeds were germinated in Com-Pack D812 trays filled with autoclaved soil:sand:Jiffy Mix (1:1:1) and fertilized daily with 1.0× Hoagland's solution. Seven days after sowing, one side of each fully expanded cotyledon was wounded by puncturing with a 24 gauge needle. The wound site was immediately inoculated with 10 μl of 10^7 pycnidiospore per milliliter suspension obtained from the *L. maculans* single ascospore-derived isolate PHW 1245 (supplied by the Crucifer Genetics Cooperative). PHW 1245 was recovered from asci originally collected from *B. napus*-infected plants in France and belongs to *L. maculans* pathogenicity group 2 (PG2) (10).

Pycnidiospore suspensions were prepared from pycnidia harvested from 8-day-old cultures grown at 22 C on V8 medium under continuous cool-white fluorescent light with an irradiance of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. Pycnidia were scraped from the media surface, and pycnidiospores were suspended in sterile deionized water, filtered through a 150-μm mesh screen, and quantified by hemacytometer. Pycnidiospore suspensions were stored in 1.5-ml microcentrifuge tubes, frozen at −20 C, and thawed just before inoculation.

A randomized complete block design (RCB) was used to evaluate the plant-pathogen interaction phenotype. Each plot consisted of two plants of a DH line replicated six times, and the plot mean constituted the experimental unit. In order to delay cotyledon senescence, true leaves were cut at the petiole insertion every other day. The interaction phenotype of each plant was evaluated 14 days after inoculation by three procedures: discrete classification of the lines as resistant or susceptible; scoring based on a qualitative scale from 0 to 9, where 0 = immune and 9 = very susceptible, based on descriptive comparisons of increasing injury to the cotyledon accompanied by increased sporulation of the fungus (24); and measurement of the lesion diameter (in millimeters).

Stem interaction. The stem inoculation experiment was conducted in a greenhouse with minimum cool-white continuous fluorescent irradiance of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 22 ± 2 C. Seeds were germinated in Rootrainers model 645d filled with autoclaved soil:sand:Jiffy Mix (1:1:1) and fertilized twice a week with 1.0× Hoagland's solution. Four weeks after germination, each plant stem was wounded at the cotyledon node with a 24 gauge needle adjusted to give maximum tissue penetrance of 2 mm. Wounded plants were immediately inoculated with 10 μl of a 1×10^7 pycnidiospore per milliliter suspension of the *L. maculans* isolate PHW 1245, prepared as described above. Plants were held in the horizontal position for 40 min before another 10 μl of the spore suspension was dispensed to the wound.

An RCB design was used to evaluate the plant-pathogen interaction phenotype. Each plot consisted of two plants of a DH line replicated three times, and the plot mean was the experimental unit. The interaction phenotype of each plant was evaluated 8 wk after inoculation by three procedures: scoring based on a qualitative scale from 0 to 9, where 0 = no response and 9 = very high susceptibility evidenced by stem girdling and plant death (4); estimating the percentage of discoloration on a transverse section of the stem 5 mm above the inoculation point; and measuring the length of the lesion (in millimeters) on an internal longitudinal section of the stem from the point of inoculation upward.

Field interaction. The field experiment was located in Souris, Manitoba, Canada. The experiment relied on natural inoculation in a field used for *L. maculans* resistance screening trials for the last 8 yr. Infected plant stubble from the previous year was scattered in the field at the beginning of spring to increase inoculum pressure. *L. maculans* isolates from Canada belong predominantly to PG2 (17,19), the same pathogenicity group as PHW 1245. One hundred seeds of each DH line were sowed in early May in single-row plots in an RCB design with three replicates. The interaction phenotype of each plant was evaluated 4 mo after sowing during ripening using a scoring scale of 0 (resistant) to 9 (susceptible) (23) based on the discoloration of a transverse section at the crown of the plant. The mean of 15 plants per plot was the experimental unit.

RFLP data analysis. A genetic map based on RFLPs was constructed using the 105 DH lines (5). Segregating RFLP loci were scored as homozygous M/M for RFLP alleles coming from the parent Major or as homozygous S/S if coming from Stellar. The map included 138 loci on 22 linkage groups plus six pairs of linked loci and covered 1,016 centimorgans (cM) of the *B. napus* genome with an average interlocus distance of 7.7 cM. In addition, six other unlinked RFLP marker loci were used in the analysis. Procedures for DNA clone selection, probe hybridization, RFLP segregation analysis, and map construction are described elsewhere (5).

Statistical analysis. A single gene model for resistance to *L. maculans* and for linkage of resistance to 138 RFLP marker loci was tested by chi-square analysis using orthogonal functions (15). The putative resistance locus and RFLP loci were mapped using the computer program MAPMAKER v2.0 (13). A minimum LOD (log of the odds ratio of linkage vs. no linkage) score of 3.0 and maximum recombination frequency of 0.35 was used to group loci, and the most probable locus order was determined by three point followed by multipoint analyses. Recombination frequencies were corrected based on Kosambi's map distance function (11).

Differences between the interaction phenotype means of the DH lines for all measurements in the stem and field experiments were tested by using generalized linear models (21) to partition total variation into effects due to lines, replications, and error. Kendall's τ (9) was calculated to determine correlations among trait measurements.

Interval mapping analyses (12) were performed for all quantitative measures of resistance in the three experiments using the computer program MAPMAKER/QTL (14). Putative quantitative trait loci (QTL) for resistance were identified using an LOD (log of the odds likelihood ratio that a QTL is present vs. absent) threshold of 2.0. Only the additive effect of allelic substitution on resistance could be estimated since there were no heterozygous lines in the DH population. The percentage of the variance explained by an interval associated with resistance (R^2) also was estimated using MAPMAKER/QTL.

RESULTS

Cotyledon interaction. Inoculated cotyledons of Stellar were highly susceptible to *L. maculans* isolate PHW 1245 (Table 1), developing large gray-brown necrotic lesions and extensive pycnidia. Major and the F_1 hybrid were highly resistant (Table 1), showing a hypersensitive reaction to the pathogen characterized by the death of the cells surrounding the inoculation point.

The three kinds of measurement of interaction phenotype, dis-

TABLE 1. Mean interaction phenotype (± standard deviation) for *Brassica napus* cultivars and populations with *Leptosphaeria maculans* isolate PHW 1245 in three experiments

Experiment	Interaction phenotype measurements[a]	Major	Stellar	F_1	DH (range)	Glacier	Quinta	Westar
Cotyledon	Discrete classification	R	S	R	R or S	R	I	S
	Scoring scale	1.64 ± 0.64	8.83 ± 0.69	1.39 ± 0.58	1.00 to 8.67	1.78 ± 0.80	5.87 ± 1.94	8.87 ± 0.34
	Lesion diameter	2.07 ± 0.73	15.22 ± 2.52	2.71 ± 0.52	1.35 to 18.42	1.57 ± 0.46	6.80 ± 2.87	8.28 ± 1.44
Stem	Scoring scale	1.62 ± 1.18	8.55 ± 0.88	2.58 ± 1.16	1.25 to 8.33	2.57 ± 0.97	...[b]	8.28 ± 1.44
	%DTS	2.62 ± 2.38	67.50 ± 27.34	2.75 ± 1.42	0.75 to 74.00	2.71 ± 1.46	...	43.33 ± 23.09
	ILL	18.61 ± 15.01	109.00 ± 34.02	24.69 ± 15.42	8.45 to 113.32	35.31 ± 31.18	...	56.00 ± 8.54
Field	Scoring scale	0.04 ± 0.07	2.61 ± 0.71	0.26 ± 0.36	0.00 to 2.28	0.06 ± 0.06	0.13 ± 0.15	4.58 ± 1.4

[a]Interaction phenotypes measured on cotyledons as discrete classifications (R = resistant, S = susceptible, I = intermediate), scoring scale of 0 (resistant) to 9 (susceptible), and lesion diameter (mm); on stems as scoring scale of 0 (resistant) to 9 (susceptible), percent discoloration of the transverse section of the stem (%DTS) 5 mm above the inoculation point, and internal length of the lesion (ILL) in mm; in the field as scoring scale of 0 (resistant) to 5 (susceptible) based on the percent discoloration in a transverse section of the plant crown.
[b]Not determined.

crete classification as resistant or susceptible, scoring scale, and lesion size were highly correlated (Kendall's $\tau > 0.97$). Lesion diameter, for example, exhibited a bimodal distribution (Fig. 1A). When classified in discrete susceptible or resistant categories, the DH lines segregated as 61 resistant and 43 susceptible, which fit a 1:1 ratio ($\chi^2 = 3.11$, $P > 0.05$), indicating that alternate alleles at a single locus, designated LEM1, controlled cotyledon resistance to *L. maculans* in this population. The reaction of the F_1 population to the pathogen indicated that the allele for resistance from Major was dominant to the allele for susceptibility from Stellar. LEM1 was linked to seven RFLP marker loci ($\chi^2 > 26.38$, $P < 0.001$) in linkage group (LG) 6. An additional marker locus, *pcr155*, identified by bulked segregant analysis using RAPD markers (4), was linked to LEM1 ($\chi^2 = 78.87$, $P < 0.001$). LEM1 was mapped to the marker locus interval *wg2a3b–tg5d9b* of LG 6 (Fig. 2).

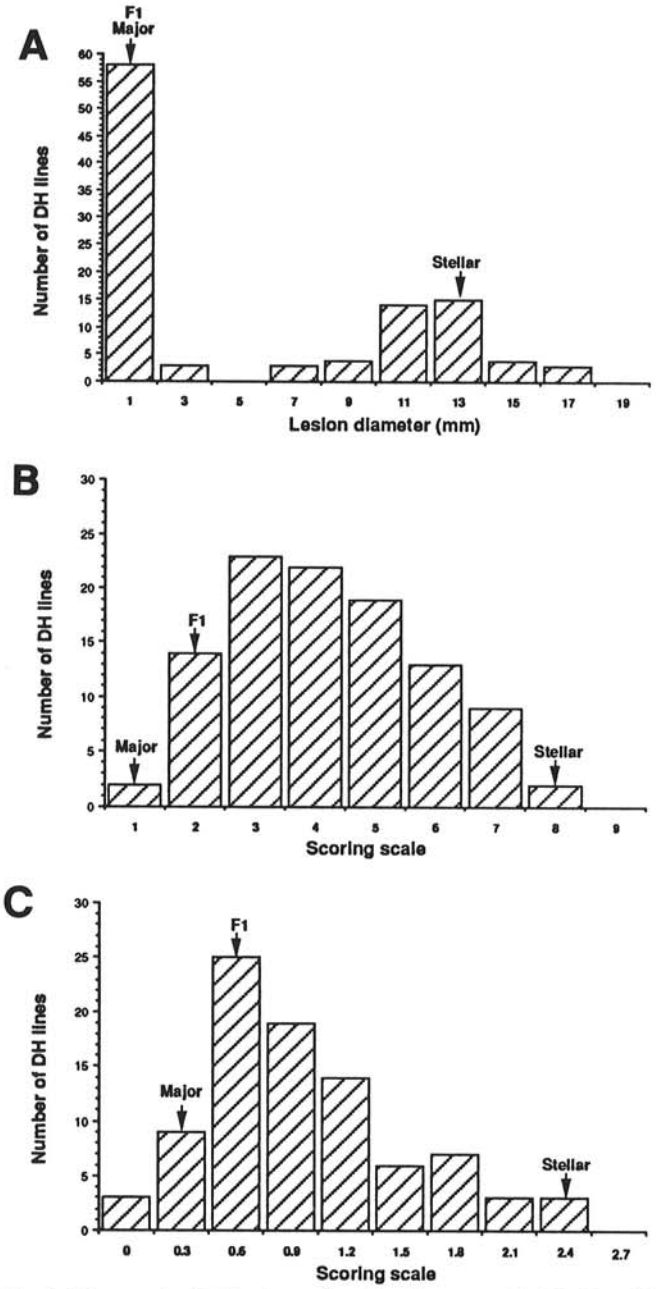

Fig. 1. Phenotypic distributions of a *Brassica napus* doubled haploid population for some interaction phenotypes (IP) with *Leptosphaeria maculans* isolate PHW 1245 in three experiments. **A,** cotyledon experiment, IP based on the diameter of the lesion; **B,** stem experiment, IP based on a scoring scale of increasing susceptibility (0 to 9); and **C,** field experiment, IP based on scoring scale of increasing susceptibility (0 to 5).

This same region in LG 6 was strongly associated with the quantitative measures of resistance (scoring scale and lesion diameter) and explained most of the variation ($R^2 = 90\%$) for resistance (Table 2). In addition to this interval, putative QTL for resistance were identified in LG 8, LG 17, and pair 4 (Table 2). The peak LOD score in LG 17 was only 5 cM from PR2, a locus homologous to a pathogenesis-related gene cloned from *Arabidopsis thaliana* (Fig. 3). The allelic contribution to resistance of all intervals identified in the cotyledon experiment was from the parent Major, except for the interval in LG 8 (Table 2), where a small contribution to resistance came from the parent Stellar.

Stem interaction. Stellar plants inoculated at the stem were highly susceptible to *L. maculans* isolate PHW 1245 (Table 1). A few plants died in the course of the experiment and most showed stem girdling, wilting, and widespread blackening and pycnidia development around the inoculation point. Major and the F_1 hybrid were resistant to the pathogen (Table 1) and showed restricted tissue discoloration around the inoculation point and lack of pycnidia. The three measurements of the DH population interaction phenotype (scoring scale, percentage of discoloration, and internal length of the lesion) were positively correlated. The magnitude of the correlation between the first two measurements was high (Kendall's $\tau = 0.80$), but both were poorly correlated with internal length of the lesion (Kendall's $\tau < 0.37$). The DH population was normally distributed for all measurements of the interaction phenotype (Fig. 1B showing scoring scale as an example), except for percentage of discoloration, which was normalized with a square-root transformation. The analyses of variance indicated significant differences between the means of the DH lines for all these measurements ($P < 0.0001$).

Putative QTL for resistance based on the scoring scale and percentage of discoloration were identified in LG 6 and LG 17 (Table 2). In LG 6, the peak LOD score was in the marker interval adjacent to the marker interval containing LEM1 (Table 2, Fig. 2), and in LG 17 the interval PR2-*tg5b2* was associated with stem resistance (Table 2, Fig. 3). Only one interval on LG 9 was significantly associated with resistance based on internal length of the lesion (Table 2). In all three intervals, the allelic contribution to resistance came from the parent Major.

Field experiment. Stellar was susceptible to *L. maculans* in the field (Table 1), showing grayish lesions in the stem and significant discoloration of the vascular tissue in the transverse section of the crown. Major and the F_1 were resistant (Table 1), showing very restricted vascular tissue discoloration or no disease symptoms.

The interaction phenotype distribution of the DH lines was not normal (skewness = 1.12; kurtosis = 1.53) (Fig. 1C), but a logarithm transformation normalized the data (skewness =

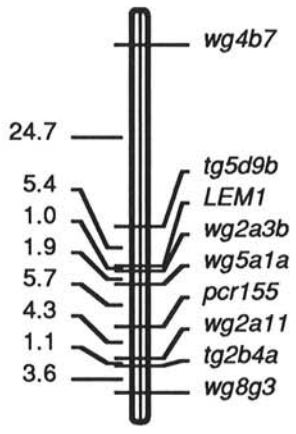

Fig. 2. Linkage group 6 of *Brassica napus* based on analysis of doubled haploid lines for segregation of molecular markers and disease resistance. LEM1 is the major locus controlling resistance to *Leptosphaeria maculans* isolate PHW 1245 in cotyledon-inoculated plants. *tg* and *wg* designate restriction fragment length polymorphism (RFLP) loci, and *pcr155* is a RAPD locus. Genetic distances (left) are in centimorgans.

TABLE 2. Peak LOD (log of odds ratio) scores (and R^2 values) for marker locus intervals in linkage groups (LG) of *Brassica napus* significantly associated with resistance to *Leptosphaeria maculans* based on different interaction phenotype measurements in three experiments

Experiment	Interaction phenotype measurement[a]	LG6 WG5A1a–WG2A3b–TG5D9b	LG8 WG1G3a–WG6A11	LG9 WG5a5–WG8G1b	LG12 WG1Ga–wg7b3	LG17 PR2–TG5B2	LG21 WG6F3–WG7B5b	Pair 4 WG5A1b–TG5D9a
Cotyledon	Scoring scale	50.32 (89.7) 51.29 (90.70)	...[b]	...	...	3.32 (18.10)	...	4.91 (21.00)
	Lesion diameter	50.01 (89.4) 51.38 (90.70)	2.01 (10.20)	...	...	3.71 (20.30)	...	3.67 (17.20)
Stem	Scoring scale	8.89 (32.80) 8.74 (31.02)	...	...	...	2.18 (11.40)	...	...
	%DTS	11.37 (40.80) 10.87 (39.46)	...	...	...	...	...	...
	ILL	...	...	3.58 (15.80)	...	...	...	...
Field	Scoring scale	...	...	...	3.62 (19.20)	...	3.10 (14.80)	...

[a]Interaction phenotypes measured on cotyledons as scoring scale of 0 (resistant) to 9 (susceptible), and lesion diameter (mm); on stems as scoring scale of 0 (resistant) to 9 (susceptible), percent discoloration of the transverse section of the stem (%DTS) 5 mm above the inoculation point, and internal length of the lesion (LL) in mm; in the field as scoring scale of 0 (resistant) to 5 (susceptible) based on the percent discoloration of a transverse section of the plant crown.
[b]Indicates interval was not significantly associated with trait.

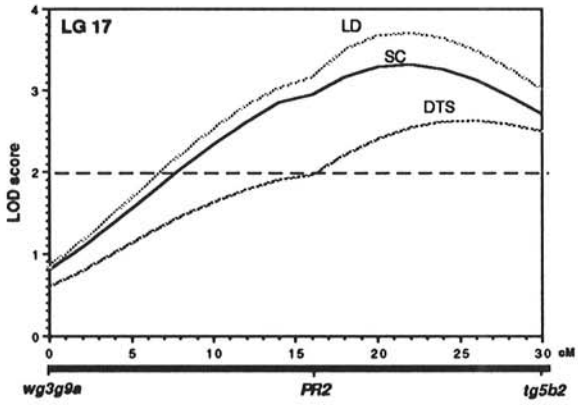

Fig. 3. LOD (log of the odds ratio) profiles for loci controlling resistance to the *Leptosphaeria maculans* isolate PHW1245 on LG 17 of *Brassica napus*. Resistance assayed by measuring lesion diameter (LD) and by using a scoring scale (SC) on cotyledon-inoculated plants, and by estimating the percent discoloration in a transverse stem section (DTS) on stem-inoculated plants. The y axis gives LOD scores ($\log_{10}$ of the likelihood odds ratio that a QTL is present vs. absent) and the x axis shows position of marker loci and their map distances in centimorgans (cM).

0.127; kurtosis = 0.546). The analysis of variance indicated that there were significant differences between the means of the DH lines ($P < 0.0001$). Intervals in LGs 12 and 21 were significantly associated with field resistance to *L. maculans* (Table 2). For both regions, alleles from Major increased resistance.

DISCUSSION

The use of a DH population for RFLP mapping and disease screening was effective in identifying loci controlling resistance to *L. maculans* in *B. napus*. Since each of the DH lines was completely homozygous, several different measurements of the plant-pathogen interaction phenotype, some of which were destructive, could be taken. Various developmental stages of *B. napus* and different disease screening protocols were used, allowing the comparison of results under different experimental conditions. Overall, seven genomic regions were associated with resistance. Within the cotyledon and stem experiments, the same genomic regions usually were associated with highly correlated interaction phenotype measurements. Interaction phenotype measurements that were poorly correlated within the stem experiment were associated with different marker intervals.

A major locus controlling resistance, designated LEM1, was identified in LG 6 by scoring plants as resistant or susceptible in the cotyledon experiment. The region containing this locus explained a large proportion of the variation for the other interaction phenotype measurements in the cotyledon experiment, and it may be associated with the control of specific resistance. The distribution of interaction phenotype measurements in the stem experiment suggested polygenic control of resistance in stem-inoculated plants. Three marker intervals with different effects on the interaction phenotype variation were identified, and the interval with the highest effect included the LEM1 locus. Marker loci clustered around LEM1 could be used for more detailed genetic analysis of the locus.

A marker interval in LG 17 (PR2-*tg5b2*) also was identified by interaction phenotype measurements in both cotyledon and stem experiments. This interval includes a locus homologous to PR2, a pathogenesis-related gene structurally similar to β-1,3-glucanase and probably associated with the enhancement of chitinase production (22). Chitinases are reported to be involved in in vitro antifungal activity (16,20) and could function in resistance by hydrolysis of fungal cell walls. In *A. thaliana,* PR2 expression is increased by inoculation with *Pseudomonas solanacearum* pv. *tomato* or with exposure to 2,6-dichloroisotonic acid (INA) or salicylic acid (22). A chitinase gene recently cloned from *B. napus* is associated with the control of resistance to *L. maculans* by differential expression in resistant and susceptible infected cultivars (18). Fine structure mapping of resistance genes with marker loci in this region and studies on PR2 expression in resistant and susceptible plants may provide further information on the role of PR2 in the interaction phenotype.

Some of the other regions associated with resistance contributed interesting information about the nature of the host-pathogen interaction. Marker intervals in pair 4 and LG 8 were significantly associated with interaction phenotype measurements taken in the cotyledon experiment but were not in the other experiments. The interval in LG 8 was the only interval significantly associated with resistance where the contribution for resistance came from the susceptible parent Stellar. An interval in LG 9 was associated only with the measurement of the internal length of the lesion in the stem experiment and was not associated with resistance in the other experiments. This interval also was associated with the control of days to flower and vernalization requirement in *B. napus* (4). It is possible that the internal length of the lesion was reduced by the high number of plant nodes (short internodes or rosette), typical of vernalization requiring *Brassica* types, causing the interaction phenotype to appear more resistant. Plant morphology can be an important component of resistance to *L. maculans,* particularly for field resistance (6).

The LEM1 locus, which had a major effect on resistance in the cotyledon and stem experiments, did not seem to be important for resistance in the field experiment. In fact, none of the intervals associated with resistance in the cotyledon and stem experiments were found to be associated with resistance in the field experiment. Genes in at least two other regions of the genome appeared to control resistance in the field. Different resistance genes may be operating in the field due to differences in plant development stage and environment, or to differences in virulence in the field pathogen population and in isolate PHW 1245. Isolates of *L. maculans* collected in the field at the end of the season were classified as PG2, the same pathogenicity group as PHW 1245 (4). However, some of the isolates classified as PG2 were highly virulent to Major as well as to Stellar, and mixtures of some

of these isolates with PHW 1245 significantly altered the interaction phenotype measurements of Major and Stellar in controlled experiments (4). This suggests that the results of the field experiment were different from those of the cotyledon and stem experiments, due at least in part to genetic variation for virulence in the *L. maculans* field population.

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