Genetic Analysis and Identification of Amplified Fragment Length Polymorphism Markers Linked to the *alm1* Avirulence Gene of *Leptosphaeria maculans*

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ABSTRACT

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A gene-for-gene interaction was previously suggested by mapping of a single major locus (*LEM 1*) controlling cotyledon resistance to *Leptosphaeria maculans* isolate PHW1245 in *Brassica napus* cv. Major. In this study, we obtained further evidence of a gene-for-gene interaction by studying the inheritance of the corresponding avirulence gene in *L. maculans* isolate PHW1245. The analysis of segregating F₁ progenies and 14 test

crosses suggested that a single major gene is involved in the interaction. This putative avirulence gene was designated *alm1* after the resistance locus identified in *B. napus*. Amplified fragment length polymorphism (AFLP) markers were used to generate a rudimentary genetic linkage map of the *L. maculans* genome and to locate markers linked to the putative avirulence locus. Two flanking AFLP markers, AC/TCC-1 and AC/CAG-5, were linked to *alm1* at 3.1 and 8.1 cM, respectively. Identification of markers linked to the avirulence gene indicated that the differential interaction is controlled by a single gene difference between parental isolates and provides further support for the gene-for-gene relationship in the *Leptosphaeria-Brassica* system.

Blackleg disease caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not, anamorph *Phoma lingam* (Tode:Fr.) Desmaz., is one of the major diseases of crucifers, especially rapeseed (*Brassica napus*). Based on commonly shared phenotypic characters including host specificity, growth characters, physical and chemical phenotypes, and DNA characteristics, *L. maculans* isolates are consistently divided into two distinct groups widely referred to in the literature as aggressive group and nonaggressive group. Much effort has been devoted to distinguishing between these two groups, and it has been proposed that they might actually represent different species (2,6,11-14,20,23-29,31,34,36). Among aggressive group isolates, there is also a range of pathogenic variation with respect to their interaction phenotype (IP) on differential host cultivars. Aggressive group isolates have been further differentiated into three pathogenicity groups (PG) based on cotyledon reactions on the *B. napus* cvs. Westar, Quinta, and Glacier (14,21). By using different sets of host lines, aggressive group isolates have been identified as having 5, 6, or even as many as 19 distinctive virulence phenotypes (3,15,16). However, little is known about the genetics of virulence of the fungus.

The gene-for-gene hypothesis has been either suggested or demonstrated in many pathosystems and has provided the foundation for the molecular cloning of pathogen avirulence (*avr*) genes and their corresponding host resistance (*R*) genes (5,9,32). The mechanisms underlying such interactions have been proposed to involve specific recognition of a ligand (generated directly or indirectly by an *avr* gene) by a plant receptor encoded by an *R* gene that leads to a defense response (32). Recently, direct interaction between the protein encoded by the *avrPto* gene of *Pseudomonas syringae* pv. *tomato* and the protein encoded by the *Pto R* gene of tomato was

demonstrated using the yeast two-hybrid system (30,33). A number of *avr* genes that have been cloned and studied are from bacteria, and only a few *avr* genes have been cloned from fungi.

In the *Leptosphaeria-Brassica* system, a gene-for-gene interaction has been previously suggested by genetic analysis of *B. napus* cv. Major resistance to *L. maculans*. A single major locus (*LEM 1*) controlling resistance to *L. maculans* isolate PHW1245 (PG2) in *B. napus* cv. Major was mapped based on the interaction phenotype on inoculated cotyledons using a segregating population of doubled haploid lines (8). However, the genetics of the corresponding *avr* gene in *L. maculans* isolate PHW1245 have not been elucidated.

Amplified fragment length polymorphisms (AFLPs) are dominant genetic markers that have several advantages over other molecular markers such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) (35). The AFLP technique has been used to study the population structure of the fungus *Pyrenopeziza brassicae*, the causal agent of light leaf spot of *Brassica* spp. (18), and to analyze gene pool similarities of potato cyst nematode populations, *Globodera rostochiensis* and *G. pallida* (10). We demonstrate here the use of AFLP techniques for detection and linkage analysis of polymorphisms between *L. maculans* isolates.

The objective of this study was to elucidate the inheritance of avirulence in *L. maculans* isolate PHW1245. Segregation of the interaction phenotype in *L. maculans* and identification of AFLP markers linked to an *avr* gene provide more complete evidence of a gene-for-gene interaction in this system.

MATERIALS AND METHODS

Fungal cultures. Two isolates of *L. maculans*, PHW1245 and PHW1223, were obtained from the Crucifer Genetic Cooperative (CrGC) in the Department of Plant Pathology at the University of Wisconsin-Madison. PHW1245 and PHW1223, which belong to PG2 and PG4, respectively, differ in virulence based on their in-

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teraction phenotype on *B. napus* cv. Major. Fungal isolates were maintained on V-8 agar plates at 24°C under continuous cool-white fluorescent light (100 to 150 $\mu\text{E s}^{-1} \text{m}^{-2}$).

Mating procedure. Mycelial disks of the two fungal isolates were placed about 1 cm apart on V-8 agar plates. Mycelia intermingled after 7 days of incubation at 24°C under continuous light. At this point, mating plates were overlaid with 2% water agar and incubated at 16°C under a 12/12-h (UV light/dark) photoperiod. Pseudothecia were formed after a 4-week incubation. Progeny were obtained and inocula were prepared as previously described (22).

Pathogenicity test. Seeds of a doubled haploid line of *B. napus* cv. Major and susceptible *B. napus* cvs. Westar and Stellar were sown in plastic flats fitted with 96-celled packs containing a 1:1 peat/vermiculite soilless growing medium, Jiffy-mix (Jiffy Products, Batavia, IL). Seedlings were maintained in a growth chamber at 24°C with continuous light and fertilized with 50% Hoagland's solution or with a solution of N-P-K (20-20-20 Peter's solution; United Industries, St. Louis) at 4, 7, 14, and 21 days after sowing. Both cotyledons were wounded at the center of each half of each cotyledon 7 days after sowing. Droplets (10 μl) of pycnidiospore suspensions (1.5×10^7 spores per ml) were placed on the wounds, and the plants were returned to the growth chamber. At 14 days after inoculation, IPs were rated on the 0 to 9 scale described previously (14). The experiment was conducted using a randomized complete block design. There were four plants in each of three blocks, and two cotyledon sites were inoculated on each plant. For analysis, an average of the 16 readings (two on each cotyledon of four plants) constituted the experimental unit. Progeny with mean IP ratings between 0 and 3 were classified as avirulent, between 4 and 6 were classified as intermediate, and between 7 and 9 were classified as virulent. No intermediate phenotypes were observed among the progeny tested on 'Major'. Thus, the ratio of avirulent/virulent progeny was tested for deviation from Mendelian expectation by chi-square analysis.

DNA preparation. *L. maculans* isolates were cultured in Czapek's medium supplemented with 0.2% yeast extract with agitation at room temperature for 2 to 5 days. Mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England), washed with sterile water, and then kept at -80°C for several hours and lyophilized. Freeze-dried mycelia (200 mg) was ground in liquid nitrogen using a mortar and pestle, and then suspended in 4 ml of extraction buffer (50 mM

Tris-HCl [pH 8.0], 850 mM NaCl, 100 mM EDTA, and 1% sodium dodecyl sulfate) and 0.4 ml of 10% (wt/vol) cetyltrimethylammonium bromide (in 0.7 M NaCl). After incubation at 65°C for 15 min, the mixture was extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then with one volume of chloroform/isoamyl alcohol (24:1). The DNA was then precipitated with two volumes of ice-cold 100% ethanol, washed with 70% ethanol, and suspended in 1 ml of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). All DNA extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng/ μl for AFLP analysis.

AFLP and linkage analysis. AFLP markers were generated by methods described previously (35). Total genomic DNAs were digested with *Hind*III and *Mse*I restriction enzymes and ligated with adapters. These fragments served as templates for the polymerase chain reaction (PCR). Primer combinations used in the PCR contained complimentary sequences to the adapters plus two and three additional bases at the 3' end of the *Hind*III and *Mse*I adapters, respectively. Each amplified fragment that showed polymorphism between the two parents and segregated in the progeny was treated as an independent genetic locus. AFLP analysis using each primer combination was repeated on the parents and a subset of progeny, and only marker loci that gave repeatable results were used in the map.

Linkage analysis among AFLP loci and the avirulence locus was performed using the MAPMAKER v2.0 computer program (8). A minimum log of the odds ratio of linkage versus no linkage (LOD) score of 4.0 and maximum recombination frequency of 0.20 was used to group loci. The order was determined by a three-point analysis followed by a multi-point analysis. Recombination frequencies were corrected based on Kosambi's map distance function. The validity of linked markers was confirmed by chi-square analysis at 95% confidence (19).

RESULTS

Disease symptoms on cotyledons of *B. napus* cvs. Major, Westar, and Stellar were evaluated 14 days after inoculation, and the analysis of variance was performed. The results indicated that there was no significant difference between the three blocks of the experiment. Thus, data are presented as averages of 24 inoculation sites

TABLE 1. Parental isolates, interaction phenotype (IP), observed ratio of avirulent/virulent (avr/vir) progeny, and the chi-square values for segregation ratios on *Brassica napus* cv. Major

Parents	Parents	IP ^a	avr/vir	χ^2 ^b	
				1:1	1:0
PHW1245 × PHW1223	3	9	52:48	0.16	...
1-040 × PHW1245	3	3	11:0	...	0
1-040 × 1-054	3	3	24:0	...	0
1-037 × 1-040	3	3	38:0	...	0
2-012 × 2-006	3	3	32:0	...	0
2-027 × 2-006	3	3	10:0	...	0
2-019 × 2-006	3	3	26:0	...	0
2-032 × 2-006	3	3	35:0	...	0
2-005 × 2-006	3	3	30:0	...	0
2-005 × PHW1223	3	9	9:9	0	...
2-032 × 2-013	3	9	5:4	0.11	...
2-019 × 2-010	3	9	16:11	0.92	...
2-032 × 2-026	3	9	10:4	2.57	...
2-032 × 2-004	3	9	14:6	3.2	...
2-012 × 2-013	3	9	12:12	0	...

^a Interaction phenotype was evaluated on a 0 to 9 scale that was an average of 24 inoculation sites.

^b The 1:1 expected ratio for crosses between avirulent and virulent isolates; the 1:0 expected ratio for crosses between two avirulent isolates. There is no significant difference. ... = Model not used.

Fig. 1. Interaction phenotype distribution of progeny (on *Brassica napus* cv. Major) from a cross between PHW1223 and PHW1245 scored using a previously described 0 to 9 rating scale (14) at 14 days postinoculation.

from 12 plants. Both *L. maculans* isolates PHW1223 and PHW1245 were virulent on cvs. Westar and Stellar, producing large areas of gray-green collapsed tissue with abundant pycnidia (IP 8 to 9). These two parental isolates differed in virulence based on their interaction phenotype on a double haploid line of 'Major'. Isolate PHW1223 was virulent (IP 9), whereas PHW1245 was avirulent (IP 3) on 'Major' and consistently caused a susceptible reaction and a restricted necrotic lesion, respectively.

A total of 100 single-ascospore progeny isolates were obtained from the cross between these two isolates, and virulence tests were performed on cotyledons of 'Major'. The interaction phenotypes of the progeny on 'Major' were either avirulent (IP 0 to 3) or virulent (IP 7 to 9). The progeny population segregated 52 avirulent isolates to 48 virulent isolates, suggesting that the interaction is controlled by a single avirulence gene (Table 1 and Fig. 1). Backcrosses and sibcrosses were used to further test the single avirulence gene model. The avirulent progeny 1-040 was backcrossed to the avirulent parent PHW1245, and all 11 progeny were avirulent. Sibcrosses between avirulent isolates all gave avirulent progeny on 'Major'. Progeny from backcrossing the avirulent F₁

progeny 2-005 to the virulent parent PHW1223 and the intercrosses between avirulent and virulent F₁ progeny segregated for avirulent/virulent in ratios not significantly different than 1:1 (Table 1). The results are consistent with the hypothesis of a single avirulence gene, and the putative avirulence gene was named *alm1* after the corresponding mapped resistance gene, *LEM1* (8).

Sixty-seven randomly selected progeny from the cross between PHW1245 and PHW1223 were analyzed with 14 primer combinations in the AFLP analysis. Each primer combination yielded 50 to 100 amplified DNA fragments. For each primer combination, an average of four to five bands were polymorphic between the parents and segregated consistently in the progeny. A total of 62 AFLP markers were used for linkage analysis, and these were given locus names indicating the additional sequences of the primer combinations used in the PCR. At the minimum LOD score of 4.0 and maximum recombination fraction of 0.2, these AFLP markers sorted into nine linkage groups, five pairs, and seven unlinked loci that covered a total of 340 cM (Fig. 2). Of the 62 markers, 18 (33%) deviated significantly ($P < 0.05$) from the expected 1:1 segregation ratio, and these were clustered in six genomic regions (Fig. 2). The *alm1* avirulence locus was located in linkage group 1 flanked by two AFLP markers, AC/TCC-1 and AC/CAG-5, at 3.1 and 8.1 cM, respectively. The validity of the linkage of markers on linkage group 1 to *alm1* was confirmed by chi-square analysis (Table 2).

DISCUSSION

Many host-pathogen relationships are genetically complex. Variation in the procedures for inoculation, disease rating, and interpretation among laboratories might affect the conclusions of genetic

TABLE 2. Linkage test between amplified fragment length polymorphism marker loci and the *alm1* avirulence locus using chi-square analysis (19)

Marker comparison	n ^a	AA:AB:BA:BB ^b	Chi-square ^c
<i>alm1</i> -AC/CCG1	54	20:4:2:29	32.46***
<i>alm1</i> -AC/CAG5	57	23:5:0:29	39.94***
<i>alm1</i> -AC/TCC1	57	26:2:0:29	49.51***
<i>alm1</i> -AC/TCG2	57	27:1:3:26	42.34***

^a Number of isolates used in comparison.

^b Class of genotype observed.

^c *** indicates $P < 0.001$.

Fig. 2. Amplified fragment length polymorphism linkage map of *Leptosphaeria maculans*. Map distances in cM are indicated on the left and marker number and locus name on the right. Markers exhibiting distorted segregation ratios are indicated with an asterisk (*).

analysis (7). In studying the genetics of pathogenicity, it is important to have genetically uniform hosts and environmental conditions to accurately assess genetic variation in the pathogen. The use of homozygous plant lines and single-spore-derived pathogen isolates can simplify and facilitate the study of the genetics of plant-pathogen relationships (8). In this study, the host was a doubled haploid line of *B. napus* cv. Major and was presumed to be homozygous for every locus. Therefore, the difference in IP observed was assumed to be the result of differences in the genetic background of fungal isolates growing in a relatively defined environment. Since the single-ascospore-derived progeny isolates obtained from a cross between virulent and avirulent parental isolates segregated for the parental phenotypes in a 1:1 ratio, it was tentatively assumed that a single avirulence gene is involved in the interaction.

However, the 1:1 ratio of avirulence/virulence progeny from the crosses alone may not be sufficient evidence to conclude that a single *avr* gene is operating. In *Magnaporthe grisea*, for instance, additional genes were identified in a cross that segregated in a 1:1 ratio (17). To gain reasonable evidence that the population segregates for a single gene that controls avirulence, several test crosses were performed. The results (Table 1 and Fig. 1) are consistent with a single-gene model.

Use of molecular markers to locate a genetic locus determining avirulence versus virulence provides additional evidence for a single-gene model (Fig. 2). One-third of the markers used in the analysis showed skewed segregation. The distorted segregation could be due to failure of amplification of the particular bands or to misscoring errors. However, distorted segregation occurred in both directions, absence or presence of fragments, and the results were reproducible. Hence, it is assumed that it is not the result of experimental errors; rather, it may have been due to differential viability of ascospores isolated from different progeny genotypes. Including markers with segregation distortion may generate false linkages. To reduce type I errors in linkage analysis using MAPMAKER, we used a relatively high threshold of 0.2 maximum recombination and a 4.0 minimum LOD score for grouping loci. Chi-square analysis (4,19) was used to verify the linkage of markers to *alm1* (Table 2).

Another *avr* gene in *L. maculans* isolate a.2, *AvrLm1*, controlling cultivar specificity on 'Quinta', has been described (1). The isolate a.2 belongs to PG3, while the isolate PHW1245 from this study belongs to PG2. High variability in *L. maculans* with respect to host and cultivar specificity suggests that there are several *avr* genes in this fungus. Future efforts to identify other *avr* and *R* genes in the system will advance our genetic understanding of the interactions and evolutionary process in the *L. maculans*-*B. napus* system.

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