Assessment of Genetic Variation Among *Leptosphaeria maculans* Isolates Using Pathogenicity Data and AFLP Analysis

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ABSTRACT

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Increased production of rapeseed in the north central and southeastern regions of the United States has been accompanied by an increase in the incidence of blackleg disease caused by *Leptosphaeria maculans*. In order to assess the genetic variability and relatedness of isolates from these regions to others from around the world, we analyzed 49 aggressive isolates representing three pathogenicity groups (PG2, 3, and 4) of *L. maculans* from North Dakota, Georgia, Ontario, Western Canada, the United Kingdom (UK), France, Germany, and Australia using pathogenicity data and amplified fragment length polymorphism (AFLP). Approximately 10% of the 400 amplified fragments were polymorphic, scored as discrete characters and analyzed by cluster analysis. The isolates from North Dakota, Western Canada, Georgia, and PHW1252 from the UK formed one tightly clustered group and were mostly of the same pathogenicity group; whereas isolates from Ontario, Australia, France, Germany, and the remaining isolates from the UK formed a second group, which exhibited greater variation and consisted of three pathogenicity groups. The similarity between North Dakota isolates collected in 1995-96 and Western Canadian isolates collected in the 1980s suggests that *L. maculans* was introduced into North Dakota from Western Canada, and that the populations have remained relatively unchanged over the past 10 years.

Since the United States Food and Drug Administration granted "generally recognized as safe" status to low erucic acid rapeseed (canola) in 1985, the consumption of canola oil in the United States, imported mostly from Canada, has increased rapidly. In response to the increasing demand for edible and specialized industrial oils, rapeseed production has increased in the United States. Rapeseed is grown as a winter crop in Kentucky and neighboring states and as a spring crop in northeastern North Dakota, Minnesota, Idaho, and Montana (5,10). In 1988, there were 15,176 ha (37,500 acres) of rapeseed in the United States, and by 1995, the area had expanded to 180,089 ha (445,000 acres). Total production reached 248 million kg (547 million pounds) and nearly equaled imports of 253 million kg (558 million pounds) (26).

Blackleg disease, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not., anamorph *Phoma lingam* (Tode:Fr.) Desmaz., is a major disease of crucifers,

including rapeseed (*Brassica napus* and *B. rapa*). It is a serious concern in all countries where rapeseed is extensively grown (24). Blackleg was first reported in 1989 in approximately 100 ha representing eight fields in south central Kentucky and was responsible for yield losses of up to 80%. Three isolates recovered from stubble collected from the Kentucky fields were from pathogenicity group (PG) 4 of the aggressive strain (16). During 1989 to 1991, disease surveys revealed that 13 of 37 fields, representing the bulk of rapeseed acreage in Kentucky, had blackleg. Among 38 isolates recovered from infected fields, one was classified as PG1, none as PG2, 18% as PG3, and 79% as PG4 (5). In 1991, blackleg disease was reported in North Dakota, with the incidence ranging from 8 to 68% in the 23 fields examined. Among the isolates recovered from six fields, PG2 isolates were detected in four fields and PG1 was detected in the other two fields (11). Since PG4 is common in Europe and Australia and PG2 is common in Canada, the outbreaks in Kentucky and North Dakota are likely to have come from separate introductions of infested seeds, the former from Europe (5) and the latter from Western Canada (10).

The appearance of *L. maculans* in these two areas of the United States will have an important impact on production of this relatively new crop. Knowledge of the fungus's virulence characteristics could lead to the development of better disease

management strategies. Variation in virulence is the most widely used marker for characterizing the pathogen population. However, the genes involved in host-specificity represent a small fraction of genes in the pathogen that may be subjected to host selection (12). Therefore, analysis of *L. maculans* field isolates with molecular markers may give a better measure of their genetic relatedness and origin. In this study, virulence analysis on differential *B. napus* cultivars and amplified fragment length polymorphism (AFLP) markers were used to evaluate the genetic relatedness of *L. maculans* field isolates from North Dakota with isolates from other geographical areas.

MATERIALS AND METHODS

Fungal cultures. Forty-nine isolates of *L. maculans* collected from rapeseed fields were used in this study (Table 1). Ten isolates from Europe and Australia were obtained from the Crucifer Genetic Cooperative (CrGC, in the Department of Plant Pathology, University of Wisconsin-Madison) (15), and five isolates from Ontario and four isolates from Manitoba, Canada, were provided by Paul Goodwin and Roger Rimmer, respectively. Five single pycnidiospore isolates from Georgia were obtained from infected residue collected in 1993 by Curtis Hill. Twenty-five single pycnidiospore isolates from North Dakota were obtained from *B. napus*-infected residue collected from three commercial fields in 1995 and 1996 by Arthur Lamey. Fields were as far as 160 km (100 miles) apart. Isolates 21A-J were collected in 1995 from field 21 of the susceptible cultivar Hyola 401 with disease incidence of 90% in Cavalier County, North Dakota. Isolates ND1-5 were collected in 1996 from a field immediately southeast of field 21. There were three *B. napus* cultivars in this field, including Hyola 401. Isolates 75A-J were collected from a single field of the moderately susceptible cultivar Trojan in Bottineau County, North Dakota, with a disease incidence of 72.5%.

Single pycnidiospore isolation. Small pieces of diseased stems were rinsed with water, surface-sterilized for 60 s in 10% commercial bleach, and rinsed. The stems were then immersed in sterile water for a few minutes, and with the aid of a dissecting microscope, single pycnidia were transferred to V8 agar plates. Cultures were grown for 7 days on V8 agar plates at

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Table 1. *Leptosphaeria maculans* isolates, country of origin, interaction phenotypes on cultivars Westar, Quinta, and Glacier, and pathogenicity grouping

Isolates ^a	Origin ^b	Westar	Quinta	Glacier	PG ^c	AFLP ^d
21A	ND	+	I	—	2	+
21B	ND	+	I	—	2	+
21C	ND	+	I	—	2	+
21D	ND	+	I	—	2	+
21E	ND	+	I	—	2	+
21F	ND	+	I	—	2	+
21G	ND	+	I	—	2	+
21H	ND	+	I	—	2	+
21I	ND	+	I	—	2	+
21J	ND	+	I	—	2	+
75A	ND	+	I	—	2	+
75B	ND	+	I	—	2	+
75C	ND	+	I	—	2	+
75D	ND	+	I	—	2	+
75E	ND	+	I	—	2	+
75F	ND	+	I	—	2	+
75G	ND	+	I	—	2	+
75H	ND	+	I	—	2	+
75I	ND	+	I	—	2	+
75J	ND	+	I	—	2	+
ND1	ND	+	I	—	2	—
ND2	ND	+	I	—	2	—
ND3	ND	+	I	—	2	—
ND4	ND	+	I	—	2	—
ND5	ND	+	I	—	2	—
IIA	Georgia	+	I	—	2	+
IIB	Georgia	+	—	I	?	+
IIC1	Georgia	+	I	—	2	+
IIC2	Georgia	+	I	—	2	+
IID	Georgia	+	—	I	?	+
PHW1223	Australia	+	+	+	4	+
PHW1227	Australia	+	+	+	4	+
PHW1230	Germany	+	I	+	3	+
PHW1236	Germany	+	I	+	3	+
PHW1237	Germany	+	I	+	3	+
PHW1244	France	+	I	+	3	+
PHW1245	France	+	I	—	2	+
PHW1246	UK	+	I	—	2	+
PHW1251	UK	+	+	+	4	+
PHW1252	UK	+	I	—	2	+
85-7	Manitoba	+	I	—	2	+
86-15	Manitoba	+	I	—	2	+
87-15	Manitoba	+	I	—	2	+
87-21	Manitoba	+	I	—	2	+
2A1	Ontario	nd	nd	nd	nd	+
2A2	Ontario	nd	nd	nd	nd	+
2C3	Ontario	nd	nd	nd	nd	+
2C4	Ontario	nd	nd	nd	nd	+
2L6	Ontario	nd	nd	nd	nd	+

^a All isolates are from *Brassica napus* host except PHW1245, which is from *B. oleracea*.

^b ND, North Dakota isolates obtained from Arthur Lamey; Georgia, isolates from Curtis Hill; Australia, Germany, France, and the UK, isolates from Crucifer Genetic Cooperative, University of Wisconsin-Madison; Manitoba, isolates from Roger Rimmer; Ontario, isolates from Paul Goodwin.

^c Pathogenicity grouping (PG) based on interaction phenotypes on three differential cultivars (+ = virulent, — = avirulent, I = intermediate); ? = undesignated; nd = not determined.

^d + = included in amplified fragment length polymorphism (AFLP) analysis; — = not included in AFLP analysis.

Table 2. Sequence of *Hind*III and *Mse*I amplification primers used in amplified fragment length polymorphism (AFLP) analysis

Primer	Sequence	No. of bands ^a
Hind-AC	5' GACTGCGTACCAGCTT-AC 3'	—
Mse-CAA	5' GATGAGTCCTGAGTAA-CAA 3'	7
Mse-CAG	5' GATGAGTCCTGAGTAA-CAG 3'	8
Mse-CAC	5' GATGAGTCCTGAGTAA-CAC 3'	8
Mse-CAT	5' GATGAGTCCTGAGTAA-CAT 3'	9
Mse-TCC	5' GATGAGTCCTGAGTAA-TCC 3'	3
Mse-GAG	5' GATGAGTCCTGAGTAA-GAG 3'	4

^a Number of polymorphic bands detected among *Leptosphaeria maculans* isolates.

24°C and irradiated under continuous cool-white fluorescent light (100 to 150 $\mu\text{E s}^{-1} \text{m}^{-2}$) to induce pycnidia formation. Water suspensions of pycnidiospores were diluted to a concentration of 10^3 to 10^4 spores per ml. One hundred μl of the pycnidiospore suspension was spread on 2% water plates, and after 2 days of incubation at room temperature, single germinating pycnidiospores were transferred to V8 agar and maintained under the conditions described above.

Inoculum preparation and pathogenicity tests. Thirty single pycnidiospore isolates from infected residue collected in North Dakota and five from Georgia were tested on *B. napus* cvs. Westar, Quinta, and Glacier to determine their PG. Inoculum preparation and pathogenicity tests were performed as previously described (14).

DNA preparation. Forty-four of the 49 *L. maculans* isolates were selected for AFLP analysis (Table 1). Isolates were grown 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 and rinsed with sterile water, then kept at -80°C for several hours and lyophilized. Two hundred mg of freeze-dried mycelia was ground in liquid nitrogen, using a mortar and pestle, and suspended in 4 ml of extraction buffer (50 mM Tris-HCl [pH 8.0], 850 mM NaCl, 100 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and 0.4 ml of 10% (wt/vol) CTAB (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 TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). All DNA extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng/ μl for AFLP analysis.

AFLP analysis. Restriction, ligation, amplification, and gel analysis were performed as described (27). The minor modification was that the primer pair used in the first amplification complementary to adapter sequence had one additional nucleotide at 3' end. The sequence of the *Hind*III adapter was:

5' CTCGTAGACTGCGTACC 3'
CATCTGACGCATGGTCCA

The sequence of the *Mse*I adapter was:
5' GACGATGAGTCCTGAG 3'
TACTCAGGACTCAT

Genomic DNA (0.5 μg) was incubated for 1 h at 37°C with 5 U each of *Hind*III and *Mse*I restriction enzymes in 40 μl of reaction mixture (20 mM Tris-HAc [pH 7.9], 10 mM MgAc, 50 mM KAc, 1 mM DTT). These DNA fragments were then

Fig. 1. Amplified fragment length polymorphism (AFLP) fingerprints of *Leptosphaeria maculans* isolates after second amplification with primer combination Hind-AC/Mse-CAT. Scored polymorphic markers are indicated as marks (*). Molecular weight markers (lane 22) are indicated in base pairs. Lanes 1 to 7 are isolates 21D-J; lanes 8 to 17 are isolates 75A-J from North Dakota encoded in Table 1; lanes 18 to 21 and 23 are isolates from Ontario 2A1, 2A2, 2C3, 2C4, and 2L6; lanes 24 to 33 are isolates from Crucifer Genetic Cooperative, University of Wisconsin-Madison, encoded in Table 1; lanes 34 to 38 are isolates from Georgia, encoded in Table 1; lanes 39 to 41 are isolates from Western Canada 86-9, 86-21, and 87-15, respectively.

ligated with the adapters by adding 10 µl of ligation mixture (5 pMol *Hind*III adapter, 50 pMol *Mse*I adapter, 1 U T4 DNA Ligase, 0.2 mM ATP, 20 mM Tris-HAc [pH 7.9], 10 mM MgAc, 50 mM KAc, 1 mM DTT) to the restriction mixture and continuing the incubation for another 3 h at 37°C. After ligation, the reaction mixture was diluted 10-fold with TE buffer and stored at -20°C. These ligated fragments served as templates in the amplification reaction.

The first amplification reaction was performed in 20 µl reaction (5 µl DNA, 50 ng each of primers, 0.08 U of *Taq* polymerase [Perkin Elmer, Norwalk, CT], 100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl₂, 0.01% [wt/vol] gelatin, 0.2 mM each dNTPs) using the following temperature profile: 30 s at 94°C; 30 s at 60°C; 60 s at 72°C for 20 cycles. The amplification product was diluted 10-fold with TE buffer and used as templates in the second amplification. The second amplification

was performed using primers derived from the first set of primers with additional nucleotides at the 3' end (Table 2). The *Hind*III primer was labeled using 100 µCi [γ -³²P] ATP and 0.2 U of T4 kinase. Amplification was performed using 5 ng of labeled primer and 50 ng of *Mse*I primer in the reaction mixture described above. The following temperature profile was used: 12 cycles of 30 s at 94°C; 30 s at 65°C; 60 s at 72°C, where the annealing temperature was subsequently reduced by 0.7°C each cycle. The amplification was continued for 23 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C. All amplification reactions were performed in a PE-9600 thermocycler (Perkin Elmer). Reaction products were analyzed by electrophoresis in a 5% denaturing acrylamide gel at the constant power of 45 W for 3 h. The gels were fixed for 30 min in 10% acetic acid, dried on the gel plate, and exposed to X-ray films at room temperature.

Data analysis. Each polymorphic AFLP fragment was treated as a unit character and scored as presence (1) or absence (0) across all isolates. The experiments were repeated, and only reproducible bands were scored. The relationships among isolates were analyzed by phenetic (cluster) analysis using computer software package NTSYS 1.8 (23). In the cluster analysis, the binary matrix was used to construct a matrix of similarities between all pairs of isolates based on Simple Matching (SM) and Dice similarity coefficients using the SIMQUAL program. The unweighted paired group method using arithmetic means (UPGMA) was used to construct dendrograms by the SAHN clustering program. Dendrograms were generated using the TREE program.

RESULTS

Pathogenicity tests. Pathogenicity data for 25 isolates from North Dakota and five isolates from Georgia based on cotyledon reactions on *B. napus* cvs. Westar, Quinta, and Glacier are presented in Table 1. All isolates tested produced a typical susceptible reaction on the cotyledons of *B. napus* cv. Westar, which appeared as large areas of gray-green collapsed tissue with abundant pycnidia (IP 8-9), indicating that they are all of the aggressive strain. The 25 isolates from North Dakota produced intermediate interaction phenotypes (IP 4-6) on Quinta and were avirulent (IP 1-3) on Glacier (Table 1), indicating they conform to PG2. Three isolates from Georgia, IIA, IIC1, and IIC2, were also classified as PG2. The other two isolates, IIB and IID, produced an intermediate IP on Glacier (IP 5-6) and were avirulent (IP 1-3) on Quinta (Table 1), and as such did not belong to any of the pathogenicity groups previously defined by the differentials Westar, Quinta, and Glacier (7,14).

AFLP analysis. AFLP fingerprints of the *L. maculans* isolates revealed clearly detected polymorphisms (Fig. 1). The experiments were performed twice, and the polymorphic DNA fragments detected were identical. The number of amplified fragments ranged from 60 to 80 for the six primer combinations, and the size of the DNA fragments ranged from 50 to 600 bp. Among these amplified fragments, 39 polymorphic DNA fragments were scored as discrete character data (presence-absence). The combined character data matrix was analyzed based on the assumption that co-migrating bands in an AFLP gel are homologous.

The 44 isolates were clustered as two distinct groups (AFLP I and AFLP II) using UPGMA with the SM coefficient (Fig. 2). AFLP I contained all isolates from North Dakota, Georgia, and Western Canada, and PHW1252 from the UK. Two of the isolates from Georgia, IIB and IID, were located further from the rest of the group at a similarity level below 75%. All

Fig. 2. Dendrogram generated from cluster analysis of 44 *Leptosphaeria maculans* isolates using simple matching similarity coefficients. Origin denotes location of *L. maculans* isolation with ND = North Dakota, GA = Georgia, WC = Western Canada, ON = Ontario, A = Australia, G = Germany, UK = United Kingdom, and F = France. PG = pathogenicity grouping based on interaction phenotypes on the three differential cultivars as shown in Table 1, with ? = undesignated and nd = not determined.

isolates in this group belong to PG2 except the two Georgia isolates, which have unassigned pathogenicity. The AFLP II group included all isolates from Ontario, Australia, France, Germany, and the remaining isolates from the UK. Isolates in AFLP II were clustered more loosely and were further divided below the 75% similarity level into two subgroups, AFLP IIA and AFLP IIB. In AFLP IIA, isolates PHW1223 and PHW1227, which belong to PG4 from Australia, clustered together and were relatively farther from the rest in the subgroup. In AFLP IIB, isolate PHW1251 from the UK was placed relatively farther from the three Ontario isolates and from the French and German isolates. The dendrogram generated via the Dice similarity coefficient was topologically similar to that obtained from the SM coefficient (data not shown).

DISCUSSION

Based on pathogenicity data, all isolates from North Dakota were classified as PG2. Three of five Georgia isolates also belong to PG2. The other two isolates did not belong to any previously described pathogenicity grouping. Previous study of *L. maculans* isolates in Kentucky revealed that PG3 and PG4 were predominant (5). The absence of PG3 and PG4 isolates from Georgia may be due to the small sample of the population surveyed. Although some isolates from Georgia may be related to those found in North Dakota, the Georgia population was more variable.

The absence of observed variation in pathogenicity among North Dakota isolates suggests that the *L. maculans* population in that state is relatively homogeneous. Previous studies have identified more variation in pathogenicity when a wider set of genetic differential host lines was used (2,8,9). A general lack of standardization of differentials, the heterogeneous consistency of lines, and the unknown genetic control of the resistance in the differentiating hosts together with variation in procedures for evaluating pathogenicity have complicated the interpretation and comparisons between pathogenicity studies (22). There is a substantial need for the development of genetically defined differentials, such as near-isogenic lines with single resistance gene differences.

The pathogenicity data alone may not reflect the true genetic variability and evolutionary history of the isolates studied. For example, isolates that are genetically distinct may have similar or identical pathogenicity patterns because they have been subjected to the same selection pressure by a common set of hosts. Molecular markers that are selectively neutral and randomly distributed in the genome can provide additional useful information on genetic variation and origin of isolates in a new crop production area. DNA marker

technologies have been used to distinguish between aggressive and nonaggressive strains of *L. maculans* (3,6,7,17,18,20,25,28). Previous phylogeny based on restriction fragment length polymorphisms led to grouping of aggressive isolates into more closely related phylogenetic groups (7).

This study showed that the AFLP technique is useful for the characterization of intraspecific variation among *L. maculans* populations. Small amounts of genomic DNA (50 ng) were sufficient as templates for an unlimited amplification of restriction fragments. Six primer combinations enabled the amplification of 400 fragments, of which 10% were polymorphic. The AFLP approach is objective, repeatable, and unaffected by environmental factors, and it provides a direct assessment of the genetic relationships of the fungus at the DNA level. The data generated are amenable to mathematically robust phylogeny reconstruction.

In cluster analysis, all North Dakota and Western Canadian isolates formed a distinct and rather uniformly clustered group supporting the hypothesis that *L. maculans* was introduced into North Dakota from Western Canada. This is likely because seeds planted in North Dakota have been imported from Western Canada. Isolate PHW1252, collected in the UK, was also placed in PG2, suggesting that it may be a possible ancestor of the Western Canadian isolates or vice versa.

Pathogenicity and cluster analysis of the Georgia isolates revealed considerable variation among isolates. It is interesting to note that some of the Georgia isolates were closely related to North Dakota and Western Canadian isolates. In spite of the small number of Georgia isolates analyzed, there was more variation observed among these isolates. Further research on the genetic relatedness of more isolates of *L. maculans* from the southeastern United States with isolates from the northern plains will provide information of use for disease management of rapeseed production in the United States.

In Canada, the aggressive isolates were first reported in Saskatchewan in 1975 (19), and the incidence increased 10-fold between 1978 and 1981 (21). Aggressive isolates were first detected in Alberta in 1983 and in Ontario in 1986 (1,19,21). Previous studies revealed relatively high similarity coefficients and the same glucose phosphate isomerase isozyme patterns among isolates studied from Western Canada and Ontario (3,19). Recently, variation in pathogenicity and random amplified polymorphic DNA (RAPD) patterns of *L. maculans* isolates within and between oilseed rape fields in southern Ontario has been reported. Eighty percent of the isolates studied were in PG4, and 11% were in PG3. The remaining 9% were assigned to the new PG5 based on intermediate reaction on the three differential cultivars.

In RAPD analysis, there was no two isolates that had identical patterns, suggesting that the populations of the fungus were produced mostly by sexual reproduction. Cluster analysis using RAPD patterns grouped isolates into two populations corresponding to the field of collection (13). In this study, Ontario isolates were clustered with isolates from Europe and were distinct from Western Canada and North Dakota isolates, all of which belong to PG2. A relatively high number of AFLP markers from this study revealed genetic variation between Western Canadian and Ontario isolates, suggesting the possibility that *L. maculans* was introduced into different regions of Canada from different source populations rather than having originated from a common Canadian ancestor, as previous studies have suggested (3,19).

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