APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2482–2486
0099-2240/91/092482-05$02.00/0

Vol. 57, No. 9

Rapid Identification of Genetic Variation and Pathotype of *Leptosphaeria maculans* by Random Amplified Polymorphic DNA Assay

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Received 22 April 1991/Accepted 11 July 1991

Canadian isolates of *Leptosphaeria maculans*, the causal agent of blackleg of crucifers, were examined for genetic relatedness by the random amplified polymorphic DNA assay. DNA polymorphisms amplified with random decamer primers were used to distinguish three groups of isolates. Group 1 contained all isolates of the virulent pathotype, group 2 contained isolates of the avirulent pathotype from western Canada, and group 3 contained avirulent pathotype isolates from Ontario. These results agreed with other reports which showed many genetic differences between pathotypes and were consistent with the hypothesis that the virulent pathotype was recently introduced into Canada and has diverged relatively little. In contrast, the avirulent pathotype has probably been present in Canada for a longer time and has diverged with geographic isolation. In addition to establishing genetic relationships, DNA fingerprints generated by the random amplified polymorphic DNA assay have potential applications in pathotype identification and blackleg disease management.

Leptosphaeria maculans (Desm.) Ces. et de Not [imperfect state, *Phoma lingam* (Tode ex Fr.) Desm.] is the causal agent of blackleg disease of crucifers (6). The fungus is variable in many traits, including cultural characteristics and pathogenicity (9). Strains from *Brassica* spp. have been divided into two distinct pathotypes which are classified as virulent (V) and avirulent (A) (12). The A isolates produce few or no symptoms on their hosts, whereas V isolates cause severe symptoms, including large lesions on leaves and stems (12). Stem cankers are important because they girdle the stems, resulting in lodging and death of plants, thus causing yield loss (6). In Canada, the V pathotype has become a serious problem on rapeseed (17).

To distinguish pathotypes, certain cultural characteristics may be used. The A pathotype generally grows faster in culture, forms few pycnidia, and produces a brown, water-soluble pigment in Czapek's broth (12). As more reliable markers, isozymes have been used to distinguish pathotypes. The A and V pathotypes differ in their isozymes of malate dehydrogenase, glucose phosphate isomerase, and pectinase (8, 10, 21). Restriction fragment length polymorphisms (RFLPs) have several advantages over isozymes as molecular markers (13). The numbers of RFLP markers are effectively unlimited and do not appear to be developmentally regulated (13). However, detection of RFLPs by Southern blot hybridization is laborious and relatively costly. To avoid these problems, the random amplified polymorphic DNA (RAPD) assay has been developed (22). In this technique, single primers with arbitrary nucleotide sequences are employed in a polymerase chain reaction to amplify genomic DNA and the resulting polymorphisms provide a simple means to construct genetic maps and perform DNA fingerprinting. The purpose of this research was to use RAPD markers to distinguish pathotypes and determine genetic variation in *L. maculans*.

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MATERIALS AND METHODS

Fungal isolates. Isolates of *L. maculans* and their sources are listed in Table 1. All were originally isolated from rapeseed, and pathotypes were determined by inoculation of adult rapeseed and isozyme analysis of glucose phosphate isomerase (21). Cultures were maintained on potato dextrose agar at 22°C.

DNA isolation. Fungal isolates were cultured for 5 to 7 days at 21°C on V8 agar covered with sterile transparent film (Flexel, Covington, Ind.). The mycelium was removed with the transparent film, and both were ground to a fine powder under liquid N_2 in a mortar and pestle. DNA was purified as described by Cryer et al. (4), with the following minor modifications: samples were incubated with 6 μg of proteinase K (Sigma) for an additional 3 to 4 h at 37°C prior to addition of chloroform, and the precipitated DNA was collected by centrifugation at 20,000 × *g* for 10 min.

Amplification conditions. Amplification conditions were modified slightly from those of Williams et al. (22). A buffer containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% (vol/vol) Triton X-100, 1 μM primer, and 0.5 U of *Taq* DNA polymerase (Pharmacia) was used. Amplification was performed in a BioOven Thermal Cycler (Biotherm, Arlington, Va.) programmed for 45 cycles of 45 s at 92°C, 60 s at 34°C, and 90 s at 72°C and a final cycle of 10 min at 72°C. The fastest available transitions (a maximum of 1°C/s) were used between temperatures. Amplification products were electrophoresed in 1.4% agarose gels with 1× TBE (0.089 M Tris-borate, 0.002 M EDTA) (20) and stained with ethidium bromide.

The primer sequences tested were R2,5′-AGTACAGGTC; R3,5′-TTCCTCTAGG; R4,5′TCCTACGCAC; R5,5′CGAC ATAATC; R6,5′-AGTTCTGTTC; and R28,5′-ATGGATC CGC. Primer synthesis was performed with a Milligen Biosurge 8600 synthesizer using standard phosphoramidite chemistry. Protecting groups were removed in 30% ammonium hydroxide at 55°C for 5 h, and then the samples were

TABLE 1. Isolates of *L. maculans* used in this study

Designation	Pathotype	Source[a]
Leroy	V	Saskatchewan[b]; G. A. Petrie
LM1	V	Alberta[b]; P. Kharbanda
LM26	V	Manitoba[b]; R. Rimmer
LM48	V	Saskatchewan[b]; G. Jesperson
A1988	V	Bruce County, Ontario; R. Assabgui
P1988	V	Grey County, Ontario; R. Assabgui
T1988	V	Wellington County, Ontario; R. Assabgui
Unity	A	Saskatchewan[b]; G. A. Petrie
LM5	A	Saskatchewan[b]; G. A. Petrie
LM6	A	Saskatchewan[b]; G. A. Petrie
LM102	A	Alberta[b]; P. Kharbanda
2358	A	Bruce County, Ontario; J. Chigogora
2374	A	Bruce County, Ontario; J. Chigogora
2379	A	Wellington County, Ontario; J. Chigogora
2382	A	Wellington County, Ontario; J. Chigogora

[a] Location where isolate was collected and original collector. Obtained from the culture collection of R. Hall, Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada.
[b] Western Canada.

dried under vacuum, dissolved in water, and diluted in TE (10 mM Tris-Cl [pH 8], 1 mM EDTA) (20).

For data analysis, similarity coefficients were calculated as described by Gabriel et al. (5) for RFLPs of repetitive sequences.

RESULTS

Amplification of *L. maculans* DNA. Six different oligonucleotide decamers were tested as primers to amplify *L. maculans* DNA. No amplification products were observed with primers R3, R5, and R6. These primers have also been found to be ineffective in the amplification of DNAs of other filamentous fungi (1). Successful amplifications of DNAs from isolates of *L. maculans* were observed with primers R2, R4, and R28 (see Fig. 1, 2, and 3). Depending on the isolate-primer combination, between 1 and 20 DNA segments were amplified, ranging in size from 0.16 to 3.32 kb. All amplifications were repeated two to five times, and the results were similar even when different DNA preparations were used for an isolate. However, variation was observed in the relative amounts of amplification of certain DNA segments. This appeared to be the result of DNA segments competing for primers, since it was found that when some minor bands were amplified to a greater amount, other DNA bands were reduced in intensity. Strongly amplified DNA segments, however, were consistently observed.

Fingerprinting isolates with RAPD markers. For the R2, R4, and R28 primers, amplified polymorphic DNA was observed (see Fig. 1, 2, and 3). In general, amplifications with these primers produced little or no polymorphism between isolates of the V pathotype, whereas isolates of the A pathotype showed greater variability, although all were clearly distinguishable from the V pathotype. Polymorphisms among the A pathotype isolates were principally related to the geographic origin of the isolate (i.e., western Canada or Ontario).

For the R2 primer, unique DNA segments of 0.95, 0.54, and 0.35 kb were amplified from V pathotype isolates (Fig. 1). Two larger amplified segments, 1.78 and 1.73 kb, were observed from most of the V pathotype isolates, but a 1.73-kb segment was also amplified from isolates of the A pathotype from western Canada. This fragment, however, was absent from amplified DNA of A pathotype isolates from Ontario, which were distinguished by amplified segments of 0.70 and/or 0.63 kb.

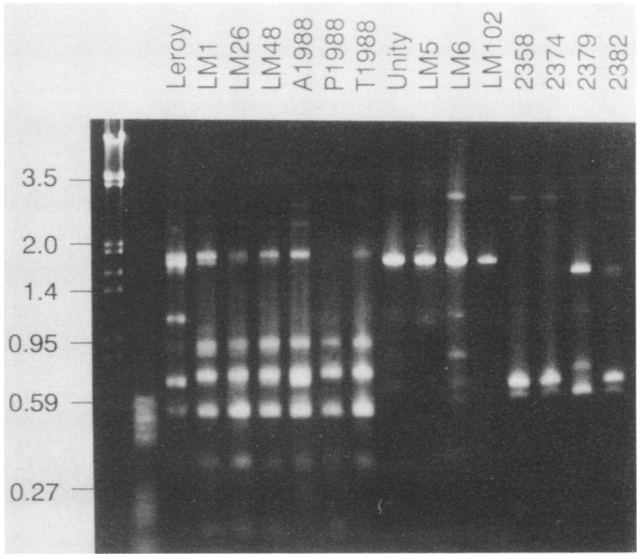

FIG. 1. Amplified DNA polymorphisms of *L. maculans* with primer R2. Lanes, left to right: λ *Hin*dIII-*Eco*RI, pBR322 *Hae*III, and the *L. maculans* isolates described in Table 1. The numbers on the left are lengths of marker bands in kilobases.

For the R4 primer, a unique amplified DNA pattern was observed for the V pathotype, with characteristic segments of 1.38, 0.76, and 0.33 kb (Fig. 2). The two A pathotype isolates, Unity and LM6, had several amplified segments, with sizes similar to those of certain V pathotype isolates. This included the 1.38-kb amplified segment, but the 0.76 and 0.33 kb segments and several lesser amplified segments for the V pathotype were absent from these A pathotype

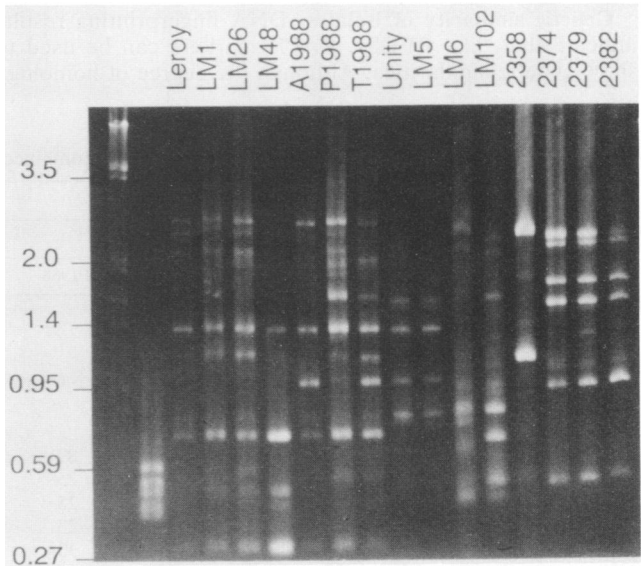

FIG. 2. Amplified DNA polymorphisms of *L. maculans* with primer R4. Lanes, left to right: λ *Hin*dIII-*Eco*RI, pBR322 *Hae*III, and the *L. maculans* isolates described in Table 1. The numbers on the left are lengths of marker bands in kilobases.

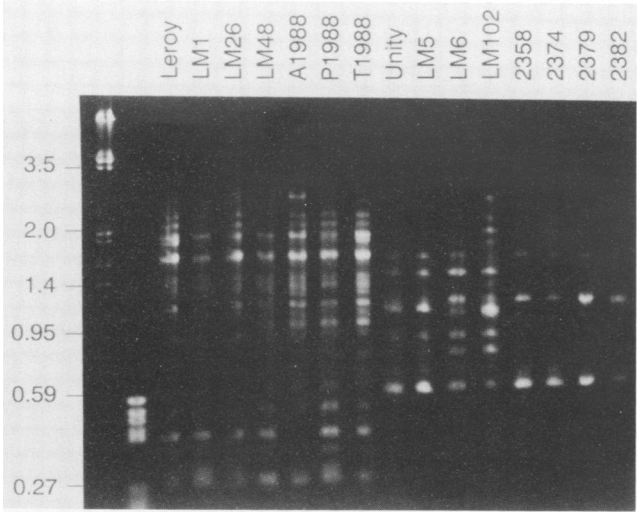

FIG. 3. Amplified DNA polymorphisms of *L. maculans* with primer R28. Lanes, left to right: λ *Hin*dIII-*Eco*RI, pBR322 *Hae*III, and the *L. maculans* isolates described in Table 1. The numbers on the left are lengths of marker bands in kilobases.

isolates. The A pathotype isolates 2374, 2379, and 2382 had a characteristic pattern of six major amplified segments. The remaining A pathotype isolates had differing patterns, although the patterns for isolates LM6 and LM102 were relatively similar.

Of the primers tested, R28 gave the greatest number of amplified segments, especially for the V pathotype (Fig. 3). All V pathotype isolates had very similar banding patterns which differed from the A pathotype by the presence of an extra three to four relatively short amplified segments. Although many of the amplified segments of the A pathotype isolates were similar, isolates from western Canada had additional segments of 1.54, 0.95, and 0.85 kb. Several segments of the same size were amplified in all isolates, including strongly amplified segments of 1.65 and 1.21 kb.

Genetic similarity of isolates. DNA fingerprinting results indicate that, like RFLPs, RAPD markers can be used to show genetic similarities. Although the degree of homology between amplified segments of the same size has not been demonstrated, the correlation of patterns with features such as pathotype and geographic origin implies that they show genetic relationships and thus could be analyzed in a manner similar to RFLPs. Use of the method of Gabriel et al. (5) to calculate similarity coefficients for RFLPs of repetitive sequences gives double weight to a match of strongly hybridizing fragments and only single weight to a match of weakly hybridizing ones. Such an approach is suited for analysis of data obtained with RAPD markers, since major (strongly amplified) DNA segments were more consistently amplified than minor (weakly amplified) segments.

Three groups were distinguishable on the basis of similarity coefficients from the results obtained by using the R2, R4, and R28 primers (Table 2). Group 1 comprised the V pathotype isolates, all of which had relatively high degrees of similarity, as evidenced by similarity coefficients of 76% or greater. No distinction could be made between these isolates on the basis of geographic origin. The A pathotype was divided into groups 2 and 3. Group 2 isolates were from western Canada, and group 3 isolates originated in Ontario. The difference between groups 2 and 3 was most obvious when the R2 primer was used (Fig. 1). Within groups 2 and 3, the similarity coefficients were relatively low (as low as 55%), whereas some of the correlation coefficients between isolates of groups 2 and 3 were as high as 44%, indicating that these groups are less homogeneous and distinct than group 1, which is composed of V pathotype isolates (Table 2).

DISCUSSION

Williams et al. (22) described several possible sources of DNA polymorphisms revealed by the RAPD assay. These include deletions of priming sites, insertions and deletions between priming sites, and single-base changes which cause mismatches in priming sites. Their study shows that RAPD results correspond to those of RFLPs. By probing Southern blots with labelled amplified DNA, they found cosegregation of the amplified polymorphisms with their corresponding RFLPs (22). For RFLPs, similarity coefficients are frequently employed to analyze the banding patterns (14). A modified form of this analysis was used in our study to

TABLE 2. Similarity coefficients of combined data from Fig. 1, 2, and 3, expressed as percentages

Isolate	% Similarity														
	Group 1							Group 2				Group 3			
	Leroy	LM1	LM26	LM48	A1988	P1988	T1988	Unity	LM5	LM6	LM102	2358	2374	2379	2382
Leroy															
LM1	92														
LM26	90	100													
LM48	82	94	89												
A1988	78	76	84	81											
P1988	85	88	94	86	79										
T1988	86	83	81	78	86	89									
Unity	30	20	23	21	25	25	21								
LM5	30	20	23	21	25	25	21	100							
LM6	23	18	25	17	29	23	13	67	67						
LM102	21	21	38	28	38	33	20	55	55	82					
2358	29	19	22	21	23	25	21	30	30	28	18				
2374	29	19	22	18	17	19	13	25	25	40	35	76			
2379	22	20	20	14	18	18	17	21	21	44	34	57	79		
2382	22	21	14	14	19	19	30	20	20	43	34	68	96	82	

examine the combined data for several RAPD markers. Conclusions from our results should be considered preliminary in view of the limited number of isolates and markers tested. To examine the genome of *Neurospora crassa*, 88 RAPD markers were employed (22). Despite the limitations, certain genetic relationships were apparent.

L. maculans is distributed worldwide. In Canada, the A pathotype has been identified since the early 1960s, but it was not until 1975 that isolates, first reported in east-central Saskatchewan, were found that resembled V pathotype isolates from other countries (16). The incidence of the V pathotype increased 10-fold in Saskatchewan between 1978 and 1981, and it was first detected in Alberta in 1983 and in Ontario in 1986 (2, 15, 19). It is not known how long the V pathotype has been in Ontario, but the Ontario isolates shared the same glucose phosphate isomerase isozyme patterns and degree of virulence as V pathotype isolates from western Canada (15). The V pathotype isolates from Ontario, however, differed from those of western Canada in certain cultural characteristics (15).

The relatively high similarity coefficients for all of the V pathotype isolates in this study indicate that they have not been geographically separated long enough to diverge significantly. This is consistent with the hypothesis that the V pathotype was introduced into Canada relatively recently (2). *L. maculans* can quickly spread long distances by movement of infected seed (6). The genetic similarity of isolates from western Canada and Ontario suggests a recent common ancestor for the V pathotype in these regions. Use of additional primers and isolates, however, may show more genetic variation among isolates of this pathotype.

In contrast, our A pathotype isolates from western Canada and Ontario make up separate groups based on their similarity coefficients. These geographically isolated groups either do not have a recent common ancestor or have developed separately with little or no recent intermixing. Localized populations of *L. maculans* can exist because the fungus survives in crop stubble and spreads to nearby fields as wind- or rain-borne spores (7). Unless seeds infected with the A pathotype were moved between these regions, the populations would remain isolated since little rapeseed or other crucifer hosts are grown in the upper Great Lakes region separating southern Ontario and western Canada.

On the basis of key morphological features, both pathotypes are classified as *L. maculans*. However, matings between A and V pathotype isolates have never been successful (3). It has been suggested that the two pathotypes represent different species (9). The amplified DNA polymorphisms reported here cannot address this question, but they are consistent with a multitude of other differences that distinguish pathotypes (9). The fact that all three primers showed differences between the pathotypes supports the suggestion that the variation in pathogenicity reflects just one of many genetic differences between pathotypes.

One practical application of the RAPD assay is that of organism identification. Identification of pathotypes and variants within pathotypes of *L. maculans* would be very useful in population studies and disease management. Although both pathotypes are seed transmitted, disease management recommendations emphasize preventing the introduction of the V pathotype through seed (2). Therefore, seed lots should be tested for the V pathotype. For Alberta alone, over 2,000 samples from rapeseed lots were tested for *L. maculans* in 1989 (2). Current assays for the pathotypes of *L. maculans*, such as measurement of germ tube length (18) or inoculation of crucifer seedlings (3), are time-consuming and can be affected by environmental conditions. The RAPD assay is relatively rapid and independent of gene expression. Quick DNA miniprep methods are available for fungi (11), and the remainder of the assay can be completed in 1 day. The R2 marker seems to be especially promising in this function. We have already found one use for the RAPD assay. Amplified DNA polymorphisms were used to discover that an isolate of *L. maculans* in a culture collection was mislabelled as to pathotype. The results of the RAPD assay were confirmed by a pathogenicity test and the isozyme pattern of glucose phosphate isomerase (data not shown). Identifying organisms by DNA fingerprinting is proving to be very useful and should be equally beneficial for classifying isolates of *L. maculans*.

ACKNOWLEDGMENTS

We thank J. B. Anderson and D. J. Hamilton for technical assistance and R. Hall for providing fungal isolates.

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada.

ADDENDUM IN PROOF

Recent publications on the genome size and RFLP patterns of isolates of *L. maculans* demonstrate that the A and V pathotypes are genetically very different and suggest that they are distinct species (10a, 21a).

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