An Unusual Repetitive Element from Highly Virulent Isolates of *Leptosphaeria maculans* and Evidence of Its Transfer to a Weakly Virulent Isolate

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A 5,238-bp repetitive DNA element from a highly virulent isolate of *Leptosphaeria maculans* has been cloned and sequenced. The element is present in approximately 80 copies per haploid genome and hybridizes to every chromosome resolved by pulse field gel electrophoresis. The sequence is composed of 66% A + T and has numerous, very short, direct and inverted repeats. No RNA complementary to the element was detected in log phase cultures, and no open reading frames of significant length are present in the sequence. It has no structural similarity to other repetitive elements or significant homology to database sequences. We have designated the element LMR1. Southern blot hybridization indicated that the element is present in all isolates of *L. maculans* that are highly virulent to *Brassica napus* and *B. rapa*. The general structure of the element was conserved among isolates of different mating type, pathogenicity group, and geographic origin, as determined by both Southern blot analysis and primer-directed DNA amplification. LMR1 did not hybridize to DNA from weakly virulent strains of *L. maculans*, with the exception of one isolate. Phylogenetic analyses of restriction fragment length polymorphism and rDNA sequence indicated that the highly virulent and weakly virulent strains of *L. maculans* are not monophyletic. Therefore, the presence of the LMR1 element in a weakly virulent isolate may indicate that a rare transfer event has occurred. Surprisingly, the weakly virulent isolate that contains LMR1 is more pathogenic on *B. napus* and *B. juncea* than a similar isolate that lacks the element.

Additional keywords: blackleg, host-pathogen interaction, *Phoma lingam*, stem canker.

Repetitive DNA is a common feature of both prokaryotic and eukaryotic genomes (Lin *et al.* 1984; Hutchison *et al.* 1989). Some of these sequences represent transposable elements (Hutchison *et al.* 1989), others serve chromosome structural functions (Fitzgerald-Hayes *et al.* 1982; Szostak and Blackburn 1982; Nakaseko *et al.* 1986), and still others appear to mediate recombination (Petes and Hill 1988). It has also been postulated that repetitive elements serve as origins of replication or as signals for RNA processing (Georgiev *et al.* 1983), stability (Clemens 1987), or transcription (Britten and Davidson 1969). Alternatively, it has been proposed that they have no function (Deininger 1989).

In recent years a number of dispersed repetitive DNA sequences have been isolated from phytopathogenic fungi. These cloned repetitive elements include a family of long, dispersed repeats from the rice blast fungus, *Magnaporthe grisea* (Hamer *et al.* 1989), and shorter repeats from strains of *Fusarium oxysporum* that infect crucifers (Kistler *et al.* 1991). Probes based on repetitive sequences have proven to be useful for differentiating formae speciales of *Erysiphe graminis* (O'Dell *et al.* 1989), detecting pathogens in plant tissue (Rollo *et al.* 1987), and genetic mapping (Romao and Hamer 1992).

Leptosphaeria maculans (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.) is a heterothallic ascomycete that causes blackleg disease in many crucifers (Venn 1979; Punithalingam and Holliday 1972). The relationship between isolates from the vegetable crucifers and rape is unclear. Humpherson-Jones (1986) reported finding isolates that were virulent on oilseed rape, swede, and cabbage. Delwiche and Williams (1979) found a cabbage isolate and a rape isolate that were host-specific. Adding to this complexity, the isolates from rapeseed are divided into two strains, highly virulent and weakly virulent (McGee and Petrie 1978). Several years ago we isolated a λ genomic clone containing repetitive DNA from a highly virulent rapeseed isolate for use in determining relationships between isolates on the basis of restriction fragment length polymorphisms (RFLPs). The results presented here illustrate why this DNA fragment is of interest for more than strain identification. We have designated the element LMR1 (GenBank accession M77515), for *L. maculans* repeat.

RESULTS

Cloning of LMR1.

Approximately 4×10^4 phage clones of DNA from the highly virulent *L. maculans* isolate Leroy were screened by plaque hybridization with nick-translated ^{32}P-labeled DNA from the same isolate. Twenty-three strongly hybridizing plaques were purified and analyzed. Seventeen of the clones

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cross-hybridized with an rDNA clone from *Neurospora crassa* and were not further analyzed. DNA from the six remaining phage clones was ^{32}P-labeled and hybridized to *Hin*dIII-digested fungal DNA. One phage insert hybridized to 18–20 *Hin*dIII fragments, while the number of fragments that hybridized to the remaining clones varied from two to 10. The phage clone that contained the highly repeated sequence was restriction mapped, and the extent of the repetitive element was determined. This was done by hybridizing ^{32}P-labeled nick-translated fungal DNA to Southern blots of restricted phage DNA, and phage DNA fragments to Southerns of fungal genomic DNA. The restriction map of the 5.2-kb repetitive element is shown in Figure 1. Overlapping subclones containing various restriction enzyme fragments from the region of the phage clone containing the repetitive DNA were inserted into pTZ18 and 19R (Pharmacia). The subclones were sequenced on both strands, and the 5,238-bp sequence was submitted to GenBank (accession M77515).

Structural analysis of LMR1.

The element is extremely A-T rich (data not shown): these bases composed 66% of the sequence. An unusual feature of the base composition of the element is the occurrence of the dinucleotides TA and TG at frequencies four times greater and 5.6 times less than normal, respectively, in noncoding eukaryotic sequences (Nussinov 1991). The TA dinucleotide occurred most frequently in the context of TAA, TAG, and TTA. The lack of restriction sites within the element for many of the enzymes tested (Fig. 1) is most likely due to its relatively simple repeated structure and high A-T content. Southern blot analysis of *Mbo*I and *Sau*3AI fungal genomic digests did not indicate the presence of N^6-methyladenine.

Only six very small open reading frames were found, in sizes ranging from 11 to 23 codons. No RNA complementary to the element was detected in northern analysis of RNA from log phase cultures. A search of the sequence databases did not reveal any sequences with extensive homology.

Analysis of the LMR1 sequence by means of the Compare and Dot Plot programs from the University of Wisconsin Genetics Computer Group (UWGCG) revealed that it is composed of numerous, very short, direct and inverted repeats. The majority of the direct repeats do not extend beyond 6 bp, and the highest concentrations of them are found at base pairs 3,600–3,800, 4,000–4,200, and 4,800–5,200. The inverted repeats (IRs) also do not generally extend beyond 6 bp. The highest concentrations of IRs overlapped with the direct repeats at base pairs 3,500–3,700 and 4,000–4,200.

Genome distribution and copy number of LMR1.

The chromosomes from several highly virulent isolates were separated on pulse field gels and probed with radiolabeled LMR1. The results for the three isolates WA51, Saskatoon 6, and Leroy are shown in Figure 2. The probe hybridized to each band in the gel. The hybridization signals generally correlated with the ethidium bromide stain UV fluorescence of the chromosomes, with one or more exceptions (indicated by arrows in Fig. 2) in each isolate. The hybridization signals of the 2.5-megabase (Mb) chromosome of isolate WA51, the 1.8- and 1.1-Mb chromosomes of Saskatoon 6, and the 1.7-Mb chromosome of Leroy were all weaker than expected from their UV fluorescence. The differences in the hybridization signals could be due to the presence of fewer copies of the element on these chromosomes than on the adjacent ones, or it could be due to sequence polymorphisms that reduce hybridization.

The copy number per haploid genome was determined by hybridizing ^{32}P-labeled probe to slot blots of fungal DNA. Plasmid DNA containing the insert was loaded adjacent to the fungal DNA in genome copy number equivalents of 1, 5, 10, 20, 50, 100, 250, 500, and 1,000. The autoradiograms were scanned by densitometer, and the peak areas were compared (data not shown). The copy number estimate on the basis of peak area was 80. This estimate was further supported by the results of a scan of a genomic reconstruction Southern blot with *Bam*HI-digested fungal and plasmid DNA.

RFLPs for LMR1 among *L. maculans* isolates.

DNA from 23 highly virulent rapeseed isolates was digested with *Bam*HI or *Xba*I, blotted, and hybridized to LMR1 (Fig. 3). A single *Bam*HI fragment, approximately 4.0 kb in size, showed strong hybridization to the element in all the isolates examined (Fig. 3A, top gel). This band corresponded to the 3.6-kb *Bam*HI fragment that composes most of LMR1 (Fig. 1). Difficulties in obtaining unsheared DNA from some of the isolates made hybridization to this fragment difficult to discern, but it was still evident. Two *Xba*I fragments, approximately 3.5 and 2.0 kb in size, were found to have the strongest hybridization to the element in all the isolates (Fig. 3A, bottom gel). The 3.5-kb fragment corresponds to the portion of LMR1 from the *Xba*I site at 1,731 bp to the *Xba*I site

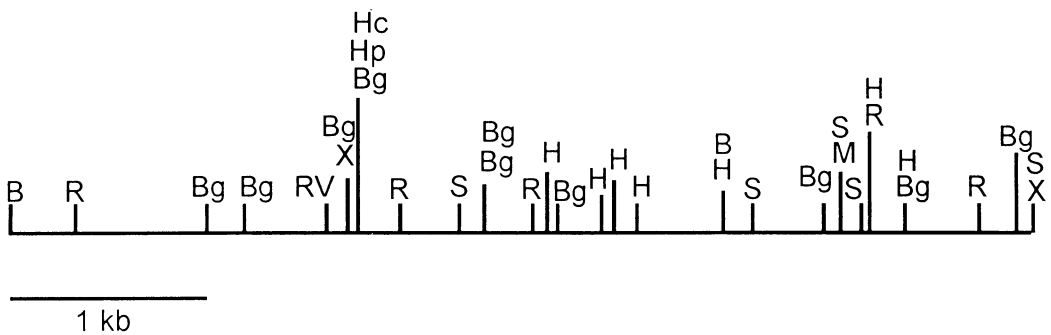

Fig. 1. Restriction map of the LMR1 repetitive element cloned from the highly virulent *Leptosphaeria maculans* isolate Leroy. The enzyme abbreviations are as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; M, *Mlu*I; R, *Rsa*I; RV, *Eco*RV; S, *Sac*I; and X, *Xba*I. The enzymes that did not cut were *Bcl*I, *Eco*RI, *Hae*III, *Kpn*I, *Nco*I, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*II, *Sal*I, *Sca*I, *Sfi*I, *Sma*I, *Sph*I, and *Stu*I.

at 5,238 bp (Fig. 1). The strongly hybridizing 2.0-kb fragment indicates that there is an *Xba*I site approximately 300 bp to the left of the initial *Bam*HI site in these isolates. A 5-day exposure of the Southern blots to X-ray film revealed hybridization to multiple *Bam*HI and *Xba*I fragments in each isolate. A sample of four lanes from each digest is shown in Figure 3B. The sizes of the additional *Bam*HI fragments evident were 12.2, 11.7, 8.8, 7.8, 7.3, 6.9, 5.5, 3.3, 2.9, 2.4, 1.9, 1.4, and 1.0 kb. The *Xba*I fragments, discernible after long exposure, were a doublet at 6.4 kb and single bands at 4.0, 2.5, 1.3, 1.0, and 0.6 kb. There is no apparent periodicity in the sizes of the hybridizing fragments that would indicate tandem repeats of the element. The additional bands represent the genomic junction fragments of the element and possibly related repeats. The unusual aspect of these results is the lack of RFLPs that are expected to result from changes in the repetitive element sequence or its surrounding genomic sequences. This result cannot be attributed to clonal lineage, since the PHW1275 and PHW1276 isolates (Fig. 3B) are of opposite mating types.

We further investigated the extent of the element sequence conservation by performing polymerase chain reaction (PCR) amplifications with two sets of primers derived from the LMR1 sequence (Fig. 4). The primers were designed to amplify the portion of the element from base pairs 881 to 2,026 and from base pairs 4,259 to 4,839. The DNA came from isolates of different mating types, different pathogenicity groups (Koch *et al.* 1991; Rimmer and van den Berg 1992), and diverse geographic origins (noted in the legend of Fig. 4). Fragments of the expected sizes of 1,145 and 580 bp were amplified from the DNA of the 10 highly virulent isolates (Fig. 4, lanes 1–10), confirming that copies similar to the cloned LMR1 element are present in these isolates. However, the presence of additional fragments, slightly larger than 580 bp, in reactions containing DNA from three of the Australian isolates indicates that sequence divergence has occurred in some of the copies of the element in these isolates. Products of the expected size were also obtained with DNA from the weakly virulent isolate Laird 2 but not with DNA from the weakly virulent isolate Unity (Fig. 4, lanes 10 and 11). These results are discussed in the next section.

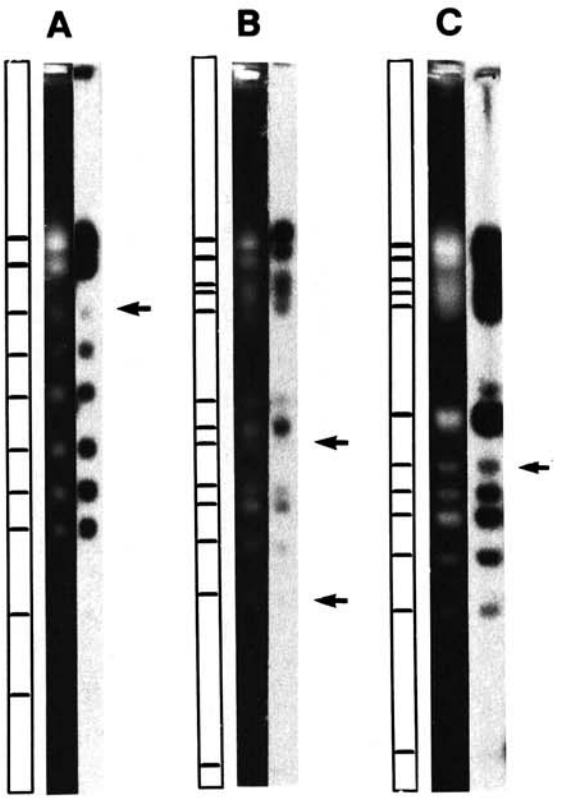

Fig. 2. Transverse alternating-field electrophoresis of chromosomes from three isolates of *Leptosphaeria maculans* highly virulent on rapeseed: for each isolate, diagram of ethidium bromide staining of chromosomes (left), photograph of ethidium bromide–stained chromosomes (center), and autoradiograph of LMR1 hybridization to the chromosomes (right). **A,** Isolate WA51, from Australia; 2.5-Mb chromosome (arrow). **B,** Isolate Saskatoon 6; 1.8- and 1.1-Mb chromosomes (arrows). **C,** Isolate Leroy; 1.7-Mb chromosome (arrow). These chromosomes showed reduced hybridization signals in comparison to ethidium bromide stain UV fluorescence.

A more detailed restriction enzyme analysis of the element's copies and junction fragments was performed by Southern blot hybridization to DNA from four isolates. The DNA from each of two Canadian and two Australian isolates was digested with 25 different restriction enzymes. The LMR1 sequence contained sites for nine of the 25 enzymes (Fig. 1). The results of the Southern blots of one of the Canadian and one of the Australian isolates are shown in Figure 5. The hybridization of LMR1 to digestions with enzymes not having sites in the cloned element (with the exception of *Sca*I) gave a continuous smear of high molecular weight DNA. These results indicated a more diverse set of genomic environments for copies of the element than was evident in the *Bam*HI and *Xba*I digests. In the light of these results, it seems likely that the majority of the less intensely hybridizing bands in the *Bam*HI and *Xba*I digests came from related repeats. The LMR1 hybridization to a smear in the lower molecular weight range following *Sca*I digestion is interesting when considered in relation to the element's trinucleotide base composition. The two half-sites of the palindromic recognition site for this enzyme, AGTACT, occur at moderately high frequencies in the element. Only minor rearrangements would be required to form *Sca*I sites within the element. In the digestions that gave discreet banding patterns, the similarity between the Australian and Canadian isolates was very high. Only a single band in each of the *Mlu*I, *Pst*I, *Sac*I, and *Nco*I digests was different for the different isolates (Fig. 5). However, the presence of hybridizing bands of less than 5.2 kb in the *Pst*I and *Sca*I digests indicates that restriction site polymorphism does exist in copies of the repeat, since no sites for these enzymes were found in the sequenced clone. Furthermore, only one *Eco*RV site was present in the sequenced repeat, but the genomic digests had three strongly hybridizing bands of less than 2.5 kb, indicating that an additional *Eco*RV site is present in some copies.

Hybridization of LMR1 to isolates of the strain weakly virulent on rapeseed.

The results of the PCR amplification with LMR1 primers indicated that the element might not be present in all weakly virulent isolates of *L. maculans* (Fig. 4, lanes 10 and 11). We examined this possibility by performing a hybridization to a Southern blot of DNA, digested with *Bam*HI or *Xba*I, from 28 weakly virulent isolates (data not shown). The DNA from

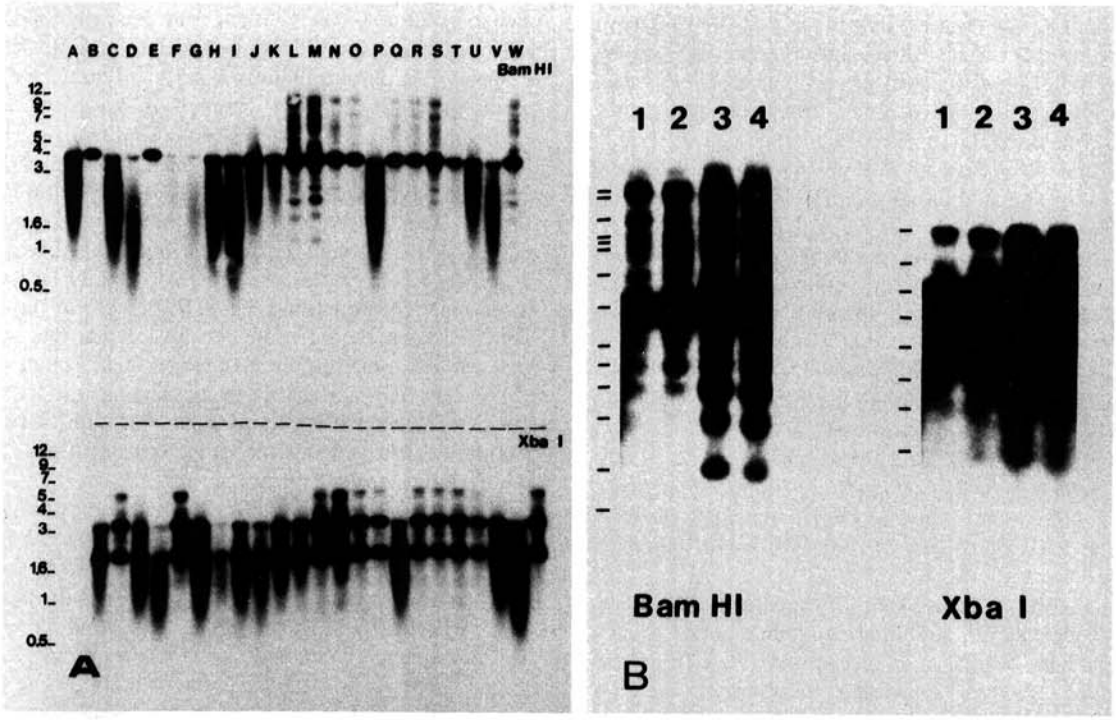

Fig. 3. Southern blot analysis of genomic DNA from isolates of *Leptosphaeria maculans* highly virulent on rapeseed that were hybridized to the LMR1 probe. **A,** DNA from the following 23 isolates was digested with *Bam*HI (top gel) or *Xba*I (bottom gel): lane A, Wainwright 1; lane B, Wakaw 1; lane C, WA74-4 (Australia); lane D, WA51 (Australia); lane E, Scott 2; lane F, Scott 1; lane G, Saskatoon (SK) 11; lane H, SK 10; lane I, SK 8; lane J, SK 7; lane K, SK 6; lane L, PHW1276 (Australia); lane M, PHW1275 (Australia); lane N, Phippen 1; lane O, North Battleford 4; lane P, North Battleford 1; lane Q, Melfort 4; lane R, Melfort 3; lane S, Mayfair 1; lane T, Leroy; lane U, Fairview 2; lane V, Fairview 1; and lane W, Arborfield 2. **B,** Five-day X-ray film exposure of four lanes from each of the above Southern blots: lane 1, North Battleford 4; lane 2, Phippen 1; lane 3, PHW1275 (Australia); and lane 4, PHW1276 (Australia). The molecular sizes of the marked bands are given in the text. All isolates were collected in Canada unless otherwise noted above.

only one isolate, Laird 2, gave distinct hybridization signals. The molecular sizes of the hybridizing bands corresponded to those found in highly virulent isolates. A genomic reconstruction Southern blot indicated that fewer than five copies of the element are present in Laird 2 (data not shown).

The isolate was collected from rapeseed stubble in Saskatchewan. It had been assigned to the weakly virulent pathogenicity group because it showed certain cultural characteristics common to those isolates. The actual pathogenicity of the isolate on rapeseed was not determined before the classification. The cultural characteristics that are common to weakly virulent isolates are the production of a brownish yellow pigment in modified Czapek media (highly virulent isolates do not produce any pigment) and a faster growth rate and the production of fewer pycnidia in V8 juice agar, compared to the growth rate and pycnidial production of highly virulent isolates (McGee and Petrie 1978). The assignment of this isolate to the weakly virulent strain was further supported by molecular genetic analysis. The electrophoretic karyotype and the sequences of the rDNA internal transcribed spacers (ITS) have been shown to correlate with the pathogenicity group of a particular isolate (Morales *et al.* 1993a,b). Both the karyotype and the ITS sequences (GenBank accession LO7735) of this isolate were identical to those of other weakly virulent isolates.

After the discovery that LMR1 hybridized to Laird 2 DNA, the pathogenicity of the isolate was tested on cotyledons of *B. napus* and *B. juncea* (Table 1). The mean diameter of the lesions caused by this isolate on *B. napus* cotyledons was approximately one-half that produced by a highly virulent isolate but twice that produced by another weakly virulent isolate. Surprisingly, the mean lesion diameter produced by this isolate on two brown mustard varieties (*B. juncea*) was greater than twice the diameters of lesions produced by the isolates highly or weakly virulent on rapeseed. Previously, Gugel *et al.* (1990) found that *Brassica* spp. containing the b genome are not susceptible to Canadian isolates of *L. maculans*. Out of nearly 100 isolates examined thus far, we have

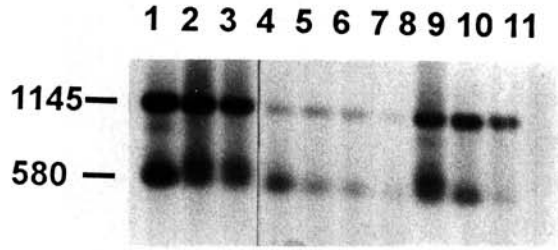

Fig. 4. Polymerase chain reaction amplification products from DNA from *Leptosphaeria maculans* isolates using LMR1 primers. The DNA from 11 different isolates was amplified with two sets of primers derived from the LMR1 sequence (see Materials and Methods). The two expected product sizes were 1,145 and 580 bp. The isolates and their mating types, pathogenicity groups (PGs), and geographic origins, if known, were: lane 1, Leroy (+, PG2, Canada); lane 2, PHW1275 (–, Australia); lane 3, PHW1276 (+, Australia); lane 4, NSW12 (+, Australia); lane 5, NSW33 (–, Australia); lane 6, Eng53 (Great Britain); lane 7, Fr1 (France); lane 8, WA74-4 (PG2, Australia); lane 9, WA51 (PG4, Australia); lane 10, Laird 2 (weakly virulent [PG1], Canada); and lane 11, Unity (PG1, Canada).

not found another isolate that, like Laird 2, has the growth behavior, electrophoretic karyotype, and ITS sequence common to weakly virulent isolates and also has copies of the LMR1 element.

DISCUSSION

The LMR1 repetitive element of highly virulent isolates of *L. maculans* has a unique structure. It shares few characteristics with either of the two general categories of eukaryotic repeats, that is, short interspersed repeats (SINEs) and long interspersed repeats (LINEs). A description of the "generic" SINE sequence states that they are 75–500 bp in length, occur in copy numbers of approximately 10^5 per genome, contain an RNA polymerase III promoter, have an A-rich 3′ end but no open reading frames, and are flanked by short direct repeats (Deininger 1989). There are only two similarities between LMR1 and SINEs: the lack of open reading frames and an A-rich region (70 out of the first 150 bases of LMR1 are A). The available sequence analysis programs found no portion of the LMR1 sequence with extensive homology to the RNA polymerase III promoter. Nor was the genomic copy number of the element near the range found for SINEs. LINEs are greater than 5 kb in length, occur in approximately 10^4 copies per genome, have poly(A) tails and open reading frames with sequence motifs characteristic of a reverse transcriptase, and are flanked by short direct repeats (Hutchison *et al.* 1989). The overall length and the possible flanking by short direct repeats are the only similarities between LINEs and LMR1. LMR1 also does not fit into the retroposon or transposon category of eukaryotic repetitive sequences. It lacks the long terminal repeats and open reading frame for

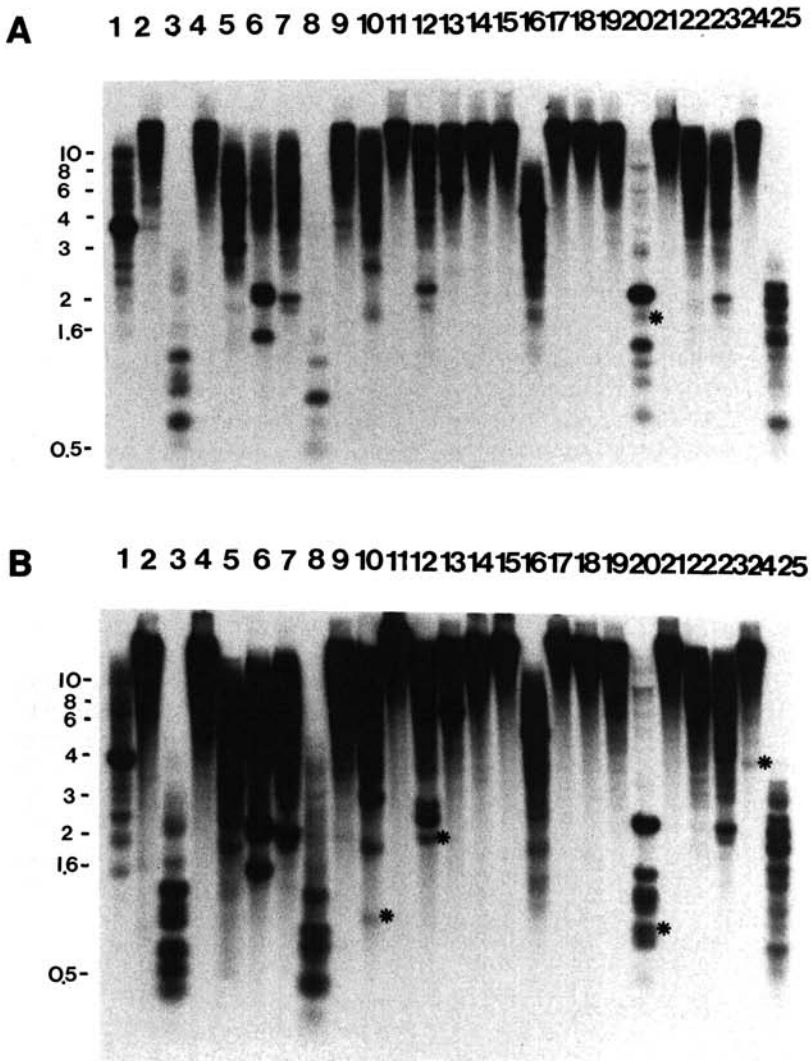

Fig. 5. Southern blot analysis of genomic DNA from the Canadian isolate Melfort 3 (**A**) and the Australian isolate PHW1276 (**B**) of *Leptosphaeria maculans* digested with 25 restriction enzymes and hybridized to the LMR1 probe. The positions of the molecular weight markers are shown beside the gels. The bands that showed restriction fragment length polymorphisms between isolates are indicated by asterisks. The enzymes used and the number of sites for each enzyme within the LMR1 sequence were: lane 1, *Bam*HI (2); lane 2, *Bcl*I (0); lane 3, *Bgl*II (10); lane 4, *Eco*RI (0); lane 5, *Hae*III (0); lane 6, *Eco*RV (1); lane 7, *Hinc*II (1); lane 8, *Hin*dIII (16); lane 9, *Kpn*I (0); lane 10, *Mlu*I (1); lane 11, *Not*I (0); lane 12, *Pst*I (0); lane 13, *Pvu*I (0); lane 14, *Pvu*II (0); lane 15, *Sal*I (0); lane 16, *Sca*I (0); lane 17, *Sfi*I (0); lane 18, *Sma*I (0); lane 19, *Sph*I (0); lane 20, *Sac*I (5); lane 21, *Sac*II (0); lane 22, *Stu*I (0); lane 23, *Hpa*I (1); lane 24, *Nco*I (0); and lane 25, *Rsa*I (5).

either a reverse transcriptase or a transposase characteristic of those types of element (Hutchison *et al.* 1989).

The comparison dot plots and base composition of LMR1 indicate that it may have evolved from simple repeat arrays that consisted of the triplet TAA. This triplet could then have diverged, predominantly into the triplets TAG, TTA, ATA, and CTA, based on the element's base composition. This would make this repeat somewhat similar to the TAA-like tandem repeats flanking *Trypanosoma* intrachromosomal variant surface glycoprotein genes (Aline *et al.* 1985) or the IR3 repeat (GGA) found in mammals (Heller *et al.* 1985). The stringency of the conditions used for hybridization and DNA amplification indicate that the majority of the copies in all isolates were more than 90% homologous to each other. Since isolates of both mating types, distinct pathogenicity groups, and diverse geographic origins were examined, the observed conservation cannot be attributed to clonal lineage. Yet the Southern blots and PCR results also indicate that sequence divergence is beginning to occur in some copies of the element within a particular isolate. The lack of open reading frames plus the evidence of sequence divergence argues against a vital biological function for LMR1. Therefore, the observed conservation in the element is most likely due to a recent evolutionary origin. Plummer and Howlett (1993) reported finding a high degree of chromosome length polymorphism in *L. maculans* after meiosis. With regard to discovering if LMR1 does have a biological function, we are investigating whether or not the element is involved in generating this chromosome length polymorphism.

Since the time of the initial cloning of LMR1, many markers, such as single-copy genomic clones, random amplified polymorphic DNA (RAPD), and rDNA, have been used to distinguish highly virulent from weakly virulent rapeseed isolates (Goodwin and Annis 1991; Koch *et al.* 1991; Morales *et al.* 1993a,b). However, because the very presence of LMR1 distinguishes highly virulent from weakly virulent isolates, it can be used in simple dot blot hybridizations for strain identification. It is also a highly sensitive diagnostic marker for detecting virulent *L. maculans* in plant tissue or seed, because of its high copy number per haploid genome. Taking advantage of these aspects of the element, a simple and sensitive PCR diagnostic test for seed contaminated with virulent *L. maculans* has been developed (Taylor 1993).

The phylogenetic analyses of RFLP data (Koch *et al.* 1991) and ITS sequence data (Morales *et al.* 1993a) indicate that the highly virulent and the weakly virulent strains of *L. maculans* are not monophyletic, and all attempts to mate the strains have failed. Therefore, the presence of the LMR1 element in the Laird 2 isolate would seem to indicate that a rare transfer event has occurred. The basis for the difference in pathogenicity of Laird 2 and the closely related isolate Unity remains to be determined. It is possible that some gene or genes encoding pathogenicity factors of the highly virulent strain are coincidentally attached to the element or elements that were transferred. We are exploring this possibility.

Table 1. Mean diameters of lesions caused by *Leptosphaeria maculans* isolates on cotyledons of selected rapeseed and mustard cultivars

		Test 1		Test 2	
Isolate[a]	Cultivar[b]	Mean lesion diameter (mm)	SE	Mean lesion diameter (mm)	SE
Unity	W	0.8	0.16	0.9	0.14
	Cr	0.6	0.06	0.9	0.06
	C	0.5	0.06	0.5	0.01
	CB	0.6	0.15	0.5	0.03
Laird 2	W	2.1	0.35	2.4	0.19
	Cr	2.1	0.46	3.2	0.53
	C	1.5	0.14	2.2	0.51
	CB	1.9	0.31	2.2	0.41
Leroy	W	5.2	0.46	3.5	0.38
	Cr	3.7	0.39	3.4	0.45
	C	0.5	0.01	0.7	0.05
	CB	0.6	0.07	1.0	0.17

[a] Unity and Laird 2 are weakly virulent and Leroy is highly virulent on rapeseed.

[b] C = *Brassica juncea* cv. Cutlass; CB = *B. juncea* cv. Commercial Brown; Cr = *B. napus* cv. Crésor; W = *B. napus* cv. Westar.

MATERIALS AND METHODS

Fungal and bacterial strains.

The majority of the fungal isolates were obtained from G. A. Petrie and R. K. Gugel (Agriculture Canada Research Station, Saskatoon, SK). WA51 and WA74-4 were obtained from S. R. Rimmer (Department of Plant Sciences, University of Manitoba, Winnipeg, MN, Canada). PHW1275 and PHW1276 were provided by P. H. Williams (Department of Plant Pathology, University of Wisconsin, Madison). The geographic origins of the isolates are noted in the legends of Figures 3 and 4. The fungal cultures were maintained on V8 juice agar (Gugel *et al.* 1990). For DNA isolation, plugs from plates were transferred to liquid minimal medium (Tinline *et al.* 1960) and grown for 1 week at room temperature with shaking. The bacterial strain LE392 was host for the bacteriophage genomic library, and plasmid subclones were maintained in XL1-blue (Stratagene).

Pathogenicity testing.

The pathogenicity of the fungal isolates Unity, Laird 2, and Leroy on cotyledons of *B. napus* and *B. juncea* cultivars was tested as described previously (Gugel *et al.* 1990).

DNA isolation, cloning, and sequencing.

Freeze-dried mycelia were vortexed in the presence of glass beads, and the resulting powders were suspended in extraction buffer. The buffers, containing cetyltrimethylammonium bromide (CTAB), and the DNA isolation protocol were as described by Murray and Thompson (1980). The DNA from isolate Leroy (highly virulent on rapeseed) was purified by CsCl-bisbenzimide centrifugation (Garber and Yoder 1983), partially digested with *Sau*3AI, size-fractionated, and ligated to *Bam*HI-digested λ2001 DNA (Stratagene) as described by Maniatis et al. (1982). The genomic library was screened with Leroy DNA that was ^{32}P-labeled by nick translation (Maniatis *et al.* 1982). The most strongly hybridizing phage clones were isolated for further analysis. Purified phages were screened with a *Neurospora crassa* rDNA clone (Free *et al.* 1979) obtained from R. L. Metzenberg. The bacteriophage clones were restriction mapped, and the repetitive DNA sequences were subcloned into plasmids TZ18 and 19R (Pharmacia). Plasmid subclones were sequenced in both directions with an Applied Biosystems 370A sequencer using

the Taq Dye Deoxy Terminator cycle system. The preparation of samples for pulse field gel electrophoresis and the electrophoretic running buffer were as described previously (Taylor *et al.* 1991). These samples, however, were electrophoresed in 1.1% rapid agarose gels (Life Technologies) at 12° C for 48 hr at 78 V with a 25-min pulse and then for 161 hr at 95 V with a 10-min pulse in a Beckman Geneline II apparatus.

Southern blot analysis and genome copy number estimation.

The fungal DNA used for Southern analysis was prepared as stated above, with the omission of the CsCl centrifugation. These DNA preparations were further purified by dissolving the CTAB precipitates in 1.2 M NaCl, precipitating in ethanol, redissolving in Tris-EDTA (Del Sal and Schneider 1987), and then digesting with RNase A (50 µg/ml) and RNase T_1 (200 U/ml) before a final phenol/chloroform extraction, ethanol precipitation, and dissolution in Tris-EDTA. Then 2 µg of fungal DNA was digested to completion with each of the restriction enzymes noted in the legend of Figure 5. The samples were electrophoresed in 0.8% agarose plus 1× Tris-acetate-EDTA gels, transferred to nylon membranes (Hybond N, Amersham) by capillary action, and hybridized under stringent conditions with random primer (Life Technologies) ^{32}P-labeled probes. All blots were washed once with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.5% sodium dodecyl sulfate (SDS) at room temperature for 10 min and twice with 0.1× SSC plus 0.5% SDS at 60° C for 30 min. The genome reconstruction was performed by binding 0.1, 0.5, and 1.0 µg of fungal DNA to nylon membranes by means of a slot blot apparatus (Tyler Research, Edmonton, AL, Canada). Plasmid DNA in amounts equal to copy number equivalents of 1–1,000 was bound to the membrane next to the fungal DNA. The fungal haploid genome size was estimated to be 28 Mb (Morales *et al.* 1993b). The slot blots were hybridized, washed, and exposed to X-ray film, and the autoradiograms were scanned with a densitometer (BioRad, Model 620) to determine peak area. The genome reconstruction Southern blot contained 2 µg of *Bam*HI-digested fungal DNA loaded adjacent to *Bam*HI-digested LMR1 plasmid DNA in copy number equivalents of 2, 10, 20, 35, 50, 70, 85, 100, 120, 135, 150, 170, 250, and 350. The blot was hybridized and the autoradiogram was scanned as described above.

Analysis of DNA sequences.

Programs from the UWGCG sequence analysis software package, versions 6.0 and 7.0, were used to analyze the structure of the repetitive element LMR1 (Devereux *et al.* 1984).

PCR amplification.

The amplification reactions contained 2.0 ng of fungal DNA; 200 µM deoxynucleoside triphosphates; primers (165 nM each); 20 mM Tris-HCl, pH 8.8; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 27.5 µM tetramethylammonium chloride; and 2.5 units of *Taq* polymerase (Life Technologies). The primers used in the amplifications were

881 = 5′—GCGCTATTACACATGCCTAACAGG—3′
2026C = 5′—TCCTCTATGCTAAGCTAGCTGTGC—3′
4259 = 5′—GCGTAAGAAGCGTGCCTTAGAGTC—3′
4839C = 5′—TCCTGCTCCTACTCCTTCTCTAGC—3′

The primer designations above denote the base-pair positions within LMR1 at which the primer originates. The Primer Designer, version 1.0 (Scientific & Educational Software), was used to select the optimal primers from the sequence. The amplifications were performed in a Barnstead Thermolyne thermal cycler with the following program: 1) 96° C dwell for 2 min; 2) 35 ramped cycles consisting of 94° C for 30 sec, 71° C for 30 sec, and 72° C for 4 min; and 3) final extension at 72° C for 7 min.

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