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The inheritance of restriction fragment length polymorphisms in the flax rust *Melampsora lini*

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Summary. Random cDNA sequences synthesized from poly A^+ RNA extracted from germinated urediospores of the flax rust fungus, *Melampsora lini*, were used as probes to detect restriction fragment length polymorphisms (RFLPs) in three races of *M. lini* originating from cultivated flax, *Linum usitatissimum*, and one race originating from Australian native flax, *L. marginale*. Fourteen out of 22 probes tested detected RFLPs in the three races from cultivated flax while 19 of the probes detected polymorphisms between these three races and the race from *L. marginale*. The segregation of seven RFLPs was determined in a family of 19 F_2 progeny derived from a cross between two of the rust races. With six of these the inheritance was consistent, in each case, with the segregation of alleles at a single locus. Inheritance of the seventh was unusual and an explanation involving two loci with null alleles at each was proposed. No linkage was detected between any of the RFLP loci and nine unlinked loci specifying avirulence.

Key words: Flax — Rust – RFLP – Genetic segregation

Introduction

Genetic studies of phytopathogenic fungi, including rusts, are frequently directed towards understanding the extent and mechanisms involved in the generation of new virulence specificities. Genetic markers for such studies have been largely limited to an analysis of virulence specificities and more recently to isozyme variation. In rust fungi these markers have been used to study the sexual transmission of genes (Johnson 1954; Flor 1955; Wilcoxson and Paharia 1958; Luig and Watson 1961; Samborski and Dyck 1968, 1976; Haggag et al. 1973; Burdon et al. 1986), as well as somatic events, such as heterokaryosis and nuclear exchange (Flor 1964; Watson and Luig 1959; Burdon et al. 1981) and parasexual recombination (Watson and Luig 1959; Ellingboe 1961). In general, isozyme markers have shown relatively little useful genetic variation.

Restriction fragment length polymorphisms (RFLPs) present an alternative source of markers for the measurement of genetic variation in phytopathogenic fungi (Michelmore and Hulbert 1987). The inheritance of RFLP markers and the construction of linkage maps have been reported for the downy mildew of lettuce, *Bremia lactucae* (Hulbert and Michelmore 1988), and the powdery mildew of wheat, *Erysiphe graminis* (Christiansen and Giese 1990). RFLPs have been observed in both maize rust (*Puccinia sorghi*) and wheat-stem rust (*P. graminis tritici*) using cDNA sequences derived from polyA$^+$ RNA purified from germinated urediospores, as probes to detect RFLPs (Anderson and Pryor 1991 b, c). This work demonstrated that cDNA probes showed little cross species homology and no hybridization between genera.

The present paper reports the use of cDNA probes in detecting RFLPs in three races of flax rust (*Melampsora lini* (Ehrenb) Lév) originating from cultivated flax (*Linum usitatissimum* L.) and one race originating from Australian native flax, *L. marginale* Cunn. The segregation for seven of the RFLPs amongst F_2 progeny derived from a cross of two of the races from cultivated flax is also described. Since these F_2 progeny were segregating for avirulence at nine unlinked loci (Lawrence et al. 1981) a test for linkage between the RFLP loci and the avirulence loci was undertaken.

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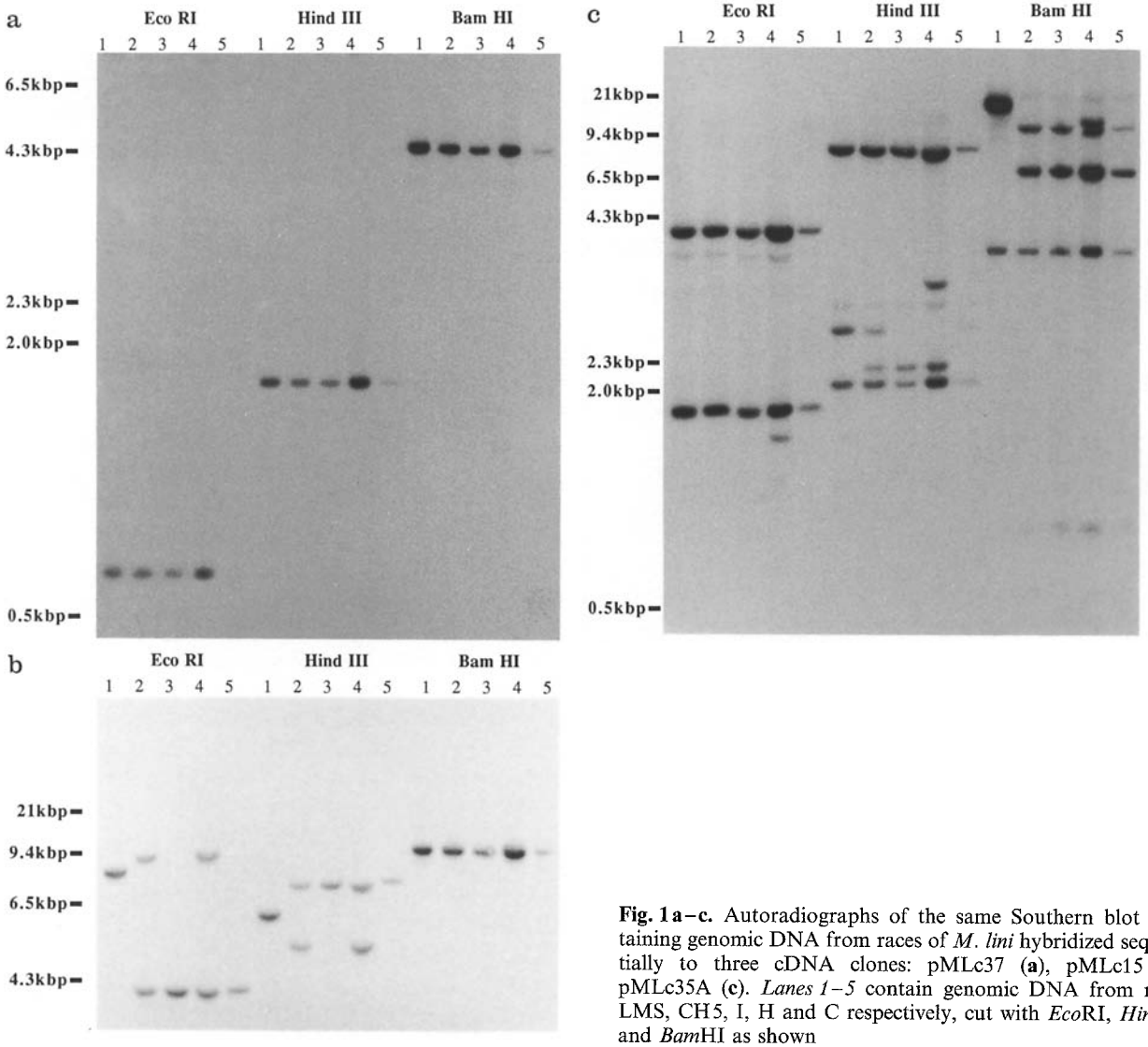

Fig. 1 a–c. Autoradiographs of the same Southern blot containing genomic DNA from races of *M. lini* hybridized sequentially to three cDNA clones: pMLc37 (**a**), pMLc15 (**b**), pMLc35A (**c**). *Lanes 1–5* contain genomic DNA from races LMS, CH5, I, H and C respectively, cut with *Eco*RI, *Hin*dIII and *Bam*HI as shown

Materials and methods

Rust races

Four races of *M. lini*, designated C, H, I and LMS, were used in this study. The first three originated from cultivated flax, *L. usitatissimum*, whereas LMS was isolated from the Australian native flax, *L. marginale*. The origin of races C, H and I is described by Lawrence et al. (1981). The races C and H were crossed to produce the hybrids CH4, CH5 and CH6. The hybrid, CH5, was self-fertilized to produce a family of F_2 individuals (Lawrence et al. 1981), 19 of which were used in this study. The method of crossing *M. lini*, and the maintenance of the races, is described by Lawrence (1988). All the races of *M. lini*, including the hybrids and the F_2 progeny, were individually increased on the universally susceptible flax variety, 'Hoshangabad'.

Extraction of DNA, poly A^+ RNA and cDNA synthesis

Rust germination and collection, DNA and RNA extraction, poly A^+ RNA purification, and cDNA synthesis, were all carried out according to the methods described for the maize rust, *P. sorghi* (Anderson and Pryor 1991 a, b). The cDNA library was synthesized from 5 µg of polyA^+ RNA extracted from race CH5 and cloned with *Eco*RI linkers into the bacterial plasmid vector pGEM 3Zf(+) (Promega). The total library was stored as aliquots of bacterial cells in 7% DMSO at –80 °C.

Identification of RFLP loci

Gel electrophoresis of digested DNAs, blotting, hybridization and labelling of DNA probes were conducted as previously described (Anderson and Pryor 1991 b). Probes were made from the cDNA insert isolated from each clone. Genomic DNA from the various rust races was digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Bam*HI (Pharmacia). Those restriction enzymes which detected RFLPs in the parent races C and H were used to digest DNA from the hybrids and F_2 progeny for segregation analysis.

Analysis of data

To compare the complexity of the hybridization patterns, the number of digests which contained one, two, three, and more

Table 1. Results of the RFLP analysis of the races LMS, CH5, I, H and C of *M. lini*. Numbers indicate the number of genomic bands hybridizing to the cDNA probes. The data for probes pMLc 15, 35A and 37 are shown in Fig. 1

Enzyme		Restriction enzyme/DNA combination														
		*Eco*RI					*Hin*dIII					*Bam*HI				
DNA		LMS	CH5	I	H	C	LMS	CH5	I	H	C	LMS	CH5	I	H	C
pMLc	insert size (bp)															
3	1,400	1	1	1	1	1	1	1	1[a]	1[b]	1[c]	1	2	2[a]	2[b,d]	2[c,e]
4	800	1	1	1	1	1	1	1	1[a]	1[b]	1[c]	1	2	2[a]	2[b]	2[c,e,f]
8	1,800	1	2	2[a]	2[b,d]	2[c,e,f]	1	2	2[a]	2[b]	1[e,f]	1	1	1	2[b,d]	1[f]
15	200	1	2	1[a]	2[b,d]	1[c,f]	1	2	1[a]	2[b,d]	1[c,f]	1	1	1	1	1
16A	1,300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16B	400	2	2	2[a]	2[b]	2[c]	1	1	1	1	1	1	1	1	1	1
16C	200	2	2	2	2	2	2	2	2	2	2	1	1	1[a]	1[b]	1[c]
17	600	1	1	1	1	1	1	1	2[a]	1[d]	1[e]	1	1	1	1	1
18	800	1	2	2[a]	2[b]	2[c]	1	1	1	1	1	1	1	1	1	1
22	700	2	4	2	2	2[c,e,f]	2	3	2	2	2[c,e,f]	2	2	2[a]	2[b,d]	2[c,e,f]
23	900	1	1	1[a]	1[b]	1[c,e,f]	1	2	1[a]	1[b]	1[f]	1	1	1[a]	1[b]	1[c]
28	500	2	3	3[a]	3[b]	3[c]	2	2	2	2	2	2	2	2	2	2
30	400	2	3	3[a]	4[b,d]	3[c,f]	2	3	2	2	3[c,e,f]	1	2	2[a]	2[b,d]	1[e,f]
31	600	1	2	2[a]	2[b]	1[c,e,f]	1	2	2[a]	2[b,d]	1[c,e,f]	1	1	1[a]	2[b,d]	1[c,e,f]
33	400	1	2	2[a]	2[b]	1[e,f]	1	1	1[a]	1[b]	1[c]	1	1	1[a]	1[b,d]	1[c,e]
35A	900	3	3	3	4[b,d]	3[f]	3	4	3[a]	4[b,d]	3[e,f]	2	2	2[a]	3[b,d]	2[c,f]
35B	800	2	2	2	3[b,d]	2	3	4	3[a]	4[b,d]	3[e]	2	2	2[a]	2[b]	2[c]
36	1,100	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
37	600	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
38	1,100	2	2	2[a]	2[b]	2[c]	2	2	2	2	2	1	1	1	1	1
39	1,150	1	1	1[a,e]	2	1[f]	2	3	2[a]	3[b,d]	2[e,f]	2	2	2[a]	3[b,d]	2[c,f]
40	350	1	1	1	1	1	2	2	2[a]	2[b]	3[c,e,f]	1	2	3[a]	2[b,d]	3[c,e,f]

[a] RFLP between LMS and I
[b] RFLP between LMS and H
[c] RFLP between LMS and C
[d] RFLP between I and H
[e] RFLP between I and C
[f] RFLP between H and C
A, B, and C, several *Eco*RI inserts were contained within the one pMLc clone

than three, bands were pooled into classes and expressed as a percentage of the total number of probe/enzyme combinations that were tested. The inheritance of seven RFLP loci detected between C and H was determined by the segregation of bands in the hybrids and F_2 progeny. The genotype of each of the F_2 progeny at the seven RFLP loci, and at the nine avirulence loci also segregating in the cross, was analysed by the Mapmaker program (Lander et al. 1987) to detect possible genetic linkage relationships.

Results

Of 60 colonies selected from the *M. lini* cDNA library, 44 were found to contain inserts, ranging in size from 300 to 2,500 bp. Twenty-four cDNAs were randomly selected and 22 of these detected different hybridization patterns when hybridized to blots of *M. lini* genomic DNA cut with three different restriction endonucleases. Two cDNA clones were each represented twice. Figure 1 a, b and c shows the autoradiographs of Southern blots hybridized with three different cDNA probes demonstrating increasing levels of complexity. The data collected from the 22 different hybridization patterns are summarized in Table 1 in which the number of hybridizing bands in each probe/restriction enzyme combination is recorded. These data were pooled and standardized as described in Materials and methods, and compared with the equivalent data from the hybridization patterns of *P. sorghi* genomic DNA (Anderson and Pryor 1991 b). From this comparison (see Table 2) it can be seen that the incidence of multiple bands was greater in *P. sorghi* than in *M. lini*.

Fourteen of 22 probes detected RFLPs between the three races originating from cultivated flax, whereas 19 of the 22 detected RFLPs between the rusts of cultivated flax and race LMS isolated from native Australian flax.

Eleven probes detected RFLPs between race C and race H. The pattern of inheritance of the polymorphisms

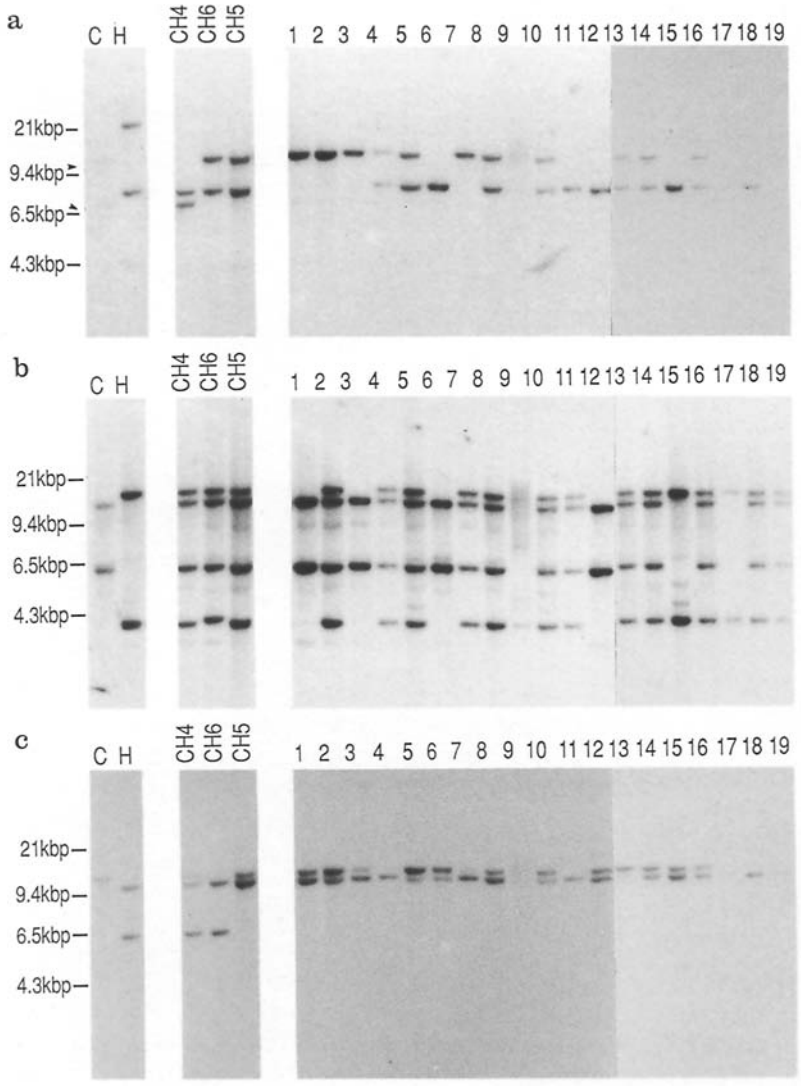

Fig. 2a–c. Autoradiographs from a Southern blot hybridized sequentially to pMLc8 (**a**), pMLc22 (**b**), pMLc33 (**c**). All DNAs were restricted with *Eco*RI. The first two lanes (*C, H*) contain DNA from the parental races C and H respectively. The next three lanes (*C, H*) contain DNA from three hybrids between these races. The remaining lanes (*numbered 1–19*) contain DNA from the F_2 progeny from the self fertilization of the CH5 hybrid. Genomic DNA from progeny 9 digested poorly and was not included in the analysis. *Arrow heads* in **a** indicate the position of bands in the race C parent

detected by seven of these probes was determined in hybrids between these two races and in 19 of the F_2 progeny generated from hybrid CH5. Four of the probes each detected two bands in the CH5 hybrid and, in each case, the pair of bands segregated in agreement with a 1:2:1 ratio among the F_2 progeny, consistent with allelism (see Fig. 2a for example). Probe pMLc22 detected four bands in CH5, two being inherited from parent C and two from parent H. In the F_2 progeny these pairs of bands segregated in a 1:2:1 ratio for the C:CH5:H patterns respectively (see Fig. 2b) and is consistent with the segregation of two alleles each of which specify a two-banded pattern. These "two-banded" alleles cannot be accounted for by the presence of an *Eco*R1 site in the probe sequence since the pMLc22 cDNA clone does not contain an internal *Eco*R1 restriction site (data not shown). Since the cDNA clones are presumably derived from transcribed sequences it is possible that this clone hybridizes to a genomic DNA that includes an intron which contains an *Eco*R1 site. Alternatively, there could be a closely linked duplication of the pMLc22 sequence in the genome.

The sixth probe detected three bands in the CH5 hybrid. Two of these bands segregated in a 1:2:1 ratio amongst F_2 progeny while the third did not segregate and was present in all the progeny.

The inheritance of the RFLP bands detected by the last probe tested, pMLc33, could not be explained by segregation at a single locus. As shown in Fig. 2c the pMLc33 probe detected one band in race C and two bands in race H. All three parental bands were found in the CH4 hybrid but only the race H bands were transmitted to the hybrid CH6. The third hybrid, CH5, possessed only one of the race H bands together with the C band. These two bands segregated in a 1:2:1 ratio in the F_2 progeny ($\chi^2 = 3.8$, $0.2 < P < 0.3$) consistent with allelism. These observations can be accounted for by a two locus model with "null" alleles segregating at each locus. If the

Table 2. Summary of the hybridization patterns of random cDNA probes from *M. lini* and *P. sorghi*. The number of hybridizing bands in each digest from all probe/DNA/enzyme combinations were grouped into 1, 2, 3, and >3 bands per digest, and expressed as a percentage of the total number of probe/enzyme combinations. Data from *M. lini* were collected from 22 probes, five DNA samples cut with three restriction enzymes. *P. sorghi* DNA extracted from two races was analysed with 24 probes and in most cases with six restriction enzymes (Anderson and Pryor 1991 b)

No. of bands per digest	% of digests	
	P. sorghi	*M. lini*
1	14	51
2	59	38
3	15	9
>3	12	2

loci are designated *A* and *B* with null alleles being designated by a dash (–) then the observations are consistent with the following genotypes: Race C = *A2–,—*; Race H = *A1A1*, *B1–*; hybrid CH4 = *A1A2,B1–*; hybrid CH6 = *A1–*, *B1–*; hybrid CH5 = *A1A2,—*. The single band in race C corresponds to the *A2* allele with the two bands in race H corresponding to the *A1* and *B1* alleles with the smaller restriction band being specified by the *B1* allele (Fig. 2c).

The segregation data of nine unlinked avirulence gene loci combined with the segregation data of the RFLP loci were analysed with the Mapmaker program (Lander et al. 1987). No linkage was detected between any of the loci.

Discussion

The high frequency with which clones selected from a cDNA library of *M. lini* detected RFLPs between races which infect cultivated and Australian native flax, indicated the potential usefulness of the clones in genetic studies of *M. lini*.

The frequency with which multiple restriction bands were observed in *M. lini* was not as high as had previously been reported in *P. sorghi*. Whereas 86% of all restriction enzyme/probe combinations gave multiple bands in *P. sorghi*, only 49% of the *M. lini* patterns contained multiple bands. In *P. sorghi* it was argued that heterozygosity of alleles present in the different nuclei of the dikaryon could explain some of the complexity, but some form of DNA duplication was required to explain more complex patterns (Anderson and Pryor 1991 b).

In *M. lini*, where the level of complexity was lower, seven multiple-banded pattern RFLPs were investigated genetically and six could be accounted for by heterozygosity at a single locus. In only one case, defined by the the pMLc33 probe, was it necessary to propose the involvement of two loci or some other form of nucleotide sequence duplication. A model which can account for this last case also requires the postulation of "null" alleles.

Null alleles are occasionally observed at loci defined by isozymes and can be explained simply as a loss of gene function. However, a null allele for a DNA sequence requires the deletion of nucleotide sequences. Hartley and Williams (1971) have argued that mitotic non-disjunction of chromosomes may be important in generating genetic variability in flax rust. A deficiency for the homologue containing the pMLc33 gene in race C could explain the unusual inheritance of this marker. Since segregation for six RFLP and nine avirulence loci were normal this would imply that only the postulated pMLc33 loci were located in this deleted region. Clearly, the segregation of null alleles as a consequence of deletion of part or a whole chromosome is only one explanation for the observed unusual inheritance, and these results may reflect some other aspect of the rust genetic system.

The segregation analysis provided no evidence of linkage between any of the 16 loci (seven RFLP and nine avirulence loci) segregating in the F_2 progeny. The length of the genetic maps of the ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa* are 1,800 and 620 cM respectively (Caten 1987). Assuming a similar in length in *M. lini*, then on average 16 random loci would be separated by around 75 cM recombination. This and the small F_2 family size tested in this study make it unlikely that linkage would have been observed.

Burdon and Roelfs (1985) using 13 isozyme markers in nine races of wheat stem rust detected six polymorphisms. In the present study 14 of 22 random cDNA probes detected RFLPs in three races of flax rust originating from cultivated flax. Thus random cDNA probes used to detect RFLPs will be a useful means for measuring the levels of genetic heterogeneity in populations of agronomically important rust pathogens. The results of this study also suggest that it should be possible to construct a high density genetic map of *M. lini* using RFLP markers. Such a map would provide a starting point for the cloning of rust genes of interest (e.g., avirulence genes) via chromosome walking methods (Young 1990) and would also be useful in characterizing the genetic events that might be involved in the generation of pathogen variation.

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