Theor Appl Genet (1990) 79:705–712

Genetic analysis of the obligate parasitic barley powdery mildew fungus based on RFLP and virulence loci

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Received December 5, 1989; Accepted February 9, 1990
Communicated by F. Salamini

Summary. Genome organization of the biotrophic barley powdery mildew fungus was studied using restriction fragment length polymorphism (RFLP). Genomic DNA clones containing either low- or multiple-copy sequences appeared to be the best RFLP markers, as they frequently revealed polymorphisms that could be readily detected. A total of 31 loci were identified using 11 genomic DNA clones as probes. Linkage analysis of the 31 RFLP loci and five virulence loci resulted in the construction of seven groups of linked loci. Two of these contained both RFLP markers and virulence genes. RFLP markers were found to be very efficient in characterizing mildew isolates, as only three markers were necessary to differentiate 28 isolates. The DNA of the barley powdery mildew fungus appeared to contain a considerable number of repetitive sequences dispersed throughout the genome.

Key words: *Erysiphe graminis* – Genetic map – Repetitive DNA – RFLP – Virulence genes

Introduction

The obligate parasitic fungus *Erysiphe graminis* f. sp. *hordei* is the causal agent of the powdery mildew disease in barley. The fungus, which is an Ascomycete, causes considerable yield losses in Europa and has been studied extensively. The interaction between barley and powdery mildew conforms to the gene-for-gene hypothesis (Flor 1955). Corresponding resistance genes in the plant and virulence genes in the fungus have been genetically determined (Moseman 1959). As with most other phytopathogenic fungi, genetical analysis has been restricted to virulence gene studies because of the lack of other markers (Jørgensen 1988). Restriction fragment length polymorphism (RFLP) analysis offers a very attractive method for obtaining neutral markers for studies of the pathogen population (O'Dell et al. 1989) and for making a detailed genetic linkage map.

An importance step in RFLP anlysis of eukaryotic organisms is to obtain clones containing nonrepetitive sequences. As repetitive DNA is generally noncoding, and noncoding DNA of many eukaryotic organisms is methylated, this has been exploited in plant RFLP analysis (Burr et al. 1988; Tanksley et al. 1987) by use of the methylation-sensitive enzyme PstI to construct genomic libraries.

This paper reports on the use of random clones from PstI and EcoRI genomic libraries of barley powdery mildew fungal DNA for RFLP analysis. The segregation of 31 RFLP and five virulence loci has been analyzed in a cross with 80 progeny isolates. Linkage analysis was performed and a tentative genetic map was constructed.

Materials and methods

Fungal and plant material

Conidia of the isolates C15 and EmA30 (Table 1) were harvested from infected barley leaf sections as described in Giese et al. (1990).

The isolates listed in Table 1 were grown on a susceptible barley line 'Pallas'. The infected leaves were harvested 10 days after inoculation, frozen in liquid nitrogen, and stored at −70 °C.

The mildew isolates C15 and TY4 (Table 1) were crossed as described in Giese et al. (1990). The parental isolates were selected to represent different geographical origins (Sweden and Denmark), virulence phenotypes, and mating types.

Progeny isolates were propagated on barley plants and leaves were harvested after 10 days, frozen in liquid nitrogen, and stored at −70 °C.

DNA preparations and cloning

Pure mildew DNA was prepared by gel electrophoresis as described in Giese et al. (1990). High-molecular-weight DNA was electroeluted (Maniatis et al. 1982) prior to restriction enzyme analysis and cloning. DNA from mildew-infected leaves was extracted as described in Sharp et al. (1988).

Purified high-molecular-weight mildew DNA was digested to completion with EcoRI or the methylation-sensitive enzyme PstI and cloned as described in Giese et al. (1990). Transformants were analyzed using the alkaline lysis minipreparation procedure of Birmboim and Doly (1979).

Selection of clones

Plasmids containing inserts (approximately 100 ng from the minipreparation) were individually spotted onto Hybond-N membranes (Amersham) using a dot blot apparatus (Manufacturer's procedures). Total mildew DNA was labelled with [α^{32} P] dATP (Amersham International) to a specific activity of 2–5 × 10^8 cpm/μg by random priming (Feinberg and Vogelstein 1983) and hybridized to the membranes. Clones showing strong hybridization were assumed to carry repetitive DNA sequences and were therefore discarded, and the remainder were selected as carrying potentially low-copy-number sequences. The final selection for copy number and polymorphism was carried out by digesting barley, infected with each of the two parental mildew isolates with five different restriction enzymes, and hybridizing each clone to the resulting fragment. Pure barley DNA was included as control.

Isolation of plasmid DNA inserts

Plasmids from clones showing weak hybridization signals on dot blots were isolated using a Qiagen Kit according to the manufacturer's instructions (Qiagen, Inc.). Inserts of DNA were separated from vector sequences by digestion with the appropriate restriction enzyme, followed by electrophoresis in 0.6%–1% agarose gels in Tris-borate-EDTA (TBE) buffer. The insert DNA was purified from the gel using the freeze-squeeze method (Tauts and Renz 1983).

Southern blot analysis

Restriction endonuclease digestions, were carried out using enzymes and buffers from Bethesda Research Laboratories. Spermidine (4 m*M*) was added to digests to promote complete digestion. Digested DNA samples (20 μg/lane) were electrophoresed in tris-borate buffer, and Southern blotting was performed according to the manufacturer's recommendations (Amersham) for Hybond-N membranes.

Prehybridization and hybridization reactions were performed as described in Giese et al. (1990). Gel-isolated DNA inserts from genomic DNA clones were labelled with ^{32}P by random priming. Membranes were washed in 2 × SSC (0.3 *M* NaCl, 0.03 *M* Na citrate, pH 7.0), 0.1% sodium dodecyl sulphate (SDS) for 3 × 15 min at 42 °C. The filters were exposed to Hyperfilm –MP (Amersham Int.) from 16 h h to 7 days at 70 °C. ^{32}P-labelled DNA was removed from membranes before reprobing according to the manufacturer's recommendation. The filters were reused 10–15 times.

Test for virulence

The virulence spectrum of the progeny isolates was determined by inoculating leaf sections of 14 isogenic 'Pallas' lines (P01, P02, P03, P04B, P08B, P09, P10, P11, P12, P17, P20, P21, P23, P24), each with its specific resistance genes (Kølster et al. 1986). The leaf sections were maintained on 0.5% water agar plates containing 40 mg l^{-1} benzimidazol. Infection types 0–4 were scored twice 6 and 7 days after inoculation (Torp et al. 1978).

Segregation analysis

Chi-square goodness-of-fit values for segregation and maximum likelihood estimates of recombination frequency of all pairs of polymorphic loci and virulence loci were calculated using the LINKAGE-1 program (Suiter et al. 1983).

Nomenclature

Segregating loci are listed in Table 2. Loci detected by an EcoRI-cloned probe were given the letter code GEE (referring to: genomic, *Erysiphe graminis*, EcoRI), while loci detected by PstI-cloned probes were given the code GEP. When a single clone detected several loci, the individual loci were designated by the same number but distinguished by a lowercase letter (e.g., *GEE-367a, GEE-367b,* etc.), in order of decreasing size of the segregating EcoRI fragments. The alphabetical order was continued for additional loci revealed by the enzyme BamHI, HindIII, EcoRV, and BglII.

The virulence genes were named after their corresponding resistance genes. Thus, the virulence genes corresponding to the resistance genes *Mla3, Mla10, Mla12, Mlc,* and *Mlh* were named *Ia3, Ia10, Ia12, Ic,* and *Ih,* respectively (J. Jensen, personal communication).

Results

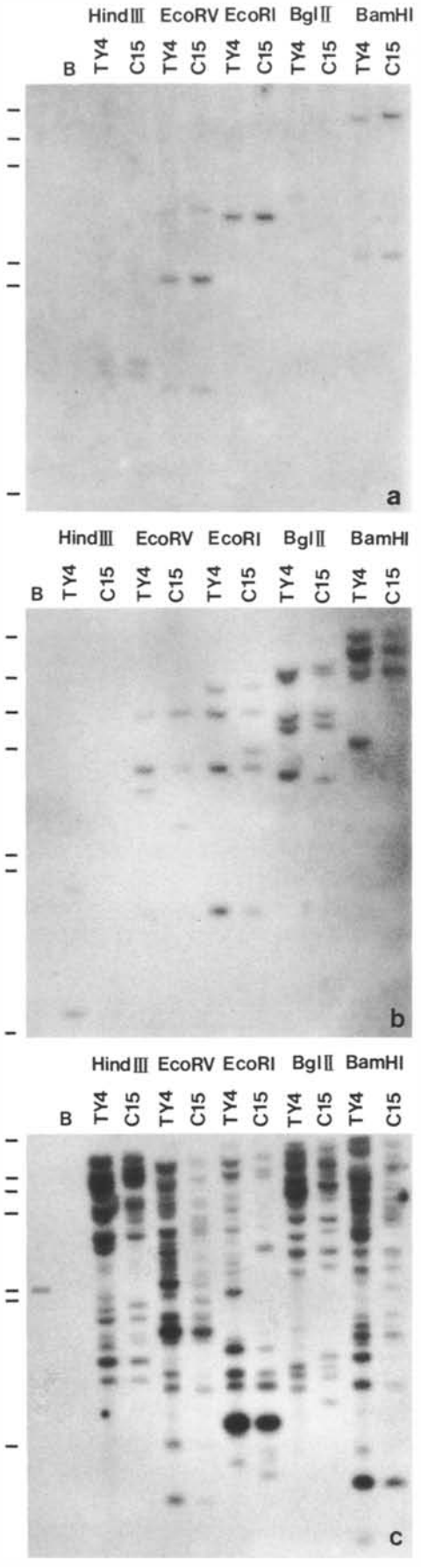
Genome characterization

Purified mildew DNA digested with the restriction enzyme EcoRI showed a large number of bands, indicating that the barley powdery mildew fungus contains a substantial amount of repetitive DNA. This was supported by dot blot analysis of the random genomic DNA libraries, where 50% of the clones appeared to contain repetitive sequences.

A mixture of mildew and barley DNA digested with the methylation-sensitive enzyme PstI indicated that DNA from both organisms is methylated. Most of the DNA appeared to be uncut, in contrast to digestion with the methylation-insensitive enzyme EcoRI. However, cloning with PstI did not result in a higher frequency of clones contraining low-copy-number sequences compared to cloning with EcoRI.

Library characterization

A total of 350 clones was tested for copy number by means of the dot blot method, which revealed three categories of clones: (1) single/low copy, (2) multiple copy, and (3) repetitive. About 50% of the clones from both PstI and EcoRI random genomic libraries was discarded as they contained repetitive sequences. The frequency of clones containing repetitive sequences increased significantly when larger fragments were cloned. For small fragments (0.5–2.5 kb) approximately 30% carried repetitive sequences, while this frequency increased to 80% for larger fragments (2.5–5 kb).



The remaining clones gave either no hybridization signal or weak signals. Results from hybridization to restriction-digested mixtures of mildew and barley DNA revealed that the clones giving no hybridization signal on dot blot represented single- or low-copy-sequences, while the clones giving a weak signal represented multiple-copy sequences (Fig. 1). Only few single-copy clones detected polymorphism, while many low-copy clones and most multiple-copy clones detected polymorphism. The dot blot method proved very efficient for discarding repetitive clones.

Hybridization patterns

As shown in Fig. 1, different hybridization patterns were obtained for the mildew clones. Single-copy clones gave one or two discrete bands (Fig. 1 a), while low-copy clones were defined as giving more than two bands (Fig. 1 b). These banding patterns were characterized by few distinct bands where polymorphism could be easily detected.

Different types of hybridization patterns were obtained with the multiple-copy clones. Some of the multiple-copy clones gave a clear signal for only a few major fragments, while the rest of the bands appeared as a smear, which could not be scored. Other multiple-copy clones showed polymorphisms that were too complex to analyze in segregation studies, but may be usable for population studies. The majority, however, showed polymorphisms that could be scored readily (Fig. 1 c).

To determine if RFLP marker could be useful in identifying mildew isolates, three low-copy-number clones (GEE-160, GEE-161, and GEP-91) were tested against an EcoRI digest of DNA from the 28 different isolates listed in Table 1. The isolates are primarily from Europe with the addition of two Japanese isolates, RaceI and RaceIX, and one American isolate, CR3. Hybridization with clones GEE-160, GEE-161, and GEP-91 resulted in 13, 10, and 18 different banding patterns, respectively. All 28 isolates could be differentiated with these three clones. The hybridization patterns of GEP-91 are shown in Fig. 2.

Fig. 1 a–c. Hybridization patterns obtained with four types of genomic mildew DNA clones. Southern blots of genomic DNA from barley leaves infected with each of C15 and TY4 digested with five different restriction enzymes and probed with **a** a single-copy clone (pGEE-151) detected no polymorphism, **b** a low-copy clone (pGEE-161) detecting polymorphism with BamHI, EcoRI, EcoRV, and HindIII, **c** a multiple-copy clone (pGEE-381) detecting polymorphism with all five enzymes. *B* – Pure barley DNA digested with BamHI. HindIII-digested λ DNA was used as marker 23130, 9416, 6557, 4361, 2322, 2027 bp

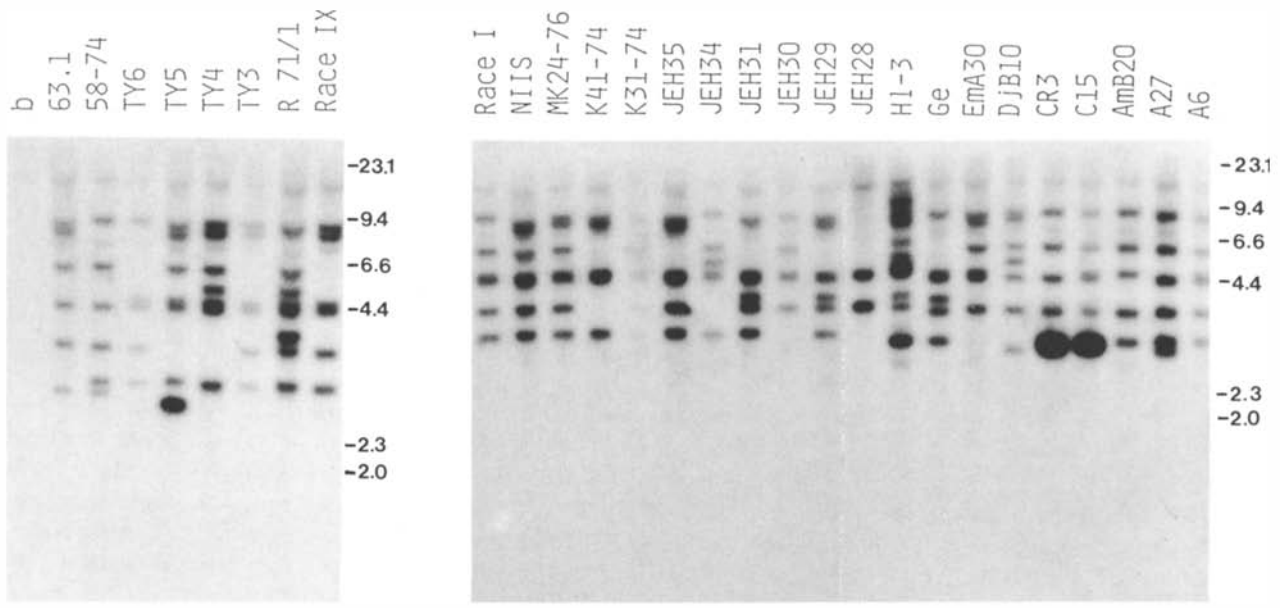


Fig. 2. Autoradiogram from Southern analysis of EcoRI-digested DNA from barley infected with each of the 28 mildew isolates listed in Table 1, probed with a low-copy-number clone pGEP-91. *b* Pure barley DNA. Molecular size markers in kilobase pairs are indicated to the *right* of the blots

Table 1. List of barley mildew isolates examined

Isolate	Country and year of sampling		Reference
A6	S	1958	Wiberg 1974
A27	S	1972	Wiberg 1974
AmB20	S	1973	Wiberg 1974
C15	S/DK	1964	Wiberg 1974
CR3	USA	1951	Moseman 1968
DjB10	NL	(1978)	A. Balkema-Boomstra, pers. comm.
EmA30	S	1972	Wiberg 1974
Ge	D	1979	Swarzbach 1979
Hl-3	D	1979	Swarzbach 1979
JEH28	DK	1975	Hermansen 1980
JEH29	DK	1976	Hermansen 1980
JEH30	DK	1976	J. E. Hermansen, pers. comm.
JEH31	DK	1972	J. E. Hermansen, pers. comm.
JEH34	DK	1977	J. E. Hermansen, pers. comm.
JEH35	DK	1975	J. E. Hermansen, pers. comm.
K31-74	S	1974	J. Meyer, pers. comm.
K41-74	S	1974	J. Meyer, pers. comm.
MK24-76	S	1976	J. Meyer, pers. comm.
NIIS	IL	(1979)	E. Swarzbach, pers. comm.
RaceI	J	(1980)	Hiura and Heta 1955
RaceIX	J	(1980)	Hiura and Heta 1955
R 71/1	DK	1976	Jensen and Jørgensen 1981
TY3	DK	(1978)	C. H. Nielsen, pers. comm.
TY4	DK	(1978)	C. H. Nielsen, pers. comm.
TY5	DK	(1978)	C. H. Nielsen, pers. comm.
TY6	DK	(1981)	C. H. Nielsen, pers. comm.
58-74	S	1974	J. Meyer, pers. comm.
63.1	IRL	1962	Moseman 1968

( ) – Year received at Risø

Restriction enzymes

Different restriction enzymes were analyzed for their ability to reveal polymorphism. Enzymes recognizing 4 bp revealed the least variability, while those recognizing 6 bp revealed the most variability. The enzymes BamHI, BglII, EcoRI, EcoRV, and HindIII were selected as they cut mildew DNA efficiently and detected polymorphism.

Number of loci per clone

Each of the clone/enzyme combinations that detected polymorphism was tested on ten progeny isolates to get an indication of whether more than one segregation locus was involved. All segreating loci were finally scored on all progeny isolates. Most of the multiple-copy clones revealed several segregating loci, while most single- and low-copy-number clones only revealed one segregating locus (Table 2). Using the multiple-copy clone GEP-53, polymorphism could be detected in BglII, EcoRI, and EcoRV digests (Fig. 3). BglII segregated in one band, while the EcoRI and EcoRV each revealed four segregating bands. Two of the segregating bands in EcoRI cosegregated (locus *GEP-53a*) (Fig. 3 a), and the lower-segregating band in EcoRI (locus *GEP-53c*) cosegregated with the lower-segregating band in EcoRV (Fig. 3 a and b). The upper-segregating band in EcoRV cosegregated with the BglII-segregating band (Fig. 3 b and c). Thus, six loci could be detected with GEP-53.

The multicopy clone GEE-367 detected polymorphisms in all five enzymes tested. With the enzymes

Table 2. Observed segregation and Chi-square goodness-of-fit analyses to 1:1 ratio for the 31 RFLP loci and 5 virulence loci

Locus name[a]	Restriction enzyme[b]	Observed ratio C15:TY4	χ^2 (1:1)	Prob
GEP-53a	EcoRI	44:29	3.08	0.08
GEP-53b	EcoRI	36:40	0.21	0.65
GEP-53c	EcoRI, EcoRV	37:38	0.01	0.91
GEP-53d	EcoRV, BglII	41:38	0.11	0.74
GEP-53e	EcoRV	34:47	9.00	0.003*
GEP-53f	EcoRV	35:46	1.49	0.22
GEP-58	EcoRI, BglII	43:33	1.32	0.25
GEP-65	EcoRI, BamHI, BglII, HindIII	35:41	0.47	0.49
GEP-91a	EcoRI	39:37	0.05	0.82
GEP-91b	BamHI	41:39	0.05	0.82
GEE-160	EcoRI, BamHI	42:37	0.31	0.57
GEE-161	EcoRI, BamHI	44:36	0.80	0.37
GEE-292	EcoRI, HindIII	35:44	1.03	0.31
GEE-307a	EcoRI	45:31	2.58	0.11
GEE-307b	BamHI	45:34	2.14	0.14
GEE-365a	EcoRI, BglII	36:41	0.32	0.56
GEE-365b	EcoRI	42:36	0.46	0.49
GEE-365c	EcoRI, HindIII	43:36	0.62	0.43
GEE-365d	HindIII	42:38	0.20	0.65
GEE-365e	HindIII	43:37	0.45	0.50
GEE-365f	BglII	41:37	0.21	0.65
GEE-365g	BglII	42:34	3.37	0.07
GEE-365h	BglII	43:35	0.82	0.37
GEE-367a	EcoRI	42:34	0.84	0.36
GEE-367b	EcoRI	41:33	0.86	0.35
GEE-367c	EcoRI	55:22	14.14	0.0002*
GEE-367d	EcoRV, HindIII, BglII	47:32	2.85	0.09
GEE-367e	HindIII, BamHI	42:37	0.32	0.57
GEE-373a	EcoRI	34:45	1.53	0.22
GEE-373b	EcoRI	34:41	0.65	0.41
GEE-373c	EcoRI	35:37 A:V[c]	0.06	0.81
I-a3		39:51	1.60	0.21
I-a10		44:46	0.04	0.83
I-a12		30:60	10.0	0.002*
I-c		39:48	0.93	0.33
I-h		35:55	4.44	0.04*

[a] See 'Materials and methods' for nomenclature of loci
[b] The restriction enzymes refer to the ones used to digest the genomic DNA
[c] A – avirulence; V – virulence
* Indicates significant ($P<0.05$) deviation from the expected (1:1) segregation

BamHI, BglII, EcoRI, EcoRV, and HindIII, 1, 1, 5, 1, and 2 segregating bands could be detected, respectively. The five bands from the EcoRI digest segregated in three groups (*GEE-367a, GEE-367b, GEE-367c*). One of the segregating bands in HindIII cosegregated with each of the segregating bands in BamHI, BglII, and EcoRV (locus *GEE-367d*). Thus, five loci could be detected with GEE-367.

With the low-copy-number clone GEP-65, one segregating band was detected with each of the enzymes

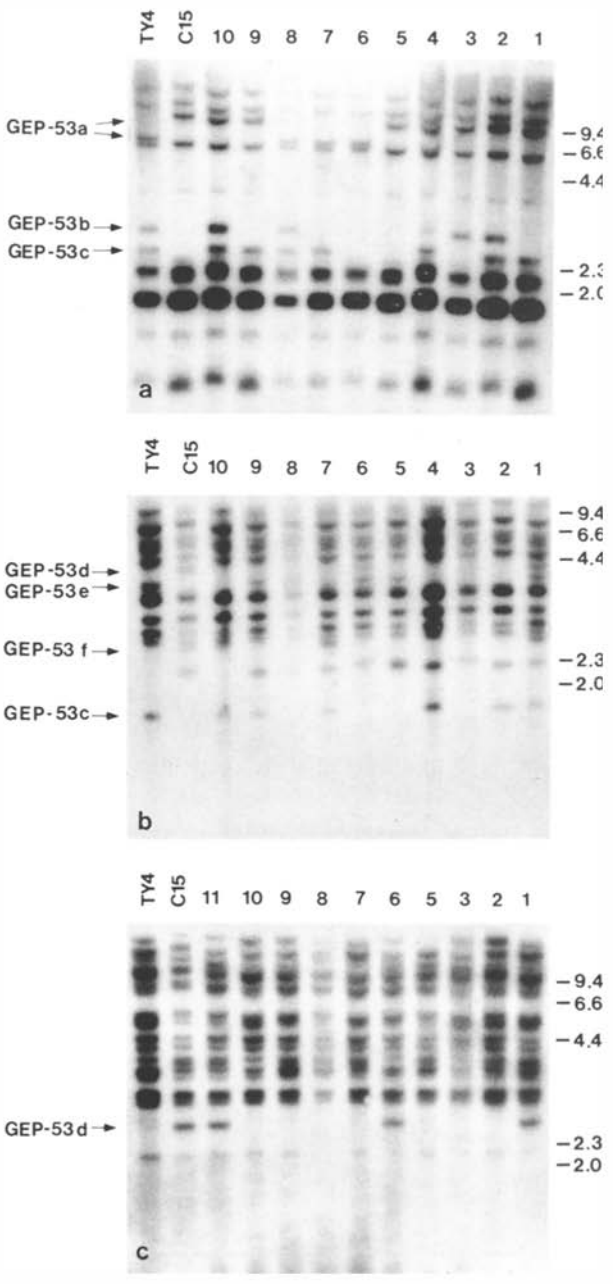


Fig. 3a–c. Segregation of nine RFLPs detected with clone pGEP-53. **a** Hybridization to EcoRI-digested DNA from ten progeny isolates revealed four segregating fragments, two of which cosegregate, thus three loci are uncovered (*GEP-53a, GEP-53b, GEP-53c*). **b** Hybridization to EcoRV-digested DNA revealed four segregation fragments, one of which cosegregates with locus *GEP 53c*, thus three new loci (*GEP-53d, GEP-53e, GEP-53f*) are uncovered. **c** Hybridization to BglII-digested progeny isolates revealed one segregating fragment, which cosegregates with locus *GEP-53d*, thus no further loci are uncovered by BglII. Molecular size markers in kilobase pairs are indicated to the *right* of the blots

BamHI, BglII, EcoRI, and HindIII. As the four bands cosegregated, only one locus (*GEP-65*) was revealed.

All RFLP markers were analyzed in this fashion. A total of 31 loci was identified using 11 different clones.

Virulence spectrum

The parental mildew isolates C15 and TY4 differ in virulence character in 8 [*Ia3, Ia7, Ia10, Ia12, Ic, Ih, Ig+ (CP), I(La)*] of the 14 virulence loci tested. However, the segregation of 3 virulence loci [*Ia7, Ig+ (CP), I(La)*] could not be scored with certainty, as the resistant and susceptible infection types of the parental lines were too similar (2 to 4 or 3 to 4). Thus, these virulence loci were not included in the linkage analysis. The segregation of the 5 remaining virulence loci (*Ia3, Ia10, Ia12, Ic, Ih*) was readily scorable and was included in the linkage analysis.

As expected, the virulence loci [*Ia, Ia6, Ia9, Ia13, Ik, I41/145*] in which the corresponding infection types were similar for the parental isolates did not segregate. This confirms that the correct cross has been analyzed.

Segregation and linkage analysis

The segregation data for the 31 RFLP loci and 5 virulence loci are listed in Table 2. Segregation of 29 RFLP and 3 virulence loci fit the expected 1:1 ratio for single genes in haploid organisms, while the segregation of the remaining 2 RFLP loci (*GEP-53e* and *GEE-367c*) and virulence loci (*Ia12* and *Ih*) deviated significantly ($P < 0.05$).

Linkage analysis performed using the LINKAGE-I program resulted in the construction of seven groups of linked loci, and a tentative chromosome map is presented in Fig. 4. Distances between loci are estimated from the recombination frequencies between pairs of loci. No attempt was made to correct for distances by considering all loci in a linkage group. The order of the loci in linkage group A was determined using log linear models (Edwards and Kreiner 1983). In linkage group B the present data do not allow a final determination of the order of the loci *Ih, GEE-307a*, and *GEE-160*. The loci are tentatively ordered according to the map distances estimated.

Only the linked loci of which we were absolutely certain are presented. The remaining loci analyzed also displayed some probable linkage groups not shown here. Further studies will be performed to confirm these results.

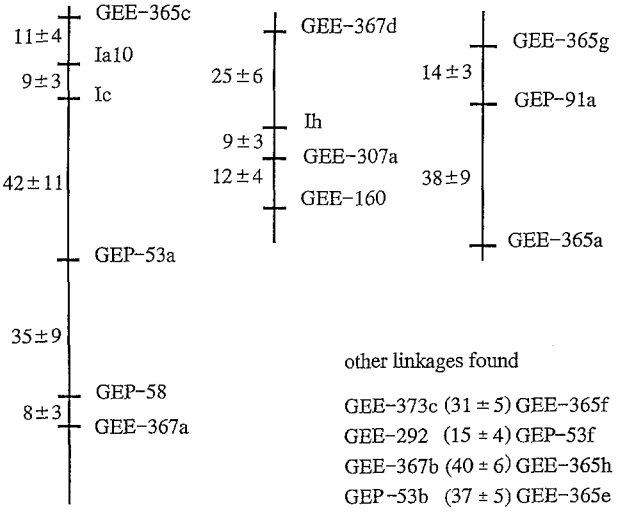


Fig. 4. Groups of linked RFLP and virulence loci. Loci names are shown to the *right* and estimated map distances (in cM) to the *left* of the vertical line

Discussion

Restriction digests of barley powdery mildew DNA and the large number of cloned sequences that are repetitive indicate that the mildew genome consists of relatively large proportions of noncoding DNA. This has previously been established by O'Dell et al. (1989). Filamentous fungi are generally reported to have low amounts of repetitive DNA contrary to higher plants, which may contain up to 75% repetitive DNA. *Neurospora crassa* is reported to contain 8% repetitive DNA (Krumlauf and Marzluf 1979) and *Aspergillus nidulans* only 2%–3% (Timberlake 1978). In both cases, the reiterated DNA is believed to consist mainly of ribosomal sequences. The hybridization patterns obtained using the the ribosomal clones show a distinct banding pattern in mildew, whereas repetitive clones give diffuse patterns similar to those derived for plants (Apuya et al. 1988). This indicates that only a proportion of the reiterated sequences can be ascribed to ribosomal sequences. Furthermore, the fact that the frequency of cloned repetitve sequences increased with the size of the cloned fragment indicates that the repetitve sequences are dispersed throughout the genome. Another biotrophic fungus, *Bremia lactucae* (Hulbert et al. 1988), has been found to contain considerable amounts of repetitive DNA dispersed throughout the genome. This, together with our results, indicates that obligate parasites might have genomes that differ in organization from other filamentous fungi.

Methylation of cytosine residues in eukaryotic DNA is common, but poorly understood. It has empirically been found that sequences in and adjacent to transcribed regions are generally cleaved by methylation-sensitive enzymes (Burr et al. 1988). Thus, in a number of papers concerning plant RFLP analysis (Figdore et al. 1988; Helentjaris et al. 1988), the methylation-sensitive enzyme PstI has been used to enrich for transcribed sequences that might be single or low copy. PstI is usually selected because it recognizes two sensitive sites (Gruenbaum et al. 1981). Although mildew DNA appears to be methylated,

cloning with PstI does not reduce the number of repetitive sequences obtained. Similar results were obtained in RFLP analysis on lentil (Havey and Muehlbauer 1989) and rice (McCouch et al. 1988), where a significant fraction of a PstI-constructed library consisted of repeated sequences. There may, therefore, be variation in the degree of methylation in different organisms, as also concluded by McCouch et al (1988).

The selection procedure used in this study circumvented the problems of using libraries with a high proportion of clones containing repetitive sequences. These clones were recognized and omitted from further analysis at an early stage, and clones with single-, low-, and multiple-copy sequences were maintained. The clones containing multiple-copy sequences turned out to be very useful as they detected frequent polymorphism.

The use of multicopy clones as RFLP markers gives rise to a nomenclature problem for the different loci detected by one clone. As a rule, all cosegregating fragments are assumed to belong to the same locus. If a clone is tested on different isolates or on restriction digests using different enzymes, fragments that cosegregate with a previously identified locus are regarded as matching the same locus. Since the combinations of clone/enzyme/isolates are infinite, it is difficult to name the loci systematically according to restriction fragment size. This nomenclature problem has not been discussed in other RFLP papers, as each set of progeny has only been tested using the same enzyme.

When a cloned fragment shows polymorphism for sites generated by one restriction enzyme, other enzymes often show polymorphism as well. For some markers all progeny isolates were tested with five different enzymes. This can reveal additional loci but, in general, it will be more fruitful to continue with a new potential RFLP marker. The enzyme EcoRI revealed most polymorphism in the powdery mildew fungus followed by the enzymes HindIII and EcoRV. Future analysis of progeny will concentrate on digests with EcoRI and HindIII.

We have given the avirulence/virulence loci the genetical designate I for infection type. The use of V (virulence) is a description of alleles of the same locus, for which reason we prefer to use the more neutral designation I.

Previous work on genetical mapping of the powdery mildew fungus has been confined to virulence genes towards particular resistance genes (H.P. Jensen, personal communication) and isozyme markers, which show very little polymorphism (G. Koch, personal communication). This study and the study of O'Dell et al. (1989) have revealed extensive RFLP polymorphism. With only three low-copy RFLP markers, we were able to differentiate between 28 different isolates (Fig. 2). The establishment of RFLP markers opens the possibility of constructing a comprehensive map of the fungal genome. With 31 RFLP markers and 5 virulence loci we identified seven groups of linked loci, two of which include virulence genes and RFLP markers.

It is interesting that the multicopy clones in general recognize sequences spread throughout the genome and that no close linkage was detected between any of these loci, i.e., they do not appear to be the result of tandem duplication. Clones that recognize duplicated loci dispersed throughout the genome have been reported earlier for *Bremia latuca* (Hulbert et al. 1988) and tomato (Bernatsky and Tanksley 1986).

In agreement with previous data (Jørgensen 1988), no close linkage was detected between the virulence loci tested that corresponds to the tightly linked or allelic resistance genes in the barley *Mla* locus (Giese 1981). This is characteristic for most plant-parasite systems where gene-for-gene relations have been demonstrated (Christ et al. 1987), and it supports the general perception that it is advantageous for the pathogen to have independently inherited virulences genes.

The segregation of four markers was found to deviate significantly from the expected 1:1 ratio. Only the *Ih* locus is included in our present map, and the data clearly shows that this locus belongs to coupling group B.

More markers are needed to get a more complete linkage map. In the present linkage analysis we detected a distance of 9 and 11 cM between virulence loci and RFLP loci. Identification of closer linkages between RFLP and virulence loci opens the possibility for chromosome walking, which can lead to the cloning of a virulence gene.

Acknowledgements. Rikke Sillesen is gratefully acknowledged for her excellent technical assistance. H.P. Jensen is thanked for his help and support throughout this study and for showing us how to cross the mildew isolates. K.A. Suiter is thanked for providing the LINKAGE-1 program. D. Edwards is acknowledged for his advice concerning statistical analysis.

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