Theor Appl Genet (1990) 79:411–416

THEORETICAL AND APPLIED GENETICS

Deletion mutation as a means of isolating avirulence genes in flax rust

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Received September 5, 1989; Accepted November 30, 1989
Communicated by G. Wenzel

Summary. The interaction between flax rust, *Melampsora lini*, and its host, flax, *Linum usitatissimum*, has been extensively studied, and certain genetic features make the system an appropriate choice to utilize in isolating genes conferring avirulence in rust. A mutant that was selected for virulence on Lx plants was isolated, after treatment with gamma rays, from a strain that is genotypically *A-L5, A-L6, A-L7, A-Lx/A-L5, A-L6, a-L7, a-Lx*. These four specificities are tightly linked. Breeding tests showed that this mutant was genotypically *A-L5, A-L6, a-L7, a-Lx/a-L5, a-L6, a-L7, a-Lx* and, when made homozygous for the mutant chromosome, was virulent on *L5, L6, L7,* and *Lx*. This result excludes somatic recombination as a source of the mutation and indicates deletion as a likely cause. A 250 bp genomic sequence from a strain of rust homozygous for these four linked avirulence genes (*A-L5, A-L6, A-L7, A-Lx*) was isolated, using a method that allows the differential cloning of the specific DNA sequences located within a deletion in the mutant genome. This clone hybridized to two EcoRI bands in genomic DNA from the strain homozygous for the four linked avirulence genes and from the strain homozygous *A-L5* and *A-L6* and heterozygous *A-L7* and *A-Lx*, but showed no homology to DNA from the strain carrying the putative chromosomal deletion. The correlation between the genetically characterized deletion mutation and the isolation of a sequence from within a region of chromosome missing from this strain of rust suggests that this 250 bp tract may be part of, or closely linked to, the defined set of avirulence genes.

Key words: Avirulence genes – Molecular clones – *Melampsora lini* – *Linum usitatissimum*

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Introduction

Rust fungi are important plant pathogens, which in some species interact so closely with their obligate host that they conform to Flor's (1956) complementary gene system between a host and its parasite. The most extensive studies of the genetics of this interaction have been reported in flax, *Linum usitatissimum*, and its rust, *Melampsora lini* (Shepherd and Mayo 1972; Lawrence et al. 1981). Consequently, although wheat rusts are commercially more important, flax and its rust provide unique experimental material with which to study the molecular events occurring during rust infection. In addition, the size of the flax genome is small, namely, 7×10^8 bp compared with that of wheat, which is 2.2×10^{10} bp (Jordan et al. 1980).

In flax rust, genes for pathogenicity have been identified corresponding to at least 29 resistance genes in the host plant. In flax, the resistance genes are clustered in five groups, *K, L, M, N,* and *P* (Flor 1971), where the members of each group may behave functionally as closely linked genes, e.g., the *M* group (Mayo and Shepherd 1980) or as multiple alleles, e.g., the *L* group (Shepherd and Mayo 1972). The avirulence genes in the parasite are not correspondingly grouped. Eleven of these genes segregate independently of each other and the remaining 18 genes show varying degrees of linkage. Thirteen of these are in four groups of apparently closely linked genes that behave as units during segregation (Lawrence 1987).

In studying the effects of UV and X-rays on the mutation of pathogenicity genes by measuring the change in phenotype from avirulent (A) to virulent (a) in "heterozygotes" (actually dikaryotes), Flor (1956, 1958) found that a mutation to virulence in any one of *A-L5, A-L6,* or *A-L7* was always accompanied by similar muta-

tion in the other two. Likewise, X-ray induced mutation in *A-P* was always accompanied by mutation in *A-P1, A-P2,* and *A-P3* (Flor 1958). He accounted for this behavior by postulating a deletion of the respective three or four closely linked genes, and suggested that deletion gave rise to the recessive phenotype.

Little is known of the molecular interactions between plant hosts and their fungal parasites and, before molecular studies of the action of genes controlling the relationship can be initiated, it is necessary to isolate one or more of these genes. Our aim is to isolate molecular clones encompassing four tightly linked members of the *A-L* series of avirulence genes in *M. lini,* using a strain carrying a deletion for these genes characterized by genetic means.

In this paper, we describe the production of a deletion mutant involving *A-L5, A-L6, A-L7,* and *A-Lx.* In addition, as an important first step in isolating rust avirulence genes, we report the cloning, by a method based on differential hybridization, of a 250-bp genomic sequence located within a deletion of this mutant.

Materials and methods

Host plants

The varieties used to test the genotype and phenotype of the four target avirulence genes of the *L* group, together with their respective resistance genes are shown below.

Variety	Resistance gene	Comments on the expression of avirulence
Wilden	*L5*	Chlorotic lesions, no pustules
Birio	*L6*	Immune flecks
Barnes	*L7*	Restricted growth of pustules
Lx	*Lx**	Immune flecks
Hoshangbad	–	No recognized resistance genes in the *L* group

* Derived by recombination within *L6* (Mayo and Shepherd 1980). The gene has not been differentiated from *L7*, but *L7* and *Lx* recombine (M. J. Mayo, unpublished results)

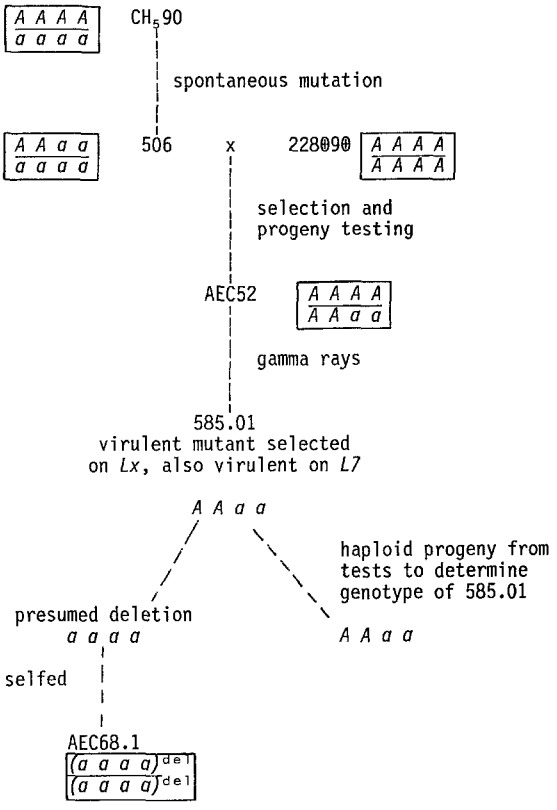
A full description of the 29 differential host varieties is given by Lawrence et al. (1981).

Rust strains

(i) 228⓿9⓿. Strain 228 (Lawrence et al. 1981) was selfed and progeny no. 9 was in turn selfed (Jones 1988)
(ii) AEC52. One of the progeny from crossing 506, a spontaneous mutant of CH5.90 (Lawrence et al. 1981) and 228⓿9⓿.

The relationships of these strains and those derived in the present work are shown in Table 1 and their relevant reaction spectra and genotypes are shown in Table 2. Rust propagation, pathogenicity testing, and scoring of reactions were as described by Lawrence et al. (1981).

Table 1. Origin of rust strains



Boxed characters indicate genotypes for avirulence for genes *A-L5, A-L6, A-L7,* and *A-Lx,* respectively. Unboxed characters are phenotypes
A = avirulence; a = virulence; a^{del} = virulence caused by deletion

Preparation of the deletion mutant

Mutagenesis by gamma-irradiation. To ensure that mutation of the four linked *A-L* genes could not originate via somatic recombination, a strain AEC52 was used, with the genotype *A-L5, A-L6, A-L7, A-Lx/A-L5, A-L6, a-L7, a-Lx.* This was derived (Table 1) from a cross between 228⓿9⓿, homozygous for the above four avirulence genes, and strain 506, a spontaneous mutant of CH5.90 (J. Mayo, unpublished results). The specificities of reaction of AEC52 and its progenitors on four flax varieties monogenic for the resistance genes *L5, L6, L7,* and *Lx* are shown in Table 2, together with their complete or partial genotypes deduced from genetic data. The genotype of AEC52 allows mutants virulent on *Lx* to be selected on *Lx* plants after gamma-irradiation.

Single pustules of AEC52 were grown in isolation on a susceptible flax variety (Hoshangabad), and the vegetative progeny of three individual pustules were tested on 29 differential host strains; all three showed the expected spectrum of specificities. One clone was further grown also in isolation until 5 g (3.5×10^9) of uredospores was produced on two separate occasions. Uredospore samples were irradiated (1,024 rads/min) in a [^{60}Co] gamma-emitting radiotherapy machine, and monitored for viability by light microscope determination of the proportion of spores germinating on a water/air interface on glass slides. A radiation dose/viability curve, produced from triplicate samples from a single harvest of uredospores, indicated that

Table 2. Phenotypes and deduced genotypes of rust strains

Host variety[a]	Resistance gene	Rust strain, genotype (G) and phenotype (P)											
		$CH_5$90		506		228⓿9⓿		AEC52		585.01		AEC68.1	
		P	G	P	G	P	G	P	G	P	G	P	G
Wilden	*L5*	−	*Aa*	−	*Aa*	−	*AA*	−	*AA*	−	*AA*	+	aa^{del}
Birio	*L6*	−	*Aa*	−	*Aa*	−	*AA*	−	*AA*	−	*AA*	+	aa^{del}
Barnes	*L7*	+/−	*Aa*	+	*aa*	−	*AA*	+/−	*Aa*	+	*aa*	+	aa^{del}
Lx	*Lx*	−	*Aa*	+	*aa*	−	*AA*	−	*Aa*	+	*aa*	+	aa^{del}

P = phenotype; + = growth; − = no growth; +/− = slight growth
G = genotype; a^{del} = virulence caused by deletion; *A* = avirulence; *a* = virulence
[a] see Lawrence et al. (1981) for a full description of the differential hosts

90% of spores were dead after receiving 100,000 rads (data not shown). Bulk spore samples were irradiated with this dose of gamma rays and immediately inoculated onto 5,000 *Lx* plants in a glasshouse with high humidity maintained for 12 h after inoculation. Two and 3 weeks later, the plants were searched for uredospore pustules whose host reactions were tested on differentials as described below.

Isolation and genetic characterization of mutants. A single uredospore pustule was recovered in the first irradiation experiment and six in a second. When tested on full sets of differentials, six of the seven mutants selected for growth on *Lx* plants were consistent with the infection spectrum of AEC52, but virulent on *L7* as well as *Lx* plants. The seventh was discarded as a probable contaminant and two of the six mutants could not be induced to form basidiospores by temperature cycling. Basidiospores from the four meiotically competent mutants were inoculated onto *L5, L6, L7,* and *Lx* plants at 13 °C in a plant growth cabinet, and the reactions produced are shown in Table 3. Each of the four mutants produced both + (growth of pathogen) and − (resistant reaction of the host) reactions on *L5* and *L6* plants, and only + reactions on *L7* and *Lx* plants. Further inoculation of basidiospores from each of the four individual mutants onto Hoshangabad plants allowed pycnia within each family to be intercrossed (Lawrence et al. 1981), so that strains homozygous for the four specificities could be isolated. These yielded aeciospores in 131 cases from a total of 351 attempted intercrosses.

Uredospores were produced on isolated Hoshangabad plants from each of the 131 aecial pustules, and these vegetative clones were individually inoculated onto Hoshangabad and sets of eight differential plants, to test their reactions at *L5, L6, L7, Lx, P, P1, P2,* and *P3* specificities. Two of the four initial mutants, 586.11 and 586.23 (Table 3), gave progeny which were apparently incompatible with the AEC52 parent, and these were set aside for further study. The progeny of the third mutant, 586.18 (Table 3), were all − − + +, respectively, on *L5, L6, L7,* and *Lx* plants testing the status of the four targeted genes. However, this latter mutant is unlikely to have arisen by mitotic recombination because of its + and − basidiospore reactions on both *L5* and *L6* differential hosts shown in Table 3.

The family of mutant 585.01 contained phenotypes consistent with a deletion from AEC52 of all four target genes of the *A-L* series. A total of 184 intercrosses of this mutant gave rise to only 39 aecial pustules, whose spectra of infection on the eight differential hosts are shown in Table 4. One family member (AEC68.1) fulfilled the requirements for a rust strain homozygous for a deletion of the four target genes. The siblings of AEC68.1

Table 3. Reactions of basidiospores from mutant strains on differential flax hosts.

Mutant strains	*L5*	*L6*	*L7*	*Lx*
585.01	+&−	+&−	+	+
586.11	+&−	+&−	+	+
586.18	+&−	+&−	+	+[a]
586.23	+&−	+&−	+	+

+ = pycnia; − = immune flecks
586.16 and 586.25 did not yield basidiospores
[a] (but necrotic)

Table 4. Phenotypes on eight differential flax varieties of 39 intercross progeny of mutant 585.01 pycnia after aeciospore production and amplification of uredospores on Hoshangabad (H) plants

No. of progeny with reaction	Host differential specificity								
	H	*L5*	*L6*	*L7*	*Lx*	*P*	*P1*	*P2*	*P3*
1	+	+	+	+	+	+	−	−	−(AEC68.1)
32	+	−	−	+	+	+	−	−	−
2	+	+	+	+	+	−	−	−	−
1	+	+/−	−	+	+	−	−	−	−
3	incompletely scored								

+ = growth; − = no growth; +/− = slight growth

in 32 cases showed the spectrum characteristic of the AEC52 chromosome containing *A-L5, A-L6, a-L7,* and *a-Lx,* and three were excluded because of incomplete scoring or lack of success in vegetatively propagating uredospores from aeciospores. Because they were unable to grow on plants testing the *A-P/P* specificity, a reaction precluded by the genotype of AEC52, 3 isolates of the 39 were attributed to contamination. This level of contamination is considered acceptable during an experiment where 131 individual rust lines were simultaneously under test. Thus, only 1 of 39 progeny was homozygous for the mutated chromosome, whereas about 10 (25%) would be expected in this class. The low number of this genotype is consistent with the relative inviability of the chromosome bearing the deletion, either in its homozygous state in binucleate aeciospores and

uredospores or in the uninucleate haploid pycnospores or basidiospores. Nevertheless, AEC68.1 is a vigorous rust line showing large uredospore pustules and normal vegetative growth.

Uredospores of AEC68.1 were amplified on Birio plants (*L6*) to exclude contamination by 585.01, which was amplified at the same time in isolation on Hoshangabad. The spectra of infection on all the 29 differential lines of flax were tested. Both rust strains were phenotypically, and 585.01 was genotypically, consistent with their origins in AEC52.

It was concluded that AEC68.1 was a strain of flax rust homozygous for a deletion involving *A-L5, A-L6, A-L7,* and *A-Lx,* induced by gamma-irradiation of strain AEC52.

Isolation of genomic sequences deleted from AEC68.1

Molecular methods. Flax rust DNA was prepared from fresh uredospores germinated overnight on an air/water interface when germination exceeded 90%. First and second harvests of spores only were used to avoid contamination by other organisms. After extensive washing of the germinated spores on a glass fiber filter, checks for contamination by light microscopy and counts of viable microorganisms indicated very low contamination of the uredospore samples. Germinated spores were homogenized with or without ground glass in sterile mortars and pestles in liquid nitrogen in 0.3 *M* NaCl, 30 m*M* Na-citrate, 20 m*M* EDTA, pH 8.0, and 4% sarkosyl. Samples were spun at 8,000 × g, 1 g of CsCl and 80 µl of ethidium bromide (10 mg/ml) were added per 1 ml of supernatant, and the solution was centrifuged to equilibrium at 130,000 × g. The visible band of DNA was removed by side puncture of the tube and after ethanol precipitation and dissolution in 10 m*M* TRIS-Cl, pH 8.0, and 1 m*M* EDTA, the DNA was monitored for integrity by electrophoresis on 0.8% agarose gels. Higher molecular weight DNA but lower yield was available when no ground glass was added during the initial homogenization. Samples were spectrophotometrically scanned and stored at −20 °C at 1 mg/ml.

The rust strain 228⓪9⓪ is one of the parents of AEC52 (Table 2) and is homozygous (*A*) at the four loci deleted from AEC68.1. Although 228⓪9⓪ is not isogenic with AEC52 or AEC68.1, it is the best related material (Table 2) available from which to attempt isolation of molecular clones of these avirulence genes. Complete isogenicity of this strain with AEC68.1 is precluded by the requirement for heterozygosity at the selective specificity (*A-Lx*) and the necessity to exclude somatic recombination as the origin of mutant strains.

Restriction fragments of total cellular DNA enriched for sequences within the deletion were prepared from rust DNA by the phenol emulsion reassociation technique (PERT) (Kohne et al. 1977; Kunkel et al. 1985). A 200-fold excess of the homozygous deletion mutant (AEC68.1) DNA to DNA from 228⓪9⓪ was used in the renaturation reaction in a phenol/aqueous emulsion (Kohne et al. 1977). TaqI-digested AEC68.1 genomic DNA (60 µg) and EcoRI-digested AEC68.1 DNA (6 µg) were mixed and combined with 0.33 µg of MboI-digested 228⓪9⓪ DNA in a volume of 300-µl low salt buffer. The DNA solution was boiled for 5 min, mixed, reboiled for 5 min, cooled in ice, and placed in a 2-ml dark-brown stoppered vial. The mixture was made to 7% phenol [redistilled and saturated with 0.1 *M* TRIS-Cl (pH 7.6), 1.25 *M* $NaClO_4$, and 125 m*M* sodium phosphate, pH 6.8 (Kunkel et al. 1985)] in a final volume of 670 µl (0.1 µg/µl DNA). In order to create an emulsion, the contents of the vial were stirred with a magnetic stirrer. After 12 days the phenol was removed from the reassociated DNA by chloroform: isoamylalcohol extraction, the excess salts were removed by centrifugation through an Amicon *Centricon 30* filter, and washed with 10 m*M* TRIS-Cl and 1 m*M* EDTA (pH 8.0).

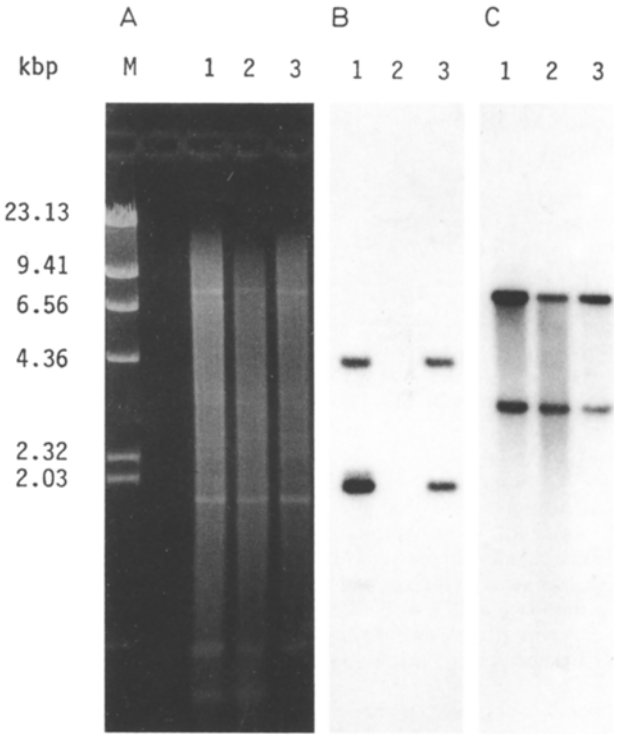


Fig. 1 A–C. Hybridization of specific probes to DNA from three flax rust strains. Samples of total genomic DNA (2 µg) from 228⓪9⓪ (*track 1*), AEC68.1 (*track 2*), and AEC52 (*track 3*) were digested with EcoRI (10 units/µg DNA) and resolved on a 0.8% agarose gel, with HindIII fragments of bacteriophage lambda included as standards (*track M*). The gel was stained with ethidium bromide and photographed (**A**). After transfer to nitrocellulose (Southern 1975), the fragments were hybridized with a gel-purified, ^{32}P oligo-labelled fragment from clone pERT5.8 (**B**). The probe was removed from (**B**) and the filter was further hybridized with the nuclear ribosomal RNA gene repeat unit from flax rust (**C**)

The DNA was then precipitated with ethanol, recovered by centrifugation, checked for reassociation by agarose gel electrophoresis, and stored frozen in 40 µl 10 m*M* TRIS-Cl and 1 m*M* EDTA (pH 7.2).

The PERT-associated DNA (1 µg) was ligated into BamHI-digested and phosphatased pUC19 (2.6 µg) in a volume of 100 µl with 800 units T4 DNA ligase (New England Biolabs). Several hundred short MboI fragments were cloned in pUC19 (Norrander et al. 1983) and selected as white colonies from X-gal plates. Unrestricted single-colony plasmid preparations (Holmes and Quigley 1981) from the deletion-enriched clones were resolved on agarose gels and transferred to nitrocellulose. Duplicate filters were hybridized with 228⓪9⓪ or AEC68.1 genomic DNAs labelled to high specific activity ($>10^8$ cpm/µg) at a concentration of 4×10^6 cpm/ml hybridization mix. Other conditions were not suitable to indicate the presence of differentially hybridizing plasmids.

Restriction enzymes and other DNA modifying enzymes were utilized in conditions specified by the supplier (Boehringer-Mannheim).

Identification of a sequence within the deletion. From several hundred clones enriched by the PERT method for the deleted

region of AEC68.1 genome, six plasmids that appeared to differentially hybridize the two genomic probes were individually nick translated and hybridized to Southern blots of EcoRI-digested genomic DNAs from 228⓪9⓪ and AEC68.1 (Fig. 1 A). One clone, pERT5.8, showed a clear signal to two bands of 4.3 and 1.9 kbp in 228⓪9⓪ DNA and none to AEC68.1 DNA (Fig. 1 B), while others showed hybridization to both genomes (results not shown). As would be expected, pERT5.8 also hybridized to corresponding bands in AEC52 genomic DNA (Fig. 1 B), indicating that it originates from a sequence deleted in the genome of AEC68.1. On removal of the probe used in Fig. 1 B and rehybridization with the cloned flax rust nuclear rDNA repeat unit (T. K. Franks and J. N. Timmis, unpublished results), homology was present to the expected 8.1 and 3.3 kbp EcoRI fragments in all three genomes (Fig. 1 C). The insert in pERT5.8 is 250 bp and no EcoRI site is present, contrary to what is expected from the presence of two bands in EcoRI-digested genomic DNA (Fig. 1 B). It is, therefore, assumed that the sequence is present at least twice in the rust genome.

Discussion

Several other approaches to this problem have been unsuccessful in our hands. Analysis, by SDS polyacrylamide gel electrophoresis, of the translation products of mRNAs failed to provide evidence of differential expression of specific genes during a time course after compatible infection. The possibility of obtaining cDNAs specific to the fungal-plant compatibility reaction was therefore not pursued. Extensive efforts were also made to derive sequences from the deletion in AEC68.1 by differential screening of a lambda bacteriophage genomic library of 228⓪9⓪ with the deleted and undeleted genomic DNAs. Several recombinants apparently hybridizing differentially were subsequently shown to hybridize to identical bands when used as probes to Southern blots of EcoRI-digested 228⓪9⓪ and AEC68.1 DNA.

Genetic evidence, central to the experimental approach described here, is diagnostic of the deletion of the linked set of four avirulence genes from AEC68.1. The results are consistent with the hypothesis that the 250 bp fragment is within a deletion in AEC68.1 and may, therefore, be a part of, or closely linked to, these specific avirulence genes of flax rust. If this hypothesis proves to be correct, the isolated sequence would provide a unique key to elucidating molecular mechanisms determining compatability in obligate parasitism. The approach shows promise of success not available in any other comparable system, because the activity of DNA isolated may eventually be unequivocally associated with the biological expression of the avirulence genes themselves. However, we do not as yet know the size of the deletion, so clone pERT5.8 could be a considerable distance from the avirulence genes themselves, even if they are a part of the characterized deletion. Many other important questions pertaining to this 250 bp clone remain to be answered.

The less rigorous approach of identifying genes whose expression is turned on or off in either host or parasite during infection is much less direct and is also less attractive, because it may lead to time-consuming characterization of many genes unrelated to those controlling the specific genetic interaction between host and parasite. Such genes may include those expressed as abundant plant mRNAs during wounding, disturbances of secondary metabolism, and fungal growth and development within the plant, as well as those more directly related to microbial attack and plant defense (Lamb et al. 1989). Isolation of sequences from a characterized deletion is also attractive compared with the less directed method of searching for linkage of pathogenicity characters or host resistance to a variety of DNA hybridization bands (O'Dell et al. 1989; Michelmore and Hulbert 1987), followed by chromosome walking to the target genes. The tagging of genes with transposons (Bennetzen et al. 1988) is a very direct but so far unsuccessful approach to resistance gene isolation.

Little progress has been made in elucidating the molecular interactions between plant pathogens and their hosts, even though empirical approaches (Harrison et al. 1987; Gerlach et al. 1987; Abel et al. 1986) have allowed the manipulation of virus resistance in tobacco. Genomic sequences controlling various stages of plant infection of commercially important bacteria have been isolated. Compared with fungal genomes, those of bacteria are readily handled by molecular techniques, and Staskawicz et al. (1984) have cloned, by transformation and complementation, avirulence genes from *Pseudomonas syringae,* the causal agent of bacterial blight of soybean. In subsequent experiments (Staskawicz et al. 1987), a specific avirulence gene was cloned that corresponds to a genetically characterized resistance gene in soybean. Although the isolated avirulence genes change race specificity of the pathogen from virulent to avirulent, the opposite has not been possible.

As the genetics of plant resistance to *Xanthomonas campestris* pv *malvacearum* are well described with 16 specific *R* genes identified in cotton, this system should prove valuable in the study of host/pathogen interactions (Gabriel et al. 1986). Ten different cosmid clones conferring race-specific avirulence have been isolated, and five of the avirulence genes were shown to interact specifically, gene-for-gene, with individual resistance genes in differential hosts. Evidence from the *Xanthomonas*/cotton system also suggests that at least some avirulence genes in the bacterium have corresponding virulence genes (Gabriel et al. 1986).

Although no sequences controlling the specificity of reaction between fungal parasites and their hosts have been isolated, some progress relevant to fungal molecular interactions with plants has been made. Soliday et al. (1984) prepared cDNA clones from mRNA from cul-

tures of *Fusarium solani* in which the cutinase gene had been induced by cutin. The primary structure of cutinase has been determined from the DNA sequence, and this was the first fungal sequence to be cloned that is associated with pathogenicity. Weltring et al. (1988) cloned a gene from *Nectria haematococcus* whose product is able to detoxify, by demethylation, a phytoalexin of pea called pisatin. A molecular library was made of the fungal genome in a cosmid vector carrying the tryptophan synthetase gene (*trpC*) from *Aspergillus nidulans*. This enabled selection of the cosmid following transformation into *trp*$^-$ *Aspergillus nidulans*. One transformant out of 1,250 tested was able to demethylate pisatin and contained the *N. haematococcus* gene responsible. So far the critical experiment which converts an avirulent strain of *N. haematococcus* to virulent by transformation with the gene causing pisatin methylation ability has not been achieved. Better progress may be possible when more fungal transformation systems have been developed, allowing the complementation approach to be used as in bacteria.

In our laboratory, work is in progress to isolate and characterize sequences flanking pERT5.8, and several approaches aim to strengthen the correlation between the sequence isolated and the four target avirulence genes. The isolation and analysis of overlapping genomic clones from a genomic library will allow determination of the size and nucleotide sequence of the DNA deleted from AEC68.1. Transformation of a virulent rust strain to confer avirulence will be the ultimate test of isolation of these avirulence genes. So far, however, no transformation system is available for rust fungi.

Acknowledgements. We are grateful to Dr. V. J. Hyland and Dr. A. J. Pryor for discussions. We thank the CSIRO, Division of Horticultural Research, and the Royal Adelaide Hospital for research facilities, and we acknowledge the Australian Research Council and the University of Adelaide for grants to support this work.

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