Rice Blast as a Model System for Plant Pathology

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I want to acknowledge the members of the Molecular Plant Pathology Group in the Central Research and Development Department at Du Pont who have contributed to the work described here. The Genetics and Molecular Biology Section is headed by Forrest G. Chumley and me and includes staff scientists James A. Sweigard, Leonard Farrall, and Anne Walter. The visiting scientists who have contributed include John E. Hamer (now at Purdue University), Marc J. Orbach, Don G. Ennis, and Kenneth A. Parsons. The Cell Biology Section is headed by Richard J. Howard and now includes staff scientists Timothy M. Bourett, Margaret A. Ferrari, and Michael A. Picollelli.

I believe that the field of plant pathology would benefit if a small number of important fungal plant pathogens were developed as "model systems." Model system, a well-used term, refers here to a single system in which a critical mass of effort is focused on understanding all aspects of pathogenicity and host specificity. All identified factors influencing the infection process can then be weighed and understood relative to one another. The value of having a critical mass of effort focused on one system is illustrated by the detailed understanding of cellular processes that has emerged for common baker's yeast, *Saccharomyces cerevisiae*. Major efforts have also gone into studies of two filamentous Ascomycetes, *Neurospora crassa* and *Aspergillus nidulans*. In contrast, in plant pathology, typically one laboratory or a small number of laboratories have worked on a particular plant-pathogen system. This has evolved from the necessity to study the many diverse pathogens that have an impact on agricultural production in various areas of the world. However, detailed studies of a few well chosen host-pathogen systems would complement these other important studies by providing hypotheses to be tested on a broad range of important pathogens.

Interdisciplinary teams of plant pathologists are required for gaining a detailed understanding of a host-pathogen system. These teams must include field pathologists, breeders, epidemiologists, geneticists, molecular biologists, physiologists, biochemists, and cell biologists. As always, biology should lead the way. A major emphasis must be put on collaboration between pathologists who know the disease in the field, and those who work mainly in the laboratory, in order to ensure that studies have relevance to the disease in the field. The need for maintaining relevance adds a new challenge that has not previously been considered in systems such as yeast, *Neurospora,* or *Aspergillus*, because studies with these fungi have been concerned with growth and differentiation in the laboratory.

Fungal plant pathogen systems are notoriously difficult. This is seen in the extreme with obligate parasites such as rusts and mildews where axenic growth is very limited at best. This difficulty in handling fungal plant pathogens increases the importance of developing model systems, systems in which techniques for detailed molecular genetic analysis can be fine-tuned as they have been for the better-studied fungi mentioned above. Because a host-pathogen system deals with an interaction between two organisms, attributes of the host must also be considered in choosing a system to develop. It is important that both the pathogen and its host plant be easy to handle in the laboratory. Both the pathogen and the host must undergo a manipulable sexual cycle. An additional attribute of a valuable model system for plant pathology is that it be an agronomically important system. This will mean that much is known about the biology of the system in the field, including some understanding of resistance in the host. This wealth of field experience will define the relevant, interesting problems for laboratory research.

One purpose of the present discourse is to outline features of rice blast disease that make it an excellent candidate for development as a model system for plant pathology. I will describe basic research on rice blast that is under way in the Molecular Plant Pathology Group at Du Pont. The present consideration, in three parts, will emphasize roles for molecular biology in understanding host-pathogen interactions, but roles for classical genetics and cell biology will also be discussed. First, I will discuss progress toward understanding host specificity in the rice blast system. Genes that determine both host cultivar and host species specificity have been identified by classical genetic analysis. Approaches to cloning these genes will be discussed. The second part will include approaches to understanding mechanisms of pathogenesis by genetic engineering of mutants. Work underway in our laboratory to construct cutinase-deficient mutants will be described. And in the third part, I will discuss DNA probes for the study of fungal population dynamics and what these probes have told us about the origin of wheat blast in Brazil.

RICE BLAST DISEASE

The heterothallic Ascomycete, *Magnaporthe grisea* Barr (anamorph, *Pyricularia oryzae* Cav. or *P. grisea*) includes pathogens of many grasses. Individual isolates, however, have a limited host range, parasitizing one or a few grass species. Strains of the fungus that parasitize rice (*Oryza sativa*) are subdivided into races, depending on the rice cultivars they can successfully infect. Pathogens of rice show a high degree of variability in the field; new races frequently appear with the ability to attack previously resistant rice cultivars. Thus, the study of rice blast will potentially further our understanding of host species specificity and host cultivar specificity, as well as the mechanisms of pathogenesis in an important plant pathogen.

Additional factors contribute to the choice of the rice blast system for detailed study. 1) The fungus can be grown on defined media and mutants including auxotrophs can be easily obtained (3). 2) Rigorous classical genetic analyses of host cultivar specificity, of host species specificity, and of mechanisms of pathogenesis are now possible with fertile laboratory strains that have been developed. Classical genetic studies of the rice blast fungus have previously been hampered by the low fertility, specifically female sterility, of field isolates of the fungus that infect rice (18). Hermaphroditic laboratory strains that infect rice were developed by selection of progeny from genetic crosses between female sterile rice pathogens and female fertile pathogens of weeping lovegrass (*Eragrostis curvula*), goosegrass (*Eleusine indica*), and finger millet (*Eleusine coracana*) (Valent, Farrall, and Chumley, *unpublished*). In addition, a unique field isolate that is both female fertile and a virulent pathogen of rice, isolated in French Guyana by J. L. Notteghem, may further expand the potential for genetic analysis. 3) The sexual cycle is relatively short. Tetrads or random ascospore progeny can be isolated two to three weeks after strains of opposite mating type are paired on oatmeal agar medium. 4) The disease cycle is rapid and the

disease is easy to contain in the laboratory under controlled conditions. Full-sized lesions develop five to seven days after inoculation. Sporulation and reinoculation in the growth chamber can be prevented by maintaining humidity at 85% or below, thus decreasing problems of cross contamination. 5) The fungus is amenable to molecular biological analysis (14, *unpublished results described below*). 6) Several dominant blast resistance genes in rice have been identified by genetic analysis (19). 7) Rice is becoming accessible to molecular genetic analysis. Useful transformation procedures are being developed (15), and a molecular linkage (RFLP) map is being generated (12).

GENES THAT CONTROL HOST SPECIFICITY

Major genes, "avirulence genes," determine rice cultivar specificity. Classical genetic analysis has identified single genes that have an all-or-nothing ("major") effect on the ability of a strain of the rice pathogen to infect specific cultivars of rice (16). By definition, we have called genes that determine cultivar specificity "avirulence genes." Some avirulence genes that determine specificity toward cultivars of rice were derived from field isolates that infect rice. Three interesting avirulence genes, specific to different rice cultivars, appear to have been derived from a strain of *M. grisea* that infects weeping lovegrass, but not rice. One gene, *Avr1-CO39*, is specific for the rice cultivar CO39, the second, *Avr1-M201*, is specific for cultivar M201 and the third, *Avr1-YM*, is specific for cultivar Yashiro-mochi. This result with the rice blast fungus is similar to the results obtained with a bacterial plant pathogen. That is, *Pseudomonas syringae* pv. *tomato*, a pathogen of tomato but not of soybean, contains avirulence genes specific for certain cultivars of soybean (11). Our experiments suggest that avirulence genes are abundant in *M. grisea*. This makes it even more intriguing to ask what these genes are and how they function in determining cultivar specificity.

One particularly interesting avirulence gene, *Avr2-YM*, specific toward rice cultivar Yashiro-mochi, has been obtained from the Chinese rice pathogen field isolate, O-137. This avirulence gene appears to be unstable. That is, spontaneous virulent mutants derived from strains carrying the avirulence allele of this gene appear frequently in standard infection assays. "MGR fingerprints" (see below) as well as morphological, pathogenicity, and fertility characteristics confirm that mutants and not contaminants have been obtained. The analysis of this unstable gene should give insight into a problem for which rice blast is noted, the frequent appearance of new races of the rice pathogen in the field.

The identification of single avirulence genes controlling cultivar specificity is only the first step in determining if the rice blast system represents a classic "gene-for-gene" system as first defined by Flor (5). Classic avirulence genes are dominant, suggesting that the expressed form of the gene confers the avirulence phenotype. Dominance cannot be determined for avirulence genes of *M. grisea* by classic methods because this haploid fungus does not form the stable vegetative diploid required for dominance testing (3). Therefore, cloning these avirulence genes will be required to determine dominance. The additional prediction of the gene-for-gene model that each avirulence gene identified in the pathogen will correspond to a dominant resistance gene in the host is now being tested in our system. If rice blast is a gene-for-gene system, our results suggest that rice cultivars contain many previously unidentified resistance genes.

Major genes for pathogenicity to weeping lovegrass. Two unlinked major genes have been clearly documented that determine pathogenicity versus nonpathogenicity toward a second host, weeping lovegrass. The nonpathogenicity allele of one of these genes, named *Pwl1*, was derived from a finger millet and goosegrass pathogen (17,18). The nonpathogenicity allele of the second gene, *Pwl2*, appears to be derived from the same Chinese rice pathogen, O-137, that contained the avirulence gene specific for rice cultivar Yashiro-mochi, *Avr2-YM*. Interestingly, the nonpathogenicity phenotype associated with the gene *Pwl2* is unstable, just as is avirulence conferred by *Avr2-YM* described above.

The genes *Pwl1* and *Pwl2* must be considered "pathogenicity genes," with no cultivar specific effects, because cultivars of weeping lovegrass are not available. However, it remains an intriguing possibility that these genes may function in a manner analogous to avirulence genes.

"Minor genes" determine pathogenicity toward rice. Genetic crosses to determine the differences between a rice pathogen and a nonpathogen of rice have identified a second type of gene, in addition to the avirulence genes described above. Pathogens of *M. grisea* and nonpathogens of rice appear to differ by polygenic factors, that is, by several genes of individually small effect, that determine the extent of tissue colonization by strains that infect rice. These minor genes that determine lesion size on rice have no effect on pathogenicity toward weeping lovegrass.

METHODS FOR CLONING GENES THAT CONTROL CULTIVAR SPECIFICITY

Cloning genes that control host specificity should provide clues to how these genes function. These clues could come from cytological experiments to localize the product of the cloned gene, or from comparisons of the sequence of the cloned gene to sequences of known genes in a sequence library. Certainly, cloning genes that control host cultivar specificity should provide insight into how new races of the pathogen arise. We hope that understanding how pathogen genes function will provide insight on how plant resistance genes function.

Two approaches exist for cloning genes for which the gene product is unknown: 1) Cloning genes by function, transforming a recipient strain of *M. grisea* with library DNA, selecting or screening for expression of the gene, and recovering the cloned gene in *E. coli*, and 2) cloning genes by map position through "chromosome walking," isolating overlapping chromosomal DNA segments that span the region from a previously cloned linked marker to the gene of interest.

Cloning genes by function. A transformation system for *M. grisea*, required for cloning by function, has been developed (14). As with other fungi, protoplasts are exposed to plasmid DNA in the presence of Ca^{++} and polyethylene glycol. Several selectable markers have been used for transformation of *M. grisea*. Selection for prototrophy in a recipient arginine auxotroph using plasmids carrying the Aspergillus *ArgB+* gene routinely results in a transformation frequency of several hundred transformants per microgram of plasmid DNA. Similar results are obtained when an isoleucine-valine auxotroph is transformed with plasmids carrying the *M. grisea ILV1+* gene. Transformation and selection for resistance to a sulfonylurea or to hygromycin B have also been accomplished. In these cases, selecting for drug resistance, the transformation frequencies routinely obtained are from 1 to 15 transformants per microgram of donor DNA.

Experiments for cloning avirulence genes are under way as follows. Since only the dominant form of a gene can be cloned by this method and since we do not know if avirulence or virulence is dominant for the genes we have identified, the experiments will cover both possibilities. For cloning dominant avirulence genes, a recipient has been chosen that is virulent on rice cultivars of interest. A cosmid library is made from genomic DNA of a strain that is avirulent on the cultivars of interest. The recipient strain will be transformed with the cosmid library and the transformants will be selected for hygromycin resistance. Individual transformants will be screened for acquired avirulence on all rice cultivars. Cosmids have a special feature that allows selection of plasmids that carry large fragments (approximately 35 kb) of DNA of *M. grisea*. We estimate that the genome size of *M. grisea* is about 38,000 kb (6). Therefore, about five thousand individual transformants would need to be screened to have a 95% chance of detecting a single avirulence gene. The experiments have been planned so that any of five different avirulence genes may be cloned, thus increasing chances of success and reducing

the effort for cloning a single gene. A similar strategy will be undertaken for cloning genes if virulence should be dominant, except that the task is made easier because virulent transformants can be selected from pools.

Cloning by map position. Cloning genes by isolating overlapping DNA fragments between the gene and a linked marker is easiest if the two are close together. This happy circumstance is most likely if a densely marked physical map is available. Maps of restriction fragment length polymorphisms (RFLPs) can be rapidly assembled in many organisms to provide starting points for chromosome walks. A RFLP is defined by a DNA probe that is homologous to different size restriction fragments in two parents. RFLPs segregate as normal Mendelian markers in genetic crosses and can be mapped relative to each other and relative to genes of interest. Closely linked RFLPs that flank the gene of interest will speed progress in gene cloning by this technique. A RFLP map for *M. grisea* is being produced by Dan Skinner and Sally Leong at the University of Wisconsin and by Hei Leung at Washington State University.

New technology now allows the separation of intact chromosomal DNAs in filamentous fungi (13). In our laboratory, Marc Orbach has used contour-clamped homogeneous electric field (CHEF) gel electrophoresis to separate chromosome-size DNAs of *M. grisea*. These DNAs range in size from 1 to 10 megabases. This technology allows us to place cloned genes on chromosomes by blotting the chromosome-size DNAs to a membrane and hybridizing with radiolabeled probes containing the cloned gene. In addition, separation of chromosomal DNAs permits construction of chromosome-specific gene libraries.

GENETIC ENGINEERING OF MUTANTS FOR UNDERSTANDING MECHANISMS OF PATHOGENESIS

Genes controlling mechanisms of pathogenesis include those genes controlling the elaborate developmental pathway that the blast fungus undergoes during infection (1,8,9), as well as genes encoding enzymes that might be directly involved in the process of infection (enzymes that degrade plant cell walls, for example). Infection proceeds as follows. A conidium has a mechanism, spore tip mucilage (STM), for attachment to the host surface (7). The germinating spore produces a germ tube that differentiates into an appressorium. The appressorium elaborates a penetration peg that pierces the cuticle and epidermal cell wall. The fungus then grows intracellularly within the host tissue. *M. grisea* is able to undergo this differentiation process on various artificial membranes (1,9), making it possible to study the differentiation sequence in the pathogen in isolation from the host plant.

A major focus of our Molecular Plant Pathology Group is to understand the formation and function of the appressorium during penetration. My colleague, Richard Howard, has now clearly shown that penetration involves a critical mechanical component. Genes encoding enzymes involved in the biosynthesis of the gray pigment, DHN-melanin, elaborated by the rice blast fungus are known to be critical for the penetration process (2). Howard and Ferrari (10) have presented convincing evidence that DHN-melanin mediates the build-up of hydrostatic pressure in the appressorium and that this high pressure provides essential driving force for a mechanical penetration component. The physical force that an appressorium can exert is illustrated by the observation that penetration pegs of *M. grisea* can indent artificial surfaces such as mylar or polyvinylchloride (9,10). On these surfaces a mechanical force must be operating without aid from fungal enzymes.

An important question remains. That is, are enzymes such as cutinase also important for penetration of host tissue? Genetic engineering of fungal mutants that lack cutinase should answer this question.

Genetic engineering of cutinase defective mutants of *M. grisea*. James Sweigard in our laboratory has succeeded in producing cutinase deficient mutants by the following technique. The cutinase gene of *M. grisea* was cloned by homology to a cutinase cDNA clone of *Colletotrichum capsici* (the generous gift of P. E. Kolattukudy, Ohio State University). The gene is highly homologous to the *Colletotrichum* and *Fusarium* cutinase genes. The gene of *M. grisea* shows interesting developmental regulation. It is highly expressed in cultures that are forming appressoria, but not expressed by undifferentiating hyphal cultures growing in liquid culture. Southern analysis shows that there is only one copy of the gene per genome, making mutant production by genetic engineering possible.

The cutinase defective mutants of *M. grisea* were constructed by "gene replacement techniques" in which the normal copy of the cutinase gene in the genome of *M. grisea* was replaced with a disrupted copy of the gene. The disrupted cutinase gene was constructed in vitro by replacing an internal 650-bp *Sst*II restriction fragment in the cutinase gene of *M. grisea* with a 5-kb *Sst*II fragment of Aspergillus DNA, which included the selectable marker, the *ArgB*+ gene. The plasmid contained 5 kb of homologous *M. grisea* DNA, with approximately 2.5-kb DNA segments flanking the inserted Aspergillus DNA. During transformation experiments, a low frequency (about 3%) of the transformants showed a neat replacement of the resident copy of the cutinase gene with the engineered copy. The potential mutants were shown to have a disrupted cutinase gene by Southern analysis. For example, the 650-bp internal *Sst*II fragment was missing in the engineered mutants. In addition, Northern analysis in which RNA was electrophoresed, blotted onto a membrane and probed with a radiolabeled cutinase gene probe showed that under conditions where the wild-type strain expressed the gene, the mutants did not. Pathogenicity tests are in progress to determine if the cutinase defective mutants show any defect in pathogenicity. This work illustrates reverse genetic techniques that can be applied to other enzymes that may play a role in pathogenicity.

USE OF REPEATED DNA SEQUENCES FOR IDENTIFICATION OF STRAINS

We have discovered a class of middle repetitive DNA sequences named MGR, for *Magnaporthe grisea* repeat sequences (6). Interestingly, these repeated sequences show host species specific conservation of sequence homology. That is, strains of *M. grisea* that infect rice from all over the world have 40–50 copies of MGR sequences per genome, and strains that infect grasses other than rice have only one to a few copies per genome. Several lines of evidence, including blotting and probing CHEF gels in which intact chromosome-size DNAs have been separated, suggest that these sequences are dispersed in the genome of rice pathogens.

The MGR sequences found within a single rice pathogen are highly polymorphic, both with respect to restriction sites and to sequence arrangement. This polymorphism also holds between rice pathogens. Every rice pathogen we have examined has a different "MGR fingerprint," the pattern obtained by cutting genomic DNA with a restriction enzyme, electrophoresing the DNA, blotting the DNA, and probing with a MGR probe. Thus, MGR fingerprints can be used to differentiate one rice pathogen from another, as well as to differentiate rice pathogens from nonpathogens of rice.

On the origin of wheat blast in Brazil. Blast has become a serious disease on wheat in Brazil during the 1980s. Because the disease first occurred in an area of Brazil where rice blast was prevalent, it seemed possible that the rice blast fungus had mutated to infect wheat. MGR fingerprinting of Brazilian rice pathogens and wheat pathogens has clearly demonstrated that the Brazilian wheat pathogens are not derived from rice pathogens indigenous to the area. The MGR patterns of Brazilian rice pathogens resembled the patterns of rice pathogens collected from various parts of the world in having many bands with homology to the MGR probe. The wheat pathogen pattern, on the other hand, contained very few bands with homology to the MGR probe.

CONCLUSIONS

I have summarized contributions that molecular biology, classical genetics, and cell biology are making to understanding host specificity and mechanisms of pathogenesis in the rice blast system. The value of DNA probes for epidemiological studies in this system has been demonstrated. These initial studies confirm the enormous potential that exists in the rice blast system.

Wouldn't it be great to understand the genetics, physiology, and cell biology of an important fungal plant pathogen with the precision that these are now understood for yeast? The potential that exists in the rice blast system could be realized, if the resources could be found to mount a serious interdisciplinary effort on this single system. Obviously, not everything learned about a model pathogen will apply to every other plant pathogen. Still we would have a point from which to initiate studies with other systems (4), by asking "Is it like rice blast?" And some mechanisms and themes will generalize. Interdisciplinary research efforts in plant pathology will reward us with an understanding of some of the most intriguing biological phenomena. It must be taken as a matter of faith that these understandings will lead to new methods of controlling plant diseases, whether these new methods involve novel, environmentally safe fungicides or stably resistant cultivars of crop plants.

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