The Induction and Analysis of Two Classes of Mutations Affecting Pathogenicity in an Obligate Parasite

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ABSTRACT

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Both temperature-sensitive and unconditional mutations were used to study separately the effects of genes controlling gene-for-gene relationships and those controlling the ability of the pathogen *Erysiphe graminis* f. sp. *tritici* to invade its *Triticum aestivum* host. Twenty-nine mutations to increased virulence were induced in the pathogen. Selection was made for a change from infection type 0 to infection type 4 on congenic lines of wheat with either of two unlinked *R* genes, *Pm*1a or *Pm*4a. Since no other changes in pathogenic specificity were detected, the mutations were interpreted as affecting the corresponding gene loci *P*1a and *P*4a in the pathogen. When the mutants were inoculated onto congenic susceptible plants, the rate of infection and the final infection type were indistinguishable from the wild type. These loci therefore appear to function for avirulence and not for virulence. Nineteen temperature-sensitive mutations were also induced in the pathogen. Growth and development of the mutant isolates could be stopped by raising the temperature from 20 to 25 C. Growth could be resumed by lowering the temperature, even after a week at the high (restrictive) temperature. The infection types on plants with *Pm* genes were unaffected by the temperature-sensitive mutations. These mutations appear to affect genes that control basic parasitic growth and development.

Additional key words: gene-for-gene specificity, disease resistance.

Erysiphe graminis f. sp. *tritici*, an obligate parasite of wheat (*Triticum aestivum*), can be induced infect a plant with high efficiency and with reasonable synchrony of development during the first 58 hr after inoculation (7,9). Genetic studies of the variability in host-parasite interactions with powdery mildew have been made in both host and parasite. The interactions have been shown to follow the gene-for-gene pattern (11). *E. graminis* f. sp. *tritici* was chosen for this study because it has morphologically well-defined stages of development and the effects of variation in genes controlling early events crucial to parasitism can be studied in detail.

Induced mutations to increased virulence have been reported in at least seven different pathogens (2). Mutations to avirulence on host plants with genes conferring specific resistance (specific *R* genes) have not been reported. A trivial explanation for this observation is that mutations to avirulence are more difficult to select than mutations to virulence, which may account for their apparent rarity. We favor another explanation. Since mutations almost always have deleterious effects on genes, the frequency of forward mutations (losses of function) is always higher than that of reverse mutations (regaining of function). If genes function for specific avirulence, then mutations to increased virulence against specific *R* genes should be more frequent than mutations to decreased virulence against specific *R* genes.

The gene-for-gene hypothesis successfully predicts the patterns of interactions between *R* genes in a host and genes conferring specific avirulence (*P* genes) in a pathogen. Although this genetic hypothesis offers limited insight into physiological mechanisms for pathogenic restriction, it suggests that certain hypotheses of mechanisms of interaction are improbable. For example, the genetically specific interaction between a host *R* gene and a pathogen *P* gene is for incompatibility. Only the *R* and *P* alleles are involved. The alternate alleles, *r* and *p*, are not genetically specific (3). The simplest interpretation of the genetic data is that the *P* gene codes for a product active in promoting avirulence. Since *P* genes do not appear to actively promote pathogenicity, then other genes must be involved in that function.

This investigation was undertaken with three objectives in mind. The first was to induce mutations in the pathogen with an increase in virulence on host plants with single *R* genes. We also wanted to determine if mutations of this type affected the interactions between the mutant parasite and host plants with recessive *r* alleles. The second objective was to induce temperature-sensitive mutations that allow the development of the parasite on susceptible cultivars at the normal (permissive) temperature, but not at a higher (nonpermissive) temperature. This type of mutation is presumed to involve genes whose function is crucial to successful parasitism. The third objective was to observe the characteristics of any mutations obtained with a view towards distinguishing between alternative hypotheses of physiological function of *P* genes.

If *P* genes function for avirulence, then: 1) mutations to increased virulence on plants with single *R* genes should result in loss of function and should be easily obtained; 2) mutants with increased virulence should still be able to parasitize plants with *r* genes as well as the wild type; and 3) mutations of genes that are crucial for parasitism should not show *P*-gene specificity. On the other hand, if *P* genes function for virulence, then: 1) mutations to increased virulence should result in gain of function and should be difficult to obtain; 2) some of the mutants with increased virulence should be more virulent than others on host plants with the same *R* gene, depending on the degree of the specificity conferred by the forward mutation; and 3) at least some mutations of genes which are thought to be crucial to parasitism could show *P* gene specificity to as yet unidentified (cryptic) host *R* genes.

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MATERIALS AND METHODS

Stock cultures. The wild-type culture of *E. graminis* f. sp. *tritici* (MS-1) was maintained as follows. Conidia collected from plants inoculated 7 days earlier were used to inoculate 6- to 7-day-old wheat seedlings (cultivar Little Club) growing in 10-cm-diameter pots. Inoculated plants were kept in a growth chamber at 18–20 C during the light period and 16–18 C during the dark period. Light intensity was 75–86 hlx during a 15-hr photoperiod. Relative humidity ranged from 70 to 90% during the light period and from 90 to 100% during the dark period. These conditions will be referred to as "standard conditions" throughout the paper.

Mutant cultures were maintained as described above in separate growth chambers, or under 21.6-cm-long glass chimneys (#845310, Corning Glass Works, Central Falls, RI 02863) topped with four layers of cheesecloth. Stock cultures were also maintained for up to 4 wk on detached leaves, in 10-cm-long test tubes with the base of the leaf immersed in 1 ml of benzimidazole (125 μg/ml) in water. The tubes were capped with ribbed test-tube caps to allow for gas exchange and were stored in growth chambers under standard conditions. Isolates were also stored for up to 2 mo on seedlings growing in 5 cm (depth) of vermiculite in 30.5-cm-long test tubes (one to three per tube) capped with a cotton plug. Seven days after inoculation, these were placed in a refrigerator at 4–10 C in continuous light from a single 32W fluorescent lamp (Sylvania F24T12 HO) ~30.5 cm (~12 inches) from the tubes.

Induction of mutations. Seven-day-old leaves of Little Club plants that had been lightly inoculated with conidia 15 hr previously were cut at the base and placed in 15-cm-long test tubes containing benzimidazole (100 μg/ml) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (125 μg/ml) (Aldrich Chemical Company, Milwaukee, WI 53233) in 2 ml of water. The tubes were placed uncapped in a growth chamber with the lights on for 8 hr under standard conditions to promote transpiration.

Isolation of mutants with increased virulence. After 7–10 days of NTG treatment, the leaves were removed from the tubes and conidia from treated mildew colonies were rubbed onto leaves of 7-day-old seedlings of cultivar Chancellor wheat. Chancellor contains no known *R* genes for resistance to culture MS-1. The inoculated plants of Chancellor were then placed under standard conditions used for induction of synchronous development of the parasite (9). A 10-cm-diameter pot of uninoculated wheat seedlings with gene *Pm*1a and another pot of uninoculated seedlings with gene *Pm*4a were placed next to the inoculated Chancellor plants in each experiment. The wheat lines (isolines) with *Pm*1a or *Pm*4a were derived from eight backcrosses to cultivar Chancellor (1) and are therefore congenic to each other and to Chancellor. *Pm*1a and *Pm*4a are unlinked (10).

The wild-type culture MS-1 contains the corresponding genes *P*1a and *P*4a, and on both the *Pm*1a and *Pm*4a isolines gives an infection rating of 0 on a 0–4 infection-type scale. MS-1 is compatible (infection type 4) with Chancellor. After 7 days of growth, the conidia produced on Chancellor were dusted daily onto the *Pm*1a and *Pm*4a isolines in the growth chamber. After a further 7 days, the *Pm*1a and *Pm*4a isolines were examined for mildew development. Any colonies growing on these lines were increased on the same line, and isolates that could be continuously maintained on plants with *Pm*1a or *Pm*4a were isolated and considered to be mutants. Only one mutant from each host line was isolated during each experiment to insure the independence of origin of each mutant culture.

Several precautions were taken to guard against the possibility of contamination by other virulent isolates. First, all experiments were conducted in a building physically isolated from any known sources of mildew. Some of the mutants were obtained in the winter, when contamination from outdoor sources was impossible. Second, all mutants were tested for their reactions on four host lines with *Pm*1a, *Pm*2a, *Pm*3a, or *Pm*4a. These differentials could distinguish isolates of mildew kept in culture in our laboratory. Two of the mutant isolates obtained, one virulent on *Pm*1a and the other virulent on *Pm*4a, were also tested against isolines *Pm*4b, *Pm*6, *Pm*7, *Pm*8, Lehmi, 15889, 17339, and *Triticum sphaerococcum*. We detected no contaminants in our tests with the mutants during the course of this work. Third, different field isolates of mildew show unique polypeptide maps by two-dimensional electrophoresis. Three of the mutants were extensively examined by two-dimensional electrophoresis and their polypeptide maps were indistinguishable from each other and from the wild-type culture MS-1.

Isolation of temperature-sensitive mutants. After 4 days of NTG treatment, the leaves were removed from the tubes and as few conidial chains as possible (usually one to five) were removed from well-separated single pustules. A fine platinum wire was used to transfer the chains of spores onto a leaf of Chancellor. The leaf was then cut at the base and placed in 1 ml of aqueous benzimidazole (125 μg/ml) solution in 10-cm-long test tubes. The tubes were capped and placed in a growth chamber under standard conditions. After 8 days, spores from individual pustules were transferred by gently brushing the pustule against two leaves. Each leaf was cut at the base and placed in the benzimidazole solution in 10-cm-long capped test tubes. One of the inoculated leaves was kept at 20 C under standard conditions, and the other kept at 25 C under otherwise standard conditions. After 1 wk the isolates were scored for growth. Isolates were considered temperature sensitive if growth appeared normal or nearly normal at 20 C (the permissive temperature) and if no macroscopically visible growth was observed at 25 C (the restrictive temperature). These tests were performed three times in test tubes. Isolates that were scored as temperature-sensitive in three trials were transferred each week to plants of Chancellor or Little Club wheat and grown in 10-cm-diameter pots under glass chimneys for the duration of the experiment.

RESULTS

Mutants with increased virulence. Twenty-nine constitutive mutations with increased virulence were induced. Sixteen had increased virulence on plants with *Pm*1a and 13 had increased virulence on plants with *Pm*4a. The wild-type culture MS-1 gave an infection type 0 on these isolines, and all mutants gave an infection type 4 on the isoline on which they were selected. All mutants were tested for infection type against host lines with other *Pm* genes and all gave reactions indistinguishable from MS-1 except for the selected change in virulence. When the mutants were grown on Chancellor wheat, which has the recessive *pm*1a and *pm*4a alleles, no differences were observed between the mutants and MS-1 in rates of infection and final infection types.

Temperature-sensitive mutants. A total of 530 single pustule isolations were made following treatment with NTG. Based on temperature-sensitivity on detached leaves in test tubes, 46 mutants (8.6%) were selected for increase of inoculum and further study. Of these, 19 (3.6%) continued to express temperature-sensitivity when grown on plants in 10 cm pots under chimneys. Growth and development of the isolates could be stopped by raising the temperature from 20 to 25 C. Growth resumed after the temperature was lowered, even when plants were held at the restrictive temperature for 1 wk. Microscopic observations of the mycelium of these mutants after 1 wk at 25 C revealed that most of the hyphae, but not all, were lysed. All 19 temperature-sensitive mutants were evaluated for reactions with the four host isolines *Pm*1a, *Pm*2a, *Pm*3a, and *Pm*4a. No changes in virulence from that of MS-1 were found.

DISCUSSION

In all gene-for-gene systems, resistance conditioned by a host *R* gene is completely dependent upon the presence of a specific corresponding parasite *P* gene. The simplest genetic interpretation of the interactions with all possible combinations of host and parasite alleles involved in a gene-for-gene interaction is that specific recognition is for an incompatible relationship. Resistance and avirulence are considered to be active functions (3,5). Therefore, a mutation from avirulence to virulence would be from one particular specificity to a loss of that specificity. Mutations of

any particular *P* gene should be easily obtained, unless the gene has some other function essential to the organism (6).

Twenty-nine mutations of independent origin to increased virulence on host lines with single *Pm* genes were obtained. Each mutant had increased virulence only on plants with the selected *Pm* gene. Because the change in reaction is so specific to plants with a particular *Pm* gene, it is considered likely that the mutation in the parasite is at the corresponding *P* locus and not at a suppressor locus. Also, when we examined the protein mobility patterns of three mutant isolates by two-dimensional electrophoresis, we found no differences between the mutants and the wild-type culture. Suppressor mutations might be expected to alter many polypeptides rather than a single polypeptide. We tentatively conclude that the mutational event altered the specificity of a *P* gene (Table 1).

Isolate MS-1, with genes *P*1a and *P*4a, gave an infection type 0 on plants with either *Pm*1a or *Pm*4a. MS-1 was easily mutated to give an infection type 4 on plants with either of these two genes. If the recessive *p* alleles functioned to specifically recognize and overcome particular *Pm* genes, then a mutation from avirulence to virulence would be from no function to a gain of specific function. Such mutations should be difficult to obtain, because the creation of a specific function requires nonrandom, highly specific changes. Mutations which by chance *completely* negate the effect of the *Pm* gene and restore maximum, as opposed to partial, compatibility should be rare indeed. At least some of the mutants might have been expected to give an intermediate infection type, but none was recovered. The ease with which MS-1 was mutated 29 independent times from incompatibility (infection type 0) to compatibility (infection type 4) on host lines with either of two unlinked *Pm* genes argues against the idea that the *p* allele actively interacts with the *Pm* allele to give a compatible reaction.

Parasite strains with the mutant *p* gene appeared to grow and develop on plants with the recessive *pm* genes similarly to parasite strains with the wild-type *P* gene. Since the mutations to increased virulence did not measurably affect compatibility of the mutants with plants with recessive *pm* genes, it appears that the *P* loci do not affect basic ability to parasitize.

Since *P* genes did not appear to be involved in the basic ability of *E. graminis* f. sp. *tritici* to parasitize, a search was initiated to identify genes that might be. With the notable exception of the host-specific toxins, we know of no examples of naturally occurring, major variability in genes whose function(s) promote basic parasitic capability. This may be because variability in genes whose function is necessary for parasitism is selected against in nature. Several researchers studying stabilizing selection have discovered that there are quantitative differences in fitness among pathogen isolates, which are not considered attributable to gene-for-gene interactions (8). These "fitness" genes may be examples of naturally occurring variability in genes crucial to the basic ability to parasitize, crucial to basic life functions unrelated to parasitism, or both. The effects of fitness genes are slight, however, and the assay is tedious. One way to overcome the problem may be to artificially generate major variability in genes essential for parasitism.

One of the best ways to study variation in essential genes is to use conditional, temperature-sensitive mutations. The mutant phenotype is expressed only at the restrictive temperature (usually slightly elevated above normal) and is not expressed at the permissive temperature, usually a temperature normal for the organism. Thus, a mutation affecting parasitic growth of even an obligate parasite can be maintained, and the mutation will be expressed by raising the temperature.

TABLE 1. Reactions[a] of isolate MS-1 of *Erysiphe graminis* f. sp. *tritici* and a mutant derived from MS-1 on three congenic isolines of wheat

Inferred pathogen genotype	Host genotype		
	*Pm*1a *Pm*1a *pm*4a *pm*4a	*pm*1a *pm*1a *Pm*4a *Pm*4a	*pm*1a *pm*1a *pm*4a *pm*4a
*P*1a *P*4a (MS-1)	−	−	+
*p*1a *P*4a (mutant)	+	−	+

[a]+ Indicates a compatible interaction and − indicates an incompatible interaction.

The temperature-sensitive mutations isolated in this study did not affect a gene-for-gene specificity. If the hypothesis that virulence is a specific function is correct, then some of the temperature-sensitive mutants selected on Chancellor might not be temperature-sensitive on Little Club. That is, if a pathogen carries a battery of specific virulence genes against many cryptic host resistance genes, then mutations in some of these should differentially affect the interactions on previously susceptible hosts. On the other hand, if ability to parasitize is nonspecific, all susceptible hosts should be equally affected. At the restrictive temperature, growth of all temperature-sensitive mutants on Chancellor or Little Club was equally affected, indicating that the primary defect was in a gene whose product was generally necessary for growth and development of the parasite.

It is possible that none of these mutations affected the processes involved in parasitism per se, but rather that they affected only basic life functions, such as protein or DNA synthesis. For example, mutations of genes apparently essential for parasitism have been obtained and distinguished from those affecting basic life functions in *Colletotrichum lindemuthianum* (4). Observations of all mutants obtained in this study revealed that conidial germination and limited mycelial growth occurred in cultures continuously exposed to the restrictive temperature immediately after inoculation. Since the enzymes themselves (whether stored or not) are affected in a temperature-sensitive mutant, this observation suggests that enzymes involved in organismal development or parasitism per se are affected in these mutants.

Prolonged exposure to restrictive temperatures caused lysis of most of the mycelium of the temperature-sensitive mutants. Lowering the temperature to permissive levels, even after 1 wk at restrictive levels, allowed resumption of normal growth of at least some of the parasite units. Temperature-sensitive mutants with the ability to resume normal growth after cessation of growth should prove to be of immediate use in applications where regulating the growth of the pathogen is desired. For example, hypotheses involving rates of accumulation and efficacy of phytoalexins at disease sites could be tested by using these conditionally expressed mutants.

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