Correlations Between Cytologically Detected Plant-Fungal Interactions and Pathogenicity of *Magnaporthe grisea* Toward Weeping Lovegrass

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ABSTRACT

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Genetic analysis previously demonstrated that two strains of the ascomycetous plant pathogen, *Magnaporthe grisea*, differ in a single gene controlling pathogenicity to weeping lovegrass. This study was undertaken to determine the feasibility of identifying a microscopic feature that segregates with the gene for pathogenicity to weeping lovegrass. Cytological results are reported for weeping lovegrass inoculated with the parental strains and progeny from two tetrads obtained from crossing these strains. Many features observed at infection sites of the nonpathogenic parent clearly were not correlated with host specificity because they were observed at some infection sites of both pathogenic and nonpathogenic progeny. The identification of a strain as pathogenic or nonpathogenic to weeping lovegrass appeared to depend on whether brown cells developed around the margins of developing colonies. Because the fungus was never seen to grow beyond these brown cells, the ability to induce this plant response may play a role in determining host species specificity. Analysis of a statistically significant number of progeny will be required to determine whether this cytological feature cosegregates with the gene for host specificity.

Additional keywords: *Pyricularia*, resistance determinants, rice blast fungus.

Fungal plant parasites show specificity for certain host species and often for certain genotypes within their host species. In some cases, such cultivar specificity is controlled by single genes in the parasite that "match" genes for resistance in the plant (a gene-for-gene interaction) (2). However, the number of genes controlling host species specificity rarely has been investigated. The ascomycete *Magnaporthe grisea* (Hebert) Barr, which includes strains pathogenic to rice and to a wide variety of other grasses, presents opportunities for understanding the molecular basis of such specificity. A genetic cross between a strain that attacks weeping lovegrass (*Eragrostis curvula* (Schrad.) Ness) and one that attacks goosegrass (*Eleusine indica* (L.) Gaertn.) has demonstrated previously that these strains differ by two unlinked genes: one critical for infection of weeping lovegrass, *Pwl1*, and one critical for infection of goosegrass, *Pgg1* (8,9).

There is no information concerning the function of any fungal gene identified as being involved in host specificity, with the exception of those from fungal pathogens with virulence dependent on the production of host-selective toxins (7). Fungal invasion of resistant or susceptible higher plants commonly is accompanied by characteristically different growth patterns and plant responses that can be detected cytologically. However, to our knowledge, no one has tried to relate cytological features to the activity of fungal genes controlling host species specificity. The efforts required for cytological studies of this nature preclude the analysis of large numbers of progeny until specific and relevant features have been identified. The present study was initiated to determine if any such relevant features can be identified in the interaction between weeping lovegrass and *M. grisea*.

MATERIALS AND METHODS

Plants and fungi. The sources of plants and fungi are described by Valent et al (9). Parental fungal isolates were K76-79, a weeping lovegrass pathogen, and WGG-FA40, a goosegrass pathogen. The ascospore progeny were from ascus 1 and 5 of a cross (assigned the serial number 4091) between the two parents. One progeny from each of the pairs of identical (sister) spores in each ascus was chosen for this study (5-1, 5-4, 5-7, 5-8; 1-2, 1-3, 1-5, 1-6). Ascospore 5-3, the sister spore to 5-4, was used in one experiment. Pairs of sister spores were identified by their pathogenic profiles, mating type, fertility, and colony morphology characteristics. In all of our studies, the two strains derived from sister spores were indistinguishable.

Weeping lovegrass was grown in vermiculite (about 20 plants per 10-cm-diameter pot) in a growth room maintained at 25 C, 80% RH, and a 14-hr photoperiod. Light provided by a mixture of tungsten and cool-white fluorescent bulbs ranged in intensity from 700 to 900 μEin$\cdot$m$^{-2}\cdot$s^{-1}. Plants were fertilized and inoculated as described previously (5). Inoculated plants were sealed in plastic bags and left in the dark for 24 hr at 24 C before being returned to the growth room. The plastic bags were removed at 24 hr after inoculation. One leaf per plant was harvested at 24, 48, and 72 hr after inoculation, and macroscopic symptom development was noted for another 4 days. The macroscopic symptoms of rare limited lesions reported here on weeping lovegrass for the nonpathogenic parent and progeny differ from those reported earlier (8,9), when no visible symptoms could be observed. This difference is likely due to the very different environments provided by the plant growth facilities that were used in the two sets of experiments because most of the earlier experiments were conducted in a rather poorly regulated greenhouse in Boulder, CO. However, in this and the previous study, "pathogenic" strains were clearly differentiated from "nonpathogenic" strains by the ability to cause total withering of inoculated tissue.

Microscopical examination. Harvested leaves were cleared and the adaxial surfaces were examined for fungal growth and plant responses as described elsewhere (4). Autofluorescence was determined by epifluorescence microscopy and blue light irradiation (4). Twenty infection sites at which the fungus had penetrated an epidermal cell were examined in detail from each of five leaves, at each time interval for each fungal strain. Total length of mycelium was measured with the aid of a micrometer eyepiece.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) and arcsine transformation, where appropriate. Significant differences between means were assessed by calculating the

least significant difference if the ANOVA gave a significant F value.

RESULTS

Interactions between weeping lovegrass and the parental strains. By 7 days after inoculation, the entire area of leaf inoculated with the pathogenic parent, K76-79, became straw colored and totally withered. The macroscopic symptoms induced by the nonpathogenic parent, WGG-FA40, were rare limited lesions (1 mm in diameter) ringed with brown. Microscopically, fungal development and plant responses varied not only with the strain of the fungus but also between individual infection sites in the same leaf. In general, the range of plant-fungal interactions seen with each parental strain closely resembled those described in detail in a previous study involving this fungus (5). Tables 1 and 2 record those features of fungal growth and plant responses that either differed qualitatively between the two strains or exhibited statistically significant quantitative differences.

As shown in Tables 1 and 2, the nonpathogen of weeping lovegrass, strain WGG-FA40, clearly infected weeping lovegrass more slowly than the weeping lovegrass pathogen, K76-79. At 24 hr after inoculation, fewer appressoria of the nonpathogen had developed infection hyphae. This difference had almost disappeared by 48 hr (data not shown). At 24 hr, the nonpathogen had formed on average only half as much mycelium at successful

TABLE 1. Growth characteristics of, and plant responses induced by, parental strains and ascus 5 progeny in weeping lovegrass

	Fungal strain							
	K76-79[a]	FA40	5-1	5-4	(5-3)[b]	5-7[a]	(5-7)[a,b]	5-8[a]
Percentage appressoria with an infection hypha by 24 hr	49 A[c]	30 B	31 B	10 C	(5 C)	62 A	(50 A)	23 B
Mean hyphal length (μm) at 24 hr	54 A	21 B	23 B	22 B	(21 B)	49 A	(44 A)	15 B
Percentage infection sites at 48 hr with fungus exiting epidermal cell	100 A	3 B	35 C	69 D	(46 CD)	92 E	(96 E)	69 D
Percentage infection sites with large colonies at 72 hr	100 A	4 B	20 C	50 D	(60 D)	90[d]	(90[d])	100 A
Percentage infection sites with fluorescent epidermal walls								
24 hr	1 A	70 B	10 C	45 D	(0 A)	14 C	(3 A)	6 AC
48 hr	0 A	100 B	100 B	89 C	(38 E)	8 D	(4 D)	9 D
Percentage infection sites with only undifferentiated hyphae at 48 hr	0 A	49 B	18 C	14 C	(18 C)	2 A	(0 A)	0 A
Percentage infection sites with necrotic fungus restricted to one fluorescent epidermal cell at 48 hr	0 A	97 B	65 C	33 D	(37 D)	8 E	(5 E)	5 E
Presence of brown plant cells around fungal colonies at 72 hr[e]	−	+	+	+	(+)	−	(−)	−
Percentage fungal colonies surrounded by brown cells at 72 hr[e]	0 A	100 B	100 B	66 C	(55 C)	0 A	(0 A)	0 A

[a] Weeping lovegrass pathogens from macroscopic observations.
[b] From different experiments.
[c] Values in each row with the same letter do not differ significantly at $P = 0.05$.
[d] Estimated due to coalescence of large lesions.
[e] Only colonies where the fungus spread from the initially invaded epidermal cell counted.

TABLE 2. Growth characteristics of, and plant responses induced by, parental strains and ascus 1 progeny in weeping lovegrass

	Fungal strain					
	K76-79[a]	FA40	1-6	1-3	1-5[a]	1-2[a]
Percentage appressoria with an infection hypha by 24 hr	86 A[b]	22 B	30 B	32 B	53 C	64 C
Mean hyphal length (μm) at 24 hr	46 A	16 B	19 B	16 B	24 BC	26 C
Percentage infection sites at 48 hr with fungus exiting epidermal cell	100 A	13 B	3 C	16 B	79 D	67 E
Percentage infection sites with large colonies at 72 hr	100 A	11 B	6 C	11 B	100 A	80 C
Percentage infection sites with fluorescent epidermal walls						
24 hr	0 A	61 B	55 B	40 C	3 A	1 A
48 hr	25 A	89 B	96 B	94 B	22 A	14 A
Percentage infection sites with only undifferentiated hyphae at 48 hr	0 A	41 B	61 C	53 D	1 A	0 A
Percentage infection sites with necrotic fungus restricted on one fluorescent epidermal cell at 48 hr	0 A	74 B	88 C	74 B	0 A	0 A
Percentage infection sites with brown contents of first-invaded cell	0	0	0	0	1 A	38 B
Presence of brown plant cells around fungal colonies at 72 hr[c]	−	+	+	+	−	−
Percentage fungal colonies surrounded by brown cells at 72 hr[c]	0 A	100 B	100 B	80 C	0 A	0 A

[a] Weeping lovegrass pathogens from macroscopic observations.
[b] Values in each row with the same letter do not differ significantly at $P = 0.05$.
[c] Only colonies where the fungus spread from the initially invaded epidermal cell counted.

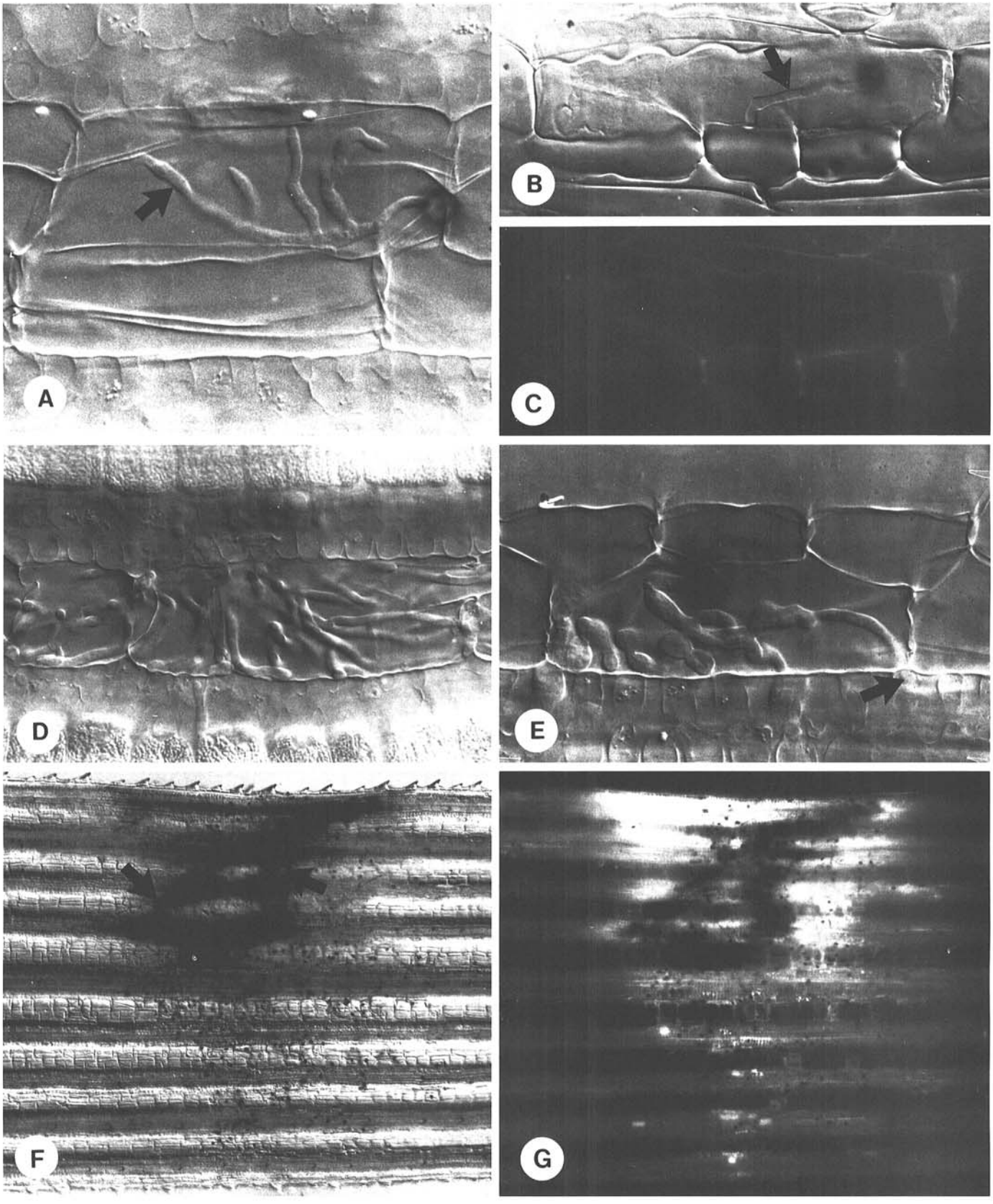

Fig. 1. Interactions between strains of *Magnaporthe grisea* and weeping lovegrass viewed in cleared leaves by differential interference contrast optics (A, B, D–F) or blue light epifluorescent irradiation (C, G). **A,** Strain K76-79 (arrow) 24 hr after inoculation. ×872. **B,** Undifferentiated infection hypha of strain WGG-FA40 (arrow) 24 hr after inoculation. ×614. **C,** Autofluorescence of the cell walls of the fungus-invaded epidermal cell shown in B. ×614. **D,** Strain K76-79 48 hr after inoculation. Fungal hyphae have spread from the first-invaded epidermal cell to surrounding epidermal and mesophyll cells. ×570. **E,** Strain FA40 48 hr after inoculation. The fungus has begun to spread from the epidermal cell into surrounding mesophyll cells (arrow). ×783. **F,** Strain FA40 72 hr after inoculation. Two fungal colonies (arrows) limited by brown host cells can be seen amid the many appressoria (small dark spots) which developed no further than the infection hypha. ×62. **G,** Autofluorescence of uninvaded cells around the limited lesions shown in F as well as epidermal cells underlying appressoria from which the fungus has not escaped. ×62.

penetration sites as the pathogen (compare Fig. 1A and 1B). By 48 hr, the nonpathogen had spread from the initially penetrated epidermal cell at 13% or fewer of infection sites, whereas the pathogen had spread from this cell at all infection sites. Many of the epidermal cells invaded by the nonpathogen had autofluorescent cell walls by 24 hr after inoculation (Fig. 1C) and almost all had autofluorescent walls by 48 hr. In other systems, similar autofluorescence is indicative of the presence of phenolic materials (3). At this time the fungus typically was autofluorescent (often a sign of death [4]) and/or appeared "empty," and the contents of the invaded cell had a granular appearance. At almost half of these infection sites, the nonpathogen was unbranched and uniformly thin (Fig. 1B), representing infection hyphae that had not differentiated to form secondary hyphae (5).

At the rare infection sites where the nonpathogen had spread from the initially invaded epidermal cell by 48 hr after inoculation, colonies formed that were smaller than those formed by the pathogen (compare Fig. 1D and 1E) but otherwise resembled colonies formed in the compatible combination. However, by 72 hr after inoculation, all nonpathogen colonies were ringed with plant cells with dark brown contents, and the fungus was never observed to grow beyond the brown zone. The leaves infected with the nonpathogen were characterized, therefore, by a few scattered limited lesions surrounded by large numbers of infection sites at which the fungus had been restricted to a single, autofluorescent cell (Fig. 1F and G). In contrast, the pathogen formed unrestricted growing colonies at all infection sites, and these engulfed the entire inoculated area of the leaf.

Interactions between weeping lovegrass and ascus 5 progeny. Of these ascospore progeny, two (5-7 and 5-8) produced macroscopic symptoms identical to those produced by the weeping lovegrass pathogenic parent, that is, total shriveling of inoculated tissue. "Nonpathogenic" progeny 5-1, 5-3, and 5-4 produced limited, brown-rimmed lesions similar to those of the nonpathogenic parent. However, the lesions produced by these progeny appeared slightly larger and more numerous than those produced by the nonpathogenic parent. This was particularly true of progeny 5-3 and 5-4, the genotypically identical sister-spore strains.

In general, ascospore progeny strains tended to resemble their respective pathogenic or nonpathogenic parents in terms of the frequency of infection sites exhibiting specific features of either fungal growth or plant responses (Table 1). However, macroscopically pathogenic strains 5-7 and 5-8 elicited responses typical of the nonpathogenic parent (for example, autofluorescence of epidermal walls) at a significant proportion of infection sites, a feature not seen for the weeping lovegrass pathogenic parent. Similarly, strains 5-1, 5-4, and 5-3, which failed to cause withering, elicited fewer signs of microscopic incompatibility than the nonpathogenic parent. For example, autofluorescence of epidermal cells occurred more slowly with the progeny than with the nonpathogenic parent, and the progeny strains more often formed growing colonies.

Progeny strains with similar host specificities showed significant differences in occurrence of certain microscopic features. For example, the nonpathogenic sister progeny strains 5-3 and 5-4 differed from their nonpathogenic sibling 5-1 in their much slower penetration from appressoria, greater incidence of colony growth, and slower development of a brown lesion margin. Pathogenic progeny strain 5-8 differed from 5-7 in its slower growth rate (resulting in a shorter average hyphal length at 24 hr and fewer cases of spread from the first epidermal cell by 48 hr). In contrast, 5-7 differed from both 5-8 and the weeping lovegrass pathogen parent by the presence of infection sites (average, 5–8%) at which the fungus was restricted to one epidermal cell.

One cytologically detectable character invariably was associated with macroscopically determined nonpathogenicity to weeping lovegrass (Table 1). This was the presence of brown cells rimming growing colonies of the fungus. Even though brown cells segregated in an all-or-nothing manner, the timing of this feature differed among the nonpathogenic strains. Brown cells were slower to develop around colonies of 5-4 and 5-3 than around those of 5-1.

Interactions between weeping lovegrass and ascus 1 progeny. Of these progeny strains, two (1-5 and 1-2) induced total withering of inoculated tissue identical to that induced by the weeping lovegrass pathogenic parent, and two (1-6 and 1-3) induced limited, brown-rimmed lesions similar to those produced by the nonpathogenic parent. As with the ascus 5 progeny, one of the nonpathogenic progeny of ascus 1 (1-3) induced more and larger lesions than the other (1-6).

Ascus 1 progeny tended to resemble the respective pathogenic or nonpathogenic parents in terms of fungal growth and plant responses; however, as with ascus 5 progeny, there were statistically significant, quantitative differences (Table 2). These differences were not identical to those seen for the ascus 5 progeny. The pathogenic progeny strains 1-5 and 1-2 differed from the weeping lovegrass pathogenic parent in the rate of development within weeping lovegrass tissue (specifically in a lower incidence of penetration from appressoria at 24 hr), shorter infection hyphae at successful penetration sites at 24 hr, and slower growth from the first-invaded cell. The pathogenic progeny (1-5 and 1-2) from ascus 1 closely resembled the weeping lovegrass pathogenic parent in many of the features listed. However, they also induced a new microscopic feature not seen with either parent or with any other progeny strains. Especially in infection sites of strain 1-2, a significant number of the first-invaded cells developed brown contents.

The nonpathogenic progeny from ascus 1 differed from the nonpathogenic parent, particularly in the greater incidence of thin, undifferentiated infection hyphae (Table 2). As observed for ascus 5, there also were significant differences between the two strains representing each pair of nonpathogenic progeny. For example, compared with strain 1-6, more than twice as many individuals of strain 1-3 had exited the first-invaded cell by 48 hr after inoculation and had formed large growing colonies by 72 hr; the resulting lesions were slower to develop brown margins.

As with ascus 5 progeny, brown plant cells surrounded and appeared to limit the growth of fungal colonies formed by the nonpathogenic progeny 1-3 and 1-6. No such brown cells were observed at infection sites formed by the pathogenic progeny 1-2 and 1-5.

Reproducibility of results. Tables 1 and 2 each show the data from one experiment (except for the data in parentheses in Table 1) in which all inoculations with parental strains and the progeny from one ascus were performed concurrently on the same batch of plants. All fungal strains from ascus 5, except 5-1 and 5-4, also were tested on separate occasions. The values obtained either were statistically identical to those reported here or, if they differed, the relative differences between strains remained the same. One such set of results for progeny strain 5-7 is shown in Table 1. The two sets of values for this strain, obtained from different experiments, are statistically identical except that fewer epidermal walls were autofluorescent by 24 hr in the separate test. The high degree of reproducibility of results also is illustrated by the similarity in values for the sister-spore strains 5-4 and 5-3 (Table 1). Again, the only statistically significant difference between them was the slower induction of fluorescent epidermal walls by strain 5-3. Parental strain K76-79 also exhibited variability in this feature between the experiments reported in Tables 1 and 2. However, the relatively high frequency of fluorescent epidermal walls at 48 hr shown in Table 2 was the result of a very high value in one leaf, a feature not seen in all other leaves or experiments.

DISCUSSION

In a previous study, analysis of seven complete tetrads of a cross (4091) between K76-79 and WGG-FA40 suggested the existence of the gene, *Pwl1*, that controlled pathogenicity to weeping lovegrass (8,9). Random ascospore analysis and tetrad analysis from several subsequent crosses confirmed this segregation. For both asci from cross 4091 used in this study, half the ascospore progeny could be clearly classified as pathogenic, causing total withering of weeping lovegrass, and the other half

could be classified nonpathogenic, causing relatively few isolated limited lesions.

Microscopically, the parental strains could be clearly differentiated by qualitative and quantitative differences in fungal growth and plant responses. The quantitative differences represent differences in the frequencies of infection sites exhibiting certain features of fungal growth or plant response. Differences between infection sites are the norm in microscopical investigations of plant-fungal interactions (3,6) and presumably reflect physiological differences between individual plant cells and fungal propagules. However, any study that compares the frequencies of specific features must take into account their reproducibility. In the present study, the frequencies of most measured features proved remarkably similar between temporally repeated experiments and between the genetically identical sister-spore progeny strains 5-4 and 5-3. This observation provides a high degree of confidence that the statistically significant differences in the frequencies of certain features are also of biological significance.

Although microscopical features of infection sites of the progeny tended to resemble those of the parental strain with the same specificity toward weeping lovegrass, significant quantitative differences were seen. Pathogenic progeny from both asci exhibited features at some infection sites, such as slow escape from the first-invaded cell and the browning or autofluorescence of plant cell walls, that typified the interaction with the nonpathogenic parent. Nonpathogenic progeny strains from ascus 5 were restricted to one autofluorescent epidermal cell at fewer infection sites than the nonpathogenic parent. In addition, progeny from ascus 1 showed a unique browning of the contents of the first-invaded cell. Overall, therefore, progeny strains of different pathogenicities from both asci did not show the same magnitude of differences as the parental strains; moreover the types of differences between progeny and parental strains were not the same for the two asci. These observations suggest that fungal control of its growth in the plant and the induction of plant responses may be genetically complex. Only the restriction of colony growth in association with the browning of uninvaded cells at the lesion margin clearly distinguished all nonpathogenic and pathogenic progeny as well as parental strains. This browning was observed with all colonies of all nonpathogenic strains and with no colonies of pathogenic strains and was, therefore, the only cytological feature that was observed to clearly correlate with the gene controlling host specificity.

An interesting conclusion from this work is that relatively few of the microscopical features of the plant-fungus interaction have a significant effect on macroscopic symptom development. For example, the fast rate of initial development of the infection hypha and the fast spread from the first-invaded cell, both typical of the pathogenic parent, were not necessarily characteristic of the pathogenic progeny. Moreover, growing colonies formed in at least some infection sites of all the parental and progeny strains. Because relatively few unrestricted colonies are necessary to engulf the small weeping lovegrass leaf, specificity toward weeping lovegrass appears to depend not on the fact that nonpathogenic strains formed fewer growing colonies, but rather on the continued spread of colonies formed by the pathogenic strains. Such observations support the primary importance of factor(s) affecting colony growth, rather than initial establishment of the fungus, in determining whether the fungus is pathogenic on weeping lovegrass.

We cannot make any conclusions concerning the segregation of cytological features with the gene determining pathogenicity to weeping lovegrass because progeny from only two asci (two meiotic events) were examined. However, the data raise the possibility that the single gene difference between strains K76-79 and WGG-FA40 governing specificity for weeping lovegrass controls the elicitation of a plant response that limits colony growth after escape from the first-invaded cell. Alternatively, the gene may act in the pathogen to suppress this response. Tests to determine if pathogenicity or nonpathogenicity is dominant are not possible with *M. grisea* because the fungus does not form a stable vegetative diploid (1). Therefore, we cannot yet determine if the gene is active in the pathogen or the nonpathogen. These two alternatives should be distinguishable by determining whether loss of function mutations turn the nonpathogen into a pathogen, or vice versa.

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