A Method for Genetic Analysis of *Glomerella graminicola* (*Colletotrichum graminicola*) from Maize

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ABSTRACT

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Strains derived from nine different isolates of *Colletotrichum graminicola* from maize participated in the production of perithecia when incubated on pieces of autoclaved corn leaves in a humidity chamber. Matings occurred between self-fertile and self-sterile strains, and also between certain self-sterile strains. As many as 200 ascospore progeny were recovered easily from individual perithecia. Characterization of progeny showed that sexual recombination and Mendelian segregation of distinct traits could be detected. Segregation of markers for chlorate resistance (Chl^R), benomyl resistance (Bml^R), and melanin deficiency (Mel^-) approximated a 1:1 ratio and defined three separate linkage groups. Crosses involving a pyrimidine auxotroph (Pyr^-) showed 2:1 segregation (Pyr^+:Pyr^-) and linkage between markers for Pyr^- and Chl^R. Attempts to combine multiple markers resulted in successful construction of a Mel^- Pyr^- self-fertile strain that was crossed with a Bml^R strain to produce offspring with a triple-mutant Mel^- Pyr^- Bml^R phenotype.

Many important advances in plant pathology have come from genetic analyses of fungi. For example, detailed studies of the inheritance of virulence in the flax rust pathogen *Melampsora lini* and of resistance in flax led Flor to propose the gene-for-gene hypothesis for specificity in host-pathogen interactions (8). Genetic analysis of *Erisyphe graminis* on wheat and barley (23), *Phytophthora infestans* on potato (9), *Venturia inaequalis* on apple (1,2) and *Bremia lactucae* on lettuce (15,17), among others, has supported the existence of a gene-for-gene relationship between these fungi and their respective hosts. Studies of the rice blast pathogen, *Magnaporthe grisea*, have demonstrated that host species and cultivar specificity are controlled by single genes that are inherited in a simple Mendelian fashion (13,29,30). In another study of *M. grisea*, genes involved in melanin biosynthesis that affect pathogenicity were identified and characterized (4). Detailed genetic analysis of *Nectria haematococca* has established the importance of the enzyme pisatin demethylase for expression of virulence on pea (11,25). Genetic analysis has also been used to identify single genes controlling host-specific toxins in *Cochliobolus heterostrophus* (31). Each of these discoveries required sexually fertile isolates of the pathogen and procedures for performing controlled crosses.

Colletotrichum graminicola (Ces.) Wils. causes anthracnose leaf blight and stalk, root, and kernel rots of corn; some isolates of the fungus infect other cereals including sorghum, wheat, barley, and oats (5,10,12,26–28). *C. graminicola* is typical of many foliar pathogens in the subdivision Deuteromycotina. Regulation of conidial development and appressorium formation in *C. graminicola*, as well as genetic determinants of host specificity, may be representative of the same processes in other important plant-pathogenic fungi. Although *C. graminicola* can be cultured easily under a variety of conditions and is well suited for physiological and biochemical studies in the laboratory and greenhouse, there have been no genetic studies using its sexual stage, *Glomerella graminicola* Politis. Presumably this is because the sexual stage is rare; it has never been found in nature and only one group has previously observed it in culture (19). The purpose of our study was to examine the sexual stage of *C. graminicola* and to determine its utility for gene transfer and genetic analysis.

MATERIALS AND METHODS

Fungal strains, culture, and selection of mutants. All strains of *C. graminicola* used in this study (Table 1) are derived from field isolates collected from maize. Medium for routine culture and germination of spores was Difco potato-dextrose agar amended with 100 mg/L of ampicillin sulfate (PDA-amp). Mutant phenotypes were determined on PDA-amp containing potassium chlorate (20 g/L), benomyl (500 ng/L), or uridine (5 mM). All cultures grown on agar medium were maintained under continuous fluorescent light (about 50 $\mu E/m^2/sec$) at 25 C.

TABLE 1. Strains of *Glomerella graminicola* used this study

Strain	Origin	Relevant phenotype[a]
M1.001	USA[b]	WT
M1.201	M1.001	Pyr^-
M1.301	M1.001	Bml^R
M1.401	M1.001	Chl^R
M1.501	M1.001	Mel^-
M1.502	M1.001	Mel^-
M2.001	North Carolina	WT
M3.001	Indiana	WT
M4.001	New York	WT
M5.001	Brazil	WT
M5.401	M5.001	Chl^R
M6.001	Brazil	WT
M7.001	Brazil	WT
M9.000	Indiana	WT
M9.001	M9.000	WT
M9.002	M9.000	WT
M9.401	M9.001	Chl^R
M9.402	M9.002	Chl^R
M10.001	Indiana	WT
M11.001	Illinois	WT

[a] WT = wild type; Pyr^- = pyrimidine auxotroph; Bml^R = benomyl resistant; Chl^R = chlorate resistant; Mel^- = melanin deficient.
[b] Precise origin unknown.

Benomyl-resistant (Bml^R) strain M1.301, formerly CgM2BmlR3 (18), melanin-deficient (Mel^-) mutants M1.501 and M1.502, formerly M5001 and M5002, respectively (21), and the pyrimidine auxotroph (Pyr^-) M1.201, formerly M2001 (22) were described previously. A modification of the procedure by Puhalla and Speith (20) was used to recover chlorate-resistant (Chl^R) mutants. The chlorate-sensitive (Chl^S) parental strains were first grown on minimal medium (2% glucose, 0.1% calcium nitrate, 0.02% potassium monophosphate, 0.025% magnesium sulfate · $7H_2O$, 0.015% sodium chloride, 1.5% Difco Bacto agar), and then a piece of agar (about 1 cm^2) was taken from the advancing edge of a colony and transferred to minimal medium containing potassium chlorate (2%) and glutamate (0.16%). Spontaneous Chl^R sectors usually appeared and these were used for monoconidial isolations of Chl^R strains.

Formation of perithecia and matings. Perithecia were formed on detached, nonsenescent leaves of the maize (*Zea mays* L.) inbred Mo940. Only the first, second, and third leaves (excluding the primordial leaf) were used from 5- to 6-wk-old plants (five- to six-leaf stage) that had been grown in 10-cm pots in the greenhouse. Three leaf pieces (i.e., basal sections 5–8 cm long, including a portion of the leaf sheath 0.5–1.0 cm long) were placed with the adaxial surface down on a layer of undyed aquarium gravel in a glass petri dish (100 mm diameter). Distilled water (about 10 ml) was added to each petri dish, and the dishes were autoclaved for 30 min. Leaves were inoculated with small (1–2 mm^2) agar plugs containing mycelium from the advancing edge of colonies grown on PDA-amp. In the case of a single strain, inoculum was placed in the center of the leaf section. For matings, parental strains were placed about 1 cm from the edge at opposite ends of the leaf. Dishes containing the inoculated leaves were then kept under continuous fluorescent light (about 25 $\mu E/m^2/sec$) at 18–20 C for 18–25 days. Sterile water was replenished as needed.

Collection of ascospores and analysis of marker segregation. Droplets containing ascospores were recovered from mature perithecia with drawn-out 100-μl glass micropipettes. Ascospore droplets were immediately delivered into 200 μl of sterile distilled water, and the suspension was agitated on a vortex mixer for 10–20 sec and then spread onto a single petri dish containing PDA-amp (or PDA-amp plus uridine in crosses involving Pyr^- strains). After 2 days, individual colonies were transferred to the

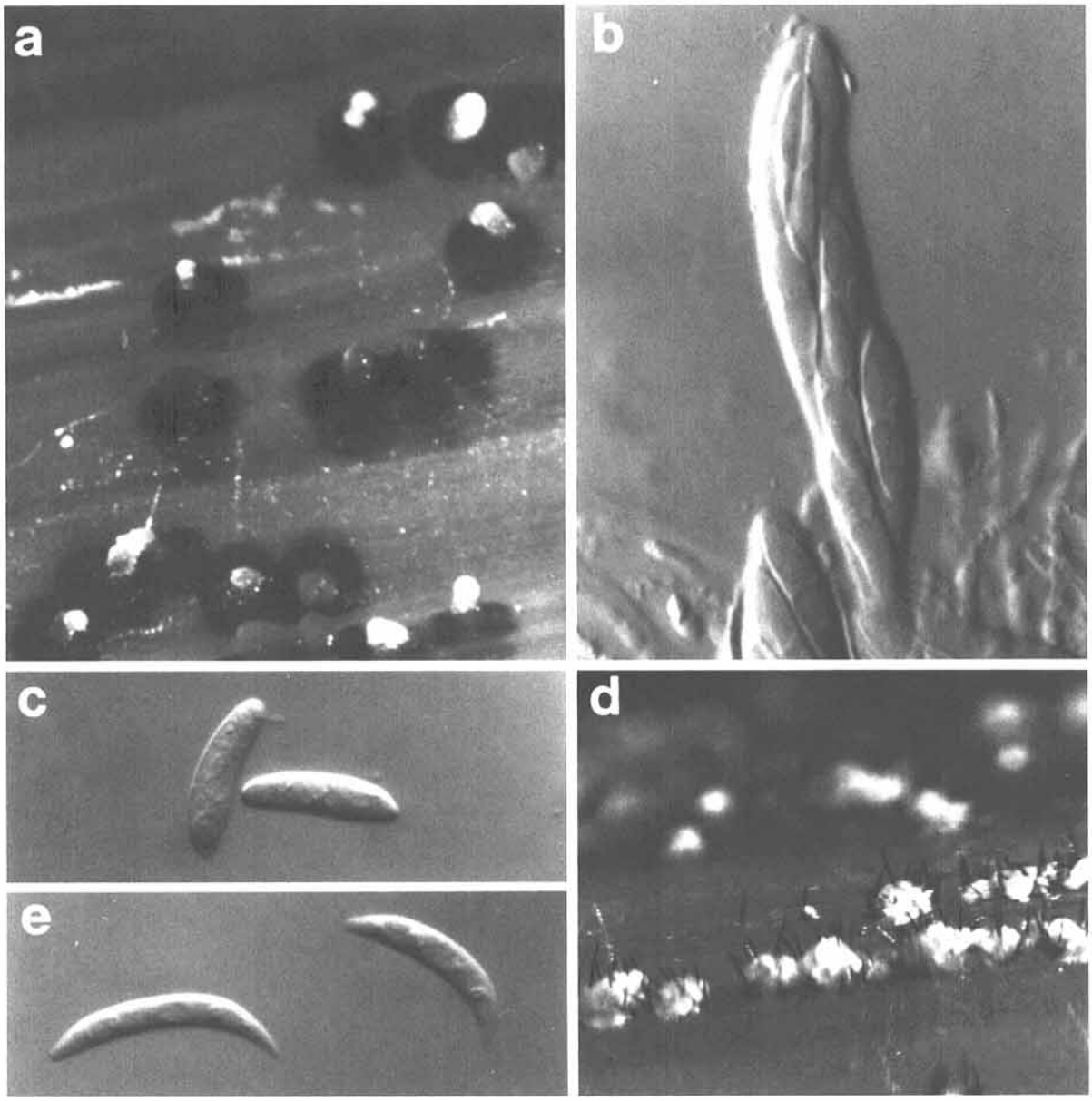

Fig. 1. Sexual and asexual fruiting bodies and spores produced by *Glomerella graminicola (Colletotrichum graminicola).* **A,** Perithecia. The white and lighter shaded apices are ascospore droplets which have begun to desiccate. ×70. **B,** Ascus. ×660. **C,** Ascospores. ×720. **D,** Acervuli. ×70. **E,** Conidia. ×720.

appropriate selective medium and their phenotypes were determined 2 to 3 days later.

RESULTS

Production and characteristics of perithecia and ascospores. M9.000 and the monoascospore siblings M9.001 and M9.002 derived from M9.000 were each capable of producing fertile perithecia (Fig. 1A–C) on autoclaved corn leaves. The number of perithecia was greater when strains M9.001 and M9.002 were grown on corn leaves together than if either strain was grown separately. Production of perithecia was always erratic; usually only one or two of every three leaves inoculated supported development of perithecia, and the numbers of perithecia produced on those leaves varied widely. All isolates (Table 1) produced acervuli bearing conidia and setae characteristic of *C. graminicola* (16,24) when grown on autoclaved corn leaves (Fig. 1D and E).

Typically, perithecia were black, immersed in the substrate, and without associated bristles or setae (Fig. 1A). When M9.001 and M9.002 were placed at opposite ends of a corn leaf, perithecia were concentrated at the junction of the two colonies. In some cases, a double line of perithecia was observed. Perithecia were 160–400 μm (mean 241 μm) in height and 130–350 μm (mean 221 μm) in diameter.

Acervuli were usually not produced on areas of leaves that contained perithecia. Therefore, the ascospores in perithecia were easily sampled without contamination by conidia. The accuracy of this recovery procedure was verified by routine microscopic analysis of samples that indicated the presence of ascospores but no conidia. Asci were 55–92 μm long (mean 76 μm) and 7–10 μm wide (mean 9 μm) (Fig. 1B). As many as 200 individual ascospores (Fig. 1C) could be recovered from a mature perithecium in a salmon-colored mass 18–25 days after inoculation of corn leaves. Ascospores were easily distinguished from conidia by their size and shape (compare Fig. 1C and E). Ascospores were hyaline, single celled, and varied in shape from cylindrical to curved. They were 13–28 μm long (mean 21 μm) and 2–7 μm wide (mean 5 μm). Single ascospores gave rise to colonies on PDA-amp that produced conidiomata typical of *C. graminicola*; mycelium and monoconidial isolates from these colonies gave rise to both acervuli and perithecia when used to inoculate pieces of autoclaved corn leaves.

Interfertility. Strains other than those derived from M9.000 (Table 1) never produced fertile perithecia when grown alone on autoclaved corn leaves, although M1.001 sometimes developed structures that resembled immature perithecia. These structures did not contain asci but contained numerous spherical droplets of various sizes. However, when self-sterile strains were paired with M9.001, M9.002, or with one another, most combinations tested produced fertile perithecia at the junction of the parental colonies (Table 2), and ascospores from these perithecia showed marker segregation (data not shown). When Mel$^-$ strain M1.502 was paired with other strains, most combinations produced both light-brown and black perithecia; in two pairings, only the light-brown perithecia were produced (Table 2). The Mel$^-$ phenotype segregated among ascospore progeny from both light-brown and black perithecia (data not shown).

Segregation of single markers. Crosses were made between M9.001 and M9.402, a Chl^R mutant derived from the self-fertile strain M9.002. Perithecia developed at the junction of the parental colonies and chlorate resistance segregated among the progeny fitting a 1:1 ratio by a chi-square goodness of fit test (Table 3). Under identical conditions with Chl^S parents (i.e., strains M9.001 and M9.002), no Chl^R progeny were recovered among 200 ascospores from a total of four perithecia.

Perithecia also formed at the junction of the parental colonies in crosses between M9.002 and M1.301, a Bml^R mutant derived from the self-sterile strain M1.001. Benomyl resistance segregated 1:1 among the progeny (Table 4). No Bml^R progeny were recovered from control crosses between M9.001 and M9.002, or M9.002 and M1.001.

Table 5 shows results from other crosses involving single markers in strains derived from the self-sterile strain M1.001.

TABLE 2. Interfertility of various *Glomerella graminicola* strains

	Parent 1		
Parent 2	M9.402	M1.502	M5.401
M1.001	+[a]	NA[b]	+
M2.001	+	ND[c]	+
M3.001	+	+ (BR)[d]	+
M4.001	+	+	+
M5.001	+	+	NA
N6.001	–[e]	ND	ND
M7.001	+	+	ND
M10.001	+	+	+
M11.001	+	+ (BR)	ND

[a] Interfertile.
[b] Not applicable.
[c] Not determined.
[d] Brown perithecia only.
[e] Not interfertile.

TABLE 3. Segregation of chlorate resistance among progeny from six perithecia from matings between *Glomerella graminicola* strains M9.402 (Chl^R)[a] and M9.001 (Chl^S)[b]

Perithecium	Progeny	Chl^R	Chl^S
1	50	34	16
2	26	15	11
3	50	23	27
4	50	26	24
5	50	25	25
6	24	14	10
Total[c]	250	137	113

[a] Chlorate-resistant phenotype.
[b] Chlorate-sensitive phenotype.
[c] χ^2 for a 1:1 ratio = 2.304; probability of a greater χ^2 = 0.129.

TABLE 4. Segregation of benomyl resistance among progeny from eight perithecia from matings between *Glomerella graminicola* strains M1.301 (Bml^R)[a] and M9.002 (Bml^S)[b]

Perithecium	Progeny	Bml^R	Bml^S
1	48	23	25
2	96	59	37
3	96	43	53
4	96	40	56
5	48	16	32
6	17	8	9
7	34	20	14
8	88	40	48
Total[c]	523	249	274

[a] Benomyl-resistant phenotype.
[b] Benomyl-sensitive phenotype.
[c] χ^2 for a 1:1 ratio = 1.195; probability of a greater χ^2 = 0.274.

TABLE 5. Segregation of single markers among progeny from various crosses of *Glomerella graminicola*

Parents	Total progeny	Total WT[a]	χ^{2b}	P^c
M1.301 (Bml^R) × M9.001	240	120	...	
M1.501 (Mel$^-$) × M9.002	159	73	1.06	0.303
M1.502 (Mel$^-$) × M9.001	353	189	1.77	0.183
M1.201 (Pyr$^-$) × M9.002	200	126	13.52[d]	0.0002
M1.401 (Chl^R) × M9.001	654	279	14.09	0.0002
M1.401 (Chl^R) × M9.002	768	346	7.52	0.006

[a] Wild type.
[b] χ^2 for 1:1 ratios.
[c] Probability of a greater χ^2.
[d] χ^2 for a 2:1 ratio (Pyr$^+$:Pyr$^-$) = 1.22; probability of a greater χ^2 = 0.269.

Benomyl resistance again segregated 1:1 when M1.301 was crossed with M9.001, and the Mel$^-$ phenotype segregated 1:1 when strains M1.501 and M1.502 were crossed with M9.002 and M9.001, respectively. Crosses involving strains M1.201 and M1.401 did not show a 1:1 segregation of their respective Pyr$^-$ and Chl^R phenotypes.

Additional crosses were made in which both parents carried a detectable marker. When M1.502 was crossed with either M9.401 or M9.402, and when M1.301 was crossed with M9.402, the parental markers segregated independently giving all possible combinations in a ratio of 1:1:1:1 (Table 6). In crosses between M1.201 and M9.402, the markers segregated to yield all possible combinations, but the pattern of segregation deviated significantly from a 1:1:1:1 ratio (Table 6).

Construction and analysis of strains with multiple markers. Two out of 20 Mel$^-$ progeny from a cross between M1.502 and M9.002 produced fertile perithecia when tested alone on autoclaved corn leaves. All ascospores recovered from these perithecia gave rise to the Mel$^-$ phenotype. One of these self-fertile Mel$^-$ strains was crossed with the Pyr$^-$ strain M1.201 and a total of 192 progeny were analyzed. Fifty-four progeny were wild type for both characters, 61 were Mel$^-$ Pyr$^+$, 34 were Mel$^+$ Pyr$^-$, and 43 had the Mel$^-$Pyr$^-$ double-mutant phenotype. After testing 15 of the double mutants, one was found to be self-fertile, and it was crossed with strain M1.301. Results from the cross showed that each marker segregated independently, and that the numbers of progeny in each phenotypic class conformed to a 1:1:1:1:1:1:1:1 ratio (Table 7).

DISCUSSION

Fertility and sexuality. The behavior of our strains confirms that *C. graminicola* has a sexual stage. Although M9.001 and M9.002 are both homothallic, production of perithecia is greater if they are grown in combination with one another, or if either is combined with certain self-sterile strains. This is similar to *Glomerella cingulata,* in which ascospore progeny from homothallic field isolates preferred to mate with one another rather than self-fertilize (14). The studies of *G. cingulata* also indicated that ascospore isolates that preferentially outcrossed could usually be distinguished from one another based on characteristics of colony morphology and habit. Although ascospore progeny recovered from the original field isolate M9.000 exhibited at least two distinct colony morphologies on PDA-amp, these were not comparable to the types described for *G. cingulata*, and they seemed not to be correlated with the ability of the strains to cross with other progeny having similar or different morphologies (data not shown).

Edgarton (6) described the ability of a hermaphroditic self-fertile strain of *G. cingulata* to cross with a self-sterile strain. The self-fertile and self-sterile strains were referred to as "plus" and "minus" strains, respectively. Our results with *C. graminicola* are similar and clearly show that self-sterile strains will cross with self-fertile ones. We also found that some self-sterile strains of *C. graminicola* will cross with one another. Therefore, genetic analysis may be possible for many isolates, regardless of whether they display self-fertility. In addition, the finding that self-sterile strains cross with one another indicates that sexual recombination may occur in nature.

The fertility of strains described in our study and in studies of *G. cingulata* (6,14) lead us to question the origin and genetic basis of self-sterility in *Glomerella* spp. Whether sterility is common among naturally occurring isolates of *C. graminicola*, or is a consequence of mutation and selection during repeated subculture, may be addressed by screening larger numbers of isolates for the presence of fertility. The question may also be addressed by direct genetic analysis of strains that differ in fertility.

In many ascomycetes, the perithecial wall consists entirely of tissue from the female parent (7). Crosses involving Mel$^-$ strain M1.502 usually resulted in the normal black perithecia and a type that was light-brown. Both types appeared identical except for color, and progeny recovered from both types segregated normally for the Mel$^-$ phenotype. Evidence based on restriction fragment length polymorphisms of mitochondrial DNA indicates that the light-brown perithecia occur when the Mel$^-$ mutant serves as the female parent (L. J. Vaillancourt and R. M. Hanau, *unpublished*). Apparently, since both types of perithecia are produced, the crosses between M1.502 and most of the other strains tested are reciprocal. However, in two pairings, only light-brown perithecia were produced, suggesting that some strains may behave only as males in combination with M1.502 (Table 2).

Marker segregation. Large numbers of progeny could be collected and tested for segregation of traits with relative ease. The results indicate that the Bml^R and Mel$^-$ phenotypes are each probably due to mutation in single genes. Segregation of chlorate resistance in crosses involving strain M1.401 did not fit a 1:1 ratio (Table 5), although crosses involving M9.402 did (Table 3). This discrepency may be an artifact since the sampling size from crosses involving M9.402 was smaller than that from crosses involving M1.401 (compare Tables 3 and 5). Indeed, when additional data (not shown) from other crosses involving M9.402 are pooled (more than 1,000 progeny), the segregation of Chl^R and Chl^S phenotypes is similar to that observed with M1.401 and does not statistically fit a 1:1 ratio. Although not 1:1, the segregation did not fit another ratio better. It is possible that Chl^R progeny are more likely to survive, thus causing them to be recovered more frequently (3). It is also possible that chlorate resistance in M9.402 and M1.401 is controlled by two closely linked genes, either of which can confer resistance. If so, the additional Chl^R progeny may represent recombinants.

In crosses involving strain M1.201 (Table 5), the Pyr$^-$ phenotype also did not segregate 1:1 but statistically did fit a 2:1 segregation (Pyr$^+$:Pyr$^-$). Crosses performed in the construction of the Mel$^-$ Pyr$^-$ double mutant showed the same aberrant segregation of the Pyr$^-$ phenotype. These results may be explained by higher mortality of progeny with the Pyr$^-$ phenotype (3). However, the constancy of the 2:1 ratio suggests that there may be another gene segregating in these crosses, one allele of which

TABLE 6. Segregation of two markers among progeny from various crosses of *Glomerella graminicola*

Parents	Number of progeny in each phenotypic class	χ^2[a]	P[b]
M9.401 (Chl^R) × M1.502 (Mel$^-$)	31 Chl^S Mel$^+$, 44 Chl^S Mel$^-$, 38 Chl^R Mel$^+$, 41 Chl^R Mel$^-$	2.41	0.492
M9.402 (Chl^R) × M1.502 (Mel$^-$)	149 Chl^S Mel$^+$, 153 Chl^S Mel$^-$, 190 Chl^R Mel$^+$, 161 Chl^R Mel$^-$	6.30	0.098
M9.402 (Chl^R) × M1.301 (Bml^R)	75 Chl^S Bml^S, 56 Chl^S Bml^R, 80 Chl^R Bml^S, 77 Chl^R Bml^R	4.91	0.179
M9.402 (Chl^R) × M1.201 (Pyr$^-$)	15 Chl^S Pyr$^+$, 143 Chl^S Pyr$^-$, 292 Chl^R Pyr$^+$, 18 Chl^R Pyr$^-$	440.21	0

[a] Accept 1:1:1:1 only for $\chi^2 < 7.81$.
[b] Probability of a greater χ^2 for a 1:1:1:1 ratio.

TABLE 7. Segregation of three markers among progeny from a cross of *Glomerella graminicola*

Parental phenotypes	Phenotypes and number of progeny	
Mel$^-$Pyr$^-$ Bml^s × Mel$^+$ Pyr$^+$ Bml^R	Mel$^+$ Pyr$^+$ Bml^S	12
	Mel$^+$ Pyr$^+$ Bml^R	8
	Mel$^+$ Pyr$^-$ Bml^R	6
	Mel$^-$ Pyr$^-$ Bml^R	3
	Mel$^-$ Pyr$^-$ Bml^S	9
	Mel$^-$ Pyr$^+$ Bml^S	12
	Mel$^+$ Pyr$^-$ Bml^S	9
	Mel$^-$ Pyr$^+$ Bml^R	7
	Total	66[a]

[a] χ^2 for a 1:1:1:1:1:1:1:1 ratio = 7.69; probability of a greater χ^2 = 0.361.

is lethal when in combination with the *pyr*$^-$ gene. Thus, 50% of the expected Pyr$^-$ progeny would be lost. We have begun to apply tetrad analysis so that we can determine the genetic basis of this interesting phenomenon.

Crosses involving two markers generally resulted in segregation patterns indicating unlinked genes. The exception was the cross between M9.402 and M1.201. The results of this cross indicate that genes for Pyr$^-$ and Chl^R are closely linked (Table 6). The 2:1 segregation of the Pyr$^-$ phenotype was also observed in this cross and, because the two loci are linked, segregation of the Chl^R phenotype also approximated a 2:1 ratio. Because of the aberrant segregation pattern, it is not possible based on these data to calculate a precise map distance between these loci, although it is approximately seven units.

Crosses resulting in the construction of a self-fertile strain having the Mel$^-$ and Pyr$^-$ markers, and crosses between this strain and M1.301, demonstrate the feasibility of transferring traits of interest between different genetic backgrounds (Table 7). Furthermore, data from these crosses suggest that the genes responsible for the Mel$^-$, Pyr$^-$, and Bml^R phenotypes are unlinked (Table 7).

Based on our observations that certain traits segregate and recombine in a Mendelian fashion, we conclude that *C. graminicola* is amenable to sexual gene transfer and genetic analysis. Potential application of this methodology includes backcrosses to obtain isogenic strains differing only in a particular trait of interest. In addition to strain construction, genetic crosses can be used to determine the number of genes responsible for a particular phenotype of interest, their relationship to one another, and their position on a linkage map relative to known markers. Information of this kind can be extremely useful in situations when transformation is to be used to clone a gene by complementation. Hence, the ability to perform transmission genetics and to conduct genetic analyses should expedite future research employing *C. graminicola* as a system to study determinants of pathogenicity.

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