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Genetic Analysis of Melanin-Deficient, Nonpathogenic Mutants of *Magnaporthe grisea*

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The fungus *Magnaporthe grisea* causes rice blast disease and gray leaf spot disease of other grasses. Numerous *M. grisea* mutants that fail to produce the dark gray pigment typical of wild-type mycelia have been isolated and analyzed. Three classes of mutants have been distinguished based on pigmentation phenotypes: albino (Alb$^-$), rosy (Rsy$^-$), and buff (Buf$^-$). Some pigment mutants were recovered following mutagenesis, and others appeared spontaneously. Spontaneous Buf$^-$ mutants have been particularly common. Genetic analysis has shown that the three mutant phenotypes are due to single gene defects at unlinked loci. Genetic crosses have yielded the three possible classes of double mutants. Analysis of the double mutants has revealed epistasis relationships: *alb*$^-$ *rsy*$^-$ and *alb*$^-$ *buf*$^-$ mutants are Alb$^-$, whereas *rsy*$^-$ *buf*$^-$ mutants are Rsy$^-$. These epistasis relationships are consistent with the order of function *ALB*$^+$ → *RSY*$^+$ → *BUF*$^+$ in melanin biosynthesis. All the pigment mutants tested failed to infect intact host plants, but the same mutants successfully infected plants that had been wounded by abrading the leaf epidermis. When scytalone, an intermediate in the biosynthetic pathway leading to dihydroxynaphthalene-based fungal melanins, was added to the growth medium, petri plate cultures of *alb*$^-$ mutants, but not *rsy*$^-$ or *buf*$^-$ mutants, darkened noticeably. When scytalone was incorporated in spore suspensions sprayed onto unwounded host plants, pathogenicity was restored to *alb*$^-$, but not to *rsy*$^-$ or *buf*$^-$ mutants.

The ascomycete *Magnaporthe grisea* (Hebert) Barr (anamorph, *Pyricularia oryzae* Cav. or *P. grisea*) causes a devastating fungal disease of rice plants known as rice blast disease (Ou 1985). The fungus causes a similar disease, known as gray leaf spot, on many grasses other than rice. Fungi produce a variety of dark pigments known generically as melanins (reviewed by Bell and Wheeler 1986). *M. grisea* produces a distinctive gray pigment, an example of the class of fungal melanins produced by polyketide biosynthesis, ending in polymerization of 1,8-dihydroxynaphthalene (DHN). DHN melanin has been implicated as a pathogenicity factor in some fungal plant diseases and animal mycoses (Kubo *et al.* 1982; Woloshuk *et al.* 1983; Wolkow *et al.* 1983; Geis *et al.* 1984; Wheeler and Stipanovic 1985), as reviewed by Wheeler and Bell (1987).

Rice blast disease can be controlled by the application of fungicides, such as tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole; Froyd *et al.* 1976), which do not significantly affect growth of the fungus, but interfere with DHN melanin biosynthesis (Tokousbalides and Sisler 1978; Woloshuk *et al.* 1980a; Woloshuk and Sisler 1982; Woloshuk *et al.* 1983; Wheeler and Greenblatt 1988). Tricyclazole blocks the *M. grisea* disease cycle by inhibiting the penetration step in which the fungus pierces the outer protective barriers of the host and gains entry into an epidermal cell (Woloshuk *et al.* 1983). This observation suggests a role for DHN melanin in the penetration process, which is executed by a specialized fungal cell known as the appressorium. DHN melanin is produced at detectable levels in a variety of fungal cell types, but the pigment is produced most abundantly just before penetration when a thick melanin layer is deposited in the inner appressorial cell wall. The role of DHN melanin in appressorial function has been the subject of considerable interest (Woloshuk *et al.* 1983; Wolkow *et al.* 1983; Sisler 1986).

Howard and Ferrari (1989) have now presented convincing evidence that DHN melanin mediates the buildup of hydrostatic pressure in the appressorium and that this high pressure provides the essential driving force for a mechanical penetration component. In part, their studies used mutants we describe here.

M. grisea offers many advantanges as an experimental system for molecular genetic investigation of host-pathogen interactions. The fungus grows on defined media, which facilitates, for example, the isolation of mutants with altered nutritional requirements, drug resistances, or pigmentation phenotypes (Crawford *et al.* 1986; Valent *et al.* 1986). Neufeld *et al.* (1958) and Latterell (1975) noted that *M. grisea* variants with buff pigmentation lacked pathogenicity. This observation was confirmed by Woloshuk *et al.* (1980b), who further determined that buff-pigmented variants were deficient in the melanin biosynthetic activity inhibited by tricyclazole (1,3,8-trihydroxynaphthalene [3HN] reductase). Such studies with buff variants did not include genetic analysis, which could determine, for example, whether or not melanin and pathogenicity defects are due to the same genetic lesions.

In this study, we report the isolation of three phenotypic classes of *M. grisea* mutants with altered pigmentation (albino, Alb$^-$; rosy, Rsy$^-$; and buff, Buf$^-$). All three classes of mutants were nonpathogenic, although they retained the ability to infect wounded host plants. The three

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phenotypes corresponded to mutations in each of three unlinked genes (*ALB1*$^+$, *RSY1*$^+$, and *BUF1*$^+$). Epistasis relationships indicated by the phenotypes of the double mutants were consistent with the order of function *ALB*$^+$ → *RSY*$^+$ → *BUF*$^+$. An intermediate in DHN melanin biosynthesis, scytalone, restored pigmentation and pathogenicity to *alb*$^-$ mutants, but not to *rsy*$^-$ or *buf*$^-$ mutants. These genetic results provide additional strong support for an essential role of DHN melanin in penetration of the host epidermis by *M. grisea* appressoria.

MATERIALS AND METHODS

Strains, media, and strain storage. The *M. grisea* field isolates and laboratory strains used in this study are listed in Table 1. The strain names of field isolates pathogenic on rice begin with the prefix "O-," except in cases in which a strain has been named in another laboratory (for example, Ken60-19). Most of the strains given a number preceded by the letters "CP" were isolated following mutagenesis. Strains designated by a three-part number are single ascospore progeny of genetic crosses; the first part of the strain number indicates the cross serial number, the second is the ascus number, and the third is the ascospore number. Strain names in which the ascus number has been replaced with "R" indicate random ascospore cultures.

Genetic nomenclature generally agrees with the recommendations of Yoder *et al.* (1986). Genotype designations are presented in italics (uppercase for wild type, lowercase for mutant forms). Allele numbers were assigned to independent *alb*$^-$, *rsy*$^-$, and *buf*$^-$ mutations in order of isolation (for example, *buf-1*, *buf-2*, and *buf-3*), and genetic locus numbers (for example, *BUF1*) were assigned when genetic analysis defined a gene. Phenotype designations are presented as an uppercase letter followed by two lowercase letters, followed by a superscript plus or minus sign (plus indicates wild type; for example, strains with an *rsy*$^-$ or an *rsy*$^-$ *buf*$^-$ genotype have an Rsy$^-$ phenotype).

Oatmeal agar, minimal medium, and complete medium have been described previously (Crawford *et al.* 1986; Valent *et al.* 1986). 2YEG medium consisted of 0.2% yeast extract and 1.0% glucose.

The fungus was stored in a nonmetabolizing state by freezing dried infected host tissue or cellulose filter paper disks (13-mm diameter, Schleicher and Schuell, Keene, NH, No. 597) on which the fungus had been cultured. In addition, key strains were stored at −70° C as mycelial fragment suspensions in 15% glycerol.

Isolation of mutants. Many pigment defective mutants were isolated following mutagenesis with UV light. Conidia were diluted to 1 × 10^6 per milliliter in sterile 0.025%

Table 1. *Magnaporthe grisea* strains used in this study

Designation	Mating type	Genotype, origin[a]	Designation	Mating type	Genotype, origin[a]
Ken60-19	*Mat1-2*	Rice pathogen, Japan (H. Yaegashi)	CP725	*Mat1-2*	*buf1-239*, UV, 4091-5-8
			281-9-2	*Mat1-1*	Wild-type laboratory strain
WGG-FA40	*Mat1-1*	Goosegrass pathogen, Japan (H. Yaegashi)	859-22-5	*Mat1-2*	Wild-type laboratory strain
			3597-17-5	*Mat1-2*	Wild-type laboratory strain
O-42	*Mat1-1*	Rice pathogen, Japan	4091-5-8	*Mat1-2*	Wild-type laboratory strain
O-111	*Mat1-1*	Rice pathogen, China	4134-11-2	*Mat1-2*	Wild-type laboratory strain
O-135	*Mat1-1*	Rice pathogen, China	4136-1-2	*Mat1-1*	Wild-type laboratory strain
O-137	*Mat1-2*	Rice pathogen, China	4136-4-3	*Mat1-1*	Wild-type laboratory strain
			4157-12-2	*Mat1-1*	*buf1-168*, Mut. Cross[b]
CP53	*Mat1-2*	*buf1-1*, SP, 859-22-5	4157-12-3	*Mat1-1*	*buf1-169*, Mut. Cross
CP60	*Mat1-1*	*buf1-2*, UV, 281-9-2	4157-22-2	*Mat1-2*	*buf1-170*, Mut. Cross
CP61	*Mat1-1*	*buf1-3*, UV, 281-9-2	4157-26-3	*Mat1-1*	*buf1-171*, Mut. Cross
CP62	*Mat1-1*	*buf1-4*, UV, 281-9-2	4170-1-3	*Mat1-1*	*arg3-12*
CP63	*Mat1-1*	*buf1-5*, UV, 281-9-2	4174-1-5	*Mat1-1*	*alb1-1*[c]
CP64	*Mat1-2*	*buf1-6*, UV, Ken60-19	4174-3-5	*Mat1-2*	*alb1-1*
CP65	*Mat1-2*	*buf1-7*, UV, Ken60-19	4175-2-1	*Mat1-2*	*alb1-2*
CP129	*Mat1-1*	*buf1-40*, UV, O-42	4175-2-2	*Mat1-1*	*alb1-2*
CP143	*Mat1-2*	Wild-type laboratory strain	4175-4-3	*Mat1-2*	*alb1-2*
CP159	*Mat1-2*	*buf1-49*, DEO, CP143	4176-5-3	*Mat1-1*	*alb1-3*
CP162	*Mat1-2*	*buf1-52*, DEO, CP143	4176-6-5	*Mat1-2*	*alb1-3*
CP220	*Mat1-1*	*buf1-39*, UV, O-42	4178-R-1	*Mat1-1*	*alb1-8*
CP280	*Mat1-2*	*alb1-1*, UV, 4091-5-8	4179-R-3	*Mat1-1*	*alb1-4*
CP283	*Mat1-2*	*alb1-2*, UV, 4091-5-8	4180-R-3	*Mat1-1*	*alb1-9*
CP412	*Mat1-1*	*alb1-5*, UV, O-42	4181-R-1	*Mat1-1*	*alb1-16*
CP413	*Mat1-1*	*alb1-6*, UV, O-42	4182-R-3	*Mat1-2*	*alb1-15*
CP461	*Mat1-2*	*buf1-138*, UV, 4091-5-8	4184-R-1	*Mat1-2*	*alb1-14*
CP471	*Mat1-2*	*buf1-148*, UV, 4091-5-8	4218-6-3	*Mat1-2*	*rsy1-1*
CP483	*Mat1-2*	*alb1-15*, UV, 4091-5-8	4218-6-5	*Mat1-1*	*rsy1-1 buf1-3*
CP485	*Mat1-2*	*rsy1-1*, UV, 4091-5-8	4218-6-6	*Mat1-2*	*rsy1-1*
CP718	*Mat1-1*	*buf1-234*, SP, O-42	4218-6-8	*Mat1-1*	*rsy1-1 buf1-3*

[a]For wild-type strains, the geographical origin is indicated. For mutant strains, the operation that yielded the mutant and the parent strain is indicated. SP indicates spontaneous appearance; UV indicates UV light mutagenesis; and DEO indicates diepoxyoctane mutagenesis (see text).
[b]Mut. Cross indicates spontaneous appearance among the progeny of a mutagenic cross (see text). Mutants appeared in a cross between 4134-11-2 and O-111, two strains that are wild-type for pigment production. Note that the mutants isolated from the same ascus, 4157-12-2 and 4157-12-3, may not be independent.
[c]Strain 4174-1-5 and those strains listed after 4174-1-5 were derived from genetic crosses of pigment mutants listed earlier in the table.

Tween 20. Five milliliters of spore suspension was stirred in a 3.5-cm diameter petri dish under a UV light for 4 min (Ultraviolet Products, San Gabriel, CA, Mineralight Model UVG-11, placed 9 cm above the conidial suspension). This treatment routinely killed approximately 95% of the conidia. Plating and testing survivors in these experiments typically yielded 0.35% auxotrophs. Other pigment mutants were isolated following mutagenesis with diepoxyoctane (DEO) as described by Rambosek and Kinsey (1983). Conidia were incubated for 90 min at room temperature in 100 mM phosphate-buffered DEO, a treatment that resulted in 0.01–0.1% survival. Many pigment mutants appeared spontaneously as papillae or sectors in mycelial cultures or as colonies on plates seeded with conidia or ascospores. All mutants were purified by propagating cultures from single conidia.

Genetic crosses. Crosses were performed by pairing strains of opposite mating type on oatmeal agar. Cross plates were incubated at 20° C under continuous cool-white fluorescent light. Asci containing viable ascospores appeared 13 to 21 days after the strains were paired. For tetrad analysis, asci were released from a perithecium using a pair of fine tweezers (A. Dumont & Fils Cie., Style 5, Biological). In fertile crosses, ascus walls typically lost their integrity about 30 min after the asci were released from the perithecium. The eight spores of an ascus were separated by hand on 2YEG medium solidified with 4.0% agar using a finely drawn glass needle and a 50× Wild stereomicroscope. In crosses between highly fertile strains, mature perithecia contained loose ascospores, facilitating random ascospore analysis. Germinated ascospores were transferred to complete medium in wells of Corning 25820 tissue culture plates for immediate storage as described by Crawford *et al.* (1986).

Pathogenicity tests. Conidia were collected from cultures growing on oatmeal agar plates by washing with a sterile 0.25% gelatin solution (Sigma, St. Louis, MO, Type IV from calfskin). Six rice (*Oryza sativa* L.) seedlings growing in a single 10-cm diameter plastic pot were inoculated at the four-leaf stage. Approximately 25 weeping lovegrass (WLG; *Eragrostis curvula* (Schrad.) Ness) or goosegrass (GG; *Eleusine indica* (L.) Gaertn.) seedlings growing in a 10-cm diameter pot were inoculated 2 wk after planting. Growth chamber conditions were 25° C with 70% relative humidity and 14 hr of light (ranging from 700 to 900 $\mu E \cdot m^{-2} \cdot s^{-1}$). Plants were grown in vermiculite and watered with a nutrient solution. A 4.0-ml suspension containing 5×10^5 conidia per milliliter was sprayed onto the plants using an artist's airbrush (Paasche No. 1; Paasche Airbrush Co., Harwood Heights, IL). Infected plants were then sealed inside a clear plastic bag and left in dim light for 24 hr at room temperature, about 22° C. The bags were removed and the plants were placed in the growth chamber. Infection responses were scored 7 days after inoculation as described by Valent *et al.* (in press).

WLG seed was obtained from Valley Seed Service, Fresno, CA. GG seed was obtained from Azlin Weed Seed Service, Leland, MS. Rice seed, cultivars M9 and M201, was generously provided by the California Cooperative Rice Research Foundation, Biggs.

In some experiments, plants were wounded before inoculation by abrading the leaves with an emery board. The melanin biosynthetic intermediate, scytalone (kindly provided by H. D. Sisler, University of Maryland, College Park), was added to the inoculum in some experiments at a final concentration of 20 μg/ml.

RESULTS

Pigment mutants isolated. We have identified and collected pigment mutants over a period of several years during the course of genetic studies involving a number of *M. grisea* strains. Petri plate cultures of wild-type *M. grisea* strains are gray, but a range in gray color intensity is noted from strain to strain. Three distinctive phenotypic classes of pigment mutants have been obtained: albino (Alb^-), rosy (Rsy^-), and buff (Buf^-). Mutants have been isolated from a number of different parent strains, including rice pathogens and pathogens of other grasses. Some mutants appeared spontaneously, and others were isolated following mutagenesis. Table 1 lists *M. grisea* strains used in these studies, including many, but not all, of the pigment mutants isolated.

Our collection includes 352 Buf^- mutants. The reddish-tan pigmentation of these mutants closely resembles that of wild-type strains grown in the presence of tricyclazole, which is known to inhibit the melanin biosynthetic enzyme, 3HN reductase, as shown by Woloshuk *et al.* (1980b). One hundred fifteen independently isolated Buf^- mutants were recovered following UV mutagenesis, and four were recovered following treatment with DEO. In most experiments, Buf^- mutants represented about 0.1 to 0.01% of the survivors of mutagenesis. Sixty-six independent Buf^- mutants occurred spontaneously as sectors or papillae on vegetatively growing cultures of the fungus. Buf^- mutants appeared at an extraordinarily high frequency (5–20%) among the progeny of some genetic crosses (see below; B. Valent, unpublished results). One hundred sixty-seven Buf^- mutants, 45 of which are clearly independent, were isolated as progeny of such genetic crosses. Thus, we have retained 233 spontaneous Buf^- mutants, but we have observed many more.

The Rsy^- class of pigment mutants is represented by a lone individual, strain CP485, which appeared as one of approximately 5,000 survivors of UV mutagenesis of strain 4091-5-8. The Rsy^- mutant secretes a rosy pigment into the medium. Rsy^- colonies growing on oatmeal agar at first appear white, but later develop a light tan appearance somewhat similar to Buf^- mutants.

The Alb^- class of pigment mutants includes 22 isolates. Seven mutants appeared spontaneously, and 15 were isolated following UV mutagenesis. Typically, 0.05–0.01% of UV-mutagenized colonies were Alb^- mutants. Spontaneous Alb^- mutants have occasionally appeared as papillae in petri plate cultures of wild-type strains. However, spontaneous Alb^- mutants are far more rare than Buf^- mutants. Alb^- mutants produce conidia normally and undergo genetic crosses with a proficiency equivalent to that of their parents. Alb^- mutants form white colonies, secrete a yellow pigment when grown on oatmeal agar,

and produce pale perithecia when they mate as females in genetic crosses. These properties, together with genetic segregation data (see results below), distinguish Alb$^-$ mutants from nonpigmented, nonconidia-producing "degraded" cultures of *M. grisea* generated by multiple rounds of subculturing in the laboratory (Valent *et al.* 1986).

Frequency of appearance and stability of Buf$^-$ mutants. Because Buf$^-$ mutants were common, we attempted to measure the frequency with which Buf$^-$ mutants arose and reverted to wild type. Table 2 shows the frequency of appearance of Buf$^-$ mutants for several strains. Spontaneous Buf$^-$ mutants were common during vegetative growth with a Chinese rice pathogen, O-137; about 0.25% of the conidia taken from O-137 cultures gave rise to Buf$^-$ colonies. A second rice pathogen from the same area of China, O-135, did not give rise to spontaneous Buf$^-$ mutants at the same high frequency as O-137 during vegetative growth. Unexpected Buf$^-$ mutant progeny were common in some genetic crosses between wild-type strains. Crosses between O-137, O-135, or O-111 (all Chinese rice pathogen field isolates; see Table 1) and any of several standard laboratory strains that infect grasses other than rice (all wild-type for pigment production) resulted in Buf$^-$ progeny at a frequency of 5–20% (B. Valent, unpublished results). Crosses between WGG-FA40 or 859-22-5 and the same laboratory strains did not produce Buf$^-$ progeny. Similarly, crosses involving two Japanese rice pathogens, Ken60-19 and O-42, did not produce Buf$^-$ progeny.

Three Buf$^-$ mutants were tested for spontaneous or UV-induced reversion to wild type, as shown in Table 3. Conidia were plated on minimal medium with 3% sorbose, which promotes colonial growth (Crawford *et al.* 1986). No gray revertants were detected for a spontaneous (CP53), a UV-induced (CP62), or a DEO-induced (CP159) Buf$^-$ mutant. DEO mutagenesis is known to result in deletion mutations (Rambosek and Kinsey 1983). Thus, DEO-induced mutants might be expected to be stable. In control experiments, wild-type conidia were mixed with excess Buf$^-$ mutant conidia and plated on minimal sorbose agar medium. In such experiments, one gray colony could easily be identified on a plate with 1,000 Buf$^-$ colonies. We conclude that Buf$^-$ mutants are relatively stable. During the course of many genetic experiments involving a variety of Buf$^-$ mutants, we have never observed a wild-type revertant, even though similar experiments have, from time to time, yielded possible revertants of Alb$^-$ mutants (see below).

Genetic analysis of pigment mutants. To determine the genetic basis of the pigmentation defects, Alb$^-$, Rsy$^-$, and Buf$^-$ mutants were crossed to strains with wild-type pigmentation, and segregation of the melanin-deficient phenotypes was scored among the progeny. Ten of the Alb$^-$ mutants, the lone Rsy$^-$ mutant, and 10 Buf$^-$ mutants were crossed in this manner. In all cases, mutant and wild-type phenotypes segregated 1:1 among the progeny, indicating the presence of single gene mutations in the pigment mutants. We previously reported single gene segregation of Buf$^-$ pigment defects (Crawford *et al.* 1986). None of the pigment mutants that we studied had a complex or epigenetic basis.

Typically, pigment mutants caused no visible symptoms when inoculated on rice, WLG, or GG (Fig. 1). Progeny from several crosses between pigment mutants and wild-type strains were tested, along with their parents, for pathogenicity. For example, wild-type strain 4136-4-3 (a WLG pathogen) was mated in separate crosses with strain CP471 (*buf-148*), with strain CP483 (*alb-15*), and with

Table 2. Frequency of Buf$^-$ mutants

Parent strain	Treatment	Buf$^-$ colonies per total colonies	Frequency of Buf$^-$
WGG-FA40	UV	4/36,750	1.1×10^{-4}
	Spontaneous	0/23,760	$<4.2 \times 10^{-5}$
859-22-5	UV	4/33,250	1.2×10^{-4}
	Spontaneous	0/8,625	$<1.2 \times 10^{-4}$
O-135	Spontaneous	0/2,075	$<4.8 \times 10^{-4}$
O137	Spontaneous	6/2,388	2.5×10^{-3}

Table 3. Reversion of Buf$^-$ mutants[a]

Buf$^-$ mutant strain	Treatment	Gray colonies per total colonies	Buf$^-$ reversion frequency
CP53 (*buf-1*)	Spontaneous	$0/6.0 \times 10^5$	$<1.6 \times 10^{-6}$
	UV	$0/4.2 \times 10^5$	$<2.4 \times 10^{-6}$
CP62 (*buf-4*)	Spontaneous	$0/7.0 \times 10^5$	$<1.4 \times 10^{-6}$
	UV	$0/5.0 \times 10^5$	$<2.0 \times 10^{-6}$
CP159 (*buf-49*)	Spontaneous	$0/1.1 \times 10^6$	$<9.0 \times 10^{-7}$
	UV	$0/3.3 \times 10^5$	$<3.0 \times 10^{-6}$

[a]For each strain, 10 independent cultures were started on oatmeal agar using single conidia as inoculum. Conidia harvested from those cultures were plated on minimal 3% sorbose agar medium at a density of approximately 1,000 viable spores per plate. After one week of incubation, pigmentation of the colonies was observed.

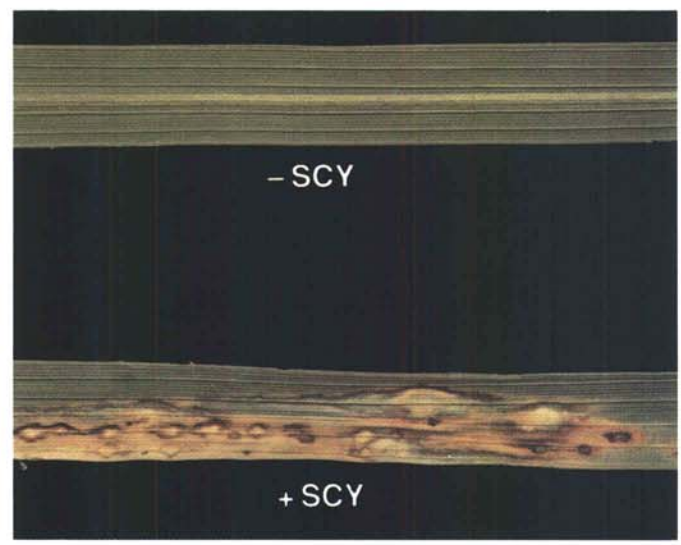

Fig. 1. Rice leaves inoculated with conidia of a melanin-deficient albino mutant. Rice seedlings at the three-leaf stage were inoculated with spores of the Alb$^-$ mutant CP412 (*alb1-5*), as described in the text. The third leaf is shown 7 days after infection for a seedling inoculated in the absence (−SCY; top) or in the presence (+SCY; bottom) of 20 μg/ml of scytalone (see text). In the presence of scytalone, *alb1-5* spores caused standard rice blast lesions, as shown. Typically, *alb*$^-$, *buf*$^-$, and *rsy*$^-$ pigment mutants caused no visible symptoms when inoculated on unwounded host plants in the absence of melanin biosynthetic intermediates.

strain CP485 (*rsy-1*). These three pigment-deficient mutants were derived from strain 4091-5-8, which is also a WLG pathogen. From each cross, 10 full tetrads were tested for pigment production and pathogenicity toward WLG. In each tetrad, four progeny were gray and pathogenic, and four progeny were pigment deficient and nonpathogenic. Thus, nonpathogenicity and lack of melanin production appear to be due to the same single gene defect.

Tests of complementation and allelism. We have previously shown that heterokaryons formed between *M. grisea* strains can be used for complementation testing of nutritional mutants (Crawford *et al.* 1986). However, heterokaryons formed in the usual manner between pigment mutants have not been useful for complementation tests. For example, heterokaryons formed between various Buf$^-$ and Alb$^-$ mutants were not gray, even though genetic analysis of the mutants indicated that they should complement. Failure to detect complementation may be due to poor diffusion of melanin biosynthetic intermediates and to the nature of *M. grisea* heterokaryons in which relatively rare heterokaryotic cells lag behind growing hyphal tips (Crawford *et al.* 1986).

A number of *buf*$^-$ and *alb*$^-$ mutations were tested for allelism. No wild-type recombinants appeared among the progeny of crosses in which 12 *buf*$^-$ mutations (11 of which were clearly independent) were tested in various pairwise combinations (Table 4), indicating that these mutations are all tightly linked. Similarly, intercrosses between strains carrying nine independent *alb*$^-$ mutations yielded no or very few wild-type recombinants (Table 5), again indicating linkage. The wild-type progeny reported in Table 5 may have arisen by intragenic recombination between the *alb-1* and *alb-3* mutations. The results of these crosses lead us to suggest that the *buf*$^-$ mutations define a single gene, *BUF1*, and the *alb*$^-$ mutations define a gene, *ALB1*.

Linkage and epistasis relationships between mutations that block melanin biosynthesis. To determine linkage relationships between the *ALB1*, *RSY1*, and *BUF1* genes, we conducted genetic crosses between strains carrying the mutations indicated in Table 6. Tetrad analysis of the progeny from these crosses showed that the three genes are not linked to one another, nor to the mating type locus, *Mat1*. The aberrant ("odd") tetrads indicated for the *buf-4* × *alb-1* cross were tetrads in which gene conversion events may have occurred at the *ALB1* locus, resulting in only two Alb$^-$ progeny.

The nonparental ditype (NPD) tetrads indicated in the first three lines of Table 6 each contained four wild-type gray spores and four spores that could be expected to carry both of the pigment mutations indicated. We examined the pigment phenotypes of these double mutants to determine epistasis relationships: *rsy-1 buf-3* double mutants appeared Rsy$^-$, *alb-1 buf-4* double mutants appeared Alb$^-$, and *alb-1 rsy-1* double mutants appeared Alb$^-$

To confirm the phenotypes and genotypes of the double mutants, we conducted genetic analysis of progeny contained in tetratype (T) tetrads indicated in Table 6. For example, tetrad 4218-6, from a cross between *rsy-1* and *buf-3* parents, contained two gray, two Buf$^-$, and four Rsy$^-$ progeny. No phenotypic differences could be detected between the four Rsy$^-$ progeny, even though two of them could be expected to carry the *rsy-1* mutation and two could be expected to carry both the *rsy-1* and *buf-3* mutations. All four of the Rsy$^-$ progeny were crossed with wild-type tester strains, and the pigment phenotypes of the progeny were determined as shown in Table 7. As we expected, two of the crosses yielded only gray and rosy progeny, but the other two yielded gray, rosy, and buff progeny, indicating the presence of the *buf-3* mutation in the double mutants. In a similar fashion, we confirmed that *rsy-1 buf-4* and *rsy-1 buf-239* double mutants had the

Table 4. Crosses to test allelism of *buf*$^-$ mutations

Parent 1[a]	Parent 2[b]	Gray progeny	Total progeny
CP60 (*buf-2*)	CP53 (*buf-1*)	0	400
CP61 (*buf-3*)	CP53 (*buf-1*)	0	400
CP62 (*buf-4*)	CP53 (*buf-1*)	0	400
CP63 (*buf-5*)	CP53 (*buf-1*)	0	400
CP60 (*buf-2*)	CP159 (*buf-49*)	0	400
CP62 (*buf-4*)	CP162 (*buf-52*)	0	400
CP62 (*buf-4*)	CP461 (*buf-138*)	0	500
4157-12-2 (*buf-168*)	CP461 (*buf-138*)	0	300
4157-12-3 (*buf-169*)	CP461 (*buf-138*)	0	300
CP62 (*buf-4*)	4157-22-2 (*buf-170*)	0	400
4157-26-3 (*buf-171*)	CP461 (*buf-138*)	0	2,500

[a] All Parent 1 strains are *Mat1-1*.
[b] All Parent 2 strains are *Mat1-2*.

Table 5. Crosses to test allelism of *alb*$^-$ mutations

Parent 1[a]	Parent 2[b]	Gray progeny	Total progeny
4174-1-5 (*alb-1*)	4174-3-5 (*alb-1*)	0	400
4175-2-2 (*alb-2*)	4175-2-1 (*alb-2*)	0	400
4176-5-3 (*alb-3*)	4176-6-5 (*alb-3*)	0	400
4174-1-5 (*alb-1*)	4175-4-3 (*alb-2*)	0	400
4175-2-2 (*alb-2*)	4174-3-5 (*alb-1*)	0	400
4174-1-5 (*alb-1*)	4176-6-5 (*alb-3*)	1	400
4176-5-3 (*alb-3*)	4174-3-5 (*alb-1*)	1	400
4175-2-2 (*alb-2*)	4176-6-5 (*alb-3*)	0	400
4176-5-3 (*alb-3*)	4175-2-2 (*alb-2*)	0	400
4179-R-3 (*alb-4*)	4174-3-5 (*alb-1*)	0	800
4178-R-1 (*alb-8*)	4174-3-5 (*alb-1*)	0	600
4180-R-3 (*alb-9*)	4174-3-5 (*alb-1*)	0	600
4174-1-5 (*alb-1*)	4184-R-1 (*alb-14*)	0	250
4174-1-5 (*alb-1*)	4182-R-3 (*alb-15*)	0	400
4181-R-1 (*alb-16*)	4174-3-5 (*alb-1*)	0	400

[a] All Parent 1 strains are *Mat1-1*.
[b] All Parent 2 strains are *Mat1-2*.

Rsy$^-$ phenotype. The phenotypes and genotypes of *alb-1 rsy-1* and *alb-1 buf-3* double mutants were also verified by analogous genetic crosses.

Other genetic crosses have shown that the *RSY1*$^+$ gene is centromere-linked. In fungi with unordered tetrads, like *M. grisea*, centromere linkage is reflected in a deficiency of T tetrads for two markers, if both are centromere-linked. Previous genetic crosses showed that the *arg3-12* mutation, which abolishes ornithine carbamoyl transferase activity and causes arginine auxotrophy (Parsons *et al.* 1987), is tightly linked to a centromere (K. Parsons, B. Valent, and F. Chumley, unpublished data). In cross 4273, strain 4170-1-3 (*Mat1-1 arg3-12*) was mated with strain 4218-6-6 (*Mat1-2 rsy1-1*). Progeny were analyzed in 46 tetrads. The *ARG3 RSY1* gene pair segregated 20 PD:22 NPD:3 T (PD, parental ditype). These results do not permit precise determination of the map distance from *ARG3* or *RSY1* to the centromeres of the chromosomes on which they reside, but the data are consistent with an average map distance of about 1.6 centimorgans. Similar crosses showed no indication of centromere linkage for *BUF1* or for *ALB1*.

Table 6. Tetrad analysis of crosses between pigment mutants[a]

Markers analyzed	PD[b]	NPD[c]	T[d]	Odd
buf-3 × *rsy-1*	6	6	32	0
buf-4 × *alb-1*	11	5	43	3
alb-1 × *rsy-1*	6	9	23	0
alb-1 × *Mat1*	5	4	7	0
buf-4 × *Mat1*	3	2	6	0
rsy-1 × *Mat1*	4	2	10	0

[a]The table includes segregation data from the following genetic crosses. Crosses 4218 and 4225: CP485 (*Mat1-2 rsy-1*) × CP61 (*Mat1-1 buf-3*). Crosses 4141 and 4226: CP62 (*Mat1-1 buf-4*) × CP280 (*Mat1-2 alb-1*). Crosses 4202 and 4256: CP485 (*Mat1-2 rsy-1*) × 4174-1-5 (*Mat1-1 alb-1*). Cross 4164: CP62 (*Mat1-1 buf-4*) × 3597-17-5 (*Mat1-2*, a wild-type laboratory strain). Cross 4219: CP485 (*Mat1-2 rsy-1*) × CP62 (*Mat1-1 buf-4*). To determine linkage between melanin biosynthetic genes and the mating type locus, mating type was determined for progeny in tetrads from crosses 4141, 4164, 4218, 4219, and 4225.
[b]PD, parental ditype.
[c]NPD, nonparental ditype.
[d]T, tetratypes.

Table 7. Identification of *rsy-1 buf-3* double mutants from a tetratype tetrad

Rosy parent[a]	Progeny[b]	Genotype inferred
4218-6-6	Gray and rosy (6 PD)	*rsy-1*
4218-6-3	Gray and rosy (9 PD)	*rsy-1*
4218-6-8	Gray, rosy, and buff (1 PD:1 NPD:5 T)	*rsy-1 buf-3*
4218-6-5	Gray, rosy, and buff (2 NPD:1 T)	*rsy-1 buf-3*

[a]The indicated Rsy$^-$ parent was crossed with a wild-type tester strain. Strains 4218-6-6 and 4218-6-3, which are both *Mat1-2*, were mated with *Mat1-1* tester 4136-1-2. Strains 4218-6-8 and 4218-6-5, which are both *Mat1-1*, were mated with the *Mat1-2* tester, strain 4091-5-8.
[b]Progeny were analyzed in tetrads. Parental ditype (PD) tetrads contained four gray and four rosy progeny; nonparental ditypes (NPDs) contained four rosy and four buff progeny; and tetratypes (Ts) contained two gray, two buff, and four rosy progeny.

Pathogenicity of pigment mutants. We tested the ability of various pigment mutants to infect rice as a function of whether or not leaves of the host plants had been wounded before inoculation (discussed previously in the text). The Japanese field isolates, Ken60-19 and O-42, are virulent pathogens of rice variety M9. The pigment mutants CP65 (*buf-7*) and CP413 (*alb-6*), derived from Ken60-19 and O-42, respectively, caused no symptoms on intact M9 seedlings. However, CP65 and CP413 caused extensive blast symptoms on wounded M201 seedlings. Under high humidity conditions (>95%), lesions produced by pigment mutants on wounded tissue enlarged to the normal size expected for the wild-type parent and sporulated normally. Fungus recovered from these lesions showed the expected Buf$^-$ and Alb$^-$ phenotypes. These observations lend support to the argument that melanin biosynthesis is required only for initial penetration into the host plant.

In one experiment, a few rare lesions were detected on intact M9 seedlings inoculated with spores of the *alb-6* mutant, CP413. Fungus recovered from these lesions showed wild-type gray pigmentation. Thus, we may have selected wild-type revertants of strain CP413, although we did not verify this possibility through further genetic analysis. Similarly, Bustamam and Sisler (1987) reported a putative wild-type revertant of one of the Alb$^-$ mutants, CP283 (*alb-2*), that we supplied to them. We never observed possible revertants of strains with *buf*$^-$ defects.

We also tested the effect of incorporating the melanin biosynthetic intermediate, scytalone, into spore suspensions that were used to inoculate host plants. The field isolate O-42 is a virulent pathogen of rice cultivar M201. Pigment mutants CP412 (*alb-5*) and CP129 (*buf-40*), both derived from O-42, were inoculated on M201 seedlings with or without scytalone. Only in the presence of scytalone did extensive blast symptoms develop on M9 seedlings inoculated with spores of the *alb-5* strain (see Fig. 1). Fungus recovered from infected leaves showed the expected Alb$^-$ phenotype. The *buf-40* strain caused no symptoms in the presence or absence of scytalone. We also incorporated scytalone into minimal agar medium at concentrations ranging from 10 to 100 μg/ml. Cultures of CP412 (*alb-5*) darkened slightly on medium with 10 μg/ml of scytalone and darkened dramatically on medium with 100 μg/ml of scytalone. CP129 (*buf-40*) cultures did not darken in response to scytalone. These observations suggest that the *alb-5* strain, but not the *buf-40* strain, is able to take up scytalone from the medium and convert it to melanin required for successful penetration of the host's outer barriers.

The results in the previous paragraph were obtained with mutants derived from rice pathogens. We have obtained similar results with mutants derived from strain 4091-5-8, a fertile laboratory strain that is a pathogen of both WLG and GG. Experiments were conducted incorporating scytalone into inoculum with spores of mutants carrying the *alb-15*, *rsy-1*, or *buf-148* mutations. The *alb-15* strain was pathogenic on WLG and on GG only in the presence of scytalone. The *buf-148* and *rsy-1* strains were nonpathogenic in the presence or absence of scytalone.

DISCUSSION

The DHN melanin biosynthetic pathway presented in Figure 2 was determined by studies involving primarily the plant pathogenic fungus *Verticillium dahliae* Klebahn (Bell *et al.* 1976a, 1976b; Stipanovic and Bell 1976, 1977). Sisler and his colleagues proposed that the same reactions lead to the production of DHN melanin in *M. grisea* (Woloshuk *et al.* 1980b).

In this study, we have described the isolation and characterization of three classes of *M. grisea* mutants deficient in DHN melanin. Genetic analysis of the mutants has defined three unlinked genes, $ALB1^+$, $RSY1^+$, and $BUF1^+$. We infer that these three genes encode enzymes essential for melanin biosynthesis, and we have inferred gene-enzyme relationships as indicated in Figure 2. The absence of complementation data limits our confidence in concluding that all the alb^- or buf^- mutations we have detected affect the single genes, $ALB1^+$ or $BUF1^+$. The apparent allelism of alb^- mutations and the overall phenotypic similarity of Alb^- mutants lead us to think it is most likely that the mutations define a single gene rather than two or more tightly linked genes. Similar reasoning leads us to think that the buf^- mutations define a single gene, $BUF1^+$. Cloning genes that complement alb^- and buf^- mutations will resolve the issue.

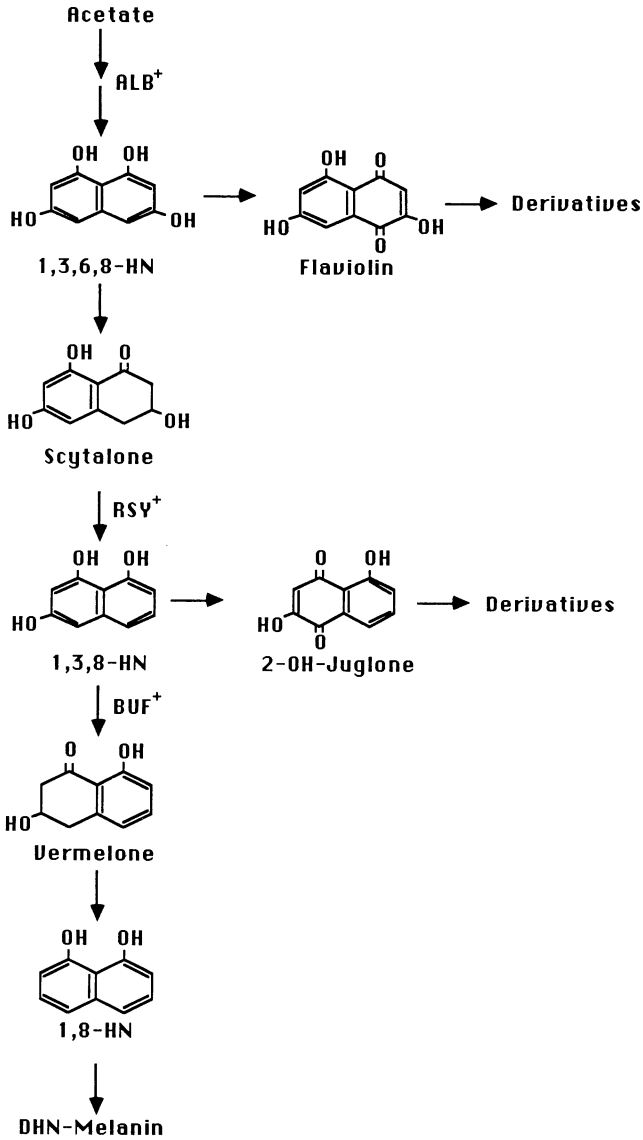

Fig. 2. *Magnaporthe grisea* polyketide biosynthetic pathway leading from acetate to DHN melanin (Stipanovic and Bell 1976, and Woloshuk *et al.* 1980b). The first intermediate detected is 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-HN; 4HN). Alternating reduction and dehydration reactions lead to scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-HN; 3HN), vermelone, and 1,8-dihydroxynaphthalene (1,8-HN; DHN). DHN is then polymerized via oxidation to yield a complex three-dimensional matrix. Branch points at 4HN and 3HN are indicated, leading to the secondary metabolites flaviolin and 2-hydroxyjuglone and to derivatives of those compounds. The gene symbols ALB^+, RSY^+, and BUF^+ are placed beside steps likely to be catalyzed by their respective gene products (see text).

We have assigned gene-enzyme relationships based on the following evidence and lines of reasoning. The Buf^- mutants that we have isolated are very similar in appearance to wild-type fungus grown in the presence of tricyclazole. In studies with *M. grisea*, Woloshuk *et al.* (1980b) showed that tricyclazole inhibits 3HN reductase strongly and 1,3,6,8-tetrahydroxynaphthalene (4HN) reductase much less strongly. They also showed that 3HN reductase was absent from Buf^- variants derived from their wild-type strain. We observed that several $buf1^-$ mutants were unable to convert scytalone to DHN melanin, indicating a block downstream of scytalone. It therefore seemed likely that the $BUF1^+$ gene encodes 3HN reductase (Fig. 2).

The following results led us to assign scytalone dehydratase to the $RSY1^+$ gene (Fig. 2). The epistasis relationship defined by the double mutants suggested that the *rsy-1* block occurs upstream of the block associated with each of three independent buf^- mutations, and we observed that the Rsy^- mutant does not convert exogenously added scytalone to melanin. Definitive evidence resulted from the biochemical analysis of a mutant carrying *rsy-1*: our colleague John Pierce showed that the *rsy-1* mutant lacks scytalone dehydratase enzyme activity (J. Pierce, personal communication).

Epistasis relationships suggested that $ALB1^+$ must act upstream of $RSY1^+$ and $BUF1^+$. The absence of colored intermediates in cultures of $alb1^-$ mutants argues for a block in melanin biosynthesis upstream of the production of 4HN. The ability of $alb1^-$ mutants to convert scytalone to melanin indicates that the downstream melanin biosynthetic enzymes are present in $alb1^-$ mutants. We consider it most likely that $ALB1^+$ encodes an enzyme essential for the earliest steps of DHN melanin biosynthesis. Nagakubo *et al.* (1983) previously reported an Alb^- mutant of *M. grisea*; we do not know if the mutation they described is allelic with $ALB1^+$.

Recognition of the alternating reductase and dehydratase steps following the formation of 4HN led to the suggestion that a single reductase might serve in the production of both scytalone and vermelone, while a single dehydratase might serve in the production of both 3HN and DHN. These suggestions received limited support from studies of *Cochliobolus miyabeanus* (Ito & Kuribayashi) Drechsler and *V. dahliae*. In *C. miyabeanus*, Kubo *et al.* (1989) showed that a single gene mutation, scy^-, eliminated both dehydratase activities, although another single gene

mutation, *brm*$^-$, apparently eliminated only 3HN reductase, leaving 4HN reductase intact. Melanin-deficient variants of *V. dahliae* (reviewed by Bell and Wheeler 1986) have not been analyzed by genetic crosses, and studies with such variants must therefore be regarded with caution. Nevertheless, the *brm-4* variant of *V. dahliae* has been reported to lack both 4HN and 3HN reductase. Whether or not the *brm-4* defect is a single gene mutation is not known. Another *V. dahliae* variant, *brm-2*, lacks the 3HN reductase, but not the 4HN reductase. Whether or not the *brm-2* and *brm-4* phenotypes are due to changes in the same or different genes is not known.

In our genetic studies, double mutants containing *rsy-1* and any of three independent *buf*$^-$ mutations had the Rsy$^-$ phenotype. It therefore seems unlikely that these *buf1*$^-$ mutations result in the loss of 4HN reductase. The Buf$^-$ variants studied by Woloshuk *et al.* (1980b) also retained 4HN reductase. Our observations thus do not support the hypothesis of a single reductase enzyme, but they also do not disprove the model. A critical test of the hypothesis could be provided by the identification and analysis of null mutations of *BUF1*$^+$.

The genetic analysis reported here accounts for three of the six steps indicated in Figure 2, with 4HN reduction, vermelone dehydration, and DHN polymerization remaining genetically undefined. Strains with the *rsy-1* mutation have yet to be tested for vermelone dehydratase, which they may lack. The oxidative polymerization of DHN to form melanin has been suggested to be catalyzed by a phenol oxidase, but this oxidase has not been characterized in any system (Bell and Wheeler 1986). Our collection of pigment mutants includes only one *rsy*$^-$ isolate, indicating that the melanin biosynthetic pathway has not been saturated genetically: other classes of *M. grisea* melanin-deficient mutants may yet be discovered.

We have observed that *alb*$^-$ mutants form normal lesions when host tissue has been wounded before inoculation. Thus, we would argue that the branch point metabolites shown in Figure 2, flaviolin, 2-hydroxyjuglone, and derivative compounds, are not required for pathogenicity after the host's outer barriers have been penetrated. Chida and Sisler (1987) showed that exogenously supplied DHN restored the ability of appressoria blocked in 3HN reductase to penetrate a membrane *in vitro*. Taking this observation together with ours, we would argue that DHN melanin is likely to be the only metabolite shown in Figure 2 which plays an important role in pathogenicity.

M. grisea resembles *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. (Wolkow *et al.* 1983) and *Colletotrichum lagenarium* (Passerini) Ellis & Halsted (Kubo *et al.* 1982) in requiring DHN melanin for function of the appressorium in penetration. DHN melanin does not appear to function in penetration of *Cochliobolus miyabeanus* into rice tissue, because pigment-defective mutants are all fully pathogenic (Kubo *et al.* 1989). The reason for such differences in the requirement of DHN melanin for pathogenicity remains to be determined. In plant pathogens such as *V. dahliae*, which has no DHN melanin requirement for pathogenicity, the pigment is believed to be required for long-term survival of propagative structures such as microsclerotia (Bell *et al.* 1976a).

The abundance of *buf1*$^-$ mutants of *M. grisea* is remarkable. As mentioned previously in the text, some strains from nature spontaneously yield Buf$^-$ conidia and ascospores at very high rates, ranging from one in 200 to one in 20 (B. Valent, unpublished results). In such strains, this genetic instability does not extend to other loci (B. Valent and F. Chumley, unpublished results), not even to other pigment biosynthetic genes. The *buf1*$^-$ mutations that we have characterized are stable. A molecular genetic explanation of these intriguing observations must await the cloning of the *BUF1*$^+$ gene.

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