Site-Directed Mutagenesis of the *magB* Gene Affects Growth and Development in *Magnaporthe grisea*

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G protein signaling is commonly involved in regulating growth and differentiation of eukaryotic cells. We previously identified *MAGB*, encoding a G α subunit, from *Magnaporthe grisea*, and disruption of *MAGB* led to defects in a number of cellular responses, including appressorium formation, conidiation, sexual development, mycelial growth, and surface sensing. In this study, site-directed mutagenesis was used to further dissect the pleiotropic effects controlled by *MAGB*. Conversion of glycine 42 to arginine was predicted to abolish GTPase activity, which in turn would constitutively activate G protein signaling in *magB*^{G42R}. This dominant mutation caused autolysis of aged colonies, mischeduled melanization, reduction in both sexual and asexual reproduction, and reduced virulence. Furthermore, *magB*^{G42R} mutants were able to produce appressoria on both hydrophobic and hydrophilic surfaces, although development on the hydrophilic surface was delayed. A second dominant mutation, *magB*^{G203R} (glycine 203 converted to arginine), was expected to block dissociation of the G $\beta\gamma$ from the G α subunit, thus producing a constitutively inactive G protein complex. This mutation did not cause drastic phenotypic changes in the wild-type genetic background, other than increased sensitivity to repression of conidiation by osmotic stress. However, *magB*^{G203R} is able to complement phenotypic defects in *magB* mutants. Comparative analyses of the phenotypical effects of different *magB* mutations are consistent with the involvement of the G $\beta\gamma$ subunit in the signaling pathways regulating cellular development in *M. grisea*.

Additional keywords: rice blast, signal transduction.

The process used by eukaryotic cells to sense and transduce environmental signals to the cell interior resulting in cellular responses involves complex interactions at the cell membrane. Cell membrane-associated heterotrimeric G proteins are key components in the signal transduction pathways for a variety of developmental processes and physiological responses in

both simple and higher eukaryotes. Binding of signal ligands to cell-surface receptors promotes an exchange of GDP for GTP on the G α subunit, which then triggers a reciprocal conformational change and dissociation from the G $\beta\gamma$ heterodimer. Both G α and G $\beta\gamma$ subunits interact with appropriate target effectors such as phosphodiesterase, protein kinases, adenylyl cyclases, phospholipases, and ion channels (Clapham and Neer 1997; Hamm 1998; Neer 1995; Simon et al. 1991) to trigger downstream signaling pathways for cellular responses. The activated G proteins are later desensitized by the GTPase activity of the G α subunit, followed by reassociation with the G $\beta\gamma$ complex.

Despite the tremendous growth in understanding the participation of G proteins in the development of mammalian systems, the role of G protein signaling in simple eukaryotes, such as filamentous fungi, is only now beginning to emerge. G proteins are well known to regulate the pheromone response for mating in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kurjan 1992). In filamentous fungi, several genes encoding the G α subunit have been cloned over the past few years and their possible roles in growth and development examined through gene disruption studies. In *Neurospora crassa*, *GNA-1* and *GNA-2* have overlapping functions and regulate mycelium growth, female fertility, and tolerance to osmotic stress (Baasiri et al. 1997; Ivey et al. 1996; Turner and Borkovich 1993). The *FADA* gene of *Aspergillus* (= *Emericella*) *nidulans* appears to interact with the *FLBA* gene to modulate mycelial growth and conidiation (Yu et al. 1996). In *Ustilago maydis*, the G α gene *GNA-3* regulates the pheromone response for mating (Regenfelder et al. 1997). *CPG-1* and *CPGB-1*, encoding G protein α and β subunits, respectively, in the chestnut blight fungus *Cryphonectria parasitica*, regulate multiple developmental processes including pigmentation, conidiation, female fertility, and virulence (Chen et al. 1996; Choi et al. 1995; Gao and Nuss 1996; Kasahara and Nuss 1997).

We previously cloned the *MAGB* gene, which encodes a G α subunit in *Magnaporthe grisea*. Sequence analysis of the deduced polypeptide of *MAGB* revealed high homology (>93%) with other fungal G α subunits, including the *CPG-1* of *C. parasitica*, *GNA-1* of *N. crassa*, and *FADA* of *A. nidulans*. Disruption of the *MAGB* gene caused pleiotropic effects, including reduced appressorium formation, conidiation, sexual

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development, mycelial growth, surface sensing, and virulence, indicating a common involvement of G protein signaling in regulating cellular activities. *MAGB* also contains some features found in members of the mammalian Gi family, includ-

ing a conserved myristylation site (MGXXX) and the pertussis toxin-responsive ADP-ribosylation site (CXXX) at the N and C termini, respectively (Liu and Dean 1997). However, *MAGB* appears to be distinct from mammalian Gi family

Fig. 1. Site-directed mutagenesis of the *MAGB* gene. **A**, Restriction map of the *MAGB* locus. The *MAGB* gene indicated by arrow is contained within a 4.3-kb *HindIII* fragment. Both start codon ATG and the stop codon TAA are shown. The novel *SmaI* site created in G42R mutation is marked as *SmaI**, and the endogenous *BalI* site removed by G203R mutation is shown by *BalI*#. Restriction enzyme sites are abbreviated as follows: H = *HindIII*, B = *BamHI*, E = *EcoRI*, S = *SalI*, Sma = *SmaI*, X = *XbaI*, and P = *PstI*. **B**, Oligomers used for site-directed mutagenesis of the *MAGB* gene. The *magB*^{G42R} mutation changes the Gly 42 to Arg and leads to a constitutively active G protein signaling by disrupting the GTPase activity of the G α subunit. The *magB*^{G203R} mutation replaces the Gly 203 with Arg and blocks dissociation of G $\beta\gamma$ subunit from G α and, therefore, inactivates the G protein signaling. Both parental and mutation sequences are shown. The modified nucleotides are highlighted. * indicates the G→C changes with resulting Gly→Arg mutation. A second silent mutation T→G in the G42R oligomer is indicated, thereby introducing a novel *SmaI* site (underlined). The mutation in G203R results in the loss of a *BalI* site (underlined). **C**, Deduced amino acid sequence of the *MAGB* gene. Both *magB*^{G42R} and *magB*^{G203R} mutations are highlighted.

members, which are commonly involved in negative regulation of the adenylyl cyclases. Repression of appressorium formation resulting from disruption of *MAGB* can be complemented by exogenous cAMP.

The identification and characterization of more than 20 mammalian G α subunits and several from simple eukaryotes, including *A. nidulans*, *N. crassa*, *S. cerevisiae*, and *Schizosaccharomyces pombe*, have provided insight into understanding the functional domains involved in GTP hydrolysis as well as the mechanisms involved in G α interaction with membrane receptors, the G $\beta\gamma$ complex, and its effectors (Berlot and Bourne 1992; Choi et al. 1995; Conklin and Bourne 1993; Rens-Domiano and Hamm 1995; Sprang 1997; Hamm 1998; Wall et al. 1995; Yang and Borkovich 1999; Yu et al. 1996). In this research, we proposed to further dissect the effect of G proteins on cellular development in *M. grisea* by modifying two functional domains in the *MAGB* protein. Two point mutations, *magB*^{G42R} and *magB*^{G203R}, were created by site-directed mutagenesis. The mutation *magB*^{G42R} converted glycine 42 to arginine in the G-3 box of the G α subunit and was expected to disrupt the endogenous GTPase activity in the G α subunit and lead to a constitutively active G protein signaling. In contrast, *magB*^{G203R}, in which the conserved glycine 203 was replaced with arginine in the α 2 helix of the G α subunit, was expected to interfere with the conformational change of the G α subunit upon GTP binding and block the dissociation of the G α from G $\beta\gamma$ subunit. The effects of these dominant mutations on appressorium formation, vegetative growth, and both asexual and sexual development are presented. Based on our findings, a possible role of G $\beta\gamma$ heterodimers in regulating development is also suggested.

Fig. 2. Genomic Southern hybridization analysis of the *magB*^{G42R} and *magB*^{G203R} transformants. Five independent transformants containing each mutation construct are shown. Genomic DNA (5 μ g) was digested with *Sma*I, size-separated on a 1% agarose gel and probed with the 4.3-kb *Hind*III fragment of *MAGB*. Lanes 1 to 5, *magB*^{G42R} transformants; lanes 6 to 10, *magB*^{G203R} transformants; and lane 11, the wild-type strain, 70-15, as control.

RESULTS

Site-directed mutagenesis of the *MAGB* gene and *M. grisea* transformation.

Site-directed mutagenesis was performed directly on plasmid p12₂₋₃, a plasmid that contained the *MAGB* gene in a 4.3-kilobase (kb) *Hind*III fragment in bluescript KSII(+) vector (Fig. 1A). The oligomers for site-directed mutagenesis reactions contained modifications of restriction sites to facilitate rapid screening for the desired mutations. For example, the oligomer designed for the *magB*^{G42R} mutation introduced a new *Sma*I site, whereas the oligomer for the *magB*^{G203R} mutation removed an endogenous *Bal*I site from *MAGB* (Fig. 1B). Resulting plasmids (pG42R-I and pG203R-I) putatively containing mutations were examined by *Sma*I and *Bal*I digestion, respectively. Of the putative G42R clones, 8% exhibited the expected *Sma*I site, and 30% of the putative G203R clones had lost the endogenous *Bal*I site. The inserts of the entire point-mutation constructs were sequenced to confirm they contained no additional changes. The mutated 4.3-kb *Hind*III fragments were subcloned into pAN7-1, a vector that contains the hygromycin-resistant cassette for fungal transformation, to create the plasmids pG42R-II and pG203R-II.

Transformation of *M. grisea* with each construct produced more than 100 hygromycin-resistant transformants. Five independent transformants of each pG42R-II and pG203R-II were further analyzed by determining the (i) presence of the hygromycin phosphotransferase gene (*HPH*) and (ii) presence or absence of the appropriate *Sma*I and *Bal*I fragments by Southern blot hybridization.

Integration of the plasmids carrying dominant mutations of the *MAGB* gene.

All pG42R-II and pG203R-II transformants revealed the presence of the *HPH* gene in Southern blot analyses (data not shown). When genomic DNA digested with *Sma*I was probed with the 4.3-kb *Hind*III fragment of *MAGB*, all pG42R-II transformants exhibited hybridization bands other than the endogenous 8.5-kb fragment, indicating the presence of the pG42R-II plasmid. The various sizes of the hybridization fragments also suggested different integration events (Fig. 2). Interestingly, the endogenous 8.5-kb *Sma*I fragment was absent from four (*magB*^{G42R}-C2, *magB*^{G42R}-T2, *magB*^{G42R}-T5, and *magB*^{G203R}-T6) of the transformants analyzed, suggesting a single homologous recombination event occurred in these transformants. Transformants *magB*^{G203R}-C2 and *magB*^{G203R}-C5 revealed the approximately 8.5-kb band; however, a second band expected for ectopic transformants was not readily observable. When hybridized with *HPH*, a second, higher molecular weight band was clearly observed. Southern blot analysis of *Bal*I digests revealed that all of the putative *magB*^{G203R} mutants contained additional bands other than the endogenous 0.8-, 1.3-, and 3.5-kb fragments when probed with the 4.3-kb *Hind*III fragment of the *MAGB* gene, indicating the presence of the pG203R-II plasmid (data not shown). Nevertheless, *magB*^{G203R}-C2 and *magB*^{G203R}-C5 were not considered for further analysis. Transformant *magB*^{G203R}-T6 contained a 4.4-kb fragment expected from a single homologous transformation event. Three independent transformants of each plasmid, including *magB*^{G42R}-C2, *magB*^{G42R}-T2, and *magB*^{G203R}-T6, which ap-

peared to show homologous recombination, and *magB*^{G42R}-T1, *magB*^{G203R}-C1, and *magB*^{G203R}-T5, in which the transforming plasmids were ectopically integrated, were chosen for phenotype studies.

Effects of *magB*^{G42R} and *magB*^{G203R} mutations on colony morphology.

The integration of both *magB*^{G42R} and *magB*^{G203R} mutation plasmids into the *M. grisea* genome did not significantly af-

Fig. 3. Colony morphology of different *magB* mutation strains. **A**, Growth on V8 agar medium without (top row) and with (bottom row) 3% NaCl. **B**, Growth on complete medium without (top row) and with (bottom row) 3% NaCl. Plates from left to right contain *magB*^{G42R}, *magB*^{G203R}, 70-15, and *magB* strains.

fect colony growth rate on either V8 or complete agar medium (data not shown). However, like the *magB* strains, *magB*^{G42R} colonies were lightly colored on V8 medium, perhaps due to the reduction in conidiation (described below). The *magB*^{G42R} mutants also exhibited autolysis, which started from the center of the colony and expanded outward, when grown on V8 medium (Fig. 3A). Autolysis could be suppressed by the addition of 3% NaCl. Autolysis was not evident on nutrient-rich agar medium; instead, the colony produced fluffy aerial mycelia. Addition of 3% NaCl to complete medium suppressed aerial mycelium development and caused the mycelia to grow beneath the medium surface (Fig. 3B). Unlike the *magB*^{G42R}

mutants, the *magB* strains did not display autolysis when growing on V8 medium and the colonies on nutrient-rich complete medium were less fluffy. No differences in mycelium growth patterns were observed between *magB*^{G203R} mu-

Table 1. Effects of *magB*^{G42R}, *magB*^{G203R}, and *magB* null mutations on conidiation

Strains	Relevant genotype	Conidiation ($\times 10^4$ conidia per plate) ^a
G42R	<i>magB</i> ^{G42R}	8.0 $\pm$ 3.0
G203R	<i>magB</i> ^{G203R}	174.0 $\pm$ 24.0
2-11	<i>magB</i>	5.5 $\pm$ 5.0
K-03 ^b	Wild type	143.0 $\pm$ 13.0
70-15	Wild type	152.0 $\pm$ 54.0

^a Mean $\pm$ standard deviation. Average from three isolates/replicates and three independent experiments. Number of conidia were counted after 14 days.

^b A 70-15 transformant containing the hygromycin resistance gene cassette.

Table 2. Effects of salt stress on conidiation by *magB*^{G203R}

Strain	Conidiation ($\times 10^4$ conidia per plate) ^a		
	– NaCl	+ NaCl	Reduction (%)
G203R-C1B	124.8 $\pm$ 20.4	2.7 $\pm$ 2.0	97.8
G203R-T5A	204.0 $\pm$ 28.6	2.0 $\pm$ 1.9	99.0
G203R-T6A	179.7 $\pm$ 19.4	36.4 $\pm$ 13.7	79.8
K-03 ^b	193.0 $\pm$ 20.0	104.1 $\pm$ 13.5	46.1
70-15	165.6 $\pm$ 17.0	71.0 $\pm$ 8.5	57.1

^a Cultures were grown on V8 agar plates with or without 3% NaCl and incubated in a 20°C light incubator for 14 days. The average and standard deviation were calculated from three isolates/replicates. Three independent experiments were performed with consistent results.

^b A 70-15 transformant containing the hygromycin resistance gene cassette.

Table 3. Appressorium development of different *magB* stains^a

Time, strain ^b	Hydrophobic		Hydrophilic		
	H ₂ O	H ₂ O	N6-cAMP	IBMX	Diol
At 24 h					
(70-15) + G42R	90.7 $\pm$ 3.8	50.7 $\pm$ 4.2	55.5 $\pm$ 9.0	76.0 $\pm$ 10.3	91.0 $\pm$ 5.7
(2-11) + G42R	87.8 $\pm$ 4.6	47.0 $\pm$ 7.8	56.7 $\pm$ 5.8	77.4 $\pm$ 8.0	80.3 $\pm$ 5.5
(70-15) + G203R	98.8 $\pm$ 1.2	5.0 $\pm$ 2.9	59.3 $\pm$ 5.5	82.0 $\pm$ 9.2	90.0 $\pm$ 1.0
(2-11) + G203R	98.3 $\pm$ 1.6	6.9 $\pm$ 4.4	70.3 $\pm$ 10.0	81.3 $\pm$ 7.7	97.7 $\pm$ 1.5
2-11	13.6 $\pm$ 5.4	7.8 $\pm$ 5.8	65.7 $\pm$ 8.6	88.0 $\pm$ 2.9	91.5 $\pm$ 3.5
70-15	99.6 $\pm$ 0.7	0.6 $\pm$ 1.2	85.0 $\pm$ 6.7	84.8 $\pm$ 4.2	89.0 $\pm$ 1.0
At 48 h					
(70-15) + G42R	93.8 $\pm$ 5.1	82.5 $\pm$ 1.4	>95.0	>95.0	>95.0
(2-11) + G42R	88.3 $\pm$ 6.1	83.3 $\pm$ 0.8	>95.0	>95.0	>95.0
(70-15) + G203R	99.2 $\pm$ 0.8	8.6 $\pm$ 3.1	>95.0	>95.0	>95.0
(2-11) + G203R	99.8 $\pm$ 0.4	9.9 $\pm$ 14.0	>95.0	>95.0	>95.0
2-11	23.4 $\pm$ 4.0	12.6 $\pm$ 6.9	>95.0	>95.0	>95.0
70-15	100 $\pm$ 0.0	0.6 $\pm$ 0.9	>95.0	>95.0	>95.0

^a Data represent the percentage of germinating conidia that were actively producing appressoria; means $\pm$ standard deviation were averaged from three replicates/isolate. N6-cAMP = N⁶-monobutyl cAMP, IBMX = 3-isobutyl-1-methylxanthine, and Diol = 1,16-hexadecanediol. Conidia were harvested from 7- to 10-day-old cultures and 1×10^3 conidia in 60- μ l drops were placed on GelBond membranes in a moisture chamber for 24 and 48 h. Experiments were repeated with consistent results.

^b Each strain represents three independent transformants. 70-15 is a wild-type strain, and 2-11 is a *magB* strain. G42R indicates strain transformed with G α ^{G42R} and G203R indicates strain transformed with G α ^{G203R}.

Fig. 4. The *magB*^{G42R} mutants exhibit misscheduled melanization at the tips of conidiophores. **A**, Tips of putative conidiophores of the *magB*^{G42R} mutants are melanized (arrow) and produce no conidia. **B**, Conidiophores of the wild-type 70-15 strain bearing normal conidia (arrow) when grown under the same conditions.

tants and the wild type. No autolysis was observed on V8 medium, and aerial mycelium growth on complete medium was unaffected by 3% NaCl.

Effects of *magB*^{G42R} and *magB*^{G203R} mutations on conidiation.

The *magB*^{G42R} mutation resulted in a 95% reduction in conidiation when grown on V8 agar medium with constant light (Table 1). As previously reported, the *magB* null mutation also repressed conidiation by 95% (Table 1) (Liu and Dean 1997). Conidiation was also greatly reduced in lesions on infected rice leaves (data not shown). By contrast, the *magB*^{G203R} mutation had no significant effect on conidiation. On complete medium, like the wild type, conidiation remained suppressed for both mutants. Neither mutation affected conidium morphology; conidia appeared tear-drop shaped, with three cells and thick, melanized cell walls.

Interestingly, *magB*^{G42R} mutants also exhibited misscheduled melanization at the tips of what resembled conidiophores in cultures grown in solid and liquid V8 medium (Fig. 4). Misscheduled melanization was light independent and occurred in cultures grown under both light or dark conditions. However, conidiation was still light dependent and no conidia were produced in cultures grown in the dark.

The presence of NaCl (3%) suppressed conidiation by *magB*^{G203R} mutants as well as in the wild type. However, conidiation was suppressed to a greater extent in *magB*^{G203R} (80 to 99%) compared with the wild type (57%) (Table 2).

Effects of *magB*^{G42R} and *magB*^{G203R} mutations on appressorium formation.

The *magB*^{G42R} mutants, in the absence of inductive chemical cues, bypassed the requirement of surface hydrophobicity and elaborated appressoria on both hydrophilic and hydrophobic

Fig. 5. Appressoria development by *magB*^{G42R} mutant and the wild-type strain on GelBond membranes. The *magB*^{G42R} mutants are able to form long and straight germ tubes and develop appressorium on both **A**, hydrophobic and **B**, hydrophilic surfaces of GelBond membranes. **C**, N6-cAMP treatment shortens germ tubes. **D**, Wild-type strain 70-15 develops appressoria from short germ tubes on the hydrophobic surface of GelBond. **E**, When germinated on the hydrophilic surface of GelBond, the wild-type strain only produces wavy germ tubes and rarely develop appressoria, unless **E**, an inducing agent such as N6-cAMP is supplemented.

surfaces of GelBond (FMC Bioproducts; Rockland, ME, U.S.A.) (Table 3). Unlike the wild type, *magB*^{G42R} mutants produced long, straight germ tubes, approximately 250 μ m in length, compared with 25- μ m lengths produced by the wild type, on the hydrophobic surface. The development of appressoria on the hydrophilic surface appeared delayed. At 24 h postincubation, fewer than 50% of germinated conidia produced appressoria on the hydrophilic surface, compared with >90% on the hydrophobic surface; however, after 48 h, more than 80% of the germinated conidia produced appressoria from the tips of the germ tubes and hyphal branches on the hydrophilic surface (Table 3). Appressoria appeared mature and were darkly pigmented on both hydrophobic and hydrophilic surfaces. The inductive chemicals 1,16-hexadecanediol (diol), N⁶-monobutyl cAMP (N6-cAMP), and 3-isobutyl-1-methylxanthine (IBMX) further promoted appressorium development and the resulting appressoria were produced on short (approximately 25 μ m) germ tubes (Table 3).

The *magB*^{G203R} mutants developed typical appressoria on short germ tubes within 8 h after germination on the hydro-

phobic surface of GelBond. Like the wild type, most germinating conidia only produced wavy germ tubes that then branched to form vegetative mycelia instead of developing appressoria on the hydrophilic surface (Fig. 5). The addition of the diol, N6-cAMP, or IBMX stimulated the development of appressoria on short germ tubes in *magB*^{G203R} mutants in a manner indistinguishable from the wild-type and *magB*^{G42R} mutants.

As previously reported, the *magB* null mutant was greatly repressed in its ability to form appressoria on a hydrophobic surface and, even after 48 h, fewer than 25% of germinating conidia were able to form appressoria (Table 3) (Liu and Dean 1997). However, like the *magB*^{G203R} and *magB*^{G42R} strains, the *magB* mutant was still responsive to diol, N6-cAMP, and IBMX (Table 3).

Both *magB*^{G42R} and *magB*^{G203R} mutants were able to develop appressoria on onion skin (data not shown). The *magB*^{G42R} mutants produced long, straight germ tubes before producing appressoria. In contrast, the *magB*^{G203R} mutants and the wild type developed appressoria on short germ tubes. The *magB* mutant only produced long, straight germ tubes and rarely developed appressoria on onion skin.

Effects of *magB*^{G42R} and *magB*^{G203R} mutations on virulence.

The *magB*^{G42R} mutants were less virulent as reflected by fewer and smaller necrotic lesions on susceptible rice plants, compared with the wild-type and *magB*^{G203R} mutants (Fig. 6). Conidia of *magB*^{G42R} mutants germinated within 8 h when sprayed on the surface of rice leaves. However, the germ tubes were long and straight compared with the short germ tubes of the wild-type and *magB*^{G203R} mutants. Seven days after spray inoculation, the percentage of surface area covered by lesions was estimated from the middle one-third of the leaf immediately below the flag leaf. Less than 10% of the leaf area was covered with lesions when inoculated with *magB*^{G42R} strains, compared with more than 50% for plants inoculated with *magB*^{G203R} and wild-type strains. The *magB* null mutants produced disease symptoms similar to those produced by *magB*^{G42R} mutants.

Similar observations were revealed from leaf sheath injection experiments. In most cases, no symptoms developed following inoculations by the *magB*^{G42R} and *magB* strains, although, in a few instances, small lesions developed. The strain *magB*^{G203R} produced typical necrotic lesions indistinguishable from the wild type. Thus, *magB*^{G42R} mutants were severely reduced in their ability to infect and grow invasively in rice leaves.

Conidia were recovered from lesions, streaked for single colonies, and examined to confirm their identity. All colonies recovered from mutant-infected lesions were hygromycin resistant and were reduced in conidiation. Colonies isolated from *magB*^{G42R}-infected lesions exhibited reduced conidiation, mischeduled melanization, and autolysis and were hygromycin resistant, whereas colonies originated from *magB*^{G203R}-infected lesions were hygromycin resistant and were able to produce wild-type levels of conidia. Colonies derived from the wild-type-inoculated lesions were all hygromycin sensitive (data not shown).

Effects of *magB*^{G42R} and *magB*^{G203R} mutations on sexual reproduction.

Growth inhibition zones developed at the junctions between colonies of opposite mating types (i.e., 70-6/70-15, 70-6/*magB*^{G42R}, and 70-6/*magB*^{G203R}) approximately 2 weeks after

Fig. 6. Lesion development by different *magB* mutation strains on susceptible rice cultivar S-201. Rice seedlings were individually sprayed with spore suspensions prepared from each strain. From left to right: *magB*^{G42R} in 70-15, *magB* 2-11, *magB*^{G203R} in *magB* 2-11, *magB*^{G203R} in 70-15, K-03 in 70-15, 70-15, and Tween-20 control. The K-03 is a transformant carrying the hygromycin resistance gene cassette. Photograph was taken 7 days postinoculation.

incubation on oatmeal agar plates. All colonies developed abundant aerial hyphae, except in the inhibition zones. Abundant dark-pigmented perithecia developed approximately 1 week later in these areas, except with crosses involving the *magB*^{G42R}

mutants (Fig. 7A). The perithecia from the crosses of *magB*^{G203R}/70-6 and 70-15/70-6 produced abundant asci, which contained six to eight ascospores in most mature asci (Fig. 7B). Interestingly, perithecia remained intact for less than 3 days (ap-

Fig. 7. Perithecia development by different *magB* mutation strains. **A**, Strains were inoculated on oatmeal agar medium. From top and clockwise: tester strain 70-6 (MAT1-2), 70-15 (MAT1-1), and *magB*^{G203R} (left plate) or *magB*^{G42R} (right plate). Photo was taken 3 weeks postinoculation. **B**, Perithecia from the cross of *magB*^{G203R}/70-6 produced abundant asci that contain six to eight ascospores in most cases.

proximately day 21 to 24 postinoculation). After this time, perithecia degraded rapidly and very few asci and ascospores could be found.

Previously, it was found that *magB* strains were unable to cross and produce perithecia. In the experiments reported here, crosses with *magB* strains occasionally resulted in pseudothecia. In the vast majority of cases, no asci were visible, but on rare occasions, asci were observed containing immature ascospores. In no instances were viable ascospores recovered.

Phenotypes of the *magB*^{G42R} and *magB*^{G203R} mutations in the *magB* genetic background.

Although G α ^{G42R} and G α ^{G203R} are reported as dominant mutations (Coleman and Sprang 1996; Hicks et al. 1997; Sprang 1997; Yu et al. 1996), experiments were performed to validate this assumption. To determine whether the presence of the wild-type copy of the *MAGB* affected the phenotype of transformants containing the *magB* dominant mutations, plasmids pG42R-I and pG203R-I were cotransformed with pCB1265 (which contains the *bar* gene and confers resistance to bialaphos) into the *magB* strain 2-11 (Liu and Dean 1997). This mutant strain is repressed for conidiation and displays lightly colored colonies; therefore, putative bialaphos-resistant cotransformants were initially evaluated for the presence of melanized conidiophore tips or complementation for conidiation caused by pG42R-I and pG203R-I, respectively. The presence of the mutant *magB* plasmids was further confirmed by genomic Southern hybridization analyses (data not shown). Four transformants of each type were evaluated for the variety of phenotypes described above. In all cases, the phenotypes were the same in wild-type and *magB* backgrounds, confirming the dominant nature of these mutations.

Determination of the cellular levels of cAMP in different *magB* mutants.

The ability of exogenous cAMP to restore essentially normal appressorium formation in both *magB* and *magB*^{G42R} mutants suggests a connection between G protein signaling and cAMP signaling pathways (Adachi and Hamer 1998; Dean 1997). To investigate a possible connection, cellular levels of cAMP were measured in different mutant backgrounds. Cultures were grown in complete medium, from which the cell-free extracts were prepared and analyzed for cAMP content. Results from the analysis revealed no significant differences in cAMP content between any of the strains (levels ranged from 2.0 $\pm$ 0.9 to 2.4 $\pm$ 1.3 pmol per microgram of protein). However, in complete media, all strains were morphologically similar and, thus, the effects of *magB* mutations on cAMP levels may not be evident. We further monitored cAMP levels in cultures growing in V8 medium, in which *magB*^{G42R} mutants show misscheduled melanization in both liquid and solid cultures. No significant differences in cAMP levels were detected (levels ranged from 1.2 $\pm$ 0.5 to 1.8 $\pm$ 1.1 pmol per microgram of protein).

DISCUSSION

We previously identified the *MAGB* gene, encoding a G α subunit, from *M. grisea*. Disruption of the gene caused pleiotropic effects, including reduction in vegetative growth, conidiation, appressorium formation, and pathogenicity (Liu

and Dean 1997). The results suggest a common involvement of MAGB and G protein signaling in a variety of developmental processes in this important fungal pathogen. In this research, we explored the effect of manipulating G protein signaling by making single base point mutations that should affect the ability of the G α subunit to dissociate from the G $\beta\gamma$ complex. A mutation expected to generate a constitutively active dominant allele (*magB*^{G42R}) affected virtually all developmental stages analyzed in this research. These included bypassing the requirement for surface hydrophobicity for appressorium development, autolysis of colonies, elevated osmosensitivity for mycelial growth, misscheduled melanization, reduction in conidiation, repression of sexual reproduction, and reduced virulence. In comparison, a mutation expected to prevent dissociation (i.e., generates a constitutively inactive dominant allele, *magB*^{G203R}) resulted in less drastic effects and mostly affected asexual reproduction. These results not only further support our previous results that G protein signaling is ubiquitously involved in regulating cellular development, but also implicate a role for the G $\beta\gamma$ subunit in these processes as described below.

Both *magB*^{G42R} and *magB* null mutants exhibited similar phenotypes, including reduced conidiation, sexual reproduction, and virulence. G $\beta\gamma$ heterocomplexes have been shown to be functionally active in null G α mutants (Hermouet et al. 1993; Simon et al. 1991; Yang and Borkovich, 1999). Thus, it is possible that G $\beta\gamma$ in *M. grisea* remains active in both null and constitutively active *magB*^{G42R} mutants and may be responsible for negatively regulating development. This hypothesis is further supported by our observations from mutants expressing the *magB*^{G203R} mutation, in which the putative negative regulator, the G $\beta\gamma$ subunit, is presumably unable to release from the G α subunit. The phenotype of the *magB*^{G203R} mutants was very similar to that of the wild type in most aspects. Whether the G $\beta\gamma$ subunit remains active and dissociated from other G α subunits, *MAGA* and *MAGC*, in *M. grisea* remains to be determined. Other possibilities for the limited morphological effects of the *magB*^{G203R} point mutation are that (i) the *magB*^{G203R} mutation does not drastically interfere with the dissociation of G α from G $\beta\gamma$; therefore, sufficient G α and G $\beta\gamma$ subunits remain free to function normally; and (ii) the phenotype differences that are observed may be due to the dosage effect of the introduced copy of *magB*^{G42R}. Recently, it has been recognized in higher eukaryotes that the G $\beta\gamma$ subunits function independently from the G α subunit and each regulates respective effectors, including the adenylate cyclases, phospholipases, and different ion channels (Clapham and Neer 1997; Hamm 1998). In filamentous fungi, such as *C. parasitica*, the G β subunit has been demonstrated to regulate several biological processes, including pigmentation, asexual reproduction, and fungal virulence (Kasahara and Nuss 1997). This may suggest that G protein signaling pathways in filamentous fungi involve components in addition to G α to regulate downstream effectors, as previously described. Studies on the G protein signaling in mammalian cells indicate that G $\beta\gamma$ is involved in communicating G protein-coupled receptors to intracellular MAPK and Jun kinase pathways (Gutkind 1998; Koch et al. 1994). Similarly, *STE4* (G β) and *STE18* (G γ) have been shown to be involved in pheromone or mating responses in *S. cerevisiae* by activating a MAPK cascade (Akada et al. 1996; Inouye et al. 1997; Kurjan 1992; Leberer et al. 1997; Leeuw et al. 1995; Whiteway et al. 1995). In *M. grisea*, two

MAPK genes, *PMK1* and *MPS1*, have been identified (Xu and Hamer 1996; Xu et al. 1998). Gene disruption resulted in impaired appressorium formation or appressorium penetration with a reduction in virulence. Interestingly, both mutants were able to respond to exogenous cAMP for at least the early stages of appressorium development, indicating potential interactions between the cAMP/PKA pathway and the MAPK signaling cascades (Kronstad et al. 1998). The G β subunit has been demonstrated to regulate cellular levels of cAMP by direct interaction with adenylate cyclases (Chen et al. 1995, 1997; Yan and Gautam 1996). Both *magB*^{G42R} and *magB* null mutations yield phenotypes similar to the *mac1* null mutants (Choi and Dean 1997), and exogenous cAMP is able to restore the defect in appressorium development in the *magB* null mutants; therefore, it is tempting to suggest that G protein signaling is mediated through the cAMP/PKA signaling pathways in *M. grisea*. cAMP/PKA signaling pathways have been demonstrated to regulate developmental processes in filamentous fungi, including dimorphic development in *U. maydis* (Durrenberger et al. 1998; Gold et al. 1994, 1997; Regenfelder et al. 1997) and hypovirulence in *C. parasitica* (Chen et al. 1996; Gao and Nuss 1996; Kasahara and Nuss 1997). However, we were unable to demonstrate significant and reproducible changes in endogenous levels of cAMP in *magB*, *magB*^{G42R}, and *magB*^{G203R} mutants compared with the wild type. Thus, the mechanism or mechanisms connecting the cAMP/PKA pathway with upstream regulatory elements and with MAPK pathways remains to be clearly defined.

Results from this current study also provide deeper insight into the mechanisms regulating appressorium formation. Mutations presumably leading to constitutively active G α signaling bypassed the requirement for surface hydrophobicity to induce appressorium formation, although infection-related development was delayed. Furthermore, *magB*^{G203R} mutants, in which the G protein signaling is expected to be constitutively off, were still able to respond to surface hydrophobicity for appressorium development. Thus, although G protein signaling plays a major role in surface hydrophobicity sensing for appressorium formation, it appears that other signaling pathways that interact and coordinate together with the G protein signaling for detecting contact surfaces for the infection development are involved. Alternatively, surface sensing involving other G protein signaling that involves other G α subunits, such as *MAGA* and *MAGC*, may be activated to compensate for *magB* mutations, although disruption of each gene failed to reveal significant effects on appressorium formation.

It is also interesting that, unlike wild-type germ tubes, the germ tubes of the *magB*^{G42R} mutants were long and straight on both hydrophobic and hydrophilic contact surfaces. A similar phenomenon was also observed from studies of *magB* mutants. This may suggest that, as in yeast (Cabib et al. 1998), the development of the actin microfilaments in *M. grisea* is also regulated by the G protein-signaling pathway. The effect on hyphal growth is further supported by observations of vegetative growth on solid media. The *magB*^{G42R} mutants show elevated aerial hyphae development on nutrient-rich complete medium, whereas, on nutrient-poor V8 agar medium, aerial hyphae were less abundant and the center of colonies autolysed. These results suggest that constitutive G protein signaling not only promotes vegetative development but also likely modifies the cell wall composition of the

vegetative mycelia. Regulation of vegetative development by G protein signaling has been reported from several filamentous fungi. For example, in *A. nidulans*, mutational activation of the G α leads to an abundant mycelial growth and sporulation inhibition; in contrast, inactivating G protein signaling reverses the effects by repressing mycelial growth but promotes conidiation (Yu et al. 1996). Disruption of G α -encoding *CPG-1* in *C. parasitica* represses mycelial growth; whereas disruption of G β -encoding *CPGB-1* stimulates vegetative development (Gao and Nuss 1996; Kasahara and Nuss 1997). Deletion of G α -encoding *GNA-1* gene in *N. crassa* appears to disrupt apical extension, whereas constitutive G α signaling promotes aerial hyphae production (Ivey et al. 1996; Yang and Borkovich 1999). However, the effect of the G protein signaling on the cell wall composition has been rarely addressed. Alternatively, it is possible that constitutive G protein signaling in nutrient-poor conditions induces higher levels of cell wall-degradation enzymes that lead to suicide-mimic autolysis of mycelia. It has been demonstrated in filamentous fungi that G protein signaling is involved in regulating enzyme activity and the biosynthesis of the secondary metabolites (e.g., *GDPI* in *Schizosaccaromyces pombe* and β -1,3-glucan synthase in *S. cerevisiae*) (Cabib et al. 1998), mycotoxin production in *A. nidulans* (Hicks et al. 1997), and pigmentation of the vegetative cells in *U. hordei* (Lichter and Mills 1998).

Our studies with different *MAGB* alleles also allow further dissection of involvement of the G protein signaling in conidiation. Mutations that are presumed to lead to constitutive G protein signaling in the *magB*^{G42R} mutants reduces conidiation, whereas mutations presumed to repress G protein signaling through interference with the dissociation of the α subunit from the $\beta\gamma$ subunit in *magB*^{G203R} mutants allow normal conidiation. Thus, as in *A. nidulans* (Yu et al. 1996), G protein signaling in *M. grisea* appears to function as a negative regulator of asexual differentiation. However, the role of G protein signaling in conidiation appears complex. For example, *magB*^{G42R} mutants appear to produce conidiophores, which are highly melanized at their tips, even under growth conditions that normally repress conidiation, such as in liquid media and dark growth cultures. Thus, asexual reproduction in *M. grisea* appears to be modulated by a collaboration of complex regulatory mechanisms in which G protein signaling may play an important role in initiating conidiation and promoting spore maturation (including melanization) while repressing the formation of conidia from conidiophores. Regulation of conidiation by G protein signaling may be through the cAMP/PKA signaling pathway or the MAPK signaling cascades because mutants deficient in the adenylate cyclase (encoded by *MAC1*) and a mitogen-activated protein kinase (encoded by *MPS1*) are also repressed in conidiation (Choi and Dean 1997; Xu et al. 1998).

In summary, results from this study not only implicate the significant role that G $\beta\gamma$ may play in the G protein signaling but also highlight the complexity of the regulation of developmental processes even in so-called simple eukaryotes. A number of questions remain to be resolved. How does G protein signaling play apparently contradictory roles during different developmental stages? What other regulatory mechanisms are also involved? How do these different regulatory pathways interact with each other? More genes associated with development in *M. grisea* are continuing to emerge.

Further analyses of the expression of these genes in different genetic backgrounds should reveal a more comprehensive and potentially clearer picture about development in this important phytopathogenic fungus.

MATERIALS AND METHODS

Culture strains, maintenance, and growth.

M. grisea strains 70-15 (MAT1-1) and 70-6 (MAT1-2) were maintained on complete agar medium (CM). Cultures for genomic DNA isolation and protoplast preparation were grown in liquid complete medium in a 150-rpm shaker at room temperature for 3 to 4 days. The *magB* 2-11 strain previously created from 70-15 (Liu and Dean 1997) was maintained on CM supplemented with hygromycinB (200 µg/ml). The same medium was also used for maintaining the *magB*^{G42R} and *magB*^{G203R} mutants created in 70-15. Three independent transformants for each mutation, including *magB*^{G42R}-C2, *magB*^{G42R}-T1, and *magB*^{G42R}-T2, and *magB*^{G203R}-C1, *magB*^{G203R}-T5, and *magB*^{G203R}-T6, were used for phenotype analyses. The *magB*^{G42R} and *magB*^{G203R} mutants created in the *magB* 2-11 strain were maintained in CM supplemented with Ignite (bialaphos; 600 µg/ml) (Avalos et al. 1989; Liu and Dean 1997). Phenotype analyses of the mutants from *magB* background included four independent transformants of each type of mutation. Conidia were prepared from 7- to 10-day-old cultures grown on V8 agar medium with constant fluorescent light in a 20°C incubator.

Cultures prepared for cellular cAMP assay were grown in liquid CM or V8 medium for 16 and 48 h for late germling and mycelia stages, respectively. Harvested biomass was quickly frozen in liquid N₂ and stored at -80°C. Colonies from different *magB* strains were also grown on CM or V8 plates at 20°C with constant fluorescent light for 7 days. Mycelia and conidia were scraped from plate surfaces and quick frozen with liquid nitrogen and stored at -80°C.

Site-directed mutagenesis of the *magB* gene.

Two dominant single nucleotide point mutations were created from the *MAGB* gene by using the GeneEditor in vitro site-directed mutagenesis system (Promega Corp., Madison, WI, U.S.A.) (Fig. 1). The first construct, G42R, replaced the nucleotide G 1091 with C and therefore converted Gly 42 to Arg. This particular glycine is required for GTPase activity so that this mutation was expected to disrupt the GTPase and lead to a constitutively active G protein. A second silent mutation replaced T 1093 with G, thereby introducing a novel *Sma*I site. The second construct, G203R, replaced G 1659 with C and converted the corresponding Gly 203 to Arg. Gly 203 is in the conserved DXXG domain of the Gα subunit, which is required for the conformational switch accompanying GTP binding and for dissociation from the Gβγ complex. This mutation was expected to inhibit dissociation of βγ from the α subunit and cause a constitutively inactive G protein. This mutation also resulted in the loss of a *Bal*I site.

The two oligomers used to introduce mutations are G42R (GACTTTCGGATTCCCGGGCTCCTGTAGACCGAGG), which introduces a novel *Sma*I site (underlined), and G203R (CGCTCAGAACGTTGGCGACCAACATCGAACATTTCG), which eliminates the endogenous *Bal*I site (underlined). Both 35-mers were purified by polyacrylamide gel electrophoresis and 5'-phosphorylated (Integrated DNA Technologies, Inc.,

Corvalville, IA, U.S.A.). The plasmid p12_{2,3}, which includes a 4.3-kb *Hind*III fragment containing *MAGB* in the Bluescript KSII(+) vector, was prepared from *Escherichia coli* JM109. This host is modification (+) and protected the plasmid from being restricted by the modification (+) BMH71-18 *mutS* strain during the site-directed mutagenesis process.

The mutagenesis reaction utilized T4 DNA polymerase, which has 3' to 5' exonuclease proofreading activity for high fidelity and reduced undesired nucleotide-misincorporation errors. The mutagenesis reaction was modified from the procedure recommended by Promega. In brief, *MAGB* plasmid DNA (approximately 2 µg) was alkaline-denatured followed by neutralization and then annealed with a mutagenic oligomer and the selection oligomer (included in the kit) by heating at 80°C for 10 min followed by slow cooling (1.5°C/min) to room temperature. T4 DNA polymerase and T4 DNA ligase were then introduced for synthesizing the mutant strand DNA. The in vitro synthesized DNA (heteroduplex) was then electroporated into BMH71-18 *mutS* cells followed by growing overnight in 5 ml of Luria broth in the presence of selection. Plasmid DNA was prepared from the culture and diluted to 5 pg/µl, after which 10 pg of each DNA was used for transformation by heat shock procedure into JM109 cells. Thirty-six drug-resistant clones from each G42R and G203R transformation were randomly chosen for plasmid miniprep followed by restriction digest analysis. In all, three G42R clones showed the expected additional *Sma*I site and 11 G203R clones had lost the *Bal*I site. The desired mutations were confirmed by sequencing the entire coding region of the *MAGB* gene using sequence specific primers on ABI 377 instruments (Applied Biosystems, Foster City, CA, U.S.A.). pG42R-I and pG203R-I, which contained the desired *magB*^{G42R} and *magB*^{G203R} mutations, respectively, were digested with *Hind*III and the modified 4.3-kb fragments were subcloned into a unique *Hind*III site in pAN7-1 vector to create pG42R-II and pG203R-II.

Transformation of *M. grisea*.

The plasmids pG42R-II and pG203R-II were independently transformed into *M. grisea* by following a modified polyethylene glycol transformation procedure (Liu and Dean 1997). In most experiments, protoplasts were prepared by Novozyme 234 digestion (20 mg/ml; InterSpex Products, Foster City, CA, U.S.A.), whereas, in some cases, β-D-glucanase (10 mg/ml; InterSpex Products) in a solution of 50 mM potassium citrate, 600 mM potassium chloride, and 10 mM EDTA, pH 5.8, with gentle shaking for 2 h at room temperature, was used. Protoplasts made from either enzyme appeared to have similar transformation efficiencies and no significant increase in mutation rate was observed using protoplasts released by glucanase. Protoplasts (2 × 10⁶ cells) were transformed with 10 µg of non-linearized mutation plasmid DNA, and mixed with 40 ml of molten TB3 agar medium containing hygromycin at 200 µg/ml before being plated onto three plates (9 cm in diameter). Cultures were incubated at 20°C in the dark for 4 to 6 days, and putative hygromycin-resistant transformants were subcultured on CM and V8 medium for further characterization. Hygromycin was maintained in the media to suppress spontaneous reversion events.

In independent experiments, the effects of the dominant mutations were examined in a *magB* null mutant genetic background previously created by gene disruption (Liu and

Dean 1997). Both mutation plasmids, pG42R-I and pG203R-I, were independently cotransformed with 5 µg of pCB1269 into the protoplasts made from the *magB* strain 2-11. The cotransformation procedure was the same as described above, except that 40 ml of the defined complex medium supplemented with Ignite (bialaphos) at 300 µg/ml was used (Leung et al. 1995). Putative transformants were transferred to V8 agar medium containing Ignite at 300 µg/ml for further analyses. All transformants were purified by single spore isolation.

Fungal DNA isolation and manipulation.

Fungal genomic DNA was isolated from independent transformants by following the procedure of Weiland (1997). DNA concentrations were estimated by comparing band intensity with DNA standards on agarose gels. *Sma*I and *Bal*I were used to digest 5 µg of each genomic DNA in separate reactions followed by electrophoresis in a 1.0% agarose gel in 1× Tris-acetate-EDTA buffer. Following transfer to Hybond N(+) (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.), membranes were probed with the vector plasmid pAN7-1 and the 4.3-kb *Hind*III fragment of the *MAGB* gene using standard high-stringency conditions. The membranes containing DNA isolated from the mutants created from the *magB* 2-11 by cotransformation were hybridized with both the *bar* gene and the 4.3-kb *Hind*III fragment of the *MAGB* gene as described above. Membranes were washed with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min followed by another wash with 1× SSC and 0.1% SDS at 65°C for 30 min, before being exposed to Fuji X-Ray films (Fuji, Tokyo, Japan).

cAMP assay.

Fungal tissue was ground to powder by mortar and pestle in liquid nitrogen, mixed with 10 volumes of 6% (wt/vol) trichloroacetic acid, and incubated on ice for 30 min with periodic mixing by vortex. Cell-free extracts were prepared by centrifugation at $2,000 \times g$ at 4°C for 15 min. Supernatants were further extracted four times with five volumes of water-saturated ethyl ether. Samples were dried by lyophilization and stored at -20°C. The level of cAMP in each sample was quantified by using the cAMP enzymeimmunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) following procedures recommended by the manufacturer. Protein quantitation of the sample was determined by the method of Bradford (1976) using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Osmosensitivity assay.

Small agar blocks were cut from the edge of 10- to 14-day-old colonies of each strain and point-inoculated onto the center of a freshly prepared CM and V8 medium in petri plates (9 cm in diameter) with or without 3% NaCl. Cultures were incubated at 20°C with constant fluorescent light. The size and morphology of the colonies were examined every 5 days for 15 days. Three independent transformants of each mutant and the control strains, including the wild-type strain 70-15 and one hygromycin-resistant ectopic transformant from an unrelated experiment, were tested in three separate experiments.

Conidiation assay.

Small agar blocks were prepared as described above and point-inoculated onto the center of a freshly prepared V8 agar plate (9 cm in diameter) and incubated as previously described for 14 days. Conidia were harvested by flooding the culture with sterile distilled H₂O, released using a rubber policeman, filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.), rinsed with distilled H₂O, concentrated by centrifugation ($7,649 \times g$ [max] at 4°C for 10 min in a Sorval SS-34 rotor [Sorval, Newtown, CT, U.S.A.]), and resuspended with 0.5 ml of H₂O. Spore concentration was counted using a hemocytometer. Five plates were used for each strain, including controls as described above. The experiments were repeated three times.

To test the light requirement for conidiation, two identical sets of plates from three independent isolates of each transformation treatment and the appropriate controls were initiated by point inoculation in V8 agar medium. One set was kept from light in a dark box and the other was light exposed in the same 20°C incubator for 14 days. Conidia were examined as described above.

Appressorium formation assay.

Conidia were collected from 7- to 10-day-old cultures on V8 agar medium as previously described. For each assay, 1×10^3 conidia was included in a 60-µl drop assay on GelBond membranes. To test the induction of appressorium formation on the hydrophobic surface, conidia were mixed with sterile distilled H₂O. On the hydrophilic surface, other treatments included 5 mM N6-cAMP, 2.5 mM IBMX, and 1 µM diol. Ethanol (10 and 1%) was used as the controls for IBMX and diol treatments, respectively. All assays were incubated in a moisture chamber at room temperature for 20 h unless otherwise indicated, and appressorium formation was observed under a microscope. Appressorium formation was also assayed on the surface of 1 × 1-cm pieces of onion peel as described by Mitchell and Dean (1995). Each experiment was done in triplicate and was repeated at least three times.

Pathogenicity assay.

Seedlings (2 to 3 weeks old) of the blast-susceptible rice variety S-201 were grown in the greenhouse. Spore suspensions were made from 10-day-old cultures of each strain in 0.05% Tween 20. Three independent isolates of each transformant were tested. Controls included the *magB* 2-11 and 70-15 strains.

For spray inoculation, spore suspensions were adjusted to 1×10^5 per milliliter in 0.05% Tween 20 and 5 ml of each treatment was evenly sprayed onto plants with a fine mist aspirator. The inoculated plants were then covered with plastic bags and incubated in a growth chamber at 25°C and 90% humidity in the dark for the first 24 h, followed by a 12/12-h light/dark cycle. Lesion development was observed daily. Photographs were taken 7 days after inoculation.

Spore suspensions (50 µl) were also injected into the leaf sheath in between the first and second leaf using a 26 gauge 1/2-inch needle and 1-ml syringe. Injected plants were incubated at 90% humidity, with a 12/12-h dark/light cycle in a growth chamber at 25°C. Disease symptoms were observed daily and scored 7 days postinjection. Each treatment included 25 seedlings, and the experiments were repeated at least three times.

To examine the identity of *M. grisea* from infected leaves, 10 conidia were further isolated from each lesion and five

lesions were analyzed from each mutation. The single spore colonies were examined for hygromycin sensitivity, conidiation, mischeduled melanization, and autolysis.

Sexual crosses.

Three-way crosses were performed to examine the effect of the *magB*^{G42R} and *magB*^{G203R} mutations on sexual reproduction. Both mating type strains, represented by 70-15 and 70-6 isolates, and the mutants (five isolates from each mutation) were point-inoculated, 5 cm apart, on oatmeal agar medium and incubated at 20°C under constant fluorescent light for 3 to 4 weeks. To avoid potential cross-contamination of the cultures inside each plate, plates were inverted. Mature perithecia were crushed to examine the asci and ascospores approximately 20 to 25 days postinoculation. Each cross was done in triplicate and the experiments were repeated three times.

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