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G Protein α Subunit Genes Control Growth, Development, and Pathogenicity of *Magnaporthe grisea*

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Three G protein α subunit genes have been cloned and characterized from *Magnaporthe grisea: mag*A is very similar to *CPG-2* of *Cryphonectria parasitica; mag*B is virtually identical to *CPG-1* of *Cryphonectria parasitica,* to *gna1* of *Neurospora crassa,* and to *fadA* of *Emericella nidulans;* and *mag*C is most similar to *gna2* of *Neurospora crassa*. Homologous recombination resulting in targeted deletion of *mag*A had no effect on vegetative growth, conidiation, or appressorium formation. Deletion of *mag*C reduced conidiation, but did not affect vegetative growth or appressorium formation. However, disruption of *mag*B significantly reduced vegetative growth, conidiation, and appressorium formation. *mag*B$^-$ transformants, unlike *mag*A$^-$ and *mag*C$^-$ transformants, exhibited a reduced ability to infect and colonize susceptible rice leaves. G protein α subunit genes are required for *M. grisea* mating. *mag*B$^-$ transformants failed to form perithecia, whereas *mag*A$^-$ and *mag*C$^-$ transformants did not produce mature asci. These results suggest that G protein α subunit genes are involved in signal transduction pathways in *M. grisea* that control vegetative growth, conidiation, conidium attachment, appressorium formation, mating, and pathogenicity.

Additional keywords: cAMP, *Pyricularia oryzae*.

Magnaporthe grisea (T. T. Hebert) Yaegashi & Udagawa (anamorph *Pyricularia oryzae* Cavara) causes rice blast disease, which severely reduces rice (*Oryza sativa* L.) production and continuously threatens food supplies worldwide, especially in many Asian nations (Ou 1985; Teng 1994). Controlling this fungal disease is an ongoing priority of plant pathologists (Ou 1985; Dobinson and Hamer 1992; Ford et al. 1994; Teng 1994). Recently, considerable research has been focused on the molecular mechanisms of rice blast infection (Valent 1990; Bourett and Howard 1990; Lee and Dean 1993; Talbot et al. 1993; Mitchell and Dean 1995; Shi and Leung 1994; Xu and Hamer 1996). The infection process is composed of several key steps: conidiation, conidia dispersal, conidium attachment to the host surface, appressorium formation, penetration, and invasive growth. The goal is to fully understand the mechanisms crucial for each key step and use this information to develop durable and environmentally sound new strategies to control this devastating disease.

Previous research has demonstrated that the formation of the appressorium, a specialized infection cell, produced by *M. grisea* can be induced by environmental signals, including both physical (Bourett and Howard 1990; Dean 1992; Lee and Dean 1994; Gilbert et al. 1996) and/or chemical (Gilbert et al. 1996) cues. cAMP and signal transduction pathways have been shown to be central to appressorium formation (Lee and Dean 1993; Mitchell and Dean 1995; Xu and Hamer 1996). Taken together, these findings suggest that *M. grisea* conidia and/or germ tubes possess receptor(s) that sense suitable host plants in order to initiate the infection process. Regulatory guanine nucleotide-binding proteins (G proteins) are likely candidates to play an important role in the signal transduction cascade.

G proteins are involved in the transduction of signals from activated cell-surface receptors to intracellular effectors, such as adenylate cyclase, phospholipases, kinases, and ion channels (Stryer and Bourne 1986; Gilman 1987; Simon et al. 1991). Many of these G protein–mediated signals are essential for proper regulation of cell function, division, and differentiation.

The structure and function of heterotrimeric G proteins has been extensively reviewed (Gilman 1987; Strathmann et al. 1989; Simon et al. 1991; Coleman and Sprang 1996). The G protein complex of α, β, and γ subunits is anchored to the cytosolic face of the plasma membrane through hydrophobic regions on the β and γ subunits. The interaction of a G protein with an activated agonist-receptor complex promotes the exchange of GTP for bound GDP on the α subunit. This exchange causes a conformational change by dissociation of the α and βγ subunits that enables the α subunit (or in some instances the βγ complex) to modulate the activity of the appropriate effector protein. Therefore, the α subunit is responsible for the specific interactions with both the receptor and effector molecules. The βγ subunit complex is usually responsible for inactivation of the α subunit and is also necessary for interaction of the G protein with receptors (Gilman 1987; Strathmann et al. 1989). The lifetime of the activated α subunit is inherently set by its GTPase activity. The cycle is thus reset when GTP is hydrolyzed to GDP.

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession numbers AF011340 (*magA*), AF011341 (*mag*B), and AF011342 (*mag*C).

Numerous genes encoding the G protein subunits have been cloned, especially for the α subunits. Sequence comparisons have identified three major groups of Gα genes, termed GPA, Gα-I, and Gα-II. The GPA group is primarily composed of Gα genes from fungi and plants. Within both Gα-I and Gα-II, four classes have been identified that have functional similarities: Gi, Gq, G12, and Gs (Wilkie and Yokoyama 1994). Certain Gαs appear to be restricted to a particular cell type and are involved in a single function (Brown and Birnbaumer 1990). In contrast, other α subunits are ubiquitous and serve multiple functions (Lerea et al. 1988). Sequence alignment of α subunit genes from fungi, plants, and animals reveals several highly conserved amino acid motifs that are thought to contribute to GTP binding. Degenerate polymerase chain reaction (PCR) primers, which target these motifs, have been used to selectively amplify genes encoding G protein α subunits from many organisms (Strathmann et al. 1989; Yatani et al. 1988; Tolkacheva et al. 1994). Several G protein α subunit genes have been isolated from filamentous fungi such as *Emericella nidulans* and *Neurospora crassa* as well as from plant pathogens such as *Cryphonectria parasitica* and *Ustilago maydis* (Choi et al. 1995; Regenfelder et al. 1997; Turner and Borkovich 1993; Yu et al. 1996). Growing evidence has revealed that Gα proteins are involved in regulating both development and pathogenicity. Choi et al. 1995) and Chen et al. 1996) reported that hypovirulence of the chestnut blight fungus *Cryphonectria parasitica,* which results from harboring RNA viruses of the genus *Hypovirus,* is mimicked by suppressing the expression of or deleting the G protein α subunit gene *CPG-1.* CPG-1 appeared to function as a negative modulator of adenylate cyclase. In *U. maydis,* disruption of the G protein α subunit gene *gpa*3 resulted in strains that were unable to respond to pheromone and were thus mating deficient. Gpa3 is also required for pathogen development (Regenfelder et al. 1997).

Here, we report on the identification of three G protein α subunit genes from *M. grisea* and present the effects of targeted deletion or disruption of these genes on growth, development, and pathogenicity.

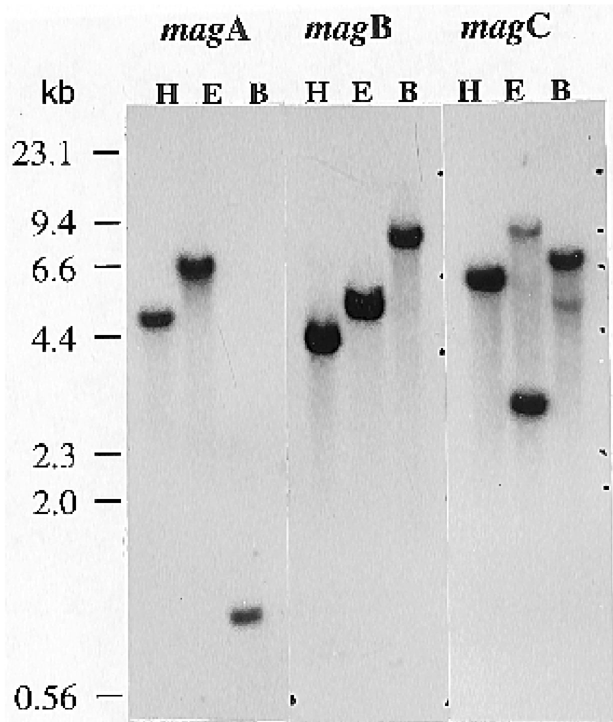

Fig. 1. Southern blots of *M. grisea* wild-type strain 70-15 genomic DNA, which had been digested with *Hin*dIII (H), *Eco*RI (E), or *Bam*HI (B) and probed with ^{32}P-labeled *p*SL 1-1 (*mag*A), *p*SL 2-3 (*mag*B), and *p*SL 8 (*mag*C), respectively. Blots were hybridized at 55°C as described in Materials and Methods.

RESULTS

Identification of G protein α subunit genes in *Magnaporthe grisea*.

Putative G protein α subunit gene fragments were obtained by PCR amplification of *M. grisea* genomic DNA with degenerate oligonucleotide primers to conserved regions in mammalian G protein α subunit genes. One forward and two different reverse primers were used. The PCR products were cloned into *p*Bluescript II KS$^+$. The relationship among the ≈200 recombinant colonies was determined by Southern hybridization with both the PCR products and representative clones as probes. Three distinctive groups of clones were found. Representative clones were sequenced, and three G protein α subunits were identified:, *p*SL 1-1, *p*SL 2-3, and *p*SL 8. All three were highly similar to the conserved portion of mammalian subunits, but were different from each other. Percent identities of amino acid sequence between *p*SL 1-1 and *p*SL 8, *p*SL 1-1 and *p*SL 2-3, and *p*SL 8 and *p*SL 2-3, were about 60% (40/67), 70% (47/67), and 67% (45/67), respectively.

Southern blot analysis of restriction enzyme–digested *M. grisea* genomic DNA confirmed that the cloned PCR DNA fragments were present in the *M. grisea* genome (Fig. 1). Clamped homogeneous electric field (CHEF) gel analysis revealed that the three G protein α subunit clones are located on three separate chromosomes (data not shown). All three genes are single copy in the *M. grisea* genome.

Nucleotide sequence and features of *mag*A, *mag*B, and *mag*C.

Cosmid clones corresponding to the three G protein α subunit PCR fragments were identified by Southern hybridization analysis and *Hin*dIII fragments were subcloned into *p*Bluescript II KS$^+$ (*p*B11 1-1, *p*E12 2-3, and *p*D10 8). The nucleotide sequence and deduced amino acid sequence for three G protein α subunit genes named *mag*A, *mag*B, and *mag*C are shown in Figure 2. Amino acid sequence comparison between *mag*A and *mag*B, *mag*A and *mag*C, and *mag*B and *mag*C revealed relatively low levels of similarity: 48, 40, and 48%, respectively. However, as described below, each deduced polypeptide is closely related to other G protein α subunit genes found in fungi.

*M. grisea mag*A encodes a polypeptide of 356 amino acids, contains five introns, and has 91% amino acid identity to *CPG*-2 of *Cryphonectria parasitica; mag*B encodes a poly- peptide of 353 amino acids, contains three introns, and has 99% identity to *CPG*-1 of *C. parasitica,* 98% identity to *gna*1 of *Neurospora crassa,* and 93% identity to *fad*A of *Emericella nidulans; mag*C encodes a polypeptide of 355 amino acids, contains four introns, and has 81% identity to *gna*2 of *N. crassa*.

***mag*A**

```
       ctgtactact taggtctagg gacccagtag attgttttct tatcctcgaa acgagaccgc caaatttacg gtcgcactgc tcgtttgctc tctgctccgt
       tgtcgcatct tgcagagtgc actagattga cacagcaatt cccaccagcc acccgccccc ccaagggttt gtttccgatc gagcgggact gattaatcag
       gattgtcgtc caattcctgc tgcaaaccgg ccgtacatac acaaccgact ctgtcatcaa ccaatacgtc tttgtcttgg tgccgctcac taaaccgcct
       atacctccga gccatcgaat ttctggcccg ggacctatcc ggctaaaata tccccactcc gacgacgtat ttggcgtctt gcctcgctgg cgaaagtcta
       cttgaccccc acgccctggc acagttggcg atcaagttgc tatcccgggc gacccgtgtc cgcccgtcta tcacaatcgc tcgccagccg tttgttgcac
       agtcctgctg tcacaggcca gctacgtgct gtcttaacaa ttcttccacg acttgcgatc cgatcgctgc agccggcagc cttccatctc gggtaatcga
       acgaacgacg aagcatatcc catccgctct cgacaacaaa gcatccgcaa cgggggactt gcatctccta cgttctgcta gacagaaagg tttatacgcg
       acgactcgtc gatgggatcc gattaggctg ggtgcgttgg ccattgtaag ccgcccgggc aacgaccttt gccagcgcag cgcgcagccc ctcgagtccc
-80    aggccccacg ccattaaggg cgcaacgaaa gaggccttga aacccaatac aattgggttc cctggccaac aggaccattc ATGATGGGCG CTTGCATGAG
1                                                                                          M  M  G  A    C  M  S
21     TGCGAGCTCG GAGGAGTCGG AACAGAAGAA ACGCAGTCAA AAGATTGATA AGGATCTCGA GGAGGACTCA AAGAGATTGA GGCGAGAATG CAAGATTCTA
8       A  S  S    E  E  S  E    Q  K  K    R  S  Q    K  I  D  K    D  L  E    E  D  S    K  R  L  R    R  E  C    K  I  L
121    CTGCTTGgta agtttacctc gctctgtctt gacgagaggt tggaaacgac gaggggtcat ttctcttaac aaattcacta caggttggaa acgacgaggg
41     L  L  G                           INTRON I
221    tcatttctct taacaattca ctacagGTTC TGGCGAGAGT GGCAAGTCCA CAATCGTCAA GCAGATGAAG ATTATACATC TGAAAGGCTA CTCGGATGAC
43                                  S    G  E  S    G  K  S  T    I  V  K    Q  M  K    I  I  H  L    K  G  Y    S  D  D
321    GAACTTTACA ACTACCGGCC AACGGTCTTC AAGAATTTAA TTGAATGTGC GAAAGCCGTG ATCAGCGCCA TGAGGCAATT CGACATAGAA CCGCAGCTGG
68     E  L  Y  N    Y  R  P    T  V  F    K  N  L  I    E  C  A    K  A  V    I  S  A  M    R  Q  F    D  I  E    P  Q  L  E

421    AAGGGAACAA AGAACTTTGC GACTATCTGC TCGACTACAT GGTCGAGTCG GGACCACAGG CAGAGATAGA CCCCAAGGTC GGTCAAGCTG TGCAGTCAAT
102     G  N  K    E  L  C    D  Y  L  L    D  Y  M    V  E  S    G  P  Q  A    E  I  D    P  K  V    G  Q  A  V    Q  S  I
521    ATGGAATGAT CCTGCAAGGG AACAGTTGAT GGACAGGCAG ACCGAGTTTT ACCTGATGGA CTCGGCAGAA TAgtgagttt cggcttggaa atgctggaga
135    W  N  D    P  A  R  E    Q  L  M    D  R  Q    T  E  F  Y    L  M  D    S  A  E    Y                INTRON II
621    taccccgcga cagtatcgca tttggtcgca aagctaacat tggtcgttct cacccaacca gCTTCTTCCA AGAGGTTCTG AGGATTGTCT CTCCCGACTA
159                                                                         F  F  Q    E  V  L    R  I  V  S    P  D  Y
721    CCTGCCCAAT GAGATGGATG TTCTTCGCGC AAGAACAAAA ACTACAGGCA TATACGAGAC ACGCTTTCAG ATGGGCCAAC TGAGCATACA gtaagcaagc
172     L  P  N    E  M  D  V    L  R  A    R  T  K    T  T  G  I    Y  E  T    R  F  Q    M  G  Q  L    S  I  H
821    aaagaatcct gtccgaatat gcacgtgata aatcagatta ctgacttgca ttgcgcgcca cagCATGTTT GATGTAGGAG GCCAGAGGAG TGAGCGTAAG
202              INTRON III                                                      M  F  D    V  G  G    Q  R  S    E  R  K
921    AAGTGGATCC ACTGCTTCGA GAACGTGACA TCCATCATCT TTTGCGTTGC ACTGAGTGAA TACGATCAAG TTTTACTTGA AGAAAGCAGT CAAgtacgtt
214    K  W  I  H    C  F  E    N  V  T    S  I  I  F    C  V  A    L  S  E    Y  D  Q  V    L  L  E    E  S  S    Q
1021   tagcaatagg cccccaggcc aaaaagtgca gttactgctt tgtgacagcc caatgcattc ctattggcgc gcatgctaac ctaatatcgg acatacagAA
245                   INTRON IV                                                                                     N
1121   CCGCATGATG GAAAGCCTTC TGCTCTTCGA TTCGGTGGTA AATTCGAGGT GGTTCATGAG GACCAGTATC ATATTGTTCC TCAACAAGGT GGATATTTTC
246     R  M  M    E  S  L  L    L  F  D    S  V  V    N  S  R  W    F  M  R    T  S  I    I  L  F  L    N  K  V    D  I  F
1221   AAGCAGAAAC TTGGCCGATC GCCTCTCGGC AACTACTTCC CAGATTACTC TGGAGGCAAC GATGTTAACA AGGCAGACAA GTACTTGCTG TGGCGTTTTA
279    K  Q  K  L    G  R  S    P  L  G    N  Y  F  P    D  Y  S    G  G  N    D  V  N  K    A  D  K    Y  L  L    W  R  F  N
1321   ACCAAGTTAA CAGGGCTCAC CTAAACCTAT ATCCTCAgtg agtccaccct agaccccagt gctgctactt aatgatgttg ctgactttta taatcaccca
313     Q  V  N    R  A  H    L  N  L  Y    P  H                      INTRON V
1421   gTCTTACGCA AGCTACCGAC ACCTCGAACA TTCGACTTGT CTTTGCGGCA GTAAAAGAGA CTATACTCAA CAATGCCCTG AAGGACTCAG GTATTCTGTA
325     L  T  Q    A  T  D    T  S  N  I    R  L  V    F  A  A    V  K  E  T    I  L  N    N  A  L    K  D  S  G    I  L  *
1521   Atccgcagca attctgacct acatttcat tctagttcct tccttatacg cccaaccttt ggaccttatg tttttattg ataccacgat caacttcata
       ctgaatctat cttgccacgg gacttatatt atactagcat cagctcggag ttatggtggg gcggcttctg ctgttatgac tacaaaacaa tttccctcaa
       ttcttttcgg tcaaccttat
```

***mag*B**

```
       cgatttgatt ctgtcgccgc gatattctcc tctccctcct aacagtcaaa atattgcgat aacacatttt aggtacgaat gtcaatcgag gttataagag
       ggaatcatac caaaataaaa ataaaaagac aaaaacatca gactgaatga ctgagagctg gcccccccga tcagcagtcg cccatcgaat taattgactt
       gttgacctgg ttcgagctgg gttgcaaaga acaattgggt ggagacgacc aaggatccgc aacgcaggag aagtcgctgt acctagcgca gattggccag
       accctggccc acgcccggcc cccctctca aattacagtc ctttgttctc tccaccaatt ttcgcagtcc ccatttcaag ttcattcctc ccggcagcac
       cctattgatt cgtgtgccga acaccttatt cttccgaaga agacgaaacc acgccgtctt cagccatcgc gtgcttcacc atcaccttcc ttttgaattt
       ttcctacttt gtttgtttgt aaacccgcta ccaagccgtc accggatagg ctcctgttgg aattcgggcg acgctggcta cttgtcaatt gcgcttattg
       taggcctgcc caattcccaa ttccccccct tccggtcgca tgcacaaaca attcagctgc cgtccacctc cctgcgaccc tgtcactagg tcgacgtttt
       gatgccgagc caaatcgaaa agcaaatcct ctcgccacac tggacacgga cacccgctga cacgccattg cgaacagtgt aatactctgg gtttctcgaa
-52    tctacctaac catcgcttgt attaattatc gttcgacaaa caatcggcca caATGGGTTG CGGAATGAGC ACGGAGGAGA AGGAGGGCAA GGCCCGGAAC
1                                                            M  G  C    G  M  S    T  E  E  K    E  G  K    A  R  N
49     GAGGAGATTG AGAACCAGCT CAAGAGGGAC AGGTTGCAAC AGCGCAATGA GATCAAGATG CTTCTGCTCG gtacgttgaa gcgaggacgc tcatcgacgg
17     E  E  I  E    N  Q  L    K  R  D    R  L  Q  Q    R  N  E    I  K  M    L  L  L  G
149    gggccacgat gcccgcgatc aaggtttttt ttttctttat gtcgctaaca tcctcggtct acagGAGCCG GTGAATCCGG AAAGTCCACC ATTCTCAAGC
41                        INTRON I                                       A  G    E  S  G    K  S  T    I  L  K  Q
249    AGATGAAGCT CATTCACGAA GGCGGCTACT CACGGGACGA GCGCGAATCT TTCAAAGAAA TCATCTTCAG CAACACTGTA CAGTCTATGC GTGTCATCTT
53      M  K  L    I  H  E    G  G  Y  S    R  D  E    R  E  S    F  K  E  I    I  F  S    N  T  V    Q  S  M  R    V  I  L
349    GGAGGCCATG GAGTCGCTCG AGCTGCCTCT GGAGGACCAA AGGATGGAGT ACCACGTACA GACCATCTTC ATGCAGCCTG CTCAGATCGA AGGCGACGTC
86     E  A  M    E  S  L  E    L  P  L    E  D  Q    R  M  E  Y    H  V  Q    T  I  F    M  Q  P  A    Q  I  E    G  D  V
449    TTGCCACCAG AGGTCGGCAA CGCCATCGAG GCCCTGTGGA AGGATCGTGG TGTCCAGGAG TGCTTCAAGC GTTCAAGGGA ATACCAGTTG AACGACTCTG
119    L  P  P  E    V  G  N    A  I  E    A  L  W  K    D  R  G    V  Q  E    C  F  K  R    S  R  E    Y  Q  L    N  D  S  A

549    CCAGATAgta ggttttaccg ttggctcgag gaaaccttat gctgcagcta cccctctgct tgaacgttga atgatgctaa cgcacaatga agCTACTTCG
153     R  Y              INTRON II                                                                            Y  F  D

649    ACAATATTGC TCGTATTGCT GCACCAGACT ACATGCCTAA CGACCAGGAC GTGCTTCGAT CTCGCGTCAA GACAACCGGT ATCACTGAGA CGACTTTCAT
158     N  I  A    R  I  A    A  P  D  Y    M  P  N    D  Q  D    V  L  R  S    R  V  K    T  T  G    I  T  E  T    T  F  I
749    CATTGGCGAC CTCACCTACC GAATGTTCGA TGTTGGTGGC CAACGTTCTG AGCGAAAGAA GTGGATTCAC TGCTTCGAGA ACGTTACAAC GATTCTCTTC
191     I  G  D    L  T  Y  R    M  F  D    V  G  G    Q  R  S  E    R  K  K    W  I  H    C  F  E  N    V  T  T    I  L  F
849    CTCGTCGCCA TTTCCGAGTA CGATCAGCTA TTGTTCGAGG ACGAGACGGT CAACCGTATG CAGGAGGCTC TCACACTATT CGACTCCATC TGCAACTCGA
224    L  V  A  I    S  E  Y    D  Q  L    L  F  E  D    E  T  V    N  R  M    Q  E  A  L    T  L  F    D  S  I    C  N  S  R
949    GGTGGTTCAT CAAAACCTCC ATCACTCTCT TCCTGAACAA GATCGATCGC TTTAAGGAGA AGCTGCCCAT CAGCCCGATG AAGAACTACT TCCCCGACTA
258     W  F  I    K  T  S    I  T  L  F    L  N  K    I  D  R    F  K  E  K    L  P  I    S  P  M    K  N  Y  F    P  D  Y
1049   CGAGGGTGGC GACGACTACG CTGCAGCCTG CGACTACATC CTCAACCGAT TCGTCAGCTT AAACCAGCAC GAGACAAAGC AAATTTACAC ACATTTCACC
291     E  G  G    D  D  Y  A    A  A  C    D  Y  I    L  N  R  F    V  S  L    N  Q  H    E  T  K  Q    I  Y  T    H  F  T
1149   TGCGCCACAG ACACCACACA GATCCGCTTC GTCATGGCGG CTGTTAATGg taagaaatca tgcgtcccgt gattggaatt actgtatgcg cagacattct
324    C  A  T  D    T  T  Q    I  R  F    V  M  A  A    V  N  D                          INTRON III
1249   aactgttaca aatagACATC ATCATACAAG AGAACCTCCG TCTCTGTGGT CTGATCTAAa atcagtgccc gctagagcac catcactacc accctcattt
341                      I    I  I  Q  E    N  L  R    L  C  G    L  I  •
       ctcagctttt ccctcggata cccaatatgc atcaataatc gacgagcctt caggcgatct ggtcctcttg tgagcctctc tgccaacgga gtcacaggcg
       tcttgtactc caccccaaag aagccgggaa aacaaggtcc acggattctt ccaaacgttc ttacttcttt aaacttctgg gtcatctggt cctgtttttg
       tggggttttt tgtggtgttt ttttttactt caaaatcttg ttggattttt attcttttgg gcacccg
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Fig. 2. Nucleic acid and deduced amino acid (italic letters) sequences for *mag*A, *mag*B, and *mag*C. Introns are in underlined lowercase letters. The GenBank accession numbers are AF011340, AF011341, and AF011342, respectively.

Figure 2 continued on next page.

Like *CPG*-1 of *C. parasitica, gna*1 of *N. crassa,* and *fad*A of *E. nidulans, mag*B contains a consensus myristylation site (MGXXXS) (Buss et al. 1987) and pertussis toxin-labeling site (CXXX) (West et al. 1985) at its N and C termini, respectively: features characteristic of members of the inhibitory G (Gi) protein α subunit family of mammals (West et al. 1985; Simon et al. 1991; Spiegel et al. 1991). *mag*A contains a potential consensus myristylation site (MMGXXXS) at its N terminus but does not have a pertussis toxin-labeling site at its C terminus. *mag*C does not contain either site.

Gene deletion of *mag*A, *mag*B, and *mag*C.

To determine the functions of *mag*A and *mag*C, homologous recombination was employed to replace the target gene with a selectable marker. For *mag*B, a fragment internal to the target gene was used for disruption (Timberlake and Marshall 1989). *mag*A was replaced by plasmid *p*B11pan, which contains the hygromycin phosphor transferase (*hph*) gene cassette flanked by 5′ and 3′ sequences from the *mag*A gene (Fig. 3A). Southern analysis confirmed that six out of 65 hygromycin-resistant transformants had undergone the double crossover event to delete the *mag*A gene (Fig. 4A). *mag*C was replaced in a similar manner by plasmid *p*8-8pan, which contains the *hph* cassette flanked by 5′ and 3′ sequences from the *mag*C gene (Fig. 3C). Six out of 54 hygromycin-resistant transformants were confirmed by Southern analysis to be deleted for *mag*C (Fig, 4C). *mag*B was disrupted by plasmid *p*AN2-3-6, which contains an internal ≈0.7-kbp fragment (amino acids 15 to 196) of *mag*B and the *hph* (Fig. 3B). Four out of 92 hygromycin-resistant transformants were shown to be disrupted for *mag*B (Figure 4B). Based on additional genomic Southern analysis, two of these transformants (2-9 and 2-11) had undergone a single plasmid DNA insertion event, whereas the other two (2-2 and 2-12) contained multiple insertions.

Effects of disruption of *mag*A, *mag*B, and *mag*C on fungal growth, conidiation, and appressorium formation.

The effects of gene disruption on fungal growth, conidiation, and appressorium formation are shown in Table 1 and Figure 5, unless otherwise indicated. Targeted deletion of *mag*A did not affect vegetative growth on different media (V8 or CM; data not shown), conidiation, or appressorium formation. Deletion of *mag*C reduced conidia formation by greater than twofold, but did not affect vegetative growth on different media (CM data not shown) or appressorium formation. However, disruption of *mag*B significantly reduced vegetative growth on different growth media, conidiation, and appressorium formation. Following 72 h of incubation, appressorium formation by *mag*B⁻ transformants was still less than 5%.

Targeted deletion/disruption of G protein α subunit genes (*mag*A, *mag*B, or *mag*C) did not affect conidium germination on the hydrophobic surface of GelBond (data not shown). The morphology of germ tubes of *mag*A⁻ and *mag*C⁻ transformants appeared similar to that of wild type (Fig. 5A, C, and D), i.e., the germ tubes were short and curvy; however, the germ tubes of *mag*B⁻ transformants were long and straight (Fig. 5B).

Conidium attachment of *mag*B⁻ transformants was reduced on the hydrophobic surface of GelBond, compared with that

Figure 2 continued from previous page.

***mag*C**

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      tggaggctgg acagataatg tcagcgcaca tacataattt tactgtcctt tactttgggt acacaacacc ggaagagatg tccgttgtag caagccgaag
      caaagccaat ctcccacggc ccgcacacac ctaccgccct ggccctccca gacccttggc atttaaccca gcctaaaatt ctggtgctat ctagtctggt
      tccaggtacc cacctaggta cctatgtatc ttcgtaggct tgcaaggctt ggtaggtaaa cttgcacacc accacggtgc ttgtccttac ccaccttgtc
      caccttgtcc acctatccac ccgctccgta ccttgccgtg cttacatcca ctcccacaaa aacccgtacg gttagcttcc ttgtgtattt cgcttcccgc
      gcctggtatc cgctgcatca cttcatcgac gtgcattgga gatccctcat cctcttatcc gcgccccgct cgcccaagaa caagcaaaag aataataata
      atagggggag ccgcttcctg cagctacccg gattgcgact aacgccatcg cgacccccac ccgtcaattt gtttcattcc atcaccaatt ggttctcctc
      ccatagcagg caagaccgac ccggacctgt cagaagacgt gcgacacaca caaatctcga ccaattatta cctaggtagc cacaaagcga caaacaagtc
      gcacccgaga aatcgaccta gaataggtct ttttactcgt tagttaatac aagcactcag ttgcttcgac ctcatcgaac cacttgtcgg gagcaatccg
-19   ccaattgccc tctggcaagA TGTGCTTCGG CAGCCGCAAC GTCCACGATG AGGCGGGGGC TGCCCGCAGC AGGGAGCTCG ACAAGGCCATC CGCGCCGAC
1                        M  C  F  G   S  R  N    V  H  D  E   A  G  A    A  R  S    R  E  L  D   K  A  I    R  A  D
81    GAGAAGCGCT TGCAAAAGGA GGTCAAACTG CTGCTGCTGG gtatgtcgcc gagcaatgtt tacattcgag gccactggtg atctggtcca aatcggaagc
28    E  K  R  L   Q  K  E    V  K  L   L  L  L  G                       INTRON I
181   actctgctaa ccaagttgtg tttttcgata ccatagGCGC CGGTGAATCG GGAAAATCGA CCGTCCTCAA GCAAATGAAG CTGATATATG CTCAAGGCTT
42                                          A   G  E  S   G  K  S  T   V  L  K   Q  M  K   L  I  Y  A   Q  G  F
281   CAGCAAGAAC GAAAAGATCG AATGGAAGCC TGTCGTGTTT CAACACATTG TGCAATCTTT CAGGCTTATC CATGATGCCA TGCAAGAGTT GGACATCGAA
63     S  K  N   E  K  I  E   W  K  P   V  V  F   Q  H  I  V   Q  S  F   R  L  I   H  D  A  M   Q  E  L   D  I  E
381    TTCGAGAACA AGGAGAACGA Ggtatgttaa aactctgcac tttcctattt catgacaaat aggtctcgcg cactcgattc cctaccggac tcgcccccat
96    F  E  N  K   E  N  E                   INTRON II
481   catcctgagc tccatccttc cgccgccacg aatgcactac aagacaacaa agagcctgcc gggtttgaaa catataacta acatcccatt ttggctacag
581   AGGAACATAA CCCTGATCAT GGAGGAGCAG GAACTCGGCG CGCATGACGC CTTACCAATC GAGTTTCTAG ACCCAATCAA AGCACTATGG GTGGATGCTG
103   R  N  I  T   L  I  M   E  E  Q   E  L  G  A   H  D  A   L  P  I   E  F  L  D   P  I  K   A  L  W   V  D  A  G
681   GCGTGAAGAA GGCGATTGCC AAGGGCAACG AGTTTGCGCT ACATGACAAC CTGGACTAgt gagtgcatta tccccaacct tcggctgatc atgccttgat
137    V  K  K   A  I  A   K  G  N  E   F  A  L   H  D  N   L  D  Y                 INTRON III
781   ttgtgttgct tacgcaggcc ctaatacaca gTTTCTGTGA CGACCTCGAC CGTATTTGGG ATAAGAGCTA CGTTCCGACA GACCAAGATT TGCTCCGCTC
156                                  F  C  D   D  L  D   R  I  W  D   K  S  Y   V  P  T   D  Q  D  L   L  R  S
881   GAGACTCAGA ACGACCGGCA TTACCGAGAC CGTGTTTGAC TTGGGCCAGC TCACATACCG AATGTTCGAT GTCGGTGGCC AAAGGTCGGA AAGGAAGAAG
179    R  L  R   T  T  G  I   T  E  T   V  F  D   L  G  Q  L   T  Y  R   M  F  D   V  G  G  Q   R  S  E   R  K  K
981   TGGATACATT GTTTCGAGAA CGTCAACTGT CTTCTTTTCC TAGTCGCAAT TTCCGGCTAC GATCAGTGTT TAGTTGAGGA TAAGGACGGA gtaagtctca
212   W  I  H  C   F  E  N   V  N  C   L  L  F  L   V  A  I   S  G  Y   D  Q  C  L   V  E  D   K  D  G

1081  acaccgcaag cacaacttta taaaacagca cagcttggca acagttttgg tcagccctag gagctcatat ctaaccataa cccggctgga cagAACCAAA
242               INTRON IV                                                                                  N  Q  M
1181  TGAACGAAGC CTTGATGCTG TGGGAGTCGA TCGCCAACTC GCATTGGTTC ACAAAATCGG CGCTGATCTT GTTCCTCAAC AAGATGGACC TGTTCAAGGA
245    N  E  A   L  M  L   W  E  S  I   A  N  S   H  W  F   T  K  S  A   L  I  L   F  L  N   K  M  D  L   F  K  E
1281  GAAAATCACA AAGAGTCCCA TTACCGAATA TGGGTTCACC GACTATCACG GACCATCAGA CGACTGGAAG CAGACTAGCA AGTACTTTAT GGACAAGTTC
278    K  I  T   K  S  P  I   T  E  Y   G  F  T   D  Y  H  G   P  S  D   D  W  K   Q  T  S  K   Y  F  M   D  K  F
1381  CGAGCACTTA ACCGAAACCC AGAGAAAGAG ATATACGGCC ACTTCACAAA CGCTACGGAC ACGAACCTAC TCAAGATCAC GATGACGTCT GTGCAGGACA

311   R  A  L  N   R  N  P   E  K  E   I  Y  G  H   F  T  N   A  T  D   T  N  L  L   K  I  T   M  T  S   V  Q  D  M
1481  TGATTATACA GCGCAACCTT AAGCAACTCA TCTTGTGAgt gctactccgg cggtcccgtt taataaccta actggcggtc gaatcttcac tatcaccacg
345    I  I  Q   R  N  L   K  Q  L  I   L  •
      acactcacac ctctggacac cattctagcg tttgattaat cctagacggg gcaaattctg ccacatcggc ggtcttgtta cttcgacaaa ccaactgcat
      catctaccgc gctgtccgtc accgctttct tgaatacctc cagataccca aggttcttat tgcccagcca cttgtttttt atttcgtttt tttcattttt
      tttctggctt tgttcttatt ttgtccatta tcacattgga cttttcgcgg aagctacctg gctgcattgg tctggttaaa ttgtatgtat tggtgtattc
      ctctttcagg gtgtacatac atggccgtgc accagcatct tgttctt
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of controls (Table 2). Fewer than 20% conidia of *mag*B$^-$ transformants remained attached to GelBond whereas about 50% of conidia from controls remained attached when rinsed with water after a 2-h incubation.

Appressorium formation by *mag*B$^-$ transformants could be restored by adding cAMP (Table 3; Fig. 6A) or 1,16-hexadecanediol (Table 3; Fig. 6B). Chemically induced appressoria formed on the tips of long, straight germ tubes.

Effect of deletion of *mag*B on the ability to infect and colonize susceptible rice.

Deletion of *mag*B reduced *M. grisea* virulence, as shown in Figure 7. Disease symptoms (necrotic lesions) on rice leaves were greatly reduced when inoculated with 10^4 conidia either by injection (Fig. 7B,C) or by spray, compared with the wild-type (Fig. 7E) and an ectopic integration control (Fig. 7D). A few spreading lesions were observed when leaf sheaths were injected with *mag*B$^-$ transformants, although wound sites on the emerging leaves were clearly visible.

Direct observation of inoculated rice leaves under the microscope revealed that appressorium formation of *mag*B$^-$ transformants was reduced. When young rice seedlings (2 weeks old) were spray inoculated, germinated conidia of *mag*B$^-$ transformants formed very few appressoria (<3%) on leaves (Fig. 8B), compared with wild-type and ectopic integration controls (>95; Fig. 8A). However, when older rice plants (4 to 5 weeks old) were spray inoculated with *mag*B$^-$ transformants, appressorium formation increased with the age of the leaves; only 20 to 30% of germinated conidia produced appressoria on the young leaves (Fig. 8D), compared with >50% on the oldest leaf (Fig. 8C). For wild-type and ectopic integration transformant controls, no difference in appressorium formation was observed on either young or old leaves; appressorium formation always exceeded 95%. Appressoria produced by *mag*B$^-$ transformants on rice leaves always formed on the tips of long, straight germ tubes and were very similar to those induced by chemicals on the hydrophobic surface of GelBond (Fig. 6).

The deletion of *mag*A or *mag*C did not appear to affect the ability to infect and spread with the host tissues (data not shown), both gene deletion transformants caused typical disease symptoms indistinguishable from wild-type and ectopic integration controls.

Recovery of the wild-type phenotype by restoring *mag*B to a gene-disrupted transformant.

*mag*B$^-$ transformant 2-12 was transformed with a plasmid (pE12Bar) containing the *mag*B gene with the *bar* gene as a selective marker. Southern blot analysis confirmed the introduction of the plasmid (Fig. 9, lanes 9 to 11). Resulting transformants exhibited typical wild-type characteristics, including normal vegetative growth on different media, normal conidiation, normal appressorium formation on the hydrophobic surface of GelBond, and pathogenicity indistinguishable from that of the wild type (Table 4; Fig. 7F,G). *mag*B-restored transformants produced appressoria on short, curved germ tubes.

G protein α subunits are required for *M. grisea* mating.

In repeated three-way crossing experiments with 70-6, 70-15, and *mag*B$^-$ transformants, perithecia failed to form between 70-6 and *mag*B$^-$ strains, whereas 70-6 and 70-15 formed perithecia and visual asci that germinated normally. Similar crosses involving *mag*A$^-$ and *mag*C$^-$ transformants formed perithecia; asci were visible but ascospores were indistinct and failed to germinate.

DISCUSSION

Our results indicate that *M. grisea* has multiple G protein α subunit genes. Each gene appears to be involved in different signal transduction pathways, as deletion of each gene affected different aspects of growth, development, and pathogenicity.

*mag*B was disrupted with a DNA fragment encoding an internal part of the protein (amino acids 16 to 196). Transformants that had undergone a single homologous recombination event thus contained 2 truncated copies of the gene, one lacking the promoter and the first 192 amino acids, and the second lacking amino acids 193 to 353. It is highly unlikely that disruption strains would retain functional *mag*B activity because

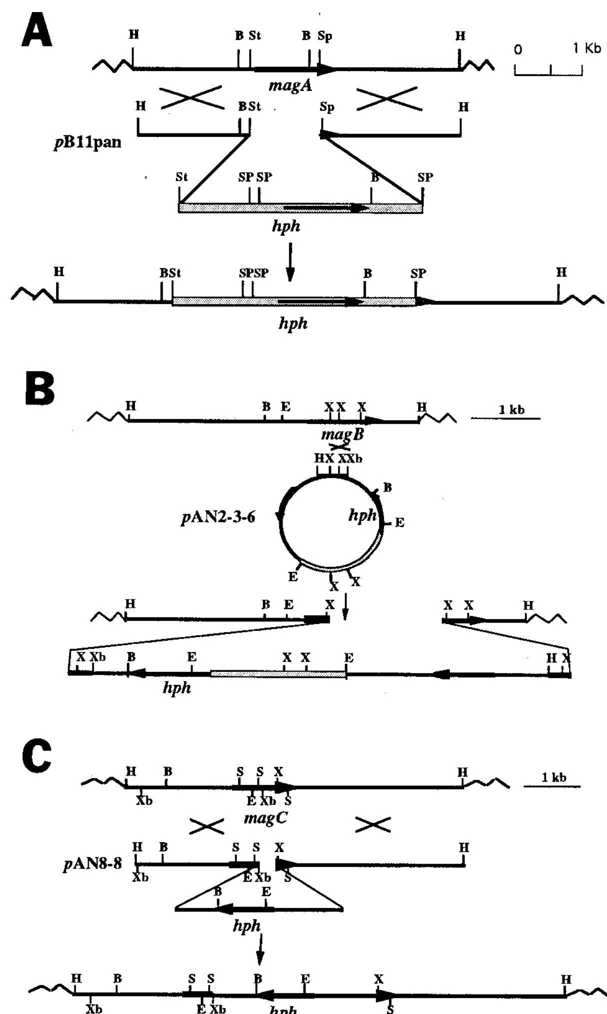

Fig. 3. Strategy for disruption of G protein α subunit genes in *Magnaporthe grisea*. **A,** Deletion of *mag*A; **B,** Disruption of *mag*B; **C,** Deletion of *mag*C. B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, S = *Sac*I, Sp = *Sph*I, St = *Stu*I, Xb = *Xba*I, X = *Xho*I.

essential features including GTP-binding domains were lacking in both truncated copies. Indeed, *mag*B apparently controls multiple functions, including vegetative growth, conidium formation, mating, and appressorium formation. Reduction of appressorium formation in *mag*B⁻ transformants clearly indicates *mag*B contributes to reduced pathogenicity, but based on the sheath injection experiments, *mag*B also contributes to invasive growth.

Upon amino acid sequence inspection, *mag*B contains certain features common to Gi protein α subunits. In mammalian systems, release of Gi protein α subunit lowers intracellular cAMP levels by inhibition of adenylate cyclase activity (Childers and Deadwyler 1996; Chen et al. 1996). Lee and Dean 1993, 1994) found that cAMP induces appressorium formation in *M. grisea*. Therefore, we may have expected that the deletion of *mag*B might stimulate appressorium formation by removing an inhibitor of adenylate cyclase, and consequently increasing the intracellular cAMP level. However, deletion of *mag*B in *M. grisea* blocked appressorium formation. Furthermore, adding external cAMP (or IBMX) induced appressorium formation of *mag*B⁻ transformants. An adenylate cyclase gene of *M. grisea* has been cloned in our laboratory (W. Choi and R. A. Dean, *unpublished data*). The relationship between *mag*B and adenylate cyclase in *M. grisea* is under investigation. It should be noted that, based on sequence alignments, the G protein α subunits of simple eukaryotes form a unique cluster (GPA) distinct from any mammalian Gα class genes (Wilkie and Yokoyama 1994). Therefore, the function of G α subunit genes in simple eukaryotes may differ from that in their mammalian counterparts.

The attachment of conidia of *mag*B⁻ to hydrophobic surface was substantially reduced; whether this is related to the failure to form appressoria is unknown. Surface proteins with possible adhesive properties such as mucilage, hydrophobin, or other secreted proteins have been postulated to be involved in recognizing environmental signals (Hamer et al. 1988; Beckerman and Ebbole 1996). These signals may be transduced into cells through *mag*B, which then activates the differentiation pathway resulting in appressorium formation. How deletion of *mag*B could affect surface attachment remains to be determined.

We checked whether *mag*B⁻ transformants have an easily wettable phenotype, as described by Talbot et al. 1993) for strains lacking the hydrophobin MPG1. All *mag*B⁻ mutant colonies on CM agar showed no wettability; drops (200 μl) of H_2O remained in place on colonies up to 7 days without any visible change. Beckerman and Ebbole 1996) reported that, when mixed with wild-type conidia, strains deleted for *MPG1* could produce appressoria by utilizing the hydrophobin from wild-type conidia. When mixed with wild-type (70-15) conidia, *mag*B⁻ transformants were clearly identifiable and continued to form distinctive straight, long germ tubes without appressoria. Apparently, deletion of *mag*B did not affect hydrophobin production, and hydrophobin did not compensate the loss of *mag*B. Thus, *mag*B⁻ transformants may be defective in attachment independent of hydrophobins.

The difference between young and old rice leaves for inducing appressorium formation of *mag*B⁻ transformants suggests that there is a difference in surface cues (either chemical or physical) presented between young and older leaves. Young

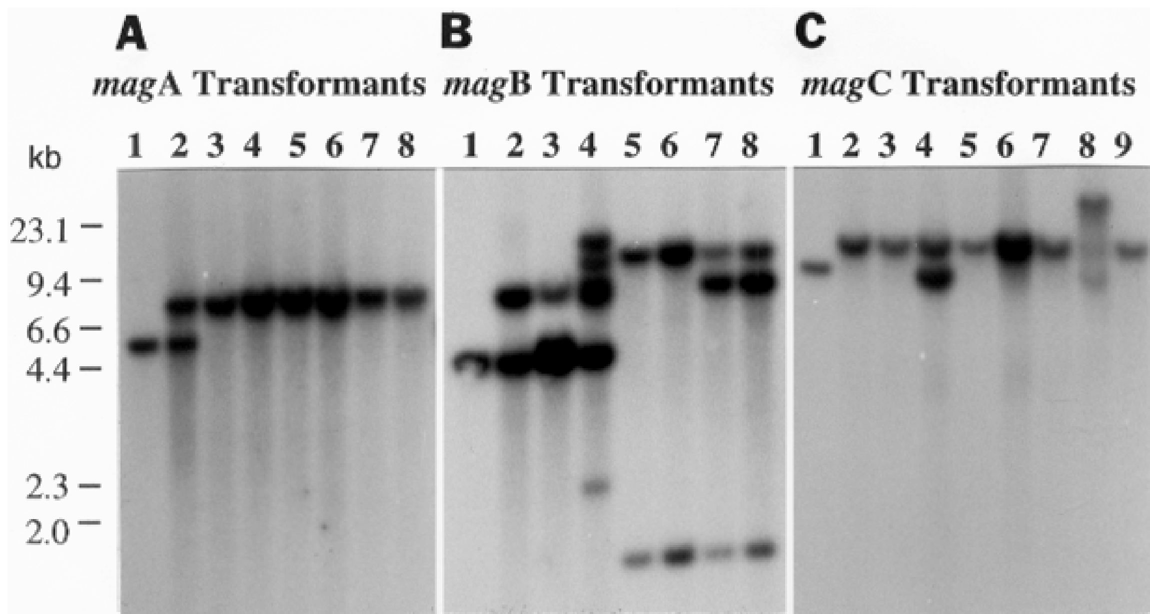

Fig. 4. Southern blots of G protein α subunit disruptions. All genomic DNA was digested with *Hin*dIII, and probed with a *Hin*dIII fragment of *mag*A (4.8 kbp) for blot **A,** *mag*B (4.3 kbp) for blot **B,** or *mag*C (6.8 kbp) for blot **C,** respectively. **A,** Lane 1 is wild-type strain DNA (70-15); lane 2, ectopic integration strain (B1); lanes 3–8, *mag*A⁻ transformants (B3, B6, B8, B14, B17, and B18). As expected, the 4.8-kbp band from *mag*A⁻ transformants is no longer present. **B,** Lane 1, wild-type (70-15); lanes 2–4, ectopic integrations (2-3, 2-5, and 2-10); lanes 5–8, *mag*B⁻ transformants (2-9, 2-11, 2-2, and 2-12). As expected, the 4.3-kbp band disappeared in *mag*B⁻ transformants. For 2-9 and 2-11, a single integration event had occurred, whereas for 2-2 and 2-12, multiple insertion events occurred, as evidenced by an additional 7.5-kbp band (the size of the disruption plasmid). **C,** Lane 1, wild-type; lanes 4 and 8, ectopic integrations (8-8 and 8-18); lanes 2, 3, 5–7, and 9, *mag*C⁻ transformants (8-2, 8-7, 8-10, 8-12, 8-17, and 8-19). As expected, the 6.8-kbp band is missing in *mag*C⁻ transformants.

leaves may contain some chemical inhibitor(s), which can prevent appressorium formation of *mag*B⁻ transformants. More likely, young leaves lack certain chemical stimulatory compounds. As leaves mature, the appropriate chemicals may be produced and deposited on to the surface. Potential candidates may be chemically similar to 1,16-hexadecanediol. This molecule, a minor cutin component, induced appressorium formation in *mag*B⁻ transformants with a morphology similar to that observed on mature rice leaves; appressoria developed at the tips of long, straight germ tubes. Gilbert et al. 1996) reported that 1,16-hexadecanediol induced appressorium formation of *M. grisea* at nanomolar levels. This molecule and related chemicals commonly exist on the surface of plants, including on mature rice leaves. Further investigation is required to examine the presence of stimulating chemicals from young and mature leaves before firm conclusions can be drawn.

The mechanism of action of 1,16-hexadecanediol is unknown. Regardless, it appears to be independent of *mag*B. *mag*B, on the other hand, appears to be linked to the reception and processing of physical signals, such as hydrophobicity. These data reinforce previous evidence that there are at least dual pathways regulating the induction of appressoria (Gilbert et al. 1996).

Table 1. Vegetative growth, conidiation, and appressorium formation assay for G protein α subunit gene transformants[u]

Strain	Colony diameter (cm) 5-day-old	7-day-old	10-day-old	Conidia (×10⁴)	Appress. (%)
Transformants lacking *mag*A[v]					
Ck[w] 70-15	2.2 ± 0.3	3.4 ± 0.3	5.0 ± 0.3	65.0 ± 12.0	>95
E[x] B1	2.2 ± 0.3	3.3 ± 0.3	4.7 ± 0.4	82.2 ± 5.8	>95
E B2	2.2 ± 0.3	3.3 ± 0.3	4.6 ± 0.3	55.8 ± 6.5	>95
M[y] B3	2.5 ± 0.3	3.1 ± 0.3	4.6 ± 0.3	67.6 ± 5.5	>95
M B6	2.3 ± 0.3	3.5 ± 0.3	4.9 ± 0.3	72.8 ± 2.3	>95
M B8	2.3 ± 0.3	3.4 ± 0.3	4.9 ± 0.3	53.4 ± 1.0	>95
M B14	2.3 ± 0.2	3.4 ± 0.2	5.0 ± 0.3	70.6 ± 11.2	>95
M B17	2.1 ± 0.3	3.3 ± 0.3	4.7 ± 0.3	70.2 ± 10.6	>95
M B18	2.3 ± 0.3	3.4 ± 0.3	4.8 ± 0.3	70.2 ± 9.9	>95
Transformants lacking *mag*B					
Ck 70-15	2.7 ± 0.3 a[z]	4.2 ± 0.3 a	6.0 ± 0.3 a	61.3 ± 4.6 a	>95 a
E 2-3	2.6 ± 0.3 a	4.0 ± 0.3 a	5.9 ± 0.3 a	68.1 ± 8.0 a	>95 a
E 2-10	2.7 ± 0.3 a	4.0 ± 0.3 a	5.9 ± 0.3 a	75.5 ± 6.9 a	>95 a
M 2-2	2.3 ± 0.3 b	3.4 ± 0.3 b	4.8 ± 0.3 b	0.3 ± 0.2 c	2.5 ± 0.6 b
M 2-9	2.3 ± 0.4 b	3.5 ± 0.4 b	5.0 ± 0.3 b	21.7 ± 4.0 b	1.8 ± 0.5 b
M 2-11	2.4 ± 0.3 b	3.6 ± 0.2 b	5.1 ± 0.3 b	1.2 ± 0.6 c	3.7 ± 0.9 b
M 2-12	2.3 ± 0.3 b	3.4 ± 0.2 b	5.0 ± 0.3 b	1.9 ± 0.6 c	1.2 ± 0.9 b
Transformants lacking *mag*C					
Ck 70-15	2.3 ± 0.3	3.4 ± 0.3	5.1 ± 0.3	76.8 ± 7.0 a	>95
E 8-8	2.2 ± 0.3	3.1 ± 0.4	4.7 ± 0.3	71.5 ± 5.0 a	>95
E 8-18	2.5 ± 0.3	3.5 ± 0.3	5.2 ± 0.3	76.3 ± 13.1 a	>95
M 8-7	2.2 ± 0.3	3.1 ± 0.3	4.7 ± 0.5	31.4 ± 8.6 b	>95
M 8-17	2.1 ± 0.4	3.0 ± 0.4	4.5 ± 0.4	21.9 ± 7.9 b	>95
M 8-19	2.4 ± 0.3	3.5 ± 0.3	5.1 ± 0.3	10.2 ± 5.2 b	>95

[u] Colonies were grown on V8 media under fluorescent light. For appressorium assays, conidia were collected from 7-day-old cultures and appressorium formation was recorded after 20 h on the hydrophobic surface of GelBond. The data from three independent experiments, each with two or three replicates; mean ± standard deviation.
[v] No statistically significant differences were observed between strains as determined by Student's *t* test.
[w] Ck is the wild-type control strain 70-15.
[x] E designates ectopic integration transformants.
[y] M designates gene disrupted mutants.
[z] Different letters in each data column indicate significant differences at $P = 0.05$.

In a recent report, Beckerman et al. 1997) discovered that the sex pheromone, α-mating factor from *Saccharomyces cerevisiae,* and a cell-free extract from *M. grisea* inhibit appressorium formation in a mating type–specific manner. The evidence reveals a possible relationship between the signal pathways for mating and appressorium formation.

Deletion of *mag*B blocked sexual development as well as inhibiting appressorium formation. All *mag*B⁻ transformants tested failed to form perithecia when crossed with the appropriate mating-type strain. This may suggest that *mag*B interacts with sex pheromone receptor(s). Thus, removing *mag*B might break the pathway between the receptor and effector(s), and prevent the initiation of the mating process. Whether the putative sex pheromone receptor(s) in *M. grisea* have dual functions will require further investigation.

It is also noteworthy that deletion of *mag*B homologs in other filamentous fungi has similar phenotypic consequences. Deletion of *CPG*1 of *C. parasitica* resulted in a marked reduction in growth and a loss of virulence, asexual reproduction, female fertility, and laccase activity (Gao and Nuss 1996); deletion of a G protein α subunit gene with high homology to *mag*B in the corn pathogen *Cochliobolus heterostrophus* resulted in a loss of the ability to form appressoria and the production of infertile pseudothecia (Horowitz 1997). In *N. crassa,* deletion of *gna*-1 caused abnormal vegetative growth and aerial hyphae as well as female sterility (Ivey et al. 1996). Disruption of *fad*A in *A. nidulans* also reduced vegetative growth, but did not affect conidiation or sexual competency (Yu et al. 1996).

Deletion of *mag*A or *mag*C on the other hand had minimal effect, although loss of these genes clearly affected a later stage of sexual development: perithecia formed but ascospores failed to germinate. Therefore these genes appear to function in ascospore maturation and/or germination.

Overall, our results indicate that different G protein α subunits control unique signal transduction pathways in *M. grisea,* involving vegetative growth, conidiation, appressorium formation, mating, and pathogenicity. It is possible the deletion of one gene may be compensated for by another, i.e., functional redundancy or pathway cross-talk. Research is presently addressing this possibility. Future insight into the role of G

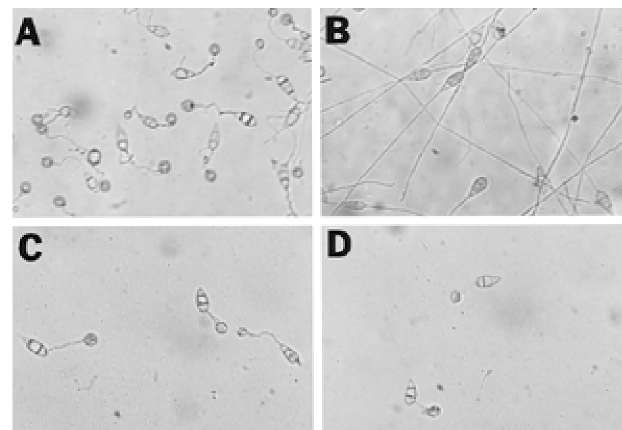

Fig. 5. Appressorium formation (at 24 h) on the hydrophobic surface of GelBond. **A,** Wild-type strain (70-15); **B,** *mag*B⁻ transformant; **C,** *mag*A⁻ transformant; and **D,** *mag*C⁻ transformant. Magnification = ×200.

proteins in mediating morphogenesis will be provided by specific mutagenesis of the α subunits affecting their activity.

MATERIALS AND METHODS

Strains, growth, and maintenance of *Magnaporthe grisea.*

Strains of *M. grisea*, 70-15 (MAT1-1) and 70-6 (MAT1-2), used in this study were originally obtained from A. Ellingboe (University of Wisconsin-Madison) and maintained on complete medium (CM) with 1.5% agar (Lau et al. 1993; Mitchell and Dean 1995). All transformant isolates were maintained on CM agar with 200 µg of hygromycin B per ml or 500 µg of basta per ml.

M. grisea conidia were produced on V8 agar medium (4% vol/vol V8 juice, 15 g of agar per liter, pH 7.0, with or without 200 µg of hygromycin B per ml), and grown under fluorescent light to induce conidiation.

Mycelia were produced by transferring conidia of *M. grisea* isolates into 20 ml of liquid CM in petri dishes (1.0×10^6 conidia per petri dish), and grown at 23°C in the dark for 4 to 5 days.

PCR cloning of G protein α subunit genes.

The degenerate oligonucleotide primers oMP 19, oMP 20, and T α 29 [oMP 19: CGGATCCAA(AG)TGGAT(ACT)CA(CT)TG(CT)TT; oMP 20: GGAATTC (AG)TC(CT)TT(CT)TT(AC)TT(ACGT)AG(AG)AA; T α 29: GGGATCC(ACT)GT(AG)TC(CT)GT(CT)GC(AG)CA(CT)GT], complementary to a conserved portion of mammalian G protein α subunits, were provided by T. M. Wilkie (University of Texas Southwestern Medical Center, Dallas). The underlined regions identify added restriction sites. PCR was performed on *M. grisea* genomic DNA with primer pairs oMP 19 × oMP 20 or oMP 19 × T α 29.

Fifty-microliter PCR reactions contained 5 µl of 10× PCR buffer (Perkin-Elmer Applied Biosystems Division, Foster City, CA), 8 µl of 25 mM $MgCl_2$, 1 µl each of dATP, dCTP, dGTP, and dTTP (10 mM stock), 1 µl of *M. grisea* genomic DNA (5 ng of stock per µl), 1 µl each of forward or reverse primer (50 ng of stock per µl), 30 µl of distilled H_2O, and 0.2 µl of AmpliTaq Polymerase (Perkin-Elmer Applied Biosystems Division). PCR conditions were as follows: 3 min at 94°C, followed by 35 cycles (1 min at 94°C; 1.5 min at 45°C; 2 min at 72°C), with a final extension at 72°C for 10 min.

PCR products were digested with *Bam*HI and *Eco*RI and cloned into *p*Bluescript II KS^+, and then transformed into *Escherichia coli* strain DH 5 α. The relationship among all white colonies was determined by Southern hybridization. Nucleotide sequence of representative clones was determined

Table 2. Attachment of conidia of *mag*B⁻ transformants to the hydrophobic surface of GelBond[x]

Strain	Attachment (%)
Ck 70-15[y]	38.8 ± 17.4 a[z]
E 2-3	55.8 ± 13.0 a
E 2-10	58.0 ± 7.8 a
M 2-2	17.7 ± 4.4 ab
M 2-9	9.7 ± 4.7 b
M 2-11	16.9 ± 5.4 b
M 2-12	10.8 ± 6.6 b

[x] Conidial suspension was placed on GelBond. After incubation in a moisture chamber at room temperature for 2 h, all conidia in a marked area were counted with a light compound microscope. The GelBond was rinsed three times with a gentle stream of double-distilled H_2O from a squeeze bottle and conidia in the marked areas were recounted. Results are the combined data from two experiments, each with three replicates; mean ± standard deviation.
[y] Ck = wild-type control strain. E = ectopic integration transformant. M = gene disrupted mutant.
[z] The same letter indicates no significant effect of treatment at $P = 0.05$ as determined by Student's *t* test.

Table 3. Induction of appressorium formation (%) by *mag*B⁻ transformants by cAMP, or 1,16-hexadecanediol

	GelBond[y]		Glass slide[z]	
Strain	H_2O	10 mM cAMP	Ethanol	1 µM Diol
Ck 70-15	12.1 ± 7.8	>95	0	>95
E 2-5	19.4 ± 3.5	93.4 ± 1.7	0	>95
E 2-10	. . .	. . .	0	>95
M 2-2	1.1 ± 0.7	62.1 ± 18.7	0	90.0 ± 5.0
M 2-9	2.1 ± 1.0	83.0 ± 10.7	0	75.0 ± 25.0
M 2-11	1.0 ± 0.6	76.0 ± 3.4	0	75.0 ± 25.0
M 2-12	0.8 ± 0.5	70.7 ± 3.7	0	75.0 ± 17.5

[y] cAMP assay was conducted on GelBond; hydrophobic surface for gene disrupted mutants (M), and hydrophilic for controls: Ck = wild type; E = ectopic transformants (Lee and Dean 1993).
[z] 1,16-hexadecanediol (Diol) assay was conducted on glass slides (Gilbert et al. 1996). Ethanol (5%) was used as the control for 1,16-hexadecanediol treatment. Results are the combined data from two experiments, each with three replicates; mean ± standard deviation. Over 100 conidia were counted for each replicate.

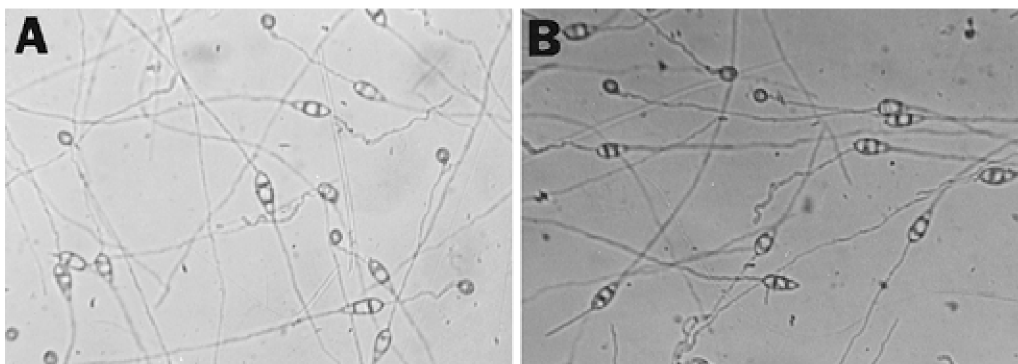

Fig. 6. cAMP (**A**) and 1,16-hexadecanediol (**B**) restore the ability to form appressorium (at 24 h) by *mag*B⁻ transformant (2-12) on the hydrophobic surface of GelBond. Magnification = ×200.

by the Taq DyeDeoxy terminator method (Amersham LifeScience, Arlington Heights, IL) and products separated on an ABI373 sequencer (Perkin-Elmer Applied Biosystems Division). Genomic clones of putative G α subunit genes were identified in a *p*UI-NM554 cosmid library of *M. grisea* strain 70-15 (Lee and Dean 1993).

Nucleotide sequencing and alignments of *mag*A, *mag*B, and *mag*C genes.

Cosmid clones corresponding to the three PCR clones were digested with *Hin*dIII and the appropriate fragments subcloned into *p*Bluescript II KS$^+$. A restriction map of each subclone was also constructed to localize the position of the putative

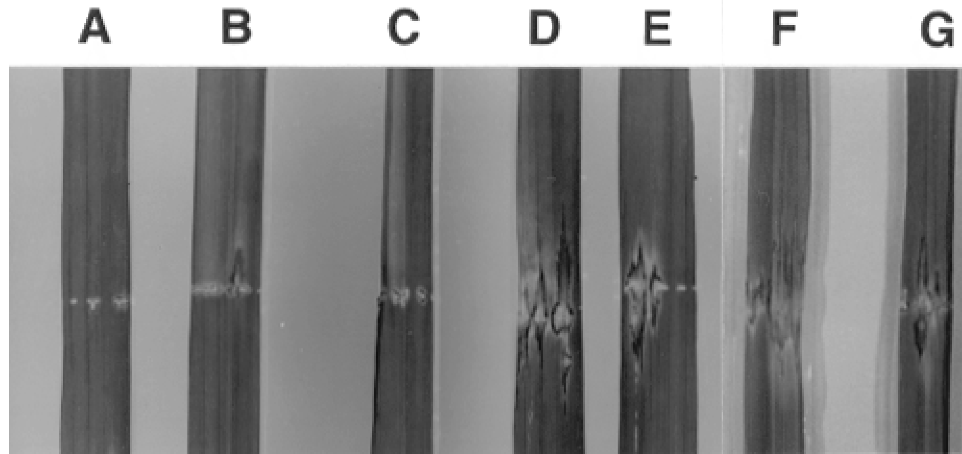

Fig. 7. Disease symptoms of *Magnaporthe grisea* on rice leaves after 7 days. Rice stem sheath was injected with 100 µl of 1×10^5 conidia/ml or water, with a hypodermic needle and syringe. Inoculated plants were checked daily for symptom development; symptoms typically began to appear by 4 days. **A,** H_2O control; **B,** *mag*B$^-$ transformant (2-12); **C,** *mag*B$^-$ transformant (2-2); **D,** ectopic integration strain (2-10); **E,** Wild-type (70-15); **F,** *mag*B rescued transformant (E2); **G,** *mag*B rescued transformant (E4).

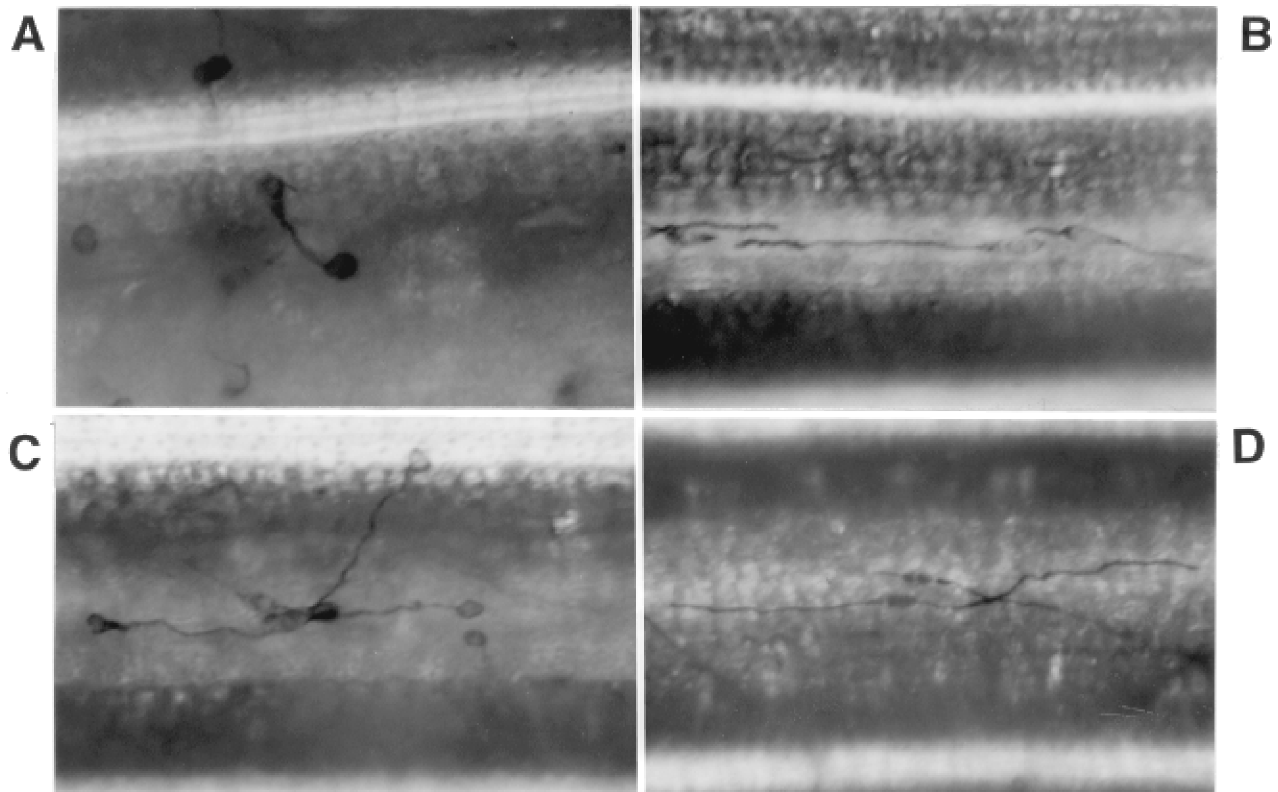

Fig. 8. Appressorium formation (at 24 h) on rice leaves. Ectopic integration strain (2-10) (**A**) or *mag*B$^-$ transformant (2-12) (**B**) inoculated on the leaves from 2-week-old rice seedlings; *mag*B$^-$ transformant (2-12) inoculated on an old leaf (**C**) or a young leaf (**D**) from a 4-week-old rice plant. Magnification = ×300.

gene. The nucleotide sequence was determined and genes were named *mag*A, *mag*B, and *mag*C, respectively. The data have been deposited at GenBank, with the following accession numbers: *mag*A, AF011340; *mag*B, AF011341; and *mag*C, AF011342. On-line data base comparisons were performed with the BLAST algorithm (Altschul et al. 1990). Additional nucleotide and amino acid sequence comparisons and alignments were made with Genejockey (Biosoft, Cambridge, UK) and GeneWorks (v2.3) (IntelliGenetics, Mountain View, CA) software packages.

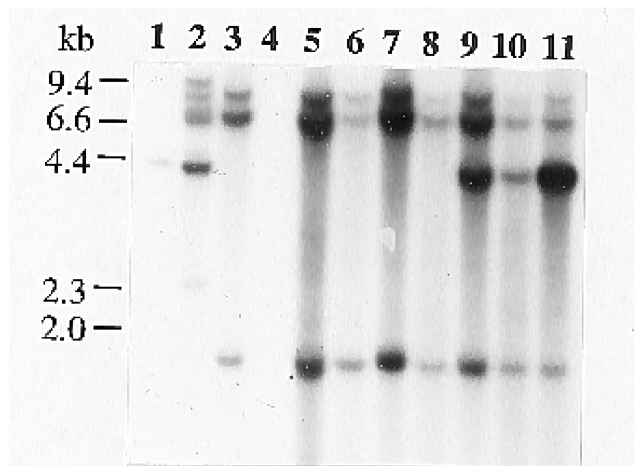

Fig. 9. Southern blots of *mag*B rescue. All genomic DNA was digested with *Hin*dIII and probed with a *Hin*dIII fragment of *mag*B (4.3 kbp). Lane 1, wild-type (70-15); lane 2, ectopic integration strain (2-10); lane 3, *mag*B⁻ transformant (2-12); lanes 5–8, transformants from 2-12 containing the *bar* gene only (P1, P2, P3, and P7); lanes 9–11, transformants from 2-12 containing *mag*B and *bar* genes (E2, E4, and E6). As expected, the 4.3-kbp band reappeared in *mag*B rescued transformants (E2, E4, and E6), and is lacking in *mag*B⁻ transformant (2-12) and *mag*B-transformants containing only the *bar* gene (P1, P2, P3, and P7).

Table 4. Vegetative growth, conidiation, and appressorium formation assay for strains restored for *mag*B[x]

Strain	Colony diameter (cm) CM	Colony diameter (cm) V8	Conidia ($\times 10^4$)	Appress. (%)
Ck 70-15[y]	3.6 ± 0.1 a[z]	4.9 ± 0.1 a	68.3 ± 14.1 a	>95 a
E 2-10	3.7 ± 0.1 a	4.9 ± 0.1 a	70.0 ± 10.5 a	>95 a
E 2-3	3.6 ± 0.1 a	4.9 ± 0.1 a	71.3 ± 7.8 a	>95 a
M 2-2	2.9 ± 0.1 b	4.1 ± 0.1 b	0.4 ± 0.2 b	2.2 ± 0.6 b
M 2-12	2.8 ± 0.1 b	4.0 ± 0.1 b	0.9 ± 0.4 b	2.5 ± 1.2 b
ME P1	2.8 ± 0.1 b	3.9 ± 0.1 b	0.6 ± 0.4 b	1.6 ± 0.5 b
ME P3	2.7 ± 0.1 b	3.9 ± 0.1 b	0.8 ± 0.4 b	0.4 ± 0.2 b
MR E2	3.6 ± 0.1 a	5.0 ± 0.1 a	64.7 ± 4.8 a	>95 a
MR E4	3.5 ± 0.1 a	4.9 ± 0.1 a	71.6 ± 10.4 a	>95 a

[x] Colonies were grown on complete medium (CM) or V8 medium under fluorescent light for 7 days before colony diameters (cm) were measured. Conidia were collected from V8 plates and counted. Appressorium formation on the hydrophobic surface of GelBond was measured at 20 h. Data are from two independent experiments, each with two or three replicates; mean ± standard deviation.

[y] Ck = wild-type control strain. E = ectopic integration transformant. M = gene disrupted mutant. ME = transformants of the *mag*B⁻ strain 2-12 containing the *bar* gene alone. MR = transformants of the *mag*B⁻ mutant 2-12 containing both *mag*B and *bar* genes.

[z] Different letters in each data column indicate significant differences at $P = 0.05$ as determined by Student's *t* test.

DNA and RNA extraction and manipulation.

Plasmid DNA was isolated by the standard alkaline lysis method (Sambrook et al. 1989). Restriction site mapping and subcloning were performed according to standard methods (Sambrook et al. 1989). For nucleotide sequencing, plasmid DNA was isolated with a Wizard DNA miniprep kit (Promega, Madison, WI). Fungal DNA was extracted from 4- to 5-day-old mycelia following the protocol of Mitchell and Dean 1995). RNA was extracted from mycelia or spores with the Qiagen RNeasy Plant mini kit (Qiagen, Chatsworth, CA). DNA digestion, agarose gel fractionation, RNA formaldehyde denatured gel electrophoresis, radiolabeling, and hybridization were according to the manufacturers' instructions and standard methods (Sambrook et al. 1989). Hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.002% Ficoll 400. 0.02% polyvinylpyrrolidone [PVP], 0.02% bovine serum albumin [BSA]), 0.1% sodium dodecyl sulfate (SDS), and 50 mM phosphate buffer, pH 6.6, at 65°C. Low-stringency hybridization was carried out at 55°C. Membranes were washed down to between 2× and 0.1× SSC, 0.1 SDS, 55 to 65°C, before being exposed to autoradiographic film.

Construction of gene replacement vectors *p*B11pan and *p*8-8pan and gene disruption vector *p*AN2-3-6.

Plasmid *p*B11pan was constructed by ligating a 3.6-kb *Stu*I-*Sph*I fragment from a partial digest of *p*AN 7-1 containing the complete hygromycin phosphor transferase (*hph*) gene (Punt et al. 1987) into *p*B11-12, digested with *Stu*I-*Sph*I. This resulted in the replacement of a 1.2-kb *Stu*I-*Sph*I fragment that contained the majority of the *mag*A gene with the *hph* gene cassette.

Plasmid *p*8-8pan was constructed by first inserting a 2.3-kb *Xba*I fragment from the 5′ end of *mag*C into the *Xba*I site of pAN 7-1 to produce plasmid *p*8pan. After the correct orientation of the *Xba*I fragment in *p*8pan was confirmed, a 3.4-kb *Xho*I-*Bam*HI fragment from the 3′ end of *mag*C was ligated into *Xho*I- and *Bgl*II-digested *p*8pan to produce *p*8-8pan.

Plasmid *p*AN2-3-6 (7.5 kbp) was constructed by inserting a 0.7-kb *Hin*dIII-*Xba*I PCR-amplified fragment of *mag*B containing the amino half of the Gα subunit. The PCR product was obtained with primers 2-3PF and 2-3PR (2-3PF <u>GGGAAGCTT</u>[44]GGAACGAGGAGATTGAGGAGAACC[64], 2-3PR 5′ <u>GGATCTAGA</u>[760]AGG'TCGCCAATGATGAAAGTCG[439]) with the conditions described above, except the annealing temperature was raised to 55°C.

Construction of plasmid for restoring *mag*B.

Plasmid *p*E12Bar was constructed by inserting a 1.9-kb *Xba*I fragment containing the complete phosphinothricin acetyltransferase (*bar*) gene (Avalos et al. 1989) from *p*BarKS1 (Pall and Brunelli 1993) into a unique *p*E12 2-3 *Xba*I that contained the complete *mag*B gene.

Transformation of *M. grisea.*

Transformation protocols were adapted from established procedures (Leung et al. 1990; Sweigard et al. 1992). Conidia (5.0×10^6) were germinated in liquid CM overnight (18 h) at room temperature with agitation. Mycelium was harvested

with Miracloth (Calbiochem, San Diego, CA), rinsed, and wrung dry. The pellet was suspended in 30 ml of 1.0 M sorbitol to which 1 ml of Novozyme 234 (20 mg/ml; InterSpex Products, Foster City, CA) was added. After 90 min of agitation at room temperature, protoplasts were collected by filtering through cheesecloth and pelleted by centrifugation at 4,800 × *g* for 10 min. Protoplasts were resuspended in STC (1.0 M sorbitol, 50 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$) and were counted and adjusted to 5×10^7 cells/ml. Five micrograms of plasmid DNA (linearized DNA for gene replacement, or nonlinearized DNA for gene disruption or restoration) was mixed with 1.0×10^7 protoplasts in 200 µl of STC for 10 min before 1 ml of PTC (40% polyethylene glycol 4000 in STC) was added. After 20 min, 3 ml of TB3 (3 g of yeast extract, 3 g of casein acid hydrolysate, 10 g of glucose, and 200 g of sucrose per liter, containing 200 µg of hygromycin B or 500 µg of basta per ml) was added and incubated for 6 h at room temperature. Twenty milliliters of molten TB3 agar with hygromycin B (200 µg/ml) or basta (500 µg/ml) was mixed with 0.5 ml of treated protoplast mixture, and plated onto a petri dish. After culturing at 23°C in the dark for 5 to 7 days, hygromycin B or basta resistant clones were transferred to CM and V8 plates containing hygromycin B or basta. In CM plates containing basta, casamino acids were replaced with 0.5% L-proline. Conidia were streaked for single colonies.

Sexual crosses.

Crosses were performed by established procedures (Lau et al. 1993). Plugs of 0.5 cm^2 of 70-6 (MAT1-2), 70-15 (MAT1-1), and a strain of interest were placed 5 cm from each other on an oatmeal agar plate and incubated at 23 ± 1°C under fluorescent light. Between 14 and 28 days, asci were released from crushed perithecia on 2% distilled H_2O agar. Individual germinated asci were transferred to V8 agar plates and allowed to conidiate. Conidia were streaked for single colonies.

Vegetative growth and conidiation assays.

A 0.5-cm^2 agar block of *M. grisea* strains, grown on CM agar, was transferred onto CM or V8 agar, and grown under fluorescent light. Colony diameters were measured in centimeters at 5, 7, and 10 days. Conidia formed on V8 agar were harvested and counted with a hemacytometer. Each treatment contained 2 to 3 replicates, and the assays were conducted at least three times.

Appressorium formation assays.

Appressorium development was assayed by placing conidia on GelBond (Lee and Dean 1993; Mitchell and Dean 1995), or on rice leaf surfaces. Three 50-µl droplets of a conidial suspensions (1×10^5 conidia/ml) from 7- to 10-day-old cultures were placed on each piece of prewashed GelBond. After incubation in a moisture chamber at room temperature for a period of time, the germination and appressorium formation rates were scored with a light compound microscope (Mitchell and Dean 1995).

For observation of appressorium formation on the surface of rice leaves, freshly cut rice leaves (8 cm long) from 2- to 5-week-old rice plants were taped at their tips to glass slides and placed face up on a layer of moistened paper towel in a clear plastic box. Multiple 10-µl droplets of conidia (2×10^5 conidia/ml with 500 ppm Tween 20) were placed on the surface. Conidia, germ tubes, and appressoria on the rice leaves were stained with cotton blue in lactophenol solution (5 ml of lactose, 5 ml of phenol, 15 ml of H_2O, and 10 mg of cotton blue) for 2 min, and counted with a light compound microscope. Germination and appressorium formation rates were checked periodically up to 72 h.

cAMP and 1,16-hexadecanediol treatments.

Fifty microliters of conidia suspension containing 10 mM cAMP or 1 µM 1,16-hexadecanediol was placed on the hydrophobic and hydrophilic surfaces of GelBond (controls only had H_2O or 5% ethanol) and allowed to germinate and form appressoria in a moisture chamber at room temperature (Lee and Dean 1993). Percent appressoria was recorded after 20 h of incubation.

Pathogenicity assays.

Conidia suspensions were prepared from 7-day-old V8 plates and adjusted to 1.0×10^5 conidia/ml in a 500-ppm Tween 20 solution. Approximately 8 ml was sprayed evenly onto 3-week-old rice plants (susceptible rice variety S-201), with a fine mist aspirator. The inoculated rice plants were placed in an unlit dew chamber at 25 ± 2°C. After 24 h, a photoperiod of 15 h with fluorescent and incandescent lighting was resumed. Plants were rated for disease at 5 to 7 days after inoculation.

Conidia suspensions (100 µl) were also injected into the leaf with a 26 gauge, 1.27-cm-long needle and 3-ml syringe (Xu and Hamer 1996). After injection, the rice plants were covered with a plastic bag. After 24 h, the bag was removed and the plants were incubated for 5 to 7 days before being examined for disease.

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