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Two Novel Fungal Virulence Genes Specifically Expressed in Appressoria of the Rice Blast Fungus

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The *PMK1* mitogen-activated protein kinase gene regulates appressorium formation and infectious hyphae growth in the rice blast fungus. To further characterize this mitogen-activated protein kinase pathway, we constructed a subtraction library enriched for genes regulated by *PMK1*. Two genes identified in this library, *GAS1* and *GAS2*, encode small proteins that are homologous with *gEgh16* of the powdery mildew fungus. Both were expressed specifically during appressorium formation in the wild-type strains, but neither was expressed in the *pmk1* mutant. Mutants deleted in *GAS1* and *GAS2* had no defect in vegetative growth, conidiation, or appressoria formation, but they were reduced in appressorial penetration and lesion development. Interestingly, deletion of both *GAS1* and *GAS2* did not have an additive effect on appressorial penetration and lesion formation. The *GAS1*–green fluorescent protein and *GAS2*–green fluorescent protein fusion proteins were expressed only in appressoria and localized in the cytoplasm. These two genes may belong to a class of proteins specific for filamentous fungi and function as novel virulence factors in fungal pathogens.

INTRODUCTION

The ascomycete *Magnaporthe grisea* is pathogenic to economically important crops such as barley, wheat, rice, and millet. Rice blast, which is caused by this heterothallic haploid fungus, is one of the most severe fungal diseases of rice throughout the world (Valent, 1990). Genetic studies of this pathogen during the past decade have made the Magnaporthe–rice pathosystem an excellent model for investigating fungus–plant interactions.

Magnaporthe infects rice plants with specialized infection structures called appressoria. Enormous turgor pressure generated in appressoria by the accumulation of high concentrations of glycerol is the force used to penetrate the underlying plant surface (de Jong et al., 1997). Mutants blocked at appressorium formation or appressorial turgor generation fail to infect healthy rice plants (Valent et al., 1991). After penetration, infectious hyphae grow in and between plant cells and eventually result in lesion formation. The blast fungus attacks all aboveground parts of the rice plant, and seedlings can be killed during epidemics.

Appressorium formation and penetration processes have been studied extensively in Magnaporthe (Dean, 1997; Hamer and Talbot, 1998). One mitogen-activated protein (MAP) kinase gene, *PMK1*, was found to be essential for appressorium formation and infectious hyphae growth in Magnaporthe (Xu and Hamer, 1996). Although *PMK1* is nonessential for vegetative growth and conidiation in culture, *pmk1* mutants fail to form appressoria and infect rice leaves. Recently, *PMK1* homologs were characterized in several phytopathogenic fungi (reviewed by Xu, 2000). In all four appressorium-forming fungal pathogens examined to date (Magnaporthe, *Colletotrichum lagenarium*, *Cochliobolus heterostrophus*, and *Pyrenophora teres*), the *PMK1* homologs are essential for appressorium formation (Xu and Hamer, 1996; Lev et al., 1999; Takano et al., 2000; Ruiz-Roldan et al., 2001).

Like *pmk1* mutants in Magnaporthe, gene replacement mutants of *PTK1* in *P. teres* and *CMK1* in *C. lagenarium* are nonpathogenic and fail to colonize healthy or wounded host tissues (Takano et al., 2000; Ruiz-Roldan et al., 2001). In *C. heterostrophus*, a *PMK1* homolog is necessary for appressorium formation and lesion formation but dispensable for colonizing plant tissues (Lev et al., 1999). The *PMK1* homologs *BMP1* and *FMK1* also are essential for fungal pathogenicity in the necrotrophic pathogen *Botrytis cinerea* (Zheng et al., 2000) and the vascular wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Di Pietro et al., 2001). The *B. cinerea bmp1* mutants fail to penetrate and kill plant cells and are nonpathogenic on a variety of plants tested (Zheng et al., 2000). *GMK1* from *Gaeumannomyces graminis* fully complemented the defects of the Magnaporthe *pmk1* mutant in appressorial development and invasive growth, indicating that the function of *PMK1* MAP kinase may be

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conserved in this root pathogen (Dufresne and Osbourn, 2001). Even in the biotrophic, nonappressorium-forming pathogen *Claviceps purpurea*, the *PMK1* homolog is essential for colonizing rye ovarian tissues (Mey et al., 2002). These data suggest that the *PMK1* pathway is conserved in many, if not all, fungi for appressorium formation and other plant infection processes.

Although *PMK1* homologs have been identified in several fungi, it is not clear how *PMK1* is activated and what kinds of genes are regulated by this MAP kinase pathway. In this study, we constructed a subtraction library enriched for genes regulated by *PMK1* during appressorium formation. Two genes identified in this library, *GAS1* and *GAS2*, also were isolated by screening of an appressorium cDNA library by differential hybridization. Both of them were expressed specifically during appressorium formation and localized in the cytoplasm of appressoria. Gene deletion mutants of *GAS1* and *GAS2* had no defect in growth, conidiation, germination, or appressorium formation but were reduced in penetration and virulence on rice and barley seedlings.

RESULTS

Identification of the gEgh16 Homologs *GAS1* and *GAS2*

To identify genes regulated by *PMK1* during appressorium formation, we constructed a subtraction library using Guy11 cDNA as the tester and nn78 cDNA as the driver. Sequence analysis with the first 96 clones of this library revealed that two of them, MBC4 and MBE5, are homologous with the *Erysiphe graminis* EST clone gEgh16, a protein expressed during the early infection stage (Justesen et al., 1996). Interestingly, MBC4 and MBE5 were found to correspond to MAS1 and MAS3, respectively. MAS1 and MAS3 are the most redundant and the third most redundant MAS (Magnaporthe appressoria specific) sequences identified in an independent differential hybridization analysis of an appressorium cDNA library constructed from strain 70-15.

Among 621 cDNA clones that exhibited upregulated expression during appressorium formation, 77 and 53 clones are represented by MAS1 and MAS3 sequences, respectively. The 0.35- and 0.4-kb inserts amplified from clones MBE5 and MBC4, respectively, were used as probes to screen a Guy11 cosmid genomic library. Two corresponding open reading frames were identified after sequencing of cosmid clones that hybridized to these two probes; they were named *GAS1* and *GAS2* (gEgh16 homologs expressed in appressorium stage).

The *GAS1* gene contains one 63-bp intron and encodes a 251–amino acid protein. The *GAS2* gene has no intron and encodes a 290–amino acid protein. The amino acid sequences of *GAS1* and *GAS2* share 42% identity, and both are homologous with gEgh16 and its homolog Egh16H1. The highest homology is between *GAS2* and Egh16H1 (67% identity). The Gas1 and Gas2 proteins are rich in Gly (12.7 and 12.6%, respectively) and Ala (12.0 and 15.8%, respectively) residues. Many Gly and Ala residues are conserved among *GAS1*, *GAS2*, and their homologs (Figure 1).

Putative homologs of *GAS1* and *GAS2* also were found in the *Neurospora crassa* genome sequenced at the Whitehead Research Institute (www-genome.wi.mit.edu/annotation/fungi/neurospora/), in the *Aspergillus fumigatus* genome (tigrblast.tigr.org/ufmg), and in the ESTs of the entomopathogenic fungus *Metarhizium anisopliae* from the cDNA library of infected insect cuticles. However, none of these Gas1/Gas2 homologs has been characterized functionally, and no other close homologs of Gas1/Gas2 with an identified function were found in BLAST searches. Interestingly, there is no homolog of *GAS1* or *GAS2* in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, or other sequenced eukaryote genomes, indicating that these two genes are specific for filamentous fungi.

Both *GAS1* and *GAS2* Are Expressed Specifically at the Appressorium Formation Stage

RNA gel blots of RNAs isolated from vegetative hyphae and appressoria formed on wax paper for 36 h were hybridized with *GAS1* and *GAS2*. Bands of 1.7 and 1.9 kb were detected with the *GAS1* and *GAS2* probes, respectively, in RNA isolated from Guy11 appressoria (Figure 2). No expression of *GAS1* and *GAS2* was detected in RNA isolated from Guy11 vegetative hyphae grown in 5 × YEG (see Methods). In the *pmk1* deletion mutant nn78, no detectable signal was observed for *GAS1* or *GAS2* in conidia germinated on wax paper for 36 h or in vegetative hyphae growing in 5 × YEG, suggesting that the transcription of *GAS1* and *GAS2* is regulated by *PMK1*.

To further confirm that *GAS1* and *GAS2* are expressed specifically during appressorium formation, RNAs from ungerminated conidia, conidia germinated in liquid 5 × YEG for 4 h, and rice leaves infected with Guy11 for 60 h and 5 days were isolated. No detectable hybridization signal was observed in RNA gel blots of these RNAs hybridized with *GAS1* and *GAS2* (data not shown). Most likely, *GAS1* and *GAS2* are expressed only during appressorium formation in Magnaporthe.

Approximately 1.2- and 1.4-kb upstream sequences of *GAS1* and *GAS2* were sequenced and analyzed with several programs, including TRES (www.bioportal.bic.nus.edu.sg/tres), Expasy (www.expasy.org), and SoftBerry (www.softberry.com). Several pyrimidine-rich repeat sequences (CT box) were located downstream from the putative TATA boxes at −257 in *GAS1* and −230 in *GAS2*. The promoter region of *GAS1* also has a putative CAAT box (−276) upstream from the start codon. Even though both *GAS1* and *GAS2* are expressed specifically in appressoria, no other known sequence element was found to be common in their promoter areas.

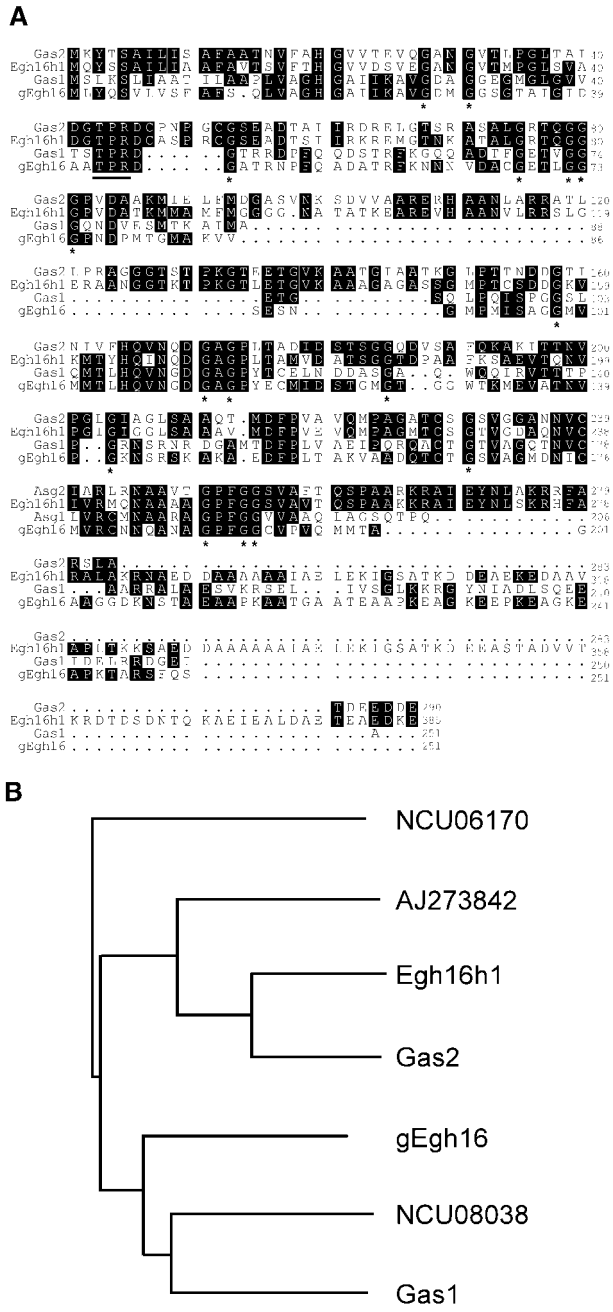


Figure 1. Amino Acid Sequences and Relatedness of *GAS1* and *GAS2*.

(A) Alignment of the predicted amino acid sequences of *GAS1* and *GAS2* with their homologs from *E. graminis* (*gEgh16* and *Egh16h*). Identical residues are shown in black boxes. Gly residues conserved in all of these proteins are marked with asterisks underneath. The putative protein kinase C phosphorylation site is underlined.

(B) Phylogenetic relationship among Gas1, Gas2, and their homologs. The amino acid sequences of these fungal proteins were analyzed using the Clustal X algorithm to create the dendrogram. NCU08038 and NCU06170 are two hypothetical proteins from *N. crassa*. AJ273824 is an EST from the infected insect cuticle library of *M. anisopliae*.

gas1 Mutants Are Defective in Penetration

The gene replacement vector pCX4 was linearized with BstXI and transformed into Guy11. Seventy hygromycin-resistant transformants were isolated and screened by PCR with primers C5F and H854. A 1.4-kb fragment was amplified in two putative *gas1* deletion transformants, BC7 and BC46 (data not shown). DNA gel blot analysis indicated that BC7 and BC46 had the 2- and 3-kb NcoI bands but lost the wild-type 4.2-kb NcoI band when hybridized with a 1.6-kb AflII fragment from pCX4 as the probe (Figure 3). One of the randomly selected transformants, BC32, contained the wild-type 4.2-kb band and two additional bands, of 1.5 and 2.5 kb, resulting from ectopically integrated pCX4.

The *gas1* deletion mutants BC7 and BC46 had no obvious defects in vegetative growth, conidiation, or sexual reproduction, and they formed typical grayish wild-type colonies. Conidia produced by these mutants were normal in germination and appressorium formation (Table 1). Appressoria formed by *gas1* mutants were melanized and regular in shape (Figure 4). However, the percentage of appressoria that penetrated onion epidermal cells was reduced in the *gas1* mutants. Although >70% of Guy11 and BC32 appressoria penetrated and developed infectious hyphae after 48 h of incubation, only ~25% of appressoria formed by *gas1* mutants penetrated under the same conditions (Table 1, Figure 4). Thus, the majority of appressoria formed by *gas1* mutants were defective in appressorial penetration.

gas1 Mutants Are Reduced in Virulence

Rice leaves of CO39 seedlings sprayed with conidia of *gas1* mutants developed blast lesions 7 days after inoculation (Figure 5A). However, the number of lesions caused by the *gas1* mutant BC46 was much lower than that caused by Guy11 under the same conditions (Table 1). On average, Guy11 caused ~45 lesions per 5-cm leaf tip, whereas leaves inoculated with BC46 conidia had ~13 lesions per 5-cm leaf tip (Table 1), an ~70% reduction compared with Guy11. On barley leaves inoculated with BC46, the number of lesions formed also was reduced (Figure 5B). In addition, lesions formed by BC46 on rice or barley leaves usually were smaller and less spreading than the lesions formed by Guy11 (Figure 5A). A similar reduction in lesion formation and lesion size also was observed in the *gas1* deletion mutant BC7 (data not shown).

For cosegregation assays, we isolated and characterized 24 progeny from a cross between BC46 and 2539. Although all 12 hygromycin-resistant progeny were reduced in appressorial penetration and lesion formation on rice leaves, the 12 hygromycin-sensitive progeny all were phenotypically similar to Guy11. These data indicated that the hygromycin resistance cosegregated with the reduced virulence of *gas1* mutants.

We also transformed pCX16 containing the full-length

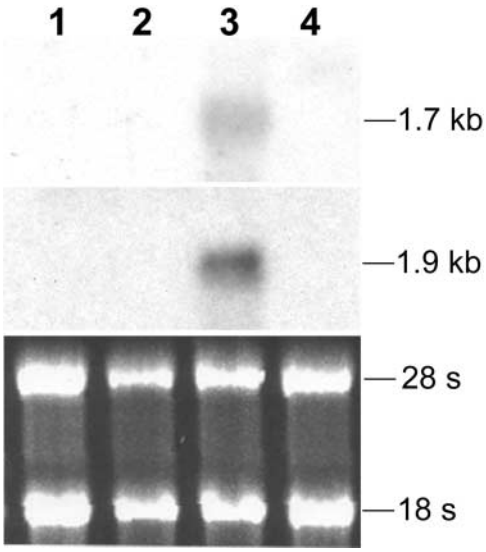


Figure 2. Expression Patterns of *GAS1* and *GAS2*.

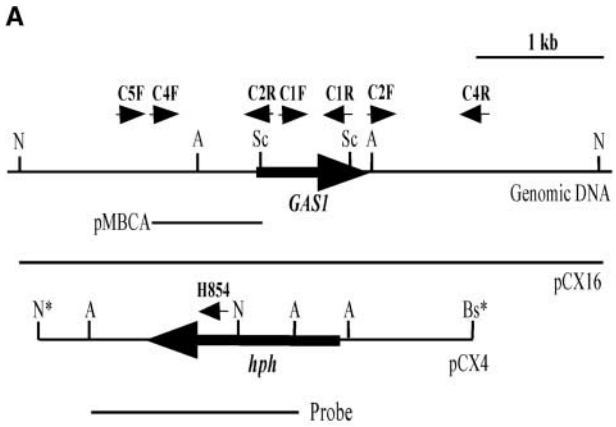
The top gel shows an RNA gel blot hybridized with *GAS1*. The middle gel shows the same blot stripped and rehybridized with *GAS2*. The bottom gel shows the RNA gel stained with ethidium bromide. Approximately 10 μg of total RNA was loaded in each well. Lanes 1 and 2 contained RNAs isolated from vegetative hyphae of Guy11 (wild type) and nn78 (*pmk1*), respectively. Lanes 3 and 4 contained RNAs isolated from Guy11 and nn78 conidia germinated on wax paper for 36 h. Both *GAS1* and *GAS2* were detectable in Guy11 only under the appressorium formation condition.

GAS1 gene (Figure 3) into *gas1* mutant BC46 by cotransformation with pAC905. Among 24 zeocin-resistant transformants screened by PCR with primers C1F and C1R, four with the *GAS1* gene integrated ectopically were identified and confirmed further by DNA gel blot analysis (data not shown). These four complemented *GAS1* transformants were as virulent as Guy11. Thus, reintroduction of the wild-type *GAS1* allele restored the appressorial penetration and lesion development defects in *gas1* mutants.

gas2 Mutants Have Phenotypes Similar to Those of *gas1* Mutants

Three putative *gas2* gene replacement mutants, BE55, BE68, and BE80, were identified by screening with the PCR primers E6F and H853 (data not shown). When genomic DNAs were digested with EcoRI and hybridized with the 2.6-kb fragment amplified from pCX3 as the probe, the wild-type strain Guy11 had an 8.5-kb band (Figure 6). The *gas2* gene replacement mutants BE55, BE68, and BE80 all had the 2.7- and 6.5-kb bands but not the wild-type 8.5-kb band (Figure 6). A randomly selected transformant, BE71, contained both the wild-type 8.5-kb EcoRI fragment and the 0.6- and 2.2-kb EcoRI fragments resulting from ectopically integrated pCX3. Because BE80 had hybridization bands resulting from both homologous recombination and ectopic integration (Figure 6), only BE68 and BE55 were used in further analyses.

The *gas2* mutants also were normal in vegetative growth and sexual and asexual reproduction, but they were reduced in appressorial penetration and lesion formation (Table 1, Figure 4). On average, the *gas2* mutants usually produced ~10 lesions on the 5-cm leaf tip, an ~77% reduction compared with Guy11 under the same conditions (Table 1). Morphologically, *gas1* and *gas2* mutants were not distin-



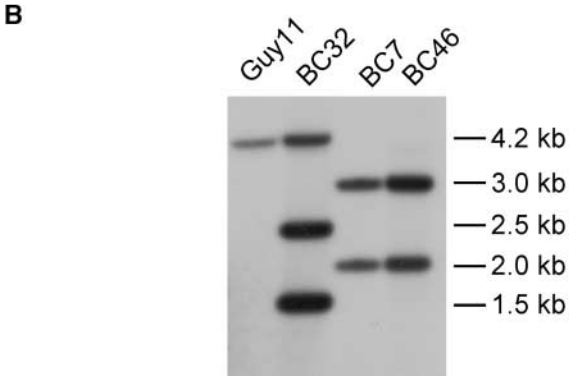


Figure 3. *GAS1* Gene Replacement Vector and Transformants.

(A) Physical map of the *GAS1* genomic region and gene replacement vector pCX4. Large arrows indicate orientations of the *GAS1* and *hph* genes. Asterisks mark the restriction enzymes derived from the cloning vectors. The positions and orientations of primers C5F, C4F, C2R, C1F, C1R, C2F, C4R, and H854 are labeled with small arrows. A, AflII; Bs, BstXI; N, NcoI; Sc, SacI.

(B) DNA gel blot hybridized with the 1.6-kb AflII fragment (probe) from pCX4. Lanes from left to right represent genomic DNAs from wild-type strain Guy11, ectopically integrated transformant BC32, and *gas1* deletion mutants BC7 and BC46. All DNA samples were digested with NcoI. The estimated size of each band is labeled at right (in kb).

Table 1. Appressorial Penetration and Lesion Development in *gas1* and *gas2* Mutants

Variable	Guy11 (Wild Type)	BE71 (Ectopic)	BC46 (*gas1*)	BE55 (*gas2*)	DS21 (*gas1 gas2*)
Appressorium formation[a] (%)	93.8 ± 4.1	90.2 ± 6.1	90.5 ± 3.6	87.7 ± 6.4	91.2 ± 2.7
Appressorium penetration (%)	77.3 ± 3.8	72.2 ± 5.4	25.7 ± 10.8	20.1 ± 11.6	19.9 ± 16.8
Lesion formation[b] (lesions/5-cm leaf tip)	46.7 ± 5.7	47.9 ± 4.9	13.0 ± 10.2	10.8 ± 7.0	9.7 ± 3.0

[a] Appressorium formation and penetration were assayed on onion epidermis. The percentage of germ tubes differentiated into appressoria and the percentage of appressoria that developed infectious hyphae were determined at 48 h after inoculation.
[b] Lesion formation was examined on infected rice leaves 7 days after inoculation. Means and SD values were calculated from at least three independent experiments.

guishable. Twenty-six progeny were isolated from a cross between BE55 and 2539. Reduction in lesion formation was observed in all 10 hygromycin-resistant progeny but not in the 16 hygromycin-sensitive progeny (data not shown). Thus, the phenotypes observed in *gas2* mutants were associated directly with the deletion of *GAS2*. We also generated *gas1* deletion mutants in strain 70-15 and found that they had phenotypes similar to those of *gas1* mutants in Guy11 (data not shown), indicating that the function of *GAS2* is not strain specific.

gas1 gas2 Double Mutants Have Phenotypes Similar to Those of *gas1* or *gas2* Mutants

One *MAT1-1 gas2* progeny (MS12) derived from the cross BE55 × 2539 was crossed with the *gas1* mutant BC46 (*MAT1-2*). Among 24 progeny isolated from this cross, four *gas1 gas2* double mutants, DS14, DS18, DS21, and DS32, were identified by PCR screening with primers C5F/H854 and E6F/H853 and confirmed further by DNA gel blot analysis (data not shown). All four of these double mutants had normal hyphal growth, colony morphology, and conidiation. Conidia germination and appressoria morphology also were normal (Table 1).

To our surprise, the reduction in the appressoria penetration and lesion formation of *gas1 gas2* double mutants was similar to that of the *gas2* or *gas1* mutants (Table 1). We repeated the infection and appressorial penetration assays several times and observed no significant difference between *gas2* and *gas1 gas2* double mutants. It is likely that the deletion of both *GAS1* and *GAS2* did not have any additive effect in *gas1 gas2* double mutants.

Both *GAS1* and *GAS2* Localize to Cytoplasm in the Appressoria

To determine the localization pattern of Gas1 and Gas2 proteins, the *GAS1*–green fluorescent protein (GFP) fusion construct pCX13 and the *GAS2*-GFP fusion construct pCX14 were transformed into Guy11. Two pCX13 transformants (GC22 and GC24) and two pCX14 transformants (GE6 and GE19) were identified by PCR and confirmed by DNA gel blot analysis (data not shown). In transformants expressing *GAS1*-GFP and *GAS2*-GFP, no obvious change in growth or virulence was observed. In both pCX13 and pCX14 transformants, green fluorescence was observed in the majority of appressoria after 24 h of incubation (Figure 7A). However, no detectable signals were observed in the germ tubes, conidia, or vegetative hyphae (Figure 7A).

We also examined conidiophores, young conidia, perithecia, and asci produced by GC22 and GE19 and did not observe any GFP signals. These data indicate that the Gas1-GFP and Gas2-GFP fusion proteins were expressed specifically in appressoria. The GFP fusion constructs pCX13 and pCX14 also were transformed into the *gas1* mutant BC46 and the *gas2* mutant BE55, respectively. MCX13 and MCX14 were two resulting transformants that were confirmed by DNA gel blot analysis to contain a single-copy integration of transforming vector pCX13 or pCX14. Both MCX13 and MCX14 were as efficient as Guy11 in appressorial penetration and lesion formation (data not shown), indicating that the Gas1-GFP and Gas2-GFP fusion proteins function normally in Magnaporthe.

When examined by epifluorescence microscopy, the majority of appressoria expressing Gas1-GFP and Gas2-GFP fusion proteins had green fluorescence in the cytoplasm but not in the cell wall. We further confirmed these observations by examining the localization of GFP fusion proteins under confocal microscopy. Appressoria formed by both GE19 and GC22 had uneven fluorescent peripheries and strong green fluorescence in the cytoplasm (Figure 7B), indicating that Gas1-GFP and Gas2-GFP fusion proteins were localized in the cytoplasm but not in the cell wall or cytoplasm membrane.

These GFP fusion proteins were not distributed evenly in the cytoplasm, however, and there were certain areas in the appressorium cytoplasm, possibly the lipid or glycogen bodies and nucleus, that had no detectable green fluorescence. Close examination indicated that the cellular localizations of

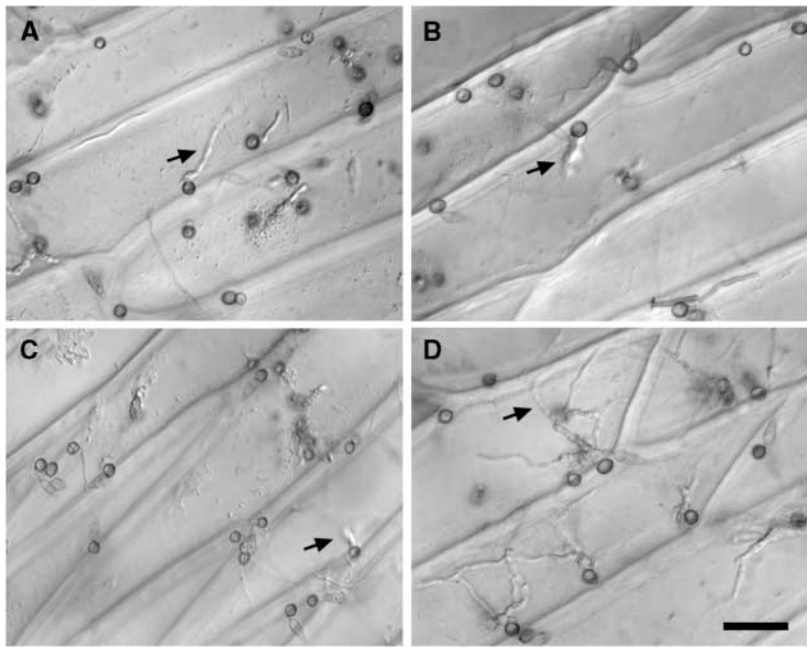


Figure 4. Appressorial Penetration Assays on Onion Epidermal Cells.

Conidia from *gas1* mutant BC46 **(A)** and *gas2* mutant BE55 **(B)** germinated and formed melanized appressoria, but only a few of them penetrated onion epidermal cells and produced infectious hyphae. The *gas1 gas2* double mutant DS21 **(C)** also was reduced in appressorial penetration. Most appressoria formed by the wild-type strain Guy11 **(D)** penetrated and produced infectious hyphae. Photographs were taken 48 h after inoculation. Arrows indicate infectious hyphae. Bar in **(D)** = 15 μm for **(A)** to **(D)**.

Gas1 and Gas2 were slightly different. Although Gas2-GFP fusion proteins were distributed somewhat evenly throughout the cytoplasm, Gas1 proteins were localized preferentially in the vacuole in appressoria (Figure 7B).

Interestingly, not all of the appressoria produced by transformants GE19 or GC22 displayed green fluorescence (Figure 7A). We then transformed pCX35 into GC22 and isolated transformants that contained *GAS1*-GFP and *GAS2*–yellow fluorescent protein (YFP) fusion constructs. In DT2, one of the transformants expressing both *GAS1*-GFP and *GAS2*-YFP, growth and virulence were normal (data not shown). However, even in DT2, there was always a small portion of appressoria that displayed no detectable fluorescence under epifluorescence microscopy (Figure 8). It is possible that the fluorescent fusion proteins were not expressed or were expressed in these appressoria at a level lower than the detection limit of the epifluorescence microscopy.

We also transformed pCX27, a *GAS2*-GFP fusion construct with the 3′ untranslated region sequence of *GAS2*, into Guy11. In MCX27 and other transformants expressing this *GAS2*-GFP-T_{GAS2} construct, we also observed variation of the GFP signal among appressoria, and some appressoria had no detectable GFP signal at all (data not shown). No obvious difference in GFP signals was observed between appressoria formed by MCX27 and GE19 (Figure 7), indicating that the 3′ untranslated region of *GAS2* has no significant effect on the accumulation and localization of Gas2-GFP fusion proteins.

Expression Patterns of *GAS1* and *GAS2* Are Different during Appressorium Formation

For appressoria produced by GC22, only very faint green fluorescence was detectable after incubation for 9 h (Table 2). Green fluorescence became stronger in the majority of appressoria when examined at 12 and 24 h. At 48 h, the green fluorescence in most appressoria was less intense, and only a small percentage of appressoria had green fluorescence after 4 days (Table 2), suggesting that the Gas1-GFP fusion protein accumulated in appressoria at the earlier stages and then was degraded later (after 24 h). The Gas2-GFP fusion protein, however, was expressed earlier than Gas1-GFP and could be detected as early as 4 h (Table 2) in appressoria formed by GE19.

At 6 h, >40% of appressoria formed by GE19 had strong green fluorescence. When examined from 12 to 48 h, the majority of appressoria (>80%) had strong green fluores-

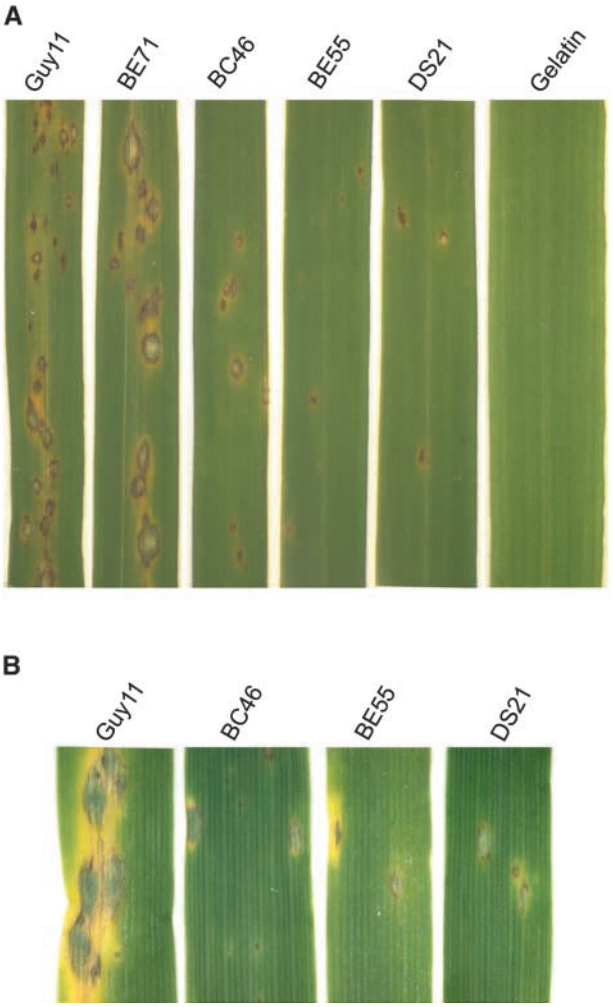


Figure 5. Infection Assays on Rice and Barley Leaves.

(A) Seedlings from rice cv CO39 were spray inoculated with wild-type Guy11, ectopic integration transformant BE71, *gas1* mutant BC46, *gas2* mutant BE55, or *gas1 gas2* double mutant DS21. For controls, leaves were sprayed with 0.25% gelatin solution.
(B) From left to right are barley leaves inoculated with Guy11, *gas1*, *gas2*, and the *gas1 gas2* double mutant. Typical leaves are shown 7 days after inoculation.

cence. The Gas2-GFP fusion protein appeared to be more stable than Gas1-GFP, because ~45% of GE19 appressoria remained fluorescent after 4 days of incubation. Interestingly, appressoria formed by transformants expressing *GAS2*-GFP usually had stronger fluorescence than those formed by transformants expressing *GAS1*-GFP (Figure 7) at any time points examined (data not shown), indicating that the expression level of *GAS2*-GFP was higher than that of *GAS1*-GFP in appressoria.

To determine whether *GAS1* and *GAS2* can complement each other, we introduced pCX14 into BC46 and pCX13 into BE55. The resulting transformants had phenotypes similar to those of the original *gas1* and *gas2* mutants BC46 and BE55. No cross-complementation effect on appressorial penetration or lesion development was observed in *gas1* and *gas2* mutants transformed with pCX14 and pCX13, respectively. It is likely that *GAS1* and *GAS2* play some overlapping but different functions at different stages of appressorium formation.

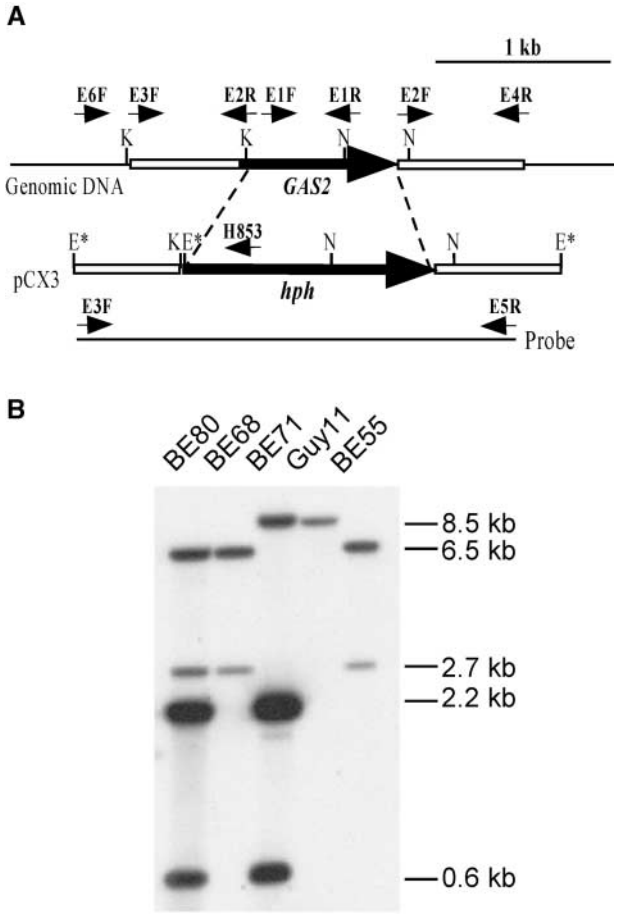


Figure 6. Gene Replacement of *GAS2*.

(A) Physical map of *GAS2* and the gene replacement vector pCX3. Large arrows indicate orientations of the *GAS2* and *hph* genes. The EcoRI sites derived from the cloning vectors are marked with asterisks. The positions and orientations of primers E6F, E3F, E2R, E2F, E4R, E5R, and H853 are labeled with small arrows. E, EcoRI; K, KpnI; N, NcoI.
(B) DNA gel blot hybridized with the 2.6-kb fragment (probe) amplified with primers E3F and E5R. Genomic DNAs isolated from Guy11 and four transformants (BE80, BE68, BE71, and BE55) were digested with EcoRI. The *gas2* deletion mutants BE80, BE68, and BE55 had the 2.7- and 6.5-kb hybridization bands but not the wild-type 8.5-kb band.

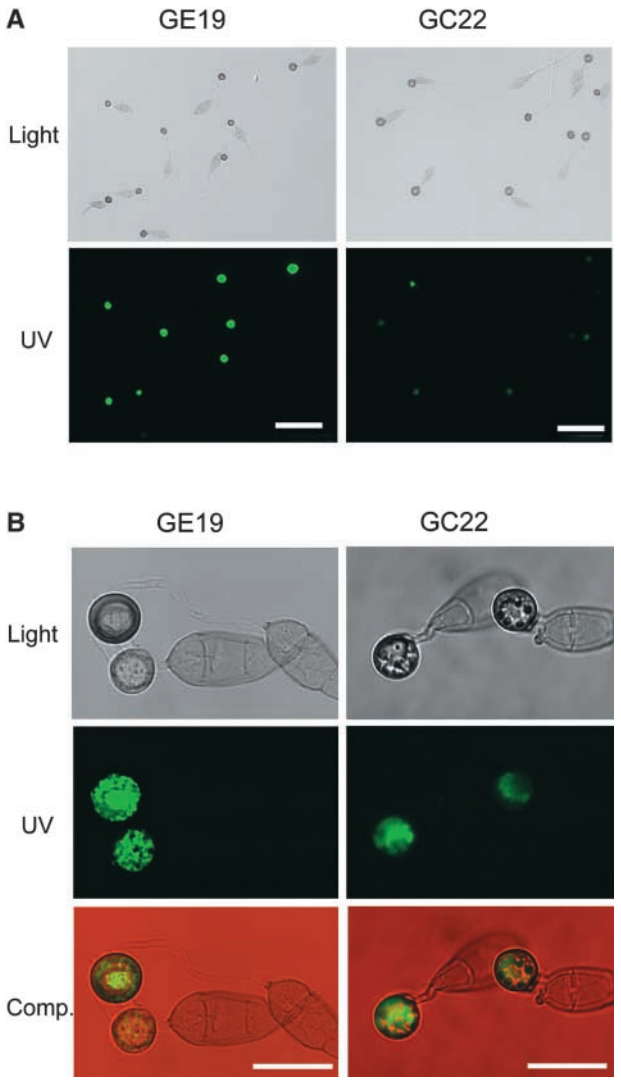


Figure 7. Expression and Localization of Gas1 and Gas2.

(A) GFP tagging of *GAS1* and *GAS2*. Appressoria formed by transformants expressing *GAS1*-GFP (GC22) and *GAS2*-GFP (GE19) were normally melanized when examined using Nomarski microscopy (top) at 24 h. Most of these appressoria had green fluorescence, but the intensity of fluorescence varied among different appressoria when examined using epifluorescence microscopy (bottom). Bars = 50 μm.

(B) Confocal microscopy examination of the Gas1-GFP and Gas2-GFP fusion proteins. GFP fusion proteins were localized in the cytoplasm of appressoria formed by GE19 (left) and GC22 (right) at 24 h. Micrographs of the same field taken under bright-field illumination (Light) and UV light (UV) with a confocal laser fluorescence microscope were used to generate the composite images (Comp.) with the Thumbsplus 4 program (Cerious Software, Charlotte, NC). Bars = 15 μm.

GAS1 and *GAS2* Are Not Expressed in Infectious Hyphae

To determine whether *GAS1* and *GAS2* were expressed in the infectious hyphae, conidia from DT2 were used to infect onion epidermal cells and barley leaves. Although appressoria formed by DT2 on onion epidermal cells had strong green fluorescence, there was no detectable fluorescence in well-developed infectious hyphae (Figure 8). Similar results were obtained with infectious hyphae formed by DT2 in barley epidermal cells (data not shown). These data indicated that *GAS1* and *GAS2* were not expressed in infectious hyphae.

In some but not all primary infectious hyphae or pegs that penetrated into onion epidermal cells, weak fluorescence was observed (Figure 8), suggesting that the fluorescent fusion proteins accumulated in appressoria may be transported into the primary infectious hyphae or that the fusion constructs were expressed transiently at this penetration stage. Interestingly, many appressoria that failed to penetrate remained fluorescent, but none of the appressoria that produced infectious hyphae had any detectable fluorescence (Figure 8).

It is likely that Gas1 and Gas2 proteins are functional only in appressoria and possibly are involved in penetration peg and primary infectious hyphae development. After penetration, these fusion proteins are either degraded rapidly or transported into penetration pegs. Data from these assays are consistent with our earlier observation that *GAS1* and *GAS2* were not detectable by RNA gel blot analysis or reverse transcriptase–mediated PCR with RNAs isolated from rice leaves collected at 60 h or 5 days after inoculation.

DISCUSSION

To further characterize the *PMK1* MAP kinase pathway in Magnaporthe, two genes identified in a subtraction library enriched for genes regulated by *PMK1* were characterized functionally in this study. Both *GAS1* and *GAS2* are highly expressed during appressorium formation and rich in Ala and Gly. Interestingly, there is a putative protein kinase C phosphorylation site (Figure 1) that is well conserved among *GAS1*, *GAS2*, and their homologs from other fungi. In Magnaporthe, pharmacological studies had indicated that protein kinase C may be involved in appressorium formation (Eckhard et al., 1998).

GAS1 or *GAS2* or both are dispensable for mycelial growth, conidiation, and sexual reproduction, but they are important for appressorial penetration and lesion development. In Magnaporthe, several genes, such as *MPG1*, *PTH11*, and *ABC1*, are dispensable for vegetative growth and sexual or asexual reproduction but play important roles in appressorium formation and plant infection. Mutants disrupted in *MPG1*, a hydrophobin gene, were reduced by 74 and 80% in appressorium formation and lesion develop-

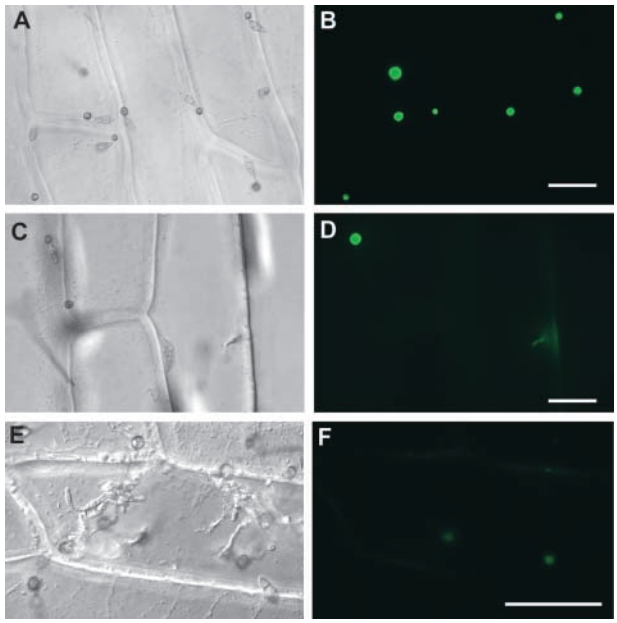


Figure 8. Expression of *GAS1* and *GAS2* in Infectious Hyphae.

Conidia from DT2, a strain expressing both Gas1-GFP and Gas2-YFP fusion proteins, were inoculated onto onion epidermal cells. At left are photographs taken using bright-field microscopy. At right are the same fields photographed using epifluorescence microscopy. Fluorescence in appressoria (**[A]** and **[B]**), penetration pegs and primary infectious hyphae (**[C]** and **[D]**), and well-developed infectious hyphae (**[E]** and **[F]**) were examined at 24, 48, and 60 h, respectively. After penetration, only the penetration pegs and primary infectious hyphae, but not the well-developed infectious hyphae, had faint fluorescence. Many appressoria that failed to penetrate remained fluorescent, but those that penetrated were no longer fluorescent. Bars = 50 μm.

ment, respectively (Talbot et al., 1993). Both *abc1* and *pth11* deletion mutants are reduced substantially in virulence, but they still form appressoria and cause rare lesions on infected rice leaves (DeZwaan et al., 1999; Urban et al., 1999).

Because the expression pattern and cellular localization of the Gas1-GFP and Gas2-GFP fusion proteins are slightly different, it is reasonable to assume that *GAS1* and *GAS2* may have different functions during appressorial penetration and infectious hyphal growth. Surprisingly, the *gas1 gas2* double mutants have phenotypes similar to those of *gas2* or *gas1* mutants. No additive effect of *gas1* and *gas2* deletions was observed in the *gas1 gas2* double mutants. One possible explanation for this finding is that *GAS1* and *GAS2* function sequentially in the penetration and lesion development processes. Mutants deleted in either the *GAS1* or *GAS2* gene will block the infection process to a level similar to that of the *gas1 gas2* double mutants.

The other possibility is that *GAS1* and *GAS2* may interact with each other and form a complex that is required for efficient penetration and lesion development. However, the Magnaporthe genome may contain additional *GAS1* or *GAS2* homolog(s) that can compensate partially for the deletion of *GAS1* or *GAS2*. In *E. graminis*, two isoforms of *Egh16H1* (A and B) that differ in only a few amino acid residues have been isolated as genes expressed in early infection stages. The exact biological function of *gEgh16* and *Egh16H1* isoforms in *E. graminis* is not clear.

Both *GAS1* and *GAS2* are expressed specifically in appressoria and play important roles in appressorium penetration. A few fungal genes are known to be expressed specifically in appressoria and are critical for plant infection. One of them is *PLS1* in Magnaporthe, which encodes a transmembrane protein expressed only in appressoria, as determined by GFP-tagging assays (Clergeot et al., 2001). The *pls1* deletion mutants fail to form penetration pegs and thus are nonpathogenic on rice (Clergeot et al., 2001).

Two other Magnaporthe genes were reported to be expressed specifically in appressoria but were not characterized functionally (Lee and Dean, 1993). In *Colletotrichum gloeosporioides*, *CAP20* was isolated as a gene expressed specifically during appressorium formation (Hwang et al., 1995). The *cap20* gene disruption mutants were reduced in virulence on avocado and tomato fruits. Like *GAS1* and *GAS2* in Magnaporthe, *CAP20* encodes a protein that is specific for filamentous fungi but with no functionally characterized homolog in GenBank.

Both Gas1-GFP and Gas2-GFP fusion proteins localized to the cytoplasm in appressoria. When analyzed with various programs available on the World Wide Web for extracellular or subcellular localization, no consistent predictions were obtained for either Gas1 or Gas2. Interestingly, putative signal peptides at the N termini of Gas1 and Gas2 were identified by SignalP version 2.0 (Nielsen et al., 1997). However, no signal peptide sequence was detected in either Gas1 or Gas2 when analyzed with PSORT II (psort.nibb.ac.jp).

Table 2. Expression of GFP Fusion Proteins in Transformants of Magnaporthe

	Appressoria with Detectable GFP Signal (%)	
Time (h)	GE19 (Gas1-GFP)	GC22 (Gas2-GFP)
2	ND[a]	ND
4	Faint[b]	ND
6	42.6 ± 2.6	ND
9	64.4 ± 13.2	Faint
12	82.3 ± 5.5	85.4 ± 12.3
24	90.8 ± 0.8	80.5 ± 1.9
36	81.1 ± 3.8	82.7 ± 1.3
48	84.2 ± 7.6	73.1 ± 8.5
96	45.9 ± 2.7	16.4 ± 1.0

[a] ND, no detectable fluorescence.

[b] Faint fluorescence was observed in some appressoria, but the signal was too weak to count reliably.

Table 3. Wild-Type Strains and Mutants of Magnaporthe Used in This Study

Strain	Brief Description	Reference
Guy11	Wild-type *MAT1-2*	Leung et al., 1988
nn78	Δ*pmk1* of Guy11	Xu and Hamer, 1996
2359	Wild-type *MAT1-1*	Leung et al., 1988
70-15	Wild-type *MAT1-2*	Chao and Ellingboe, 1991
BC32	Ectopic transformant of Guy11 transformed with pCX4	This study
BE71	Ectopic transformant of Guy11 transformed with pCX3	This study
BC7	Δ*gas1* of Guy11	This study
BC46	Δ*gas1* of Guy11	This study
BE55	Δ*gas2* of Guy11	This study
BE68	Δ*gas2* of Guy11	This study
DS21	Δ*gas1* Δ*gas2* double mutant of Guy11	This study
GC22	Guy11 transformed with pCX14 (*GAS1*-GFP)	This study
GC24	Guy11 transformed with pCX14 (*GAS1*-GFP)	This study
GE6	Guy11 transformed with pCX13 (*GAS2*-GFP)	This study
GE19	Guy11 transformed with pCX13 (*GAS2*-GFP)	This study
DT2	GC22 transformed with pCX35 (*GAS2*-YFP)	This study
MCX13	BC46 transformed with pCX13 (*GAS1*-GFP)	This study
MCX14	BE55 transformed with pCX14 (*GAS2*-GFP)	This study
MCX27	Guy11 transformed with pCX27 (*GAS2*-GFP + 3′ untranslated region)	This study

We were unable to observe green fluorescence in the surrounding areas of either fluorescent or nonfluorescent appressoria formed by GE19, GC22, or DT2 (Figures 7 and 8). After removing appressoria by rubbing with gloved fingers, no green fluorescence was observed in the appressorial mucilage left behind (data not shown). Most likely, the Gas1 and Gas2 fusion proteins were not secreted from these appressoria. We were unable to detect green fluorescence in infectious hyphae or the transcript of *GAS1* or *GAS2* in infected rice leaves, indicating that these two genes were not expressed in infectious hyphae.

However, it remains possible that the fluorescent fusion proteins may be expressed and secreted into plant cells at a level lower than the detection limit. Plant cells penetrated by Magnaporthe usually accumulate autofluorescent materials as part of the defense response, making it impossible to detect small amounts of secreted fluorescent fusion proteins. It may be necessary to isolate extracellular proteins secreted by Magnaporthe and analyze them by protein gel blot analysis.

In the GFP-tagging experiments with *GAS1* and *GAS2*, there were always some normally shaped appressoria without any detectable green fluorescence. This is similar to what has been reported in transformants expressing the Pls1-GFP fusion protein, which had strong fluorescence only in 80% of appressoria (Clergeot et al., 2001). We also observed that the strength of fluorescent signals varied among appressoria formed by transformants expressing Gas1-GFP, Gas2-GFP, or both. In Magnaporthe, appressorium formation and maturation involve the deposition of additional cell wall layers, mobilization and use of carbohydrate stores, generation of turgor, cytoskeleton reorganization, and other steps (Bourett and Howard, 1990).

The expression of *GAS1* and *GAS2* may be regulated developmentally in these processes. The variation in GFP signal among appressoria may reflect different developmental stages of the appressoria we observed. *MST12* is one of the transcription factors regulated by *PMK1* to control appressorial penetration and infectious hyphae growth (Park et al., 2002). Our preliminary data indicated that the expression of Gas2-GFP is normal in appressoria formed by the *mst12* mutant MK23 transformed with pCX14. Thus, the expression of *GAS2* is not regulated by *MST12*. *GAS1* and *GAS2* may be controlled by other transcription factors specific for appressorium formation and maturation and infectious hyphal growth. Although their homologs exist in the saprophytic fungus *N. crassa*, *GAS1* and *GAS2* may belong to a class of genes that are specific for filamentous fungi and that play important roles during the early infection stage in fungal pathogens. It will be interesting to isolate and characterize transcription factors that may regulate their expression during appressorium formation.

METHODS

Strains and Culture Conditions

Wild-type *Magnaporthe grisea* strains and various transformants generated in this study (Table 3) were cultured at 25°C on oatmeal agar plates or V8 juice agar under fluorescent light to induce conidiation (Xu and Hamer, 1996; Choi and Dean, 1997). Mycelia collected from 2-day-old 5 × YEG (5 mg/ml yeast extract and 10 mg/ml glucose; Zheng et al., 2000) cultures shaken at 150 rpm at room temperature were used for the isolation of fungal DNA

and protoplasts. Genetic crosses and isolation of progeny were performed as described (Xu and Hamer, 1996). The mating type of each progeny was determined by mating tests and PCR with the *MAT1-1*–specific primers MATA1 (5′-AGCCTCATCAACGGCAA-3′) and MATA5 (5′-GGCACGAACATGCGATG-3′) and the *MAT1-2*–specific primers MATB15 (5′-CTCAATCTCCGTAGTAG-3′) and MATB16 (5′-ACAGCAGTATAGCCTAC-3′).

Molecular Manipulations with DNA and RNA

Fungal DNAs were extracted using the cetyl-trimethyl-ammonium bromide protocol (Xu and Hamer, 1996). Plasmid DNAs were isolated with Qiagen plasmid preparation kits (Valencia, CA) and sequenced with the ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA). Standard molecular biology procedures were followed for RNA and DNA gel blot analyses and enzymatic manipulations with DNAs and RNAs (Sambrook et al., 1989). Homolog searches of DNA/protein sequence databases were performed with the BLAST programs (Altschul et al., 1997). Amino acid sequence comparisons and alignments were made with the BESTFIT, PILEUP, T-COFFEE, and BOXSHADE programs in the GCG Wisconsin software package (Accelrys, San Diego, CA).

Construction of a Subtraction Library Enriched for Genes Regulated by *PMK1*

RNAs were prepared with the Trizol reagent (Invitrogen, Carlsbad, CA) from the wild-type strain Guy11 and *pmk1* mutant nn78 conidia germinated on wax paper (Reynolds, Richmond, VA) for 18 h. Under these conditions, the wild-type strains form appressoria, but *pmk1* mutants form only subapical swollen bodies (Xu and Hamer, 1996). A subtraction library was constructed with the PCR-Select kit (Clontech, Palo Alto, CA) using the cDNA synthesized from Guy11 RNA as the tester and the cDNA synthesized from nn78 as the driver. Subtractive PCR products were cloned in the pGEM-T Easy vector (Promega, Madison, WI). After transformation into *Escherichia coli* DH5α, 550 white colonies were picked and preserved individually as the subtraction library.

Construction and Differential Screening of an Appressorium-Stage cDNA Library

RNA isolated using the guanidine isothiocyanate method (Sambrook et al., 1989) from conidia of strain 70-15 germinated on cellophane membranes for 6 h was used to synthesize cDNA with a cDNA synthesis kit (Stratagene, La Jolla, CA). After digestion with EcoRI and XhoI, the resulting cDNA was ligated to pBluescript II SK+ (Stratagene) and transformed into *E. coli* DH10B. A total of 18,432 individual recombinant clones were transferred by a Q-Bot robotic work station (Genetix, New Milton, UK) to 384-well microplates and preserved. High-density filters of this library were hybridized differentially with cDNA probes prepared from total RNAs extracted from vegetative hyphae grown in liquid complete medium and from the appressorial stage. The cDNA clones that hybridized exclusively to the appressorium cDNA probe were selected for sequencing and RNA gel blot analysis.

Construction of the *GAS1* and *GAS2* Gene Replacement Vectors

The genomic sequences of *GAS1* and *GAS2* were obtained by sequencing the corresponding cosmid clones isolated from a Guy11 pMOcosX cosmid genomic library (Xu and Hamer, 1996) by primer walking. A 0.8-kb upstream sequence of *GAS1* was amplified with PCR primers C4F (5′-AAGGTCCTCAGAGCAGCTTG-3′) and C2R (5′-ATGCTCTCAACATCGTTCTG-3′) and cloned into the pGEM-T Easy vector as pMBCA. The 1.4-kb SalI fragment containing the bacterial hygromycin phosphotransferase gene (*hph*) was released from plasmid pCB1003 (Carroll et al., 1994) and cloned into the SalI site on pMBCA. The resulting construct was linearized with SacI and ligated with a downstream fragment of *GAS1* amplified with primers C2F (5′-TTAAGCGCCTTTCACCTCAA-3′) and C4R (5′-TAGAGCTCTGTAGGAGCCTCAAGTC-3′) to generate the gene replacement vector pCX4.

A similar approach was used to construct the *GAS2* gene replacement vector pCX3. The 0.6-kb upstream sequence amplified with primers E3F (5′-CAATGTGCAAGAGAGTCAGCA-3′) and E2R (5′-AGTGCCATCGATGGCGGTCA-3′) was cloned into pGEM-T Easy as pMBEA. The *hph* cassette released from pCB1003 and the downstream flanking sequence of *GAS2* amplified with primers E2F (5′-GAGGATGACGAGTAGAGTGT-3′) and E4R (5′-GAGTGTTGAGAGTTGAGACA-3′) were cloned between the KpnI and BamHI sites and the BamHI and BstXI sites on pMBEA, respectively, to generate pCX3.

Isolation of *gas1* and *gas2* Deletion Mutants

The gene replacement vectors pCX4 and pCX3 were linearized with BstXI and transformed into Guy11 protoplasts as described (Sweigard et al., 1992). Monoconidial cultures of transformants resistant to 150 μg/mL hygromycin B (Calbiochem, La Jolla, CA) were screened by PCR with primers C5F (5′-CTTCACCCGACCTCTCCTAAC-3′) and H854 (5′-AACAATGTCCTGACGGACAA-3′) or E6F (5′-TGGACGTACAAGCATGACTA-3′) and H853 (5′-GACAGACGTCGCGGTGAGTT-3′). Because the C5F primer sequence was not present in pCX4 (Figure 3), only transformants that had undergone homologous recombination between pCX4 and endogenous *GAS1* would have the 1.4-kb PCR product. Similarly, primers E6F and H853 will amplify a 1.1-kb product only in *gas2* gene replacement transformants with homologous recombination between pCX3 and *GAS2*.

All putative gene replacement mutants identified in PCR screens were confirmed further by DNA gel blot analyses. For complementation assays, a 4.2-kb NcoI fragment containing the full-length *GAS1* gene (Figure 3) was cloned into pGEM-5Zf (Promega) and cotransformed into the *gas1* mutant BC46 with the bleomycin-resistant vector pAC905 (Zheng et al., 2000). Transformants resistant to 200 μg/mL zeocin (Invitrogen) were isolated and screened by PCR with primers C1F and C1R, which are located in the *GAS1* region deleted in pCX4 (Figure 3).

Assaying Germination, Appressorium Formation, and Penetration

Conidia were harvested from 10-day-old oatmeal agar cultures, filtered once through Miracloth (Calbiochem), and resuspended to 5×10^4 conidia/mL in sterile distilled water. Drops of conidial

suspensions (50 μL) were placed on plastic microscope cover slips (Fisher Scientific, Pittsburgh, PA) and incubated in a moist chamber at room temperature. Conidial germination and appressorium formation were examined after incubating for 0.5, 2, 6, 12, and 24 h. Appressorial penetration on onion epidermal cells was assayed as described previously (Xu et al., 1997). Appressorium formation and infectious hyphal growth were examined after incubation at room temperature for 24 to 72 h.

Plant Infection Assays

Conidia were collected from 10-day-old oatmeal agar cultures and resuspended to 2×10^4 conidia/mL in a 0.25% gelatin solution. Two-week-old seedlings of rice (*Oryza sativa* cv CO39) and 8-day-old seedlings of barley (*Hordeum vulgare* cv Golden Promise) were used for infection assays. Plant incubation and inoculation were performed as described (Valent et al., 1991; Xu et al., 1997). Lesion formation was examined 7 days after inoculation. The mean number of lesions formed on 5-cm leaf tips was determined as described previously (Talbot et al., 1993, 1996).

Construction and Expression of Gas1–Green Fluorescent Protein and Gas2–Green Fluorescent Protein Fusion Proteins

The *GAS1* coding region and its 1.2-kb upstream sequence were amplified with primers CU3F (5′-TCAAGCTTCCAGTGCTGGTCT-AGTTC-3′) and CU2R (5′-ATGGATCCGCTTTAGCGATCTCG-3′) using Vent DNA polymerase (New England Biolabs, Beverly, MA) and cloned between the HindIII and BamHI sites on pEGFP (Clontech) as pCX13. The stop codon TAA of *GAS1* was changed to AAA in the primer CU2R, and the resulting *GAS1*–green fluorescent protein (GFP) fusion construct was under the control of the native *GAS1* promoter. A similar procedure was used to generate the *GAS2*-GFP fusion construct. The *GAS2* open reading frame and its 1.4-kb upstream region were amplified with primers EU2F (5′-ATGGGC-CCTTGCAAATAGAGCTAG-3′) and EU2R (5′-ATGGATCCTTCTCGT-CATCCTCCTC-3′) and cloned into pEGFP as pCX14.

We also cloned the PCR product amplified by EU2F and EU2R into pEYFP (Clontech) as pCX26. The *GAS2*–yellow fluorescent protein (YFP) fusion construct was released from pCX26 with HindIII and NotI digestion and cloned into pCB1004 (Carroll et al., 1994) as pCX35. An 825-bp region downstream from the *GAS2* open reading frame was amplified with primers BE5F (5′-TCGCGGCCGCAGGAT-GACGAGTAGAGTGT-3′) and BE5R (5′-ACGGGCCCGAGTGTT-GAGAGTTGAGACA-3′) and cloned between the NotI and ApaI sites on pCX14 as pCX27. All of the fusion constructs were confirmed by sequencing to be correct and in frame.

pCX13 and pCX14 were introduced individually into Guy11 by cotransformation with pAC905. Zeocin-resistant transformants were screened by PCR with primers GP1F (5′-CATCCTGGTCGAGCT-GGA-3′) and GP1R (5′-CTTGTACAGCTCGTCCATG-3′) and confirmed by DNA gel blot analysis. The expression of GFP was examined on both glass cover slips (Fisher Scientific) and onion epidermis with a Nikon Eclipse E800 epifluorescence microscope (Tokyo, Japan). The Chroma Endow GFP filter on this microscope is suitable to detect both GFP and YFP but not to distinguish them. The subcellular GFP localization also was examined with a Bio-Rad MRC 1024 UV/Vis System confocal laser scanning microscopy (Hercules, CA) on infected barley leaves and glass cover slips. Transformants expressing both *GAS1*-GFP and *GAS2*-YFP fusion constructs were generated by transforming pCX35 into GC22, a Guy11 transformant carrying *GAS1*-GFP.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The GenBank accession numbers for *GAS1* and *GAS2* are AF363065 and AF264035, respectively.

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