Mir1 is Highly Upregulated and Localized to Nuclei During Infectious Hyphal Growth in the Rice Blast Fungus

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Rice blast, caused by *Magnaporthe grisea*, is a devastating disease of rice throughout the world. Many recent molecular studies have focused on the early infection stages, but our knowledge about molecular events at the infectious hyphae stage is limited. In this study, 750 hygromycin-resistant transformants were isolated by transforming *M. grisea* Guy11 with a promoterless enhanced green fluorescent protein (EGFP) construct. In one of the transformants, L1320, EGFP signals were observed in the nuclei of infectious hyphae. The transforming vector was inserted in a predicted gene named *MIR1* and resulted in a *Mir1*¹⁻¹⁰⁷-EGFP fusion. *Mir1* is a low-complexity protein with no known protein domain and has no homolog in GenBank or other sequenced fungal genomes. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis and expression assays of *MIR1-EGFP* fusion constructs indicated that the expression of *MIR1* was highly induced during plant infection. Deletion analyses identified a 458-bp region that was sufficient for the *MIR1* promoter activity. Further characterization revealed that a 96-bp sequence was essential for the enhanced in planta expression. *MIR1* is an *M. grisea*-specific gene that is highly conserved among the field isolates belonging to the *M. grisea* species complex. The *mir1* mutants had no obvious defects in appressorial penetration and rice infection. When overexpressed with the RP27 promoter, nuclear localization of the *Mir1*-EGFP fusion was observed in conidia and vegetative hyphae. These data suggest that the expression but not the nuclear localization of *MIR1* is specific to infectious hyphae and that reporter genes based on *MIR1* may be suitable for monitoring infectious growth in *M. grisea*.

Additional keywords: appressorium, fungus-plant interaction, pathogenesis.

Rice blast, caused by *Magnaporthe grisea*, is one of the most severe fungal diseases of rice throughout the world (Talbot

2003; Valent and Chumley 1991). *M. grisea* has been developed as a model system to study fungus–plant interactions and is the first fungal plant pathogen to have its genome sequence published (Dean et al. 2005). The disease is initiated by the attachment and germination of conidia on rice leaves. The hardness and hydrophobicity of the rice leaf surface triggers the formation of a specialized infection structure called an appressorium. Enormous turgor pressure is generated inside appressoria as the physical force for plant penetration (de Jong et al. 1997). After penetration, the fungus forms bulbous, highly vacuolated infectious hyphae inside plant cells and further colonizes the rice tissues.

During the past decade, there have been extensive studies on molecular mechanisms involved in turgor generation and appressorium formation in *M. grisea* (Tucker and Talbot 2001). The cyclic AMP signaling and *PMK1* mitogen-activated protein kinase pathways are critical for appressorium formation and plant penetration (Adachi and Hamer 1998; Talbot 2003). Over a dozen pathogenicity genes important for appressorial penetration have been functionally characterized, including *PLS1*, *PDE1*, *MST12*, *GAS1*, and *GAS2* (Balhadere and Talbot 2001; Clergeot et al. 2001; Gilbert et al. 2006; Park et al. 2002; Xue et al. 2002). Apart from avirulence (*AVR*) genes (Bohnert et al. 2004; Farman and Leong 1998; Jia et al. 2000; Orbach et al. 2000), only a few genes, including *ABC1* and *HEX1*, are known to be required for fungus–plant interactions after penetration (Soundararajan et al. 2004; Urban et al. 1999). Recently, a P-type ATPase gene (*MgAPT2*) involved in exocytosis and rapid induction of host defense responses has been identified (Gilbert et al. 2006). *MgApt2*-dependent exocytotic processes may play a role during plant infection. However, many of these genes are expressed and functional in vegetative hyphae. Except for the *avr* gene *AVR-Pita* (Jia et al. 2000), no other functionally characterized *M. grisea* genes are known to be specifically expressed in the infectious hyphae. Recently, serial analysis of gene expression (SAGE) and microarray analyses have been used to identify genes with altered expression levels in germinating conidia, appressoria, and mycelia under different growth conditions (Dean et al. 2005; Irie et al. 2003; Kamakura et al. 2002). However, there is no report on applying functional genomic approaches to identify *M. grisea* genes specifically expressed during plant infection. Reporter genes that are specific for infectious hyphae and suitable for monitoring infectious growth have not been identified in *M. grisea*.

In this study, an enhanced green fluorescent protein (EGFP)-based promoter-trapping or gene-tagging approach was used to identify fungal genes that are expressed in different plant infection processes in *M. grisea*. In one transformant, L1320,

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Nucleotide sequence data is available in the GenBank database under accession numbers XP_369115 for *MIR1* (MG00129), XP_369114 for MG00130, XP_369113 for MG00131, and XP_369112 for MG00132.

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EGFP signals were observed in the nuclei of infectious hyphae. Molecular characterization revealed that EGFP was fused in-frame to the *MIR1* gene in L1320. The expression of *MIR1* was significantly induced in infected plant tissues. Mutational analyses of the *MIR1* promoter revealed a 96-bp region that was essential for enhanced in planta expression of *MIR1*. *MIR1* is an *M. grisea*-specific nuclear protein that is well conserved in various rice blast isolates. When overexpressed with the RP27 promoter, nuclear localization of the Mir1-EGFP fusion was observed in conidia and vegetative hyphae of *M. grisea*, suggesting that the expression but not the nuclear localization of *MIR1* is specific to infectious hyphae. Therefore, the *MIR1* gene is suitable as a reporter gene for monitoring infectious growth and its promoter can be used to express genes in infectious hyphae.

RESULTS

Identification of transformants expressing EGFP in infectious hyphae.

Plasmid pCX12 carrying the promoter-less EGFP construct was linearized with *HindIII* and transformed into a wild-type strain Guy11. Among the 750 hygromycin-resistant transformants screened by onion epidermal penetration and rice seedling infection assays, 4 mutants were found to be defective in plant infection (Table 1). Six transformants had EGFP signals in appressoria or infectious hyphae (Table 1). In transformant L1306, EGFP signals were relatively strong in infectious hyphae but had no specific subcellular localization pattern (Fig. 1A). In contrast, nuclear localization of EGFP signals was observed in infectious hyphae of transformant L1320 (Fig. 1A), which had no detectable EGFP signals in conidia, germ tubes, appressoria, and vegetative hyphae (data not shown). Both L1306 and L1320 were normal in appressorium formation and

still pathogenic on rice seedlings (Fig. 1B). To verify the nuclear localization in L1320, we transformed a histone H1-EGFP fusion construct pMF280 (Freitag et al. 2004) into Guy11. In the resulting transformant mhg280 (Table 2), the same pattern of nuclear localization for EGFP signals was observed in infectious hyphae (data not shown). However, unlike L1320, vegetative hyphae and conidia of mhg280 also had strong fluorescence in nuclei.

Transformant L1320 has EGFP inserted in the *MIR1* gene.

To identify the *M. grisea* sequence conferring the EGFP expression and localization in L1320, plasmid rescue was used to recover the flanking sequences of the transforming vector pCX12. Plasmid pLL21 rescued from L1320 carried a 4-kb *M. grisea* genomic DNA fragment (Fig. 2A). Sequence analysis of pLL21 revealed that pCX12 was inserted in the open reading frame (ORF) of a predicted gene MG00129, which encodes a protein of 364 amino acids and was designated *MIR1* (for *Magnaporthe* infection-related gene 1). In transformant L1320, EGFP was fused in-frame with the first 107 amino acids of Mir1, and the expression of *MIR1*¹⁻¹⁰⁷-EGFP fusion likely was regulated by the *MIR1* promoter (Fig. 2A). Southern hybridization with a *MIR1* fragment detected polymorphisms between Guy11 and transformant L1320 when digested with *EcoRV* and *StuI* but not with *SalI* (Fig. 2B). These data further confirmed that pCX12 was inserted at the N-terminal region of the *MIR1* gene in L1320.

To verify that the expression and nuclear localization of EGFP in transformant L1320 were conferred by its fusion with *MIR1*, we generated a *MIR1*-EGFP fusion construct (pLL36) and a truncated *MIR1*¹⁻¹²²-EGFP fusion construct (pLL37) that are both under the control of the *MIR1* promoter. After transforming pLL36 and pLL37 into Guy11, a total of 30 hygromycin-resistant transformants of each were isolated. Transformants

Table 1. Mutants of *Magnaporthe grisea* identified by penetration and infection assays

Transformant	Phenotype	Green fluorescent protein expression
L210	No appressorium formation, nonpathogenic	In cytoplasm of infectious hyphae like structures
L279	No defect in plant infection	Weakly in infectious hyphae
L324	No defect in plant infection	In appressoria and infectious hyphae
L325	No defect in plant infection	Weakly in infectious hyphae
L370	Nonpathogenic, form appressoria	No enhanced green fluorescent protein (EGFP) signal
L398	No appressorium formation, nonpathogenic	No EGFP signal
L1287	Reduced in virulence, rare lesions	No EGFP signal
L1306	No defect in plant infection	Only in infectious hyphae
L1320	No defect in plant infection	In the nuclei of infectious hyphae

Table 2. Wild-type strains and transformants of *Magnaporthe grisea* used in this study

Strains	Description	Reference
Guy11	Wild type, <i>MAT1</i> -2	Valent and Chumley 1991
mhg280	Guy11 transformed with pMF280 (histone H1-EGFP ^a)	This study
mir16	<i>mir1</i> gene replacement mutant of Guy11	This study
mir22	<i>mir1</i> gene replacement mutant of Guy11	This study
mir30	<i>mir1</i> gene replacement mutant of Guy11	This study
mir41	<i>mir1</i> gene replacement mutant of Guy11	This study
mir3	Ectopic transformant of pLL44 of Guy11	This study
M36-6	Complemented transformant of mir16	This study
ML36-1	Full-length Mir1-EGFP fusion (pLL36) in Guy11	This study
M37-30	1.5-kb <i>MIR1</i> promoter- <i>MIR1</i> ¹⁻¹²² -EGFP (pLL37) in mir16	This study
ML46-11	RP27-Mir1-EGFP (pLL46) in Guy11	This study
ML47-1	1.5-kb <i>MIR1</i> promoter-EGFP (pLL47) in Guy11	This study
ML48	1.1-kb <i>MIR1</i> promoter-EGFP (pLL48) in Guy11	This study
ML49	756-bp <i>MIR1</i> promoter-EGFP (pLL49) in Guy11	This study
ML50	458-bp <i>MIR1</i> promoter-EGFP (pLL50) in Guy11	This study
ML51	209-bp <i>MIR1</i> promoter-EGFP (pLL51) in Guy11	This study
ML60	Region A deletion in the 458-bp <i>MIR1</i> promoter (pLL60)	This study
ML61	Region B deletion in the 458-bp <i>MIR1</i> promoter (pLL61)	This study

^a EGFP = enhanced green fluorescent protein.

ML36-1, ML36-10, and ML37-30 (Table 2) contained a single copy of pLL36 or pLL37 according to Southern blot analyses (data not shown). In onion epidermal penetration assays, all these transformants had strong fluorescence signals in the nuclei of infectious hyphae (Fig. 2C). Similar to transformant L1320, EGFP signals were either not detectable or very weak in conidia (Fig. 2C), appressoria, and vegetative hyphae of ML36-1, ML36-10, and ML37-30 (data not shown). Enhanced EGFP signals in nuclei were observed only in infectious hyphae formed inside plant cells. Thus, *MIR1* encodes a nuclear protein that is highly expressed in infectious hyphae. The N-terminal portion of Mir1 protein must be sufficient for

directing its nuclear localization in infectious hyphae because the Mir1-EGFP fusion in L1320 or pLL37 was truncated and lacked the downstream predicted bipartite nuclear localization signal (NLS) (Fig. 2A).

The expression of *MIR1* is enhanced during plant infection.

To further confirm the upregulation of *MIR1* expression during rice infection, quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to determine relative transcriptional levels of *MIR1* in infected rice leaves and vegetative hyphae grown in complete medium (CM). The amplification of the *M. grisea* actin gene *MgACT1*

Fig. 1. Onion epidermis penetration and rice infection assays. **A**, Onion epidermis were inoculated with conidia of the transformants L1306 and L1320. Both mutants L1306 and L1320 penetrated onion epidermal cells and expressed enhanced green fluorescent protein (EGFP) in infectious hyphae. EGFP signals were detected in the cytoplasm in L1306 but localized to the nuclei in L1320. Bar = 10 μ m. **B**, Infectious hyphae formed by L1320 inside rice leaf sheath epidermal cells. EGFP signals were observed in nuclei. **C**, Rice infection assays. Seedlings of rice cv. CO39 were sprayed with conidia of the wild-type strain Guy11 and mutants L210, L1306, L1320, and L398. Similar to Guy11, mutants L1306 and L1320 caused numerous blast lesions on rice leaves. Lesions were not observed on leaves inoculated with mutants L210 and L398.

Fig. 2. Disruption of the *MIR1* gene in mutant L1320. **A**, Schematic diagram of the genomic sequence of the *MIR1* gene (empty arrow). The filled arrow bars marked the enhanced green fluorescent protein (EGFP). Two shaded boxes were putative bipartite nuclear localization signal sequences (residues 230 to 247 and 289 to 306). The insertion site of the transforming vector pCX12 is 321 bp downstream from the start codon of *MIR1*. E, *EcoRV*; S, *Sall*; Su, *StuI*. Plasmid pLL21 is the plasmid rescued from L1320. **B**, Southern blot of genomic DNA of Guy11 (lanes 1, 3, and 5) and L1320 (lane 2, 4, and 6) was hybridized with probe 1 amplified with primers MG129F1 and MG129R2. **C**, Transformant ML36 was observed by DIC and epifluorescent microscopy. Strong EGFP signals were observed in the nuclei of infectious hyphae that developed inside onion epidermal cells but no or only weak EGFP signals were detectable in asexual spores. Bar = 10 μ m.

was used as the endogenous reference for normalization. In comparison with the expression level of *MIR1* in the CM cultures, a 165-fold increase of *MIR1* expression, on average, was detected in the infected rice tissues at 5 days postinoculation (dpi) in three replicates (Fig. 3A).

To experimentally examine the *MIR1* promoter activity, a 1.5-kb fragment upstream of the *MIR1* gene was cloned in front of the EGFP reporter as pLL47 and transformed into Guy11. In transformants carrying a single copy of pLL47 (ML47-1) (Table 2), EGFP signals were very weak or not visible in the conidia and germ tubes (Fig. 3B) when germinated on artificial surfaces. However, strong EGFP signals were observed in the infectious hyphae formed by ML47-1 in the onion epidermal cells (Fig. 3B), indicating that this 1.5-kb fragment contained the promoter elements required for the upregulation of *MIR1* in infectious hyphae. Because the *MIR1* ORF sequence was absent in pLL47, nuclear localization was not observed in ML47 (Fig. 3B).

The 458-bp upstream sequence is sufficient for enhanced in planta expression of *MIR1*.

Plasmids pLL48, pLL49, pLL50, and pLL51 were constructed by fusing the EGFP reporter gene with a 1.1-kb, 756-bp, 458-bp, or 209-bp fragment upstream of the *MIR1* ORF, respectively (Fig. 4A). All these plasmids were constructed by yeast gap repair (Bourett et al. 2002; Raymond et al. 2002) and

transformed into Guy11. Over 10 transformants from each construct were analyzed by Southern blot hybridization (data not shown). At least one transformant with a single copy of the corresponding transforming vector (Table 2) was selected and assayed for promoter activities. Transformants ML48, ML49, and ML50 had EGFP expression levels similar to ML47 in infectious hyphae that developed inside onion epidermal cells (Fig. 4B). In transformant ML51, however, no or only very weak EGFP signals were detected in infectious hyphae (Fig. 4B). In all these experiments, autofluorescence related to plant defense responses was observed in plant cell walls and at the penetration point where papilla was developed (Fig. 4B). In all these transformants, vegetative hyphae and conidia had no or

Fig. 3. Induced expression of *MIR1* during plant infection. **A**, Relative quantity of *MIR1* transcripts in vegetative hyphae grown on complete medium (CM) and in infected rice leaves (IF) were quantified by real-time reverse-transcriptase polymerase chain reaction. **B**, Conidia of ML47 were germinated on a slide glass (upper panel) or on onion epidermis (lower panel) and observed under bright-field or UV light of an epifluorescence microscope. Strong enhanced green fluorescent protein (EGFP) signals were observed in infectious hyphae inside onion epidermal cells but no or only weak EGFP signals were detectable in conidia and germ tubes.

Fig. 4. Functional characterization of the *MIR1* promoter. **A**, Schematic diagrams of constructs containing different upstream sequences of *MIR1* fused with enhanced green fluorescent protein (EGFP). Black arrow indicates the *MIR1* gene. Open boxes mark the EGFP open reading frame. The length of each promoter region was labeled on the left in reference to the start codon of *MIR1*. **B**, Infectious hyphae developed by the wild-type strain Guy11 and transformants containing a single copy of pLL47 (ML47), pLL48 (ML48), pLL49 (ML49), pLL50 (ML50), and pLL51 (ML51) inside onion epidermal cells were examined by epifluorescent microscopy. The strength of EGFP signals in transformants ML48, ML49, and ML50 was similar to that of ML47. In ML51, only very weak or no EGFP signals were observed. Bar = 10 μ m.

only very weak EGFP signals (data not shown). These results suggest that the upstream sequence of *MIR1* (–1 to –458) carried by pLL50 contains essential regulatory elements of the *MIR1* promoter.

Fig. 5. Mutational analysis of the *MIR1* promoter. **A**, Direct repeats of attgac (in lower case) and inverted repeats of CAATGA (underlined), GGTGGA (double lined), and TTCCCA (in bold) were identified in the *MIR1* promoter sequence. The start codon of *MIR1* is designated '+1'. The transcription initiation site is shaded in gray and the CCA triplets are in italics. **B**, Plasmids pLL60 and pLL61 were constructs generated by deleting region A (–361 to –433) and region B (–209 to –305), respectively, in the 458-bp *MIR1* promoter sequence. Open boxes indicate enhanced green fluorescent protein (EGFP) open reading frame. **C**, In infectious hyphae that formed inside rice sheath epidermal cells, EGFP signals were not detectable in ML61. In ML60, EGFP signals were observed but at a reduced level in comparison to that of ML50.

A 96-bp promoter region is critical for the *MIR1* promoter activity.

To identify the transcription initiation site of *MIR1*, we conducted 5'-RACE (rapid amplification of cDNA ends) with RNA isolated from infected rice leaves harvested at 5 dpi. Sequence analysis of the resulting 5'-RACE PCR products revealed that the transcription initiation site is 156 bp upstream from the start codon of *MIR1* (Fig. 5A). Thus, the critical elements of the *MIR1* promoter must be between –157 and –458. Based on the nucleotide sequence, we then generated EGFP reporter constructs pLL60 and pLL61 (Fig. 5B) in which region A (–361 to –433) and region B (–209 to –305), respectively, were deleted from the 458-bp minimal *MIR1* promoter carried by pLL50. Region A contained two inverted repeats of CAATGA and two direct repeats of ATTGAC. Region B had inverted repeats of TTCCCA and TCCACC (Fig. 5A). In penetration assays with rice leaf sheath epidermal cells, EGFP signals were still detectable but markedly reduced in infectious hyphae formed by transformant ML60 expressing pLL60 (Fig. 5C). However, in transformant ML61 expressing pLL61, only very weak or no EGFP signals were observed in infectious hyphae that developed inside rice leaf sheath epidermal cells (Fig. 5C). These data indicate that region B but not region A contains critical regulatory elements of the *MIR1* promoter.

MIR1 is a novel gene unique to *M. grisea*.

MIR1 had no significant homolog (*e* value < 1e-5) in the GenBank or other fungal genomes that have been sequenced, including *Fusarium graminearum*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Neurospora crassa*. A Southern blot containing DNA samples from 28 *M. grisea* strains isolated from different hosts (Kang et al. 1995; Sweigard et al. 1995) was probed with the *MIR1* gene. In all the isolates except G-231 (a *Cyperus rotundus* isolate), at least one hybridizing band was detected in the *EcoRI* digests (Fig. 6). All the rice isolates and strains isolated from *Eleusine* and *Panicum* spp. had the same 6-kb band (Fig. 6). When the same blot was stripped and reprobed with the *MgACT1* actin gene amplified with primer Act5F and Act6R, G-231 had a much weaker hybridization band than the other strains (Fig. 6). All but one rice isolate (A598, lane 27) had a band larger than 12 kb. The fragment of the *MIR1* ORF and its 1.5 upstream region was sequenced from P-131 (Peng et al. 1994), a rice blast field isolate from Japan. Only two nucleotide differences between Guy11 and P-131 were identified. The single base pair difference located in the *MIR1* ORF, however, does not affect the amino acid sequence. These data indicate that *MIR1* is highly conserved among the rice blast isolates and widely distributed in *M. grisea* strains isolated from different hosts or regions.

The Mir1 protein sequence was analyzed with various programs, including PSORT and PFSCAN (Nakai and Horton

Fig. 6. Southern blot of genomic DNA from different *Magnaporthe grisea* field isolates hybridized with the *MIR1* gene (upper panel) and the *MgACT1* actin gene (lower panel). Lanes 1–28 were loaded with *EcoRI*-digested DNA from isolates O-137, KJ201, Guy11, O-42, O-17, O-190, O-224, O-315, G-158, G-201, G-176, G-219, G-220, G-221, G-156, G-11, G-163, G-223, G-189, G-81, G-222, G-224, G-231, G-1, G-17, T-47, A598, and LO3-8, respectively.

1999; Pagni et al. 2004). No known domain or motif was identified except the putative NLS sequences (Fig. 2A). Mir1 is a protein of low sequence complexity. It has a low content of hydrophobic amino acids (15%) and a high proportion of polar and charged amino acids (64%). These features resemble the characteristics of “intrinsic disordered” or “nonstructured” proteins (Dyson and Wright 2005) that lack properly folded and organized three-dimensional structures. Several programs, including FoldIndex, DisEMBL, GLOBPROT 2, and

DISOPRED2, all predicted that Mir1 contained large segments of disordered regions.

MIR1 is not essential for pathogenesis in *M. grisea*.

To determine the function of *MIR1* in *M. grisea*, the *MIR1* gene replacement construct pLL44 (7A) was transformed into Guy11. Among 60 hygromycin-resistant transformants screened, 4 *mir1* gene-replacement mutants (*mir16*, *mir22*, *mir30*, and *mir41*) (Table 2) were identified and confirmed by Southern blot analysis (Fig. 7B). The *mir1* mutants were normal in vegetative growth and conidiation. In penetration assays with onion and rice leaf sheath epidermal cells, the *mir1* mutants were able to form melanized appressoria, penetrate plant cells, and develop infectious hyphae as efficiently as the wild-type strain Guy11 (data not shown). The *mir1* mutants caused typical blast lesions on seedlings of rice cv. CO-39. No statistically significant changes in virulence were observed in the *mir1* mutants (data not shown). The *mir1* mutants also were able to infect barley cvs. Golden Promise and Kindred (Fig. 7C). Therefore, *MIR1* is dispensable for pathogenesis in *M. grisea*.

Nuclear localization of the Mir1-EGFP fusion in overexpression transformants.

We also generated an overexpression vector pLL46, in which the *MIR1*-EGFP fusion was controlled by the strong constitutive RP27 promoter (Bourett et al. 2002). In Guy11 transformants expressing pLL46, EGFP signals were observed in the nuclei of conidia (Fig. 8) and vegetative hyphae (data not shown), indicating that the expression of *MIR1* but not its nuclear localization is specific to infectious hyphae. To determine whether *MIR1* localizes to nuclei when expressed in other plant-pathogenic fungi, the *MIR1*-EGFP fusion construct pLL46 was transformed into the *F. graminearum* PH-1 and *Colletotrichum gloeosporioides* Cga3.1.3. In the resulting transformants of PH-1 and Cga3.1.3 expressing pLL46, EGFP signals were observed in the nuclei of conidia (Fig. 8) and vegetative hyphae (data not shown). These data further indicate that the localization of Mir1 to nuclei, when expressed by the RP27 promoter, is not specific to infectious hyphae of *M. grisea*. Therefore, the function of Mir1 must be

Fig. 7. *MIR1* gene replacement construct and mutants. **A**, Schematic diagram of the genomic region of *MIR1* and the gene replacement construct pLL44. The black arrow indicated the open reading frame of *MIR1*. **B**, Southern blot analysis of the *mir1* knockout mutants. Lanes 1 through 7 were *NcoI*-digested genomic DNA of the wild-type Guy11, ectopic transformant *mir3*, four *mir1* knockout mutants (*mir16*, *mir30*, *mir22*, and *mir41*), and a complementation strain (M36-6). A 3.4-kb band was detected in Guy11 and *mir3* but not in *mir16*, *mir30*, *mir22*, or *mir41* when hybridized with probe A. When hybridized with probe B (right), a 2.6-kb but not the wild-type 3.4-kb band was detected in the *mir1* mutants. **C**, Barley seedlings of cvs. Golden Promise and Kindred Lesion were inoculated with conidia of Guy11, *mir16*, *mir30*, and M36-6. Lesion formation was examined on infected rice leaves 7 days postinoculation. Means and standard errors were calculated from at least three independent experiments.

Fig. 8. Overexpression of the Mir1-enhanced green fluorescent protein (EGFP) fusion with the strong constitutive RP27 promoter. ML46, NLS1, and Cga313 were transformants of *Magnaporthe grisea*, *Fusarium graminearum*, and *Colletotrichum gloeosporioides*, respectively, expressing pLL46 (RP27-*MIR1*-EGFP). Nuclear localization of EGFP signals was observed in the conidia and vegetative hyphae of ML46, NLS1, and Cga3.1.3 when examined under epifluorescence microscopy. Scale bar = 10 μ m.

regulated at the transcriptional level. The *Mir1* promoter is not active during vegetative growth and could be used as a reporter for infectious hyphae.

DISCUSSION

Several transformants with defects in plant infection or expression of EGFP during plant infection were identified in this study. In transformant L1320 that was characterized in detail, EGFP signals were observed in infectious hyphae and localized to nuclei. The *MIR1* gene disrupted in L1320 is unique to *M. grisea*. The expression level of *MIR1* was increased significantly during plant infection. Although it is dispensable for plant infection under laboratory conditions, *MIR1* may be important for the fitness and virulence of *M. grisea* in field conditions because of its upregulation during plant infection and well-conserved homologs in *M. grisea* isolates. Among the field isolates examined, only G-231 had no hybridization band when probed with the *MIR1* gene (Fig. 6). However, recent studies have indicated that G-231 may not be a member of the *M. grisea* species complex (S. Kang, *personal communication*).

MIR1 has no homologous sequences in GenBank and it contains no known protein domain or motif other than the NLSs. Therefore, it is impossible to predict the exact biological function of *Mir1*. However, several short peptide repeats such as direct repeats of ASKR, KROR, and SDESxxDDD (Supplemental Fig. 1) were identified in the *Mir1* sequence by the SAPS program (Brendel et al. 1992). The predicted NCU07751 protein of *N. crassa* has a low homology (e value = $1e-5$) with *MIR1* but it lacks these repeats. We also found that *Mir1* meets the criteria of a group of intrinsically unstructured proteins (Dyson and Wright 2005). These intrinsically unstructured proteins appear as long stretches of amino acids that likely are unfolded in solution or adopt nonglobular structures (Dyson and Wright 2005). Other unstructured proteins have been implicated in different biological functions in various organisms, such as regulation of transcription and translation, cellular signaling, protein phosphorylation, the regulation of the self-assembly of large multiprotein complexes, and chaperones for other proteins or RNA molecules (Wright and Dyson 1999). Some of these unstructured proteins can undergo coupled folding with interacting proteins and transform themselves into more ordered states (Dyson and Wright 2005). In *M. grisea*, *Mir1* may undergo similar conformation changes by interacting with proteins that facilitate its nuclear localization or co-localize to nuclei in infectious hyphae. Unfortunately, we failed to identify any *MIR1*-interacting clones by screening yeast two-hybrid libraries constructed with RNA isolated from appressoria and vegetative hyphae (data not shown).

To date, there are no reporter constructs in *M. grisea* that are specific for infectious hyphae. Because the expression of *MIR1* is not detectable in vegetative hyphae and increased over 150-fold during plant infection (Fig. 3), the *MIR1*-EGFP fusion is well suitable as a reporter to monitor infectious hyphal growth. In other pathogenic fungi, dozens of genes that are upregulated during infection have been isolated by subtractive hybridization, differential display, and promoter trapping (Aichinger et al. 2003; Basse et al. 2000; Cramer and Lawrence 2004; Thara et al. 2003). In the maize pathogen *Ustilago maydis*, a group of genes encoding secreted proteins, including *mig1* and the *mig2* cluster, are highly induced in leaf tumors (Basse et al. 2000). The *mig2* cluster contains five similar genes known as *mig2-1* to *mig2-5* (Basse et al. 2002). The *mig2-6* gene is unlinked to the *mig2* cluster but shares the homology and expression pattern with the *mig2* genes (Farfsing et al. 2005). However, deletions of these *mig* genes individually or in com-

bination have no effect on pathogenicity in *U. maydis* (Basse et al. 2000, 2002; Farfsing et al. 2005). In addition to the *mig2* gene cluster, other plant-regulated genes were identified by promoter trapping in *U. maydis* (Aichinger et al. 2003). The *pig2* gene, encoding a putative protein disulfide isomerase, and several other *pig* genes (*pig3*, *pig4*, *pig5*, and *pig7*) located in the *p* locus also are upregulated during plant infection (Aichinger et al. 2003). Similar to the *mig* genes, deletion of *pig2* or other individual *pig* genes or the entire *p* locus (with ectopic introduction of an essential gene in the locus) had no effect on the virulence of the fungus (Aichinger et al. 2003). In the *M. grisea* genome, three predicted genes adjacent to *MIR1*, MG00130, MG00131, and MG00132, have no homolog in GenBank or other sequenced fungal genomes. This cluster of *M. grisea*-specific genes is flanked by MG000128 and MG00133 that encode conserved hypothetical proteins.

In *M. grisea*, promoter elements responsible for in planta-specific or induced expression have not been characterized previously. In this study, we identified a 458-bp fragment that is sufficient to control the *MIR1* expression in infectious hyphae. Unlike region A (Fig. 5, 72 bp) that played a minor role in regulating *MIR1* expression, the 97-bp region B is critical for its induced expression during plant infection. When the region B sequence (–305 to –209) was analyzed with the Tfssearch program, several putative TF binding sites were identified (Supplemental Fig. 2). These and additional putative transcription factor binding sites also were identified by the TESS program. Interestingly, the predicted gene MG00130 and *MIR1* are opposite to each other (head to head) and only 900 bp apart. The sequence located between *MIR1* and MG000130 may contain promoter elements, such as the inverted repeats (Fig. 5) identified in region B, that are common for regulating the expression of these two genes. Similar to *MIR1*, MG00130 has no matching expressed sequence tag sequences, suggesting that they are not expressed in conidia and vegetative hyphae grown under various conditions (Ebbole et al. 2004). Our preliminary data indicated that the expression level of MG000130, similar to that of *MIR1*, increased over 100-fold in infectious hyphae (S.-L. Ding and J.-R. Xu, *unpublished data*).

We also identified seven repeats of 5'-CCA-3' in region B of the *MIR1* promoter. Two of them are tandem repeats and the others were evenly distributed (Fig. 5). Many CCA repeats also were found in the promoter regions of the *ABC1*, *AVR-Pita*, and *MHP1* genes that have induced or enhanced expression levels during plant infection (Kim et al. 2005; Orbach et al. 2000; Urban et al. 1999). In *U. maydis*, promoters of the *mig1*, *mig2-1*, and *mig2-5* genes are subject to both positive and negative regulation during in planta growth (Basse et al. 2002). The *mig2-5* promoter has several repeats of 5'-CCA-3' motifs that are critical for the induced expression of *mig2-5* during infection of maize plants (Farfsing et al. 2005). However, the transcription factor binding to 5'-CCA-3' has not been identified (Farfsing et al. 2005). In *M. grisea*, the 5'-CCA-3' repeats in the 96-bp region upstream of *MIR1* also probably play important roles in the induced expression of *MIR1* in planta. Both inverted repeats found in region B contain 5'-CCA-3'. To date, the only transcription factor known to be involved in infectious hyphal growth in *M. grisea* is Mst12 (Park et al. 2002). Unfortunately, no genes regulated by Mst12 have been identified and its binding site is not clear. Our data indicate that the expression of *MIR1* is highly induced during plant infection. Further characterization of the *MIR1* promoter may lead to the identification of the transcription factor binding site and corresponding transformation factor that is responsible for the enhanced expression of *MIR1* in infectious hyphae.

MATERIALS AND METHODS

Fungal strains, transformation, and growth conditions.

The wild-type strain Guy11 and transformants used in this study (Table 2) were cultured on oatmeal agar plates at 25°C under fluorescent light (Xue et al. 2002). Mycelium was collected by filtration through Miracloth (Calbiochem, La Jolla, CA, U.S.A.) from 2-day-old 5× YEG (0.5% yeast extract, 1% glucose) or complete medium cultures (Zhao et al. 2005) and used for isolation of fungal DNA and protoplasts. Media were supplemented with hygromycin B at 250 µg/ml or zeocin at 100 µg/ml (Invitrogen, Carlsbad, CA, U.S.A.) for selecting hygromycin-resistant and zeocin-resistant transformants, respectively. Transformation, monoconidial culture isolation, and genetic crosses of *M. grisea* were performed as described previously (Talbot et al. 1993). Cultivation and transformation of the *F. graminearum* wild-type strain PH-1 (Trail and Common 2000) and related transformants were performed as described previously (Hou et al. 2002). The *C. gloeosporioides* f. sp. *aeschyromene* wild-type strain Cga3.1.3 and the transformants generated in this study were cultured at 28°C on EMS agar (Barhoom and Sharon 2004).

A Southern blot containing *Eco*RI-digested genomic DNA samples from 28 *Magnaporthe* isolates (Kang et al. 1995; Sweigard et al. 1995) was provided by S. Kang at Pennsylvania State University. These representative *Magnaporthe* strains included 10 rice isolates: O-137 (China), KJ201 (South Korea), Guy11 (French Guyana), O-42 (Japan), O-17 (Japan), O-190 (South Korea), O-224 (Nigeria), O-315 (Cameroon), A598 (U.S.A.), and LO3-8 (U.S.A.); 5 *Digitaria* isolates: G-156 (Brazil), G-11 (Japan), G-163 (Philippines), G-189 (U.S.A.), and G-1 (U.S.A.); 5 *Pennisetum* isolates: G-221 (Japan), G-223 (Brukina Faso), G-81 (U.S.A.), G-222

(Mali), and G-224 (Ivory Coast); 2 *Panicum* isolates: G-219 (India) and G-220 (India); 2 *Eleusine* isolates: G-201 (Brazil) and G-176 (Nepal); 1 wheat isolate: T-47 (Brazil); 1 Feral Triticale isolate: G-158 (Brazil); 1 *Eragrostis curvula* isolate: G-17 (Japan); and 1 *Cyperus rotundus* isolate: G-231 (Philippines).

Construction of the promoter-trapping vector.

To construct the promoter-trapping vector pCX12, a 0.9-kb *trpC* terminator from *Aspergillus nidulans* was released from pAC905 (Zheng et al. 2000) and cloned between the *Bam*HI and *Xba*I sites of pCB1004 (Carroll et al. 1994) as pCB1004T. The 0.7-kb EGFP ORF from pEGFP (BD Bioscience Clontech, San Jose, CA, U.S.A.) then was cloned upstream from the *trpC* terminator on pCB1004T to generate pCX12. Plasmid pCX12 was linearized with *Hind*III and transformed into protoplasts of Guy11.

Molecular manipulations and sequence analyses.

Standard molecular biology procedures were followed for DNA gel blot analyses and enzymatic manipulations with DNAs (Sambrook and Russell 2001). For plasmid rescue, the self-ligation mixture of *Pst*I-digested L1320 genomic DNA was transformed into the XL1-Blue cells. For determining the copy number of transforming vector in the genome, genomic DNA samples were digested with *Sac*I and hybridized with EGFP as the probe. RNA was isolated from mycelia or infected rice leaves with the TRIzol reagent (Invitrogen) and mRNA was purified with the PolyA Ttract mRNA isolation kit (Promega Corp., Madison, WI, U.S.A.). The SMART RACE cDNA Amplification Kit (BD Bioscience Clontech) was used for cDNA synthesis and 5' RACE PCR. The specific primer used for 5' RACE was reverse primer NRace (Table 3). Pro-

Table 3. Polymerase chain reaction primers used in this study

Names	Sequence (5'→3')
NRace	GCGGCGTCTCGCCGTGTGGATTGATT
PMR7	ATTGGGTACTCAAATTGGTTTCGACAAAGGTCATAATGT
PMF8	CAGTCTCTCGCCCTTGCTCACCTTTAGCAAAAAGCCACC
PMF13	CAGTCTCTCGCCCTTGCTCACGATTTCAGATGCGTCTT
PMF17	GTCGAGCTCTCGAGTGGATTGGTAAGTTT
PMR18	ATAAGAATGCGGCCGCGCCGCGCCCTAGAGTATACCCAGCTA
PMF19	CGCGGATCCGGCGCGCCTAAAGATTGTGTAAATT
PMR20	AAAAGTGCAGATGACGAGACCGAGATTG
PMF21	CAGTCTCTCGCCCTTGCTCACCATTTTGGTTGCCAAGTT
PMR22	AGGAACCCAATCTTCAAAATGTGCGCCAAAAAGACAA
PMF23	ATTGGGTACTCAAATTGGTTACAGCAGCAGGAGGTGAT
PMF24	ATTGGGTACTCAAATTGGTTGTTCATCTCTTGATTGA
PMF25	ATTGGGTACTCAAATTGGTTTCGTCGCCATCCAACATT
PMF28	TTCGCCCCGAATTAGCTTGGCTGCAGGATTTCCAGATGCGTCTT
PMR29	CAGTTGACTGTATCGCCGGAATTCTGCGCCAAAAAGACAACCAA
PMF30	TCGCCCGGAATTAGCTTGGCTGCAGTCACTTTAGCAAAAAGCCA
PMR32	AGATCGAATTAGGATCCTCTGCTAGCCTCAGCAGTGCCAGGAAG
PMF33	TGCAGTAATACGACTCACTATAGGGTCACTTTAGCAAAAAGCCA
PMR34	ATCGAATTAGGATCCTCTGCTAGCTGCGCCAAAAAGACAACCAA
PMF36	ATTGGGTACTCAAATTGGGACGTGATTGTCTCGCAA
PMF37	CTTGATTGATAGACAGTACAACCTCGAGGTTCAAAATACAAATGGTTG
PMR38	CAACCATTGTATTTGAACCTCGAGTTGTACTGTCTATCAATCAAG
PMF39	CAGATCATTGACCTGAACCTCGAGCTAACTCACATGAAGGC
PMR40	GCCTTCATGTGAGTTTGTAGCTCGAGTTCAGGTCATGATCTG
PMF45	CAACCCCGTACGCGTTTTGTGCGCCATCCAACAT
PMR46	ATGTTGGATGGCGACAAAACGCGTACGGGGTTG
PMF47	CTTGATTGATAGACAGTACTACATGAAGGCTCTCGTA
PMR48	TACGAGAGCCTTCATGTGAGATCTGTCTATCAATCAAG
PMF49	GCTACCAGCCCTGAGGACGTACAGAGTTT
MG129Q1	CGGCCGAGTTTCTCGAGT
MG129Q2	CGTCTCGAATTGATCTTCTTGG
ActQ1	TCTTCGAGACCTTCAACGCC
ActQ2	ACCGGAGTCGAGCACGATAC
Act5F	ACAGGGACACAGAGCTT
Act6R	GTGGTAGAGAAGGTGTAA

grams FoldIndex, DisEMBL, GLOBPROT 2, and DISOPRED2 were used to search for unstructured protein regions. PFSCAN, SAPS, and Pfam were used for protein domain and motif searches.

Assays for germination, appressorium formation, penetration, and plant infection.

Conidia harvested from 10-day-old oatmeal agar cultures were used for appressorium formation on plastic microscope coverslips (Fisher Scientific Co., Pittsburgh) or GelBond membranes (Cambrex, East Rutherford, NJ, U.S.A.) as described (Bruno et al. 2004; Li et al. 2004; Tucker et al. 2004). Appressorial penetration and infectious hyphal growth were assayed as previously described (Koga 1994; Koga et al. 2004; Xue et al. 2002). Expression of EGFP in appressoria and infectious hyphae was examined after 48 h of incubation with a Nikon Eclipse E800 epifluorescence microscope (Tokyo) or a Bio-Rad MRC 1024 UV/Vis System confocal laser scanning microscopy (Hercules, CA, U.S.A.). Two-week-old seedlings of rice cvs. CO-39, M201, and M202 and 8-day-old seedlings of barley cv. Golden Promise and Kindred were used for infection assays (Park et al. 2004). Plant incubation and inoculation were performed as described (Talbot et al. 1993; Valent et al. 1991). Lesion formation was examined at 7 dpi as described (Talbot et al. 1993; Xue et al. 2002).

Quantitative real-time RT-PCR.

RNAs were isolated from Guy11 mycelia grown in CM for 2 days and infected rice leaves at 5 dpi. First-strand cDNAs were synthesized with the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, U.S.A.) and used as templates for real-time RT-PCR. Primers MG129Q1 and MG129Q2 (Table 3) were used to amplify a 100-bp amplicon of *MIR1*. As an endogenous control, the *M. grisea* actin gene *MgACT1* (MG03982) was amplified as a 101-bp amplicon with primers ActQ1 and ActQ2 (Table 3). Quantitative real-time RT-PCR was performed with the ABI 7700 Sequence detection system (Applied Biosystem, Foster City, CA, U.S.A.) using the QuantiTect SYBR-green PCR Master mix (Qiagen) as described previously (Flaherty and Dunkle 2005). To verify that the efficiencies of the *MIR1* and *MgACT1* amplifications were approximately equal, PCR reactions were performed with serial dilutions of cDNA templates. After the amplification efficiencies of *MIR1* and *MgACT1* were verified to be acceptable, relative quantification of the *MIR1* transcripts was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001), with *MgACT1* as the endogenous reference and the level of *MIR1* expression in the CM cultures assigned the value of 1.

Construction of the *MIR1* gene replacement and EGFP-fusion vectors.

A 758-bp fragment upstream from the *MIR1* gene was amplified with primers PMF17 and PMR18 (Table 3) and cloned between the *SacI* and *NotI* sites of pYK11 (Zhao et al. 2004) as pLL42. The 778-bp downstream fragment was amplified with primers PMF19 and PMR20 and cloned between the *BamHI* and *PstI* sites of pLL42 as pLL43. The *MIR1* gene replacement construct pLL44 was generated by cloning the *hph* cassette from pCX62 (Zhao et al. 2004) into the *AscI* and *FseI* sites of pLL43.

The yeast gap repair approach (Bourett et al. 2002; Raymond et al. 2002) was used to generate the EGFP fusion constructs. To generate the *MIR1*-EGFP fusion construct, a 2.7-kb fragment containing the entire *MIR1* gene and its 1.5-kb upstream region was amplified with primers PMR7 and PMF8 (Table 3) and cotransformed with *XhoI*-digested pDL2 into

Saccharomyces cerevisiae XK1-25 (Bourett et al. 2002; Bruno et al. 2004). Plasmid pLL36 rescued from the resulting Trp⁺ yeast transformants was sequenced to confirm that the *MIR1* gene was fused in-frame with EGFP. A similar approach was used to generate the *MIR1*¹⁻¹²²-EGFP fusion construct pLL37 and a *MIR1*-EGFP overexpression construct pLL46. To generate pLL37, primers PMF13 and PMR7 were used to amplify a 1.9-kb fragment containing the native promoter region and the first 122 amino acid residues of *MIR1*. To construct pLL46, primers PMF8 and PMR22 were used to amplify the *MIR1* ORF.

Promoter analyses of *MIR1*.

We also used the yeast gap repair approach to generate all the EGFP reporter constructs for *MIR1* promoter analysis. Primer pairs PMF21-PMR7, PMF21-PMF22, PMF21-PMF23, PMF21-PMF24, and PMF21-PMF25 were used to generate pLL47, pLL48, pLL49, pLL50, and pLL51 (Fig. 4), respectively, in which EGFP was under the control of a 1,487-, 1,066-, 756-, 458-, and 209-bp sequence upstream from *MIR1*. PCR products amplified with primer pairs PMF49-PMR48 and PMF47-PMF21 were used as the templates for overlapping PCR (Horton et al. 1990) to delete region A (−433 to −361) of the *MIR1* promoter (Fig. 5). The same approach was used to delete region B (−305 to −209) of the *MIR1* promoter with primer pairs PMF24-PMR46 and PMF45-PMF21. The resulting overlapping PCR fragments were cotransformed with *XhoI*-digested pDL2 into yeast strain XK1-25 to generate pLL60 and pLL61 (Fig. 5), in which EGFP was under the control of the *MIR1* promoter deleted of region A and region B, respectively. All of the EGFP fusion constructs were confirmed by sequence analysis and linearized with *PstI* before fungal transformation.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

AIST Computational Biology Research Center's Tfsearch program:
www.cbrc.jp/research/db/TFSEARCH.html
European Molecular Biology Laboratory's (EMBL) intrinsic protein disorder prediction DisEMBL server: dis.embl.de
EMBL's intrinsic protein disorder, domain, and globularity prediction GLOBPROT 2 server: globplot.embl.de
Sanger Institute's Pfam database: www.sanger.ac.uk/Software/Pfam
Stanford University's SAPS (statistical analysis of protein sequences) server: www.isrec.isb-sib.ch/software/SAPS_form.html

Swiss Institute of Bioinformatics PFSCAN database:
hits.isb-sib.ch/cgi-bin/index
University College London Bioinformatics Unit's DISOPRED2 disorder prediction server: bioinf.cs.ucl.ac.uk/disopred
University of Pennsylvania's Transcription Element Search System (TESS) program: www.cbil.upenn.edu/cgi-bin/tess/tess
University of Tokyo Human Genome Center's PSORT server: psort.ims.u-tokyo.ac.jp
Weizmann Institute of Science's FoldIndex website:
bip.weizmann.ac.il/fldbin/findex