A Homeobox Gene Is Essential for Conidiogenesis of the Rice Blast Fungus *Magnaporthe oryzae*

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Magnaporthe oryzae starts its infection by the attachment of pyriform conidia on rice tissues, and severity of the disease epidemic is proportional to the quantity of conidia produced in the rice blast lesions. However, the mechanism of conidial production is not well understood. Homeodomain proteins play critical roles in regulating various growth and developmental processes in fungi and other eukaryotes. Through targeted gene replacement, we find that deletion of *HTF1*, one of seven homeobox genes in the fungal genome, does not affect mycelial growth but causes total defect of conidial production. Further observation revealed that the $\Delta htf1$ mutant produces significantly more conidiophores, which curve slightly near the tip but could not develop sterigmata-like structures. Although the $\Delta htf1$ mutant fails to form conidia, it could still develop melanized appressoria from hyphal tips and infect plants. The expression level of *HTF1* is significantly reduced in the $\Delta mgb1$ G- β and $\Delta cpkA$ deletion mutant, and the *ACR1* but not *CON7* gene that encodes transcription factor required for normal conidiogenesis is significantly downregulated in the $\Delta htf1$ mutant. These data suggest that the *HTF1* gene is essential for conidiogenesis, and may be functionally related to the trimeric G-protein signaling and other transcriptional regulators that are known to be important for conidiation in *M. oryzae*.

The ascomycete *Magnaporthe oryzae* causes blast disease in economically important crops such as rice, barley, wheat, and millet and has been developed as a model system to study fungus–plant interactions (Talbot 2003; Ebbole 2007; Xu et al. 2007b). The infection process of this pathogen is initiated by the attachment of pyriform conidia to the plant surface and differentiation of appressoria. After appressorial penetration, infectious hyphae of *M. oryzae* initially grow biotrophically in infected plant tissues (Kankanala et al. 2007) but eventually cause cell death and lesion formation on a susceptible host. When environmental conditions are suitable, the fungus produces and releases more conidia from blast lesions to reinstate the infection cycle. The severity of the rice blast disease epidemics is proportional to the quantity of spores produced in the lesions (Teng et al. 1991). Therefore, conidium production and dispersal of conidia play critical roles in the epidemiology of rice blast as well as many other plant fungal diseases.

In the model filamentous fungus *Aspergillus nidulans*, several transcription factors regulating different developmental stages of conidiation have been characterized, including Br1A, StuA, MedA, and AbaA (Yu et al. 2006). However, the regulatory network controlling conidiation appears to be different in another model filamentous fungus, *Neurospora crassa*. The *StuA* homolog in *N. crassa*, *ASM-1*, is involved in ascospore development but dispensable for asexual reproduction (Aramayo and Metzenberg 1996). In *M. oryzae*, disruption of the *ACR1* gene that encodes a protein with limited homology to MedA results in the production of head-to-tail (acropetal) arrays of elongated conidia. The *ACR1* gene seems to be a stage-specific negative regulator that is required to establish a sympodial pattern of conidiation (Nishimura et al. 2000). Another transcription factor gene that may be involved in regulating conidiation in *M. oryzae* is *CON7*. The $\Delta con7$ mutant produces conidia with abnormal cell shape and is reduced in sporulation by approximately 35% compared with that of the wild-type strain (Shi et al. 1998). Deletion of *CON7* affected the expression of several genes that are important for plant infection (Odenbach et al. 2007).

In eukaryotic cells, homeobox genes encode transcription factors with the homeodomain for recognizing and binding to specific DNA sequences. The homeodomain proteins or homeoproteins are evolutionarily conserved and play regulatory roles during different developmental stages in plants, animals, and fungi (Gehring and Hiromi 1986; Scott et al. 1989; Svingen and Tonissen 2006). In fungi, homeoproteins are best studied in the budding yeast *Saccharomyces cerevisiae*, which has nine homeobox genes. Two of them, *HML α 2* and *HMR α 1*, are not expressed. Among the other expressed homeobox genes, *MAT α 2* and *MAT α 1* are part of the mating type locus. *Mata2p* acts with the MADS-box transcription factor *Mcm1p* to suppress α -specific genes in haploid cells and binds with *Mata1p* to repress haploid-specific genes in diploid cells (Houston et al. 2002). Like *MAT α 2* and *MAT α 1*, the other five yeast homeobox genes, *YOX1*, *YHP1*, *TOS8*, *CUP9*, and *PHO2*, are not essential for growth. *Yox1p* and *Yhp1p* are two transcriptional repressors that interact with *Mcm1p* and bind to the promoters of some cell-cycle-regulated genes expressed in M/G1 phase (Kunoh et al. 2000). *Yhp1p* also negatively regulates the transcription of the *IME1* gene that is required for the initiation of meiosis (Pramila et al. 2002). *Tos8*, a Swi4-Swi6 cell cycle binding factor (SBF)-regulated transcription factor, plays a role in transcriptional regulation of genes that are functional in the G1/S phase events, such as bud site selection, bud emergence, and cell cycle progression (Horak et al. 2002). *CUP9* is a repressor of *PTR2* (Xia et al. 2008), which encodes a major peptide transporter. *Pho2p* interacts with *Pho4p* to regulate genes involved in phos-

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phate metabolism. It also binds cooperatively with Swi5 to activate Ho (a homothallic switching endonuclease) and interacts with coregulator protein Bas1 to activate genes related to purine and histidine biosynthesis (Bhoite et al. 2002).

In *Schizosaccharomyces pombe* and *Candida albicans*, the mating locus also contains homeoproteins. In addition to mating, the interaction between *C. albicans* Mtl1p and Mtl2p homeoproteins is important for the white-opaque switching and virulence (Miller and Johnson 2002; Lockhart et al. 2005). Another homeobox gene in *C. albicans* is *FIL2*, a regulator of filamentation (Xu et al. 2007a). In *Yarrowia lipolytica*, the Hoy1p homeoprotein also is required for hyphal formation (Torres-Guzman and Dominguez 1997). Like the ascomycetous yeasts, many basidiomycetes contain homeobox genes in their mating type loci. In *Ustilago maydis*, the bE and bW homeobox genes are important for mating, filamentous growth, and pathogenesis (Bakkeren et al. 2008). Interestingly, the homeoproteins of the mating type locus are insufficient to regulate sexual differentiation in *Cryptococcus neoformans*. Two homeobox genes *SX11* and *SX12a* that are unlinked to the mating type locus have been shown to be required for determining cell identity and regulating sexual differentiation (Hull et al. 2005).

The first homeobox gene characterized in filamentous ascomycetes is *pah1* in *Podospora anserina* (Arnaise et al. 2001). Deletion of the *pah1* gene increased the production of microconidia (male gametes), indicating that it may function as a repressor of genes involved in microconidiation. The *pah1* deletion mutant had a reduced growth rate and formed colonies with a wavy appearance. Vegetative hyphae of the *pah1* mutant branched more frequently than those of the wild type (Arnaise et al. 2001). In *N. crassa*, mutants in which the *pah1* homolog *KAL-1* was deleted were generated in a high-throughput functional genomics project (Colot et al. 2006). The *kal-1* deletion mutant also had colony morphology defects and produced shorter aerial hyphae and fewer conidia. Hyphal branching was increased in the *kal-1* mutant but its growth rate was reduced only when incubated at temperatures higher than 25°C. Interestingly, the growth and conidiation defects of the *kal-1* mutant can be partially restored by 2% yeast extract, indicating a possible role of *KAL-1* in nutrient sensing. Two other *N. crassa* homeobox genes, *BEK-1* and NCU03070, were characterized in the study (Colot et al. 2006). Although the function of NCU03070 was not described, *BEK-1* (NCU00097) was required for normal development of perithecial beaks during sexual development in *N. crassa* (Colot et al. 2006; Krystofova and Borkovich 2006).

The *M. oryzae* genome has seven predicted homeobox genes. One of them is *PTH12* that has been reported to be important for plant infection (Sweigard et al. 1998). Through deletion of the other six homeobox genes, we found, in this study, that the $\Delta htf1$ mutant had normal colony morphology and produced abundant conidiophores but was blocked in conidium formation. Appressoria formed by hyphal tips of the $\Delta htf1$ mutant were able to penetrate and infect plant cells. Our data indicated that the *HTF1* gene may specifically regulate the differentiation of conidia. Moreover, gene expression analysis suggested that Htf1 may be functionally regulated through the trimeric G-protein signaling pathway and other transcriptional regulators such as Acr1 and Con7 that are known to be important for conidiation in *M. oryzae*.

RESULTS

Homeobox genes in *M. oryzae*.

The *M. oryzae* genome has seven predicted genes encoding proteins with the homeodomain. They are MGG_00184, MGG_01730, MGG_04853, MGG_06285, MGG_07437,

MGG_11712, and MGG_12865 (Fig. 1A). The last one is the *PTH12* gene that was disrupted in one of the insertional mutants with reduced virulence (Sweigard et al. 1998). The *Mst12* (MGG_12958) gene was originally regarded to have a homeobox domain (residues 61 to 205) (Park et al. 2002) but this region is considered to be an STE-like transcription factor domain rather than homeodomain in the fungal genome annotation. Therefore, we characterized the other six homeobox genes, designated herein as Htf1 to Htf6 (for homeodomain transcription factor genes).

The position of the homeodomain varies among these homeobox genes (Fig. 1A). In Htf1, Htf2, Htf3, and Htf6, the homeodomain is near the N-terminus regions. It is in the central region of Htf5 and C-terminal regions of Htf4 and Pth12 (Fig. 1A). The homology among these seven homeobox genes in *M. oryzae* is limited to the homeodomain that is responsible for DNA binding. Sequence alignment (Fig. 1B) and phylogenetic analysis (Fig. 1C) with the homeodomain sequences revealed that these homeobox genes could be divided into three subgroups. Htf2, Htf4, and Pth12 formed one clade, and Htf3, Htf5, and Htf6 were in another one. The homeodomain of Htf1 appeared to be more distantly related to that of the other homeobox genes (Fig. 1C).

HTF1 is dispensable for vegetative growth but essential for conidiation.

To determine the function of these predicted homeobox genes in *M. oryzae*, we generated targeted deletion mutants by gene replacement approach (Table 1; Fig. 2A). Mutants with gene deletion were selected on hygromycin-containing me-

Fig. 1. Homeobox genes in *Magnaporthe oryzae*. **A**, Schematic diagram of seven predicted homeobox genes: *HTF1* (MGG_00184), *HTF2* (MGG_01730), *HTF3* (MGG_04853), *HTF4* (MGG_06285), *HTF5* (MGG_07437), *HTF6* (MGG_11712), and *PTH12* (MGG_12865). Empty box, homeodomain (HD). **B**, Sequence alignment of the homeodomains. Three predicted helices labeled on the top. **C**, Phylogenetic analysis with the homeodomain sequences of seven homeobox genes. A bootstrapped analysis was performed with 1,000 iterations and bootstrap value were indicated on the branches.

dium and then confirmed by polymerase chain reaction (PCR) and Southern blot analyses (Fig. 2B). When cultured on complete medium (CM) and oatmeal agar plates, the $\Delta htf1$ mutants had no obvious difference in vegetative growth in comparison with the wild-type strain Guy11 (Fig. 3A; Table 2); however, the $\Delta htf1$ mutants failed to form conidia (Table 2, HK11 and HK12).

Although the $\Delta htf1$ mutant was blocked in conidiation, it still formed grayish colonies (Fig. 3A). Examination by light microscopy of the aerial cultures stained with lactophenol cotton blue revealed that $\Delta htf1$ mutant developed conidiophore stalks very well (Fig. 3B). This result was consistent with the observation from scanning electron microscopy, showing that only conidiophores but no conidia existed in the culture of the $\Delta htf1$ mutants.

When the $\Delta htf1$ mutant was examined under a dissecting microscope, abundant conidiophores were observed. However, unlike the wild-type strain, conidiophores of the $\Delta htf1$ mutant failed to differentiate conidia (Fig. 4A). We also examined colonies formed by the $\Delta htf1$ mutant by scanning electron microscopy (SEM). In comparison with the wild-type strain, conidiophores of the $\Delta htf1$ mutant had no conidia and did not display the typical sympodial (zigzag) pattern (Fig. 4B). However, tips of $\Delta htf1$ conidiophores were often curved to various

degrees. Unlike sympodial conidiophores formed by the wild-type strain, curving conidiophores of the $\Delta htf1$ mutant had a smooth appearance and lacked sterigmata-like structures (Fig. 4B). These results indicate that the $\Delta htf1$ mutant was not defective in conidiophore development but was blocked in subsequent conidiogenesis (differentiation of sterigmata and conidium formation).

When mated with the wild-type strain 70-15, the $\Delta htf1$ mutant produced fertile perithecia, suggesting that *HTF1* is not required for the fungal sexual reproduction. We then isolated 32 ascospore progeny from this cross. All 19 hygromy-

Fig. 2. Generation of *HTF1* mutants. **A**, *HTF1* gene replacement construct was constructed by amplifying the upstream and downstream flanking sequences with primers 1AF/1AR and 1BF/1BR and ligated with the *hph* cassette. Probe is fragments amplified by polymerase chain reaction with primers 1NF/1NR. Restriction enzymes were as follows: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; S, *Sac*I; St, *Stu*I; X, *Xho*I. **B**, DNA gel blot analysis of genomic DNA from Guy11 and 2 transformants digested with *Stu*I using the radiolabeled probes as shown in A. The results show a single 6.8-kb band for the wild type (Guy11) and no band for gene replacement (HK11, and HK12) when using probe 1 (left); and a single 6.8-kb band for the wild-type (Guy11) and another single 10.3-kb band for gene replacement (HK11 and HK12) when using probe 2 (right).

Fig. 3. Colony morphology and lactophenol cotton blue staining of the wild type and the $\Delta htf1$ mutant strains. **A**, Colony morphology of wild-type Guy11 and the *HTF1* mutant grown on oatmeal agar plates for 10 days. **B**, Aerial cultures stained with lactophenol cotton blue observed under a light microscope. Hyphae are stained blue, whereas conidiophore stalks are in gray. Bar = 20 μ m. Con, conidiophores; H, hyphae.

Table 1. Wild-type and mutant strains of *Magnaporthe oryzae* used in this study

Strain	Brief description	Reference
Guy11	Wild type, <i>MAT1</i> -2	Chao and Ellingboe 1991
70-15	Wild type, <i>MAT1</i> -1	Chao and Ellingboe 1991
nn78	$\Delta pmk1$ mutant of Guy11	Xu and Hamer 1996
nw96	$\Delta mgb1$ mutant of Guy11	Nishimura et al. 2003
I27	$\Delta cpkA$ mutant of Guy11	Mitchell and Dean 1995
HK11	$\Delta htf1$ (MGG_00184) mutant of Guy11	This study
HK12	$\Delta htf1$ mutant of Guy11	This study
HE1	Ectopic transformant of the <i>HTF1</i> knockout construct	This study
HC1	HK11 transformed with pYH1 (wild-type <i>HTF1</i>)	This study
HD12	<i>HTF1</i> ^{ΔHD} in HK11	This study
HD14	<i>HTF1</i> ^{ΔHD} in HK11	This study
HD15	<i>HTF1</i> ^{ΔHD} in HK11	This study
HD17	<i>HTF1</i> ^{ΔHD} in HK11	This study
N184-12	HK11 transformed with the <i>HTF1</i> -EGFP construct	This study

cin-resistant progeny failed to produce conidia but the 13 hygromycin-sensitive progeny were normal in conidium production, suggesting that there is only one gene locus inserted with the *HPH* gene. We also cloned the entire *HTF1* gene to complement the $\Delta htf1$. Transformants of the $\Delta htf1$ mutant expressing the wild-type *HTF1* allele restored the ability to conidiate normally (Table 2, HC1). Therefore, deletion of *HTF1* was directly responsible for the defects observed in the $\Delta htf1$ mutant.

The $\Delta htf1$ mutant could still develop functional appressoria from hyphal tips.

In *M. oryzae*, appressorium formation is normally assayed with conidia on hydrophobic surfaces. Because the $\Delta htf1$ mutant did not produce conidia, we assayed appressorium formation by hyphal tips by inserting plastic coverslips in front of the edge of fungal colonies. After incubation for 48 h, melanized appressoria were formed by approximately 10% of the hyphal tips examined in both the wild-type and $\Delta htf1$ mutant strains (Fig. 5A). Some hyphae developed swollen or deformed tips, which likely represent early stages of appressorium formation. We also examined appressorium formation on rice leaves with a similar approach. Abundant appressoria were observed in both the $\Delta htf1$ mutant and wild type Guy11 (Fig. 5B). These appressoria formed by hyphal tips were melanized and morphologically similar to those formed by germ tubes of Guy11 conidia. These results indicated that the *HTF1* gene is required for conidiation but dispensable for appressorium formation.

Table 2. Growth rate and conidiation by *Magnaporthe oryzae* strains^a

Strain	Growth rate (mm/day) ^b	Conidiation (×10 ⁵ conidia/plate) ^c	Conidiophores per microscope field
Guy11	5.63 ± 0.06	47.60 ± 9.28	11.10 ± 2.05
HK11	5.60 ± 0.20	0	39.27 ± 1.27
HK12	5.70 ± 0.10	0	39.87 ± 2.96
HE1	5.47 ± 0.12	49.90 ± 6.64	ND
HC1	5.70 ± 0.10	42.60 ± 12.15	ND
HD17	5.67 ± 0.15	0	ND

^a Mean and standard deviations were calculated from three independent experiments; ND = no data.
^b Colony diameter (mm) was measured after incubating for 5 days at 25°C.
^c Number of conidia formed on each oatmeal agar plate (diameter 9 cm) examined after incubation for 14 days.

Fig. 4. Examination for conidiation in the wild-type and $\Delta htf1$ mutant strains. **A**, Images of 10-day old cultures examined under a dissecting microscope and **B**, by scanning electronic microscopy. Arrows point to conidiophores (Con), conidia (C), and sterigmata (S).

Appressoria formed by the $\Delta htf1$ mutant were normal in plant penetration and infection.

To further assay whether appressoria formed by the $\Delta htf1$ mutant were able to penetrate plant cells, we conducted onion epidermal cell penetration assays with blocks of mycelia. After incubation at room temperature for 36 h, agar blocks were removed. Appressorium formation was examined after another 24 h. Hyphal tips of the $\Delta htf1$ mutant formed melanized appressoria that could penetrate onion epidermal cells and formed normal infectious hyphae (Fig. 6A). No obvious difference was observed between the $\Delta htf1$ mutant and Guy11 in appressorium formation and plant penetration by hyphal tips.

We also conducted infection assays with blocks of mycelium from oatmeal agar cultures. On rice and barley leaves inoculated with Guy11 and the $\Delta htf1$ mutant, blast lesions were observed at the inoculation sites 7 days postinoculation (dpi) (Fig. 6B). The $\Delta htf1$ mutant also was pathogenic in infection assays with rice roots (Fig. 6B). When rice leaf segments with blast lesions were excised and incubated on water agar after surface sterilization, conidia and conidiophores were formed on leaves infected by the wild-type strain after incubation for 3 days in a moisture chamber at 25°C. Under the same conditions, only conidiophores were observed on the lesions caused by the $\Delta htf1$ mutant (Fig. 6C), indicating that *HTF1* is also required for conidiation on the host plant. Although it is dispensable for plant infection under laboratory conditions, the *HTF1* gene regulates a critical step in the disease cycle of *M. oryzae*.

The homeodomain is essential for the function of *HTF1*.

The homeodomain is the only conserved motif that was identified in the Htf1 protein. To determine its function, we generated the *HTF1*^{ΔHD} allele, deleting residues 86 to 146, and transformed it into the $\Delta htf1$ mutant (Fig. 7A). Six transformants, including HD12 and HD14 (Table 1), were confirmed by Southern analyses to contain the *HTF1*^{ΔHD} construct in the genome (data not shown). The expression of the *HTF1*^{ΔHD} construct in transformant HD12 and HD14 was verified by reverse-transcription (RT)-PCR (Fig. 7B). Similar to the $\Delta htf1$ mutant, HD17 (Table 2) and other *HTF1*^{ΔHD} transformants produced conidiophores but not conidia on rice polish and oat-

Fig. 5. Appressorium formation assays with the $\Delta htf1$ mutant. **A**, Appressoria formed on plastic cover slips by hyphal tips of Guy11 and the $\Delta htf1$ mutant; hyphal tips of the $\Delta pmk1$ mutant failed to form appressoria as a control. **B**, Appressoria formed on rice leaf surface by hyphal tips of Guy11 and the $\Delta htf1$ mutant. Bar = 10 μ m. Arrows point to hypha (H) and appressorium (A).

meal agar media (Fig. 7C). On barley or leaves inoculated with mycelia of transformants expressing the *HTF1*^{AHD} allele, blast lesions were observed after 7 dpi (data not shown). However, these lesions failed to produce any conidia after incubation on water agar for 48 h (data not shown). Thus, we conclude that deletion of the homeodomain abolished the function of the *HTF1*^{AHD} allele.

Expression of the Htf1-EGFP fusion construct.

A Htf1-EGFP (enhanced green fluorescent protein) in-frame fusion construct under the control of its native promoter was generated and transformed into the $\Delta htf1$ mutant. Strain N184-12 (Table 1) was one of the resulting transformants that were confirmed to contain the *HTF1-EGFP* construct by Southern analysis. It produced normal conidia (Fig. 8), suggesting that *HTF1-EGFP* functionally complemented the defect of the $\Delta htf1$ mutant. However, GFP signals were not detectable or extremely weak in vegetative hyphae and mature conidia. Although faint fluorescent signals were observed in some of the conidiophores and developing conidia, nuclear localization of Htf1-EGFP fusion proteins was difficult to observe (Fig. 8). Considering that C-terminal fusion might interfere with detection of GFP, we also generated an N-terminal fusion construct (*EGFP-HTF1*) and transformed it into the $\Delta htf1$ mutant. All 10 resulting transformants produced normal conidia but the GFP signal was too weak to determine the nuclear localization of Htf1 protein (data not shown). It is likely that the Htf1 protein may be activated and localized to the nucleus but its nuclear localization may be too transient to be detected during conidiation in *M. oryzae*.

Fig. 6. Penetration and plant infection assays with mycelial blocks of the $\Delta htf1$ mutant. **A**, Penetration of onion epidermal cells by appressoria formed by hyphal tips of the wild-type and $\Delta htf1$ mutant strains. **A**, appressoria; **IF**, infectious hyphae. Bar = 10 μ m. **B**, Lesions formed by mycelial blocks of Guy11 and the $\Delta htf1$ mutant on rice and barley leaves (left) and rice roots (right). **C**, Lesions caused by Guy11 and the $\Delta htf1$ mutant were surface sterilized and examined for conidiation after incubating for 3 days on 2% water agar. The $\Delta htf1$ mutant formed conidiophores but failed to produce conidia.

HTF1 is not involved in the repression of conidium development under nonconductive conditions.

Because the $\Delta htf1$ mutant appeared to produce more conidiophores than the wild-type strain on culture plates (Fig. 4A and B), *HTF1* may function as a negative regulator of conidiophore development. Quantification of conidiophore development showed that the $\Delta htf1$ mutant formed significantly ($P < 0.01$) more conidiophore than the wild-type strain Guy11 (Table 2) from the same size of oatmeal culture block under conditions with light. In cultures maintained in the dark, no obvious differences in conidiophore development were observed between the $\Delta htf1$ mutant and Guy11 (data not shown). We then assayed conidiophore development in liquid CM cultures. Similar to Guy11, the $\Delta htf1$ mutant failed to develop conidiophores in vegetative hyphae grown in liquid CM (data not shown). These data indicate that the $\Delta htf1$ mutant is not derepressed in conidiophore development under nonconductive conditions.

The *HTF1* expression was detected at all growth stages, including conidium, mycelium, appressorium, and infectious hypha, by quantitative real-time (q)RT-PCR, with the lowest level at the conidiophore stage (Fig. 9A). To further determine whether *HTF1* expression is affected at different growing conditions, we conducted qRT-PCR with RNA samples isolated from the wild-type strain cultures on rice polish agar media or submerged cultures in liquid CM under constant light or dark conditions. Data from three independent experiments indicated that the expression level of *HTF1* was increased significantly ($P < 0.01$) in cultures under constant-dark compared with constant-light conditions. To our surprise, the expression level of *HTF1* was increased over 38-fold ($P < 0.01$) more in submerged cultures in liquid CM under the constantly dark condition (Fig. 9B) than the cultures on rice polish agar medium with constant light. It is known that constant light and rice polish agar medium are conducive for conidiation in *M. oryzae*. Therefore, the elevated expression level of *HTF1* gene in non-

Fig. 7. Function analysis of the homeodomain of the *HTF1* gene. **A**, Diagram of the *HTF1*^{AHD} allele. The homeodomain (empty box) was deleted in pYH12. The position of the only intron of the *HTF1* gene is labeled with a filled triangle. Primers D1F and D1R were used to amplify *HTF1*. **B**, Reverse-transcription polymerase chain reaction with RNA isolated from the $\Delta htf1$ mutant and *HTF1*^{AHD} transformants HD12 and HD14. Genomic DNA (gDNA) was used as the control. **C**, Conidiophores formed by the complementation strain HC1 and *HTF1*^{AHD} transformants HD12 and HD14. Bar = 10 μ m.

conductive conditions might suggest that *Htf1* functions as a negative regulator of the fungal conidiophore formation. Another possibility is that feedback signals from conidia may inhibit further conidiophore development through modulating the *HTF1* expression in *M. oryzae*.

The expression of *HTF1* was downregulated in the $\Delta mgb1$ and $\Delta cpkA$ mutant.

In *M. oryzae*, several genes have been implicated in regulating conidiation, including components of the trimeric G-proteins. The $\Delta mgb1$ G- β mutant rarely produces conidia and forms fluffy colonies with abundant conidiophores (Nishimura et al. 2003). The gene encoding the catalytic subunit of the cAMP-dependent protein kinase (CpkA) is a downstream component of the G-protein pathway (Fang and Dean 2000; Nishimura et al. 2003). To determine the expression level of *HTF1*, we conducted qRT-PCR with RNA samples isolated from aerial hyphae and conidiophores of the wild-type, $\Delta mgb1$, and $\Delta cpkA$ mutant strains grown on oatmeal agar for 10 days. Data from three independent experiments indicated that the expression level of *HTF1* was reduced over fivefold ($P < 0.01$) in the $\Delta mgb1$ mutant and approximately twofold in the $\Delta cpkA$ mutant ($P < 0.05$) (Fig. 10A). Therefore, the *HTF1* gene may function downstream from the trimeric G-protein signaling to regulate conidium formation in *M. oryzae*. However, the exogenous cAMP could not rescue the mutant defect in conidiation by growing the *htf1* mutant strain on the oatmeal agar plate with supplement of 10 mM cAMP (data not shown).

Deletion of *HTF1* reduced the expression of the *ACR1* gene.

HTF1 is specifically involved in the regulation of conidiation but not conidiophore development. In *M. oryzae*, two other transcription factor genes, *ACR1* and *CON7*, are known to regulate conidiogenesis and conidium morphology (Lau and Hamer

1998; Odenbach et al. 2007). To determine the effect of $\Delta htf1$ mutation on *ACR1* and *CON7*, we assayed the expression of these two genes by qRT-PCR. In comparison with that of Guy11, the expression level of *ACR1* was reduced significantly ($P < 0.05$) in the $\Delta htf1$ mutant (Fig. 10B). However, expression of the *CON7* gene was not significantly ($P > 0.05$) reduced in the $\Delta htf1$ mutant (approximately 80% of the wild type). As a control, the expression level of *BUF1* involved in melanin synthesis but not conidiation was not affected under the same experimental conditions (Fig. 10B).

DISCUSSION

Homeodomain proteins are well conserved for regulating development and differentiation in various eukaryotic organisms. In *Saccharomyces cerevisiae*, four of the nine homeobox genes are related to the mating type or switching locus (Houston et al. 2002). Like many other filamentous ascomycetes, *M. oryzae* does not contain homeobox genes in the mating type loci and lacks putative homologs of yeast *MATa2* and *MATa1* (Kanamori et al. 2007). Therefore, it is rational to expect that homeobox genes may play a different but important role in the growth, development, and plant infection of the fungus. Indeed, Sweigard and associates (1998) already indicated the important role of PTH12. In this study, we demonstrated that *HTF1* is a key regulator for the fungal conidiogenesis in *M. oryzae*. To our knowledge, *HTF1* is the only gene in *M. oryzae* that is known to be specifically and absolutely required for conidia-

Fig. 8. Expression and localization of HTF1-EGFP (enhanced green fluorescent protein) fusion proteins. Weak GFP signals were observed in conidiophores and young developing conidia from transformants expressing the *HTF1*-EGFP fusion construct. A, appressoria; Con, conidiophores; VH, vegetative hyphae; MC, mature conidia; YC, young conidia.

Fig. 9. Real-time reverse-transcription polymerase chain reaction quantifying *HTF1* expression at different developmental stages in *Magnaporthe oryzae*. **A**, Expression at the conidiophore stage showed the lowest level compared with conidium, mycelium, appressorium, or infectious hyphae stage. Con, conidium; Myc, mycelium; App, appressorium; Cph, conidiophore; Inf, infectious hypha. **B**, Expression level of *HTF1* was increased significantly ($P < 0.01$) in cultures under constant dark compared with constant light condition, reaching the maximum in submerged cultures in liquid complete media under the constant dark condition. LL, light liquid; DL, dark liquid; LS, light solid; DS, dark solid. Mean and standard deviation were calculated from data of three replicates.

tion, and also the first homeobox gene reported to be involved in conidia development in filamentous ascomycete fungi.

Interestingly, we found that deletion of *HTF1* blocked conidiation but not the development of conidiophores in *M. oryzae*. This is different from the role of *COS1*, in which gene deletion also causes defect in conidiation, but it is shown that *Cos1* is essential for conidiophore development (Zhou et al. 2009). It seems that *HTF1* may function as an essential positive regulator for switching from conidiophore growth to the initiation of conidium development. Therefore, conidiophores fail to produce conidia when *HTF1* is deleted. Because the $\Delta htf1$ mutant appeared to produce more abundant conidiophores than the wild-type strain under suitable conditions, *HTF1* may also function as a negative regulator of conidiophore development in *M. oryzae*. However, the $\Delta htf1$ mutant did not form conidiophore-like structures in darkness or in liquid CM cultures. It is likely that *HTF1* does not directly regulate conidiophore formation. Increased conidiophore development in the $\Delta htf1$ mutant may be an indirect consequence of its defects in conidium formation.

Conidiation has been best studied in *A. nidulans* and *N. crassa*. However, unlike *M. oryzae*, these two model filamentous fungi do not form conidia sympodially on conidiophores. In *N. crassa*, the predicted gene NCU03070.3 shared 33% identity with *HTF1* but its function was not described in the original high-throughput functional genomics study (Colot et al. 2006). The mutant deleted in NCU03070.3 (Fungal Genetics Stock Center, Kansas City, MO, U.S.A.) had no obvious changes in vegetative growth. Normal macroconidia were formed in chains on short, branched conidiophores (data not shown). These results suggest that the conidiogenesis processes are regulated by different transcription factors in *N. crassa* and *M. oryzae*. In *A. nidulans*, the *brlA* mutant forms conidiophores but rarely produces conidia (Mah and Yu 2006). The *M. oryzae* genome lacks a distinct homolog of *brlA*. Mutants deleted for MGG_00501, a predicted gene with limited similarity to *brlA*, were reduced in conidiation but still formed normal sympodial conidiophores and pyriform conidia (S.-L.

Ding and J.-R. Xu, unpublished). An08g02580 is a predicted homeobox gene that is homologous to *HTF1* but has not been functionally characterized in *A. nidulans*.

A number of mutants that are defective in conidiation or have altered conidium morphology have been reported in *M. oryzae*, such as $\Delta acr1$, $\Delta con1$, $\Delta con7$, $\Delta MoATG8$, $\Delta Mgrac1$, $\Delta mgb1$, and $\Delta cos1$ (Chen et al. 2008; Deng et al. 2009; Lau and Hamer 1998; Nishimura et al. 2000; Odenbach et al. 2007; Shi et al. 1998; Zhou et al. 2009). However, most of the conidiation-related genes characterized in *M. oryzae* are not specific or essential for conidiogenesis. In fact, many of these mutants have pleiotropic defects in other developmental or plant-infection processes. In a large-scale random insertional mutagenesis project, MGG_00184 was tagged by the transforming T-DNA (Jeon et al. 2007). The disruptant was reported to be blocked in conidiation but no further characterization of its phenotype was reported. Our efforts to characterize the homeobox genes in *M. oryzae* were initiated in 2005. Our studies indicated that the $\Delta htf1$ mutant had no defects in appressorium formation. Appressoria formed by hyphal tips were able to penetrate plant cells, and the $\Delta htf1$ mutant caused typical blast lesions on leaves inoculated with mycelial blocks. Because rice blast is a polycyclic disease, blocking conidiation can serve as an efficient disease control strategy. Therefore, it will be important to determine molecular mechanisms regulating the Htf1 transcription factor and genes regulated by *HTF1* in *M. oryzae*. *HTF1* may function downstream from *MGB1* because the expression level of *HTF1* was significantly ($P < 0.01$) reduced in the $\Delta mgb1$ mutant (Fig. 10A). Trimeric G-proteins are known to regulate conidiation in several filamentous ascomycetes, including *Cochliobolus heterostrophus* and *A. nidulans* (Ganem et al. 2004; Yu et al. 2006). In *M. oryzae*, the cAMP-protein kinase A (PKA) pathway functions downstream of the trimeric G-proteins (Fang and Dean 2000; Nishimura et al. 2003). The Htf1 protein has two putative PKA phosphorylation sites outside the homeodomain but their role in its activation is not clear.

The homeodomain of *HTF1* is essential for its function. By searching the homeodomain resource database, we found that the homeodomain of *HTF1* shared limited homology with that of the *OTX2* gene, which has the recognition site CCTAAT CCTGGGTTATC (Briata et al. 1999). The promoter region of the *ACR1* gene has the CCTAATCGGCGGTTTTC sequence (−901 to −885, identical sequences underlined) that may function as the Htf1 binding site. Our qRT-PCR data indicated that the expression level of *ACR1* was reduced approximately two-fold in the $\Delta htf1$ mutant (Fig. 10B). The *CON7* gene lacks a similar site in the promoter region, and its transcription level was not reduced significantly in the $\Delta htf1$ mutant (Fig. 10B). Further studies are necessary to determine the actual binding site of the homeodomain of Htf1 and the relationship among conidiation-related transcription factors in *M. oryzae*. Some

Fig. 10. Real-time reverse-transcription polymerase chain reaction analyses of the *HTF1*, *ACR1*, *CON7*, and *BUF1* transcripts. The relative quantity of the transcripts of each gene was compared between the wild-type strain (arbitrarily set to 1) and the $\Delta mgb1$ and $\Delta cpka$ mutant. **A**, Expression level of *HTF1* was reduced in the $\Delta mgb1$ and $\Delta cpka$ mutant. **B**, *ACR1* and *CON7* genes but not the *BUF1* gene had a reduced transcription level in the $\Delta htf1$ mutant (filled boxes) in comparison with that of Guy11 (empty box). Mean and standard deviation were calculated from data of three replicates.

Fig. 11. Model scheme for the role of Htf1 in conidiogenesis.

homeoproteins are known to exert regulatory roles via combinatorial interaction with other transcription factors (Bhoite et al. 2002). In *M. oryzae*, Htf1 may interact with Acr1 or other transcription factors during conidiogenesis.

Taken together, we proposed a model to interpret how Htf1 might regulate conidiation (Fig. 11). Conidiation is stimulated by light and inhibited in submerged cultures in *M. oryzae*. Young conidia formed at the tip of conidiophores are single celled. Mature conidia have three conidial cells that are formed by two asymmetric mitotic divisions. When the first conidium matures, the active apical tip moves to the side to produce the next conidium. This process is repeated to give rise to multiple conidia borne sympodially on conidiophores. Trimeric G-protein signaling is involved in regulating conidiophore development and conidium formation. Recently, Zhou and associates (2009) showed that Cos1 may be a key transcriptional regulator controlling genes responsible for conidiation but it is essential for conidiophore stalk development. We have demonstrated that Htf1 is a positive regulator required for the differentiation of conidia and may also function as a negative regulator of conidiophore development. Trimeric G-proteins may function upstream from Htf1 but other mechanisms must exist to activate Htf1 because the $\Delta magB$ ($G\alpha$) and $\Delta mgb1$ ($G\beta$) deletion mutants occasionally form rare conidia. Con7 and Con1 are required for normal conidium morphology and are functional in the maturation of conidia. Acr1 is required for the termination of further development in mature conidia and formation of individual conidia sympodially on conidiophores.

MATERIALS AND METHODS

Culture conditions.

The wild-type strains Guy11 and 70-15, $\Delta pmk1$ (Xu and Hamer 1996) and $\Delta mgb1$ (Nishimura et al. 2003) mutants, and all the transformants (Table 1) generated in this study were

cultured on oatmeal agar, CM, or rice polish medium plates at 25°C as described (Park et al. 2006; Zhao et al. 2005). Media were supplemented with hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) at 250 µg/ml or zeocin (Invitrogen, Carlsbad, CA, U.S.A.) at 200 µg/ml for selecting hygromycin-resistant and zeocin-resistant transformants, respectively. Monoconidial culture isolation, genetic crosses, and measurements of conidiation and growth rate were performed as previously described (Park et al. 2006; Zhao et al. 2005). For appressorium formation from hyphal tips, plastic coverslips were inserted in front of the edge of fungal colonies, and the coverslips were examined with a Nikon Eclipse 800 Epifluorescence microscope after 48 h of incubation.

Quantification of conidiophores.

When the fungus grew for 5 days on the oatmeal agar plate, the mycelia were pushed down with a glass slide. The oatmeal culture blocks (4 mm²) were sliced out from the edge of colonies and then placed onto a glass slide. The slides were put onto a moisture plastid plate, then incubated in a chamber with constant-light conditions for 48 h. Conidiophores were then examined with a Nikon SMZ1000 dissecting microscope by counting 10 microscope fields for each sample. Three samples were examined for the $\Delta htf1$ mutant and the wild-type Guy11, respectively.

Statistical analysis.

Statistical analysis was performed with the data from three replicates. Mean and standard deviation were calculated using SPSS V13.0, and one-way analysis of variance was performed on the data for significant differences between genotypes.

Plant infection assays.

Two-week-old seedlings of rice cv. CO39 and 10-day-old seedlings of barley cv. Golden Promise were used for plant

Table 3. Polymerase chain reaction primers used in this study

Name	Sequence(5'→3')
1AF	CGGAGCTCCCCAGCTATGGTCAAATCA
1AR	CCCAAGCTTCCTTCTATCTCCTTGGTCGT
1BF	CCGCTCGAGGCAAGTCAAGCACATACAT
1BR	GGGGTACCACCAAATGCTCGCTCACTA
1NF	GCTACTTCGTCCCTCAGTA
1NR	CCATCGCTACCTTCTCCAT
1DB	TTGGACGCTCTAGGGATGCC
H853	ACAGACGTCGCGGTGAGTT
1H1F	CCGCTCGAGTCCCGGCTGGCTTCATTG
1H1R	GGACTAGTCTGGTCTGCTTCGACGTTG
1H2F	GGACTAGTGGAATCAAAAAGACGCAAG
1H2R	GCTCTAGAAGGACCTAATAAAGCACGAC
D1F	TGTCACCAACCGACTCCTT
D1R	TGTTCTCCCTGGTTCCCT
1NGF	CGACTCACTATAGGGCGAATTGGGTAATGAAATTGGCTCGAACACCAAGAAGTGA
1NGR	CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCTTCTCGACACCTGTGTT
BTF	TCCGTGGAAAAGGTTTCCATG
BTR	ATCCAATCGACGAAGTACGA
1QF	GGAGAAGGTAGCGATGGTGAA
1QR	GGTGTGGCTGTTGGTCGTAG
AQF	GGACCAAGTCGGAACAAGAGTG
AQR	GACCCAAACGAGACGCAAA
BQF	TGAGGAATTTGACCGTGTCTTTAC
BQR	AATCAGGCGACCAACATCT
CQF	TCATAGTGCGCAGCAGTGGAG
CQR	CGGTTGGGCATAGAGGTTG
1612 PF	CGGAGCTCGGATTGACAACGACCATT
1612 PR	TCCCCGCGGGCTTTTTTGTGTCGCTTG
GFP 717F	TCCCCGCGGATGGTGAGCAAGGGCGA
GFP 717R	GCTCTAGACTTGTACAGCTCGTCCAT
3175 OF	GCTCTAGAATGGACTACATGAACATG
3175 OR	CCGCTCGAGGGAATAGCAGGTCTTTAGTG

infection assays as described (Park et al. 2004). Rice root infection assays were carried out as described in Dufresne and Osbourn (2001). For infection assays with mycelial blocks of oatmeal agar, rice and barley seedlings were removed from pots and laid over a moist Whatman no. 1 filter paper with the adaxial surface of the second leaf exposed. Another layer of moist filter paper was used to cover the root areas to keep plants in position and maintain moisture. Blocks (4 mm²) of mycelia were excised from the edge of fungal colonies and placed over the second leaf of each plant. After incubating for 48 h in a moisture chamber, the blocks were removed. Lesions were examined after 5 days of incubation at room temperature. Segments of plant leaves with blast lesions were excised, surface sterilized, and incubated on water agar for 2 to 3 days to assay conidiophore development as described (Xu and Hamer 1996).

Molecular manipulations and sequence analyses.

Standard molecular biology procedures were followed for enzymatic manipulations of DNA (Sambrook and Russell 2001). Genomic DNA was extracted from mycelia by the cetyltrimethylammonium bromide protocol (Xu and Hamer 1996). Programs ClustalW and TreeView 32 were used for sequence alignment and construction of the phylogenetic tree. For qRT-PCR analysis, RNA samples were isolated from mycelia and conidiophores harvested from 10-day-old oatmeal cultures with the SV total RNA isolation system (Promega Corp., Madison, WI, U.S.A.). First-strand cDNA was synthesized with AMV reverse transcriptase (Promega Corp.). Real-time PCR was performed with the MJ Research OPTICON Real-Time Detection System using the TaKaRa SYBR Premix Ex Taq (Takara, Shiga, Japan). Primer pairs 1QF/1QR, AQF/AQR, BQF/BQR, CQF/CQR, and BTF/BTR were used to amplify the *HTF1*, *ACR1*, *BUF1*, *CON7*, and β -tubulin genes, respectively. The relative quantification of each transcript was calculated by the 2^{- $\Delta\Delta C_t$} method (Livak and Schmittgen 2001) with the *M. oryzae* β -tubulin gene (MGG_00604) as the internal control.

Gene replacement vectors and mutants.

To generate the *HTF1* gene-replacement vector, a 0.91-kb fragment upstream from *HTF1* was amplified with primers 1AF and 1AR and cloned between the *SacI* and *HindIII* sites on pCSN43 as pHT1a. A 0.81-kb fragment downstream from *HTF1* was amplified with primers 1BF and 1BR and cloned between the *XhoI* and *KpnI* sites on pHT1a as pHT1, which was transformed into protoplasts of the wild-type strain Guy11 as described (Zhao and Xu 2007). Hygromycin-resistant transformants were screened by PCR with primers 1DB and H853 (Table 3). The complementation vector pYH1 was constructed by cloning a 4.7-kb fragment of *HTF1* amplified with primers 1H1F and 1H2R between the *XhoI* and *XbaI* sites of pYK11 (Zhao and Xu 2007). Primers used to generate the constructs and screen for gene replacement mutants are listed in Table 3.

SEM.

Blocks of 10-day-old oatmeal agar cultures (5 mm²) were fixed in 4% glutaraldehyde at 4°C for 16 h. The samples were then dehydrated in a graded ethanol series and dried in a critical point dryer as described (Zange et al. 2005). After being sputter-coated with gold, conidia and conidiophores were observed with a JSM-6360LV (Jeol Ltd., Tokyo) scanning electron microscope.

Construction of the *HTF1*^{AHD} allele.

To generate the *HTF1*^{AHD} allele, a 2.2-kb fragment amplified with primers 1H1F and 1H1R (Table 3) was cloned into pYK11 between the *XhoI* and *SpeI* site as pYK11-H1. A 2.3-kb downstream fragment of *HTF1* was amplified with primers 1H2F and 1H2R and cloned into the *SpeI* and *SacI* site of pYK11-H1. In

the resulting construct, pYH12, the *HTF1* gene was under the control of its native promoter but amino acid residues 86 to 146 were deleted. All PCR reactions were performed with the *La* DNA polymerase (Takara). The pYH12 construct was confirmed by sequencing and transformed into the $\Delta htf1$ mutant. Primers D1F and D1R were used for RT-PCR to determine the expression of *HTF1*^{AHD} in the resulting transformants.

Generation of the *HTF1*-EGFP fusion construct.

For constructing the *HTF1*-EGFP fusion vector, pHN1, the entire *HTF1* open reading frame (ORF) and a 1.1-kb upstream promoter sequence were amplified with primers 1NGF and 1NGR (Table 3). The resulting PCR product was cloned into the bleomycin-resistance vector pKB04 (Bruno et al. 2004) by the in vivo yeast homologous recombination approach (Bourett et al. 2002). To generate the *EGFP-HTF1* fusion construct pHN2, a 1,612-bp fragment upstream from *HTF1* as its native promoter was amplified with primers 1612 PF and 1612 PR and cloned between the *SacI* and *SacII* sites on pYK11 as pHN2a. A 711-bp fragment of EGFP without stop codon was amplified with primers GFP 717F and GFP 717R and cloned between the *SacII* and *XbaI* sites on pHN2a as pHN2b and, then, a 3,175-bp fragment of *HTF1* ORF and its terminator was amplified with primers 3175 OF and 3175 OR and cloned between the *XbaI* and *XhoI* sites on pHN2b as pHN2. Plasmid pHN1 and pHN2 was confirmed by sequencing analysis to carry the *HTF1*-EGFP and *EGFP-HTF1* fusion construct under the control of its native promoter. pHN1 and pHN2 were transformed into the $\Delta htf1$ mutant by selecting for zeocin-resistant transformants (Zhao and Xu 2007).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Magnaporthe oryzae* database:
www.broadinstitute.org/annotation/fungi/magnaporthe
 National Institutes of Health National Human Genome Research Institute
 website: research.nhgri.nih.gov