Penetration Peg Formation and Invasive Hyphae Development Require Stage-Specific Activation of *MoGti1* in *Magnaporthe oryzae*

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The hemibiotrophic pathogen *Magnaporthe oryzae* causes one of the most destructive diseases in cultivated rice. Complex infection-related morphogenesis and production of various effectors are known to be important for successful colonization and disease development. In this study, we characterized the activation of the *MoGti1* transcription factor and its role in infection-related morphogenesis and effector gene expression. The *Mogti1* mutant was nonpathogenic, although it was normal in appressorium formation and turgor generation. Close examination showed that *Mogti1* was defective in penetration and growth of normal invasive hyphae. Deletion of *MoGti1* affected the expression of the majority of effector genes. The expression of *MoGti1* appeared to be controlled by the *Mps1* but not *Pmk1* mitogen-activated protein kinase (MAPK), and the *mps1* and *Mogti1* mutants had similar phenotypes in plant infection and cell wall integrity defects. However, lack of MAPK phosphorylation sites and dispensability of the putative MAPK docking site suggested that *MoGti1* is not a direct target of *Mps1*. Site-specific mutagenesis analyses showed that the putative protein kinase A phosphorylation site was not essential for localization of *MoGti1* to the nucleus but important for its normal function. Although the cyclin-dependent kinase (CDK) phosphorylation site of *MoGti1* is dispensable during vegetative growth and appressorium formation, the *S77A* mutation affected penetration and invasive growth. Localization of *MoGti1*^{S77A}-green fluorescent protein to the nucleus in late stages of appressorium formation and during invasive growth was not observed, suggesting a stage-specific CDK phosphorylation of *MoGti1*. Overall, our data indicate that *Mps1* may indirectly regulate the expression of *MoGti1* in maintaining cell wall integrity, conidiation, and plant infection. *MoGti1* is likely a stage-specific target of CDK and plays a crucial role in effector gene expression and morphogenesis related to the development of penetration pegs and invasive hyphae.

Rice blast, caused by *Magnaporthe oryzae*, is a destructive disease of cultivated rice, which is the most important food source in the world. Although planting resistant cultivars is a

major disease control method, the rice blast fungus is known to be adept at overcoming plant resistance (Dean et al. 2005). In addition, a wheat-adapted population of *M. oryzae* causing wheat blast disease in South America has recently emerged and poses a major threat to global wheat production (Cruz et al. 2012; Giraldo et al. 2013). Because of its economic importance and experimental tractability, *M. oryzae* has been developed as a model for studying fungus–plant interactions (Wilson and Talbot 2009). When conidia attach to plant surfaces, germ tubes emerge and differentiate into melanized appressoria for penetration. Inside plant cells, penetration pegs differentiate into primary infectious hyphae, which further develop into invasive hyphae (IH). Under favorable environmental conditions, blast lesions are formed on infected leaves due to extensive IH growth, and conidia are released to spread the disease.

As a hemibiotrophic fungus, the IH of *M. oryzae* are enclosed in an extrinvasive hyphal membrane (EIHM) system (Kankanala et al. 2007). The bulbous IH are morphologically distinct from vegetative hyphae (Mosquera et al. 2009) and lack cortical actin patches (Zhou et al. 2011b). They are constricted at septal junctions and, thus, resemble pseudohyphal growth in yeasts (Giraldo et al. 2013; Sudbery et al. 2004). This morphological switch from filamentous to pseudohyphal growth appears to be critical for disease development in *M. oryzae* (Giraldo and Valent 2013). Similar observations of differences in hyphal morphology between saprophytic and infectious growth have been observed in other plant-pathogenic fungi such as *Fusarium graminearum*, *Colletotrichum higginsianum*, and *Zymoseptoria tritici* (O’Connell et al. 2012). In some dimorphic fungi, such as *Ustilago maydis* and *Taphrina deformans*, the switch from yeast-like growth to filamentous growth is intricately connected with mating and plant infection (Nadal et al. 2008).

To successfully invade living cells and manipulate plant immunity, *M. oryzae* expresses and delivers various effectors into plant cells (Valent and Khang 2010; Zhang and Xu 2014). Recent results suggest that *M. oryzae* deploys distinct secretion systems for translocation of effectors across the plant plasma membrane. Cytoplasmic effectors preferentially accumulate in the biotrophic interfacial complex (BIC), a novel plant membrane-rich structure associated with invasive hyphae. They are delivered into plant cells by a different pathway involving the exocyst complex and target (t)-SNAREs, which are independent of the Golgi-dependent secretory system (Giraldo et al. 2013). The apoplastic effectors accumulate extracellularly at the host–pathogen interface and are secreted from IH by the conserved endoplasmic reticulum to a Golgi secretory pathway that is independent of the BIC (Giraldo et al. 2013). A number of cytoplasmic and apoplastic effectors were found by microarray

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analysis to be highly or specifically expressed in invasive hyphae (Mosquera et al. 2009; Zhang and Xu 2014). However, the underlying molecular mechanisms controlling their transcriptional regulation are not clear.

Recently, orthologs of the *WOR1/GTI1* transcription factor gene were shown to be important for morphogenesis and pathogenesis in fungal pathogens. *WOR1/GTI1* was first identified in the human pathogen *Candida albicans* and shown to be involved in the white to opaque cell type switch, a transition that is required for mating and pathogenicity (Huang et al. 2006). In *Histoplasma capsulatum*, its ortholog controls the morphological switch from filamentous growth in the soil to parasitic budding-yeast growth in humans (Nguyen and Sil 2008). Even in the budding yeast *Saccharomyces cerevisiae*, *MIT1*, an ortholog of *WOR1/GTI1*, is a regulator of pseudohyphal growth (Cain et al. 2012). Although *Wor1/Gti1* and *Mit1* bind to the same motif in DNA, they appear to regulate different sets of genes that are related to different developmental processes in *C. albicans* and *S. cerevisiae* (Cain et al. 2012).

In plant-pathogenic fungi, including *F. oxysporum* f. sp. *lycopersici* (FoSGE1), *F. graminearum* (FGP1), *Botrytis cinerea* (BcREG1), *Verticillium dahliae* (VdSGE1), *Cladosporium fulvum* (CfWOR1), *M. oryzae* (Chen et al. 2014), *F. verticillioides* (Michielse et al. 2009), and *Zymoseptoria tritici* (ZtWOR1), deletion of the *WOR1/GTI1* ortholog resulted in reduced virulence and species-specific defects in metabolic and developmental processes (Gohari et al. 2014; Jonkers et al. 2012; Michielse et al. 2011; Ökmen et al. 2014; Santhanam and Thomma 2013). For example, FGP1 and BcREG1 regulate phytotoxin production in *F. graminearum* and *B. cinerea* (Jonkers et al. 2012; Michielse et al. 2011). In *F. oxysporum*, FoSGE1 was shown to regulate parasitic growth and is essential for pathogenicity (Michielse et al. 2009). In *V. dahlia*, *C. fulvum*, and *Z. tritici*, *WOR1/GTI1* orthologs regulate the expression of putative effector genes (Gohari et al. 2014; Ökmen et al. 2014; Santhanam and Thomma 2013). In *Z. tritici*, ZtWOR1 is also involved in cell fusion and anastomosis morphogenesis (Gohari et al. 2014).

To determine the regulatory role of the *Wor1/Gti1* ortholog in effector gene expression and mechanisms of its activation in *M. oryzae*, we functionally characterized the *MoGTII* (= *MoWOR1*) gene. Similar to the report on the same gene published during the course of this study, *MoGTII* was found to be important for conidiation and plant infection but dispensable for appressorium formation (Chen et al. 2014). Although the *Mogti1* mutant was nonpathogenic, in this study we found that approximately 1% of mutant appressoria were able to penetrate plant cells. However, only limited growth of invasive hyphae with less branching and narrower width than those of the wild type were observed in plant cells penetrated by the mutant. We further showed that deletion of *MoGTII* affected some but not all of the known *M. oryzae* effector genes assayed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The expression and activation of *MoGti1* appeared to be controlled by *Mps1* but not *Pmk1* or *CpkA*. Although the putative cyclin-dependent kinase (CDK) phosphorylation site of *MoGti1* is dispensable during vegetative growth and appressorium formation, the S77A mutation affected penetration and invasive growth. Localization of *MoGti1*^{S77A}-green fluorescent protein (GFP) to the nucleus, which normally occurs during late stages of appressorium formation and invasive growth, was not observed. These results indicate that *Mps1* may indirectly regulate the expression and activation of *MoGti1* in maintaining cell wall integrity, conidiation, and plant infection. *MoGti1* likely functions as a stage-specific target of CDK and plays a role in effector gene expression and development of penetration pegs and invasive hyphae.

RESULTS

MoGTII is dispensable for vegetative growth but essential for plant infection.

The *MoGTII* (MGG_08850.6) gene replacement mutants of Guy11 (Table 1) were generated with the split-marker approach and confirmed by Southern blot analysis (Supplementary Fig. S1). Similar to the earlier report (Chen et al. 2014), the *Mogti1* mutants had no significant growth defects but were reduced in conidiation (Table 2) and nonpathogenic (Supplementary Fig. S2). We also found that the mutant failed to infect through wounds, indicating that *MoGTII* is required for invasive growth.

In comparison with the wild type, the *Mogti1* mutant was delayed in appressorium formation and produced long germ tubes on hydrophobic surfaces (Fig. 1A). By 12 h postinoculation (hpi), only 44% of germ tubes in the *Mogti1* mutant formed appressoria, compared with 94% in Guy11. By 24 hpi, 97% of Guy11 germ tubes formed appressoria but only 81% of *Mogti1* formed appressoria (Fig. 1B). Although the *Mogti1* mutant still formed melanized appressoria, it was significantly reduced in the penetration efficiency. Only approximately 1% of appressoria formed by the mutant were able to penetrate the epidermal cells of barley. However, no significant difference in appressorium turgor was observed between Guy11 and *Mogti1* by cytorrhysis assays (Fig. 1C). Therefore, the penetration defect is not caused by reduced appressorium turgor pressure. Because penetration peg formation is known to elicit strong autofluorescence in onion epidermal cells (Park et al. 2004), we conducted penetration assays with onion epidermis. At 24 h, appressoria formed by the wild-type strain efficiently penetrated or elicited autofluorescence and papilla formation in underlying onion epidermal cells (Fig. 1D). Similar to the *mst12* mutant, the *Mogti1* mutant rarely elicited autofluorescence in onion epidermal cells (Fig. 1D), even after prolonged incubation up to 72 h. These results suggested that *MoGTII* plays a critical role in the formation of penetration pegs or eliciting autofluorescence.

The *Mogti1* mutant is defective in invasive hyphae growth.

In penetration assays with barley epidermal cells, appressoria formed by the *Mogti1* mutant rarely (1%) were successful in developing invasive hyphae in underlying plant cells. However, in comparison with the wild type, the extent of invasive growth also was reduced in the mutant. In addition, unlike typical bulbous invasive hyphae formed by the wild type, the *Mogti1* mutant produced invasive hyphae that were narrower and less branching (Fig. 2A; Supplementary Fig. S3). We examined over 40 infection sites with invasive hyphae developed inside plant cells by the *Mogti1* mutant but failed to observe typical pseudohyphal-like growth. Similar results were observed in penetration assays with rice leaf sheaths, with over 40 infection sites examined (Fig. 2B). These results indicate that *MoGTII* is important for the differentiation and growth of normal bulbous pseudohyphae in *M. oryzae*.

MoGTII localizes to the nucleus.

The *MoGTII*-GFP construct was generated and transformed into the *Mogti1* mutant. The resulting *Mogti1/MoGTII*-GFP transformant WC15 was normal in conidiation and virulence (Supplementary Fig. S4). When examined by epifluorescence microscopy, weak GFP signals were observed in the nucleus of conidia and appressoria (Fig. 3A).

MoGti1 contains a predicted nuclear localization signal sequence (NLS), PPGEKKR, at its N-terminal region. To determine its role in subcellular localization, we generated the *MoGTII*^{ANLS}-GFP construct and transformed it in to the *Mogti1* mutant. In the resulting transformant WN2, fluorescent signals

were still observed in the nucleus (Fig. 3B). Expression of *MoGTII*^{ΔNLS}-GFP also restored the defects of the *Mogti1* mutant in conidiation and pathogenicity (Fig. 3C), suggesting that the PPGEKKR sequence is not important for the localization of MoGti1 to the nucleus. Because we failed to identify other putative NLS, MoGti1 may interact with other proteins to form a complex for its localization to the nucleus.

MoGTII expression is regulated by Mps1 but not Pmk1.

In *M. oryzae*, two mitogen-activated protein kinase (MAPK) genes, *PMK1* and *MPS1*, have been implicated in regulating plant infection processes. To determine whether *MoGTII* is regulated by Pmk1 and Mps1, the expression of *MoGTII* was assayed by qRT-PCR in the *pmk1* and *mps1* mutants (Xu and Hamer 1996; Xu et al. 1998), respectively. In RNA samples isolated from 48-h complete medium (CM) cultures, the expression level of *MoGTII* was twofold lower in the *mps1* mutant than in Guy11. However, the expression level of *MoGTII* was similar between Guy11 and the *pmk1* mutant (Fig. 4A). Thus, it appears that *MoGTII* expression is regulated by *MPS1* but not *PMK1*.

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

Strain	Genotype description	Reference
Guy11	Wild type (<i>MAT1</i> -2)	(Leung et al. 1988)
nn78	<i>pmk1</i> deletion mutant	(Xu and Hamer 1996)
M3H51	<i>mps1</i> deletion mutant	(Xu et al. 1998)
MW1	<i>Mogti1</i> deletion mutant	This study
MW4	<i>Mogti1</i> deletion mutant	This study
MW12	<i>Mogti1</i> deletion mutant	This study
WC15	<i>MoGTII</i> -GFP transformant of the <i>Mogti1</i> mutant	This study
WN2	<i>MoGTII</i> ^{ΔNLS} -GFP transformant of the <i>Mogti1</i> mutant	This study
WM4	<i>MoGTII</i> ^{ΔDOCK} -GFP transformant of the <i>Mogti1</i> mutant	This study
WA2	<i>MoGTII</i> ^{T71A} -GFP transformant of the <i>Mogti1</i> mutant	This study
WA12	<i>MoGTII</i> ^{T71A} -GFP transformant of the <i>Mogti1</i> mutant	This study
WA20	<i>MoGTII</i> ^{T71A} -GFP transformant of the <i>Mogti1</i> mutant	This study
WS5	<i>MoGTII</i> ^{S77A} -GFP transformant of the <i>Mogti1</i> mutant	This study
WGC4	<i>MoGTII</i> -GFP transformant of Guy11	This study
WGS9	<i>MoGTII</i> ^{S77A} -GFP transformant of Guy11	This study
BC12	<i>MoGTII</i> -NYFP and <i>CPKA</i> -CYFP transformant of Guy11	This study
PBC2	<i>MCNA</i> -NYFP and <i>MCNB</i> -CYFP transformant of Guy11	This study
BSG1-5	<i>BAS1</i> -GFP transformant of Guy11	This study
BSG4-4	<i>BAS4</i> -GFP transformant of Guy11	This study
BSW1-27	<i>Mogti1 BAS1</i> -GFP	This study
BSW4-4	<i>Mogti1 BAS4</i> -GFP	This study

Table 2. Growth rate and conidiation of the wild type and *Mogti1* strains

Strain	Growth rate (mm/day) ^y				Conidiation (×10 ⁵ conidia/plate)	Penetration rate (%) ^z
	CM	0.5 M NaCl	0.01% SDS	5 mM H ₂ O ₂		
Guy11 (WT)	3.8 ± 0.0a	2.4 ± 0.0a	1.5 ± 0.1a	2.8 ± 0.2a	127 ± 38a	86.0 ± 6.6a
MW1 (<i>Mogti1</i>)	2.8 ± 0.1b	1.5 ± 0.0b	0.7 ± 0.1b	1.5 ± 0.1b	13 ± 1b	0.8 ± 0.2b

^y For growth rate and conidiation measurements, means ± standard errors were calculated from at least three independent measurements. CM = complete medium and SDS = sodium dodecyl sulfate. Data were analyzed with Duncan's pairwise comparison. Different letters mark statistically significant differences (*P* = 0.05).

^z For penetration rate measurements, means ± standard errors were calculated from three independent replicates, with at least 500 mature appressoria examined for each replicate.

We also introduced the *MoGTII*-GFP fusion construct into the *mps1* and *pmk1* mutants. In the resulting transformants, GFP signals were significantly weaker in the nucleus in conidia and appressoria in the *mps1* background than the wild type (Fig. 4B). In contrast, the strength of GFP signals was not obviously affected in transformants of the *pmk1* mutant expressing the *MoGTII*-GFP fusion (Fig. 4B).

Fig. 1. Appressorium formation and turgor pressure assays. **A**, Appressoria formed by the wild-type Guy11 (WT) and *Mogti1* mutant strains on hydropobic surfaces after incubation for 24 h. Bar = 10 μm. **B**, Percentage of germ tubes that formed appressoria at 6, 12, and 24 h. **C**, Percentage of appressoria that underwent cytorrhysis when treated with 25, 30, 35, and 40% (wt/vol) PEG-8000. Mean and standard errors were calculated from three independent replicates with at least 100 mature appressoria examined for each replicate. No significant difference between the WT and *Mogti1* mutant was identified by Wilcoxon rank-sum test. **D**, Onion epidermal strips inoculated with conidia of the WT and the *mst12* and *Mogti1* mutants were examined by differential interference contrast and epifluorescence microscopy incubation for 24 h. Papilla formation was marked with arrows.

MPS1 is involved in the cell wall integrity pathway (Xu et al. 1998). To determine whether the *Mogti1* mutant also has defects in cell wall integrity, we assayed the growth rate of the *Mogti1* mutant on CM containing 0.01% sodium dodecyl sulfate (SDS). The growth rate of the *Mogti1* mutant was more significantly reduced than that of the wild type in the presence of SDS (Fig. 4C). In addition, the *Mogti1* mutant was hypersensitive to cell-wall-degrading enzymes, because the *Mogti1* mutant released a large amount of spheroplasts when vegetative hyphae were treated with lysing enzymes at 5 mg/ml for 30 min (Fig. 4D). These results indicated that *MoGti1*, like *MPS1*, plays a role in cell wall integrity in *M. oryzae*.

Fig. 2. Distinct morphology of invasive hyphae formed by the *Mogti1* mutant. **A**, Invasive hyphae developed by the wild-type Guy11 (WT) and *Mogti1* mutant strains inside barley epidermal cells at 36 h postinoculation (hpi). **B**, Invasive hyphae of the WT and *Mogti1* mutant in epidermal cells of rice leaf sheaths at 36 hpi. Bar = 10 μ m.

Fig. 3. Subcellular localization of MoGti1-green fluorescent protein (GFP) and MoGti1^{ANLS}-GFP. **A**, Conidia and appressoria of the *MoGti1*-GFP transformant were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by differential interference contrast and epifluorescence microscopy with the GFP- or DAPI-specific filter. Merged images showed that MoGti1-GFP localized to the nucleus. Bar = 10 μ m. **B**, Appressoria of the *MoGti1*^{ANLS}-GFP transformant showing that Gti1^{ANLS}-GFP still localized to the nucleus. Bar = 10 μ m. **C**, Rice leaves inoculated with conidia of the *MoGti1*- and *MoGti1*^{ANLS}-GFP transformants were examined 7 days postinoculation.

Although it lacks any consensus MAPK phosphorylation sites (Reynolds et al. 2000), MoGti1 contains a putative MAPK docking site (RRHSTHMTLPI). To determine its function, we generated the *MoGti1*^{ADOCK}-GFP construct and transformed it into the *Mogti1* mutant. The resulting transformants had similar phenotypes and subcellular localization of GFP fusion proteins with the complemented *MoGti1*-GFP transformants (Fig. 4E). Therefore, deletion of this putative MAPK docking site had no obvious effect on *MoGti1* function. It is likely that MoGti1 is not directly phosphorylated by Mps1 or a direct target of Mps1.

The conserved protein kinase A activation site in MoGti1 is not essential for its transcription activity.

Based on CLUSTALW2 alignment analysis, MoGti1 contains a putative protein kinase A (PKA) phosphorylation site (T71) (Fig. 5A) that is conserved in its orthologs in other fungi (Huang et al. 2006; Jonkers et al. 2012). To determine the role of the PKA phosphorylation site in MoGti1 activation, we generated the *MoGti1*^{T71A}-GFP construct by changing the T71 residue to A and transformed this construct into the *Mogti1*

Fig. 4. Assays for the expression and activation of MoGti1. **A**, Abundance of *MoGti1* transcripts was assayed by quantitative reverse-transcription polymerase chain reaction with RNA isolated from 2-day-old complete medium (CM) cultures of the wild-type strain Guy11 (WT) and the *pmk1* or *mps1* deletion mutant. Relative expression of *MoGti1* in the WT was arbitrarily set to 1. A one-way analysis of variance with a post hoc Bonferroni test was performed. Error bars represent standard deviation of three biological repeats. The asterisk marks a significant difference at $P = 0.05$. **B**, Conidia of the *MoGti1*-green fluorescent protein (GFP) transformants of the *pmk1* and *mps1* mutants were examined by epifluorescence microscopy. Bar = 10 μ m. **C**, Seven-day-old CM cultures of the WT and *womor1* mutant with 0.01% sodium dodecyl sulfate (SDS). **D**, Vegetative hyphae of the WT and *Mogti1* mutant harvested from 2-day-old 5 $\times$ YEG (yeast extract, glucose) cultures were photographed after treatment with cell wall lytic enzyme at 5 mg/ml for 30 min. The *Mogti1* mutant had increased sensitivity to cell-wall-degrading enzymes. **E**, Appressoria formed by the *MoGti1*^{ADOCK}-GFP transformant WM4 still expressed GFP signals in the nucleus. Bar = 10 μ m.

mutant. In the resulting transformants, GFP signals were still mainly observed in the nucleus (Fig. 5B). No obvious differences were observed in signal strength between the *MoGtiI*- and *MoGtiI*^{T71A}-GFP transformants, suggesting that the T71A mutation had no effect on the expression and localization of MoGtiI.

In comparison with the original *MogtiI* mutant, conidiation and appressorium formation in all three *MogtiI*/*MoGtiI*^{T71A}-GFP transformants were increased (Fig. 5C; Table 3). In infection assays, the *MoGtiI*^{T71A}-GFP transformants caused lesions on rice leaves (Fig. 5D). However, all of these *MoGtiI*^{T71A} transformants were still reduced in conidiation, appressorium formation, and virulence when compared with the wild type, indicating that expression of *MoGtiI*^{T71A} only partially restored the defects of the *MogtiI* mutant. Because *CPKA*, the major catalytic subunit of PKA, is involved in surface recognition and plant infection (Kang et al. 1999; Mitchell and Dean 1995; Xu et al. 1997), we generated the MoGtiI-NYFP and CpkA-CYFP

fusion constructs and assayed their interactions by bimolecular fluorescence complementation (BiFC) assays. In transformants of Guy11 expressing these two constructs, yellow fluorescent protein (YFP) signals were not observed in conidia, appressoria, or invasive hyphae (Supplementary Fig. S5), indicating that Gti1 and CpkA may not directly interact with each other in vivo. Therefore, it is unlikely that MoGtiI is a direct substrate of CpkA and T71 is a PKA phosphorylation site.

The S77 CDK phosphorylation site is specifically required for *MoGtiI* during plant infection.

MoGtiI also has a putative CDK phosphorylation site (S⁷⁷PSR) (Holmes and Solomon 1996) that is conserved among its orthologs in Ascomycetes (Fig. 5A). To determine whether this CDK phosphorylation site contributes to the *MoGtiI* function, we generated the *MoGtiI*^{S77A}-GFP mutant allele and transformed it into the *MogtiI* mutant. Surprisingly, even though conidiation was rescued to the wild-type level in the resulting transformant WS5, and MoGtiI^{S77A}-GFP still localized to the nucleus (Fig. 5B), it failed to cause typical lesions on intact rice leaves (Fig. 5D). Therefore, this putative CDK phosphorylation site must be important for *MoGtiI* function during invasive growth, although it is dispensable for conidiation and appressorium formation.

To further determine the function of S77, we transformed *MoGtiI*-GFP and *MoGtiI*^{S77A}-GFP into the wild-type strain Guy11. We observed a dynamic localization pattern of MoGtiI^{S77A}-GFP in appressoria and invasive hyphae in the resulting transformants WGC4 and WGS9. MoGtiI^{S77A}-GFP localized to the nucleus at 12 hpi but fluorescence signals became weaker after 12 hpi. At 24 hpi, no MoGtiI^{S77A}-GFP fluorescence was observed in appressoria (Fig. 6A). Under the same conditions, GFP signals were still visible in the *MoGtiI*-GFP transformant (Fig. 6A).

In invasive hyphae formed by the *MoGtiI*^{S77A}-GFP transformants, we failed to observe GFP signals in the nucleus (Fig. 6B). Under the same experimental conditions, strong GFP signals were observed in the nucleus in invasive hyphae formed by the *MoGtiI*-GFP transformants (Fig. 6B), indicating that the CDK phosphorylation of MoGtiI may be specifically required for late stages of appressorium morphogenesis and invasive hyphae formation. Phosphorylation by CDK may activate MoGtiI and affect its localization to the nucleus during appressorium penetration and invasive growth.

MoGtiI regulates the expression of certain effector genes during plant infection.

To determine whether *MoGtiI* regulates the expression of effector genes in *M. oryzae*, we assayed the expression of the *BAS1*, *BAS2*, *BAS4*, *BAS107*, *AvrPita*, *AvrPIZ*, *PWL2*, *SLP1*,

Fig. 5. Site-directed mutagenesis of the putative protein kinase A (PKA) and cyclin-dependent kinase (CDK) phosphorylation sites of MoGtiI. **A**, Sequence alignment of the R loop of MoGtiI and its orthologs from *Fusarium graminearum* (FgFgp1), *Botrytis cinerea* (BcReg1), *Cladosporium fulvum* (CfWor1), *Schizosaccharomyces pombe* (SpGti1), and *Candida albicans* (CaWor1). Identical and similar amino acid residues are shaded in black and gray, respectively. Residues known to be important for the transcription activity of Wor1 in *C. albicans* are marked with asterisks. **B**, Conidia and appressoria of the *MoGtiI*^{T71A}-green fluorescent protein (GFP) (WA2) and *MoGtiI*^{S77A}-GFP (WS5) transformants were examined by differential interference contrast and epifluorescence microscopy. Neither T71A nor S77A had obvious effects on the localization of the fusion proteins to the nucleus. Bar = 10 μ m. **C**, Conidiation and appressorium formation assays with the *MogtiI* mutant and transformants WA2 and WS5. Conidiation was assayed with 10-day-old oatmeal agar cultures, and appressorium formation was examined on hydrophobic plastic coverslips for 12 h. Different letters mark statistically significant differences ($P = 0.05$). **D**, Rice leaves spray inoculated with conidia of Guy11 (WT), WA2 (*MoGtiI*^{T71A}-GFP), WS5 (*MoGtiI*^{S77A}-GFP), and *MogtiI* mutant were examined 7 days postinoculation.

Table 3. Conidiation and appressorium formation in the *MogtiI*/*MoGtiI*^{T71A}-GFP transformants^y

Strain	Conidiation (×10 ⁵ conidia/plate)	Appressorium formation (%) ^z	Virulence (lesions/5 cm of leaf tip)
Guy11 (WT)	110 ± 19 ^A	98.3 ± 1.2 ^A	24.3 ± 4.2 ^A
MW1 (<i>MogtiI</i>)	11 ± 3 ^C	44.0 ± 3.6 ^C	0.0 ± 0.0 ^C
WA2	63 ± 8 ^B	79.0 ± 4.0 ^B	3.3 ± 0.6 ^B
WA12	57 ± 6 ^B	66.0 ± 6.6 ^B	2.7 ± 0.6 ^B
WA20	61 ± 11 ^B	68.0 ± 10.8 ^B	3.3 ± 0.6 ^B

^y Means ± standard errors were estimated from at least three independent experiments. Data were analyzed with Duncan's pairwise comparison. Different letters mark statistically significant differences ($P = 0.05$).

^z Percentage of germ tubes that formed appressoria on the hydrophobic side of GelBond membranes.

and *MC69* effector genes (Giraldo and Valent 2013). In qRT-PCR assays with RNA isolated from barley leaves harvested at 48 hpi, the expression levels of *BAS1*, *BAS4*, *AVR-Pita*, and *PWL2* were reduced 83-, 33-, 30-, and 90-fold, respectively, in the *Mogti1* mutant in comparison with the wild type (Fig. 7A). The expression of *BAS107* was not detectable in the *Mogti1* mutant but it was expressed in Guy11. In contrast, *MoGTII* showed negative regulation of *BAS2* and *SLP1* because their expression levels increased nine- and threefold, respectively, in *Mogti1*. The expression of *AVR-Piz* and *MC69* was not affected by deletion of *MoGTII* (Fig. 7A).

To assay the expression and translation of effectors in invasive hyphae, we generated the *BAS1*-GFP and *BAS4*-GFP constructs and transformed them into Guy11 and the *Mogti1* mutant individually. Invasive hyphae formed inside epidermal cells of barley leaves at 48 hpi were examined as described (Kong et al. 2012). In the transformants of Guy11 (wild type), Bas1-GFP was observed in the BIC structures and Bas4-GFP accumulated in the EIHM outlining the IH (Fig. 7B). In the transformants of the *Mogti1* mutant, successful penetration and invasive hyphae were rarely observed. However, GFP signals were never observed in *BAS1*-GFP or *BAS4*-GFP transformants (Fig. 7B). These results suggested that the transcription or translation of *BAS1* and *BAS4* is directly or indirectly regulated by *MoGTII* during invasive growth.

DISCUSSION

Wor1/Gti1-like proteins are transcriptional regulators with the conserved WOPR box (Lohse et al. 2010) that are involved in morphological switches in yeast species (Cain et al. 2012;

Lohse et al. 2010). Recent studies in several plant-pathogenic fungi showed that *WOR1/GTI1* orthologs are involved in virulence by regulating the expression of effector genes and phytoxin production during infection (Jonkers et al. 2012; Michielse et al. 2011; Santhanam and Thomma 2013). In *M. oryzae*, deletion of *MoGTII* resulted in a more dramatic phenotype than other fungi, including conidiation, resistance to environment stress, and loss of pathogenicity (Chen et al. 2014). In addition to the phenotypes reported by Chen and colleagues (2014), we showed that appressorium turgor generation was not affected in the *Mogti1* mutant. However, deletion of *MoGTII* affected penetration peg formation and the differentiation of invasive hyphae, which may be responsible for the loss of pathogenicity and account for the failure of *Mogti1* to infect through wounds.

In *M. oryzae*, penetration peg formation and development of primary infectious hyphae involve the switching from apical growth to the polarized growth, which is also involved in the actual penetration process to enter plant cells (Giraldo et al. 2013). Even for the rare events of successful penetration, invasive hyphae formed by the *Mogti1* mutant in plant cells had restricted growth and were morphologically different from typical bulbous invasive hyphae. These results suggest that MoGti1 performs the conserved role of Wor1/Gti1 orthologs in morphological or developmental switching in *M. oryzae*.

Fig. 6. Expression of *MoGTII* and *MoGTII*^{S77A} during appressorium formation and invasive growth. **A**, Appressoria formed on hydrophobic surfaces by the *MoGTII*-green fluorescent protein (GFP) and *MoGTII*^{S77A}-GFP transformants of the wild-type strain after incubation for 12 or 24 h were examined by differential interference contrast and epifluorescence microscopy. Bar = 10 µm. **B**, Invasive hyphae formed by the *MoGTII*-GFP and *MoGTII*^{S77A}-GFP transformants in barley epidermal cells at 48 h postinoculation were examined for GFP signals. Bar = 10 µm.

Fig. 7. Assays for the expression of selected effector genes in the *Mogti1* mutant. **A**, Expression levels of marked effector genes were assayed by quantitative reverse-transcription polymerase chain reaction with RNA isolated from barley leaves inoculated with the wild-type Guy11 strain (WT) and *Mogti1* mutant and harvested at 48 h postinoculation. **B**, Invasive hyphae formed by the *BAS1*-green fluorescent protein (GFP) and *BAS4*-GFP transformants of the WT or *Mogti1* mutant inside epidermal cells of barley were examined for the expression and localization of GFP fusion proteins. Whereas, in the WT, Bas1-GFP and Bas4-GFP localized to the BIC and apoplasts, respectively, no GFP signals of these two fusion proteins were observed in the *Mogti1* background. Bar = 10 µm.

Deletion of *MoGTII* may block the formation of penetration pegs and differentiation and growth of normal invasive hyphae.

In *M. oryzae*, two MAPK genes, *PMK1* and *MPS1*, have been implicated in regulating plant infection processes (Mehrabani et al. 2008). In the previous study, deletion of *PMK1* was found to affect the localization of MoGti1 in conidia (Chen et al. 2014). However, we failed to detect dispersed GFP signals of MoGti1 in the *pmk1* mutant with conidia harvested from 10-day-old oatmeal agar (OTA) cultures incubated on constant illumination. The localization of MoGti1-GFP to the nucleus was not affected by deletion of *PMK1* in our mutant strain (Xu and Hamer 1996). It is possible that different culture conditions or different strains are responsible for this discrepancy. In the previous study (Chen et al. 2014), conidia were harvested from straw decoction and corn plates of a different *Mopmk1/MoGTII*-GFP transformant cultured under the dark-light switching conditions. Nevertheless, we failed to observe dispersed localization of MoGti1-GFP in repeated examinations of the *pmk1 MoGTII*-GFP transformants. The *Mogti1* mutant was normal in appressorium formation, a differentiation process regulated by *Pmk1*. In addition, our data showed that *MPS1* but not *PMK1* is important in regulating the expression of *MoGTII* and the localization of MoGti1 to the nucleus. The *Mogti1* mutant and the *mps1* mutant also had similar phenotypes. Like the *mps1* mutant, the *Mogti1* mutant has defects in cell wall integrity. Both the *mps1* and *Mogti1* mutants formed melanized appressoria but failed to infect living plant cells (Chen et al. 2014; Xu et al. 1998). Nevertheless, the deletion of the putative MAPK-docking site had no effect on the localization and function of *MoGTII*. The MoGti1 protein lacks a predictable or conserved MAPK phosphorylation site. Therefore, the regulation of its expression and activation of MoGti1 by *MPS1* needs to be further characterized.

In *Candida albicans*, activation of Wor1/Gti1 involves its phosphorylation by Tpk2, a catalytic subunit of the PKA (Huang et al. 2006). In *M. oryzae*, CpkA, an ortholog of Tpk2, is also known to regulate growth, differentiation, appressoria formation, and pathogenesis (Xu et al. 1997). *MoGTII* possesses a putative PKA phosphorylation site T71 that is conserved among its fungal orthologs. However, the T71A mutation had no effect on the localization of MoGti1 to the nucleus, and *MoGTII*^{T71A} still partially suppressed the defects of *Mogti1* in conidiation and plant infection, indicating that this putative PKA site is not essential for its activation and function. Furthermore, we failed to detect an interaction between CpkA and MoGti1 by BiFC assays. In *F. oxysporum*, the putative PKA phosphorylation site is essential for the activation and function of Sge1 (Michielse et al. 2009), which is different from our observation. However, recent studies in *C. albicans* have indicated that the putative PKA site in the conserved KRWTD motif is part of the R loop (Fig. 5A) that is important for DNA binding (Zhang et al. 2014). Several residues in the R loop are highly conserved among the MoGti1 orthologs in other fungi. In *C. albicans*, seven residues of this loop, including R65, W66, T67, D68, W72, S75, and R76 (Fig. 5A), are known to be important for the function of Wor1/Gti1 in transcriptional activation (Zhang et al. 2014). Moreover, the putative T67D or T67E dominant active mutation failed to activate CaWor1 and initiate the white to opaque switching in *C. albicans* (Zhang et al. 2014). Therefore, although it is important for the transcriptional activity of MoGti1, T71 is unlikely the PKA phosphorylation site in *M. oryzae*.

Interestingly, we found that the putative S77 CDK phosphorylation site of MoGti1 is important for its function during invasive growth but dispensable for vegetative growth. In *C. albicans*, mutation at the same site had no significant effect on DNA binding and its proper functions in the activation of

white to opaque switching (Zhang et al. 2014). Appressoria formed by the *MoGTII*^{S77A} transformant were melanized but they were defective in penetrating and infecting rice leaves. The S77A mutation had stage-specific effects on the localization and activation of MoGti1 during late stages of appressorium morphogenesis and invasive growth. In *M. oryzae*, the later stages of appressorium formation include the formation of appressorium pores and septin rings at the bottom and initiation of penetration pegs (Dagdas et al. 2012), and invasive growth is similar to pseudohyphal growth (Saunders et al. 2010). This infection-related morphogenesis in *M. oryzae* likely involves changes in cell cycle regulation (Saunders et al. 2010). Mitotic entry is considered to be sufficient to initiate appressorium maturation but exit from mitosis is required only for plant infection (Saunders et al. 2010). In *U. maydis*, cell cycle regulation has been shown to be tightly regulated during plant infection (Castanheira et al. 2014). Recently, we found that the wheat scab fungus *F. graminearum* has two *CDC2* kinase genes. Although they have overlapping functions in vegetative growth, only Cdc2A is required for invasive growth and sexual reproduction (Liu et al. 2015). Invasive hyphae with distinct morphology from vegetative hyphae may differ from the latter in cell cycle regulation in *M. oryzae* and other hemibiotrophic pathogens. Our data suggested that *MoGTII* functions as the stage-specific target of CDK to regulate the cell-cycle-dependent morphological switches during plant infection. Considering that the function of this CDK phosphorylation site is not involved in cell-cycle-mediated morphological switches in *C. albicans*, it is likely that CDK phosphorylation of Wor1/Gti1 orthologs may have species-specific functions.

In several plant-pathogenic fungi, *WOR1/GTI1* orthologs have been implicated in the regulation of putative effector genes such as the *SIX* genes in *F. oxysporum* (Jonkers et al. 2012; Santhanam and Thomma 2013). In *M. oryzae*, deletion of *MoGTII1* also resulted in the upregulation of two and downregulation of five effector genes during invasive growth. However, two effector genes, *AvrPiz* and *MC69*, were not affected in the *Mogti1* mutant. Therefore, it is likely that *MoGTII* is involved in the regulation of some but not all the *M. oryzae* effector genes. In *C. albicans*, a 14-nucleotide-pair motif (WVHWDTARRSTTT) has been described as the Wor1/Gti1 recognition sequence (Lohse et al. 2010). Wor1/Gti1-like proteins in yeast species recognize the same DNA sequence but control different set of genes (Cain et al. 2012). Nevertheless, similar Wor1/Gti1-binding motifs were not identified or conserved in genes regulated by the Wor1/Gti1 orthologs in plant-pathogenic fungi (Jonkers et al. 2012; Michielse et al. 2011). In *M. oryzae*, MEME (available online) analyses with the 1,000-bp upstream regions of the effector genes regulated by *MoGTII1* failed to identify similar *cis*-elements. Therefore, although the WOPR DNA binding domain is conserved among all the fungal species, its binding motif in the promoters of its target genes may have undergone considerable diversification in different species.

MATERIALS AND METHODS

Strains and culture conditions.

The wild-type strain Guy11 (*MAT1-2*) and all transformants generated in this study (Table 1) were cultured on OTA plates at 25°C and stored on desiccated Whatman number 1 filter paper at -20°C (Park et al. 2002). Protoplast preparation and polyethylene glycol-mediated transformation were performed as described (Park et al. 2006). Spheroplast release and cell-wall degradation were examined with a Nikon E800 light microscope after treatment with lysing enzymes from *Trichoderma harzianum* (Sigma) at 5 mg/ml and 30°C. Transformants were selected on regeneration medium with hygromycin B (CalBiochem)

at 250 µg/ml, geneticin G418 (Sigma) at 250 µg/ml, or zeocin (Invitrogen) at 200 µg/ml. Growth rate and conidiation were assayed with OTA cultures, as described (Li et al. 2004; Park et al. 2006). Defects in stress responses were assayed on CM with 0.01% SDS or 5 mM H₂O₂, as described (Guo et al. 2011).

Generation of the *Mogtil* mutant.

The double-joint PCR method (Yu et al. 2004) was used to generate the *MoGTII* gene replacement vector. Flanking sequences of *MoGTII*, 1.2 kb upstream and 1.2 kb downstream, were amplified with primer pairs WOR1-1F/WOR1-2R and WOR1-3F/ WOR1-4R, respectively. The *hph* cassette was amplified with primers G418/F and G418/R from pBC1003. The resulting products of double-joint PCR were transformed into protoplasts of Guy11. Putative *gti1* deletion mutants were screened by PCR and confirmed by Southern blot analysis. Genomic DNA was extracted from mycelia by the cetyltrimethylammonium bromide protocol (Xu and Hamer 1996). Standard molecular biology procedures were followed for Southern blot analysis and enzymatic manipulations with DNA.

Appressoria formation, cytorrhysis, penetration, and plant infection assays.

Conidia were harvested from 10-day-old OTA cultures and resuspended to 5×10^4 conidia/ml in sterile distilled water. For appressorium formation assays, droplets of 50-µl conidial suspensions were placed on glass cover slips (Fisher Scientific) or GelBond membranes (Cambrex) and incubated at 25°C, as described (Zhou et al. 2012). Mature appressoria formed on GelBond membranes for 24 h were used for cytorrhysis assays, as described (Howard et al. 1991). Appressorial penetration and invasive growth were assayed with barley epidermal cells and rice leaf sheaths, as described. Over 500 appressoria were examined in penetration assays with barley epidermal cells and more than 40 infection sites were examined in both barley and rice penetration assays for invasive hyphae growth observation. For infection assays, conidia were resuspended to 5×10^4 conidia/ml in 0.25% gelatin. Two-week-old seedlings of ‘CO-39’ rice were used for spray or injection infection assays, as described (Li et al. 2004; Zhou et al. 2011b). Lesion formation was examined 7 days postinoculation.

Construction of the *MoGTII*-GFP, *MoGTII*^{ΔDOCK}-GFP, *MoGTII*^{T71A}-GFP, *MoGTII*^{ΔNLS}-GFP, and *MoGTII*^{S77A}-GFP fusion vectors.

All *MoGTII*-GFP fusion constructs were generated by the yeast gap repair approach (Bourett et al. 2002). To generate the *GTII*-GFP fusion construct, PCR products amplified with primers Wor1ser1F and Wor1ser4R were cotransformed with *Xho*I-digested pFL2 into XK1-25. Similar approaches were used to generate other GFP fusion vectors. Overlapping PCR-mediated methods were used to generate *MoGTII*^{ΔDOCK}, *MoGTII*^{T71A}, *MoGTII*^{ΔNLS}, and *MoGTII*^{S77A} alleles (Heckman and Pease 2007). All of the primers used in the construction of these mutant alleles are listed in Supplementary Table S1. All of the resulting fusion constructs were confirmed by sequence analysis and transformed into the *Mogtil* mutant.

BiFC assays for the *MoGti1*-Cpka interaction.

The MoGti1-NYFP fusion construct was generated by cloning the *MoGTII* fragment amplified with primers Wor1BiNT/F and Wor1BiNT/R into pHZ65 (Zhao and Xu 2007). The *CPKA* fragment was amplified with primers 6368BiCT/F and 6368BiCT/R into pHZ68 (Zhao and Xu 2007) to generate the *CPKA*-CYFP fusion construct. The resulting *MoGTII*-NYFP

and *CPKA*-CYFP fusion constructs were cotransformed into protoplasts of Guy11. Transformants resistant to both hygromycin and zeocin were isolated and confirmed by PCR and Southern blot analyses. YFP signals were examined with a Nikon E800 epifluorescence microscope. Transformant PBC2 (Table 1) expressing the *MCNA*-NYFP and *MCNB*-CYFP constructs was used as the positive control.

qRT-PCR analyses.

Total RNAs were isolated from vegetative hyphae and infected barley leaves with the TRIzol reagent (Invitrogen) (Kong et al. 2013). The resulting RNA samples were used to synthesize first-strand cDNA with the AccuScript first-strand cDNA synthesis kit (Agilent). RT-PCR was performed with the Gene MX 3000 PM (Agilent) using the RT2 Real-Time SYBR Green/ROX PCR master mixture (SABiosciences). Primers used for qRT-PCR assays are listed in Supplementary Table S1. The relative expression level of each gene was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), with the β -tubulin gene MGG_00604.6 as the internal control. Mean and standard deviation were calculated from qRT-PCR results of three independent biological replicates.

Generation of transformants expressing the *BAS1*-GFP and *BAS4*-GFP constructs.

The *BAS1*-GFP was generated by cloning the *BAS1* fragment amplified with primers Bas1F and Bas1R into the geneticin-resistance vector pFL2 (Zhou et al. 2011a) by the yeast gap repair approach (Bruno et al. 2004). Similar strategies were used to generate the *BAS4*-GFP construct by cloning the *BAS4* fragment amplified with primers Bas4F/Bas4R into pFL2. The resulting fusion constructs were confirmed by sequence analysis and individually transformed into protoplasts of Guy11 and the *Mogtil* mutant. Transformants resistant to G418 were isolated and confirmed by PCR to contain the transforming GFP fusion constructs.

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