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 Mutant was nonpathogenic, although it was normal in appressorium formation 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 MoGTI1S77A-green fluorescent protein to the nucleus in late stages of appressorium formation and 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.

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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, GTI1, 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 dahlia (VdSGE1), Cladosporium fulvum (CIWOR1), 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; Okmen et al. 2014; Santhanam and Thomma 2013). For example, FGP1 and BcREG1 regulate phytoxin 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; Okmen 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 MoGTI1 (= MoWOR1) gene. Similar to the report on the same gene published during the course of this study, MoGTI1 was found to be important for conidiation and plant infection but dispensable for appressorium formation (Chen et al. 2014). Although the Mobgt1 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 MoGTI1 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 MoGt1 appeared to be controlled by Mps1 but not Pmk1 or CpkA. Although the putative cyclin-dependent kinase (CDK) phosphorylation site of MoGt1 is dispensable during vegetative growth and appressorium formation, the S77A mutation affected penetration and invasive growth. Localization of MoGt1S77A-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 MoGt1 in maintaining cell wall integrity, conidiation, and plant infection. MoGt1 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**

MoGTI1 is dispensable for vegetative growth but essential for plant infection.

The MoGTI1 (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 Mobgt1 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 MoGTI1 is required for invasive growth.

In comparison with the wild type, the Mobgt1 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 Mobgt1 mutant formed appressoria, compared with 94% in Guy11. By 24 hpi, 97% of Guy11 germ tubes formed appressoria but only 81% of Mobgt1 formed appressoria (Fig. 1B). Although the Mobgt1 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 Mobgt1 by cytology 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 null mutant, the Mobgt1 mutant rarely elicited autofluorescence in onion epidermal cells (Fig. 1D), even after prolonged incubation up to 72 h. These results suggested that MoGTI1 plays a critical role in the formation of penetration pegs or eliciting autofluorescence.

The Mobgt1 mutant is defective in invasive hyphae growth.

In penetration assays with barley epidermal cells, appressoria formed by the Mobgt1 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 Mobgt1 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 Mobgt1 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 MoGTI1 is important for the differentiation and growth of normal bulbous pseudohyphae in M. oryzae.

MoGTI1 localizes to the nucleus.

The MoGTI1-GFP construct was generated and transformed into the Mobgt1 mutant. The resulting Mobgt1/MoGTI1-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).

MoGt1 contains a predicted nuclear localization signal sequence (NLS), PPGEKKR, at its N-terminal region. To determine its role in subcellular localization, we generated the MoGTI1NLS2-GFP construct and transformed it in to the Mobgt1 mutant. In the resulting transformant WN2, fluorescent signals
were still observed in the nucleus (Fig. 3B). Expression of MoGTI1\(^{\text{ALS}}\)-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.

**MoGTI1 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 MoGTI1 is regulated by Pmk1 and Mps1, the expression of MoGTI1 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 MoGTI1 was twofold lower in the *mps1* mutant than in Guy11. However, the expression level of MoGTI1 was similar between Guy11 and the *pmk1* mutant (Fig. 4A). Thus, it appears that MoGTI1 expression is regulated by MPS1 but not PMK1.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guy11</td>
<td>Wild type (MAT1-2)</td>
<td>(Leung et al. 1988)</td>
</tr>
<tr>
<td>nn78</td>
<td><em>pmk1</em> deletion mutant</td>
<td>(Xu and Hamer 1996)</td>
</tr>
<tr>
<td>M3H51</td>
<td><em>mps1</em> deletion mutant</td>
<td>(Xu et al. 1998)</td>
</tr>
<tr>
<td>MW1</td>
<td><em>Mogti1</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>MW4</td>
<td><em>Mogti1</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>MW12</td>
<td><em>Mogti1</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WC15</td>
<td>MoGTI1-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WN2</td>
<td>MoGTI1(^{\text{ALS}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WM4</td>
<td>MoGTI1(^{\text{DOCK}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WA2</td>
<td>MoGTI1(^{\text{T11A}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WA12</td>
<td>MoGTI1(^{\text{T11A}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WA20</td>
<td>MoGTI1(^{\text{T11A}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WS5</td>
<td>MoGTI1(^{\text{S77A}})-GFP transformant of the <em>Mogti1</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WGC4</td>
<td>MoGTI1-GFP transformant of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>WGS9</td>
<td>MoGTI1(^{\text{S77A}})-GFP transformant of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>BC12</td>
<td>MoGTI1-NYFP and CPKA-CYFP transformant of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>PBC2</td>
<td>MCNA-NYFP and MCNB-CYFP transformant of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>BSGI-5</td>
<td>BAS1-GFP transformant of Guy11</td>
<td>This study</td>
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<tr>
<td>BSG4-4</td>
<td>BAS4-GFP transformant of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>BSW1-27</td>
<td><em>Mogti1</em> BAS1-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>BSW4-4</td>
<td><em>Mogti1</em> BAS4-GFP</td>
<td>This study</td>
</tr>
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**Fig. 1.** Appressorium formation and turgor pressure assays. A, Appressoria formed by the wild-type Guy11 (WT) and *Mogti1* mutant strains on hydrophobic 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>CM</th>
<th>0.5 M NaCl</th>
<th>0.01% SDS</th>
<th>5 mM H(_2)O(_2)</th>
<th>Conidiation (×10(^5) conidia/plate)</th>
<th>Penetration rate (%) (^{#})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guy11 (WT)</td>
<td>3.8 ± 0.0a</td>
<td>2.4 ± 0.0a</td>
<td>1.5 ± 0.1a</td>
<td>2.8 ± 0.2a</td>
<td>127 ± 38a</td>
<td>86.0 ± 6.6a</td>
</tr>
<tr>
<td>MW1 (<em>Mogti1</em>)</td>
<td>2.8 ± 0.1b</td>
<td>1.5 ± 0.0b</td>
<td>0.7 ± 0.1b</td>
<td>1.5 ± 0.1b</td>
<td>13 ± 1b</td>
<td>0.8 ± 0.2b</td>
</tr>
</tbody>
</table>

\(^{\#}\) For penetration rate measurements, means ± standard errors were calculated from three independent replicates, with at least 500 mature appressoria examined for each replicate.
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.

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 MoGTI1DOCK-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 MoGTI1T71A-GFP construct by changing the T71 residue to A and transformed this construct into the Mogti1 mutant. The resulting transformants had similar phenotypes to each other with moGTI1-GFP transformants (Fig. 4F). 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.

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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 MoGTI1- and MoGTI1T71A-GFP transformants, suggesting that the T71A mutation had no effect on the expression and localization of MoGti1.

In comparison with the original Mogti1 mutant, conidiation and appressorium formation in all three Mogti1/MoGTI1T71A-GFP transformants were increased (Fig. 5C; Table 3). In infection assays, the MoGTI1T71A-GFP transformants caused lesions on rice leaves (Fig. 5D). However, all of these MoGTI1T71A transformants were still reduced in conidiation, appressorium formation, and virulence when compared with the wild type, indicating that expression of MoGTI1T71A only partially restored the defects of the Mogti1 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 MoGti1-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, appressorium, 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 MoGti1 is a direct substrate of CPKA and T71 is a PKA phosphorylation site.

The S77 CDK phosphorylation site is specifically required for MoGTI1 during plant infection.

MoGti1 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 MoGTI1 function, we generated the MoGTI1S77A-GFP mutant allele and transformed it into the Mogti1 mutant. Surprisingly, even though conidiation was rescued to the wild-type level in the resulting transformant WS5, and MoGti1S77A-GFP still localized to the nucleus (Fig. 5B), it failed to cause typical lesions in intact rice leaves (Fig. 5D). Therefore, this putative CDK phosphorylation site must be important for MoGTI1 function during invasive growth, although it is dispensable for conidiation and appressorium formation.

To further determine the function of S77, we transformed MoGTI1-GFP and MoGTI1S77A-GFP into the wild-type strain Guy11. We observed a dynamic localization pattern of MoGti1S77A-GFP in appressoria and invasive hyphae in the resulting transformants WGC4 and WGS9. MoGti1S77A-GFP localized to the nucleus at 12 hpi but fluorescence signals became weaker after 12 hpi. At 24 hpi, no MoGti1S77A-GFP fluorescence was observed in appressoria (Fig. 6A). Under the same conditions, GFP signals were still visible in the MoGTI1-GFP transformant (Fig. 6A).

In invasive hyphae formed by the MoGTI1S77A-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 MoGTI1-GFP transformants (Fig. 6B), indicating that the CDK phosphorylation of MoGti1 may be specifically required for late stages of appressorium morphogenesis and invasive hyphae formation. Phosphorylation by CDK may activate MoGti1 and affect its localization to the nucleus during appressorium penetration and invasive growth.

MoGTI1 regulates the expression of certain effector genes during plant infection.

To determine whether MoGTI1 regulates the expression of effector genes in M. oryzae, we assayed the expression of the BAS1, BAS2, BAS4, BAS107, AvrPita, AvrPiz, PWL2, SLP1,
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, MoGTI1 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 MoGTI1 (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 MoGTI1 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 phytoalexin production during infection (Jonkers et al. 2012; Michielse et al. 2011; Santhanam and Thomma 2013). In *M. oryzae*, deletion of MoGTI1 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 MoGTI1 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 MoGTI1 and MoGTI1<sup>577A</sup> during appressorium formation and invasive growth. A, Appressoria formed on hydrophobic surfaces by the MoGTI1-green fluorescent protein (GFP) and MoGTI1<sup>577A</sup>-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 MoGTI1-GFP and MoGTI1<sup>577A</sup>-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-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 MoGTI1 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 (Mehrabi 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 *Mpmk1/*MoGTI1-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 MoGTI1-GFP transformants. The Mogt1 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 MoGTI1 and the localization of MoGti1 to the nucleus. The Mogt1 mutant and the mps1 mutant also had similar phenotypes. Like the mps1 mutant, the Mogt1 mutant has defects in cell wall integrity. Both the mps1 and Mo gt1 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 MoGTI1. 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, appressorium formation, and pathogenesis (Xu et al. 1997). *MoGTI1* 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 *MoGTI1* 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 KRWD 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 *MoGTI1* 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 *MoGTI1* 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 *MoGTI1* 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 *MoGTI1* is involved in the regulation of some but not all the *M. oryzae* effector genes. In *C. albicans*, a 14-nucleotide-pair motif (WVHWDTW ARS STT) 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 *MoGTI1* 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 (*MATI-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, genetin 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 H$_2$O$_2$, as described (Guo et al. 2011).

**Generation of the Mogti1 mutant.**

The double-joint PCR method (Yu et al. 2004) was used to generate the MoGTT1 gene replacement vector. Flanking sequences of MoGTT1, 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.

**Appressora formation, cytorrhysis, penetration, and plant infection assays.**

Conidia were harvested from 10-day-old OTA cultures and resuspended to 5 × 10$^5$ 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 appressora 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 appressora 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 hyphal growth observation. For infection assays, conidia were resuspended to 5 × 10$^5$ 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 MoGTT1-GFP, MoGTT1$^{ADock}$-GFP, MoGTT1$^{T14A}$-GFP, MoGTT1$^{NL}$-GFP, and MoGTT1$^{S77A}$-GFP fusion vectors.**

All MoGTT1-GFP fusion constructs were generated by the yeast gap repair approach (Bourett et al. 2002). To generate the GTI1-GFP fusion construct, PCR products amplified with primers Wor1Ser1F and Wor1Ser4R were cotransformed with XhoI-digested pFL2 into XKL-25. Similar approaches were used to generate other GFP fusion vectors. Overlapping PCR-mediated methods were used to generate MoGTT1$^{ADock}$, MoGTT1$^{T14A}$, MoGTT1$^{NL}$, and MoGTT1$^{S77A}$ 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 Mogti1 mutant.

**BiFC assays for the MoGti1-CpKA interaction.**

The MoGti1-NYFP fusion construct was generated by cloning the MoGTT1 fragment amplified with primers Wor1BinT/F and Wor1BinR into pHZ65 (Zhao and Xu 2007). The CPKA fragment was amplified with primers 636BiCT/F and 636BiCT/R into pHZ68 (Zhao and Xu 2007) to generate the CPKA-CYFP fusion construct. The resulting MoGTT1-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 (SA Biosciences). 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 genetin-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 Mogti1 mutant. Transformants resistant to G418 were isolated and confirmed by PCR to contain the transforming GFP fusion constructs.

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**LITERATURE CITED**


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