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System-wide characterization of bZIP transcription factor proteins involved in infection-related morphogenesis of *Magnaporthe oryzae*

Wei Tang¹, Yanyan Ru¹, Li Hong¹, Qian Zhu¹, Rongfang Zuo¹, Xianxian Guo¹, Jingzhen Wang¹, Haifeng Zhang¹, Xiaobo Zheng¹, Ping Wang², and Zhengguang Zhang^{1,*}

¹Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, and Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Nanjing 210095, China

²Department of Pediatrics, and the Research Institute for Children, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112 USA

Abstract

The basic-leucine zipper (bZIP) domain-containing transcription factors (TFs) function as key regulators of cellular growth and differentiation in eukaryotic organisms including fungi. We have previously identified MoAp1 and MoAtf1 as bZIP TFs in *Magnaporthe oryzae* and demonstrated that they regulate the oxidative stress response and are critical in conidiogenesis and pathogenicity. Studies of bZIP proteins could provide a novel strategy for controlling rice blast, but a systematic examination of the bZIP proteins has not been carried out. Here, we identified 19 additional bZIP TFs and characterized their functions. We found that the majority of these TFs exhibit active functions, most notably, in conidiogenesis. We showed that MoHac1 regulates the endoplasmic reticulum (ER)-stress response through a conserved unfolded protein response (UPR) pathway, MoMetR controls amino acid metabolism to govern growth and differentiation, and MoBzip10 governs appressorium function and invasive hyphal growth. Moreover, MoBzip5 participates in appressorium formation through a pathway distinct from that MoBzip10, and MoMeaB appears to exert a regulatory role through nutrient uptake and nitrogen utilization. Collectively, our results provide insights into shared and specific functions associated with each of these TFs and link the regulatory roles to the fungal growth, conidiation, appressorium formation, host penetration, and pathogenicity.

Introduction

Magnaporthe oryzae is the causal agent of rice blast, the most destructive disease of cultivated rice worldwide. Consequently, *M. oryzae* is becoming one of the important species for studies of plant-pathogen interactions because of its economical significance. The fungus produces three-celled pyriform conidia that can spread through wind, rainwater, or human activities. To cause infection, conidia attach to rice leaves produce germ tubes that

*Corresponding author: Zhengguang Zhang, Dept. of Plant Pathology, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, China, Tel: 86-25-84396972, Fax: 86-25-84395325, zhgzhang@njau.edu.cn.

can form dome-shaped infection cells called appressoria (Talbot, 2003; Ebbole, 2007). Accumulation of osmolytes such as glycerol in the mature appressoria results in generation of enormous turgor pressure (8 MPa) that allows the fungus to penetrate into and colonize the host (Howard et al., 1991; DeJong, 1997; Talbot, 2003). Hundred of thousands of conidia are produced on the lesions and then released to initiate new disease cycles on new plant tissues within 3–5 days.

Cellular growth and differentiation are developmental processes that are tightly regulated by transcription factors (TFs), whose characteristics, including specific DNA binding, transcriptional activation or inhibition, the presence/absence of a nuclear localization signal, interactions with other TFs or molecular chaperones, and posttranslational modifications, are all important for the transcriptional regulation of specific target genes (Schwechheimer and Bevan, 1998). TFs are generally classified in structural categories based on conserved DNA-binding domains. These include bZIP proteins, MYB-like proteins, MADS-box proteins, helix-loop-helix proteins, zinc-finger proteins, and homeobox proteins (Pabo and Sauer, 1992; Meshi and Iwabuchi, 1995).

The bZIP proteins that contain a basic region that mediates sequence-specific DNA binding and a leucine-zipper region required for dimerization are one of the most widely distributed and conserved TF classes in eukaryotes. The plant *Arabidopsis thaliana* genome contains 75 distinct members of the bZIP family that regulate pathogen defense, light and stress signaling, seed maturation, and flower development (Jakoby et al., 2002). The rice *Oryza sativa* genome contains 89 bZIP TF-encoding genes (Nijhawan et al., 2008), and 14 were found to function in ABA-mediated signaling (Nakagawa et al., 1996; Hobo et al., 1999), cold sensing (Aguan et al., 1993; Shimizu et al., 2005), and symptom development of the tungro disease (Yin et al., 1997; Dai et al., 2003; Dai et al., 2004). In fungi such as *Aspergillus nidulans* and *A. fumigatus*, the bZIP TF FlbB protein is required for proper asexual development (Ettxebeste et al., 2008; Xiao et al., 2010). In addition, the AnMetR (*A. nidulans*) and NcCyc3 (*Neurospora crassa*) proteins activate the expression of various sulfur-related genes and contribute to the regulation of sulfur metabolism (Fu et al., 1989; Natorff et al., 2003). Moreover, *Aspergillus* NapA and AfYap1 have a general role in mediating the oxidative stress response (Asano et al., 2007; Lessing et al., 2007). Thus, members of the bZIP TF family also play a diverse variety of regulatory roles in fungi.

Recent studies have showed that several TFs including three homeobox TFs (MoHox2, MoHox4, and MoHox6), two zinc finger TFs (MoCos1, MoCon7), and one putative TF (MoCom1) are all important in conidiogenesis of *M. oryzae* (Odenbach et al., 2007; Kim et al., 2009; Zhou et al., 2009; Yang et al., 2010). The MADS-box TFs MoMcm1 and MoMst12 are important in appressorium formation and infectious hyphae growth (Park et al., 2002; Zhou et al., 2011), whereas the C₂H₂ TF MoCrz1 is essential for growth and full pathogenicity (Zhang et al., 2009). Loss of the APSES family TF MoSwi6, which functions as a target of the MoMps1-mediated signaling pathway, caused a reduction in appressorial turgor pressure and pathogenicity (Qi et al., 2012).

We have previously showed that MoAp1 and MoAtf1 mediate the oxidative stress response and are required for pathogenicity (Guo et al., 2010; Guo et al., 2011). We found that

MoAp1 targets MoGti1 and MoPac2, the paralogs of the fungal Gti1/Pac2 family proteins, to regulate hyphal growth, conidiation, stress responses, and pathogenicity (Chen et al., 2013). The findings of important and specialized functions by MoAp1 and other TFs prompted us to explore additional bZIP family proteins in a system-wide scale. We here report the identification and characterization of additional 19 bZIP TFs from *M. oryzae* based on the whole genomic sequence. We found that most of proteins are functional. Particularly, MoHac1, MoMetR, and MoBzip10 are required for growth, conidiogenesis, and pathogenicity. MoBzip5 is required for appressorium formation, while MoBzip12 is essential for conidium morphology. We also provided evidence indicating that MoHac1 and MoMetR respectively play important roles in the unfolded protein response (UPR) pathway and methionine biosynthesis. Our findings help to discern the specific functions associated with each of the bZIP TFs and link the regulation of these proteins to the pathogenicity of *M. oryzae*.

Results

Identification of the bZIP domain containing proteins and mutants generation in *M. oryzae*

Previous characterization of MoAp1 and MoAtf1 illustrated the importance of bZIP TFs in the growth and virulence of *M. oryzae* (Guo et al., 2010; Guo et al., 2011). To identify additional bZIP proteins, we carried out a BLAST search of the *M. oryzae* genome (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/Home.html) for proteins containing the bZIP domain. Twenty-one candidates, including MoAp1 and MoAtf1, were found. Based on protein sequences of the bZIP domains, phylogenetic relationship of the bZIP candidates with those found in other fungi such as *A. nidulans* (17 proteins), *Botrytis cinerea* (13 proteins), *Stagonospora nodorum* (13 proteins), *Saccharomyce cerevisiae* (12 proteins), *Fusarium graminearum* (21 proteins), and *N. crassa* (13 proteins) is presented in Fig. 1. According to the bootstrap values (more than 60%), we generally diverged them into twenty separate clades. The colored branch indicates the different clade and the black represent for unclassified clade (Fig. 1). The phylogenetic dendrogram shows that, for most cases, *M. oryzae* bZIP proteins are evenly distributed among these clades, implying that genes in the clade may have evolved from a copy of their common fungal ancestor. MoBzip3 is highly homologous with MoBzip6 in a clade; moreover, MoBzip4 and MoBzip9 show low similarity with other proteins in the fungus, suggesting that these genes may be specific to *M. oryzae*.

Expression patterns of *MoBZIP* genes at different developmental stages

To gain insight into the potential functions of these *MoBZIP* genes, we examined the gene transcription profiles at different developmental stages by quantitative real-time PCR (qRT-PCR). In the conidium stage, *MoHAC1*, *MoMETR*, *MoBZIP5*, *MoBZIP8*, *MoBZIP11*, *MoFCR3* and *MoIDI4* showed higher transcription levels than those of the mycelial stage (range from 11.32 to 1.09 fold). In the infection stage, the expression of most *MoBZIP* genes showed low transcription levels, except for *MoHAC1* (highest level in 2.44 fold at 8 h infection stage) (Table S1). These observations, while helping us to define the potential functions of *MoBZIP* genes, suggested that *MoBZIP* genes may play potentially various roles at different developmental stages.

To characterize the functions of *MoBZIP* genes of *M. oryzae*, constructs for gene replacement were generated and transformed into either the wild-type (WT) Guy11 strain or a Ku80 derivative strain allowing easy mutant generation. At least two independent null mutants with identical phenotypes were obtained for each *MoBZIP* gene, and all deletion mutants were confirmed by polymerase chain reaction (PCR) and Southern blot analysis using a gene-specific probe and sets of PCR primers (Fig. S1, Table S2). None of *Mogcn4* was obtained as *MoGCN4* disruption resulted in a near-lethal effect. In addition, *Mohac1*, *MometR*, *Mobzip10*, and *Mobzip12* were complemented with their respective wild type alleles.

We also examined the impact of individual *MoBZIP* gene deletion on expressions of various other *MoBZIP* genes. Deletion of each *MoBZIP* gene resulted in an alternative effect on the expression of other *MoBZIP* TFs (Table S3). For example, all *MoBZIP* candidate genes were down-regulated in the *Mohac1* and *MometR* mutants but up-regulated in the *Mobzip1* and *Mobzip2* mutants. Disruption of one TF gene resulted in pleiotropic effect on the expression of other TF genes. This suggests that each MoBzip TF did not function in isolation but rather cooperation, and the phenomenon controlled by each single *MoBZIP* gene was not a linear sequence of discrete events, but a result of cross-talk between various pathways.

Characterization of vegetative growth

All of the knockout mutants (two independent mutants each) were examined for phenotypic changes in vegetative growth, conidiation, conidium germination, appressorium formation and penetration. As summarized in Table 1, the deletion mutants of each *MoBZIP* gene displayed distinctive effects on vegetative growth, conidial morphology, conidiation, appressorium formation, and infection-related morphogenesis.

Among 18 mutants, *Mohac1*, *Mobzip10*, and *Momeab* mutants displayed, respectively, 41%, 22%, and 22% reductions in the growth rate on complete medium (CM) (Fig. 2A and Table 1). The *MometR* mutant showed apparent reduction in aerial hyphae formation on CM and loss of vegetative growth on minimal medium (MM), straw decoction & corn (SDC) medium, and oatmeal medium (OM) (Fig. 2A). Similar results were observed when each of these mutants was cultured in liquid CM for 48 h (Fig. 2B). These results indicated that MoHac1, MoMetR, MoBzip10, and MoMeab are involved in the regulation of vegetative growth in *M. oryzae*. The growth defect shown by the *MometR* mutant was likely due to restricted amino acids in growth media.

Conidium production and germination and appressorium formation

Conidia play an important role in the disease cycle of the rice blast fungus (Lee et al., 2006). Eleven *MoBZIP* genes were found here to be involved in conidiation. The *Mohac1*, *MometR*, *Momeab*, *Mobzip5*, *Mobzip9*, *Mobzip10*, and *Mobzip12* were markedly reduced in conidiation compared to wild-type Guy11 (Table 1). For the *Mohac1* mutant, conidia were very rare with only two or three seen following examination of more than ten 10-day-old culture plates. The *MometR* mutant was unable to sporulate due to lack of

vegetative growth. The *Mobzip10* mutant showed over 95% reduction in conidiation (Fig. 3A).

On the other hand, the *Mobzip1*, *Mobzip2*, *Mobzip3*, and *Mobzip11* mutants showed increased conidia production (Fig. S2A, and Table 1). Apart from *Mobzip12*, whose conidia were abnormal, elongated, and spindly shaped, all of the other *Mobzip* mutants produced three-celled conidia with normal morphology (Fig. S3). These results suggested that most members of the *MoBZIP* TFs family regulate conidiation. Since MoHac1, MoMetR, and MoBzip10 are important in conidiogenesis, we examined the *MoCOM1*, *MoHOX2*, *MoCON2*, *MoCON7*, *MoCOS1*, and *MoSTUA* genes that were shown previously to be involved in this process (Shi and Leung, 1995; Kim et al., 2009; Nishimura et al., 2009; Zhou et al., 2009; Yang et al., 2010). The expression of *MoCOM1* and *MoCOS1* was significantly reduced in all three mutants, while the expression of the other genes tended to be different with each individual mutant (Fig. 3B). This result indicated that MoHac1, MoMetR, and MoBzip10 govern the expression of *MoCOM1* and *MoCOS1*, although it is not known if the regulation is through a common mechanism or independent ones.

None of the 16 *MoBZIP* mutants that produced conidia was blocked in conidium germination and most did not show any changes in appressorium formation. However, the *Mobzip10* mutant displayed a lower percentage of appressorium formation compared to wild-type and the other *Mobzip* mutants (Table 1), and the *Mobzip5* mutant produced two appressoria ($20\% \pm 1.5$) either on branched germ tubes or on two germ tubes that emerged from one conidial cell after an extended incubation on artificial inductive surfaces of more than 24 h.

Pathogenicity assay

When spraying with the same amount of conidia, the *Mobzip10* mutant was significantly reduced in virulence on rice seedlings, which was consistent with the result of the penetration test on onion epidermal cells (Fig. 4A, S2B and Table 1). Similar results of fewer lesions surrounding the infection sites were also observed when using the injection method on rice (Fig. 4B).

Due to lack of sporulation, mycelia of the *Mohac1* and *MometR* mutants were placed on detached rice leaves. No obvious disease symptoms were found five days after inoculation (Fig. 4C). However, both mutants could cause lesions when mycelia were placed on the wounded leaves (Fig. 4D). These results indicated that MoBzip10, MoHac1, and MoMetR are important for pathogenesis.

Overall, MoHac1, MoMetR, MoMeaB, MoBzip5, and MoBzip10 appear to have more of a pleiotropic role in conidiation and pathogenicity in *M. oryzae* (Table 1). We therefore subjected the *Mohac1*, *MometR*, *Mobzip10*, *Mobzip5*, and *Momeab* mutants for further studies.

MoHac1 shares a conserved functional mechanism with *S. cerevisiae* ScHac1

Fungal Hac1 proteins are transcription activators involved in the endoplasmic reticulum (ER)-stress response that is mediated by the unfolded protein response (UPR) signaling pathway.

In *S. cerevisiae*, the *ScHAC1* mRNA nucleotide sequence contains a 252-nt atypical intron whose removal is mediated by ScIre1, an endoribonuclease residing in the ER membrane and the primary signal transducer of UPR. The intron blocks translation due to base pairing with a sequence in the 5'-untranslated region of its mRNA. This alternative splicing reaction is a required step in the signaling pathway, allowing for the translation of ScHac1 that then regulates the expression of UPR target genes (Cox and Walter, 1996; Ron and Walter, 2007).

To examine whether *MoHAC1* has the splicing mechanism similar to *ScHAC1*, we assessed the presence of spliced and un-spliced forms of *MoHAC1* mRNA under the presence of dithiothreitol (DTT) and tunicamycin (TM) that induce the unfolded protein due to ER stress. Two bands, one unspliced (*MoHAC1^u*) and the other spliced (*MoHAC1^s*), were detected. The presence of *MoHAC1^s* was significantly more abundant than *MoHAC1^u* upon ER stress (Fig. S4A). DNA sequencing confirmed that a 23-nt fragment was absent in the spliced form in comparison to the unspliced one (Fig. 5). These findings indicated that a similar splicing mechanism exists for MoHac1.

To test if MoHac1 shares conserved functions with ScHac1, the *Schac1* mutant was transformed with *MoHAC1* and *MoHAC1^S* cDNA and the resulting transformants were tested for their sensitivity to DTT treatment. While the *Schac1* mutant was hypersensitive to DTT, the hypersensitivity was partially suppressed by the expression of *MoHAC1^S*, but not *MoHAC1*, in comparison to the strain transformed with the pYES2 vector alone and wild-type *S. cerevisiae* BY4741 strain (Fig. S5). This indicated that MoHac1 is a partial functional paralog of ScHac1. The partial suppression of hypersensitivity to ER stress suggested that *S. cerevisiae* may not effectively process the non-spliceosomal intron of the *MoHAC1* gene.

MoHac1 is involved in the endoplasmic reticulum (ER) stress response through the conserved unfolded protein response (UPR)

Since *MoHAC1* was expressed throughout all developmental stages and the *Mohac1* mutant exhibited pleiotropic defect, we explored its function in more details. To determine whether MoHac1 has a role in ER-stress response, the *Mohac1* mutants were tested for sensitivity to TM and DTT. When exposure to 1.0 mM DTT or 0.15 µg/ml TM, the *Mohac1*, *Mohac1/MoHAC1^{mut intron}* (a mutant carrying several site-directed mutations in the unconventional intron) (Fig. S6A), and *Mohac1/MoHAC1^S* (a mutant lacking the unconventional intron) (Fig. S6B) mutants heightened significantly sensitivity to DTT (an average 21.6%, 26.9% and 24.6% decrease, respectively) and TM (an average 72.8%, 57.2% and 46.1% decrease, respectively) compared to wild-type strain (Fig. 6). This data suggests that MoHac1 has a role in the ER-stress response through the conserved UPR pathway.

To characterize the functions of the *MoHAC1* gene variants, we examined the phenotypes of *Mohac1* mutants during growth, conidiation, and pathogenesis. After 7 days cultured on CM medium, the *Mohac1/MoHAC1^{mut intron}* and *Mohac1/MoHAC1^S* exhibited no apparent defect in radial growth in comparison to the *Mohac1* mutant (Fig. 7A and Fig. S7A). However, growth of the mutants with defective UPR appeared as a flat colony due to the reduced aerial hyphal growth (Fig. 7B). On SDC medium, conidium was rarely observed

after 10 days in the *Mohac1*, *Mohac1/MoHAC1^{mut intron}*, and *Mohac1/MoHAC1^S* strains compared to Guy11 (Fig. S7B). The microscopic observation result was consistent with that of the statistical analysis (Fig. 7C). As sporulation is abolished in the UPR defective mutants, we hypothesized that ER-stress may be induced to activate the UPR during the conidial stage in *M. oryzae*. We then examined the expression of *MoHAC1^u* and *MoHAC1^S* at this stage and found that the expression of *MoHAC1^u* and *MoHAC1^S* was significantly increased during the conidial stage (Fig. S4A), and the conversion of *MoHAC1^u* into *MoHAC1^S* was also intensively induced at this stage (Fig. S4B). Collectively, these data validate our hypothesis that UPR activation is required for conidiogenesis in *M. oryzae*.

Since mutant strains were not able to provide enough conidia for inoculation, the mycelium was prepared to inoculate the detached wounded and unwounded rice leaves for pathogenicity assay. After 5 days, no obvious disease symptoms caused by the mutant strains were found on surfaces in comparison with the wild-type Guy11. While, smaller extend symptoms were observed on wounded sites that inoculated with mutant strains (Fig. 7D).

To investigate possible reasons for the lost pathogenicity, we examined the infection process by inoculating barley epidermal cells with mycelia of the wild-type, *Mohac1*, *Mohac1/MoHAC1^{mut intron}*, and *Mohac1/MoHAC1^S*. At 48 h post-inoculation, all strains formed normal appressoria. The invasive hyphae of wild-type and complemented strain *Mohac1/MoHAC1* expanded freely into the host cells, but the appressoria of the *Mohac1* and *Mohac1/MoHAC1^{mut intron}* mutants failed to develop any penetration structures. The *Mohac1/MoHAC1^S* mutant partly complemented the deficiency on penetrating into cells, but the invasive hyphae growth was restricted to host cells and branching was limited (Fig. S8). Thus, ER stress appears to be induced during infection and extension of the invasive hyphae, and a constitutive UPR could only partly relieve the stress then successfully infects the cells, but not supports the subsequent invasive hyphae growth. These results suggested that a fully functional UPR is required for penetration and invasive hyphae growth in *M. oryzae*.

MoHac1 functions as a transcription factor in the UPR pathway

To examine whether MoHac1 is a transcription factor under ER stress, the transcript levels of putative UPR-regulated genes were measured by qRT-PCR. Expression of all of the genes detected was markedly increased in wild-type Guy11 in response to ER stress, with the exception of *MoSOD2* (mitochondrial superoxide dismutase). In contrast, several putative UPR target genes, such as *MoSIL* (a nucleotide exchange factor), *MoPMT1* (protein *O*-mannosyltransferase), *MoERV29* (ER-derived vesicles protein), *MoWBPI* (lipid-linked *N*-oligosaccharyltransferase), *MoSEC61* (translocation of misfolded proteins out of ER), *MoALG7* (lipid-linked *N*-oligosaccharide biosynthesis), *MoSCJ1* (chaperone dnaJ 2), and *MoPDII* (protein disulfide isomerase1) showed no significant changes in expression upon DTT treatment of the *Mohac1* strain, indicating that the expression of these genes acts in a MoHac1-dependent manner (Fig. S9). Additionally, the expression of other genes such as *MoKAR2* (ER protein chaperone BiP), *MoPMT4* (protein *O*-mannosyltransferase), *MoOST1* (lipid-linked *N*-oligosaccharyltransferase), *MoDER1* (ER-associated degradation), *MoPMT2*

(dolichyl-phosphate-mannose-protein mannosyltransferase 2), and *MoPPS1* (protein phosphatase S phase) were markedly increased under ER stress in both *Mohac1* mutant and Guy11. Since both *Mohac1* and *Mohac1/MoHAC1^{mut intron}* result in a blocked UPR, the expression of these genes in *Mohac1/MoHAC1^{mut intron}* was low and could not be increased to the levels of the wild-type upon DTT treatment (Fig. S9). In addition, the expression of *MoOST1* and *MoPPS1* was significantly up-regulated to the level of the wild-type in response to DTT treatment in *Mohac1/MoHAC1^S* mutant but not as well as the other UPR-regulated genes, suggesting that the MoHac1^S only could partly restore the UPR pathway. These results collectively indicated that the absence of UPR generates more negative effects on the expression of some UPR-regulated genes, and that the alternate intron splicing is necessary to activate the downstream pathway leading to up-regulation of the putative UPR-regulated genes in response to ER-stress.

MoMetR is required for normal mycelial growth and is involved in amino acid metabolism

In fungi, loss of the transcriptional factors such as *N. crassa* NcCyc3, *A. nidulans* AnMetR, and *S. cerevisiae* ScMet4 and ScMet28 leads to a methionine auxotrophic phenotype (Fu et al., 1989; Thomas et al., 1992; Kuras et al., 1996; Natorff et al., 2003). In *M. oryzae*, the MGG_14561 gene was predicted to encode a 303-amino acid (aa) protein, named MoMetR, with substantial similarity to MetR proteins in other fungi (AnMetR, 48%; NcCyc3, 51%; ScMet4, 19%; and ScMet28, 29%). Its bZIP domain is 81% and 90% identical to the bZIP domains of AnMetR and NcCyc3, respectively. In comparison, MoMetR shares less similarity to ScMet4 and ScMet28, which are involved in the regulation of sulfur metabolism, within the bZIP domain (59% and 56% identity, respectively).

Given that MoMetR was identified based on its homology to others, we investigated whether MoMetR has a role in methionine metabolism. Exogenous methionine (1.0 mM) supplement compensated for the defect of the *MometR* mutant when grown on MM, SDC, and OM media. In addition, the *MometR* mutant was restored to produce wild-type like dense aerial hyphae (Fig. 8A).

To identify whether the components of CM could remediate the growth of the *MometR* mutant, we added individual supplement of CM to MM and found that the peptide and amino acid sources, but not vitamins or yeast extract without amino acids, allow the growth of the *MometR* strain (Fig. S10).

To confirm that the auxotrophic phenotype was due only to the block of methionine biosynthesis, a unique amino acid assay was performed to validate the presupposition. We added amino acid dropout mixes (DO Supplement -Met/-Trp) to MM media and supplemented with Trp to create methionine selective media. The *MometR* deletion strain was unable to grow on MM lacking methionine but could grow thin aerial hyphae on MM with methionine supplement (Fig. S11A). This result indicated that the effect of MoMetR on growth lies solely in its function in methionine biosynthesis.

To further analyze the role of MoMetR in utilizing different sulphur sources, the *MometR* mutant, Guy11, and complemented strains were cultured on various S-containing media. The *MometR* mutant could not grow on media supplemented with inorganic sulphate

(SO_4^{2-}) and sulphite (SO_3^{2-}) as a sole sulphur source, but appear to have poor and restricted growth on MM supplemented with sulphide (S^{2-}). In addition, the *MometR* mutant could grow on media supplemented with organic S sources such as cysteine or homocysteine, albeit poorly (Fig. S11B). The observation is consistent with that seen for MetR homologs in *A. nidulans* and *A. fumigatus* (Natorff et al., 2003; Amich et al., 2013). These results suggested that MoMetR functions as a global regulatory factor and plays a role in utilization of inorganic and organic sulphur sources.

MoMetR is required for conidial formation, morphology, and pathogenicity

Because the *MometR* mutant is a methionine-requiring strain, we tested the role of *MoMETR* in conidiogenesis. The *MometR* mutant produced no conidia on SDC medium without exogenous methionine. In contrast, the mutant developed aerial hyphae and produced conidia on SDC with methionine (Fig. 8B). The defect in conidia formation of the *MometR* mutant was likely due to the function of MoMetR in methionine biosynthesis.

Notably, we found that some conidia formed by the *MometR* mutant on the SDC supplemented with 5.0 mM methionine showed an abnormal morphology (Fig. S12A). About 45% of conidia from the *MometR* mutant had only one or two cells in comparison to 14% and 20% in that of wild-type and wild-type supplemented with 5.0 mM methionine, respectively (Fig. S12B). However, the *MometR* mutant showed no obvious defects in conidium germination or appressorium formation (Fig. S13). These results suggested that the loss of MoMetR affects conidial morphology, and also it is possible that some of the aerial hypha cannot get sufficient methionine to support full and normal development of conidia in the *MometR* mutant.

To confirm that MoMetR mediated methionine biosynthesis contributes to the pathogenicity of *M. oryzae*, conidia (5×10^4 spores/ml) from the wild type and the *MometR* mutant grown with methionine supplementation were sprayed onto susceptible rice (CO-39 cv. *oryzae*). After 7 days, typical lesions were formed on rice leaves inoculated with wild type conidia. In contrast, only small dark brown spots were found occasionally on leaves inoculated with the *MometR* conidia (Fig. 8C). An injection assay showed that Guy11 caused more lesions surrounding the injection sites than those by the *MometR* mutant (Fig. S14A). We also tested the ability of penetration and the expansion of invasion hyphae in rice sheath cells and barley epidermal cells. The results showed that the invasive hyphae growth was restricted for the *MometR* mutant when compared with wild type and the complemented strains (Fig. 8D). The similar result was observed when inoculations were made on barley epidermal cells (Fig. S14B). 66% of cells infected by Guy11 showed invasive hyphal growth of type 4 and 15% showed type 1 and type 2 growth. In contrast, 2% of cells in the *MometR* mutant infected host showed type 4 and 64% showed type 1 and type 2 growth. These results demonstrated that loss of *MoMETR* led to a defect in the extension of invasive hyphae, resulting in eventual reduction in pathogenicity.

To determine whether the impaired pathogenicity of *MometR* mutant is caused by a secondary effect of the reduced fitness, we next added additional different concentration of exogenous methionine into the conidial suspensions (harvested from SDC medium with 5.0 mM Met), and then sprayed onto rice seedlings. After 7 days, only small dark brown spots

were observed on rice leaves inoculated with the *MometR* mutant (Fig. S14C), indicating that adding exogenous methionine could not rescue pathogenicity caused by deletion of *MoMETR*. Furthermore, conidial suspensions with different concentrations of methionine from the same set of strains were drop-inoculated onto the detached barley leaves to create continual methionine supplements during infection. But impaired pathogenicity was still evident when compared with the wild-type (Fig. S14D). These results demonstrated that *MoMetR* directly contributes to the pathogenicity, and meanwhile the exogenous methionine is likely not sufficiently or readily available to the strain at the infection sites where invasive growth is required.

MoBzip10 is required for appressorium function

We found that MoBzip10 is required for appressorium formation and full virulence. In *M. oryzae*, appressorium-mediated penetration requires very high internal turgor pressure to create sufficient mechanical forces to breach the rice leaf cuticle (Wang et al., 2007). Appressorium turgor can be measured using an incipient cytorrhysis assay, which uses hyperosmotic concentrations of a solution to collapse the appressoria (DeJong, 1997). To determine whether the reduced pathogenicity of the *Mobzip10* mutant was due to a defect in the appressorium, an appressorium turgor assay was conducted with a 1–4 M glycerol solution. The appressoria of the *Mobzip10* mutant showed an increased collapse rate in 1–4 M glycerol solution (Fig. S15). We further examined the ability of the appressoria to penetrate onion epidermal cells. The *Mobzip10* mutant showed decreased penetration efficiency ($48\% \pm 2.6$) compared to the wild-type ($81\% \pm 3.5$) (Table 1). These results revealed that the reduced pathogenicity of the *Mobzip10* mutant was caused, at least in part, by defects in appressorium turgor pressure and subsequent host penetration.

MoBzip10 plays an important role in infectious hyphal growth in planta

Since the *Mobzip10* mutant formed appressoria but had a reduced virulence, we examined the invasive hyphal growth on both barley leaves and rice leaf sheaths. After incubation with spore suspensions for 24 h, all four types of invasive hyphae were observed in barley tissues. In Guy11 and the complemented strains, about 55% of cells showed type 4 and 20% showed type 1 and type 2 invasive hyphal growth. In contrast, less than 25% of cells showed type 4 and 40% showed type 1 and type 2 in the *Mobzip10* mutant (Fig. S16A). While Guy11 and the complemented strain exhibited hyphal growth extension into neighboring cells, the *Mobzip10* mutant showed restricted hyphal growth limiting to single cells (Fig. S16B). These results suggested that MoBzip10 plays an important role in infectious hyphal growth and plant colonization.

To investigate the cellular localization of MoBzip10 during infection-related development, a 1.54-kb promoter fragment upstream of the gene and the entire MoBzip10 protein coding sequence were fused in-frame with the GFP-encoding gene and transformed into the *Mobzip10* mutant. Punctate green fluorescence signals were observed in vegetative hyphae, conidia, appressorium and invasive hyphae with each cell containing one spot of fluorescence, suggesting that MoBzip10 is expressed in all developmental stages of *M. oryzae*. Further examination by DAPI staining showed that MoBzip10-GFP is localized to the nucleus, consistent with its regulatory functional mechanism (Fig. 9).

Effects of MoBzip5 on appressorium formation and intracellular cAMP

As the *Mobzip5* mutant often produced two appressoria (as high as $20\% \pm 1.5$) either on branched germ tubes or on two germ tubes that emerged from one conidial cell on the artificial inductive surfaces, we observed appressorium formation on natural inductive surfaces with similar results (onion epidermal cells and barley leaves) (Fig. 10). Our previous studies showed that most of the regulators of G-protein signaling (RGS) proteins were involved in appressoria formation and these mutants exhibited a similar phenotype to the *Mobzip5* mutant (Zhang et al., 2011c). Thus, qRT-PCR was performed to detect the expression of *MoRGS* genes in the *Mobzip5* mutant. The results showed that the expression of all RGS genes, except *MoRgs5* and *MoRgs8*, was significantly down-regulated in the *Mobzip5* mutant (#5). Reductions in the expression levels were <2.7-fold (*MoRGS1*), <4.1-fold (*MoRGS2*), <6.9-fold (*MoRGS3*), <2.7-fold (*MoRGS4*), <3.5-fold (*MoRGS6*), and <2.1-fold (*MoRGS7*). This is in contrast to >3.2-fold (*MoRGS5*) and >1.5-fold (*MoRGS8*) (Fig. S17A). These findings indicated that MoBzip5 could differentially regulate *MoRGS* gene expression. Since our previously results showed that intracellular cAMP levels were altered in the *Morgs* mutants, we measured the intracellular cAMP levels in the *Mobzip5* mutant. The *Mobzip5* mutant strains #5, #27, and #47 accumulated 1.4-, 1.5-, and 1.7-fold higher levels of cAMP, respectively, when compared with the wild type strain (Fig. S17B). The *Morgs3* mutant showed a ~2.8-fold higher intracellular cAMP level, which was consistent with previous studies (Zhang et al., 2011c). These results suggested that the MoBzip5 transcription factor might be involved in cross talk with MoRgs proteins to participate in appressorium formation.

MoMeaB is involved in nitrogen utilization

In *A. nidulans*, AnMeaB mediates nitrogen metabolite repression that is controlled by the nitrogen signal (Wong et al., 2007; Wong et al., 2008). The *F. oxysporum* ortholog FoMeaB also plays an important role in nitrogen utilization (Lopez-Berges et al., 2010). To test if MoMeaB has a similar regulatory function, we tested the growth of the *Momeab* mutant on various nitrogen sources media. The *Momeab* mutant showed significantly reduced growth on nitrogen sources such as Gln, nitrite or ammonium (Fig. S18). Particularly, the *Momeab* mutant could not grow on nitrite (5 mM NaNO₂) as the sole nitrogen source, indicating MoMeaB is essential for utilization of nitrite. These phenotypes are quite similar to those reported for the *meab* mutants of *A. nidulans* and *F. oxysporum* (Polley and Caddick, 1996; Wong et al., 2007; Lopez-Berges et al., 2010). We also found that the *Momeab* mutant displayed poor growth on ammonium nitrate with ammonium sulfate or ammonium tartrate (Fig. S18). These results suggested that MoMeaB shares a conserved role in nutrient uptake with those of other MeaB proteins. However, no significantly change was observed for pathogenicity of the *Momeab* mutant.

Discussion

In fungi, the bZIP TF family proteins are closely linked to various developmental and physiological processes. In *A. nidulans*, the bZIP TFs play important roles in secondary metabolism, sexual development, and stress responses (Natorff et al., 2003; Yin et al., 2013). In *N. crassa*, the bZIP TFs are involved in responses to amino acid starvation, sulfur

sources, and the oxidative stress (Fu et al., 1989; Tian et al., 2007; Tian et al., 2011). In *A. fumigatus*, the bZIP TFs regulate asexual development, gliotoxin production, sulphur assimilation, iron starvation, and virulence (Schrettl et al., 2010; Xiao et al., 2010; Amich et al., 2013). In *F. graminearum*, bZIP TF FgAp1 mediates the oxidative stress response and trichothecene biosynthesis but not virulence (Montibus et al., 2013), while *F. oxysporum* FoMeab is important for the nitrogen response pathway (Lopez-Berges et al., 2010). Here, our data show that bZIP proteins of *M. oryzae* including MoHac1, MoMetR, and MoMeab, all have important regulatory functions governing various developmental processes of the fungus. Our previous studies demonstrated that the bZIP TFs MoAp1 and MoAtf1 regulate stress responses that are important for pathogenicity. In this study, we systemically searched for bZIP TFs and identified additional 19 proteins that contain the characteristic bZIP structures in their ORFs. We showed that these additional members of the bZIP gene family have important shared and distinct effects on infection-related morphogenesis and pathogenicity. A summarizing model for *MoBZIPs* function is presented in Fig. 11. Among them, MoHac1, MoMetR, and MoBzip10 are associated with vegetative growth, conidiation, and pathogenicity, similar to MoAp1 and MoAtf1. MoBzip1, MoBzip2, MoBzip3, and MoBzip11 are negative regulators of conidiation. In addition, MoMetR and MoBzip12 are required for conidial morphology, and MoBzip5 contributes to the appressorium formation. Moreover, MoHac1 plays an important role in the UPR via alternate splicing of its mRNA. MoMetR regulates methionine metabolism and loss of *MoMETR* results in methionine-deficient mutation. Finally, MoBzip10 participates in pathogenicity through the involvement in the generation of appressorium turgor pressure. Our findings revealed that most of the bZIP TFs have general roles during growth and development, which may be due to their conserved bZIP domains. However, the distinct phenotypes of these bZIP TFs suggested that they may also function via distinct pathways or mechanisms during normal or infection-related morphogenesis and development.

In eukaryotic cells, membrane and secretory proteins are folded, assembled, and modified in the ER with the assistance of numerous foldases and molecular chaperones. To cope with the dynamic environment, cells had evolved the UPR pathway as a conserved quality control system. When protein folding is disturbed, unfolded or misfolded proteins accumulate in the ER, resulting in ER stress, and the UPR pathway is activated. Here, we identified MoHac1 and found that loss of *MoHAC1* impacts vegetative hyphal growth, conidiation, and pathogenicity. We showed that *MoHAC1* contains a very short unconventional intron with highly conserved border sequences that is spliced out in response to ER stress. Our results are quite in agreement with the previous findings that an *A. niger hacA* mutant exhibits poor growth and is nearly absent in sporulation (Mulder and Nikolaev, 2009), and that deletion of *HACA* in *A. brassicicola* dramatically reduces growth rates, conidiation, and a complete loss of virulence (Joubert et al., 2011). These results indicated that Hac1 proteins are responsible for hyphal growth, conidiation, and virulence in various fungi.

As in most fungal pathogens, conidiogenesis is a key step in the colonization of host plants by *M. oryzae*. *Mohac1*, *MometR*, *Momeab*, *Mobzip5*, *Mobzip9*, *Mobzip10*, and *Mobzip12* all showed reduced conidiation, whereas the *Mobzip1*, *Mobzip2*, *Mobzip3*, and *Mobzip11* mutants showed increased conidia production (Fig. 11). While the

mechanism of negative regulation remains unclear, we examined the mechanism by which MoHac1 regulates conidiogenesis. We found that MoHac1 regulates a large number of specific-expressed proteins participating in the conidia morphogenesis through the conserved UPR.

Like many other fungal pathogens, effectors of *M. oryzae* are secreted into host cells and then suppress host immunity to sustain infection (Zhang and Xu, 2014). Two distinct effector secretion systems were identified to deliver respective target effectors. Cytoplasmic effectors are transported into host cells via the biotrophic interfacial complex (BIC), and specifically, the apoplastic effectors are secreted from IH by the conventional fungal ER-Golgi secretion pathway (Giraldo et al., 2013). Thus, genes associated with the ER to Golgi secretory pathway and post-translational modifications are also likely essential for the secretion of apoplastic effectors in *M. oryzae* (Zhang and Xu, 2014). For instance, recent studies showed that the ER chaperone MoLhs1 is involved in asexual development and functions in the UPR, also is essential for proper processing of apoplastic effectors in *M. oryzae* (Yi et al., 2009); an α -1,3-mannosyltransferase for protein *N*-glycosylation MoAlg3 mediated *N*-glycosylation of an apoplastic effector MoSlp1 is required for evade host innate immunity (Chen et al., 2014). We are also interested in determining whether the UPR has a role in the secretion of effectors, but unable to achieve due to that the *Mohac1* mutant could not develop any penetration structures in the host cells, as well as the *Mohac1/MoHAC1^{mut intron}* mutant. Consequently, it is suggested that the UPR may affect the proper function of the effectors, as fold and modification of secreted proteins during the specifically expressed stage like infection in the host cell are intimately associated with the UPR pathway.

Poor nutritional conditions may result in ER stress (Xu et al., 2005; Palorini et al., 2013), and pathogenic fungi must cope with this challenge to cause infection. Nitrogen is an important requirement for growth, and fungi are able to use a wide variety of compounds as nitrogen sources. In our studies, MoMeaB was identified to be involved in nitrogen utilization, however, it did not affect the pathogenicity.

Moreover, amino acids are important components in the metabolism of a variety of pathogens, plants, and animals. Sulfur-containing amino acids are also the key component of proteins. In fungi, *de novo* biosynthesis of methionine consists of two processes: the sulphate assimilation and methionine synthesis. Sulphate is subsequently reduced to sulfide by a series of enzymatic steps containing ATP sulfurylase, adenosine 5'-phosphosulfate kinase, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) sulfotransferase, and sulfite reductase. An O-acetyl-L-serine thiol-lyase condenses sulfide and O-acetylserine to form cysteine (Guillouard et al., 2002). Then, cysteine is catalyzed by cystathionine-synthase (CGS) and cystathionine-lyase (CBL) to generate homocysteine. Finally, homocysteine generates methionine under the effect of methionine synthase (MS). Methionine biosynthesis plays an important role in fungal growth and pathogenicity. Previous studies showed that deletion of the transcription activator ScMet4 in *S. cerevisiae*, the regulatory protein NcCyc3 in *N. crassa*, and the transcription regulator AnMetR in *A. nidulans* all resulted in methionine auxotrophy (Fu et al., 1989; Thomas et al., 1992; Natorff et al., 2003). Recently, a study of the transcriptional activator AfMetR in *A. fumigatus* showed that

the MetR regulator is essential for growth on a variety of sulphur sources and is important for virulence in both leukopenic mice and an alternative mini-host model of aspergillosis virulence (Amich et al., 2013). Here, we showed that MoMetR, a MetR protein, plays an important role in growth, conidiation, and pathogenicity. Consistent with previous results in other fungi, when medium was supplemented with methionine, the *MometR* mutant could sustain hyphal growth and conidiation but not conidium morphology and pathogenicity, indicate that MoMetR not only participates in the methionine biosynthesis but also is required for conidium morphology and pathogenicity. As the *MometR* mutant could not sustain growth on media supplemented with inorganic S-sources such as SO_4^{2-} and SO_3^{2-} as a sole sulphur source, but appear to have poor and restricted growth on minimal medium supplemented with S^{2-} and organic S-sources, we concluded that *M. oryzae* requires MoMetR for assimilation of various sulphur sources.

Our results showed that nutrient supplementation plays an important role in the process of colonization and invasion by *M. oryzae* and that supplemental methionine is required for growth on media, conidiation, and infection, but is not sufficient for conidium germination or appressorium formation on host. This work is consistent with results of a study on the cystathionine beta-lyase gene *MoSTR3* showing that a methionine-requiring *Mostr3* mutant could not establish disease in rice leaves and failed to develop invasive hyphae (Wilson et al., 2012). Our research is in agreement with an appressorial transcriptional profiling study that suggests amino acid uptake not being an important process during appressorium development or the initial stages of plant infection (Soanes et al., 2012).

The bZIP MoBzip10 appears to be novel, as similar proteins have not been found previously. Disruption of *MoBZIP10* resulted in defective hyphal growth, conidiogenesis, and pathogenicity, despite that the *Mobzip10* mutant still incited a few small lesions on rice and barley leaves. We showed that reduced virulence of *Mobzip10* mutant is directly related to defective appressorium turgor generation. Thus, MoBzip10 may represent a new bZIP TF that directly contributes to virulence.

In *M. oryzae*, appressoria can be induced on non-inductive surfaces in the presence of exogenous cAMP or inhibitors of cAMP phosphodiesterase. In addition, loss of the adenylyl cyclase MoMac1 leads to failure of appressorium formation, indicating that cAMP plays an important role in appressorium formation (Lee and Dean, 1993; Zhang et al., 2011c). Our studies showed that *MoRGS1-MoRGS4*, *MoRGS6*, and *MoRGS7* expression was significantly down-regulated in the *Mobzip5* mutant and that loss of MoBzip5 increased intracellular cAMP levels. Moreover, the *Mobzip5* mutant displayed an abnormal appressoria formation phenotype that was also observed in *Morgs* mutants in which the intracellular cAMP level was significantly increased. Integrating these phenotypes, we propose that MoBzip5 may participate in the regulation of *MoRGS* gene expression.

In summary, we have shown that 20 members of the bZIP transcription factor family are involved in the growth, differentiation, and pathogenicity of *M. oryzae* through either shared or distinct mechanisms. Our findings revealed MoHac1, MoMetR, and MoBzip10 as important virulence regulators, each through distinct regulatory circuitry. Given the importance of these proteins and the urgency of developing novel fungicides for blast

control, further investigation of the bZIP transcription factors and their respective regulatory networks are warranted.

Materials and Methods

Strains and culture conditions

The *M. oryzae* Guy11 and Ku80 strains were used as wild type for transformation in this study. All strains were cultured on complete medium (CM) agar plates (Zhang et al., 2011b). Liquid CM medium was used to prepare the mycelia for DNA and RNA extraction. For conidiation, strain blocks were maintained on straw decoction and corn (SDC: 100 g of straw, 40 g of corn powder, 15 g of agar in 1 L of distilled water) agar media (Zhang et al., 2011b) at 28°C for 7 days in the dark followed 3 days continuous illumination under fluorescent light.

Targeted gene deletion and complementation

The gene-deletion mutants were generated using the standard one-step gene replacement strategy. First, two 1.0 kb of sequences flanking of targeted gene were PCR amplified with primer pairs (Table S2). Then, the resulting PCR products of each bZIP gene were digested with restriction endonucleases and ligated with the hph cassette released from pCX62. Finally, the completed inserts were sequenced. The ~3.4-kb fragments, which contain the flanking sequences and hygromycin resistance cassette, were amplified and transformed into protoplasts of wild type Guy11 or Ku80. Putative mutants were screened by PCR and confirmed by Southern blotting analysis (Fig. S1). The complement fragments, which contain the entire bZIP genes and their native promoter regions, were amplified by PCR with primers (Table S2) and inserted into pYF11 (bleomycin resistance) to complement the respective mutant strains.

Assays for vegetative growth, conidiation, appressorium formation, and cuticle penetration

Small agar blocks were cut from the edge of 4-day-old cultures and placed onto fresh media (CM, MM, OM and SDC) (Zhang et al., 2010) for culturing in the dark at 28°C.

To test hyphal growth on different sulphur sources, strains were cultured on MM [1% glucose minimal medium: 6 g/L NaNO₃, 0.52 g/L KCl, 0.52 g/L MgSO₄·7H₂O, 1.52 g/L KH₂PO₄, 10 g/L glucose, 0.001% (W/V) thiamine and 0.1% (W/V) trace elements]. For sulphur-free medium, MgCl₂ was substituted for MgSO₄ and a modified mixture of trace elements lacking any sulphate salt was used. For inorganic S-sources, the same amount of sulphate (SO₄²⁻), sulphite (SO₃²⁻) and sulphide (S²⁻) are added into the sulphur-free medium. For organic S-sources, 1 mM cysteine or homocysteine is added into the sulphur-free medium. The radial growth was measured after incubation for 7 days and then photographed. Similar methods are used to test hyphal growth on different nitrogen sources, strains are cultured on MM agar containing either sodium Glu, Gln, NaNO₃, NH₄NO₃, (NH₄)₂C₄H₄O₆, (NH₄)₂SO₄ (all 25 mM) and NaNO₂ (1 mM and 5 mM, the wild type could not grow on 25 mM) as nitrogen source. All experiments were repeated three times, each with three replicates. For conidia production, mycelia were grown in the dark on SDC

medium at 28 °C for 7 days, followed by constant illumination for 3–4 days. Conidia were harvested from 10-day-old cultures, filtered through three layers of lens paper, and resuspended to a concentration of 5×10^4 spores per milliliter in sterile water. For appressorium formation and cuticle penetration assays, droplets (30 μ l) of conidial suspension were placed on cover slips (hydrophobic) and epidermal cells (onion, barley) and incubated under humid conditions at room temperature as described previously (Zhang et al., 2009). Appressorium formation and development of invasive hyphae were examined after incubation for 24 hours.

Plant infection and rice sheath penetration assays

Plant infection and injection assays were performed as described (Zhang et al., 2011a). For determining the pathogenicity of the mutants without conidia, mycelia cultured in CM liquid medium for 2 days were harvested and inoculated on the healthy or wounded rice leaves or barley leaves for 5 days and kept in the same condition as described above (Dou et al., 2011). For microscopic observation of penetration and infectious hyphae expansion on rice tissue, rice was inoculated with 100 μ l of conidial suspension (5×10^4 spores/ml) on the inner leaf sheath cuticle cells. After incubation for 48 h under humid conditions at 28°C, the leaf sheaths were observed under a microscope.

Generation of the *MoHAC1^{mut intron}*, *MoHAC1^S*, *P_{RP27}-GFP-MoHAC1* and *MoBZIP10-GFP* constructs

For generating *MoHAC1^{mut intron}*, *MoHAC1^S* constructs, PCR products containing the native promoter of *MoHAC1* were amplified with primers FL12298/FL15974 (Table S2), and co-transformed with fragments amplified with primers FL15975/ FL12299 (Table S2) into the yeast strain XK1-25 with *Xho*I digested vector pYW11 that contains the bleomycin-resistant gene (Bruno et al., 2004). Plasmid pYW11::*MoHAC1^{mut intron}* was rescued from the resulting Trp+ yeast transformants. The same strategy was used to generate the pYW11::*MoHAC1^S* vector (PCR products amplified with primers FL12298/ FL15107 and FL15108/ FL15109, respectively).

For generating the *P_{RP27}-GFP-MoHAC1* fusion construct, a 0.7 kb GFP fragment (amplified by FL15141 and FL15142) and a 1.7 kb fragment of the *MoHAC1* gene (amplified by FL15143 and FL15144) were co-transformed with *Xho*I-digested pYF11 into yeast strain XK1-25 (Bruno et al., 2004). Plasmid pYF11::GFP::*MoHAC1* was rescued from the resulting Trp+ yeast transformants.

For generating the *MoBZIP10-GFP* construct, a 2.8 kb fragment of the *MoBZIP10* native promoter and the gene coding sequence (amplified by FL14051 and FL14052) was co-transformed with *Xho*I-digested pYF11 into yeast strain XK1-25 (Bruno et al., 2004). Plasmid pYF11::*MoBZIP10*::GFP was rescued from the resulting Trp+ yeast transformants.

Quantitative RT-PCR, RT-PCR, and gene expression analysis

For RT-PCR and quantitative real time RT-PCR (qRT-PCR), total RNA were reverse transcribed into first-strand cDNA using the oligo(dT) primer and M-MLV Reverse Transcriptase (Invitrogen). Confirmation of the spliced machinery of *MoHAC1* mRNA

under the UPR-induced conditions was made with primer pairs FL12131/ FL12132 (Table S2). 32 cycles of RT-PCR were run on a Bio-Rad PTC0200 Peltier Thermal Cycler. The stable expression actin gene (MGG_03982.5) amplified by primer pairs FL4362/ FL4363 (Table S2) was used as internal control.

Quantitative Real-time PCR (qRT-PCR) was run on the Applied Biosystems 7500 Real Time PCR System with SYBR *Premix Ex Taq*TM (Perfect Real Time, Takara, Japan). Normalization and comparison of mean Ct values were performed as previously described (Livak and Schmittgen, 2001; Guo et al., 2010). The experiment was conducted twice with three independent biological replicates.

DAPI staining

For the localization of *MoBZIP10*, conidia of strains transformed with plasmid pYF11::*MoBZIP10* were stained with DAPI (50 µg/ml, Sigma) for 10 min, and visualized under a microscope (Olympus IX71). GFP fluorescence was detected using a 450 to 490 nm excitation filter and a 520 nm barrier filter and DAPI fluorescence was detected under UV light using a 360 to 400 nm excitation filter.

Intracellular cAMP assay

Two-day-old liquid mycelial cultures were harvested, frozen in liquid nitrogen and lyophilized for 16 hours. Intracellular cAMP extraction was followed as previously described (Liu et al., 2007). The cAMP levels were quantified according to the cAMP Biotrak Immunoassay System (BD Bioscience, NJ, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 11.

Table 1

Comparison of mycological characteristics among strains.

Strain	Growth (cm) ^a				Conidiation (×100/cm ²) ^b	Germination rate (%) ^c	Appressorium formation (%) ^d	Penetration (%) ^e
	CM	MM	SDC	OM				
Guy11	4.6±0.2 ^{Cg}	4.2±0.1 ^D	4.5±0.0 ^D	4.3±0.1 ^D	466.8±8.7 ^D	97±1.6 ^{AB}	98±1.7 ^B	81±3.5 ^C
<i>Mohac1</i>	2.7±0.1 ^A	2.5±0.1 ^B	2.6±0.1 ^B	3.1±0.1 ^{BC}	RO ^{HA}	ND ^f	ND	ND
<i>MomeR</i>	4.4±0.1 ^C	0 ^A	0 ^A	0 ^A	0 ^A	ND	ND	ND
<i>Moidi4</i>	4.5±0.3 ^C	3.9±0.1 ^{CD}	4.7±0.2 ^{DE}	4.5±0.1 ^D	461.1±1.7 ^D	96±1.2 ^{AB}	97±1.8 ^B	79±3.8 ^{BC}
<i>Mofrr3</i>	4.1±0.6 ^{BC}	3.3±0.2 ^C	3.8±0.2 ^C	3.1±0.1 ^{BC}	393.0±7.1 ^D	96±1.3 ^{AB}	96±1.6 ^B	74±3.5 ^B
<i>Momeab</i>	3.6±0.2 ^B	3.1±0.2 ^C	4.7±0.2 ^{DE}	2.8±0.3 ^B	216.9±7.1 ^C	96±1.1 ^{AB}	95±1.6 ^B	77±1.4 ^B
<i>Mohapx</i>	4.2±0.2 ^{BC}	3.3±0.1 ^C	4.3±0.1 ^D	4.1±0.0 ^{CD}	478.8±5.8 ^D	96±1.2 ^{AB}	97±1.6 ^B	79±1.8 ^{BC}
<i>Mobzip1</i>	5.3±0.4 ^D	3.5±0.2 ^C	3.4±0.4 ^B	3.7±0.5 ^C	3645.1±6.5 ^F	97±1.3 ^{AB}	96±1.3 ^B	81±3.1 ^C
<i>Mobzip2</i>	4.5±0.2 ^C	3.9±0.2 ^{CD}	4.0±0.3 ^{CD}	4.6±0.00 ^D	3236.2±2.4 ^F	97±1.9 ^{AB}	95±1.5 ^B	78±2.5 ^{BC}
<i>Mobzip3</i>	5.2±0.3 ^D	3.5±0.1 ^C	5.0±0.1 ^E	5.4±0.2 ^E	3359.6±4.3 ^F	96±1.6 ^{AB}	96±1.7 ^B	75±1.6 ^B
<i>Mobzip4</i>	4.7±0.1 ^C	3.7±0.1 ^{CD}	4.1±0.2 ^{CD}	4.7±0.2 ^D	426.2±4.3 ^D	96±1.2 ^{AB}	97±1.6 ^B	80±2.2 ^C
<i>Mobzip5</i>	4.7±0.4 ^C	3.8±0.2 ^{CD}	4.7±0.1 ^{DE}	4.7±0.1 ^D	239.8±8.0 ^{CD}	97±1.6 ^{AB}	94±1.6 ^{AB}	74±3.4 ^B
<i>Mobzip6</i>	4.8±0.3 ^C	3.9±0.1 ^{CD}	4.2±0.3 ^{CD}	3.9±0.1 ^{9C}	540.4±1.5 ^D	95±1.2 ^A	93±1.7 ^{AB}	79±1.8 ^{BC}
<i>Mobzip7</i>	5.2±0.1 ^D	4.2±0.1 ^D	5.1±0.1 ^E	4.9±0.2 ^{DE}	400.1±8.3 ^D	97±1.6 ^{AB}	95±1.2 ^B	76±0.9 ^B
<i>Mobzip8</i>	4.7±0.1 ^C	4.1±0.1 ^{CD}	4.6±0.2 ^{DE}	4.9±0.2 ^{DE}	466.9±3.1 ^D	97±1.4 ^{AB}	97±1.4 ^B	81±1.1 ^C
<i>Mobzip9</i>	4.1±0.1 ^{BC}	3.6±0.1 ^C	4.3±0.1 ^D	4.2±0.00 ^{CD}	143.7±5.1 ^C	96±1.3 ^{AB}	98±1.2 ^B	79±2.4 ^{BC}
<i>Mobzip10</i>	3.6±0.1 ^B	3.8±0.1 ^{CD}	3.7±0.1 ^C	3.7±0.1 ^C	13.4±1.9 ^B	97±1.8 ^{AB}	90±3.1 ^A	48±2.6 ^A
<i>Mobzip11</i>	4.4±0.3 ^C	4.1±0.2 ^{CD}	4.3±0.1 ^D	4.2±0.2 ^{CD}	1094.2±6.2 ^E	97±1.5 ^{AB}	97±1.3 ^B	77±1.9 ^B
<i>Mobzip12</i>	5.3±0.1 ^D	3.8±0.2 ^{CD}	4.6±0.2 ^{DE}	5.1±0.0 ^E	10.6±6.8 ^B	94±1.5 ^A	98±1.7 ^B	80±2.1 ^C

^a Diameter of hyphal radii at day 7 after incubation on CM, MM, SDC and OM agar plates at room temperature.^b Number of conidia harvested from a 9 cm SDC plate at day 10 after incubation at room temperature.^c Percentage of conidial germination on artificial surface at 4 h post-inoculation.^d Percentage of appressorium formation on artificial surface at 24 h post-inoculation.^e Percentage of appressoria penetrated onion epidermal cells at 24 h post-inoculation.

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f_{ND} = not determined.

g Significant changes compared to the wild type. The same letters in a column showed no significant difference.

h RO = rarely observed.

The experiments were repeated three times.