Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2017) 18(7), 1001–1011 DOI: 10.1111/mpp.12455

MoYcp4 is required for growth, conidiogenesis and pathogenicity in *Magnaporthe oryzae*

YUE CHEN[1,2], XINYI LE[2], YI SUN[2], MENGYING LI[2], HAIFENG ZHANG[2], XINQIU TAN[1], DEYONG ZHANG[1], YONG LIU[1,*] AND ZHENGGUANG ZHANG[2,*]

[1]*Hunan Academy of Agricultural Sciences, Institute of Plant Protection, Changsha, Hunan 410125, China*

[2]*Department 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*

SUMMARY

The transcription factor MoAP1 has been shown previously to be required for pathogenicity in *Magnaporthe oryzae* via mediation of the oxidative stress response. In the serial analysis gene expression database, it was found that expression of MoYcp4, a homologue of the *Saccharomyces cerevisiae* flavodoxin-like protein ScYcp4, was affected by MoAP1. Transcriptional analysis demonstrated that *MoYCP4* was significantly up-regulated during conidiation, appressorium formation and infection. The growth rate of a Δ*Moycp4* mutant was reduced slightly, but conidial production was increased significantly (more than 10-fold), compared with the wild-type strain. Although the rate of appressorium formation was unaffected, the appressorial turgor was abnormal and the ability to infect rice and barley was reduced, resulting in decreased pathogenicity. In summary, MoYcp4, a target of MoAP1, is involved in the growth, conidiogenesis and pathogenicity of *M. oryzae*. Our studies provide a comprehensive analysis of flavodoxin-like proteins and will aid in the study of pathogen-related molecular mechanisms.

Keywords: conidiogenesis, growth, MoYcp4, pathogenicity.

INTRODUCTION

Magnaporthe oryzae is an important pathogenic fungus which causes great damage on rice and a variety of other cereal crops. The annual grain losses caused by rice blast would be sufficient to feed 60 million people; therefore, pathogenic morphogenesis has important economic and social significance (Ou, 1985). Considering the important economic impact and genetic tractability of the pathogen and host, rice blast is considered to be an important model for the study of plant–pathogen interactions at the molecular level (Caracuel-Rios and Talbot, 2007).

Like most other fungal pathogens, the conidia of *M. oryzae* play a central role in the disease cycle. When attached to the plant surface, conidia begin to germinate and form a specialized infection structure, known as an appressorium, at the end of the germ tube (Bourett and Howard, 1991). Once mature, the appressorium accumulates enormous turgor pressure (as high as 8 MPa) to help penetrate the strong leaf cuticle and to enter plant cells (Howard *et al.*, 1991). After penetration, infectious hyphae spread and cause necrotic lesions on the plants. Once conditions are suitable, thousands of conidia are produced in the lesions, which are then released to initiate a new infection cycle on plant tissues within 3–5 days. Therefore, the characterization of the molecular mechanisms involved in conidiation and appressorium penetration-mediated plant infection is pivotal to the production of new strategies for disease management.

We have shown previously that the transcription factor MoAP1 is required for the pathogenicity of *M. oryzae* via mediation of the oxidative stress response. In the serial analysis gene expression (SAGE) database, many of the genes are affected by MoAP1, including those required for pathogenicity (Guo *et al.*, 2011). MoYcp4, a homologue of the *Saccharomyces cerevisiae* flavodoxin-like protein (FLP) ScYcp4, has also been shown to be affected by MoAP1. In *S. cerevisiae*, *ScYCP4* and its homologue *ScRFS1* regulate the expression of a series of genes during late growth, and the deletion of *ScYCP4* leads to the expression of genes associated with hexose transport and glycolysis, as well as altered expression of genes involved in mating and pheromone responses. In addition, the response to oxidative and osmotic stress is affected in the Δ*Scycp4*Δ*Scrfs1* double mutant (Cardona *et al.*, 2011), indicating that *ScYCP4* is involved in a series of biological processes and plays an important role in the growth of *S. cerevisiae*. Moreover, *Paracoccidioides brasiliensis PBY20*, the homologue of *YCP4*, has been shown to play an important role in cell differentiation or even in the maintenance of the yeast form (Daher *et al.*, 2005). Deletion of four FLPs brought about an increased sensitivity to benzoquinone and oxidants, reduced ubiquinone synthesis and loss of virulence to the host in *Candida albicans* (Morschhäuser *et al.*, 2015).

Here, we define a pivotal FLP, MoYcp4, and, for the first time, elucidate its function in phytopathogenic fungi by the analysis of its role in the growth, conidiogenesis and pathogenicity of *M. oryzae*.

* *Correspondence*: Email: haoasliu@163.com; zhgzhang@njau.edu.cn

RESULTS

Identification of *MoYCP4*

MoAP1 is a positive transcription factor which regulates the transcription of a series of genes important for the growth, development and pathogenicity of *M. oryzae* (Guo *et al.*, 2011). To identify target genes affected by MoAP1, we screened the Δ*Moap1* mutant SAGE database and identified a gene (MGG_01569) with an encoded protein that showed 55% identity and 65% similarity to the *S. cerevisiae* FLP ScYcp4; therefore, we named the protein MoYcp4. Considering that *ScYCP4* and its homologue *ScRFS1* are involved in the osmotic stress response in *S. cerevisiae* (Cardona *et al.*, 2011), we expressed *MoYCP4* in a Δ*Scycp4* mutant through the yeast expression vector pYES2 to determine whether MoYcp4 can complement ScYcp4 function. Transformants carrying the *MoYCP4* gene exhibited similar growth to the wild-type on medium containing 0.7 M NaCl and 0.6 M KCl, Growth of the transformants is much better when compared with the Δ*Scycp4* mutant (Fig. S1, see Supporting Information), suggesting that MoYcp4 is a functional paralogue of ScYcp4.

We have shown previously that the transcription of *MoYCP4* is reduced in the Δ*Moap1* mutant (Guo *et al.*, 2011). We confirmed the expression levels of *MoYCP4* using quantitative real-time polymerase chain reaction (qRT-PCR) and found that our result was consistent with that in the Solexa SAGE database. The expression of *MoYCP4* was reduced significantly in the Δ*Moap1* mutant (Fig. 1A). To further validate that *MoYCP4* is regulated directly by the transcription factor MoAP1, we performed an agarose gel electrophoretic mobility shift assay (EMSA). We found that the digoxigenin (DIG)-labelled promoter of *MoYCP4* was retarded by the addition of MoAP1 protein in EMSA, but not by the addition of glutathione *S*-transferase (GST) (Fig. 1B). The addition of proteinase K caused the DIG-labelled DNA to shift back to the fast-mobility band (Fig. 1B). Further, specificity and competition assays were performed; we found that the DIG-labelled promoter of other gene *MoLYS2* (Chen et al., 2014b) was not retarded by the addition of MoAP1 protein (Fig. S3A, see Supporting Information), and the concentrations of the binding complexes of MoAP1 and the DIG-labelled promoter were progressively reduced with the addition of increasing amounts of unlabelled promoter of *MoYCP4* (Fig. S3B), These results suggest that MoAP1 is able to bind to the promoter of *MoYCP4*.

MoYCP4 is highly expressed during various stages of infection

Before exploring the functions of *MoYCP4*, we evaluated the transcriptional profile in various growth and infection stages. The expression of *MoYCP4* was higher in the conidial, appressorial and infection stages than in the mycelium stage, with the highest level occurring during the conidial stage (>20-fold increase; Fig. 2). These observations suggest that *MoYCP4* plays an important role in infection-related morphogenesis.

MoYCP4 deletion affects hyphal growth

A *MoYCP4* deletion mutant was generated by replacing the *MoYCP4* coding region with the hygromycin resistance cassette (*HPH*) (Fig. S2A, see Supporting Information). The putative mutant (Δ*Moycp4*) was screened and confirmed by Southern blot analysis (Fig. S2B). Furthermore, a complementation strain (Δ*Moycp4*/*MoYCP4*) containing the open reading frame (ORF) encoded by *MoYCP4* and green fluorescent protein (GFP) under the control of

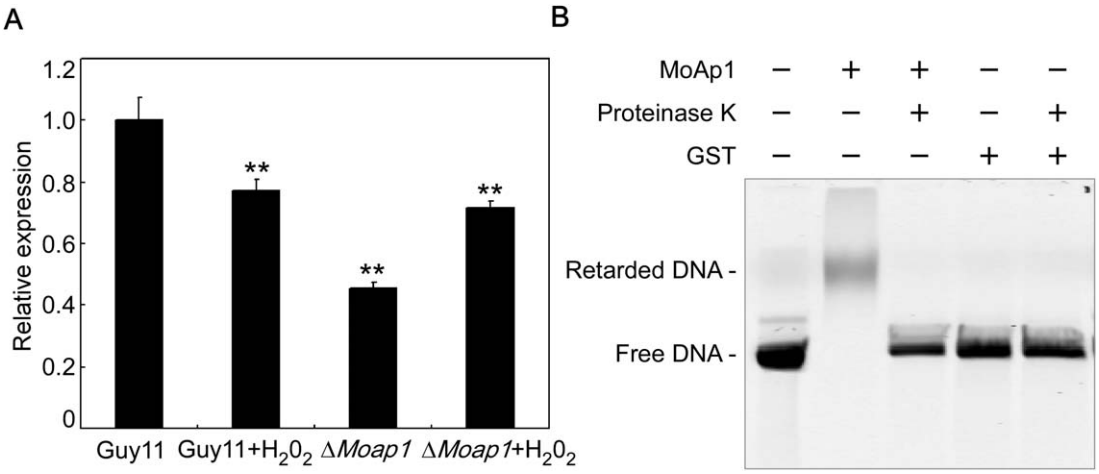

Fig. 1 Expression analysis of MoYCP4 in the ΔMoap1 mutant and the DNA-binding activity of MoAP1. (A) Transcriptional levels of MoYCP4 in the wild-type and ΔMoap1 mutant before and after exposure to H_2O_2 measured by quantitative real-time polymerase chain reaction (qRT-PCR). (B) The digoxigenin (DIG)-labelled promoter DNA of MoYCP4 was incubated in the absence or presence of purified MoAp1 protein. Proteinase K was added after the incubation of MoAp1 with the probe, and the sample was incubated for 10 min at room temperature. GST, glutathione *S*-transferase. Double asterisks represent significant differences ($P < 0.01$).

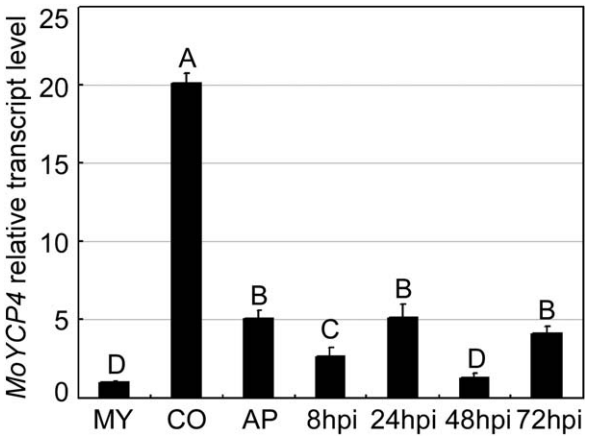

Fig. 2 Transcription profiles of MoYCP4 at different stages of fungal development. The phase-specific expression of MoYCP4 was quantified by quantitative real-time polymerase chain reaction (qRT-PCR), with the synthesis of cDNA from each sample including infectious growth, vegetative growth and conidia. MY, mycelia; CO, conidia; AP, appressoria; hpi, hour post-inoculation. Capital letters represent significant differences ($P < 0.01$).

the *MoYCP4* native promoter was also generated (Fig. S2C). The resulting transformant showed normal growth, conidiation and infection (Figs 3–10) and was considered as the complemented strain. Under epifluorescence microscopy, the GFP signal of MoYcp4-GFP was visible in the cytoplasm of the conidia, appressoria and infectious hyphae (Fig. S4, see Supporting Information).

We evaluated the growth of the Δ*Moycp4* mutant on complete (CM), minimal (MM), oatmeal (OM), and straw decoction and corn (SDC) media. The Δ*Moycp4* mutants showed slightly smaller colony diameter than the wild-type strain (Guy11) and the complemented strain Δ*Moycp4/MoYCP4* on all media types, except for CM (Fig. 3A). These results were further supported by statistical analysis (Table 1), indicating that MoYcp4 plays a role in hyphal growth.

MoYcp4 is important in stress responses

To investigate whether Δ*Moycp4* exhibited any defects under different conditions of stress, the Δ*Moycp4* mutant was exposed to 0.7 M NaCl and 0.6 M KCl. The Δ*Moycp4* mutant showed stronger growth inhibition than Guy11 and the complemented strain (Fig. 4A). Inhibition of the Δ*Moycp4* mutant was 17% and 12% higher than Guy11 in NaCl- and KCl-containing CM, respectively (Fig. 4B). These findings suggest that MoYcp4 contributes to the osmotic stress response of the fungus.

Considering the involvement of FLPs in the response to quinone and oxidants in *C. albicans*, and the ubiquinone reduction of the *FLP* deletion mutant (Morschhäuser *et al.*, 2015), we examined the sensitivity of the Δ*Moycp4* mutant to these agents. Consistent with the previous study, the Δ*Moycp4* mutant showed a higher inhibition rate on exposure to *p*-benzoquinone (BZQ) and menadione (MND) than did Guy11 and the complemented strain (Fig. 5), indicating that the Δ*Moycp4* mutant is more sensitive to quinone. However, when treated with 2.5 or 5 mM H_2O_2, the growth inhibition rates were not significantly different between the Δ*Moycp4* mutant and Guy11 (Fig. 5). Consistently, the transcription level of *MoYCP4* was also down-regulated in Guy11 in the presence vs. absence of H_2O_2 treatment (Fig. 1A). We also examined the sensitivity of the Δ*Moycp4* mutant to linolenic acid (LNA), a polyunsaturated fatty acid which induces lipid peroxidation (Do *et al.*, 1996); no significant difference was observed between the Δ*Moycp4* mutant and Guy11 (Fig. 5). These results indicate that the deletion of *MoYCP4* does not affect the oxidation stress response in *M. oryzae*.

MoYcp4 is involved in cell wall integrity

To examine the role of MoYcp4 in cell wall integrity, mycelial growth was measured on CM containing sodium dodecylsulfate (SDS), calcofluor white (CFW) and Congo red (CR), all of which are cell wall-perturbing agents. The sensitivity of the Δ*Moycp4* mutant to SDS, CFW and CR at low concentrations was not significantly different from that of the wild-type strain Guy11 (Table 2); however, the mutant was more sensitive to these agents at high concentrations (Table 2). We further examined the effects of lytic enzymes (10 mg/mL) on the Δ*Moycp4* mutant. More protoplasts were found in the Δ*Moycp4* mutant than in the controls after incubation for 30, 60 and 90 min (Fig. 6A,B). Mycelial fragments were still found after 60 min of incubation (Fig. 6A). In contrast, no mycelial fragments were found in the Δ*Moycp4* mutant (Fig. 6A). These results indicate that MoYcp4 plays a role in cell wall integrity.

MoYcp4 is involved in conidiogenesis, but not in appressorium formation

As conidia play an important role during *M. oryzae* infection, we measured the conidial production of the Δ*Moycp4* mutant. We found that conidia production was significantly increased on SDC medium (Fig. 3B). The Δ*Moycp4* mutant produced normal-shaped conidia; the production was increased by over 10-fold compared with the wild-type Guy11 (Table 1). We further examined the expression of six conidiation-related genes. The expression levels of *MoCON2* and *MoSTUA* (Nishimura *et al.*, 2009; Shi *et al.*, 1998) were significantly lower in the Δ*Moycp4* mutant than in the Guy11 strain (Fig. 3C). In contrast, the transcript levels of *MoCOS1*, *MoCOM1*, *MoCON7* and *MoHOX2* (Kim *et al.*, 2009; Liu *et al.*, 2010; Shi *et al.*, 1998; Yang *et al.*, 2010; Zhou *et al.*, 2009) were significantly increased in the Δ*Moycp4* mutant (Fig. 3C), indicating that MoYcp4 is involved in the regulation of the expression of conidiation-related genes. Next, we examined appressorium formation in the Δ*Moycp4* mutant. The Δ*Moycp4* mutant formed normal germ tubes. Microscopic examination revealed that the rate of appressorium formation in Δ*Moycp4* was not significantly different from that in Guy11, as well as in the complemented strain

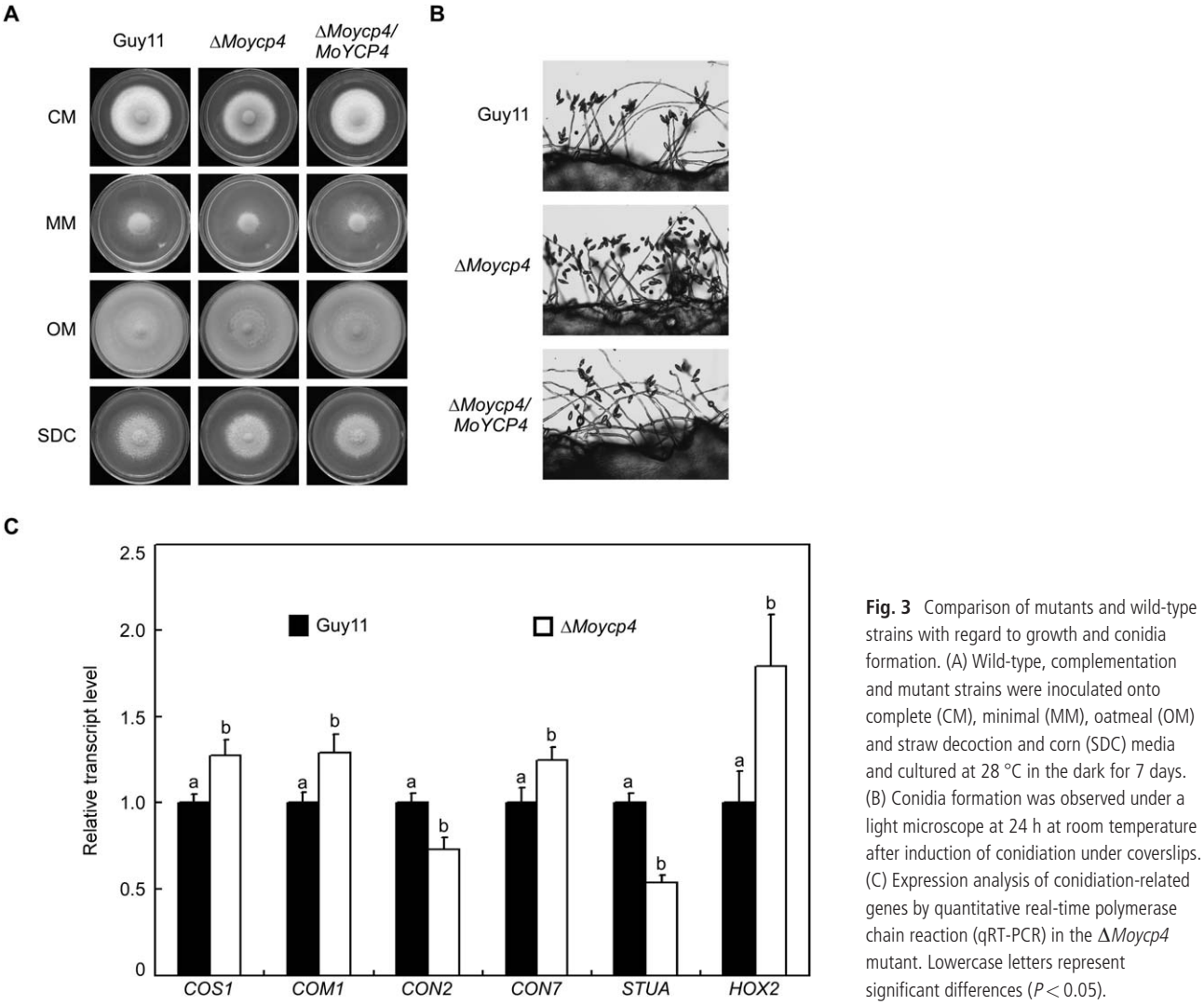

Fig. 3 Comparison of mutants and wild-type strains with regard to growth and conidia formation. (A) Wild-type, complementation and mutant strains were inoculated onto complete (CM), minimal (MM), oatmeal (OM) and straw decoction and corn (SDC) media and cultured at 28 °C in the dark for 7 days. (B) Conidia formation was observed under a light microscope at 24 h at room temperature after induction of conidiation under coverslips. (C) Expression analysis of conidiation-related genes by quantitative real-time polymerase chain reaction (qRT-PCR) in the Δ*Moycp4* mutant. Lowercase letters represent significant differences ($P < 0.05$).

Δ*Moycp4/MoYCP4* (Table 1). These results suggest that MoYcp4 negatively regulates the conidiogenesis of *M. oryzae*.

MoYcp4 is required for full virulence

To determine whether MoYcp4 is involved in pathogenicity, conidial suspensions of the Δ*Moycp4* mutant, wild-type and complemented strain were sprayed onto 2-week-old rice seedlings (cv. CO-39) and detached barley leaves. When observed at 7 days post-infection in rice, the Δ*Moycp4* mutant produced disease lesions, but a remarkable reduction was observed when compared with the lesions caused by the control strain in both size and number (Fig. 7A). Signs of disease on rice plants were also quantified using a 'lesion-type' scoring assay (Valent *et al.*, 1991). The Δ*Moycp4* mutant mainly produced type 1–3 lesions with fewer lesions of types 4 and 5 (severe, coalescing) (Fig. 7B). On detached barley leaves, which were inoculated with three different concentrations of a conidial suspension (10^5, 10^4 and 10^3 spores/mL), the lesions caused by the Δ*Moycp4* mutant were obviously less severe (Fig. 7C). Taken together, these results indicate that MoYcp4 is involved in pathogenicity.

MoYcp4 is required for normal appressorium turgor pressure

To penetrate the rice leaf cuticle and cause infection, a high appressorium internal turgor pressure is required (Talbot and Foster, 2001). Appressorium turgor can be measured using an incipient cytorrhysis assay, which applies hyperosmotic concentrations of a solute to collapse the appressoria (Howard *et al.*, 1991; de Jong *et al.*, 1997). To further elucidate the mechanism underlying virulence in the Δ*Moycp4* mutant, we examined the appressorium turgor in the Δ*Moycp4* mutant and wild-type strain. Surprisingly, the appressoria of the Δ*Moycp4* mutant showed a reduced collapse rate in 2, 3 and 4 M glycerol compared with the appressoria

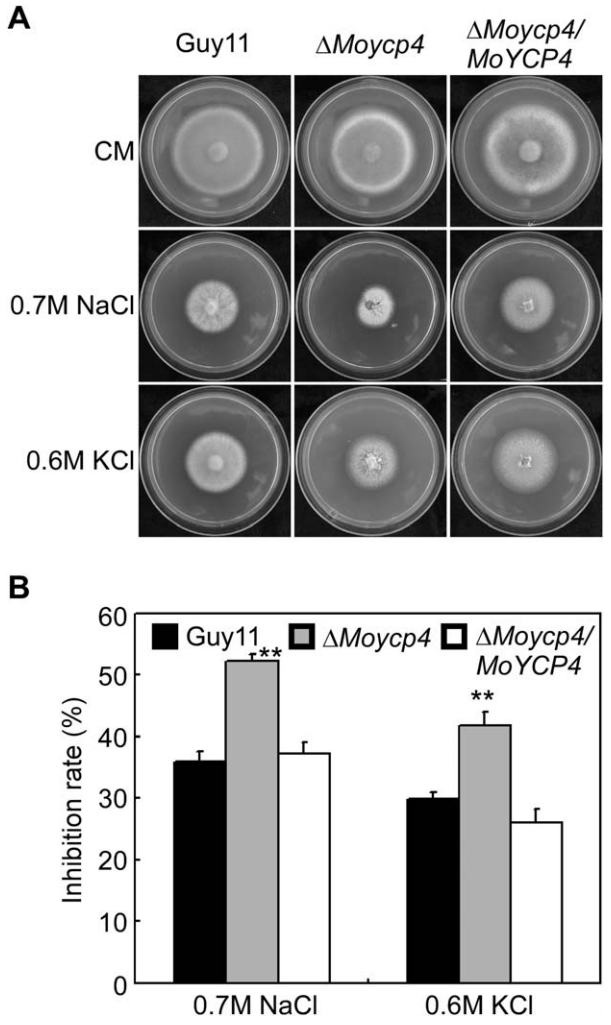

Fig. 4 Δ*Moycp4* mutant ion stress assessment. (A) The Δ*Moycp4* mutant is more sensitive than Guy11 to ion stress. Colonies of the wild-type Guy11, the Δ*Moycp4* mutant and the complemented strains were grown on complete medium (CM) plates with 0.7 M NaCl or 0.6 M KCl, and cultured at 28 °C for 7 days. (B) The growth inhibition rate is estimated relative to the growth rate of each untreated control [inhibition rate = (diameter of untreated strain − diameter of treated strain)/(diameter of untreated strain × 100%)]. Three repeats were performed and similar results were obtained. Error bars represent the standard deviations and double asterisks represents significant differences ($P < 0.01$).

of the wild-type (Fig. 8), indicating that the appressoria of the Δ*Moycp4* mutant produced an abnormal turgor pressure.

Δ*Moycp4* mutant is defective in infectious hyphal growth on plants

To further explore why the Δ*Moycp4* mutant showed reduced virulence on host plants, we performed an infection assay to examine infectious hyphal growth on barley leaves and rice leaf sheaths. Infectious hyphal growth on barley was also evaluated

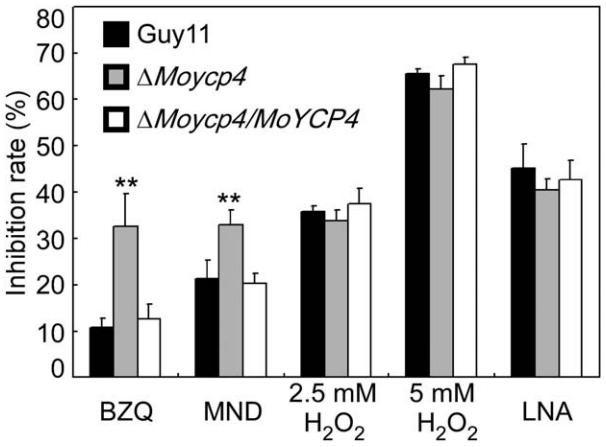

Fig. 5 Δ*Moycp4* mutant oxidation stress assessment. Equal plugs of mycelia from 5-day-old complete medium (CM) plates were transferred into liquid CM containing *p*-benzoquinone (BZQ), menadione (MND), 2.5 mM H_2O_2, 5 mM H_2O_2 and linolenic acid (LNA); the dry weight of mycelia was determined by culture with shaking (150 rpm) at 28 °C for 2 days. The inhibition rate is estimated relative to the growth rate of each untreated control [inhibition rate = (dry weight of untreated strain − dry weight of treated strain)/(dry weight of untreated strain × 100%)]. Three repeats were performed and similar results were obtained. Error bars represent the standard deviations and double asterisks represents significant differences ($P < 0.01$).

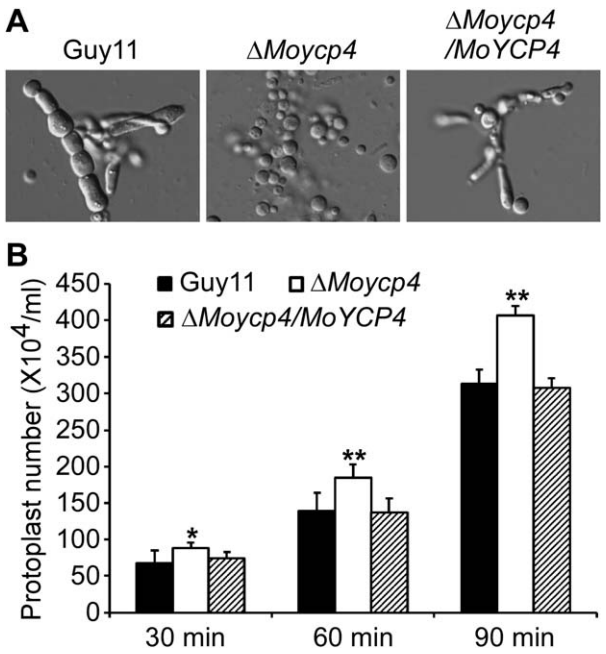

Fig. 6 Protoplast release assay. (A) Protoplasts released on treatment with cell wall-degrading enzymes. Light microscopic examination after 40 min. (B) The protoplasts released were quantified at 30-min intervals. Asterisks represent significant differences ($P < 0.05$)

using an 'invasive hypha type' assay (Wang *et al.*, 2013) at 48 h post-inoculation (hpi) employing spore suspensions; four grades (grade 1, no penetration; grade 2, with a penetration peg; grade

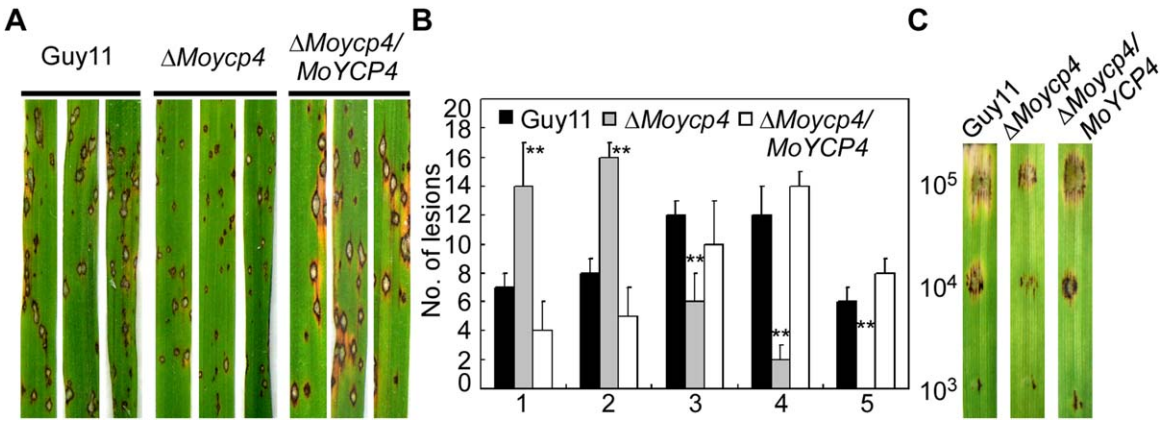

Fig. 7 Pathogenicity assay of the mutant. (A) Leaf spraying assay. Four millilitres of conidial suspension (5×10^4 spores/mL) of each strain were sprayed onto 2-week-old rice seedlings. Diseased leaves were photographed at 7 days after inoculation. (B) Quantification of lesion type (0, no symptom; 1, pinhead-sized brown specks; 2, 1.5-mm brown spots; 3, 2–3-mm grey spots with brown margins; 4, many elliptical grey spots longer than 3 mm; 5, coalesced lesions infecting 50% or more of the leaf area). Lesions were photographed and measured or scored at 7 days post-inoculation (dpi) and experiments were repeated twice with similar results. (C) Conidia of different concentrations were drop inoculated onto barley; the Δ*Moycp4* mutant showed a virulence defect compared with the wild-type and complemented strains, as manifested by smaller lesions at 5 dpi. Double asterisks represent significant differences ($P < 0.01$).

3, with a single invasive hypha; grade 4, with extensive hyphal growth) of invasive hyphae were observed in barley tissues (Fig. 9A). In the wild-type and complemented strains, about 60% of the cells showed grade 4 growth; few strains showed grades 1 and 2 invasive hyphal growth. In contrast, 20% of the cells showed grade 4 and more than 40% of the cells showed grades 1 and 2 invasive hyphal growth in the Δ*Moycp4* mutant (Fig. 9B). A similar result was observed in rice leaf sheaths. In the Δ*Moycp4* mutant, invasive hyphae were mostly restricted to the primary infected leaf sheath cells, in contrast with the free spread of invasive hyphae in the wild-type Guy11 (Fig. 10A). We also examined the expression of the plant defence genes *PR1a* and *PBZ1*, and observed higher transcription levels of *PBZ1* in rice infected with the Δ*Moycp4* mutant when compared with that infected with wild-type Guy11 (Fig. 10B,C). Finally, 3,3′-diaminobenzidine (DAB) staining was performed to detect the reactive oxygen species (ROS) burst at the infection site of Guy11 and the Δ*Moycp4* mutant; however, no distinguishable difference between Guy11 and the Δ*Moycp4* mutant was observed (data not shown).

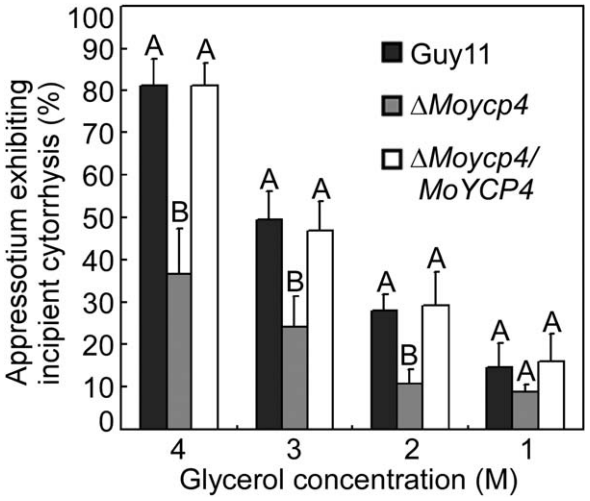

Fig. 8 Collapsed appressoria were observed in the mutant strain. For each glycerol concentration, at least 100 appressoria were observed and the numbers of collapsed appressoria were counted. Capital letters represent significant differences ($P < 0.01$).

DISCUSSION

Our previous studies have shown that the transcription factor MoAP1 mediates the oxidative stress response and is required for *M. oryzae* pathogenicity (Guo *et al.*, 2011). In the Δ*Moap1* mutant SAGE database, many MoAP1-regulated genes, including *MoYCP4* encoding an FLP, play important roles in infection-related morphogenesis. This study increases our understanding of the mechanism involved in the pleiotropic functions of MoAP1 and the molecular mechanisms involved in rice blast.

MoYcp4 is involved in vegetative growth and conidiation in *M. oryzae*. In *S. cerevisiae*, the expression of metabolism genes related to hexose transport and glycolysis is affected, suggesting that the Δ*Scycp4* mutant possesses defects in nutrient absorption and glyco-metabolism (Cardona *et al.*, 2011). In this study, we found that the growth rate of the Δ*Moycp4* mutant was decreased in CM, MM, OM and SDC media; in particular, the colony diameter of the Δ*Moycp4* mutant was significantly lower than that of wild-type Guy11, suggesting that the disruption of *MoYCP4* reduces the ability of the fungus to absorb and utilize nutrients. Further, the Δ*Moycp4* mutant showed defects in cell wall integrity, as it exhibited increased sensitivity to cell wall stress agents and lysis. It is well known that

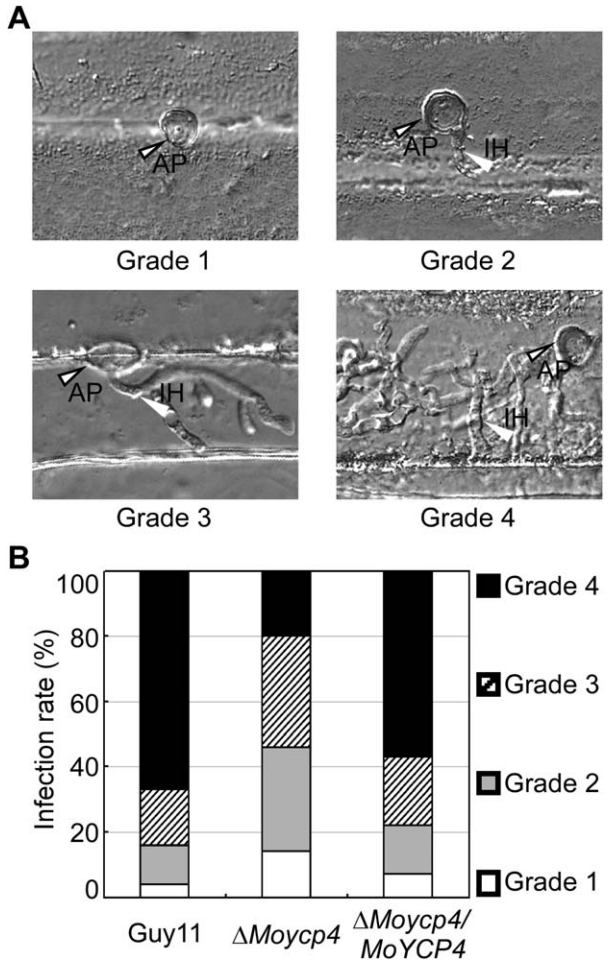

Fig. 9 Infectious hyphal growth on barley leaves. (A) Excised barley leaves from 7-day-old barley seedlings were inoculated with conidial suspension (5×10^4 spores/mL). Infectious growth was observed at 24 h post-inoculation (hpi). (B) Statistical analysis of each type of infectious hyphal shape for each tested strain; 100 infecting hyphae were counted per replicate and the experiment was repeated three times. AP, appressorium; IH, infected hypha. Grade 1, no penetration; grade 2, with a penetration peg; grade 3, with a single invasive hypha; grade 4, with extensive hyphal growth.

the fungal cell wall plays an important role in the maintenance of cell morphogenesis and adaptation to the environment (Levin, 2005). Moreover, previous studies have shown that cell wall integrity-defective mutants exhibit multiple defects in growth and stress responses (Li *et al.*, 2006; Qi *et al.*, 2012). Therefore, it is reasonable to speculate that the defects of growth and osmotic response of the Δ*Moycp4* mutant result from a breach in cell wall integrity.

Previous studies have indicated that FLPs are involved in the oxidative stress response, and *YCP4*, a member of the FLP family, is up-regulated under oxidative stress (Daher *et al.*, 2005; Gasch *et al.*, 2001; Kudo *et al.*, 1999). However, our data showed that the expression level of *MoYCP4* was decreased and the inhibition rate was no different between the Δ*Moycp4* mutant and wild-type when treated with H_2O_2. In addition, MoYcp4 was dispensable for the elimination of ROS of host cells, indicating that MoYcp4 was not sensitive to oxidation stress and such a function might be differentiated in *M. oryzae*. Considering that MoAP1 functioned as a redox sensor and was involved in the regulation of the oxidative stress response (Guo *et al.*, 2011), our further study will focus on the elucidation of why MoYcp4 does not participate in the oxidation response.

The Δ*Moycp4* mutant showed markedly increased conidial production. Combined with our transcript level analysis, *MoYCP4* was obviously up-regulated during the conidial stage, indicating that MoYcp4 is a negative regulator of conidiation. These results are similar to those obtained for MoPac2, which is also regulated by MoAP1. The Δ*Mopac2* mutant produced normal conidia, but the number was increased by about two-fold when compared with wild-type Guy11 (Chen *et al.*, 2014a). Although Δ*Moap1* showed reduced production of conidia and affected conidial morphology, *MoYCP4* and *MoPAC2*, both of which function downstream of MoAP1, showed a different regulatory mechanism to MoAP1. These results indicate that other genes positively control conidiation downstream of MoAP1; a number of genes are known to be involved in this process, including *MoSSADH*, *MoACT1* and *MoGTI1* (Chen *et al.*, 2014a; Guo *et al.*, 2011).

Disruption of *MoYCP4* leads to a defect in infection-related morphogenesis in *M. oryzae*. The Δ*Moycp4* mutant showed reduced pathogenicity in rice and barley. To elucidate the mechanism of reduced pathogenicity, we examined appressorium turgor pressure. We found that the collapse rate of appressoria of the Δ*Moycp4* mutant was less than that of Guy11, suggesting that appressorium turgor in the Δ*Moycp4* mutant was increased. Previous studies have shown that the broken balance of appressorium turgor decreases the infectivity of a fungus (Zhang *et al.*, 2009). As expected, invasive growth of the Δ*Moycp4* mutant was inhibited in rice sheaths, and the expansion ratio was markedly lower than that for wild-type Guy11 in barley epidermis. Moreover, PBZ1, a key component in the jasmonic acid (JA) pathway and involved in JA-induced plant defence (Mei *et al.*, 2006), showed a stronger activation in mutant-inoculated plants compared with controls. We conclude that these factors contribute to the decreased pathogenicity of the Δ*Moycp4* mutant.

Collectively, we have identified an important pathogenic factor, MoYcp4, which functions downstream of MoAP1. Our results indicate that MoYcp4 plays an important role in growth, conidiation, infection and pathogenicity in *M. oryzae*.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Magnaporthe oryzae Guy11 strain was used as the wild-type in this study. All strains were cultured on CM agar plates for 3–15 days at 28 °C (Talbot *et al.*, 1993). Fungal mycelia were harvested from liquid CM and used for

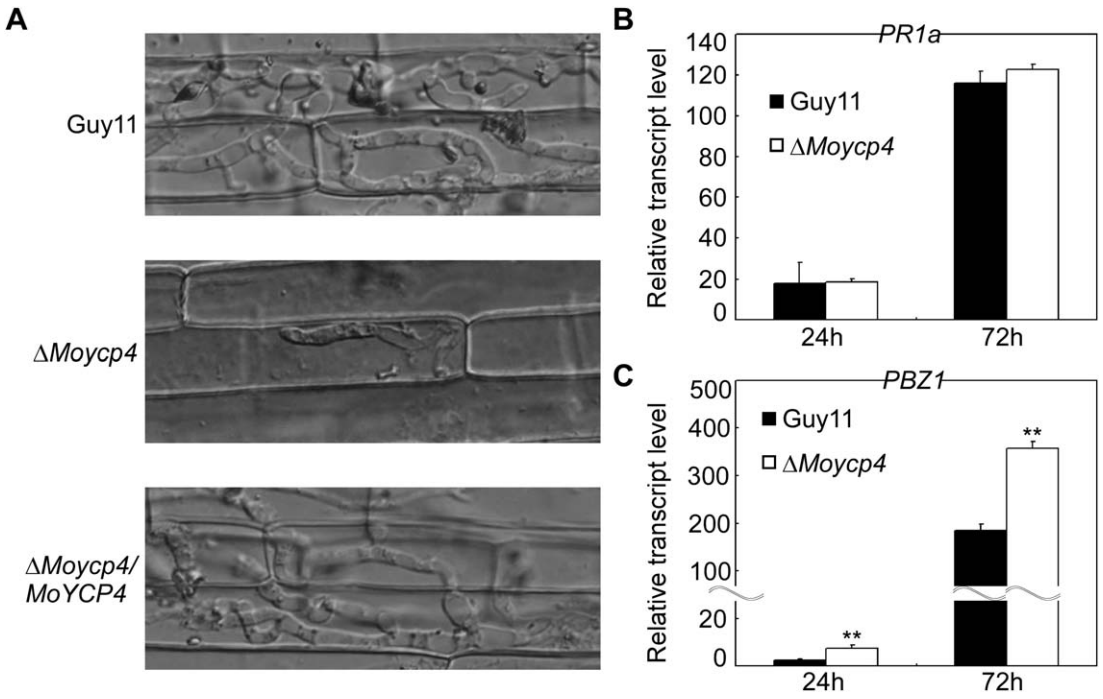

Fig. 10 Close observation of infectious growth. (A) Excised rice sheath from 4-week-old rice seedling was inoculated with conidial suspension (5×10^4 spores/mL). Infectious growth was observed 48 h after inoculation. (B, C) The transcription of *PR1a* and *PBZ1* in the infected host was assayed using quantitative real-time polymerase chain reaction (qRT-PCR). Three independent biological experiments with three replicates each time were performed, and similar results were obtained each time. Double asterisks represent significant differences ($P < 0.01$).

genomic DNA and RNA extractions. Protoplasts were prepared and transformed as described by Sweigard *et al.* (1992). Transformants were selected on TB3 medium (3 g of yeast extract, 3 g of casamino acids, 200 g of sucrose, 7.5 g of agar in 1 L of distilled water) with 300 μg/mL hygromycin B (Roche, San Francisco, California, USA) or 200 μg/mL zeocin (Invitrogen, Shanghai, China). For conidiation, strain blocks were maintained on RDC medium at 28 °C for 7 days in the dark, followed by 3 days of continuous illumination under fluorescent light (Zhang *et al.*, 2009).

Yeast Δ*Scycp4* mutant complementation

The full lengths of *MoYCP4* and *ScYCP4* cDNA were amplified using primers FL13475/FL13476 and FL13477/FL13478 (Table S1, see Supporting Information), respectively. The PCR products were digested with *EcoRI*/*SphI* and *XhoI*/*SphI*, respectively, cloned into the pYES2 vector (Invitrogen) and transformed into the Δ*Scycp4* mutant. Colonies were selected on synthetic drop-out (SD) medium without uracil. As a control, the wild-type strain BY4742 and the Δ*Scycp4* mutant were transformed with the empty pYES2 vector. Yeast cells were incubated on liquid YPD medium (2% glucose, 2% peptone and 1% yeast extract) and aliquots (5 μL) of a 10-fold serial dilution were grown on SD-NaCl (galactose + 0.7 M NaCl) and SD-KCl (galactose + 0.6 M KCl) plates at 30 °C for 4 days and photographed.

MoYCP4 gene disruption and Δ*Moycp4* mutant complementation

The ligation PCR approach (Zhao *et al.*, 2004) was used to generate the *MoYCP4* gene replacement constructs. Approximately 1-kb upstream and

Table 1 Comparison of mycological characteristics among strains.

	Mycelial growth* (cm)					
Strain	CM	MM	OM	SDC	Conidiation† ($\times 10^4$/cm^2)	Appressorial formation‡(%)
Guy11	3.90 ± 0.10^A	3.53 ± 0.05^A	3.87 ± 0.15^A	4.03 ± 0.15^A	3.72 ± 0.39^A	96.00 ± 2.79^A
Δ*Moycp4*	3.60 ± 0.10^A	3.1 ± 0.10^B	3.03 ± 0.15^B	3.70 ± 0.10^B	50.22 ± 2.79^B	92.67 ± 2.79^A
Δ*Moycp4/MoYCP4*	4.00 ± 0.20^A	3.63 ± 0.12^A	3.63 ± 0.12^A	4.10 ± 0.10^A	4.34 ± 0.80^A	93.33 ± 2.36^A

*Diameter of hyphal radii at day 7 after incubation on complete (CM), minimal (MM), oatmeal (OM) and straw decoction and corn (SDC) media plates at room temperature.

†Number of conidia harvested from a 9-cm SDC plate at day 10 after incubation at room temperature.

‡Percentage of appressorium formation on an artificial surface at 24 h post-incubation at room temperature.

Capital letters represent significant differences ($P < 0.01$).

Table 2 Δ*Moycp4* mutant cell wall stress response (inhibition rate).

	Guy11	Δ*Moycp4*	Δ*Moycp4/MoYCP4*
0.05% SDS	34.69 ± 0.59A	35.04 ± 3.37A	35.01 ± 1.71A
0.08% SDS	45.54 ± 1.46A	54.99 ± 1.67B	47.55 ± 2.17A
0.10% SDS	54.33 ± 1.77A	69.48 ± 1.31B	55.35 ± 2.10A
200 μg/mL CFW	5.70 ± 0.51A	7.94 ± 1.48A	5.39 ± 1.37A
400 μg/mL CFW	9.04 ± 0.42A	10.69 ± 1.31A	9.20 ± 0.48A
600 μg/mL CFW	15.05 ± 0.85A	23.59 ± 2.04B	17.54 ± 1.06A
200 μg/mL CR	10.78 ± 0.61A	11.11 ± 1.42A	10.12 ± 2.25A
400 μg/mL CR	15.80 ± 0.25A	23.94 ± 1.66B	15.87 ± 1.22A
600 μg/mL CR	21.00 ± 1.04A	27.24 ± 1.90B	21.27 ± 1.58A

CFW, calcofluor white; CR, Congo red; SDS, sodium dodecylsulfate.
Capital letters represent significant differences ($P < 0.01$)

downstream flanking sequences of the *MoYCP4* gene were amplified by PCR with the primer pairs FL12705/FL12706 and FL12707/FL12708 (Table S1), respectively. The resulting PCR products of primer pairs FL12705/FL12706 and primer pairs FL12707/FL12708 were digested with *XhoI*/*HindIII* and *SpeI*/*SacI*, respectively, and then purified and orderly ligated to vector pCX62. The *MoYCP4* gene replacement constructs were transformed into protoplasts of Guy11. Putative Δ*Moycp4* mutants were identified by PCR and further confirmed by Southern blot analyses. For complementation assays, the full length (except stop codon) of the *MoYCP4* gene, including the native promoter, was amplified and cloned into the bleomycin-resistant vector pYF11 by the yeast *in vivo* recombination approach (Bruno *et al.*, 2004; Zhou *et al.*, 2011) and transformed into the Δ*Moycp4* mutant.

Vegetative growth, stress response and protoplast release assay

Vegetative growth of Δ*Moycp4* and Guy11 was measured on CM (50 mL 20 × nitrate salts, 1 mL trace elements, 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 mL vitamin solution and 15 g agar in 1 L distilled water), MM (6 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO_4$, 1.52 g KH_2PO_4, 10 g glucose, 0.5% biotin and 15 g agar in 1 L distilled water), OM (30 g oatmeal and 10 g agar in 1 L distilled water) and SDC medium (100 g straw, 40 g corn powder and 15 g agar in 1 L distilled water) for 7 days. Mycelial plugs of equal size, from 5-day-old CM plates, were transferred into liquid CM. The mycelia were cultured with shaking (150 rpm) at 28 °C for 2 days. All growth assays were repeated three times, with three replicates each time.

Mycelial plugs (5 mm × 5 mm) were placed onto freshly prepared CM agar plates with NaCl (0.7 M), KCl (0.6 M), SDS (0.05%, 0.08% and 0.1%), CFW (200, 400 and 600 μg/mL) and CR (200, 400 and 600 μg/mL) and cultured in the dark at 28 °C to determine their effects on fungal growth. The size of the colonies were measured and photographed after 7 days of incubation. The inhibition rate was determined by the percentage decrease in the colony diameter (Zhang *et al.*, 2014). The experiment was repeated three times with three replicates each time.

For protoplast release assays, mycelia were cultured in liquid CM for 48 h and harvested by filtration; they were then resuspended in 0.7 M NaCl with lysing enzyme (7.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and placed in a shaker (70 rpm) at 28 °C. Lysis activity was stopped after 30, 60 and 90 min of incubation, and protoplasts were counted under a light microscope using a haemocytometer.

Nucleic acid manipulation, Southern blotting and EMSA

The standard Southern blot protocol was utilized (Sambrook and Russell, 2001). The target gene probe and HPH probe were amplified with primer pairs FL6084/FL6085 (for *MoYCP4*) (Table S1) and FL1111/FL1112 (for *HPH*), respectively. Probe labelling, hybridization and detection were performed with the DIG High Prime DNA Labelling and Detection Starter Kit (Roche Applied Science, Penzberg, Germany). Total RNA was isolated from frozen fungal mycelia using the RNA extraction kit (Invitrogen, Carlsbad, California, USA). To measure the relative abundance of gene transcripts, RNAs were extracted from mycelia grown in CM liquid medium for 2 days at 28 °C in a 150 rpm orbital shaker.

The MoAP1 protein was expressed and purified from *Escherichia coli* strain Rosetta using the pGEX4T-2 construct. The DNA fragment from the *MoYCP4* promoter was end labelled with Alex660 by PCR amplification using the 5′ Alex660-labelled primer. The purified protein was mixed with Alex660-labelled DNA and incubated for 20 min at 25 °C in binding buffer, and then 10× loading buffer was added. The reaction mixtures were separated by electrophoresis in 1% agarose with 1 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Gels were visualized using a LI-COR Odyssey scanner (Pleasanton, California, USA) with excitation at 700 nm.

Conidiation, appressorium formation, turgor analysis and examination of the *pathogenesis-related* (*PR*) gene transcription level

For conidiation, 10-day-old conidia were collected with 5 mL of distilled water, filtered through three layers of lens paper and counted with a haemocytometer under a microscope. Conidial germination and appressorium formation were measured on a hydrophobic surface. Conidial suspensions of 30 μL (5×10^4 spores/mL) were dropped onto a hydrophobic surface and placed in a moistened box at 28 °C (Zhang *et al.*, 2011). The appressorium formation rate was counted at 24 hpi under the microscope; more than 200 appressoria were counted for each strain. Photographs were taken at 24 hpi. The appressorium turgor was measured using an incipient cytorrhysis (cell collapse) assay and a 1–4 M glycerol solution (Howard *et al.*, 1991). Droplets (20 μL) of the conidial suspensions (5×10^4 spores/mL) were placed on plastic coverslips and incubated in a humid chamber for 24 h at room temperature. The water surrounding the conidia was removed carefully and then replaced with an equal volume (20 μL) of glycerol in the concentration range 1–4 M. The number of appressoria that had collapsed after 10 min was recorded. The experiment was repeated three times, and >100 appressoria were observed for each replicate. To measure the relative abundance of rice *PR* gene transcripts, conidial suspensions of the wild-type and Δ*Moycp4* mutant were inoculated onto rice leaves for 24 and 72 h, respectively, and plant total RNA samples were extracted according to a previous method (Chen *et al.*, 2014b).

Plant infection and penetration assays

Plant infection assays were performed on 4-week-old susceptible rice seedlings (*Oryza sativa*) CO-39 by spraying 4 mL of a conidial suspension (5×10^4 conidia/mL in 0.2% gelatin) with a sprayer. Inoculated plants were placed in a moist chamber at 28 °C for the first 24 h in darkness, and then transferred to another moist chamber with a photoperiod of 12 h under fluorescent light. The disease severity was assessed at 7 days after inoculation. Approximately 6-cm-long diseased rice blades were photographed to evaluate the virulence of the mutants (Chen *et al.*, 2014b). For microscopic observation of penetration and infectious hyphal expansion on rice and barley tissue, rice was inoculated with 100 μL of conidial suspension (5×10^4 spores/mL) on inner leaf sheath cuticle cells and barley was inoculated with 30 μL of conidial suspension (5×10^4 spores/mL) on the underside of the leaves; after 48 h (rice) and 24 h (barley) of incubation under humid conditions at 28 °C, the leaf sheaths and barley leaves were observed under a microscope (Chen *et al.*, 2015).

ACKNOWLEDGEMENTS

This research was supported by the Natural Science Foundation of China (Grant No. 31271998 to Z.Z. and Grant No. 31501599 to Y.C.). We thank Yan Wang of Nanjing Agricultural University for critical review of the manuscript and for providing helpful suggestions.

REFERENCES

Bourett, T.M. and Howard, R.J. (1991) *In vitro* development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can. J. Bot.* **68**, 329–342.

Bruno, K.S., Tenjo, F., Li, L., Hamer, J.E. and Xu, J.R. (2004) Cellular localization and role of kinase activity of *PMK1* in *Magnaporthe grisea*. *Eukaryot. Cell*, **3**, 1525–1532.

Caracuel-Rios, Z. and Talbot, N.J. (2007) Cellular differentiation and host invasion by the rice blast fungus *Magnaporthe grisea*. *Curr. Opin. Microbiol.* **10**, 339–345.

Cardona, F., Orozco, H., Friant, S., Aranda, A. and del Olmo, M. (2011) The *Saccharomyces cerevisiae* flavodoxin-like proteins Ycp4 and Rfs1 play a role in stress response and in the regulation of genes related to metabolism. *Arch. Microbiol.* **193**, 515–525.

Chen, Y., Zhai, S., Zhang, H., Zuo, R., Wang, J., Guo, M., Zheng, X., Wang, P. and Zhang, Z. (2014a) Shared and distinct functions of two Gti1/Pac2 family proteins in growth, morphogenesis and pathogenicity of *Magnaporthe oryzae*. *Environ. Microbiol.* **16**, 788–801.

Chen, Y., Zuo, R., Zhu, Q., Sun, Y., Li, M., Dong, Y., Ru, Y., Zhang, H., Zheng, X. and Zhang, Z. (2014b) MoLys2 is necessary for growth, conidiogenesis, lysine biosynthesis, and pathogenicity in *Magnaporthe oryzae*. *Fungal Genet. Biol.* **67**, 51–57.

Chen, Y., Zhai, S., Sun, Y., Li, M., Dong, Y., Wang, X., Zhang, H., Zheng, X., Wang, P. and Zhang, Z. (2015) MoTup1 is required for growth, conidiogenesis and pathogenicity of *Magnaporthe oryzae*. *Mol. Plant Pathol.* **16**, 799–810.

Daher, B.S., Venancio, E.J., de Freitas, S.M., Bao, S.N., Vianney, P.V., Andrade, R.V., Dantas, A.S., Soares, C.M., Silva-Pereira, I. and Felipe, M.S. (2005) The highly expressed yeast gene pby20 from *Paracoccidioides brasiliensis* encodes a flavodoxin-like protein. *Fungal Genet. Biol.* **42**, 434–443.

Do, T.Q., Schultz, J.R. and Clarke, C.F. (1996) Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. USA*, **93**, 7534–7539.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmelharel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2001) Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell*, **11**, 4241–4257.

Guo, M., Chen, Y., Du, Y., Dong, Y., Guo, W., Zhai, S., Zhang, H., Dong, S., Zhang, Z., Wang, Y., Wang, P. and Zheng, X. (2011) The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog.* **7**, e1001302.

Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, N.P. (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA*, **88**, 11 281–11 284.

de Jong, J.C., McCormack, B.J., Smirnoff, N. and Talbot, N.J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244–245.

Kim, S., Park, S.Y., Kim, K.S., Rho, H.S., Chi, M.H., Choi, J., Park, J., Kong, S., Park, J., Goh, J. and Lee, Y.H. (2009) Homeobox transcription factors are required for conidiation and appressorium development in the rice blast fungus *Magnaporthe oryzae*. *PLoS Genet.* **5**, e1000757.

Kudo, N., Taoka, H., Toda, T., Yoshida, M. and Horinouchi, S. (1999) A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *J. Biol. Chem.* **274**, 15 151–15 158.

Levin, D.E. (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **69**, 262–291.

Li, S., Myung, K., Guse, D., Donkin, B., Proctor, R.H., Grayburn, W.S. and Calvo, A.M. (2006) FvVE1 regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in *Fusarium verticillioides*. *Mol. Microbiol.* **62**, 1418–1432.

Liu, W., Xie, S., Zhao, X., Chen, X., Zheng, W., Lu, G., Xu, J.R. and Wang, Z. (2010) A homeobox gene is essential for conidiogenesis of the rice blast fungus *Magnaporthe oryzae*. *Mol. Plant–Microbe Interact.* **23**, 366–375.

Mei, C., Qi, M., Sheng, G. and Yang, Y. (2006) Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. *Mol. Plant–Microbe Interact.* **19**, 1127–1137.

Morschhäuser, J., Li, L., Naseem, S., Sharma, S. and Konopka, J.B. (2015) Flavodoxin-like proteins protect *Candida albicans* from oxidative stress and promote virulence. *PLOS Pathog.* **11**, e1005147.

Nishimura, M., Fukada, J., Moriwaki, A., Fujikawa, T., Ohashi, M., Hibi, T. and Hayashi, N. (2009) Mstu1, an APSES transcription factor, is required for appressorium-mediated infection in *Magnaporthe grisea*. *Biosci. Biotechnol. Biochem.* **73**, 1779–1786.

Ou, S.H. (1985) *Rice Diseases*. Kew, Surrey: Commonwealth Mycological Institute.

Qi, Z., Wang, Q., Dou, X., Wang, W., Zhao, Q., Lv, R., Zhang, H., Zheng, X., Wang, P. and Zhang, Z. (2012) MoSwi6, an APSES family transcription factor, interacts with MoMps1 and is required for hyphal and conidial morphogenesis, appressorial function and pathogenicity of *Magnaporthe oryzae*. *Mol. Plant Pathol.* **13**, 677–689.

Sambrook, J. and Russell, D. (2001) *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Shi, Z., Christian, D., Fau-Leung, H. and Leung, H. (1998) Interactions between spore morphogenetic mutations affect cell types, sporulation, and pathogenesis in *Magnaporthe grisea*. *Mol. Plant–Microbe Interact.* **11**, 199–207.

Sweigard, J.A., Chumley, F.G. and Valent, B. (1992) Disruption of a *Magnaporthe grisea* cutinase gene. *Mol. Gen. Genet.* **232**, 183–190.

Talbot, N.J. and Foster, A.J. (2001) Genetics and genomics of the rice blast fungus *Magnaporthe grisea*: developing an experimental model for understanding fungal diseases of cereals. In: *Advances in Botanical Research Incorporating Advances in Plant Pathology* (Jacqot, J.-P., ed.) Vol.**34**, pp. 263–287, Elsevier.

Talbot, N.J., Ebbole, D.J. and Hamer, J.E. (1993) Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell*, **5**, 1575–1590.

Valent, B., Farral, L. and Chumley, F.G. (1991) *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics*, **127**, 87–101.

Wang, J., Du, Y., Zhang, H., Zhou, C., Qi, Z., Zheng, X., Wang, P. and Zhang, Z. (2013) The actin-regulating kinase homologue MoArk1 plays a pleiotropic function in *Magnaporthe oryzae*. *Mol. Plant Pathol.* **14**, 470–482.

Yang, J., Zhao, X., Sun, J., Kang, Z., Ding, S., Xu, J.R. and Peng, Y.L. (2010) A novel protein Com1 is required for normal conidium morphology and full virulence in *Magnaporthe oryzae*. *Mol. Plant–Microbe Interact.* **23**, 112–123.

Zhang, H., Tang, W., Liu, K., Huang, Q., Zhang, X., Yan, X., Chen, Y., Wang, J., Qi, Z., Wang, Z., Zheng, X., Wang, P. and Zhang, Z. (2011) Eight RGS and RGS-like

proteins orchestrate growth, differentiation, and pathogenicity of *Magnaporthe oryzae*. *PLoS Pathog.* **7**, e1002450.

Zhang, H., Zhao, Q., Guo, X., Guo, M., Qi, Z., Tang, W., Dong, Y., Ye, W., Zheng, X., Wang, P. and Zhang, Z. (2014) Pleiotropic function of the putative zinc-finger protein MoMsn2 in *Magnaporthe oryzae*. *Mol. Plant–Microbe Interact.* **27**, 446–460.

Zhang, H.F., Zhao, Q., Liu, K.Y., Zhang, Z.G., Wang, Y.C. and Zheng, X.B. (2009) *MgCRZ1*, a transcription factor of *Magnaporthe grisea*, controls growth, development and is involved in full virulence. *FEMS Microbiol. Lett.* **293**, 160–169.

Zhao, X.H., Xue, C., Kim, Y. and Xu, J.R. (2004) A ligation-PCR approach for generating gene replacement constructs in *Magnaporthe grisea*. *Fungal Genet. Newsl.* **51**, 17–18.

Zhou, X., Liu, W., Wang, C., Xu, Q., Wang, Y., Ding, S. and Xu, JR. (2011) A MADS-box transcription factor MoMcm1 is required for male fertility, microconidium production and virulence in *Magnaporthe oryzae*. *Mol Microbiol.* **80**, 33–53.

Zhou, Z., Li, G., Lin, C. and He, C. (2009) Conidiophore stalk-less1 encodes a putative zinc-finger protein involved in the early stage of conidiation and mycelial infection in *Magnaporthe oryzae*. *Mol. Plant–Microbe Interact.* **22**, 402–410.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 The *MoYCP4* gene rescued the defect of the Δ*Scycp4* mutant. The Δ*Scycp4* mutant was transformed with the pYES2::*MoYCP4* construct encoding MoYcp4 and pYES2::*ScYCP4* construct encoding ScYcp4. Serial dilutions of cultures were grown overnight on SD (galactose+0.7M NaCl) and SD (galactose+0.6M KCl) plates, and grown at 30 °C for 4 days and photographed. The experiment was repeated three times and representative results were obtained.

Fig. S2 Targeted gene replacement and complementation. (A) A 955-bp fragment of the *MoYCP4* coding region were replaced by a 1.4-kb fragment containing the hygromycin B resistance cassette to create the Δ*Moycp4* a alleles, respectively. (B) Southern hybridization analysis was used to validate the deletion of *MoYCP4* gene and the addition of a single copy integration of the *HPH* gene. (C) Semiquantitative RT-PCR was carried out to confirm the deletion and reintroduction of *MoYCP4* gene.

Fig. S3 Specificity and competition assays of MoAP1. (A) The DIG-labeled promoter DNA of *MoYCP4* and other gene *MoLYS2* was incubated in the absence or presence of purified MoAp1 protein, respectively. (B) Increasing amounts of unlabeled competitor (promoter DNA of *MoYCP4*) were mixed with the DIG-labeled promoter DNA of *MoYCP4*, then the complex were incubated in the purified MoAP1 protein.

Fig. S4 Expression and localization of *MoYCP4-GFP* fusion gene. Conidium, appressoria and infectious hyphae of the Δ*Moycp4/MoYCP4-GFP* transformant examined under an epifluorescence microscope.

Table S1 Primers used in this study.