Fungal Genetics and Biology 61 (2013) 133–141

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

Two conidiation-related $Zn(II)_2Cys_6$ transcription factor genes in the rice blast fungus

Hyunjung Chung [a,1], Jaehyuk Choi [b,1], Sook-Young Park [b], Junhyun Jeon [a], Yong-Hwan Lee [a,b,c,*]

[a] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea
[b] Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Republic of Korea
[c] Center for Fungal Genetic Resources, Plant Genomics and Breeding Institute, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea

ARTICLE INFO

Article history:
Received 26 July 2013
Accepted 8 October 2013
Available online 15 October 2013

Keywords:
Binucleate zinc cluster
Conidiation
Magnaporthe oryzae
Rice blast
Transcription factor

ABSTRACT

Regulation of gene expression by transcription factors (TFs) helps plant pathogens to interact with the host plants and to sustain a pathogenic lifestyle in the environmental changes. Elucidating novel functions of TFs is, therefore, crucial for understanding pathogenesis mechanisms of plant pathogens. *Magnaporthe oryzae*, the rice blast pathogen, undergoes a series of developmental morphogenesis to complete its infection cycle. To understand TF genes implicated in pathogenic development of this fungus, two $Zn(II)_2Cys_6$ TF genes, *MoCOD1* and *MoCOD2*, whose expression was notably induced during conidiation, were functionally characterized. Targeted deletion of *MoCOD1* resulted in defects in conidiation and pathogenicity due to defects in appressorium formation and invasive growth within the host cells. MoCOD2 was also a critical regulator in conidiation and pathogenicity, but not in conidial germination and appressorium formation. When rice plants were inoculated with conidia of the Δ*Mocod2* mutant, rapid accumulation of dark brown granules was observed around the infection sites in the plant cells and no visible disease symptom was incited. Taken together, both MoCOD1 and MoCOD2 play important roles in conidiation and pathogenicity of the rice blast fungus.

1. Introduction

Rice blast caused by *Magnaporthe oryzae* has been the most serious disease in all rice-growing areas worldwide. The annual yield loss of rice by blast disease would be enough to feed more than 60 million people (Khush and Jena, 2009). The *Oryza sativa–M. oryzae* pathosystem has been a model to study plant–fungal interactions not only due to socioeconomic importance but also genetic tractability of this fungus (Dean et al., 2005; Ebbole, 2007).

Conidia (asexual spores) play an important role in the disease cycle of *M. oryzae*. Three to five conidia are produced successively on conidiophore in a sympodial manner (Howard, 1994). In general, each conidium has the three-celled and pyriform structure (Howard and Valent, 1996; Ou, 1985). Mature conidia are released by dew or rain and are dispersed to new hosts via wind or splash. Upon landing on the waxy surface of a rice leaf, the conidium starts to germinate and, at the tip of the germ tube, develops a dome-shaped infection structure, called an appressorium. For appressorium formation, hydrophobicity on the leaf surface is recognized by the germ tube tip and signals are transduced through cyclic AMP-dependent protein kinase A pathway (Choi et al., 1998; Lee and Dean, 1993; Mitchell and Dean, 1995; Xu et al., 1997). When the appressorium is melanized, high turgor pressure (>8 MPa) is generated by accumulation of solutes, such as glycerol (Bourett and Howard, 1990; de Jong et al., 1997). A penetration peg emerges at the bottom of the appressorium with high turgor pressure (Bourett and Howard, 1990) and then differentiates to bulbous invasive hyphae that occupy the infected cell (Kankanala et al., 2007). After filling the first-invaded cell, infectious hyphae extensively colonize the neighboring cells (Koga et al., 2004). In general, visible lesions are observed at 5–7 days post inoculation. This infection process of *M. oryzae* is complex and not yet fully understood. However, thousands of proteins might be involved in this process and be orchestrated by transcription factors (TFs) in *M. oryzae*.

TFs are essential for modulating such a variety of biological processes by promoting or repressing gene expression. In an effort to understand fungal TFs, an informatics pipeline of Fungal Transcription Factor Database (FTFD; http://ftfd.snu.ac.kr) was developed

* Corresponding author at: Department of Agricultural Biotechnology, Center for Fungal Pathogenesis, Center for Fungal Genetic Resources, Plant Genomics and Breeding Institute, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea. Fax: +82 2 873 2317.
E-mail address: yonglee@snu.ac.kr (Y.-H. Lee).
[1] These authors contributed equally to the paper.

1087-1845
http://dx.doi.org/10.1016/j.fgb.2013.10.004

where 66,355 putative fungal TFs (61 families) were identified from 163 fungi and 6 Oomycetes (Park et al., 2008b). In the *M. oryzae* 70–15 genome (ver. 8), 481 putative TF genes are identified and correspond to 4.02% of 12,991 genes. The largest group of TFs is the $Zn(II)_2Cys_6$ family that has been found exclusively in fungi. This TF family has DNA-binding domain containing six cysteine residues which bind two zinc atoms (MacPherson et al., 2006; Park et al., 2008b). Many TF genes have been reported to be involved in pathogenesis in *M. oryzae* (described in detail in Section 4). For example, the mutation of the *COS1* gene encoding C_2H_2 type zinc finger TF, exhibited a conidiophore-less phenotype resulting in no conidia (Zhou et al., 2009). Another C_2H_2 type zinc finger TF mutant, *con7*$^-$ produced two types of abnormal conidia and showed no pathogenicity due to defects in appressorium formation (Odenbach et al., 2007). The homeobox TF mutant, Δ*Mohox2*, can produce conidiophores without forming any conidium on the tips of its conidiophores (Kim et al., 2009). However, only one $Zn(II)_2Cys_6$ TF gene, *PIG1* (MGG_07215), has been characterized as a regulator of melanin biosynthesis in *M. oryzae* (Tsuji et al., 2000), although 174 (36.2%) TF genes belong to the family (Park et al., 2008b). Recently, we performed a genome-wide profiling analysis for gene expression during conidiation using a DNA microarray system. In the gene expression profiles, 1160 genes (8.4% of 13,666 probes) were differentially regulated in response to aeration. Interestingly, two $Zn(II)_2Cys_6$ TF genes, MGG_05343 and MGG_09263, were highly up-regulated during conidiation RNA samples (Kim and Lee, 2012). The genes were named *MoCOD1* (*M. oryzae* COnidia Development) and *MoCOD2*, respectively. To understand roles of the $Zn(II)_2Cys_6$ TF family, two $Zn(II)_2Cys_6$ TF genes were functionally characterized in *M. oryzae*. *MoCOD1* and *MoCOD2* are involved in conidiation and pathogenicity by modulating appressorium formation or inducing strong plant defense. This would be the first report on important roles of $Zn(II)_2Cys_6$ TF genes in fungal pathogenicity in plants.

2. Materials and methods

2.1. Strains and culture conditions

M. oryzae wild-type strain KJ201 and all mutants used in this study were incubated on oatmeal agar medium (OMA, 5% oat meal (w/v), 2.5% agar powder (w/v)) or V8 juice agar medium (8% V8 juice, 1.5% agar powder (w/v), pH 6.7) at 25 °C under the constant fluorescent light. Complete medium (CM) broth (0.6% yeast extract (w/v), 0.6% tryptone (w/v), 1% sucrose (w/v)) was used for mycelial harvest.

2.2. Fungal transformation

Fungal transformation was carried out as previously described (Goh et al., 2011). In brief, the gene deletion mutants were generated by gene replacement with hygromycin B phosphotranferase gene (*HPH*) cassette via homologous recombination. The *HPH* cassette amplified from pBCATPH (Choi et al., 2009) and fused with both flanking regions of *MoCOD1* and *MoCOD2* genes (Yu et al., 2004). The primers used for PCR are listed in Supplementary Table 1. In detail, UF and UR primers were used for amplification of the upstream flanking region, and DF and DR primers amplified downstream region. PEG-mediated transformation was performed using wild-type protoplasts. Hygromycin-resistant transformants were selected on TB3 agar medium (0.3% yeast extract (w/v), 0.3% casamino acids (w/v), 1% glucose (w/v), 20% sucrose (w/v) and 0.8% agar powder (w/v)) supplemented with hygromycin B (200 ppm in final concentration) or geneticin (800 ppm in final concentration). Genetic complementation was performed by transforming both mutant protoplasts with their original genes and promoters fused with the geneticin resistance cassette amplified from pII99 (Yi et al., 2009). The Δ*Mocod1*/Δ*Mocod2* double mutant was generated using the Δ*Mocod2* competent cells with the geneticin resistance cassette for *MoCOD1*. All strains were deposited in the Center for Fungal Genetic Resources at Seoul National University, Seoul, Korea (http://genebank.snu.ac.kr).

2.3. Southern blot analysis

Genomic DNA was extracted by quick and safe method (Chi et al., 2009b) or standard protocols (Choi et al., 2007). Agarose gel separation, restriction enzyme digestion and Southern hybridization analysis were performed following the standard procedures (Sambrook and Russel, 2001). DNA fragments for DNA hybridization probes were labeled with ^{32}P by using Rediprime™ II Random Prime Labeling System kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's manuals.

2.4. Real-time quantitative reverse transcription PCR

Total RNA was extracted by using the Easy-Spin™ total RNA extraction kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturer's instruction. For real-time quantitative reverse transcription PCR (qRT-PCR), 5 μg of total RNA was used and cDNA synthesis was performed using the oligo dT primer with the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's instruction. qRT-PCR reactions were performed in 10 μl solution containing 2 μl of cDNA template (12.5 ng/μl), 3 μl of forward and reverse primers (100 nM concentration for each) and 5 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Samples were run for 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C after 3 m of denaturation at 95 °C on AB7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The average of threshold cycle (Ct) was normalized to that of *β-tubulin* gene for each of the treated samples as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (C_{t,\ \text{target gene}} - C_{t,\ \beta\text{-tubulin}})_{\text{treated}} - (C_{t,\ \text{target gene}} - C_{t,\ \beta\text{-tubulin}})_{\text{control}}$ (Kwon et al., 2010).

2.5. Sequence analysis

Nucleotide and protein sequences were analyzed by using the computer programs provided at the Comparative Fungal Genomics Platform (http://cfgp.snu.ac.kr/) (Park et al., 2008a) and the BLAST program provided at the National Center for Biotechnology Information (NCBI), Bethesda, USA (http://www.ncbi.nlm.nih.gov/blast/) (McGinnis and Madden, 2004). Sequences were aligned by ClustalW algorithm (Thompson et al., 1994) and phylogenetic trees were constructed using the neighbor-joining method at the MEGA 5.0 program. Domain architectures were drawn by using InterProScan (Mulder et al., 2005).

2.6. Mycelial growth, conidiation, conidial germination and appressorium formation

Mycelial growth was measured on minimal medium (MM; 1% glucose (w/v), 0.1% trace element, 0.1% vitamin supplement, 0.6% $NaNO_3$ (w/v), 0.05% KCl (w/v), 0.05% $MgSO_4$ (w/v), 0.15% KH_2PO_4 (w/v), pH 6.5) and modified complete medium (CM; 1% glucose (w/v), 0.2% peptone (w/v), 0.1% yeast extract (w/v), 1% casamino acid (w/v), 0.1% trace element, 0.1% vitamin supplement, 0.6% $NaNO_3$ (w/v), 0.05% KCl (w/v), 0.05% $MgSO_4$ (w/v), 0.15% KH_2PO_4 (w/v)) as previously described (Talbot et al., 1993). For the sole carbon source test, glucose was replaced by monosaccharides (galactose, fructose, and xylose), disaccharides (sucrose, trehalose, and maltose), and polysaccharides (starch, pectin, and cellulose). The

same concentration (1% w/v) was used for all carbons. Conidiation was measured by counting the number of asexual spores with a haemacytometer under a microscope. Conidiophore development was monitored under a microscope at 24, 36 and 48 h after incubation (Lau and Hamer, 1998). Conidia was collected from 7-day-old V8 juice agar plates with sterilized distilled water and passed through two layers of Miracloth (CalBiochem, San Diego, CA, USA). Conidial germination and appressorium formation were performed on hydrophobic microscope coverslips. Conidial suspension adjusted to 2×10^4 conidia/ml was dropped onto coverslips with three replicates and incubated in moistened box at room temperature. At 2 and 4 h after incubation, conidial germination was determined by counting at least one hundred conidia per replicate under a microscope. The frequency of appressorium formation was measured from germinated conidia at 8 and 16 h after incubation. 10 mM of cAMP was added on conidial suspensions and incubated on hydrophilic surfaces of GelBond (Pharmacia Biotech AB, Uppsala, Sweden) and hydrophobic microscope coverslips for 24 h. These assays were performed with at least three replicates in three independent experiments.

2.7. Pathogenicity and sheath inoculation assays

For the pathogenicity assay by spray inoculation, conidia were collected from 7-day-old V8 juice agar medium and 10 ml of conidial suspension adjusted to 5×10^4 conidia/ml containing Tween 20 (250 ppm, final concentration) was sprayed onto the rice seedlings (*Oryza sativa* cv. Nakdongbyeo) in three to four leaf stage. Inoculated rice seedlings were placed in a dew chamber for 24 h under the dark condition at 25 °C. They were transferred to the growth chamber at 25 °C, 80% humidity with photoperiod of 16 h with fluorescent lights (Valent et al., 1991). Drops of conidial suspension (10^5 conidia/ml) and mycelial agar plugs were also used for the pathogenicity assay on the detached rice leaves. Leaves were inoculated with 20 μl of drops of conidial suspension or 6 mm mycelial agar plugs and incubated in moistened box for 7 days at room temperature (Kim et al., 2009).

Penetration assays were carried out using the rice leaf sheaths and onion epidermal cells. For the leaf sheath assay, conidial suspension (2×10^4 conidia/ml) was injected in excised rice sheaths and incubated in moistened box at room temperature (Koga et al., 2004). The infected rice sheaths were trimmed to remove chlorophyll enriched plant parts at 48 and 72 h after inoculation. Left epidermal layers of mid vein (three to four cell layers thick) were used for microscopic observation. For penetration assay using the onion epidermis, mycelial plugs were placed onto the onion cells and incubated in moistened box at room temperature. Onion epidermis was observed under a microscope at 48 h after inoculation.

3. Results

3.1. Bioinformatic analysis for the MoCOD1 and MoCOD2 genes

Homologs of the *MoCOD1* and *MoCOD2* genes were identified using BLASTP ($\leqslant e^{-10}$) and InterPro analyses from the Fungal Genome Gold Standard consisting of a standard set of 29 fungal and 6 non-fungal genomes (http://fggs.riceblast.snu.ac.kr). Homologs of the *MoCOD1* gene were found in 15 fungal species (Fig. 1A). All species belong to Ascomycota except one, *Ustilago maydis* (Basidiomycota). $Zn(II)_2Cys_6$ binuclear DNA-binding (IPR001138) and fungal specific TF (IPR007219) domains were identified in all homologs. Among them, functions of *BGLR* in *Trichoderma reesei*, *MAL13* in *Saccharomyces cerevisiae*, and *AMYR* in *Aspergillus nidulans* were characterized as regulators of β-glucosidase, maltase and α-amylase, respectively (Nitta et al., 2012; Tani et al., 2001; Charron et al., 1986). The homologs of *MoCOD2* were found in only 10 species belonging to Pezizomycotina in Ascomycota (Fig. 1B), indicating that this gene is more specifically conserved in filamentous fungi. Only one domain (IPR001138) was commonly identified and no protein has been functionally characterized in these homologs. Thus, *MoCOD1* and *MoCOD2* are structurally homologous to their homologs and specifically conserved in Ascomycota (or Pezizomycotina).

3.2. Mutant generation for two putative $Zn(II)_2Cys_6$ TF genes

Deletion mutants for two putative $Zn(II)_2Cys_6$ TF genes, *MoCOD1* and *MoCOD2*, were generated by homologous recombination with the DNA constructs shown in Fig. 2A and C. Correct integration in the genome of *M. oryzae* strain KJ201 was determined by PCR and Southern hybridization (Fig. 2B and D). Complemented strains for the deletion mutants were generated by transformation with the 5.7 Kb and 6.5 Kb DNA fragments corresponding *MoCOD1* and *MoCOD2*, respectively. To understand the relationship between *MoCOD1* and *MoCOD2*, the double deletion mutant, Δ*Mocod1*/Δ*Mocod2*, was also generated.

Because the homologs of *MoCOD1* were known as regulating genes involved in oligo- or polysaccharides degradation, wild-type and mutant strains were incubated on minimal media containing 10 different carbons including maltose and galactose as the sole carbon sources. Significant growth reduction was observed in the Δ*Mocod1* mutant on glucose, maltose, and galactose-containing media, compared to wild-type ($P < 0.01$, Supplementary Fig. 1). Interestingly, however, Δ*Mocod1* also grew faster on starch-containing minimal media ($P < 0.01$), suggesting that the role of *MoCOD1* was different from that of *AMYR* in *A. nidulans* (Tani et al., 2001). Instead, *MoCOD1* plays a broader role in utilization of glucose and galactose as suggested in *T. reesei* and *Aspergillus niger* (Nitta et al., 2012; vanKuyk et al., 2012).

3.3. Gene expressions during life cycle of the rice blast fungus

The expression levels of *MoCOD1* and *MoCOD2* were examined by qRT-PCR using total RNA samples harvested from six developmental stages in *M. oryzae* (mycelia, conidiation, conidial germination, appressorium formation, 72 hours post inoculation (hpi), and 150 hpi (Fig. 3). Relative expression was calculated based on mRNA levels of the mycelial stage. The *MoCOD1* gene exhibited significant up-regulation during conidiation, appressorium formation, and 72 hpi stages (5.8, 2.3, and 3.6-fold, respectively). Expression of *MoCOD2* was up-regulated exclusively in conidiation by 11.3-fold. These results are consistent with the data from microarray experiment (Kim and Lee, 2012), suggesting that both genes were expected to be involved in conidial development. In addition, *MoCOD1* may be related to appressorium formation and pathogenicity of the rice blast fungus.

3.4. Conidiation of the ΔMocod1 and ΔMocod2 mutants

The numbers of conidia produced by the Δ*Mocod1* and Δ*Mocod2* mutants were significantly reduced by 22.5 and 1000 times, compared to that of wild-type, respectively (Fig. 4A). A reduced number of conidiophores were also observed in both mutants after 24 h incubation while wild-type developed dense conidiophores and typical sympodial conidia (Fig. 4B). Especially, the Δ*Mocod2* mutant exhibited greatly reduced conidiophore development compared to the Δ*Mocod1* mutant. Defects in development of conidia and conidiophores in the mutants were restored by genetic complementation with their original ORFs (Fig. 4). The number of conidia of the Δ*Mocod1*/Δ*Mocod2* mutant was even less

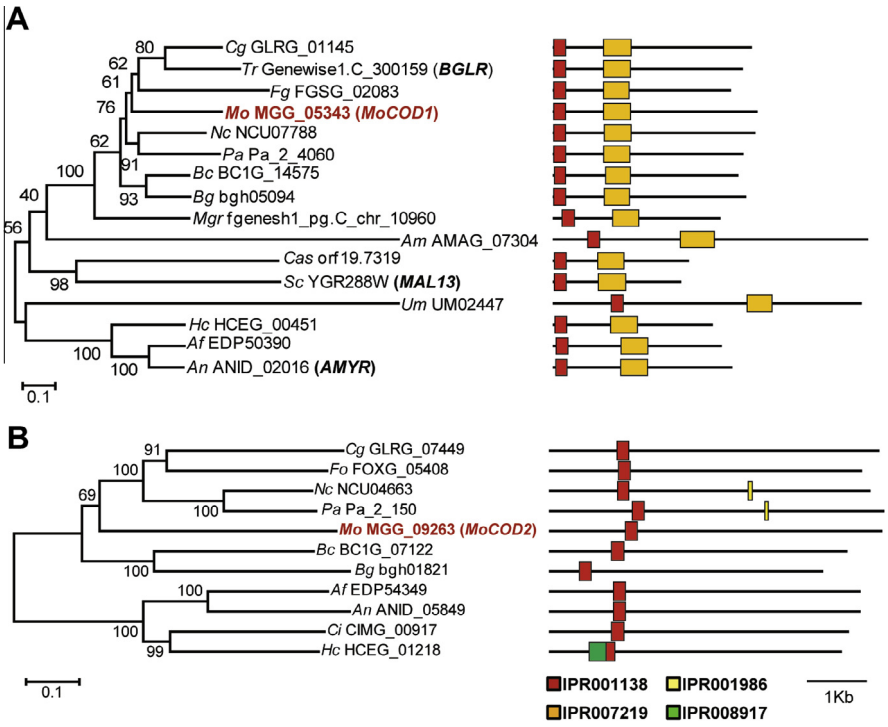

Fig. 1. *MoCOD1* and *MoCOD2* encoding $Zn(II)_2Cys_6$ transcription factor. (A and B) Phylogenetic analysis of *MoCOD1* (MGG_05343) and *MoCOD2* (MGG_09263), respectively. The trees were constructed using MEGA with 1000 bootstraps based on alignment of full sequences of homologs. Red box indicates $Zn(II)_2Cys_6$ binuclear DNA-binding domain (IPR001138) and orange box indicates fungal specific transcription factor domain (IPR007219). Yellow and green boxes indicate EPSP synthase (IPR001986) and eukaryotic transcription factor (IPR008917) domains, respectively. Abbreviations for fungal species are as follows: Af, *Aspergillus fumigatus*; Am, *Allomyces macrogynus*; An, *Aspergillus nidulans*; Bg, *Blumeria graminis*; Bc, *Botrytis cinerea*; Ca, *Candida albicans*; Ci, *Coccidioides immitis*; Cg, *Collectotrichum graminicola*; Fg, *Fusarium graminearum*; Fo, *Fusarium oxysporum* f. sp. *lycopersici*; Hc, *Histoplasma capsulatum*; Mo, *Magnaporthe oryzae*; Mg, *Mycosphaerella graminicola*; Nc, *Neurospora crassa*; Pa, *Podospora anserina*; Sc, *Saccharomyces cerevisiae*; Tr, *Trichoderma reesei*; Um, *Ustilago maydis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than that of Δ*Mocod2* (~30%), indicating different role of *MoCOD1* from *MoCOD2* in conidiation.

In addition, expression of *MoCOD1* and *MoCOD2* was examined in both mutants. The expression of *MoCOD2* was down-regulated in the transcripts of the Δ*Mocod1* mutant during conidiation (Fig. 5A), and *MoCOD1* was also greatly reduced in the Δ*Mocod2* mutant (Fig. 5B). The similar transcriptional reduction in both genes indicates the close functional relationship of *MoCOD1* and *MoCOD2* during conidiation. We further analyzed transcriptional changes of the previously characterized TF genes in conidiation (Table 1). Interestingly, the expressions of *CON7*, *COS1*, *MoHOX2*, and *MoMCM1* genes in both mutants were similar, supporting that they might be closely related to *MoCOD1* and *MoCOD2* during conidiation (Fig. 5). The Δ*Mocod1* mutant exhibited significant up-regulation of *MSTU1* and *ACR1* whereas significant reduction of *ACR1*, *COM1* and *MoSWI6* expressions was observed in the Δ*Mocod2* mutant (Fig. 5). Taken together, *MoCOD1* and *MoCOD2*, in combination, play important roles in conidiation and regulate conidiation-related TF genes during conidiation.

3.5. Pathogenicity of the ΔMocod1 and ΔMocod2 mutants

The pathogenicity assay was conducted for the Δ*Mocod1* and Δ*Mocod2* mutants (Fig. 6). The conidial suspension spray method was applicable to the Δ*Mocod1* mutant (Fig. 6A), but not to Δ*Mocod2*. Because the Δ*Mocod2* mutant has much less conidia than Δ*Mocod1*, detached leaves with conidial suspension drops (10^3 conidia in 20 μl drop) (Fig. 6B) and mycelial plugs (Fig. 6C) were alternatively used. The leaves inoculated with the Δ*Mocod1* mutant showed reduced lesions in number and size when they were compared to control leaves inoculated with wild-type (Fig. 6A). However, no lesions were found in the leaves inoculated with the Δ*Mocod2* mutant in the conidial drop or mycelial plug forms (Fig. 6B and C). Compared to the mutants, wild-type and each complemented strain produced typical lesions on rice leaves. Therefore, these results indicate that *MoCOD1* and *MoCOD2* are responsible for fungal pathogenicity to rice.

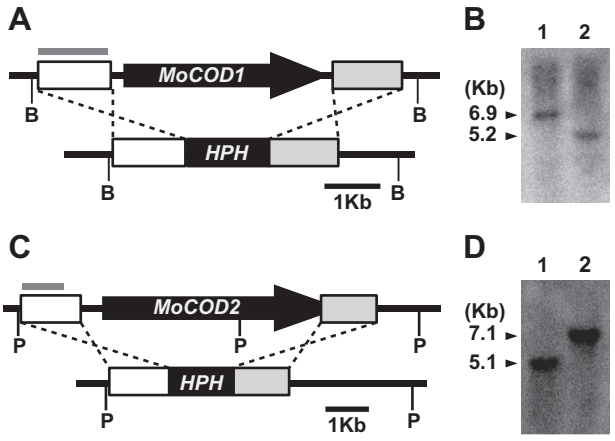

Fig. 2. Generation of Δ*Mocod1* and Δ*Mocod2* mutants in *M. oryzae*. (A and C) Gene replacement strategy of *MoCOD1* and *MoCOD2* (MGG_05343 and MGG_09263, respectively). B and P: *Bgl*II and *Pvu*II restriction enzyme sites. HPH: the hygromycin-resistant gene cassette. The gray bars indicate the position of the used probes in Southern blot analyses. (B and D) Southern blot analyses of the Δ*Mocod1* and Δ*Mocod2* mutants, respectively. Lane 1: wild-type. Lane 2: deletion mutant.

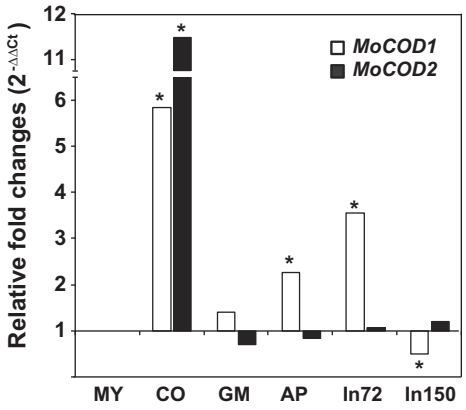

Fig. 3. Transcription levels of *MoCOD1* and *MoCOD2* in different development stages. Relative expressions of *MoCOD1* and *MoCOD2* in the conidiation (CO), conidial germination (GM), appressorium formation (AP), 72 h post inoculation (In72) and 150 h post inoculation (In150) conditions were calculated based on those in mycelia (MY). The asterisk indicates a significant difference from the mycelia condition by more than two folds.

3.6. Conidial germination and appressorium formation of the ΔMocod1 and ΔMocod2 mutants

We further investigated phenotypes of conidial germination and appressorium formation (Fig. 7). When incubated on hydrophobic surfaces such as cover slips, more than 80% of wild-type conidia germinated at 4-h incubation (Fig. 7A). However, only

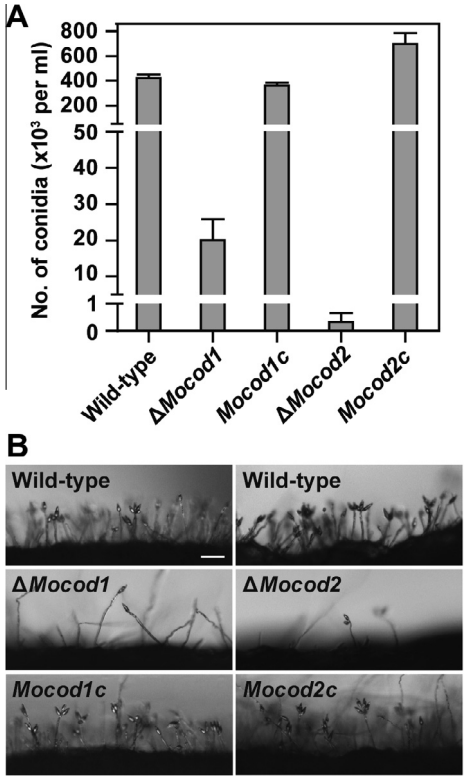

Fig. 4. Conidiation and conidiophore development of Δ*Mocod1* and Δ*Mocod2*. (A) Conidiation of Δ*Mocod1* and Δ*Mocod2*. Conidia were collected from 7-day-old cultures on V8 juice agar plates with 5 ml water. The number of conidia was counted in a haemacytometer under the microscope. (B) Conidiophore development of Δ*Mocod1* and Δ*Mocod2*. Strains were grown on oatmeal agar plates for 20 days. Scraped mycelial agar blocks were incubated for 24 h and then observed under the microscope. Scale bar indicates 100 μm.

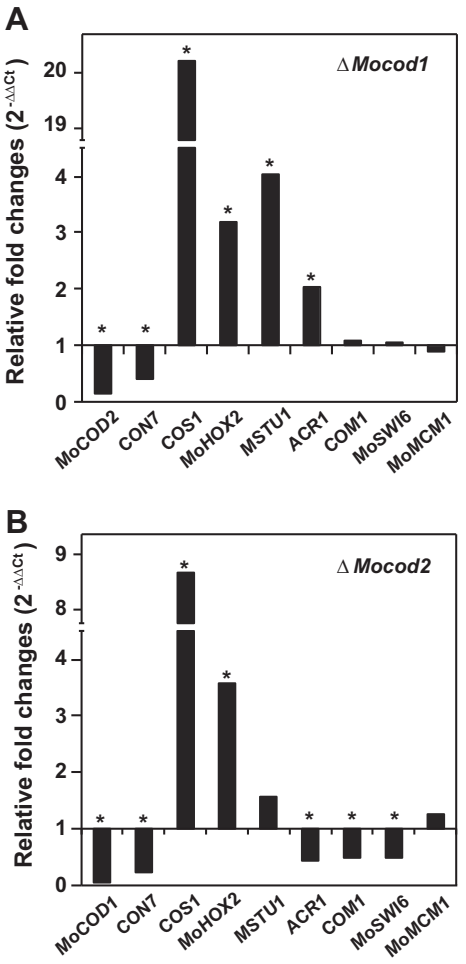

Fig. 5. Transcriptional expression patterns of conidiation-related TF genes in the Δ*Mocod1* and Δ*Mocod2* mutants. Relative expressions of *CON7*, *COS1*, *MSTU1*, *ACR1*, *COM1*, *MoSWI6*, *MoCOD1* and *MoCOD2* were measured in the Δ*Mocod1* (A) and Δ*Mocod2* (B) mutants. Transcript levels were normalized to β-tubulin and relative values were calculated by fold change ($2^{-\Delta\Delta Ct}$) compared to each transcript in conidiation of wild-type. Total RNA was prepared from conidiation stages of the Δ*Mocod1* and Δ*Mocod2* mutants. The asterisk indicates a significant difference from the mycelia condition by more than two folds.

14% of Δ*Mocod1* conidia developed germ tubes at the same time (Fig. 7A). More than 80% of the germinated conidia of wild-type formed appressoria at 8-h incubation, whereas no appressorium was formed at 8-h incubation in the Δ*Mocod1* mutant (Fig. 7B). The germination rate of the mutant reached to 80% at 8-h incubation but appressorium formation rate was just ~40% even after 16-h incubation. These defects of Δ*Mocod1* were fully recovered by genetic complementation, indicating that *MoCOD1* plays important roles not only in conidiation but also conidial germination and appressorium formation (Fig. 7A and B). In contrast to Δ*Mocod1*, the Δ*Mocod2* mutant did not show any differences in conidial germination and appressorium formation. The Δ*Mocod1*/Δ*Mocod2* exhibited about 40% of appressoria which is a similar rate of appressorium formation of Δ*Mocod1*.

Interestingly, the Δ*Mocod1* mutant developed long and convoluted germ tubes (Fig. 7C), which resembles phenotypes of the cAMP-dependent protein kinase A mutant (Δ*cpkA*) (Mitchell and Dean, 1995; Xu et al., 1997) and the homeobox transcription factor mutant (Δ*Mohox7*) (Kim et al., 2009). Addition of exogenous cAMP partially complemented such defects in appressorium formation on both inductive and non-inductive surfaces in the Δ*Mocod1* mutant (Fig. 8), suggesting that *MoCOD1* may be involved in recog-

Table 1
Conidiation-related TF genes in *M. oryzae*.

Genes	Loci	TF types	Phenotypes in conidiation	References
CON7	MGG_05287	C_2H_2 zinc finger	Abnormal conidial morphology	Odenbach et al. (2007)
COS1	MGG_03977	C_2H_2 zinc finger	No conidiophores stalk	Zhou et al. (2009)
MoHOX2	MGG_00184	Homeobox	No conidia production	Kim et al. (2009)
COM1	MGG_01215	bHLH	Abnormal conidial morphology	Yang et al. (2010)
MSTU1	MGG_00692	APSES	Reduced number of conidia	Nishimura et al. (2009)
MoSWI6	MGG_09869	APSES	Reduced number of conidia	Qi et al. (2012)
MoMCM1	MGG_02773	MADS-box	Reduced number of conidia	Zhou et al. (2011)
ACR1	MGG_09847	–	Head-to-tail array of conidia	Lau and Hamer (1998)

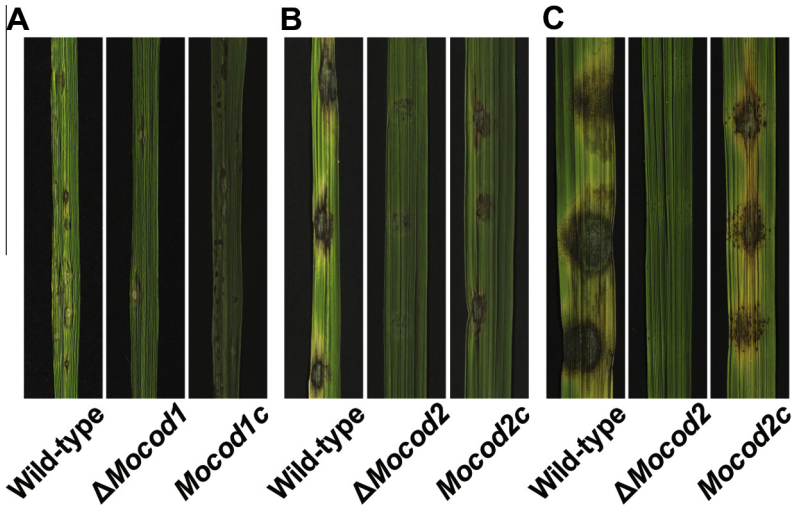

Fig. 6. Pathogenicity of Δ*Mocod1* and Δ*Mocod2*. (A) Spray inoculation for pathogenicity assay of the Δ*Mocod1* mutant. Susceptible rice seedlings were sprayed with conidia suspension (5×10^4 conidia/ml) of each strain from 7-day-old V8 agar plates. (B) Assay for Δ*Mocod2* mutant using drops of conidia suspension. Conidia suspension (10^3 cells in 20 μl) was dropped onto detached rice leaves. (C) Infection assay for Δ*Mocod2* mutant using mycelia agar plugs. Strains were grown on oatmeal agar plates for 10 days. Activated mycelia agar plugs (6 mm in diameter) were placed onto detached rice leaves. Photographs were taken at 7 dpi (day post-inoculation).

nition of hydrophobic surface or cAMP-mediated signal transduction.

We further examined appressorial turgor using a cytorrhysis assay to determine functionality of appressoria (Supplementary Fig. 2). The proportions of collapsed appressoria in the Δ*Mocod1* and Δ*Mocod2* mutants were not much different from that of wild-type, suggesting that appressoria produced by the mutants were functional and neither TF is required for appressorium turgor generation, that is, maturation of appressorium.

3.7. Infectious growth in the ΔMocod1 and ΔMocod2 mutants

Rice sheath infiltration assay was performed to investigate the roles of *MoCOD1* and *MoCOD2* within rice cells. Infection hyphae of wild-type filled the first-invaded cell and moved to adjacent cells within 48 h after inoculation while those of the Δ*Mocod1* and Δ*Mocod2* mutants were remained in the first-invaded cell (Fig. 9). The Δ*Mocod1* mutant moved to adjacent cells later whereas the Δ*Mocod2* mutant was restricted to the first-invaded cell even at 72 hpi. Interestingly, dark-brown granules were accumulated around the infection hyphae of the Δ*Mocod2* mutant (Fig. 9B). Mycelial growth under oxidative stress conditions was tested because the accumulation of granules was thought to be an evidence of strong induction of plant defense reactions. However, no significant difference between the Δ*Mocod2* mutant and wild-type strains was observed in mycelial growth on the media containing hydrogen peroxide (1 and 5 mM) and Congo Red (Supplementary Fig. 3). The growth defects of the mutants within the plant cells were recovered in the *Mocod1c* and *Mocod2c* strains (Fig. 9). Therefore, both *MoCOD1* and *MoCOD2* play essential roles in invasive growth, and *MoCOD2* may induce plant defensive responses in a ROS-independent manner.

4. Discussion

In this study, two $Zn(II)_2Cys_6$ TF genes, *MoCOD1* and *MoCOD2*, were identified and functionally characterized in the rice blast fungus. Conidiation was substantially reduced in both mutants where significant transcriptional changes of other conidiation-related TF genes were also observed. The *MoCOD1* gene is involved in signal transduction to activate appressorium formation, and invasive growth after penetration. The *MoCOD2* gene contributes to invasive growth and loss of the gene induces plant defense responses such as accumulation of dark brown granules. Therefore, the $Zn(II)_2Cys_6$ TFs MoCOD1 and MoCOD2 play essential roles in conidiation and pathogenicity in the rice blast fungus.

The Δ*Mocod1* and Δ*Mocod2* mutants produce 5% and 0.1% conidia (22.5- and 1000-time reduction in conidiation, respectively) compared to wild-type (Fig. 4A), suggesting that *MoCOD1* and *MoCOD2* contribute greatly to conidiation of *M. oryzae*. In comparison with other TF mutants, the *Momcm1* and *Mstu1* mutants produced 10% of conidia compared to wild-type (Zhou et al., 2011; Zhou et al., 2009). No conidium was found only in the *cos1* and *Mohox2* mutants (Kim et al., 2009; Nishimura et al., 2009). *MoCOD1* and *MoCOD2* have a unique relationship in conidiation. Firstly, both genes are activated when conidia are formed as shown in the microarray (Kim and Lee, 2012) and qRT-PCR analyses (Fig. 3). Secondly, the expression of both genes was significantly decreased in each mutant (Fig. 5). Thirdly, expression patterns of

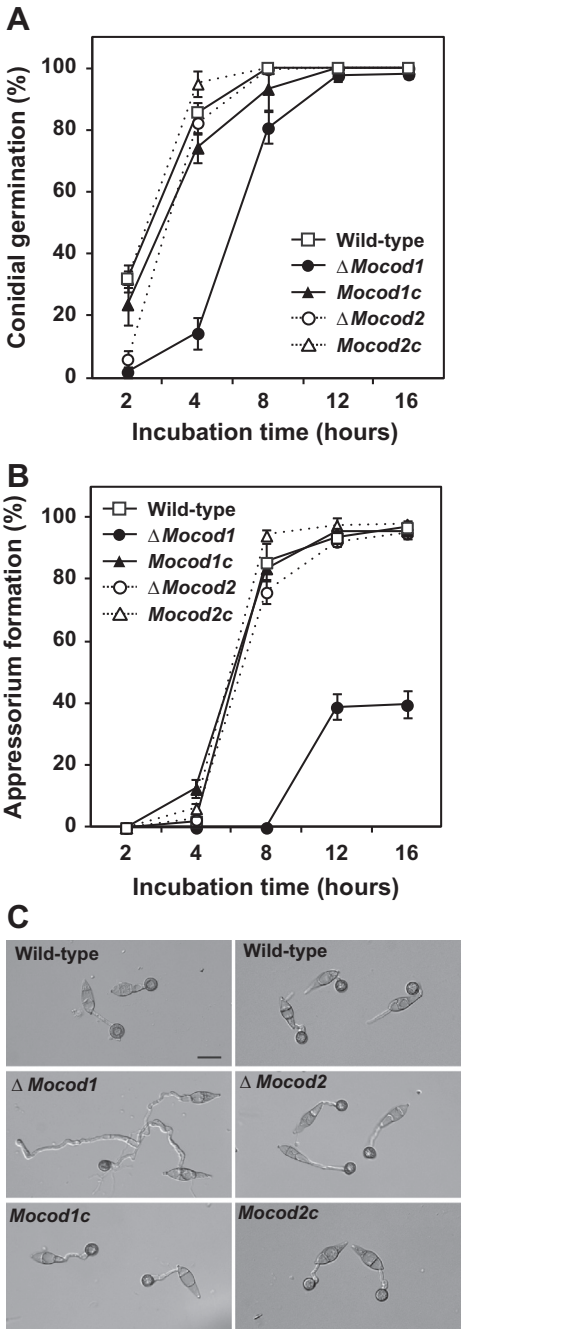

Fig. 7. Germination and appressorium formation of Δ*Mocod1* and Δ*Mocod2*. Germination (A) and appressorium formation (B) rates were recorded at 2, 4, 8, 12, and 16 h after incubation on hydrophobic coverslips. (C) Appressorium formation at the tips of germ tubes on the hydrophobic surface. Photos were taken at 16 h after incubation. Scale bar indicates 20 μm.

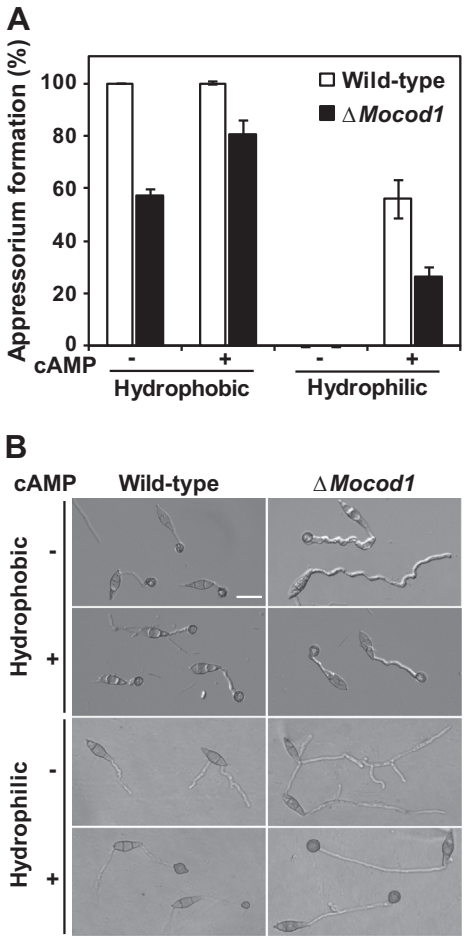

Fig. 8. cAMP treatment for appressorium formation of the Δ*Mocod1* mutant. (A) The cAMP assay was conducted with conidia suspension (2×10^4 conidia/ml) on hydrophobic (coverslip) and hydrophilic surfaces (GelBond). Conidia of each strain were collected from 7-day-old V8 agar plates and treated with 10 mM cAMP. (B) Appressorium formation of Δ*Mocod1* on both of hydrophobic and hydrophilic surfaces with or without 10 mM cAMP. Photographs were taken at 24 h after inoculation. Scale bar indicates 50 μm.

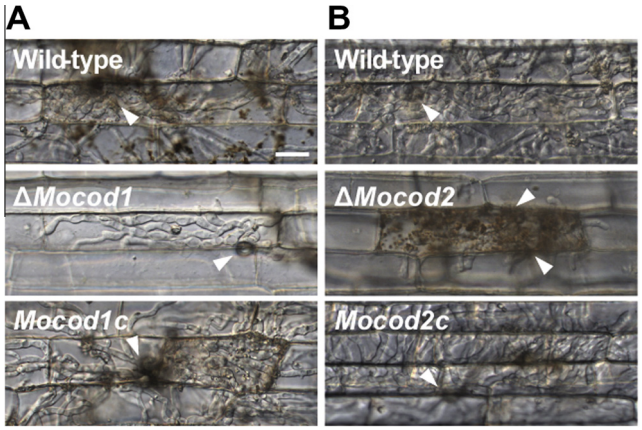

Fig. 9. Invasive growth of Δ*Mocod1* and Δ*Mocod2*. Infectious growth was observed in of the Δ*Mocod1* (A) and Δ*Mocod2* (B) mutants in rice sheath cells. Conidial suspension (2×10^4 conidia/ml) was infiltrated into the excised rice sheath. Photographs were taken at 48 and 72 h after incubation in the dew chamber (A and B, respectively). White arrows indicate appressorium. Scale bar indicates 20 μm.

other TF genes were quite similar in both mutants. *COS1* and *MoHOX2*, for example, were significantly up-regulated and *CON7* was down-
regulated, compared to the wild-type (Fig. 5). Fourthly, both genes seem to play additive roles in conidiation when Δ*Mocod1*/Δ*Mocod2* was compared to Δ*Mocod1* and Δ*Mocod2*. Therefore, both *MoCOD1* and *MoCOD2* are positive regulators for conidiation and might work independently by regulating expression of conidiation-related genes.

Reduction in conidiophores was observed in both mutants (Fig. 4B). We hypothesized that such defects might be caused by reduced expression of *COS1* encoding regulator for conidiophore stalk development (Zhou et al., 2009). Contrary to our expectation, however, the *COS1* expression was highly up-regulated in both

Δ*Mocod1* and Δ*Mocod2* mutants (Fig. 5). The *ACR1* gene is a presumed transcription regulator in conidiation and the *acr1*$^{-}$ mutant exhibits reduction in conidiophore formation (Lau and Hamer, 1998). Interestingly, *ACR1* expression was increased in Δ*Mocod1* and decreased in Δ*Mocod2* when compared to the same wild-type (Fig. 5). Given that *COS1* expression was much higher in Δ*Mocod1* (20.8 folds) than in Δ*Mocod2* (8.5 folds), relatively more transcripts of *COS1* and *ACR1* might explain more conidiophores in the Δ*Mocod1* mutant than Δ*Mocod2*.

The Δ*Mocod1* mutant showed defects in appressorium formation. At the 60% of germinated tubes, no appressorium was formed at 16 h of incubation (Fig. 7B). In addition, the elongated and hooked germ tubes were observed on hydrophobic surface (Fig. 7C). Similar germ tubes were observed in the Δ*Mohox7* mutant where a homeobox TF gene was disrupted (Kim et al., 2009). However, the Δ*Mohox7* mutant failed to develop any appressorium even after treatment of signal molecules such as cAMP (Kim et al., 2009) while appressorium formation of Δ*Mocod1* was partially recovered by cAMP on both hydrophobic and hydrophilic surfaces (Fig. 8A). Therefore these evidences suggest that sufficient signal molecules were not generated by surface recognition or signal molecules were not properly transduced to activate TFs such as *MoHOX7* in the Δ*Mocod1* mutant.

The lack of pathogenicity exhibited by the Δ*Mocod2* mutant could be explained by restricted growth of infection hyphae and granule formation in host cells (Fig. 6). Infection hyphae were limited the first-invaded cell even after 72 h and many dark brown granules were formed around the hyphae in the Δ*Mocod2* mutant. Similar reactions were observed in a loss of *DES1* and *MoATF1*, encoding a serine-rich protein and basic leucine zipper TF, respectively, in *M. oryzae* (Chi et al., 2009a; Guo et al., 2010). When the plant cells were treated with the flavoprotein inhibitor diphenylene iodonium (DPI), no granule was formed and infection hyphae of the Δ*des1* and Δ*Moatf1* mutants grew like wild-type. However, in the Δ*Mocod2* mutant, some hyphae were still restricted to the first-invaded cell without the granule accumulation and Δ*Mocod2* mutant did not show any significant difference in mycelial growth on the media containing hydrogen peroxide (1 and 5 mM) and Congo Red (Supplementary Fig. 3) compared to wild-type, suggesting that *MoCOD2* contributes to induction of host defense in a ROS-independent manner. In addition, *COM1* was significantly down-regulated in Δ*Mocod2* (Fig. 5B). Given that the Δ*com1* mutant exhibited retardation in infectious growth (Yang et al., 2010), reduced expression of *COM1* might contribute to limited infectious growth of the Δ*Mocod2* mutant.

Taken together, both *MoCOD1* and *MoCOD2* play important roles in conidia production and conidiophore development, but not conidial morphology. Both *MoCOD1* and *MoCOD2* are also involved in invasive growth in host cells that result in reduced pathogenicity. Additionally, *MoCOD1* is involved in hydrophobic surface recognition and/or cAMP-mediated signal transduction while *MoCOD2* induces host defense response in a ROS-independent manner. Understanding of the functional roles of $Zn(II)_2Cys_6$ transcription factor genes on fungal developments would provide new insights on deciphering molecular mechanisms of fungal pathogenicity on plants.

Acknowledgments

This work was supported by the National Research Foundation of Korea Grant funded by the Korean government (2008-0061897 and 2013-003196) and the Next-Generation BioGreen 21 Program of Rural Development Administration in Korea (PJ00821201). H. Chung is grateful for the graduate fellowship through the Brain Korea 21 Program.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.10.004.

References

Bourett, T.M., Howard, R.J., 1990. *In vitro* development of penetration structures in the rice blast fungus *Magnaporthe grisea*. Can. J. Bot./Rev. Can. Bot. 68, 329–342.
Charroni, M.J. et al., 1986. Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6, 3891–3899.
Chi, M.H. et al., 2009a. A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host. PLoS Pathog. 5, e1000401.
Chi, M.H. et al., 2009b. A quick and safe method for fungal DNA extraction. Plant Pathog. J. 25, 108–111.
Choi, J. et al., 2009. *MoCRZ1*, a gene encoding a calcineurin-responsive transcription factor, regulates fungal growth and pathogenicity of *Magnaporthe oryzae*. Fungal Genet. Biol. 46, 243–254.
Choi, J. et al., 2007. Genome-wide analysis of T-DNA integration into the chromosomes of *Magnaporthe oryzae*. Mol. Microbiol. 66, 371–382.
Choi, W.B. et al., 1998. Cyclic AMP restores appressorium formation inhibited by polyamines in *Magnaporthe grisea*. Phytopathology 88, 58–62.
de Jong, J.C. et al., 1997. Glycerol generates turgor in rice blast. Nature 389, 244–245.
Dean, R.A. et al., 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 434, 980–986.
Ebbole, D.J., 2007. Magnaporthe as a model for understanding host-pathogen interactions. Annu. Rev. Phytopathol. 45, 437–456.
Goh, J. et al., 2011. The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in *Magnaporthe oryzae*. PLoS One 6, e28220.
Guo, M. et al., 2010. The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. Mol. Plant–Microbe Interact. 23, 1053–1068.
Howard, R., 1994. Cell biology of pathogenesis. In: Zeigler, R. et al. (Eds.), Rice Blast Disease. CAB International, Wallingford, UK, pp. 3–22.
Howard, R.J., Valent, B., 1996. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu. Rev. Microbiol. 50, 491–512.
Kankanala, P. et al., 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. Plant Cell 19, 706–724.
Khush, G., Jena, K.K., 2009. Current status and future prospects for research on blast resistance in rice (*Oryza sativa* L.). In: Wang, G.-L., Valent, B. (Eds.), Advances in Genetics, Genomics and Control of Rice Blast Disease. Springer, Netherlands, pp. 1–10.
Kim, K.S., Lee, Y.H., 2012. Gene expression profiling during conidiation in the rice blast pathogen *Magnaporthe oryzae*. PLoS One 7, e43202.
Kim, S. et al., 2009. Homeobox transcription factors are required for conidiation and appressorium development in the rice blast fungus *Magnaporthe oryzae*. PLoS Genet. 5, e1000757.
Koga, H. et al., 2004. A novel inoculation method of *Magnaporthe grisea* for cytological observation of the infection process using intact leaf sheaths of rice plants. Physiol. Mol. Plant Pathol. 64, 67–72.
Kwon, M. et al., 2010. A short-chain dehydrogenase/reductase gene is required for infection-related development and pathogenicity in *Magnaporthe oryzae*. Plant Pathol. J. 26, 8–16.
Lau, G.W., Hamer, J.E., 1998. *Acropetal*: a genetic locus required for conidiophore architecture and pathogenicity in the rice blast fungus. Fungal Genet. Biol. 24, 228–239.
Lee, Y.H., Dean, R.A., 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. Plant Cell 5, 693–700.
MacPherson, S. et al., 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol. Mol. Biol. Rev. 70, 583–604.
McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucl. Acids Res. 32, W20–W25.
Mitchell, T.K., Dean, R.A., 1995. The cAMP-dependent protein-kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. Plant Cell 7, 1869–1878.
Mulder, N.J. et al., 2005. InterPro, progress and status in 2005. Nucl. Acids Res. 33, D201–D205.
Nishimura, M. et al., 2009. Mstu1, an APSES transcription factor, is required for appressorium-mediated infection in *Magnaporthe grisea*. Biosci. Biotechnol. Biochem. 73, 1779–1786.
Nitta, M. et al., 2012. A new $Zn(II)_2Cys_6$-type transcription factor BglR regulates *β-glucosidase* expression in *Trichoderma reesei*. Fungal Genet. Biol. 49, 388–397.
Odenbach, D. et al., 2007. The transcription factor Con7p is a central regulator of infection-related morphogenesis in the rice blast fungus *Magnaporthe grisea*. Mol. Microbiol. 64, 293–307.
Ou, S.H., 1985. Rice Diseases. Commonwealth Agricultural Bureaux, Wallingford, UK.
Park, J. et al., 2008a. CFGP: a web-based, comparative fungal genomics platform. Nucl. Acids Res. 36, D562–D571.
Park, J. et al., 2008b. FTFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. Bioinformatics 24, 1024–1025.

Qi, Z.Q. et al., 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., Russel, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Talbot, N.J. et al., 1993. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. Plant Cell 5, 1575–1590.

Tani, S. et al., 2001. Characterization of the *amyR* gene encoding a transcriptional activator for the amylase genes in *Aspergillus nidulans*. Curr. Genet. 39, 10–15.

Thompson, J.D. et al., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Tsuji, G. et al., 2000. Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and Pig1p of *Magnaporthe grisea*, contain Cys_2His_2 zinc finger and $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. Mol. Microbiol. 38, 940–954.

Valent, B. et al., 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics 127, 87–101.

vanKuyk, P.A. et al., 2012. A broader role for AmyR in *Aspergillus niger*: regulation of the utilisation of D-glucose or D-galactose containing oligo- and polysaccharides. Appl. Microbiol. Biotechnol. 93, 285–293.

Xu, J.-R. et al., 1997. The CPKA gene of *Magnaporthe grisea* is essential for appressorial penetration. Mol. Plant–Microbe Interact. 10, 187–194.

Yang, J. et al., 2010. A novel protein Com1 is required for normal conidium morphology and full virulence in *Magnaporthe oryzae*. Mol. Plant–Microbe Interact. 23, 112–123.

Yi, M. et al., 2009. The ER chaperone LHS1 is involved in asexual development and rice infection by the blast fungus *Magnaporthe oryzae*. Plant Cell 21, 681–695.

Yu, J.H. et al., 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41, 973–981.

Zhou, X.Y. et al., 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.Z. et al., 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.