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

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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

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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