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Two different subcellular-localized Acetoacetyl-CoA acetyltransferases differentiate diverse functions in *Magnaporthe oryzae*

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ABSTRACT

The mevalonate pathway is an efficient biosynthesis pathway that yields isoprenoids for promoting different crucial cellular functions, including ergosterol synthesis and growth regulation. Acetoacetyl-CoA acetyltransferase (EC2.3.1.9) is the first major catalytic enzyme constituting the mevalonate pathway and catalyzes the transformation of Acetoacetyl-CoA from two molecules of acetyl-CoA enroute ergosterol production in fungi. We identified two homologous genes encoding Acetoacetyl-CoA acetyltransferase (MoAcat1 and MoAcat2) in *Magnaporthe oryzae*, the rice blast fungus. Phylogenetic analysis indicates these two genes have different evolutionary history. We subsequently, conducted targeted gene deletion using homologous recombination technology to ascertain the unique roles of the two *MoAcat* homologues during the fungal morphogenesis and pathogenesis. The findings from our investigations showed that the activity of MoAcat1 promoted virulence in the rice blast fungus as such, the *ΔMoacat1* mutants generated exhibited defect in virulence, whilst *ΔMoacat1* mutants did not portray growth defects. *ΔMoacat2* mutants on the other hand were characterized by reduction in growth and virulence. Furthermore, MoAcat1 and MoAcat2 showed different expression patterns and subcellular localizations in *M. oryzae*. From our investigations we came to the conclusion that, different subcellular localization contributes to the diverse functions of MoAcat1 and MoAcat2, which helps the successful establishment of blast disease by promoting efficient development of cell morphology and effective colonization of host tissue.

1. Introduction

Acetyl-CoA acetyltransferase which has been variously referred to as ERG10, Acetoacetyl-CoA thiolase or simply as thiolases (EC2.3.1.9) is highly conserved in prokaryotes and eukaryotes and occurs as multiple isozyme in most organisms (Wilding et al., 2000). Acetyl-CoA acetyltransferase is the first catalytic enzyme required for processing two molecules of acetyl-CoA into Acetoacetyl-CoA within the mevalonate pathway (Bock, 2007; Groot et al., 1977). The mevalonate pathway is a biosynthetic route for producing mevalonate as well as, the biosynthesis of cholesterol (Goldstein and Brown, 1990; Hunter, 2007). In fungi, Acetoacetyl-CoA acetyltransferase contributes immensely towards the biosynthesis of isopropanoid to facilitate the generation of ergosterol from mevalonate. Although the precise physiological functions of ergosterol in most organisms still remains elusive (Palermo et al., 1997; Parks and Casey, 1995), it has however being implicated and cited as a crucial component required for synthesizing and strengthening cell membrane alongside the promotion of growth in bacteria, fungi and other eukaryotes (Hsueh et al., 2005; Wilcox et al., 2002). Previous investigations have adequately shown the relevance of mevalonate and mevalonate pathway in pathogenesis and have deeply exploited it in developing potent strategies for controlling diseases caused by pathogenic fungi and bacteria (Gazzerro et al., 2012), and these strategies were deployed successfully in controlling diseases in human, animals and plants by depleting and preventing the accumulation of ergosterol in cell membrane of invading pathogens through the inhibition of Acetoacetyl-CoA acetyltransferase which results in the inhibition

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of the mevalonate pathway (Dayan and Watson, 2011; Geng et al., 2014). The inhibition of the mevalonate pathway affects the activities of cytochrome P450 which has been cited as a major system contributing to resistance in plants and virulence in pathogens (Morant et al., 2003; Wittstock and Gershenzon, 2002).

Magnaporthe oryzae, a filamentous ascomycete fungus, is the causal agent of rice blast disease and has emerged as the most destructive disease of rice worldwide (Dean et al., 2012; Ou, 1980). The cosmopolitan rice blast fungus is undisputedly the most important plant pathogenic fungus, owing to the fact that rice remains the primary source of calorie for over one-half of the world's population (Maclean et al., 2002). *M. oryzae* can infect all foliar tissues of susceptible host plants; however, infection of the panicle can lead to complete loss of grain. Although, losses of 10–30% are typical, regional epidemics can be devastating (Dean et al., 2012). Insight gained from plant-pathogen interaction suggests that, both innate immunity (PTI) and effector triggered immunity (ETI) involves diverse mechanisms including the rapid accumulation of terpenes, diterpenes and other plant natural products (Dixon, 2001; Gershenzon and Dudareva, 2007). In plants, the mevalonate pathway serves as an essential route for synthesizing isoprenoids required for generating the backbone of plant terpenes and diterpenes (Moses et al., 2013), and most of these terpenes and diterpenes possess an antimicrobial property and effectively inhibits the growth of pathogenic fungi (Guggisberg et al., 2014). It is reasonable to predict that, the relevance of the mevalonate pathway as a biosynthetic route for producing isoprenoids that are needed for vital cellular functions including the production of mevalonate required for the subsequent generation of cholesterol and ergosterol that are largely regulated by the first and indispensable catalytic enzyme acetyl-CoA acetyltransferase (Yasmin et al., 2012). The biosynthesis of mevalonate is vital in the progress of diverse and crucial functions including the production of ergosterol as a growth promoting factor of yeast cell (Alvarez-Vasquez et al., 2011; Motley and Hettema, 2007) and also enhances the pathogenicity of certain pathogenic fungi (Asakura et al., 2009; Bittel and Robatzek, 2007; Elliott and Howlett, 2006). More so, findings from recent investigations showed that fungal pathogen *M. oryzae* modifies similar defense mechanisms in host plants to suppress host resistance for successful establishment of the blast disease (Ferreira et al., 2005; Kubo, 2013), which has prompted our curiosity to access the function of acetyl-CoA acetyltransferase in the rice blast fungus. We further premise that, genetic evaluation of acetyl-CoA acetyltransferase in *M. oryzae* would help in laying solid foundation for further research works and invariably enhance our understating on the developmental and infectious stages of the rice blast fungus with regards to nutrition, morphology, pathogenicity and virulence.

2. Materials and methods

2.1. Strains and culture conditions

M. oryzae strain Ku80 served as the wild type strain in this study, and all the fungal strains were cultured at 26 °C using complete medium (CM: 0.6% yeast extract, 0.6% casein hydrolysate, 1% sucrose, 1.5% agar). Protoplast preparation and fungal transformation were performed as described (Wang et al., 1999; Wendland, 2003), and transformants were selected by using TB3 medium with 250 μg/mL hygromycin B (Roche Applied Science) or 200 μg/mL G418 (Invitrogen). Genomic DNA and total RNA were extracted from mycelia cultured in liquid CM medium with 130 rpm shaking at 26 °C for 3–4 days. 10 mM glucose, sodium acetate, olive oil, glycerol, alcohol, pyruvate or mevalonolactone (Sigma) was respectively added to the minimum medium (MM: $NaNO_3$ 6 g, KCl 0.52 g, $MgSO_4{\cdot}7H_2O$ 0.152 g, KH_2PO_4 1.52 g, Thiamine VB1 0.001%, Trace elements 0.1% ($CuSO_4{\cdot}5H_2O$ 400 mg/L, $ZnSO_4{\cdot}7H_2O$ 8 g/L, $MnSO_4{\cdot}2H_2O$ 800 mg/L, $FeSO_4{\cdot}7H_2O$ 1180 mg/L, Na_2MoO_4 800 mg/L, $Na_2B_4O_7{\cdot}10H_2O$ 40 mg/L, 15 g agar in 1 L of distilled water) and adjust pH value to 7.0 as sole carbon source in our quest to determine the effects on fungal growth (Foster et al., 2003). Conidiation was examined by harvesting conidia from 10-day-old mutants and wild type colonies cultured on rice-bran agar medium (2% rice-polish, 1.5% agar, and pH 6.5) at 26 °C under constant light to promote conidial development.

2.2. In vivo expression pattern of MoAcat at different developmental and infectious stages

To assess the in vivo expression level of *MoAcat* genes during mycelium (MYC.) stage, we accordingly cultured Ku80 wild type in liquid CM medium and incubated at 130 rpm shaking under 26 °C for 3–4 days before filtering, drying and proceeding to extract RNA following standard protocol. Also, the expression level of *MoAcat* genes in conidia (CON.) was achieved by extracting RNA from conidium harvested from 10-days-old Ku80 cultured on rice-bran agar medium at 26 °C under constant light. Furthermore, to investigate the expression level of *MoAcat* genes during conidial germination (GER.) and appressorium development (APP.), RNA was extracted from conidial suspensions inoculated on an artificial hydrophobic surface for 2 and 4 h to induce germination and appressorium formation respectively. During this process, conidial suspensions were collected from artificial hydrophobic surface and centrifuged for one minute at 12,000 rpm to allow the coagulation of the conidia, the supernatant was discarded and the coagulated germinating conidia for 2 h post exposure to hydrophobic surface and developed appressorium from 4 h post exposure to hydrophobic surface were collected respectively. The extracted RNA was used to investigate the expression level of *MoAcat* genes during conidia germination (GER.) and appressorium development (APP.). More so, RNA was extracted from leaf tissues of 1, 2, 3 and 4 days post inoculated susceptible rice plants and proceeding to examine the expression level of *MoAcat* genes with uniquely designed *MoAcat* genes specific primers with no homologue in rice. Fold changes in expression during fungal developmental and infectious stages were calculated as described (Livak and Schmittgen, 2001).

2.3. Targeted gene disruption

For constructing the gene replacement vector, a 983-bp upstream and a 1183-bp downstream flanking sequence of *MoAcat1*, and a 1190-bp upstream and 1178-bp downstream flanking sequence of *MoAcat2* were separately amplified from *M. oryzae* genomic DNA. Upstream flanking sequence digested with *Xho* I and *Eco*R I and downstream flanking sequence digested with *Bam*H I and *Xba* I were successively cloned into plasmid pCX62 containing a hygromycin-resistance gene. After transformation, potential targeted gene replacement candidates were confirmed by PCR, Southern blot and qRT-PCR. All the primers used in this study are shown in Table S1.

2.4. Complementation and GFP/mCherry fusion constructs

Complementation assays were simultaneously conducted with the subcellular localization analysis. To construct MoAcat1-GFP and MoAcat2-GFP fusion vectors, a 3204-bp and a 3202-bp fragment containing the native promoter were amplified from genomic DNA, respectively. Purified fragments were digested with *Apa* I and *Xho* I before cloning into plasmid pKNTG containing a GFP tag and a neomycin-resistance gene. The precise GFP fusion vectors were then transformed into corresponding deletion mutants.