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MoARG1, *MoARG5,6* and *MoARG7* involved in arginine biosynthesis are essential for growth, conidiogenesis, sexual reproduction, and pathogenicity in *Magnaporthe oryzae*

Yong Zhang[a,b], Huanbin Shi[a], Shuang Liang[a], Guoao Ning[a], Nanchang Xu[b], Jianping Lu[c], Xiaohong Liu[a,*], Fucheng Lin[a,d,*]

[a] State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University, Hangzhou 310058, China
[b] Quzhou Municipal Plant Protection and Quarantine Station, Quzhou Municipal Bureau of Agriculture, Quzhou 324000, China
[c] College of Life Sciences, Zhejiang University, Hangzhou 310058, China
[d] China Tobacco Gene Research Center, Zhengzhou Tobacco Institute of CNTC, Zhengzhou 450001, China

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ABSTRACT

Arginine is one of the most versatile amino acids in eukaryote cells, which plays important roles in a multitude of processes such as protein synthesis, nitrogen metabolism, nitric oxide (NO) and urea biosynthesis. The de novo arginine biosynthesis pathway is conserved among fungal kingdom, but poorly understood in plant pathogenic fungi. Here, we characterized the functions of three synthetic enzyme-encoding genes *MoARG1*, *MoARG5,6*, and *MoARG7*, which involved the seventh step, second–third step and fifth step of arginine biosynthesis in *Magnaporthe oryzae*, respectively. Deletion of *MoARG1* or *MoARG5,6*, resulted in arginine auxotrophic mutants, which had a strict requirement for arginine on minimal medium (MM). Both *ΔMoarg1* and *ΔMoarg5,6* severely reduced in aerial hyphal growth, pigmentation, conidiogenesis, sexual reproduction and pathogenicity. Interestingly, like *Saccharomyces cerevisiae*, deletion of *MoARG7* caused a leaky arginine auxotrophy, and attenuated pathogenicity. Limited appressorium-mediated penetration and restricted invasive hyphae growth in host cells are responsible for the severely attenuated pathogenicity of the Arg^- mutants. Additionally, we monitored the NO generation during conidial germination and appressorial formation in both Arg^- mutants and wild type, and demonstrated that NO generation may not occur via arginine-dependent pathway in *M. oryzae*. In summary, *MoARG1*, *MoARG5,6*, and *MoARG7* are required for growth, conidiogenesis, sexual reproduction, and pathogenicity in *M. oryzae*.

Introduction

Magnaporthe oryzae is the causal agent of rice blast disease, which causes huge yield losses in rice production globally each year. Owing to its economic importance and genetic tractability, *M. oryzae* has recently emerged as a model organism for studying the mechanisms of plant–fungal pathogen interactions (Talbot, 2003; Ebbole, 2007; Wilson and Talbot, 2009). Numerous extensive studies have focused on the molecular mechanisms of pathogenicity in *M. oryzae* (Caracuel-Rios and Talbot, 2007; Kershaw and Talbot, 2009; Li et al., 2012; Lu et al., 2014). However, recently, many studies have indicated that nutrient acquisition during infection and the synthesis of primary metabolite components such as amino acids are crucial for full pathogenicity in *M. oryzae* (Wilson and Talbot, 2009; Wilson et al., 2012; Yan et al., 2013; Chen et al., 2014).

Amino acids are biologically important organic compounds and play fundamental roles in a multitude of functions including protein synthesis, cell growth, development and production of energy. Arginine as one of the most versatile amino acids in animal cells is important not only for protein synthesis, but also serves as a precursor for the synthesis of nitric oxide, urea, polyamines, proline, creatine, and agmatine. Arginine plays very important roles in a multitude of processes, including protein synthesis, cell growth, sexual reproduction, hormone metabolism, signal transduction, osmotic pressure homeostasis, production of metabolic energy, nitrogen metabolism, and urea biosynthesis (Wipf et al., 2002; Bedford and Richard, 2005; Wu et al., 2009). The de novo biosynthesis of arginine occurs via an eight-step reaction that includes two main processes: the synthesis of ornithine from glutamate, and

* Corresponding authors at: State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University, Hangzhou 310058, China. Tel.: +86 571 88982291; fax: +86 571 88982183.
E-mail addresses: xhliu@zju.edu.cn (X. Liu), fuchenglin@zju.edu.cn (F. Lin).

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the conversion from this ornithine intermediate to arginine (Cunin et al., 1986; Davis, 1986; Crabeel et al., 1997; Slocum, 2005). Additionally, in animals, arginine is well known as a precursor in NO synthesis (Appleton, 2002). The discovery of the biological relevance of NO has stimulated renewed interest in the biochemistry, physiology, and nutrition of arginine in humans and other organisms (Wu and Morris, 1998; Appleton, 2002). It has been reported that NO may be produced from arginine by NOS-like activity and NO plays important roles in growth, morphogenesis, appressorium formation, reproduction, and apoptosis in fungi (Prats et al., 2008; Mur et al., 2006; Wang and Higgins, 2005; Nishimura et al., 2010; Rőszer, 2012).

Previously, numerous studies have demonstrated that amino acid metabolism plays an important role in the growth and virulence in many pathogenic fungi (Namiki et al., 2001; Seong et al., 2005; Gong et al., 2007; Patel et al., 2010; Takahara et al., 2012). In *M. oryzae*, two REMI transformants *pth3* and *met1* have been identified, which exhibit histidine auxotrophy and methionine auxotrophy, respectively (Sweigard et al., 1998; Balhadère et al., 1999). Recently, the functions of several synthetic enzyme genes involved in methionine (*MET12*, *MET13* and *STR3*), leucine-isoleucine-valine (*ILV1*, *ILV2* and *ILV6*), lysine (*LYS2*), and purine (*ADE1*) biosynthesis were investigated in *M. oryzae* (Yan et al., 2013; Wilson et al., 2012; Du et al., 2013, 2014; Chen et al., 2014; Fernandez et al., 2013). A link between auxotrophy and the loss of, or a reduction in pathogenicity was found in most of these cases.

To date, little is known about the roles of arginine biosynthesis in plant pathogenic fungi. Here, we identified three genes (*MoARG1*, *MoARG5,6*, and *MoARG7*) involved in arginine biosynthesis of *M. oryzae*, and characterized their functions in fungal development and pathogenicity. The results showed that *MoARG1*, *MoARG5,6*, and *MoARG7* are essential for growth, conidiogenesis, sexual reproduction, and pathogenicity in *M. oryzae*.

Materials and methods

Fungal strains and culture conditions

M. oryzae wild type strain Guy11 was used in this study; mutants were derived from Guy11. All *M. oryzae* strains were routinely cultured at 25 °C with a 16 h light/8 h dark-photoperiod on complete medium (CM) agar plates (Petri dish, 9 cm diameter). Strain maintenance protocols and medium compositions as described previously (Talbot et al., 1993; Zeng et al., 2014). Minimal medium (MM: CM lacking peptone, yeast extract, and casamino acids) and oatmeal agar [OMA: 5% oatmeal (w/v), 1.5% agar (w/v)] were used for phenotypic assays.

Nucleic acids manipulation, Southern blotting, and gene expression analysis

Fungal genomic DNA extractions were performed as described previously (Talbot et al., 1993), and general procedures for nucleic acid manipulation followed standard protocols (Sambrook and Russell, 2001). Southern blot analysis was performed by using a digoxigenin (DIG) high prime DNA labeling and detection starter Kit I (Roche, Germany), following the manufacturer's instructions. Total RNA was extracted with Trizol® Reagent (Invitrogen, USA) from fungal tissues, including fresh mycelia cultured in liquid CM for 3 days, conidia harvested from 10-day-old colonies from CM agar plates, appressoria incubated on hydrophobic surfaces at 6, 12, and 24 h post-incubation (hpi). The *M. oryzae* β-tubulin (MGG_00604.7) gene was used as an endogenous control; the relative expression analysis was performed by determining the Ct value of each gene and normalized by the Ct value of β-tubulin. All primers used in this study are listed in Supplementary Table S1.

Target gene deletion and complementation

Target gene deletion in *M. oryzae* was performed using the homologous recombination strategy. For constructing the gene deletion vector of *MoARG1*, the 1.2-kb upstream and downstream flanking fragments of *MoARG1* were amplified from *M. oryzae* genomic DNA with gene specific primer pairs *ARG1*-uF1/*ARG1*-uR1 and *ARG1*-dF1/*ARG1*-dR1, respectively. A sulfonylurea resistance gene cassette (*SUR*) was amplified with the *SUR*-F1 and *SUR*-R1 primer pair using pKD5 vector as a template. Through enzyme digestion and ligation reactions, the resulting PCR products were successively inserted into the pCAMBIA1300 vector (Cambia, Australia) to generate the gene deletion vector pKO-*MoARG1*. Using a similar construction strategy, target gene deletion vectors for *MoARG5,6* (pKO-*MoARG5,6*, *SUR*) and *MoARG7* [pKO-*MoARG7*, hygromycin resistance (*HPH*)] were constructed. For mutant complementation, a complementary fragment containing the entire target gene coding region with its native promoter (~1.5 kb) and terminator region (~0.5 kb) was cloned and inserted into the pKD8 (G418 resistance, *NEO*) successively. In this way, gene complementation vectors were generated, and named as pCOM-*MoARG1*, pCOM-*MoARG5,6*, and pCOM-*MoARG7*, respectively. The vector of target gene deletion/complementation was transformed into *Agrobacterium tumefaciens* strain AGL-1. Transformants were generated via *A. tumefaciens*-mediated transformation (ATMT), as described previously (Rho et al., 2001). The gene deletion mutants and complemented strains were selected through antibiotic resistance screening and PCR genotyping with target gene internal primers and finally confirmed by Southern blot analysis. All related primers are listed in Supplementary Table S1.

Fungal growth, conidiation, appressorium formation, and chemical treatment assays

For vegetative growth, conidiation assays, and evaluation of colony characteristics, mycelial plugs of 3 mm × 3 mm were inoculated on fresh CM and MM agar plates at 25 °C for 10 days. Colony diameters were measured at 10 days. Conidial germination and appressorium formation were measured on a hydrophobic surface (Zeng et al., 2014). Conidial germination and appressorium formation were examined at 4, 8, 12, and 24 h post-incubation (hpi); at least 200 conidia or appressoria were counted for each strain. Exogenous arginine or citrulline (Sigma–Aldrich, USA) was supplemented to culture medium at different concentrations (0, 0.5, 2.5, and 5 mM) to evaluate the effects on the growth, conidiation and pathogenicity of the Arg⁻ mutants. Sodium nitroprusside dihydrate (SNP, a nitric oxide donor) and N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME, an inhibitor of NOS) (Sigma–Aldrich, USA) (Ninnemann and Maier, 1996; Gong et al., 2007) were used to treat mutants and wild type fungal samples, using working concentrations of 0.01–100 μM and 5–2500 μM, respectively. All experiments were repeated three times, with three replicates for each treatment each time.

Fertility assays

Mating tests were carried out by pairing the wild type strain Guy11 (*MAT1-2*) and Arg⁻ mutants with a standard tester strain TH3 (*MAT1-1*) on oatmeal agar (OMA) plates, as described previously (Yan et al., 2013). The plates were incubated at 25 °C until the colony margins contacted each other, and then placed under continuous white fluorescent light at 18 °C for 4 weeks. The junctions