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# Inactivation of the global regulator LaeA in *Monascus ruber* results in a species-dependent response in sporulation and secondary metabolism

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ABSTRACT

The nuclear regulator LaeA has been proven to globally govern fungal development and secondary metabolism, but its function may be species-dependent, even though its amino acid sequences are well conserved in numerous fungi. Herein we identified the LaeA in *Monascus ruber* M7 (MrLaeA), and verified its role to mediate growth, sporulation and secondary metabolism. Results showed that the radial growth rate of the selected *MrlaeA* knock-out mutant (MrΔ*laeA*-22) was significantly faster than that of the parental strain *M. ruber* M7, and growth was accompanied by the formation of an abnormal colony phenotype with more abundant aerial hyphae. Interestingly, conidia production of the MrΔ*laeA*-22 strain was about thrice that of *M. ruber* M7, but ascospores were not observed in the MrΔ*laeA*-22 strain. Additionally, compared to *M. ruber* M7, MrΔ*laeA*-22 exhibited drastically reduced production of multiple secondary metabolites, especially those of the six well-known *Monascus* pigments and citrinin. Simultaneously, the selected *MrlaeA* complementation strain (MrΔ*laeA*::*laeA*-45) nearly recovered the capacity for sporulation and secondary metabolism observed in the parental strain. These results demonstrate that MrLaeA regulates not only secondary metabolism, but also asexual and sexual differentiation in *M. ruber*, but some of its regulation appears to differ from other fungi.



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*Abbreviations; CIT, citrinin; GMM, glucose minimal medium; HPLC, high performance liquid chromatography; MPs,* Monascus *pigments; MrLaeA,* Monascus ruber *LaeA; ORF, open reading frame; PDA, potato dextrose agar medium; PDB, potato dextrose broth medium; RMR, red mould rice*

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## Introduction

One of the fascinating aspects of filamentous fungi is their ability to produce secondary metabolites (Lin et al. 2008). Fungi of the genus *Monascus* can produce various bioactive substances, such as *Monascus* pigments (MPs) (Feng et al. 2012; Shao et al. 2014) and monacolins (Endo 1979). MPs have been used as food additives for several centuries in Asian countries (Dufossé et al. 2005). However, the safety of *Monascus*-fermented products has been questioned because a mycotoxin, citrinin (CIT), with nephrotoxic and hepatotoxic properties, is coproduced with the MPs in some *Monascus* strains (Lin et al. 2008). The wild-type parental strain used in this study, *Monascus ruber* M7, also coproduces MPs and CIT (Yang et al. 2012; Li et al. 2014).

Several researches have shown that the biosynthesis of the fungal secondary metabolites is regulated by not only the pathway-specific regulators, but also a complex network of global regulators (Bok & Keller 2004; Georgianna & Payne 2009; Sarikaya-Bayram et al. 2015). One of the global regulators is the LaeA protein, a putative methyltransferase that controls the synthesis of many different secondary metabolites in Aspergilli including aflatoxins, sterigmatocystin, penicillin, emericellamide, terrequinone, gliotoxin, and lovastatin (Brakhage & Schroeckh 2011). It is worth mentioning that Kosalková et al. (2009) studied the differences in overall metabolite secretion between the wild-type strain *Penicillium chrysogenum* Wis54-1255 and the *laeA* knock-down mutant AT92 grown for 48 h and 72 h in defined production medium (Esmahan et al. 1994) by high performance liquid chromatography (HPLC). They found that the disruption of *laeA* resulted in the decreased generation of metabolite L, other metabolites increased in both the hydrophobic and hydrophilic fractions.

Besides regulating the secondary metabolism, LaeA can regulate the sporulation in some *Aspergillus* strains. Bok et al. (2005) found that the disruption of *laeA* could decrease conidia formation in *Aspergillus fumigatus* AF293 on glucose minimal medium (GMM). Kale et al. (2008) reported that the *laeA* deletion mutant of *Aspergillus flavus* decreased conidial production on yeast extract sucrose medium, GMM medium and on peanut seeds. However, Chang et al. (2012) discovered that the *laeA* deletion mutant of *A. flavus* CA14 showed increased production of conidia on potato dextrose agar (PDA) medium. Additionally, Sarikaya Bayram et al. (2010) reported that the *laeA* null mutant of *Aspergillus nidulans* resulted in constitutive sexual differentiation, indicating that LaeA plays a pivotal role in inhibiting sexual development in response to light.

The pathway-specific regulators of MPs and CIT in *M. ruber* synthesis have been identified as PigR and CtnA, respectively (Shimizu et al. 2007; Xie et al. 2013). Additionally, two reports about the *laeA* gene in *Monascus pilosus* have been published. Zhang & Miyake (2009) discovered that the fungal growth stage and the principal nutrients could regulate the alternative splicing patterns of *MpLaeA* (*d*- and *l*-*MpLaeA*), and *d*-*MpLaeA* mRNA was an ineffectively spliced mRNA. Lee et al. (2013) reported that the overexpression of *laeA* could upregulate the production of many secondary metabolites, such as MPs, monacolin K, and other unknown substances. However, whether LaeA regulates the sporulation in *Monascus* is unknown.

In the current study, we investigated the effects of LaeA inactivation on secondary metabolism and on growth and sporulation in *M. ruber* M7. Our results indicated that MrLaeA positively regulated sexual reproduction and secondary metabolism, but negatively controlled the vegetative development and the asexual reproduction, which are effects that may differ from those observed in other fungi. Notably, this is the first report that the disruption of *laeA* in *Monascus* spp. could lead to more abundant aerial hyphae, absence of cleistothecia, and significantly decreased yield of CIT.

## Materials and methods

### *Fungal strains, culture media, and growth conditions*

*Monascus ruber* M7 (CCAM 070120, Culture Collection of State Key Laboratory of Agricultural Microbiology, part of the China Center for Type Culture Collection (CCTCC), Wuhan, China) (Chen & Hu 2005) was used to generate the MrΔ*laeA* strains. The MrΔ*laeA* strain was exploited for the generation of MrΔ*laeA*::*laeA* strains. Hygromycin B-resistant transformants and neomycin-resistant transformants were selected on potato dextrose agar (PDA) medium containing 30 μg $mL^{-1}$ hygromycin B (Sigma-Aldrich, Shanghai, China) and 15 μg $mL^{-1}$ G418 (Sigma-Aldrich, Shanghai, China), respectively. All strains were maintained on PDA slants at 28 °C. For analysis of secondary metabolites, strains were grown in potato dextrose broth (PDB) medium. Every 50 mL of the culture medium was inoculated with $5 \times 10^4$ freshly harvested spores and cultivated at 28 °C with continuous shaking at 120 rpm.

### *Genomic DNA and RNA extraction*

Fungal genomic DNA and RNA from *M. ruber* M7 and all mutant strains were isolated from mycelia grown on cellophane membranes covering PDA plates. The genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Shao et al. 2009), and the total RNA was abstracted according to Xie et al. (2013).

### *Bioinformatics analysis of the MrLaeA sequence*

The amino acid sequences were aligned with the CLUSTAL program at the EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the DNAMAN software 6.0.3 (Lynnon Biosoft, Quebec, Canada). The sequences were highlighted according to the similarity (≥50 %).

### *Deletion and complementation of the* MrlaeA *gene*

To verify the function of *MrlaeA*, the gene was deleted and complemented according to the homologous recombination strategy as previously described (Liu et al. 2014). The gene disruption construct carried 5′ flanking regions (782 bp, amplified with the primer pair P1—P2 (Table 1)), hygromycin B resistance