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Proteome analysis reveals global response to deletion of *mrflbA* in *Monascus ruber*[§]

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***Monascus* spp. are commonly used for a wide variety of applications in the food and pharmaceutical industries. In previous studies, the knock-out of *mrflbA* (a putative regulator of the G protein α subunit) in *M. ruber* led to autolysis of the mycelia, decreased pigmentation and lowered mycotoxin production. Therefore, we aimed to obtain a comprehensive overview of the underlying mechanism of *mrflbA* deletion at the proteome level. A two-dimensional gel electrophoresis analysis of mycelial proteins indicated that the abundance of 178 proteins was altered in the *ΔmrflbA* strain, 33 of which were identified with high confidence. The identified proteins are involved in a range of activities, including carbohydrate and amino acid metabolism, hyphal development and the oxidative stress response, protein modification, and the regulation of cell signaling. Consistent with these findings, the activity of antioxidative enzymes and chitinase was elevated in the supernatant of the *ΔmrflbA* strain. Furthermore, deletion of *mrflbA* resulted in the transcriptional reduction of secondary metabolites (pigment and mycotoxin). In short, the mutant phenotypes induced by the deletion of *mrflbA* were consistent with changes in the expression levels of associated proteins, providing direct evidence of the regulatory functions mediated by *mrflbA* in *M. ruber*.**

Keywords: *Monasucs ruber*, comparative proteomics, development, modulation, regulator of G protein α subunit, secondary metabolites

Introduction

Regulators of G protein signaling (RGSs) are a group of proteins governing the intensity and duration of G protein signaling by interacting with an activated G protein α subunit (Gα). Currently, six RGS protein families, including the orthologs of RgsA, RgsB, RgsC, RgsD, FlbA, and Gprk, have been identified in *Aspergillus* spp. During the past two decades, several investigations have demonstrated that FlbA plays a role in a variety of biological processes in fungi (Shin *et al*., 2013).

Monascus spp., are intensely cultivated in Asian countries for their production of bioactive metabolites, which are widely used as food or food additives, in folk medicine, and in starter cultures for fermentation (Patakova, 2013; Shao *et al*., 2014). However, some *Monascus* strains can secret mycotoxin (citrinin). In recent years, attention has been increasingly drawn to the secondary metabolism and development of *Monascus* spp. In our previous study, the deletion of *flbA* in *M. ruber* (*mrflbA*) accelerated autolysis of the mecylia, increased conidia production and significantly reduced pigment and mycotoxin levels (Yang *et al*., 2012). However, it is unknown how the absence of MrflbA causes these phenotypical changes. The identification of molecular targets by proteomic profiling may provide an insight into understanding these mechanisms in *M. ruber*.

Comparative proteomic analysis is a powerful tool, providing a qualitative and quantitative expression profiling of total proteins as well as a detailed and comprehensive understanding of molecular events. In filamentous fungi, proteomic analyses have been employed to identify various pathogenic factors, diagnostic markers, immune-reactive fungal antigens, resistance mechanisms against antifungal drugs, and responses to environmental stresses (Shin *et al*., 2013). In *Monascus* spp., proteomic analyses have been used to evaluate the protein profile in *M. pilosus* responding to environmental growth factors (Lin *et al*., 2007a, 2007b, 2007c, 2008). In this study, we explored the effect of the deletion of MrflbA on protein expression in *M. ruber*. The results revealed that the absence of MrflbA perturbed the expression of multiple proteins involved in the metabolism of carbohydrates and amino acids, hyphal development and the oxidative stress response, protein modification, cell signaling regulation, and secondary metabolism, which provided direct evidence for MrflbA-mediated regulation of multiple bioprocesses at the protein level.

Materials and Methods

Fungal strain and culture conditions

In this study, *M. ruber* M7 (WT, CCAM 070120 collected in the Culture Collection of the State Key Laboratory of Agricultural Microbiology, which is part of the China Center

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for Type Culture Collection [CCTCC], Wuhan, China) and ΔMrflbA strain (preserved in our lab, which was generated by knocking out the gene *mrflbA* from WT) were used for the following investigations (Yang *et al*., 2012). Potato Dextrose Agar (1 L of PDA, consisting of 4 g potato infusion, 20 g dextrose, and 20 g agar powder) was used to culture the fungal strains. For submerged culture, approximately 3 × 10^5 /ml of conidia were inoculated into Potato Dextrose Broth (PDB, identical to PDA, omitting the agar) and incubated at 28°C with continuous shaking at 180 rpm.

Extraction of mycelia proteins

Mycelia of the WT and *ΔmrflbA* strains were collected from triplicate cultures, which were grown for 5 days in PDB and washed with PBS (pH 7.4). The collected mycelia samples were submerged in liquid nitrogen and ground by mortar-pestle. The prepared powder was resuspended in ice-cold acetone containing 10% (w/v) trichloroacetic acid. The samples were kept at -20°C overnight and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in ice-cold 70% acetone. This centrifugation- and-resuspension process was repeated three times. The final pellets were vacuum-dried and resuspended in lysis solution (7 M urea, 2 M thiourea containing 4% [w/v] CHAPS, 1% [w/v] DTT, 2% [v/v] pharmalyte, and 1 mM benzamidine) at 4°C overnight. After adequate vortexing, the samples were centrifuged at 12,000 rpm for 30 min at 4°C. Insoluble material was discarded, and the soluble fraction was subjected to two-dimensional gel electrophoresis (2-DE) analysis. The total protein concentration of each sample was assayed by the Bradford method using the Bio-Rad DC Protein Assay kit according to the manufacturer's instruction.

2-DE and protein identification

Proteins were separated by 2-DE analysis using the Ettan IPG phor 3 System (GE Healthcare) for isoelectric focusing (IEF) and the Ettan DALTSix System (GE Healthcare) to perform SDS-PAGE. IEF was performed with DryStrip IPG strips of 24 cm (pH 4–7), which were rehydrated overnight at 20°C with 120 μl (1 μg/μl of protein) of the protein sample in the rehydration buffer (Bio-Rad). The IEF was performed at 20°C as follows: step 1: 300 V for 0.5 h, step 2: 700 V for 0.5 h, step 3: 1,500 V for 1.5 h, step 4: 9,000 V for 3 h, step 5: 9,000 V for 5 h, for a total of 64 kVh. After completion of the IEF program, the strips were stored at -20°C. Prior to two-dimensional (2-D) analysis, the individual strips were equilibrated twice, to re-denature the proteins and reduce the disulfide bonds, for 15 min in 10 ml denaturing solution (6 M urea, 30% glycerol, 2% SDS, 0.375 M Tris; pH 8.8 containing 0.1 M DTT, a trace of bromophenol blue) and subsequently for 15 min in 10 ml equilibration buffer containing 250 mM iodoacetamide (IAA). After equilibration, the 2-D electrophoresis was performed on a 12.5% SDS polyacrylamide gel. The gels were run at 2 W per gel for the first 45 min and followed by 17 W per gel for approximately 4.5 h or until the dye front reached the bottom of the gel. The protein spots were visualized via silver staining. Subsequently, gel evaluation and data analysis were carried out using the ImageMaster v 7.0 program (GE Healthcare). The differences in 2-DE data were evaluated by a one-way ANOVA and a T-test ($P < 0.05$) using SPSS 13.0 (SPSS).

To analyze the protein patterns, stained gels were scanned and calibrated using a PowerLook 1100 scanner (UMAX), followed by analysis of the protein spots via the ImageMaster v 7.0 program (GE Healthcare). Only those with significant and reproducible changes ($P < 0.05$) were considered to be differentially expressed proteins. The target protein spots were manually excised from the stained gels and washed, then digested with sequencing-grade trypsin (Promega). MALDI-TOF spectra were calibrated using trypsin autodigestive peptide signals and matrix ion signals. The protein sample in an equivalent matrix solution was further analyzed by MALDI-TOF MS/MS, which was performed using a fuzzy logic feedback control system (Ultraflex MALDI-TOF-TOF mass spectrometer Bruker) equipped with delayed ion extraction. Proteins were identified by searching the peptide masses using the Mascot program (http://www.matrixscience.com). Search parameters of Mascot were as follows: trypsin with 1 missed cleavage, precursor mass tolerance of 50 ppm, fragment ions mass tolerance of 0.6 Da, carbamidomethyl (C) as a fixed modification and oxidation (M) as a variable modification. The Proteome Discoverer program was used with the following settings: peptide score filter; Mascot score > 55; and a peptide mass deviation of 10 ppm. The function and functional category of each protein was assigned based on published literature and public databases.

Chitinase activity

Chitinases are enzymes that degrade the cell wall. The chitinase activity of the *ΔmrflbA* strain was detected following a published method with minor modification (Rojas-Avelizapa *et al*., 1999). Briefly, 1 ml of filtered fermentation broth was added to 1 ml of 1% colloidal chitin and incubated for 1 h at 50°C. Then, the reaction was terminated at 100°C for 10 min, and 1 ml of supernatant was added to 1 ml of DNS reagent and boiled for 10 min. Next, 3 ml distilled H_2O was supplemented in DNS-contained system. Finally, this mixture was centrifuged at 5,000 rpm for 5 min. The OD was measured by UV-Vis spectrophotometery (UV-1700, Shimadzu) at 540 nm. One unit of chitinase was defined as the amount of the enzyme releasing 1 μmol/h of N-acetyl-D-glucosamine under the conditions described above.

Comparison of menadione resistance

To compare the antioxidative activity of the *ΔmrflbA* strain to that of the WT, menadione resistance was evaluated. The procedure was as follows: 100 μl of fresh spores (10^5/ml) were spread onto PDA punched with 7-mm-diameter micropores, each filled with 100 μl of menadione (the final concentrations of 5 mM, 10 mM, 25 mM, and 50 mM, respectively, in each micropore). The zone of inhibition was measured from the second to the sixth day.

Real-time quantitative PCR analysis

The transcript levels of genes associated with the production of pigment and citrinin were analyzed by a real-time quantitative PCR (RT-qPCR) analysis. Hyphae were harvested

Table 1. Primers used in RT-qPCR[a]

Primers	Sequence (5′-3′)	Length (bp)
pksA-f	AAGGGTTCTGGGACTTGCT	138
pksA-r	CATCGTGGTCTCGGATAAAG	
pksCT-f	AAGATTAGCCACCATTTCGG	125
pksCT-r	AGTGCCCCGTTACATTCC	
ctnR-f	CGACAGTCCTACGAAACCC	101
ctnR-r	TGAGCAGGTGGTCCAAGAA	
GAPDH-f	CAAGCTCACTGGCATGTCTATG	162
GAPDH-r	AAGTTCGAGTTGAGGGCGATA	

f, forward primer; r, reverse primer.

at the 4th day, the 7th day, and the 9th day. RNA was extracted from mycelia both of WT and *ΔmrflbA* strain using the EZ-10 Spin Column RNA Purification Kit (BBI) according to the manufacturer's protocol. RT-qPCR was performed following a previous description (Liu *et al.*, 2016a). GAPDH was used as the reference gene. The primers used in this study are listed in Table 1.

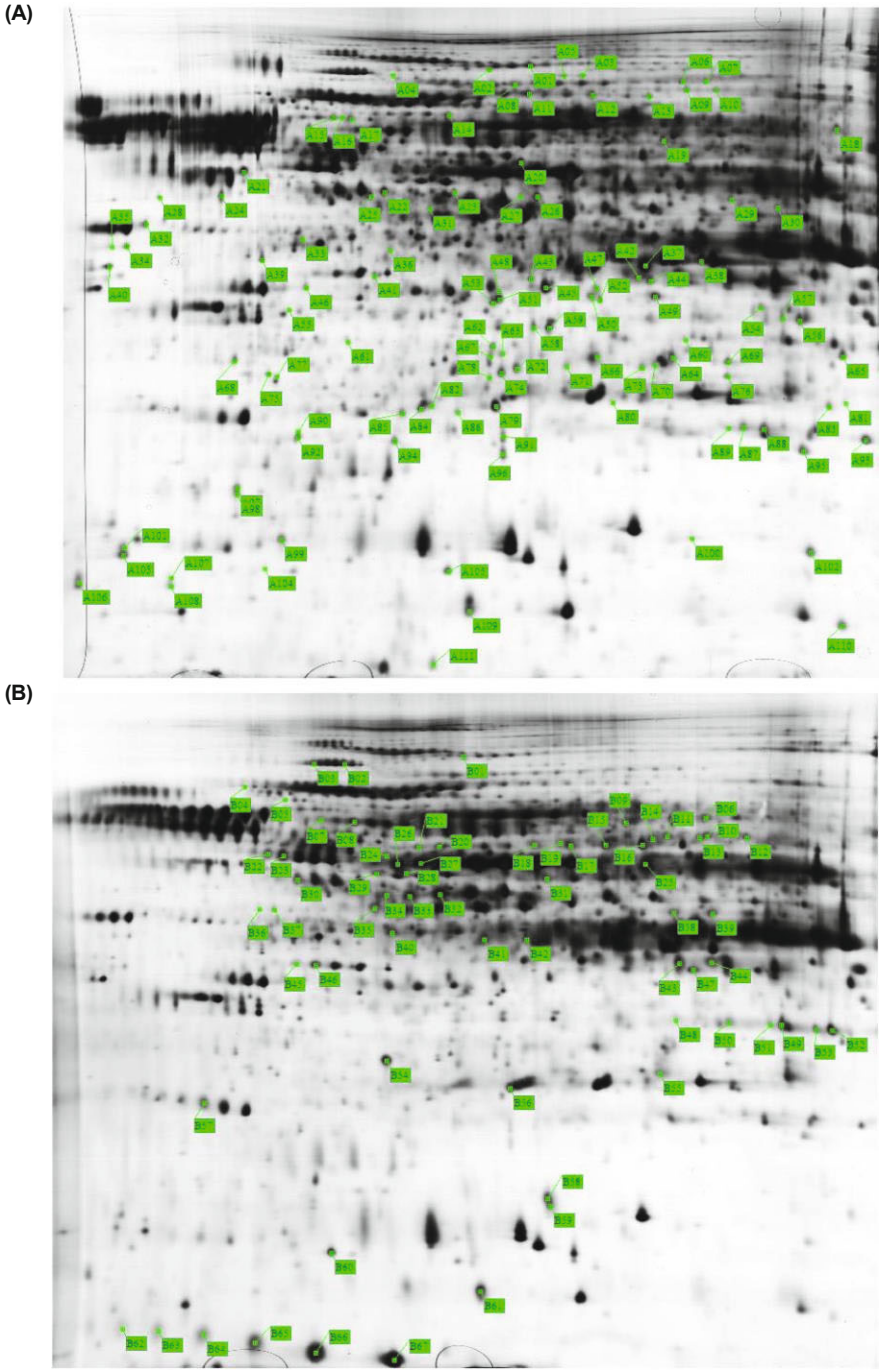

Fig. 1. Comparative analysis of the mycelia proteome of WT and *ΔmrflbA* strains. (A) 2-DE images of total proteins from WT grown in liquid medium for 5 days; (B) 2-DE images of total proteins from the *ΔmrflbA* strain grown in liquid medium for 5 days. In all, 111 spots and 67 spots exhibited upregulated and downregulated levels in the *ΔmrflbA* strain.

Results

Overall mycelia proteome analysis

To understand the mechanisms underlying the response of *M. ruber* to the absence of MrflbA protein, intracellular proteins were extracted from the mycelia of 5-day-old cultures, and a total of 120 μg (1 mg/ml) of protein was separated via 2-DE. The silver-stained 2-DE images of the WT and *ΔmrflbA* strains in Fig. 1 revealed 178 protein spots with statistically significant alteration of expression level, including 111 upregulated and 67 downregulated spots. Spots with an intensity of ≥ 2.0 relative spot volume (82 spots) were manually excised and identified by MALDI-TOF/TOF. All the proteins identified in the study are listed in Supplementary data Table S1. Within the parameters of our Mascot search, 33 proteins were identified with high confidence. The identified proteins and their Mascot score distributions, ion score, number of peptides matched, % sequence coverage, and molecular weight are listed in Fig. 2. Most of the proteins identified were within the molecular weight range of 20–60 kDa and sequence coverage was greater than 20% using identified peptides fragments of > 6 amino residues. The identified proteins were categorized by their biological function based on published documents and public databases and were found to primarily play roles in carbohydrate, protein, amino acid and energy metabolism, hyphal development and the cell stress response, protein modification, and cell signaling regulation (Table 2).

Carbohydrate and energy metabolism response to MrflbA depletion

It has been demonstrated that hyphal autolysis is an energy-dependent and nutrient-dependent process occurring during the stationary phase of fungal growth (Emri *et al.*, 2008). In this study, the expression levels of proteins participating in carbohydrate metabolism significantly changed. Several enzymes responsible for glycolytic processes were downregulated; meanwhile, 2 proteins presumed to be involved in energy metabolism were downregulated, including the putative NADH-ubiquinone oxidoreductase subunit (B39) and ATP synthase subunit E (B53). Notably, acetate-CoA ligase (A07 and A09), also called acetyl-CoA synthetase involved in the synthesis of acetyl-CoA from acetate, was upregulated. In addition, triosephosphate isomerase (A80) increased in abundance. These results suggest that deletion of *mrflbA* disturbed carbohydrate and energy metabolism and that MrflbA-mediated autolysis is a carbon-source and energy-associated process.

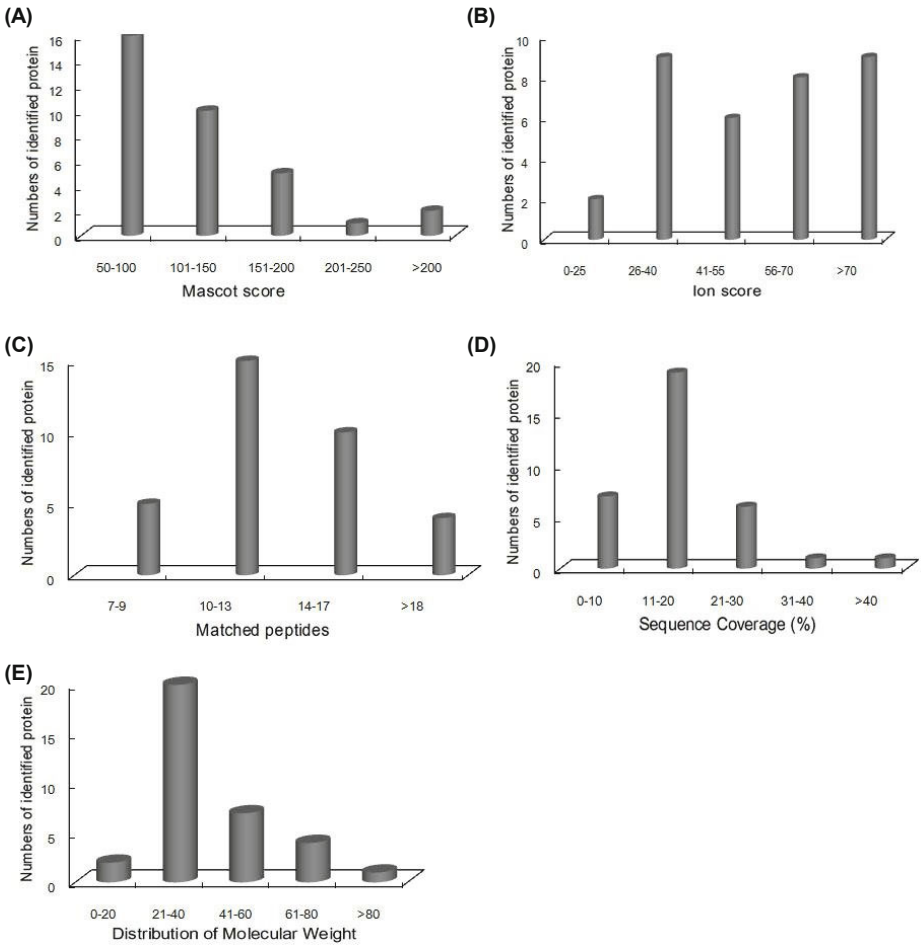

Fig. 2. Proteins and their distribution of molecular weight, % sequence coverage, Mascot score, matched peptides, and ion score. (A) Mascot score, (B) Ion score, (C) Matched peptides, (D) % Sequence coverage, (E) Molecular weight.

The role of MrflbA in the regulation of mycelia development

In many filamentous fungi, deletion of FlbA can lead to fluffy hyphae (Shin *et al*., 2013). In this study, 5 proteins closely associated with *Monascus* hyphal development were identified with significantly different expression in *ΔmrflbA* strain. Among of them, actin (A31), which is involved in many different key cellular processes, such as cell motility, cytokinesis, secretion, and the control of cell morphology, was more abundant in the *ΔmrflbA* strain than in the WT (González-Rodríguez *et al*., 2016). Elongation factor 2 (A52), which has demonstrated involvement in hyphal elongation and normal branching in *Neurospora crassa* (Propheta *et al*., 2001), was increased more than 3-fold in the *ΔmrflbA* strain. However, 3 identified actin-interacting proteins were significantly downregulated, including the putative Septin AspA (B35), F-actin capping protein beta subunit (B45), and cofilin (B61). One possible explanation for this effect is that the overexpression of actin hampered its normal interaction with these proteins. These results suggested that the aberrant expression of elongation factor 2, actin, and actin-associated proteins leads to abnormal hyphal development due to deletion of *mrflbA*.

The role of MrflbA in regulation of pigment and citrinin synthesis

Proteomic analysis revealed that proteins participating in the synthesis of pigment and citrinin were downregulated in the *ΔmrflbA*, such as the putative fatty acid synthesase beta

Table 2. The differentially expressed proteins in WT and *ΔmrflbA* strains identified with high confidence

Spot IDa	Protein Description	Fold changeb	pIc	Mrd (kDa)	NCBI ID
Carbohydrate and energy metabolism					
A07	acetate-CoA ligase	4.40599	5.84	74649	145258043
A09	acetate-CoA ligase	4.23001	5.84	74649	145258043
A80	triosephosphate isomerase	4.49918	5.46	27511	52783473
B41	Enolase	3.25435	5.3	47432	114194476
B42	fructose-bisphosphate aldolase, class II	3.34231	5.55	39937	159127469
B39	NADH-ubiquinone oxidoreductase 39 kDa subunit, putative	2.99421	8.3	43759	119406573
B53	ATP synthase subunit E, putative	14.1633	5.57	25971	119398473
Hyphal development					
A31	Actin	1000000	5.84	40340	114190657
A52	elongation factor 2 (EF-2) (Colonial temperature-sensitive 3)	3.83544	6.45	94043	358365542
B35	septin AspA, putative	2.81052	5.06	43912	218718192
B45	F-actin capping protein beta subunit	4.11122	4.95	32294	159130536
B61	cofilin	1000000	5.48	16796	218715384
Oxidative response					
A79	mitochondrial peroxiredoxin PRX1	5.59311	5.88	24770	114192711
A84	mitochondrial peroxiredoxin PRX1	1000000	5.88	24770	114192711
Amino and protein metabolism and protein modification					
A27	S-adenosylmethionine synthetase	1000000	5.4	42264	119397343
A21	carboxypeptidase Y homolog A; Flags: Precursor	3.366	4.79	62486	332313322
A104	ubiquitin-like modifier SUMO, putative	1000000	4.87	10296	220693706
A73	proteasome component Y13	4.09229	5.63	28582	525587570
A74	transcription initiation factor subunit	3.44562	5.52	26304	358366625
B58	TPA: 30 kDa heat shock protein (Broad)	1000000	5.8	20334	19399550
B59	TPA: 30 kDa heat shock protein (Broad)	1000000	5.8	20334	119399550
Secondary metabolism and signaling regulation					
B38	aflatoxin B1 aldehyde reductase member 3	2.95428	6.02	38882	511262094
A15	karyopherin alpha subunit	3.56737	4.92	60770	358374928
A23	stomatin family protein	3.50072	6.49	47490	358372986
A55	multifunctional chaperone	1000000	4.8	30840	391863504
A77	eukaryotic translation initiation factor 6	1000000	4.89	26588	114188867
Others					
A72	HAD superfamily hydrolase	10.8659	5	27374	358374694
A89	Hypothetical protein ASPNIDRAFT_212618	3.0379	7	28933	350639981
A19	Pc21g02650	3.24807	5.66	51963	211589040
A56	Pc13g03260	5.58569	6.04	32415	211583384
B50	Hypothetical protein ASPNIDRAFT_50815	7.40474	5.44	23591	350638899
B51	Hypothetical protein ASPNIDRAFT_50815	8.26613	5.44	23591	350638899
B63	Hypothetical protein AN1478.2	1000000	5.62	377489	40745452

A and B represented upregulated and downregulated proteins in Δ*mrflbA* strain, respectively.
[a] Spot number in Fig. 1; [b] Fold change from statistical analysis of silver-stained gels by PowerLook 1,100 scanner and ImageMaster v 7.0 program; [c and d] Theoretical values.

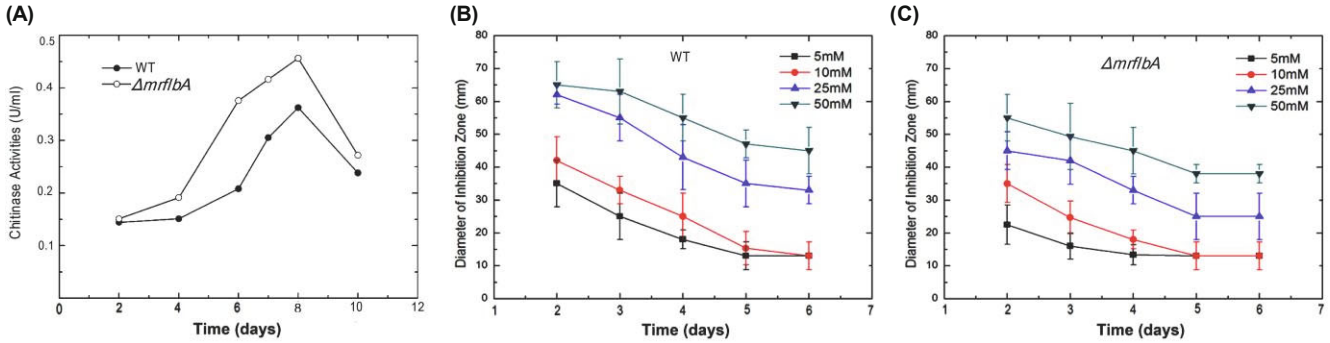

Fig. 3. Comparison of the transcriptional level of *ctn R*, *pks CT*, and *pks A* between WT and *ΔmrflbA* strains. (A) *ctn R*, (B) *pks CT*, (C) *pks A*.

subunit (B49) and the transcriptional activators Ctn R (B55) and Ctn G (B60). Although the confidence in the identity of these proteins was lower than the initial Mascot search parameters, the observed sequence variation was in line with previous reports of pigment and citrinin protein identification (Yang *et al*., 2012). An analysis of transcript levels by RT-qPCR indicated that the expression of the polyketide synthetase for pigment synthesis and for citrinin synthesis (*pks A* and *pks CT*, respectively), as well as *ctn R*, were also downregulated (Fig. 3). At the protein expression and transcriptional level, the proteins and mRNA involved in the synthesis of pigment and citrinin tended to be downregulated, which was in accordance with our previous study (Yang *et al*., 2012).

Chitinase activity and tolerance against menadione

Chitinases are specific enzymes responsible for degrading fungal cell walls, but we did not identify chitinase proteins in mycelia. In fact, the chitinase activity of the *ΔmrflbA* strain in the supernatant was higher than that of WT in the tested period, suggesting that chitinases contributed to autolysis of the *ΔmrflbA* strain's hyphae (Fig. 4A).

Reactive oxygen species (ROS) will accumulate when autolysis occurs; correspondingly, the activities of antioxidative enzymes increase to prevent their harm to cells. In this study, the protein expression level of mitochondrial peroxiredoxin PRX1 (A79 and A84) was significantly upregulated in the *ΔmrflbA* strain. Figure 4B shows that the diameter of the growth inhibition zone became larger with increasing concentrations of menadione both for the *ΔmrflbA* and WT strains. However, the recorded inhibition zone diameter of the *ΔmrflbA* strain was smaller than that of WT at the same concentration of menadione, demonstrating that the *ΔmrflbA* strain had much stronger resistance to menadione than WT (Fig. 4C), which we attribute to the enhanced activities of antioxidative enzymes. Our previous study also demonstrated that the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase were enhanced in fermentation broth of the *ΔmrflbA* strain (Zhang *et al*., 2014).

Discussion

FlbA, a regulator of the G protein α subunit, is involved in multiple biological processes, including growth, development, conidiation, hyphae morphogenesis, pathogenicity, and virulence, hydrophobin biosynthesis, and secondary metabolism, as well as regulation of pheromone and cAMP signaling (Segers *et al*., 2004; Fang *et al*., 2007, 2008; Liu *et al*., 2007; Shen *et al*., 2008; Xue *et al*., 2008; Mukherjee *et al*., 2011; Zhang *et al*., 2011; Park *et al*., 2012; Ramanujam *et al*., 2012; Yang *et al*., 2012). Our previous study focused on the phenotypic effects of the absence of *mrflbA* on the development of *M. ruber* and the production of secondary metabolites. To better understand the mechanisms underlying the

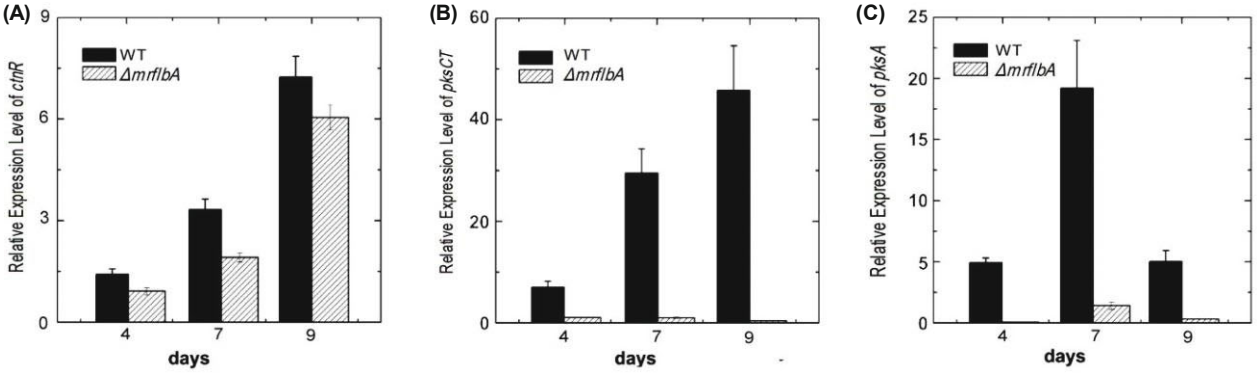

Fig. 4. Comparison of chitinase activity and the resistance against menadione between WT and *ΔmrflbA* strains. (A) Chitinase activities, (B) Resistance against menadione by WT, (C) Resistance against menadione by *ΔmrflbA* strain.

response to the absence of MrflbA, a proteomic approach was applied to identify the hyphal proteins of *M. ruber*. The 5-day cultures was chosen because the two strains began to show differences in autolysis and pigmentation at this time. Overall, 178 protein spots showed alterations in abundance between WT and the *ΔmrflbA* strains, and function was predicted for 39 of the identified protein spots (Supplementary data Table S1). Among these, 24 and 15 spots were significantly upregulated and downregulated, respectively, and were predicted to be involved in carbohydrate, energy, amino acid and protein metabolism, cell morphogenesis, oxidative stress response, protein modification, DNA repair, secondary metabolism, and signaling regulation. These data are consistent with the physiological characteristics and phenotypic variation observed from the knock-out of MrflbA (Yang *et al*., 2012).

A typical phenotypic response to *flbA* deletion is the development of fluffy aerial hyphae in several different fungi. In most fungi, hyphae are the main mode of vegetative growth. Comparative proteomic analyses reveal that the intensities of 5 proteins associated with hyphae development are significantly altered in the *ΔmrflbA* strain. Among these, the levels of actin (A31) and elongation factor 2 (A52) in *ΔmrflbA* were more abundant than in the WT. Meanwhile, the levels of Septin AspA (SepA) (B35), F-actin capping protein beta subunit (B45), and cofilin (B61) were significantly downregulated. Actin is a highly conserved protein found in all eukaryotes with two forms: globular (G-) actin and filamentous (F-) actin. F-actin exists in three higher-order structures containing actin rings, patches, and cables, playing a vital role in the cell morphogenesis of filamentous fungi (González-Rodríguez *et al*., 2016). These 3 downregulated proteins can interact with F-actin to affect hyphae development. For instance, SepA is necessary for normal development and morphogenesis to polymerize actin cables and target cargo delivery in *Aspergillus nidulans* (Lindsey *et al*., 2010). F-actin capping protein, composed of alpha and beta subunits, binds to the fast growing ends of F-actin cables to stabilize them (González-Rodríguez *et al*., 2016). Cofilin binds the minus end of actin and inhibits the formation of actin filaments (Bravo-Cordero *et al*., 2013; Bao *et al*., 2015). In *A. nidulans*, mutation of Cot 3 (a homolog of elongation factor 2) led to curled hyphae and lagged conidia germination (Propheta *et al*., 2001). In *M. ruber,* however, the variance in expression of these proteins did not affect the biomass of the *ΔmrflbA* strain (Yang *et al*., 2012). These current data suggest that the FlbA-mediated signaling pathway can independently regulate the expression of proteins involved in hyphae development.

Generally, cell autolysis is regarded as a natural process of self-digestion in aged hyphal cultures, leading to the accumulation of ROS and cell wall degradation. In *Aspergillus fumigatus*, catalase Cat1 (EF1) was secreted at higher levels in the culture supernatants of the ΔflbA strain, while β-D-glucoside glucohydrolase (endo-chitosanase precursor), and Cu/Zn SOD were secreted at higher levels in WT (Shin *et al*., 2013). In *M. ruber*, the depletion of MrflbA leads to accelerated hyphal autolysis and, consistently, the chitinase activity and activity of antioxidative enzymes including SOD, CAT, and glutathione reductase in the supernatant of the *ΔmrflbA* strain were higher than those of WT (Yang *et al*., 2012). Furthermore, the resistance of the *ΔmrflbA* strain to menadione was much stronger than that of WT, demonstrating the antioxidative ability of *ΔmrflbA* strain was enhanced. In addition, we observed higher expression of mitochondrial peroxiredoxin PRX1 (A79 and A84) in the *ΔmrflbA* strain. All these data suggested that hydrolase and antioxidative enzymes are necessary to cell autolysis in filamentous fungi.

In this study, we also identified proteins differentially expressed between WT and *ΔmrflbA* strains involved in carbohydrate and energy metabolism. Acetate-CoA ligase (also known as acetyl-CoA synthetase and acetyl-activating enzyme) is the predominant enzyme for the activation of acetate to acetyl-CoA. In yeast, there are two isoforms of acetate-CoA ligase differing with respect to localization, regulation, and kinetic properties (Liu *et al*., 2016b). Here, we also identified 2 acetate-CoA ligases (A07 and A09) with higher expression levels in the *ΔmrflbA* strain, possibly playing different roles in *M. ruber*. Notably, the expression level of several proteins involved in glycolysis process altered. Triosephosphate isomerase (A80), the glycolytic enzyme that catalyses the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, was upregulated. Putative enolase (B41), catalysing the reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate as part of the glycolysis and gluconeogenesis pathways, was downregulated. Fructose- bisphosphate aldolase (B42), catalysing the reversible aldol cleavage or condensation of fructose-1, 6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate, was also downregulated. Pyruvate decarboxylase (B52) catalyses the decarboxylation of pyruvic acid to acetdehyde and CO_2 in mitochondria of eukaryotes. Meanwhile, proteins implicated in energy metabolism, NADH-ubiquinone oxidoreductase (B39) and the putative ATP synthase subunit E (B53), were downregulated in the *ΔmrflbA* strain. Taken together, these data indicate that the absence of *mrflbA* attenuated glycolysis and energy metabolism in *M. ruber*.

In addition to involvement in fungal development, FlbA has also been implicated in the regulation of secondary metabolite production (Shin *et al*., 2013). Previous work revealed that *mrflbA*-mediated signaling negatively regulates the production of pigment and citrinin (Yang *et al*., 2012). Notably, this study found that the knockout of *mrflbA* resulted in reduced expression of Fas β (B49), Ctn R (B55), and Ctn G (B60). Fas β, a putative fatty acid synthetase beta subunit, was demonstrated to produce the side chain fatty acyl moiety of *Monascus* pigment in both *M. ruber* and *M. purpureus* (Balakrishnan *et al*., 2014; Chen *et al*., 2017). The deletion of Ctn A (a homolog of Ctn R, a transcriptional activator of citrinin synthesis) led to a sharp reduction of citrinin production in *M. purpureus* (Shimizu *et al*., 2007). The deletion of Ctn G (a homolog of β-carbonic anhydrase) led to a reduced production of pigment and citrinin in *M. aurantiacus* (Li *et al*., 2015). In *Aspergillus fumigates*, expression and secretion of GliT (which detoxifies exogenous mycotoxin) was elevated in the absence of *flbA* (Shin *et al*., 2013). Li *et al.* (2010) reported that *mga1* (a putative G protein α subunit in *M. ruber*) negatively regulates the production of pigment and citrinin in *M. ruber*. The deletion of *mrflbA*

can increase the transcription of *mga1*. In combination, this evidence suggests that the reduction of pigment and citrinin production upon deletion of *mrflbA* could be attributed to an increase in the activity of *mga1*.

Notably, additional proteins identified with abnormal expression in the *ΔmrflbA* strain were involved in DNA repair (A83 and A102), protein modification (A104, A74, and A28), signaling regulation (A15, A23, A77, and 320 A96) (Manfiolli *et al*., 2017), and pyridoxine biosynthesis (B57), all of which play very important roles in maintaining normal vital cell processes (Carvalho *et al*., 2010; Lamoth *et al*., 2015; Droescher *et al*., 2011). These data suggest that MrflbA can globally perturb the expression of proteins responsible for *M. ruber* development and metabolism. In summary, this study revealed that *mrflbA*-mediated signaling can directly lead to the altered expression of proteins involved in multiple biological processes, connecting the morphological and physiological differences with associated proteins.

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