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Naftifine inhibits pigmentation through down-regulation on expression of phytoene desaturase gene *CAR1* in *Rhodotorula mucilaginosa*

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Naftifine, an antifungal drug, inhibits pigmentation in *Rhodotorula mucilaginosa*. However, the relative mechanism is minutely understood. In this study, regulation of gene expression by naftifine was investigated to elucidate mechanism of yeast de-pigmentation. RNA-sequencing (RNA-seq) was used to screen differentially expressed genes (DEGs), followed by quantitative PCR (qPCR). The qPCR results showed that mRNA expression of phytoene desaturase gene *CAR1* was reduced to 37% of its original level, after one day's naftifine treatment. Since CAR1 acts at the immediate upstream of carotenoid biosynthesis pathway, it was concluded that naftifine involves in the process to inhibit the activity of phytoene desaturase, and that the down-regulation of gene *CAR1* by naftifine contributes to de-pigmentation in *R. mucilaginosa*.

Key word: Naftifine, carotenoid, *Rhodotorula mucilaginosa*, phytoene desaturase.

INTRODUCTION

Naftifine is a topical allylamine antifungal drug that is commonly used to treat dermatophytes infections (Carrillo-Munoz et al., 1999; Gupta et al., 2008; Ghannoum et al., 2013). Previously, it was known that naftifine increased the level of squalene and decreased that of ergosterol through inhibiting the activity of squalene epoxidase in fungi. Changes in the above mentioned steroid levels at opposite directions might increase permeability of fungal cells, thus triggered death of their cells (Paltauf et al., 1982). In *Staphylococcus aureus*, naftifine at low concentrations inhibited production of the virulence factor Staphyloxanthin, a carotenoid pigment, with a IC_{50} = 0.088 mg/L and had no effect to inhibit bacterial growth. This inhibitory effect was not through regulating the expression of operon crtOPQMN or by inhibiting isoprenoid biosynthetic pathway, but by inhibiting CrtN enzyme directly (Chen et al., 2016).

Rhodotorula mucilaginosa is a common species of environmental yeasts existing in soil, water and air. Although rarely infecting humans as a conditional pathogen, *R. mucilaginosa* is known to cause diseases under special situations (Mot et al., 2017; Peretz et al.,

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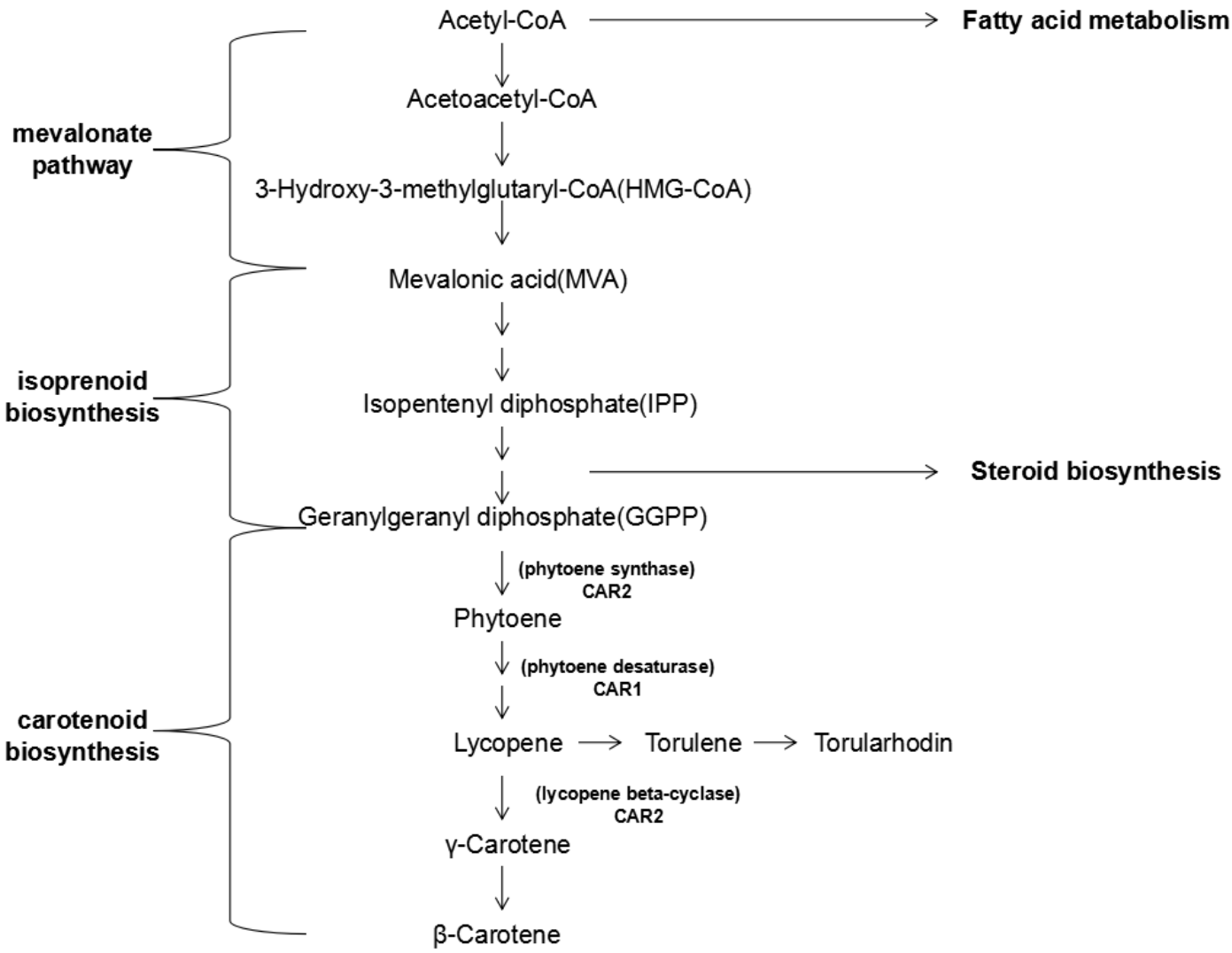

Figure 1. Diagram of the carotenoid biosynthetic pathway.

2018; Idris et al., 2019). Phospholipase was found to be a possible virulence factor in Rhodotorula genus. Rhodotorula showed more phospholipase activity than *Candida albicans* (Mayser et al., 1996). Phospholipase may increase the adhesion capacity of pathogenic microorganisms and increase the mortality of laboratory animals. Some strains of Rhodotorula have significant aspartyl peptide kinase activity (Krzyściak and Macura, 2010) which has been proposed as a virulence factor in opportunistic pathogens of Candida (Schaller et al., 2005). *R. mucilaginosa* is not sensitive to conventional antifungal drugs such as naftifine. However, its pigmentation is inhibited by naftifine at low concentrations (IC_{50} < 0.1 mg/L) as demonstrated by the decoloration of yeast. Decoloration is a reversible process (Mot et al., 2017). Carotenoids are natural apolar pigments, most of them are C40 terpenoids and some of them have oxygen-containing functional groups (Mata-Gómez et al., 2014). Carotenoids and steroids are produced in parallel pathways downstream to isoprenoid biosynthesis (Figure 1). Carotenoids are widely found in plants, fungi, and bacteria. Biosynthesis of carotenoids begins at acetyl-CoA. In *R. mucilaginosa*, acetyl-CoA sequentially converts into mevalonic acid, isopentenyl pyrophosphate, and the carotenoid precursor geranylgeranyl pyrophosphate (GGPP) (Buzzini et al., 2007; Moliné et al., 2012; Mata-Gómez et al., 2014; Kot et al., 2018; Landolfo et al., 2018). Subsequently, two molecules of GGPP are coupled by phytoene synthesase ([EC:2.5.1.32], a function of *CAR2* product) to form phytoene, a C40 carotene (Schmidhauser et al., 1994; Díaz-Sánchez et al., 2011). Phytoene thereafter produces lycopene and 3.4 dehydrolycopene by phytoene desaturase ([EC:1.3.99.30], CAR1) (Schmidhauser et al., 1990; Hausmann and

Sandmann, 2000). Finally, lycopene beta-cyclase (EC: 5.5.1.19], the other function of *CAR2* product) catalyzes production of cyclic carotenoids such as β-carotene, γ-carotene, Torulene (Figure 1) (Schmidhauser et al., 1994; Díaz-Sánchez et al., 2011).

The research aimed at identifying the targets of naftifine and understanding the mechanism of yeast decoloration through naftifine activations. Up to now, the effect of any antifungal drugs at gene expression level was rarely reported. In order to explore gene candidates that are regulated by naftifine as an antifungal drug, RNA-seq was used to screen the DEGs with focus on DNA replication and pigment synthesis pathways, as well as to quantify the mRNA levels of selected genes in qPCR assay.

MATERIALS AND METHODS

Yeast strain

The *R. mucilaginosa* strain was isolated from the nails of a healthy 41-year-old Chinese man (Idris et al., 2019).

Culture of *R. mucilaginosa*

Culture media: YPD (2% glucose, 2% peptone, 1% yeast extract); SDA agar (4% glucose, 1% peptone, 2% agar). *R. mucilaginosa* was inoculated on SDA plates and incubated at 28°C. Single colonies were picked into YPD medium and incubated 160 rpm at 28°C overnight. Log growth phase yeast was then transferred into 10 ml YPD medium in 50 ml flasks with different concentrations of naftifine. The liquid cultured *R. mucilaginosa* was in the log phase before 36 h, and entered the stationary phase after 40 h (Landolfo et al., 2018). The culture was exposed to lab lights.

Pigment extraction

1.5 ml culture, 10,000 ×g 1 min, mixed with 500 μL 2 mol/L hydrochloric acid, 60 min, boiling water 5 min, 4000 ×g 5 min. The pellet was washed, resuspended in 1 mL acetone and vortexed well for 30 min, 10,000 ×g 1 min (Michelon et al., 2012). The supernatant was used for absorbance measurement in Thermo Scientific™ Multiskan™FC.

RNA-seq

R. mucilaginosa was streaked on SDA agar and cultured at 28°C. There were three samples: "Rh_ctrl" grown for 3 days, "Rh_+naftifine" grown with 200 mg/L of naftifine for 3 days, sample "Rh_-naftifine" grown with 200 mg/L of naftifine for 3 days, followed by naftifine-free for 3 days. The samples were crushed with liquid nitrogen. Total RNA was extracted using ESscience Tissue RNA Purification Kit (ESscience, Shanghai, China) according to the manufacturer's instructions. Nano Drop ND2000® spectrophotometry was used to measure RNA purity. RNA was enriched by oligo (dT) beads, fragmented and reverse-transcribed into cDNA, purified with QiaQuick PCR extraction kit, end repaired, poly (A) added, and ligated to Illumina sequencing adapters. The products were selected by size using agarose gel electrophoresis; PCR amplified, and sequenced using Illumina HiSeqTM2500 by Gene Denovo Biotechnology Co.

Bioinformatics analysis

RNA-seq data was submitted to the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database with the accession number: PRJNA590855. Alignment tool, Bowtie2, was used for mapping (rRNA reads were removed). The reconstruction of transcripts was carried out with software Cufflinks together with TopHat2 (version 2.0.3.12). To identify new gene transcripts, all reconstructed transcripts were aligned to reference genome and were divided into twelve categories by using Cuff compare (a method of cufflinks, version 2.2.1). Genes with class code “u” (the transcripts was either unknown or in intergenic spacer region) were defined as novel genes. Novel genes were then aligned to the Nr and Kyoto Encyclopedia of Genes and Genomes (KEGG) database to obtain protein functional annotation. Gene abundances were quantified by software RSEM. The gene expression level was normalized by using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method. To identify differentially expressed genes (DEGs) across samples, the edgeR package (http://www.r-project.org/) was used. Genes with a fold change ≥ 2 and a false discovery rate (FDR) < 0.05 in a comparison as significant DEGs were identified. DEGs were then subjected to enrichment analysis of Gene Ontology (GO) functions and KEGG pathways.

Real-time qPCR

The qPCR primers were designed using the NCBI primer designing tool (Table 1). Two internal controls were selected, ltv1 (a cytoplasmic protein) and DLD2 (D-lactate dehydrogenase), since their fluctuation in expression level between different samples of RNA-seq was insignificant and their FPKM values were moderate (Van et al., 2017). The qPCR results showed that the relative expression of the internal control genes was stable between the naftifine-treated and control groups.

Yeast was cultured in liquid medium and treated with 4 mg/L naftifine for various days. Total RNA was extracted the same way as in RNA-seq. The RNA samples were reverse-transcribed into cDNA by PrimeScript™ RT reagent Kit (Takara, Dalian, China), and qPCR was performed using SYBR Premix Ex Taq GC kit (Takara, Dalian, China). The cycles were: 95°C 2 min, 95°C 20 s, 60°C 20 s, 72°C 15 s, 39 cycles. Dissolution curve conditions were: 65°C 5 s, 95°C 5 s, 4°C 30 s. Each sample was processed in triplicates using the CFX-96 TouchTM Real-Time PCR Detection System (BioRad, USA). In calculating the relative expression level of *CAR1* and *CAR2* genes, the two internal controls were used to calculate their ΔΔct values, and average with $2^{-\Delta\Delta Ct}$ method.

Data analysis software

GraphPadPrism7 was used to calculate IC_{50}. Bio-Rad CFX Manager and GraphPadPrism7 software were used for qPCR analysis and plotting.

RESULTS

Decoloration of *R. mucilaginosa* was induced by low concentration of naftifine

The drug sensitivity of the selected *R. mucilaginosa* strain was first examined. The 50% inhibiting concentration (IC_{50})

Table 1. RT-qPCR primer design.

Gene name	Sequence (5' - 3')	PCR product size (bp)	Annealing temp (℃)
DLD (internal control 1)	Forward primer : GGCTACTCCAAGACGGAACC Reverse primer : TCACTCTGGAACGGCAACTC	125	60
ltv1 (internal control 2)	Forward primer : CCGGAACACCACGATGAAGA Reverse primer : AGTCGCCGGTTGTGAGATTT	149	60
CAR1	Forward primer :CGTGACGGTCCTCGAAAAGA Reverse primer : GTCCTTGAACGCCTCCTCAA	136	60
CAR2	Forward primer : TCCGAGTTCCTCCCGATTCT Reverse primer : CTCTTCGAATGCGCCGAAAG	104	60

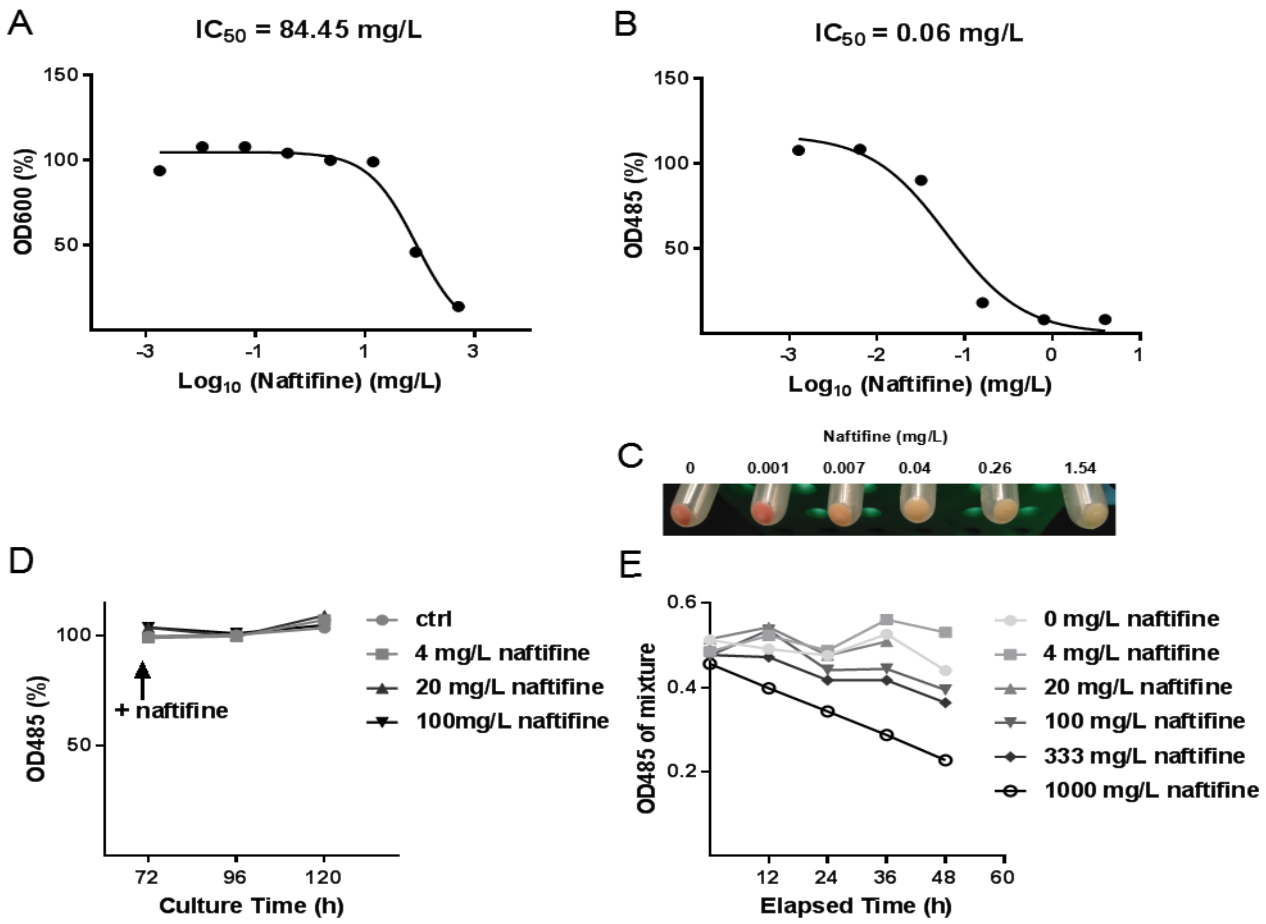

Figure 2. (A) 50% inhibitory concentration (IC_{50}) of naftifine in inhibiting yeast growth. (B) 50% inhibitory concentration of naftifine in inhibiting pigmentation. (C) Decoloration with different concentrations of naftifine. (D) Absorbance of pigment extract with naftifine added at stationary phase (day 3) for 1-2 day treatment. (E) Time-elapsed absorbance of pigment extract mixed with different concentrations of naftifine.

of allylamine antifungals for growth was: naftifine (IC_{50} = 84.45 mg/L) (Figure 2A), butenaphthol (IC_{50} = 16.75 mg/L), terbinaphthol (IC_{50} = 6.38 mg/L). The concentration of naftifine in inhibiting pigmentation was IC_{50} = 0.06 mg/L (Figure 2B and C), which was far lower than its growth inhibitory concentration (about 1, 400-fold lower). Remarkably, butenaphthol and terbinafine, the naftifine analogs, did not decolorize *R. mucilaginosa* at the same

Table 2. Differentially expressed genes between three groups.

Sample comparison	Number of genes up-regulated	Number of genes down-regulated	Total
Rh_+naftifine vs Rh_ctrl	975	279	1254
Rh_-naftifine vs Rh_ctrl	268	150	418
Rh_-naftifine vs Rh_+naftifine	417	885	1302

or even higher mass concentrations even though their growth inhibitory IC_{50} is lower than naftifine's. Based on the dramatic difference in IC_{50}, the mechanisms of growth inhibition and decoloration through naftifine treatments were likely independent. In addition, decoloration depends on the yeast growth phase. No decoloration was observed when 4 mg/mL of naftifine was added to the yeasts that already grew in liquid medium for 3 days, not even with much higher concentrations (Figure 2D).

Naftifine did not accelerate pigment degradation *in vitro*

Next, the mechanism of decoloration induced by naftifine was studied through assessing the possibility that naftifine accelerated pigment degradation. The hypothesis is that whether pigments are prone to degradation when they interact with naftifine. Pigment extract from yeast was mixed with various concentrations of naftifine and monitored for 48 h for light absorbance by a spectrophotometer. Results showed that only at extremely high concentration of 1, 000 mg/L, naftifine could reduce light absorbance at a significant level (Figure 2E). There was no significant acceleration of pigment degradation with concentration up to 100 mg/L, that is > 1, 000-fold of the decoloration IC_{50}. The results suggested that the major yeast decoloration was not due to naftifine-facilitated pigment degradation.

RNA-seq identified the candidate genes in carotenoid biosynthesis pathway

It is reasonable to hypothesize that decoloration might be due to the decrease of carotenoid synthesis. In order to screen candidate DEGs, *R. mucilaginosa* was cultured on SDA plates for different treatment scenarios for 3 days, which yielded three samples for RNA-seq analysis (Rh_ctrl, Rh_+naftifine, and Rh_-naftifine, see Materials and Methods). By aligning with the reference (JGI Rhomuc1_GeneCatalog_20160519), a total of 7, 618 known genes and 98 new genes were identified from these three samples (Table 2). Results indicated that none of the new genes was among carotenoid, isoprene, and steroid biosynthesis pathways based on annotation.

Gene Ontology function analysis showed that DEGs were significantly enriched in terms of both DNA replication and protein-DNA complex (Figure 3A). KEGG pathway analysis showed that DEGs between Rh_ctrl and Rh_+naftifine were enriched in DNA replication, material metabolism (especially fatty acid metabolism), and oxidation pathways (Figure 3B). Out of the expectation, no DEGs were found in the steroid biosynthetic pathway. However, two genes of carotenoid synthesis, *CAR1* and *CAR2*, were marginally at the higher expression levels after 3 days' naftifine treatments. Since *CAR1* and *CAR2* were found to play important roles in carotenoid synthesis in *R. mucilaginosa* (Figure 1) (Landolfo et al., 2018), their expressional regulations were further studied in qPCR assay.

Real-time qPCR showed down-regulation of *CAR1* gene expression after naftifine treatment

The above RNA-seq analysis was from solid culture and the concentration of nafitifine was higher than growth inhibitory IC_{50}. To focus on studying the effect of naftifine on decoloration and to investigate the effect at a time-dependent manner, we switched to liquid culture and used a much lower concentration (4 mg/ml vs 200 mg/ml) for real-time qPCR assay. At 4 mg/ml, nafitifine decolorized yeast, but had little effect on growth. After treatment for 1 day, the expression level of *CAR1* gene decreased to 37% when compared to control (Figure 4A, B). The t-test of three experiments showed the reduction was significant ($p = 0.007$). The expression was also reduced after treatment for 3 days, but not statistically significant. Similarly, the reduction of expression for CAR2 was also not statistically significance after treatment for 1 or 3 days. After treatment for 5 days, both genes showed no change in relative expression level. Since decoloration was not detectable anymore if naftifine was not introduced until the yeast had grown for 3 days and afterward (Figure 2D), whether the *CAR1* down-regulation was also dependent of yeast growth phase in a similar way of decoloration dynamics was further analyzed. Results of real-time qPCR assay indicated that expression levels of both *CAR1* and *CAR2* did not change after 1-day's naftifine treatment when yeast had reached stationary phase (Figure 4C). This result further supported that

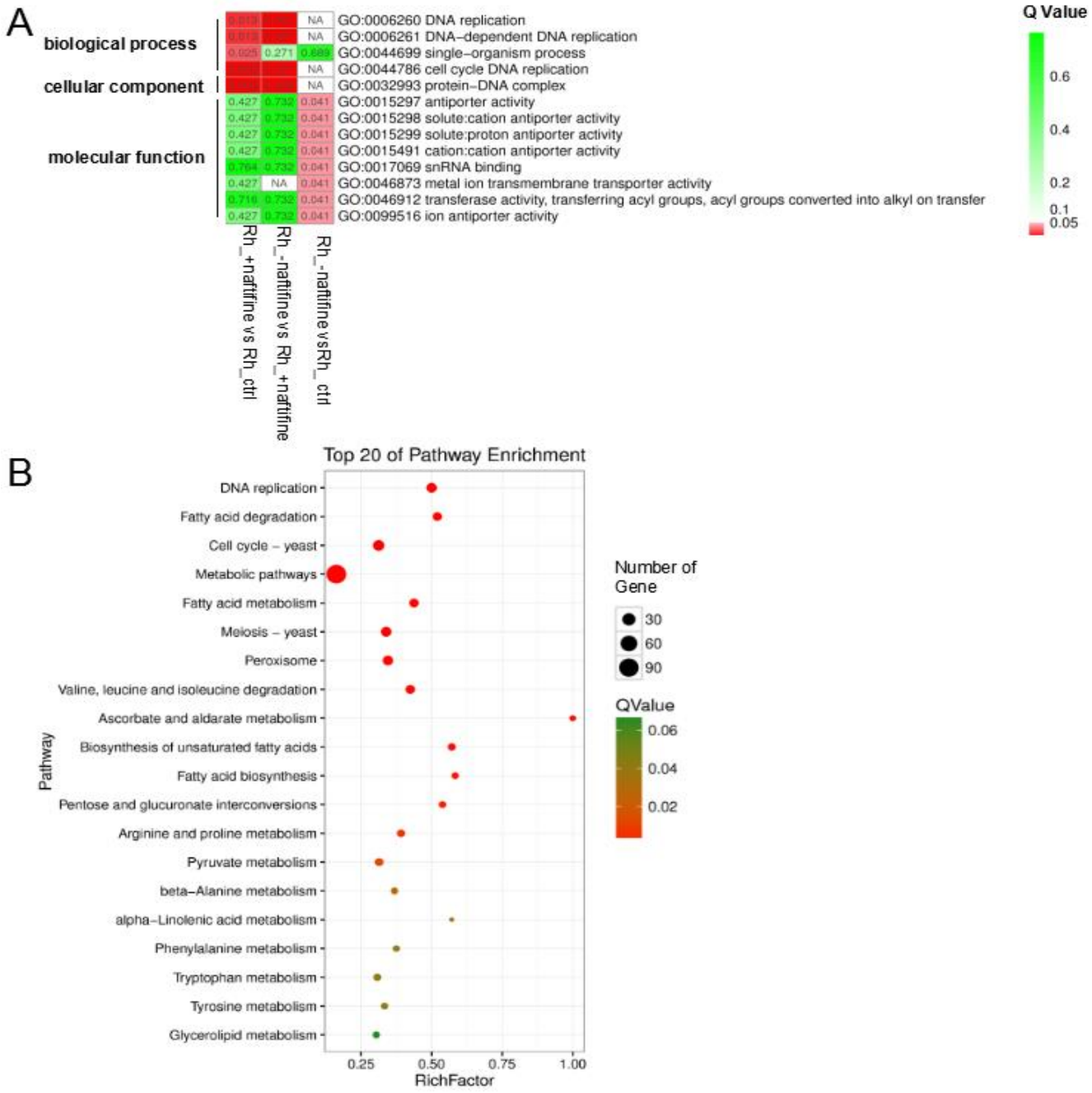

Figure 3. Enrichment analysis of differentially expressed genes: (A) Most significant Gene Ontology terms in molecular function, cellular component and biological process, evaluated by Qvalue. (B) Top 20 biological functions of KEGG between ctrl and + naftifine, evaluated by Qvalue.

decoloration correlated to *CAR1* down-regulation.

Bioinformatical analysis on inhibition of phytoene desaturase activity by naftifine

In addition to analysis of the effect on gene expressions, further evaluation was done to know whether naftifine acted on their protein levels. Since naftifine was a potent inhibitor of CrtN (diapophytoene desaturase) in *S. aureus*, homologous proteins of CrtN in *R. mucilaginosa* was searched. Interestingly, phytoene desaturase encoded by *CAR1* had the highest similarity with CrtN (Figure 5). Functionally, both of them are a membrane-peripheral and

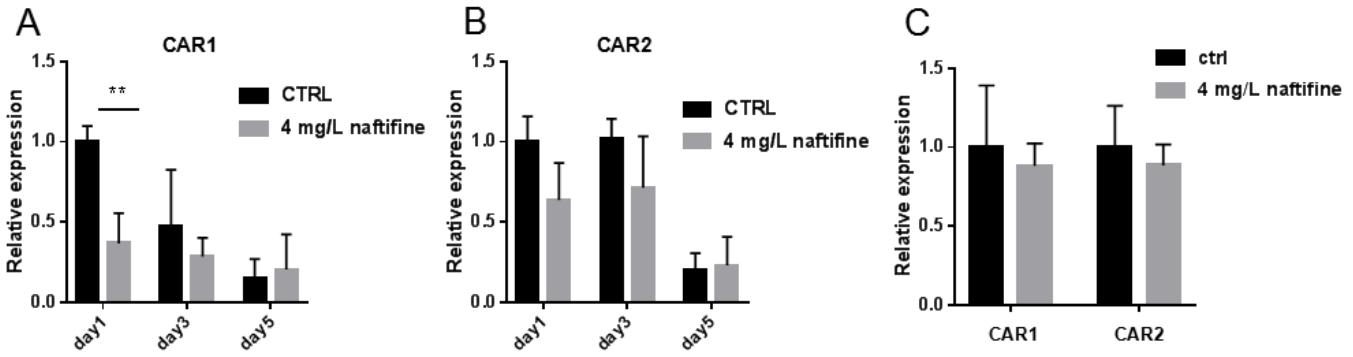

Figure 4. (A), (B) Relative expression levels of *CAR1* and *CAR2* by qPCR with naftifine treatment for 1, 3, 5 days starting at day 0. (C) Relative expression levels of *CAR1* and *CAR2* by qPCR with naftifine added at stationary phase for 1 day treatment.

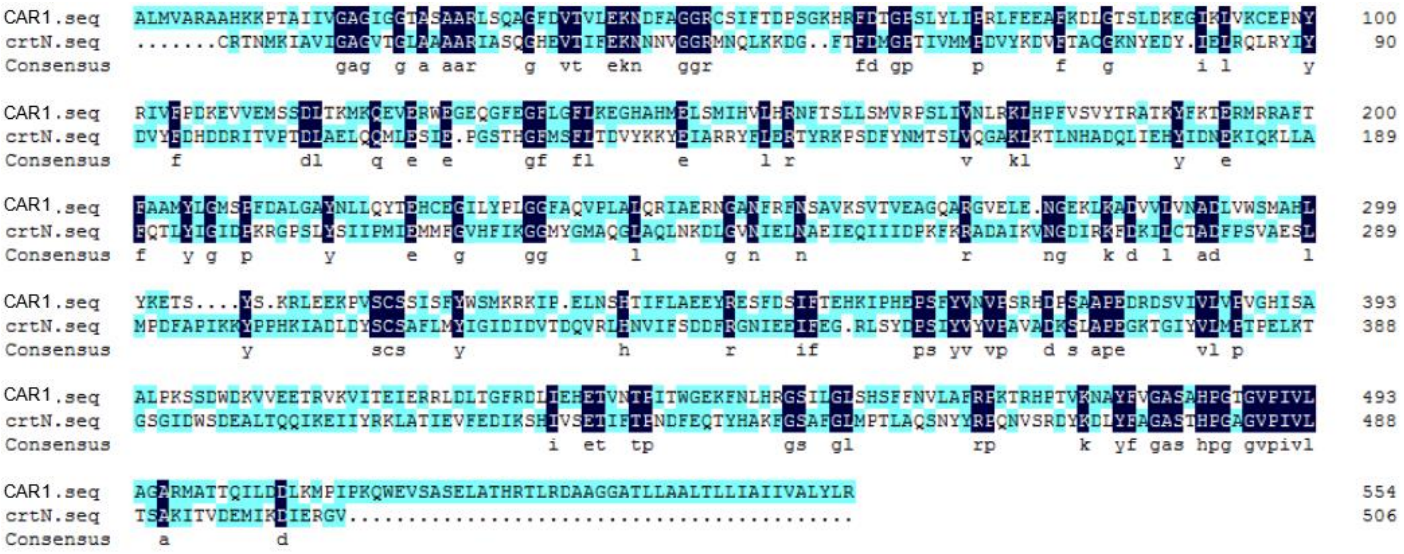

Figure 5. Sequence alignment of yeast lycopene desaturase and *S.aureus* CrtN protein.

FAD-dependent oxidase/isomerase that catalyzes the formation of multiple unsaturated double bonds of carotenoids (Schaub et al., 2012). Based on their high sequence homology, the results suggested that naftifine involved into the process of inhibiting the activity of phytoene desaturase in yeast.

DISCUSSION

Many non-phototrophic bacteria and fungi rely on carotenoids for protection from harmful radicals (Chi et al., 2015; Llansola et al., 2017). In humans, carotenoids are precursors of vitamin A, an effective antioxidant supplied from food (Bohn et al., 2017). As a non-photosynthetic fungus, *R. mucilaginosa* is a carotenoid producer and is protected by carotenoids against oxidative damage from UVB (Moliné et al., 2009, 2010). This study focused on understanding the mechanism of yeast decoloration by naftifine. Pigmentation was reduced when low concentration of naftifine was added to early phase yeast (Figure 2B). For the first time, it was shown further that the reduction was not due to faster degradation in the presence of naftifine. No significant change happened in degradation rate when pigment extract was mixed with up to 333 mg/L naftifine, > 1, 000 fold higher than IC_{50}.

For decolorization in *R. mucilaginosa*, naftifine is much more potent (IC_{50} = 0.30 umol/L) than drug diphenylamine (IC_{50} = 20 umol/L) (Raisig and Sandmann, 2001; Ghannoum et al., 2013; Mot et al., 2017). Diphenylamine reduces carotenoid accumulation by inhibiting desaturation of phytoene. Naftifine may reduce carotenoid levels partially by inhibiting phytoene desaturase in similarity to diphenylamine. Among annotated proteins of

R. mucilaginosa, the phytoene desaturase has the highest homology in sequence with bacterial CrtN, a desaturase inhibited directly by naftifine in bacteria. However, the change of carotenoid level under naftifine treatment was not measured directly in this study. The results suggested that de-pigmentation by naftifine was largely due to regulation at gene expression level of *CAR1*. It was expected that diphenylamine is unlikely down-regulating *CAR1* gene expression since it is much less potent in decoloration.

The study indicated that RNA-seq was an effective method to successfully screen DEGs. Naftifine regulated the expression of genes related to DNA replication and metabolism (Figure 3A and B). Interestingly, *CAR1* and *CAR2* had a slightly higher FPKM values, suggesting that naftifine might regulate gene expression in carotenoid synthesis pathway. To further study naftifine regulation in gene expression, real-time qPCR was used to specifically quantify relative gene expression levels of *CAR1* and *CAR2* with treatment of lower naftifine concentration in liquid culture. Naftifine down-regulated the relative expression of *CAR1* to 37% of control level after one-day treatment. Apparently, the *CAR1* down-regulation was not related to the effect of naftifine in yeast growth. We further suspect that the other antifungal drugs without inducing de-pigmentation have no regulating effect in *CAR1* expression. In conclusion, yeast decoloration by naftifine might be large through down-regulation of *CAR1* expression.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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