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The acyl-CoA-binding protein 2 exhibited the highest affinity for palmitoyl-CoA and promoted *Monascus* pigment production

Jingjing Cui[1,2†], Mengmeng Liu[3†], Weiwei Wu[3], Chuannan Long[1,3*] and Bin Zeng[1,3*]

Abstract

Purpose The present study aimed to explore the binding ability of acyl-CoA binding protein 2 to fatty acid acyl-CoA esters and its effect on *Monascus* pigment production in *M. ruber* CICC41233.

Methods The *Mracbp2* gene from *M. ruber* CICC41233 was cloned with a total DNA and cDNA as the templates through the polymerase chain reaction. The cDNA of the *Mracbp2* gene fragment was ligated to expression vector pGEX-6P-1 to construct pGEX-MrACBP2, which was expressed in *Escherichia coli* BL21 to obtain the fusion protein GST-*Mr*ACBP2 and then measure the binding ability of fatty acid acyl-CoA esters. Additionally, the DNA of the *Mracbp2* gene fragment was ligated to expression vector pNeo0380 to construct pNeo0380-MrACBP2, which was homologously over-expressed in *M. ruber* CICC41233 to evaluate *Monascus* pigment production and fatty acid.

Results The cloned *Mracbp2* gene of the DNA and cDNA sequence was 1525 bp and 1329 bp in length, respectively. The microscale thermophoresis binding assay revealed that the purified GST-*Mr*ACBP2 had the highest affinity for palmitoyl-CoA (Kd =70.57 nM). Further, the *Mracbp2* gene was homologously overexpressed in *M. ruber* CICC41233, and a positive transformant *M. ruber* ACBP-E was isolated. In the *Monascus* pigments fermentation, the expression level of the *Mracbp2* gene was increased by 1.74-fold after 2 days and 2.38-fold after 6 days. The palmitic acid content and biomass in *M. ruber* ACBP2-E were significantly lower than that in *M. ruber* CICC41233 on 2 days and 6 days. However, compared with *M. ruber* CICC41233, the yields of total pigment, ethanol-soluble pigment, and water-soluble pigment in *M. ruber* ACBP2-E increased by 63.61%, 71.61%, and 29.70%, respectively.

Conclusions The purified fusion protein GST-*Mr*ACBP2 exhibited the highest affinity for palmitoyl-CoA. The *Mracbp2* gene was overexpressed in *M. ruber* CICC41233, which resulted in a decrease in palmitic acid and an increase in *Monascus* pigments. Overall, the effect of *Mr*ACBP2 on the synthesis of fatty acid and *Monascus* pigment was explored. This paper explored the effect of *Mr*ACBP2 on the fatty acid synthesis and the synthesis of *Monascus* pigment. The results indicated the regulation of fatty acid synthesis could affect *Monascus* pigment synthesis, providing a novel strategy for improving the yield of *Monascus* pigment.

Keywords Acyl-CoA binding protein, *M. ruber* CICC41233, *Mracbp2* gene, Affinity, *Monascus* pigments

†Jingjing Cui and Mengmeng Liu equally contributed to the manuscript.

*Correspondence:
Chuannan Long
longcn2004@126.com
Bin Zeng
zengtx001@aliyun.com
[1] Jiangxi Key Laboratory of Bioprocess Engineering, Jiangxi Science & Technology Normal University, Nanchang 330013, People's Republic of China
[2] Jiangxi Key Laboratory of Organic Chemistry, Jiangxi Science and Technology Normal University, Nanchang 330013, People's Republic of China
[3] School of Life Science, Jiangxi Science & Technology Normal University, Nanchang 330013, People's Republic of China

Introduction

Monascus pigment, belonging to natural edible pigments, is a red mold rice fermented by inoculating *Monascus* spp. with rice as a raw material. *Monascus* pigments have been widely used to brew rice wine and in fermented bean curd, vinegar, food coloring, meat products, Chinese medicine, etc. (Chen et al. 2015). Hajjaj et al. firstly reported the *Monascus* pigment synthesis pathway involving fatty acids and polyketide metabolism using isotope analysis (Hajjaj et al. 1999). The *Monascus* pigment biosynthesis in *Monascus* spp. was carried out using acyl-coenzyme A as a precursor through the polyketide pathway, which was catalyzed by polyketide synthase (PKS) and fatty acid catalyzed by fatty acid synthase (FAS) (Hajjaj et al. 2000). In 2000, Hajjaj et al. reported that the effects of medium-chain fatty acids on citrinin production and found that fatty acids, such as octanoic acid could increase the yield of *Monascus* pigment by 30 to 50% (Hajjaj et al. 2000). This result revealed the association between fatty acid and *Monascus* pigment synthesis. Recently, *Monascus* pigments biosynthesis gene cluster has been identified in *Monascus purpureus* (Balakrishnan et al. 2013) and *Monascus ruber* M7 (Feng et al. 2012; Chen et al. 2015), comprising the core elements, such as PKS, FAS, dehydrogenase, transporter, regulator, etc.

Acyl-CoA binding protein (ACBP) is a highly conserved protein found in all eukaryotic and prokaryotic cells (Qiu and Zeng 2020). Two genes encoding ACBP are found in filamentous fungi like *Aspergillus oryzae* (Kwon et al. 2017). ACBP could bind with long-chain fatty acyl-CoA of different lengths with high specificity and high affinity and play multiple functions in the organism (Qiu and Zeng 2020). ACBP can mediate intracellular and extracellular Acyl-CoA transport, maintain the stability of intracellular fatty Acyl-CoA library, protect it from hydrolysis, and participate in organelle biosynthesis, biofilm assembly, and the regulation of lipid metabolism-related genes and enzyme activities.

According to a previous study, MrACBP from *Monascus ruber* CICC41233 has the highest binding affinity for myristoyl-CoA. *Monascus ruber* ACBP5, the homologously overexpressed *Mracbp* gene, showed the fatty acid C14:0 decreased while the *Monascus* pigment increased (Long et al. 2018).

In this study, a second *acbp* gene from *M. ruber* CICC41233, named *Mracbp2*, which was completely different from *Mracbp* was cloned. The keywords "acyl-coenzyme A binding protein" were used to search, and an *acbp* gene (transcript Id 440789) encoding ACBP protein (protein ID 440654) was obtained from the *Monascus ruber* NRRL1597 genome database. Therefore, it was interesting to identify the biochemical function of MrACBP2 in *M. ruber*. The gene *Mracbp2* was expressed in *Escherichia coli* to test the affinity of acyl-CoA esters and over-expressed in *M. ruber* CICC41233 to measure *Monascus* pigment yield.

The study of *Mracbp2* gene could further reveal the relationship between fatty acid synthesis and *Monascus* pigment synthesis.

Materials and methods

Strains

Monascus pigment production strain *Monascus ruber* CICC41233 was cultured on malt–peptone–starch (MPS) agar. *Escherichia coli* DH5a was used to construct the vector. *Escherichia coli* BL21 was used to express the target protein. *Agrobacterium tumefaciens* EHA105 was used to transform the binary plasmid into *M. ruber*.

Construction of gene expression vectors

The DNA and cDNA sequences of *Mracbp2* gene (MONRU_440654 of *M. ruber* NRRL1597 genome database) were cloned from *M. ruber* CICC41233 using DNA and cDNA as the templates through polymerase chain reaction (PCR), using primers *Mracbp2*-F-*Pst*Iand *Mracbp2*-R-*Kpn*I, *Mracbp2*-F-*Eco*RI, and *Mracbp2*-R-*Not*, respectively (Supplementary Table S1). And then the cloned gene sequences were sequenced by Beijing Genomics Institute (BGI) (China).

Both DNA of the *Mracbp2* gene fragment and the binary expression vector pNeo0380 were digested with *Pst*Iand *Kpn*I, respectively, and then ligated to construct the vector pNeo0380-MrACBP2.

Both cDNA of the *Mracbp2* gene fragment and the protein expression vector pGEX-6P-1 were digested with *Eco*RIand *Not*I, respectively, and then ligated to construct the vector pGEX-MrACBP2.

Phylogenetic tree analysis

The amino-acid sequence of *Mr*ACBP2 from *M. ruber* CICC41233 was analyzed by Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) to obtain the homologous sequences. These sequences were aligned using CLUSTALX, and then, a phylogenetic tree was constructed by MEGA 4 software using the Neighbor-Joining method .

Protein expression and purification

The expression vector pGEX-MrACBP2 was transformed into *E. coli* BL21. One of the positive clones were selected and activated overnight in 30-mL TB medium (containing 100 μg/mL ampicillin). Then, the 20-mL microbial was transferred to 2 L TB medium (containing 100 μg/mL ampicillin) and incubated at 37°C until OD_{600}=0.4~0.6. The isopropyl β-d-1-thiogalactopyranoside (IPTG) (final

concentration of 0.5 mM) was added as an inducer and incubated for 16 h at 16 °C.

The bacteria were collected at 8000 rpm/min for 10 min with 4°C. Later, 10 mL of GST binding buffer and 10 µL of PMSF (100 mM) were added, and the cells were resuspended by soaking, followed by centrifugation in an ice bath. Afterward, the supernatant was passed through a 0.45-µm microporous membrane filter to remove the impurities and obtain the filtrate. The protein of interest was purified using the GST-tagged protein purification kit (BBI Life Sciences), and the eluted protein of interest was stored in a −80 °C refrigerator for further use.

The protein concentration was determined using a Bradford protein assay kit (Sangon Biotech Co. Ltd., Shanghai, China). The protein was denatured by incubating in boiling water for 10 min and directly analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel stained with Coomassie brilliant blue.

Microscale thermophoresis binding analysis

The microscale thermophoresis (MST) with a NanoTemper monolith NT.115 (CA, USA) was used to measure the binding ability of the recombinant protein pGEX-MrACBP2 for fatty acid acyl-CoA esters (from C4 to C20). The recombinant protein was mixed with the gradient-diluted ligand 1:1, and the light emitting diode (LED) power of microthermophoresis was 20% and the microscale thermophoresis power was 40%. The Kd value was analyzed by NanoTemper software (Jerabek-Willemsen et al. 2011; Parker and Newstead 2014).

Screening positive *M. ruber* transformants

A. tumefaciens EHA105, containing a binary vector (pNeo0380-MrACBP2), was introduced into *M. ruber* CICC41233 through ATMT with G418 (80 µg/mL) as a screening marker (Balakrishnan et al. 2013). The transformants were picked from the MPS agar (containing G418) plate and then cultured thrice on MPS agar plates without G418. Single strains were transferred to MPS plates containing G418 to determine the stability. The total genomic DNA was extracted from the mycelia following a previously reported method by Balakrishnan et al. (2017). Positive transformants were identified by PCR amplification using the primers PgpdA-OE-F and TtrpC-OE-R.

Monascus pigment production analysis

After 7–10 days cultivation, the spores of *M. ruber* CICC41233 were obtained. The final concentration of freshly harvested spores used for inoculation was 10^5 conidia/mL. The flasks were shaken at 30°C with a rotation speed of 180 rpm/min. The fermentation experiment was carried out in a 250-mL flask, containing 50-mL fermentation medium (9.0% rice powder, 0.2% sodium nitrate, 0.1% potassium dihydrogen phosphate, 0.2% magnesium sulfate heptahydrate, and 0.2% acetate, pH 3.2). Each strain was tested in triplicate, and the samples were taken 2 days and 6 days of fermentation (Long et al. 2018).

After fermentation, 25 mL of culture broth was centrifuged at 16 °C for 30 min at 9000 × *g*. The supernatant was the extracellular pigments (water-soluble pigment). The precipitate was resuspended in 25 mL of 70% (v/v) ethanol and incubated at 60 °C for 1 h with shaking at 90 × *g* and then centrifuged at 16 °C for 15 min at 9000 × *g*. The supernatant contained the intracellular pigment (ethanol-soluble pigment). The residual mycelial precipitate was dried to a constant weight at 60 °C to determine its biomass.

The absorbance spectrum of the pigment sample was adjusted to 0.1–1.0 using a spectrophotometer at 510 nm. The result was expressed in the units of absorbance (U) at a given wavelength, multiplied by the dilution factor (Shi et al. 2015). The total MPs comprised water-soluble and ethanol-soluble pigments.

Transcription analysis

Transcription analysis was performed according to a previously reported method (Long et al. 2018). The primers of gene *GAPDH*, *pks*, *mppr1*, *fasA*, and *fasB* are listed in Supplementary Table S1.

Fatty acid content analysis

The fatty acid analysis was conducted by GuangZhou Chemical Union Quality Testing Technology Co., Ltd (China).

Results and discussion

Cloning of *Mracbp2* gene and phylogenetic tree analysis of MrACBP2 protein

The *Mracbp2* was subjected to PCR to obtain the DNA fragments and cDNA fragments (Fig. 1) and sequenced by BGI. Subsequently, the DNA and cDNA fragments of 1525 bp and 1329 bp in length were obtained along with three introns, respectively. This was different from the previously reported *Mracbp* gene from *M. ruber* CICC41233, which has the DNA and cDNA of 629 bp and 450 bp in length, respectively, with two introns (Long et al. 2018).

The deduced 442 amino-acid sequence of MrACBP2 was aligned by BLAST on NCBI to obtain the homologous sequences (Supplementary Sequence S1). These homologous sequences were used to construct a phylogenetic tree, as depicted in Fig. 2. The phylogenetic analysis revealed that the *Mr*ACBP2 from *M. ruber* CICC41233 was 100% confidence level to *M. ruber* NRRL1597.

Expression and purification of MrACBP2 protein

In the TB broth without IPTG, *Mr*ACBP2 was not fully expressed in *E. coli* BL21 (Fig. 3A, lane 1). However,

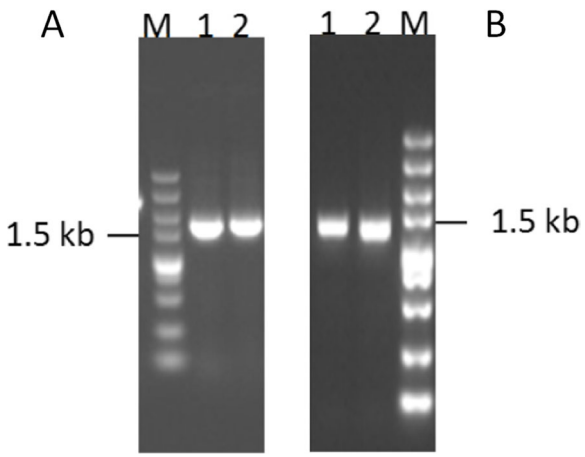

Fig. 1 PCR amplification of *Mracbp2* gene fragment from *M.ruber* CICC41233 (**A** *Mracbp2* gene fragment from DNA. **B** *Mracbp2* gene fragment from cDNA)

with the addition of IPTG as an inducer, *Mr*ACBP2 was expressed more (Fig. 3A, lane 2). The predicted molecular weight of *Mr*ACBP2 was 51 kDa, and the molecular weight of GST-tag was 26 kDa. Thus, the purified fusion protein GST-ACBP2 was approximately 77 kDa, which was consistent with the expected size by SDS-PAGE analysis (Fig. 2B, lanes 1–4).

The MrACBP2 exhibited binding preference for palmityl-CoA

The binding ability of the purified GST-ACBP2 fusion protein and acyl-CoA esters from C4 to C20 was detected by MST (Supplementary Fig. S1). As depicted in Fig. 4, the Kd value of GST-MrACBP2 for palmityl-CoA (C16-CoA) was 70.57 nM, which was the lowest value among of the all acyl-CoA esters. Thus, the MrACBP2 clearly showed a binding preference for palmityl-CoA.

The MrACBP2 from *M.ruber* CICC41233 was consistent with AoACBP of *A. oryzae* 3.042, exhibiting a binding preference for palmityl-CoA(Hao et al. 2016). However, it was different from the previously reported MrACBP from *M.ruber* CICC41233, which exhibited a binding preference for myristoyl-CoA (Long et al. 2018).

Comparison of Monascus pigment production between *M.ruber* CICC41233 and *M.ruber* ACBP2-E

The pNeo0380-*Mracbp2* vector was transformed into *M.ruber* CICC41233. A total of 11 transformants were obtained and verified by PCR to be positive

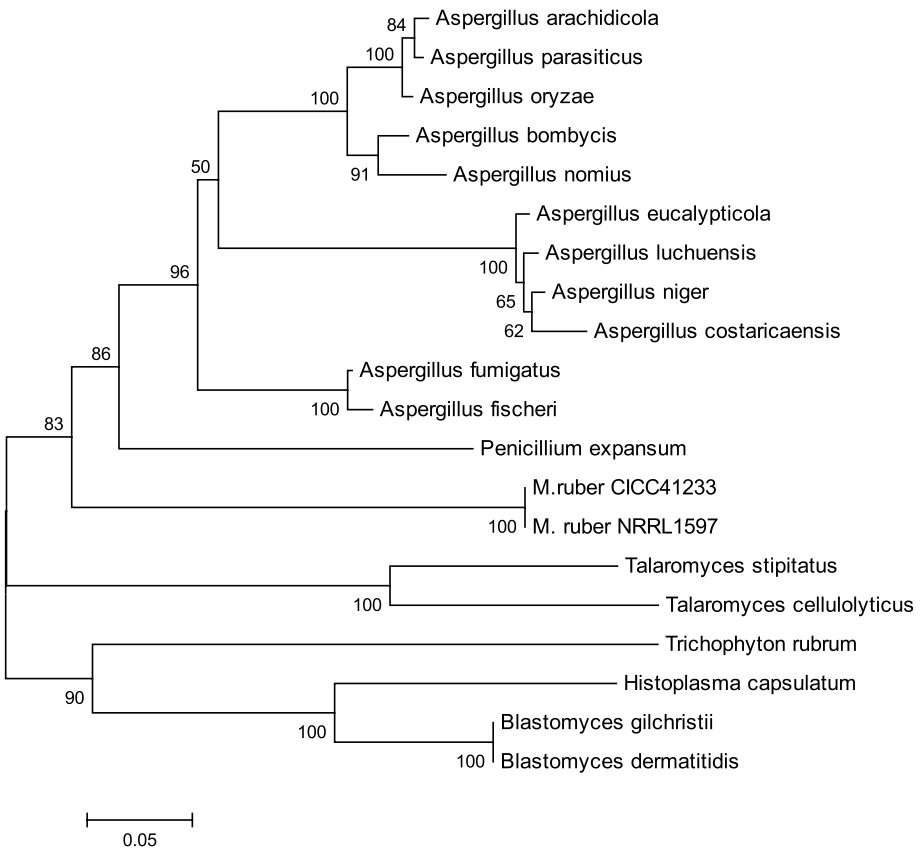

Fig. 2 Phylogeny analysis of *Mr*ACBP2 protein

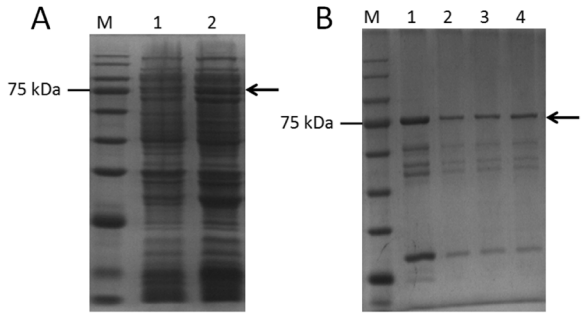

Fig. 3 SDS-PAGE analysis of GST-MrACBP2 protein. (**A** Lane 1. No IPTG inducer; Lane 2. IPTG inducer; **B** Lanes 1–4. Purified target protein)

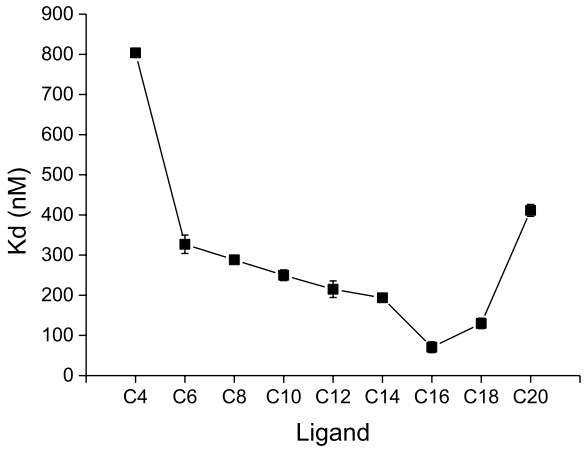

Fig. 4 The affinity of Kd value between GST-MrACBP2 and fatty acyl-CoA analyzed by MST (error bars represent SD from duplicate replicates)

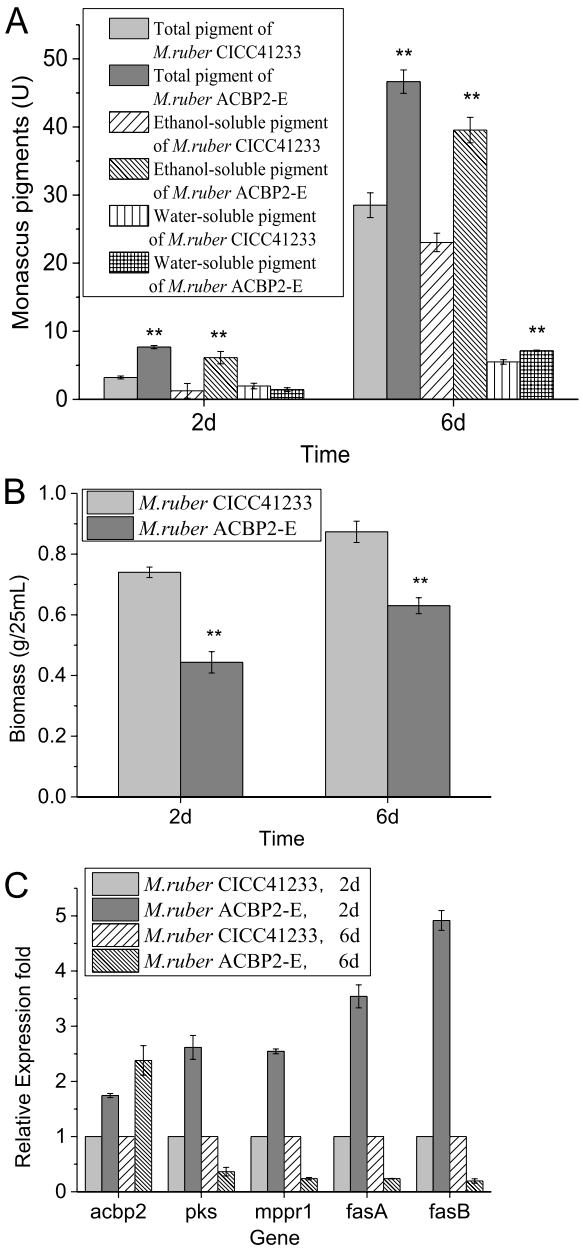

Fig. 5 *Monascus* pigment production (**A**), biomass (**B**), and relative expression fold (**C**) of *M. ruber* CICC41233, and *M. ruber* ACBP2-E

transformants. After initial screening, one of the transformants which named *M.ruber* ACBP2-E was selected for subsequent experiments.

As depicted in Fig. 5A, the pigment yield of *M.ruber* CICC41233 and *M.ruber* ACBP2-E increased with time. On the 6th day of fermentation, the yields of total pigment, ethanol-soluble pigment, and water-soluble pigment increased by 63.61%, 71.61%, and 29.70%, respectively, *M. ruber* ACBP2-E compared with *M.ruber* CICC41233.

The biomasses of the two strains after fermentation are depicted in Fig. 5B. The biomass of *M.ruber* ACBP2-E was always lower than that of *M.ruber* CICC41233 on 2 days (40.1%) and 6 days (27.9%), respectively. It was different from the *Mracbp* overexpressed strain *M.ruber* ACBP5. There was no significant increase in the biomasses on 2 days and 4 days, but there was an increase in the biomasses on 6 days (Long et al. 2018). Kwon et al. (2017) reported that the Aoacb2 encoding acyl-CoA binding protein from *A.oryzae* is an essential gene for growth and undergoes unconventional secretion. The MrACBP2 from *M.ruber* CICC41233 showed 56.0% homology with the AoAcb2 protein.

The expression levels of the genes are depicted in Fig. 5C. The expression of *Mracbp2* gene was increased by 1.74-fold after a 2-day fermentation, and the expression of the *pks*, *mppr1*, *fasA*, and *fasB* genes increased by 2.62-, 2.54-, 3.54-, and 4.92-fold, respectively. After 6 days of fermentation, the expression of *Mracbp2* gene was increased by 2.38-fold, and the expression

Table 1 Cellular fatty acid composition of *M.ruber* CICC41233

Strain Fatty acid	*M.ruber* CICC41233 48 h (%)	*M.ruber* ACBP2-E 48 h (%)	*M.ruber* CICC41233 144 h (%)	*M.ruber* ACBP2-E 144 h (%)
C16:0	18.88±0.85	16.83±0.44*	19.36±0.06	15.22±0.46**
C17:0	33.05±0.79	33.35±0.90	18.00±0.55	18.52±1.59
C18:0	4.84±0.15	4.86±0.07	11.98±0.02	11.30±0.41
C18:1	17.14±0.17	17.35±0.49	25.13±0.22	32.20±0.65**
C18:2	26.09±0.20	27.60±0.10**	25.54±0.40	22.75±0.57**

* $p < 0.05$, ** $p < 0.01$

of the *pks*, *mppr1*, *fasA*, and *fasB* genes decreased by 63.7%, 76.2%, 76.1%, and 80.4%, respectively.

Fatty acid content analysis

The contents of four fatty acids, including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), were measured and summarized in Table 1. The palmitic acid content in *M.ruber* ACBP2-E was significantly lower than that in *M.ruber* CICC41233 at 48 h and 144 h. This phenomenon was consistent with the previous study results. The fatty acid C14:0 content in *M. ruber* ACBP5 was lower than that in the *M. ruber* CICC41233 (Long et al. 2018). This indicated that MrACBP2 was responsible for the transport of intracellular acyl-CoA and the formation of an acyl-CoA ester pool (Knudsen et al. 1994; Færgeman et al. 2004; Xiao and Chye 2011; Yao et al. 2016). This was consistent with the strong binding selectivity of *Mr*ACBP2 to long-chain acyl-CoA, especially palmityl-CoA, as shown by MST analysis.

There were no significant differences in stearic acid content at 48 h and 144 h, and oleic acid content at 48h between *M.ruber* CICC41233 and *M.ruber* ACBP2-E. However, there were significant differences in linoleic acid content at 48 h and 144 h, and oleic acid content at 144h between *M.ruber* CICC41233 and *M.ruber* ACBP2-E.

Conclusions

In this study, a second *acbp* gene named as *Mracbp2* from *M. ruber* CICC41233 was cloned and showed the purified fusion protein GST-*Mr*ACBP2 exhibited the highest affinity for palmitoyl-CoA. Compared with *M.ruber* CICC41233, the palmitic acid content decreased, but the total *Monascus* pigments increased in the *Mracbp2* gene overexpressed strain of *M.ruber* ACBP2-E. The results showed a significant difference between *Mracbp* and *Mracbp2* on the affinity of fatty acid for acyl-CoA esters. However, these two genes could adjust the fatty acid synthesis and *Monascus* pigment syntheses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-023-01710-1.

Additional file 1 The following supporting information can be downloaded at: ***Figure S1.** The Kd values for ligand to bind of GST-MrACBP2 by MST. **Table S1.** Primers used in this study for polymerase chain reaction. **Sequence S1.** The sequences were used for phylogenetic analysis of ACBP2.

Authors' contributions

Jingjing Cui and Mengmeng Liu performed the experiments, analyzed the data, and wrote the manuscripts. Weiwei Wu performed the validation data. Chuannan Long modified the paper. Chuannan Long and Bin Zeng conceived, designed the experiments, and provided financial support. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study did not violate ethics, and all participants agreed to publish the paper.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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