Abstract: Monascus pigments (MPs) have been used as food colorants for several centuries in Asian countries and are currently used around the world via Asian catering. The MPs biosynthetic pathway has been well-illustrated; however, the functions of a few genes including mrpigH in the MPs gene cluster of M. ruber M7 are still unclear. In the current study, mrpigH was disrupted in ΔmrpigHΔmrpyrG, a highly efficient gene modification system, using mrpyrG as a selection marker, and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG and ΔmrpigHΔmrlig4ΔmrpyrG have been obtained. Subsequently, their morphologies, biomasses, MPs and citrinin (CIT) production were analyzed, respectively. These results have revealed that the deletion of mrpigH has significant effects on the morphology and growth of M. ruber M7. Moreover, compared with M. ruber M7, the yields of MPs and CIT were drastically increased and decreased in mrpigH mutants, respectively.

Keywords: Monascus ruber; mrpigH; Monascus pigments; citrinin

1. Introduction

Monascus species are famous medicinal and edible filamentous fungi used in traditional fermentation in Asian countries, such as China, Japan, and the Korean Peninsula for nearly 2000 years [1,2]. At present, their fermented products, such as Hongqu, also called red fermented rice, red yeast rice and red mold rice, are widely used as food additives and nutraceutical supplements worldwide owing to their production of abundant beneficial secondary metabolites (SMs), such as Monascus pigments (MPs), monacolin K (MK) and γ-amino butyric acid (GABA) [1,3]. However, citrinin (CIT), a nephrotoxic mycotoxin produced by some strains of Monascus spp., restricts the application of Monascus fermented products [4].

M. ruber M7, which can produce MPs and CIT, without MK, was subjected to whole-genome sequencing analysis [5]. And the functions of most genes in the MPs gene cluster of M. ruber M7 have been investigated by gene manipulation [6]. However, there are a few genes in the MPs gene cluster of M. ruber M7, such as mrpigH and mrpigI, which have not been investigated [6,7]. In 2017, Balakrishnan et al. predicted that the mppE in M. purpureus KACC (highly homologous to mrpigH in M. ruber M7) encoded a reductase which can decrease orange pigments (OPs) and red pigments (RPs) in the biosynthesis of MPs [8]. In 2019, Chen et al. also guessed that MrPigH might contribute to reducing the carbon double bond of the precursor compounds to the typical yellow pigments (YPs) monascin and ankaflavin [7].

In this study, we firstly cloned mrpigH from M. ruber M7. Subsequently, mrpigH was disrupted in the highly efficient system Δmrlig4ΔmrpyrG [9] using the mrpyrG selection marker, and two mrpigH deletion strains ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG and...
ΔmrpigHΔmrlig4ΔmrpyrG have been constructed. Finally, the morphologies, biomasses, MPs and CIT production of these mrpigH mutants were assessed. The results revealed that the deletion of mrpigH led to a dramatic reduction in biomass accumulation. Crucially, the inactivation of MrPigH resulted in an increase of MPs and a decrease of CIT.

2. Materials and Methods
2.1. Fungal Strains, Culture Media, and Growth Conditions

M. ruber M7 (CCAM 070120, Culture Collection of State Key Laboratory of Agricultural Microbiology, Wuhan, China), which can produce MPs and CIT, but no MK, is an original strain, which was isolated from Hongqu and preserved in our laboratory [10]. Δmrlig4ΔmrpyrG, the highly efficient gene modification system for M. ruber M7, also named as the markerless disruption strain [9] was used to generate ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG and ΔmrpigHΔmrlig4ΔmrpyrG. All strains used in this study are described in Table 1. Strains were cultivated in PDA (potato dextrose agar) or minimal medium (MM, 2.0 g NH₄Cl, 1 g (NH₄)₂SO₄, 0.5 g KCl, 0.5 g NaCl, 1.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.02 g FeSO₄·7H₂O, 20.0 g glucose, distilled water to 1 L, pH 5.5). When required, 10 mM uridine and/or 0.75 mg/mL 5-fluoroorotic acid (5-FOA) were added. For observation of colonial and microscopic morphologies, four different types of media, PDA, malt extract agar (MA), Czapek yeast extract agar (CYA) and 25% glycerol nitrate agar (G25N) were utilized [11]. PDA was used for the analysis of MPs and CIT production. All strains were maintained on PDA slants at 28 °C.

Table 1. M. ruber strains constructed and used in this study.

| Strain | Parent | Source |
|--------|--------|--------|
| M. ruber M7 | M7 [10] | Red fermented rice |
| ΔmrpigHΔmrlig4 | Δmrlig4ΔmrpyrG [9] | This study |
| ΔmrpyrG::mrpyrG | ΔmrpigHΔmrlig4 | This study |
| ΔmrpigHΔmrlig4ΔmrpyrG | ΔmrpyrG::mrpyrG | This study |

2.2. Cloning and Analysis of mrpigH

A pair of primers, pigH F1–pigH R1 (Table 2), was designed to amplify mrpigH using Oligo 6 software (http://www.oligo.net/, accessed on 24 March 2021). PCR was carried out to amplify mrpigH from the genome of M. ruber M7. Amino acid sequence encoded by mrpigH was predicted using Softberry’s FGENESH program (http://www.softberry.com/, accessed on 24 March 2021), and the MrPigH functional regions were analyzed using the Pfam 33.1 program (http://pfam.xfam.org/, accessed on 24 March 2021). Homology of the deduced amino acid sequence was analyzed using the BLASTP program on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 24 March 2021).

Table 2. Primers used in this study.

| Names | Sequences (5′→3′) | Descriptions |
|-------|------------------|-------------|
| pigHpipyrG 5F | GATATCGAATTCCCAATACT | For amplification of the 993 bp of 5′ flanking regions of the mrpigH |
| pigHpipyrG 5R | CGGTCGAGCTCAGAAGGGCA | |
| pigHpipyrG pyrGeF | TGCCCCATGCAGCTGCCACC | |
| pigHpipyrG pyrGeR | TGCCCCATGCAGCTGCCACC | |
| pigHpipyrG 5-1F | CCACAGATGGCGCATGAAAGAAGGGCA | |
| pigHpipyrG 5-1R | CGGTCGAGCTCAGAAGGGCA | For amplification of the 531 bp of 5′-1 flanking regions of the mrpigH |
| pigHpipyrG 3F | GCCATATCGGCTGACATGAGCCATCGA | |
| pigHpipyrG 3R | GCCATATCGGCTGACATGAGCCATCGA | |

For amplification of the 775 bp of 3′ flanking regions of the mrpigH.
Table 2. Cont.

| Names      | Sequences (5'→3')                      | Descriptions                                      |
|------------|----------------------------------------|---------------------------------------------------|
| pigH F     | GTGCTGGTGGCCCGACCTGAC                  | For amplification of the 583 bp of the             |
| pigH R     | CGAAGATGAAATTCGACTTGA                  | partial mrpigH                                    |
| pyrG F2    | GTGCCATACCTCAGAT                      | For amplification of the 498 bp of the             |
| pyrG R2    | CCAAAAGAACAGAATGTA                    | partial mrpigG                                    |

Labeled with dotted lines letters are nucleotide sequences of pBLUE-T vector; labeled with single underline letters are nucleotide sequences of 5'-UTR of mrpigH; labeled with double underline letters are nucleotide sequences of mrpigG gene; labeled with wavy line letters are nucleotide sequences of 5'-1UTR of mrpigH.

2.3. Construction of Deletion Cassettes and Plasmids

The genomic DNA of *M. ruber* M7 used for PCR was isolated as described previously [12]. The mutant strains Δmrlig4ΔmrpyrG::mrpigH and Δmrlig4ΔmrpyrG were constructed using site-directed homologous recombination. The *mrpigH* gene markerless deletion cassette (5'-UTR-mrpigG-5'-UTR-3'UTR) was constructed by seamless cloning, and shown schematically in Figure 1a. The relative primer pairs were shown in Table 2.

![Figure 1](image_url)

**Figure 1.** Markerless deletion of *mrpigH* in Δmrlig4ΔmrpyrG. (a) Schematic representation of the homologous recombination strategy yielding *mrpigH* markerless deletion strains. (b) Construction of *mrpigH* disruption construct by Seamless Cloning and assembly method. Lane 1, 5' flanking region of *mrpigH*; lane 2, 5' flanking region of *mrpyrG* plus *mrpyrG* ORF regions; lane 3, 3'-1 flanking region of *mrpigH*; lane 4, 3' flanking region of *mrpigH*; lane 5, deletion cassette product. (c) Confirmation of *mrpigH* homologous recombination events. Three primer pairs were used and PCR amplifications showed distinct bands in different strains. Lane 1, the Δmrlig4ΔmrpyrG::mrpigG strain; lane 2, the Δmrlig4ΔmrpyrG strain. (d) Confirmation of *mrpyrG* homologous recombination events in Δmrlig4ΔmrpyrG::mrpigG strain. Lane 1, the Δmrlig4ΔmrpyrG::mrpigG strain; lane 2, the Δmrlig4ΔmrpyrG::mrpigG strain. (e) Confirmation of *mrpigH* markerless deletion in Δmrlig4ΔmrpyrG::mrpigG strain. Lane 1, the Δmrlig4ΔmrpyrG::mrpigG strain; lane 2, the wild-type strain.
The 5′ and 3′ flanking regions (993 bp and 775 bp, respectively) and the 5′-1 flanking region (531 bp) of \textit{mrpigH} were amplified with the primers \textit{pigHpyrG 5F–pigHpyrG 5R}, \textit{pigHpyrG 3F–pigHpyrG 3R} and \textit{pigHpyrG 5F–1–pigHpyrG 5R-1}, respectively. The 1.28-kb \textit{mrpyrG} marker cassette was amplified from \textit{M. ruber} M7 genomic DNA with the primer pair \textit{pigHpyrG pyrGeF–pigHpyrG pyrGeR}. Then the four amplicons (5′ and 3′ regions, 5′-1 regions and \textit{mrpyrG} expression fragment) were mixed at a 1:1:1:1 molar ratio and cloned into vector pBLUE-T using the seamless cloning and assembly kit (Beijing Zoman Biotechnology). Subsequently, both the cloned DNA fragment and the pCAMBIA3300 plasmid were digested with \textit{HindIII} and \textit{XbaI}, and ligated by T4 DNA ligase to generate plasmid pCPGPIGH for the \textit{mrpigH} knock-out harbouring \textit{mrpyrG} selection marker.

2.4. Deletion of \textit{mrpigH} in \textit{Δmrlig4ΔmrpyrG} Strain

The plasmid pCPGPIGH was transformed into \textit{Agrobacterium tumefaciens} EHA105 using a freeze-thaw method [13]. \textit{Δmrlig4ΔmrpyrG}, a markerless disruption strain, was used as a host strain to delete \textit{mrpigH} with the \textit{mrpyrG} recyclable marker [9]. The \textit{A. tumefaciens} clones containing pCPGPIGH were incubated for transformation with \textit{Δmrlig4ΔmrpyrG} to generate a \textit{mrpigH} gene deletion mutant (\textit{ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG}) by minimal medium without uridine/uracil. The conidia of \textit{ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG} were collected and spread onto PDA with 0.75 mg/mL 5-FOA and 10 mM uridine. After incubated at 28 °C for 6 days, the surviving colonies were transferred to a new PDA under the same conditions for 4 days. The final surviving colonies were selected and verified by PCR. Selected transformants were designated as \textit{ΔmrpigHΔmrlig4ΔmrpyrG}.

2.5. MPs and CIT Analyses

\textit{M. ruber} M7 can produce MPs and CIT, but no MK. Previous researches have shown that MPs mainly accumulate in the mycelia, while CIT exists in the media [14]. Therefore, the intracellular MPs and extracellular CIT were detected. 1 mL spores suspension (10^5 cfu/mL) of each strain were inoculated on PDA plate coated with cellophane membranes and incubated at 28 °C for 11 days. 20 mg freeze-dried mycelia or media powder were suspended in 1 mL 80% (v/v) methanol solution, and subjected to 30 min ultrasonication treatment (KQ-250B, Kunshan, China). Then, the extraction solutions were separated by centrifugation at 10,000 \times g for 15 min and filtered with a 0.22 µm filter membrane for further analysis. The pigments groups concentration was measured using a UV–vis UV-1700 spectrophotometer (Shimadzu, Tokyo, Japan) at 380, 470 and 520 nm which are the maximal absorption of yellow, orange, and red pigments, respectively. The results were expressed as optical density (OD) units per gram of dried mycelia multiplied by a dilution factor [15].

The CIT was detected on Waters ACQUITY UPLC BEH C18 column (2.1 mm \times 100 mm, 1.7 µm, Waters) by fluorescence detector (Waters, Milford, MA, USA) in accordance with a previously described method [16].

2.6. Detection of the Relative Gene Expression Level in MPs and CIT Gene Clusters by RT-qPCR

To analyze the influence of \textit{mrpigH} deletion on gene expression in MPs and CIT gene cluster, \textit{ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG} and the wild-type strain (\textit{M. ruber} M7) were selected for quantitative real-time PCR (RT-qPCR) detection. The \textit{ΔmrpigHΔmrlig4ΔmrpyrG} was lacked of \textit{mrpyrG} and had to supply uridine, which might have had an effect on the yields of MPs and CIT.

One milliliter freshly harvested spores (10^5 cfu/mL) of each strain were inoculated on PDA plate and incubated at 28 °C and samples were taken every other day from the third day to the ninth day. RT-qPCR was performed according to the method described by Liu et al. [13]. Beta-actin was used as a reference gene. The primers used in these analyses were listed in Table S1.
3. Results
3.1. Sequence Analysis of mrpigH in M. ruber M7

A 1.24-kb fragment containing the putative mrpigH homolog was successfully amplified from the genomic DNA of M. ruber M7. Sequence prediction of mrpigH by Softberry’s FGENESH program has revealed that the putative mrpigH gene consists of a 1110 bp open reading frame (ORF) which consists of one exon and encodes 369-amino acids. A database search with Pfam 33.1 program has shown that MrPigH pertains to the alcohol dehydrogenase GroES-like domain. Besides, a database searched with NCBI-BLAST has been demonstrated that the deduced 369-amino acid sequences encoded by mrpigH share 65.31% similarity with the enoyl reductase (GenBank: PCH03974.1), 57.84% similarity with oxireductase of Glonium stellatum (GenBank: OCL03635.1), and 56.64% similarity with dehydrogenase of Hyphodiscus hymeniophilus (GenBank: KAG0646231.1). The specific function of mrpigH is still unclear.

3.2. Verification of the mrpigH Deletion in Δmrlig4ΔmrpyrG

The Δmrlig4ΔmrpyrG strain is a promising host for efficient gene targeting in M. ruber M7 and analysis of biosynthesis of SMs. The deletion of mrpigH was executed in Δmrlig4ΔmrpyrG with the mrpyrG marker (Figure 1a). After the plasmid pCFPG1427 harbouring mrpyrG was transformed into Δmrlig4ΔmrpyrG, transformants without uridine/uracil auxotrophic were obtained and verified by PCR, and five of 28 transformants were mrpigH-deleted strains. As shown in Figure 1c, a 0.5-kb product was amplified when the genomic DNA of Δmrlig4ΔmrpyrG::mrpyrG was used as template with primers pyrG F2-pyrG R2, while no DNA band was amplified using genome of the Δmrlig4ΔmrpyrG. A 0.58-kb fragment of the mrpigH gene could be amplified from Δmrlig4ΔmrpyrG using primers pigH F-pigH R, while no band was obtained from Δmrlig4ΔmrpyrG::mrpyrG.

Meanwhile, amplicons of Δmrlig4ΔmrpyrG::mrpyrG (3.54 kb and 1.73 kb) and Δmrlig4ΔmrpyrG (2.85 kb) differed in size when primers pigH-pyrG 5′-pigHpyrG 3R annealing to homologous arms were used.

In the ΔmrpyrGΔmrlig4ΔmrpyrG::mrpyrG strain, the mrpigH deletion cassette contains two completely homologous sequences (5′UTR and 5′-1UTR) between the mrpyrG expression fragments. If ΔmrpyrGΔmrlig4ΔmrpyrG::mrpyrG was incubated on PDA plates containing 5-FOA, the strains that lost the mrpyrG expression fragments by homologous recombination should be selected. As predicted, ΔmrpyrGΔmrlig4ΔmrpyrG strains without the mrpyrG fragment could be isolated from ΔmrpyrGΔmrlig4ΔmrpyrG::mrpyrG after growth on PDA containing 0.75 mg/mL 5-FOA and 10 mM uridine. Total 12 putative ΔmrpyrGΔmrlig4ΔmrpyrG strains with 5-FOA resistance were obtained and analyzed, and one of them was as follows. In PCR analysis as shown in Figure 1e, a 0.58-kb fragment of the mrpigH gene and a 0.5-kb product of the mrpyrG gene could be amplified from M. ruber M7 using primers pigH F-pigH R and pyrG F2-pyrG R2, while nothing was obtained from ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG, respectively. Meanwhile, amplicons of ΔmrpyrGΔmrlig4ΔmrpyrG (1.73 kb) and M. ruber M7 (2.85 kb) differed in size when primers pigH-pyrG 5′-pigHpyrG 3R annealing to homologous arms were used. Those results indicated that there was mrpigH markerless deletion was constructed in Δmrlig4ΔmrpyrG.

3.3. Morphologies and Biomasses of mrpigH Mutants and M. ruber M7

To investigate whether the mrpigH was markerless deleted by mrpyrG in Δmrlig4ΔmrpyrG, M. ruber M7 and its mrpigH markerless mutants were cultivated on PDA supplemented the appropriate additive (10 mM uridine for the uridine/uracil auxotrophy). The results (Figure 2a) revealed that ΔmrpigHΔmrlig4ΔmrpyrG showed no growth on PDA, but it was able to grow on PDA with 0.75 mg/mL 5-FOA and 10 mM uridine, while the growths of M. ruber M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG were inhibited by the addition of 0.75 mg/mL 5-FOA to PDA, but could grow on PDA. Those results indicated that the mrpigH markerless mutants were successfully constructed.
Figure 2. Morphologies and biomasses of mrpigH mutants and M. ruber M7. (a) Growth of M. ruber M7 and mrpigH mutants on PDA, PDA with 10 mM uridine, PDA with 10 mM uridine and 0.75 mg/mL 5-FOA for 5 days at 28 °C. A, M. ruber M7; B, ΔmrpigH, mrpigH was disrupted in M. ruber M7 with the hph selection marker [9]; C, ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG; D, ΔmrpigHΔmrlig4ΔmrpyrG. (b) Colonial morphologies of M. ruber M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG on PDA, CYA, MA and G25N plates for 10 days at 28 °C; (c) Cleistothecia and conidia formation of M. ruber M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG on different plates (PDA, CYA, G25N, and MA) for 7 days at 28 °C; (d) Biomass (dry cell weight) of M. ruber M7 and mrpigH mutants on PDA plates at 28 °C. The error bar represents the standard deviation between the three repeats.
To test the influence of deleting mrpigH on developmental processes, *M. ruber* M7 and ΔmrpigHmrlig4ΔmrpyrG::mrpyrG were cultivated on different media (PDA, CYA, MA and G25N) to observe their colonial and microscopic characteristics. The results showed that the colonial morphologies of mrpigH mutants (Figure 2b) were obviously different from those of *M. ruber* M7 on different culture plates, especially on PDA plate, the ΔmrpigHmrlig4ΔmrpyrG::mrpyrG showed slower growth rate and darker color. However, the microscopic morphologies, including conidia and cleistothecia, of ΔmrpigHmrlig4 ΔmrpyrG::mrpyrG was not dramatically different from those of *M. ruber* M7 on different culture plates (Figure 2c). Moreover, the biomasses of two mrpigH mutants apparently decreased compared with that of M7 on PDA in 5–11 d (Figure 2d).

3.4. Analysis of MPs and CIT Production

In order to evaluate the effect of detecting mrpigH on MPs and CITs during fermentation, the samples cultured for 3, 5, 7, 9 and 11 days on PDA were obtained and each test was repeated independently three times. OD values representing yellow, orange and red pigment production were determined using a spectrophotometer at 380 nm, 470 nm and 520 nm, respectively. As shown in Figure 3a–c, from the seventh day to the 11th day, *M. ruber* M7 produced much fewer MPs (including YPs, OPs and RPs) than 2 mrpigH mutants (ΔmrpigHmrlig4ΔmrpyrG::mrpyrG and ΔmrpigHmrlig4ΔmrpyrG), whereas the ability of producing MPs among 2 mrpigH mutants showed no obvious difference. After 11 days of cultivation, the YPs, OPs and RPs production in ΔmrpigHmrlig4ΔmrpyrG::mrpyrG and ΔmrpigHmrlig4ΔmrpyrG were 2.04–2.22, 8.76–10.06, 4.29–4.69 times those of *M. ruber* M7, respectively.

As to CIT, the UPLC has been performed to detect the production during fermentation. As shown in Figure 3d, CIT produced by *M. ruber* M7 and all mrpigH mutants showed an obvious difference. At the end of the 11 days of fermentation, CIT production in ΔmrpigHmrlig4ΔmrpyrG::mrpyrG and ΔmrpigHmrlig4ΔmrpyrG decreased dramatically, and was two to three orders of magnitude less than that of *M. ruber* M7. Among 2 mrpigH mutants, CIT production in ΔmrpigHmrlig4ΔmrpyrG was higher than that of ΔmrpigHmrlig4ΔmrpyrG::mrpyrG. The possible cause was that the ΔmrpigHmrlig4ΔmrpyrG lacked mrpyrG and had to supply uridine, which might have an effect on the yields of CIT.

![Figure 3](image-url)

**Figure 3.** Production of intracellular MPs and extracellular CIT by *M. ruber* M7 and mrpigH mutants on the PDA supplied with/without uridine. (a) The yield of yellow pigments; (b) The yield of orange pigments; (c) The yield of red pigments; (d) The yield of CIT. The error bar represents the standard deviation between the three repeats.
3.5. The Genes’ Expression in MPs and CIT Gene Clusters from mrpigH Mutants

The gene expression in MPs and CIT gene clusters in ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG and *M. ruber* M7, were analyzed by RT-qPCR. As shown in Figure 4, the relative expression levels of *mrpigA*, *mrpigB*, *mrpigC*, *mrpigD*, *mrpigE*, *mrpigF*, *mrpigG*, *mrpigJ*, *mrpigK*, *mrpigM*, *mrpigN*, *mrpigO* and *mrpigP* in ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG were obviously higher than that of *M. ruber* M7 on the fifth to ninth days. Therefore, the deletion of *mrpigH* increased the majority of MPs gene expression level, which might correspond with the enhanced MPs production.

In a previous study, Li et al. [17] considered the CIT biosynthetic gene cluster in *M. aurantiacus* including 16 genes. He and Cox [18] demonstrated that a minimal set of conserved genes were involving in CIT biosynthesis, including: oxydoreductase (*mrl7*), dehydrogenase (*mrl6*), glyoxylase-like domain (*mrl5*), NAD(P)+ dependent aldehyde dehydrogenase (*mrl4*), transcriptional regulator (*mrl3*), Fe(II)-dependent oxygenase (*mrl2*), serine hydrolase (*mrl1*), non-reducing polyketide synthase (*mrpks*),and major facilitator superfamily (MFS) protein (*mrr1*). As shown in Figure 5, the relative expression levels of *mrl6*, *mrl5*, *mrl4*, *mrl2*, *mrl1*, *mrpks* and *mrr1* were dramatically lower than those of *M. ruber* M7 at 3rd and 5th day. Therefore, the deletion of *mrpigH* decreased the expression level of core CIT genes, which might correspond with the reduced CIT production.
Figure 4. RT-qPCR analysis of the mrpigA-mrpigP in M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG. mrpigA: Non-reducing polyketide synthase; mrpigB: Transcription factor; mrpigC: C-11-Ketoreductase; mrpigD: 4-O-Acyltransferase; mrpigE: NAD(P)H-dependent oxidoreductase; mrpigF: FAD-dependent oxidoreductase; mrpigG: Serine hydrolase; mrpigH: Enoyl reductase; mrpigI: Transcription factor; mrpigJ: FAS subunit alpha; mrpigK: FAS subunit beta; mrpigL: Ankyrin repeat protein; mrpigM: O-Acetyltransferase; mrpigN: FAD-dependent monooxygenase; mrpigO: Deacetylase; mrpigP: MFS multidrug transporter; M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG were incubated in PDA on the 3rd, 5th, 7th, 9th day at 28 °C. The beta-actin gene was used as control. The error bars indicate the standard deviations of three independent cultures.
Figure 5. RT-qPCR analysis of the mrl7-mrr8 in M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG. mrl7: Oxydoreductase; mrl6: Dehydrogenase; mrl5: Glyoxylase-like domain; mrl4: NAD(P)+ dependent aldehyde dehydrogenase; mrl3: Transcriptional regulator; mrl2: Fe(II)-dependent oxygenase; mrl1: Serine hydrolase; mrpks: Non-reducing PKS; mrr1: Major facilitator superfamily (MFS) protein; mrr2: Histidine phosphatase; mrr3: Unknown protein; mrr4: WD40 protein; mrr5: Carbonic anhydrase; mrr6: Unknown protein; mrr7: Enoyl-(acyl carrier protein) reductase; mrr8: AMP-binding enzyme; M7 and ΔmrpigHΔmrlig4ΔmrpyrG::mrpyrG were incubated in PDA on the 3rd, 5th, 7th, 9th day at 28 °C. The beta-actin gene was used as control. The error bars indicate the standard deviations of three independent cultures.
4. Discussion

Monascus spp. have been widely used in food fermentation for nearly 20 centuries in East and Southeast Asian countries due to their ability of producing vivid MPs [11,19], which are a complex mixture of secondary metabolites (SMs) with a tricyclic azaphilone scaffold, produced via the polyketide pathway by a few filamentous fungi such as Monascus spp. and Penicillium spp. [3,20,21]. MPs are biosynthesized by their gene cluster, which contains 16 genes (mrpigA-mrpigP) in M. ruber M7 [6,7].

In the current study, in order to explore the function of mrpigH, we used the ∆mrlig4 ∆mrpyrG as the starting strain to generate two kinds of mrpigH disruptants, namely ∆mrpigH∆mrlig4∆mrpyrG::mrpyrG and ∆mrpigH∆mrlig4∆mrpyrG. The colonial and microbiological phenotypes and biomass of the mrpigH mutants showed an obviously difference from those of M. ruber M7 (Figure 2b, d). In particular, the biomass of mrpigH mutants accumulated more slowly than that of M. ruber M7. Our laboratory previously constructed MPs gene knockout strains with the resistance selection markers (hph/neo) in M7, and found that the morphologies and biomass of mrpigC, mrpigE, mrpigF, mrpigM and mrpigO knockout were comparable to those of M. ruber M7, whereas the growth rates of mrpigA, mrpigI, and mrpigK knockout were increased, and the deletion of mrpigN resulted in a reduction in biomass accumulation compared with M. ruber M7 [22].

Moreover, we have discovered that the deletion of mrpigH in M. ruber M7 can enhance YPs, OPs and RPs (Figure 3a–c), which is mostly consistent with the results obtained in M. purpureus [8]. We also found that deletion of mrpigH dramatically decreased the CIT production (Figure 3d), which is a promising scheme for control of CIT. CIT is a kind of mycotoxin produced via the polyketide pathway by filamentous fungi, mainly by Monascus spp. and Penicillium spp. [23–25]. To this end, we investigated the changes in the expression levels of MPs and CIT genes in their gene clusters of mrpigH mutants, and found that the relative gene expression levels almost corresponded with the increase of MPs and decrease of CIT (Figures 4 and 5).

The deep study about the reason for increasing the pigment content while reducing the CIT production by knocking out mrpigH need to be investigated further. In early research, both MPs and CIT were considered to be derived from polyketide pathways [26]. Lately, two hypotheses about the biosynthetic pathways of MPs and CIT were put forward. One of them is based on metabolic pathways; the MPs and CIT shared a common pathway to a certain branch [26]. The other is based on the analysis of the whole genome sequence; their biosynthetic gene clusters had been found separately, thereby, they belonged to two different pathways [6,18].

Previous results suggested that the yields of CIT and MPs in Monascus spp. could affect each other. For example, Xie et al. (2013) and Liu et al. (2014) separately identified that the overexpression of mrpigB and mrpigE in MPs gene cluster of M. ruber M7 resulted in a reduction of CIT production [13,27]. Meanwhile, Liang et al. (2017) obtained a mutant of a putative glyoxalase (orf6) in CIT gene cluster of M. purpureus, and found that the deletion of orf6 could improve the MPs and CIT yields at the same time [28]. A recent study on the biosynthesis of MPs found that acetyl-CoA and malonyl-CoA are catalyzed by a sequence of enzymes to produce MPs precursors. Then the pathway bifurcates into two branches. First, the MPs precursors were reduced by a reductase (MrPigH and/or GME3457, which is encoded outside of the MPs gene cluster in M. ruber). The other branch of the pathway produces the typical OPs rubropunctatin and monascorubrin by a FAD-dependent oxidoreductase (MrPigF in M. ruber M7). Furthermore, rubropunctatin and monascorubrin were converted into RP’s rubropunctamine and monascorubramine through an amination reaction [6,7]. Due to the deletion of mrpigH, the relative expression of mrpigF dramatically increased (Figure 4), which was beneficial for the OPs and RPs production, and the increase in relative yields of OPs and RPs was greater than that of YPs. Recently, Li et al. (2020) reported that MP’s biosynthetic gene cluster was a composite supercluster, and the naphthoquinone (monaseone) gene cluster was embedded in the MPs gene cluster, and speculated that
MrPigH was essential in the biosynthesis of naphthoquinone, but it was a supplemental enzyme in the biosynthesis of MPs [29]. As a result, the mrpigH knockout’s blocking of the naphthoquinone biosynthesis pathway might result in naphthoquinone reduction, and more substrates and intermediates were utilized to synthesize MPs, including YPs. In terms of CIT production, He Yi et al. [18] revealed a minimal set of conserved genes involved in CIT biosynthesis, which included nine genes (mrl7, mrl6, mrl5, mrl4, mrl3, mrl2, mrl1, mrpks and mrr1). When mrpigH was deleted, the majority of the genes in the cluster (mrl6, mrl5, mrl4, mrl2, mrl1, mrpks and mrr1) were obviously down-regulated (Figure 5), which could explain why CIT production has decreased in this study.

In conclusion, the mrpigH gene pertains to the MPs biosynthetic gene cluster of M. ruber M7 and plays a remarkable role in the biosynthesis of MPs and CIT. The disruption of mrpigH had very little effect on the microscopic morphologies, while the mrpigH mutants showed slower biomass accumulation and darker color on PDA. Compared with M. ruber M7, the YPs, OPs and RPs production in the mrpigH mutants (ΔmrpigHΔmrlig4ΔmrpyrG:: mrpyrG and ΔmrpigHΔmrlig4ΔmrpyrG) increased dramatically. However, the CIT production of the mrpigH mutants decreased drastically. This work will make some contribution to the regulation of MPs and CIT production in M. ruber M7.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7121094/s1, Table S1: Primers used in RT-qPCR.

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