Regulation of MareA gene on monascus growth and metabolism under different nitrogen sources

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Abstract

Monascus is a widely used natural microorganism in our country, which can produce useful secondary metabolites. Studies have shown that medium composition plays an important role in the primary and secondary metabolism of Monascus, especially the nitrogen source directly affects the growth, reproduction and secondary metabolites of Monascus. As a global transcriptional regulator of nitrogen metabolism, *AreA* gene is involved in the regulation of secondary metabolism. In this study, a MareA gene with high sequence homology to *AreA* gene was found by alignment analysis of the C100 genome. C100 knockout and overexpression mutants were obtained by Agrobacterium tumefaciens transformation. In this study, four nitrogen sources (Glutamine, Urea, $NaNO_3$, $(NH_4)_2SO_4$) were used to grow C100 and mutant strains. The yield of monascus rice under different nitrogen sources was compared by solid-state fermentation, and the types and yields of Mps produced were analyzed. HPLC and RT-qPCR were used to analyze the secondary metabolites of the three strains. Notably, growth differences between these monochorus strains were most pronounced when $NaNO_3$ medium was added. The fermentation product yield and gene expression level of the knockout strain were significantly lower than those of the C100 strain, while the opposite was observed for the overexpression strain. In conclusion, *MareA* gene had different regulatory effects on monochorus growth and metabolism under different nitrogen sources.

Introduction

The filamentous fungus *Monascus* spp. was initially identified by the French scientist Van Tieghem in 1884 (Van Tieghem, 1884). The growth of *Monascus* spp. occurs within a temperature range of 26°C to 42°C, with the most favorable temperatures falling between 30°C and 35°C. Optimal pH for growth ranges from 3.5 to 5.0, showing a specific preference for lactophilic conditions(Park et al., 2017). Therefore,*Monascus* spp. has been extensively utilized in China since ancient times, with red yeast rice being the most prevalent form of application. Red yeast rice is typically derived from solid-state fermentation of the *Monascus* spp. on a rice substrate, resulting in a delightful flavor and distinctive color during the fermentation process (Zhang et al., 2023). Meanwhile, *Monascu* s is known to produce a range of beneficial secondary metabolites, such as*Mps* s, M*onacolin* K, and *γ-αμινοβυτψρις* acid, during fermentation. However, it should be noted that certain strains of*Monascus* may also generate the *mycotoxin citrinin* under specific fermentation conditions.

The nitrogen source is an indispensable element in the growth process of fungi, serving as a crucial constituent of nucleotides, amino acids, and other vital components. It exerts a profound influence on fungal growth and development, spores production capacity, as well as the synthesis of secondary metabolites(Tudzynski, 2014). Fungi possess the capacity to utilize a diverse array of nitrogen sources, albeit with varying degrees of proficiency. In environments where ammonium nitrogen and glutamine are available, fungi exhibit a preferential utilization for these nitrogen sources. Conversely, in the absence of such nitrogen sources, uptake of nitrate nitrogen, urea, uric acid, and purine-pyrimidine as alternative nitrogen sources is observed(Marzluf, 1997). In certain fungal species, the processes of response, uptake, and assimilation of nitrogen sources

necessitate the involvement of multiple regulators in nitrogen metabolism. This enables the activation or inhibition of specific structural genes involved in nitrogen metabolism based on the form of available nitrogen sources to meet the organism's demand for this essential element.(Stajić et al., 2006).

The expression of genes involved in nitrogen metabolism is subject to global regulatory mechanisms, while simultaneously being specifically regulated by individual substrates.(Qian et al., 2019). The GATA family of transcription factors serves as the primary regulators of nitrogen metabolism in fungi. These GATA transcription factors were initially discovered within the promoter region of the chicken bead protein.(Evans et al., 1988). The DNA-binding proteins, characterized by a conserved zinc-finger amino acid sequence of Cys-X2 -Cys-X17 -Cys-X2 -Cys, exhibit a strong affinity towards the (T/A) GATA (A/G) motif.(Lentjes et al., 2016). The GATA transcription factors in fungi have been extensively investigated and found to play crucial roles in fungal nitrogen metabolism, sexual and asexual development, as well as iron carrier biosynthesis(Liu et al., 2018). The yeast saccharomyces cerevisiae has identified four GATA transcription factors involved in nitrogen metabolism, namely Gatl1p and Gatl3p, which exert positive regulation on nitrogen metabolism, and Gzf3p and Dal80p, which exert negative regulation on nitrogen metabolism. (Magasanik and Kaiser, 2002) . The filamentous fungi possess only two GATA-type nitrogen regulators, namely*AreA* and *AreB* .

The two GATA global transcriptional regulators of nitrogen metabolism in filamentous fungi, *AreA* and *AreB* , play distinct roles:*AreA* activates genes associated with the utilization of secondary nitrogen sources, while *AreB* inhibits the activation of*AreA* .(Tudzynski, 2014). The *AreA* gene was previously investigated in *Aspergillus nidulans* (Hynes, 1975), thereby confirming its involvement in the regulation of nitrogen metabolism in filamentous fungi and its impact on secondary metabolites as well as pathogenicity.

The red *Monsacus* pigments exhibit a chemical similarity to the orange ones, differing only in the substitution of an eNHe group for the eOe group. This substitution is believed to occur through the amination reaction between NH_3 units and orange pigments(Haws et al., 1959; Kumasaki et al., 1962).The water-soluble red pigment derivatives are primarily located extracellularly and are synthesized through the reaction between the orange pigment and amino acids(Jung et al., 2003; Lin and Demain, 1994). Therefore, the nitrogen source is considered a crucial regulator, and its impact on red pigment production has been extensively investigated by numerous researchers. It has been demonstrated that specific nitrogen sources significantly influence the composition of *MPs* . Ammonium chloride and ammonium sulfate facilitate the formation of orange or yellow pigments(Kang et al., 2014; Liang, 2009).The formation of red pigments is facilitated by nitrates and organic nitrogen sources, such as monosodium glutamate (MSG), amino acids, or yeast extracts (Jung et al., 2003; Lee et al., 2001).

In this study, the original C100 strain was used to screen out the knockout and overexpression strains by homologous recombination and co-culture with Agrobacterium tumefaciens. Subsequently, the three strains were cultured by solid state fermentation, and the products were analyzed. In this study, the expression of key genes related to growth and development and pigment synthesis was analyzed to elucidate the function of the MareA gene.

Materials and Methods

2.1 Strains, media and culture conditions

Monascus ruber C100: a gift from Prof. Mianhua Chen, the original strain for gene knockout and overexpression; *Escherichia coli* DH5α: kept in our lab for plasmid transformation;*Agrobacterium tumefaciens* EHA105: Preserved in this laboratory for use in transformation; p59 plasmid: kept in this lab for the construction of knockout plasmid vectors; p1301 plasmid: kept in our lab for the construction of overexpression plasmid vectors.

Inoculate the C100 with Z-shaped wavy lines onto the inactivated MEA slant, and incubate it at a constant temperature of 30 for about 7 days. Store it in the refrigerator at 4 and reactivate every six months.

2.2. Extraction of C100 genome

Genomic DNA was extracted as described by Yu et al(Yu et al., 2004)with some modifications. Weigh the mycelium into a centrifuge tube, add 1mL of a 5% CTAB solution and 20μL of β-mercaptoethanol, then use a crusher to crush it for 1 minute. After crushing, place it in a 65 incubator for 30 minutes to maintain warmth. Mix well by adding an equal volume of chloroform, followed by centrifugation at 10,000rpm/min at 4 for 5 minutes. Take the supernatant and add isopropanol (0.6 times the volume) and NaAC (0.1 times the volume). Leave it at -20 for 2 hours, then wash with fresh aliquots of ethanol (75%) for a duration of 5 minutes to remove the supernatant. Remove ethanol after resting for another minute using fresh aliquots of ethanol (75%). Centrifuge again at -20 with NaAC present during stationary phase lasting two hours at a speed of 10000 rpm/min followed by removal of supernatant. Finally, add TE buffer (50μL) to obtain the genome extract from red currant bacteria.

Extraction of total RNA from monascus was performed using the Fungal RNA Kit (OMEGA) procedure. The obtained total RNA from Monascus was then converted into cDNA using the HiFiScript cDNA Synthesis Kit.The reaction system and procedures are shown in Tables 2-1 and 2-2.

2.3 Construction of knockout and overexpressed recombinant plasmids

Specific primers were designed using C5.761 of the M. purpurus YY-1 genome as the reference sequence. Primers were designed to amplify the upstream and downstream homology arms of the target gene using the original strain DNA as a template. Using p59 as the cloning vector, the recombinant plasmid p59s was obtained after first double digestion of the vector using PstI and HindIII and ligation according to the instructions of the seamless cloning kit. Similarly, p59s was double digested using KpnI and XbaI followed by ligation, resulting in the final recombinant plasmid p59ss. Primers for the target gene were designed using C100 cDNA as a template and amplified. The target fragment and the vector were digested with NocI and AflII, and then ligated with the DNATAKARA kit to obtain the overexpression vector p1301s.

2.4 *Agrobacterium-mediated* transformation

Agrobacterium-mediated transformation was referenced to W. Li et al.(Li et al., 2023) with fine-tuned corrections. To compare the gene expression of the MareA gene in the original strain and the knockout and overexpression strains, total RNA was extracted from both strains and converted into cDNA. RT-qPCR was performed using the SYBR® Premix Ex TaqTM II kit from TAKARA for relative quantification. The C100 setup served as the template in the assay as the calibrator, withβ-actin used as the internal reference gene. Three replicates were conducted for each gene. At the end of the reaction, amplification efficiencies of genes were observed, and when their efficiencies were essentially equal, comparisons and analyses could be made by comparing and analyzing Ct values at which gene amplification reached threshold levels. The relative expression of genes could be analyzed using the $2^{-\Delta\Delta C_t}$ method. The RT-qPCR reaction system is shown in Tables 2-3, and the RT-qPCR reaction procedure is shown in Tables 2-4.

-ΔΔCt=-[(experimental group gene Ct value - experimental group internal reference Ct value) - (control group gene Ct value - control group internal reference Ct value)]

Equation (2-1)

2.5 Observation and Determination of Colonies

Spore suspensions of the original strain *C100* , knockout strain*ΔMapϵA* , and overexpression strain *OE-MareA* were inoculated onto PDA, PDA-Gln, PDA-$(NH_4)_2SO_4$, PDA-$NaNO_3$, and PDA-Urea media centers with 5μL each. The bacterial growth was observed after constant incubation at 30 for 7 days.(Liu et al., 2019) .

C100 ,*ΔMapϵA* and *OE-MareA* were cultured on media supplemented with different nitrogen sources and their diameters were measured using the criss-cross method.

Cultured *C100,* Δ*MareA* , and OE-*MareA* were incubated at 30 for 7 days. The mycelium and spores on the surface of the medium were washed off with aseptic water. Then, a 20μL drop was collected on a slide and

observed under a fluorescence inverted microscope (20×) to assess the growth of the mycelium and spores as well as the intensity of fluorescence.

˜100, ΔMaρϵA , and *OE-MareA* were cultured on PDA medium supplemented with different nitrogen sources, respectively. After 7 days of incubation at 30, mycelia and spores were washed off the surface of the medium with aseptic water, centrifuged at 8,000 rpm for 10 minutes. After removing the supernatant, they were soaked in 2.5% glutaraldehyde and fixed overnight in a refrigerator at 4. The fixative was then removed and the mycelium and spores were rinsed three times with PBS buffer at pH=7.0 for each time interval of 10 minutes. Then the samples were dehydrated step by step using ethanol concentrations of 30%,50%,70%,80%,90% and finally 100%. Each concentration was treated for a duration of ten minutes. The processed samples underwent vacuum freeze-drying process. The samples were glued onto carbon conductive adhesive, sprayed with gold, and observed under scanning electron microscope to examine the mycelium and spores.(Zhang et al., 2019).

2.6 Determination of the color valence of the *Mps* s(MPs)

Weigh 0.1g of red yeast rice powder in a 10mL centrifuge tube, and add 75% ethanol three times according to the ratio of 4:3:3. Sonicate for 30 minutes each time. Centrifuge at 8000rpm/min for 10 minutes, transfer the supernatant into a new centrifuge tube, and finally adjust the volume to 10mL with 75% ethanol. Take 1mL of extracted MPs for appropriate dilution, add it into a quartz cuvette, and measure its absorbance at wavelengths of 385nm (yellow pigment),475nm (orange pigment),505nm (red pigment),and 410nm(total pigment). Use blank solution containing only75% ethanol to set absorbance between 0.2-0.8 range. Calculate color value using equation(2-2).

Equation (2-2)

Where: S- color value of the sample;

A - Absorbance value of the dilution;

V-80% methanol volume (mL);

m-Dry weight of monascus mycelium (g);

n-dilution times;

2.7 HPLC Determination of MPs

After extracting the pigments, they were diluted to OD_{410} [?] 10 and filtered using a 0.22μm organic filter membrane before determining the pigment composition through high-performance liquid chromatography (HPLC). The mobile phases for pigment detection were as follows: phase A consisted of 0.1% formic acid in water, while phase B was pure acetonitrile. Both aqueous and organic phases were filtered with a 0.45μm filter membrane and degassed by ultrasonic treatment for 30 minutes. HPLC conditions are shown in Tables 2-5. Pigment detection methods are shown in Tables 2-6.

2.8 Analysis of relative gene expression level

The expression of four key genes involved in *MPs* biosynthesis (*MpPKS5* , *mppG* , *mppD* , *mppE*) and four genes related to growth and reproduction (*veA, vosA, laeA, gprD*) were analyzed in the original strain of *M. ruber* C100, the knockout strain *ΔMaρϵA* , and the overexpression strain *OE-MareA* . Primers were designed according to RT-qPCR primer design principles. cDNAs from C100, *ΔMaρϵA* , and *OE-MareA* were used as templates for RT-qPCR analysis of the above eight related genes. Primers were designed according to the RT-qPCR primer design principles, as shown in Tables 2-7.

RESULTS

3.1 Construction and validation of the vectors

Upstream and downstream homology arms were designed at both ends of the*MareA* gene and inserted into both ends of the *hyg* gene on the p59 plasmid, respectively, to achieve gene knockdown. The schematic diagram illustrating the construction of its knockdown plasmid is shown in Figure 3-1.

The *MareA* target gene was inserted between the strong promoter and terminator of tobacco mosaic virus CaMV 35S on the p1301 plasmid for the purpose of gene overexpression. The schematic diagram illustrating the construction of its overexpression plasmid is shown in Figure 3-2.

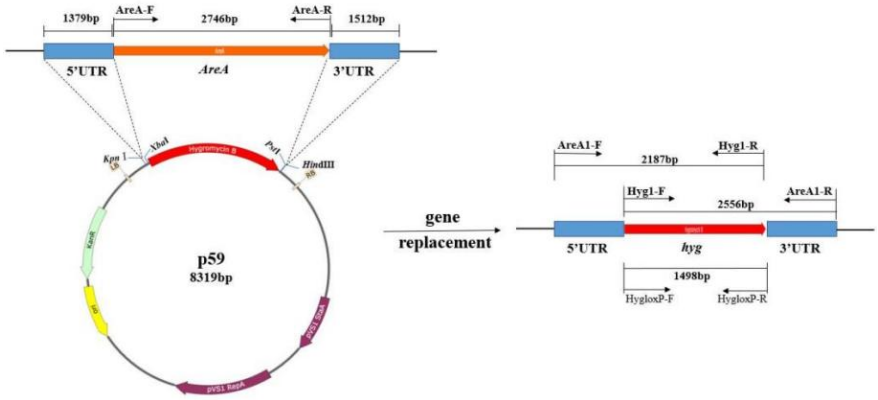

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Fig.3-1 Construction diagram of *MareA* gene knockout vector

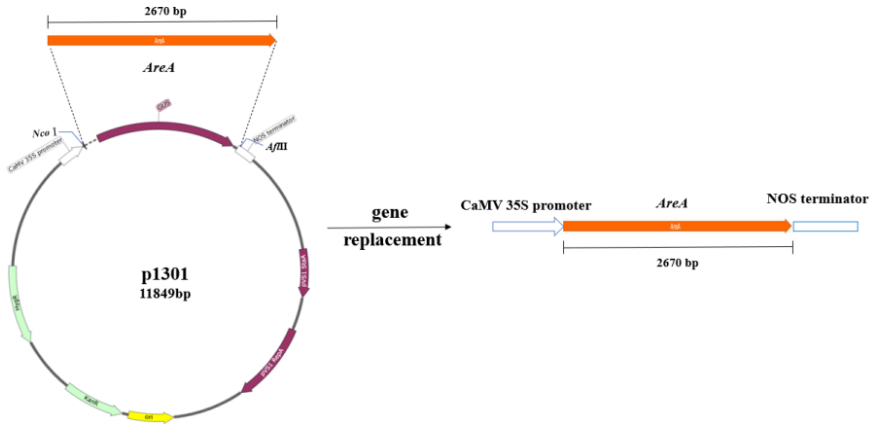

Fig.3-2 Construction diagram of *MareA* gene overexpression vector

The RT-qPCR was performed using the cDNA of C100 and *ΔMaρϵA* as templates, with qMareA-F and qMareA-R serving as primers. The expression of the *MareA* gene in both strains was compared through calculation. By comparing the expression levels of the *MareA* gene in C100 and Δ*MareA* strains, it was observed that the gene could be expressed normally in the original strain C100, while its expression in the knockout strain Δ*MareA* approached zero. This suggests that there is almost no transcriptome of the *MareA* gene in Δ*MareA,* further confirming successful knockdown of the*MareA* gene.

The cDNAs of C100 and OE-*MareA* were used as templates for RT-qPCR, with qMareA-F and qMareA-R serving as primers. The expression of the *MareA* gene in both strains was compared through calculation. The relative expression level of the *MareA* gene in the overexpression strain *OE-MareA* was found to be 3.41

times higher than that in the original strain C100, indicating successful insertion of the *MareA* gene fragment into the genome of monascus C100 and successful screening of the overexpression transformant *OE-MareA* . The results are presented in Figure 3-3.

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Fig.3-3 The expression levels of *MareA* gene in C100, Δ*MareA* and OE-*MareA* strains

3.2 Phenotypic analysis of Δ*MareA* and OE-*MareA* strains

3.2.1 Morphological analysis of colonies

Based on the combined data presented above, it is evident that the knockout strains *ΔMaρϵA* exhibit a more pronounced difference in colony morphology compared to the original strain C100. Regarding aerial mycelium production, the overexpression strain OE-*MareA* demonstrates increased levels, while the Δ*MareA* strain displays significantly reduced aerial mycelium formation. There is minimal variation in colony color among the three strains. When comparing different nitrogen sources, Gln and $(NH_4)_2SO_4$ have negligible impact on colony morphology, whereas $NaNO_3$and Urea exert a more significant effect. Specifically, growth of the Δ*MareA* strain is markedly inhibited on PDA plates supplemented with $NaNO_3$ and Urea, while colonies of OE-*MareA* strain exhibit normal growth. In conclusion, it can be inferred that knockdown or overexpression of *MareA* gene influences colony morphology without substantial impact on pigmentation species. Additionally, different nitrogen sources exert varying degrees of influence on colony morphology for all three strains. As shown in Figure 3-4.

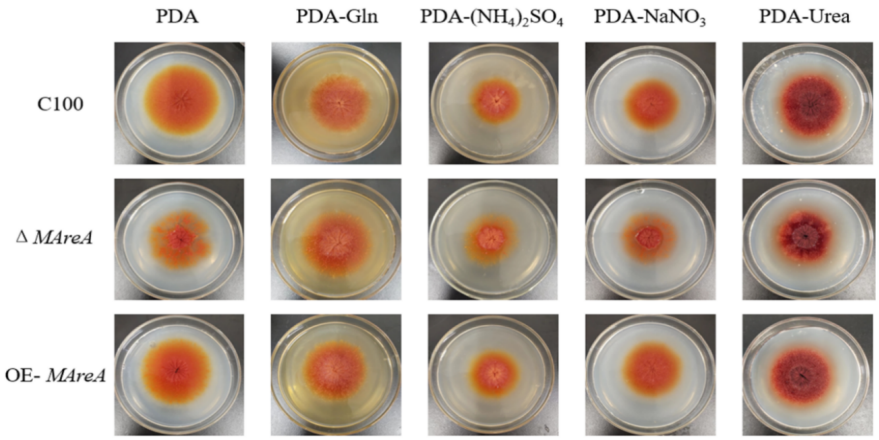

Fig.3-4 Colony phenotypes of C100, Δ*MareA* and OE-*MareA* in different nitrogen sources media

3.2.2 Colony diameter analysis

On PDA medium, the growth of the knockout strain *ΔMaρϵA* was significantly inhibited, with its colony diameter only 33.5% of that of the original strain C100. In contrast, the overexpression strain OE-*MareA* exhibited faster growth. This indicates that the*AreA* gene has an impact on the growth and development of C100. On PDA-Gln medium, all three strains showed a faster and essentially equal growth rate, suggesting that Gln as a nitrogen source can compensate for the effect of the *AreA* gene on C100 growth and development. However, when $(NH_4)_2SO_4$was added in the PDA medium, Δ*MareA* strain's ability to utilize it was significantly reduced, consistent with findings in*Penicillium oxalicum* (Rabha and Jha, 2018). In PDA-$NaNO_3$ medium, Δ*MareA* strain exhibited poor utilization of this nitrogen source resulting in slowed down growth rate; whereas *OE-MareA* strain was not affected due to presence of*MareA* gene and showed

slightly higher growth rate than C100. In PDA-Urea medium, Δ*MareA* strain's growth was higher compared to $NaNO_3$. Overall, the *MareA* gene had a significant impact on monascus' growth rate. Furthermore,the *MareA* gene also plays an important regulatory role in utilizing different nitrogen sources. The function of *MareA* gulatory varies among different fungi,which demonstrates diversity in regulatory functions performed by the *MareA* gene. As shown in Figure 3-5.

Fig.3-5 Colony diameters of C100, Δ*MareA* and OE-*MareA* in PDA medium with different nitrogen addition

3.2.3 Analysis of conidial production

The conidial yield of the knockout strain Δ*MareA* on PDA medium was significantly lower than that of the original strain C100, while the spore yield of the overexpression strain increased. The spore production of Δ*MareA* was relatively high on PDA-Gln medium, while it was the least on PDA-$NaNO_3$ medium. The spore production of the overexpression strain OE-*MareA* was higher than that of the original strain C100 on PDA-Gln, PDA-$NaNO_3$, and PDA-Urea media, while it was lower than that on PDA-$(NH_4)_2SO_4$ media. It is speculated that a higher concentration of ammonium salt would inhibit conidial production in *O* E-*MareA* . In summary, the*MareA* gene affects monascus' conidial production by inhibiting it when deleted and increasing it when overexpressed. Additionally, there is a difference in how *MareA* gene utilizes different nitrogen sources: Gln as a nitrogen source can offset *AreA* gene's effect on conidia to some extent, while $NaNO_3$ has the greatest impact on *Monascus* . As shown in Figure 3-6.

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Fig.3-6 Conidial numbers of C100, Δ*MareA* and OE-*MareA* in medium with different nitrogen sources

3.2.4 Microscopic Morphological Analysis

In PDA medium, C100 mycelium had septa and a few nuclei. Δ*MareA* mycelium was slender, with a long distance between septa and few nuclei. OE-*MareA* mycelium was thicker and had more nuclei, and OE-*MareA* mycelium exhibited a stronger fluorescence signal. In PDA-Gln medium, both Δ*MareA* and OE-*MareA* mycelium were thicker compared to C100, and all three strains showed stronger fluorescence signals. On PDA-$(NH_4)_2SO_4$ medium, the three strains did not differ significantly, but the fluorescence intensity of C100 was slightly stronger than that of Δ*MareA* and OE-*MareA* strains. In PDA-$NaNO_3$ medium, the mycelium of C100 and OE-*MareA* was thicker than that of Δ*MareA* . The fluorescence intensity of C100 was the strongest, followed by OE-*MareA* , while Δ*MareA* had the weakest intensity. The fluorescence intensity of C100 and OE- *MareA* in PDA-Urea medium was significantly stronger than that of Δ*MareA* strain. Overall,the three strains exhibited different morphologies under different culture conditions along with varying levels of fluorescence intensities,with the most significant differences observed in the strain supplemented with $NaNO_3$.This suggests that the *MareA* gene can influence monascus' growth and development,and at the same time,the *MareA* gene utilizes different nitrogen sources differently which subsequently affects monascus' growth. As shown in Figure 3-7.

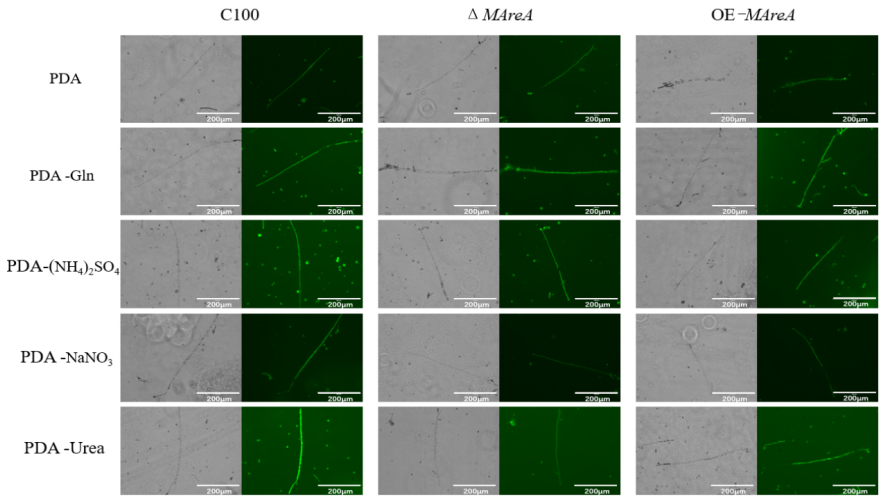

Fig.3-7 Morphology of C100, Δ*MareA* and OE-*MareA* in mycelium from different nitrogen sources

3.2.5 Morphological analysis of mycelium and spores under scanning electron microscope

In PDA medium, the mycelium of C100 exhibited a lamellar shape, while the spores showed folds. The Δ*MareA* mycelium and spore surface were smooth. The OE-*MareA* mycelium collapsed severely, and the spores had depressions in the middle. Under PDA-Gln culture conditions, both C100 and Δ*MareA* mycelia were columnar with an uneven spore surface. On the other hand, *OE-MareA* showed a lamellar shape with a flatter and smoother spore surface. When grown on PDA-$(NH_4)_2SO_4$ medium, both C100 mycelium and spores had full and smooth surfaces. However, Δ*MareA* and OE-*MareA* spores were collapsed to varying degrees. Under PDA-$NaNO_3$ medium condition, C100 mycelium was cylindrical with a non-smooth surface. Δ*MareA* mycelium was flaky and twisted, while OE-*MareA* mycelium was very smooth. Additionally, the volume of C100 and Δ*MareA* spores was smaller than that of OE-*MareA* . In PDA-Urea medium, the mycelium of C100 was smooth but slightly depressed, Δ*MareA* mycelium and spores were smooth and full with relatively small volumes, whereas OE-*MareA* displayed a rough lamellar surface for its myelum along with fuller-spored characteristics. Overall, the *AreA* gene had an effect on the growth and morphology of mycelium and spores, but had little impact on their thickness and size. The most significant difference between the three strains was observed when Gln and $NaNO_3$ were used as nitrogen sources. As shown in Figure 3-8.

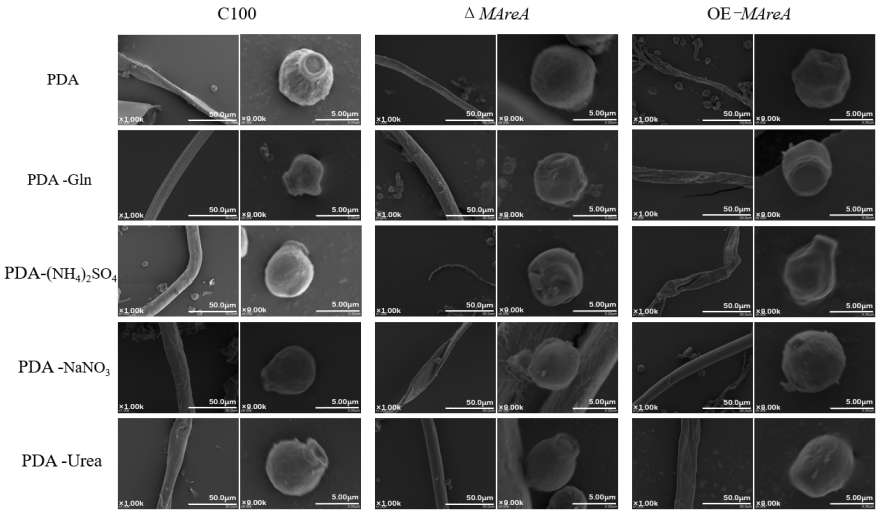

Fig.3-8 Mycelium and spore morphology of C100, Δ*MareA* and OE-*MareA* in media with different nitrogen sources

3.3 Solid-state fermentation of *C100,* Δ*MareA* and OE-*MareA*

3.3.1 Yield of red yeast rice by solid state fermentation

Monascus consumes nutrients in rice to meet its own growth and development needs during solid-state fermentation. Different strains of bacteria utilize rice to varying degrees, so the dry weight of red yeast rice can reflect the growth and metabolism of different *Monascus* strains in the solid-state fermentation process. In the rice fermentation medium without adding any nitrogen source, the knockout strain Δ*MareA* weighed the heaviest at 10.48g, indicating slower growth in the rice medium, while the overexpression strain OE-*MareA* weighed the lightest at 9.67g, suggesting better utilization of nutrients in rice. When comparing the dry weights of red yeast rice with different nitrogen sources added, it was observed that OE-*MareA* had the lightest weight after fermentation, followed by C100, and Δ*MareA* had the heaviest weight. The addition of Gln resulted in a consistent lighter weight for red yeast rice around 9.10 g, indicating that Gln addition was most favorable for *Monascus* ' fermentation of rice and was not affected by *AreA* gene. The weight of *Monascus* was the heaviest compared to other nitrogen sources, reaching more than 15g, as it could not effectively utilize $(NH_4)_2SO_4$. However, when $NaNO_3$ was added, Δ*MareA* showed poor utilization resulting in heavier red yeast rice. On the other hand, OE-*MareA* red yeast rice was lighter in weight. When Urea was added to the fermentation medium, both Δ*MareA* and OE-*MareA* fermented red yeast rice weighed less compared to $NaNO_3$. In conclusion, the addition of Gln had no effect on red yeast fermentation regardless of deletion or overexpression of the *AreA* gene, whereas $NaNO_3$ best demonstrated the impact of this gene on red yeast fermentation. As shown in Figure 3-9.

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Fig.3-9 Dry weight of C100, Δ*MareA* and OE-*MareA* in media with different nitrogen sources

3.3.2 Total color value of solid fermented red yeast rice

Color value is one of the main quality indicators of natural pigments, which can reflect the pigment production. In the fermentation medium of rice without an additional nitrogen source, the total color value of Δ*MareA* decreased compared to that of C100, while the color value of OE-*MareA* increased. Under the fermentation culture conditions with the addition of Gln, there was little difference in the total color values

among the three strains and they were largely unaffected by the*AreA* gene. In rice fermentation medium with $(NH_4)_2SO_4$ added, all three strains had very low total color values. When $NaNO_3$ was added to the medium, Δ*MareA* strain had a lower color value than C100 while OE-*MareA* strain had a higher color value than that of the original strain. This effect was most pronounced. In fermentation medium with Urea added, similar but less significant results were observed compared to $NaNO_3$addition. In summary, *MareA* gene affects monascus' color value mainly through reducing MPs' color value when *MareA* gene is deleted and accelerating pigment accumulation when *MareA* gene is overexpressed, under different nitrogen sources culture conditions adding different sources of nitrogen, overexpression of *MareA* gene increases MPs' color value . Among them, the most significant effect occurred in culture medium with $NaNO_3$. As shown in Figure 3-10.

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Fig.3-10 Total color values of C100, Δ*MareA* and OE-*MareA* under different nitrogen sources

3.3.3 Red, orange and yellow values of monascus rice fermented by solid state fermentation

Under the condition of rice fermentation culture without an additional nitrogen source, the color valence of pigments from the original strain C100 was highest at 475 nm. However, the knockout strain Δ*MareA* and overexpression strain OE-*MareA* exhibited a higher color value at 505 nm. After adding Gln for fermentation culture, the color values of all three strains at three wavelengths were similar to those of the original strain, indicating that Gln addition could restore red wine growth and reproduction to its original state unaffected by the*AreA* gene. Upon adding $(NH_4)_2SO_4$, there was not much difference in color values among the three strains at three wavelengths; however, their overall color values decreased significantly. The most significant difference in color price among the three strains was observed after adding $NaNO_3$, suggesting that $NaNO_3$ had pronounced effects on both knockout and overexpression strains' color prices. When Urea was used as a nitrogen source, changes in color price for all three strains were similar to those obtained with $NaNO_3$ but less effective.

In general, knocking out the *MareA* gene resulted in a decrease in the color values of yellow and orange pigments, but had little effect on red pigments; overexpression of the *MareA* gene led to an increase in the color values of all three pigments. The addition of Gln restored the growth and reproduction of red pigment to its original state, which was unaffected by the *AreA* gene. When $NaNO_3$ was added, the utilization of $NaNO_3$ by the strain was regulated by the *MareA* gene, which prevented the strain from using nitrogen as a nitrogen source, resulting in significantly lower color values than the original strain. Conversely, overexpression strains exhibited significantly higher color values than the original strain with noticeable effects. As shown in Figure 3-11.

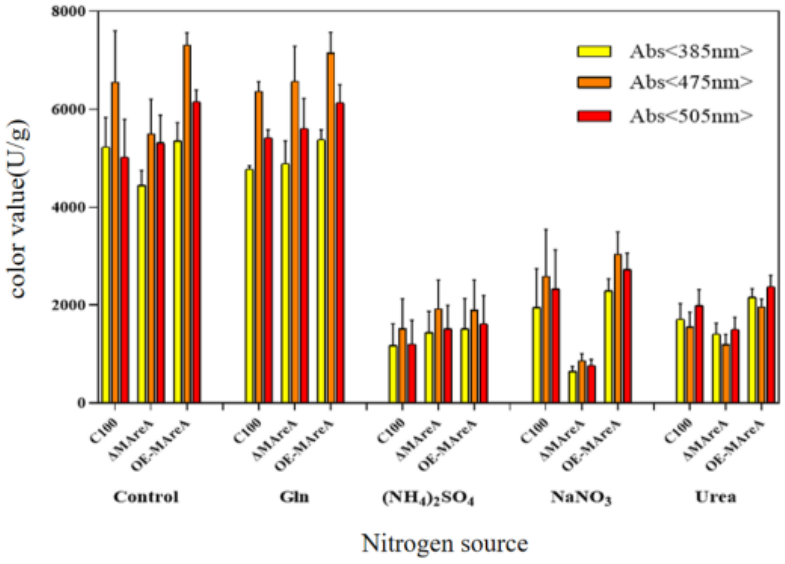

Fig.3-11 Red, orange and yellow values of C100, Δ*MareA* and OE-*MareA* under different nitrogen sources

3.4 HPLC analysis of pigments produced by solid state fermentation of C100, Δ*MareA* and OE-*MareA* strains

As shown in Figure 3-12.In the rice fermentation medium without additional nitrogen source, the pigment species of all three strains were dominated by Y1, O1 and O2, among which, O2 was the most abundant. The contents of yellow pigments of the three strains did not differ much, and they contained more Y1 than Y2.The contents of C100 red pigments R1 and R2 were significantly lower relative to orange and yellow pigments. The content comparison of Δ*MareA* red pigments R1 and R2 were lower than that of C100.Peak area comparison of OE-*MareA* in R1 was higher than that of C100 and R2 peak area was lower than that of C100.As shown in Tables 3-1

As shown in Figure 3-13.After the addition of Gln to the rice fermentation medium, there was not much difference in the peak area share of the six pigments C100, Δ*MareA* , and OE-*MareA* . The contents of all three strains were dominated by Y1, O1, and O2. Among them, O1 and O2 were the most abundant while Y2 was the least abundant. Compared with the control group without an added nitrogen source, there was an increase in the peak area share of R1 and R2 while a decrease in the peak area share of O1 and O2. This indicates that after adding Gln, it promotes the transformation from orange pigment amination to red pigment during red pigment synthesis. As shown in Tables 3-1.

As shown in Figure 3-14.After the addition of $(NH_4)_2SO_4$, there was no significant difference in pigment type and content between the original strain C100 and the knockout strain Δ*MareA* . However, the red pigment content of the overexpression strain OE-*MareA* increased significantly. There was no significant difference in Y1 and Y2 contents between OE-*MareA* and C100. Compared to the control group without nitrogen source, there was an exponential increase in peak area occupied by R1 and R2 while orange pigment decreased; however, Y1 and Y2 contents did not change much. The only strain that showed a significant change towards red pigment synthesis with this addition was OE-*MareA*. This indicates that $(NH_4)_2SO_4$ promotes amination of orange pigment to red during red pigment synthesis. As shown in Tables 3-2.

As shown in Figure 3-15. Using $NaNO_3$ as the nitrogen source, there was no significant difference in pigment types and contents between the original strain C100 and the overexpression strain OE-*MareA* . The red

pigments R1 and R2 were very low in C100, while the knockout strain Δ*MareA* had slightly higher red pigment content compared to C100. The percentage of yellow pigments Y1 and Y2 in the peak area of Δ*MareA* was reduced compared to that of C100, with a relatively large reduction observed for Y1. The orange pigment remained most abundant in C100, with a slightly increased O1 content observed in Δ*MareA* compared to the original strain, while O2 content did not differ significantly. As shown in Tables 3-3.

As shown in Figure 3-16. The addition of Urea as a nitrogen source resulted in the most noticeable change, which was the undetectable levels of orange pigments O1 and O2 in the original strain C100, knockout strain Δ*MareA* , and overexpression strain OE-*MareA* due to their extremely low levels. The other four major alcohol-soluble pigments in these three strains had the highest percentage of R1 and R2 and the lowest percentage of Y2. As shown in Tables 3-4.

In summary, knockdown and overexpression of the *MareA* gene do not affect the types of pigments but only their contents. Among them, the most noticeable changes were observed in orange and red pigments, while the content of yellow pigment remained relatively unchanged. Knocking down the *MareA* gene resulted in a decrease in red pigment content, particularly R2. Conversely, overexpressing the *MareA* gene led to an increase in orange pigment content, especially O2. It can be hypothesized that the *MareA* gene may influence the conversion of orange pigment to red pigment through amination in the synthesis pathway. Deletion of this gene inhibits this process, whereas its overexpression promotes it. After adding Gln and $(NH_4)_2SO_4$, even without presence of the *MareA* gene, preferred nitrogen sources are still utilized resulting in similar pigmentation as observed in original strain. These two preferred nitrogen sources facilitate ammonification process. The addition of $NaNO_3$ did not promote or inhibit the conversion from orange to red pigment mediated by*MareA* gene. When Urea is added and heated for decomposition into NH_3 with high concentration of $(NH_4)^{2+}$ present in medium, sufficient ammonification occurs leading to substantial conversion from orange to red pigment and consequently significant increase in red pigment content.

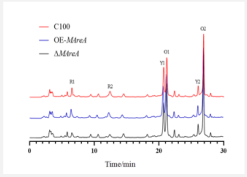

Fig.3-12 Chromatograms of C100, Δ*MareA* and OE-*MareA* in rice solid state fermentation by HPLC

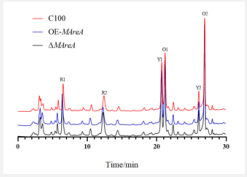

Fig.3-13 Chromatograms of pigments C100, Δ*MareA* and OE-*MareA* in rice solid state fermentation by HPLC with Gln

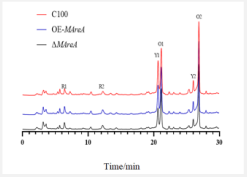

Fig.3-14 Chromatograms of pigments C100, Δ*MareA* and OE-MareA in rice solid state fermentation by HPLC with $NaNO_3$

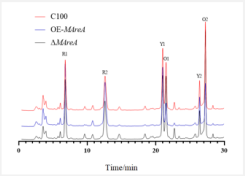

Fig.3-15 Chromatograms of pigments C100, Δ*MareA* and OE-*MareA* in rice solid state fermentation by HPLC with $(NH_4)_2SO_4$

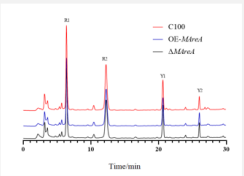

Fig.3-16 Chromatograms of pigments C100, Δ*MareA* and OE-*MareA* in rice solid state fermentation by HPLC with Urea

3.11 Analysis of relative expression levels of genes

Based on the measurements of Monascus growth and development through different experiments using various nitrogen sources, it can be concluded that the addition of glutamine as a nitrogen source in the medium does not affect the growth and development of monascus. However, when $NaNO_3$ is used as the nitrogen source, knockdown and overexpression of the *AreA* gene have a significant impact on monascus growth and development. Therefore, we analyzed the expression of four key genes involved in red currant pigment biosynthesis (*MpPKS5, mppG, mppD, mppE*) and four genes related to growth and reproduction (*VeA, VosA, LaeA, GprD*) in Aspergillus rubra's original strain C100 as well as in the knockout strain Δ*MareA* and overexpression strain OE-*MareA* .

There are 17 key genes on the MPs synthesis gene cluster. Among them,*MpPKS5* and *mppD* are structural genes in the erythro-pigment polyketide synthesis pathway, playing crucial roles in the process of pigment synthesis. *MppD* is a homolog of*MpPKS5* and has been suggested to have a specialized but undetermined auxiliary role to *MpPKS5. MppE* acts as a reductase in the polyketide pathway, controlling the biosynthesis of yellow pigment. *MppG* is closely involved in synthesizing reddish currant orange and red pigments(Chen et al., 2017).

As shown in Figures 3-17, the relative expression of MpPKS5 and mppD genes in the knockout strain Δ*MareA* was decreased under control conditions without the addition of nitrogen source. The expression of mppE gene was significantly up-regulated. The relative expression levels of MpPKS5, mppG, mppD and mppE genes in the OE-*MareA* were significantly down-regulated. These show that the quantity of key gene expression *MareA* gene of red kojic rice fermentation have played an important role in the pigment metabolism. In solid fermentation medium supplemented with Gln, the expression of MpPKS5 gene in Δ*MareA* was essentially unchanged, whereas the relative expression of mppG, mppD and mppE genes all decreased. The relative expression levels of MpPKS5, mppG, mppD and mppE genes in OE-*MareA* were up-regulated to varying degrees. After addition of $NaNO_3$, the expression of MpPKS5 gene in Δ*MareA* was close to that in C100, while the relative expression of mppG, mppD and mppE genes was significantly up-regulated. In OE-*MareA* , the expression of MpPKS5 and mppE genes was down-regulated, while that of mppG and mppD genes was up-regulated. This indicates that the loss of*MareA* gene resulted in increased expression of pigmentation genes after addition of $NaNO_3$, and that overexpression of these genes resulted in a significant reduction in yellow pigment accumulation.

In summary, the *MareA* gene affects the expression of pigment genes. When Gln was added, the deletion of *MareA* gene suppressed the expression of key pigmentation genes, while overexpression promoted it. When the nitrogen source $NaNO_3$ was added, the deletion of *MareA* gene promoted it more obviously compared to overexpression.

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Fig.3-17 Expression levels of pigment genes of Monascus C100, Δ*MareA* and OE-*MareA* in solid-state fermentation under different nitrogen sources

Morphological development and secondary metabolism in fungi are regulated by a diverse array of genes or gene families(Wang et al., 2015). In numerous fungal species, the regulation of these processes has been attributed to two velvet protein complexes known as *VeA* and*VosA* *VeA* is highly conserved in a variety of fungi and controls sexual/asexual development and secondary metabolism in filamentous fungi(Wang et al., 2019).The *VosA* gene was initially identified in Aspergillus globulus and plays a crucial role in long-term spore viability along with the regulation of conidial maturation and conidiogenesis (Ni and Yu, 2007). *LaeA* serves as a global transcription factor that orchestrates fungal growth, development, and metabolism(Pomraning et al., 2022). Fungi perceive external signals to modulate growth, development, and metabolic signaling pathways; within this context, *GprD* participates in G protein signaling pathway exerting significant influence on this intricate process(Wang et al., 2013).

As shown in Figure 3-18, under the control condition without added nitrogen source, the expression of *VeA* and *GprD* genes of the knockout strain Δ*MareA* did not differ much from that of the original strain C100, while the expression of *VosA* and*LaeA* genes was significantly down-regulated. The expression of*VeA* and *VosA* genes of overexpression strain OE-*MareA* did not change. It indicates that the deletion of*MareA* gene affects the growth and conidial production of*Monascus* , while the overexpression of the gene has its promoting effect to a certain extent. When Gln was added to the medium, the gene expression of the three strains did not differ much, indicating that even the deletion of the *MareA* gene would not affect the growth of monascus too much when Gln was present in the environment. The expression of *VeA, VosA, LaeA* and *GprD* genes of the knockout strain Δ*MareA* was severely suppressed by the addition of $NaNO_3$ to the

medium, while the expression of the four genes of the overexpression strain OE-*MareA* was significantly up-regulated. It indicates that when $NaNO_3$ is present in the environment, the *MareA* gene-deficient strain cannot utilize secondary nitrogen sources for growth and reproduction, whereas overexpression of the genes promotes the growth and spore production of monascus.

In summary, deletion of the *MareA* gene slowed down the growth of*Monascus* and also reduced spore production, while overexpression of the gene promoted the growth and spore production of *Monascus* . The presence of Gln in the environment did not affect the growth and reproduction of *Monascus* when the *MareA* gene was present. However, in an environment with $NaNO_3$, deletion of the*MareA* gene resulted in an inability to utilize $NaNO_3$, leading to inhibition of Monascus growth. Conversely, overexpression of the gene promoted *Monascus* growth and development.

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Fig.3-18 Expression levels of growth and metabolism genes of*Monascus* C100, Δ*MareA* and OE-*MareA* in solid-state fermentation under different nitrogen sources

This study used M. ruber C100 as the starting strain and amplified the*Mare* A gene through PCR. *MareA* gene knockout and overexpression vectors were constructed. The study then used Agrobacterium-mediated transformation to infect Monascus purpureus with the vectors, and selected the correct Δ*MareA* and OE-*MareA* strains based on the principle of homologous recombination. The study then compared the changes in colony morphology, spore production, and mycelial morphology among the three strains to investigate the impact of the *MareA* gene on the mycelial morphology of Monascus purpureus.We conducted a preliminary study on the impact of the*MareA* gene on growth and reproduction in *monascus.* The regulation of the *MareA* gene on monascus (a secondary metabolite) in *monascus* was examined through solid-state fermentation by analyzing expression differences in red currant pigment and genes related to growth and development. This analysis aimed to understand the role played by the *MareA* gene in monascus' metabolic process. The research results of this thesis are as follows:

The DNA of *M. ruber* C100 was used as a template, and the*MareA* gene homologous sequence C5.761 in the genome of M. purpureus YY-1 was used as a reference to design primers. The full length of the *MareA* gene fragment, 2746 bp, was obtained through PCR amplification and sequencing. The sequencing results of C100 were compared with those of C5.761 in the YY-1 genome using DNAMAN software for analysis, resulting in a 99.15% similarity. This fragment contained 889 amino acids and exhibited the conserved GATA-type zinc finger structure (C-X(2)-C-X(17)-C-X(2)-C).

Using p59 as the original vector, the homologous arms of the MareA gene on both ends were amplified by PCR and then inserted into the two ends of the p59 vector with the selection marker gene Hyg. The successfully reconstructed vector p59ss was screened and verified. Using p1301 as the original vector, a fragment of the MareA gene was amplified by PCR and then inserted into the CaMV 35S strong promoter and CaMV 35S terminator between the p1301 vector. The correct overexpression recombinant plasmid p1301s was screened and verified. The obtained recombinant vectors p59ss and p1301s were introduced into *Agrobacterium rhizogenes* separately, followed by co-culturing with monascus through*Agrobacterium-mediated* transformation to screen and validate Δ*MareA* knockout strain and OE-*MareA* overexpression strain.

The monascus were cultivated through solid-state fermentation under different nitrogen source conditions, and the yield of MPs was tested after 13 days of fermentation. The results showed that the Δ*MareA* strain reduced the color values of red, orange, and yellow pigments, while OE-*MareA* increased the color values of these pigments. Meanwhile, the *MareA* gene does not affect the species of the six major pigments but influences their contents. Specifically, it has little effect on Y1 and Y2 contents but mainly affects the percentage of peak area occupied by R1, R2, O1, and O2. Comparing fermentations under different nitrogen

source conditions revealed that the ΔMareA strain could preferentially utilize Gln and $(NH_4)_2SO_4$ but had limited utilization ability for $NaNO_3$ and Urea. Additionally, it had varying effects on pigment color values. In summary, these results demonstrate that knocking down the *MareA* gene decreases total pigment color value in red yeast while overexpressing this gene increases it. This indicates that metabolism of red yeast pigment is regulated by *MareA* gene expression levels. Furthermore, different nitrogen sources are utilized differently by*MareA* gene. Gln can counteract its effects on growth and development in monascus whereas $NaNO_3$ most prominently reflects its impact during fermentation.

RT-qPCR was used to analyze the expression of four MPs genes and four growth-regulated genes. From the expression of the four key genes involved in MPs biosynthesis, it can be observed that after deleting the*MareA* gene, the gene expression of *mppE* was significantly up-regulated, while the relative expressions of *MpPKS5, mppG* , and*mppD* genes were down-regulated to varying degrees. On the other hand, overexpressing the *MareA* gene suppressed the expression of all four key pigmentation genes. Regarding growth regulation, deletion of *MareA* gene notably reduced *VosA* and *LaeA* gene expressions, whereas overexpression promoted *GprD* gene significantly. In presence of Gln (glutamine), deletion of *MareA* did not affect monascus growth, however, in presence $NaNO_3$ (sodium nitrate), its deletion had a clear inhibitory effect on both growth and reproduction. These results indicate that *MareA* gene influences sexual and asexual development as well as metabolism in monascus.

Discussion

In this study, overgene knockout and overexpression techniques were used to achieve the knockout and overexpression of *MAreA* gene, the global transcriptional regulatory factor of monascus, and the*MAreA* knockout strain Δ*MAreA* and the overexpression strain OE-*MAreA* were successfully obtained through agrobacterium-mediated transformation technology. Through solid state fermentation, it was found that overexpression of *MAreA* gene would increase the yield of Mps, and different types of Mps would be obtained due to different utilization of nitrogen source by *MAreA* gene. Especially, the yield of red pigment R1 and R2 increased significantly after urea was added. The effects of *MAreA* gene on the sexual and asexual development and pigment metabolism of monascus were studied. The study results provided a reference for determining the functional characteristics of *AreA* gene, theoretical guidance and technical support for monascus large-scale fermentation of Mps in the future, and provided a basis for the production of Mps strains for industrial use. However, due to time constraints, only RT-qPCR was used to achieve the difference analysis of gene expression in the functional prediction of *MAreA* gene, and the number of genes for difference analysis was small. In subsequent studies, high-throughput sequencing can be used to analyze the function of *MAreA* gene on the transcript data. In addition, due to the hygromycin antibiotic gene in the strain genome, it can not be used for industrial scale fermentation of Mps. After removing the hygromycin resistance gene, it should be considered as a high-yield pigment engineering strain. Previous studies have shown that there is no secondary metabolite citrinin in Monascus rubrus C100 or the content is very small. Therefore, the effect of MAreA gene on the yield of citrinin was not studied in this paper. In future studies, it is possible to select monascus strains with high yield of citrinin, such as monascus purple M8, for *MAreA* gene knockout and overexpression, so as to study the effect of MAreA gene on citrinin metabolism. Finally, the optimal process of industrial production and preparation of Mps can be achieved by cultivation under different nitrogen source conditions, so as to achieve large-scale production of Mps and improve its application in various industries.

Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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