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Effects of blue light on pigment biosynthesis of *Monascus*[§]

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The influence of different illumination levels of blue light on the growth and intracellular pigment yields of *Monascus* strain M9 was investigated. Compared with darkness, constant exposure to blue light of 100 lux reduced the yields of six pigments, namely, rubropunctatamine (RUM), monascorubramine (MOM), rubropunctatin (RUN), monascorubrin (MON), monascin (MS), and ankaflavin (AK). However, exposure to varying levels of blue light had different effects on pigment production. Exposure to 100 lux of blue light once for 30 min/day and to 100 lux of blue light once and twice for 15 min/day could enhance RUM, MOM, MS, and AK production and reduce RUN and MON compared with non-exposure. Exposure to 100 lux twice for 30 min/day and to 200 lux once for 45 min/day decreased the RUM, MOM, MS, and AK yields and increased the RUN and MON. Meanwhile, the expression levels of pigment biosynthetic genes were analyzed by real-time quantitative PCR. Results indicated that gene *MpPKS5, mppR1, mppA, mppB, mmpC, mppD , MpFasA, MpFasB,* and *mppF* were positively correlated with the yields of RUN and MON, whereas *mppE* and *mppR2* were associated with RUM, MOM, MS, and AK production.

Keywords: *Monascus*, blue light, illumination level, pigments, biosynthetic genes

Introduction

Light is a crucial environmental signal that influences fungi in many aspects, including mycelium development, conidia formation (Lee *et al.*, 2006), and secondary metabolism biosynthesis (Miyake *et al.*, 2005). Blue light regulates circadian rhythms, spore formation, and synthesis of carotenoids in *Neurospora crassa* (Linden, 2002). The mold *Aspergillus nidulans* forms asexual spores in the presence of light but preferentially undergoes sexual reproduction in the dark (Häggblom and Unestam, 1979). Additionally, blue light also inhibits mycotoxin production in *A. flavus*, *A. parasiticus*, and *Alternaria alternate* (Mooney and Yager, 1990).

Monascus species have been used in Chinese food, medicine, and industry for more than 1,000 years (Chen *et al.*, 2015). They produce many secondary metabolites, such as *Monascus* pigments (MPs), monacolins, γ-aminobutyric acid, and dimerumic acid (Jůzlová *et al.*, 1996). As natural food colorants, MPs have been widely utilized in food industries in eastern Asia. Moreover, MPs exhibit several biological activities, such as anti-mutagenic, anticancer (Akihisa *et al.*, 2005), antimicrobial (Kim *et al.*, 2006), and potential anti-obesity properties (Kim *et al.*, 2007). Up to now, more than 50 kinds of MPs have been identified (Feng *et al.*, 2012); and investigations focus on six well-known MPs, namely, monascin (MS), ankaflavin (AK), rubropunctatin (RUN), monascorubrin (MON), rubropunctamine (RUM), and monascorubramine (MOM) (Blanc *et al.*, 1994). MS and AK are yellow pigments from the same chromophores and are distinguished by the lengths of their saturated side chains. The same structural relationship was observed for the orange (RUN and MON) and red pigments (RUM and MOM) (Teng and Feldheim, 1998). Until today, the biosynthetic pathway of MPs is still unclear and controversial, although it is generally considered to follow a polyketide pathway. Recently, Balakrishnan *et al.* (2013) identified the biosynthetic gene cluster of azaphilone pigments in the *M. pilosus* genome and proposed a pigment biosynthetic pathway through targeted gene inactivation and homologous analysis. In this study, the expression levels of pigment biosynthetic genes under various blue light conditions were measured, and our results confirm the rationality of the proposed biosynthetic pathway.

Several reports have reported the effect of light on pigment production of *Monascus*. (Miyake *et al.*, 2005) reported that red light enhanced pigment production but blue light inhibited it. Babitha *et al.* (2008) found that direct blue illumination totally suppressed pigment production in *M. purpureus*. However, studies that focus on the influences of blue light on the three kinds of MPs, red, orange and yellow pigments, respectively, are scarce. In this study, the effects of various intensities of blue light and illumination time on the production of six well-known MPs (RUN and MON, MS and AK, RUM and MOM) were determined. Different changes in the yield of red, orange, and yellow pigments, respectively, exposed to varying blue light conditions were observed. We proposed a new hypothesis that the biosynthetic transformation among MPs is induced by blue light. Meanwhile, gene cluster expression analyses of pigment synthesis under varying blue light conditions were carried out.

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Materials and Methods

Strain and growth conditions

Monascus strain M9, was maintained on malt extract agar (MEA) for 7 days at 30°C. Spores were harvested with 3 ml of sterile water and inoculated in 250-ml flasks seed medium (100 ml; glucose, 60 g/L; peptone, 20 g/L; KH_2PO_4, 10 g/L; $NaNO_3$, 10 g/L, and $MgSO_4$, 5 g/L; pH 4.5). Cultures were incubated for 30 h at 30°C and 180 r/min. For pigment production, 3 ml of spore suspension (adjusted to 10^6 spores /ml) was inoculated into 50 ml of rice medium (rice powder, 50 g/L; KH_2PO_4, 1.5 g/L; $NaNO_3$, 3 g/L and $MgSO_4 \cdot 7H_2O$, 1 g/L) in a 12-mm culture dish. The dishes were incubated at 30°C without shaking for 8 days in the darkness or exposed to blue light.

Light exposure conditions

Two light chambers were constructed to enable incubation of cultures under different intensities of blue light. Each chamber was equipped with 9 W LEDs (460 nm) with the following conditions: chamber 1, light intensity 100 lux; chamber 2, 200 lux. Another dark chamber was used as control. Heating effect produced by LEDs could not be detected. A time switch was fixed on each chamber to control exposure times.

Growth assessment

To observe the effects of blue light on mycelium growth, 20 µl of spore suspension was inoculated on MEA at one point exposed to blue light or left in the darkness for 8 days at 30°C. The mycelium morphology was observed by microscope.

Pigment extraction and estimation

Light- and dark-grown mycelium was obtained from rice medium when the incubation finished, dried, and ground into powder. Then the powder (0.5 g) was transferred into a 10-ml centrifuge tube. Pigments were extracted three times with 3 ml of 75% ethanol for 30 min incubated in an ultrasonic bath followed by centrifugation (2,862 × *g*, 10 min). The supernatant was merged and filtered through 0.45-µm membranes.

Pigments were analyzed quantitatively by HPLC (Agilent 1200 system; Agilent Technologies) equipped with a diode array detector (DAD) on a column (Agilent XDB C18, 150 mm × 4.6 mm, 5 µm) at 25°C. For yellow and orange pigments analysis, the isocratic elution was carried out with the mobile phase [acetonitrile/water/trifluoroacetic acid (62.5/37.5/0.05, v/v/v)]. The wavelength of DAD was set at 234 nm. The red pigment was analyzed with a gradient of mobile phase of acetonitrile/water from 35:65 (v/v) to 65:35(v/v) and the chromatogram detected at 394 nm. The flow rate was 1 ml/min. 20 µl of samples was used for each experiment.

Pigments were analyzed qualitatively by Waters AB SCIEX 3500 LC/MS System (Waters) equipped with an electrospray ionization (ESI) source. Full scans were performed between *m/z* 300 and 500. Electrospray conditions were as follows: capillary voltage, 3.5 kV; nebulizer pressure, 40 psi; drying gas flow, 10 ml/min; temperature, 350°C.

Real-time quantitative PCR analysis

Total RNA was extracted from mycelia using the Plant RNA Kit (Omega). First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa), with the Oligo dT Primer 15. Gene expression was monitored by RT-qPCR and carried out using the SYBR Premix Ex Taq II (TaKaRa). RT-qPCR was performed using the Stratagen Mx3000P (Agilent) with the following cycling program: hold at 95°C for 30 sec, followed by a three-step PCR (42 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec) and dissociation curve analysis (at 95°C for 15 sec, annealing at 60°C for 30 sec, then collecting the dissociation curve from 60°C to 95°C, finally at 95°C for 15 sec).

Statistical analysis

Each experiment was repeated at least three times. Numerical data are presented as mean ± SD. The differences among different treatments were analyzed using one-way ANOVA. All statistical analyses were performed by using SPSS 17.0 software. $P < 0.05$, $P < 0.01$ was considered statistically significant.

Results

Effects of constant blue light exposure on colony morphology and mycelium growth

To observe the influence of blue light on the growth of *Monascus*, we inoculated M9 in liquid rice powder medium at 30°C under continuous exposure to blue light (100 lux) and in the darkness for eight days (Fig. 1A and B). A significant difference was observed on colony morphology. Fresh mycelium cultivated in the darkness almost covered the entire

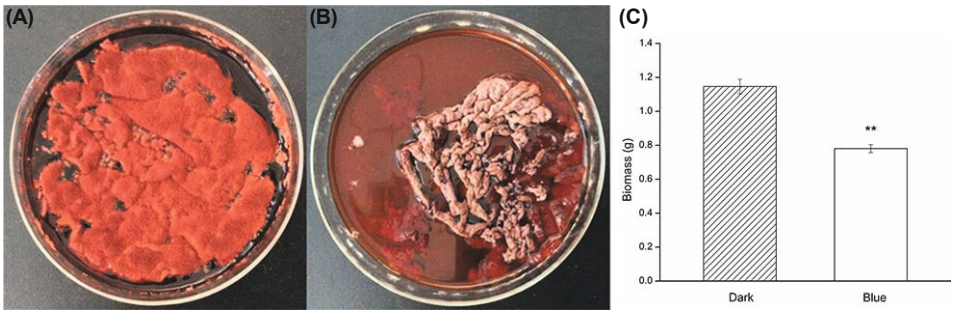

Fig. 1. Effects of blue light on colony morphology and biomass of M9. Images of colonies after eight days inoculated in liquid rice medium at 30°C (A) in the darkness or (B) exposed to constant blue light (100 lux). (C) Biomass in the darkness and under blue light was estimated by determining the dry weight of the mycelium. The data are represented as the mean ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$ compared with darkness.

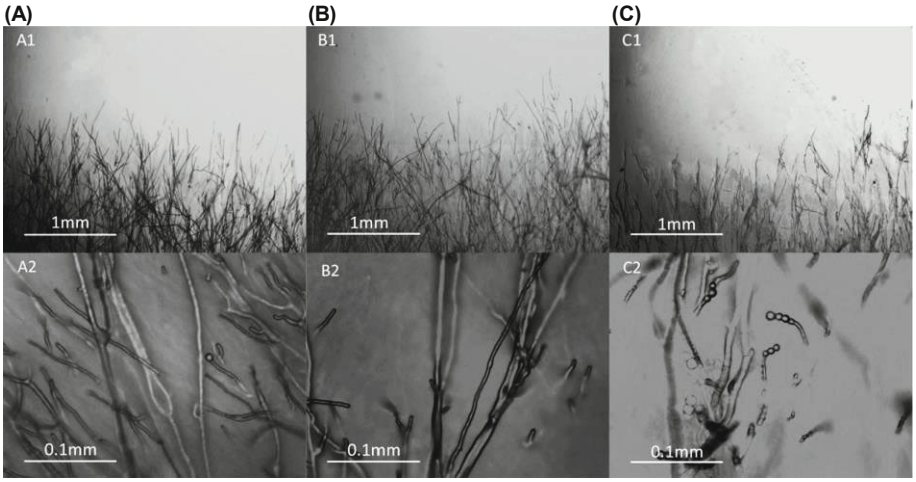

Fig. 2. Effects of blue light on mycelium growth. Spore suspension was inoculated at one single point on MEA (A) under darkness, (B) exposed to blue light for 12 h/day, or (C) kept under constant blue light for eight days at 30°C. Images of mycelium on the edge of colonies were observed by a microscope.

surface of the medium. Colonies exposure to constant blue light were curled and sparse, whereas those in the dark were gray in color instead of bright orange. Fig. 1C shows that the biomass of M9 under blue light was significantly less than that in the darkness, which was consistent with the observation above (Fig. 1A and B).

After eight days of cultivation on the MEA, the mycelia exposed to blue light were shorter and looser compared with those in the darkness. It indicated constant blue light might be a disadvantage to the growth of mycelia.

Effects of constant blue light on MP production

The qualitative analysis of pigments from M9 was performed by HPLC-MS. RUM, MOM, RUN, MON, MS, and AK had molecular weights of 353, 381, 354, 382, 358, 386, respectively; almost the same spectra of these pigments have already been described by Teng and Feldheim (1998) (Supplementary data Figs. S1 and S2).

The effect of constant blue light exposure on the yields of these MPs was analyzed by HPLC (Fig. 3). Evidently, the content of pigments exposed to blue light was significantly lower than those kept in the dark. Yields of RUM, MOM, RUN, MON, MS, and AK were reduced by 93.7%, 86.5%, 71.4%, 29.0%, 32.4%, and 28.6%, respectively. This indicated that constant exposure to blue light is a disadvantage to pigment biosynthesis.

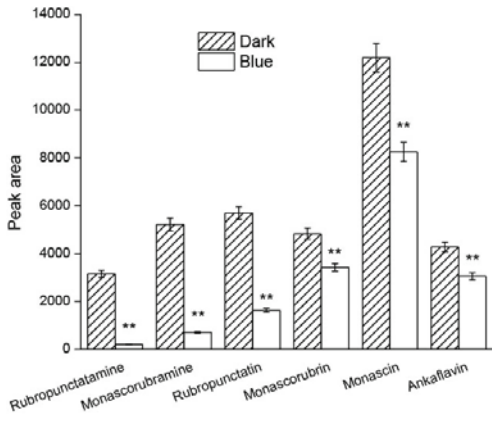

Fig. 3. Effects of darkness and constant blue light on MPs production. M9 was inoculated in rice medium in the darkness or exposed to constant blue light (100 lux). Yields of rubropunctatamine, monascorubramine, rubropunctatin, monascorubrin, monascin and ankaflavin were assessed by HPLC. Relative peak areas of intracellular MPs divided by weight of dry mycelium were chosen to measure MPs production. The data are represented as the mean ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$ compared with darkness.

Effects of blue light with various intensities, illumination time, and number of exposures on MP production

In our previous studies, we found that the intensity of blue light, illumination time, and number of exposures were the main factors that influenced the pigment production of M9. Thus, five different experimental conditions of blue light illumination were designed as follows: 100 lux, one 15 min per 24 h exposure [marked as 100 15 (1)]; 100 lux, two 15 min per 24 h exposure [marked as 100 15 (2)]; 100 lux, one 30 min per 24 h exposure [marked as 100 30 (1)];100 lux, two 30 min per 24 h exposure [marked as 100 30 (2)]; and 200 lux, one 45 min per 24 h exposure [marked as 200 45 (1)]. The mycelium cultured in the darkness served as the control.

The production of the red pigments RUM and MOM increased by 115% and 46.9% under 100 30 (1), respectively; 76.6% and 42% under 100 15 (1), respectively; and 52.9% and 35.0% under 100 15 (2), respectively. RUM and MOM decreased by 87.1% and 6.2% under 100 30 (2), respectively; and by 87.6% and 7.7% under 200 45 (1), respectively (Fig. 4). A similar trend in the two yellow pigments MS and AK was observed under different blue light conditions. MS and AK production were increased by 17.5% and 14.2% under 100 30 (1), respectively; by 14.4% and 14.0% under 100 15 (1), respectively; and 2.8% and 2.9% under 100 15 (2), respectively. However, MS was reduced under 100 30 (2) and 200 45 (1) by 4.0% and 18.5%, respectively; whereas AK was decreased by 4.5% and 11.3 % under 100 30 (2) and 200 45 (1), respectively. On the contrary, blue light with 100 30 (1), 100 15 (1), and 100 15 (2) reduced the yields of the orange pigment RUN by 34.7%, 28.1%, and 24.3%; and blue light with 100 30 (1), 100 15 (1), and 100 15 (2) reduced the yield of MON

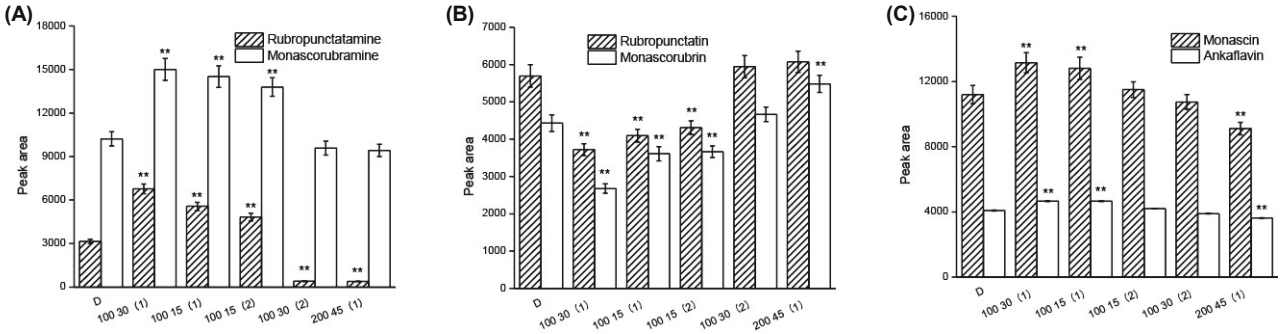

Fig. 4. Effects of blue light intensity, illumination time, and number of exposures on MP production. M9 was incubated in rice medium at 30°C for eight days in the darkness or under different blue light conditions of 100 lux, one 15-min per 24 h exposure [marked as 100 15 (1)]; 100 lux, two 15-min per 24 h exposure [marked as 100 15 (2)]; 100 lux, one 30-min per 24 h exposure [marked as 100 30 (1)]; 100 lux, two 30-min per 24 h exposure [marked as 100 30 (2)]; and 200 lux, one 45-min per 24 h exposure [marked as 200 45 (1)]. Yields of (A) rubropunctatamine and monascorubramine, (B) rubropunctatin and monascorubrin, and (C) monascin and ankaflavin were assessed by HPLC. Relative peak areas of intracellular MPs divided by weight of dry mycelium were chosen to measure MP production. Data are presented as the mean ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$ compared with darkness.

by 39.6%, 18.5%, and 17.3% compared with dark, respectively. The production of RUN was enhanced by 4.4% and 6.7% under 100 30 (2) and 200 45 (1), respectively; and the production of MON was increased by 5.4% and 23.7% under 100 30 (2) and 200 45 (1) condition, respectively. The production of red and yellow pigments was enhanced/reduced along with the decrease/increase of orange pigment yield upon exposure to certain blue light conditions. Blue light with various illumination levels caused different changes in the production of red, orange, and yellow pigment, respectively.

Effects of blue light on the expression of pigment biosynthetic gene

To understand the effects of blue light on MP production at the molecular level, we analyzed the expression levels of pigment biosynthetic gene clusters by RT-qPCR. This gene cluster was a homolog of the azaphilone pigment biosynthesis genes (GenBank accession no. KC148521) existing in the *M. pilosus* genome (Balakrishnan *et al.*, 2013). According to the aforementioned pigment biosynthesis genes, eleven targeted fragments were identified in M9 by colony screening with eleven pairs of primers, namely, *MpPKS5, mppR1, mppA, mppB, mppC, mppD, mppE, mppR2, MpFasA2, MpFasB2,* and *mppF*.

As shown in Fig. 5, the expression levels of *MpPKS5, mppR1, mppA, mppB, mppC, mppD, MpFasA2, MpFasB2,* and *mppF* under different illumination levels was in the sequence 200 45(1) > 100 30(2) > D > 100 15(2) > 100 15(1) > 100 30(1), which was contrary to that of *mppE* and *mppR2*. In combination with Fig. 4, we found that the expression levels of *MpPKS5, mppR1, mppA, mppB, mppC, mppD, MpFasA2, MpFasB2*, and *mppF* were positively correlated with the production of orange pigments RUN and MON; and negatively correlated with red pigments RUM and MOM and yellow pigments MS and AK. The expression levels of *mppE* and *mppR2* coincided with those of the red and yellow pigment yields but were contrary with the yields of orange pigments.

Discussion

The effects of light on the production of MPs have been reported in the past few years. Miyake *et al.* (2005) reported that red light enhanced pigment production but blue light inhibited it. Babitha *et al.* (2008) found the red light only slightly affected pigment production, whereas green and blue wavelengths significantly inhibited the pigment production. Although these studies have discussed the effects of light on MPs production, minimal attention has been paid to the influences of blue light on the three kinds of MPs, red, orange and yellow pigments, respectively. In fact MPs is a mixture of azaphilones mainly composed of red, orange and yellow pigments. The different colors show different absorption wavelengths of light, so the effect of blue light on each pigment should be considered, respectively. This study is the first report about the influences of blue light on the red, orange, and yellow pigments, respectively.

In this study we found that constant exposure to blue light reduced red (RUM and MOM), orange (RUN and MON), and yellow pigment (MS and AK) production compared with dark. However, varying blue light levels caused different changes of production for red, orange, and yellow ones.

The red, yellow, and orange pigments have very similar chemical structures. Several investigations showed that the oxygen atom of the orange pigments RUN and MON was replaced by a NH group to form the red pigments RUM and MOM (Carels and Shepherd, 1977). RUN and MON can also be oxidized with hydrogen peroxide to give the yellow pigments MS and AK (Yongsmith *et al.*, 1993). Carels and Shepherd (1977) proposed that only orange pigment components were biosynthetic whereas the others were transformed from them through chemical transformations. Thus, it is existed that the interconversion between orange (RUN and MON), red (RUM and MOM), and yellow pigments (MS and AK). In this study, the production of red and yellow pigments was enhanced/reduced together with the decrease/increase of orange pigments under different blue light conditions. A biosynthetic transformation may exist between orange pigments and red/yellow pigments through the action of blue light.

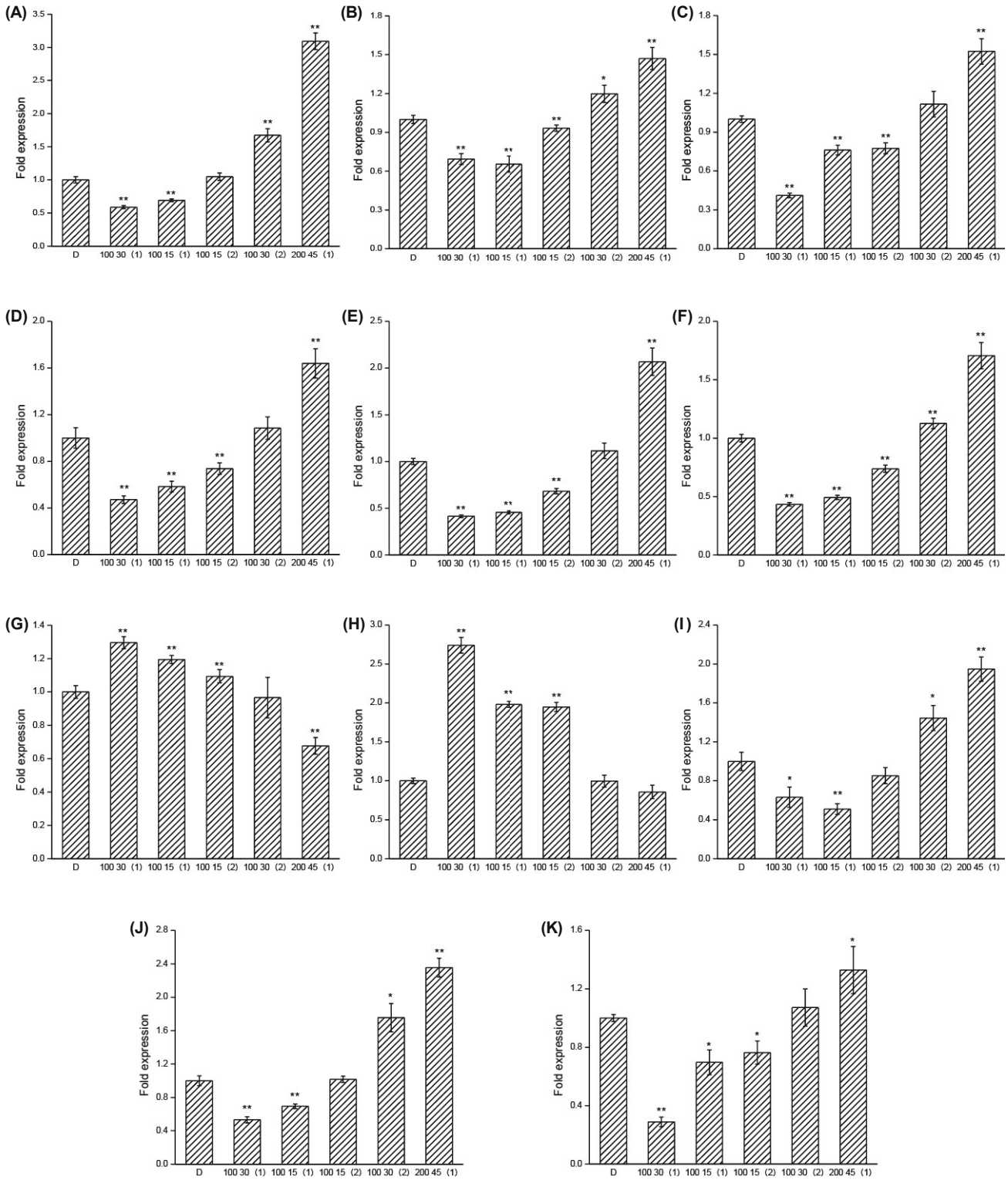

Fig. 5. Expression of pigment biosynthetic genes (A) *MpPKS5*, (B) *mppR1*, (C) *mppA*, (D) *mppB*, (E) *mppC*, (F) *mppD*, (G) *mppE*, (H) *mppR2*, (I) *MpFasA2*, (J) *MpFasB2*, and (K) *mppF* in the darkness and under blue light illumination conditions of 100 15 (1), 100 15 (2), 100 30 (1), 100 30 (2), and 100 45 (1). Gene expression test samples exhibited a one-to-one correspondence with samples used for MP content tests under different blue light conditions. The transcriptional levels were normalized to those of the *actin* gene. The mRNA levels in the darkness were used as the reference value. Data were expressed as the relative mRNA level for each gene and represented as the mean ± SD (n=3). * $P < 0.05$, ** $P < 0.01$ compared with mRNA ratio in the dark.

Moreover, the expression levels of pigment biosynthetic genes under the same blue light conditions were also discussed. Balakrishnan *et al.* (2013) identified the azaphilone pigment biosynthetic gene cluster in the *M. pilosus* genome and proposed a pigment biosynthetic pathway, which indicates that *MpPKS5*, *mppD,* and *mppF* are responsible for the formation of a pyranoquinone structure. *MpFasA2/B2* generates short chain fatty acyl-CoA, whereas *mppB* transfers the acyl group on the pyranoquinone structure for the biosynthesis of orange pigments. Simply put, *MpPKS5, MpFasA2/B2*, and *mppB/D/F* are responsible for the biosynthesis of orange pigments. In this study, the production of orange pigments RUN and MON increased along with the upregulation of *MpPKS5*, *MpFasA2/B2*, and *mppA/B/C/D/F* expression levels, confirming the rationality of the proposed biosynthetic pathway.

However, the upregulation of *mppE* occurred with the increase of yellow pigments (MS and AK) and the decrease of orange pigments. According to Balakrishnan *et al.* (2013), *mppE* is a putative oxidoreductase encoding gene. Moreover, orange pigments can also be oxidized to yellow pigments, so we supposed that *mppE* plays an important role in the transformation of orange and yellow pigments. Thus, *mppE* gene expression was positively correlated to yellow pigment production and negatively correlated to orange pigment production. The expression levels of pigment biosynthetic genes under different blue light conditions further proved that the effect of blue light on the production of red, orange, and yellow pigments was a biosynthetic process.

Acknowledgements

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