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ORIGINAL RESEARCH PAPER

Real-time quantitative analysis of the influence of blue light on citrinin biosynthetic gene cluster expression in *Monascus*

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Abstract When *Monascus* MX was grown under blue light instead of in the dark, citrinin production increased from 478 mg l^{-1} to 698 mg l^{-1}. To explain this, the expression of the *pksCT* gene, which encodes citrinin polyketide synthase, and of 5 ORFs around it, were monitored. Blue light enhanced citrinin production by upregulating the expression of *orf1*, *orf3,* and *orf4*, indicating that *pksCT* was not the key gene responsible for the quantity of citrinin production in blue light.

Keywords Blue light · Citrinin · Monascus · RT-qPCR

Introduction

The effects of light on the metabolic pathways of carbohydrate, fatty acid, polysaccharide, and secondary metabolites in fungi are diverse (Tisch and Schmoll 2010). *Neurospora crassa* is used as a paradigm for genetic studies of blue light perception and signal transduction. About 60–80 genes are regulated by blue light in *Neurospora crassa* (Nawrath and Russo 1990). Besides *Neurospora crassa*, the *DBB1a* gene in *Arabidopsis*, the *SigB* gene in *Bacillus subtilis*, and the cellulase genes in *Trichoderma reesei* can be stimulated by blue light (Wang et al. 2011; Ondrusch and Kreft 2011; Castellanos et al. 2010).

Citrinin is a mycotoxin that has nephrotoxic activity in mammals and is produced by *Aspergillus*, *Penicillium,* and *Monascus* species (Malmstrøm et al. 2000; Blanc et al. 1995). *Monascus* is a traditional fungus often found in food fermentation in Asian countries, but the existence of citrinin poses a serious safety problem in *Monascus*-fermented food. Previous studies have shown that blue light can influence the processes of mycelium, spore formation, and the production of secondary metabolites in *Monascus*; for example, citrinin production by *Monascus* decreased in blue light culture conditions (Babitha et al. 2008; Miyake et al. 2005). However, citrinin biosynthesis in *Penicillium verrucosum* and *P. expansum* was found to be enhanced by blue light (Schmidt-Heydt et al. 2011).

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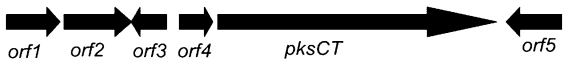

Fig. 1 Map of the citrinin biosynthetic gene cluster. *Arrows* show the genes and their direction of transcription. The gene *pksCT* (7,838 bp) is the first-characterized gene, encoding citrinin polyketide synthase, which is responsible for citrinin biosynthesis. Four ORFs: *orf1* (1,506 bp), *ctnA* (1,731 bp), *orf3* (990 bp), and *orf4* (942 bp), encoding a homolog of a dehydrogenase, a regulator, an oxygenase, an oxidoreductase, respectively, are present in the 5′-flanking region, and 1 ORF: *orf5* (1,566 bp), encoding a transporter, is present in the 3′-flanking region (Shimizu et al. 2005, 2007)

The citrinin biosynthetic gene cluster has been identified (Fig. 1). To understand the mechanism of blue light on citrinin biosynthesis in *Monascus*, we analyzed the gene expression patterns of the citrinin biosynthetic gene cluster together with changes in citrinin yield.

Materials and methods

Fungal strain and culture conditions

Monascus strain MX, a high producer of citrinin, was maintained on potato/dextrose/agar (PDA) for 5 days at 30 °C. Spores were harvested with 3 ml sterile water and inoculated into 75 ml seed medium (30 g rice powder l^{-1}, 2.5 g KH_2PO_4 l^{-1}, 3 g $NaNO_3$ l^{-1}, and 1 g $MgSO_4 \cdot 7H_2O$ l^{-1}; the initial pH of the medium was adjusted to 4.5 with lactic acid.) in 200 ml flasks. The cultures were incubated at 30 °C for 30 h with shaking at 180 rpm. For citrinin production and gene expression testing, 5 ml (10 % v/v) of the seed fermentation broth was inoculated into 50 ml YES medium (40 g yeast extract l^{-1} and 160 g sucrose l^{-1}) in a 250 ml flask, and incubated under static conditions at 30 °C (Wang et al. 2009; Davis et al. 1975).

Analysis of citrinin production

Citrinin production was determined by HPLC on a C_{18} column at 25 °C (5 μm, 250 × 4.6 mm) after filtration of the fermentation broth through a 0.45 μm filter. The mobile phase was acetonitrile/water/methanol (7:2:1, v/v) with the pH adjusted to 2.68 with H_3PO_4, running at 1 ml min^{-1}. The eluate was monitored by fluorescence; the excitation and emission wavelengths were 331 and 500 nm, respectively. Citrinin from Sigma was used as the standard.

Real-time quantitative PCR analysis (RT-qPCR)

Mycelia were harvested from the citrinin production medium and stored in liquid N_2 for RNA extraction. Total RNA was extracted from mycelia using the RNeasy Plant Mini-Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio), with the Oligo dT-Adaptor Primer. Gene expression was monitored by RT-qPCR, carried out using the SYBR Green PCR master mix (Invitrogen). Primers for *orf1*, *ctnA*, *orf3*, *pksCT*, *orf4*, *orf5* (DDBJ accession no. AB243687) and actin gene (Gen Bank accession no. AJ417880) were designed (Supplementary Table 1). RT-qPCR was performed using the ABI step one plus system (applied biosystems) followed by melting curve analysis with the following cycling program: initial activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min.

Results and discussion

Effects of blue light on citrinin production

Figure 2 shows citrinin accumulation by *Monascus* MX grown in the dark and in blue light. Under blue light, maximal production of citrinin (698 mg l^{-1}) was seen on the 6th day. In the dark, maximal citrinin production (478 mg l^{-1}) was seen on the 5th day. No significant effect of blue light on the biomass of *Monascus* MX was detected.

Effects of blue light on expression of citrinin biosynthetic genes

Figure 3 shows the expression profiles of citrinin biosynthetic genes in the dark and in blue light. The expression levels of *orf1*, *ctnA*, *orf3*, *orf4*, *orf5* and *pksCT* were positively correlated with citrinin accumulation when cultured in the dark. On the 5th day, gene expression as well as citrinin production reached their highest points.

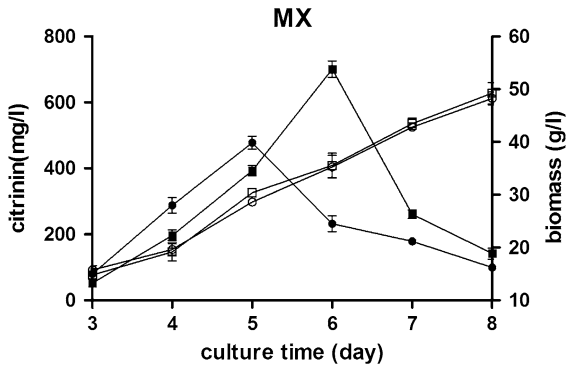

Fig. 2 Effect of blue light on citrinin production and biomass. Citrinin content of *Monascus* MX in YES broth grown in the dark (*filled circle*) or blue light (*open circle*), assessed by HPLC. Samples were taken every 24 h from the 3rd to 8th day. In the meantime, 500 mg (wet weight) of mycelium was weighed for RNA extraction, and the rest was used for determining the biomass. Biomass in the dark (*filled square*) and in blue light (*open square*) was estimated by determining the dry weight of the mycelium. Blue light was generated by a blue-LED box, which provided stable and uniform pure blue light (470 nm). The light intensity was 130 Lux. Values are the average of three independent experiments. *Error bars* represent the standard deviation ($n = 3$)

In blue light, the expression of *orf1*, *orf3,* and *orf4* were significantly up-regulated. Unlike the other genes, when *Monascus* was cultured for 4, 5, and 6 days in blue light, the expression of *pksCT* decreased by 80, 68, and 44 %, respectively, compared to its expression in the dark.

The expression levels of *orf1*, *orf3*, *orf4*, which were predicted to participate in citrinin biosynthesis as structural genes, were highest on the 6th day. This trend is same as that of citrinin production in blue light, indicating that blue light stimulates citrinin production via the up-regulation of the expression levels of these three genes. The expression levels of *pksCT* and *ctnA* were highest on the 5th day in blue light. This trend does not mirror that of citrinin production in blue light. Although *pksCT* is the key gene encoding citrinin polyketide synthase Shimizu et al. (2005), the data shown in this study indicate that *pksCT* is not the key gene responsible for the quantity of citrinin production in blue light.

Contrary to the results of the present study, production of citrinin in *Monascus* was previously

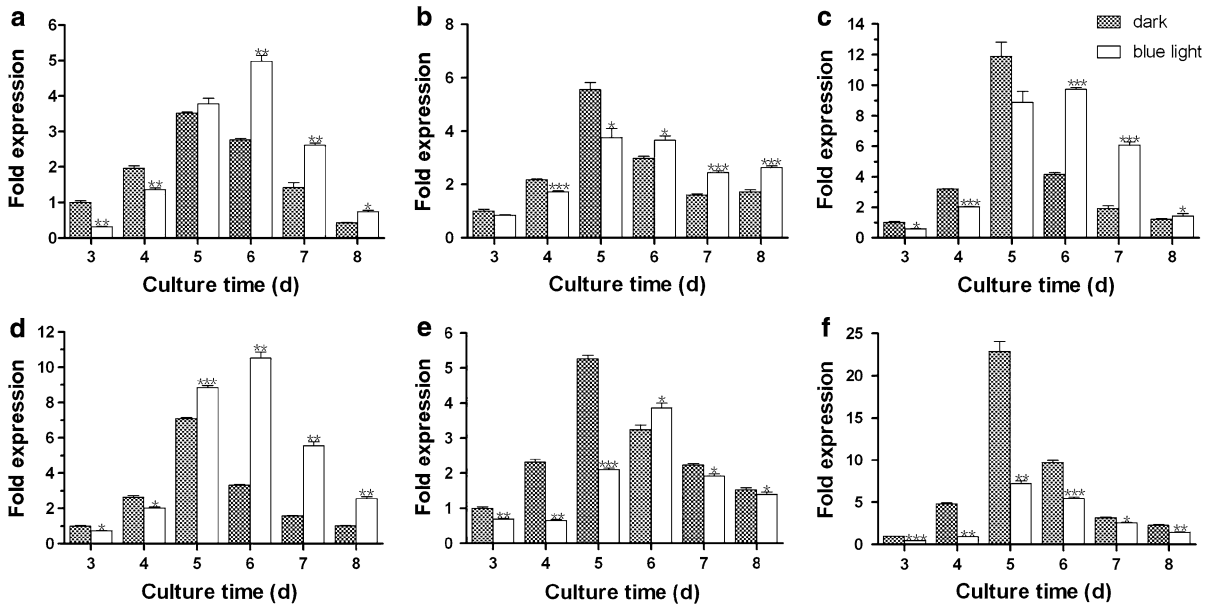

Fig. 3 Expression of *orf1* (**a**), *ctnA* (**b**), *orf3* (**c**), *orf4* (**d**), *orf5* (**e**) and *pksCT* (**f**) during fermentation, monitored by RT-qPCR. Gene expression test samples corresponded one-to-one with the samples used for citrinin testing. Transcriptional levels were normalized to that of the actin gene. In order to standardize the results, we took the mRNA levels accumulated on the 3rd day in the dark as the reference value (value 1). Data are expressed as the relative mRNA level for each gene and represent the average values from three separate experiments. *Error bars* represent the standard deviation ($n = 3$). $^{***}P < 0.001$, $^{*}P < 0.05$ compared with mRNA ratio in dark

found to be negatively influenced by blue light (Wang et al. 2009; Miyake et al. 2005). This discrepancy may be due to the diversity of citrinin biosynthetic genes in different strains or species. In species that produce little or no citrinin, some citrinin biosynthetic functional genes are either absent or not transcribed (Chen et al. 2008; Shimizu et al. 2007). On the other hand, citrinin can be directly degraded by blue light (Wang et al. 2009). The highest citrinin yield of *Monascus* MX seen in this study was much higher than that seen with *Monascus ruber* N and IFO4478 (Wang et al. 2009; Miyake et al. 2005). The degradation effect of blue light on citrinin may be responsible for the negative influence of blue light on citrinin accumulation in *Monascus ruber* N (a low citrinin-producing strain). On the other hand, the up-regulation of some citrinin biosynthetic genes (*orf1*, *orf3*, *orf4*) may be the major reason for the positive effect of blue light on citrinin production in *Monascus* MX (a high citrinin-producing strain). However, further comprehensive systematic analyses of the effect of light on the genus *Monascus* are needed.

Conclusion

Compared with cultivation in the dark, blue light has a transient enhancing effect on citrinin production in *Monascus* MX. Citrinin accumulation is positively correlated with the expression of the citrinin biosynthetic gene cluster in the dark, but not in blue light. Blue light can enhance citrinin production by upregulating the expression of *orf1*, *orf3,* and *orf4*.

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References

Babitha S, Carvahlo JC, Soccol CR et al (2008) Effect of light on growth, pigment production and culture morphology of *Monascus purpureus* in solid-state fermentation. World J Microbiol Biotechnol 24:2671–2675

Blanc PJ, Laussac JP, Le Bars J et al (1995) Characterization of monascidin A from *Monascus* as citrinin. Int J Food Microbiol 27:201–213

Castellanos F, Schmoll M, Martínez P et al (2010) Crucial factors of the light perception machinery and their impact on growth and cellulase gene transcription in *Trichoderma reesei*. Fungal Genet Biol 47:468–476

Chen YP, Tseng CP, Chien IL et al (2008) Exploring the distribution of citrinin biosynthesis related genes among *Monascus* species. J Agric Food Chem 56:11767–11772

Davis ND, Dalby DK, Diener UL et al (1975) Medium-Scale production of citrinin by *Penicillium citrinum* in a semi-synthetic medium. Appl Microbiol 29:118–120

Malmstrøm J, Christophersen C, Frisvad JC (2000) Secondary metabolites characteristic of *Penicillium citrinum*, *Penicillium steckii* and related species. Phytochemistry 54: 301–309

Miyake T, Mori A, Kii T et al (2005) Light effects on cell development and secondary metabolism in *Monascus*. J Ind Microbiol Biotechnol 32:103–108

Nawrath C, Russo VE (1990) Fast induction of translatable mRNA by blue light in *Neurospora crassa* wt: the wc-1 and wc-2 mutants are blind. J Photochem Photobiol B 4:261–271

Ondrusch N, Kreft J (2011) Blue and red light modulates SigB-dependent gene transcription, swimming motility and invasiveness in *Listeria monocytogenes*. PLoS ONE 6:e16151

Schmidt-Heydt M, Rüfer C, Raupp F et al (2011) Influence of light on food relevant fungi with emphasis on ochratoxin producing species. Int J Food Microbiol 145:229–237

Shimizu T, Kinoshita H, Ishihara S et al (2005) Polyketide synthase gene responsible for citrinin biosynthesis in *Monascus purpureus*. Appl Environ Microbiol 71:3453–3457

Shimizu T, Kinoshita H, Nihira T (2007) Identification and in vivo functional analysis by gene disruption of *ctnA*, an activator gene involved in citrinin biosynthesis in *Monascus purpureus*. Appl Environ Microbiol 73:5097–5103

Tisch D, Schmoll M (2010) Light regulation of metabolic pathways in fungi. Appl Microbiol Biotechnol 85:1259–1277

Wang CL, Fu ZL, Chen MH et al. (2009) Blue light effects on pigment and citrinin production in *Monascus*. 3rd International conference bioinformatics and biomedical engineering, ICBBE, Beijing, 11–16 June 2009, pp 1–4

Wang Q, Zeng J, Deng K et al (2011) *DBB1a*, involved in gibberellin homeostasis, functions as a negative regulator of blue light-mediated hypocotyl elongation in *Arabidopsis*. Planta 233:13–23