JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3493-3499

Impact of Blue Light from LED on Astaxanthin Accumulation and Transcription of Key Carotenogenetics in *Haematococcus pluvialis*

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(Received: 15 June 2014; accepted: 29 July 2014)

Haematococcus pluvialis **can produce a high yield of astaxanthin under stresses, but this induced-biosynthesis is a relatively slow process and low productivity. As blue light can quickly promote astaxanthin accumulation in *H. pluvialis,* this study further investigated that the impact of blue light illumination from LED (3500 µmol photons $m^{-2}sec^{-1}$) on astaxanthin content, mRNA expression and regulation profiles of eight astaxanthin-synthesis-related carotenogentic genes in *H. pluvialis*. The results showed that (1) the astaxanthin content was enhanced to 3.03 mg/L in 18 days after blue light induction; (2) the expression levels of carotenoid genes *ipi-1*, *ipi-2*, *psy*, *pds*, *lyc*, *crt*R-b, *bkt2* and *crt*O were up-regulated in different time points during the cultivation; (3) the transcriptional expression of *crt*R-b, *bkt2* and *crt*O was more efficient than other five genes under blue light induction. It was indicated that genes of *crt*R-b, *bkt2* and *crt*O might be vital to astaxanthin production in blue light treatment for *H. pluvialis*. This study suggested that blue-light illumination in *H. pluvialis* is an effective stimulator to induce astaxanthin production in *H. pluvialis.***

Key words: *Haematococcus pluvialis*; Astaxanthin; Blue light; APE; Carotenogenic genes; Transcriptional expression.

Due to a high level of astaxanthin yield which has been used widely in aquaculture, poultry industry and pharmaceutical applications as a feed additive for the pigmentation of cultured salmon, egg yolk and/or an effective antioxidant bioactivator, photosynthetic microalga *Haematococcus pluvialis* has been attracting more and more attention[1]. As the astaxathin production is happened along with the *H. pluvialis* cell transformation from green flagellates to un-motile red cysts, the two-stage cultivation process is widely used for its large-scaled cultivation using the stress induction for astaxanthin accumulation in the second stage.

At present, high light radiation and /or nutrient depletion are major approaches to stimulate astaxanthin biosynthesis in *H. pluvialis* as in a photoautotrophic culture mode[2]. However, this induction process for astaxanthin accumulation is not very efficient mainly because sun-light is not constant and continuous strength[3]. There are also several other induction methods, such as manipulating nutrient concentration, pH control and applying artificial illumination on *H. pluvialis.* LEDs emitting light of short wavelengths (380–470 nm) were found to induce astaxanthin

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accumulation of up to 5–6% per dry cell[1, 4]. Illumination with blue light emitting diode lamps and nutrient starvation induced accumulation of astaxanthin, and the interactive effects of these two increased the astaxanthin concentration to 76 μg.cm^{-3} [4]. More importantly, aligned with the fed-batch addition method, the cell density also can increase simultaneously under the blue light. Using the fed-batch addition method, the cell concentration increased above 1 mg-dry cell/cm^{-3}, and under illumination with blue LEDs, the astaxanthin concentration reached approximately 70 μg/cm^{-3} which was more efficient than ordinary methods[5]. Therefore, LED blue light has been considered as a proper illumination source and effective astaxanthin-induced approach for a fast conversion from vegetative growth (green fagellate) to inductive growth (red cyst) produced by blue light from LEDs in *Haematococcus* cultivation and would be an alternative solution to improve astaxanthin production under fad batch or photobioreactor[3].

LED light is an economical light source for large-scale indoor industrial farming[6]. It was reported that 11μmol photon m-2 sec-1 of blue LEDs (380–470 nm) could achieve a high level (5-6 % of dry-cell weight) of astaxanthin yield in *H. pluvialis*[1, 7]. Moreover, they reported the effects of flashing blue light emitting diodes (LEDs could also increase the cells productivity and it therefore has been highly recommended for indoor algal cultivation, in particular of algal photobioreactors[7]. The cultivation of *H. pluvialis* was established as a two-stage process and the productiveness of astaxanthin were shown to be maximum when the cells were illuminated by blue light LEDs[8]. This method was much simpler to operate than the medium replacement method in operation and enabled us to attain a higher total yield of astaxanthin[7].

Despite of many literatures reporting the effectiveness of blue light on *H. pluvialis* growth and/or astaxanthin accumulation[3], the impact of blue light on biosynthesis molecular mechanism of astaxanthin has not been investigated in detail. In present study, we used the blue light LEDs to illuminate the *H. pluvialis* cultures, and then monitored the correlations between the transcription of its key carotenogenetic genes (*ipi-1*, *ipi-2*, *psy*, *pds*, *lyc*, *crt*R-b, *bkt2* and *crt*O) and astaxanthin accumulation to target rate-limiting genes in the carotenoid biosynthesis pathway.

MATERIALS AND METHODS

Cell culture and blue-light induction

Similar to our previous studies[9, 10], the strain 712 of *H. pluvialis* was used for this study. These algal cells at logarithmic growth were cultured in 500mL of beakers in Bold's Basal Medium (BBM) with a constant aeration via filtering (0.22 ¼m). The cultures were illuminated from one side at the light intensities of 660lx with light cycle of 12h: 12h light/dark from blue light LEDs based on the method of Rodríguez-Romero and Corrochano[11]). For the controls, similar triplicated cultures and the same light density were illuminated by the normal light from fluorescent lamps (referred to as fluorescents treatments in the following text) parallel to the test of blue light radiation. The cultivation temperature was maintained at 22±1°C during the test. After illumination treatment 18 days, all the samples were collected at 0h, 1h, 2h, 6h and on day 1, 2, 3, 6, 9, 12, 15, and 18. The algal solutions were centrifuged (30 mL algal solution for RNA extraction and 30 mL for astaxanthin measurement every time**)**, then frozen in liquid nitrogen and stored at 80°C for subsequent analyses.

Astaxanthin measurment and gene expression analysis

The cell morphology and color observation was conducted using Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Measurements of astaxanthin content and gene transcription expression were performed following the previously described protocols [9, 10]. Real-time RT-PCR analysis was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA) using actin gene as the internal control[12]. All exposure experiments were repeated three times independently, and data were recorded as the mean with standard deviation (SD).

RESULTS

Color change and astaxanthin accumulation in *H. pluvialis*

Under the illumination of blue light LEDs, the culture color change from green to red was observed on day 3 and became distinguished from

the control green color after day 6 (Figure 1). On day 18, the algal cells with blue light illumination became red entirely and none dead cells were observed in the cultures. In contrast, there was no much color change in the controls under the normal light illumination (Figure 1). Similar to the microscopy observation, results from astaxanthin measurements also showed that the increase of astaxanthin in the blue-light treatment started on day 3 and gradually built up afterwards (Figure 2). In 18 days, the astaxanthin content in the blue-light treatment reached 3.03 mg/L^{-1} vs 1.65 mg/L in the control. This was in line with previous results, which indicated that blue light illumination could

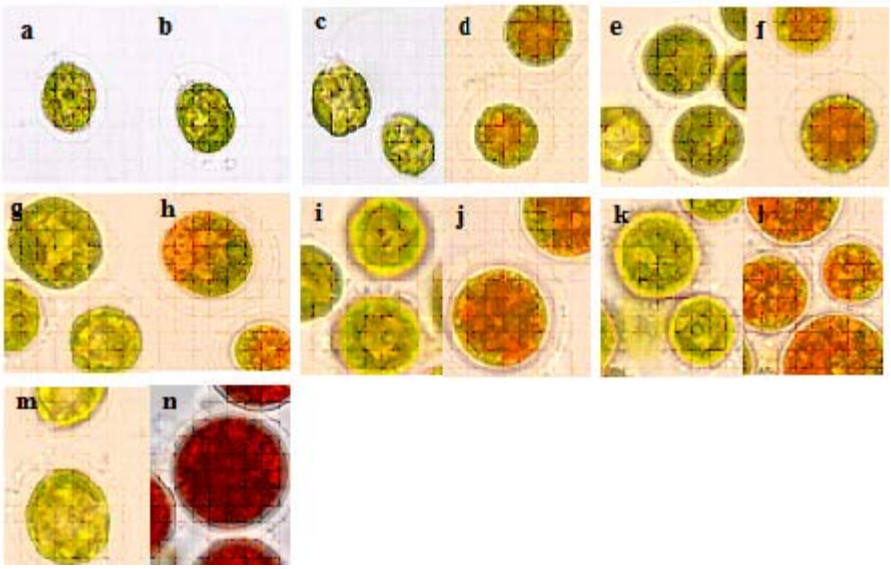

Fig. 1. Microscopic images (400×) of *H. pluvialis* cells culture samples day 1, 3, 6, 9, 12, 15, 18. a, c, e, g, i, k, m represent the controls illuminated by ordinary light from fluorescent lamp, b, d, f, h, j, l, n represent samples illuminated by blue light, respectively

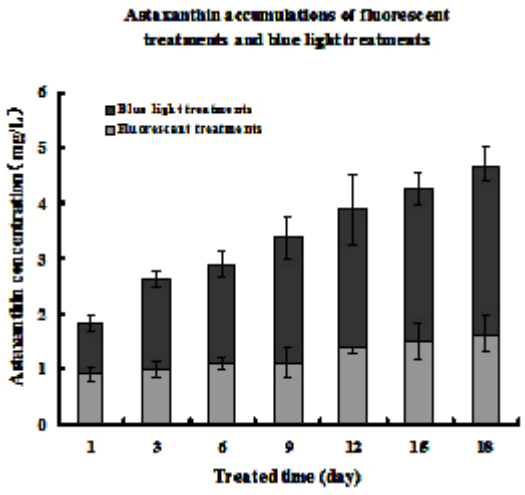

Fig. 2. Astaxanthin accumulations of controls and blue light illumilated samples during cultivation. OD_{490} represents relative astaxanthin content in alga culture solution.

stimulate *H. pluvialis* to accumulate astaxanthin[1, 4, 5, 7, 20].

Transcription profile of carotenogenic genes

The mRNA levels of eight genes (*ipi-1*, *ipi-2*, *psy*, *pds*, *lyc*, *crt*R-b, *bkt2* and *crt*O) were significantly up-regulated with different patterns over 18 days (Figure 3). Under blue light induction, mRNA levels of *ipi*-1, *ipi*-2, *psy* and *pds* initially increased significantly than fluorescents treatments on days 9, 3, 3 and 2, respectively. But *crt*R-b, *bkt2* and *crt*O initially increased significantly than fluorescents treatments at 1st hour, and *lyc* on 6h. Maximum transcriptional levels of *ipi-1* and *ipi*-2 were also found on day 9 and 3, increasing 2.0- and 1.5-fold compared to the fluorescents treatments (*ipi-1* and *ipi*-2 of blue light treatments displayed relative transcriptional expression with 3.2 and 1.7, respectively, Figure 3a, 3b). The mRNA expression of *psy* increased sharply with 2.0-fold of controls on day 3 and

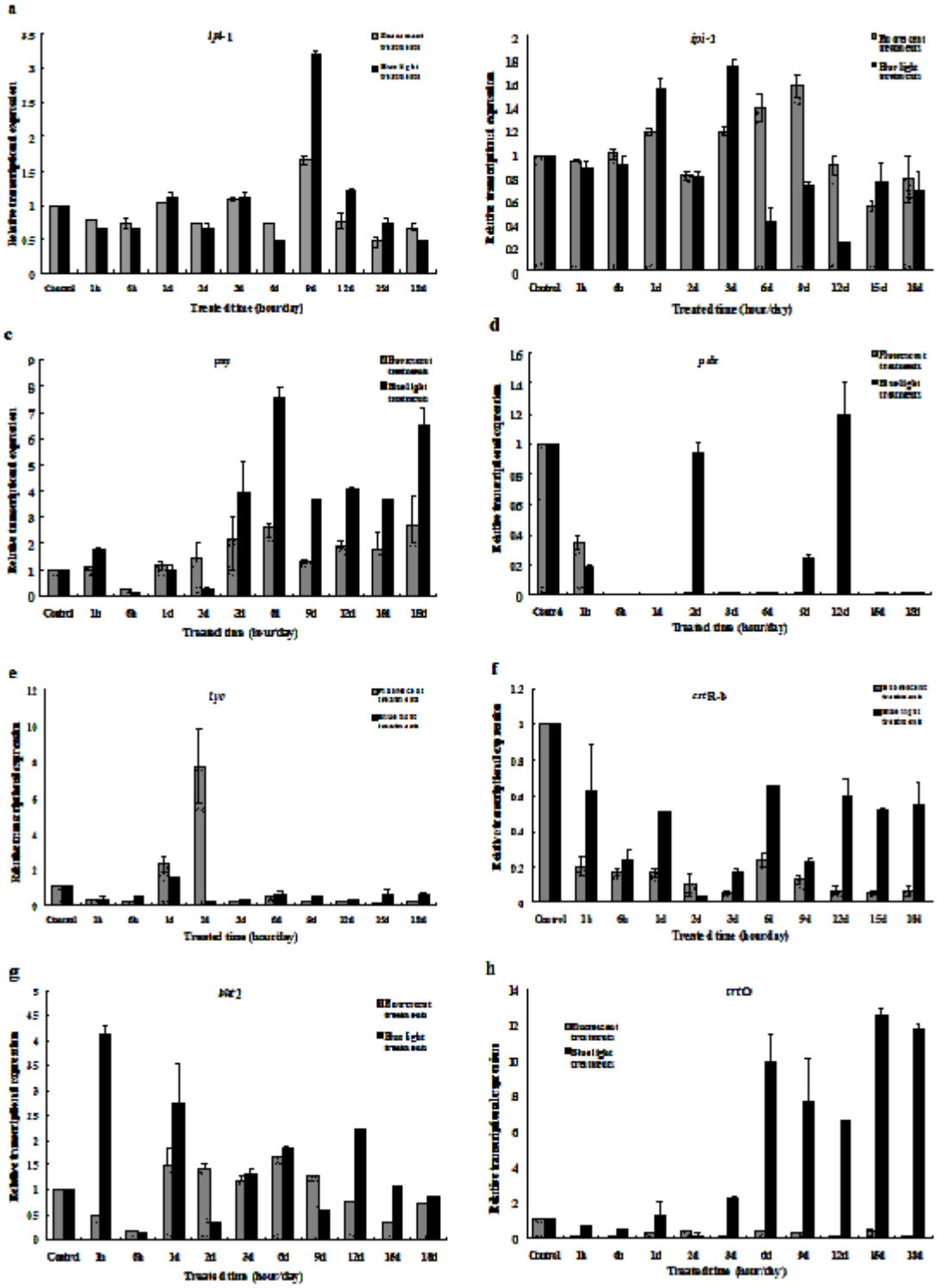

Fig. 3. The effects of ET on the transcript levels expression kinetics of eight carotenogenic genes in *H. pluvialis* during incubation. a, b, c, d, e, f, g and h represent transcript levels expression kinetics of *ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-b, *bkt* and *crt*O respectively

reached the highest level of 2.9-fold of fluorescents treatments on day 6 (*psy* of blue light treatments displayed relative transcriptional expression with 7.5, Figure 3c), then gradually decreased until day 18. The transcription of *pds* was up to 57.0-fold of fluorescents treatments on day 2 but declined on day 3. However, it rose again on day 9 and became maximum of 170.0-fold of fluorescents treatments on day 12 (*pds* of blue light treatments displayed relative transcriptional expression with 1.2, Figure 3d). Based on the initial increase, *lyc* transcriptional level increased 2.2-fold of fluorescents treatments on 6th hour and become the highest level of 7.6-fold of controls on day 15 (*lyc* of blue light and fluorescent treatments displayed highest relative transcriptional expression with 1.5 and 7.7 on day 2 and 1, respectively, Figure 3e). The initial mRNA expression increase of *crtR*-b, *bkt*2 and *crt*O were observed at the 1st hour with 3.1-, 8.7- and 4.4-fold of fluorescents treatments, while they showed the highest level at 1st hour, 1st hour and on day 15 with 3.1-, 8.7- and 41.0-fold of fluorescents treatments (*crtR*-b, *bkt*2 and *crt*O of blue light treatments displayed relative transcriptional expression with 0.6, 4.1 and 12.5, respectively, Figure 3f, 3g, 3h).

DISSCUSION

During extreme environmental conditions, such as nutrient limitation, increased light intensity or high salt concentrations, *H. pluvialis* form red cysts by increasing lipids biosynthesis and carotenogenesis, particularly accumulation of orange-red pigment astaxanthin esters as survival strategies[8].

A *cis* element, APE sequence (al proximal element, consensus sequence GAANNTTGCC), involved in blue light regulation of *al-3* gene expression, was shown to be essential for the promoter of other genes in *N.crassa*[13], *carB* genes in *Phycomyces blakesleeanus*[14], *carRA* and *carB* and *carG* in Mucor circinelloides[15-17]. In our previous studies, results of the site-directed mutagenesis of a C-repeat/DRE and two APE-like motifs in a promoter-like region (–630/–308) suggested that two APE-like motifs might be essential for transcriptional regulation of the *bkt2* gene[18, 19]. All the fact listed suggested blue light was likely to be a potential regulator of genes related to astaxanthin biosynthsis in *H. Pluvialis*. Therefore, similar kinetics to those described for the structural genes involved in carotenogenesis might lie in astaxanthin synthesis of *H. Pluvialis*. In this paper, we want to target eight key genes responsible for high astaxanthin accumulation and evaluate the correlations between gene expression and astaxanthin profile.

LEDs have the advantage of lower energy consumption and higher duration to a high frequency of on-off switching[20]. Similar to other studies [1, 4, 5, 7, 20], we also found that the blue light illumination could stimulate *H. pluvialis* producing more astaxanthin. Even in the green stage of *H. pluvialis*, 80 ¼mol photons $m^{-2}sec^{-1}$ of blue light illumination also could induce a gradual increase of carotenoids and up to 56.0-fold in 23 days compared to day 0 of growth in *H. pluvialis*[8]. However, their data suggest LED lights are not effective for the production of carotenoids during red-phase of *H. pluvialis*, which contradicted the previous finding by Lababpour et al who reported that blue LEDs enhance astaxanthin production[4]. Nevertheless, in our paper, *H. pluvialis* accumulated astaxanthin up to 3.0 mg/L (with 1.8-fold control's production, Figure 2) in 18 days after illumination of blue light LEDs.

In previous literatures, numerous genes have been cloned that show an increase in mRNA steady state levels in response to blue light due to transcriptional regulation instead of post-transcriptional control mechanisms[21]. The up-regulation of carotenogenetic genes by blue light was also reported in fungus *Phycomyces* for ²-carotene induction[14]. Based on the mode of Mucoral fungus *Phycomyces*, it is believed that the putative control motifs (also named APE) are essential for the blue-light induction on biosynthesis of carotene[22]. Upon blue light illumination, concomitant with astaxanthin accumulation increasing on day 3, the expression levels of 8 carotenoid genes *ipi-1*, *ipi-2*, *psy*, *pds*, *lyc*, *crt*R-b, *bkt2* and *crt*O were up-regulated but with different transcriptional profiles in *H. pluvialis* (Figure 3). Firstly, the induction of blue light caused delayed up-regulation of *ipi-1*, *ipi-2*, *psy* and *crt*R-b since their mRNA levels initially increased on days 9, 3 and 3, whereas *pds*, *lyc*, *crt*R-b, *bkt2* and *crt*O expression were early up-regulated on day 2, at 6th hour, 1st hour, 1st hour and 1st hour (Figure 3a, 3b, 3d, 3f). Secondly, the initial increase and

maximal expression level of *pds* of blue light treatments occurred on day 2 and 12 with 57.0- and 170.0-fold of fluorescents treatments, which was superior to other 7 genes (Figure 3d). Thirdly, the quick astaxanthin accumulation observed in *H. pluvialis* cells from day 3 seems to be correlated with fast increasing expressions of *crt*R-b, *bkt*2 and *crt*O (*crtR*-b, *bkt*2 and *crt*O of blue light treatments displayed 3.1-, 8.7- and 4.4-fold relative transcriptional expression of fluorescent treatments, respectively, Figure 3f, 3g, 3h) on 1st hour of blue light irradiation. Therefore, *crt*R-b, *bkt*2 and *crt*O seem to be more efficient at transcriptional expression among eight astaxanthin biosynthetic related genes in *H. pluvialis*. Further, it is worth noting that *bkt*2 expression was early up-regulated on 1st hour with 8.7-fold (Figure 3g), which was correlated with the transcriptional regulation from two APE sequence at the promoter region of *bkt*2 according to our previous reports[18, 19]. Meanwhile, it has been highlighted that studying the relationship between the regulation of key genes and the APE sequence which is very important for investigating astaxanthin biosynthesis mechanism in *Haematococcus*[18,19].

In this study, we investigated the relationship between astaxanthin concentration and the transcriptional profiles of 8 related genes with blue-light challenge. This study reveals that these candidate genes, *crt*R-b, *bkt*2 and *crt*O, might be vital to for metabolic engineering for more astaxanthin production with high quality and less production cost under blue-light stress. However, these primary results may not be efficient to reveal all transcriptional mechanism in *H. pluvialis* with blue light stress. Therefore, further study is required to distinguish or confirm the presence of APE-like sequence in other genes (except for *bkt2*) involved in astaxanthin synthesis.

ACKNOWLEDGEMENTS

The present study was supported by the National Natural Science Foundation of China (41106124, 31170279), the National Natural Science Foundation of Shandong province (ZR2011DM006, ZR2011CQ010), Hi-Tech Research and Development Program (863) of China (2014AA022003) and the James Cook University Miscellaneous Research Fund 2013 (JCU20732) and the supporting project for young teachers in Shandong University of Technology.

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