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Melatonin enhances astaxanthin accumulation in the green microalga *Haematococcus pluvialis* by mechanisms possibly related to abiotic stress tolerance

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ABSTRACT

Melatonin (*N*-acetyl-5-methoxytryptamine) is widely regarded as an important messenger in higher plants and mammals in their resistance to various biotic and abiotic stresses. However, the role of melatonin in microalgae has been rarely investigated. In this study, melatonin was first used to promote astaxanthin biosynthesis under limited nitrogen and high light conditions. The interactions between melatonin and the secondary messengers, namely, nitric oxide (NO) and salicylic acid (SA), during the stress response were also investigated. Moreover, fatty acid biosynthesis was explored. Finally, the expression levels of astaxanthin biosynthesis genes in *Haematococcus pluvialis* LUGU supplemented with melatonin were simultaneously monitored through quantitative real-time PCR. The astaxanthin content of the microalgae increased 2.36-fold after treatment with 10 μM of melatonin. The maximal astaxanthin content achieved was 31.32 mg g^{-1}. The increased NO and SA production caused by melatonin occurred in parallel with the up-regulation of the expression of astaxanthin biosynthesis genes and the enhancement in astaxanthin biosynthesis in *H. pluvialis* LUGU. The analysis of fatty acid composition showed that melatonin stimulated the production of C16:0 (palmitic acid); thus, the percentage of astaxanthin esters was higher than that observed in the control algae. Furthermore, the inhibitors, namely, carboxy-PTIO, paclobutrazol, and *N*-acetyl-L-cysteine, significantly suppressed the levels of secondary messengers. Meanwhile, the role of melatonin in mediating the rise in astaxanthin content was prevented when the SA- and NO-dependent pathways were inhibited. These findings indicate that melatonin plays a physiological role in direct and indirect responses to abiotic stresses in *H. pluvialis*.

1. Introduction

Astaxanthin (3, 3′-dihydroxy-β, β-carotene-4, 4′-dione), a red ketocarotenoid, has been observed in plants, bacterial, and green algae [1–3]. This compound is widely used in the aquaculture, nutrition and pharmaceutical industries due to its pigmentation, high antioxidant activity, and pharmaceutical and nutraceutical value [4,5].

The unicellular green alga *Haematococcus pluvialis* synthesizes large amounts of astaxanthin under unfavorable environmental conditions [6], considerably more than other astaxanthin-producing microorganisms, such as *Phaffa rhodozyma* and *Chlorella zofingiensis* [7,8]. Thus, a number of studies have investigated the strategies of the enhanced production of astaxanthin in *H. pluvialis*. Recently, a new strategy that uses a combination of stress factors and/or the addition of phytohormones and chemicals was developed in an attempt to overcome the inadequate of conventional approaches and to achieve maximal deliverable astaxanthin content [9,10]. For example, exposure to butylated hydroxyanisole (BHA), an antioxidant, accelerates astaxanthin accumulation in *H. pluvialis* under conditions of high light stress [11]. However, further study is warranted to determine more effective strategies in which the conditions used in microalgae cultivation are customized to improve the astaxanthin content of the cells.

Melatonin, a methoxylated indoleamine that was discovered in bovine pineal fissure [12], is a molecule with numerous and diverse

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functions [13–15]. In plants, melatonin is a potent signaling molecule that has regulatory functions in flowering, circadian rhythm regulation, photosynthesis, free radical scavenging, senescence, developmental processes and multiple biotic and abiotic stress responses [16–20]. Conversely, melatonin has been found to regulate plant innate immunity against bacterial pathogens positively through the salicylic acid (SA) - and nitric oxide (NO)-dependent pathways [17]. Reactive oxygen species (ROS), signaling molecules that are generated in many cellular metabolic processes, plays an important role in regulating astaxanthin accumulation in both *H. pluvialis* and *C. zofingiensis* [21]. Li et al. demonstrated that extraneous melatonin can significantly influences the levels of intracellular ROS in green microalgae [22]. Several studies documented that melatonin improves stress resistance in the microalga *Chlamydomonas reinhardtii* and promotes the accumulation of lipids by *Monoraphidium* [22,23]. However, the effects of melatonin on comprehensive metabolic homeostasis and the mechanisms of these effects in *H. pluvialis* in response to this indole and stress conditions remain unclear.

Previous research has indicated that astaxanthin biosynthetic pathway is catalyzed by several enzymes that are encoded by astaxanthin biosynthesis genes in *H. pluvialis*, and the majority of the involved genes have been cloned [24,25]. Studying carotenoid genes at the transcriptional level thus offers a helpful approach to understanding the mechanism of astaxanthin biosynthesis in *H. pluvialis* under abiotic stress conditions and in the presence of inducers [10,11,26].

In addition, in *H. pluvialis*, astaxanthin has been shown to be stockpiled in triacylglycerol (TAG)-rich lipid bodies (LBs). Chen et al. indicated that astaxanthin esterification may stimulate enzymatic reactions in which astaxanthin is formed from β-carotene by relieving the end-product feedback inhibition of carotenogenesis [27]. It has also been reported that the increase in astaxanthin content that occurs under stress conditions is strongly correlated with fatty acid synthesis [28].

This study was conducted to develop and test a novel induction strategy for astaxanthin accumulation in *H. pluvialis* LUGU involving combined nitrogen limitation, photoinduction and treatment with melatonin. Subsequently, the possible mechanisms involved in astaxanthin biosynthesis and accumulation, including ROS, NO, SA, fatty acid composition and transcription of carotenogenic genes were investigated. These findings provide valuable insight into the mechanism of astaxanthin accumulation in microalgae and may also be helpful in designing novel strategies for the overproduction of specific secondary metabolites by microalgae.

2. Materials and methods

2.1. Algal culture, growth conditions, and melatonin treatment

The *H. pluvialis* strain LUGU (18 s GenBank: KM115647.1) was obtained through filtration from Lake Lugu, a plateau freshwater lake in Yunnan Province, China [10]. The strain was preserved and cultivated in a bubbling column photobioreactor (0.2 m diameter, 0.3 m height, for 3 L) with 2 L of Bold's basal medium (BBM). The initial algal culture was maintained under continuous lumination at 30 $\mu mol\, m^{-2}\, s^{-1}$ intensity and continuous pumping of 0.1 vvm of sterile air at 25 ± 1 °C.

For the melatonin treatment experiments, melatonin was dissolved in dimethylsulfoxide (DMSO). Stock solutions were added to 300 mL of modified BBM containing 2.5 $g\, L^{-1}$ NaNO3 (MBBM) to obtain 5, 10, and 15 μM concentrations (control, added with an equal amount of DMSO). The cells in the late exponential growth phase (approximately 9.0×10^5 cells mL^{-1}) were centrifuged at 3800 ×*g* for 5 min and washed with aseptic water to remove residual nutrients. The pelleted cells were resuspended in MBBM and cultured under stress conditions at a high illumination intensity of 150 $\mu mol\, m^{-2}\, s^{-1}$, which was provided by a white-light fluorescent lamp. The temperature of the cultures was maintained at 28 ± 1 °C, and the initial cell concentration was adjusted to 2×10^5 cells mL^{-1}. Each treatment was conducted in triplicate. The samples were harvested at 1-day intervals.

2.2. Analyses of dry cell weight and astaxanthin content

To measure the dry cell weight (DCW) of the *H. pluvialis* LUGU, 10 mL of the cultured cells was centrifuged at 3800 ×*g* for 5 min; the cell pellet was washed, collected in an Eppendorf micro test tube and dried in a vacuum freeze dryer at −80 °C for 24 h until its weight was constant. To measure the content of astaxanthin in acetone–chloroform (1:1) extracts, an HPLC apparatus with a photodiode array detector (Waters 996, USA) and a reverse-phase C18 column (Waters, 25 cm × 4.6 mm) was used. The elution gradient consisted of eluent A (acetone) and eluent B (methanol: water, 9:1, v/v) as follows: phase B from 80% to 20% for 25 min, phase B at 20% for 10 min, and phase B at 20% to 80% for 15 min. The flow rate was 1.25 $mL\, min^{-1}$. The detection wavelengths used for integration were 445 and 476 nm. Free astaxanthin and astaxanthin esters (Sigma) were used as standards to calculate the proportion of each of these compounds in the samples. Optical observation of the morphology, color, and pigment accumulation in *H. pluvialis* was performed with an Olympus FV1000 confocal laser scanning Eclipse microscope.

2.3. Determination of chlorophyll, protein and carbohydrate content

To estimate cellular physiology based on the chlorophyll, protein and carbohydrate content of the cells, 5 mL of cell culture was harvested by centrifugation during the induction phase, and the cell pellet was washed twice with distilled water. The samples were subsequently pulverized using a mortar and pestle. Next, the algal cells were extracted repeatedly with 90% acetone until the pellet became colorless. The absorbance of the pooled extract was read at 663 and 645 nm, and the chlorophyll content was calculated [29]. Total protein was extracted and measured as previously described with bovine serum albumin (BSA) as a standard [30]. Lyophilized algal powders from the control and melatonin-induced cultures were used for the analysis of total carbohydrate content; glucose was used to prepare a standard curve for analysis of the total carbohydrate content [31].

2.4. Treatment with inhibitors and assays of intracellular ROS, NO and SA

To determine the potential crosstalk among melatonin and NO, SA and ROS in response to environmental stress, algae were cultured in induction medium (MBBM) containing 10 μM melatonin with the addition of 200 μM carboxy-PTIO (an NO scavenger), 100 μM paclobutrazol (Pac, an SA inhibitor), and 10 mM *N*-acetyl-L-cysteine (NAC, an ROS scavenger), respectively. The algal cells were harvested at 1-day intervals.

The intracellular ROS levels were monitored using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, China) as probe, as previously described [32]. To detect intracellular ROS, 10 μM (final concentration) of DCFH-DA was added to the cultures that were then incubated on a shaker at 37 °C in the dark for 30 min. Thereafter, the cells were washed three times with the same buffer. The fluorescence of the samples was measured with a spectrofluorophotometer (RF-540, Shimadzu, Japan) using an excitation wavelength of 488 nm and an emission band between 500 and 600 nm. The average fluorescence density of the intracellular areas was measured to index the ROS level.

To analyze the endogenous SA and NO levels, 10 mL of the cell culture was harvested by centrifugation during the induction phase, and the pelleted cells were washed twice with distilled water. Next, the samples were pulverized using a mortar and pestle. The endogenous SA level was estimated by means of an SA colorimetric assay kit (Bio Vision, America) [33]. The endogenous NO level was analyzed using the Total Nitric Oxide Assay Kit (Beyotime, China) [34].

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