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Metabolomic profiling of the astaxanthin accumulation process induced by high light in *Haematococcus pluvialis*

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ABSTRACT

The growth of *Haematococcus pluvialis* exposed to high light was divided into three pigmentation phases: a green phase, a yellow phase and a red phase. Classification was based on astaxanthin and chlorophyll content and cell morphology. Metabolic profiling of the three pigmentation phases was performed. A total of 81 metabolites were identified and quantified by gas chromatography–mass spectrometry, including 23 amino acids, 19 sugars, 15 organic acids, 8 alcohols, 7 amines, 4 nucleic acids and 5 others. These were subdivided into four groups according to their changes during the three phases. The clustering of metabolites was revealed, and potential biomarkers were identified by principal component analysis, partial least squares analysis and hierarchical clustering analysis, suggesting details of the metabolic pathways of cells. The contents of some cytoprotective metabolites were increased in the yellow phase, such as sucrose, proline and glutamic acid. The precursors of these metabolites are the intermediates of the Calvin cycle and the TCA cycle, indicated those two cycles provided more precursors for the synthesis of the cytoprotective metabolites. A hypothetical metabolic regulation model of *H. pluvialis* exposed to high light was proposed. Our study provides the first metabolomics view of the astaxanthin accumulation process that is induced by high light in *H. pluvialis*.

1. Introduction

The xanthophyll carotenoid astaxanthin is a type of antioxidant that is mainly found in marine animals and in many microorganisms, including microalgae [1]. Astaxanthin is called 'super vitamin E' because of its strong antioxidant properties. It is estimated that astaxanthin shows ten times greater antioxidant activity than β-carotene, which is another commercialized antioxidant product [2]. Nutraceutical and pharmacological studies have shown that astaxanthin has anti-aging effects [3], pro-immunity effects [4], anti-cancer effects [5], and effects against cardiovascular disease [6] and diabetes [7]. The global market size of astaxanthin is estimated to reach $253 million by 2015 [8].

The unicellular green alga *Haematococcus pluvialis* can accumulate large quantities of astaxanthin under various stress conditions, including high light, nutrient starvation and osmotic stress. *H. pluvialis* cells can be divided into four types that occur during its life cycle, including stationary microzooids, large flagellated swarming macrozooids, non-motile palmella forms and resting hematocysts [9]. Astaxanthin biosynthesis in *H. pluvialis* occurs in two steps: β-carotene synthesis occurs first, followed by production of astaxanthin through oxidation and hydroxylation reactions of β-carotene [10]. The key genes involved in astaxanthin synthesis have been cloned and metabolically engineered successfully into tobacco and other species to produce astaxanthin [11,12]. However, the astaxanthin levels in metabolically engineered plants are too low for them to be used for industrial scale production. Therefore, further exploration and exploitation of the underlying regulatory mechanisms and components of the astaxanthin biosynthesis pathway in *H. pluvialis* are needed.

Metabolomics is a powerful tool for systematically analyzing the dynamic responses to stimulation and small molecule metabolite changes in many organisms [13]. Recently, many studies on microalgae using metabolomics have been performed. For example, a metabolomics approach was used to study the metabolite responses and phytotoxic effects of the herbicide prometryn on the growth of the green alga *Scenedesmus vacuolatus* [14]. The diatom *Skeletonema marinoi* was studied in different growth phases by using comparative metabolomics [15]. Metabolomics analysis of *H. pluvialis* was used to study responses to stress factors including acetate (Ac), Fe^{2+} and high light stress [16]. Until now, metabolomic analysis of astaxanthin accumulation process in *H. pluvialis* has not been performed. In the present study of astaxanthin and chlorophyll contents, growth and astaxanthin accumulation in *H. pluvialis* were divided into three phases, i.e., a green phase with higher chlorophyll content and lower astaxanthin content (contents equal to those of microzooids and macrozooids), a yellow phase with intermediate levels of chlorophyll and astaxanthin (equal to those of the non-motile palmella forms), and a red phase with lower chlorophyll and higher astaxanthin (equal to the contents found in hematocysts). The metabolomics of the three phases induced by high light in *H. pluvialis* were profiled.

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2. Materials and methods

2.1. Algal culture and stress conditions

H. pluvialis were cultured in liquid Bold's basal medium (BBM) [17] in 500 mL Erlenmeyer flasks under a light intensity of 24 μmol/m^2 s for 16 h/8 h light/dark cycle at 24 °C without aeration. Log phase cells (OD_{669} = 0.4) were cultured under a light intensity of 24 μmol/m^2 s for 7 days (OD_{669} = 0.7) and collected as green phase cells, Yellow phase cells were generated by culturing green phase cells under a light intensity of 80 μmol/m^2 s for 14 days, Red phase cells were generated by culturing green phase cells under a light intensity of 80 μmol/m^2 s for 28 days. All experiments were carried out in four replicates and repeated at least thrice in this study.

2.2. Analytical methods

2.2.1. Pigment analysis

H. pluvialis cells were harvested by centrifuge at 4000 rpm for 5 min and washed with Milli-Q water. The dry cell weight of the algal biomass was measured repeatedly until a constant weight was obtained by drying at 90 °C in a hot-air oven. Pigments of algal biomass used for astaxanthin HPLC analyses were extracted by acetone. For astaxanthin saponification, 1 mL NaOH-CH_3OH (0.1 M) was added into 5 mL pigment extract and kept at 55 °C for 12 h. The astaxanthin content was analyzed by HPLC using a reverse-phase BDS HYPERSIL C18 (250 × 4.6 mm, 5.0 μm). Samples were eluted using acetonitrile/methanol (75/25, v/v) as mobile phases. Elution was carried out at 1 mL/min with a 20 μL injection volume loop with micro-syringe with a detection wavelength and a column temperature of 476 nm and 25 °C, respectively. Pigments of algal biomass used for chlorophyll and total carotenoids analyses were extracted with 5 mL DMSO for 12 h at room temperature in the dark. The contents of chlorophyll and total carotenoids were determined as follows [18]: Chl *a* (mg/L) = 12.19 A_{665}–3.45A_{649}, Chl *b* (mg/L) = 21.99 A_{649}–5.32A_{665}, Total carotenoids (mg/L) = (1000A_{480} − 2.14C_a − 70.16C_b) / 220. An analytical electronic balance (Mettler-Toledo, ME204, Greifensee, Switzerland) with an accuracy of ±0.0001 g was used for all weightings in the experiments.

2.2.2. Microscopy

H. pluvialis cells were observed under an optical microscope (OM) (Olympus BX53, Tokyo, Japan) with an Olympus DP72 digital color camera (Olympus, Tokyo, Japan). The morphology of the cell surface was investigated using a Hitachi SU1510 scanning electronic microscope (SEM) (Hitachi, Tokyo, Japan). For SEM observation, *H. pluvialis* cells were harvested by centrifugation at 4000 rpm for 5 min and washed with Milli-Q water. Cell fixation, dehydration and coating were performed according to previously reported methods [19].

2.2.3. Extraction of intracellular metabolites

The quenching and extraction processes were performed according to previously reported methods [20]. In detail, samples were immediately quenched with pre-chilled −40 °C 60% (v/v, methanol/water) methanol solution for 5 min. Cells were harvested by centrifugation at 8000 rpm for 5 min at 4 °C and washed with PBS (pH 7.0,0.1 mM) twice, then washed with ultrapure water once. The cell pellets were immediately frozen in liquid nitrogen and ground into powder. The intracellular metabolites were extracted according to previously reported methods [21,22]. Briefly, 50 mg cell powder was suspended in 1 mL of pre-chilled −20 °C extraction buffer (1:1, v/v, methanol/water) and 5 μL of internal standard solution (succinic acid, 2,2,3,3-*d4*, 1.5 mg/mL, Sigma), then thoroughly mixed by vortexing. The mixture was frozen in liquid nitrogen for 2 min and then thawed, and the cycle was repeated five times. The supernatant was collected by centrifugation at 10,000 rpm for 5 min and was then used for lyophilization.

2.2.4. Derivatization and gas chromatography–mass spectrometry (GC–MS) analysis

Prior to GC–MS analysis, a two-stage chemical derivatization was performed. First, 50 μL of methoxamine hydrochloride (20 mg/mL in pyridine, Sigma) was added to the lyophilizate, and the mixture was incubated at 40 °C for 80 min. Then, 80 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma) was added to the samples, which were subsequently incubated at 40 °C for 80 min [23,24].

GC–MS analysis was performed by using a GC–MS system (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-5 capillary column (60 m × 320 μm i.d., 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA, USA). 1 μL of sample was injected without a split ratio. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The electron impact ionization (70 eV) was set to full scan mode (*m/z*: 50–800). The GC oven temperature for metabonomics was set to 70 °C for 2 min, then raised to 290 °C at a rate of 5 °C/min, and maintained at 290 °C for 6 min. The GC oven temperature for fatty acid methyl esters analysis was set to 70 °C for 2 min, followed by an increasing rate of 8 °C/min to 200 °C, which was held for 2 min, then increased at a rate 3 °C/min to 245 °C and maintained at 245 °C for 3 min.

2.2.5. Fatty acid methyl esters extract

The fatty acid of three different phases were extracted according to a modified method [25]. Briefly, 10 mg algae powder was suspended in 2 mL 2 M NaOH-CH_3OH solution and the 40 μL 2 mg/mL nonadecanoic acid (internal standard solution) was added and mixed thoroughly.

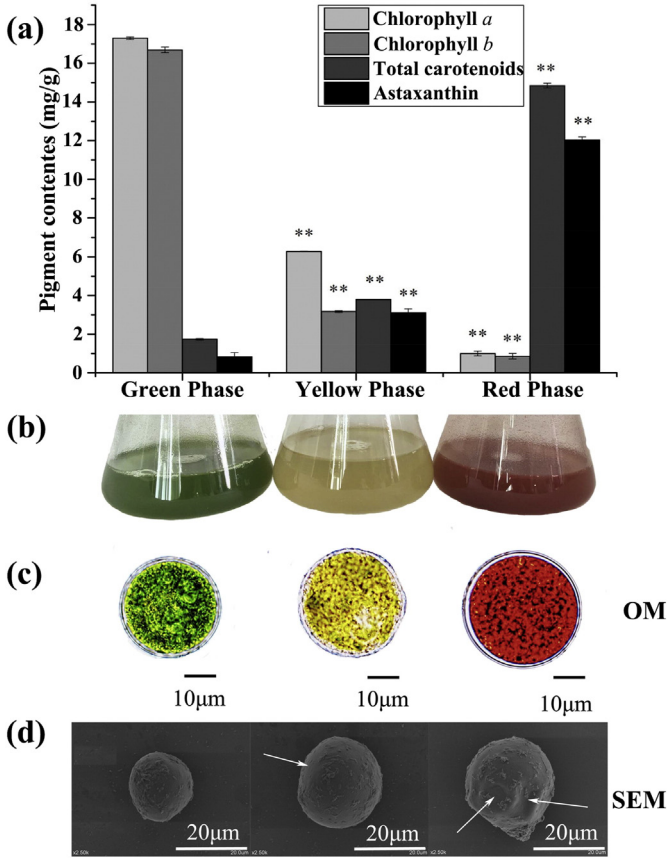

Fig. 1. The contents of chlorophylls, total carotenoids, astaxanthin and microscopy of cells in three phases. The data shown are the averages ± SE of three replicates. $^{**}P < 0.01$ compared with green phase. Changes in culture color of three pigmentation phases (b). Changes in cell color of three pigmentation phases (c). Changes in cell surface of three pigmentation phases, irregular concave features are marked by arrows (d). OM, optical microscope; SEM, scanning electron microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)