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Inhibition of autophagy modulates astaxanthin and total fatty acid biosynthesis in *Chlorella zofingiensis* under nitrogen starvation

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ABSTRACT

The present study showed that inhibition of autophagy significantly increased cellular levels of reactive oxygen species in *Chlorella zofingiensis* under nitrogen starvation. This was accompanied with increased expression of PSY, and enhanced accumulation of astaxanthin after 48 h of cultivation. Nevertheless, the proportion of astaxanthin in secondary carotenoids remained unchanged. Meanwhile, the expression level of ACCase was also elevated in the 3-MA-treated cells compared to the control despite a > 20% lower content of fatty acid in the former than the latter. This phenomenon might be due to inhibition of recycling of cellular components by 3-MA and suggests the potential involvement of post-transcriptional regulation in fatty acid biosynthesis. In summary, our work has been the first to report a potentially important role of autophagy in fatty acid and astaxanthin accumulation in *C. zofingiensis* under stress conditions. The findings might provide valuable insights to guide further research in this area.

1. Introduction

Microalgae are unicellular species which consist of an extremely diverse polyphyletic group, and can be typically found in freshwater and marine systems (Fu et al., 2016). Depending on the species or strains and cultivation conditions, the chemical composition of microalgae, such as proteins, lipids and carotenoids, varies widely (Finkel et al., 2016; Fu et al., 2016). Adjustment of environmental conditions such as temperature, illumination, pH, CO_2 supply, salt and nutrients has been one of the most common approaches to accumulating desired cellular products in microalgae (Fu et al., 2016; Liu et al., 2014; Zhang et al., 2017). Meanwhile, microalgae grow fast and can be cultivated on non-arable land (Hu et al., 2008). These properties make microalgae a promising source of biomass feedstock for biofuels and a range of value-added products (Hu et al., 2008; Liu et al., 2014; Scaife et al., 2015; Zhu, 2015).

Lipids and astaxanthin are two of the major products of microalgae. Lipids can be directly converted to biodiesel (Scaife et al., 2015). As the strongest natural antioxidant in the world, astaxanthin can be applied in cosmetics, nutraceuticals and even pharmaceuticals (Zhang et al., 2016). *Chlorella zofingiensis*, a green microalga, has been considered as a promising candidate for both biodiesel and astaxanthin production in recent years owing to its ability to accumulate certain lipids and astaxanthin (Liu et al., 2011, 2014). Abiotic stress conditions, such as high-intensity light, nitrogen starvation and salt stress, have been reported to efficiently promote the accumulation of these microalgal products in *Chlorella* (Fan et al., 2014; Liu et al., 2016). Previous researches showed that, nitrogen starvation could efficiently increase the expression level of ACCase, a key enzyme in the de novo TAG biosynthesis pathway, and further increase the lipid accumulation (Fan et al., 2014). Besides, high light and nitrogen starvation were found to be efficient in increasing the expression of PSY and BKT, the first and last steps in astaxanthin biosynthesis pathway, which further promoted astaxanthin accumulation in *C. zofingiensis* (Zhang et al., 2017). Furthermore, ROS has been proposed to play an important role in regulating lipid and astaxanthin accumulation under stress conditions (Baxter et al., 2014; Ip and Chen, 2005; Kuchitsu, 2013; Ma et al., 2012; O'Brien et al., 2012; Zhang et al., 2016).

Autophagy, a regulated destructive cellular process which allows the orderly degradation and recycling of cellular components, is believed to play a crucial role in cell metabolism under certain stress conditions (Hou et al., 2013; Klionsky, 2008; Kobayashi, 2015; Liu and Bassham, 2012; Mizushima and Komatsu, 2011; Perez-Martin et al., 2015, 2014). Interestingly, autophagy is often accompanied with changes, especially elevation in cellular ROS. Meanwhile, nutrient deficiency might lead to elevation in ROS level, which could trigger autophagy (Hongjuan et al., 2013; Hou et al., 2013). Reduction of ROS levels with antioxidants was found to attenuate autophagy (Hongjuan

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et al., 2013). The interrelationship between autophagy and ROS may be summarized as follows: (1) ROS can induce autophagy; (2) Autophagy can reduce ROS toxicity of cells by eliminating ROS damaged lipids, proteins and organelles (Reumann et al., 2010; Xiong et al., 2007). In higher plants, functional studies have revealed that autophagy plays a key prole in cellular response to abiotic stresses, senescence, and pathogen infection (Liu and Bassham, 2012).

Compared to yeasts, plants and mammalian cells, there has been very limited research on the role of autophagy in microalgae cellular metabolism. Recent studies showed that, in *Chlamydomonas*, autophagy could be elicited under various stress conditions, such as deprivation of nutrients (nitrogen, carbon, sulfur), oxidative stress (carotenoid deficiency, high light) and high concentrations of certain metals (copper, cobalt) (Davey et al., 2014; Hou et al., 2013; Perez-Martin et al., 2015, 2014). Besides, a loss of chloroplast integrity due to depletion of the chloroplastic ClpP protease can also trigger autophagy in *Chlamydomonas* (Ramundo et al., 2014). It was reported that in the green alga *Auxenochlorella protothecoides*, the lipid droplets in heterotrophic cells was degraded by autophagy after switching to photoautotrophic conditions (Zhao et al., 2014).

It has been well established that astaxanthin and fatty acids often accumulate in microalgae under stress conditions and this is usually followed by changes in cellular ROS levels (Ip and Chen, 2005; Liu et al., 2014; Shemi et al., 2015; Zhang et al., 2017, 2016). The present study therefore aimed to investigate the role of autophagy in fatty acid and astaxanthin accumulation in *C. zofingiensis* under stress conditions. To this end, we employed 3-methyladenine (3-MA), a widely used autophagy inhibitor (Jiang et al., 2012), to evaluate its effects on cell growth, ROS levels, astaxanthin and fatty acid accumulation in *C. zofingiensis* under nitrogen starvation condition. Besides, the differential expression of key genes in astaxanthin and fatty acid biosynthesis pathways under nitrogen starvation was also examined. Our study has been the first to report that autophagy likely plays an important role in astaxanthin and FA metabolism in *C. zofingiensis* under certain stress condition and might shed light on future research aiming to enhance astaxanthin and fatty acid accumulation in this microalga.

2. Materials and methods

2.1. Strains and culture conditions

C. zofingiensis (ATCC 30412) was purchased from American Type Culture Collection (ATCC, Rockville, USA). For cell activation, 10 mL of stock culture was inoculated into 100 mL Kuhl growth medium in a 500 mL Erlenmeyer flask (with filter cap), and cultured under white light (50 μmol m^{-2} s^{-1}) with orbital shaking at 150 rpm (Lorenzen, 1964; Zhang et al., 2016) in an incubator which was maintained 25 °C. After 6 days when the cells have entered the late exponential phase, the cells were collected, and re-suspended in 200 mL of Kuhl medium at a concentration of 0.75 g L^{-1} in a tubular photobioreactor maintained at 25 °C with CO_2 aeration (1.5% CO_2 mixed in air) to produce the seed culture. The light intensity was 100 μmol m^{-2} s^{-1}. After 4 days when the seed culture reached the late exponential phase, the cells were collected, and re-suspended in the tubular photobioreactor containing 200 mL of nitrogen-free Kuhl medium at a concentration of 0.75 g L^{-1} with CO_2 aeration (1.5% CO_2 mixed in air). This culture was used to evaluate the effect of 3-MA on fatty acid and astaxanthin accumulation in cells. The temperature and light intensity were the same as those for the seed culture.

2.2. Dry weight (DW) measurement

Two mL of the culture was collected at each of the following time points: 12, 24, 48, and 96 h and centrifuged at 5000*g* for 3 min. The supernatants were discarded, and the cell pellets were washed 3 times with distilled water, dried at 80 °C for 12 h and weighed.

2.3. ROS measurement

ROS levels were determined with an ROS assay kit (Beyotime Institute of Biotechnology, China) by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to a fluorescent compound dichlorofluorescin (DCF) (Jia et al., 2006; Lin et al., 2006). Briefly, 1 mL each of the culture samples was harvested by centrifugation at 4000*g* for 3 min and the cell pellets were re-suspended in 200 μL of Kuhl medium with 10 μM DCFH-DA. Cell pellets re-suspended in Rosup reagent was used as a positive control. After incubation (20 min), washing, and resuspension in the original culture medium, fluorescence was measured (excitation, 485 nm; emission, 520 nm) on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All measurements were done in triplicate.

2.4. Fatty acid analysis

Cells were collected by centrifugation at 4000*g* for 3 min at 4 °C and lyophilized in a DW3 freeze-drier (Heto Dry Winner, Denmark). The lyophilized cell samples were directly methylated to fatty acid methyl esters (FAMEs) by incubating with 1% sulphuric acid in methanol according to the procedures described by Liu et al. (2012). The FAMEs were analyzed on a GC-MS-QP 2010 SE (Electron Ionization type) gas chromatograph-mass spectrometer (SHIMADZU, Japan) equipped with an Rtx-2330 capillary column (30 m × 0.25 mm) (Restek, Guangzhou, China). Helium was used as the carrier gas. The injection temperature, ion temperature and interface temperature were set at 250 °C, 200 °C and 260 °C, respectively. The initial column temperature was set at 150 °C. The column temperature subsequently rose to 200 °C at 10 °C/min and then to 220 °C at 6 °C/min, followed by holding at 220 °C for 10 min. Quantification of FAMEs was performed according to Liu et al. (2011). All measurements were done in triplicate.

2.5. Pigment extraction and analysis

The lyophilized microalgal cell samples were ground with a mortar and pestle under liquid nitrogen and then extracted three times with acetone. The acetone layers were collected by centrifugation and dried under nitrogen gas. The dried pigments were dissolved in 1 mL acetone and filtered with a 0.22-μm Millipore membrane before HPLC analysis. Free astaxanthin and astaxanthin esters were quantified on a Waters 2695 HPLC system equipped with a 2998 detector (Waters, Milford, MA, USA). The method was adopted from Ip et al. (2004). All measurements were done in triplicate.

2.6. RNA isolation, cDNA synthesis, and real-time PCR (RT-PCR)

RNA was extracted from about 10^8 cells using the RNA plant plus Reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm. Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA with QuantScript RT Kit reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. RT-PCR amplification was carried out using One Step SYBR PrimeScript PLUS RT-PCR Kit reagent (TaKaRa, Tokyo, Japan). The primers used are listed in Table 1. The mRNA expression level was

Table 1
PCR primers used for RT-PCR to quantify the genes' expression level.

Gene	Forward (5′–3′)	Reverse (5′–3′)
PSY	CACCAGGTTGTCAGAGTCCA	ACTAGTGTGTTGCTGACTCT
BKT	GGTGCTCAAAGTGGGGTGGT	CCATTTCCCACATATTGCACCT
ACCase	CGTGGCAGCTACGGCTTAAC	TGCACCAACTCCTGGAATGA
ACT	TGCCGAGCGTGAAATTGTGA	CGTGAATGCCAGCAGCCTCCA