# High light boosts salinity stress-induced biosynthesis of astaxanthin and lipids in the green alga *Chromochloris zofingiensis*

Yaping Kou, Meijing Liu, Peipei Sun, Zhaoqi Dong, Jin Liu*

*Laboratory for Algae Biotechnology & Innovation, College of Engineering, Peking University, Beijing 100871, China*

A B S T R A C T

*Chromochloris zofingiensis*, an astaxanthin-producing oleaginous green alga, is emerging as a model for studying carotenogenesis and lipogenesis. While the stimulated biosynthesis of secondary carotenoids and storage lipids by abiotic stresses has long been perceived in *C. zofingiensis,* the underlying mechanisms particularly under combinatorial stresses remain to be explored. In the present study, the synthesis of carotenoids and lipids was comparatively investigated in *C. zofingiensis* under high light (HL), salinity stress (SS) and the combinatorial HL and SS (HL + SS) conditions. While HL or SS alone enhanced astaxanthin and lipids moderately, HL + SS had synergistic effect and promoted these compounds considerably. HL + SS also gave rise to the highest productivities. Carotenoid profiling and transcriptional analysis supported the diversion of carotenoid flux from primary carotenoids to secondary carotenoids particularly astaxanthin and violaxanthin cycle that produces zeaxanthin likely contributed to astaxanthin synthesis. Lipid profiling and transcriptional analysis, on the other hand, suggested that both de novo fatty acid synthesis and membrane lipid turnover contributed fatty acyls for supporting triacylglycerol assembly via the acyl CoA-dependent and acyl CoA-independent routes. Astaxanthin and triacylglycerol that share common carbon precursors were induced to synthesize in a coordinated way regulated at the transcriptional level, yet the carbon flux was allocated predominantly to the latter. Taken together, our results demonstrated the beneficial effect of combinatorial stresses on both astaxanthin and triacylglycerol production, identified critical genes involved in carotenogenesis and lipogenesis, and provided new insights into algal astaxanthin biosynthesis.

## 1. Introduction

Carotenoids represent a diverse group of terpenoid pigments widely present in photosynthetic organisms [1]. In addition to the essential functions in photosynthesis and photoprotection of phototrophs, carotenoids serve as important components of human diets and play critical roles in human health [2]. The ketocarotenoid astaxanthin is of particular interest, as it possesses potent antioxidant activity and has broad applications in food, feed and nutraceutical industries [3]. There are only a limited number of organisms that are capable of synthesizing astaxanthin, including some green algae, marine bacteria and the yeast *Xanthophyllomyces dendrorhous* [1]. *Haematococcus pluvialis*, a unicellular green alga able to accumulate the highest level of astaxanthin found in nature (4% of dry weight), is the predominant source of natural astaxanthin in the current market [4]. Nevertheless, *H. pluvialis* astaxnathin production is compromised by its slow growth and vulnerability to contamination. By contrast, the green alga *Chromochloris zofingiensis* can grow robustly under multiple trophic conditions to achieve high cell densities [5–10] and has been considered as a promising alternative producer of astaxanthin [11].

*C. zofingiensis* is also able to synthesize substantial amounts of lipids under photoautotrophic, heterotrophic and mixotrophic modes [7–10,12]. Algal lipids, superior to plant oils in many ways, are considered to be the next-generation biofuel feedstock [13]. Although substantial progress has been achieved during the past decades, challenges remain to be addressed for algae-based biofuels [13,14]. Integrated production of lipids with value-added products from algae represents a promising strategy to bring down the cost of algal biofuels. *C. zofingiensis* is considered as one of the leading algal candidates for such co-production, because of its ability to synthesize asataxanthin, a high-value carotenoid, and triacylglycerol (TAG), an ideal lipid for making biodiesel [7,8,12]. Moreover, the chromosome-level genome sequence and genetic tools are available [15,16], allowing deep investigation of the mechanisms underlying astaxanthin and TAG biosynthesis and manipulation of *C. zofingiensis* for trait improvements.

The production potential of *C. zofingiensis* for astaxanthin and/or

* Corresponding author.
*E-mail addresses:* kou_yaping@163.pku.edu.cn (Y. Kou), 1801111694@pku.edu.cn (M. Liu), 1801111691@pku.edu.cn (Z. Dong), gjinliu@pku.edu.cn (J. Liu).

TAG has been evaluated by independent research groups, from photo-autotrophy [5,7,8,17,18] to heterotrophy [6,12,19], mixotrophy [9,20] and trophic transition [10,21,22]. To understand the global regulation of astaxanthin and TAG syntheses, omics studies have been conducted for *C. zofingiensis* particularly under the conditions of nitrogen deprivation [16,23], high light stress [15] and glucose induction [22,24]. As the utilization of seawater for algal cultivation helps reduce the footprint of freshwater, a scare resource on the earth, the response of *C. zofingiensis* to salinity levels has been studied recently, with biomass production severely compromised [25]. Light is important to algal growth and metabolite production and the increase of light intensity benefits biomass production as well as certain products such as astaxanthin in *C. zofingiensis* [10]. Yet the combination of salt and light treatment, which may have potential to maximize the accumulation of secondary metabolites while not compromising biomass production, has not been applied to *C. zofingiensis*. In this study, the main objective is to comparatively investigate the synthesis and accumulation of astaxanthin and TAG and the transcriptional regulation of their biosynthesis in *C. zofingiensis* under four different culture conditions, namely the normal growth condition (referred to as control), high light illumination (HL), salinity stress (SS) and the combination of HL and SS (HL + SS). The integrated analysis of chemical changes (carotenoids and lipids) and transcript dynamics of key genes suggested that HL + SS had synergistic effect on the accumulation of both astaxanthin and TAG, likely regulated at the transcriptional level. Carotenoid flux was diverted from primary carotenoids to secondary carotenoids particularly astaxanthin and violaxanthin cycle that produces zeaxanthin was likely involved in astaxanthin synthesis. Both de novo fatty acid synthesis and membrane lipid turnover contributed fatty acyls for supporting TAG assembly. Our work demonstrated that HL boosted the SS-induced production of astaxanthin and lipids and provided new insights into the understanding of their biosynthesis in *C. zofingiensis*.

## 2. Materials and methods

### 2.1. Algal strain and culture conditions

*Chromochloris zofingiensis* (ATCC 30412) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The maintaining of the alga was described by our previous study [23], using Kuhl medium that consists of (per liter) 1.01 g $KNO_3$, 0.62 g $NaH_2PO_4 \cdot H_2O$, 0.089 g $Na_2HPO_4 \cdot 2H_2O$, 0.247 g $MgSO_4 \cdot 7H_2O$, 14.7 mg $CaCl_2 \cdot 2H_2O$, 6.95 mg $FeSO4 \cdot 7H_2O$, 0.061 mg $H_3BO_3$, 0.169 mg $MnSO_4 \cdot H_2O$, 0.287 mg $ZnSO_4 \cdot 7H_2O$, 0.0025 mg $CuSO_4 \cdot 5H_2O$, and 0.01235 mg $(NH_4)_6MO_7O_{24} \cdot 4H_2O$. The pH of the medium was adjusted to pH 7.0 prior to autoclaving. To recover algal activity, the cells from the agar plate was inoculated into a flask grown aerobically at 25 °C with orbital shaking (150 rpm) and continuous illumination (30 µE $m^{-2} s^{-1}$). The cells were then inoculated at 10% ($v/v$) into a 200-mL column (3-cm diameter), and grown to exponential phase under constant illumination of 80 µE $m^{-2} s^{-1}$ and aeration of 1.5% $CO_2$ enriched air (normal growth condition).

The algal cells in exponential phase were harvested by centrifugation (5000 *g*, 5 min) and resuspended in fresh medium with an initial cell density of 0.5 g $L^{-1}$. For high light (HL) treatment, the algal culture was exposed to continuous illumination of 400 µE $m^{-2} s^{-1}$. For salinity stress (SS) treatment, the algal culture was supplemented with 0.25 M NaCl (80 µE $m^{-2} s^{-1}$). For the combined treatment of HL and SS (HL + SS), the algal culture was supplemented with 0.25 M NaCl and constantly illuminated with 400 µE $m^{-2} s^{-1}$. The algal culture under the normal growth condition was used as the control (CT). All cultures were aerated with 1.5% $CO_2$ enriched air.

### 2.2. Dry weight determination of C. zofingiensis cells

The algal dry weight was determined as previously described [17].

Briefly, algal cells harvested from each sampling time point were washed twice with deionized water and filtered through a pre-weighted dry Whatman GF/C filter paper (1.2 µm pore size), followed by drying at 90 °C in a vacuum oven overnight till constant weight. The filter papers were then placed in a desiccator and allowed to cool down prior to dry weight determination.

### 2.3. Extraction of pigments and lipids from C. zofingiensis biomass

Algal samples were harvested by centrifugation (5000 *g*, 5 min) and washed twice with deionized water, followed by the freeze-drying in a DW3 freeze-drier (Heto Dry Winner, Denmark) till constant weight. The lyophilized algal biomass was disrupted by full homogenization in the presence of liquid nitrogen and subjected to extraction by a solvent mixture of chloroform/methanol/0.75% aqueous NaCl solution (2:1:0.75, by volume). After phase separation, the lower chloroform layer containing pigments and lipids was collected and evaporated under nitrogen gas. The dried extracts were dissolved either in acetone for pigment analysis or in chloroform for lipid analysis.

### 2.4. Pigment analysis

For thin layer chromatography (TLC) separation of pigments, the algal extracts were loaded on a TLC silica gel 60 plate (EMD Millipore, Billerica, MA) and developed with hexane/tert-butyl methyl ether/acetic acid (80:2:2, by volume) for 30 min. For the quantification of pigments, 20 µL of each extract was separated by high performance liquid chromatography (HPLC) on a Waters Spherisorb® 5 µm ODS2 4.6250 mm analytical column with a Waters 2695 HPLC system, according to our previously described procedures [23]. Pigments were eluted at a flow rate of 1.2 mL $min^{-1}$. Individual carotenoid was identified by the absorption spectra and typical retention time compared to the standard of pure carotenoids.

### 2.5. Lipid analysis

Neutral lipids were separated on a TLC plate using hexane/tert-butyl methyl ether/acetic acid (80:2:2, by volume) as the development system [16]. For lipid visualization by charring, the TLC plate was sprayed uniformly with 8% ($w/v$) $H_3PO_4$ containing 10% (w/v) copper (II) sulfate pentahydrate, air dried, and baked at 180 °C for 3 min. For quantification, TAG on TLC plates was visualized with iodine vapor, recovered, transesterified and analyzed by the gas chromatography–mass spectrometry (GC–MS) method.

Fatty acid methyl esters (FAMEs) were prepared by direct transmethylation of total lipids or recovered TAG according our previously described procedures [16]. The FAMEs were separated by GC–MS using the Agilent 7890 capillary gas chromatograph equipped with a 5975C mass spectrometry detector and a HP-88 capillary column (60 m × 0.25 mm) (Agilent Technologies, Wilmington, DE). The temperature program consisted of an initial hold at 100 °C for 5 min, ramping at 3.5 °C $min^{-1}$ to 240 °C, and a final hold at 240 °C for 5 min. The injector was kept as 250 °C with an injection volume of 2 µL in a split mode (19:1 split ratio). The flow rate of the carrier gas (helium) was 1.5 mL $min^{-1}$, and the ionization energy was 70 eV (EI, full scan mode). The individual FAME in each sample was quantified by using a FAME standard (Sigma-Aldrich, St. Louis, MO, USA).

### 2.6. RNA isolation, cDNA synthesis, and quantitative real-time PCR assay

The algal samples at 1, 3, 6 and 12 h under different culture conditions (CT, HL, SS and HL + SS) were harvested for quantitative real-time PCR (qPCR) assay. Total RNA was extracted using the plant RNA extraction kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The RNA samples were treated with RNase-free DNase I (TaKaRa), checked by gel electrophoresis and determined by

**Table 1**
Primers used for qPCR in this study.

| Genes | Forwards (5′ - 3′) | Reverse (5′ - 3′) |
|---|---|---|
| BKT1 | ACCTCAAGCCGCACTCAAAT | GCCAGCAGCCATGGTAAAAG |
| BKT2 | GCCTTCAACAACAGGCGATT | CGCATTGCCCTTGTGAAAGT |
| CHYb | CGTTACGCACACAAGGCATT | CACAGGCTGAAGGCAGGTACA |
| LCYb | TTCCACTGGCATGGCTTCTT | CTGCCGAGCTCACCATCTG |
| LCYe | CAGTGTTTCGCGGTCTTTGA | ACGCGTTGGTAGCTGACAGA |
| VDE | GTTGACCGTGGCTTTGATCTG | TTGAGTTTGCCCACCAGCTT |
| ZEP | CTGGGTCCCTTGTCTTGGAT | TTGAAGAACTCCGCCTCAGT |
| NXS | GTGTGGCATACACTGTGCTG | GCCATGCCATAGCTGTCAAT |
| AAT | GGTTGGTGAAGAAGGCCAAA | CATGGATCAGCCCACTCAATG |
| PDAT | AAGGTGTCGGCTGTGAAGGA | CATCCACGGATTGGGTAGCT |
| DGAT1A | GGCATCCGAATGAACCTCAT | AACTCCTCGTCCTGCTTCATG |
| DGTT5 | CCAGCAGCGTGTTCTCCATT | GCAATACCCCCCACAATCAC |
| SAD | GATGAGGGACGGCATGAAAT | GTGTGCGGGCATCACAATC |
| MCT | GTGTGACGCTGTTGAGAAATTAG | GGTAGCTTTGAGGGCTTCTT |
| PGD1 | TACTTGCCGCTGGTACAGGA | GGTGTACACGGGTGCGATTT |
| MLDP | CTTCATGGACTCCTTCCCATAC | TGCTTCACATACTTGGACTTCT |
| β-actin | GCTGGCATTCACGACACAAC | TGCCACCACCTTGATCTTCA |

BKT, β-carotenoid ketolase; CHYb, β-carotenoid hydroxylase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase; AAT, astaxanthin acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; DGAT1A, diacylglycerol acyltransferase type IA; DGTT5, diacylglycerol acyltransferase type II5; SAD, stearoyl-ACP desaturase; PGD1, plastid galactoglycerolipid degradation1; MLDP, major lipid droplet protein.

spectrophotometrically at 260 nm. The total RNA (1 μg) from each sample was reverse transcribed to cDNA using Prime Script™ RT Master Mix (TaKaRa) according to the manufacturer's instructions. qPCR assay was conducted by using SYBR® Premix Ex Taq™ II (Tli RNase H Plus) (TaKaRa), and the relative gene expression level was calculated according to Liu et al. [16] using the endogenous β-actin gene as the internal control for normalization. The primers used for qPCR are listed in Table 1.

### 2.7. Statistical analysis

Experiments were conducted in three biological replicates and the data were expressed as mean value ± SD. The statistical significance of the results was tested by student *t*-test.

## 3. Results and discussion

### 3.1. Differential effects of stress conditions on the growth of C. zofingiensis

The growth of *C. zofingiensis* over a six-day period was assessed under four different culture conditions, namely, the normal growth condition (designated as control, CT), the high light illumination (HL, 400 μmol m$^{-2}$ s$^{-1}$), the salinity stress (SS, 0.25 M NaCl), and the combination of HL and SS (HL + SS). *C. zofingiensis* showed a gradual increase in cell density under the CT condition and reached 6.9 g L$^{-1}$ after six days of cultivation (Fig. 1a). The algal cell density was promoted considerably by HL during the whole culture period (Fig. 1a), which was 2.4-, 1.6- and 1.5-fold higher than that under the CT condition on day 2, day 4 and day 6, respectively (Fig. 1b). Probably, the light intensity under CT (80 μmol m$^{-2}$ s$^{-1}$) is not enough for maximal algal growth (photolimitation), whereas under HL the light intensity is no longer limiting, as is the case previously reported in algae [26]. Upon SS treatment, although the algal growth was observed (Fig. 1a), the cell density was lower than that under the CT condition, which was particularly obvious on day 6 (40% less, Fig. 1b), confirming the SS-associated growth inhibition. Accordingly, HL + SS gave rise to a lower cell density than HL, yet comparable to CT (Fig. 1a and b). Albeit exhibiting differential effects on algal growth, the three stress conditions all led to a severe decline in chlorophyll level, with HL + SS being most
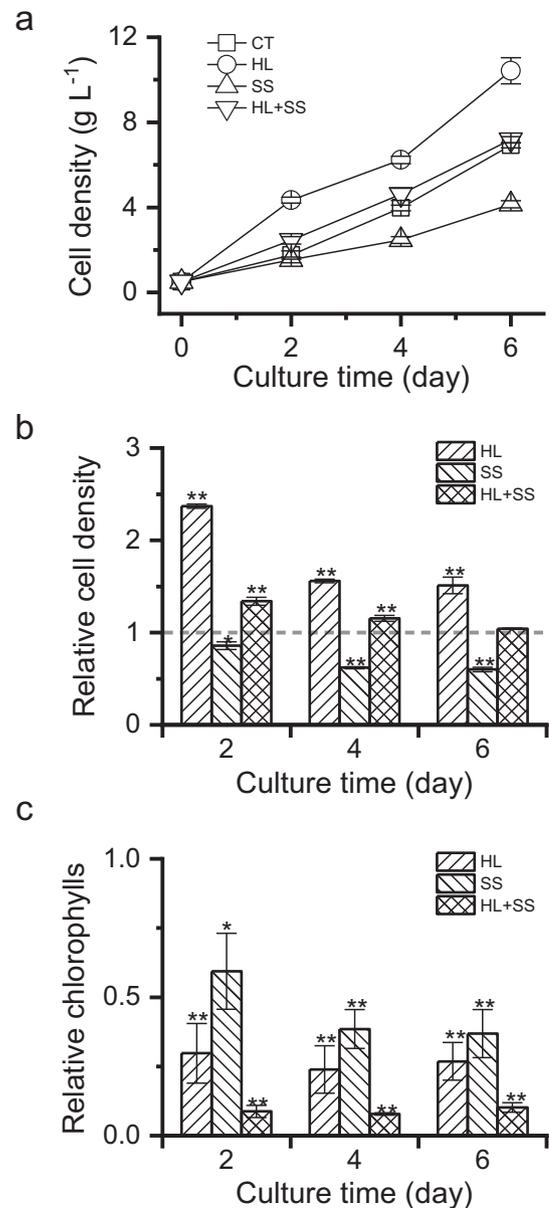


**Fig. 1.** Effect of stress conditions on the growth of *C. zofingiensis*. (a) Time course of cell density. (b) Relative cell density under stress conditions, which were normalized to CT (set as 1). (c) Relative chlorophylls under stress conditions, which were normalized to CT (set as 1). CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS. Data are expressed as mean ± SD (*n* = 3). Significant difference (compared to CT; student *t*-test) at *p* < 0.01 and *p* < 0.05 level is indicated with * and **, respectively.

evident (Fig. 1c). It is worth noting that HL, compared to CT, gave rise to a considerable higher cell density but a severely lower chlorophyll level (Fig. 1a and c). Probably, when light is limited, algal cells tend to synthesize more chlorophylls to enhance light capture, whereas when light is in excess (but not that high to cause cell deaths), chlorophylls are degraded but photosynthesis capacity is still maintained, allowing easy penetration of light into the cultures for biomass production. In line with this, it has been reported that reduction of light harvesting pigments including chlorophylls could improve biomass production particularly in dense cultures [27].

### 3.2. Salinity stress boosts the high light-induced accumulation of astaxanthin

Under the CT condition, *C. zofingiensis* synthesized a basal level of astaxanthin, which was maintained at a stable level during the whole culture period (Fig. 2a). By contrast, astaxanthin increased as the culture time lasted under the HL condition and reached 2.8 mg g$^{-1}$ dry weight (DW) on day 6, 4.0-fold higher than that achieved under the CT condition (Fig. 2a). Similarly, SS stimulated the synthesis of astaxanthin over the whole culture period and astaxanthin level was comparable to that under the HL condition (Fig. 2a). HL + SS, on the other hand, made *C. zofingiensis* to accumulate the highest level of astaxanthin, which reached 6.0 mg g$^{-1}$ DW after six days of cultivation and was 8.5-fold higher than that under the CT condition (Fig. 2a). It is worth noting that astaxanthin level was even greater than the sum of that under the HL and SS conditions (day 4 and day 6), indicative of a synergistic effect of the combination of HL and SS on astaxanthin synthesis. Similar to *H. pluvialis*, *C. zofingiensis* has three forms of astaxanthin, free, mono-ester and di-ester [11,28–30]. Interestingly, except HL on day 2, the three stress conditions only slightly affected the accumulation of free astaxanthin (Fig. 2b). By contrast, astaxanthin mono-ester and di-ester under stress conditions were considerably higher than that under the CT condition (Fig. 2c and d; Supplemental Fig. S1). Therefore, it is the ester form that contributes to the stress-associated increase of astaxanthin. Notably, astaxanthin ester dominated over free astaxanthin regardless
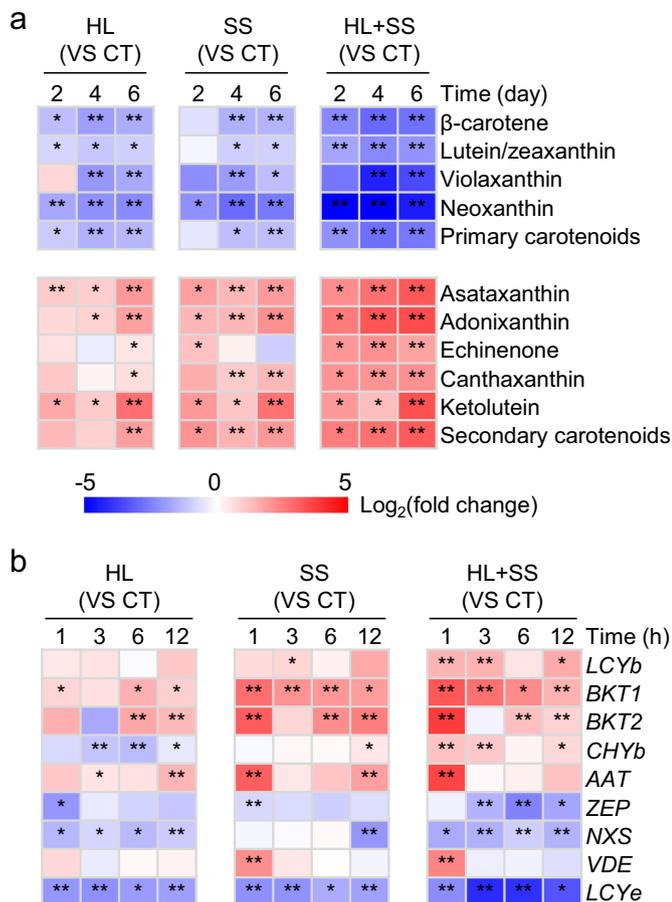
of culture conditions and time points, yet a higher portion of ester was observed under stress conditions (Fig. 2e). Consistent with the dominant portion of astaxanthin ester to total astaxanthin (~96%) in *C. zofingiensis*, *H. pluvialis* accumulates as high as over 90% of astaxanthin ester [4,28]. This suggests that the enzyme responsible for astaxanthin esterification may be highly efficient in the two algae. The mono-ester level was much lower than the di-ester level (mono-ester/di-ester ratio < 0.6) in *C. zofingiensis* under the CT condition, yet the ratio was promoted by stress conditions (Fig. 2f).

### 3.3. Violaxanthin cycle is likely involved in astaxanthin biosynthesis

Similar in higher plants, lycopene serves as the branching intermediate for synthesizing α-carotene and β-carotene and their downstream primary carotenoids [1]. The formation of α-carotene from lycopene requires two cyclases, lycopene β-cyclase (LCYb) and lycopene ε-cyclase (LCYe), while β-carotene formation needs only LCYb. Hydroxylation of α-carotene and β-carotene leads to the formation of lutein and zeaxanthin, respectively; the former is catalyzed by cytochrome P450 dependent hydroxylases while the latter is mediated by β-carotenoid hydroxylase (CHYb). Zeaxanthin can be converted stepwisely to violaxanthin and neoxanthin, catalyzed by zeaxanthin epoxidase (ZEP) and neoxanthin synthase (NXS), respectively. Violaxanthin, on the other hand, can be converted back to zeaxanthin by the action of violaxanthin de-epoxidase (VDE). Except the primary
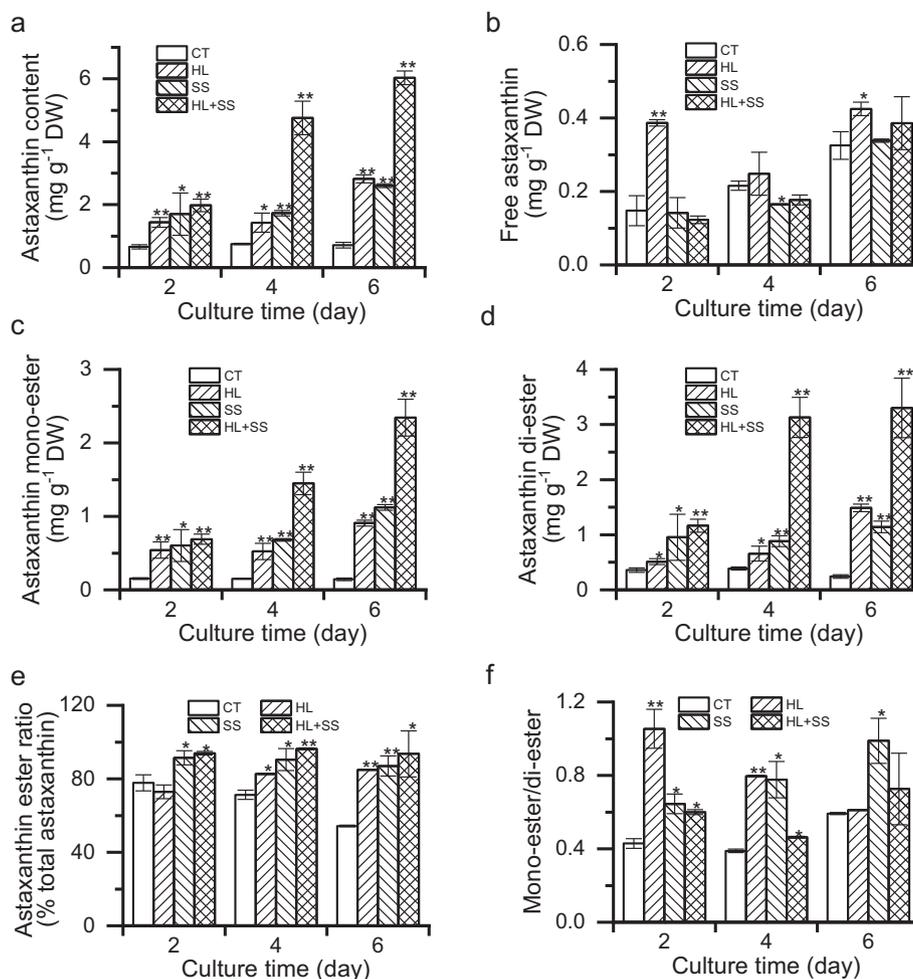


**Fig. 2.** Effect of stress conditions on astaxanthin profiles in *C. zofingiensis*. (a) Astaxanthin content. (b-d) Content of free astaxanthin (b), mono-ester (c) and di-ester (d). (e) Percentage of astaxanthin ester based on total astaxanthin. (f) Mono-ester/di-ester ratio. CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS. Data are expressed as mean ± SD (*n* = 3). Significant difference (compared to CT; student *t*-test) at *p* < 0.01 and *p* < 0.05 level is indicated with * and **, respectively.

**a**

|  | HL (VS CT) | | | SS (VS CT) | | | HL+SS (VS CT) | | | Time (day) |
|---|---|---|---|---|---|---|---|---|---|---|
|  | 2 | 4 | 6 | 2 | 4 | 6 | 2 | 4 | 6 |  |
| β-carotene | * | ** | ** |  | ** | ** | ** | ** | ** |  |
| Lutein/zeaxanthin | * | * | * |  | * | * | ** | ** | ** |  |
| Violaxanthin |  | ** | ** |  | ** | * |  | ** | ** |  |
| Neoxanthin | ** | ** | ** | * | ** | ** | ** | ** | ** |  |
| Primary carotenoids | * | ** | ** |  | * | ** | ** | ** | ** |  |
| Asataxanthin | ** | * | ** | * | ** | ** | * | ** | ** |  |
| Adonixanthin |  | * | ** | * | ** | ** | * | ** | ** |  |
| Echinenone |  |  | * | * |  |  | * | ** | ** |  |
| Canthaxanthin |  |  | * |  | ** | ** | * | ** | ** |  |
| Ketolutein | * | * | ** | * | * | ** | * | * | ** |  |
| Secondary carotenoids |  |  | ** | * | ** | ** | * | ** | ** |  |

-5    0    5   Log₂(fold change)

**b**

|  | HL (VS CT) | | | | SS (VS CT) | | | | HL+SS (VS CT) | | | | Time (h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 1 | 3 | 6 | 12 | 1 | 3 | 6 | 12 | 1 | 3 | 6 | 12 |  |
| LCYb |  |  |  |  |  | * |  |  | ** | ** |  | * |  |
| BKT1 | * |  | * | * | ** | ** | ** | * | ** | ** | * | ** |  |
| BKT2 |  |  | ** | ** | ** |  | ** | ** | ** |  | ** | ** |  |
| CHYb |  | ** | ** | * |  |  |  | * | ** | ** |  | * |  |
| AAT |  | * |  | ** | ** |  |  | ** | ** |  |  |  |  |
| ZEP | * |  |  |  | ** |  |  |  |  | ** | ** | * |  |
| NXS | * | * | * | ** |  |  |  | ** | * | ** | ** | ** |  |
| VDE |  |  |  |  | ** |  |  |  | ** |  |  |  |  |
| LCYe | ** | ** | * | ** | ** | ** | * | ** | ** | ** | ** | * |  |

**Fig. 3.** Dynamic changes of carotenoids (a) and expression of selected carotenogenic genes (b) in *C. zofingiensis* under different conditions. Heat map showing log₂ (fold change) values of carotenoids relative to CT (VS CT) under different stress conditions (a) and of gene transcripts relative to CT (VS CT) under different stress conditions (b). CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS; LCYb, lycopene β-cyclase; BKT, β-carotenoid ketolase; CHYb, β-carotenoid hydroxylase; AAT, astaxanthin acyltransferase; ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase; VDE, violaxanthin de-epoxidase; LCYe, lycopene ε-cyclase. Data are expressed as mean ± SD (*n* = 3). Significant difference (student *t*-test) at p < 0.01 and p < 0.05 level is indicated with * and **, respectively.

carotenoids, *C. zofingiensis* and *H. pluvialis*, attributed to the presence of β-carotenoid ketolase (BKT), can synthesize secondary carotenoids, such as echinenone, canthaxanthin, adonirubin, adonixanthin, astaxanthin and ketolutein [11,31]. The genes encoding these enzymes have all been identified in *C. zofingiensis* [15,23].

To understand the underlying mechanisms for astaxanthin biosynthesis in *C. zofingiensis*, the profile of carotenoids and transcriptional expression pattern of carogenogenic genes were monitored under different induction conditions (Fig. 3). Clearly, the primary carotenoids β-carotene, lutein, zeaxanthin, violaxanthin and neoxanthin all declined in response to HL, although to varying extents (Fig. 3a). Similarly, these primary carotenoids all showed a decrease under the SS condition (Fig. 3a). In comparison with HL or SS, HL + SS led to a more severe reduction of primary carotenoids (Fig. 3a). Secondary carotenoids including echinenone, canthaxanthin, adonixanthin, astaxanthin and ketolutein, on the other hand, were all induced to accumulate under the HL, SS and HL + SS conditions, with HL + SS being most effective (Fig. 3a). It is worth mentioning that the amount of decreased primary carotenoids was close to the amount of increased secondary carotenoids, consistent with the observation occurring in *C. zofingiensis* under the nitrogen deprived (ND) condition [23]. These results suggest

that in this alga, regardless of induction conditions, secondary carotenoids are mainly derived from the conversion of pre-existing primary carotenoids.

Quantitative real-time PCR (qPCR) was used to examine the transcriptional expression of nine carotenogenic genes, namely, *LCYb*, *LCYe*, *BKT1*, *BKT2*, *CHYb*, *AAT*, *ZEP*, *VDE* and *NXS*. Generally, the expression pattern of these carotenogenic genes was consistent among the three stress conditions of HL, SS, and HL + SS, yet the latter triggered a greater change in the gene's transcripts than the other two conditions did (Fig. 3b). Considering that the change of carotenoids followed a similar pattern among the three stress conditions and HL + SS induced a bigger variation in the carotenoid levels (Fig. 3a), the transcriptional regulation of carotenoid synthesis may occur in *C. zofingiensis*. *LCYb* was up-regulated while *LCYe* was down-regulated (Fig. 3b), indicative of the enhanced synthesis of β-carotene and attenuated synthesis of lutein. Probably, upon stress conditions, carotenoid flux is rerouted from lutein to β-carotene for sustaining astaxanthin biosynthesis. The decrease of lutein may also be contributed by the up-regulation of *BKT2* (Fig. 3b), which encodes a proposed enzyme responsible for the formation of ketolutein from lutein [32]. Accordingly, ketolutein accumulated under stress conditions (Fig. 3a). BKT1 and CHYb that work together to convert β-carotene to astaxnathin in *C. zofingiensis* [15,23,32] and AAT, an acyltransferase possibly involved in the formation of astaxanthin ester [15], were up-regulated by stress conditions particularly HL + SS (Fig. 3b), supporting the considerably augmented synthesis and accumulation of astaxanthin (mainly in ester form) (Fig. 2). It is worth mentioning that *C. zofingiensis* utilizes a route different from *H. pluvialis* for astaxanthin synthesis, namely, ketolation of zeaxanthin rather than hydroxylation of canthaxanthin [23]. Therefore, as is in the astaxnathin biosynthetic pathway-reconstructed Arabidopsis [33], tomato [34] or *Chlamydomonas reinhardtii* [35], the availability of zeaxanthin may represent a bottleneck for astaxnathin synthesis in *C. zofingiensis*. Considering that *BKT1* was more up-regulated than *CHYb* (Fig. 3a), the de novo synthesis of zeaxanthin may not be sufficient to support the downstream ketolation reactions for astaxanthin production. To compensate for this, zeaxanthin synthesis via the violaxanthin cycle was likely stimulated under stress conditions particular HL + SS, as indicated by the down-regulation of *ZEP* and *NXS* and the up-regulation of *VDE* (Fig. 3b). Accordingly, *C. zofingiensis* exhibited a sizable decline in violaxanthin and neoxanthin levels under stress conditions (Fig. 3a). The reversible conversion between zeaxanthin and violaxanthin catalyzed by ZEP and VDE, referred to as the violaxanthin cycle, is widely present in higher plants and algae and plays important roles in adapting to environmental changes [36]. Under favorable growth conditions, violaxanthin synthesis via zeaxanthin epoxidation is stimulated; when exposed to environmental stresses such as HL illumination, de-epoxidation of violaxanthin is switched on to synthesize zeaxanthin for protecting against photooxidative damages [37]. This also occurs in *C. zofingiensis* [38]. Nevertheless, because of the action of BKT, *C. zofingiensis* zeaxanthin is transformed to astaxanthin, which accumulates and contributes to protecting algal cells from stress-associated damages [4,10,39]. Therefore, the violaxanthin cycle that produces zeaxanthin from de-epoxidation of violaxanthin is likely involved in astaxanthin biosynthesis in *C. zofingiensis*. This differs from *H. pluvialis* in which astaxanthin is dominantly derived from canthaxanthin hydroxylation instead of zeaxanthin ketolation [4].

### 3.4. Salinity stress enhances the high light-induced production of lipids

Compared with CT, HL promoted total fatty acid (TFA) accumulation in *C. zofingiensis* (Fig. 4a). Similarly, SS enhanced TFA production, but in a greater extent; TFA content reached 284 mg g⁻¹ DW on day 6 and was 2.8-fold higher than that achieved under the CT condition (Fig. 4a). HL + SS, on the other hand, gave rise to the highest TFA level, which reached 423 mg g⁻¹ DW on day 6 and was 4.0-fold greater than that under the CT condition (Fig. 4a). Notably, the increased
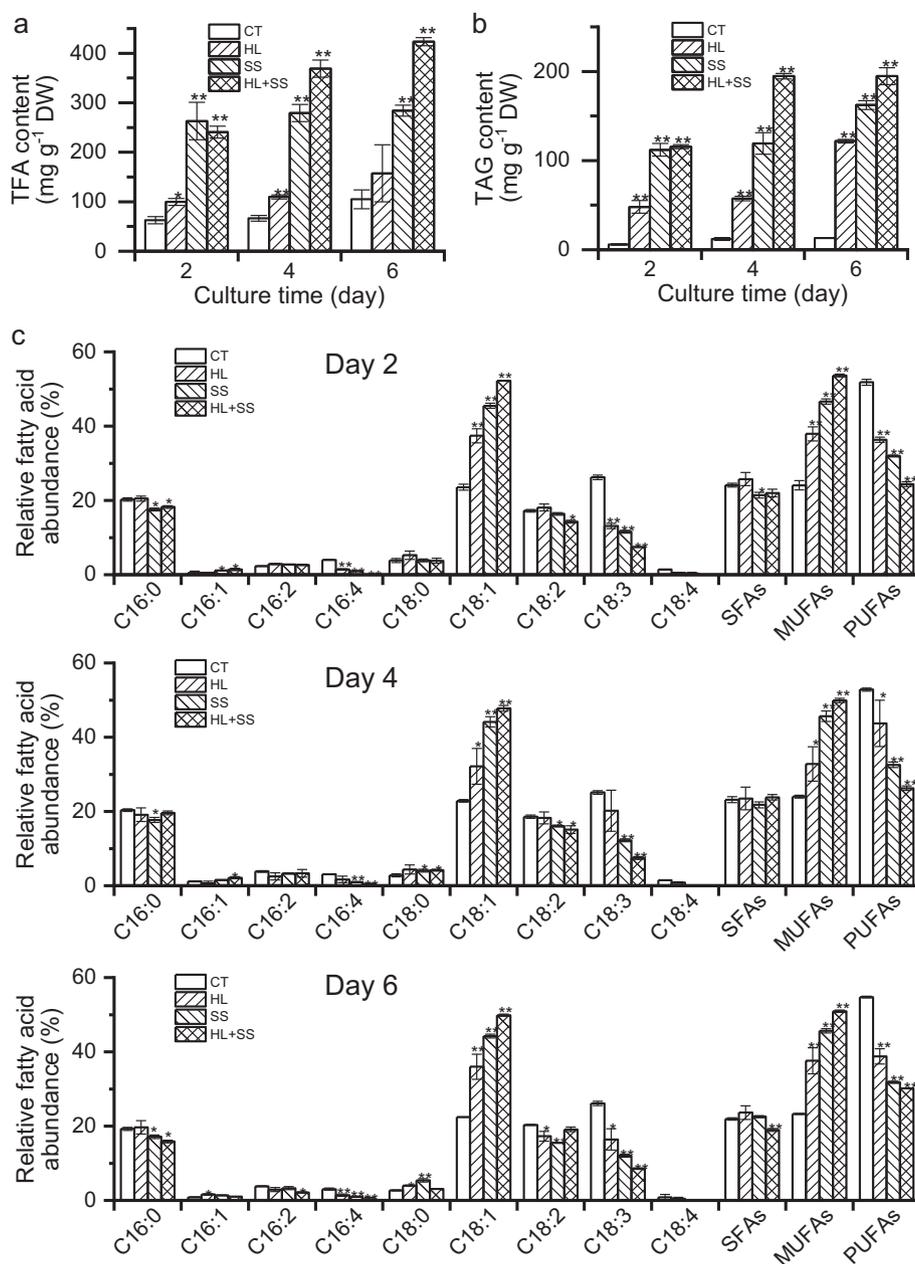
**Fig. 4.** Effect of stress conditions on lipid profiles in *C. zofingiensis*. (a) TFA content. (b) TAG content. (c) Relative fatty abundance in TFA. CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS; TAG, triacylglycerol; TFA, total fatty acid; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Data are expressed as mean ± SD ($n$ = 3). Significant difference (compared to CT; student *t*-test) at p < 0.01 and p < 0.05 level is indicated with * and **, respectively.

amount of TFA caused by HL + SS exceeded the sum of that caused by HL and SS (day 4 and day 6), suggesting that the combination of HL and SS showed a synergistic effect on fatty acid production. TAG, the highest energy-dense lipid, was only present in a trace amount under the CT condition (Fig. 4b). By contrast, stress conditions triggered a considerable increase in TAG content over the culture period, with HL + SS being most effective followed by SS and HL (Fig. 4b; Supplemental Fig. S1). Specifically, TAG content on day 6 were 122, 162 and 195 mg g$^{-1}$ DW for HL, SS and HL + SS conditions, 9.4-, 12.5-, and 15.0-fold greater than that under the CT condition, respectively.

Considering that fatty acid composition determines the quality of biodiesel [40], *C. zofingiensis* fatty acids under different culture conditions and time points were profiled in this study. Under the CT condition, C18:3, C18:2, C18:1 and C16:0 were the major fatty acids and together accounted for over 86% of TFA over the culture period

(Fig. 4c). It is reasonable as under the favorable growth conditions, the lipid pool is dominated by the membrane polar lipids particularly monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), which are rich in polyunsaturated fatty acids (PUFAs) especially C18:3 [16,22,41]. Upon stress conditions, C18:1 showed a drastic increase and reached up to 50% of TFA, accompanied by a severe decline in PUFAs particularly C18:3, while the saturated fatty acids (SFAs) including C16:0 and C18:0 only changed slightly (Fig. 4a). The increase of C18:1 abundance at the expense of PUFAs particularly C18:3 seems to be ubiquitous for *C. zofingiensis* under various stress conditions (Fig. 4c) [17,23,26]. It is believed that C18:1 helps balance the oxidative stability and low-temperature properties of biodiesel, and a high proportion of C18:1 is desirable [40]. In this context, *C. zofingiensis* lipids that are produced under stress conditions (rich in C18:1) are suitable for biodiesel production.
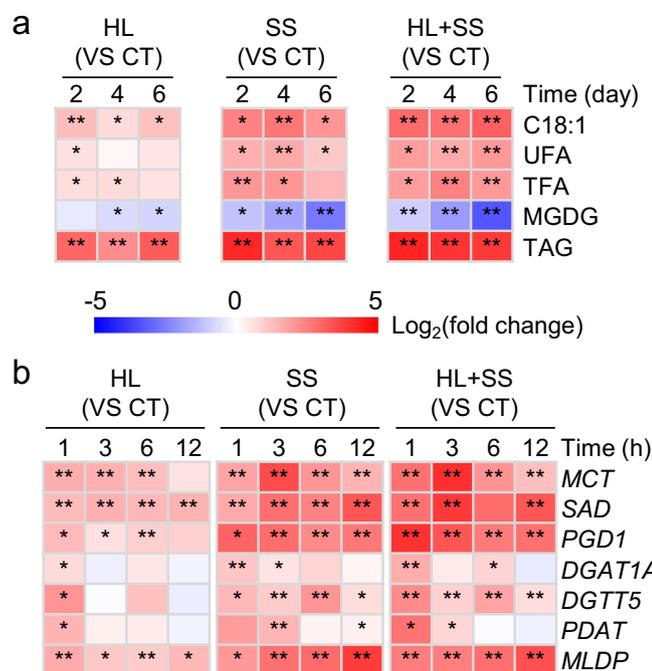
**Fig. 5.** Dynamic changes of lipids (a) and expression of selected lipogenic genes (b) in *C. zofingiensis* under different conditions. Heat map showing log₂ (fold change) values of lipids relative to CT (VS CT) under different stress conditions (a) and of gene transcripts relative to CT (VS CT) under different stress conditions (b). CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS; UFA, unsaturated fatty acids; TFA, total fatty acid; TAG, triacylglycerol; MGDG, monogalactosyl diacylglycerol; MCT, malonyl-CoA:acyl carrier protein transacylase; SAD, stearoyl-ACP desaturase; PGD1, plastid galactoglycerolipid degradation1; DGAT1A, diacylglycerol acyltransferase type IA; DGTT5, diacylglycerol acyltransferase type II5; PDAT, phospholipid:diacylglycerol acyltransferase; MLDP, major lipid droplet protein. Data are expressed as mean ± SD ($n$ = 3). Significant difference (student $t$-test) at p < 0.01 and p < 0.05 level is indicated with * and **, respectively.

### 3.5. De novo fatty acid synthesis and membrane lipid turnover contribute to storage lipid synthesis

To understand the stress-associated lipogenesis in *C. zofingiensis*, the profile of lipids and transcriptional expression pattern of key selected lipogenic genes were monitored under different induction conditions (Fig. 5). Generally, algae and higher plants follow similar pathways for lipid metabolism, such as de novo fatty acid synthesis, fatty acid desaturation, synthesis and turnover of membrane lipids, TAG assembly, etc. [42]. Based on the high-quality genome sequence of *C. zofigiensis* [15], we have recently reconstructed the pathways of lipid metabolism [16]. De novo fatty acid synthesis starts from acetyl-CoA, which is converted to malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACCase) and further to malonyl-ACP by malonyl-CoA:acyl carrier protein

transacylase (MCT).

Malonyl-ACP enters the type II FAS reaction and the reaction repeats eight times to form C18:0-ACP, which can be further converted to C18:1-ACP via the action of stearoyl-ACP desaturase (SAD). Consistent with the increase of TFA (Fig. 5a), the up-regulation of *MCT* and *SAD* was triggered by stress conditions in the order of HL + SS, SS and HL (Fig. 5B), suggesting the stimulation of de novo fatty acid synthesis occurs in *C. zofingiensis*, yet to different extents in response to these stresses. SAD is believed to control the synthesis of C18:1 as well as other unsaturated fatty acids (UFAs) [43]. Accordingly, both C18:1 and UFAs showed an increase in their levels regardless of stress conditions (Fig. 5a). In addition to de novo synthesized fatty acids, the fatty acids recycled from membrane polar lipids also contribute to TAG synthesis in algae [41,44]. It has been demonstrated that PGD1, a lipase with strong preference on MGDG, is involved in the turnover of thylakoid membrane for C18:1 salvation and TAG assembly [44,45]. In *C. zofingiensis*, MGDG was the major membrane lipid and decreased severely in response to stress conditions (Fig. 5a). The extent of MGDG decrease generally agreed with the degree of *PGD1* up-regulation (Fig. 5b), pointing to the involvement of PGD1 in MGDG turnover in *C. zofingiensis*. As is in *C. reinhardtii* [45]. *C. zofingiensis* PDG1 likely plays a crucial role in the reorganization of thylakoid membrane composition and thereby coping with the reactive oxygen species (ROS) generated under adverse environmental conditions. It is believed that algal TAG is mainly assembled from two routes, the acyl CoA-independent route mediated by phospholipid:diacylglycerol acyltransferase (PDAT) and the acyl CoA-dependent route catalyzed by diacylglycerol acyltransferase (DGAT) [42]. Of the ten *C. zofingiensis* DGAT genes, *DGAT1A* and *DGTT5* contribute to the stress-associated accumulation of TAG [46]. *DGAT1A*, *DGTT5* and *PDAT* were all up-regulated under the three stress conditions, consistent with the drastic increase of TAG in *C. zonfigiensis* (Fig. 5). TAG, once synthesized, is packed into lipid droplets (LDs) for storage, stabilized by the major lipid droplet protein (MLDP), which is ubiquitous in green algae [47,48]. *C. zofingiensis MLDP* showed a remarkable up-regulation in response to stress conditions (Fig. 5B), supporting the need of MLDP for reserving increased TAG. Collectively, our data suggest that both de novo fatty acid synthesis and MGDG turnover contribute to synthesis and accumulation of the storage lipid TAG in *C. zofingiensis*.

### 3.6. Astaxanthin and TAG are synthesized in a coordinated way

The production of astaxanthin and lipids depends not only their contents but also biomass concentration. As HL gave rise to a higher biomass concentration and content of astaxanthin and lipids (TFA and TAG) than CT did (Figs. 1, 2 and 4), greater yield of either astaxanthin or lipids was achieved (Table 2). SS also enhanced the yield of both astaxanthin and lipids, yet to a less extent than HL did (Table 1), due to the adverse effect on algal growth (Fig. 1). By contrast, HL + SS led to the highest yield, which reached 41.8 mg L$^{-1}$, 3.03 g L$^{-1}$, and 1.39 g L$^{-1}$ for astaxanthin, TFA and TAG, respectively (Table 2). Accordingly, the greatest productivities for astaxanthin

**Table 2**
Production of TFA, TAG and astaxanthin by *C. zofingiensis* under different conditions.

| | Yield | | | Productivity | | | |
|---|---|---|---|---|---|---|---|
| | Astaxanthin (mg L$^{-1}$) | TFA (g L$^{-1}$) | TAG (g L$^{-1}$) | Biomass (g L$^{-1}$ day$^{-1}$) | Astaxanthin (mg L$^{-1}$ day$^{-1}$) | TFA (g L$^{-1}$ day$^{-1}$) | TAG (g L$^{-1}$ day$^{-1}$) |
| CT | 4.9 ± 0.60 | 0.73 ± 0.05 | 0.09 ± 0.00 | 1.06 ± 0.01 | 0.8 ± 0.11 | 0.12 ± 0.01 | 0.02 ± 0.00 |
| HL | 29.9 ± 3.47 | 1.78 ± 0.31 | 1.27 ± 0.12 | 1.65 ± 0.10 | 5.0 ± 0.58 | 0.30 ± 0.05 | 0.21 ± 0.02 |
| SS | 10.9 ± 0.80 | 1.00 ± 0.04 | 0.67 ± 0.08 | 0.61 ± 0.03 | 1.8 ± 0.13 | 0.17 ± 0.01 | 0.12 ± 0.01 |
| HL + SS | 41.8 ± 3.02 | 3.03 ± 0.07 | 1.39 ± 0.14 | 1.11 ± 0.01 | 7.0 ± 0.50 | 0.51 ± 0.02 | 0.24 ± 0.02 |

TFA, total fatty acid; TAG, triacylglycerol; CT, control; HL, high light; SS, salinity stress; HL + SS, the combination of HL and SS. The six-day cultures were used for analysis. Data are expressed as mean ± SD ($n$ = 3).

**Table 3**
Comparison of astaxanthin and TAG production by *C. zofingiensis* with previous reports.

| Stain | Culture conditions | Biomass (g L$^{-1}$) | Biomass productivity (g L$^{-1}$ day$^{-1}$) | Astaxanthin content (mg g$^{-1}$) | Astaxanthin yield (mg L$^{-1}$) | Astaxanthin productivity (mg L$^{-1}$ day$^{-1}$) | TAG productivity (g L$^{-1}$ day$^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|
| ATCC30412 | P, Batch | 7.2 | 1.11 | 6.0 | 41.8 | 7.0 | 0.24 | This study |
| ATCC30412 | P, T-S | 6.9 | 0.57 | 13.1 | 89.9 | 7.5 | – | [18] |
| ATCC30412 | H, FB | 4.4 | 5.80 | 0.7 | 47.3 | 4.0 | – | [10] |
| | P, T-S | 71.1 | 4.80 | 2.7 | 194.5 | 9.9 | – | |
| ATCC30412 | P, Batch | 1.2 | 0.12 | 3.9 | 4.5 | 0.6 | 0.04 | [17] |
| ATCC30412 | M, T-S | 98.4 | 7.03 | 0.8 | 73.3 | 5.2 | – | [21] |
| ATCC30412 | M, Batch | 6.0 | 0.50 | 6.5 | 38.9 | 3.2 | – | [9] |
| UTEX32 | P, S-C | – | 1.04 | 3.2 | – | 3.3 | 0.32 | [8] |
| | P, Batch | 7.5 | 1.40 | 1.7 | 12.8 | 2.0 | 0.17 | |
| UTEX32 | P, Batch | 8.2 | 0.58 | 2.4 | 19.6 | 1.4 | 0.32 | [7] |
| ATCC30412 | M, Batch | 11.9 | 0.59 | 2.2 | 25.8 | 1.3 | – | [20] |
| ATCC30412 | H, FB | 45.6 | 4.56 | 1.2 | 56.1 | 5.6 | – | [6] |
| ATCC30412 | H, Batch | 12.9 | 1.55 | 1.1 | 13.6 | 1.7 | 0.53 | [12] |
| ATCC30412 | H, FB | 53.0 | 3.31 | 1.0 | 32.0 | 2.0 | – | [19] |
| CCAP 211/14 | P, Batch | 7.0 | 0.96 | 1.5 | 15.0 | – | – | [5] |

TAG, triacylglycerol; P, photoautotrophic culture; M, mixotrophic culture; H, heterotrophic culture; S–C, semi-continuous culture; FB, fed-batch culture; T-S, two-stage; –, not available.

(7.0 mg L$^{-1}$ day$^{-1}$), TFA (0.51 g L$^{-1}$ day$^{-1}$) and TAG (0.24 g L$^{-1}$ day$^{-1}$) were all achieved under the HL + SS condition (Table 2). Generally, astaxanthin and TAG productivities are higher or comparable to the values achieved in *C. zofingiensis* in previous reports (Table 3). Therefore, HL + SS represents a feasible strategy that can be applied to *C. zofingiensis* for production uses, as it triggers the synthesis of substantial amounts of astaxanthin and lipids and meanwhile reduces the footprint of freshwater, a scarce natural resource on the earth.

Clearly, astaxanthin and TAG (or C18:1) were induced to accumulate simultaneously under HL, SS and HL + SS conditions (Figs. 2 and 4). This also occurs in *C. zofingiensis* under other conditions such as ND, ND + HL and glucose induction [7,8,22,49] or in *H. pluvialis* [50], indicative of the coordinated synthesis of astaxanthin and TAG in *C. zofingiensis*. Nevertheless, TAG/astaxanthin or C18:1/astaxanthin ratio may vary greatly under different stress conditions, e.g., TAG/astaxanthin ratio among ND, HL, ND + HL and glucose induction varies from 1.4–3.8 folds [49]. Probably, astaxanthin and TAG (or C18:1) are less correlated across different stress conditions in *C. zofingiensis*. Therefore, it is not surprising to observe that SS gave rise to higher levels of TAG and C18:1 than HL (Fig. 4), yet the astaxanthin level was comparable between the two conditions (Fig. 2). It is worth noting that astaxanthin and TAG share and may compete for the carbon precursor pyruvate, which is derived from the photosynthetically fixed $CO_2$ under our culture conditions. Nevertheless, the ratio of astaxanthin to TFA is normally < 1.5% (Figs. 2 and 4), suggesting that the carbon flux is allocated predominantly to the synthesis of lipids particularly TAG. A mechanistic model for carotenogenesis (astaxanthin synthesis) and lipogenesis (TAG synthesis) in *C. zofingiensis* was proposed (Fig. 6): the key genes involved in both pathways were up-regulated to different extents in response to HL, SS and HL + SS, allowing the concurrent synthesis of astaxanthin and TAG, which were both packed into LDs for storage. It is likely that the transcriptional regulation of astaxanthin and TAG synthesis is involved, which may be stimulated by ROS generated under adverse conditions [8,39,45,50]. While serving as the secondary messenger at a certain level, excess ROS may induce oxidative stress and is thus detrimental to organisms [17,39,44]. To cope with the excess ROS-associated adverse effect, algae may have developed complex mechanisms including the elicited synthesis of TAG and/or astaxanthin, of which the former is believed to sequestrate excess electrons moving through the photosynthetic electron transport chain and therefore preventing the reduction of molecular oxygen for ROS generation [44], while the latter may serve as a strong antioxidant for the sequestration of ROS [4,17,50]. The specific mechanisms, however, remain to be disclosed.

## 4. Conclusions

Taken together, among HL, SS and HL + SS conditions, the latter was most effective in inducing the synthesis of astaxanthin and TAG in *C. zofingiensis*. HL + SS also gave rise to the highest productivities, which were 7.0 mg L$^{-1}$ day$^{-1}$ and 0.24 g L$^{-1}$ day$^{-1}$ for astaxanthin and TAG, respectively. Carotenogenesis and lipogenesis were regulated in a coordinated way, leading to the concurrent synthesis of astaxanthin and TAG regardless of stress conditions, yet predominant carbon flux was allocated to the latter. Our findings shed new light on understanding astaxanthin and TAG synthesis and provide a feasible way for simultaneous production by *C. zofingiensis*.

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### Credit author statement

Yaping Kou: Investigation, Formal analysis, Methodology, Writing - original draft. Meijing Liu: Formal analysis, Methodology. Peipei Sun: Methodology. Zhaoqi Dong: Methodology. Jin Liu: Conceptualization, Methodology, Supervision, Writing - review & editing.

### Author contributions

Yaping Kou and Jin Liu conceived the study. Yaping Kou, Meijing Liu, Peipei Sun and Zhaoqi Dong performed the experiments. Yaping Kou drafted the manuscript. Jin Liu reviewed and edited the manuscript. All authors read and approved the final manuscript.

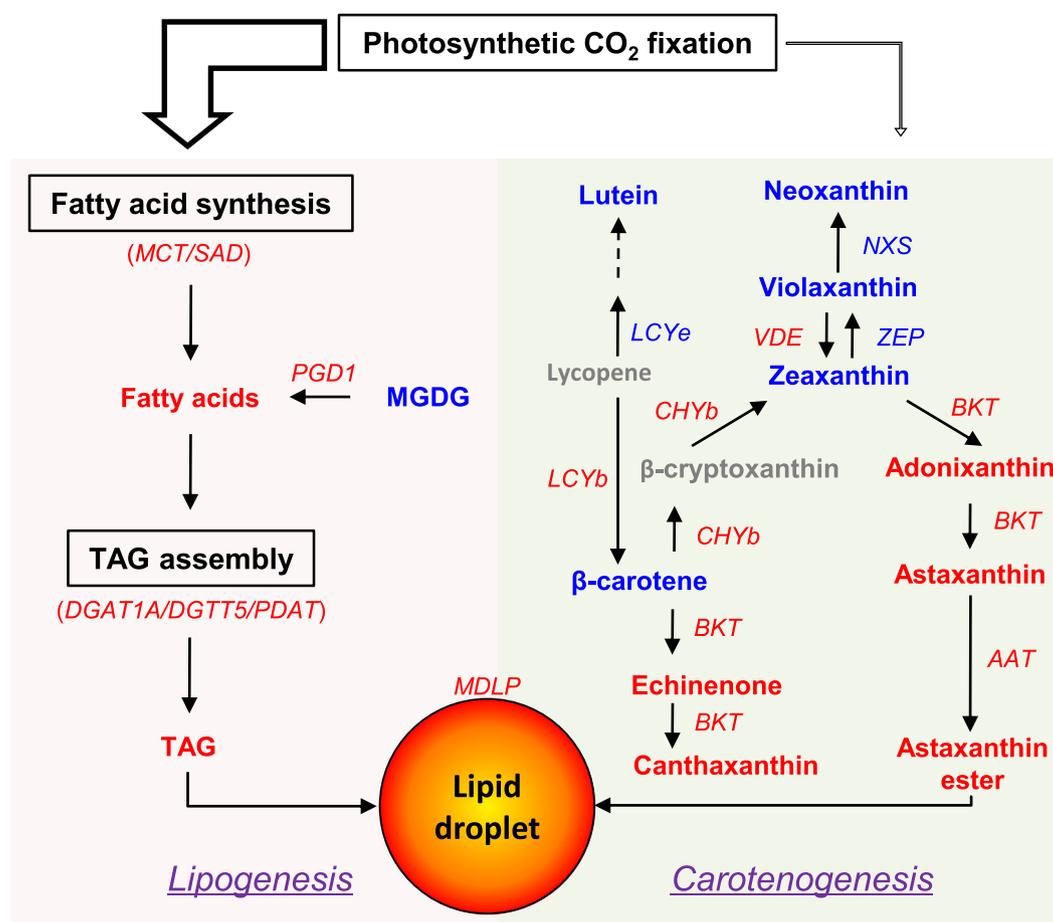### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Statement of informed consent, human/animal right

No conflicts, informed consent, human or animal rights applicable.

**Fig. 6.** A mechanistic model for the stress-induced carotenogensis and lipogenesis in *C. zofingiensis*. Compounds are showed in bold: red and blue designate the increase and decrease in response to stress conditions, respectively, while gray indicates not detected compounds. Enzymes are showed in italics: red and blue indicate the up-regulation and down-regulation in response to stress conditions, respectively. AAT, astaxanthin acyltransferase; BKT, β-carotenoid ketolase; CHYb, β-carotenoid hydroxylase; DGAT1A, diacylglycerol acyltransferase type IA; DGTT5, diacylglycerol acyltransferase type II5; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; MGDG, monogalactosyl diacylglycerol; MLDP, major lipid droplet protein; NXS, neoxanthin synthase; PDAT, phospholipid:diacylglycerol acyltransferase; PGD1, plastid galactoglycerolipid degradation1; SAD, stearoyl-ACP desaturase; TAG, triacylglycerol; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase. The thickness of the right angle arrow designates the flux of carbon allocated for lipogenesis and carotenogenesis (not to scale). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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