Enhancement of lutein biosynthesis in *chlorella vulgaris-1068* under low temperature and luminosity stress conditions

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Abstract: Lutein, a high-value primary carotenoid with a broad range of applications in food, feed, nutraceutical, and pharmaceutical industries, has been widely studied in recent years. The green microalgae *Chlorella vulgaris-1068*(*C.v-1068*) screened by our lab showed both a high content in this carotenoid and a high growth rate and has a good profile for lutein production. This work studies the variety of environmental stimulants which enhance cellular accumulation of lutein from *C.v-1068*. The lutein accumulation and its biosynthesis pathway genes expression were analyzed under low temperature and high luminosity. We find that both cold and high light stresses can induce lutein biosynthesis in *C.v-1068*. In addition, during dark to light transition also leads to higher lutein productivity and the maximal lutein content (2.86 mg/g DW) was attained after six days dark-cultivated then exposed to 1000 μ photons m^{-2} s^{-1} for two days treatment. Consistently, the transcript levels of lutein biosynthesis genes *PSY* and *ZDS* were largely enhanced by cold, high light and dark to light transition treatments. And except *PSY* and *ZDS*, 1000 μ photons m^{-2} s^{-1} luminosity stress also highly increased expression of *PDS* as compared with low irradiance condition.

Keywords: lutein, microalgae, cold and luminosity stress, gene expression

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1 Introduction

Carotenoids are an important group of natural pigments that are synthesized by all photosynthetic organisms as well as some non-photosynthetic bacteria and fungi (Goodwin, 1980). Lutein, as primary carotenoids, plays essential roles in photosynthesis, nutrition and protection against photooxidative damage in higher plants (Boussiba, 2000; Dweyer et al., 2001; Olmedilla et al., 2003; Stahl et al., 2005). In recent years, additional applications for lutein, especially in the field of human health, have been found. Lutein is known to play a critical function in prevention of certain cancers and chronic diseases (Nkondjock et al., 2005). Currently the commercial source of lutein is French marigold (*Tagetespatula*) (Campo et al., 2007). However, mass plantation of marigold occupies a large land area and is easily influenced by season and climate. Therefore, there is an increasing interest in microalgae as an alternative source of this carotenoid. *ChlorellaVulgaris-1068* screened by our lab is a strain that produces a high yield of lutein, which is proposed as potential source of lutein. Nevertheless, its lutein values are not high enough to be economically feasible on an industrial scale. To further improve lutein accumulation and productivity, the mechanism for molecular regulation of lutein levels is needed to be understood.

The lutein biosynthesis pathway in *ChlorellaVulgaris-1068* is similar to that of higher plants and is highly conserved in all carotene genic organisms (Liang et al.,

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2006; Sieiro et al., 2003). The process starts with the condensation of two geranyl pyrophosphate (GGPP) molecules to form the first carotene, phytoene, catalyzed by phytoene synthase (PSY) (Figure 1). Then, four double bonds are introduced into the phytoene by phytoene desaturases (PDS) and ζ-carotene desaturases (ZDS) to produce pro-lycopene, which is isomerized by a specific isomerase to all-*tans* lycopene. The cyclation of lycopene by lycopene ε-cyclase (LYCE) and lycopene β-cyclase (LYCB) introduces ε- and β-ionone end groups, respectively, yielding α- and β-carotenes (Harjes et al., 2008). α-carotene is modified to lutein by hydroxylation of one β- and one ε-ring, catalyzed by two heme-containing cytochrome, β-carotene hydroxylase (BHY) and ε-carotene hydroxylase (EHY), respectively. Although the biosynthesis pathway is well known by us, the regulatory mechanisms that control lutein biosynthesis are poorly understood.

In this study, we provide biochemical and molecular evidence to demonstrate: (1) the effect of some environmental factors on lutein production in *ChlorellaVulgaris-1068*; (2) the molecular mechanism for environmental regulation of lutein biosynthesis.

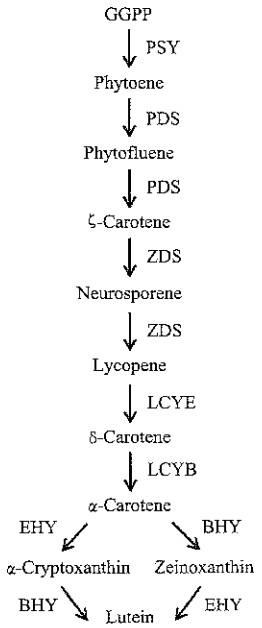


Figure 1 Schematic diagram of the lutein biosynthetic pathway and enzymes in plants and microalgae.

2 Materials and methods

2.1 Microalgae strain and culture conditions

The microalgae used in this study was *ChlorellaVulgaris-1068*, purchased from the Freshwater Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Science, Wuhan, China.

2.2 Cultivation and stress conditions

2.2.1 Medium

C.v-1068 was cultivated in an algae culture medium, which composition per liter of distilled water is: 400 mg NH_4Cl, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, 108 mg K_2HPO_4, 56 mg KH_2PO_4, 2420 mg Tris (hydroxymethyl) aminomethane, 1 mL Glacial acetic acid, 1 mL trace elements solution consisted of 50 g Na_2EDTA, 22 g $ZnSO_4 \cdot 7H_2O$ (/L), 11.4 g H_3BO_3, 5.06 g $MnCl_2 \cdot 4H_2O$, 4.99 g $FeSO_4 \cdot 7H_2O$, 1.61 g $CoCl_2 \cdot 6H_2O$, 1.57 g $CuSO_4 \cdot 5H_2O$, 1.10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 16 g KOH.*C.v-1068* strain was grown in 1 L Erlenmeyer flasks and continuously shaken at 80 r/min, 25°C in an incubator, illuminated with LED lamp at 150 μmol photons m^{-2} s^{-1} (μE) for 12 h per day.

2.2.2 Stressed phase: effect of temperature and illumination

C.v-1068 was grown in the algae medium for two weeks for biomass growth, and then the temperature and irradiances were changed. The temperature was decreased to 15°C and 4°C and the two high irradiances, 1000 μE and 2000 μE were treated. The experiments were carried out for three days.

2.2.3 Photoperiod effect

Two weeks' growth *C.v-1068* were transferred to various time periods of darkness, two days, four days or six days, then separately exposed to 150 μE for 6 d, 4 d and 2 d to induce lutein biosynthesis.

2.3 Harvesting

The microalgae were harvested by decantation prior to centrifugation at 10,000 rpm, at 15°C for 15 min (Beckman, Avanti J-251), then freeze-dried and ground and dry weight using glass fiber filters (GF/C, 47 mm, Whatman) at 80°C overnight.

2.4 Lutein extraction and HPLC analysis

Cells of *C.v-1068* were harvested by centrifugation and washed twice with distilled water. The pigment was extracted with 80% of acetone (v/v) according to leon et al (2005). Then the sample was centrifuged (2862×g, 5 min), and the supernatant was obtained for HPLC

analysis.

Lutein extracts were analyzed by HPLC (Agilent 1260series) as described by Wang, et al. (2010) equipped with a quaternary pump, automatic injector, degasser, column heater, ultraviolet detector and a data acquisition system. The extracts were separated with C18 column (Shim-packVP-ODS, 5 μm, 4.6 mm×250 mm) and the eluted compounds were analyzed at 445 nm. Mobile phase was the mixture of methanol and acetonitrile in a binary isocratic elution, keeping the ratio of 90:10 for 15 min. Flow rate was 1.0 mL/min and the column temperature was set to 20°C. The solvents were of HPLC-grade and filtered in 0.20 μm organic membranes before use. Samples were also filtered before injection.

2.5 Ribonucleic acid (RNA) extraction and quantitative real-time PCR (qRT-PCR)

The microalgae total RNA was extracted by RNA extraction kit (Tiangen). The first-strand cDNA was synthesized by reverse transcriptase (Invitrogen). Real-time PCR was performed with the SYBR Premix ExTaq kit (Takara) in a 15 mL reaction system following the manufacturer's instructions. Three biological replicates were performed for each sample, and the expression levels were normalized to those of *CBLP*, which encodes a G-protein β-subunit-like polypeptide. All primers sequences are listed in Table 1.

Table 1 Nucleotide sequences of primer pairs used for PCR amplification

Primer	Sequence(5'→3')
PSY-F	ATTCAGGGACATGATTGGG
qPSY-R	CCCTTGTACGCTTTGTCAAC
qZDS-F	AGCTCGACTTCCGCTTCTAC
qZDS-R	CACCTTGTCGGTGAAGGAC
qLYCE-F	AGGGCTGCTCATCCAGAG
qLYCE-R	AGATGCACGAGGAGGAGTG
qLYCB-F	CCTTTGAGCTGGACACCAT
qLYCB-R	CTTCAGGTCAGGGAAGTCAAC
qPDS-F	TGCTGGAGCTGGTCTTTGCA
qPDS-R	CGCACTCGGGCACCGTCTTG
qCBLP-F	CGCCACCAGTCCTCCATCAAGA
qCBLP-F	CTAGGCGCGGCTGGGCATTTAC

3 Results and discussion

3.1 Cold stress induces lutein biosynthesis in *Chlorella vulgaris-1068*

Temperature is one of the most important environmental factors affecting many biosynthetic pathways, including lutein biosynthesis (Hayman et al., 1974). To assess the influence of temperature on lutein level of *C.v-1068*, two-week-old normal cultured cells were transferred to cold stress, and the cellular lutein contents were recorded after three days. We found that lutein accumulation increased by about 40% with temperature down to 4°C and the maximum cellular lutein content (2.27 mg/g DW) was obtained at 15°C in the early deceleration phase of growth (Figure 2). These data indicate that temperature plays an important role in lutein biosynthesis of *C.v-1068*, and it maybe control the concentration of enzymes to regulate lutein level in algae cell.

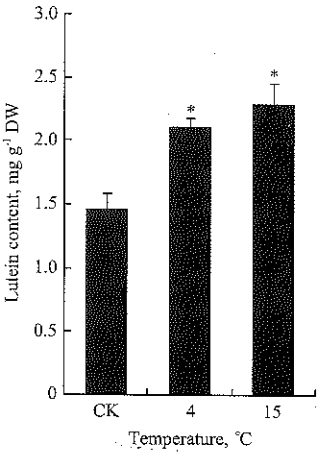


Figure 2 Effect of temperature on lutein accumulation in *Chlorella vulgaris-1068* 2-w-old normal cultured cells were grown at the indicated temperature and 25°C as a control (CK), the rest of culture conditions being the standard ones described in methods. Lutein content data are the mean values of three independent measurements recorded after 3 days. Asterisks indicate significant differences of 4°C and 15°C compared with CK based on Student's t test, $P<0.05$ (*)

3.2 Higher irradiance promotes lutein accumulation in *C.v-1068*

Lutein production and accumulation are reported to be positively affected by light irradiation in algae (Boussiba and Vanshak, 1991). We investigated lutein accumulation in *C.v-1068* bath cultures at different irradiances. Similar to cold treatment, 2-w-old normal cultured cells were transferred to light intensities of 1000 μmol photons m^{-2} s^{-1} or higher irradiance for three days. Lutein content in the culture was enhanced by 1.5-fold as irradiance increased from 150 to 1000 μE, decreasing at higher irradiance value (Figure 3). The

maximum cellular lutein content (2.32 mg g^{-1} DW) was reached at 1000 μE.

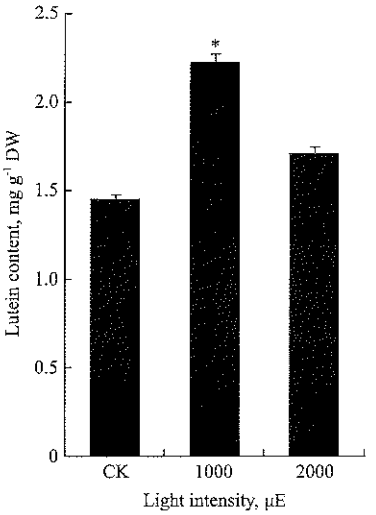


Figure 3 Effect of irradiance on lutein accumulation in *Chlorella vulgaris-1068*

Lutein contents of 2-w-old normal cultured cells transferred to high light (1000 μmol m^{-1} s^{-1}, 2000 μmol m^{-1} s^{-1}) for 3 days and 150 μmol photons m^{-2} s^{-1} as a control (CK). Error bars indicate the standard deviations of three independent measurements. Asterisks indicate significant differences of 1000 μE compared with CK based on Student's t test, $P<0.05$ (*).

3.3 Dark to light transition boost lutein biosynthesis in*C.v-1068*

Reactive oxygen species (ROS), generated by excess photooxidation caused by high light irradiance, has been found to play a crucial role upon lutein synthesis (Fernández et al., 2010). In darkness, the algae cells accumulate excess free protochlorophyllide (pchlide). When upon light illumination, excess pchlide may generate ROS or free radicals to enhance lutein biosynthesis and protect cell from ROS damage (Wagner et al., 2004). To investigate the influence of ROS on lutein level of *C.v-1068*, 2-w-old algae cells grown in different periods of darkness were illuminated with high intensity white light (1000 μE), and the lutein contents were menstruated after an additional two days. We found that the lutein accumulation increased by about 62% after four days or longer of dark treatment and the maximum cellular lutein content (2.61 mg/g DW) was obtained after six days treatment (Figure 4). We thus conclude that lutein holds a protection role conveyed by its antioxidant features.

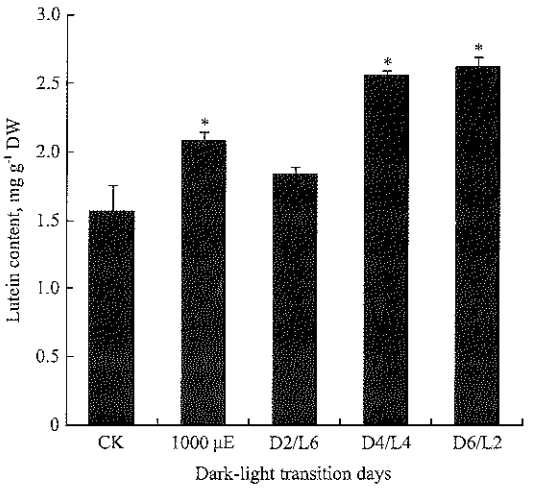


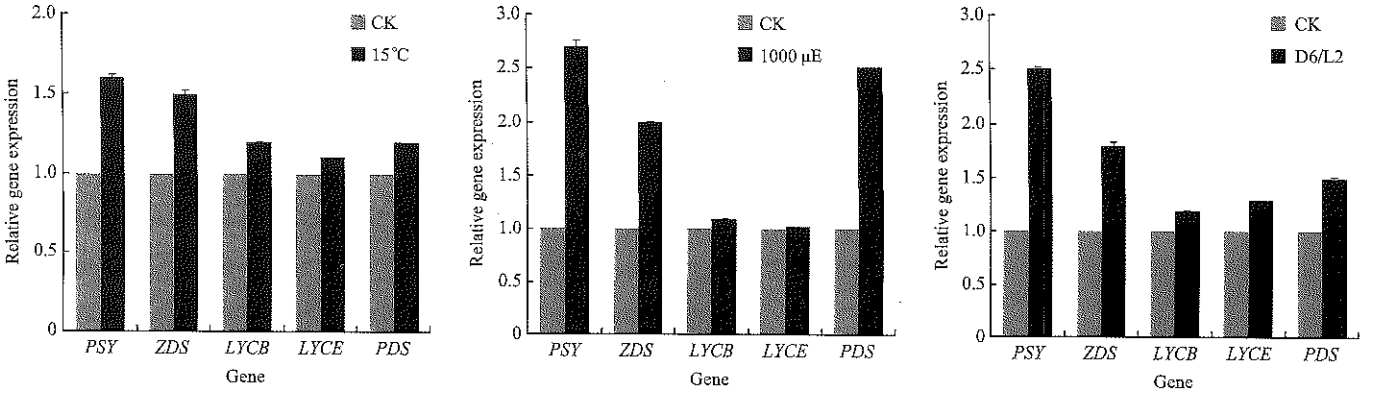
Figure 4 Effect of photoperiod on lutein accumulation in *Chlorella vulgaris-1068*

Two weeks' growth *C.v-1068* were transferred to various time periods of darkness, 2d, 4d or 6d, then separately exposed to 150 μE for 6 d, 4 d and 2 d to detect lutein contents. Cells cultivated at 150 μmol photons m^{-2} s^{-1} (μE) for 12h per day as a control (CK). Lutein content data are the mean values of three independent measurements. Asterisks indicate significant differences of 1000 μE, D4/L4 and D6/L2 compared with CK based on Student's t test, $P<0.05$ (*).

3.4 Effect of cold and irradiance on the expression of *C.v-1068* gene

To establish how the cold and luminosity stress affect the lutein biosynthesis, we analyzed the relative gene expression by quantitative RT-PCR (qRT-PCR). Cells of *C.v-1068* were grown at low irradiance and suitable temperature (as indicated in materials and methods) for two weeks. Then cells were kept in darkness for 18 h, in order to make the transcript levels come down to basal values. After this dark period, cells were subjected to either high irradiance or cold temperature. As shown in Figure 5a, under low temperature, the relative transcript levels of *PSY* and *ZDS* increased significantly, attaining 1.5-fold higher values than basal ones after 48 h. Similarly, high irradiance also had a strong effect on transcript levels of *PSY*, *ZDS* and *PDS*, which increased more than 2-flod higher as compared to basal level after 48 h (Figure 5b). To assess how expressions of lutein biosynthetic genes were regulated during dark to light transition, four days dark-grown cells was transferred to high light. Real-time RT-PCR analysis shown that the

PSY and *ZDS* transcript level were also largely elevated after light treatment for 48 h (Figure 5c). These findings indeed support the important role of environment in regulating lutein biosynthesis.



a. Cold stress treatment (15°C) for 2 days

b. 1000 μE high light treatment for 2 days

c. Six-day-darkness growth algae were transferred to 1000 μE high light for 2 days

Figure 5 Effect of temperature, irradiance and photoperiod on the mRNA levels of *PSY, ZDS, LYCB, LYCE, PDS*
Relative expression levels are normalized to the housekeeping control gene CFBLP. Error bars indicate the standard deviations of three independent measurements.

4 Conclusions

Microalgae as a new source of safe antioxidants has been the focus of extensive research. The microbial production of lutein has significant market and industrial potential. In this paper, we study the variety of environmental stimulants which can enhance volumetric production and cellular accumulation of lutein in green microalgae *C.v-1068*. Both cold and high light stresses can highly induce lutein biosynthesis and the transcript levels of lutein biosynthesis genes *PSY* and *ZDS* were consistently enhanced by cold, high light and dark to light transition treatments. Though manipulation of external stimulants can allow lutein production to be scaled-up for commercialization, the lutein synthesis also needs to be explored to the fullest extent. Modern technology, such as the use of recombinant DNA, coupled with the isolation of some of the vital genes involved in lutein biosynthesis have encouraged research into lutein production. Maybe the *PSY* and *ZDS* genes will be the suitable candidate factors.

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