Gene Expression Analysis of Zeaxanthin Epoxidase from the Marine Microalga *Dunaliella tertiolecta* in Response to Light/Dark Cycle and Salinity

Minjae Kim¹, Yongsoo Kang², and EonSeon Jin^{1,3*}

¹Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 04763, Republic of Korea

²Eugene Biotech Co. Ltd., Daejeon 34051, Republic of Korea

³Research Institute of Natural Sciences, Hanyang University, Seoul 04763, Republic of Korea

Received: April 26, 2019
Revised: August 2, 2019
Accepted: August 2, 2019

First published online
August 7, 2019

*Corresponding author
Phone: +82-2-2220-2561;
Fax: +82-2-2299-2561;
E-mail: esjin@hanyang.ac.kr

pISSN 1017-7825, eISSN 1738-8872

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Zeaxanthin is an important pigment in the photo-protection mechanism of microalgae. However, zeaxanthin epoxidase, an enzyme involved in the accumulation and conversion of zeaxanthin, has not been extensively studied in microalgae. In this work, we report the expression pattern of zeaxanthin epoxidase in *Dunaliella tertiolecta* (DtZEP) at different light and diverse salinity conditions. To confirm the responsiveness to light conditions, the ZEP expression pattern was investigated in photoperiodic (16 h of light and 8 h of dark) and continuous (24 h of light and 0 h of dark) light conditions. mRNA expression levels in photoperiodic conditions fluctuated along with the light/dark cycle, whereas those in continuous light remained unchanged. In varying salinity conditions, the highest mRNA and protein levels were detected in cells cultured in 1.5 M NaCl, and ZEP expression levels in cells shifted from 0.6 M NaCl to 1.5 M NaCl increased gradually. These results show that mRNA expression of DtZEP responds rapidly to the light/dark cycle or increased salinity, whereas changes in protein synthesis do not occur within a short period. Taken together, we show that DtZEP gene expression responds rapidly to light irradiation and hyperosmotic stress. In addition, ZEP expression patterns in light or salinity conditions are similar to those of higher plants, even though the habitat of *D. tertiolecta* is different.

Keywords: Zeaxanthin epoxidase, *Dunaliella tertiolecta*, carotenoid biosynthetic pathway, photoperiodic condition, continuous light condition, salinity condition

Introduction

Zeaxanthin is an important pigment in the photo-protection mechanism of microalgae and higher plants [1, 2]. In these organisms, zeaxanthin is accumulated under excessive light [2, 3] or dehydration stress conditions [4–6]. To understand how zeaxanthin is accumulated in response to these environmental conditions, the xanthophyll enzymes have been extensively studied in higher plants [7–9].

After the first report of zeaxanthin epoxidase (ZEP) from *Nicotiana plumbaginifolia* [9], a variety of ZEPs have been isolated and investigated in higher plants [5, 7, 10–15]. Generally, ZEP is located on the stromal side of the

chloroplast thylakoid membrane, and is involved in the conversion of the xanthophyll pigments together with violaxanthin de-epoxidase (VDE), which is located on the luminal side [8, 16]. When light energy is lower than that required for the saturation of the photosynthetic apparatus, zeaxanthin is converted to violaxanthin by ZEP [2]. On the other hand, when light energy is above the saturation levels of photosynthesis, a reversible reaction is initiated by VDE in the chloroplast, resulting in the accumulation of zeaxanthin [17]. ZEP expression increases in leaves under light conditions, but decreases significantly at drought stress conditions [5, 7, 18, 19]. Interestingly, ZEP shows different expression patterns in roots and leaves [5]. ZEP

expression levels in roots are increased significantly under drought [5, 19] or salt stress conditions [20]. These tissue-specific expression patterns of *ZEP* are thought to be related to different roles of *ZEP* in various tissues [5].

In microalgae, most studies have focused on identifying the role of the xanthophyll cycle in photo-protection using *ZEP* mutants [21–23], not on *ZEP* itself. However, some microalgal *ZEP*s have been described recently, and their characteristics have begun to be investigated [24, 25]. Although *ZEP* expression has been extensively studied in higher plants, microalgae and higher plants are morphologically different and are found in completely different environments. Microalgal *ZEP*s may thus show different expression patterns in response to environmental conditions than those in higher plants, and investigation is warranted.

Zeaxanthin accumulation in response to excessive light and salinity has been reported in *Dunaliella tertiolecta* [6] and other *Dunaliella* species [4, 27, 28]. We have previously characterized the DNA sequence and encoded protein of *ZEP* from *D. tertiolecta* of the phylum Chlorophyta [24]. In this study, we evaluated the expression pattern of *ZEP* in response to the light/dark cycle and salinity conditions. We also investigated the expression of carotenoid biosynthetic genes to understand the mechanism of zeaxanthin accumulation under salinity conditions. The characteristics of *D. tertiolecta* *ZEP* response to light/dark cycles and salinity conditions were compared to those of higher plant *ZEP*s.

Materials and Methods

Strains and Culture Medium

Dunaliella tertiolecta CCAP 19/42 was used for this study. The composition of specific culture media for *Dunaliella* species (D media) was described in the previous paper [6]. To produce diverse salinity conditions, the concentration of NaCl was differently applied to the culture medium according to the purpose of each experiment (0.3 M, 0.6 M, and 1.5 M).

Experimental Light/Dark Cycle and Salinity Conditions

For the light/dark cycle experiments, cells were grown on 0.6 M NaCl D media under two different conditions; a photoperiodic condition (16 h of light and 8 h of dark) and a continuous light condition (24 h of light and 0 h of dark). The irradiance and temperature were maintained to $150 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 25°C.

For the salinity condition experiments, cells were grown respectively on 0.3 M, 0.6 M, and 1.5 M NaCl D media. Light and temperature conditions were maintained at $150 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of continuous light and 25°C. The cells were sub-

cultured more than twice to let them adapt to each salinity level. Also, the cells grown on 0.6 M NaCl D media were transfer into 1.5 M NaCl D media to investigate the response to increased salinity condition.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from the cells using RNeasy Plant Mini Kit (Qiagen, Germany), and cDNA was synthesized using 2× Reverse Transcription Master Premix (ELPiS Biotech, Republic of Korea). To perform the quantitative real-time PCR (qRT-PCR), synthesized cDNA was mixed with SYBR Premix (Takara, Japan) and amplified by the Thermal Cycler Dice Real-Time System TP 8200 (Takara). The gene of 60S ribosomal protein large subunit (60S) was used for reference. Results were analyzed by the Ct method. Sequences of primers for qRT-PCR were as follows; *ZEP_Fwd* (5'-ACAAGCATGGTACGGGGATG-3'), *ZEP_Rev* (5'-CATGGCCTGCATGACCACTA-3'), *GGPPS_Fwd* (5'-CATTGC CATTCTTGCCGGAG-3'), *GGPPS_Rev* (5'-CACCTCCTGGTTTC GCTCT-3'), *PSY_Fwd* (5'-CAGCTCACCAACATTCTGCG-3'), *PSY_Rev* (5'-TACCCCATCCTCTGCTTCTCT-3'), *PDS_Fwd* (5'-ACGGAGAGATTGTGGAGGGA-3'), *PDS_Rev* (5'-ACTGTGGAT AGCTTGCGGTC-3'), *LCYB_Fwd* (5'-GGTGTGACCTTCCTCACC AG-3'), *LCYB_Rev* (5'-AGCTCAAAGGGATGCGACTC-3'), *60S_Fwd* (5'-CCAAGCTCACCTACGAGCAG-3'), *60S_Rev* (5'-GTCCACCAA CCCGAATCCAT-3').

Western Blot Analysis

Lanes were equally loaded with crude extracts on the basis of cell number (100×10^4 cells per lane). Western blot analysis was carried out following the same process as described in the previous paper [24]. Primary antibodies used for western blot analysis; the custom-made polyclonal antibody of *ZEP* (1:5,000) used in our previous paper [24] and the commercial antibody of ATP synthase β -subunit (ATP- β ; Agrisera AS05 085, 1:10,000) as control protein. Secondary HRP-conjugated goat anti-rabbit IgG (H+L) antibody (Invitrogen, USA, 1:20,000) was used too.

Carotenoid Analysis

Samples of 0.5 ml each were harvested by centrifugation ($14,000 \times g$, 2 min), and the number of cells in the samples was counted for calculation of pigment content as the same cell density (10^6 cells). Pigment analysis was performed with high-performance liquid chromatography (HPLC) following the method as described in the previous paper [6].

Results

Pigment Composition and DtZEP Expression Are Regulated under Light/Dark Conditions

To examine the effects of the light/dark cycle on *DtZEP* expression, cells were cultured in photoperiodic (16 h of light and 8 h of dark) and continuous light (24 h of light

Fig. 1. Changes in xanthophyll pigment content in *Dunaliella tertiolecta* under different light conditions.

(A) Cells cultured under photoperiodic conditions (16 h of light and 8 h of dark); (B) Cells cultured under continuous light condition (24 h of light and 0 h of dark). Vx, violaxanthin; Ax, antheraxanthin; Zx, zeaxanthin.

and 0 h of dark) conditions. Using fully adapted cells, the composition of xanthophyll pigments and the expression levels of *DtZEP* were analyzed every 4 h. In cells cultured under photoperiodic conditions, zeaxanthin and violaxanthin levels increased and decreased, respectively, when cells were exposed to light (Fig. 1A). In contrast, zeaxanthin and violaxanthin levels in cells cultured under continuous light remained almost unchanged (Fig. 1B).

DtZEP mRNA levels in cells cultured under photoperiodic conditions showed light-responsiveness and increased after light exposure (Fig. 2A). In contrast, no significant

change in mRNA levels was observed in cells adapted to continuous light (Fig. 2B). Protein levels were similar in cells cultured under photoperiodic and continuous light conditions (Figs. 2C and 2D). In both conditions, protein expression patterns were different from those of mRNA expression.

Carotenoid Content and *DtZEP* Expression Are Affected by Salinity

Cells were cultured under three different salinity conditions for adaptation; 0.3 M, 0.6 M, and 1.5 M NaCl D

Fig. 2. Changes in *DtZEP* expression under different light conditions.

(A) mRNA expression pattern in cells under photoperiodic conditions; (B) mRNA expression pattern in cells under continuous light conditions; (C) Protein expression pattern in cells under photoperiodic conditions; (D) Protein expression pattern in cells under continuous light conditions. mRNA levels are expressed relative to the value corresponding to 0.5 h. Error bars indicate the standard errors of triplicated samples. ATP-β protein was used as a loading control.

Fig. 3. Effects of salinity on carotenoid content and *DtZEP* expression.

(A) β -Branch carotenoid content (Vx, violaxanthin; Ax, antheraxanthin; Zx, zeaxanthin; β -car, β -carotene); (B) mRNA expression levels calculated relative to the value corresponding to 0.3 M; (C) Protein expression levels of *DtZEP*. Error bars indicate the standard errors of triplicated samples. Statistical analysis was performed using Student's *t*-test; **p* < 0.05. ATP- β protein was used as a loading control.

media. Pigment content and expression levels of *DtZEP* were analyzed in cells at the stationary stage (10 days from initial inoculation). Pigment analysis was conducted of β -branch carotenoids; β -carotene, zeaxanthin, antheraxanthin, and violaxanthin (Fig. 3A). Cells cultured in 1.5 M NaCl D media contained high content of β -carotene and zeaxanthin. The amount of zeaxanthin in 1.5 M NaCl was approximately 2-fold higher than that in 0.3 M NaCl. No difference in antheraxanthin was observed in various salinity conditions, whereas violaxanthin content decreased in response to an increase in salinity.

DtZEP expression in different salinity conditions was analyzed at the mRNA and protein levels. The highest gene expression levels were observed in cells cultured at 1.5 M NaCl D media, which was more than 70% higher than those of in cells cultured at 0.3 M NaCl (Fig. 3B). Similar patterns were observed in protein expression levels. Western blotting results show that there was significant accumulation of *DtZEP* protein in cells cultured at a higher salinity than 0.3 M NaCl D media (Fig. 3C).

Hyperosmotic Stress Affects Carotenoid Content and *DtZEP* Expression

After cells cultured at 0.6 M NaCl were transferred to 1.5 M NaCl, the kinetics of pigment content and *DtZEP* expression levels were determined. Zeaxanthin was significantly accumulated after the 6 h time point, whereas violaxanthin content decreased steadily during the experiments. In addition, the content of antheraxanthin

Fig. 4. Effects of hyperosmotic stress on β -branch carotenoid content and *DtZEP* expression.

(A) Changing pattern in β -branch carotenoid content (Vx, violaxanthin; Ax, antheraxanthin; Zx, zeaxanthin; β -car, β -carotene); (B) mRNA expression levels calculated relative to the value corresponding to 0 h; (C) Protein expression levels of *DtZEP*. Error bars indicate the standard errors of triplicated samples. Statistical analysis was performed using Student's *t*-test; **p* < 0.05. ATP- β protein was used as a loading control.

and β -carotene gradually increased after the 6 h time point (Fig. 4A).

The kinetics of *DtZEP* expression were also determined at the mRNA and protein levels. mRNA levels of *DtZEP* increased gradually until 24 h, and the maximal levels were approximately 1.7-fold higher than the initial levels (Fig. 4B). However, the protein expression patterns were different from those of mRNA expression (Fig. 4C).

Zeaxanthin and β -Carotene Accumulation in Response to Hyperosmotic Stress Is Related to Upregulation of the Carotenoid Biosynthetic Pathway

In higher plants, the carotenoid content is closely related to the expression level of carotenoid biosynthetic genes [29, 30]. We confirmed the varying carotenoid content in response to changes in salinity (Figs. 3A and 4A), and then investigated the mRNA expression levels of carotenoid biosynthetic genes, especially the genes of the β -branch synthetic pathway, under different salinity conditions (circled on Fig. 5A). Cells cultured at 0.3 M, 0.6 M, and 1.5 M NaCl were used for analysis. mRNA expression levels of GGPS, PSY, LCYB and ZEP at 1.5 M NaCl were approximately 1.5-fold higher than those at 0.3 M NaCl (Fig. 5B). In contrast, *PDS* expression levels at 1.5 M NaCl were approximately 10% higher than those at 0.3 M NaCl.

Discussion

Zeaxanthin epoxidase (*ZEP*) has been studied in many species of Chlorophyta, but most of them are higher plants [5, 7, 9–13]. Recently, we reported the *ZEP* sequence of the marine microalga, *Dunaliella tertiolecta* CCAP 19/42 [24]. This microalgal species is classified as a Chlorophyta and is able to grow at diverse salinity conditions [26], unlike higher plants. Gene expression in living organisms can be different according to their environments. Therefore, we studied marine microalgal *ZEP* and compared it to higher plant *ZEP*s. This is the first report of an investigation of *ZEP* expression in marine microalgae at the environmental conditions affecting the expression of higher plant *ZEP*s.

Light and dehydrating conditions are representative triggering factors of *ZEP* expression in higher plants [5, 15, 19]. Characterization of *ZEP* expression in response to light conditions has been extensively examined in leaves of higher plants. The mRNA expression of *ZEP* in leaves shows an oscillating pattern following the diurnal rhythm [7, 18, 19], and is similar to the variation pattern of xanthophyll pigments [18]. In this study, the zeaxanthin and violaxanthin content in *D. tertiolecta* increased and decreased, respectively, immediately after light exposure (Fig. 1A), similar to plant leaves. In addition, the *DtZEP*

Fig. 5. Gene expression levels of the β -branch carotenoid synthetic pathway in *Dunaliella tertiolecta* under different salinity conditions.

(A) Schematic outline of the carotenoid biosynthetic pathway; (B) mRNA expression levels calculated relative to the value corresponding to 0.3 M NaCl. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl pyrophosphate; GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; LCYB, lycopene β -cyclase; LCYE, lycopene ϵ -cyclase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase. Error bars indicate the standard errors of three independent measurements. Statistical analysis was performed using Student's *t*-test; **p* < 0.05.

expression showed a pronounced difference between photoperiodic (16 h of light and 8 h of dark) and continuous light conditions (Fig. 2). Cells adapted to a light/dark cycle showed an oscillating pattern, whereas cells cultured under continuous light showed a mostly unchanging pattern. The expression of the DtZEP protein varies with light/dark cycle, but unlike the level of DtZEP mRNA, the degree of the protein level change is moderate. These results indicate that transcription of DtZEP is rapidly regulated by light, but the translation of DtZEP may not be responsive to light.

In dehydrating conditions, ZEP expression in roots is strongly induced. Unlike ZEP in leaves, the expression of ZEP in roots is enhanced significantly by drought stress [5, 19] or salt stress [20] conditions. In this study, we examined the expression levels of DtZEP at diverse salinity conditions (0.3 M, 0.6 M, and 1.5 M NaCl). Pigment analysis confirmed that the cells contained higher amounts of zeaxanthin and β -carotene in higher salinity conditions (Fig. 3A). In addition, high expression levels of DtZEP were detected in cells cultured at 1.5 M NaCl (Fig. 3B). To investigate the effect of hyperosmotic stress, we analyzed the kinetics of changes in pigment content and mRNA expression of DtZEP. When cells at 0.6 M NaCl were transferred to 1.5 M NaCl, zeaxanthin and β -carotene began to accumulate after 6 h (Fig. 4A). Gene expression levels also increased gradually for 24 h until reaching 1.7-fold the initial levels (Fig. 4B). However, protein expression of DtZEP did not follow mRNA expression, similarly to the results at light/dark cycle conditions (Fig. 2) and those from studies in higher plants [5]. These results suggest that enzyme reaction and transcription of DtZEP rapidly respond to hyperosmotic stress, but the changes in transcription levels are not reflected in the protein levels in a short time.

Zeaxanthin and β -carotene accumulation in response to salinity conditions have been reported in other *Dunaliella* species [4, 6, 27, 28]. Borowizka *et al.* (1990) reported that the proportion of zeaxanthin as a percentage of total carotenoids increases following a salinity up-shock [27]. Kim *et al.* (2017) also reported that cells cultured at 3.0 M NaCl have 2-fold higher zeaxanthin content than at 0.3 M NaCl [6]. As expected, the mRNA levels of carotenoid biosynthetic genes were the highest in cells at 1.5 M NaCl (Fig. 5). Especially, the expression levels of phytoene synthase, a rate-limiting step of carotenoid biosynthesis [30], was 1.5-fold higher in cells at 1.5 M NaCl than at 0.3 M NaCl. Our results show that zeaxanthin and β -carotene accumulation in *Dunaliella* species seems to be associated with up-regulation of carotenoid biosynthesis when cells

are exposed to increased salinity.

In summary, DtZEP gene expression was modulated in response to light similarly to ZEPs in leaves of higher plants. DtZEP expression also markedly increased in response to increased salinity, similar to ZEP expression in roots. Consequently, our results show that the expression patterns of ZEP at light or salinity conditions are similar to those of higher plants, even if the habitat of *D. tertiolecta* is completely different. In addition, our results suggest the possibility of increasing zeaxanthin content in *D. tertiolecta* through modulation of the light/dark cycle and salinity conditions.

Acknowledgments

This research was supported by the Business for Cooperative R&D between Industry, Academy, and Research Institute (S2449124) funded by the Korea Government (Ministry of SMEs and Startups) and the Korea CCS R&D Center (KCRC) (NRF-2014M1A8A1049273).

Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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