JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 2503-2511

Effect of Exogenous Ethylene (ET) on Regulating Carotenogenesis Expression and Astaxanthin Accumulation in *Haematococcus pluvialis*

Zhengquan Gao[1], Chunxiao Meng[1*], Hongzheng Gao[1], Naihao Ye[2], Xiaowen Zhang[2], Yuanfeng Su[1], Yuren Zhao[1] and Yuanyuan Wang[1]

[1]School of Life Sciences, Shandong University of Technology, Zibo 255049, China.
[2]Yellow Sea Fishery Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China.

(Received: 12 April 2013; accepted: 25 June 2013)

The freshwater eukaryotic unicellular alga Haematococcus pluvialis is one of the best sources to produce natural astaxanthin in nature. However, the regulatory role of carotenogenesis leading to astaxanthin formation is not well understood. In this study, the effects of ET on transcriptional expressions of eight carotenoid genes in *H. pluvialis* were measured using qRT-PCR. Results showed that eight carotenogenic genes were up-regulated by ET with different expression profiles. Astaxanthin accumulation was promoted efficiently by ET, which might result from the up-regulating of ipi-1, ipi-2, pds, lyc, bkt, crtR-b and crtO at the transcriptional level, and by psy at both transcriptional and post-transcriptional levels. Moreover, fourteen proteins were expressed differentially in ET treatments compared to controls. Furthermore, algal cell division accompanying accompanied by astaxanthin accumulation for the first time during the whole course of ethylene induction. The knowledge in the present paper might help to optimize the physiological conditions needed to produce a high yield of astaxanthin with ET.

Key words: *Haematococcus pluvialis*, Astaxanthin, Ethylene (ET), Regulation, Carotenoid genes, Real-time fluorescence quantitative PCR (qRT-PCR).

Among the natural ketocarotenoids found in higher plants, algae, fungi or bacteria, astaxanthin (3, 3'-dihydroxy-β, β-carotene-4, 4-dione) is the most important from the biotechnological and economic viewpoint[1]. It is used widely in aquaculture as a color additive, nutraceutical ascribed to free-radical scavenging, pharmaceutical for its immunomodulation and cancer prevention, and cosmetic industries due to its antioxidant activity[2, 3]. The freshwater unicellular algae Haematococcus pluvialis is one of the most promising natural sources of astaxanthin, producing up to 4% (w/w) of dry weight under a variety of environmental stresses and its maximum production of astaxanthin was up to 4% (w/w) of dry weight[4].

Plant growth, development and reproduction are regulated by phytohormones. When stressed by adversity, phytohormones content in plants change, resulting in a series of physiological activities to resist intimidation[5, 6]. And vice versa, when certain phytohormone changes to a certain concentration, plant itself will show similar behaviors encountering adversity; some scientists already have already used these

* To whom all correspondence should be addressed.
Tel.: +86-5332762265 ; Fax: +86-5332781832;
E-mail : mengchunxiao@126.com

phytohormones simulating adversity stress in signal transduction of adversity[7]. However, little is known about the putative role of phytohormones in algae physiology, especially their role in the regulation of secondary metabolism in algae.

Jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) serve as regulatory signaling molecules that regulate diverse stress responses in higher plants[8]. The regulatory effects of JA and SA at the transcriptional level were reported in our most recently studies[9, 10]. Results indicated that both the two phytohormones could enhance transcriptional expression of eight carotenoid genes and astaxanthin productivity in *H. pluvialis*. However, little is known about the effects of ET on algae, as it was not detected in algae until 2007[11]. Our previous studies have reported that there might be ET-responsive elements (ERE) in the 5'-flanking region of astaxanthin biosynthesis related genes, such as bkt, crtO, ipi, which suggested that ET might be used as effective regulators to produce ketocarotenoids in *H. pluvialis*[12-16]. It was proved that exogenous ET induced *H. pluvialis* to accumulate large amounts of astaxanthin[17]. Lu *et al*. also reported that the 5'-flanking regions of bkt contain regulatory element that are sensitive to (ET) and alga cells treated separately with GA3 accumulated more astaxanthin than the controls. ET treatment increased the transcription of three β-carotene ketolase genes (bkts)[18]. However, regulatory patterns and physiological roles of carotenogenesis leading to astaxanthin formation exposed to ET are still unknown. The objectives of this study were to investigate the effects of ET on transcriptional expression of eight of carotenoid genes (ipi-1, ipi-2, psy, pds, lcy, bkt, crtR-b and crtO) in *H. pluvialis* using real-time fluorescence quantitative PCR (qRT-PCR). This study will also attempt to evaluate relationship between application of exogenous ET and astaxanthin accumulation.

MATERIALS AND METHODS

Source of algal strain of *H. pluvialis* and cultivation condition

H. pluvialis strain 712 was acquired from Institute of Oceanology, Chinese Academy of Sciences and preserved in our laboratory. The *H. pluvialis* were grown in liquid medium (MCM)[19] in cultivation conditions as described in Gao *et al*. [9, 10].

ET inducing *H. pluvialis*

Algae at the logarithmic phase were divided into three treatments, three replicates for each treatment: the final concentrations of ET were 0 mg/L (control), 12 mg/L ET (ET12, 30µl 40% ethephon solution purchased from Shandong Dacheng of the Co., Ltd., Honggou road, Zibo, Shandong, China) and 24 mg/L ET (ET24 with 60µl 40% ethephon solution). An equal amount of deionized water was added to the controls. The algal cells were harvested on day 1, 2, 4, 8, 12, 16, 18 of the course of 18 d.

Observation of optical microscope and measurement of astaxanthin content

Microscope observations were done using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and astaxanthin content measured with a spectrophotometer (T6 new century, Beijing General Instrument Ltd, China) according to references[9, 10, 20].

RNA isolation and RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) and gene-specific primers for eight genes were designed according to Gao *et al*. [9, 10]. PCR products were quantified continuously with ABI StepOne Plus Real-Time PCR System (Applied Biosys-tems USA) using SYBR green fluorescence (Takara) according to the manufacturer's instructions. qRT-PCR analysis of related genes was performed according to Gao *et al*. (2012a, 2012b)[9, 10]. The actin gene was used as a reference for total RNA. Dissociation curve analysis of the amplification products was conducted at the end of each round of PCR to confirm that only one specific PCR product had been amplified and detected. Triplicate qRT-PCRs were conducted for each sample. After the PCR program was conducted, data were analyzed using the comparative Ct method[21]. To maintain consistency, the baseline was set automatically by the software. The mean ± SD was calculated for each experiment and analyzed using the SPSS 17.0 data processing system software.

SDS-PAGE

Preparation of cell fractions was performed according to reference[22] with alteration to extraction buffer and step (0.05 mmol/L Tris-HCl, 0.005 mol/l $MgCl_2$, 0.005 $CaCl_2$, 5 mm Na_2-

EDTA, 0.1 mm phenylmethylsulfonyl fluoride (PMSF). Samples were frozen in liquid nitrogen, ground into a fine powder and then centrifuged for 10 min at 16,000g (Eppendorf 5804R, German) with extraction buffer. The supernatant contains the protein samples. SDS-PAGE was used to examine differential polypeptides compositions during the different induction time according to reference[23].

RESULTS

Observation of microscope and astaxanthin determination in *H. pluvialis* exposed to ET

A significant difference in astaxanthin accumulation was observed between the controls and two ET treatments ($p<0.01$). Results from microscopy observations showed the initial color change from green to red was 4th day after application of ET. On day 24, about 12.5% and 44.3% of the total algae numbers were whitened or autolysed, which meant these cells died. The percentage of totally red cells was about 56.2% and 88.1% of living algae cells in ET12 and ET24 treatments, respectively (Figure 1). Impressively, extremely individual algae cells were found being divided into two new cells when accumulating astaxanthin during the whole course of induction. Figure 2 showed the algae cells being divided on day 4, 8, 16 20 of ET induction process along with the astaxanthin accumulation in ET12 treatments. ET12 treatments resulted in the highest astaxanthin production (3.3 mg/L alga culture solution), which reached the maximum peak on day 24. The maximum peak of ET24 treatments occurred on day 12 with 2.4 mg/L and astaxanthin content of the controls which was 0.05 mg/L after culture for 24 d (Figure 3). However, too higher concentration ET seemed to have side effects for the astaxanthin production since ET12 and ET24 treatments resulted in more than 12.5% and 44.3% of the total algae numbers died on day 24.

Differential expression profiles of total soluble proteins under ET treatments

Figure 4 showed the profiles of differential expression of proteins in two ET treatments. Compared with controls, there were fourteen proteins expressed differentially in the four ET samples. Although there were six proteins, including protein 3 (20 kD), 4 (23 kD), 5 (27 kD), 9 (50 kD), 10 (52kD) and 14 (130 kD) also expressed in controls, they expressed in two ET samples much higher. Protein 1 (16 kD) was only detected in two ET12 samples and protein 7(38 kD) was only detected in two ET24 samples. Protein 13 (98 kD) appeared only in ET24 sample on day 24. Protein 2

Table 1. Gene-specific primers and annealing temperatures used for qRT-PCR

Primer	Primer sequence (5'-3')	Annealing temp(°C)	GenBank ID
psyF	CGATACCAGACCTTCGACG	55	AF305430
psyR	TGCCTTATAGACCACATCCAT		
pdsF	ACCACGTCGAAGGAATATCG	58	X86783
pdsR	TCTGTCGGGAACAGCCG		
lycF	TGGAGCTGCTGCTGTCCCT	61	AY182008
lycR	GAAGAAGAGCGTGATGCCGA		
crtR-bF	ACACCTCGCACTGGACCCT	62	AF162276
crtR-bR	GTATAGCGTGATGCCCAGCC		
bkt2F	CAATCTTGTCAGCATTCCGC	61	AY603347
bkt2R	CAGGAAGCTCATCACATCAGAT		
ipi-1F	GCGAGCACGAAATGGACTAC	61	AF082325
ipi-1R	GCTGCATCATCTGCCGCA		
ipi-2F	AGTACCTGGCGCAAAAGCTG	62	AF082326
ipi-2R	GTTGGCCCGGATGAATAAGA		
crtOF	ACGTACATGCCCCACAAG	55	X86782
crtOR	CAGGTCGAAGTGGTAGCAGGT		
actF	TGCCGAGCGTGAAATTGTGAGG	55	[30]
actR	CGTGAATGCCAGCAGCCTCCA		

(18 kD), 6 (33 kD), 8 (44 kD), 11(61 kD) and 12 expressed more in ET24 treatments than in ET12 treatments but protein 2 expressed (63 kD) expressed in both two ET treatments. Among of them, protein 6, 8, 11, 12 expressed equally in two ET treatments.

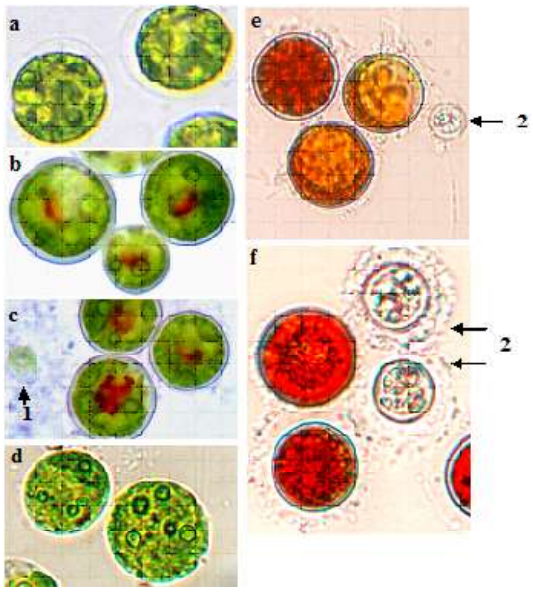

Fig. 1. Microscopic images (400×) of *H. pluvialis* cells culture samples day 4 and 24 after treatments with ET. a, b and c represent the controls, ET12 sample and ET24 sample after 4 days of treatments, respectively; d, e, f represent the controls, ET12 sample and ET24 sample after 24 days of treatments, respectively; arrow 1 show the algae cells were autolysed and arrow 2 represented whitened algae cells

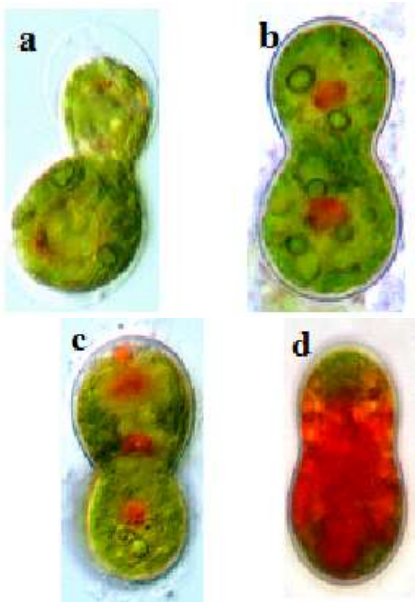

Fig. 2. The algae cells being divided on day 4, 8, 16 20 (a, b, c, and d) of ethylene induction process along with the astaxanthin accumulation in ET12 treatments.

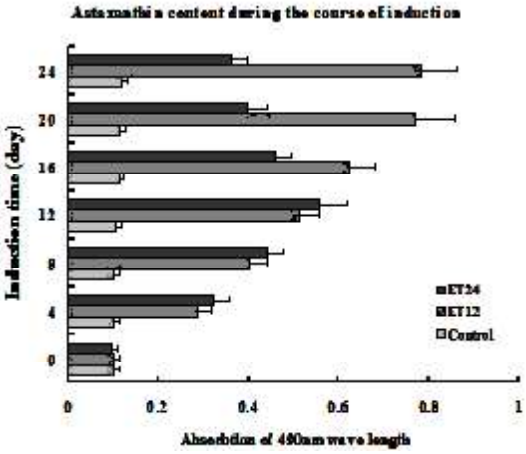

Fig. 3. Astaxanthin accumulations of controls, ET12 samples and ET24 samples during cultivation. OD_{490} represents relative astaxanthin content in alga culture solution.

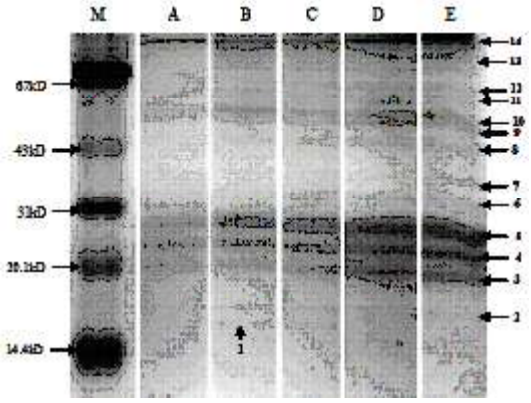

Fig. 4. Differential expression profiles of total soluble proteins two ET treatments in the induction course. M was molecular marker. A, B, C, D and E lane represented the total soluble profiles of controls, ET12 samples on day 12 and 24, ET24 samples on day 12 and 24 of induction course, respectively.

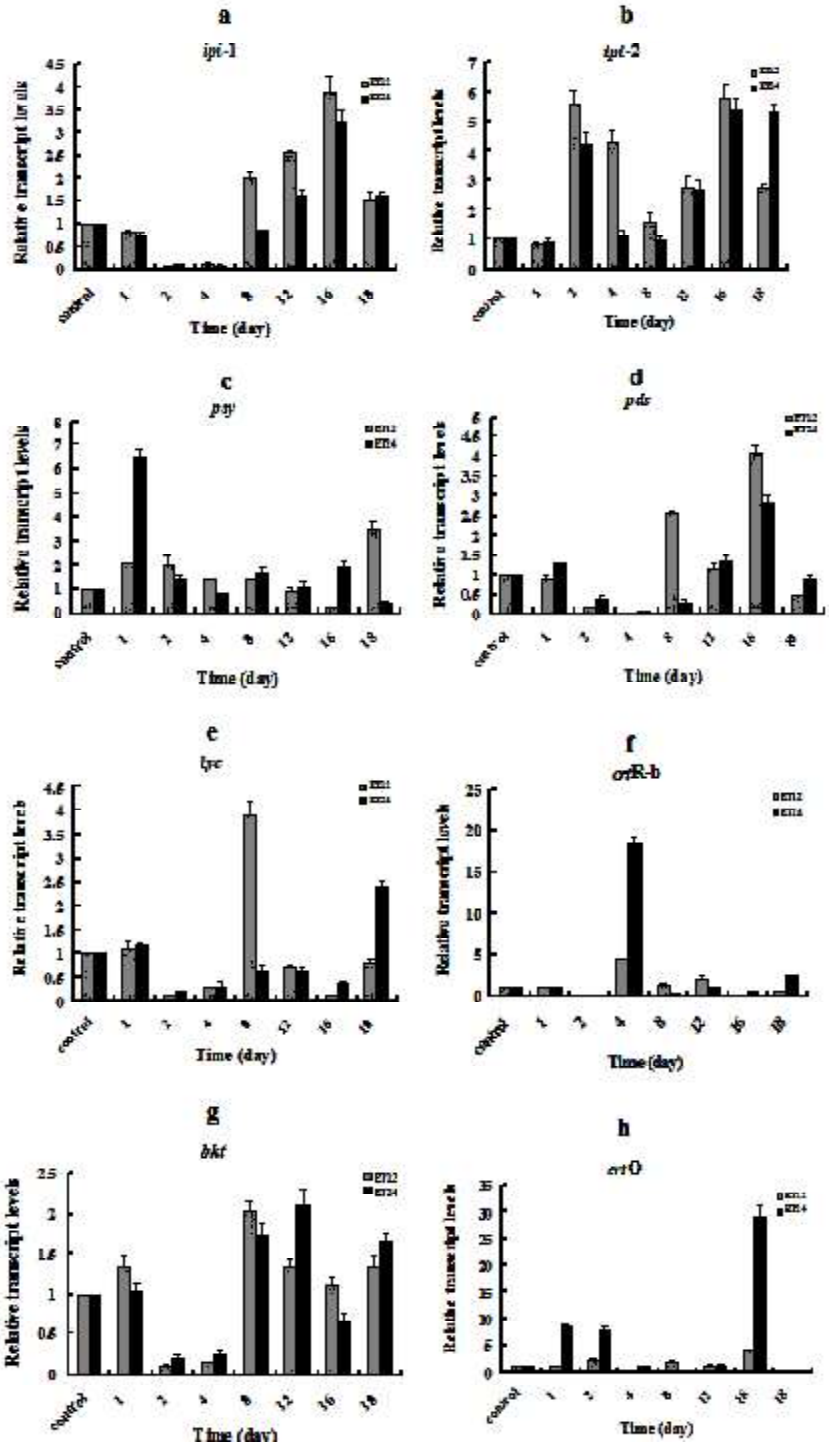

Fig. 5. The effects of ET on the transcript levels expression kinetics of eight carotenogenic genes in *H. pluvialis* during incubation. a, b, c, d, e, f, g and h represent transcript levels expression kinetics of *ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O respectively

Transcriptional patterns of carotenogenic genes induced by ET

The initial increased transcriptional expression of ipi-1 and ipi-2 in ET12 treatments were observed on day 8 and 2 with 2.0- and 5.6-fold of controls, respectively. While both the maximum transcript levels of them occurred on day 16 with 3.9- and 5.8-fold, respectively. Meanwhile, the initial increased and maximum values of ipi-1 in ET24 treatments occurred on day 12 and 16 with 1.6- and 3.2-fold compared to controls, respectively. The initial enhancement and highest mRNA expression of ipi-2 in ET24 treatments occurred on day 2 and 16, with 4.3- and 5.4-fold, respectively (Figure 5a and 5b).

In ET12 treatments, the first increasing and highest transcriptional levels of psy (2.1- and 3.4-fold, respectively) occurred on day 1 and 18. In ET24 treatments, both the initial increased and highest psy mRNA levels occurred on day 1, with 6.5-fold of transcriptional expression of controls (Figure 5c). The first increased and maximum transcriptional level of pds in ET12 treatments occurred on day 8 and 16, with 2.5- and 4.1-fold, respectively. In ET24 treatments, both the initial increasing and highest pds mRNA expression occurred on day 16, with 2.8-fold (Figure 5d). Both the initial increased and maximum transcriptional level of lyc occurred on day 8 in ET12 and on day 18 in ET24 treatments with 3.9- and 2.4-fold, respectively (Figure 5e).

With the similar pattern of lyc, the first increased and maximum mRNA level of crtR-b in both ET treatments occurred on day 4, with 4.3- and 18.3-fold, respectively (Figure 5f). The patterns observed for bkt were similar to those for pds. In ET12 treatments, both the initial increased and highest bkt mRNA levels occurred on day 8, with 2.0-fold. In ET24 treatments, the initial increased and the highest bkt mRNA values occurred on day 8 and 12, with 1.7- and 2.1-fold, respectively (Figure 5g). In ET12 treatments, the initial increased and the highest transcriptional levels of crtO occurred on day 2 and 16 with 2.1- and 4.0-fold. While, in ET24 treatments, the initial increased crtO levels occurred on day 1 with 8.3-fold, then decreased sharply, and climbed to the highest on day 16 with 28.0-fold (Figure 5h). These data above suggested that the strong up-regulation of these eight enzymes in *H. pluvialis* resulted from the inductive effects of ET, which resulted in astaxanthin accumulation of *H. pluvialis*.

DISCUSSION

Phytohormone is one of several systems controlling plant growth and development at cellular, tissue, and organismal levels[11]. ET is a defensive phytohormone involved in senescence, fruit ripening, abscission and physiological responses to environmental. In higher plants, ET modulates gene expression via a variety of mechanisms, in which for certain genes ET affects transcriptional processes, while for another gene it affects both transcriptional and post-transcriptional processes[24]. Lawton *et al*. indicated that ET induction increased transcription of senescence-related genes[25]. Some plant defense and pathogenesis-related proteins genes also could be differentially up-regulated by ET[26], and other ET-regulated genes identified were known defense signaling pathways[27].

In present study, astaxanthin content of both two ET treatments was stimulated significantly on 4th day. According to the correlation between transcriptional peaks of eight carotenogenic genes and initiative time of fast astaxanthin accumulation, it could be inferred that they up-regulate astaxanthin accumulation at transcriptional level, post-transcriptional level, or both levels[9, 10, 28, 29]. The maximum transcript expressions of ipi-1, ipi-2, psy, pds, lyc, bkt, crtR-b and crtO of ET12 samples occurred on day 16, 16, 18, 16, 8, 8, 4 and 16 with 3.9-, 5.8-, 3.4-, 4.1-, 3.9-, 2.0-, 4.3- and 4.0-fold in ET12 treatment, respectively, which lagged behind or in line with the starting time of fast astaxanthin accumulation. Therefore, the eight genes might up-regulate astaxanthin accumulation at transcriptional level in ET12 treatment. These results also indicated that ET12 induction had a greater effect on the transcriptional expression of ipi-2, pds, crtR-b and crtO (> 4-fold up-regulation) than on ipi-1, psy, lyc and bkt.

With respect to ET24 treatment, their transcriptional expressions attained to maximum peaks on day 16, 16, 1, 16, 18, 12, 4 and 16 with 3.2-, 5.4-, 6.5-, 2.8-, 2.1-, 2.3-, 18.3- and 29.0-fold of controls, which showed that ET24 treatment had a greater impact on the transcriptional expression of ipi-2, psy, crtR-b and crtO (> 4-fold up-regulation)

than on ipi-1, pds, lyc and bkt. The mRNA expression maximum level time point of ipi-1, ipi-2, pds, lyc, bkt, crtR-b and crtO fell behind or in line with the starting time of fast astaxanthin accumulation, which suggested these seven genes might up-regulate astaxanthin accumulation at transcriptional level. However, psy displayed the maximum expression on day 1, preceding the initiative time of fast astaxanthin accumulation. Therefore it might up-regulate astaxanthin biosynthesis at post-transcriptional level. In conclusion, astaxanthin biosynthesis of *H. pluvialis* under ET might be up-regulated mainly by ipi-1, ipi-2, pds, crtR-b, lyc, bkt and crtO genes at transcriptional level, but by psy gene at both transcriptional and post - transcriptional levels.

Results from microscopy and astaxanthin concentration measurements indicated application of exogenous ET quickened the cell color change from green to red and excite *H. pluvialis* accumulate astaxanthin. The most interest part of the study is the finding of individual alga cell division accompanying astaxanthin accumulation. Although it was observed in our previous works occasionally (data not shown), it was not attract our enough attention. This finding is in conflict with the classical viewpoint that astaxanthin accumulation occurs under the condition that was not suitable for cells of *H. pluvialis* division and breeding, which was accepted by most algologists. Therefore, the finding may be a necessary supplement of the classical viewpoint.

SDS-PAGE profiles of the differential expression showed fourteen proteins expressed differentially under the induction of ET. Six proteins enhanced the quantity of expression exposed to ET compared with controls. Only one protein (16kD) expressed specifically in ET12 samples and two (38 and 98 kD) specifically in ET24 samples. These proteins expressed differentially might be the enzymes or regulators related to carotenoid biosynthesis, which needed be identified further in the following research.

Astaxanthin accumulation is a defensive reaction of *H. pluvialis* to stresses. Therefore, defensive phytohormones are very likely to serve as the imitator of stress inducing *H. pluvialis* to accumulate astaxanthin efficiently. The conjecture of was confirmed using two other defensive phytohormones, SA and JA, by our teamet al[9, 10]. Application of defensive phytohormones might mislead *H. pluvialis* accumulate astaxanthin efficiently to withstand the imaginary stress, simulating adversity by defensive phytohormones. Although the present study was preliminary and the results need to clarify further, knowledge about the regulation of ET on carotenogenic genes will help to optimize the physiological conditions needed to produce a high yield of astaxanthin with ET and may provide important information for future genetic manipulation of carotenogenesis in *H. pluvialis*.

In conclusion, astaxanthin accumulation of *H. pluvialis* was induced effectively by ET. Though all eight carotenogenic genes were up-regulated by ET treatments, they exhibited different mRNA expression profiles. And cell division accompanying astaxanthin accumulation was detected in the presented.

ACKNOWLEDGMENTS

The present study was supported by the National Natural Science Foundation of China (41106124), the National Natural Science Foundation of Shandong province (ZR2011DM006, ZR2011CQ010) and the open funds of State Key Laboratory of Agricultural Microbiology (AMLKF201003).

REFERENCES

1. Margalith, P.Z. Production of ketocarotenoids by microalgae. *Appl. Microbiol. Biotechnol.,* 1999; **51**: 431-438.
2. Kim, Z.H., Kim, S.H., Lee, H.S., Lee, C.G. Enhanced production of astaxanthin by flashing light using Haematococcus pluvialis. *Enzy. and Microb. Technol.*, 2006; **39**; 414-419.
3. Jin, E., Lee, C.G., Polle, J.E.W. Secondary carotenoid accumulation in Haematococcus (Chlorophyceae): biosynthesis, regulation, and biotechnology. *J. Microbiol. Biotechnol*., 2006; **16**: 821-831.
4. Boussiba, S. Carotenogenesis in the green alga Haematococcus pluvialis: cellular physiology and stress response. *Physiol. Plant.,* 2000; **108**: 111-117.
5. Hiron, R.W.P., Wright, S.T.C. The role of endogenous abscisic acid in response of plants stress. *Exp. Bot*., 1973; **24**: 769-781.

6. Griffiths, H., Parry, M.A. Plant responses to water stress. *Ann. Bot.* (Lond). 2002; **89**: 801-802.
7. Hedden,P., Thomas, S.G. Plant Hormone Signaling: Annual Plant Reviews. Blackwell Publishing Ltd. Oxford, 2006.
8. Anerson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R., Kazan, K. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *The Plant Cell*, 2004; **16**: 3460-3479.
9. Gao, Z.Q., Meng, C.X., Zhang, X.W., Xu, D., Miao, X.X., Wang, Y.T., Yang, L.M., Lv, H.X., Chen, L.L., Ye, N.H. Induction of salicylic acid (SA) on transcriptional expression of eight carotenoid genes and astaxanthin accumulation in *Haematococcus pluvialis*. Enzy. *MicroB. Techno*. , 2012; **51**: 225-230.
10. Gao, Z.Q., Meng, C.X., Zhang,X.W., Xu, D., Zhao, Y.F., Wang, Y.T., Yang, L.M., Lv, H.X., Chen, L.L., Ye, N.H. Differential expression of carotenogenic genes, associated changes on astaxanthin production and photosynthesis features induced by JA in *H. pluvialis*. *PLoS ONE*, 2012; **7**: 1-7.
11. Tarakhovskaya, E.R., Massloy, Y.I., Shishov, M.F. Phytohormones in Algae. *Russ. J. Plant. Physiol*. 2007; **54**: 163-170.
12. Meng, C.X., Teng, C.Y., Jiang, P., Qin, S., Tseng, C.K. Cloning and characterization of β-carotene ketolase gene promoter in *Haematococcus pluvialis*. Acta Biochim. *Biophys. Sinica*., 2005; **37**: 270-275.
13. Meng, C.X., Liang, C.W., Qin, S., Tseng, C.K. There are two 5'-flanking regions of bkt encoding beta-carotene ketolase in the *Haematococcus pluvialis*. *Phycologia*., 2006; **45**: 218-224.
14. Meng, C.X., WEI, W., SU, Z.L., Qin, S., Tseng, C.K. Characterization of carotenoid hydroxylase gene promoter in *Haematococcus pluvialis. Ind. J. Biochem. Biophy*., 2006; **43**: 284-288.
15. Gao, Z.Q., Meng, C.X., Ye, N.H. Regulatory analysis of IPP isomerase gene in *Haematococcus pluvialis. Mar. Sci. Bull.*, 2009; **11**: 37-42.
16. Gao, Z.Q., Meng, C.X., Ye, N.H. Three 5'-flanking regions of crtO encoding ?-carotene oxygenase in Haematococcus pluvialis. Mar .*Sci. Bull*., 2010; **12**: 59-64.
17. Gao, Z.Q., Meng, C.X. Impact of Extraneous Ethylene Concentrations to Astaxanthin Accumulation of *Haematoccus pluvialis*. *Food Sci*., 2007, **28**: 376-380 (in Chinese).
18. Lu, Y.D., Jiang, P., Liu, S.F., Gan, Q.H., Cui, H.L., Qin S. Methyl jasmonate or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of b-carotene ketolase genes (bkts) in microalga *Haematococcus pluvialis. Bioresou. Tech.*, 2010; **101**: 6468-6474.
19. Borowitzka, M.A., Huisman, J.M., Osborn, A. Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. Effects of nutrients on growth and cell type. *J. Appl. Phyco*., 1991; **3**: 295-304.
20. Boussiba, S., Vonshak, A. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant and Cell Physiol*., 1991; **32**: 1077-1082.
21. Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Meth*., 2001; **25**: 402-408.
22. Grünewald, K., Eckert, M., Hirschberg, J., Hagen, C. Phytoene desaturase is localized exclusively in the chloroplast and up-regulated at the mRNA level during accumulation of secondary carotenoids in *Haematococcus pluvialis* (Volvocales, Chlorophyceae). *Plant Physiol*., 2000; **12**: 1261-1268.
23. Gao ZQ, G.C., Wang, Tseng, C.K. Isolation and characterization of photosystem of *Porphyra yezoensis* Udea. *Acta Biochim. Biophy. Sinica*., 2004; **36**: 780-785.
24. Lincoln,J.E., Fischer, R.L. Diverse mechanisms for the regulation of ethylene-inducible gene expression. *Mol. Gen. Genet.,* 1988; **212**: 71-75.
25. Lawton, K.A., Raghotham, K.G., Goldsbroug, P.B., Woodson, W.R. Regulation of senescence-related gene expression in Carnation flower petals by ethylene. *Plant Physiol*., 1990; **93**: 1370-1375.
26. Sato, F., Kitajima, S., Koyama, T., Yamada, Y. Ethylene-indueed gene expression of osmotin-Like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC cis-Sequence. *Plant Cell Physiol.,* 1996; **37**: 249-255.
27. Zhong, G.V., Burns, J.K. Profiling ethylene-regulated gene expression in *Arabidopsis thaliana* by microarray analysis. *Plant Mol. Biol.*, 2003; **53**: 117-131.
28. Li, Y.T., Sommerfelda, M. Chen, F., Hu, Q. Consumption of oxygen by astaxanthin biosynthesis: A protective mechanism against oxidative stress in *Haematococcus pluvialis*(Chlorophyceae). *J. Plant Physiol*., **165**; 1783-1797 (2008).
29. Li, Y.T., Sommerfelda, M. Chen, F., Hu, Q. Effect of photon flux densities on regulation of

carotenogenesis and cell viability of *Haematococcus pluvialis* (Chlorophyceae). *J. Appl. Phycol*., **22**; 253, 263 (2010).

30. Huang, J.C., Chen, F., Sandmann, G. Stress-related differential expression ofmultiple b-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. *J. Biotechnol.,* 2006; **122**: 176-185.