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Proteomic Analysis of Protein Expression Patterns Associated with Astaxanthin Accumulation by Green Alga *Haematococcus pluvialis* (Chlorophyceae) Under High Light Stress

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Abstract Two kinds of *Haematococcus pluvialis* cells (green vegetative cells cultivated under optimal cell culture conditions and red cyst cells maintained under high light stress conditions to induce astaxanthin production) were used to investigate the protein expression profiles by two-dimensional electrophoresis, image analysis, and peptide mass fingerprinting. The cellular accumulation of astaxanthin was evident after exposure to high light intensity and reached the maximum cellular level after 78 h of high light stress. In a 2-D electrophoresis analysis, 22 proteins were upregulated over 2-fold in the red cyst cells when compared with the green vegetative cells and selected for further analysis by chemically assisted fragmentation (CAF)-MALDI-TOF sequencing to identify the protein functions. Among 22 different spots, several key enzymes specific to the carotenoid pathway, including isopentenyl pyrophosphate isomerase (IPP) and lycopene β-cyclase, appeared in *H. pluvialis* after exposure to high light intensity. Therefore, IPP and lycopene β-cyclase would appear to be involved with carotenoid accumulation in the cytoplasm, as these peptides were preferentially upregulated by high light intensity preceding an increase in carotenoid, and only these forms were detected in the red cyst cells.

Key words: Astaxanthin, high light intensity, isopentenyl pyrophosphate isomerase, lycopene β-cyclase

Microalgae have an extensive biotechnological potential for producing valuable substances for feed, food, lipids, vitamins, pigments, cosmetics, and pharmaceuticals, as well as the treatment of waste materials [4, 11, 26]. For example, the high-value red carotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is used as a source of pigment in the diets of fish and animals, and has potential clinical applications because of its higher antioxidant activity [16, 21]. The cyst form of *H. pluvialis* is known to contain large amounts of valuable carotenoid pigments, such as astaxanthin, and many factors have been found to affect the cell concentration in *H. pluvialis*. Among such factors, light energy is the major factor producing a high cell concentration [26], thus the growth rate of *H. pluvialis* depends on the light energy absorbed by the cells.

A number of carotenoid biosynthesis genes, such as isopentenyl diphosphate isomerase, phytoene synthase, phytoene desaturase, lycopene β-cyclase, β-carotene ketolase, and carotenoid hydroxylase, have already been cloned and at least partially characterized in *H. pluvialis* [2–4, 20]. A common feature of these carotenoid genes is that their expression undergoes transient upregulation, with the maximum mRNA transcript levels occurring 24–48 h after the onset of stress induction, followed by vigorous biosynthesis and cellular accumulation of astaxanthin, with the maximum level reached after 6–12 days of stress [10, 19, 39]. It is generally assumed that the accumulation of astaxanthin is a survival strategy of *H. pluvialis* and other related organisms in response to stressful environments.

Accordingly, the present study investigated the accumulation of astaxanthin, as distinct from morphological changes, in *Haematococcus pluvialis* cells suddenly exposed to high light intensity. In addition, 2-DE revealed that the induction of astaxanthin biosynthesis and change in the protein expression profile in response to increased light intensity were correlated with the accumulation of astaxanthin.

Materials and Methods

Microorganism and Culture Conditions

The unicellular green alga, *Haematococcus pluvialis* UTEX 16, was obtained from the Culture Collection of Algae at the University of Texas at Austin, U.S.A. The *H.*

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pluvialis was cultivated in a modified Bold's basal medium (MBBM), consisting of $NaNO_3$, 246.5 mg/l; $CaCl_2 \cdot 2H_2O$, 24.99 mg/l; $MgSO_4 \cdot 7H_2O$, 73.95 mg/l; $FeSO_4 \cdot 7H_2O$, 4.98 mg/l; K_2HPO_4, 74.9 mg/l; KH_2PO_4, 175.57 mg/l; NaCl, 25.13 mg/l; $C_{10}H_{16}N_2O_8$ (EDTA), 49.68 mg/l; $CuSO_4 \cdot 5H_2O$, 1.57 mg/l; $Na_2MoO_4 \cdot 2H_2O$, 1.19 mg/l; H_3BO_3, 11.13 mg/l; $MnCl_2 \cdot 4H_2O$, 1.44 mg/l; $ZnSO_4 \cdot 7H_2O$, 8.83 mg/l; $Co(NO_3)_2 \cdot 6H_2O$, 0.49 mg/l; MoO_3, 6.06 mg/l ; KOH, 30.86 mg/l; and H_2SO_4, 0.98 mg/l. The cells were grown in 2.5-l bubble column photobioreactors, each containing 2 l of the growth medium, at 25°C under a continuous 40 $\mu E/m^2/s$ light intensity to grow immature green cells. For stress induction, exponentially growing cultures (cell density of approximately 1.5×10 cells/ml) were exposed to a continuous light intensity of 250 $\mu E/m^2/s$ illumination.

Analytical Methods

The cell concentration and cell size were measured using a Coulter counter (Model Z2; Beckman, Inc., Hialeah, FL, U.S.A.), and the astaxanthin concentration was measured using a spectrophotometer (Model HP8453B, Hewlett Packard, Waldbronn, Germany) and calculated based on a calibration curve obtained using synthetic astaxanthin (Product number A9335, Sigma Chemical Co., St Louis, MO, U.S.A.) as the standard. For an astaxanthin concentration less than 10 mg/l, the following calibration was used.

$$\text{Astaxanthin concentration (mg/l)} = 0.0045 \times OD_{475} \quad [5]$$

Extraction of Proteins

The culture suspension (250 ml) was collected at various time intervals and the cells harvested by centrifugation at 3,000 rpm for 10 min, and then washed three times with cold deinonized water. The harvested cells were homogenized for 40 min in a mortar in liquid nitrogen using a lysis buffer (Urea, 7 M; Thiourea, 2 M; CHAPS, 1%; and Tris-base, 40 mM), and then the homogenized cells were centrifuged at 50,000 rpm for 1 h to separate the cell debris and the supernatants used for the protein analysis.

Two-Dimensional Electrophoresis

A total of 300 µl of a rehydration sample buffer containing the protein sample (250 µl) was incubated with IPG (immobilized pH gradient) gel strips (pH 4–10 linear gradient, 18 cm, Bio-Rad, Hercules, CA, U.S.A.) for 12–16 h at 20°C. Isoelectric focusing was initiated at 250 V for 15 min, ramped by degrees to 10,000 V over 5 h, and retained at 10,000 V for an additional 6 h. Before the second dimension, the IPG strips were incubated for 15 min in equilibration buffer I [containing Urea, 6 M; Tris-HCl, 0.375 M (pH 8.8); glycerol, 20%; SDS, 2%; and DTT, 2% (w/v)] and then incubated for 15 min in equilibration buffer II [containing Urea, 6 M; Tris-HCl, 0.375 M (pH 8.8); Urea, 6 M; glycerol, 20%; SDS, 2%; and Iodoacetamide, 2.5% (w/v)]. The second-dimensional SDS-PAGE was performed on 7.5–20% gradient separating gels, which ran for 2 h at 8 mA, followed by 4 h at 15 mA/gel, and then 3 h at 20 mA/gel. The gels were stained with an alkaline silver staining solution and scanned using an Image Reader FLA-5000 (Fuji Photo Film Co. Ltd., Japan).

In-Gel Digestion and Peptide Extraction

The in-gel digestion was carried out as described by Rosenfeld *et al.* [23] with modifications. All the pipetting and liquid transfer were performed using a Multiprobe II automatic liquid handler (Packard Instrument). The gel pieces were placed in a 96-well PCR plate (Advanced Biotechnologies, Epsom, Surry, U.K.), washed in water for 2×20 min, then in 35% acetonitrile/25 mM ammonium carbonate (pH 8.0) for 3×20 min, and dehydrated in a SpeedVac vacuum evaporator for 20–30 min. The gel pieces were reswollen by adding 15 µl of a trypsin solution that was 5 ng/µl modified trypsin (Sigma) in 25 mM ammonium carbonate (pH 8.0). The samples were then incubated overnight for 16 h and the protein fragments extracted by the addition of 30 µl of 5% formic acid.

Chemically Assisted Fragmentation (CAF)-MALDI-TOF

The derivatization reactions of the tryptic peptides were performed using the instructions and reagents in an Ettan CAF-MALDI Sequencing Kit. The additional reagents included C_{18} ZipTip (Millipore), ACH-cinnamic acid, trifluoroacetic acid, acetonitrile (Sigma), and ultrapure water (18 MΩ/cm). The samples and matrix were loaded on the Ettan MALDI-TOF Pro target as described in the kit instructions. All the spectra were obtained using the ion gate set for the precursor ion (*i.e.*, parent ion) in the post-source decay (PSD) analysis mode. The fragment masses of the CAF-labeled peptides were determined using the Ettan MALDI-TOF Pro software package, and the proteins were identified using the automated protein identification program included in the system.

Database Searching

The peptide mass fingerprints generated by the CAF-MALDI-TOF MS for the samples were matched to theoretical tryptic digests of proteins in the ExPaSy Proteomics Server (Expert Protein Analysis System) using the MS-Fit software through the USCF Mass Spectrometry facility, San Francisco (http://prospertor.ucsf.edu), and ProFound software (http://prowl.rockefeller.edu/cgi-bin/ProFound). First, the taxonomy category was determined for green algae and green plants. Compute pI/Mw is a tool that computes the theoretical pI (isoelectric point) and Mw (molecular weight) based on the protein sequence used to compare the pI and Mw of individual protein spots on the 2-DE gel. The mass accuracy was set at ±50–80 ppm for

all the MALDI-TOF MS analyses. Possible post translational modifications, like N-terminal acetylation, an N-terminal pyroglutamic acid, the oxidation of methionine, and modification of cysteine by acrylamide, were also taken into consideration through queries.

RESULTS AND DISCUSSION

Cell Growth and Pigment Profiling of *H. pluvialis* Under High Light Intensity

Figure 1 shows the change in the cell population of *H. pluvialis* starting as 7-day-old green vegetative cell cultures and exposed to high light intensity on the surface of a bubble column photobioreactor. The cell number generally increased from 1.5×10^5 cells/ml to 2.2×10^5 cells/ml during 30 h of exposure to high light stress, and then leveled off. After 48 h of persistent stress, the cell concentration dramatically reduced to 67% of the initial cell concentration after 84 h of stress. Some ruptured cells were also observed under the stress conditions, as the cells were bleached and the cell wall skeletons collapsed under the stress of the high light intensity. Therefore, the results indicated that the cell numbers slightly decreased after 30 h of stress. Similar cell death under stress conditions was also reported by Kobayashi *et al.* [18], where a 20% decrease in cell numbers was observed after 5 days of exposure to stress. Wang *et al.* [34] reported an 18% decrease in the cell concentration of *H. pluvialis* after 6 days of oxidative stress. The cellular accumulation of astaxanthin in *H. pluvialis* is also shown in Fig. 1 during the transformation of the green vegetative cells into red cysts under high light intensity. Only a little astaxanthin was present in the cells during the 12 h of incubation, even though cells were cultured under stress conditions. However, astaxanthin was slowly induced in the cells between 6 h and 42 h, and dramatically accumulated after 78 h of stress. It is assumed that the induction of astaxanthin accumulation in the cells was related to a key enzyme involved in astaxanthin biosynthesis, such as a carotenoid hydroxylase, corresponding to the form of astaxanthin in the *H. pluvialis* cells during the initial (early time/6 h) exposure to high light intensity [25]. Tjahjono *et al.* [31] reported a similar induction pattern, where astaxanthin production in *H. pluvialis* cultivated at 30°C was 3-fold higher than that at 20°C, suggesting that endogenously generated active oxygen may be responsible for the highly stimulated carotenogenesis.

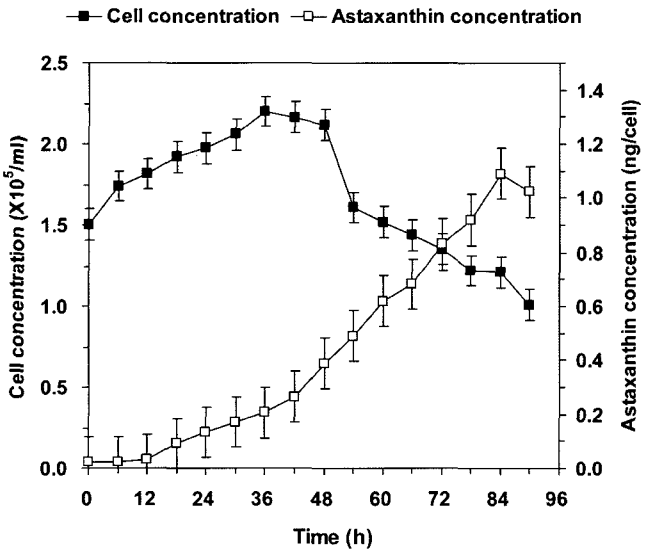

Fig. 1. Cell concentration and cellular astaxanthin accumulation by *H. pluvialis* during exposure to high light intensity on the surface of photobioreactors.

Data represent mean values of three independent cultures. Arrows indicate the time of the high light intensity exposure.

Analysis of Cellular Protein Expression Profiles Following High Light Stress

Green vegetative cells before any high light stress (at 0 h of incubation) and red cysts after 78 h of incubation under

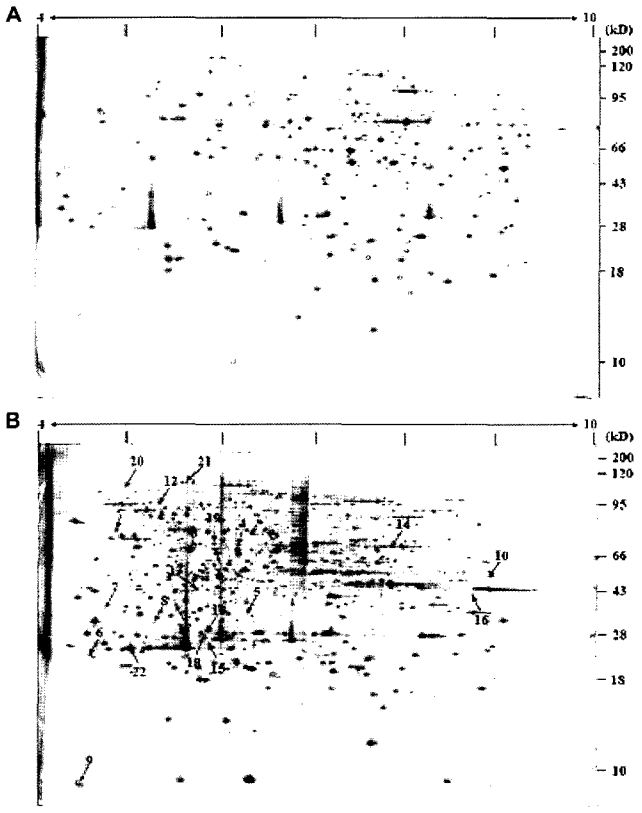

Fig. 2. Representative 2-DE maps of 78-h-old *H. pluvialis* cells under optimal culture conditions (at 25°C under continuous 40 μE/m²/s light intensity).

Arrows indicate proteins detected in red cyst cells under high light intensity (250 μE/m²/s). Numbers correlate with the protein identification listed in Table 1. **A.** Immature cells; **B.** mature cells of *H. pluvialis*.

Table 1. Protein expression profiles for *Haematococcus pluvialis* after exposure to high light intensity, as resolved by 2-DE.

Protein expression patterns	No. of protein spots excised	No. of proteins identified
Constantly expressed at all times	1,120	-
Expression downregulated[a]	225	-
Expression upregulated[a]	155	22

[a]Proteins whose abundance became at least two-fold higher or lower than the basal level in the green vegetative cells were regarded as upregulated or downregulated, respectively, under the stress culture conditions.

high light intensity on the surface of a photobioreactor were collected for water-soluble protein extraction to analyze the protein expression profiles by 2-DE. Figure 2 represents a 2-D map of the proteins extracted from the green vegetative cells, in which approximately 1,500 well-defined protein spots were detected in 4–10 pI regions by alkaline silver staining. To classify the cellular protein expression profiles, systemic compression of the water-soluble proteins revealed in the 2-D maps was carried out using PDQuest software (Bio-Rad, Hercules, CA, U.S.A.) during the transformation of the green vegetative cells into red cyst cells under high light stress. Table 1 shows three types of soluble protein expression profiles in response to high light intensity, where 225 proteins were downregulated, 155 proteins were upregulated, and 1,120 proteins occurred at all stages of the cell cycle throughout the 78 h of high light stress.

Among all the proteins, 30 spots (found on the 2-D gel in five independent 2-D electophoreis assays) associated with upregulation were selected for identification. The spots were excised from the 2-D gels and subjected to in-gel digestion and MALDI-TOF MS analysis. The selected proteins were reproducibly visible and clearly assigned in different gels. Table 2 lists the identified proteins with an apparently altered abundance under high light intensity. Overall, 22 proteins that were transiently upregulated during the 78-h high light stress were identified (Tables 1 and 2), among which 20 were from the green algae *Chlamydomonas reinhardtii* and *Haematococcus pluvialis* [32, 34]. It is believed these proteins are related to cellular processes, stress responses, metabolism, and the biosynthesis of carbohydrates and carotenoids, as well as protein translation, transport, and binding.

Heat-Shock Proteins

In response to sudden changes resulting from adverse environmental stimuli, algal cells exhibit an enhanced protein expression, collectively referred to as heat-shock proteins

Table 2. Proteins induced in red cyst cells of *H. pluvialis* under high light stress.

Spot No.	Protein	Accession No.	MW (kDa)	pI	NMP	SC (%)	Mowse score
1	Peroxidase precursor	P08071	36.9	5.9	6	18.0	5.34e+03
2	60 kD Chaperonin 2	2129459	59.1	4.9	6	28.0	1.92e+04
3	ABC transporter ATP-binding protein (ycf24)	16331744	53.4	5.8	6	18.0	1.18e+03
4	Purple acid phosphate-like protein	15231682	56.2	6.2	6	28.0	1.31e+04
5	UDP-*N*-acetylmuramate dehydrogenase	Q9A5A7	34.4	6.3	4	18.0	1.10e+04
6	Heat-shock 22-kDa protein-like A	15242086	23.4	4.6	3	15.0	1.47e+03
7	Isopentenyl pyrophosphate (IPP) isomerase	Q99RS7	33.5	4.8	4	12.0	4.32e+03
8	Light harvesting chlorophyll *a/b*-banding protein associated with photosystem I	10308399	30.4	5.3	6	20.0	3.96e+04
9	Lipooxygenase	10764845	8.2	4.4	3	10.0	2.41e+04
10	Protein kinase family protein	7488240	43.1	8.9	5	16.0	1.21e+04
11	Oxygen-evolving enhancer protein 2 chloroplast	P12852	28.9	5.8	5	18.0	2.18e+03
12	Oligopeptidase A	P44573	88.4	5.3	5	10.5	5.22e+04
13	Actin	P53498	38.5	5.8	7	17.0	1.39e+04
14	Lycopene β-cyclase	Q55276	55.7	7.8	6	11.0	4.38e+04
15	Hypothetical protein CT852	O84860	26.9	5.8	4	13.0	2.63e+04
16	Fe-S-cluster redox enzyme	P87027	38.4	8.7	3	10.0	6.38e+03
17	ABC transporter ATP-binding protein (ycf16)	17228136	37.8	5.5	6	10.0	3.26e+03
18	ATP synthase α-subunit	P38382	26.4	5.8	6	13.0	1.02e+04
19	Potassium-transporting P-type ATPase C chain KdpC	16330520	55.0	5.9	6	17.0	1.08e+04
20	Protein kinase	6755156	99.3	5.0	3	7.5	1.10e+03
21	ATP synthase β-subunit, mitochondrial	P38482	106.9	5.7	5	11.0	1.20e+03
22	Putative 33-kDa oxygen-evolving protein of photosystem II	Q943W1	26.8	5.6	8	28.0	3.62e+04

(HSPs) [36]. In the present study, spots 2 and 6 were identified as heat-shock proteins, and based on their molecular weights, classified as HSP60 and a low-molecular-weight HSP, respectively. These two major heat-shock proteins have already been detected in the cell well of *H. pluvialis* [35], where Wang *et al.* [34, 35] identified HSP60 in cell wall extracts, suggesting that these proteins may also be related to cell wall biogenesis in addition to their role responding to high light stress.

In *Arabidopsis*, HSP transcripts are slightly accumulated under normal growth conditions [37], yet expressed at high levels under chemical stress, thereby suggesting that the expression of HSPs is necessary at all times to supply sufficient protection.

Putative Oxidoreductases

A number of putative oxidoreductases have already been identified in *H. pluvialis* cell wall extracts [35]. Here, two oxidoreductases of *H. pluvialis*, peroxidase (spot 1) and UDP-*N*-acetylmuramate dehydrogenase (spot 5), were identified as being induced with exposure to high light intensity, although the expressions levels were only enhanced (data not shown), suggesting that oxidoreductases, particularly peroxidase, may cause a reduction in the cell wall extensibility [17]. In *Chlamydomonas reinhardtii* [33], the H_2O_2 that accompanies peroxidase activity is produced when the cell wall becomes insoluble.

Putative Transporters/Binding Proteins, ATP Synthase, and Ion Channel

Two putative ATP-binding proteins of an ABC transporter were identified (spots 3 and 17). ABC transporter ATP-binding proteins are membrane proteins involved in the active transfer of a broad range of substances across the membrane [34]. In *Arabidopsis* [24], there are 129 open reading frames (ORFs) capable of encoding ABC transporters. As several ATP-binding ABC transporters responsible for the uptake of almost all inorganic nutrients have already been deduced from the genome of *Synechocystis* [22], more putative ABC transporter ATP-binding proteins are anticipated. Wang *et al.* [35] previously reported six putative transporter/binding proteins present in the cell wall of *H. pluvialis*; however, this difference with the present study was possibly caused by the following: (i) the concentration of binding proteins was rather low, meaning no visible spots in the 2-D gels; (ii) binding proteins are much less conserved than other subunits of ABC transporters; and (iii) additional binding proteins may have existed among the unidentified proteins. In the present study, the two contiguous ATP-binding proteins of an ABC transporter (spots 3 and 17), involved in as yet unidentified processes in the assembly of various iron-sulfur centers (spot 16), were commonly induced by high light intensity, suggesting polycistronic transcription [28].

There have also been several reports on the presence of ATP synthase and respiratory chain components in *H. pluvialis* [32, 34, 36], plus the mitochondrial ATP synthase β-subunit is upregulated, which is analogous with physiological evidence that the respiration rate gradually increases during the transformation of the vegetative cells into red cyst cells in *H. pluvialis* under stress conditions [29]. In the present study, subunits α (spot 18) and β (spot 21) were found that were related to the mitochondrial respiratory proteins of ATP synthase. This increase in the mitochondrial respiratory activities in the cells may have been caused by the energy-defendant build-up of the antioxidative defense pathway.

In the *Synechocystis* genome [14], four subunits of putative K^+-transporting P-type ATPase are found as the operon *kdpABCD*, where KdpA is involved in the binding and transport of K^+, KdpB is the catalytic subunit in *Synechocystis*, and KdpC (spot 19), which is present in the plasma membrane of *Synechocystis*, has the same predicted secondary structure as a transmembrane helix in the amino-terminal end anchored in the plasma membrane exposing the C-terminus to the cytoplasm [1, 12, 14].

Subunits of Photosystems I and II

Plants produce metabolic energy by collecting light energy and transferring it to photosynthetic reaction centers. This process is facilitated by antennae or light-harvesting complexes (LHCs) composed of light-harvesting complex proteins that bind light-harvesting pigments. Several LHCs have already been characterized from various organisms, including chlorophyll *a/b*-containing chlorophyta, chlorophyll *a/c*-containing chromophyta, and chlorophyll *a*-containing rhodophyta [9]. The light-harvesting chlorophyll *a/b*-binding protein associated with photosystem I (spot 8) and the genes encoding this protein have been characterized in *Chlamydomonas reinhardtii* [30]. The putative 33-kDa oxygen-evolving protein of photosystem II (spot 22) and oxygen-evolving enhancer protein (OEE; spot 11) are auxiliary components of the photosystem II manganese cluster that facilitates algal photosynthesis. In particular, an OEE protein, which is a heat-stable protein, is known as a thioredoxin-like chloroplast protein of the fructosebisphosphatase-stimulation f-type in *Scenedesmus* and *Chlamydomonas* [13]. Phosphogylycerate kinase was also upregulated after 78 h of high light stress, which is similar to the experimental results of Wang *et al.* [34].

Carotenoid Biosynthetic Enzymes

Isopentenyl pyrophosphate (IPP) isomerase (spot 70) was detected and it catalyzes the reversible isomerization of IPP to produce dimethylallyl pyrophosphate, the initial substrate leading to the biosynthesis of carotenoids [6]. An increase in IPP activity is correlated with an increased biosynthesis of carotenoids, and regarded as a crucial rate-

limiting step leading to the biosynthesis of carotenoids [6, 15]. In the present study, the expression of IPP isomerase and the two enzymes specific to the carotenoid pathway, lycopene-β-cyclase (spot 14) and β-carotene-C-4-oxygenase, occurred in the green alga *H. pluvialis* after exposure to high light intensity [27], Thus, it is suggested that IPP isomerase is involved in carotenoid accumulation in the cytoplasm of *H. pluvialis*, since IPP isomerase was preferentially upregulated by high light intensity preceding an increase in carotenoid, and only this form was detected in the red cyst cells.

Lycopene β-cyclase (spot 14) was identified and is also known to be involved in carotenoid accumulation [15]. Carotenoids with a cyclic end group are essential compounds in the photosynthetic membranes of algae, including the genus *Haematococcus*. The cyclization of lycopene (ψ,ψ-carotene) is a key branch point in the pathway of carotenoid biosynthesis. There are two types of cyclic end groups, a β and ε ring, in carotenoids [7]. Carotenoids with two β rings (β-carotene; β,β-carotene) are ubiquitous in oxygen-evolving photosynthetic organisms, and those with one β and one ε ring are also common, but two ε rings are rare. β-Carotene is intimately associated with chlorophyll in the photosynthetic reaction center [34]. Two lycopene β- and ε-cyclases are encoded, and both enzymes use a linear symmetrical lycopene as a substrate. However, the ε-cyclase only adds one ring to form a monocyclic δ-carotene, whereas the β-cyclase introduces a ring at both ends of lycopene to form a bicyclic β-carotene [7]. Therefore, an increased lycopene β-cyclase activity catalyzes the production of β-carotene (β,β-carotene), instead of the formation of inappropriate ε,ε-carotenoids. The enzyme also converts neurosporene into a monocyclic β-zeacarotene, yet does not cyclize ζ-carotene, indicating that desaturation of the 7–8 or 7'–8' carbon-carbon bond is required for cyclization [8]. Therefore, the present results prove that β-carotene was synthesized *de novo* during the transformation of green vegetative cells into red cyst cells by the lycopene β-cyclase (Fig. 3) and suggest that in

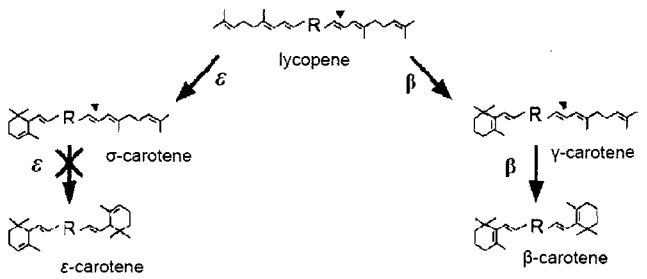

Fig. 3. Putative routes of carotenoid cyclization in *H. pluvialis*. The demonstrated activities of the lycopene β- and ε-cyclase enzymes are indicated by boldface arrows labeled with β or ε, respectively. The × over the arrow leading to ε-carotene indicates that the enzymatic activity was examined, yet no product was detected.

Haematococcus, the specific astaxanthin pathway and general carotenoid biosynthesis are both subject to control by light.

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