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Astaxanthin Biosynthesis Enhanced by Reactive Oxygen Species in the Green Alga *Haematococcus pluvialis*

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Abstract The unicellular green alga *Haematococcus pluvialis* has recently attracted great interest due to its large amounts of ketocarotenoid astaxanthin, 3,3'-dihydroxy-β,β-carotene-4,4'-dione, widely used commercially as a source of pigment for aquaculture. In the life cycle of *H. pluvialis*, astaxanthin biosynthesis is associated with a remarkable morphological change from green motile vegetative cells into red immotile cyst cells as the resting stage. In recent years we have studied this morphological process from two aspects: defining conditions governing astaxanthin biosynthesis and questioning the possible function of astaxanthin in protecting algal cells against environmental stress. Astaxanthin accumulation in cysts was induced by a variety of environmental conditions of oxidative stress caused by reactive oxygen species, intense light, drought, high salinity, and high temperature. In the adaptation to stress, abscisic acid induced by reactive oxygen species, would function as a hormone in algal morphogenesis from vegetative to cyst cells. Furthermore, measurements of both *in vitro* and *in vivo* antioxidative activities of astaxanthin clearly demonstrated that tolerance to excessive reactive oxygen species is greater in astaxanthin-rich cysts than in astaxanthin-poor cysts or astaxanthin-less vegetative cells. Therefore, reactive oxygen species are involved in the regulation of both algal morphogenesis and carotenogenesis, and the accumulated astaxanthin in cysts can function as a protective agent against oxidative stress damage. In this study, the physiological roles of astaxanthin in stress response and cell protection are reviewed.

Keywords: antioxidant, astaxanthin, cyst cell, environmental stress, *Haematococcus pluvialis*, reactive oxygen species

INTRODUCTION

The red ketocarotenoid astaxanthin, 3,3'-dihydroxy-β, β-carotene-4,4'-dione, is used not only as a source of pigment in the diets of fish and animals [1], but also has potential clinical applications due to its higher antioxidative activity than β-carotene and α-tocopherol [2-5]. The principal microbiological sources of astaxanthin are the yeast *Phaffia rhodozyma* [6] and the green alga *Haematococcus pluvialis* [7]. The content of astaxanthin in *P. rhodozyma* is low (0.4 mg/g dry cell), while that in *H. pluvialis* is very high (50 mg/g dry cell) [8]. Interestingly, astaxanthin occurs freely in *P. rhodozyma* but is predominately found as esters in *H. pluvialis* [4,6]. The algal astaxanthin is composed of monoesters (70-80% of total astaxanthin) and diesters (20-30%) with higher fatty acid levels [4,7,9].

In the life cycle of *H. pluvialis*, a deficiency of nutrients such as nitrogen induces cyst formation, with a morphological change from vegetative to cyst cells as the resting stage [10-13]. Astaxanthin biosynthesis in cyst cells is enhanced by conditions of oxidative stress induced by the addition of reactive oxygen species (ROS) [14], intense light [15], drought [16], high salt [17], and high temperature [18]. Therefore, oxidative stress was considered to be involved in the regulation of astaxanthin biosynthesis in *H. pluvialis*. Since ROS also regulate carotenoid biosynthesis in some microorganisms such as the yeast *P. rhodozyma* [19] and the green alga *Dunaliella bardawil* [20], the accumulated carotenoids would provide protection *in vivo* against oxidative stress damage as we suggested for *H. pluvialis* [4,21,22]. In this paper, on *H. pluvialis*, the morphology, physiology, and the biosynthesis of carotenoid will be discussed. The interrelationships between ROS and astaxanthin in stress response and cell protection will also be reviewed.

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CELL MORPHOLOGY

The unicellular green alga *H. pluvialis* lives primarily in small fresh water holes or on the surface of snow on high mountains [23]. The red coloring caused by the accumulation of astaxanthin in the alga is a natural phenomenon known as "blood rain" and "red snow". Fig. 1 shows photographs of *H. pluvialis*. In nature as shown in Fig. 1

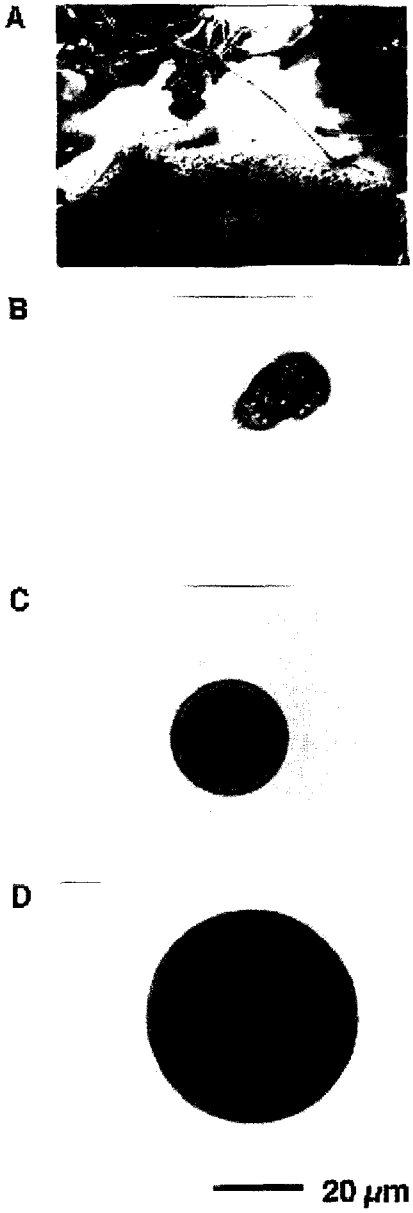

Fig. 1. Photographs of *H. pluvialis* in "red snow" (a), vegetative cell (b), astaxanthin-poor immature cyst (c), and astaxanthin-rich mature cyst (d), quoted from Kobayashi [23]. Reproduced with permission from the publisher.

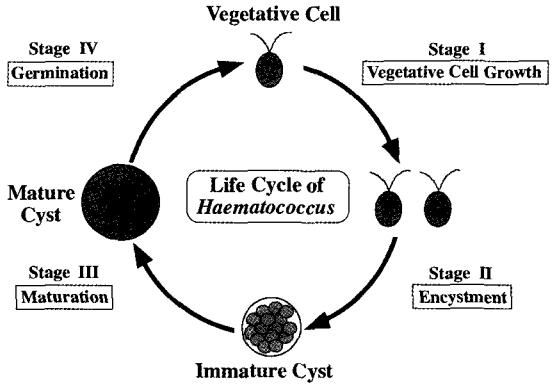

Fig. 2. Schematic diagram of the life cycle of *H. pluvialis*, quoted from Kobayashi *et al.* [13]. Reproduced with permission from the publisher.

(a), the algal life cycle depends on environmental conditions such as the availability of nutrients, temperature, drought, light intensity, and so on. In our laboratory, factors such as the medium, temperature, and illumination were optimized to simplify and shorten the incubation period [7,13]. The life cycle of *H. pluvialis* on acetate medium was divided into four stages [13], as illustrated in Fig. 2: I, vegetative cell growth; II, encystment (vegetative to immature cyst cells); III, maturation (immature to mature cyst cells); IV, germination (mature cyst to vegetative cells). In this way, the life cycle of *H. pluvialis* was completed within only 2 weeks in our culture system [13]. Although the morphological change from vegetative to enlarged thick-walled cyst cells has been reported to take several weeks under autotrophic conditions, and in either nitrogen- or phosphate-deficient media, we showed that a high acetate content (*i.e.*, effect of a high carbon/nitrogen ratio) in the medium induced encystment within several days under mixotrophic conditions because of the relative deficiency of nitrogen [7]. Under optimal growth conditions, ellipsoidal vegetative cells were capable of actively swimming with two flagella and of increasing in number as shown in Fig. 1(b) [13]. In response to adverse environmental conditions, the green flagellated cells cease to be motile and gradually transform into spherical immotile cyst cells (aplanospores) as shown in Fig. 1(c), developing a distinct red color due to the accumulation of astaxanthin during maturation as shown in Fig. 1(d) [13]. This phase is considered the resting stage. When mature cysts were transferred to fresh medium, they germinated releasing intracellular daughter cells into the medium, and the vegetative cells grew again [13]. Although most algae generally grew autotrophically on CO_2, vegetative cells of *H. pluvialis* were able to grow heterotrophically on acetate in the dark as well as mixotrophically on acetate in the light [13,24]. However, light is essential for the life cycle of *H. pluvialis* particularly for cell differentiation (encystment and germination) and maturation (carotenogenesis) [13].

ASTAXANTHIN BIOSYNTHESIS ENHANCED BY ROS

The increase in the level of astaxanthin in cyst cells of *H. pluvialis* was closely associated with the maturation of cysts [13]. Interestingly, we showed that the biosynthesis of astaxanthin was enhanced by the addition of Fe^{2+} in 1991 [7]. At that time, while a number of articles had been published on the photoinhibition of carotenoid formation in microbial secondary metabolism [25], only a few studies had focused on the involvement of oxidative stress in carotenoid production. In 1993, we demon-

strated that the Fe^{2+}-enhanced synthesis of astaxanthin was inhibited by potassium iodide (a scavenger for hydroxyl radicals), suggesting that hydroxyl radicals formed by an iron-catalyzed Fenton reaction may be require for enhanced carotenogenesis [14]. Moreover, astaxanthin biosynthesis in cysts was enhanced by the addition of ROS such as singlet oxygen ($^1O_2$), superoxide anion radicals (O_2^-), H_2O_2, hydroxyl radicals, and peroxy radicals [14]. The ROS-regulated production of carotenoid in two microorganisms, the yeast *P. rhodozyma* [19] and the green alga *D. bardawil* [20], was also reported in 1993. Since the Fe^{2+}-enhanced biosynthesis of astaxanthin did not require the *de novo* synthesis of protein [14], oxidative stress would be involved in the post-translational activation of astaxanthin production. In 1997, Fraser *et al.* [26] purified carotenogenic enzymes from *Escherichia coli* strains expressing the genes for astaxanthin biosynthesis, and clearly demonstrated that the biosynthetic reaction using the purified enzymes strictly required the addition of Fe^{2+} *in vitro*. Since astaxanthin biosynthesis required oxygen, NADPH and Fe^{2+}, Cunningham and Gantt [27] proposed that a cytochrome P450-dependent enzyme, hydroxylase, is involved in the transformation of β-carotene to astaxanthin.

Astaxanthin biosynthesis in cysts was also enhanced by several environmental factors involved in the generation of ROS, namely intense light [15], drought [16], high salt [17], and high temperature [18]. Under conditions of either drought [16] or high temperature [18], astaxanthin production in cysts was inhibited by the addition of a scavenger or quencher for ROS, suggesting that ROS regulated algal carotenogenesis. In plants, endogenous ROS is considered to cause plant senescence in response to such stress [28], and for example, O_2^- in chloroplasts of drought-stressed wheat has been directly detected [29]. In higher plants, abscisic acid (ABA) is best known for its function as a stress hormone in the adaptation to drought and other stress [30]. Hirsh *et al.* [31] first reported that ABA is universally distributed within the algal kingdom, and identified it in salt-stress-dependent ABA synthesis and metabolism in the green alga *Dunaliella*. They also indicated that further investigations into the more specialized functions of ABA in algae would be necessary to confirm its action as a hormone in lower organisms [31]. Recently, in the green alga *Chlamydomonas reinhardtii*, Yoshida *et al.* [32] reported that ABA would play a role in the enhancement of tolerance to oxidative stress by increasing the activity of antioxidant enzymes such as catalase and ascorbate peroxidase. In *H. pluvialis*, when exogenous ABA was added to vegetative cells under conditions of drought, red mature cyst cells with increased levels of astaxanthin rapidly appeared [16]. ABA biosynthesis in vegetative cells was enhanced by light, and mixotrophic vegetative cells contained 3-fold more ABA than heterotrophic vegetative cells [16]. Encystment coincided with a decrease in ABA, which remained low in cyst cells during maturation [16]. It was also suggested that β-carotene-rich yellow cells that ceased to divide, contained significantly less ABA than green cells in *Dunaliella* [31]. The relationship between ABA levels and morphogenesis in *H. pluvialis* indicated that the decreased ABA level coincides with the cell stage of aging or maturation. In stressed cultures such as drought [16] and salt [31], ABA might function as a hormone in algal metabolism such as during encystment of *H. pluvialis*. However, it is possible that the decline in ABA content is fortuitous due to accumulation of astaxanthin which limits supply of carbon for 9'-*cis*-neoxanthin formation – the carotenoid precursor to ABA [16]. Since algal ABA biosynthetic gene or enzyme has not been identified, ABA biosynthetic pathway in green algae is not clear.

It seems likely that both cyst formation and astaxanthin biosynthesis in cysts are important adaptive responses against oxidative stress in the life cycle of *H. pluvialis*. The alga has developed an efficient defense system that has helped it to survive under environmentally adverse conditions during evolution.

ANTIOXIDANT ROLE OF ASTAXANTHIN

Antioxidant Defense System in *H. pluvialis*

Generally, carotenoids have two important roles in photosynthetic organisms [33]. First, they act as accessory light-harvesting pigments, trapping light energy and passing it on to chlorophylls. Second and more importantly, carotenoids protect the photosynthetic apparatus from light-mediated stress, for example by quenching $^1O_2$ generated by photooxidation. In *D. bardawil*, Shaish *et al.* [20] suggested that photosynthetically produced ROS, O_2^- (and its products) and $^1O_2$, are involved in triggering the biosynthesis of β-carotene, and the massive amount of carotenoid accumulated can protect the photosynthetic apparatus against oxidative stress. Since adding azide, an inhibitor of superoxide dismutase (SOD), to *D. bardawil* greatly enhanced β-carotene production with the accumulation of ROS, intracellular β-carotene may function together with SOD to preserve viability in the face of the continued production of ROS in aging cells [20].

Similarly, tolerance to excessive methyl viologen (MV, O_2^- generator) was higher in astaxanthin-rich mature cysts than in astaxanthin-poor immature cysts of *H. pluvialis* [21]. We determined the SOD-like antioxidant activities *in vivo* against O_2^-, using the whole-cell *in vitro* assay system to elucidate the physiological role of astaxanthin in cyst cells of *H. pluvialis* [21]. Astaxanthin-rich cysts showed high antioxidant activity against O_2^- in permeabilized whole cells, but not in astaxanthin-free cell extracts, while astaxanthin-poor cysts had very low antioxidant activities against O_2^- in both [21]. The results suggested that astaxanthin accumulated in the cyst cells functions as an antioxidant against excessive oxidative stress. In contrast, the same levels of antioxidant activities against O_2^- in both permeabilized whole cells and cell extracts from vegetative cells suggested the presence of antioxidative enzymes [21]. Therefore, cyst cells might be deficient in the enzymes or low-molecular-mass antioxidants, such as ascorbate and glutathione, necessary to detoxify ROS. Astaxanthin located in cytosolic lipid glob-

ules and membraneous regions of cysts [34] could compensate for the lack of these enzymes and antioxidants. *H. pluvialis* has two antioxidative mechanisms involving antioxidative enzymes such as SOD and catalase in vegetative cells and the antioxidative ketocarotenoid astaxanthin in cyst cells, and might change from one of these systems of defense against environmental oxidative stress to the other during its life cycle. Moreover, the tolerance of astaxanthin-rich mature cysts to UV-B was 6-fold higher than that of astaxanthin-poor immature cysts [35]. Since increasing exposure to UV-B irradiation in the environment involved endogenous photosensitization and ROS formation in the alga, astaxanthin in cyst cells would function as an all-round protective agent against environmental stress.

In Vitro Antioxidant Activities

Carotenoids are well known to detoxify $^1O_2$ catalytically, and a single β-carotene molecule can detoxify 250-1,000 molecules of O_2 [36]. $^1O_2$ is O_2 in an excited electronic state which causes the peroxidation of lipids, cellular membrane damage, and damage to DNA when it decays to its ground state [37]. Although astaxanthin is more effective against $^1O_2$ than β-carotene both *in vitro* and *in vivo* [2,3,37,38], only free astaxanthin was used to evaluate the antioxidant ability. We measured the $^1O_2$ quenching activities of carotenoids, β-carotene from carrot, free astaxanthin from *P. rhodozyma*, and its esters (monoester and diester) from *H. pluvialis*, *in vitro* by a simple and rapid method for the measurement of methylene blue-sensitized photooxidation of linoleic acid in the hexane/ethanol (hydrophobic/hydrophilic) solvent system [4]. As the hydrophobicity of the solvent increased, the quenching activity of free astaxanthin was reduced, while the activity of β-carotene was increased [4]. Thus, the quenching activities of β-carotene and free astaxanthin varied depending on the hydrophobicity of the solvent. However, the two algal astaxanthin esters maintained strong antioxidant activities independent of the hydrophobicity [4]. In *H. pluvialis*, most astaxanthin is present in an esterified form with fatty acid (mainly $C_{18:1}$), which has both hydrophilic groups (C=O and OH) and hydrophobic esters in the β-ionone rings [4,7,9]. Esterification of free astaxanthin would compensate for the decreased antioxidant activity of free astaxanthin under hydrophobic conditions. In other words, the ester part of astaxanthin esters might function as a stabilizer to maintain the antioxidant ability under hydrophilic and hydrophobic conditions. In the resting cyst cells of *H. pluvialis*, the accumulated astaxanthin esters would efficiently function as antioxidant agents in both cytoplasmic and membraneous regions to protect cyst cells against environmental oxidative stress.

In Vivo Antioxidant Activities

Although we showed that astaxanthin functions as a powerful antioxidant in two experiments to measure extracellular or exogenous ROS-reacted products *in vitro* [4,21], the physiological role of astaxanthin *in vivo* in cyst cells of *H. pluvialis* was not clear. ROS are highly reactive and short lived, which complicates the measurement of their intracellular production [39]. The general approach has been to measure the products of ROS damage of membrane lipids, proteins and DNA, or the decrease in levels of ROS-scavenging/quenching substances such as ascorbate and glutathione. The quantification of ROS in *H. pluvialis* is complicated since ROS diffuse out of the cells and the measurement of damage does not provide a direct measure of intracellular ROS production because of the presence of protective enzymes and antioxidants. As an alternative, we studied the intracellular production of ROS by measuring *in vivo*, the capacity to convert 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) to the fluorescent dye dichlorofluorescein (DCF) in different algal cell types (*i.e.*, vegetative, immature cyst and mature cyst cells) [22]. DCFH-DA is a non-polar, non-fluorescent compound that diffuses across membranes, and the incorporated DCFH-DA is deacetylated by intracellular esterase to give the polar compound 2',7'-dichlorohydrofluorescein (DCFH), which is trapped in the cell [40-42]. In contrast to DCFH-DA, DCFH can be oxidized by ROS and converted to the fluorescent compound DCF [40-42]. The increase in formation of DCF by MV (O_2^- generator) was linear for 2 h in astaxanthin-poor immature cyst cells in a MV-concentration-dependent manner, while no production was detected in astaxanthin-rich mature cysts [22]. The formation of DCF in cyst cells decreased as the astaxanthin content increased under excessive oxidative stress [22]. Therefore, astaxanthin in cyst cells appeared to function as an antioxidant *in vivo* against oxidative stress. Compared to that in cyst cells, the DCF production in vegetative cells (no astaxanthin) was markedly increased by MV [22]. Vegetative cells might be more sensitive to ROS than cyst cells, and thus the morphological change from vegetative to cyst cells would be important for survival under adverse conditions. DCF production is an indicator of oxidative stress; *i.e.*, the net formation of ROS in excess of the capacity of cellular protective mechanisms, rather than a function of absolute (gross) production of these metabolites. The formation of DCF from DCFH-DA is potentially a very powerful tool for the study of oxidative stress in organisms and can complement other techniques. Recently, the effect of ABA treatment of *C. reinhardtii* on the generation of ROS was investigated by flow cytometric analysis using DCFH-DA as an indicator [32].

RECENT TOPICS IN *HAEMATOCOCCUS*

Astaxanthin Biosynthesis

During the past ten years, the pathway of astaxanthin biosynthesis has been well studied, the intermediates clearly identified using excellent analytical procedures and the genes encoding carotenogenic enzymes involved in the pathways have been cloned as described in detail [25,27,43-46], but the regulatory mechanism of astaxan-

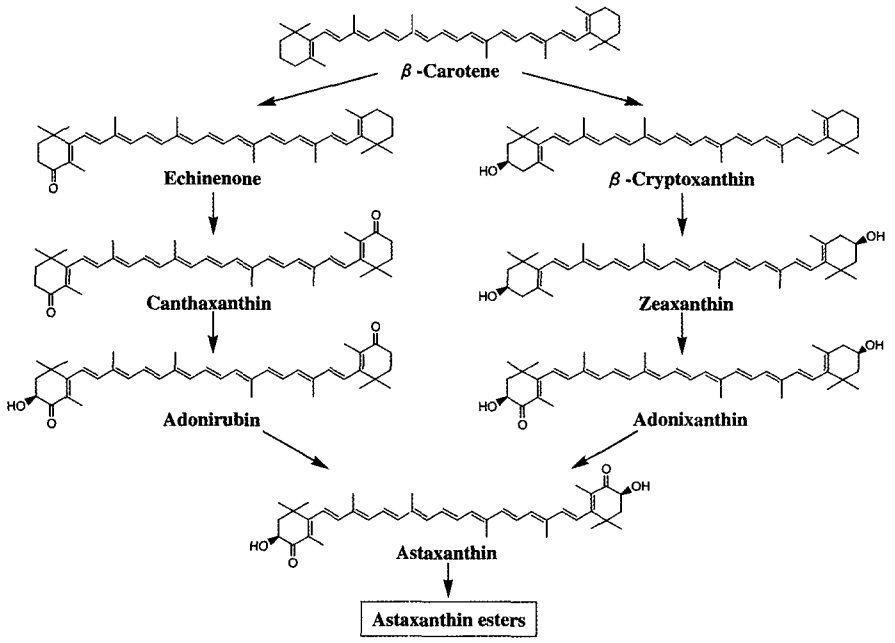

Fig. 3. Biosynthetic pathway of astaxanthin from β-carotene in *H. pluvialis*.

thin biosynthesis has not been investigated thoroughly. In this review, the pathway of astaxanthin biosynthesis from β-carotene is focused on (Fig. 3). Astaxanthin is produced in *H. pluvialis* first at the expense of pre-existing β-carotene, and two pathways have been identified: the first one would start with the oxidation of β-carotene and have echinenone, canthaxanthin and adonirubin as intermediates, whereas the second would begin with the hydroxylation of β-carotene and have β-cryptoxanthin, zeaxanthin and adonixanthin as intermediates [47]. Although the genes encoding the enzymes, β-carotene oxygenase (another name, β-carotene ketolase) and β-carotene hydroxylase, involved in these pathways have been cloned [48-50], the nature of the enzymes remains partially undetermined. Experiments using inhibitors and *in vitro/in vivo* analyses of these two enzymes suggested that the route of astaxanthin production is through the initial formation of canthaxanthin via echinenone, which is then converted into astaxanthin [25,27,43-46].

Using immunogold labeling and Western blot analysis, Grünewald *et al.* [51] showed that β-carotene oxygenase in *H. pluvialis* was localized both to chloroplasts and to cytoplasmic lipid vesicles, which are derived from cytoplasmic membranes, while β-carotene oxygenase activity was confined to the lipid vesicle compartment only. Because an early carotenogenic enzyme in the pathway, phytoene desaturase, was found only in the chloroplast, the transport of intermediates from the site of biosynthesis in the chloroplast to the site of oxygenation and accumulation in cytoplasmic lipid vesicles is proposed to take place [51].

From the expression of phytoene synthase and β-carotene hydroxylase, two key enzymes in the production of astaxanthin, Steinbrenner and Linden [52] suggested that ROS did not directly influence transcript levels of the two genes although levels of expression increased in response to environmental stress such as strong light and salt. Furthermore, they showed that Fe^{2+}-enhanced astaxanthin biosynthesis in cyst cells [7,14] would be clearly independent of *de novo* protein synthesis because of the function of ROS at the post-translational level. However, Bouvier *et al.* [53] reported that ROS are potent inducers of carotenogenic gene expression during chromoplast development in pepper. Although the interrelationships between ROS and carotenoid biosynthesis in the response to stress and photosynthesis are not clear, complex regulatory mechanisms might function at both the gene and protein level to coordinate antioxidant responses.

In relation to photosynthesis-dependent carotenoid biosynthesis, it was reported that plastoquinone can be substituted for molecular oxygen as a terminal electron acceptor in phytoene desaturation in chromoplasts of daffodil [54], and cytochrome *f* was shown to be selectively lost in red cyst cells of *H. pluvialis* [55]. Therefore, instead of impairing the linear electron flow between PS II and PS I, the plastoquinone pool might function as an electron crossover point between photosynthesis and astaxanthin biosynthesis in cyst cells [13]. Recently, Steinbrenner and Linden [56] indicated that the light-regulation of astaxanthin biosynthesis in *H. pluvialis* is under photosynthetic redox control, and the plastoquinone pool seems to function as a redox sensor: reduction of the plastoquinone pool subsequently leads to the transcriptional activation of genes involved in astaxanthin production. The redox control of carotenogenic genes seems to have two different physiological functions as follows [56]. The transient induction of the genes under moderate light is part of the acclimation process of the

alga to intense light. On the other hand, the redox regulation of carotenogenic genes is a prerequisite for the production of astaxanthin under stressful conditions such as high light and in the presence of ROS, which might affect the post-translational regulation of astaxanthin biosynthesis. *H. pluvialis* would perceive changes in light intensity via the redox state of components of the photosynthetic electron transport system.

Improvement of High Producing Strains

Mutants of *H. pluvialis* have been obtained through UV exposure and ethyl methanesulphonate treatment for hyperproduction of astaxanthin [57-60]. These mutants were further screened using several inhibitors of carotenoid biosynthesis, such as compactin [58,59], diphenylamine [59,60], norflurazon [57,60], fluridone [57,60], and nicotine [57,59,60]. These resistant mutants accumulated two- to three-fold more astaxanthin than the wild type, and also exhibited stronger carotenogenic enzyme activity corresponding to the inhibitor's specific site. Although mutagenesis is a classic and conventional method for strain improvement, the mutants obtained steadily maintained resistance against the inhibitors after routine transfer to inhibitor-free medium for more than one year [59]. Furthermore, with the establishment of an effective method of preparing osmotically labile protoplasts from algae by protease treatment [61], hybrid strains have been generated by the protoplast fusion of herbicide-resistant mutants that possess two-fold the level of ploidy, and three-fold the astaxanthin content of the parental strains [57].

Recently, a genetic transformation system for *Haematococcus* was developed to study the regulatory mechanism of astaxanthin biosynthesis, and to enhance astaxanthin production using genetic manipulation [62]. Presently, the genetic transformation of unicellular green algae has only been reported for cell wall-deficient mutants of *C. reinhardtii* and some species of *Chlorella* [62]. However, *H. pluvialis* has an intact cell wall and no cell wall-deficient mutant has been obtained [62]. Upon use of the micro-particle bombardment method to introduce a foreign reporter gene into *H. pluvialis*, vegetative cells in the exponential phase showed a transient expression of the β-galactosidase gene (*lac*Z) driven by the SV40 promoter, but cyst cells did not [62]. This result suggests that the SV40 promoter and the *lac*Z reporter gene have potential applications in the genetic engineering of algae.

Mixed cultures of wild-type *Dunaliella salina* contained two distinct populations: larger cells containing large amounts of β-carotene and smaller cells containing small amounts of β-carotene, and very few cells were as large as the hyperproducing strains [63]. Similarly, larger cyst cells of *H. pluvialis* produced more astaxanthin in culture than smaller cells, and could thrive in mass culture because of their higher tolerance to environmental oxidative stress [64]. In *P. rhodozyma*, astaxanthin-hyperproducing strains could be selected as viable cells by flow cytometry because astaxanthin is a fluorescent cellular constituent [65]. However, as algal cells contain chlorophyll that is also a major fluorescent constituent, this technique would not apply to *H. pluvialis*. As described above, DCFH-DA is useful as a fluorescent marker for the generation of ROS in viable cells *in vivo*, because DCFH-DA and DCF have no toxicity and the fluorescence of chlorophyll did not interfere with that of DCF [22]. The production of DCF in cyst cells decreased the larger the cell diameter, and this decline was also correlated with increases in astaxanthin content [64]. Therefore, both the diameter and fluorescent DCF content of cyst cells would be good parameters with which to select astaxanthin-hyperproducing strains from native populations of *H. pluvialis*.

The solubility of carotenoids in lipid is generally very low: at 30°C the solubilities of β-carotene in corn oil and olive oil are only 0.08% and 0.1% respectively [36]. Assuming a lipid concentration in the cell of 25% and a carotenoid solubility of 0.1%, the maximum concentration of β-carotene would be 0.25 mg/g cells [36]. Although numerous methods exist to increase the concentration of carotenoid such as the screening of herbicide-resistant strains [57-60], limited carotenoid solubility in the cell and phase separation in the cytoplasm may be major limitations to strain improvement. One mechanism to increase astaxanthin solubility may be through esterification [36]. In fact, the amount of astaxanthin in *P. rhodozyma* is low (0.4 mg/g dry cell), while that in *H. pluvialis* is very high (50 mg/g dry cell) [8]. Interestingly, astaxanthin occurs freely in *P. rhodozyma* but is predominately found as esters in *H. pluvialis* [4,6]. The algal astaxanthin is composed of monoesters (70-80% of total astaxanthin) and diesters (20~30%) with higher fatty acid levels [4,7,9]. In *H. pluvialis*, Schoefs *et al.* [47] showed that the availability of fatty acids could constitute a *sine qua non* for secondary carotenoid accumulation, like in *Dunaliella* [66], where the fatty acids participate in the formation of β-carotene droplets but not for any esterification. Furthermore, they reported that astaxanthin production also required the active synthesis of fatty acids, and isolated new strains, which accumulated only non-esterified or only monoesterified or diesterified astaxanthin molecules. In *H. pluvialis*, it would be more important to determine whether the synthesis of fatty acids is required for the esterification of the astaxanthin molecules and/or for the accumulation of astaxanthin droplets in cytoplasm.

CONCLUDING REMARKS

Recently, in the Hawaiian Islands, a commercial large-scale cultivation of *H. pluvialis* was achieved in the production of an algal mass containing astaxanthin using a two-stage system [67]. The two stages comprised vegetative cell growth in a closed reactor, then encystment and astaxanthin biosynthesis in open ponds. The safety of the astaxanthin-rich algal extract of *H. pluvialis* was confirmed by a randomized clinical trial [68], and the algal extract is actively being applied as a potential natural source of astaxanthin in the nutraceutical, cosmetics, food, and feed industries [5]. Although algal astaxanthin

is now available as crude extract, astaxanthin esters will be purified commercially in future, and the specific biological activities will need to be investigated further.

During the past ten years, most of the interest in *H. pluvialis* has centered on three algal events; the growth of vegetative cells, the formation of cysts, and the biosynthesis of astaxanthin in cyst cells as described in this review (Fig. 2). For the commercial production of algal astaxanthin, it is important to determine how to obtain a high concentration of vegetative cells, induce a quick encystment, and enhance astaxanthin production in laboratory studies. However, in nature, the cyst stage is presumably too long and more importantly, cysts have greater tolerance than vegetative cells to adverse conditions such as "hibernation" in animals. We happened to isolate hyper-astaxanthin-producing strains of *H. pluvialis* from common water holes, and surprisingly, found that the strains accumulated 200~300 mg/g dry cell of astaxanthin [69]. One must actively pursue "field work". In nature, whenever cysts of *H. pluvialis* are carried to favorable environments by wind or melting snow, the vegetative growth of daughter cells starts after germination. Since germination might be the most important of the algal events, further study is necessary for a complete understanding of this process.

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