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Minireview

Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response

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The unicellular green alga *Haematococcus pluvialis* Flotow has recently aroused considerable interest due to its capacity to amass large amounts of the ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), widely used commercially to color flesh of salmon. Astaxanthin accumulation in *Haematococcus* is induced by a variety of environmental stresses which limit cell growth in the presence of light. This is accompanied by a remarkable morphological and biochemical 'transformation' from green motile cells into inert red cysts. In recent years we have studied this transformation process from several aspects: defining conditions governing pigment accumulation, working out the biosynthetic pathway of astaxanthin accumulation and questioning the possible function of this secondary ketocarotenoid in protecting *Haematococcus* cells against oxidative damage. Our results suggest that astaxanthin synthesis proceeds via cantaxanthin and that this exceptional stress response is mediated by reactive oxygen species (ROS) through a mechanism which is not yet understood. The results do not support in vivo chemical quenching of ROS by the pigment, although in vitro it was shown to quench radicals very efficiently. The finding that most of the pigment produced is esterified and deposited in lipid globules outside the chloroplast further supports this assumption. We have suggested that astaxanthin is the by-product of a defense mechanism rather than the defending substance itself, although at this stage one cannot rule out other protective mechanisms. Further work is required for complete understanding of this transformation process. It is suggested that *Haematococcus* may serve as a simple model system to study response to oxidative stress and mechanisms evolved to cope with this harmful situation.

Introduction

The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red pigment common to many marine animals contributing to the pinkish color of their flesh (Johnson and An 1991). Only a few animals can synthesize it de novo from other carotenoids, and most of them acquire it in their food (Katayama et al. 1970). There has been growing interest in the use of this pigment as a food coloring agent, natural feed additive for the poultry industry and for aquaculture, especially as a feed supplement in the culture of salmon, trout and shrimp (Johnson and Schroeder 1996). Recently, there have also been reports concerning its application in medicine due to its powerful antioxidant capacity (Palozza and Krinsky 1992). Though astaxanthin can be synthesized by plants, bacteria, a few fungi (Yokoyama and Miki 1995, Johnson and Schroeder 1996, Tsubokura et al. 1999) and green algae (Fjerdingstad et al. 1974), the amounts accumulated by the green alga *Haematococcus pluvialis* (Boussiba et al. 1999) surpass any other reported source. Thus, the research on *H. pluvialis* has been accelerated by the hope that it may become an important natural source for the mass production of astaxanthin. In this study, aspects concerning growth, physiology and the carotenogenesis process will be discussed. The interrelationships between reactive oxygen species (ROS) and astaxanthin in the stress response and photoprotection will be reviewed.

Abbreviations – DPA, diphenylamine; PSI (II), photosystem I (II); ROS, reactive oxygen species; SOD, superoxide dismutase.

Cellular morphology and vegetative growth

The fresh-water unicellular alga *H. pluvialis* Flotow (Volvocales) occurs primarily in temporary, small fresh water pools (Droop 1954, Czygan 1970). In its growth stages, it has both motile and non-motile forms. In the former, a pear-shaped cell ranges from 8 to 50 μm in diameter. The cellular structure of this stage is similar to most of its family members: a cup-shaped chloroplast with numerous and scattered pyrenoids, contractile vacuoles which are often numerous and apparently quite irregularly distributed near the surface of the protoplast, a nucleus and 2 flagella of equal length emerging from the anterior papilla which perforate the cellulose wall at a wide angle. The structure's uniqueness is marked by its cell wall which is strongly thickened, gelatinous, and is usually connected to its protoplast by simple or branched strands.

In its non-motile form, the so-called 'palmella' stage, the spherical protoplast is enveloped within a closely adherent palmella membrane, and, with the exception of the flagella, the cellular structure remains the same as its motile form. Once growing conditions become unfavorable, cells increase their volume drastically and enter a resting stage in which the cell is surrounded by a heavy resistant cellulose wall, comprised in part by sporopolinine-like substances (S. Boussiba, unpublished). This overall process is termed 'encystment'. The protoplast is then a markedly red color, determined to be a secondary carotenoid, astaxanthin (Goodwin and Jamikorn 1954).

The recent growing interest in this alga as an alternative source for the red astaxanthin pigment has generated several research projects towards the optimization and characterization of its growth conditions. Although contradictory data came from different groups concerning the demand for light to maintain vegetative growth and the media composition (Borowitzka et al. 1991, Hagen et al. 1992), our group was able to define conditions to support optimal photoautotrophic vegetative growth (Boussiba and Vonshak 1991, Lu et al. 1994). These conditions are summarized in Table 1.

The specific growth rate of this alga under these growth conditions (one of the highest reported) is 0.054 h^{-1}, corresponding to a doubling time of 13 h.

Environmental stresses and astaxanthin accumulation

Effect of different environmental growth conditions on astaxanthin accumulation

Factors inducing astaxanthin accumulation in *H. pluvialis* are defined as inductive conditions. Accordingly, factors tending to maintain vegetative growth are defined as non-inductive conditions. Under inductive conditions, the green vegetative cells of *H. pluvialis* gradually convert into red, non-motile resting cysts (Droop 1954).

Table 1. Optimal conditions for vegetative growth of *H. pluvialis*

Medium: BG-11 containing 17.6 m*M* nitrogen and 0.22 m*M* phosphate
Light intensity: 90–150 μmol photons m^{-2} s^{-1}
Aeration: Air enriched with 1.5% of CO_2
Temperature: 25–28°C
pH: 6–7

Inductive conditions for astaxanthin accumulation in *H. pluvialis* have been studied intensively since the 1950s but with contradictory results, particularly concerning the role of light on astaxanthin synthesis (Droop 1955, Yong and Lee 1991). The claim that light is necessary for the synthesis of astaxanthin (Goodwin and Jamikorn 1954) was questioned by Droop (1955) who showed that astaxanthin can be synthesized by *H. pluvialis* in the dark in the presence of sodium acetate (500 mg l^{-1} or 6 m*M*). Droop's result was confirmed recently by Kobayashi et al. (1992). The role of nutritional conditions on the induction and accumulation of astaxanthin has also been investigated by a number of researchers. Several hypotheses have been put forward to elucidate the nutrient requirements for astaxanthin accumulation in *H. pluvialis*. The hypothesis that the carbon-nitrogen balance in the medium determines the degree of carotenoid formation was dismissed by Droop (1954) who pointed out that carotenogenesis may be independent of available nitrogen but not of the carbon supply and that cell multiplication precludes the accumulation of carotenoids. Boussiba et al. (1992) summarized conditions governing astaxanthin accumulation in *H. pluvialis*: high light intensity (175 μmol photons m^{-2} s^{-1}), nitrogen limitation, phosphate or sulfate starvation, salt stress (0.8% of NaCl). Similar results were also reported by others (Borowitzka et al. 1991, Yong and Lee 1991, Kobayashi et al. 1992). Most recently, Aflalo et al. (1999) have shown that inhibition of the glutamine synthetase activity by the herbicide BASTA (Na^+ glufosinate) could also lead to astaxanthin accumulation, similarly to the effect of nitrogen starvation. Since, in most of these cases, accumulation was accompanied with the inhibition of cell division, it was concluded that this is an imperative condition for the pigment accumulation in this alga. Indeed, Boussiba and Vonshak (1991) were able to demonstrate that the addition of vinblastin (an inhibitor of cell division) to a vegetative green culture inhibits cell division and promotes astaxanthin accumulation.

Physiological changes occurring during astaxanthin accumulation

While most studies on *H. pluvialis* have elaborated the inductive conditions for astaxanthin accumulation (see previous section), relatively few studies have focused on physiological changes during the conversion of *H. pluvialis* green cells to red ones. These could be divided into 2 major categories: changes in cell constituents and changes in cellular activities.

Changes in cell constituents

The conversion of green motile cells to red aplanospores is accompanied by a massive increase in carbohydrate content which may reach up to 63% of the cell dry weight (Boussiba and Vonshak 1991). This reflects entrance to a resting stage (lower metabolism) and the synthesis of compounds requiring less energy than proteins whose synthesis requires higher

energy input and whose content decreases during this process (Boussiba and Vonshak 1991). An increase in lipid content which correlates with the pigment accumulation was also observed (Boussiba and Vonshak 1991). This probably represents the need for pigment accommodation in the lipid globules, suggesting that accumulation of astaxanthin is closely related to the synthesis of fatty acids. A similar phenomenon was reported in *Ankistroclosmus braunii* (Czygan and Eichenberger 1971) and *Dunaliella* (Ben-Amotz et al. 1982). Carotenoids are hydrophobic compounds dissolved in oil. It is possible that the fatty acids accumulated serve as a matrix for solubilizing the esterified pigment. This allows astaxanthin esters to 'float' and accumulate throughout the cytoplasm (Lang 1968, Santos and Mesquita 1984).

The most pronounced change during the encystment process is, of course, the massive accumulation of the red ketocarotenoid astaxanthin. In the green cells, chlorophylls followed by the carotenoid lutein and β-carotene dominate the total pigment content; thus cells appear green (Lu et al. 1995). As encystment is induced, the amount of astaxanthin increases dramatically from a few picograms per cell to a few hundreds at the end of the process (Boussiba et al. 1999). At this stage, astaxanthin esters (mono and di) constitute up to 98% of the total carotenoids profile and reach up to 4% of total cellular dry weight (Boussiba et al. 1999).

Changes in cellular activities

The most remarkable physiological change in *H. pluvialis* during the accumulation of astaxanthin is the decrease in photosynthesis as demonstrated by several groups (Yong and Lee 1991, Hagen et al. 1992, Zlotnik et al. 1993). This decrease was attributed mainly to the damage to the photosystem II (PSII) complex reflected by the decrease in O_2 evolution rate, or comparable decrease in the variable fluorescence F_v/F_m. Lu (F. Lu 1994. Thesis, Ben-Gurion University, Beer-Sheva, Israel) demonstrated that the amount of cellular astaxanthin is inversely related to cellular photosynthetic activities, although the amount of chlorophyll and PSII reaction center remain stable during astaxanthin accumulation (Tan et al. 1995, Boussiba et al. 1999). Tan et al. (1995) attributed the decline in photosynthetic activity mainly to the lack of cytochrome *f* and the expected absence of linear electron flow from PSII to PSI and, to a lesser extent, to the decrease of some of the PSI and PSII components. Biochemical and molecular studies are required to identify the exact location(s) where damage occurs in the photosynthetic apparatus upon exposure to astaxanthin synthesis conditions.

Involvement of ROS in the carotenogenesis process

It is generally believed that the production of ROS in photosynthetic organisms is an unavoidable consequence of the operation of the photosynthetic electron transport chain in an oxygen atmosphere. The chloroplast is a particularly rich source of ROS since it contains the highly energetic reactions of photosynthesis and an abundant oxygen supply (Alscher et al. 1997). Under optimal growth conditions, light absorbed by antenna pigments is converted to chemical energy forming ATP and NADPH through a photosynthetic electron transport chain. This chemical energy is finally stored in starch by fixing CO_2 through the Calvin cycle. Under stress environmental conditions, however, ROS will be produced when the photosynthetic energy balance between input (absorption of solar energy by antenna pigments) and output (CO_2 fixation) is quenched. For example, excessive irradiance can lead to an over-reduction of PSI. This results in the formation of $O_2^{\bullet -}$ through the Mehler reaction (Mehler 1951). The $O_2^{\bullet -}$ can be sequentially converted to H_2O_2 and $HO^{\bullet}$ (Elstner 1982). In addition, excessive irradiance may also cause the generation of triplet chlorophyll (^{3}Chl) that can interact with molecular oxygen (triplet ground state) in a bimolecular collision reaction which forms $^1O_2$ as long as ^{3}Chl is not quenched by carotenoids. On the other hand, when CO_2 fixation is limited by stress environmental conditions such as nutrient starvation, high salinity, cold temperatures or low CO_2 availability, the production of these ROS can occur even at moderate light intensity because of an energy surplus (Allen 1995). In summary, ROS will be produced whenever there is an excessive reducing power in photosynthesis.

Observations that generators of ROS induce astaxanthin accumulation in *H. pluvialis* under normal growth conditions have suggested that the carotenogenic response is mediated by ROS (Kobayashi et al. 1993, Lu et al. 1998). Our recent study suggested that under nutrient starvation, $^1O_2$ is probably the most effective ROS species involved in astaxanthin accumulation (Lu et al. 1998).

The interrelationships between ROS and astaxanthin in the stress response and photoprotection are not clear. New exciting studies indicate that ROS play a role in signaling defense response systems (Bouvier et al. 1998, Demple 1998). Current data suggest that complex regulatory mechanisms function at both the gene and protein level to coordinate antioxidant responses and that a critical role is played by organellar localization and the inter-compartment coordination (Alscher et al. 1997).

The mechanism by which $^1O_2$ and other ROS activate astaxanthin biosynthesis is not known. ROS or their products may regulate astaxanthin accumulation by direct activation of biosynthetic latent enzymes as glutathione transferase (Aniya and Anders 1992) and glutathione reductase (Miller and Claiborne 1991). Alternatively ROS may activate the expression of genes coding for carotenogenesis enzymes (Bouvier et al. 1998). Such a mechanism of oxidative stress response has been well characterized in *Escherichia coli* for the OxyR SoxR system (Demple 1991, 1998).

Kobayashi et al. (1993) have shown that ROS other than $^1O_2$ may also be involved in astaxanthin accumulation. Since carotenoids are not involved in the detoxification of these molecules, we thus assume the involvement of other antioxidant systems such as enzymes (superoxide dismutase (SOD), catalase, etc.; see next section) or antioxidant molecules to quench these substances. It is also possible that both of these ROS are produced under certain oxidative growth conditions and convert to singlet oxygen which can react with carotenoids.

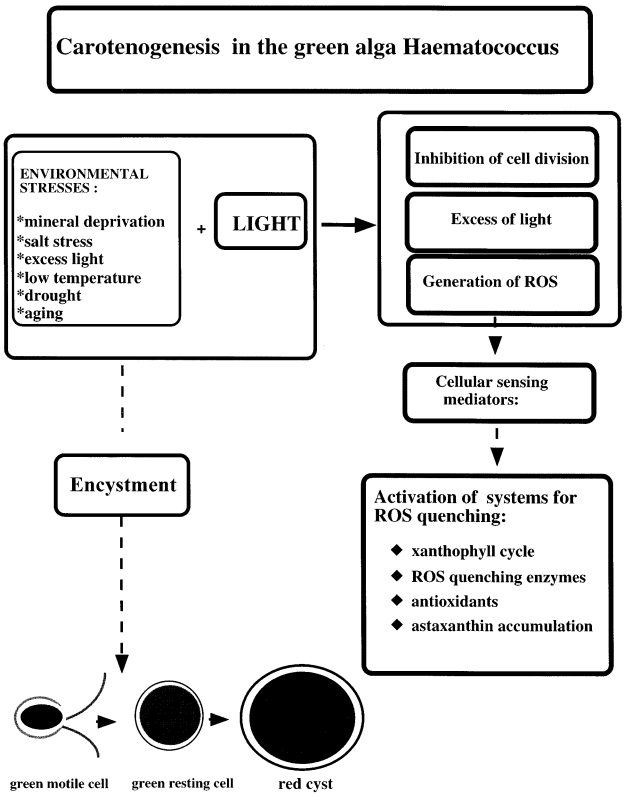

Fig. 1. Carotenogenesis in the green alga *H. pluvialis*. – A schematic model.

Further, it is possible that $^1O_2$ acts both as messenger and excessive energy carrier to inhibit photosynthesis and cell division, to initiate the expression of carotenoid biosynthesis genes (Bouvier et al. 1998) and to drive carotenogenesis process.

Mechanisms of protection against ROS damage

If not kept under strict control in living cells, ROS can cause damage to cellular constituents or metabolic processes, e.g. destruction of membranes, inhibition of enzyme activity, or oxidation of amino acids from proteins (Herbert et al. 1992). Consequently, they will lead to the death of the organism or cause mutagenicity or carcinogenicity (Toyokuni et al. 1995). To cope with these damaging activities of ROS, cells have evolved various antioxidant defense systems. These include anti-oxidative enzymes such as SOD, catalase, ascorbate peroxidases, and some small antioxidant molecules (glutathione, ascorbic acid, hydroquinones and α-tocopherol) (Asada 1994). In *Haematococcus*, neither the activities of such defense mechanisms nor the presence of antioxidant molecules have been worked out in detail. Recently, however, Kobayashi et al. (1997) and Boussiba (unpublished data) were able to demonstrate the activity of SOD and catalase in green *Haematococcus* cells. A schematic model summarizing the different steps in the carotenogenesis process is depicted in Fig. 1.

Astaxanthin biosynthesis

The pathway as elucidated via diphenylamine (DPA) inhibition

The inhibition of carotenoid synthesis by DPA has been observed in bacteria, fungi and algae. It was first demonstrated in photosynthetic bacterium *Rhodospirillum rubrum* by Goodwin and Osman (1953). Cohen-Bazire and Stanier (1958) observed an accumulation of phytoene in *R. rubrum* by using 70 μM diphenylamine. The mode of action of DPA is still not clear (Goodwin 1980) since it is not known whether diphenylamine inhibits the transcription of carotenogenesis genes, the translation of related enzymes, or inactivates these enzymes. In *Haematococcus*, addition of low concentration of DPA (30 μM) inhibited the conversion of β-carotene to ketocarotenoids (Lu et al. 1995). Since no deleterious effects of DPA were detected on growth, metabolism, or carotenoid composition of *H. pluvialis* grown under optimal conditions, it was concluded that under these conditions astaxanthin biosynthesis is specifically inhibited in this alga.

The identification of small but significant amounts of echinenone and canthaxanthin in *H. pluvialis* (Grung et al. 1992) has led to the hypothesis that in algae the pathway of (3S,3S′)-astaxanthin biosynthesis is different since it occurs via canthaxathin. It has been shown that diphenylamine inhibition of astaxanthin biosynthesis is associated with β-carotene accumulation and decrease of canthaxanthin. Our results support this hypothesis by providing evidence that the C-4 keto groups are introduced to β-carotene first. These 2 steps are likely to be carried out by the same enzyme. This latter conclusion is based upon the detection of a sharp decrease in the concentration of canthaxanthin that occurs in diphenylamine-treated cells, while β-carotene is the only carotenoid that accumulates. Under these conditions, the steady-state level of echinenone is slightly reduced, namely by additions of 2 keto groups, both of which are sensitive to diphenylamine. A proposed pathway of astaxanthin biosynthesis from β-carotene is depicted in Fig. 2.

The enzymes and genes involved in astaxanthin biosynthesis

Starting from geranylgeranyl pyrophosphate, a phytoene synthase, up to 2 desaturases and 1 cyclase are involved in the synthesis of β-carotene which is the most universal carotenoid found in nature. This hydrocarbon is a precursor of the synthesis of astaxanthin in bacteria, fungi and *Haematococcus* (Lu et al. 1995). The 2 remaining reactions are the introduction of keto groups at positions 4 and 4′ as well as hydroxy groups at positions 3 and 3′ to the β-carotene molecule. Genes encoding ketolases which introduce 2 keto groups, in contrast to the gene product of crtO from *Synechocystis*, a monoketolase, are crtW from bacteria (Misawa et al. 1995) and the bkt cDNA from *Haematococcus* (Lotan and Hirschberg 1995). Recently, Sandman and coworkers have shown that the ketolase from *Haematococcus* recognize only β-carotene but not zeaxanthin (Breitenbach et al. 1996). Therefore, the puitative hydroxylase from *Haematococcus* should use canthaxanthin as the major sub-

strate like the bacterial enzyme (H. Sandmann and P. Böger, personal communication). This assumption is supported by the most recent report of Linden (1999) who demonstrated that β-carotene hydroxylase from *Haematococcus* can convert cantaxanthin to astaxanthin. This new finding further supports the suggested pathway of astaxanthin biosynthesis in *Haematococcus* as suggested by Lu et al. (1995).

Site of synthesis and accumulation

The carotenoid pigments are synthesized in the chloroplasts of plants and algae. In these organelles they accumulate primarily in the photosynthetic membranes in association with the light harvesting and reaction center complexes. Our previous finding revealed the biosynthetic pathway of astaxanthin biosynthesis in *Haematococcus* (Lu et al. 1995). From this report we can also suggest that the chloroplasts of *Haematococcus* contain 1 set of enzymes responsible for the biosynthesis of carotenes and some xantophylls including the β-carotene hydroxylase, responsible for the conversion of β-carotene to zeaxanthin. However, the enzymes responsible for the oxygenation of β-carotene to cantaxanthin and the hydroxylation of the later to astaxanthin have not been localized yet. This is of particular interest as the pigment accumulates in the cytoplasm outside the chloroplast (Lang 1968, Santos and Mesquita 1984). Since the addition of the keto group most likely precedes hydroxylation (see above paragraph) (Lu et al. 1995), an extraplastidic site for the ketolase would entail a location outside of the chloroplast for hydroxylase as well. It is tempting to postulate that in *Haematococcus* 2 sites of carotenoid synthesis may operate. One is responsible for the synthesis of these carotenes and xantophylls involved in the reaction center and the xantophyll cycle located in the chloroplast. A second site contains another set of enzymes or their precursors present in the cytoplasm in their latent form, awaiting activation by ROS when *Haematococcus* encounters environmental stresses. This suggestion is partially supported by the fact that astaxanthin accumulation does not require new protein synthesis.

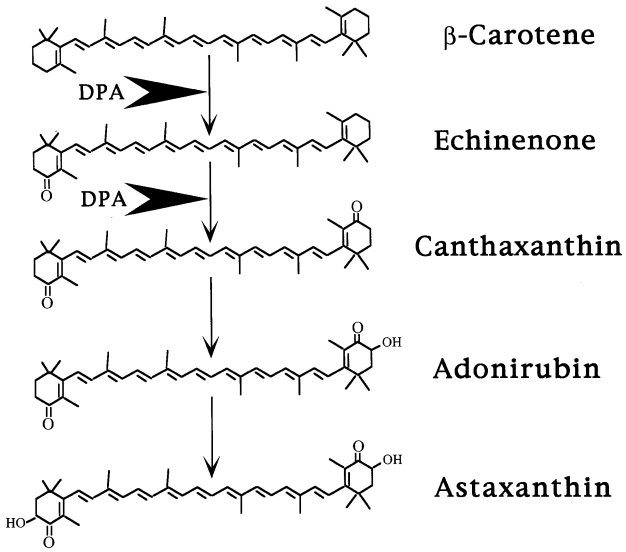

Fig. 2. Proposed pathway for astaxanthin biosynthesis in *H. pluvialis* as revealed by the utilization of low concentration (30 μM) of DPA which specifically blocks the conversion of β-carotene to astaxanthin.

Role of astaxanthin in photoprotection

Indirect involvement in ROS quenching

Carotenoids could forestall damage of excessive irradiance by directly quenching triplet chlorophyll (^{3}Chl) or singlet oxygen ($^1O_2$) produced from photodynamic reactions (Krinsky 1979). This quenching mechanism requires close proximity between the quencher (carotenoids) and the photosensitive (chlorophyll). Thus, this type of photoprotection can be provided by those carotenoids closely associated with chlorophylls, namely the primary carotenoids which are integrated into the complexes of the photosystems.

Another mechanism of carotenoid photoprotection was described by Ben-Amotz et al. (1989) who concluded that the large amount of β-carotene accumulated in the interthylakoid space of green alga *D. bardawil* could protect the cells from high irradiance injury by acting as a screen preventing excessive irradiance of blue light from reaching the antenna chlorophylls. This 'filter effect' of β-carotene is attributed to the large overlap between the absorption spectra of β-carotene and chlorophyll in the blue light regime.

The massively accumulated astaxanthin in *H. pluvialis* is located in the cytoplasm (Lang 1968, Santos and Mesquita 1984). It is, therefore, unlikely that astaxanthin exerts a photoprotective role in this alga by quenching excited triplet chlorophylls (^{3}Chl), due to the distance between the two. In addition, the overlap between the absorption spectra of astaxanthin and chlorophyll is rather small; hence, it is difficult to imagine how astaxanthin can act as a screen filtering excessive blue light absorbed by the chlorophylls. What, then, is the role of this pigment in *Haematococcus*? We have suggested that astaxanthin itself is not the protective agent, and that protection is provided only if astaxanthin biosynthesis is not interrupted since inhibition of the latter caused cell death (Lu et al. 1998). This assumption is not in agreement with previous studies which suggested direct involvement of astaxanthin in the photoprotection mechanism as sunshade (Yong and Lee 1991, Hagen et al. 1994) (see next section).

Direct involvement in ROS quenching

Our suggestion regarding the indirect involvement of astaxanthin in the photoprotection process needs further support. One cannot exclude the possibility of direct quenching of some radicals and especially $^1O_2$ by the accumulated astaxanthin esters, which recently were shown to retain high antioxidative activity (Kobayashi and Sakamoto 1999).

As mentioned previously (section 'Changes in cell constituents'), singlet oxygen is the dominant ROS molecule involved in astaxanthin accumulation. Since this molecule is not charged it can cross biological membranes and when its concentration rises inside the chloroplast may leak to the cytoplasm where it can be quenched chemically by astaxan-

thin. However, no indication of such quenching of ROS by astaxanthin has been reported so far (no oxidation products of the oxidized pigment were ever detected). It is possible that the detoxified oxygen molecule resulting from physical reaction of ROS with astaxanthin may serve as a substrate to the conversion of β-carotene to astaxanthin. This hypothesis still needs supportive data.

Concluding remarks

Model organism to study oxidative stress response

During their entire life cycle plants are exposed to environmental changes which result, in many cases, in reduction of their productivity. Understanding the mechanisms which plants have evolved to cope with these harmful changes is a major challenge to modern agriculture. In some cases, the damage to plant organs is the result of the toxic oxygen molecules often referred as ROS. Plants have evolved different mechanisms to detoxify these harmful molecules. Understanding these stress response reactions and protection mechanisms is thus imperative.

The unicellular green alga *H. pluvialis* which responds in the same manner when exposed to a variety of environmental stress conditions via production of ROS (Fig. 1) can serve as an excellent tool to reveal these kinds of cell responses (signals transduction) and protection mechanisms.

Cellular and molecular aspects

Although there are many exciting results concerning the mechanisms governing the production of astaxanthin and its function in *Haematococcus*, there is still much to be done to define this transformation process. Below are a few examples of research areas of particular interest which may help in further understanding this carotenogenesis process.

Astaxanthin biosynthesis site

An exciting area of research in carotenoids is the ability of this molecule to traverse a biological membrane. The mechanism of this translocation, if it exists at all, is still obscure and a subject for further investigation. In *H. pluvialis* it is of particular interest since this secondary metabolite accumulates in the cytoplasm but so far, there is no evidence of the site of its synthesis. So, one can speculate on the synthesis of this molecule inside the chloroplast and its excretion and accumulation in the cytoplasm. Elucidating this question will no doubt shed light on another interesting aspect related to the function of this pigment in *Haematococcus*.

The encystment process

Astaxanthin accumulation is accompanied by cell enlargement and the formation of a heavy wall consisting of sporopolinine-like substances. The relationship between these 2 processes awaits further assessment. It is tempting to suggest that pigment accumulation and wall formation are affected via the same signal transduction system. The other possibility of these 2 processes running in parallel and being indirectly related cannot be dismissed at this stage either.

The interrelationship between pigment accumulation and lipid biosynthesis

It is possible that the main reason for massive accumulation of astaxanthin in *Haematococcus* is related to its esterification and deposition in the lipid globules. This will suggest the close interaction between these 2 processes. Indeed, in *D. bardawil* it was shown that the main limiting factor of β-carotene accumulation is regulated via the enzyme acetyl CoA carboxylase (a key enzyme in lipid biosynthesis) (Rabbani et al. 1998). This interesting hypothesis should be tested also in *Haematococcus*, since our preliminary results already showed the dependency of pigment accumulation on fatty acid biosynthesis.

Genetic transformation

The possibility of developing a genetic transformation system for this alga, a close relative to *Chlamydomonas*, a well-studied organism in this regard, will be of critical importance to further understand some of the problems described above. Since *Haematococcus* possesses most of the different valuable carotenoids such as lycopene, lutein, β-carotene and astaxanthin, regulation of their production via genetic manipulation will be of great value for basic research and will no doubt promote the production recombinant strains of this alga as a natural source for these valuable pigments.

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