RESEARCH PAPER

Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga Haematococcus pluvialis under nutrient stress conditions

Raman Vidhyavathi, Lakshmanan Venkatachalam, Ravi Sarada* and Gokare Aswathanarayana Ravishankar

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore – 570 020, India

Received 11 December 2007; Revised 21 January 2008; Accepted 30 January 2008

Abstract

Haematococcus pluvialis, a green alga, accumulates carotenoids, predominantly astaxanthin, when exposed to stress conditions. In the present work, changes in the pigment profile and expression of carotenogenic genes under various nutrient stress conditions and their regulation were studied. Nutrient stress and higher light intensity in combination with NaCl/sodium acetate (SA) enhanced total carotenoid and total astaxanthin content to 32.0 and 24.5 mg g⁻¹ of dry biomass, respectively. Expression of carotenogenic genes, phytoene synthase (PSY), phytoene desaturase (PDS), lycopene cyclase (LCY), β-carotene ketolase (BKT), and β-carotene hydroxylase (CHY) were up-regulated under all the stress conditions studied. However, the extent of expression of carotenogenic genes varied with stress conditions. Nutrient stress and high light intensity induced expression of astaxanthin biosynthetic genes, BKT and CHY, transiently. Enhanced expression of these genes was observed with SA and NaCl/SA, while expression was delayed with NaCl. The maximum content of astaxanthin recorded in cells grown in medium with SA and NaCl/SA correlated with the expression profile of the astaxanthin biosynthetic genes. Studies using various inhibitors indicated that general carotenogenesis and secondary carotenoid induction were regulated at both the transcriptional and the cytoplasmic translational levels. The induction of general carotenoid synthesis genes was independent of cytoplasmic protein synthesis while BKT gene expression was dependent on de novo protein synthesis.

Key words: Astaxanthin, carotenoid, Haematococcus pluvialis, NaCl, nutrient stress, sodium acetate.

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), is a commercially important, high value ketocarotenoid, used as a pigmentation source in the aquaculture and poultry industries. It has also found applications in the nutraceutical, pharmaceutical, and cosmetic industries (Guerin et al., 2003; Jin et al., 2006). Haematococcus pluvialis is a commercially promising source among the astaxanthin-producing organisms, owing to its ability to accumulate astaxanthin up to 4% (w/w) on a dry weight basis (Boussiba, 2000). It accumulates astaxanthin in response to stress conditions such as high light intensity, salinity, acetate addition, nutrient depletion, influence of reactive oxygen species (ROS), and increased C/N ratio (Kobayashi et al., 1993; Sarada et al., 2002).

Carotenoids are ubiquitous and essential components of the photosynthetic tissues in plants, algae, and cyanobacteria wherein they participate in the light-harvesting process and protect the photosynthetic apparatus from photo-oxidative damage (Bartley and Scolnik, 1995). In Haematococcus, astaxanthin accumulation occurs in extra-plastidic lipid globules as a secondary carotenoid (Grünewald et al., 2001). In the biosynthesis of carotenoids, the first committed step, the head-to-head condensation of geranylgeranyl diphosphate (GGPP) to phytoene, is mediated by the soluble enzyme phytoene synthase (PSY) (Fig. 1). The subsequent steps of the pathway leading to the synthesis of colored carotenoids are carried out...
Fig. 1. Pathway of secondary carotenoid synthesis in *H. pluvialis*. Enzyme designation is according to the corresponding gene: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, \( \zeta \)-carotene desaturase; CRTISO, carotenoid isomerase; LCY, lycopene cyclase; BKT, \( \beta \)-carotene ketolase; CHY, \( \beta \)-carotene hydroxylase. Several intermediates were omitted for simplification. The gene expression of the boxed enzymes were analysed in this study.

by membrane-localized enzymes such as phytoene desaturase (PDS) and lycopene \( \beta \)-cyclase (LCY) (Cunningham and Gantt, 1998). The biosynthesis of astaxanthin in *Haematococcus* follows the general carotenoid pathway up to \( \beta \)-carotene formation. Studies using carotenogenic inhibitors and *in vitro* and *in vivo* analyses of astaxanthin synthesis in *Haematococcus* revealed the involvement of two enzymes \( \beta \)-carotene ketolase (BKT) (synonym: \( \beta \)-carotene oxygenase, CRTO) and \( \beta \)-carotene hydroxylase (CHY or CRTR-B) in the conversion of \( \beta \)-carotene to astaxanthin. BKT converts \( \beta \)-carotene to canthaxanthin via echinenone which is further acted upon by CHY resulting in the formation of astaxanthin (Fan *et al.*, 1995; Lotan and Hirschberg, 1995; Fraser *et al.*, 1998). The genes for \( \zeta \)-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) have not yet been reported in *Haematococcus* (Jin *et al.*, 2006). Although the specific steps of astaxanthin biosynthesis are carried out in the cytoplasm, the enzymes of the general carotenoid pathway appears to be localized in the chloroplast (Grünewald *et al.*, 2000; Jin *et al.*, 2006).

Expression of carotenogenic genes has been studied in several plants including green algae, and transcription of carotenogenic genes were shown to be up-regulated by light (Bohne and Linden, 2002; Simkin *et al.*, 2003; Steinbrenner and Linden, 2003; Romer and Fraser, 2005) or a combination of light with N-deprivation (Grünewald *et al.*, 2000). Although there are reports available on the influence of light on the expression of carotenogenic genes in *Haematococcus*, studies on the influences of other stresses on the expression of carotenogenic genes are limited (Grünewald *et al.*, 2000; Huang *et al.*, 2006). The life cycle of *H. pluvialis* contains two distinct phases, namely a green motile vegetative phase and a non-motile astaxanthin-accumulating cyst phase (Sarada *et al.*, 2006). This favours the use of *Haematococcus* as a model system to study the regulation of secondary carotenogenesis. Under nutrient-limiting conditions, the induction of astaxanthin accumulation occurs in flagellated cells (Brinda *et al.*, 2004) which is more advantageous for biochemical analysis and the extraction of pigments as the cells are fragile. Understanding the molecular basis of stress-induced astaxanthin accumulation in *Haematococcus* will be useful for the optimization of astaxanthin production. Therefore, the present work focused on establishing a relationship between pigment profile and the corresponding expression profile of carotenogenic genes under the influence of nutrient depletion combined with high light intensity, and sodium acetate and NaCl. In order to understand the regulation of formation of general and secondary carotenoids, transcriptional and translational inhibitors were used.

**Materials and methods**

**Algal culture, growth conditions and inhibitors** *Haematococcus pluvialis* (SAG 19-a) was obtained from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, Universität Göttingen, Göttingen, Germany and grown in autotrophic Bold’s Basal medium (Usha *et al.*, 1999). The cultures were incubated at 25±1 °C under 16/8 h light/dark cycle with 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) intensity for a period of 7 d. Then cultures were harvested by centrifugation at 3500 g for 5 min and resuspended in fresh nutrient-limiting medium containing 1/10 of the nitrogen and phosphorus of the original medium. The initial cell concentration was adjusted to \( 15 \times 10^6 \) cells ml\(^{-1} \). The cultures were subjected to the following treatments (i) control (without NaCl and SA addition), (ii) NaCl 17.1 mM, (iii) sodium acetate 4.4 mM (SA), and (iv) NaCl 17.1 mM and SA 4.4 mM (NaCl/SA). The cultures were then incubated under a continuous light intensity of 60 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The transcriptional inhibitor actinomycin D (SRL, Mumbai, India), the cytoplasmic translational inhibitor, cycloheximide (Sigma-Aldrich, Bangalore, India), and the organelar translational inhibitor, chloramphenicol (HiMedia, Mumbai, India) were added at concentrations of 10 \( \mu \text{g ml}^{-1} \), 300 \( \text{ng ml}^{-1} \), and 50 \( \mu \text{g ml}^{-1} \), respectively.

**Growth and pigment analyses**

Growth was measured by counting cell numbers using a haemacytometer. Dry weight of the algal biomass was estimated after drying at 60 °C in a hot-air oven until a constant weight was obtained. For
pigment analysis, an aliquot of culture was harvested and freeze-dried. A known quantity of biomass was extracted with 90% acetone and, chlorophyll and carotenoids were quantified as per the procedure given by Vidhyavathi et al. (2007).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction, reverse transcription, and PCR were carried out according to Vidhyavathi et al. (2007). The primers used for amplification of PSY, PDS, LCY, BKT, and CHY (Vidhyavathi et al., 2007), and actin (Huang et al., 2006) are listed in Table 1. Following the separation of the PCR products on ethidium bromide-stained 1.8% agarose gels, the bands were quantified. Each band was normalized against the intensity obtained with the same cDNA using the actin-specific primers. For calculating the transcript abundance under stress conditions, the transcript levels of each gene in green motile cells were taken for comparison, while under inhibitor-added conditions, transcripts of the respective control (without inhibitors) were used for comparison.

Experimental design and data analysis

Each experiment was repeated three times with at least three replications. All the observations and calculations were made separately for each set of experiments and were expressed as mean ± SD. The significance (P ≤ 0.05) of the variables studied was assessed by one-way analysis of variance (ANOVA) using Microsoft Excel XP. The mean separations were performed by Duncan’s Multiple Range Test (DMRT) for segregating means where the level of significance was set at P ≤ 0.05 (Harter, 1960).

Results

Haematococcus growth under nutrient stress and continuous high light

Cells exposed to nutrient stress and high light retained their flagella for longer periods whereas the addition of NaCl and SA induced early cyst formation. On the second day of stress induction, 18, 40, 82, and 89% of cells were transformed to cysts in control, NaCl-, SA-, and NaCl/SA-added cultures respectively. Nine days after stress induction, only 42% of cells in control culture retained flagella whereas cells in NaCl-, SA-, and NaCl/SA-added cultures lost their flagella completely. As the stress advanced, all the treatments showed an increase in biomass yield and the SA- and NaCl/SA-added cultures produced significantly more biomass (Fig. 2A).

Changes in pigment profile during stress induction

Haematococcus cells collected at different intervals of stress induction were analysed for the changes in content and composition of both carotenoids and chlorophylls. The initial carotenoid content of green motile cells was 12.1 mg g⁻¹ dry weight (DW) (Fig. 2B) with lutein and β-carotene as major constituents, followed by neoxanthin and violaxanthin occurring in traces, but there were no secondary carotenoids. In our preliminary study, it was revealed that exposure of H. pluvialis cells to nutrient-stress and high-light conditions increased astaxanthin production by 3.5-fold over nutrient-sufficient and high-light-exposed cells and 1.5-fold over nutrient-stress and low-light-exposed cells. Upon stress induction, the carotenoid content increased with a concomitant decrease in the chlorophyll content (Fig. 2B, C). After 9 d of stress, the total chlorophyll content in all the treatments was 90% less than that of green motile cells (Fig. 2C). The addition of NaCl decreased the total carotenoid content, however, addition of SA and NaCl/SA increased the total carotenoid content showing the synergistic effect of SA and NaCl (Fig. 2B).

In all the treatments, there was an overall decrease in primary carotenoids and especially lutein content under NaCl stress (Fig. 2F). β-carotene constituted 1.7, 1.3, 1.9, and 2.0 mg g⁻¹ DW and lutein constituted 9.3, 3.9, 6.1, and 5.1 mg g⁻¹ DW in control, NaCl, SA, and NaCl/SA cultures, respectively (Fig. 2E, F). Astaxanthin monoester (6.08–22.03 mg g⁻¹ DW) and diester (0.7–2.4 mg g⁻¹ DW) contents increased while free astaxanthin, canthaxanthin, and echinenone (intermediates in the astaxanthin biosynthetic pathway) were present in traces throughout the experimental period (data not shown). The fluctuations

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'–3')</th>
<th>Annealing temperature (°C)</th>
<th>Total number of amplification cycles</th>
<th>GenBank ID/reference Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSY- forward</td>
<td>ATGTACCATCCCAAGGCAAG</td>
<td>60</td>
<td>30</td>
<td>NY835634</td>
</tr>
<tr>
<td>PSY- reverse</td>
<td>CTGGACCGAGGCTCAAGC</td>
<td>60</td>
<td>30</td>
<td>NY835634</td>
</tr>
<tr>
<td>PDS- forward</td>
<td>TCCATGATCTTGGCATG</td>
<td>60</td>
<td>30</td>
<td>NY768691</td>
</tr>
<tr>
<td>PDS- reverse</td>
<td>CCGAGTGGAACATGAGGTC</td>
<td>60</td>
<td>30</td>
<td>NY835634</td>
</tr>
<tr>
<td>LCY- forward</td>
<td>CTTCTTCGCCCTTCCTCA</td>
<td>60</td>
<td>30</td>
<td>NY835634</td>
</tr>
<tr>
<td>LCY- reverse</td>
<td>GCTACCTACCCGCTCAAGAAA</td>
<td>55</td>
<td>30</td>
<td>NY835634</td>
</tr>
<tr>
<td>BKT- forward</td>
<td>CATCCTCTGGTGACTCTGG</td>
<td>55</td>
<td>30</td>
<td>X86782</td>
</tr>
<tr>
<td>BKT- reverse</td>
<td>CAGTGACGTCGAAGTGGTA</td>
<td>55</td>
<td>30</td>
<td>X86782</td>
</tr>
<tr>
<td>CHY- forward</td>
<td>CTACACACAGCAGCAGTA</td>
<td>55</td>
<td>30</td>
<td>AF162276</td>
</tr>
<tr>
<td>CHY- reverse</td>
<td>GCTACCTACCTAGTCTACCA</td>
<td>55</td>
<td>30</td>
<td>AF162276</td>
</tr>
<tr>
<td>ACT- forward</td>
<td>AGGCAGTAGTGTCGCCGA</td>
<td>61</td>
<td>22</td>
<td>Huang et al., 2006</td>
</tr>
<tr>
<td>ACT- reverse</td>
<td>ATGCCACCCGCTCCATGC</td>
<td>61</td>
<td>22</td>
<td>Huang et al., 2006</td>
</tr>
</tbody>
</table>
in their concentration indicated their faster conversion to astaxanthin and its ester forms. At the end of the experimental period, total astaxanthin contents were 15.7, 6.8, 21.8, and 24.5 mg g\(^{-1}\) DW produced by control, NaCl-, SA-, and NaCl/SA-added cultures, respectively (Fig. 2D). Astaxanthin monoester constituted 88–90% while the diester constituted 8–10% of the total astaxanthin under all the stress conditions studied.

**Expression analysis of carotenoid genes during stress induction**

Transcripts of PSY, PDS, LCY, BKT, and CHY genes were detected in all stages of stress induction. Both general carotenogenic genes and specific astaxanthin biosynthetic genes were found to be up-regulated upon exposure to various stresses (Fig. 3A, B). Maximum transcript levels of PSY, PDS, LCY, BKT, and CHY were found to be 158–277, 5–9, 470–674, 28–40, and 451–673-fold higher, respectively, than green vegetative cells. Exposure to nutrient stress and high light (control) resulted in the early up-regulation of PSY, PDS, and LCY transcripts that reached a maximum on the second day of stress induction. The induction was found to be transient except for PDS. As the stress progressed, transcript levels of these genes increased. Maximum levels of PSY and PDS transcripts were observed on the ninth day when compared with the second day. NaCl addition, delayed the up-regulation of PSY, PDS, and LCY expression, but the transcript levels were similar or higher when compared with other stresses. PSY transcripts reached a maximum level on the sixth day while PDS and LCY transcripts reached their maximum on the ninth day. Addition of SA and NaCl/SA produced an early up-regulation of PSY, PDS, and LCY, which reached a maximum on the ninth day (Fig. 3A, B). However, their levels were comparatively lower than both control and NaCl-treated cultures.

The increase in transcripts of the astaxanthin biosynthetic genes, BKT and CHY, in response to nutrient stress and high light intensity was similar to mRNA levels of LCY. The addition of NaCl caused delayed up-regulation of BKT and CHY with their levels reaching a maximum on the third day and the ninth day, respectively. Addition of SA and NaCl/SA produced an early up-regulation and maximum transcripts, compared with control and NaCl treatments. Both BKT and CHY transcripts reached a maximum on the fourth day in SA-treated cultures. NaCl/SA-treated cultures produced maximum BKT transcripts on the third day and CHY on the ninth day (Fig. 3A, B).

**Effect of transcriptional and translational inhibitor on carotenoid formation and carotenoid biosynthesis genes expression under nutrient stress conditions**

The concentration and composition of carotenoids and the expression of carotenoid genes were studied in stressed

---

**Fig. 2.** Growth and pigment contents of *H. pluvialis* cultures during induction of secondary carotenoid synthesis. After one week of initial growth in autotrophic media, *H. pluvialis* cultures were transferred to nutrient-limiting media and high light (control) along with NaCl, SA, and NaCl/SA addition. Cells were harvested at different periods of incubation and, changes in biomass production (A), total carotenoid (B), total chlorophyll (C), total astaxanthin (D), \(\beta\)-carotene (E), and lutein (F) content were analysed. Values are means ± SD of three independent determinations.
Fig. 3. Expression of carotenoid biosynthetic genes in *H. pluvialis* during the induction of secondary carotenoid synthesis. The *H. pluvialis* cells used for the preparation of RNA were harvested after one week of growth in autotrophic media (G) and after additional growth under various secondary carotenoid-inducing conditions for 0.5, 1, 2, 3, 4, 6, and 9 d. Secondary carotenoid-inducing conditions used were nutrient deficiency and high light (control), combined with NaCl, SA, and NaCl/SA. RT-PCR was performed as described in the Materials and methods with 5 μg of total RNA. (A) The PCR products were analysed by agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (lower panel). (B) The band intensity of each gene was adjusted with the band intensity of actin. Data shown are mean ± SD of three independent experiments expressed as the fold increase in PSY, PDS, LCY, BKT, and CHY genes of control, NaCl, SA, and NaCl/SA added to *H. pluvialis* cultures compared with the value for green vegetative cells (G).
cultures treated with transcription and translation inhibitors. Actinomycin D, a transcriptional inhibitor, caused a reduction in total carotenoid and astaxanthin content, although the overall carotenoid composition remained unchanged (Fig. 4). While cycloheximide, a cytoplasmic translational inhibitor, on addition to stressed cultures also caused a reduction in total carotenoids, but with a marked differences in astaxanthin content (Fig. 4). It caused a reduction in astaxanthin content in control cultures and completely inhibited astaxanthin synthesis in other stress cultures. The translation inhibitor at the organelar level, chloramphenicol, exhibited a reduction in overall carotenoid and astaxanthin production in all stress cultures except in NaCl-treated cells. Carotenoid inhibition was maximum in NaCl/SA-added cultures. However, chloramphenicol increased the accumulation of lutein (as analysed by HPLC) in all stress conditions except for the NaCl-added cultures where the astaxanthin content was higher. Traces of neoxanthin, violaxanthin, canthaxanthin, and echinenone were detected in all treated cultures.

As addition of SA and NaCl/SA hastens transcription of carotenoid genes and carotenoid accumulation, the influence of transcriptional and translational inhibitors were studied by adding inhibitors to cultures after 3 d of stress induction. Actinomycin D reduced the total carotenoid and astaxanthin content in all treatments except in the SA-added culture. The extent of reduction was prominent in control and NaCl-treated cultures. However, the decrease in astaxanthin content in the NaCl/SA culture was less (Fig. 5). The addition of cycloheximide did not reduce total carotenoid in SA-added culture and a considerable reduction was observed in control, NaCl-, and NaCl/SA-added cultures. Total astaxanthin production was completely inhibited in the control and NaCl-added cultures and significantly reduced in SA- and NaCl/SA-treated cultures (Fig. 5). This indicated the role of acetate in enhancing total carotenoid and astaxanthin production through post-translational activation. No significant reduction in total carotenoid content was observed when chloramphenicol was added after 3 d of stress induction. However, there was a noticeable decrease in astaxanthin content in control and NaCl/SA-added cultures. It is interesting to note that the decrease in astaxanthin content correlated with the increase in lutein content in the control and NaCl/SA-added cultures (data not shown) indicates the diversion of the carotenoid pathway towards protection of the organism from the inhibitory effect caused by chloramphenicol.

Actinomycin D reduced expression of all carotenoid biosynthesis genes studied (Fig. 6A, B). Cycloheximide
reduced the expression of only the BKT gene under all the conditions studied. There were no noticeable changes in the expression levels of other carotenoid genes in the control and NaCl-treated cultures. However, significant increase in the expression levels of these genes was observed in the presence of SA and NaCl/SA. Chloramphenicol significantly reduced the expression of all genes studied in control and NaCl-added cultures and increased the carotenogenic genes expression in SA- and NaCl/SA-added cultures (Fig. 6). It is evident from the results that both organellar and cytoplasmic translational inhibitors in the presence of acetate (SA or NaCl/SA) influenced the expression levels of all carotenogenic genes except BKT by cycloheximide. To obtain more information on stability of carotenoid mRNAs, transcripts were studied after 2 d and 3 d of actinomycin D addition and stress induction. Carotenogenic gene transcripts exhibited faster degradation under these conditions (Fig. 7).

**Discussion**

In the present work, attempts were made to compare the changes in pigment profile with expression of carotenogenic genes under various stress conditions in *H. pluvialis*. The influence of transcriptional and translational inhibitors on carotenoid accumulation and carotenogenic genes expression was also studied in order to understand the regulation of carotenogenesis. Enhanced cyst formation and astaxanthin accumulation by acetate addition (Fig. 2D), indicated the role of acetate in increasing C/N ratio thereby enhancing astaxanthin accumulation, as suggested by Kakizono *et al.* (1992). The addition of NaCl resulted in the reduction of total carotenoid content and astaxanthin content when compared with the control. However, addition of NaCl/SA enhanced the astaxanthin content. Another significant feature observed during stress-induced astaxanthin formation was the decrease in the chlorophyll content (Fig. 2C), which may be due to nutrient deficiency-induced chlorophyll breakdown (Boussiba *et al.*, 1999).

Generally, secondary carotenoids, namely astaxanthin, canthaxanthin, and echinenone, were detected only after stress induction. However, in the present study, genes for astaxanthin biosynthesis, BKT and CHY, were found to be expressed at a basal level even in green flagellated harvested 6 d after stress induction. RT-PCR was performed as described in the Materials and methods with 0.2 μg of total RNA. (A) The PCR products were analysed by agarose gel electrophoresis. (B) Relative transcript level of each gene in each treatment is calculated by comparing the band intensity with the respective control. Data shown are mean ±SD of three independent experiments expressed as the fold increase in PSY, PDS, LCY, BKT, and CHY genes of actinomycin D-, cycloheximide-, and chloramphenicol-treated *H. pluvialis* cultures compared with the value for the respective control cells.
cells. During stress, these genes were found to be up-regulated along with other carotenoid genes, namely, PSY, PDS, and LCY (Fig. 3A, B) and these carotenogenic gene transcripts were detected even in 3-month-old cysts (Vidhyavathi et al., 2007). Differential expression of carotenoid genes during carotenogenesis indicates their probable regulation at different stages of carotenoid accumulation. Among the treatments studied, the addition of SA and NaCl/SA showed the maximum up-regulation of carotenogenic genes. Although NaCl addition favoured up-regulation of carotenogenic genes, expressions were delayed (Fig. 3). In contrast to this, Steinbrenner and Linden (2001) reported a high level of expression for PSY and CHY genes, when cells were exposed to high light and NaCl. This difference in expression by NaCl may be due to the difference in mode of cultivation (heterotrophic and autotrophic) and stress induction (nutrient sufficient and deficient). It was also reported that NaCl had a similar effect as SA in inducing the expression of PSY, CHY, and BKT, interpreting that SA initiates a salt stress as does NaCl (Steinbrenner and Linden, 2001; Huang et al., 2006). But the contrasting result revealed in the present study suggests the involvement of a mechanism other than salt stress in SA-induced transcription of carotenogenic genes. Kovacs et al. (2000) reported that, in Chlamydo- botrys stellata, the involvement of acetate in the regulation of the redox state of photosystem components thereby affected transcription of nuclear and chloroplast genes. Endo and Asada (1996) also reported plastoquinone reduction stimulated by acetate, thereby affecting photosynthetic activity. These effects of acetate have considerable importance since both general carotenogenic and specific astaxanthin biosynthetic genes were reported to be under photosynthetic redox control (Steinbrenner and Linden, 2003). Based on the results from the present study and previous reports, it may be suggested that acetate has a role in enhancing astaxanthin accumulation through photosynthetic redox control.

Increased production of astaxanthin by the addition of SA and NaCl/SA is correlated with early up-regulation and higher expression of astaxanthin biosynthetic genes. This is further supported by the absence or less inhibitory effect on carotenoid and astaxanthin accumulation by actinomycin D when added 3 d after SA and NaCl/SA stress induction (Fig. 5). The absence or reduced inhibition of total carotenoid and astaxanthin production by later addition of cycloheximide in SA and NaCl/SA cultures (Fig. 5) indicated post-translational activation of carotenoid biosynthesis by acetate. Similar to this, post-translational activation of carotenoid biosynthesis by oxidative stress was reported in acetate-induced cyst cells of H. pluvialis (Kobayashi et al., 1993).

The reduction in total carotenoid and astaxanthin contents by transcriptional and cytoplasmic translational inhibitors (Fig. 4) indicates the regulation of primary and secondary carotenoid formation at both the transcriptional and the cytoplasmic translational levels. Carotenoid genes expression in response to high light and nutrient stress combined with NaCl and SA additions were found to be related to transcriptional activation rather than to the stability of mRNAs as indicated by actinomycin D treatment (Fig. 7). Inhibition of astaxanthin by cycloheximide substantiates the cytoplasmic translational regulation of secondary carotenogenesis. Cycloheximide addition did not affect the transcription of PSY, PDS, LCY, and CHY genes (Fig. 6). Therefore induction of carotenogenic genes expression in response to stress conditions may be independent of cytoplasmic protein synthesis, at least for general carotenoid synthesis genes. Similar to this, induction of a higher expression of PSY and CHY genes of Haematococcus by sodium acetate, Fe^{2+} and high light (Steinbrenner and Linden, 2001), and mRNA levels of PSY gene in corollas of Cucumis sativus were shown to be independent of de novo protein synthesis (Vishnevetsky, 1997). Significant reduction in the expression of BKT by cycloheximide showed that regulation of this gene expression differs from other carotenoid genes and induction is dependent on de novo protein synthesis in the cytosol. The decrease in BKT gene expression by cycloheximide is also reflected in the significant reduction in astaxanthin content. The enhanced expression of some carotenoid genes transcripts upon cycloheximide treatment suggests the involvement of post-transcriptional modifications and stabilization of mRNAs by a translational arrest linked process or by
preventing the synthesis of labile nucleases (Price et al., 2004). Although the role of acetate (SA and NaCl/SA) in further enhancing the expression of carotenoid genes (except BKT) in the presence of cycloheximide and chloramphenicol is not clear, it suggests a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes. Chloramphenicol reduced astaxanthin production except in NaCl-added cultures (Fig. 4), which is similar to the report of Brinda et al. (2004). This indicates the involvement of the translation of organellar genes for the enhanced production of secondary carotenoids. This is the first report of its kind where regulation of carotenogenesis, both general and astaxanthin-specific, under the influence of nutrient and other stress conditions has been studied at the expression level and the metabolite level using transcriptional and translational inhibitors. It is evident from this study that acetate plays a crucial role in the enhancement of astaxanthin accumulation. This study will be helpful in understanding the regulation of carotenogenesis and expression of these genes in other organisms.

Acknowledgements

The authors RV and LV acknowledge CSIR, India, for the financial support in the form of a Senior Research Fellowship. Encouragement by Dr V Prakash, Director, CFTRI for research activities is gratefully acknowledged. The authors are extremely grateful to Dr Richard Joseph and Dr N Kumaresan, for their valuable suggestions in revising the manuscript.

References


Steinbrenner J, Linden H. 2003. Light induction of carotenoid biosynthesis genes in the green alga Haematococcus

