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Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation

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Abstract

A cDNA homologous to β-carotene hydroxylase from *Arabidopsis thaliana* was isolated from the green alga *Haematococcus pluvialis*. The predicted amino acid sequence for this enzyme shows homology to the three known plant β-carotene hydroxylases from *Arabidopsis thaliana* and from *Capsicum annuum* (38% identity) and to prokaryote carotenoid hydroxylases (32–34% identities). Heterologous complementation using *E. coli* strains which were genetically engineered to produce carotenoids indicated that the *H. pluvialis* β-carotene hydroxylase was able to catalyse not only the conversion of β-carotene to zeaxanthin but also the conversion of canthaxanthin to astaxanthin. Furthermore, Northern blot analysis revealed increased β-carotene hydroxylase mRNA steady state levels after induction of astaxanthin biosynthesis. In accordance with the latter results, it is proposed that the carotenoid hydroxylase characterized in the present publication is involved in the biosynthesis of astaxanthin during cyst cell formation of *H. pluvialis*.

Keywords: Astaxanthin; β-Carotene; Green alga; Hydroxylase; Zeaxanthin; (*Haematococcus pluvialis*)

1. Introduction

In some green algae such as *Dunaliella bardawil* and *Haematococcus pluvialis*, accumulation of carotenoids is induced by various environmental stress factors including high light, high salinity and nutrient deprivation [1,2]. Thus, large amounts of the keto carotenoid astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) are produced in the green alga *Haematococcus pluvialis* under unfavourable culture conditions such as nitrogen or phosphate deficiency and increased light intensities [3,4]. This massive production of astaxanthin in *H. pluvialis* (up to 3% of the dry weight [4]) is normally but not necessarily accompanied by morphological changes of biflagellate vegetative cells into non-mobile cyst cells. In contrast to the situation in higher plants where the carotenoids are produced in chloroplasts and chromoplasts, the accumulation of astaxanthin in *H. pluvialis* seems to occur in the cytoplasm [5]. During this extraplastidic accumulation of astaxanthin, the general structure of the chloroplast remains intact as shown by electron microscopy. It is not clear at present whether astaxanthin (or the precursors for astaxanthin biosynthesis) are synthesized in the chloroplast and then transported to the cytoplasm or whether biosynthesis occurs both in the chloroplast and in the cytoplasm. The induction and regulation of astaxanthin biosyn-

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thesis in *H. pluvialis* has recently received considerable attention due to the increasing use of astaxanthin as a source for pigmentation for fish aquacultures and due to a putative function in cancer prevention and as free radical quencher [4].

Carotenoids are synthesized by all photosynthetic organisms. They play an important role as light harvesting pigments and protect the photosynthetic apparatus from photooxidative damage under excess light conditions (reviewed in [6]). The early steps of carotenoid biosynthesis which consist of the biosynthesis of phytoene from isoprenoid precursors as well as the desaturation of phytoene to lycopene, followed by two cyclization reactions converting lycopene into β-carotene have been extensively studied and the corresponding genes have been isolated from bacteria and plants [6,7]. Several of the enzymes involved in xanthophyll biosynthesis, carotenoids modified with oxygen containing groups, have also been characterized, e.g., the β-carotene hydroxylase which converts β-carotene into β-cryptoxanthin and zeaxanthin from bacteria [8–10] and higher plants [11,12]. Moreover, some genes encoding β-carotene ketolase which catalyses the conversion of β-carotene to canthaxanthin via echinenone have been isolated not only from bacteria but also from *H. pluvialis* [13–15]. In enzymatic studies both in vitro and in vivo in *Escherichia coli* using the *Haematococcus* β-carotene ketolase, it was shown that the latter enzyme essentially converted β-carotene into canthaxanthin [14,16–18]. In contrast, the presence of hydroxy groups (β-cryptoxanthin and zeaxanthin) either reduced or prevented the formation of keto groups. These findings led to a putative biosynthetic pathway with the most favourable route to astaxanthin being via echinenone, canthaxanthin and adonirubin as outlined in Fig. 1. The same route has also been proposed previously after the detection of the latter intermediates following the inhibition of astaxanthin biosynthesis by diphenylamine [19]. Consequently, the existence of a carotenoid hydroxylase in *H. pluvialis* has been predicted which is capable of converting canthaxanthin into astaxanthin and which is active during the induction of astaxanthin biosynthesis in response to environmental stress situations [14].

In spite of many reports published on the accumulation of carotenoids in *H. pluvialis* in response to high light and unfavourable culture conditions, little research has been carried out on the molecular process of the regulation of astaxanthin biosynthesis. Only recently has the expression of two IPP isomerases as well as the expression of lycopene β-cyclase and β-carotene ketolase during the induction of astaxanthin biosynthesis by light been examined [20]. The lycopene cyclase did not show higher protein levels, whereas the mRNA steady state levels of β-carotene ketolase and of the IPP isomerases were upregulated in response to higher light intensities.

In the present publication, the isolation and characterization of a cDNA coding for a *Haematococcus* carotenoid hydroxylase is described. Heterologous complementation and astaxanthin production in *E. coli* as well as the increased mRNA steady state levels during cyst cell formation indicated that this carotenoid hydroxylase is involved in the biosynthesis of astaxanthin in *H. pluvialis*.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

Haematococcus pluvialis Flotow NIES-144 was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. The basal medium (pH 6.8) for growth of *H. pluvialis* contained 1.2 g sodium acetate, 2.0 g yeast extract, 0.4 g L-asparagine, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.02 g $CaCl_2 \cdot 2H_2O$ per litre [3]. *H. pluvialis* was grown at 20°C under a dark/light cycle of 12 h light (20 μE/m^2 s) and 12 h dark for 4 days. For induction of astaxanthin biosynthesis and cyst cell formation, sodium acetate and $FeSO_4$ were added after 4 days at a final concentration of 45 mM and 450 μM, respectively. After addition, light conditions were changed to continuous light at 125 μE/m^2 s according to [13].

E. coli strain JM101 was used as a host for the complementation experiments with plasmids pACCAR16ΔcrtX, pRKbkt1 and β-carotene hydroxylase. Plasmid pACCAR16ΔcrtX harbours the carotenoid biosynthesis genes *crtE*, *crtB*, *crtI*, and *crtY* from *Erwinia uredovora* and resulted in biosynthesis of β-carotene [8,15]. Plasmid pRKbkt1 carries the β-carotene ketolase (*bkt*) cDNA from *H. pluvialis* [13]. Cultures of *E. coli* JM101 containing the different

plasmids were grown in LB medium at 28°C for 48 h and ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), tetracycline (10 μg/ml) and isopropyl-β-D-thiogalactopyranoside (0.5 mM) were added as required [21].

2.2. Construction of H. pluvialis λcDNA expression libraries, screening and DNA sequencing

For the construction of cDNA libraries, *H. pluvialis* RNAs were extracted either from vegetative cells or from cyst cells (induction of cyst cell formation for 8 h) as described below. After purification of poly(A)RNA and synthesis of cDNAs, two λZAP expression libraries were constructed with the cDNA Synthesis and ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene). A cDNA fragment of β-carotene hydroxylase from *Arabidopsis thaliana* [11] (bases 121 to 743; kindly provided by Dr. S. Römer, Konstanz, Germany) which was amplified by reverse transcription–polymerase chain reaction using specific oligonucleotides was employed as a heterologous probe in the screening procedure. Probe labelling and plaque hybridization was carried out according to the instructions in the DIG Nonradioactive Nucleic Acid Labelling and Detection system (Boehringer Mannheim). After purification and in vivo excision using the ExAssist/SOLR system (Stratagene), the positive cDNA clone was further utilized for complementation experiments and DNA sequencing. The nucleotide sequence of the *H. pluvialis* β-carotene hydroxylase cDNA was determined for both strands using the Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer). The analysis of nucleotide and derived amino acid sequences as well as multiple alignments were carried out using the PC/Gene program (IntelliGenetics). Minor changes for multiple alignments were subsequently introduced.

2.3. Northern blot analysis

After 4 days of growth, the *H. pluvialis* cells were collected by centrifugation either directly or after varying induction times of astaxanthin biosynthesis. The cells were frozen and subsequently powdered under liquid nitrogen. RNA was then isolated according to the miniprep RNA extraction procedure described by Sokolowsky et al. [22]. For Northern blot analysis, total RNA (10 μg) was denatured in formaldehyde, electrophoresed on a 1% agarose gel containing 6% formaldehyde, transferred to Hybond N+ membrane (Amersham) and hybridized in the presence of 50% formamide after addition of 1.5×10^6 cpm/ml of ^{32}P-labelled probes.

2.4. Carotenoid extraction and HPLC analysis

For the isolation of carotenoids (carotenes and hydroxylated products) from *E. coli*, the freeze-dried cells were extracted twice with acetone at 55°C for 15 min. The combined extracts were then partitioned into diethylether/petrol (b.p. 35–80°C) (1:9, v/v), evaporated to dryness and separated on a nucleosil 100-5 C18 column (Macherey–Nagel) with acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as the eluent for the separation of β-carotene and hydroxylated products. For the separation of keto carotenoids, acetonitrile/methanol/H_2O (50:44:6, v/v/v) was applied as an eluent 1 for 22 min and methanol as the eluent 2. Spectra were recorded directly from elution peaks using a Waters 994 diode array detector. Total carotenoids from *Haematococcus* cells were isolated as already described and astaxanthin as well as astaxanthin esters were quantified after HPLC separation. Standards for HPLC analysis such as β-carotene, astaxanthin and zeaxanthin were purchased either from Sigma or Roth.

2.5. Nucleotide sequence accession number

The EMBL GenBank accession number for the β-carotene hydroxylase cDNA reported in this paper is AF162276.

3. Results and discussion

3.1. Isolation and DNA- and amino acid sequence of β-carotene hydroxylase from H. pluvialis

For the isolation of carotenoid biosynthesis genes from *H. pluvialis*, two cDNA libraries were constructed in the λZap vector. For the vegetative cell library, RNA was extracted after 4 days of growth under low light conditions. The cyst cell library was

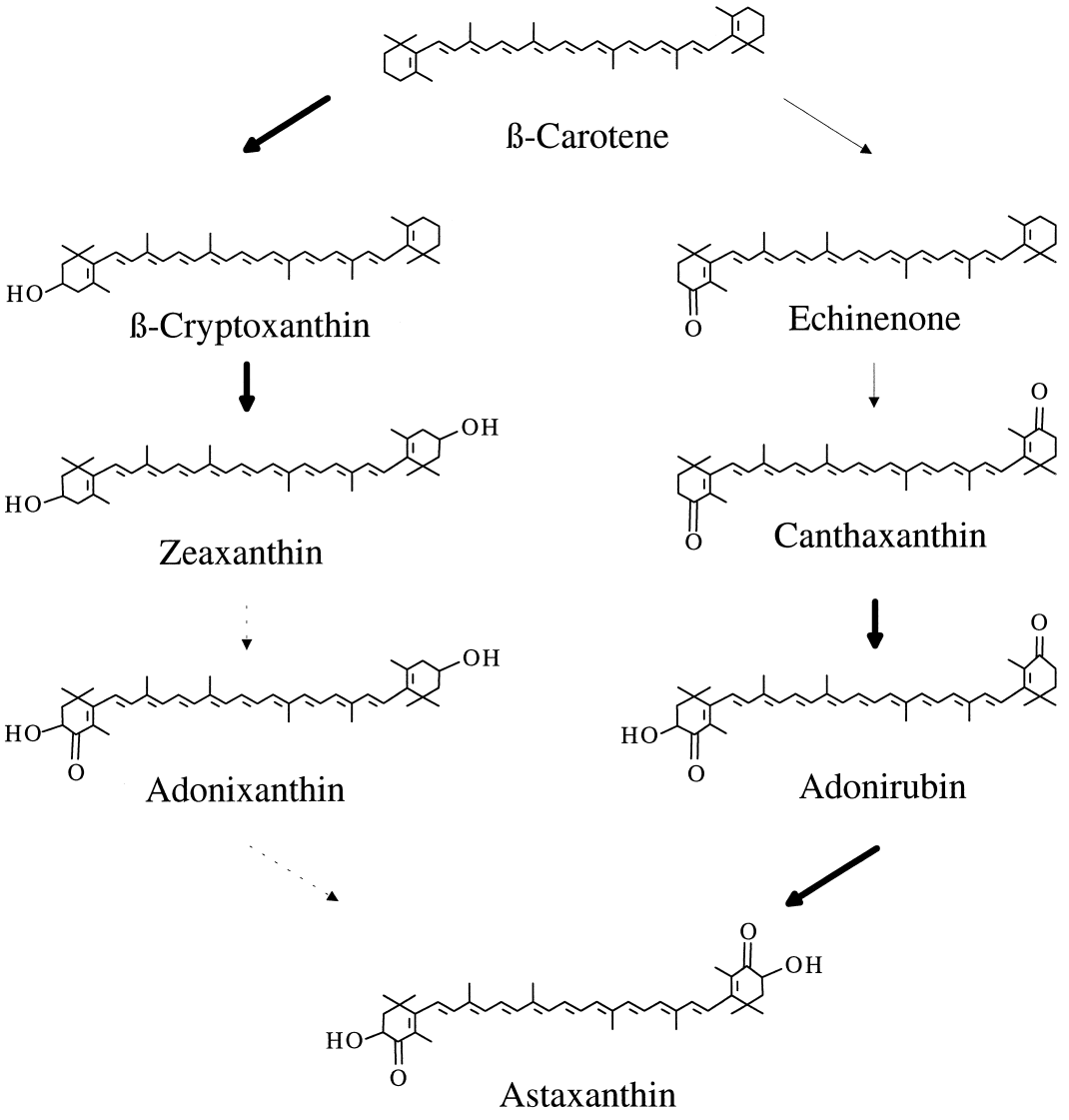

Fig. 1. Proposed astaxanthin biosynthetic pathway in *H. pluvialis* according to in vivo complementation studies [16]. Bold arrows indicate enzymatic reactions carried out by β-carotene hydroxylase. All the other reactions are either carried out by β-carotene ketolase (solid arrows) or indicate biosynthetic steps unlikely to occur in *H. pluvialis* (broken arrows). Additional non-accumulating intermediates (e.g., hydroxyechinenone) were omitted.

constructed using RNA which was extracted after induction of cyst cell formation and astaxanthin biosynthesis for an additional 8 h. Induction was carried out by the addition of acetate and ferrous sulfate according to [3]. The addition of acetate seems to cause a high carbon/nitrogen ratio and consequently a relative deficiency in nitrogen. The addition of Fe^{2+} is presumed to result in the formation of active oxygen species via the Fenton reaction [3]. Under these conditions, the morphological change of vegetative cells into cyst cells and the biosynthesis of astaxanthin were shown to proceed rapidly. Therefore, the mRNA steady state levels of genes specifically involved in the biosynthesis of astaxanthin could be expected to be high at this stage. For the screening procedure, a cDNA fragment of *A. thaliana* β-carotene hydroxylase was used which had been isolated and characterized previously [11]. In spite of extensive screening of both the vegetative and the cyst cell library, only one positive plaque was isolated from the cyst cell library. Sequence analysis of the entire cDNA insert revealed a 1608 base pair (bp) long

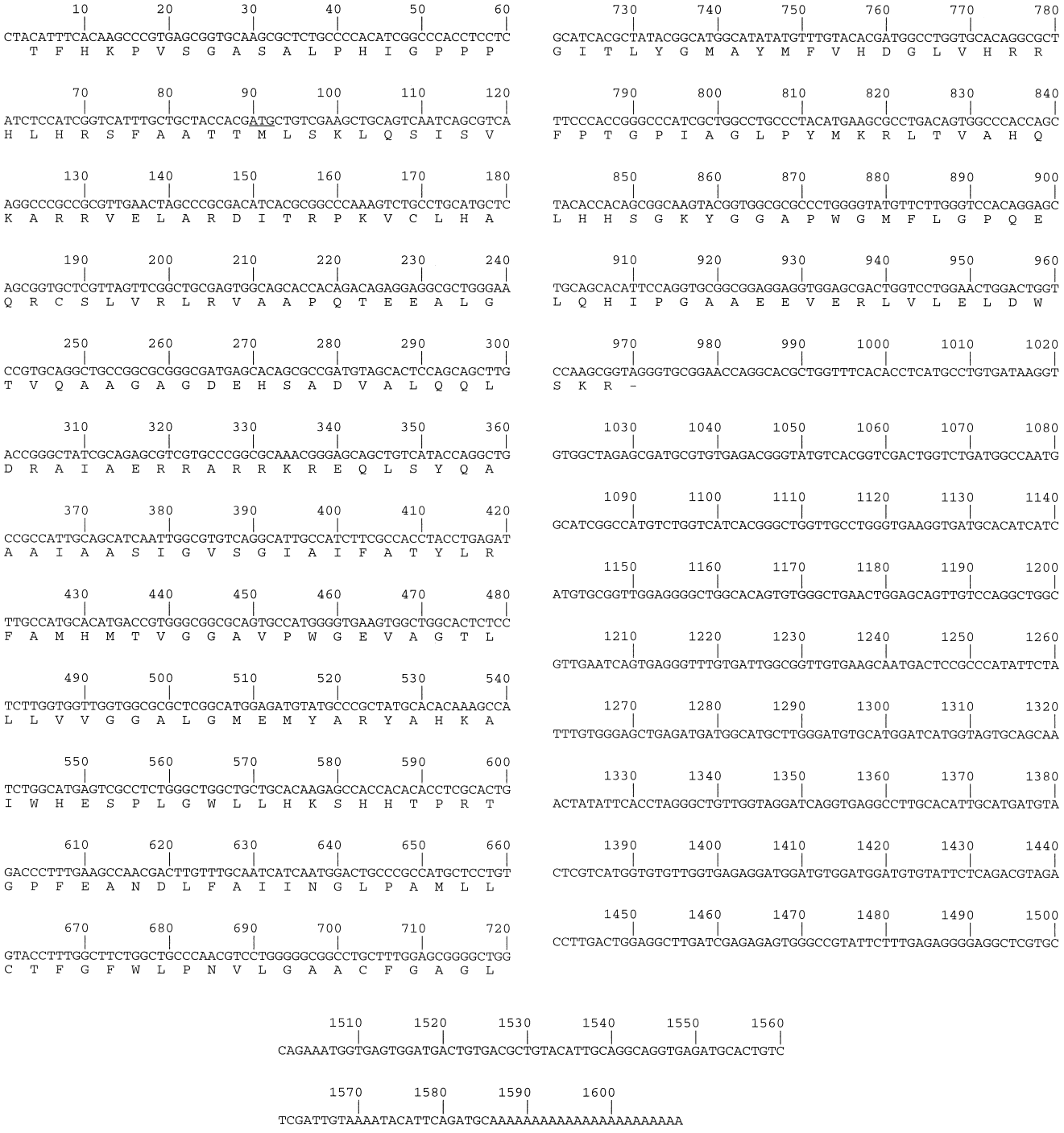

Fig. 2. Nucleotide and deduced amino acid sequences of the cDNA encoding β-carotene hydroxylase from *H. pluvialis*. The first ATG codon in frame is underlined.

fragment including a 23 bp poly(A) tail. The DNA sequence shared significant identity with the *A. thaliana* β-carotene hydroxylase cDNA (59% identity over 346 bp). The cDNA revealed one long open reading frame which extends from the beginning of the cDNA to a stop codon at base 969 (Fig. 2), indicating that this cDNA may not represent the entire open reading frame. The first ATG start codon

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H.pluv.      ----TFHKPVSGASALPHIGPPPHLHRS-FAATTMLSKLQSI-SVKARRV    44
A.thal.      --------------SFSSSSTDFRLRL-----PKS---------LSGFSP    22
Cap. Ca1     ---------MAAEISISASSRAICLQRNPFPAPKYFATAPPLLFFSPLTC    41
Cap. Ca2     TTGRYHYQLVWCQISFSSTSRTSYYRHSPFLGPKPTPTTPSVYPITPFSP    50
Alical.      --------------------------------------------------
A.aurant.    --------------------------------------------------
E.herb.      --------------------------------------------------
E.ured.      --------------------------------------------------

H.pluv.      ELARDITRPKVCLHAQRCSLVRLRVAAPQTEEALGTVQAAGAGDEHSADV    94
A.thal.      SL--------RFKRFSVCYVVEERRQNSPIENDERPESTSSTNAIDAEY-    63
Cap. Ca1     NLD-AILRSRRKPRLAACFVLKDDKLYTA-QSGKQSDTEAIGDEIEVETN    89
Cap. Ca2     NLG-SILRCRRRPSFTVCFVLEDDKF--------KTQFEAGEEDIEMKIE    91
Alical.      --------------------------------------------------
A.aurant.    --------------------------------------------------
E.herb.      --------------------------------------------------
E.ured.      --------------------------------------------------

H.pluv.      ALQQLDRAIAERRARRKREQLSYQAAIAASIGVSGIAIFATYLRFAMHM   144
A.thal.      ----LALRLAEKLERKKSERSTYLIAAMLSSFGITSMAVMAVYYRFSWQM   109
Cap. Ca1     EEKSLAVRLAEKFARKKSERFTYLVAAVMSSLGITSMAVISVYYRFSWQM   139
Cap. Ca2     EQIS-ATRLAEKLARKKSERFTYLVAAVMSSFGITSMAVMAVYYRFYWQM   140
Alical.      --------------------------------------------------
A.aurant.    --------------------------------------------------
E.herb.      --------------------------------------------------
E.ured.      --------------------------------------------------

                                          H----H        H--H
H.pluv.      TVGGAVPWGEVAGTLLLVVGGALGMEMYARYAHKAIWHESPLGWLLHKSH   194
A.thal.      E-GGEISMLEMFGTFALSVGAAVGMEFWARWAHRALWHASL--WNMHESH   156
Cap. Ca1     E-GGEMPFSEMFCTFALAFGAAIGMEYWARWAHRALWHASL--WHMHESH   186
Cap. Ca2     E-GGEVPFSEMFGTFALSVGAAVGMEFWARWAHKALWHASL--WHMHESH   187
Alical.      ----------MTQFLIVVATVLVMELTAYSVHRWIMHGP-LGWGWHKSH    38
A.aurant.    ----------MTNFLIVVATVLVMELTAYSVHRWIMHGP-LGWGWHKSH    38
E.herb.      ----------MLVNSLIVILSVIAMEGIAAFTHRYIMHG--WGWRWHESH    38
E.ured.      --------MLWIWNALIVFVTVIGMEVIAALAHKYIMHG--WGWGWHLSH    40
                           .   ... **  *  .*. . *.    *  * **

             H
H.pluv.      HTPRTGPFEANDLFAIINGLPAMLLCTFGFWLPNVLGAACFGAGLGITLY   244
A.thal.      HKPREGPFELNDVFAIVNAGPAIGLLSYGFFNKGLVPGLCFGAGLGITVF   206
Cap. Ca1     HRPREGPFELNDIFAIINAVPAIAFFSFGFNHKGLIPGICFGAGLGITVF   236
Cap. Ca2     HKPREGPFELNDVFAIINAVPAIALLDYGFFHKGLIPGLCFGAGLGITVF   237
Alical.      HEEHDHALEKNDLYGVVFAVLATILFTVG--A--YWWPVLWWIALGMTVY    84
A.aurant.    HEEHDHALEKNDLYGLVFAVIATVLFTVG--W--IWAPVLWWIALGMTVY    84
E.herb.      HTPRKGVFELNDLFAVVFAGVAIALIAVG--TAGVW--PLQWIGCGMTVY    84
E.ured.      HEPRKGAFEVNDLYAVVFAALSILLIYLG--STGMW--PLQWIGAGMTAY    86
             *  ..  .* **..... .  .  .   *             . *.* .

                    H----H                    H--HH
H.pluv.      GMAYMFVHDGLVHRRFPTGPIAGLPYMKRLTVAHQLHHS--GKYGGAPWG   292
A.thal.      GIAYMFVHDGLVHKRFPVGPIADVPYLRKVAAAHQLHHT--DKFNGVPYG   254
Cap. Ca1     GMAYMFVHDGLVHKRFPVGPIAKVPYFQRVAAAHQLHHS--DKFDGVPYG   284
Cap. Ca2     GMAYMFVHDGLVHKRFPVGPVANVPYLRKVAAAHSLHHS--EKFNGVPYG   285
Alical.      GLIYFILHDGLVHQRWPFRYIPRRGYFRRLYQAHRLHHAVEGRDHCVSFG   134
A.aurant.    GLIYFVLHDGLVHQRWPFRYIPRKGYARRLYQAHRLHHAVEGRDHCVSFG   134
E.herb.      GLLYFLVHDGLVHQRWPFHWIPRRGYLKRLYVAHRLHHAVRGREGCVSFG   134
E.ured.      GLLYFMVHDGLVHQRWPFRYIPRKGYLKRLYMAHRMHHAVRGKEGCVSFG   136
             *. *. .******.*.*   ..   * ...  ** .**.  ..   ...*

H. pluv.     MFLGPQELQHIPGAAEEVERLVLELDWSKR------------   322
A.thal.      LFLGPKELEEVGGN--EELDKEISRRIKSYKKASGSGSSSSS   294
Cap. Ca1     LFLGPKELEEVGVIEELEKEVNRRIKSLKRL-----------   315
Cap. Ca2     LFLGPKELEEVGGLEELEKEVNRRTRYIKGS-----------   316
Alical.      FIYAPPVDKLKQDLKRSGVLRPQDERPS--------------   162
A. aurant.   FIYAPPVDKLKQDLKMSGVLRAEAQERT--------------   162
E.herb.      FIYARKPADLQAILRERHGRPPKRDAAKDRPDAASPSSSSPE   176
E.ured.      FLYAPPLSKLQATLRERHGARAGAARDAQGGEDEPASGK---   175
             .. ..   .
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Fig. 3. Amino acid sequence alignment of the *H. pluvialis* β-carotene hydroxylase with the known bacterial and plant β-carotene hydroxylases. Amino acid residues which were either well or perfectly conserved in all sequences are indicated by (.) and (*) below the alignment, respectively. Identical amino acids are printed in bold. Conserved histidine motifs are shown above the sequence. A highly conserved motif is underlined. GenBank accession numbers: *A. thaliana*, U58919; *C. annuum* Ca1, Y09225; *C. annuum* Ca2, Y09722; *Alcaligenes* sp., D58422; *Agrobacterium aurianticum*, D58420; *Erwinia herbicola*, M87280; *Erwinia uredovora*, D90087.

for this open reading frame was found at nucleotide position 90. In contrast to the higher plant cDNAs encoding β-carotene hydroxylases, the *H. pluvialis* cDNA showed a very long nucleotide sequence which is due to a long 3′ non-coding region (*A. thaliana*, 1156 bp; *C. annuum*, 1112 bp and 1044 bp; *H. pluvialis*, 1608 bp). An alignment of the deduced amino acid sequence of *H. pluvialis* β-carotene hydroxylase with other carotene hydroxylases from bacteria and higher plants is shown in Fig. 3. The only carotene hydroxylase sequence which has been omitted in Fig. 3 is the β-carotene hydroxylase of the cyanobacteria *Synechocystis* sp. PCC6803 [15]. This protein revealed only limited sequence homologies to the β-carotene hydroxylases presented in Fig. 3. In contrast, it was reported to show sequence similarities with the β-carotene ketolase of *H. pluvialis*, an enzyme which is also involved in astaxanthin biosynthesis (Fig. 1). The predicted amino acid sequence for the *H. pluvialis* β-carotene hydroxylase revealed an overall homology to the three known plant β-carotene hydroxylases from *A. thaliana* and from *C. annuum* (38% identity). A lower homology was found to the prokaryote carotenoid hydroxylases (32–34% identities). The four conserved histidine motifs which have been described [12] and the highly conserved so called motif 1 (amino acids HDGLVHXRXP; [11]) are also present in the *H. pluvialis* protein. These histidine residues were proposed to be involved in iron binding and their importance for the hydroxylation reaction was proven by site-directed mutagenesis [12]. The *H. pluvialis* β-carotene hydroxylase revealed a N-terminal extension when compared to the bacterial hydroxylases which has also been observed for the higher plant enzymes. Due to putative localization of the higher plant enzymes in the chloroplast, a function as chloroplast targeting sequence has been assumed [11]. However, the sequence similarities between the algal and plant enzymes extend for about 50 amino acid residues towards the N-terminus indicating an involvement of this sequence in the enzymatic activity (Fig. 3). In support of this idea, it has been shown for the *Arabidopsis* β-carotene hydroxylase that a part of this N-terminal region is necessary for a fully functional enzyme. Furthermore, a carotenoid hydroxylase implicated in astaxanthin biosynthesis may be expected to be localized in the cytosol as has been proposed for the *H. pluvialis* β-carotene ketolase [13]. Further experiments are required to address these questions.

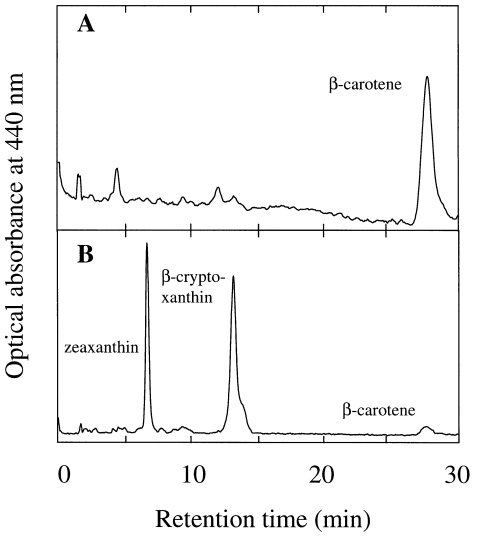

Fig. 4. HPLC separation of carotenoid pigments extracted from *E. coli* cells carrying either plasmid pACCAR16ΔcrtX only (A) or plasmid pACCAR16ΔcrtX together with β-carotene hydroxylase from *H. pluvialis* (B). The following carotenoids were identified by their absorption spectra and their retention times, as compared to standards: β-carotene, β-cryptoxanthin and zeaxanthin, all revealing absorption maxima at 425 nm, 452 nm and 480 nm.

3.2. Heterologous complementation of β-carotene hydroxylase in E. coli

In order to examine the catalytic activities of the *H. pluvialis* β-carotene hydroxylase, *E. coli* strains were used which had been genetically engineered to produce various carotenoids [13,15]. Transformation of plasmid pACCAR16ΔcrtX which contained the *crtE*, *crtB*, *crtI* and *crtY* genes from *E. uredovora* in *E. coli* resulted in the accumulation of β-carotene (Fig. 4A). After co-transformation of plasmids pACCAR16ΔcrtX and the plasmid containing the entire β-carotene hydroxylase cDNA from *H. pluvialis*, the corresponding *E. coli* transformants were shown to produce β-cryptoxanthin and zeaxanthin (Fig. 4B). The conversion ratio between β-carotene and the hydroxylated products was similar to the conversion ratio which has previously been described for the higher plant hydroxylases from *Arabidopsis* and *Capsicum* [11,12].

In enzyme studies using the β-carotene ketolase

from *Haematococcus*, it was shown that the latter enzyme mainly converted β-carotene into canthaxanthin (Fig. 1, [14,16–18]). However, the hydroxylated carotenoids such as β-cryptoxanthin and zeaxanthin were only poor substrates for the ketolase. Therefore, a carotenoid hydroxylase which is involved in astaxanthin biosynthesis should also be capable of catalysing the conversion from canthaxanthin to astaxanthin as previously proposed [14]. To investigate this hypothesis, a second co-transformation experiment was designed. Co-transformation of plasmid pACCAR16ΔcrtX and plasmid pRKbkt1 (containing the *Haematococcus* β-carotene ketolase [13]) in *E. coli* resulted in the biosynthesis of the keto carotenoid canthaxanthin in addition to small amounts of β-carotene (Fig. 5A, peaks 4 and 6). After transformation of this *E. coli* strain with a third plasmid containing the *Haematococcus* carotenoid hydroxylase cDNA fragment, several carotenoids were detected by HPLC analysis (Fig. 5B). First of all, canthaxanthin (peak 4), β-cryptoxanthin (peak 5) and zeaxanthin (peak 3) were identified. These xanthophylls correspond to the intermediate and end products of β-carotene ketolase and hydroxylase, respectively, as shown in Figs. 4B and 5A. In addition, the *E. coli* transformant produced astaxanthin (Fig. 5B, peak 1). Astaxanthin was identified by the absorbance maximum of 480 nm and the retention time which corresponded to the retention time of a purchased astaxanthin standard (Fig. 5C, peak 1). Furthermore, a second additional xanthophyll was

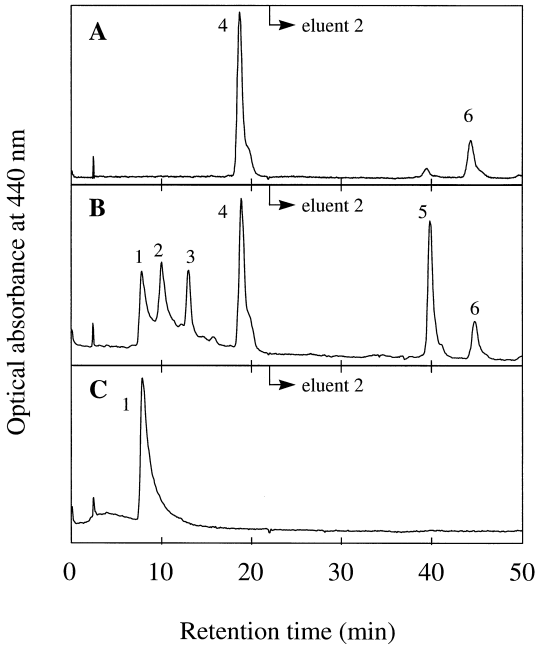

Fig. 5. HPLC separation of carotenoid pigments extracted from *E. coli* cells after transformation with plasmids pACCAR16ΔcrtX and pRKbkt1 (A) and with plasmids pACCAR16ΔcrtX, pRKbkt1 and β-carotene hydroxylase from *H. pluvialis* (B). For comparison, an astaxanthin standard was also separated (C). HPLC separation was carried out using acetonitrile/methanol/H_2O (50:44:6, v/v/v) as eluent 1 for 22 min and methanol as eluent 2. The following carotenoids were identified by their absorption spectra and their retention times, as compared to standards: 1, astaxanthin (absorbance maximum, 480 nm); 2, probably adonixanthin (470 nm); 3, zeaxanthin (425 nm, 452 nm, 480 nm); 4, canthaxanthin (475 nm); 5, β-cryptoxanthin (425 nm, 452 nm, 480 nm), 6, β-carotene (425 nm, 452 nm, 480 nm).

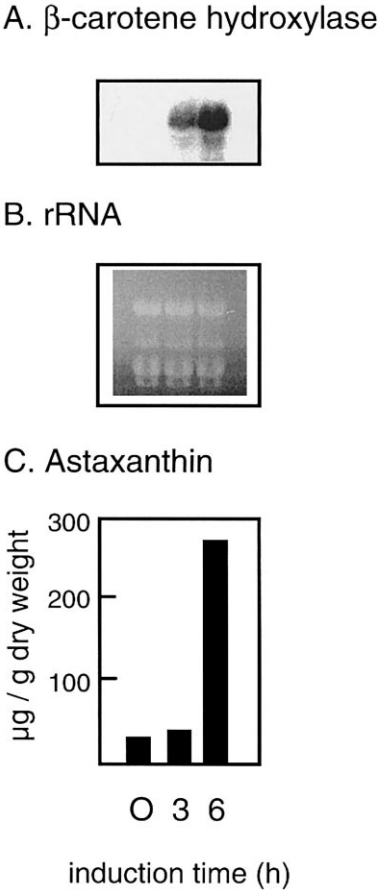

Fig. 6. Expression of β-carotene hydroxylase cDNA in *H. pluvialis* and astaxanthin biosynthesis before and after induction of cyst cell formation. The *H. pluvialis* β-carotene hydroxylase was used as specific probe (A). For comparison, total RNA on the agarose gel was stained with ethidium bromide (B). In addition, the accumulation of astaxanthin was examined (C).

found which was identified as adonixanthin because of a higher retention time in comparison to astaxanthin and the spectrum with only one maximum at 470 nm (Fig. 5B, peak 2). In consequence, the *Haematococcus* carotenoid hydroxylase seems to be a multifunctional enzyme, which accepts not only β-carotene but also keto carotenoids such as canthaxanthin as possible substrates. This is in accordance with results from in vitro studies of β-carotene ketolase from *H. pluvialis* which showed that the ketolase has a higher catalytic specificity for non-hydroxylated substrates such as β-carotene and echinenone [17]. Due to this higher catalytic specifity of β-carotene ketolase, adonixanthin and zeaxanthin detected in Fig. 5 are proposed to represent final products which can not be further converted to astaxanthin. This also means that adonixanthin is not derived from zeaxanthin but is produced via other keto carotenoid intermediates (hydroxyechinenone).

The biosynthesis of astaxanthin in *E. coli* has also been reported using bacterial carotenoid hydroxylases from *E. uredovora* which revealed the same enzymatic activities as the carotenoid hydroxylase from *H. pluvialis*. [9,13,14]. In contrast, the hydroxylation of keto carotenoids has not yet been described for the higher plant hydroxylases from *Arabidopsis* and *Capsicum* [11,12].

3.3. Expression of β-carotene hydroxylase during the induction of astaxanthin biosynthesis in H. pluvialis

In Northern blot analysis, the expression pattern of β-carotene hydroxylase was examined using RNA extracted from cells at different stages of cyst cell and astaxanthin biosynthesis induction (Fig. 6). No transcript was detected in the vegetative cells (Fig. 6A). In contrast, the *Haematococcus* cells clearly revealed a hydroxylase transcript 3 h after induction of astaxanthin biosynthesis and cyst cell formation by high light and by the addition of ferrous sulfate and acetate. An additional strong increase in the hydroxylase mRNA steady state levels was observed after 6 h induction time. For comparison, the accumulation of astaxanthin was also examined over the same period of induction (Fig. 6C). Only a very small increase was observed after 3 h induction, whereas a significant increase in astaxanthin was found after an induction for 6 h. The latter result is a confirmation of the kinetics of astaxanthin accumulation already published in which a first significant increase of astaxanthin was observed several hours after induction [3]. The maximum astaxanthin accumulation was reported to occur only 2 to 3 days after the induction of cyst cell formation.

In conclusion, the *Haematococcus* β-carotene hydroxylase characterized in the present publication reveals all the features of a carotenoid hydroxylase involved in astaxanthin biosynthesis. Firstly, the enzyme was shown to be capable of astaxanthin biosynthesis in complementation experiments. Furthermore, the transcript of the carotenoid hydroxylase revealed a strong induction during the process of astaxanthin accumulation. An additional function in the biosynthesis of zeaxanthin during vegetative growth can be presumed although no transcript was detected (Fig. 6A). A minor undetectable expression of the same transcript in the vegetative cells may be sufficient for xanthophyll production. The fact that the *Haematococcus* carotenoid hydroxylase revealed a bifunctional catalytic activity hydroxylating not only canthaxanthin but also β-carotene, as shown in Fig. 4, supports the latter presumption. Alternatively, another as yet not identified hydroxylase may exist which is transcribed only in vegetative cells and which is responsible for the biosynthesis of the xanthophylls involved in photosynthesis.

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