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Gene cloning and expression profile of a novel carotenoid hydroxylase (CYP97C) from the green alga *Haematococcus pluvialis*

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Abstract A full-length complementary DNA (cDNA) sequence of ε-ring CHY (designated *Haecyp97c*) was cloned from the green alga *Haematococcus pluvialis* by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends methods. The *Haecyp97c* cDNA sequence was 1,995 base pairs (bp) in length, which contained a 1,620-bp open reading frame, a 46-bp 5′-untranslated region (UTR), and a 329-bp 3′-UTR with the characteristic of the poly (A) tail. The deduced protein had a calculated molecular mass of 58.71 kDa with an estimated isoelectric point of 7.94. Multiple alignment analysis revealed that the deduced amino acid sequence of HaeCYP97C shared high identity of 72–85 % with corresponding CYP97Cs from other eukaryotes. The catalytic motifs of cytochrome P450s were detected in the amino acid sequence of HaeCYP97C. The transcriptional levels of *Haecyp97c* and xanthophylls accumulation under high light (HL) stress have been examined. The results revealed that *Haecyp97c* transcript was strongly increased after 13–28 h under HL stress. Meanwhile, the concentrations of chlorophylls, carotenes, and lutein were decreased, and zeaxanthin and astaxanthin concentrations were increased rapidly, respectively. These facts indicated that HaeCYP97C was perhaps involved in xanthophyll biosynthesis, which plays an important role in adaption to HL for *H. pluvialis*.

Keywords Xanthophylls biosynthesis · Carotenoid hydroxylase · *Haematococcus pluvialis* · Gene cloning · High light

H. Cui and X. Yu contributed equally.

X. Yu is the co-first author and has the same contribution to this paper.

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Introduction

Carotenoid hydroxylase (CHY) is considered as the rate-limiting enzyme performing entry reaction into the α- and β-branch xanthophyll biosynthesis in photosynthetic organisms. In cyanobacteria, the production of β-branch xanthophylls (zeaxanthin) is catalyzed by β-CHY (CrtR-type), while the enzyme involved in the biosynthesis of α-branch xanthophylls is unclear (Lagarde and Vermaas 1999; Takaichi 2011). In higher plants, the β-carotene is hydroxylated mainly by the non-heme di-iron enzymes, BCH1 and BCH2, to produce zeaxanthin (Sun et al. 1996; Tian and DellaPenna 2001), while α-carotene is mainly hydroxylated

by P450 enzymes, CYP97A3 for the β-end group and CYP97C1 for the ε-end group, to produce lutein (Dall'Osto et al. 2006; Kim and DellaPenna 2006; Tian and DellaPenna 2004; Tian et al. 2003, 2004). In the past, biosynthesis of α- and β-branch xanthophylls of higher plants has been studied extensively, whereas research in algae is still in its infancy. For unicellular green algae, only genes coding BCHs have been cloned and functionally characterized from *Haematococcus pluvialis* and *Chlamydomonas reinhardtii* (Linden 1999; Tan et al. 2007; Zheng et al. 2007). Although lutein and its derivatives are found in Rhodophyta (macrophytic type), Cryptophyta, Euglenophyta, Chlorarachniophyta, and Chlorophyta, enzymes involved in the hydroxylation of α-carotene are still unknown (Takaichi 2011). Recently, Li Tian and colleagues reported that a new P450 protein is responsible for the hydroxylation of the ε-ring of α-carotene in *Arabidopsis thaliana* (Inoue 2004; Tian et al. 2004), which not only identifies one of the missing pieces of carotenoid biosynthetic enzyme but also provides valuable clues for further research on the carotenoid hydroxylation in algae.

H. pluvialis, a green alga with high commercial value, has the ability to synthesize and accumulate large amounts of the red ketocarotenoid astaxanthin under various stress conditions (Boussiba 2000; Teng et al. 2002; Wang et al. 2012b; Zhang et al. 2012). Astaxanthin can be synthesized from β-carotene with the introduction of keto and hydroxyl moieties at the C-4,4′ and C-3,3′ positions via many hydroxylated or ketolated carotenoid intermediates (Choi et al. 2006; Wang et al. 2012a). Previous studies have demonstrated that the CHY involved in the astaxanthin biosynthetic pathway in *H. pluvialis* contains a cytochrome P450 dependent enzyme by the use of ellipticine (Schoefs et al. 2001), an inhibitor of cytochrome P450-dependent enzymes (Lemaire and Livingstone 1995). When cells were treated prior to the stress with this compound, canthaxanthin, echinenone, and β-carotene accumulated, indicating that the astaxanthin biosynthetic pathway via canthaxanthin is the main one (Schoefs et al. 2001). At present, such a cytochrome P450 hydroxylase remains to be isolated from *H. pluvialis* (Lemoine and Schoefs 2010). In addition, some studies have suggested that the cooperation of appropriate hydroxylase and ketolase enzymes or their conversion capacity toward various substrates may be crucial for the efficient production of astaxanthin in a heterologous host (Fraser et al. 1997; Misawa et al. 1995; Yokoyama and Miki 2006). It is therefore necessary to find and identify such hydroxylase and ketolase that have high conversion efficiency from β-carotene to astaxanthin.

In this study, homologous cloning coupled with the rapid amplification of complementary DNA ends (RACE) was used to clone full-length cDNA sequence of *Haecyp97c*. The sequence analysis for CYP97Cs from green algae and higher plants were finished, focusing on their phylogeny, evolution, and conserved domains. The transcriptional expression profiles of *Haecyp97c* and the composition of xanthophylls under different high light (HL) stresses were investigated by quantitative reverse transcription PCR (qRT-PCR) and high-performance liquid chromatography (HPLC), respectively.

Materials and methods

Haematococcus pluvialis strain Flotow 1844 was obtained from Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory) and maintained at the Biological Resources Laboratory, Yantai Institute of Costal Zone Research, Chinese Academy of Science. Algae were incubated in 250-mL Erlenmeyer flasks, each containing 100 mL Bold's basal medium, and placed in an illuminating incubator under a light intensity of 25 μmol photons m^{-2} s^{-1} with a diurnal cycle of 14 h light and 10 h dark at temperature of 22±1 °C without aeration. All flasks were shaken manually twice at a fixed time every day.

For high light stress conditions, the exponentially growing cultures (cell density approximately 5×10^7 cells mL^{-1}) were harvested and transferred cells to fresh medium under continuous white light (390–770 nm) or blue light (420–500 nm) with light intensity of 1,000 μmol photons m^{-2} s^{-1} without a day/night cycle. Collected algal cells were rinsed with phosphate-buffered saline and divided into replicate parts, one for qRT-PCR analysis, and the other for pigments profiling, stored at −80 °C if not immediately used. All experimental chemicals and reagents were analytical grade.

DNA, RNA isolation, and cDNA synthesis

H. pluvialis in the exponential growth phase was harvested. Genomic DNA was isolated following the protocol described by Sambrook and Russell (2001). Total RNA was extracted from fresh cells of *H. pluvialis* using Trizol reagent (TaKaRa D9108B, China) according to the user manual. In this protocol, RNA was treated with DNaseI. Nucleic acids were quantified by NanoDrop 2000c (Thermo Scientific, USA). Both DNA and RNA solutions were stored at −80 °C, if not immediately used. First-strand cDNAs were synthesized from 2 μg total RNA with PrimeScript® RT Enzyme Mix I (TaKaRa DRR047A, China) according to the manufacturer's instructions.

PCR with degenerate primers

First-strand cDNA was used as a template. The degenerate primers (F1 and R1) for homologous cloning of core partial sequences of *Haecyp97c* were designed by CODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primers) (Rose et al. 2003) based on the highly conserved regions of predicted putative genes encoding CYP97Cs from some

green algae with available genomes database, including *C. reinhardtii*, *Chlorella* sp. NC64A, V*olvox carteri*, *Chlorella vulgaris*, and *Coccomyxa* sp. C-169 (Online resources ESM_1.pdf). The primers and conserved peptide sequences were listed in Table 1. All primers were biosynthesized by Sangon Biotech (China).

PCR amplification was conducted with TaKaRa LA Taq® (TaKaRa DRR002A, China) according to the manufacturer's instructions and was processed with the following parameters: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min (according to the length of products, 1,000 bp min^{-1}), with a final extension at 72 °C for 7 min and cooling to 4 °C. The PCR products were resolved by electrophoresis on 1 % agarose gel. Then, the fragment of interest was excised and purified using an agarose gel DNA fragment recovery kit (TaKaRa D823A, China). Finally, the fragment was cloned into pMD-18T vector (TaKaRa D101A, China) and sequenced (Invitrogen, China).

Full-length cDNA and genomic sequences isolation

The nucleotide sequences of the 3′- and 5′-ends of *Haecyp97c* were amplified by RACE method. The gene-specific primers 3GSP1, 3GSP2, 5GSP1, and 5GSP2 (Table 1) were designed from the amplified core cDNA sequence of *Haecyp97c* and its 3′- and 5′-ends were obtained using SMARTTM RACE cDNA Amplification Kit (Clontech) according to the manual. The RACE reactions were performed using cDNA, primers and TaKaRa LA Taq® enzymes (TaKaRa DRR02AM, China). Nested PCR was carried out using the nested universal primers and gene-specific primers. Secondary PCR fragments were performed the same as described above.

Based on the information of 3′- and 5′-ends sequences, two gene-specific primers F2 and R2 (Table 1) were designed to amplify the genomic sequence. PCR was performed with the GC-Rich PCR system (TaKaRa DRR02AG, China). Reaction conditions were as follows: 5 min denaturation at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min kb^{-1}, finally extended at 72 °C for 7 min. The PCR fragments of the expected length (3.1 kb) were cloned into pMD-18-T vector and sequenced.

Bioinformatics analysis

The full-length cDNA sequence of *Haecyp97c* was spliced according to the RACE-PCR results by the SeqMan software of DNAStar 7.1 (DNASTAR Inc., USA). The theoretical molecular weight (M_w) and isoelectronic point (p*I*) of HaeCYP97C protein were computed by ExPASy Compute pI/Mw tool (Bjellqvist et al. 1993). Transmembrane regions were predicted by "DAS"-Transmembrane Prediction server (Cserzö et al. 2002). Prediction of subcellular localization of the deduced amino acid was conducted by using ChloroP

Table 1 Primers used in experiment. Note: *F* forward, *R* reverse, *GSP* gene-specific primer

Primers	Oligonucleotide sequence 5′–3′
Partial *Haecyp97c* gene fragment	
F1	CCCTGTTCCAGCCCCTGTWYAARTGGAT (LXKWMXEXGXXYLLPP)
R1	CGGGTGGGGGTACAGGCKCATNSWYTC (ESMRLYPXPPV)
5′ and 3′ RACE	
3GSP1	CGCTGAAGGAGACGGAGACAC
3GSP2	AAGGCGATGGTGGATGAAGAA
5GSP1	CAGTGGCTCGTGTCTCCGTCT
5GSP2	CCACCTGAGATGGCAAACCC
Genomic sequences cloning	
F2	CGTTTCGTTGTTGTTT
R2	TGCTTGGGAATGTTCTCTA
Real-time PCR	
F3	AAGGGGTTGGTGGCAGAGG
R3	GGACGATGGCACAAAGAAATG
F4	AGCGGGAGATAGTGCGGGACA
R4	ATGCCCACCGCCTCCATGC
Sequencing primer	
M13-47	CGCCAGGGTTTTCCAGTCACGAC
RV-M	GAGCGGATAACAATTTCACACAGG

and TargetP Server (Emanuelsson et al. 2007). HaeCYP97C proteins and those identified by the BLAST searches were aligned using ClustalW (Larkin et al. 2007; Thompson et al. 1994) with a gap opening penalty of 10, a gap extension penalty of 0.2, and Gonnet as the weight matrix. Maximum likelihood trees of some CYP97 proteins were constructed using PhyML (Guindon and Gascuel 2003). The Le and Gascuel evolutionary model (Le and Gascuel 2008) was selected for analysis of the protein phylogenies assuming an estimated proportion of invariant sites and a gamma correction (four categories). Bootstrap (BS) values were inferred from 400 replicates. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Chevenet et al. 2006).

Quantification analysis of *Haecyp97c* transcriptional expression under HL stress conditions

Quantitative real-time PCR was performed on an ABI fast 7500 Sequence Detection System (Applied Biosystems) to investigate the expression of *Haecyp97c*. Two pairs of gene-specific primers were used to amplify a 384-bp PCR product of *Haecyp97c* (F3 and R3, Table 1) and a 114 bp of *actin* gene (F4 and R4, Table 1) gene. The product of actin was used to as an internal control for RT-PCR. The qRT-PCR amplifications were carried out in triplicate in a total volume of 20 μL according to the manufacturer's instructions of SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa DRR420A, China). The qRT-PCR program was holding stage, 50 °C for 20 s and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and melt curve stage, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 60 °C for 15 s. DEPC-treated water for the replacement of template was used as negative control. All analyses were based on the C_T values of the PCR products. The amplification specificity for *Haecyp97c* and *actin* genes was determined by analyzing the dissociation curves. The comparative C_T method was used to investigate the transcriptional expression levels of *Haecyp97c* gene.

Pigment analysis under HL stress conditions

For cell dry biomass (mg L^{-1}) determination, cells from 40-mL culture were collected by centrifugation at 12,000 rpm at 4 °C for 5 min and washed three times. The EP tubes containing cells were dried in a freeze-dryer.

For pigment analyses, HPLC method was applied to quantify the content of total pigments as per the procedure given by Sun et al. (2008). Briefly, the freeze-dried cells (0.01 g) were ground with nitrogen and extracted with acetone until the cells became colorless. After centrifugation at 12,000 rpm at 4 °C for 5 min, the supernatant was collected and evaporated under nitrogen gas and the residue was redissolved in 1 mL acetone and filtered through a 0.22-mm Millipore organic membrane prior to HPLC analysis. Pigments were eluted at a flow rate of 1.2 mL min^{-1} with a linear gradient from 100 % solvent A [acetonitrile/methanol/0.1 M Tris–HCl, pH 8.0 (84:2:14)] to 100 % solvent B [methanol/ethyl acetate (68:32)] for 15 min, followed by 12 min of solvent B. The absorption spectra of the pigments were shown between 300 and 700 nm. Peaks were measured at 450 nm. The concentration of individual carotenoids (carotenes, lutein, zeaxanthin, and astaxanthin) was determined using standard curves of standard pigments at known concentrations.

Statistical analysis

All exposure experiments were repeated three times independently, and data are recorded as the mean with standard deviation (SD). For gene expression experiments, quantitative real-time PCR analysis was performed using the BioRAD iQ5 software. For each gene, the fold change expressed as the mean±SD (% control) was calculated using the (standard curve) approximation corrected for primer efficiency and normalized to housekeeping gene *actin* expression values.

Nucleotide sequencing and accession number

The nucleotide sequences of *Haecyp97c* genomic and cDNA have been deposited and assigned the accession number KC182114 and JX232405 in the EMB/GenBank/DDBJ database.

Results

Cloning and characterization of *Haecyp97c* gene

A 996-bp cDNA fragment was generated by RT-PCR with degenerate primers F1and R1 (Table 1). The *Haecyp97c* fragments shared 69 % sequence similarity with *cyp97c3* genes from *C. reinhardtii* and 54 % with *cyp97c3* genes from *A. thaliana*. It indicated a partial putative *cyp97c* was isolated from *H. pluvialis*. On the basis of this sequence information, two pairs of specific primers (3GSP1 and 3GSP2, 5GSP1 and 5GSP2) were designed for 3′ and 5′ RACEs. A 857-bp 5′-RACE product and 1,169-bp 3′-RACE product were amplified. The nucleotide sequences of both products from RACE experiments shared an overlap on flanking regions of the 5′- or 3′-ends of the partial putative *Haecyp97c* cDNA fragment, suggesting that these fragments were portions of the same gene. Sequence analysis revealed that the cloned *Haecyp97c* cDNA was 1,995 bp in length, which contained a 1,620-bp open reading frame, a 46-bp 5′-untranslated region (UTR), and a 329-bp 3′-UTR

with the characteristics of the poly (A) tail. An ATG translation initiation codon was identified in the sequence of the 5′ terminus (47–49 bp), and a TGA termination codon was found 1,618 nucleotides downstream of the initiation site (Fig. 1a).

To elucidate the genomic organization, the genomic DNA sequence of *Haecyp97c* was cloned with primers F2 and R2 (Table 1) and aligned with cDNA sequence. BlastX and BlastN searches combined with alignments between *Haecyp97c* genome and *Haecyp97c* cDNA sequences allowed us to identify the genomic organization. The result showed that *Haecyp97c* genomic sequence of 3,144 bp was interrupted by six introns of 72, 80, 100, 562, 176, and 157 bp, respectively (Fig. 1b). Intron/exon splice sites of the *Haecyp97c* gene are highly conserved and all introns start with GT and end with AG.

The deduced HaeCYP97C protein is composed of 539 amino acids with a calculated molecular mass of 58.71 kDa and a theoretical isoelectric point of 7.94. A BLAST search revealed that the primary structure of HaeCYP97C shared 85/82/72 % sequence similarity with CYP97C3/C3/C1 genes from *C. reinhardtii* (ABQ59243), *C. subellipsoidea* C-169 (EIE23687), and *A. thaliana* (NP_190881), respectively. The ChloroP server predicts the presence of possible chloroplast transit peptides (cTP, 23 aa, Fig. 1a) in amino acid sequence of HaeCYP97C. The TargetP program predicted the chloroplast sublocation of HaeCYP97C. No TM helices were predicted by "DAS"-Transmembrane Prediction and TMHMM servers in protein sequences of HaeCYP97C. All these observations suggested that *Haecyp97c* encoded a soluble enzyme localized in the chloroplast of *H. pluvialis* strain Flotow 1844.

Conserved domains in CYP97C from green algae

P450s all share a common catalytic center, heme with iron coordinated to the thiolate of a conserved cysteine (Bak et al. 2011). Despite a low sequence identity at the amino acid level, P450s display a common overall topology and tridimensional fold (Graham and Peterson 1999; Werck-Reichhart and Feyereisen 2000). The deduced amino acid sequence of HaeCYP97C were aligned with other known sequences using the ClustalX program, and the results revealed that eight conserved domains (CD) were discovered (Fig. 2).

Figure 2 shows an alignment of the deduced amino acid sequences of CYP97Cs from ten green algae with available genome database. There are eight domains with strong conserved amino acid sequences in all CYP97C protein sequences. Three conserved domains are discovered in the N-terminal of the protein sequences, including CD1 (GPVYLLPTGPXXS), CD2 (WXXRRKAVXPSLH), and CD3 (LDIIGXXVFNYDE), which the forward degenerate primers were designed. In addition, the P450s active site components were found as expected in the C-terminal of amino acid sequence, including CD4 (*I*-helix, LVAGHETT) involved in oxygen binding, CD5 (ERR triad, ESMRLYPXPPVXXRRA) involved in locking the heme pockets into position and to assure stabilization of the conserved core structure, and CD7 (PFXXGPRRXXGD) involved in heme binding. Otherwise conserved domains, CD6 (clade signature, PXRF), and CD8 (MTXGATIHT) were found to locate near the active sites. As expected, the primary amino acid sequence of HaeCYP97C contained all the conserved domains involved in the catalytic mechanisms of P450s (CD4, 378–385, CD5, 437–450, and CD7, 514–525) (Fig. 1a). It is likely that conserved domains involved in the active sites supply an important clue that HaeCYP97C is a member of P450s and that domains that are conserved within the CYP97C proteins are responsible for the specificity with respect to the β- or ε-ring of the different carotenes.

Phylogeny of CYP97 between green algae and higher plants

In order to explore the phylogeny and evolution of CYP97 in viridiplantae group, 35 genes encoding putative CYP97s were predicted from ten green algae with genome database, including *C. reinhardtii*, *Chlorella* sp. NC64A, *C. vulgaris*, *Coccomyxa* sp. C-169, *V. carteri*, *Micromonas pusilla*, *Micromonas* sp. RCC299, *Ostreococcus* sp. RCC809, *Ostreococcus tauri*, and *Ostreococcus lucimarinus* by BLASTp program with a query including HaeCYP97C. Moreover, ten genes encoding CYP97s from higher plants were also downloaded from NCBI GenBank. All sequence information was summarized in Online Resources ESM_1.pdf.

A phylogentic tree of CYP97s from Viridiplantae group was constructed by maximum likelihood method (Fig. 3). Genes encoding CYP97A/C/B from green algae and higher plants build three monophyletic groups (BS: 96/100/46 %), respectively. Two clades were observed comprising genes encoding CYP97A/C as sister groups. The CYP97B homologues from green algae and higher plants were found at the base of all CYP97 family when gene encoding CYP86A from *A. thaliana* was used as outgroup. It should be mentioned that in each monophyletic group, the phylogeny of genes encoding CYP97 corresponds well with the phylogeny of organisms based on the 16 s rRNA sequences from chloroplasts of eukaryotic phototrophs. Several interesting results were discovered from further analyses of the phylogenetic tree. The formation of CYP97A/B/C ancestral genes occurred before the higher plant/green algae split. The further lineage-specific gene duplications occurred in the evolution of CYP97A in green algae (*C. reinhardtii* and *O. lucimarinus*) and CYP97B in green

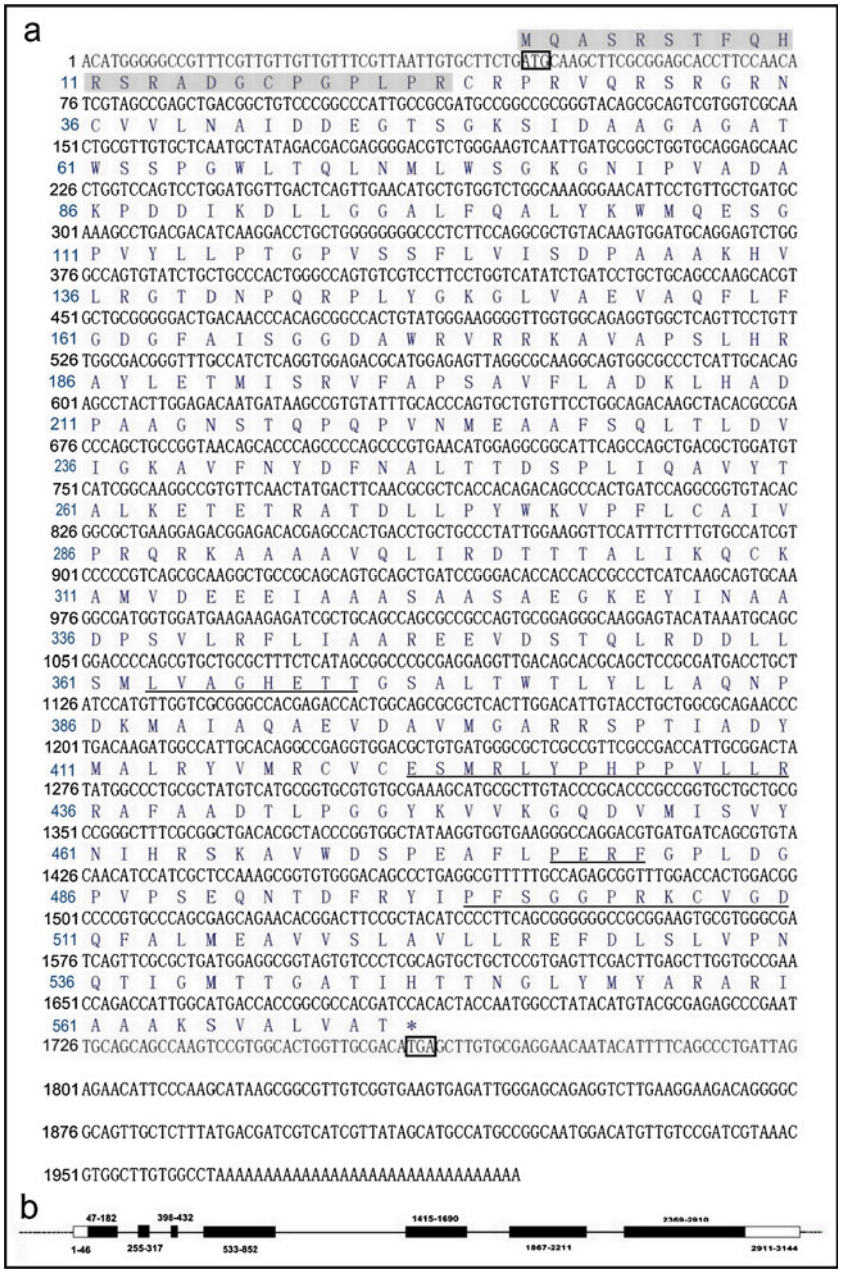


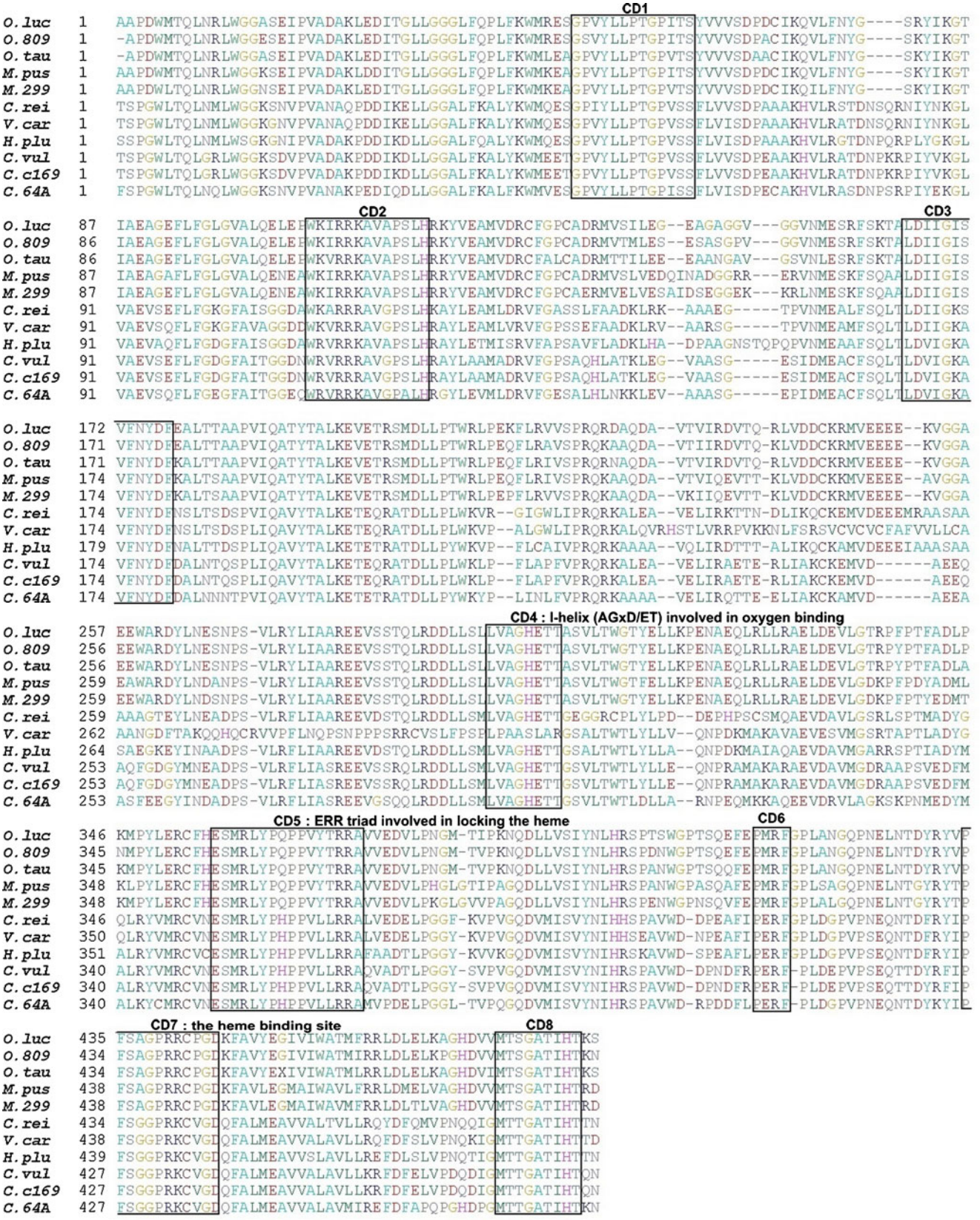
Fig. 1 The nucleotide and deduced amino acids sequence of *Haecyp97c* from *H. pluvialis*. **a** The *letters in box* indicate the start codon (ATG) and the stop codon (TAG) sequence. The predicted N-terminal chloroplast transit peptide (by ChloroP serve) was indicated by *shadow* in the deduced amino acids sequence of HaeCYP97C. The four conserved domains involved in catalytic mechanisms of all cytochrome P450 proteins are *underlined*, including I-helix (LVAGHETT) involved in oxygen binding, ERR triad (ESMRLYPHPPVLLRRA) involved in locking the heme pockets into position and assuring stabilization of the conserved core structure and heme binding site (PFSGGPRKCVGD and PERF). **b** The complete genomic structures of *Haecyp97c*. Exons are shown as *boxes*: *black boxes* are open reading frames, *empty boxes* are 5′- or 3′-untranslated regions

algae (*O. lucimarinus*, *Micromonas* sp. RCC299, *Ostreococcus* RCC809, and *O. tauri*).

High light upregulate the transcripts of *Haecyp97c* and enhance the biosynthesis of xanthophylls

Time-course patterns of cell growth in photoautotrophic *H. pluvialis* with two different high light (HL) stresses are shown in Fig. 4a. The results signify that HL (white or blue) stress has completely different influence on biomass concentration of *H. pluvialis* during the early 54 h exposure. During this period, the HL (white) improved the growth of *H. pluvialis*, while HL (blue) inhibited the growth of *H.*

Fig. 2 Multiple alignment of the deduced amino acids of the HaeCYP97C from *H. pluvialis* and other predicted CYP97Cs from ten green algae with available genome database. The query with HaeCYP97C and the BLASTp program were applied to search for putative CYP97C homologs in ten green algae, including *Chlamydomonas reinhardtii*, *Chlorella* sp. NC64A, *Chlorella vulgaris*, *Coccomyxa* sp. C-169, *Volvox carteri*, *M. pusilla*, *Micromonas* sp. RCC299, *Ostreococcus* sp. RCC809, *Ostreococcus tauri*, and *Ostreococcus lucimarinus*. All the sequences information was summarized in Online Resources ESM_1.pdf. The three typical conserved domains of all cytochrome P450s were discovered, including the oxygen binding site signature (CD4, LVAGHETT), the ERR triad involved in locking the heme (CD5, ESMRLYPXPPVXXRRA), and the heme binding signature sequence (CD7, PFXXGPRRXXGD)


CD1
CD2
CD3
CD4 : I-helix (AGxD/ET) involved in oxygen binding
CD5 : ERR triad involved in locking the heme
CD6
CD7 : the heme binding site
CD8

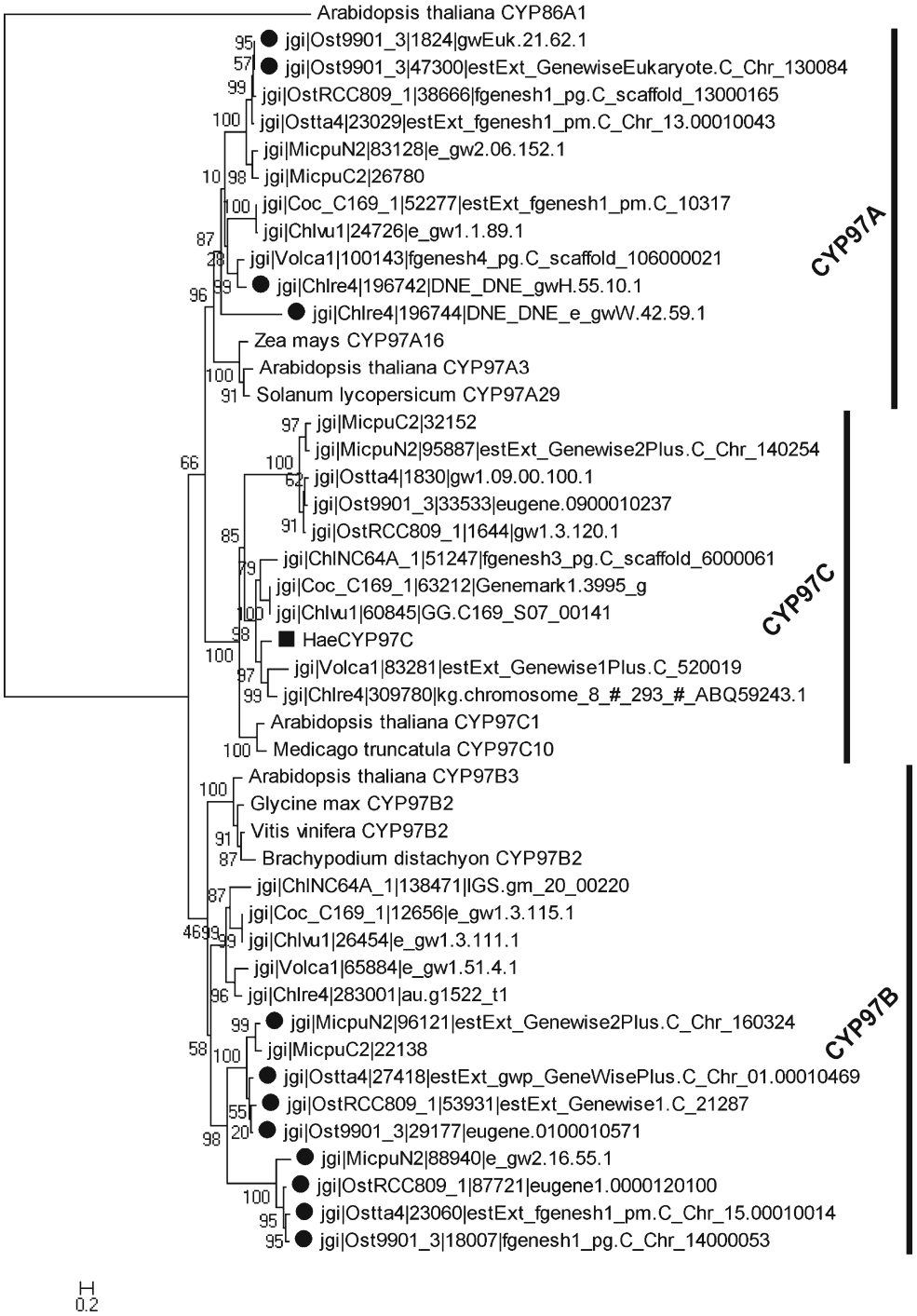


Fig. 3 Phylogenetic tree of CYP97 homologues from algae and higher plants. The alignment was generated by the ClustalW program and the tree was constructed with maximum likelihood method using PHYML software as described in "Materials and methods." The BLASTp program was applied to search the putative HaeCYP97C homologue sequences of ten green algae with genome database. Other HaeCYP97C homologues of higher plants were collected from GeneBank by BLAST program. All the sequences information was summarized in Online Resources ESM_1.pdf. Note: the *square box with black* is the HaeCYP97C sequences from *H. pluvialis*. The *circle boxes with black* indicated species- or linage-specific gene duplication in the evolution of CYP97 in algae

pluvialis. With the extension of induction time, the biomass concentrations were lower under both white and blue HL stresses, respectively, than that of control (Fig. 4a).

As revealed by qRT-PCR results (Fig. 4b), upon exposure of *H. pluvialis* cells to white HL (390–770 nm and 1,000 μmol photons m^{-2} s^{-1}), an initial decrease messenger DNA (mRNA) level of *Haecyp97c* was observed in the prophase of the treatment (0–6 h). Then, the mRNA level increased and reached its maximum at 10 h exposure, with 2.75-fold higher than that of the control. The transcriptional level of *Haecyp97c* was higher than that of control throughout the following 29 h (6–34 h), and then it remained stable until 72 h (Fig. 4b). Blue HL (420–500 nm and 1,000 μmol photons m^{-2} s^{-1}) treatment also upregulated *Haecyp97c* transcripts to much stronger extent as compared to HL (white) treatment (Fig. 4b). Similarly, the *Haecyp97c* mRNA level also began to decline in the early stage of exposure (0–4 h). However, transcript accumulation

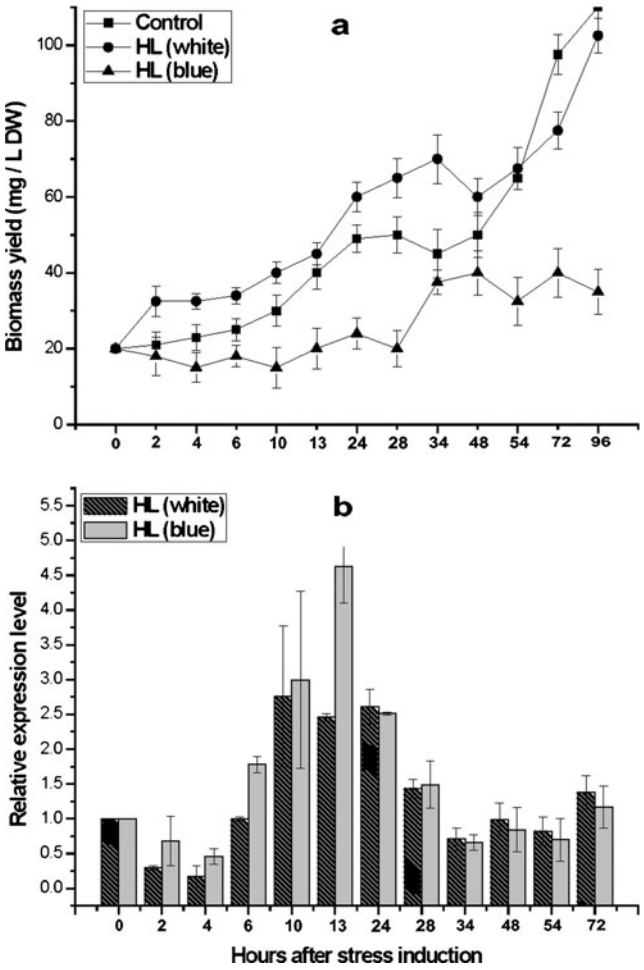


Fig. 4 Growth and *Haecyp97c* expression of *H. pluvialis* cultures during induction of high light. **a** The growth curve of *H. pluvialis* under different HL stresses. Algal cells at the logarithmic phase were transformed with different treatments: HL (*white or blue*), with the starting optical density (approximately 5×10^7 cells mL^{-1}), each of these in triplicate. Cells were harvested at different periods of induction and cell dry weight was monitored as descripted in methods. **b** gene expression profile of *Haecyp97c* was detected by real time RT-PCR. Values are mean±SD of three independent determinations

increased significantly from 6 to 28 h, and the maximum transcriptional levels occurred at 13 h exposure, with 4-fold higher than that of the control. Transcript accumulations remained relatively constant when exposure during 34–72 h under blue HL stress. These above observed expression patterns of *Haecyp97c* under HL stresses suggested that this enzyme may be involved in the adaptation of HL intensity for *H. pluvialis*.

To explore the relationship between the transcriptional level of *Haecyp97c* gene and the biosynthesis of xanthophylls, the accumulation of chlorophylls, lutein, zeaxanthin, α-carotene, β-carotene, and astaxanthin was examined over the period of induction (Fig. 5). Upon white and blue HL stress inductions, the total chlorophylls concentration decreased, and it was less in the former than that in the latter (Fig. 5a).

The lutein concentration slowly increased during the early stage (0–6) under both white and blue HL stresses, respectively, and it then started decline during the following exposure (6–96) (Fig. 5b). The lutein concentrations were lower under both HL stress than that of the control. Similarly, the changing trends of the α-carotene concentration were similar with those results observed in lutein concentration (Fig. 5c). The concentrations of zeaxanthin under all treatments increased markedly and were higher than that of the control (Fig. 5d). In addition, the cells accumulated more zeaxanthin under blue HL than that under white HL during 0–48 h exposure (Fig. 5d), suggesting that the *Haematococcus* cells was more sensitive and react quickly under blue HL stress than that of the white HL stress. During the early stress inductions (0–6 h), the concentrations of β-carotene were higher than that measured at control, while they decreased with the extension of stress conditions (6–96 h) (Fig. 5e). It is interesting to mention that the concentration of β-carotene under blue HL was less than that under white HL at induction time 6–34 h (Fig. 5e). The astaxanthin concentration under blue or white HL stress was higher than that of the control, reaching a maximum level at 34 and 54 h exposure, respectively (Fig. 5f). These results indicated that the coordinated upregulation of one of xanthophyll biosynthetic gene, *Haecyp97c*, leads to a reduction in the concentration of substrate (α-c and β-carotene) and the main product (lutein), while rapid synthesis of zeaxanthin and astaxanthin, although zeaxanthin may be also derived from the conversion of violaxanthin under HL irradiation. As a consequence, we can draw a speculation that, besides the roles in producing α-branch xanthophylls (lutein), the HaeCYP97C may involve in synthesizing β-branch xanthophylls (zeaxanthin) and astaxanthin under HL irradiation.

Discussion

A novel chloroplast location carotenoid ε-ring hydroxylase from the economic green alga *H. pluvialis* Flotow 1844 was isolated using RT-PCR with degenerate primers designed from conserved motifs and RACE methods. To our knowledge, it is the first time that a *cyp97c* homologue gene has been isolated from eukaryotic algae, and the expression profiles have been established under different HL stresses. Previously, only the genes encoding members (CYP97A/B/C) of CYP97 family have been isolated and functionally investigated from higher plants (Fiore et al. 2012; Kim and DellaPenna 2006; Kim et al. 2009; Lv et al. 2012; Stigliani et al. 2012; Tian and DellaPenna 2004; Tian et al. 2003, 2004). It is reasonable to speculate that

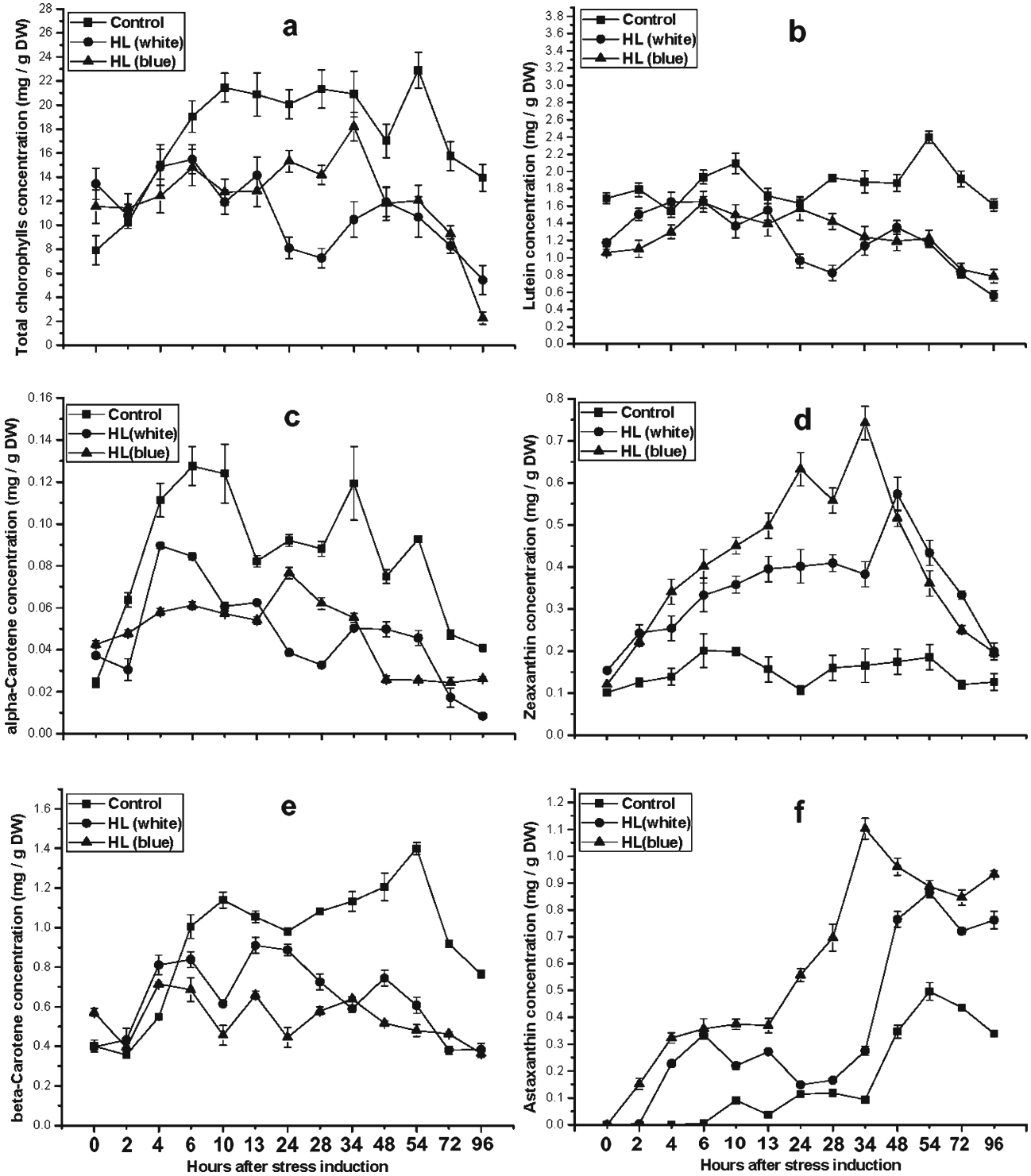


Fig. 5 Changes in total chlorophylls and carotenoids concentrations of *H. pluvialis* under different high light stresses (*white or blue*). Cells grown in autotrophic medium were harvested and transferred to fresh medium with different high light intensity. Cells were harvested at different periods of induction and changes in total chlorophylls (**a**), total chlorophylls (**b**), lutein (**c**), α-carotene (**d**), β-carotene (**e**), and astaxanthin (**f**). Values are mean±SD of three independent determinations

there were also three members of CYP97 family in eukaryotic algae especially in green algae result from the close phylogenetic relationship at evolution level (Adl et al. 2005). Bacterial P450s are soluble proteins, but all plant P450s described so far are bound to membranes, usually anchored on the cytoplasmic surface of the endoplasmic

reticulum through a short hydrophobic segment of their N-terminus, and possibly a hydrophobic loop of the protein (Williams et al. 2000). However, analysis of a few *Arabidopsis* and other plant P450 sequences, predicts potential signal peptides that should target some of them to the plastids or to the mitochondria (Schuler et al. 2006). The isolated *Haecyp97c* was predicted to be a nonmembrane and chloroplast-targeted protein. This result is consistent with previous studies from higher plants, in which three CYP97 family members (CYP97A3, CYP97B3, and CYP97C1) were predicted in *Arabidopsis* genome (Tian et al. 2004), and CYP97A3 and CYP97C1 are predicted to be chloroplast-targeted (Schuler and Werck–Reichhart 2003; Tian and DellaPenna 2004). This predicted that chloroplast localization of CYP97A/C from algae and higher plants is coincident with the subcellular localization of carotenoid biosynthesis.

P450s are hemethiolate enzymes involved in numerous biosynthetic and xenobiotic pathways found in all organisms from bacteria to humans (Nelson 2009) and all share a common catalytic center, heme with iron coordinated to the thiolate of a conserved cysteine (Bak et al. 2011). The catalytic motifs of the P450s were predicted in the primary amino acid sequence of HaeCYP97C and indicated that it is a member of P450s. In addition, there are some other conserved domains in the deduced proteins sequences of genes encoding homologues of CYP97C from ten green algae. It is likely that these conserved domains may be responsible for the specificity with respect to the β- or ε-ring of the different carotenes.

The CYP97 family is a carotenoid-metabolizing family (Nelson and Werck-Reichhart 2011). CYP97s are among the most ancient gene and plant-specific family consists of three distinct subfamilies (CYP97A/B/C) that emerged before the higher plant/green algae split (Bak et al. 2011). Our results from the phylogenetic analysis of CYP97 between green algae and higher plants support this previous conclusion. All three subfamilies in CYP97 are represented in *Arabidopsis* and all other land plants, usually in single copy in each subfamily, which indicates critical functions (Kim et al. 2009). On the contrary, there are some paralog genes encoding CYP97A/B in some green algae, which indicated recent gene duplication may have occurred. Among the three subfamilies, CYP97B represents the oldest of these three as it shares the highest phylogenetic identity with the CYP97E and CYP97F sequences of diatoms and other protists (Nelson et al. 2008). Our results show that genes encoding CYP97A and CYP97C are evolutionarily more closely than those genes encoding CYP97B.

High light is considered an effective stimulus to induce accumulation of astaxanthin in the economic green alga *H. pluvialis* (Li et al. 2010; Zhekisheva et al. 2005). Quantitative RT-PCR analysis clarified that the transcripts of *Haecyp97c* decreased at the early stage and increased markedly at the following stage under both blue and white HL stress conditions. In higher plants, mutant studies have suggested that the CYP97A3 and CYP97C1 enzymes are primarily responsible for catalyzing hydroxylation of the β- and ε-rings of α-carotene to produce lutein, which is most abundant in the light-harvesting complexes where they are key structural and functional components for light harvesting (Dall'Osto et al. 2007, 2006; Kim and DellaPenna 2006; Tian et al. 2003, 2004). During early stages (0–6 h) of HL irradiation, lutein concentrations were lower under both treatments than under the control (Fig. 5b), which is consistent with the decreased transcriptional levels of *Haecyp97c*. However, along with the extension of induction, the concentrations of lutein under treatments were decreased and were still slightly less than that of the control though the transcriptional level of *Haecyp97c* was increased (Figs. 4b and 5b), indicating that the HaeCYP97C enzyme may play additional role (besides the role in hydroxylation of ε-ring of α-carotene) under HL stress. The similar results were reported in previous studies, in which the lutein concentrations in cells exposed to 460 μmol or 920 μmol photons m^{-2} s^{-1} was less than half that of cells maintained at 90 μmol photons m^{-2} s^{-1} in green alga *Chlorella zofingiensis* (Del Campo et al. 2004). The zeaxanthin concentration rapidly increased (Fig. 5d) under both HL stress conditions. Two reasons may be responsible for this result: first, the zeaxanthin may be produced from β-carotene by BCH enzymes; secondly, zeaxanthin may be also derived from the conversion of violaxanthin by the xanthophyll cycles under high light (Li et al. 2009). The apparent conflicting results between the transcriptional level of *Haecyp97c* and the lutein concentration under HL treatments indicated that HaeCYP97C may play roles in producing zeaxanthin by hydroxylation of β-carotene or perform other unknown functions under HL stress conditions. In addition, it is worth mentioning that *Haematococcus* cells respond differently to stresses, at least in different degree. For example, our results indicated that *Haematococcus* cells may react quickly and more sensitive to blue HL induction compared to white HL stress resulting in zeaxanthin and astaxanthin concentrations under the former than in the latter. This conclusion is similar to a previous study, in which red light emitting diodes (LEDs) operated at a relatively low light intensity were suitable for cell growth with replacement of medium, and LEDs emitting short wavelengths (380–470 nm) the morphological change of *H. pluvialis* and enhanced the accumulation of astaxanthin (Katsuda et al. 2006; Lababpour et al. 2004). Previous studies have demonstrated that the CHY involved in the astaxanthin biosynthetic pathway in *H. pluvialis* contains a cytochrome P450 by the use of ellipticine and that the main biosynthetic pathway is via canthaxanthin (Schoefs et al. 2001). In this study, although the *Haematococcus* cells accumulated much higher concentrations of astaxanthin under white or blue HL

stresses, it is not clear whether the HaeCYP97C is the only P450 protein playing roles in astaxanthin biosynthesis under these stress conditions. There is only very limited knowledge on the hydroxylation of rings in carotenes from eukaryotic algae. While it is difficult to completely understand the two structurally unrelated carotenoid hydroxylases (non-heme di-iron hydroxylases and cytochrome P450 monooxygenases) in algae from such a limited data set, we can propose some hypotheses based on the results presented in this study. We speculate that, besides the roles in lutein biosynthesis (hydroxylation of ε-ring of α-carotene), HaeCYP97C may play additional roles in zeaxanthin or astaxanthin biosynthesis by hydroxylation of β-rings in β-carotene, echinenone, or canthaxanthin in the chloroplast. As a consequence, HaeCYP97C may be a candidate gene that can be further utilized in genetic engineering to produce astaxanthin from β-carotene. Isolation, functional investigation and expression profiles of other two members of CYP97 (CYP97A/B) family from *H. pluvialis* and other distinct eukaryotic algae are a topic of ongoing research.

In conclusion, we isolated a novel carotenoid ε-ring hydroxylase (*Haecyp97c*) gene involved in xanthophyll biosynthesis in the green alga *H. pluvialis*. *Haecyp97c* is a nuclear gene encoding chloroplast-targeted protein. Conserved domain analyses revealed that HaeCYP97C is a member of P450s. qRT-PCR analysis revealed that *Haecyp97c* was involved in different stress responses in a different manner. Although the changing profiles of *Haecyp97c* mRNA levels are not completely coincident with the change of α-branch xanthophylls (lutein), we believe that the response process of *H. pluvialis* to HL stresses is a network where HaeCYP97C involved. It may play some critical roles (i.e., an additional role in hydroxylation of β-rings in β-carotene, echinenone, or canthaxanthin) in the acclimation to dramatic changes of light intensity by regulating xanthophyll biosynthesis or perform other unknown functions under different stress conditions. Stresses alter gene expression positively or negatively in a regulatory network with synergistic and antagonistic effects. Further research should consider the non-heme diiron (BCH) and other two members of CYP97 family from gene cloning, functional investigation, and expression profiles, which is necessary to further elucidate the detailed biosynthetic mechanisms of xanthophylls under different stress conditions.

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