Cloning, identification and functional characterization of two cytochrome P450 carotenoids hydroxylases from the diatom Phaeodactylium tricornutum

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The diatom microalgal Phaeodactylium tricornutum accumulates a large amount of fucoxanthin. Carotenoids hydroxylases (CHYs) play key roles in fucoxanthin biosynthesis in diatoms. However, not any type of CHYs had been identified in P. tricornutum. In this study, two genes (designated Ptcyp97b1 and Ptcyp97b2) were cloned, identified and functionally characterized. They shared high sequence identity (50–94 %) with lutein deficient 1-like proteins from other eukaryotes. The typical catalytic active motifs of cytochrome P450s (CYP) were detected in the amino acid sequences of Ptcyp97b1 and Ptcyp97b2. The two genes were probably due to gene duplication. Ptcyp97b1 and Ptcyp97b2 transcriptional expression was up-regulated with distinct patterns under high light conditions. The metabolic profiles of the major carotenoids (β-carotene, zeaxanthin, diadinoxanthin, diatoxanthin and fucoxanthin) were determined based on the high performance liquid chromatography method. The fucoxanthin and diatoxanthin contents were increased, while the β-carotene content was decreased. By truncation of the N-terminal trans-membrane anchor or chloroplast transit peptide and addition of a 6×His-tag, Ptcyp97b1 and Ptcyp97b2 were separately heterologously expressed in E. coli BL21(DE3) cells. Ptcyp97b1 might have the ability to catalyze the hydroxylation of other substrates other than β-carotene. These results contribute to the further elucidation of xanthophyll biosynthesis in diatoms.

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Key words: Cytochrome P450; Carotenoids hydroxylase; Phaeodactylium tricornutum; Cloning; Identification; Functional characterization

Microalgae are important potential sources of diverse carotenoids which are divided into two major groups that include carotenoids (Cars) and xanthophylls (Xans). Cars are enriched in the center of the photosystem reaction. Xans are oxygenated Cars that serve various functions in photosynthetic organisms and are essential for the survival of the organism (1–3). Fucoxanthin (Fcx), diadinoxanthin (Ddx) and diatoxanthin (Dtx) are the most abundant and special Xans (contributing more than 10 %) in the marine environment and reveal remarkable biological properties with many applications (4). For instance, Fcx is a significant component of Fcx-chlorophyll-protein-complexes (FCPs) that are functionally related to the light-harvesting-complexes (LHCs) of green microalgae and higher plants. Fcx acts as an antenna and transmits excitation energy to chlorophyll a/c. The light induced conversion of Dtx and Ddx protects the organisms from photodamage (5). Fcx excitation energy to chlorophyll a/c. The light induced conversion of Dtx and Ddx protects the organisms from photodamage (5).

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important enzyme for carotene hydroxylation (21). In microalgae, it has been implied that CYP97B (PuCHY1) from red algae (Porphyra) might be able to hydroxylate the β-rings of β-carotene to produce zeaxanthin (22).

At present, three hypothetical pathways for Fcx biosynthesis in diatoms have been proposed and discussed (Fig. S1). First, the pathway is summarized as follows, β-carotene (Zea) → Axr → Vlx → Dcx or Dtx. Second, the pathway is summarized as follows, β-carotene (Zea) → Axr → Vlx. Finally, Vlx is synthesized from β-carotene through intermediate metabolites that include in turn, zeaxanthin (Zea), antheraxanthin (Atx) and violaxanthin (Vlx). Then neoaxanthin, as the branch point, is converted to Fcx or Ddx (3). The intermediate metabolites that include in turn, zeaxanthin (Zea), β-cryptoxanthin-5,6-epoxide and β-carotene hydroxylation (21). In microalgae, it has been implied that CYP97B (PuCHY1) from red algae (Porphyra) is an important enzyme for carotene hydroxylation (21).

To identify and clone the putative PtrCYP97B1 and PtrCYP97B2 cDNAs, the full-length cDNAs of PtrCYP97B1 and PtrCYP97B2 were amplified by the RACEs method according to the manual of the SMARTMRT RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). The gene-specific primers (Table S1: PtrCYP97B1-1-F/PtrCYP97B1-1-R and PtrCYP97B2-2-F/PtrCYP97B2-2-R) were designed from the amplified full-length cDNAs sequences of PtrCYP97B1 and PtrCYP97B2. Nested PCR were carried out using the nested universal primers and gene-specific primers. The ORFs of PtrCYP97B1 and PtrCYP97B2 were cloned by the primers (Table S1: PtrCYP97B1-1-C/PtrCYP97B1-1-C’; and PtrCYP97B2-2-C/PtrCYP97B2-2-C’). The obtained nucleotide sequences corresponding to the corresponding reading frames were submitted to the NCBI database with the accession numbers MK595592 and MK595953.

Bioinformatics analysis The molecular weight (Mw), isoelectric point (pI), sub-cellular localization, signal peptides, trans-membrane regions, secondary and tertiary structures of PtrCYP97B1 and PtrCYP97B2 were computed and predicted by ExPaSy (https://www.expasy.org/). These proteins and others were aligned using ClustalX (25). Maximum likelihood trees (Le and Gascuel evolutionary model) of some CYP97 proteins were constructed using PhyML (26, 27). Bootstrap (BS) values were inferred from 400 replicates.

Transcriptional analysis under HL conditions qRT-PCR was performed on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster, CA, USA). qRT-PCR primers (Table S1: PtrCYP97B1-1-Q/PtrCYP97B1-1-R and PtrCYP97B2-2-Q/PtrCYP97B2-2-R) were used. The gene histone primers (Table S1: Prhistone-Q/R) were selected from the amplified cDNA sequences of Prhistone-Ptrcyp97b1 and Prhistone-Ptrcyp97b2 and were selected as an internal control (23). The amplifications were carried out in triplicate in a total volume of 20 μL according to the manufacturer’s instructions of SYBR Premix Ex Taq Kit (Takara). The 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 15 °C for 15 s. DEFPC-treated water for the replacement of template was used as negative control. The comparative CT method (2-ΔΔCt) was used to investigate the transcription expression of genes (28). The specific gene primers are listed in Supplementary Table S1 and were designed with Primer 5.0 software.

Carotenoids analysis under HL conditions For cell dry biomass determination, dry cells were collected by centrifugation at 13,100 × g for 4 min and the collection of cells washed three times. The EP tubes containing cells were dried in a DW3 freeze-drier (Heto Dry Winner, Thermo Fisher Scientific, Shanghai, China). For carotenoids analysis, the high performance liquid chromatography (HPLC) method was applied to quantify the contents of all the carotenoids (16). Briefly, the freeze-dried dry cells of P. tricornutum were homogenized in 35 cm3 of nitrogen and extracted with acetone until the cells became colorless. After centrifugation at 13,100 × g for 15 min, the supernatant was collected and evaporated under nitrogen gas. Finally, the residue was re-dissolved in 1 mL acetone and filtered through a 0.22 mm Millipore organic membrane filter (Millipore Co., Burlington, MA, USA) prior to HPLC analysis. Carotenoids were eluted at a flow rate of 12 ml min-1 with a linear gradient from 100 % solvent A [acetonitrile/methanol (0.1 M Tris–HCl (pH 8.0)] to 100 % solvent B [methanol/ethyl acetate (68:32)] for 15 min, followed by 12 min of solvent B.

MATERIALS AND METHODS

Algal strain and culture conditions P. tricornutum strain CCMP 1055 was maintained in our laboratory and was cultivated in 100 mL flasks in liquid medium and placed in an incubating incubator under light intensity of 75 μmol photons m-2 s-1, with a photoperiod of 12 h/12 h (light/dark) at temperature of 25 ± 1 °C. For the HL conditions, the cultures were dark-adapted for 48 h, the later exponentially growing cultures (2 L and cell density of approximately 5 × 106 cells mL−1) were further transferred into continuous white light (AMDOB-90-770, TITUEIT, Beijing, China: 390–770 nm) or blue light (AMDOB-5710-20-420-500, TITUEIT: 420–500 nm) with a light intensity of 500 μmol photons m−2 s−1 without a light/dark cycle. The cultures under white light intensity of 75 μmol photons m−2 s−1 without a light/dark cycle were used as the control Samples were collected at the end of the dark period and after the onset of light by centrifugation and the cells were washed with PBS prior to storage in liquid nitrogen.

Molecular cloning of PtrCYP97B1 and PtrCYP97B2 cDNAs Total RNA was extracted from P. tricornutum CCMP 1055 cells using the TRIzol reagent (Takara, Dalian, China) according to the user’s manual. In this protocol, RNA was treated with DNase I quantified by Nanopore 2000x (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNAs were synthesized from 2 μg of total RNA with RT Enzyme Mix I (Takara) according to the manufacturer’s instructions. Two pairs of degenerate primers (Table S1: PtrCYP97B1-1-F/PtrCYP97B1-1-R and PtrCYP97B2-2-F/PtrCYP97B2-2-R) for homologous cloning were designed by CODEHOP (http://dyndna.biols.uic.edu/), and PCR were conducted. Primers were designed to have highly conserved regions of putative genes encoding CYP97B and LUT-like proteins predicted from higher plants and microalgae (Table S2). First-strand cDNAs were used as a template. PCR amplification was conducted with LATaq Kit (Takara) according to the manufacturer’s instructions, and the PCR was processed with the following parameters: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58 °C (according to the Tm values of primers) for 30 s, and 72 °C for 1 min (according to the length of products at 1000 bp min−1), with a final extension at 72 °C for 7 min and cooling to 4 °C. The fragments of interest were purified and cloned into the pMD-18T vector (Takara) and sequenced (Invitrogen, Shanghai, China). The full-length cDNAs of PtrCYP97B1 and PtrCYP97B2 were annotated with the NCBI database with the accession numbers MK595592 and MK595953.

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Cloning and identification of two genes encoding PtrCYP97Bs

Detailed information about the cDNA sequences of PtrCyp97b1 (MK955592) and PtrCyp97b2 (MK955593) is summarized in Table S3. The PtrCyp97b1 cDNA sequence was 2605 base pairs (bp) in length, which contained a 2268 bp open reading frame (ORF), a 220 bp 5′-untranslated region (UTR), and a 117 bp 3′-UTR with the characteristic of the poly(A) tail. The deduced protein had a calculated molecular weight of 85.26 kDa with an estimated isoelectric point of 5.25. The cloned PtrCyp97b2 cDNA was 2194 bp in length and contained an ORF of 1875-bp with a 5′-UTR of 217-bp and 3′-UTR of 102-bp and encoded a protein of 624 amino acids. The deduced protein had a calculated molecular weight of 70.48 kDa with an estimated isoelectric point (pI) of 5.23.

A BLASTp search revealed that PtrCyp97b1 and PtrCyp97b2 shared high sequences similarity (49–94 %) with some predicted lutein-deficient 1-like or CYP97B proteins from diatom, green microalgae and higher plants (Table S4). Analyses of the trans-membrane helices, chloroplast transit peptides and signal peptides suggested that PtrCyp97b1 might be a nuclear gene encoded and cytoplasm-targeted enzyme (Fig. S2 and Table S3). On the contrary, chloroplast transit peptides, but not trans-membrane helices were predicted in the amino acid sequence of PtrCyp97b2 (Fig. S3 and Table S3), which implied a soluble enzyme localized in the chloroplast. The distinct subcellular localization for different PtrCyp97Bs is consistent with the localization of PtrlUTL-like1 and PtrlUTL-like2 predicted from the genome sequences of *P. tricornutum* CCAP1055/1 (23).

The PtrCyp97Bs and other CYP97B or LUT-like proteins were aligned using the ClustalX program. The results revealed that eight conserved domains (CDs) were discovered in the amino acid sequences of PtrCyp97b1 and PtrCyp97b2 (Fig. 1), which is consistent with previous study (2,23). Briefly, the CYP450s active site components, e.g., CD4 (1-helix) involved in oxygen binding, CD5 (ERR triad) involved in locking the heme and assuring stabilization of the conserved core structure, and CD7 involved in heme binding were found. In addition, there were some CYP97B subfamily-specific CDs including YC/DKM/VAILE, FSCV/IT-X-ESPI/VKAVY-X4-EAHE5, DSLR/FLFLD/GMRE/AD and LYS/NE-X3-DAF.

The phylogenetic analysis of the CYP97s from eukaryotic microalgae and higher plants is illustrated in Fig. 2, which was also consistent with previous results (2). Briefly, the cloned PtrCyp97b1 and PtrCyp97b2 formed separate cluster (BS: 100/100) among those of other predicted LUT-like proteins from Bacillariophyta. The CYP97Bs and LUT-like proteins from Bacillariophyta constituted a monophyletic group (BS: 97), which further formed another monophyletic group (BS: 72) with CYP97Bs from green microalgae and higher plants as a sister group. Interestingly, gene encoding CYP97b from each organism of green microalgae and higher plants was single copy while genes encoding CYP97Bs or LUT-like proteins were double copies in each organism from Bacillariophyta. In particular, lineage-specific gene duplication event might occur during the evolution of CYP97Bs or LUT-like proteins in Bacillariophyta.

Transcriptional expression of PtrCyp97Bs under HL conditions

Time-course transcriptional patterns of PtrCyp97b1 and PtrCyp97b2 in photoautotrophic *P. tricornutum* CY-H9 under different HL conditions were determined (Fig. 3). The transcriptional level of PtrCyp97b1 increased at 12–60 h exposure under blue HL and reached the maximum was measured at 36 h exposure, which was 5.84-fold higher than that of the control. A steady and strong increase of PtrCyp97b1 transcript level (2.48-fold higher than that of the control) at 24–60 h exposure was found under white HL condition (Fig. 3A). The transcript level of PtrlCyp97b1 gene in both blue and white HL was similar, but the kinetics of increase were faster in blue HL than in white HL (Fig. 3A). We found a transitory and strong increase of PtrCyp97b2 transcript levels after 12–24 h of blue and white HL, and after this period the levels decreased again (Fig. 3B). As shown in Fig. 3B, the highest steady state transcript levels were measured after 12 h and reached approximately 5.02-fold and 4.75-fold higher under blue and white HL respectively than that of the control. Then it decreased sharply and remained relatively constant when exposure duration 36–72 h under both blue and white HL conditions. The corresponding patterns of PtrCyp97b2 expression under blue and white HL conditions were essentially identical. The effect of blue HL on PtrlCyp97b2 transcript level was stronger than white HL. Blue HL appeared to have a stronger effect on both PtrlCyp97b2 and PtrlCyp97b2 transcription than white HL, even though the response patterns were distinct.

Carotenoids accumulation of Xans under HL conditions

The biomass yield and accumulation of β-Car, Zea, Ddx, Dtx and Fcx were examined over the period of induction (Fig. 4). The results implied that white HL condition slightly promoted growth during the (0–72 h) exposure, while blue HL condition mildly inhibited growth (Fig. 4A). In summary, different HL conditions had no obvious effect on growth and biomass yield. The later exponentially growing cultures were used for different HL conditions, which was responsible for the above phenomenon. Upon blue and white HL conditions, in the early stress induction period (0–12 h), the content of β-Car decreased sharply and remained stable through the 12–72 h exposure, had a lower value than that of measured at control. The final β-Car content under blue HL was less than it was under white HL condition (Fig. 4B). It was interesting to mention that the Zea content increased rapidly and reached its maximum at 48 h and 36 h exposure under blue and

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FIG. 1. Multiple alignments of CYP97 A/B/Cs. All the sequences information is summarized in Table S2. There are common eight conserved domains (black boxes: CD1-CD8) in the protein sequences of CYP97 A/B/Cs. The three typical conserved domains of all cytochrome P450s were discovered, including the oxygen binding site signature (CD4, LI/VAGHETT), the ERR triad involved in locking the heme (CD5, EXXRLYPXPPV/LLI/LRR), and the heme binding signature sequence (CD7, PFG/SGGPRKCXGDXFA). The CYP98B-specific CDs are indicated by black asterisks.

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white HL conditions respectively. Then, it decreased sharply along the extension of induction (48—72 h), with the lower content value than that of measured at control (Fig. 4C). However, the content of Fcx under treatments increased markedly and reached the max values at 72 h exposure, which was higher than that of measured at control (Fig. 4D). The content of Ddx under treatment was relatively stable (Fig. 4E). In addition, the content of Dtx under both blue and white HL conditions increased slowly and reached its maximum at 72 h exposure (Fig. 4F). These above results suggested that compared with white HL, blue HL was more effective in inducing the synthesis of carotenoids in *P. tricornutum* GY-H9. These results also indicated that the coordinated up-regulation of *Ptrcyp97b1* and *Ptrcyp97b2* genes might give rise to a decrease in the content of the substrate (**b**-Car) and an increase in the contents of the major products (Fcx and Dtx) generated through the intermediate metabolite (Zea).

**Heterologous expression of PtrCYP97Bs in *E. coli* BL21(DE3) cells** To further characterize the catalytic properties ofPtrCYP97Bs, the genes were separately heterologously expressed in *E. coli* BL21(DE3) and purified by Ni-NTA affinity chromatography. To improve the solubility of the recombinant proteins, *PtrCYP97B1* and *PtrCYP97B2* were truncated to exclude the N-terminal amino acid residues including the predicted transmembrane regions or the chloroplast transit peptides. SDS-PAGE analysis showed that *PtrCYP97B1* and *PtrCYP97B2* were expressed in a soluble form after induction with IPTG (Fig. 5). The recombinant *PtrCYP97B1* and *PtrCYP97B2* proteins had approximate sizes of 79.64 and 68.28 kDa, respectively. These results suggested that two *PtrCYP97Bs* enzymes were successful expressed in *E. coli* BL21(DE3).

**Functional characterization of PtrCYP97Bs in β-Car-accumulating *E. coli* BL21(DE3) cells** In the present study, we used the β-Car-accumulating *E. coli* BL21(DE3) cells to investigate the functions of the two carotene hydroxylase genes (*Ptrcyp97b1* and *Ptrcyp97b2*) of *P. tricornutum* GY-H9. The results showed that only *PtrCYP97B2* catalyzed the hydroxylation of the **b**-rings of β-Car to produce Zea in β-Car-accumulating *E. coli* BL21(DE3) cells, which supported the finding that *PtrCYP97B2* might be a key gene for **b**-Xans accumulation in *P. tricornutum* GY-H9 (Fig. 6A—D,F). It was surprising that only Zea but not β-Cpx was detected. Usually, the
be responsible for the hydroxylation of Cars in diatoms (23). In addition, two putative genes encoding CYP97B (LUT-like1 and LUT-like2) have been predicted in *P. tricornutum* CCAP1055/1 (2, 23). Our previous study indicated that cyp97b paralogs were present in *P. tricornutum* CCAP1055/1 (2). It is reasonable to speculate that CYP97B may be the possible CHYs in the diatom. To our knowledge, it is the first time that CHYs genes have been isolated and characterized from *P. tricornutum* CY-H9. PtrCYP97B1 and PtrCYP97B2 shared the highest sequence similarity (94/93 %) with the predicted lutein deficient 1-like proteins (LUT-like1 and LUT-like2) in *P. tricornutum* CCAP1055/1 (Table S4). After careful comparative analysis, we think that there are only two genes encoding LUT-like1 and LUT-like2 in *P. tricornutum* CCAP1055/1, which are the two genes most similar to genes of this kind in *P. tricornutum* CY-H9. The isolated PtrCYP97B2 was a chloroplast-targeted protein (Fig. S3), which is consistent with the sub-cellular localization of CYP97A and CYP97C in higher plants and green microalgae and is coincide with the Xans biosynthesis location (16,17,23,30). However, PtrCYP97B1 might be a cytoplasm-targeted enzyme encoded by nuclear gene (Fig. S2). The different sub-cellular localization of PtrCYP97B1 and PtrCYP97B2 provides important clues for further functional studies.

CYP450s are defined by the 450 nm light absorption of their heme cofactor and are involved in numerous biosynthetic pathways found in almost all organisms (33). All CYP450s share a common catalytic center, i.e., heme with iron coordinated with the thiolate of a conserved cysteine (34). The CYP450 catalytic motifs were discovered in the protein sequences of PtrCYP97B1 and PtrCYP97B2, indicating that they are two members of the CYP450s family (Fig. 1). In addition, there are some other CYP97B subfamily specific conserved domains (Fig. 1). It is likely that these subfamily specific conserved domains are responsible for the substrate specificity with respect to the β- or ε-ring of the different carotenoids (2). At present, gene encoding CYP97 A/B/C subfamily protein from *Arabidopsis* and other land plants is single copy in each organism, which indicating critical function (15). Interesting, in the diatom, there are two genes encoding CYP97B building two monophyletic groups. This finding indicates that lineage-specific gene duplication phenomenon may occur before the formation of various algal strains from the diatom (Fig. 2). The Duplication and subsequent functional divergence of genes have been recognized increasingly as an important mechanism of evolution (2,23).

Light is considered an effective stimulus for inducing carotenoids biosynthesis related genes expression and accumulation in *P. tricornutum* (23,36–38). Our results suggested that *Ptrcyp97b1* and *Ptrcyp97b2* transcription was mainly influenced by different HL conditions. As shown in Fig. 3, the response of both *Ptrcyp97b1* and *Ptrcyp97b2* genes began 12 h later under HL conditions in this study, which is not consistent with the short respond time (2–6 h) of *psy*, *pds*, *zep1*, *zep2*, *zep3*, *vde*, *vdfl1* and *vdfl2* genes under white or blue HL conditions (23). One possible reason for above phenomenon is the missing sampling time points from 0 to 12 h. In addition, continuous respond over time (12–60 h) is the characteristic for *Ptrcyp97b1*. However, transitory respond short time (12–24 h) is the prototypical properties for *Ptrcyp97b2* which is similar with other CHYs (Haecyp97a, Haecyp97b, Haecyp97c and Ckecyp97a) in *Haematococcus pluvialis* and *Chlorella kessleri* under both white and blue HL conditions (2,16,17). The distinct kinetics of increase for *Ptrcyp97b1* and *Ptrcyp97b2* under different HL conditions implied that they have various function. Moreover, our results indicated that blue HL is more effective for inducing the expression of *Ptrcyp97b1* and *Ptrcyp97b2* than white HL stress. This phenomenon is similar to that found in our previous studies which indicated that blue HL is more effective for inducing the expression of *Haebch*, *Haecyp97a* and *Haecyp97c* and the accumulation of astaxanthin in *H. pluvialis* (2,16). Generally, blue light can be absorbed almost

**DISCUSSION**

Previous studies have demonstrated that a Xans cycle (*Zea-Atx-Vlx*), with Vlx as the putative precursor of Ddx, Dtx and Fcx biosynthesis, was present in *P. tricornutum* (19), which implies that there must be an enzyme that catalyzes the hydroxylation of β-Car. However, the homologous genes of four known types of CHYs (CrtR, BCH, CYP97A and CYP97C) were absent in *P. tricornutum* (2,23). Fortunately, studies have indicated that LUT-like P450 proteins may have evolved from CHY family (Fig. 1). In addition, there are some other CYP97B subfamily specific conserved domains (Fig. 1). It is likely that these subfamily specific conserved domains are responsible for the substrate specificity with respect to the β- or ε-ring of the different carotenoids (2). At present, gene encoding CYP97 A/B/C subfamily protein from *Arabidopsis* and other land plants is single copy in each organism, which indicating critical function (15). Interestingly, in the diatom, there are two genes encoding CYP97B building two monophyletic groups. This finding indicates that lineage-specific gene duplication phenomenon may occur before the formation of various algal strains from the diatom (Fig. 2). The Duplication and subsequent functional divergence of genes have been recognized increasingly as an important mechanism of evolution (2,23).

Light is considered an effective stimulus for inducing carotenoids biosynthesis related genes expression and accumulation in *P. tricornutum* (23,36–38). Our results suggested that *Ptrcyp97b1* and *Ptrcyp97b2* transcription was mainly influenced by different HL conditions. As shown in Fig. 3, the responses of both *Ptrcyp97b1* and *Ptrcyp97b2* genes began 12 h later under HL conditions in this study, which is not consistent with the short respond time (2–6 h) of *psy*, *pds*, *zep1*, *zep2*, *zep3*, *vde*, *vdfl1* and *vdfl2* genes under white or blue HL conditions (23). One possible reason for above phenomenon is the missing sampling time points from 0 to 12 h. In addition, continuous respond over time (12–60 h) is the characteristic for *Ptrcyp97b1*. However, transitory respond short time (12–24 h) is the prototypical properties for *Ptrcyp97b2* which is similar with other CHYs (Haecyp97a, Haecyp97b, Haecyp97c and Ckecyp97a) in *Haematococcus pluvialis* and *Chlorella kessleri* under both white and blue HL conditions (2,16,17). The distinct kinetics of increase for *Ptrcyp97b1* and *Ptrcyp97b2* under different HL conditions implied that they have various function. Moreover, our results indicated that blue HL is more effective for inducing the expression of *Ptrcyp97b1* and *Ptrcyp97b2* than white HL stress. This phenomenon is similar to that found in our previous studies which indicated that blue HL is more effective for inducing the expression of *Haebch*, *Haecyp97a* and *Haecyp97c* and the accumulation of astaxanthin in *H. pluvialis* (2,16). Generally, blue light can be absorbed almost

![FIG. 3.](image-url)
FIG. 4. The growth and contents of β-Car, Zea, Fcx Ddx and Dtx upon white and blue HL conditions. The biomass yield of *P. tricornutum* GY-H9 (A), the content of β-Car (B), the content of Zea (C), the content of Fcx (D), the content of Ddx (E) and the content of Dtx (F) were determined after 12, 24, 36, 48, 60 and 72 h. The values were normalized to the level in the normal light condition.
were indicated by red arrow and green arrow, respectively.

In our study, both blue and white HL conditions decreased the content of a HL condition than white light (39). In addition, previous studies have shown that blue light is essential for HL acclimation in the marine diatom *P. tricornutum*. Light acclimation is a complex process mediated by blue light receptor auxochrome or photoreceptor-triggered transcription factors (40). Total 7193 genes (58% of all genes) were significantly differentially regulated by plant-like cryptochrome under blue HL condition in *P. tricornutum* (41). Thus, blue light had a more up-regulating effect on gene expression (41). According to above results, we speculate that carotenoids biosynthetic genes expression for *P. tricornutum* depends on not only light intensity, but also light quality. In this present study, for HL conditions, the later exponentially growing cells were further used to investigate the effects of different HL light conditions. Under the circumstances, the nutrient limitation and cell status might play an important role besides high light stress. Previous studies have indicated that nitrogen limitation inhibits the growth and biosynthesis of Fcx in *P. tricornutum* (38). In general, cells seem to react quickly to the increase in light intensity. For instance, the contents of Ddx and Dtx increased dramatically after 6–24 h and increased steadily during 24–72 h and reached its maximum (3.4-fold) on the 72 h under sine HL (500 μmol photons m<sup>-2</sup> s<sup>-1</sup>) condition in four strains of *P. tricornutum* (CCAP1055/1, Pt1sil, Pt4 and Pt4ov) (45). However, under fluctuating HL (500 or 1000 μmol photons m<sup>-2</sup> s<sup>-1</sup>) condition, the increase of Ddx and Dtx pigments was only 1.7-fold higher than the control at the last time 72 h and a trend of slow increase was observed (45). In our results, although the maximal contents of Dtx and Fcx were reached at last time (72 h), a trend of slow increase was also observed in the early stress induction (0–36 h), which was similar with the previous results from *P. tricornutum* under fluctuating HL condition. In addition, the later exponentially growing cells were used to investigate the effects of different HL conditions in this study. Under the circumstances, the nutrient limitation and cell status might play an important role besides HL stress. Therefore, the physiological state of cells (i.e., cells reach stationary phase) and the pattern of HL (light quality, light intensity, light model and photoperiod) may be responsible for this phenomenon. According to the results from transcriptional expression of *Ptrcyp97bs* genes and carotenoids accumulation of Xans under HL conditions, it is reasonable to speculate that the *PtrCYP97B1* and *PtrCYP97B2* enzymes may co-involve in Xans biosynthesis by hydroxylation of β-Car into intermediate metabolite Zea, which testified the previous predicted biosynthetic pathway (3,23).

Usually, *E. coli* cells accumulating different carotenoids have been identified to be an efficient platform for investigating the functions of algal carotenoid metabolic genes (46–49). In the
FIG. 6. HPLC analysis of carotenoids in β-carotene-accumulating E. coli BL21(DE3) cells. The Zea standard (A), the β-Cpx standard (B), the Zea and β-Cpx standards (C), the E. coli BL21(DE3) cells harboring pACCAR16ΔcrtX (D), the E. coli BL21(DE3) cells harboring pACCAR16ΔcrtX and pET-28a (+)-Ptrcyp97b1 (E), the E. coli BL21(DE3) cells harboring pACCAR16ΔcrtX and pET-28a (+)-Ptrcyp97b2 (F).
The functional analysis results showed that PtrCYP97B2 could catalyze the hydroxylation of the β-rings of β-Car to produce Zea in β-Car-accumulating E. coli BL21(DE3) cells (Fig. 6). It is surprising that only Zea but not β-Cpx was detected, this finding might be interpreted to mean that all the β-Cpx was transformed into Zea by the time of the E. coli BL21(DE3) cells were analyzed. Moreover, the carotene hydroxylation activity of PtrCYP97B1 was not detected in β-Car-accumulating E. coli BL21(DE3) cells (Fig. 6), which indicated that PtrCYP97B1 might be able to catalyze the hydroxylation of other substrates except for β-Car. The E. coli BL21(DE3) cells that accumulate other carotenoids (e.g., α-Car) should be used for further investigation into the function of PtrCYP97B1. The functions of CYP97 A/B/C members are various and species-specific. In higher plants (Arabidopsis and rice), it was reported that CYP97A could hydroxylase β-rings of β-Car to produce β-Cpx and Zea in turn. Meanwhile, it is mainly responsible for the hydroxylation of β-ring of α-Car to produce zeinoxanthin, and then zeinoxanthin was further hydroxylated by CYP97C to produce lutein (31). However, in Satsuma mandarin, CitBCH and CitCYP97C were participated in the biosynthesis of lutein (47). The carotene hydroxylation activities of CitCYP97A and CitCYP97B were not detected in β-Car-accumulating E. coli BL21(DE3) cells (48). To date, few CYP97B genes have been functionally characterized. Fortunately, the hydroxylation of the β-rings of β-Car to produce Zea was detected, which is consistent with the function of CYP97B (PuchY1) from red algae (Porphyra) (22). Although it is difficult to completely understand the functions of the two PtrCYP97B genes in vivo and in vitro, we supply two candidate genes encoding CYP97B involved in the hydroxylation of β-Car. This study provides information on gene cloning and functional investigation of CYP97B in P. tricornutum in the future. In addition, over-expression and knock-down of the two PtrCYP97B1 and Ptrcyp97b2 genes are necessary to elaborate the true function of P. tricornutum in vivo. Although carotene hydroxylation is the crucial step in the Fcx upstream biosynthetic pathway but is not a direct step in its production (3,19,23). The downstream biosynthetic pathway of Fcx in P. tricornutum seems to be very complicated, and requires additional enzymes including PtxVE, PtxDE, PtxVLD1, PtxVLD2, PtxZEP-1, PtxZEP-2 and PtxZEP-3 (14,50,51). The further synthetic biology studies focusing on assembling these genes to create new metabolic pathways in β-Car-accumulating E. coli BL21(DE3) are expected to be powerful tools for uncovering the Fcx biosynthesis pathway.

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