Cloning and characterization of a novel β-carotene hydroxylase gene from *Lycium barbarum* and its expression in *Escherichia coli*

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Abstract

Lycium barbarum contains high levels of zeaxanthin, which is produced by the conversion of β-carotene into zeaxanthin. β-Carotene hydroxylase catalyzes this reaction. We cloned a cDNA (*chyb*) encoding β-carotene hydroxylase (Chyb) from the *L. barbarum* leaf. A 939-bp full-length cDNA sequence was determined with 3′-rapid amplification of cDNA end assay encoding a deduced Chyb protein (34.8 kDa) with a theoretical isoelectric point of 8.36. A bioinformatics analysis showed that the *L. barbarum* Chyb was located in the chloroplast. Further, to investigate the catalytic activity of the *L. barbarum* Chyb, a complementation analysis was conducted in *Escherichia coli*. The results strongly demonstrated that Chyb can catalyze β-carotene to produce zeaxanthin. Thus, this study suggests that *L. barbarum* β-carotene hydroxylase could be a means of zeaxanthin production by genetic manipulation in *E. coli*.

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Keywords: *Lycium barbarum, β-carotene hydroxylase, carotenoids, zeaxanthin*

1. Introduction

Carotenoids are naturally occurring fat-soluble and pigmented compounds because of their structure and level of desaturation. Owing to their role in photosynthesis, carotenoids are ubiquitous in photosynthetic organisms. Carotenoids also perform essential functions within nonphotosynthetic organisms—quenching reactive oxygen species and preventing DNA damage—as a precursor to vitamin A. Composed of C_5 subunits, isopentenyl diphosphate and dimethylallyl diphosphate, carotenoids have a C_{40} backbone and exhibit colors ranging from yellow to red [1–3]. Some carotenoids such as β-carotene and zeaxanthin are very important to humans. β-Carotene is a vital precursor of vitamin A, which has a therapeutic effect on vitamin A deficiency-related diseases [4]. Zeaxanthin (3R, 3R′-β, β-carotene-3, and 3′-diol) is a kind of xanthophyll (oxygenated carotenoid) found chiefly in egg yolks and dark-green leafy vegetables [5]. Many studies show that zeaxanthin displays essential roles in resistance to some diseases such as age-related macular degeneration (AMD), which is the main cause of irreversible blindness for people, although zeaxanthin is not an essential nutrient for people's health [5–10]. The critical enzymatic step for zeaxanthin biosynthesis is hydroxylation reaction catalyzed by β-carotene hydroxylase [11–14], which introduces two hydroxy groups into the β-ionone rings of β-carotene to produce zeaxanthin. The first key step for zeaxanthin biosynthesis is the cyclization of lycopene to form β-carotene (Fig. 1). The production of β-rings is catalyzed by lycopene β-cyclases. The

Abbreviations: *IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; VAD, vitamin A deficiency; AMD, age-related macular degeneration; IPTG, isopropyl-β-D-1-thiogalactopyranoside; qPCR, quantitative real-time PCR.*

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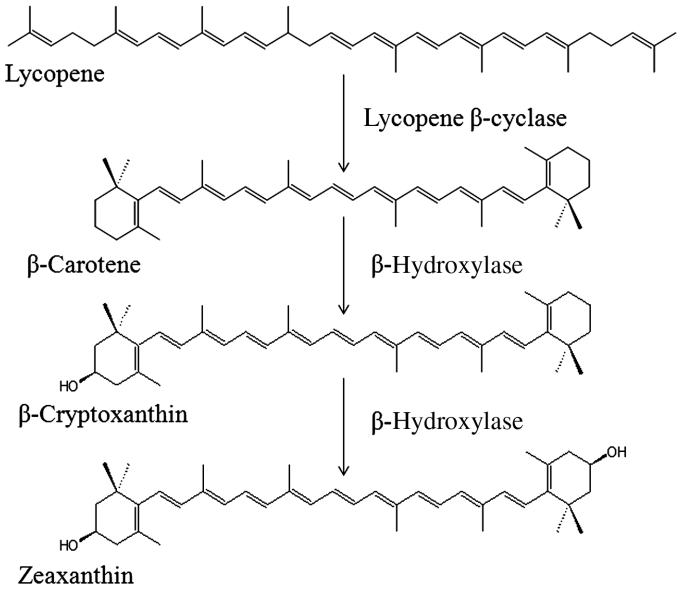

FIG. 1 *Zeaxanthin biosynthesis in higher plants. Lycium barbarum β-carotene hydroxylase is shown.*

committed enzymatic step for zeaxanthin biosynthesis is hydroxylation reactions, performed by β-carotene hydroxylase, which directly adds a hydroxyl group to the β-ionone ring [15].

All of β-carotene hydroxylases are non-heme di-iron monooxygenases, which catalyze the hydroxylation of β-carotene. Many β-carotene hydroxylase genes (*chyb*) have been cloned from eukaryotes (plants, green algae) and prokaryotes (bacteria), and characterized by the functional complementation assay in *Escherichia coli* or expression in plants [2, 16–23]. Although these β-carotene hydroxylases have low protein identity, they include highly conserved histidine regions that have been proven to be the membrane fatty acid desaturases [24]. Genetic engineering of the carotenoid biosynthetic pathway by introducing β-carotene hydroxylase into plants can enhance abiotic stress tolerance in plants [23].

Lycium barbarum L. (Family: Solanaceae) is a kind of traditional Chinese medicine with many important biological activities, such as anticancer, antiaging, antioxidation, and prevention of AMD [25–28]. Various functional chemical constituents in *L. barbarum*, such as flavonoids, polysaccharides, and carotenoids, account for these biological activities [25]. Among them, carotenoids have been proven to be abundant in *L. barbarum* fruits [29]. Moreover, some studies have shown that zeaxanthin dipalmitate, which is derived from zeaxanthin, is the main carotenoid in *L. barbarum* fruits (1143.7 μg/g) [30, 31]. However, no studies have been conducted to clone the *chyb* gene from *L. barbarum*. Accordingly, this paper focuses on the cloning and expression of *L. barbarum chyb* gene. To analyze the biological function of *L. barbarum chyb* gene, we have cloned the gene and investigated its functional properties by functional complementation assay.

2. Materials and Methods

2.1. Plant materials

L. barbarum was cultured in a greenhouse under 16 H light/8 H dark cycles at 22 °C. The plants were watered normally. Various plant tissues including roots, stems, leaves, flowers, and fruits were separated from mature plants. Then, collected plant tissues were quickly placed in liquid nitrogen followed by immediate storage at −80 °C for RNA isolation.

2.2. Isolation of total RNA and synthesis of the first-strand cDNA

Total *L. barbarum* RNA was isolated by using the RNeasy® Plant Mini Kit (QIAGEN, Shanghai, People's Republic of China) according to the manufacturer's guidelines. The total RNA was quantified by SMA1000 UV Spectrophotometer (Merinton Technology Co., Beijing, People's Republic of China), and 1 μg of total RNA was used as a template for the first-strand cDNA synthesis with 3′-Full RACE Core Set Ver.2.0 (Takara Biotechnology, Dalian, People's Republic of China) following the manufacturer's guidelines.

2.3. Transcriptome sequencing

For the purpose of cloning *chyb* gene and further studying the functional genes in *L. barbarum*, the transcriptome sequencing of *L. barbarum* was performed by Beijing Genome Institute-Shenzhen (Shenzhen, People's Republic of China) using the *L. barbarum* RNA.

2.4. Cloning, bioinformatics, and phylogenic analysis

To clone the *chyb* gene, the 5′-end primer (5′-ATGGCTGCCGGAATTTCAGGC-3′) of *chyb* gene was designed on the basis of the *L. barbarum* transcriptomic sequence, and the 3′-end primer (5′-TACCGTCGTTCCACTAGTGATTT-3′) was the 3′ RACE Outer Primer of 3′-Full RACE Core Set Ver.2.0 (Takara Biotechnology). The first-strand cDNA was synthesized from 1 μg of total RNA using 3′-Full RACE Core Set Ver.2.0 (Takara Biotechnology) according to the manufacturer's instructions. A negative control (water instead of RNA template) was used for the reverse-transcriptase PCR (RT-PCR). In this study, the PCR products were cloned into pMD18-T vector (Takara Biotechnology) and transformed in *E. coli* Top 10 (TIANGEN, Beijing, People's Republic of China) and sequenced by Beijing Genome Institute-Shenzhen.

The homology analysis of the protein encoded by *chyb* and other β-carotene hydroxylase proteins was made using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html) was employed for the analysis of the molecular weight and isoelectric point (p*I*) of the protein. Signal peptides and the conserved domains of the protein were predicted with SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [32] and Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [33], respectively. ProtScale, TMHMM server version 2.0, and WoLF PSORT were likewise employed for the analysis of

L. barbarum Chyb [34, 35]. The three-dimensional (3D) structure of *L. barbarum* Chyb was predicted by I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [36, 37]. MEGA 4 software was utilized for the phylogenic analysis based on the neighbor-joining method with 22 β-carotene hydroxylase proteins of other species (all are of the same type of non-heme di-iron monooxygenases) [38].

2.5. Construction of expression plasmids for functional assays in *E. coli*

To determine the enzymatic function of the β-carotene hydroxylase encoded by the *chyb* gene, the open reading frame (ORF) of *chyb* was amplified by PCR using the forward primer, 5′-CGCGGATCCATGGCTGCCGGAATTTCAGGC-3′, which contained *Bam*HI site (underline) and the reverse primer, 5′-CCGCTCGAGTCACCTCTGTGGCTCTG-3′, which contained *Xho*I site (underline). The PCR products were digested with *Xho*I and *Bam*HI, and then inserted into the same sites in vector pET-28a(+) (kanamycin resistant) to construct pET-28a-*Lbchyb*. Vector pACCAR16ΔcrtX (chloramphenicol resistant) constructed by Misawa et al. [39] to produce β-carotene was kindly provided by Changfu Zhu (Northeast Normal University, Jilin, People's Republic of China). Plasmids pACCAR16ΔcrtX and pET-28a-*Lbchyb* were used to cotransform into *E. coli* BL21 (DE3) (TIANGEN) for the synthesis of zeaxanthin; meanwhile, the plasmid pACCAR16ΔcrtX and an empty plasmid pET-28a(+) were cotransformed into *E. coli* BL21 (DE3) as a negative control. *E. coli* transformants were grown in Luria–Bertani (LB) medium with antibiotics (34 μg/mL chloramphenicol and 100 μg/mL kanamycin) and incubated in the dark at 37 °C. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM when the OD_{600} of the culture had reached about 0.6, and these cultures were further shaken in the dark at 28 °C for 12 H. After cultivation, the cells were harvested by centrifugation at 4,000*g* and 4 °C for 15 Min, and the pellets were either used for carotenoid extraction and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assay or stored at −80 °C.

2.6. Purification of the recombinant β-carotene hydroxylase (Chyb) and SDS-PAGE analysis

E. coli transformant containing pET-28a-*Lbchyb*, in which *chyb* gene was successfully expressed, was cultivated in LB medium in the dark at 28 °C for 12 H with 0.5 mM (final concentration) IPTG. The cells were harvested by centrifugation at 4,000*g* and 4 °C for 15 min. The total protein was extracted following the instructions of the Bacterial Protein Extraction Kit (CWBIO, Beijing, People's Republic of China), and the Chyb protein was purified with the 6× His-Tagged Protein Purification Kit (CWBIO). SDS-PAGE was used to detect the expression production of the total and purified protein. SDS-PAGE was carried out on a 12% separating gel [40, 41].

2.7. Carotenoid extraction and analysis

The β-carotene and zeaxanthin standards were obtained from Sigma−Aldrich. Carotenoid was extracted from *E. coli* pellet with methyl alcohol containing 6% KOH and incubated in a thermostat water bath for 20 Min at 60 °C. Extraction was performed using a mixture of diethyl ether and petroleum ether (1:1, v/v). When the precipitate was nearly colorless [6], the top phase was transferred into an amber bottle and dried under a N_2 gas stream (all the extraction processes were performed in darkness). The dried extract was then dissolved in acetone and separated on a standard C-18 reversed-phase column (3.9 × 300 mm^2) and a 20 × 4.6 mm^2 i.d. YMC C-30 guard (YMC Europe GmbH, Schermbeck, Germany) with a LabAlliance high-performance liquid chromatography (HPLC) system. The mobile phase consisted of an isocratic flow of acetonitrile, methanol, and 2-propanol (85:10:5, v/v/v) and the flow rate was 1 mL/Min [42]. For detecting the carotenoid, the eluate was measured at 450 nm [43].

2.8. Gene expression analysis

To detect the tissue-specific expression patterns of *chyb* in *L. barbarum* under normal conditions, semiquantitative RT-PCR and quantitative real-time PCR (qPCR) were performed. Total RNA was extracted from the roots, stems, leaves, flowers, and fruits of *L. barbarum* as described above. The RNA samples were quantified by SMA1000 UV Spectrophotometer (Merinton Technology) at 260 nm. First-strand cDNA was synthesized from 1-μg total RNA using PrimeScriptTM RT Master Mix (Takara Biotechnology). All protocols were performed in accordance with the manufacturer's instructions. The constitutively expressed *L. barbarum β-actin* gene was used as the internal control gene.

For semiquantitative RT-PCR, the primers of *L. barbarum chyb* gene (forward, 5′-TTCAGGCATTGCTACCCC-3′; reverse, 5′-CACTTCTCCACCCTCCATTT-3′) and *L. barbarum β-actin* gene (forward, 5′-GGGAATTGCTGATAGAATG-3′; reverse, 5′-AGGGAAGCCAAGATAGAG-3′) were used. The specificity of the RT-PCR product of *L. barbarum chyb* gene was verified by sequencing. The reproducibility of the semiquantitative RT-PCR was tested at least three times by independent assays. The amplification program was carried out as follows: 94 °C for 3 Min, then 26 cycles at 94 °C for 30 Sec, 55 °C for 30 Sec, 72 °C for 1 Min, and a final extension of 72 °C for 7 Min. PCR products were loaded into wells of 0.75% Tris-acetate–EDTA–agarose gels and stained with ethidium bromide.

For qPCR, the specific primers of *L. barbarum chyb* gene (forward, 5′-CTCATCCCTGGACTCTGT-3′; reverse, 5′-GCTACCCTCCGAAAATAA-3′) and *L. barbarum β-actin* gene (forward, 5′-GGGAATTGCTGATAGAATG-3′; reverse, 5′-AGGGAAGCCAAGATAGAG-3′) were used. qPCR assays were performed on Mx3000P QPCR Systems (StrataGene Mx3000; Agilent, Santa Clara, CA, USA) using SuperReal PreMix Plus (SYBR Green) (TIANGEN) following the manufacturer's guidelines. The PCR program was carried out in triplicate, under the following program for amplification: 95 °C for 3 Min, 40 cycles at 95 °C for 20 Sec, 60 °C for 15 Sec, and 72 °C for 20 Sec. The MxPro QPCR system software was adopted for the calculation of the threshold cycle values. The X-fold changes of *L. barbarum*

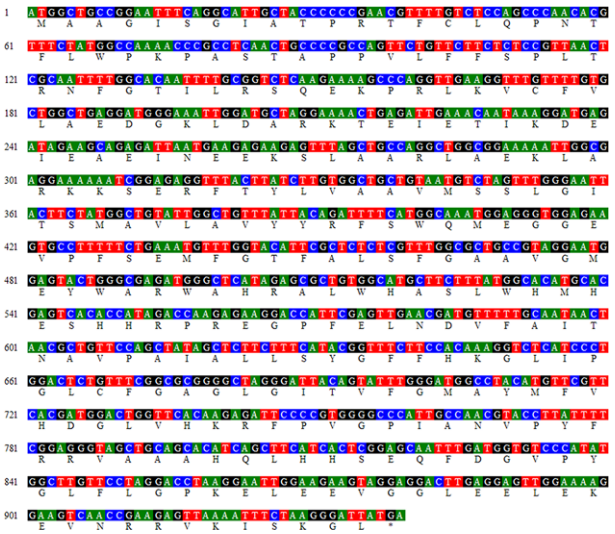

FIG. 2 *Nucleotide sequence and deduced amino acid sequence for the chyb cloned from Lycium barbarum.*

chyb gene transcripts relative to the control samples were used to calculate relative expression levels.

2.9. Nucleotide sequence accession number

These *chyb* sequence data have been submitted to the GenBank databases under accession number KF430643.

3. Results

3.1. Cloning and characterization of *chyb* cDNA

By using the *chyb* 5′-end primer, which was designed based on the *L. barbarum* transcriptomic sequence, and the 3′ RACE outer primer, an ORF of 939 bp encoding Chyb was cloned by PCR reaction (Fig. 2). The full-length cDNA of the *L. barbarum chyb* encoded a predicted protein of 312 amino acids. Alignment of the Chyb peptides from other plants showed two highly conserved peptide motifs: VGAAVGME and AHQLHHSDK (Fig. 3A). Five conserved histidine motifs, HXXXXH and HXXH, were also revealed by the multiple sequence alignment of the five β-carotene hydroxylases (Fig. 3B) [44]. These histidine motifs, which possess characteristics of membrane hydrocarbon hydroxylases, play an important role in electron transfer during the catalytic process [13, 44]. In fact, these two conserved peptide motifs and five conserved histidine motifs can be considered essential for the enzymatic activity of β-carotene hydroxylases. These motifs should be the important composition of the active center of β-carotene hydroxylases.

3.2. Characteristics of the *L. barbarum* Chyb

On the basis of ProtParam tool and Compute pI/Mw tool analyses, the 312-amino acid polypeptide encoded by a 939-bp ORF contained 34 positively charged amino acid residues and 32 negatively charged amino acid residues; the chemical formula of the *L. barbarum* Chyb was $C_{1601}H_{2452}N_{424}O_{427}S_{12}$, the instability index was computed to be 48.06 (unstable), the aliphatic index was 86.35, the predicted molecular weight was 34,856.3 Da, and the theoretical p*I* was 8.36. Prediction results of SignalP 4.0 Server displayed that the signal peptide was not contained in this Chyb; therefore, *L. barbarum* Chyb was not a secretory protein. Several hydrophilic and hydrophobic regions were observed by the ProtScale analysis tool. There were two deduced transmembrane regions predicted by the TMHMM server (version 2.0). This result suggests that the *L. barbarum* Chyb was a membrane protein. The WoLF PSORT tool predicted that the *L. barbarum* Chyb was located in the chloroplast. Accordingly, these results implied that the *L. barbarum* Chyb was a kind of chloroplast protein.

3.3. Protein structure of the *L. barbarum* Chyb

Analyzed by CDD, it was suggested that the *L. barbarum* Chyb belonged to the fatty acid hydroxylase superfamily, and consequently, this putative protein was a β-carotene hydroxylase. I-TASSER server was employed to predict the 3D structure of the *L. barbarum* Chyb (Fig. 4). The structure shown in Fig. 4 was the most probable structure predicted by I-TASSER server.

3.4. Phylogenetic analysis

Phylogenetic analysis results of 22 β-carotene hydroxylase proteins shown in Fig. 5 indicated that the *L. barbarum* Chyb had a closer relationship with the β-carotene hydroxylase of *Lycopersicon esculentum*, *Capsicum annuum*, and *Solanum tuberosum*, which are Solanaceae species. It is worth noting that the *L. barbarum* Chyb sequence has a high similarity with other species of plants, especially with Solanaceae plants, but not with Saccharomycetes. Hence, these results indicated an evolutionary link among the plants.

3.5. Expression of the *chyb* gene in *E. coli*

The ORF of *L. barbarum chyb* was cloned into pET-28a(+) to construct a recombinant vector pET-28a-*Lbchyb*. The total proteins of *E. coli* cells and the purified protein were subjected to SDS-PAGE analysis (Fig. 6).

SDS-PAGE results showed that the proteins extracted from *E. coli* BL21 (DE3) harboring pET-28a-*Lbchyb* contained a distinctive protein band with a molecular weight about 37 kDa (Fig. 6, lane 3). In view of the hexa–his fusion peptide in plasmid pET-28a(+), the molecular weight of 37 kDa was close to the expected protein molecular weight. After purification by Ni-NTA, a single protein band was identified by SDS-PAGE (Fig. 6, lane 5).

3.6. Functional complementation expression of *L. barbarum chyb* in *E. coli*

To find out whether *L. barbarum* Chyb possessed the enzymatic activity of β-carotene hydroxylase, a functional complementation assay was performed in *E. coli* (Fig. 7). *E. coli* control strain harboring empty vector pET-28a(+) and pACCAR16ΔcrtX-accumulated β-carotene (Fig. 7A), whereas *E. coli* carrying plasmid pET-28a-*Lbchyb* and pACCAR16ΔcrtX synthesized

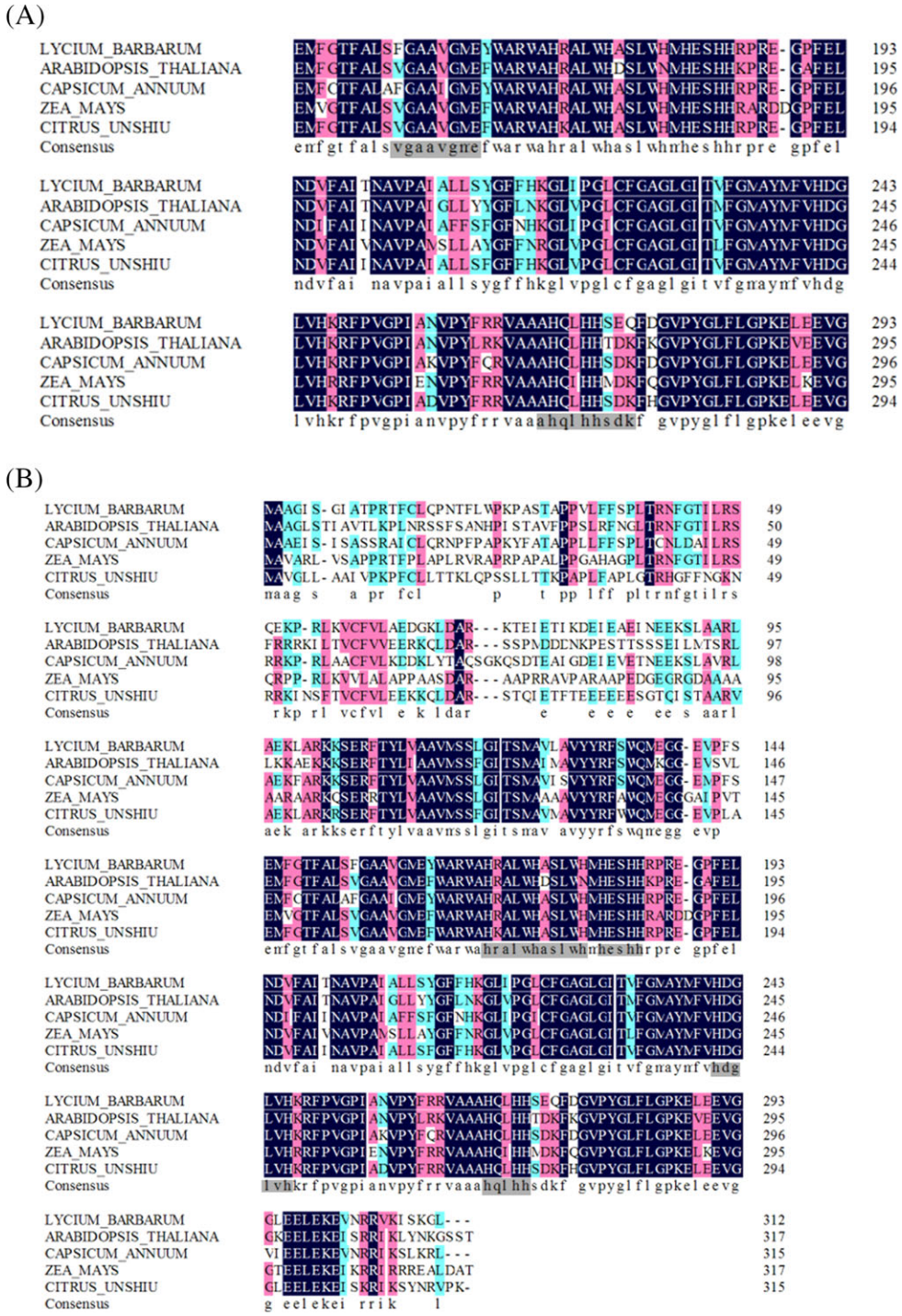

FIG. 3 *Alignment of amino acids of β-carotene hydroxylases: (A) highly conserved six-peptide sequence of the five proteins is shown by shadow; (B) alignment of the predicted peptide of Chyb to those of β-carotene hydroxylases of four other organisms. Shadings in the "Consensus" line are the motifs mentioned in the paper. GenBank accession numbers of the four β-carotene hydroxylases are as follows: Arabidopsis thaliana (AAM51300), Capsicum annuum (CAA70427), Zea mays (NP_001148085), and Citrus unshiu (AF315289_1).*

FIG. 4 *The 3D model of the Lycium barbarum Chyb predicted by I-TASSER server. In this picture, the ribbons and arrows denote the α-helix and β-sheet regions, respectively. The white regions indicate the loop regions of Chyb.*

zeaxanthin except for β-carotene (Fig. 7B). Of the total carotenoid pigments, β-carotene and zeaxanthin accounted for 45.1% (±0.5%) and 54.9% (±0.9%), respectively. The phenomenon of color complementation analysis (Fig. 7D) showed that *E. coli* containing pET-28a-*Lbchyb* and pACCAR16ΔcrtX (Fig. 7D,i) presented as slightly yellow, whereas the *E. coli*

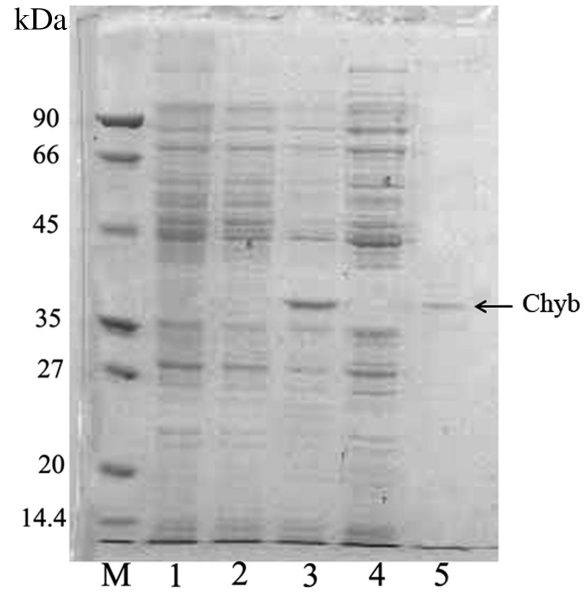

FIG. 6 *SDS-PAGE analysis of various protein samples. Lane M, standard-molecular-weight protein marker; lane 1, Escherichia coli BL21 (DE3) harboring pET-28a(+); lane 2, E. coli BL21 (DE3) harboring pET-28a-Lbchyb; lane 3, E. coli BL21 (DE3) harboring pET-28a-Lbchyb with IPTG induction; lane 4, proteins in washing buffer; lane 5, purified Lycium barbarum Chyb by Ni-NTA.*

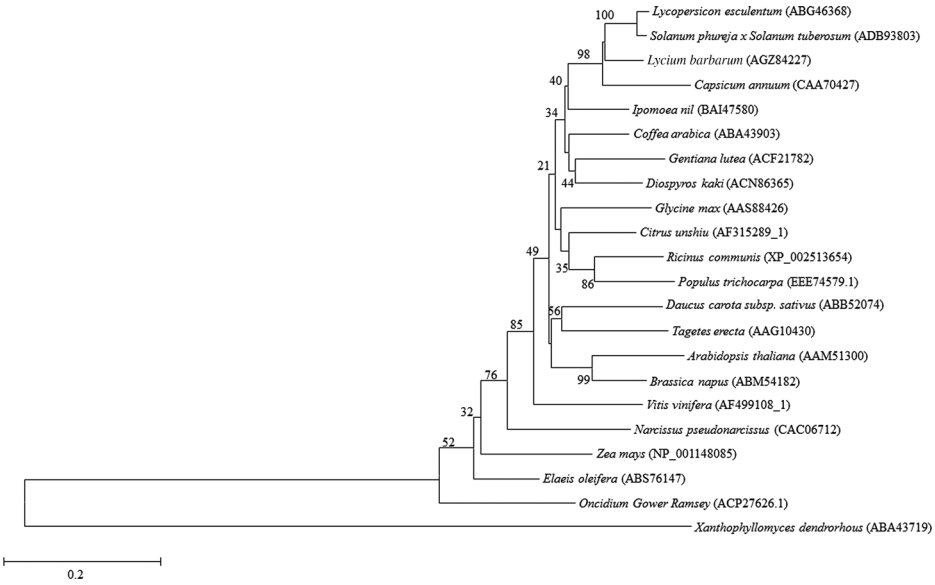

FIG. 5 *Phylogenetic tree (NJ) between the Lycium barbarum Chyb and the other organisms β-carotene hydroxylases. This phylogenetic analysis result is based on the predicted amino acid sequences of β-carotene hydroxylases from various organisms. Clustal X and MEGA4 are utilized to produce the phylogenetic tree. GenBank accession numbers of the proteins follow the name of each organism.*

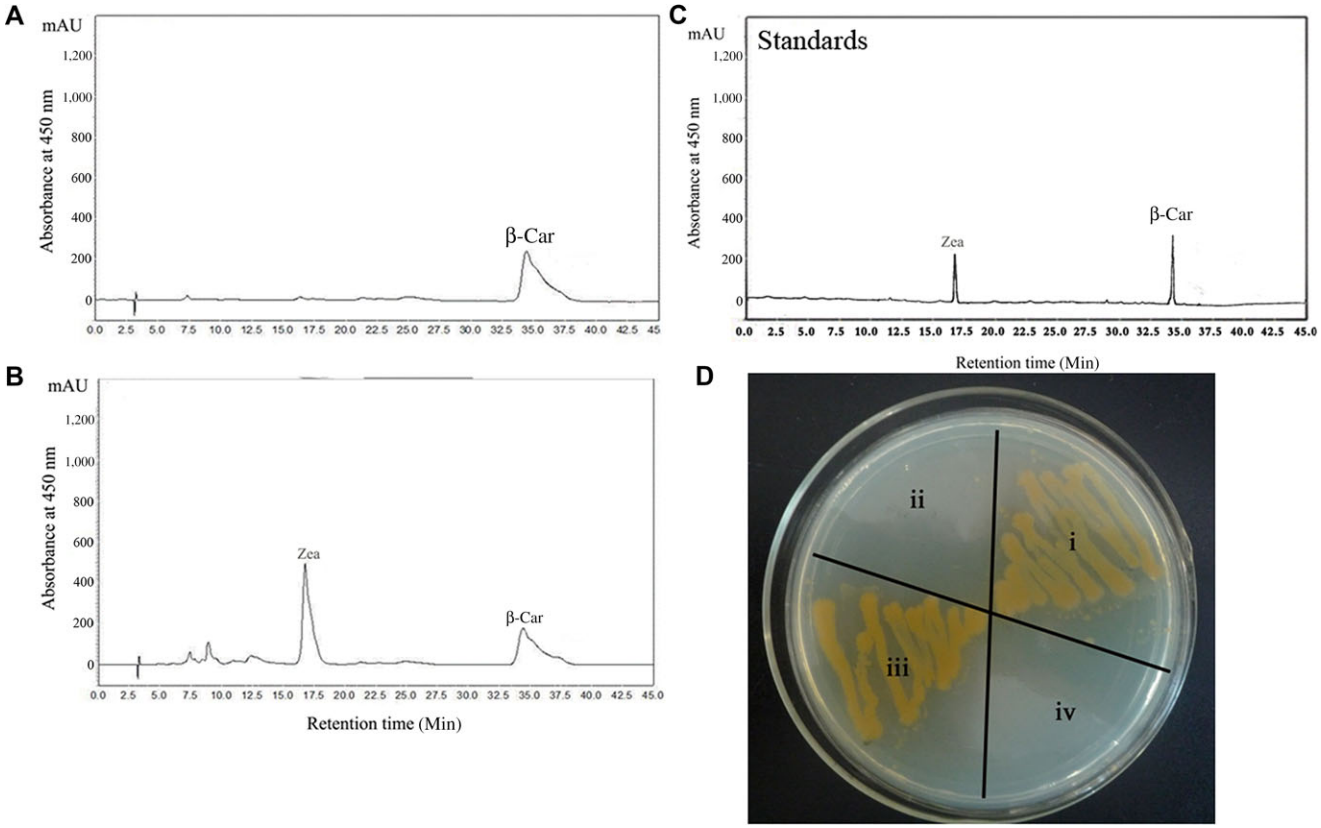

FIG. 7 *HPLC chromatogram analysis results of carotenoids biosynthesized in Escherichia coli cells carrying plasmids: (A) empty expression vector pET-28a(+) and pACCAR16ΔcrtX; (B) pET-28a-Lbchyb and pACCAR16ΔcrtX. Peaks β-car and Zea were identified as β-carotene and zeaxanthin, respectively; (C) β-carotene and zeaxanthin standards; (D) color complementation analysis of chyb gene in E. coli cells. i, pET-28a-Lbchyb and pACCAR16ΔcrtX; ii, pET-28a-Lbchyb; iii, pET-28a(+) and pACCAR16ΔcrtX; iv, pACCAR16ΔcrtX. All the colonies were propagated on LB–kanamycin (100 μg/mL) agar plates supplemented with 34 μg/mL chloramphenicol.*

containing pET-28a(+) and pACCAR16ΔcrtX (Fig. 7D,iii) presented as slightly red. *E. coli* containing only pET-28a-*Lbchyb* (Fig. 7D,ii) or pACCAR16ΔcrtX (Fig. 7D,iv) could not survive on the LB solid medium plate containing 34 μg/mL chloramphenicol and 100 μg/mL kanamycin.

The results acquired by using the plasmid pACCAR16ΔcrtX accumulating β-carotene revealed that Chyb from *L. barbarum* catalyzed the reaction of β-carotene to zeaxanthin [12, 44, 45]. Catalytic properties of *L. barbarum* Chyb are similar to *Arabidopsis thaliana* β-carotene hydroxylases, which catalyzed β-carotene to produce about 90% zeaxanthin and about 6% β-cryptoxanthin, but different from the β-carotene hydroxylase of *Adonis aestivalis*, which could convert β-carotene to ~38.9% zeaxanthin and ~24.5% β-cryptoxanthin using similar systems in *E. coli*. It should be noted that the enzymatic reaction kinetics of Chyb were not determined in this paper. Nevertheless, this study does demonstrate that the *chyb* gene cloned from *L. barbarum* possesses the biological function of β-carotene hydroxylase.

3.7. Tissue expression analysis

To investigate the tissue-specific expression level, semiquantitative RT-PCR and qPCR were carried out using total RNA extracted from different tissues of *L. barbarum*. Different expression levels of *chyb* gene were observed among different tissues as shown in Fig. 8. The expression profile of *chyb* gene in the examined different tissues seemed predominant in fruits and then in leaves and flowers; however, few expression patterns in roots and stems were detected. The expression level of *chyb* gene in fruits was strongest among the five tissues, which was about 19-fold higher than that in stems.

4. Discussion

L. barbarum is rich in carotenoids in its fruits and flowers [46]. In view of the carotenoid pathway, the carotenogenic genes in *L. barbarum* should be closely studied. However, to our knowledge, there have been few studies on the presence of carotenogenic genes in *L. barbarum*. In this study, in an effort to develop tools for operating the carotenoid profiles in microorganisms and plants, we cloned the cDNA encoding β-carotene hydroxylase from *L. barbarum* using the 3′ RACE

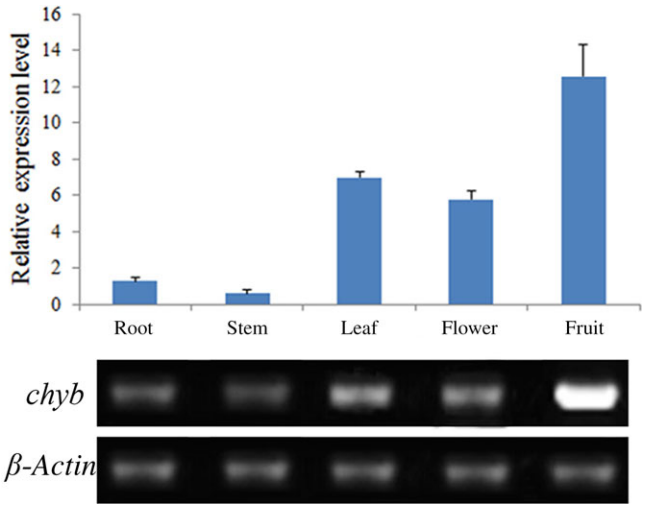

FIG. 8 *Quantitative real-time PCR and semiquantitative reverse-transcriptase PCR results of the Lycium barbarum chyb in specific tissues. L. barbarum β-actin gene was used as the internal control gene. The X-fold changes of L. barbarum chyb gene transcripts relative to the control samples were used to calculate relative expression levels.*

method. β-Carotene hydroxylases have been cloned from many organisms [18, 19, 47–49]; however, this is the first time that these hydroxylases have been cloned from *L. barbarum*.

A full-length cDNA sequence with an ORF of 939 bp, encoding a putative *β*-carotene hydroxylase (Chyb), has been cloned and characterized. It is a key enzyme catalyzing the hydroxylation of *β*-carotene to produce zeaxanthin. The putative protein had a sequence of 312 amino acids with a molecular mass of 34.8 kDa. The phylogenetic tree by the alignment of plant and microorganism *β*-carotene hydroxylase amino acid sequences was in accordance with hypotheses of evolutionary relationships. Also, the bioinformatics analysis showed that *L. barbarum* Chyb contains two transmembrane regions and predicted that it is a membrane protein of the chloroplast [18].

Sequence alignments at the protein level showed that *L. barbarum β*-carotene hydroxylase contains two conserved peptide motifs, VGAAVGME and AHQLHHSDK, and five conserved histidine motifs, HXXXXH and HXXH (Fig. 3). These results suggest that *L. barbarum* Chyb shares the same functional region with other β-carotene hydroxylases.

Functional complementation analyses of *L. barbarum chyb* in *E. coli* indicated that *L. barbarum* Chyb could produce zeaxanthin by catalyzing the hydroxylation of β-carotene (Fig. 7). HPLC analysis of the carotenoid showed that *E. coli* strains containing plasmids pET-28a-*Lbchyb* and pACCAR16ΔcrtX produced β-carotene and zeaxanthin (Fig. 7). The negative control *E. coli* cells containing pET-28a(+) and pACCAR16ΔcrtX produced only β-carotene (Fig. 7). The elution peaks were identified by the retention time compared with the standard samples. The catalytic properties of *L. barbarum* Chyb showed symmetric hydroxylation activity toward β-carotene as well as *A. thaliana* β-carotene hydroxylases. These results are consistent with much past research [6, 11, 12, 14]. In this study, five tissues of *L. barbarum* were used to determine tissue-specific expression patterns of *chyb* gene. The expression level of fruits was strongest among five tissues analyzed for *chyb* gene; this can explain why the zeaxanthin content of *L. barbarum* was very high [50].

5. Conclusions

cDNA sequences encoding hydroxylase for the hydroxylation reaction in carotenoid biosynthesis pathway, β-carotene hydroxylase, have been cloned and characterized from *L. barbarum*. The identification of *L. barbarum chyb* gene provides useful molecular materials for more in-depth studies on the carotenoid biosynthesis pathway. This genetic approach of the zeaxanthin biosynthetic pathway reveals the possibility of increasing the productivity of zeaxanthin using the *E. coli* expression system rather than chemical synthesis. Also, *L. barbarum chyb* can be used as a tool to alter the pathway of carotenoid biosynthesis in plants, through which plants enhance tolerance to abiotic stress. In the future, genetic modification of this gene will provide more insight into the physiological function of zeaxanthin biosynthesis.

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