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Identification and expression profile of an alpha-COPI homologous gene (COPA1) involved in high irradiance and salinity stress in *Haematococcus pluvialis*

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ABSTRACT

In response to stresses, *Haematococcus pluvialis* accumulates triacylglycerols (TAGs) and astaxanthin, which are highly positively correlated in cells. Coatomer protein I (COPI) complexes play important roles in protein and lipid transportation between endoplasmic reticulum (ER) and Golgi apparatus. The proposed functions of COPI alpha-subunit (COPA1) were to maintain the integrity of endomembrane system and to regulate cellular physiological activities (such as autophagy, protein sorting, secretory trafficking and lipid metabolism). However, only scarce information has been reported on this subunit. In this study, the full-length cDNA encoding *H. pluvialis* COPA1 (*HpCOPA1*) and its promoter were cloned and further analyzed by bioinformatics tools. The predicted length of HpCOPA1 was 1234 amino acids (AAs), including WDR, Coatomer-WDAD and COPI-C motifs. The AA sequence of HpCOPA1 was highly identical (78%–99%) to other algal COPA1 and had a highly conserved WDR domain at the N terminus. The promoter of HpCOPA1 contained *cis*-elements in relation to light and salt stresses, such as AE-box, MNF1, CCAAT-box and TCA-element. Moreover, real-time qPCR analyses showed that the mRNA level of *HpCOPA1* was strongly induced by treatments with either high irradiance, or high salinity (45 mM sodium acetate) or in combination. *HpCOPA1* revealed a delayed but higher peak expression level in treatments with high salinity (12.5-fold at 4 h) compared with high irradiance (4.7-fold at 2 h). To investigate its subcellular localization, HpCOPA1 was expressed in tobacco lower epidermal leaf cells in fusion with GFP. The results showed that HpCOPA1 was localized to plasma membrane and endomembrane system, partly similar to the localization of COPA1 in higher plants and animals. Taken together, the present results would contribute to the further elucidation of the regulatory mechanisms underlying the biosynthesis and transportation of lipids and astaxanthin in *H. pluvialis*.

1. Introduction

The unicellular green alga *Haematococcus pluvialis* has attracted increasing interests due to its ability to produce astaxanthin, an economical additive widely used in food, pharmaceutical, cosmetic, and health industries. In response to various environmental stresses, astaxanthin is biosynthesized and accumulates in *H. pluvialis* to avoid damages of reactive oxygen species (ROS) [1]. Among these stresses, high light (HL) is the most widely applied, due to its high efficiency to induce the synthesis of astaxanthin. Salinity stress is also effective to promote astaxanthin accumulation in *H. pluvialis*. The enhanced accumulation of fatty acids, primarily triacylglycerols (TAGs), is linearly correlated with the level of astaxanthin in *H. pluvialis* in response to stressful conditions, such as high irradiation, nitrogen deprivation and high salinity [2]. During the biosynthesis of astaxanthin, TAG might serve as the ‘solvent’ for astaxanthin in lipid bodies (LBs) [3]. De novo

Abbreviations: COPI, coatomer protein I; COPA1, alpha subunit of COPI; TAG, triacylglycerol; RACE, rapid amplification of cDNA ends; HL, high light; WDR, WD repeat domain; ER, endoplasmic reticulum; ROS, reactive oxygen species; LBs, lipid bodies

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fatty acid synthesis occurs in the chloroplasts, but TAGs are synthesized in the endoplasmic reticulum (ER) and then transported to and stored in the cytosolic LBs [3]. Cytoplasmic proteins that participate in lipid trafficking include clathrin, vesicle associated myosin, coatomer protein (COP) I and II complexes [4].

COPI complexes mediate the bidirectional transport of transmembrane proteins and lipids between ER and Golgi apparatus, which are essential to the development of eukaryotic cells [5]. Seven subunits (α/β/β′/γ/δ/ε/ζ) involved in the COPI complex are structurally and functionally organized into two subcomplexes according to their motifs. The β/γ/δ/ζ F-subcomplex forms a heterotetramer that acts as AP clathrin adaptor complexes, whereas the α/β′/ε B-subcomplexes, containing N-terminal WD repeat domains and C-terminal α-solenoid domains, compose a heterotrimer.

Recently, the genetic function and protein structure of the alpha-COPI subunit (COPA1) were illustrated in several species. The functional loss of alpha2-COPI in *Arabidopsis* caused abnormal morphology of Golgi apparatus and resulted in a dwarf phenotype [6]. RNA-interference silencing of COPA1 disintegrated the endomembrane system and further killed *Tetranychus urticae* [7]. These studies suggested that COPA1 might play important roles in maintaining the integrity of the endomembrane system and regulating the physiological activation of autophagy, protein sorting, trafficking and secretion. COPI complexes regulated lipid droplet growth via establishing connections between lipid droplets and the ER, thereby facilitating the relocalization of key enzymes involved in triacylglycerol synthesis [8]. As a subunit of COPI, COPA1 is also involved in lipid metabolism in *Drosophila* [9]. Up to date, the functions of COPA1 in *H. pluvialis* have not been thoroughly elucidated. To resolve this question, the first step should be the cloning and characterization of *H. pluvialis* COPA1.

Our previous RNA-Seq analyses of *H. pluvialis* revealed that the expression of *HpCOPA1* gene was significantly induced by treatments with high irradiation and salinity for 1.5 h (unpublished data). To characterize the gene and protein sequences of COPA1 in *H. pluvialis*, the full-length cDNA and promoter of *HpCOPA1* were isolated and then analyzed using bioinformatics tools. The evolutionary relationship of *HpCOPA1* from various organisms was also investigated. The changes in mRNA level of *HpCOPA1* in response to high irradiance and/or salinity (45 mM sodium acetate) were assessed using qRT-PCR. To the best of our knowledge, this is the first report to describe the COPA1 protein in *H. pluvialis*, a genetically uncharacterized organism. The results would provide new insights to the regulatory mechanisms underlying the biosynthesis and transportation of lipid and astaxanthin in *H. pluvialis*.

2. Materials and methods

2.1. Algal strains and culture conditions

H. pluvialis 192.80 was purchased from the SAG Culture Collection of Algae (www.epsag.uni-goettingen.de). The ESP medium (pH 7.0) for growing *H. pluvialis* contained 1.0 g of peptone, 0.2 g of KNO_3, 0.02 g of K_2HPO_4, 0.02 g of $MgSO_4·7H_2O$, 35 mg of $FeSO_4·7H_2O$, 40 mg of EDTA, 5 μg of $ZnSO_4·7H_2O$, 10 μg of $MnSO_4·4H_2O$, 50 μg of H_3BO_3, 5 μg of $Co(NO_3)_2·6H_2O$, 5 μg of $Na_2MoO_4·2H_2O$, 0.025 μg of $CuSO_4·5H_2O$, and 30 mL of soil extract per liter. *H. pluvialis* was grown in 250-mL Erlenmeyer flasks, coved by permeable sealing membrane and then statically incubated at 22 °C under a dark/light cycle of 12 h: 12 h (25 μM photons·m^{-2}·s^{-1}) for 4 days. For high salinity treatments, 45 mM sodium acetate was used. In the high irradiation experiments, a light intensity of 550 μM photons·m^{-2}·s^{-1} was applied.

2.2. Total RNA and genomic DNA extraction

H. pluvialis cells were collected and stored at − 80 °C. The total RNA was prepared using a modified Trizol method. In brief, samples were suspended in Trizol reagent (Invitrogen, USA), containing 2% (w/v) polyvinylpolypyrrolidone (PVP40) and 1% (v/v) β-mercaptoethanol. After cells lysed, chloroform was added and the supernatant was cleaned using step-by-step solutions of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). Finally, RNA was precipitated using isopropanol, washed with 75% ethanol and dissolved in water. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's instruction. The qualities of nucleic acid were examined using electrophoresis, and their concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA).

2.3. Cloning of full-length cDNA by RACE

The 5′- and 3′-RACE was performed to obtain the 5′- and 3′-ends of the *H. pluvialis* alpha-COPI subunit (*HpCOPA1*) using a SMARTerRACE 5′/3′ Kit (Clontech, USA) according to the manual manufacturer's instruction. 5′- or 3′-GSP primers (Table S1) were designed based on the *HpCOPA1* fragment revealed by BLAST using the coatomer (COPI) alpha subunit C terminus as a seed. RACE PCRs were performed using the following protocol: 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. The PCR products were in-fusion cloned into a pRACE vector and sequenced. After RACE, the primers c-*HpCOPA1*-F(R) (Table S1) were designed to amplify the full-length *HpCOPA1* cDNA.

2.4. Bioinformatic analyses

Based on the full-length cDNA sequences of *HpCOPA1* obtained by RT-PCR, the open reading frame (ORF) and deduced amino acid sequences were predicted using the ORFfinder software (https://www.ncbi.nlm.nih.gov/orffinder/). The conserved protein motifs of HpCOPA1 were identified using the SMART (http://smart.embl-heidelberg.de/). The theoretical molecular weight (Mw) and isoelectric point (pI) of the HpCOPA1 protein were computed using the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The transmembrane structure and signal protein prediction of HpCOPA1 were performed via the 'DAS' - Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/) and the SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP/), respectively. Secondary structure was predicted using the SAS software (http://www.ebi.ac.uk/thorntonsrv/databases/sas) and the PredictProtein server (https://www.predictprotein.org). Comparative 3D protein predictions were performed using the Phyre^2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html). Homologous proteins of HpCOPA1 were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments were clustered using ClustalX 2.1, and the alignments were edited using the GeneDoc 2.7 sequence editor. The phylogenetic tree of HpCOPA1 was constructed using the Maximum Likelihood (ML) method that was implemented in Phyml 3.0 (http://www.atgc-montpellier.fr/phyml/). The optimal substitution model (LG + I + G) was selected by the Smart Model Selection in PhyML (SMS). The proportion of invariable sites and gamma shape parameter was 0.946 and 0, respectively. The starting tree was calculated using BIONJ [10], and fast nearest neighbor interchange (NNI) was used for improvement. Bootstrap values (BS) were based on 1000 replicates. Graphical representation and edition of phylogenetic tree were performed using the FigTree 1.43 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Isolation and characterization of the HpCOPA1 promoter

The promoter of *HpCOPA1* was obtained using the Universal GenomeWalker™ kit (Clontech, USA) according to the manufacturer's instruction. *H. pluvialis* genomic DNA was digested with *Dra*I, *Eco*RV, *Pvu*II or *Stu*I restriction enzyme. After purification, adaptors were ligated to the corresponding blunt-end digestion product using the T4

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