Algal Research 31 (2018) 291–297

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Over-accumulation of astaxanthin in *Haematococcus pluvialis* through chloroplast genetic engineering

Janeth I. Galarza[a], Javier A. Gimpel[b], Verónica Rojas[c], Bertha O. Arredondo-Vega[d], Vitalia Henríquez[c,e,*]

[a] Laboratorio de Biotecnología, Facultad de Ciencias del Mar, Universidad Estatal Península de Santa Elena, La Libertad, Ecuador
[b] Departamento de Ingeniería Química y Biotecnología, Centro para Biotecnología y Bioengeniería (CEBIB), Universidad de Chile, Santiago, Chile
[c] Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[d] Laboratorio de Biotecnología de Microalgas, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico
[e] Fraunhofer Chile Research Foundation, Center for System Biotechnology, Santiago, Chile

ABSTRACT

The carotenoid biosynthesis pathway of *Haematococcus pluvialis* has been genetically modified to overproduce astaxanthin, a red pigment of high commercial value. The endogenous phytoene desaturase coding sequence (*pds*) has been codon optimized and overexpressed in the chloroplast of *H. pluvialis* under the control of the *psbA* promoter/UTR. After biolistic transformation and selection, PDS mRNA and protein were detected in transformants. Astaxanthin accumulated up to 67% higher in transformed strains than in wild-type upon induction with high light intensity and nitrogen depletion. This is the first report of plastid transformation of a microalgae with its endogenous *pds* nuclear gene.

1. Introduction

Many species of microalgae have been exploited as sources of food additives, animal feeds and cosmetics as well as for potential health beneficial compounds [1]. Particularly, microalgae carotenoids have broad applications in aquaculture, pharmaceutical, nutraceutical, and cosmetic industries [2–4]. The fresh water chlorophyte *Haematococcus pluvialis*, is the main source of natural astaxanthin (3,3′-dihydroxy-di-keto-β,β′-carotene-4, 4-dione), a high-value secondary carotenoid that belongs to the xanthophylls family [1,5]. Its unique structure, containing a keto and a hydroxyl group on each end of the molecule, contribute to its enhanced antioxidant properties. Its standardized antioxidant activity is 65–fold higher than vitamin C, 54-fold more powerful than beta-carotene, and 14-fold higher than vitamin E [6]. The astaxanthin is accumulated as a response to stress such as nutrient starvation, high salinity, high light, etc. [for reviews see 7,8].

Hence, it has an extraordinary potential for applications in human health such as in chronic inflammatory diseases, anti-aging, cancers, metabolic syndrome, neurodegenerative diseases as well as protection against UV light damage [9–12]. On the other hand, astaxanthin is also widely implemented as a pigment and protein ingredient for the fish and poultry feed industry [10].

In *H. pluvialis* the precursors of astaxanthin are synthesized in the chloroplast but its synthesis occurs in the cytosol and accumulates as cytoplasmic lipid droplets [9,13]. Under stress conditions the unicellular green microalgae *H. pluvialis* accumulates astaxanthin up to 1–4% dry weight, thus becoming the most widely used source for natural astaxanthin production [1].

In *H. pluvialis* the carotenoid biosynthesis uses the universal isoprenoid precursor Isopentenyl pyrophosphate (IPP) and is allylic isomer dimethilallyl pyrophosphate (DMAPP) through the non-mevalonate pathway located in the chloroplast [for reviews, see 14,15]. The first committed step for carotenoid biosynthesis consists in the sequential addition of three IPP molecules to DMAPP to yield the C20 geranylgeranyl pyrophosphate (GGPP). Then after, the phytoene synthase (PSY) catalyzes the head-to-tail condensation of two GGPP molecules to form the first tetraterpene carotenoid, the phytoene. The successive desaturation reactions of phytoene, catalyzed by two structurally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), converting the pale yellow phytoene into ζ-carotene and the latter into lycopene, respectively. PDS catalyzes the first two-dehydrogenation reactions to form phytofluene and ζ-carotene, which are among the rate-limiting steps of the pathway [16–19]. ZDS catalyzes two further reactions converting ζ-carotene to neurosporene and

* Corresponding author at: Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile.
E-mail address: vitalia.henriquez@pucv.cl (V. Henríquez).

https://doi.org/10.1016/j.algal.2018.02.024
Received 3 March 2017; Received in revised form 19 February 2018; Accepted 19 February 2018
Available online 23 February 2018

lycopene. Cyclization of lycopene by the membrane-localized lycopene β-cyclase yields α-carotene (lutein precursor) and β-carotene (astaxanthin precursor). The astaxanthin formation from β-carotene will need the introduction of two hydroxy groups and two-keto groups by hydroxylase and ketolase enzymes, respectively. Additionally, two isomerase enzymes (*Z*-ISO and CRTISO) intervene in the synthesis of carotene in cyanobacteria and plants, but their function is not well established in *H. pluvialis*. Their *H. pluvialis* homologs have not been reported, and their differential expression has not been detected in astaxanthin accumulation conditions [20,21]. Notwithstanding, since both enzymes have been identified in *Chlamydomonas reinhardtii* we can infer that they are also present in *Haematococcus pluvialis*.

Although metabolic pathways in microalgae are complex with many regulatory inputs and interplay with other pathways [22,23], genetic engineering has been established as a promising approach for enhancing carotenoid accumulation in algae [24,25]. Metabolic engineering of the astaxanthin biosynthesis pathway has received special attention given its economical significance [26]. *H. pluvialis* β-carotene ketolases *bkt3* [27] and *bkt1* [28] have been overexpressed in the nucleus of *Chlamydomonas reinhardtii* without an increase in astaxanthin accumulation. In contrast nuclear co-transformation of β-carotene ketolase along with the β-carotene hydroxylase from *H. pluvialis* in *C. reinhardtii* resulted in 30% higher astaxanthin accumulation than wild-type [29]. Kathiresan's studies reported over-expression of *H. pluvialis bkt* in the same host through Agrobacterium-mediated nuclear transformation [30]. The transgenic strains showed 2 to 3-fold increase in the astaxanthin content under sodium acetate stress.

Phytoene desaturase (PDS) is an early enzyme in the carotenogenic pathway localized exclusively in the chloroplast that catalyzes the desaturation of phytoene, a rate-limiting step in the pathway [31,32]. Steinbrenner and Sandman (2006) overexpressed a modified version of the *H. pluvialis pds* gene containing a point mutation for norflurazon resistance, in the nucleus of *H. pluvialis* [16]. The transgenic lines accumulated up to 36% more astaxanthin than the wild type strain after 48 h of high light induction. Similarly, a norflurazon-resistant *pds* gene from *Chlorella zofingiensis* was overexpressed in the nucleus of the same host resulting in 54% higher astaxanthin accumulation [17].

The aforementioned studies have dealt exclusively with nuclear transformation. Only one research group has attempted to target carotenogenesis genes directly to the chloroplast of microalgae [33]. These authors transformed the *H. pluvialis* β-carotene hydroxylase nuclear gene without codon optimization into the *C. reinhardtii* chloroplast. The results showed an increase in xanthophyll pigments upon high-light treatment.

The key enzymes for carotenogenesis are targeted and localized exclusively in the chloroplast [31]. Therefore, the high expression levels achieved by chloroplast genetic engineering would be a major advantage for astaxanthin overproduction [1]. This work proposes to enhance the accumulation of astaxanthin through chloroplast transformation of *H. pluvialis* with a plasmid based on pHpluS1, including a new expression cassette for a plastid codon-optimized *H. pluvialis pds* coding sequence driven by the endogenous *psbA* promoter/UTR [34].

2. Materials and methods

2.1. Strains, growth media and culture conditions

H. pluvialis (CCAP 34/7) was purchased from the Culture Collection of Alga and Protozoa (CCAP), Windermere, United Kingdom. *H. pluvialis* was grown in modified liquid medium EG/JM (*Euglena gracilis* Medium: Jaworski's Medium (http://www.ccap.ac.uk/) with trace elements and vitamins. Cultures were incubated at 22–25 ± 2 °C with continuous aeration and light (30 μmol photon × m^{-2} × s^{-1}), and were also shaken manually twice daily. Cell biomass dry weights were measured as in Gomez and coworkers [35].

For induction of astaxanthin biosynthesis and red cyst formation, strains were grown for four days until stationary phase. Transgenic cultures contained 200 μg × mL^{-1} spectinomycin at this step. Cultures were then exposed to continuous illumination at 400 μmol photon × m^{-2} × s^{-1} in nitrogen free EG/JM medium. Cultures were maintained on these conditions for 15 days until complete aplanospore induction occurred. As a control, wild-type cells (WT) were maintained throughout the experimental period under the same conditions. All carotenoid induction experiments were performed in triplicates.

2.2. Codon optimization of the pds gene

The *pds* nuclear gene (Genbank Accession number X86783.1) was initially codon-optimized for *C. reinhardtii* chloroplast codon bias (http://www.kazusa.or.jp/codon/) using OPTIMIZER (http://genomes.urv.is/CAIcal/). The generated sequence was then manually cured using CAIcal (http://genomes.urv.is/CAIcal/) for avoiding too many repetitions of single codons, while maintaining a high Codon Adaption Index (CAI) [36]. Quickfold was then used for eliminating potential secondary structures (http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold). The first 73 amino acids were not included in the synthetic gene since they are predicted as the N-terminal transit peptide according to Genbank X86783.1. The final coding sequence was submitted to GeneScript (Piscataway, NJ, USA) for chemical synthesis flanked with suitable restriction sites for cloning (*Nde*I and *Xho*I).

2.3. Construction of vector pSM382/PDS and chloroplast transformation

The plasmid pHpS1 specifically designed for the chloroplast transformation of *H. pluvialis* [34] was used as backbone for the cloning of a USER multi-cloning-site with restriction sites *Bbv*CI-*Pac*I-*Bbv*CI [37]. The USER multi-cloning-site was cloned into the *Sal*I site generating the plasmid pSM382-USER. A new *H. pluvialis* promoter-terminator was cloned as a USER fusion 5′psbA/*Nde*I/*Xho*I/3′rbcL to drive the expression of the *pds* gene. The synthetic *pds* CDS was PCR amplified with and without a C-terminal 1xFlag-tag sequence, and cloned using restriction/ligation, thus generating plasmids pSM382-PDS-Flag and pSM382-PDS. All recombinant DNA manipulations were in accordance with standard techniques. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid propagation.

Transformations were carried as in [34]. Transformants were selected on solid EG/JM containing 200 μg × mL^{-1} spectinomycin.

2.4. Verification of construct integration by PCR

Each colony representing a putative transformant was analyzed by colony picking PCR to confirm that transgenes were incorporated into the chloroplast genome. Specific primers TH127/TH118 (5′-TGGCCTT GGATCACCTCCTTCTCTTAA-3′/5′-TATAAGTAAGTAAGTAAGTAAGCC AGTACC-3′) that hybridize with the endogenous 16S and 23S flanking regions were used to verify integration of the transgenic DNA into the chloroplast genome (Fig. 1). The amplification program was performed according to Gutiérrez and coworkers [34] using a thermocycler T100 Thermal Cycler (Bio-Rad). PCR reaction conditions were as follows: initial denaturation at 95 °C by 5 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 3 min at 72 °C, ending with a final extension of 10 min at 72 °C. Untransformed *H. pluvialis* was used as positive control and distilled water as a negative PCR control. The PCR fragments were separated on 1% agarose gel.

2.5. RT-PCR

Reverse transcription PCR experiments were carried out using total RNA extracted from wild-type and modified microalgae using the TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. First strand cDNA was synthesized from 2 μg of the DNase-

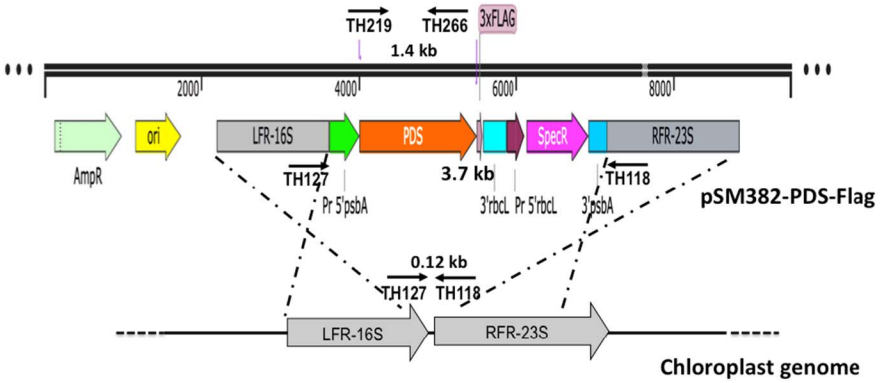

Fig. 1. Map of the plasmid pSM382-PDS-Flag transformation plasmid. Integration of the *pds* and *aadA* genes is achieved by homologous recombination of the RFR-16S and LFR-23S flanking sequences. Primers pair TH219/TH266 for PDS cDNA amplification (Fig. 3) and primers pair TH127/TH118 for transgene integration verification (Fig. 2) are indicated.

treated RNA using the M-MLV Reverse Transcriptase and random primers according to the manufacturer's instruction (Promega, Madison, WI, USA). cDNAs were diluted 100 times and 1 μL of each dilution was used as a template in a final PCR volume of 10 μL, using primers TH219/TH266 (5′-GTTGTCGACATGCGTGTTGTTATTGCTGGT-3′/5′-GTTTTTACAACCACCAGCAATAGCACCTAC-3′). The PCR fragments were separated on 1% agarose gel.

2.6. Western blot analysis

Total proteins were isolated from 30 mg of transgenic algae. Samples were resuspended in 500 μL dH_2O containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich) and lysed by strong vortex with a mix of metal beads of 1 and 2.38 mm. 10 μL of this mix plus loading buffer were separated on a 10% SDS-PAGE gel. Western blots were performed as described by Barrera [38] using a mouse anti-Flag M2 (1:1000) (Sigma, St. Louis, MO, USA) and an HRP-conjugated goat anti-mouse secondary antibody (1:7500) (Thermo Fisher, Waltham, MA, USA). Membranes were developed using the Westar Supernova kit (Cyanagen, Bologna, Italy).

Total carotenoids, astaxanthin and chlorophyll extraction and quantification.

A known quantity of cysts biomass of *H. pluvialis* (20 mg) was lysed with strong vortex with a mix of 1 and 2.38 mm metal beads. Total carotenoids were extracted with 50 mL 100% acetone and kept at −20 °C for 24 h. Samples were sonicated and centrifuged at 4 °C repeatedly until the pellets became colorless. Extracts were measured at 474 nm for total carotenoids, and 663 and 645 nm for chlorophyll using an Espectrostar Nano spectrophotometer (BMG Labtech, Offenburg, Germany). Total carotenoids and chlorophyll were calculated using Fuji Chemical Industry and Becker equations, respectively [39,40].

For astaxanthin extraction, 3 mL of total carotenoid were mixed with 1 mL of cholesterol esterase enzyme 4 U/mg (Waco, USA) and incubated at 37 °C for 45 min. Then, 1 g sodium sulphate decahydrate and 2 mL of petroleum ether were added, vortexed for 30 s and centrifuged for 5 min at 3000 RPM. The petroleum ether layer including the carotenoids was transferred to a new tube containing 1 g of anhydrous sodium sulphate. The petroleum ether carotenoid extract was decanted and dried under N_2 gas and re-dissolved in 3 mL 100% acetone [35]. Aliquots of extracts were stored in amber vials at −80 °C prior to HPLC analysis. Extracts were subjected to High-Performance Liquid Chromatography in Jasco PU-2089-plus instrument (Easton, MD, USA), fitted with a UV–Vis intelligent detector (model 2075) using a 3.5 μm C8 column (4.6 × 100 mm, Water, Milford, MA, USA). A gradient solvent system consisting of methanol:ammonium acetate 70% (70:30 v/v) and 100% methanol at a flow rate of 1.0 mL/min was used. Astaxanthin was identified at 425 nm by comparing the retention times and spectra against a known standard. Astaxanthin was used as the standard (Dr. Ehrenstorfer CAS N° 7542-45-2 GmbH, Germany).

2.7. Cell productivity in H. pluvialis clones

Clones of *H. pluvialis* PDS and PDS-flag and wild type *H. pluvialis* were submitted to vegetative cell growth in 300 mL EG/JM medium up to the end of exponential growth phase. Assays were performed in triplicate. Growth rate was determined by cell counting using Neubauer chamber 0.1 mm and an Olympus inverted microscope. Specific growth rate was calculated using the equation $\mu = \mathrm{Ln}\,(X2 - X1)/(T2 - T1)$, where X2 and X1 are biomass (preferably dry wt) at the beginning (T1) and at the end (T2) of the exponential growth phase [41]. Cellular productivity (mg/L/day) was calculated by the formula applied by Sorokin [42].

3. Results and discussion

3.1. pds codon optimization and cloning

It is well established that in microalgal chloroplasts genomes, codon bias is an important determinant for protein expression [43,44]. In *C. reinhardtii*, nuclear and plastid genes have very different codon composition biases [45]. The full-length nuclear cDNA encoding the *H. pluvialis pds* gene (1494 bp) was codon optimized for *C. reinhardtii* chloroplast codon bias, since this alga belongs to the same order (Chlamydomonadales) as *H. pluvialis*, and its chloroplast genome is sequenced, unlike the one from the latter. Expressing a nuclear gene in the chloroplast genome avoid gene silencing and also takes advantage of the high translation levels that can be achieved in this organelle [46].

The optimized *pds* gene flanked by the restriction enzymes *Nde*I and *Xho*I, with and without the Flag tag was cloned into pSM382-USER, thus placing it under the control of 5′*psbA* promoter/UTR and 3′*rbcL* UTR. The latter vector allows the cloning of any gene flanked by these two restriction sites for expression, driven by the *psbA* promoter/UTR for future experiments. The accompanying spectinomycin resistance gene (*aadA*) used as the selectable marker is driven by 5′*rbcL* promoter/UTR and 3′*psbA* UTR sequences, thus avoiding the possibility of homologous recombination with the *pds* cassette. The flanking 16S and 23S regions of *Haematococcus pluvialis* allow the integration of both cassettes within the chloroplast genome by homologous recombination [34]. These cloning yielded the expression plasmids pSM382-PDS and pSM382-PDS-Flag (Fig. 1).

3.2. Chloroplast transformation

Chloroplast transformation of *H. pluvialis* with both plasmids was done according to the method described by Gutierrez [34]. After 6 weeks of cultivation in the selection medium (EG/JM spectinomycin

200 μg × mL^{-1}) single colonies appeared providing a transformation efficiency of 8.5 transformants/μg DNA for pSM382-PDS and 9.5 transformants/μg DNA for the vector pSM382-PDS-Flag. Transformation efficiencies were rather low compared to the ones observed by Gutierrez and coworkers [34] that could be due to the larger construct that needed to be integrated, or because of the increased metabolic burden caused by the overexpression of *pds*. These efficiencies are still higher than the ones from Sharon-Gojman et al. and Steinbrenner and Sandmann using the same target gene [16,47]. These authors obtained efficiencies between $10^{-5} - 10^{-6}$ cells/ug DNA when they targeted the *pds* gene to the nuclear genome.

3.3. Analysis of gene integration into the chloroplast of H. pluvialis

Algae chloroplasts exhibit genome polyploidy. Therefore, transformation event results in an initial heteroplasmic state in which only some copies of the chloroplast genome within an individual cell have been modified [48,49]. Growth of transformant lines under selective pressure should eventually result in homoplasmic cells. Integration of the transgene into the chloroplast genome of transformed *H. pluvialis* was assessed after 5 and 10 months of post-bombardment selection and antibiotic plate re-streaking. Using primers TH127/TH118 a fragment of 3.7 Kb is generated when *pds* and *aadA* expression cassettes are integrated into the plastome, whereas a fragment of 0.12 Kb would show no integration, corresponding to the native sequence of the ribosomal operon (Fig. 1). Wild type *H. pluvialis* showed only the 0.12 Kb fragment indicating the native sequence of the ribosomal operon. Although several rounds of single-colony propagation under selective pressure were done to allow the recovery of homoplasmic clones, none of the tested transgenic lines (PDS and PDS-flag) showed an individual 3.7 Kb amplicon and no 0.12 Kb fragment, which would represent homoplasmy. All strains were heteroplasmic showing both bands, indicating that the transgenes have been inserted into some copies of the chloroplast genome (Fig. 2A and B). Evidence for persistent heteroplasmic state has been seen before in *C. reinhardtii* and *Platymonas subcordiformes* [48,50]. The persistent heteroplasmic state of the chloroplast genome could be an indicative of instability of the transgene due to the inherent polar effect within the rRNA operon. It seems likely that the genetic change is disrupting the ribosomal operon function, and therefore both types of plastoma are maintained [51]. Interestingly, two PDS-flag transformants resulted in the amplification of only the 0.12 Kb amplicon showing apparently no integration of the transgene (Fig. 2B). Nonetheless, the positive RT-PCR and Western blots for these strains (Fig. 3), indicate that the smaller PCR fragment probably outcompeted the amplification of the much larger 3.7 Kb band.

3.4. Expression confirmation by RT-PCR and Western blot

In order to confirm expression from the transformation constructs, we have determined the transcriptional activity of the optimized *pds* gene by RT-PCR for all clones selected. Specific primers (TH219/TH266) against the codon optimized *pds* gene were designed in order to distinguish it from the nuclear *pds* copy. The result demonstrated that the optimized *pds* gene (1494 bp) was constitutively transcribed in all transgenic clones of PDS-Flag (Fig. 3A). Data from putative PDS transformants are not shown. This is in accordance with the activity of *C. reinhardtii psbA* promoter, which is also constitutively transcribed [52].

Total proteins were extracted and analyzed by SDS-polyacrylamide gel electrophoresis. Immunological detection using a monoclonal antibody against Flag tag showed a band with molecular mass of about 50 kDa in all transformed clones containing the tag, the band was not present in *H. pluvialis* WT (Fig. 3B), suggesting that *pds*-flag mRNA (Predicted MW 55.7 kDa) is translated. This is the first time that the nuclear *pds* gene has been successfully expressed in an algal chloroplast; all previous efforts have targeted nuclear overexpression of this gene [16–18]. This work also contributes as another example of the feasibility of relocating nuclear genes into the chloroplast genome when removing their transit peptides and performing proper codon optimization. The latter seems highly reasonable given that the evolutionary

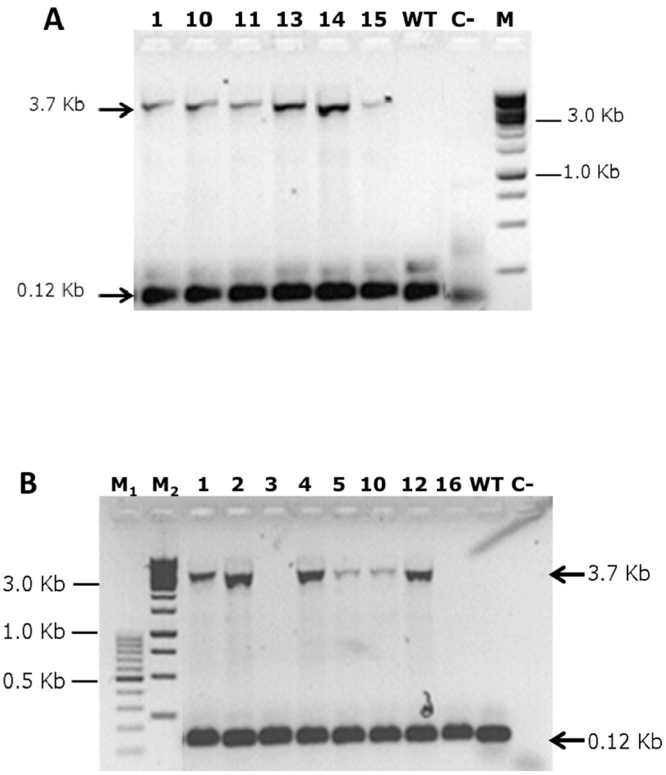

Fig. 2. Analysis of integration by homologous recombination of *pds* and *aadA* genes in the plastome of *H. pluvialis*. PCR with primers TH127/TH118 flanking the 16S–23S integration site (Fig. 1). Amplification of the wild-type untransformed site results in a 120 bp amplicon. Amplification of the integrated construct results in a 3700 bp amplicon.
A. PDS transformed strains: 1, 10, 11, 13, 14, 15; WT: *H. pluvialis* wild-type; C-: no DNA PCR control; M: 1 Kb ladder.
B. PDS-flag transformed strains: 1, 2, 3, 10, 12, 16; WT: *H. pluvialis* wild-type; C-: no DNA PCR control; M_1: 100 bp ladder; M_2: 1 Kb ladder.

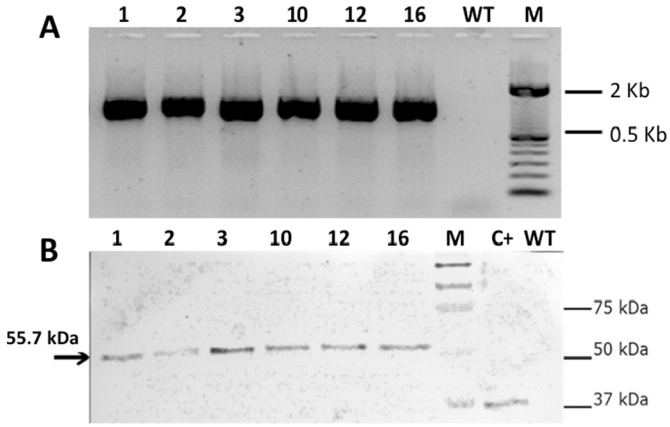

Fig. 3. Analysis of PDS-Flag transformed colonies.
A. Reverse transcribed *pds* cDNA amplification with primers TH219/TH266 (Fig. 1). Clones 1, 2, 3, 10, 12, 16: *H. pluvialis*/pSM382-Flag; WT: *H. pluvialis* wild-type cDNA. M: 1 Kb ladder. The expected size of the PCR product is 1494 bp.
B. Western blot analysis probed with anti-FLAG M2 primary antibody, and HRP-conjugated secondary antibody. Total protein was extracted as indicated in the experimental procedures and separated by electrophoresis on 10% SDS-PAGE gel. Clones 1, 2, 3, 10, 12, 16: *H. pluvialis*/pSM382-Flag; M: Molecular weight standard; C+: Flag Positive control protein; WT: *H. pluvialis* wild-type. The expected molecular weight of PDS is indicated (55.7 kDa).

Table 1
Astaxanthin, chlorophyll and biomass accumulation of transformed *H. pluvialis* strains.

Strain	Carotenoid/ chlorophyll ratio	Astaxanthin/ DW %	Cell biomass mg/L	Astaxanthin yield mg/L
WT	1,41	1.58 ± 0.06*	1136 ± 43	18.0 ± 1.0
PDS-1	4,96	2.64 ± 0.06*	1300 ± 46	34.3 ± 1.4
PDS-Flag-12	2,62	2.20 ± 0.04*	1212 ± 62	26.7 ± 1.5

* ($p < 0.05$).

evidence shows that several nuclear genes derive from horizontal gene transfer from the chloroplast genome, which was formerly the photosynthetic endosymbiont genome [53].

3.5. Analysis of astaxanthin in H. pluvialis transformants

Phytoene desaturase (PDS) is a rate-limiting enzyme in carotenoid biosynthesis and is restricted to the chloroplast [31]. The nuclear transformation of *pds* gene demonstrated that the increase of transcripts of PDS increase the protein level and enable the microalgae to maximize the secondary carotenoid biosynthesis [16,17].

After a preliminary astaxanthin accumulation screening of the putative transformants, PDS-1 and PDS-Flag-12 strains were selected for detailed carotenoid production characterization (Table 1). Following four days of vegetative growth, both transgenic strains grew to a comparable cell density to the wild type. This indicates that the transgenes are not toxic and do not represent a significant metabolic burden to the cells. Astaxanthin accumulation per dry weight was enhanced by 67% and 39% for PDS-1 and PDS-Flag-12, respectively. These differences were considered to be statistical significant ($p < 0.05$). The lower value attained by the tagged strain could be due to the Flag-tag obstructing the conserved C-terminal carotenoid binding motif found in PDS, thus resulting in lower activity [54,55]. The enhancement of astaxanthin accumulation from this report is within range of previous experiments for nuclear PDS overexpression, resulting in 36% and 54% higher build-up per dry weight in *H. pluvialis* and *C. zofingiensis*, correspondingly [16,17]. Accordingly, delivering the *pds* gene directly within the plastome would be acting the same, increasing the transcripts and protein level for interacting in the accumulation of a higher amount of astaxanthin.

Furthermore, the calculated astaxanthin accumulation per culture volume was 90% and 48% higher in PDS-1 and PDS-Flag-12 than in the *H. pluvialis* non-transformed. Higher increments in astaxanthin per culture volume compared to per dry weight relates to the higher biomass density attained by the transgenic strains. Higher cell densities could be due to a higher amount of total carotenoids available for photoprotection during growth of the transformants [56]. PDS-1 and PDS-Flag-12 had total carotenoids to chlorophyll ratios 252% and 86% higher than the control after induction, respectively. The apparent difference between total carotenoids and astaxanthin accumulation is probably due to the fact that PDS generates the precursor for all major carotenoids that accumulate in the cell (e.g. β-carotene, lutein, lycopene, zeaxanthin, astaxanthin), thus resulting in overall enhanced carotenoid accumulation, and not just that of astaxanthin [32]. Although transformants remain heteroplasmic, the second copy of the PDS gene allowed higher accumulation of astaxanthin in the transgenic lines assessed.

3.6. Biomass and astaxanthin productivity analysis for H. pluvialis transformants

In order to assess if transgene expression affects cellular viability, the growth rate and cellular productivity of PDS-1 and PDS-flag-12 strains were compared with the wild-type strain. Transgenic and non-transgenic lines appeared similar with respect to morphology, mobility, and cell growth kinetics. The cells in vegetative state remained suspended in the culture medium, with a green translucent color and two long flagella in constant movement. These characteristics suggested that cultures were viable. The stationary cell concentration of the wild-type strain was 6.82×10^5 cells/mL with a growth rate of $\mu = 0.64$ div/day, while the transformed strains reached 6.88×10^5 and 6.74×10^5 cells/mL with a growth rate of $\mu = 0.64$ div/day and $\mu = 0.62$ div/day for PDS-1 strains and PDS-flag-12 respectively (Fig. 4). These values are in accordance with previously recorded growth rates of *H. pluvialis* [57]. The fourth day of culture corresponds to the end of the exponential growth phase and the statistical analysis did not show significant differences ($p > 0.05$) between wild type and transformed *H. pluvialis* growth rates (Table 2). Cell productivity reached 3.25×10^2 mg/L/day in strain PDS-1 and 3.03×10^2 mg/L/day for PDS-flag-12, while wild type strain reached 2.84×10^2 mg/L/day (Table 2). These results clearly demonstrate that the transformation event of *H. pluvialis* does not affect cell viability.

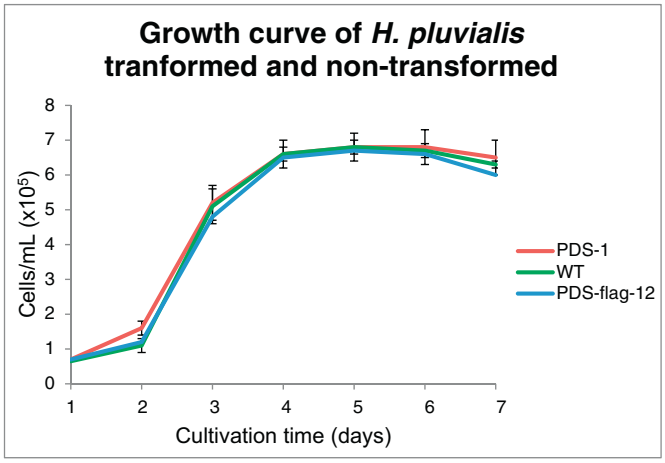

Fig. 4. Growth curve of *H. pluvialis* transformed and non-transformed strains
WT: *H. pluvialis* non-transformed used as control: PDS-1 and PDS-flag-12 strains of transformed *H. pluvialis*.

4. Conclusions

This is the first report demonstrating that a nuclear gene (*pds*) is expressed in the chloroplast of *H. pluvialis*. The latter has allowed for successful metabolic engineering of the carotenoid biosynthesis pathway resulting in up to 90% higher astaxanthin accumulation per culture volume. This has confirmed previous results suggesting that the phytoene desaturating step is rate limiting for carotenoid accumulation [16–19]. In the future, the developed strategy could be implemented in *H. pluvialis* industrial strains for reducing natural astaxanthin production costs, given that the transgenes do not impair growth rates or biomass productivity.

Table 2
Cell productivity of transformed *H. pluvialis* strains.

Strain	Cellular density cell/mL	Growth rate division/day	Cell productivity mg/L/day
WT	6.82×10^5 ± 3.28	0.64 ± 0.01	2.84×10^2 ± 10.70
PDS-1	6.88×10^5 ± 8.30	0.65 ± 0.01	3.25×10^2 ± 11.40
PDS-Flag-12	6.74×10^5 ± 6.77	0.62 ± 0.01	3.03×10^2 ± 15.58

Acknowledgments

The authors thank Dr. Laura Carreon Palau for her instructional support regarding the pigments extraction and quantification process by HPLC. This research was supported by Fondef IDeA grant # CA12I10214.

Author contributions

J. Galarza performed mostly all the experiments. J. Gimpel designed the expression vector. B.O. Arredondo helped carry out the quantification process by HPLC. V. Rojas helped supervised the project. V. Henríquez directed the project and wrote the manuscript with support from J. Gimpel and J. Galarza. All the authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

References

[1] M.R. Shah, Y. Liang, J.J. Cheng, M. Daroch, Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products, Front. Plant Sci. 7 (2016) 531 https://doi.org/10.3389/fpls.2016.00531.
[2] M.A. Borowitzka, High-value products from microalgae—their development and commercialisation, J. Appl. Phycol. 25 (2013) 743–756.
[3] M. Guerin, M.E. Huntley, M. Olaizola, Haematococcus astaxanthin: applications for human health and nutrition, Trends Biotechnol. 21 (2003) 210–221.
[4] M. Olaizola, Commercial development of microalgal biotechnology: from the test tube to the marketplace, Biomol. Eng. 20 (2003) 459–466.
[5] A.M. Collins, H.D.T. Jones, D. Han, Q. Hu, T.E. Beaechem, J.A. Timlin, Carotenoid distribution in living cells of *Haematococcus pluvialis* (chlorophyceae), PLoS One 6 (2011).
[6] S. Biswal, Oxidative stress and astaxanthin: the novel supernutrient carotenoid, Int. J. Health Allied Sci. 3 (2014) 147–153.
[7] Y. Lemoine, N. Rmiki, A. Creach, J. Rachidi, B. Schoefs, Cytoplasmic accumulation of astaxanthin by the green alga *Haematococcus pluvialis* (flotow), in: B. Schoefs (Ed.), Plant Cell Organelles-Selected Topics, Research Signpost, Trivandrum, 2008, pp. 251–284.
[8] P. Moulin, Y. Lemoine, B. Schoefs, Carotenoids and stress in higher plants and algae, in: M. Pessarakli (Ed.), Handbook of Plant and Crop Stress, 3rd ed., Taylor and Francis, New York, 2010.
[9] A.C. Guedes, H.M. Amaro, F.X. Malcata, Microalgae as sources of carotenoids, Mar. Drugs 9 (2011) 625–644.
[10] D. Han, Y. Li, Q. Hu, Astaxanthin in microalgae: pathways, functions and biotechnological implications, Algae 28 (2013) 131–147.
[11] S. Leu, S. Boussiba, Advances in the production of high value products by microalgae, Ind. Biotechnol. 10 (2014) 169–183.
[12] R.R. Ambati, P.S. Moi, S. Ravi, R.G. Aswathanarayana, Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review, Mar. Drugs. 12 (2014) 128–152 http://dx.http://dx.doi.org/10.3390/md12010128.
[13] K. Chekanov, E. Lobakove, I. Selyakh, L. Semenova, R. Sidorof, A. Solovchencko, Accumulation of astaxanthin by a new *Haematococcus pluvialis* strain BM1 form the white sea coastal rocks (Russia), Mar. Drugs. 12 (8) (2014) 4504–4520, http://dx.doi.org/10.3390/md12084504.
[14] H. Lichtenthaler, The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 47–65.
[15] Y. Lemoine, B. Schoefs, Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress, Photosynth. Res. 106 (2010) 155–177.
[16] J. Steinbrenner, G. Sandmann, Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis, Appl. Environ. Microbiol. 72 (2006) 7477–7484 http://dx.http://dx.doi.org/10.1128/AEM.01461-06.
[17] J. Liu, Z. Sun, H. Gerken, J. Huang, Y. Jiang, F. Chen, Genetic engineering of the green alga *Chlorella zofingiensis*: a modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker, Appl. Microbiol. Biotechnol. 98 (2014) 5069–5079 http://dx.http://dx.doi.org/10.1007/s00253-014-5593-y.
[18] L. Liu, H. Gerken, J. Huang, F. Chen, Engineering of an endogenous phytoene desaturase gene as a dominant selectable marker for *Chlamydomonas reinhardtii* transformation and enhanced biosynthesis of carotenoids, Process Biochem. 48 (2013) 788–79595 http://dx.http://dx.doi.org/10.1016/j.procbio.2013.04.020.
[19] D. Chamovitz, G. Sandmann, J. Hirschberg, Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis, J. Biol. Chem. 268 (1993) 17348–17353.
[20] H. Eom, C.G. Lee, E. Jin, Gene expression profile analysis in astaxanthin-induced *Haematococcus pluvialis* using a cDNA microarray, Planta 223 (2006) 1231–1242.
[21] Z. Gao, Y. Li, G. Wu, G. Li, H. Sun, S. Deng, Y. Shen, G. Chen, R. Zhang, C. Meng, X. Zhang, Transcriptome analysis in *Haematococcus pluvialis*: Astaxanthin induction by Salicylic Acid (SA) and Jasmonic Acid (JA), PLoS ONE 10 (2015), http://dx.doi.org/10.1371/journal.pone.0140609.
[22] G. Farré, D. Blancquaert, T. Capell, D. Van Der Straeten, P. Christou, C. Zhu, Engineering complex metabolic pathways in plants, Annu. Rev. Plant Biol. 65 (2014) 187–223.
[23] G. Farré, R.M. Twyman, P. Christou, T. Capell, C. Zhu, Knowledge-driven approaches for engineering complex metabolic pathways in plants, Curr. Opin. Biotechnol. 32 (2015) 54–60 http://dx.http://dx.doi.org/10.1016/j.copbio.2014.11.004.
[24] A.A. Saei, P. Ghanbari, A. Barzegari, *Haematococcus* as a promising cell factory to produce recombinant pharmaceutical proteins, Mol. Biol. Rep. 39 (2012) 9931–9939.
[25] V. Henríquez, C. Escobar, J. Galarza, J. Gimpel, Carotenoids in microalgae, in: C. Stange (Ed.), Carotenoids in Nature: Biosynthesis, Regulation and Function, 2014, pp. 219–237.
[26] J.A. Gimpel, V. Henríquez, S.P. Mayfield, In metabolic engineering of eukaryotic microalgae: potential and challenges come with great diversity, Front. Microbiol. 6 (2015) 1376, http://dx.doi.org/10.3389/fmicb.2015.01376.
[27] K.H. Wong, Transgenic *Chlamydomonas reinhardtii* as an Experimental System to Study the Regulation of Carotenoid Biosynthesis in Green Microalgae, The University of Hong Kong, Hong Kong, 2006 Ph.D. Thesis.
[28] R. León, I. Couso, E. Fernandez, Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*, J. Biotechnol. 130 (2007) 143–152.
[29] K.J. Zheng, C.G. Wang, M. Xiao, J. Chen, J.C. Li, Z.L. Hu, Expression of *bkt* and *bch* genes from *Haematococcus pluvialis* in transgenic Chlamydomonas, Sci. China Life Sci. 57 (2014) 1028–1033 http://dx.http://dx.doi.org/10.1007/s11427-014-4729-8.
[30] S. Kathiresan, A. Chandrashekar, G.A. Ravishankar, R. Sarada, Regulation of astaxanthin and its intermediates through cloning and genetic transformation of β-carotene ketolase in *Haematococcus pluvialis*, J. Biotechnol. 196–197 (2015) 33–41 http://dx.doi https://doi.org/10.1016/j.jbiotec.2015.01.006.
[31] K. Grünewald, M. Eckert, J. Hirschberg, C. Hagen, Phytoene desaturase is localized exclusively in the chloroplast and upregulated at the mRNA level during accumulation of secondary carotenoids in *Haematococcus pluvialis* (Volvocales, Chlorophyceae), Plant Physiol. 122 (2000) 1261–1268.
[32] A.E. Solovchenko, Physiology and adaptive significance of secondary carotenogenesis in green microalgae, Russian, J. Plant Physiol. 60 (2013) 1–13.
[33] C.P. Tan, F.Q. Zhao, Z.L. Su, C.W. Liang, S. Qin, Expression of B-carotene hydroxylase gene (*crtR-B*) from the green alga *Haematococcus pluvialis* in chloroplasts of *Chlamydomonas reinhardtii*, J. Appl. Phycol. 19 (2007) 347–355.
[34] C.L. Gutiérrez, J. Gimpel, C. Escobar, S.H. Marshall, V. Henríquez, Chloroplast genetic tool for the green microalgae *Haematococcus pluvialis* (chlorophyceae, volvocales), J. Phycol. 48 (2012) 976–983, http://dx.doi.org/10.1111/j.1529-8817.2012.01178.x.
[35] P.I. Gómez, I. Inostroza, M. Pizarro, J. Pérez, From genetic improvement to commercial-scale mass culture of a Chilean strain of the green microalga *Haematococcus pluvialis* with enhanced productivity of the red ketocarotenoid astaxanthin, AOB Plants 5 (2013) 1–7, http://dx.doi.org/10.1093/aobpla/plt026.
[36] P.M. Sharp, W.H. Li, The codon Adaptation Index—a measure of directional synonymous codon usage bias, and its potential applications, Nucleic Acids Res. 15 (1987) 1281–1295.
[37] F. Geu-Flores, H.H. Nour-Eldin, M.T. Nielsen, B.A. Halkier, USER fusion: a rapid and efficient method for simultaneous fusion and cloning of multiple PCR products, Nucleic Acids Res. 35 (2007) e55http://dx.http://dx.doi.org/10.1093/nar/gkm106.
[38] D. Barrera, J. Gimpel, S. Mayfield, Rapid screening for the robust expression of recombinant proteins in algal plastids, in: P. Maliga (Ed.), Chloroplast Biotechnology, Methods in Molecular Biology, 1132 2014, pp. 391–399.
[39] Fuji Chemical Industry Co. Ltd., Spectrophotometric and HPLC Analysis Method for Determining Astaxanthin Content in AstaREAL-L10, Report, Japan (2009).
[40] E.W. Becker, Microalgae: Biotechnology and Microbiology, Cambridge University Press, Cambridge, UK, 1994.
[41] B. Arredondo, D. Voltolina, Evaluación de la biomasa microalgal: concentración, recuento celular y tasa de crecimiento, CIBNOR (Ed.), Métodos y herramientas analíticas en el análisis de la biomasa microalgal, 2007, pp. 21–32.
[42] C. Sorokin, Dry weight, packed cell volume and optical density, in: J.R. Stein (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements, 1973, pp. 321–343.
[43] S. Franklin, B. Ngo, E. Efuet, S. Mayfield, Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast, Plant J. 30 (2002) 733–744.
[44] R. Surzycki, K. Greenham, K. Kitayama, F. Dibal, R. Wagner, J.D. Rochaix, T. Ajam, S. Surzycki, Factors effecting expression of vaccines in microalgae, Biologicals 37 (2009) 133–138.
[45] E.S. Specht, S. Miyake-Stoner, S. Mayfield, Micro-algae come of age as a platform for recombinant protein production, Biotechnol. Lett. 32 (2010) 1373–1383.
[46] D.J. Barrera, S.P. Mayfield, High-value recombinant protein production in microalgae, in: A. Richmond, Q. Hu (Eds.), Handbook of Microalgal Culture: Applied Phycology and Biotechnology, 2013, pp. 532–544.

[47] R. Sharon-Gojman, E. Maimon, S. Leu, A. Zarka, S. Boussiba, Advanced methods for genetic engineering of *Haematococcus pluvialis* (Chlorophyceae, Volvocales), Algal Res. 10 (2015) 8–15, http://dx.doi.org/10.1016/j.algal.2015.03.022.
[48] A. Day, M. Goldschmidt-Clermont, The chloroplast transformation toolbox: selectable markers and marker removal, Plant Biotechnol. J. 9 (2011) 540–553.
[49] S. Purton, J.B. Szaub, T. Wannathong, R. Young, C.K. Economou, Genetic engineering of algal chloroplasts: progress and prospects, Russ. J. Plant Physiol. 60 (2013) 521–528, http://dx.doi.org/10.1134/S1021443713040146.
[50] Y. Cui, S. Qin, P. Jiang, Chloroplast transformation of *Platymonas* (*Tetraselmis*) *subcordiformis* with the *bar* gene as selectable marker, PLoS One 9 (6) (2014) e98607http://dx.http://dx.doi.org/10.1371/journal.pone.0098607.
[51] S. Purton, Tools and techniques for chloroplast transformation of *Chlamydomonas*, Adv. Exp. Med. Biol. 616 (2007) 34–45.
[52] P. Mulo, I. Sakurai, E.M. Aro, Strategies for *psbA* gene expression in cyanobacteria, green algae and higher plants: from transcription to PSII repair, Biochim. Biophys. Acta 1817 (2012) 247–257 http://dx.http://dx.doi.org/10.1016/j.bbabio.2011.04.011.
[53] A.C. Barbrook, C.J. Howe, S. Purton, Why are plastid genomes retained in non-photosynthetic organisms? Trends Plant Sci. 11 (2006) 101–108.
[54] A.B. Lopez, Y. Yang, T.W. Thannhauser, L. Li, Phytoene desaturase is present in a large protein complex in the plastid membrane, Physiol. Plant. 133 (2008) 190–198.
[55] M. Li, Z. Gan, Y. Cui, C. Shi, X. Shi, Structure and function characterization of the phytoene desaturase related to the lutein biosynthesis in *Chlorella protothecoides* CS-41, Mol. Biol. Rep. 40 (2013) 3351–3361.
[56] A.D.D. Baroli, T. Yamane, K.K. Niyogi, Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects *Chlamydomonas reinhardtii* from photo-oxidative stress, Plant Cell 15 (2003) 992–1008.
[57] A. Cifuentes, M. González, S. Vargas, Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions, Biol. Res. 36 (2003) 343–357.