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Genetic engineering of the green alga *Chlorella zofingiensis*: a modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker

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Abstract The unicellular green alga *Chlorella zofingiensis* has been proposed as a promising producer of natural astaxanthin, a commercially important ketocarotenoid. But the genetic toolbox for this alga is not available. In the present study, an efficient transformation system was established for *C. zofingiensis*. The transformation system utilized a modified norflurazon-resistant phytoene desaturase (*PDS*-L516F, with an leucine-phenylalanine change at position 516) as the selectable marker. Three promoters from endogenous *PDS*, nitrate reductase (*NIT*), and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*) genes were tested, with the *RBCS* promoter demonstrating the highest transformation efficiency. Inclusion of the first intron of the *PDS* gene further enhanced the efficiency by 91 %. Both particle bombardment and electroporation methods were examined, and the latter gave a fourfold higher transformation efficiency. The introduction of *PDS*-L516F, which exhibited a 33 % higher desaturation activity than the unaltered enzyme, enabled *C. zofingiensis* to produce 32.1 % more total carotenoids (TCs) and 54.1 % more astaxanthin. The enhanced accumulation of astaxanthin in transformants was revealed to be related to the increase in the transcripts of *PDS*, β-carotenoid ketolase (*BKT*), and hydroxylase (*CHYb*) genes. Our study clearly shows that the modified *PDS* gene is a dominant selectable marker for the transformation of *C. zofingiensis* and possibly for the genetic engineering of the carotenoid biosynthetic pathway. In addition, the engineered *C. zofingiensis* might serve as an improved source of natural astaxanthin.

Keywords Astaxanthin · *Chlorella zofingiensis* · Genetic engineering · Norflurazon · Phytoene desaturase · Transformation

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Introduction

Astaxanthin is a high-value ketocarotenoid found in some algae, bacteria, yeasts, and many marine organisms (Schmidt et al. 2011). It possesses a broad range of applications in food, feed, nutraceutical, and pharmaceutical industries because of its pigmentation property and strong antioxidative activity (Fraser and Bramley 2004; Guerin et al. 2003). Currently, natural astaxanthin is primarily produced from the green alga

Haematococcus pluvialis (Guerin et al. 2003). The production capacity of *H. pluvialis*, however, is restricted by its slow growth, low biomass density, and ease of contamination. In contrast, *Chlorella zofingiensis*, another astaxanthin-producing green alga, is able to grow fast and achieve high cell density under photoautotrophic (Del Campo et al. 2004), mixotrophic (Ip et al. 2004), and heterotrophic (Ip and Chen 2005) conditions and, thus, has been suggested to be a promising alternative for astaxanthin production (Liu et al. 2012a, 2013a; Sun et al. 2008). The major constraint of this alga is its relatively low cellular content of astaxanthin. One strategy to overcome the low astaxanthin content is to manipulate the carotenoid biosynthetic pathway in *C. zofingiensis* through genetic engineering.

Over the past decades, the carotenoid biosynthetic pathway and carotenogenic genes have been thoroughly investigated, which has greatly facilitated the genetic manipulation of carotenoid biosynthesis for special purposes, such as the enhancement of preexisting carotenoids (Romer et al. 2000; Shewmaker et al. 1999; Steinbrenner and Sandmann 2006) and the production of new pigments (Huang et al. 2013; Jayaraj et al. 2008; Mann et al. 2000; Morris et al. 2006; Ralley et al. 2004; Zhong et al. 2011). While the engineering of carotenoid biosynthesis in higher plants has achieved great progress, it still remains at the very preliminary stage in many commercially important algae due to the shortage of suitable selectable markers for the development of functional transformation systems. Exogenous genes are likely to undergo epigenetic silencing in microalgae (Cerutti et al. 1997; Ohresser et al. 1997; Poulsen and Kröger 2005). So, modified endogenous genes are sought as selectable markers and have proven effective in several algae (Dawson et al. 1997; Kovar et al. 2002; Liu et al. 2013b; Randolph-Anderson et al. 1998; Steinbrenner and Sandmann 2006).

Phytoene desaturase catalyzes a rate-limiting step in carotenoid biosynthesis (Chamovitz et al. 1993; Liu et al. 2010a; Steinbrenner and Sandmann 2006). Overexpression of *PDS*- or *crt*I-type phytoene desaturase genes has been demonstrated to elevate the production of carotenoids in transgenic plants and algae (Liu et al. 2013b; Misawa et al. 1993; Romer et al. 2000; Steinbrenner and Sandmann 2006). PDS is also a target of various bleaching herbicides such as norflurazon and fluridone (Chamovitz et al. 1993). We reported previously the isolation and characterization of the *PDS* gene from the *C. zofingiensis* mutant E17 (Liu et al. 2010a). This *PDS* mutant, with an amino acid change from leucine (L) to phenylalanine (F) at position 516 (L516F), was revealed to encode an enzyme with norflurazon resistance and improved desaturation activity. Since *C. zofingiensis* is very sensitive to the herbicide norflurazon, *PDS*-L516F may be adopted as a dominant selectable marker for the transformation of this alga. It is also reasonable to expect that the expression of *PDS*-L516F gene would enhance the flux of colored carotenoids in transgenic *C. zofingiensis*.

In the present study, we described, for the first time, the establishment of a stable nuclear transformation of *C. zofingiensis*. The transformation system utilizes *PDS*-L516F as the selectable marker, which is driven by endogenously derived promoters. Both particle bombardment and electroporation methods proved effective in achieving efficient transformation of *C. zofingiensis*. The introduction of *PDS*-L516F resulted in not only norflurazon resistance but also the enhanced accumulation of carotenoids including astaxanthin. Our results will expand the understanding of genetic engineering of *C. zofingiensis* and may allow the direct manipulation of the astaxanthin biosynthetic pathway to further increase astaxanthin content. The establishment of a stable transformation system may also facilitate the engineering of *C. zofingiensis*, or other oleaginous microalgae, for better oil production.

Materials and methods

Algal strain and culture conditions

The green microalga *C. zofingiensis* (ATCC 30412) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). This alga was maintained at 4 °C on an agar slant of CZM1 medium (Ip et al. 2004) consisting of (per liter) 0.5 g KNO_3, 0.62 g $NaH_2PO_4\ H_2O$, 0.089 g $Na_2HPO_4\ 2H_2O$, 0.247 g $MgSO_4\ 7H_2O$, 14.7 mg $CaCl_2\ 2H_2O$, 6.95 mg FeSO4 $7H_2O$, 0.061 mg H_3BO_3, 0.169 mg $MnSO_4\ H_2O$, 0.287 mg $ZnSO_4\ 7H_2O$, 0.0025 mg $CuSO_4\ 5H_2O$, and 0.01235 mg $(NH_4)_6MO_7O_{24}\ 4H_2O$. The pH of the medium was adjusted to 6.8 prior to autoclaving.

Ten milliliters of liquid medium was inoculated with cells from slants, and the alga was grown aerobically in 100-mL flasks at 25 °C for 4 days with orbital shaking at 150 rpm and illuminated with continuous light of 25 μmol photons $m^{-2}\ s^{-1}$. The cells were then inoculated at 10 % (*v*/*v*) into 250-mL flasks provided with illumination of 50 μmol photons $m^{-2}\ s^{-1}$ and aeration of 1.5 % CO_2-enriched air (normal growth condition), grown to late exponential phase, and used as seed cultures for subsequent experiments.

For the transformation experiments, the seed culture was inoculated into a new 250-mL flask (5×10^5 cells mL^{-1}) illuminated with a light intensity of 50 μmol photons $m^{-2}\ s^{-1}$ and allowed to grow to 5×10^6 cells mL^{-1}.

For the molecular characterization and growth comparison, seed cultures of wild type (WT) and transformants of *C. zofingiensis* were inoculated at a density of 1×10^6 cells mL^{-1} into 250-mL flasks. Cells were cultured under normal growth conditions for 4 days.

For astaxanthin induction, seed cultures of WT and transformants of *C. zofingiensis* were inoculated into 250-mL flasks at a density of 1×10^6 cells mL^{-1} and allowed

to grow for 4 days under normal growth conditions. The cells were then centrifuged, resuspended in nitrogen-deprived medium (giving about 0.5 g L^{-1}), and grown under high light at 300 μmol photons m^{-2} s^{-1} (HL-N) for another 4 days.

Site-directed mutagenesis and *E. coli* expression of PDS

The previously constructed plasmid pUC-czPDS, which contains the *C. zofingiensis PDS* coding sequence (Huang et al. 2008, GenBank accession number EF621406), was used as the template for site-directed mutagenesis. The single-amino acid substitutions of R279P, L399P, and V483G were obtained by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) with the primers shown in Table S1. The L516R and L516F mutations were generated by Liu et al. (2010a). Mutations were verified by sequencing. The plasmids were introduced into *E. coli* JM109 individually for PDS enzyme expression.

In vitro PDS assay

The *E. coli* transformant cells were grown in SOB medium containing 50 μg mL^{-1} ampicillin at 37 °C with vigorous shaking, and 1 mM IPTG was added when the optical density at 600 nm reached 0.5. After a 5-h induction period, the cells were harvested for preparation of crude PDS proteins.

The *E. coli* cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.2, containing 1 mM DTT) and then passed through a French pressure cell (Spectronic Instruments, Rochester, NY, USA) at an internal pressure of 20 MPa. To the broken cell extract, 10 μg mL^{-1} DNase was added, and the mixture was incubated on ice for 15 min. Cell debris was removed from the suspension by centrifugation at 10,000*g*, and the resultant supernatant was adjusted to 1 mg protein mL^{-1} and used as a source of crude PDS enzyme. The reaction mixture contained 990 μL of crude PDS enzyme, 5 μL of the substrate phytoene (2 μg) in acetone, and 5 μL of decylplastoquinone (10 mM solution in methanol; Sigma, St. Louis, MO, USA). Phytoene was extracted from *E. coli* JM109/pACCRT-EB freeze-dried cells. The assays were carried out at 28 °C with vigorous shaking for 6 h and terminated by the addition of 1 mL of methanol. The residual phytoene and enzymatically ζ-carotene formed were extracted from the incubation mixture with diethyl ether/petroleum ether (1:9, *v*/*v*), evaporated to dryness under a nitrogen stream, and resuspended in acetone for HPLC analysis. PDS activity was calculated in terms of formed ζ-carotene and expressed as micrograms ζ-carotene per milligram protein) per hour. The herbicide resistance of PDS was conducted according to Liu et al. (2010a).

Construction of the transformation vectors

The primers PDS_f1 and PDS_r1 (Table S1) were deployed to amplify a *PDS* fragment containing a 1.08-kb promoter and a 0.24-kb coding sequence, using genomic DNA as the template. The primers PDS_f2 and PDS_r2 were used to amplify another *PDS* fragment that contains a 1.46-kb coding sequence and a 0.4-kb terminator, using the cDNA prepared from the mutant strain E17 (Liu et al. 2010a; containing the L516F mutation) as the template. The two fragments were digested with *Hin*dIII and ligated. The ligated product was then digested with *Xba*I/*Bam*HI and inserted into the corresponding restriction sites of the pBluescript SKII(+) vector (Stratagene, La Jolla, CA, USA), resulting in the transformation vector pCZT1 (Fig. 1a).

Three additional transformation vectors were constructed (Fig. 1b), by using a restriction enzyme-independent overlap extension PCR cloning strategy (Bryksin and Matsumura 2010). pCZT2 and pCZT3 are derived from pCZT1, with the *PDS* promoter substituted with a 1.14-kb nitrate reductase (*NIT*, GenBank accession number KC316012) promoter and a 0.9-kb ribulose bisphosphate carboxylase/oxygenase small subunit (*RBCS*, GenBank accession number KC316010) promoter, respectively. pCZT4 is derived from pCZT3, containing the first intron sequence (0.45 kb) of the *PDS* gene (GenBank accession number EF621406).

C. zofingiensis transformation protocol

Both particle bombardment and electroporation methods were employed for *C. zofingiensis* transformation. The *C. zofingiensis* cells prepared for transformation were collected and resuspended to a density of 1×10^8 cells mL^{-1} in liquid medium. For each bombardment, 0.2 mL of the concentrated cells was plated on filters on agar plates, using a Biolistic PDS-1000/He system (Bio-Rad, Hercules, CA, USA). Fifty microliters of a gold particle solution (0.6 μm, 60 mg mL^{-1}) was mixed with 5 μL of plasmid solution (1 μg μL^{-1}), 50 μL of 2.5 M $CaCl_2$, and 20 μL of 0.1 M spermidine (Sigma). The mixture was incubated at room temperature for 10 min and centrifuged for 10 s. The pellet was washed once with 70 % ethanol and twice with 100 % ethanol and resuspended in 50 μL ethanol. Each 10 μL of the DNA-coated gold particles was layered on a macrocarrier. Plates were bombarded from a distance of 6 cm under vacuum of 28 mmHg using 1,350-lb in^{-2} rupture disks. The bombarded cells were then washed off the filters with culture medium and allowed to recover for 24 h.

For electroporation transformation, the *C. zofingiensis* cells prepared for transformation were washed twice with deionized water (sterile, ice-cold) and resuspended to give a density of 1×10^8 cells mL^{-1}. For each electroporation, 0.2 mL of the concentrated cells containing 1 μg pCZT4 (linearized by *Xba*I) and 10 μg salmon sperm DNA (Invitrogen) were used. Electroporation was conducted using Gene Pulser® II (Bio-

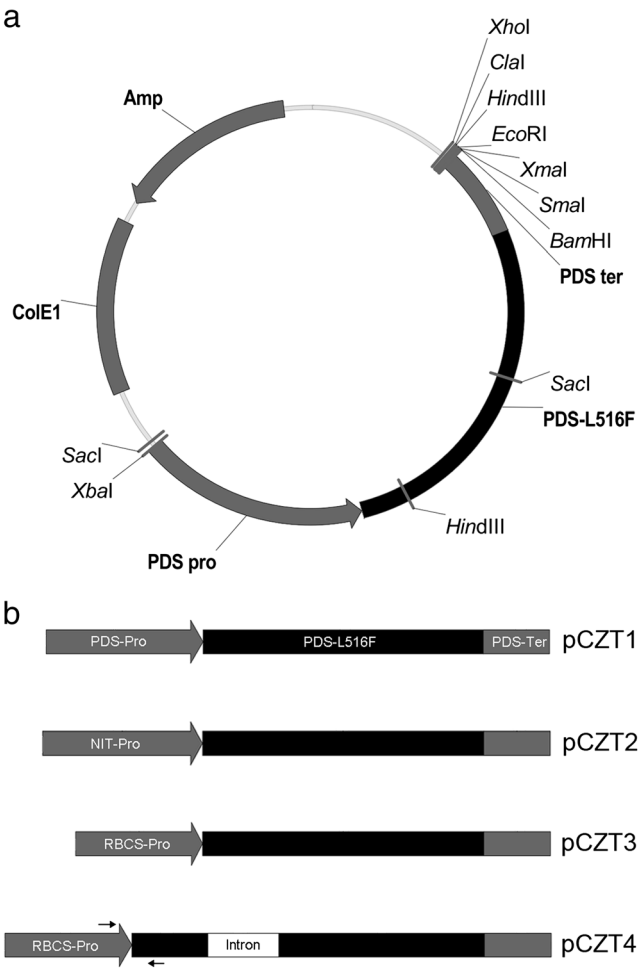


Fig. 1 **a** Map of *C. zofingiensis* transformation vector pCZT1 with restriction sites indicated. The E17 *PDS* cDNA (*PDS*-L516F) with its promoter and terminator is inserted into the vector pBluescript SKII(+). The construct also contains the ampicillin resistance gene, the *E. coli* origin of replication (ColE1). **b** Schematic illustration of four transformation vectors for *C. zofingiensis*. pCZT1 contains a 1.08-kb *PDS* promoter, the coding sequence of *PDS-L516F*, and a 0.4-kb *PDS* promoter. pCZT2 and pCZT3 are derived from pCZT1, with the *PDS* promoter substituted with a 1.14-kb *NIT* promoter and a 0.9-kb *RBCS* promoter, respectively. pCZT4 is derived from pCZT3, containing the first intron sequence (0.45 kb) of the *PDS* gene. *Arrows* on pCZT4 indicate the location of primers for PCR determination of *C. zofingiensis* transformants

Rad) with capacitance at 50 μF, resistance at 200 ohms, and field strength ranging between 0.5 and 12 kV cm^{-1}. The electroporated cells were then allowed to recover in culture medium for 24 h.

After a 24-h recovery, the transformed cells were spread on agar plates containing 0.5 μM norflurazon for selection. Colonies appearing after 3 to 4 weeks were picked up and restreaked three times on selective plates.

Genomic DNA extraction and Southern blot

DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Stewart and Via 1993). DNA concentration was determined spectrophotometrically at 260 nm, and the quality was checked by electrophoresis. Eight milligrams of genomic DNA was digested with *Sac*I+*Xba*I, which have no recognition sites in the probed region of the *PDS* gene. The digested DNA was separated on a 0.8 % agarose gel, transferred to a positively charged nylon membrane (Roche, Mannheim, Germany), and hybridized with digoxigenin (DIG)-labeled DNA probes in the presence of 50 % (*v*/*v*) formamide at 47 °C. DNA probe was prepared by amplifying a 339-bp fragment of the *PDS* gene with a pair of specific primers (Probe_f and Probe_r, Table S1) from the genomic DNA (30 cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 30 s). Probe labeling and hybridization were carried out according to the instructions in the DIG Nonradioactive Nucleic Acid Labeling and Detection System (Roche). After hybridization, the membrane was washed twice with 0.1× standard saline citrate (SSC) containing 0.1 % sodium dodecyl sulfate (SDS) at 68 °C for 15 min. The membrane was developed with an X-ray film in a dark room.

RNA isolation, reverse transcription (RT)-PCR, and quantitative real-time PCR assays

RNA was isolated from aliquots of about 10^8 cells harvested under different conditions using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm, and the quality was checked by electrophoresis. Total RNA (1 μg) extracted from different samples was reverse-transcribed to cDNA by using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) primed with oligo(dT) according to the manufacturer's instructions. RT-PCR was carried out as described by Liu et al. (2012a, b), with *C. zofingiensis* actin gene being used to demonstrate equal amounts of templates and loading. Quantitative real-time PCR was performed in a Bio-Rad iCycler IQ Multicolor Real-Time PCR Detection System (Bio-Rad) in the presence of a SYBR Green PCR Master Mix (Invitrogen). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using actin gene for normalization. The GenBank accession numbers for *PSY*, *PDS*, *BKT*, and *CHYb* were FR670783, EF621405, AY772713, and EU016205, respectively. The primers used for expression level analysis of these genes were shown in Table S1.

Extraction and analysis of pigments

Cell samples were harvested and freeze-dried on a DW3 freeze drier (Heto Dry Winner, Denmark). Extraction was carried out with acetone and liquid nitrogen until the cell debris was almost colorless. The extracts were filtered through

a 0.22-μm Millipore organic membrane. On a Waters Spherisorb® ODS2 analytical column (5 μm, 4.6×250 mm) with a Waters HPLC system (Waters, Milford, MA, USA), 20 μL of each extract was separated by HPLC. Pigments were eluted at a flow rate of 1.2 mL min^{-1} with a linear gradient from 100 % solvent A [acetonitrile/methanol/0.1 M Tris-HCl (pH 8.0), 84:2:14, by volume] to 100 % solvent B (methanol/ethyl acetate, 68:32, *v*/*v*) over a 15-min period, followed by 10 min of solvent B. Individual carotenoids were identified by their absorption spectra, and their typical retention times were compared to standard samples of pure carotenoids.

Statistical analyses

All experiments were determined in biological triplicate to ensure the reproducibility. Experimental results were obtained as mean value ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Paired-samples *T* test was applied. The statistical significances were achieved when $p<0.05$.

Results

PDS engineering for herbicide resistance

PDS is inhibited by herbicides, e.g., norflurazon, fluridone, and flurochloridone, causing the accumulation of phytoene at the cost of colored carotenoids and a concurrent bleaching of algal cells. Certain mutations occurring on *PDS* give the cyanobacterium *Synechococcus* sp. PCC 7942 resistance against herbicides (Chamovitz et al. 1993). Four of the mutations located in the coding sequence correspond to R279P, L399P, V483G, and L516R of *C. zofingiensis* PDS. These four mutants, plus L516F (Liu et al. 2010a), were expressed in *E. coli* for in vitro desaturation assay and herbicide resistance determination. As shown in Table 1, all five mutants exhibited greater resistance against the herbicide norflurazon, of which L516F gave the highest resistance, 31-fold higher as compared to WT. Some mutants also showed resistance against other herbicides, but to a lesser extent (Table 1). Intriguingly, L516F enhanced the desaturation activity by 33 %, while the other four mutants attenuated the activity to different extents (Table 1). Therefore, PDS with L516F mutation may be a promising selectable marker for *C. zofingiensis* transformation.

C. zofingiensis transformation

Endogenous promoters have proven to be important for the efficient expression of genes in microalgae (Cerutti et al. 1997; Niu et al. 2011). In this study, three promoters, namely, *PDS*, *NIT*, and *RBCS*, were cloned and fused to the upstream of the *PDS*-L516F coding sequence, resulting in pCZT1, pCZT2, and pCZT3, respectively (Fig. 1). In addition, it has been reported that inclusion of introns from endogenous genes helps to enhance gene expression in microalgae (Lumbreras et al. 1998). Thus, a fourth transformation vector, pCZT4, was employed, which includes the first intron of the *PDS* gene (Fig. 1).

Particle bombardment has been demonstrated to be an effective method to deliver genes into the intact cells of microalgae (Dawson et al. 1997; Radakovits et al. 2011; Steinbrenner and Sandmann 2006). Using particle bombardment delivery, pCZT3 gave a significantly higher transformation efficiency than pCZT1 and pCZT2 (Fig. 2a), indicating that *RBCS* promoter can drive the expression of the selectable marker more efficiently. Inclusion of the first intron of the *PDS* gene further increased the transformation efficiency by 91 % (Fig. 2a), which is consistent with the previous study indicating that introns can improve stable expression of genes in microalgae (Lumbreras et al. 1998). However, the overall efficiency of transformation using particle bombardment is relatively low (Fig. 2a, 3×10^{-6}).

Electroporation is another frequently used transformation method, but less costly and simpler as compared to particle bombardment. The electroporation field strengths varying from 0.5 to 12 kV cm^{-1} were examined, using pCZT4 as the transformation vector. The highest transformation efficiency, 14×10^{-6}, was achieved with a field strength of 4,000 kV cm^{-1} (Fig. 2b), which is 3.7-fold higher than that obtained with the particle bombardment method.

Table 1 Herbicide resistance and desaturation activity of *C. zofingiensis* PDS as affected by site-directed mutations

	Desaturation activity	Herbicide resistance		
		Norflurazon	Fluridone	Flurochloridone
WT	1±0.08 a	1±0.1 a	1±0.2 a	1±0.1 a
R279P	0.32±0.03 b	18.1±1.3 b	1.6±0.2 ab	4.9±0.5 b
L399P	0.94±0.06 a	13.5±1.1 c	11.3±1.0 c	8.2±0.6 c
V483G	0.81±0.05 a	16.7±1.2 bc	2.3±0.3 b	2.6±0.2 d
L516F	1.33±0.07 c	31.4±2.4 d	1.3±0.2 a	1.9±0.1 e
L516R	0.67±0.04 d	28.3±2. 5 d	2.2±0.2 b	0.8±0.1 a

The desaturation activity and herbicide resistance were normalized to those of WT, which were set as 1. The values followed by the same letter in each column are not significantly different ($p>0.05$)

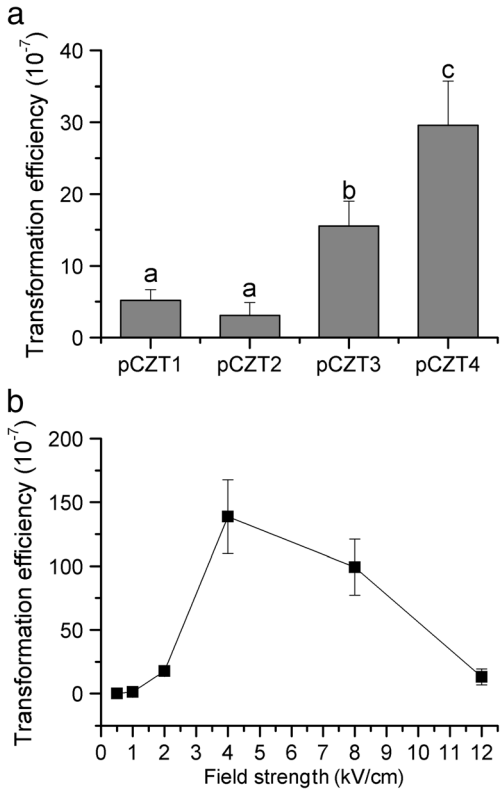


Fig. 2 The transformation efficiency of *C. zofingiensis* as affected by various transformation vectors using particle bombardment method (**a**) and by different electroporation field strengths using electro-transformation method (**b**). The values given the same letter are not significantly different ($p>0.05$)

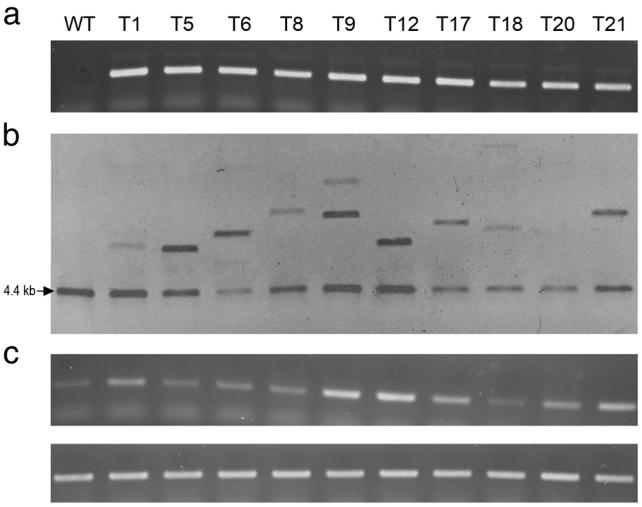


Fig. 3 Molecular characterization of *C. zofingiensis* transformants. **a** PCR determination of *C. zofingiensis* transformants using the primer set as shown in Table S1. **b** Southern blot analysis of *C. zofingiensis* transformants; the DNA from WT and transformants were digested with *Sac*I and *Xba*I and electrophoresed on a 0.8 % agarose gel; the 4.4-kb fragment indicates the endogenous *PDS*, while the larger fragments represent the *PDS*-L516F inserts. **c** Transcript analyses of *PDS* (*upper*) and *ACT* (*lower*) in *C. zofingiensis* transformants. The primers are shown in Table S1. Cells were harvested after 2-day cultivation with a continuous irradiation of 50 μmol m^{-2} s^{-1}

Characterization of PDS-L516F transformants

After transformation of *C. zofingiensis* with the vector pCZT4 and a regeneration period, the cells were spread on selective agar plates containing 0.5 μM norflurazon. Colonies showed up after an incubation period of 3–4 weeks. The colonies were restreaked three times on selective agar plates and then inoculated into liquid media. For transformant determination, PCR analysis was performed with primers (Table S1) specific to the *RBCS*::*PDS* chimeric fusion on the transformation vector (Fig. 1b). A total of ten randomly picked colonies from selective agar plates were compared to WT strain. While WT lacked the PCR product, all putative transformants examined yielded PCR products of the expected size (Fig. 3a), indicating successful nuclear integration of the introduced gene.

In addition, southern blot was used to further confirm the nuclear integration of *PDS*-L516F. DNA was isolated from the cultures of WT and transformants, digested with restriction enzymes, and hybridized with a 0.34-kb probe for the *PDS* gene. The endogenous *PDS* gene, corresponding to a 4.4-kb *Xba*I/*Sac*I excised fragment, was detected in WT and all tested transformants (Fig. 3b). All transformants possessed additional copies of *PDS*-L516F, which are larger than the endogenous *PDS* fragment (Fig. 3b).

To survey the effect of the transgene in the genome of transformants on the expression level of the *PDS* mRNA, RT-PCR analysis was performed. Cell samples were harvested from 2-day cultures under continuous illumination of 50 μmol photons m^{-2} s^{-1}. WT exhibited a basal expression of the *PDS* gene (Fig. 2c). In contrast, transformants, T9, T12, and T21 in particular, showed elevated levels of *PDS* transcripts (Fig. 2c). It is worth mentioning that the *PDS* expression levels do not correlate well with the number of integration events of the transgene (Fig. 2b, c).

To obtain further information about the expression of transgenic *PDS*-L516F and the functionality of the 0.9-kb *RBCS* promoter, cDNA synthesis and subsequent sequencing of the *PDS* transcript pool were conducted. A mixture of CTT (for WT *PDS*) and TTT (for *PDS*-L516F) at codon position 516 was observed for transformants' *PDS* cDNA pool. This finding indicates that the 0.9-kb promoter is sufficient to drive the expression of transgene in *C. zofingiensis*.

Six transformants, namely, T1, T9, T12, T17, T20, and T21 with elevated *PDS* transcripts, were chosen for pigment analysis, using 2-day cultures under HL-N condition. Only two transformants, i.e., T9 and T21, showed a significantly higher accumulation of total carotenoids (TCs) than WT (Table 2). T9 and T21 also produced more astaxanthin. As for chlorophylls, no significant difference was observed between transformants and WT (Table 2).

Table 2 Pigment contents of *C. zofingiensis* WT and transformants exposed to HL-N for 2 days

	Pigment content (mg g^{-1} dry weight)		
	TCs	Astaxanthin	Chlorophylls a+b
WT	7.0±0.4	3.4±0.2	4.5±0.2
T1	7.5±0.5	3.8±0.1	4.2±0.1
T9	9.3±0.4**	4.6±0.2**	4.3±0.3
T12	7.9±0.3	3.9±0.2	5.1±0.2
T17	6.9±0.4	3.3±0.1	4.5±0.3
T20	7.2±0.4	3.0±0.2	3.9±0.2
T21	8.8±0.3*	4.2±0.3*	4.4±0.1

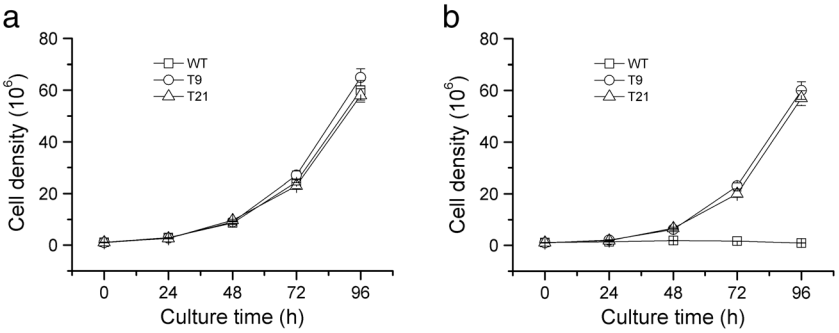
*$p<0.05$; **$p<0.01$ (significant difference when compared to the WT)

Resistance of transformants to the bleaching herbicide norflurazon

T9 and T21 were employed to examine the effect of norflurazon on growth and carotenoid accumulation. Cultures of transformants or WT with or without 0.5 μM norflurazon were grown under normal growth conditions (see "Materials and methods") for 4 days. In the absence of 0.5 μM norflurazon, transformants showed a similar growth rate to WT (Fig. 4a), indicating that the introduction of *PDS*-L516F does not affect algal growth. In the presence of norflurazon, the growth of WT was blocked, while transformants were just slightly inhibited (Fig. 4a, b). Norflurazon blocked TC biosynthesis in WT, resulting in the accumulation of phytoene, degradation of chlorophylls, and bleaching of algal cells (Fig. 4c). TC synthesis in transformants was also attenuated, but not significantly as compared to norflurazon-free cultures (Fig. 4c). These results indicate that transformants exhibited a strong resistance to norflurazon. In addition, the stability of transformants was analyzed, and after more than 50 subcultures under nonselective conditions, transformants retained the integrated transgene in the genome and showed no loss of norflurazon resistance (data not shown).

Enhanced accumulation of astaxanthin in transformants

C. zofingiensis accumulates astaxanthin under stress conditions such as high light and/or nitrogen deprivation (Del Campo et al. 2004; Liu et al. 2010a). The time course of TC and astaxanthin accumulation in WT and transformants cultured under HL-N conditions (see "Materials and methods") were examined, and the results are shown in Fig. 5. Upon induction of HL-N, TC content in both WT and transformants gradually increased, reaching a maximum at 48 h (Fig. 5a). During the first 48 h, transformants accumulated more (up to 32.1 % more) TCs than WT; thereafter, no significant difference was observed between transformants and WT (Fig. 5a). Unlike TCs, astaxanthin showed a drastic increase during the first 48 h of HL-N induction (Fig. 5a). Although there was no difference at 0 h, transformants produced significantly higher amounts of astaxanthin (up to 54.3 % higher, achieved in T21 after 24-h induction) than WT after 24- and 48-h induction of HL-N (Fig. 5b). Longer induction (e.g., 96 h), however, gave no significant difference in astaxanthin content between transformants and WT (Fig. 5b). In correlation with the higher content of astaxanthin, the cells of transformants exhibited a deeper orange color than WT (data not shown).



c

	Colored carotenoids (mg g^{-1})		Phytoene (mg g^{-1})		Chlorophylls a+b (mg g^{-1})	
	-	+	-	+	-	+
WT	5.1 ± 0.3	0.2 ± 0.0	ND	1.3 ± 0.1	19.1 ± 1.1	0.1 ± 0.0
T9	6.9 ± 0.4 *	6.2 ± 0.3 **	ND	0.1 ± 0.0 **	18.2 ± 0.9	16.6 ± 0.9 **
T21	6.5 ± 0.2 *	5.9 ± 0.2 **	ND	0.2 ± 0.0 **	20.9 ± 0.8	17.4 ± 0.8 **

Fig. 4 **a**, **b** Cell growth of WT and transformants under normal growth conditions without and with 0.5 μM norflurazon. **c** Contents of colored carotenoids, phytoene, and chlorophylls in cells of WT and transformants cultured for 4 days under normal growth conditions without (−) or with (+) 0.5 μM norflurazon. Values followed by *single* and *double asterisks* are significantly different at the levels of 0.05 and 0.01, respectively, when compared to WT. *ND* not detected

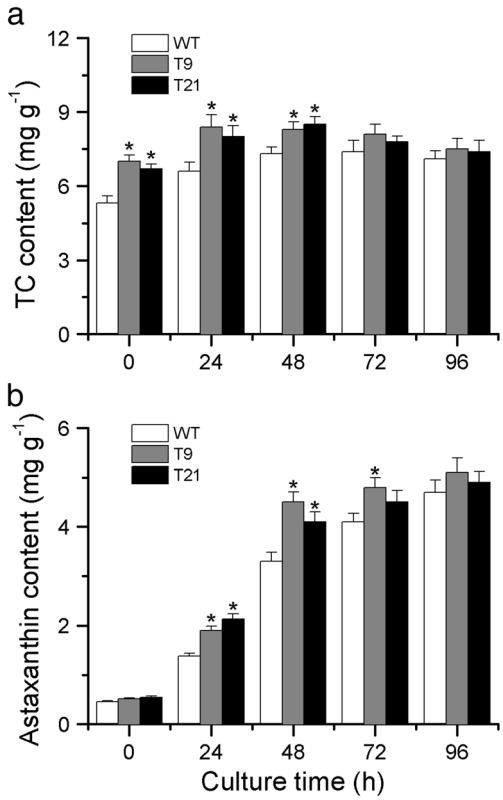


Fig. 5 Contents of TCs (**a**) and astaxanthin (**b**) of WT and transformants under the astaxanthin induction condition (HL-N). Values followed by *single asterisk* are significantly different ($p<0.05$) compared to WT

Transcription analysis of carotenogenic genes

It has been reported that the enhanced biosynthesis of carotenoids including astaxanthin in *C. zofingiensis* was correlated to the elevated transcript levels of carotenogenic genes (Liu et al. 2010a, 2012a). To survey if the enhanced accumulation of carotenoids in transformants was attributed to the upregulation of *PDS* and/or other carotenogenic genes, the transcript levels of *PSY*, *PDS*, *BKT*, and *CHYb* were quantified by using the quantitative real-time PCR approach. Cell samples after HL-N induction of 0, 12, 24, and 48 h were collected for analyses. All four genes examined were upregulated by HL-N and reached their maximums at 24 h (Fig. 6). Transformants accumulated more *PDS* transcripts than WT under both favorable and stress conditions (Fig. 6b; 0 h, 12–24 h). The overexpression of *PDS* in transformants may drive more carbon flux to carotenoid biosynthesis and thus result in enhanced accumulation of TCs in transformants (Fig. 5a). Transcription levels were also higher for *BKT* and *CHYb* in transformants, but only at 12 h of HL-N induction; further induction to 24 or 48 h gave no significant difference (Fig. 6c, d). These results correlate well with the higher amounts of astaxanthin accumulating in transformants at early stages of HL-N induction (Fig. 5b, 24–48 h). The expression of *PSY*, however, showed no significant difference between transformants and WT under the induction period of 0–48 h (Fig. 6a).

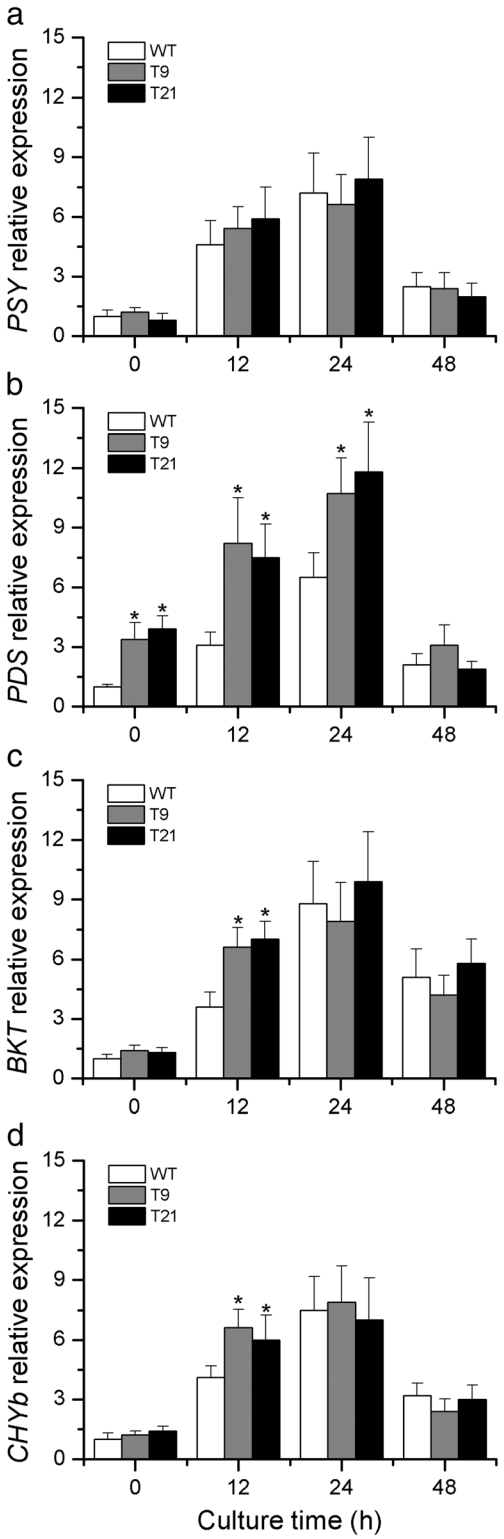


Fig. 6 Transcriptional expression analysis of carotenogenic genes of *PSY* (**a**), *PDS* (**b**), *BKT* (**c**), and *CHYb* (**d**) in WT and transformants under HL-N conditions. Values followed by *single asterisk* are significantly different ($p<0.05$) compared to WT

Discussion

While it is now easy to generate a transgenic plant, there are still significant technical challenges to develop a functional transgenic system for many commercially important microalgae including *Chlorella*. The efforts to transform *Chlorella* strains have achieved some successes during the past 20 years (Chow and Tung 1999; Hawkins and Nakamura 1999; Kim et al. 2002; Niu et al. 2011). The selectable markers used for *Chlorella* transformation consisted mainly of bacteria-derived bleomycin-binding protein, chloramphenicol acetyltransferase, hygromycin B phosphotransferase, and neomycin phosphotransferase II. These heterologous genes undergo likely epigenetic silencing in *Chlorella*, and proper endogenous promoters and/or enhancers are commonly required for stable expression. *Chlorella* transformation by functional complementation of endogenous genes (e.g., nitrate reductase) can also be achieved, provided that the corresponding gene-deficient mutant is available (Dawson et al. 1997). But there is no such a mutant available for *C. zofingiensis*. Engineering an endogenous *PDS* gene as the selectable marker may be a promising alternative choice (Liu et al. 2013b; Steinbrenner and Sandmann 2006).

In the present study, five PDS mutants were generated and PDS-L516F exhibited the highest resistance against the herbicide norflurazon (Table 1). This resistance, which is less than *H. pluvialis* PDS-L504R (Steinbrenner and Sandmann 2006) but comparable to *Chlamydomonas reinhardtii* PDS-L505F (Liu et al. 2013b), is sufficient for the selection of *C. zofingiensis*, as growth of this alga is very sensitive to norflurazon and can be completely inhibited by the presence of 0.25 μM norflurazon (Liu et al. 2010a). The introduction of *PDS*-L516F enabled *C. zofingiensis* transformants, but not WT, to grow under selection of norflurazon (Fig. 4b), indicating that this engineered *PDS* can be employed as a dominant selectable marker. The efficacy of promoters from endogenous genes, such as *NIT* (Dawson et al. 1997; Niu et al. 2011), *PDS* (Steinbrenner and Sandmann 2006), and *RBCS* (Cerutti et al. 1997; Lumbreras et al. 1998), has been demonstrated for transgene's expression in microalgae. Among the three promoters, *RBCS* promoter gave the highest transformation efficiency for *C. zofingiensis* (Fig. 2a), which may be explained by the fact that this promoter gives the highest expression of *PDS*-L516F. Even higher transformation efficiency can be achieved by including the first intron of the *PDS* gene in the selectable marker (Fig. 2a), indicating that the presence of an intron(s) helps to improve stable expression of the transgene (Lumbreras et al. 1998).

Microalgae generally possess a thick cell wall, which serves as a major barrier for the efficient delivery of foreign DNA. Particle bombardment has proven to be an effective method to introduce genes into microalgae (Dawson et al. 1997; Steinbrenner and Sandmann 2006; Radakovits et al. 2011), but it requires specific instruments and involves the expensive consumables such as gold or tungsten particles, making it neither easy to conduct nor cost-effective. In addition, those DNA-carrier particles are commonly over 0.6 μm in diameter and may cause a high off-target rate leading to relatively low transformation efficiency, in particular for the small-sized algae such as *Chlorella* and *Nannochloropsis*. In contrast, electroporation is convenient to perform and less costly. The transformation efficiency of electroporation is linked mainly to the field strength, and the optimal field strength is species/strain-dependent, for example, 1.8 kV cm^{-1} for *Chlorella vulgaris* (Chow and Tung 1999), 11 kV cm^{-1} for *Nannochloropsis* sp. (Kilian et al. 2011), and 4 kV cm^{-1} for *C. zofingiensis* in this study (Fig. 2b). With optimized electroporation conditions, the transformation

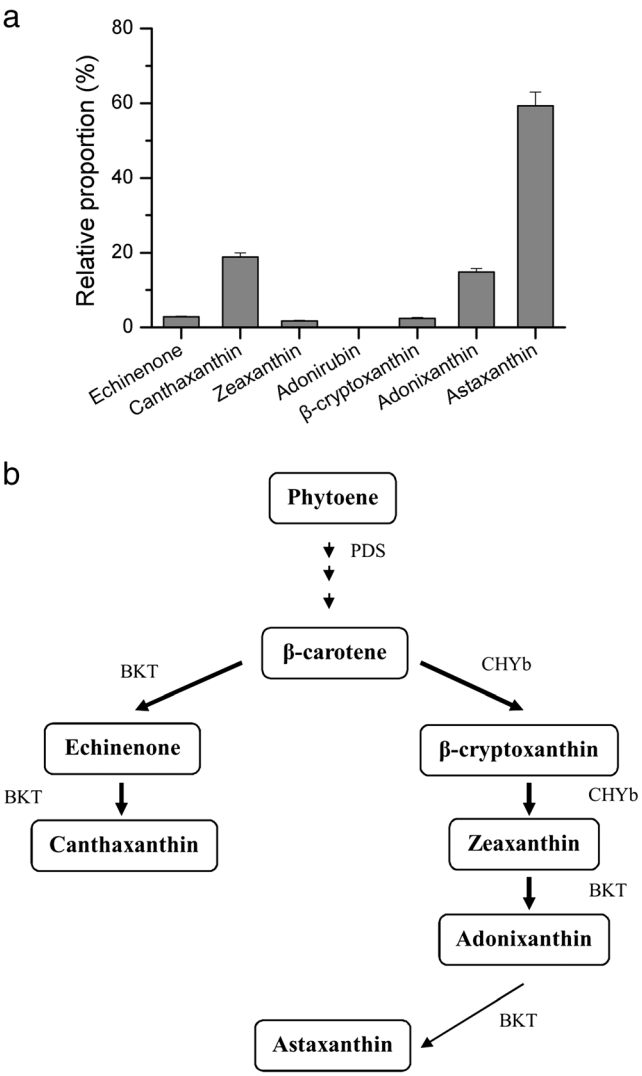


Fig. 7 **a** The relative proportion of carotenoids in *C. zofingiensis* under astaxanthin induction conditions (4-day induction with HL-N). Each carotenoid is expressed as percentage of the sum of echinenone, canthaxanthin, adonirubin, cryptoxanthin, zeaxanthin, adonixanthin, and astaxanthin, which is set as 100 %. Adonirubin is not detected. **b** The proposed astaxanthin biosynthetic pathway in *C. zofingiensis*

efficiency of *C. zofingiensis* reached up to 14×10^{-6}, which is acceptable for insertional mutagenesis purposes.

The integration of *PDS*-L516F in the *C. zofingiensis* genome was confirmed by both PCR and Southern blot analysis (Fig. 3a, b). All transformants contained additional copies of the *PDS* gene varying in sizes (Fig. 3b), suggesting that the integration of transgene occurred at different loci of the algal genome. The altered expression level of the *PDS* gene is most likely due to the presence of multiple gene copies in transformants (Fig. 3b, c). It is worth noting that the strength of expression was partially linked to the copy number of transgene, and there might be additional factors involved such as positional effect, which occurs commonly in transgenic higher plants and algae.

The enhanced expression level of the *PDS* gene not only conferred norflurazon resistance on transformants (Fig. 4) but also influenced their carotenoid composition (Fig. 5). Carotenoid analysis showed that transformants could accumulate higher amounts of TCs than WT under both favorable and stress conditions (Fig. 5a). This is in accordance with the previous findings that overexpression of the *PDS* gene resulted in elevated production of carotenoids in transgenic *H. pluvialis* (Steinbrenner and Sandmann 2006) and *C. reinhardtii* (Liu et al. 2013b). In addition to TCs, more astaxanthin was found in transformants (Fig. 5b). There is a possibility that the increased expression of *PDS* causes an increase in the flux of colored carotenoids, which in turn upregulates the expression of downstream astaxanthin biosynthetic genes such as *BKT* and *CHYb*, leading to increased astaxanthin production (Figs. 5 and 6). A similar phenomenon was observed in transgenic tomato plants in which overexpression of a *crt*I-type phytoene desaturase induced the upregulation of ζ-carotene desaturase and lycopene β-cyclase genes, leading to the elevation of β-carotene synthesis (Romer et al. 2000). Still, in *C. reinhardtii*, *PDS* overexpression does not upregulate the expression of other carotenogenic genes (Liu et al. 2013b). These results suggest that the phytoene desaturation step is rate-limiting for carotenoid biosynthesis in *C. zofingiensis*.

PDS overexpression drives more carbon toward carotenoid biosynthesis, which can enhance astaxanthin accumulation in *C. zofingiensis* but only under certain conditions such as at early stress stages (Fig. 5b). Directed engineering to pull carbon into astaxanthin biosynthesis may be a more efficient way to increase astaxanthin accumulation in *C. zofingiensis*. Considering the presence of substantial amounts of the end product canthaxanthin and the intermediate product adonixanthin (Fig. 7a), CHYb may not accept canthaxanthin as a substrate to produce astaxanthin and BKT might be insufficient to catalyze the formation of astaxanthin from adonixanthin in *C. zofingiensis* (Fig. 7b). It has been reported that CHYb from *H. pluvialis* can utilize canthaxanthin as the substrate for efficient synthesis of astaxanthin (Linden 1999) and BKT from *C. reinhardtii* has a high activity of converting adonixanthin to astaxanthin (Huang et al. 2012). Therefore, the manipulation of specific astaxanthin biosynthetic steps by introducing these two genes into *C. zofingiensis* may provide a pulling force for astaxanthin synthesis at the cost of both canthaxanthin and adonixanthin, which, when coupled with the pushing force from *PDS* overexpression, may represent a feasible strategy to further increase astaxanthin content and purity.

In conclusion, the results reported here clearly demonstrate that *PDS*-L516F gene can be adopted as a dominant selectable marker for the stable nuclear transformation of *C. zofingiensis*. The transformants overexpressing the *PDS* gene could enhance the flux of colored carotenoids, providing a proof of concept for genetic engineering of carotenoid biosynthesis in *C. zofingiensis* toward elevated astaxanthin production. *C. zofingiensis* is also able to accumulate a high level of oils (Liu et al. 2010b, 2011, 2012a, b), so the establishment of a transformation system for *C. zofingiensis* may facilitate the further exploration of the alga as a promising feedstock for biofuel production. To this end, "omics" analyses (genomics, transcriptomics, and proteomics) of *C. zofingiensis* are currently underway, which will help to understand the biosyntheses of both astaxanthin and oil and to develop a more sophisticated molecular toolbox for the manipulation of this strain toward economically feasible production of astaxanthin and/or oil.

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