Planta (2010) 232:61–67
DOI 10.1007/s00425-010-1132-y

ORIGINAL ARTICLE

One amino acid substitution in phytoene desaturase makes *Chlorella zofingiensis* resistant to norflurazon and enhances the biosynthesis of astaxanthin

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Received: 5 January 2010 / Accepted: 10 February 2010 / Published online: 10 March 2010

Abstract A stable *Chlorella zofingiensis* mutant (E17) produced by chemical mutagen was characterized with respect to growth, astaxanthin biosynthesis, and phytoene desaturation. The mutant E17 could grow well and produce normal levels of colored carotenoids in the presence of 0.25 μM norflurazon, in which the growth of wild type (WT) cells was greatly limited due to inhibited carotenoid formation. Induced by high-light irradiation or glucose, E17 produced 44 or 36% more astaxanthin than WT when cultured in media without norflurazon. A point mutation (C–T) was revealed to occur in the *PDS* gene of E17, leading to an amino acid change (L516F) in its coding region. The mutated PDS exhibited 31-fold resistance to norflurazon when compared to WT as determined by an in vitro assay. Surprisingly, the mutated PDS exhibited higher efficiency in converting phytoene to ζ-carotene. No difference in *PDS* transcripts was found between E17 and WT cells cultured either in normal or induced conditions. In contrast, higher transcript levels of *β*-carotene ketolase and hydroxylase were found in the E17 cells. Taken together, we conclude that a point mutation in *Chlorella PDS* gene makes E17 resistant to norflurazon and synthesize higher amounts of carotenoids including astaxanthin.

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Keywords Astaxanthin · *Chlorella* · Norflurazon · Phytoene desaturase

Abbreviations

BKT	*β*-Carotene ketolase
CHYb	*β*-Carotene hydroxylase
HL	High-light
HPLC	High-performance liquid chromatography
NPQ	Non-photochemical quenching
PDS	Phytoene desaturase
RT-PCR	Reverse transcriptase-PCR
TC	Total carotenoids
WT	Wild type

Introduction

Astaxanthin is a red keto carotenoid found in some microalgae, bacteria, yeasts, and many marine animals (Lorenz and Cysewski 2000; Zhang et al. 2009). It has important applications in food, feed, nutraceutical, and pharmaceutical industries because of its powerful antioxidative activity and beneficial effects on human health (Guerin et al. 2003; Fraser and Bramley 2004). Currently, natural astaxanthin is primarily obtained from the unicellular green alga *Haematococcus pluvialis.* However, due to the limited production by microorganisms, natural astaxanthin contributes only to a very small fraction to its market (Higuera-Ciapara et al. 2006).

H. pluvialis is able to accumulate astaxanthin up to 4% of its dry biomass, the highest content in nature (Boussiba 2000). One limitation of this green microalga is its relatively slow growth rate in combination with low-biomass yield (Olaizola 2000; Hata et al. 2001). *Chlorella zofingiensis* is a potential alternative host for mass production of

astaxanthin due to its fast growth and astaxanthin accumulation in the dark using glucose as the sole carbon and energy source (Ip and Chen 2005). Furthermore, high yields of astaxanthin (up to 32 mg L^{-1}) can be obtained by this alga with a fed-batch fermentation strategy (Sun et al. 2008). However, the relatively low content of astaxanthin per dry biomass of about 0.6% in *C. zofingiensis* hampers its commercial application (Orosa et al. 2001).

One strategy for increasing the astaxanthin content of *C. zofingiensis* is to generate mutants whose carotenoid flux is directed to astaxanthin due to mutations in carotenogenic genes. Chemical mutagenesis coupled with herbicide selection has created astaxanthin over-producing mutants of *H. pluvialis* (Tripathi et al. 2001; Chen et al. 2003; Hu et al. 2008; Sandesh Kamath et al. 2008). We previously found that a low concentration of norflurazon (0.25 μM) bleached *C. zofingiensis* cells. This herbicide inhibits the activity of plant-type phytoene desaturase (PDS), a key enzyme involved in catalyzing the rate-limiting step of carotenoid biosynthesis (Chamovitz et al. 1993). Mutants resistant to norflurazon may result from the increased amount of *PDS* gene product or from structural change of PDS that lowers its affinity to the herbicide (Chamovitz et al. 1991, 1993; Michel et al. 2004). Therefore it may be possible to isolate *C. zofingiensis* mutants with increased astaxanthin biosynthesis due to certain mutations in its *PDS* gene.

We have generated a number of norflurazon-resistant mutants of *C. zofingiensis* by chemical mutagenesis (unpublished data). One of them, named as E17, is a stable mutant that had a higher content of astaxanthin than the WT. In this study, we characterized E17 with respect to its growth, carotenogenesis and PDS activity. This mutated PDS is the first example for a point mutation in PDS, which not only makes the alga resistant to the herbicide norflurazon, but also enhances the biosynthesis of total carotenoids (TC) including astaxanthin.

Materials and methods

Strain and culture conditions

The green microalga *C. zofingiensis* (ATCC 30412) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The algal strain was maintained and cultured in modified Bristol's medium (referred to as CZ-M1) at 25°C under continuous illumination (25 μmol photon m^{-2} s^{-1}; standard growth condition) (Ip et al. 2004). The *C. zofingiensis* E17 mutant was isolated by treating the algal cells with ethyl methanesulfonate (EMS, Sigma, St Louis, MO, USA) followed by selecting the herbicide norflurazon. For herbicide treatment, the cell cultures were grown in CZ-M1 medium supplemented with 5 g L^{-1} glucose for 4 days and then inoculated into the same medium containing various concentrations of norflurazon (Sigma), at an inoculum of 10% (by volume, average cell concentration of 0.5 g L^{-1}). For induction of astaxanthin biosynthesis, the 4-day cultures mentioned above were exposed to continuous illumination of 250 μmol photon m^{-2} s^{-1} or inoculated into fresh CZ-M1 medium containing 30 g L^{-1} glucose and maintained in the dark.

Cell dry weight determination

The cells were centrifuged at 3,800*g* for 5 min. The pellet was re-suspended in distilled water and filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size). The algal cells on the filter paper discs were dried at 70°C in a vacuum oven until constant weight and were cooled down to room temperature in a desiccator before weighing.

Extraction and analysis of pigments

Cell samples from different stages were harvested and freeze-dried on a DW3 freeze-drier (Dry Winner, Heto-Holten, Allerød, 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. Twenty microliters of each extract was separated by HPLC on a Waters Spherisorb 5 μm ODS2 analytical column (4.6 × 250 mm) with a Waters HPLC system (Waters, Milford, MA, USA) using a modified method previously described by Baroli et al. (2004). 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 vol] 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 as compared to standard samples of pure carotenoids (Sigma).

Chlorophyll fluorescence measurement

Cells were harvested by centrifugation and re-suspended in fresh CZ-M1 medium. Chlorophyll fluorescence was measured using a multi-mode chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA) at room temperature. Non-photochemical quenching (NPQ) was calculated using $(F_m–F_m')/F_m'$, where F_m is the maximum fluorescence in the dark-adapted state and F_m' is the maximum fluorescence in the light-adapted state.

RNA isolation and RT-PCR assay

RNA was isolated from aliquots of about 1×10^8 cells harvested at different stages 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. The 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). Reverse transcription was performed by priming with oligo(dT) according to the manufacturer's instructions. PCR amplification and product detection were carried out according to the protocol described in Li et al. (2008).

PDS expression in *Escherichia coli*

The coding region of *PDS* cDNA from E17 was amplified by RT-PCR with specific primers as described previously (Huang et al. 2008), and was then sequenced and found to harbor a point mutation. The mutated *PDS* coding sequence was cloned into the plasmid pUC18 (Stratagene, La Jolla, CA, USA) as an in-frame fusion to the *lac*Z gene, resulting in plasmid pUC-czPDS-L516F (a leucine codon at position 516 changed to a phenylalanine codon). This plasmid and pUC-czPDS constructed previously (Huang et al. 2008) were introduced into *E. coli* JM109 for PDS enzyme expression. The *E. coli* 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.

In vitro PDS assay

The *E. coli* cells collected above were re-suspended in 0.1 M sodium phosphate buffer (pH 7.2, containing 1 mM DTT), and then passed through a French pressure cell (Spectronics Instruments, Rochester, NY, USA) at an internal pressure of 20 MPa; 10 μg mL^{-1} DNase was added to the broken cell extract 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 mL^{-1} of protein and used as the source of the PDS enzyme. The reaction mixture contained 1 mL of enzyme extract, 5 μL of the substrate phytoene (2 μg) in acetone, and 5 μL of decyl plastoquinone (10 mM solution in methanol, Sigma). Phytoene was extracted from *E. coli* JM109/pACCRT-EB freeze-dried cells (Breitenbach et al. 2001). 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 formed ζ-carotene were extracted from the incubation mixture with diethyl ether/petroleum ether (1:9, v/v), evaporated to dryness under a nitrogen stream and re-suspended in acetone for HPLC analysis. PDS activity was calculated in terms of formed ζ-carotene and expressed as μg ζ-carotene (mg protein)$^{-1}$ h^{-1}.

Results

The growth and carotenogenesis of E17

We treated *C. zofingiensis* cells with chemical mutagen EMS followed by selecting the bleaching herbicide norflurazon. A number of mutants resistant to norflurazon were obtained and analyzed for their astaxanthin production. A stable mutant, E17, was found to produce much more astaxanthin. The E17 showed no loss of resistance to norflurazon after more than 100 times of subculture without norflurazon selection (data not shown). The E17 grew well and produced nearly normal levels of TC in the presence of 0.25 μM norflurazon, in which the growth of wild type (WT) cells was greatly limited due to total blocking of carotenoid formation. Norflurazon suppressed carotenogenesis, leading to a decreased formation of TC including astaxanthin in both WT and E17 cells (Fig. 1). WT cells showed high sensitivity to norflurazon as indicated by a decreased TC content (from 1.69 to 0.83 mg g^{-1}) in the present of 0.05 μM norflurazon. Higher concentrations of norflurazon resulted in much lower amounts of TC (Fig. 1). In addition, the formation of astaxanthin in WT almost ceased when the cultures were treated with up to 0.25 μM norflurazon. In contrast, E17 synthesized significant amounts of TC and astaxanthin even at 0.5 μM norflurazon, a lethal concentration for the WT. Values of half-maximum inhibitory concentration (I_{50}) of norflurazon on TC and astaxanthin formation in WT and E17 were determined from (Fig. 1). The E17 exhibited 22.7- and 25.9-fold resistance to norflurazon with respect to TC accumulation and astaxanthin formation as compared to WT.

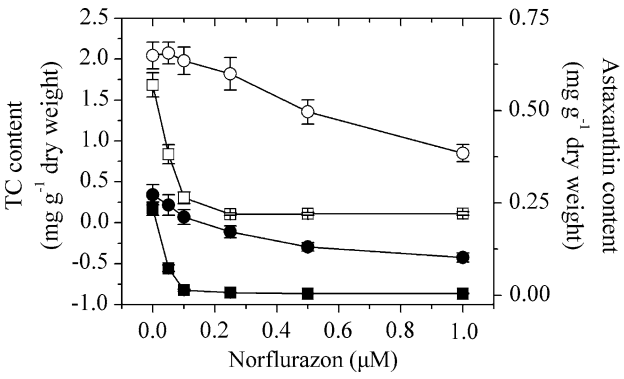

Fig. 1 Effect of norflurazon concentration on TC and astaxanthin contents of WT and E17. *Open square* WT TC, *filled square* WT astaxanthin, *open circle* E17 TC, *filled circle* E17 astaxanthin. Cells were harvested from a 4-day culture under standard growth conditions

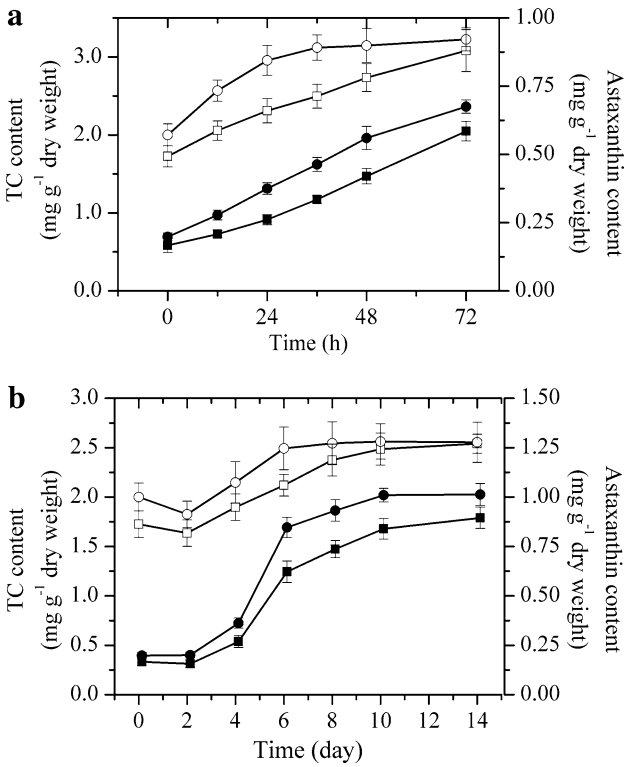

Fig. 2 Accumulation of TC and astaxanthin in WT and E17 under continuous illumination of HL **a** or under induction of 30 g L^{-1} glucose in the dark **b**. *Open square* WT TC, *filled square* WT astaxanthin, *open circle* E17 TC, *filled circle* E17 astaxanthin

Enhanced production of TC and astaxanthin by E17 under high-light stress or glucose induction

High-light (HL) irradiation was reported to trigger keto carotenogenesis in *C. zofingiensis* cells (Orosa et al. 2000, 2001; Del Campo et al. 2004; Li et al. 2009). Here a comparative study of WT and E17, in terms of TC and astaxanthin contents under HL irradiation, was conducted. Consistent with previous reports, *C. zofingiensis* WT and E17 synthesized much higher amounts of TC and astaxanthin under HL than normal light (Fig. 2a). The E17 produced apparently greater amounts of both TC and astaxanthin than WT during the whole illumination period (Fig. 2a). The carotenoid contents of WT and E17 cells exposed to HL for 24 h are shown in Table 1. Lutein was the major carotenoid, followed by zeaxanthin and astaxanthin. Significant higher amounts of *β*-carotene, lutein, zeaxanthin, as well as the keto carotenoids adonixanthin, canthaxanthin and astaxanthin were found to accumulate in E17 cells. The TC and astaxanthin contents (2.96 and 0.38 mg g^{-1}) of E17 were 27.9 and 43.7% higher than that of WT, respectively. Consistent with the higher content of zeaxanthin, the NPQ value of E17 was 33.3% higher than that of WT.

In addition to light, glucose is another inducer of astaxanthin biosynthesis in *C. zofingiensis* cells (Ip et al. 2004).

Table 1 Pigment contents of green cells of WT and E17 exposed to HL for 24 h

	Total carotenoids (mg g^{-1})	Chlorophyll (mg g^{-1})	Carotenoid composition (μg g^{-1})										Zea/(Vio + Ant + Zea)
			Neo	Vio	Ant	Ast	Ado	Lut	Zea	Can	*β*-Car	Vio + Ant + Zea	
WT	2.31 ± 0.09	6.23 ± 0.21	64 ± 2.1	38 ± 1.3	67 ± 3.1	261 ± 11	219 ± 8	1132 ± 48	300 ± 11	128 ± 4	101 ± 5	405	0.74
E17	2.96 ± 0.12	6.05 ± 0.27	75 ± 3.4	32 ± 1.5	64 ± 1.8	375 ± 15	297 ± 13	1397 ± 55	414 ± 17	160 ± 6	121 ± 3	510	0.81

Mean values ± SD of three independent measurements

Ado adonixanthin, *Ant* antheraxanthin, *Ast* astaxanthin, *Can* canthaxanthin, *β-Car β*-carotene, *Lut* lutein, *Neo* neoxanthin, *Vio* violaxanthin, *Zea* zeaxanthin

With glucose as the sole carbon and energy source, *C. zofingiensis* grew fast and synthesized astaxanthin (Ip and Chen 2005; Sun et al. 2008). The time course of TC and astaxanthin accumulation in heterotrophic WT and E17 cells cultured in CZ-M1 medium with 30 g L^{-1} is shown in Fig. 2b. Compared with the non-induced cells on day 0, the TC and astaxanthin contents of WT were slightly decreased on day 2. Thereafter both TC and astaxanthin increased and the astaxanthin content reached its maximum in 14 days. The E17 exhibited the same induction pattern of TC and astaxanthin as that of WT. However, E17 produced apparently greater amounts of carotenoids, e.g., on day 6 the TC and astaxanthin contents were 2.50 and 0.85 mg g^{-1}, 18 and 36% higher than that of WT, respectively. Even on day 10 the astaxanthin content of WT was slightly lower than that of E17 on day 6. It is worth noting that after day 6 the difference in TC and astaxanthin contents between WT and E17 became smaller. In correlation with the earlier enhanced astaxanthin accumulation, E17 exhibited an earlier color change from green to orange (data not shown).

Characterization of E17 PDS

Norflurazon can be coped with by increased amounts of the target enzyme or by a mutation that affects the binding properties. The sequencing of the *PDS* gene from E17 revealed a single point mutation (C–T), which resulted in an amino acid change from leucine (L) to phenylalanine (F) at codon 516 (from CTC to TTC). This L residue is located in a conserved motif in the PDS polypeptides. Another substitution of this L residue by arginine (R) was earlier revealed by us to confer herbicide resistance on *Chlorella* PDS (Huang et al. 2008). To verify whether PDS-L516F is related to norflurazon resistance, we carried out in vitro assays of the *Chlorella* PDS and PDS-L516F. In vitro desaturation activities of WT PDS and PDS-L516F were measured by determining the conversion of phytoene to ζ-carotene in the presence of various concentrations of norflurazon. The results were expressed as units of activity relative to that of the control without the herbicide. A Dixon plot (Dixon 1953) of the reciprocal of PDS desaturation activity versus the concentration of norflurazon is shown in Fig. 3. The concentrations for 50% inhibition (I_{50}) of PDS desaturation activity were calculated to be 0.072 μM for WT PDS and 2.242 μM for PDS-L516F, respectively. Compared with the WT PDS, the PDS-L516F showed 31-fold greater resistance to the inhibitor norflurazon, which is comparable with the PDS-L516R as revealed in a previous study (Huang et al. 2008). Surprisingly, PDS-L516F exhibited higher activity in converting phytoene to ζ-carotene than its WT counterpart as well as the PDS-L516R that was shown to have attenuated desaturation activity (Chamovitz et al. 1993; Steinbrenner and Sandmann 2006; Huang et al. 2008). The specific

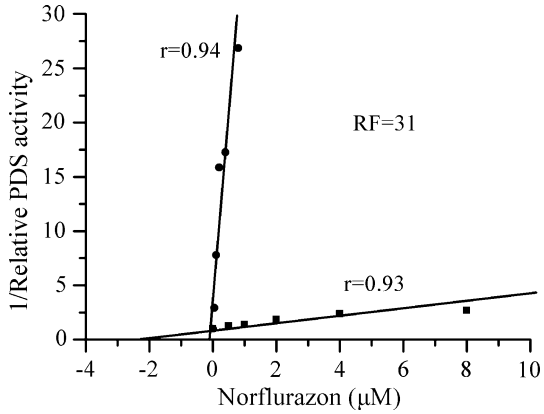

Fig. 3 Plot of the reciprocal of in vitro phytoene desaturase activities versus concentrations of norflurazon for the *E. coli*-based recombinant WT PDS (*filled circle*) and PDS-L516F (*filled square*)

activity of PDS-L516F was 0.0315 μg ζ-carotene formed (mg protein)$^{-1}$ h^{-1}, about 29% higher than that of the WT PDS and 50% more than PDS-L516R (Table 2).

Transcription analysis of *PDS*, *BKT* and *CHYb* genes

We reported earlier that the enhanced biosynthesis of carotenoids including astaxanthin in *C. zofingiensis* by HL or glucose induction was correlated to the increased transcript levels of carotenogenic genes (Li et al. 2008, 2009). To further investigate whether the enhanced production of TC and astaxanthin by E17 was attributed to the up-regulation of *PDS* and/or other carotenogenic genes, we quantified the transcript levels of PDS together with BKT and CHYb, two enzymes directly involved in astaxanthin formation from β-carotene. RT-PCR results showed no apparent differences in *PDS* transcripts between WT and E17 either under standard growth conditions or induced conditions (Fig. 4). In contrast, higher transcript levels of BKT and CHYb were found in E17 cells in response to 1-day HL treatment or induced by high concentration of glucose (30 g L^{-1}) for 4 days. This result correlated well to the higher amounts of astaxanthin accumulated in E17 than in WT cells under HL stress (Fig. 2a) or glucose induction conditions (Fig. 2b).

Table 2 In vitro phytoene desaturation activity of *E. coli* expressing PDS proteins

Crude enzymes	Specific activity (μg ζ-carotene formed (mg protein)$^{-1}$ h^{-1})
PDS	0.0245 ± 0.0015
PDS-L516R	0.0193 ± 0.0012
PDS-L516F	0.0315 ± 0.0021

Mean values ± SD of three independent experiments

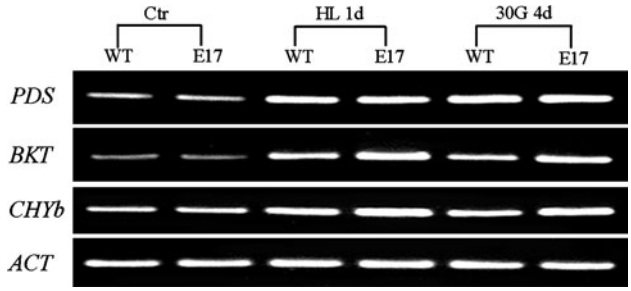

Fig. 4 Transcript levels of phytoene desaturase (*PDS*), β-carotene ketolase (*BKT*), and β-carotene hydroxylase (*CHYb*) in WT and E17 cells cultured under standard growth conditions (*Ctr*), high-light induction for 1d (*HL* 1d) or 30 g L^{-1} glucose induction for 4d (30G 4d). Actin gene (*ACT*) was used as control

Discussion

Chlorella zofingiensis synthesizes astaxanthin as secondary carotenoid that is stored in lipid vesicles outside the chloroplasts under stress conditions such as HL irradiation or nitrogen deficiency (Rise et al. 1994; Bar et al. 1995). A mutation in PDS revealed that this enzyme is one of the limiting steps of carotenoid biosynthesis. An amino acid substitution (L516F) in PDS cannot only make *C. zofingiensis* resistant to norflurazon (Fig. 1), but also enhance the biosynthesis of TC and astaxanthin (Fig. 2). Norflurazon-resistant phenotypes had been found in *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 mutants resulting from either a point mutation or a deletion in the upstream region of *PDS* gene (Chamovitz et al. 1991, 1993; Martínez-Férez and Vioque 1992). Point mutations in PDS give rise to a modified structure for the herbicide-binding site; whereas the deletion in regulating region causes up-regulation of *PDS* transcription and consequently a great increase of PDS protein (Chamovitz et al. 1993). In addition, point mutations in PDS resulted in plant mutants resistant to fluridone, another bleaching herbicide that also specifically inhibits the desaturation activity of PDS (Michel et al. 2004; Arias et al. 2005). Some mutations in PDS conferred cross-resistance to fluridone and norflurazon (Arias et al. 2006), which is not the case for E17 cells (data not shown). Norflurazon was reported to inhibit PDS by competition with the cofactor plastoquinone (Breitenbach et al. 2001). Therefore, bleaching herbicides including norflurazon can be regarded as plastoquinone analogs targeting the same binding region (Sandmann 2002).

The E17 harbors a point mutation (C–T) in *PDS*, causing an amino acid substitution (L–F) at codon position 516. Surrounding the L residue is a conserved motif in PDS polypeptides from plants (Pecker et al. 1992; Scolnik and Bartley 1993), algae (Harker and Hirschberg 1997; McCarthy et al. 2004; Huang et al. 2008) and cyanobacteria (Chamovitz et al. 1993). Previous mutations including the substitution of the L residue to arginine (R) in PDS had been found to confer norflurazon-resistance on *Synechococcus* sp. PCC 7942 but meanwhile lower the catalytic activity in PDS and consequently reduce the production of downstream carotenoids (Sandmann et al. 1993). Similar results were obtained when the same mutation occurred in the PDS from *H. pluvialis* and *C. zofingiensis* (Steinbrenner and Sandmann 2006; Huang et al. 2008). We assume that the L residue may play a decisive role in the binding of herbicide and the binding of plastoquinone. In contrast to the substitution of L–R, the L–F exchange may favor the kinetics for plastoquinone binding, resulting in higher PDS specific activity (Table 2). This leads to a higher TC accumulation in E17 (Fig. 2). Transgenic *H. pluvialis* overexpressing an additional *PDS* gene also enhanced TC content (Steinbrenner and Sandmann 2006). Determination of transcript levels of carotenogenic genes showed no changes for *PDS* in E17 (Fig. 4). However, E17 accumulated higher levels not only of TC but also of astaxanthin than WT (Fig. 2; Table 1). This is well-explained by the increased transcripts of *BKT* and *CHYb* in E17 (Fig. 4). However, the regulatory mechanism leading to an up-regulation of both transcripts in E17 as a consequence of higher PDS activity remains open.

In conclusion, the *PDS* gene of *C. zofingiensis* E17 harbors a point mutation, which leads to one amino acid substitution and makes the mutated cells resist norflurazon. In contrast to all other mutations conferring norflurazon resistance, PDS in E17 shows increased specific activity leading to enhanced biosynthesis of carotenoids including the high-value astaxanthin. The mutated *PDS* gene can serve as a dominant selectable marker for genetic engineering of *C. zofingiensis* and even other green algae to enhance the biosynthesis of astaxanthin.

Acknowledgments The work described in this paper was fully supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. HKU 7501/06M).

References

Arias RS, Netherland MD, Scheffler BE, Puri A, Dayan FE (2005) Molecular evolution of herbicide resistance to phytoene desaturase inhibitors in *Hydrilia verticillata* and its potential use to generate herbicide-resistant crops. Pest Manag Sci 61:258–268

Arias RS, Dayan FE, Michel A, Howell J, Scheffler BE (2006) Characterization of a higher plant herbicide-resistant phytoene desaturase and its use as a selectable marker. Plant Biotechnol J 4:263–273

Bar E, Rise M, Vishkautsan M, Arad S (1995) Pigments and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. J Plant Physiol 146:527–534

Baroli I, Gutman BL, Ledford HK, Shin JW, Chin BL, Havaux M, Niyogi KK (2004) Photo-oxidative stress in a xanthophyll-deficient mutant of *Chlamydomonas*. J Biol Chem 279:6337–6344

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. Physiol Plant 108:111–117

Breitenbach J, Zhu CF, Sandmann G (2001) Bleaching herbicide norflurazon inhibits phytoene desaturase by competition with the cofactors. J Agric Food Chem 49:5270–5272

Chamovitz D, Pecker I, Hirschberg J (1991) The molecular-basis of resistance to the herbicide norflurazon. Plant Mol Biol 16:967–974

Chamovitz D, Sandmann G, Hirschberg J (1993) 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 268:17348–17353

Chen Y, Li D, Lu W, Xing J, Hui B, Han Y (2003) Screening and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. Biotechnol Lett 25:527–529

Del Campo JA, Rodriguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2004) Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 64:848–854

Dixon M (1953) The determination of enzyme inhibitor constants. Biochem J 55:170–171

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends Biotechnol 21:210–216

Harker M, Hirschberg J (1997) Biosynthesis of ketocarotenoids in transgenic cyanobacteria expressing the algal gene for β-C-4-oxygenase, *crt*O. FEBS Lett 404:129–134

Hata N, Ogbonna JC, Hasegawa Y, Taroda H, Tanaka H (2001) Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic-photoautotrophic culture. J Appl Phycol 13:395–402

Higuera-Ciapara I, Félix-Valenzuela L, Goycoolea FM (2006) Astaxanthin: a review of its chemistry and applications. Crit Rev Food Sci Nutr 46:185–196

Hu ZY, Li YT, Sommerfeld M, Chen F, Hu Q (2008) Enhanced protection against oxidative stress in an astaxanthin-overproduction *Haematococcus* mutant (Chlorophyceae). Eur J Phycol 43:365–376

Huang JC, Liu J, Li YT, Chen F (2008) Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 44:684–690

Ip PF, Chen F (2005) Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. Process Biochem 40:733–738

Ip PF, Wong KH, Chen F (2004) Enhanced production of astaxanthin by the green microalga *Chlorella zofingiensis* in mixotrophic culture. Process Biochem 39:1761–1766

Li Y, Huang J, Sandmann G, Chen F (2008) Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). Planta 228:735–743

Li Y, Huang J, Sandmann G, Chen F (2009) High-light and sodium chloride stress differently regulate the biosynthesis of astaxanthin in *Chlorella zofingiensis* (Chlorophyceae). J Phycol 45:635–641

Lorenz RT, Cysewski GR (2000) Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol 18:160–167

Martínez-Férez IM, Vioque A (1992) Nucleotide sequence of the phytoene desaturase gene from *Synechocystis* sp. PCC 6803 and characterization of a new mutation which confers resistance to the herbicide norflurazon. Plant Mol Biol 18:981–983

McCarthy SS, Kobayashi MC, Niyogi KK (2004) White mutants of *Chlamydomonas reinhardtii* are defective in phytoene synthase. Genetics 168:1249–1257

Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M, Dayan FE (2004) Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). Mol Ecol 13:3229–3237

Olaizola M (2000) Commercial production of astaxanthin from *Haematococcus pluvialis* using 25, 000-liter outdoor photobioreactors. J Appl Phycol 12:499–506

Orosa M, Torres E, Fidalgo P, Abalde J (2000) Production and analysis of secondary carotenoids in green algae. J Appl Phycol 12:553–556

Orosa M, Valero JF, Herrero C, Abalde J (2001) Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under N-starvation and high light conditions. Biotechnol Lett 23:1079–1085

Pecker I, Chamovitz D, Linden H, Sandmann G, Hirschberg J (1992) A single polypeptide catalyzing the conversion of phytoene to ξ-carotene is transcriptionally regulated during tomato fruit ripening. Proc Natl Acad Sci USA 89:4962–4966

Rise M, Cohen E, Vishkautsan M, Cojocaru M, Gottlieb HE, Arad SM (1994) Accumulation of secondary carotenoids in *Chlorella zofingiensis*. J Plant Physiol 144:287–292

Sandesh Kamath B, Vidhyavathi R, Sarada R, Ravishankar GA (2008) Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants. Bioresour Technol 99:8667–8673

Sandmann G (2002) Bleaching herbicides: action mechanism in carotenoid biosynthesis, structural requirements and engineering of resistance. In: Böger P, Wakabayashi K, Hirai K (eds) Herbicide classes in development. Springer, Berlin, pp 43–57

Sandmann G, Kuhn M, Böger P (1993) Carotenoids in photosynthesis: protection of D1 degradation in the light. Photosynth Res 35:185–190

Scolnik PA, Bartley GE (1993) Phytoene desaturase from *Arabidopsis*. Plant Physiol 103:1475

Steinbrenner J, Sandmann G (2006) Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. Appl Environ Microbiol 72:7477–7484

Sun N, Wang Y, Li Y-T, Huang J-C, Chen F (2008) Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). Process Biochem 43:1288–1292

Tripathi U, Venkateshwaran G, Sarada R, Ravishankar GA (2001) Studies on *Haematococcus pluvialis* for improved production of astaxanthin by mutagenesis. World J Microbiol Biotechnol 17:143–148

Zhang BY, Geng YH, Li ZK, Hu HJ, Li YG (2009) Production of astaxanthin from *Haematococcus* in open pond by two-stage growth one-step process. Aquaculture 295:275–281