Overexpression of an Exogenous Phytoene Synthase Gene in the Unicellular Alga *Chlamydomonas reinhardtii* Leads to an Increase in the Content of Carotenoids

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Phytoene synthase (PSY) catalyses the first step in the production of carotenoids, which has been described as a key regulatory step in the carotenoids biosynthetic pathway. PSY gene from Dunaliella salina *was constitutively expressed in* Chlamydomonas reinhardtii *under the control of the* RBCS2 *and* HSP70A *promoters and targeted to the chloroplast by the RBCS2 transit peptide.* DsPSY *overexpression resulted in a stable increase in the corresponding* PSY *transcript level and in the content of carotenoids such as violaxanthin, lutein, and β-carotene, reaching between 125 and 260% the levels in control untransformed cells.*
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Keywords: phytoene synthase, PSY, Chlamydomonas reinhardtii*, carotenoid biosynthetic pathway, genetically modified microalgae*

Introduction

Carotenoids are the most diverse and widespread pigments found in nature.[1] They are the wide group of lipophylic isoprenoids synthesized by all photosynthetic organisms and also by some nonphotosynthetic bacteria and fungi. They have been described as indispensable in light harvesting and energy transference during photosynthesis[2] and in the protection of the photosynthetic apparatus against the photooxidative damage.[3,4] Since animals do not synthesize carotenoids *de novo*, they must include them in their diet as precursors for essential compounds, such as vitamin A or the visual pigment retinal.[5] The important colorant, antioxidant and provitamin properties of carotenoids, have made of them an important group of high-added value compounds, massively commercialized as dietetic and food/feed additives.[6] Restrictive regulations on the use of synthetic dyes in food and feed industries has contributed to increase the interest in natural carotenoids and has stimulated research about the production of carotenoids from microalgae, which are their main natural source.

In microalgae, carotenoids are synthesized in the plastids from isopentenylpyrophosphate originated through the mevalonic independent pathway also known as methylerythriol 4-phosphate pathway.[7–9] The first committing step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranylpyrophosphate (GGPP) to yield the first uncolored carotenoid, phytoene, catalysed by the phytoene synthase enzyme (PSY). From this step on, phytoene is subjected to four sequential desaturations and an isomerization producing the colored carotene lycopene. At this level, the pathway splits into two branches. In one branch, the lycopene is cycled by lycopene cyclase β yielding β-carotene, which can be further hydroxylated and/or epoxidated to yield zeaxanthin and violaxanthin. In the other branch, the concentrated action of β and ε cyclases results in the formation of α-carotene, which hydroxylation leads to the formation of lutein (Figure 1). Reviews of Cunningham and Gantt[10] or Grossman et al.,[11] and the more recently published by Botella-Pavia and Rodriguez-Concepcion,[6] Sandmann et al.,[12] and Giuliano et al.[13] offer a good general view of this metabolic pathway.

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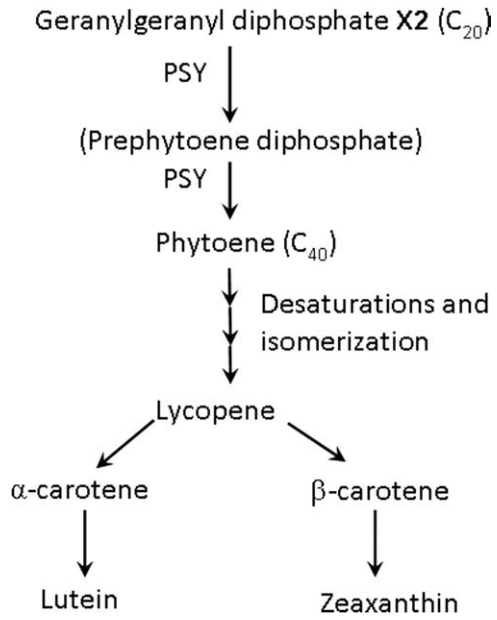

Figure 1. Schematic representation of the carotenoid biosynthetic pathway.

The first step is catalyzed by phytoene synthase (PSY), enzyme leading the carbon flux towards the carotenes and xanthophylls production.

Despite the importance of microalgae as a source for natural carotenoids, there are few reports about the genetic manipulation of the carotenoid biosynthetic pathway in these microorganisms.[14,15] Most efforts have focused on higher plants, trying to obtain transgenic species with enhanced nutritional characteristics. There are a couple of works describing silencing via RNA interference of phytoene desaturase in *Dunaliella*[16] and *Chlamydomonas*[17] and one report about the production of a new carotenoid not previously synthetized in *Chlamydomonas* through the expression of a foreign β-carotene oxygenase (*BKT*) gene.[18] But the only report about overexpression of an enzyme from the carotenoid biosynthetic pathway in microalgae is, to our knowledge, the one of Steinbrenner and Sandmann (2006), who observed higher carotenoid content in some transformants of *H. pluvialis* which expressed a modified *PDS* gene with higher resistance to the herbicide norflurazon.[15]

PSY is considered as a rate limiting key enzyme in the carotenogenic pathway[19,20] and is believed to play an important role in regulation of the carbon source flux towards carotenoids route.[21–23] Overexpression of bacterial or plant *PSY* genes in higher plants has resulted in a significant increase in total carotenoid levels in tomato fruit,[24,25] Canola and *Arabidopsis* seed,[21,26] rice endosperm,[27,28] potato tuber,[29] and carrot.[30] In this study, a *PSY* gene from *Dunaliella salina,* which accumulates high quantities of β-carotene under certain stress conditions, was constitutively expressed in the freshwater microalga *C. reinhardtii* under the control of the strong *RBCS2* and *HSP70A* promoters. Expression of the exogenous *PSY* in *Chlamydomonas* resulted in an increase on the corresponding *PSY* transcript level and on the content of carotenoids.

Materials and Methods

Strains and culture conditions

Chlamydomonas reinhardtii 704 strain (Cw15, Arg7, mt +) was kindly donated by Dr. Roland Loppes[31] and cultured photomixotrophically in liquid or agar solidified Tris-acetate phosphate (TAP) medium[32] under continuous white light irradiance (50 μE m^{-2} s^{-1} PAR) at 25°C in a culture chamber. *Dunaliella salina* 19/18 was obtained from the CCAP collection (Scotland, UK) and cultured in the liquid medium described by Johnson et al.,[33] with the same light source, irradiance and temperature as *Chlamydomonas*. The *Escherichia coli* strain used for in vivo amplification of DNA and functional complementation experiments was DH5α, cultured in LB medium at 37°C as previously described[34] supplemented with either ampicillin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}) or both of them.

Complementation experiments

Functional analysis of the isolated *PSY* cDNA was performed in *E. coli* cells that carried the plasmid pAC-85b.[35] This plasmid contains c*rtE, crtI*, and c*rtY* genes from the soil bacteria *E. herbicola* which encode the GGPP synthase, the phytoene desaturase and the lycopene cyclase enzymes, providing all the enzymes necessary for the synthesis of *β-carotene*, excepting the PSY and harbours the cm^r gene that confers resistance to chloramphenicol.

Construction of expression vectors for Chlamydomonas

The plasmid pSI105 is based on the plasmid pSI104-PLK, derived from the pSI103[36] in which the *APHVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3′phosphotransferase that confers resistance to the antibiotic paromomycin, is expressed under the control of the strong constitutive promoters *RBCS2* and *HSP70A* and terminated by the 3′ untranslated region of *RBCS2*. PSI103 was cut with NotI and religated to eliminate unwanted restriction sites. In pSI104-PLK the *APHVIII* gene has been substituted by a polylinker region. To obtain pSI105, the NaeI/XbaI region (538bp) of pSI104-PLK has been eliminated and replaced by the *RBCS2: APHVIII* cassette. This plasmid allows the integration in the same construction of the marker gene, *APHVIII*, and functional genes, which can be inserted in the polylinker (Figure 2a).

The DNA fragment encoding the desired transit peptide was synthesized by Genescript Co (NJ) and cloned in the EcoRV site of the multiple cloning region of the plasmid pUC57, from where they were cut, purified and inserted between BstBI and XhoI sites in the polylinker of pSI105.

RNA extraction and reverse transcription-PCR

Large scale isolation of the total RNA was performed using phenol:chlorophorm extraction and salt precipitation (3 M of sodium acetate). Total RNA used for Quantitative Real-Time PCR, was obtained from RNAeasy plant minikit from Qiagen according to the instructions of the manufacturer. The RNA concentrations were determined with Nanodrop 1000 Spectrophotometer from Thermo Scientific (Wilmington, DE) and the integrity of the RNA confirmed by agarose gel electrophoresis. Residual genomic DNA was removed by incubation with the DNAase (Qiagen) as indicated by the manufacturer. Single strand cDNA was synthesized from total RNA according to the Superscript II RNaseH-reverse transcriptase manual (Invitrogene).

The PCR amplification was performed from 1 μL of the resulting extraction of cDNA in a total volume of 25 μL

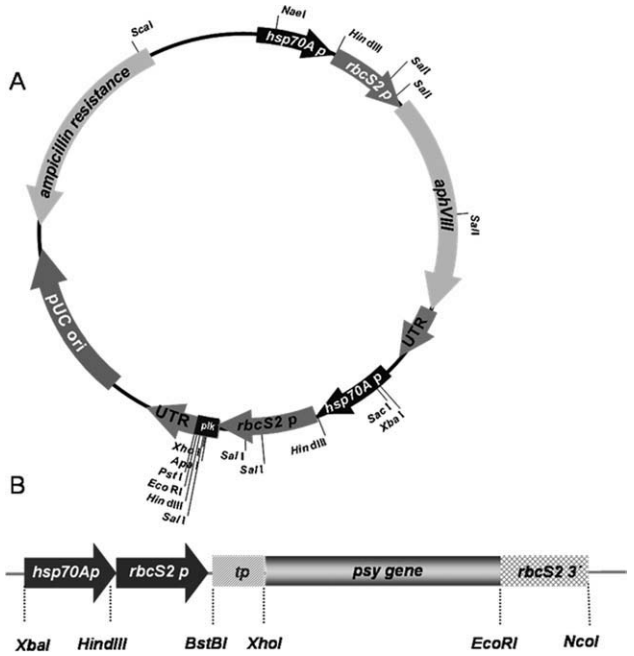

Figure 2. Structure of pSI105 plasmid (A) and detailed schematic representation of the *PSY* gen inserted in the expression cassette of pSI105 plasmid (B).

containing a final concentration of 0.5 μM each primer (Forward and Reverse), 200 μM dNTPs, 3% of DMSO and 0.5U of Taq polymerase from Biotools (Spain). The PCR program used was: (i) 5 min at 98°C; (ii) 30 cycles of 0.5 min at 98°C, 1.5 min at the melting temperature of the different primers used (Table 1), 0.5 min at 72°C (iii); and a final amplification of 5 min at 72°C.

Chlamydomonas *nuclear transformation*

Nuclear transformation of *C. reinhardtii* was carried out using the glass beads method of Kindle[37] with minor modifications. *Chlamydomonas* cultures were grown to a cell density of about 10^7 cells per mL, spinned down and resuspended to get a final 100-fold concentrated cell suspension. 0.3 g of sterile glass beads (0.4–0.6 mm Ø), were added to 0.6 mL of concentrated cell suspension, 0.2 mL of 20% polyethylene glycol (MW8000) and about 1 μg of the desired plasmid. This mixture was agitated during 10s and then cells were resuspended in 50 mL of fresh TAP medium and left in dim light conditions overnight. After this incubation, cells were spread onto the selective solid medium carrying 30 μg mL^{-1} of paromomicyn. Transformed colonies were visible after 4 or 5 days.

Dry weight determination

Dry weight was determined by filtering an exact volume of microalgae culture (30 mL) on pretared glass-fiber filters (GF/F Whatman). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts and dried at 100°C for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference.

Pigments extraction and analysis

Samples from *E. coli* or *C. reinhardtii* cultures were concentrated and used for the extraction of carotenoids with 80% of acetone (v/v) as previously described.[38] The chromatographic analysis were performed in a Merck Hitachi HPLC equipped with a DAD detector as described by Young et al.,[39] using a RP-18 column, a flow rate of 1 mL min^{-1} and a final injection volume of 100 μL. Two mobile phases were used solvent A, ethyl acetate 100% and solvent B acetonitrile: H_2O (9:1 v/v). The gradient applied was: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100% A. Standards were supplied by DHI (Hoersholm, Denmark). Measurements were done in triplicate and the mean was then calculated.

Quantitative RT-PCR

qPCR experiments were performed in triplicate on a Mx3000P Multiplex Quantitative PCR System (Stratagene) using 1 μL of the cDNA mixture added as template and Brilliant SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies, La Jolla, CA). Cycling conditions were: 10 min at 95°C for activation of the Hot start Taq polymerase and 40 cycles for the melting (30s at 95°C), annealing (30s at 60°C) and extension (30 s at 72°C). Each qPCR measurement was carried out in triplicate using primers for either *PSY* from *Chlamydomonas* or *Dunaliella* (Table 1). The *CBLP* gene, encoding a G-protein β-subunit-like polypeptide,[40] which expression was previously shown to be constitutive under the different conditions used,[41] was used for the standardization of the different samples.

Results

Isolation and functional analysis of PSY gene from D. salina

Dunaliella PSY cDNA was obtained by RT-PCR from total mRNA, using forward and reverse primers designed to contain BamHI and HindIII restriction sites as shown in Table 1. The 1.26 Kb cDNA fragment obtained was inserted between BamHI and HindIII sites of the pQE80-L bacterial expression vector. The difference between codon usage of

Table 1. Primers Designed for the Insertion of the *PSY* gen into Different Vectors (Enzymes Restriction Sites Underlined) Either for its Expression in *Chlamydomonas* (1,2) or *E.coli* (3,4) and Primers Designed for the Real-Time PCR Analysis (5–8).

Primer Num	Primer Name	Sequence	T_m (°C)
1	UpDSPSYXhoI	CTCGAGATGCCCTCCACTTCCG	57.3
2	RevDSPSYEcoRI	CTACTGTGAATTCTTGGGCATCA	48
3	UpDSPSYBamHI	GGATCCATGCCCTCCACTTC	50
4	RevDSPSYHindIII	CTGTGGGCTCTTAAGCTTCATG	49
5	qFCRPSY	CACTCGCGCCCGGCAATACTT	62.8
6	qRCRPSY	CCACGGGCAGCGACACCATC	63.0
7	qFDSPSY	CCTTGGATGAGCTACGGGAGTTTG	59.9
8	qRDSPSY	GCAGGGAGGCCAGCTTCTTTGA	61.4

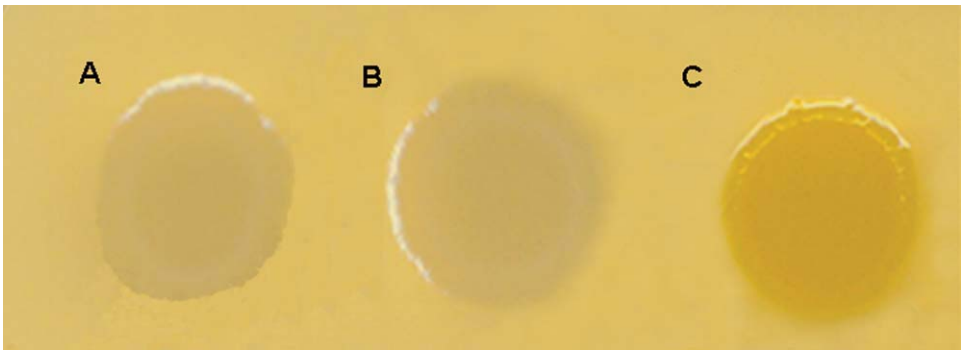

Figure 3. Colonies of IPTG-induced *E. coli* transformants carrying the pAC85b plasmid (A); the pAC85b plasmid with the empty pQE80-L vector (B); and the pAC85b plasmid together with pQE80-L vector carrying the gene *PSY* from *Dunaliella salina* (C).

the *PSY* gene isolated from *Dunaliella salina* and the genome of *C. reinhardtii* was 14.25%, as calculated using the Graphical codon usage analyser v. 2.0 (http://gcua.schoedl.de/index.html), which is not a significant difference to influence on the efficiency of the translation of this gene.

Functionality of the isolated cDNA was checked by complementation experiments in *E. coli* cells that carried the plasmid pAC-85b.[35] This plasmid contains c*rtE*, c*rtI*, and c*rtY* genes from *E. herbicola* which encode the GGPP synthase, the phytoene desaturase and the lycopene cyclase enzymes, providing all the enzymes necessary for the synthesis of β-carotene, excepting the PSY and harbours the cm^r gene that confers resistance to chloramphenicol. After cotransformation and selection with chloramphenicol plus ampicillin, the obtained colonies were subjected to induction with IPTG (1 mM) during 12 h at 37°C and 48 h at room temperature in the dark. Expression of *DsPSY* gene, together with the pAC-85b plasmid in *E. coli* resulted in the production of β-carotene, as could be appreciated in the yellow–orange color of the colonies (Figure 3). Extraction of the carotenoids from the colored colonies and their HPLC analysis confirmed the presence of β-carotene and the functionality of *DsPSY* gene.

Insertion of PSY gene in a microalgal expression vector and nuclear transformation of Chlamydomonas *with the obtained construction*

The obtained PCR product was reamplified with specific primers containing XhoI and EcoRI restriction sites and subcloned between the XhoI and EcoRI restriction sites of the *Chlamydomonas* expression vector pSI105. This plasmid, obtained as described in the "Materials and Methods" section, contains the *HSP70A* and *RBCS2* promoters and the 3′untraslated terminator region of *Chlamydomonas RBCS2* gene,[42] and harbours the *APHVIII* gene that confers resistance to the antibiotic paromomycin, flanked by the same *Chlamydomonas reinhardtii RBCS2* 3′ and 5′ untranslated region (Figure 2a).

Before insertion of the *PSY* genes, a 96 bp sequence encoding the RBCS2 transit peptide was inserted immediately before the polylinker region, between BstBI and XhoI sites (Figure 2b). The *PSY* gene was inserted in the same reading frame as the sequence encoding the transit peptide. Transformants were first selected on the basis of paromomycin resistance, and then submitted to a screening by PCR to test the insertion of *DsPSY* cDNA in Chlamydomonas nuclear genome using the UpDSPSYXhoI and RevDSPSYEcoRI primers (Table 1). About 30% of total paromomycin resistants were harbouring both *APHVIII* and *Dunaliella salina PSY* genes in their genomes. Expression of the inserted gene in the isolated transformants and their phenotypic characteristics were further analyzed.

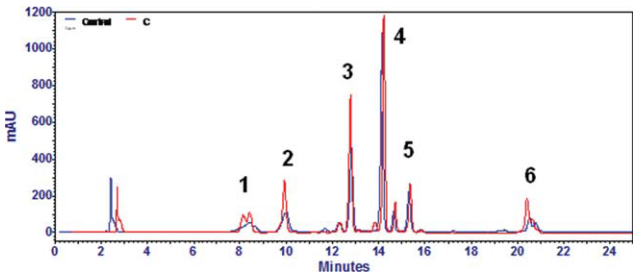

Figure 4. Carotenoids profiles of Control (blue) and one of *DsPSY*-transformants obtained (red).

Numbers correspond to neoxanthin,[1] violaxanthin,[2] lutein,[3] chlorophyll b,[4] chlorophyll a,[5] and β-carotene[6] peaks. All data are the mean of two independent replicates.

Analysis of DsPSY-transformed Chlamydomonas reinhardtii

The positive transformants that showed to contain the exogenous *Dunaliella* PSY adequately inserted in the nuclear genome were further analysed for their carotenoids content. Typical HPLC chromatogram comparing pigment composition of control and transformant cells is shown in Figure 4. We selected about 18 transformants designated from "A" to "M" in which overexpression of *Dunaliella PSY* (*DsPSY*) resulted in a significant increase in the total carotenoids content. The detailed carotenoids profile and the intracellular *PSY* mRNA content of two random examples of these transformants are shown in Figure 5.

Exogenous *DsPSY* was constitutively expressed under the control of the strong promoters *RBCS2* and *HSP70A* and high levels of *DsPSY* mRNA were detected in the two transformants analysed. In transformant "C" *DsPSY* transcript level reached threefold the level of the endogenous *PSY*, which expression level was of the same order as in wild type cells. The intracellular level of violaxanthin and lutein were 2- and 2.6-fold the level in control wild type cells and the content in β-carotene and neoxanthin showed a 1.25- and a 1.8-fold increase, respectively, upon expression of exogenous *DsPSY*. For transformant "H", a significant but slightly minor increase in the intracellular level of violaxanthin and lutein (1.6- and 2.2-fold increase) and a similar increase for β-carotene and neoxanthin intracellular levels was observed.

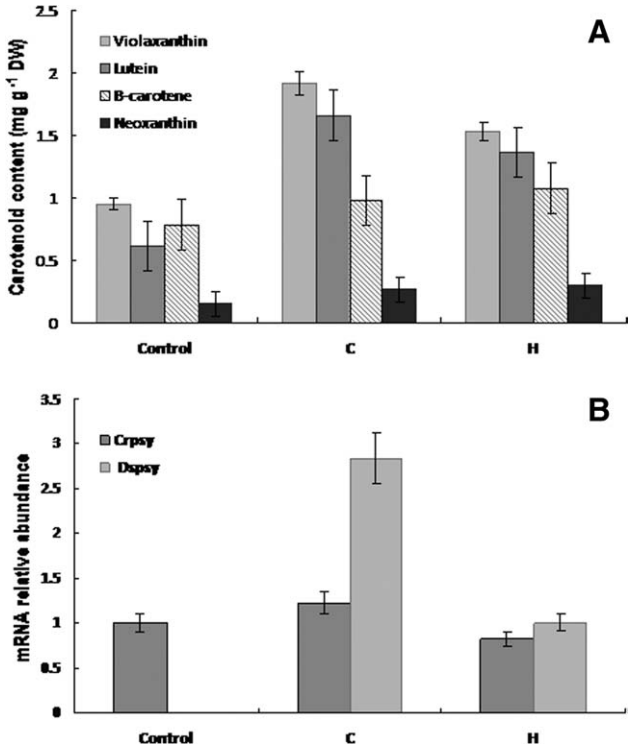

Figure 5. Carotenoids content (A) and mRNA relative abundance of endogenous (■) and foreign (□) *PSY* mRNA in *Chlamydomonas reinhardtii* transformed with *PSY* cDNA from *Dunaliella salina*.

(B). Levels of *PSY* transcripts are normalized respect to the housekeeping control gene (*CBLP*) and expressed as relative fold to the normalized *PSY* level of the control untransformed cells. All data were obtained in triplicate.

In transformant "H" *DsPSY* transcript level was about 33% the level observed in transformant "C." After several months, the phenotype and expression of exogenous *PSY* remained the same, as confirmed by chromatographic analysis and quantitative Real Time PCR quantification of *Dunaliella salina PSY* transcript level.

Discussion

PSY genes have been isolated from a wide variety of organisms including bacteria,[43,44] cyanobacteria,[45] higher plants such as tomato,[46] and corn;[47] green microalgae, such as *Haematococcus pluvialis*,[48] *Dunaliella salina*,[49] *Chlamydomonas reinhardtii*,[50] and *Micromonas* sp. RCC299[51] and diatoms such as *Phaeodactylum tricornutum* CCAP 1055/1.[52] In *Chlamydomonas*, PSY is a polypeptide of 382 aminoacids with a molecular weight of 43.53 KD very similar to the corresponding PSY of *Dunaliella* salina with 420 aminoacids. All plant, algal and cyanobacterial PSY sequences are well conserved resemble those of the analogous bacterial PSY enzymes (CRTB) and share an extensive prenyl transferase domain with squalene synthase enzymes.[10] Comparision of *C. reinhardtii* and *D. salina* PSY sequences yields 75% of identity and a similar codon usage. In this work *PSY* gene from *Dunaliella salina*, which has an extraordinary ability to produce carotenoids under certain stress conditions,[53] was isolated and, after checking its functionality by complementation assays in bacteria (Figure 3), expressed in *Chlamydomonas* nucleus and targeted to the chloroplast.

We observed that expression of the *PSY* from *Dunaliella* in *Chlamydomonas* caused the stable production of the corresponding *PSY* transcript and a significant increase in the content of carotenoids, such as violaxanthin, lutein and neoxanthin (Figure 5), which reached between 1.8- and 2.6-fold the level in control untransformed cells. Differences in the *DsPSY* transcript level observed in the different transformants obtained could be related with the insertion position in the genome and with the gene copy number, but this point has not been confirmed.

Similar strategy to increase the synthesis of carotenoids, based on the expression of *PSY* genes, has been successfully employed in higher plants. For example seed-specific overexpression of bacterial *PSY* led to an increase in phytoene, α and β-carotene reaching a 50-fold increment in the total content of carotenoids in mature seeds of *Brassica napus*.[21] Fraser et al.[25] reported the expression of a bacterial *PSY* in the fruit of tomato yielding elevated levels of phytoene, lycopene, lutein, and β-carotene (two–fourfold higher than the controls). In a similar way, Ducreux et al.[29] have obtained potato tubers with higher content of violaxanthin and other carotenoids, and Hauptmann et al.[54] reported a 1.5–2-fold carotenoids increment in carrot root, in both cases due to the expression of the bacterial *crtB* gene.

The largest carotenoids increases in genetically modified plants have been reported in plants or tissues with no carotenoids or with very low original levels, such as canola seeds.[21] In some cases the *PSY* product, phytoene, was found in the *PSY*-overexpressing tranformants.[21,25] In our study no phytoene was detected (Figure 4), showing that other enzymes downstream in the pathway are not limited in *Chlamydomonas*. Phytoene has not either been found in potato[29] or carrot[54] overexpressing foreign *PSY* genes.

In most cases the bacterial *phytoene synthase* encoding gene *crtB* from *Erwinia herbicola* has been used as inserted gene. The approximation used by Lindgren et al.[26] in *Arabidopsis* was, however, based on an endogenous *PSY* gene. They reported that overexpression of the own *PSY* enzyme under the control of a seed specific promoter resulted in transgenic *Arabidopsis thaliana* plants with darker seeds that had a 43-fold average increase of β-carotene. They also showed higher amounts of lutein, violaxanthin and substantial level of lycopene and α-carotene. Similar approach was used in tomato by Enfissi et al.[55] who expressed the own tomato *PHY1* cDNA and obtained transgenic lines with almost complete reduction in fruit carotenoids due to cosuppression phenomena. Our previous attempts to overexpress the own *PSY* cDNA from *C. reinhardtii* resulted in a transient increase in the synthesis of violaxanthin, lutein, β-carotene and neoxanthin but they did not yield a stable phenotype (data not shown).

Despite the progresses achieved, poor understanding of the regulation of the pathway in plants and microalgae limits the extent to which carotenoids levels can be increased by insertion of structural genes and causes relatively low carotenoids increments, usually under twofold the carotenoids in control cells, and in some occasions difficulties for stable expression of transgenes. *Chlamydomonas reinhardtii* can be an excellent host for foreign genes of the carotenogenic pathway. The genetic modification of the carotenogenic pathway in microalgae opens up the possibility of enhancing the productivity of commercial microalgal-based systems to obtain carotenoids and offers an excellent tool to gain basic knowledge about an important pathway, not fully understood yet.

Acknowledgments

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