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Departamento de Química y Ciencias de los Materiales

Estudio y manipulación genética de la ruta de carotenoides en "Chlamydomonas Reinhardtii" mediante la expresión de genes exógenos

Memoria para optar al grado de doctora
presentada por:

Inmaculada Couso Liañez

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Rosa María León Bañares

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ESTUDIO Y MANIPULACIÓN GENÉTICA DE LA RUTA DE SÍNTESIS CAROTENOIDES EN *CHLAMYDOMONAS REINHARDTII* MEDIANTE LA EXPRESIÓN DE GENES EXÓGENOS

Memoria presentada para optar al grado de Doctora en Biología con Mención Europea por la Licenciada

Inmaculada Couso Liañez

Dirigida por: **Dra. Rosa Mª León Bañares**

Profesora titular de Bioquímica y Biología Molecular

Dept. Química y CC. De los Materiales
"Profesor José Carlos Vílchez Martín"

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PREFACIO

El trabajo que se recoge en esta tesis ha sido realizado en el laboratorio del área de Bioquímica y Biología Molecular de la Facultad de CC. Experimentales de la Universidad de Huelva. Esta área lleva años inmersa en la investigación de la producción de carotenoides en microalgas tanto a un nivel fisiológico como molecular. Proyectos como "Biosíntesis de astaxantina y β-caroteno en *Haematococcus pluvialis*, *Dunaliella salina* y cepas transgénicas de *Chlamydomonas reinhardtii* (AGL2004-08215-C03-01)", "Biosíntesis de carotenoides de interés comercial en microalgas: regulación y manipulación genética de la ruta (AGL2007-65303-C02-01)" o el más reciente, "Mejora de las características nutricionales de las microalgas mediante manipulación genética de la ruta de síntesis de carotenoides (P09-CVI-5053)" han aportado la financiación y el apoyo al trabajo realizado por este grupo en los últimos años.

Esta tesis consiste en una introducción general al tema que se ha estado estudiando más cuatro capítulos en formato artículo que describen el trabajo realizado por la doctoranda durante el desarrollo de su tesis doctoral. Además, se han incluido al comienzo de cada capítulo su correspondiente resumen en español para clarificar más aún, si cabe, el objetivo propuesto en cada caso. De los cuatro capítulos propuestos, el primero fue publicado en 2007 en la revista Journal of Biotechnology, el segundo capítulo ha sido fue publicado en la revista Biotechnlology Progress en Febrero del 2011 y el tercer capítulo ha sido enviado a otra revista este mismo año.

Publicaciones

Inmaculada Couso Liañez and Rosa León Bañares (2011). Overexpression of an exogenous phytoene synthase gene in the unicellular alga *Chlamydomonas reinhardtii* leads to an increase in the content of carotenoids. Biotechnology Progress 27(1), 54-60.

Rosa León Bañares, Inmaculada Couso Liañez, Emilio Fernández (2007). Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*. Journal of Biotechnology. 130, 143-152.

Inmaculada Couso Liañez, Rosa León Bañares (2008).Targeting of exogenous β-carotene oxygenase into the chloroplast is essential for its efficient function in the microalga *Chlamydomonas reinhardtii*. In: Enviromental, Industrial and Applied Microbiology. Formatex: Badajoz. Vol. 1 pp. 607-610.

Otras colaboraciones

Baldo F. Cordero, Irina Obraztsova, Lucía Martín, Inmaculada Couso, Rosa León, M. Ángeles Vargas and Herminia Rodríguez (2010). Isolation and characterization of a lycopene β-cyclase gene from the astaxanthin producing green alga *Chlorella zofingiensis* (Chlorophyta). Journal of Phycology. (En prensa).

Marta Vila Spinola, Inmaculada Couso Liañez, Rosa León Bañares (2008). Carotenoid content in mutants of the chlorophyte *Chlamydomonas Reinhardtii* with low expression levels of phytoene desaturase. Process Biochemistry. 43, 1147-1152.

Abbreviations:

ATP: Adenosine-5'-triphosphate.
AMD: Age related macular degeneration.
BKT: β-carotene-C4-ketolase.
CRTA: Spheroidene monooxygenases.
CRTB: Phytoene synthase.
CRTISO: Carotene isomerase.
CRTW/CRTO: β-carotene oxygenases.
DMAPP: Dimethylallyl pyrophosphate.
DOXP: 1-Deoxy-D-xylulose 5-phosphate.
FAD: Flavin adenine dinucleotide.
FDFT: Plant squalene synthases.
FPP: Farnesyl pyrophosphate.
GGPP: Geranylgeranyl pyrophosphate.
IPI: Isopentenyl pyrophosphate isomerase.
IPP: Isopentenyl pyrophosphate.
LCYB/CRTLB: Lycopene cyclase β.
LCYE/CRTLE: Lycopene cyclase ε.
LHC: Light harvesting complex.
MEP: 2-C-Methyl-D-erythritol 4-phosphate.
MVA: Mevalonate pathway.
NADPH: Nicotinamide adenine dinucleotide phosphate.
NPQ: Non photochemical quenching.
PDS: Phytoene desaturase.
PSY: Phytoene synthase.
VDE: Violaxanthin de-epoxidase.
ZDS: ζ-carotene desaturase.
ZEP: Zeaxanthin epoxidase.

INTRODUCTION

1. Carotenoids

Carotenoids are a wide group of lipophylic isoprenoid compounds synthesised by all photosynthetic organisms and also by some non photosynthetic bacteria and fungi. They bind to membrane integral proteins where they participate in light harvesting and in the protection of the photosynthetic apparatus against the photooxidative damage (Demming-Adams et al., 1996; Baroli and Niyogi, 2000). Since only photosynthetic organisms and certain bacteria and yeasts are able to synthesize carotenoids, animals and other organisms that do not synthesise them *de novo* must include them in their diet to acquire their characteristic colours (this is the case of salmons, shrimps o some birds) or as precursors for essential compounds, such as vitamin A or the visual pigment retinal.

Carotenoids have been industrially exploited as natural pigments and pro-vitamin factors for a long time. For instance some ketocarotenoids, such as astaxanthin and canthaxanthin are essential feed additives to provide aquacultured-grown salmon, trout or shrimp their characteristic pigmentation (Lorenz and Cysewski, 2000). These animals acquire its characteristic pink colour when grown in nature due to the carotenoids-rich phytoplacton (microalgae and marine bacteria) that they take as feed. Astaxanthin is principally consumed by the salmon feed industry and its annual worldwide market is estimated at US$ 200 million with an average price of US$ 2500/kg. But, nowadays, the global market of this carotenoid is dominated by the synthetic forms of the pigment which is produced by BASF and Hoffman–La Roche (Fig. 1).

The xanthophylls, lutein and zeaxanthin, are used to enhance the yellow pigmentation of eggs and poultry (Botella-Pavía and Rodríguez-Concepción, 2006) and β-carotene is used as colorant and antioxidant in many human foods, such as margarines, and in cosmetic and pharmaceutical industries. Carotenoids intake has shown to offer protection against macular degeneration, UV-induced skin damage and some age-related degenerative diseases (Guerin et al., 2003). Recent studies suggest that their antioxidant properties and other unexpected biological functions related to gene regulation or juntional communication could provide additional health benefits, such as tumour suppressing, immune system protective and anti-

inflammatory activities (Bertram, 1999; Demming-Adams, 2002; Stahl and Sies, 2005). Carotenoids can be considered functional foods or nutraceuticals, which are object of growing interest.

The global market of carotenoids mainly corresponds to *in vitro* synthesis, but the growing demand on natural additives and the fact that some of them have proven even more active than the ones from the *in vitro* synthesis are making those natural products more attractive to consumers.

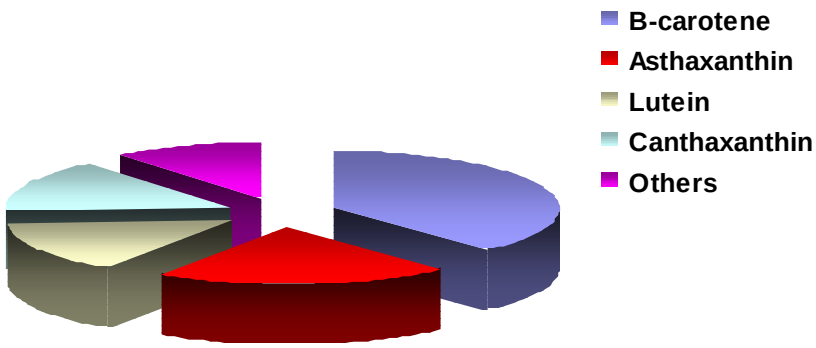

Figure 1: Representation of global market value for specific carotenoids.

In general, a distinction can be made between primary and secondary carotenoids. Primary carotenoids are defined as structural and functional components of the photosynthetic apparatus of the cell, while secondary carotenoids are produced by microalgae after being exposed to different stresses, production. Their localization is typically associated with membranes and usually bound to specific proteins. In general terms primary carotenoids are localized in the thylakoid membrane, while secondary ones are found in lipids vesicles even in the plastid stroma or in the cytosol (Eonseon et al., 2002).

Carotenoids are split into two different types, carotenes, which are purely hydrocarbons and contain no oxygen, and xantophylls, which contains oxygenated groups. Carotene is a terpene, synthesized biochemically from eight isoprene units.

The most abundant isomers are alpha-carotene (α-carotene) and beta-carotene (β-carotene). Gamma, delta, epsilon, and zeta (γ, δ, ε, and ζ-carotene) also exist. Since they are hydrocarbons, and therefore do not contain oxygen, carotenes are fat-soluble and insoluble in water, while other carotenoids, such as xanthophylls, are slightly less chemically hydrophobic. Carotenes are responsible for the orange colour of the carrot, for which it is named, and many other fruits and vegetables (for example, sweet potatoes and orange cantaloupe melon). Carotenes are also responsible for the orange colours in dry foliage. They also (in lower concentrations) impart the yellow colouration to milk-fat and butter. One of the most important carotenes is β-carotene which is composed of two retinyl groups, and is broken down in the mucosa of the small intestine by β-carotene 15, 15'-monooxygenase to retinal, a form of vitamin A. β-carotene can be stored in the liver and body fat and converted to retinal as needed, thus making it a form of vitamin A for humans and some other mammals. The carotenes α-carotene and γ-carotene, due to their single retinyl group (β-ionone ring), also have some vitamin A activity (though less than β-carotene), as does the xanthophyll carotenoid β-cryptoxanthin. All other carotenoids, including lycopene, have no β-ring and thus no pro-vitamin A activity, although they have antioxidant activity and other biological activity.

Xanthophylls are oxygenated carotenoids that serve a variety of functions in photosynthetic organisms. Microalgae, yeast or other microorganisms produce some of the xanthophylls that are being commercially used due to their colour and antioxidant capacity. Many functions of xanthophylls have been reported from accessory light harvesting pigments, structural entities within the light harvesting complexes (LHC), molecules required for the protection of the photosynthethic organisms from the potentially toxic effects of light and antioxidants in membranes to mitigate lipid peroxidation (Baroli and Niyogi , 2000). In the next sections some of the major functions and applications of carotenoids are going to be described.

1.1 Carotenoids in Photosynthesis

The main function of carotenoid pigments is related to the cellular process of photosynthesis, the photoautotrophic process in which carbon dioxide and water are converted into carbohydrates with molecular oxygen as a by-product (Berg and Stryer, 2002). In photosynthetic organisms a number of protein complexes have been identified that complete the reactions necessary for photosynthesis to take place, these include reaction centres and light harvesting complexes I and II (Telfer et al., 1994; Tracewell et al., 2001; Berg and Stryer, 2002). Carotenoids act principally as accessory light harvesting pigments within these distinct complexes and also serve to prevent cellular damage, via the absorption or quenching of radicals (Noguchi et al., 2001). In photosystem II complexes, the primary function of carotenoids is in the quenching and scavenging of singlet oxygen (Telfer et al., 1994; Tracewell et al., 2001). Therefore the functions of carotenoids, in the photosynthetic apparatus, in general, include the protection of the apparatus via the direct quenching of chlorophyll reactive intermediates and/or singlet oxygen molecules, the dissipation of absorbed energy, beyond that required for photosynthesis (Niyogi, 1999; Li et al., 2009), light absorption in regions of the spectra where other pigments are less efficient, such as the blue-green and yellow regions of the visible spectrum, and as integral structural elements of various photosynthetic complexes (Freer et al., 1996). In order to successfully function as antioxidants, carotenoids must have a lower excited triplet energy level than that of singlet oxygen. This essentially means that to function as an antioxidant compound, a carotenoid molecule must contain a conjugated system that is composed of more than nine double bonds.

1.2 Carotenoids in photoprotection; the xantophyll cycle

Carotenoids are distributed in the thylakoid pigment-protein complexes in close proximity to the chlorophylls, and thus to the potential sites of singlet oxygen formation. They are the most important quenchers of electronically excited states in the thylakoid membrane. The interaction between carotenoids and chlorophyll is undoubtedly complex but it is clear that other factors than direct de-excitation by carotenoids are important in the control of quenching of chlorophylls fluorescence *in vivo*. Chlorophyll triplet state or singlet oxygen transfers its excitation energy to a

nearby carotenoid molecule to form a carotenoid triplet that decays harmlessly to the ground state by thermal dissipation. Carotenoids can also protect against lipid peroxidation by reacting with free radicals directly (Palozza and Krinsky, 1992), forming a carotenoid radical that could be regenerated by interaction with tocopherols and ascorbate in the lipid phase of the membrane (Edge et al., 1997).

Carotenoids are involved in the de-excitation of chlorophyll reactive states (Demmig-Adams et al., 1992; Demmig-Adams et al., 1996; Gilmore, 1997; Grunëwald et al., 2001; Horton et al., 1996). This deexitation, measured as non-photochemical quenching of chlorophyll fluorescence (NPQ), depends on a large trans-thylakoid proton gradient that is established in excessive light. In general terms the developement of a NPQ correlates the biosynthesis of zeaxanthin and antheraxanthin from violaxanthin via the xanthophyll cycle.

The light–dependent xanthophyll conversion is essential for the adaptation of plants and algae to different light conditions and allows a reversible switch of photosynthetic light harvesting complexes between a light harvesting state under low light and a dissipative state under high light (Jahns et al., 2009). The photoprotective functions of zeaxanthin have been intensively studied and a number of roles have been proposed for the xantophyll cycle over the past 20-30 years (Young et al., 1997) but it was not until 1989 when Demming-Adams and coworkers first proposed a now widely accepted role linking zeaxanthin formation with photoprotection. The process of zeaxanthin mediated dissipation of excess excitation energy is a key process in the photosynthetic systems of higher plants and microalgae. Among all the xanthophylls, zeaxanthin is the only one that accumulates exclusively under excess light, by deepoxidation of existing violaxanthin in the so-called xanthophyll cycle (Eskling et al., 1997) (Fig. 2). It is widely thought that the main function of zeaxanthin is as a quencher of the chlorophyll singlet state through the process of NPQ (Müller et al., 2001). However, it has been suggested that zeaxanthin may protect from light stress by directly quenching singlet oxygen and free radicals (Havaux and Niyogi, 1999) and by making the thylakoid membrane less permeable to oxygen (Gruszecki, 1999).

When the irradiance is greater than required for photosynthesis in the chloroplasts of plants and green algae, a reversible violaxanthin de-epoxidation reaction occurs to form antheraxanthin subsequently resulting in the accumulation of

zeaxanthin in chloroplast thylakoids. In chloroplasts the extent of the de-epoxidation depends on at least two factors, namely, the pigment pool size and the fraction of the violaxanthin pool that is available for de-epoxidation (Sieffermann et al., 1974; Siefermann et al., 1975). Violaxanthin de-epoxidase (VDE) carries out the stepwise removal of the 5-6 epoxides from violaxanthin and antheraxanthin to form zeaxanthin on the thylakoid lumen side of the membrane. De-epoxidase activity requires induction of a lumen pH and the presence of ascorbate. In the dark when the pH is neutral, the de-epoxidase enzyme is unbound. Under high light the photosynthetic proton pump increases the acidity of the lumen and the de-epoxidase enzyme binds to the thylakoid membrane. Purification of the VDE from lettuce was firstly achieved by an anion exchange chromatography step using the ability of the epoxidase to bind the thylakoid lipid monogalactosylglycerol (MGDG) at pH 5.2 and release at pH 7.2 (Rockholm et al., 1996). Using an internal sequence of *vde* in lettuce as a probe, other *vde* genes from tobacco and *Arabidopsis* were amplified as well. The *vde* gene has not been found in *Chlamydomonas reinhardtii*, although a violaxanthin de-epoxidase-related protein (VDR1), which functionality has not been reported yet, has been found in the genome of this microalga (Merchant et al., 2007).

When the absorbed irradiance is lower than that required for the saturation of photosynthesis, zeaxanthin is converted back to violaxanthin by the enzyme zeaxanthin epoxidase (ZEP) with the intermediate antheraxanthin, this occurs on the chloroplast stromal side of the membrane and it is relatively slower than the de-epoxidation reaction. ZEP has specificity for carotenoids with a 3-hydroxy-β-hexenyl ring and requires the presence of NADPH and FAD as cofactors, with ferredoxin and ferredoxin-like reductives also required for its activity (Büch et al., 1995). The first isolation of this gene was done in *Nicotiana plubaginifolia* (Marin et al., 1996) and many genetic studies in *Arabidopsis thaliana* (Rock et al., 1991) or the green algae *Chlamydomonas reinhardtii* (Niyogi et al., 1997) have been made.

VDE and ZEP are the first reported lipocalin proteins identified from plants and only the second example of lipocalin proteins with enzymatic activity (Hieber et al., 2000). Lipocalins are characterized as having three motifs from structurally conserved regions within a β-barrel structure; both the de-epoxidase and epoxidase enzymes exhibit longer N- and C-terminal regions when compared to other lipocalin molecules and it is realistic to predict that those enzymes has the same tertiary structure as they

both function on the same intermediate antheraxanthin. However, apart from the lipocalin core structure there are no common sequences that can predict any homology between these two enzymes.

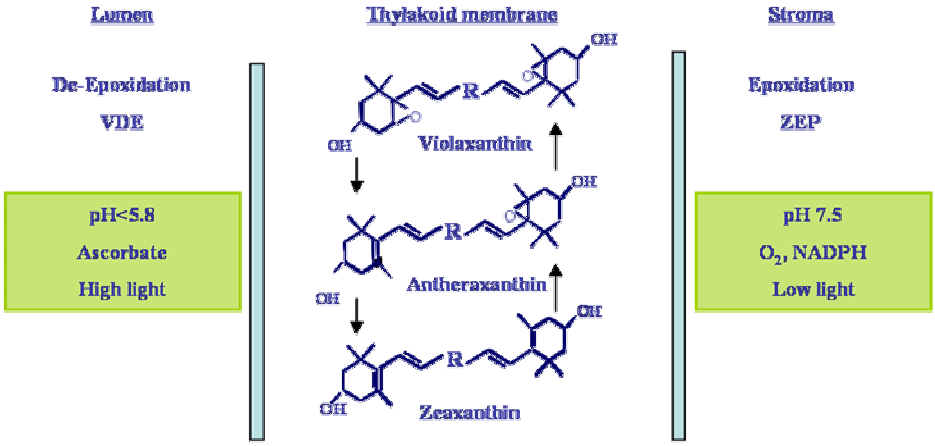

Figure 2: Scheme of the reactions of the xanthophyll cycle (Jahns et al., 2009).

1.3 Carotenoids are beneficial for non-photosynthetic organisms as well

The benefitial properties of carotenoids are not limited to photosynthetic organisms, as in non-photosynthetic organisms, including invertebrate and vertebrates, carotenoids are also essential, functioning as colorants, pro-vitamin compounds and antioxidants. This is despite the inability of most of these organisms to biosynthesis carotenoid compounds *de novo* (Johnson, 2003; Bhosale and Berstain, 2007). Thus carotenoids are obtained from dietary sources, such as fruits and vegetables, rich in carotenoid compounds. In mammalian cells, as with all eukaryotic cells, chemical energy adenosine triphosphate (ATP) is generated in the mitochondria via a series of oxidative phosphorylation reactions, by-products of these reactions are large amounts of reactive oxygen species. Additionally, in mammals, activities of various parts of the immune system also results in the production of reactive oxygen species. This is of significance as these by-products include hydrogen peroxide, hydroxyl radicals, superoxide radicals, myeloperoxidase and peroxinitrate. As with

antioxidant activity in photosynthesis, it has been demonstrated that carotenoids are able to quench these oxidant by-products, thereby reducing instances of oxidative cellular damage and macromolecule degradation (Edge et al., 1997).

1.4 Importance in human's health

The antioxidant capacity of carotenoids has received much interest due to potential applications in human health and nutrition. Oxidative stress has been identified as a key causative agent in a range of prolific human diseases, including numerous cancers, cardiovascular and coronary diseases (Mayne, 1996), as well as ocular dystrophies such as age related macular degeneration (Bernstein et al., 2002; Zhao et al.,2003) and cataracts. Antioxidants, such as vitamin A, C and D, as well as carotenoids, are under investigation to determine there functions in prevention and/or treatment of these devastating pathologies (Goodman and Pardee, 2003; Polidori, 2004). In addition to disease, the antioxidant capacity of carotenoids has also been linked to the protection of mammals from UV radiation. Damaging solar radiation is predominantly in the form of UVA and UVB (Sies and Stahl, 2004), excessive exposure of the skin to this damaging radiation results in the production of reactive oxygen species within the tissue , leading to the induction of inflammatory responses, and more long term to photo-carcinogenesis and photo-induced ageing. Antioxidant compounds have been proven to limit UV-induced damage to cellular DNA and epidermal cells, and indeed a strong correlation is observed between dermal carotenoid content and endogenous UV photo-protection.

The age related macular degeneration (AMD) is caused by the degeneration of the retina and macula, which ultimately culminates in the loss of vision. AMD is cited here, in part due to its prevalence, affecting more than 14 million people globally, but also because a clear link between carotenoids and AMD has been established. In particular two carotenoids, lutein and zeaxanthin are relevant. These are present in high concentrations within ocular material, including the macula region of the retina, and their levels have been linked directly to disease on set and its prevention (Landrum et al., 1999). Zeaxanthin and lutein function both as antioxidants and as light absorbing pigments, thereby protecting the eye from both oxidation and the damaging UVB energies of the sun, as zeaxanthin and lutein predominantly absorb high blue light energy. Interestingly studies on other known antioxidants, vitamin A, E

and C revealed no relationship between consumption and reduced instances of AMD. In addition to AMD, carotenoids are also known to have benefitial influences on a number of other ocular diseases, including cataracts and atherosclerosis (Mares-Perlman et al., 1995).

2. Carotenoid Biosynthesis

2.1 Mevalonate versus non-Mevalonate pathway for the synthesis of IPP

Carotenoids are formed from a 5C building block, precursor of all isoprenoids, the isopentenyl pyrophosphate (IPP). For many years, it has been assumed that in all organisms IPP was synthetized through the well-known mevalonate pathway (MVA), but an alternative MVA independent pathway, also called non-mevalonate , 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Ramos -Valdivia et al., 1997), seems to be the main responsible for the synthesis of carotenoids in photosynthetic tissues. This pathway was identified in eubacteria and plastids, when unexpected labelling patterns that did not agree with the MVA route, were observed in bacteria and microalgae grown with 13C labelled acetate (Eisenreich et al., 2004, Lichtenhaler, 1999).

The main difference between both routes, apart from their location, is the starting condensation step. In the MVA pathway, the IPP is synthesised by the condensation of three acetyl-coA molecules, while the MEP pathway occurs via the condensation of pyruvate and D-glyceraldehyde-3-phosphate. In higher plants it has been demonstrated that IPP is synthesised via the mevalonate pathway, located in the cytoplasm, forming the precursor for cytokinins, polyprenoids and sesquiterpene biosynthesis, while the mevalonate independent pathway is responsible for IPP biosynthesis in the chloroplasts, providing precursors for photosynthetic terpenoids, carotenoids, phytol, and prenyl side chain of plastoquinone, as well as mono and diterpene products of the plant and algae cells (Rohmer, 1999) (Fig. 3). Green algae, in turn, seem to have lost their cytosolic MVA pathway of IPP formation.

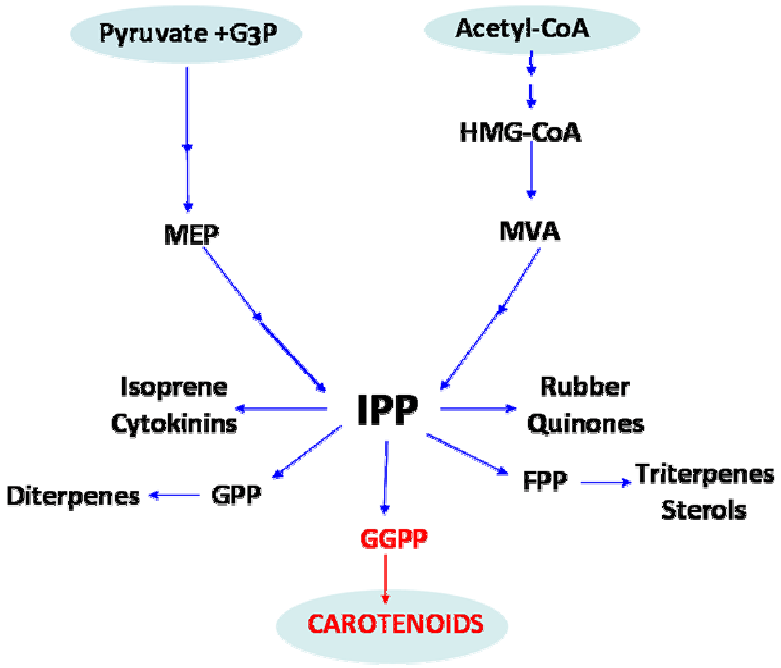

Figure 3: Schematic representation of isopentenyl pyrophosphate (IPP) formation by either mevalonate (MVA) or 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways. IPP serves as the central metabolite leading to an immense variety of different isoprenoid compounds. G3P, D-glyceraldehyde-3-phosphate; HMG-CoA, hydroxymethylglutaryl-CoA; GPP, geranylpyrophosphate; GGPP, geranylgeranylpyrophophate; FPP, farnesylpyrophosphate.

2.2 Biosynthesis of carotenoids from IPP

The formed IPP is isomerised by the isopentenyl pyrophosphate isomerase (IPI) to a dimethylallyl pyrophosphate (DMAPP) that is converted to GGPP (Geranylgeranyl pyrophosphate) by the successive addition of three molecules of IPP. The condensation of two GGPP molecules, catalyzed by the enzyme phytoene synthase (PSY) yields the first 40C lineal uncoloured carotenoid, phytoene. From phytoene the rest of the carotenoids are synthetized by a series of desaturations and cyclations;
and the xanthophylls by hydroxylation, oxygenation or epoxidation of the corresponding carotenoids. Reviews of Sandmann in 1994, Amstrong in 1997 or Cunningham and Gantt in 1998 and the more recent ones of Botella-Pavía and Rodriguez- Concepción in 2006 and Sandmann and co-workers in 2006, offer a good

general view of this metabolic pathway in plants and microorganisms. Most of the carotenoids biosynthesis genes in *Chlamydomonas* have been cloned or assigned by homology with the sequences of genes from other similar microalgae (Grossman et al., 2004; Lohr et al., 2005).

The phytoene undergoes a series of four desaturation reactions that increase the number of conjugated double bonds of phytoene and transform it into lycopene. In bacteria one unique enzyme (CRTI) catalyses the four desaturation steps, while in plants two related enzymes are involved, the phytoene desaturase (PDS) and ζ-phytoene desaturase (ZDS) are involved in the synthesis of pre-lycopene, or poly cislycopene that is isomerised to all-trans lycopene thanks to the enzyme carotenoid isomerase (CRTISO). It seems that in the presence of light this isomerization can take place spontaneously, but CRTISO has been found in many higher plants and microalgae (Isaacson et al., 2002). Also, the role of Z-ISO in isomerization of the 15-cis-bond present in the PDS product, 9,15,9´-tri-cis-ζ-carotene, to form the ZDS substrate 9,9´-di-cis-ζ carotene has been recently reported by Chen and co-workers (2010). Both PDS and ZDS follow the same reaction mechanism and use plastoquinone as hydrogen acceptor, connecting in this way carotenoids desaturation and the photosynthetic electron transport chain.

At this point, the pathway split into two branches. Lycopene can be cycled by the action of the beta enzyme (LCYB, CRTLB) to yield gamma-carotene with a β-ring or by the action of lycopene cyclase epsilon (LCYE, CRTLE) to yield delta-carotene with a ε-ring. Each of the mono-cycled carotenoids formed can be newly cycled by any of the cyclases, so β-carotene with two β-rings, α-carotene with a ε- and β-ring or ε-carotene with two ε-rings can be formed. ε- and β-rings only differ in the position of a double bond. A diagram with all these steps is shown in figure 4.

The hydroxylation of the C3s of each ring of β-carotene produces zeaxanthin and the hydroxylation of the C3s of α-carotene produces lutein. The carotenoid hydroxilases in *Chlamydomonas* and also in *Arabidopsis*, are significantly less conserved than the other enzymes of the pathway. In *Arabidopsis*, there are two classes of structurally unrelated hydroxylases that catalyze these ring hydroxylations: two heme-containing cytochrome P450 monooxygenases compromising β- and ε-ring hydroxylases (CHYBP450/CYP97A3, CHYEP450/CYP97C1) (Kim and DellaPenna, 2006;

Tian et al. 2004) and two non-heme di-iron monooxygenases that catalyze only the hydroxylation of β-ionone rings (CHYB) (Sun et al., 1996). In *Chlamydomonas,* the existence of candidate genes for CHYB and as well as for the P450 dependent enzymes has been reported, but no functional analysis has been carried out (Lohr, 2009). The enzyme β-carotene-C4-ketolase (BKT) is the main responsible for the synthesis of astaxanthin. Ketolase enzymes does not exist in higher plants, only in some microalgae, such as *Haematococcus* or *Muriella zofingiensis*, some photosynthetic bacteria such as *Agrobacterium aurantiacum* and some yeasts. Astaxanthin is synthesized from β-carotene through two reactions, one of oxygenation and other of hydroxylation. In all studied organisms, except in *Xanthophyllomyces dendrorhous*, these two steps are independent (Ojima et al., 2006). Two different pathways for the biosynthesis of astaxanthin have been proposed: one starts with the oxidation of β-carotene and has equinenone, canthaxanthin and adonirubin as intermediates, and the other starts with the hydroxylation of β-carotene and involves the intermediates β-criptoxanthin, zeaxanthin and adonixanthin. The first route has been shown to work in the marine bacteria *A. aurantiacum* and the green algae *H. pluvialis* through functional analysis experiments (Lotan and Hirschberg, 1995; Lu et al., 1995; Misawa et al., 1995a; Breitenbach et al., 1996; Linden, 1999). These ketolases have been reported to accept preferently β-carotene as substrate. Recently, the second possible route has been confirmed in *Muriella zofingiensis* by Huang et al. 2006. Table 1 shows a summary of the different carotenoid genes called differently in different organisms. Interestingly, genes encoding BKT and CHYB are contiguous on the *Chlamydomonas reinhardtii* genome, with *bkt* located in the same strand and just upstream. Despite this fact, no astaxanthin has been detected in *C. reinhardtii* (Lohr et al., 2005).

Table I: Principal genes and enzymes related to carotenes and xanthophyll biosynthesis.

Enzyme	Plant	Bacteria	Cianobacteria	Function
		Biosynthesis of acyclic carotenoids		
Phytoene synthase	*psy*	*crtB*	*crtB*	Condensation of two GGPP
Phytoene desaturase	*pds*	*crtI**	*crtP*	Desturation of phytoene.
ζ-Carotene desaturase	*zds*	*crtI**	*crtQ*	Desaturation of ζ-carotene
Carotene isomerase	*crtIso*	-	*crtH*	Isomerization of pro-lycopene.
15-cis-ζ-Carotene isomerase	*ziso*	-	*ziso*	Isomerization of 15-cis-ζ-carotene
		Cyclation of carotenoids		
Lycopene β-cyclase	*lcyB*	*crtY*	*crtL-b*	Introduction of β rings
Lycopene-ε-cyclase	*lcyE*	-	*crtL-e*	Introduction of ε rings
		Biosynthesis of xanthophylls		
Carotene β-ring hydroxylase	*chy B*	*crtZ*	*crtR*	Hydroxylation of β rings
Carotene ε-ring hydroxylase	*chy E*	-	-	Hydroxylation of ε rings
Zeaxanthin epoxidase	*zep*	-	-	Epoxidation of β rings
Violaxanthin deepoxidase	*vde*	-	-	De-epoxidation of β rings
Neoxanthin synthase	*nsy*	-	-	Conversion of violaxanthin to neoxanthin.
β-carotene (C4) ketolase	*bkt*	*crtW/crtO*	*crtO/crtW*	Introduction of a keto–group in the C4 position of β rings.

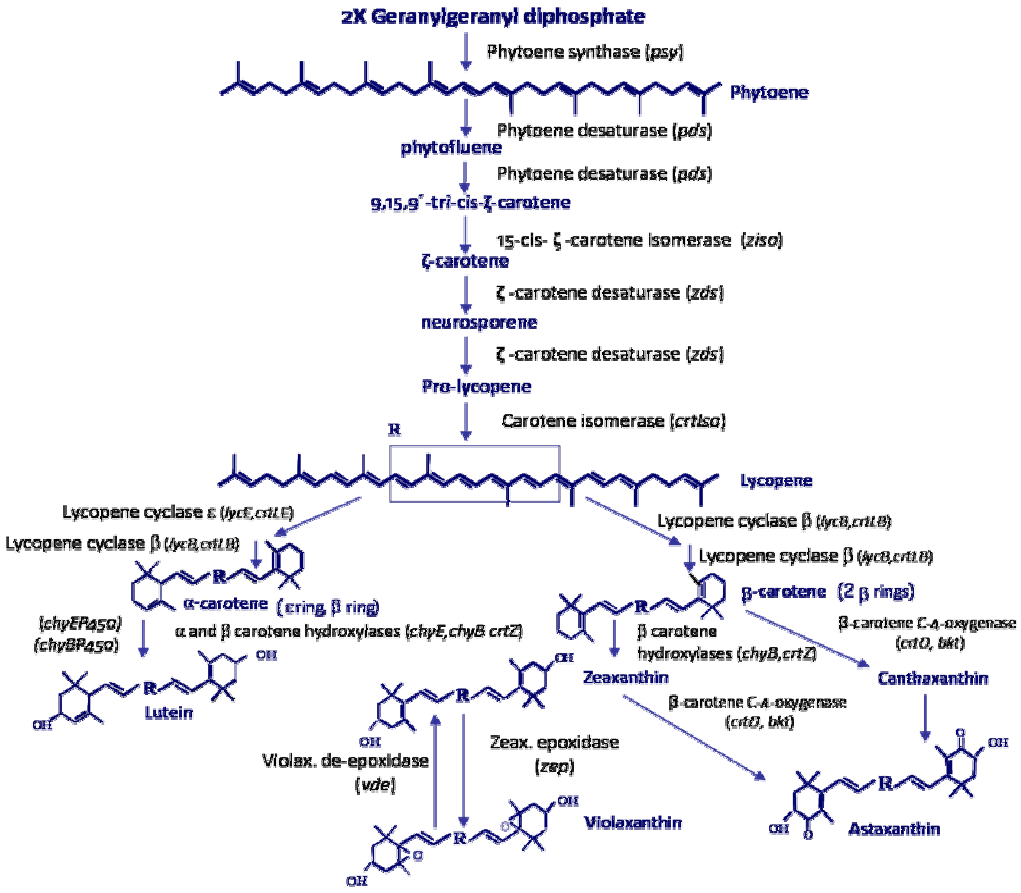

Figure 4: Diagrammatic representation of carotenoid biosynthesis proceeding from phytoene to astaxanthin.

In the following sections we will analyze in detail two enzymes of the pathway (Fig.4) that will be especially important during the development of this work.

2.3 Phytoene Synthase

The first committed step in carotenogenesis is the condensation of two GGPP molecules, resulting in the synthesis of phytoene, (Chamovitz et al., 1992) the first carotenoid of the pathway. This reaction and all subsequent steps are hydrophobic and membrane associated. This reaction is catalysed by a conserved monomeric protein, phytoene synthase (CRTB, PSY), and proceeds in two stages. First, the phytoene synthase catalyses the formation of prephytoene diphosphate, via the C1`-2-3 (head-to-middle) condensation of two GGPP molecules. In this step, a bond is formed between the first carbon of one allylic molecule, and the C2-C3 double bond of the second, producing a cyclopropylcarbinyl diphosphate molecule with a C1`-2-3 structure, often referred to as prephytoene diphosphate (Fig. 5). This reaction is quickly followed by a second, in which the pre-phytoene diphosphate intermediate undergoes a rearrangement to a 1`-1 structure, which contains a double bond between the two original GGPP molecules. In this second step phosphate is lost, as there are two protons in the form of hydrogen from the first carbon of each original GGPP molecule (Fig. 5). Early investigations revealed that the mechanism of synthesis for phytoene was similar to that for squalene, as this also proceeds via the 1`-2-3 condensation of two molecules, in this case of FPP, through an intermediate prior to reaching the final product squalene. Synthesis of phytoene results in the synthesis of two isomers, in which stereochemistry about the central double bond is observed. These isomers are termed either *Z*- or *E*-phytoene, or alternatively, *cis*- or *trans*-phytoene, respectively. Most downstream carotenoid compounds possess an *E*-type central double bond (Misawa et al., 1990; Misawa et al., 1995b). A number of potential regulatory roles have been identified for this protein; several experimental evidences support the expected role of *psy* as a key regulatory step. Overexpression of the *psy* gene in tomato plants resulted in dwarf plants because elevated phytoene production caused a reduction in gibberellin synthesis (Fray et al., 1995). In higher plants expression of the phytoene synthase gene has been demonstrated to be positively regulated by light (Bartley et al., 1999; Steinbrenner and Linden, 2001).

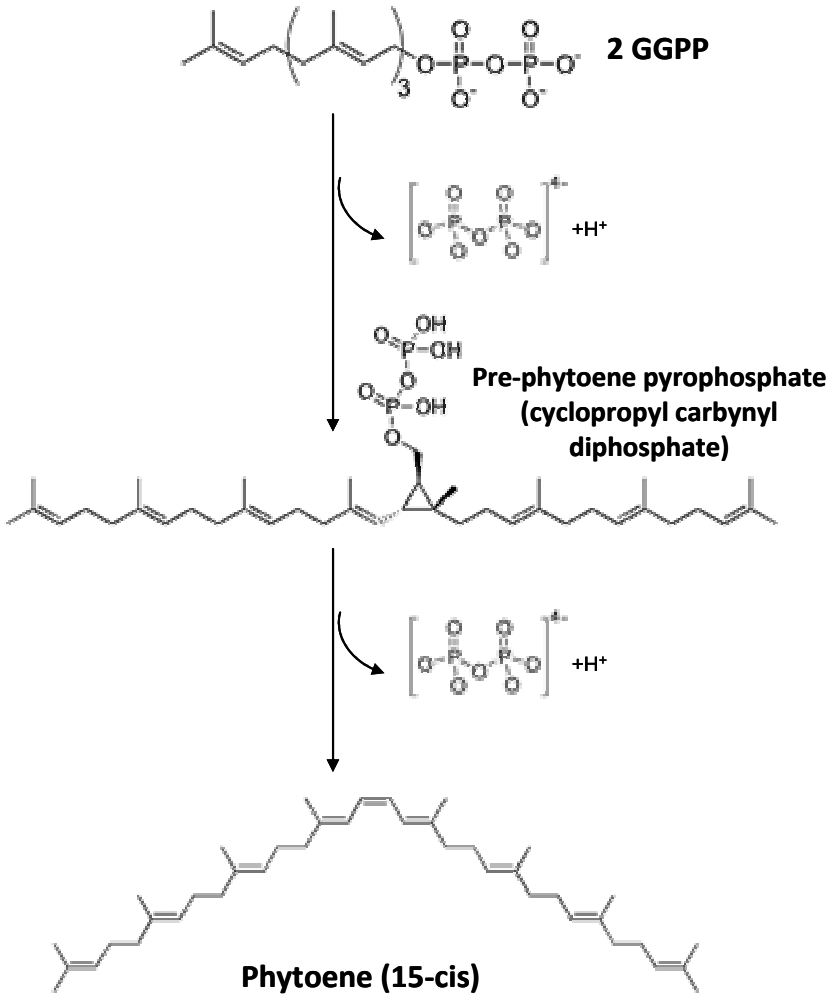

Figure 5: Condensation of the GGPP molecules by phytoene synthase to yield phytoene.

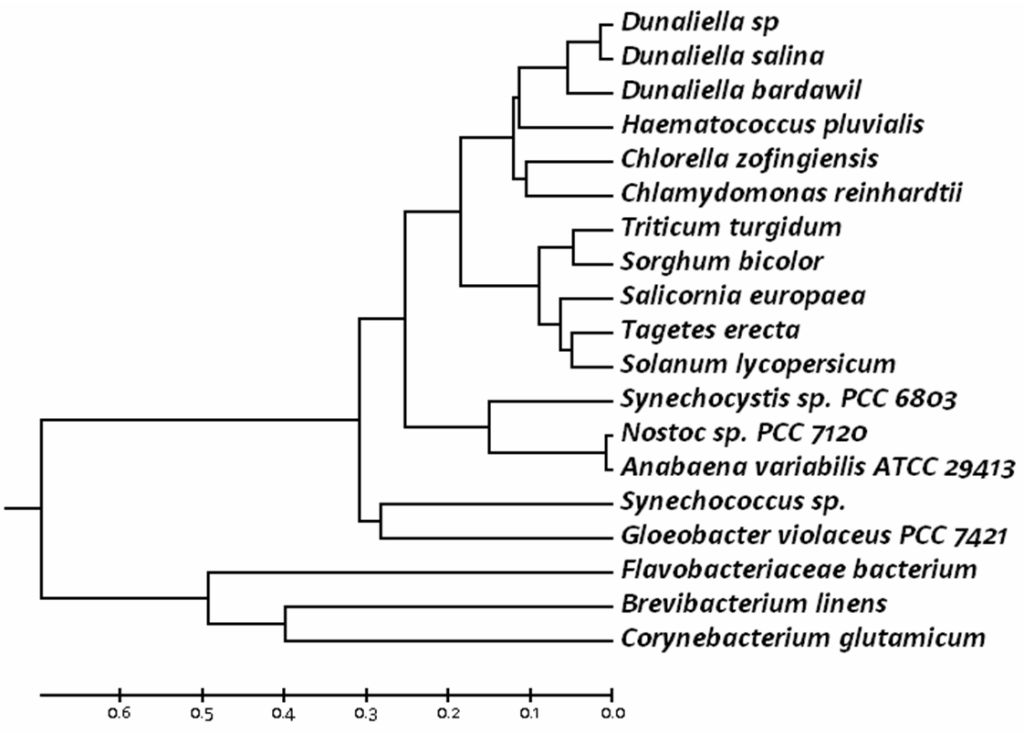

Figure 6: Neighbour joining tree calculated based on a clustalW aligment of different *psy* sequences from microalgae, plant, cyanobacteria and bacteria.

The aminoacid sequences of plant, algal and cyanobacterial PSY, resemble those of the analogous bacterial phytoene synthase enzymes (CRTB) (Fig. 6) and share an extensive prenyl transferase domain with squalene synthase enzymes. The PSY and CRTB enzymes share an additional conserved sequence domain not found in squalene synthases, and the known plant PSY enzymes have a third conserved sequence region that is not found in the algal, cyanobacterial or bacterial phytoene synthases (Fig. 7) (Cunningham and Gantt, 1998).

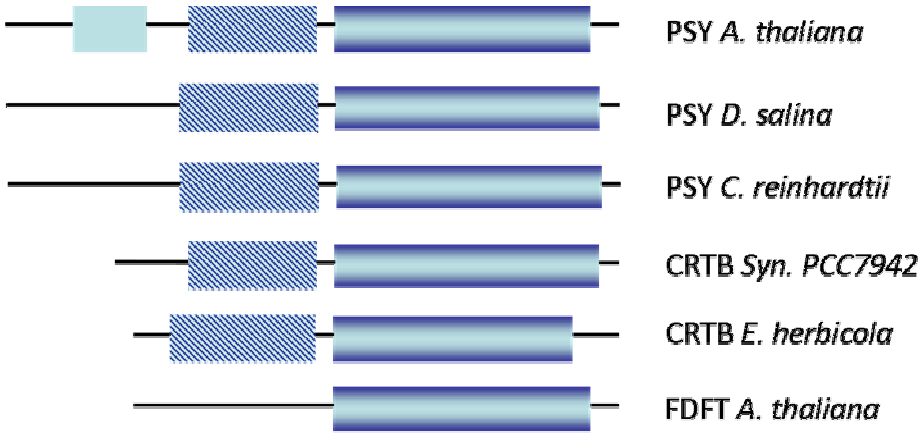

Figure 7: Plant, algal and cyanobacterial phytoene synthase enzymes (PSY) share conserved amino acid sequence regions with bacterial phytoene synthases (CRTB) and with plant squalene synthases (FDFT). Boxes with the same grids belong to the same conserved domains determined by ProDom 34.1.

The main biological significance of phytoene is to be an intermediate in the synthesis of later carotenoid compounds. Furthermore, phytoene is transparent and has a short system of conjugated double bonds (n9). Therefore is of no significant use in species colouration and posses only limited antioxidant ability, but has important uses in cosmetics as a non-color antioxidant additive in moisturising creams and other products such as the ones related to the skin whittening.

2.4 β-carotene ketolase

In addition to hydroxylation, xanthophylls may also, or alternatively, be modified via the addition of a ketone group (Sieiro et al., 2003). This reaction is catalysed by a group of proteins commonly referred to as β-carotene ketolases, due to the fact that to date ketolase proteins have only been identified that are capable of catalysing the ketolation of ionone rings of the β-ring conformation. Different types of β-carotene ketolase proteins are known, commonly referred to as the BKT (in plants and algae), CRTW (in bacteria and cyanobacteria as well) and CRTO types (found mainly in cyanobacteria and some bacteria as well), these are structurally very different and differ in the predominant ketolated carotenoid synthesised (Misawa et al., 1995a; Misawa et al., 1995b; Kajiwara et al., 1995; Fernández-González et al., 1997).

Recently, *Anabaena* sp. PCC 7120 (also known as *Nostoc*) was found to have both, CRTW and CRTO ketolases which functioned in two distinct biosynthetic pathways. CRTO catalyses the conversion of β-carotene to echinenone and canthaxanthin while CRTW catalyses myxol to keto-myxol synthesis (Mochimaru et al., 2005).

In general terms using β-carotene as a substrate, BKT and CRTW type ketolases predominantly produce the di-keto carotenoid, canthaxanthin, with trace amounts of the intermediate mono-keto carotenoid echinenone, while CRTO type ketolases produce preferently the mono-keto compound echinenone, as the main product. Even though the reactions of CRTO and BKT/CRTW involve the same β-carotene ketolation, the characteristics of enzymes are different. Although, BKT and CRTW types ketolases share significant levels of sequence identity with a family of proteins containing fatty acid desaturases and non-heme di-iron monooxygenases (β-carotene hydroxylases) (Masamoto et al., 1998), the CRTO type ketolase show homology to bacterial phytoene desaturase proteins and they are almost twice the size of the first ones, showing no significant amino acid sequence homology with BKT/CRTW enzymes (Tao and Cheng, 2004).

BKT and CRTW type ketolases contain 4 motifs rich in histidines useful for the coordination of iron atoms that are also present in β-hydroxylases (Kajiwara et al., 1995). Alternatively, CRTO type ketolases contain six highly conserved motifs, two of which are shared with fatty acids desaturases (FADs) (Tao and Cheng, 2004). β-carotene ketolases catalyse the stereo specific replacement of a hydrogen atom, of the 4 or 4` carbon of the β-ionone ring containing carotenoid molecule, with a ketone group (=O). Relatively little is known about the reaction mechanisms employed by ketolases, due to the sequence similarities and shared conserved domains of BKT and CRTW, non-heme di-iron mono-oxygenases (β-hydroxylases) and fatty acid desaturases, it is postulated that BKT/CRTW type ketolation proceeds via a similar reaction mechanism, in which the introduced oxygen atom is in the form of a ketone group, rather than a hydroxyl group, or alternatively, the mechanism may be identical, but quickly followed by a dehydrogenation reaction, producing the ketone functional group (Fernández-González et al., 1997). A similar mechanism may also be proposed for the CRTO type ketolase. The two kinds of ketolases structurally unrelated may have independently originated along evolution to catalyze the

introduction of a keto-group in the C4 position of β-rings by a similar mechanism but until now there are no reports of direct evolution of carotenoid oxygenases or post-cyclase carotenoid-modifying enzymes so further analyses are needed (Umeno et al., 2005; Mochimaru et al., 2005).

BKT and CRTW type β-carotene ketolase

β-carotene ketolase and β-carotene hydroxylase are carotenoid biosynthetic enzymes responsible for astaxanthin and zeaxanthin formation, respectively. This superfamily domain has extensive hydrophobic regions that would be capable of spanning the membrane bilayer at least twice. Comparison of these sequences also reveals three regions of conserved histidine cluster motifs that contain eight histidine residues: HXXX(X)H, HXX(X)HH, and HXXHH (an additional conserved histidine residue is seen between clusters 2 and 3). These histidines clusters commonly appear in enzymes that contain non-heme iron (Fraser et al., 1997) (Fig.9a). The eight histidine residues are reported to be catalytically essential and proposed to be the ligands for the iron atoms contained. On the basis of hydrophobicity studies and prediction models it has been reported that BKT can be formed by four transmembrane domains and other two hydrophobic regions. It will also include at least two histidines rich-motives which help in the iron coordination (Fig. 8).

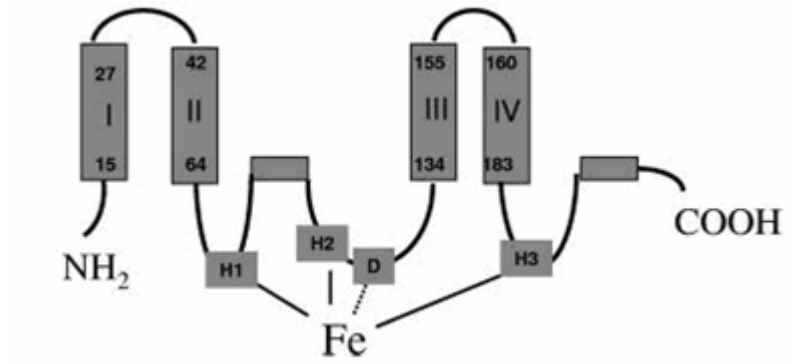

Figure 8. Schematic structure of β-carotene ketolase (CRTW) from *Paracoccus* sp. strain N81106 (taken from Ye et al., 2006).

BKT and CRTW type β-carotene ketolase genes have been isolated from a wide range of predominantly marine organisms, including three genes isolated from *H. pluvialis.* These have been nominated *bkt*1, 2 and 3, and share extensive sequence identity with CRTW type ketolase encoding genes from other sources (Kajiwara et al., 1995; Huang et al., 2006). Other examples include those of the marine bacteria *Paracoccus* sp. N81106, previously *Agrobacterium aurantiacum, Paracoccus* sp. PC-1, previously *Algaligenes* sp. PC-1 (Misawa et al., 1995a; Misawa et al.,1995b), and *Brevundimonas* sp. SD212 (Nishida et al., 2005) , and the photosynthetic bacterium *Bradyrhizobium* sp. strain ORS278 (Hannibal et al., 2000) (Fig. 9b).

Additionally, putative examples have been identified in a number of other species such as the microalgae *Chlamydomonas.* Although, there is no evidence that *Chlamydomonas* can produce ketocarotenoids in vegetative cells some recent studies showed that *Chlamydomonas´* zygospores can produce some kind of ketocarotenoid called ketolutein (Lohr et al., 2005). A β-carotene ketolase gene has also been isolated from the eukaryotic green algae *Muriella zofingiensis* (Huang et al., 2006). Although they erroneously name it CRTO, the protein from *Muriella zofingiensis* shares extensive sequence homology with the *crtW* homologous *bkt* genes of *H. pluvialis*, and possesses the capacity to convert zeaxanthin to astaxanthin (Huang et al., 2006; Choi et al., 2007). This therefore suggests that the β-carotene ketolase gene isolated from *M. zofingiensis* (Huang et al., 2006) is in fact a CRTW/BKT type ketolase.

CRTO type β-carotene ketolase

CRTO type β-carotene ketolase genes are less abundant in nature, than are BKT/CRTW type ketolases. The first example was isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 (Fernández-González et al., 1997), since then further examples have been experimentally characterised from *Anabaena* sp. PCC7120 (Mochimaru et al., 2005), and putative examples have been identified in a number of other cyanobacterial species (Liang et al., 2007). CRTO type ketolases are not unique to the cyanobacterial phyla, as examples have also been elucidated from the actinomycetales, *Rhodococcus erythropolis* AN12 and *Deinococcus radiodurans* R1 (Tao and Cheng, 2004). All of these carotene ketolase genes function to add a single ketone functional group to the fourth carbon of either one or both β-ionone rings of β-carotene. Substrate specificity is not limited to β-carotene, as two β-carotene

ketolase genes have been isolated, that, when present in their native host catalyse the ketolation of the single β-ionone ring of α-carotene, leading to the synthesis of 4-keto-α-carotene. However, when expressed heterologously in β-carotene accumulating bacterial cells, these proteins are able to utilise β-carotene as a substrate (Tao and Cheng, 2004). Although, in *Synechocystis* sp. PCC 6803 CRTO mediates the formation of the mono-keto product echinenone, it was demonstrated that higher oxygen supply and higher levels of the protein favour the formation of the di-keto products (Gerjets and Sandmann, 2006) and also in the case of *Rhodococcus* and *Deinococcus* CRTO can add two keto groups to produce canthaxanthin (Tao and Cheng, 2004). These examples serve to demonstrate that the β-carotene ketolase proteins do not recognise the entire carotenoid compound, as prior modification, or even presence/absence of a second β-ionone ring does not alter substrate preference. A further example is known in which the ketolation reaction is not specific to a β-ionone ring, as the enzyme spheroidene monooxygenases (CRTA) catalyses the ketolation of a linear carotenoid. This protein is found in some photosynthetic bacteria, specifically of the *Rhodobacter* and *Rubrivivax genus* compound (Takaichi et al., 2001).

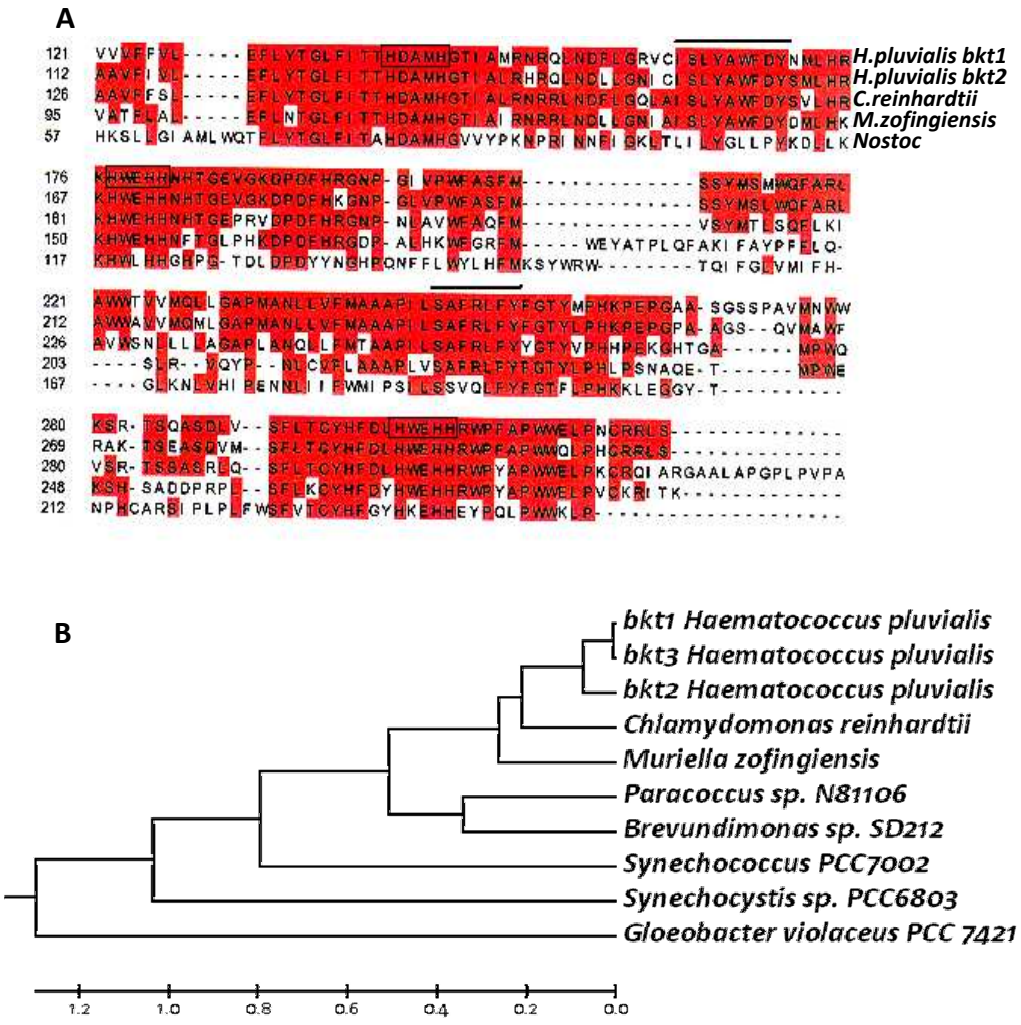

Figure 9: (A) Histidines conserved motifs and highly conserved domains in different ketolases (B) neighbour joining tree including BKT and CRTW type ketolases.

2.5 Biological significance of ketocarotenoids

Ketocarotenoids are relatively rare in higher plants, but are abundant in a number of cyanobacterial and some microalgae species (Cunningham and Gantt, 2005; Takaichi and Mochimaru, 2007). Despite this, relatively little is known about the physiological function of these compounds within the native host. Regardless, a number of general biological functions can be assigned, primarily due to the extended conjugated double bond present within this molecule, canthaxanthin is assumed to function as an antioxidant compound scavenging free radicals and quenching reactive oxygen species, indeed this has been demonstrated (Giraud et al., 2004). However, due to the modification of its β-ionone rings with ketone groups, the antioxidant potential of this compound is not as great as it is for other carotenoids such as lycopene and β-carotene. It has been demonstrated that canthaxanthin, when present in microalgal cells, is able to form an integral part of the photosynthetic apparatus, and can accumulate at the thylakoid membrane. In this position, research has demonstrated that one of the functions of canthaxanthin is to reduce the affection of excessive photo irradiation, protecting the photosynthetic tissues from photo-induced oxidation (Albrecht et al., 2001). Furthermore, it was demonstrated that within this role canthaxanthin was better able to complete this function than the di-hydroxylated carotenoid, zeaxanthin, thus demonstrating how the diversity of carotenoids is benefitial in nature. Additionally, it is known that in mammals, canthaxanthin does not function as a pro-vitamin A compound, and thus is not biologically active in this respect (Chew and Park, 2004). However, a number of studies have suggested that within mammals' canthaxanthin holds potential as both an immuno-enhancing and anticancer agent. Additionally, canthaxanthin is highly valued as a natural colorant, and is widely exploited in the poultry and aquaculture industries for its preferable pigmentation effects when provided as a component of feed (Tang et al., 2007).

3. Genetically modified microalgae

Algae are of commercial importance in the food industry and in aquaculture and a natural source of high-value products such as carotenoids, long chain polyunsaturated fatty acids, and phyco-colloids (Walker et al., 2005).
Although many bacteria, yeasts and even higher plants have been genetically modified, only a few microalgae strains have been successfully transformed with efficiency. The nuclear, chloroplast and mitochondrial transformation of *Chlamydomonas reinhardtii* has been established and widely described (León and Fernández, 2007; León et al., 2004). In the nearer future, this problem has to be sorted out to get transgenic microalgae exploited as cell factories of valuable recombinant products such as vaccines, specialty oils, and novel carotenoids (León et al., 2004). The feasibility of microalgae to be genetically modified and express heterologous genes would open up the possibility of enhancing the productivity of traditional algal compounds and producing new bioactive products for industrial and pharmaceutical applications through metabolic engineering.

3.1 Different methods for the nuclear transformation in microalgae

Microalgae are phylogenetically very heterogeneous. Until now, there are reports of stable nuclear transformations in the three eukaryotic microalgal groups: chlorophytes, diatoms and dinoflagellates. The main important step in every designed method for the nuclear transformation in microalgae is the changing of the permeability in the cell membrane allowing DNA molecules getting inside the cells without any loss of the cell viability. There are several methods for the different species. Particularly, *Chlamydomonas* has been transformed with several methods such as glass beads method in the presence of polyethylene glycol (PEG) (Kindle, 1990), electroporation (Shimogawara et al., 1998), particle bombardment (Debuchy et al., 1989; Kindle, 1989) and using *Agrobacterium* (Kumar et al., 2004). Electroporation method makes a temporal permeabilization of the cell membrane due to the external application of an electric field. It has been very useful for the transformation of different species such as *Chlamydomonas reinhardtii* (Shimogawara et al., 1998), *Chlorella elipsoidea* (Chen et al., 2001), *Chlorella saccharophila* (Maruyama et al., 1994) and *Dunaliella salina* (Geng et al., 2003). Particle

bombardment method has also been successful in diatoms (Apt et al., 1996; Dunahay et al., 1995; Zaslavskaia et al., 2000; Falciatore et al., 1999) and *Dunaliella salina* (Tan et al., 2005; Lü et al., 2005). The base of this method is the acceleration of the DNA carrying particles by helium driven gun into the target cells. This method is commonly used for the transformation of *Chlamydomonas* chloroplasts which is going be explained in the next section.

The use of *Agrobacterium tumefaciens* is a well-known method for the transformation of higher plants and so far only one report about the transformation of the chlorophyte *Chlamydomonas* based on the capacity of this bacterium to transfer its DNA (called Ti plasmid) to the host cell has been reported (Gelvin, 2003).

Agitation in the presence of glass beads method has also been proven successful in some dinoflagelates such as *Amphidinium* and *Symbiodinium* (Lohuis and Miller, 1998). It was originally designed for yeast transformation and has been successfully used for the transformation of cell-wall deficient *Chlamydomonas* strains. The symplicity and efficiency of this method make it interesting to be applied to other species of microalgae. A similar method using silicon carbide whiskers was described by Dunahay in 1993. This method has been proven successful in the transformation of some dinoflagellates such as *Amphidinium* and *Symbiodinium* as well as in *Chlamydomonas´* transformation without disrupting the cell wall.

There is an emergent interest in transforming different species specially those with commercial applications. Novel transformation methods, such as microinjection (Langridge et al., 1985) and the use of recombinant viruses that carry exogenous DNA (León and Fernández, 2007) are also under study.

The best studied genetically modified eukaryotic microalga is, so far, the freshwater chlorophyte *Chlamydomonas reinhardtii.* Which was first transformed in 1989 by the complementation of *nit1* and *arg7* mutations with the homologous nitrate reductase and argininosuccinate lyase genes, respectively (Fernández et al., 1989). Since then, a significant number of selectable markers, promoters and new procedures for efficient introduction of DNA into the nucleus, have been developed for the dramatically improvement of the nucleus transformation methodology.

In the beginning, using mutants was the only way of selection available for the transformation of the microalgae but nowadays we have a wide variety of reporter and marker genes (León et al., 2004) that enable us to transform wild type strains. The best considered marker genes are antibiotic and herbicides resistance ones such as *Ble, NptI*, *aadA* and *aphVIII* that confer resistance to bleomycine, neomycin, spectinomycin and paromomycine, respectively or *als* that confers resistance to sulfonylurea herbicides (León and Fernández, 2007).

3.2 Chloroplast transformation in *Chlamydomonas*

The chloroplast compartment is the site of a number of important biosynthetic pathways and can also serve as a storage organelle. Unlike higher plants, *Chlamydomonas* cells contain a single cup-shaped chloroplast that occupies nearly 40% of the cell volume. In case of higher plants, cells may have as many as 100 chloroplasts which make the screening for a transformation event, far more complicated than in this microalga. The chloroplast genome consists of a 196kb circular double stranded DNA with approximately 80 copies per cell (Harris, 1989). *Chlamydomonas* is a particularly favorable organism for chloroplast transformation, not only for its large single chloroplast but for the availability of photosynthetic mutants which are viable if a reduced carbon is provided in the form of acetate (Kindle, 1990).

Stable transformation of the chloroplast involves three main steps: the introduction of DNA into the organelle, the expression of a marker gene for selection and the replication of the introduced DNA. The high level of ploidy implies that in the initial stages after transformation the plastid is heteroplasmic: it contains both wild-type and modified copies of the genome. This may hamper the expression of recessive markers, but the problem is alleviated by the rapid segregation that usually occurs.

Foreign gene expression in the chloroplast is an atractive strategy for different reasons. The amplification of the transgenes, fused to appropiate cis elements, is considerably higher than in the nucleus. This is because of the hundred copies of the plastome holding highly expressed photosynthetic genes. The second advantage is the homologous recombination that occurs almost exclusively, avoiding the

unwanted “side-position effects” that usually occur in nuclear transformation. Also, no post-transcriptional silencing has been reported in chloroplast transformation. All these advantages make chloroplast transformation a great tool for the transgenes expression as well as for the commercial applications of recombinant products (Purton, 2007).

“Biolistic” bombardment with DNA-coated microprojectiles remains the method of choice for chloroplast transformation (Boynton et al., 1988). The alternative is vortexing in the presence of glass beads (Kindle et al., 1991), but this is less efficient and requires cell wall deficient algae or a pre-treatment with a preparation of autolysin (gamete lytic enzyme) to degrade the cell wall. Biolistic transformation involves the bombardment with micron-sized microprojectiles carrying the transforming DNA (as well known as microcarriers). Acceleration of the microparticles is commonly based on one of two principles. In the first, the microprojectiles are deposited on macrocarrier (a plastic bullet), or on a disk, which is accelerated and then stopped in flight by a perforated stopping plate or a mesh that allows the microprojectiles to continue their trajectory to the target cells. In the first generation of gene guns, the macrocarrier was accelerated by the explosion of a charge of gunpowder but the more recent versions use a burst of compressed air or helium to propel the macrocarrier or a plastic membrane disk (Sandford et al., 1993).

Many of the corresponding chloroplast genes have been identified and cloned, providing a source of selectable markers for transformation of the corresponding mutant hosts: transformants can be selected for phototrophy on minimal medium. Another approach has been to use mutations that confer resistance to various inhibitors. Mutations on the rRNA genes that bestow resistance to spectinomycin, streptomycin or erythromycin have been used to transform wild-type cells (Newman et al., 1990; Kindle et al., 1991).

The obvious advantage of drug resistance over photosynthetic activity is that the host does not need to carry a mutation. The markers are integrated by homologous recombination, replacing the wild-type (drug sensitive) copies of the corresponding genes. However, herbicide and also spectinomycin resistance mutations can have effects on photosynthetic activity that should be taken into account by using

appropriate control strains in structure-function studies of the photosynthetic complexes (Goldschmidt-Clermont, 1998).

If region of homology are provided for homologous recombination, foreign DNA can be stably integrated into the chloroplast genome. Transgenic expression of a foreign gene in the chloroplast to produce functional protein was first demonstrated in *Chlamydomonas* with the *aadA* cassette by showing AAD activity in crude extracts of transformants (Goldschmidt-Clermont, 1991). Using constructs with the *uidA* gene, β-glucuronidase (GUS) activity can also be measured in extracts from transformed cells (Sakamoto et al., 1993). These genes can thus be used as reporters of gene expression for the analysis of transcription, RNA processing, RNA stability and translation under the control of a variety of promoters as well as 5´and 3´-untranslated sequences (Sakamoto et al., 1993). With the *aadA* cassette, the level of resistance of the transformants of different concentrations of spectinomycin for growth on solid media can provide a rough indication of the expression level of the AAD reporter (Goldschmidt-Clermont, 1998).

3.3 Main problems for the expression of transgenes in microalgae

In most microalgae, attemps to express genes under the control of heterologous promoters has not been successful. It was only reported some successful experiments using *gus* gene and the promoter from 35S from the Cauliflower Mosaic Virus and using p1`2` from *Agrobacterium* in dinoflagelates such as *Amphidinium* and *Symbiodinium* (Lohuis and Miller, 1998). More in concrete, having a stable heterologous expression in microalgae and diatoms, depends on the correct use of the promoter and untranslated regulatory regions. Actually the expression of *ble* and *aphVIII* genes under the control of *rbcS2* promoter were increased by using the first intron of the RuBisCO Small subunit gene, *rbcS2* (Lumbreras et al., 1998; Sizova et al., 2001).

Once the transgene has been introduced in the cell and integrated in the chromosome of the microalgae it should be expressed. The codon usage between the transcript and the host cell is an important aspect to take care of (León et al., 2004; Heitzer et al., 2007). As the genetic code is degenerated in some cases there are multiple codons for the same aminoacid. The fact that the codon bias is different

between species, it is important to be considered in the case of using heterologous genes for transformations because while in the host cell one aminoacid is codified mainly by one codon in our transcript can be codified by different codons. This problem cause a reasonably loss of expression of the gene as there is no sufficient tRNA of the required codon that can lead to translational stalling, premature translation termination, translation frame shifting and amino acid misincorporation. In the case of *Chlamydomonas reinhardtii* and its well known rich GC-content genomes (GC~61%), the codon bias may significantly influence the expression efficiency of foreing genes after integration into any of the genomes. Nowadays, this problem can be overcome by using an optimised version of the gene, but even in the case of using this codon optimised version sometimes it cannot be functional. The Codon Adaptation Index (CAI) is a way of seeing how different from the natural one is the codon optimised version of a gene. The index uses a reference set of highly expressed genes from species to assess the relative merits of each codon and a score for a gene is calculated from the frequency of use of all codons in that gene (Sharp and Li, 1987). At the moment, several applications are able to calculate CAI values for defined coding sequences. The most representative examples are J. Peden's CodonW and A. Carbone's CAIJava. In those programs, the codon usage tables are generated on the fly based on different estimators of codon bias. Other interesting resources are represented by the JCat server (Grote et al., 2005) and the OPTIMIZER server (Puigbó et al., 2007), in which the CAI is calculated starting from pre-computed codon usage tables in order to improve coding sequences for heterologous expression.

Although every single required element for the correct transcription and expression of the heterologous gene have been added to the vector in use, the expression can be very low or cannot be any expression at all. Sometimes epigenetic, transcription and post-transcription factors and also iRNA can cause the silencing of the gene expression.

4. Carotenoids production in genetically modified organisms

Many studies have been trying to increase the nutritional value of some higher plants by manipulating the carotenoids route (Botella-Pavía and Rodriguez-Concepción, 2006; Giuliano et al., 2000; Sandmann, 2001 and 2006; Giuliano 2008).
One of the most important achievements in this field was the well-known golden rice (Ye et al., 2000). Even though all required genes to produce pro-vitamin A are present in the grain, some of them are turned off during development. In rice-based societies, the absence of β-carotene in rice grains manifests itself in a marked incidence of blindness, disease susceptibility and premature death of small children. To obtain a functioning pro-vitamin A (β-carotene) biosynthetic pathway in rice endosperm, the cDNA coding for phytoene synthase (*psy*) and lycopene β-cyclase (*lcyB*), both from *Narcissus pseudonarcissus* and both under the control of the endosperm-specific glutelin promoter together with a bacterial phytoene desaturase (*crtI*, from *Erwinia uredovora* under constitutive 35S promoter control) were introduced in the rice genome. As a result of the transformation, they got an important increase in the total carotenoids (Ye et al., 2000). Substituting *Narcisus* by maize *psy* in the initial gene construction increased the total amount of carotenoids in the endosperm of the seeds from 1.6μg g^{-1} to 37μg g^{-1} (Paine et al., 2005) (Fig. 10). Therefore, sometimes there are some side effects in the transforming specie. In the case of transgenic tomato plants overexpressing *psy,* they got small size and decreased in the chlorophyll content phenotypes due to some alterations in the synthesis of abcisic acid and phytol from GGPP (Fraser et al., 2002).

Figure 10: Noticeable colour change between wild type rice seeds (A) and rice seeds accumulating β-carotene (B).

In vivo production of some ketocarotenoids, such as asthaxanthin, has also been achieved by using genetically modified higher plants (Zhu et al., 2009). Constitutive expression of *bkt1* from *H. pluvialis* in tobacco resulted in the production of astaxanthin, canthaxanthin, adonaxanthin and 3´-hydroxyl-ehinenone (Mann et al., 2000). The same gene was also expressed in *Arabidopsis thaliana´*s seeds resulting in the production of 4-ketolutein, adonirubin and canthaxanthin (Stalberg et al., 2003). In order to enhance the levels of carotenoids, the overexpression of the endogenous phytoene synthase gene (*psy*) was carried out in *Arabidopsis thaliana* (Lindgren et al., 2003) and the simultaneous constitutive expression of *Paracoccus crtW* and *crtZ* genes were detected in *Nicotiana tabacum* by the production of 9-fold increase of ketocarotenoids levels in nectarines (Ralley et al., 2004). These are some examples of the importance of manipulating the route in higher plants.

Carotenogenic pathway has not only been manipulated in higher plants but also in cyanobacteria and yeasts (Sandmann, 2001; Schmidt-Dannert et al., 2000) but the study and the manipulation of the route in microalgae are still being developed. As a part of this thesis we got the expression of the *bkt1* gene from *H. pluvialis* in *Chlamydomonas* that was the first achievement of manipulating the route to obtain ketolutein as a final product (León et al., 2007) and the overproduction of carotenoids by expressing a foreign *psy* gene in *Chlamydomonas* (Couso et al., 2010) (*in press*).

4.1 Expression of carotenoid genes in transformed *Escherichia coli* strains

Carotenoids have been successfully synthesized in non-carotenogenic bacteria and yeast using recombinant gene techniques. Considerable progress has been made, especially in the expression of all the necessary genes to synthesize structurally different carotenoids in *E. coli* (Ruther et al., 1997; Cunningham and Gantt, 2007). The expression of carotenoid genes in *E. coli* has been useful for identifying functions of gene products (Chamovitz et al., 1992; Cunningham et al., 1993), dissection of the pathways, the study of transcriptional regulators of carotenogenic genes and isolation of new genes encoding enzymes of the carotenoids biosynthetic pathways (Kajiwara et al., 1995; Lotan and Hirschberg, 1995) or enzymes catalyzing the synthesis of carotenoid precursors (Ohnuma et al., 1994). The potential of this system is determined by several factors. First, sufficient precursors should be available. Second, a balanced level of carotenogenic enzymes should be expressed, to enable

efficient precursors conversion without the formation of intermediate metabolite pools. Third, the correct plasmid combination is important to minimize the accumulation of intermediates and to increase the yield of the end product as in the zeaxanthin formation. Last, the host organism should exhibit an active central terpenoid pathway and possess a high storage capacity for carotenoids (Sandmann et al., 1999). *E. coli* can accumulate carotenoids coupling an endogenous isoprenoid biosynthetic pathway with enzymes encoded by transformed genes of carotenogenic organisms. Carotenogenic genes or cDNAs are available from bacteria, algae, fungi and higher plants most of which can be functionally expressed in *E. coli*. One advantage to using *E. coli* for carotenoid production is its capacity for transformation with several plasmids as long as they all possess a different origin of replication. Furthermore, it is essential that each plasmid carries a different antibiotic resistance marker and that selection pressure has to be maintained at a high level to prevent spontaneous plasmid loss (Sandmann et al., 1999).

Carotenoid biosynthesis mediated by foreign genes in *E. coli* must compete with their endogenous pathways for terpenoid precursors. This diversion of early prenyl pyrophosphates from endogenous pathways into carotenoids can be alleviated by genetic engeneering of the metabolic pathway of *E. coli*. But the expression of heterologous carotenoid genes has a couple of limiting steps due to the dependence on the endogenous terpenoid pathway in *E. coli* which is involved in the bacterial cell-wall synthesis. The first one is at the isopentenyl pyrophosphate (IPP) isomerization to dimethylallyl pyrophosphate. As IPP is a substrate for the chain elongation, the amounts of carotenoids synthesized are dependent on the activity of the IPP isomerase expressed in *E. coli*. In addition to this, there is also a competition for geranylgeranyl pyrophosphate (GGPP), which is the substrate of the phytoene synthase. This means that in the case of using a high level expression phytoene synthase gene in *E. coli*, it may cause a decrease on the growth of the expressing strain by draining off GGPP from the endogenous metabolic processes (Neudert et al., 1998; Sandmann et al., 1999) because the capacity for GGPP synthesis is very low in *E. coli.* When both GGPP synthase and IPP isomerase levels were increased by simultaneous introduction of the corresponding genes (Neudert et al., 1998) enough GGPP and other precursors are provided for the synthesis of essential housekeeping terpenoid compounds in *E. coli*.

4.2 Microalgae vs bacteria and yeasts

Microalgae are considered one of the best model organisms for the study of plant cells as they can be ideal host cells for the expression of foreign genes. They are either important for basic studies and for the biotechnological applications such as higher productivity in carotenoids. Apart from this, microalgae have better analogies with higher plants than bacteria and yeasts and on the top of this they grow easily and faster than higher plants. Most of them have an active terpenoid metabolism that guaranties a high synthesis of carotenoids and a great capacity of accumulating them into chloroplasts and plastids. These two factors limit the use of bacteria and yeasts for this purpose. Most of the microalgae species are generally regarded as safe (GRAS) something very useful in the production at industrial scale of pharmaceutical or nutraceutical products.

Although genetic manipulation is a good alternative to get better productivity and the best way of knowing the carotenoids synthesis route, there is a general negative view of genetically modified organisms (GMO) and also nowadays, the genetic manipulation in microalgae is quite limited and only well-known in a very few organisms (León et al., 2004; León et al., 2007). The random DNA integration and non-homologous recombination in microalgae nucleus are the main problems for the expression of heterologous genes as they have made it difficult to carry out metabolic studies on reverse genetics and also to obtain genetically modified organisms. This is a problem to face up in the nearer future, as modifying species such as *Dunaliella salina*, which is able to accumulate β-carotene until 10% of its dry weight, should be interesting for large scale production of those metabolites. Although there are some references supporting methods for its transformation, there are still lots of doubts of how to get stable transformants (Sun et al., 2005; Tan et al., 2005; Geng et al., 2003; Lü et al., 2005). *Haematococcus* is also an important specie for the production of ketocarotenoids. There have been several tries to achieve its nuclear transformation (Teng et al., 2002) obtained by using particle bombardment with some positives results obtained (Steinbrenner and Sandmann, 2006).

The choice of selective conditions is very complicated in those microalgae. In the case of *Dunaliella* the salinity of the media make antiobiotics precipitate and *Haematococcus* is even worse due to the ability of this microalga to encyst in any stressing condition which makes very difficult to analyse the toxicity of the antibiotics or herbicides tested.

Chlamydomonas reinhardtii is still the best studied microalga (Harris, 2001). Its nuclear manipulation is well-known and stablished and there are plenty of constructions for its stable transformation (Lumbreras et al., 1998; León and Fernández, 2007). On the top of this, we have several molecular and genomics tools such as EST data base (http://www.chlamy.org), a microarray of about 10000 elements that cover about 87% of the total transcriptoms (Eberhard et al., 2006) and the whole sequenced genome (Version 4) (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) (Grossman et al., 2003; Merchant et al., 2007). For those reasons, nowadays *Chlamydomonas* is the best candidate for expressing heterologous genes from the carotenogenic route. Most of those genes have been assigned by homology with genes in other microalgae, this means that the functionality of some of them has not already been checked (Grossman et al, 2004; Lohr et al., 2005). The modifications in this microalga can be the overexpression of the gene in use or the inactivation of the gene by the use of the recent discovered iRNA methodology (Schroda, 2006).

5. Objetivos

Las microalgas son la principal fuente natural de un buen número de carotenoides naturales, pero su producción no está exenta de problemas y su productividad no es siempre tan alta como sería deseable. La falta de conocimientos sobre la ruta de síntesis de los carotenoides y de métodos estandarizados para la manipulación genética de las microalgas son los principales inconvenientes para su aprovechamiento biotecnológico. El propósito de esta Tesis Doctoral es obtener un mejor conocimiento de la ruta de la carotenogénesis de microalgas y su regulación y conseguir estirpes genéticamente modificadas de *Chlamydomonas reinhardtii* que produzcan nuevos carotenoides o mayores cantidades de los carotenoides que ya producen. Para esto vamos a abordar una serie de objetivos concretos:

- Obtener transformantes de *Chlamydomonas* capaces de producir cetocarotenoides, que las células vegetativas no producen de forma natural, gracias a la expresión del gen que codifica la enzima β-caroteno cetolasa de *Haematococcus pluvialis*.

- Obtener transformantes de *Chlamydomonas reinhardtii* con alto contenido en carotenoides mediante la expresión del gen que codifica una de las enzimas limitantes de la ruta, la fitoeno sintasa, obtenido a partir de *Dunaliella salina*.

- Realizar la caracterización del nivel de expresión de algunos genes de la ruta de biosíntesis de carotenoides en distintas condiciones nutricionales y de iluminación y su correlación con los niveles intracelulares de carotenoides en esas mismas condiciones.

- Desarrollar nuevas herramientas moleculares para la expresión de genes exógenos en el cloroplasto de *Chlamydomonas*.

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RESUMEN I

PRODUCCIÓN DE CETOCAROTENOIDES EN *CHLAMYDOMONAS REINHARDTII*

Plantemiento y objetivos del trabajo

La enzima BKT cataliza la adicción de oxígeno a los anillos β del β-caroteno y en ocasiones de sus derivados hidroxilados. La clorofita *Haematococcus pluvialis* es una de las principales productoras de astaxantina. De hecho, la producción del cetocarotenoide astaxantina por *Haematococcus* es una excepción poco común porque la mayoría de las microalgas y plantas superiores no poseen la β-caroteno cetolasa (BKT). Los ceto-carotenoides, como la astaxantina y la cantaxantina, solo se sintetizan en una serie de bacterias, cianobacterias u hongos; o en determinadas microalgas clorofitas, tales como *Haematococcus pluvialis* o *Chlorella zofingiensis* y en algunas especies de plantas superiores del género *Adonis*, como *Adonis aestibalis.*

En condiciones normales, el perfil de carotenoides de la clorofita *Haematococcus pluvialis* es como el de la mayoría de los tejidos vegetales, pero cuando es sometida a ciertas condiciones de estrés, pierde su movilidad, se transforma gradualmente en quistes y acumula grandes cantidades del cetocarotenoide astaxantina y sus ésteres, que pueden llegar a suponer hasta el 5% de su peso seco. En *H. pluvialis* se han encontrado varios genes *bkt.* También se han encontrado cantidades importantes de astaxantina y otros productos del gen β-caroteno oxigenasa en ciertas bacterias marinas y un gen muy relacionado, capaz de oxigenar un solo anillo del β-caroteno, en cianobaterias.

La nomenclatura se complica ya que las cetolasas bacterianas se denominan CRTW. Estas CRTW catalizan la oxigenación de ambos anillos del β-caroteno, y son muy similares en funcionalidad y secuencia a las BKT encontradas en plantas y algas. Mientras que las cetolasas tipo CRTO encontradas originalmente en la cianobacteria *Synechocystis*, añaden asimétricamente un grupo ceto al β-caroteno para formar equinenona y parecen haber aparecido de forma independiente en la evolución, dada la baja similitud que tienen con las cetolasas tipo CRTW. Tanto las cetolasas tipo CRTW como las BKT están muy relacionadas con las hidroxilasas de plantas (BHY) y bacterias (CRTZ). Recientemente, se ha aislado en la levadura *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) un gen que codifica una enzima con ambas

funciones 4-cetolasa y 3-hidroxilasa, confirmando la relación filogenética entre ambos genes.

Cuando se inició este trabajo publicado en el 2007 los *bkt* de *Haematococcus* eran los únicos genes *bkt* publicados en microalgas, con posterioridad se han clonado en otras microalgas, como *C. zofingiensis* que también puede acumular importantes cantidades de astaxantina en ciertas condiciones y sorprendentemente se ha descubierto que algunas clorofitas, como *Chlamydomonas* y *Volvox*, tienen en sus genomas genes que presentan una importante homología con los genes *bkt* de *H. pluvialis* y *C. zofingiensis.* Aunque las células vegetativas de estas especies no sintetizan cetocarotenoides, parece ser que estos genes podrían inducirse en ciertas formas de resistencia que pueden aparecer tras la reproducción sexual, las zigoesporas (M. Lohr, comunicación personal).

Los genes que codifican productos génicos con actividad BKT aislados en *Haematococcus* son muy parecidos entre si (más del 80% en las secuencias proteícas) y guardan también mucha homología con otras BKTs encontradas en otras clorofitas capaces de producir cetocarotenoides, como *Chlorella zofingiensis*, y con las cetolasas bacterianas. Las cetolasas tienen también mucha homología con las β-caroteno hidroxilasas y algunas ácido graso desaturasas, de hecho todas son miembros de una clase de oxigenasas dependientes de hierro con motivos ricos en histidinas que se conservan en todas las cetolasas, hidroxilasas y ácido graso desaturasas (como se indicaba en la introducción). Se ha demostrado mediantes estudios de mutagénesis dirigida que 6 de las 8 histidinas conservadas en todas las cetolasas son imprescindibles para la actividad de la enzima. Estos *clusters* de motivos de histidina (HX_4H, HX_3HH and HX_2HH) son típicos de enzimas que contienen hierro no hemo y podrían ser los ligandos para los átomos de hierro.

El objetivo de este trabajo era expresar el gen *bkt* de *Haematococcus* en *Chlamydomonas* (cuyas células vegetativas no producen astaxantina) y así obtener cepas de *Chlamydomonas* transgénicas capaces de sintetizar cetocarotenoides.

Metodología y resultados obtenidos

El gen *bkt1* fue aislado de *H. pluvialis*, cultivada en condiciones de ausencia de fuente de nitrógeno en el medio de cultivo para provocar la inducción de la transcripción de enzimas pertenecientes a la ruta de la carotenogénesis. El fragmento de cDNA correspondiete al *bkt1* de *Haematococcus*, obtenido del RNA mensajero total mediante RT-PCR, se subclonó primero en un vector de expresión bacteriano pQE80-L (Qiagen). El plásmido pQE80L-bkt1 fue introducido en células de *E. coli*, que portaban a su vez el plásmido de pACCAR16ΔcrtX (Misawa et al., 1995) responsable de la síntesis de β-caroteno. El resultado fue la producción de cantaxantina y cantidades traza del intermediario equinenona, confirmándose así la funcionalidad del gen aislado.

Una vez comprobada la funcionalidad el gen *bkt1*, éste fue insertado en el vector pSI-104PLK, construcción que incluye las regiones del promotor y de terminación para la subunidad pequeña de la RuBisCO (*rbcS2)*, además de un segundo promotor fuerte *hsp70A* y las secuencias de los péptidos señal del cloroplasto RBCS2. Alternativamente, se diseñó otra construcción que difería en el péptido señal, en este caso el de la proteína cloroplástica ferredoxina (FD). El gen fue insertado guardando la pauta de lectura adecuada con las secuencias codificantes de estos péptidos y los vectores resultantes se utilizaron para la transformación del núcleo y expresión en *C. reinhardtii*. Ésta fue co-transformada con estas nuevas construcciones, pSI104PLK-tp-bkt1, y el plásmido pSI103 preparado por Sizova y colaboradores que contiene el gen *aphVIII* de *Streptomyces rimosus*. Este gen codifica una aminoglicósido 3`fosfotransferasa que confiere resistencia al antibiótico paromomicina. Los transformantes fueron seleccionados en primer lugar en base a su resistencia a este antibiótico, y posteriormente sometidos a un *screening* con un test de PCR sobre el DNA genómico, para comprobar la inserción del cDNA *bkt1* en el genoma del alga.

Aproximadamente ciento cincuenta colonias con resistencia a paromomicina (50 para cada uno de los péptidos señal utilizados) fueron obtenidas tras la co-transformación con los plásmidos pSI104-tp*bkt1* y pSI103 y chequeadas por PCR para confirmar la integración en su genoma del gen *bkt1* fusionado a los péptidos señal de RBCS2 y FD, respectivamente. Alrededor del 40% de las microalgas transformadas con la construcción que contenía el péptido señal RBCS2 y el 31% que portaban el péptido

FD resultaron positivas tras el análisis mediante PCR. Los pigmentos de las microalgas seleccionadas fueron aislados y analizados mediante HPLC-MS.

En realidad el perfil de carotenoides de las células control no transformadas y las nuevas estirpes transgénicas obtenidas es muy parecido excepto por la presencia de un nuevo pico, cuyo espectro de absorción coincide con el típico espectro de los cetocarotenoides, tal como pudimos comprobar mediante el uso de diodo array. Este pequeño pico aparecía a un tiempo de retención de unos 8.4 min en los extractos de las células de *Chlamydomonas* transformadas con el gen *bkt1*. Este tiempo de retención es menor que el de otros cetocarotenoides como cantaxantina (RT≈13.2min) o astaxantina (RT≈12.7 min). El análisis en un HPLC con un detector de espectro de masas (HPLC-MS) nos permitió obtener el espectro de masas de este nuevo pigmento, en el que se apreciaba el pico mayoritario a un valor m/z de 583.4 (M^+H). Esto significa que el nuevo cetocarotenoide formado en las cepas transgénicas es 4-ceto-luteína o su isómero 4-ceto-zeaxantina.

La luteína es el carotenoide más abundante en *Chlamydomonas* y tiene un solo anillo tipo β, susceptible de ser oxigenado por la cetolasa, mientras que la zeaxantina está solo presente en menores cantidades y en el caso de que fuera el sustrato para la cetolasa sería esperable que sus dos anillos tipo β fueran oxigenados. Esto nos hace pensar que muy probablemente el nuevo pico encontrado en las cepas transgénicas corresponde a 4-ceto-luteína. El contenido en este cetocarotenoide era aproximadamente el 0.75 % del contenido en luteína, lo que significa unos 20 $\mu g\ g^{-1}$ DW. No encontramos formas esterificadas de los cetocarotenoides, astaxantina o cantaxantina, como sería en principio esperable.

En este trabajo mostramos por primera vez la expresión de un gen exógeno de la carotenogénesis en *Chlamydomonas* y la producción del cetocarotenoide ceto-luteína. La rígida organización de los carotenoides y las enzimas carotenogénicas en la membrana tilacoidal puede causar limitaciones en la disponibilidad de los substratos para las nuevas enzimas transgénicas e influir sobre la baja conversión conseguida por las cetolasas exógenas producidas por genes recombinantes. Si la enzima cetolasa es expresada y dirigida hacia las membranas tilacoidales, pero no es adecuadamente integrada en los complejos carotenogénicos, tendrá un pobre acceso a sus sustratos. Los sustratos para las cetolasas, por su parte, también están

integrados en estructuras funcionales ordenadas, los fotosistemas, que contienen cientos de moléculas de clorofila y carotenoides unidas a proteínas integrales de membrana. Varios estudios proponen que los carotenos estarían preferentemente ligados a los centros de reacción de los fotosistemas, mientras que las xantofilas se encontrarían preferentemente en los complejos colectores de luz (LHC) y serían consecuentemente más accesibles que los anteriores.

CHAPTER I

Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*

Rosa León [a,*], Inmaculada Couso [a], Emilio Fernández´ [b]

[a] *Departamento de Química y Ciencias de los Materiales, Facultad de Ciencias Experimentales, Avda. Fuerzas Armadas s/n, Universidad de Huelva, 21007 Huelva, Spain*

[b] *Departamento de Bioquímica y Biología Molecular, Campus de Rabanales, Edif. Severo Ochoa, p.B-E, Universidad de Córdoba, 14071 Córdoba, Spain*

Abstract

Most higher plants and microalgae are not able to synthesize ketocarotenoids. In this study the unicellular chlorophyte *Chlamydomonas reinhardtii* has been genetically engineered with the β-carotene ketolase cDNA from *Haematococcus pluvialis*, *bkt1* (GeneBank accession no. X86782), involved in the synthesis of astaxanthin, to obtain a transgenic microalga able to synthesize ketocarotenoids. The expression of *bkt1* was driven by the *Chlamydomonas* constitutive promoter of the RuBisCO small subunit (*rbcS2*) and the resulting protein was directed to the chloroplast by the *Chlamydomonas* transit peptide sequences of RuBisCO small subunit (RBCS2) or Ferredoxin (FD). In all transformants containing the *bkt1* gene fused to the RBCS2 or the FD transit peptides a new pigment with the typical ketocarotenoid spectrum was detected. Surprisingly, this ketocarotenoid was not astaxanthin nor canthaxanthin. The ketocarotenoid was identified on the basis of its mass spectrum as 3,3´-dihydroxy-β,ε-carotene-4-one (4-keto-lutein) or its isomer ketozeaxanthin.

1. Introduction

Carotenoids are a wide group of isoprenoids present in all photosynthetic organisms and in some non-photosynthetic ones. They bind to integral proteins of the thylakoid membranes where they participate in light harvesting processes and prevent photoxidative damage of the photosynthetic apparatus (Demming-Adams et al., 1996; Baroli and Niyogi, 2000).

The synthesis of α- and β-carotene from the first uncoloured carotenoid phytoene is a universal pathway in higher plants and green algae (Fig. 1) that involves phytoene desaturase (*pds*), ζ-carotene desaturase (*zds*), carotenoid isomerase (*crtiso*) and lycopene cyclases (*lcy-ε* and *lcy-β*). The hydroxylated derivates lutein and zeaxanthin and the epoxidated violaxanthin are also found in most green tissues (Cunningham and Gantt, 1998; Botella-Pavía and Rodríguez-Concepción, 2006). Not so the ketocarotenoids, such as astaxanthin and canthaxanthin, which are only synthesized in a number of bacteria (Misawa et al., 1990, 1995), cyanobacteria (Fernández-González et al., 1997) or fungi (Johnson, 2003; Visser et al., 2003); in certain microalgae such as the chlorophytes *Haematococcus pluvialis* and *Chlorella zofigiensis* (Boussiba, 2000; Huang, 2005); and in some species of the higher plant genus *Adonis*, such as *Adonis aestibalis* (Cunningham and Gantt, 2005).

Carotenoids have been industrially exploited for a long time as natural pigments and pro-vitamin factors. Animals and other organisms that not synthesize carotenoids *de novo* must include then in their diet to acquire their characteristic colours, this is the case of salmons, shrimps and many birds. Carotenoids are also precursors for essential compounds such as vitamin A or the visual pigment retinal. The intake of carotenoids has proved to offer protection against macular degeneration, UV-induced skin damage and some age-related degenerative diseases (Guerin et al., 2003).

Recent studies suggest that their antioxidant properties and other unexpected biological functions related to gene regulation or junctional communication could provide additional health benefits, such as tumour suppressing activity (Bertram,1999; Demming-Adams and Adams, 2002; Stahl and Sies, 2005). All these reasons have stimulated research to improve the nutritional value of several crop plants by the genetic manipulation of the carotenogenic pathway (Botella-Pavía and Rodríguez-Concepción, 2006; Sandmann, 2001; Giuliano et al., 2000; Sandmann et al., 2006). The genetic manipulation of the carotenogenic route in plants has not been free of difficulties but many of them have been solved thanks to creativity and hard work of different researchers. For example the low productivity of ζ-carotene in the endosperm of original golden rice (Ye et al., 2000) has been partially overcome by substituting daffodil by maize *psy* in the initial gene construction, this has increased total carotenoid amount in the rice endosperm from 1.6 to 37 μg g^{-1} DW (Paine et al., 2005). Or the undesirable collateral effects observed in transgenic tomato overexpressing the *psy* gene (Fray et al., 1995) low size and reduced chlorophyll content due to the alterations in the synthesis of abscisic acid and phytol from its precursor GGPP, have been circumvented by targeting the *psy* to the fruit chromoplast fusing it to the specific transient sequence (Fraser et al., 2002).

Carotenoid synthesis has also been achieved in transgenic bacteria, cyanobacteria and yeast (Sandmann, 2001; Schmidt-Dannert, 2000), but the genetic manipulation of this pathway in eukaryotic microalgae has not been accomplished yet; excepting the very recent report of Steinbrenner and Sandmann (2006), who have expressed a modified *Haematococcus pds* gene with higher resistance to the herbicide norflurazon using *Haematococcus* itself as host.

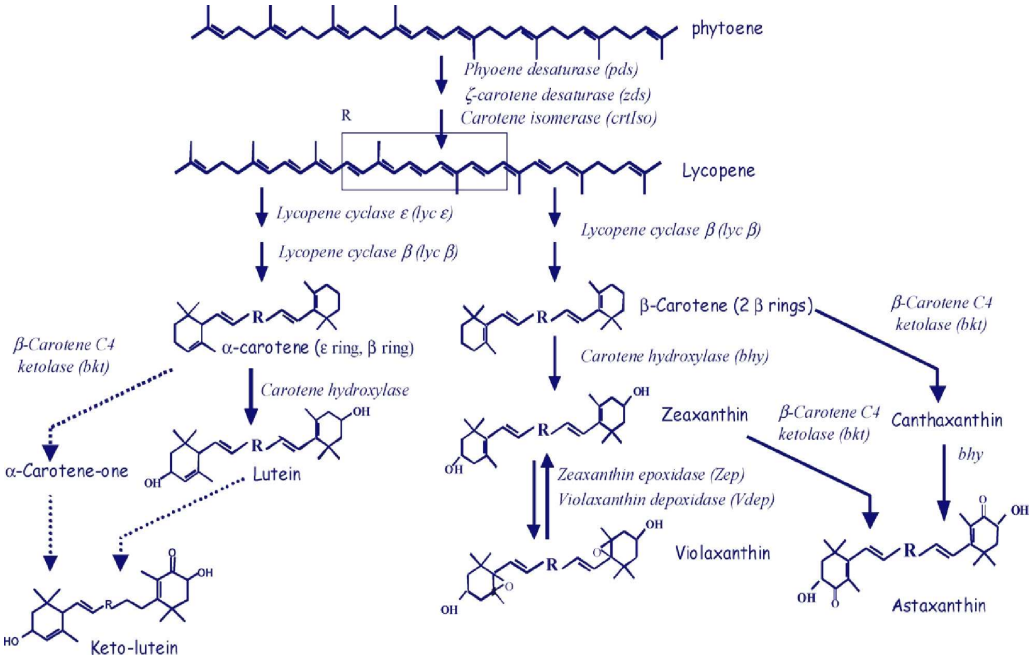

Fig. 1. Metabolic pathway for the synthesis of carotenoids and ketocarotenoids in higher plants and microalgae. *psy* (phytoene synthase); *pds* (phytoene desaturase); *crtiso* (carotenoid isomerase); *lyc-β* (lycopene cyclase *β*); *lyc-ε* (lycopene cyclase *ε*); *bhy* (β-carotene hydroxylase); *zep* (Zeaxanthin epoxidase); *vde* (Violaxanthin de-epoxidase); *bkt* (β-carotene ketolase). Dashed arrows indicate possible reactions for the synthesis of ketolutein.

They propose that this modified *pds* gene can be used as reporter gene for transformants isolation in the presence of norflurazon.

Microalgae combine the fast and easy growth of bacteria and other unicellular microorganisms with an active isoprenoid metabolism that ensures enough precursors for carotenogenic pathway and adequate storage capacity. For these reasons they can be adequate hosts for heterologous genes encoding carotenoid biosynthetic enzymes that allow them to overproduce typical carotenoids or produce new ones not usually synthesized by microalgae. In addition, many microalgal strains are considered GRAS organisms, which is useful for the production of carotenoids or carotenoids-enriched microalgae for pharmaceutical and feed applications (León et al., 2001). Unfortunately, the nuclear transformation of microalgae is still limited to a small number of species (León-Bañares et al., 2004). In most chlorophytes the levels of carotenoids and their composition is very similar to that of higher plants, but there are important exceptions, such as the halophilic green microalga *Dunaliella salina* and the freshwater one *H. pluvialis*, which are the main source of natural β-carotene and astaxanthin, respectively. In normal conditions, the carotenoids concentration in these two strains is similar to that of the rest of the photosynthetic tissues,

but it can be extraordinarily increased when they are subjected to stressing conditions.

The production of the ketocarotenoid astaxanthin by *Haematococcus* is an unusual exception because most microalgae and higher plants do not posses the β-carotene ketolase (*bkt*) activity. Ketocarotenoids, such as astaxanthin and canthaxanthin are highly demanded as feed supplements for fish aquaculture (Lorenz and Cysewski, 2000) and as nutraceutical for human nutrition (Guerin et al., 2003). Their production by *Haematococcus* at industrial scale is not free of problems. *Haematococcus* grows slowly and can be easily contaminated (Lee and Zhang, 1999); it is difficult to obtain high cell density cultures of this microalga and astaxanthin production is linked to the development of red inert aplanospores (haematocysts) (Lorenz and Cysewski, 2000). *In vivo* production of astaxanthin and other ketocarotenoids by organisms that do not synthesize it in a natural way has been achieved by metabolic engineering in *Escherichia coli* (Breitenbach et al., 1996; Lotan and Hirschberg, 1995), cyanobacteria (Harker and Hirschberg, 1997) and several higher plants (Ralley and Fraser, 2004; Stalberg et al., 2003; Mann et al., 2000; Gerjets and Sandmann, 2006; Morris et al., 2006). All these transgenic hosts expressed *bkt* genes from *H. pluvialis*, *crtW* genes from several strains of bacteria or *crtO* gene from cyanobacteria. Expression of the enzyme β-carotene ketolase in *Dunaliella*, which can reach intracellular β-carotene concentrations of about 10% its dry weigh, should be very interesting; but despite several reports (Sun et al., 2005; Tan et al., 2005; Geng et al., 2003) its stable genetic transformation is far from being well stated.

The unicellular microalgae *Chlamydomonas reinhardtii* is the first and best studied transformed chlorophyte, grows at high rates under photoautotrophic, heterotrophic or mixotrophic conditions and its nuclear genetic manipulation is easy and well established. A great variety of transformation methods and constructions have been designed for this microalga that counts with other interesting molecular and genomic tools, such as a commercial microarray with about 10.000 unique array elements covering around 87% of known transcriptome (Eberjard et al., 2006), an EST database (http://www.chlamy.org), and a draft of the complete genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) (Harris, 2001; Grossman et al., 2003). All these reasons make of *C. reinhardtii* a good candidate to express foreign carotenogenic genes and synthesize new carotenoids, for both carrying out basic metabolic and regulatory studies of the pathway and for the biotechnological production of interesting carotenoids.

In the present work we have isolated the *bkt* gene from *H. pluvialis*, inserted it in adequate constructions including the promoter and terminator regions and chloroplast transit peptides sequences, and expressed it in *C. reinhardtii*. This is the first example of manipulation of the carotenogenic pathway in eukaryotic microalgae, which can be an interesting tool to develope basic studies on the synthesis of new carotenoids in transgenic plant cells and a promising approach for the massive production of ketocarotenoids in transgenic microalgae.

2. Materials and methods

2.1. Microorganisms and standard culture conditions

H. pluvialis (SAG 192-80) was kindly provided by the culture service of the Plant Biochemistry and Photosynthesis Institute (CSIC, Seville).

Standard cultures were grown in mineral liquid medium at 25ºC, bubbled with air containing 3% (v/v) CO_2 and continuously illuminated with cool white and daylight from fluorescent lamps (100 µE m^{-2} s^{-1}), unless other conditions are indicated. The composition of the liquid medium was the described by Sueoka et al. (1967) for *C. reihardtii*. *C. reinhardtii* cell-wall deficient strain 704 was kindly provided by Loppes et al. (1999) and cultured photomixotrophically in liquid or agar solidified TAP medium (Gorman and Levine, 1965) at 25°C under continuous white light irradiation (100 µE m^{-2} s^{-1}). The *E. coli* strain used for *in vivo* amplification of DNA and functional complementation experiments was DH5α, cultured in LB medium as previously described (Sambrook et al., 1989) supplemented with either ampicillin (100 µg mL^{-1}), chloramphenicol (30 µg mL^{-1}) or both of them.

2.2. Complementation experiments

Functional analysis of the isolated *bkt1* transcripts was carried out in a β-carotene accumulating strain of *E. coli* This bacteria produces β-carotene due to the plasmid pACCAR16ΔcrtX (Misawa et al., 1995) that contains the soil bacteria *Erwinia* sp. carotenoid biosynthetic genes, *crtE*, *crtB*, *crtI* and *crtY*, which are responsible for the conversion of farnesyl pyrophospate (FPP) to β-carotene via phytoene and lycopene and harbours the cm^r gene that confers resistance to chloramphenicol.

2.3. RNA extraction and reverse transcription-PCR

H. pluvialis cells were subjected to nitrogen starvation and high light intensity (400 µE m^{-2} s^{-1}) to induce the synthesis of astaxanthin. Cell cultures (200 mL) were harvested by centrifugation and resuspended in 3 mL of a buffered solution containing 50 mM Tris–HCl, pH 8, 0.3 M NaCl, 5 mM EDTA and 2% SDS. Isolation of total RNA was performed by phenol–chloroform–isoamyl alcohol (50:48:2) extraction and selective precipitation with 4M LiCl, according to previously described protocols (Schloss et al., 1984; Sambrook et al., 1989). All solutions were DEPC treated. Single strand *bkt1* cDNA was synthesized from total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen) and used as substrate for PCR reactions.

The PCR amplification was performed from 2 µl of the RT reaction mixture in a total volume of 50 µl containing 20 pmol of each primer, 0.2 mM dNTPs, 1 U pfu *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 5 µl of specific 10x buffer (containing 2.5mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96ºC, 0.5 min at 42ºC, and 1.5 min at 72ºC for 30 cycles.

2.4. Genomic DNA preparation for PCR screening of transformants

One loopful of *Chlamydomonas* cells was scrapped from a plate, resuspended in 10 µl of lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3% SDS) and incubated at room temperature for 15 min. After the incubation, 500 ?l of TE (10 mM Tris–HCl, pH 8.0,
1 mM EDTA) and 60 µl of 3 M sodium acetate, pH 5.2, are added. DNA is extracted with phenol/chloroform and precipitated with isopropanol. The pellet is washed with ethanol 70%, dried and resuspended in 5 mM Tris–HCl, pH 8. The genomic DNA solution (1 µl) is used to carry out the PCR reaction as described before (see http://www.chlamy.org/methods/dna.html for more details).

2.5. Construction of expression vectors for Chlamydomonas

Plasmid pSI104PLK was obtained from plasmid pSI103 prepared by Sizova et al. (2001) that contains the *aphVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3´-phosphotransferase that confers resistance to the antibiotic paromomycin, under the control of the strong constitutive promoters *rbcS2* and *hsp70A* and terminated by the 3´-untranslated region of *rbcS2*. PSI103 was cut with *Not*I and religated to eliminate unwanted restriction sites, then the *AphVIII* gene was cut with *Bst*BI and *Bam*HI and substituted by a polylinker region obtained by PCR amplification from pBluescript II KS+ (Promega). We called the obtained plasmid pSI104PLK.

The β-carotene oxygenase cDNA (*bkt1*) was amplified by RT-PCR based on the sequence (GeneBank accession no. X86782) published by Harker and Hirschberg (1997). The obtained PCR product (1.4 kb) was ligated to pGEM-T vector (Promega), sequenced and subcloned between the *Xho*I and *Pst*I restriction sites of the *Chlamydomonas* expression vector pSI104PLK.

DNA fragments encoding the desired transit peptides were synthesized by Genescript Co. (NJ, USA) and cloned in the *Eco*RV site of the multiple cloning region of the plasmid pUC57, from where they were cut, purified and fused to the *bkt1* gene to drive the encoded protein into chloroplast, as explained in Section 3.

2.6. Nuclear transformation of C. reinhardtii

Transformation was carried out using the glass-bead method of Kindle (1990) with minor modifications. *C. reihardtii* cells grown to a cell density of about 10^7 cells/mL, were harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100 fold concentrated cell suspension. The concentrated cell suspension (0.6 mL) was added to a conical tube contain-ing 0.3 g of sterile glass beads (Ø0.4–0.6 mm), 0.2 mL of 20% polyethylene glycol 8000 and about 1 μg of the desired plasmid. Cells were vortexed for 8 s and resuspended in 50 mL of fresh sterile TAP medium where they were incubated in the dark overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP medium with paromomycin (30 μg mL^{-1}). Transformed colonies were visible after 4 or 5 days.

2.7. Pigments extraction and analysis

Carotenoids and chlorophylls were extracted with 80% acetone as previously described (León et al., 2005). The separation and chromatographic analysis of pigments was performed in Merck Hitachi HPLC equipped with a UV–vis detector as described by Young et al. (1997), using a RP-18 column and a flow rate of 1 mL min^{-1}. The mobile phase consisted on: solvent A, ethyl acetate; solvent B acetonitrile/water (9:1, v/v) and the gradient programme applied was: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100%. Injection volume 100 μl. Pigments detection was carried out at 450 nm. Pigments standards were supplied by SIGMA or DHI (Hoersholm, Denmark). The Ketocarotenoid was identified using an Agilent 1100 series chromatograph equipped with a diode array and a mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA).

2.8. Dry weight determination

Dry weight was determined by filtering an exact volume of microalgae culture (30 mL) on

pre-targeted glass-fiber filters (1 µm pore size). The filter was washed with a solution of ammonium formate (0.5M) to remove salts and dried at 100ºC for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference.

2.9. DNA analysis

Sequences were analysed using the Lasergene program (DNASTAR, Inc.) and the NCBI Blast server (http://www.ncbi. nlm.nih.gov/BLAST/).

3. Results and discussion

3.1. Isolation and functionality of β-carotene ketolase (bkt1) gene from H. pluvialis

It has been reported the existence of several classes of carotenoid ketolases. Those ketolases encoded by *crtW* genes, found in bacteria (Misawa et al., 1990; Misawa et al., 1995) that catalyse the oxygenation of both ione rings of β-carotene and are very similar in functionality and sequence to the *bkt* genes found in *Haematococcus*, and *crtO* type found in the cyanobacteria *Synechocystis* (Fernández-González et al., 1997), which asymmetrically adds one keto group to β-carotene to form echinenone, that seems to have arisen independently as they share very little sequence similarity with the CrtW type. CrtW type ketolases are related to the plant (*bhy*) and bacteria (*crtZ*) β-ring hydroxylases. They all are members of a large class of membrane integral, di-iron oxygenase enzymes (Cunningham and Gantt, 1998). Recently, a gen coding a multifunctional enzyme with both 4-ketolase and 3-hydroxylase activities has been cloned in the yeast *Xanthophyllomyces dendrorhous*(*Phaffia rhodozyma*) (Ojima et al., 2006), confirming the relation between these two genes.

Originally two different carotene β-ione ring ketolase cDNAs were isolated from two different strains of *H. pluvialis* grown in different induction conditions, *bkt1* (GeneBank accession no. X86782), originally named as *crtO* (Lotan and Hirschberg, 1995; Harker and Hirschberg, 1997) and *bkt2* (GeneBank accession no. D45881) (Kajiwara et al., 1995). The genomic sequences DQ086233, that generates the *bkt1* mRNA, and AF534876 which transcripted sequence is very similar to *bkt2*, were isolated and deposited in the NCBI databank in 2005 and 2002, respectively. Other versions of *bkt* cDNA deposited in the gene bank database since then are highly homologous to *bkt2* (GeneBank accession nos. AY603347; DQ257290). A

Recent study (Huang et al., 2006) demonstrates the presence of at least three of the *bkt* transcripts in the same *H. pluvialis* strain. They propose the existence of multiple *bkt* genes in *H. pluvialis*, that are up-regulated by different stress conditions. Different *bkt* genes can also have different degrees of affinity for different substrates, such as β-carotene and zeaxanthin.

We chose *bkt1* gene from *H. pluvialis* to express β-carotene ketolase activity in *C. reinhardtii* due to the close phylogenetic relationship between both green microalgae and after analysing the codon usage of several *bkt*/*crtW* genes. The mean difference between the codons present in *bkt1* gene and in *Chlamydomonas* genome was only 11.09%, this difference was increased to 18.48% for the β-carotene ketolase of *Agrobacterium aurantiacum* and to 26.14% in the case of β-carotene ketolase from *Synechocystis* (Table 1). Codon usage comparison was performed using Graphical codon usage analyser v. 2.0

(http://gcua.schoedl.de/index.html).

C. reinhardtii codon usage was calculated from 602 genes. A 1.4 kb cDNA fragment was obtained by RT-PCR from total mRNA of *H. pluvialis* (SAG-192-80). This mRNA was isolated from cells grown during 48 h without nitrogen source and at high light intensity (400 µE $m^{-2}s^{-1}$), conditions that have been reported to stimulate the synthesis of astaxanthin (Boussiba et al., 1999; Boussiba, 2000) and to induce the expression of carotenogenic genes (Eom et al., 2005; Steinbrenner and Linden, 2003). The forward (5´-TGCCGCTCGAGAGCCTCAAATAA-3´) and reverse primers (5´CACTCCTGCAG-ACGCAAGACATC-3´) were based on the sequence published by Harker and Hirschberg (1997) and were designed to contain *Xho*I and *Pst*I restriction sites, respectively (underlined).

Sequencing of the obtained product confirmed that it was *bkt1* cDNA (GeneBank accession no. X86782), following the nomenclature proposed by Huang et al. (2006). Functionality of the isolated cDNA was checked by complementation experiments in a β-carotene accumulating bacterium. This *bkt1* cDNA was subcloned into a bacterial expression vector pQE80-L (Qiagen), introduced in *E. coli* cells that carried the plasmid pACCAR16ΔcrtX (Misawa et al., 1995) responsible for the synthesis of β-carotene and its expression induced with IPTG (1mM, 12h). Expression of BKT1 protein in β-carotene accumulating *E. coli* resulted in the production of canthaxanthin with trace amounts of the monoketolated intermediate echinenone (Fig. 2A) confirming the functionality of the obtained cDNA.

3.2. Fusion of the β-carotene ketolase (bkt1) gene with DNA sequences encoding different transit peptides

The obtained PCR product was then subcloned between the *Xho*I and *Pst*I restriction sites of the *Chlamydomonas* expression vector pSI104PLK. This plasmid, obtained as described in Section 2.5, contains the *hsp70A* and *rbcS2* promoters and the *rbcS2* 3´untranslated terminator region of *Chlamydomonas* (Schroda et al., 2000).

C. reinhardtii was co-transformed by this new construction, that we called pSI104PLK-*bkt1*, and the plasmid pSI103 prepared by Sizova et al. (2001) that contains the *aphVIII* gene from *S. rimosus*, coding for an aminoglycoside 3´-phosphotransferase that confers resistance to the antibiotic paromomycin. Transformants were first selected on the basis of paromomycin resistance and submitted to a screening with genomic DNA PCR to test the insertion of *bkt1* cDNA in the algal genome. About 40% of the transformants contained both *aphVIII* and *bkt1* genes. But none of the transformants that had the *btk1* gene adequately inserted in its genome was able to produce ketocarotenoids.

Carotenoids synthesis occurs within the chloroplast and is catalysed by enzymes encoded by nuclear genes. These enzymes are thus synthesized in the cytoplasm as precursor polypeptides with an amino terminal extension, the transit peptide (tp) that targets them to their final location in the chloroplast. Rubisco small subunit (RBCS2) and Ferredoxin (FD) are targeted to the stroma and the interthylakoid lumen of the chloroplasts, respectively.

Gene (GeneBank accession no.)	Organism	Reference	Difference (%)
Bkt1 (X86782)	*H. pluvialis*	Harker and Hirschberg (1997)	11.09
Bkt2 (D45881)	*H. pluvialis*	Kajiwara et al. (1995)	15.95
Bkt3 (AY603347)	*H. pluvialis*	Huang et al. (2006)	16.08
Bkt4 (DQ257290)	*H. pluvialis*	---	16.13
CrtW (D58420)	*Agrobacterium aurantiacum* (*Paracoccus* sp. *N81106*)	Misawa et al. (1995)	18.48
CrtW (D58422)	*Alcaligenenis PCI* (*Paracoccus PC1*)	Misawa et al. (1995)	17.45
CrtO (BA000022)	*Synechocystis PCC 6803*	Fernández-González et al. (1997)	26.14

Table 1: Comparison of codon usage of several β-carotene ketolases encoded by genes from different sources with the usual codon usage in *Chlamydomonas reinhardtii.* The percentage corresponds to the mean difference between codon usages of the indicated gene and the genome of *C. reinhardtii*. Codon usage comparison was performed using Graphical codon usage analyser v. 2.0 (http://gcua.schoedl.de/index.html). *Chlamydomonas* codon usage was calculated from 602 genes.

The transit sequences of the *Chlamydomonas* RuBisCO and of the intrathylakoid lumen protein ferredoxin are peptides with 32 amino acids, well known at both primary sequence and structural levels (Roesler and Ogren, 1990; Krimm et al., 1999). Detailed sequences of each transit peptide are shown in Fig. 3.

The DNA sequences encoding these two transit peptides flanked by the *BstBI* and *Xho*I restriction sites (Fig. 3) were synthesized and fused in the same reading frame to *bkt1*, previously inserted in the polylinker region of the plasmid pSI104PLK. The structure of this construction carrying the *bkt1* gene and the corresponding transit peptides are detailed in Fig. 3.

C. reinhardtii cells were cotransformed with plasmid pSI104PLK-tpbkt1 that harbours the described construction and the plasmid pSI103 (Sizova et al., 2001). Transformants were newly selected by their resistance to paromomycin and by testing the insertion of *bkt1* cDNA sequence in their genomes by PCR, as detailed in Section 2.4.

3.3. Screening of bkt1 transformed C. reinhardtii

One hundred colonies (50 for each of the two transit peptides used) obtained after co-transformation with the plasmids pSI104-tp *bkt1*

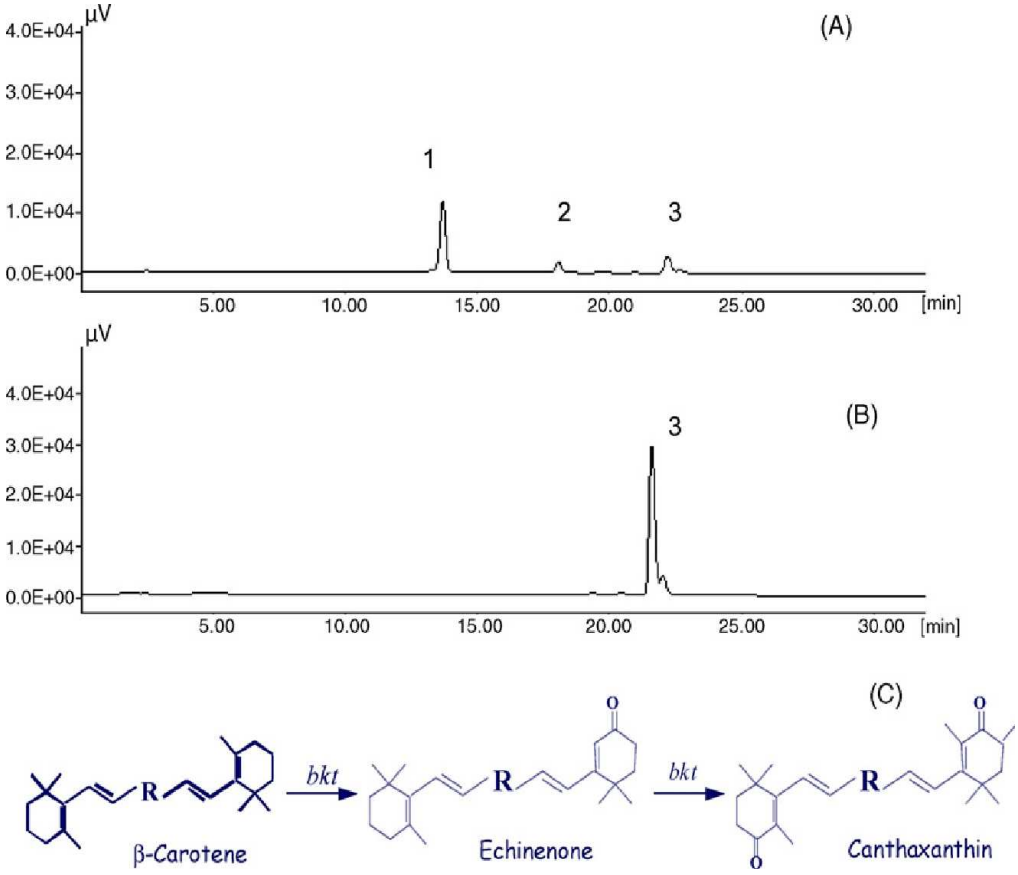

Fig. 2. Chromatographic analysis of pigment extracts from *Escherichia coli* transformed with the plasmid pACCAR16ΔcrtX (responsible for the accumulation of β-carotene) (B) and cotransformed with the plasmids pACCAR16ΔcrtX + pQE80-bkt1 (A) *E. coli* cells transformed with the indicated plasmids were isolated in the presence or chloramphenicol + ampicillin (A) or chloramphenicol (B), grown in LB liquid medium until a OD_{600} of 0.5–0.7 and incubated for 12 h in the presence of IPTG (1 mM). The cells were then harvested by centrifugation and their pigments extracted with acetone and analysed by HPLC. Peaks identification:(1) canthaxanthin; (2) echinenone; (3) β-carotene. β-Carotene ketolase catalyses the transformation of β-carotene to canthaxanthin via the intermediate echinenone, as shown in panel (C).

and pSI103 that showed resistance to paromomycine were screened by PCR to confirm integration in their genome of the *bkt1* gene fused to RBCS2 and FD transit peptides, respectively (Fig. 4). About 40% of the microalgae transformed with the construction that contained the RBCS2 transit peptide and 31% of those transformed with the construction that contained the FD one had integrated the foreign construction in their genomes. The primers designed for PCR analysis of the *rbcS2*:*tp*:*bkt1* constructions

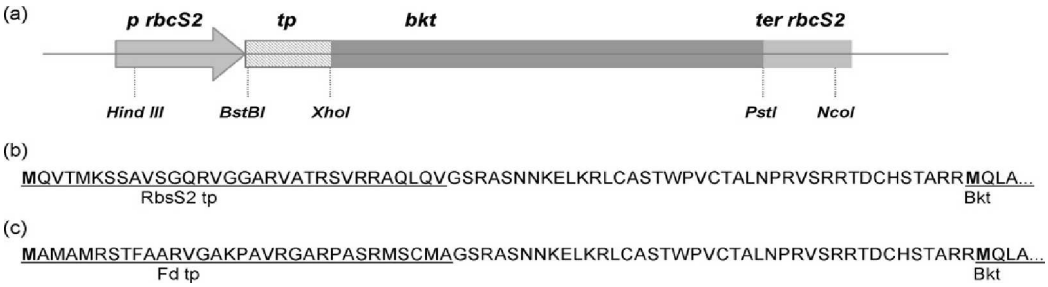

Fig. 3. (a) Schematic representation of the construction used to express the *bkt1* gene in *Chlamydomonas*. *p rbcS2*, ribulose 1,5 biphosphate carboxylase small subunit promoter; *ter rbcS2*, ribulose 1,5 biphosphate carboxylase small subunit terminator region; *phsp70A*, heat shock protein 70A promoter; *tp*, transit peptide; *bkt*, β-carotene ketolase. Detailed sequence of the constructions containing RuBisCO (b) and ferredoxin (c) transit peptides are also shown. The sequences corresponding to the transit peptides and to the beginning of the *bkt1* product are underlined. The whole construction has 2550 bp.

were: AGCGGTGCCCTCCTGATAAAC (forward) and TTCCGGTAAGCTGCTCCAAC
AT (reverse) that anneal with the *rbcS2* promoter and the *bkt1* gene respectively, and amplify a fragment of 547 bp (Fig. 4A).

The positive co-transformants selected by PCR were grown in TAP liquid medium transferred to nitrogen-free medium where they were incubated during 48h and analysed for their pigments content by HPLC. In all the transformants bearing the *bkt1* cDNA fused to either RBCS2 or FD transit peptides, the presence of a new pigment with the typical ketocarotenoid spectrum was detected. Both RBCS2 and FD transit peptides seemed to be equally effective to target *bkt1* gene to its final location in the chloroplast. *Bkt1* preceded by either of these tp are probably directed to the chloroplast and finally integrated into the thylakoid membrane due to its own structure in which several intramembrane helices are present (Cunningham and Gantt, 1998).
There are many cases where it has been shown that the ultimate location of proteins produced by exogenous genes is determined by its own structural information (Misawa et al., 1993).

3.4. Carotenoids analysis of parental and bkt1-transformant strains of Chlamydomonas

The main carotenoids found in *C. reinhardtii* cells subjected to nitrogen starvation during 48h were lutein and β–carotene followed by violaxanthin (Fig. 5). The carotenoid profile of parental and transgenic *Chlamydomonas* were very similar. The most significant difference is the presence of a small new peak, with the typical ketocarotenoid absorption spectrum and a retention time of about 8.4 min, in the extracts of *bkt1*-transformats (Fig. 5). This retention time is lower than that of the pigments canthaxathin (RT ≈ 13.2 min) or astaxanthin (RT ≈ 12.7 min). HPLC-MS analysis of the pigment extracts showed a mass spectra for the new ketocarotenoid with a major peak at *m/z* 583.4 (*M* + *H*). This means that the ketocarotenoid formed in transgenic *Chlamydomonas* is 4-keto-lutein or its isomer 4-keto-zeaxanthin. Lutein is the most abundant carotenoid in *Chlamydomonas* and has only one β-type ring susceptible of being oxygenated by the ketolase, while zeaxanthin is only present in minor quantities, and in the case it were substrate for the ketolase it would be expected that its both β-ione rings were oxygenated to yield astaxanthin, so this new peak correspond very probably to the unusual ketocarotenoid 4-keto-lutein.

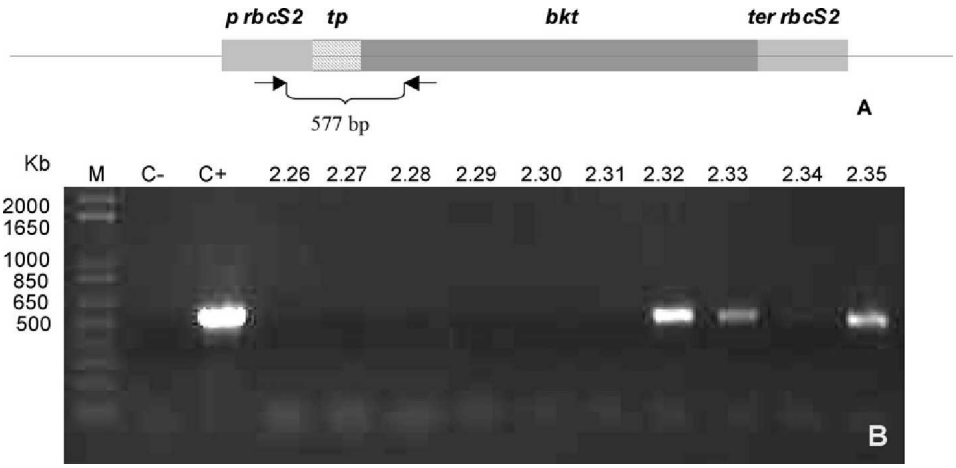

Fig. 4. PCR to screen the insertion of the constructions rbcS2:tp:bkt1 in the genome of *C. reihardtii*. PCR primers were specifically designed to detect rbcS2:tp:bkt1 constructions (indicated with arrows in panel A), being the transit peptide either *RbcS2* or *Fd* corresponding transit peptides. In panel B an example of screening over 10 paromomycin resistant colonies isolated after co-transformation with pSI104PLK:Fdtp:bkt1 and pSI103 is shown. The M lane is the1Kb plus DNA ladder. Transformants 2.32, 2.33 and 2.34 showed a band of 577 bp and correspond to positive transformants with the desired construction inserted in their genomes.

The content of the ketocarotenoid was about 0.75% of the lutein content of the cells, this means about 20 μg g^{-1} DW. Non esterified forms of the ketocarotenoid were found.

There are not many data about the ability of 4-β-ketolases to oxygenate the β-ione-ring of β-carotene or lutein to produce 4-ketolutein, also called α–doradexanthin. It has been found as a minor carotenoid in the red tilefish *Branchiostegus japonicus* (Tsushima and Matsuno, 1998), and in transgenic *Arabidopsis thaliana* expressing the *bkt* gene of *H. pluvialis* (Stalberg et al., 2003).

Here we have also shown that *C. reinhardtii* cells transformed by *bkt1* cDNA from *H. pluvialis* synthesize ketolutein or its isomer keto-zeaxanthin and no astaxanthin or canthaxanthin. The levels of ketolutein in transformant seeds of *Arabidopsis* (Stalberg et al., 2003) were, as we have observed in *Chlamydomonas*, very low. Only in plants obtained by crossings *bkt* transformants with phytoene synthase overexpressing plants, considerable amounts of ketocarotenoids were observed. The content of total ketocarotenoids obtained in transgenic tobacco or tomato leafs (Ralley and Fraser, 2004) was also very low, not higher than 2% of the total carotenoids. On the other hand, in transgenic tobacco flowers expressing *H. pluvialis bkt1* gene targeted to the chloroplast by the transit peptide of tomato *pds* (Mann et al., 2000) about 50% of the total carotenoids were ketocarotenoids, which means a much more efficient transformation of carotenoids to the corresponding ketocarotenoids by the recombinant ketolase.

Stalberg et al. (2003) proposed that limitations on the availability of substrates more than low activity of the enzyme could be the cause for such low quantities of ketocarotenoids obtained in transgenic seeds of *Arabidopsis*. Ralley and Fraser (2004) also propose lack of substrate accessibility in chromoplasts and chloroplasts of certain plant tissues as the possible cause for poor metabolic conversion of carotenoids by ketolases genes. Cunningham and Gantt (1998) have postulated the existence of multisubunit enzyme aggregates associated with the plastid membranes of plant cells where the carotenogenic enzymes should be integrated. Phytoene, the product of soluble phytoene synthase should be the initial substrate for these complexes, through which the rest of the carotenoids would be channeled. This postulate was previously proposed to operate in fungi (Candau et al., 1991) and could explain the low activity observed for exogenous carotenogenic genes introduced by genetic engineering in plant cells. If the foreign ketolase enzyme is expressed and directed to the thylakoid membrane, but is not integrated in the carotenogenic complexes, it will have poor access to its substrates. This could explain higher levels of ketocarotenoides observed by Mann et al. (2000) in transgenic tobacco flowers were the *bkt* enzyme was directed to the chloroplast by the transit peptide of other carotenogenic enzyme, phytoene desaturase (*pds*), and presumably integrated in these carotenogenic complexes. Substrates for carotenogenic enzymes are also integrated in functional ordered structures, the photosystems, which contain hundreds of chlorophyll and carotenoid molecules bounded to integral membrane proteins. Several reports propose that in most photosynthetic tissues carotenes are preferentially ligated to the reaction centres of photosystems, while xanthophylls are more usually found in the light harvesting complexes and consequently are more accessible than carotenes (Grossman et al., 2004). Nevertheless, hydroxylated xanthophylls seem to be not as good substrates to *bkt1* as carotenes (Lotan and Hirschberg, 1995). This could explain the low levels of keto derivates observed in many transformants systems.

The present work corroborates that carotenoid ketolases can act *in vivo* over other substrates than □-carotene, such as the one β-ione-ring lutein or β-carotene, to synthesize new ketocarotenoids such as keto-lutein. Conversion yields are nevertheless very low in most cases. The rigid organization of carotenoids and carotenogenic enzymes in the thylakoid membrane can influence the low conversions achieved by exogenous carotenogenic enzymes produced by recombinant genes. Strategies such as overexpressing endogenous enzymes of the initial steps of the route or directing the *bkt1* gene to the chloroplast by transit peptides of carotenogenic enzymes, such as phytoene desaturase, have allowed to increase the yield of ketocarotenoids production in *Arabidopsis* seeds (Stalberg et al., 2003) and tobacco (Mann et al., 2000). It is interesting to know whether these strategies might also work in *Chlamydomonas* and other plant species. This is the first example of a microalga expressing an exogenous carotenogenic enzyme.

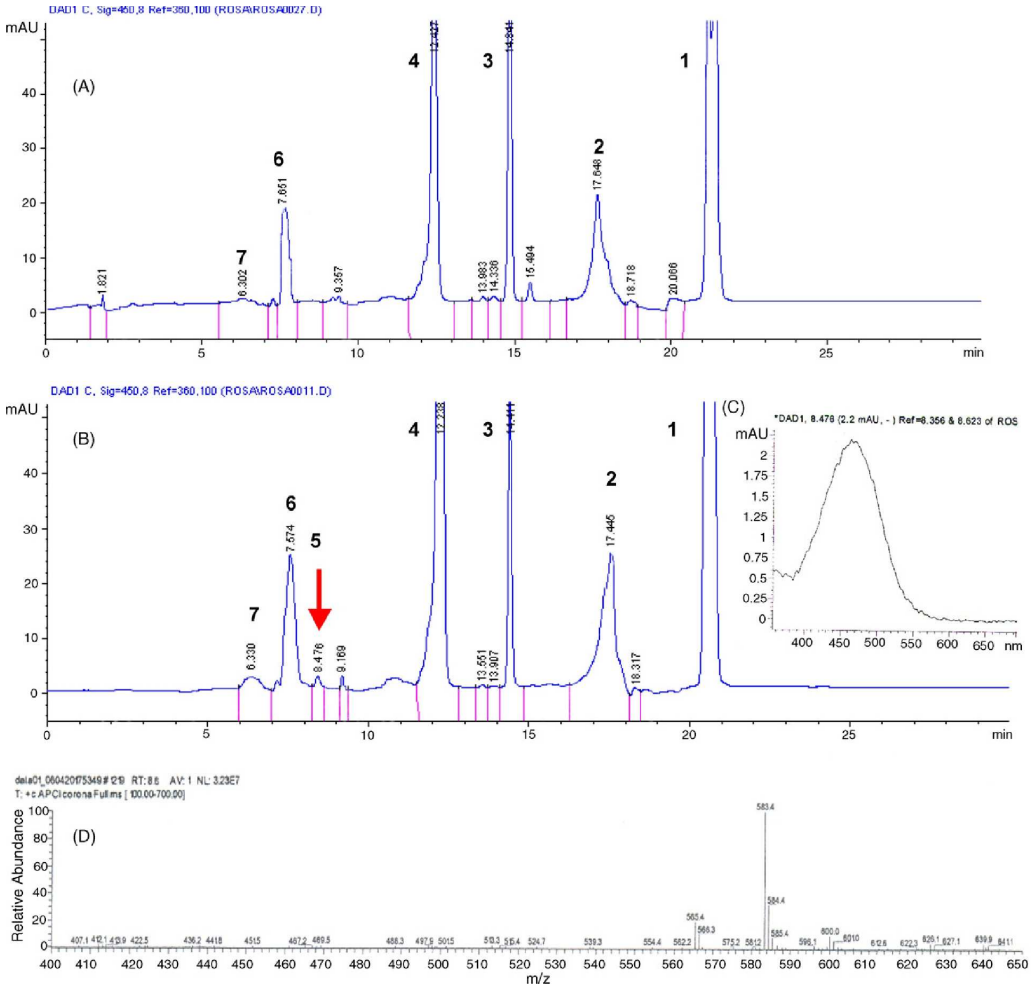

Fig. 5. HPLC profiles of carotenoids present in acetone extracts from control non-transformed (A) and from transgenic *Chlamydomonas* cells (transformant 2.35) containing the *H. pluvialis bkt1* cDNA fused to ferredoxin transit peptide (B). The arrow indicates the new pigment present in the transformed cells. The absorption spectra (C) and the mass spectra (D)corresponding to the new peak were obtained by a diode array detector and a mass spectrometer detector, respectively. Peaks identification: (1) □-carotene; (2) chlorophyll A; (3) chlorophyll B; (4) lutein; (5) keto-lutein; (6) violaxanthin;(7) neoxanthin.

Although the presence of ketocarotenoids in *Chlamydomonas* has never been reported, a gene coding a protein with high homology to the ketolases from *H. pluvialis* has been surprisingly found in a comparative genome study of chlorophyll and carotenoid biosynthetic metabolic pathways in *Chlamydomonas* (Lohr et al., 2005). Lohr et al. (2005) did not detect ketocarotenoids neither in control nor in nutrient limited cultures of *Chlamydomonas*. Our attempts to detect ketocarotenoids in wild *Chlamydomonas* pigment extracts also corroborate these observations. The possible function of this gene in a non-ketocarotenoid producing microalga is not clear. Grossman et al. (2004) propose the possibility that this gene product had acquired a different catalytic function.

4. Conclusions

We have shown that the expression of exogenous carotenogenic genes in *C. reinhardtii* and the production of new carotenoids not synthesized by the parental strains is feasible. Nevertheless, there are limits to obtain high yield productions. Not only in microalgae, also in higher plants, our limited knowledge of the mechanisms and signals that control carotenoids biosynthesis, modification and storage is the main challenge to obtain higher levels of carotenoids through metabolic engineering of the carotenoid biosynthetic pathway.

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RESUMEN II

LA SOBREEXPRESIÓN DE UNA FITOENO SINTASA EXÓGENA EN EL ALGA UNICELULAR *CHLAMYDOMONAS REINHARDTII* CONDUCE A UN INCREMENTO EN EL CONTENIDO EN CAROTENOIDES.

Plantemiento y objetivos del trabajo

La enzima fitoeno sintasa (PSY) cataliza el primer paso de la ruta de biosíntesis de los carotenoides, la condensación de dos moléculas de geranilgeranilpirofosfato (GGPP) para obtener el primer carotenoide incoloro llamado fitoeno. Este paso se ha descrito como un importante punto regulatorio de la ruta. De hecho, la sobreexpresión de genes *psy* bacterianos o vegetales en plantas superiores ha dado lugar a la obtención de un buen número de plantas transgénicas con altos niveles de carotenoides. Por ejemplo, se han descrito plantas de tomate, *Arabidopsis*, arroz, colza o zanahoria con elevados niveles de carotenoides en frutos, semillas o tubérculos gracias a la sobreexpresión del gen *crtB* de *Erwinia.*

A pesar de la importancia de las microalgas como fuente de carotenoides naturales, prácticamente no hay datos sobre la manipulación genética de la ruta de síntesis de carotenoides en estos microorganismos. La mayoría de los estudios se han centrado en plantas superiores, tratando de obtener especies transgénicas con mejores características nutricionales.

La microalga *Dunaliella salina* acumula bajo ciertas condiciones de estrés altas concentraciones de β-caroteno, que pueden llegar hasta el 10% de su peso seco y se considera que la fitoeno sintasa juega un papel importante en la regulación del flujo de carbono hacia la síntesis de carotenoides en esta microalga. En este estudio, el gen *psy* de *Dunaliella salina* fue aislado y expresado constitutivamente bajo el control de promotores fuertes como el de la subunidad pequeña de la RuBisCO, *rbsS2*, o el de la proteína de choque térmico, *hsp70A* en la microalga unicelular *Chlamydomonas reinhardtii*. Su expresión permitió un incremento en el correspondiente nivel de transcrito de la fitoeno sintasa y un incremento de unas 1.9 veces en el contenido de carotenoides.

Metodología y resultados obtenidos

A partir del mRNA total de *Dunaliella salina*, aislamos el cDNA correspondiente al gen que codifica para la enzima fitoeno sintasa y mediante estudios de complementación en bacterias comprobamos su funcionalidad. Para estos estudios de análisis funcional, el cDNA de *Dspsy* aislado se unió a un vector de expresión bacteriano, el pQE80-L, y se expresó en células de *E. coli* que portaban el plásmido pAC-85b. Este plásmido contiene los genes *crtE*, *crtI* y *crtY* de la bacteria *E. herbicola*, que codifican las enzimas GGPP sintasa, fitoeno desaturasa y licopeno ciclasa, proporcionando todas las enzimas necesarias para la síntesis de β-caroteno excepto la fitoeno sintasa y lleva el gen cm^r que confiere resistencia al antibiótico cloranfenicol que es el que nos permite seleccionar en un medio suplementado con dicho antibiótico. El análisis mediante HPLC de las colonias resistentes a ampicilina y cloranfenicol, revelaron la presencia de β-caroteno y confirmaron la funcionalidad del gen aislado.

El cDNA de *Dspsy* fue entonces subclonado en un vector de expresión para microalgas, el pSI105, basado en el pSI103 de Sizova y colaboradores. El plásmido pSI105 tiene una región polilinker precedida de los promotores de la subunidad pequeña de la RuBisCO y el de la proteína de choque térmico HSP70A, y el gen *aphVIII* que confiere resitancia al antibiótico paromomicina. Además, una secuencia de 96 pb que codifica el péptido señal de la subunidad pequeña de la RuBisCO se insertó inmediatamente antes de la región polilinker en la misma fase de lectura que el gen *Dspsy* para asegurar que la nueva enzima sintetizada a partir del cDNA de *Dspsy* se dirigiera a su ubicación final en el cloroplasto.

La transformación nuclear de *Chlamydomonas* cw^- 704 con la construcción obtenida, fue realizada por el método de agitación con perlas de vidrio, tal como lo describió Kindle en 1990. Los transformantes seleccionados en primer lugar, en base a su resistencia a paromomicina, se chequearon mediante PCR para confirmar la adecuada inserción de la construcción *rbcS2*:*tp*:*Dspsy* en su genoma. Los transformantes positivos fueron entonces analizados mediante HPLC para determinar su contenido en carotenoides y por PCR cuantitativa para determinar el nivel de expresión tanto de la *psy* endógena de *Chlamydomonas* como de la *psy* de *Dunaliella* introducido en el núcleo de *Chlamydomonas.*

De este modo hemos podido aislar transformantes con mayor concentración de todos los carotenoides, especialmente violaxantina y luteína. La relación carotenoides/clorofila en los transformantes que sobreexpresan la *Dspsy* era un 40%

mayor que en las estirpes control y los fenotipos siguen estables en la actualidad casi 8 meses después de su obtención.

CHAPTER II

OVEREXPRESSION OF AN EXOGENOUS PHYTOENE SYNTHASE GENE IN THE UNICELLULAR ALGA *CHLAMYDOMONAS REINHARDTII* LEADS TO AN INCREASE IN THE CONTENT OF CAROTENOIDS.

ABSTRACT

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. Phytoene synthase 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 to 260 % the levels in control untransformed cells.

Keywords: phytoene synthase; PSY; *Chlamydomonas reinhardtii;* carotenoid biosynthetic pathway; genetically modified microalgae.

Abbreviations: IPP, isopentenylpyrophosphate; MVA, the mevalonic independent pathway; MEP, methylerythriol 4-phosphate; GGPP, geranylgeranylpyrophosphate; PSY, phytoene synthase; mt, mating type; *rbcS2* RuBisCO small subunit*; hsp70A,* Heat Shock Protein 70A; DAD, Diodo Array Detector.

INTRODUCTION

Carotenoids are the most diverse and widespread pigments found in nature (1). They are a wide group of lipophylic isoprenoids synthesised by all photosynthetic organisms and also by some non photosynthetic 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 synthesise 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). Some of them need carotenoids to acquire their characteristic colours, this is the case of salmons, shrimps o some birds (6). Regarding human nutrition, in addition to their paper as pro-vitaminic factors, carotenoids have been described to play an important role in the prevention of cardiovascular diseases, UV-induced skin damage, some cancers and degenerative diseases. The clearest link has been established between the intake of the carotenoids lutein and zeaxanthin and the age related macular degeneration.

These health benefits, together with their colorant, antioxidant and pro-vitamin properties, have made of carotenoids an important group of high-added value compounds, massively commercialized for dietetic and cosmetic products and food/feed additives. Restrictive regulations on the use of synthetic dyes in the food, feed, aquaculture, cosmetics and other

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 synthesised in the plastids from isopentenylpyrophosphate (IPP) originated through the mevalonic (MVA) independent pathway also known as methylerythriol 4-phosphate (MEP) pathway (7; 8; 9). The first committing step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranylpyrophosphate (GGPP) to yield the first uncoloured carotenoid, phytoene, catalysed by the phytoene synthase enzyme (PSY). From this step on, phytoene is subjected to four desaturations and a couple of isomerizations producing the coloured 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 (Fig.1). Reviews of Cunningham and Gantt (10) or Grossman and co-workers (11), and the more recently published by Botella-Pavía and Rodriguez-Concepción (6), Sandmann (12) and Giuliano and co-workers (13) offer a good general view of this metabolic pathway.

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 focussed on higher plants, trying to obtain transgenic species with enhanced nutritional characteristics. There is 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).

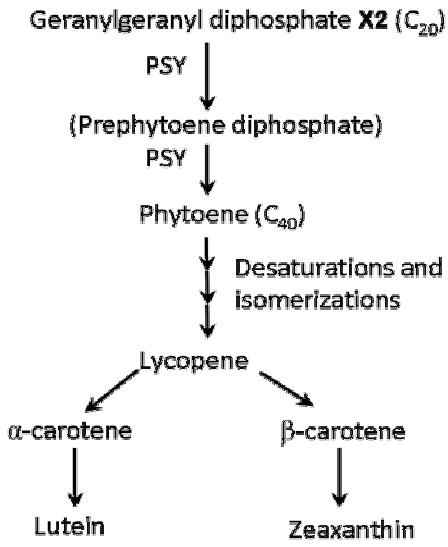

Figure 1: Schematic representation of the carotenoid biosynthetic pathway. The first step is catalyzed by phytoene synthase (PSY), enzyme leading the carbon flux towards the carotenes and xanthophylls production.

Phytoene synthase 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,

22, 23). Overexpression of bacterial or plant phytoene synthase 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 phytoene synthase 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 irradiation (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), at the same light, irradiation 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) and 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 carried out in *E. coli* cells that carried the plasmid pAC-85b (35). This plasmid contains *crtE, crtI* and *crtY* 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 phytoene synthase 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 (Fig.2a), 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 region. This plasmid allows the integration in the same construction of the marker gene, *aphVIII*, and functional genes, which can be inserted in the polylinker (Fig. 2a).

The DNA fragment encoding the desired transit peptide was synthesized by Genescript Co (NJ, USA) 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 polilynker of pSI105.

RNA extraction and reverse transcription-PCR

Large scale isolation of the total RNA was performed using phenol:chlorophorm extraction and salt precipitation (3M 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, USA) 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 1µL of the resulting extraction of cDNA in a total volume of 25µL containing a final concentration of 0.5µM each primer (Forward and Reverse), 200µM dNTPs, 3% of DMSO and 0.5U of Taq polymerase from Biotools (Spain). The PCR program used was 0.5min at 98°C, 0.5min at melting temperature of the different primers used (Table I), 0.5 min at 72°C and a final amplification of 5min 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 0.3g of sterile glass beads (Ø 0.4-0.6mm), were added to 0.6mL of concentrated cell suspension, 0.2mL of 20% polyethylene glycol (MW8000) and about 1µg of the desired plasmid.
This mixture was agitated during 10s and then cells were resuspended in 50mL of fresh TAP medium and left in dim light conditions overnight. After this incubation, cells were spread onto the selective solid medium carrying 30µg mL^{-1} of paromomycin. 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 pre-tared glass-fiber filters (GF/F Whatman).

Table I: Primers designed for the insertion of the genes into different vectors either for their expression in *Chlamydomonas* (1,2) or *E.coli* (3,4) and primers designed for the real-time PCR analysis (5-8).

Primer number	Primer name	Sequence	Tm(°C)
1	UpDSPSIXhoI	ctcgagIATGCCCTCCACTTCCG	57.3
2	RevDSPSIEcoRI	CTACTGTgaattcTTGGGCATCA	48
3	UpDSpQEBamHI	ggatccATGCCCTCCACTTC	50
4	RevDSpQEHindIII	CTGTGGGCTCTTaagcttCATG	49
5	qFCRPSY	CACTCGCGCCCGGCAATACTT	62.8
6	qRCRPSY	CCACGGGCAGCGACACCATC	63.0
7	qFDSPSY	CCTTGGATGAGCTACGGGAGTTTG	59.9
8	qRDSPSY	GCAGGGAGGCCAGCTTCTTTGA	61.4

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 1mL min^{-1} and a final injection volume of 100µL. Two mobile phases were used; solvent A, ethyl acetate 100% and solvent B acetonitrile: H2O (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 1µL of the cDNA mixture added as template and Brilliant SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies, La Jolla, CA, USA). 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 I. 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 the phytoene synthase 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 *crtE, crtI* and *crtY* 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 phytoene synthase and harbours the cm^r gene that confers resistance to chloramphenicol. After co-transformation and selection with chloramphenicol plus ampicillin, the obtained colonies were subjected to induction with IPTG (1mM) during 12h at 37°C and 48h at room temperature in the dark.

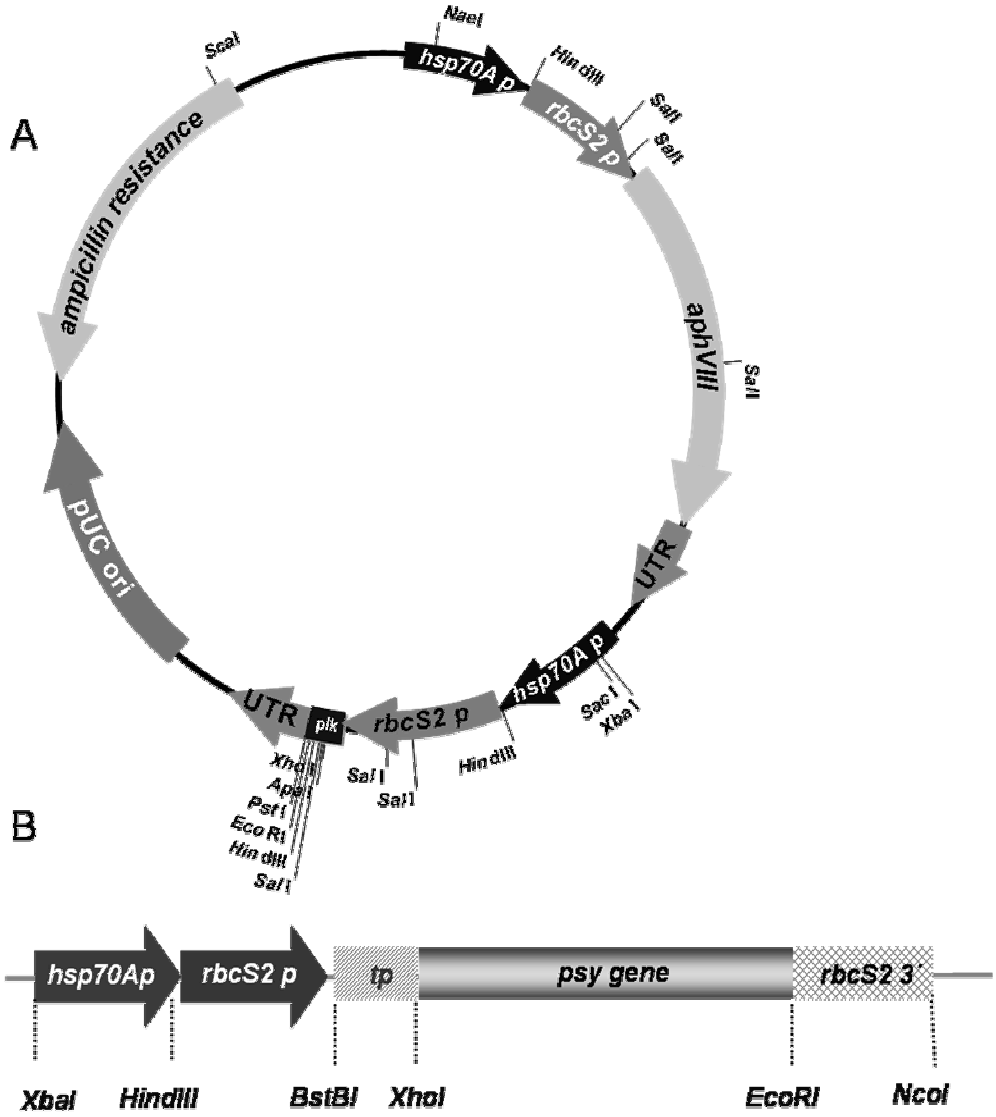

Figure 2: Structure of psI105 plasmid (A) and detailed schematic representation of the regulation regions flanking the polylinker site for insertion of the desired gene (B).

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 colour of the colonies (Figure 3).

Extraction of the carotenoids from the coloured colonies and their HPLC analysis confirmed the presence of β-carotene and the functionality of *Dspsy* gene.

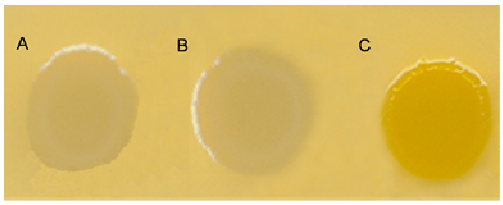

Figure 3: Colonies of IPTG-induced *E. coli* transformants carrying the pAC85b plasmid (A), *E. coli* carrying the pAC85b plasmid with the empty pQE80-L vector (B) and the pAC85b plasmid together with pQE80-L vector carrying *psy* from *Dunaliella salina*(C).

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 (Fig. 2a).
Before insertion of the *psy* gene, a 96 bp sequence encoding the RBCS2 transit peptide was inserted immediately before the polylinker region, between BstBI and XhoI sites (Fig. 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 submitted to a screening by PCR to test the insertion of *psy* cDNA in the algal genome. For analysis of the *rbcS2:tp:Dspsy* the UpDSPSIXhoI and RevDSPSIEcoRI primers (Table I) previously used for amplification of the *Dspsy* gene were used. 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 analysed.

Analysis of Dspsy-transformed Chlamydomonas reinhardtii

The positive transformants that showed to contain the exogenous *Dunaliella* phytoene synthase adequately inserted in the nuclear genome were further analysed for their carotenoids content. We selected those transformants in which overexpression of *Dunaliella psy* (*Dspsy*) resulted in a significant increase in the carotenoids content. The detailed carotenoids profile and the intracellular *psy* mRNA content of two of these transformants are shown in figure 4.
Exogenous *Dspsy* was constitutively expressed under the control of the strong promoters *rbcS2* and *hsp70A* and high levels of *Dspsy* mRNA were detected in both transformants analysed. In transformant “C” *Dpsy* transcript level reached 3 fold 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. After several months, the phenotype remained the same and

expression of exogenous *psy* was so, as confirmed by quantitative Real Time PCR quantification of *Dunaliella salina psy* transcript level.

DISCUSSION

Phytoene synthase 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) *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 43.53kDa 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 phytoene synthase enzymes (CRTB) and share an extensive prenyl transferase domain with squalene synthase enzymes (10).

Comparison of *C.reinhardtii* and *D. salina psy* sequences yields 75% of identity and a similar codon usage. In the present 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 phytoene synthase 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 to 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 phytoene synthase 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 and co-workers (25) reported the expression of a bacterial *psy* in the fruit of tomato yielding elevated levels of phytoene, lycopene, lutein, and β-carotene (2-4 fold 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.

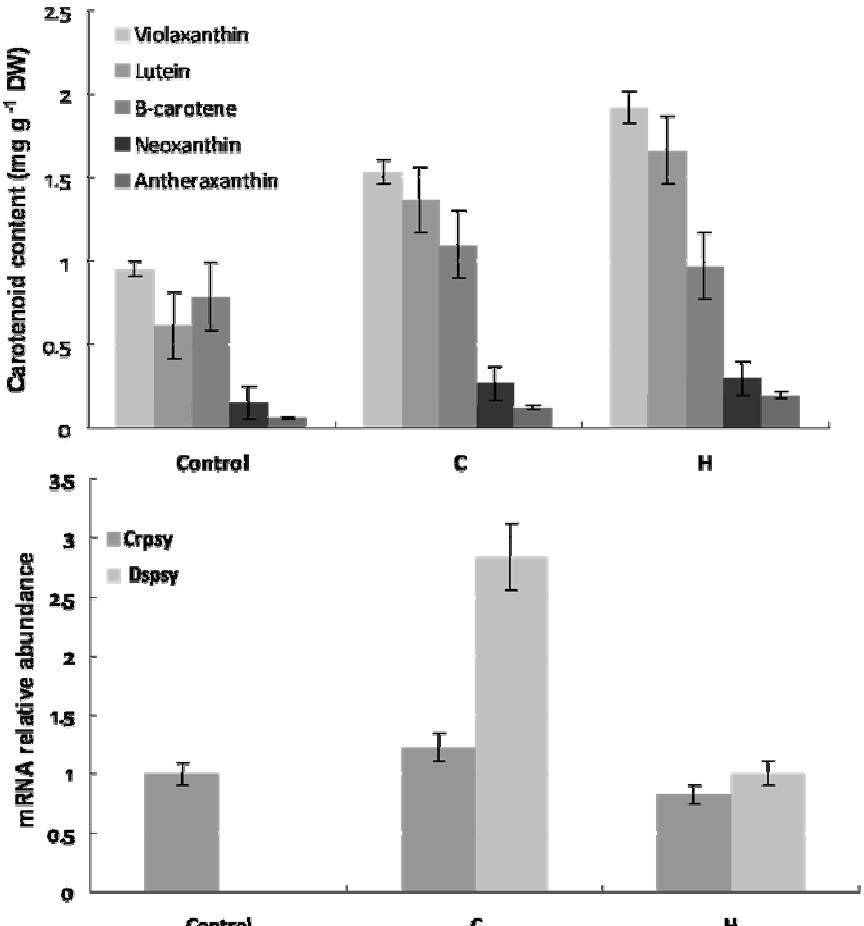

Figure 4. Carotenoids content (A) and mRNA relative abundance of endogenous *Chlamydomonas psy* (■) and foreign *Dunaliella psy* (■) mRNA (B) in *Chlamydomonas reinhardtii* transformed with *psy* cDNA from *Dunaliella salina*. Levels of *psy* transcripts are normalized respect to the housekeeping control gene (*cblp*) and expressed as relative fold to the normalized *psy* level of the control untransformed cells.

The approximation used by Lindgren and co-workers (26) in *Arabidopsis* was, however, based on an endogenous phytoene synthase 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 and co-workers (55) who expressed the own tomato *phy1* cDNA and obtained transgenic lines with almost complete reduction in fruit carotenoids due to co-suppression 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 extend to which carotenoids levels can be increased by insertion of structural genes and causes relatively low carotenoids increments, usually under 2-fold 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.

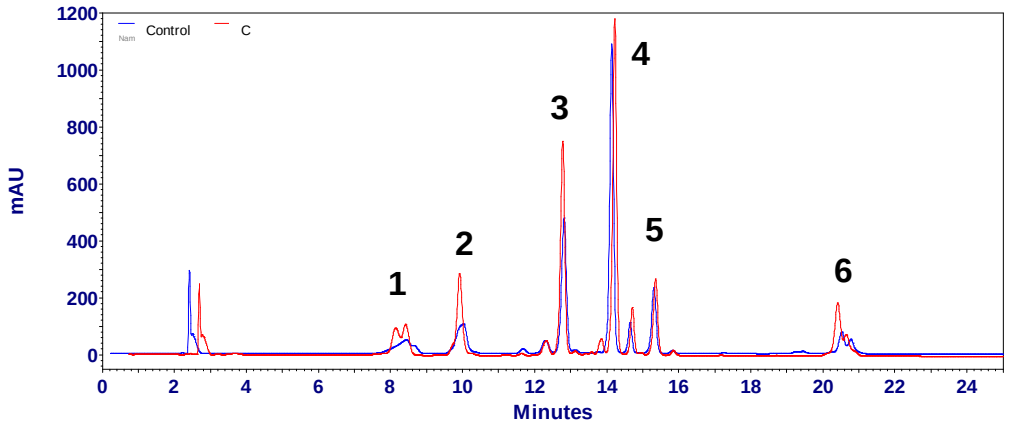

Figure 5. Carotenoids profiles of control (blue) and *Dspsy*-transformant "C" (red). Numbers correspond to (1) neoxanthin, (2) violaxanthin, (3) lutein, (4) chlorophyll B, (5) chlorophyll A and (6) β-carotene peaks.

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RESUMEN III

SÍNTESIS DE CAROTENOIDES Y REGULACIÓN DE SU RUTA BIOSINTÉTICA EN RESPUESTA AL ESTRÉS POR ALTA INTENSIDAD DE LUZ EN LA MICROALGA UNICELULAR C*HLAMYDOMONAS REINHARDTII*

Plantemiento y objetivos del trabajo

A pesar de la importancia fisiológica y económica de la ruta de síntesis de carotenoides, muchos de los aspectos regulatorios de esta vía no están claros aún. Obtener un conocimiento más profundo de las enzimas involucradas en esta ruta y su regulación es esencial para poder abordar su manipulación genética de forma eficiente.

Los organismos fotosintéticos reaccionan ante el exceso de luz con una variedad de mecanismos que incluyen la reparación de las proteínas y estructuras dañadas por las condiciones de estrés foto-oxidativo, la síntesis de especies antioxidantes (Krieger-Liszkay y Trebst, 2006) o la disipación del exceso de luz absorbida por mecanismos NPQ ("non photochemical quenching"). Todas estas respuestas implican en mayor o menor medida cambios en la composición de los carotenoides, ya que éstos son componentes de los complejos captadores de luz (LHC) o los centros de reacción, que necesitan ser reparados cuando se dañan los fotosistemas. Pero el papel de los carotenoides es particularmente importante en el componente térmico de la disipación no fotoquímica del exceso de energía absorbida (qE-NPQ), que implica la activación del ciclo de las xantofilas.

Además, se ha demostrado que la síntesis de los carotenoides puede ser extraordinariamente incrementada en determinadas condiciones ambientales, tales como la alta irradiancia, la carencia de nutrientes u otras condiciones de estrés. Aunque *Chlamydomonas* ha sido ampliamente utilizada como modelo en la investigación de los procesos fotosintéticos, los datos sobre el contenido en carotenoides en diferentes condiciones ambientales son escasos y la regulación de la biosíntesis de carotenoides en esta alga no ha sido rigurosamente examinada.

Utilizando *Chlamydomonas reinhardtii*, este trabajo pretende arrojar luz sobre la ruta de biosíntesis de carotenoides en las células vegetales bajo determinadas condiciones de estrés. Para ello, se han estudiado los cambios en el perfil de

carotenoides de *Chlamydomonas* cuando las células son sometidas a alta luz en presencia de inhibidores de la síntesis de carotenoides y proteínas. Hemos identificado los pasos más significativos de la vía que participan en esta respuesta y hemos analizado el nivel de expresión de los genes que codifican las enzimas que catalizan estos pasos clave.

Metodología y resultados obtenidos

En este trabajo hemos analizado la evolución temporal de los carotenoides en células de *C. reinhardtii* cultivadas en carencia de nitrógeno y a diferentes intensidades de luz. Esto nos ha permitido concluir que hay una activación del ciclo de las xantofilas en respuesta a las altas intensidades luminosas, de acuerdo con datos previos, y en respuesta a otras situaciones de estrés como la carencia de nitrógeno o la presencia del herbicida norflurazón, que no había sido descrito hasta la fecha. Después de sólo seis horas de exposición a una intensidad de luz muy alta (1000 µE $m^{-2}s^{-1}$) casi la mitad de la reserva total de xantofilas se había convertido en zeaxantina.

Además, estudiamos el efecto sobre la evolución a lo largo del tiempo del contenido en carotenoides en células de *Chlamydomonas* transferidas a altas intensidades luminosas en la presencia del herbicida norflurazón y del antibiótico cicloheximida. El norflurazón es un herbicida blanqueante que bloquea la síntesis de carotenoides a nivel del fitoeno, inhibiendo la enzima fitoeno desaturasa. Mientras que la cicloheximda es un inhibidor de la síntesis proteíca en eucariotas, que actúa interfiriendo la actividad peptidil transferasa del ribosoma 60S y bloqueando por tanto la elongación traduccional.

Gracias a estos estudios concluimos que en células de *Chlamydomonas* sometidas a un estrés por alta luz, aunque la zeaxantina es sintetizada principalmente por de-epoxidación de la violaxantina, hay una contribución importante de síntesis de zeaxantina a partir del β-caroteno. En presencia de norflurazón, condiciones en que la síntesis *de novo* de carotenoides está bloqueada, toda la zeaxantina se sintetiza a partir de las reservas de violaxantina. Por otra parte, observamos que cuando las células son transferidas a alta luz en presencia de cicloheximida, la acumulación de zeaxantina sigue teniendo lugar pero su contenido

es menor que en los controles sin antibiótico. Esto demuestra que al menos una de las dos posibles vías para la síntesis de zeaxantina en alta luz, la síntesis *de novo* de carotenoides o la interconversión de violaxantina en zeaxantina, es dependiente de síntesis proteíca.

La gran variedad de respuestas al estrés foto-oxidativo implican la inducción de los genes que codifican proteínas muy diversas, incluyendo las proteínas que forman los complejos captadores de la luz, del metabolismo del carbono y proteínas de choque térmico. En este trabajo hemos estudiado mediante, PCR cuantitativa, el nivel de transcripción de un buen número de genes de la ruta de síntesis de carotenoides tras la transferencia de los cultivos de oscuridad a luz de alta intensidad (800µE m^{-2} s^{-1}). Estudiamos el nivel de expresión de los genes que codifican las enzimas que catalizan los primeros pasos de la ruta, PSY y PDS, las enzimas responsables de la ciclación de licopeno, LCYE y LCYB, las hidroxilasas implicadas en la síntesis de las xantofilas zeaxantina y luteína, CHYB, CHYE-P450 (CYP97C3) y CHYB-P450 (CYP97A5), y la enzima zeaxantina epoxidasa (ZEP).

Los resultados más interesantes se observaron en el análisis de la expresión de las caroteno hidroxilasas dependientes de citocromo P450. El nivel de la transcripción de ambas hidroxilasas dependientes de citocromo es de diez y ocho veces superior en alta luz que en luz normal. La inducción máxima de ambos genes se observó después de una hora de exposición a luz alta, pero un alto nivel, de alrededor de 5 veces el valor inicial, se mantuvo incluso después de 6 horas. La inducción extraordinaria de los genes *chyb-P450* y *chye-P450* implicados en la síntesis de luteína que hemos observado al exponer las células de *Chlamydomonas reinhardtii* a la luz alta indica que las hidroxilasas β y ε son pasos clave en las respuestas al estrés foto-oxidativo.

CHAPTER III

SYNTHESIS OF CAROTENOIDS AND REGULATION OF THE CAROTENOID BIOSYNTHESIS PATHWAY IN RESPONSE TO HIGH LIGHT STRESS IN THE UNICELULLAR MICROALGA C*HLAMYDOMONAS REINHARDTII*

ABSTRACT

The carotenoid biosynthesis pathway catalyses the synthesis of pigments essential for light harvesting and photoprotection in photosynthetic organisms. It allows the production of several commercially important compounds and is the target of many herbicides. In the present work we have studied the influence of light on the carotenoid composition and on the expression of the genes encoding the main steps of the pathway, in the freshwater microalga *Chlamydomonas reinhardtii*. We have observed that there is an activation of the xanthophyll cycle in response to high light, but also in response to other stressing conditions such as nitrogen starvation, which has not been reported so far. We have analysed the expression level of the genes encoding the two first enzymes of the pathway, phytoene synthase and phytoene desaturase; the enzymes responsible for the cyclization of lycopene, Lycopene cyclase ε and Lycopene cyclase β; Zeaxanthin epoxidase, which catalyses the epoxidation of zeaxanthin; and the three known carotene hydroxylases, directly involved in the synthesis of xanthopylls from α and β-carotene. Measurements of carotenoids intracellular concentration in the presence of inhibitors of protein and carotenoid synthesis suggest that the synthesis of zeaxanthin is subjected to regulation at protein synthesis and activity levels, and show that the synthesis of zeaxanthin could take place without *de novo* synthesis of carotenoids. The high increase in the transcript levels of the cytochrome dependent β– and ε–hydroxylases in response to high light suggest an important role of these enzymes in regulation of xanthophyll synthesis upon light stress. These conclusions can be of high interest to accomplish an efficient engineering of the pathway.

Keywords: Carotene hydroxylase; carotenoids; microalgae biotechnology*;* high light stress; xanthophyll cycle.

Abbreviations: *bkt*, β-carotene ketolase gene; CPTA, 2-(4-chlorophenylthio) triethylamine hydrochloride; DW, dry weight; NPQ, non-photochemical quenching; LHCII, light harvesting complex II; PSII, photosystem II; HPLC, high performance liquid chromatography; LHC, light harvesting complex; *psy*, phytoene synthase gene; *pds*, phytoene desaturase gene; *zep*, zeaxanthin epoxidase; *vde*, violaxanthin de-epoxidase gene; *lycb*, lycopene cyclase β; *lcye*, lycopene cyclase ε gene; *chyb*, β-carotene hydroxylase; *chyb P450*, β-carotene hydroxylase P450 cytochrome dependent gene; *chye P450*, ε-carotene hydroxylase P450 cytochrome dependent gene.

1. INTRODUCTION

Carotenoids are isoprenoid pigments with high commercial value due to their antioxidant, colorant and pro-vitamin properties and with important physiological functions. In plant cells, this pathway is the target of many herbicides and catalyses the synthesis of pigments essential for light harvesting, maintenance of the phostosystems structure and photoprotection mechanisms. Animals and other non-photosynthetic organisms, that do not synthesize carotenoids *de novo,* must include them in their diet to acquire their characteristic colours or as precursors for essential compounds such as vitamin A or the visual pigment retinal. Furthermore, the intake of carotenoids has proved to offer protection against macular degeneration, UV-induced skin damage and some age-related degenerative diseases [1, 2]. Reviews of Cunningham and Gantt [3], Botella-Pavía and Rodriguez-Concepción [4], Sandmann [5] or Lohr and co-workers [6], offer a good general view of this metabolic pathway in plants and microorganisms.

Nutritional benefits of these terpenoids have stimulated the work to obtain genetically modified plants with new carotenoids or higher quantities of the already synthesized ones. But, in many cases, unexpected collateral effects, low functionality or silencing of the exogenous genes has withdrawn the productivity or viability of the new transgenic strains obtained [7, 8, 9, 10]. Despite the physiological and economical importance of this metabolic route in microalgae, many regulatory aspects are not clear and several genes remain unidentified. It is essential to get a deep understanding of the enzymes involved in this pathway and to identify their key regulatory steps to accomplish an efficient metabolic engineering of this pathway for improving the production of the different added-value carotenoids [11]. Furthermore, it has been shown that the synthesis of carotenoids can be extraordinarily increased under certain environmental conditions, such as high light, nutrient starvation or other stressing conditions. Changes in the expression of the key enzymatic steps of the pathway upon stressing conditions are also essential to understand the response of the cells and eventually to find the best conditions to synthesize carotenoids.

Microalgae are one of the main sources of natural carotenoids. They combine the fast and easy growth of bacteria and other unicellular microorganisms with an active isoprenoid metabolism, which ensures enough precursors for carotenogenic pathway, and adequate storage capacity. All these reasons make of microalgae adequate cell factories for the biotechnological production of high added value carotenoids [12]. Unfortunately, the nuclear transformation of microalgae is limited to a small number of species. *Chlamydomonas* was the first genetically modified microalga [13] and, even now, is one of the easiest and most stable eukaryotic system for genetic manipulation. This microalga has already become a powerful model system due to its easy growth in the lab under different environmental and nutritional conditions, short generation time (about 6h), and the availability of molecular and bioinformatic tools, including plenty of specific promoter and marker genes and a draft of the complete genome sequence (http://genome.jgi.psf.org/Chlre4/Chlre4.home.html) [14].
Chlamydomonas can be an excellent biotechnological system for the massive production of high added value carotenoids

if adequately engineered [12]. It has already been shown that the expression of an exogenous β-carotene oxygenase (*bkt*) gene in *C. reinhardtii* allows the synthesis of new ketocarotenoids that the microalga did not previously produced [15]; but there are still serious difficulties for high productivity. Our limited knowledge about the mechanisms and signals that control carotenoids biosynthesis and storage is the main challenge to obtain higher levels of carotenoids through metabolic engineering of the carotenoid biosynthetic pathway in *Chlamydomonas*, but also in other microalgae and higher plants.

In this work we try, using the model cell *C. reinhardtii*, to gain knowledge about the regulation of carotenoid biosynthesis pathway in green algae. We have studied the changes in the carotenoid profile when *Chlamydomonas* cells are submitted to high light stress in the presence of inhibitors of protein and carotenoid synthesis, and we have analysed the expression level of the genes encoding the enzymes that catalyze the main steps of the pathway.

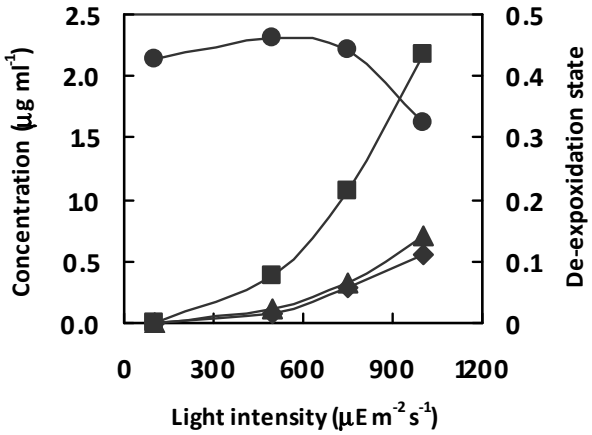

Figure 1. De-epoxidation state of the xanthophyll cycle in *Chlamydomonas reinhardtii* cells after 6h of exposure to light of increasing intensities. The concentration of violaxanthin (●), zeaxanthin (▲), antheraxanthin (♦) and the de-epoxidation state (■), expressed as the ratio [(ant + zea) / (ant + zea + viol)], for *C. reinhardtii* cells grown during 6h at the indicated light intensities are shown.

2. RESULTS

2.1 Activation of the xanthophyll cycle in response to high irradiance and other stressing conditions

Photosynthetic organisms exhibit a variety of response mechanisms when they are exposed to high light or standard light in combination with other stressing conditions. The role of carotenoids, and specially xanthophylls, is particularly important in thermal NPQ dissipation, which is triggered by an acidification of the thylakoid lumen and involves activation of the xanthophylls cycle, in which violaxanthin is reversibly converted into zeaxanthin [16, 17].

To check the ability of high light stress to trigger the xanthophylls cycle, we submitted

Chlamydomonas cells to increasing light intensities between 50 and 1000 µE m^{-2} s^{-1} and analysed the concentration of zeaxanthin, antheraxanthin and violaxanthin after several hours of growth in these conditions. In figure 1, the concentration of the xanthophyll cycle components and the de-epoxidation state observed after 6 hours of growth at the indicated light intensities are plotted. The de-epoxidation state is expressed as the (ant + zea) / (ant + zea + viol) ratio.

Accumulation of zeaxanthin and antheraxanthin, and the corresponding decrease in violaxanthin are correlated with the high light intensity.

After only six hours of exposure to very high light intensity (1000 µE $m^{-2}s^{-1}$) almost half of the total xanthophyll pool is de-epoxidized. There is a slight increase in the total pool of xanthophylls and the (zea + ant) / (zea + ant + viol) ratio increases significantly with the light intensity, in agreement with data previously reported for other microalgae and higher plants [18, 19, 20].

No zeaxanthin was found in control cells cultured at light intensities of 100 µE $m^{-2}s^{-1}$, but when these moderate-light cultured cells were submitted to other stressing conditions an activation of the xanthophyll cycle and accumulation of zeaxanthin was also observed. In Table I, the zeaxanthin intracellular concentration for *C. reinhardtii* cells cultured during 48h at moderate light in the presence of the herbicide norflurazon (5µg mL^{-1}) and at nitrogen starvation is shown.

The presence of zeaxanthin started to be significant only after 24h of growth at the indicated conditions and practically no antheraxanthin was found.

We confirmed that high light intensity (800µE$m^{-2}s^{-1}$), but also depletion of nitrogen, were able to trigger the synthesis of zeaxanthin. Since the response to high light stress was much faster, we used high irradiance (800µE$m^{-2}s^{-1}$) to induce the xanthophylls cycle in the presence of protein and carotenoid synthesis inhibitors, as described in the following experiments.

Table I. Concentration of the xanthophylls cycle components and the de-expoxidation state of the cycle in *C. reinhardtii* cells grown at the indicated conditions. *C. reinhardtii* cells grown at standard conditions were harvested at the exponential phase of growth and resuspended in fresh medium (a,b); nitrogen depleted medium (c) or in completed medium with 5 µg mL^{-1} of the herbicide norflurazon (d). All cultures were kept at normal irradiance excepting the high light stressed one (b), which was submitted to 1000 µE m^{-2} s^{-1}. Analysis of violaxanthin, zeaxanthin and antheraxantin was performed at the indicated times.

	Culture conditions and time	Violaxanthin (mg g^{-1}DW) ±SD	Zeaxanthin (mg g^{-1}DW) ±SD	Antheraxanthin (mg g^{-1}DW) ±SD	De-epoxidation state
(a)	Control (6h)	1.41 ±0.02	0.00	0.00	0.00
(b)	High light (6h)	0.68±0.01	0.32±0.05	0.30±0.03	0.47
(c)	- N (48h)	0.81±0.02	0.22±0.04	0.05±0.01	0.25
(d)	+ Norflurazon (48h)	0.84±0.02	0.30±0.05	0.04±0.01	0.29

2.2 Time course evolution of the carotenoid content in the presence of protein and carotenoid synthesis inhibitors

Upon transition of *Chlamydomonas* cells to high light conditions, not only changes in the concentration of the xantophylls cycle components, but also alterations in the content of other intermediates of the pathway take place. We have analysed the time-course evolution of the main carotenoid pigments in *C. reinhardtii* cells cultured at different light intensities, and we have studied the effect of norflurazon and cycloheximide on this evolution (Fig. 2).

Cycloheximide is an inhibitor of eukaryotic protein translation which has been previously used for successful inhibition of protein expression in *Chlamydomonas* [21]. Norflurazon is a well known bleaching herbicide that inhibits carotenoids biosynthesis in higher plants [22] and microalgae [23, 24]. To optimize the concentration of norflurazon able to inhibit the synthesis of carotenoids in *C. reinhardtii*, the microalga was cultured with increasing quantities of the herbicide during 24h (Table II).

Norflurazon concentrations of 1, 2, 5 and 10 $\mu g\ mL^{-1}$ caused a decrease in the β-carotene content and a proportional increase in the concentration of phytoene. The highest accumulation of phytoene was observed for a norflurazon concentration of 5 $\mu g\ mL^{-1}$. Higher concentrations of norflurazon caused certain inhibition of photosynthetic activity and lower production of carotenoids. So, for the time-course evolution experiments a norflurazon concentration of 5 $\mu g\ mL^{-1}$ was used.

C. reinhardtii cells grown in the standard conditions described in Material and Methods were harvested at the beginning of the exponential phase of growth, resuspended in fresh medium and cultured at high light intensity (800 $\mu E\ m^{-2}s^{-1}$) in the presence of the protein translation inhibitor, cycloheximide (2 $\mu g\ mL^{-1}$), in the presence of the carotenoid synthesis inhibitor, norflurazon (5$\mu g\ mL^{-1}$), and with no inhibitor. After 2, 4, 6 and 8 hours of growth in these conditions, zeaxanthin, antheraxanthin, neoxanthin, β-carotene and violaxanthin were quantified by HPLC. The pigments content was also analysed in control cells cultured at a light intensity of 100$\mu E\ m^{-2}\ s^{-1}$. The de-epoxidation state of the xanthophylls cycle *in C. reinhardtii* cells cultured in these conditions was determined and is shown in figure 3.

As it is shown in figure 2, zeaxanthin and antheraxanthin are undetectable in *Chlamydomonas* cells grown at low light ($50\mu Em^{-2}s^{-1}$) intensity, while for high light intensity the concentration of both zeaxanthin and the intermediate antheraxanthin is about 0.3mg g^{-1} DW. At this high light intensity, zeaxanthin and antheraxanthin are, at least partially, synthetized at the expense of violaxanthin, which internal concentration decreases drastically in high light stressed cells while it stays constant or increases in control low light-irradiated cells.

The intracellular level of zeaxanthin and antheraxanthin in the high-light stressed cells treated with norflurazon is much higher than in cells grown at low light intensity, but not as high as in the stressed cells without inhibitor. The same can be observed in the de-epoxidation state of the cycle (Fig. 3).

This means that, although the zeaxanthin is mainly synthetized by de-epoxidaton of violaxanthin, there is a certain percentage of zeaxanthin synthetized from β-carotene. The increase in the concentration of zeaxanthin observed at high light intensity in *Chlamydomonas* in the presence of norflurazon is due to the interconversion

Table II. Effect of the herbicide norflurazon on the carotenoids content in *Chlamydomonas reinhardtii*. The concentration of phytoene and β–carotene, were determined in *C. reinhardtii* cells grown during 24h at the standard conditions described in Materials and Methods in the presence of increasing quantities of norflurazon. Photosynthetic activity is also shown.

Norflurazon ($\mu g\ mL^{-1}$)	β-carotene ($\mu g\ mL^{-1}$) ±SD	Phytoene ($\mu g\ mL^{-1}$) ±SD	Photosynthetic activity ($\mu moles\ O_2\ mgChl^{-1}\ h^{-1}$) ± SD
0	1.21±0.05	0.00	248.0 ± 0.04
1	0.25±0.03	0.28±0.01	245.4 ± 0.05
2	0.13±0.01	0.32±0.02	210.3 ± 0.03
5	0.14±0.01	0.32±0.02	178.0 ± 0.04
10	0.12±0.01	0.29±0.02	135.5 ± 0.05

between violaxanthin and zeaxanthin and not to the *de novo* synthesis of carotenoids from their precursors, which is inhibited in the presence of the herbicide. Accumulation of zeaxanthin upon transference to high light in the presence of the protein synthesis inhibitor, cycloheximide, is not as high as in the absence of the antibiotic, but is not completely prevented (Fig.3).

This means that only one of the two possible routes for the synthesis of zeaxanthin upon transference to high light, the *de novo* synthesis of carotenoids or the interconversion of violaxanthin and zeaxanthin is dependent of protein synthesis. β-carotene shows an important decrease in the light-stressed cultures, while lutein increases slightly. Norflurazon and cycloheximide caused important decrease in the content of lutein but had no influence on the evolution of β–carotene (Fig. 2).

2.3 Expression patterns of key genes of the carotenoid biosynthesis pathway upon exposure to high light intensity

Several previous studies have tried to identify, high light-induced genes and proteins in *Chlamydomonas* using differential display library screening [25] microarray [26, 27, 28] and proteomic approaches [29]. The large variety of protective responses that photooxidative stress is able to induce involves genes encoding very diverse proteins, including light harvesting complexes proteins, C-metabolism enzymes and heat shock proteins, among others. Although carotenogenic genes and/or proteins are very important in the response to light/oxidative stress, they are poorly represented in these studies. Microarray or proteomic approaches offer a global picture of the changes in expression of genes involved in responses to high light, but are not the most appropriate to detect low-abundant mRNAs/proteins and small changes in gene expression.

In this work we have studied the transcription level of a wide range of carotenogenic genes upon transference from the dark to high intensity light. *C. reinhardtii* cells were collected at the beginning of the exponential phase of growth and cultured in the dark for 16h.

After this dark treatment the cells were transferred to high light intensity (800 $\mu E\ m^{-2}\ s^{-1}$) where they were cultured for 6 hours.

Samples were taken after 1, 3 and 6 hours of exposure to the described conditions. Total RNA was extracted from each of the samples and transcript levels of the enzymes

catalysing the main steps of the pathway were analyzed by qPCR.

As it shown in figure 4, we studied the first steps of the pathway (*psy* and *pds*), the expression of the enzymes responsible for the cyclization of lycopene (*lcye* and *lcyb*), the hydroxylases involved in the synthesis of the xanthophylls zeaxanthin and lutein, *chyb*, *chyε-P450 (CYP97C3)* and *chyβ-P450 (CYP97A5) and* the enzyme zeaxanthin epoxidase (*zep*).

Levels of each transcript are normalized to the housekeeping gene *cblp* level and expressed as a relative fold to the normalized level of the corresponding gene at zero time. Data about the transcription of *vde* would complete the picture of the synthesis of xanthophylls but no orthologue to this gene has been found in the last version of the genome of *Chlamydomonas*.

Exposition of dark pre-grown cells to high light intensity (800$\mu Em^{-2}s^{-1}$) seems to trigger induction of both *psy* and *pds*, which transcripts levels are 2 and 4 fold increased after one hour of exposure to very high light. Lycopene ε- and β-cyclase, involved in the cyclization of lycopene to yield β-carotene and/or α-carotene, show a slight decrease in their mRNA level. For zeaxanthin epoxidase, *zep*, which catalyzes the synthesis of violaxanthin and is directly involved in the xanthophylls cycle, we observed a very slight decrease of its transcript along the time of exposure to high light (Fig. 4).

Regarding carotene hydroxylase β (*chyb*), the non-heme di-iron monoxygenase, which is supposed to be involved in the conversion of β-carotene into zeaxanthin, we observed that its expression is slightly and progressively induced during the 3 first hours of exposure to light stress.

The most interesting results are observed when analysing the expression of the P450 cytochrome dependent ε- and β-ring carotene hydroxylases. The transcript level of both cytochrome dependent hydroxylases in high light stressed cells is ten and eight fold the level of control cells. The maximum induction for both genes was observed after one hour of exposure to high light, but a high level of about 5 fold the initial value was maintained even after 6 hours. The hydroxylases *chyb-P450 (CYP97A5)* and *chye-P450 (CYP97C3)* are orthologes which the corresponding cytochrome dependent hydroxylases, which have shown to be necessary for the synthesis of lutein in *Arabidopsis* [30]. The extraordinary induction in these two hydroxylases, that we have observed when submitting *C. reinhardtii* cells to high light, indicates that they are key steps in the regulatory responses to high light.

3. **DISCUSSION**

Photosynthetic organisms react to excessive light by a variety of mechanisms that include the repair of the proteins and structures damaged by photo-oxidative conditions, synthesis of antioxidant species [31] or dissipation of excess absorbed light by non-photochemical quenching (NPQ) [32].

The two best known NPQ mechanisms of response to excess light are state transition (qT), which involves the migration of a fraction of LHCII between both photosystems, and the thermal dissipation (qE) also known as feedback de-excitation, which involves de-excitation of singlet excited chlorophyll molecules (^{1}Chl*) in the PSII [33].

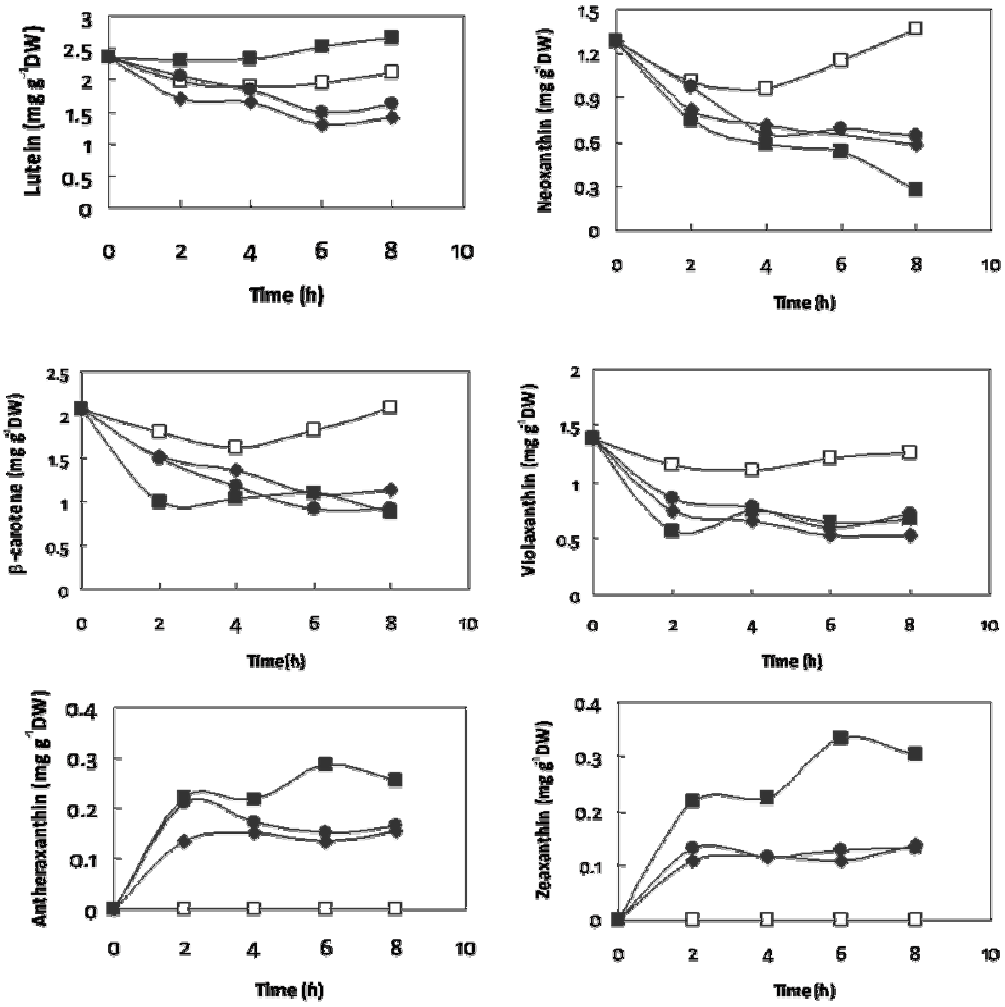

Figure 2. Time-course evolution of the main carotenoid pigments in *C. reinhardtii* cells cultured at high light in the presence of carotenoids and proteins synthesis inhibitors. *C. reinhardtii* cells harvested at the begining of the exponential phase of growth were resuspended in fresh medium and cultured at high light intensity (800 µE $m^{-2}s^{-1}$) in the presence of 2 µg mL^{-1} of the protein translation inhibitor, cycloheximide (●), in the presence of 5 µg mL^{-1} of the carotenoid synthesis inhibitor, norflurazon (♦), and with no inhibitor (■). The pigments content was also analysed in control cells cultured at a light intensity of 100 µE $m^{-2}s^{-1}$ (□). After 2, 4, 6 and 8 hours of growth in these conditions, zeaxanthin, antheraxanthin, neoxanthin, β–carotene, lutein and violxanthin were quantified by HPLC.

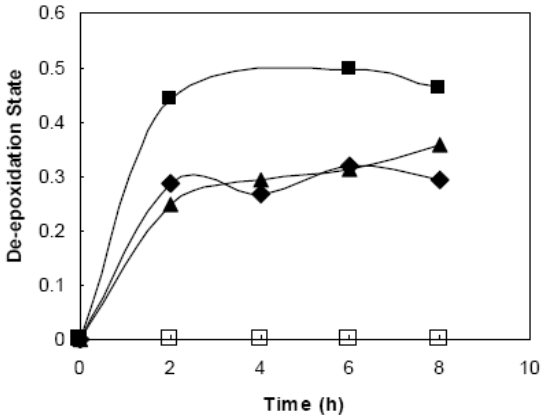

Figure 3. De-epoxidation state of the xanthophylls cycle in *C. reinhardtii* cells exposed to high light in the presence of carotenoids and proteins synthesis inhibitors. The de-epoxidation state of *C. reinhardtii* cells treated as described in figure 2 was plotted for cells cultured at the standard light intensity of 100 µE m^{-2} s^{-1} (□); at a high light intensity of 800 µE $m^{-2}s^{-1}$ (■); and at this high light intensity in the presence of cycloheximide (2 µg $mL^{-1,}$ ▲), and in the presence of norflurazon (5 µg mL^{-1} ♦).

All these responses involve changes in the carotenoid composition, since carotenoids are component of the LHC or the reaction centres that need to be repaired when the photosystems are damaged, but their role is particularly important in thermal NPQ dissipation, which involves activation of the xanthophylls cycle [16, 17].

In some chlorophytes, such as *Dunaliella* or *Haematococcus*, photoxidative stress induces the synthesis of very high quantities of carotenoids and this has been used for massive commercial production of β-carotone and astaxanthin in large scale plants based on these algae. In *C. reinhardtii*, as in higher plants, photooxidative stress has been shown to induce the xanthophylls cycle [17, 19]. In this work we have observed that there is an activation of the cycle in response to high light, in agreement with data previously reported [34,28] but also in response to other stressing situations such as nitrogen starvation, or the presence of the herbicide norflurazon (Table II), which had not been reported so far. Previous work has reported an accumulation of de-epoxidated xanthophylls following exposure of *Chlamydomonas* cells to phosphorus and sulphur limitation [35] The effect of the inhibitor of lycopene cyclases (CPTA) on the changes in zeaxanthin content during high light illumination, described by Depka et al [36], were very similar to the changes that we have observed in the presence of norflurazon, which inhibits the pathway at the level of phytoene. Confirming that part of the zeaxanthin synthetized at high light intensities comes from the hydroxylation of β-carotene.

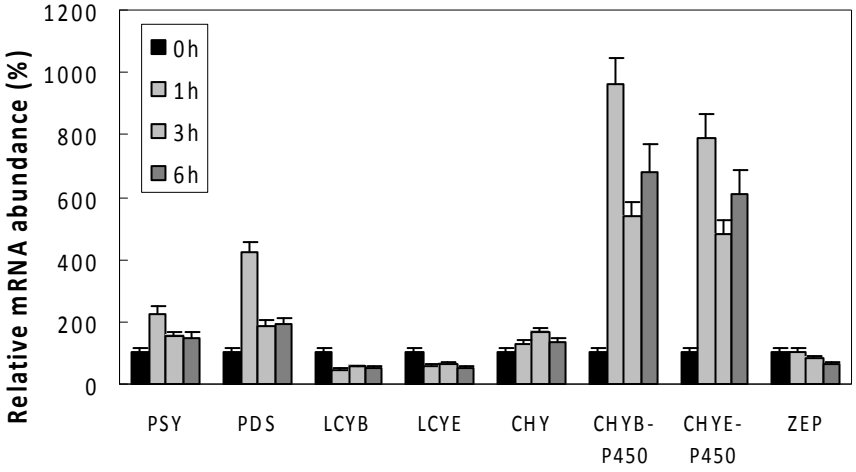

Figure 4. Transcript levels profile of key carotenoid biosynthetic genes in *C. reinhardtii* submitted to strong light shift. *C. reinhardtii* cells cultured in the dark for 16h were transferred to high light intensity (800 μE m^{-2} s^{-1}). Total RNA was extracted at the indicated times and the transcript levels of phytoene synthase (*psy*), phytoene desaturase (*pds*), zeaxanthin epoxidase (*zep*), non heme di-iron enzyme carotene hydroxylase β (*chyb*), the cytochome P450 β and ε hydroxylases (*chyb-P450* and *chye-P450*) and β- and ε-lycopene cyclases (*lcyb* and *lcye*) were analyzed by qPCR. Levels of each transcript are normalized to the housekeeping gene *cblp* level and expressed as a relative fold to the normalized level of the corresponding gene at zero time in the dark. All qPCR data are the mean of measures obtained in triplicate and the result of two independent identical experiments.

Furthermore, we have observed that the synthesis of other xanthophyll not directly involved in the cycle, lutein, is also induced under light stress and that the transcription of the two hydroxylases involved in its synthesis is extraordinarily induced upon high light shifts. The function of xanthophylls in protection against photoinhibiton has been widely studied. Zeaxanthin seems to have multiple functions which can in many occasions be substituted or complemented by antheraxanthin or lutein. Zeaxanthin with a longer conjugated double bond system (11 double conjugated bonds against the 10 of lutein or antheraxanthin or the 9 present in violaxanthin) has been probed to be an efficient free radical quencher and better antioxidant than its epoxidated precursor. Zeaxanthin and/or lutein have been proposed as the quenching species responsible for de-excitation of singlet excited chlorophyll during qE by forming a chlorophyll-xanthophyll heterodimer which allows the thermal dissipation of excitation energy [37, 38].

Other models propose that acidification of the thylakoid lumen and the de-epoxidation of violaxanthin may induce conformational changes in LHC II which should be the direct chlorophyll quencher.

Jin et al., [39] probed, comparing the wild type and mutant lacking epoxycarotenoids of *Dunaliella salina*, that zeaxanthin plays a role for the protection of photodamaged and disassembled PSII reaction centers. The work

with *Chlamydomonas* non-photochemical-quenching-mutants, defective in VDE (npq1) or ZEP (npq2) activities or lor1 mutant, that lacks lutein and its derivatives, has allowed establishing the role of lutein in thermal NPQ. Niyogi et al., [40] showed that mutants of *Chlamydomonas* defective in either alpha or beta carotenoids were able to tolerate high light, but not so the double mutant (npq1 lor1) defective in both lutein and zeaxanthin. The same has been observed in *Arabidopsis*. Similar mutants were obtained for *Dunaliella salina* [39] or *Arabidopsis* [41]. Although many questions remain unclear, it is well established that xanthophylls are essential for NPQ dissipation of excess excitation energy as heat and to quench singlet or triplet chlorophyll.

The role that carotenoids, and specially the xanthophylls cycle components, play on the protection of the photosynthetic apparatus against photooxidative damage has been extensively studied [19] but little is known about the light regulation of carotenogenesis on a molecular level. Here we offer the complete pattern of carotenoids when dark-pregrown *C. reinhartii* are submitted to high light and we compare this pattern with the expression level of practically all the genes of the pathway (Fig.4). To our knowledge this is the first detailed study about the regulation of carotenoid biosynthesis genes in response to light stress in *Chlamydomonas*. The few reports that have focussed on the molecular response of carotenogenic pathway to light, either by qPCR or northern-blot analysis, have exclusively studied a limited number of genes.

Bohne and Linden [42] observed a clear induction of *psy* and *pds* when submitting dark or low light-pregrown *Chlamydomonas* cells to moderate (200 µE $m^{-2}s^{-1}$) white or blue light. They observed a transient induction of *psy* and *pds* expression, reaching a maximum increase 1-2 hours after the transition to light and recovery of the original levels after 4 hours. Similar up-regulation was observed when cells were exposed to very low intensity light (0.01 µE $m^{-2}s^{-1}$) [43, 6]. It is interesting to note that the up-regulation induced by such a low light intensity is similar to the increase that we have observed for these two genes, suggesting that their control is at least partially dependent on specific photoreceptors and confirm their important regulatory role.

Regarding ZEP, which catalyzes the synthesis of violaxanthin and is directly involved in the xanthophylls cycle, both a slight up [6] and down [43] regulation upon exposure to light has been described. The small decrease that we have observed in *zep* mRNA level makes us think that regulation at expression level of *zep* must not be critical when cells submitted to a high light shock. Regulation in the synthesis of the other component of the xanthophylls cycle, the violaxanthin de-epoxidase, should be of high interest but, as we have already indicated, the sequence corresponding to this gene is still unknown in *Chlamydomonas*. An incomplete sequencing could explain the lack of an orthologe for *vde* in *Chlamydomonas* genome, but the fact that it has not either been found in other related algae which genome has also been sequenced, such as *Volvox*, suggests the possibility that a structurally unrelated enzyme was the responsible for the epoxidation of zeaxanthin. This hypothesis would be supported by the fact that common plant violaxanthin de-epoxidases inhibitors are not effective on *Chlamydomonas* [6].

The non-heme di-iron hydroxylase of *Chlamydomonas*, *chyb*, has an identity of 76% with its orthologe in *Arabidopsis* [44].

On the other hand, the heme-containing cytochrome P450 ε- and β- hydroxylases found in *Chlamydomonas* genome have high similarity with the corresponding ε- (CYP97C1) and β- (CYP97A3) hydroxylases of *Arabidopsis* [45,46]. Identity/similarity between the putative hydroxylases found in *Chlamydomonas* and the corresponding ones of *Arabidopsis* was 56%/72% for β-P450 hydroxylase and 61%/74% for ε–P450 hydroxylase [6, 47]. In *Arabidopsis*, the function of these two unrelated hydroxylases families has been well studied using multiple mutants affected in the different hydroxylases and performing substrate specificity studies by complementation experiments [30]. It is generally considered that the cytochrome dependent hydroxylases are the main responsible for the hydroxylation of ε and β rings of α–carotene to yield lutein, while the non-heme di-iron hydroxylase is mainly involved in the hydroxylation of β–carotene to yield zeaxanthin [48], although certain degree of functional overlapping between the different hydroxylases seems to exist [44,49]. In *Chlamydomonas* there are no data about the activity and substrate preference of these hydroxylases. No previous studies about expression of Lycopene cyclases or carotene hydroxylases in *C. reinhardtii* high light stressed cells exist.

4 MATERIALS AND METHODS

4.1 Microorganisms and standard culture conditions

Chlamydomonas reinhardtii wild strain 21gr from Dr. R. Sager (Sidney Faber Cancer Center, Boston, MA) was grown at 25ºC in 15mM K-phosphate (pH 7) buffered medium [50] under continuous white light irradiation of 100 μE $m^{-2}s^{-1}$, unless other intensity is indicated. The cultures were bubbled with air containing 5% CO_2. When indicated the inhibitors cycloheximide (4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione) or norflurazon (4-chloro-5-methylamino-2-(α,α,α-trifluoro-m-tolyl) pyridazin-3(2H**)**-one), supplied by SIGMA (St. Louis, MO, USA), were added to the culture medium. The *Escherichia coli* strain used for *in vivo* amplification of DNA was DH5α, cultured in LB medium as previously described [51].

4.2 High irradiance experiments

For high irradiance experiments *C. reinhardtii* cultures contained in 0.5L flasks were illuminated with an adjustable halogen lamp (Philips 500W). The flasks were immersed in a glass thermostatic bath. The bath was connected with a refrigerated circulator (LAUDA E300) to ensure culture temperatures of 25ºC even at very high light intensity. Light intensity was measured by a Delta OHM quantum photo radiometer equipped with a PAR sensor.

4.3 RNA isolation and retrotranscription reaction

Isolation of total RNA was performed with the RNAeasy plant MiniKit of Qiagen (Hilden, Germany) and residual genomic DNA was removed by incubation with the DNAase (Quiagen) during 10min, as indicated by the manufacturer. The RNA concentrations were determined at 260nm with the Nanodrop 1000 spectrophotometer (Thermo Sicentific).
Single strand cDNA was synthesized from total RNA with oligodT (0.5 μM) and the SuperScript II RNaseH- reverse transcriptase (Invitrogen) in 20μL of reaction mixture, at

25ºC for 10min, followed by 50min incubation at 42ºC. The reaction was stopped by heat inactivation at 70ºC for 15 min, and RNA was removed with RNase H treatment at 37ºC for 20 min. The obtained cDNA was used as substrate for Real Time PCR reactions.

4.4 Quantitative RT-PCR

Real time PCR was performed on a Mx3000P Multiplex Quantitative PCR System from Stratagene using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, La Jolla, CA, USA). Each determination was carried out in triplicate using as template the cDNA synthesized from total RNA, as previously described, and 10 pmoles of the indicated primers (see Table I) in a final volume of 25 µL. Cycling conditions were: 10 min at 95ºC for activation of the hot start Taq polymerase and 40 cycles for the melting (30 s at 95 ºC), annealing (30s at 60 ºC) and extension (30s at 72 ºC). The fluorescence measurement was made at the end of the annealing step. A dissociation curve (30s at 95 ºC, 30s at 55 ºC and 30s at 95 ºC) was applied at the end of the amplification reaction to check possible formation of dimers.

All primers were designed to yield amplicons between 150 and 280 bp (Table III) and in such a way that they anneal with regions of the mRNA encoded by different exons. Eventual amplification of genomic DNA with these primers would yield longer PCR product that could be easily detected both in agarose gels and in the dissociation curve of Real Time PCR. This avoids interference in the mRNA quantification by possible genomic contaminations. The *cblp* gene (GenBank X53574), which encodes a protein that shows sequence similarity with the beta subunit of G-binding proteins from mammals, was used as housekeeping gene. Expression of this gene was previously shown to be constitutive under the different conditions used [25, 26]. Primers *cblpfor* and *cblprev* amplify a 219 bp fragment. Eventual amplification of genomic DNA with these primers would generate a fragment of 449 bp.

4.5 Dry weight determination.

Dry weight was determined by filtering an exact volume of microalgae culture (10 mL) on pre-tared glass-fiber filters (1µm pore size). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts and dried at 100 ºC for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference between the pre-tared and the cell-containing dried filters. All measurements were done in triplicate.

4.6 Pigments extraction and analysis

Carotenoids and chlorophylls were extracted with 80 % acetone. The separation and chromatographic analysis of pigments were performed on a Merck Hitachi HPLC equipped with a diode array detector as described by Young and co-workers [53], using a RP-18 column and a flow rate of 1 mL min^{-1}. The mobile phase consisted of: solvent A, ethyl acetate; solvent B acetonitrile/water (9:1, v/v) and the gradient programme applied was: 0-16 min 0-60%A; 16-30 min 60%A; 30-35 min 100%A. Injection volume was 100 µL. Pigments detection was carried out at 450 nm, excepting phytoene that was detected at 288nm. Pigments standards were supplied by SIGMA or DHI (Hoersholm, Denmark).
The results are the average of at least two replicates.

Table III. Sequence of the primers used for Real-Time RT-PCR experiments

Primer name	Target gen	Sequence	Amplicon	Annealing Tª
qCrCBLPf	*cblp*	CGCCACCCAGTCCTCCATCAAGA	219	63,9
qCrCBLPr		CTAGGCGCGGCTGGGCATTTAC		63,4
qCrPSYf	*psy*	CACTCGCGCCCGGCAATACTT	193	62.8
qCrPSYr		CCACGGGCAGCGACACCATC		63.0
qCrPDSf	*pds*	GCCGCTCACCGCTGCTGTC	145	61.4
qCrPDSr		GGTGGCGGCGATGATGTCCTC		63.2
qCrLCYBf	*lcyb*	GCATTGTGGCGGAGGTTGAGTCT	147	61.1
qCrLCYBr		AAGGGCATGGCGTACAGGAAGGT		61.6
qCrLCYEf	*lcye*	TGGTGAAGGAGCAGGCGAACA	281	60.2
qCrLCYEr		CGAAGTAGCACACGGCATCAGG		59.3
qCrCHYBf	*chyb*	CGCGGGTCTGGGCATCAC	152	59.5
qCrCHYBr		CCGAACTTGTTGGTGTGGTGGAT		59.5
qCrCHYBP450f	*chyp450b*	AGGAGGCGCTGGTGCTGGTAAAT	201	61.8
qCrCHYBP450r		CCCGCAATCAGCATAGTCATCAGG		61.4
qCrCHYEP450f	*chyp450e*	CCGGCCCGCTCTGTTTGTTC	230	61.2
qCrCHYEP450r		CCGCCCAGCAGCTCCTTGAT		61.0
qCrZEPf	*zep*	CTGCGCCGCGACATCTTTGAC	166	62.4
qCrZEPr		GACACGGCGCGGCTGAGGT		62.9

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CONCLUSIONS

CONCLUSIONS

1. We have shown by functional studies in bacteria that the enzyme β-carotene ketolase, codified by *bk1* gene (X86782) from *Haematococcus pluvialis*, is able to oxygenate the position C4 of the β-rings of the β-carotene and its hydroxylated derivate zeaxanthin. In the same way, the phytoene synthase enzyme (PSY) encoded by the *psy* gene (AY601075.1) isolted from *Dunaliella salina* is able to efficiently complete the production of β-carotene in *psy* deficient *E.coli* cells.

2. *Bkt1* gene can be expressed *in vivo* in *Chlamydomonas*, due to the RuBisCO small subunit gene promoter and the gene product obtained targeted to its final location in the chloroplast thanks to the transit peptides of RuBisCO small subunit or ferredoxin, both chloroplast proteins which are encoded by nuclear genes. In this way, we managed to get the first *Chlamydomonas* transgenic strains able to constitutively synthesise ketocarotenoids in vegetative cells.

3. Analogously, the expression in *Chlamydomonas* nucleus of the gene that encodes one of the first enzymes of the carotenogenic route, the phytoene synthase from *Dunaliella salina*, enabled us to isolate *Chlamydomonas* transformants with high levels of *Dspsy* mRNA and a chlorophyll/carotenoid relation 40% higher than the control strains. In these transformant strains, an important increment in β-carotene and violaxanthin production is observed.

4. We analysed the time-course evolution of carotenoid pigments in *Chlamydomonas reinhardtii* in the absence of nitrogen source and at different light intensities. From those studies we can conclude that there is an activation of the xanthophyll cycle in *Chlamydomonas* in response to the high light intensities, as previously reported, and also in response to other stressing conditions, such as nitrogen depletion and the presence of the herbicide norflurazon, not previously reported.

5. We also studied the effect of light on the content of carotenoids in *Chlamydomonas* cells transferred to high light in the presence of norflurazon and the antibiotic cycloheximide. From these results, we can conclude that in high light stressed *Chlamydomonas* cells, although the zeaxanthin is principally synthesised by violaxanthin de-epoxidation, there is an important contribution of the synthesised zeaxanthin that comes from the β-carotene hydroxylation.

6. Quantitative Real Time PCR enabled us to quantify the expression levels of the main key enzymes of the carotenoids pathway. We studied the mRNA levels of the genes codifying the enzymes from the early steps of the pathway *psy* and *pds*, enzymes responsible for the lycopene cyclization, *lcye*, *lcyb*, hydroxylases implied in the synthesis of the xanthophylls zeaxanthin and lutein, *chyb*, *chye-P450*,(CYP97C3) and *chyb-P450* (CYP97A5) to zeaxanthin epoxidase (*zep*). The most interesting results are observed in the analysis of the P450 dependent hydroxylases expression as their transcript levels are increased between eight and ten fold in high light conditions than in standard light. This extraordinary induction of the *chyb-P450* and *chye-P450,* probably involved in the synthesis of lutein, suggests that β- and ε- hydroxylases should be important steps in the photo-oxidative light response.

CONCLUSIONES

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1. Mediante estudios de análisis funcional en bacterias, demostramos que la enzima β-caroteno oxigenasa, codificada por el gen *bkt1* (X86782) de *H. pluviales,* es capaz de catalizar la oxigenación de la posición C4 de los anillos β tanto del β-caroteno, como la de su derivado hidroxilado zeaxantina. De la misma forma, la enzima fitoeno sintasa (PSY) codificada por el gen *psy* (AY601075.1), aislada de *Dunaliella salina*, es capaz de complementar eficientemente la producción de β-caroteno en células de *E.coli* deficientes en fitoeno sintasa.

2. El gen bkt1 puede expresarse *in vivo* en *Chlamydomonas,* gracias al promotor de la subunidad pequeña de la RuBisCO, y el producto génico obtenido trasladarse hasta su ubicación definitiva en el cloroplasto, gracias a los péptidos señal de la propia subunidad pequeña de la RuBisCO o de la ferredoxina, ambas proteínas del cloroplasto codificadas por genes nucleares. De este modo hemos conseguido las primeras primeras cepas transgénicas de *Chlamydomonas* capaces de sintetizar cetocarotenoides de forma constitutiva en sus células vegetativas.

3. De forma análoga la expresiónen el núcleo de *Chlamydomonas* del gen que codifica una de las primeras enzimas de la ruta de la carotenogénesis, la fitoeno sintasa (*psy)* de *Dunaliella salina,* nos ha permitido aislar transformantes de *Chlamydomonas* con altos niveles de *dspsy* mRNA en los que la relación carotenoides/clorofila era un 40% mayor que en las estirpes control. En estos transfomantes se observó un importante aumento de la producción de β-caroteno y violaxantina.

4. Hemos analizado la evolución temporal de los carotenoides en células de *C. reinhardtii* cultivadas en carencia de nitrógeno y a diferentes intensidades de luz. Esto nos ha permitido concluir que hay una activación del ciclo de las xantofilas en respuesta a las altas intensidades luminosas, de acuerdo con datos previos, y en respuesta a otras situaciones de estrés como la carencia de nitrógeno o la presencia del herbicida norflurazón, que no había sido descrito hasta la fecha.

5. Además estudiamos el efecto sobre el contenido en carotenoides en células de *Chlamydomonas* transferidas a altas intensidades luminosas en presencia

del herbicida norflurazón y del antibiótico cicloheximida. Gracias a estos estudios concluimos que en células de *Chlamydomonas* sometidas a un estrés por alta luz, aunque la zeaxantina es sintetizada principalmente por de-epoxidación de la violaxantina, hay una contribución importante de síntesis de zeaxantina a partir del β-caroteno.

6. La técnica de qPCR cuantitativa nos ha permitido cuantificar el nivel de expresión de las principales enzimas clave de la ruta. Estudiamos el nivel de expresión de los genes que codifican las enzimas que catalizan los primeros pasos de la ruta, *psy* y *pds*, las enzimas responsables de la ciclación de licopeno, *lcyε* y *lcyβ*, las hidroxilasas implicadas en la síntesis de las xantofilas zeaxantina y luteína, *chyβ*, *chyε*-P450 (CYP97C3) y *chyβ*-P450 (CYP97A5), y la enzima zeaxantina epoxidasa (*zep*). Los resultados más interesantes se observaron en el análisis de la expresión de las caroteno hidroxilasas dependientes de citocromo P450, cuyo nivel de expresión se incrementó entre diez y ocho veces respecto a las células cultivadas en luz normal. Esta extraordinaria inducción de *chyβ-p450* y *chyε-p450* presuntamente implicados en la síntesis de luteína indica que las hidroxilasas β y ε son clave en la respuesta al estrés fotooxidativo.