PRODUCCIÓN DE CAROTENOIDES POR MICROALGAS Y CARACTERIZACIÓN DE LA RUTA CAROTENOGÉNICA EN *Chlorella zofingiensis*

Trabajo presentado para optar al grado de Doctor en Biología por el licenciado

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A mi gente…

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Abreviaturas y Símbolos

aas	Aminoácidos
ABA	Ácido abscísico
AMD	Degeneración macular asociada a la edad
bkt	β-caroteno C-4-oxigenasa/cetolasa eucariota
cDNA	ADN copia
chyB	Caroteno β-hidroxilasa tipo no-hemo di-hierro-monooxigenasa
Crpsy	Fitoeno sintasa de *Chlamydomonas reinhardtii*
crtB	Fitoeno sintasa procariota
crtH	Caroteno isomerasa de cianobacteria
crtI	Fitoeno desaturasa bacteriana
crtIso	Caroteno isomerasa eucariota
*crt*L	Licopeno ciclasa de cianobacteria
crtLb	Licopeno β-ciclasa de cianobacteria
crtLe	Licopeno ε-ciclasa de cianobacteria
crtO	β-caroteno C-4-oxigenasa/cetolasa procariota
crtP	Fitoeno desaturasa de cianobacteria
crtQ	ζ-fitoeno desaturasa de cianobacteria
crtR	Caroteno β-hidroxilasa de cianobacteria
crtW	β-caroteno C-4-oxigenasa/cetolasa procariota
crtY	Licopeno β-ciclasa bacteriana
crtYB	Licopeno ciclasa bifuncional
*crtY*c	Licopeno β-ciclasa heterodimérica
*crtY*d	Licopeno β-ciclasa heterodimérica
crtZ	Caroteno β-hidroxilasa bacteriana
Czbkt	β-caroteno C-4-oxigenasa/cetolasa de *Chlorella zofingiensis*
CzchyB	Caroteno β-hidroxilasa de *C. zofingiensis*
CzlcyB	Licopeno β-ciclasa de *C. zofingiensis*
CzlcyE	Licopeno ε-ciclasa de *C. zofingiensis*
Czpds	Fitoeno desaturasa de *C. zofingiensis*
Czpsy	Fitoeno sintasa de *C. zofingiensis*
DMAPP	Dimetilalil pirofosfato
DNA	Ácido desoxirribonucleico
dNTP	Desoxinucleótido-5'-fosfato
DOXP	Ruta del 1-deoxi-D-xilulosa 5-fosfato
EDTA	Ácido etilendiamina tetraacético
EMS	Etil metano sulfonato
FPP	Farnesil pirofosfato
g	Gramo
G3P	Gliceraldehido 3-fosfato
GPP	Geranil pirofosfato
GGPP	Geranilgeranil pirofosfato
GGPPs	Geranilgeranil pirofosfato sintasa
h	Hora
HMG-CoA	Hidroximetilglutaril-CoA
HPLC	Cromatografía líquida de alta precisión
IPi	Isopentenil pirofosfato isomerasa
IPP	Isopentenil pirofosfato
IPTG	isopropil-β-D-1-tiogalactopiranósido
kb	Kilobase
kDa	KiloDalton
L	Litro
LB	Medio de cultivo Luria-Bertani
lcyB	Licopeno β-ciclasa eucariota
lcyE	Licopeno ε-ciclasa eucariota
μ	Velocidad específica de crecimiento
μE	Microeinstein
μg	Microgramo
μm	Micrometro
M	Molar
MEP	Ruta del no mevalonato (2-C-metil-d-eritritol 4-fosfato)
mg	Miligramo
mL	Mililitro
mM	Micromolar
MNNG	N-metil-N´-nitro-N-nitrosoguanidina

mRNA	ARN mensajero
MVA	Ruta del mevalonato
NADPH	Nicotinamida adenina dinucleótido fosfato
NF	Norflurazón
ng	Nanogramo
NH-di-hierro	Monooxigenasa tipo no-hemo di-hierro
NIC	Nicotina
nm	Nanómetro
nsy	Neoxantina sintasa
ORF	Fase de lectura abierta
P450chyE	Caroteno ε-hidroxilasa tipo hemo-monooxigenasa citocromo P450
P450chyB	Caroteno β-hidroxilasa tipo hemo-monooxigenasa citocromo P450
pb	Pares de bases
PCR	Reacción en cadena de la polimerasa
pds	Fitoeno desaturasa eucariota
ps	Peso seco
psy	Fitoeno sintasa eucariota
qPCR	PCR cuantitativa a tiempo real
RACE	Amplificación rápida de los extremos de cDNA
RNA	Ácido ribonucleico
rpm	Revoluciones por minuto
ROS	Especies reactivas de oxígeno
RT-PCR	Retrotranscripción de RNA
SD	Desviación típica
SDS	Laurilsulfato sódico
t	Tiempo
Tª	Temperatura
TAP	Medio de cultivo Tris-acetato-fosfato
TMD	Dominios transmembranas
V	volumen
vde	Violaxantina de-epoxidasa
WT	Organismo silvestre
X-gal	5-Bromo-4-cloro-3-indolil-β-D-galactopiranósido
zep	Zeaxantina epoxidasa
zds	ζ-fitoeno desaturasa eucariota

Bases Nitrogenadas

A	Adenina
C	Citosina
G	Guanina
T	Timina

Aminoácidos

A	Ala	Alanina	L	Leu	Leucina
R	Arg	Arginina	K	Lys	Lisina
N	Asn	Asparagina	M	Met	Metionina
D	Asp	Aspartato	F	Phe	Fenilalanina
C	Cys	Cisteína	P	Pro	Prolina
E	Glu	Glutamato	S	Ser	Serina
Q	Gln	Glutamina	T	Thr	Treonina
G	Gly	Glicina	W	Trp	Triptófano
H	His	Histidina	Y	Tyr	Tirosina
I	Ile	Isoleucina	V	Val	Valina

Código Genético

		Segunda base					
		U	C	A	G		
Primera base	U	Phe UUU	Ser UCU	Tyr UAU	Cys UGU	U	Tercera base
		Phe UUC	Ser UCC	Tyr UAC	Cys UGC	C	
		Leu UUA	Ser UCA	**Stop UAA**	**Stop UGA**	A	
		Leu UUG	Ser UCG	**Stop UAG**	Trp UGG	G	
	C	Leu CUU	Pro CCU	His CAU	Arg CGU	U	
		Leu CUC	Pro CCC	His CAC	Arg CGC	C	
		Leu CUA	Pro CCA	Gln CAA	Arg CGA	A	
		Leu CUG	Pro CCG	Gln CAG	Arg CGG	G	
	A	Ile AUU	Thr ACU	Asn AAU	Ser AGU	U	
		Ile AUC	Thr ACC	Asn AAC	Ser AGC	C	
		Ile AUA	Thr ACA	Lys AAA	Arg AGA	A	
		Met AUG	Thr ACG	Lys AAG	Arg AGG	G	
	G	Val GUU	Ala GCU	Asp GAU	Gly GGU	U	
		Val GUC	Ala GCC	Asp GAC	Gly GGC	C	
		Val GUA	Ala GCA	Glu GAA	Gly GGA	A	
		Val GUG	Ala GCG	Glu GAG	Gly GGG	G	

Introducción

1. CAROTENOIDES

Los carotenoides son una amplia familia de isoprenoides que contienen una serie de dobles enlaces conjugados que constituyen el grupo cromóforo de la molécula, responsable del color y de las propiedades de absorción de estos compuestos. Los carotenoides son sintetizados por todos los organismos fotosintéticos y por algunas bacterias no fotosintéticas y hongos. En los organismos fotosintéticos, los carotenoides son componentes esenciales del aparato fotosíntético y se localizan en los centros de reacción de los fotosistemas y en los complejos antena, unidos a proteínas integrales de membrana, donde participan en la absorción de luz y en la protección del aparato fotosintético frente a daños fotooxidativos (Demming-Adams et al., 1996; Baroli and Niyogi, 2000). Ya que sólo los organismos fotosintéticos y ciertas bacterias y hongos son capaces de sintetizar carotenoides, los animales, que no pueden sintetizarlos *de novo*, deben incluirlos en su dieta para adquirir sus colores característicos (como es el caso de salmones, gambas y crustáceos en general o algunos pájaros) o como precursores de compuestos esenciales, tales como la vitamina A o el pigmento visual retinal (Eonseon et al., 2003).

Los carotenoides se dividen en dos grupos: carotenos y xantofilas. Los carotenos son hidrocarburos lineales o cíclicos en uno o ambos extremos de la molécula, siendo los más abundantes el α-caroteno y el β-caroteno (Figura 1). Los carotenos son responsables del color anaranjado de muchas frutas, verduras, plumaje de aves, y del color amarillento de margarinas y derivados. Además, el β-caroteno es el precursor de la vitamina A (retinal) en vertebrados. Debido a que el β-caroteno está compuesto de dos grupos retinilo, su rotura enzimática produce dos moléculas de vitamina A. Los carotenos α-caroteno y γ-caroteno también poseen actividad vitamina A, aunque en menor medida que el β-caroteno, debido a que tienen un único grupo retinilo (anillo β-ionona). Por otro lado, las xantofilas son derivados oxigenados de los carotenos (Figura 1), y presentan una gran variedad de funciones en los organismos fotosintéticos. Las microalgas, levaduras y otros microorganismos, producen algunas de las xantofilas que actualmente se comercializan debido a su color y capacidad antioxidante, como es el caso de la astaxantina.

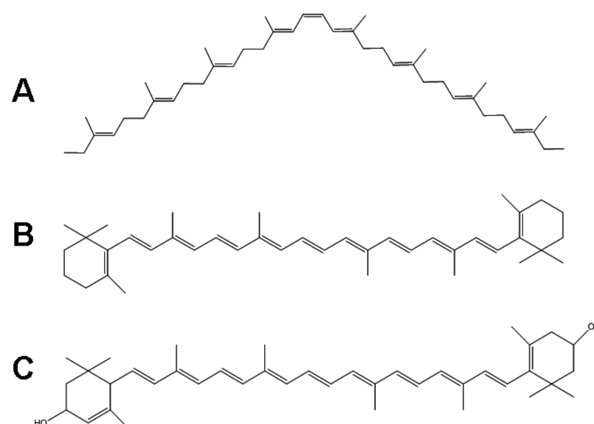

Figura 1. Estructura de los carotenos fitoeno (A) y β-caroteno, y de la xantofila luteína (C).

Los carotenoides también se pueden clasificar en primarios y secundarios. En microalgas, los carotenoides primarios, como la luteína, actúan como componentes estructurales y funcionales del aparato fotosintético de las células y, por tanto, son esenciales para su supervivencia en condiciones de cultivo fotoautotrófico (Demming-Adams et al., 1996; Baroli and Niyogi, 2000). Los carotenoides secundarios, como la astaxantina, se acumulan en grandes cantidades en cuerpos lipídicos dentro o fuera de los cloroplastos cuando las células se exponen a diferentes condiciones de estrés. La función de los carotenoides secundarios en microalgas no se conoce. Actualmente, se cree que pueden actuar como filtros fotoprotectores y como antioxidantes, previniendo la acumulación de radicales de oxígeno (Boussiba 2000; Demming-Adams and Adams 2002; Jin et al., 2003).

1.1. Biosíntesis de carotenoides

1.1.1. Biosíntesis de IPP y DMAPP: Ruta del Mevalonato y del no Mevalonato

Los carotenoides se forman a partir de los isoprenos activos de 5 átomos de carbono, isopentenil pirofosfato (IPP) y dimetilalil pirofosfato (DMAPP). Durante muchos años, se ha asumido que en todos los organismos el IPP se sintetizaba mediante la ruta del mevalonato (MVA) y posteriormente se isomerizaba por la isopentenil pirofosfato isomerasa (IPI) a DMAPP. Sin embargo, una ruta alternativa independiente de la del MVA, denominada ruta del no mevalonato, ruta del 1-deoxi-D-xilulosa 5-fosfato (DOXP) o ruta del 2-C-metil-d-eritritol 4-fosfato (MEP) (Ramos-Valdivia et al., 1997), definida completamente hace tan sólo unos años, es la principal responsable de la síntesis de carotenoides en tejidos fotosintéticos (Ramos-Valdivia et al., 1997; Eisenreich et al., 2004; Lichtenhaler, 1999; Lohr et al., 2012). La diferencia principal entre ambas rutas, aparte de su localización, reside en el paso de condensación inicial. En la ruta MVA, el IPP se sintetiza por condensación de tres moléculas de acetil-CoA, mientras que la ruta MEP se produce por la condensación de piruvato y D-gliceraldehido-3-fosfato. Además, la ruta MEP, a diferencia de la ruta MVA, produce en el último paso IPP y DMAPP (Figura 2). En plantas y algunas clases de algas la ruta MVA, localizada en el citoplasma, suministra precursores para la biosíntesis de esteroles citosólicos, citoquininas, poliprenoides y ubiquinona mitocondrial, mientras que la ruta MEP, localizada en los cloroplastos, produce precursores para isoprenoides plastídicos tales como carotenoides, fitol, fitohormonas, cadena lateral de la plastoquinona y productos mono y diterpenos de plantas y algas (Rohmer, 1999). Existen numerosas evidencias que indican que las algas verdes han perdido la ruta MVA citosólica para la formación de IPP y la ruta MEP suministra los bloques constructores para la biosíntesis de todos los isoprenoides celulares (Rohmer, 1999; Lohr et al., 2012).

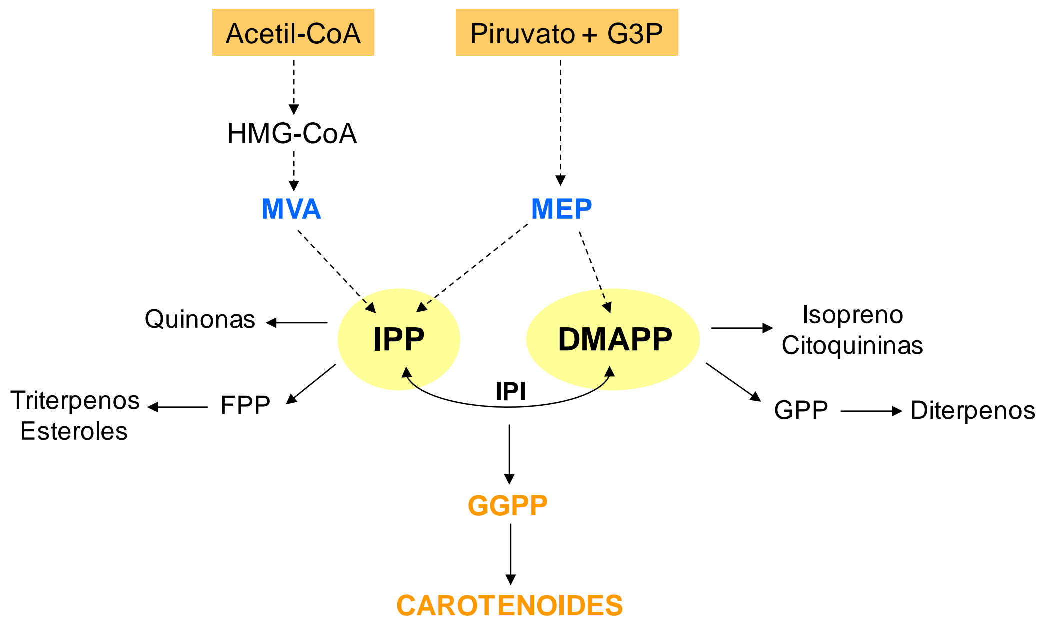

Figura 2. Representación esquemática de la síntesis de IPP por las rutas MVA o MEP, y de DMAPP por la ruta MEP. G3P, gliceraldehido 3-fosfato; HMG-CoA, hidroximetilglutaril-CoA; GPP, geranil pirofosfato; GGPP, geranilgeranil pirofosfato; FPP, farnesil pirofosfato.

1.1.2. Biosíntesis de carotenoides a partir de IPP y DMAPP

Aunque la ruta MEP produce IPP y DMAPP como productos finales, los cloroplastos también contienen la enzima que interconvierte ambos isómeros (IPI), indicando el papel esencial de este enzima en proporcionar un suplemento balanceado de IPP y DMAPP. El DMAPP se convierte en geranilgeranil pirofosfato (GGPP) por la adición consecutiva de tres moléculas de IPP. La condensación de dos moléculas de GGPP catalizada por la fitoeno sintasa (PSY) produce el primer carotenoide lineal de 40 átomos de carbono, el fitoeno (15-*cis*-fitoeno). A partir del fitoeno se sintetizan el resto de carotenoides por una serie de desaturaciones y ciclaciones; y las xantofilas por hidroxilación, oxidación o epoxidación de los correspondientes carotenoides (Figura 3).

El fitoeno, que es incoloro, sufre cuatro desaturaciones consecutivas que incrementan el número de dobles enlaces conjugados y se transforma en licopeno, de color rojizo. En bacterias una única enzima (CRTI) cataliza los cuatro pasos de desaturación, mientras que en plantas y microalgas dos enzimas relacionadas, la fitoeno desaturasa (PDS) y la ζ-fitoeno desaturasa (ZDS) están implicadas en la síntesis de pro-licopeno (7,9,7′,9′ tetra-*cis* prolicopeno), que es isomerizado a todo-*trans* licopeno por la caroteno isomerasa (CRTISO). Además, Chen y colaboradores (2010) han descrito el papel de la Z-isomerasa (Z-ISO) en la isomerización del doble enlace 15-*cis* presente en el producto de PDS (9,15,9’-tri-*cis*-ζ-caroteno) para formar el sustrato de ZDS (9,9’-di-*cis*-ζ-caroteno). Tanto PDS como ZDS

presentan el mismo mecanismo de acción y usan plastoquinona como aceptor de hidrógeno, conectando de esta forma la desaturación de carotenoides y la cadena fotosintética de transporte de electrones.

El licopeno puede ciclarse en uno de sus extremos por la acción de la licopeno β-ciclasa (LCYb) para producir γ-caroteno, o por la acción de la licopeno ε-ciclasa (LCYe) para formar δ-caroteno. Ambos carotenoides monociclados formados se ciclan nuevamente por la LCYb para producir β-caroteno o α-caroteno. La hidroxilación del carbono 3 de cada anillo del β-caroteno y α-caroteno produce zeaxantina (3,3´-hidroxi-β-caroteno) y luteína (3,3´-hidroxi-α-caroteno), respectivamente (Sandmann et al., 2006). En *Arabidopsis*, estas hidroxilaciones están catalizadas por dos clases de hidroxilasas no relacionadas estructuralmente: dos monooxigenasas P450, P450CHYb/CYP97A3 y P450CHYe/CYP97C1, que hidroxilan los anillos β y ε del α-caroteno, respectivamente (Kim and DellaPenna, 2006; Tian et al., 2004); y dos monooxigenasas no hemínicas, CHYb/BCH1 y BCH2, que hidroxilan los anillos β del β-caroteno (Sun et al., 1996). P450CHYb es también activa en la hidroxilación del β-caroteno. En *Chlamydomonas* se ha descrito la existencia de genes candidatos a codificar las enzimas *chyB* y *P450chyB*, y en *C. zofingiensis chyB*, pero aún no se ha confirmado mediante análisis funcional (Lohr, 2009). La zeaxantina se convierte en violaxantina por la zeaxantina epoxidasa (ZEP) con anteraxantina como intermediario. En condiciones de alta irradiancia, la violaxantina es de-epoxidada a zeaxantina por la violaxantina de-epoxidasa (VDE). Esta interconversión entre zeaxantina y violaxantina se denomina Ciclo de las Xantofilas y es esencial para la disipación del exceso de energía de excitación en la fotosíntesis de plantas, y microalgas clorofíceas y feofíceas (Goss and Jakob, 2010).

Solamente un número limitado de organismos entre los que se incluyen algunas microalgas verdes como *Haematococcus pluvialis* o *Chlorella zofingiensis*, algunas bacterias fotosintéticas como *Agrobacterium aurantiacum* y algunos hongos pueden sintetizar astaxantina (3,3′-hidroxi-4,4′-ceto-β-caroteno) a partir de β-caroteno, mediante dos reacciones, una de oxigenación catalizada por la β-caroteno cetolasa (β-caroteno-C4-oxigenasa, BKT) y otra de hidroxilación catalizada por la hidroxilasa CHYb (Lotan and Hirschberg, 1995). Se han descrito dos rutas diferentes de biosíntesis de la astaxantina: una que comienza con la oxigenación del β-caroteno pasando por equinona, cantaxantina y adonirubina y otra que empieza con la hidroxilación del β-caroteno con β-criptoxantina, zeaxantina y adonixantina como intermediarios (Cunningham and Gantt, 1998; Margalith, 1999; Sandmann, 1994). La primera ruta ha sido demostrada mediante análisis funcional de las cetolasas tipo CrtW/BKT de la bacteria marina *A. aurantiacum* y la microalga *H. pluvialis*, que sólo aceptan β-caroteno como sustrato (Breitenbach et al., 1996; Linden, 1999; Lotan and Hirschberg 1995; Lu et al., 1995; Misawa et al., 1995). Además, mediante análisis funcional de la cetolasa de *C. zofingiensis* se ha confirmado la segunda ruta (Huang et al., 2006), habiéndose demostrado que el producto del gen *bkt* de esta microalga puede actuar tanto sobre β-caroteno como sobre zeaxantina, y habiéndose propuesto que la astaxantina se sintetiza en esta microalga

preferentemente mediante oxidación de zeaxantina. La Tabla 1 muestra un resumen de los diferentes genes carotenogénicos denominados de forma diferente en diferentes organismos.

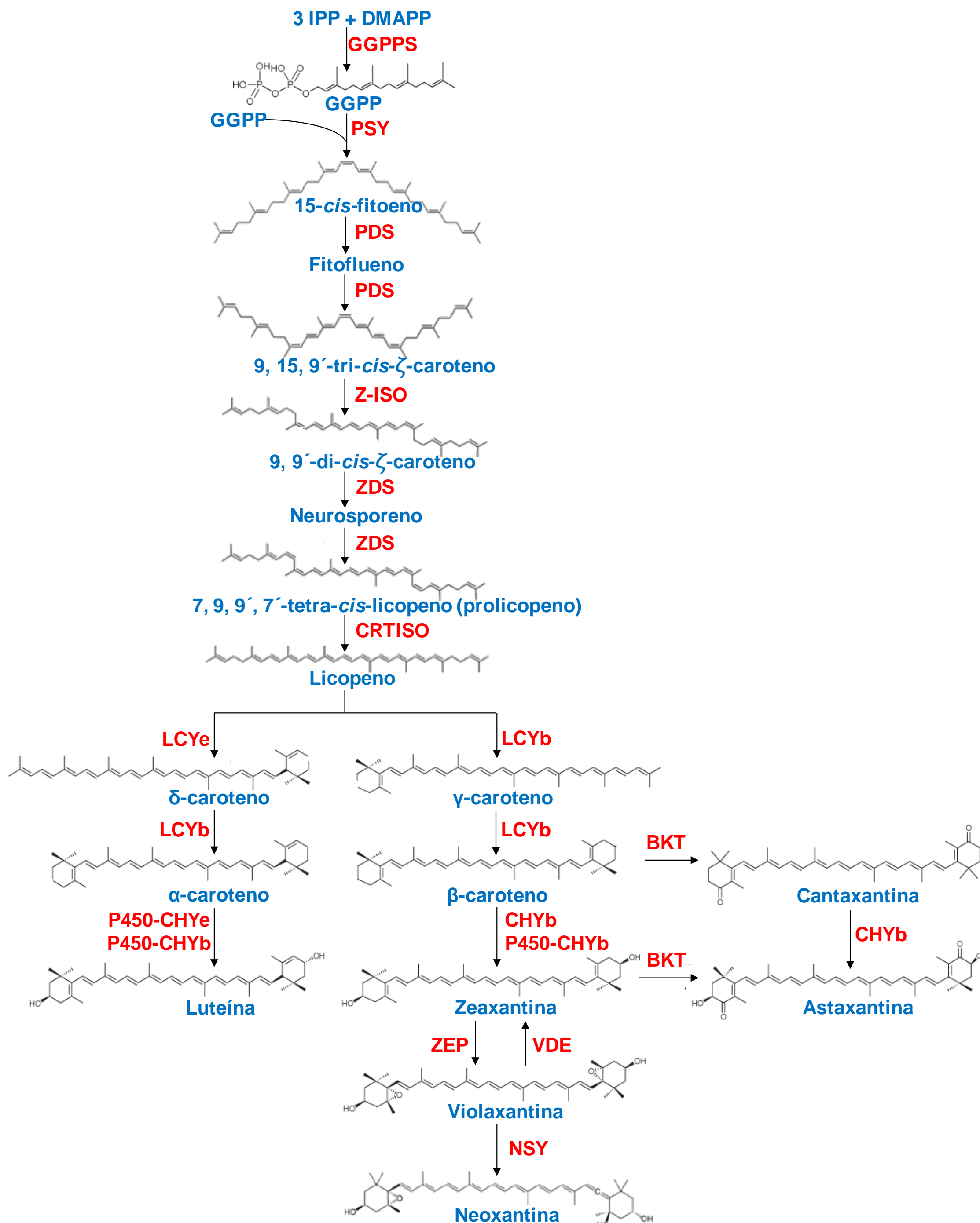

Figura 3. Ruta de biosíntesis de carotenoides en plantas y microalgas.

Las revisiones de Sandmann (1994), Armstrong (1997) o Cunningham and Gantt (1998), y las más recientes de Botella-Pavía and Rodríguez-Concepción (2006), Sandmann et al. (2006), Farré et al. (2010) y Takaichi (2011), ofrecen una buena visión general de la ruta

carotenogénica en plantas y microorganismos. La ruta carotenogénica se ha estudiado principalmente en plantas y cianobacterias entre los fotótrofos oxigénicos. En algas solamente unos cuantos enzimas de la ruta se han confirmado funcionalmente y principalmente en clorofíceas.

Tabla 1. Principales genes y enzimas de la ruta biosintética de carotenoides en plantas, cianobacterias y bacterias.

Enzima	Planta	Cianobacteria	Bacteria	Función
Biosíntesis de Carotenoides Acíclicos				
Fitoeno sintasa	*psy*	*crtB*	*crtB*	Condensación de dos GGPP
Fitoeno desaturasa	*pds*	*crtP*	*crtI**	Desaturación del fitoeno
15-*cis*-ζ-caroteno isomerasa	*ziso*	*ziso*	-	Isomerización de 15-*cis*-ζ-caroteno
ζ-caroteno desaturasa	*zds*	*crtQ*	*crtI**	Desaturación del ζ-caroteno
Caroteno isomerasa	*crtIso*	*crtH*	-	Isomerización del pro-licopeno
Ciclación de Carotenoides				
Licopeno β-ciclasa	*lcyB*	*crtLb*	*crtY*	Introducción de anillos β
Licopeno ε-ciclasa	*lcyE*	*crtLe*	-	Introducción de anillos ε
Biosíntesis de Xantofilas				
Caroteno β-hidroxilasa	*P450chyB* *chyB*	*crtR*	*crtZ*	Hidroxilación de anillos β
Caroteno ε-hidroxilasa	*P450chyE*	-	-	Hidroxilación de anillos ε
Zeaxantina epoxidasa	*zep*	-	-	Epoxidación de anillos β
Violaxantina de-epoxidasa	*vde*	-	-	De-epoxidación de anillos β
Neoxantina sintasa	*nsy*	-	-	Conversión de violaxantina a neoxantina
β-caroteno (C4) oxigenasa/cetolasa	*bkt*	*crtO/crtW*	*crtW/crtO*	Introducción de un grupo ceto en posición C4 de anillos β

* La misma enzima realiza las cuatro desaturaciones.

En las siguientes secciones se analizan en detalle tres enzimas de la ruta que son especialmente relevantes en el desarrollo de este Trabajo.

1.1.3. Fitoeno Sintasa

El primer paso específico en la biosíntesis de carotenoides es la condensación de dos moléculas de GGPP para producir fitoeno (Chamovitz et al., 1992). Esta reacción está catalizada por la fitoeno sintasa (PSY), una proteína monomérica muy conservada, y tiene lugar en dos pasos. En el primer paso, la PSY cataliza la formación de un enlace entre el carbono 1 de una molécula de GGPP y el doble enlace entre los carbonos 2 y 3 de la segunda molécula de GGPP, produciendo una molécula de ciclopropilcarbonil difosfato, denominada también prefitoeno difosfato (Figura 4). En el segundo paso, el prefitoeno pirofosfato se convierte en fitoeno tras la eliminación del pirofosfato. En plantas, algas y algunos hongos el

fitoeno aparece como 15-*cis*-fitoeno, con un doble enlace entre las dos moléculas de GGPP originales, mientras que algunas bacterias producen mezclas de isómeros que pueden incluir todo-*trans* y 9-*cis*-fitoeno. En el segundo paso, se elimina el pirofosfato y el prefitoeno pirofosfato se convierte en 15-*cis*-fitoeno con un doble enlace *cis* entre las dos moléculas de GGPP originales (Misawa et al., 1990, 1995).

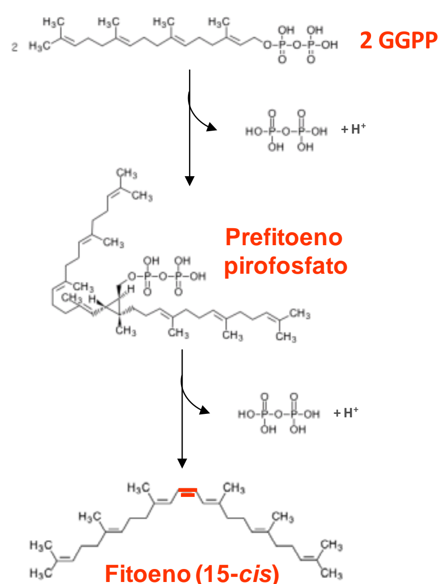

Figura 4. Síntesis de fitoeno a partir de dos moléculas de GGPP.

Aunque se conoce bastante poco sobre los mecanismos reguladores que controlan la biosíntesis de carotenoides, hay abundantes evidencias que indican que la reacción catalizada por la PSY es un importante punto de control que limita la velocidad de la ruta y determina el flujo de carbono hacia la biosíntesis de carotenoides (Cunningham and Gantt 1998; Shewmaker et al., 1999). Por ese motivo, la PSY ha sido objeto de manipulación genética en muchas plantas de interés agronómico, para incrementar la biosíntesis de carotenoides (Fraser et al., 2002; Zang et al., 2009). La sobreexpresión del gen *psy* en plantas de tomate resultó en la formación de plantas enanas debido a que la producción elevada de fitoeno causó una reducción en la síntesis de giberelinas (Fray et al., 1995). En plantas y algunas clorofíceas como *Dunaliella*, *Haematococcus* y *Chlamydomonas*, se ha demostrado que la expresión del gen *psy* está regulada positivamente por la luz (Bartley et al., 1999; Coesel et al., 2008; Steinbrenner and Linden, 2001; Vidhyavathi et al., 2008).

1.1.4. Licopeno Ciclasas

La ruta biosintética de carotenoides se divide en dos ramas divergentes a nivel del licopeno en plantas, algunas clases de algas, como las algas verdes, y ciertas cianobacterias. En una rama, la LCYb introduce un anillo β en ambos extremos de la molécula lineal de licopeno para formar β-caroteno, que es transformado en zeaxantina, violaxantina y, sólo en algunas microalgas verdes, en astaxantina. En la otra rama, la LCYe introduce un anillo ε en un extremo del licopeno para formar δ-caroteno, que es transformado por la LCYb en α-caroteno, que es posteriormente hidroxilado a luteína. La ciclación del licopeno a α-caroteno (con un anillo ε y un anillo β) o β-caroteno (con dos anillos β) representa, por tanto, un importante punto de ramificación en la biosíntesis de carotenoides y se ha propuesto como un punto de regulación; las actividades relativas de LCYb y LCYe determinan la proporción de carotenoides dirigidos hacia cada rama de la ruta.

El mecanismo propuesto para la formación de los anillos β y ε a partir del grupo final lineal ψ del licopeno se muestra en la Figura 5. Los anillos β y ε se diferencian únicamente en la posición del doble enlace carbono-carbono dentro del anillo de ciclohexano, y se ha sugerido que su formación transcurre a través de un ion carbonio intermediario común. Dependiendo de la elección del protón eliminado del ion carbonio se formará un anillo β o un anillo ε. Por tanto, las reacciones catalizadas por LCYb y LCYe son similares. Cada enzima utiliza un grupo final ψ como sustrato, los productos difieren únicamente en la posición del doble enlace carbono-carbono del anillo de seis átomos de carbono, y los mecanismos de reacción propuestos son esencialmente iguales.

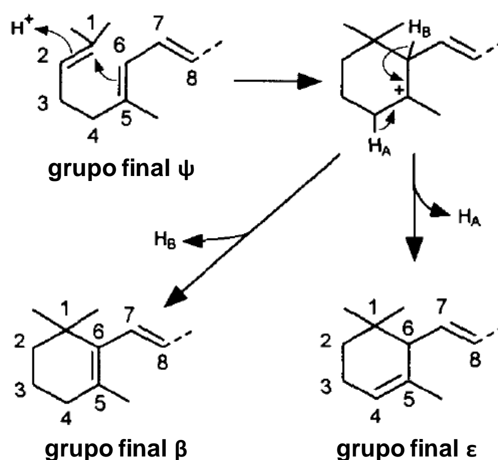

Figura 5. Formación de los anillos β y ε a partir del grupo final lineal ψ. Los grupos finales ψ del licopeno se convierten en los anillos β ó ε presentes en muchos carotenoides. Se ha propuesto que estas reacciones de ciclación ocurren a través de un ion carbonio intermediario común. Dependiendo del átomo de hidrógeno, H_A o H_B, que participe en la reacción se formará un anillo ε o un anillo β (Britton, 1988; Hornero-Mendez and Britton, 2002).

Todos los organismos fotosintéticos oxigénicos sintetizan carotenoides que contienen dos anillos β; sin embargo, carotenoides que contienen un anillo β y un anillo ε como el α-caroteno y la luteína son sintetizados únicamente por plantas, algunas clases de algas como las rodofitas (tipo macrofitas), criptofitas, euglenofitas, cloraracniofitas y clorofitas (Takaichi, 2011), y por cianobacterias de los géneros *Prochlorococcus* y *Acaryochloris* (Stickforth et al., 2003). Carotenoides con dos anillos ε se han detectado rara vez y a muy baja concentración en plantas y algas.

Cuatro familias de licopeno ciclasas se han identificado en los organismos carotenogénicos. Una familia contiene las monoméricas licopeno β- y ε-ciclasas de plantas y algunas clases de algas (LCYb y LCYe) y cianobacterias (CRTLb y CRTLe). La segunda familia incluye la monomérica licopeno β-ciclasa de bacterias (CRTY). Recientemente, se ha identificado otra familia de licopeno β-ciclasas, CRUA y CRUP, en bacterias sulfuradas verdes y algunas cianobacterias. Genes ortólogos a *cruP* también se han encontrado en genomas de plantas. La cuarta familia de licopeno ciclasas incluye las heterodiméricas licopeno β-ciclasas formadas por dos pequeños polipéptidos (CRTYc y CRTYd) de algunas bacterias, que están relacionadas con la licopeno ciclasa bifuncional de hongos (CRTYB) y que presenta tanto actividad licopeno β-ciclasa como PSY. Estas familias están poco relacionadas entre sí y comparten solamente unos pocos motivos conservados, incluyendo un motivo de unión a dinucleótidos, que está presente en los tres primeros grupos, pero que parece que se ha perdido en el cuarto. Este motivo se define por una configuración característica de hoja β-hélice α-lazo- hoja β. Polipéptidos con este motivo unen dinucleótidos tales como NADP, NAD, ADP y FAD. Para las ciclasas esta secuencia se parece a la encontrada en el extremo amino terminal de enzimas que unen FAD. No se conoce si las licopeno ciclasas unen FAD (o NAD, NADP o ADP) ni tampoco el papel de estos cofactores en la reacción catalizada por estas enzimas. No hay oxidación ni reducción (las fórmulas moleculares de los productos son las mismas que la de los sustratos) y el mecanismo de reacción propuesto para la reacción de ciclación no requiere la presencia de un cofactor rédox. Se ha sugerido que el FAD podría estabilizar el ion carbonio intermediario o ser simplemente una reliquia del enzima ancestral del que las ciclasas evolucionaron. Hay otras enzimas que unen FAD y catalizan reacciones sin oxidación o reducción neta (Cunningham et al., 1996).

Las licopeno β-ciclasas son en su mayoría bicíclicas, mientras que las licopeno ε-ciclasas son mayoritariamente monocíclicas. Dos de los pocos ejemplos de licopeno ε-ciclasas que muestran actividad biciclasa son una LCYe de *Lactuca sativa* que introduce dos anillos ε en el licopeno para formar ε-caroteno que posteriormente se hidroxila para formar lactucaxantina (Cunningham and Gantt, 2001), y una CRTLe de *Prochlorococcus marinus* MED4 que introduce anillos ε y β al licopeno para producir α-, β- y ε-caroteno (Stickforth et al., 2003). En estas dos ciclasas se ha identificado en una región próxima al extremo carboxilo terminal un aminoácido básico histidina o lisina en lugar del aminoácido leucina presente en todas las licopeno ε-ciclasas monocíciclas estudiadas (Cunningham and Gantt, 2001; Stickforth et al., 2003).

1.2. Aplicaciones comerciales de los carotenoides

Los carotenoides presentan numerosas aplicaciones en el campo de la industria alimentaria y de piensos, así como en nutracéutica y cosmética. Así, el β-caroteno se utiliza como colorante y antioxidante en muchos alimentos humanos, como las margarinas o mayonesas, así como en las industrias cosmética y farmacéutica. Algunos cetocarotenoides como astaxantina y cantaxantina se usan como aditivos alimentarios esenciales para la adecuada pigmentación de salmones, truchas o mariscos en acuicultura (Guerin et al., 2003; Lorenz and Cysewski, 2000). Estos animales adquieren su característico color rosado cuando crecen en libertad gracias a su alimentación a base de fitoplancton (microalgas y bacterias marinas) rico en estos compuestos. A pesar de que en acuicultura la astaxantina se utiliza principalmente como fuente de pigmentación, aporta también otra serie de beneficios, constituyendo un nutriente necesario para el adecuado crecimiento y reproducción de estas especies de peces y crustáceos con alto valor comercial (Becker 2004; Higuera-Ciapara et al., 2006). El mercado mundial de la astaxantina se ha evaluado recientemente en torno a 234 millones de dólares USA al año, con un precio aproximado de 2500 dólares USA por kg, principalmente como aditivo alimentario en acuicultura. Aunque, el mercado actual de este carotenoide está dominado por la forma sintética del pigmento producido por BASF y Hoffman-La Roche. Las xantofilas, luteína y zeaxantina, se utilizan como aditivos en avicultura para incrementar la pigmentación de la yema de huevo, así como de la piel y tejido muscular de aves de corral (Botella-Pavía and Rodríguez-Concepción, 2006). Se ha comprobado que la luteína no solo proporciona pigmentación, sino que también mejora la salud y fertilidad de las aves (Cysewski and Lorenz, 2004). La luteína también se usa, aunque en menor medida, como colorante en alimentos humanos y en las industrias cosmética y farmacéutica (Johnson and Schroeder, 1995).

La capacidad antioxidante de los carotenoides es actualmente objeto de gran interés debido a sus aplicaciones en la salud humana y nutrición. El estrés oxidativo parece ser la base de distintas enfermedades como la degeneración macular de la retina asociada a la edad, otras retinopatías como cataratas, carcinogénesis, aterosclerosis o enfermedad de Alzheimer, entre otras (Maher, 2000; Mayne, 1996; Bernstein et al., 2002; Zhao et al., 2003). Antioxidantes, tales como las vitaminas A, C y D, así como los carotenoides, se están investigando para determinar sus funciones en la prevención y/o tratamiento de estas enfermedades. La ingesta de carotenoides ha demostrado ofrecer protección frente a la degeneración macular, los daños inducidos en la piel por la luz UV y algunas enfermedades degenerativas asociadas a la edad avanzada (Guerin et al., 2003). Diversos estudios sugieren que las propiedades antioxidantes y otras funciones biológicas inesperadas de los carotenoides, relacionadas con la regulación génica y la comunicación intercelular, pueden proporcionar beneficios adicionales para la salud, como actividad anticancerígena, estimuladora del sistema inmunitario o antiinflamatoria (Demming-Adams, 2002; Stahl and Sies, 2005).

La degeneración macular asociada a la edad (AMD) es producida por la degeneración de la retina y mácula, y finalmente culmina en la pérdida de la visión. Esta enfermedad afecta

mundialmente a más de 14 millones de personas, y se ha establecido una clara conexión entre AMD y los carotenoides luteína y zeaxantina. Estas xantofilas se encuentran a altas concentraciones en el material ocular, incluyendo la región mácula de la retina y sus niveles se han relacionado directamente con la enfermedad y su prevención (Landrum et al., 1999). La luteína y zeaxantina funcionan como antioxidantes y como pigmentos que absorben luz, y puede proteger, por tanto, el ojo de daños tanto oxidativos como inducidos por la luz azul de alta energía, ya que la luteína y zeaxantina absorben predominantemente luz azul de baja longitud de onda. Se ha descrito que especies reactivas de oxígeno inducen apoptosis de fotorreceptores y la luteína es capaz de bloquear la apoptosis inducida por H_2O_2 o paraquat de células fotorreceptores de la retina cultivadas (Chucair et al., 2007; Kijlstra et al., 2012). Recientes evidencias indican que la AMD tiene características de un estado crónico de inflamación de baja intensidad (Kijlstra et al., 2012). Estudios con otros antioxidantes como las vitamina A, E y C han revelado que no existe una relación entre su consumo y la reducción de AMD. La luteína también presenta efectos beneficiosos sobre otras enfermedades oculares, incluyendo cataratas (Mares-Perlman et al., 2002).

Estudios *in vitro* con células humanas e *in vivo* en ratas y pollos han revelado que la luteína y astaxantina inhiben procesos inflamatorios bloqueando la expresión de genes pro-inflamatorios, como consecuencia de la supresión del factor nuclear NF-kB. También inhiben la producción de óxido nítrico y prostaglandina E2, así como las citoquinas pro-inflamatorias TNF-alfa (factor de necrosis tumoral alfa), y la interleuquina-1B e interleuquina-6, quimioquina (motivo C-C) ligando 2 (CCL2) y quimioquina (motivo C-X-C) ligando 2 (CXCL2) (Hussein et al., 2006; Kijlstra et al., 2012). El efecto inhibidor de la luteína y astaxantina sobre los efectos pro-inflamatorios mediados por NF-kB podría estar relacionado con la capacidad de estos carotenoides de neutralizar especies reactivas de oxígeno que se piensa que activan la ruta NF-kB. Diversos estudios han mostrado que la inclusión de ciertos carotenoides como la luteína o la astaxantina en la dieta de ratas, reduce la incidencia de carcinogénesis inducida en vejiga, cavidad oral o colon, entre otros. Este efecto podría estar relacionado con la capacidad de neutralizar especies reactivas, presuntos protagonistas de los procesos de iniciación y propagación de diversas formas de cáncer. Se ha observado también que la actividad anticancerígena de estos carotenoides está relacionada con su papel inductor en la comunicación celular en las uniones gap, necesaria para la modulación del crecimiento celular, así como con la estimulación del sistema inmune (Guerin et al., 2003). También se ha observado que la luteína inhibe la liberación de ácido araquidónico de una línea celular de macrófagos de ratón y este efecto se ha atribuido a una inhibición de la fosfolipasa A_2 citosólica (PLA_2) por la luteína (Song et al., 2010; Kijlstra et al., 2012). La activación de PLA_2 es importante en la generación de mediadores inflamatorios tales como prostaglandinas, tromboxanos y leucotrienos. Por otro lado, se ha descrito que la luteína impide la progresión de la aterosclerosis (Dwyer et al., 2001) y se ha propuesto para proteger la piel de los daños inducidos por la luz UV (Alves-Rodrigues and Shao, 2004).

El uso de la luteína en los sectores nutracéutico/farmacéutico para la prevención de AMD y otras retinopatías como cataratas, está actualmente muy extendido, resultando en un negocio en auge que se considera que representa un mercado de unos mil millones de dólares USA al año (Kijlstra et al., 2012). En la actualidad, la principal fuente comercial de luteína es Marigold (*Tagetes erecta* y *Tagetes patula*). Sin embargo, el contenido en luteína de los pétalos de Marigold es muy bajo (0,3 mg g^{-1} peso seco) y, por tanto, existe un interés creciente en las microalgas como fuente alternativa de este carotenoide. Algunas microalgas clorofíceas como *Muriellopsis* sp. (Del Campo et al., 2000, 2001), *Chlorella zofingiensis* (Del Campo et al., 2004), *Cocomyxa acidophyla* (Casal et al. 2011), *Scenedesmus almeriensis* (Sánchez et al., 2008), y *Chlorella protothecoides* (Shi et al., 2002) se han propuesto como fuentes alternativas de luteína para la Industria.

2. PRODUCCIÓN DE CAROTENOIDES POR MICROALGAS

En la actualidad, el mercado mundial de la mayoría de los carotenoides corresponde a los obtenidos mediante síntesis química. Sin embargo, la creciente demanda de aditivos naturales, la observación de que los isómeros naturales parecen ser más activos que los sintetizados químicamente, y una legislación cada vez más restrictiva sobre el uso de colorantes artificiales, hacen muy atractiva su producción a partir de fuentes naturales. Las microalgas son una de las principales fuentes naturales de carotenoides, sin embargo su producción a escala industrial no está exenta de problemas y su productividad no es siempre tan alta como sería deseable. La excasez de conocimiento de la ruta de la carotenogénesis de microalgas y de los mecanismos y señales que la controlan es uno de los principales obstáculos para obtener mayores productividades y para manipular genéticamente la ruta con garantías de obtener transformantes con las características deseadas. Además, el número de carotenoides producidos con fines comerciales y de especies cultivadas para ello a gran escala es muy limitado. Esto hace que sea esencial la búsqueda de especies más productivas, bien nuevas especies aisladas del medio natural, o bien obteniendo mutantes super-productores mediante Mutagénesis al Azar o Ingeniería Genética.

La comercialización de carotenoides a partir de microalgas se halla en la actualidad restringida a β-caroteno a partir de *Dunaliella* (Spolaore et al., 2006), y astaxantina a partir de *Haematococcus pluvialis* (Cyanotech, Mera Pharmaceuticals), pero con importantes limitaciones en esta última (Lee and Zhang, 1999). La potencialidad de otros carotenoides, tales como luteína que ha demostrado tener un papel fundamental en la prevención de la degeneración macular, así como de otras especies para la producción de astaxantina (Orosa et al., 2001; Del Campo et al., 2004) están siendo objeto de intenso estudio en la actualidad por diversos grupos. Aunque *H. pluvialis* presenta el contenido más alto en astaxantina descrito hasta la fecha (hasta un 4 % del peso seco) (Boussiba, 2000), muestra un crecimiento lento y la densidad celular que se alcanza en los cultivos es baja. Además, la síntesis de astaxantina

conlleva el enquistamiento de las células y, por tanto, la parada del crecimiento. Esto hace que las productividades no sean muy altas y obliga a que la producción industrial de astaxantina tenga que realizarse en dos fases, limitando su aplicación a gran escala. Nuestro grupo ha seleccionado otra microalga clorofita, *Chlorella zofingiensis* SAG 211/14, que también acumula altas cantidades de astaxantina además de luteína (hasta 20 mg L^{-1} cultivo para ambos pigmentos) y que presenta una mayor velocidad de crecimiento que *H. pluvialis*, además de alcanzar densidades celulares muy altas en los cultivos (hasta $1,3 \cdot 10^{11}$ células L^{-1} o 7 g peso seco L^{-1}) cuando se cultiva fotoautotróficamente (Del Campo et al., 2000, 2004; Orosa et al., 2001). Por otra parte, *C. zofingiensis* muestra altas productividades de astaxantina (hasta 32,4 mg L^{-1}) en cultivos heterotróficos con glucosa (Sun et al., 2008) y la acumulación de astaxantina en esta microalga no va asociada al enquistamiento de las células, por lo que su cultivo resultaría más fácil desde el punto de vista industrial. Estas características indican que esta microalga podría ser utilizada para la producción comercial de astaxantina y/o luteína a gran escala.

Además de su capacidad de producción de astaxantina, *C. zofingiensis* representa un organismo modelo excepcional para estudiar la regulación de la ruta biosintética de carotenoides, ya que produce tanto el carotenoide primario luteína, como el carotenoide secundario astaxantina. Sin embargo, no existe aún un método de transformación establecido para esta microalga y además es muy poco lo que se conoce de su ruta carotenogénica. Solamente se han aislado y caracterizado los genes carotenogénicos *bkt*, *pds* y *chyB* en esta microalga (Huang et al., 2006, 2008; Li et al., 2008a; Sun et al. 2008). Con respecto a su regulación, altas irradiancias aumentan los transcritos de *pds*, *chyB* y *bkt*, mientras que el NaCl solamente aumenta los transcritos de *bkt* (Huang et al., 2008; Li et al., 2008b). La expresión del gen *bkt* se induce transitoriamente por glucosa en cultivos mixotróficos (Huang et al., 2006), y la glucosa, sacarosa y manosa inducen la expresión de *pds*, *chyB* y *bkt* en células cultivadas en oscuridad (Li et al., 2008a; Sun et al., 2008). Un mayor conocimiento de la ruta carotenogénica y su regulación en *C. zofingiensis* permitirá encontrar los puntos regulatorios o limitantes de la ruta e incrementar la acumulación y productividad de carotenoides de interés comercial en este organismo.

Como se ha mencionado anteriormente otras microalgas clorofíceas como *Muriellopsis* sp y *Scenedesmus almeriensis* se han propuesto como fuentes potenciales para la producción de luteína. No obstante, los valores de luteína descritos y las productividades obtenidas no son lo suficientemente altas para ser rentables económicamente a escala industrial. Existe, por tanto, gran interés en mejorar la acumulación y productividad de luteína mediante la selección de las especies adecuadas, la optimización de las condiciones de cultivo y la obtención de mutantes super-productores de luteína. Especies de microalgas con mayores velocidades de crecimiento y acumulación de carotenoides hacen el proceso comercial de producción de luteína más viable.

2.1. Aislamiento de mutantes super-productores de carotenoides mediante Mutagénesis al Azar

A pesar de que la manipulación genética mediante Ingeniería Genética es una metodología necesaria para el estudio de la ruta biosintética de carotenoides, así como para el desarrollo de estirpes altamente productivas para usos biotecnológicos, en la actualidad aún existe cierto rechazo social y reticencia por parte de las empresas hacia los productos procedentes de organismos modificados genéticamente, por lo que es necesario investigar en mutagénesis al azar (genética clásica), especialmente en el caso de los organismos en los que aún no se han establecido métodos de transformación.

La mutagénesis al azar ha sido una técnica ampliamente utilizada para la mejora de estirpes, así como para el estudio de los mecanismos de procesos metabólicos (Doan and Obbard, 2012). La introducción de una mutación en un cierto gen carotenogénico mediante el aislamiento de mutantes resistentes a un inhibidor específico de la ruta biosintética de carotenoides ha permitido obtener mutantes de ciertas microalgas que muestran altos contenidos en carotenoides. La mayoría de los estudios encaminados a la obtención de microalgas mutantes super-productoras de xantofilas de interés comercial mediante mutagénesis clásica o al azar se han llevado a cabo con *H. pluvialis*. En estos trabajos se han utilizado como agentes mutagénicos etil metanosulfonato (EMS) o luz UV y como criterio de selección la resistencia a los inhibidores de la biosíntesis de carotenoides norflurazón, fluridona, nicotina, difenilamina y compactina. Sin embargo, los mutantes resistentes obtenidos, bien no presentaron un contenido en astaxantina significativamente mayor que el silvestre (Tjahjono et al., 1994; Tripathi et al., 2001) o aunque mostraron un incremento en el contenido en astaxantina del orden de 1,4 a 2,1 veces respecto al tipo silvestre, la velocidad de crecimiento y la densidad celular alcanzada fueron menores en los mutantes que en el silvestre. En consecuencia, el contenido volumétrico de carotenoides en los cultivos no era significativamente superior que en los mutantes (Chen et al., 2003; Chumpolkulwong et al., 1997). Por otro lado, se ha aislado un mutante del alga verde *Dunaliella salina*, usando EMS como agente mutagénico y como criterio de selección el color de las colonias distinto al amarillo. Este mutante era incapaz de sintetizar neoxantina, anteraxantina y violaxantina, acumulando zeaxantina constitutivamente a expensas de aquellos pigmentos, por lo que el total de carotenoides era prácticamente igual que en el silvestre (Jin et al., 2002). Estos resultados se deben posiblemente a que el criterio de selección de los mutantes se basaba únicamente en el contenido en pigmentos sin tener en cuenta la velocidad de crecimiento.

2.2. Obtención de transformantes super-productores de carotenoides mediante Ingeniería Genética

Las propiedades nutricionales y terapéuticas de los carotenoides han estimulado a muchos investigadores a intentar aumentar el valor nutricional de varias plantas de interés agronómico mediante la manipulación genética de la ruta carotenogénica. Hay muchos ejemplos de plantas transgénicas que expresan genes carotenogénicos exógenos (Botella-Pavía and Rodríguez Concepción, 2006; Giuliano et al., 2000; Sandmann, 2001, 2006). La sobreexpresión de los genes fitoeno sintasa de bacterias o plantas en plantas ha resultado en un incremento significativo en los niveles de carotenoides totales en tomate y Hongkong kumquat (*Fortunella hindrii*) (Fraser et al., 2002; Fray et al., 1995; Zhang et al., 2009), semillas de *Canola* y *Arabidopsis* (Lindgren et al., 2003; Shewmaker et al., 1999), endospermo de arroz (Paine et al., 2005), tubérculos de patata (Ducreux et al., 2005) y zanahoria (Baranski, 2008). La producción *in vivo* de astaxantina y otros carotenoides por plantas que no los sintetizan de forma natural se ha conseguido mediante Ingeniería Genética en varias especies (Gerjets and Sandmann, 2006; Ralley et al., 2004; Stalberg et al., 2003; Zhu et al., 2009). No obstante, la falta de información sobre muchos de los aspectos de la regulación de la carotenogénesis limita su manipulación genética y ocasiona problemas de baja productividad y efectos colaterales no deseados. Por ejemplo, en el caso de plantas de tomate transgénico que sobreexpresan el gen *psy* acumulan carotenoides en numerosos tejidos de la planta, pero la alteración de la ruta desequilibró la producción de giberelinas y fitol, que también se sintetizan por la ruta común de los terpenoides a partir de GGPP, provocando la producción de plantas enanas y con reducidos contenidos en clorofila (Fray et al., 1995). Varios autores han conseguido evitar estos problemas usando genes específicos de ciertos tejidos y evitando la expresión del gen exógeno en las partes vegetativas (Fraser et al., 2002; Lindgren et al., 2003). No obstante en la mayoría de los casos el rendimiento de la producción de carotenoides en organismos transgénicos es bajo debido a que la viabilidad de los transformantes obtenidos puede ser limitada por el desequilibrio inducido indirectamente en otras vías metabólicas relacionadas, por el déficit en el aporte de precursores, o la falta de estructuras adecuadas para el almacenamiento de los productos (Britton et al., 1998; Sandmann, 2001). Es por ello que un conocimiento más profundo de los procesos bioquímicos y fisiológicos y su regulación a nivel genético resulta altamente deseable (Britton et al., 1998; Sandmann, 2001; Vermaas, 1996).

Hasta ahora sólo se ha conseguido la transformación nuclear estable en tres grupos de microalgas eucariotas: clorofitas, diatomeas y dinoflageladas. Uno de los pasos más importantes en cada método diseñado para la transformación nuclear en microalgas es el cambio de permeabilidad en la membrana celular que permita a las moléculas de DNA entrar en las células sin pérdida de viabilidad celular. Hay varios métodos para las diferentes especies. Así, *Chlamydomonas* se ha transformado con varios métodos tales como el método de perlas de vidrio en la presencia de polietilenglicol (PEG) (Kindle et al., 1990), electroporación (Shimogawara et al., 1998), bombardeo de partículas (Debuchy et al., 1989) y

usando *Agrobacterium* (Kumar et al., 2004). Existe un interés creciente en la transformación de diferentes microalgas, especialmente aquellas con aplicaciones comerciales. La microalga eucariota modificada genéticamente y mejor estudiada es la clorofita *Chlamydomonas reinhardtii* (León et al., 2004, 2007a).

En microalgas, solamente unos pocos trabajos describen la manipulación genética de la ruta carotenógénica. Algunos ejemplos son: el silenciamiento, mediante RNA de interferencia, del gen *pds* en *Dunaliella* (Sun et al., 2007) y *Chlamydomonas* (Vila et al., 2007); el silenciamiento del gen *psy* de *Chlamydomonas* por microRNAs (Molnar et al., 2009); la transformación de *H. pluvialis* con un gen *pds* modificado (Steinbrenner and Sandmann 2006); y la producción de un nuevo cetocarotenoide en *Chlamydomonas* por la expresión del gen *bkt* de *Haematococcus* (León et al. 2007b).

Las microalgas no sólo constituyen un excelente modelo para el estudio de la carotenogénesis en plantas, sino que pueden ser hospedadores ideales para la expresión de genes de la ruta carotenogénica de otras especies, tanto con fines de investigación básica, como para la obtención de transgénicos con mayores productividades de carotenoides que las estirpes naturales. Las microalgas, además de presentar más analogías fisiológicas y morfológicas con las células de plantas que las bacterias, tienen casi las mismas ventajas que estas últimas en cuanto a facilidad y rapidez de crecimiento. Muchas tienen un activo metabolismo de los terpenoides, lo que garantiza el suficiente aporte de precursores para la síntesis de carotenoides y poseen alta capacidad de almacenamiento de carotenoides en cloroplastos y plastidios. Estos dos factores pueden limitar la productividad de carotenoides en bacterias y levaduras. Además, en algas eucarióticas es mucho más simple seguir la expresión de las enzimas de la síntesis de carotenoides que en plantas, en las que los carotenoides se acumulan en tejidos u órganos especializados. Muchas especies de microalgas están consideradas como organismos GRAS (Generally Regarded as Safe), lo que es útil para la producción de carotenoides o microalgas enriquecidas en ellos para aplicaciones alimentarias, farmacéuticas o cosméticas. Sin embargo, a diferencia de lo que ocurre en procariotas, en el genoma nuclear de microalgas y otros eucariotas, el DNA foráneo se integra preferentemente al azar por recombinación no homóloga. Esto ha dificultado la expresión de genes heterólogos y los estudios metabólicos por aproximaciones de genética reversa. Aunque hay algunos resultados positivos, la transformación genética de *Haematococcus* y *Dunaliella* no está aún bien establecida, sobre todo en lo referente a la estabilidad de los transformantes (Geng et al., 2003; Lu et al., 2005; Steinbrenner and Sandmann, 2006; Sun et al., 2005; Tan et al., 2005; Teng et al. 2002). La elección de genes de resistencia para la selección de transformantes es también complicada. En el caso de *Dunaliella* la salinidad del medio hace precipitar los antibióticos y en *Haematococcus* las células se enquistan ante la presencia de la mayoría de los antibióticos y herbicidas, enmascarando su efecto letal.

Chlamydomonas reinhardtii es la primera y mejor estudiada de las clorofitas transformadas (Harris, 2001); crece a altas tasas de crecimiento en condiciones fotoautotróficas, heterotróficas y mixotróficas; su manipulación genética nuclear es fácil y está

bien establecida. Se han diseñado una gran variedad de métodos de transformación y construcciones para esta microalga (León et al., 2007a; Lumbreras et al., 1998), habiéndose establecido para esta especie herramientas moleculares y genómicas muy interesantes, como un microarray con cerca de 10000 elementos que cubren alrededor del 87% de su transcriptoma (Eberhard et al., 2006), una base de datos de EST (http://www.chlamy.org) y su genoma se ha secuenciado completamente (http://www.genome.jqc.psf.org/Chlre3/Chlre3.home.html) (Grossman et al., 2003; Harris, 2001), lo que la convierten en un organismo modelo para la Ingeniería Genética de microalgas. Y aunque no es buena productora de ningún carotenoide de interés comercial, posee gran valor para expresar genes carotenogénicos foráneos que codifican carotenoides de interés aplicado y para llevar a cabo estudios metabólicos y regulatorios de la ruta (León et al., 2004).

Objetivos

Las microalgas son fuente natural de un buen número de carotenoides de interés comercial. Sin embargo, su producción a nivel industrial está limitada principalmente por el desconocimiento de su ruta biosintética y de su regulación, así como por los bajos niveles de acumulación de dichos pigmentos en la célula y bajas productividades de los cultivos. El propósito de esta Tesis Doctoral es obtener un mejor conocimiento de la ruta carotenogénica en microalgas y de su regulación, y conseguir especies más productivas mediante la selección de estirpes más adecuadas, la optimización de las condiciones de cultivo, y la obtención de estirpes modificadas genéticamente por Ingeniería Genética o mutagénesis al azar. Dentro de este objetivo global se pueden establecer los siguientes objetivos concretos:

1) Llevar a cabo una selección de microalgas para la producción de luteína.

2) Incrementar la producción de luteína en la(s) microalga(s) seleccionada(s) mediante la optimización de las condiciones de cultivo y mutagénesis al azar.

3) Aislar y caracterizar los genes de la fitoeno sintasa, licopeno β-ciclasa y licopeno ε-ciclasa de la ruta biosintética de carotenoides en *Chlorella zofingiensis.*

4) Obtener transformantes de *Chlamydomonas reinhardtii* con alto contenido en carotenoides mediante la sobreexpresión del gen que codifica una de las enzimas limitantes de la ruta, la fitoeno sintasa, obtenido a partir de *Chlorella zofingiensis*.

5) Estudiar el efecto de la luz y del nitrógeno sobre el nivel de expresión de algunos genes de la ruta de biosíntesis de carotenoides en *Chlorella zofingiensis* y su correlación con los niveles intracelulares de carotenoides.

Resumen Global de los Resultados

Las microalgas son una de las principales fuentes naturales de carotenoides de interés comercial, sin embargo su productividad no es siempre tan alta como se requeriría para que sea económicamente rentable. Para superar esta limitación es esencial seleccionar las estirpes más adecuadas para la producción, identificar las condiciones que conducen a las máximas productividades, obtener mutantes super-productores mediante mutagénesis clásica o Ingeniería Genética en las estirpes seleccionadas, y estudiar la ruta carotenogénica y su regulación.

En el primer capítulo de esta Tesis se ha realizado un estudio de 13 microalgas clorofíceas y se ha seleccionado *Chlorella sorokiniana* para la producción de luteína, ya que presentaba un alto contenido en este carotenoide (24 mg L^{-1}) y la más alta velocidad de crecimiento (0,12 h^{-1}). En esta microalga se ha estudiado el efecto de varios factores nutricionales y ambientales sobre el crecimiento y acumulación de luteína, con el fin de optimizar la producción de dicho carotenoide. Los resultados obtenidos indican que, en general, las condiciones óptimas para el crecimiento también conducen a la máxima productividad de luteína. La mayor velocidad específica de crecimiento y el máximo contenido en luteína (35,0 mg L^{-1} y 5,2 mg g^{-1} de peso seco) se alcanzaron a 690 μmoles de fotones m^{-2} s^{-1}, 28 ºC, 2 mM de NaCl, 40 mM de nitrato y bajo condiciones mixotróficas con 40 mM de acetato y 100 mM de glucosa. Por otro lado, se han obtenido mutantes de *C. sorokiniana* super-productores de luteína mediante mutagénesis al azar y selección de los mutantes por su resistencia a inhibidores de la ruta carotenogénica, además de por su alta velocidad de crecimiento y alto contenido en luteína. Así, el mutante MR-16 mostró un contenido volumétrico en luteína 2 veces mayor que el de la estirpe silvestre, alcanzándose valores de 42 mg L^{-1} y los mutantes DMR-5 y DMR-8 presentaron un contenido celular en luteína de 7 mg g^{-1} de peso seco. Estos valores son superiores a los descritos para otras microalgas productoras de luteína bajo condiciones fotoautotróficas a escala de laboratorio. Por tanto, *C. sorokiniana* es una excelente candidata para la producción de este pigmento de gran interés comercial.

En el segundo capítulo de esta Tesis se ha llevado a cabo el aislamiento y caracterización del gen de la fitoeno sintasa de *Chlorella zofingiensis* (*Czpsy*), implicada en el primer paso de la ruta biosintética de los carotenoides. La funcionalidad del gen *Czpsy* se ha comprobado por complementación genética heteróloga en *E. coli* y el análisis por Southern blot ha revelado que *C. zofingiensis* contiene una única copia del gen *Czpsy*. Este gen recién aislado codifica una proteína de 420 aminoácidos que contiene un dominio transmembrana de 20 aminoácidos y una secuencia señal para su exportación al cloroplasto, situada en el extremo amino terminal. Además, el análisis filogenético ha mostrado que la fitoeno sintasa de *C. zofingiensis* está muy relacionada evolutivamente con las fitoeno sintasas de plantas y microalgas. Por otro lado, el gen *Czpsy* fue insertado adecuadamente en un vector y expresado constitutivamente bajo el control de los promotores fuertes *rbsS2*, de la subunidad pequeña de la RuBisco, y *hsp70A*, de la proteína de choque térmico, en la microalga *Chlamydomonas reinhardtii*. Su sobreexpresión por tansformación nuclear permitió un incremento en los niveles de transcrito de la fitoeno sintasa y un incremento en el contenido de los carotenoides

violaxantina y luteína, que alcanzaron niveles 2,0 y 2,2 veces mayores, respectivamente que los de las células no transformadas. Además, no se observó fenotipo atípico en los transformantes y sus tasas de crecimiento eran similares a la de la estirpe silvestre. Estos resultados suponen uno de los pocos ejemplos descritos de manipulación genética estable de la ruta carotenogénica en microalgas y abren la posibilidad de aumentar la productividad de carotenoides de interés comercial por Ingeniería Genética.

En los capítulos tercero y cuarto de esta Tesis Doctoral se ha llevado a cabo el aislamiento y caracterización de los genes de la licopeno β-ciclasa (*CzlcyB*) y de la licopeno ε-ciclasa (*CzlcyE*) de *C. zofingiensis*, implicados en la formación de carotenoides con anillos β y ε. El análisis por Southern blot ha sugerido la presencia de una única copia de estos genes en el genoma de *C. zofingiensis*. El gen *CzlcyB* codifica una proteína de 546 aminoácidos que contiene dos dominios transmembrana de 21 aminoácidos cada uno en el extremo carboxilo terminal. El análisis funcional por complementación heteróloga en *E. coli* mostró que esta proteína cataliza la ciclación del licopeno y del δ-caroteno para producir β- y α-caroteno, respectivamente, al igual que las LCYb de plantas y microalgas estudiadas. El gen *CzlcyE* codifica una proteína de 654 aminoácidos que contiene cinco dominios transmembrana de 20 aminoácidos cada uno distribuidos uniformemente en la proteína y una secuencia señal para la exportación al cloroplasto, localizada en el extremo amino terminal. Mediante complementación genética en *E. coli* se ha comprobado la funcionalidad del gen *CzlcyE* y se ha determinado que la proteína codificada por dicho gen cataliza la conversión de licopeno en δ-caroteno, pero no la formación de α-caroteno a partir de δ-caroteno, presentando, por tanto, actividad monociclasa como la mayoría de las LCYe de plantas y la de la microalga verde *Auxenochlorella protothecoides*.

Por otro lado, se ha estudiado la regulación por luz y nitrógeno de la ruta carotenogénica en *C. zofingiensis*, determinando los niveles de mRNA de los genes *psy*, *lcyB*, *lcyE*, *pds*, *chyB* y *bkt*, así como los contenidos celulares en α-caroteno y su producto luteína, y en β-caroteno y sus derivados cantaxantina, astaxantina, zeaxantina y violaxantina. El estrés por alta irradiancia no incrementó los niveles de mRNA de los genes *lcyB* y *lcyE* en comparación con los niveles registrados en condiciones de baja irradiancia, mientras que los niveles de transcritos de los demás genes estudiados aumentaron significativamente; activando, sin embargo, la síntesis de los carotenoides secundarios astaxantina, cantaxantina y zeaxantina y disminuyendo los niveles de los carotenoides primarios α- y β-caroteno, violaxantina y luteína. El estrés por deficiencia de nitrógeno *per se* aumentó los niveles de mRNA de todos los genes estudiados, excepto los de los genes *lcyE* y *pds*, sin embargo no desencadenó la síntesis de cantaxantina, zeaxantina ni astaxantina. No obstante, la combinación de ambos factores de estrés, alta irradiancia y deficiencia de nitrógeno, incrementó significativamente los niveles de estos carotenoides, así como los niveles de los transcritos del gen *bkt*, en comparación con los registrados en los cultivos sometidos únicamente a estrés de alta irradiancia.

Capítulo I

Aumento de la producción de luteína en *Chlorella sorokiniana* (Chlorophyta) por optimización de las condiciones de cultivo y mutagénesis al azar

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Article

Enhancement of Lutein Production in *Chlorella sorokiniana* (Chorophyta) by Improvement of Culture Conditions and Random Mutagenesis

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Abstract: *Chlorella sorokiniana* has been selected for lutein production, after a screening of thirteen species of microalgae, since it showed both a high content in this carotenoid and a high growth rate. The effects of several nutritional and environmental factors on cell growth and lutein accumulation have been studied. Maximal specific growth rate and lutein content were attained at 690 μmol photons m^{-2} s^{-1}, 28 °C, 2 mM NaCl, 40 mM nitrate and under mixotrophic conditions. In general, optimal conditions for the growth of this strain also lead to maximal lutein productivity. High lutein yielding mutants of *C. sorokiniana* have been obtained by random mutagenesis, using *N*-methyl-*N′*-nitro-nitrosoguanidine (MNNG) as a mutagen and selecting mutants by their resistance to the inhibitors of the carotenogenic pathway nicotine and norflurazon. Among the mutants resistant to the herbicides, those exhibiting both high content in lutein and high growth rate were chosen. Several mutants exhibited higher contents in this carotenoid than the wild type, showing, in addition, either a similar or higher growth rate than the latter strain. The mutant MR-16 exhibited a 2.0-fold higher volumetric lutein content than that of the wild type, attaining values of 42.0 mg L^{-1} and mutants DMR-5 and DMR-8 attained a lutein cellular content of 7.0 mg g^{-1} dry weight.

The high lutein yield exhibited by *C. sorokiniana* makes this microalga an excellent candidate for the production of this commercially interesting pigment.

Keywords: microalgae; carotenoids; batch culture; mixotrophic culture; *N*-methyl-*N*′-nitro-nitrosoguanidine; high lutein yielding mutants

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1. Introduction

Carotenoids are synthesized by all photosynthetic organisms as well as by some non-photosynthetic bacteria and fungi. There are two main classes of naturally occurring carotenoids: carotenes, which are hydrocarbons, either linear or cyclic in either one or both ends of the molecule, and xanthophylls, which are oxygenated derivatives of carotenes. In microalgae, a distinction can be made between primary and secondary carotenoids. Primary carotenoids, as lutein, function as accessory pigments in the photosystems, as structural components of light harvesting complexes in chloroplasts, as well as photoprotective agents and therefore are essential for cell survival. Secondary carotenoids, such as astaxanthin, accumulate in large quantities in lipid bodies outside the chloroplasts, after subjecting cells to stress conditions. The role of secondary carotenoids in algal cells is not fully understood. They could function as photoprotective filters and as antioxidants preventing accumulation of oxygen radicals [1–3].

Lutein is used as a food dye and especially as a feed additive in aquaculture and poultry farming; it is also used for the coloration of pharmaceutical products and cosmetics [4]. Recently, additional applications for lutein, especially in the field of human health, have been found. Lutein is used as a nutraceutical against macular degeneration; lutein and zeaxanthin are known to play a critical function in maintaining a normal visual function [5–8]. In addition to the development of cataracts, also the progression of early atherosclerosis seems to be hampered by lutein [9,10]. In canines and cats, it has been proved that lutein enhances both cell mediated and humoral immune response [11,12]. Lutein has also been proposed for the prevention of certain cancers [13] and to protect skin from UV-induced damage [5]. Global lutein market has been increasing markedly in the last years [14]. In the US only, sales amount to $150 million [14]. Currently the commercial source of lutein is Marigold (*Tagetes erecta* and *Tagetes patula*) [14]. However, the lutein content of Marigold flowers is low (0.3 mg g^{-1} DW), and therefore there is an increasing interest in microalgae as an alternative source of this carotenoid [14,15]. The microalgae *Muriellopsis* sp. [16,17], *Chlorella zofingiensis* [18], *Coccomyxa acidophila* [19], *Scenedesmus almeriensis* [20] and *Chlorella protothecoides* [21] have been proposed as potential sources of lutein. Nevertheless, the described lutein values are not high enough to be economically feasible on an industrial scale. There is a need to improve lutein accumulation and productivity, by selecting an adequate species, optimizing culture conditions and obtaining high lutein yielding mutants.

Algal species with improved growth rate and enhanced carotenoid accumulation make the commercial process of lutein production more feasible. Induction and selection of mutants has been a technique widely employed for strain improvement as well as for studying the mechanisms of metabolic processes [22]. The introduction of a mutation in a certain carotenoid biosynthetic gene by isolating mutants

resistant to a specific inhibitor for the carotenoid biosynthesis has been a method commonly used to obtain mutants of certain microalgae exhibiting high carotenoid contents. There are several reports describing the isolation of mutants of *Haematococcus pluvialis*, showing higher astaxanthin content per cell than the wild type by random mutagenesis with ethyl methane sulfonate (EMS) or UV and subsequent mutant selection on carotenoid biosynthesis inhibitors, such as compactin, nicotine, diphenylamine or norflurazon [23–25]. However, there is no available information on the obtention of high lutein yielding mutants most probably because cell growth is negatively affected in these mutants.

The present study describes: (1) The screening of thirteen different microalgae to select a good candidate for lutein production; (2) The effect of some nutritional and environmental factors on growth and lutein content in *Chlorella sorokiniana*, the selected species; and (3) The obtention of mutants of *C. sorokiniana* with high yields of lutein in comparison to the wild type strain by random chemical mutagenesis using MNNG.

2. Results

2.1. Screening of Different Species of Chlorophycean Microalgae for Lutein Production

Cell growth and carotenoid content of cultures of 13 chlorophycean microalgae are shown in Table 1. In all species lutein was the most abundant carotenoid. The highest lutein levels (24 mg L^{-1}) were found in *C. sorokiniana*, *Monoraphidium braunii*, *Scenedesmus armatus* and *Scenedesmus vacuolatus* (Table 1). Other carotenoids like antheraxanthin, α-carotene, β-carotene, violaxanthin and zeaxanthin were also produced in all the tested species albeit at concentrations lesser than lutein. Astaxanthin and canthaxanthin were present only in *Chlorella fusca*, *Chlorella zofingiensis*, *Chlorococcum* sp. and *S. vacuolatus*. With regard to growth, *C. sorokiniana* and *S. armatus* exhibited the highest specific growth rates of 0.11 and 0.09 h^{-1}, respectively, and for most species the maximum biomass value attained in the culture ranged between 7.5 to 8.5 g L^{-1}, except for *Chlamydomonas reinhardtii*, *Chlorella luteoviridis* and *Chlorella stigmatofora*, which showed lower biomass values.

Table 1. Specific growth rate (μ), biomass and carotenoids levels in cultures of several species of chlorophycean microalgae. Culture conditions for all the species were the standard ones as reported in the Experimental Section. Data correspond to the maximal values attained in the culture at the deceleration phase: 9–10 days ([a]), 11–12 days ([b]) and 13–14 days ([c]) of culture, being the means of three independent measurements. The standard deviation (SD) is omitted since it was lower than 10% of the mean values. Carotenoids: Ant, antheraxanthin; Ast, astaxanthin; C, canthaxanthin; α-c, α-carotene; β-c, β-carotene; L, lutein; V, violaxanthin; Z, zeaxanthin. nd: not detected.

Species	μ (h^{-1})	Biomass (g L^{-1})	Carotenoid Content (mg L^{-1})							
			Ant	Ast	C	α-c	β-c	L	V	Z
Chlamydomonas reinhardtii [b]	0.05	4.5	0.4	nd	nd	0.1	3.0	12.4	1.0	0.2
Chlorella fusca [b]	0.04	8.5	1.5	6.5	1.2	0.3	4.6	22.0	1.5	6.0
Chlorella luteoviridis [b]	0.03	2.7	0.3	nd	nd	nd	0.5	5.8	0.2	0.6
Chlorella sorokiniana [a]	0.11	8.0	0.3	nd	nd	0.2	1.6	24.0	0.9	0.4

Table 1. *Cont.*

Chlorella stigmatofora [b]	0.02	4.1	0.1	nd	nd	0.1	nd	3.2	0.2	0.4
Chlorella vulgaris [c]	0.08	8.5	0.9	nd	nd	1.8	2.3	22.2	2.2	3.3
Chlorella zofingiensis [c]	0.03	7.6	0.9	5.6	1.2	0.4	3.0	20.0	1.3	3.0
Chlorococcum sp. [a]	0.03	7.5	0.8	nd	0.8	0.2	2.2	15.0	2.5	0.5
Monoraphidium braunii [c]	0.07	8.4	2.4	nd	nd	0.9	3.8	24.0	2.1	8.4
Scenedesmus armatus [b]	0.09	8.5	0.5	nd	nd	0.3	4.4	24.0	2.4	nd
Scenedesmus quadricauda [c]	0.06	8.4	0.6	nd	nd	0.3	3.0	22.0	5.0	nd
Scenedesmus obliquus [b]	0.08	8.0	0.5	nd	nd	0.2	2.5	15.0	1.5	1.2
Scenedesmus vacuolatus [c]	0.08	8.2	1.6	5.3	1.7	0.5	4.2	24.0	2.4	3.8

Some of the species considered in this work, such as *C. sorokiniana*, *M. braunii*, *S. armatus* and *S. vacuolatus* are of potential practical interest on the basis of their high lutein level. Among these, *C. sorokiniana* has been selected for further work focused on the production of lutein, since this microalga also shows the highest specific growth rate (0.11 h^{-1}) and a high biomass value (8.0 g L^{-1}).

2.2. *Effect of Some Environmental and Nutritional Factors on Growth and Lutein Content in C. sorokiniana*

2.2.1. Growth and Lutein Accumulation under Standard Conditions

Figure 1 shows the evolution with time of growth and lutein content in a photoautotrophic batch culture of *C. sorokiniana*. Both volumetric and cellular lutein content increased with cell biomass, attaining a maximum of 24 mg L^{-1} at the end of the deceleration phase, and 4.2 mg g^{-1} DW in the early deceleration phase, respectively. However, whereas the volumetric content was kept constant in the deceleration phase, the cellular content decreased markedly to 2.8 mg g^{-1} DW. The accumulation of the rest of carotenoids followed the same trend as lutein (data not shown).

2.2.2. Effect of Irradiance

Cell growth and lutein accumulation in *C. sorokiniana* batch cultures at different irradiances, in the range from 92 to 1495 µmol photons m^{-2} s^{-1}, was studied. Both specific growth rate and biomass in the culture increased with irradiance until 690 µmol photons m^{-2} s^{-1}, by 2.6-fold and 1.5-fold, respectively, keeping constant at higher intensity values. Lutein content in the culture was enhanced by 87% as irradiance increased from 92 to 690 µmol photons m^{-2} s^{-1}, decreasing at higher irradiance values. Cellular lutein content exhibited an optimum in an irradiance range between 368 and 690 µmol photons m^{-2} s^{-1}, decreasing at higher and lower irradiances by 22 and 19%, respectively (Table 2). The maximum cellular lutein content (4.3 mg g^{-1} DW) was reached at 690 µmol photons m^{-2} s^{-1}, in the early deceleration phase of growth (data not shown).

Figure 1. Kinetics of growth and lutein accumulation in a batch culture of *C. sorokiniana* under standard conditions. Culture conditions were the standard ones reported in Experimental Section. Data shown represent the mean values of three independent measurements, SD being lower than 10% of the means. Symbols: (closed circles) dry cell weight; (open circles) volumetric lutein content in the culture; (closed triangles) cellular lutein content.

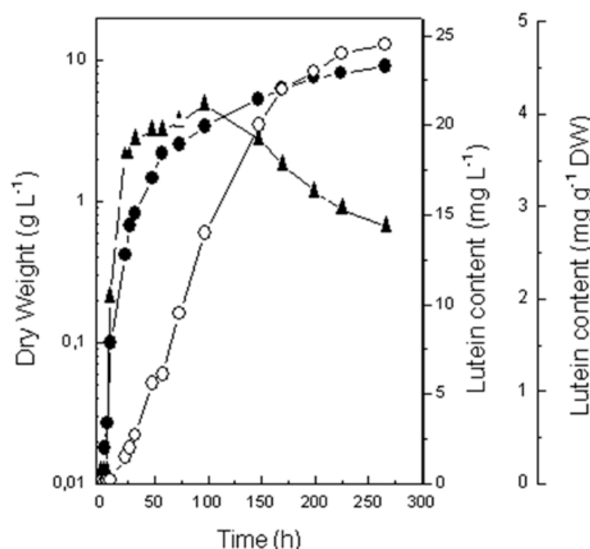


Table 2. Effect of irradiance on growth and lutein accumulation in *C. sorokiniana*. Cells were grown at the indicated irradiances, the rest of culture conditions being the standard ones described in Experimental Section. Biomass and lutein content data correspond to the mean values of three independent measurements recorded after 10 days, at the end of the deceleration phase, when the maximal volumetric lutein contents and biomass values were attained in the cultures. The SD were lower than 10% of the means.

Irradiance (μmol photons m^{-2} s^{-1})	μ (h^{-1})	Biomass (g L^{-1})	Lutein Content mg L^{-1}	Lutein Content mg g^{-1} DW
92	0.05	6.0	15.0	2.5
230	0.08	7.2	19.0	2.6
368	0.11	7.8	23.0	3.0
460	0.11	8.0	24.0	3.0
690	0.13	9.0	28.0	3.1
920	0.13	8.5	24.0	2.8
1495	0.13	8.5	20.0	2.4

2.2.3. Effect of Temperature

The influence of temperature on lutein level and growth of *C. sorokiniana* has also been examined. Cell growth, in terms of specific growth rate, increased by 50% when the temperature was raised from 25 to 28 °C, keeping constant at higher temperatures. Biomass in the culture increased by about 10% with temperatures up to 28 °C, decreasing by 44% at higher temperatures. Optimal volumetric and cellular lutein contents were recorded in a range from 28 to 32 °C, decreased values being registered at lower and higher temperatures (Table 3). The maximum cellular lutein content (4.2 mg g^{-1} DW) was achieved at 28 °C in the early deceleration phase of growth (data not shown).

Table 3. Effect of temperature on growth and lutein accumulation in *C. sorokiniana*. Cells were grown at the indicated temperatures, the rest of culture conditions being the standard ones described in Experimental Section. Biomass and lutein content data are the mean values of three independent measurements recorded after 9 days, when cultures were at the end of deceleration phase, which corresponds to the maximal volumetric lutein contents and biomass attained in the cultures. The SD were lower than 10% of the means.

Temperature (°C)	μ (h^{-1})	Biomass ($g\ L^{-1}$)	Lutein Content	
			$mg\ L^{-1}$	$mg\ g^{-1}$ DW
22	0.08	7.5	19.0	2.4
25	0.08	7.9	20.0	2.4
28	0.12	8.2	25.0	3.0
32	0.12	6.6	24.0	3.6
36	0.11	5.6	19.0	3.4
40	0.11	4.6	16.0	3.4

2.2.4. Effect of Nitrogen

Nitrogen availability affects carotenoid accumulation in some microalgae [14], therefore the effect of nitrate concentration on lutein production was also assayed. Both specific growth rate and biomass in the culture increased by 30% when nitrate concentration in the medium was enhanced from 10 to 40 mM, decreasing by about 15% at higher concentrations. In addition, volumetric and cellular lutein contents increased 2.9 and 2.3-fold, respectively, when nitrate concentration in the medium was raised from 10 to 40 mM, decreasing by about 10% at higher nitrate concentrations (Table 4). Nevertheless, maximum cellular lutein content did not change significantly with nitrogen concentration in the range from 20 to 80 mM (data not shown).

Table 4. Effect of nitrate concentration on growth and lutein accumulation in *C. sorokiniana*. Cells were grown at the indicated concentrations of $NaNO_3$, the rest of culture conditions being the standard ones described in Experimental Section. Biomass and lutein content data are the mean values of three independent measurements recorded after 10 days, when cultures were at the end of the deceleration phase, which corresponds to the maximal volumetric lutein contents and biomass attained in the cultures. The SD were lower than 10% of the means.

$NaNO_3$ concentration (mM)	μ (h^{-1})	Biomass ($g\ L^{-1}$)	Lutein Content	
			$mg\ L^{-1}$	$mg\ g^{-1}$ DW
10	0.10	6.5	9.0	1.4
20	0.10	8.0	24.0	3.0
30	0.12	8.3	25.0	3.0
40	0.13	8.2	26.0	3.2
60	0.12	7.9	25.0	3.2
80	0.12	7.4	24.0	3.2
100	0.11	7.7	24.0	3.1
120	0.11	7.2	22.0	3.0

2.2.5. Effect of NaCl

The influence of NaCl concentration in the medium was also analyzed in a range from 2 to 200 mM. Both growth and lutein content decreased drastically at NaCl concentrations higher than 2 mM (data not shown).

2.2.6. Effect of Acetate and Glucose (Mixotrophic Culture)

The main limiting factor for biomass productivity in photoautotrophic cultures of microalgae is imposed by light availability. To solve this problem mixotrophic cultures can be used. For that reason the effect of acetate, as an extra source of carbon, on growth and lutein accumulation was assayed in *C. sorokiniana*. As shown in Table 5, specific growth rate and biomass in the culture were enhanced by 33% and 20%, respectively, when acetate concentration in the medium was raised from 0 to either 40 or 50 mM, decreasing only by 8% thereafter. The volumetric and cellular lutein contents increased by 45% and 20%, respectively, when acetate was added to the medium at concentrations up to 40 mM, decreasing by a 25% at higher acetate concentrations. The maximum cellular lutein content followed the same trend as cellular lutein content at the end of the deceleration phase, attaining maximum values of 5.2 mg g^{-1} DW at 40 mM of acetate (data not shown).

Table 5. Effect of acetate concentration on growth and lutein accumulation in *C. sorokiniana*. Cells were grown mixotrophically by the addition of sodium acetate at the indicated concentrations at the beginning of the culture, the rest of culture conditions being as described in Experimental Section. Biomass and lutein content data are the mean values of three independent measurements recorded after 10 days, when cultures were at the end of deceleration phase, which corresponds to the maximal volumetric lutein contents and biomass attained in the cultures. The SD were lower than 10% of the means.

Acetate concentration (mM)	μ (h^{-1})	Biomass (g L^{-1})	Lutein Content	
			mg L^{-1}	mg g^{-1} DW
0	0.09	7.5	22.0	3.0
20	0.11	7.6	26.0	3.4
30	0.11	8.0	28.0	3.5
40	0.12	9.0	32.0	3.6
50	0.12	9.0	26.0	2.9
60	0.11	9.2	25.0	2.7

The addition of glucose, as the only extra carbon source, to cultures of *C. sorokiniana* increased by 60% the biomass in the culture and by 12% the volumetric lutein content, which attained a level of 33.5 mg L^{-1}, the cellular lutein content decreasing by 30% (Table 6). A similar effect was observed when glucose was added to cultures containing 40 mM acetate before the end of the exponential phase, reaching a volumetric lutein content of 35.0 mg L^{-1}. On the other hand, an extra addition of acetate or sodium nitrate before the end of the exponential phase did not affect either biomass or lutein content significantly. Moreover, when sodium nitrate was replaced by urea, the specific growth rate and lutein content were decreased by 17% and 20%, respectively.

Table 6. Effect of carbon or nitrogen extra supply on growth and lutein accumulation in *C. sorokiniana*. Cells were grown mixotrophically at an irradiance of 690 µmol photons m^{-2} s^{-1} in Arnon medium modified to contain 4 mM K_2HPO_4, 40 mM sodium nitrate and 40 mM sodium acetate (AM), except where indicated ([b,c]). [a] Addition of glucose, extra nitrate or acetate during exponential phase after 24 h of culture. [b] Nitrate was replaced by urea at the beginning of the culture. [c] Acetate was replaced by glucose at the beginning of the culture. The rest of culture conditions were as described in Experimental Section. Biomass and lutein content data are the mean values of three independent measurements recorded after 10 days, when cultures were at the end of the deceleration phase, which corresponds to maximal volumetric lutein contents and biomass attained in the cultures. The SD were lower than 10% of the means.

Culture medium	µ	Biomass	Lutein Content	
	(h^{-1})	(g L^{-1})	mg L^{-1}	mg g^{-1} DW
AM	0.12	8.8	30.0	3.4
AM + 100 mM Glucose [a]	0.11	13.5	35.0	2.6
AM + 40 mM Acetate [a]	0.11	9.0	29.0	3.2
AM + 40 mM Nitrate [a]	0.12	8.8	29.0	3.3
AM + 60 mM Urea [b]	0.10	8.5	23.0	2.7
AM + 100 mM Glucose [c]	0.11	14.0	33.5	2.4

Similar values of growth and lutein content were registered when sodium nitrate was replaced by ammonium nitrate. When *C. sorokiniana* was grown heterotrophically in the dark with glucose as the only carbon source, specific growth rate was similar and biomass decreased only slightly with regard to the values obtained under mixotrophic conditions, however lutein accumulation was much lower than that measured in mixotrophic or photoautotrophic conditions (data not shown).

2.3. Isolation of High Lutein Yielding Mutants of C. sorokiniana

To obtain high lutein producing mutants of *C. sorokiniana*, cells were subjected to random chemical mutagenesis with MNNG and mutants were screened on the basis of their resistance either norflurazon or nicotine, growth rate and lutein content. First, the survival curve for mutagenesis with MNNG was performed to determine the MNNG concentration which resulted in around 5–10% of cells viability (Figure 2), and afterwards, a wide range of concentrations of the herbicides were tested to find out the minimal concentration which inhibited the grown of the wild strain, resulting in 400 µM for nicotine and 4 µM for norflurazon (data not shown).

According to the first step of selection, 745 herbicide-resistant colonies were obtained, from which only 222 (210 resistant to norflurazon and 12 resistant to nicotine) were selected according to the second step criterion, which consisted of discarding mutants exhibiting either low growth or unstable resistance to the herbicides. The selected mutants were grown in shaken liquid cultures under photoautotrophic standard conditions to determine lutein content and growth rate. Increases in volumetric and cellular lutein contents of the best mutants as compared to the wild type are shown in Table 7. The mutant MR-16, resistant to nicotine, exhibited volumetric and cellular lutein contents

2.0- and 1.4-fold higher, respectively, than those of the wild strain. This mutant also showed a 3.0-fold higher volumetric α-carotene and antheraxanthin levels and 2.0-fold higher β-carotene and zeaxanthin contents as compared with those of the wild type, being the total volumetric carotenoid content 2.0-fold higher than in the wild strain (data not shown). The mutants DMR-5 and DMR-8, both resistant to norflurazon, stood out in terms of cellular content in lutein, showing 53–55% increase relative to the wild type, reaching values of 7.0 mg lutein g^{-1} DW.

Figure 2. Survival curve of *C. sorokiniana* to the chemical mutagen MNNG. Data shown represents mean values of three independent measurements, SD being lower than 10%.

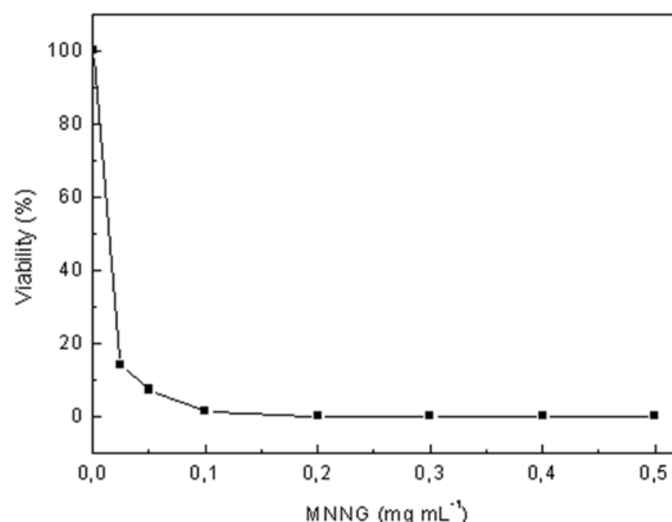
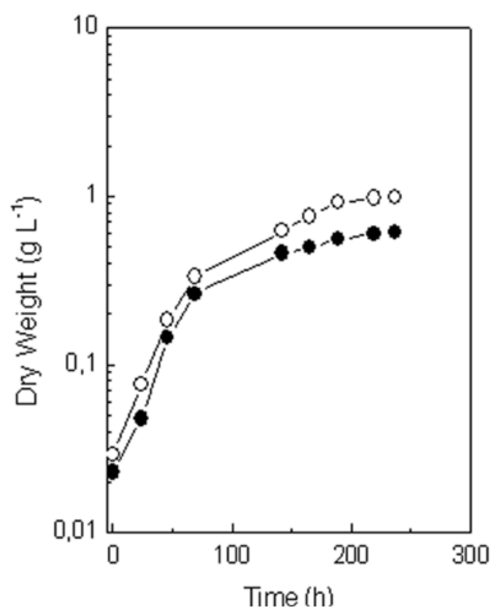


Table 7. Increase in lutein content of the best mutants of *C. sorokiniana* relative to the wild type. Cells were grown photoautotrophically in shaken cultures at 50 µmol photons m^{-2} s^{-1}, the rest of conditions being the standard ones described in Experimental Section. Data are mean values of three independent measurements in the deceleration phase after 13 days of growth, SD being lower than 10%. NF: resistant to norflurazon; NIC: resistant to nicotine.

Strain	Volumetric Lutein Content (mg L^{-1})	Cellular Lutein Content (mg g^{-1} DW)
	(% with respect to the wild strain)	
MR-3 (NF)	52	17
MR-14 (NIC)	63	29
MR-16 (NIC)	101	42
DMR-4 (NIC)	62	38
DMR-11 (NIC)	49	49
DMR-5 (NF)	29	55
DMR-8 (NF)	29	53

Moreover, the growth of the selected high lutein producing mutants under photoautotrophic conditions was similar or even higher than that of the parental strain, as shown in the Figure 3 for MR-16.

Figure 3. Kinetics of growth of *C. sorokiniana* wild type and the mutant MR-16. Cells were grown as indicated in Table 7. Data shown represent mean values of three independent measurements, SD being lower than 10%. Symbols: (closed circle) wild type; (open circles) mutant MR-16.



All mutants of *C. sorokiniana* obtained in the processes of mutagenesis were analyzed for their viability and stability by consecutive sub-cultures in nonselective and selective media with herbicides to check their resistance.

2.4. Comparison of Growth and Lutein Accumulation in the Selected Mutant (MR-16) and in the Wild Type of C. sorokiniana under Best Photoautotrophic and Mixotrophic Culture Conditions

MR-16 mutant exhibited both a growth and lutein content higher than the wild strain under photoautotrophic conditions (Figure 3, Table 7). For this reason, cells of the wild type and the MR-16 mutant were grown under the best photoautotrophic and mixotrophic conditions found previously in the wild strain of *C. sorokiniana* for lutein accumulation in order to know accurately the improvement of the MR-16 mutant as compared to the wild type. As shown in Table 8, the volumetric and cellular lutein contents increased both by 68% in the mutant MR-16 with respect to the wild strain when the mutant was grown photoautotrophically, attaining maximum values of 42.0 mg L^{-1} and 5.0 mg g^{-1} DW at the end of the deceleration phase. On the other hand, specific growth rate was either constant or very similar at the different conditions assayed in both strains. However when the mutant was grown mixotrophically, biomass decreased 40% as compared to the wild strain. The addition of acetate and glucose to cells of the MR-16 mutant had a negative effect decreasing a 48% the maximum lutein content in the culture, and a 33% the cellular content with regard to the parental strain. A difference between the wild and the mutant *C. sorokiniana* strains was that the wild type showed the maximum values of cellular lutein during the early deceleration phase, whereas MR-16 mutant attained these contents at the end of the deceleration phase, due to a continuous and higher lutein accumulation.

Table 8. Growth and lutein accumulation in MR-16 mutant and wild strain of *C. sorokiniana* under photoautotrophic and mixotrophic conditions. Cells were grown photoautotrophically at an irradiance of 690 μmol photons m^{-2} s^{-1} in Arnon medium modified to contain 4 mM K_2HPO_4 and 40 mM sodium nitrate, and mixotrophycally by the addition of both 40 mM sodium acetate and 100 mM glucose after 24 h of culture (by the middle of the exponential phase). The rest of culture conditions were as described in Experimental Section. Biomass and lutein content data are the mean values of three independent measurements recorded after 10 days, when cultures were at the end of the deceleration phase, which corresponds to maximal volumetric lutein content and biomass attained in the cultures. The SD were lower than 10% of the means.

Culture condition	μ (h^{-1})	Biomass ($g\ L^{-1}$)	Lutein Content	
			mg L^{-1}	mg g^{-1} DW
Wild type				
Photoautotrophic	0.11	8.4	25.0	3.0
Mixotrophic	0.11	13.0	33.0	2.6
MR-16 mutant				
Photoautotrophic	0.12	8.3	42.0	5.0
Mixotrophic	0.11	7.8	22.0	2.8

3. Discussion

3.1. Effect of Several Environmental and Nutritional Factors on Lutein Production by C. sorokiniana under Photoautotrophic Conditions

Since irradiance, temperature, NaCl and nitrate concentration are known to affect the levels of different carotenoids in microalgae, the effect of these factors on cell growth and lutein accumulation was analyzed in *C. sorokiniana* which has been selected among thirteen chlorophycean microalgae, since it showed the highest growth rate and lutein accumulation. In this selected microalga lutein content in the culture was optimal at moderate irradiances, since both biomass and cellular content in lutein decreased at both high and low irradiance (Table 2). A similar trend with regard to the cellular content has also been observed for lutein and β-carotene in different species of *C. zofingiensis* and *Muriellopsis* sp. [26,16,18]. In contrast, in *H. pluvialis* and *C. zofingiensis* the cellular content of secondary carotenoids, such as astaxanthin and canthaxanthin, followed the opposite trend to lutein, being enhanced at high irradiance [18,27]. *C. sorokiniana* exhibited maximal volumetric and cellular lutein content in the range of 28 to 32 °C decreasing at lower and higher temperatures (Table 3); these results being in agreement with those obtained in the lutein-producing microalga *C. zofingiensis* [16] and *Scenedesmus almeriensis* [20,28]. Contrastingly, extreme temperatures triggered carotenogenesis in *H. pluvialis*. It has been suggested that endogenously generated active oxygen is responsible for the stimulation of astaxanthin synthesis at high temperature in this microalga [24]. Nitrogen limitation enhances the synthesis of secondary carotenoids such as astaxanthin, but reduces biomass yield. However, nitrogen at none limiting concentrations is required for primary carotenoids accumulation due possibly to the need of a continued synthesis of light-

harvesting proteins and structural xanthophylls under optimal growth conditions [3,16,18]. This agrees with the results obtained in *C. sorokiniana* since both volumetric and cellular lutein content increased when nitrate concentration in the culture medium was raised from 10 to 40 mM, decreasing slightly at higher nitrate concentrations (Table 4). On the other hand, although in heterotrophic cultures of *C. protothecoides* maximal lutein productivities were achieved using urea as the nitrogen source [29], in *C. sorokiniana* grown mixotrophically either a slight decrease or no effect in growth and lutein content was registered when sodium nitrate was replaced by either urea (Table 6) or ammonium nitrate (data not shown). NaCl stress, which has been described to induce the biosynthesis of secondary carotenoids [30], seems not to trigger the biosynthesis of the primary ones. Thus in *C. sorokiniana*, both growth and lutein content decreased drastically when NaCl concentrations were increased (data not shown), and in the cases of other chlorophyta as *Muriellopsis* sp., *C. zofingiensis* and *S. almeriensis*, the lutein levels per cell remained practically constant at the different NaCl concentrations assayed [16,18,20]. Therefore, although stress factors, such as high irradiance, extreme temperatures, high NaCl concentration or nutrients limitation enhance the cellular accumulation of secondary carotenoids, such as astaxanthin [1,18,31], these factors do not increase the cellular levels of lutein in *C. sorokiniana*, since lutein is a primary carotenoid, being required for the structure and function of the light-harvesting complexes in photosynthesis [2], and accordingly, conditions that increase photoautotrophic growth of this microalga are also those enhancing lutein accumulation.

3.2. Lutein Production by C. sorokiniana under Mixotrophic Conditions

In photoautotrophic mass cultures of microalgae for the production of biomass and valuable compounds, the main limiting factor is usually light availability, which in many cases limits cell density and productivity of the cultures, making it unprofitable for industry. An alternative to overcome this problem is to use mixotrophic cultures. In *C. sorokiniana*, the addition of acetate to the cultures enhanced both growth and volumetric and cellular lutein contents (Table 5). Although it is known that acetate enhances growth and synthesis of astaxanthin in *H. pluvialis* [32], the effect of this carbon source on the biosynthesis of lutein has not been studied in other microalgae. Even though the addition of glucose decreased the cellular content in lutein, it increased biomass considerably; therefore, the volumetric lutein content was enhanced and the highest volumetric lutein levels (35 mg L^{-1}) were achieved in cultures supplemented with this carbon source (Table 6). Therefore the supply of an extra carbon source to cultures supported higher growth and productivity, overcoming the limitation by light. Recently, it has been shown that in heterotrophically grown *C. zofingiensis*, glucose increased the cellular accumulation of astaxanthin and zeaxanthin by increasing the transcription levels of both β-carotene ketolase and β-carotene hydroxylase involved in the synthesis of these carotenoids, but decreased the cellular content of lutein and chlorophyll [33,34] and in *C. protothecoides* lutein productivity was much higher in heterotrophic cultures supplemented with glucose than in photoautotrophic cultures, since in the former very high cell densities were achieved [35]. Therefore, our results are in agreement with these findings, which indicate that although glucose decreases the celular content of lutein, it increases considerably the biomass, therefore resulting in a higher lutein production in mixotrophic cultures as compared to those performed photoautotrophically.

3.3. Enhancement of Lutein Yield in C. sorokiniana by Random Mutagenesis

There are few reports concerning the isolation of high carotenoid yielding mutants of microalgae by random mutagenesis. A mutant of *Chlorella regularis* showing a high cellular content of lutein has been isolated by Ishikawa *et al.* (2004) [36], although it exhibited lower growth than the wild strain and no volumetric lutein content data have been reported. Some mutants with enhanced accumulation of astaxanthin have also been described, however their growth rates are usually lower than that of the wild type [37,23,38]. In our experiments with *C. sorokiniana*, mutants were selected not only on the basis of high lutein content, but also according to a high growth rate. Thus, the mutant MR-16 exhibited a growth and cellular lutein content higher than those of the wild type and, as a consequence, lutein yields 2.0-fold higher than those measured in the wild strain were achieved (Table 7 and Figure 3). In addition, this mutant shows the highest lutein content described in the literature under photoautotrophic conditions of growth at laboratory scale [14,16,18–20], attaining a value of 42.0 mg L^{-1} at the end of the deceleration phase (Table 8). The mutant MR-16 was resistant to nicotine, a specific inhibitor for the enzyme lycopene β-cyclase, involved in lutein biosynthesis, which could possibly have an altered specific activity in this mutant, for instance a modified structure for the herbicide-binding site, and/or a different expression of this enzyme, which means a higher enzyme activity, and would result into an improved lutein production under photoautotrophic growth. In addition, the higher growth rate of this mutant with respect to the wild type strain under photoautotrophic conditions could be due to the higher cellular lutein content, since this carotenoid plays important roles in the function, structure and photo-protection of the photosynthetic apparatus, leading to a higher photosynthetic efficiency. It is relevant to mention that the addition of acetate and/or glucose to the culture medium decreased the biomass in the cultures of MR-16. The mechanism of this effect is not understood yet. It is possible that the mutation has provoked a metabolic alteration causing a weak repression of growth by glucose or acetate.

3.4. C. sorokiniana as a Promising Lutein Producer for Commercial Applications

Although microalgae are not used yet as a lutein source at industrial scale, first steps have been made at laboratory and pilot scales in the recent years [16,14,28,21]. The data here reported point to *C. sorokiniana* as an attractive candidate for the production of lutein, since it shows a high growth rate (0.12 h^{-1}), a volumetric lutein content of 35 mg L^{-1} and cellular lutein contents of 5.2 mg g^{-1} DW, which are enhanced by random mutagenesis up to 42.0 mg L^{-1} and 7.0 mg g^{-1} DW. These values are similar or higher than those reported for other lutein producing microalgae grown at laboratory scale under photoautotrophic conditions. Thus, *C. zofingiensis* shows a specific growth rate of 0.04 h^{-1} and a volumetric and cellular lutein content of 20.0 mg L^{-1} and 3.0 mg g^{-1} DW, respectively [18,14]; *Scenedesmus almeriensis* has been reported to accumulate 5.5 mg g^{-1} DW and a specific growth rate of 0.07 h^{-1} [20]; *Muriellopsis* exhibited a growth rate of 0.17 h^{-1} and a volumetric and cellular lutein content of 29.0 mg L^{-1} and 5.5 mg g^{-1} DW, respectively [16]; and *Coccomyxa acidophila* has been reported to accumulate 6.1 mg g^{-1} DW under extreme culture conditions [19]. From all this information, we can propose C. *sorokiniana* as a promising microalgal species for the production of lutein for commercial applications.

4. Experimental Section

4.1. Organisms

The species of microalgae used in this work were: *Chlorella fusca* 211-8b, *Chlorella sorokiniana* 211-32, *Chlorella zofingiensis* 211-14, *Monoraphidium braunii* 202-7d, *Scenedesmus vacuolatus* 211-15 and *Chlorella stigmatofora* 9-86 from SAG, Culture Collection of Göttingen University (Germany); *Chlorella luteoviridis* 258, *Chlorococcum* sp. 2438, *Scenedesmus armatus* 2533, *Scenedesmus quadricauda* 76 and *Scenedesmus obliquus* 393 from UTEX, Culture Collection of Algae of the University of Texas (USA); *Chlorella vulgaris* 101 from UAM, Culture Collection of Microalgae of Universidad Autónoma de Madrid (Spain); and *Chlamydomonas reinhardtii* CC621(−) from Culture Collection of Institut für Biologie III at the University of Freigburg (Germany).

4.2. Culture Conditions

4.2.1. Standard Culture Conditions

Cells were grown photoautotrophically by bubbling through the cell suspension air supplemented with 1% (v/v) CO_2 as the only source of carbon. The culture medium of Arnon *et al.* (1974) [39] modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$, was used. The cells were grown in batch culture at 28 °C, in Roux flasks of 1 L capacity, laterally illuminated with mercury halide lamps at 460 µmol photons m^{-2} s^{-1}, measured at the surfaces of the flasks using a LI-COR quantum sensor (model L1-1905B, Li-Cor, Inc. Lincoln, NE, USA) connected to a quantum photometer.

4.2.2. Mixotrophic Culture Conditions

Cells were grown mixotrophically by the addition to Arnon medium (modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$) sodium acetate and/or glucose, either at the beginning of the culture or after 24 h (by the middle of the exponential phase) and at an irradiance of either 460 or 690 µmol photons m^{-2} s^{-1}. The rest of culture conditions were the same as the standard ones.

4.3. Random Mutagenesis and Selection of High Lutein Yielding Mutants of C. sorokiniana

Cells of *C. sorokiniana* in the exponential phase of growth (10^6 cells mL^{-1}) were harvested by centrifugation (2700× *g*, 10 min), washed with sterile water and treated with 0.1 mg mL^{-1} of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (survival rate 5–10%) for 1 h. This mutagen is known to induce nucleotides substitutions at high frequencies and little lethality, and the inhibitors nicotine and norflurazon were used as the selection method. These herbicides inhibit the carotenogenic enzymes lycopene β-cyclase and phytoene desaturase, respectively. Therefore, the screening of the mutants was performed by their resistance to the carotenoid biosynthesis inhibitors nicotine and norflurazon.

The treated cells were washed with sterile water, resuspended in Arnon modified medium and incubated under dim light during 24 h. After the incubation, cells were spread on solid modified Arnon medium containing, either 4 µM norflurazon (Supelco, Bellefonte, PA, USA) or 400 µM nicotine (Sigma-Aldrich, Steinheim, Germany) and incubated at 25 °C and 50 µmol photons m^{-2} s^{-1} for

3–4 weeks. Then, the herbicide-resistant colonies were sub-cultivated several times in solid medium containing either norflurazon or nicotine to check their resistance to the herbicide and growth in solid medium. Herbicide-resistant mutants that showed good growth were grown in 100 mL-capacity erlenmeyers under photoautotrophic conditions, shaken at 100 rpm and illuminated from the top at 50 µmol photons m^{-2} s^{-1}, in order to analyze carotenoids content and growth.

4.4. Analytical Methods

For dry weight (DW) determinations, 5 mL aliquots of the cell culture were filtered through pre-dried Whatman GF/C paper (Whatman International Ltd., Maidstone, England), washed three times with distilled water, and the filters containing the algae were dried at 80 °C for 24 h.

Specific growth rate (µ) was calculated from the measured DW during the exponential phase of growth, using the equation: $\mu = (\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x_2 and x_1 represent DW values in terms of g L^{-1} at times t_2 and t_1, respectively.

For carotenoid analysis, pigments were extracted with methanol at 70 °C, centrifuged, the supernatant evaporated under N_2 and the pellet dissolved in methanol. Then the samples were centrifuged and analyzed by HPLC using a Waters Spherisorb ODS2 column (4.6 × 250 mm, 5 µm particle size) (Waters, Mildford, MA, USA). The chromatographic method described by Cordero *et al.* [40] was used. The pigments were eluted at a flow rate of 1.2 mL min^{-1} and detected at 440 nm using a Waters 2996 photodiode-array detector. Identification of carotenoids was achieved by comparison of the individual characteristic absorption spectrum and the retention time with known standards. Quantification was performed using a calibration curve generated with commercially available carotenoids standards from Sigma-Aldrich (St. Louis, MO, USA) and DHI (Holsholm, Germany).

Samples analyzed were withdrawn along the curve of growth. Independent triplicate analyses were carried out for each sample, the results representing the mean values. The standard deviation (SD) is omitted since it was lower than 10% of the mean values.

5. Conclusions

Chlorella sorokiniana has been selected for lutein production after a screening of thirteen species of microalgae. The effects of several nutritional and environmental factors on cell growth and lutein content have been studied. Maximal specific growth rate and lutein accumulation were attained at 690 µmol photons m^{-2} s^{-1}, 28 °C, 2 mM NaCl, 40 mM nitrate and under mixotrophic conditions, attaining values of lutein of 35.0 mg L^{-1} and 5.2 mg g^{-1} DW. In general, optimal conditions for the growth of this species also lead to maximal lutein productivity, since lutein is a primary carotenoid, being required for the structure and function of the light-harvesting complexes in photosynthesis. These lutein values were further enhanced by chemical random mutagenesis up to 42.0 mg L^{-1} and 7.0 mg g^{-1} DW, using MNNG and selecting mutants by: (1) their resistance to the inhibitors of the carotenogenic pathway nicotine and norflurazon; (2) their high growth rate; and (3) high lutein content. From all our results we can propose *C. sorokiniana* as an interesting and promising microalga for the production of lutein for commercial and industrial applications.

Acknowledgments

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References

1. Boussiba, S. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. *Physiol. Plant.* **2000**, *108*, 111–117.
2. Demming-Adams, B.; Adams, W.W., III. Antioxidants in photosynthesis and human nutrition. *Science* **2002**, *298*, 2149–2153.
3. Jin, E.; Juergen, E.W.P.; Hong, K.L.; Sang, M.H.; Man, C. Xanthophylls in microalgae: From biosynthesis to biotechnological mass production and application. *J. Microbiol. Biotechnol*. **2003**, *13*, 165–174.
4. Johnson, E.A.; Schroeder, W.A. Advances in Biochemical Engineering, Biotechnology. In *Microbial Carotenoids*; Fiechter, A., Ed.; Springer: Berlin, Germany, 1995; Volume 53; pp. 119–178.
5. Alves-Rodrigues, A.; Shao, A. The science behing lutein. *Toxicol. Lett.* **2004**, *150*, 57–83.
6. Carpentier, S.; Knaus, M.; Such, M. Associations between lutein, zeaxanthin, and age-related macular degeneration: An overview. *Crit. Rev. Food Sci. Nutr*. **2009**, *49*, 313–326.
7. Chin, C.J.; Taylor, A. Nutritional antioxidants and age-related cataract and macular degeneration. *Exp. Eye Res.* **2007**, *84*, 229–245.
8. Whitehead, A.J.; Mares, J.A.; Danis, R.P. Macular pigment: A review of current knowledge. *Arch. Ophtalmol*. **2006**, *124*, 1038–1045.
9. Arnal, E.; Miranda, M.; Almansa, I.; Muriach, M.; Barcia, J.M.; Romero, F.J.; Díaz-Llopis, M.; Bosch Moreer, F. Lutein prevents cataract development and progression in diabetic rats. *Graefes Arch. Clin. Exp. Ophthalmol*. **2009**, *247*, 115–120.
10. Dwyer, J.H.; Navab, M.; Dwyer, K.M.; Hassan, K.; Sun, P.; Shircore, A.; Hama-Levy, S.; Hough, G.; Wang, X.; Drake, T.; Merz, N.B.; Fogelman, A.M. Oxygenated carotenoid lutein and the progression of early atherosclerosis. *Circulation* **2001**, *103*, 2922–2977.
11. Kim, H.W.; Chew, B.P.; Wong, T.S.; Park, J.S.; Weng, B.B.C.; Byrne, K.M.; Hayek, M.G.; Reinhart, G.A. Modulation of humoral and cell-mediated immune responses by dietary lutein in cats. *Vet. Immunol. Immunopathol*. **2000**, *73*, 331–341.
12. Kim, H.W.; Chew, B.P.; Wong, T.S.; Park, J.S.; Weng, B.B.C.; Byrne, K.M.; Hayek, M.G.; Reinhart, G.A. Dietary lutein stimulates immune response in the canine. *Vet. Immunol. Immunopathol*. **2000**, *74*, 315–327.
13. Basu, H.N.; Del Vecchio, A.J.; Flider, F.; Orthoefer, F.T. Nutritional and potential disease prevention properties of Carotenoids. *J. Am. Oil Chem. Soc*. **2001**, *78*, 665–675.
14. Del Campo, J.A.; Garcia-Gonzalez, M.; Guerrero, M.G. Outdoor cultivation of microalgae for carotenoid production: Current state and perspectives. *Appl. Microbiol. Biotechnol*. **2007**, *74*, 1163–1174.

15. Piccaglia, R.; Marotti, M.; Grandi, S. Lutein and lutein ester content in different types of *Tagetes patula* and *T. erecta*. *Ind. Crops Prod*. **1998**, *8*, 45–51.
16. Del Campo, J.A.; Moreno, J.; Rodriguez, H.; Vargas, M.A.; Rivas, J.; Guerrero, M.G. Carotenoid content of chlorophycean microalgae: Factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). *J. Biotechnol*. **2000**, *76*, 51–59.
17. Del Campo, J.A.; Rodriguez, H.; Moreno, J.; Vargas, M.A.; Rivas, J.; Guerrero, M.G. Lutein production by *Muriellopsis* sp. in an outdoor tubular photobioreactor. *J. Biotechnol.* **2001**, *85*, 289–295.
18. Del Campo, J.A.; Rodriguez, H.; Moreno, J.; Vargas, M.A.; Rivas, J.; Guerrero, M.G. Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*. **2004**, *64*, 848–854.
19. Casal, C.; Cuaresma, M.; Vega, J.M.; Vilchez, C. Enhanced productivity of a lutein-enriched novel acidophile microalga grown on urea. *Mar. Drugs* **2011**, *9*, 29–42.
20. Sanchez, J.F.; Fernandez, J.M.; Acien, F.G.; Rueda, A.; Perez-Parra, J.; Molina, E. Influence of culture conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*. *Process Biochem*. **2008**, *43*, 398–405.
21. Shi, X.M.; Jiang, Y.; Chen, F. High-yield production of lutein by the green microalga *Chlorella protothecoides* in heterotrophic fed-batch culture. *Biotechnol. Prog*. **2002**, *18*, 723–727.
22. Fischer, R. Isolation of Mutants, a Key for the Analysis of Complex Pathways and for Strain Improvement. In *Microbes for Health, Wealth and Sustainable Environment*; Verma, A., Ed.; Malhotra Publishing House: New Delhi, India, 1998; pp. 739–751.
23. Chen, Y.; Li, D.; Lu, W.; Xing, J.; Hui, B.; Han, Y. Screening and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. *Biotechnol. Lett*. **2003**, *25*, 527–529.
24. Tjahjono, A.E.; Hayama, Y.; Kakizono, T.; Terada, Y.; Nishio, N.; Nagai, S. Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. *Biotechnol. Lett*. **1994**, *16*, 133–138.
25. Jin, E.; Melis, A. A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. *Biotechnol. Bioeng*. **2003**, *81*, 115–124.
26. Bar, E.; Rise, M.; Vishkantsan, H.; Arad, S. Pigment and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. *J. Plant Physiol.* **1995**, *146*, 527–534.
27. Bousiba, S.; Vonshak, A. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol*. **1991**, *32*, 1077–1082.
28. Fernandez-Sevilla, J.M.; Acien, F.G.; Molina, E. Biotechnological production of lutein and its applications. *Appl. Microbiol. Biotechnol*. **2010**, *86*, 27–40.
29. Shi, X.M.; Zhang, X.W.; Chen, F. Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. *Enzym. Microb*. *Technol*. **2000**, *27*, 312–318.
30. Harker, M.; Tsavalos, A.J.; Young, A.J. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. *Bioresour. Technol.* **1996**, *55*, 207–214.
31. Vidhyavathi, R.; Venkatachalam, L.; Sarada, R.; Ravishankar, G.A. Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *J. Exp. Bot.* **2008**, *59*, 1409–1418.

32. Borowitzka, M.A.; Huisman, J.M.; Osborn, A. Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. *J. Appl. Phycol.* **1991**, *3*, 295–304.
33. Li, Y.; Huang, J.; Sandmann, G.; Chen, F. Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). *Planta* **2008**, *228*, 735–743.
34. Huang, J.C.; Wang, Y.; Sandmann, G.; Chen, F. Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*. **2006**, *71*, 473–479.
35. Shi, X.M.; Liu, H.J.; Zhang, X.W.; Chen, F. Production of biomass and lutein by *Chlorella protothecoides* at various glucose concentrations in heterotrophic cultures. *Process Biochem.* **1999**, *34*, 341–347.
36. Ishikawa, E.; Sansawa, H.; Abe, H. Isolation and characterization of a *Chlorella* mutant producing high amounts of chlorophyll and Carotenoids. *J. Appl. Phycol*. **2004**, *16*, 385–393.
37. Zhang, D.H.; Lee, Y.K. Enhanced accumulation of secondary carotenoids in a mutant of the green alga, *Chlorococcum* sp. *J. Appl. Phycol*. **1997**, *9*, 459–463.
38. Kamath, B.S.; Vidhyavathi, R.; Sarada, R.; Ravishankar, G.A. Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants. *Bioresour. Technol.* **2008**, *99*, 8667–8673.
39. Arnon, D.I.; McSwain, B.D.; Tsujimoto, H.Y.; Wada, K. Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim. Biophys. Acta* **1974**, *357*, 231–245.
40. Cordero, B.F.; Obraztsova, I.; Martin, L.; Couso, I.; Leon, R.; Vargas, M.A.; Rodriguez, H. Isolation and characterization of a lycopene β-cyclase gene from the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). *J. Phycol.* **2010**, *46*, 1229–1238.

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Capítulo II

Aumento de la biosíntesis de carotenoides en *Chlamydomonas reinhardtii* mediante transformación nuclear, utilizando el gen de la fitoeno sintasa aislado de *Chlorella zofingiensis*

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*

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Abstract The isolation and characterization of the phytoene synthase gene from the green microalga *Chlorella zofingiensis* (*CzPSY*), involved in the first step of the carotenoids biosynthetic pathway, have been performed. *CzPSY* gene encodes a polypeptide of 420 amino acids. A single copy of *CzPSY* has been found in *C. zofingiensis* by Southern blot analysis. Heterologous genetic complementation in *Escherichia coli* showed the ability of the predicted protein to catalyze the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene. Phylogenetic analysis has shown that the deduced protein forms a cluster with the rest of the phytoene synthases (PSY) of the chlorophycean microalgae studied, being very closely related to PSY of plants. This new isolated gene has been adequately inserted in a vector and expressed in *Chlamydomonas reinhardtii*. The over-expression of *CzPSY* in *C. reinhardtii*, by nuclear transformation, has led to an increase in the corresponding *CzPSY* transcript level as well as in the content of the carotenoids violaxanthin and lutein which were 2.0- and 2.2-fold higher than in untransformed cells. This is an example of manipulation of the carotenogenic pathway in eukaryotic microalgae, which can open up the possibility of enhancing the productivity of commercial carotenoids by molecular engineering.

Keywords Carotenoids · *Chlorella zofingiensis* · Phytoene synthase · Transgenic microalgae · *Chlamydomonas reinhardtii*

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Introduction

Carotenoids are isoprenoids synthesised by all photosynthetic organisms and by some fungi and non-photosynthetic bacteria. In photosynthetic organisms, carotenoids bind to integral membrane proteins of the thylakoid where they participate in light harvesting and in the protection of the photosynthetic apparatus against the photo-oxidative damage (Frank and Cogdell 1996; Varkonyi et al. 2002). Carotenoids are high-value compounds, being used as nutraceuticals and as natural dyes and additives in food, feed, aquaculture, and cosmetic industries. They are considered effective agents for the prevention of a variety of age-related, degenerative and chronic diseases such as cataracts, macular degeneration, atherosclerosis, cardiovascular diseases, and some types of cancer (Dweyer et al. 2001; Demming-Adams and Adams 2002; Guerin et al. 2003; Olmedilla et al. 2003; Stahl and Sies 2005).

In chloroplasts of plants and algae, the carotenoids precursor, geranylgeranyl pyrophosphate (GGPP), is formed by the action of GGPP synthase from isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are derived from deoxyxylulose 5-phosphate pathway. GGPP is not only the precursor for carotenoids but also participate in the synthesis of other terpenoid compound, such as phytol, plastoquinones, and

tocopherols. The condensation of two GGPP molecules produces the first carotene, phytoene, catalyzed by PSY (Fig. 1). Phytoene is desaturated by phytoene and ζ-carotene desaturases (PDS and ZDS) and isomerized by *15-cis*-ζ-carotene isomerase (Z-ISO) (Chen et al. 2010) and carotene isomerase (CRTISO) to form the linear *all trans*-lycopene. The cyclation of lycopene by lycopene ε-cyclase (LCYe) and lycopene β-cyclase (LCYb) introduces ε- and β-ionone end groups, respectively, yielding α- and β-carotenes. α- and β-carotene are hydroxylated into lutein and zeaxanthin, respectively. Two P450 hydroxylases (P450b-CHY and P450e-CHY) are active in α-carotene hydroxylation. P450b-CHY is also active in β-carotene hydroxylation, together with two non-heme di-iron hydroxylases (CHYb). Zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) catalyze the interconversion of zeaxanthin and violaxanthin (Kim et al. 2009; Sandmann et al. 2006). A limited number of organisms including some green algae as *Haematococcus pluvialis* and *Chlorella zofingiensis* can synthesize astaxanthin from β-carotene by the action of a ketolase/oxygenase (BKT) and the hydroxylase (CHYb) (Fan et al. 1995; Huang et al. 2006; Li et al. 2008a).

Although the regulatory mechanisms that control carotenoid biosynthesis are poorly understood, there is abundant evidence to indicate that the reaction catalyzed by PSY, first committed step of the carotenoid synthesis, is an important control point for the regulation of carbon flux into and through the pathway (Fraser et al. 2002; Sandmann et al. 2006). The high economic value of carotenoids as nutritional sources of vitamin A and health- promoting compounds has stimulated research to increase carotenoid biosynthesis in crop plants through genetic manipulation of the pathway. Over-expression of bacterial or plant phytoene synthase genes in higher plants has resulted in a significant increase in total carotenoid levels in tomato and Hongkong kumquat (*Fortunella hindsii*) Swingle fruit (Fray et al. 1995; Fraser et al. 2002; Zhang et al. 2009), *Canola* and *Arabidopsis* seeds (Shewmaker et al. 1999; Lindgren et al. 2003), rice endosperm (Paine et al. 2005), potato tuber (Ducreux et al. 2005), and carrot (Baranski 2008). In microalgae, only a few works describe genetic manipulation of the carotenogenic pathway. Silencing, via RNA interference, of PDS in *Dunaliella* (Sun et al. 2007) and *Chlamydomonas* (Vila et al. 2007), silencing of the *Chlamydomonas PSY* gene by artificial microRNAs (Molnar et al. 2009), transformation of *H. pluvialis* with a modified *PDS* (Steinbrenner and Sandmann 2006), and the production of a new ketocarotenoid in *Chlamydomonas* through the expression of a foreign β-carotene oxygenase (*BKT*) gene (León et al. 2007) are some examples. The chlorophyta *C. zofingiensis* accumulates high amounts of astaxanthin and lutein (Del Campo et al. 2004; Sun et al. 2008) and is considered as a model organism to study the regulation of the carotenoids biosynthetic pathway, since it produces the primary carotenoid lutein as well as the secondary carotenoid astaxanthin. However, only the carotenogenic genes *BKT* (Huang et al. 2006), *PDS* (Huang et al. 2008), *CHYb* (Li et al. 2008b), and *LCYb* (Cordero et al. 2010) have been isolated and characterized in this microalga until now. In addition, nuclear transformation in this microalga has never been accomplished. *Chlamydomonas reinhardtii* is the first and best studied transformed chlorophyte, it grows at high rates, and its nuclear genetic manipulation is easy and well established. This makes *C. reinhardtii* a good candidate to express foreign carotenogenic genes for the biotechnological production of commercially interesting carotenoids and for carrying out basic metabolic and regulatory studies of the pathway (León et al. 2004).

In the present work, we report the isolation and characterization of the *PSY* gene from *C. zofingiensis*, as well as its ability to convert two GGPP molecules into phytoene. Moreover, this novel gene has been inserted in an adequate vector and expressed in *C. reinhardtii*. This is an example of the over-expression of an exogenous gene (*PSY*) in an eukaryotic microalgae, which can be an interesting tool for the massive production of carotenoids in transgenic microalgae by genetic engineering.

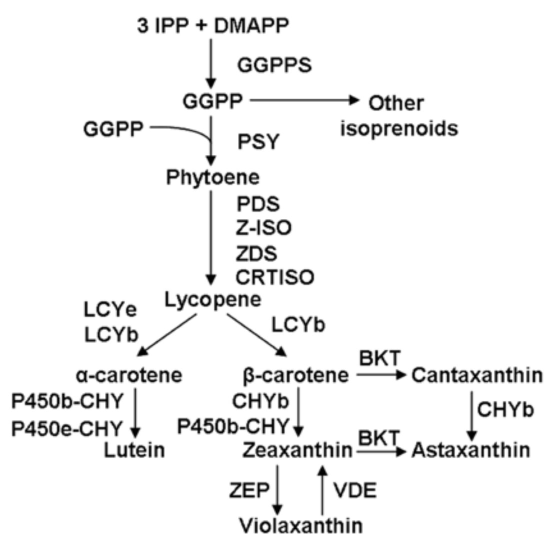

Fig. 1 Schematic diagram of the carotenoid biosynthetic pathway in plants and microalgae. Phytoene synthase (*PSY*) catalyses the first step in the carotenoid specific pathway, which leads the carbon flux towards carotenes and xantophylls production. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *GGPPS* geranylgeranyl pyrophosphate synthase, *PDS* phytoene desaturase, *Z-ISO 15-cis*-ζ-carotene isomerase, *ZDS* ζ-carotene desaturase, *CRTISO* carotene isomerase, *LCYb* lycopene β-cyclase, *LCYe* lycopene ε-cyclase, *P450b-CHY* cytochrome P450 β-hydroxylase, *P450e-CHY* cytochrome P450 ε-hydroxylase, *CHYb* carotene β-hydroxylase, *BKT* β-carotene oxygenase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase.

Materials and methods

Strains and culture conditions

The green microalga strain *C. zofingiensis* SAG 211–14 was obtained from the Culture Collection of Göttingen University (SAG, Germany). This microalga was grown photoautotrophically in Arnon medium (Arnon et al. 1974) modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$, at 25°C under continuous illumination (50 µmol photons m^{-2} s^{-1}). The light intensity was measured at the surface of the flasks using a LI-COR quantum sensor (model L1-1905B, Li-Cor, Inc. Lincoln, NE, USA). The liquid cultures were continuously bubbled with air supplemented with 1% (v/v) CO_2 as the only source of carbon. *C. reinhardtii* cell-wall-deficient strain 704 was kindly provided by Dr. Roland Loppes (Loppes et al. 1999) and cultured mixotrophically in either liquid or agar solidified Tris-acetate phosphate (TAP) medium (Gorman and Levine 1965) at 25°C under a continuous irradiance of 50 µmol photons m^{-2} s^{-1}. *Escherichia coli* DH5α and BL21 (DE3) strains were used as the hosts for DNA manipulation and for heterologous expression of *PSY* gene, respectively.

For the analysis of transformants, cells were grown in Erlenmeyer flasks of 100 mL capacity at 25°C under continuous illumination (50 µmol photons m^{-2} s^{-1}) in liquid TAP medium.

Genomic DNA and RNA isolation and cDNA preparation

DNA and total RNA were isolated using DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen, Düsseldorf, Germany), respectively. For genomic DNA isolation for PCR screening of transformants from *C. reinhardtii*, a loopful of cells was scrapped from a plate and resuspended in 150 µL of cold distilled water and 350 µL of a buffered solution containing 50 mM Tris–HCl, pH 8, 0.3 M NaCl, 5 mM EDTA, and 2% SDS. The DNA isolation was performed by phenol-chloroform-isoamyl alcohol (50:48:2) extraction and selective precipitation with ethanol, according to previously described protocols (Anwaruzzaman et al. 2004). For quantitative real-time PCR analysis (qRT-PCR), first-strand cDNA synthesis was obtained from total RNA treated with DNase as recommended by the manufacturer, by using the SuperScript First-Strand Synthesis System (Invitrogen, Barcelona, Spain) primed with oligo(dT)18 according to the manufacturer's instructions.

Cloning of *C. zofingiensis* PSY cDNA and genomic gene

For isolating the cDNA clone coding for the *C. zofingiensis* PSY homologue, amino acid sequences deduced from previously cloned *PSY* genes from different kinds of algae, cyanobacteria, and plants were aligned. Highly conserved regions were identified, and different pairs of degenerated primers were designed. The PCR product was cloned in the pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's manual and then sequenced. The cDNA fragment obtained corresponding to partial *PSY* clone provided sequence information for the designing of genespecific primers for amplification of 5′ and 3′ cDNA ends by RACE-PCR. All reactions were performed with kits according to the manufacturer's instructions (Smart RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA). 5′ and 3′ RACE products were cloned into pGEM-T vector and sequenced. Specific primers were synthesized for genomic DNA amplification based on cDNA sequence. The primers sets used in this study are listed in Table 1.

Nucleotide sequence accession numbers

The *CzPSY* cDNA and genomic DNA sequences have been registered in the EMBL database (EMBL, FR670783 and EMBL, FR670784, respectively).

Sequencing and phylogenetic analysis

The deduced amino acid sequence of the *C. zofingiensis* PSY was compared with other PSY sequences of algae, plants, cyanobacteria, and bacteria. The sequence analysis and alignments were done with CLUSTAL_W software. The deduced amino acid sequence was subjected to the ProtParam application at the ExPASy server (Gasteiger et al. 2005) for physical and chemical parameters, program Predotar v. 1.03 (Caboche 2003) for the prediction of possible plastid localization, and ChloroP 1.1 server (Emanuelsson et al. 1999) for the identification of a chloroplast transit peptide. For transmembrane analysis, ProtScale (Gasteiger et al. 2005) and TopPred (von Heijne 1992) servers were used. The construction of a phylogenetic tree was performed in MEGA4 (Tamura et al. 2007) using the UPGMA method.

Southern blot analysis

Genomic DNA was digested with *Hinc*II and *Hind*III, which showed one and no recognition site, respectively, in the probed region of the *PSY* gene. The probe was prepared by amplifying genomic DNA with the primers Czpsy-S-F and Czpsy-S-R, resulting in a 752-bp fragment of *CzPSY* gene. The digested DNA was transferred to a Hybond-N membrane (GE Healthcare, Little Chanfont, UK) by capillary transfer and hybridized with the 32P labeled DNA probe at both low and high stringency overnight. After hybridization, the radioactivity of the membrane was monitored by the Cyclone Phosphor System (Perkin-Elmer, Waltham, MA, USA).

Table 1 Nucleotide sequences of primer pairs used for PCR amplification

Primer	Sequence (5′→ 3′)
Partial PSY fragment	
psy-1F	GAYATGATHGARGGNATG
psy-1R	AARTTRTCRTARTCRTT
5′and 3′ RACE	
GSP-F	ATGAATTTAGTCAAGTCACGG
GSP-R	TTTGGGCACATAAGCACG
NGSP-F	CTGGATGAGAAGGCAAGG
NGSP-R	CCTTGCCTTCTCATCCAG
Genomic DNA amplification	
psy-2F	ACATGGGGGCGAATTTGTTGGT
psy-2R	CCCGTGCCTGCTTAGGAGTCAT
psy-3F	ATGCGGTGCGGTTACCAGTGAA
psy-3R	ACCTGTCATCTATCTTGCCTGT
psy-4F	ACAGGCAAGATAGATGACAGGT
psy-4R	CTCTCCAAAGTTAAGATGAACA
psy-5F	TGTTCATCTTAACTTTGGAGAGTTT
psy-5R	AGCACGCATTACAAGGCTCC
PCR for probe preparation	
Czpsy-S-F	ATGGATTTAGTCAAGTCACGG
Czpsy-S-R	TTTGGGCACATAAGCACG
Genetic complementation-pQE-80L[a]	
pQE-psy-F	ggatccATGGCGTCGTTTAGCACCAGG
pQE-psy-R	ggtaccTTAGCTGCTGGTTGCCGCAGC
PCR for *Chamydomonas reinhardtii* transformation[b]	
CzpsyCr105-F	cgcctcgagTATGGCGTCGTTTAGCACCAG
CzpsyCr105-R	cgcgaattcTTAGCTGCTGGTTGCC
CzPSY expression	
RT-Czpsy-F	CACCAGGTTGTCAGAGTCCA
RT-Czpsy-R	ACTAGTGTGTTGCTGACTCT
CrPSY expression	
RT-Crpsy-F	CACTCGCGCCCGGCAATACTT
RT-Crpsy-R	CCACGGGCAGCGACACCATC
CBLP expression	
RT-cblp-F	CGCCACCCAGTCCTCCATCAAGA
RT-cblp-R	CTAGGCGCGGCTGGGCATTTAC

F forward, *R* reverse

[a] *Bam*HI and *Kpn*I sites (lowercases underlined) were added for cloning the gene into the corresponding cut sites of pQE-80L vector

[b] *Xho*I and *Eco*RI sites (lowercases underlined) were added for cloning the gene into the corresponding cut sites of pSI105 plasmid

Functional analysis of *CzPSY* cDNA by heterologous expression in *E. coli*

The *CzPSY* ORF was amplified by PCR with the primers pQE-psy-F and pQE-psy-R, which were designed to contain *Bam*HI and *Kpn*I restriction sites, respectively, and cloned between the *Bam*HI and *Kpn*I restriction sites of the pQE-80L expression vector (Qiagen), resulting in plasmid pQE-CzPSY, which carries ampicillin resistance.

Plasmid pAC-85b, a gift from Prof. Cunningham, contained the carotenoid pathway genes responsible for the synthesis of β-carotene except *PSY* gene (*crtE*, *crtI*, and *crtY* of *Erwinia herbicola*) (Cunningham and Gantt 2007). Transformation of *E. coli* BL21 (DE3) with either pAC-85b (carrying chloramphenicol resistance) or pAC-85b and one of the two plasmids, pQE-*CzPSY* or pQE-80L (empty vector), was made by electroporation. Transformed cells were plated on Luria–Bertani solid medium (Sambrook et al. 1989), supplemented with 100 μg mL^{-1} ampicillin and/or 40 μg mL^{-1} chloramphenicol, and grown at 37°C for 1 day. The inducer isopropyl-β-D-1-thiogalactopyranoside was added at a final concentration of 1 mM.

Chlamydomonas nuclear transformation

The complete coding region of *CzPSY* was amplified by PCR with primers CzpsyCr105-F and CzpsyCr105-R, which were designed to contain *Xho*I and *Eco*RI restriction sites respectively, and cloned into pGEM-T vector (Promega), which carries ampicillin resistance. The PCR product was digested with *Xho*I and *Eco*RI and cloned in the pSI105-Tp1 vector, resulting in the plasmid pSI105-Tp1-psy that was used to transform *Chlamydomonas* cells. The plasmid pSI105-Tp1 is based on the plasmid pSI104-PLK (León et al. 2007), derived from the pSI103 (Sizova et al. 2001), 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*. The construction pSI105-Tp1 also carries a second expression cassette driven by the same two constitutive promoters and terminator region. This second cassette carries the transit peptide of RuBisCO small subunit (*rbcS2*) to target the final peptide into the chloroplast stroma and also carries a polilynker region in which the cDNA from *C. zofingiensis PSY* was subcloned in frame with the transit peptide sequence.

Nuclear transformation was carried out using the glass beads method of Kindle (1990) with some modifications (León et al. 2007). *C. reinhardtii* cells grown to about 107 cells mL^{-1} 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 containing 0.3 g of sterile glass beads (Ø 0.4–0.6 mm), 0.2 mL of 20% polyethylene glycol (MW8000) and 1 μg of the desired plasmid. Cells were vortexed and resuspended in 50 mL of fresh sterile TAP medium where they were incubated in the dark overnight. After this incubation, cells were centrifuged

and spread onto solid TAP medium with paromomycin (30 µg mL^{-1}). Transformed colonies were visible after 4 to 5 days.

Quantitative RT-PCR

The mRNA relative abundance of endogenous *C. reinhardtii PSY* and foreign *C. zofingiensis PSY* was examined by qRT-PCR on an IQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA), according to Cordero et al. (2010). In each experiment, a series of standard dilutions containing a specific concentration of a PCR fragment or a cDNA template was amplified in 20 µL of reaction containing 1×SYBR Green PCR Master Mix (Quantimix Easy SYG kit, BioTools B&M Labs, Madrid, Spain) and corresponding primers for either *PSY* from *C. zofingiensis* or *C. reinhardtii* (Table 1). After heating at 95°C for 10 min, cycling parameters were: 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Finally, the specificity of the qRT-PCR products was confirmed by performing a melting temperature analysis at temperatures ranging from 55°C to 95°C at 0.5°C/min and also by electrophoresis on a 2% agarose gel. Data were captured as amplification plots. Transcription levels of the target genes were calculated from the threshold cycle by interpolation from the standard curve. To standardize the results, the relative abundance of *CBLP* gene, which encodes a G-protein β-subunit-like polypeptide (von Kampen et al. 1994), was also determined and used as the internal standard. The complete experiments (RNA isolation, cDNA synthesis followed with qRT-PCR) were repeated twice independently, and the data were averaged.

Analytical methods

Cell concentration and dry weight determinations

Cell number was determined with a Neubauer hemocytometer. For dry weight measurements, aliquots (5 mL) of the cell culture were filtered through Whatman GF/C paper (Whatman plc, Kent, UK), washed three times, and dried at 80°C for 24 h.

Carotenoid extraction and HPLC analysis

Total pigments were extracted with 80% of acetone (v/v) according to León et al. (2005). Then the samples were centrifuged and analysed by HPLC using a Waters Spherisorb ODS2 column (4.6×250 mm, 5 µm particle size) (Waters, Mildford, MA, USA). The chromatographic method described by Cordero et al. (2010) was used. Pigments were eluted at a flow rate of 1.0 mL min^{-1} and were detected at 440 nm using a Waters 2996 photodiode-array detector.

Identification of carotenoids was achieved by comparison of the individual characteristic absorption spectrum and the retention time with known standards. Quantification was performed using a calibration curve generated with commercially available carotenoids standards from Sigma-Aldrich (St. Louis, MO, USA) and DHI (Holsholm, Germany).

Results

Isolation and characterization of the *PSY* gene and deduced protein from *C. zofingiensis*

Different pairs of degenerate primers were designed on the basis of the conserved motifs present in *PSY* from microalgae, cyanobacteria, and plants. A partial cDNA fragment of 1,337 bp was isolated by PCR amplification using degenerate primers (psy-1F and psy-1R) (Table 1). A complete BLAST homologous search in the Genbank database showed that this fragment had enough similarity with the *PSY* gene from other species, and provided sequence information for designing specific primers for rapid amplification of 5′and 3′ cDNA ends (RACE-PCR). This analysis generated a full-length cDNA of 2,944 bp, which contained an ORF of 1,263 bp, 310-nucleotides of 5 -untranslated region (UTR), and a long 3 UTR of 1,340 nucleotides. A typical algal polyadenylation signal TGTAAA (Gruber et al. 1992) was present in the ′3 UTR at 18 nucleotides upstream from the beginning of the poly(A) tail. The predicted protein has 420 amino acids residues, with an estimated molecular weight of 47.64 kDa, a theoretical isoelectric point of 8.53 and an instability index of 48.41 (data obtained with ProtParam program).

The differences between the *C. zofingiensis PSY* gene and the cDNA sequence were compared and revealed the presence of four exons and three introns (Fig. 2).

To determine the copy number of *PSY* gene in the genome of *C. zofingiensis*, genomic DNA was digested with two different restriction enzymes (either *Hinc*II or *Hind*III) and subjected to Southern blot analysis at different conditions of stringency. Using a 752-bp fragment of *CzPSY* as a probe, strong hybridization signals have been obtained with both digestions. The digestion with *Hinc*II enzyme, which cuts once inside the probe sequence, showed two bands, while digestion with *Hind*III, with no restriction site in the probe, exhibited only one band (Fig. 3). These results have suggested the presence of a single copy of the *PSY* gene in the genome of *C. zofingiensis*.

The BlastP search results demonstrated that the cloned CzPSY showed the highest overall homology sequence with other PSY from green algae, such as *Dunaliella salina* and *Dunaliella* sp. (identity, 74% and similarity, 84%) and *C. reinhardtii* (identity, 70% and similarity, 84%).

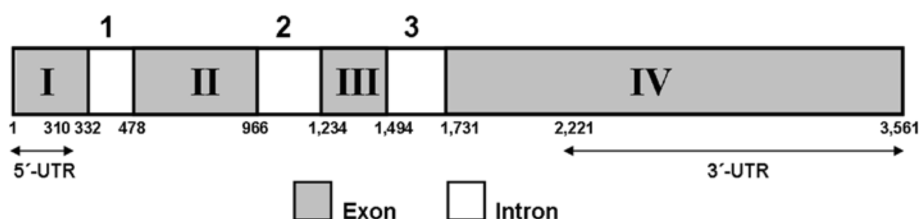

Fig. 2 *CzPSY* gene organization. The diagram shows exons (*I–IV*) and introns (*1–3*) location. The 5′ UTR and 3′ UTR sequences are indicated with arrows. *Numbers* indicate cDNA sequence coordinates (bp)

The GC content of the *CzPSY* coding region was 53%, which was lower than that of *D. salina* (64%) or of *C. reinhardtii* (59%). The phylogenetic analysis of the PSY from microalgae, cyanobacteria, plants, and bacteria is illustrated in Fig. 4. Analysis was conducted in MEGA4 (Tamura et al. 2007) using the UPGMA method. The predicted CzPSY forms a cluster with the rest of the microalgae studied, which are phylogenetically closely related to PSY of plants (between 65% and 70% of identity and around 75–80% similarity). The degree of homology was lower with cyanobacterial phytoene synthases (55–60% identity and 65–70% similarity). As other algal PSY, CzPSY, was distantly related to bacterial PSY (CRTB), sharing with them only a few conserved motifs and less than 40% identity.

Since in microalgae and plants PSY is located in the chloroplast, we analysed the CzPSY sequence with two different programs to determine the presence of a signal peptide. The Predotar v. 1.03 program predicted putative plastid localization for the CzPSY, and ChloroP 1.1 server identified an N-terminal chloroplast transit peptide at position 45. Analysis with ProtScale and TopPred servers identified a deduced transmembrane domain of CzPSY located between amino acids 236 and 256.

Functional analysis of the *CzPSY* in *E. coli*

The use of *E. coli* engineered to produce different carotenogenic substrates is an efficient and frequently used method for the functional characterization of the enzymes of the carotenoid biosynthetic pathway (Cunningham and Gantt 2005; Cunningham et al. 1996), due to the complexity of determining the activity of these membrane-associate and low-abundant enzymes.

In order to check the functionality of the recently isolated gene, the full-length ORF of *CzPSY* was amplified and cloned into pQE-80L vector under the control of the β-galactosidase promoter. The resulting plasmid (pQE-*CzPSY*) was introduced in *E. coli* that carried the plasmid pAC-85b, which contained the carotenogenic genes responsible for the synthesis of β-carotene as final product except *PSY* gene. HPLC analysis of carotenoids extracted from *E. coli* showed that cells containing pAC-85b and pQE-*CzPSY* produced β-carotene (Fig. 5). As negative controls, *E. coli* co-transformed with pAC-85b or pAC-85b and empty pQE-80L were used, resulting in no accumulation of carotenoids. These results confirmed the functionality of *CzPSY* gene product, which could catalyze the conversion of two molecules of GGPP into phytoene, as most PSY previously isolated.

Nuclear transformation of *C. reinhardtii* with *PSY* gene from *C. zofingiensis* and screening of transformants obtained

The complete coding region of *CzPSY* gene was amplified by PCR and cloned between the *Xho*I and *Eco*RI restriction sites of the *Chlamydomonas* expression vector pSI105-Tp1, resulting in the plasmid pSI105-*psy*. *C. reinhardtii* cells were

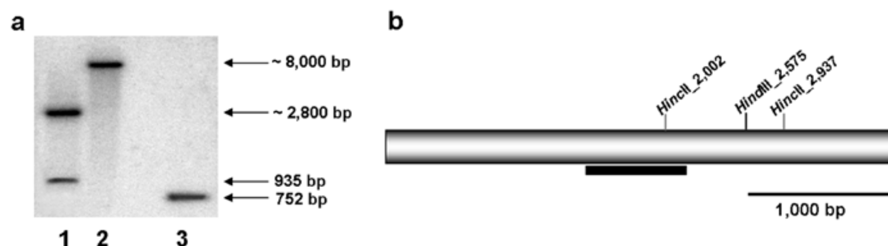

Fig. 3 Southern blot analysis of genomic DNA from *C. zofingiensis*. **a** DNA was digested with *Hinc*II (lane 1) and *Hind*III (lane 2), electrophoretically separated on a 0.8% agarose gel, blotted and hybridized at high stringency with a probe of 752 bp of the *PSY* gene amplified by PCR. A plasmid containing the *PSY* gene was used as a positive control (lane 3). **b** *Hinc*II and *Hind*III restriction sites present in the *CzPSY* gene. The black bar indicates the probe location.

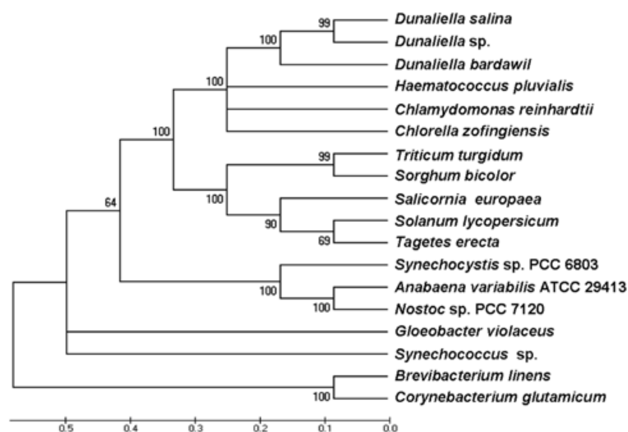

Fig. 4 UPGMA tree analysis of the indicated plant, algal, cyanobacterial, and bacterial PSY amino acid sequences. Analysis was performed in MEGA4 (Tamura et al. 2007). The GenBank accession numbers for other species are as follows: *Dunaliella salina*, AAT46069; *Dunaliella* sp., ABE97388.1; *Dunaliella bardawil*, AAB51287.1; *Haematococcus pluvialis*, AAW28851.1; *Chamydomonas reinhardti*i, XP_001701192.1; *Triticum turgidum*, ACQ59152.1; *Sorghum bicolor*, ACY70869; *Salicornia europaea*, AAX19898.1; *Solanum lycopersicum*, ABM45873.1; *Tagetes erecta*, AAG10427.1; *Synechocystis* sp. PCC 6803, BAA17848.1; *Anabaena variabilis* PCC 29413, YP_325286.1; *Nostoc* sp. PCC 7120, BAB73532.1; *Gloeobacter violaceus* PCC 7421, NP_924690.1; *Synechococcus* sp. JA-3-3-Ab, YP_473801.1; *Brevibacterium linens*, AAF65581.1; *Corynebacterium glutamicum*, AAK64298.1. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances correspond to the number of amino acid substitutions per site and were computed using the Poisson correction method. Numbers at nodes indicate bootstrap values calculated over 500 replicates.

transformed with plasmid pSI105-*psy* and the transformants were firstly selected on the basis of their paromomycin resistance. The colonies obtained after transformation that showed resistance to paromomycin were screened for the insertion of *CzPSY* cDNA in their genome by PCR test. Figure 6 shows some of the positive transformants analyzed exhibiting a band of 147 bp, which correspond to the *CzPSY* cDNA integrated in their genome. The primers used for PCR analysis to confirm this integration were the same used for the expression analysis of *CzPSY* (Table 1). More than 100 colonies resistant to paromomycin were analysed and 80% of them were found positives. Some of these positive transformants selected by PCR were grown in liquid TAP medium and their carotenoids content analysed by HPLC and the relative mRNA levels of *C. reinhardtii PSY* (*CrPSY*) and *CzPSY* genes determined by qRT-PCR.

Analysis of carotenoids content and mRNA levels of *CrPSY* and *CzPSY* genes in both parental and *CzPSY*-transformed strains of *C. reinhardtii*

The mRNA relative abundance of endogenous (*CrPSY*) and exogenous (*CzPSY*) phytoene synthase genes of parental and *CzPSY*-transformant strains of *C. reinhardtii* was monitored by qRT-PCR, and changes in cellular carotenoids content were determined in order to correlate transcript levels with the biosynthesis of those specific carotenoids. Figure 7 shows carotenoids content and mRNA levels of *CrPSY* and *CzPSY* genes in parental and six selected *CzPSY* transformants. The carotenoid profile of parental and *CzPSY* transformed *C. reinhardtii* cells were very similar, being violaxanthin the major carotenoid, followed by lutein and β-carotene, α-carotene showing the lowest levels. However, some of the transformants (approximately 10% of the positives found) exhibited a violaxanthin and lutein content of 1.8- to 2.0- and 2.0- to 2.2-fold higher than the parental strain, respectively, whereas β-carotene and α-carotene levels were virtually identical to those of the wild type strain. T10 and T11 are two representative examples of these violaxanthin and lutein hyperproducing mutants (Fig. 7a). Other transformants

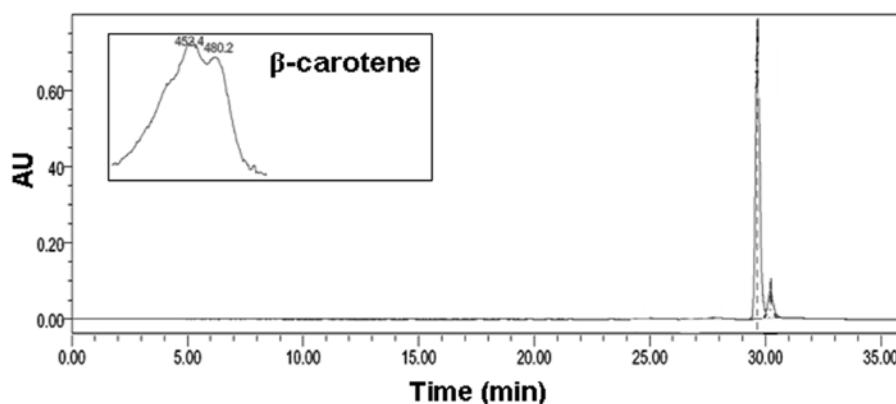

Fig. 5 HPLC elution profile of carotenoids extracted from cultures of *E. coli* carrying plasmids pAC-85b+pQE-*CzPSY*. The absorption spectrum of β-carotene is also shown. *E. coli* BL21 (DE3) colonies transformed with the indicated plasmids were isolated in the presence of chloramphenicol+ampicillin. Peak identification, β-carotene

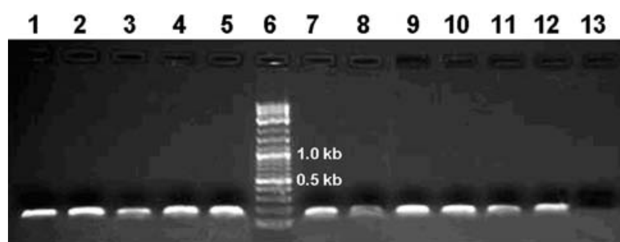

Fig. 6 Verification of the insertion of the plasmid pSI105-*CzPSY* in the genome of *C. reinhardtii* by PCR. *C. reinhardtii* cells transformed with plasmid pSI105-*CzPSY* were grown in TAP medium with paromomycin (30 $\mu g\ ml^{-1}$), and paromomycin -resistant colonies were tested by PCR. Line 6 is the 2-log DNA ladder (0.1–10 kb, Biolabs). Lines 1–5 and 7–11 are transformants analysed. Lines 12 and 13 are wild strain from *C. zofingiensis* and *C. reinhardtii*, respectively.

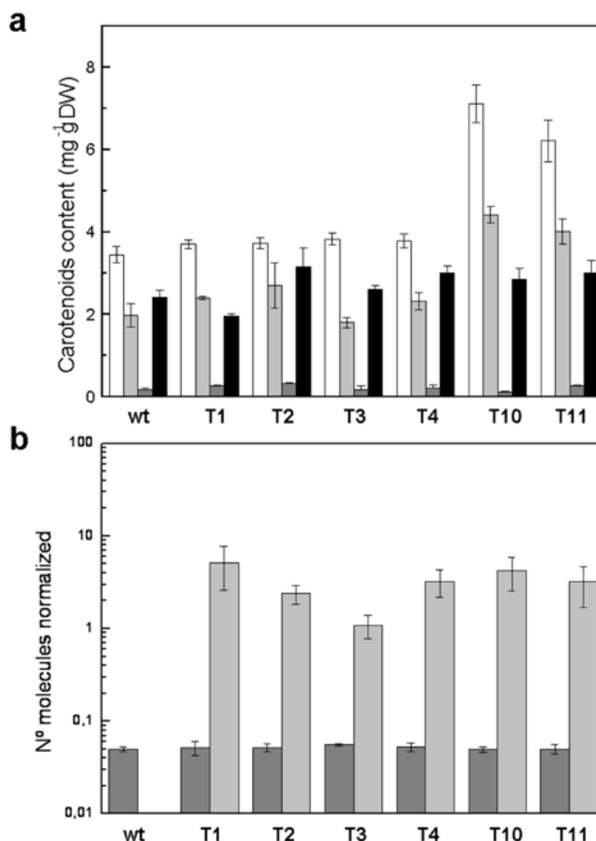

Fig. 7 Carotenoids content **(a)** and mRNA relative abundance of endogenous *CrPSY* and foreign *CzPSY* **(b)** in cells of *C. reinhardtii* wild type (wt) and six selected transformants (T1, T2, T3, T4, T10, and T11). **a** Violaxanthin (white bar), lutein (light-grey bar), α-carotene (dark grey bar), and β-carotene (black bar). **b** Levels of *PSY* transcripts were normalized respect to the housekeeping control gene (*CBLP*). Endogenous *CrPSY* (dark-grey bar); foreign *CzPSY* (light-grey bar). Error bars indicate the standard deviations of four independent measurements

showed only a slight increase in the levels of violaxanthin and lutein, although in all of them the exogenous *PSY* gene was adequately transcribed and high levels of *CzPSY* mRNA were found.

Regarding the expression analysis, in all the transformants analysed the levels of *CzPSY* mRNA were higher than those of the endogenous *PSY*, since exogenous *CzPSY* was constitutively expressed under the control of the strong promoters *rbcS2* and *hsp70A*. Transformants T1 and T10 showed the highest *CzPSY* levels, which reached 140-fold the level of the endogenous *PSY*. Expression levels of endogenous *PSY* in transformants were quite similar to that of the wild type cells. Levels of *PSY* transcripts were standardized respect to the housekeeping control gene (*CBLP*) and expressed as numbers of normalized molecules.

Discussion

Phytoene synthase catalyzes the first step of the carotenoid biosynthetic pathway and is considered as a rate-limiting key enzyme in this pathway (Cunningham and Gantt 1998) and as the branching enzyme that determines the carbon flux towards carotenoids production (Shewmaker et al. 1999). Therefore, PSY has been the target of genetic manipulation in many crop plants to increase carotenoid biosynthesis (Fray et al. 1995; Fraser et al. 2002; Lindgren et al. 2003). The *CzPSY* gene isolated in this work, as well as the known *PSY* genes of eukaryotic microalgae, have a smaller number of exons than the common five or seven exons in plants. Southern blot analysis has demonstrated that in *C. zofingiensis*, as in other microalgae as *C. reinhardtii* and *D. salina* (McCarthy et al. 2004; Lohr et al. 2005; Yan et al. 2005), only a single gene coding for PSY is present. However, recently, it has been described that other algae as *Dunaliella bardawil* and *Micromonas pusilla* contain either multiple paralogous or orthologous copies of the *PSY*, which could be expected that analogous to the diversity of *PSY* genes and their differential expression in higher plants, some algae also differentially could regulate expression of their multiple copies of *PSY* gene. The comparative analyses of various algal genomes for PSY in combination with phylogenetic analyses have suggested an ancient gene duplication creating two classes of PSY (Tran et al. 2009). Our results indicate that only one class of PSY seems to be present in *C. zofingiensis*. Alignments at the protein level have indicated that CzPSY has the essential characteristics of both classes of PSY including predicted substrate-Mg^{2+}-binding sites (Aspartate-rich regions) and catalytic residues (data not shown). Moreover, the alignments have showed that the sequence differences between plant and bacterial PSY are mainly found at the N terminus due to the presence of a signal peptide responsible for the localization of these enzymes in chloroplasts and chromoplasts (Cunningham and Gantt 1998). The high homology degree found between the predicted protein encoded by the new gene isolated from *C. zofingiensis* and plant phytoene synthases, specially with

the known PSY of other green microalgae, would have been probably enough to consider this new gene as a PSY, but functional analysis has definitely confirmed this hypothesis. It has been shown by functional complementation in *E. coli* that this gene encodes phytoene synthase. Although the environment and the processing of the putative *PSY* gene in the prokaryotic system probably differ from those in the algal original system, the obtained gene product was functional and able to catalyze the synthesis of phytoene, complementing plasmid pAC85 and resulting in the accumulation of β-carotene (Fig. 5). This confirms that the isolated gene is responsible for the synthesis of phytoene from two GGPP molecules.

Overexpression of bacterial or plant *PSY* genes in crop plants has resulted in increases in total carotenoid content of about 1.8–6.3-fold in carrot roots (Hauptmann et al. 1997), tomato fruit (Fraser et al. 2002), and potato tubers (Ducreux et al. 2005). Higher carotenoids increases have been reported in plant or tissues with no carotenoids or with very original levels (Farré et al. 2010), such a 50-fold increase in *canola* seeds (Shewmaker et al. 1999). Microalgae combine the fast and easy growth of bacteria with an active isoprenoid metabolism that ensures enough precursors for carotenogenic pathway and adequate storage capacity. The unicellular microalgae *C. reinhardtii* is the first and best studied transformed chlorophyta. It grows at high rates under photoautotrophic, heterotrophic or mixotrophic conditions and its nuclear genetic manipulation is easy and well established. All these reasons make *C. reinhardtii* a good candidate to express foreign carotenogenic genes, for both carrying out basic metabolic and regulatory studies of the pathway as well as for the biotechnological production of commercially interesting carotenoids. As the sequence of the *PSY* from *C. zofingiensis* shares a 76% of identity with that reported for *Chlamydomonas PSY*, the expression of such a gene in the nuclear genome of this microalga is a step forward both in the characterization of the gene and in the increased production of interesting carotenoids in microalgae. In this study, the nuclear transformation of *C. reinhardtii* with *CzPSY* gene resulted in a significant accumulation of violaxanthin and lutein content that reached 2.0- and 2.2-fold, respectively, as compared with the parental level (Fig. 7a). Moreover, transformants showed an overexpression of *C. zofingiensis PSY* gene due to the strong promoters used (Fig. 7b). These results suggest that the higher carbon flux from GGPP to carotenoid synthesis was promoted by a combination of the expression of *CzPSY* gene product in *C. reinhardtii* as well as the expression of the endogenous copy of the gene. The *CzPSY* transcript level reached 140-fold the level of the endogenous *PSY* of the wild type in the transformants T1 and T10, however the violaxanthin and lutein contents in these transformants were only 2.0- and 2.2-fold higher than the parental strain, respectively, in T10 transformant, and 7% and 21% higher, respectively, in T1. Although transcriptional control has been shown to be the most important regulation factor for carotenogenic genes, a possible explanation for these results is that post-transcriptional and translational controls play also important roles, as wells as the stability of RNA, since silencing of the *PSY* endogenous gene by artificial miRNAs has been described in *Chlamydomonas* (Molnar et al. 2009). Moreover, an increase in the levels of PSY cannot be directly correlated with an increase in carotenoids content, since the protein could not be correctly targeted, processed or assembled into a fully functional complex. In transgenic tomato and potato plants expressing a bacterial phytoene synthase (CRTB), despite CRTB enzyme activity being substantially elevated, there was only a moderate increase in total carotenoid content and no linear correlation between the levels of transcript, protein, enzyme activity, and total carotenoids (Fraser et al. 2002; Ducreux et al. 2005).

Overexpression of an exogenous *PSY* in tomato resulted in an accumulation of phytoene, and in a decrease in the flux control coefficient for PSY. Then, the increase in PSY activity shifts probably the regulatory step from PSY to a later enzyme (Shewmaker et al. 1999; Fraser et al. 2002). Accumulation of β-carotene in higher plants over-expressing exogenous *PSY*, probably indicates that β-carotene hydroxylases become rate-limiting as the carotenogenic flux increases (Ducreux et al. 2005). In our study no increase in the content of phytoene or other carotenoids intermediates were detected, showing that other enzymes in the pathway are not limited in *Chlamydomonas*, and that phytoene, β-carotene or other intermediates are not inaccessible to the downstream enzymes of the pathway in the single cell of *Chlamydomonas*. Phytoene was consecutively converted to the downstream metabolites α-carotene, lutein, β-carotene and violaxanthin, catalyzed by endogenous carotenoid biosynthetic enzymes such as PDS, ZDS, LCYb, LCYe, CHYb, and ZEP.

In plants and microalgae, carotenoid biosynthesis is part of the plastidic terpenoid metabolim. GGPP is a common intermediate to the different terpenoid biosynthetic pathway such as carotenoids, chlorophylls, gibberellins and quinones. The engineering of any terpenoid pathway may have a direct effect on other branches of the pathway. In the case of carotenoid overproduction, the increase of carbon flux into the carotenoid pathway can produce limitations especially in the synthesis of gibberellins, chlorophylls, and quinines with

negative effects on growth and photosynthesis, respectively. In some higher plants overexpressing *PSY* genes undesired collateral effects or low carotenoids increase were observed (Farré et al. 2010). The constitutive expression of phytoene synthase genes in tomato (*Lycopersicum esculentum*) and tobacco (*Nicotiana tabacum*) resulted in dwarfism and chlorosis in the plants (Fray et al. 1995; Busch et al. 2002) correlated with a decrease of gibberellins. But so far, in our case, no atypical phenotype was observed in the *Chlamydomonas* transformants, showing even a similar growth rate than the wild type cultures (data not shown).

In conclusion, *C. zofingiensis* is a model green microalga to study the regulation of the carotenoid biosynthetic pathway, since it accumulates both lutein and astaxanthin. In this study the phytoene synthase gene from *C. zofingiensis* has been isolated and functionally characterized. The over-expression of *CzPSY* in *Chlamydomonas* cells under the control of strong constitutive promoters has resulted in a significant enhancement in the content in violaxanthin and lutein, reaching 2.0- and 2.2-fold the values of control untransformed cells, respectively. This is a successful case of manipulation of the carotenogenic pathway in eukaryotic microalgae, which opens up the possibility of enhancing the productivity of microalgal based systems to produce carotenoids and offers an excellent tool to gain basic knowledge about an important pathway that at present is not yet completely characterized and a promising alga for industrial applications.

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References

Anwaruzzaman N, Chin BL, Li XP, Lohr M, Martínez DA, Niyogi KK (2004) Genomic analysis of mutants affecting xanthophylls biosynthesis and regulation of photosynthetic light harvesting in *Chlamydomonas reinhardtii*. Photosynth Res 82:265–276

Arnon DI, McSwain BD, Tsujimoto HY, Wada K (1974) Photochemical activity and components of membrane preparations from blue-green algae.I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. Biochim Biophys Acta 357:231–245

Baranski R (2008) Genetic transformation of carrot (*Daucus carota*) and other apiaceae species. Trans Plant J 2(1):18–38

Busch M, Seuter A, Hain R (2002) Functional analysis of the early steps of carotenoid biosynthesis in tobacco. Plant Physiol 128:439–453

Caboche M (2003) A prediction service for identifying putative N-terminal targeting sequences. Available at: http://urgi.versailles.inra.fr/predotar/predotar.html. Accessed 15 October 2003

Chen Y, Li F, Wurtzel ET (2010) Isolation and characterization of the *Z-ISO* gene encoding a missing component of carotenoid biosynthesis in plants. Plant Physiol 153:66–79

Cordero BF, Obraztsova I, Martín L, Couso I, León R, Vargas MA, Rodríguez H (2010) Isolation and characterization of a lycopene β-cyclase gene from the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 46:1229–1238

Cunningham FX Jr, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:55–583

Cunningham FX Jr, Gantt E (2005) A study in scarlet: enzymes of ketocarotenoid byosinthesis in the flowers of *Adonis eastivalis*. Plant Cell 41:478–492

Cunningham FX Jr, Gantt E (2007) A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. Photosynth Res 92:245–259

Cunningham FX Jr, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E (1996) Functional analysis of the β- and ε-lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8:1613–1626

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

Demming-Adams B, Adams WW III (2002) Antioxidants in photosynthesis nutrition. Science 298:2149–2153

Ducreux JM, Morris WL, Hedley PE, Shepherd T, Davies HV, Millan S, Taylor MA (2005) Metabolic engineering of high carotenoids potato tubers containing enhanced levels of β-carotene and lutein. J Exp Bot 56:81–89

Dweyer JH, Navab M, Dwyer KM, Hassan K, Sun P, Shircore A, Hama-Levy S, Hough G, Wang X, Drake T, Merz NB, Fogelman AM (2001) Oxygenated carotenoid lutein and the progression of early atherosclerosis. Circulation 103:2922–2927

Emanuelsson O, Nielsen H, von Hejne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8:978–984

Fan L, Vonshak A, Rachel G, Hirshberg J, Cohen Z, Boussiba S (1995) The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. Plant Cell Physiol 36:1519–1524

Farré G, Sanahuja G, Naqvi S, Bai C, Capell T, Zhu C, Christou P (2010) Travel advice on the road to Carotenoids in plants. Plant Sci 179:28–48

Frank HA, Cogdell RJ (1996) Carotenoids in photosynthesis. Photochem Photobiol 63(3):257–264

Fraser PD, Römer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit specific-manner. Proc Natl Acad Sci USA 99:1092–1097

Fray W, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J 8:693–701

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker J, Walker M (eds) The proteomics protocols handbook. Humana Press, Totowa, pp 571–607

Gorman DS, Levine RP (1965) Cytocrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 54:1665–1669

Gruber H, Goetinck SD, Kirk DL, Schmitt R (1992) The nitrate reductase-encoding gene of *Volvox carteri*: map location, sequence and induction kinetics. Gene 120:75–83

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

Hauptmann R, Eschenfeldt WH, English J, Brinkhaus FL (1997) Enhanced carotenoid accumulation in storage organs of genetically engineered plants. US Patent 5:618–988

Huang JC, Wang Y, Sandmann G, Chen F (2006) Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 71:473–479

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

Kim J, Smith JJ, Tian L, DellaPenna D (2009) The evolution and function of carotenoid hydroxylases in *Arabidopsis*. Plant Cell Physiol 50:463–479

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA 87:1228–1232

León R, González D, Galván A, Fernández E (2004) Transgenic microalgae as green cell factories. Trends Biotechnol 22(1):45–52

León R, Vila M, Herranz D, Vilches C (2005) Production of phytoene by herbicide-treated microalga *Dunaliella bardawil* in two phase systems. Biotechnol Bioeng 92:695–701

León R, Couso I, Fernández E (2007) Metabolic engineering of ketocarotenoids biosynthesis in the unicellular microalga *Chlamydomonas reinhardtii*. J Biotechnol 130:143–152

Li Y, Sommerfeld M, Chen F (2008a) Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). J Plant Physiol 165:1783–1797

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

Lindgren LO, Stalberg KG, Höglund AS (2003) Seed-specific over-expression if an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. Plant Physiol 132:779–785

Lohr M, Im CS, Grossma AR (2005) Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. Plant Physiol 138(1):490–515

Loppes R, Radoux M, Ohresser MC, Matagne RF (1999) Transcriptional regulation of the Nia1 gene encoding nitrate reductase in *Chlamydomonas reinhardtii*: effects of the various environmental factors on the expression of a reporter gene under the control of the *Nia1* promoter. Plant Mol Biol 41:701–711

McCarthy SS, Kobayashi MV, Niyogi KK (2004) White mutants of *Chlamydomonas reinhardtii* are defective in phytoene synthase. Genetics 168(3):1249–1257

Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii.* Plant J 58:165–174

Olmedilla B, Granado F, Blanco I, Vaquero M (2003) Lutein, but not α-tocopherol, supplementation improves visual function in patients with age-related cataracts: a 2-y double blind, placebo-controlled study. Nutrition 19:21–25

Paine JA, Shipton CA, Chaggar S, Howell RM, Kennedy MJ, Vernon G, Susan Y, Wright SY, Hinchliffe E, Adams JL, Aron L, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sandmann G, Römer S, Fraser PD (2006) Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metab Eng 8:291–302

Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DE (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412

Sizova I, Fuhrman M, Hegemann P (2001) A Streptomyces rimosus aphVIII gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. Gene 277:221–229

Stahl W, Sies H (2005) Bioactivity and protective effects of natural carotenoids. Biochim Biophys Acta 1740:101–107

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

Sun G, Zhang X, Sui Z, Mao Y (2007) Inhibition of pds gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). Mar Biotechnol 10(3):219–226

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

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tran D, Haven J, Qiu WG, Polle JEW (2009) An update on carotenoid biosynthesis in algae: phylogenetic evidence for the existence of two classes of phytoene synthase. Planta 229:723–729

Varkonyi Z, Masamoto K, Debreczeny M, Zsiros O, Ughy B, Gombos Z, Domonkos I, Farkas T, Wada H, Szalontai B (2002) Low-temperature-induced accumulation of xantho- phylls and its structural consequences in the photosynthetic apparatus of the cyanobacterium *Cylindrospermopsis raciborskii:* an FTIR spectroscopic study. Proc Natl Acad Sci USA 99:2410–2415

Vila M, Couso I, León R (2007) Carotenoid content in mutants of the chlorophyte *Chlamydomonas reinhardtii* with low expression levels of phytoene desaturase. Process Biochem 43(10):1147–1152

von Heijne G (1992) Membrane protein structure prediction: hydrophobicity analysis and the 'Positive inside' rule. J Mol Biol 225:487–494

von Kampen J, Nieländer U, von Wettern M (1994) Stress-dependent transcription of a gene encoding a Gb-like polypeptide from *Chlamydomonas reinhardtii*. J Plant Physiol 143:756–758

Yan Y, Zhu YH, Jiang JG, Song DL (2005) Cloning and sequence analysis of the phytoene synthase gene from a unicellular Chlorophyte, *Dunaliella salina*. J Agric Food Chem 53:1466–1469

Zhang J, Tao N, Xu Q, Zhou W, Cao H, Xu J, Deng X (2009) Functional characterization of citrus *PSY* gene in Hongkong kumquat (*Fortunella hindsii* Swingle). Plant Cell Rep 28:1737–1746

Capítulo III

Aislamiento y caracterización del gen licopeno β-ciclasa del alga verde productora de astaxantina *Chlorella zofingiensis* (Chlorophyta)

ISOLATION AND CHARACTERIZATION OF A LYCOPENE β-CYCLASE GENE FROM THE ASTAXANTHIN-PRODUCING GREEN ALGA *CHLORELLA ZOFINGIENSIS* (CHLOROPHYTA) [1]

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The isolation, characterization, and regulation by light and nitrogen of the lycopene β-cyclase gene from *Chlorella zofingiensis* Donz (*CzlcyB*), involved in the biosynthesis of astaxanthin and lutein, have been per- formed in this work. These carotenoids are of high commercial value as dyes in food and as nutraceuticals. The open reading frame (ORF) of *CzlcyB* encoded a polypeptide of 546 amino acids. A single copy of *CzlcyB* has been found in *C. zofingiensis*. The chararacteristic Rossmann or dinucleotide binding fold, present in most lycopene cyclases, has been also identified in the LCYb of *C. zofingiensis* (CzLCYb). Heterologous genetic complementation in *Escherichia coli* showed the ability of the predicted protein to cycle both lycopene and δ-carotene. Phylogenetic analysis has shown that the deduced protein forms a cluster with the rest of the lycopene β-cyclases (LCYb) of the chlorophycean microalgae studied, being very closely related to LCYb of plants. Transcript levels of *CzlcyB* were increased under nitrogen deprivation, but no increase was observed under high-light conditions. However, high irradiance triggered astaxanthin synthesis, while nitrogen deprivation by itself could not induce it. The combination of high irradiance and nitrogen deprivation led to a significant enhancement of the astaxathin accumulation.

***Key index words*: astaxanthin; *Chlorella zofingiensis*; gene expression; lutein; lycopene b-cyclase**

***Abbreviations*: CzLCYb, lycopene β-cyclase from *C. zofingiensis*; *CzlcyB*, lycopene β-cyclase gene from *C. zofingiensis*; LCYb, lycopene β-cyclase; *lcyB*, lycopene β-cyclase gene; ORF, open reading frame; qRT-PCR, quantitative real-time PCR; UTR, untranslated region**

Carotenoids are produced by all photosynthetic organisms and several nonphotosynthetic ones and play roles in light harvesting, photoprotection of cellular components, structural maintenance of pigment-protein complexes, and membrane structure and fluidity (Frank and Cogdell 1996, Varkonyi et al. 2002).

In chloroplasts of plants and algae, the precursor for carotenoids, geranylgeranyl diphosphate (GGPP), is derived from the desoxyxylulose 5-phosphate pathway. The condensation of two GGPP molecules produces the first carotene, phytoene, catalyzed by phytoene synthase (PSY). Then, four double bonds are introduced into the phytoene molecule by phytoene and ζ-carotene desaturases (PDS and ZDS), resulting in the formation of pro-lycopene, which is isomerized by a specific isomerase (CRTISO) to all-*trans* lycopene. Lycopene is converted into either β-carotene by the action of LCYb or into δ-carotene by the action of lycopene ε-cyclase (LCYe). δ-carotene is then modified to α-carotene by LCYb action. The cyclation of lycopene into either α- or β-carotene is a key branch point in the pathway of carotenoid biosynthesis in plants and algae and has been proposed as a control step (Harjes et al. 2008); the relative activities of LCYe and LCYb determine the proportion of carotenes directed to each branch of the pathway. α-carotene is modified to lutein by hydroxylation of one β- and one ε-ring, catalyzed by two heme-containing cytochrome P450 hydroxylases (P450b-CHY and P450e-CHY, respectively). β-carotene with two β-ionone end groups is hydroxylated by a nonheme di-iron hydroxylase (CHYb) at C-3 and C-3´ to zeaxanthin (Sandmann et al. 2006, Kim et al. 2009). A limited number of organisms, including some marine bacteria, the red yeast *Xanthophyllomyces dendrorhous*, and some green algae such as *Haematococcus pluvialis* and *Chlorella zofingiensis*, can synthesize astaxanthin from β-carotene by the action of a

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ketolase/oxygenase (BKT) and the hydroxylase (CHYb) (Fan et al. 1995, Johnson and Schroeder 1995, Huang et al. 2006, Li et al. 2008b).

Astaxanthin and lutein are high-value carotenoids that are not only used as food dyes and especially as feed additives in aquaculture and poultry farming, but also considered as effective agents for the prevention of a variety of age-related, degenerative, and chronic diseases, such as cataracts, macular degeneration, atherosclerosis, and some types of cancer, because of their antioxidant properties (Dweyer et al. 2001, Demming-Adams and Adams 2002, Guerin et al. 2003, Olmedilla et al. 2003).

Although *H. pluvialis* shows the highest reported astaxanthin content (up to 4% of dry biomass; Boussiba 2000), it exhibits a slow growth rate, which restricts its application on a large scale. On the other hand, *C. zofingiensis* accumulates high amounts of astaxanthin or lutein (up to 20 mg L^{-1} culture, for both pigments), shows excellent growth (specific growth rate is 0.04 h^{-1}), and reaches high values of biomass when grown photoautotrophically in batch culture (Orosa et al. 2001, Del Campo et al. 2004). Moreover, this microalga has shown high yields of astaxanthin (up to 32.4 mg L^{-1}) in heterotrophic cultures using glucose-fed batch fermentation (Sun et al. 2008a). These characteristics suggest that this microalga might be potentially used for commercial production of astaxanthin and/or lutein on a large scale.

Besides its potential for production of astaxanthin, *C. zofingiensis* is a model organism to study the regulation of the carotenoid biosynthetic pathway since it produces the primary carotenoid lutein as well as the secondary carotenoid astaxanthin. However, only the carotenogenic genes *bkt*, *pds*, and *chyB* have been isolated and characterized in this microalga (Huang et al. 2006, 2008, Li et al. 2008a, Sun et al. 2008a). The regulation of these genes by light, NaCl, and different organic carbon sources has been studied. High light up-regulated the transcripts of *pds*, *chyB*, and *bkt*, and NaCl only up-regulated the transcript levels of *bkt* (Huang et al. 2008, Li et al. 2009). The expression of the *bkt* gene was transiently up-regulated by glucose in mixotrophic cultures (Huang et al. 2006), and glucose, sucrose, and mannose induced the expression of *pds*, *chyB*, and *bkt* in dark-grown cells (Li et al. 2008a, Sun et al. 2008a).

In the present study, we report the isolation and characterization of the *CzlcyB* gene. The regulation of this gene in response to light and nitrogen in cells grown photoautotrophically has been studied, as well as its ability to convert lycopene into β-carotene, and δ-carotene into α-carotene in vivo.

MATERIALS AND METHODS

Strains and culture conditions. The green microalga strain *C. zofingiensis* SAG 211-14 was obtained from the Culture Collection of Gottingen University (SAG, Gottingen, Germany). The microalga was maintained and grown photoautotrophically in Arnon medium (Arnon et al. 1974) modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$, at 25°C under continuous illumination (50 µmol photons m^{-2} s^{-1}), except where indicated. The liquid cultures were continuously bubbled with air, supplemented with 1% (v/v) CO_2 as the only source of carbon. For the expression experiments, cells were grown in Roux flasks of 1 L capacity laterally and continuously illuminated with mercury halide lamps at either 20 or 300 µmol photons m^{-2} s^{-1}) either in the presence or in the absence of nitrate. To keep constant the nitrate in the medium, the nitrate concentration was measured daily (see Analytical methods), and the nitrate consumed by the cells was added to the cultures. The light intensity was measured at the surface of the flasks using a LI-COR quantum sensor (model L1-1905B, Li-Cor Inc., Lincoln, NE, USA).

Escherichia coli DH5a and BL21 strains were used as the hosts for DNA manipulation and for heterologous expression of *lcyB* gene, respectively.

Isolation of genomic DNA and RNA, and cDNA preparation. DNA and total RNA were isolated using DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany), respectively. For quantitative real-time PCR (qRT-PCR) analysis, first -strand cDNA synthesis was obtained from total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Barcelona, Spain) primed with oligo$(dT)_{18}$ according to the manufacturer's instructions.

Cloning of full-length lcyB cDNA and the genomic gene of C. zofingiensis. Degenerate primers, 2F and 2R, were designed for the amplification of a partial cDNA fragment of *CzlcyB*, which provided sequence information for designing gene-specific primers for rapid amplification of 5´ and 3´ cDNA ends (RACE). RACE-PCR synthesis was performed according to the manufacturer's manual (Smart RACE cDNA Amplification Kit; Clontech, Mountain View, CA, USA). The 5´ and 3´ RACE products were cloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced. The full-length *CzlcyB* cDNA was amplified with the sense primer pQE-1F and the antisense primer pQE-1R, used also for functional analysis. Specific primers were synthesized for genomic DNA amplfication based on cDNA sequence. The primer sets used in this study are listed in Table 1.

Nucleotide sequence accession numbers. The *CzlcyB* cDNA and genomic DNA sequences have been registered in the European Molecular Biology Laboratory (EMBL) database under the accession numbers FN563998 and FN563999, respectively.

Functional analysis of CzlcyB cDNA. The *CzlcyB* ORF was amplified by PCR with the primers pQE-1F and pQE-1R, which were designed to contain *Bam*HI and *Sal*I restriction sites, respectively, and cloned into pQE-80L expression vector (Qiagen) resulting in plasmid pQE-*CzlcyB*, which carries ampicillin resistance. The plasmids pAC-LYC and pAC-DELTA, a gift from Prof. F. Cunningham, carried the carotenoid pathway genes responsible for the synthesis of lycopene (*crtE*, *crtB*, and *crtI* of *Erwinia herbicola*) and δ-carotene (*crtE*, *crtB*, and *crtI* of *E. herbicola* + *lcyE* of *Arabidopsis*), respectively (Cunningham et al. 1994, Cunningham and Gantt 2007). Transformation of *E. coli* BL21 with pQE-*CzlcyB* and/or one of the two plasmids pAC-LYC or pAC-DELTA (both carrying chloramphenicol resistance) was made by electroporation. Transformed cells were plated on Luria-Bertani (LB) (Sambrook et al. 1989), supplemented with 100 µg ml^{-1} ampicillin and/or

TABLE 1. Nucleotide sequences of primer pairs used for PCR amplification.

Primer	Sequence (5´→ 3´)
Partial *lcyB* fragment	
2F	TGGCCCAACAACTAYGG
2R	GTKGAKGGATGMACCAT
cDNA amplification	
e-1F	TGAACAATAGCAACGCAGGC
e-1RG	ATCAAGCAGTACTCCTCCTC
5´and 3´ RACE	
GSP-1F	ACTGCCCAAGCACCCCCAGCGTGT
GSP-1R	TGGCTGCACATCTCTCCAGTAACTTCCGT
NGSP-1F	GCAGAGGTCGAGTCCCATCCGTTTGAGGTT
NGSP-1R	GACCTCCAAACAGTCATCCAAGCCCATTGC
Genomic DNA amplification	
4F	ATGTGGCGACGTTCACGC
4R	GCTTGAAACTCATCCACCCAG
5F	AGCGTGTGTTGGGTATTGGTGG
5R	CTCACCATCTGGGATCTTGTAGTA
6F	TACTACAAGATCCCAGATGGTGAG
6R	TTACAAAAAGGGCATTTACAGGGGG
PCR for probe preparation	
Czlcy-S-F	CTGGGTGGATGAGTTTCAAGC
Czlcy-S-R	CATCTCTCCAGTAACTTCCG
Genetic complementation-pQE-80L[a]	
pQE-1F	cccggatccttcATGGAGTCAAAACTGCTGCGC
pQE-1R	ccagtcgacCTAGCTCTTCACCTGTGCCCT
lcyB expression	
RT-2F	CGCAGGCGAAAAATTCCTGTCA
RT-2R	TAAGGAATGTCACACCGCTGGC

F, forward; R, reverse.
[a] *Bam*HI and *Sal*I sites (lowercase letters underlined) were added for cloning the gene into the corresponding cut sites of pQE-80L vector.

40 µg mL^{-1} chloramphenicol, and grown at 37°C for 1 d. The inducer isopropyl-β-d-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM.

qRT-PCR. qRT-PCR was performed in triplicate on cDNA samples or control samples, which were used to confirm the absence of DNA contamination, on an IQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). In each experiment, a standard dilution series of plasmid containing specific PCR fragments or a known cDNA concentration of each sample were ampfi ed in a 20 µL of final volume reaction containing 1 x SYBR Green PCR Master Mix (Quantimix Easy SYG kit; BioTools B&M Labs, Madrid, Spain) and corresponding primers (Table 1). After heating at 95°C for 10 min, cycling parameters were as follows: 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Finally, the specficity of the qRT-PCR products was confirmed by performing a melting temperature analysis at temperatures ranging from 55°C to 95°C at 0.5°C min^{-1} and also by electrophoresis on a 2% agarose gel. Data were captured as amplification plots. Transcription levels of the target genes were calculated from the threshold cycle by interpolation from the standard curve. To standardize the results, the relative abundance of the actin gene was also determined and used as the internal standard (Sun et al. 2008b, Vidhyavathi et al. 2008).

All calculations and statistical analyses were performed as described in the IQ5 Optical System Software 1.0 (BioRad). The complete experiments (RNA isolation, cDNA synthesis followed with qRT-PCR) were repeated twice independently, and the data were averaged.

Southern blot. Genomic DNA was digested with *Hind*III and *Sph*I, which show one recognition site in the probed region of the *lcyB* gene. The probe was prepared by amplifying genomic DNA with the primers Czlcy-S-F and Czlcy-S-R, resulting in a 464 bp fragment of the *CzlcyB* gene. The digested DNA was transferred to a Hybond-N membrane (GE Healthcare, Little Chanfont, UK) by capillary transfer and hybridized with the ^{32}P-labeled DNA probe at different stringencies (42, 50, and 65°C) overnight. Washes were also performed at different stringencies (42, 50, and 65°C). After hybridization, the radioactivity of the membrane was detected by the Cyclone Phosphore System (Perkin Elmer, Waltham, MA, USA).

Analytical methods. *Determination of cell concentration*: Cell concentration was measured by absorbance at 450 nm. A correlation between absorbance and cell number, determined with a Neubauer hemocytometer (Paul Marienfeld, LaudaKonigshofen, Germany), was established.

For dry weight determination, aliquots (5 mL) of the cell culture were filtered through Whatman GF /C paper (Whatman plc, Kent, UK), washed three times, and dried at 80°C for 24 h.

Nitrate concentration determination: Nitrate concentration in the medium was determined by HPLC, using a Whatman Partisil SAX 10 column (4.6 x 250 mm), chromatography being performed in the isocratic mode at a flow rate of 1 mL min^{-1}. Mobile phase consisted of a mixture of 50 mM H_3PO_4 with 2% tetrahydrofuran (pH 1.9). Nitrate was detected at 210 nm using a Waters 2996 photodiode-array detector (Waters, Mildford, MA, USA). For nitrate quantification of the samples, a calibration curve with known nitrate concentrations was previously performed (Romero et al. 1989).

Carotenoid determination: For carotenoid analysis, pigments were extracted with methanol at 70°C and centrifuged, the supernatant evaporated under N_2, and the pellet resuspended in methanol. For this strain, the carotenoid extraction with methanol at 70°C proved to be a more efficient method than the extraction with pure acetone. The samples were then saponified with ethyl ether and KOH 2% in methanol (Del Campo et al. 2004) and then centrifuged (Eppendorf, Hamburg, Germany) and analyzed by HPLC using a Waters Spherisorb ODS2 column (4.6 x 250 mm, 5 µm particle size). The chromatographic method described by Baroli et al. (2003) and modified by us was used. Pigments were eluted at a flow rate of 1.0 mL min^{-1} with a linear gradient from 100% solvent A (acetonitrile:methanol:0.1mM Tris-HCl pH 8.0 [84:2:14]) to 100% solvent B (methanol:ethyl acetate [68:32]) for 20 min, followed by 7 min of solvent B, then 1 min with a linear gradient from 100% solvent B to 100% solvent A, and finally 6 min with solvent A. The carotenoids were detected at 440 nm using a Waters 2996 photodiode-array detector. The different carotenoids were idenfied using standards from Sigma (St. Louis, MO, USA) and DHI (Holsholm, Germany).

RESULTS

Isolation and characterization of the lcyB gene and deduced protein sequence of C. zofingiensis.

Different pairs of degenerate primers were designed on the basis of the conserved motifs present in *lcyB* from microalgae, cyanobacteria, and plants. A partial *CzlcyB* cDNA fragment of 1,715 bp was isolated by PCR amplification using degenerate primers, providing sequence information for designing specific primers for RACE that generated a full-length cDNA of 2,131 bp. cDNA sequence analysis revealed an ORF of 1641 bp encoding a deduced *lcyB* gene preceded by 97 pb of 5´ untranslated region (UTR) and followed by 393 bp of 3´ UTR. A typical algal polyadenylation signal TGTAAA (Gruber et al. 1992) was present in the 3´ UTR at 18 nucleotides upstream from the beginning of the poly (A) tail. The ORF of this cDNA was predicted to encode a hypothetical protein of 546 amino acids, with an estimated molecular weight of 60 kDa and a theoretical isoelectric point of 9.15 (data obtained with ProtParam program of ExPASy server, Gasteiger et al. 2005). The instability index was computed to be 31.40, which allowed the classification of this protein as stable. The amino-acid sequence of CzLCYb showed the highest overall homology sequence with other LCYb from green algae, such as *H. pluvialis* and *C. reinhardtii* (identity 70%, similarity 82%) and *D. salina* (identity 62%, similarity 76%). The GC content of the *CzlcyB* coding region was 54%, which was much lower than that of *C. reinhardtii* (67%) or of *H. pluvialis* (62%), being very similar to that of *D. salina* (58%). Alignment analysis of genomic and the full-length cDNA sequences revealed the presence of seven exons and six introns uniformly distributed in the *CzlcyB* gene.

Exon size ranged between 78 (exon II) and 501 bp (exon I), and intron size between 189 (intron II) and 349 bp (intron V). Approximately 57% (2.1 out of 3.7 kb) of the *CzlcyB* gene corresponded to exon sequences. In the introns 1, 3, and 5, the consensus GT donor and AG acceptor sequences at the 5´ and 3´ termini were found (Fig. 1).

To determine the copy number of *lcyB* gene in the genome of *C. zofingiensis*, genomic DNA was digested with two different restriction enzymes (*Hind*III and *Sph*I) and subjected to Southern blot analysis under different stringency conditions, both in hybridization and washing (42, 50, and 65°C). Using a 464 bp fragment of *CzlcyB* as a probe, strong hybridization signals have been obtained, suggesting the presence of a single copy of the *lcyB* gene in the genome. The digestion with *Hind*III enzyme, which cuts at one extreme of the probe sequence, showed only one band, while digestion with *Sph*I, which cuts once inside the probe, exhibited two bands for the different conditions of stringency tested (Fig. 2).

The phylogenetic analysis of the LCYb from microalgae, cyanobacteria, plants, and bacteria is illustrated in Figure 3. Analysis was conducted in MEGA4 (Tamura et al. 2007) using the UPGMA method. The predicted CzLCYb forms a cluster with the rest of the microalgae studied, which are phylogenetically very closely related to LCYb of plants (between 50% and 55% of identity, and ≈70% similarity). The degree of homology was lower with cyanobacterial cyclases (30% identity and 47% similarity). As other algal LCYb, CzLCYb was distantly related to bacterial CRTY cyclases, sharing with them only a few conserved motifs and <25% identity. Practically, there was no similarity with the CRU

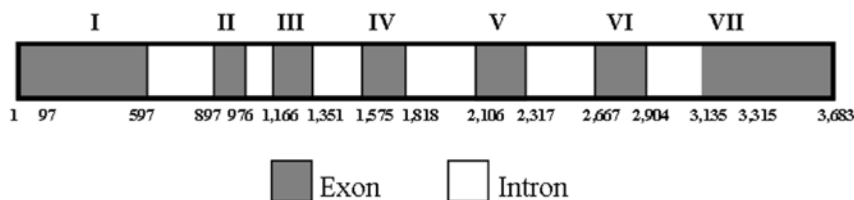

Fig. 1. *CzlcyB* gene organization. The diagram shows that the *CzlcyB* gene consists of seven exons (I–VII) and six introns. The 5´ UTR and 3´ UTR sequences correspond to the positions 1–97 and 3,315–3,683, respectively. Numbers represent the cDNA coordinates (bp). UTR, untranslated region.

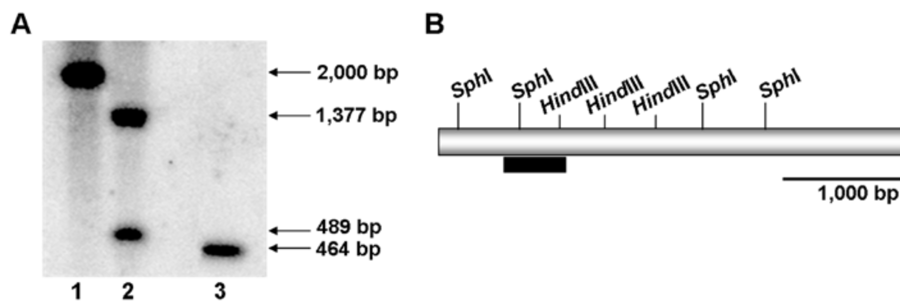

FIG. 2. Southern analysis of genomic DNA from *Chlorella zofingiensis*. (A) DNA was digested with *Hind*III (lane 1) and *Sph*I (lane 2), electrophoretically separated on a 0.8% agarose gel, blotted, and hybridized at 65°C with a probe of 464 bp of the *lcyB* gene amplified by PCR. A plasmid containing the *lcyB* gene was used as a positive control (lane 3). (B) Restriction sites of *Hind*III and *Sph*I in the *CzlcyB* gene and the probe (black bar).

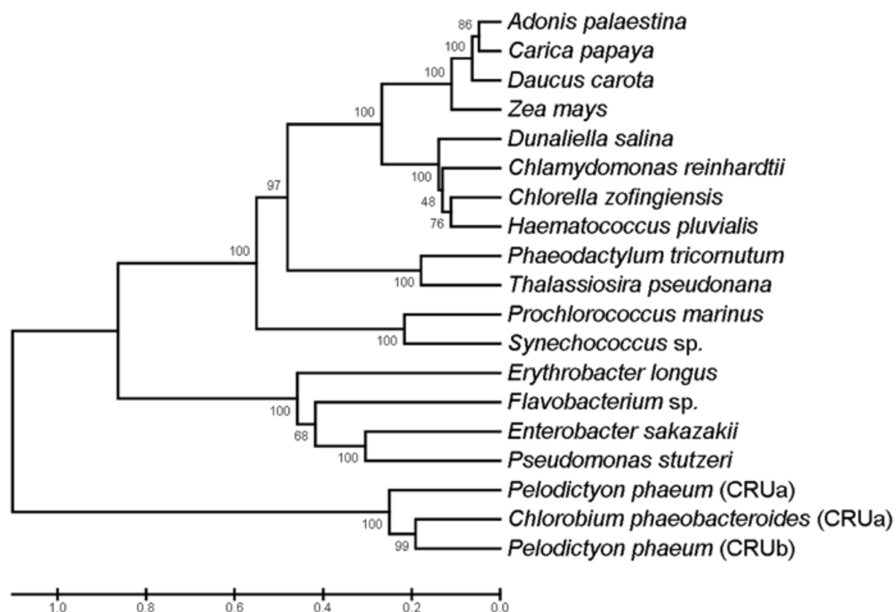

FIG. 3. Neighbor-joining tree analysis from LCYb of the indicated plant, algal, cyanobacterial, and bacterial amino-acid sequences. The accession numbers for other species are as follows: *Adonis palaestina*, AAK07430; *Carica papaya*, ABD91578; *Daucus carota*, ABB52071; *Zea mays*, AAO18661; *Dunaliella salina*, ACA34344; *Chlamydomonas reinhartii*, AAX54906; *Haematococcus pluvialis*, AAO64977; *Phaeodactylum tricornutum*, EEC51075.1; *Thalassiosira pseudonana*, EED95313; *Prochlorococcus marinus*, CAE21298; *Synechococcus* sp., CAE07243; *Erythrobacter longus*, BAA20275; *Flavobacterium* sp., AAC44851; *Enterobacter sakazakii*, CAL34120; *Pseudomonas stutzeri*, ABP81497; *Pelodictyon phaeum* (CRUa), ABQ40352; *Chlorobium phaeobacteroides* (CRUa), ABQ40354; *Pelodictyon phaeum* (CRUb), ABQ40353. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino-acid substitutions per site. Numbers at nodes indicate bootstrap values calculated >500 replicates.

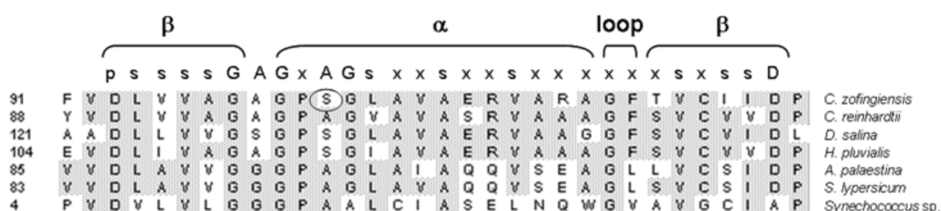

FIG. 4. Comparison of the Rossmann or dinucleotide binding fold of CzLCYb and other organisms. The consensus sequence of the motif is also shown at the top of thefigure. The accession numbers for other species are as follows: *Chlamydomonas reinhartii*, AAX54906; *Dunaliella salina*, ACA34344; *Haematococcus pluvialis*, AAO64977; *Adonis palaestina*, AAK07430; *Solanum lycopersicum,* CAJ57430.1; *Synechococcus* sp., CAE07243. p, polar or charged amino acid: D, E, K, R, H, S, T, Q, N; s, small hydrophobic amino acid: A, I, L, V, M, C; x, any amino acid.

family, a new type of bacterial cyclase recently reported (Maresca et al. 2007).

As shown in Figure 4, the characteristic Rossmann or dinucleotide binding fold, constituted by a β sheet–α helix–loop–β sheet (Rossmann et al. 1974), present in microalgal and plant LCYb (Cunningham et al. 1994), was identified in the LCYb of *C. zofingiensis* between amino acids 93 and 121. The only exception to the consensus sequence was position 10 of the motif, which was a serine in *C. zofingiensis* instead of the usual alanine. Interestingly, this substitution has been observed in other microalgae, but not in cyanobacteria. Several authors have proposed that these conserved exceptions are due to the flexible and evolving character of the motif.

As microalgae and plant LCYb is localized in chloroplasts, we analyzed the CzLCYb with different programs to determine the presence of signal peptides. The program Predotar v. 1.03 (Caboche 2003) predicted possible plastid localization for the CzLCYb; using TargetP v.1.1 (Emanuelsson et al. 2007) and iPSORT (Bannai et al. 2002), the mitochondrial targeting peptide was predicted; and SignalP program (Bendtsen et al. 2004) identified a signal peptide at position 22. The hydrophobic profile of CzLCYb obtained by ProtScale (Gasteiger et al. 2005), TMHMM (Krogh et al. 2001), and TMAP (Persson and Argos 1994) programs showed that this protein had two putative transmembrane domains in the carboxyl terminal end: FLSARLSFPQLIGFGLSLFTK and LAMGLPGLLSMLAGLAPTL GQ (data not shown).

Functional analysis of the CzLCYb in E. coli. The use of *E. coli* engineered to produce different carotenogenic substrates is an efficient and frequently used method for the functional characterization of the enzymes of the carotenoid biosynthetic pathway (Cunningham et al. 1996, Cunningham and Gantt 2005), due to the difficulties of the enzymatic determinations of these membrane-associated enzymes in low abundance.

In order to assay the enzymatic activity of the CzLCYb, the ORF of this gene was amplified and inserted in vector pQE-80L under the control of the β-galactosidase promoter. The resulting plasmid (pQE-*CzlcyB*) was introduced in *E. coli* engineered to accumulate either lycopene or δ-carotene, due to the presence of the plasmids pAC-LYC or pAC-DELTA, respectively. HPLC analysis of carotenoids extracted from *E.coli* showed that cells containing either pAC-LYC or pAC-DELTA produced lycopene (Fig. 5A) or δ-carotene (Fig. 5B), respectively, while cells cotransformed with both pAC-LYC and pQE-*CzlcyB* or pAC-DELTA and pQE-*CzlcyB* accumulated either β-carotene (Fig. 5C) or α-carotene (Fig. 5D), respectively. As negative controls, E. coli cotransformed with pAC-LYC or pAC-DELTA and empty pQE-80L were used, resulting in the same accumulation of carotenoids as when *E. coli* was transformed only with pAC-LYC or pAC-DELTA (data not shown). Therefore, CzLCYb allowed both the double cyclation of lycopene to yield β-carotene and the conversion of δ-carotene into α-carotene, as most LCYb previously isolated.

Expression of the CzlcyB gene: effect of irradiance and nitrogen. Cells of *C. zofingiensis* were grown photoautotrophically at low irradiance and constant nitrate (as indicated in Materials and Methods) until the middle of the exponential phase. Then cells were kept in darkness for 18 h, in order to make the transcript levels come down to basal values. After this dark period, cells were subjected to either low or high irradiance, both under constant nitrogen or nitrogen-deprivation conditions. The nitrate concentration was measured daily (data not shown), and the nitrate consumed by the cells was added to the cultures. The evolution of transcriptional expression of the *CzlcyB* gene as affected by irradiance and nitrogen availability was monitored by qRT-PCR, changes in cellular lutein and astaxanthin being also determined in order to correlate transcript levels with the biosynthesis of those specific carotenoids. As shown in Figure 6, A and C, high irradiance did not increase the levels of transcript in the presence of nitrate. On the contrary, nitrogen depletion, both at low and high irradiance (Fig. 6, B and D), had a strong effect on transcript levels of *CzlcyB*, which increased with time, reaching maximum levels after 24–48 h (about 4-fold higher as compared to basal level). At low irradiance under nitrogen-depletion conditions (Fig. 6B), the maximum transcript levels, attained after 24 h, decreased significantly thereafter, whereas when combining high irradiance and nitrogen starvation (Fig. 6D), the levels of mRNA were kept practically constant during the studied period.

With regard to the cellular content in carotenoids, astaxanthin was only synthesized at high irradiance, the levels of this secondary carotenoid increasing with time, much higher accumulation taking place under nitrogen depletion (Fig. 6D) than under constant nitrogen (Fig. 6C). However, the concentration of the primary carotenoid lutein concentration decreased with time at high irradiance, only slightly in the presence of nitrogen (Fig. 6C) and drastically under nitrogen starvation (Fig 6D).

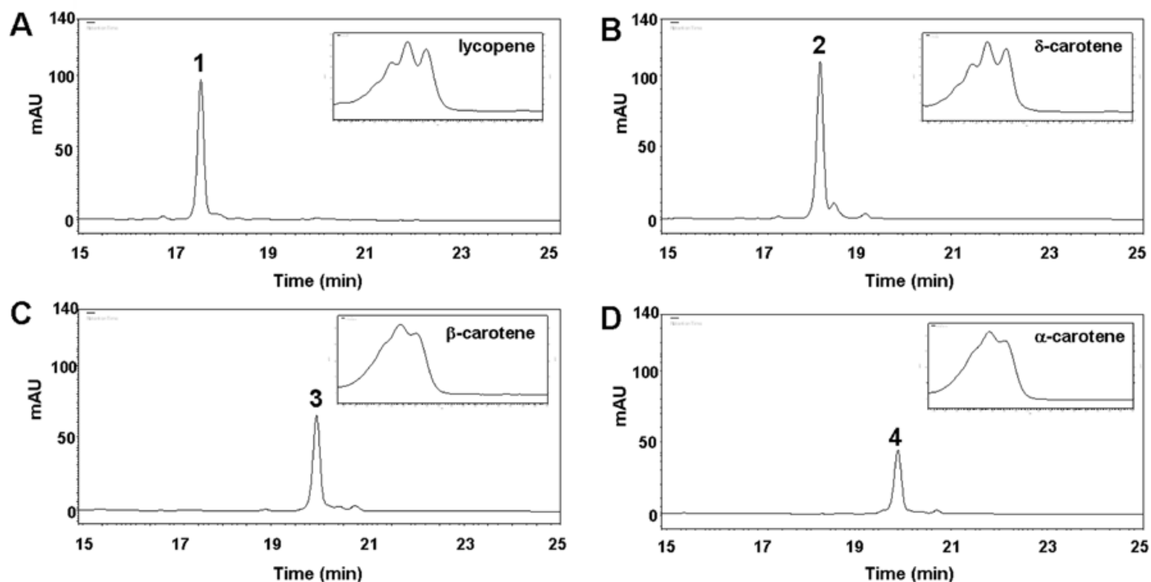

FIG. 5. HPLC elution profiles of carotenoids extracted from cultures of *Escherichia coli* carrying plasmids pAC-LYC (A), pAC-DELTA (B), pAC-LYC+pQE-*CzlcyB* (C), and pAC-DELTA+pQE-*CzlcyB* (D). As a negative control, E. coli cotransformed with pAC-LYC or pAC-DELTA and empty pQE-80L was used, resulting in the same elution profiles as (A) and (B), respectively. The absorption spectra of the corresponding carotenoids are also shown. *E. coli* BL21 cells transformed with the indicated plasmids were isolated in the presence of chloramphenicol (A, B) or chloramphenicol + ampicillin (C, D). The y-axis (mAU) represents units of absorbance ($x10^{-3}$) at 440 nm. Identification of peaks: 1, lycopene; 2, δ-carotene; 3, β-carotene; 4, α-carotene.

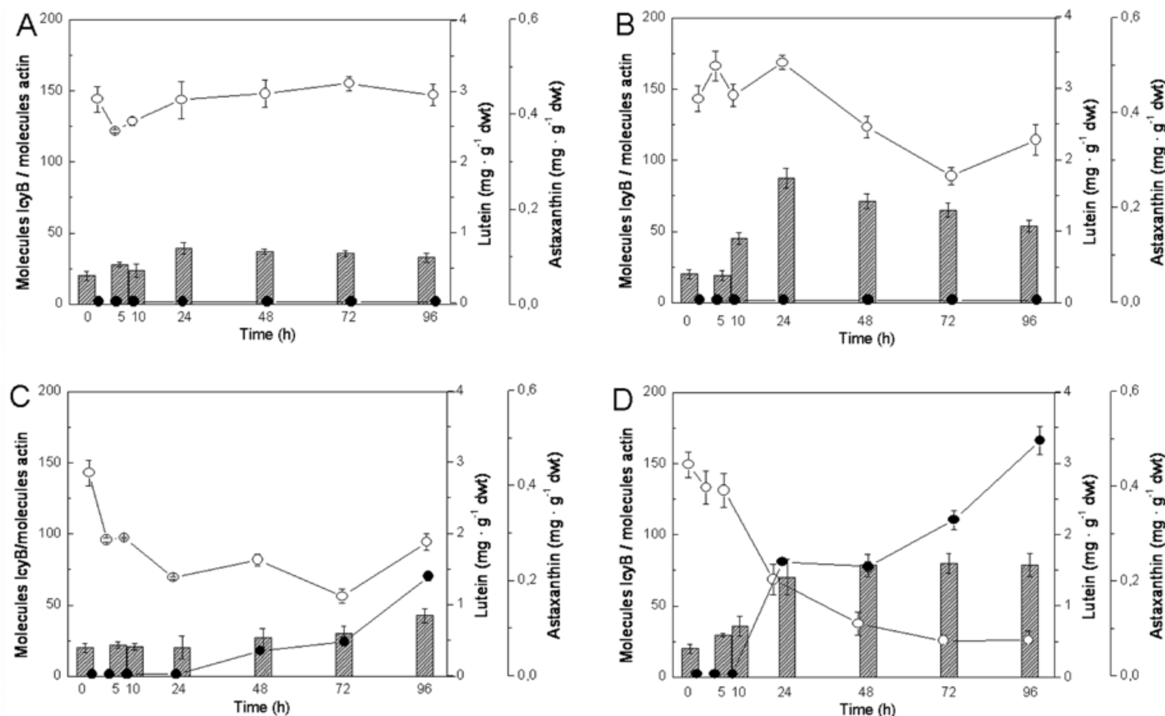

FIG. 6. Effect of irradiance and nitrogen availability on the mRNA levels of the lycopene β-cyclase gene (*lcyB*) and cellular content of lutein and astaxanthin in *Chlorella zofingiensis*. (A) Low irradiance (20 µmol photons m^{-2} s^{-1}) and constant nitrate concentration; (B) low irradiance and nitrate deprivation; (C) high irradiance (300 µmol photons m^{-2} s^{-1}) and constant nitrate concentration; and (D) high irradiance and nitrate deprivation. The nitrate concentration was measured daily in the cultures, and the nitrate consumed by the cells was added to the cultures (up to 20 mM). Columns indicate mRNA levels of the *lcyB* gene; (•), astaxanthin content (mg g^{-1} dwt); (o), lutein content (mg g^{-1} dwt). Error bars indicate the standard deviations of four independent measurements. dwt, dry weight.

At low irradiance, lutein was kept constant with time in the presence of nitrate (Fig. 6A) but decreased under nitrogen deprivation (Fig. 6B). Astaxanthin was not detected under these low-irradiance conditions.

DISCUSSION

The *CzlcyB* gene showed seven exons and six introns, the ORF of the cDNA encoding a hypothetical protein of 546 amino acids (Fig. 1). By comparing the intron positions of *lcyB* genes of *C. zofingiensis* and *D. salina*, we determined that five introns of the *C. zofingiensis* gene matched positions with that of *D. salina* (Ramos et al. 2008). The length of the putative protein agrees with those described for microalgae and plants, being that bacterial proteins are ≈150 amino acids shorter, according to alignments performed with BLOCKS PROGRAM (data not shown). This could indicate the existence of a signal peptide in the 5´ end of eukaryotic proteins. In fact, different programs have confirmed the presence of a signal peptide in the CzLCYb. As other LCYb proteins (Cunningham et al. 1994), the predicted CzLCYb is not as hydrophobic as would be expected for an enzyme localized in membranes. It has 21% of charged amino acids, and 29% are hydrophobic. In a ddition, by using different programs, only two transmembrane domains of 21 amino acids at the carboxyl end have been found.

Several kinds of proteins with lycopene cyclase activity have been found. Plant and microalgal LCY are related to cyanobacterial CRTL and share only certain conserved motifs in their amino-acid sequences with the bacterial lycopene cyclases, referred to as CRTY. A common phylogenetic origin for all these genes has been proposed. These families of lycopene cyclases comprise β- and ε-cyclases, either mono- or bicyclases, and even some bifunctional lycopene β-ε-biciclases (Bouvier et al. 2005). Another family of lycopene cyclases has been recently identified in bacteria, including cyclases encoded by *cruA* and *cruP* genes, found in green sulfur bacteria and in cyanobacteria that lack genes encoding *CrtL* or *CrtY*. Orthologous genes to *CruP* also occur in plant genomes. Practically no similarity can be determined between LCY/CRTL or CRTY peptide sequences and the CRU-type cyclases, but they all are integral membrane proteins and conserve the dinucleotide binding motif (Maresca et al. 2007).

Significant examples of all these kinds of β-cyclases have been aligned with the CzLCYb, and the resulting phylogenetic tree is shown in Figure 2. Additional types of cyclases, such as the heterodimeric cyclases formed by two small polypeptides encoded by *crtYc* and *crtYd* genes,

originally described in the gram-positive bacteria *Brevibacterium linens*, or the bifunctional fungal lycopene cyclases with both PSY and lycopene cyclase activity, have not been considered in our analysis. The high degree of homology that we have observed between the predicted protein encoded by the new gene isolated from *C. zofingiensis* and plant cyclases, especially with the known cyclases of other green microalgae, would probably be enough to consider this new gene as a LCYb, but functional analysis has definitely confirmed this hypothesis.

At the lycopene level, the pathway is divided into two divergent branches. In one branch, the lineal lycopene is converted by the LCYb into the β-bicycled β-carotene (two β-rings) via the monocycled intermediate γ-carotene (one β-ring), β-carotene being transformed into astaxanthin. In the other branch, the lycopene is transformed by the concerted action of LCYe and LCYb and in α-carotene, which has one β- and one ε-ring, which is hydroxylated to yield lutein. The cyclase that acts first has not been well defined yet, but given the inability of the plant and algal LCYb to add only one β-ring to the lycopene, it is more probable that the LCYe monocyclase acts first to catalyze the formation of δ-carotene, which will then be transformed into α-carotene by the action of the LCYb. Some authors have proposed that both cyclations would take place simultaneously by the action of cyclase dimers. LCYb homodimers would catalyze the synthesis of β-carotene, and a hypothetical LCYb–LCYe heterodimer would transform lycopene into α-carotene (Grossman et al. 2004).

In this study, we have checked the substrate specificity of the new CzLCYb by complementation experiments in bacteria. Even though the environment and the processing of the putative *lcyB* gene in the prokaryotic system probably differ from those in the original algal system, the gene product obtained was functional and able to catalyze the cyclation of not only the lineal lycopene, but also the ε-monocycled δ-carotene, as shown in Figure 5. This finding confirms that the catalytic properties of CzLCYb are similar to that of the rest of LCYb previously cloned in plants (Cunningham et al. 1996) and microalgae (Cunningham and Gantt 2007, Ramos et al. 2008). The CzLCYb is responsible for the synthesis of β-carotene from lycopene and is also involved in the production of α-carotene. The fact that CzLCYb is able to perform the cyclation of both lineal lycopene and monocycled δ-carotene means that the formation of cyclase dimers is not essential for the catalytic activity of this enzyme, at least in a heterologous system. In the thylakoid membranes, the environment around the enzyme and the substrates may be very different, and dimmers can play an important role in the regulation and in the equilibrium between the α and β branches of the pathway.

In photoautotrophically grown *C. zofingiensis*, it is known that the combination of both high irradiance and nitrogen starvation causes a drop in the cellular content in lutein and a concomitant accumulation of astaxanthin (Rise et al. 1994, Bar et al. 1995, Orosa et al. 2001). However, the molecular basis of the astaxanthin accumulation under those conditions has not been studied yet. Recently, it has been shown that high irradiance up-regulates the *pds*, *chyB*, and *bkt* genes, significantly enhancing astaxanthin synthesis in *C. zofingiensis* (Huang et al. 2008, Li et al. 2009). Nevertheless, the effect of nitrogen deprivation per se has not been investigated in this green alga yet, neither with respect to the expression of the carotenogenic genes nor in terms of carotenoid accumulation.

We have investigated the regulation of the *CzlcyB* gene by irradiance and nitrogen, determining the RNA levels as well as the cellular content in lutein and astaxanthin. The transcript levels of *CzlcyB* were virtually not enhanced by increasing light irradiance from 20 to 300 µmol photons m^{-2} s^{-1}; on the contrary, under nitrogen deprivation, mRNA levels were significantly higher (≈2 -fold) than under nitrogen-constant conditions, at both high and low irradiance. However, only at high irradiance was astaxanthin biosynthesis triggered, accompanied by a decrease in lutein levels. Although high irradiance was required for astaxanthin synthesis, when combined with nitrogen deprivation, astaxanthin accumulation was enhanced significantly (>2-fold) (Fig. 6).

According to our results, high irradiance does not increase mRNA levels of *CzlcyB*, although it triggers astaxanthin synthesis. However, in *H. pluvialis*, unlike in *C. zofingiensis*, an up-regulation of *lcyB* by high light has been shown, astaxanthin synthesis also being induced under this irradiance condition (Vidhyavathi et al. 2008). Therefore, in *C. zofingiensis*, high irradiance triggered astaxanthin synthesis by up-regulation of *pds*, *chyB*, and *bkt* genes, but not *lcyB*. Nitrogen starvation per se increased mRNA levels of *lcyB* but did not trigger the synthesis of astaxanthin. Nevertheless, the combination of both factors, high irradiance and nitrogen starvation, increased the levels of astaxanthin significantly. A possible explanation for these results is that the up-regulation of *lcyB* by nitrogen starvation shifts the carbon flow in the pathway from lycopene to β-carotene and astaxanthin, instead to α-carotene and lutein, under high irradiance, when other carotenogenic genes are up-regulated. In addition, nitrogen starvation could also up-regulate genes of the specific biosynthetic pathway of astaxanthin or other genes previous to *lcyB* in the carotenogenic pathway. Changes in the mRNA levels have been shown to be the most important regulation level for carotenoid genes but not the only one; posttranscriptional and translational levels also playing important roles, as well as the stability of RNA, as has been shown for *H. pluvialis* (Steinbrenner and Linden 2003, Vidhyavathi et al. 2008). Other levels of regulation of *CzlcyB*, not only changes in the transcript levels, could be taking place.

Our data on the characterization and regulation of the *CzlcyB* gene by light and nitrogen can contribute to the understanding of the regulatory mechanisms of the biosynthesis of carotenoids at a molecular level, which can be helpful for the optimization of the physiological conditions

for high astaxantin and/or lutein production by *C. zofingiensis*, and eventually for performing metabolic and genetic engineering in this microalga.

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Arnon, D. I., McSwain, B. D., Tsujimoto, H. Y. & Wada, K. 1974. Photochemical activity and components of membrane reparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim. Biophys. Acta* 357:231–45.

Bannai, H., Tamada, Y., Maruyama, O., Nakai, K. & Miyano, S. 2002. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* 18:298–305.

Bar, E., Rise, M., Vishkautsan, M. & Arad, S. 1995. Pigment and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. *J. Plant Physiol*. 146:527–34.

Baroli, I., Do, A. D., Yamane, T. & Niyogi, K. K. 2003. Zeaxanthin accumulation in the absence of a functional xanthophylls cycle protects *Chlamydomonas reinhardtii* from photooxidative stress. *Plant Cell* 15:992–1008.

Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol.Biol*. 340:783–95.

Boussiba, S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiol. Plant.* 108:111–7.

Bouvier, F., Rahier, A. & Camara, B. 2005. Biogenesis, molecular regulation and function of plant isoprenoids. *Prog. Lipid Res.* 44:357–429.

Caboche, M. 2003. *A Prediction Service for Identifying Putative N-terminal Targeting Sequences*. INRA, Evry Cedex, France. Available at: http://urgi.versailles.inra.fr/predotar/predotar.html (last accessed 15 October 2003).

Cunningham, F. X., Jr. & Gantt, E. 2005. A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis eastivalis. Plant Cell* 41:478–92.

Cunningham, F. X., Jr. & Gantt, E. 2007. A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. *Photosynth. Res*. 92:245–59.

Cunningham, F. X., Jr., Pogson, B., Sun, Z., McDonald, K. A., DellaPenna, D. & Gantt, E. 1996. Functional analysis of the β- and ε-lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* 8:1613–26.

Cunningham, F. X., Jr., Sun, Z., Chamovitz, D., Hirschberg, J. & Gantt, E. 1994. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Plant Cell* 6:1107–21.

Del Campo, J. A., Rodrıguez, H., Moreno, J., Vargas, M. A., Rivas, J. & Guerrero, M. G. 2004. Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotech*. 64:848–54.

Demming-Adams, B. & Adams, W. W., III 2002. Antioxidants in photosynthesis nutrition. *Science* 298:2149–53.

Dweyer, J. H., Navab, M., Dwyer, K. M., Hassan, K., Sun, P., Shircore, A., Hama-Levy, S., et al. 2001. Oxygenated carotenoid lutein and the progression of early atherosclerosis. The Los Angeles atherosclerosis study. *Circulation* 103:2922–7.

Emanuelsson, O., Brunak, S., von Heijne, G. & Nielsen, H. 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. *Nat. Protocols* 2:953–71.

Fan, L., Vonshak, A., Rachel, G., Hirshberg, J., Cohen, Z. & Boussiba, S. 1995. The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant Cell Physiol.* 36:1519–24.

Frank, H. A. & Cogdell, R. J. 1996. Carotenoids in photosynthesis. *Photochem. Photobiol.* 63:257–64.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy server. In Walker, J. M. [Ed.] *The Proteomics Protocols Handbook.* Humana Press, Totowa, New Jersey, pp. 571–607.

Grossman, A. R., Lohr, M. & Im, C. S. 2004. *C. reinhardtii* in the landscape of pigments. *Annu. Rev. Genet*. 38:119–73.

Gruber, H., Goetinck, S. D., Kirk, D. L. & Schmitt, R. 1992. The nitrate reductase-encoding gene of *Volvox carteri*: map location, sequence and induction kinetics. *Gene* 120:75–83.

Guerin, M., Huntley, M. E. & Olaizola, M. 2003. *Haematococcus* astaxanthin applications for human health and nutrition. *Trends Biotechnol*. 21:210–16.

Harjes, C. E., Rocheford, T. R., Bai, L., Brutnell, T. P., Kandianis, C. B., Sowinski, S. G., Stapleton, A. E., Vallabhaneni, R., Williams, M. & Wurtzel, E. T. 2008. Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. *Science* 319:330–33.

Huang, J. C., Liu, J., Li, Y. & Chen, F. 2008. Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). *J. Phycol.* 44:684–90.

Huang, J. C., Wang, Y., Sandmann, G. & Chen, F. 2006. Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*. 71:473–79.

Johnson, E. A. & Schroeder, W. A. 1995. Microbial carotenoids. *Adv. Biochem. Eng. Biotechnol.* 53:119–78.

Kim, J., Smith, J. J., Tian, L. & DellaPenna, D. 2009. The evolution and function of carotenoid hydroxylases in *Arabidopsis*. *Plant Cell Physiol.* 50:463–79.

Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. L. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305:567–80.

Li, Y., Huang, J., Sandmann, G. & Chen, F. 2008a. Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). *Planta* 228:735–43.

Li, Y., Huang, J., Sandmann, G. & Chen, F. 2009. High-light and sodium chloride stress differentially regulate the biosynthesis of astaxanthin in *Chlorella zofingiensis* (Chlorophyceae). *J. Phycol.* 45:635–41.

Li, Y., Sommerfeld, M. & Chen, F. 2008b. Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). *J. Plant Physiol.* 165:1783–97.

Maresca, J. A., Graham, J. E., Wu, M., Eisen, J. & Bryant, D. A. 2007. Identification of a new family of lycopene cyclases in photosynthetic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 104:11784– 89.

Olmedilla, B., Granado, F., Blanco, I. & Vaquero, M. 2003. Lutein, but not α-tocopherol, supplementation improves visual function in patients with age-related cataracts: a 2-y double blind, placebo-controlled study. *Nutrition* 19:21–25.

Orosa, M., Valero, J. F., Herrero, C. & Abalde, J. 2001. Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other microalgae under N-starvation and high light conditions. *Biotechnol. Lett.* 23:1079–85.

Persson, B. & Argos, P. 1994. Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J. Mol. Biol*. 237:182–92.

Ramos, A., Coesel, S., Marques, A., Rodrigues, M., Baumgartner, A., Noronha, J., Rauter, A., Brenig, B. & Varela, J. 2008. Isolation and characterization of a stress-inducible *Dunaliella salina Lcy-β* gene encoding a functional lycopene β-cyclase. *Appl. Microbiol. Biotechnol.* 79:819–28.

Rise, M., Cohen, E., Vishkautsan, M., Cojocaru, M., Gottlieb, H. E. & Arad, S. M. 1994. Accumulation of secondary carotenoids in *Chlorella zofingiensis. J. Plant Physiol.* 144:287–92.

Romero, J. M., Lara, C. & Guerrero, M. G. 1989. Determination of intracellular nitrate. *Biochem. J.* 259:545–48.

Rossmann, M. G., Moras, D. & Olsen, K. W. 1974. Chemical and biological evolution of a nucleotide-binding protein. *Nature* 250:194–99.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. *Molecular Cloning*. *A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, A.1, 1.74–1.84 pp.

Sandmann, G., Romer, S. & Fraser, P. D. 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng*. 8:291–302.

Steinbrenner, J. & Linden, H. 2003. Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. *Plant Mol. Biol.* 52:343–56.

Sun, N., Wang, Y., Huang, J. C. & Chen, F. 2008a. Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). *Process Biochem*. 43:1288–92.

Sun, G., Zhang, X., Sui, Z. & Mao, Y. 2008b. Inhibition of *pds* gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). *Mar. Biotechnol.* 10:219–26.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. 24:1596–99.

Varkonyi, Z., Masamoto, K., Debreczeny, M., Zsiros, O., Ughy, B., Gombos, Z., Domonkos, I., Farkas, T., Wada, H. & Szalontai, B. 2002. Low-temperature-induced accumulation of xanthophylls and its structural consequences in the photosynthetic apparatus of the cyanobacterium *Cylindrospermopsis raciborskii*: an FTIR spectroscopic study. *Proc. Natl. Acad. Sci. U.S.A.* 99:2410–15.

Vidhyavathi, R., Venkatachalam, L., Sarada, R. & Ravishankar, G. A. 2008. Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *J. Exp. Bot*. 59:1409–18.

Capítulo IV

Aislamiento y caracterización del gen licopeno ε-ciclasa de *Chlorella* (*Chromochloris*) *zofingiensis*. Regulación de la ruta carotenogénica por luz y nitrógeno

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Article

Isolation and Characterization of a Lycopene ε-Cyclase Gene of *Chlorella* (*Chromochloris*) *zofingiensis*. Regulation of the Carotenogenic Pathway by Nitrogen and Light

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Abstract: The isolation and characterization of the lycopene ε-cyclase gene from the green microalga *Chlorella* (*Chromochloris*) *zofingiensis* (*Czlcy-e*) was performed. This gene is involved in the formation of the carotenoids α-carotene and lutein. *Czlcy-e* gene encoded a polypeptide of 654 amino acids. A single copy of *Czlcy-e* was found in *C. zofingiensis*. Functional analysis by heterologous complementation in *Escherichia coli* showed the ability of this protein to convert lycopene to δ-carotene. In addition, the regulation of the carotenogenic pathway by light and nitrogen was also studied in *C. zofingiensis*. High irradiance stress did not increase mRNA levels of neither lycopene β-cyclase gene (*lcy-b*) nor lycopene ε-cyclase gene (*lcy-e*) as compared with low irradiance conditions, whereas the transcript levels of *psy*, *pds*, *chyB* and *bkt* genes were enhanced, nevertheless triggering the synthesis of the secondary carotenoids astaxanthin, canthaxanthin and zeaxanthin and decreasing the levels of the primary carotenoids α-carotene, lutein, violaxanthin and β-carotene. Nitrogen starvation *per se* enhanced mRNA levels of all genes considered, except *lcy-e and pds*, but did not trigger the synthesis of astaxanthin, canthaxanthin nor zeaxanthin. The combined effect of both high light and nitrogen starvation stresses enhanced

significantly the accumulation of these carotenoids as well as the transcript levels of *bkt* gene, as compared with the effect of only high irradiance stress.

Keywords: carotenoids; microalgae; lycopene cyclase genes; gene expression; gene regulation

1. Introduction

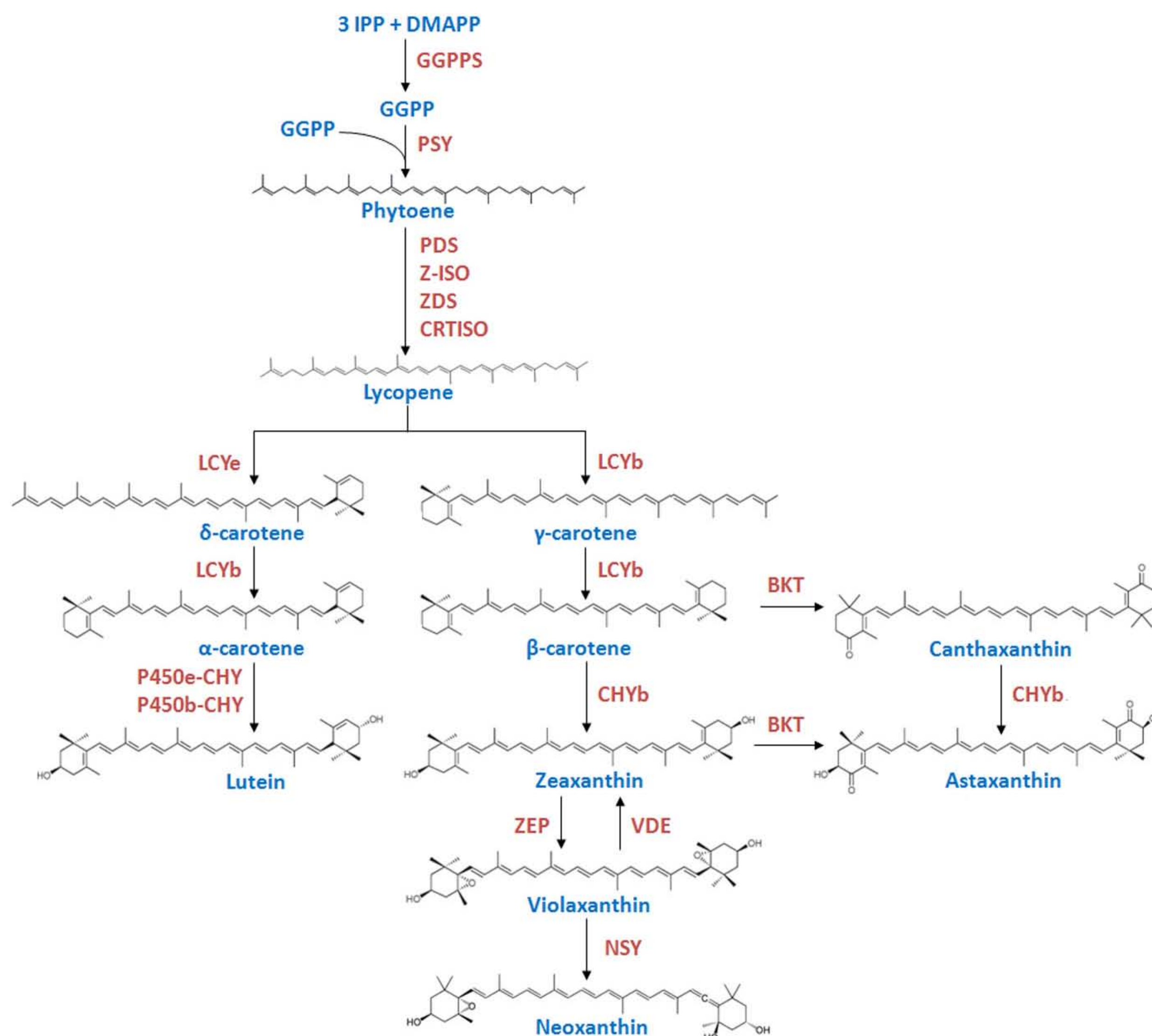
Carotenoids are essential pigments for all photosynthetic organisms as concerns participation in light harvesting, photoprotection, structural maintenance of pigment-protein complexes and membrane structure and fluidity [1–3]. In chloroplasts of plants and algae, the carotenoids precursor, geranylgeranyl pyrophosphate (GGPP), is synthesized by the action of the GGPP synthase from isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are derived from deoxyxylulose 5-phosphate pathway. The condensation of two GGPP molecules produces the first carotene, phytoene, catalyzed by phytoene synthase (PSY) (Figure 1).

PSY has been shown to be rate-limiting for carotenoid synthesis in plants [4,5] and algae [6]. Phytoene is desaturated by phytoene and ζ-carotene desaturases (PDS and ZDS) and isomerized by 15-*cis*-ζ-carotene isomerase (Z-ISO) [7] and carotene isomerase (CRTISO) to form the linear all *trans*-lycopene. Lycopene is converted into either β-carotene by the action of lycopene β-cyclase (LCYb) or into α-carotene by the action of lycopene ε-cyclase (LCYe) and LCYb. The cyclation of lycopene into either α- or β-carotene is a key branch point in the pathway of carotenoid biosynthesis in plants and some algal classes and has been proposed as a control step [8]; the relative activities of LCYe and LCYb determine the proportion of carotenoids directed to each branch of the pathway. α-carotene is modified into lutein by the hydroxylases P450b-CHY and P450e-CHY, and β-carotene is hydroxylated by CHYb to zeaxanthin. Zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) catalyze the interconversion of zeaxanthin and violaxanthin [5,9], and neoxanthin is formed from violaxanthin by the action of neoxanthin synthase (NSY). A limited number of organisms including some green algae such as *Haematococcus pluvialis* and *C. zofingiensis* can synthetize astaxanthin from β-carotene by the action of a ketolase/oxygenase (BKT) and the hydroxylase (CHYb) [10–14].

Currently, lutein and astaxanthin are widely used as feed additives in poultry farming and aquaculture. They have also important applications in food, nutraceutical and pharmaceutical industries because of their antioxidant activity and beneficial effects on human health [15,16].

C. zofingiensis is a model organism for the study of the regulation of the carotenoids biosynthetic pathway, since it produces both the primary carotenoids lutein and violaxanthin under standard growth conditions, as well as the secondary carotenoids astaxanthin, canthaxanthin and zeaxanthin under stress conditions such as high irradiance and nitrogen starvation or NaCl stress [17–21]. In recent years, some carotenogenic genes, namely *pds*, *bkt*, and *chyB* have been isolated and characterized in this microalga [12,13,22,23] and their regulation by light, NaCl, and different organic carbon sources has also been studied. High light stress up-regulated the transcripts of *pds*, *chyB* and *bkt* and NaCl stress only up-regulated the transcript levels of *bkt* [22,24]. The astaxanthin contents and the expression of *pds*, *chyB* and *bkt* genes were increased by glucose, sucrose or mannose addition to cells grown either at low irradiance [12,22] or in the dark [13,22].

Figure 1. Schematic diagram of the carotenoid biosynthetic pathway in plants and green algae. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGPPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; ZDS, ζ-carotene isomerase; CRTISO, carotene isomerase; LCYe, lycopene ε-cyclase; LCYb, lycopene β-cyclase; P450e-CHY, cytochrome P450 ε-hydroxylase; P450b-CHY, cytochrome P450 β-hydroxylase; CHYb, carotene β-hydroxylase; BKT, β-carotene oxygenase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase.



Recently, we have isolated and characterized the carotenogenic genes *psy* and *lcy-b* from *C. zofingiensis* and have studied the effect of light and nitrogen on transcript levels of *lcy-b* gene, as well as on lutein and astaxanthin accumulation [6,25]. High irradiance stress did not up-regulate the transcripts of *lcy-b*, although it triggered astaxanthin synthesis. In contrast, nitrogen starvation increased mRNA levels of *lcy-b* but did not trigger the synthesis of astaxanthin. Nevertheless, the combination of high irradiance and nitrogen deprivation led to a significant enhancement of the astaxanthin accumulation accompanied by a decrease in lutein levels.

In this paper, we report the isolation and characterization of the *lcy-e* gene from *C. zofingiensis*, a very unknown gene in algae, as well as confirm its ability to convert lycopene into δ-carotene. A general regulation study of the carotenogenic pathway, considering the new isolated *Czlcy-e* gene and other genes of the pathway in response to light and nitrogen has also been performed.

2. Results

2.1. Isolation and Characterization of the lcy-e Gene and Deduced Protein from C. zofingiensis

Different pairs of degenerate primers were designed on the basis of the conserved motifs present in *lcy-e* from microalgae. A partial cDNA fragment of 669 bp was isolated by PCR amplification using degenerate primers (*lcy-e*-1F and *lcy-e*-1R) (Table 1). A complete BLAST homology searches in the Genbank database showed that this fragment had enough similarity with the *lcy-e* gene from other species and provided sequence information for designing specific primers for rapid amplification of 5′ and 3′ cDNA ends (RACE-PCR). This analysis generated a full-length cDNA of 2204 bp, which contained an ORF of 1965 bp, 2-nucleotides of 5′-untranslated region (UTR), and a long 3´ UTR of 237 nucleotides. A typical algal polyadenylation signal TGTAAA [26] was present in the 3´ UTR at 82 nucleotides upstream from the beginning of the poly(A) tail. The predicted protein has 654 amino acids residues, with an estimated molecular weight of 71.1 kDa, a theoretical isoelectric point of 8.45 and an instability index of 43.6 (data obtained with ProtParam program, [27]). The differences between the *C. zofingiensis lcy-e* gene and the cDNA sequence were compared and revealed the presence of 8 exons and 7 introns. Exon size ranged between 124 (exon III) and 488 bp (exon I), and intron size between 159 (intron 1) and 331 bp (intron 3). Approximately 56% (2.2 out of 3.9 kb) of the *Czlcy-e* gene corresponded to exon sequences. In all introns, the consensus GT donor and AG acceptor sequences at the 5´and 3´termini were found (Figure 2).

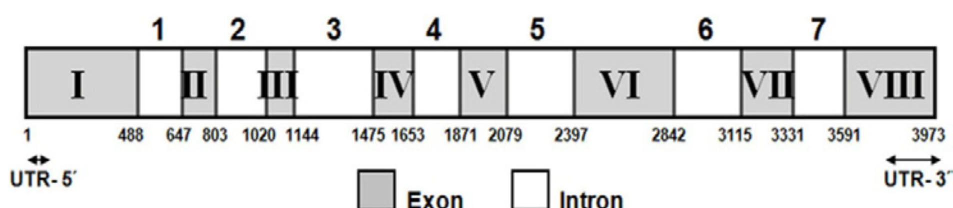
Table 1. Nucleotide sequences of primer pairs used for PCR amplification. F: forward; R: reverse. [a] *Sac*I and *Hind*III sites (lowercase letters underlined) were added for cloning the gene into the corresponding cut sites of pQE-80L vector.

Primer	Sequence (5′→3′)
Partial *lcy-e* fragment	
lcy-e-1F	AACCGCGTGTTCCTGGARGARACNTG
lcy-e-1R	TGGCACAGCAGCTCCATNCCRAA
5′ and 3′ RACE	
GSP-F	GGCATCAAAGTCACACGCATACACG
GSP-R	ACTGAACCCTGTGGCGGGATGCACC
NGSP-F	ACCCTCAGCAAACCAGCCTATTACAGCT
NGSP-R	CTTGAAAGGCAGTGCTGGCTTAGCTA
Genomic DNA amplification	
lcy-e-2F	ACATGGGGACACCAGCAGCAACTG
lcy-e-2R	GCGAGGGGGTTGTGACTGCATCT
lcy-e-3F	CCAGCAAGACAAGCTCGCAGCAATG
lcy-e-3R	TGCACAGATCCACGAGGTGCTGGC

Table 1. *Cont.*

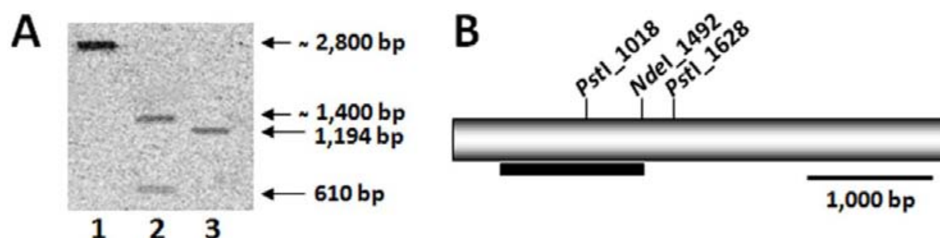
lcy-e-4F	GTGTTACTTTGGTGAGGGCAATCAGGTC
lcy-e-4R	CCACAAGCCATCATTAGCATTCGGGTGG
lcy-e-5F	GTTCATGGATTACAGAAGGCACCACACAGG
lcy-e-5R	TGACTCCCTGACAATGCTTGCACCGC
lcy-e-6F	TGGTGCATCCTGCCACAGGGTTCA
lcy-e-6R	CACCAGTCATAGCTGATTCCTTACTGCTCC
lcy-e-7F	GATCCTGCTGGCAGATACCTAATCAGTC
lcy-e-7R	GCAACTCTTGGCTTAAAGCTAGGTGC
PCR for probe preparation	
Czlcy-e-S-F	CCAGCAAGACAAGCTCGCAGCAATG
Czlcy-e-S-R	TGCACAGATCCACGAGGTGCTGGC
Genetic complementation-pQE-80L [a]	
pQE-lcy-e-F	gagctcATGGGGACACCAGCAGCAACTGTA
pQE-lcy-e-R	aagcttTCACTGTTGCACCTGTGTTGCTGC
***Czlcy-e* expression**	
RT-Czlcy-e-F	TCAAAGCACAGGCGAACAAACA
RT-Czlcy-e-R	AACGTCGGGACCTATAAGTCCG
***Czlcy-b* expression**	
RT-Czlcy-b-F	CGCAGGCGAAAAATTCCTGTCA
RT-Czlcy-b-R	TAAGGAATGTCACACCGCTGGC
***Czpsy* expression**	
RT-Czpsy-F	CACCAGGTTGTCAGAGTCCA
RT-Czpsy-R	ACTAGTGTGTTGCTGACTCT
***Czpds* expression**	
RT-Czpds-F	GCCAGAAAAGATCCAATTTG
RT-Czpds-R	CATGCTTCTCCCGCAAGAAC
***CzchyB* expression**	
RT-CzchyB-F	ATTGGAGGAGTGTTTGGCATGGAG
RT-CzchyB-R	AGATATCGTTGGCCTCGAATGGTC
***Czbkt* expression**	
RT-Czbkt-F	GTGGTTTGGCAGGTTTATGT
RT-Czbkt-R	AGAACAATCGGAACGCACTG

Figure 2. *Czlcy-e* gene organization. The diagram shows that the *Czlcy-e* gene consists of eight exons (I-VIII) and seven introns (1-7). The 5´ UTR and 3´ UTR sequences are indicated with arrows. Numbers indicate cDNA sequence coordinates (bp). UTR, unstranslated region.



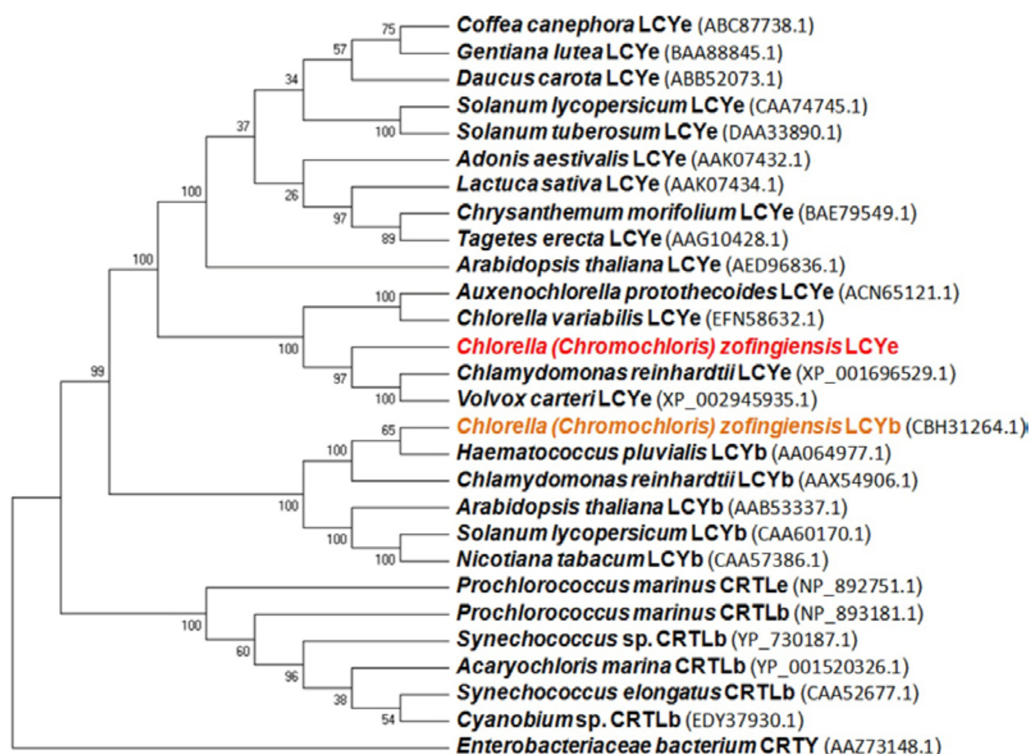
To determine the copy number of *lcy-e* gene in the genome of *C. zofingiensis*, genomic DNA was digested with two different restriction enzymes (either *Nde*I or *Pst*I) and subjected to Southern blot analysis at two different conditions of stringency, both in hibridization and washing (42 and 65 °C). Using a 1194-bp fragment of *Czlcy-e* as a probe, strong hybridization signals were obtained with both digestions. The digestion with *Pst*I enzyme, which cuts once inside the probe sequence, showed two bands, while digestion with *Nde*I, which cuts at one extreme of the probe, exhibited only one band for the two conditions of stringency tested (Figure 3). These results suggested the presence of a single copy of the *lcy-e* gene in the genome of *C. zofingiensis*.

Figure 3. Southern blot analysis of genomic DNA from C. zofingiensis. (A) DNA was digested with NdeI (lane 1) or PstI (lane 2), electrophoretically separated on a 0.8% agarose gel, blotted and hybridized at 65 °C with a probe of 1194 bp of the lcy-e gene amplified by PCR. A plasmid containing the lcy-e gene was used as a positive control (lane 3). (B) NdeI and PstI restriction sites present in the Czlcy-e gene. The black bar indicates the probe location.



The BlastP search results demonstrated that the cloned CzLCYe showed the highest overall homology sequence with other LCYe from green algae, such as *Chlamydomonas reinhardtii* and *Volvox carteri* (identity 66%, similarity 77%) and *Auxenochlorella protothecoides* and *Chlorella variabilis* (identity 62%, similarity 74%). The GC content of the *Czlcy-e* coding region was 53%, which was lower than that of *C. reinhardtii* and *V. carteri* (63%) or of *A. protothecoides* (61%). The phylogenetic analysis of lycopene ε- and β-cyclases from green algae, cyanobacteria, plants and bacteria is illustrated in Figure 4. Analysis was conducted in MEGA5 using UPGMA method [28]. The predicted CzLCYe forms a cluster with the LCYe of green algae, which are phylogenetically close to LCYe of plants (~44% of identity, and 61% similarity). The degree of homology was lower with the LCYb of green algae, including *C. zofingiensis*, and plants (about 39% identity and 55% similarity) and with both cyanobacterial cyclases (CRTLe and CRTLb) (around 35% identity and 51% similarity). As other algal LCYe, CzLCYe was distantly related to bacterial CRTY cyclases, sharing with them only a few conserved motifs and about 23% identity. The characteristic Rossmann or dinucleotide binding fold and two cyclase motifs, present in LCYe and LCYb of green algae and plants, CRTLe and CRTLb of cyanobacteria and CRTY of bacteria, were also identified in the LCYe of *C. zofingiensis*, between amino acids 187 and 215, 405 and 421, and 480 and 489, respectively. In addition, a leucine in a region near the C-terminus, which was demonstrated to determine ε-monocyclase activity, was also identified in LCYe of *C. zofingiensis*. One basic amino acid histidine or lysine in that position was found in lycopene ε-cyclases from *Lactuca sativa* and *Prochlorococcus marinus* MED4 both of them showing ε-bicyclase activity [29,30].

Figure 4. UPGMA tree analysis of the indicated plant, algal, cyanobacterial and bacterial lycopene cyclase amino acids sequences. Analysis was performed in MEGA5 [28]. The GenBank accession numbers for these species are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances correspond to the number of amino acid substitutions per site and were computed using the Poisson correction method. Numbers at nodes indicate bootstrap values calculated over 500 replicates.



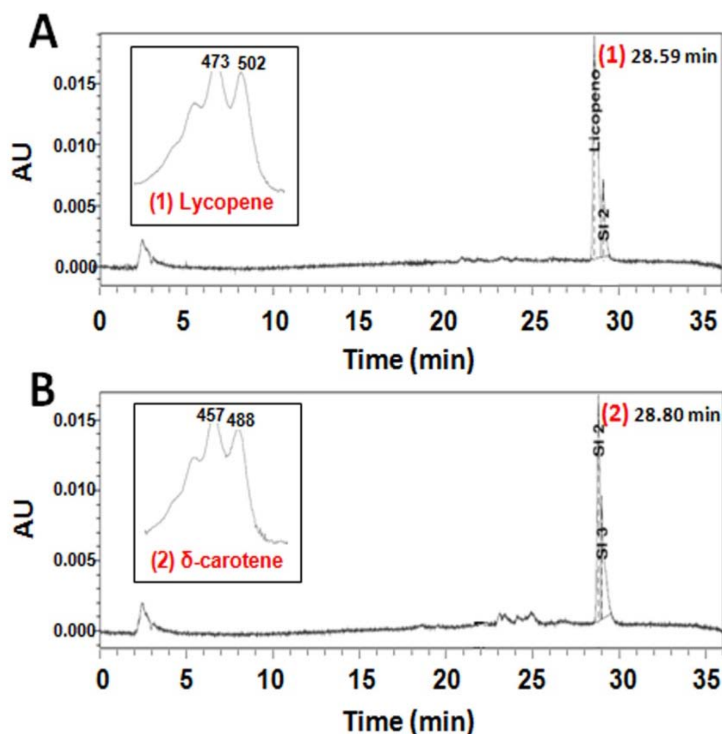
Since microalgae and plants LCYe is located in the chloroplast membranes, we analyzed the CzLCYe sequence with different programs to determine both the presence of a signal peptide and transmembrane domains. The iPSORT program [31] predicted a putative plastid localization for the CzLCYe, and ChloroP 1.1 server [32] identified a chloroplast transit peptide at *N*-terminal end. Analysis with ProtScale [27] and TopPred [33] servers identified five deduced transmembrane domains of CzLCYe located between amino acids 1–21, 186–206, 472–492, 576–596 and 633–656 (data not shown). In addition, the predicted CzLCYe was highly hydrophobic. It had 46% of hydrophobic and non polar amino acids and 19% of charged amino acids.

2.2. Functional Analysis of the CzLCYe *in E. coli*

In order to check the functionality of the recently isolated gene, the full-length ORF of *Czlcy-e* was amplified and cloned into pQE-80L vector under the control of the β-galactosidase promoter. The resulting plasmid (pQE-*Czlcy-e*) was introduced in *E. coli* engineered to accumulate either lycopene or δ-carotene as final products, due to the presence of the plasmids pAC-LYC or pAC-DELTA, respectively. HPLC analysis of carotenoids extracted from *E. coli* showed that cells containing pAC-LYC produced

lycopene (Figure 5A), while cells co-transformed with both pAC-LYC and pQE-*Czlcy-e* accumulated δ-carotene (Figure 5B). δ-carotene was also the only carotenoid synthesized by *E. coli* cells containing pAC-DELTA or both pAC-DELTA and pQE-*Czlcy-e* (data not shown). As negative controls, *E. coli* co-transformed with either pAC-LYC or pAC-DELTA and empty pQE-80L were used, resulting in the accumulation of lycopene or δ-carotene, respectively (data not shown). These results indicated that CzLCYe could catalyze the formation of one ε-ring at one of the ends of lineal lycopene to yield δ-carotene, but not the conversion of δ-carotene into ε-carotene (with one ε-ring in each end of lycopene), exhibiting, therefore, monocyclase activity.

Figure 5. HPLC elution profiles of carotenoids extracted from cultures of *E. coli* carrying plasmids pAC-LYC (**A**) and pAC-LYC + pQE-*Czlcy-e* (**B**). The absorption spectra and retention time of the corresponding carotenoids are also shown. *E. coli* BL21 (DE3) cells transformed with the indicated plasmids were isolated in the presence of chloramphenicol (**A**) or chloramphenicol + ampicillin (**B**). Lycopene and δ-carotene were identified as described in Experimental Section. Peaks identification: (1) lycopene; (2) δ-carotene.



2.3. Expression of Carotenogenic Genes: Effect of Irradiance and Nitrogen

C. zofingiensis cells were grown photoautotrophically at low irradiance (20 µmol photon m^{-2} s^{-1}) and a nitrogen replete concentration (nitrate 20 mM), as indicated in Experimental Section, until the middle of the exponential phase. Cells were then kept in the dark for 18 h, in order to make the mRNA levels come down to basal values. After this dark period, cells were subjected to either low (20 µmol photon m^{-2} s^{-1}) or high irradiance (300 µmol photon m^{-2} s^{-1}), under either nitrogen replete or nitrogen-deprivation conditions. Under conditions of nitrogen replete, nitrate concentration was measured

daily (data not shown) and the nitrate consumed by the cells was added to the cultures. The evolution with time of transcriptional expression of the carotenogenic gene *lcy-e* as well as *psy*, *pds*, *lcy-b*, *chyB* and *bkt* as affected by irradiance and nitrogen availability was monitored by qRT-PCR. Changes in content of both the primary carotenoids lutein and α-carotene, β-carotene and violaxanthin and the secondary carotenoids astaxanthin, canthaxanthin and zeaxanthin were also determined in order to correlate transcript levels with the biosynthesis of them. As shown in Figure 6, under nitrogen replete concentrations and both at low and high irradiance, the relative transcript levels of *lcy-e* increased significantly, attaining 14-fold higher values than basal ones after 48 h, decreasing thereafter. On the contrary, nitrogen starvation did not affect the transcript levels of *lcy-e*, registering similar values to basal ones at both low and high irradiance. As previously described [25], the transcriptional expression of the *Czlcy-b* gene increased under nitrogen starvation (4-fold higher than basal levels), attaining a maximum after 24 h at low irradiance and decreasing later, similar maximal values being reached later, at 48 h, at high irradiance and being kept with time. Under nitrogen replete, no difference in maximal transcript levels either at high or low irradiance was observed. In the case of *psy*, mRNA levels at low irradiance and nitrogen replete were similar to those in the dark, increasing by about 5-fold when irradiance was raised from 20 to 300 µmol photons m^{-2} s^{-1}, both under nitrogen starvation and nitrogen replete, attaining a peak after 24 h and decreasing slightly with time. At low irradiance, nitrogen starvation enhanced *psy* transcript levels by 3-fold with respect to the basal level after 96 h. The *pds* transcript levels, regardless of nitrogen availability, were two-fold higher at low irradiance after 24 h and three-fold higher at high irradiance after 5 h, when compared to basal levels, decreasing with time in both cases. The relative mRNA levels of *chyB* were about 4- and 7-fold higher than basal levels at low and high irradiance, respectively, under nitrogen replete, attaining a peak after 24 h. Nitrate deprivation increased slightly the transcript levels of *chyB* at low irradiance, showing similar values at high light intensity. In the case of *bkt*, mRNA levels at low irradiance and nitrogen replete were similar to basal values, increasing by about 4-fold when irradiance was raised from low to high light intensity, attaining a peak after 24 h and remaining constant with time. At low irradiance, nitrogen starvation enhanced *bkt* transcript levels by 3-fold with respect to the basal level after 24–48 h, remaining constant thereafter. The highest values were attained at high irradiance and nitrogen deprivation, reaching mRNA levels of *bkt* 7-fold higher as compared to the basal ones after 24 h, decreasing with time.

With regard to cell primary carotenoid contents such as lutein, α-carotene, β-carotene and violaxanthin (Figure 7), they accumulated at low irradiance and enough nitrogen availability, cell contents decreasing at high irradiance and more significantly under conditions of both high irradiance and nitrogen starvation. An opposite trend was observed for secondary carotenoids, such as canthaxanthin, zeaxanthin and astaxanthin, their synthesis being triggered at high irradiance and the highest accumulations of these carotenoids being registered under conditions of nitrogen starvation. In addition, cells were lacking these carotenoids at low irradiance, regardless of the nitrogen availability. Total carotenoids and chlorophylls *a* and *b* contents showed a similar response than that registered for lutein under the different conditions studied (data not shown).

Figure 6. Effect of irradiance and nitrogen availability on the mRNA levels of the phytoene synthase (*psy*), phytoene desaturase (*pds*), lycopene β-cyclase (*lcy-b*), lycopene ε-cyclase (*lcy-e*), carotene β-hydroxylase (*chyB*) and β-carotene oxygenase (*bkt*) genes in *C. zofingiensis*. Culture conditions: low irradiance (20 μmol photons m^{-2} s^{-1}) and nitrate replete (black line); low irradiance and nitrate deprivation (red line); high irradiance (300 μmol photons m^{-2} s^{-1}) and nitrate replete (green line); high irradiance and nitrate deprivation (blue line). Error bars indicate the standard deviations of four independent measurements.

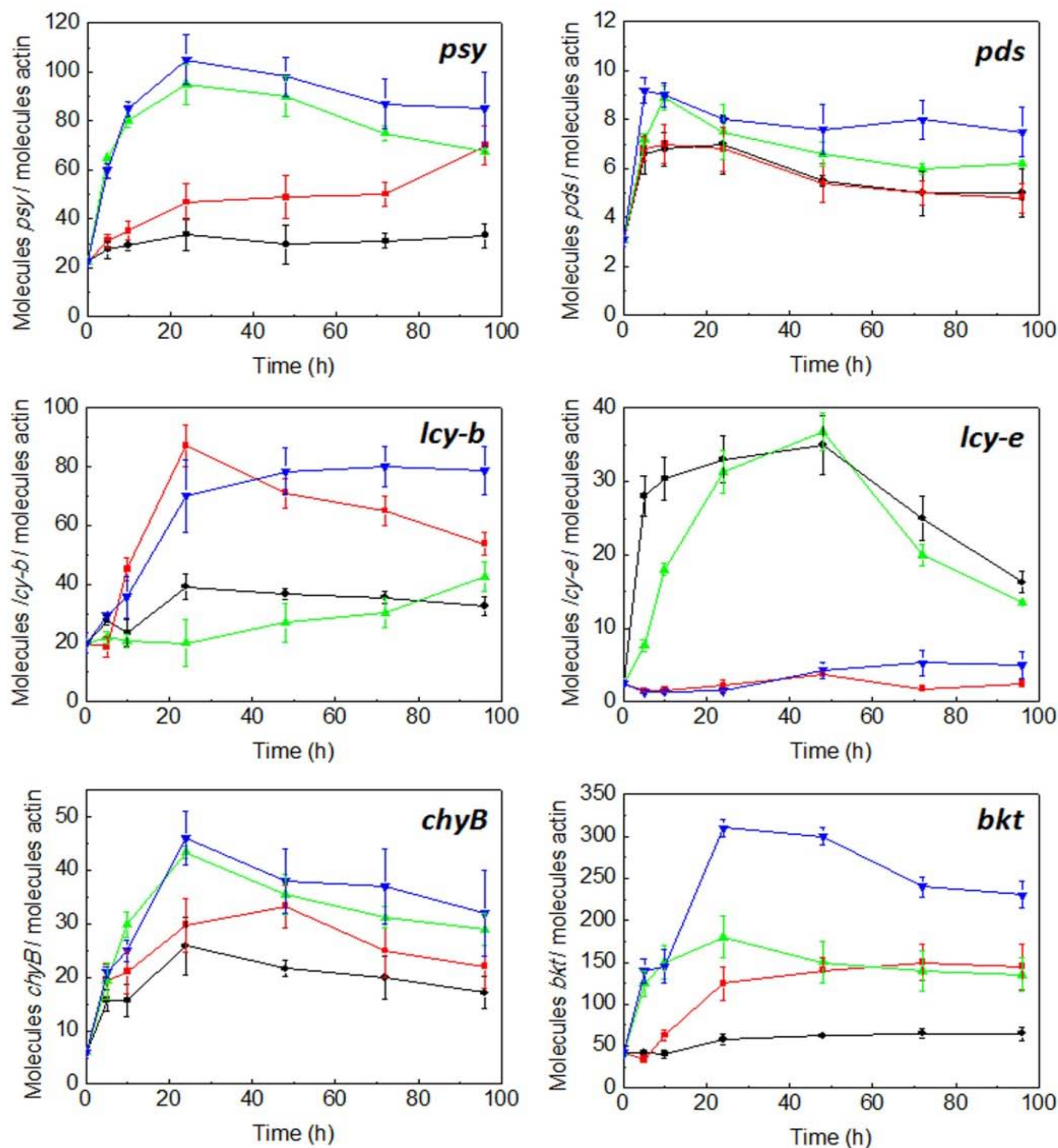
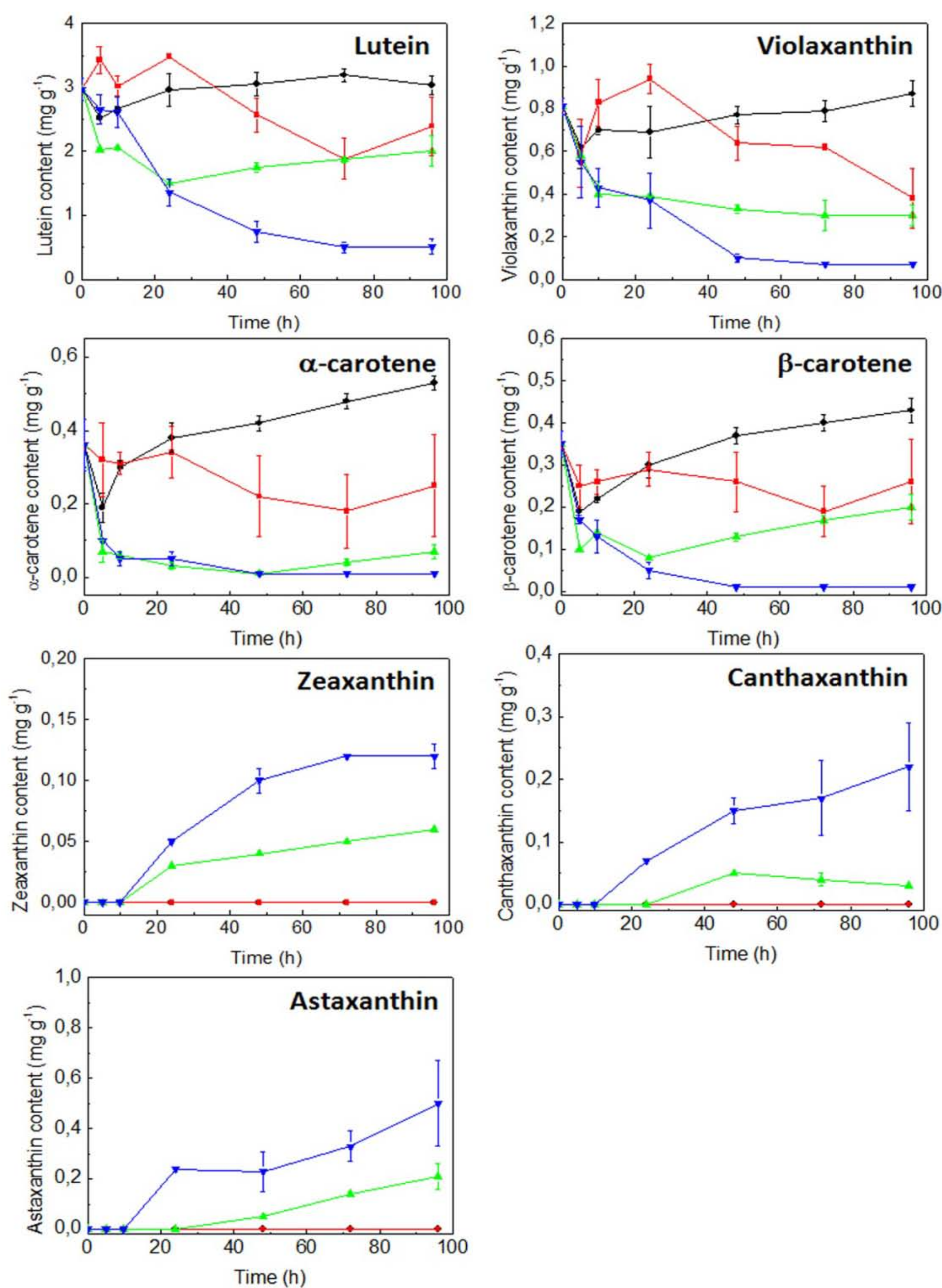


Figure 7. Effect of irradiance and nitrogen availability on the cellular content of lutein, violaxanthin, α-carotene, β-carotene, zeaxanthin, canthaxanthin and astaxanthin in *C. zofingiensis*. Culture conditions: low irradiance (20 µmol photons m^{-2} s^{-1}) and nitrate replete (black line); low irradiance and nitrate deprivation (red line); high irradiance (300 µmol photons m^{-2} s^{-1}) and nitrate replete (green line); high irradiance and nitrate deprivation (blue line). Carotenoids were identified as described in Experimental Section. Error bars indicate the standard deviations of four independent measurements. dw, dry weight.



3. Discussion

The carotenoid biosynthetic pathway is divided into two divergent branches at lycopene level in plants, some algae classes, such as green algae and certain cyanobacteria. In one branch, LCYb adds one β-ring at both ends of linear lycopene to form the β-β-carotenoid (β-carotene), which is transformed into zeaxanthin, violaxanthin and, only in some green algae, into astaxanthin (as *H. pluvialis* and *C. zofingiensis*). In the other branch, LCYe adds one ε-ring at one end of lycopene to form δ-carotene, which is transformed by LCYb into the β-ε-carotenoid, α-carotene, which is hydroxylated to lutein [3,29,34,35] (Figure 1).

In algae, *lcy-e* gene has only been functionally characterized in the green alga *Auxenochlorella protothecoides* CS-41 [36] and, in cyanobacteria, in the divinyl-Chl *a*/*b*-containing cyanobacterium *Prochlorococcus marinus* MED4 [30]. We report here the isolation and characterization of the *lcy-e* gene isolated from *C. zofingiensis*. This new gene exhibited 8 exons and 7 introns, the ORF of the cDNA encoding a hypothetical protein of 654 amino acids (Figure 2). The length of this putative protein was slightly higher than those described for green algae and plants, being the cyanobacterial protein about 150 amino acids shorter, according to alignments performed with Blocks program (data not shown). This could indicate the existence of a signal peptide at the 5´end of eukaryotic proteins. In fact, ChloroP program has confirmed the presence of a signal peptide in the CzLCYe. Although LCYe is an enzyme localized in chloroplast membranes, the predicted amino acid sequence of CzLCYe is not particularly hydrophobic. It has an average hydrophobic index of −0.078 and 19% of charged amino acids, and 46% are hydrophobic. However, by using Protscale and TopPred programs, five transmembrane domains of 20 amino acids each one uniformly distributed in the protein were found. A 42%–50% of hydrophobic amino acids were described for LCYe of plants and other green algae, as well as for other enzymes of the carotenogenic pathway, which were similar to that present in CzLCYe. However, the average hydrophobic index of these enzymes showed a higher variability, ranging from +0.116 for LCYe of *Volvox carteri* to −0.421 for PSY of *C. zofingiensis*

Four families of lycopene cyclases were identified: the monomeric lycopene β- and ε-cyclases from plants and some algae classes (LCYb and LCYe) and cyanobacteria (CRTLb and CRTLe); the usual monomeric bacterial lycopene β-cyclase (CRTY); the lycopene β-cyclases cruA and cruP, recently found in green sulphur bacteria and some cyanobacteria; and the heterodimeric lycopene β-cyclases (CRTYc and CRTYd) of some bacteria, which are related to the fungal bifunctional one (CRTYB) and include both PSY and lycopene β-cyclase activity. These families are distantly related to each other and share only a few conserved motifs, including a dinucleotide binding motif that is found in the first three groups but appears to be missing in the fourth [35,37–39]. This dinucleotide binding motif, as well as two cyclases motifs and a leucine residue in a region near the *C*-terminus indicative of ε-monocyclase activity were found in the predicted protein encoded by the new gene isolated from *C. zofingiensis* (data not shown). The predicted CzLCYe showed a high degree of homology with LCYe of plants, especially with the already known LCYe of other green algae, being the homology degree lower with the algae and plant LCYb and cyanobacterial CRTLe and CRTLb (Figure 4). These results would probably be enough to consider this new gene as a LCYe, but the functional analysis definitely confirmed this hypothesis. By complementation in *E. coli*, it was demonstrated that CzLCYe was able to catalyse the conversion of

lycopene into δ-carotene, but not the formation of α-carotene from δ-carotene (Figure 5), exhibiting, therefore, monocyclase activity, as most LCYe of plant and algae previously studied. Two of the few examples of ε-cyclases that show bicyclase activity are a LCYe of romaine lettuce which adds two ε-rings to lycopene to form ε-carotene [29], and a CRTLe from *Prochlorococcus marinus* MED4 that adds not only ε- but also β-rings to lycopene to form α-, β- and ε-carotene [30]. In these two cyclases one basic amino acid histidine or lysine was identified in a region near the *C*-terminus instead of a leucine, which is found in all ε-monocyclases studied [29,30].

The cyclation of lycopene into either α- or β-carotene has been proposed as a control step in the carotenogenic pathway of plants. The relative activities of LCYe and LCYb may determine the flow of carbon through the carotenoids pathway from lycopene to either α- or β-carotene and their derivatives [29,34]. Indeed, by reducing the expression of *lcy-e* in *Arabidopsis*, *Brassica* and potato by mutation, either by using RNAi or by introducing an antisense fragment of this gene, the ratio of β- to α-carotene and their products increased [40–42]. A study of the natural carotenoid variation in maize uncovered alleles of *lcy-e* that are expressed at low levels also correlated with an increase in β-carotene and its derivatives [8]. On the other hand, overexpression of either the endogenous *lcy-b* gene or the equivalent heterologous genes in crop plants, such as *Brassica* seeds and tomato fruits increased β-carotene levels [43], and *lcy-e* overexpression in *Arabidopsis* increased lutein content up to 180% of wild type [44].

In photoautotrophically grown *C. zofingiensis*, it is known that the combination of both high irradiance and nitrogen starvation causes a drop in the cellular content in primary carotenoids, such as α-carotene and its derivative lutein, and a concomitant accumulation of secondary carotenoids, such as the β-carotene products astaxanthin and canthaxanthin [17–19]. However, the molecular basis of this regulation under those conditions is not yet well understood. Recently, it has been shown that high irradiance up-regulated the *pds*, *chyB and bkt* genes, enhancing significantly the synthesis of the β-carotene derivatives canthaxanthin, zeaxanthin and astaxanthin in this microalga [12,22,24], but did not affect the transcription of *lcy-b* gene [25]. On the other hand, nitrogen deprivation, regardless of irradiance, induced the transcription of *lcy-b* gene, however astaxanthin content increased at the expense of lutein under nitrogen deprivation but only at high irradiance [25]. In this work, we have investigated the regulation by irradiance and nitrogen of the carotenogenic pathway of *C. zofingiensis* by determining the mRNA levels of *lcy-b*, *psy*, *pds*, *chyB*, *bkt* in addition to the *lcy-e* gene, just now isolated by us, as well as the cellular contents in α-carotene and its product lutein and β-carotene and its derivatives canthaxanthin, astaxanthin, zeaxanthin and violaxanthin. According to our results, high irradiance stress did not increase mRNA levels of neither *lcy-b* nor *lcy-e* genes as compared to low irradiance conditions, whereas the transcript levels of *psy*, *pds*, *chyB* and *bkt* genes were enhanced significantly. However, high light stress triggered the synthesis of the secondary carotenoids astaxanthin, canthaxanthin and zeaxanthin and decreased the levels of the primary carotenoids α-carotene, lutein, β-carotene and violaxanthin. Therefore, in *C. zofingiensis*, high irradiance triggered the synthesis of astaxanthin, canthaxanthin and zeaxanthin by transcriptional up-regulation of the *psy*, *pds*, *chyB* and *bkt* genes, but not *lcy-b*, and, consequently, the regulation of this last gene must take place at a post-transcriptional level under the conditions mentioned above. In *H. pluvialis*, unlike in *C. zofingiensis*, an up-regulation of *lcy-b* by high light was shown astaxanthin synthesis also being induced under this high irradiance condition [45,46]. In *Dunaliella salina*, high irradiance stress increased slightly mRNA levels of *lcy-b*, *psy* and *pds* genes as well as the cellular β-carotene content, and

the highest levels of these genes transcripts and β-carotene were obtained under high light combined with nutrient depletion. Nutrient limitation seems to be essential for β-carotene accumulation in this microalga [3,47,48].

In *C. zofingiensis*, nitrogen starvation per se enhanced mRNA levels of all genes considered, except *lcy-e* and *pds*, but did not trigger the synthesis of canthaxanthin, zeaxanthin nor astaxanthin. Nevertheless, the combination of both factors, high irradiance and nitrogen starvation, increased the levels of those carotenoids significantly. Therefore, the up-regulation of *lcy-b* but not of *lcy-e* by nitrogen starvation, regardless of irradiance, addressed the carbon flow from lycopene to β-carotene, canthaxanthin, astaxanthin and zeaxanthin instead of to α-carotene and lutein. However, nitrogen starvation under low irradiance did not trigger canthaxanthin, zeaxanthin and astaxanthin synthesis, although all up-stream and down-stream genes related to that branch of the pathway (except *pds*) were up-regulated, indicating either that the synthesis of all these carotenoids under the conditions mentioned above should be controlled at a post-transcriptional level or that pds is the main regulatory gene. The combined effect of both high light and nitrogen starvation stresses enhanced significantly the synthesis of canthaxanthin, zeaxanthin and astaxanthin, possibly due to a higher expression of *bkt*; on the contrary, lutein, α-carotene, β-carotene and violaxanthin decreased under these conditions. The decrease of violaxanthin content at high irradiance, especially under nitrogen deprivation, could be due to the increase in the accumulation of zeaxanthin through the xanthophylls cycle [49]. Therefore, although changes in the mRNA levels have been shown to be the most important regulation for carotenoid genes [3], post-transcriptional and translational levels also playing important roles, as well as the stability of RNA, as has been shown for *H. pluvialis* [3,45,46].

4. Experimental Section

4.1. Strains and Culture Conditions

The green microalga strain *Chlorella zofingiensis* SAG 211-14 (recently classified as *Chromochloris zofingiensis*, [50]) was obtained from the Culture Collection of Göttingen University (SAG, Germany). This microalga was maintained and grown photoautotrophically in Arnon medium [51] modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$, at 25 °C under continuous illumination (50 µmol photons m^{-2} s^{-1}), except where indicated. The liquid cultures were continuously bubbled with air, supplemented with 1% (v/v) CO_2 as the only source of carbon. For the expression experiments, cells were grown in Roux flaks of 1 L capacity laterally and continuously illuminated with mercury halide lamps at either 20 (low irradiance) or 300 µmol photons m^{-2} s^{-1} (high irradiance) either in the presence or in the absence of nitrate. To keep constant the nitrate in the medium, the nitrate concentration was measured daily by HPLC according to Cordero *et al.* [25], and the nitrate consumed by the cells was added to the cultures. The light intensity was measured at the surface of the flasks using a LI-COR quantum sensor (model L1-1905B, Li-Cor, Inc. Lincoln, NE, USA).

Escherichia coli DH5α and BL21 strains were used as the hosts for DNA manipulation and heterologous expression of *lcy-e* gene, respectively.

4.2. Genomic DNA and RNA Isolation and cDNA Preparation

DNA and total RNA were isolated using DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen, Düsseldorf, Germany), respectively. For Quantitative Real-Time PCR analysis (qRT-PCR), first-strand cDNA synthesis was obtained from total RNA treated with DNase as recommended by the manufacturer, by using the SuperScript First-Strand Synthesis System (Invitrogen, Barcelona, Spain) primed with oligo(dT)$_{18}$ according to the manufacturer's instructions.

4.3. Cloning of C. zofingiensis lcy-e cDNA and Genomic Gene

For isolating the *lcy-e* cDNA from *C. zofingiensis*, degenerate primers were designed from the conserved regions of LCYe from different species of algae. The PCR product was cloned in the pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's manual and then sequenced. The cDNA fragment obtained corresponding to partial *lcy-e* clone provided sequence information for the designing of gene-specific primers for amplification of 5´and 3´ cDNA ends by RACE-PCR. All reactions were performed with kits according to the manufacturer's instructions (Smart RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA). 5´and 3´ RACE products were cloned into pGEM-T vector and sequenced. Specific primers were synthesized for genomic DNA amplification based on cDNA sequence. The primers sets used in this study are listed in Table 1.

4.4. Nucleotide Sequence Accession Numbers

The *Czlcy-e* cDNA and genomic DNA sequences have been registered in the EMBL database under the accession numbers HE664109 and HE664108, respectively.

4.5. Southern Blot Analysis

Genomic DNA was digested with *Nde*I and *Pst*I, which showed one recognition site in the probed region of the *lcy-e* gene. The probe was prepared by amplifying genomic DNA with the primers Czlcy-e-S-F and Czlcy-e-S-R, resulting in a 1194-bp fragment of *Czlcy-e* gene. The digested DNA was transferred to a Hybond-N membrane (GE Healthcare, Little Chanfont, UK) by capillary transfer and hybridized with the ^{32}P labelled DNA probe at both low and high stringency overnight. After hybridization, the radioactivity of the membrane was monitored by the Cyclone Phosphor System (Perkin Elmer, Waltham, MA, USA).

4.6. Functional Analysis of Czlcy-e cDNA by Heterologous Expression in E. coli

The *Czlcy-e* ORF was amplified by PCR with the primers pQE-lcy-e-F and pQE-lcy-e-R, which were designed to contain *Sac*I and *Hind*III restriction sites, respectively, and cloned into pQE-80L expression vector (Qiagen) resulting in plasmid pQE-*Czlcy-e*, which carries ampicillin resistance. The plasmids pAC-LYC and pAC-DELTA, kindly donated by Prof. Cunningham, carried the carotenoid pathway genes responsible for the synthesis of lycopene (*crtE*, *crtB*, and *crtI* of *Erwinia herbicola*) and δ-carotene (*crtE*, *crtB*, and *crtI* of *E. herbicola* + *lcy-e* of *Arabidopsis*), respectively [52,53]. Transformation of *E. coli*

BL21 (DE3) with pQE-*Czlcy-e* and/or one of the two plasmids pAC-LYC or pAC-DELTA (both carrying chloramphenicol resistance) was made by electroporation. Transformed cells were plated on Luria-Bertani (LB) [54], supplemented with 100 μg mL^{-1} ampicillin and/or 40 μg mL^{-1} chloramphenicol, and grown at 37 °C for 1 day. The inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM.

4.7. Quantitative RT-PCR

The mRNA relative abundance of *psy*, *pds*, *lcy-b*, *lcy-e*, *chyB* and *bkt* genes of *C. zofingiensis* was examined by qRT-PCR on an IQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA), according to Cordero *et al.* (2010) [25]. In each experiment, a series of standard dilutions containing a specific concentration of a PCR fragment or a cDNA template was amplified in 20 µL of reaction containing 1× SYBR Green PCR Master Mix (Quantimix Easy SYG kit, BioTools B&M Labs, Madrid, Spain) and corresponding primers (Table 1). After heating at 95 °C for 10 min, cycling parameters were: 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Finally, the specificity of the qRT-PCR products was confirmed by performing a melting temperature analysis at temperatures ranging from 55 to 95 °C at 0.5 °C per min and also by electrophoresis on a 2% agarose gel. Data were captured as amplification plots. Transcription levels of the target genes were calculated from the threshold cycle by interpolation from the standard curve. To standardize the results, the relative abundance of actin gene was also determined and used as the internal standard [45,55]. All calculations and statistical analyses were performed as described in the IQ5 Optical System Software 1.0 (BioRad). The complete experiments (RNA isolation, cDNA synthesis followed with qRT-PCR) were independently repeated twice, and the data were averaged.

4.8. Analytical Methods

4.8.1. Cell Concentration and Dry Weight Determinations

Cell number was determined with a Neubauer hemocytometer. For dry weight measurements, aliquots (5 mL) of the cell culture were filtered through Whatman GF/C paper (Whatman plc, Kent, UK), washed three times, and dried at 80 °C for 24 h.

4.8.2. Carotenoid Extraction and HPLC Analysis

Pigments were extracted with methanol according to Cordero *et al.* (2010) [25]. The samples were then saponified with ethyl ether and KOH 2% in methanol [20] and then centrifuged and analyzed by HPLC using a Waters Spherisorb ODS2 column (4.6 × 250 mm, 5 µm particle size) (Waters, Mildford, MA, USA). The chromatographic method described by Cordero *et al.* (2010) was used [25]. Pigments were eluted at a flow rate of 1.0 mL min^{-1} and were detected at 440 nm using a Waters 2996 photodiode-array detector. Identification of carotenoids was achieved by comparison of the individual characteristic absorption spectrum and the retention time with known standards. The retention times of carotenoid analysed were: violaxanthin, 13.26 min; astaxanthin, 15.54 min; lutein, 18.10 min; zeaxanthin, 18.51 min; canthaxanthin, 20.51 min; lycopene, 28.59 min; δ-carotene, 28.80 min; α-carotene, 29.24 min; β-carotene,

29.52 min. Quantification was performed using a calibration curve generated with commercially available carotenoids standards from Sigma-Aldrich (St. Louis, MO, USA) and DHI (Holsholm, Germany).

5. Conclusions

Our results on the characterization of the *Czlcy-e* gene and the regulation by light and nitrogen of lycopene cyclase genes and other carotenogenic genes such as *psy*, *pds*, *chyB* and *bkt* from *C. zofingiensis* could contribute to the understanding of the regulatory mechanisms of the biosynthesis of carotenoids at the molecular level, which can be helpful for the optimization of the physiological conditions for high carotenoids production by *C. zofingiensis* (specially the very rare and of high industrial interest astaxanthin), and for performing metabolic and genetic engineering in this microalga, when transformation methods are well established.

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References

1. Frank, H.A.; Cogdell, R.J. Carotenoids in photosynthesis. *Photochem. Photobiol*. **1996**, *63*, 257–264.
2. Cazzonelli, C. Carotenoids in nature: Insights from plants and beyond. *Funct. Plant Biol.* **2011**, *38*, 833–847.
3. Ramos, A.; Polle, J.; Tran, D.; Cushman, J.C.; Jin, E.; Valera, J. The unicellular green alga *Dunaliella salina* Teod. as a model for abiotic stress tolerance: Genetic advances and future perspectives. *Algae* **2011**, *26*, 3–20.
4. Fraser, P.D.; Romer, S.; Shipton, C.A.; Mills, P.B.; Kiano, J.W.; Misawa, N.; Drake, R.G.; Schuch, W.; Bramley, P.M. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit specific-manner. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1092–1097.
5. Sandmann, G.; Romer, S.; Fraser, P.D. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng.* **2006**, *8*, 291–302.
6. Cordero, B.F.; Couso, I.; Leon, R.; Rodriguez, H.; Vargas, M.A. Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 341–351.
7. Chen, Y.; Li, F.; Wurtzel, E.T. Isolation and characterization of the *Z-ISO* gene encoding a missing component of carotenoid biosynthesis in plants. *Plant Physiol.* **2010**, *153*, 66–79.
8. Harjes, C.E.; Rocheford, T.R.; Bai, L.; Brutnell, T.P.; Kandianis, C.B.; Sowinski, S.G.; Stapleton, A.E.; Vallabhaneni, R.; Williams, M.; Wurtzel, E.T. Natural genetic variation in lycopene ε-cyclase tapped for maize biofortification. *Science* **2008**, *319*, 330–333.

9. Kim, J.; Smith, J.J.; Tian, L.; DellaPenna, D. The evolution and function of carotenoid hydroxylases in *Arabidopsis*. *Plant Cell Physiol.* **2009**, *50*, 463–479.
10. Fan, L.; Vonshak, A.; Rachel, G.; Hirshberg, J.; Cohen, Z.; Boussiba, S. The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant Cell Physiol.* **1995**, *36*, 1519–1524.
11. Johnson, E.A.; Schroeder, W.A. Microbial carotenoids. *Adv. Biochem. Eng. Biotechnol.* **1995**, *53*, 119–178.
12. Huang, J.C.; Wang, Y.; Sandmann, G.; Chen, F. Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*. **2006**, *71*, 473–479.
13. Li, Y.; Huang, J.; Sandmann, G.; Chen, F. Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). *Planta* **2008**, *228*, 735–743.
14. Ojima, K.; Breitenbach, J.; Visser, H.; Setoguchi, Y.; Tabata, K.; Hoshino, T.; van den Berg, J.; Sandmann, G. Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and its assignment as a β-carotene 3-hydroxylase/4-ketolase. *Mol. Gen. Genomics* **2006**, *275*, 148–158.
15. Guerin, M.; Huntley, M.E.; Olaizola, M. *Haematococcus* astaxanthin applications for human health and nutrition. *Trends Biotechnol*. **2003**, *21*, 210–216.
16. Olmedilla, B.; Granado, F.; Blanco, I.; Vaquero, M. Lutein, but not α-tocopherol, supplementation improves visual function in patients with age-related cataracts: A 2-y double blind, placebo-controlled study. *Nutrition* **2003**, *19*, 21–25.
17. Rise, M.; Cohen, E.; Vishkautsan, M.; Cojocaru, M.; Gottlieb, H.E.; Arad, S.M. Accumulation of secondary carotenoids in *Chlorella zofingiensis*. *J. Plant Physiol*. **1994**, *144*, 287–292.
18. Bar, E.; Rise, M.; Vishkautsan, M.; Arad, S. Pigment and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. *J. Plant Physiol*. **1995**, *146*, 527–534.
19. Orosa, M.; Valero, J.F.; Herrero, C.; Abalde, J. Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other microalgae under *N*-starvation and high light conditions. *Biotechnol. Lett*. **2001**, *23*, 1079–1085.
20. Del Campo, J.A.; Rodríguez, H.; Moreno, J.; Vargas, M.A.; Rivas, J.; Guerrero, M.G. Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*. **2004**, *64*, 848–854.
21. Cordero, B.F.; Obraztsova, I.; Couso, I.; Leon, R.; Vargas, M.A.; Rodriguez, H. Enhancement of lutein production in *Chlorella sorokiniana* (Chorophyta) by improvement of culture conditions and random mutagenesis. *Mar. Drugs* **2011**, *9*, 1607–1624.
22. Huang, J.C.; Liu, J.; Li, Y.; Chen, F. Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). *J. Phycol.* **2008**, *44*, 684–690.
23. Sun, N.; Wang, Y.; Huang, J.C.; Chen, F. Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). *Proc. Biochem.* **2008**, *43*, 1288–1292.

24. Li, Y.; Huang, J.; Sandmann, G.; Chen, F. High-Light and sodium chloride stress differentially regulate the biosynthesis of astaxanthin in *Chlorella zofingiensis* (Chlorophyceae). *J. Phycol.* **2009**, *45*, 635–641.
25. Cordero, B.F.; Obraztsova, I.; Martin, L.; Couso, I.; Leon, R.; Vargas, M.A.; Rodriguez, H. Isolation and characterization of a lycopene β-cyclase gene from the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). *J. Phycol.* **2010**, *46*, 1229–1238.
26. Gruber, H.; Goetinck, S.D.; Kirk, D.L.; Schmitt, R. The nitrate reductase-encoding gene of *Volvox carteri*: Map location, sequence and induction kinetics. *Gene* **1992**, *120*, 75–83.
27. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.; Wilkins, M.R.; Appel, R.D.; Bairoch, A. Protein Identification and Analysis Tools on the ExPASy Server. In *The Proteomics Protocols Handbook*; Walker, J., Walker, M., Eds.; Humana Press: New Jersey, NJ, USA, **2005**; pp. 571–607.
28. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739.
29. Cunningham, F.X., Jr.; Gantt, E. One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2905–2910.
30. Stickforth, P.; Steiger, S.; Hess, W.R.; Sandmann, G. A novel type of lycopene ε-cyclase in the marine cyanobacterium *Prochlorococcus marinus* MED4. *Arch*. *Microbiol.* **2003**, *179*, 409–415.
31. Bannai, H.; Tamada, Y.; Maruyama, O.; Nakai, K.; Miyano, S. Extensive feature detection of *N*-terminal protein sorting signals. *Bioinformatics* **2002**, *18*, 298–305.
32. Emanuelsson, O.; Nielsen, H.; von Hejne, G. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci*. **1999**, *8*, 978–984.
33. Von Heijne, G. Membrane protein structure prediction: Hydrophobicity analysis and the "positive inside" rule. *J. Mol. Biol.* **1992**, *225*, 487–494.
34. Cunningham, F.X., Jr.; Pogson, B.; Sun, Z.; McDonald, K.A.; DellaPenna, D.; Gantt, E. Functional analysis of the β- and ε-lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* **1996**, *8*, 1613–1626.
35. Takaichi, S. Carotenoids in algae: Distributions, biosyntheses and functions. *Mar. Drugs* **2011**, *9*, 1101–1118.
36. Li, T.; Shi, C.; Gan, Z.; Shi, X. Cloning and analysis of the gene encoding lycopene epsilon cyclase in *Chlorella protothecoides* CS-41. *Wei Sheng Wu Xue Bao* **2009**, *49*, 1180–1189.
37. Sandmann, G. Molecular evolution of carotenoid biosynthesis from bacteria to plants. *Physiol. Plant* **2002**, *116*, 431–440.
38. Krubasik, P.; Sandmann, G. A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. *Mol. Gen. Genet.* **2000**, *263*, 423–432.
39. Maresca, J.A.; Graham, J.E.; Wu, M.; Eisen, J.; Bryant, D.A. Identification of a new family of lycopene cyclases in photosynthetic bacteria. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11784–11789.
40. Pogson, B.J.; McDonald, K.; Truong, M.; Britton, G.; DellaPenna, D. *Arabidopsis* carotenoid mutants demonstrate lutein is not essential for photosynthesis in higher plants. *Plant Cell* **1996**, *8*, 1627–1639.
41. Yu, B.; Lydiate, D.J.; Young, L.W.; Schafer, U.A.; Hannoufa, A. Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene ε-cyclase. *Transgenic Res.* **2008**, *17*, 573–585.

42. Diretto, G.; Tavazza, R.; Welsch, R.; Pizzichini, D.; Mourgues, F.; Papacchioli, V.; Beyer, P.; Giuliano, G. Metabolic engineering of potato tuber carotenoids through tuberspecific silencing of lycopene ε-cyclase. *BMC Plant Biol.* **2006**, *6*, 13.
43. Farre, G.; Sanahuja, G.; Naqvi, S.; Bai, C.; Capell, T.; Zhu, C.; Christou, P. Travel advice on the road to carotenoids in plants. *Plant Sci.* **2010**, *179*, 28–48.
44. Pogson, B.J.; Rissler, M. Genetic manipulation of carotenoid biosynthesis and photoprotection. *Phil. Trans. R. Soc. Lond. B* **2000**, *355*, 1395–1403.
45. Vidhyavathi, R.; Venkatachalam, L.; Sarada, R.; Ravishankar, G.A. Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *J. Exp. Bot.* **2008**, *59*, 1409–1418.
46. Steinbrenner, J.; Linden, H. Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: Regulation by photosynthetic redox control. *Plant Mol. Biol.* **2003**, *52*, 343–356.
47. Ramos, A.; Coesel, A.; Marques, A.; Rodrigues, M.; Baumgartner, A.; Noronha, J.; Rauter, A.; Brenig, B.; Varela, J. Isolation and characterization of a stress-inducible *Dunaliella salina Lcy*-β gene encoding a functional lycopene β-cyclase. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 819–828.
48. Coesel, S.N.; Baumgartner, A.C.; Teles, L.M.; Ramos, A.; Henriques, N.M.; Cancela, L.; Valera, J. Nutrient limitation is the main regulatory factor for carotenoid accumulation and for *Psy* and *Pds* steady state transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. *Mar. Biotechnol*. **2008**, *10*, 602–611.
49. Goss, R.; Jakob, T. Regulation and function of xanthophylls cycle-dependent photoprotection in algae. *Photosynth. Res.* **2010**, *106*, 103–122.
50. Fucikova, K.; Lewis, L.A. Intersection of *Chlorella*, *Muriella* and *Bracteacoccus*: Resurrecting the genus *Chromochloris* KOL et CHODAT (Chlorophyceae, Chlorophyta). *Fottea* **2012**, *12*, 83–93.
51. Arnon, D.I.; McSwain, B.D.; Tsujimoto, H.Y.; Wada, K. Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim. Biophys. Acta* **1974**, *357*, 231–245.
52. Cunningham, F.X., Jr.; Sun, Z.; Chamovitz, D.; Hirschberg, J.; Gantt, E. Molecular structure and enzymatic function of lycopene cyclase from the cyanbacterium *Synechococcus* sp. strain PCC 7942. *Plant Cell* **1994**, *6*, 1107–1121.
53. Cunningham, F.X., Jr.; Gantt, E. A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. *Photosynth. Res.* **2007**, *92*, 245–259.
54. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Nolan, C., Ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 1989.
55. Sun, G.; Zhang, X.; Sui, Z.; Mao, Y. Inhibition of *pds* gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). *Mar. Biotechnol.* **2008**, *10*, 219–226.

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Discusión

1. Selección de microalgas para la producción de luteína y aumento de la producción de dicho carotenoide en *Chlorella sorokiniana* por optimización de las condiciones de cultivo y mutagénesis al azar

En un estudio de 13 microalgas clorofíceas se ha seleccionado *Chlorella sorokiniana* para la producción de luteína, ya que presentaba la velocidad de crecimiento más alta (0,11 h^{-1}), además de un alto contenido en luteína (24 mg L^{-1}) (Tabla 1, Capítulo I). En esta microalga la luteína es el principal carotenoide (80-90 % del total de carotenoides), mientras que violaxantina, zeaxantina, anteraxantina, α- y β-caroteno están presentes en muy bajas concentraciones (Tabla 1, Capítulo I).

Puesto que se ha descrito que la irradiancia, la temperatura y las concentraciones de NaCl y nitrato afectan los niveles de diferentes carotenoides en microalgas (Jin et al., 2003; Bhosale, 2004; Del Campo et al., 2000, 2004; Masojidek et al., 2004; Sánchez et al., 2008), se estudió el efecto de estos factores sobre el crecimiento y acumulación de luteína en cultivos batch de *C. sorokiniana* bajo condiciones fotoautotróficas.

Los estudios realizados a diferentes irradiancias mostraron que en *C. sorokiniana* el contenido en luteína en el cultivo alcanzaba un valor óptimo a irradiancias moderadas, ya que tanto la biomasa como el contenido celular en luteína decrecieron a mayores y menores irradiancias (Tabla 2, Capítulo I). Una tendencia similar con respecto al contenido celular en luteína y β-caroteno se ha descrito en diferentes especies de *C. zofingiensis* y *Muriellopsis* sp. (Bar et al., 1995; Del Campo et al., 2000, 2004). Por el contrario, en *H. pluvialis* y *C. zofingiensis* el contenido celular en los carotenoides secundarios astaxantina y cantaxantina mostró una respuesta opuesta a la de la luteína, incrementándose a altas irradiancias (Del Campo et al., 2004; Boussiba and Vonshak, 1991).

El análisis del efecto de la temperatura sobre el crecimiento y acumulación de luteína en *C. sorokiniana* mostró que los contenidos volumétrico y celular en luteína máximos se encontraban dentro del rango de 28 a 32 ºC, disminuyendo a temperaturas menores y mayores (Tabla 3, Capítulo I). Estos resultados coinciden con los descritos en *C. zofingiensis* (Del Campo et al., 2000) y *S. almeriensis* (Sánchez et al., 2008; Fernández-Sevilla et al., 2010). Por el contrario, en *H. pluvialis* las temperaturas extremas inducían la carotenogénesis. Se ha sugerido que en esta última microalga, especies reactivas de oxígeno generadas endógenamente son las responsables de la estimulación de la síntesis de astaxantina a altas temperaturas (Tjahjono et al., 1994).

La limitación de nitrógeno incrementa la síntesis de carotenoides secundarios como la astaxantina, pero disminuye la producción de biomasa. Sin embargo, el nitrógeno se requiere a concentraciones no limitantes para la acumulación de carotenoides primarios, debido posiblemente a la necesidad de una síntesis continua de proteínas y carotenoides, componentes estructurales y funcionales del aparato fotosintético bajo condiciones óptimas de crecimiento (Jin et al., 2003; Del Campo et al., 2000, 2004). Esto está de acuerdo con los resultados obtenidos en *C. sorokiniana*, ya que tanto el contenido celular como el contenido

volumétrico en luteína incrementaron cuando la concentración de nitrato en el medio de cultivo aumentó de 10 a 40 mM, decreciendo ligeramente a mayores concentraciones de nitrato (Tabla 4, Capítulo I). Por otro lado, aunque en cultivos heterotróficos de *C. protothecoides* los valores máximos de productividad de luteína se alcanzaban usando urea como fuente de nitrógeno (Shi et al., 2000), en cultivos mixotróficos de *C. zofingiensis* prácticamente no se registraron cambios ni en el crecimiento ni en el contenido en luteína cuando el nitrato sódico fue sustituido por urea (Tabla 6, Capítulo I) o nitrato amónico (datos no mostrados).

Aunque el estrés de NaCl induce la biosíntesis de carotenoides secundarios (Harker et al., 1996), no incrementa la biosíntesis de carotenoides primarios. Así, en *C. sorokiniana* el crecimiento y contenido en luteína disminuyeron drásticamente cuando las concentraciones de NaCl se incrementaron por encima de 2 mM (datos no mostrados). En el caso de otras clorofitas como *Muriellopsis* sp., *C. zofingiensis* y *S. almeriensis*, los niveles de luteína por célula permanecieron prácticamente constantes a las diferentes concentraciones de NaCl ensayadas (2-400 mM) (Del Campo et al., 2000, 2004; Sánchez et al., 2008).

Por tanto, aunque factores de estrés, tales como alta irradiancia, temperaturas extremas, alta concentración de NaCl o limitación de nutrientes aumentan la acumulación celular de los carotenoides secundarios como la astaxantina (Boussiba, 2000; Del Campo et al., 2004; Vidhyavathi et al., 2008), estos factores no incrementan los niveles celulares de luteína en *C. sorokiniana*, ya que la luteína es un carotenoide primario necesario en fotosíntesis para la estructura y función de los complejos de captura de luz. En consecuencia, las condiciones que incrementan el crecimiento de esta microalga bajo condiciones fotoautotróficas coinciden con aquellas que aumentan la acumulación de luteína.

En cultivos fotoautotróficos de microalgas para la producción de biomasa y de compuestos de interés a partir de la misma, el principal factor que limita la densidad celular y la productividad de los cultivos es, generalmente, la disponibilidad de la luz. Una alternativa para superar este problema es el uso de cultivos mixotróficos. Aunque se ha descrito que el acetato aumenta el crecimiento y la síntesis de astaxantina en *H. pluvialis* (Borowitzka et al., 1991), el efecto de esta fuente de carbono sobre la biosíntesis de luteína no se ha estudiado en microalgas. En *C. sorokiniana*, la adición de acetato a los cultivos incrementó tanto el crecimiento como los contenidos volumétrico y celular en luteína (Tabla 5, Capítulo I). La adición de glucosa, aunque disminuyó el contenido celular en luteína, incrementó considerablemente la biomasa y, por tanto, el contenido volumétrico en luteína. El contenido volumétrico en luteína más alto (35 mg L^{-1}) se alcanzó en cultivos mixotróficos suplementados con glucosa (Tabla 6, Capítulo I).

Por tanto, el suplemento de una fuente de carbono extra a los cultivos fotoautotróficos incrementó el crecimiento y la productividad, superándose de esta forma la limitación impuesta por la disponibilidad de la luz. Recientemente, se ha descrito que en cultivos heterotróficos de *C. zofingiensis*, la glucosa incrementó los niveles de transcritos de los genes *bkt* y *chyB*, así como la acumulación celular de astaxantina y zeaxantina, pero disminuyó el contenido celular en luteína y clorofila (Li et al., 2008a; Huang et al., 2006). En *C. protothecoides*, se ha

observado que la densidad celular y la productividad de luteína eran mucho mayores en cultivos heterotróficos con glucosa que en cultivos fotoautotróficos (Shi et al., 1999). Por tanto, nuestros resultados están de acuerdo con los obtenidos en *C. zofingiensis* y *C. protothecoides*, que indican que aunque la glucosa disminuye el contenido celular en luteína, incrementa considerablemente la biomasa, resultando en una mayor producción de luteína en cultivos mixotróficos que en cultivos fotoautotróficos.

Existe escasa información sobre el aislamiento de mutantes superproductores de carotenoides de microalgas mediante mutagénesis clásica o al azar. Ishikawa et al. (2004) aislaron un mutante de *Chlorella regularis* que presentaba un alto contenido celular de luteína, aunque su crecimiento era menor que el de la estirpe silvestre. Estos autores, sin embargo, no suministraron información sobre el contenido volumétrico en luteína de dicho mutante (Ishikawa et al., 2004). También se han descrito algunos mutantes de *Chlorococcum* sp. y *H. pluvialis* que presentan un incremento en el contenido celular de astaxantina, pero sus tasas de crecimiento son generalmente menores que la de la estirpe silvestre (Chen et al., 2003; Zhang et al., 1997; Kamath et al., 2008).

En nuestros experimentos con *C. sorokiniana*, los mutantes se seleccionaron no sólo en base a un alto contenido en luteína sino también en base a una alta tasa de crecimiento. Así, el mutante MR-16 presentó un crecimiento y contenido celular en luteína mayores que los de la estirpe silvestre y, en consecuencia, alcanzó una producción de luteína doble de la obtenida con la estirpe silvestre (Tabla 7 y Figura 3, Capítulo I). Además, este mutante presenta el mayor contenido volumétrico en luteína (42,0 mg L^{-1} al final de la fase de desaceleración) descrito en la literatura en condiciones fotoautotróficas de crecimiento a escala de laboratorio (Tabla 8, Capítulo I). MR-16 también mostraba contenidos volumétricos en β-caroteno, anteraxantina y zeaxantina más de dos veces superiores que los de la estirpe silvestre (datos nos mostrados). El mutante MR-16 presentaba resistencia a nicotina, un inhibidor específico de la enzima carotenogénica LCYb implicada en la biosíntesis de luteína. La resistencia a nicotina que se observa en MR-16 podría deberse a una mutación en LCYb que afecte directamente a su interacción con el inhibidor, a una mutación en el promotor del gen *lcyB* produciéndose un incremento en su expresión y/o algún otro mecanismo indirecto que conduce a una mayor actividad enzimática, resultando en un aumento en la producción de luteína en condiciones fotoautotróficas. Además, la mayor tasa de crecimiento de este mutante con respecto a la estirpe silvestre en condiciones fotoautotróficas podría ser debido al mayor contenido en luteína, ya que este carotenoide tiene importantes papeles en la función, estructura y fotoprotección del aparato fotosintético, conduciendo a una mayor eficiencia fotosintética. Es interesante destacar que el incremento en biomasa que muestra la estirpe silvestre cuando se cultiva mixotróficamente con acetato y/o glucosa no se observaba en el mutante MR-16 (Tabla 8, Capítulo I). El mecanismo de este efecto no se conoce. Es posible que la mutación hubiera provocado una alteración metabólica causando una débil represión del crecimiento por glucosa o acetato.

Aunque las microalgas no se utilizan todavía como fuente de luteína a escala industrial, en los últimos años se han realizado los primeros pasos necesarios para ello tanto a nivel de laboratorio como al exterior a escala piloto (Del Campo et al., 2000, 2007; Shi et al., 2002; Blanco et al., 2007; Fernández-Sevilla et al., 2010). Los resultados obtenidos en este trabajo indican que *C. sorokiniana* es una candidata atractiva para la producción de luteína, ya que presenta una alta velocidad de crecimiento (0,12 h^{-1}), contenidos volumétrico y celular en luteína de 35,0 mg L^{-1} y 5,2 mg g^{-1} peso seco, que se incrementan por mutagénesis al azar hasta 42,0 mg L^{-1} y 7,0 mg g^{-1} peso seco, siendo estos valores los mayores descritos en la bibliografía bajo condiciones fotoautotróficas a escala de laboratorio (Del Campo et al., 2000, 2004, 2007; Sánchez et al., 2008; Casal et al., 2011).

2. Aumento de la biosíntesis de carotenoides en *Chlamydomonas reinhardtii* mediante transformación nuclear, utilizando el gen de la fitoeno sintasa aislado de *Chlorella zofingiensis*

La enzima PSY cataliza el primer paso específico de la ruta biosintética de carotenoides, la condensación de dos moléculas de GGPP para producir fitoeno, y actualmente se considera que juega un papel importante en la regulación del flujo de carbono hacia la síntesis de carotenoides.

En este trabajo se ha aislado el gen de la fitoeno sintasa de *C. zofingiensis* (*Czpsy*) y se ha comprobado su funcionalidad por complementación en *E. coli* transformada con el plásmido pAC85 que contiene los genes carotenogénicos implicados en la síntesis del β-caroteno excepto el gen *psy*. El gen *Czpsy*, de 3561 pb, consiste de 4 exones y 3 intrones (Figura 2, Capítulo II) y codifica una proteína de 420 aminoácidos. Al igual que los genes *psy* conocidos de otras microalgas eucarióticas, el gen *Czpsy* tiene un número de exones menor que los 5 ó 7 exones presentes comúnmente en los genes *psy* de plantas.

El análisis por Southern blot ha revelado que *C. zofingiensis*, al igual que otras microalgas como *C. reinhardtii* y *D. salina* (McCarthy et al., 2004; Lohr et al., 2005; Yan et al., 2005) contiene únicamente un solo gen que codifica la fitoeno sintasa (Figura 3, Capítulo II). Sin embargo, recientemente se ha descrito que otras algas como *Dunaliella bardawil* y *Micromonas pusilla* poseen múltiples copias del gen *psy*, y se ha sugerido que, de forma análoga a la diversidad de genes *psy* y su expresión diferencial en plantas, algunas algas también podrían regular diferencialmente la expresión de sus múltiples copias del gen *psy.* Análisis comparativos de varios genomas de algas para PSY en combinación con análisis filogenéticos han sugerido que una duplicación de un gen ancestral originó dos clases de PSY (Tran et al., 2009). Nuestros resultados indican que *C. zofingiensis* parece tener solamente una clase de PSY.

Alineamientos a nivel de proteína han indicado que la enzima CzPSY tiene las características esenciales de ambas clases de PSY, incluyendo los posibles sitios de unión a los complejos sustrato-Mg^{2+} (regiones ricas en aspartato) y residuos catalíticos (datos no mostrados). Además, los alineamientos mostraron que las diferencias en secuencia entre las PSY eucariotas y bacterianas se encuentran principalmente en el extremo amino terminal, debido a la presencia de un péptido señal responsable de la localización de estas enzimas en los cloroplastos (Cunningham and Gantt, 1998).

La sobreexpresión de genes fitoeno sintasa bacterianos o vegetales en plantas superiores ha permitido incrementar de 1,8 a 6,3 veces el contenido total en carotenoides en raíces de zanahoria (Hauptman et al., 1997), frutos de tomate (Fraser et al., 2002), y tubérculos de patata (Ducreux et al., 2005). Incrementos mayores en el contenido en carotenoides se han descrito en plantas o tejidos que no contienen carotenoides o tienen niveles muy bajos. Así, se ha descrito un incremento de 50 veces en semillas de canola (Farré et al., 2010). A pesar de la importancia de las microalgas como fuente de carotenoides naturales, hay muy pocos datos sobre la manipulación genética de la ruta biosintética de carotenoides en estos microorganismos. La mayoría de los estudios se han centrado en plantas, con el objetivo de obtener especies transgénicas con mayores características nutricionales.

En este trabajo, la transformación nuclear de *C. reinhardtii* con el gen *psy* aislado de *C. zofingiensis* resultó en un significativo incremento en los contenidos en violaxantina y luteína que alcanzaron niveles 2,0 y 2,2 veces mayores, respectivamente, que los de las células no transformadas (Figura 7a, Capítulo II). Además, los transformantes mostraron una sobreexpresión del gen *psy* de *C. zofingiensis* debido a los promotores fuertes usados (Figura 7b, Capítulo II). Estos resultados sugieren que el mayor flujo de carbono desde el GGPP a la síntesis de carotenoides estaba promovido por una combinación de las expresiones de los productos del gen *Czpsy* en *C. reinhardtii* y de la copia endógena del gen. En los transformantes T1 y T10 los niveles de transcritos del gen *Czpsy* fueron 140 veces superiores a los del gen *psy* endógeno de la estirpe silvestre, sin embargo, los contenidos en violaxantina y luteína en estos transformantes fueron solamente 2,0 y 2,2 veces mayores que los de la estirpe silvestre, respectivamente, en el transformante T10, y 7 % y 21 % mayores, respectivamente, en T1. Aunque se ha descrito que el control transcripcional es el factor regulador más importante de los genes carotenogénicos, una posible explicación para estos resultados es que la regulación post-transcripcional y traduccional jueguen también papeles importantes, así como la estabilidad del mRNA (Molnar et al., 2009). Además, un incremento en los niveles de PSY no tiene por qué estar directamente correlacionado con un aumento en el contenido en carotenoides, ya que el destino, procesamiento o ensamblaje de la proteína para formar un complejo completamente funcional puede no ser el correcto. En plantas transgénicas de tomate y patata que expresaban una fitoeno sintasa bacteriana (CRTB), a pesar de que la actividad enzimática de la CRTB era sustancialmente alta, sólo se detectaba un ligero incremento en el contenido total de carotenoides y no se apreciaba una correlación lineal entre los niveles de

transcritos, proteína, actividad enzimática y carotenoides totales (Fraser et al., 2002; Ducreux et al., 2005).

En plantas de tomate se ha descrito que la sobreexpresión de un gen *psy* exógeno producía una acumulación de fitoeno, y se ha sugerido que el incremento en la actividad de la PSY probablemente provoca un cambio en el paso regulador de la ruta, siendo una enzima posterior el nuevo punto regulador (Shewmaker et al., 1999; Fraser et al., 2002). La acumulación de β-caroteno también se ha observado en plantas que sobreexpresan genes *psy* exógenos, indicando que la reacción catalizada por laβ -caroteno hidroxilasa podría convertirse en un paso limitante de la ruta cuando incrementa el flujo carotenogénico (Ducreux et al., 2005). En los transformantes de *Chlamydomonas* obtenidos en este trabajo no se detectaron incrementos en los contenidos en fitoeno u otros carotenoides intermediarios, indicando que en esta microalga otras enzimas de la ruta no eran limitantes, y que el fitoeno, β-caroteno u otros intermediarios eran accesibles a enzimas posteriores de la ruta.

En plantas y microalgas, la biosíntesis de carotenoides forma parte del metabolismo de los terpenoides de los cloroplastos. El GGPP es un intermediario común de las diferentes rutas biosintéticas de los terpenoides, tales como carotenoides, clorofilas, giberelinas y quinonas. La alteración de cualquiera de estas rutas puede tener un efecto directo sobre las demás. En el caso de una sobreproducción de carotenoides, el incremento del flujo de carbono hacia la ruta carotenogénica puede producir limitaciones, especialmente en la síntesis de giberelinas, clorofilas y quinonas, con efectos negativos sobre el crecimiento y fotosíntesis, respectivamente. En algunas plantas se ha observado que la sobreexpresión de genes *psy* provocaba efectos colaterales no deseados (Farré et al., 2010). Así, la expresión constitutiva de genes de la fitoeno sintasa en tomate (*Lycopersicum esculentum*) y tabaco (*Nicotiana tabacum*) provocó enanismo y clorosis en las plantas que iban acompañados por una disminución de giberelinas (Fray et al., 1995; Busch et al., 2002). En los transformantes de *Chlamydomonas* obtenidos en este trabajo no se observó fenotipo atípico y sus tasas de crecimiento eran similares a la de la estirpe silvestre (datos no mostrados).

3. Aislamiento y caracterización de los genes licopeno β-ciclasa y licopeno ε-ciclasa de *Chlorella zofingiensis*

Las enzimas licopeno β-ciclasa y licopeno ε-ciclasa catalizan la formación de anillosβ y ε en los extremos de la molécula lineal de licopeno para producir β- y α-caroteno y, en muy raras ocasiones ε-caroteno. En plantas, se ha sugerido que estas enzimas desempeñan un papel regulador en la ruta biosintética de carotenoides, dirigiendo el flujo de carbono a través de la ruta desde el licopeno al β-caroteno y sus derivados o alternativamente al α-caroteno y sus derivados. Así, en *Arabidopsis, Brassica* y patata la reducción en la expresión del gen *lcyE* mediante mutación, usando RNAi o introduciendo un fragmento antisentido de este gen,

incrementó la proporción de β- a α-caroteno y sus productos (Pogson et al., 1996; Yu et al., 2008; Diretto et al. 2006). Un estudio sobre la variación natural de carotenoides en maíz descubrió alelos del gen *lcyE* que se expresan a niveles bajos y también van acompañados por un incremento en β-caroteno y sus derivados (Harjes et al., 2008). Por otro lado, la sobreexpresión del gen *lcyB* endógeno o de genes heterólogos equivalentes en plantas de cultivo, tales como *Brassica* y frutos de tomate, incrementaron los niveles de β-caroteno (Farré et al., 2010), y la sobreexpresión del gen *lcyE* en *Arabidopsis* incrementó el contenido en luteína hasta un 180 % con respecto a la estirpe silvestre (Pogson et al., 2000).

En este trabajo se han aislado y caracterizado los genes de la licopeno β-ciclasa (*CzlcyB*) y de la licopeno ε-ciclasa (*CzlcyE*) de *C. zofingiensis*. El gen *CzlcyB* consta de 7 exones y 6 intrones. La fase de lectura abierta (ORF) de este gen codificó una proteína de 546 aminoácidos (Figura 1, Capítulo III). Al comparar las posiciones de los intrones de los genes *lcyB* de *C. zofingiensis* y *D. salina*, se determinó que 5 intrones del gen *CzlcyB* igualaban posiciones con las del gen de *D. salina* (Ramos et al., 2008). Alineamientos llevados a cabo con el programa Blocks indicaron que la longitud de la hipotética proteína coincide con las de otras proteínas LCYb descritas para microalgas y plantas, siendo las licopeno β-ciclasas bacterianas unos 150 aminoácidos más cortas (datos no mostrados). Esto podría indicar la existencia de un péptido señal en el extremo amino terminal de las proteínas eucarióticas. De hecho, diferentes programas han confirmado la presencia de un péptido señal en la CzLCYb. Al igual que otras proteínas LCYb (Cunningham et al., 1994), la CzLCYb no es tan hidrofóbica como se podría esperar para una enzima localizada en membranas. Contiene 21 % y 29 % de aminoácidos cargados e hidrofóbicos, respectivamente, y usando diferentes programas solamente se han encontrado dos dominios transmembrana de 21 aminoácidos cada uno situados en el extremo carboxilo terminal.

Mediante experimentos de complementación genética en *E. coli* se ha determinado que la CzLCYb es capaz de catalizar la ciclación no sólo del licopeno, sino también del δ-caroteno (Figura 5, Capítulo III). Estos resultados confirman que las propiedades catalíticas de la CzLCYb son similares a las de las LCYb de plantas (Cunningham et al., 1996) y microalgas (Cunningham and Gantt, 2007; Ramos et al., 2008). La CzLCYb, por tanto, es responsable de la síntesis de β-caroteno a partir de licopeno y también está implicada en la síntesis de α-caroteno.

Por otro lado, el gen *CzlcyE* aislado en este trabajo consta de 8 exones y 7 intrones y codifica una proteína de 654 aminoácidos (Figura 2, Capítulo IV). La longitud de esta proteína es ligeramente mayor que la descrita para las LCYe de otras algas verdes y plantas. Aunque la LCYe es una enzima localizada en las membranas de los cloroplastos, la CzLCYe no es particularmente hidrofóbica. Tiene un 19 % y un 46 % de aminoácidos cargados e hidrofóbicos, respectivamente. Sin embargo, usando los programas ProtScale y TopPred se han encontrado cinco dominios transmembrana de 20 aminoácidos cada uno distribuidos uniformemente en la proteína (datos no mostrados). El porcentaje de aminoácidos hidrofóbicos de CzLCYe fue

similar al de las LCYe de plantas y otras algas verdes, así como al de otras enzimas de la ruta carotenogénica.

El característico dominio de unión a nucleótidos y dos motivos ciclasas presentes en las LCYe y LCYb de algas verdes y plantas, las CRTLe y CRTLb de cianobacterias y las CRTY de bacterias, así como un residuo de leucina en una región cerca del grupo carboxilo terminal indicativo de una actividad ε-monociclasa, se encontraron en la CzLCYe (datos no mostrados). La CzLCYe mostró un alto grado de homología con la LCYe de plantas, especialmente con las LCYe de otras microalgas verdes, siendo el grado de homología menor con las LCYb de plantas y microalgas, así como con las CRTLe y CRTLb de cianobacterias (Figura 4, Capítulo IV).

Por complementación en *E. coli* se ha demostrado que la CzLCYe era capaz de catalizar la conversión de licopeno en δ-caroteno, pero no la formación de α-caroteno a partir de δ-caroteno (Figura 5, Capítulo IV). Estos resultados indican, por tanto, que la CzLCYe presenta actividad monociclasa como la mayoría de las LCYe de plantas previamente estudiadas. En algas, hasta la fecha, el gen *lcyE* solamente se ha caracterizado funcionalmente en la microalga verde *Auxenochlorella protothecoides* CS-41 (Li T et al., 2009), y en cianobacterias, en *Prochlorococcus marinus* MED4 (Stickforth et al., 2003). El producto del gen *lcyE* de *A. protothecoides*, al igual que el del gen *lcyE* aislado en este trabajo presentó actividad monociclasa. Sin embargo, la CRTLe de *P. marinus* MED4 fue capaz de introducir en el licopeno no solamente anillos ε, sino también anillosβ para formar α-, β- y ε-caroteno (Stickforth et al., 2003).

4. Regulación de la ruta carotenogénica por luz y nitrógeno en *Chlorella zofingiensis*

En *C. zofingiensis* cultivada fotoautotróficamente la combinación del estrés de alta irradiancia y del de ausencia de nitrógeno en el medio de cultivo provoca una disminución en el contenido celular en los carotenoides primarios α-caroteno y su derivado luteína, y una acumulación de los carotenoides secundarios derivados del β-caroteno astaxantina y cantaxantina (Rise et al., 1994; Bar et al., 1995; Orosa et al., 2001; Del Campo et al., 2004). Sin embargo, la base molecular de esta regulación no se ha estudiado aún. Recientemente, se ha descrito que los transcritos de los genes *pds*, *chyB* y *bkt* de *C. zofingiensis* aumentan en condiciones de alta irradiancia, incrementándose también la síntesis de cantaxantina, zeaxantina y astaxantina (Huang et al., 2008; Li et al., 2009). Sin embargo, el efecto de la ausencia de nitrógeno *per se* sobre la expresión de los genes carotenogénicos y sobre la acumulación de carotenoides no se ha investigado en esta microalga.

En este trabajo se ha estudiado la regulación por luz y nitrógeno de la ruta carotenogénica de *C. zofingiensis* determinando los niveles de los mRNA de los genes *pds*,

chyB, *bkt*, *psy*, *lcyB* y *lcyE*, así como los contenidos celulares en α-caroteno y su producto luteína, y en β-caroteno y sus derivados cantaxantina, astaxantina, zeaxantina y violaxantina. Los niveles de los mRNA de los genes *lcyB* y *lcyE* no aumentaron cuando la irradiancia incrementó de 20 a 300 µmol fotones m^2 s^{-1}, mientras que los niveles de los transcritos de los genes *psy*, *pds*, *chyB* y *bkt* aumentaron significativamente (Figura 6, Capítulo IV). Sin embargo, el estrés de alta irradiancia desencadenó la síntesis de los carotenoides secundarios astaxantina, cantaxantina y zeaxantina, y disminuyó los niveles de los carotenoides primarios α-caroteno, luteína, β-caroteno y violaxantina (Figura 7, Capítulo IV). Por tanto, en *C. zofingiensis*, el estrés de alta irradiancia desencadenó la síntesis de astaxantina, cantaxantina y zeaxantina debido posiblemente a la activación transcripcional de los genes *psy*, *pds*, *chyB* y *bkt*, pero no del gen *lcyB*, y, en consecuencia, este último gen parece no estar regulado a nivel transcripcional bajo estas condiciones. En *H. pluvialis*, a diferencia de *C. zofingiensis*, se ha observado un aumento en los transcritos del gen *lcyB* en condiciones de alta irradiancia, siendo también la síntesis de astaxantina inducida bajo estas condiciones (Vidhyavathi et al., 2008; Steinbrenner and Linden, 2003). En el caso de *Dunaliella salina*, el estrés provocado por la alta irradiancia incrementó ligeramente los mRNA de los genes *lcyB*, *psy* y *pds*, así como el contenido celular en β-caroteno, aunque los mayores niveles de los transcritos de estos genes y de β-caroteno se obtuvieron en condiciones de alta irradiancia y deficiencia de nutrientes. La limitación de nutrientes parece ser esencial para la acumulación de β-caroteno en esta microalga (Ramos et al., 2008, 2011; Coesel et al., 2008).

En *C. zofingiensis*, la deficiencia de nitrógeno *per se* aumentó los mRNA de todos los genes considerados, excepto los de los genes *lcyE* y *pds* (Figura 6, Capítulo IV), sin embargo no desencadenó la síntesis de cantaxantina, zeaxantina ni astaxantina (Figura 7, Capítulo IV). No obstante, la combinación de ambos factores de estrés, alta irradiancia y deficiencia de nitrógeno, incrementó marcadamente los niveles de estos carotenoides en comparación con los niveles registrados en cultivos sometidos únicamente a estrés por alta irradiancia (Figura 7, Capítulo IV). Por tanto, el aumento de los transcritos del gen *lcyB*, pero no del gen *lcyE*, por deficiencia de nitrógeno, independientemente de la irradiancia, dirigió el flujo de carbono desde el licopeno al β-caroteno, cantaxantina, astaxantina y zeaxantina en lugar de al α-caroteno y luteína. Sin embargo, la deficiencia de nitrógeno a baja irradiancia no desencadenó la síntesis de cantaxantina, zeaxantina y astaxantina, aunque incrementó los transcritos de todos los genes implicados en la síntesis de esos carotenoides (excepto los del gen *pds*), indicando que la síntesis de todos estos carotenoides en estas condiciones parece estar controlada a nivel post-transcripcional o que el gen *pds* es el principal gen regulador. El efecto combinado de los dos tipos de estrés, alta irradiancia y deficiencia de nitrógeno aumentó marcadamente los niveles de cantaxantina y astaxantina en comparación con los niveles registrados en células expuestas solamente a estrés de alta irradiancia, debido posiblemente a la mayor expresión del gen *bkt*; por el contrario la síntesis de luteína, α-caroteno, β-caroteno y violaxantina disminuyó en estas condiciones. La disminución del contenido en violaxantina a alta irradiancia, especialmente bajo deficiencia de nitrógeno, podría ser debida al incremento en la acumulación

de zeaxantina a través del ciclo de las xantofilas (Goss y Jakob, 2010). Por tanto, aunque se ha descrito que la regulación de la síntesis de mRNA es el principal nivel de regulación de los genes carotenogénicos, las regulaciones a niveles post-transcripcional y traduccional también parecen tener papeles importantes, así como la estabilidad del mRNA, como se ha observado en *H. pluvialis* (Steinbrenner and Linden, 2003; Vidhyavathi et al., 2008; Ramos et al., 2011).

Conclusiones

1. Se ha seleccionado *Chlorella sorokiniana* para la producción de luteína, a partir del estudio de diferentes estirpes de microalgas clorofíceas, en base a su rápido crecimiento y alto contenido en dicho carotenoide.

2. Se han establecido las condiciones óptimas tanto para el crecimiento como para la producción de luteína por *Chlorella sorokiniana*. En general, las condiciones más favorables para el crecimiento, también lo son para la producción de luteína. Las mayores productividades de luteína se alcanzaron a 690 µmol de fotones m^{-2} s^{-1}, 28 ºC, 2 mM de NaCl, 40 mM de nitrato y condiciones mixotróficas de cultivo.

3. Se han obtenido mutantes de *Chlorella sorokiniana* super-productores de luteína mediante mutagénesis al azar y selección de los mutantes por su resistencia a inhibidores de la ruta carotenogénica. Así, el mutante MR-16 muestra una productividad en luteína 2 veces mayor que la estirpe silvestre. Además, los mutantes DMR-5 y DMR-8 destacaron por su alto contenido celular en luteína de 7 mg g^{-1} peso seco. Estos valores son superiores a los descritos hasta ahora para otras microalgas productoras de luteína en cultivos fotoautotróficos a escala de laboratorio. Por tanto, *Chlorella sorokiniana* es una candidata excelente para la producción de luteína a escala industrial.

4. A partir de *Chlorella zofingiensis*, de gran potencial para la producción de astaxantina, carotenoide poco común y de gran interés comercial, se han aislado y caracterizado los genes de la fitoeno sintasa, licopeno β-ciclasa y licopeno ε-ciclasa implicados en la síntesis de astaxantina y/o luteína. La funcionalidad de estos genes se ha determinado mediante complementación genética en *E. coli*, y el análisis filogenético ha mostrado que la fitoeno sintasa y licopeno β- y ε-ciclasas de *C. zofingiensis* están muy relacionados evolutivamente con los de plantas y microalgas y escasamente con los de bacterias.

5. La sobreexpresión en *Chlamydomonas* del gen que codifica la primera enzima específica de la ruta carotenogénica, la fitoeno sintasa de *Chlorella zofingiensis*, ha permitido aislar transformantes de *Chlamydomonas* con altos niveles de transcritos de la fitoeno sintasa y con niveles de violaxantina y luteína 2,0 y 2,2 veces superiores, respectivamente, a los de las células no transformadas. Estos resultados suponen un claro ejemplo del gran potencial de la Ingeniería Genética en microalgas eucarióticas para aumentar su producción de carotenoides de interés comercial.

6. En *Chlorella zofingiensis*, el estrés por alta irradiancia incrementa los niveles de los transcritos de los genes *psy* y *pds*, implicados en las primeras fases de la ruta carotenogénica, y de los genes *chyB* y *bkt*, implicados en los últimos pasos de la síntesis de astaxantina, pero no afecta los niveles de transcritos de los genes *lcyB* y *lcyE*, responsables de la ciclación del licopeno. No obstante, dicho estrés por alta irradiancia desencadena la

síntesis de los carotenoides secundarios astaxantina, cantaxantina y zeaxantina y disminuye los niveles de los carotenoides primarios α-caroteno, luteína, β-caroteno y violaxantina.

7. La deficiencia de nitrógeno *per se*, independientemente de la irradiancia a la que se sometan los cultivos, aumenta los niveles de los transcritos de los genes *psy*, *chyB*, *bkt* y *lcyB* en *C. zofingiensis*, sin embargo no afecta a los niveles de transcritos de los genes *lcyE* y *pds*, ni induce la síntesis de astaxantina, zeaxantina ni cantaxantina.

8. En *Chlorella zofingiensis*, el estrés por alta irradiancia combinado con la deficiencia de nitrógeno aumenta significativamente la acumulación de astaxantina, zeaxantina y cantaxantina, así como los niveles de transcritos del gen *bkt*, en comparación con los niveles registrados en cultivos sometidos a estrés por alta irradiancia únicamente.

Bibliografía

Alves-Rodrígues A, Shao A (2004) The science behing lutein. *Toxicol Lett*, 150: 57-83.

Armstrong GA (1997) Genetics of eubacterial carotenoid biosynthesis: a colorful tale. *Annu Rev Microbiol*, 51: 629-659.

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

Baranski R (2008) Genetic transformation of carrot (*Daucus carota*) and other apiaceae species. *Trans Plant J*, 2(1):18–38.

Baroli I, Niyogi KK (2000) Molecular genetics of xanthophyll-dependent photoprotection in green algae plants. *Philos Trans R Soc Lond B Biol Sci*, 355(1402): 1385-94.

Bartley GE, Scolnik PA, Beyer P (1999) Two *Arabidopsis thaliana* carotene desaturases, phytoene desaturase and ζ-carotene desaturase, expressed in *Escherichia coli*, catalyze a poly-*cis* pathway to yield pro-lycopene. *Eur J Biochem*, 259: 396-403.

Becker W (2004) Microalgae in human and animal nutrition. *In*: Richmond A (ed) *Handbook of microalgal culture*. Blackwell, Oxford, pp 312-351.

Bernstein PS, Zhao DY, Wintch SW, Ermakov IV, McClane RW, Gellermann W (2002) Resonance Raman measurement of macular carotenoids in normal subjects and in age-related macular degeneration patients. *Ophthalmology*, 109: 1780-1787.

Bhosale P (2004) Environmental and cultural stimulants in the production of carotenoids from microorganisms. *Appl Microbiol Biotechnol*, 63(4): 351-361.

Blanco AM, Moreno J, Del Campo JA, Rivas J, Guerrero MG (2007) Outdoor cultivation of lutein-rich cells of *Muriellopsis* sp. in open ponds. *Appl Microbiol Biotechnol*, 73:1259-1266.

Borowitzka MA, Huisman JM, Osborn A (1991) Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. *J Appl Phycol*, 3: 295-304.

Botella-Pavía P, Rodríguez-Concepción M (2006) Carotenid biotechnology in plants for nutritionally improved foods. *Physiol Plant*, 126: 396-381.

Boussiba S, Vonshak A (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol*, 32: 1077-1082.

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

Breitenbach J, Misawa N, Kajiwara S, Sandmann G (1996) Expression in *Escherichia coli* and properties of the carotene ketolase from *Haematococcus pluvialis*. *FEMS Microbiol Lett*, 140(2-3): 241-6.

Britton (1988) Biosynthesis of carotenoids. *In*: TW Goodwin (ed) *Plant Pigments*. Academic Press, London, pp 133–180.

Britton G, Liaaen-Jensen S, Pfander H (1998) Synthesis from a different perspective: How nature does It? *In*: Britton G, Liaaen-Jensen S, Pfander H (eds) *Carotenoids*, Vol. 3: *Biosynthesis and Metabolism*. Birkhäuser Verlag Basel, Switzerland, pp 1-12.

Busch M, Seuter A, Hain R (2002) Functional analysis of the early steps of carotenoid biosynthesis in tobacco. *Plant Physiol*, 128: 439–453.

Casal C, Cuaresma M, Vega JM, Vilchez C (2011) Enhanced productivity of a lutein-enriched novel acidophile microalga grown on urea. *Mar Drugs*, 9: 29-42.

Chamovitz D, Misawa N, Sandmann G, Hirschberg J (1992) Molecular cloning and expression in *Escherichia coli* of a cyanobacterial gene coding for phytoene synthase, a carotenoid biosynthesis enzyme. *FEBS Lett*, 296(3): 305-10.

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

Chen Y, Li F, Wurtzel ET (2010) Isolation and characterization of the *Z-ISO* gene encoding a missing component of carotenoid biosynthesis in plants. *Plant Physiol,* 153: 66–79.

Chucair AJ, Rotstein NP, Sangiovanni JP, During A, Chew EY, Politi LE (2007) Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: relation with docosahexaenoic acid. *Invest Ophthalmol Vis Sci*, 48(11): 5168-5177.

Chumpolkulwong N, Kakizono T, Handa T, Nishio N (1997) Isolation and characterization of compactin resistant mutants of an astaxanthin synthesizing green alga *Haematococcus pluvialis*. *Biotechnol Letters*, 19: 299-302.

Coesel SN, Baumgartner AC, Teles LM, Ramos A, Henriques NM, Cancela L, Valera J (2008) Nutrient limitation is the main regulatory factor for carotenoid accumulation and for *Psy* and *Pds* steady state transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. *Mar Biotech*, 10: 602-611.

Cunningham FX Jr, Sun Z, Chamovitz D, Hirschberg J, Gantt E (1994) Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus sp.* strain PCC 7942. *Plant Cell*, 6: 1107-1121.

Cunningham FX Jr, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E (1996) Functional analysis of the β- and ε-lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell*, 8: 1613-1626.

Cunningham FX Jr, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol,* 49: 557-583.

Cunningham FX Jr, Gantt E (2001) One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases. *Proc Natl Acad Sci USA*, 98: 2905–2910.

Cunningham FX Jr, Gantt E (2007) A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. *Photosynth Re*s, 92: 245–259.

Cysewski GR, Lorenz RT (2004) Industrial production of microalgal cell-mass and secondary products -species of high potential. *Haematococcus*. *In*: Richmond, A. (ed.). *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*. Oxford, Blackwell Publ, pp. 281-288.

Debuchy R, Purton S, Rochaix JD (1989) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. *The EMBO Journal*, 8: 2803-2809.

Del Campo JA, Moreno J, Rodríguez H, Vargas MA, Rivas J, Guerrero MG (2000) Carotenoid content of chlorophycean microalgae. Factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). *J Biotechnol*, 76: 51-59.

Del Campo JA, Rodríguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2001) Lutein production by *Muriellopsis* sp. in an outdoor tubular photobioreactor. *J Biotechnol*, 85: 289-295.

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

Del Campo JA, García-González M, Guerrero MG (2007) Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Appl Microbiol Biotechnol,* 74: 1163-1174.

Demming-Adams B, Gilmore AM, Adams WW (1996) In vivo functions of carotenoids in higher plants. *FASEB J*, 10: 2149-2153.

Demming-Adams B, Adams WW (2002) Antioxidants in photosynthesis and human nutrition. *Science*, 298: 2149-2153.

Diretto G, Tavazza R, Welsch R, Pizzichini D, Mourgues F, Papacchioli V, Beyer P, Giuliano G (2006) Metabolic engineering of potato tuber carotenoids through tuber specific silencing of lycopene ε-cyclase. *BMC Plant Biol*, 6:13.

Doan TTD, Obbard JP (2012) Enhanced intracellular lipid in *Nannochloropsis* sp. via random mutagenesis and flow cytometric cell sorting. *Algal Research*, 1: 17-21.

Ducreux JM, Morris WL, Hedley PE, Shepherd T, Davies HV, MIllan S, Taylor MA (2005) Metabolic engineering of high carotenoids potato tubers containing enhanced levels of β-carotene and lutein. *J Exp Bot*, 56: 81-89.

Dwyer JH, Navab M, Dwyer KM, Hassan K, Sun P, Shircore A, Hama-Levy S, Hough G, Wang X, Drake T, Merz NB, Fogelman AM (2001) Oxygenated carotenoid lutein and the progression of early atherosclerosis. *Circulation* 103: 2922-2977.

Eberhard S, Jain M, Im CS, Pollock S, Shrager J, Lin Y, Peek AS, Grossman AR (2006) Generation of an oligonucleotide array for analysis of gene expression in *Chlamydomonas reinhardtii*. *Curr Genet*, 49: 106-124.

Eisenreich W, Bacher A, Arigoni D, Rohdich F (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell Mol Life Sci*, 61: 1401-1426.

Eonseon J, Polle JEW, Lee HK, Hyum SM, Chang M (2003) Xanthophylls in Microalgae: From Biosynthesis to Biotechnological mass production and application. *J Microbiol Biotechnol*, 13(2): 165-174.

Farré G, Sanahuja G, Naqvi S, Bai C, Capell T, Zhu C, Christou P (2010) Travel advice on the road to carotenoids in plants. *Plant Science*, 179: 28-48.

Fernández-Sevilla JM, Acién FG, Molina E (2010) Biotechnological production of lutein and its applications. *Appl Microbiol Biotechnol*, 86: 27-40.

Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit- specific manner. *Proc Natl Acad Sci USA*, 99: 1092-97.

Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J*, 8: 693-701.

Geng D, Wang Y, Wang P, Li W, Sun Y (2003) Stable expression of hepatitis B surface antigen gene in *Dunaliella salina*. *J Appl Phycol*, 15: 451-456.

Gerjets T, Sandmann G (2006) Ketocarotenoid formation in transgenic potato. *J Exp Bot*, 57: 3639-3645.

Giuliano G, Aquilani R, Dharmapuri S (2000). Metabolic engineering of plant carotenoids. *Trends Plant Sci*, 5(10): 406-9.

Goss R, Jakob T (2010) Regulation and function of xanthophylls cycle-dependent photoprotection in algae. *Photosynth Res*, 106: 103-122.

Grossman AR, Harris EE, Hauser C, Lefebvre PA, Martinez D, Rokhsar D, Shrager J, Silflow CD, Stern D, Vallon O, Zhang Z (2003) *Chlamydomonas reinhardtii* at the crossroads of genomics. *Eucaryotic cell*, 2(6): 1137-1150.

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends Biotechnol*, 21(5): 210-216.

Harjes CE, Rocheford TR, Bai L, Brutnell TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET (2008) Natural genetic variation in lycopene ε-cyclase tapped for maize biofortification. *Science*, 319: 330-333.

Harker M, Tsavalos AJ, Young AJ (1996) Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis. Bioresour Technol*, 55: 207-214.

Harris E (2001) *Chlamydomonas* as a model organism. *Ann Rev Plant Physiol Plant Mol Biol*, 52: 363-406.

Hauptmann R, Eschenfeldt WH, English J, Brinkhaus FL (1997) Enhanced carotenoid accumulation in storage organs of genetically engineered plants. US Patent 5: 618-988.

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

Hornero-Mendez D, Britton G (2002) Involvement of NADPH in the cyclization reaction of carotenoid biosynthesis. *FEBS Lett*, 515: 133-136.

Huang JC, Wang Y, Sandmann G, Chen F (2006) Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). *Appl Microbiol Biotechnol*, 71: 473-479.

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

Hussein G, Goto H, Oda S, Sankawa U, Matsumoto K, Watanabe H (2007) Antihypertensive potential and mechanism of action of astaxanthin: III. Antioxidant and histopathological effects in spontaneously hypertensive rats. *Biol Pharm Bull*, 29: 684-688.

Ishikawa E, Sansawa H, Abe H (2004) Isolation and characterization of a *Chlorella* mutant producing high amounts of chlorophyll and carotenoids. *J Appl Phycol*, 16: 385-393.

Jin ES, Feth B, Melis A (2002) A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. *Biotechnol Bioeng*, 81: 115-124.

Jin ES, Polle JEW, Lee HK, Hyun SM, Chang M (2003) Xanthophylls in microalgae, from biosynthesis to biotechnological mass production and application. *J Microbiol Biotechnol*, 13: 165-174.

Johnson EA, Schroeder WA (1995) Microbial carotenoids. *Adv Biochem Eng Biotechnol*, 53: 119-178.

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

Kijlstra A, Tian Y, Kelly ER, Berendschot TJM (2012) Lutein: More than just a filter for blue light. *Progress in Retinal and Eye Research*, 31: 303-315.

Kim J, DellaPenna D (2006) Defining the primary route for lutein synthesis in plants: the role of *Arabidopsis* carotenoid β-ring hydroxylase *CYP97A3. Proc Natl Acad Sci USA*, 103(9): 3474-3479.

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii. Proc Natl Acad Sci USA*, 87: 1228-1232.

Kumar SV, Misqitta RW, Reddy VS, Rao BJ, Rajam MV (2004) Genetic transformation of the green alga *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens. Plant Sci*, 166: 731-738.

Landrum JT, Bone RA, Moore LL, Gomez CM (1999) Analysis of zeaxanthin distribution within individual human retinas. *Methods Enzymol*, 299: 457-467.

Lee YK, Zhang DH (1999) Production of astaxanthin by *Haematococcus. In*: Cohen Z (ed) *Chemicals from Microalgae*. Taylor & Francis Ltd, London, pp 173-195.

León R, Gónzalez D, Galván A, Férnandez E (2004) Transgenic microalgae as green cell factories. *Trends Biotechnol*, 22(1): 45-52.

León R, Fernández E (2007a) Nuclear transformation of eukaryotic microalgae: historical overview, achievements and problems. *In*: León R, Galván A and Fernández E (eds) *Transgenic Microalgae as green cell factories.* Springer Science and Business Media, NY. Adv Exp Med Biol, 616, 1-11.

León R, Couso I, Fernández E (2007b) Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii. J Biotech*, 130(2): 143-152.

Li T, Shi C, Gan Z, Shi X (2009) Cloning and analysis of the gene encoding lycopene ε-cyclase in *Chlorella protothecoides* CS-41. *Wei Sheng Wu Xue Bao*, 49: 1180-1189.

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

Li Y, Sommerfeld M, Chen F (2008b) Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). *J Plant Physiol*, 165: 1783–1797.

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

Lichtenthaler H (1999) The 1-deoxy-d-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol*, 50: 47-65.

Linden H (1999) Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. *Biochim Biophys Acta*, 1446(3):203-12.

Lindgren LO, Stalberg KG, Höglund AS (2003) Seed-specific overexpression if an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant Physiol*, 132: 779-785.

Lohr M, Chung-Soon I, Grossman AR (2005) Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii. Plant Physiol*, 138: 490-515.

Lohr M (2009) Carotenoids. *In*: Stern D (ed). *The Chlamydomonas Sourcebook. Vol 2: Organellar and Metabolic Processes*. San Diego: Elsevier, pp. 799-817.

Lohr M, Schwender J, Polle JEW (2012) Isoprenoid biosynthesis in eukaryotic phototrophs: A spotlight on algae. *Plant Science,* 185-186: 9-22.

Lorenz RT, Cysewski GR (2000) Commercial potential for *Haemotococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol*, 18: 433-435.

Lotan T, Hirschberg J (1995) Cloning and expression in *E. coli* of the gene encoding β-C-4-oxygenase that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. *FEBS Lett*, 364: 125-128.

Lu F, Vonshak A, Gabbay R, Hirschberg J, Boussiba S (1995) The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant Cell Physiol*, 36: 1519-1524.

Lü YM, Jiang GZ, Niu XL, Hou GQ, Zhang GX, Xue LX (2005) Cloning and functional analyses of two carbonic anhydrase genes from *Dunaliella salina. Acta Genet Sin*, 31: 1157-1166.

Lumbreras V, Stevens DR, Purton S (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J*, 14: 441-447.

Maher P, Schubert D (2000) Signaling by reactive oxygen species in the nervous system. *Cell Mol Life Sci*, 57: 1287-1305.

Mares-Perlman JA, Millen AE, Ficek TL, Hankinson SE (2002) The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. Overview. *J Nutr*, 132: 5185-5245.

Margalith PZ (1999) Production of ketocarotenoids by microalgae. *Appl Microbiol Biotechnol*, 51:431–438.

Masojidek J, Kopecky J, Koblizek M, Torzillo G (2004) The xanthophyll cycle in green algae (chlorophyta): its role in the photosynthetic apparatus. *Plant Biol*, 6(3):342-9.

Mayne, S.T. 1996. Beta-carotene, carotenoids, and disease prevention in humans. *The Faseb Journal*, 10: 690-701.

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

Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by funcitional analysis of gene products expressed in *Escherichia coli*. *J Bacteriol,* 172: 6704-6712.

Misawa N, Kajiwara S, Kondo K, Yokoyama A, Satomi Y, Saito T, Miki W, Ohtani T (1995) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon β-carotene by a single gene. *Biochem Biophys Res Commun*, 209(3): 867-76.

Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J*, 58: 165-174.

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

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright (2005) Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nat Biotechnol*, 23: 482-487.

Pogson B, MacDonald K, Truong M, Britton G, DellaPenna D (1996) *Arabidopsis* carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. *Plant Cell,* 8: 1627-1639.

Pogson B, Rissler H (2000) Genetic manipulation of carotenoid biosynthesis and photoprotection. *Phil Trans R Soc Lond B,* 355: 1395-1403.

Ralley L, Fraser PD (2004) Metabolic engineering of ketocarotenoid formation in higher plants. *Plant J*, 39: 477-486.

Ramos A, Coesel S, Marques A, Rodrigues M, Baumgartner A, Noronha J, Rauter A, Brenig B, Varela J (2008) Isolation and characterization of a stress-inducible *Dunaliella salina Lcy*-β gene encoding a functional lycopene β-cyclase. *Appl Microbiol Biotechnol*, 79: 819-828.

Ramos A, Polle J, Tran D, Cushman JC, Jin E, Valera J (2011) The unicellular green alga *Dunaliella salina* Teod. as a model for abiotic stress tolerance: genetic advances and future perspectives. *Algae*, 26: 3-20.

Ramos-Valdivia AC, van der Heijden R, Verpoorte R (1997) Isopentenyl diphosphate isomerase: a core enzyme in isoprenoid biosynthesis. A review of its biochemistry and function. *Nat Prod Rep*, 14(6),591-603.

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

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat Prod Rep*, 16(5): 565-74.

Sánchez JF, Fernández JM, Acién FG, Rueda A, Pérez-Parra J, Molina E (2008) Influence of culture conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*. *Process Biochem*, 43: 398-405.

Sandmann G (1994) Carotenoid biosynthesis in microorganisms and plants. *Trends Biochem*, 223: 7-24.

Sandmann G (2001) Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements.*Trends Plant Sci*, 6(1):14-17.

Sandmann G, Römer S, Fraser PD (2006) Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab Eng*, 8: 291–302.

Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DE (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J*, 20: 401-412.

Shi XM, Lu HJ, Zhang XW, Chen F (1999) Production of biomass and lutein by *Chlorella protothecoides* at various glucose concentrations in heterotrophic cultures. *Process Biochem*, 34: 341-347.

Shi XM, Zhang XW, Chen F (2000) Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. *Enzym Microb Technol*, 27: 312-318.

Shi XM, Jiang Y, Chen F (2002) High-yield production of lutein by the green microalga *Chlorella protothecoides* in heterotrophic fed-batch culture. *Biotechnol Prog*, 18: 723-727.

Shimogawara K, Fujiwara S, Grossman A and Usuda H (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, 148: 1821-1828.

Song HS, Kim HR, Kim MC, Hwang YH, Sim SS (2010) Lutein is a competitive inhibitor of cytosolic Ca2+-dependent phospholipase A(2). Journal of Pharmacy and Pharmacology, 62: 1711–1716.

Spolaore P, Joammis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. *J Biosci Bioeng*, 101(2): 87-96.

Stahl W, Sies H (2005) Bioactivity and protective effects of natural carotenoids. *Biochim Biophys Acta*, 1740:101-107.

Stalberg K, Lindgren O, Ek B, Hoglund AS (2003) Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. *Plant J*, 36 (6): 771-779.

Steinbrenner J, Linden H (2001) Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. *Plant Physiol,* 125:810-817.

Steinbrenner J, Linden H (2003) Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. *Plant Mol Biol*, 52: 343-356.

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

Stickforth P, Steiger S, Hess WR, Sandmann G (2003) A novel type of lycopene ε-cyclase in the marine cyanobacterium *Prochlorococcus marinus* MED4. *Arch Microbiol*, 179:409-415.

Sun G, Zhang X, Sui Z, Mao Y (2007) Inhibition of *pds* gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). *Mar Biotechnol*, 10: 219-226.

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

Sun Y, Yang Z, Gao X, Li Q, Zhang Q and Xu Z (2005) Expression of foreign genes in *Dunaliella* by electroporation. *Mol Biotechnol*, 30(3): 185-92.

Sun Z, Gantt E, Cunningham FX Jr (1996) Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. *J Biol Chem*, 271: 24349-34352.

Tan C, Quin S, Zhang Q, Jiang P, Zhao F (2005). Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *J Microbiol*, 43(4) : 361-365.

Takaichi S (2011) Carotenoids in algae: Distributions, biosynthesis and functions. *Mar Drugs*, 9 : 1101-1118.

Teng C, Qin S, Liu J, Yu D, Liang C, Tseng C (2002) Transient expression of *lacZ* in bombarded unicellular green alga *Haematococcus pluvialis*. *J Appl Phycol*, 14: 495-500.

Tian L, Musetti V, Kim J, Magallanes-Lundback M, DellaPenna D (2004) The *Arabidopsis* LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid ε-ring hydroxylation activity. *Proc Natl Acad Sci USA*, 101(1): 402-407.

Tjahjono AE, Hayama Y, Kakizono T, Terada Y, Nishio N, Nagai S (1994) Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. *Biotechnol Lett*, 16: 133-138.

Tran D, Haven J, Qiu WG, Polle JEW (2009) An update on carotenoid biosynthesis in algae: phylogenetic evidence for the existence of two classes of phytoene synthase. *Planta*, 229: 723-729.

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

Vila M, Couso I, León R (2007) Carotenoid content in mutants of the chlorophyte *Chlamydomonas reinhardtii* with low expression levels of phytoene desaturase. *Proccess Biochem*, 43: 1147-1152.

Vermaas W (1996) Molecular genetics of the cyanobacterium *Synechocystis* sp. PCC 6803: Principles and possible biotechnology applications. *J Appl Phycol*, 8: 263-273.

Vidhyavathi R, Venkatachakam L, Sarada R, Ravishankar GA (2008) Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *J Exp Bot*, 59: 1409-1418.

Yan Y, Zhu YH, Jiang JG, Song DL (2005) Cloning and sequence analysis of the phytoene synthase gene from a unicellular Chlorophyte, *Dunaliella salina*. *J Agric Food Chem*, 53: 1466-1469.

Yu B, Lydiate DJ, Young LW, Schäfer UA, Hannoufa A (2008) Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene ε-cyclase. *Transgenic Res*, 17:573–585.

Zhang DH, Lee YK, Ng ML, Phang SM (1997) Composition and accumulation of secondary carotenoids in *Chlorococcum* sp. *J Appl Phycol*, 9: 147-55.

Zhang J, Tao N, Xu Q, Zhou W, Cao H, Xu J, Deng X (2009) Functional characterization of citrus *PSY* gene in Hongkong kumquat (*Fortunella hindsii* Swingle). *Plant Cell Rep*, 28: 1737-1746.

Zhao DY, Wintch SW, Ermakov IV, Gellermann W, Bernstein PS (2003) Resonance Raman measurement of macular carotenoids in retinal, choroidal, and macular dystrophies. *Archives of Ophthalmology*, 121: 967-972.

Zhu C, Naqvi S, Capell T, Christou P (2009). Metabolic engineering of ketocarotenoid biosynthesis in higher plants. *Arch Biochem Biophys*, 483: 182-190.

Publicaciones y Patentes

Publicaciones

Cordero BF, Obraztsova I, Martín L, Couso I, León R, Vargas MA, Rodríguez H (2010) Isolation and characterization of a lycopene β-cyclase gene from the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). *J Phycol*, 46: 1229-1238.

Cordero BF, Couso I, León R, Rodríguez H, Vargas MA (2011) Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. *Appl Microbiol Biotechnol*, 91: 341-351.

Cordero BF, Obraztsova I, Couso I, León R, Vargas MA, Rodríguez H (2011) Enhancement of lutein production in *Chlorella sorokiniana* (Chlorophyta) by improvement of culture conditions and random mutagenesis. *Mar Drugs*, 9: 1607-1624.

Cordero BF, Couso I, León R, Rodríguez H, Vargas MA (2012) Isolation and characterization of a lycopene ε-cyclase gene of *Chlorella zofingiensis*. Regulation of the carotenogenic pathway by nitrogen and light. *Mar Drugs*, 10: 2069-2088.

Otras Colaboraciones

Couso I, Cordero BF, Vargas MA, Rodríguez H (2012) Efficient heterologous transformation of *Chlamydomonas reinhardtii npq2* mutant with the zeaxanthin epoxidase gene isolated and characterized from *Chlorella zofingiensis*. *Mar Drugs*, 10: 1955-1976.

Couso I, Vila M, Vigara J, Cordero BF, Vargas MA, Rodríguez H, León R (2012) Synthesis of carotenoids and regulation of the carotenoid biosynthesis pathway in response to high light stress in the unicellular microalga *Chlamydomonas reinhardtii*. *Eur J Phycol*, 47: 223-232.

Patentes

Weiss A, Johannisbauer W, Gutsche B, Martín L, Cordero BF, Rodríguez H, Vargas MA, Obraztsova I (2006) Zeaxanthin producing microalgal strain. Universidad de Sevilla y Cognis Deutschland GMBH and Co. KG. Nº Solicitud: EP/06000158.3

Weiss A, Johannisbauer W, Gutsche B, Cordero BF, Martín L , Rodríguez H, Vargas MA, Obraztsova I (2006) Process for obtaining lutein from algae. Universidad de Sevilla y Cognis Deutschland GMBH and Co. KG. Nº Solicitud: EP/06000599.8

Weiss A, Johannisbauer W, Gutsche B, Cordero BF, Martín L, Rodríguez H, Vargas MA, Obraztsova I (2007) Efficient astaxanthin production strains derived from *Haematococcus pluvialis*. Universidad de Sevilla y Cognis Deutschland GMBH and Co. KG. Nº Solicitud: EP/07010258.7

Cordero BF, Obraztsova I, Rodríguez H, Vargas MA, Martín L, Verseck S, Martínez C, Gutsche B, Weiss A (2008) Process for obtaining lutein from green algae. Universidad de Sevilla y Cognis Deutschland GMBH and Co. KG. Nº Solicitud: E08014415