UNIVERSITAT DE BARCELONA

Facultad de Farmacia

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Biosíntesis de carotenoides en *Escherichia coli* y tejidos no fotosintéticos de *Arabidopsis thaliana*

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Biosíntesis de carotenoides en *Escherichia coli* y en tejidos no fotosintéticos de *Arabidopsis thaliana*

Memoria presentada por **Antía Rodríguez Villalón** para optar al título de doctor por la Universidad de Barcelona

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Aos meus. Por estardes sempre ao meu carón, aínda desde lonxe. Sen o voso agarimo, isto non tería sido posible.

Índice general

Introducción general

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1.1. Isoprenoides y carotenoides

Los isoprenoides, también llamados terpenoides, son la mayor familia de productos naturales conocida, con una amplia variedad de funciones fisiológicas (Croteau 2000). Alrededor de 40000 moléculas de isoprenoides diferentes se han identificado en plantas, animales, bacterias y otros organismos (Rodriguez-Concepcion y Boronat, 2002; Rohdich *et al.*, 2005; Withers y Keasling, 2007). La mayor diversidad de isoprenoides se encuentra en plantas, en donde despliegan funciones esenciales (primarias) y secundarias. Los isoprenoides más abundantes en células vegetales (esteroles y carotenoides) están involucrados en el mantenimiento de la fluidez de la membrana (función desempeñada por los esteroles en la mayoría de las eucariotas), así como en la estabilización de las membranas tilacoidales y protección de los components fotosintéticos contra el daño foto-oxidativo (función desempeñada por los carotenoides) (Hirschberg, 2001; Fraser y Bramley, 2004). Otros isoprenoides intervienen en procesos de modificación post-traduccional de proteínas como la prenilación (grupos prenil) y la glicosilación (dolicoles) y forman parte de las cadenas de transporte electrónico (la cadena lateral de los tocoferoles y de las quinonas ubiquinona, plastoquinona y filoquinona). Por otro lado, componentes esenciales del aparato fotosintético de los organismos fotoautotróficos como los ya mencionados carotenoides y la cadena lateral de las clorofilas son isoprenoides. Asimismo, algunos reguladores del desarrollo como citoquininas, brasinoesteroides, giberelinas, ácido abcísico y estrigolactonas son de naturaleza isoprenoides (Figura 1).

No obstante, la mayoría de los isoprenoides presentes en las plantas actúan como metabolitos secundarios en la interacción de las plantas con su entorno. Algunos terpenos, como los mono- o los sesquiterpenos, desempeñan un importante papel en la defensa de la planta frente a patógenos y herbívoros. Algunos monoterpenos volátiles son los responsables de participar en la atracción de insectos para favorecer la polinización, siendo la acumulación de carotenoides en flores y frutos la responsable de la atracción de animales para la dispersión de las semillas (Croteau *et al*., 2000; Chappell, 2002). Además, varios grupos de isoprenoides son empleados comercialmente como fuente natural de pigmentos, aromas, fibras o ceras, así como por sus aplicaciones farmacéuticas (por ejemplo, artemisina y taxol), agroquímicas o como fitonutrientes beneficiosos para la salud humana.

Como ya se ha indicado, un grupo particularmente importante de isoprenoides son los carotenoides. En las plantas, los carotenoides se sintetizan y se localizan en todos los tipos de plastos. En cloroplastos se encuentran asociados a las proteínas que forman los complejos fotosintéticos, contribuyendo a la captación de la luz y actuando como antioxidantes esenciales para la fotoprotección. En algunos tejidos no fotosintéticos los carotenoides se acumulan en plastos especializados llamados cromoplastos. En estos plastos, los carotenoides son secuestrados en distintos tipos de estructuras lipoproteicas y cristalinas (Vishnevetsky *et al.*, 1999a). Además de colorear flores y frutos, los productors de degradación de los carotenoides

proporcionan aromas y sabores característicos. Algunas moléculas hormonales como el ácido abcísico o las estrigolactonas, esenciales en la regulación del desarrollo vegetal, son también apocarotenoides (Nambara y Marion-Poll, 2005; Klee, 2008).

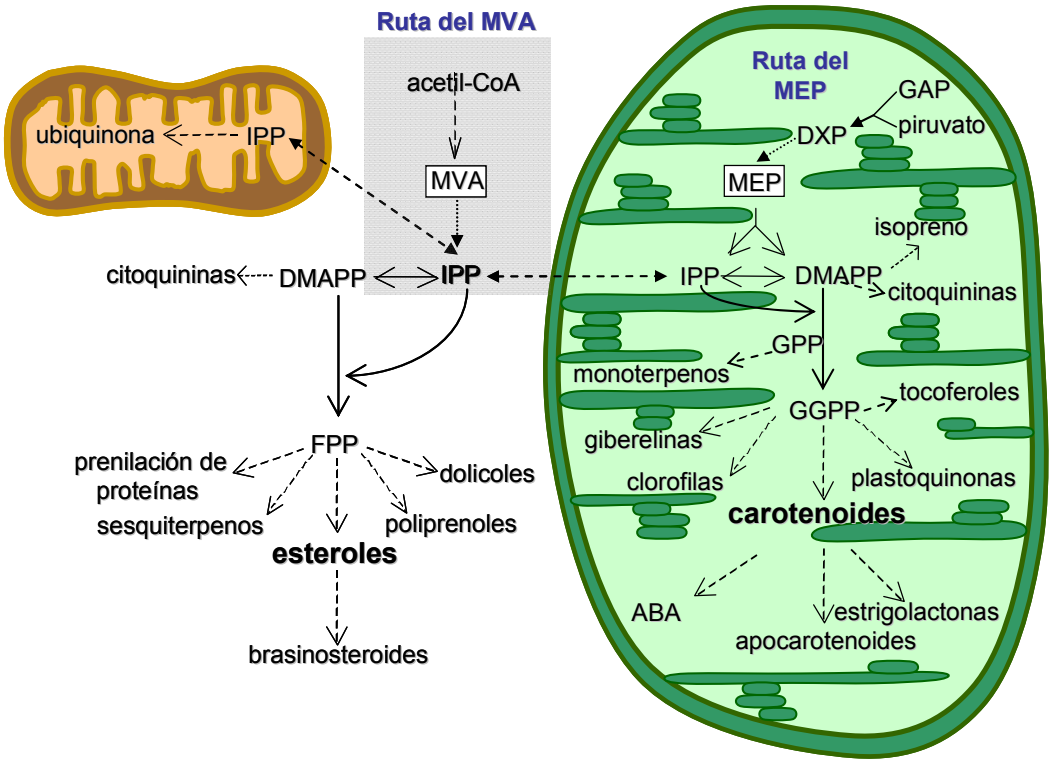

Figura 1. Esquema de las rutas biosintéticas de isoprenoides y sus precursores en una célula vegetal. MVA, mevalonato; FPP, farnesil difosfato; GAP, gliceraldehído 3-fosfato; DXP, desoxixilulosa 5-fosfato; MEP, 2-C-metil-D-eritritol-4-fosfato; IPP, isopentenil difosfato; DMAPP; dimetilalil difosfato; GPP, geranil difosfato; GGPP, geranilgeranil difosfato; ABA, ácido abscísico.

Los animales no pueden sintetizar carotenoides "de novo" pero necesitan ingerirlos a través de su dieta para la producción esencial de retinoides como el retinol o la vitamina A. Recientemente ha sido reportado que una ingesta diaria elevada de carotenoides en la dieta (frutas y vegetales, salsas de tomate) se correlaciona con la prevención de la aparición de ciertos tipos de cánceres y enfermedades coronarias. Una dieta pobre en β-caroteno aumenta el riesgo de padecer cataratas y degeneración macular (Christen *et al*., 2003), cáncer de pulmón (aunque dosis muy elevadas en pacientes de riesgo como fumadores presenta efectos adversos) del mismo modo que se relaciona con la aparición de enfermedades cardiovasculares (Kohlmeier, 1995). Diversos estudios relacionan dosis elevadas de licopeno con la prevención del cáncer de pecho (Dorgan *et al*., 1998), del cáncer de próstata (Giovannucci *et al*., 1995) y en el caso de ingestas elevadas de luteína aún queda por determinar su efecto positivo en la disminución del riesgo de cáncer colón (Slattery *et al*., 2000). Pero sin duda el mayor beneficio que presenta una dieta rica en carotenoides, en concreto en β-caroteno, es su papel como provitamina A. Este hecho cobra relevancia en

países en vías de desarrollo, donde el β-caroteno se encuentra ausente en la mayor parte de los alimentos que constituyen la dieta básica de la mayoría de la población (arroz blanco principalmente) causando una elevada incidencia de ceguera infantil.

Debido a sus propiedades antioxidantes beneficiosas para la salud humana y a sus propiedades colorantes empleadas en el mundo de la industria como pigmentos naturales, se ha generado una fuerte demanda en la producción de carotenoides. Sin embargo, las técnicas empleadas hasta el momento, la síntesis química y la extracción a partir de fuentes naturales han demostrado ser insuficientes, por lo que aparece como una alternativa muy atractiva la producción biotecnológica de carotenoides en sistemas bacterianos y vegetales. En las últimas décadas se han realizado numerosos esfuerzos para manipular genéticamente microorganismos y plantas para enriquecerlos en su producción de carotenoides (Giuliano *et al*., 2008) o su empleo como factorías celulares. Especialmente notorio es el caso del "golden rice", donde la introducción en arroz de los genes biosintéticos apropiados para la formación de β-caroteno permitió obtener un arroz enriquecido en este pigmento, cuya ingesta se preveé que pueda ayudar a prevenir la ceguera infantil antes comentada en los países en vías de desarrollo (Ye *et al*., 2000).

Sin embargo, pese a los avances realizados en esta área, aún son muchas las incógnitas por resolver en cuanto a cómo se regula la biosíntesis de estos pigmentos para la consecución de una producción biotecnológica de elevado rendimiento. El limitado conocimiento de los pasos limitantes en la biosíntesis de carotenoides así como otros factores relacionados con su almacenamiento o su relación con otras vías biosintéticas, impiden la conversión de esta incipiente industria biotecnológica en una industria madura, constituyendo estos factores por su importancia, un área de estudio de elevado interés.

1.2. Biosíntesis de carotenoides

Los carotenoides son moléculas de 40 carbonos cuya principal característica es la presencia de una larga cadena polieno (en donde la presencia de dobles enlaces puede variar de 3 a 15) responsable del color percibido por el ojo humano.

La ruta biosintética de carotenoides comenzó a ser desvelada entre 1950 y 1960 a partir de aproximaciones bioquímicas clásicas, usando inhibidores específicos de la ruta y mutantes que presentaban ciertas reacciones enzimáticas bloqueadas. A principios de los años 1970 estudios *in vitro* permitieron abordar el estudio de los enzimas biosintéticos (Bramley y Mackenzie, 1988), así como posteriormente se profundizó en el conocimiento en la ruta carotenogénica, en la regulación de la ruta biosintética y en los genes implicados en este proceso.

1.2.1 Formación del isopentenil difosfato (IPP)

Los carotenoides se sintetizan a partir de precursores de cinco carbonos, el isopentenil difosfato (IPP) y su isómero el dimetilalil difosfato (DMAPP), precursores que comparten con el resto de compuestos de naturaleza isoprenoide como giberelinas, tocoferoles, clorofilas y filoquinonas (Figura 1). Actualmente se conoce la existencia de dos rutas biosintéticas para la formación de precursores isoprenoides, la vía del mevalonato (MVA) y la vía del 2-*C*-metil-D-eritritol-4-fosfato (MEP) (Figura 1). Desde que se descubrió la vía del MVA se asumió que el IPP se sintetizaba únicamente a partir de acetil-CoA, sustrato de la ruta (Figura 2). No obstante, datos experimentales demostraron que la vía del MVA no podía abastecer únicamente los precursores necesarios para la formación de carotenoides en plantas. A principios de los años noventa estudios retro-biosintéticos establecieron la existencia de una ruta biosintética independiente de la ruta del MVA (Rodriguez-Concepcion y Boronat, 2002) que se conoce con el nombre de ruta del MEP (Figura 2). Los genes codificantes para los enzimas de esta última fueron primero identificados en *E. coli* descubriéndose sus homólogos en plantas poco tiempo después (Tabla 1).

Actividad enzimática	Enzima	Gen Arabidopsis	Gen *E. coli*
1-desoxixilulosa 5-fosfato sintasa	DXS	*DXS*	*dxs /dxs*
1-desoxixilulosa 5-fosfato reductoisomerasa	DXR	*DXR*	*yaeM/ispC*
2-metileritritol 4-fosfato citidililtransferasa	MCT	*MCT*	*ygbP /ispD*
4-(citidina 5-difosfato) 2-metileritritol kinasa	CMK	*CMK*	*ychB/ispE*
2-metileritritol 2,4-ciclodifosfato sintasa	MDS	*MDS*	*ygbB/ispF*
4-hidroxi 3-metil 2-butenil difosfato sintasa	HDS	*HDS*	*gcpE/ispG*
4-hidroxi 3-metil 2-butenil difosfato reductasa	HDR	*HDR*	*lytB/ispH*

Tabla 1. Relación de las diversas nomenclaturas empleadas para referirse a cada uno de los genes codificantes para los enzimas de la vía del MEP, en plantas (*Arabidopsis*) y bacterias (*E. coli)*.

La vía del MVA está presente en algunos procariotas Gram-positivos y en arqueobacterias, hongos, algas, plantas y animales. En las plantas, los enzimas de la ruta del MVA se localizan en varios compartimentos subcelulares incluyendo los peroxisomas y el retículo endoplasmático (Leivar *et al*., 2005; Carrie *et al*., 2007). Actualmente se asume que esta ruta genera como producto final IPP citosólico que después se utiliza para la producción de esteroles, brasinosteroides, sesquiterpenos, dolicoles y grupos prenil (Figura 1). Además, parte del IPP derivado de la ruta del MVA es transportado a las mitocondrias para la síntesis de ubiquinona (Figura 1; Disch *et al*., 1998). Aunque se cree que la mayor parte del IPP y DMAPP destinado a la síntesis de isoprenoides plastídicos en plantas procede de la vía MEP, el

intercambio de prenil difosfatos entre compartimentos celulares se ha demostrado en diversos sistemas vegetales (Bouvier *et al*., 2005).

La ruta del MVA comienza con la conversión de tres moléculas de acetil-CoA a β-hidroxi-β-metilglutaril coenzima A (HMG-CoA) (Figura 2). El primer paso específico de la ruta es la síntesis de MVA a partir de HMG-CoA, catalizado por la HMGR-CoA reductasa (HMGR). Posteriormente el MVA es fosforilado por el enzima MVA quinasa (MVK) a MVA-5-fosfato (MVP) y la fosforilación de esta molécula por la MVP quinasa (PMK) da lugar a MVA-5-difosfato (MVPP). La descarboxilación de este intermediario por la MVPP descarboxilasa (PMD) origina el IPP. En la ruta del MVA, el DMAPP se forma a partir de IPP por la acción del enzima IPP-DMAPP isomerasa (IDI) (Figura 2). Además de hallarse en el citoplasma, también existen isoformas de IDI en las mitocondrias (a donde se importa IPP derivado de MVA para la síntesis de ubiquinona y otros isoprenoides mitocondriales) y en los plastos, en donde no sería indispensable puesto que se sintetizan simultáneamente IPP y DMAPP plastídicos a partir de la ruta del MEP (Phillips *et al.*, 2008a).

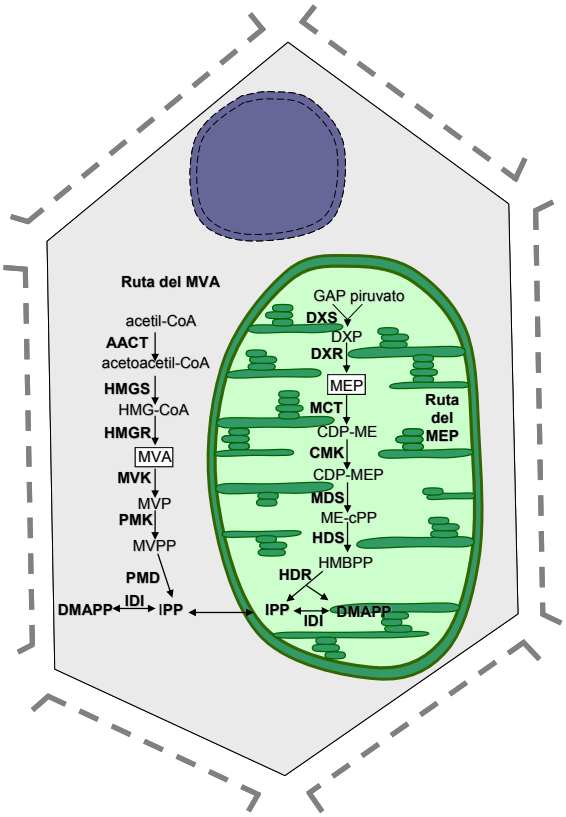

Figura 2. Rutas biosintéticas de los precursores isoprenoides. Enzimas y sustratos de la ruta del MVA: AACT, acetoacetil-CoA tiolasa; HMG-CoA, hidroximetil glutaril CoA; HMGS, HMG-CoA sintasa; HMGR, HMG-CoA reductasa; MVK; MVA quinasa; MVP, mevalonato-5-fosfato; PMK, MVP quinasa; MVPP, mevalonato-5-difosfato; PMD; MVPP descarboxilasa. Enzimas y sustratos de la ruta del MEP: GAP, gliceraldehído 3-fosfato; DXP, desoxixilulosa 5-fosfato; DXS, DXP sintasa; DXR, DXP reductoisomerasa; CDP-ME, 4-(citidina 5´difosfo)- 2-metileritritol ; MCT, CDP-ME transferasa; CDP-MEP, 4-(citidina-5-difosfato) 2-metileritritol 2-fosfato; CMK, CDP-MEP quinasa; ME-cPP, 2-metileritritol 2,4-ciclodifosfato; MDS, ME-cPP sintasa; HMBPP, 4-hidroxi-3-metil-2-butenil difosfato; HDS, HMBPP sintasa; HDR, HMBPP reductasa. IPP, isopentenil difosfato; DMAPP, dimetilalil difosfato; IDI, IPP isomerasa. Figura adaptada de Whiters y Keasling, 2007. Nomenclatura adaptada de Phillips *et al*., 2008b.

La ruta del MEP está presente en la mayoría de eubacterias (incluyendo *Escherichia coli* y la mayoría de las bacterias patogénicas), algas verdes, plantas y algunos protozoarios (como el parásito de la malaria). En contraste con la mayoría de los organismos, que sólo poseen una única vía para sintetizar los precursores de sus isoprenoides, las plantas usan tanto la ruta del MEP como la ruta del MVA para la síntesis de IPP y DMAPP aunque ambas rutas se localizan en diferentes compartimentos celulares (Roberts, 2007; Withers y Keasling,

2007). Los enzimas de la ruta del MEP (Tabla 1) son codificados por genes nucleares y transportados a los plastos, donde participan en la producción de los precursores de los carotenoides, ácido abscísico, giberelinas, monoterpenos, isoprenos y la cadena lateral de las clorofilas, tocoferoles, filoquinonas y plastoquinona (Figura 1). La ausencia de la ruta del MEP en arqueobacerias, hongos y animales hace de esta ruta bioquímica una importante diana para el desarrollo de nuevos herbicidas y nuevos fármacos antimicrobianos y antimaláricos con un amplio espectro de actividad y potencialmente sin efectos tóxicos para los humanos (Rodriguez-Concepcion *et al*., 2004).

La ruta del MEP comienza con la producción de desoxixilulosa 5-fosfato (DXP) en una reacción catalizada por DXP sintasa (DXS) (Lange *et al.*, 1998; Lois *et al.*, 1998). DXS cataliza una secuencia de dos reacciones dependientes de pirofosfato de tiamina (TPP). En un primer momento se produce la formación del intermediario hidroximetiltiamina por unión de la tiamina al piruvato con la posterior descarboxilación de este último. A continuación tiene lugar la condensación del intermediario sobre el D-gliceraldehido-3-fosfato (GAP), resultando en la formación de DXP. En bacterias, DXP no solo actúa como intermediario de la ruta del MEP sino que también es una molécula precursora de la biosíntesis de vitamina B_1 (tiamina) (Julliard y Douce, 1991) y B_6 (piridoxal) (Rohmer, 1999). Estudios recientes sugieren que la ruta biosintética de la vitamina B_6 independiente de DXP es la vía biosintética originaria de esta vitamina en la mayoría de los organismos y durante la evolución, algunos linajes bacterianos como *E.coli* la han ido perdiendo (Fitzpatrick *et al*., 2007; Cordoba *et al*., 2009).

En plantas, la mayoría de las especies presentan dos o más genes codificantes para DXS que normalmente pertenecen a dos clases diferentes (I y II), que aparentemente fueron originados antes de la diversificación de las mono y dicotiledóneas (Krushkal *et al.*, 2003; Kim *et al.*, 2006b; Phillips *et al.*, 2007; Phillips *et al.*, 2008b). La funcionalidad de ambos tipos de DXS se ha demostrado bien por complementación y/o por actividad *in vitro* (Fraser and Bramley, 2004). La existencia de múltiples genes codificantes para DXS potenciales se ha descrito en muchas especies, incluyendo *Zea mays, Medicago truncatula, Oryza sativa, Picea abies, Gingo biloba* y *Pinus densiflora* (Walter *et al*., 2002; Kim *et al*., 2006b; Phillips *et al*., 2007). La perdurancia de dos clases diferentes de genes con homología para DXS a lo largo de la evolución en diferentes especies y con un patrón de expresión específico para cada isogen sugiere una especialización funcional. La presencia de tránscritos de genes de tipo I en tejidos fotosintéticos de maíz, tomate y tabaco (Walter *et al*., 2002) se asoció con la síntesis específica de clorofilas y carotenoides. Por otra parte, se observó que la acumulación de tránscritos de genes de clase II en raíces de maíz, arroz y Medicago después de la colonización con hongos micorriza concordaba con un aumento en la producción de apocarotenoides (Walter *et al*., 2002). Estos datos sugieren que los isogenes de tipo II deben estar implicados en la biosíntesis tejido-específica de metabolitos secundarios derivados de isoprenoides (Córdoba *et al*., 2009). *Arabidopsis thaliana* presenta únicamente un gen codificante para una DXS funcional de tipo I, sin embargo se encontraron dos genes más con homología de secuencia a DXS, *DXL1* y *DXL2* (Phillips *et al*., 2008b). No obstante, estudios experimentales con *DXL1* (Carretero-Paulet,

2003) y estudios de secuencia para *DXL2* (Rodriguez-Concepcion y Boronat, 2002; Xiang *et al.*, 2007) sugieren que ninguno de los dos genes codifica para un enzima DXS funcional. El fenotipo albino presentado por el mutante de pérdida de función de DXS en Arabidopsis (llamado *cla1*) apoya la hipótesis de que ninguno de los otros dos genes con homología de secuencia a DXS presentes en *Arabidopsis* es redundante con *DXS* (Phillips *et al.*, 2008b).

El primer paso específico de la ruta del MEP es el rearreglo intramolecular seguido de un proceso inespecífico de reducción de DXP (Rohmer, 1999) para originar MEP en una reacción catalizada por el enzima DXP reductoisomerasa (DXR) que requiere NADPH y Mn^{+2} /Mg^{+2} como cofactores (Takahashi *et al*., 1998). A continuación se produce la conversión del MEP en 4-(citidina 5´difosfo)- 2-metileritritol (CDP-ME) catalizada por el enzima CDP-ME transferasa (MCT). Inmediatamente después, el CDP-ME es fosforilado en una reacción dependiente de ATP por el enzima 4-(citidina 5´difosfo)- 2-metileritritol kinasa (CMK) dando lugar al CDP-ME 2-fosfato (CDP-MEP) que es seguidamente transformado en 2-metileritritol 2,4-ciclodifosfato (ME-cPP) por la intervención del enzima ME-cPP sintasa (MDS). Los últimos pasos enzimáticos de la vía consisten en la reducción catalizada por la 4-hidroxi-3-metil-2-butenil difosfato (HMBPP) sintasa (HDS) para la producción de HMBPP, que es convertido finalmente en una mezcla de IPP y DMAPP (en una proporción aproximada de 5:1) por el enzima HMBPP reductasa (HDR) (Lange *et al*., 1998; Withers y Keasling, 2007). Tanto el enzima HDS como el enzima HDR son reductasas de ferro-azufre que requieren un grupo (4Fe-4S) $^{+1}$para su actividad enzimática (Rohdich *et al*., 2003; Wolff *et al*., 2003). A diferencia de lo que ocurre con *DXS*, en el genoma de *Arabidopsis thaliana* sólo se encuentra una única copia para los genes que codifican para los restantes enzimas de la ruta del MEP (Rodriguez-Concepcion y Boronat, 2002; Cordoba *et al.*, 2009) No obstante, estudios recientes en la angiosperma *Hevea brasiliensis* (Seetang-Nun *et al*., 2008) sugieren la existencia de al menos dos genes para el enzima DXR, al igual que para HDR en gimnospermas (Kim *et al*., 2008). Estos datos abren la posibilidad de una expresión tejido-específica de algunos isogenes de la vía del MEPque podría contribuir a la regulación de la vía en plantas.

El control de la ruta MEP es ejercido por varios enzimas, siendo mayoritario el control del primer enzima, DXS, y en menor medida DXR o HDR (Veau *et al.*, 2000; Mahmoud y Croteau, 2001; Walter *et al.*, 2002; Botella-Pavia *et al.*, 2004; Carretero-Paulet *et al.*, 2006). Sin embargo, el grado de control ejercido por cada enzima depende del organismo y las condiciones de crecimiento (Córdoba *et al*., 2009). Se cree que la ruta del MEP podría estar controlada en diferentes puntos para compensar así las fluctuaciones en el equilibrio entre precursores y productos y para redistribuir el balance en el control dentro de la ruta.

1.2.2 Formación de fitoeno a partir de IPP

El IPP y el DMAPP constituyen los bloques de cinco carbonos esenciales a partir de los cuales se generará la gran variedad de isoprenoides. Precursores prenildifosfatos de mayor longitud se generan a su vez por la condensación de 1 molécula de DMAPP con 1, 2, y hasta 3

moléculas de IPP, obteniéndose respectivamante geranil pirofosfato (GPP), C_{10}, precursor de los monoterpenos, farnesil pirofosfato (FPP), C_{15}, precursor de sesquiterpenos y triterpenos y geranil geranil difosfato (GGPP), C_{20}, precursor común de diterpenos y tetraterpenos como las clorofilas, carotenoides y giberelinas (Figura 1). En las plantas, a diferencia de lo que ocurre con los sesquiterpenos y triterpenos que se sintetizan en el citosol a partir de precursors generados fundamentalmente por la vía del MVA, la síntesis de los carotenoides, al igual que la de las clorofilas y monoterpenos se produce en los plastos a partir de precursores generados en su mayoría por la vía del MEP (Figura 1). GGPP sintasa (GDS) es el enzima responsable de la formación de GGPP, a partir del cual derivará la formación de fitoeno, C_{40}, precursor incoloro de los carotenoides (Figura 3). Se sabe que este enzima requiere de la presencia del ión divalente Mg^{+2} para realizar su actividad. Varias isoformas de GDS codificadas por pequeñas familias génicas existen en la mayoría de las plantas. Por ejemplo, el genoma de *Arabidopsis* contiene una familia multigénica de 12 miembros, la mayoría de los cuales codifican para enzimas plastídicos (Lange y Ghassemian, 2003; Joyard *et al.*, 2009). Sin embargo, aún se desconoce si existen isoformas de GDS específicas para la producción de carotenoides.

La fitoeno sintasa (PSY en plantas, CrtB en bacterias) cataliza el primer paso específico de la ruta carotenogénica, llevando a cabo la condensación de dos moléculas de GGPP en una molécula de fitoeno (Figura 3). Los genes que codifican para este enzima han sido identificados y aislados variando su número entre las diferentes plantas. En *Arabidopsis* se ha identificado un único gen mientras que dos fitoeno sintasas han sido descritas en tabaco (Busch *et al*., 2002) y en tomate (Fray y Grierson, 1993; Giorio *et al.*, 2008), encontrándose tres genes en arroz y maíz (Li *et al*., 2008a; Welsch *et al*., 2008). Todos estos enzimas se sitúan en los plastos, donde son importados después de ser sintetizados (Dogbo *et al.*, 1988; Misawa *et al.*, 1994; Fraser y Bramley, 2004). Se creía que el enzima PSY era una proteína integral de membrana, sin embargo, a partir de estudios bioquímicos llevados a cabo en fitoeno sintasas aisladas de cromoplastos (PSY1) y de cloroplastos (PSY2) de tomate (Fraser *et al*., 2000) se llegó a la conclusión que este enzima estaba laxamente asociado a membranas e incluso se llegó a postular la formación de un complejo proteico soluble formado por GDS y PSY que catalizaría la formación de fitoeno en el estroma plastídico (Cunningham y Gantt, 1998). En cuanto al enzima bacteriano CrtB, su actividad fitoeno sintasa se demostró en células de *E. coli* (Neudert *et al*., 1998) requiriendo de la presencia de los mismos cofactores que su homólogo en plantas (Mn^{+2} y ATP). La capacidad de este enzima bacteriano de formar fitoeno en sistemas heterólogos fue ampliamente empleada en plantas superiores para enriquecer su contenido en carotenoides (Fraser y Bramley, 2004). Por ejemplo, la sobreexpresión del gen *CrtB* de *Erwinia uredovora* en semillas de colza permitió incrementar el contenido de fitoeno y de algunos carotenoides en éstas de forma espectacular (Shewmaker *et al*., 1999). Del mismo modo la sobreexpresión de este gen en tubérculos de patata producía un efecto similar provocando un aumento en la acumulación de los carotenoides finales (Ducreux *et al*., 2005).

1.2.3 Formación de carotenoides a partir de fitoeno

El cromóforo característico que presentan todos los carotenoides está constituído por una serie de dobles enlaces conjugados que se van introduciendo en el fitoeno a través de diversas reacciones de desaturación consecutivas (Figura 3). La longitud del cromóforo determina el espectro de absorción de la molécula y por lo tanto el color percibido por el ojo humano.

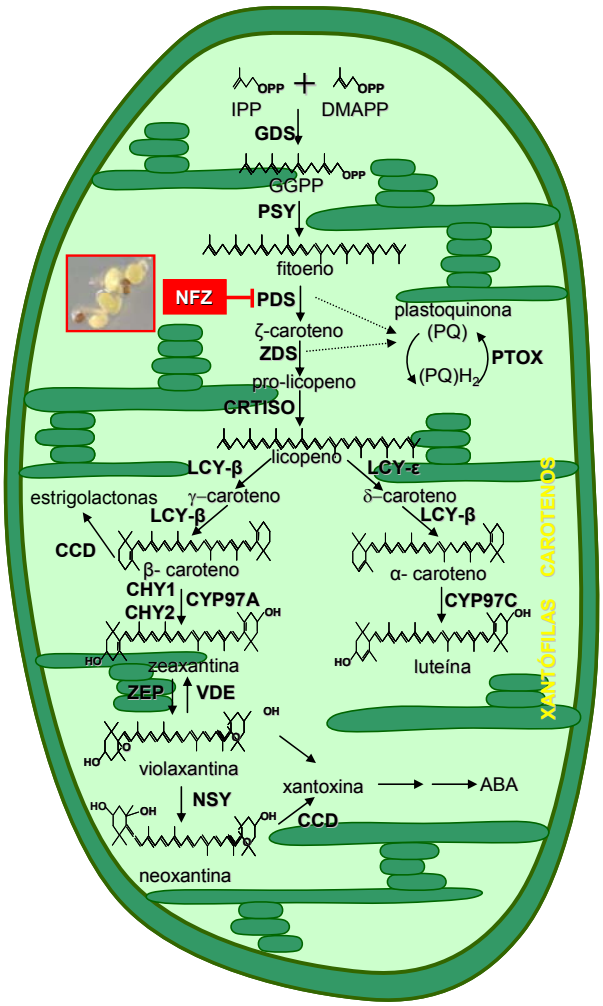

Figura 3. Biosíntesis de carotenoides a partir de isopentenil difosfato (IPP) y dimetilalil difosfato (DMAPP). GGPP, geranilgeranil difosfato; GDS, GGPP sintasa; PSY, fitoeno sintasa; PDS, fitoeno desaturasa; ZDS, ζ-caroteno desturasa; PTOX, oxidasa terminal plastídica; CRTISO, isomerasa carotenogénica; LCY-β, licopeno ciclasa beta; LCY-ε, licopeno ciclasa epsilon; CHY1, CHY2, no-hemo hidroxilasas; CYP97A, CYP97C, hidroxilasas citocromo P450; ZEP, zeaxantina epoxidasa; VDE; violaxantina desepoxidasa; NSY, neoxantina sintasa; CCD, dioxigenasas de carotenoides; NFZ, norflurazon.

Éstos se basan en siete grupos terminales diferentes, sin embargo únicamente cuatro (β,ε,κ,ψ) se encuentran en los carotenoides presentes en las plantas (Weedon, 1969). Gracias a sucesivas reacciones de desaturación del fitoeno se acaba generando el licopeno, pasando de un pigmento incoloro a un pigmento de coloración rosada. Los enzimas responsables de la desaturación del fitoeno son la fitoeno desaturasa (PDS, que cataliza la primera reacción de desaturación del fitoeno) y la ζ-caroteno desaturasa (ZDS). La actividad del enzima PDS puede ser inhibida por la acción del norflurazón (NFZ), que provoca la aparición de un fenotipo albino en las plantas cuando éstas crecen en su presencia.

Todos los genes codifcantes para PDS que han sido aislados e identificados en *Arabidopsis thaliana* (Lange y Ghassemian, 2003), tomate (Pecker *et al*., 1992), maíz y tabaco (Busch *et al*., 2002) presentan un dominio conservado de unión a un dinucleótido (FAD/NADP) en el extremo amino terminal. Cada reacción de desaturación conlleva la desaparición de dos

átomos de hidrógeno y la reducción de moléculas de plastoquinona que actúan como aceptoras. La regeneración de estas moléculas aceptoras tiene lugar gracias a la cadena de transporte de electrones de la fotosíntesis en cloroplastos (Figura 3). Los componentes de esta cadena redox en otros tipos de plastos han sido elucidados gracias a mutantes de *Arabidopsis* (Josse *et al*., 2000). La reoxidación de las quinonas se realiza gracias a la cadena de transporte de electrones cloroplastídica que emplea como aceptor final de electrones el oxígeno, con la consiguiente formación de agua. Trabajos realizados con el mutante *immutans* en *Arabidopsis* (defectivo en la biogénesis de plastos por deficiencias en la biosíntesis de carotenoides) demostraron la importancia de la oxidasa plastídica terminal PTOX, que se veía afectada en este mutante, codificada por un único gen en *Arabidopsis*. En plastos no fotosintéticos, como los cromoplastos o etioplastos de plántulas de *Arabidopsis* crecidas en la oscuridad, se vio como la reoxidación de las quinonas era llevada a cabo por este enzima, empleando como aceptor final de electrones el oxígeno. Se observó que la actividad de este enzima es crucial en los primeros estadíos de desarrollo de las hojas, sin embargo, pese a continuar activa, va perdiendo relevancia a medida que la biogénesis de los cloroplastos se complementa (Carol y Kuntz, 2001).

Para la completa desaturación del fitoeno en licopeno se requiere la actividad del enzima ZDS. Genes codificantes para esta actividad enzimática han sido identificados en pimiento (Albrecht *et al*., 1995), en *Arabidopsis* (Scolnik y Bartley, 1993) y tomate (Bartley *et al*., 1999). Todos los genes contienen un dominio de unión al dinucleótido FAD/NADP en su posición amino terminal y se ha demostrado que el enzima requiere de la presencia de oxígeno y plastoquinonas para desarrollar su actividad (Figura 3). La conversión de la forma predominante en plantas de 15-*cis* fitoeno en *trans*-licopeno sugería la existencia de una actividad isomerasa que posteriormente, mediante varios estudios experimentales, se demostró que realizaba la isomerasa de carotenoides CRTISO (Isaacson *et al*., 2002) junto con la acción del enzima ζ-caroteno isomerasa (Z-ISO). La existencia de este enzima se ha corroborado en trabajos realizados en maíz (Li *et al*., 2007). No obstante, aún no ha sido demostrada a nivel molecular.

Sorprendentemente, todas las reacciones necesarias para la conversión de fitoeno en licopeno pueden ser llevadas a cabo por la fitoeno desaturasa bacteriana (CrtI), quien es capaz de realizar todas las reacciones de isomerización y desaturación antes mencionadas. Por esta razón este enzima bacteriano ha sido ampliamente utilizado en abordajes de ingeniería genética que pretendían incrementar los niveles de carotenoides en diferentes especies de plantas (Fraser y Bramley, 2004). La co-expresión de los genes bacterianos CrtB y CrtI en tubérculos de patata conllevaba un aumento de los niveles de pigmentos (Diretto *et al*., 2007). Del mismo modo, la coexpresión de ambos genes en endospermo de arroz inducía la acumulación de β-caroteno, un pigmento de color anaranjado, dando lugar al conocido “golden rice” (Ye *et al*., 2000). El enzima CrtI, que presenta poca similitud con las desaturasas e isomerasas de carotenoides vegetales, no es inhibida por NFZ (Misawa *et al*., 1993).

A continuación el licopeno, que es una molécula lineal, sufre una reacción de ciclación que le genera un anillo de seis carbonos en cada uno de sus extremos (Figura 3). La formación de un anillo ε o un anillo β en el primer paso de ciclación depende únicamente del tipo de ciclasa que actúe sobre el licopeno, ya que la actividad del enzima β-ciclasa (LCY-β) difiere de la actividad del enzima ε-ciclasa (LCY-ε) únicamente en la posición donde introducen el doble enlace en anillo ciclohexano. Experimentos realizados en cromoplastos de pimientos (Camara y Dogbo, 1986) y de tomate (Fraser *et al.*, 2000) demostraron que ambas ciclasas requerían de NADPH como cofactor para desarrollar su actividad (Beyer *et al*., 1991). A diferencia de LCY-β que es capaz de catalizar la conversión de licopeno en β-caroteno introduciendo dos anillos β, para la formación de α-caroteno se requiere la actuación consecutiva de ambas ciclasas. La incapacidad de la ε-ciclasa de añadir más de un anillo ε se ha sugerido como un mecanismo para controlar la formación de carotenoides cíclicos en *Arabidopsis*, ya que su homólogo en ciertas especies de *Lactuca sativa* ha demostrado poder introducir dos anillos ε pese a presentar un 80% de homología proteica (Pecker *et al*., 1996). A partir del β-caroteno se cree que tiene lugar el comienzo de la ruta biosintética de las estrigolactonas (Booker *et al*., 2004), un grupo de hormonas descubiertas recientemente esenciales en la regulación de la arquitectura de las plantas y cuyos genes implicados en su biosíntesis solo se conocen parcialmente.

Las xantófilas son moléculas derivadas de la oxidación de los carotenos. La hidroxilación del α y del β-caroteno produce la luteína y la zeaxantina respectivamente. La hidroxilación redundante del α y del β-caroteno puede llevarse a cabo o bien a través de la acción de dos no-hemo hidroxilasas (CHY1 y CHY2) o bien a través de las hidroxilasas tipo citocromo P450, CYP97A y CYP97C. La hidroxilación del anillo β la llevan a cabo tanto CHY1 y CHY2 como CYP97A y genera zeaxantina a partir de β-caroteno. Sin embargo, la hidroxilación del anillo ε del α-caroteno para producir luteína tiene lugar gracias a la acción de la hidroxilasa CYP97C (Tian *et al.*, 2004; Galpaz *et al.*, 2006; Kim y DellaPenna, 2006). El nivel de redundancia varía según el tejido (Galpaz *et al.*, 2006).

La actividad del enzima violaxantina desepoxidasa (VDE) conlleva la conversión de zeaxantina en violaxantina a través de un intermediario mono-epoxidado, la anteraxantina. Esta secuencia de reacciones es reversible y gracias a la acción de la zeaxantina epoxidasa (ZEP) se puede reconvertir la violaxantina en zeaxantina. La formación de la zeaxantina tiene lugar cuando la planta se ve sometida a una gran irradiación de luz, con lo que la acción de este pigmento impide el daño fotoxidativo de las clorofilas y protege las membranas lipídicas de la peroxidación (Havaux y Niyogi, 1999). La neoxantina se sintetiza a partir de la violaxantina por la acción del enzima neoxantina sintasa (NSY). Estudios recientes sugieren que el producto del gen ABA4 es imprescindible para la síntesis de este pigmento (North HM *et al*., 2007).

Como se ha indicado anteriormente, los carotenoides pueden ser catabolizados para la formación de apocarotenoides por la acción de dioxigenasas de carotenoides (CCD) (Auldridge *et al.*, 2006b). La neoxantina y la violaxantina pueden ser catabolizadas por la 9-epoxicarotenoide dioxigenasa para la formación de xantoxina, el sustrato directo de la

fitohormona ácido abscísico (ABA) (Schwartz *et al*., 2003), molécula implicada en regular numerosos procesos del crecimiento vegetal así como la coordinación a las diferentes respuestas a estrés abiótico (De Smet *et al.*, 2003; Nambara y Marion-Poll, 2005; Li *et al.*, 2008a; Welsch *et al.*, 2008).

El conocimiento sobre cada una de las reacciones enzimáticas implicadas en la biosíntesis de carotenoides ha sido esencial en el desarrollo de diferentes aproximaciones biotecnológicas para modificar el contenido de pigmentos en organismos bacterianos y vegetales.

1.3. Ingeniería metabólica en sistemas bacterianos

Los carotenoides, al igual que otros isoprenoides, presentan numerosas aplicaciones en medicina y agricultura, del mismo modo que están siendo ampliamente empleados en al actualidad como nutracéticos y como precursores de apocarotenoides con propiedades como sabores y aromas. Diferentes aproximaciones biotecnológicas han sido llevadas a cabo con el objetivo de conseguir en algunos casos abaratar los costes de la producción de los carotenoides, igual que de otros isoprenoides. Hasta el momento, los isoprenoides derivados de las plantas se extraían directamente de su fuente natural (Brahmachari *et al*., 2004), su síntesis química parcial o total (Horwitz, 1994) o mejorando su contenido en plantas de cultivo (Croteau *et al*., 2005). Éstas están siendo reemplazadas por la ingeniería genética de plantas (Ye *et al*., 2000), por el cultivo celular (Roberts, 2007) y por su producción en huéspedes microbianos (Ajikumar *et al*., 2008). Un claro ejemplo es el caso de la artemisina. El aislamiento de la artemisina, un sesquiterpeno producido en las hojas de *Artemisia annua* y la demostración de su uso como antimalárico (Nosten y White, 2007) se consiguió gracias al estudio realizado sobre antiguos escritos de la medicina tradicional china. El coste de fármaco es hoy en día inalcanzable para la mayor parte de la población de los países en vías de desarrollo. La producción biotecnológica de este compuesto usando una fuente productora microbiológica, asegura un abastecimiento de artemisina a un coste mucho menor que el actual método de extracción a partir de la planta (Hale *et al*., 2007). Otro conocido caso es la producción de un diterpeno aislado de *Taxus brevifola* que permitió el desarrollo de un importante anticancerígeno, el Taxol, que es empleado en numerosos tratamientos contra el cáncer (Tabata, 2006). Sin embargo, la demanda de Taxol para abastecer el creciente mercado de aplicaciones clínicas generó una gran controversia a raíz del impacto ambiental que dicha producción ocasionaba. Para generar un 1kg de Taxol se requiere la corteza de 2000-3000 árboles; ésto es el equivalente de un árbol de 100 años para extraer una única dosis de fármaco. No obstante, la elaboración de una ruta semisintética y la producción de taxol en cultivos celulares (http://www.epa.gov/greenchemistry/pubs/pgcc/winners/gspa04.html) pueden sustituir el antiguo proceso de extracción. No obstante, pese a los numerosos avances realizados en el campo de la ingeniería metabólica vegetal, son muchos los factores que limitan una producción eficiente de isoprenoides e impiden su conversión en una técnica

biotecnológica madura (Giuliano *et al*., 2008). En comparación con la ingenería metabólica en plantas, el empleo de los sistemas bacterianos permite una mejor aplicación a gran escala de proyectos de ingeniería metabólica, ya que, a diferencia de lo que sucede en sistemas vegetales, se pueden combinar y comparar diferentes modificaciones al mismo tiempo.

En el caso concreto de los carotenoides, la producción microbiológica de carotenoides a gran escala se presenta como una alternativa ecológica a la síntesis química que se venía empleando hasta ahora. Microorganismos carotenogénicos han sido investigados para una producción de pigmentos a gran escala (Jacobson *et al*., 2000) al igual que organismos no productores de pigmentos como *Escherichia coli, Sacharomyces cerevisiae* y *Candida utilis* mediante su transformaicón con genes apropiados de microorganismos carotenogénicos (Misawa y Shimada, 1997). En concreto *E. coli* ha demostrado ser un huésped excelente para la producción de pigmentos (Figura 4) ya que existe una gran variedad de herramientas de ingeniería metabólica que permiten la síntesis de una gran variedad de carotenoides como fitoeno, licopeno, β-caroteno, cantaxantina, zeaxantina y astaxantina (Misawa *et al*., 1990; Cunningham *et al*., 1993). La combinación de genes carotenogénicos procedentes de diferentes organismos en secuencias de reacciones biosintéticas nuevas y la evolución de nuevas funciones catalíticas *in vitro* permitió la exploración de diferentes estrategias de ingeniería para generar una amplia diversidad química. Además numerosos enzimas de carotenoides son capaces de aceptar más de un sustrato diferente, lo cual permite generar una mayor diversidad de arquitecturas moleculares. La optimización de las condiciones de cultivo así como el escalado de cultivos a biorreactores de laboratorio han permitido mejorar la productividad en los sistemas bacterianos.

Figura 4. Ejemplo de organismo no carotenogénico modificado genéticamente para la síntesis de carotenoides. La imagen representa células de *E. coli* transformadas con los genes biosintéticos procedentes de *Erwinia uredevora* para la síntesis (en el sentido de las agujas del reloj) de fitoeno (incoloro), licopeno (rojo) y β-caroteno (naranja).

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Sin embargo, aún existen problemas importantes por resolver para la obtención de una producción biotecnológica de carotenoides eficaz. Los más relevantes se resumen en dos puntos: i) desajustes metabólicos causados por la disponibilidad limitada de sustratos y la acumulación de intermediarios tóxicos para las células; ii) alteraciones morfológicas por la necesidad de almacenar productos lipofílicos en membranas dada la inexistencia de mecanismos naturales para el secuestro y acumulación de estos compuestos (Klein-Marcuschamer *et al*., 2007).

1.3.1 Aporte de sustratos.

Una de las principales estrategias empleadas para aumentar la producción de carotenoides en *E. coli* modificadas genéticamente es aumentar los niveles de precursores IPP y DMAPP. Una disminución en los niveles de IPP puede resultar perjudicial para la célula ya que éste es indispensable en la prenilación del tRNA y en la biosíntesis de quinonas y paredes celulares (Caillet y Droogmans, 1988; Maury *et al.*, 2005). Tanto la modificación de compuestos esenciales para la síntesis de vías centrales metabólicas, como la sobreexpresión de los genes codificantes para enzimas limitantes de la vía del MEP, como la introducción de vías heterólogas com la del MVA han sido estrategias utilizadas para aumentar la acumulación de pigmentos.

1.3.1.1 Modificación del metabolismo central.

Tanto el sustrato inicial de la vía del MVA, el acetil-CoA, como los sutratos de la vía del MEP, el piruvato y GAP, son sustratos compartidos por la vía glicolítica. La vía del MEP requiere cantidades exactamente iguales de piruvato que de GAP para la síntesis de IPP, sin embargo se ha sugerido que la disponibilidad de GAP podría ser un factor limitante en la producción de licopeno (Kim y Keasling, 2001). Éste hecho puede deberse a que el piruvato es el precursor empleado en numerosas vías metabólicas y se encuentra en la célula mucho más disponible que el GAP. En concordancia con estos datos se ha demostrado que la sobreexpresión o inactivación de enzimas que dan como resultado un redireccionamiento del piruvato hacia el GAP conlleva un incremento en la producción de licopeno en *E. coli* (Farmer y Liao, 2001). Se ha descrito que la inactivación de vías metabólicas que compiten por el acetil-CoA y el piruvato pueden aumentar hasta un 45% la producción de licopeno (Vadali *et al.*, 2005).

1.3.1.2 Sobreexpresión de los genes codificantes para enzimas del MEP

La estrategia más sencilla para incrementar los niveles de precursores IPP y DMAPP en células de *E. coli* es la sobreexpresión de genes codificantes para los enzimas de la vía del MEP. Numerosos trabajos se centraron principalmente en la sobreexpresión del enzima DXS, que cataliza el primer paso de la vía del MEP, observándose que la producción de licopeno mejoraba en células carotenogénicas de *E. coli* (Harker y Bramley, 1999; Matthews y Wurtzel, 2000; Yuan *et al.*, 2006). La sobreexpresión del gen DXS, sin embargo, puede tener efectos deletéros para el crecimiento celular al acabar con la disponibilidad de su precursor glicolítico, el GAP. Aunque la sobreexpresión individual de otros genes codificantes para los enzimas del MEP ha demostrado producir un incremento en la producción de carotenoides en *E. coli*, este aumento es menor que el alcanzado mediante la sobreexpresión de DXS (Abrecht *et a*l., 1999; Cunnigham *et al*., 2000; Kim y Keasling, 2001; Yuan *et al*., 2006).

La sustitución de los promotores endógenos de los genes codificantes para los enzimas de la ruta del MEP por el promotor del bacteriófago T5 (P_{T5}) y el análisis de la producción de β-

caroteno en células de *E. coli* modificadas genéticamente para la producción de carotenoides, permitió establecer como enzimas moduladores del flujo de la vía, además de DXS e IDI, a MCT y MDS. La producción de este pigmento en las células P_{T5}-*ispD* y P_{T5}-*ispF* era mayor que las células silvestres, lo cual sugería que ambos enzimas, eran limitantes para la producción de carotenoides (Yuan *et al*., 2006).

En este mismo estudio se observó que la sobreexpresión de los genes *ispE*, *ispG* y *ispH* no tenían ningún efecto en la producción de β-caroteno. Poco más se sabe acerca del papel regulador que puedan tener los otros enzimas de la vía del MEP.

La isomerización de IPP en DMAPP también parecen ser pasos limitantes en la producción de carotenoides ya que la síntesis de pigmentos aumenta al incrementar la actividad de IDI y GDS (Kajiwara *et al.*, 1997; Albrecht *et al*., 1999; Cunningham *et al*., 2000).

1.3.1.3 Introducción de una vía foránea: la vía del MVA

Un modo alternativo a aumentar el aporte de IPP y DMAPP incrementando el flujo de la vía del MEP es la introducción de una vía foránea como la vía del MVA (Figura 5). Grandes avances han sido realizados en la ingeniería de los isoprenoides con la expresión heteróloga de la ruta biosintética del MVA en células de *E. coli* y *S. cerevisiae*. La producción de licopeno se vio incrementada significativamente en cepas de *E. coli* en las que se les había introducido la vía del MVA en comparación con aquellas cepas que presentaban únicamente la vía del MEP (Yoon *et al*., 2006; Rodriguez-Villalon *et al*., 2008). De modo similar la producción de β-caroteno aumentaba en células de *E.coli* carotenogénicas que presentaban el operón del MVA además de sobreexpresar el gen *dxs*, conseguiéndose una producción de este pigmento 503 mg/l al añadirle al medio una concentración de MVA exógeno de 16.5 mM MVA y un 2.5 % (p/v) de glicerol como fuente de carbono (Yoon *et al*., 2007). El hecho de emplear una vía foránea en *E. coli* permitía evitar la propia regulación endógena de la célula, la cual se vio que sí afectaba a la vía del MEP. Se ha demostrado que un paso limitante en el control del flujo metabólico de la ruta del MVA es el paso catalizado por el enzima HMGR. Trabajos realizados en *S. cerevisiae*, que sintetiza sus isoprenoides exclusivamente a partir de la ruta del MVA, se centraron mayoritariamente en la regulación del enzima HMGR (Figura 3), y en concreto en una forma truncada que dejaba de degradarse al perder su regulación, viéndose así aumentados los niveles de MVA y por tanto, de IPP (Kirby y Keasling, 2009). Sin embargo, la producción de un exceso de IPP a partir del acetil-CoA causaba una inhibición en el crecimiento celular, sugiriendo que un aumento desaforado de los niveles de IPP podría resultar tóxico para el crecimiento. En células de *E. coli* sobreexpresoras de *dxs* se vio que las células podían ser rescatadas mediante la co-expresión de un enzima que transformaba este exceso de precursor en un producto final (Martin *et al*., 2003) como el licopeno (Kim and Keasling, 2001).

Otro modo de incrementar la producción de IPP y DMAPP era aumentar la cantidad del propio MVA que llegaba a la vía aumentando los niveles de su propio sustrato, el acetil-CoA. El acetato puede ser transformado directamente en acetil-CoA por el enzima acetil-CoA sintasa y

a su vez el acetato es generado por la conversión del acetaldehído en acetato mediante la acción del enzima acetaldehído deshidrogenada. La sobreexpresión de ambos enzimas en células de *S. cerevisiae* supuso un incremento en los niveles de precursores isoprenoides (Shiba *et al*., 2007) y de productos finales.

Todos estos trabajos demostraban que la manipulación metabólica de las vías que suministran los precursores IPP y DMAPP podían aumentar los niveles de carotenoides. Sin embargo se requiere de un preciso control de los niveles de expresión de los enzimas para evitar la acumulación de intermediaros metabólicos tóxicos para las células.

1.3.1.4 Otras aproximaciones de ingeniería metabólica

Algunas aproximaciones diferentes a la sobreexpresión de los enzimas limitantes de la vía del MEP o la introducción de una vía heteróloga fueron llevadas a cabo para incrementar la producción de licopeno en bacterias. En estas aproximaciones no se tiene en cuenta tan sólo la disponibilidad de precursores, sino que se intentó combinar el estado energético de la célula, el balance transcripcional de los principales enzimas reguladores así como otros procesos que pudieran estar relacionados con una mayor acumulación de carotenoides. Algunas de estas estrategias consistieron en la delección (knockout) al azar o dirigida de algunos genes que pudieran interferir o cuya ausencia podría incrementar la producción de pigmentos, la búsqueda al azar de otros genes que pudieran intervenir en la producción de licopeno (shotgun) y otros factores como la disponibilidad de carbono.

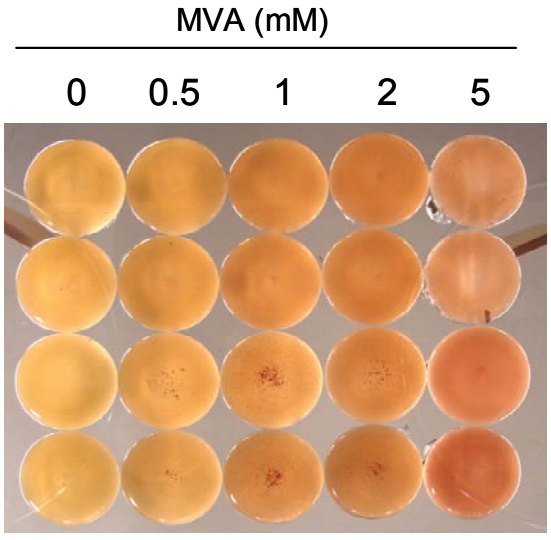


Figura 5. Ejemplo de la influencia de precursores en la síntesis de carotenoides. La imagen representa la parte inferior de erlenmeyers que contienen 25 ml de medio inoculado con células de *E. coli* modificadas genéticamente con los genes necesarios para la producción de licopeno y las cantidades indicadas de mevalonato (MVA), que se emplea para la síntesis de precursores difosfatos necesarios para la formación de licopeno

Knockout. Alper y colaboradores demostraron mediante un estudio estequiométrico exhaustivo combinado con un análisis de flujo metabólico que la ausencia de ciertos genes era suficiente para aumentar la producción de licopeno sin que la tasa de crecimiento celular se viera afectada. El análisis estequiométrico de flujos permitió determinar que la producción de licopeno era directamente proporcional a la ingesta de glucosa por parte de las células e inversamente proporcional al crecimiento de éstas. Además se llegó a identificar aquellos

enzimas que al verse suprimida su actividad se producía un aumento en los niveles de precursores IPP y DMAPP y, por consiguiente, de licopeno: *ace*E (pyruvato deshidrogenasa), *gdhA* (glutamato deshidrogenasa), *gpmA* (fosfoglucomutasa), *gpmB* (fosfoglucomutasa) y *ppc* (fosfoenolpiruvato carboxilasa) (Alper *et al*., 2005). Sin embargo este análisis estequiométrico no podía predecir la regulación y la complejidad cinética de los flujos, así que se llevó a cabo una estrategia complementaria que permitía la búsqueda de genes knockout por mutagénesis al azar con transposones (Alper *et al*., 2005).

La combinación de ambas estrategias sugirió la construcción de un triple mutante (Δ*gdhA* Δ*aceE* Δ*fdhF*). Esta cepa fue testada experimentalmente y se vio que su producción de licopeno era un 37% más elevada que la de la cepa silvestre (Klein-Marcuschamer *et al*., 2007), sugiriendo que otros procesos además de la síntesis de precursores podrían estar afectando la producción de licopeno.

Shotgun. Genes que producían un aumento en la producción de licopeno fueron identificados gracias al cribado colorimétrico que se realizó en una librería de clones construída a partir de ADN cromosómico de *E. coli* (Kang *et al*., 2005). La co-expresión del principal enzima regulador de la vía del MEP, DXS, con los tres genes que fueron identificados, *crl, rpoS* y *appY* demostraron aumentar la acumulación de licopeno. Sorprendentemente, los genes aislados estaban relacionados con la limitaciones energéticas que podría sufrir la célula y la protección de ésta ante la hidrofobicidad del licopeno; *appY*, que demostró ser un activador transcripcional del metabolismo energético de los operones y *crl,* regulador transcripcional asociado con la síntesis de fibras en las superficies celulares. El gen *rpoS* codifica para un factor sigma S que estimula la transcripción de los genes que se expresan durante la fase estacionaria o relacionados con las respuestas a estrés (Kang *et al*., 2005), apoyando de nuevo la consideración de factores energéticos o de almacenamiento a la hora de intentar optimizar la producción de carotenoides.

Disponibilidad de fuentes de carbono. En otras estrategias se consideró la disponibilidad de fuente de carbono a la hora de mejorar la producción de precursores. El gen codificante para IDI fue expresado bajo el control del promotor glnAp2 que responde a un incremento de los niveles de carbono típico de la fase estacionaria (Farmer y Liao, 2000). En estas condiciones se vio que las cepas producían mayores niveles de licopeno que las cepas control probablemente debido no sólo a la mayor disponiblidad de precursores sino también a las características del crecimiento en fase estacionaria que presentaban las bacterias. Estos datos concuerdan con lo observado por Yuan y colaboradores, donde conseguían incrementar la producción de licopeno al añadir glicerol a los cultivos celulares (Yuan *et al*., 2006).

1.3.2 Capacidad de almacenamiento.

Se cree que los carotenoides, una vez sintetizados en *E. coli*, se acumulan en las membranas celulares dada su hidrofobicidad. Niveles elevados de estos pigmentos pueden llegar a resultar tóxicos para las células llegando a inhibir incluso su crecimiento celular

(Rodriguez-Villalon *et al*., 2008; Kim y Keasling, 2001). Se cree que esto es debido a la ausencia de estructuras presentes en células de organismos fotosintéticos como las plantas, donde aparecen unidos a proteínas del aparato fotosintético (en el caso de los cloroplastos) o lipoproteínas (en el caso de cromoplastos). En algunos estudios se ha demostrado que la adición al medio de cultivo de un detergente (Tween 80, SDS) ayuda a prevenir la aparición de agregados y por tanto mejora el crecimiento bacteriano bajo unas condiciones de sobreproducción de licopeno (Yoon *et al*., 2006). Sin embargo, poco más se sabe acerca de cómo se produce el almacenamiento de estos pigmentos o cómo se puede mejorar en organismos modificados genéticamente para la producción de pigmentos.

Futuras aproximaciones para una producción de licopeno a gran escala requerirán modificar la capacidad de almacenamiento de *E. coli* o bien dotar a las bacterias de proteínas de unión a carotenoides solubles como las presentes en bacterias fotosintéticas (Wu y Krogmann, 1997).

En resumen podríamos decir que, pese a que muchos estudios demostraron con éxito un incremento en la producción de carotenoides aumentado el aporte de IPP y DMAPP, otros factores limitantes como las condiciones energéticas y de almacenamiento celulares, además de un preciso equilibrio entre los niveles de expresión de genes heterólogos y el acúmulo de intermediarios tóxicos, han de tenerse en cuenta a la hora de unificar todas estas aproximaciones en un único sistema que permita una producción eficiente de diferentes isoprenoides en microorganismos.

1.4. Mecanismos moleculares reguladores de la biosíntesis de carotenoides en plantas.

En las últimas décadas se han realizado numerosas aproximaciones biotecnológicas con el fin de mejorar el contenido de carotenoides en plantas consiguiéndose éxitos importantes en varios sistemas (Botella-Pavia *et al.*, 2004; DellaPenna y Pogson, 2006; Sandmann *et al.*, 2006; Giuliano *et al.*, 2008). Pese a los grandes avances que se han realizado en este área, aún es necesario mejorar nuestro limitado conocimiento sobre la regulación de la síntesis de carotenoides y de sus precursores en diferentes tejidos y órganos de las plantas, así como su interacción con otras vías de síntesis de isoprenoides. Los carotenoides se encuentran presentes en todo tipo de plastos, aunque éstos difieren en las cantidades de pigmentos que presentan y la función que en ellos desempeñan. Grandes cantidades de carotenoides pueden ser encontradas en cromoplastos de flores y frutos, donde proporcionan colores y aromas que ayudan a la atracción de animales para su polinización y dispersión de las semillas. Asimismo, los carotenoides se encuentran en niveles elevados en cloroplastos, donde se encuentran asociados a las membranas tilacoidales protegiendo a las clorofilas del daño producido por radicales libres generados por un exceso de luz (Niyogi, 1999). Pero también podemos encontrarlos en cantidades mucho menores en etioplastos presentes en plántulas crecidas en la oscuridad (Park *et al*., 2002) y leucoplastos (plastos de tejdos no fotosintéticos), donde su función está menos clara. Aunque se conocen los

principales mecanismos que regulan la síntesis de carotenoides y de sus precursores en cloroplastos y cromoplastos, donde la luz desempeña un papel fundamental en su regulación, poco se sabe acerca de la regulación en otros plastos de tejidos no fotosintéticos.

1.4.1 Control del aporte de precursores por la ruta MEP

La síntesis de carotenoides se abastece en la mayoría de plastos a partir de los precursores generados por la vía del MEP. Los dos primeros enzimas de la ruta del MEP, DXS y DXR, han sido objeto de numerosos estudios como potenciales enzimas reguladores del flujo metabólico en la producción de IPP y DMAPP plastídicos (Estevez *et al*., 2001; Botella-Pavia *et al*., 2004; Enfissi *et al*., 2005; Carretero-Paulet *et al*., 2006). En *Arabidopsis* se ha mostrado que la sobreexpresión de *DXS* o la disminución de su actividad se acompaña de cambios correlativos en los niveles de productos isoprenoides derivados de la ruta del MEP como clorofilas y carotenoides (Estevez *et al*., 2001; Botella-Pavia *et al*., 2004; Carretero-Paulet *et al*., 2006). Del mismo modo, en frutos de tomate transgénico, la sobreexpresión de un enzima DXS bacteriano genera incrementos significativos en la producción de carotenoides (Enfissi *et al*., 2005). Además del efecto descrito en cloroplastos de *Arabidopsis* y cromoplastos de tomate, el papel clave de DXS en el control del flujo metabólico hacia isoprenoides se ha demostrado también en otros tipos de plastos no fotosintéticos (Morris *et al*., 2006; Munoz-Bertomeu *et al*., 2006).

En plantas parece que la gran acumulación de carotenoides que tiene lugar durante la maduración del fruto de tomate no se correlaciona positivamente con una inducción del gen *DXR* ni de un aumento en los niveles proteicos (Rodriguez-Concepcion *et al*., 2001). De manera opuesta a *DXS,* la expresión de *DXR* en flores de *Anthirrhinum majus* no muestra el patrón de oscilación diurna en la biosíntesis y emisión de isoprenoides volátiles derivados de la ruta del MEP (Dudareva *et al*., 2005). Aunque existen otros ejemplos de la falta de correlación entre la expresión de *DXR* y los patrones de acumulación de productos isoprenoides plastídicos finales derivados del MEP, también se ha descrito una correlación positiva entre incrementos de biosíntesis de isoprenoides y acumulación de tránscritos codificantes para *DXR* en plántulas de *Arabidopsis* durante la desetiolación (Carretero-Paulet *et al*., 2002; Carretero-Paulet *et al*., 2006) y en raíces micorrizadas de monocotiledóneas (Walter *et al*., 2002; Hans *et al*., 2004). Es más, plantas sobreexpresoras de *DXR* presentaban una acumulación mayor de carotenoides y clorofilas en Arabidopsis (Carretero-Paulet *et al*., 2006) y de monoterpenos en menta (Mahmoud y Croteau, 2001). Asimismo, la regulación negativa de la actividad DXR se correlaciona con una baja acumulación de productos finales (Mahmoud y Croteau, 2001; Carretero-Paulet *et al.*, 2006), sugiriendo que la producción de MEP catalizada por DXR podría controlar, junto con DXS, la biosíntesis de isoprenoides plastídicos al menos en algunas plantas.

Los dos pasos finales de la ruta del MEP también se han propuesto como puntos de control potenciales. La forma oxidada $(4Fe\text{-}4S)^{+1}$ de las dos enzimas puede ser reducida *in vitro*

por un sistema flavodoxina/flavodoxina NADPH/reductasa (probablemente el sistema reductor *in vivo* en *E. coli*) o dezaflavina foto-reducida. En plantas se ha mostrado que ambas son enzimas blanco de la tiorredoxina (Balmer y Schurmann, 2001; Lemaire *et al.*, 2004), un miembro del sistema ferredoxina/tiorredoxina que es reducido en cloroplastos fotosintéticamente activos para regular positivamente la actividad de las proteínas blanco a través de la reducción de grupos disulfuro específicos (Buchanan *et al*., 2002). Además, recientemente se ha observado que el flujo electrónico de la fotosíntesis puede proveer directamente los electrones requeridos para la actividad de HDS en los cloroplastos vía ferredoxina en ausencia de cofactores reductores (Seemann *et al*., 2006). Basándose en la regulación post-transcripcional de los niveles de este enzima en los plastos (Gil *et al.*, 2005; Sauret-Gueto *et al.*, 2006a; Oudin *et al.*, 2007), su participación en mecanismos de defensa (Gil *et al*., 2005) y en el control dependiente de la fotosíntesis de su actividad enzimática en las células vegetales (Seemann *et al*., 2006) se propuso un papel para este enzima en la regulación del flujo de la vía MEP. Sin embargo, el análisis de la expresión de *HDS* durante la maduración del fruto del tomate no muestra una mayor acumulación de tránscritos durante la inducción de la biosíntesis de carotenoides sugiriendo que la actividad HDS podría no ser limitante en la producción de los precursores isoprenoides (Rodriguez-Concepcion *et al*., 2003). De acuerdo con esta posibilidad, la sobreexpresión de HDS en Arabidopsis no provocó una mayor producción de clorofilas y carotenoides (Flores-Perez *et al*., 2008a). La sobreexpresión del gen codificante para HDS tampoco tiene un efecto positivo en la acumulación de carotenoides en *E. coli* (Yuan *et al*., 2006; Flores-Perez *et al*., 2008a). A diferencia de HDS, la expresión del gen codificante para HDR, el enzima que cataliza el último paso enzimático de la ruta del MEP, se induce significativamente durante la maduración del tomate pero también durante la desetiolación de plántulas de *Arabidopsis*, de manera similar al fenómeno descrito para el gen codificante de DXS (Lois *et al*., 2000; Botella-Pavia *et al*., 2004). Estudios de sobreexpresión de isoformas de HDR bacterianas (*Synechocystis*) y vegetal (*Adonis aestivalis*) han mostrado que la actividad del enzima es limitante para la producción de isoprenoides en *E. coli* (Cunningham *et al*., 2000). En *Arabidopsis*, la sobreexpresión de HDR en plantas transgénicas también genera niveles elevados de isoprenoides derivados de MEP como carotenoides (Botella-Pavia *et al*., 2004). Además plantas transgénicas dobles para la sobreexpresión de DXS, DXR o HDR y de taxadieno sintasa (TXS), un enzima de tejo (*Taxus baccata*) que convierte directamente GGPP en taxadieno, acumulan mayores niveles de este isoprenoide no nativo de *Arabidopsis* comparados con los de plantas transgénicas sobreexpresoras únicamente de TXS (Botella-Pavia *et al*., 2004; Carretero-Paulet *et al*., 2006). Estos resultados confirman que los mayores niveles de isoprenoides plastídicos observados en plantes sobreexpresoras de estas enzimas de la ruta del MEP derivan del aumento en la producción de sus precursores. En resumen, DXS, DXR y HDR (pero no HDS) tendrían un papel en la regulación del flujo metabólico de la ruta del MEP.

En cuanto a la regulación de los niveles de enzimas de la vía del MEP, la mayor parte de los estudios se han centrado en analizar los mecanismos moleculares de la regulación de DXS y DXR, observándose que ésta tiene lugar tanto a nivel transcripcional como a nivel post-transcripcional en respuesta a la alteración del metabolismo de isoprenoides, el ambiente y señales del desarrollo (Rodríguez-Concepción *et al*., 2006; Córdoba *et al*., 2009). A nivel transcripcional se ha visto que la luz es uno de los factores más importantes en la regulación positiva de los enzimas de la ruta del MEP. Aumentos en los niveles de tránscritos de todos los genes de la vía tenían lugar en plántulas de *Arabidopsis* durante el proceso de desetiolación (Botella-Pavia *et al*., 2004; Córdoba *et al*., 2009) y en el desarrollo de las primeras hojas verdaderas (Carretero-Paulet *et al*., 2002; Guevara-Garcia *et al*., 2005). Del mismo modo se vio que la luz tenía una importancia fundamental en el control del metabolismo fotosintético y en la consiguiente disponibilidad de sustratos de la ruta del MEP (piruvato y gliceraldehído-3-fosfato), que también podrían contribuir al incremento en la producción de IPP y DMAPP en los cloroplastos. Una regulación positiva de los niveles de tránscritos de todos los genes de la vía MEP por la luz confiere una ventaja importante para el desarrollo de la plántula, durante el cual se producirá una fuerte demanda de pigmentos fotosintéticos derivados de esta vía. No obstante, esta regulación positiva ejercida por la luz no parece un mecanismo universal entre todas las especies de plantas. Es el caso del árbol del caucho, donde la expresión de DXR no se veía inducida por luz (Seetang-Nun *et al*., 2008). En *Arabidopsis* se ha observado la existencia de una oscilación diurna para todos los genes de la vía del MEP, observándose la misma fluctuación en los niveles de tránscritos para todos los genes de la vía, que presentan la mayor inducción justo antes del amanecer (Hsieh y Goodman, 2005; Cordoba *et al.*, 2009). No obstante, análisis más profundos deben llevarse a cabo para finalmente comprender la regulación ejercida sobre la vía del MEP a manos del reloj circadiano. Por último, la luz podría regular negativamente el intercambio de prenil difosfatos entre los plastos y el citoplasma en plántulas de *Arabidopsis* (Rodriguez-Concepcion *et al*., 2004). Sin embargo, otros factores como disponibilidad de azúcares (Cordoba *et al*., 2009), heridas mecánicas o la interacción con hongos podrían regular la acumulación transcripcional de DXS, DXR y HDR (Phillips *et al*., 2007).

La regulación post-transcripcional de los principales enzimas de la vía del MEP es especialmente relevante ya que se ha descrito que cambios en la expresión génica no siempre están correlacionados con cambios similares en los niveles proteicos, más aún en las actividades enzimáticas o en la producción de metabolitos. Se ha postulado que su acumulación post-transcripcional podría estar regulada por cambios en los niveles de los metabolitos de la ruta. Experimentos de aportación de 1-deoxi-D-xilulosa (DX) concuerdan con la idea de que niveles alterados del intermediario DXP podrían ser los responsables de una regulación por retroalimentación de la expresión de DXS en tomate (Lois *et al*., 2000; Rodriguez-Concepcion *et al*., 2001) y de la actividad de DXS en hojas de *Eucalyptus globulus* (Wolfertz *et al*., 2004). También ha sido descrita una acumulación en los niveles proteicos de DXS al inhibirse farmacológica o genéticamente la conversión de DXP en MEP (Guevara-

Garcia *et al*., 2005). La acumulación post-transcripcional de DXS podría ser resultado de una acumulación de DXP o de una disminución en los niveles de prenil difosfatos derivados del MEP. Apoyando la segunda posibilidad, se ha observado que durante las primeras etapas del desarrollo de plántulas de *Arabidopsis*, en las que hay una fuerte demanda de precursores para la síntesis de isoprenoides plastídicos implicados en el metabolismo fotosintético (como clorofilas y carotenoides), se acumulan altos niveles de proteína DXS sin cambios a nivel de expresión génica (Guevara-Garcia *et al*., 2005). Asimismo, estudios recientes con mutantes de *Arabidopsis* (*rif)* resistentes a la inhibición por fosfomidomicina (un inhibidor del enzima DXR) han demostrado la importancia de las señales plastídicas en la acumulación y degradación proteica de los enzimas reguladores de flujo de la ruta del MEP: DXS, DXR y HDR (Sauret-Gueto *et al.*, 2006a; Flores-Perez *et al.*, 2008b). En concreto, el estudio del mutante *rif1* permitió establecer el papel de una proteasa plastídica estromal, Clp (Koussevitzky *et al*., 2007) en la acumulación post-transcripcional de DXS y DXR en cloroplastos (Flores-Perez *et al*., 2008b). La degradación proteica mediada por la proteasa Clp se ha sugerido como un mecanismo de regulación de los nieveles proteicos de los principales enzimas de la vía MEP probablemente para ajustar la biosíntesis de isoprenoides al estado fisiológico y funcional de los cloroplastos (Flores-Perez *et al*., 2008b). Sin embargo, poco más se sabe acerca de la regulación de estos enzimas en tejidos no fotosintéticos.

4.2 Regulación de la ruta biosintética

Los precursores de naturaleza isoprenoide son canalizados hacia la biosíntesis de carotenoides por el enzima fitoeno sintasa (PSY). El primer enzima de la ruta carotenogénica ha sido propuesto como enzima clave en la regulación del flujo metabólico hacia carotenoides tanto en cloroplastos como en cromoplastos (Hirschberg, 2001; Giuliano *et al*., 2008). Se ha descrito que la biogénesis de los cromoplastos es un proceso asociado a la deposición de pigmentos y con una inducción de los principales enzimas carotenogénicos. Durante el proceso de maduración del fruto de tomate se ha visto como un descenso de los niveles de clorofilas y un progresivo aumento de licopeno se correlacionaba positivamente con un aumento en los niveles de transcritos de *PSY1* y *PDS* (Giuliano *et al*., 1993; Fraser *et al*., 1994; Corona *et al*., 1996; Lois *et al*., 2000). El papel esencial que desempeña la luz en la regulación transcripcional del enzima PSY se vio en plántulas de *Arabidopsis* y en maíz durante el proceso de deseetiolación. Durante este proceso, la explosión en la producción de clorofilas y carotenoides que tiene lugar se correlacionó positivamente con un incremento en los tránscritos de *PSY* (von Lintig *et al*., 1997; Botella-Pavia *et al*., 2004). El efecto que ejerce la luz, su naturaleza e intensidad en la regulación de la carotenogénesis, el papel de los fitocromos (Alba *et al*., 2005), criptocromos (Giliberto *et al*., 2005) y componentes en la mediación de la señal (Liu *et al*., 2004; Davuluri *et al*., 2005) han sido profundamente estudiados. Tratamientos con luz roja en frutos de tomate provocaban un aumento en el licopeno acumulado. En este proceso estaban implicados los fotocromos (receptores de luz roja) cuya expresión se inducía progresivamente

durante la maduración del fruto y de forma coordinada con la acumulación de licopeno (Alba *et al*., 2005), del mismo modo que se había observado en *Arabidopsis,* donde los fitorcromos mediaban la acumulación de carotenoides dependiente de luz roja mediante su regulación a nivel de PSY (von Lintig *et al*., 1997). Por otra parte, otros regímenes de luz, como la luz azul, demostraron inducir la acumulación de carotenoides en frutos de tomate y plántulas durante la desetiolación. Es más, la sobreexpresión del criptocromo (receptor de la luz azul) CRY2 provoca un aumento en la producción del licopeno consecuencia de una disminución en la expresión de Le-LCY-β (Giliberto *et al*., 2005). Del mismo modo, alteraciones en los mecanismos implicados en el control del desarrollo fotomorfogénico por luz se correlacionaron con cambios en los niveles de carotenoides. Líneas transgénicas de RNAi interferente en plantas de tomate para los genes LeHY5 y LeCOP1LIKE presentaban alteraciones en los niveles de carotenoides acumulados en el fruto. Una disminución de la actividad de HY5, regulador positivo de la transducción de la señal por luz que actúa después de los fotorreceptores, conllevaba una disminución de la producción total de carotenoides (Liu *et al*., 2004). Un descenso de la actividad de COP1, que es un represor de la fotomorfogénesis que interacciona con HY5 causando su degradación, provocaba un aumento de hasta el 40% de los niveles de pigmentos (Liu *et al*., 2004). No obstante, pese al papel clave que tiene la luz en la regulación de la carotenogénesis, se desconocen los factores de transcripción implicados directamente en la regulación de PSY mediada por luz. Recientemente en nuestro laboratorio ha sido descrita la unión directa de un factor de transcripción que interacciona con los fitocromos (PIF1) con el promotor del gen *PSY* (Toledo-Ortiz *et al*., 2010). Cuando éste se encuentra activo (en la oscuridad) provoca una represión en la expresión de *PSY* que desaparece al degradarse PIF1 en presencia de luz. Otro factor de transcripción capaz de unirse al promotor de *PSY* es RAP2.2 (Welsch *et al*., 2007). Sin embargo, su implicación en la regulación de la carotenogénesis no ha podido ser claramente establecida.

Aunque el control a nivel transcripcional de *PSY* es un mecanismo clave en la regulación de la carotenogénesis, mecanismos post-transcripcionales han sido descritos para regular los niveles proteicos de este enzima. Mientras que formas inactivas de los enzimas PSY y PDS fueron detectadas en la fracción soluble de cromoplastos de *Narcissus*, su activación tan sólo ocurrría cuando éstas se unían a las membranas de los cromoplastos (Al-Babili *et al*., 1996). Este mecanismo se vio que estaba asociado al estado redox de los aceptores de electrones de la membrana en el caso concreto de PDS en cromoplastos de dadófila (Fraser y Bramley, 2004). En etioplastos, se sabe que los carotenoides desempeñan un papel esencial en el mantenimiento de la estructura del cuerpo prolamelar (PLB), que con la percepción de la luz dará lugar a los tilacoides de los cloroplastos (Park *et al*., 2002). Welsch y co-trabajadores demostraron que durante el proceso de desetiolación se producía un incremento de los niveles proteicos de PSY y consecuentemente de su actividad enzimática, que requería de la relocalización del enzima en la membrana tilacoidal de los cloroplastos. Sin embargo, en este mismo estudio observaron que bajo determinados regímenes de luz, un aumento de PSY tanto a nivel transcripcional como proteica no se correlacionaba

positivamente con un aumento en su actividad, ya que el enzima permanecía unido al PLB de forma enzimáticamente inactiva (Welsch *et al*., 2000; Welsch *et al*., 2003). Sin embargo, poco más se sabe acerca de la regulación de este enzima en plántulas crecidas en la oscuridad y la regulación de sus precursores. En cuanto a la regulación del enzima PSY en leucoplastos, la mayor parte de los estudios han sido realizados en gramíneas. A diferencia de *Arabidopsis thaliana*, que presenta un único gen, en maíz se ha visto que PSY está codificada por tres isogenes con una subfuncionalidad específica que les permite un control preciso de la carotenogénesis bajo diferentes estímulos externos y de desarrollo (Li *et al*., 2009). Tránscritos de *PSY3* fueron encontrados principalmente en raíces de maíz a diferencia de las otras dos isoformas, las cuales se localizaban principalmente en el endospermo (PSY1) y en los tejidos fotosintéticos (PSY2) (Li *et al*., 2008a; Li *et al*., 2008b). Éstos autores demostraban una inducción a nivel transcripcional de *PSY3* únicamente en raíces bajo condiciones de sequía, salinidad y la aplicación de ABA exógeno. El reciente descubrimiento de la isoforma PSY3 en raíces de arroz implicada en la biosíntesis de ABA bajo diferentes condiciones de estrés abiótico (Welsch *et al*., 2008) refuerza la idea de una especialización de las distintas isoformas de PSY en gramíneas, sin embargo, poco se sabe acerca de los mecanismos que regulan dicha inducción ni qué ocurre con el único gen codificante para PSY en *Arabidopsis* en estos tejidos no fotosintéticos.

En cuanto a la regulación de los restantes genes biosintéticos de la ruta se desconoce qué factores están implicados. A diferencia de *PSY* y *PDS*, se sabe que la regulación transcripcional de los restantes genes de la vía no es dependiente de luz en frutos de tomate (Fraser y Bramley, 2004; Lu y Li, 2008). Se observó que el proceso de maduración iba acompañado de una disminución de los tránscritos, de proteína y de actividad de los enzimas LCY-β y LCY-ε. La acumulación de licopeno en frutos de tomate maduro conlleva un bloqueo en la formación de productos finales de la vía con la consiguiente represión de los genes biosintéticos correspondientes, mecanismo compartido por el fruto de pimiento también (Hirschberg, 2001). Por otra parte, durante el desarrollo de la flor se observó una correlación entre el aumento de carotenoides y una inducción transcripcional de la mayoría de los genes biosintéticos (Al-Babili *et al*., 1996; Moehs *et al*., 2001).

Por otra parte, la degradación oxidativa de los carotenoides por los enzimas CCD no sólo produce apocarotenoides para la formación de importantes hormonas si no que también tiene la finalidad de regular los niveles de carotenoides en plantas. Cambios en la expresión de varios genes codificantes para enzimas CCD correlacionaban inversamente con la acumulación de estos pigmentos en pétalos de crisantemo (Ohmiya *et al*., 2006). Este proceso se observó que estaba regulado por el estrés ambiental y por el reloj circadiano (Simkin *et al*., 2004).

Mecanismos de retroalimentación también han sido descritos para la regulación de algunos enzimas de la vía carotenogénica. Inhibición de la ciclación del licopeno en hojas de tomate provocaba un aumento en la expresión de los genes *PSY1* y *PDS* (Giuliano *et al*., 1993; Corona *et al*., 1996). Estudios con inhibidores de la carotenogénesis apoyaron estos resultados donde los tejidos tratados acumulaban mayor cantidad de carotenoides que los tejidos control

(Bramley, 1994). Otros mecanismos como la biogénesis de los plastos (Cookson *et al*., 2003; Liu *et al*., 2004) o la acción del ABA han estado también implicados en la regulación de la carotenogénesis en frutos de tomate, aunque mutantes *aba* en *Arabidopsis* no mostraron un aumento en la producción de carotenoides (Fraser *et al*., 1999).

Otro mecanismo propuesto recientemente en la regulación de la acumulación de carotenoides es el control en la biogénesis de los plastos. Trabajos realizados con mutantes de tomate *high pigment* (*hp1*, *hp2* y *hp3*) demostraron que un aumento en el número y tamaño de los plastos provocaba un incremento en la acumulación de licopeno (Liu *et al*., 2004; Kolotilin *et al*., 2007). Del mismo modo, tomates con una mutación en el gen *ZEP* presentaban niveles elevados de licopeno debido a un aumento en el tamaño de los plastos y de su capacidad de almacenamiento (Galpaz *et al*., 2008). Un aumento en la deposición de carotenides debido a un desarrollo alterado de los plastos se encontró también al estudiar el mutante en tomate para HSP21, que además de fotoproteger el fotosistema II promueve la conversión de los cloroplastos en cromoplastos (Neta-Sharir *et al.*, 2005) igual que el gen *Or* procedente de coliflor, que expresado en un sistema heterólogo promueve la transformación de proplastos en cromoplastos con la consecuente acumulación de carotenoides (Lu *et al.*, 2006; Lopez *et al.*, 2008). La biosíntesis de los carotenoides parece estar regulada también por el secuestro de los productos finales que difiere notablemente entre cloroplastos y cromoplastos. En el primer caso, los carotenoides se localizan en las membranas fotosintéticas y se integran con las proteínas de unión a clorofilas formando complejos pigmento-proteína, mientras que en los cromoplastos los carotenoides se asocian con lípidos polares y proteínas, formando lipoproteína-carotenoides, estructuras que han demostrado ser muy efectivas en la retención de grandes cantidades de pigmentos (Deruere *et al.*, 1994; Vishnevetsky *et al.*, 1999b). En concreto se sabe que más del 80% de estas proteínas en pimiento son fibrilinas, observándose una relación directa entre la presencia de estas proteínas y el desarrollo de los cromoplastos con la consecuente acumulación de carotenoides (Leitner-Dagan *et al.*, 2006; Simkin *et al.*, 2007). En colza (Shewmaker *et al*., 1999) y en tomate (Romer *et al*., 2000) se vio que la ultraestructura de los plastos variaba en respuesta al contenido en carotenoides. Se cree que la formación de estructuras secuestradoras de carotenoides puede prevenir la retroalimentación negativa observada en la ruta biosintética por los productos finales (Lu y Li, 2008).

Objetivos

Objetivos

El principal objetivo de este trabajo de tesis ha sido profundizar en nuestro conocimiento de la carotenogénesis en bacterias (con un enfoque biotecnológico) y plantas (centrándose en tejidos no fotosintéticos). Para ello se desarrollaron los siguientes apartados:

1. Ensayar nuevas estrategias biotecnológicas para una mayor acumulación de carotenoides en *E. coli*.
    - 1.1 Aumentar el aporte de precursores mediante la modificación de vías endógenas y exógenas.
    - 1.2 Mejorar la capacidad de almacenamiento mediante la expresión de genes de cianobacterias.

2. Estudiar la síntesis de carotenoides en tejidos no fotosintéticos de *Arabidopsis*.
    - 2.1 Identificar factores que controlan la producción de carotenoides en etioplastos de plántulas crecidas en la oscuridad.
    - 2.2 Analizar la distribución de la expresión del gen codificante para PSY y de la síntesis de carotenoides en la planta, con especial atención a la raíz.

Resumen global

Resumen global: resultados y discusión

El presente trabajo de investigación doctoral se ha centrado en el estudio de algunos pasos críticos en la regulación de la biosíntesis de carotenoides en sistemas bacterianos y en tejidos no fotosintéticos de *Arabidopsis thaliana*. Para ello se han llevado a cabo dos estrategias independientes que serán expuestas y discutidas separadamente a continuación: (1) estudios de algunos factores limitantes en la producción de carotenoides en huéspedes no carotenogénicos como *Escherichia coli* (estudio de la influencia del aporte de precursores y la capacidad de almacenamiento), y (2) estudio de la carotenogénesis en tejidos no fotosintéticos de *Arabidopsis thaliana* (estudio de los mecanismos reguladores de la biosíntesis de carotenoides en etioplastos de plántulas germinadas en la oscuridad y leucoplastos de raíz).

3.1 Nuevas estrategias biotecnológicas para una mayor acumulación de carotenoides en *E. coli*.

3.1.1 Aumento del aporte de precursores mediante la modificación de vías endógenas y sintéticas.

Aunque *E. coli* puede ser modificada genéticamente para la producción de carotenoides, tan sólo dispone de pequeñas cantidades de precursores para la síntesis de isoprenoides que puedan ser utilizados para producir carotenoides. El IPP es una molécula esencial involucrada en la síntesis de quinonas y hopanoides (reguladores de las propiedades de las membranas) y bactoprenol (necesario para la producción de peptidoglicano de la pared celular). En varios trabajos se ha reportado un aumento en la producción de carotenoides al aumentar la cantidad de IPP y DMAPP disponible en huéspedes no carotenogénicos modificados biotecnológicamente mediante la sobreexpresión de DXS, el principal enzima regulador de la ruta MEP (Harker y Bramley, 1999; Matthews y Wurtzel, 2000). Sin embargo, el papel de otros enzimas de la ruta en el control del flujo metabólico de la misma ha sido poco explorado.

Con este objetivo se propuso sobreexpresar cada uno de los genes codificantes para los diferentes enzimas de la vía MEP utilizando las células EcAB4-1(Sauret-Gueto *et al*., 2003) como ejemplo de organismo bacteriano no carotenogénico. Estas células (fondo genético K12 MG1655) presentan en su genoma un operón sintético que contiene todos los genes de la vía del MVA necesarios para la conversión de esta molécula en IPP y DMAPP bajo el control de un promotor inducible por arabinosa lo cual permite un elevado control del nivel transcripcional de sus enzimas (Campos *et al*., 2001). A continuación los genes que codifican para los enzimas individuales de la vía del MEP, DXS, DXR, MCT, CMK, MDS, HDS y HDR (Figura 2), fueron clonados en el plásmido pQE-30. Este vector presenta un gen que confiere resistencia a ampicilina, un epítopo de 6 Histidinas localizado en N-terminal y un promotor inducible por IPTG. Para comprobar que la sobreexpresión de los diferentes genes producía una proteína funcional, células deficientes para genes individuales de la ruta del MEP fueron transformadas

con los plásmidos que contenían el gen del cual carecían. Dichas cepas presentan una disrupción en el gen codificante para los enzimas, de modo que sólo serán viables mediante la expresión de una proteína funcional. A diferencia de las células control (células transformadas con el plásmido pQE-30 vacío) las células transformadas con el plásmido correspondiente presentaban un crecimiento normal en placa, lo cual demostraba la funcionalidad de los distintos enzimas recombinantes. Con el objetivo de evaluar su papel en la producción de carotenoides, los plásmidos generados se usaron para transformar células *E. coli* EcAB4-1 conjuntamente con el plásmido pLYC (resistencia a cloramfenicol) (Rodriguez-Villalon *et al*., 2008) que contiene los genes procedentes de *Erwinia uredevora CrtE* (GGPP sintasa), *CrtB* (fitoeno sintasa) y *CrtI* (fitoeno desaturasa) los cuales conferían la capacidad de sintetizar licopeno a partir de IPP y DMAPP (Cunningham *et al*., 1993). Las células cotransformadas con pLYC y cada uno de los plásmidos con genes de la ruta MEP se seleccionaron en placas suplementadas con cloramfenicol y ampicilina. Tres colonias transformantes para cada uno de los enzimas individuales de la ruta del MEP (que presentaban una coloración rosada característica del licopeno) se emplearon para inocular cultivos líquidos de 3 ml que crecieron a 37ºC durante 15 horas. Al día siguiente, aquellos dos cultivos cuya acumulación de licopeno era mayor (coloración rosada más intensa) se seleccionaban para inocular de nuevo medios suplementados con los antiobióticos correspondientes con una concentración del 1% y en presencia o ausencia de IPTG (concentración final 0.5 mM) que se dejaron crecer durante 15 horas más.

En cada caso se realizaron dos réplicas biológicas para cada condición de cultivo. Pese a la gran variabilidad que se observaba en los cultivos líquidos de la misma cepa en cuanto a la producción de licopeno, se observaba como las células sobreexpresoras de DXS llegaban a acumular entre 5 y 8 veces más licopeno en ausencia de IPTG que las células control (transformadas con el plásmido pQE-30 vacío) crecidas exactamente en las mismas condiciones (Figura 6). La sobreexpresión del enzima DXR en ausencia de IPTG producía un ligero aunque significativo incremento en la producción de licopeno en comparación con la tasa de producción de las células control, lo mismo que ocurría en el caso de HDR (Figura 6). Sin embargo, pese a un estudio reciente donde se sugería un papel regulador para los enzimas MCT y MDS en el control del flujo metabólico de la vía del MEP (Yuan *et al*., 2006), en nuestro estudio observamos que la sobreexpresión de los restantes enzimas de la ruta no variaba la tasa de acumulación de licopeno. Todos estos datos confirman que DXS es el principal enzima regulador de la ruta MEP en cuanto al abastecimiento de isoprenoides para las células, lo cual ya había sido sugerido en trabajos anteriores. No obstante, de nuestros datos se desprende que el control del flujo podría estar compartido con otros enzimas (DXR y HDR) aunque de forma minoritaria, igual que había sido descrito en plantas (Botella-Pavia *et al*., 2004; Carretero-Paulet *et al*., 2006).

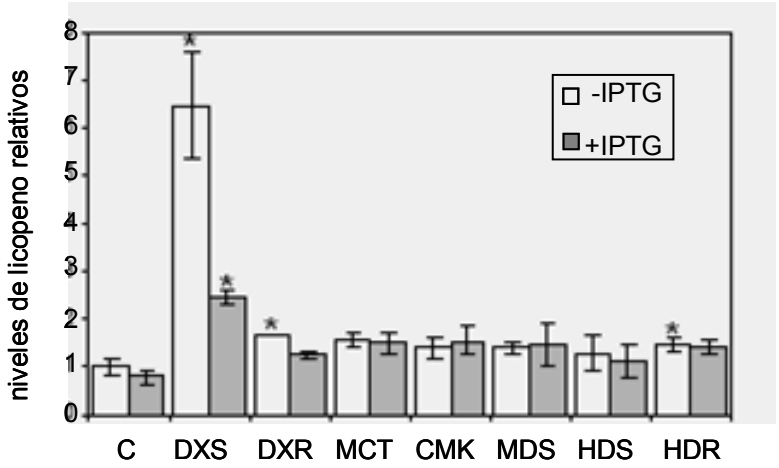


Figura 6. Niveles de producción de licopeno de células transformadas con plásmidos con los genes codificantes para los enzimas de la ruta del MEP. Los cultivos se realizaron en medio líquido (10 ml) en presencia de IPTG 0.5 mM (barras grises) o en ausencia de IPTG (barrras blancas) a 37ºC en agitación durante 15 horas. La producción de pigmentos se representa normalizada al crecimiento celular y relativa a los niveles de células control transformados con un plásmido pQE-30 vacío. Los asteriscos indican valores significativamente distintos (T-test) a los del control.

A diferencia de lo que ocurre en sistemas vegetales, en nuestro estudio no se observa una correlación positiva entre la sobreexpresión de estos enzimas y un incremento en la producción de carotenoides. El descenso en los niveles de licopeno que vemos cuando sobreexpresamos DXS y DXR al añadir al medio IPTG pueda deberse a la disminución que se produce en las células de gliceraldehído 3-fosfato (GAP), produciéndose una inhibición en el crecimiento celular. El GAP, al igual que el piruvato, además de tratarse de un sustrato de la vía del MEP, está implicado en numerosos procesos metabólicos celulares. Una disminución de la disponibilidad de piruvato y GAP como consecuencia de la sobreexpresión de *dxs* puede tener efectos deletéreos en el crecimiento celular, como ya había sido descrito anteriormente (Jones *et al*., 2000; Kim y Keasling, 2001). Esto unido a los requerimientos energéticos que supone la sobreexpresión de un enzima que puedan afectar al metabolismo de la célula puede explicar la falta de una producción de licopeno incrementada en nuestras células. El uso de vectores de baja copia inducibles en vez de copia mútiple permitiría una precisa expresión de los genes de la ruta sin causar ningún tipo de interferencia en el crecimiento (Kim y Keasling, 2001), obteniéndose un rendimiento mayor en la producción de licopeno. Una modulación en los niveles de expresión de los genes que codifican para estos enzimas implicaría una optimización en la producción de licopeno posiblemente.

Como estrategia alternativa para aumentar los niveles de precursores isoprenoides decidimos emplear la cepa EcAB4-1 (Sauret-Gueto *et al*., 2006b) tranformada con el vector pLYC, lo que nos permitía aumentar los niveles de IPP y DMAPP mediante la adición exógena al medio de MVA y estudiar el efecto de la disponibilidad de precursores en la síntesis de carotenoides. Siguiendo el mismo proceso experimental que en el caso anterior, se escogieron

aquellas tres colonias que presentaban la mayor coloración rosada para realizar los precultivos e inóculos celulares a paritr de los cuales se analizaría la producción final de licopeno. Como se esperaba, en presencia de MVA las células producían una mayor cantidad de licopeno que las células control (crecidas en ausencia de MVA) ya que al no ser éste producido por las células de forma endógena los enzimas del operón del MVA no debían de interferir negativamente con el metabolismo celular. Sin embargo, cantidades crecientes de arabinosa se correlacionaban negativamente con la producción de este pigmento. Este efecto negativo podía deberse bien a la interferencia del operón del MVA con el metabolismo celular o bien a los efectos tóxicos que podría tener el acúmulo de IPP al no poder ser canalizado para su conversión en licopeno, opción que ya había sido descrita anteriormente (Campos *et al*., 2001; Martin *et al*., 2003). Para distinguir entre ambas posibilidades se estudió la producción de licopeno a lo largo del crecimiento celular en presencia de dos cantidades de MVA (1 y 5 mM) a la concentración de arabinosa 0.1%, la cual no provocaba ningún tipo de inhibición en el crecimiento celular. A diferencia de las células control (crecidas en ausencia de MVA) que presentaban su máxima producción de licopeno en la fase exponencial, aquellas que crecían en presencia de MVA continuaban sintetizando licopeno hasta en la fase estacionaria de su crecimiento, observándose una ligera inhibición en el crecimiento celular cuando crecían en presencia de una concentración 5mM de MVA (Figura 4, Artículo 1). La correlación positiva observada entre la adición de MVA al medio y la acumulación de licopeno, además de la relación inversamente proporcional entre arbinosa y pigmento, sugerían que un exceso en los niveles de enzimas codificados por el operón podrían interferir negativamente con el metabolismo celular. También se podía deducir que un incremento en la producción de precursores podría ser eficientemente canalizado hacia la formación de productos finales. Con el propósito de analizar el nivel de acumulación máxima de licopeno que se podía alcanzar en las células EcAB4-1 durante su fase estacionaria, se añadieron cantidades crecientes de MVA (0-5-25-50 mM) al medio de cultivo, consiguiéndose con 25 mM de mVA una producción de licopeno 10 veces mayor que en ausencia de esta molécula (Figura 5, Artículo1), sugiriendo que la inhibición en el crecimiento celular observada no se debía a un exceso de IPP y DMAPP tóxicos para la célula. Sin embargo, este rendimiento no puede superarse incrementando la cantidad de MVA exógeno, ya que cuando la concentración de éste se duplicaba (50 mM) no se observaba una mejoría en la acumulación del pigmento. Estos datos sugerían la existencia de otros factores limitantes además del aporte de precursores en la producción de licopeno. Una posible explicación sería la carencia de sistemas de transporte para el MVA por parte de las células bacterianas, de modo que al entrar el MVA en la célula por simple difusión, no todo el producto añadido al medio podría ser metabolizado.

Dado que el fondo genético bacteriano puede afectar sustancialmente a la producción de carotenoides (Matthews y Wurtzel, 2000; Vadali *et al*., 2005; Yuan *et al*., 2006) el mismo planteamiento experimental se llevó a cabo en células de *E. coli* BL21 (DE3) a las que se les había introducido el operón del MVA por transducción con el fago P1, la cepa EcAM5-1 (Rodriguez-Villalon *et al*., 2008). En comparación con la producción de licopeno alcanzada por

la cepa EcAB4-1, el cambio de fondo genético provocaba una duplicación en los niveles alcanzados de pigmento (Figura 5, Artículo 1). Es posible que el fondo genético presente en las células EcAM5-1 sea más apropiado para la sobreexpresión de enzimas y proteínas recombinantes así como puede disponer de más recursos tanto energéticos como físicos (almacenamiento) para la síntesis de carotenoides. Todos estos datos sugieren que el empleo de una vía heteróloga como el operón del MVA puede incrementar la producción de licopeno a través de un incremento de los niveles de IPP y DMAPP. Estos datos concuerdan con datos ya publicados (Martin *et al*., 2003; Yoon *et al*., 2007), donde se demuestra la correlación positiva entre la incorporación de MVA y la producción de licopeno. El aumento de los niveles de sustrato de la propia vía del MVA, el acetil-CoA, bien mediante la inactivación de las vías de conversión del acetato en otros productos (Vadali *et al*., 2005) o bien mediante la introducción de una acetil-CoA sintasa (Shiba *et al*., 2007) se presenta como una estrategia útil para optimizar la producción de carotenoides, así como la combinación con la sobreexpresión del principal enzima regulador de la vía del MEP, DXS y la adición de una fuente de carbono (Yoon *et al*., 2007). Sin embargo, cómo mejorar la expresión de los genes heterólogos de las vías sintéticas o cómo reducir la interferencia con el metabolismo energético de las células son factores que aún hoy se desconocen.

Todos estos datos sugieren que, factores como un preciso balance en la expresión de proteínas recombinantes o la capacidad de almacenamiento han de tenerse presentes a la hora de optimizar la producción de carotenoides en bacterias modificadas genéticamente. Sin embargo, la importancia de los precursores IPP y DMAPP como factor limitante en la producción de carotenoides se confirma por los elevados niveles de licopeno producidos al introducir una vía heteróloga como el MVA o al sobreexpresar enzimas como DXS, DXR o HDR. Estos resultados se correlacionan positivamente con lo observado en plantas, donde la sobreexpresión de DXS, DXR y HDR producía un incremento en la producción de carotenoides (Carretero-Paulet *et al*., 2006; Botella-Pavia *et al*., 2004). Por otro lado, la falta de efecto de la sobreexpresión de otros genes de la ruta del MEP como *HDS* sobre la acumulación de carotenoides en *E. coli* (Figura 6) sugiere que estos enzimas tienen un papel relevante en el control del flujo metabólico de la vía. De acuerdo con esta posibilidad, la sobreexpresión de *HDS* en plantas de *Arabidopsis thaliana* no alteraba la síntesis de carotenoides (Flores-Perez *et al*., 2008a). Mi contribución a este trabajo (Anexo 1) consistió en la realización de las medidas de pigmentos en los cultivos bacterianos. La correlación positiva entre los datos obtenidos en *Arabidopsis* y células de *E. coli* validan el estudio de la carotenogénesis en sistemas bacterianos, un sistema mucho menos complejo que organismos superiores como las plantas.

3.1.2 Mejora de la capacidad de almacenamiento mediante la expresión de genes de cianobacterias.

Escherichia coli, al igual que otros huéspedes no carotenogénicos, ha sido modificada genéticamente para la producción de carotenoides. Sin embargo, la naturaleza hidrofóbica y la

baja solubilidad de los carotenoides en el entorno celular puede afectar a la integridad de las membranas bacterianas cuando la cantidad de pigmentos es muy elevada afectando a su tasa de crecimiento (Kim y Keasling, 2001). Posiblemente este efecto se deba a la ausencia de los mecanismos de almacenamiento presentes en las células fotosintéticas donde los carotenoides se acomplejan con proteínas asociadas a las membranas o en ambientes hidrofóbicos generalmente. En las cianobacterias carotenogénicas los carotenoides se encuentran también asociados a proteínas solubles que unen carotenoides, como Orange Carotenoid Protein (OCP), codificada por el gen *slr1963* de *Synechocystis PCC 6803* (Wu y Krogmann, 1997). Esta proteína desempeña un papel esencial de fotoprotección y como sensor de luz (Wilson *et al*., 2006; Wilson *et al*., 2008). Pese a ser su sustrato natural la 3-hidroxiquinononona en cianobacterias, su capacidad de unir otros carotenoides ha sido descrita recientemente (Kerfeld, 2004; Punginelli *et al*., 2009).

Con el propósito de intentar mejorar la capacidad de almacenamiento de carotenoides en células de *E. coli* modificadas genéticamente, se propuso la sobreexpresión de genes procedentes de la cianobacteria *Synechocystiys* y estudiar su efecto en la acumulación de carotenoides. La primera aproximación llevada a cabo fue la sobreexpresión de una proteína OCP recombinante en células de *E. coli* modificadas genéticamente para producir licopeno. Con tal propósito, el gen codificante para OCP fue clonado en un vector pET bajo un promotor inducible por IPTG y se le añadió una cola de histidinas en su extremo C-terminal (construcción pET-OCP). La transformación de células BL21 (DE3) permitió su expresión e inducción con IPTG y la posterior purficación de la proteína recombinante mediante una columna de afinidad iónica metálica (IMAC) de Ni^{+2}. Para testar su capacidad de unión a otros carotenoides se incubó 0.2 mg de proteína recombinante con exactamente la misma cantidad de β-caroteno y licopeno (Figura 3, Artículo 2). A diferencia de lo que ocurría con la proteína control, la albúmina de suero bovino (BSA), al cabo de 90 minutos a temperatura ambiente se observaba la formación de un precipitado con la coloración característica de cada pigmento, demostrando la capacidad de esta proteína de unir otros carotenoides. Para evaluar si la presencia de OCP podía mejorar la producción de carotenoides, decidimos emplear la cepa EcAM5-1, previamente descrita y co-transformarla con pET-OCP y pLYC. Sorprendentemente, la presencia de OCP no sólo no mejoraba la producción de licopeno en comparación con las células control (que carecían de plásmido pET-OCP), sino que se observaba una ligera disminución. A diferencia de lo que se observaba al sobreexpresar algunos enzimas de la vía del MEP, la sobreexpresión de OCP no afectaba al crecimiento bacteriano, probablemente porque no suponía el acúmulo de ningún intermediario tóxico para la célula (Kim y Keasling, 2001) o porque no suponía el secuestro de ningún sustrato limitante para la célula, como el GAP. Con el propósito de analizar si la presencia de OCP estaba afectando la síntesis de licopeno o bien su acumulación, decidimos incrementar la producción de licopeno mediante la adición de MVA. En concordancia con resultados anteriores (Yoon *et al*., 2006; Rodriguez-Villalon *et al*., 2008) la adición de una fuente exógeno de precursores como el MVA aumentaba la producción de licopeno. Sin embargo, en las células co-transformadas con pET-OCP los

niveles en la producción de licopeno alcanzados eran ligeramente inferiores que los de las células control (transformados únicamente con el plásmido pLYC). Estos resultados sugerían que la presencia de OCP no interfería con la síntesis de licopeno pero su presencia no mejoraba la acumulación de este pigmento lipofílico. Probablemente la presencia de OCP evite la unión del licopeno a las membranas celulares, siendo ésta unión de naturaleza más débil y por tanto, disminuyendo la cantidad de licopeno acumulado. En todo caso, nuestros datos muestran que la sobreexpresión de OCP no es la estrategia indicada para mejorar la capacidad de almacenamiento en células de *E. coli* modificadas genéticamente para la producción de carotenoides, y esfuerzos en otras direcciones, como la generaicón de estructuras semejantes a plastoglóbulos o el impedimento de la formación agregados celulares por la presencia de licopeno (Yoon *et al*., 2006) han de considerarse a la hora de optimizar la síntesis de carotenoides.

Una estrategia alternativa empleada para mejorar la capacidad de almacenamiento en *E. coli* fue la expresión de genes de la cianobacteria *Synechocystis* mediante el cribado de una genoteca generada con su genoma y clonada en pBluescript (Linden *et al*., 1993). Células DH5-α fueron co-transformadas con el plásmido pLYC y la genoteca, y aquellas que presentaban una coloración rosada más intensa fueron seleccionadas. Entre 120000 clones independientes, 400 presentaban una coloración rosada más intensa y éstas se crecieron en nuevas placas a fin de realizar una nueva selección. El análisis de la producción de licopeno de 85 colonias seleccionadas permitió aislar tres clones independientes que presentaban una producción de licopeno mayor que las células control (transformadas con el vector vacío) de manera reproducible y consistente. A fin de analizar el gen de cianobacteria que producía este incremento en la síntesis de carotenoides, los clones S82, S91 y S92 fueron secuenciados. Las secuencias presentes en los clones S82 y S91 eran idénticas y se correspondían con 257 pares de bases del gen *slr1779* que codifica para el enzima piridoxina 5-fosfato sintasa (PdxJ). El tercer clon positivo presentaba una secuencia de 104 pares de bases perteneciente al gen *slr0665* que codifica para la aconitato hidratasa B (acnB). En el primer caso especulamos que la presencia de este fragmento de cianobacteria pueda interferir con la expresión o actividad del gen endógeno *pdxJ* de *E. coli*. Este enzima cataliza la conversión de DXP en piridoxina 5-fosfato, un precursor de la vitamina B_6. La interferencia con la expresión o actividad del gen endógeno de *E. coli pdxJ* podría resultar en un aumento de los niveles de DXP. El carácter limitante del enzima DXS y de su producto de reacción DXP han sido puestos de manifiesto en esta tesis (Artículo 1) y en numerosos trabajos anteriores (Cunningham *et al*., 2000; Yuan *et al*., 2006). Por tanto, una mayor disponiblidad de DXP para la síntesis de pigmentos podría explicar el incremento observado en la síntesis de licopeno en los casos de S82 y S91. En cuanto al aumento de carotenoides observado con la expresión de la secuencia del gen codificante para aconitato hidratasa cianobacteriana, creemos que pueda deberse a una interferencia con el enzima AcnB de *E. coli*. Este enzima, que participa en el ciclo de los ácidos tricaboxílicos y en el ciclo del glioxilato catalizando la conversión de citrato en isocitrato, se sintetiza principalmente durante la fase exponencial, en donde se ha descrito que hay una mayor

producción de carotenoides. Una interferencia con la expresión o actividad del enzima endógeno podría aumentar la disponibilidad de piruvato hacia la vía del MEP, sustrato que ha demostrado ser limitante para la producción de carotenoides (Jones *et al*., 2000; Kim y Keasling, 2001). Es más, una canalización de piruvato hacia la vía del MEP ha demostrado ser un método eficaz para incrementar la síntesis de licopeno (Farmer y Liao, 2001; Vadali *et al*., 2005). Por otra parte, la secuencia de cianobacterias codificante pertenece al dominio 1 del enzima, que junto con el dominio 2, 3 y 4, forman el centro activo altamente conservado alrededor del grupo reducido (4Fe-4S) (Williams *et al*., 2002; Tang *et al*., 2005). AcnB desempeña un papel regulador: bajo condiciones de estrés el grupo reducido (4Fe-4S) desaparece, igual que el centro catalítico activo y la apoproteína se une a mRNA específicos para regular la transcripción o traducción (Kiley y Beinert, 2003; Tang *et al*., 2005). Los dos últimos enzimas biosintéticos de la vía del MEP son enzimas ferro-sulfurados (Rodríguez-Concepción *et al*., 2006), con lo que, un incremento de la actividad del enzima limitante HDR se podría dar al aumentar la disponibilidad del grupo reducido (4Fe-4S) produciéndose un aumento del flujo del MEP y por tanto, una mayor síntesis de licopeno (Cunningham *et al*., 2000). También es posible que la presencia de este fragmento de cianobacterias evite el ensamblaje de un grupo funcional reducido (4Fe-4S) resultando en una apoproteína AcnB que provocaría cambios a nivel transcripcional que podrían favorecer un aumento en la síntesis de licopeno.

Aunque las estrategias planteadas en este apartado no han tenido los resultados esperados y no han servido para mejorar el acúmulo de carotenoides en *E. coli*, no se puede descartar que la sobreexpresión de genes procedentes de cianobacterias pueda en un futuro funcionar para aumentar la capacidad de almacenamiento.

3.2 Síntesis de carotenoides en tejidos no fotosintéticos de *Arabidopsis.*

3.2.1 Factores que controlan la producción de carotenides en etioplastos de plántulas crecidas en la oscuridad.

En *Arabidopsis thaliana*, la biosíntesis de carotenoides está fuertemente activada cuando las plántulas pasan de germinar en la oscuridad (escotomorfogénesis) a percibir la luz (fotomorfogénesis). Además de cambios morfológicos en la planta, la fotomorfogéneis provoca la transformación de los etioplastos en cloroplastos y la transición a un metabolismo fotosintético en el que los carotenoides juegan un papel esencial como protectores frente al estrés fotooxidativo (Baroli y Niyogi 2000; Pogson y Rissler, 2000). Los carotenoides presentes en los etioplastos (luteína y violaxantina como componentes mayoritarios), se encuentran asociados al cuerpo prolamelar (PLB) y se cree que están involucrados en facilitar su ensamblaje y de esta manera facilitar el acúmulo de clorofilas después de la iluminación (Park *et al*., 2002). Experimentos realizados con norflurazón (NFZ) en plántulas crecidas en la ocuridad confirmaron que la biosíntesis de carotenoides ocurría “de novo” en los etioplastos. La coloración amarilla que presentan los cotiledones debido a la acumulación de carotenoides

desaparecía progresivamente al bloquear la ruta carotenogénica con cantidades crecientes de inhibidor (Figura 2, Artículo 3). Sin embargo, la regulación del proceso de la carotenogénesis en etioplastos era prácticamente desconocida al inicio de este trabajo. Con el próposito de estudiar los mecanismos que regulan la biosíntesis de carotenoides en la oscuridad decidimos incrementar la producción de carotenoides en ausencia de luz. Para ello decidimos inducir la fotomorfogénesis en plántulas crecidas en la oscuridad y ver si en ausencia de luz se producía igualmente un incremento en la tasa de producción de carotenoides. El mutante *cop1*-4 presenta una pérdida de función para COP1, un importante represor de la fotomorfogénesis (Wei y Deng, 1996). Asimismo algunas hormonas como las giberelinas (GA) están implicadas en la regulación de la fotomorfogénesis en *Arabidopsis*. El bloqueo de la actividad de estas hormonas con el inhibidor paclobutrazol (PAC) conlleva la aparición de un fenotipo parcialmente desetiolado (Alabadi *et al*., 2004), del mismo modo que ocurre con los mutantes *cop1*-4. Para evitar la inhibición en la germinación producida por la acción de PAC, el diseño experimental llevado a cabo con las plántulas silvestres fue su vernalización durante dos días a 4ºC en papeles de filtros y al tercer día éstos eran transferidos a nuevas placas de medio MS suplementadas con PAC. Tanto en el caso de las plántulas silvestres como en el de los mutantes (crecidos únicamente en MS), el análisis de los niveles de carotenoides producidos en plántulas crecidas en la oscuridad durante 3 días mostraba un incremento en la síntesis de carotenoides en comparación con plantas silvestres crecidas únicamente en MS. Estos datos demostraban que la desrepresión de la fotomorfogénesis conlleva un aumento en la síntesis de carotenoides incluso sin la presencia física de la luz (Figura 3, Artículo 3), lo cual no ocurre en el caso de las clorofilas (Welsch *et al*., 2000). El aumento en la producción de carotenoides podía deberse o bien a un aumento en la capacidad de almacenamiento debida a las transformaciones que tenían lugar en los plastos al desreprimir la fotomorfogénesis o bien a una activación de la vía biosintética. Análisis de la morfología de los plastos en plántulas crecidas en presencia de PAC mostraban no sólo que éstos presentaban un forma mucho más alargada que los plastos presentes en las plantas control (crecidas en ausencia de inhibidor químico), sino que el área del PLB disminuía ligeramente aumentando el número y la longitud de membranas protilacoidales (Figura 7). Este efecto producido por PAC desaparecía al crecer las plantas en presencia de inhibidor y GA, demostrándose así que la desrepresión de la fotomorfogénesis provoca una incipiente conversión morfológica de los etioplastos a cloroplastos.

Con el propósito de analizar si este cambio en la morfología era el único responsable del mayor nivel de carotenoides acumulados en las plántulas o bien estaba ocurriendo una activación de la biosíntesis de carotenoides, decidimos crecer nuestras plantas en presencia de PAC suplementadas con NFZ. El bloqueo de la ruta carotenogénica con NFZ conlleva una acumulación de fitoeno que en condiciones normales es rápidamente transformado en luteína y violaxantina, los dos componentes mayoritarios en los etioplastos.

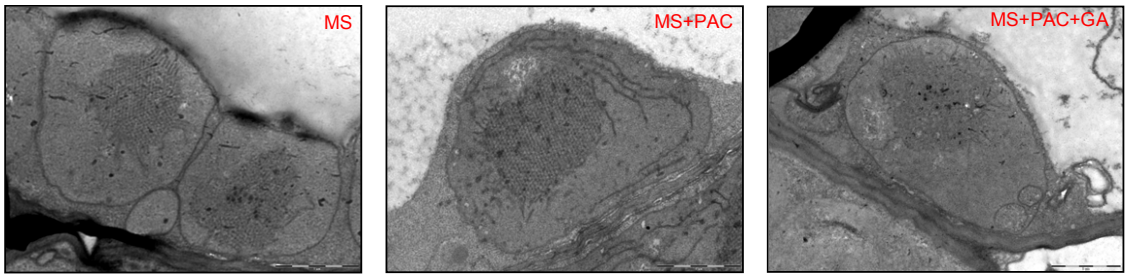


Figura 7. Análisis morfológico por microscopía electrónica de los plastos de plántulas crecidas en MS, en MS y paclobutrazol (PAC) y MS, PAC y giberelinas (GA).

La acumulación de fitoeno, producto de la reacción catalizada por PSY que se observaba en plantas tratadas con NFZ (Figura 4, Artículo 3), demostraba no sólo la existencia de una forma activa para PSY en los etioplastos, sino que ocurre una inducción de su actividad al desreprimir la fotomorfogénesis en ausencia de luz. Aunque había sido descrito que PSY se encontraba asociada al PLB de forma inactiva y requería de ciertos regímenes de luz para activarse y pasar a las membranas tilacoidales de los cloroplastos (Welsch *et al*., 2000), estos resultados demostraban que no sólo se encontraba activa en los etioplastos sino que el incremento en la producción de carotenoides que se observaba al desreprimir la carotenogénesis se correlacionaba con un aumento en su actividad. Análisis de los tránscritos del gen *PSY* demostraban que, en plantas tratadas con PAC, el incremento de la actividad de PSY observado se correlacionaba con una mayor acumulación de los tránscritos (Figura 5, Artículo 3). Más aún, mediante el análisis por tinción histoquímica y por microscopía confocal de líneas transgénicas que presentaban el promotor de *PSY* fusionado al gen reportero *GUS-GFP* crecidas en presencia de PAC, se veía como la actividad del promotor era mayor en los cotiledones, donde se acumulan de forma natural los carotenoides. Estos datos, junto con la mayor acumulación a nivel proteico de GFP que se observaron en estas líneas transgénicas al crecerlas en presencia de PAC (Figura 5, Artículo 3), demostraban que el aumento en la tasa biosintética de carotenoides que observamos al desreprimir la fotomorfogénesis se debe a una mayor actividad de PSY producida por una mayor expresión del único gen codificante para este enzima en *Arabidopsis*.

Era de esperar que la mayor tasa biosintética de carotenoides que se observaba al desreprimir la fotomorfogénesis implicase un aumento en la producción de precursores. El fenotipo albino que presentaba el mutante *cla1* (deficientes en actividad DXS) al crecerlo en la oscuridad sugería un papel mayoritario para la vía del MEP en el abastecimiento de precursores para la síntesis de carotenoides en etioplastos, al igual que ocurre en otros tipos de plastos como cloroplastos y cromoplastos. Análisis de plántulas p*DXS::GUS-GFP* mostraron un patrón de actividad GUS prácticamente idéntico al observado para las líneas p*PSY::GUS-GFP* (Figura 2, Artículo 3). Estos datos sugerían una coordinación a nivel transcripcional entre la vía del MEP y la carotenogénesis como se había observado en plántulas desetioladas por luz (Botella-Pavia *et al*., 2004) o durante la maduración del fruto de tomate (Lois *et al*., 2000).

Sin embargo, análisis de los niveles de tránscritos del gen *DXS* no mostraban una inducción a nivel transcripcional cuando las plantas eran tratadas con PAC. Lo mismo ocurría en el caso de *DXR* y *HDR* (Figura 5, Artículo 3). Trabajos recientes han mostrado que la regulación de la vía MEP puede darse a diferentes niveles (Flores-Perez *et al*., 2008b; Cordoba *et al*., 2009), siendo muy importante la regulación post-transcripcional. Para investigar si estaba ocurriendo una acumulación post-transcripcional de proteínas DXS o otras enzimas reguadoras del flujo de la ruta MEP como DXR o HDR cuando desreprimíamos la fotomorfogénesis en la oscuridad, decidimos estudiar los niveles proteicos de estos enzimas. Análisis por western blot confirmaron que tanto DXS como DXR y HDR sufrían un incremento de sus niveles proteicos en plántulas tratadas con PAC (Figura 6, Artículo 3). Recientemente ha sido descrito en nuestro laboratorio el papel de la proteasa estromal plastídica Clp en la regulación de los niveles de los enzimas de la vía MEP (Flores-Pérez *et al*., 2008). Dada la presencia de este complejo proteasa en todos los tipos de plastos, decidimos analizar su implicación en la inducción post-transcripcional que observábamos centrándonos en el enzima DXS. Los niveles proteicos de este enzima en etioplastos del mutante *clpr1*-2, que muestra una actividad proteasa Clp menor (Koussevitzky *et al*., 2007; Stanne *et al*., 2009) eran superiores a los detectados en plántulas silvestres. Este resultado demostraba un papel del complejo proteolítico Clp en la regulación de los niveles de DXS en etioplastos. Sin embargo, las plántulas mutantes mostraban, igual que el control silvestre, una inducción proteica de DXS al desreprimir la fotomorfogénesis con PAC (Figura 6, Artículo 3). Estos resultados sugerían que, aunque Clp pueda estar implicada en la regulación de los niveles de DXS en los etioplastos, no es la responsable de la inducción post-trascripcional cuando se produce la inducción de la fotomorfogénesis.

En plántulas de *Arabidopsis* se había descrito como la sobreexpresión de transgenes codificantes para DXS o HDR conllevaba un aumento en la producción de carotenoides (Estevez *et al*., 2001; Botella-Pavia *et al*., 2004; Carretero-Paulet *et al*., 2006). Con el propósito de investigar si un aumento de los niveles de precursores podría ser suficiente para incrementar la producción de carotenoides en plántulas crecidas en la oscuridad, decidimos crecer plántulas sobreexpresoras de DXS, *35S::DXS* (Carretero-Paulet *et al*., 2006), en presencia y ausencia de PAC y analizar su producción de carotenoides. A diferencia de los resultados observados en luz, un aumento en la disponibilidad de precursores demostró no tener ningún efecto en la producción de carotenoides en etioplastos, ni siquera cuando la producción de estos compuestos era aumentada al desreprimir la fotomorfogénesis (Figura 7, Artículo 3). Estos resultados sugieren que la disponibilidad de precursores es un factor limitante siempre y cuando la actividad de PSY, encargarda de canalizarlos hacia la síntesis de carotenos, no lo sea (Lois *et al*., 2000). Para comprobar si la actividad PSY era realmente un factor limitante en la producción de carotenoides en etioplastos, se generaron líneas transgénicas que presentaban el gen codificante para PSY de *Erwinia uredevora*, *CrtB*, dirigido a plastos bajo el control de un promotor inducible por dexametasona (DEX) y líneas control que presentaban el plásmido vacío. Plántulas transgénicas fueron crecidas durante 3 días en la oscuridad en presencia o ausencia de PAC y fueron transferidas a nuevas placas con y sin

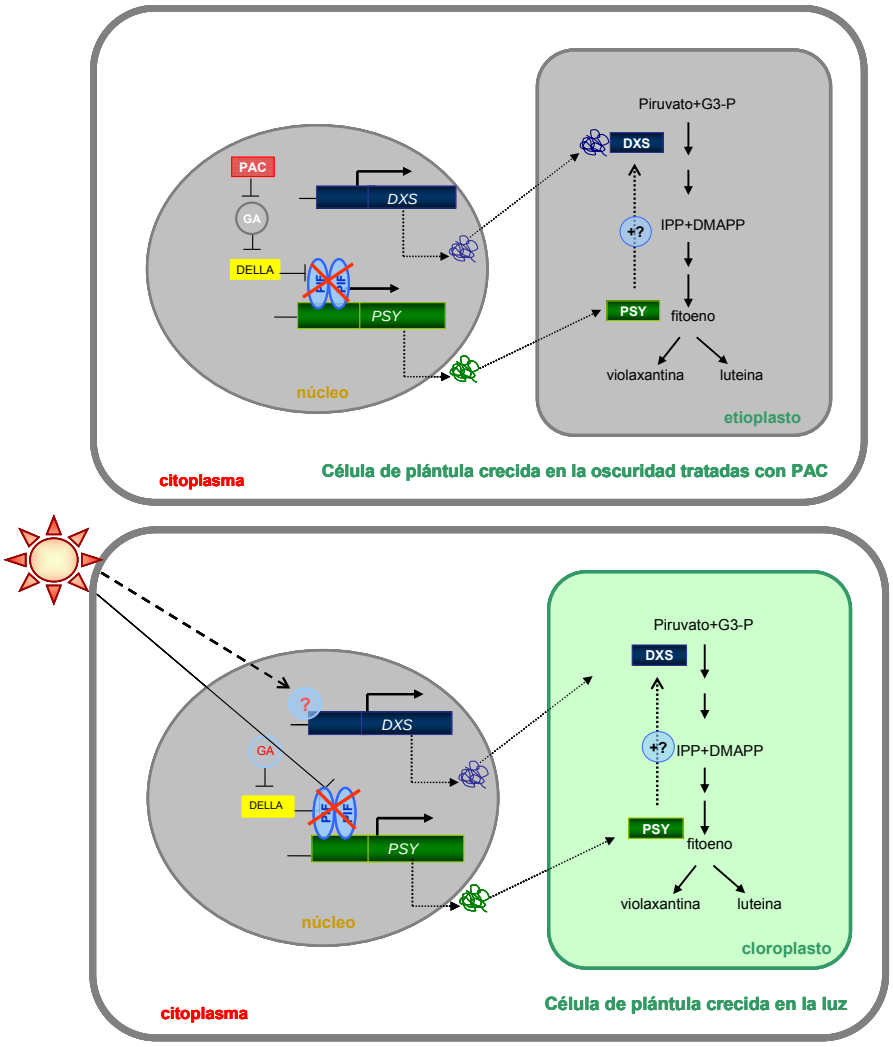
DEX creciendo en la oscuridad por 24 horas más. El análisis de los niveles de carotenoides de plántulas tratadas con DEX mostró que una inducción de la actividad PSY era suficiente para aumentar ligeramente los niveles de pigmentos producidos a diferencia de lo que ocurría al sobreexpresar los enzimas de la vía del MEP (Figura 7, Artículo 3). Más aún, este aumento incrementaba al desreprimir la fotomorfogénesis. Con la finalidad de investigar si en esta situación un incremento en la actividad de PSY era suficiente para aumentar los niveles de precursores mediante el acúmulo de los enzimas del MEP, decidimos ver qué ocurría con los niveles de proteína de DXS en las líneas transgénicas p*DEX::CrtB*. Un aumento en la actividad PSY conlleva un incremento en los niveles proteicos de DXS y DXR (y por tanto, de los precursores que puedan ser canalizados hacia la síntesis de carotenoides) sin que haya ninguna alteración en los niveles de expresión génica de estos enzimas (Figura 8, Artículo 3). Estos resultados demuestran un papel clave para PSY en la regulación de la biosíntesis de carotenoides en etioplastos, de forma similar a lo que ocurre en cloroplastos y cromoplastos. La desrepresión de la fotomorfogénesis provoca una inducción a nivel génico del primer enzima biosintético de la ruta, *PSY* y por tanto un aumento en los niveles de carotenoides. En parte esto es posible gracias al incremento en los niveles de DXS y DXR (y en consecuencia, de precursores canalizados hacia la síntesis de pigmentos) mediante un mecanismo de regulación post-transcripcional (Figura 8). De este modo, la plántula es capaz, mediante la inducción transcripcional de *PSY*, de incrementar la síntesis de carotenoides en los etioplastos, imprescindibles en su adaptación a la transición al metabolismo fotosintético. Tanto es así que plántulas crecidas en la oscuridad que presentaban un mayor nivel de carotenoides en sus etioplastos mostraron tener una acumulación de clorofilas mayor durante el proceso de desetiolación que aquellas cuyos niveles de pigmentos no habían sido alterados (Figura 2, Artículo 3).

Los componentes del mecanismo postranscripcional antes descrito así como los factores implicados en la inducción transcripcional de *PSY* se desconocen. El papel de las GA en el control de la escotomorfogénesis ha sido ampliamente estudiado. Después de la germinación, la actividad de las GA resulta en el mantenimiento de bajos niveles de las proteínas DELLA (Figura 8) que son reguladores negativos de factores de transcripción como HY5 y PIFs (phytocrome-interacting factors). Recientemente ha sido demostrado el papel regulador de PIF1 en la expresión de *PSY*, provocando la represión de este gen cuando los fitocromos están inactivos (como ocurre en plántulas crecidas en la oscuridad; Toledo-Ortiz *et al*., 2009).

En resumen, cuando las plantas germinan en la oscuridad, niveles elevados de GA y de COP1 impiden un aumento de los niveles de las proteínas DELLA y HY5, lo que junto con niveles elevados de PIF resulta en una represión de la expresión de *PSY* (Figura 8). Al desreprimir la fotomorfogénesis en los mutantes *cop1*-4 crecidos en la oscuridad, se observa un incremento en los niveles de HY5 que, por sí mismo o mediante la acción de otros factores de transcripción, podrían regular la expresión de *PSY* pero no de *DXS*. En plantas crecidas con PAC, una menor síntesis de GA resultaría en un aumento de los niveles de las proteínas DELLA, la degradación de los factores PIF y como resultado la desrepresión específica de

PSY, un mayor flujo carotenogénico y acumulación proteica de los principales enzimas de la vía MEP.

Figura 8. Modelo propuesto para la regulación de la biosíntesis de carotenoides y de sus precursores durante el proceso de desetiolación en la oscuridad y en luz en plántulas de *Arabidopsis*. Líneas de rayas significan más de una reacción enzimática.



Se desconoce si la inducción del gen *PSY* podría llevarse a cabo por otros factores de transcripción como HY5 (Figura 8). Es posible que el mecanismo anteriormente descrito pueda tener lugar en cloro y cromoplastos donde la luz juega un papel fundamental en la desrepresión de la fotomorfogénesis. La luz provoca un rápido descenso de los niveles de GA, la degradación de COP1 y de los factores PIF, lo cual podría suponer una inducción coordinada de la expresión de *DXS* y *PSY*, como ya se había descrito anteriormente (Botella-Pavía *et al*., 2004). La inducción de *DXS* y de otros genes de la vía del MEP ha sido ampliamente estudiada, sin embargo qué factores son los responsables de dicha regulación todavía se desconocen. No obstante, por nuestros datos se deduce que deben ser diferentes de los que regulan la inducción de *PSY*. Por otra parte, mecanismos post-transcripcionales han sido descritos para la regulación de los niveles proteicos de DXS en estadíos tempranos de

desarrollo (Guevara-Garcia *et al*., 2005), así que es posible que la acumulación proteica observada para DXS como consecuencia de un aumento de la actividad de PSY en cloroplastos y cromoplastos podría darse como un nivel más de regulación de la vía del MEP. No obstante, se requiere un análisis más profundo sobre la naturaleza de esta señal y sobre los mecanismos implicados para poder confirmar estos datos.

3.2.2 Distribución de la síntesis de carotenoides en la planta y su función en la raíz.

Los carotenoides se encuentran presentes en todo tipo de plastos en plantas variando tanto sus niveles de acumulación como la función desempeñada en los mismos. En tejidos fotosintéticos, los carotenoides desempeñan un papel esencial en la fotoprotección de las plantas contra el daño fotooxidativo, mientras que en tejidos no fotosintéticos modulan la respuesta a señales ambientales siendo los precursores de importantes moléculas reguladoras del crecimiento. De forma consistente con este papel multifacético de los carotenoides, ha sido descrita la existencia de numerosas isoformas para distintos enzimas carotenogénicos. En el caso concreto de la fitoeno sintasa (PSY), que como ya hemos citado anteriormente es el enzima que cataliza la primera reacción específica de la síntesis de carotenoides, han sido descritos dos isogenes en tomate (Giorio *et al*., 2008), tres en arroz (Welsch *et al*., 2008) y en maíz (Li *et al*., 2008a). Algunas isoformas están implicadas en la síntesis de carotenoides específicamente en tejidos fotosintéticos mientras que otras están especializadas en la producción de carotenoides en frutos (como en tomate PSY1), en el endospermo de semillas (como el caso del maíz PSY1) o en la raíz (en arroz y maíz, PSY3). Sin embargo, *Arabidopsis thaliana* presenta un único gen codificante para PSY. Con el propósito de investigar los mecanismos que regulan la expresión de este gen en los distintos tejidos y la función de la carotenogénesis en los mismos, decidimos analizar su patrón de expresión durante el desarrollo de las plántulas y en respuesta a ciertas condiciones ambientales.

De forma consistente con el papel de fotoprotección de los carotenoides, al analizar los niveles de expresión de PSY en las bases de datos se observó que los mayores niveles de expresión aparecían en los tejidos fotosintéticos (cotiledones, hojas y sépalos) mientras que niveles mucho menores fueron detectados en raíces (Figuras S1, Artículo 4). Estos datos fueron confirmados al analizar los niveles de tránscritos de *PSY* en las distintas partes de la plántula por qRT-PCR (Figura S2, Artículo 4). De forma inesperada se encontró una elevada expresión de este gen en pétalos y estambres.

Con el objetivo de estudiar en mayor profundidad la regulación de la expresión de *PSY* decidimos generar distintas líneas transgénicas que presentaban el promotor de *PSY* y la región 5´-UTR (1276 pares de bases antes del incio de la transcripción), la construcción -1276/+716 fusionada al gen GUS-GFP. Del mismo modo, delecciones del promotor (-300/+716, -196/+716) fueron fusionadas al mismo gen reportero con la consecuente creación de diferentes líneas transgénicas. El análisis del patrón de expresión por tinción histoquímica GUS y por microscopía confocal de dichas líneas era consistente con lo observado en las bases de datos, siendo la actividad de GUS y GFP prácticamente ubicua. Este trabajo, sin embargo,

permitió descubrir que la actividad del promotor de PSY es especialmente elevada en los haces vasculares tanto de tejidos fotosintéticos como en tejidos no fotosintéticos (raíz, pétalos y estambres; Figuras 2, 3, 6 y 7, Artículo 4). La acumulación de la expresión mayoritaria de *PSY* en los vasos fue confirmada por experimentos de hibridación *in situ* con sondas específicas antisentido para *PSY* (Figura 4, Artículo 4).

Figura 9. Análisis de la distribución de carotenoides en un pétalo por microscopía Raman (panel de la izquierda). Análisis de la expresión de GFP en un pétalo de plantas p*PSY::GUS-GFP* (izquierda) en comparación con la distribución de clorofila (en rojo, panel de la derecha).

Por otra parte, la misma distribución de actividad GUS y GFP fue observada en las distintas líneas transgénicas con fragmentos menores del promotor PSY, sugiriendo estos datos que los elementos *cis* presentes en los 0.9 kb anteriores al inicio de la traducción son los responsables del control de la expresión espacio-temporal del gen. Estudios realizados por microespectroscopía Raman de la distribución *in planta* (Baranski *et al*., 2005; Baranska *et al*., 2006) de los carotenoides mostraron una mayor acumulación de estos pigmentos en los haces vasculares, sugiriendo que una mayor expresión de *PSY* resulta en una mayor actividad carotenogénica con la consecuente acumulación de productos finales (Figura 5, artículo 4).

Con el objetivo de investigar sobre la diferente funcionalidad de la expresión de *PSY* en tejidos fotosintéticos y no fotosintéticos decidimos analizar la expresión de este gen en los pétalos. Un exhaustivo análisis de la distribución de los plastos en los pétalos demostró como la presencia de cloroplastos se encontraba en la parte basal mientras que en la parte superior éstos desaparecían dando lugar a leucoplastos (Pyke y Page, 1998). Del mismo modo, análisis de la distribución de carotenoides en pétalos de *Arabidopsis* mostraban una acumulación de estos pigmentos únicamente en la parte basal de los pétalos (Figura 9) mientras que la expresión de *PSY* se detecteba en las células con cloroplastos pero también en los haces vasculares de la parte superior del pétalo. De forma consistente con estos datos, cortes ortogonales de flores embebidas en historresina mostraban como la actividad de GUS aparecía en los haces vasculares de los pétalos y los estambres. Estos datos sugieren una especialización funcional según el tejido para *PSY.* Una elevada expresión en los cloroplastos podría relacionarse con la síntesis de carotenoides para la fotoprotección mientras que en los

tejidos no fotosintéticos podría relacionarse con el abastecimiento de precursores para la síntesis de apocarotenoides como ABA y estrigolactonas.

La expresión de *PSY* en los haces vasculares de las raíces sugiere que pueda participar en la producción de carotenoides o, más probablemente, productos de su degradación como apocarotenoides como ABA y estrigolactonas que después serán transportados a los tejidos aéreos para realizar su función. Isoformas específicas radiculares de *PSY* en arroz y maíz se relacionaron con la producción de ABA ante respuestas de estrés abiótico, el cual una vez sintetizado podría ser transportado hacia la parte aérea de la planta (Li *et al*., 2008; Welsch *et al*., 2008). Del mismo modo, las estrigolactonas son sintetizadas en la raíz y son transportadas hacia la parte aérea donde desempeñan un importante papel en el control de la arquitectura radicular (Gomez-Roldan *et al*., 2008; Klee, 2008). Un examen más profundo del patrón de distribución de la actividad GUS y de la florescencia de GFP demostró que el promotor de *PSY* era más activo en la zona de maduración y elongación radicular. Cortes ortogonales realizados por microscopía confocal de raíces previamente teñidas con yoduro de propidio (PI) que señala las paredes celulares, permitieron localizar la actividad del promotor del gen *PSY* en la estela de la raíz. Análisis de cortes ortogonales de raíces previamente teñidas por tinción histoquímica GUS y embebidas en historresina confirmó que la expresión de *PSY* era más elevada en la células de la estela, en concreto en las células acompañantes del floema (Figura 7, Artículo 4). Estos datos eran consistentes con los datos provenientes de las bases de datos donde se mostraba un enriquecimiento de tránscritos PSY en el floema.

Con el propósito de investigar la función de los carotenoides en el tejido radicular decidimos bloquear la carotenogénesis de forma específica en la raíz. La adición al medio de norflurazón (NFZ), un inhibidor de la actividad de la fitoeno desaturasa (PDS), inhibe completamente la producción de carotenoides. Sin embargo, la actividad de CrtI, fitoeno desaturasa proveniente de *Erwinia uredevora*, aparentemente no se ve afectada por este inhibidor químico (Misawa *et al*., 1993). Se generaron líneas transgénicas para este transgén bacteriano, CrtI, fusionada a un péptido de tránsito dirigido a los plastos procedente de RuBisCO de guisante (*Pisum sativum*) bajo el control de dos promotores diferentes de expresión únicamente aérea, el promotor del gen codificante para chlorophyll binding protein 3 (CAB3; At1g29910) y glutamato sintasa 1 dependiente de ferredoxina (GLU1; At5g04140). Como líneas control se emplearon las líneas de expresión constitutiva 35S::*TP-CrtI*. Era de esperar que el crecimiento de estas líneas en presencia de NFZ bloqueara la síntesis de carotenoides únicamente en raíz pero no en la parte aérea en las líneas CAB3::*TP-CrtI* y GLU1::*TP-CrtI* y que no se observara ningún efecto en las líneas 35S::*TP-CrtI*.

Dos líneas homozigóticas independientes con una única inserción de cada construcción fueron seleccionadas para comprobar su resistencia al bloqueo por NFZ. A una concentración de 50 nM de este inhibidor se comprobó que, a diferencia de las plántulas silvestres, que mostraban un fenotipo albino, las líneas transgénicas no presentaban ninguna alteración fenotípica (Figura 8A, Artículo 4). Análisis por cromatografía líquida de alta presión (HPLC)

demostró que los niveles de β-caroteno y luteína (componentes mayoritarios en la raíz) eran exactamente idénticos en plántulas transgénicas crecidas en presencia de NFZ y en MS, a diferencia de lo que ocurría con las plántulas silvestres que presentaban un fenotipo albino con la consiguiente acumulación de fitoeno al ser bloqueada la carotenogénesis con NFZ (Figura 8B, Artículo 4). En concreto se observó que los niveles de carotenoides de raíz relativos a aquellos cuantificados en la parte aérea no se veían significativamente alterados en las líneas CAB3::*TP-CrtI* y GLU1::*TP-CrtI* crecidas en presencia de NFZ. Estos resultados sugerían que una expresión residual del transgén en las raíces de CAB3::*TP-CrtI* y GLU1::*TP-CrtI* debía ser suficiente para la producción de carotenoides en presencia de inhibidor. En concordancia con estos datos, el análisis de los niveles de expresión del transgén por qPCR demostró que los tránscritos de *TP-CrtI* se encontraban mayoritariamente en tejido áereo con una expresión radicular cercana al 20% en las líneas GLU1::*TP-CrtI* y CAB3::*TP-CrtI*. En el caso de las líneas CAB3::*TP-CrtI*, la diferencia entre los niveles de expresión del gen endógeno *CAB3* en tejido radicular y en los tejidos aéreos demostró ser mucho mayor en comparación a la observada para *TP-CrtI*. Este resultado indicaba que la expresión del trangén no es tan específica como aquella de su propio gen endógeno, probablemente debido a la presencia del promotor 35S para la expresión del gen de resistencia del T-DNA como ya había sido reportado anteriormente (Yoo *et al*., 2005).

La existencia de una acumulación de carotenoides en la raíz similar en plántulas crecidas en MS y plántulas crecidas en NFZ era consistente con la ausencia de cualquier alteración en el fenotipo de la raíz, tanto en su longitud como en la aparición de raíces secundarias. Del mismo modo, experimentos realizados para estudiar la implicación de una reducción en los niveles de carotenoides con la producción de ABA o estrigolactonas no mostraron ningún fenotipo claro para ninguna de las dos hormonas, en consistencia con los niveles inalterados de carotenoides en la raíz de estas plántulas. Análisis de la germinación (proceso controlado por la actividad del ABA) de las plántulas transgénicas no mostraron diferencias significativas entre plantas tratadas con inhibidor de las crecidas en MS. De forma similar, no se observó ningún fenotipo ramificado en la parte aérea en las plantas CAB3::*TP-CrtI* y GLU1::*TP-CrtI* al crecerlas en presencia de NFZ. Todos estos resultados demostraron que una pequeña actividad de la fitoeno desaturasa por parte de *TP-CrtI* es suficiente para proveer a la raíz de los carotenoides necesarios probablemente para ser empleados en la síntesis de hormonas.

Numerosos trabajos han demostrado la síntesis de apocarotenoides en raíz implicados en la formación de hormonas reguladoras del desarrollo vegetal. En concreto, la raíz es el órgano donde se encuentra la mayor fuente de producción de ABA. Las raíces responden ante el estrés abiótico o sequía mediante un incremento en los niveles de esta hormona y su transporte hacia los tejidos aéreos. En arroz y maíz se observó que este proceso se llevaba a cabo mediante la inducción específica de PSY3 (Li *et al*., 2008a; Welsch *et al*., 2008) mostrando una clara implicación del primer enzima de la ruta carotenogénica en la síntesis de los precursores de ABA. Pese a que *Arabidopsis thaliana* presenta un único gen codificante

para *PSY*, se decidió investigar si éste respondía ante condiciones de estrés abiótico. Con tal fin se decidió crecer plántulas silvestres por 14 días sobre papeles de filtro en placas de MS y a continuación las plántulas fueron transferidas a nuevas placas de MS suplementadas con NaCl 200 mM por 0-0.5-1-2-4 horas. Tejido de la parte aérea y de la parte radicular fue recolectado separadamente para su posterior extracción de ARN. Análisis por qPCR de los tránscritos de *PSY* en la parte radicular mostró una inducción en la expresión génica al cabo de 1 hora de tratamiento salino que no fue detectado en la parte aérea (Figura 9A, Artículo 4). Tratamientos de más duración provocan igualmente una inducción génica que, aunque menor que la observada al cabo de 1 hora, continúa siendo mayor que la detectada en plántulas sin tratar. Estos datos son consistentes con lo observado para PSY3 en arroz y maíz sugiriendo que la actividad de PSY es de algún modo limitante para la producción de ABA bajo condiciones de estrés abiótico. Del mismo modo, cuando se sustituía el tratamiento salino por ABA exógeno se observaba el mismo comportamiento en la expresión del gen, sugiriendo un mecanismo de regulación de retroalimentación positiva (Figura 9B, Artículo 4) específico del tejido radicular, ya que no se observaba una inducción en la expresión de *PSY* en las hojas, a diferencia de lo descrito para otros isogenes *PSY* en maíz (Li *et al*., 2008). Para testar esta hipótesis, plántulas silvestres y plántulas del mutante *aba2*, el cual presenta una mutación en la vía de síntesis del ABA, fueron sometidas al mismo tratamiento salino previamente explicado durante 1 hora. En comparación con las plántulas silvestres, el análisis de los niveles de tránscritos de *PSY* en el tejido radicular del mutante *aba2* mostraba un ligero descenso en la inducción de la expresión del gen en respuesta a 1 hora con tratamiento salino (Figura 9C, Artículo 4) a diferencia de lo observado para el tejido aéreo, donde no se encontró ninguna variación. Todos estos datos sugerían que el ABA puede regular la expresión de *PSY* por un mecanismo de retroalimentación específico de la raíz.

Del mismo modo que el ABA, las estrigolactonas son productos sintetizados en la raíz que son transportados a la parte aérea para desarrollar su función. En este contexto, decidimos investigar si las estrigolactonas podían influír en la regulación de la expresión de *PSY* en raíces. Mutantes defectivos en la síntesis de estas hormonas (*max1*, *max3*) o en su percepción (*max2*) fueron sometidos al tratamiento salino anteriormente explicado. Al analizar la expresión génica de *PSY* en la raíz se observó que los mutantes presentaban una inducción menor en comparación con las plántulas silvestres, sin que se encontrar ningún cambio en los niveles de expresión en la parte aérea (Figura 10C, Artículo 4). Estos datos sugieren que las estrigolactonas podrían estar implicadas en la regulación de la expresión de *PSY* en las raíces de Arabidopsis en condiciones de estrés salino. Es posible que este mecanismo de retroalimentación positiva para el ABA puede verse disminuído en los mutantes defectivos en estrigolactonas. Más aún, recientemente ha sido descrito que bajo condiciones de estrés abiótico por falta de fosfatos, el ABA puede influir en la síntesis de estrigolactonas en raíces de plantas de tomate (López-Raez y Bouwmeester, 2008). No obstante, se requiere más trabajo en esta dirección para entender la relación entre ABA y estrigolactonas en el control de la expresión de *PSY* bajo condiciones de estrés salino en las raíces.

En conclusión este trabajo ha demostrado que, en concordancia con el papel multifacético que presentan los carotenoides, el único gen para *PSY* en *Arabidopsis thaliana* se expresa de forma ubicua en la planta localizándose su expresión en los haces vasculares de tejidos fotosintéticos y no-fotosintéticos. En concreto en la raíz se ha observado que su expresión se localiza en las células acompañantes del floema, probablemente para sintetizar los precursores necesarios para la formación de hormonas apocarotenoides. En concreto se ha observado, en respuesta a tratamiento salino y ABA exógeno, una inducción en su expresión génica únicamente en el tejido radicular que se veía disminuída en las raíces no sólo de mutantes *aba2* sino también de mutantes *max*, apuntando un nuevo papel para las estrigolactonas en la regulación del estrés abiótico. En definitiva, estos resultados han demostrado que la biosíntesis de carotenoides y de sus precursores está controlada por la actividad de PSY en la mayoría de los tejidos de la planta. La regulación de su expresión génica por múltiples factores podría asegurar un aporte adecuado de carotenoides en respuesta a diferentes estímulos internos (desarrollo) y externos (ambientales).

Conclusiones

Conclusiones

1. Células de *E.coli* modificadas genéticamente para la producción de carotenoides han demostrado ser un excelente sistema de estudio de los mecanismos reguladores de la producción de carotenoides y de sus precursores cuyos resultados pueden extrapolarse a sistemas vegetales como *Arabidopsis*.
2. La producción de pigmentos en células de *E. coli* modificadas genéticamente para la síntesis de licopeno puede aumentarse significativamente al incrementar los niveles de IPP y DMAPP ya sea mediante la activación de la ruta del MEP (vía endógena) o, de forma más eficaz , mediante la utilización del operón del MVA (vía exógena).
3. El primer enzima de la vía del MEP, DXS, (y en menor medida DXR y HDR) es clave para el control de la producción de precursores para la síntesis de carotenoides. No obstante, una sobreexpresión excesiva de estos enzimas puede afectar al crecimiento celular, probablemente al interferir negativamente con el metabolismo endógeno de las células.
4. El fondo genético afecta significativamente la producción de licopeno en *E. coli*, de lo cual se deriva que, además del aporte de precursores, otros factores como la capacidad de almacenamiento celular afectan considerablemente a la acumulación de carotenoides.
5. La expresión de secuencias de *Synechocystis* en células de *E. coli* productoras de carotenoides permitió incrementar los niveles de pigmentos no por una mejora en la capacidad de almacenamiento sino probablemente por una mayor disponibilidad de sustratos de la ruta del MEP como consecuencia de la interferencia con el metabolismo celular.
6. La desrepresión de la fotomorfogénesis en plántulas de *Arabidopsis thaliana* crecidas en oscuridad produce un notable incremento en la producción de carotenoides.
7. El aumento de los niveles de carotenoides en plastos de plántulas germinadas en la oscuridad facilita su adaptación a un metabolismo fotosintético tras ser iluminadas.
8. La ruta del MEP es la principal vía de síntesis de precursores de carotenoides en plántulas crecidas en oscuridad. Los niveles de enzimas clave de esta ruta (DXS, DXR y HDR) aumentan post-transcripcionalmente al desreprimir la desetiolación en la oscuridad.
9. El incremento observado en la síntesis de carotenoides al desreprimir la fotomorfogénesis en oscuridad se correlaciona con una inducción del gen codificante para el enzima PSY, produciéndose consecuentemente un aumento de actividad.
10. Un aumento de la actividad PSY es suficiente para inducir la biosíntesis de carotenoides y de sus precursores. Mediante un mecanismo aún por dilucidar, una mayor actividad PSY provoca una acumulación post-transcripcional de DXS, posiblemente aumentando el flujo de la vía MEP.

11. El gen codificante para *PSY* se expresa en todos los tejidos. Sin embargo, se observa una mayor expresión en los haces vasculares, sobre todo en el caso de tejidos no fotosintéticos, donde la concentración de carotenoides es mucho menor. En el caso concreto de la raíz, se observa una mayor acumulación de tránscritos *PSY* en la estela.
12. La expresión de *PSY* aumenta en respuesta al estrés salino pero únicamente en la raíz. Es posible que la síntesis de productos derivados de carotenoides como ABA y estrigolactonas se sinteticen en las células cercanas a los vasos tras la inducción de *PSY* y sean después transportados a los tejidos aéreos a través del sistema vascular para regular la respuesta fisiológica.
13. Tanto el ABA como las estrigolactonas, hormonas derivadas de la degradación oxidativa de los carotenoides, parecen estar implicadas a su vez en la regulación de la expresión de *PSY* en respuesta a estrés salino.
14. La regulación de la expresión de *PSY* por múltiples factores podría asegurar un aporte adecuado de carotenoides (y de sus productos) en respuesta a estímulos internos (desarrollo) y externos (ambientales).

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Publicaciones

Artículo 1:

Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways

Acumulación de carotenoides en bacterias que presentan un mayor aporte de precursores isoprenoides gracias a la inducción de vías endógenas y exógenas

Resumen

Los carotenoides son pigmentos de naturaleza isoprenoide de gran valor industrial y nutricional. Aunque pueden llegar a ser producidos en huéspedes no carotenogénicos como *Escherichia coli* modificadas genéticamente con genes carotenogénicos, éstos organismos presentan únicamente una limitada cantidad de precursores metabólicos disponible para la síntesis de pigmentos. En este trabajo hemos comparado la producción de carotenoides (licopeno) en cepas en las cuales el aporte de precursores se había incrementado bien mediante la inducción de la vía endógena por la sobreexpresión de desoxixilulosa 5-fosfato sintasa (DXS) o bien mediante la incorporación de una vía exógena como el operón del mevalonato (MVA). En cepas que expresaban DXS bajo el control de un promotor inducible por IPTG, la acumulación de licopeno se veía incrementada hasta 8 veces en ausencia de inductor. La adición de IPTG, sin embargo, afectaba negativamente a la producción de licopeno. Aunque la inducción de niveles demasiado elevados de los enzimas del operón del MVA también parecía interferir con el metabolismo celular, la adición al medio de MVA (para ser transformado en precursores de carotenoides) resultó en una producción de licopeno 10 veces mayor en células con un fondo genético parecido al silvestre. Del mismo modo, el empleo de la cepa de laboratorio BL21 duplicó la producción de carotenoides, consiguiéndose niveles de hasta 228 mg/l. Estos resultados confirmaron que la utilización del operón del MVA es mejor para conseguir un aumento en la producción de carotenoides (licopeno) en *E. coli*. No obstante, otros factores además del aporte de precursores han de tenerse en cuenta a la hora de conseguir incrementar la acumulación de pigmentos.

Experimentos específicos de la tesis doctoral:

Figura 2, Figura 3, Figura 4, Figura 5.

Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways

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ABSTRACT

Carotenoids are isoprenoid pigments of industrial and nutritional interest. Although they are produced in non-carotenogenic *Escherichia coli* engineered with the appropriate biosynthetic genes, only a limited pool of their metabolic precursors is available in these bacteria. We have compared the production of carotenoids (lycopene) in strains in which the supply of precursors was enhanced either by upregulating the endogenous pathway *via* overexpression of deoxyxylulose 5-phosphate synthase (DXS) or by incorporating an exogenous MVA+ operon. In strains expressing DXS under the control of a leaky IPTG-inducible promoter, lycopene accumulation was increased up to 8-fold in the absence of inducer. Addition of IPTG, however, negatively affected lycopene production. Although induction of too high levels of the MVA+ operon enzymes also appeared to cause interference with cell metabolism, supplementation with mevalonate (to be metabolized into carotenoid precursors) resulted in a 10-fold increase in lycopene levels in cells with a near wild type background. An additional 2-fold increase (up to 228 mg/l) was obtained using an engineered BL21 strain. These results confirm that the MVA+ pathway is most convenient to upregulate the production of carotenoids (lycopene) production in *E. coli* but that factors other than precursor supply should be considered for high pigment accumulation levels.

Keywords: carotenoids, isoprenoids, metabolic engineering, methylerythritol 4-phosphate pathway, mevalonate pathway

Abbreviations: DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonic acid.

Introduction

Carotenoids are an abundant group of isoprenoid pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. Animals do not synthesize carotenoids *de novo* but take them from their diet as an essential source of vitamin A and retinoids. Carotenoids such as lycopene also act as natural antioxidants that help to prevent some types of human cancer and degenerative diseases (Fraser and Bramley, 2004). Besides their activity as pro-vitamin A and their potential as nutraceuticals, the colorant and aromatic properties of some carotenoids and derived compounds have an important industrial value (Bartley and Scolnik, 1995; Hirschberg, 2001; Sandmann, 2001; Fraser and Bramley, 2004). Most commercial carotenoids are obtained by chemical synthesis, but the growing demand of natural additives has spurred several metabolic engineering approaches to increase carotenoid production in plants and microorganisms (Lee and Schmidt-Dannert, 2002; Fraser and Bramley, 2004; Botella-Pavía and Rodríguez-Concepción, 2006). Carotenoids have been succesfully produced even in non-carotenogenic bacteria such as *Escherichia coli* after transformation with appropriate genes from carotenogenic organisms (Figure 1). However, important problems remain to be solved to efficiently overproduce carotenoids in non-carotenogenic hosts. One of such problems is the supply of their metabolic precursors.

Structurally carotenoids are tetraterpenes derived from the 5-carbon units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoid compounds (Cunningham and Gantt, 1998). IPP and DMAPP can be interconverted by the enzyme IPP/DMAPP isomerase (IDI). The addition of three molecules of IPP to one DMAPP unit catalyzed by the enzyme geranylgeranyl diphosphate (GGPP) synthase (GDS) yields GGPP, the immediate precursor for carotenoids (Figure 1). There are two pathways for the synthesis of these prenyl diphosphates: the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002; Eisenreich *et al*., 2004). Archaebacteria, fungi and animals synthesize their isoprenoids exclusively through the operation of the MVA pathway, whereas the MEP pathway is the only one present in most eubacteria, including *E. coli* (revised in Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002).

The first reaction of the MEP pathway (Figure 1) is the production of deoxyxylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate, catalyzed by DXP synthase (DXS). Many studies have reported a positive correlation between DXS levels and carotenoid production in bacteria (Albrecht *et al*., 1999; Harker and Bramley, 1999; Cunningham *et al*., 2000; Jones *et al*., 2000; Matthews and Wurtzel, 2000; Kim and Keasling, 2001; Kang *et al*., 2005; Yuan *et al*., 2006) and plants (Estévez *et al*., 2001; Botella-Pavía *et al*., 2004; Enfissi *et al*., 2005; Carretero-Paulet *et al*., 2006). Furthermore, up-regulation of DXS levels also results in increased accumulation of other MEP-derived isoprenoids such as monoterpetenes, diterpenes, sesquiterpenes, and the prenyl tails of ubiquinone in bacteria (Harker and Bramley, 1999; Martin *et al*., 2003; Reiling *et al*., 2004; Kim *et al*., 2006) and chlorophylls, tocopherols,

taxadiene, cytokinins, gibberellins, and essential oils in plant cells (Estévez *et al*., 2001; Botella-Pavía *et al*., 2004; Carretero-Paulet *et al*., 2006; Morris *et al*., 2006; Muñoz-Bertomeu *et al*., 2006), confirming that the effect of DXS overexpression on carotenoid levels is due to an enhanced production of their metabolic precursors. Although increasing the levels of other individual MEP pathway enzymes has sometimes been reported to result in enhanced carotenoid production in *E. coli* cells, the increase was always lower than that achieved by DXS overexpression (Albrecht *et al*., 1999; Cunningham *et al*., 2000; Miller *et al*., 2000; Kim and Keasling, 2001; Yuan *et al*., 2006). DXS therefore appears to be the most appropriate enzyme to overexpress in order to increase the production of IPP and DMAPP by the endogenous MEP pathway in *E.coli*. Activities downstream the MEP pathway such as IDI and GDS (Figure 1) have also been shown to be limiting for carotenoid synthesis in engineered *E.coli* cells (Kajiwara *et al*., 1997; Albrecht *et al*., 1999; Wang *et al*., 1999; Cunningham *et al*., 2000).

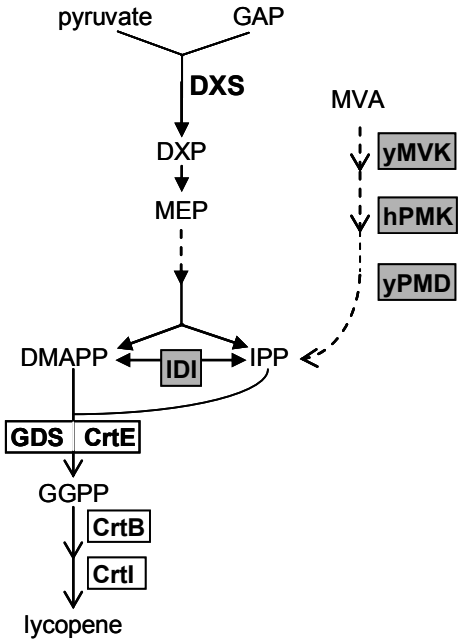

Figure1. Pathways involved in lycopene biosynthesis in engineered *E. coli* cells. GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonic acid; DXS, DXP synthase. The enzymes encoded by the MVA+ operon are boxed in gray: yMVK, yeast mevalonate kinase; hPMK, human 5-phosphomevalonate kinase; yPMD, yeast 5-diphosphomevalonate decarboxylase; IDI, *E.coli* IPP/DMAPP isomerase. The enzymes encoded by the pLYC plasmid are boxed in white: CrtE, GGPP synthase (GDS); CrtB, phytoene synthase; CrtI, phytoene desaturase.

Disruption of the gene encoding DXS or any other MEP pathway enzyme is lethal in *E. coli* cells but it can be rescued by engineering a synthetic operon harboring heterologous MVA pathway genes required to transform exogenously supplied MVA into IPP (Campos *et al*., 2001) (Figure 1). The endogenous IDI activity of *E. coli* can produce DMAPP from MVA-derived IPP. Nevetheless, the introduction of the bacterial *idi* gene into the synthetic operon to create the MVA+ operon resulted in improved growth, likely because the production of high IPP levels is toxic for bacterial cells (Campos *et al*., 2001; Martin *et al*., 2003). The MVA+ operon, under the transcriptional control of the arabinose-induced P_{BAD} promoter, is amenable to a high degree of control (Campos *et al*., 2001). A similar operon was shown to produce very high levels of IPP and DMAPP molecules from the MVA added to the growth medium and to outperform DXS overexpression for improving amorphadiene production in bacteria (Martin *et al*., 2003). It is not known, however, to what extent the accumulation of carotenoids in *E. coli* cells is limited by the supply of isoprenoid precursors. Here we have standardized the experimental conditions with *E.*

coli cells harboring the MVA+ operon and a plasmid to synthesize lycopene from IPP and DMAPP to compare the accumulation of lycopene after upregulating the supply of precursors via the endogenous MEP pathway or the synthetic MVA+ operon.

Results and discussion

Induction of too high DXS levels negatively impacts lycopene accumulation in *E. coli*.

The most direct strategy to increase the supply of precursors for carotenoid production in *E.coli* is engineering the endogenous MEP pathway. Although increased levels of several MEP pathway enzymes has been reported to result in enhanced carotenoid production in *E. coli* cells, the increase was always highest with DXS (Albrecht *et al*., 1999; Cunningham *et al*., 2000; Miller *et al*., 2000; Kim and Keasling, 2001; Yuan *et al*., 2006), supporting that DXS catalizes the main flux-controlling step of the MEP pathway. We therefore selected DXS as the most appropriate enzyme to overexpress in order to efficiently upregulate the endogenous MEP pathway for the production of IPP and DMAPP in *E.coli*. *E.coli* K12 MG1655 cells with a genomic copy of the MVA+ operon (strain EcAB4-1) (Sauret-Güeto *et al*., 2006) were transformed with the pLYC plasmid harboring the *Erwinia uredovora* genes required to synthesize lycopene from IPP and DMAPP (Table 1 and Figure 1). Succesful transformants were identified based on their double antibiotic resistance and the red color imparted by lycopene production. The presence of the *E. coli idi* gene in the MVA+ operon (Campos *et al*., 2001) and the *E. uredovora crtE* gene in the pLYC plasmid (Cunningham *et al*., 1993) ensured that carotenoid production was not limited by the endogenous IDI or GDS activities in EcAB4-1 pLYC cells. Competent EcAB4-1 pLYC cells were then transformed with the pTAC-DXS plasmid (Table 1), in which the expression of the *E.coli dxs* gene is under the control of an IPTG-inducible promoter (Lois *et al*., 1998), or an empty pTAC vector. Several overnight cultures of pigmented colonies of each strain in media without IPTG were used to inoculate fresh liquid media and quantify the production of lycopene in the presence or absence of IPTG.

In the absence of IPTG, overnight cultures of EcAB4-1 pLYC pTAC-DXS cells accumulated 8-fold higher lycopene levels compared to control cells harboring the pTAC plasmid (Figure 2). Upregulation of DXS levels in *E. coli* cells using different expression vectors has been reported to result in typically 2 to 5-fold increases in lycopene accumulation (Harker and Bramley, 1999; Cunningham *et al*., 2000; Jones *et al*., 2000; Kim and Keasling, 2001; Reiling *et al*., 2004; Kang *et al*., 2005). The use of the pTAC-DXS construct, however, was shown to lead to 7-fold higher lycopene levels in *E. coli* colonies grown in the absence of IPTG (Matthews and Wurtzel, 2000), an increase similar to that reported here in liquid cultures (Figure 2). The pTAC vectors lack tight control of the basal expression level from the strong P_{tac} promoter, leading to DXS expression in the absence of induction by IPTG. Addition of IPTG to the growth media, however, resulted in much lower increases in lycopene accumulation (Figure 2).

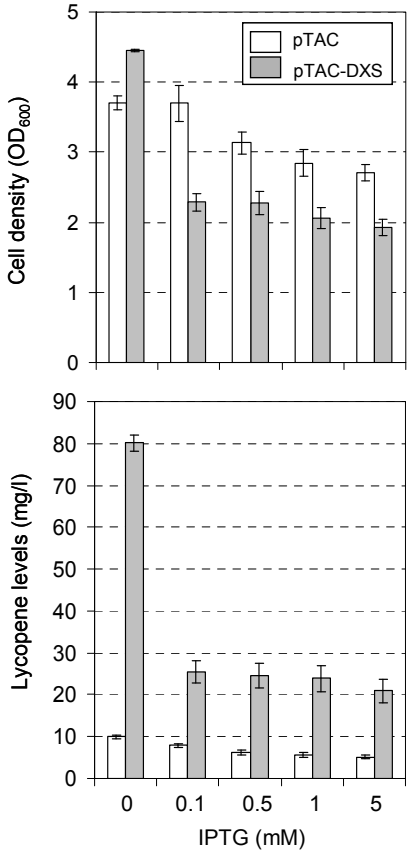

Figure2. Effect of DXS overexpression on cell growth and lycopene levels in EcAB4-1 pLYC cells. Liquid media (10 ml) supplemented with the indicated concentrations of IPTG was inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC cells harboring the pTAC-DXS plasmid (gray columns) or an empty pTAC vector (white columns) and grown overnight at 37ºC. Cell growth was determined by optical density at 600 nm (OD_{600}) and lycopene content was calculated by measuring the absorbance at 472 nm of acetone extracts of the cells and comparison with a standard curve. Mean values and standard errors corresponding to four independent experiments with several replicas each are represented.

Low concentrations of the inducer (0.1 mM) had no effect on the growth of control EcAB4-1 pLYC pTAC cells but it dramatically reduced growth of EcAB4-1 pLYC pTAC-DXS cells to 50 % compared to that in the absence of IPTG (Figure 2). Addition of higher concentrations of IPTG progressively decreased growth of both strains (Figure 2). The strong negative effect of IPTG on cell growth and lycopene production in *E.coli* cells expressing DXS under a strong inducible promoter has been repeatedly observed by other authors using different strains and constructs (Matthews and Wurtzel, 1999; Jones *et al*., 2000; Kim and Keasling, 2001). It has been suggested that production of excess DXS levels might divert a substantial amount of its substrates pyruvate and glyceraldehyde 3-phosphate to the MEP pathway and prevent their use in other central metabolic pathways. This, together with the energetic requirements associated to the production of high protein levels, might compromise the energetic and metabolic resources of the cell. Additionally, the production of a single molecule of lycopene requires 8 molecules of pyruvate, 8 glyceraldehyde 3-phosphate, 8 CTP, 8 ATP, and 16 NADPH (Alper *et al*., 2005) and the development of storage structures for such a lypophylic compound is also expensive in energetic terms. It is therefore expected that the metabolic burden likely caused by DXS overexpression has a negative impact on the production and accumulation of lycopene. Together, our data show that high increases in lycopene production can be achieved in EcAB4-1 pLYC pTAC-DXS cells, which accumulated up to 80 mg of lycopene per liter of culture. However, manipulation of the endogenous MEP pathway in *E. coli* by altering DXS levels may have effects on cell metabolism that are difficult to control, resulting in suboptimal production of lycopene.

An exogenous supply of isoprenoid precursors dramatically increases lycopene accumulation.

To better determine the influence of highly increased IPP and DMAPP levels on the production of lycopene in bacteria, we took advantage of the MVA+ operon. The transformation of exogenously supplied MVA into IPP and DMAPP by the MVA+ operon enzymes was expected to have little or no interference with the endogenous cell metabolism in EcAB4-1 pLYC cells, as bacteria do not produce MVA or have the enzymes to metabolize it into isoprenoid precursors. Because the MVA+ operon is under the control of the arabinose-induced P_{BAD} promoter (Campos *et al*., 2001), we first tested the influence of the concentration of arabinose in the accumulation of lycopene in overnight EcAB4-1 pLYC cultures.

As expected, cells grown in media supplemented with 1 mM MVA and different concentrations of arabinose (from 0.1 to 1%) accumulated more lycopene than cells grown without the inductor (Figure 3).

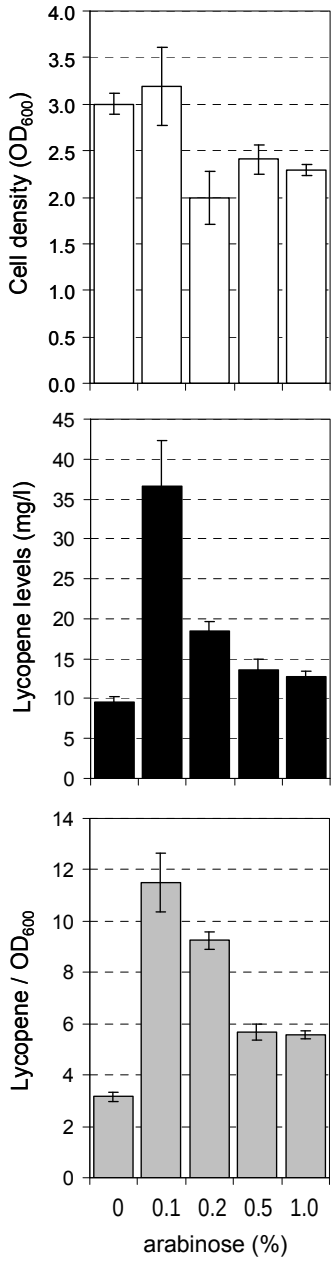

Figure 3. Influence of arabinose concentration on cell growth and lycopene accumulation in EcAB4-1 pLYC cultures. Liquid media (10 ml) containing 1 mM MVA and different concentrations of arabinose (from 0 to 1 %) were inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC cells in media lacking arabinose and MVA and grown overnight at 37ºC. Cell growth (white columns) and lycopene content (black columns) were measured as described in Figure 2. Lycopene levels normalized to cell density are also shown (gray columns). Mean values and standard errors from two replicates of two independent cultures are shown.

However, lycopene accumulation was highest at the lowest concentration of arabinose tested (0.1 %), reaching levels that were *ca.* 4-fold higher than those measured in the absence of inducer. No effect on cell growth was observed at this concentration of the inductor. Although increasing concentrations of arabinose did have an inhibitory effect on cell growth, the decrease in cell growth was not as dramatic as that observed for lycopene accumulation (Figure 3). Both total and normalized (in a per cell basis) lycopene levels decreased in an arabinose concentration-dependent manner. It is therefore possible that inducing the production of high levels of MVA+ operon enzymes could actually interfere with cell metabolism. Alternatively, the inhibitory effect on growth and lycopene

accumulation observed in EcAB4-1 pLYC cultures grown in the presence of high arabinose concentrations might be derived from the production of excess IPP and DMAPP that can not be efficiently channeled to the production of lycopene and become toxic for the cell (Campos *et al*., 2001; Martin *et al*., 2003).

To distinguish between these two possibilities, we grew EcAB4-1 pLYC cells in media supplemented with 0.1 % arabinose and two concentrations of MVA (1 and 5 mM) and monitored the time-course of growth and lycopene accumulation. As shown in Figure 4, lycopene accumulation in EcAB4-1 pLYC cultures grown in liquid medium supplemented with 0.1 % arabinose but no MVA reached a maximum level at the end of the exponential growth phase. When 1 mM MVA was included in the medium, cells accumulated significantly higher lycopene levels during their exponential growth and they continued synthesizing this carotenoid when reaching the stationary phase. No effect was observed in cell growth (Figure 4), consistent with our previous results (Figure 3).

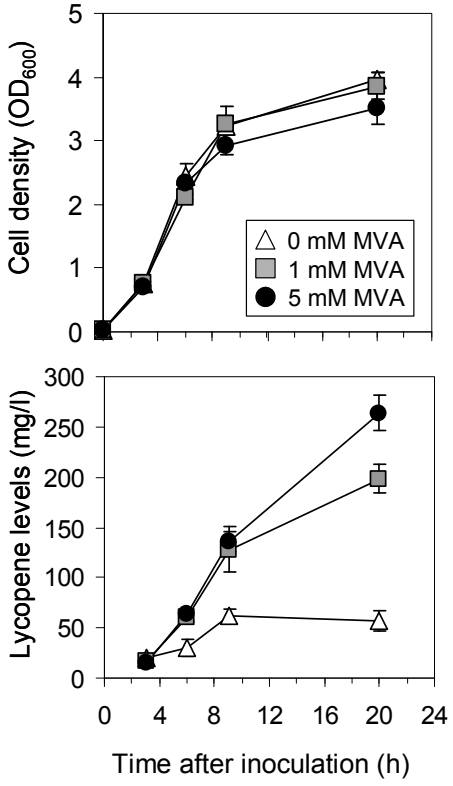

Figure 4.Time course of cell growth and lycopene accumulation in EcAB4-1 pLYC cultures. Liquid media (100 ml) containing 0.1 % arabinose and either supplemented or not with MVA were inoculated with 1 ml of overnight cultures of EcAB4-1 pLYC cells in media lacking arabinose and MVA and grown at 37ºC. At the indicated times, 1 ml samples were collected and used to measure cell growth and lycopene content as described in Figure 2. Mean values and standard errors from two replicates of two independent cultures are shown.

When the concentration of MVA added to the growth medium was increased to 5 mM, a slight inhibition of growth was observed when cells were reaching the stationary stage (Figure 4). Lycopene accumulation during the stages of active cell growth was similar in media supplemented with 1 mM or 5 mM MVA. In overnight (20 h) cultures, however, lycopene levels in cultures supplemented with 5 mM MVA were significantly higher than those measured in cultures supplemented with 1 mM MVA (Figure 4). It is therefore possible that the decrease of cell growth observed in overnight cultures might be caused by a deleterious effect of increased accumulation of hydrophobic lycopene molecules within bacterial cells. Together, the fact that lycopene accumulation was negatively correlated with arabinose concentration in the medium (Figure 3) but positively correlated with MVA concentration (Figure 4) supports that excess levels of the enzymes encoded by the MVA+ operon cause interference with cell metabolism and eventually result in a lower production of lycopene. Our results also

support that an enhanced production of MVA-derived IPP and DMAPP only marginally affects growth of EcAB4-1 pLYC cells, possibly because a large proportion of these precursors is efficiently channeled to the biosynthesis of lycopene. We next determined the maximum level of lycopene that could be produced in overnight (20 h) cultures of EcAB4-1 pLYC cells with an excess supply of MVA. As shown in Figure 5, some inhibition of cell growth was observed as the amount of MVA added to the growth medium increased. By contrast, the production of lycopene in overnight EcAB4-1 pLYC cultures was highly increased with MVA concentration up to 25 mM, reaching levels that were 10-fold higher than those measured in the absence of MVA (Figure 5).

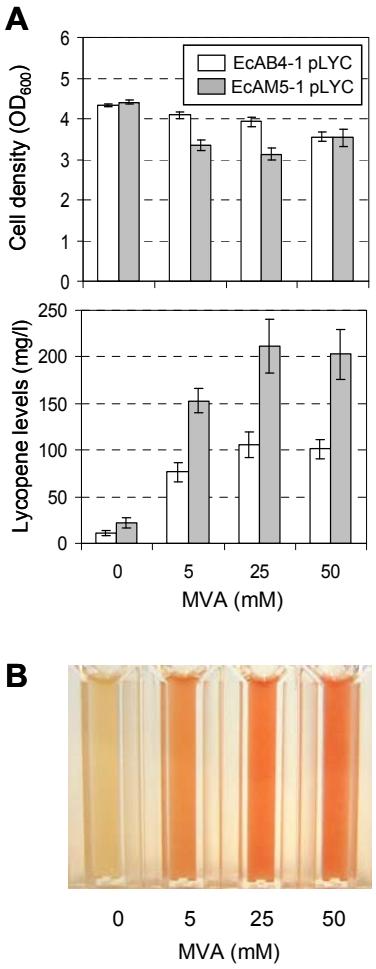

Figure 5. Cell growth and lycopene levels in MVA-supplemented cultures. Liquid media (10 ml) containing 0.1 % arabinose and the indicated concentrations of MVA were inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC (white columns) or EcAM5-1 pLYC (gray columns) strains in media lacking arabinose and MVA and grown overnight at 37ºC. Cell growth and lycopene levels (A) were measured as described in Figure 2. Mean values and standard errors correspond to two replicates of at least four independent cultures. Aliquots of EcAM5-1 pLYC cultures grown in the presence of the indicated concentrations of MVA are also shown (B).

No significant increases were observed when MVA concentration was doubled to 50 mM, suggesting that factors other than precursor supply might be limiting lycopene production under these conditions. Since only a fraction of the supplied MVA appears to be eventually metabolized into lycopene (Yoon *et al*. 2006), improved production of lycopene by increasing addition of MVA to the growth medium might be at least in part due to a facilitated import into *E.coli* cells (which do not have a transport system for MVA) by simple diffusion.

Further increases in lycopene accumulation can be achieved by optimizing factors other than precursor supply.

Because the genetic background can substantially influence lycopene accumulation in *E. coli* cells (Wurtzel *et al*., 1997; Kim and Keasling, 2001; Vadali *et al*., 2005; Yuan *et al*., 2006), we investigated the production of lycopene in a different *E. coli* strain. When the MVA+ operon was transduced from the K12 MG1655 wild-type background of the EcAB4-1 strain to BL21 (DE3) cells to create strain EcAM5-1 (Table 1), a significant increase in lycopene levels

(*ca.* 2-fold) was observed in EcAM5-1 pLYC cells compared to EcAB4-1 pLYC cells at all the concentrations of MVA tested (Figure 5). Similarly to that shown for EcAB4-1 pLYC, however, lycopene levels in EcAM5-1 pLYC cells reached a maximum at 25 mM MVA (Figure 5). These results confirm that the enzymes expressed in *E. coli* cells harboring the pLYC plasmid can channel an enhanced supply of metabolic precursors (IPP and DMAPP) to the production of lycopene until other factors become limiting for its accumulation.

A question arising from our results is why carotenoid accumulation in the BL21 background of the EcAM5-1 strain was improved relative to the near wild type background of EcAB4-1 cells. No obvious correlation has been found between genetic markers and carotenoid accumulation (Wurtzel *et al*., 1997). However, because the K12 MG1655 strain has been maintained with minimal genetic modifications, whereas BL21 is the most widely used host background for overexpression of foreign recombinant proteins and is deficient in both Lon and OmpT proteases, it is likely that the mutations in EcAM5-1 cells result in a more appropriate expression of the heterologous genes introduced in the MVA+ operon and the pLYC plasmid or/and an increased stability of the encoded proteins compared to EcAB4-1. Alternatively, EcAM5-1 cells might be able to more efficiently meet the metabolic (energy) and physical (storage) requirements associated with an upregulated production of lycopene. Attempts to control and optimize the production of the enzymes required to synthesize lycopene from its prenyl diphosphate precursors have shown that low levels of such enzymes are enough for efficient lycopene synthesis in rich media, whereas too high levels can actually be deleterious for cell growth and lycopene accumulation (Ruther *et al*., 1997; Yoon *et al*., 2006; Yoon *et al*., 2007). Much less is known about how carotenoid storage can be improved in bacteria.

Our results confirm that engineering an exogenous pathway for enhanced IPP and DMAPP biosynthesis results in higher carotenoid (lycopene) production and less interference with the metabolism of *E. coli* cells when compared to upregulating the levels of enzymes (DXS) of the endogenous MEP pathway. Under our growth conditions, EcAM5-1 pLYC cells accumulated up to 228 mg of lycopene per liter of overnight culture, an impressive 20-fold increase compared to the levels measured in EcAB4-1 pLYC cells grown in the absence of MVA (Figure 5). Further optimization of growth conditions, vectors, and host strains should result in even higher production of lycopene. In any case, the increase in lycopene accumulation observed in EcAB4-1 pLYC and EcAM5-1 pLYC cells grown in the presence of MVA is substantially higher than that reported for *E. coli* DH5α cells harboring a plasmid-bound IPTG-inducible MVA+ operon from *Streptococcus pneumoniae* (Yoon *et al*., 2006; Yoon *et al*., 2007). The use of a “complete” MVA operon with all the *Streptomyces* genes required to convert acetyl coenzyme-A (produced by the bacteria) into IPP and DMAPP (Takagi *et al*., 2000) has been found to only result in increased lycopene production in *E. coli* strains in which the acetate pathway is inactivated (Vadali *et al*., 2005). However, further work is required to establish whether a complete MVA pathway constructed with genes from other species and with a fine control of its expression might allow to significantly increase the production of lycopene in rich media with no added MVA without compromising the metabolic balance of *E. coli* cells.

Materials and methods

Bacterial strains and growth conditions

Plasmids and strains used in this study are included in Table 1. The MVA^+ synthetic operon from strain EcAB4-1 (Sauret-Güeto *et al*., 2006) was incorporated into the genome of the BL21 (DE3) strain by phage P1 transduction to construct strain EcAM5-1. Competent cells were transformed with plasmid pACCRT-EIB (here designated pLYC) harboring the *Erwinia uredovora* (currently named *Pantoea ananatis*) *crtE*, *crtB* and *crtI* genes for lycopene synthesis (Cunningham *et al*., 1993) and, when indicated, plasmid pTAC-ORF2 (here designated pTAC-DXS) containing the *E.coli dxs* gene under the control of an IPTG-inducible promoter (Lois *et al*., 1998). All strains were grown at 37ºC in Luria broth (LB) medium supplemented with antibiotics to final concentrations of 25 µg/ml kanamycin (Km, to select for the MVA+ operon), 17 µg/ml chloramphenicol (Cm, to select for the pLYC plasmid) or/and 100 µg/ml ampicillin (Ap, to select for the pTAC-DXS plasmid). When indicated, the growth medium was supplemented with arabinose and MVA prepared as described (Campos *et al*., 2001). Bacterial growth in liquid media was monitored by measuring optical density at 600 nm (OD_{600}). When required, the culture was diluted to enable photometric measurement in the linear range (OD_{600}=0.1-0.5).

Table 1. Plasmids and strains used in this study.

Name	*Description*	*Antibiotic marker*	*Reference*
Plasmids			
pLYC	pACCRT-EIB, with *crtE, crtB* and *crtI* genes	Chloramphenicol	Cunningham et al., 1993
pTAC-DXS	pTAC-ORF2, with a IPTG-inducible *dxs* gene	Ampicillin	Lois et al., 1998
Strains			
K12 MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	none	Blattner et al., 1997
EcAB4-1	K12 MG1655 with an arabinose-inducible MVA+ operon	Kanamycin	Sauret-Güeto et al., 2006
BL21(DE3)	B F– *dcm ompT hsdS*(rB- mB-) *gal* λ(DE3)	none	Stratagene
EcAM5-1	BL21(DE3) with an arabinose-inducible MVA+ operon	Kanamycin	This work

Quantification of lycopene production

Fresh LB medium containing the appropriate supplements was inoculated with 0.01 volumes of overnight cultures of several independent colonies from each *E.coli* strain and grown at 37ºC. After quantifying cell growth (OD_{600}), a 1.3 ml aliquot of the culture was centrifuged at 13,000 *g* for 3 min and the cell pellet was resuspended in 700 µl of acetone. The samples were incubated at 55ºC for 15 min in the dark and then centrifuged at 13,000 *g* for 10 min to recover the supernatant with the pigment, which was placed in a clean tube. At least two samples were taken from each culture for lycopene extraction. When necessary, up to three extractions in acetone were performed to remove all color from the cell pellet of a single sample. In this case, the supernatants of the different extractions were pooled. Lycopene was quantified as described (Harker and Bramley, 1999) by measuring absorbance at 472 nm of diluted supernatants in acetone and comparing with a standard curve made with known concentrations of a lycopene standard (Sigma).

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Artículo 2:

Carotenoid accumulation in engineered *Escherichia coli* cells expressing cyanobacterial genes

Acumulación de carotenoides en células de *Escherichia coli* que expresan genes procedentes de cianobacterias

Resumen

Los carotenoides son pigmentos de naturaleza isoprenoide de gran interés como colorantes y nutracéuticos. Aunque pueden ser producidos en células de *E. coli* modificadas genéticamente, su naturaleza hidrofóbica impide la acumulación de grandes cantidades de pigmentos sin interferir con las funciones de la membrana bacteriana y con el crecimiento celular. Con el propósito de incrementar la capacidad de almacenamiento en células de *E. coli* se analizó el efecto de la expresión de genes procedentes de la cianobacteria carotenogénica *Synechocystis*. Una proteína de unión a carotenoides producida *in vitro*, la "orange carotenoid binding protein" (OCP), era capaz de unir el carotenoide acíclico licopeno y el bicíclico β-caroteno. Sin embargo, la acumulación de estos carotenoides no aumentaba en células de *E. coli* que sobreexpresaban esta proteína recombinante, incluso cuando la disponibilidad de precursores no era un factor limitante. La transformación de células de *E. coli* modificadas genéticamente para la producción de carotenoides con una librería de fragmentos de DNA procedentes de *Synechocystis* y el análisis de los transformantes que presentaban una mayor producción de licopeno tampoco permitió identificar proteínas que pudieran mejorar el almacenamiento de pigmentos en bacterias. En su lugar, nuestros resultados sugieren que la mayor acumulación de carotenoides observada se debe a una interferencia con el metabolismo celular.

Experimentos específicos de la tesis doctoral:
Figura 2, Figura 3, Figura 4, Figura 5, Figura 6.

Carotenoid accumulation in engineered *Escherichia coli* cells expressing cyanobacterial genes.

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ABSTRACT

Carotenoids are isoprenoid pigments of huge industrial and nutraceutical interest. They can be produced in engineered *E. coli* cells but their lipophylic nature prevents their accumulation to very high levels without interfering with the bacterial membrane functions and cell growth. To try to improve the storage capacity in *E. coli* cells we analyzed the effect of expressing genes from the carotenogenic cyanobacterium *Synechocystis*. An *in vitro*-produced orange carotenoid binding protein (OCP) was able to bind the acyclic carotenoid lycopene and the bicyclic carotenoid beta-carotene. However the final accumulation of these carotenoids was not increased *in vivo*. In *E. coli* cells overexpressing this recombinant protein, even when isoprenoid precursors where not a limiting factor. Transformation of carotenoid –producing *E. coli* cells with a library of genomic *Synechocystis* DNA sequences and analysis of transformants with increased carotenoid levels was also unsuccessful to identify proteins that could improve pigment storage in bacteria. Instead, our results suggest that the carotenoid accumulation phenotype observed the selected *E. coli strains* harboring *Synechocystis* DNA fragments resulted from interference with cell metabolism.

Keywords: carotenoids, isoprenoids, metabolic engineering, orange carotenoid protein, cyanobacterial genes, MEP pathway.

Introduction

Carotenoids are an abundant family of isoprenoid pigments synthesized by all photosynthetic organisms (including plants, algae and cyanobacteria) and some non-photosynthetic bacteria and fungi. Animals do not produce carotenoids *de novo*, despite they are essential as a source of retinoids (including vitamin A). A dietary intake of carotenoid-rich foods also appears to provide health benefits due to their antioxidant nature (Fraser and Bramley, 2004; Stahl and Sies, 2005). Besides their phytonutrient properties and their use as nutraceuticals, carotenoids have an important industrial value as natural pigments (Botella-Pavía and Rodríguez-Concepción, 2006). Most commercial carotenoids are currently obtained by chemical synthesis or from natural sources. However, it is expected that the demand for these pigments will highly increase in the future, making it necessary to develop novel systems for an efficient production of carotenoids of interest. Several biotechnological approaches have successfully engineered microorganisms and plants for increased carotenoid levels (Lee and Schmidt-Dannert, 2002; Botella-Pavía and Rodríguez-Concepción, 2006; Giuliano et al., 2008; Kirby and Keasling, 2009). In particular, major advances have been reported by engineering carotenoid production in non-carotenogenic cells such as those of the rice endosperm (Golden Rice) or *Escherichia coli* after transformation with appropriate carotenoid biosynthetic genes (Albrecht *et a*l., 2000; Farmer and Liao, 2000; Schmidt-Dannert *et al*., 2000; Ye *et al*., 2000; Alper *et al*., 2005). Despite these achievements, further improvements will require to identify and manipulate the key steps controlling metabolic flux to carotenoids and to improve pigment storage capacity of the host cells.

The main pathway for carotenoid biosynthesis in plants and microorganisms is elucidated (Cunningham and Gantt, 1998). Structurally carotenoids are tetraterpenes derived from geranylgeranyl diphosphate (GGPP), a prenyl diphosphate molecule synthesized by condensation of the universal isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors can be synthesized by two different pathways: the methylerythritol 4-phophate (MEP) pathway and the mevalonate (MVA) pathway. In most eubacteria (including *E. coli*) and plant plastids carotenoid precursors are produced by the MEP pathway (Rodríguez-Concepción and Boronat, 2002). The first reaction of this pathway (Figure 1) is the production of deoxyxylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate, catalyzed by the enzyme DXP synthase (DXS). In the last step of the MEP pathway, IPP and DMAPP are simustaneously synthesized by the enzyme hydroxymethylbutenyl diphosphate reductase (HDR). It is well established that DXS and HDR are major rate-determining enzymes of the MEP pathway (Rodríguez-Concepción, 2006; Córdoba *et al*., 2009). In agreement, increased DXS or HDR levels result in an enhanced production of carotenoids in *E. coli* strains harboring carotenogenic enzymes from the phytopathogenic proteobacterium *Erwinia uredovora*, also known as *Pantoea ananas* (Harker and Bramley, 1999; Cunningham *et al*., 2000; Matthews and Wurtzel, 2000; Kim and Keasling, 2001; Rodriguez-Villalon *et al*., 2008). The limiting role of precursor supply for carotenoid production in *E. coli* is also deduced from the dramatically increased accumulation of these

compounds achieved by using strains with an artificial MVA operon (Figure 1) that allows the production of high IPP and DMAPP levels from exogenously supplied MVA (Yoon *et al*., 2006; Rodriguez-Villalon *et al*., 2008; Harada *et al*., 2009; Yoon *et al*., 2009). Much less is known about how carotenoid storage can be improved in bacteria.

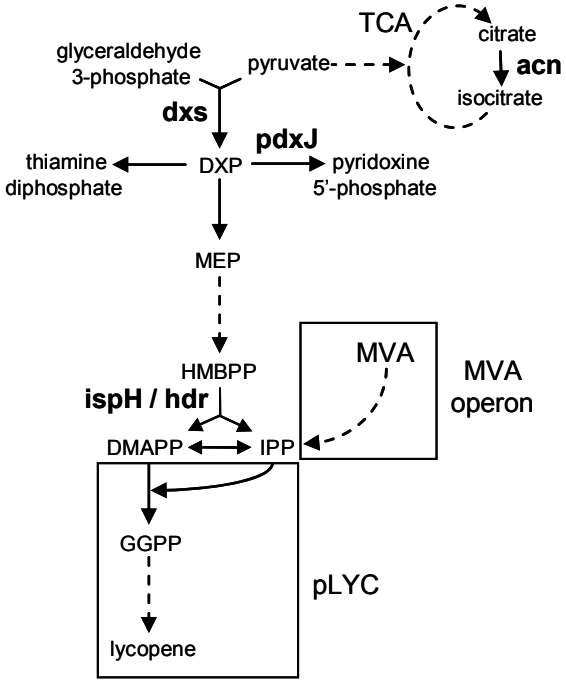


Figure1. Pathways involved in lycopene biosynthesis in engineered *E. coli* cells. Pyruvate is a common precursor of the methylerythritol 4-phosphate (MEP) pathway and the tricarboxylic acid pathway (TCA). The pathways encoded by MVA operon and pLYC plasmids are boxed. acn, aconitate hidratase; dxs, deoxyxylulose 5-phosphate (DXP) synthase; pdxJ, pyridoxine 5-phosphate synthase; ispH/hdr, hydroxymethylbutenyl diphosphate (HMBPP) reductase. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate.

Because carotenoids are highly insoluble lypophylic compounds, they are found embedded in membranes, lipid globules, and other hydrophobic environments, usually associated with proteins. The accumulation of high carotenoid levels in non-carotenogenic bacteria such as *E. coli* can therefore affect membrane structures and eventually interfere with normal cell functions, negatively impacting microbial growth (Rodriguez-Villalón *et al*., 2008) In carotenogenic cyanobacteria, carotenoids are also found associated with water-soluble carotenoid binding proteins such as the Orange Carotenoid Protein (OCP), encoded by the *slr1963* gene in *Synechocystis* PCC 6803 (Wu and Krogmann, 1997). This protein, recently shown to play an essential role in photoprotection and to function as a light sensor (Wilson *et al*., 2006; Wilson *et al*., 2008), forms homodimers inside which the non-covalently bound carotenoid molecules are almost buried (Kerfeld *et al*., 2003). OCP preferentially binds the bicyclic keto-carotenoid 3'-hydroxyechinenone in cyanobacteria, but it can also bind other non-native carotenoids (Kerfeld, 2004; Punginelli *et al*., 2009). In this work, we aimed to improve the carotenoid storage capacity of *E. coli* cells by overexpressing OCP and other *Synechocystis* genes.

Results and discussion

High levels of soluble OCP with carotenoid-binding properties can be obtained in *E. coli*

As a direct strategy to improve the carotenoid storage capacity of *E. coli* cells, we aimed to investigate whether overexpression of a soluble carotenoid-binding protein such as the cyanobacterial OCP could improve the accumulation of these lypophilic compounds in engineered bacteria. The OCP-encoding gene from *Synechocystis* PCC 6803 (*slr1963*) was cloned into an *E. coli* expression vector for the production of a His-tagged protein under the control of an IPTG-induced promoter (pET-OCP construct). After transformation of BL21 (DE3) *E.coli* cells with this construct and induction of foreign protein expression with IPTG, we confirmed that the recombinant protein (of a predicted size of *ca.* 35 kDa) could be synthesized at high levels and in soluble form (Figure 2).

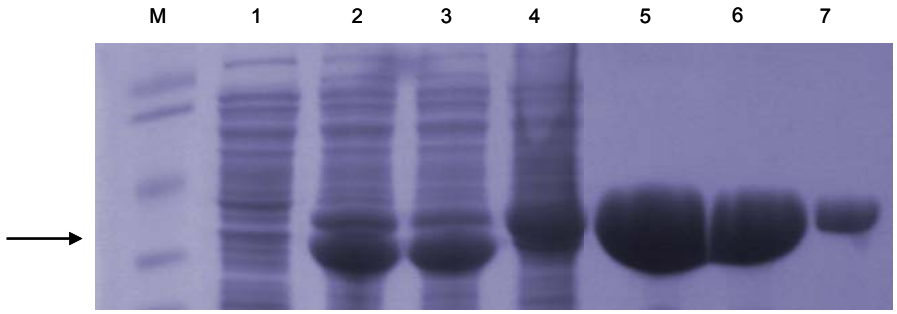
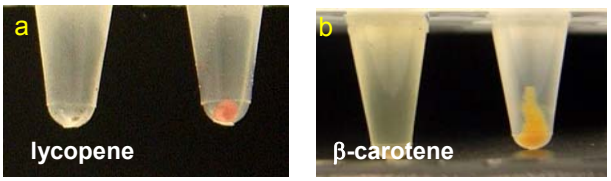


Figure 2. Protein extracts from cultures of BL21 cells transformed with the pET-OCP construct were electrophoresed by SDS-PAGE and stained with Coomassie Blue. Lanes correspond to the following: M, molecular weight marker; 1, 0 mM IPTG, total cell extract; 2, 0,4 mM IPTG, total cell extract; 3 supernatant; 4 pellet; 5, 6, 7, diferent fractions of recombinant OCP after protein purification. The arrow to the right of the gel indicates the expected size (35 kDa).

The His-tagged protein overproduced in *E. coli* cells was purified by immobilized metal ion affinity chromatography (IMAC). As shown in Figure 2, large amounts of highly pure recombinant OCP were obtained in several fractions that eluted from the column using an imidazol gradient elution.

We next evaluated whether the recombinant OCP could bind carotenoids different from 3'-hydroxyechinenone, the natural carotenoid bound to this protein in cyanobacterial cells. To test the carotenoid-binding properties of the purified OCP, 0.2 mg of the carotenoids lycopene (acyclic) and β-carotene (with one beta-ring in each end of the molecule, similar to 3'-hydroxyechinenone) were incubated with the same amounts of either OCP or BSA as a negative control. After 90 minutes at room temperature, a coloured precipitate was formed in the samples containing OCP, but not in those incubated with BSA (Figure 3). These results suggest that the recombinant OCP produced in *E. coli* was able to bind different carotenoids, including lycopene (a strong antioxidant that provides protection against some cancer types) and beta-carotene (the most efficient precursor of vitamin A).



OCP	-	+	-	+
BSA	+	-	+	-

Figure 3. Carotenoid binding assays. Incubation of 0.2 mg of recombinant OCP or bovine serum albumine (BSA) with 0.2 mg of lycopene (a) or β-carotene (b). In both cases the presence of OCP leads in a coloured precipitate.

Overexpression of OCP in *E.coli* cells does not result in higher carotenoid levels

To evaluate the influence of OCP on the accumulation of carotenoids in *E. coli* we used the EcAM5-1 strain, previously created in our lab by incorporating a MVA^+ synthetic operon into the genome of BL21 (DE3) cells (Rodriguez-Villalon *et al*., 2008). EcAM5-1 cells were co-transformed with a plasmid (pLYC) encoding the *Erwinia uredovora* enzymes required for the production of the carotenoid lycopene from the IPP and DMAPP (Figure 1) and with the pET-OCP vector. Cells transformed with pLYC only were used as controls. As shown in Figure 4, cells harbouring the pET-OCP plasmid did not show any growth defect, even when high levels of the recombinant protein were produced after induction with IPTG. However, the accumulation of lycopene was not improved but even reduced in OCP-producing cells compared to control cells lacking the pET-OCP plasmid (Figure 4). To investigate whether OCP was negatively impacting either the synthesis or the accumulation of lycopene, we next induced lycopene biosynthesis by adding MVA to the growth medium (Rodriguez-Villalon *et al*., 2008) and evaluated how the presence of OCP influenced final lycopene levels. As expected, the addition of MVA in the growth medium led to a substantial increase in the production of lycopene (Figure 5), most likely because it provides an additional supply of IPP and DMAPP precursors (Yoon *et al*., 2006; Rodriguez-Villalon *et al*., 2008; Harada *et al*., 2009; Yoon *et al*., 2009).

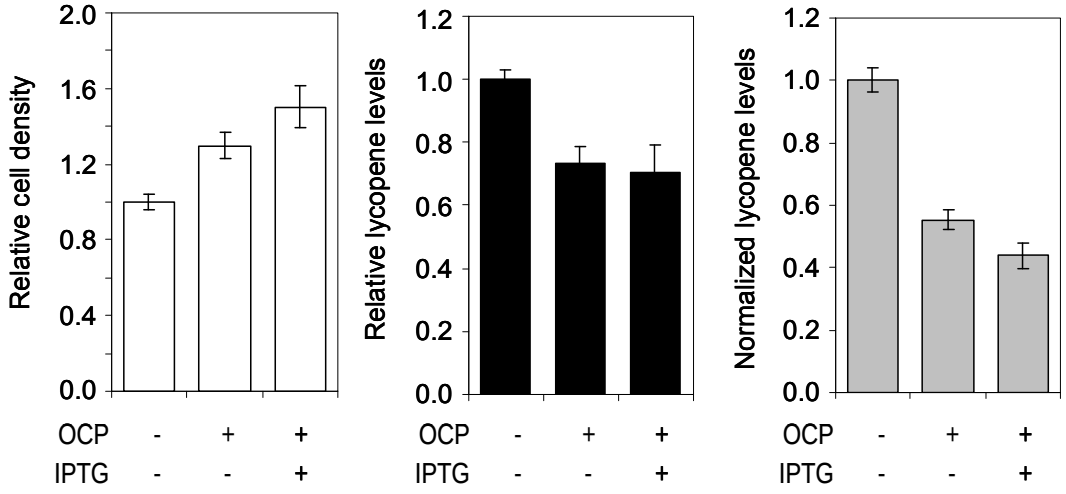


Figure 4. Lycopene production in EcAM5-1 *E. coli* cells harbouring the pLYC and pET-OCP plasmids as indicated. Cell grown and lycopene production are relative to control cells (harbouring only the pLYC plasmid). Pigment production was normalized to cell growth of overnight cultures incubated in the presence (+) or in the absence (-) of 0.4 mM IPTG that induces OCP expression.

Cells transformed with pET-OCP also accumulated more lycopene in the presence of MVA, but the final levels were slightly lower than those achieved in control cells (Figure 5).
These results suggest that the presence of OCP does not interfere with the biosynthesis of lycopene but it unexpectedly has a negative impact in the final accumulation of this lypophilic pigment. It is possible that preferential binding of lycopene to OCP prevents efficient targeting of this lipophylic pigment to cell membranes, eventually resulting in a reduced accumulation of carotenoids (which might be less stable when bound to OCP). Together, our results indicate that expression of OCP is not an appropriate strategy to improve the carotenoid storage capacity of *E. coli* cells.

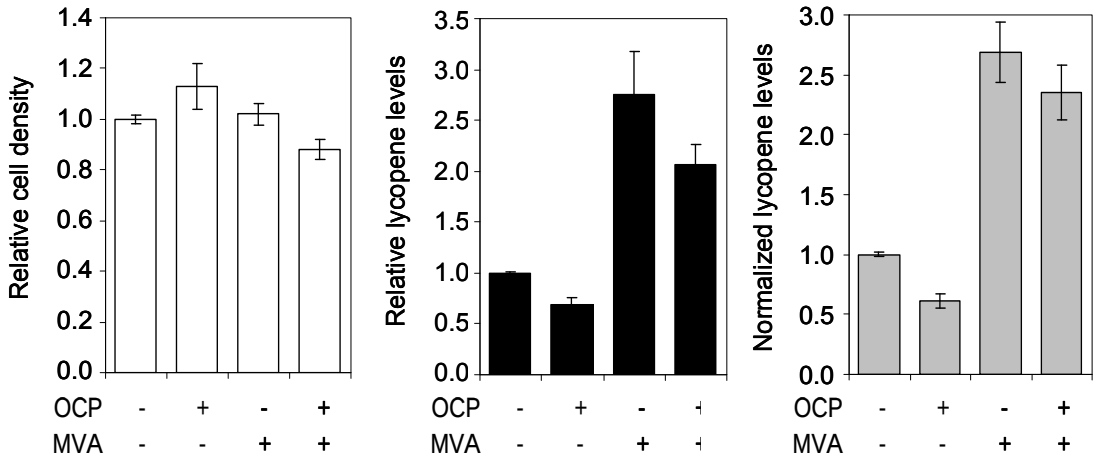


Figure 5. Lycopene production in EcAM5-1 *E.coli* cells cotransformed with pLYC and pET-OCP incubated in LB media supplemented with 5 mM MVA. Cell grown and lycopene production are relative to control cell (cells harbouring pLYC plasmid grown in the absence of MVA). Lycopene levels are normalized to cell growth. Pigment accumulation represented is the average of 4 independent experiments.

Synechocystis **sequences encoding enzyme fragments result in increased carotenoid accumulation when expressed in** ***E. coli***

As an alternative strategy to improve the accumulation of carotenoids in *E. coli* by expressing proteins of carotenoid-producing cyanobacteria, we used a *Synechocystis* PCC 6803 genomic library synthesized in pBluescript as described (Linden *et al*., 1993). DH5α *E.coli* cells harboring the pLYC plasmid were transformed with the library and positive transformants were selected on LB plates supplemented with chloramfenicol and ampicillin. Among the 120.000 independent clones that we visually screened for high lycopene levels, about 400 colonies were selected as displaying a stronger pink color. After streaking these colonies on fresh LB plates, a second round of visual screening led to the selection of 85 colonies that were darker pink compared to controls transformed with an empty plasmid. Most of these clones were undistinguishable from controls in terms of lycopene accumulation when grown in liquid media and were therefore discarded. Only 3 clones (S82, S91 and S92) were finally selected for further studies because they consistently accumulated twice the levels of lycopene found in control clones harbouring an empty vector control (Figure 6A).

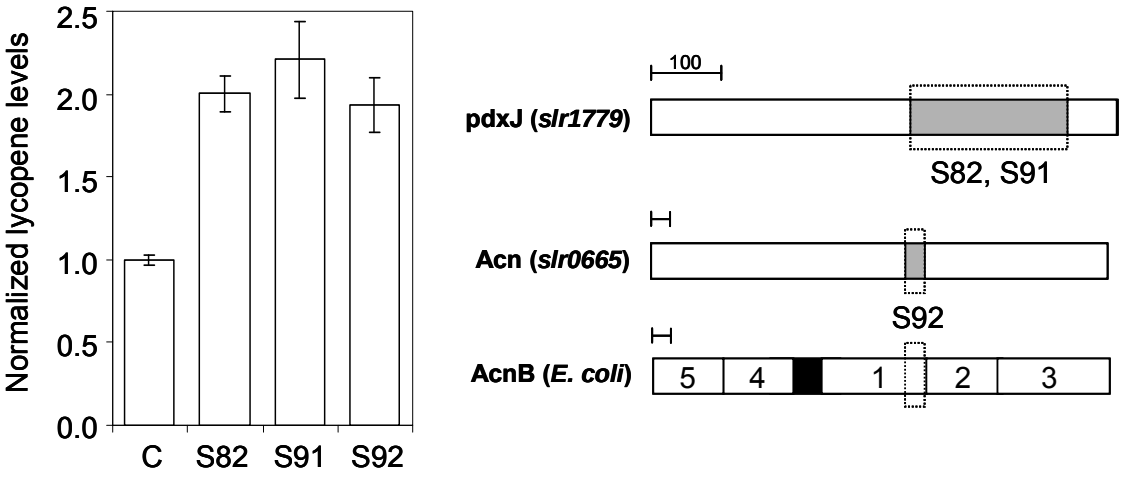


Figure 6. Analysis of *E.coli* clones showing increased lycopene levels. (A) Lycopene production in clones harbouring *Synechocystis* genes. Pigment production was normalized to cell growth and control represents lycopene production of cells cotransformed with an empty vector. (B) Schematic representation of the sequences present in lycopene-overproducing clones. Sequences from the individual *Synechocystis* genes found in clones S82, S91 and S92 are marked in grey. The position of the S92 sequence on the *E. coli* AcnB protein is also indicated. Bars correspond to 100 bp.

Sequencing of the corresponding plasmids isolated from these clones showed that they contained only short sequences from the coding region of genes encoding metabolic enzymes. The sequences cloned in S82 and S91 were identical and correspond to 257 bp of the *slr1779* gene, encoding pyridoxine 5'-phosphate synthase (Figure 6B). This enzyme, encoded by the *pdxJ* gene in *E. coli*, participates in the biosynthesis of the vitamin B_6 (pyridoxine) by catalyzing the production of pyridoxine 5'-phosphate from the condensation of 3-hydroxy-1-aminoacetone phosphate and DXP, the first intermediate of the MEP pathway (Rodríguez-Concepción and Boronat, 2002; Fitzpatrick *et al*., 2007). The fragment present in clones S82 and S91 corresponds to the region between the positions D137 and R221 of the *E. coli* PdxJ protein, which contains the residues of the active site E153, H193, G194, G215 y H216 (Garrido Franco *et al*., 2000). It is possible that the presence of a truncated PdxJ-encoding sequence in *E. coli* cells transformed with the S82 and S91 plasmids somehow disrupts the expression of the endogenous *pdxJ* gene or the activity of the corresponding protein, eventually resulting in higher DXP levels (Figure 1). Because DXS activity and therefore DXP levels are limiting for lycopene production under experimental circumstances very similar to those described here (Rodriguez-Villalon *et al*., 2008), we speculate that an enhanced availability of DXP might contribute to an enhanced carotenoid production in the S82 and S91 clones.

In the case of the S92 clone, the cloned fragment corresponded to a 104 bp sequence from the coding region of the *slr0665* gene, encoding aconitase (acn) or aconitate hydratase (Figure 6B**).** Aconitases are iron-sulfur enzymes that catalyze the reversible isomerization of citrate and isocitrate *via cis*-aconitate in the tricarboxylic acid (TCA) and glyoxylate cycles. *E. coli* possesses two genes encoding acn enzymes, *acnA* and *acnB* (Bradbury *et al*., 1996;

Cunningham *et al*., 1997). As the major TCA cycle enzyme, AcnB is synthesized during exponential growth, whereas AcnA is a stress-induced stationary-phase enzyme (Cunningham *et al*., 1997). When compared with the whole *E. coli* genome, the S92 DNA fragment was most similar to a fragment of the *acnB* gene corresponding to the positions G495 to P525 of the protein. According to a previous nomenclature of AcnB protein domains (Williams *et al*., 2002; Tang *et al*., 2005), this region belongs to domain 1 and it is located next to domain 2 (Figure 6B**)**, which together with domains 3 and 4 form a highly conserved active site around the [4Fe-4S] cluster. AcnB also fulfils a regulatory role: under stress conditions, the [4Fe-4S] cluster is destroyed, catalytic activity is lost and the resulting apoprotein binds to specific mRNAs to regulate transcript stability or translation (Kiley and Beinert, 2003; Tang *et al*., 2005). Interestingly, the last two enzymes of the MEP pathway, including the rate-determining HDR enzyme, are iron-sulfur proteins as well (Rodríguez-Concepción, 2006). Based on these observations, several possibilities might explain the observed phenotype of lycopene overaccumulation in cells harbouring the S92 clone. The cyanobacterial fragment might interfere with *E. coli* AcnB activity in the TCA cycle, resulting in a increased availability of pyruvate for the MEP pathway and carotenoid biosynthesis (Figure 1). It is also possible that this fragment could also prevent the assembly of a functional iron-sulfur cluster, which would result in AcnB apoprotein-mediated changes in gene expression that might favour lycopene accumulation. An increased HDR activity as a result of an improved availability of iron-sulfur clusters would also result in an enhanced production of lycopene as a result on an activated flux through the MEP pathway (Cunningham *et al*., 2000).

Concluding remarks

Although further experiments will be required to unveil the precise mechanisms by which transformation of *E. coli* cells with fragments of *Synechocystis* genes encoding enzymes metabolically related to the MEP pathway leads to an enhanced accumulation of lycopene, our results can be interpreted in agreement with the current model that the supply of isoprenoid precursors is limiting for the production of carotenoids in bacteria and also in plant cells. The library we used contained full genes, but we do not retrieve any clone encoding flux-controlling *Synechocystis* MEP pathway enzymes such as DXS or HDR or any carotenoid-binding protein that could improve the accumulation of lycopene in transformed *E. coli* cells. We speculate that the expression level achieved with the pBluescript vector might not be high enough for such genes to be selected with our strategy. Despite these results and those on the negative effect of OCP expression on carotenoid accumulation reported here, the cyanobacterial genomes should not be discarded as a powerful source of genes with a potential to improve the production or/and the storage of carotenoids in *E. coli*.

Materials and methods

Cloning, expression and purification of *Synechocystis* OCP.

A sequence encoding the full-length OCP was amplified by PCR using *Synechocystis* genomic DNA as a template and primers OCP-F (5´-CCTATGCCATTCACCATTGAC) and OCP-R (5´-ATTGTAGCGAGCAAAGTTGAG). The PCR product was cloned into the *Hinc*II site of the pET-23b vector and sequenced to confirm its integrity. In the resulting construct, pET-OCP, the expression of a recombinant OCP with a C-terminal His-tag was under the control of an IPTG-inducible promoter. For OCP overexpression in *E.coli*, BL21 (DE3) cells were transformed with the pET-OCP construct and isolated colonies were grown at 37ºC in 5 ml of Luria broth (LB) medium supplemented with 100 mg/L ampicillin. Overnight cultures (2.5 mL) were used to inoculate 250 ml of fresh media. After growth at 37ºC until reaching an optical density at 600 nm (OD600) of 0.5, cultures were supplemented with 0.4 mM IPTG and grown for 6 more hours. Cells were then sedimented by centrifugation and resuspended in seven volumes of lysis buffer containing 0.5 mM EDTA, 1mg/ml lysozyme (Sigma), and a protease inhibitor cocktail (Roche). Following 5 rounds of sonication (35 W pulses for 30 s) and cooling (ice-bath for 1 min), samples were centrifuged for 30 minutes at 12500 r.p.m. in a microfuge. The soluble fraction (supernatant) was filtered and loaded in a Ni^{2+} 1ml IMAC column. Protein elution was performed in a linear gradient of 10-500 mM Imidazol and the process was monitorized at 280 nm. Protein analysis was carried out by SDS-PAGE and Coomasie blue staining. Protein concentration was estimated by the Bradford method using BSA as a protein standard.

Carotenoid binding assays.

The same amount (0.2 mg) of purified recombinant OCP or bovine serum albumine (BSA; Sigma) was incubated with 0.2 mg of different carotenoids (β-carotene, lycopene, cantaxanthin) in 1 mL of PBS buffer (Sigma). After incubation for 90 min at room temperature, proteins were pelleted by centrifugation for 5 min at maximum speed in a microfuge.

Quantification of carotenoid production and cell growth in transformed cells

Competent cells of the EcAM5-1 strain, created by incorporating a MVA synthetic operon into the genome of BL21 (DE3) cells (Rodriguez-Villalon *et al*., 2008), were transformed with plasmid pLYC (Rodriguez-Villalon *et al*., 2008) harbouring the *Erwinia uredovora* genes required for the biosynthesis of lycopene from endogenous IPP and DMAPP precursors. When indicated, these cells were also cotransformed with plasmid pET-OCP. The corresponding transformants were selected on LB plates supplemented with 17 mg/L chloramphenicol (to select for the pLYC plasmid) or/and 100 mg/L ampicillin (to select for the pET-OCP vector). Several independent colonies were grown overnight in liquid media and 0.06 mL of each culture were used to inoculate 6 mL of fresh LB medium containing the appropriate supplements. After growth at 37ºC until reaching an OD600 of 0.5, IPTG was added to a final concentration of 0.4 mM and grown for 6 more hours when indicated. Cell growth and quantification of lycopene

levels were determined as described (Rodriguez-Villalon *et al*., 2008). Similar experiments were also performed with media supplemented with 0.1 % arabinose and 5 mM MVA prepared as described (Campos *et al*., 2001).

Screening and analysis of carotenoid-overproducing *E. coli* strains

A *Synechocystis* 6803 genomic library was constructed by partial digestion of genomic DNA with *Hpa*II. Fragments of 4-6 kb were cloned in the *Cla*I restriction site of pBluescript (Linden *et al*., 1993). DH5α cells were cotransformed with 1 µg of this genomic library and pLYC and grown on LB plates supplemented with ampicillin and chloramphenicol. Colonies with stronger pink colour were grown in 3 ml of LB liquid media supplemented with appropriate antibiotics for 24 hours at 37°C. Cell grown and lycopene accumulation was analyzed as described (Rodriguez-Villlalon *et al*., 2008).

Acknowledegments

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Artículo 3:

Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown Arabidopsis seedlings

La actividad fitoeno sintasa controla la biosíntesis de carotenoides y de sus precursores metabólicos en plántulas de Arabidopsis crecidas en la oscuridad.

Resumen

Los carotenoides son isoprenoides plastídicos esenciales para la vida de las plantas. En *Arabidopsis thaliana*, la síntesis de carotenoides se induce fuertemente cuando las plántulas que germinan en la oscuridad (etioladas) emergen de bajo tierra y la luz desreprime la fotomorfogénesis, provocando la conversión de los etioplastos en cloroplastos. En este trabajo hemos observado que la biosíntesis de carotenoides también se estimula cuando la fotomorfogénesis se desreprime en ausencia de luz. La mayor producción de carotenoides observada se correlaciona con un incremento en la actividad fitoeno sintasa (PSY, el primer enzima de la carotenogénesis) y con una inducción de su expresión génica en cotiledones (el lugar donde se acumulan los carotenoides en plántulas crecidas en la oscuridad). En estas condiciones, los precursores metabólicos para la síntesis de carotenoides provienen mayoritariamente de la vía del metileritritol 4-fosfato (MEP). La desrepresión de la fotomorfogénesis en la oscuridad produce una acumulación post-transcripcional de enzimas claves en el control del flujo metabólico como la desoxixilulosa 5-fosfato sintasa (DXS). A diferencia de lo observado en plántulas crecidas en luz, la sobreexpresión de DXS en plántulas crecidas en la oscuridad no produce un incremento en la acumulación de carotenoides. Por el contrario, una inducción de la actividad PSY en plántulas transgénicas etioladas produce un incremento en los niveles de carotenoides paralela a una acumulación post-transcripcional de DXS. Estos datos ponen de manifiesto la existencia de un mecanismo de retroalimentación por el cual PSY controla el flujo metabólico hacia carotenoides en plantas.

Experimentos específicos de la tesis doctoral:

Figura 2; Figura 3; Figura 4; Figura 5B; Figura 6B; Figura 7B; Figura 8, Tablas S1 y S2.

Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown Arabidopsis seedlings.

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ABSTRACT

Carotenoids are plastidial isoprenoids essential for plant life. In *Arabidopsis thaliana* carotenoid biosynthesis is strongly upregulated when seedlings germinated in the dark (etiolated) emerge from the soil and light derepresses photomorphogenesis, causing etioplasts to become chloroplasts. We found that carotenoid biosynthesis is also induced when deetiolation is derepressed in the absence of actual light, eventually resulting in improved greening (chlorophyll accumulation) upon illumination. The increased production of carotenoids in the dark correlates with an upregulated activity of phytoene synthase (PSY, the first committed enzyme of carotenogenesis) and an induction of *PSY* gene expression in cotyledons (where carotenoids accumulate in dark-grown seedlings). The metabolic precursors for carotenoid synthesis under these conditions are mostly supplied by the plastidial methylerythritol-4-phospate (MEP) pathway. Accumulation of flux-controlling MEP pathway enzymes such as deoxyxylulose-5-phosphate synthase (DXS) is posttranscriptionally increased when deetiolation is derepressed in the dark. Unlike that observed in light-grown plants, however, the sole overexpression of DXS in dark-grown seedlings does not increase carotenoid accumulation. By contrast, induced expression of a PSY-encoding transgene results in increased carotenoid levels and a concomitant posttranscriptional accumulation of DXS. These data provide evidence for a feedback mechanism by which PSY controls metabolic flux to the carotenoid pathway in plants.

Keywords: carotenoid, phytoene synthase, MEP pathway, regulation, etioplasts, greening.

Introduction

Carotenoids are isoprenoid pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. Plant carotenoids are synthesized in plastids, where they serve as precursors of important growth regulators such as abscisic acid and strigolactones (Klee 2008, Nambara and Marion-Poll 2005). In chloroplasts of photosynthetic tissues, carotenoids also act as membrane stabilizers and accessory light-harvesting pigments, and they are essential to channel excess energy away from chlorophylls for essential protection against photooxidative damage (Baroli and Niyogi 2000; Pogson and Rissler 2000). Chromoplasts typically found in flower and fruit tissues are plastids specialized in accumulating carotenoids, providing yellow to red colors that attract animals to disperse pollen and seeds. Much lower amounts of carotenoids are also synthesized in the etioplasts of dark-grown (etiolated) seedlings and in other non-photosynthetic plastids such as amyloplasts and leucoplasts (Howitt and Pogson 2006). Carotenoids have been extensively used as natural pigments, and they are an important source of retinoids (including vitamin A) and antioxidants of these health-related properties and the increasing demand for natural products have spurred an unprecedented interest in the biotechnological overproduction of carotenoids in plants. Despite important achievements in this area, a major problem still to be solved is our limited knowledge on the regulation of the carotenoid pathway in different plant organs and tissues and its cross-talk with other isoprenoid pathways (Giuliano *et al.* 2008).

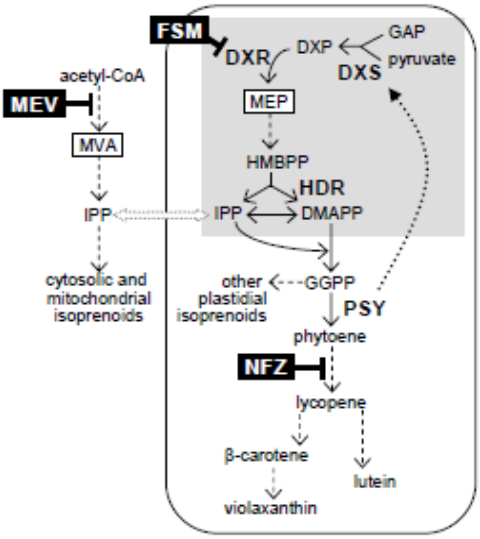

Figure1. Pathways for isoprenoid biosynthesis in plant cells. The steps inhibited by mevinolin (MEV), fosmidomycin (FSM), and norflurazon (NFZ) are indicated. MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GAP, glyceraldehyde 3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; HBMPP, hydroxymethylbutenyl diphosphate; GGPP, geranylgeranyl diphosphate. Enzymes are indicated in bold: DXS, DXP synthase; DXR, DXP reductoisomerase; HDR, HMBPP reductase; PSY, phytoene synthase. The MEP pathway is boxed in gray. Dashed arrows represent multiple enzymatic steps. A dotted line indicates the feedback loop revealed by this work.

Like all isoprenoids, carotenoids are synthesized from prenyl diphosphate precursors (Figure 1). Two pathways synthesize these precursors in plants, the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Bouvier *et al*. 2005; Rodríguez-Concepción and Boronat 2002). The latter is located in plastids and it provides virtually all precursors for the production of carotenoids in chloroplasts and chromoplasts. Consistently, overproduction of the MEP pathway enzymes deoxyxylulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), or hydroxymethylbutenyl diphosphate synthase (HDR) in transgenic *Arabidopsis thaliana* plants and tomato fruit led to increased carotenoid levels, whereas no changes were observed when flux through

the MVA pathway was upregulated (Botella-Pavía *et al.* 2004, Carretero-Paulet *et al.* 2006, Enfissi *et al.* 2005, Estévez *et al.* 2001). Although it is assumed that carotenoids are also formed from MEP-derived precursors in etioplasts, as deduced from the albino phenotype displayed by etiolated mutant seedlings with no DXS activity (Nagata *et al.* 2002), overexpression of HDR in etiolated seedlings did not increase carotenoid levels (Botella-Pavía *et al.* 2004). Prenyl diphosphate precursors are channeled to the carotenoid pathway by phytoene synthase (PSY), proposed to be the main rate-determining enzyme of the pathway (Giuliano *et al.* 2008, Hirschberg 2001). After desaturation of phytoene to lycopene in several enzymatic steps, lycopene cyclization produces carotenes (such as beta-carotene) which can be oxygenated to form xanthophylls such as violaxanthin and lutein (Figure 1). Excellent recent reviews are available for more information on the structure and regulation of biosynthetic pathways of isoprenoids in general and carotenoids in particular (Bouvier *et al*. 2005; Fraser and Bramley 2004).

In plants lacking chromoplasts, including Arabidopsis, carotenoid biosynthesis is most active during deetiolation. When germinated in the dark, seedlings show an etiolated (skotomorphogenic) phenotype of long hypocotyls, an apical hook, and closed unexpanded cotyledons with etioplasts containing chlorophyll precursors and low levels of carotenoids (lutein, violaxanthin, and smaller amounts of beta-carotene and other carotenoids) associated to the prolamellar body (PLB), a lattice of tubular membranes that appears to facilitate greening when underground seedlings emerge into the light (Park *et al.* 2002). Etioplast carotenoids have been proposed to maintain late skotomorphogenic growth and to participate in PLB assembly (Barrero *et al.* 2008, Cuttriss *et al.* 2007, Park *et al.* 2002). Upon light perception photomorphogenic development is derepressed, resulting in decreased hypocotyl elongation, apical hook opening, cotyledon expansion, and differentiation of etioplasts into chloroplasts. This light-triggered deetiolation process involves the production of high levels of chlorophylls and carotenoids in chloroplasts to support photosynthetic development. Deetiolation is paralleled by a strong and coordinated increase in the level of transcripts encoding DXS, HDR and PSY (Botella-Pavía *et al.* 2004, von Lintig *et al.* 1997). However, only light qualities allowing chlorophyll biosynthesis and differentiation of etioplasts into chloroplasts appear to result in an enhanced PSY enzyme activity (Welsch *et al.* 2000) and a concomitant activation of carotenoid production and accumulation (von Lintig *et al.* 1997).

Transformation of etioplasts into chloroplast-like organelles can also take place in the absence of light when the function of global repressors of photomorphogenic development such as COP1 is impaired (Wei and Deng 1996). When grown in darkness, partial loss-of-function mutants such as *cop1-4* show a photomorphogenic phenotype with transcriptional profiles and morphological features resembling those of light-grown plants (Ma *et al.* 2002, Ma *et al.* 2003). Hormones such as brassinosteroids (BRs) and gibberellins (GAs) have also been implicated in the regulation of photomorphogenesis in Arabidopsis. Thus, block of BR production in mutants such as *det2-1* results in partially deetiolated seedlings in the dark (Li *et al.* 1996), and a similar phenotype is observed when the production of GAs is inhibited with paclobutrazol (PAC) or genetically blocked in mutants (Alabadi *et al.* 2004). However, biochemical processes that directly require light such as chlorophyll biosynthesis are not initiated in the dark even when photomorphogenesis is derepressed in mutant or

PAC-treated seedlings. We observed that chemical or genetic derepression of deetiolation in the absence of light significantly increased the production of carotenoids in Arabidopsis and used this system to learn more about the regulation of carotenoid biosynthesis in dark-grown seedlings. The key relevance of such regulation for plant fitness is illustrated by the improved accumulation of chlorophylls (greening) observed upon illumination in seedlings in which carotenoid levels had been upregulated in the dark by PAC treatment. We show that the production of carotenoids in dark-grown seedlings relies on the supply of MEP-derived precursors and it is mostly regulated at the level of PSY activity. Furthermore, we provide evidence that PSY controls metabolic flux to the carotenoid pathway by a feedback regulation of DXS protein levels.

Results and Discussion

Carotenoid accumulation is enhanced in seedlings that deetiolate in the dark.

Etiolated Arabidopsis seedlings have a characteristic phenotype of yellow cotyledons caused by the accumulation of lutein and violaxanthin in these organs (Park *et al.* 2002). Two main lines of evidence indicate that cotyledon carotenoids are synthesized *de novo* following germination. First, the cotyledons of embryos isolated from imbibed seeds are visually devoid of carotenoid pigments (Figure 2a) (Carol and Kuntz 2001). And second, germination of Arabidopsis seeds in the dark on media supplemented with norflurazon (NFZ), an inhibitor of phytoene desaturation (Figure 1), results in etiolated seedlings with pale cotyledons due to a concentration-dependent block in the production of carotenoid pigments (Figure 2b).

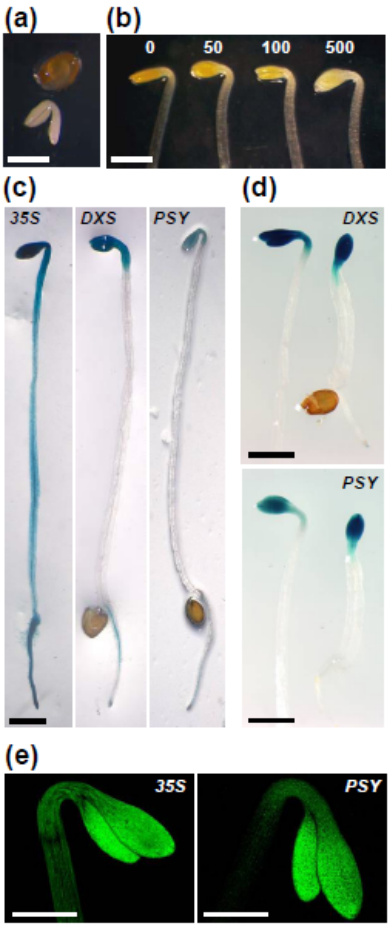

Figure 2. Distribution of carotenoid pigments and expression of biosynthetic genes in dark-grown Arabidopsis seedlings. **(a)** Wild-type (WT) embryo after removal of the seed coat following imbibition on MS plates for 2 days at 4ºC in the dark. **(b)** WT seedlings grown for 3 days in darkness on MS plates supplemented with the indicated concentrations of NFZ (nM). **(c)** Distribution of GUS staining in representative seedlings from transgenic *35S:GUS-GFP*, *DXS:GUS*, and *PSY:GUS-GFP* lines germinated and grown for 3 days in darkness on MS plates. **(d)** Distribution of GUS staining in representative seedlings from transgenic *DXS:GUS* and *PSY:GUS-GFP* lines grown in darkness for 3 days on MS plates with no inhibitors (seedlings on the left) or supplemented with 1 µM PAC (seedlings on the right). GUS staining was carried out in parallel for 3h. **(e)** Confocal laser scanning microscopy images of GFP fluorescence in the cotyledon area of representative *35S:GUS-GFP* and *PSY:GUS-GFP* seedlings grown as described in (c). Bars correspond to 1 mm (a-d) or 0.5 mm (e).

Although block of phytoene desaturation has been reported to affect several metabolic pathways at the gene expression level (Qin *et al.* 2007), the only phenotypic effect that we observed in NFZ-treated etiolated seedlings was the reduced pigmentation of the cotyledons (Figure 2b).

To get new insights into the molecular mechanisms that operate in underground seedlings to regulate carotenoid biosynthesis after germination, we first aimed to upregulate carotenoid production in the absence of light. Because -a burst in carotenoid biosynthesis takes place during light-triggered deetiolation, we tested whether carotenogenesis could be activated by depressing photomorphogenesis in dark-grown seedlings. When germinated and grown in the dark, *cop1-4, det2-1* and PAC-treated wild-type seedlings show a partially deetiolated phenotype (Figure 3a). As shown in Figure 3b, total carotenoid levels relative to fresh weight in mutant and PAC-treated seedlings germinated and grown for 3 days in the dark were substantially higher than those in mock-treated (etiolated) wild-type controls.

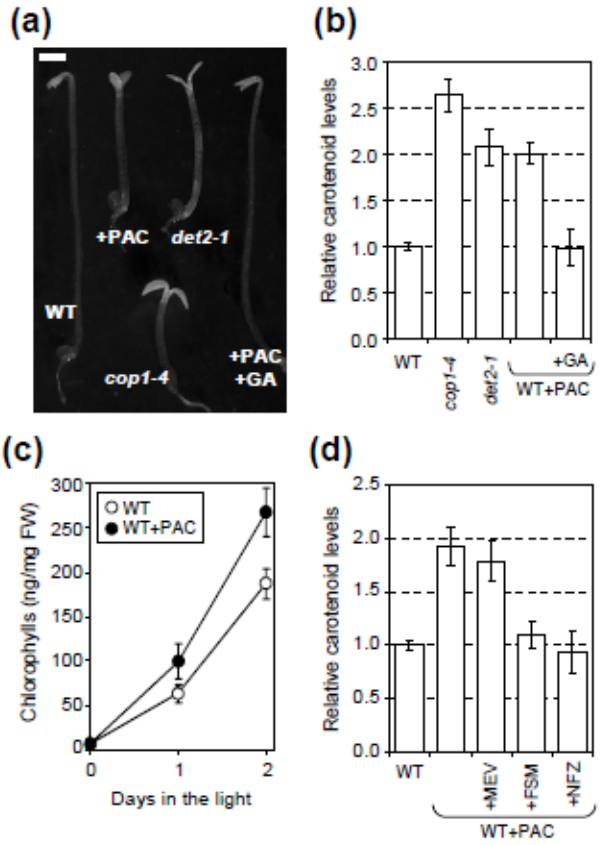

Figure 3. Photomorphogenic development and pigment accumulation in seedlings. **(a)** Representative 3-day-old WT, *cop1-4* or *det2-1* seedlings germinated and grown in the dark. Mutant seedlings were grown on MS plates, whereas WT seedlings were also grown on MS medium supplemented with 1 µM PAC and 10 µM gibberellic acid (GA) as indicated. Bar corresponds to 1 mm. **(b)** Total carotenoid levels in seedlings grown as described in (a). Levels are represented relative to those of WT seedlings grown on MS medium and correspond to the mean and standard error of five experiments (n=5). **(c)** Chlorophyll accumulation in WT seedlings grown in the dark with or without 1 µM PAC and then transferred to MS plates before incubation in the light for 2 days. FW, fresh weight. Mean and standard error correspond to five different experiments (n=5). **(d)** Total carotenoid levels in WT seedlings grown on medium supplemented with 1 µM PAC, 50 µM MEV, 500 µM FSM, or 1 µM NFZ as indicated. Levels are represented relative to those in etiolated (mock-treated) seedlings. Mean and standard error of three experiments (n=3) is shown.

Because etiolated and photomorphogenic seedlings have a similar fresh weight (Table S1), the increased accumulation of carotenoids in the latter was also observed when expressed as absolute amounts per seedling (Figure S1). Most of the observed increase in total carotenoid levels corresponded to the accumulation of lutein and violaxanthin (Figure 4 and Table S2). In the case of PAC-treated seedlings, supplementation of the growth medium with gibberellic acid restored the etiolated phenotype (Figure 3a) and abolished the increase in carotenoid accumulation (Figures 3b

and S1), confirming the association between treatment with PAC, the derepression of a deetiolation program, and the upregulation of carotenoid levels even in the absence of light.

Higher carotenoid levels in dark-grown seedlings result in improved greening upon illumination.

A defective production of lutein and violaxanthin in etioplasts of Arabidopsis seedlings was shown to negatively affect PLB assembly and, as a consequence, to delay greening upon illumination of dark-grown seedlings (Park *et al.* 2002). These results suggested that etioplast carotenoids contribute to the proper adaptation of soil-emerging seedlings to sunlight. However, it remained to be established whether greening of dark-grown seedlings could be improved by upregulating carotenoids levels. To answer this question, we monitored the accumulation of chlorophylls upon illumination of dark-grown seedlings treated with PAC to double their carotenoid levels relative to untreated controls. When wild-type seedlings grown for three days on PAC-supplemented medium in the dark were transferred to a fresh medium without PAC for incubation in the light, they became green faster than mock-treated seedlings due to a more active accumulation of chlorophylls (Figure 3c). These and previous results (Park *et al.* 2002) suggest that regulating the production of carotenoids after germination might allow underground seedlings to optimize the transition to photosynthetic development after illumination. The following experiments aimed to unveil the mechanisms involved in this control.

The increase in carotenoid levels correlates with an enhanced biosynthetic rate.

All carotenoid species detected in etiolated seedlings (lutein, violaxanthin, and beta-carotene) accumulated at higher levels in dark-grown photomorphogenic seedlings (Figure 4 and Table S2). This carotenoid profile could be the result of an increased storage capacity within plastids or/and an activated metabolic flux of the carotenoid pathway. In agreement with the latter possibility, increased accumulation of carotenoids in PAC-treated seedlings was prevented by specifically blocking the second step in carotenoid biosynthesis with NFZ (Figures 3d and 4). NFZ treatment also resulted in the accumulation of phytoene, the product of PSY activity (Figure 4). Phytoene is not detected in the absence of NFZ because it is readily converted into downstream carotenoids (such as lutein and violaxanthin) under normal conditions. Its accumulation in NFZ-treated seedlings demonstrates that it is produced during normal etiolated growth by an active PSY enzyme. Although it has been reported that PSY is mainly associated to the PLB of etioplasts in a mostly enzimatically inactive form and that it requires relocation to thylakoid membranes of developing chloroplasts during light-triggered deetiolation for enzymatic activation (Welsch *et al.* 2000), our results show that an active PSY enzyme exists in normal etioplasts. Furthermore, the fact that substantially higher levels of phytoene are detected in deetiolated *cop1-4* and *det2-1* mutants and PAC-treated seedlings grown under the same conditions (Figure 4) implies that PSY activity is upregulated after derepression of photomorphogenic development even in darkness.

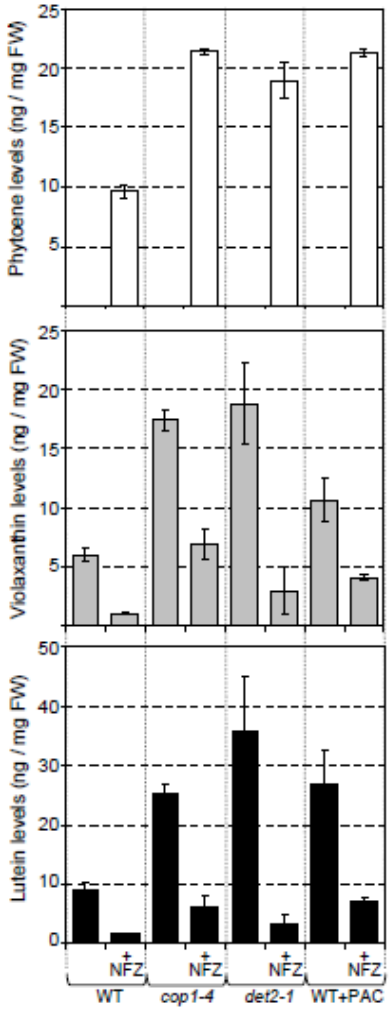

Figure 4. Level of individual carotenoids in dark-grown seedlings.WT, *cop1-4* and *det2-1* seeds were plated on MS medium for stratification at 4ºC and then transferred to MS plates either supplemented or not with 1 µM NFZ or/and 1 µM PAC as indicated. After incubation at 22ºC in the dark for 3 days, seedlings were collected and used for carotenoid extraction and analysis by HPLC after recording their fresh weight (FW). Mean and standard error of the levels of the indicated carotenoids correspond to four samples (n=4) collected in two independent experiments.

Cotyledon plastids from dark-grown photomorphogenic *cop1* mutants develop thylakoid-like membranes and lack a PLB (Deng *et al.* 1991), whereas those from PAC-treated seedlings conserve a PLB but develop more prothylakoid membranes than untreated controls (D. Alabadí and M.A. Blázquez, personal communication), similar to that observed in dark-grown *det2* seedlings (Chory *et al.* 1991). It is possible that the enhanced development of prothylakoids triggered by different ways of chemical or genetic derepression of photomorphogenic development in plastids of dark-grown seedlings might allow an increased association of PSY to thylakoid-like membranes leading to an enhanced enzyme activity and subsequent production of carotenoids. Development of new membrane structures within the etioplast could also facilitate the storage of carotenoid products and contribute to the accumulation of these lipophylic compounds in photomorphogenic mutants and PAC-treated seedlings in the dark.

***PSY* gene expression is upregulated in seedlings that deetiolate in the dark.**

To investigate whether the increased PSY activity detected in seedlings that deetiolate in the dark resulted from an enhanced association of already existing PSY polypeptides with newly formed prothylakoid membranes or/and an upregulated production of new PSY molecules, levels of *PSY* transcripts were measured by quantitative real time PCR (qPCR) using RNA isolated from whole seedlings grown in the dark for 3 days with or without PAC. Supporting the latter possibility, *PSY* transcripts were much more abundant in photomorphogenic seedlings compared to etiolated controls (Figure 5a). Similar results were obtained when comparing the level of *PSY* transcripts in dark-grown wild-type and mutant *cop1-4* and *det2-1* seedlings (Figure S2). Presence of NFZ in the growth medium did not significantly affect the enhanced accumulation of *PSY* transcripts (Figure 5a), indicating that the upregulation of *PSY* gene expression was independent of carotenoid levels.

We next investigated whether the observed changes in transcript levels were the result of transcriptional (promoter activity) events using transgenic lines harboring constructs with the promoter region of the *PSY* gene fused to a reporter with beta-glucuronidase and green fluorescent protein (GUS-GFP) activities. Plants expressing the GUS-GFP double reporter under the control of the constitutive CaMV *35S* promoter were used as controls. All organs of etiolated *35S:GUS-GFP* seedlings showed high levels of GUS staining (Figure 2c) and GFP fluorescence (Figure 2e), as expected. By contrast, all four independent *PSY:GUS-GFP* lines selected displayed the highest promoter activity in cotyledons (the organs where carotenoids accumulate), with a much weaker GUS staining detected in the radicle and the apical hook (Figure 2c). Confocal scanning laser microscopy analysis of GFP fluorescence confirmed that the activity of the *PSY* promoter was strongest in cotyledons (Figure 2e). Analysis of publicly available microarray data (Ma *et al.* 2005) also showed that levels of PSY-encoding transcripts in etiolated Arabidopsis seedlings were much higher in cotyledons than in hypocotyls or radicles (Figure S3), validating the *PSY:GUS-GFP* construct as a good reporter of the activity of the endogenous *PSY* promoter. GUS staining in transgenic PAC-treated *PSY:GUS-GFP* seedlings was more intense and it remained highest in cotyledons (Figure 2d), indicating that the observed induction of *PSY* transcript accumulation after derepression of photomorphogenic development in the dark does not result from ectopic gene expression. Moreover, immunoblot analysis of transgenic *PSY:GUS-GFP* plants with an anti-GFP serum showed a substantially enhanced accumulation of the GUS-GFP reporter protein in PAC-treated seedlings (Figure 5b). Together, our results confirm that derepressing deetiolation in the dark induces the activity of the *PSY* promoter, eventually resulting in increased transcript levels, protein accumulation, and PSY enzyme activity.

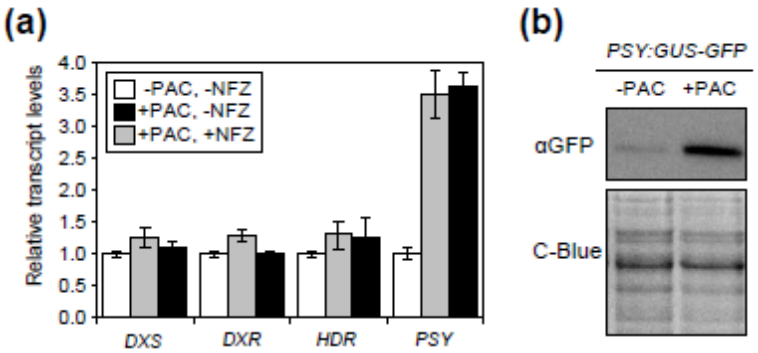

Figure 5. Transcript and protein levels of biosynthetic enzymes in PAC-treated seedlings. Seedlings were grown with (+) or without (-) PAC or NFZ as described in Figure 3 and collected for RNA and protein extraction.**(a)** Quantitative real time PCR analysis of *DXS*, *DXR*, *HDR* and *PSY* transcript levels in PAC-treated WT seedlings, represented relative to those in etiolated (mock-treated) controls. Mean and standard error of four samples (n=4) collected in two different experiments is shown. **(b)** Immunoblot analysis of GUS-GFP levels in *PSY:GUS-GFP* seedlings with an antibody against GFP. Coomassie Blue (C-Blue) staining of the gel is shown to illustrate equal protein loading.

Other genes required for carotenoid synthesis in the dark are also upregulated in PAC-treated seedlings.

Two enzymatic activities are essential for the biosynthesis of carotenoids only in non-photosynthetic plastids (Figure S4). One of them is carotenoid isomerase (CRTISO), required for the isomerization of prolycopene to lycopene (Isaacson *et al.* 2002, Park *et al.* 2002). Interestingly, a microarray analysis of Arabidopsis seedlings germinated and grown in the dark in the presence or absence of PAC showed that *PSY* and *CIS2* (encoding one of the two Arabidopsis CRTISO isoforms) were among the only 3 carotenoid pathway genes found to be upregulated more than 1.5-fold in PAC-treated seedlings (D. Alabadí and M.A. Blázquez, personal communication). Another enzyme that is needed for carotenoid biosynthesis in the dark is plastid terminal oxidase (PTOX). The energetically unfavorable reactions that desaturate phytoene require the transfer of electrons to plastoquinone (Figure S4), which can be reoxidized by the photosynthetic electron transport chain in chloroplasts. By contrast, in non-photosynthetic tissues (including cotyledons of dark-grown seedlings) plastoquinone is regenerated by PTOX activity using O_2 as a terminal acceptor (Carol and Kuntz 2001). The single gene encoding PTOX in Arabidopsis was not included in the microarray, but a strong upregulation of transcripts encoding PTOX could be detected in PAC-treated seedlings by RT-PCR (Figure S4). These results confirm that the enhanced accumulation of carotenoids that takes place in deetiolating dark-grown seedlings results, at least in part, from an activated production supported by the transcriptional upregulation of carotenoid pathway genes encoding key biosynthetic enzymes.

The MEP pathway supplies the precursors for carotenoid synthesis in dark-grown seedlings.

It is expected that the enhanced biosynthesis of carotenoids observed in seedlings that deetiolate in the dark also requires an activated supply of metabolic precursors. A major role of the MEP pathway in supplying these precursors in etiolated seedlings was deduced from the lack of carotenoid accumulation observed in mutants defective in DXS activity, which display albino cotyledons (Nagata *et al.* 2002). Consistently, the relative abundance of transcripts in different organs of etiolated Arabidopsis seedlings is similar for all the enzymes of the MEP pathway and the carotenoid pathway, with highest levels in cotyledons (Figure S3). The analysis of transgenic *DXS:GUS* seedlings harboring the DXS promoter fused to a GUS reporter (Carretero-Paulet *et al.* 2002) confirmed an almost identical spatial pattern of *DXS* and *PSY* promoter activity (Figure 2c). By contrast, the highest levels of transcripts for MVA pathway enzymes were not found in cotyledons but in radicles (Figure S3). But because an exchange of common prenyl diphosphates between the cytoplasm and the plastid appears to occur in light-hyposensitive mutants (Rodríguez-Concepción *et al.* 2004), it might be expected that MVA-derived precursors could be imported into plastids and used for the burst in carotenoid biosynthesis that takes place when deetiolation is derepressed in the dark. We tested this possibility by growing Arabidopsis seedlings in the presence of PAC and inhibitors specific for the MEP pathway or the MVA pathway. As shown in Figures 3d and S1, inhibition of the MEP pathway with fosmidomycin (FSM) prevented the increase in carotenoid accumulation in PAC-treated seedlings, whereas no significant effect was observed when the MVA pathway was blocked

with mevinolin (MEV). These data confirm that the increased production of carotenoids in deetiolating dark-grown seedlings relies mostly on the supply of MEP-derived precursors and support the conclusion that carotenoids derive mainly from precursors synthesized by MEP pathway in all plastid types.

MEP pathway enzyme levels are posttranscriptionally upregulated in dark-grown seedlings.

The expression of MEP pathway genes has been shown to either precede or parallel the activation of *PSY* and other carotenoid biosynthesis genes during fruit ripening and light-induced deetiolation, when an enhanced supply of precursors is required to fuel an activated production of carotenoids (Botella-Pavía *et al.* 2004, Bouvier *et al.* 1998, Lois *et al.* 2000). Microarray data, however, showed that none of the MEP pathway genes was significantly upregulated in PAC-treated seedlings (D. Alabadí and M.A. Blázquez, personal communication). In particular, qPCR experiments confirmed that the level of transcripts encoding the main flux-controlling enzymes of the pathway (DXS, DXR and HDR) did not substantially change when carotenoid biosynthesis was triggered by PAC treatment (Figure 5a) or in *cop1-4* and *det2-1* seedlings (Figure S2). The spatial activity of the *DXS* promoter was also unaltered by PAC treatment (Figure 2d).

Increasing evidence indicates that, besides the coarse control exerted by changes in the expression of MEP pathway genes, posttranscriptional events are central for regulating enzyme levels and eventually metabolic flux through the pathway (Guevara-Garcia *et al.* 2005, Laule *et al.* 2003, Sauret-Güeto *et al.* 2006, Wolfertz *et al.* 2004). To investigate whether this was also the case in dark-grown deetiolated seedlings, we next analyzed the levels of flux-controlling MEP pathway enzymes in PAC-treated seedlings grown in the absence of light. As shown in Figure 6a, immunoblot analysis with specific antibodies confirmed that DXS, DXR and HDR levels were strongly upregulated in PAC-treated seedlings. Block of phytoene desaturation with NFZ did not prevent the posttranscriptional accumulation of these proteins (Figure 6a), suggesting that it was not dependent on the levels of carotenoid end-products. Alternatively, it is possible that changes in plastid ultrastructure associated with PAC treatment might result in increased stability of MEP pathway proteins.

A recent work has shown a role for the stromal Clp protease in the degradation of MEP pathway enzymes (including DXS) in chloroplasts (Flores-Pérez *et al.* 2008). Because the Clp protease complex is present in all plastid types (Peltier *et al.* 2004), we next evaluated its influence in regulating DXS levels in plastids of dark-grown seedlings. Etiolated seedlings of the Arabidopsis *clpr1-2* mutant, which shows a reduced Clp proteolytic activity (Flores-Pérez *et al.* 2008, Koussevitzky *et al.* 2007, Stanne *et al.* 2009), accumulated increased DXS levels compared to wild-type controls (Figure 6b). However, the relative increase in DXS protein levels after PAC treatment was similar in wild-type and *clpr1-2* seedlings (Figure 6b). These results suggest that the Clp protease regulates DXS levels in etioplasts but it is not required for the posttranscriptional upregulation of enzyme levels that occurs when deetiolation and carotenoid accumulation take place in the dark. These results indicate that MEP pathway enzyme levels are regulated by multiple posttranscriptional mechanisms, likely to ensure that enough precursors will be produced in plastids with peak demands of carotenoids or other plastidial isoprenoids.

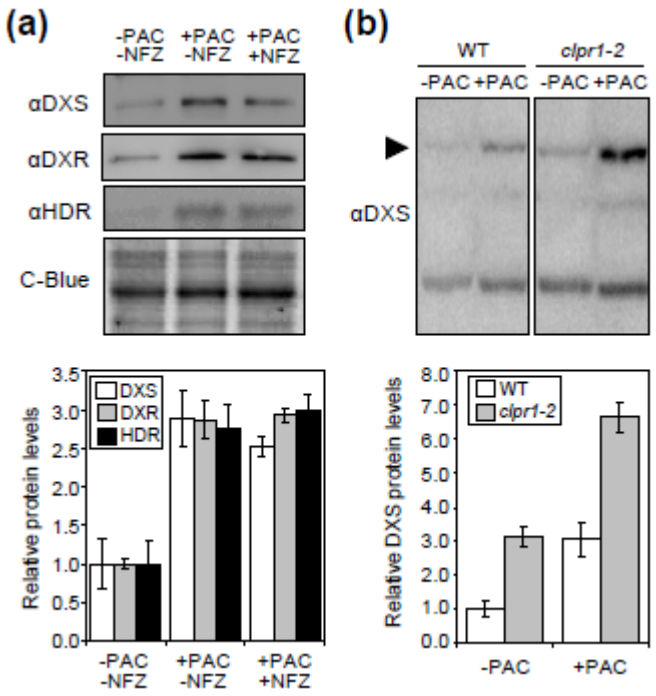

Figure 6. MEP pathway protein levels in PAC-treated WT and *clpr1-2* seedlings. Seedlings were grown with (+) or without (-) PAC or NFZ as described in Figure 3. **(a)** Immunoblot analysis of DXS, DXR and HDR levels in WT seedlings. Coomassie Blue staining of the gel is shown to illustrate equal protein loading. **(b)** Immunoblot analysis of DXS levels in WT and *clpr1-2* seedlings. The position of the DXS protein is indicated with an arrowhead. A major unspecific band recognized by the antibody is shown as a control of protein loading. The graphs show normalized protein levels (estimated from immunoblot band intensity) represented relative to those of mock-treated WT seedlings. Mean and standard errors from three (n=3) experiments are represented.

Carotenoid production in the dark can be increased by upregulating PSY activity.

The results shown above suggest that posttranscriptionally upregulated levels of flux-controlling MEP pathway enzymes in dark-grown photomorphogenic seedlings might result in an enhanced supply of isoprenoid precursors that could be efficiently channeled into the carotenoid pathway by increased levels of active PSY enzyme. However, while transgene-mediated overproduction of DXS or HDR in chloroplasts of light-grown Arabidopsis seedlings results in increased accumulation of carotenoids (Botella-Pavía *et al.* 2004, Carretero-Paulet *et al.* 2006, Estévez *et al.* 2001), HDR-overproducing lines show wild-type levels of carotenoids in etiolated seedlings (Botella-Pavía *et al.* 2004). Because the reducing power required for HDR enzymatic activity might be compromised in the absence of light and photosynthesis (Rodríguez-Concepción 2006), we aimed to ascertain the influence of an upregulated supply of MEP-derived precursors on carotenoid biosynthesis in dark-grown Arabidopsis seedlings by analyzing the effect of increasing DXS levels by using a transgenic *35S:DXS* line previously shown to increase carotenoid production in light-grown plants (Carretero-Paulet *et al.* 2006). As shown in Figure 7a, no significant differences in carotenoid levels were detected between transgenic *35S:DXS* seedlings and untransformed controls when grown in the dark with or without PAC. These data suggest that the production of increased levels of MEP-derived isoprenoid precursors is not sufficient to enhance carotenoid biosynthesis in the dark.

Precursor supply only appears to be limiting for carotenoid biosynthesis when PSY activity (i.e., the activity channelling these precursors into carotenoids) is not (Lois *et al.* 2000).

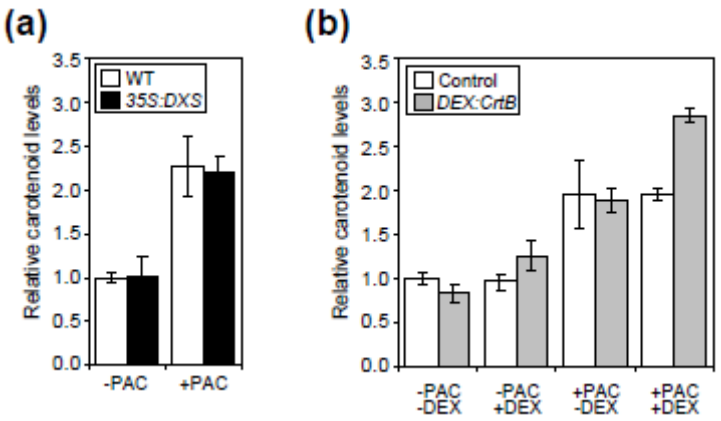

Figure 7. Carotenoid accumulation in dark-grown transgenic seedlings with upregulated DXS or PSY levels. **(a)** Carotenoid levels in seedlings from lines constitutively overproducing DXS (*35S:DXS*) or untransformed controls (WT) grown for 3 days in the dark on MS plates supplemented (+) or not (-) with 1 µM PAC. Levels are represented relative to those in WT seedlings grown without PAC. Mean and standard error of four experiments (n=4) is shown. **(b)** Seedlings harboring a construct for the production of a plastid-targeted bacterial PSY enzyme (CrtB) under the control of a DEX-induced promoter (*DEX:CrtB*) were grown for 3 days in the dark on MS plates either supplemented or not with 1 µM PAC or/and 1 µM DEX as indicated. Carotenoid levels are represented relative to those in control seedlings from a transgenic line harboring a similar construct but without the CrtB-encoding sequence grown in non-supplemented medium. Mean and standard error of five samples (n=5) is shown.

To test whether PSY activity instead of precursor availability might be the limiting step for carotenoid biosynthesis in seedlings grown in the absence of light, we generated transgenic Arabidopsis plants in which PSY activity could be upregulated. Cosuppression events were prevented by expressing a recombinant gene encoding a bacterial PSY enzyme (CrtB) fused to a plastid-targeting sequence (Fraser *et al.* 2002) under the control of a promoter that could be induced with dexamethasone (DEX), a synthetic glucocorticoid (Aoyama and Chua 1997). Control lines were generated with the empty vector. Transgenic seedlings from control and *DEX:CrtB* lines were grown for 3 days in the dark in medium either supplemented or not with PAC and/or DEX, and samples were then collected for carotenoid quantification. In the absence of PAC (etiolated seedlings), DEX treatment resulted in only a slight increase in carotenoid levels in *DEX:CrtB* lines but had no effect on control lines (Figure 7b). In the presence of PAC (partially deetiolated seedlings) DEX treatment resulted in a much clearer increase in carotenoid accumulation in *DEX:CrtB* seedlings, whereas it remained unchanged in control lines (Figure 7b). The substantial improvement in carotenoid accumulation observed in *DEX:CrtB* seedlings treated with PAC and DEX compared to those only treated with DEX could be explained by several factors. First, the level of MEP pathway enzymes such as DXS, DXR and HDR (and presumably the supply of MEP-derived carotenoid precursors) is posttranscriptionally upregulated when photomorphogenesis is derepressed with PAC (Figure 6). Second, the expression of the endogenous *PSY*, *PTOX* and *CIS2* genes is also induced in seedlings that deetiolate in the dark (Figures 5 and S4). This could result in much higher levels of proteins with PSY activity (endogenous PSY plus recombinant CrtB) in *DEX:CrtB* seedlings treated with both PAC and DEX and an increased metabolic capacity to synthesize carotenoids in the dark by enhanced PTOX and CRTISO activities. And third, the development of prothylakoid membranes in PAC-treated dark-grown seedlings likely improves PSY enzymatic activity and storage of the newly synthesized carotenoid products, as discussed above. Together, our results demonstrate that, unlike that

observed with MEP pathway enzymes, upregulation of PSY activity is sufficient to increase the production of carotenoids in dark-grown seedlings. The fact that no phytoene was detected in etiolated or photomorphogenic dark-grown seedlings (Figure 3) suggests that it was readily transformed into downstream carotenoid products under these conditions, consistent with PSY catalyzing the main rate-determining step for carotenoid production in dark-grown seedlings.

An enhanced PSY activity leads to upregulated DXS levels in etiolated seedlings.

It has been hypothesized that a high biosynthetic rate of plastidial isoprenoids (including carotenoids) during the initial stages of seedling development in the light could lead to a concomitant decrease in the levels of MEP pathway intermediates or products, which in turn would trigger a posttranscriptional accumulation of DXS to upregulate pathway flux (Guevara-Garcia *et al.* 2005). To test whether the increase in MEP pathway enzyme levels observed in PAC-treated seedlings (Figure 6) was a direct consequence of upregulating PSY activity instead of the result of derepressing the photomorphogenic program in dark-grown seedlings, we analyzed DXS protein levels in etiolated *DEX:CrtB* seedlings after inducing transgenic PSY activity with DEX. Control and *DEX:CrtB* lines were germinated and grown in the dark on filter paper soaked in MS medium. At day 2, the paper with the seedlings was transferred in the dark to plates supplemented or not with DEX. Samples were collected one day later and used for protein and RNA extraction. As shown in Figure 8a, etiolated *DEX:CrtB* seedlings accumulated higher levels of DXS protein when treated with DEX compared to mock-treated samples, whereas no differences were observed in control seedlings. Levels of DXS-encoding transcripts, however, were similar in control and *DEX:CrtB* seedlings even in the presence of DEX (Figure 8b). These results strongly suggest that the sole upregulation of PSY activity in etioplasts is sufficient to trigger an enhanced supply of MEP-derived precursors *via* posttranscriptional accumulation of flux-controlling pathway enzymes such as DXS.

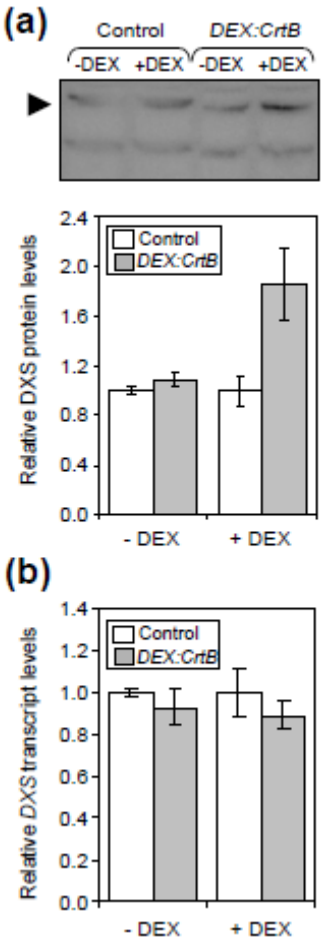

Figure 8. DXS protein and transcript levels in seedlings with induced PSY levels. In the dark, transgenic *DEX:CrtB* and control seedlings were germinated and grown for 2 days on MS plates and then transferred to plates either supplemented (+) or not (-) with 1 µM DEX as indicated. After 24 hours, samples were collected for both RNA and protein extraction. **(a)** Immunoblot analysis of DXS levels. The position of the DXS protein is indicated with an arrowhead. The lower (unspecific) band is shown as a control of protein loading. The mean and standard errors of normalized DXS levels relative to control seedlings shown in the graph correspond to data of four (n=4) immunoblots with samples from three independent experiments. **(b)** qPCR analysis of *DXS* transcript levels represented relative to those in control seedlings. Mean and standard error correspond to two different experiments (n=2).

Concluding remarks

Our data show that carotenoid biosynthesis in dark-grown seedlings relies on the availability of MEP-derived precursors and their efficient channeling to the carotenoid pathway by PSY activity. They also demonstrate that PSY upregulation is the key driver to increase carotenoid production in etioplasts, which may eventually facilitate the transition to photosynthetic development in the light (greening) and therefore influence plant fitness. Enhanced PSY levels in transgenic plants also lead to an increased production of carotenoids in chromoplasts (Fraser *et al.* 2007, Fraser *et al.* 2002) and other plastid types not specialized in carotenoid accumulation such as those of seeds (Lindgren *et al.* 2003, Shewmaker *et al.* 1999) and tubers (Ducreux *et al.* 2005). In chloroplasts, however, the production of carotenoids is limited by precursor supply rather than PSY activity. This is well exemplified by the increase in carotenoid accumulation observed in light-grown Arabidopsis seedlings overexpressing MEP pathway enzymes (Botella-Pavía *et al.* 2004, Carretero-Paulet *et al.* 2006, Estévez *et al.* 2001) and the reduced availability of precursors for plastidial isoprenoids other than carotenoids in transgenic plants constitutively overexpressing PSY (Busch *et al.* 2002, Fray *et al.* 1995). In any case, a crosstalk between the carotenoid pathway and the MEP pathway appears to be required for an efficient production of carotenoids (Fraser *et al.* 2007, Giuliano *et al.* 2008, Lois *et al.* 2000, Rodríguez-Concepción *et al.* 2001). Here we show a new mechanism by which PSY controls metabolic flux to the carotenoid pathway in plants based on a feedback regulation of DXS levels to ensure an optimal supply of MEP-derived precursors (Figure 1). Future experiments should determine whether the accumulation of other MEP pathway enzymes (including DXR and HDR) is also regulated by such a feedback mechanism. Although we describe this mechanism in etioplasts, it could also be functional in chloroplasts (Guevara-Garcia *et al.* 2005) and other plastid types. For example, the increased DXS activity (but not gene expression) detected in tomato fruit with upregulated PSY levels (Fraser *et al.* 2007) might be the result of an enhanced accumulation of active DXS enzymes in chromoplasts. Ongoing work in our lab is addressed to characterize the molecular and biochemical components of this feedback mechanism.

Materials and methods

Plant material and growth conditions

All the *Arabidopsis thaliana* lines used in this work are in the Columbia background. Seeds from *cop1-4* and *det2-1* mutants were kindly provided by J.F. Martínez-García. Homozygous *DXS:GUS* (Carretero-Paulet *et al.* 2002) and *35S:DXS* (Carretero-Paulet *et al.* 2006) lines were kindly provided by L. Carretero-Paulet, whereas *PSY:GUS-GFP* and *DEX:CrtB* lines were constructed as described below. Because seed germination is prevented when directly plated on PAC-supplemented medium, experiments were carried out as follows. Seeds were surface-sterilized and sown on sterile Whatman filter papers placed on top of solid Murashige and Skoog (MS) medium in Petri dishes. After stratification for at least two days at 4ºC in darkness, plates were incubated for 2 to 6 h under fluorescent white light (photon fluence rate of 80 µmol m^{-2} s^{-1}) at 22ºC to induce germination. The filter papers with the seeds were then transferred to plates with solid MS medium supplemented or not with PAC and other chemicals. Control and treatment plates were wrapped in several layers of aluminum

foil and incubated in darkness for 3 days at 22ºC. Chemicals (FSM, MEV, NFZ, PAC, GA_3, DEX) were prepared as described (Alabadi *et al.* 2004, Besumbes *et al.* 2004, Rodríguez-Concepción *et al.* 2004, Sauret-Güeto *et al.* 2006). For greening experiments, filter papers with dark-grown seedlings were rinsed in sterile MS liquid medium and then transferred to MS plates for incubation in the light.

Generation and analysis of transgenic lines

A DNA sequence containing 2 kb of the genomic region upstream the ATG translation initiation codon of the Arabidopsis *PSY* (At5g17230) gene was amplified by PCR and cloned in the pCAMBIA1303 vector to drive the expression of the GUS-GFP reporter protein. The sequence harboring a plastid-targeted *Erwinia uredovora crtB* gene was PCR-amplified with primers ts-Psy-1 and crtB from transgenic tomato plants (Fraser *et al.* 2002) kindly provided by A. Boronat and cloned into the pTA7002 vector under the transcriptional control of a DEX-inducible promoter (Aoyama and Chua 1997). The resulting pPSY:GUS-GFP and pDEX-CrtB constructs were used for Agrobacterium-mediated transformation of Arabidopsis plants as described (Carretero-Paulet *et al.* 2002). Control lines were generated after transformation of Arabidopsis plants with empty pCAMBIA1303 and pTA7002 vectors, respectively. For each construct, four transgenic lines harboring only one T-DNA insertion (as estimated from the analysis of hygromycin resistance segregation) were selected for further experiments. Homozygous individuals of *PSY:GUS-GFP* and *35S:GUS-GFP* lines were used for GUS staining (Carretero-Paulet *et al.* 2002) and confocal microscopy of GFP fluorescence (Roig-Villanova *et al.* 2007). The same distribution of GUS activity and GFP fluorescence was observed in all lines harboring the same construct, although at different intensity levels. The results shown in this article correspond to representative individuals of the line showing the highest GUS activity and GFP fluorescence levels. Homozygous *DEX:CrtB* lines were confirmed to express the transgene in a DEX-dependent manner by RT-PCR analysis. Representative *DEX:CrtB* and control lines showing no visible phenotypic effects after DEX treatment in the light were selected for the experiments described here.

Measurement of carotenoid and chlorophyll levels

Total carotenoids and chlorophylls were extracted and quantified spectrophotometrically as described (Fraser *et al.* 2000, Lichtenthaler 1987). For quantification of individual carotenoids, they were separated by HPLC (Rodríguez-Concepción *et al.* 2004) and eluting compounds were monitored and identified using a Waters 2996 Photodiode Array detector. Major carotenoids (lutein, violaxanthin and beta-carotene) were detected at 450 nm, whereas phytoene was detected at 287 nm. Peak areas at the indicated wavelengths were compared to that of standards for quantification as described (Rodríguez-Concepción *et al.* 2004). Unless stated otherwise, concentration in a given sample is given relative to fresh weight.

Analysis of transcript and protein levels

Total RNA extraction, cDNA synthesis, and measurement of transcript levels by qPCR were carried out as described (Carretero-Paulet *et al.* 2006). PCR reactions were performed using TaqMan

Universal PCR Master Mix (Applied Biosystems), cDNA derived from 20 ng of input RNA, and predesigned FAM-labeled TaqMan MGB probes and unlabeled primers (Applied Biosystems) for DXS (At02221413_g1), DXR (At02281385_g1), HDR (At02249877_g1), and PSY (At02201001_g1). Transcripts encoding elongation factor 1a (EF1a) were also quantified (At02337969_g1) to normalize the threshold cycle for each probe assay and calculate relative gene expression as described (Carretero-Paulet *et al.* 2006). Similar cDNA samples were used for estimation of *PTOX* transcript levels by RT-PCR with primers PTOX-F (5'-T G G T T C C T G A C G G A G A T G G C-3') and PTOX-R (5'-A A G A A C C T T G C A T A T G T T C G-3') using actin ACT8 as a control of constitutive expression as described (An *et al.* 1996). Protein extraction and immunoblot analyses were carried out as described (Flores-Pérez *et al.* 2008, Sauret-Güeto *et al.* 2006). Chemiluminescent signals of immunoblot bands were visualized and quantified using a LAS-3000 image analyzer and the Multigauge 3.0 software (Fujifilm). The levels of unspecific bands detected with the anti-DXS serum and Coomassie staining were used as a control of equal loading and quantified for normalization.

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SUPPLEMENTARY TABLES

	Seedling weight (mg)
WT	0.49 ± 0.05
WT + PAC	0.48 ± 0.01
cop1-4	0.48 ± 0.01
det2-1	0.52 ± 0.05

Table S1. Fresh weight (mg) of individual seedlings grown as described in Figure 3. The indicated mean ± standard error values were calculated from measurements of n=4 (WT, *cop1-4* and *det2-1*) and n=2 (WT+PAC) groups of 50 seedlings.

ng / mg FW

	phytoene	β-carotene	violaxanthin	lutein
WT	n.d.	0.71 ± 0.15	5.98 ± 0.59	9.14 ± 1.09
WT + NFZ	9.63 ± 0.57	tr	1.07 ± 0.06	1.62 ± 0.07
cop1-4	n.d.	3.75 ± 0.19	17.38 ± 0.84	25.17 ± 1.53
***cop1-4* + NFZ**	21.43 ± 0.23	0.72 ± 0.03	6.87 ± 1.24	6.11 ± 2.02
det2-1	n.d.	3.51 ± 0.16	18.78 ± 3.41	35.71 ± 9.39
***det2-1* + NFZ**	18.91 ± 1.48	0.63 ± 0.06	2.93 ± 1.96	3.41 ± 1.44
WT + PAC	n.d.	2.18 ± 0.52	10.62 ± 1.83	27.01 ± 5.55
WT + PAC + NFZ	21.31 ± 0.27	tr	4.064 ± 0.693	7.15 ± 0.69

Table S2. Quantification of individual carotenoids in seedlings grown as described in Figure 4. The indicated mean ± standard error values correspond to four samples (n=4) and are indicated as ng per mg of fresh weight. n.d., not detected; tr, trace amounts.

SUPPLEMENTARY FIGURES

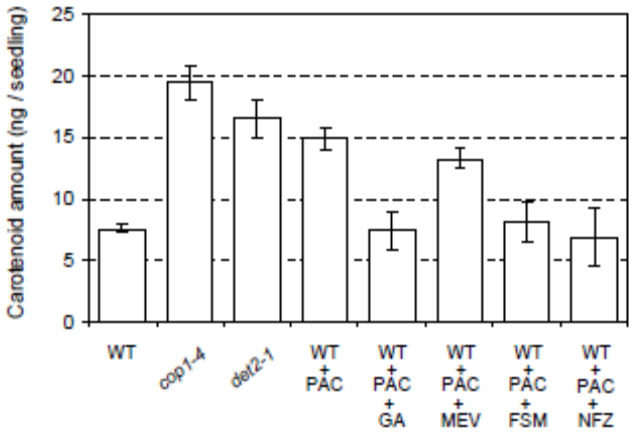

Figure S1. Carotenoid amounts in individual dark-grown seedlings. Mean and standard error of measurements from five (n=5) or three (n=3) independent samples is represented, as described in Figure 3.

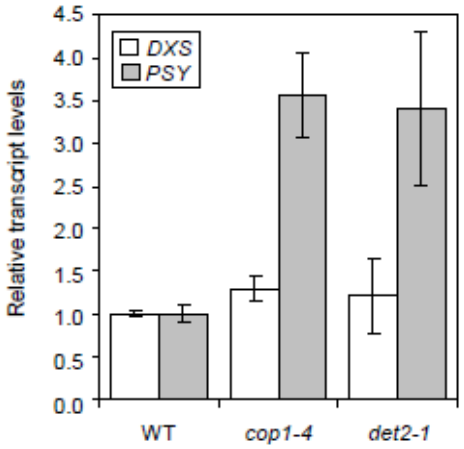

Figure S2. Quantitative real time PCR analysis of *DXS* and *PSY* transcript levels in dark- wild type, *cop1-4* and *det2-1* seedlings.Transcript levels in seedlings grown as described in Figure 3 are represented relative to those in WT seedlings

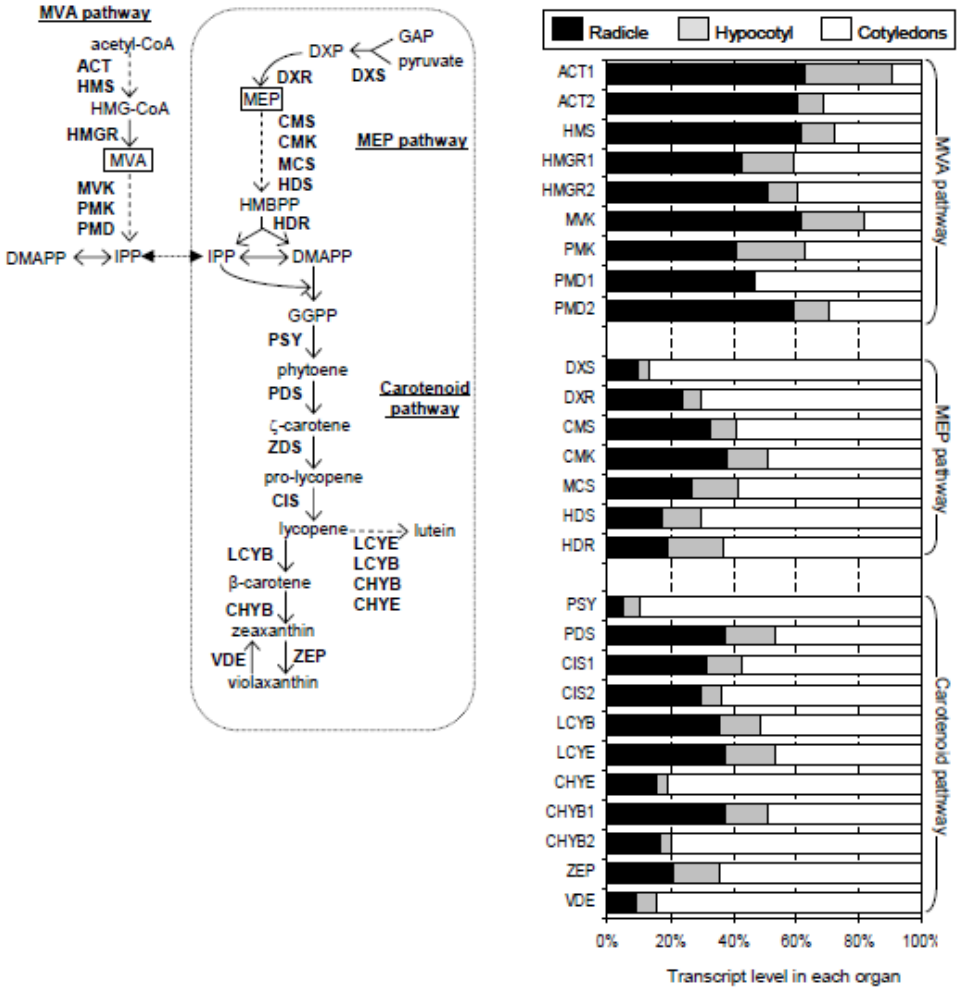

Figure S3. Transcript levels of genes involved in isoprenoid and carotenoid biosynthesis in different organs of etiolated seedlings.Data are extracted from the analysis of the transcriptome of 3 separate organs (radicle, hypocotyl, and cotyledons) of Arabidopsis seedlings germinated and grown in the dark for 6 days (Ma et al., 2005) and they are represented relative to the total amount of transcripts for each gene. Acronyms correspond to the following accessions: ACT1, At5g47720; ACT2, At5g48230; HMS, At4g11820; HMGR1, At1g76490; HMGR2, At2g17370; MVK, At5g27450; PMK, At1g31910; PMD1, At2g38700; PMD2, At3g54250; DXS, At4g15560; DXR, At5g62790; CMS, At2g02500; CMK, At2g26930; MCS, At1g63970; HDS, At5g60600; HDR, At4g34350; PSY, At5g17230; PDS, At4g14210; CIS1, At1g06820; CIS2, At1g57770; LCYB, At3g10230; LCYE, At5g57030; CHYE, At3g53130; CHYB1, At4g25700; CHYB2, At5g52570; ZEP, At5g67030; VDE, At1g08550. The steps catalyzed by the corresponding enzymes are represented in the cartoon on the left. HMG-CoA, hydroxymethylglutaryl coenzyme A; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GAP, glyceraldehyde 3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; HBMPP, hydroxymethylbutenyl diphosphate; GGPP, geranylgeranyl diphosphate.

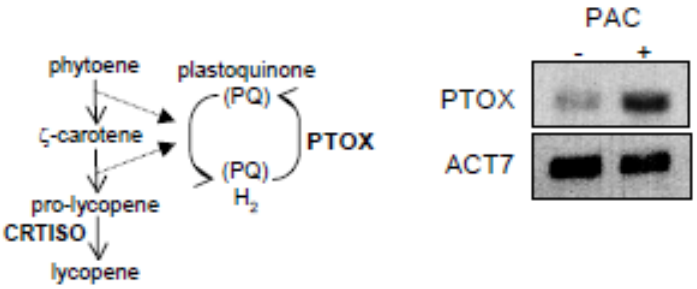

Figure S4. Levels of PTOX-encoding transcripts in dark-grown seedlings. RT-PCR was carried out using RNA isolated from wild-type seedlings grown in the dark on medium supplemented (+) or not (-) with 1 µM PAC. A constitutive control (*ACT8*) is shown for normalization. The steps catalyzed by PTOX and CRTISO, the only two enzyme activities needed for carotenoid biosynthesis in non-photosynthetic plastids, are represented in the cartoon on the left.

Artículo 4:

The only gene encoding phytoene synthase in Arabidopsis is ubiquitously expressed but responds to salt stress in a root-specific manner

El único gen codificante para fitoeno sintasa en Arabidopsis se expresa de forma ubicua pero presenta una respuesta específica de raíz ante estrés salino.

Resumen

Los carotenoides son pigmentos plastídicos de naturaleza isoprenoide esenciales para la vida de las plantas. Además de su papel esencial para la fotoprotección en tejidos fotosintéticos, estos pigmentos actúan como precursores del ácido abscísico (ABA) y de las estrigolactonas, que participan en la modulación de la respuestas de la planta ante estímulos internos de desarrollo y ambientales. En consonancia con su multifacético papel, el gen codificante para fitoeno sintasa (PSY), responsable de catalizar el primer paso específico de la ruta carotenogénica, está expresado de forma ubicua en *Arabidopsis thaliana*. Un análisis exhaustivo del patrón de expresión de este gen demostró que los mayores niveles de tránscritos se encontraban en los haces vasculares, especialmente en las raíces donde los niveles de carotenoides son menores. En condiciones de estrés salino se observó una inducción en la expresión de *PSY* en las raíces pero no en el tejido aéreo de las plántulas, sugiriendo que la producción de carotenoides (o más bien de productos derivados de su degradación y que puedan ser transportados a los tejidos aéreos por el sistema vascular) es crucial para la respuesta de la planta ante condiciones de estrés. Un tratamiento con ABA exógeno provoca igualmente un aumento de los tránscritos de *PSY* únicamente en el tejido radicular de la planta disminuyendo dicha inducción en los mutantes defectivos en ABA. Estos resultados sugieren que el ABA modula su propia producción en raíces bajo condiciones de estrés mediante el control de la expresión de *PSY* por un mecanismo de retroalimentación positiva. No obstante, una reducción en la expresión de *PSY* se observó en mutantes defectivos en la síntesis o en la percepción de estrigolactonas, sugiriendo un nuevo papel para estas hormonas derivadas de carotenoides en respuesta a estrés abiótico. Estos resultados sugieren que el único gen codificante para PSY en Arabidopsis es regulado por un mecanismo de retroalimentación controlado por diversos factores para asegurar probablemente un aporte de precursores suficiente para el desarrollo normal de la planta o en respuesta a estímulos ambientales.

Experimentos específicos de la tesis doctoral:

Figura 2; Figura 3; Figura 4; Figura 5; Figura 6; Figura 7; Figura 8; Figura 9; Figura S2; Figura S3.

The only gene encoding phytoene synthase in Arabidopsis is ubiquitously expressed but responds to salt stress in a root-specific manner.

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ABSTRACT

Carotenoids are plastidial isoprenoids essential for plant life. Besides their well-known role for photoprotection in photosynthetic-tissues, they are involved in the modulation of responses to developmental and environmental cues by serving as precursors of abscisic acid and strigolactones. Consistent with this multifaceted role, the gene encoding the main rate-determining enzyme of the carotenoid biosynthesis pathway, phytoene synthase (PSY), appears to be ubiquitously expressed in *Arabidopsis thaliana*. A careful analysis of the *PSY* expression pattern, however, showed an enriched accumulation of transcripts in the vascular area of photosynthetic and particularly non-photosynthetic organs such as roots (where carotenoid accumulation is scarce). We observed that *PSY* expression is upregulated by salt treatment in roots but not in shoots, suggesting that the production of root carotenoids (or, most likely, carotenoid-derived products that can be transported to the shoot by the vascular system) is central for the response of the whole plant to saline stress. ABA treatment also resulted in a root-specific increase in *PSY* expression levels, whereas the upregulation of *PSY* transcript levels in roots of salt-treated seedlings was partially repressed in ABA-defective mutants. These results support the hypothesis that ABA modulates its own production in stressed roots by feedback-regulating *PSY* expression. Interestingly, a reduced induction of *PSY* gene expression was also observed in roots of salt-treated mutants defective in strigolactone synthesis or perception, suggesting a new role of these carotenoid-derived hormones in the response to abiotic stress. Together our results suggest that the only gene encoding PSY in Arabidopsis is feedback-regulated by multiple factors to ensure an appropriate supply of carotenoid products during normal development or in response to environmental cues.

Introduction

Carotenoids are isoprenoid pigments that are present in photosynthetic organisms and also in some non-photosynthetic bacteria and fungi. Plant carotenoids are synthesized in plastids. Right after germination, carotenoid biosynthesis takes place in etioplasts of dark-grown seedlings, facilitating greening when underground seedlings emerge into sunlight (Park *et al.*, 2002; Rodriguez-Villalon *et al*., 2009). In chloroplasts of photosynthetic tissues, carotenoids are associated to light-harvesting complexes and photosynthetic reactions centers, where they contribute to light harvesting and have an essential role in photoprotection by dissipating the excess of light energy that cannot be used for photosynthesis (Niyogi, 1999; Demmig-Adams and Adams, 2000, Hirschberg, 2001). Carotenoids also accumulate at high levels in chromoplasts of flowers and fruits of some plant species, contributing to their colours. They are present as well, but in much lower levels, in other non-photosynthetic plastids (leucoplasts) of adult plants, including amyloplasts (starch-storing plastids) and elaioplasts (lipid-storing plastids) (Howitt and Pogson, 2006). In all these plastid types, carotenoids also serve as precursors of important growth regulators such as abscisic acid (ABA) and strigolactones, which have roles in plant development and in the interaction of plants with their environment (Nambara and Marion-Poll, 2005; Gomez-Roldan *et al.*, 2008; Arite *et al.*, 2009). Carotenoids have been extensively used as natural pigments and they are an important source of retinoids (including vitamin A) and antioxidants with health-promoting properties in the human diet (Botella-Pavia *et al*., 2004; Fraser and Bramley, 2004). The recent discovery of the nutritional benefits of a carotenoid-rich diet and the increasing demand for natural products have spurred an unprecedented interest in the biotechnological overproduction of carotenoids in plants. Despite important achievements in this area, however, a major problem still to be solved is our limited knowledge about the regulation of the carotenoid biosynthesis in different plant organs and tissues (Giuliano *et al.*, 2008).

Like all isoprenoids, carotenoids are synthesized from isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) (Cunningham and Gantt, 1998). From the two distinct pathways that can synthesize these metabolites in plants, the plastidial methylerythritol 4-phospate (MEP) pathway is the one supplying most of the metabolic precursors for carotenoid biosynthesis (Rodriguez-Concepcion and Boronat, 2002; Bouvier *et al.*, 2005) even in the dark, when the activity of the MEP pathway is much lower (Rodriguez-Villalon *et al.*, 2009). The first reaction of the MEP pathway is the production of deoxyxylulose 5-phophate (DXP) from pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase (DXS). Consecutive reactions lead the production of IPP and DMAPP, which are then transformed by geranylgeranyl diphosphate (GGPP) synthase into GGPP, a common precursor of carotenoids, gibberellins, and the side chain of chlorophylls, tocopherols and photosynthetic quinones. The first specific reaction of the carotenoid pathway is the production of phytoene from two GGPP molecules catalyzed by phytoene synthase (PSY). In most cases, PSY activity levels are regulated at the transcriptional level and determine flux to carotenoids (Hirschberg, 2001; Giuliano *et al.*, 2008). Uncoloured phytoene is then desaturated and

isomerized to lycopene (Figure 1), a red carotenoid that only accumulates in chromoplasts like those found in tomato fruit. In most tissues, cyclization of lycopene by beta or epsilon cyclises generates orange carotenes such as alpha-carotene (with one beta ring and one epsilon ring) and beta-carotene (with two beta rings). Although beta-carotene (the most important precursor of vitamin A) is an abundant carotenoid in photosynthetic tissues, carotene accumulation is more typical of some fruits and carrot roots. Hydroxylation of carotenes generates xanthophylls such as lutein (from alpha-carotene) and violaxanthin (from beta-carotene), that accumulate in photosynthetic and non-photosynthetic tissues. Along the pathway, carotenoids can be cleaved by dioxygenases into apocarotenoids with roles either as pigments and flavours that attract pollinators or seed-dispersing animals (Giuliano *et al.*, 2003) or as hormones (ABA, strigolactones) that regulate plant development and environmental interactions (Nambara and Marion-Poll, 2005; Klee, 2008; Van Norman, *et al.*, 2007).

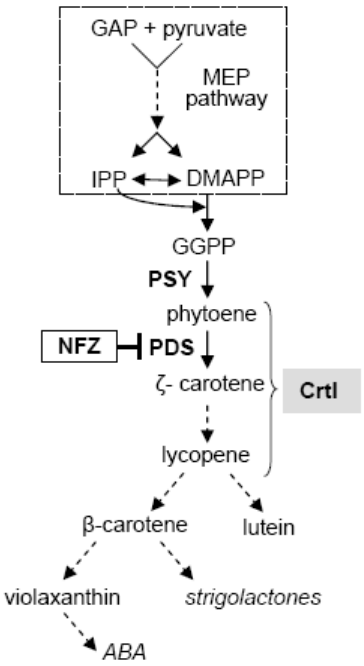


Figure 1. Carotenoid biosynthesis pathway. The MEP pathway (which provides most precursors for the production of carotenoids and other plastidial isoprenoids) is boxed. Dashed arrows represent several enzymatic steps. The step inhibited by norflurazon (NFZ) is shown. Enzymes are indicated in bold: PSY, phytoene synthase; PDS, plant phytoene desaturase; CrtI; bacterial phytoene desaturase. GAP; glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid.

The multifaceted role of carotenoids in plants (from photosynthesis and photoprotection to the modulation of plant responses to developmental and environmental cues) is consistent with the existence of different isoforms for many of the pathway enzymes in most plant species. For example, PSY is encoded by two genes in tomato (Giorio *et al*., 2008) and tobacco (Busch *et al.*, 2002) and three genes in rice (Welsch *et al.*, 2008) and maize (Li *et al.*, 2008). Some isoforms are involved in the biosynthesis of carotenoids in chloroplast-containing tissues, whereas others have more specific roles in the production of carotenoids in non-photosynthetic tissues of the fruit (tomato PSY1), the seed endosperm (maize PSY1) or the root (maize and rice PSY3). By contrast, PSY is encoded by a single gene (At5g17230) in the model species *Arabidopsis thaliana*. In this work we investigated the expression profiles of the Arabidopsis *PSY* gene throughout development and found an enriched accumulation of *PSY* transcripts in the vascular area of photosynthetic and particularly non-photosynthetic organs such as roots. Interestingly, the *PSY* gene was upregulated by salt or ABA treatments in roots but not in shoot tissues. We also investigated how this response was influenced in mutants defective in carotenoid-derived hormones (strigolactones, ABA). Together, our results suggest that the

production of carotenoid products for different functions (in the same tissue or in others after transportation by the vascular system) is controlled by multiple factors regulating the only gene encoding *PSY* in Arabidopsis.

Results and discussion

***PSY* expression is found in all Arabidopsis organs and it is highest in vascular bundles.**

The analysis of *PSY* gene expression in different tissues and developmental stages was initially performed by investigating transcript levels deduced from available microarray data (Figure S1). These data indicated that *PSY* transcripts are present in virtually all tissues and organs, consistent with a role of carotenoids in all plastid types. Also consistent with the critical role of carotenoids for photoprotection against excess light, high *PSY* transcript levels were found in photosynthetic organs (such as cotyledons, leaves, and stems), whereas much lower levels were detected in roots (Figure S1). Real-time quantitative RT-PCR (qPCR) analysis of *PSY* transcript levels in seedlings confirmed this result (Figure S2). Surprisingly, *PSY* levels were also very high in petals and stamens (Figure S1). To get more detailed experimental data, we generated transgenic lines expressing a construct with the *PSY* promoter and 5'-UTR sequence (1992 bp from the translation start codon and 1276 bp from the transcription initiation site: construct -1276/+716) fused to a sequence encoding the double GUS-GFP reporter. As expected based on the microarray data (Figure S1), the *PSY* promoter was active in virtually all organs. However, GUS activity (Figure 2) and GFP fluorescence (Figure 3) were found to be highest in vascular bundles of photosynthetic (cotyledons, leaves, sepals) and non-photosynthetic (roots, petals, stamens) organs.

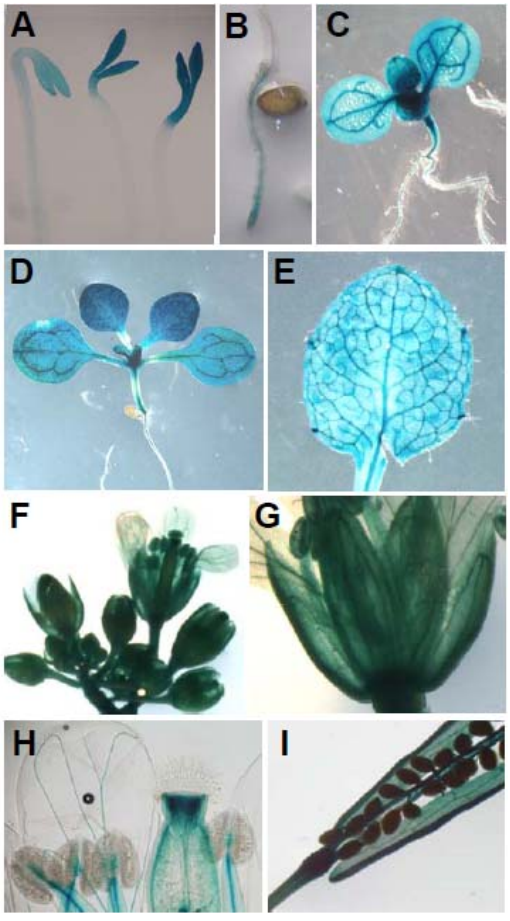


Figure 2. Analysis of *PSY* expression patterns by detection of GUS activity in *PSY:GUS-GFP* plants. Transgenic plants harbouring a construct for the expression of the double GUS-GFP reporter protein under the control of the *PSY* promoter (-1276/+716) were used for histochemical GUS staining. Similar results were found with different independent lines. Pictures correspond to representative individuals of one of the generated lines. (A) Seedlings germinated and grown in the dark for 3 days and then illuminated for 0 (left), 3 (middle) and 9 (right) hours with white light. (B) Root of 3-day-old seedlings. (C) Seedling germinated and grown for 5 days under long-day conditions. (D) Seedling germinated and grown for 8 days under long-day conditions. (E) First leaf from a 10-day-old seedling. (F) Inflorescence. (G) Lower part of mature flower. (H) Upper part of a mature flower. (I) Mature silique.

A higher accumulation of *PSY* transcripts in vascular bundles of wild type plants was confirmed by *in situ* hybridization with *PSY*-specific probes (Figure 4), validating the use of the *PSY:GUS-GFP* lines to estimate the patterns of *PSY* expression. Similar results were found when constructs with the GUS-GFP reporter fused to shorter promoter regions (constructs -300/+716 and -196/+716) were expressed in transgenic Arabidopsis lines (Figure S3). These results suggested that the *cis* elements regulating the organ, tissue and developmental patterns of *PSY* expression are found within the 0.9 kb region upstream of the translation initiation codon.

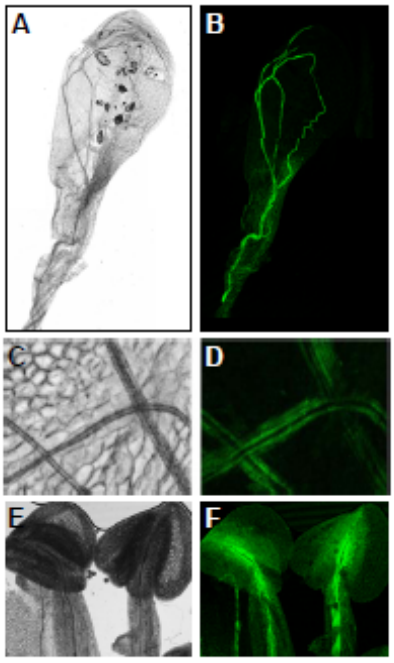


Figure 3. Analysis of *PSY* expression patterns in sepals and stamens by detection of GFP fluorescence in *PSY:GUS-GFP* plants. (A) Bright field image of a petal. (B) GFP fluorescence in the petal shown in A. (C) Magnification of a petal area showing vascular bundles. (D) GFP fluorescence in the area shown in C. (E) Upper region of stamens corresponding to the filaments and anthers. (F) GFP fluorescence in the stamens shown in E. GFP fluorescence pictures were taken under the confocal laser scanning microscope (CLSM) and represent assembled Z-stacks.

Analysis of carotenoid distribution in cotyledons by Raman microespectroscopy mapping (Baranski *et al.*, 2005; Baranska *et al.*, 2006) also showed an increased accumulation of carotenoids in areas corresponding to the vasculature (Figure 5), suggesting that increased *PSY* transcript levels result in higher PSY activity and eventually an enhanced carotenoid production in these cells. Raman mapping has previously shown that carotenoids accumulate in the vicinity of the vascular tissue in carrot roots (Baranska *et al.*, 2006).

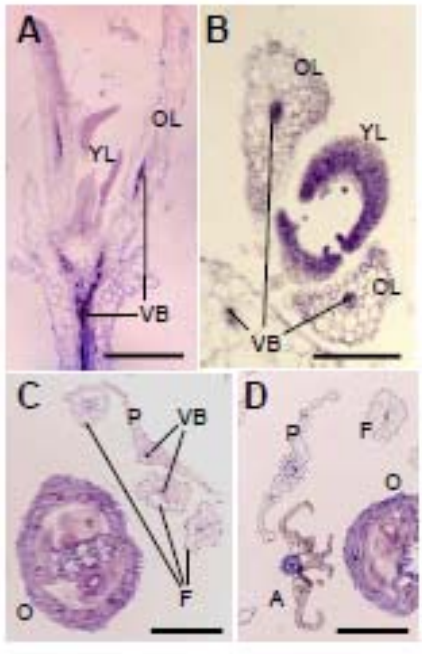


Figure 4. Analysis of *PSY* expression patterns by *in situ* hybridization. An antisense probe was used on sections from untransformed Arabidopsis plants corresponding to the apical shoot region (A), leaf primordial (B), and lower (C) and upper (D) sections of a mature flower. Hybridization was detected by the development of a purple color. A, anther; F, filament; O, ovary; P, petal; OL, older leaves; YL, young leaves; VB, vascular bundles. Bars correspond to 500 µm in A and B and 200 µm in C and D.

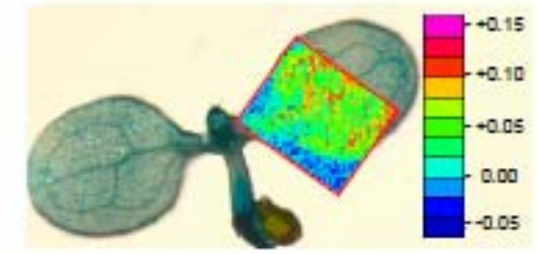
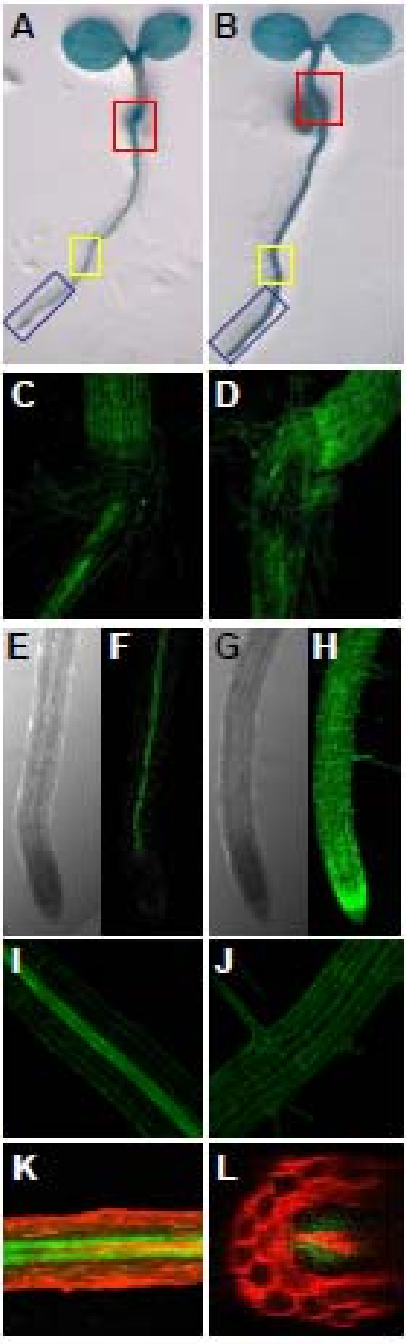


Figure 5. Raman mapping of carotenoid distribution in cotyledons. The indicated area of a cotyledon from a 5-old-day seedling was scanned to record Raman spectra at discrete points and then colored according to the intensity of the band at 1520 cm^{-1} (representing the most abundant carotenoids, beta-carotene and lutein). The distribution of GUS activity in a similar *PSY::GUS-GFP* seedling is shown for reference.

The vasculature has been involved in the transport of mobile signals from their biosynthetic location to the organs where they play their function (De Smet *et al.*, 2003). In the root, expression of specific rice and maize *PSY* isoforms has been shown to be associated with the production of ABA that could be then transported upward in the xylem to the shoot to trigger appropriate stress-related responses (Li *et al*. 2008; Welsh *et al*. 2008). Strigolactones are another group of apocarotenoids produced in roots that are transported to the shoot to regulate branching (Klee, 2008). In this context, the high level of expression of the only PSY-encoding gene in Arabidopsis in the vascular area of roots suggests that this activity might be required to produce carotenoid-derived products that can be transported elsewhere for biological activity. A careful examination of the patterns of GUS staining and GFP fluorescence in the roots of transgenic *PSY:GUS-GFP* lines indicated that the *PSY* promoter was most active in the central region of the elongation and maturation zones (Figure 6). GUS staining of cross sections of the elongation zone from transgenic roots confirmed that *PSY* expression was highest in the stele, particularly in phloem companion cells (Figure 7).

Low phytoene desaturation activity is sufficient to support carotenoid biosynthesis in roots.

In contrast to the well known function of carotenoids in photosynthesis and photoprotection in chloroplasts, much less is known about the role of these compounds in non-photosynthetic tissues, particularly in roots. To get a deeper insight into such roles and to investigate whether carotenoids produced in the root were involved in other developmental processes elsewhere, we aimed to block carotenoid biosynthesis specifically in roots and investigate the consequences on root and shoot development. In order to achieve this goal, we took advantage of the existence of bacterial CrtI enzymes that perform the desaturation and isomerization steps required to transform phytoene into lycopene (Figure 1) but show resistance to norflurazon (NFZ), an inhibitor of plant phytoene desaturase (PDS) enzymes. Thus, the addition of NFZ to the growth medium does not affect the production of carotenoids in tissues of transgenic plants producing a recombinant CrtI enzyme but it arrests carotenogenesis in wild type cells which only have the endogenous plant PDS enzyme (Misawa *et al*. 1993). A synthetic codon-optimized *TP-CrtI* gene encoding a CrtI enzyme from *Erwinia uredovora* fused to the pea (*Pisum sativum*) RuBisCO small subunit transit peptide for plastid import (Al-Babili *et al.*, 2006)



was expressed in transgenic Arabidopsis lines under the control of either the constitutive *35S* promoter or promoters from shoot-specific genes such as those encoding chlorophyll binding protein 3 (*CAB3*; At1g29910) and ferredoxin-dependent glutamate synthase 1 (*GLU1*; At5g04140). It was expected that the presence of NFZ would block the production of carotenoids in all tissues of untransformed plants, only in the roots of transgenic *CAB3:TP-CrtI* and *GLU1:TP-CrtI* lines, or nowhere in *35S:TP-CrtI* plants.

Figure 6. Analysis of *PSY* expression patterns in roots. Representative images from 3-day-old transgenic *PSY::GUS-GFP* (A, C, E, F, I, K, L) and control *35S::GUS-GFP* (B, D, G, H, J) seedlings are shown. A and B show seedlings stained for GUS activity and C, D, F, H, I, J, K and L show GFP fluorescence patterns (assembled z-stacks) detected by CLSM. Red fluorescence of cell walls in K and L results from staining with propidium iodide. (A) *PSY::GUS-GFP* seedling; (B) *35S::GUS-GFP* seedling. (C) GFP fluorescence of the region boxed in red in A. (D) GFP fluorescence of the region boxed in red in B. (E) Bright field image of the region boxed in blue in A. (F) GFP fluorescence of the root shown in E. (G) Bright field image of the region boxed in blue in B. (H) GFP fluorescence of the root shown in G. (I) GFP fluorescence of the region boxed in yellow in A. (J) GFP fluorescence of the region boxed in yellow in B. (K) GFP (green) and cell wall (red) fluorescence of a root region similar to that in i. (L) Optical cross-section through the root region shown in K.

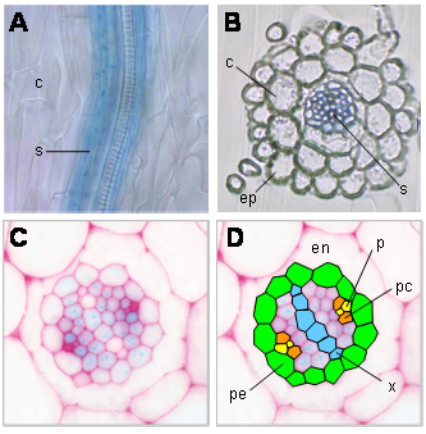


Figure 7. Analysis of *PSY* expression patterns in roots by detection of GUS activity in *PSY::GUS-GFP* plants. Root sections from 3-day-old seedlings corresponding to the region boxed in yellow in Figure 6A were used for GUS stainning. (A) Longitudinal section. (B) Cross-section. (C) Magnification of the stele area. (D) Representation of cell types in the section shown in C. ep, epidermis; c, cortex, s, stele; en, endodermis; pe, pericycle (green); p, phloem (yellow); pc, phloem companion cells (orange); x, xylem (blue).

Two independent lines harbouring a single insertion of each construct (as estimated based on the segregation of the T-DNA resistance marker) and showing a NFZ-resistance phenotype (no bleaching in the presence of the inhibitor) were selected for further experiments (Figure 8A).

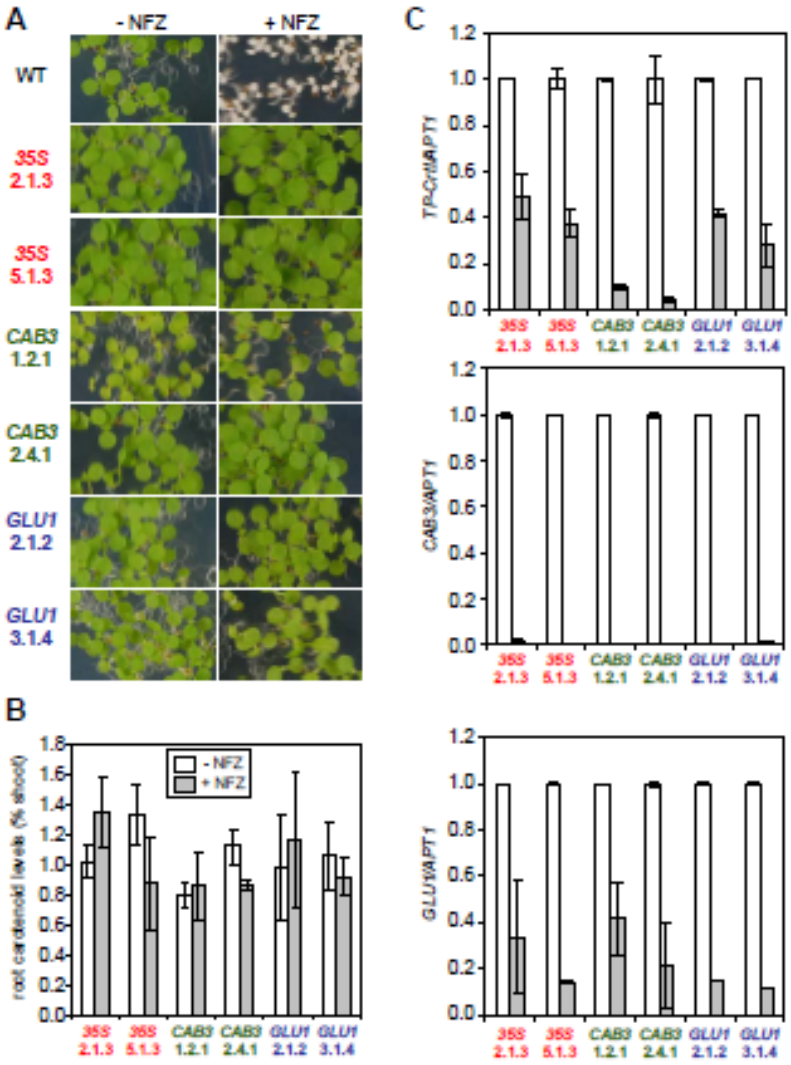


Figure 8. Characterization of transgenic lines expressing a recombinant *TP-CrtI* gene. (A) Representative images of 8-day-old seedlings of the indicated lines grown in the presence (+) or absence (-) of 50 nM NFZ. (B) Carotenoid levels in roots of the seedlings shown in A relative to the levels measured in photosynthetic (shoot) tissues of the same seedlings. (C) Transcript levels of the indicated genes in roots (gray bars) of transgenic seedlings grown without NFZ. Levels were estimated by qPCR, normalized using the *APT1* gene and represented relative to those in shoots (white bars). Bars represented mean and standard deviation of n=2 (B) or n=3 (C) experiments

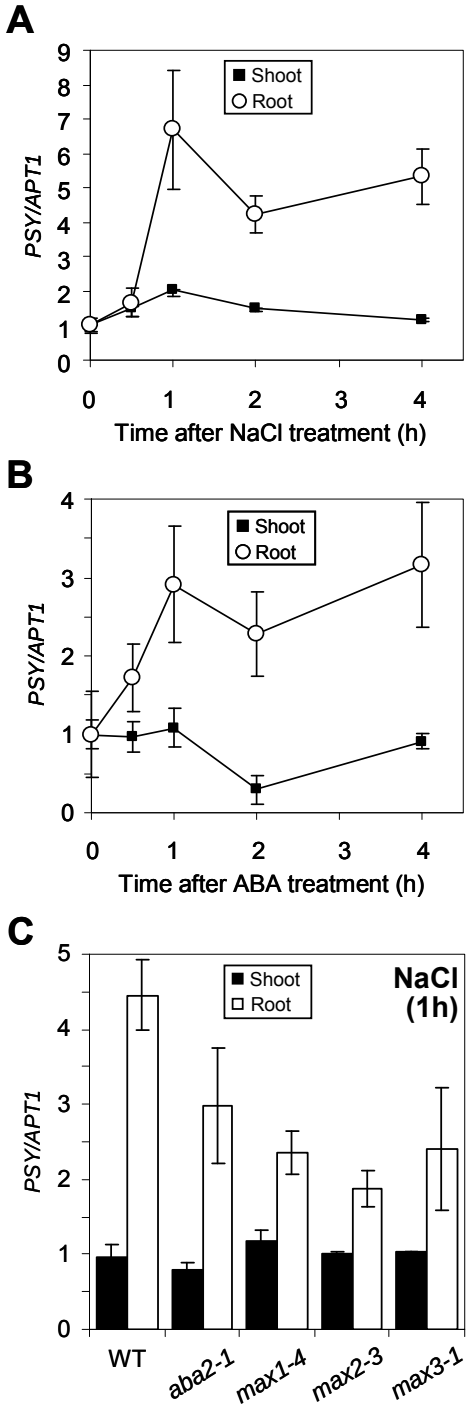
The minimum concentration of NFZ producing an albino phenotype in untransformed control seedlings but no visible bleaching in the transgenic lines selected was estimated to be 50 nM. We next quantified carotenoid levels by HPLC in shoot and root tissues of transgenic lines and untransformed controls growing with or without 50 nM NFZ to verify whether this concentration of inhibitor was sufficient to block carotenogenesis in root tissues of *CAB3:TP-CrtI* and *GLU1:TP-CrtI* seedlings without affecting the level of carotenoids in photosynthetic (shoot) tissues. As shown in Figure 8B, however, the levels of root carotenoids relative to those measured in shoots were not significantly unaffected by NFZ treatment in any transgenic line. These results suggested that a residual transgene expression in roots of *CAB3:TP-CrtI* and *GLU1:TP-CrtI* seedlings might be sufficient to support carotenoid biosynthesis in NFZ-

supplemented media. In agreement, qPCR analysis showed that *TP-CrtI* transcripts were detectable in roots of *CAB3:TP-CrtI* and *GLU1:TP-CrtI* seedlings, although at lower levels compared to shoot tissues (Figure 8C). In the case of *CAB3:TP-CrtI* lines, the difference between root and shoot *TP-CrtI* levels was not as dramatic as that observed for the endogenous *CAB3* transcripts (Figure 8C). These results indicate that the expression of the transgene is not as root-specific as the expression of the endogenous gene, perhaps due to the presence of a *35S* promoter sequence for the expression of the resistance gene in the same T-DNA (Yoo *et al.*, 2005). Despite using a constitutive promoter, levels of *TP-CrtI* transcripts in *35S:TP-CrtI* plants were different in shoots and roots (Figure 8C). In fact, the decreased accumulation of *TP-CrtI* transcripts detected in *35S:TP-CrtI* roots was similar to that observed in *GLU1:TP-CrtI* seedlings (Figure 8C). This might be due to differential promoter activity or *TP-CrtI* mRNA stability in root and shoot organs.

The existence of similar carotenoid profiles in roots of NFZ-treated and untreated transgenic seedlings is consistent with the lack of a clear inhibitor-dependent phenotype in these lines. Thus, we detected no differences in root growth (length) or root branching in NFZ-treated versus untreated plants. Analysis of phenotypes that might be caused by a reduced production of carotenoid-derived hormones in NFZ-treated plants such as ABA-related alterations in germination or strigolactone-dependent shoot branching also failed to detect significant differences relative to untreated seedlings. Concentrations of NFZ higher than 50 nM resulted in bleaching of the shoot tissues of transgenic seedlings, preventing their use to draw conclusions on the effect of decreasing carotenoid levels specifically in the root. Together, these results indicate that the low phytoene desaturation activity provided by residual *TP-CrtI* expression is likely sufficient to support carotenoid synthesis in the root of transgenic Arabidopsis plants. Our data also imply that the metabolic flux to carotenoids is probably very low in Arabidopsis roots, consistent with the low levels of carotenoid products found in these organs.

***PSY* expression is upregulated in roots in response to salt treatments.**

Despite the scarce accumulation of carotenoids in roots, these organs are a major source of ABA. This hormone plays an important role in regulating plant abiotic stress response. ABA can be synthesized in the stele and cortex of roots and move to the aerial parts of the plant where it regulates responses to water stress such as stomatal movements and the activity of shoot meristems (Saab *et al.*, 1992; Hartung *et al.*, 2002). Roots respond to osmotic or water stress by increasing ABA levels, which in maize and rice plants is achieved in part by upregulating the expression of PSY-encoding root isogenes (Li *et al.* 2008; Welsh *et al.* 2008). To investigate whether the Arabidopsis *PSY* gene was also responsive to abiotic stress, wild type plants were grown for 14 days on filter papers and then transferred to fresh solid medium supplemented with 200 mM NaCl. Samples of shoot and root tissues were collected separately right before and hours after the transfer and used for RNA extraction. As shown in Figure 9A, qPCR experiments detected a substantial upregulation of *PSY* transcript levels in roots, but not



in shoots, as soon as 1 h after exposing seedlings to osmotic stress (NaCl treatment). At longer times, *PSY* transcript accumulation decreased but it was still higher than in untreated samples. This is similar to that observed for the maize and rice *PSY3* isogenes (Li *et al*. 2008; Welsh *et al.* 2008), which led to suggest that PSY activity was limiting for the stress-triggered biosynthesis of ABA in roots. Also similarly, a lower but still important increase in *PSY* transcript levels was observed in roots (but not in shoots) from seedlings transferred to media supplemented with 50 µM ABA (Figure 9B). These results suggested a positive feedback regulation of *PSY* expression by ABA in Arabidopsis roots. To test this hypothesis, wild-type plants and ABA-deficient *aba2* mutants were grown for 14 days on filter papers and then transferred to NaCl-supplemented medium for 1 hour. As shown in Figure 9C, the upregulation of *PSY* expression was significantly reduced in the root of mutant seedlings, whereas no changes were observed in shoot tissues. All these data together confirm that ABA can feedback regulate the expression of the only Arabidopsis *PSY* gene specifically in roots.

Figure 9. Effect of salt stress and ABA application on *PSY* transcript levels. After transferring WT and mutant seedlings to plates containing either 200 mM NaCl (A, C) or 50 µM ABA (B), total RNA was extracted from shoot and root tissues and used for qPCR analysis of *PSY* expression. Transcript levels were normalized using the *APT1* gene and represented relative to those before treatment (0 h). Only data corresponding to 1 h after NaCl treatment are shown in (C). Mean and standard deviation of n=2 experiments are shown.

Similar to ABA, strigolactones are signalling molecules synthesized in roots that are transported to the shoot to regulate branching. Because both ABA and strigolactones are synthesized from carotenoids and carotenoid production in Arabidopsis roots is mainly regulated by PSY activity (Maass *et al.*, 2009), we next evaluated whether strigolactones could also influence *PSY* expression in roots. When mutants defective in strigolactone synthesis

(*max1*, *max3*) or perception (*max2*) were subjected to salt treatment as described above no changes in *PSY* transcript levels were observed in shoot tissues. By contrast, *PSY* upregulation in root tissues was decreased relative to that observed in wild-type seedlings (Figure 9C), suggesting that this novel hormone might be involved in the regulation of *PSY* expression in roots under salt stress conditions. It is possible that this feedback regulatory mechanism could involve the decrease of ABA levels in strigolactone mutants. Although no evidence is currently available that this could be the case, it is known that ABA can influence strigolactone biosynthesis when tomato roots are stressed by phosphate shortage (Lopez-Raez and Bouwmeester, 2008). Nevertheless, much work is still required to understand the crosstalk between ABA and strigolactones and the feedback mechanisms eventually resulting in the regulation of *PSY* expression.

Concluding remaks

In summary, our work demonstrates that the single gene encoding PSY in Arabidopsis is expressed ubiquitously. However, *PSY* expression in non-photosynthetic tissues, which do not accumulate high carotenoid levels, is virtually restricted to the vascular bundles (the stela in roots). The vascular system provides a route for long-distance movement, and specifically the xylem for root-to-shoot signaling. Root-to-shoot signals play roles communicating soil conditions, and these signals are important for plant growth and fitness. At least three carotenoid-derived products act as signals known to move from the root to the shoot to regulate shoot response to stress and plant growth: ABA, strigolactones, and a novel substance that requires the function of the *BYPASS1* gene (Nambara and Marion-Poll, 2005; Klee, 2008;Van Norman and Sieburth, 2007). The production of carotenoids in Arabidopsis roots is mainly controlled by PSY activity (Maass *et al.*, 2009).

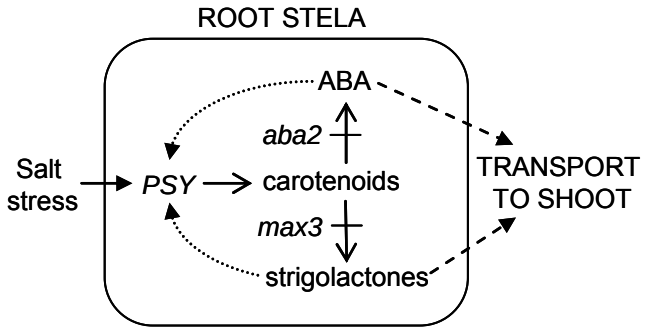


Figure 10. A model for the regulation of *PSY* expression in salt-stressed plants. Roots are known for their capability to form ABA and strigolactones that are later transported to the shoot. These metabolites are synthesized by oxidative cleavage of carotenoids, whose production in Arabidopsis roots is mainly controlled by PSY activity. We observed that NaCl treatment rapidly results in a transient peak accumulation of *PSY* transcripts in root cells. Salt-induced elevation of *PSY* expression is actually correlated with an increased production of ABA in roots (Li *et al.,* 2008; Welsch *et al*., 2008), whereas strigolactones also accumulate in response to abiotic stress (Lopez-Raez *et al.*, 2008; Lu and Li, 2008).Our results show *PSY* transcript accumulation was induced by ABA treatment but reduced in ABA-deficient mutants, suggesting a positive feedback regulation. Strigolactone mutants also showed a reduced response to NaCl treatment in terms of *PSY* upregulation, suggesting a new role for these signaling molecules in the regulation of salt stress responses.

As summarized in the model shown in Figure 10, our results show that ABA and strigolactones regulate *PSY* expression in response to salt stress specifically in the root, a feedback mechanism that could be used to finely control the levels of apocarotenoids precursors in the cells of the root stella. Together our results demonstrate that regulation of *PSY* expression is key to ensure an appropriate supply of carotenoid products during normal development or in response to environmental cues.

Materials and Methods

Plant material and growth conditions

All the *Arabidopsis thaliana* lines used in this work are in the Columbia background. Seeds from *aba2-1* (*gin1-3*) mutants were kindly provided by L.M. Lois (CRAG, Spain) and *max* mutants were kindly provided by S. Yamaguchi (RIKEN, Japan). Homozygous *PSY::GUS-GFP* (-1276/+716; -300/+716; -196/+716), *CAB3::TP-CrtI*, *GLU1::TP-CrtI* and *35S::TP-CrtI* lines were constructed as described below. Seeds were surface-sterilized and sown on solid Murashige and Skoog (MS) medium in Petri dishes. After stratification for 2 days at 4°C in the dark, plates were grown at 22°C in long-day photoperiod. When indicated, 50 nM of NFZ prepared as described (Rodriguez-Concepcion *et al.*, 2004) was included in the growth medium. For abiotic stress treatments, plants grown for 14 days on filter papers in solid MS plates were transferred to new plates supplemented or not with 200 mM NaCl or 50 µM ABA for up to 4 hours.

Generation and analysis of transgenic lines

To generate the transgenic *PSY::GUS-GFP* lines, DNA sequences containing the *PSY* promoter and 5´-UTR of the genomic region upstream the ATG translation initiation site of the Arabidopsis *PSY* (At5g17230) gene (constructs -1276/+716, -300/+716, and -196/+716) were amplified by PCR and cloned into the pCAMBIA 1303 vector to drive the expression of the GUS-GFP reporter protein. Similarly, a DNA sequence containing 1.5 kb and 1 kb of the region upstream the translation start codon of *CAB3* (At1g29910) and *GLU1* (At 5g04140), respectively, were amplified by PCR and cloned into pCAMBIA 1304 to generate plasmids pCAB3::GFP-GUS and pGLU1::GFP-GUS. These plasids were used to clone a PCR-amplified sequence encoding a plastid-targeted codon-optimized CrtI enzyme (Al-Babili *et al.*, 2006) under the control of the *CAB3* and *GLU1* promoter sequences. To generate the *35S::CrtI* construct, the amplified TP-CrtI sequence was directly cloned into pCAMBIA 1304. The resulting constructs were used for *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* plants as described (Carretero-Paulet *et al.*, 2002). Control lines were generated after the transformation with an empty pCAMBIA 1303 vector. Four *PSY::GUS-GFP* and three *CAB3::TP-CrtI*, *GLU1::TP-CrtI* and *35S::TP-CrtI* transgenic lines harbouring only 1 T-DNA insertions (as estimated from hygromycin resistance segregation) were selected for further experiments.

Histology and microscopy

Homozygous individuals of *PSY::GUS-GFP* and *35S::GUS-GFP* lines were used for GUS staining and confocal microscopy of GFP fluorescence as described (Rodriguez-Villalon *et al*., 2009). The same distribution of GUS activity and GFP florescence was observed in all lines harbouring the same construct, although at different intensity levels. The results shown here correspond to representative individuals of the lines showing the highest GUS activity and GFP fluorescence levels. Roots were stained with 20 µg/ml propidium iodure (PI) to stain cell walls. For high resolution analysis of cell structures, GUS stained samples were fixed overnight in 4% (v/v) glutaraldehyde in 25 mM $NaPO_4$ buffer, followed by embedding and polymerization in Technovit 7100 (Heraeus Kulzer). Sections (10 µM) were done using a Leica Jung Autocut microtome and visualized under a Leica Axiophot microscope.

Raman mapping

NIR-FT-Raman spectroscopy mappings were performed as described (Baranski *et al*., 2005). Two-dimensional Raman maps were obtained of an area of 4.570 x 4.855 µm, with an increment of 80 µm and 8 scans per point. Samples were mounted between two glass slides to avoid their movement and deformation during the measurement. Data obtained were represented as 2-D maps coloured according to band intensity of 1526 cm-1 as expected for carotenoids with 11 and 9 conjugated C=C bonds (lutein and β-carotene) in the polyene chain (Withnall *et al.*, 2003; Schulz *et al.*, 2005).

***In situ* hybridization analysis**

To generate specific probes for *PSY* gene, cDNA of wild-type plants was used as template to amplify by PCR a DNA sequencing containing 1.5 kb of *PSY* gene with primers PSY-F: 5´- ATTGTGGGTTGGTAAGGGTTC-3´ and PSY-R: 5´- ATTGTGGGTTGGTAAGGGTTC-3´. The resulting sequence was cloned in the *Sma*I site of pBluescript plasmid. Antisense probe was created using T3-polymerase whereas sense probe was created using T7 polymerase. Flowers and seedlings were fixed, stained and dehydrated in progressive ethanol solutions as described in (Ferrandiz *et al.*, 2000). Hybridization was performed at 49ºC degrees for more than 24 hours as described in (Ferrandiz *et al.*, 2000). Sample sections were visualized under a Leica Aixophot microscope.

Measurements of carotenoids and chlorophylls

Total carotenoids and chlorophylls were extracted as described (Fraser *et al.*, 2000) and individual carotenoids were analyzed and quantified by HPLC separation (Fraser *et al.*, 2000) monitoring and identifying the eluting compounds using a Waters 2996 photodiode array detector. Pigments were detected at 450 nm and peak areas at the indicated wavelengths were compared with those of internal standars for quantification as described (Rodriguez-Concepcion *et al.*, 2004).

Analysis of transcript levels

Total RNA extraction of plant samples was carried out as described in the RNAasy plant mini kit (QIAGEN) and digested on column for 20 min with DNAseI (QIAGEN). cDNA synthesis was carried out as described (Carretero-Paulet *et al.*, 2006). 50 ng of DNA input was used to perform SYBR Green PCR reactions using Light cycler 480 (Roche) mix buffer 2x with 10 µM final concentration of each primer. Controls were included as non-RT controls and non-template controls as PCR reactions were carried out as described (Phillips *et al.*, 2009). To detect *PSY* gene expression (At5g17230) primers qPSY-F (5´-GACACCCGAAAGGCGAAAGG-3´) and qPSY-R (5´-CAGCGAGAGCAGCATCAAGC-3´) were employed. To detect transgene *CrtI* expression, qCrtI-F: 5´-CGA CTT TAG AGA CCA ACT GAA TGC-3´ and qCrtI-R: 5´- CTA CCA ATA ACG CCA GGA ATG C-3´ primers were employed. For *CAB3* (At1g29910) and *GLU1* (At 5g04140) gene expression both couples of primers were used: qCAB3-F: 5´- GCG GAG TTG AAG GTG AAG GTG AAG GAG-3´, qCAB3-R: 5´- GGA ACA AAG TTG GTT GCG AAG G-3´and qGLU1-F: 5´- TCG GGA ATC TGG GAC C-3´ and qGLU1-R: 5´-CAA TCG GGT TTA ATG ACC AA-3´. Transcripts encoding adenine phosporibosyltransferase 1 (APT1) (At1g27450) (APT1-F: 5´-GTT GCA GGT GTT GAA GCT AGA GGT-3´and APT1-R: 5´-TGG CAC CAA TAG CCA ACG CAA TAG-3´) were also quantified to normalize the threshold cycle for each assay and calculate relative gene expression as described (Phillips *et al.*, 2009).

Acknowledegments

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SUPPLEMENTARY FIGURES

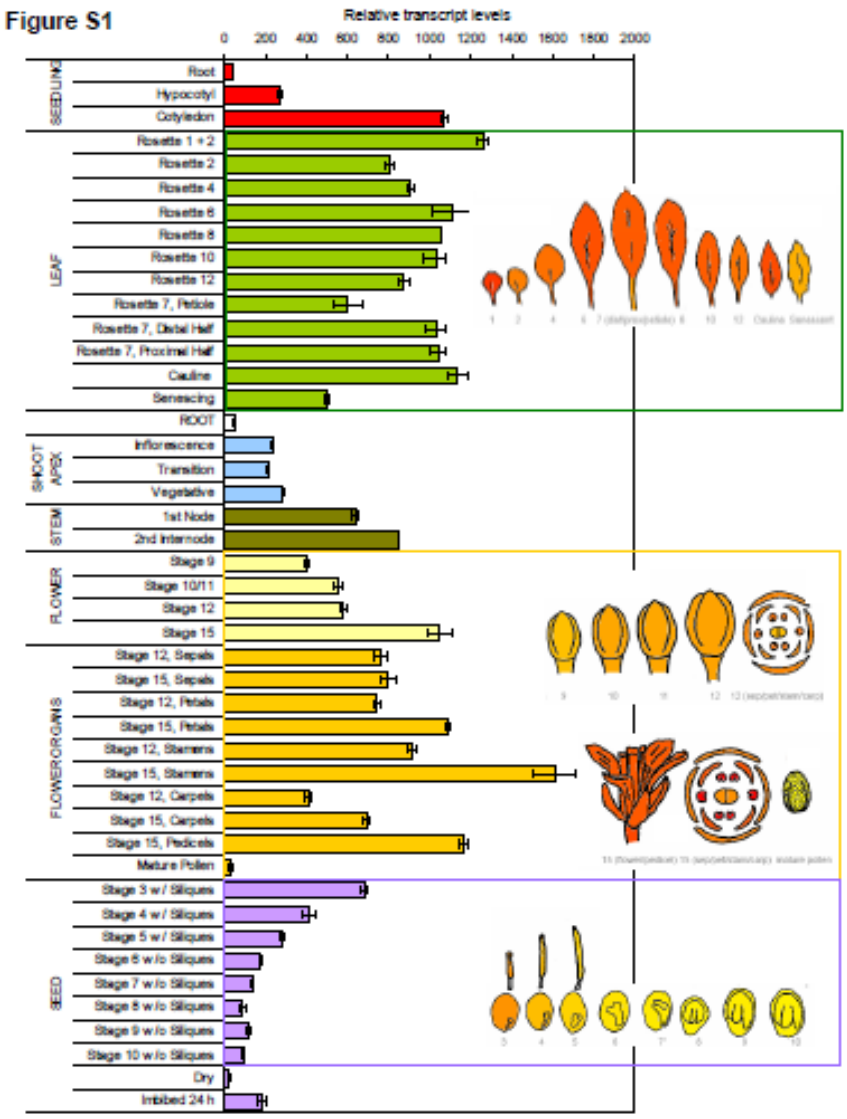

Figure S1. Levels of *PSY* transcripts in different organs of *Arabidopsis* plants. Data were obtained using the Arabidopsis eFP browser at www.bar.utoronto.ca (Winter *et al*., 2007 Plos One 2:e718).

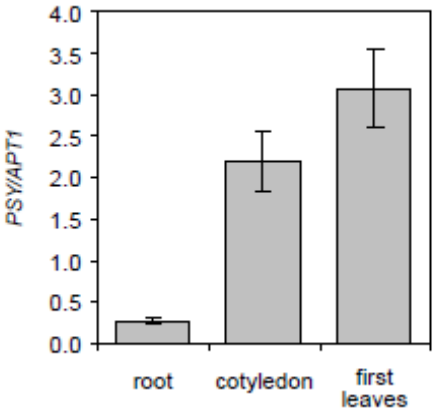

Figure S2. Levels of *PSY* transcripts in different organs of *Arabidopsis* seedlings. RNA was extracted from the indicated organs of 14-day-old seedlings and used for qPCR. The *APT1* gene was used for normalization. Bars represent mean and standard deviation of n=2 independent samples.

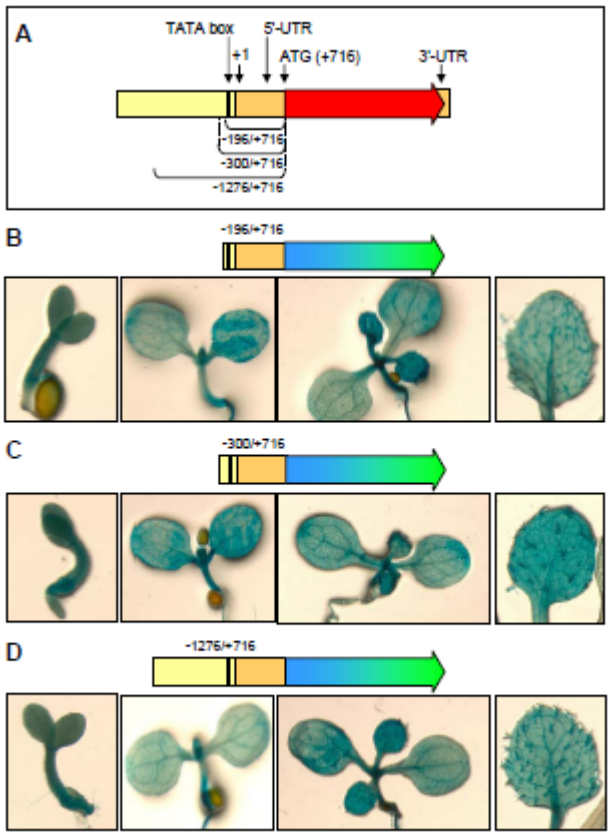

Figure S3. Analysis of *PSY* expression patterns in *PSY::GUS-GFP* plants. Transgenic lines harboring a construct for the expression of the double *GUS-GFP* reporter protein under the control of different regions of the *PSY* promoter were used for histochemical GUS staining. Pictures correspond to representative individuals of the generated lines at 2, 4 and 6 days after stratification and leaves from 10-day-old seedlings. (A) Schematic representation of the *PSY* gene structure. UTR regions are indicated in orange. (B) GUS staining of -196/+716 lines. (C) GUS staining of -300/+716 lines. (D) GUS staining of -1276/+716 lines.

Apéndices

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Contribution of hydroxymethylbutenyl diphosphate synthase to carotenoid biosynthesis in bacteria and plants

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ABSTRACT

The methylerythritol 4-phosphate (MEP) pathway synthesizes the precursors of carotenoids and other isoprenoids in bacteria and plant plastids. Despite recent progress in the identification of rate-determining steps, the relative contribution of most pathway enzymes to flux control remains to be established. In this work we investigated whether upregulated levels of hydroxymethylbutenyl diphosphate synthase (HDS) could increase the metabolic flux through this pathway, as judged by endpoint (carotenoid) measurements. Unlike other MEP pathway enzymes, however, increasing the levels of an active HDS protein in carotenoid-producing *Escherichia coli* cells and transgenic *Arabidopsis thaliana* plants did not result in an enhanced accumulation of MEP-derived isoprenoids. Our data suggest that enhanced flux through the MEP pathway for peak demand periods in bacteria and plastids does not require increased HDS activity.

Isoprenoids (also called terpenoids) are essential in all organisms but are particularly abundant and diverse in plants. Some act as primary metabolites in respiration, photosynthesis, and in the regulation of growth and development, but the bulk of plant isoprenoids are secondary metabolites that modulate the interaction of plants with their environment [1–3]. Despite their functional and structural diversity, all isoprenoids derive from the same five-carbon building blocks, isopentenyl diphosphate (IPP) and its double-bound isomer dimethylallyl diphosphate (DMAPP). Consecutive condensation of one or several IPP units with DMAPP results in the production of prenyl diphosphates of increasing size which constitute branch points for the biosynthesis of isoprenoid endproducts such as sterols, carotenoids, hormones, and the prenyl moiety of chlorophylls, tocopherols, and quinones [2].

Plants synthesize IPP and DMAPP by two independent pathways: the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerytritol 4-phosphate (MEP) pathway [1]. By contrast, the MEP pathway is the only one present in most eubacteria, including *Escherichia coli*. Upregulating the biosynthesis of MEP-derived prenyl diphosphate precursors has been shown to be a viable biotechnological strategy to increase the production of economically-important isoprenoid end-products in bacteria and plant plastids [4,5]. This has been achieved by overexpression of the enzymes catalyzing the first two and the last step of the pathway (Fig. 1), deoxyxylulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), and hydroxymethylbutenyl diphosphate (HMBPP) reductase (HDR). The enhanced accumulation of carotenoids and other MEP-derived isoprenoid products in transgenic bacteria and plants overexpressing DXS, DXR, or HDR indicates that their production is limited by the supply of precursors and that several enzymes share control over the metabolic flux of the MEP pathway, with different enzymes exhibiting different degrees of control [6]. The contribution of other enzymes of the pathway to flux control remains to be established. However, a regulatory role has been suggested for HMBPP synthase (HDS), the enzyme catalyzing the transformation of methylerythritol 2,4-cyclodiphosphate (ME-cPP) into HMBPP in the penultimate step of the MEP pathway (Fig. 1). The proposal that HDS might be a flux-controlling enzyme is based on the distinctive sequence features of the plant enzyme [7,8], its post-transcriptional regulation in plastids [9,10], its participation in defense mechanisms [11], and the photosynthesis-dependent control of its enzymatic activity in plant cells [12]. In this work we have evaluated this hypothesis by upregulating HDS levels in transgenic bacteria (*E. coli*) and

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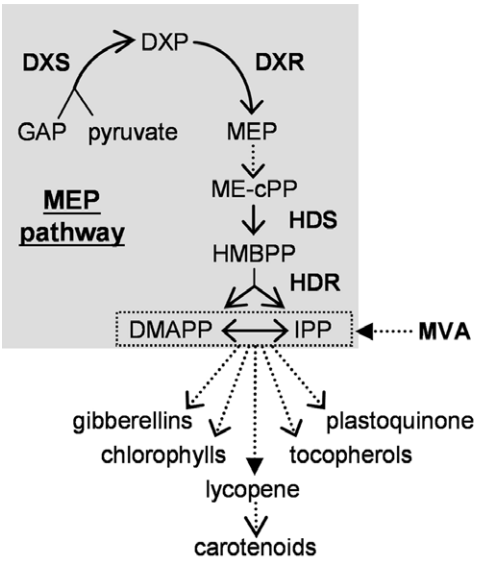

Fig. 1. Isoprenoid biosynthesis in plant cells. Dashed arrows represent multiple enzymatic steps. Pathways introduced in the *E. coli* strains used in this work are indicated with solid head arrows. GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxyxylulose 5-phosphate; MEP, 2-methylerythritol 4-phosphate; ME-cPP, 2-methylerythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MVA, mevalonic acid. MEP pathway enzymes are indicated in bold: DXS, DXP synthase; DXR, DXP reductoisomerase; HDS, HMBPP synthase; HDR, HMBPP reductase.

plants (*Arabidopsis thaliana*) and analyzed the effects on the levels of derived isoprenoids (carotenoids).

Materials and methods

Bacterial strains and growth conditions. The EcAB4-4 (*gcpE::CAT*) strain was constructed as described [13]. Briefly, after incorporating the MVA$^+$ synthetic operon into the genome of the K-12 MG1655 wild type strain, most of the coding region of the *gcpE* gene was replaced with the *CAT* gene encoding chloramphenicol acetyltransferase. Competent EcAB4-4 cells were transformed with the pQE32-GcpE plasmid [14] or an empty pQE32 vector (Qiagen) as a control and used for complementation experiments as described [8]. The same plasmids were also used to transform BL21(DE3) cells (Stratagene) together with plasmid pACCRT-EIB, which harbors the *Erwinia uredovora crtE, crtB, and crtI* genes for lycopene synthesis [15]. Double transformants were selected on plates with solid Luria broth (LB) medium supplemented with antibiotics to final concentrations of 17 μg/ml chloramphenicol (to select for the pACCRT-EIB plasmid) and 100 μg/ml ampicillin (to select for the pQE32 or pQE32-GcpE plasmids). Overnight cultures of several independent pink (lycopene-producing) colonies in antibiotic-supplemented medium were then used to inoculate fresh LB medium (1:100 dilution) containing the appropriate supplements with or without 0.5 mM IPTG. Aliquots of cultures grown at 37 °C for 15 h were used for protein and lycopene extraction as described below.

Plant material and growth conditions. Plasmid pCAMBIA-*35S-CSB3* (with the *A. thaliana* cDNA sequence encoding HDS under the transcriptional control of the constitutive *35S* promoter) was used for transformation of *Arabidopsis* plants of the Columbia-0 background harboring the *P69C*-GUS reporter or mutant *clb4-3* plants as described [11]. Plants were grown on plates and soil as described [16].

Analysis of transcript levels. Total RNA was isolated from 10-day-old seedlings grown on plates, copied to cDNA, and used for real-time quantitative RT-PCR as described [16]. A predesigned FAM-labeled TaqMan MGB probe and unlabeled primers (Applied Biosystems) were used for HDS (At02186785_g1). The threshold cycle for each probe assay was normalized using EF1α (elongation factor – 1α, At02337969_g1) and relative transcript levels were estimated as described [16].

Immunoblot analysis. After growing *E. coli* BL21(DE3) cells containing the pACCRT-EIB and pQE32-GcpE or pQE32 plasmids as described above, 1 ml aliquots were harvested and cells were pelleted by centrigugation. Protein crude extracts were made from 7.5 mg of cells and used for immunoblot analysis of RGS-His-GcpE levels as described [9,10] using a 1:2000 dilution of a commercial antibody against the RGS-His epitope (Qiagen). Immunoblot analysis of HDS levels in protein extracts of *Arabidopsis* 10-day-old seedlings was performed as described [9].

Quantification of pigment levels. For quantification of lycopene in *E. coli* strains harboring the pACCRT-EIB plasmid, the cell pellet of a 1.3 ml aliquot of cultures grown as described above was resuspended in 700 μl of acetone. Samples were then incubated at 55 °C for 15 min in the dark and centrifuged at 13,000*g* for 10 min to recover the supernatant with the pigment, which was placed in a clean tube. At least two samples were taken from each culture for lycopene extraction. Lycopene was quantified by measuring absorbance at 505 nm of diluted supernatants in acetone and comparing with a standard curve made with known concentrations of a lycopene standard (Sigma). Chlorophylls and carotenoids were extracted from 10-day-old seedlings, separated by HPLC, and quantified as described [16]. Statistical analysis of the data was performed using the Simple Interactive Statistical Analysis (SISA) *T*-test available online (http://home.clara.net/sisa/t-test.htm).

Results and discussion

Upregulated HDS levels in E. coli cells do not result in an enhanced production of isoprenoid precursors

A recent work showed that replacing the native promoter of the endogenous *E. coli gcpE* gene encoding HDS (also named GcpE or IspG in bacteria) with the strong bacteriophage T5 promoter in carotenoid-producing strains did not affect the accumulation of these MEP-derived isoprenoids [17]. Although the results suggested that the production of carotenoid precursors might not be limited by HDS activity in *E. coli* cells, no experiments were reported to confirm that increased levels of active enzyme were actually present in the engineered strains. As an alternative strategy to successfully upregulate HDS activity levels in bacteria, we used the pQE32-GcpE plasmid [14]. In this multi-copy vector, the T5 promoter (which can be induced by IPTG) drives the expression of a recombinant *E. coli* HDS protein with an N-terminal RGS-His epitope [14]. We first confirmed that the addition of the RGS-His tag did not impair enzyme activity by complementation of the lethal phenotype of HDS-defective cells. Disruption of the *gcpE* gene in the EcAB4-4 (*gcpE::CAT*) strain, which also harbors a synthetic MVA+ operon encoding heterologous MVA pathway enzymes that can transform exogenously supplied MVA into IPP and DMAPP, results in MVA auxotrophy [13]. As shown in Fig. 2A, transformation of EcAB4-4 cells with the pQE32-GcpE construct eliminated the requirement of MVA for survival, whereas cells from the same strain transformed with the empty pQE32 plasmid remained auxotrophic for MVA. These results confirmed that the recombinant RGS-His-GcpE protein retained HDS activity. We next co-transformed *E. coli* BL21(DE3) cells with the same constructs and a plasmid (pACCRT-EIB) encoding heterologous enzymes to synthesize the carotenoid lycopene from IPP and DMAPP [15] in order to eval-

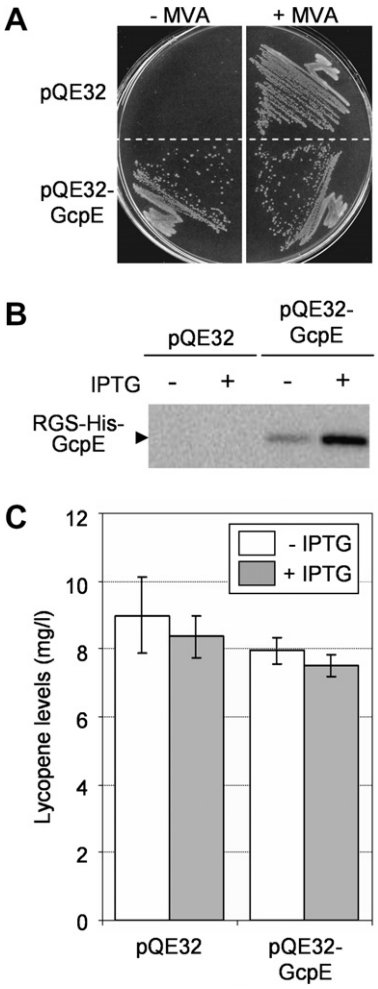

Fig. 2. Upregulation of HDS in bacteria. (A) Complementation of HDS-deficient *E. coli* cells. EcAB4-4 cells were transformed with a construct to express the recombinant RGS-His-GcpE protein (pQE32-GcpE) or an empty pQE32 vector and plated on LB medium supplemented with 1 mM MVA and antibiotics to recover transformants. Positive colonies were then streaked on new plates with (+) or without (−) MVA. (B) Immunoblot analysis of RGS-His-GcpE levels. BL21(DE3) cells were transformed with the indicated vectors together with plasmid pACCRT-EIB for lycopene synthesis. The resulting co-transformants (identified by their pink color) were grown in liquid medium supplemented (+) or not (−) with 0.5 mM IPTG for 15 h at 37 °C. Aliquots of the cultures were then used for protein extraction and immunoblot analysis with antibodies against the RGS-His epitope. Arrowhead indicates the position of the RGS-His-GcpE protein. (C) Lycopene content of the cultures described in (B). Mean values and standard errors of two replicates of at least four independent cultures are shown.

uate whether upregulated levels of HDS activity resulted in an enhanced production of isoprenoids using carotenoid (lycopene) accumulation as a reporter. Colonies of double transformants grown on plates supplemented with the appropriate antibiotics were visually identified based on the red color imparted by lycopene accumulation and used to inoculate liquid media. Grown cultures were then used to analyze the production of recombinant protein and to quantify the levels of lycopene. Immunoblot analysis of RGS-His-GcpE levels with an antibody specific for the RGS-His epitope showed that this protein was synthesized in BL21(DE3) cells harboring the pQE32-GcpE plasmid even in the absence of the IPTG inducer (Fig. 2B). However, lycopene accumulation was not increased compared to control cells transformed with the pQE32 vector (Fig. 2C). Addition of 0.5 mM IPTG to the growth medium resulted in a substantially increased production of RGS-His-GcpE protein (Fig. 2B), but no significant ($P < 0.05$) changes were observed in lycopene accumulation (Fig. 2C). Our data confirm that overproduction of an active HDS enzyme in *E. coli* cells does not result in increased carotenoid levels, consistent with a non-limiting role of this activity for isoprenoid biosynthesis in bacteria.

HDS levels are not limiting for plastid isoprenoid biosynthesis in wild type Arabidopsis plants

The control exerted by HDS over the metabolic flux to plastidial isoprenoids in plant cells was examined by upregulating its levels in *Arabidopsis* plants with a wild type *HDS* gene (At5g60600, named *GCPE*, *ISPG*, *CLB4*, or *CSB3* in the literature) and in mutants with reduced HDS activity. Complete loss of HDS function in the *chloroplast biogenesis 4* (*clb*4) null alleles *clb4-1* and *clb4-2* results in a seedling lethal albino phenotype, as expected for a knockout mutant of the MEP pathway [18]. A point mutation at this locus has been reported to result in a partial loss of function in the *constitutive subtilisin 3* (*csb3*, renamed *clb4-3*) mutant, causing a developmental delay and, unexpectedly, a strikingly enhanced resistance to biotrophic pathogens [11]. We also observed that *clb4-3* plants had a slightly pale phenotype (Fig. 3A) caused by a decreased accumulation of MEP-derived photosynthetic pigments, chlorophylls and carotenoids (Fig. 4). Transformation of *clb4-3* plants with a construct to constitutively express the *Arabidopsis* HDS enzyme under the control of the *35S* promoter restored a wild type phenotype in terms of growth and pigmentation (Fig. 3A), confirming that the mutant phenotypes were caused by reduced HDS levels and that the recombinant protein produced in these lines was active. Quantitative real-time PCR analysis of three of these *clb4-3 35S::HDS* lines (1.1, 5.6 and 4.2) confirmed that *HDS* transcripts were increased several fold in the rescued lines (Fig. 3B). Immunoblot analysis with HDS-specific antibodies demonstrated a corresponding accumulation of the encoded protein (Fig. 3C). But despite the observed upregulation of an active HDS enzyme, the levels of chlorophylls and carotenoids were unchanged ($P < 0.05$) compared to those observed in plants with wild type HDS activity levels (Fig. 4). The same construct was used for transformation of *Arabidopsis* plants with wild type HDS levels (line P69C-GUS) [11]. The generated lines (G5.A, G6.A, and G6.B) were visually undistinguishable from untransformed plants or *clb4-3 35S::HDS* lines (Fig. 3B). Also similar to *clb4-3 35S::HDS* plants, G5.A, G6.A, and G6.B lines displayed enhanced *HDS* transcript (Fig. 3B) and protein levels (Fig. 3C) but no significant ($P < 0.05$) changes in the accumulation of chlorophylls or carotenoids compared to untransformed controls (Fig. 4). Together, our data suggest that the production of carotenoids and other MEP-derived isoprenoids is not limited by the levels of HDS activity present in wild type *Arabidopsis* chloroplasts under normal growth conditions. Consistent with this observation, no major changes in the levels of HDS-encoding transcripts are detected when carotenoid biosynthesis is boosted during the transition from etioplasts to chloroplasts in deetiolating *Arabidopsis* seedlings and from chloroplasts to chromoplasts in ripening tomato fruit [6,7].

Several MEP pathway enzymes, but not HDS, control isoprenoid precursor supply during peak demand periods in bacteria and plastids

Accumulation of ME-cPP, the substrate of HDS (Fig. 1), has been observed in bacteria under oxidative stress [19], whereas in *Arabidopsis* plants it appears to be involved in resistance to some pathogens [11]. On the other hand, HMBPP, the product of HDS, acts as a potent antigen that triggers human immunological responses [20]. Furthermore, plant HDS has been shown to be an essential enzyme [18] whose activity is post-transcriptionally regulated in plastids [9,10] in part by a photosynthesis-dependent control [12]. These observations suggest that a certain level of HDS activity is maintained under normal growth conditions but it might be changed

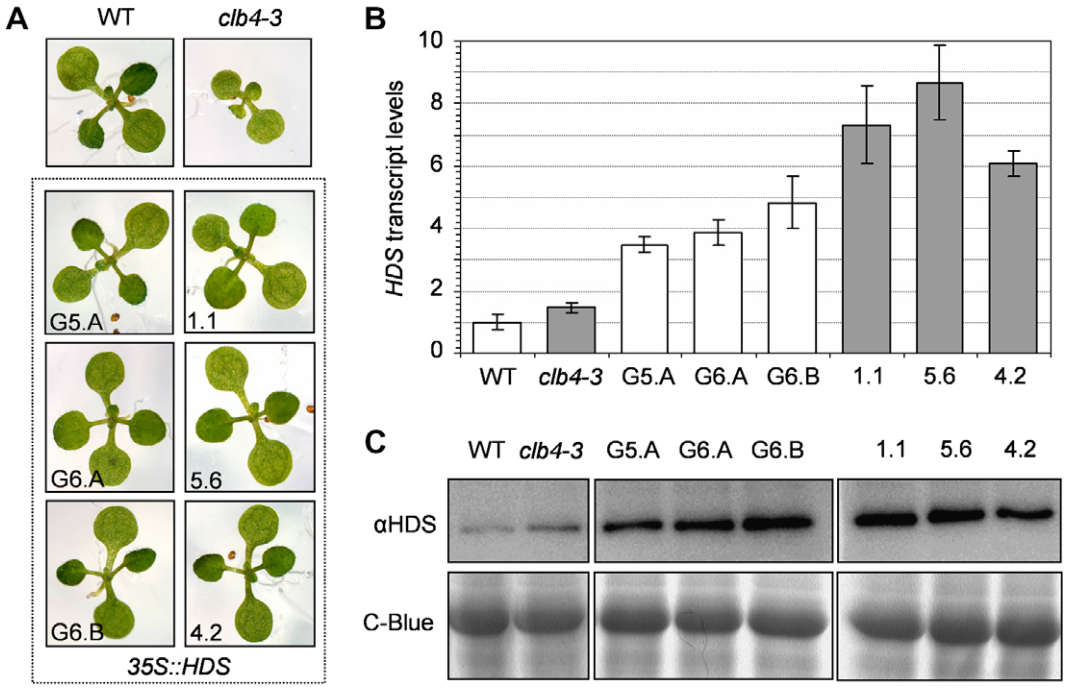

Fig. 3. Phenotype of *Arabidopsis* lines with altered levels of HDS activity. *Arabidopsis* plants with a wild type (WT) gene encoding HDS or a partial loss of function mutant allele (*clb4-3*) were transformed with a construct to constitutively overproduce the enzyme (*35S::HDS*). Several independent lines with the WT (G5.A, G6.A, G6.B) or mutant (1.1, 5.6, 4.2) genomic locus were selected. (A) Representative 10-day-old seedlings of the indicated lines. All panels are to the same scale. (B) Real-time quantitative RT-PCR analysis of *HDS* transcript levels in seedlings of lines shown in (A). Mean and standard error of duplicates of two independent experiments is shown. Levels are represented relative to those in untransformed WT seedlings. (C) Immunoblot analysis of HDS protein levels in 10-day-old seedlings of the indicated lines with a HDS-specific antibody (αHDS). Coomassie Blue (C-Blue) staining is shown as an estimate of equal protein loading.

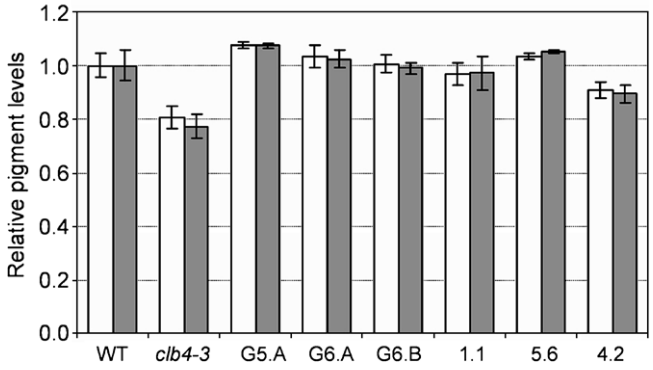

Fig. 4. Accumulation of plastidial isoprenoid pigments in transgenic *35S::HDS* lines. Total carotenoid (white columns) and chlorophyll (gray columns) levels of 10-day-old seedlings of the indicated lines are shown. Pigment levels are represented relative to those in untransformed WT plants. Mean values and standard errors correspond to two experiments.

in response to different stimuli in bacteria and plant cells. Consistently, our results with *clb4-3 35S::HDS* lines suggest that the production of wild type levels of plastidial isoprenoids such as chlorophylls and carotenoids requires a threshold level of HDS enzyme activity. However, two observations imply that the flux control coefficient of HDS could be quite low. First, the effect of the *clb4-3* mutation on carotenoid and chlorophyll production is relatively slight (Fig. 4), suggesting the flux control coefficient of this step does not rise to a significant level until HDS activity has decreased below its wild type level. By contrast, similar point mutations in the gene encoding DXS produce a far more dramatic phenotype [21,22]. Secondly, that overexpression has no effect on endproduct accumulation (Fig. 4) indicates that the flux control coefficient (under wild type expression levels) is too low to increase flux by upregulating this step.

Alternatively, it is possible that the availability of enzyme cofactors required for HDS activity might limit the production of plastidial prenyl diphosphates upon upregulation of enzyme levels. Both HDS and HDR, the last two enzymes of the MEP pathway (Fig. 1), are iron–sulfur reductases that require a reduced $[4Fe–4S]^{1+}$ cluster for enzymatic activity [23–26]. The oxidized $[4Fe–4S]^{2+}$ form of the two enzymes can be reduced *in vitro* by a flavodoxin/flavodoxin reductase/NADPH system, probably the *in vivo* reducing system in *E. coli* [24,25]. In plants, electron flow from photosynthesis can directly provide the electrons required for HDS activity in chloroplasts *via* ferredoxin in the absence of any reducing cofactor [12]. However, it is not likely that this reducing system is limiting for HDS activity or/and the production of MEP-derived isoprenoid precursors in chloroplasts because overexpression of HDR (another enzyme that most likely requires the same reducing system) successfully increased the accumulation of chlorophylls and carotenoids in *Arabidopsis* [27]. Moreover, upregulated HDR levels in *E. coli* also resulted in enhanced accumulation of carotenoids [28], indicating that the reducing power required for the activity of both HDS and HDR is also non-limiting in bacteria.

Although it is possible that another unknown factor required for the activity of HDS (but not HDR) might prevent an increased production of carotenoids in transgenic bacteria and plants with enhanced levels of active HDS protein, it is more likely that the activity of a different MEP pathway enzyme becomes limiting when HDS activity is upregulated. Here, we have shown for the first time that not all the MEP pathway enzymes cause a significant change in the pathway flux when their concentration is altered, i.e. not all of them might be considered rate-determining according to metabolic control analysis (MCA) terminology. Based on overexpression studies, the control of flux through the MEP pathway

should be shared by the rate-determining enzymes DXS, DXR, and HDR [6,16], whereas the contribution of HDS to overall flux appears to be comparatively much lower. A thorough examination of the relevant MCA parameters is now in progress in our lab to confirm this model, which has important implications for metabolic engineering of carotenoids and other isoprenoids of interest in bacterial and plant systems.

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Colors in the dark

A model for the regulation of carotenoid biosynthesis in etioplasts

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Carotenoids are plastidial isoprenoid pigments essential for plant life. High carotenoid levels are found in chloroplasts and chromoplasts, but they are also produced in the etioplasts of seedlings that germinate in the dark. Our recent work has shown that an enhanced production of carotenoids in plastids of dark-grown *Arabidopsis thaliana* seedlings results in an improved transition to photosynthetic development (greening) upon illumination, illustrating the relevance of regulating etioplast carotenoid biosynthesis for plant fitness. We showed that the biosynthesis of etioplast carotenoids is controlled at the level of phytoene synthase (PSY), the enzyme catalyzing the first committed step of the pathway. Upregulation of PSY is necessary and sufficient to increase the production of carotenoids in dark-grown seedlings, in part because it triggers a feedback mechanism leading to the post-transcriptional accumulation of flux-controlling enzymes of the methylerythritol 4-phosphate (MEP) pathway, which synthesizes the substrates for PSY activity. Based on these and other recent data on the molecular mechanisms controlling deetiolation, we propose a model for the regulation of carotenoid biosynthesis in etioplasts.

Key words: carotenoid, deetiolation, etioplast, feedback regulation, MEP pathway

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After germination, plant development can follow two different pathways depending on light conditions: skotomorphogenesis in darkness and photomorphogenesis in the light.[1] Skotomorphogenic development results in etiolated seedlings with long hypocotyls and closed unexpanded cotyledons with etioplasts containing chlorophyll precursors and relatively low levels of carotenoids associated to the prolamellar body (PLB). Etioplast carotenoids (lutein, violaxanthin, and much smaller amounts of other carotenoids) have been proposed to participate in the assembly of the PLB, a lattice of tubular membranes that facilitates greening when underground seedlings emerge into the light.[2,3] Upon light perception, photomorphogenic development is derepressed, resulting in decreased hypocotyl elongation, cotyledon expansion and differentiation of etioplasts into chloroplasts.[1] This light-induced deetiolation process involves the production of high levels of chlorophylls and carotenoids in chloroplasts to support photosynthetic development. Chloroplast carotenoids act as membrane stabilizers and accessory light-harvesting pigments, but their essential role is to channel excess energy away from chlorophylls for protection against photooxidative damage.[4] Carotenoids (but not chlorophylls) also accumulate in chromoplasts of flowers and fruits (providing distinctive yellow to red colors) and, to a much lower level, in other non-photosynthetic plastids of adult plants such as amyloplasts (starch-storing plastids), elaioplasts (lipid-storing plastids) and leucoplasts.[5]

Deetiolation can be derepressed in the absence of actual light by manipulating the levels of proteins and hormones involved in its control. For example, reduced activity of the E3 ubiquitin ligase COP1 (which interacts with HY5 and other transcription factors required for photomorphogenesis and promotes their proteasome-mediated degradation) or of the members of the PIF subfamily of bHLH transcription factors (PIF1, PIF3, PIF4 and PIF5) results in a partially deetiolated phenotype in the

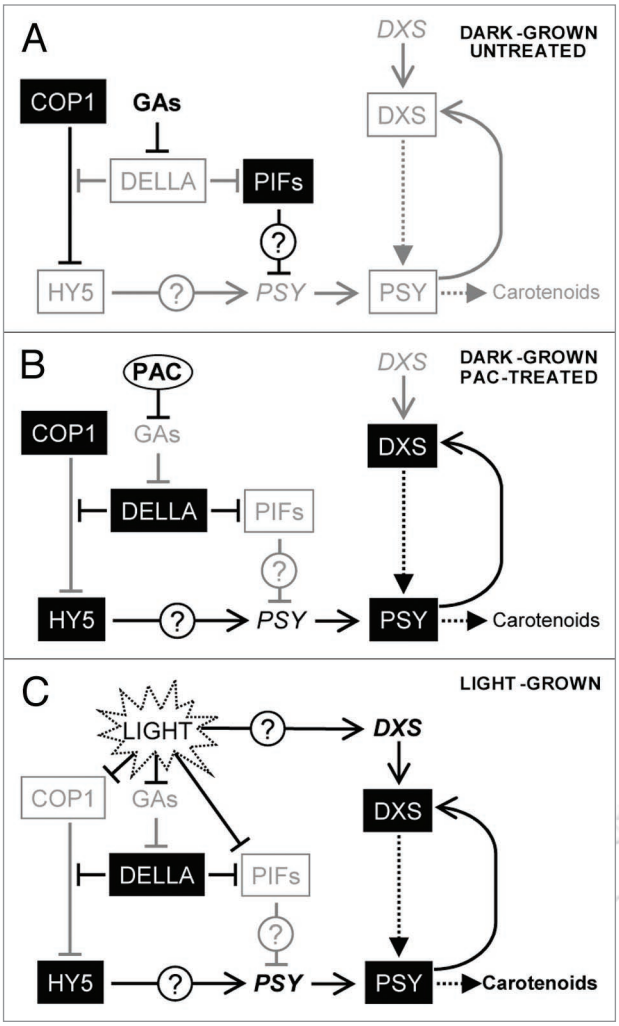

Figure 1. Model for the regulation of carotenoid biosynthesis and precursor supply during deetiolation in Arabidopsis seedlings. (A) Dark-grown, untreated seedlings (etiolated). (B) Dark-grown, PAC-treated seedlings (deetiolated). (C) Light-grown seedlings (deetiolated). Closed arrows with a dotted line indicate metabolic fluxes. Lines with open arrows and bars represent positive and negative effects, respectively. Genes are indicated in italics and proteins are boxed. Low levels are represented in gray and high levels in black.

dark.[6,7] Similary, a block in the production or signaling of hormones controlling deetiolation such as brassinosteroids (BRs) and gibberellins (GAs) also leads to a photomorphogenetic phenotype in the absence of illumination.[8-10] In particular, GAs appear to promote skotomorphogenic (etiolated) growth after germination by maintaining low levels of DELLA, GA signalling proteins that are negative regulators of HY5 and PIF function.[11,12] Derepression of deetiolation in mutants defective in COP1 (*cop1-4*) or BRs (*det2-1*) or in wild-type seedlings treated with paclobutrazol (PAC, an inhibitor of GA biosynthesis) results in a *ca.* 2-fold accumulation of carotenoids in the dark.[13] Interestingly, PAC-treated seedlings became greener faster when transferred to light in the absence of the inhibitor, indicating that carotenoids contribute to a proper adaptation of soil-emerging seedlings to sunlight. Photomorphogenic dark-grown seedlings showed an increased activity of phytoene synthase (PSY), the enzyme catalyzing the first committed step in the biosynthesis of carotenoids.[14] Such increase resulted, at least in part, from an enhanced promoter activity and transcript accumulation of the only Arabidopsis gene encoding PSY in cotyledons, the carotenoid-accumulating organs of etiolated seedlings. Furthermore, it was demonstrated that the upregulation of PSY activity was sufficient to activate carotenoid synthesis in etioplasts.[13] Analysis of gene expression and treatment with specific inhibitors demonstrated that the methylerythritol 4-phosphate (MEP) pathway supplied most of the prenyl diphosphate precursors required for carotenoid biosynthesis under these conditions. Although expression of genes encoding flux-controlling enzymes of the MEP pathway was unaltered in photomorphogenic dark-grown seedlings compared to skotomorphogenic controls, protein levels were higher in PAC-treated seedlings. Work with one of these enzymes, deoxyxylulose 5-phosphate synthase (DXS), showed that the observed post-transcriptional upregulation of MEP pathway enzyme levels was specifically triggered by the induction of PSY activity, most likely to ensure an appropriate supply of metabolic precursors.[13] These results indicate that the regulation of *PSY* expression is the main driving force controlling carotenoid biosynthesis and precursor supply in etioplasts.

Based on the available data, we propose the following model (**Fig. 1**). When seedlings germinate and grow in the absence of light, high GA and COP1 levels result in low levels of DELLA and HY5 proteins, which together with high PIF levels result in skotomorphogenic (etiolated) development.[6,12] Under these conditions, *DXS* and *PSY* gene expression and carotenoid synthesis are low (**Fig. 1A**). Derepression of photomorphogenesis (deetiolation) in dark-grown *cop1-4* seedlings results from an increased accumulation of HY5 and other related transcription factors,[7] some of which might regulate the expression of *PSY* but not *DXS* as deduced from the levels of the corresponding transcripts in mutant seedlings.[13] Because COP1 has been reported to participate in the accumulation of PIF3 in the nucleus,[15] it is possible that a decreased PIF activity in *cop1-4* seedlings might also positively influence *PSY* expression and eventually lead to an enhanced accumulation of carotenoids. In PAC-treated seedlings, decreased GA levels would cause higher DELLA accumulation, leading to an enhanced accumulation of HY5 and a decreased PIF activity,[11,16,17] as well as changes in carotenoid gene expression and accumulation similar to those described for *cop1-4*

seedlings.[13] In both mutant and PAC-treated seedlings, upregulation of *PSY* gene expression would result in higher PSY protein and activity levels, which in turn would cause an enhanced accumulation of DXS enzymes and a concomitant increase in the supply of precursors by a feedback mechanism that remains to be characterized (**Fig. 1B**). Illumination quickly decreases the levels of bioactive GAs,[18] COP1,[7] and PIFs,[19] which together could synergistically contribute to strongly upregulate *PSY* expression (**Fig. 1C**). The expression of *DXS* and other genes encoding MEP pathway enzymes is also upregulated by light, but the components of the signaling pathway involved remain unknown.[20,21] Our data suggest that these components might be different from those regulating *PSY* expression in the dark. Post-transcriptional events appear to also regulate the level of the DXS protein during early development of illuminated seedlings,[22] so it is possible that the feedback mechanism responsible for the enhanced accumulation of DXS protein in response to an induction of PSY activity in etioplasts might also be functional in chloroplasts. Because transgene-mediated upregulation of PSY levels in tomato fruit leads to an enhanced DXS activity without changes in gene expression,[23] it is tempting to speculate that some of the features of the model proposed here for the regulation of carotenoid biosynthesis during the transition of etioplasts to chloroplasts might also be conserved when chloroplasts are transformed into chromoplasts.

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