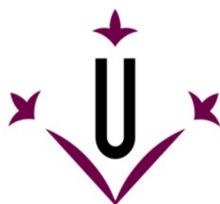

Universitat de Lleida

MECHANISTIC INVESTIGATIONS OF CAROTENOID AND KETOCAROTENOID BIOSYNTHESIS IN RICE

Chao Bai

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UNIVERSITAT DE LLEIDA

ESCOLA TÈCNICA SUPERIOR D'ENGINYERIA AGRÀRIA

PRODUCCIÓ VEGETAL I CIÈNCIA FORESTAL

MECHANISTIC INVESTIGATIONS OF CAROTENOID AND KETOCAROTENOID BIOSYNTHESIS IN RICE

CHAO BAI

DOCTORAL DISSERTATION

February 2014

PhD Thesis

MECHANISTIC INVESTIGATIONS OF CAROTENOID AND KETOCAROTENOID BIOSYNTHESIS IN RICE

PhD Supervisor:

Prof. Dr. Paul Christou

Dept. Producció Vegetal i Ciència Forestal

Universitat de Lleida

Co-supervisors:

Prof. Dr. Vicente Medina Piles

Dept. Producció Vegetal i Ciència Forestal

Universitat de Lleida

Dr. Changfu Zhu

Dept. Producció Vegetal i Ciència Forestal

Universitat de Lleida

UNIVERSITAT DE LLEIDA
ESCOLA TÈCNICA SUPERIOR D'ENGINYERIA AGRÀRIA
PRODUCCIÓ VEGETAL I CIÈNCIA FORESTAL

Paul Christou, Doctor of Plant Biochemistry, Professor of Plant Biotechnology and Head of the Applied Plant Biotechnology group at the University of Lleida, and Changfu Zhu, Doctor of Plant Physiology, Professor of Plant Biotechnology of the Applied Plant Biotechnology group at the University of Lleida, attached to the department of "Producció Vegetal i Ciència Forestal" of the University of Lleida during the course of the thesis experimental work,

We hereby state, that Chao Bai, who majored in Biology at the UdL University, has performed under our direction and supervision, and within the Applied Plant Biotechnology group from the department of "Producció Vegetal i Ciència Forestal", the experimental work entitled "Mechanistic investigations of carotenoid and ketocarotenoid biosynthesis in rice",

That the work accomplishes the adequate conditions in order to be defended before the corresponding Thesis Committee and, if the opportunity arises, to obtain the degree by the University de Lleida, and we sign the current document that this may be officially recorded, to complete formalities deemed necessary,

Lleida, January 2014

Prof. Dr. Paul Chritou **Prof. Dr. Vicente Medina Piles** **Dr. Changfu Zhu**

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my supervisor **Paul Christou** for his valuable guidance, enthusiastic encouragement and support in science and beyond. I would have not achieved this success without his endless support during my PhD. I am grateful to acknowledge my co-supervisors **Vicente Medina**, who is expert for supporting his microscopy knowledge through all my researches. I would like to express my great appreciation to my co-supervisors **Changfu Zhu** for his constructive suggestions and useful advices during my researches.

I would like to offer my special thanks to **Teresa Capell** for her kind assistance in several different documents, which would have required a lot of my time. Without her great help in all the official work, I couldn't concentrate on my research. Her excellent advocacy capacity to disseminate our scientific work to all the world.

I gratefully acknowledge the Universitat de Lleida (UDL, Spain) for providing the PhD fellowship. And a special thank you to the secretary **Nuria Gabernet** for assisting me with many official documents. I would like to thank **Jaume Capell** for looking after my rice plants.

I would like to extend my sincerest appreciation to the co-authors of our publications for the fruitful collaboration and scientific support in the diverse topics it spanned: Prof. **Gerhard Sandmann** (Goethe Universitaet Frankfurt, Germany) for providing his HPLC facility for carotenoid analysis and his stimulating discussion, **Jürgen Breitenbach** (Goethe Universitaet Frankfurt, Germany) for his assistance during HPLC analysis, Prof. **Rui Alves**, **Ester Vilaprinyo** and **Albert Sorribas** for creating and

solving the mathematical models of carotenoid biosynthesis, Prof. **Ramon Canela** for HPLC analysis guidance, and **Paquita** for HPLC analysis technical support.

I would like to thank my colleague **Ludovic Bassie** for his useful advise in understanding certain lab techniques.

I would like to thank my colleague and my best assistance **Sol M. Rivera** for setting up good condition of UHPLC system for carotenoids analysis.

I would like to thank my colleague **Dawei Yuan** for helping me getting started in the lab and supporting his technical knowledge on my project.

I would like to thank my best friend **Bing Liu**, whose encouraging remarks and help to my life is beyond measure.

I would like to thank all my lab colleagues Shaista Naqvi, Ariadna Peremarti, Koreen Ramessar, Sonia Gomez, Svetlana Dashevskaya, Maite Sabalza, Gemma Farre, Georgina Sanahuja, Bruna Miralpeix, Gemma Arjo, Raviraj Banakar, Evangelia Vamvaka, Eduard Perez, Uxue Zorrilla, Gemma Massip, Judit Berman and Daniela Zanga for their excellent friendship, assistance and encouragement through all my degree. Birthday parties, celebrating dinners, weddings, barbeques, Calagoal, turkey lunches, making cakes…many moments are valuable memorable. With you, my life was different and colorful.

Finally, I wish to thank my entire family for their love and encouragement. My grandmother, my uncle liansheng Liu, aunt xiulan Hou, cousin Jia Liu and last my kindness mother Yuzhi Liu for her remarkably support.

I will always remember all of you and also the great time in Spain.

ABSTRACT

My research project focused on the rice (*Oryza sativa*) carotenoid biosynthesis pathway. I used multigene engineering to identify and characterize bottlenecks in the pathway leading to β-carotene (pro-vitamin A) and other important carotenoids. I created transgenic lines expressing distinct combinations of transgenes and investigated the resulting diverse carotenoid profiles to determine how the integrated transgene complement influenced carotenoid production. I also developed a simple assay based on embryogenic rice callus for the functional characterization of carotenogenic transgenes. Remarkably, I discovered that diverse endosperm-specific promoters were highly active in callus tissue despite their restricted activity in mature plants. The callus system offers a unique opportunity to predict the impact of metabolic engineering in complex pathways and provides a starting point for quantitative modeling and the rational design of engineering strategies using synthetic biology. I identified and characterized the novel ketocarotenoid 4-keto-α-carotene as an unexpected byproduct during experiments targeting the biosynthesis of astaxanthin, which involved the co-expression of *ZmPSY1*, *PaCRTI* and two carotenoid ketolases (*BrCRTW*/*sCrBKT*) in rice callus. In separate experiments, I introduced the ketocarotenoid biosynthesis pathway to astaxanthin into rice endosperm. These transgenic lines accumulated canthaxanthin and adonirubin as major ketocarotenoids rather than astaxanthin, indicating the presence of a bottleneck caused by insufficient endogenous hydroxylation activity in rice endosperm tissue. Targeted metabolomics in rice endosperm co-expressing binary (*ZmPSY1* and *PaCRTI*) or tertiary (*ZmPSY1*, *PaCRTI* and *AtDXS or AtOR*) transgene combinations demonstrated that carotenoid accumulation in rice endosperm is inhibited by the limited supply of isoprenoid precursors derived from the MEP pathway and/or the absence of an effective carotenoid sink.

RESUM

El meu programa de recerca està enfocat a identificar els passos limitants de la biosíntesi de carotenoids en arròs (*Oryza sativa*) de forma que mitjançant enginyeria genètica amb múltiples gens es puguin dividir els passos i elucidar el mecanisme de acumulació de la pro-vitamina A i dels carotenoids en arròs, un cereal molt important en alimentació. Per tal d'assolir el meus objectius, he generat varies línies transgèniques de arròs que expressen combinacions específiques de gens diferents. Las plantes transgèniques presenten perfils carotenogènics diferents en funció de la combinació dels gens integrats. Durant les meves investigacions he desenvolupat un experiment que facilita la caracterització funcional de gens que representen una ruta metabòlica en un sistema de calls embriogènics d'arròs. A mes, he descobert que variïs promotors específics de l'endosperma són molt actius en calls, tot i que la seva activitat tindria que ser restringida a la llavor. El sistema d'expressió en calls proporciona una oportunitat única per a predir l'impacte de la enginyeria genètica de rutes complexes i la seva quantificació, i es converteix en el punt d'inici per al disseny racional d'estratègies d'enginyeria de rutes biosintètiques complexes utilitzant biologia sintètica. També he identificat i caracteritzat un nou ketocarotenoid, el 4 keto-alfa–carotè, com un subproducte no esperat als experiments en els quals el meu objectiu era la biosíntesi d'astaxantina mitjançant la co-expressió de *BrCRTW/sCrBKT*, *ZmPSY1* i *PaCRTI* en calls d'arròs. En un altre conjunt d'experiments he introduït la ruta biosintètica per a la producció de astaxantina en l'endosperma d'arròs. Amb les anàlisis que he dut a terme, he detectat una acumulació de cantaxantina i adonirubina, més que de astaxantina, essent aquests dos els carotenoids més abundants en l'endosperma d'arròs, la qual cosa suggereix l'existència de un pas limitant en la capacitat endògena de hidroxilació de l'endosperma d'arròs. La caracterització específica del metaboloma de l'endosperma

d'arròs co-expressant combinacions binàries (*ZmPSY1* i *PaCRTI*) o terciàries (*ZmPSY1* i *PaCRTI* i *AtDXS* o *AtOR*) de gens demostra que la quantitat subministrada de precursors isoprenoics derivats de la ruta metabòlica MEP, o la creació de un sistema efectiu de retenció o de deposició de carotenoids son dos factors clau limitants de l'acumulació de carotenoids en l'endosperma d'arròs.

RESUMEN

Mi programa de investigación está enfocado a identificar los pasos limitantes de la biosíntesis de carotenoides en arroz (*Oryza sativa*) de forma que mediante ingeniería genética con múltiples genes pueda dividir los pasos y elucidar el mecanismo de acumulación de la pro-vitamina A y de los carotenoides en arroz, un cereal muy importante en alimentación. Para conseguir mis objetivos, he generado diversas líneas transgénicas de arroz que expresan combinaciones específicas de genes diferentes. Las plantas transgénicas presentan perfiles diferentes en función de la combinación de genes integrados. Durante mis investigaciones he desarrollado un experimento que facilita la caracterización funcional de genes que de una ruta metabólica en un sistema de callos embriogénicos de arroz. Además he descubierto que varios promotores específicos del endospermo son muy activos en callos a pesar de que su actividad tendría que estar restringida a la semilla. El sistema de expresión en callos proporciona una oportunidad única para predecir el impacto de la ingeniería genética de rutas complejas y su cuantificación, y se convierte en el punto de inicio para el diseño racional de estrategias de ingeniería de rutas biosintéticas complejas utilizando biología sintética. También he identificado y caracterizado un ketocarotenoide nuevo, el 4 keto-alfa –caroteno, como un subproducto inesperado en los experimentos donde mi objetivo era la biosíntesis de astaxantina mediante la co-expresión de *BrCRTW/sCrBKT*, *ZmPSY1* y *PaCRTI* en callos de arroz. En otro conjunto de experimentos he introducido la ruta biosintética para la producción de astaxantina en el endosperma de arroz. Con mis análisis he detectado una acumulación de cantaxantina y adonirubina, más que de astaxantina, siendo estos dos los carotenoides mas abundantes en el endosperma de arroz, lo cual sugiere la existencia de un paso limitante en la capacidad endógena de hidroxilación del endosperma de arroz. La caracterización específica del metaboloma del endosperma de

arroz co-expresando combinaciones binarias (*ZmPSY1* y *PaCRTI*) o terciarias (*ZmPSY1* i *PaCRTI* y *AtDXS* o *AtOR*) de genes demuestra que la cantidad suministrada de precursores isoprenoicos derivados de la ruta metabólica MEP, o la creación de un sistema efectivo de retención o de deposición de carotenoides son dos factores clave limitantes de la acumulación de carotenoides en el endosperma de arroz.

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LIST OF ABBREVIATIONS

ACN: acetonitrile

ADPGPP: ADP-glucose pyrophosphorylase

AFLPs: amplified fragment length polymorphisms (AFLPs)

AsA/AA: Ascorbate/Ascorbic acid

BCH: β-carotene hydroxylase

BKT: β-carotene ketolase

CaMV 35S: Cauliflower Mosaic Virus 35S

cDNA: complementary DNA

CRTB: bacterial phytoene synthase

CRTI: bacterial β-carotene desaturase

CRTISO: bacterial Carotene isomerase

CRTO: bacterial β-carotene hydroxylase

CRTW: bacterial β-carotene ketolase

CRTY: bacterial lycopene cyclase

CRTZ: bacterial β-carotene hydroxylase

CYP97: carotene ε-ring hydroxylase

DAP: days after pollination

DMAPP: dimethylallyl diphosphate

DNA: deoxyribonucleic acid

DRI: dietary reference intake

DXP: 1-deoxy-D-xylulose-5-phosphate

DXS: 1-deoxy-D-xylulose 5-phosphate synthase

DW: dry weight

EMS: ethyl methanesulfonate

ER: endoplasmic reticulum

GBSS: tuber-specific granule-bound starch synthase

GGPP: geranylgeranyl diphosphate

GGPPS: geranylgeranyl diphosphate synthase

GR: golden rice

HDR: DXP reductoisomerase

HMBPP: 4-hydroxy-3-methylbut-2 enyl

HPLC: high performance liquid chromatography

HPT: Hygromycin phosphotransferase

HYDB: β-carotene hydroxylase

IPP: isopentenyl diphosphate

IPPI: isopentenyl diphosphate isomerase

LYCB: lycopene β-cyclase

LYCE: lycopene ε-cyclase

MAS: marker assisted selection

MeOH: methanol

MEP: 2-C-Methyl-D-erythritol 4-phosphate pathway

mRNA: messenger RNA

MS: mass or mass spectrometry

MVA: mevalonic acid pathway

m/z: mass-to-charge ratio

$NaBH_4$: sodium borohydride

NOS: nopaline synthase

NMR: nuclear magnetic resonance

OR: orange gene

Os: *Oryza sativa*

PCR: Polymerase chain reaction

PDS: phytoene desaturase

pGZ63: maize γ-zein gene promoter

pRP5: rice prolamin promoter

p326/LMW: wheat low molecular weight glutenin promoter

pHorp-P: barley D-hordein promoter

PSY: phytoene synthase

QTL: quantitative trait locus

RAE: retinol activity equivalent

RNA: ribonucleic acid

RNAi: RNA interference

RNA-seq: census sequencing approaches

RT-PCR: reverse transcription PCR

RT: retention time(s)

SCAR: sequence-characterized amplified region

SD: standard deviation

SSU: small subunit of ribulose bisphosphate carboxylase

T-DNA: the transfer DNA

TILLING: targeting induced local lesions in genomes

TPS: transit peptide sequence

tRNA: transfer RNA

Ubi-1: ubiquitin-1

UHPLC-MS: ultra high-performance liquid chromatography-mass spectrometry

UNICEF: United Nations Children's Fund

5'UTR: 5'-untranslated region

VAD: vitamin A deficiency

VDE: violaxanthin de-epoxidase

VIGS: virus-induced gene silencing

Wf: white-flesh

Y: yellow

ZDS: ζ-Carotene desaturase

ZEP: Zeaxanthin epoxidase

ZISO: ζ-carotene isomerase

λmax: wavelength of maximum absorption

GENERAL INTRODUCTION

1. Provitamin A enhancement in diverse crops

1.1 Dietary Vitamin A

Vitamin A is an essential nutrient in mammals that occurs in several forms known as retinoids. Each form is characterized by a common skeleton known as the retinyl group, comprising an unsubstituted β-ionone ring and an isoprenoid side chain (**Figure 1**). The aldehyde form (retinal or retinaldehyde) is necessary for the production of rhodopsin in the eyes and for the maintenance of epithelial and immune cells, whereas the acidic form (retinoic acid) is a morphogen in development. Many foods are good sources of vitamin A but usually they do not contain significant quantities of either retinal or retinoic acid (Olson, 1994). Meat and dairy sources primarily contain retinyl esters that are converted into the alcohol form (retinol) in the small intestine, and from there into retinal or retinoic acid. In contrast, plant sources contain pro-vitamin A carotenoids, the most important of which is β-carotene. This can be converted into retinal by the enzyme β-carotene 15,15'-monooxygenase. Many carnivores lack this enzyme and cannot obtain vitamin A from plants, but herbivores and omnivores (including humans) can synthesize retinal from the abundant pro-vitamin A carotenoids in dark green, yellow and orange fruits and vegetables, such as oranges, broccoli, spinach, carrots, squash, sweet potatoes and pumpkins (Harrison, 2005).

The dietary reference intake (DRI) for vitamin A is 900 RAE for males, 700 RAE for females (770 RAE in pregnancy and 1200-1300 RAE when lactating) and 400-500 RAE for children. RAE is retinol activity equivalent and is the recommended unit for vitamin A intake taking bioavailability into account. One RAE is equivalent to 1 μg of retinol, 2

μg of β-carotene supplement dissolved in oil, or 12 μg of β-carotene in food (IOM, 2001). A DRI of 900 RAE for males is therefore equivalent to 900 μg (3000 IU) of retinol, 1800 μg of β-carotene supplement or 1.08 mg of β-carotene in food (IOM, 2001).

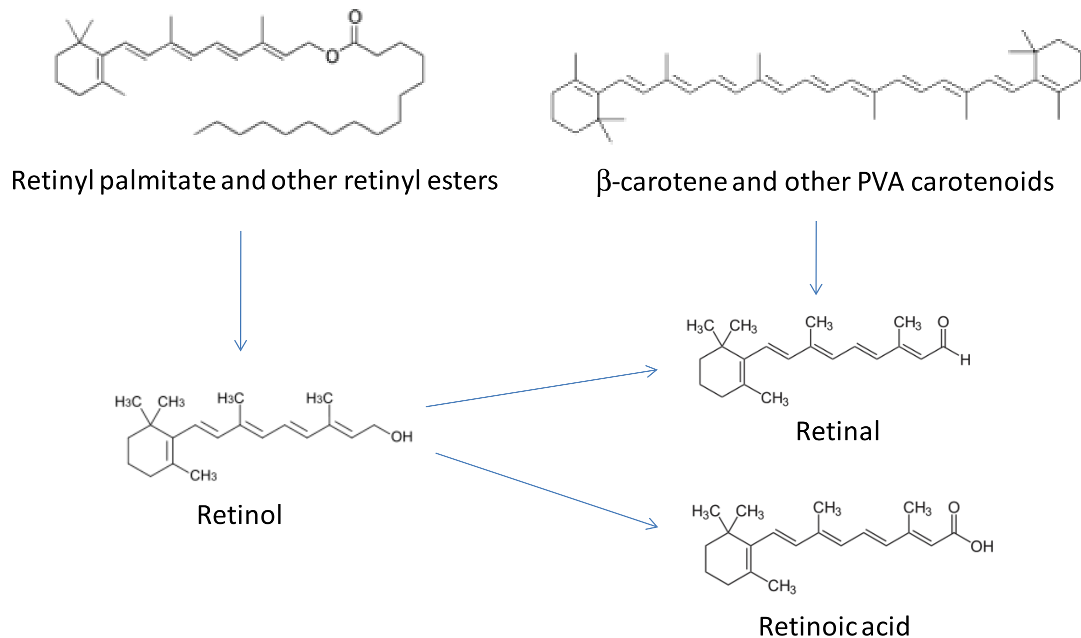


Figure 1 Vitamin A in humans is a group of molecules with a common retinyl group. The functional molecules are retinal and retinoic acid. Dietary sources are principally retinyl esters from meat and dairy products and pro-vitamin A (PVA) carotenoids from plants.

Most people in the developed world have diets of sufficient diversity to ensure they achieve the RDI for vitamin A. However, the situation in developing countries is very different, with many subsisting on a cereal-dominated diet with low levels of nutrients including β-carotene. These individuals are severely at risk of vitamin A deficiency (VAD), which weakens the immune system, causes the deterioration of light-sensitive rod cells essential for low-light vision, and in extreme cases can lead to an irreversible form of blindness called xerophthalmia (UNICEF, 2006). More than four million children worldwide exhibit signs of severe VAD (**Figure 2**), including 250,000-

–500,000 per year who become partially or totally blind. Women have a higher demand for vitamin A during pregnancy and currently more than 20 million pregnant women in developing countries suffer from VAD. Approximately 6 million of these women show clinical signs of night blindness, with half the cases in India (http://www.harvestplus.org/content/vitamin). VAD in pregnancy causes nearly 600,000 deaths every year (UNICEF, 2006).

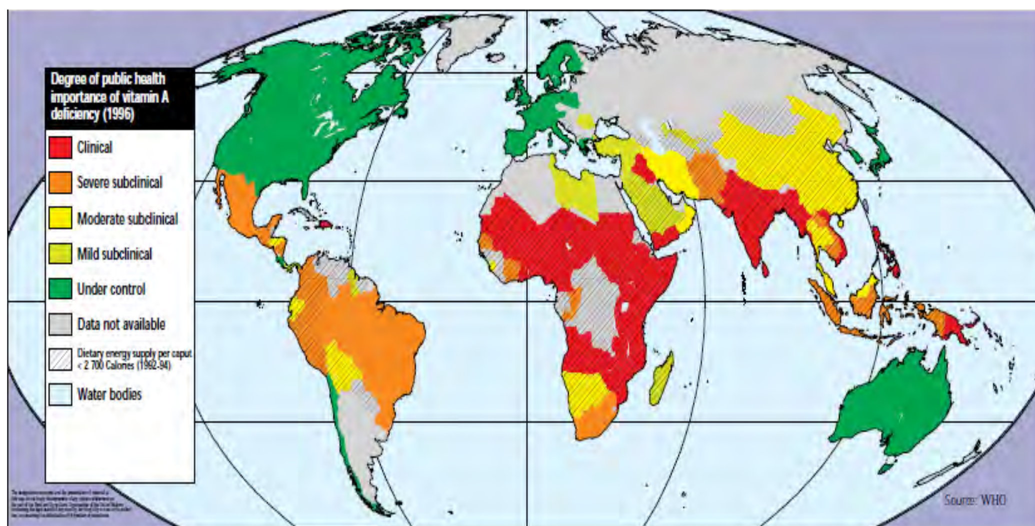


Figure 2 Prevalence of vitamin A deficiency around the world (World Health Organization data, 1996).

1.2 The carotenoid biosynthetic pathway in plants

Plants produce four pro-vitamin A carotenoids, distinguished by the possession of at least one retinyl group. Two of these molecules (α-carotene and β-carotene) accumulate in significant amounts whereas the others (γ-carotene and β-cryptoxanthin) are intermediates and tend to be converted rapidly into downstream products (Farre *et al.*, 2010; Zhu *et al.*, 2010) (**Figure 3**).

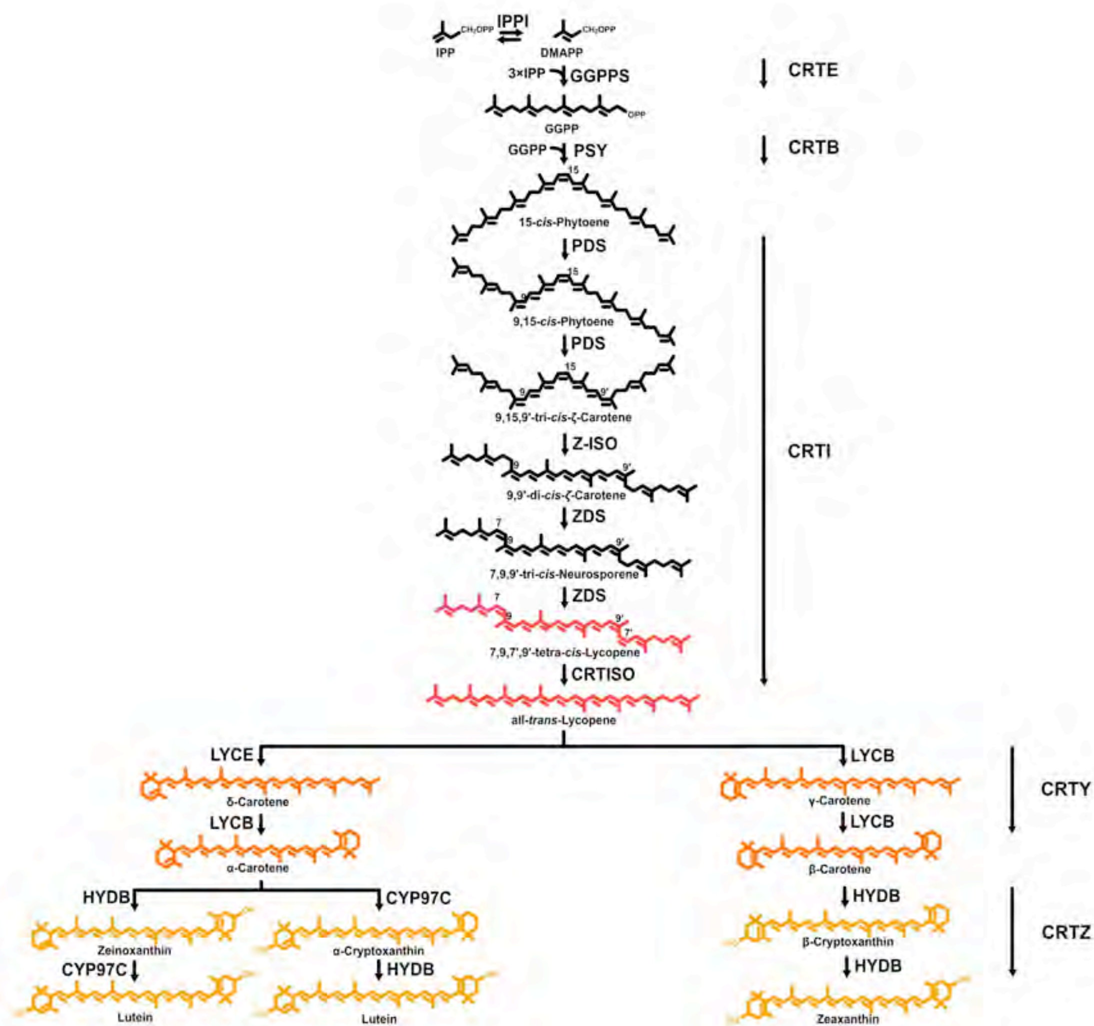


Figure 3 Carotenoid biosynthesis pathway in plants and equivalent steps in bacteria

IPP, isopentenyl diphosphate, IPPI, isopentenyl diphosphate isomerase, DMAPP, dimethylallyl diphosphate, GGPP, geranylgeranyl diphosphate, GGPPS, GGPP synthase, PSY, phytoene synthase, PDS, phytoene desaturase, Z-ISO, ζ-carotene isomerase, ZDS, ζ-carotene desaturase, CRTISO, carotenoid isomerase, LYCB, lycopene β-cyclase, LYCE, lycopene ε-cyclase, CYP97C, carotene ε-ring hydroxylase, HYDB, β-carotene hydroxylase, CRTE, bacterial geranylgeranyl diphosphate synthase, bacterial GGPP synthase, CRTB, bacterial phytoene synthase, CRTI, bacterial phytoene desaturase/isomerase, CRTY, bacterial lycopene cyclase, CRTZ, bacterial β-carotene hydroxylase (Bai *et al.*, 2011)

Carotenoids are tetraterpenoids, i.e. they comprise eight condensed C5 isoprenoid precursors generating a C40 linear backbone. In plants, this condensation reaction involves the isomeric precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and occurs de novo within plastids (Chappell, 1995). IPP and DMAPP are derived predominantly from the plastidial methylerythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat, 2002) although the same precursors are formed by the cytosolic mevalonic acid (MVA) pathway, and there is some evidence for the shuttling of intermediates between pathways (Rodriguez-Concepcion, 2006). The condensation of three IPP molecules with one molecule of DMAPP produces the C20 intermediate geranylgeranyl diphosphate (GGPP), a reaction catalyzed by GGPP synthase (GGPPS). In bacteria, GGPPS is encoded by the *CRTE* gene (**Figure 3**).

The first committed step in plant carotenoid synthesis is the condensation of two GGPP molecules into 15-cis-phytoene by the enzyme phytoene synthase (PSY) (Misawa *et al.*, 1994). The equivalent enzyme in bacteria is CRTB. A series of four desaturation reactions carried out in plants by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. The product of the first desaturation is 9,15,9´-tri-cis-ζ-carotene, which is isomerized by light and/or ζ-carotene isomerase (Z-ISO) to yield 9,9′-di-cis-ζ-carotene, the substrate of ZDS (Li *et al.*, 2007; Chen *et al.*, 2010). The end product of the desaturation reactions is converted to all-trans-lycopene by a carotenoid isomerase (CRTISO) in non-green tissue, and by light and chlorophyll (acting as a sensitizer) in green tissue (Breitenbach and Sandmann, 2005; Isaacson *et al.*, 2004; Li *et al.*, 2010). In bacteria, a single enzyme encoded by the *CRTI* gene accomplishes all the above steps and produces all-trans-lycopene from 15-cis-phytoene directly (**Figure 3**).

Lycopene is an important branch point in the carotenoid pathway because it acts as the substrate for two competing enzymes, lycopene β-cyclase (LYCB) and lycopene ε-cyclase (LYCE) (Cunningham *et al.*, 1996). Both enzymes cyclize the linear backbone to generate terminal ionone rings, but the structures of these rings are distinct. The addition of one β-ring to lycopene by LYCB generates γ-carotene, and the addition of a second β-ring to the free end by the same enzyme produces β-carotene. The bacterial gene *CRTY* encodes LYCB. Both γ- and β-carotene therefore have retinyl groups and possess pro-vitamin A activity, but β-carotene is a better source because it possesses two such groups. Furthermore, as stated above, γ-carotene is rapidly converted into β-carotene and tends not to accumulate. Alternatively, the addition of one ε-ring to lycopene by LYCE generates α-carotene (which has no pro-vitamin A activity). This is a poor substrate for LYCE so it is unusual for the second ε-cyclization to take place, but it is a good substrate for LYCB which adds a β-ring to the free end generating α-carotene, which does have pro-vitamin A activity. In the presence of the enzyme β-carotene hydroxylase (BCH), both α-carotene and β-carotene can be converted into more complex downstream carotenoids that do not possess pro-vitamin A activity (Tian *et al.*, 2003). In the case of α-carotene this downstream product is lutein, and in the case of β-carotene the downstream product is zeaxanthin although the reaction involves the intermediate β-cryptoxanthin which also has pro-vitamin A activity. The bacterial gene *CRTZ* encodes BCH. Whereas lutein represents the natural end point of the α-carotene branch, zeaxanthin enters the xanthophyll cycle and can be converted through a number of additional steps into the important plant hormone abscisic acid (Seo and Koshiba, 2002).

1.3 Strategies for enhancing β-carotene levels in plants

Addressing VAD in the developing world requires a robust strategy to increase access to foods rich in (pro)-vitamin A, and several approaches can be considered. Dietary supplements (vitamin tablets and suspensions) and fortification (artificially increasing vitamin levels by adding vitamins to processed food) campaigns have been highly successful in the developed world, and have significantly reduced the incidence of deficiency diseases. Many processed foods, including bread, packaged cereals, milk and soft drinks, are fortified with vitamins and minerals so the average diet contains micronutrients well in excess of requirements. In developing countries, the less robust and less reliable food distribution infrastructure, poor governance and the lack of funding renders such programs inefficient and unsustainable, especially when trying to reach remote areas (Darnton-Hill and Nalubola, 2002). Vitamin A supplementation campaigns have enjoyed limited success but most programs have failed to address VAD, especially at the local level when dealing with small and remote villages (Underwood, 2003). An alternative approach to adding vitamin A directly to the diet is to enhance the accumulation of pro-vitamin A carotenoids in crops. There are both conventional and biotechnological approaches to biofortification, each of which has met with some success as discussed below.

1.3.1 Conventional breeding

The limitations of conventional breeding include its dependence on a compatible gene pool (i.e. the collection of species with which the target crop can interbreed) and on the degree of available natural/inducible variation. If sufficient natural variation in vitamin A levels can be identified within a breeding population, or can be induced by mutation, then selection strategies can be used to produce nutritionally improved lines. Even so, programs to exploit such variation have met with only marginal success. For example, a

recent attempt to use variation at the *LYCE* locus in maize to increase β-carotene levels achieved a five-fold increase (the best line contained 13.6 µg β-carotene per gram dry weight of endosperm; Harjes *et al.*, 2008), whereas transgenic strategies have achieved >100-fold increases over a much shorter time-scale (the best line reported thus far contains 59.32 µg β-carotene per gram dry weight of endosperm; Naqvi *et al.*, 2009). The long time required to generate nutritionally improved lines, especially if a trait has to be introgressed into an elite local breeding line, is one of the greatest challenges in conventional breeding.

Conventional breeding can be accelerated by marker-assisted selection (MAS) and the usefulness of molecular markers is particularly evident when dealing with quantitative traits such as nutrient levels, since these tend to reflect the activities of multiple genes contributing to the same phenotype. Breeding without markers can only identify the most productive combinations of alleles by chance, whereas MAS allows particular alleles, identified by the linked markers, to be stacked in the same line without any need for phenotypic analysis. MAS has allowed the construction of saturated linkage maps for many crops and has made it possible to map the quantitative trait loci (QTLs) that control them. Understanding how QTLs affect crop performance under different environmental conditions and in different genetic backgrounds can facilitate the development of enhanced crop varieties. Consequently, the mapping of QTLs for agronomic traits is an important component of conventional nutritional improvement programs (Dwivedi *et al.*, 2007).

Conventional breeding can also be accelerated by mutagenesis, which generates new alleles more rapidly than would occur in nature. This can be achieved by irradiating seeds with X-rays or exposing them to chemical mutagens such as ethyl methanesulfonate (EMS), each of which causes random damage to DNA and usually

generates point mutations. Any phenotypic effects of such mutations are observed in subsequent generations, depending on whether the effect is dominant or recessive, and must be mapped to identify the affected gene. An advanced method for identifying such point mutations is TILLING (Targeting Induced Local Lesions In Genomes), a high-throughput method based on conformational electrophoresis for the detection of point mutations in large populations of plants (Comai and Henikoff, 2006). TILLING can identify genetic variation in elite germplasm without the need to acquire variation from exotic cultivars, thus avoiding the introduction of agriculturally undesirable traits. Once a TILLING library is set up, it can be used for the analysis of many different gene targets. TILLING is a powerful reverse genetics approach that has the unique advantage of allowing the generation of an allelic series for any target gene, including essential genes (Slade and Knauf, 2005). If a variety developed by TILLING has commercial potential, it is not subject to the same regulatory approval requirements as transgenic crops.

Even with methods such as TILLING making the process of mapping novel mutations easier, it is still preferable to be able to identify mutated genes with a unique DNA signature. This is the benefit of insertional mutagenesis using unique DNA tags such as transposons or T-DNA, the former endogenous to plants, the latter introduced artificially. In this approach, randomly integrating DNA sequences disrupt genes and cause a loss of function. The identification of desirable mutant phenotypes is followed by DNA analysis using the insertional mutagen sequence as a probe, or as the basis for PCR primer design, allowing flanking gene sequences to be identified (Maes *et al.*, 1999; Parinov and Sundaresan, 2000).

1.3.2 Genetic engineering

Biofortification at source can be achieved by transferring genes conferring enhanced nutritional traits directly into elite breeding lines, generating transgenic plants. Many crop species have been genetically engineered to produce higher levels of pro-vitamin A and these are discussed in subsequent sections. Compared to conventional breeding, transgenesis has the advantages of speed, direct modification of breeding lines, simplicity, the potential for multiple simultaneous biofortification for different nutrients and unrestricted access to genetic diversity (i.e. genes from bacteria and animals, and even completely synthetic or artificially modified genes that do not exist in nature) (Zhu *et al.*, 2007).

Because pro-vitamin A carotenoids are synthesized de novo by plants, increasing the availability of these compounds must involve metabolic engineering with the focus on β-carotene because it is the most important and potent of the four available pro-vitamin A carotenoids. However, intervention can take place at any point along the pathway and multiple strategies are available (Capell and Christou, 2004). General approaches include increasing flux through the entire carotenoid pathway by enhancing the production of GGPP, whereas more targeted approaches involve boosting the production of β-carotene specifically, or reducing the amount of α-carotene, which can be regarded as a competitor because it shares a common precursor. As well as enhancing the synthesis of β-carotene, additional approaches include the inhibition of post-β-carotene steps to prevent conversion to zeaxanthin and other derivatives, and increasing the ability of plant cells to store β-carotene, thereby providing a metabolic sink and preventing feedback inhibition. Another useful approach is RNA interference (RNAi), in which short double stranded RNA molecules are used to induce a defense pathway that destroys homologous mRNAs and leads to potent post-transcriptional gene silencing (Lawrence and Pikaard, 2003; Mansoor *et al.*, 2006). RNAi has the advantage

of being able to knock down the expression of multiple related genes with one construct if they share a homologous core sequence, whereas conventional mutagenesis/TILLING and insertional mutagenesis only affect single genes. The ability to silence several genes simultaneously using RNAi can speed up the creation of plant lines with novel or enhanced nutritional properties. All these methods have been attempted alone and in combination with some remarkable achievements, as discussed in more detail below (**Table 1**).

1.4 Vitamin A enhancement through biotechnology

1.4.1 Vitamin A enhancement in cereals

Rice, an important food staple worldwide, accumulates no carotenoids in its endosperm and therefore it is associated with vitamin A deficiency. In wild type rice, immature endosperm synthesizes geranylgeranly diphosphate (GGPP), the immediate precursor for carotenoid biosynthesis (Burkhardt *et al.*, 1997). The ability of daffodil (*Narcissus pseudonarcissus*) PSY expression alone to produce phytoene but not desaturated products (Burkhardt *et al.*, 1997) indicates that at least PDS activity is deficient in rice endosperm. Similarly, the expression of bacterial CRTI (which can replace plant PDS, Z-ISO, ZDS and CRTISO) alone did not produce rice with colored endosperm due to the lack of PSY activity (Schaub *et al.*, 2005). Therefore carotenoid accumulation in rice requires expression of both *PSY* and *CRTI* transgenes (Ye *et al.*, 2000; Paine *et al.*, 2005). Golden Rice was engineered with heterologous phytoene synthase (*PSY*) and phytoene desaturase (*CRTI*) genes and has an endosperm carotenoid content of 1.6 µg/g dry weight, demonstrating that expression of these two genes results in accumulation of

carotenoids in the endosperm (Ye *et al.*, 2000). Subsequently, Datta *et al.* (2003) extended this 'proof of concept' from the original GR japonica cultivar (T309) to several indica cultivars, producing transgenic Indica lines (Parkhi *et al.*, 2005) and also near-isogenic introgression lines of an elite Indica cultivar, also eliminating the selectable marker used in the original transformation experiments (Baisakh *et al.*, 2006). All these lines contained the same transgenes as the original GR (daffodil *PSY* and bacterial *CRTI*) and the carotenoid levels were similar (Ye *et al.*, 2000; Datta *et al.*, 2003; Parkhi *et al.*, 2005; Baisakh *et al.*, 2006). The predicted biosynthetic product of the two carotenogenic transgenes in GR is lycopene (**Figure 3**), which is red in color. However, the endosperm of GR is yellow due to the accumulation of β-carotene and xanthophylls. The absence of lycopene in GR demonstrated that the pathway continued beyond the transgenic end point and thus the endogenous pathway downstream of lycopene must be present in rice endosperm. By using qRT-PCR, Schaub *et al.*, (2005) showed that wild type rice endosperm expresses the carotenogenic genes encoding PDS, ZDS, CRTISO, LYCE and BCH, but PSY mRNA was all but absent. Through systematic testing of PSY enzymes from different plants such as daffodil (used in all previous versions of GR), carrot, tomato, rice and maize, *PSY* from maize was found to give the best results in terms of achieving maximum levels of β-carotene in the rice endosperm. The endosperm of Golden Rice 2 (GR2) accumulated up to 37 μg/g carotenoids (a 23-fold increase compared to GR) with β-carotene representing ~84% of the total (Paine *et al.*, 2005). CRTI was also regarded as a rate limiting enzyme in GR because it was barely detectable in the endosperm (Al-Babili *et al.*, 2006). The low

protein levels may reflect weak transcription from the CaMV35S promoter or suboptimal codon usage. The amount of CRTI protein was increased by expressing a synthetic *CRTI* gene, codon optimized to match rice storage proteins. The gene was expressed under the control of the endosperm-specific glutelin B1 promoter. Transgenic plants expressing the unmodified CRTI gene using the endosperm-specific glutelin B1 promoter were also generated. The endosperm-specific promoter made a significant difference to CRTI levels in T1 rice endosperm even in the absence of codon optimization, but this did not lead to a significant increase in the carotenoid content. The conclusion was that CRTI is not rate limiting in rice endosperm after all, even at very low levels (Al-Babili *et al.*, 2006).

Endosperms of maize and wheat are low in provitamin A (1-10 %) as compared with non-provitamin A carotenoids (Harjes *et al.*, 2008; Cong *et al.*, 2009). For example, Aluru *et al.* (2008) introduced the bacterial *CRTB* and *CRTI* genes under the control of an enhanced λ-zein promoter to provide strong endosperm-specific expression, increasing the total carotenoid content to 33.6 g/g dry weight. A significant advance was achieved by Zhu *et al.* (2008) with the development of a combinatorial nuclear transformation system designed to dissect and modify the carotenoid biosynthetic pathway in maize, using the white-endosperm elite South African inbred M37W. The method involved transforming plants with multiple genes encoding the enzymes involved in carotenoid biosynthesis and then screening a library of random transformants for plants with appropriate metabolic profiles. The pilot study for this technique involved the introduction of five genes (maize *PSY1*, *Gentiana lutea LYCB* and *BCH*, and bacterial *CRTI* and *CRTW*) under the control of endosperm-specific promoters. This recreates the entire pathway from GGPP to zeaxanthin (**Figure 3**) and

also adds the enzyme CRTW (β-carotene ketolase) that converts β-carotene into downstream ketocarotenoids. The M37W genetic background provided a blank template because the endosperm in this variety has no PSY activity and therefore lacks all carotenoids. The recovery of plants carrying random combinations of genes resulted in a metabolically diverse library comprising plants with a range of carotenoid profiles, revealed by easily identifiable endosperm colors ranging from yellow to scarlet. The kernels contained high levels of β-carotene, lycopene, zeaxanthin and lutein, as well as further commercially-relevant ketocarotenoids such as astaxanthin and adonixanthin (Zhu *et al.*, 2008).

Another breakthrough in this area was the development of transgenic maize plants transformed with multiple genes enabling the simultaneous modulation of three metabolic pathways in the endosperm, thereby increasing the levels of the three key nutrients: β-carotene, ascorbate and folate (Naqvi *et al.*, 2009). The same M37W line was used as the basis for these experiments because, in addition to the absence of carotenoids, it also has very low ascorbate and folate levels. Kernels from the transgenic plants contained 169-fold more β-carotene than normal (60 µg/g dry weight) as well as six times the normal level of ascorbate (110 µg/g dry weight) and twice the normal level of folate (1.94 µg/g dry weight).

Hexaploid tritordeums produce more carotenoids than their respective wheat parents or hybrids derived from crosses between wild diploid barley and durum wheat (Alvarez *et al.*, 1999). A double haploid wheat population, which was previously characterized for endosperm color (Clarke *et al.*, 2006), was used to map the *PSY1* and *PSY2* genes against four QTLs affecting endosperm color, with one showing strong linkage (Pozniak *et al.*, 2007). Carotenoid levels in elite wheat lines (*Triticum aestivum* EM12) have also been improved by genetic engineering. Transgenic wheat expressing maize

PSY1 under the control of the endosperm-specific 1Dx5 promoter in combination with constitutively expressed *CRTI* produced yellow grains containing 10.8-fold the carotenoid levels of wild type EM12 plants (Cong *et al.*, 2009).

1.4.2 Vitamin A enhancement in Brassicas

Two members of the Barassica family have been the focus for carotenoid enhancement: canola (*Brassica napus*) and cauliflower (*B. oleracea*). Canola seeds contain high total carotenoid levels (up to 23 mg/g fresh weight, including 0.2 mg/g β-carotene) and canola oil is therefore considered a valuable dietary source of pro-vitamin A and a good target for carotenoid enhancement (Fujisawa *et al.*, 2009). Despite the existence of informative high-density genetic markers, no QTLs affecting carotenoid accumulation have yet been reported (Zamir, 2001). In contrast, there has been impressive progress in carotenoid genetic engineering (Nesi *et al.*, 2008). Shewmaker *et al.* (1999) expressed bacterial *CRTB* using the seed-specific napin promoter and increased total carotenoid levels to more than 1 mg/g fresh weight, a 500-fold improvement, which included 401 µg/g fresh weight of β-carotene. This was improved by Ravanello *et al.* (2003) using the same transgene (1.34 mg/g fresh weight total carotenoids including 739 µg/g fresh weight β-carotene). The combined expression of *CRTB* and *CRTI* in the same study boosted total carotenoid levels to 1.4 mg/g fresh weight with β-carotene levels reaching 857 µg/g fresh weight. However, the further addition of *CRTY*, which might be expected to increase total carotenoid and β-carotene levels even further, caused a marginal reduction in β-carotene levels (846 µg/g fresh weight) and a significant reduction in total carotenoids (1.23 mg/g fresh weight) presumable by increasing the flux to post-carotenoid steps in the pathway (Ravanello *et al.*, 2003).

Fujisawa *et al.* (2009) introduced seven bacterial carotenogenic genes into canola. One of these genes (*ipi*) is from the MEP pathway and its role was to increase the amount of the universal carotenoid precursor GGPP. Another four of the genes represented the linear part of the bacterial carotenoid synthesis pathway as shown in **Figure 3** (*CRTE*, *CRTB*, *CRTI* and *CRTY*) and the others were *CRTW* (β-carotene ketolase) and *CRTZ* (β-carotene hydroxylase) from the marine bacterium *Brevundimonas* SD212, which enable ketocarotenoid biosynthesis. Transgenic seeds expressing all seven transgenes accumulated up to 214 μg/g fresh weigh β-carotene, a 1070-fold increase over wild type, and also produced ketocarotenoids rarely seen in the plant kingdom, such as echinenone, canthaxanthin, astaxanthin and adonixanthin.

Wei *et al.* (2010) expressed the *Arabidopsis thaliana* microRNA gene *AtmiR156b* in canola either constitutively or under the control of the napin promoter. The function of this gene in *Arabidopsis* is to regulate a family of transcription factors, some of which control the transition from vegetative to reproductive growth. Constitutive expression of *AtmiR156b* in canola increased the amount of β-carotene in the seeds by up to 4.5-fold and also doubled the number of flowering shoots, whereas seed-specific *AtmiR156b* expression had no effect. *AtmiR156b* therefore appears to affect seed quality and shoot branching, and the transgenic lines showed significant variation in seed yield and weight. A single line produced apparently normal seeds with the higher carotenoid levels.

Although cauliflower seems an unusual target for carotenoid engineering, it has made a significant impact on the field through the discovery of the *OR* allele, the result of a spontaneous mutation that causes large quantities of carotenoids to accumulate in the edible curd, turning it orange. Homozygous *OR*/*OR* plants possess small but intensely orange curds, whereas heterozygous plants have less pigmentation but the curd is

normal in size and therefore commercially viable (Crisp *et al.*, 1975; Dickson *et al.*, 1988). Li *et al.* (2001) investigated the metabolic profiles of *OR* mutants and found that the predominant carotenoid in curd tissue was β-carotene, whose concentration is hundreds of times higher than in wild type curds. This unique phenotype suggested that *OR* has a regulatory rather than enzymatic role in carotenogenesis. Investigating the phenomenon, Li *et al.* (2001) found that *OR* induces the assembly of large carotenoid-sequestering organelles by influencing the differentiation of proplastids into orange chromoplasts (Paolillo *et al.*, 2004).

Li and Garvin (2003) identified 10 amplified fragment length polymorphisms (AFLPs) closely linked to the *OR* locus, and resolved sequence-characterized amplified region (SCAR) markers to facilitate positional cloning of the gene. Lu *et al.*, (2006) showed that *OR* encodes a plastid-associated protein containing a DnaJ cysteine-rich domain, and that the dominant *OR* mutation is caused by the insertion of a long terminal repeat retrotransposon. The function of *OR* is related to the differentiation of proplastids or other non-colored plastids into chromoplasts. The dominant allele is a hypermorph which induces abnormally large numbers of proplastids to differentiate into carotenoid-sequestering chromoplasts. The creation of a carotenoid sink may release the carotenoid pathway from feedback inhibition, allowing the production of higher amounts of β-carotene. A carotenoid-rich Chinese cabbage variety has recently been discovered whose orange leaves contain seven times the amount of carotenoids found in white cabbage (Feng *et al.*, 2012). The locus has been mapped but, at the time of writing, the gene has not been cloned and its relationship to the cauliflower *OR* gene is unknown.

1.4.3 Vitamin A enhancement in root vegetables

Four major root vegetable crops have been the focus of β-carotene enhancement strategies: carrot, potato, cassava and sweet potato. Carrot root color is a result of various pigments that serve as intermediate products in the carotenoid pathway. The *rp* allele was the first recessive allele shown to cause pigment loss in carrot roots and is associated with a 96% reduction in carotenoid levels, predominantly α- and β-carotene (Goldman and Breitbach, 1996).

Genetic engineering for carotenoids in carrot roots has been reported by Maass *et al.* (2009). They expressed bacterial *CRTB* resulting in carrot roots with a 93-fold increase in carotenoid content. The increased flux through the early part of the pathway revealed bottlenecks further downstream. The overall balance between xanthophylls and β-carotene shifted in favor of the latter, and a number of carotene intermediates that are normally converted to downstream products were seen to accumulate in the transgenic roots.

The *Y* (*Yellow*) locus in potato controls tuber flesh color by influencing carotenoid accumulation, and there exists an allelic series of increasing dominance beginning with the fully recessive *y* allele (white flesh, no carotenoids), then the *Y* allele (yellow flesh) and the fully dominant *OR* allele (orange flesh, reflecting the accumulation of zeaxanthin). The *Y* locus has been mapped to a region on chromosome 3 with two candidate genes, encoding PSY and BCH, and possibly additional regulatory elements (Bonierbale *et al.*, 1988). It is therefore clear that the natural *OR* allele in potato is completely unrelated to the *OR* allele in cauliflower discussed above, and presumably represents an enzyme that has a greater activity than wild type or that is expressed at a higher level than wild type.

Three different approaches have been explored in potato to engineer increased carotenoid levels. The first, as in the other cases discussed above, involves the

introduction of carotenogenic transgenes to promote the synthesis of carotenoids in tuber flesh. Transgenic potato plants have been produced expressing bacterial *CRTB*, resulting in 4–7-fold increase in carotenoid levels, including 11- and 19-fold increases in the amounts of β-carotene and lutein, respectively (Ducreux *et al.*, 2005). Diretto *et al.* (2007a) introduced the bacterial *CRTB*, *CRTI* and *CRTY* genes under the control of tuber-specific and constitutive promoters, increasing total carotenoid levels to 114 μg/g dry weight and β-carotene to 47 μg/g dry weight.

The second approach inhibits competing enzyme activities to direct flux specifically towards the synthesis of β-carotene, preventing further conversion. Diretto *et al.* (2006) silenced the endogenous *LYCE* gene, thereby eliminating competition at the branch point between the α- and β-carotene pathways. The total carotenoid content of transgenic tubers reached 12.27 μg/g dry weight (ca. 2.5-fold increase), while the β-carotene content was 0.043 μg/g dry weight (ca. 14-fold increase). Even higher levels were achieved by silencing the *BCH* gene and thereby preventing the further metabolism of β-carotene. Transgenic plants contained 21.7 μg/g dry weight total carotenoids (ca. 4.5-fold increase) including 0.085 μg/g dry weight β-carotene (ca. 38-fold increase) (Diretto *et al.*, 2007b). In a separate study, silencing the *BCH* gene alone elevated β-carotene levels to 2.64 μg/g dry weight (Van Eck *et al.*, 2007). Silencing the endogenous *zep* gene also increased total carotenoid levels to 60.8 μg/g dry weight (ca. 5.7-fold increase) predominantly represented by higher amounts of zeaxanthin, whereas violaxanthin levels were lower than normal (Romer *et al.*, 2002).

The third approach involves the creation of a metabolic sink for carotenoids. This reflects the discovery of the cauliflower *OR* allele as discussed above and its introduction into potato as a neomorphic transgene (Li *et al.*, 2001). Expression of the cauliflower *OR* allele in potatoes under the control of the tuber-specific granule-bound

starch synthase (GBSS) promoter resulted in the production of potato plants with orange-yellow tubers, and with carotenoid-sequestering organelles similar to those seen in the original cauliflower mutation suggesting the regulation of proplastid differentiation is conserved (Lu *et al.*, 2006). The total carotenoid levels in the *OR* transgenic lines reached 24 µg/g dry weight, a six-fold increase over wild type tubers.

Cassava is a particularly important root crop in terms of carotenoid enhancement because it is the preferred staple crop in some parts of Africa, but like cereals it is generally a poor source of carotenoids. Cultivars with carotene-rich yellow roots are rare and most breeding populations have white roots (Ferreira *et al.*, 2008; Nassar *et al.*, 2009). Welsch *et al.* (2010) recently characterized the *PSY2* locus in cassava and identified a polymorphism that increased carotenoid accumulation in cassava roots and also increased the rate of carotenoid synthesis when expressed in bacteria and yeast. The authors proposed that PSY is likely to be a significant bottleneck in cassava carotenoid synthesis and therefore introduced the bacterial *CRTB* gene into cassava plants under the control of the *CP1* promoter. The resulting transgenic lines had deep orange roots containing up to 21.84 µg/g dry weight of β-carotene compared to 6.67 µg/g in wild type roots.

Sweet potatoes with yellow/orange flesh are rich in β-carotene, but in most developing countries the preferred sweet potato cultivars have cream or white flesh and a low carotenoid content. Wang *et al.* (2007) reported a mutant variety of sweet potato with orange flesh, containing up to 19.5 mg/g fresh weight of carotenoids, which is seven times more than wild type plants. Genetic analysis has revealed eight QTLs affecting β-carotene content, three with very significant positive effects on the levels of β-carotene (Cervantes-Flores, 2006).

1.4.4 Vitamin A enhancement in fruits

Significant variation in β-carotene levels has been observed in kiwifruit, citrus and tomato.

Kiwifruit shows a significant variation in flesh color associated with carotenoid content. The common commercial varieties have green flesh (*Actinidia deliciosa*) or yellow flesh (*Actinidia chinesis*) and only moderate carotenoid levels, but several non-commercial varieties that accumulate high levels of anthocyanins and carotenoids have also been investigated (Ampomah-Dwamena *et al.*, 2009). The major carotenoids in kiwifruit are β-carotene and lutein. Moderate levels of β-carotene were detected 150 days after full bloom in *Actinidia chinesis* MP161 fruit (1.12 μg/g fresh weight), but much higher levels were found in three individual fruits from a hybrid *A. macrosperma* x *A. melanandra* population (7.07, 3.45 and 6.11 μg/g fresh weight, respectively) representing 43%, 39% and 70% of total carotenoids, respectively. Recently, Kim *et al.* (2010) established an efficient genetic transformation procedure for kiwifruit using micro-cross sections of stems from the *A. deliciosa* variety Hayward. Transgenic leaves constitutively expressing mandarin (*Citrus unshiu*) GGPPS or PSY accumulated up to 1.3-fold the normal amounts of lutein or β-carotene (Kim *et al.*, 2010). Although these represent only marginal improvements, this initial study paves the way for additional metabolic engineering studies to modulate β-carotene levels specifically in fruits.

Citrus fruits vary greatly in carotenoid content, depending on species, variety and growing conditions (Gross, 1987). Mandarin varieties such as Satsuma (*Citrus unshiu* Marc.) accumulate β-cryptoxanthin predominantly in the flavedo and juice sacs of mature fruit (Goodner *et al.*, 2001; Ikoma *et al.*, 2001), whereas mature sweet orange (*Citrus sinensis* Osbeck) accumulates violaxanthin isomers, principally 9-cis-violaxanthin (Molnar and Szabolcs, 1980; Lee and Castle, 2001). The most

abundant carotenoids present in the peel and pulp of citrus fruits are β-xanthophylls, which do not have pro-vitamin A activity (Kato *et al.*, 2004). Metabolic engineering in citrus fruits has therefore focused on increasing the β-carotene content, e.g. by expressing the *PSY* gene from sweet orange (*Citrus sinensis* Osbeck) in Hong Kong kumquat (*Fortunella hindsii* Swingle). The transgenic fruits were found to contain up to 171.9 μg/g fresh weight of total carotenoids, twice the normal level (Zhang *et al.*, 2009). The concentration of β-carotene and β-cryptoxanthin were twice normal levels, and the transgenic fruits also contained three times the normal amounts of phytoene and lycopene.

Whereas moderate progress has been made in the fruit crops discussed above, much more extensive work has been done to engineer carotenoid levels in tomato (*Solanum lycopersicum*). Ripe tomato fruits contain up to 207 μg/g fresh weight total carotenoids, including large amounts of lycopene (70.5 μg/g) but lower amounts of β-carotene (36.8 μg/g), and lutein (6.4 μg/g) (Fraser *et al.*, 1994). The *Beta* mutant, which has higher LYCB activity than wild type fruits, accumulates 186 μg/g fresh weight of total carotenoids, slightly lower than the wild type levels, but this includes 132 μg/g β-carotene and 38 μg/g lycopene, resulting in a characteristic orange fruit color (Rosati *et al.*, 2000). Various transgenic strategies have been implemented to induce similar effects (**Table 1**). For example, the constitutive expression of bacterial *CRTI* nearly doubled the amount of β-carotene but reduced overall carotenoid levels (Romer *et al.*, 2000). Further investigation showed that endogenous lycopene β-cyclases were upregulated in the transgenic fruits, thus diverting flux towards β-carotene rather than lycopene as had been predicted (Romer *et al.*, 2000). A seven-fold increase in β-carotene was achieved expressing the native *LYCB* gene using the fruit-specific tomato *PDS* promoter (Rosati *et al.*, 2000). The expression of bacterial *CRTB* increased

total carotenoid levels up to 4-fold, including a 2.5-fold increase in β-carotene levels (Fraser *et al.*, 2002). Fruits of the Moneymaker variety expressing *Arabidopsis LYCB* and pepper *BCH* had 12 times the normal levels of β-carotene seen in the control lines (Dharmapuri *et al.*, 2002). One of the most significant achievements is the HighCaro (HC) tomato variety, which expresses tomato *LYCB* cDNA driven by the CaMV 35S promoter and produces orange fruits as a result of the complete conversion of lycopene to β-carotene (D'Ambrosio *et al.*, 2004). The β-carotene content of HC tomato fruits is 205 μg/g fresh weight, 1.6-fold more than the *Beta* mutant described above. HC tomato has been evaluated extensively in greenhouse and field trials and has maintained its ability to convert lycopene to β-carotene into the T6 generation without negative effects on agronomic performance (Giorio *et al.*, 2007). Transgenic fruits expressing plastid-targeted bacterial DXS produced 1.6-fold higher levels of carotenoids than wild-type fruits, including a 1.4-fold increase in β-carotene (Enfissi *et al.*, 2005).

Like potato, tomato has also been engineered using the RNAi-mediated silencing approach. Silencing of the endogenous photomorphogenesis regulator gene *DE-ETIOLATED1* (*DET1*) resulted in fruits with 8.5-fold higher β-carotene levels than wild type plants (Davuluri *et al.*, 2005). The overexpression of tomato *CRY2*, a blue-light photoreceptor, increased carotenoid levels 1.7-fold including a 1.3-fold increase in β-carotene (Giliberto *et al.*, 2005). The overexpression of pepper fibrillin, which plays a role in the formation of lipoprotein carotenoid-storage structures, resulted in a 95% increase in total carotenoids, including a 64% increase in β-carotene and a 118% increase in lycopene (Simkin *et al.*, 2007). Chloroplast transformation has also been used to increase carotenoid levels in tomato (Wurbs *et al.*, 2007; Apel and Bock, 2009). Bacterial *CRTY* driven by the *atp*l promoter was introduced into tomato plastids by particle bombardment and resulted in the conversion of lycopene to β-carotene,

increasing the amount of β-carotene four-fold in transplastomic fruits, to 28.6 µg/g fresh weight (Wurbs *et al.*, 2007). However, the expression of daffodil *LYCB* in plastids under the control of the rRNA operon promoter increased β-carotene levels to 95 µg/g fresh weight in tomato fruits, together with a >50% increase in total carotenoids, suggesting that LYCB is normally a rate limiting step (Apel and Bock, 2009)

Table 1 Total carotenoid levels and β-carotene levels in wild type and transgenic plants (DW = dry weight, FW = fresh weight, ND = not determined)

[a] Different carotenoid levels cited for each species (wild type) reflect the different varieties used in each investigation,

[b] We converted dry weight to fresh weight assuming the water content of tomato fruit is 90%.

Species	**Genes (origin)**	**Total carotenoid levels in wild type [a]**	**Total carotenoid levels (increase relative to wild type) in transgenic plants**	**β-Carotene levels in wild type [a]**	**β-Carotene levels (increase relative to wild type) in transgenic plants**	**References**
Rice	*PSY1* (*Narcissus pseudonarcissus*; daffodil) and *CRTI* (*P. ananatis*)	ND	1.6 µg/g DW	ND	1.4 µg/g DW	Ye *et al.*, 2000
	PSY1 (*Zea mays*; maize) and *CRTI* (*P. ananatis*)	ND	37 µg/g DW	ND	31 µg/g DW	Paine *et al.*, 2005

	PSY1 (*Zea mays,* maize) and *CRTI* (*P. ananatis*)	ND	1.06 µg/g DW	ND	0.41 µg/g DW	Baisakh *et al.*, 2006
Maize	*CRTB* and *CRTI* (*P. ananatis*)	0.99 µg/g DW	33.6 µg/g DW (34)	0.98 µg/g DW	9.8 µg/g DW	Aluru *et al.*, 2008
	PSY1 (*Zea mays*; maize) *CRTI* (*P. antoeaananatis*) *CRTW* (*P. aracoccus* spp) *LYCB* (*Gentiana lutea*)	1.10 µg/g DW	146.7 µg/g DW (133)	0.14 µg/g DW	57.35 µg/g DW (410)	Zhu *et al.*, 2008
	PSY1 (maize) *CRTI* (*Pananatis*)	1.45 µg/g DW	163.2 µg/g DW (112)	0.35 µg/g DW	59.32 µg/g DW (169)	Naqvi *et al.*, 2009
Wheat	*PSY1* (maize) *CRTI* (*P. ananatis*)	0.46 µg/g DW	4.96 µg/g DW (10.8)	ND	ND	Cong *et al.*, 2009
Potato	*ZEP* (*Arabidopsis thaliana*; *Arabidopsis*)	10.6 µg/g DW	60.8 µg/g DW (5.7)	0.7 µg/g DW	2.4 µg/g DW (3.4)	Romer *et al.*, 2002
	CRTB (*P. ananatis*)	5.6 µg/g DW	35.5 µg/g DW (6.3)	ND	10.3 µg/g DW	Ducreux *et al.*, 2005
	OR (*Brassica oleracea*; cauliflower)	5.41 µg/g DW	28.22 µg/g DW (6)	ND	5.01	Lopez *et al.*, 2008
	Antisense *LYCE* (*Solanum tuberosum*;	4.6 µg/g DW	9.974 µg/g DW (2.5)	0.00317 µg/g DW	0.043 µg/g DW (14)	Diretto *et al.*, 2006

	potato)					
	CRTB, CRTI and *CRTY* (*P. ananatis*)	5.8 µg/g DW	114.4 µg/g DW (20)	0.013 µg/g DW	47.4 µg/g DW (3600)	Diretto *et al.*, 2007 a
	Antisense *BCH* (potato)	4.88 µg/g DW	14.264 µg/g DW (2.9)	0.00225 µg/g DW	0.085 µg/g DW (38)	Diretto *et al.*, 2007 b
	Antisense *BCH* (potato)	22.48 µg /g DW (75% water content)	23.52 µg/g DW (104) (75% water content)	0.04 µg /g DW (75% water content)	13.24 µg/g DW (331) (75% water content)	VanEck *et al.*, 2007
Cassava	*CRTB* (*P. ananatis*)	0.65 µg/g DW	21.84 µg/g DW (336)	0.41 µg/g DW	6.67 µg/g DW (16)	Wesch et al., 2010
Carrot	*PSY* (*Arabidopsis*)	5.5 µg/g DW	514.1 µg/g DW (93)	1.265 µg/g DW	214.627 µg/g DW (178)	Maass *et al.*, 2009
Canola	*CRTB* (*P.ananatis*)	36 µg/g FW	1055 µg/g FW (29.3)	5 µg/g FW	401 µg/g FW (80.2)	Shewmaker *et al.*, 1999
	CRTB (*P. ananatis*)	ND	1341 µg/g FW	ND	739 µg/g FW	Ravanello *et al.*, 2003
	CRTE and *CRTB* (*P. ananatis*)		1023 µg/g FW		488 µg/g FW	
	CRTB (*P. ananatis*) *CRTI* (*P. ananatis)*		1412 µg/g FW		857 µg/g FW	
	CRTB and *CRTY* (*P. ananatis*)		935 µg/g FW		459 µg/g FW	
	CRTB (*P. ananatis*) and *LYCB* (*Brassica*		985 µg/g FW		488 µg/g FW	

	napus; canola)					
	CRTB, *CRTI* and *CRTY* (*P. ananatis*)		1229 µg/g FW		846 µg/g FW	
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 5' end	5.34 µg/g FW	227.78 µg/g FW (42.5)	0.49 µg/g FW	90.76 µg/g FW (185.2)	Yu *et al.*, 2007
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 3' end		94.09 µg/g FW (17.6)		27.02 µg/g FW (55)	
	idi, *CRTE*, *CRTB*, *CRTI* and *CRTY* (*P. ananatis*) *CRTZ*, *CRTW* (*Brevundimonas* sp)	21.7 µg/g FW	656.7 µg/g FW (30)	0.2 µg/g FW	214.2 µg/g FW (1070)	Fujisawa *et al.*, 2009
	microRNA miR156b (*Arabidopsis*)	3 µg/g FW (10% water content)	6.9 µg/g FW (2.45) (10% water content)	0.08 µg/g FW (10% water content)	0.38 µg/g FW (4.5) (10% water content)	Wei *et al.*, 2010
Tomato [b]	*CRTI* (*P. ananatis*)	285 µg/g FW	137.2 µg/g FW (0.5)	27.1 µg/g FW	52 µg/g FW (1.9)	Romer *et al.*, 2000
	LYCB (*Solanum lycopersicum*; tomato)	66 µg/g FW	109 µg/g FW (1.7)	7 µg/g FW	57 µg/g FW (7.1)	Rosati *et al.*, 2000
	LYCB (*Arabidopsis*) *BCH* (*Capsicum annuum*;	66.3 µg/g FW	100.7 µg/g FW (1.5)	5 µg/g FW	63 µg/g FW (12)	Dharmapuri *et al.*, 2002

	pepper)					
	CRTB (*Pananatis*)	285.7 µg/g FW	591.8 µg/g FW (2.1)	33 µg/g FW	82.5 µg/g FW (2.5)	Fraser *et al.*, 2002
	LYCB (tomato)	94.5 µg/g FW	215.2 µg/g FW (2.3)	4.4 µg/g FW	205.0 µg/g FW (46.6)	D´Ambrosio *et al.*, 2004
	DXS (*Escherichia coli*)	460 µg/g FW	720 µg/g FW (1.6)	50 µg/g FW	70.0 µg/g FW (1.4)	Enfissi *et al.*, 2005
	Antisense *DET-1* (tomato)	36.4 µg/g FW	83.8 µg/g FW (2.3)	1.63 µg/g FW	13 µg/g FW (8)	Davuluri *et al.*, 2005
	CRY2 (tomato)	87.6 µg/g FW in ripe fruit pericarps	149 µg/g FW in ripe fruit (1.7) pericarps (17)	7.8 µg/g FW in ripe fruit pericarps	10.1 µg/g FW in ripe fruit (1.3) pericarps (13)	Giliberto *et al.*, 2005
	PSY1 (tomato)	181.20 µg/g FW	227.67 µg/g FW (1.25)	58.62 µg/g FW	81.93 µg/g FW (1.4)	Fraser *et al.*, 2007
	fibrillin (pepper)	325 µg/g FW	650 µg/g FW (2.0)	90 µg/g FW	150 µg/g FW (1.6)	Simkin *et al.*, 2007
	CRTY (*Erwinia herbicola*)	372.66 µg/g FW	323.71 µg/g FW (09)	6.91 µg/g FW	28.61 µg/g FW (4)	Wurbs *et al.*, 2007
	LYCB (*Narcissus pseudonarcissus*; daffodil)	76.67 µg/g FW	115 µg/g FW (1.5)	19 µg/g FW	95 µg/g FW (5)	Apel and Block 2009
Kumquat	*PSY* (*Citrus sinensis*; orange)	84.3 µg/g FW	131.9 µg/g FW (1.6)	0.70 µg/g FW	1.72 µg/g FW (2.5)	Zhang *et al.*, 2009

1.2 Ketocarotenoid biosynthesis and accumulation in diverse crops

Ketocarotenoids are carotenoids that contain at least one keto group, either in the linear chain or on the β-ionone ring(s). Many ketocarotenoids are found in algae, fungi and bacteria, but astaxanthin (3,3´-dihydroxy-β, β-carotene-4,4´-dione) and canthaxanthin (β, β-carotene-4,4`-dione) are the most valuable (Margalith, 1999), because they are widely used in the aquaculture industry to produce pink flesh in fish and shellfish (Guerin *et al.*, 2003). Astaxanthin is a strong antioxidant (Miki *et al.*, 1991) and contributes to general eye and skin health (Guerin *et al.*, 2003). It has anti-inflammatory properties and inhibits the oxidation of low-density lipoproteins in humans (Iwamoto *et al.*, 2000). It also helps to prevent diabetic nephropathy in diabetic db/db mice (Naito *et al.*, 2004), protects against cancer (Tanaka *et al.*, 1994; Chew *et al.*, 1999) and boosts the immune system (Jyonouchi *et al.*, 1995; Chew *et al.*, 2004). The various functions of different carotenoids make them targets for genetic engineering in plants where the objective is to increase the content of valuable carotenoids and ketocarotenoids in staple crops (Sandmann *et al.*, 2006; Howitt *et al.*, 2006; Zhu *et al.*, 2007; 2008; 2009; 2010; Giuliano *et al.*, 2008; Farre *et al.*, 2010).

Astaxanthin is synthesized from β-carotene by the introduction of keto and hydroxyl moieties at the 4,4´ and 3,3´ positions of the β-ionone ring. These reactions are catalyzed by a β-carotene ketolase (CRTW, BKT) and a β-carotene hydroxylase (CRTZ, BCH), respectively (Misawa *et al.*, 1995; Zhu *et al.*, 2009) (**Figure 4**). Eight reaction products have been identified during this conversion and four possible biochemical routes elucidated (Fraser *et al.*, 1997; Misawa *et al.*, 1995) as shown in figure 4.

Competition between β-carotene hydroxylase and β-carotene ketolase for substrate synthesis in the extended pathway provides a novel way for astaxanthin formation, a molecule which is a valuable metabolite in commercial use.

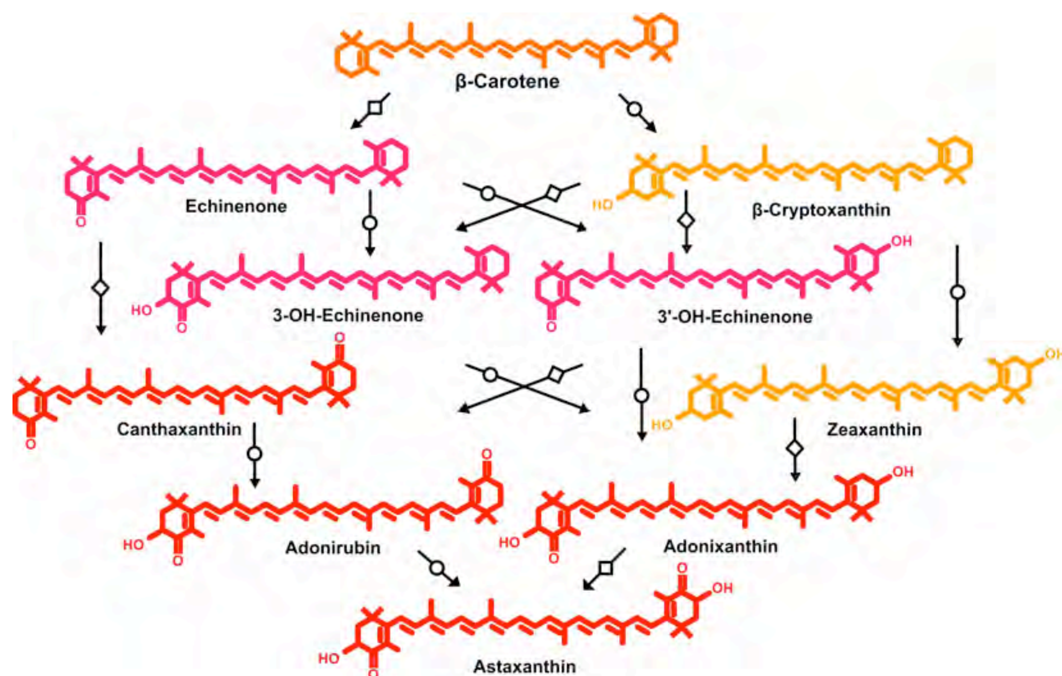


Figure 4 Astaxanthin biosynthesis pathway (Zhu *et al.*, 2009, 2013).

Astaxanthin biosynthesis pathway in astaxanthin-producing bacteria and transgenic plants, and the catalytic function of β-carotene hydroxylase (arrow with circle) and β-carotene ketolase (arrow with square).

A significant bottleneck preventing high levels accumulation of astaxanthin in transgenic plants is the inefficient conversion of zeaxanthin to astaxanthin via adonixanthin, a reaction catalyzed by β-carotene ketolases (Zhu *et al.*, 2009). There are three major classes of β-carotene ketolases, BKT, CRTO, and CRTW (Mann *et al.*, 2000; Stalberg *et al.*, 2003; Ralley *et al.*, 2004; Suzuki *et al.*, 2007; Zhu *et al.*, 2008; 2009). Previous literature reported that plants overexpressing the marine or bacterial

CRTW can synthesize astaxanthin with lower efficiency (Mann *et al.*, 2000; Stalberg *et al.*, 2003; Ralley *et al.*, 2004; Morris *et al.*, 2006; Gerjets *et al.*, 2007; Suzuki *et al.*, 2007). Transgenic tobacco expressing *Brevundimonas CRTW* and *CRT*Z accumulated more than 0.5% (dry weight) astaxanthin (ca: 70% of total carotenoids) in leaves, which developed a reddish brown color (Hasunuma *et al.*, 2008). Transgenic *Arabidopsis thaliana* expressing *BKT* from *Chlamydomonas reinhardtii* (*CrBKT*) exhibited orange pigmentation in leaves due to the accumulation of substantial amounts of astaxanthin (up to 2 mg/g dry weight with a 1.8-fold increase in total carotenoids (Zhong *et al.*, 2011). The efficiency of *CrBKT* for the accumulation of astaxanthin was therefore demonstrated to be much higher than other *BKT* genes, including *CzBKT* (*Chlorella zofingiensis BKT)* and *HpBKT3* (*Haematococcus pluvialis BKT3*) (Zhong *et al.*, 2011).

Expression of the two linked gene cassettes *Crbkt* and *Hpbch* in tomato resulted in the up-regulation of most intrinsic carotenogenic genes leading to massive accumulations of mostly free astaxanthin in leaves (3.12mg/g) and esterified astaxanthin in fruits (16.1mg/g). A 16-fold increase in total carotenoid accumulation was reported in these plants without any detrimental effects on plant growth and development (Huang *et al.*, 2013) (**Table 2**)

Table 2 Ketocarotenoid levels in diverse transgenic plants

(DW = dry weight, FW = fresh weight)

Genes origin	**Promoter**	**Plant species**	**Major ketocarotenoids (µg/g)/position of accumulation**	**References**
BKT (*Haematococcus pluvialis*)	Tomato phytoene synthase gene (*PDS*)	Tobacco	astaxanthin (23.5), adonirubin (17.1), adonixanthin (12), Canthaxanthin (10.2), 3-OH echinenone (8.5), 3´-Hydroxyechinenone (8.1)/nectaries (FW)	Mann *et al.*, 2000
BKT (*Hpluvialis*)	seed storage protein napA (oilseed rape)	*Arabidopsis thaliana*	4-Keto-lutein, canthaxanthin and adonirubin/seeds	Stålberg *et al.*, 2003
CRTW and *CRTZ* (*Paracoccus* spN81106)	Cauliflower mosaic virus (CaMV) 35S	Tobacco	ketocarotenoids/leaves (800)(DW) and nectaries (64)(DW)	Ralley *et al.*, 2004
CRTO (*Synechocystis* spPCC 6803)	CaMV 35S	Potato	echinenone (7600), 3'-hydroxyechinenone (1500) and 4-ketozeaxanthin (2700)/leaves(DW); astaxanthin (1.8), 3'-hydroxyechinenone (0.4) and 4-Ketozeaxanthin (8.5)/tubers(DW)	Gerjets *et al.*, 2006

BKT (*Hpluvialis*)	Patatin (potato)	Potato	4-ketolutein (0.5) and astaxanthin (0.6)/desiree tubers(DW)	Morris *et al.*, 2006
BKT (*Hpluvialis*)	Patatin (potato)	Potato	4-Ketolutein(9.8) and astaxanthin(9.5)/ Mayan Gold tubers(DW)	Morris *et al.*, 2006
CRTW (*Paracoccus* spN81106)	CaMV 35S		ketocarotenoids (89.8) / flower petals(DW)	Suzuki *et al.*, 2007
CRTO (*Synechocystis* spPCC 6803), *CRTZ* (*Pananatis*)		Tobacco	Echinenone(8), 3´-Hydroxyechinenone(5.2), 4-keto-lutein(8.8)/leaves(DW); echinenone(11.6), 4-keto-lutein(8.5) and 4-keto-zeaxanthin(12.9)/ nectaries(DW)	Gerjets *et al.*, 2007
CRTO (*Synechocystis* spPCC 6803)	CaMV 35S	Tobacco	echinenone (2.3) and 4-keto-lutein (2.6)/ leaves, echinenone (83.), 4-keto-lutein (6.7) and 4-keto-zeaxanthin (8.3)/nectaries(DW)	Gerjets *et al.*, 2007
CRTO (*Synechocystis* spPCC 6803)	CaMV 35S	*Nicotiana glauca*	echinenone (3.8), 4-keto-zeaxanthin (7.8), ketolutein (9.9) and 3´-hydroxyechinenone (7.0)/petals(DW); echinenone (1.8), ketolutein (3.5) and 3´-hydroxyechinenone (4.4) /nectary(DW); echinenone (7.6), 4-keto-zeaxanthin (2.2), ketolutein (3.7) and	zhu *et al.*, 2007

			3´-hydroxyechinenone (11.4)/pistils(DW); echinenone (11.0) and 3´-hydroxyechinenone (14.5) /sepal(DW); echinenone (6.7) and 3´-hydroxyechinenone (12.9) /ovary(DW)	
CRTW and *CRTZ* (*Brevundimonas* spSD212)	Tobacco rrn	Tobacco	astaxanthin (5440), 4-ketoantherxanthin (370), adonirubin (140), adonixanthin (110), canthaxanthin (80) and 3´-hydroxyechinenone (30)/leaves(DW)	Hasunuma *et al.*, 2008
BKT (*Hpluvialis*)	Double CaMV 35S promoter	Carrot	astaxanthin (91.6), adonixanthin (15.9), adonirubin (57), canthaxanthin (50.1) and echinenone (21.4)/roots(DW); astaxanthin (34.7), adonixanthin (5.0), adonirubin (5.9), canthaxanthin (4.0) and echinenone (2.7)/leaves(DW); astaxanthin (12.4), adonixanthin (3.2), adonirubin (3.1), canthaxanthin (5.8) and echinenone (5.6)/callus(DW)	Jayaraj *et al.*, 2008
CRTW (*Paracoccus*	λ-zein (maize)	Maize	astaxanthin (4.5), adonixanthin (22.4), 3'-hydroxyechinenone	zhu *et al.*, 2008

spN81106)			(3.8) and echinenone (5.1)/Maize endosperm(DW)	
Idi (*Paracoccus* spstrain N81106), *CRTW* (*Brevundimonas* spstrain SD212), *CRTZ*, *CRTE* (*Pantoea ananatis*, ATCC19321), *CRTB*, *CRTY*, *CRTI*	Pnos, the nopaline synthase gene (nos) promoter, Pnapin, the napin promoter from Bnapus,P35S, the cauliflower mosaic virus (CaMV) 35Spromoter, PFAE, the fatty acid elongase 1 gene (FAE1) seed-specific promoter from Athaliana	*Brassica napus*	echinenone (109.8), 3'-hydroxyechinenone (3.9), 3-hydroxyechinenone (10.6), astaxanthin (0.6), adonixanthin (1.5), adonirubin (7.1) and canthaxanthin (51.1)/Bnapus seeds(DW)	Fujisawa *et al.*, 2009
CrBKT (*Chlamydomonas reinhardtii*)	cauliflour mosaic virus (CaMV) 35S promoter	*Arabidopsis*	echinenone (80), 3'-hydroxyechinenone (100), astaxanthin (1370), adonixanthin (390), adonirubin (370), canthaxanthin (240), and 4-ketoantheraxanthin (190)/*Arabidopsis*	Zhong *et al.*, 2011

			leaves(DW); echinenone (2.27), 3-hydroxyechinenone (0.51), Astaxanthin (17.23), adonixanthin (16.64) and 4-Ketoantheraxanthin(0.14)/*Arabidopsis* seed(DW)	
HpBKT (*Haematococcus pluvialis*)	ibAGP1 promoter	Carrot	Astaxanthin(17.2), adonirubin(5.6)/carrot root(DW)	Ahn *et al.*, 2012
CrBKT (*Chlamydomonas reinhardtii*)	cauliflour mosaic virus (CaMV) 35S promoter	Tomato	Echinenone(60), 3'-hydroxyechinenone(190), 4-Ketoantheraxanthin(10), Astaxanthin(350), adonixanthin(320), adonirubin(530) and canthaxanthin(1690)/tomato leaves(DW), Echinenone(200), Astaxanthin(926), adonirubin(828), canthaxanthin(2249)/tomato fruits(DW)	Huang *et al.*, 2013
CrBKT (*Chlamydomonas reinhardtii*) and *HpBCH*	cauliflour mosaic virus (CaMV) 35S promoter	Tomato	Echinenone(80), Astaxanthin(3120), adonixanthin(910), adonirubin(230) and 4-Ketoantheraxanthin(250), canthaxanthin(338)/tomato leaves(DW), Echinenone(562.7),	Huang *et al.*, 2013

(*Haematococcus pluvialis*)			Astaxanthin(16104.1), adonixanthin(393), adonirubin(197) and 4-Ketoantheraxanthin(117), canthaxanthin(338)/tomato fruits(DW)	

REFERENCES

Ahn MJ, Noh SA, Ha SH, Back K, Lee SW, Bae JM. (2012) Production of ketocarotenoids in transgenic carrot plants with an enhanced level of β-carotene. Plant Biotechnol Rep 6: 133–140.

Al-Babili S, Hoa TT, Schaub P. (2006) Exploring the potential of the bacterial carotene desaturase CRTI to increase the β-carotene content in Golden Rice. J Exp Bot 57: 1007–1014.

Aluru M, Xu Y, Guo R, Wang Z, Li S, White W, Wang K, Rodermel S. (2008) Generation of transgenic maize with enhanced provitamin A content. J Exp Bot 59: 3551–3562.

Alvarez JB, Martin LM, Martin A. (1999) Genetic variation for carotenoid pigment content in the amphiploid *Hordeum chilense* x *Triticum turgidum* conv*durum.* Plant Breeding 118: 187–189.

Ampomah-Dwamena C, McGhie T, Wibisono R, Montefiori M, Hellens RP, Allan AC. (2009) The kiwifruit lycopene β-cyclase plays a significant role in carotenoid accumulation in fruit. J Exp Bot 60: 3765–3779.

Apel W, Bock R. (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitaminA conversion. Plant Physiol 151: 59–66.

Azevedo-Meleiro CH, Rodriguez-Amaya DB. (2007) Qualitative and quantitative differences in carotenoid composition among *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo.* J Agric Food Chem 55: 4027–4033.

Baisakh N, Rehana S, Rai M, Oliva N, Tan J, Mackill DJ, Khush GS, Datta K, Datta SK. (2006) Marker–free transgenic (MFT) near-isogenic introgression lines (NIILs) of ‘golden’ indica rice (cvIR64) with accumulation of provitamin A in the endosperm tissue. Plant Biotechnol J 4: 467–475.

Bonierbale MW, Plaisted RL, Tanksley SD. (1988) RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120: 1095–1103.

Breitenbach J, Sandmann G. (2005) ζ-Carotene *cis* isomers as products and substrates in the plant poly-*cis* carotenoid biosynthetic pathway to lycopene. Planta 220: 785–793.

Buishand JG, Gableman WH. (1979) Investigations of the inheritance of color and carotenoid content in phloem and xylem of carrot roots (*Daucus carota* L). Euphytica 28: 611–632.

Buckner B, Miguel PS, Janick-Buckner D, Bennetzen JL. (1996) The *y1* gene of maize codes for phytoene synthase. Genetics 143: 479–488.

Burkhardt PK, Beyer P, Wunn J, Kloti A, Armstrong GA, Schledz M, von LJ, Potrykus I. (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synt hase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J 11: 1071–1078.

Capell T, Christou P. (2004) Progress in plant metabolic engineering. Curr Opin Biotechnol 15: 148–154.

Chappell J. (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Ann Rev Plant Physiol Plant Mol Biol 46: 521–547.

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

Chew BP, Park JS. (2004) Carotenoid action on the immune response. J Nutr 134: 257S–261S.

Chew BP, Park JS, Wong MW, Wong TS. (1999) A comparison of the anticancer activities of dietary β-carotene, canthaxanthin and astaxanthin in mice *in vivo.* Anticancer Res 19: 1849–1853.

Clarke FR, Clarke JM, McCaig TN, Knox RE, DePauw RM. (2006) Inheritance of yellow pigment concentration in seven durum wheat crosses. Can J Plant Sci 86: 133–141.

Comai L, Henikoff S. (2006) TILLING: practical single-nucleotide mutation discovery. Plant J 45: 684–694.

Cong L, Wang C, Chen L, Liu H, Yang G, He G. (2009) Expression of phytoene synthase1 and carotene desaturase *CRTI* genes result in an increase in the total carotenoids content in transgenic elite wheat (*Triticum aestivum* L). J Agric Food Chem 57: 8652–8660.

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

Crisp P, Walkey DGA, Bellman E, Roberts E. (1975) A mutation affecting curd colour in cauliflower (*Brassica oleracea* L. var*botrytis* DC). Euphytica 24: 173–176.

Darnton-Hill I, Nalubola R. (2002) Fortification strategies to meet micronutrient needs: successes and failures. Proc Nutr Soc 61: 231–241.

Datta K, Baisakh N, Oliva N, Torrizo L, Abrigo E, Tan J, Rai M, Rehana S, Al–Babili S, Beyer P. (2003) Bioengineered ''golden'' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. Plant Biotechnol J 1: 81–90.

Davuluri GR, Van Tuinen A, Fraser PD, Manfredonia A, Newman R, Burgess D, Brummell DA, King SR, Palys J, Uhlig J, Bramley PM, Pennings HMJ, Bowler C. (2005) Fruit-specific RNAi-mediated suppression of *DET1* enhances carotenoid and flavonoid content in tomatoes. Nat Biotechnol 23: 890–895.

Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G. (2002) Metabolic engineering of xanthophyll content in tomato fruits. FEBS Lett 519: 30–34.

Dickson MH, Lee CY, Blamble AE. (1988) Orange-curd high carotene cauliflower inbreds. HortScience 23: 778–779.

Diretto G, Al-Babili S, Tavazza R, Papacchioli V, Beyer P, Giuliano G. (2007a) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. PLoS One 2: e350.

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

Diretto G, Welsch R, Tavazza R, Mourgues F, Pizzichini D, Beyer P, Giuliano G. (2007b) Silencing of β-carotene hydroxylase increases total carotenoid and β-carotene levels in potato tubers. BMC Plant Biol 7: 11.

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

Dwivedi SL, Crouch JH, Mackill DJ , Xu Y, Blair MW, Ragot M, Upadhyaya HD, Ortiz R. (2007) The molecularization of public sector crop breeding: progress, problems, and prospects. Adv Agron 3: 163–319.

Enfissi EM, Fraser PD, Lois LM, Boronat A, Schuch W, Bramley PM. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J 3: 17–27.

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

Farre G, Rivera SM, Alves R, Vilaprinyo E, Sorribas A, Canela R, Naqvi S, Sandmann G, Capell T, Zhu C, Christou P. (2013) Targeted transcriptomic and metabolic profiling reveals temporal bottlenecks in the maize carotenoid pathway that can be addressed by multigene engineering. Plant J 75: 441–455.

Feng H, Li Y, Liu Z, Liu J. (2012) Mapping of *or*, a gene conferring orange color on the inner leaf of the Chinese cabbage (*Brassica rapa* Lssp*pekinensis*). Mol Breed 29: 235–244.

Ferreira CF, Alves E, Pestana KN, Junghans DT, Kobayashi AK, de Jesus Santos V, Silva RP, Silva PH, Soares E, Fukuda W. (2008) Molecular characterization of cassava (*Manihot esculenta Crantz*) with yellow-orange roots for β-carotene improvement. Crop Breed Appl Biotechnol 8: 23–29.

Fraser PD, Enfissi EMA, Halket JM, Truesdale MR, Yu D, Gerrish C, Bramley PM. (2007) Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. Plant Cell 19: 3194–3211.

Fraser PD, Miura Y, Misawa N. (1997) In *vitro* characterization of astaxanthin biosynthetic enzymes. J Biol Chem 272: 6128–6135.

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

Fraser PD, Truesdale MR, Bird CR, Schuch W, Bramley PM. (1994) Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). Plant Physiol 105: 405–413.

Fujisawa M, Takita E, Harada H, Sakurai N, Suzuki H, Ohyama K, Shibata D, Misawa N. (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. J Exp Bot 60: 1319–1332.

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

Gerjets T, Sandmann M, Zhu C, Sandmann G. (2007) Metabolic engineering of

ketocarotenoid biosynthesis in leaves and flowers of tobacco species. Biotechnol J 2: 1263–1269.

Giliberto L, Perrotta G, Pallara P, Weller JL, Fraser PD, Bramley PM, Fiore A, Tavazza M, Giuliano G. (2005) Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. Plant Physiol 137: 199–208.

Giorio G, Stigliani AL, D´Ambrosio C. (2007) Agronomic performance and transcriptional analysis of carotenoid biosynthesis in fruits of transgenic HighCaro and control tomato lines under field conditions. Transgenic Res 16: 15–28.

Giuliano G, Tavazza R, Diretto G, Beyer P, Taylor MA. (2008) Metabolic engineering of carotenoid biosynthesis in plants. Trends Biotechnol 26: 139–145.

Goodner KL, Rouseff RL, Hofsommer HJ. (2001) Orange, mandarin, and hybrid classification using multivariate statistics based on carotenoid profiles. J Agric Food Chem 49: 1146–1150.

Goldman IL, Breitbach DN. (1996) Inheritance of a recessive character controlling reduced carotenoid pigmentation in carrot (*Daucus carota* L). J Hered 87: 380–382.

Gross J. (1987) Carotenoids: Pigments in Fruits. Academic Press, London.

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

Harjes CE, Rocheford TR, Bai L, Brutnell TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET, Yan J, Buckler ES. (2008) Natural

genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319: 330–333.

Harrison EH. (2005) Mechanisms of digestion and absorption of dietary vitamin A. Ann Rev Nutr 25: 87–103.

Hasunuma T, Miyazawa SI, Yoshimura S, Shinzaki Y, Tomizawa KI, Shindo K, Choi SK, Misawa N, Miyake C. (2008) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. Plant J 55: 857–868.

Howitt CA, Pogson BJ. (2006) Carotenoid accumulation and function in seeds and non–green tissues. Plant Cell Environ 29: 435–445.

Huang JC, Zhong YJ, Liu J, Sandmann G, Chen F. (2013) Metab Eng 17: 59–67.

Ikoma Y, Komatsu A, Kita M, Ogawa K, Omura M, Yano M, Moriguchi T. (2001) Expression of a phytoene synthase gene and characteristic carotenoid accumulation during citrus fruit development. Physiol Plant 111: 232–238.

Imam MK, Gabelman WH. (1968) Inheritance of carotenoids in carrots, *Daucus carota* L. Proc Am Soc Hortic Sci 93: 419–428.

IOM. (2001) Dietary reference intakes for vitamin a, vitamin k, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington, DC: National Academy Press.

Isaacson T, Ohad I, Beyer P, Hirschberg J. (2004) Analysis in vitro of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants. Plant Physiol 136: 4246–4255.

Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, Kamiyama M, Itakura H, Yamamoto S, Kondo K. (2000) Inhibition of low–density lipoprotein oxidation by astaxanthin. J Atheroscler Thromb 7: 216–222.

Jyonouchi H, Sun S, Tomita YGross MD. (1995) Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. J Nutr 125: 2483–2492.

Kato M, Ikoma Y, Matsumoto H, Sugiura M, Hyodo H, Yano M. (2004) Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol 134: 824–837.

Kim M, Kim SC, Song KJ, Kim HB, Kim IJ, Song EY, Chun SJ. (2010) Transformation of carotenoid biosynthetic genes using a micro-cross section method in kiwifruit (*Actinidia deliciosa cvHayward*). Plant Cell Rep 29: 1339–1349.

Lawrence RJ, Pikaard CS. (2003) Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. Plant J 36: 114–121.

Lee HS, Castle WS. (2001) Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd Blood orange juices. J Agric Food Chem 49: 877–882.

Li F, Murillo C, Wurtzel ET. (2007) Maize *Y9* encodes a product essential for 15-*cis*-zeta-carotene isomerization. Plant Physiol 144: 1181–1189.

Li L, Garvin DF. (2003) Molecular mapping of *Or*, a gene inducing β-carotene accumulation in cauliflower (*Brassica oleracea* Lvar*botrytis*). Genome 46: 588–594.

Li L, Paolillo DJ, Parthasarathy MV, Dimuzio EM, Garvin DF. (2001) A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* Lvar*botrytis*). Plant J 26: 59–67.

Li Q, Farre G, Naqvi S, Breitenbach J, Sanahuja G, Bai C, Sandmann G, Capell T, Christou P, Zhu C. (2010) Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. Transgen Res 19: 1053–1068.

Lu S, Van Eck J, Zhou X, Lopez AB, O'Halloran DM, Cosman KM, Conlin BJ, Paolillo DJ, Garvin DF, Vrebalov J, Kochian LV, Kupper H, Earle ED, Cao J, Li L. (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell 18: 3594–3605.

Maes T, De Keukelerie P, Gerats T. (1999) Plant tagnology. Trends Plant Sci 4: 90–96.

Maass D, Arango J, Wust F, Beyer P, Welsch R. (2009) Carotenoid crystal formation in *Arabidopsis* and carrot roots caused by increased phytoene synthase protein levels. PLoS One 4: e6373.

Mann V, Harker M, Pecker I, Hirschberg J. (2000) Metabolic engineeringof astaxanthin production in tobacco flowers. Nat Biotechnol 18: 888–892.

Mansoor S, Amin I, Hussain M, Zafar Y, Briddon RW. (2006) Engineering novel traits in plants through RNA interference. Trends Plant Sci 11: 559–565.

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

Morris WL, Ducreux LJM, Fraser PD, Millam S, Taylor MA. (2006) Engineering ketocarotenoid biosynthesis in potato tubers. Metab Eng 8: 253–263.

Miki W. (1991) Biological functions and activities of animal carotenoids. Pure Appl Chem 63: 141–146.

Misawa N, Truesdale MR, Sandmann G, Fraser PD, Bird C, Schuch W, Bramley PM. (1994) Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation in vivo and in vitro, and functional analysis of the various truncated gene products. J Biochem 116: 980–985.

Misawa N, Kajiwara S, Kondo K, Yokoyama A, Satomi Y, Saito T, Miki W, Ohtani T. (1995) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarobon β–carotene by a single gene. Biochem Biophys Res 209: 867–876.

Ralley L, Enfissi EM, Misawa N, Schuch W. (2004) Metabolic engineering of ketocarotenoid formation in higher plants. Plant J 39: 477–486.

Molnar P, Szabolcs J. (1980) β-Citraurin epoxide, a new carotenoid from valencia orange peel. Phytochemistry 19: 633–637.

Naito Y, Uchiyama K, Aoi W, Hasegawa G, Nakamura N, Yoshida N, Maoka T, Takahashi J, Yoshikawa T. (2004) Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. Biofactors 20: 49–59.

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P. (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106: 7762–7767.

Nassar NMA, Fernandes PC, Melani RD, Pires ORJr. (2009) Amarelinha do Amapa: a carotenoid-rich cassava cultivar. Genet Mol Res 8: 1051–1055.

Nesi N, Delourme R, Bregeon M, Falentin C, Renard M. (2008) Genetic and molecular approaches to improve nutritional value of *Brassica napus* L. seed. C R Biol 331: 763–771.

Olson JA. (1994) Needs and sources of carotenoids and vitamin A. Nutr Rev 52: S67–S73.

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

Paolillo DJ, Garvin DFJr, Parthasarathy MV. (2004) The chromoplasts of *Or* mutants of cauliflower (*Brassica oleracea* Lvar*botrytis*). Protoplasma 224: 245–253.

Parinov S, Sundaresan V. (2000) Functional genomics in *Arabidopsis*: large-scale insertional mutagenesis complements the genome sequencing project. Curr Opin Biotechnol 11: 157–161.

Parkhi V, Rai M, Tan J, Oliva N, Rehana S, Bandyopadhyay A, Torrizo L, Ghole V, Datta K, Datta SK. (2005) Molecular characterization of marker-free transgenic lines of indica rice that accumulate carotenoids in seed endosperm. Mol Genet Genomics 274: 325–336.

Pozniak CJ, Knox RE, Clarke FR, Clarke JM. (2007) Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. Theor Appl Genet 114: 525–537.

Ravanello MP, Ke D, Alvarez J, Huang B, Shewmaker CK. (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. Metab Eng 5: 255–263.

Rodriguez-Concepcion M. (2006) Early steps in isoprenoid biosynthesis: Multilevel regulation of the supply of common precursors in plant cells. Phytochem Rev 5: 1–15.

Rodriguez-Concepcion M, Boronat A. (2002) Elucidation of the methylerthritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids A metabolic milestone achieved through genomics. Plant Physiol 130: 1079–1089.

Romer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM. (2000) Elevation of the provitamin A content of transgenic tomato plants. Nat Biotechnol 18: 666–669.

Romer S, Lubeck J, Kauder F, Steiger S, Adomat C, Sandmann G. (2002) Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metab Eng 4: 263–272.

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G. (2000) Metabolic engineering of β-carotene and lycopene content in tomato fruit. Plant J 24: 413–419.

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

Schaub P, Al-Babili S, Drake R, Beyer P. (2005) Why is golden rice golden (yellow) instead of red? Plant Physiol 138: 441–450.

Seo M, Koshiba T. (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7: 41–48.

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

Simikin AJ, Gaffe J, Alcaraz JP, Carde JP, Bramley PM, Fraser PD, Kuntz M. (2007) Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. Photochemistry 68: 1545–1556.

Slade AJ, Knauf VC. (2005) TILLING moves beyond functional genomics into crop improvement. Transgenic Res 14: 109–115.

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

Suzuki S, Nishihara M, Nakatsuka T, Misawa N, Ogiwara I, Yamamura S. (2007) Flower color alteration in Lotus japonicas by modification of the carotenoid biosynthetic pathway. Plant Cell Rep 26:951–959.

Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, Mori H. (1994) Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15: 15–19.

Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D. (2003) Functional analysis of β- and epsilon-ring carotenoid hydroxylases in *Arabidopsis*. Plant Cell 5: 1320–1332.

UNICEF Vitamin A deficiency (2006) *http://wwwchildinfoorg/areas/vitamina/*

Underwood BA. (2003) Scientific research: essential, but is it enough to combat world food insecurities? J Nutr 133: 1434S–1437S.

Van Eck J, Conlin B, Garvin DF, Mason H, Navarre DA, Brown CR. (2007) Enhancing β-carotene content in potato by RNAi-mediated silencing of the β-carotene hydroxylase gene. Am J Potato Res 84: 331–342.

Wei S, Yu B, Gruber MY, Khachatourians GG, Hegedus DD, Hannoufa A. (2010) Enhanced seed carotenoid levels and branching in transgenic *Brassica napus* expressing the *Arabidopsis* miR156b Gene. J Agric Food Chem 58: 9572–9578.

Welsch R, Arango J, Bar C, Salazar B, Al-Babili S, Beltran J, Chavarriaga P, Ceballos H, Tohme J, Beyer P. (2010) Provitamin A accumulation in cassava (*Manihot esculenta*) roots driven by a single nucleotide polymorphism in a *phytoene synthase* gene. Plant Cell 22: 3348–3356.

Wurbs D, Ruf S, Bock R. (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. Plant J 49: 276–288.

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287: 303–305.

Yu B, Lydiate DJ, Young LW, Schafer UA, Hannoufa A. (2007) Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase. Transgenic Res 17: 573–585.

Zamir D. (2001) Improving plant breeding with exotic genetic libraries. Nat Rev Genet 2: 983–989.

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

Zhong YJ, Huang JC, Liu J, Li Y, Jiang Y, Xu ZF, Sandmann G, Chen F. (2011) Metabolic engineering of tomato for high-yield production of astaxanthin. J Exp Bot 62: 3659–3669.

Zhu C, Bai C, Sanahuja G, Yuan D, Farre G, Naqvi S, Shi L, Capell T, Christou P. (2010) The regulation of carotenoid pigmentation in flowers. Arch Biochem Biophys 504: 132–141.

Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci USA 105: 18232–18237.

Zhu C, Naqvi S, Capell T, Christou P. (2009) Metabolic engineering of ketocarotenoid biosynthesis in higher plants. ABB 483: 182–190.

Zhu C, Naqvi S, Gomez-Galera S, Pelacho AM, Capell T, Christou P. (2007) Transgenic strategies for the nutritional enhancement of plants. Trends Plant Sci 12: 548–555.

AIMS & OBJECTIVES

My research project focused on investigations to unravel the mechanism of carotenoid accumulation in rice endosperm and also to develop a facile system to functionally characterize genes involved in the carotenoid pathway with a view to validate such candidate genes for further use in metabolic engineering applications. I set out to provide a starting point for quantitative modeling and the rational design of engineering strategies using synthetic biology. A further aim was to elucidate as yet unknown bottlenecks in the ketocarotenoid pathway leading to the production of the commercially valuable antioxidant astaxanthin in rice endosperm.

Chapter 1

An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation

The Plant Journal (2014), 77: 464-475

Chapter 1 is formatted according to The Plant Journal

Chapter1

An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation

SUMMARY

We have developed an assay based on rice embryogenic callus for the rapid functional characterization of metabolic genes. We validated the assay using a selection of well-characterized genes with known functions in the carotenoid biosynthesis pathway, allowing the rapid visual screening of callus phenotypes based on tissue color. We were then able to use the system to identify the functions of two uncharacterized genes: a chemically-synthesized β-carotene ketolase gene optimized for maize codon usage; and a wild-type *Arabidopsis thaliana* ortholog of the cauliflower *Orange* gene. In contrast to previous reports, we found that the wild-type *Orange* allele was sufficient to induce chromoplast differentiation. We also found that chromoplast differentiation could be induced by increasing the availability of precursors and thus driving flux through the pathway, even in the absence of *Orange*. Remarkably, we found that diverse endosperm-specific promoters were highly active in rice callus despite their restricted activity in mature plants. Our callus system provides a unique opportunity to predict the impact of metabolic engineering in complex pathways and provides a starting point for quantitative modeling and the rational design of engineering strategies using synthetic biology. We discuss the impact of our data on the analysis and engineering of the carotenoid biosynthesis pathway.

INTRODUCTION

The advent of large-scale DNA and RNA sequencing has provided unprecedented insights into the information content of plant genomes and transcriptomes (Ozsolak and Milos, 2011). However, the functions of many of the uncharacterized sequences remain unknown because annotations based on homology searching are only approximate and the slow pace of conventional functional characterization experiments has created a major information bottleneck (Zhu and Zhao, 2007).

Several large-scale direct functional annotation approaches have been developed but these typically focus on single genes. For example, plant genes can be characterized rapidly by large-scale insertional mutagenesis using either transposons or T-DNA insertions (Myouga *et al.*, 2009), enhanced breeding approaches such as TILLING (Kurowska *et al.*, 2011), silencing approaches such as virus-induced gene silencing (VIGS) (Purkayastha *et al.*, 2009) and RNA interference (RNAi) (Purkayastha *et al.*, 2009), expression profiling using microarrays (Liu *et al.*, 2008), census sequencing approaches (RNA-seq) (Ozsolak and Milos, 2011) or quantitative proteomics (Nikolov *et al.*, 2012), or by the analysis of protein interactions using platforms such as the yeast two-hybrid systems and its derivatives (Snyder and Gallagher, 2009). These approaches provide empirical functional data but the methods are indirect, i.e. function is inferred through expression profiles, loss-of-function phenotypes and associations with other gene products rather than direct biochemical analysis.

The drawbacks of *in silico* and empirical functional annotation have created significant challenges in the characterization and engineering of plant metabolic pathways (Capell and Christou, 2004; Dafny-Yelin and Tzfira, 2007). Uncharacterized plant genes can be

annotated on the basis of homology but the assignments are often vague, e.g. a new sequence may be assigned as a cytochrome P450 monooxygenase, a glycosyltransferase or a methyltransferase, but this provides only a basic catalytic function without much information about substrate or product specificity. Similarly, a gene knockout or gene silencing experiment will show the overall impact of loss-of-function on plant metabolism and physiology, but there is no deeper insight into its role. Yeast two-hybrid screens may show potential interaction partners, but only if physical interactions occur. None of these methods, or any combination of them, can completely join the dots and show the precise metabolic role of uncharacterized sequences, and how they fit into the surrounding context of known metabolic pathways.

We had reported previously a combinatorial gene transfer system that allows the rapid analysis of metabolic genes in random combinations to provide insight into the roles of such genes, how they interact in the overall metabolic pathway, and the most optimum strategy to achieve the synthesis of particular metabolic compounds (Zhu *et al.*, 2008; Farre *et al.*, 2013). This platform was established in maize endosperm, which is most useful for assembling metabolic pathways that are relevant in seeds, such as those leading to the production of essential nutrients (Zhu *et al.*, 2008; Ramessar *et al.*, 2008). A more general platform for high-throughput functional analysis would require a less specific physiological structure and also a shorter timescale than that provided by maize endosperm. Previous studies have shown that callus cultures in species such as maize, *A. thaliana*, sweet potato, marigold and banana can be used to test gene function (Paine *et al.*, 2005; Harada *et al.*, 2009; Kim *et al.*, 2011; Vanegas-Espinoza *et al.*, 2011; Mlalazi *et al.*, 2012; Kim *et al.*, 2013a,b). However these studies have been limited to single genes driven by a constitutive

promoter because complex and time consuming strategies are required to construct cassettes carrying multiple transgenes. This makes them unsuitable for the analysis of multiple gene interactions in complex metabolic pathways.

We hypothesized that our combinatorial transformation platform could be used to investigate multiple gene functions in rice callus tissue, which can be prepared, transformed and screened much more rapidly than transgenic maize plants. Gene expression and protein accumulation in rice callus involves the same mechanisms that occur in the mature plant. Therefore, rice callus derived from zygotic embryos could be used as a surrogate platform for the rapid analysis and evaluation of multiple candidate transgenes in a metabolic pathway to predict their behavior in whole plants.

The approach was tested using well-characterized genes from the carotenoid biosynthesis pathway (**Figure 1 and Figure S1**) (Zhu *et al.*, 2009; Ruiz-Sola and Rodriguez-Concepcion, 2012; Zhu *et al.*, 2013). Having confirmed that rice callus is suitable for the functional analysis of known genes we then investigated the functions of a chemically-synthesized uncharacterized β-carotene ketolase and *Orange* ortholog, which could only be established by the simultaneous expression of other genes from the carotenoid biosynthesis pathway in rice. The callus assay provides a rapid and inexpensive platform for the functional characterization of uncharacterized genes by combinatorial expression, and can be combined with synthetic biology approaches for the analysis of complex metabolic pathways and the prediction of model-driven metabolic engineering strategies based on multigene expression (Zurbriggen *et al.*, 2012).

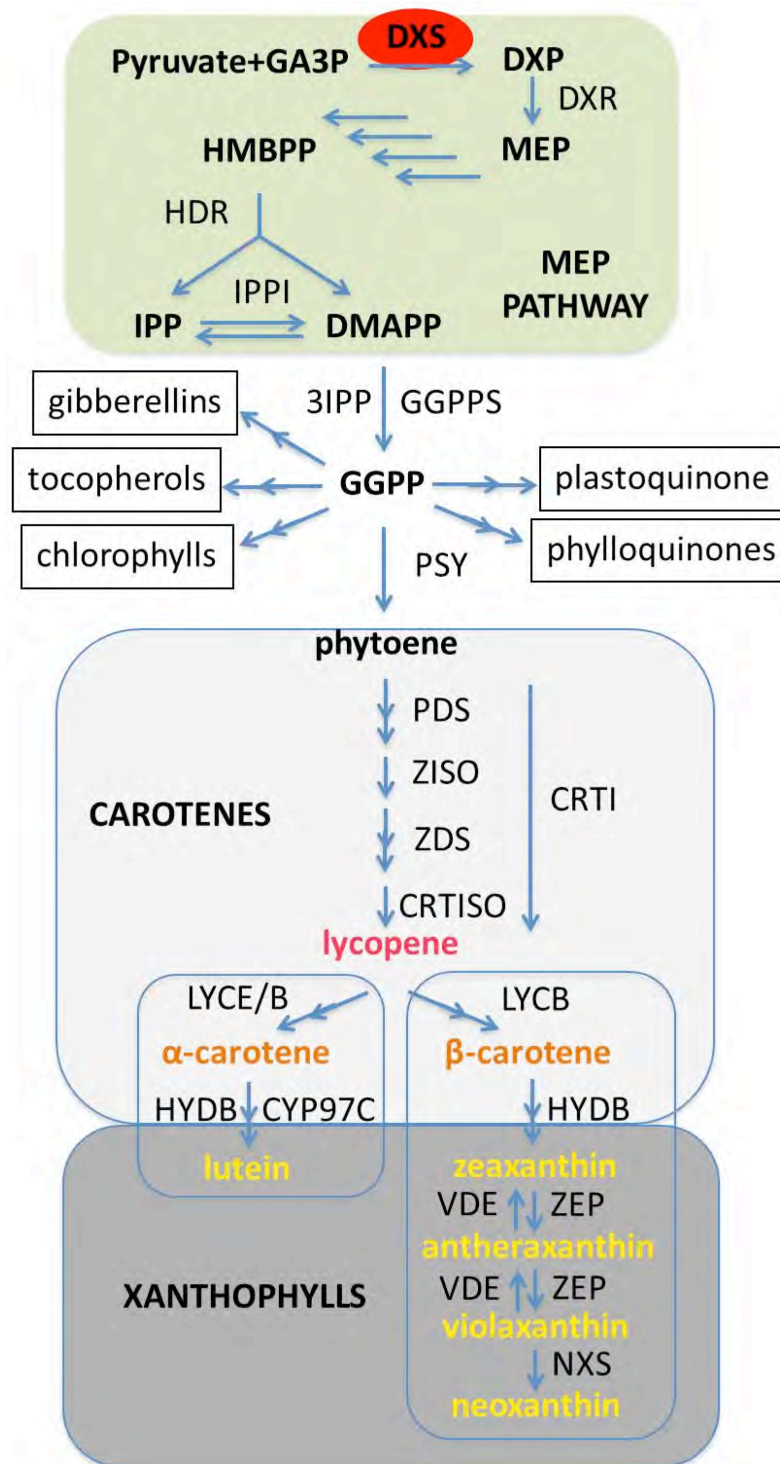


Figure 1 The carotenoid biosynthesis pathway (Zhu *et al.*, 2009; Farre *et al.*, 2010, 2011; Bai *et al.*, 2011; Ruiz-Sola and Rodriguez-Concepcion, 2012; Zhu *et al.*, 2013).

All carotenoids are synthesized from the five-carbon monomeric building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Plastidial IPP is generated via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MEP pathway begins with glyceraldehyde-3-phosphate (GA-3-P) and pyruvate. They are converted into 1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS). DXP reductoisomerase (DXR) converts DXP to MEP in the second step of the MEP pathway. MEP is then converted to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) in four sequential reactions. Hydroxymethylbutenyl diphoshate reductase (HDR) simultaneously synthesizes IPP and DMAPP from HMBPP in the last step of the MEP pathway. Three IPP molecules are added to DMAPP to produce geranylgeranyl diphosphate (GGPP), which serves as the immediate precursor not only for carotenoids but also for the biosynthesis of gibberellins and the side chain of chlorophylls, tocopherols, phylloquinones and plastoquinone. The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP by phytoene synthase (PSY) to produce phytoene. Phytoene is converted into all-*trans* lycopene by the action of two desaturases and two isomerases: phytoene desaturase (PDS), ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). The bacterial phytoene desaturase (CRTI) can apparently perform all the desaturation and isomerization reactions carried out by plant PDS, ZISO, ZDS and CRTISO. As CRTI has low homology with plant PDS and ZDS, and reduces the number of transgenes it is widely used in metabolic engineering. Lycopene represents a branch in the pathway, leading to either the α- or β-carotenes. In the α-carotene branch, the addition of one ε-ring and one β-ring to lycopene produces α-carotene by lycopene ε-cyclase (LYCE) and lycopene β-cyclase (LYCB), respectively. In turn, in the β-carotene branch, lycopene is cyclized to produce the provitamin A carotenoids γ-carotene and then β-carotene when LYCB adds β-rings to both ends of the linear lycopene molecule. β- and α-carotene are redundantly hydroxylated by non-heme di-iron β-carotene hydroxylases (BCH1 and BCH2) and cytochrome P450-type β- and ε-hydroxylases (CYP97A, CYP97B and CYP97C). β-xanthophylls are epoxidated, de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), giving rise to the xanthophylls cycle.

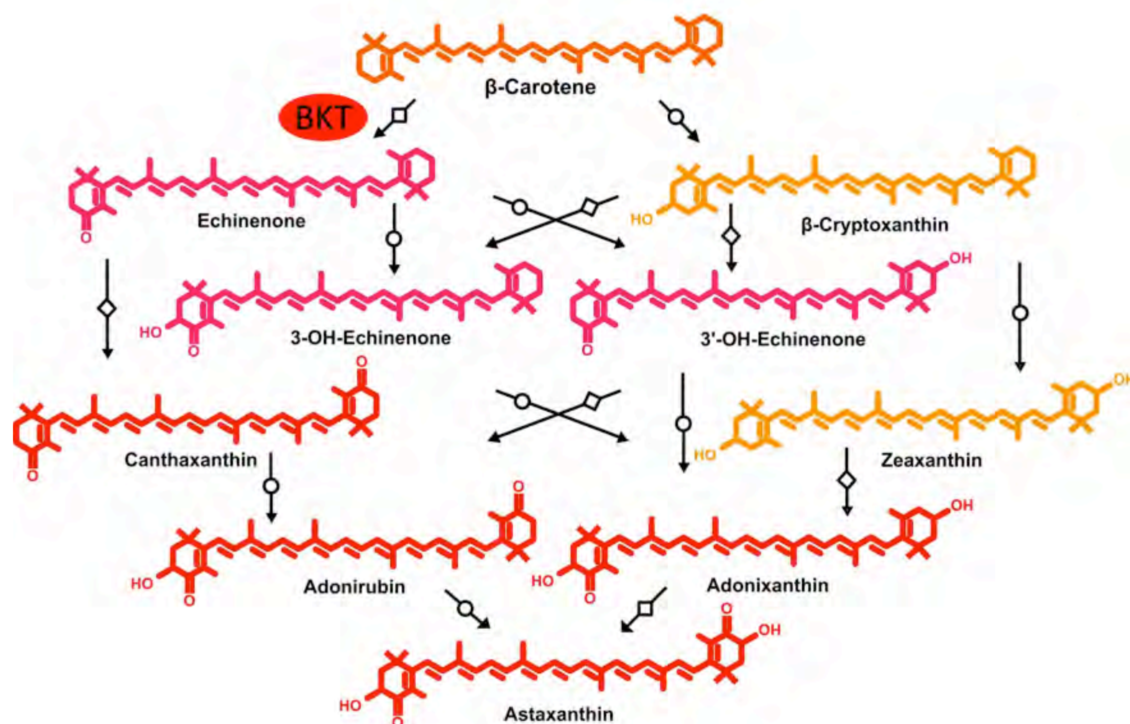


Figure S1 Astaxanthin biosynthesis pathway (Zhu *et al*., 2009, 2013)

Astaxanthin biosynthesis pathway in astaxanthin-producing bacteria and transgenic plants, and the catalytic function of β-carotene hydroxylase (arrow with circle) and β-carotene ketolase (arrow with square).

RESULTS

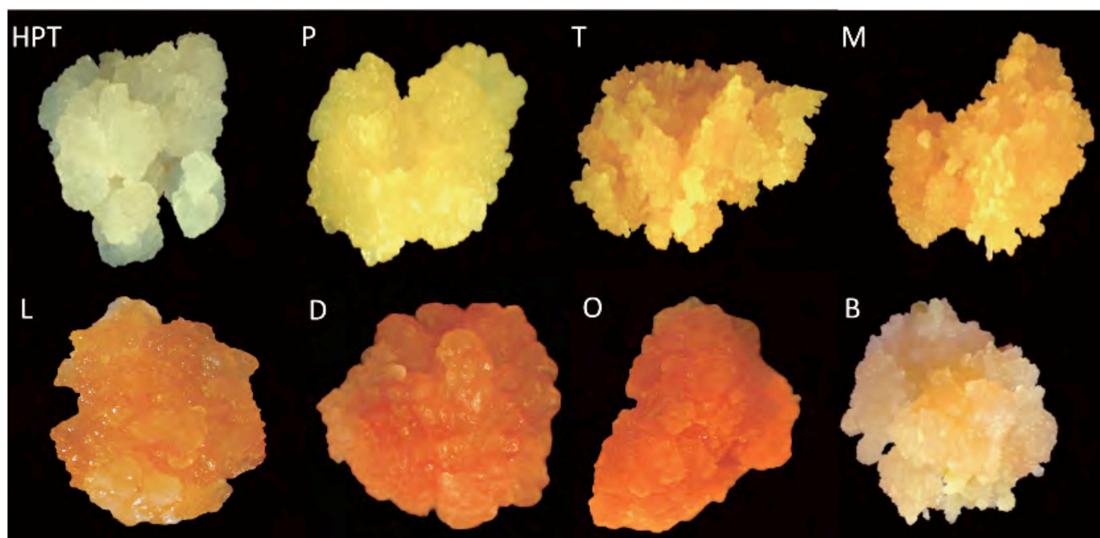
Combinatorial transformation of rice embryos with endosperm-specific carotenogenic transgenes generates rice callus lines that accumulate carotenoids

Embryo-derived rice callus is white in color and accumulates only minimal levels of carotenoids (**Figures 2a and 3**). Our analysis revealed the presence of small amounts of lutein and zeaxanthin in wild-type callus (**Figure 3**), which is similar to the carotenoid

profile of white maize endosperm (Zhu *et al.*, 2008). This indicated that rice callus could likewise be used as a platform to test the combinatorial activity of multiple carotenogenic transgenes although within a much shorter experimental timescale.

In pilot experiments to establish the platform, we transformed seven-day-old mature zygotic rice embryos with four constructs containing unlinked transgenes. These comprised the selectable marker *HPT* for hygromycin resistance, two carotenogenic transgenes with known functions in the committed carotenoid biosynthesis pathway, namely maize (*Zea mays*) phytoene synthase 1 (*ZmPSY1*) and *Pantoea ananatis* phyoene desaturase (*PaCRTI*), and the *A. thaliana* 1-deoxy-D-xylulose 5-phosphate synthase (*AtDXS*) gene, a limiting enzyme of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway which supplies carotenoid precursors. The *HPT* gene was expressed constitutively and the three carotenogenic genes were driven by endosperm-specific promoters.

a.



b.

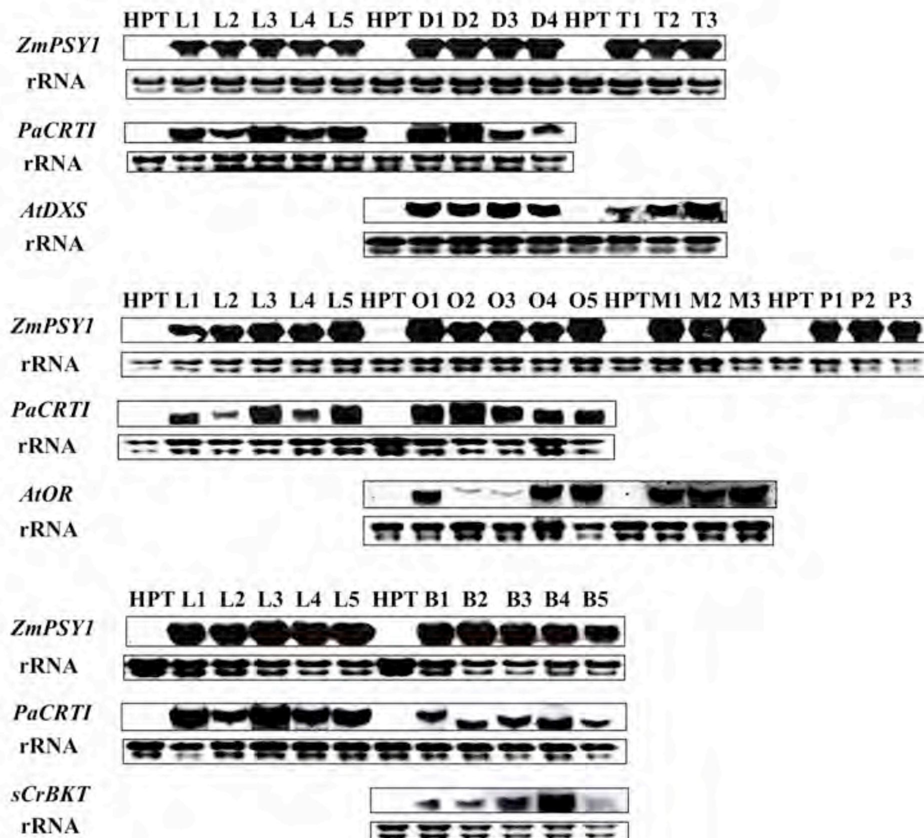


Figure 2 Phenotypes and genotypes of seven rice callus combinatorial transformants

(a) Phenotypes of seven transgene combinations expressed in rice callus. HPT, callus expressing *HPT* (white color); P, *ZmPSY1* alone results in a pale yellow color; *ZmPSY1* and *AtDXS* in T, *ZmPSY1* and *AtOR* in M, and *ZmPSY1*and *PaCRTI* in L, result in a similar yellow color; *AtDXS* in addition to *ZmPSY1* and *PaCRTI* in D, or in addition to *ZmPSY1, PaCRTI* and *AtOR* in O, result in a similar orange color; B, *ZmPSY1, PaCRTI* and *sCrBKT* generate colors ranging from pink to red depending on the accumulation of ketocarotenoids. (b) Analysis of mRNA transgenic rice callus expressing *AtOR/AtDXS*, *ZmPSY1* and/or *PaCRTI*; *ZmPSY1, PaCRTI* and *sCrBKT* (25 µg of total rRNA was loaded for each sample). Abbreviations: HPT, callus expressing *HPT*; transgenic callus expressing *ZmPSY1* and *PaCRTI* in L1-L5; expressing *AtDXS*, *ZmPSY1* and *PaCRTI* in D1-D4; expressing *ZmPSY1* and *AtDXS* in T1-T3; expressing *ZmPSY1, PaCRTI* and *AtOR* in O1-O5; expressing

ZmPSY1 and *AtOR* in M1-M3; expressing *ZmPSY1* alone in P1-P3; expressing *ZmPSY1, PaCRTI* and *sCrBKT* in B1-B5.

Remarkably, we observed during selection that the transgenic rice callus ranged in color from white through various shades of yellow to orange, representing the expression of different combinations of the three carotenogenic transgenes and thus different carotenoid profiles (**Figure 2a**). The analysis of steady-state mRNA levels showed that *ZmPSY1* was expressed in all the yellow and orange callus lines but not in the white callus (even if the other carotenogenic transgenes were expressed) confirming *ZmPSY1* is essential for carotenoid accumulation. Faint *ZmPSY1* hybridization bands in the white callus samples represented the endogenous *OsPSY1* gene, which is expressed at minimal levels in rice callus. The appearance of color in the callus tissue confirmed that the ‘endosperm-specific’ wheat low-molecular-wheat glutenin, barley D-hordein and rice prolamin promoters were each also active in dedifferentiated rice tissue, as previously reported for the maize 27-kDa γ-zein promoter (Wu and Messing, 2009).

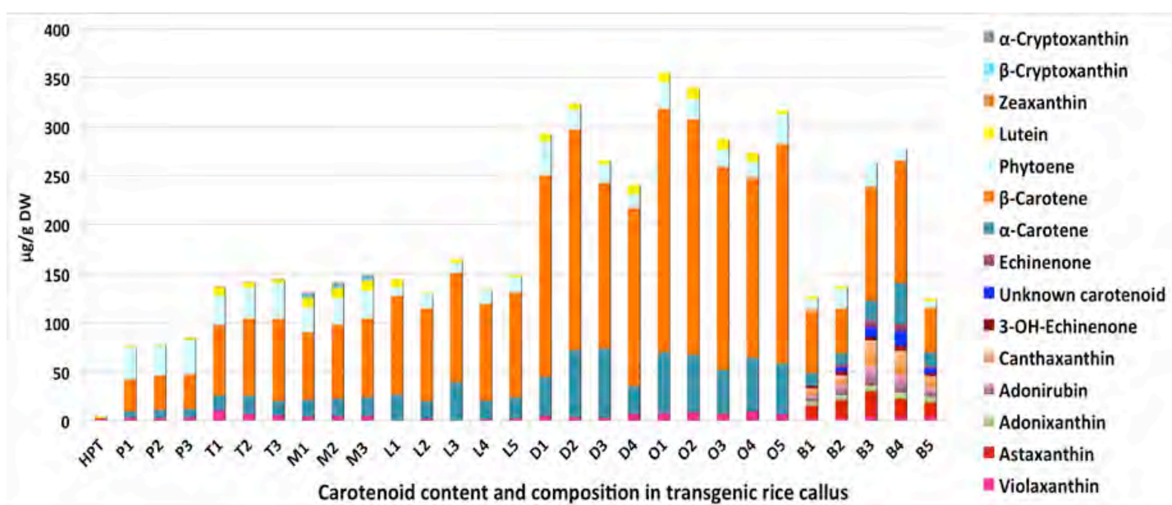


Figure 3 Carotenoid content and composition in transgenic rice callus

Carotenoid content and composition were analyzed by UHPLC. Different colored squares represent different carotenoid compositions as indicated in the key. All carotenoid content data were averaged on three independent measurements. Column names are defined in the legend to Figure 2.

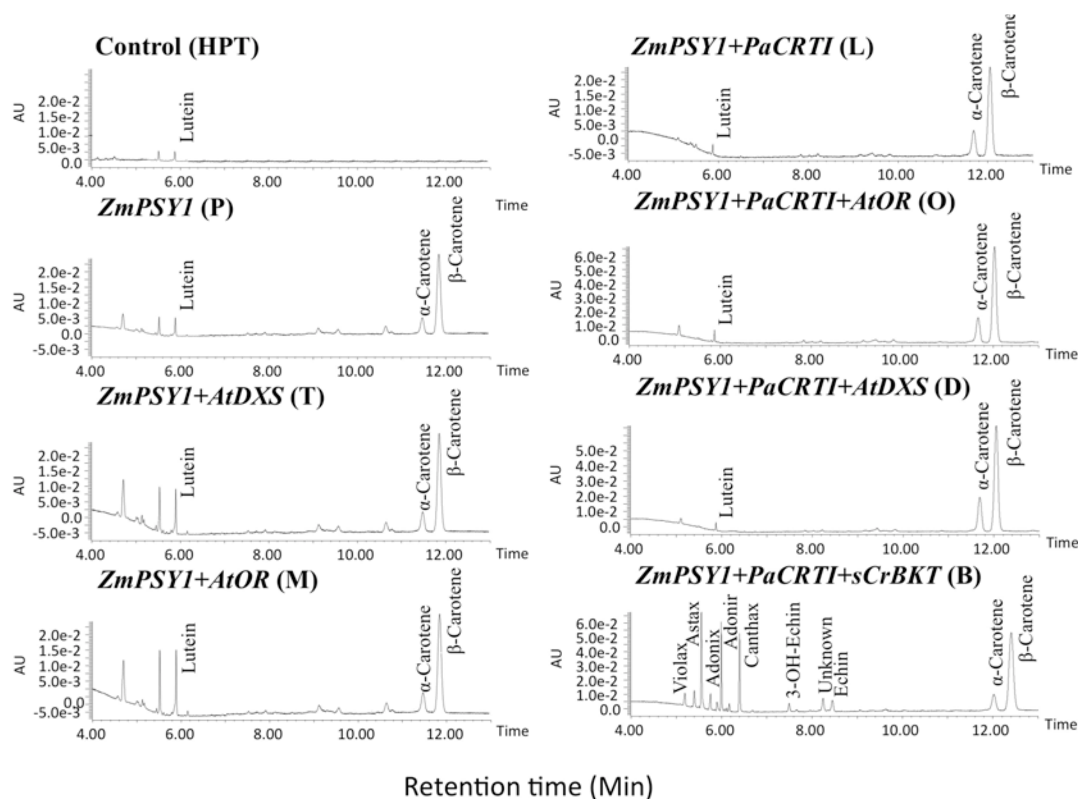


Figure S2 Carotenoid profiles in transgenic rice callus

The predominant carotenoid in each transgenic callus was β-carotene. Gene expression indicated in each transgenic line. Abbreviations: Violax, violaxanthin; Astax, Astaxanthin; Adonix, Adonixanthin; Adonir, Adonirubin; Canthax, Canthaxanthin; 3-OH-Echin; 3-OH-Echininone; Echin, Echininone.

We found a precise correlation between the phenotypes and expressed transgenes at the mRNA level (**Figures 2a and 2b**). Rice callus expressing *ZmPSY1* alone was pale yellow in color, whereas callus expressing both *ZmPSY1* and *AtDXS* was darker yellow and callus

expressing both *ZmPSY1* and *PaCRTI* was yellow-orange. Callus expressing all three carotenogenic transgenes was a darker orange color. Therefore, we were able to make accurate predictions of the transgenes expressed in each callus line by simple visual screening, allowing the straightforward selection of callus pieces for further experiments or regeneration into transgenic plants.

Metabolic profiling and mathematical modeling of transgenic callus lines reveals a correlation between transgene expression and carotenoid accumulation

The analysis of carotenoid content and composition by ultra-high-performance liquid chromatography (UHPLC) showed that the different color phenotypes reflected different metabolic profiles, confirming a direct correlation between the genotype and metabolic phenotype of the callus lines. The white callus did not express *ZmPSY1* and contained similar levels of total carotenoids regardless of the additional transgene complement, i.e. control lines expressing *HPT* alone accumulated the same level of carotenoids as callus also expressing *PaCRTI,* or *AtDXS* or both genes simultaneously. The yellow callus expressing *ZmPSY1* alone accumulated β-carotene and phytoene in similar amounts, as well as smaller amounts of α-carotene, lutein, violaxanthin and zeaxanthin (**Figure 3 and Figure S2 and Table S1**). The darker yellow callus expressing *ZmPSY1* and *AtDXS* accumulated 1.8-fold more total carotenoids than callus expressing *ZmPSY1* alone, most of which was represented by β-carotene, whereas the levels of phytoene and the minor carotenoids hardly changed. The yellow-orange callus expressing *ZmPSY1* and *PaCRTI* accumulated twice as much in total carotenoids than callus expressing *ZmPSY1* alone, but although most of this was again represented by β-carotene, the next most abundant carotenoid was α-carotene,

which was three times more abundant than phytoene. Finally the orange callus expressing all three transgenes accumulated 3.5-fold more total carotenoids than callus expressing *ZmPSY1* alone, again dominated by β-carotene, α-carotene and phytoene in similar proportions to the *ZmPSY1-PaCRTI* callus, but also with significant amounts of lutein (**Figure 3 and Figure S2 and Table S1**). The presence of *AtDXS* in addition to *ZmPSY1* and *PaCRTI* therefore increased the carotenoid content 1.9-fold suggesting that *A. thaliana DXS* gene boosts carotenoid biosynthesis by alleviating the limited supply of precursors into the pathway.

Table S1a Carotenoid content and composition in transgenic rice callus expressing *ZmPSY1*, and/or *PaCRTI* and *AtDXS/AtOR* (µg/g DW)

Name	Violaxanthin	β-Cryptoxanthin	a-Cryptoxanthin	α-Carotene	β-Carotene	Phytoene	Lutein	Zeaxanthin	Total carotenoids	β/ε ratio
HPT	2.26±0.60						2.1±0.2	0.3±0.1	4.66±0.9	1.22
P1	3.14±0.65			6.19±1.51	32.54±7.63	32.11±4.94	1.3±0.04	0.44±0.17	75.72±15.04	4.82
P2	2.59±0.44			7.96±1.14	35.53±3.76	30.97±4.92	0.89±0.06	0.37±0.15	78.31±10.48	4.35
P3	3.2±0.6			8.2±1.45	35.83±8.82	35.42±12.82	1.8±0.2	0.4±0.1	84.85±4.3	3.94
T1	10.2±0			15.5±2.23	72.01±4.1	29.8±8.68	8.4±0.2	0.9±0.1	136.81±22.42	3.48
T2	6.6±1.21			19.01±4.69	78.59±17.44	31.89±6.78	4.64±0.36	0.68±0.29	141.41±30.13	3.63
T3	5.43±2.18			14.15±2.3	84.21±10.48	37.02±5.41	3.3±0.33	0.72±0.23	144.83±19.59	5.18
M1	4 ± 0.9	2.6 ±0	2.6 ±0.2	17.3±2.4	69.3±14.8	24.98±4.17	9.7 ± 3.3	0.7 ± 1.1	131.18±26.85	2.59
M2	4.1 ± 0.6	2.6 ±0.1	2.6 ±0.1	18.3±1.5	75.6±4.4	26.95±3.15	10.3 ± 1.6	0.7± 1.2	141.15±10.11	2.66
M3	4.6 ± 0.5	2.6 ±0	2.6 ±0	18.9±1.8	80.6±8.4	28.49±1.92	10.1 ± 0.2	1.0 ± 1.4	144.83±11.44	2.81
L1				26.27±1.34	101.19±9.29	8.32±0.00	9.05		144.83±4.34	2.86
L2	2.1±2.29			17.53±1.35	95.2±6.65	15.21±2.22	0.94±0.09		130.98±10.17	5.27
L3				39.03±1.37	112.12±25.41	9.34±2.70	4±0.4		164.49±26.75	2.87
L4	2.02±0.15			18.14±2.69	98.91±11.01	14.13±1.95	0.89±0.11		134.09±15.6	5.30
L5	2.4±0.11			21.03±0.55	108.03±2.31	15.08±0.92	2.16±1.36		148.7±4.85	4.76
D1	4.78±0.93			39.55±6.84	205.53±45.41	34.58±6.52	7.99±0.98		292.43±60.02	4.42
D2	3.27±0.33			68.46±2.86	225.33±13.55	19.98±2.03	6.35±0.27		323.39±15.02	3.06
D3	2.56±0.34			71.25±7.26	168.57±26.30	19.07±2.21	3.97±0.33		265.41±34.48	2.28
D4	6.86±0.04			28.35±0.47	181.57±16.21	13.76±2.03	9.35±0.03		239.88±17.76	5.00
O1	7.28±1.65			62.80±12.84	248.11±46.42	27.14±6.84	9.99±2.87		355.31±70.62	3.51
O2	8.33±0.28			59.16±4.66	240.05±21.82	20.23±0.38	12.03±0.80		339.81±28.20	3.49
O3	7.10±0.60			44.63±6.98	207.13±40.75	17.76±5.70	10.59±1.19		287.22±55.22	3.88
O4	9.32±0.13			54.56±3.17	184.14±16.67	15.72±0.72	9.19±0.04		272.94±20.72	3.03
O5	6.55±1.21			51.85±8.96	223.82±38.07	30.38±9.86	3.97±0.72		316.57±59.13	4.13

Table S1b Carotenoid content and composition in transgenic rice callus expressing *ZmPSY1, PaCRTI* and *sCrBKT* (μg/g DW)

Name	Violaxanthin	Astaxanthin	Adonixanthin	Adonirubin	Canthaxanthin	3-OH-Echinenone	Unknown carotenoid	Echinenone	α-Carotene	β-Carotene	Phytoene	Lutein	Total carotenoids	% Ketocarotenoids	β/ε ratio
B1	3.12±0.43	11.95±1.34	4.09±0.41	6.85±0.98	6.57±0.71	2.56±0.29			13.72±1.33	64.36±4.52	10.59±1.67	3.44±0.05	127.25±11.28	25.17	5.80
B2	2.42±0.48	18.23±2.34	5.26±0.65	11.46±1.66	8.70±1.14	4.07±0.83	4.23±0.24	2.88±1.00	11.55±0.84	45.55±5.47	20.08±3.88	2.89±0.03	137.32±18.23	39.93	7.12
B3	3.69±0.46	25.83±2.60	5.97±0.20	20.81±0.41	25.42±1.30	4.07±0.30	9.55±0.43	7.26±0.43	19.93±1.03	115.72±14.34	24.73±0.24		262.98±18.42	37.61	10.95
B4	3.32±0.04	18.54±1.28	6.73±0.22	18.61±0.82	24.06±1.77	5.33±0.27	14.95±2.65	7.52±1.23	41.58±0.47	124.64±3.81	12.31±1.58		277.58±0.62	34.49	5.38
B5	3.60±0.29	14.61±0.91	5.90±0.07	10.22±0.33	10.84±0.61	2.73±0.09	5.29±0.30	2.61±0.84	13.77±1.01	45.68±3.89	6.40±0.57	3.20±0.19	124.83±6.95	41.48	5.98

Table S1 Carotenoid content and composition in transgenic rice callus

Carotenoid content and composition were analyzed by UHPLC. Line names are defined in the legend to Figure 2.

Although the transgene expression levels correlated with carotenoid accumulation (**Figures 2a and 2b**), this required a basic assumption that the expression of each gene remained approximately constant among the different lines. In support of this assumption, the quantitation of mRNA in the northern blots using ImageJ software (Schneider *et al.*, 2012) suggested that, for each transgene, the amount of expressed mRNA remained approximately constant in each transformed line (**Figure 2b and Table S2**). If we also assume a constant rate of protein synthesis, the abundance of each protein should therefore be constant across the different callus lines too, and the metabolite concentrations should therefore allow us to model the behavior of carotenoid biosynthesis. We validated these assumptions experimentally as follows:

Table S2 Relative amounts of mRNA expressed in the transformed rice callus lines

Protein	HPT	L	D	T	O	M	P	B
PSY1	15.00	179.19	190.63	192.10	219.75	228.49	195.48	170.30
CRTI	5.18	190.66	185.88		195.04			155.79
DXS	5.04		145.33	161.06				
OR	22.26				103.30	204.14		
BKT	31.21							132.82

These amounts were determined using ImageJ (Schneider *et al*., 2012) to analyze the bands on the northern blots shown in Figure 2b. Numbers indicate average intensity per pixel in the analyzed area*. Columns correspond to the transformed callus lines. This analysis was carried out to estimate whether similar amounts of mRNA per cell representing each transgene were produced in the different transgenic lines.

First, phytoene production appears to limit the production of downstream carotenoids. This can be inferred because transgenic callus expressing *ZmPSY1* increases the abundance of carotenoids by 17-fold. Second, the quantity of phytoene precursors produced in the callus appears to limit the production of downstream carotenoids, even in the presence of ZmPSY1. This can be inferred because the *ZmPSY1-AtDXS* lines accumulate almost twice the level of total carotenoids present in the *ZmPSY1* lines. Third, endogenous carotene desaturase activity in rice appears to account for a significant proportion of carotenoid production from phytoene. This can be inferred because both the *ZmPSY1* and *ZmPSY1-AtDXS* lines produce 20-30-fold more carotenoids than the control callus. However, this native activity can limit carotenoid production, because introducing the

exogenous *CRTI* boosts carotene production and reduces the amount of phytoene compared to lines that do not express *CRTI*.

In order to validate these hypotheses, we created simplified mathematical models of the callus system and investigated whether the dynamic behavior of the models was consistent with the experimental data (**Table S3**). Figure S3 shows the simplified pathway that was modeled. The modeling process is described in methods and in the text (**Method 1**). Table S4 summarizes the parameter values for the models. We found that the models quantitatively reproduced what was observed in the transgenic lines.

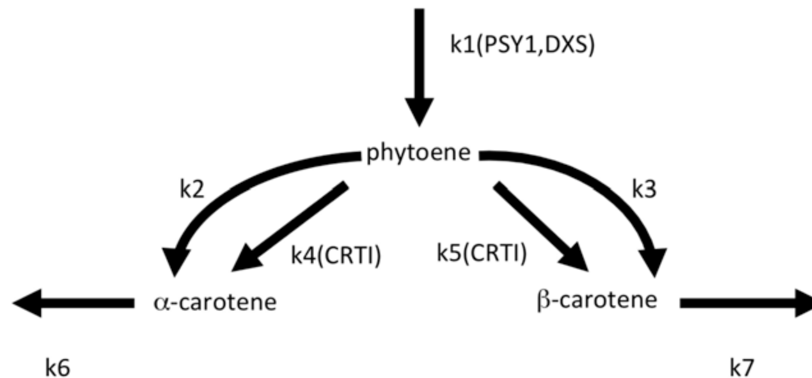


Figure S3 Simplified representation of carotene biosynthesis in transgenic rice callus lines

The flux of phytoene production depends on both PSY1 and DXS. Also, to account for the possibility that CRTI introduces another flux channel for carotene production from phytoene, we introduce the processes represented by k4 (CRTI) and k5 (CRTI).

The first hypothesis cannot be tested by the modeling process, as it represents a founding assumption for the modeling process itself. Analysis of the model parameters revealed that introducing *AtDXS* generates approximately twice the amount of flux per unit time to produce phytoene than callus lines lacking this transgene. This is consistent with the second

hypothesis. In addition, the production of carotenoids in lines that lack *CRTI* can be explained by endogenous flux channels. This activity accounts for a significant amount of α-carotene and β-carotene production even in lines expressing *CRTI*, and is consistent with the third hypothesis. A more detailed analysis can be found in the material (**Method 1**).

Table S3 Quantification of metabolites in the various transgenic rice callus lines

Carotenoids	Carotenoid content (μg/g DW)				
	HPT	PSY1	PSY1-DXS	PSY1-CRTI	PSY1-CRTI-DXS
Phytoene	0	35.4±12.8	37±5.4	9.3±2.7	20±2
Violaxanthin	2.3±0.6	3.2±0.6	5.4±2.18	0	3.3±0.3
Zeaxanthin	0.3±0.1	0.4±0.1	0.7±0.2	0	0
α-Carotene	0	8.2±1.5	14.2±2.3	39±1.4	68.5±2.9
β-Carotene	0	35.8±8.8	84.2±10.48	112.1±25.4	225.3±13.6
Lutein	2.1±0.2	1.8±0.2	3.3±0.3	4±0.4	6.3±0.3
Total	4.7±0.9	84.8±4.3	144.8±19.6	164.4±26.8	323.4±15

Table S4 Parameter estimation for the different rice callus lines

Parameter	PSY1	PSY1-DXS	PSY1-CRTI	PSY1-CRTI-DXS
k1	84.8	144.8	170.0	323.15
k2	0.43	0.62	0.43	0.62
k3	1.68	3.20	1.68	3.20
k4	0	0	4.62	3.36
k5	0	0	11.56	8.97
k6	0	0	0	0
k7	0	0	0	0

All parameter units are presented as g (DW) mg^{-1} $week^{-1}$. Values are rounded to two decimal places.

Functional characterization of a chemically-synthesized, codon optimized *Chlamydomonas reinhardtii* β-carotene ketolase gene (*sCrBKT*)

Having established the suitability of the callus platform for the analysis of carotenogenic genes with known functions, we analyzed an synthetic *Chlamydomonas reinhardtii* β-carotene ketolase gene (*sCrBKT*), codon-optimized for cereals, which was likewise controlled by an endosperm-specific promoter, in this case the γ-zein gene promoter from maize (GZ63).

We investigated the function of the chemically-synthesized *sCrBKT* gene and the interaction of its product with other carotenogenic enzymes by transforming 7-day-old rice embryos with *ZmPSY1*, *PaCRTI* and *sCrBKT*. We recovered many independent callus lines under hygromycin selection including a large proportion that were pink in color, suggesting the accumulation of ketocarotenoids (**Figure 2**). This was confirmed by UHPLC analysis, which showed not only the presence of ketocarotenoids but also higher levels of β-carotene (**Figure 3 and Figure S2 and Table S1**). The best-performing pink callus line contained 277.6 ± 0.6 μg/g dry weight in total carotenoids including 18.5 ± 1.3 μg/g astaxanthin, 6.7 ± 0.2 μg/g adonixanthin, 18.6 ± 0.8 μg/g adonirubin, 24 ± 1.8 μg/g canthaxanthin, 5.3 ± 0.3 μg/g 3-OH-echinenone, 7.5 ± 1.2 μg/g echinenone, 15 ± 2.7 μg/g unknown molecule, 124.6 ± 3.8 μg/g β-carotene, 12.3 ± 1.6 μg/g phytoene, 41.6 ± 0.5 μg/g α-carotene, and 3.3 ± 0.04 μg/g violaxanthin. These data showed that ketolation of β-carotene in combination with hydroxylation by the endogenous rice hydroxylase occurred due to the presence of *sCrBKT*,

which is functional in transgenic rice callus, and that the maize 'endosperm-specific' γ-zein gene promoter (GZ63) is active in callus tissue like the other endosperm specific promoters we used in earlier experiments.

Functional characterization of the *Arabidopsis thaliana Orange* gene (*AtOR*)

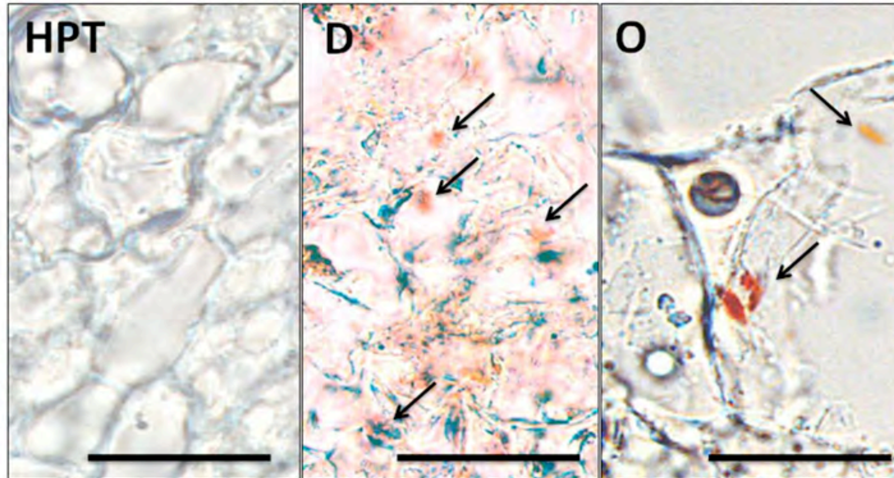
Another biological phenomenon that can be investigated using the callus platform is carotenoid accumulation in subcellular organelles. The cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* gene (*OR*) is involved in this process by regulating the development of chromoplasts, which store carotenoids and can accumulate large amounts of β-carotene. The cauliflower *OR* gene was identified as a gain-of-function mutant allele that accelerates the formation of chromoplasts, creating a metabolic sink for carotenoid accumulation. This allele also functions in a heterologous background (Lu *et al.*, 2006; Li and Van Eck, 2007; Lopez *et al.*, 2008). Recently, a sweet potato ortholog of *OR* was shown to promote the accumulation of carotenoids by inducing the expression of carotenoid biosynthesis genes in transgenic sweet potato callus (Kim *et al.*, 2013b).

We identified the *A. thaliana OR* gene through sequence comparison with the cauliflower ortholog and used this sequence to test our callus platform in more detail. We cloned the *AtOR* from *A. thaliana* leaf tissue by RT-PCR, and then introduced it into a plant expression vector under the control of the wheat LMW promoter. We transformed rice embryos with *ZmPSY1*, *PaCRTI*, *AtOR* and *HPT*, generating a number of callus lines that were orange or yellow in color, whereas the control callus remained white (**Figure 2a**). The callus expressing additional *AtOR* to the gene complement increased the carotenoid content by 2.2-fold compared to callus expressing *ZmPSY1* and *PaCRTI* (**Table S1**). Samples of the

orange and yellow callus were analyzed by UHPLC. The best-performing orange callus was shown to express all the transgenes and produced 355.3 ± 70.6 μg/g dry weight in total carotenoids, comprising 248.1 ± 46.4 μg/g β-carotene, 27.1 ± 6.8 μg/g phytoene, 62.8 ± 12.8 μg/g α-carotene, 10 ± 2.9 μg/g lutein and 7.3 ± 1.7 μg/g violaxanthin, confirming the *AtOR* gene was functional and that was able to boost the accumulation of carotenoids in the transgenic callus and particularly to enhance the accumulation of β-carotene (**Figure 3 and Figure S2 and Table S1**).

Representative transgenic callus samples were examined by microscopy. Orange, crystal-like structures were observed in the chromoplasts of orange callus samples expressing *OR* (**Figure 4a**) similar to those reported in transgenic plants expressing the cauliflower *OR* gene (Lu *et al.*, 2006; Lopez *et al.*, 2008). Interestingly, the same structures were observed in transgenic callus samples expressing *AtDXS* (**Figure 4a**). Transmission electron microscopy revealed numerous pigment-containing plastoglobuli varying in size and electron density in both of the *AtDXS* and *AtOR* transgenic lines (**Figure 4b**).

a.



b.

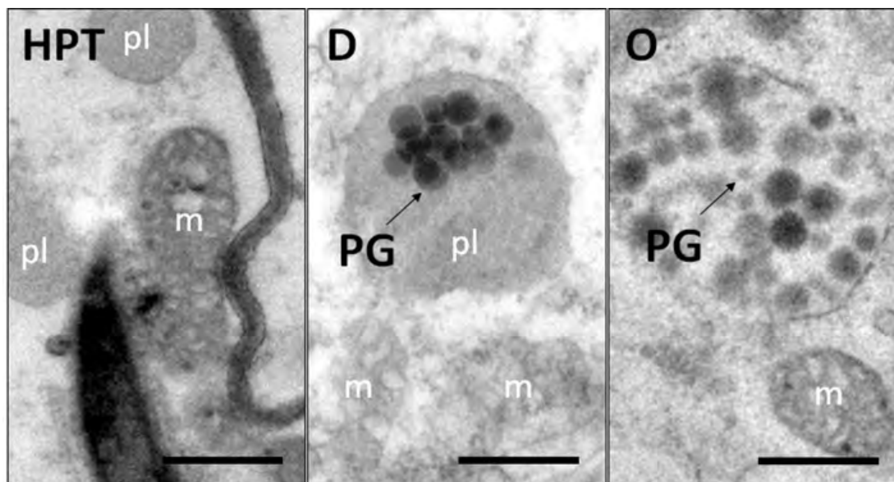


Figure 4 Microscopic cytological analysis of transgenic rice callus

(a) Light micrographs of callus expressing *HPT* in HPT; transgenic rice callus expressing *ZmPSY1, PaCRTI* and *AtDXS* in D; expressing *ZmPSY1, PaCRTI* and *AtOR* in O. Arrows indicate orange carotenoid crystal-like structures. Bars = 20 µm. (b) Transmission electron micrographs of transgenic rice callus expressing *HPT* (HPT); *ZmPSY1, PaCRTI* and *AtDXS* (D); *ZmPSY1, PaCRTI* and *AtOR* (O). Arrows indicate pigment-containing plastoglobuli (PG) in the chromoplast. Bars = 1 µm.

DISCUSSION

Rice callus provides a functional screening platform for carotenoid biosynthesis genes even if the genes are controlled by endosperm-specific promoters

We previously described a combinatorial gene transfer platform based on white maize endosperm for the functional analysis of combinations of metabolic genes (Zhu *et al.*, 2008). The endosperm of M37W maize is white because the carotenoid biosynthesis pathway is blocked at the first committed step, providing a blank canvas for the analysis of carotenogenic genes introduced by gene transfer, and further allowing the rapid analysis of diverse genotypes by visual screening for endosperm color. The endosperm tissue can also be studied in more detail by UHPLC, allowing the quantitative analysis of all carotenoids, and this means the platform can be extended to any other metabolic pathway that is also missing from endosperm tissue.

The usefulness of the maize platform in the functional characterization of metabolic genes is constrained by the time needed to regenerate transgenic plants carrying combinations of transgenes. Others have reported the use of callus tissue from maize, *A. thaliana*, sweet potato, marigold and banana to characterize gene functions and expression levels, although only individual genes were tested in these cases (Paine *et al.*, 2005; Harada *et al.*, 2009; Kim *et al.*, 2011; Kim *et al.*, 2013a; Vanegas-Espinoza *et al.*, 2011; Mlalazi *et al.*, 2012; Kim *et al.*, 2013b). We therefore considered adapting our combinatorial platform to work in rice callus thus permitting the analysis of multiple genes and their interactions over much shorter experimental timescales than possible in maize. Similarly to white maize endosperm, rice callus is also white and does not accumulate significant levels of carotenoids. However, as a relatively undifferentiated tissue it is likely that rice callus also lacks significant

components of most of the complex secondary pathways found in plants, suggesting it could be used as a platform to test many different aspects of secondary metabolism.

Although we anticipated the need to test transgenes using constitutive promoters suitable for undifferentiated tissues, we serendipitously discovered that each of the endosperm-specific promoters we used for the maize platform was also active in rice callus tissue, as revealed by the growth of callus displaying a range of yellow, orange or pink hues depending on the transgene complement, showing that carotenoids were synthesized. The promiscuous activity of endosperm-specific promoters has been reported previously, e.g. Wu and Messing (2009) showed that a maize 27-kDa γ-zein promoter was sufficient to drive high-level expression of green fluorescent protein in maize callus (they also showed that the endogenous γ-zein protein accumulated in the callus), and suggested that the tissue-specificity of storage proteins was a more recent evolutionary event and that older storage protein genes were less restricted. By confirming the activity of four different endosperm-specific promoters in the callus of a heterologous plant species, our data suggest that rice callus could be used as a platform to test transgene expression and activity in the endosperm without the need to generate mature, seed-bearing transgenic plants, therefore providing a rapid screening platform that avoids the labor-intensive process of regeneration and breeding.

The rice callus platform reveals multiple bottlenecks in the carotenoid biosynthesis pathway

One of the most important attributes of the combinatorial transformation system, either in seed endosperm or dedifferentiated embryo-derived callus, is its ability to reveal multiple

bottlenecks in metabolic pathways. Traditionally, metabolic engineering has been a trial-and-error approach where the elimination of one bottleneck often merely serves to reveal the next bottleneck, and many different experiments are required to identify the most suitable engineering strategy. In contrast, combinatorial transformation generates a library of metabolic variants allowing the best strategy to be deduced in a single step if all necessary combinations of transgenes are represented in the population (Zhu *et al.*, 2008; Naqvi *et al.*, 2010; Bai *et al.*, 2011; Zhu *et al.*, 2013). Here, we established that rice callus expressing the *ZmPSY1* gene was pale yellow and accumulated 17-fold more total carotenoids than control callus, but the addition of *AtDXS* boosted the accumulation of carotenoids by a further 1.8-fold yielding a darker yellow color compared to the callus expressing *ZmPSY1* alone. Similarly callus expressing both *ZmPSY1* and *PaCRTI* was yellow-orange in color, but the addition of *AtDXS* generated orange callus accumulating twice the amount of total carotenoids compared to the callus expressing only the two transgenes.

These data suggest that DXS eliminates a bottleneck in the supply of precursors to the carotenoid pathway, increasing the overall flux. In previous studies, the analysis of transgenic plants expressing DXS revealed higher levels of diverse isoprenoids including chlorophylls, tocopherols, carotenoids, abscisic acid and gibberellic acid, showing that DXS is a rate-limiting enzyme in the production of plastid-derived IPP (Estevez *et al.*, 2001). The expression of *A. thaliana* DXS in tomato fruits therefore increased the carotenoid content by 1.6-fold (Enfissi *et al.*, 2005) and the expression of *E. coli* DXS in potato tubers increased the carotenoid content by 2-fold (Morris *et al.*, 2006). The combined data from our callus experiments and the transgenic plants discussed above

suggest that enough DXS is normally produced to provide 50% of the maximum potential flux into the carotenoid biosynthesis pathway, but expressing a DXS transgene boosts the flux to the maximum capacity of the carotenoid pathway in the absence of further augmentation, resulting in a doubling of total carotenoid levels in several species. Hence rice callus expressing *ZmPSY1* alone produces 84.9 ± 4.3 μg/g dry weight in total carotenoids, whereas callus expressing both *ZmPSY1* and *AtDXS* doubles the flux and produces 144.8 ± 19.6 μg/g.

Our mathematical model suggests the flux channel for phytoene production is approximately twice as strong when *AtDXS* is introduced, as can be seen by comparing the k1 values of the callus line that contain *AtDXS* and *ZmPSY1* to the k1 values of the callus line that contains only *ZmPSY1* in Table S4. This indicates that phytoene production is a limiting factor for carotenoid biosynthesis and that AtDXS removes this bottleneck by increasing the availability of precursors.

The *CRTI* gene product catalyzes the entire desaturation sequence from 15-cis phytoene to all-trans lycopene thus circumpassing the rice desturation reactions. Its has been previously shown that CRTI can replace the function of inhibited desaturases (Misawa *et al.*, 1993). When *PaCRTI* is introduced to the system, the amounts of α-carotene and β-carotene increase. Our parameter estimation results are consistent with this enzyme increasing the flux capacity of the callus desaturase system per unit time by 6-fold, as can be seen by comparing the k2+k4 values in the lines expressing *PaCRTI* to the k2 values of the callus lines lacking this gene (**Table S4**). The capacity for α-carotene synthesis is also increased by more than 3-fold, as can been seen by comparing the k3+k5 values in the lines expressing *PaCRTI* to the k3 values of the callus lines lacking this gene (**Table S4**). The

lines expressing *PaCRTI* are predicted to draw more flux per unit time towards the production of carotenoids. This can be seen by comparing the k1 values in lines expressing and lacking *PaCRTI* (PSY to PSY-CRTI and PSY-DXS to PSY-CRTI-DXS). This result is consistent with a system in which the conversion of phytoene into other carotenoid precursors limits the production of those carotenoids.

The rice callus platform can functionally characterize uncharacterized genes involved in carotenoid biosynthesis and accumulation

Having established the investigative value of the callus platform by testing it against a combinatorial panel of well-characterized carotenogenic genes, we next sought to investigate the functions of genes which are absent in plants but may extend plant biosynthesis pathways, selecting a synthetic *C. reinhardtii* β-carotene ketolase (*sCrBKT*) gene optimized for maize codon usage. We co-transformed rice callus with *sCrBKT* plus *ZmPSY1* and *PaCRTI*, because these genes were required to establish the early part of the pathway and provide the necessary intermediates for ketolation. As above, it was straightforward to identify the callus expressing *sCrBKT*, *ZmPSY1* and *PaCRTI* because the accumulation of ketocarotenoids was indicated by the pink color. Ketocarotenoids represented 25–42% of total carotenoids in these lines, predominantly astaxanthin plus lower amounts of adonixanthin, adonirubin, canthaxanthin, 3-hydroxyechinenone and echinenone. The callus system therefore confirmed that the optimized synthetic gene was expressed in plants, that the enzyme was active and cooperated with endogenous carotenogenic enzymes and that the activity of *sCrBKT* depended on the concurrent activity of *ZmPSY1* and *PaCRTI*, as would be expected.

In plants, carotenoids accumulate in specialized pigment-bearing structures known as plastoglobuli, within plastid-derived organelles called chromoplasts (Vothknecht and Soll, 2005; Brehelin *et al.*, 2007). The cauliflower *Orange* (*OR*) gene was discovered following the analysis of a mutant cauliflower with an orange curd, and was shown to encode a chaperone-like protein that induced the formation of chromoplasts and thus created a metabolic sink for carotenoids (Lu *et al.*, 2006; Li and Van Eck, 2007).

Orthologs of cauliflower wild-type *OR* have been identified in other species but only the sweet potato ortholog has been shown to induce carotenoid accumulation (Kim *et al.*, 2013b). We therefore cloned the *A. thaliana OR* gene and found that the corresponding protein was 74.4% identical to the cauliflower wild-type ortholog and contained the DnaJ cysteine rich domain, which is required for chaperone activity (Miernyk, 2001). The overexpression of *AtOR* in rice callus specifically in the endosperm increased the level of total carotenoids by 2-fold. Interestingly, the wild-type cauliflower *OR* allele did not increase carotenoid levels when it was expressed in potato whereas the originally-discovered mutant allele increased β-carotene levels in the tubers by 6-fold, suggesting the mutation caused a dominant gain of function (Lu *et al.*, 2006; Lopez *et al.*, 2008). It is unclear why the wild-type cauliflower *OR* allele was unable to increase carotenoid levels in potato tubers whereas the wild-type sweet potato gene was able to induce carotenoid accumulation in sweet potato callus (Kim *et al.*, 2013b) and our experiments showed that the wild-type *A. thaliana* gene was able to increase carotenoid levels in rice callus. The lack of carotenoid accumulation in transgenic potatoes may reflect the analysis of only four lines, which may have been subject to silencing (Lu *et al.*, 2006). Further experiments will be required to identify and characterize proteins that associate

with in order to determine its precise role in chromoplast differentiation and carotenoid accumulation.

Carotenoid-lipoprotein structures may be induced either by *OR* gene expression or enhanced accumulation of carotenoids in the absence of *OR*

Chromoplasts are typically found in mature storage tissues and are categorized as globular, tubular, reticulotubular, membranous or crystalline subtypes (Sitte *et al.*, 1980). For example, crystalline bodies have been observed in carrots (Frey-Wyssling and Schwegler, 1965) and tomatoes (Harris and Spurr, 1969). We analyzed the structure and ultrastructure of rice callus expressing the *A. thaliana OR* gene and observed one or two typical orange chromoplast structures per cell, as previously described in mutant cauliflower and transgenic potato tubers expressing the dominant cauliflower *OR* allele (Lu *et al.*, 2006; Lopez *et al.*, 2008). We assumed that the ectopic chromoplasts were formed because the *OR* transgene induced the precocious differentiation of these structures from immature plastids. However, we also observed chromoplast-like structures in the callus expressing *AtDXS*, *ZmPSY1* and *PaCRTI*, but not in those expressing *ZmPSY1* and *PaCRTI*. These data suggest that chromoplast differentiation can be triggered either by the direct expression of a gene involved in the differentiation process (*OR*) or by increasing the flux through the carotenoid pathway to such an extent that the process of chromoplast differentiation is triggered by the abundance of carotenoids. This phenomenon has previously been observed in non-green *A. thaliana* tissues (callus and roots) expressing high levels of phytoene synthase (Maass *et al.*, 2009), suggesting that the chromoplast differentiation program may be a response to the accumulation of carotenoids above a certain threshold unless it is

triggered by *OR* before this threshold is reached (Maass *et al.*, 2009). There appears not to be a relationship between these independent events, because the *OR* gene does not normally induce the activity of carotenogenic genes (Li *et al.*, 2001, 2006), thus suggesting that it may act at the level of the metabolome by shifting the chemical equilibrium in the cell towards carotenogenesis (Li *et al.*, 2001; Maass *et al.*, 2009). More recently sweet potato *OR* was shown to induce carotenogenic gene expression, suggesting that *OR* might contribute towards increasing carotenoid levels to reach the threshold necessary to trigger chromoplast differentiation (Kim *et al.*, 2013b).

CONCLUSIONS

Our callus-based assay allows the rapid combinatorial testing of different expression constructs, making it an ideal platform for synthetic biology, which involves the assembly of genetic circuits from components such as promoters, genes and protein targeting signals. The callus platform will allow large numbers of constructs to be tested in parallel, in different combinations, so that ideal engineering strategies can be developed before any transgenic plants are produced. Similarly, the platform can be used to modulate promoter strength, protein synthesis and metabolite production, thus facilitating a more quantitative approach to synthetic biology and thus more refined and sophisticated strategies for metabolic engineering. The method is applicable to any pathway and any gene product that can be analyzed through standard analytical procedures such as HPLC, mass spec, NMR, etc. It is neither limited nor constraint by a color phenotype.

EXPERIMENTAL PROCEDURES

Gene cloning and vector construction

The *AtDXS* and *AtOR* cDNAs were cloned directly from *A. thaliana* mRNA by reverse transcriptase PCR based on sequence data in GenBank (accession numbers NM 203246 and U27099.1). The cDNAs were transferred to the pGEM-T easy vector (Promega, Madison, WI, USA) and the recombinants were digested with *Eco*RI. *AtDXS* was introduced into vector pRP5, containing the rice prolamin promoter and the ADPGPP terminator, whereas *AtOR* was introduced into vector p326, containing the wheat low molecular weight (LMW) glutenin gene promoter and *nos* terminator.

A truncated β-carotene ketolase gene from *Chlamydomonas reinhardtii* (Zhong *et al.*, 2011) was chemically synthesized and optimized for maize codon usage. The modified gene (*sCrBKT*) was fused with the transit peptide sequence (TPS) from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) and the 5'-untranslated region (5'UTR) of the rice alcohol dehydrogenase gene (Sugio *et al.*, 2008) under the control of the maize γ-zein promoter. The TPS and 5'UTR were also optimized for maize codon usage.

The maize *PSY1* cDNA was cloned from maize inbred line B73 by RT-PCR using forward primer 5'-AGG ATC CAT GGC CAT CAT ACT CGT ACG AG-3' and reverse primer 5'-AGA ATT CTA GGT CTG GCC ATT TCT CAA TG-3' based on the *PSY1* sequence (GenBank accession number AY324431). The product was transferred to pGEM-T (Promega) for sequencing and then to p326 containing the LWM glutenin promoter and *nos* terminator (Stoger *et al.*, 1999).

The *Pantoea ananatis* (formerly *Erwinia uredovora*) *CRTI* gene was fused in-frame with the transit peptide sequence (TPS) from the *P. vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) in plasmid pYPIET4 (Misawa *et al.*, 1993) and amplified by PCR using forward primer 5'-ATC TAG AAT GGC TTC TAT GAT ATC CTC TTC-3' and reverse primer 5'-AGA ATT CTC AAA TCA GAT CCT CCA GCA TCA-3'. The product was transferred to pGEM-T for sequencing and then to pHorp-P containing the barley D-hordein promoter (Sorensen *et al.*, 1996) and the rice ADPGPP terminator. All the transformation constructs were verified by sequencing.

Rice transformation

Seven-day-old mature rice zygotic embryos were bombarded with 10 mg gold particles coated with the carotenogenic constructs and the selectable maker *HPT* at a ratio 3:3:3:1 as previously reported (Christou *et al.*, 1991). The embryos were returned to osmoticum medium for 12 h before selection on medium supplemented with 50 mg/l hygromycin and 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark for 2–3 weeks (Christou, 1997). Callus was selected by visual screening for yellow, orange and pink coloring as appropriate, and was subcultured every 2 weeks to collect sufficient material for further analysis.

Analysis of mRNA

We separated 30 µg of denatured RNA by 1.2% (w/v) agarose-formaldehyde gel electrophoresis in 1× MOPS buffer and transferred the fractionated RNA to a membrane by

capillary blotting (Sambrook *et al.*, 1989). Procedures description in Zhu *et al.*, 2009 were used. The forward and reverse primers for each transgene are shown in Table S5.

Table S5 Oligonucleotide sequences of forward (F) and reverse (R) primers for mRNA blot analysis

Transgenes	Primers sequence
ZmPSY1.F	Forward 5´-GTGTAGGAGGACAGATGAGCTTGT-3´
ZmPSY1.R	Reverse 5´-CATCTGCTAGCCTGTGAGAGCTCA-3´
PaCRTI.F	Forward 5´-TGGAGAAGCGTTTACAGTAAGGT-3´
PaCRTI.R	Reverse 5´-GCGTGCAGATAAAGTGAGAAGTC-3´
AtDXS.F	Forward 5´-GACACAGCTCAACCACAATATCTGCTGG-3´
AtDXS.R	Reverse 5´-GGAAGACAAGCCATAAATGTCACATCGAAAG-3´
AtOR.F	Forward 5´-ATGTCATCTTTGGGTAGGATTTTGT-3´
AtOR.R	Reverse 5´-GGTTTTGGGCGGTGATAGAGA-3´
sCrBKT.F	Forward 5´-GGATCCTCAGCCAGGAGCCAGTGCAGCGCCTCT-3´
sCrBKT.R	Forward 5´-GAATTCCATGGGGCCAGGCATTCAGCCCACTTCCG-3´

Carotenoid extraction and quantitation

Carotenoids were extracted from 10 mg freeze-dried callus in the dark using 50/50 (v/v) tetrahydrofuran and methanol at 60°C for 20 min. The mixture was filtered and the residue re-extracted in acetone. Lutein and zeaxanthin were separated on a YMC C30 carotenoid 3-µm, 2.0 x 100 mm HPLC column (Waters, Milford, MA) using a mobile phase comprising solvent A (methanol:water, 80:20 v/v) and solvent B (100% *tert*-butylmethylether) at a flow rate of 0.30 mL/min. All other carotenoids were separated on a reversed-phase ACQUITY UPL BEH 300 Å C18, 1.7 mm, 2.1 × 150 mm column

(Waters) using a gradient system with the mobile phase consisting of solvent A (acetonitrile:methanol, 70:30 v/v) and solvent B (100% water) at a flow rate of 0.35 mL/min. The mixtures were analyzed using an ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) linked to a 2996 photo diode array detector (Waters). MassLynx software v4.1, (Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing (Rivera *et al.*, 2013).

Microscopy

Rice callus pieces (0.5 x 2.0 mm) were fixed in 2.5% (v/v) glutaraldehyde and 2.0% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C for light and transmission electron microscopy. After three washes with 0.1 M sodium phosphate buffer (pH 7.2), they were sectioned on a Leica CM3050 cryotome using Cryogel as the embedding medium for light microscopy. Thin sections (16 µm) were prepared with a diamond knife using a Reichter Jung Ultramicrotome Ultracut E (Scotia, NY, USA) and were mounted on glass slides for analysis under a Zeiss Axioplan light microscope (Gottingen, Germany) coupled to a Leica DC 200 digital camera (Wetzlar, Germany).

For transmission electron microscopy, the sections were washed three times in 0.1 M sodium phosphate buffer (pH 7.2) and post-fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h. They were then washed three times in redistilled water and dehydrated in an alcohol series (30–100%) before embedding in epoxy resin (Araldite, Epon, Hexion) and polymerizing at 60ºC. Ultrathin sections (80–90 nm) were prepared with a diamond knife using a Reichter Jung Ultramicrotome Ultracut E (Scotia, NY, USA) and were mounted on Formvar-carbon coated copper grids, stained with uranyl

acetate and Reynold's lead citrate prior to examination using an EM 910 transmission electron microscope (Zeiss, Oberkochen, Germany).

Mathematical modeling

Mathematica (Wolfram, 1999) was used to create and solve the mathematical models for carotenoid biosynthesis. It was also used to find the best-fit parameters for the models. Further details about the modeling process are provided in the text (**Method 1**).

Method 1 Mathematical models for the callus lines transformed with *ZmPSY1*, and/or *PaCRTI* and/or *AtDXS*

Section 1: A general mathematical model for carotene biosynthesis in the callus lines

From Table S2 and Figure 1 from the main paper we built a simplified conceptual representation of carotene biosynthesis in the transformed callus lines. Because phytoene, α- and β-carotene are below the detection limit in the control rice callus which expressing *HPT*, we can not create a model that accounts for general carotene biosynthesis in this tissue. In addition, because either lutein, violaxanthin, zeaxanthin or a combination of the later two are bellow detection limits in some of the transformed callus tissues, we can not create a model for their production in those lines either. Even when detected these metabolites together make for less than 10% of accumulated carotenes in all cases. Therefore, to create a carotene biosynthesis model that is general for all transformed callus

lines we consider only phytoene, α- and β-carotene. The conceptual representation of this simplified model is shown in Figure S3.

As explained below, the flux of phytoene production depends on both, PSY1 and DXS. Also, to account for the possibility that CRTI introduces another flux channel for carotene production from phytoene, we introduce the processes represented by k4(CRTI) and k5(CRTI). See below for full details.

To transform this conceptual scheme into a mathematical model we assume that the individual processes shown in Figure S3 can be approximately represented by apparent mass action kinetics of the form $k_i S_i$, where k_i is the apparent rate constant and S_i is the substrate of the process. This allows us to write Eq 1:

$$\frac{dphyt}{dt} = k1(PSY1, DXS) - (k2 + k3)phyt - \left(k4(CRTI) + k5(CRTI)\right)phyt$$

$$\frac{d\,\alpha car}{dt} = \left(k2 + k4(CRTI)\right)phyt \; - \; k6\,\alpha car \qquad \text{Eq. 1}$$

$$\frac{d\,\beta car}{dt} = \left(k3 + k5(CRTI)\right)phyt \; - \; k7\,\beta car$$

Eq. 1 can be solved analytically, and the time dependent concentration of the metabolites can be calculated as a function of the parameter of the system. These concentrations are given by Eq. 2 – Eq 4:

$$\alpha\text{-car}(t) = -\frac{e^{(k2+k3+k4+k5)\,t-k6\,t}\left(-1+e^{(-k2-k3-k4-k5)\,t+k6\,t}\right)k1\,(k2+k4)}{(k2+k3+k4+k5)\,(k2+k3+k4+k5-k6)} - \frac{k1\,(k2+k4)\left(\frac{e^{(k2+k3+k4+k5-k6)\,t}}{k2+k3+k4+k5}-\frac{1}{k6}\right)}{k2+k3+k4+k5-k6} + e^{-k6\,t}\,C[1] - \frac{e^{-k6\,t}\left(-1+e^{(-k2-k3-k4-k5)\,t+k6\,t}\right)(k2+k4)\,C[3]}{k2+k3+k4+k5-k6} \qquad \text{Eq. 2}$$

β-car(t)=

$$-\frac{k1\,(k3+k5)\left(\frac{e^{(k2+k3+k4+k5-k7)\,t}}{k2+k3+k4+k5}-\frac{1}{k7}\right)}{k2+k3+k4+k5-k7}+\frac{e^{(k2+k3+k4+k5)\,t-k7\,t}\left(-1+e^{(-k2-k3-k4-k5)\,t+k7\,t}\right)k1\,(k3+k5)}{(k2+k3+k4+k5)\,(-k2-k3-k4-k5+k7)}+e^{-k7\,t}\,C[2]+\frac{e^{-k7\,t}\left(-1+e^{(-k2-k3-k4-k5)\,t+k7\,t}\right)(k3+k5)\,C[3]}{-k2-k3-k4-k5+k7}$$ Eq. 3

phyt(t)= $\frac{e^{(-k2-k3-k4-k5)\,t+(k2+k3+k4+k5)\,t}\,k1}{k2+k3+k4+k5}+e^{(-k2-k3-k4-k5)\,t}\,C[3]$ Eq. 4

Assuming that all concentrations are zero at time t=0, we can calculate the integration constants C[1]-C[3] from Eqs. 2-4. This can be done by making α-car(t=0)= β-car(t=0)= phyt(t=0) = t=0 and solving the resulting equations with respect to C[1]-C[6]:

C[1]= $-\frac{k1\,(k2+k4)\left(-e^{k6}\,k2-e^{k6}\,k3-e^{k6}\,k4-e^{k6}\,k5+e^{k2+k3+k4+k5}\,k6\right)}{(k2+k3+k4+k5)\,k6\,(-k2-k3-k4-k5+k6)}$

C[2]= $-\frac{k1\,(k3+k5)\left(-e^{k7}\,k2-e^{k7}\,k3-e^{k7}\,k4-e^{k7}\,k5+e^{k2+k3+k4+k5}\,k7\right)}{(k2+k3+k4+k5)\,k7\,(-k2-k3-k4-k5+k7)}$ Eq 5

C[3]= $-\frac{e^{k2+k3+k4+k5}\,k1}{k2+k3+k4+k5}$

Section 2: Modeling the PSY1 callus line

Because phytoene, α- and β-carotene are not detected in control callus our model does not apply to this line. We start by modeling the PSY1 callus line. In this line k4(CRTI)=k5(CRTI)=0. This simplifies the system to:

$$\frac{dphyt}{dt}=k1(PSY1,DXS)-(k2+k3)phyt$$

$$\frac{dphyt}{dt}=k1(PSY1,DXS)-(k2+k3)phyt$$

$$\frac{d\,\alpha car}{dt} = (k2)phyt - k6\,\alpha car \qquad \text{Eq. 6}$$

$$\frac{d\,\beta car}{dt} = (k3)phyt - k7\,\beta car$$

Replacing the values for k4(CRTI) and k5(CRTI) in Eqs 2-5 we obtain the simplified integration constants and solutions.

Given that we have the concentrations of the three metabolites at 2 weeks (**Table S3**) we can replace phyt(t), α-car(t) and β-car(t) by their respective values and make t=2 weeks, rewriting Eqs 2-4 as

$$\left(-35.4 - \frac{e^{k2+2(-k2-k3)+k3}\,k1}{k2+k3} + \frac{e^{2(-k2-k3)+2(k2+k3)}\,k1}{k2+k3}\right)^2 +$$

$$\left(-8.2 + \frac{e^{k2+k3-2k6}\left(-1+e^{2(-k2-k3)+2k6}\right)k1\,k2}{(k2+k3)(k2+k3-k6)} - \frac{e^{2(k2+k3)-2k6}\left(-1+e^{2(-k2-k3)+2k6}\right)k1\,k2}{(k2+k3)(k2+k3-k6)} - \right.$$

$$\left.\frac{k1\,k2\left(\frac{e^{2(k2+k3-k6)}}{k2+k3} - \frac{1}{k6}\right)}{k2+k3-k6} - \frac{e^{-2k6}\,k1\,k2\left(-e^{k6}\,k2 - e^{k6}\,k3 + e^{k2+k3}\,k6\right)}{(k2+k3)\,k6\,(-k2-k3+k6)}\right)^2 +$$

$$\left(-35.8 - \frac{k1\,k3\left(\frac{e^{2(k2+k3-k7)}}{k2+k3} - \frac{1}{k7}\right)}{k2+k3-k7} - \frac{e^{k2+k3-2k7}\left(-1+e^{2(-k2-k3)+2k7}\right)k1\,k3}{(k2+k3)(-k2-k3+k7)} + \right.$$

$$\left.\frac{e^{2(k2+k3)-2k7}\left(-1+e^{2(-k2-k3)+2k7}\right)k1\,k3}{(k2+k3)(-k2-k3+k7)} - \frac{e^{-2k7}\,k1\,k3\left(-e^{k7}\,k2 - e^{k7}\,k3 + e^{k2+k3}\,k7\right)}{(k2+k3)\,k7\,(-k2-k3+k7)}\right)^2 = 0 \qquad \text{Eq. 7}$$

We can now solve Eq. 7 numerically and estimate the values for k1-k7. This was done using Mathematica (Wolfram, 1999). The tolerance for the solutions was established at 10^{-10}. In other words, if the right-hand side of Eq. 7 is smaller than 10^{-10}, we accept that we have a possible solution for the parameters that solves the equation. A similar process was followed for each line (see below). The results for all lines are given in Table S4.

Section 3: Modeling the PSY1-CRTI, PSY1-DXS, and PSY1-DXS-CRTI callus lines

In the PSY1-DXS lines we can further simplify the general model by making k4(CRTI) = k5(CRTI) = 0. These parameters are estimated in lines PSY1-CRTI and PSY1-CRTI-DXS. Following the same procedure as described in Section 2 we find the parameter values shown in Table S4.

Section 4: Interpreting the results

The basic assumption of the modeling process is that introduction of PSY1 is needed for production of phytoene, which serves as substrate for the production of α- and β-carotene. Such production is negligible in the control line. Given that the amount of mRNA being expressed for this gene remains approximately constant between the various transgenic lines and given that mRNA translation and processing should be similar in all lines, this assumption seems reasonable.

When PSY1 is introduced, a flux channel for the production of phytoene is introduced. When DXS is introduced, the flux channel for phytoene production is approximately twice as strong as in the absence of this gene, as can be seen by comparing the values of k1 in the callus line that contain DXS and PSY1 to the k1 values in the callus lines that contain only PSY1. This is fully consistent with phytoene production being a limiting factor for carotene production and with DXS increasing the production of precursors that are needed for phytoene synthesis.

In addition, when CRTI is introduced the amount of α- and β-carotenes that are produced increases. Our parameter estimation results are consistent with this gene increasing the

capacity per time unit of the callus desaturase systems to produce α-carotene by more than six fold, as can be seen by comparing k2+k4 in the lines with CRTI to k2 in the lines where no CRTI is present. β-carotene production capacity is also increased by more than three-fold, as can been seen by comparing k3+k5 in the CRTI-transformed callus lines to k3 in the lines that do not contain CRTI.

Together, the data and our model and the data show that the natural desaturases of the plant are able to use most of the material flowing into the pathway to produce carotenoids in the PSY1 and PSY1-DXS lines, accumulating a similar amount of phytoene. This is the case even though addition of DXS increases the flux through the pathway by two fold. If CRTI is also present, the total desaturase activity available to the cells increases. In the absence of DXS this activity more efficiently converts phytoene into carotenoids from the α- and β-branch and four-fold less phytoene accumulates with respect to the control line. In contrast, when CRTI and DXS are present, the amount of accumulated phytoene is about half of that in the control line, suggesting the desaturase system is more saturated with material than in the absence of DXS.

Finally, it must be noted that lines containing the CRTI gene are predicted to draw more flux per time unit towards production of phytoene. This can be seen by comparing k1 in the CRTI free lines to k1 in corresponding CRTI-containing lines (PSY to PSY-CRTI and PSY-DXS to PSY-CRTI-DXS). This result is consistent with a system where transformation of phytoene into other carotene precursors is limiting the production of those carotenes.

REFERENCES

Bai, C., Twyman, R.M., Farre, G., Sanahuja, G. Christou, P., Capell, T. and Zhu, C. (2011) A golden era –_pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol*. **47**, 205–221.

Brehelin, C., Kessler, F. and van Wijk, K.J. (2007) Plastoglobules: versatile lipoprotein particles in plastids. *Trends Plant Sci.* **12**, 260–266.

Capell, T. and Christou, P. (2004) Progress in plant metabolic engineering. *Curr. Opin. Biotech.* **15**, 148–154.

Christou, P. (1997) Rice transformation by bombardment. *Plant Mol. Biol.* 35, 197–203.

Christou, P., Ford, T.L. and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Biotechnology,* **9**, 957–962.

Dafny-Yelin, M. and Tzfira, T. (2007) Delivery of multiple transgenes to plant cells. *Plant Physiol.* **145**, 1118–1128.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W. and Bramley, P.M. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotech. J.* **3**, 17–27.

Estevez, J.M., Cantero, A., Reindl, A., Reichler, S. and Leon, P. (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* **276**, 22901–22909.

Farre, G., Sanahujaa, G., Naqvia, S., Bai, C., Capell, T., Zhu, C. and Christou, P.

(2010) Travel advice on the road to carotenoids in plants. *Plant Sci.* **179**, 28–48.

Farre, G., Bai, C., Twyman, R.M., Capell, T., Christou, P. and Zhu, C. (2011) Nutritious crops producing multiple carotenoids – a metabolic balancing act. *Trends Plant Sci*. **16**, 532–540.

Farre, G., Rivera, S.M., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Naqvi, S., Sandmann, G., Capell, T., Zhu, C. and Christou, P. (2013) Targeted transcriptomic and metabolic profiling reveals temporal bottlenecks in the maize carotenoid pathway that can be addressed by multigene engineering. *Plant J.* **75***,* 441–455.

Frey-Wyssling, A. and Schwegler, F. (1965) Ultrastructure of the chromoplasts in the carrot root. *J. Ultrastruct. Res.* **13**, 543–559.

Harada, H., Fujisawa, M., Teramoto, M., Sakurai, N., Suzuki, H., Shibata, D. and Misawa, N. (2009) Simple functional analysis of key genes involved in astaxanthin biosynthesis using Arabidopsis cultured cells. *Plant Biotechnol.* **26**, 81–92.

Harris, W.M. and Spurr, A.R. (1969) Chromoplasts of tomato fruits. II. The red tomato. *Am. J. Bot.* **56**, 380–389.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013a) Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol. Biochem.* **70**, 445–454.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Lee, H.S. and Kwak, S.S. (2011) Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato. *Phytochemistry,* **74**, 69–78.

Kim, S.H., Kim, Y.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S.

(2013b) Downregulation of the lycopene ε-cyclase gene increases carotenoid synthesis via the β-branch-specific pathway and enhances salt-stress tolerance in sweetpotato transgenic calli. *Physiol. Plant.* **147**, 432–442.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013b) Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol. Biochem.* **70**, 445–454.

Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I. and Maluszynski, M. (2011) TILLING - a shortcut in functional genomics. *J. Appl. Genet.* **52**, 371–390.

Li, L. and Van Eck J. (2007) Metabolic engineering of carotenoid accumulation by creating a metabolic sink. *Transgenic Res.* **16**, 581–585.

Li, L., Lu, S., Cosman, K.M., Earle, E.D., Garvin, D.F. and O'Neill, J. (2006) β-carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. *Phytochemistry,* **67**, 1177–1184.

Li, L., Paolillo, D.J., Parthasarathy, M.V., Dimuzio, E.M. and Garvin, D.F. (2001) A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *Plant J.* **26**, 59–67.

Liu, C.G., Calin, G.A., Volinia, S. and Croce, C.M. (2008) MicroRNA expression profiling using microarrays. *Nat. Protoc*. **3**, 563–578.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot*. **59**, 213–223.

Lu, S., Van Eck, J., Zhou, X., Lopez, A.B., O'Halloran, D.M., Cosman, K.M., Conlin,

B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Kupper, H., Earle, E.D., Cao, J. and Li, L. (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. *Plant Cell* **18**, 3594–3605.

Maass, D., Arango, J., Wu, F., Beyer, P. and Welsch, R. (2009) Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS One* **4**, e6373.

Miernyk, J.A. (2001). The J-domain proteins of Arabidopsis thaliana: An unexpectedly large and diverse family of chaperones. *Cell Stress Chaperones,* **6**, 209–218.

Misawa, N., Yamano, S., Linden, H., de Felipe, M.R., Lucas, M., Ikenaga, H. and Sandmann, G. (1993) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–840.

Mlalazi, B., Welsch, R., Namanya, P., Khanna, H., Geijskes, R.J., Harrison, M.D., Harding, R., Dale, J.L. and Bateson, M. (2012) Isolation and functional characterisation of banana *phytoene synthase* genes as potential cisgenes. *Planta*, **236**, 1585–1598.

Morris, W.L., Ducreux, L.J.M., Hedden, P., Millam, S. and Taylor, M.A. (2006) Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *J. Exp. Bot.* **57**, 3007–3018.

Myouga, F., Akiyama, K., Motohashi, R., Kuromori, T., Ito, T., Iizumi, H., Ryusui, R., Sakurai, T. and Shinozaki, K. (2009) The chloroplast function database: a large-scale collection of Arabidopsis *Ds/Spm-* or T-DNA-tagged homozygous lines for

nuclear-encoded chloroplast proteins, and their systematic phenotype analysis. *Plant J.* **61**, 529–542.

Naqvi, S., Farre, G., Sanahuja, G., Capell, T., Zhu, C. and Christou, P. (2010) When more is better: multigene engineering in plants. *Trends Plant Sci.* **15**, 48–56.

Nikolov, M., Schmidt, C. and Urlaub, H. (2012) Quantitative mass spectrometry-based proteomics: an overview. *Methods Mol. Biol.* **893**, 85–100.

Ozsolak, F. and Milos, P.M. (2011) RNA sequencing: advances, challenges and opportunities. *Nat. Rev. Genet.* **12**, 87–98.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotech.* **23**, 482–487.

Purkayastha, A. and Dasgupta, I. (2009) Virus-induced gene silencing: A versatile tool for discovery of gene function in plants. *Plant Physiol. Biochem.* **47**, 967–976.

Ramessar, K., Sabalza, M., Capell, T. and Christou, P. (2008) Maize plants: An ideal production platform for effective and safe molecular pharming. *Plant Sci.* **174**, 409–419.

Rivera, S.M., Vilaro, F., Zhu, C., Bai, C., Farre, G., Christou, P. and Canela-Garayoa, R. (2013) Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem.* **61**, 5279–5285.

Ruiz-Sola, M.A. and Rodriguez-Concepcion, M. (2012) Carotenoid biosynthesis in Arabidopsis: a colorful pathway. *The Arabidopsis Book.* 10, e0158.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press pp. 63–70.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.

Schreier, P., Seftor, E., Schell, J. and Bohnert, H. (1985) The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J.* **4**, 25–32.

Sitte, P., Falk, H. and Liedvogel, B. (1980) *Chromoplasts.* Stuttgart, NY: Gustav Fischer Verlag, pp. 117–148.

Snyder, M. and Gallagher, E.G. (2009) Systems biology from a yeast omics perspective. *FEBS lett.* **583**, 3895–3899.

Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol. Gen. Genet*. **250**, 750–60.

Stoger, E., Williams, S., Keen, D. and Christou, P. (1999) Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Res.* **8**, 73–82.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A. and Kato, K. (2008) The 5'-untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells*. J. Biosci. Bioeng.* **105**, 300–330.

Vanegas-Espinoza, P.E., Ramos-Viveros, V., Jimenez-Aparicio, A.R., Lopez-Villegas, O., Heredia-Mira, F.J., Melendez-Martinez, A.J., Quintero-Gutierrez, A.G., Paredes-Lopez, O. and Del Villar-Martinez, A.A. (2011) Plastid analysis of pigmented undifferentiated cells of marigold *Tagetes erecta* L. by transmission electron microscopy. *In Vitro Cell. Dev. Biol.* **47**, 596–603.

Vothknecht, U.C. and Soll, J. (2005) Chloroplast membrane transport: interplay of prokaryotic and eukaryotic traits. *Gene,* **354**, 99–109.

Wolfram, S. (1999) *The Mathematica Book*. 4th edn. Cambridge, UK: Cambridge, University Press.

Wu, Y. and Messing, J. (2009) Tissue-specificity of storage protein genes has evolved with younger gene copies. *Maydica,* **54**, 409–415.

Zhong, Y.J., Huang, J.C., Liu, J., Li, Y., Jiang, Y., Xu, Z.F., Sandmann, G. and Chen, F. (2011) Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic Arabidopsis. *J. Exp. Bot*. **62**, 3659–3669.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P. and Capell, T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl. Acad. Sci. USA* **105**, 18232–18237.

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

Zhu, C., Sanahuja, G., Yuan, D., Farre, G., Arjo, G., Berman, J., Zorrilla-Lopez, U., Banakar, R., Bai, C., Perez-Massot, E., Bassie, L., Capell, T. and Christou, P. (2013) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J.* **11**, 129–141.

Zhu, M.J. and Zhao, S.H. (2007) Candidate gene identification approach: progress and challenges. *Int. J. Biol. Sci.* **3**, 420–427.

Zurbriggen, M.D., Moor, A. and Weber, W. (2012) Plant and bacterial systems biology as platform for plant synthetic bio(techno)logy. *J. Biotechnol.* **160**, 80–90.

Chapter 2

A novel carotenoid, 4-keto-α-carotene, as an unexpected by-product during genetic engineering of carotenogenesis in rice callus

Published in the Phytochemistry

http://dx.doi.org/10.1016/j.phytochem.2013.12.008

Chapter 2 is formatted according to the Phytochemistry Journal

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A novel carotenoid, 4-keto-α-carotene, as an unexpected by-product during genetic engineering of carotenogenesis in rice callus

ABSTRACT

Rice endosperm is devoid of carotenoids because the initial biosynthetic steps are absent. The early carotenogenisis reactions were constituted through co-transformation of endosperm-derived rice callus with phytoene synthase and a phytoene desaturase transgene. Subsequent steps in the pathway such as cyclization and hydroxylation reactions were catalysed by endogenous rice enzymes in the endosperm. The carotenoid pathway was extended further by including a bacterial ketolase gene able to form astaxanthin, a high value carotenoid which is not a typical plant carotenoid. In addition to astaxanthin and precursors, a carotenoid accumulated in the transgenic rice callus which did not fit into the pathway to astaxanthin. This was subsequently identified as 4-keto-α-carotene by HPLC co-chromatography, chemical modification, mass spectroscopy and the reconstruction of its biosynthesis pathway in *Escherichia coli*. We postulate this keto carotenoid is formed from α-carotene which accumulates by combined reactions of heterologous gene products and endogenous rice endosperm cyclization reactions.

INTRODUCTION

Genetic engineering is a powerful tool to modulate existing biosynthetic pathway or establish novel routes in microbes and plants. Carotenogenesis is a key target for genetic engineering of staple crops due to the many nutritional and other health benefits of a number of such molecules for humans and animals (Fraser et al., 2009; Misawa, 2011). Successful examples are increase of the carotenoid yield, e.g. lycopene in tomato (Fraser et al., 2002), the accumulation of intermediates to higher levels, e.g. zeaxanthin in potato (Römer et al., 2002) or extension of an existing pathway to a novel end product, e.g. astaxanthin in maize (Zhu et al., 2008). In addition, maize was used to explore interactions between an induced transgenic pathway and the endogenous pathway in maize (Naqvi et al., 2011). On a number of occasions there have been examples of novel unexpected phenotypes due to unknown regulatory mechanisms and unpredicted enzyme interactions (Sandmann et al., 2006). Rice provides an interesting example of carotenogenesis by genetic engineering. In contrast to the endosperm of yellow maize which is pigmented due to the accumulation of lutein, zeaxanthin, and 5,6-epoxy derivatives (Quakenbush et al., 1963), rice endosperm is colourless. Nevertheless, rice endosperm possesses a hidden potential for carotenoid biosynthesis even though the initial steps in the carotenoid pathway are absent. The endogenous levels of phytoene synthase and phytoene desaturase in wild type rice are below the threshold level for carotenoid biosynthesis (Schaub et al., 2005). It has been demonstrated that the limitation of carotenogenesis can be overcome in rice endosperm by expressing genes encoding a phytoene synthase and a bacterial phytoene desaturase to replace all plant desaturation and isomerisation reactions (Ye et al., 2000). The expect product of these reactions is lycopene. Interestingly, biosynthesis proceeded beyond lycopene by cyclization to α- and β-carotene and the hydroxylation of both carotenes to

lutein and zeaxanthin, respectively (Ye et al., 2000). Thus intrinsic rice cyclases and hydroxylases are constitutively expressed in rice endosperm. A survey of phytoene synthase genes from different plant species indicated that the enzyme from maize is the most effective in rice (Paine et al., 2005). Its use led to the generation of a rice line rich in α- and β-carotene which both exhibit provitamin A activity, in addition to lutein and zeaxanthin (structures shown in **Fig. 1**). In our current carotenogenesis engineering experiments we attempted to extent the pathway beyond carotene to astaxanthin. Astaxanthin is a high priced carotenoid which is beneficial for human health (Guerin et al., 2003; Hussein et al., 2006) and is used as an essential feed additive in salmon farming (Bjerkeng, 2008).

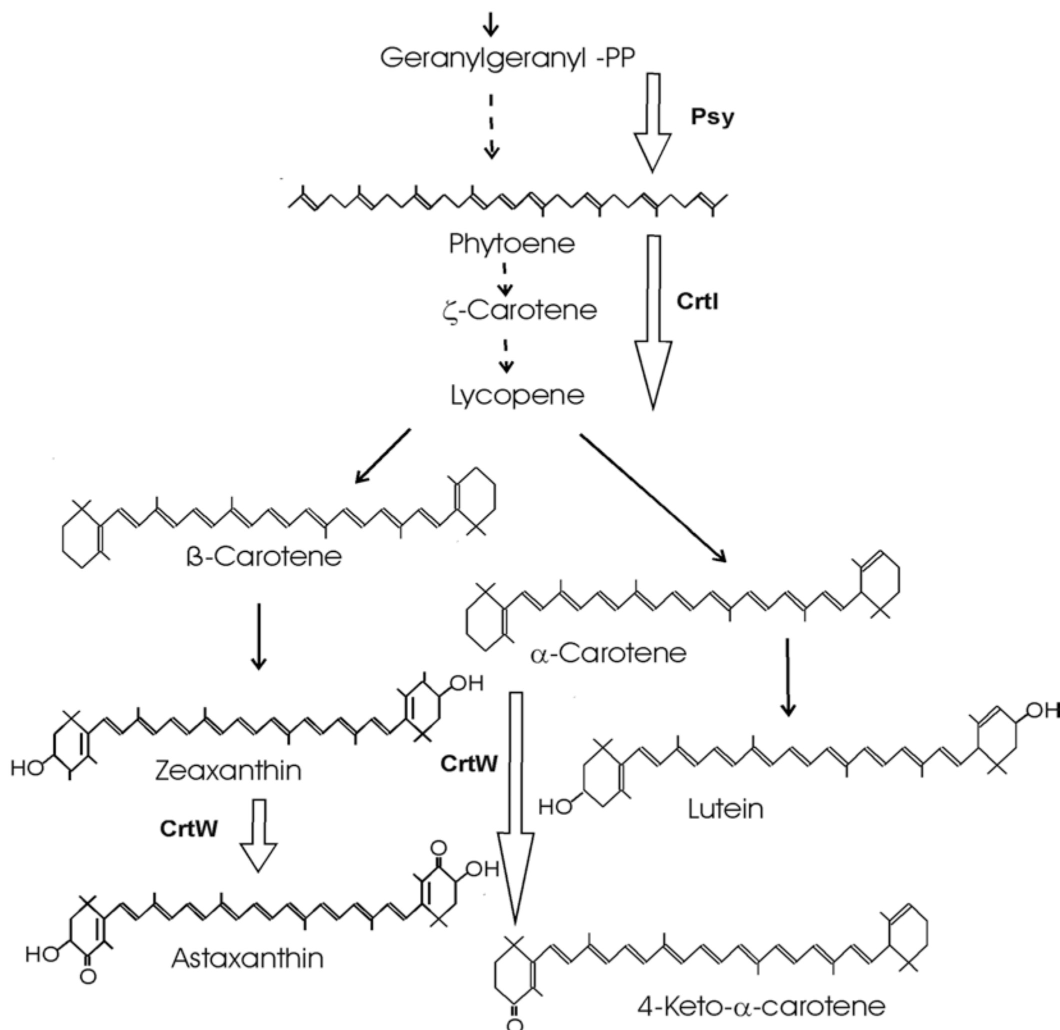

Fig. 1 The biosynthesis pathway of astaxanthin formation in transgenic endosperm-

derived rice callus

Dotted arrows indicate pathway limitations in rice, open arrows indicate the reactions catalysed by the transgenes *PSY1* (phytoene synthase), *CRTI* (phytoene desaturase) and *CRTW* (carotene ketolase), and solid arrows indicate maize-specific carotenogenic reactions.

Considerable effort has been invested to achieve sustainable and economic production of astaxanthin in microorganisms and plants (Zhu et al., 2009). Our strategy has been to engineer suitable plant material such as rice grains to synthesize astaxanthin for directly feeding or consumption (Sandmann, 2001). In previous work, we reported that transformation of maize with a ketolase and an additional hydroxylase gene resulted in the accumulation of astaxanthin (Zhu et al., 2008). However, the yields were not satisfactory. Therefore, we have chosen rice as an alternative crop for the engineering of carotenogenesis using combinatorial genetic transformation (Farre et al., 2012). The endosperm-based rice callus system allowed us to investigate the impact of pathway modulation on carotenoid composition before the time consuming and labour intensive regeneration of intact plants. Transformation was carried out simultaneously with three genes, a maize phytoene synthase, a bacterial phytoene desaturase and a bacterial β-carotene ketolase gene, which in combination with the endogenous endosperm expressed β-carotene hydroxylase should be able to synthesize astaxanthin.

Engineering of the carotenoid biosynthesis pathway in rice endosperm was successful in terms of engineering a ketolation pathway leading to astaxanthin as the end product. However in addition to the expected hydroxy and keto intermediates derived from β-carotene, we detected a novel carotenoid which did not fit directly into the pathway to astaxanthin. Its identification is the scope of this work.

RESULTS AND DISCUSSION

The initial synthesis of phytoene and its 4-step desaturation and isomerization to lycopene was engineered into rice callus in combination with a bacterial carotenoid ketolase (**Fig. 1**). Expression of carotenoid ketolase and all other transgenes has been shown previously (Bai et al., 2013). Fig. 2A shows the HPLC carotenoid profile of a typical transgenic line. Two carotenes were synthesized by the interaction of phytoene synthase and desaturase and the endogenous lycopene cyclases, α-carotene (peak 5) and β-carotene (peak 6). The prominent carotenoid peak 1 at 6.7 min with the typical bell shaped optical absorbance spectrum with its maximum at 475 nm resembles astaxanthin. This is the end product of the ketolation pathway starting from β-carotene or zeaxanthin (**Fig. 1**) which was initiated by the transgenic carotenoid ketolase. This enzyme works by interaction with the endogenous β-carotene hydroxylase which is active and specific enough for astaxanthin synthesis. Two other peaks resemble intermediates of this pathway, 4-keto-zeaxanthin peak 2 and echinenone peak 4 (**Fig. 2A**), both with an asymmetrical bell shaped optical absorbance spectrum with a maximum at 465 nm (**Fig. 3B**). The latter originated directly from ketolation of β-carotene, the other by ketolation of zeaxanthin.

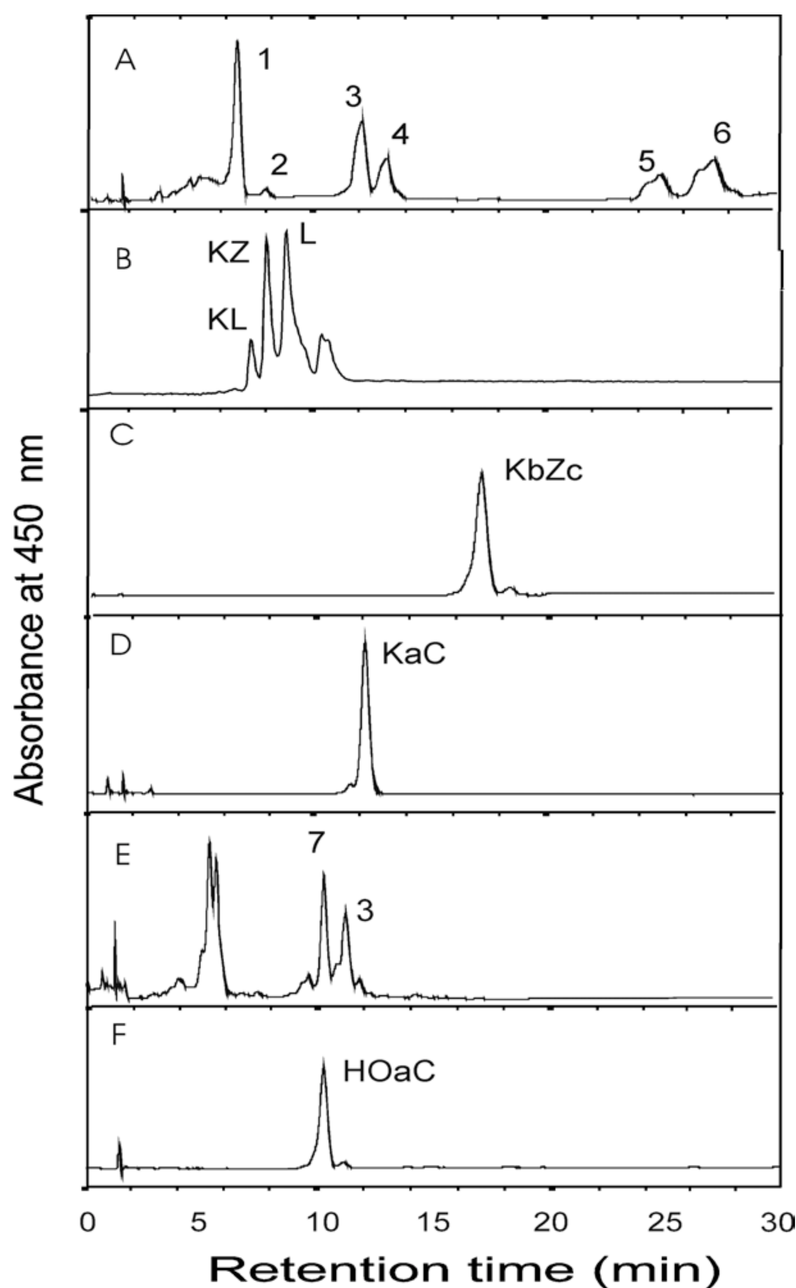

Fig. 2 HPLC separation of carotenoids from transgenic rice callus with a ketolase gene (A). (B) isolate with 4-keto-lutein, (C) 4-keto-β-zeacarotene (KbZc) standard, (D) 4-keto-α-carotene standard (KaC), (E) co-chromatography of rice extract with 4-keto-α-carotene standard, (F) carotenoids from transgenic rice callus after reduction with $NaBH_4$, (G) 4-hydroxy-a-carotene (HOaC) standard

Peak 1, axtaxanthin; 2, 4-keto-zeaxanthin; 3, novel unknown carotenoid; 4, echinenone; 5, α-carotene; 6, β-carotene; 7, 4-hydroxy-α-carotene; 8, 4-hydroxy-echinenone. L, lutein; KL, 4-keto-lutein; KZ, 4-keto-zeaxanthin.

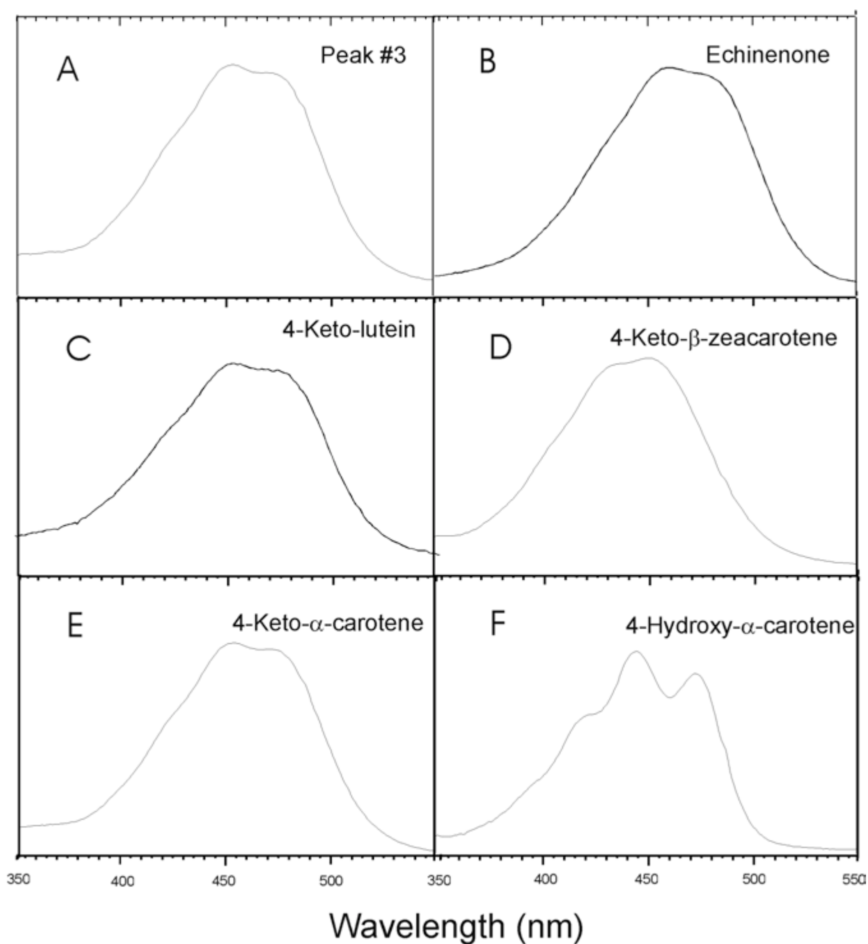

Fig. 3 Optical spectra of unknown carotenoid (A), echinenone (=4-keto-β-carotene) (B), 4-keto-lutein (C), 4-keto-β-zeacarotene (D), 4-keto-α-carotene (E) and 4-hydroxy-α-carotene (F)

One compound which does not fit into this pathway is the carotenoid represented by peak 3 (**Fig. 2A**). Its spectrum is shown in Fig. 3A. Although it has a very similar shape to the spectrum of echinenone (**Fig. 3B**) which may indicate a mono-keto carotenoid, its maximum of 452 nm is 14 nm lower than that of echinenone. The only known carotenoid with the same absorbance and shape of the spectrum is 4-ketolutein (**Fig. 3C**). This was extracted from transgenic *Nicotiana tabaccum* nectary tissue transformed with a cyanobacterial β-carotene ketolase gene (Gerjets et al., 2007). However, it is more polar with its retention time of 7.5 min compared to 12.2 min for peak 3 (**Fig. 2B**).

From the spectrum and the basic carotenoid pathway to α- and β-carotene related compounds, 4-keto-β-zeacarotene (7,8-dihydro-β,ψ-carotene-4-one) and 4-keto-α-carotene (β,ε-carotene-4-one) are the most likely candidates for the novel carotenoid molecule. Therefore, we generated these mono keto carotenoids which should exhibit not only a similar absorbance but also a similar polarity as reference compounds by combinatorial biosynthesis in *E. coli* (Sandmann, 2002). In the case of 4-keto-β-zeacarotene the absorbance maximum was the same as that of as that of the novel compound but the shape of the absorbance peak did not match (**Fig. 3D**). In addition, this carotenoid did not co-chromatograph with compound 3 due to its longer retention time (**Fig. 2C**). Only 4-keto-α-carotene matched exactly the spectrum of compound 3 (**Fig. 3E**) and it exhibited the same retention time in the HPLC diagram (**Fig. 2D**). For further confirmation of the structure of compound 3, the carotenoid extract from rice was reduced by sodium borohydride. This chemical modification of the keto group was also carried out with the 4-keto-α-carotene reference compound resulting in 4-HO-α-carotene. In each case, a more polar compound was formed (**Fig. 2D and 2F**). The spectrum of this hydroxy derivative shows the typical three maxima of non-ketolated carotenoids exhibiting a hypsochromic shift to 442 nm of the main central maximum to (**Fig. 3F**) for reduction of a keto group conjugated with the polyene chain. The resulting spectrum resembles that of α-carotene (Britton et al., 2004).

For further structure elucidation of peak 3, a mass spectrum was determined by HPLC-MS with atmospheric pressure chemical ionisation identifying the $[M+H]^+$ ions (**Fig. 4A**). The dominating mass peak in the spectrum is the ion at *m/z* 552. This may resemble the protonated molecular ion of compound 3 with a molecular mass of 551 g/mol. Several carotenoid masses fit to this value including the one for echinenone with 550.86 g/mol (Rivera et al., 2011). Starting from $[M+H]^+$ ion of 552, transitions related

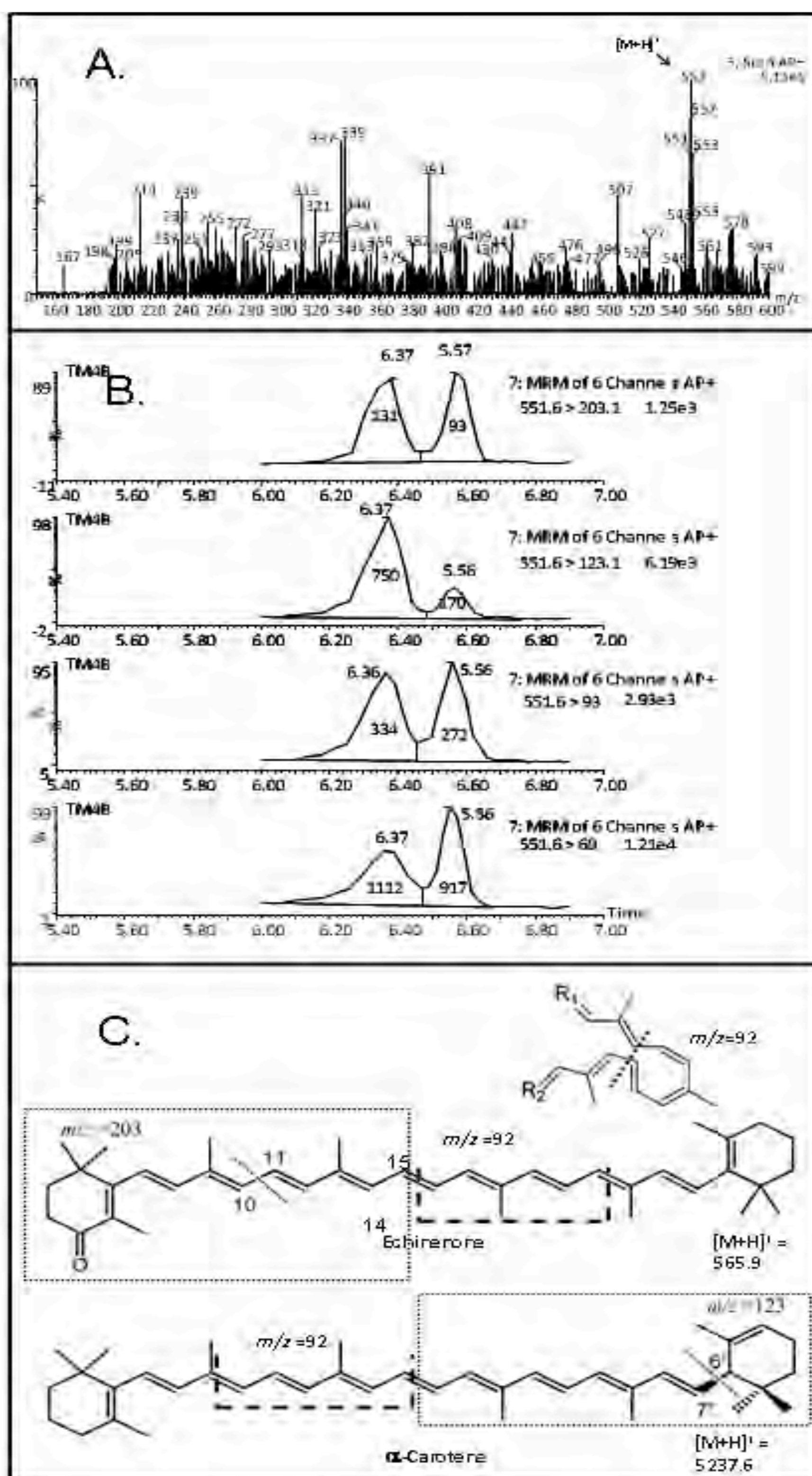

Fig. 4 Mass spectroscopy of unknown compound

(A) Atmospheric pressure chemical ionisation mass spectrum, (B) transitions from the $[M+H]^+$ ion of 552, (C) fragmentation pattern of echinenone and α-carotene. Boxes indicate the two halves of the identified molecule, 4-keto-α-carotene.

to functional groups in the carotenoid structures were built. The ones shown in Fig. 4B were those with characteristic signals for identification. The ones to identify an echinenone-related structure are the transitions 551.6>203.1 resulting from the cleavage at C-10,11 of a 4-keto fragment as indicated in Fig. 4C (van Bremen et al., 2012), transition 551.6>93 by in-chain elimination of toluene and transition 551.6>69 previously reported for echinenone (Enzell et al., 1969). One of the most intense transitions is 551.6>123.1. This transition is a specific indicator of the existence of an ε-ring in the structure of compound 3 (Enzell and Back, 1995). Taken together all results from mass spectroscopy, we can identify compound 3 as a carotenoid combining the ketolated half of echinenone and the other half with the ε-ring of α-carotene (boxed in **Fig. 4C**). This is consistent with 4-keto-a-carotene which has the same molecular mass 4-keto-α-carotene with the same molecular mass as echinenone.

HPLC co-chromatography, chemical modification and mass spectrometry all identified compound 3 as 4-keto-α-carotene. This is a unique carotenoid not reported thus far (Britton et al., 2004). A similar but different carotenoid is 2-keto-α-carotene from the stick insect *Ectatosoma tiaratum* (Kayser, 1981). Due to the position of the keto group at C-2, it does not contribute to the conjugated polyene chain which influences the optical absorbance spectrum with three pronounced peaks at 423, 446 and 475 nm, distinct from spectrum in Fig. 3E. Formation of 4-keto-α-carotene is due to the relatively high α-carotene content in the transgenic rice callus. Obviously, the carotene ketolase from *Brevundimonas* sp. possesses broad substrate specificity. This enzyme typically ketolates both β-ionone rings in β-carotene (Nishida et al., 2005). In addition, it is also able to ketolate the β-ionone end of the α-carotene molecule. This broad substrate specificity in combination with the α-carotene concentrations as substrate for the ketolase is the primary reason for the formation of 4-keto-α-carotene in the

genetically engineered rice callus.

CONCLUSIONS

Although genetic engineering of the carotenoid pathway to astaxanthin was successful, a specific side reaction occurred in rice callus. The expressed bacterial ketolase not only converted β-carotene and zeaxanthin towards astaxanthin but also accepted the β-ionone ring of α-carotene as substrate for ketolation. The resulting product was identified as 4-keto-α-carotene. Its formation branches off the desired β-carotene to astaxanthin route. This loss for the astaxanthin pathway may be avoided by further genetic engineering of carotenogenesis by antisense or RNAi down-regulation of the expression of rice specific lycopene ε-cyclase depleting the formation of α-carotene.

EXPERIMENTAL

Transgenic rice

The transgenic endosperm-derived rice callus was generated by combinatorial nuclear transformation which has been reported earlier (Zhu et al., 2008). The three plasmids contained phytoene synthase *ZmPSY1*, phytoene desaturase *PaCRTI* and β-carotene ketolase *CRTW* from *Brevundimonas* sp. Strain SD212 (Nishida et al., 2005). The latter gene was chemically synthesized according to the codon usage of *Brassica napus* and fused to the full-length rice alcohol dehydrogenase 5´-untranslated region (Sugio et al., 2008) and to the transit peptide sequence from pea ribulose 1, 5-bisphospate carboxylase small subunit (Schreier et al., 1985). This DNA fragment was inserted into plasmid GZ63 containing the maize γ-zein gene promoter and the *nos* terminator. The maize *PSY1* cDNA (Buckner et al., 1996) was transferred into plasmid p326 containing

the LWM glutenin promoter (Stoger et al., 1999) and *NOS* terminator. The *CRTI* gene from *Pantoea ananatis* (formerly known as *Erwinia uredovora*) was fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase in plasmid pYPIET4 (Misawa et al., 1994), amplified by PCR and then transferred to pHorp-P containing the barley D-hordein promoter (Sørensen et al., 1996) and the rice ADPGPP terminator. All transformation constructs are based on pUC8 plamids. Their map is shown in Fig. 5. Transgenic coloured calli were selected and cultured on MS selection medium (Farre et al., 2012).

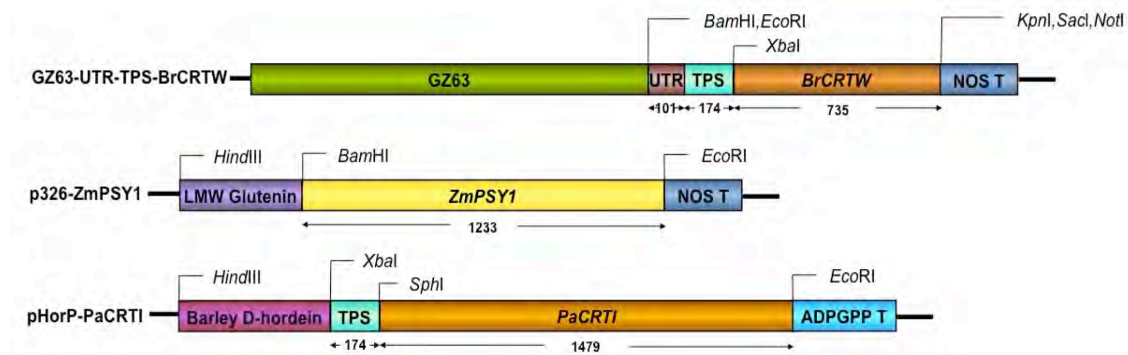

Fig. 5 pUC8-derived plasmid constructs used for rice transformation to express carotene ketolase (BrCRTW), phytoene synthase (ZmPSY1) and phytoene desaturase (PaCRTI)).

GZ63, maize γ-zein gene promoter; UTR, 5´-untranslated region from rice alcohol dehydrogenase; TPS, transit peptide from pea ribulose-1, 5-bisphospate carboxylase small subunit; NOS T, *nos* terminator; LMW Glutenin, LWM glutenin promoter; Barley D-hordein, barley D-hordein promoter; ADPGPP T, rice ADPGPP terminator.

Combinatorial carotenoid synthesis in Escherichia coli

For the synthesis of reference compounds the biosynthesis pathways for 4-keto-α-carotene and 4-keto-β-zeacarotene the following combinations of compatible plasmids

were used to transform *E. coli* strain DH5α. Plasmid pACCAR16ΔcrtX (Misawa et al., 1995) in combination with pBBRK-ara-epsilon with the lycopene ε-cyclase from *Arabidopsis thaliana* (Cunningham et al., 1996) cloned into pBBR1-MCS1 with kanamycin as selection marker (Kovach et al., 1994) mediates the formation of α-carotene. This carotene is then ketolated at the β-ionone ring to 4-keto-α-carotene by expression of pPEU30CRTO (Breitenbach et al., 2013). 4-Keto-β-zeacarotene is synthesized from neurosporene generated by pACCRT-EBI_{RC} (Linden et al., 1993), which is cyclised by expression of plasmid pRK-crtY (Hausmann and Sandmann, 2000) and finally ketolated by expression of pCRBKT (Zhong et al., 2011). The *E. coli* transformant generating 4-keto-α-carotene was grown at 37°C in the presence of ampicillin (100 µg/ml), chloramphenicol (34 µg/ml), kanamycin (25 µg/ml) and IPTG (1 mM) in LB medium (Sambrook et al., 1989). The 4-keto-β-zeacarotene producing transformant was cultured at 28°C in the same medium without IPTG replacing kanamycin with tetracyclin (25 µg/ml).

Carotenoid extraction and analysis

Carotenoids were extracted from freeze-dried rice callus, *E. coli* cells and nectary tissue from *Nicotiana tabaccum* (Gerjets et al., 2007) for 20 min with methanol at 60°C and partitioning into 10% ether in petrol. In the case of tobacco nectary tissue, the 10% ether in petrol phase was diluted 3: 1 with hexane, passed through a silica column and the adsorbed polar carotenoid fraction eluted with acetone (Steiger et al., 1999). Analytical HPLC was isocratic on a 15 cm Nucleosil C18, 3µ column with acetonitrile/ methanol/2- propanol (85: 10: 5, v/v) at 10°C and a flow 0.8 ml/min. Carotenoids were identified by their optical absorbance spectra and co-chromatography with standard carotenoids. In the case of the unknown carotenoid, its biosynthetic pathway was

reconstructed in *E. coli*. This carotenoid was reduced to 4-hydroxy-α-carotene with sodium borohydride ($NaBH_4$) (Eugster, 1995). For mass analysis, UHPLC was carried out using an ACQUITY Ultra Performance LC TM system linked to an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx™ software version 4.1 (Waters, Milford, MA, USA) was used to control the instruments, and also for data acquisition and processing. Separation was on a reverse phase ACQUITY UPL® C18 BEH 130 Å, 1.7 µm, 2.1 × 100 mm column (Waters, Milford, MA) and a gradient system with the mobile phase consisting of solvent A: acetonitrile: methanol (70: 30, v/v) and solvent B: water 100 % at a flow rate of 0.35 ml/min (Rivera et al., 2013).

REFERENCES

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P., Zhu C., 2013. An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. Plant J. http://dx.doi.org/10.1111/tpj.12384, 2013 Nov 23.

Bjerkeng, B., 2008. Carotenoids in aquaculture: fish and crustaceans. In: Britton G. Liaaen–Jensen S, Pfander H, (Eds.) Carotenoids, Vol 4, Natural Functions, Birkhäuser Verlag, Basel, pp, 237–254.

Breitenbach, J., Gerjets, T., Sandmann, G., 2013. Catalytic properties and reaction mechanism of the CrtO carotenoid ketolase from the cyanobacterium *Synechocystis* sp. PCC6803. Arch. Biochem. Biophys. 529, 86–91.

Britton, G., Liaaen-Jensen, S., Pfander, H., 2004. Carotenoids Handbook: Birkhäuser Verlag, Basel.

Buckner, B., San Miguel, P., Janick-Buckner, D., Bennetzent, J.L., 1996. The *yl* gene of maize codes for phytoene synthase. Genetics 143, 479–488.

Cunningham, F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D., Gantt, E., 1996. Functional analysis of the β and ε lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8, 1613–1626.

Enzell, C.R., Back, S., 1995. Mass spectrometry. In: Britton, G., Liaaen–Jensen, S., Pfander, H. (Eds.), Carotenoids, Spectroscopy, Vol. 1B Birkhäuser Verlag, Basel, pp, 261–320.

Enzell, C.R., Francis, G.W., Liaaen-Jensen, S., 1969. Mass spectrometric studies of

carotenoids. 2. A survey of fragmentation reactions. Acta Chem. Scand. 23, 727–750.

Eugster, C.H., 1995. Chemical derivatization: microsomale tests for the presence of common functional groups in carotenoids, In: Britton, G., Liaaen–Jensen, S., Pfander, H. (Eds.), Carotenoids, Isolation and Analysis, Vol. 1A BirkhäuserVerlag, Basel, pp. 71–80.

Farre, G., Naqvi, S., Sanahuja, G., Bai, C., Zorrilla-López, U., Rivera, S.M., Canela, R., Sandmann, G., Twyman, R.M., Capell, T., Zhu, C., Christou, P., 2012. Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. Methods Mol. Biol. 847, 419–435.

Fraser, P.D., Römer, S., Shipton, C.A., Mills, P.B., Kiano, J.W., Misawa, N., Darke, R.G., Schuch, W., Bramley, P.M., 2002. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit–specific manner. Proc. Natl. Acad. Sci. USA 99, 1092–1097.

Fraser, P.D., Enfissi, E.M., Bramley, P.M., 2009. Genetic engineering of carotenoid formation in tomato fruit and the potential application of systems and synthetic biology approaches. Arch. Biochem. Biophys. 483, 196–204.

Gerjets, T., Sandmann, M., Zhu, C., Sandmann, G., 2007. Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species. Biotechnol. J. 2, 1263–1269.

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

Hausmann, A., Sandmann, G., 2000. A single 5-tep desaturase is involved in the carotenoid biosynthesis pathway to β-carotene and torulene in *Neurospora crassa*, Fung. Gen. Biol. 30, 147–153.

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid in human health and nutrition. J. Nat. Prod. 69, 443–449.

Kayser, H., 1981. Carotenoids in the stick insect Ectatosoma tiaratum isolation of β,ε-carotene-2-ol and β,ε-carotene-2-one. Z. Naturforsch. 36c, 755–764.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., Peterson, K.M., 1994. PBBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Linden, H., Vioque, A., Sandmann, G., 1993. Isolation of a carotenoid biosynthesis gene coding for a zeta-carotene desaturase from *Anabaena* PCC7120 by heterologous complementation. FEMS Microbiol. Lett. 106, 99–104.

Misawa, N., 2011. Pathway engineering for functional isoprenoids. Curr. Opin. Biotechnol. 22, 627–633.

Misawa, N., Masamoto, K., Hori, T., Ohtani, T., Böger, P., Sandmann, G., 1994. Expression of an *Erwinia* phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. Plant J. 6, 481–489.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., Miki, W., 1995. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J. Bacteriol. 177, 6575–6584.

Naqvi, S., Zhu, C., Farre, G., Sandmann, G., Capell, T., Christou, P., 2011. Synergistic metabolism in hybrid corn reveals bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. Plant Biotechnol. J. 9, 384–393.

Nishida, Y., Adachi, K., Kasai, H., Shizuri, Y., Shindo, K., Sawabe, A., Komemushi, S.,

Miki, W., Misawa, N., 2005. Elucidation of a carotenoid biosynthesis gene cluster encoding a novel enzyme 2 2'–beta–hydroxylase from *Brevundimonas* sp. strain SD212 and combinatorial biosynthesis of new or rare xanthophylls. Appl. Environ. Microbiol. 71, 4286–4296.

Paine, J.A., Shipton, C.A., Chaggar, R.M. Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L., Drake, R., 2005. Improving the nutritional value of Golden Rice through increased pro–vitamin A content. Nat. Biotechnol. 23, 482–487.

Quackenbush, F.W., Firch, J.G., Brunson, A.M., House, L.R., 1963. Carotenoid, oil, and tocopherol content of corn inbreds. Cereal Chem. 40, 250–253.

Rivera, S., Vilaró, F., Canela, R., 2011. Determination of carotenoids by liquid chromatography/mass spectrometry: Effect of several dopants. Analyt Bioanalyt Chem 400, 1339–1346.

Rivera, S.M., Vilaró, F., Zhu, C., Bai, C., Farré, G., Christou, P., Canela-Garayoa, R., 2013. Fast quantitative method for the analysis of carotenoids in transgenic maize. J. Agric. Food Chem. 61, 5279–5285.

Römer, S., Lübeck, J., Kauder, F., Steiger, S., Adomat, C., Sandmann, G., 2002. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metabolic Engin. 4, 263–272.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manua, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandmann, G., 2001. Genetic manipulation of carotenoid biosynthesis: strategies problems and achievements. Trends Plant Sci. 6, 14–17.

Sandmann, G., 2002. Combinatorial biosynthesis of carotenoids in a heterologous host:

A powerful approach for the biosynthesis of novel structures. Chem. Bio. Chem. 3, 629–635.

Sandmann, G., Römer, S., Fraser, P.D., 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metabolic Engin. 8, 291–302.

Schaub, P., Al-Babili, S., Drake, R., Beyer, P., 2005. Why is golden rice golden (yellow) instead of red? Plant Physiol. 138, 441–450.

Schreier, P., Seftor, E., Schell, J., Bohnert, H., 1985. The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. 4, 25–32.

Sørensen, M.B., Müller, M., Skerritt, J., Simpson, D., 1996. Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. Mol. Gen. Genet. 250, 750–760.

Steiger, S., Schäfer, L., Sandmann, G., 1999. High–light upregulation of carotenoids and their antioxidative properties in the *cyanobacterium Synechocystis* PCC 6803. J. Photochem. Photobiol. 521, 14–18.

Stoger, E., Williams, S., Keen, D., Christou, P., 1999. Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. Transgenic Res. 8, 73–82.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A., Kato, K., 2008. The 5'–untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells. J. Biosci. Bioeng. 105, 300–330.

Van Breemen, R.B., Dong, L., Pajkovic, N.D., 2012. Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids. Int. J. of Mass Spectrometry 312, 163–172.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I., 2000. Engineering the provitamin A (beta–carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287, 303–305.

Zhong, Y.J., Huang, Y.C., Liu, J., Li, Y., Jiang, Y., Xu, Z.F., Sandmann, G., Chen, F., 2011. Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic *Arabidopsis*. J. Exp. Bot. 62, 3659–3669.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T., 2008. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc. Natl. Acad. Sci. 105, 18232–18237.

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

Chapter 3

Unravelling constraints in carotenoid accumulation in rice (*Oryza sativa*) endosperm through multigene engineering

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SUMMARY

The quantitative and qualitative profile of secondary metabolites in plants reflects the net result of their biosynthesis, degradation, and storage. We focused on the carotenoid pathway to test the hypothesis that as yet unknown metabolic bottlenecks in a biosynthetic pathway constrain the further accumulation of metabolites in plants and these might be unraveled by combinatorial genetic transformation and investigating their regulation in engineered plants expressing appropriate input gene combinations. We used rice as a model to ascertain the impact of co-expressing genes involved in the carotenoid pathway either directly or indirectly. Specifically we introduced the Arabidopsis 1-deoxy-D-xylulose 5-phosphate synthase gene (*AtDXS*) and *Orange* gene (*AtOR*) in separate experiments but always in combination with *ZmPSY1* and *PaCRTI*, as these genes are essential for carotenoid biosynthesis in rice endosperm. The combinatorial nature of our experiments permitted the direct comparison of subpopulations of plants with different transgene complements. Endosperm tissue co-expressing binary and tertiary transgene combinations allowed us to infer that the supply of isoprenoid precursors derived from the MEP pathway is one of the key factors limiting carotenoid accumulation in the endosperm. Experiments involving *AtOR*

clearly demonstrated that overexpression of *AtOR* could also increase substantially carotenoid accumulation in rice endosperm though upregulation of a series of endogenous carotenogenic genes. The understanding of the nature of remaining metabolic bottlenecks in the pathway will permit the design and implementation of more refined strategies for the creation of engineered plants with particular carotenoid profiles.

INTRODUCTION

Plants synthesize a wide variety of secondary metabolites using complex pathways (Miralpeix *et al.*, 2013; Richer *et al.*, 2013). Many genes involved in secondary metabolite biosynthesis, transport and storage are not well characterized. In addition, compartmentalization of the corresponding enzymes within subcellular organelles plays a critical role in the control of secondary metabolite pathways (O'Connor and Maresh, 2006). Therefore, many challenges need to be overcome before multistep secondary metabolic pathways can be engineered in heterologous plants effectively.

The endosperm tissues of cereal crops, such as rice, maize and wheat serve as major food staples world-wide, though they are deficient in nutritionally essential carotenoids (Zhu *et al.*, 2007, 2008). In humans and animals, various carotenoids derived from plant sources act as antioxidants and protect against important diseases, while other carotenoids are precursors of vitamin A and retinoid derivatives which are essential for a variety of critical functions in humans (Fraser and Bramley, 2004; Bai *et al.*, 2011).

Rice, an important food staple in developing countries worldwide, accumulates no carotenoids in its endosperm and is therefore associated with vitamin A deficiency (Underwood and Arthur, 1996; Farré *et al.*, 2010). Humans can synthesize vitamin A if provided with the precursor molecule β-carotene (also known as provitamin A; von Lintig and Vogt, 2004).

Enhancement of carotenoid content and/or composition is dependent on expression of the requisite pathway enzymes and availability of isoprenoid precursors. Generally, carotenoid levels in plants do not depend solely on carotenogenic enzyme activities. The upstream pathways supplying carotenoid precursors may also influence carotenoid accumulation (Rodríguez-Concepción, 2010), and downstream pathways that metabolize carotenoids deplete the carotenoid pool (Ohmiya *et al.*, 2006; Auldridge *et al.*, 2006; Campbell *et al.*, 2010). The characterization of *Orange* (*OR*) a cauliflower mutation that induces proplastids and/or non-colored plastids to differentiate into chromoplasts, demonstrated that the creation of a metabolic sink to sequester carotenoids can also be used to promote their accumulation in plants (Lu *et al.*, 2006; Li and van Eck, 2007; Lopez *et al.*, 2008). Thus, the final content of carotenoids in plants is the net result of their biosynthesis, degradation, and storage (Lu and Li, 2008; Cazzonelli and Pogson, 2010).

In addition to lack of a suitable sink structure for effectively sequestering and storing carotenoids the supply of isoprenoid precursors such as GGPP (geranylgeranyl diphosphate) is thought to be an additional rate-limiting step in carotenoid accumulation in rice endosperm since phytoene was absent in the endosperm of Golden Rice (Ye *et*

al., 2000; Paine *et al.*, 2005). In the present study we focused on the expression of a heterologous 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in rice endosperm in order to investigate whether modulation of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway might impact carotenoid biosynthesis and accumulation (Figure 1) (Bai *et al*., 2011, 2013). Our results indicate that expression of *AtDXS* in combination with maize (*Zea mays*) phytoene synthase 1 (*ZmPSY1*) and *Pantoea ananatis* phyoene desaturase (*PaCRTI*), significantly enhances carotenoid accumulation in rice endosperm, suggesting that the supply of isoprenoid precursors such as GGPP is a limiting factor in carotenogenesis in rice endosperm. In parallel experiments we investigated the impact of creating a metabolic sink in the endosperm by overexpression of wild type Arabidopsis *Orange* gene (*AtOR*) which has been recently demonstrated to sequester carotenoids in rice callus (Bai *et al.*, 2013). Endosperm co-expressing *AtOR*, *ZmPSY1* and *PaCRTI* exhibited greatly elevated carotenoid accumulation compared to tissues only co-expressing *ZmPSY1* and *PaCRTI*, indicating that overexpression of *AtOR* could also increase substantially carotenoid accumulation in rice endosperm through upregulation of a series of endogenous carotenogenic genes.

Figure 1 General carotenoid biosynthetic pathway (Bai *et al*., 2011, 2013)

Abbreviations: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; DXR, DXP reducoisomerase; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; HDR, hydroxymethylbutenyl diphoshate reductase; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB,

lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene hydroxylase (BCH, CYP97A or CYP97B).

RESULTS

Combinatorial transformation of carotenogenic genes into rice endosperm generates diverse genotypes and phenotypes with different qualitative and quantitative carotenoid profiles

In order to investigate how modulation of the MEP pathway impacts carotenoid biosynthesis and accumulation in rice endosperm, we introduced into seven-day-old mature zygotic rice embryos four constructs containing unlinked transgenes. These comprised two carotenogenic transgenes in the committed carotenoid biosynthesis pathway, namely *ZmPSY1* and *PaCRTI*, and *AtDXS* as well as the selectable marker *HPT* for hygromycin resistance. The *HPT* gene was expressed constitutively and the three transgenes were expressed specifically in the endosperm. *AtDXS, ZmPSY1 and PaCRTI* were driven by the rice RP5 prolamin promoter, wheat low-molecular-wheat glutenin gene promoter and barley D-hordein promoter, respectively. Two different endosperm phenotypes were obtained depending on the particular combination of integrated transgenes, namely yellow and orange (Figure 2). Similarly, in order to investigate how sequestering carotenoids in an induced metabolic sink might impact their accumulation we engineered rice embryos with four constructs. These four transgenes were *ZmPSY1*, *PaCRTI*, *AtOR* and the selectable marker *HPT*. Both *ZmPSY1* and *AtOR* were driven by the wheat low-molecular-wheat glutenin gene promoter.

PaCRTI was under the control of the barley D-hordein promoter. Similar to the co-transformation experiments with *ZmPSY1*, *PaCRTI* and *AtDXS*, experiments in which the *AtOR* was expressed in rice endosperm also resulted in yellow or orange endosperm depending on the transgene combination(s) (Figures 2 and 3).

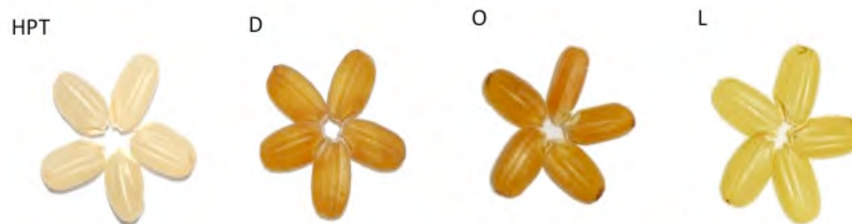


Figure 2 Phenotype of transgenic and wild type rice seeds expressing different combinations of carotenogenic genes

Two different endosperm phenotypes at 40 DAP were obtained. The seed endosperm with orange phenotype co-expressed *AtDXS*, *ZmPSY1* and *PaCRTI* in lines D and with similar orange phenotype co-expressed *AtOR*, *ZmPSY1* and *PaCRTI* in lines O, whereas the seed endosperm with yellow phenotype (L) only co-expressed *ZmPSY1* and *PaCRTI.* Abbreviations: HPT, seed endosperm expressing *HPT*; D, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtDXS*; O, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtOR*; L, seed endosperm expressing *ZmPSY1* and *PaCRTI.*

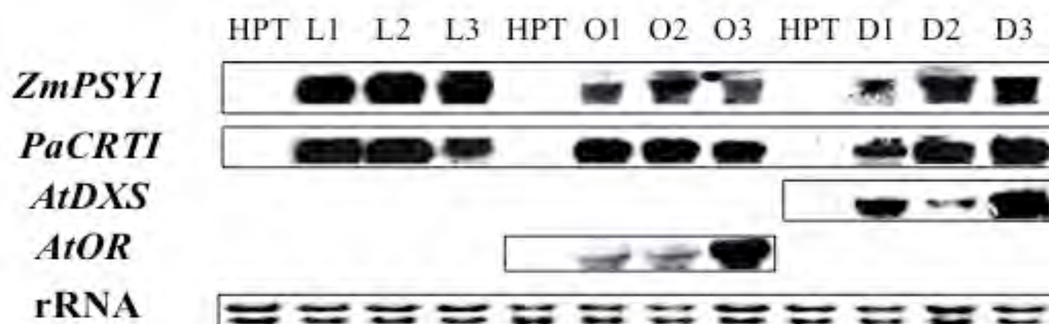


Figure 3 Transgene expression analyses in rice endosperm

Transgene expression in rice endosperm (T3) at 25 DAP (25 µg of total RNA was loaded for each sample). Line names are defined in the legend to Figure 2.

The analysis of steady-state mRNA levels showed that the seed endosperm with orange phenotype co-expressed *AtDXS*, *ZmPSY1* and *PaCRTI*, or *AtOR*, *ZmPSY1* and *PaCRTI* whereas the seed endosperm with yellow phenotype only co-expressed *ZmPSY1* and *PaCRTI* (Figure 3). Three representative independent lines from over 20 independent lines with identical genotypes with each gene combination were used to analyze the carotenoid content and composition in rice endosperm in detail. The total carotenoid content of endosperm expressing *ZmPSY1* and *PaCRTI* was 5.43, 5.51 and 4.61, µg/g DW in lines L1, L2 and L3 respectively (Table 1), while the total carotenoid content of endosperm expressing *ZmPSY1*, *PaCRTI* and *AtDXS* was 17.79, 14.94 and 31.78 µg/g DW in lines D1, D2 and D3, respectively. The total carotenoid content of endosperm expressing *ZmPSY*, *PaCRTI* and *AtOR* was 11.53, 18.59 and 25.83 µg/g DW in lines O1, O2 and O3, respectively (Table 1). The carotenoid accumulation in endosperm of lines with orange phenotype was approximately 2.1- to 4.7-fold in *AtDXS* expressing lines (D1, D2 and D3 endosperm expressing *ZmPSY1*, *PaCRTI* and *AtDXS*) and 2.7- to 5.8-fold in *AtOR* expressing lines (O1, O2 and O3 endosperm expressing *ZmPSY1*, *PaCRTI* and *AtOR*) higher than those lines with yellow endosperm phenotype (L1, L2 and L3) expressing *ZmPSY1* and *PaCRTI* only (Figures 4 and S1). The ratios of β-carotene to the total carotenoid amount in lines O1-O3 and D1-D3 (40%-50% and 47%-52%, respectively) were higher as compared to those of lines L1-L3 (25%-39%). The β-carotene levels in endosperm were much higher in lines O1-O3 and D1-D3 (an average of 8.3 and 10.8 µg/g DW, respectively) compared with the lines L1-L3 (an average of 1.6 µg/g DW) (Table 1). The levels of α-carotene were also higher in lines O1-O3 and

D1-D3 (an average of 6.0 and 6.1 µg/g DW, respectively) compared with the lines L1-L3 (an average of 1.2 µg/g DW). Lutein levels were much higher in lines O1-O3 (an average of 1.0 µg/g DW) compared with the lines D1-D3 and L1-L3 (an average of 0.4 and 0.2 µg/g DW, respectively) (Table 1).

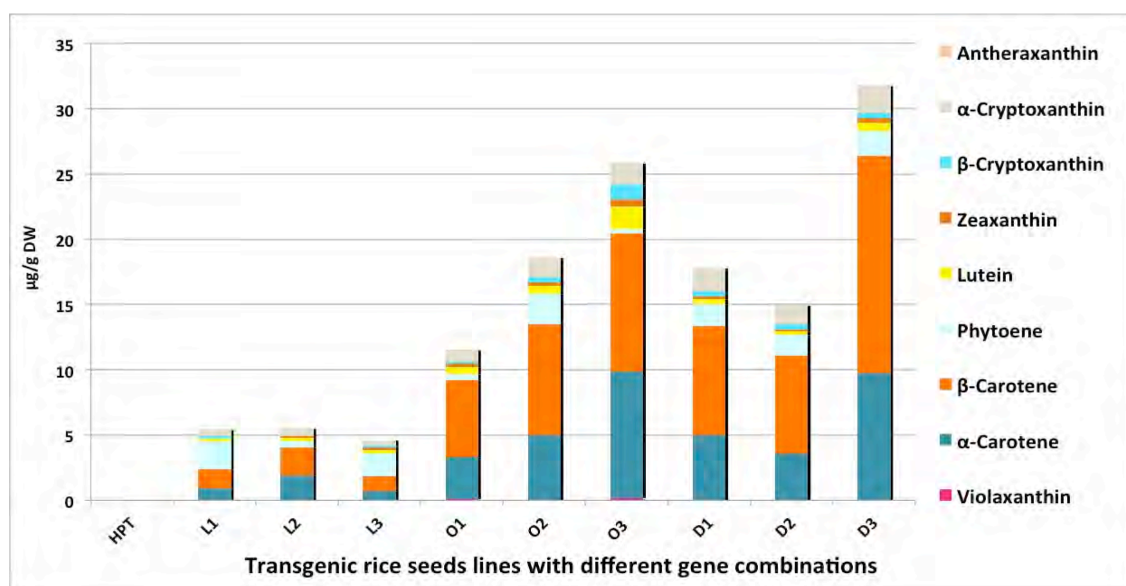


Figure 4 Carotenoid content and composition in transgenic rice endosperm

Carotenoid content and composition of all lines were analyzed by UHPLC. Bars represent different carotenoid composition in individual lines expressing different transgene combinations. All carotenoid content data were averaged on three independent measurements (T3 mature seeds at 40 DAP). Column names are defined in the legend to Figure 2.

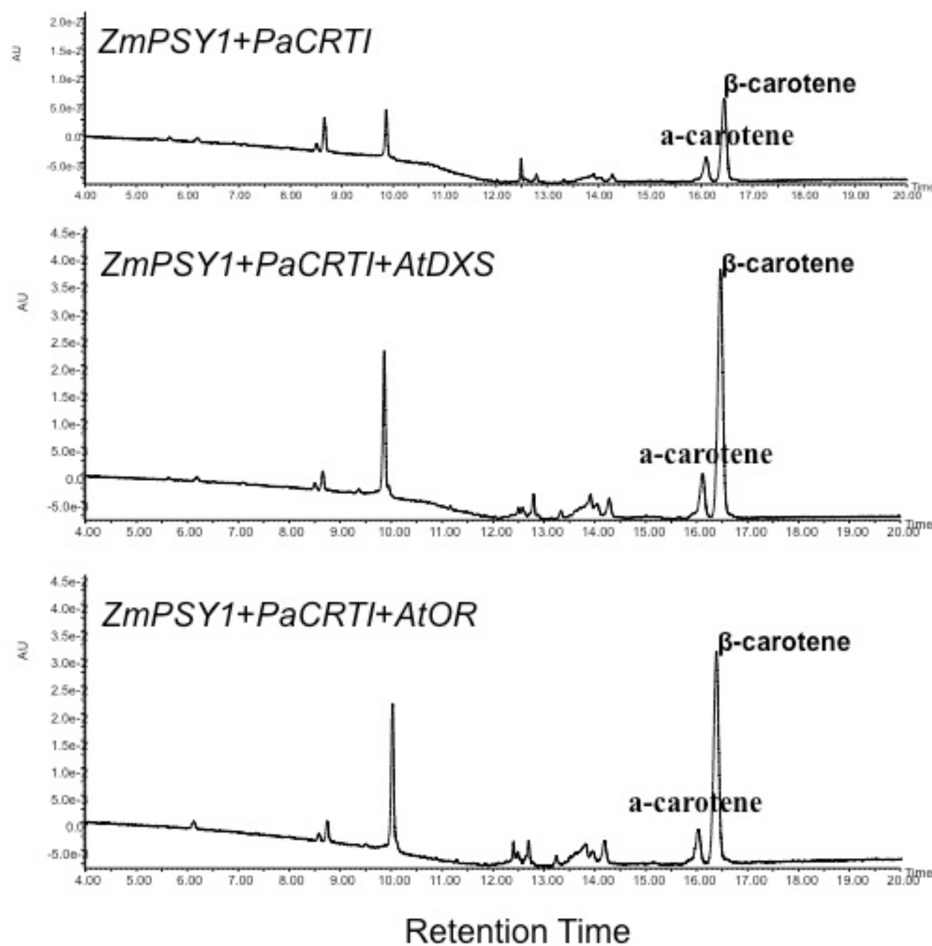


Figure S1 Carotenoid profiles of transgenic endosperm determined by HPLC

HPLC analysis shows the profile of carotenoids in the T3 endosperm (40 DAP) of transgenic rice lines L, D and O (mature seeds). Line names are defined in the legend to Figure 3. No carotenoids detected in wild type of rice endosperm.

Table 1 Carotenoid content and composition in endosperm (T3)

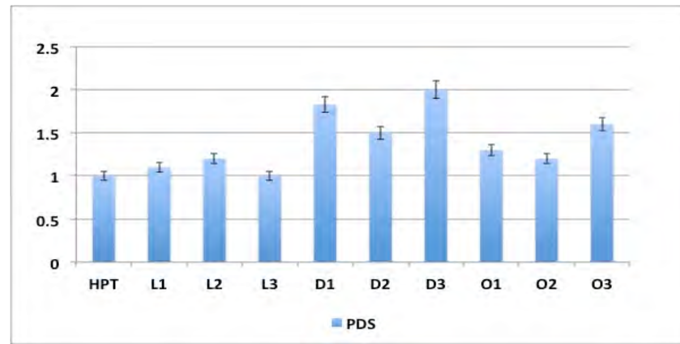
	O1	O2	O3	D1	D2	D3	L1	L2	L3
Violaxanthin	0.12±0	0	0.25±0.03	0	0	0.06±0.1	0	0	0
Antheraxanthin	0.08±0.05	0	0	0	0	0	0	0	0
Zeaxanthin	0.23±0.14	0.29±0.22	0.57±0.18	0.22±0.15	0.13±0.22	0.33±0.12	0.03±0.03	0.09±0.02	0.17±0.13
β-Cryptoxanthin	0.19±0.33	0.40±0.70	1.13±0.03	0.41±0.72	0.39±0.67	0.41±0.72	0.17±0.30	0	0.15±0.27
β-carotene	5.87±1.44	8.47±1.23	10.52±1.64	8.36±0.44	7.5±1.25	16.61±1.37	1.48±0.02	2.15±0.09	1.17±0.19
Lutein	0.55±0.07	0.58±0.20	1.73±0.16	0.39±0.2	0.26±0.16	0.69±0.39	0.16±0.09	0.26±0.02	0.26±0.09
a-Cryptoxanthin	0.82±0.13	1.49±0.10	1.63±0.16	1.74±0.08	1.48±0.10	2.09±0.68	0.53±0.01	0.61±0.04	0.42±0.08
a-carotene	3.2±0.50	5.00±1.00	9.7±0.80	5.00±0.10	3.60±0.70	9.70±1.60	0.90±0.10	1.90±0.00	0.70±0.10
Phytoene	0.47±0.06	2.36±0.35	0.35±0.30	1.67±0.52	1.58±0.12	1.89±0.28	2.16±0.05	0.50±0.01	1.74±0.06
Total Carotene	11.53±2.33	18.59±1.64	25.83±2.37	17.79±1.12	14.94±1.12	31.78±5.01	5.43±0.37	5.51±0.16	4.61±0.81
β/a	1.42	1.3	0.95	1.26	1.5	1.4	1.06	0.81	1.08
% β-carotene	50.91	45.56	40.73	46.99	50.2	52.27	27.26	39.02	25.38

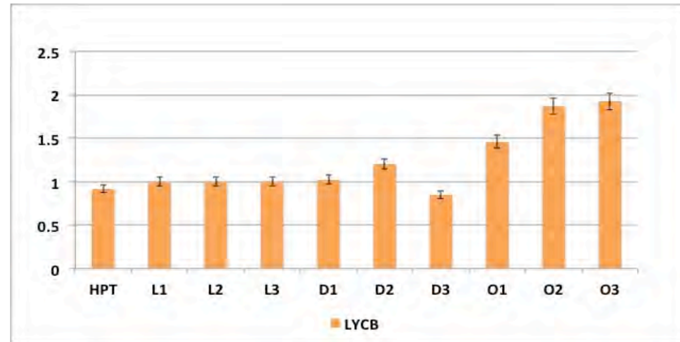
Data are means ±SD from analysis of three independent seed batches (at 40 DAP) and are expressed as µg/g DW. Values in parentheses represent the ratio of carotenoid composition to the total carotenoid amount (%). Abbreviations: HPT, seed endosperm expressing *HPT*; D, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtDXS*; O, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtOR*; L, seed endosperm expressing *ZmPSY1* and *PaCRTI.* Rice endosperm expressing *HPT* has no detectable carotenoids.

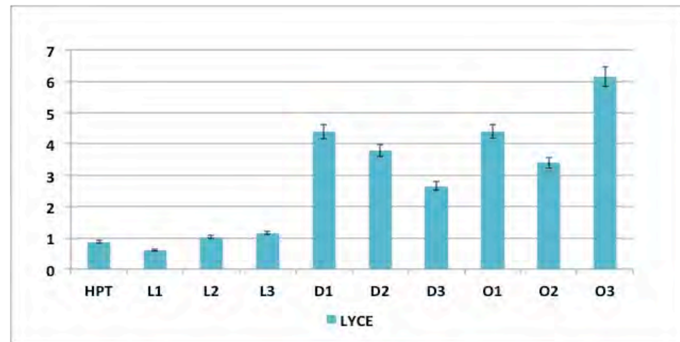
Expression of transgenes and endogenous carotenoid biosynthetic genes

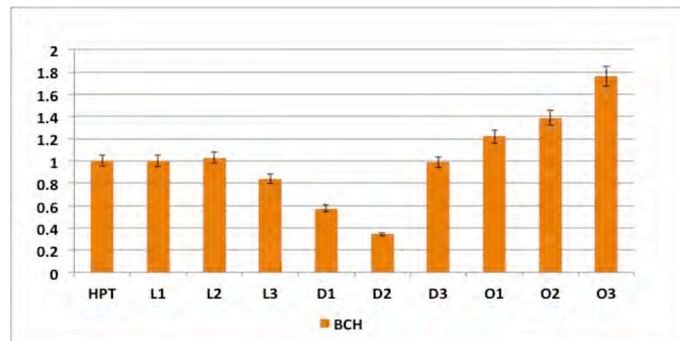
Expression analysis of transgenes (*ZmPSY1*, *PaCRTI*, *AtDXS* and *AtOR* genes) in the endosperm at 25 DAP was carried out by mRNA blot (Figure 3). Both *ZmPSY1* and *PaCRTI* mRNA accumulated in all transformants except the HPT control line, as expected. *AtDXS* mRNA accumulated in lines D1, D2 and D3 while *AtOR* mRNA accumulated in lines O1, O2 and O3 (Figure 3) in addition to *ZmPSY1* and *PaCRTI* mRNA. To further investigate whether endogenous carotenoid gene expression was influenced by the expression of the introduced transgenes, transcript levels of endogenous phytoene desaturase (*OsPDS*), lycopene β-cyclase (*OsLYCB*), lycopene ε-

cyclase (*OsLYCE*), β-carotene hydroxylase (*OsBCH*) and zeaxanthin epoxidase (*OsZEP*) genes were monitored by quantitative real-time PCR in the endosperm at 25 DAP (Figure 5). Lines (L1, L2 and L3) expressing *ZmPSY1*, *PaCRTI* exhibited similar expression levels with the *HPT* control line for all five endogenous carotenogenic genes (Figure 5). Transcript levels of *OsPDS* were upregulated in all the *AtDXS* expressing lines (D1-D3) and one *AtOR* expressing line (O3) as compared with lines L1-L3, whereas the mRNA levels were similar between O1 and O2, and lines (L1-L3) as well as HPT control (Figure 5), may result from the expression of *AtOR* in lines O1 and O2 was much lower than in line O3. That may effect on the *OsPDS* expression level. Similar expression levels for *OsLYCB* were measured in lines L1-L3, D1-D3 and HPT whereas the transcript levels were upregulated 1.4- to 1.8-fold in lines O1-O3 as compared with lines L1-L3 (Figure 5). Expression levels of endogenous *OsLYCE* were greatly upregulated in lines D1-D3 and O1-O3 as compared to lines L1-L3 (Figure 5). However, expression levels of *OsBCH* were decreased in lines D1-D3 as compared to lines L1-L3. Lines O2 and O3 had higher transcript expression of *OsBCH* than lines L1-L3, whereas line O1 had similar expression levels with lines L1-L3. For *OsZEP,* the transcript levels in lines O1 and O2 were upregulated 1- to 2-fold as compared with lines L1-L3, while the expression levels were similar among L1-L3, O3, D1-D3 (Figure 5). The expression of *AtOR* in line O3 was much higher than in lines O1 and O2, and expression of *AtOR* may down regulated expression of *OsZEP*.


2.5
2
1.5
1
0.5
0
HPT L1 L2 L3 D1 D2 D3 O1 O2 O3
PDS


2.5
2
1.5
1
0.5
0
HPT L1 L2 L3 D1 D2 D3 O1 O2 O3
LYCB


7
6
5
4
3
2
1
0
HPT L1 L2 L3 D1 D2 D3 O1 O2 O3
LYCE


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPT L1 L2 L3 D1 D2 D3 O1 O2 O3
BCH

3
2.5
2
1.5
1
0.5
0
HPT L1 L2 L3 D1 D2 D3 O1 O2 O3
ZEP

Figure 5 Expression analyses of endogenous carotenogenic genes in transgenic rice endosperm

Relative transcript levels of endogenous *OsPDS OsLYCB*, *OsLYCE*, *OsBCH* and *OsZEP* genes in rice endosperm (T3) at 25 DAP expressing different transgene combinations. Values are a mean of three quantitative real-time PCR replicates with ±SD. Expression for each gene was normalized against actin. Line names are defined in the legend to Figure 2.

DISCUSSION

Increasing the supply of isoprenoid precursors derived from MEP pathway enhances carotenoid accumulation in rice endosperm

Carotenoids are formed predominantly via the non-mevalonic acid (MVA) methylerythritol 4-phosphate (MEP) pathway (Figure 1) (Eisenreich *et al.*, 2001; Rodríguez-Concepción and Boronat, 2002; Rodríguez-Concepción, 2010; Farré *et al.*, 2010). The MEP pathway uses pyruvate and glyceraldehyde 3-phosphate (GAP) to produce 1-deoxy-D-xylulose 5-phosphate (DXP), the initial reaction catalyzed by DXP synthase (DXS). DXS has been demonstrated to catalyze the rate-limiting step in the formation of plastid-derived isoprenoids (Estévez *et al.*, 2001; Enfissi *et al.*, 2005; Morris *et al.*, 2006; Rodríguez-Concepción, 2010). Fruit specific overexpression of *Escherichia coli DXS* resulted in an increase in total carotenoid level of up to 1.6-fold in tomato fruit. Phytoene and β-carotene levels were increased by 2.4- and 2.2-fold, respectively (Enfissi *et al.*, 2005). DXS enzyme activity in these transgenic tomato lines was increased, especially in line F-EcDXS-3/19, up to 2.3-fold, but the main end-

product carotenoids (lycopene and β-carotene) were similar compared with wild type (Enfissi *et al.*, 2005). This may be due to the accumulation of phytoene (2.4-fold), suggesting that phytoene desaturation became limiting in this line, rather than endogenous PSY-1 whose activity was unchanged (Enfissi *et al.*, 2005). Tuber specific overexpression of an *E. coli DXS* led to increased total carotenoid levels (ca. 2-fold) in potato tuber, with most of the increase accounted for by a 6–7-fold increase in phytoene (Morris *et al.*, 2006). There were no significant changes in carotenoids downstream of phytoene. The endogenous *PSY* transcript level incresed aproximately 15-fold whereas the phytoene desaturase (PDS) transcript level was unchanged in the DXS expressing potato tubers compared with controls (Morris *et al.*, 2006). This result implies that phytoene desaturase activity became the rate-limiting step in transgenic tuber carotenoid biosynthesis when *PSY* expression increased, resulting in the accumulation of phytoene (Morris *et al.*, 2006).

Rice endosperm is deficient in carotenoids even though geranylgeranyl diphosphate (GGPP), the immediate precursor for carotenoid biosynthesis, accumulates in wild type rice endosperm (Burkhardt *et al.*, 1997). A heterologous daffodil (*Narcissus pseudonarcissus*) *PSY* when introduced into rice resulted in the accumulation of phytoene but not desaturated products (Burkhardt *et al.*, 1997) indicating that at least PDS activity is deficient in the endosperm. Similarly, the expression of bacterial CRTI (which replaces plant PDS, Z-ISO, ZDS and CRTISO) alone was insufficient to relieve the bottleneck and no carotenoids accumulated in the endosperm due to the absence of PSY activity (Schaub *et al.*, 2005). Therefore carotenoid accumulation in rice

endosperm requires both *PSY1* and *CRTI* expression (Ye *et al.*, 2000; Paine *et al.*, 2005). The isoprenoid precursor GGPP was thought to be another rate-limiting for further enhancement of carotenoid accumulation in rice endosperm since phytoene did not accumulate in the endosperm of Golden Rice (Ye *et al.*, 2000; Paine *et al.*, 2005) but to date there is no direct evidence to support this hypothesis at least in the case of rice. In order to investigate the effects of overexpression of DXS on isoprenoid precursor enhancement for carotenoid accumulation in rice endosperm, we generated transgenic rice lines co-expressing *AtDXS*, *ZmPSY1* and *PaCRTI* (D1, D2 and D3) and lines co-expressing *ZmPSY1* and *PaCRTI* (L1, L2 and L3). DXS expressing lines (D1, D2 and D3) exhibited an orange endosperm phenotype whereas the corresponding lines expressing *ZmPSY1* and *PaCRTI* (L1, L2 and L3) were yellow. Total carotenoid levels were approximately 2.1- to 4.7-fold higher in lines co-expressing *AtDXS*, *ZmPSY1* and *PaCRTI* than in lines only co-expressing *ZmPSY1* and *PaCRTI* (Table 1). Different from the transgenic tomato and potato as described above (Enfissi *et al.*, 2005; Morris *et al.*, 2006), the phytoene levels were very similar in the lines expressing *AtDXS, ZmPSY1* and *PaCRTI* (D1-D3) and lines expressing only *ZmPSY1* and *PaCRTI* (L1-L3) (an average of 1.5 and 1.7 µg/g DW, respectively) (Table 1). The enhanced carotenoids in *AtDXS* expressing lines mainly resulted from β-carotene, β-cryptoxanthin and α-carotene increase (Table 1). These carotenoid enhancements in rice endosperm of these *AtDXS* expressing lines might result from the overexpression of *AtDXS*, and the upregulation of endogenous phytoene desaturase (*OsPDS*) and lycopene ε-cyclase (*OsLYCE*) gene (Figure 5) since the expression of the endogenous lycopene β-cyclase

and β-carotene hydroxylase and zeaxanthin epoxidase genes was unchanged in *AtDXS* expressing lines and control lines expressing *ZmPSY1* and *PaCRTI*. Thus, similar to tomato (Enfissi *et al.*, 2005) and potato (Morris *et al.*, 2006) the phytoene desaturation step is also the limiting factor for carotenoid biosynthesis in transgenic rice endosperm although the expression of endogenous *OsPDS* was upregulated in *AtDXS* expressing lines. The total carotenoid content of endosperm expressing *ZmPSY1*, *PaCRTI* and *AtDXS* was at least 3-fold higher than that expressing only *ZmPSY1* and *PaCRTI* (Table 1, Figure 4), indicating the supply of isoprenoid precursors such as GGPP derived from the MEP pathway is critical for maximizing carotenoid accumulation in rice endosperm.

Overexpression of *AtOR* facilitates carotenoid accumulation in rice endosperm due to the upregulation of a series of endogenous carotenogenic gene expression

The cauliflower *Orange* (*OR*) gene represents the only known gene that acts as a *bona fide* molecular switch to trigger the differentiation of non-colored plastids into chromoplasts (Lu *et al.*, 2006; Giuliano and Diretto, 2007). *OR* is a natural spontaneous mutation in cauliflower, which causes low-pigmented tissues, most noticeably the edible curd, to accumulate high levels of β-carotene and turns them orange (Li *et al.*, 2001). *OR* confers high level of β-carotene accumulation by inducing the formation of a metabolic sink for carotenoids (Lu *et al.*, 2006). Expression of *OR* in potato tubers also leads to the formation of chromoplasts (Lopez *et al.*, 2008). As a result, *OR* transgenic potato accumulates substantial amounts of β-carotene, violaxanthin, lutein and also phytoene, phytofluene and ζ-carotene (Lopez *et al.*, 2008). Recently, we demonstrated

that the overexpression of wild type Arabidopsis *OR* induced chromoplast formation, carotenoid sequestration and enhanced carotenoid levels in rice callus (Bai *et al.*, 2013). In our current experiments transgenic rice lines co-expressing *AtOR*, *ZmPSY1* and *PaCRTI* (lines O1-O3) had 2.7- to 5.8-fold higher total carotenoid levels in the endosperm compared with lines (L1-L3) co-expressing *ZmPSY1* and *PaCRTI* (Table 1), resulting in an orange endosperm phenotype in lines O1-O3 whereas the corresponding phenotype in lines L1-L3 manifests as yellow endosperm (Figure 2). β-Carotene, β-cryptoxanthin and α-carotene levels were all increased by ca: 5.2-fold for these three provitamin A carotenoids (Table 1). Lutein levels were also 2.5 to 5.0-fold higher in *AtOR* expressing lines compared with the lines D1-D3 and L1-L3 (Table 1). The cauliflower *OR* gene does not normally induce the expression of carotenogenic genes (Li *et al.*, 2001, 2006; Lu *et al.*, 2006). However, more recently the orthologue of sweet potato *OR* was shown to induce carotenogenic gene expression, suggesting that *OR* might contribute towards increasing carotenoid levels to reach the threshold necessary to trigger chromoplast differentiation (Kim *et al.*, 2013). The enhancement of carotenoids (β-carotene, β-cryptoxanthin, α-carotene and lutein) in rice endosperm in *AtOR* expressing lines as described above might result from the upregulation of both lycopene cyclases and β-carotene hydroxylase genes (Figure 5). Thus, overexpression of *AtOR* could also increase substantially carotenoid accumulation in rice endosperm though upregulation of a set of endogenous carotenogenic genes.

***AtDXS* and *AtOR* regulate carotenoid biosynthesis and composition in rice endosperm through distinct mechanisms**

It appears that the expression of endogenous carotenogenic genes (*OsLYCB*, *OsLYCE*, *OsBCH* and *OsZEP*) except *OsPDS* were greatly upregulated in *AtOR* expressing lines (Figure 5) whereas in *AtDXS* expressing lines only *OsPDS* and *OsLYCE* expression was upregulated. Overexpression of *AtDXS* or *AtOR* in addition to *ZmPSY1* and *PaCRTI* enhanced the total carotenoid content in rice endosperm by more than at least 2-fold compared with the lines only expressing *ZmPSY1* and *PaCRTI* (Table 1). This enhancement mainly resulted from β-carotene and α-carotene increases in lines expressing *AtDXS* (D1-D3) and *AtOR* (O1-O3) (Table 1 and Figure 4). The expression level of endogenous *OsLYCB* was up-regulated in the *AtOR* expressing lines (O1-O3), however, it was not upregulated in the *AtDXS* expressing lines (D1-D3) (Figure 5), suggesting endogenous *OsLYCB* is sufficient for β-carotene synthesis in the *DXS* lines compared to the *OR* lines since no lycopene accumulated in the endosperm of the *DXS* lines. The level of endogenous *OsLYCE* expression was significantly up-regulated in lines expressing *AtDXS* or *AtOR* (Figure 5), which is in agreement with the enhancement of α-carotene in these *AtDXS* or *AtOR* expressing lines (more than 5-fold increase in lines D1-D3 and O1-O3 compared with lines L1-L3) (Table 1). Zeaxanthin and lutein enhancement in lines expressing *OR* was congruent with the up-regulated endogenous *OsBCH* expression (Table 1 and Figure 5); however, lines expressing *AtDXS* (D1-D3) in which the endogenous *OsBCH* expression was not up-regulated resulted in the similar enhancement of zeaxanthin and lutein in endosperm like lines

expressing *AtOR* (O1-O3) (Table 1 and Figure 5). A possible reason for this might be endogenous *OsBCH* gene expression was not limited factor for zeaxanthin and lutein synthesis in *AtDXR* expressing lines. The phytoene levels were relative lower in lines expressing *AtOR* (O1-O3, an average of 1.1 μg/g DW) compared with the lines expressing *DXS* (D1-D3) and lines expressing only *ZmPSY1* and *PaCRTI* (L1-L3) (an average of 1.5 and 1.7 μg/g DW, respectively) (Table 1). Nevertheless, phytoene accumulated in all the nine lines analyzed, suggesting PaCRTI was not efficient enough to convert phytoene to downstream carotenoids quantitatively, differently from the previous Golden Rice where there was no phytoene accumulation in endosperm (Ye *et al.*, 2000; Paine *et al.*, 2005). Very recently, phytoene, the precursor of the carotenoid pathway, was identified in the plastoglobule of chromoplasts in transgenic tomato fruits, whereas the biosynthetic enzymes were in the membranes (Nogueira *et al.*, 2013). Similar to transgenic tomato, in our transgenic rice endosperm phytoene and PaCRTI enzymes might be located in different partitions (plastoglobule, stroma, membranes etc.) of organelle or in different organelles in rice endosperm, thus resulting in residual phytoene accumulation, which reflected that an important component of metabolic regulation is compartmentation.

CONCLUSIONS

We explored factors limiting carotenoid accumulation in rice endosperm, specifically focusing on two aspects. The metabolite analyses in rice endosperm tissue co-expressing binary (*ZmPSY1* and *PaCRTI*) and tertiary (*ZmPSY1*, *PaCRTI* and *AtDXS*) transgene combinations suggested that the supply of isoprenoid precursors derived from the MEP pathway is one key factor which limits carotenoid accumulation in the endosperm. Our experiments involving the *AtOR* gene clearly demonstrated that overexpression of *AtOR* could also increase substantially carotenoid accumulation in rice endosperm through upregulation of a series of endogenous carotenogenic genes. The more than 2-fold enhancement of total carotenoid in both sets of experiments mainly resulted from at least 5-fold provitamin A carotenoids (more than 5-fold β-carotene β-cryptoxanthin and α-carotene, respectively). We found that the effect of the *AtDXS* and *AtOR* transgenes on the expression of endogenous carotenogenic genes in the transgenic plants through different mechanisms. It appears that expression of *AtOR* transgene exhibited a specific effect on inducing the expression of a series of endogenous carotenogenic genes. This in turn will activate the whole carotenoids pathway flux for their accumulation. Our understanding of the metabolic bottlenecks in the pathway in rice endosperm will permit the design and implementation of more targeted strategies for the creation of engineered plants with particular carotenoid profiles particularly by combining DXS and OR in subsequent experiments. This will take advantage of our separate findings for each transgene complement and simultaneously coexpression of *AtDXS* and *AtOR* in addition to *ZmPSY1* and bacterial

CRTI in rice might synergistically or additively facilitate further carotenoid accumulation in cereal crops.

MATERIALS AND METHODS

Growth and maintenance of rice plants

Wild type rice (*Oryza sativa* L. cv. EYI105) and transgenic rice plants were grown in the greenhouse and growth chamber at 28/20°C day/night temperature with a 10-h photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21/18°C day/night temperature with a 16-h photoperiod treatments.

Gene cloning and vector construction

The *AtDXS* and *AtOR* cDNAs were cloned directly from *A. thaliana* leaf mRNA by reverse transcriptase PCR based on sequence data in GenBank (accession numbers NM 203246 and U27099.1). The cDNAs were transferred to the pGEM-T easy vector (Promega, Madison, WI, USA) and the recombinants were digested with *Eco*RI. *AtDXS* was ligated into the same cut (*Eco*RI) of vector pRP5 (Su *et al.*, 2001) containing the endosperm specific rice prolamin promoter and the *ADPGPP* terminator, whereas *AtOR* was ligated into the same cut (*Eco*RI) of vector p326 (Stoger *et al.*, 1999) containing the endosperm specific wheat low molecular weight (LMW) glutenin gene promoter and *NOS* terminator.

The maize *PSY1* cDNA was cloned from maize inbred line B73 endosperm by RT-PCR using forward primer with *Bam*HI cut (sequence GGATCC, the restriction site is underlined below) 5′-AGGATCCATGGCCATCATACTCGTACGAG-3′ and reverse primer with *Eco*RI cut (sequence GAATTC, the restriction site is underlined below) 5′-AGAATTCTAGGTCTGGCCATTTCTCAATG-3′ based on the *PSY1* sequence (GenBank accession number AY324431). The product was transferred to pGEM-T easy (Promega) to generate pGEM-ZmPSY1 for sequencing and then inserted into p326 (Stoger et al., 1999) vector containing the LWM glutenin promoter and *NOS* terminator through digestion of both plasmids of pGEM-ZmPSY1 and p326 by *Bam*HI and *Eco*RI.

The *Pantoea ananatis* (formerly *Erwinia uredovora*) phytoene desaturase gene (*CRTI*) was fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier et al., 1985) in plasmid pYPIET4 (Misawa *et al.*, 1994) and amplified by PCR using forward primer with *Xba*I cut (sequence TCTAGA, the restriction site is underlined below) 5′-ATCTAGAATGGCTTCTATGATATCCTCTTC-3′ and reverse primer with *Eco*RI cut (sequence GAATTC, the restriction site is underlined below)5′-AGAATTCTCAAATCAGATCCTCCAGCATCA-3′, based on the *P. ananatis CRTI* gene sequence deposited in GenBank (accession number D90087). The product was transferred to pGEM-T easy vector to produce pGEM-PaCRTI for sequencing and then inserted into pHorp-P (Sorensen et al., 1996) containing the endosperm specific barley D-hordein promoter (Sorensen et al., 1996) and the rice ADPGPP terminator through *Xba*I and *Eco*RI digestion of both pGEM-PaCRTI and pHorp-P plasmids.

Transformation of rice plants

Seven-day-old mature zygotic embryos were transferred on osmoticum medium (containing 4.4 g/l MS powder supplemented with 0.3 g/l casein hydrolysate, 0.5 g/l proline, 72.8 g/l mannitol and 30 g/l sucrose) four hours before bombarding and then bombarded with 10 mg gold particles coated with the carotenogenic constructs and the selectable maker *hpt* at a 3:3:3:1 ratio as previously described (Christou *et al.*, 1991, 1997). They were returned to osmoticum medium for 12 h before selection on MS medium (containing 4.4 g/l MS powder, 0.3 g/l casein hydrolysate and 0.5g/l proline and 30 g/l sucrose) supplemented with 50 mg/l hygromycin and 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark for 2-3 weeks (Farré *et al.*, 2012). Transgenic plantlets were regenerated and hardened off in soil.

Transgene expression analysis

Total RNA was extracted from rice endosperm at 25 days after pollination (DAP). We separated 25 μg of denatured total RNA by 1.2% (w/v) agarose-formaldehyde gel electrophoresis in 1× MOPS buffer and transferred the fractionated RNA to a membrane by capillary blotting (Sambrook *et al.*, 1989). The membrane was probed with digoxigenin-labeled partial cDNAs prepared using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), with hybridization carried out at 50 °C overnight using DIG Easy Hyb (Roche, Mannheim, Germany). The membrane was washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature, twice for 20 min in 0.2× SSC, 0.1%

SDS at 68 °C, and then twice for 10 min in 0.1× SSC, 0.1% SDS at 68 °C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemiluminescence generated by disodium 3-(4-methoxyspiro{1,2-dioxetane-3, 2'-(5'-chloro) tricyclo[3.3.1.13,7] decan}-4-yl) phenyl phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma–Aldrich, St. Louis, USA) according to the manufacturer's instructions. The forward and reverse primers for each transgene were used for probe are shown in Supplementary Table 1.

Table S1 Oligonucleotide sequences of forward (F) and reverse (R) primers for mRNA blot analysis

Transgenes	Primers sequence
ZmPSY1.F	Forward 5´-GTGTAGGAGGACAGATGAGCTTGT-3´
ZmPSY1.R	Reverse 5´-CATCTGCTAGCCTGTGAGAGCTCA-3´
PaCRTI.F	Forward 5´-TGGAGAAGCGTTTACAGTAAGGT-3´
PaCRTI.R	Reverse 5´-GCGTGCAGATAAAGTGAGAAGTC-3´
AtDXS.F	Forward 5´-GACACAGCTCAACCACAATATCTGCTGG-3´
AtDXS.R	Reverse 5´-GGAAGACAAGCCATAAATGTCACATCGAAAG-3´
AtOR.F	Forward 5´-ATGTCATCTTTGGGTAGGATTTTGT-3´
AtOR.R	Reverse 5´-GGTTTTGGGCGGTGATAGAGA-3´

Carotenoid extraction and quantification

Carotenoids from 50 mg freeze-dried 25 DAP endosperm were extracted in darkness with 50/50 (vol/vol) tetrahydrofuran and methanol at 60 °C for 20 min. The mixture was filtered and the residue re-extracted in acetone. Chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA)

consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array (PDA) 2996 detector. Compounds were separated with an ACQUITY UPLC™ BEH C18 column (1.7 µm; 1 mm × 150 mm) (Waters, Manchester, UK) and a gradient system with the mobile phase consisting of solvent A, ACN–MeOH (7:3, v/v) and solvent B, H_2O 100%. The linear gradient was as follows: 0–1.0 min, 25% B, 0.5 mL/min (isocratic); 1.0–10.0 min, 4.9% B 0.5 mL/min (linear gradient); 10.0–11.4 min, 0% B, 0.7 mL/min (linear gradient); 11.4–19.2 min, 0% B, 0.7 mL/min (isocratic); 19.2–20.0 min, 25% B, 0.5 mL/min (linear gradient); and 20.0–22.0 min, 25% B, 0.5 mL/min (isocratic). Weak and strong needle solvents were ACN–MeOH (7:3, v/v) and 2-propanol, respectively. The injection volume was 5 µL and the column was kept at 32 ºC while the temperature in the sample manager was maintained at 25 ºC. The average maximum pressure in the chromatographic system was 15000 psi. Instrument control, data acquisition and processing were carried out using MassLynx™ software (version 4.1; Waters, USA) (Delpino-Rius *et al.*, 2014).

Quantitative real time PCR

Real-time PCR was performed with RNA isolated 25 DAP rice endosperm on a BIO-RAD CFX96TM system using a 25µl- mixture containing 10 ng of synthesized cDNA, 1× iQ SYBR green supermix (BIO-RAD) and 0.2 µM forward and reverse primers for the target genes. To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. PCRs were performed in triplicate using 96-well optical reaction plates, comprising a heating step for 3 min at 95 °C,

followed by 40 cycles of 95 °C for 15 s, 58.5 °C for 1 min and 72 °C for 20 s. Amplification specificity was confirmed by melt curve analysis on the final PCR products in the temperature range 50–90 °C with fluorescence acquired after each 0.5 °C increment. The fluorescence threshold value and gene expression data were calculated using the CFX96TM system software. Values represent the mean of three real time PCR replicates ±SD. The forward and reverse primers for each transgene are shown in Supplementary Table 2.

Table S2 Oligonucleotide sequences of forward (F) and reverse (R) primers for quantitative real-time PCR analysis

Transgenes	**Primers sequence**
OsACTIN-F	Forward 5´-GACTCTGGTGATGGTGTCAGC-3´
OsACTIN-R	Reverse 5´-TCATGTCCCTCACAATTT-3´
OsPDS-F	Forward 5´-GATCCAAACCGTTCAATGCTGG-3´
OsPDS-R	Reverse 5´- TGTCACTCCGTCCAACCCATT -3´
OsLYCB-F	Forward 5´-CGTCCAGTACGACAAGCCGTA-3´
OsLYCB-R	Reverse 5´-AAGGGCATGGCGTAGAGGAACG-3´
OsLYCE-F	Forward 5´-GTATGGCAGCGTTCACAGGGAC-3´
OsLYCE-R	Reverse 5´-GCCAGCGTCATAGCATCGTCTC-3´
OsBCH.F	Forward 5´-TCGAGAACGTGCCCTACTTCC-3´
OsBCH.R	Reverse 5´-ACCCACCTCCTCCAACTCCTT-3´
OsZEP-F	Forward 5´-GGATGCCATTGAGTTTGGTT-3´
OsZEP-R	Reverse 5´-CTCTCGTTCATGCTGCCTGC-3´

REFERENCES

Auldridge, M.E., Block, A., Vogel, J.T., Dabney-Smith, C., Mila, I., Bouzayen, M., Magallanes-Lundback, M., DellaPenna, D., McCarty, D.R. and Klee, H.J. (2006) Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *Plant J.* **45**, 982 –993.

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P. and Zhu, C. (2013) An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. *Plant J.* doi: 10.1111/tpj.12384.

Bai, C., Twyman, R.M., Farré, G., Sanahuja, G., Christou, P., Capell, T. and Zhu, C. (2011) A golden era-pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol–Plant* **47**, 205–221.

Burkhardt, P.K., Beyer, P., Wunn, J., Kloti, A., Armstrong, G.A., Schledz, M., von Lintig, J. and Potrykus, I. (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* **11**, 1071–1078.

Campbell, R., Ducreux, L.J., Morris, W.L., Morris, J.A., Suttle, J.C., Ramsay, G., Bryan, G.J., Hedley, P.E. and Taylor, M.A. (2010) The metabolic and developmental roles of carotenoid cleavage dioxygenase 4 form potato. *Plant Physiol.* **154,** 656–664.

Cazzonelli, C.I. and Pogson, B.J. (2010) Source to sink: regulation of carotenoids biosynthesis in plants. *Trends Plant Sci*. **15**, 266–274.

Christou, P. (1997) Rice transformation by bombardment. *Plant Mol. Biol.* **35**, 197–203.

Christou, P., Ford, T. and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nat. Biotechnol*. **9**, 957–962.

Delpino-Rius, A., Eras, J., Marsol-Vall, A., Vilaró, F., Balcells, M., Canela-Garayoa, R. (2014) Ultra performance liquid chromatography analysis to study the changes in the carotenoid profile of commercial monovarietal fruit juices. *J. Chromatogr. A* In press.

Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Deoxyxylulose phosphate pathway to terpenoids**.** *Trends Plant Sci.* **6**, 78–84.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W. and Bramley, P. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol J.* **3**, 17–27.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S. and Leon, P. (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* **276**, 22901–22909.

Fraser, P.D. and Bramley, P.M. (2004) The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* **43**, 228–265.

Farré, G., Naqvi, S., Sanahuja, G., Bai, C., Zorrilla-López, U., Rivera, S.M., Canela, R., Sandman, G., Twyman, R.M., Capell, T., Zhu, C. and Christou, P. (2012) Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. *Methods Mol. Biol.* **847**, 419–435.

Farre, G., Sanahuja, G., Naqvi, S., Bai, C., Capell, T., Zhu, C.F. and Christou, P. (2010) Travel advice on the road to carotenoids in plants. *Plant Sci.* **179**, 28–48.

Giuliano, G. and Diretto, G. (2007) Of chromoplasts and chaperones. *Trends Plant Sci.* **12**, 529–531.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013) Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol. Biochem.* **70**, 445–454.

Landrum, J.T. and Bone, A.R. (2001) Lutein, zeaxanthin, and the macular pigment. *Arch. Biochem. Biophys.* **385**, 28–40.

Li, L., Paolillo, D.J., Parthasarathy, M.V., DiMuzio, E.M. and Garvin, D.F. (2001) A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *Plant J.* **26**, 59–67.

Li, L., Lu, S., Cosman, K.M., Earle, E.D., Garvin, D.F., O'Neill, J. (2006) β-carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. *Phytochem.* **67**, 1177–1184.

Lu, S. and Li, L. (2008) Carotenoid metabolism: biosynthesis, regulation, and beyond. *J. Integr. Plant Biol.* **50**, 778–785.

Lu, S., et al. (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high-levels of β-carotene accumulation. *Plant Cell* **18**, 3594–3605.

Li, L. and van Eck, J. (2007) Metabolic engineering of carotenoids accumulation by creating a metabolic sink. *Transgenic Res.* **16**, 581–585.

Lopez, A.B., van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) Effect of the cauliflower *Or* transgene on carotenoids accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* **59**, 213–223.

Miralpeix, B., Rischer, H., Häkkinen, S.T., Ritala, A., Seppänen-Laakso, T., Oksman-Caldentey, K.M., Capell, T. and Christou, P. (2013) Metabolic engineering of plant secondary products: which way forward? *Curr. Pharm. Des.* **19**, 5622–5639.

Misawa, N., Yamano, S., Linden, H., de Felipe, M.R., Lucas, M., Ikenaga, H. and Sandmann, G. (1994) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–840.

Morris, W.L., Ducreux, L.J.M., Hedden, P., Millam, S. and Taylor, M.A. (2006) Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato

tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *J. Exp. Bot.* **57**, 3007–3018.

Nogueira, M., Mora, L., Enfissi, E.M., Bramley, P.M. and Fraser, P.D. (2013) Subchromoplast Sequestration of Carotenoids Affects Regulatory Mechanisms in Tomato Lines Expressing Different Carotenoid Gene Combinations. Plant Cell. doi: http://dx.doi.org/10.1105/tpc.113.116210.

O'Connor, S.E. and Maresh, J.J. (2006) Chemistry and biology of monoterpene indole alkaloid biosynthesis. *Nat. Prod. Rep.* **23**, 532–547.

Ohmiya, A., Kishimoto, S., Aida, R., Yoshioka, S. and Sumitomo, K. (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in Chrysanthemum Petals. *Plant Physiol.* **142**, 1193–1201.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J, Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol*. **23**, 482–487.

Rischer, H, Häkkinen, S.T., Ritala, A., Seppänen-Laakso, T., Miralpeix, B., Capell, T., Christou, P. and Oksman-Caldentey, K.M. (2013) Plant cells as pharmaceutical factories. *Curr. Pharm. Des*. **19**, 5640–5660.

Rivera, S.M., Vilaro, F., Zhu, C., Bai, C., Farre, G., Christou, P., Canela-Garayoa, R. (2013) Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem.* **61**, 5279–5285.

Rodríguez-Concepción, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol*. **130**, 1079–1089.

Rodríguez-Concepción, M. (2010) Supply of precursors for carotenoid biosynthesis in plants. *Arch. Biochem. Biophys*. **504**, 118 –122.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press, **1**, 63–70

Schaub, P., Al-Babili, S., Drake, R. and Beyer, P. (2005) Why is golden rice golden (yellow) instead of red? *Plant Physiol.* **138**, 441–450.

Schreier, P., Seftor, E., Schell, J. and Bohnert, H. (1985) The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J.* **4**, 25–32.

Sommer, A. and West, K.J. (1996) *Vitamin A Deficiency: Health, Survival and Vision.* N.Y., Oxford University Press.

Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol. Gen. Genet*. **250**, 750–60.

Stoger, E., Williams, S., Keen, D. and Christou, P. (1999) Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Res.* **8**, 73–82.

Su, PH., Yu, S.M. and Chen, C.S. (2001) Spatial and temporal expression of a rice

prolamin gene RP5 promoter in transgenic tobacco and rice. *J. Plant Physiol*. **158**, 247–254.

Underwood, B.A. and Arthur, P. (1996) The contribution of vitamin A to public health. *FASEB J.* **10**, 1040–1048.

von Lintig, J. and Vogt, K. (2004) Vitamin A formation in animals: molecular identification and functional characterization of carotene cleaving enzymes. *J. Nutr. Jan.* **134**, 251S–256S.

Welch, R.M. and Graham, R.D. (1999) A new paradigm for world agriculture: meeting human needs, productive, sustainable, nutritious. *Field Crops Res.* **60**, 1-10.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I. (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoids-free) rice endosperm. *Science,* **287**, 303–305.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P. and Capell, T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoids pathway in maize. *Proc. Natl. Acad. Sci. USA* **105**, 18232–18237.

Zhu, C., Naqvi, S., Gomez-Galera, S., Pelacho, A.M., Capell, T. and Christou, P. (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* **12**, 548–555.

Chapter 4

Mechanistic considerations for the reconstruction of the astaxanthin biosynthesis pathway in rice endosperm

Chapter 4 is formatted according to the Plant Biotechnology Journal

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Mechanistic considerations for the reconstruction of the astaxanthin biosynthesis pathway in rice endosperm

SUMMARY

Astaxanthin is a high-value ketocarotenoid rarely found in plants. It is derived from β-carotene by the 3-hydroxylation and 4-ketolation of both ionone end groups, in reactions catalyzed by β-carotene hydroxylase and β-carotene ketolase, respectively. We investigated the feasibility of introducing an extended carotenoid biosynthesis pathway into rice endosperm to achieve the production of astaxanthin. This allowed us to identify potential metabolic bottlenecks that have thus far prevented the accumulation of this valuable compound in storage tissues such as cereal grains. Rice endosperm does not usually accumulate carotenoids because phytoene synthase, the enzyme responsible for the first committed step in the pathway, is not present in this tissue. We therefore expressed maize phytoene synthase 1 (*ZmPSY1*), *Pantoea ananatis* phytoene desaturase (*PaCRTI*) and a synthetic *Chlamydomonas reinhardtii* β-carotene ketolase (*sCrBKT*) in transgenic rice plants under the control of endosperm-specific promoters. The resulting grains predominantly accumulated the diketocarotenoids canthaxanthin, adonirubin and astaxanthin as well as low levels of monoketocarotenoids. The predominance of canthaxanthin and adonirubin indicated the presence of a hydroxylation bottleneck in the ketocarotenoid pathway. This final rate-limiting step must therefore be overcome to maximize the accumulation of astaxanthin, the end product of the pathway.

INTRODUCTION

Carotenoids are natural tetraterpenoid compounds that are widely used as nutritional supplements due to their health-promoting activities. Ketocarotenoids such as astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) are particularly valuable because large quantities are used as feed additives in the aquaculture industry to impart the pink color to salmon, trout and shellfish (Meyers, 1994). They also have potential applications in the pharmaceutical, nutraceutical and cosmetic industries reflecting their potent antioxidant activity (Guerin *et al.*, 2003). Astaxanthin helps to prevent cancer (Tanaka *et al.*, 1994; Chen *et al.*, 1999), inhibits the oxidation of low-density lipoproteins (Iwamoto *et al.*, 2000), quenches singlet oxygen (Tatsuzawa *et al.*, 2000) and boosts the immune system (Jyonouchi *et al.*, 1995; Chen and Park, 2004). These properties of astaxanthin and other ketocarotenoids make them attractive targets for metabolic engineering in plants where objectives include increasing their abundance or achieving specific ketocarotenoid profiles in staple foods and/or animal feed crops (Misawa *et al.*, 2009; Zhu *et al.*, 2009).

Carotenoids are predominantly derived from the plastid methylerythritol 4-phosphate (MEP) pathway, and most of the genes required for carotenoid biosynthesis have been identified and characterized (Bai *et al.*, 2011; Farré *et al.*, 2010). The first committed step in plants is the condensation of two geranylgeranyl diphosphate (GGPP) molecules by the enzyme phytoene synthase (PSY) to form 15-*cis*-phytoene (Figure 1, Bai *et al.*, 2013). This is converted into all-*trans*-lycopene through a series of desaturation and isomerization reactions catalyzed by a single enzyme (CRTI) in bacteria, but by the four sequentially-acting enzymes phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) in plants (Bai *et al.*,

2011; Farré *et al*., 2010; Zhu *et al*., 2013). Lycopene marks a branch in the pathway leading in one case to α-carotene and in the other to β-carotene, depending on the competing activities of lycopene β-cyclase and lycopene ε-cyclase.

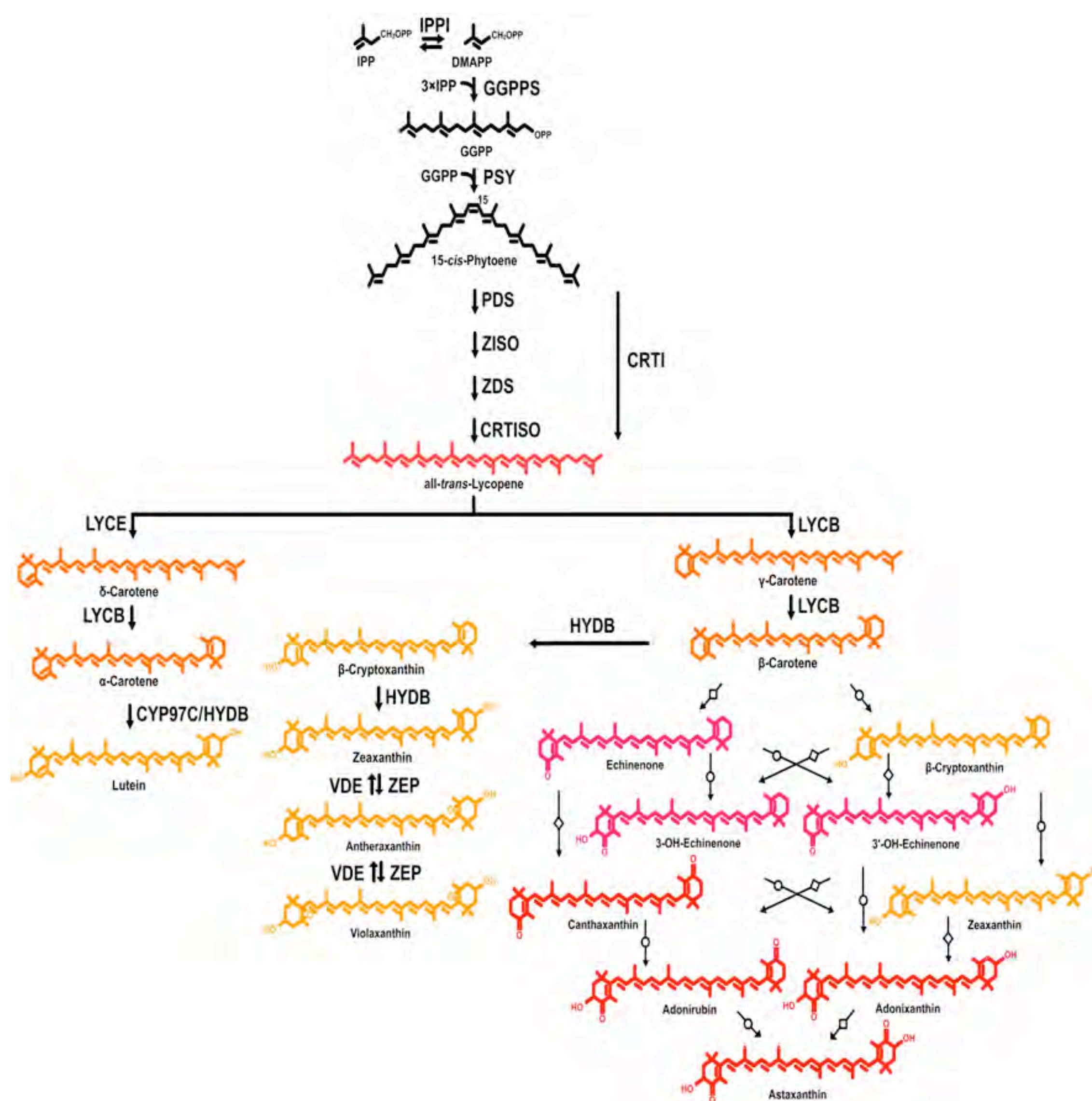

Figure 1 The extended carotenoid biosynthesis pathway in plants (Bai *et al*., 2013)

All carotenoids are synthesized from the five-carbon monomeric building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Plastidial IPP

is generated via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MEP pathway begins with glyceraldehyde-3-phosphate (GA-3-P) and pyruvate, which are converted into 1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS). DXP reductoisomerase (DXR) converts DXP to MEP, which is then converted into 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) in four sequential reactions. Hydroxymethylbutenyl diphoshate reductase (HDR) simultaneously synthesizes IPP and DMAPP from HMBPP in the last step of the MEP pathway. Three IPP molecules are added to DMAPP to produce geranylgeranyl diphosphate (GGPP). The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP by phytoene synthase (PSY) to produce phytoene. This is converted into all-*trans* lycopene by the action of two desaturases and two isomerases: phytoene desaturase (PDS), ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). The bacterial phytoene desaturase (CRTI) can apparently perform all the desaturation and isomerization reactions carried out by PDS, ZISO, ZDS and CRTISO. Because CRTI reduces the number of transgenes required to introduce a complete carotenoid pathway it is widely used for metabolic engineering. Lycopene is a branch in the pathway, leading to the α-carotenes and β-carotenes. In the α-carotene branch, the addition of one ε-ring and one β-ring to lycopene by lycopene ε-cyclase (LYCE) and lycopene β-cyclase (LYCB) produces α-carotene, whereas the addition of two β-rings by LYCB in the β-carotene produces the pro-vitamin A carotenoids γ-carotene and β-carotene. Both α-carotene and β-carotene are redundantly hydroxylated by non-heme di-iron β-carotene hydroxylases (BCH1 and BCH2) and cytochrome P450-type hydroxylases (CYP97A, CYP97B and CYP97C) to yield lutein and zeaxanthin, respecively. The β-xanthophylls are epoxidated, de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), giving rise to the xanthophyll cycle. Astaxanthin is produced from β-carotene in astaxanthin-producing bacteria and transgenic plants, catalyzed by the combined activities of β-carotene ketolase (arrow with square) and β-carotene hydroxylase (arrow with circle).

In the extended carotenoid pathway, astaxanthin is derived from β-carotene by the 3-hydroxylation and 4-ketolation of both ionone end groups (Figure 1). These reactions

are catalyzed by β-carotene hydroxylase and β-carotene ketolase, respectively. The hydroxylation reaction is widespread in higher plants, but ketolation is mostly restricted to bacteria, fungi, and some unicellular green algae (Misawa *et al*., 2009; Zhu *et al*., 2009). Therefore, whereas many higher plants can synthesize different hydroxylated carotenoids such as zeaxanthin, only a few species can synthesize ketocarotenoids, e.g. *Adonis aestivalis* which accumulates ketocarotenoids in its flowers (Cunningham and Gantt, 2005). This is because β-carotene ketolase, which is responsible for the conversion of β-carotene to canthaxanthin and zeaxanthin to astaxanthin, is not usually expressed in plants.

In order to identify metabolic bottlenecks that may hinder the accumulation of astaxanthin in rice endosperm, we expressed maize *PSY1* (phytoene synthase 1, *ZmPSY1*), *Pantoea ananatis CRTI* (phytoene desaturase, *PaCRTI*), and a synthetic *Chlamydomonas reinhardtii sCrBKT* (β-carotene ketolase) under the control of endosperm-specific promoters. All the transfrmants with pink in color derived from color of the transgenic rice callus, which allowed rapid visual screening of the transformants and correctly predicted the carotenoid profiles in the corresponding transgenic plants. These profiles revealed that the synthetic β-carotene ketolase gene was sufficient to produce ketocarotenoids in both tissues when there was strong flux through the early part of the pathway, but that the heterologous β-carotene ketolase overwhelmed the endogenous β-carotene hydroxylase activity and skewed the profiles strongly in favor in ketolation thus promoting the accumulation of canthaxanthin and adonirubin rather than astaxanthin. Therefore, the low endogenous β-carotene hydroxylase activity represented a rate-limiting step in the extended carotenoid pathway and this must be addressed in order to achieve the high-level production of astaxanthin.

RESULTS

Expression of a truncated *C. reinhardtii* β-carotene ketolase (sCrBKT) leads to the preferential accumulation of ketocarotenoids in rice endosperm

Rice endosperm lacks the metabolic capacity to produce carotenoids because the first enzyme in the pathway is not expressed. Therefore, in order to investigate the production of ketocarotenoids in this tissue it was necessary to introduce both the early steps in the pathway (represented by the maize phytoene synthase 1 (*ZmPSY1*) and *P. ananatis* phytoene desaturase (*PaCRTI*) genes) and the synthetic *C. reinhardtii* β-carotene ketolase (*sCrBKT*) gene to provide the ketolation activity that is missing in most plants. We introduced all three genes into 7-day-old zygotic rice embryos under the control of endosperm-specific promoters, along with the constitutively expressed selection marker *hpt*.

The transformants were grown on selective medium before transfer to soil, and those producing the most intense pink endosperm tissue during seed development were selected for self-pollination in the T1 generation (Figure 2). This visual screen automatically selected for plants producing ketocarotenoids and therefore expressing all three transgenes. The three best-performing lines (B1, B2 and B3) were progressed to the T3 generation to generate homozygous lines producing enough seeds for detailed carotenoid and transgene expression analysis. The transgenic plants were indistinguishable from their wild-type counterparts in terms of growth, development, morphology and leaf pigmentation.

Figure 2 Phenotype of transgenic and control rice seeds

Endosperm colors of five different transgenic rice lines at 40 DAP (B1, B2, B3, B4 and B5) expressing *ZmPSY1*, *PaCRTI*, *sCrBKT* and *hpt* compared to a control line expressing *hpt* alone. Abbreviations: *ZmPSY1,* maize phytoene synthase gene 1; *PaCRTI*, bacterial phytoene desaturase gene; *sCrBKT*, synthetic *C. reinhardtii* β-carotene ketolase gene; *hpt*, hygromycin phosphotransferase gene.

HPLC analysis of extracts from the pink endosperm tissue of lines B1–B3 at 40 DAP indicated the presence of astaxanthin, canthaxanthin, adonirubin, echinenone and 3-hydroxyechinenone (Figure 3). Quantitative analysis (Table 1a, Figure 4) revealed a similar distribution of carotenoid species in all three lines and a total carotenoid content in the endosperm that ranged from 6.42 µg/g dry weight (DW) in line B3 to 8.78 µg/g DW in line B2.

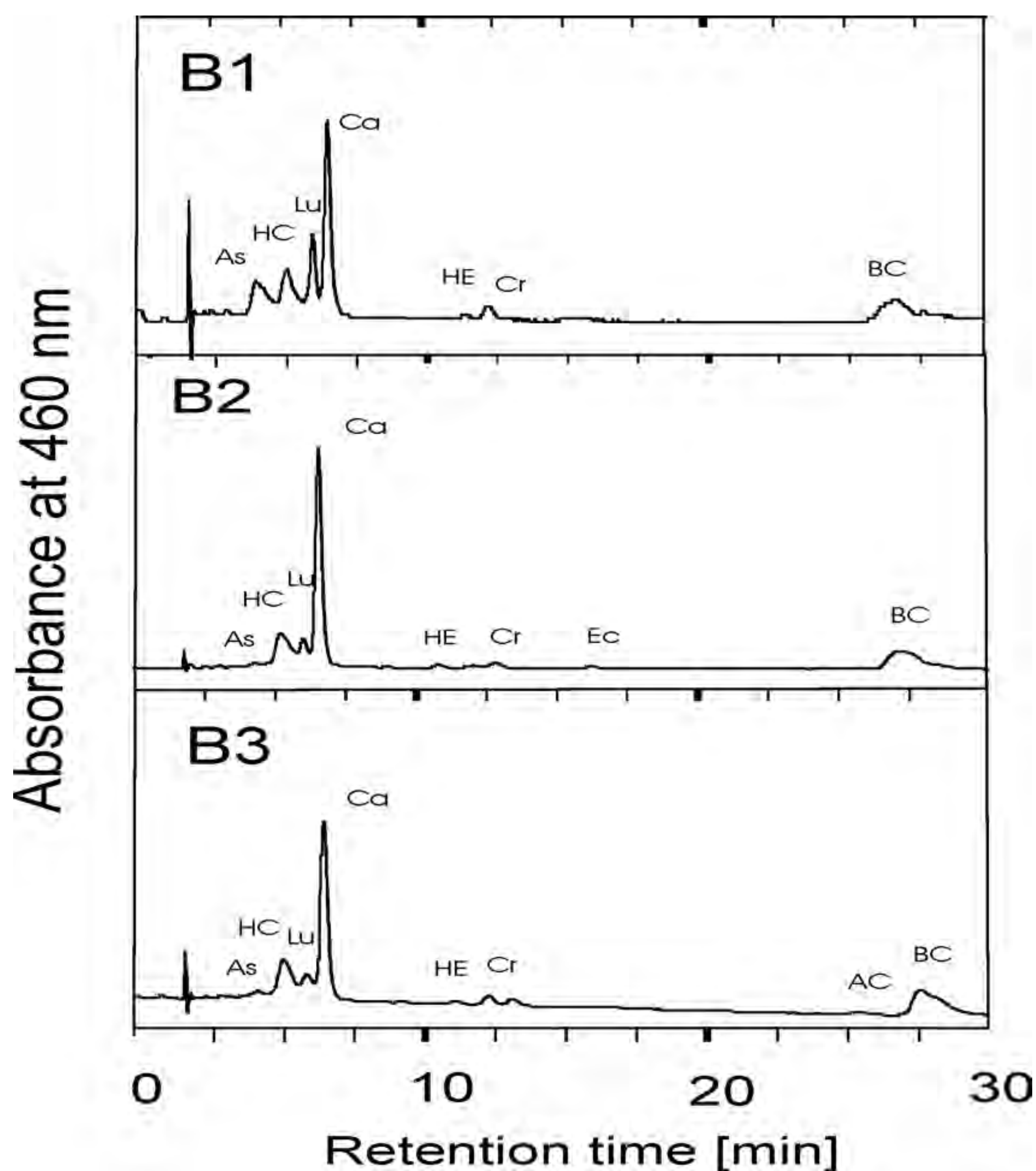

Figure 3 Carotenoid profiles of transgenic endosperm by HPLC

HPLC analysis shows the profile of carotenoids in the T3 endosperm (40 DAP) of transgenic rice lines B1, B2 and B3 (mature seeds). Abbreviations: As, astaxanthin; HC, adonirubin; Ca, canthaxanthin; HE; 3-OH-echininone; Ec, echininone, Lu, lutein, AC, α-carotene BC, β-carotene, Cr, β-crytoxanthin. No carotenoids detected in wild type of rice endosperm.

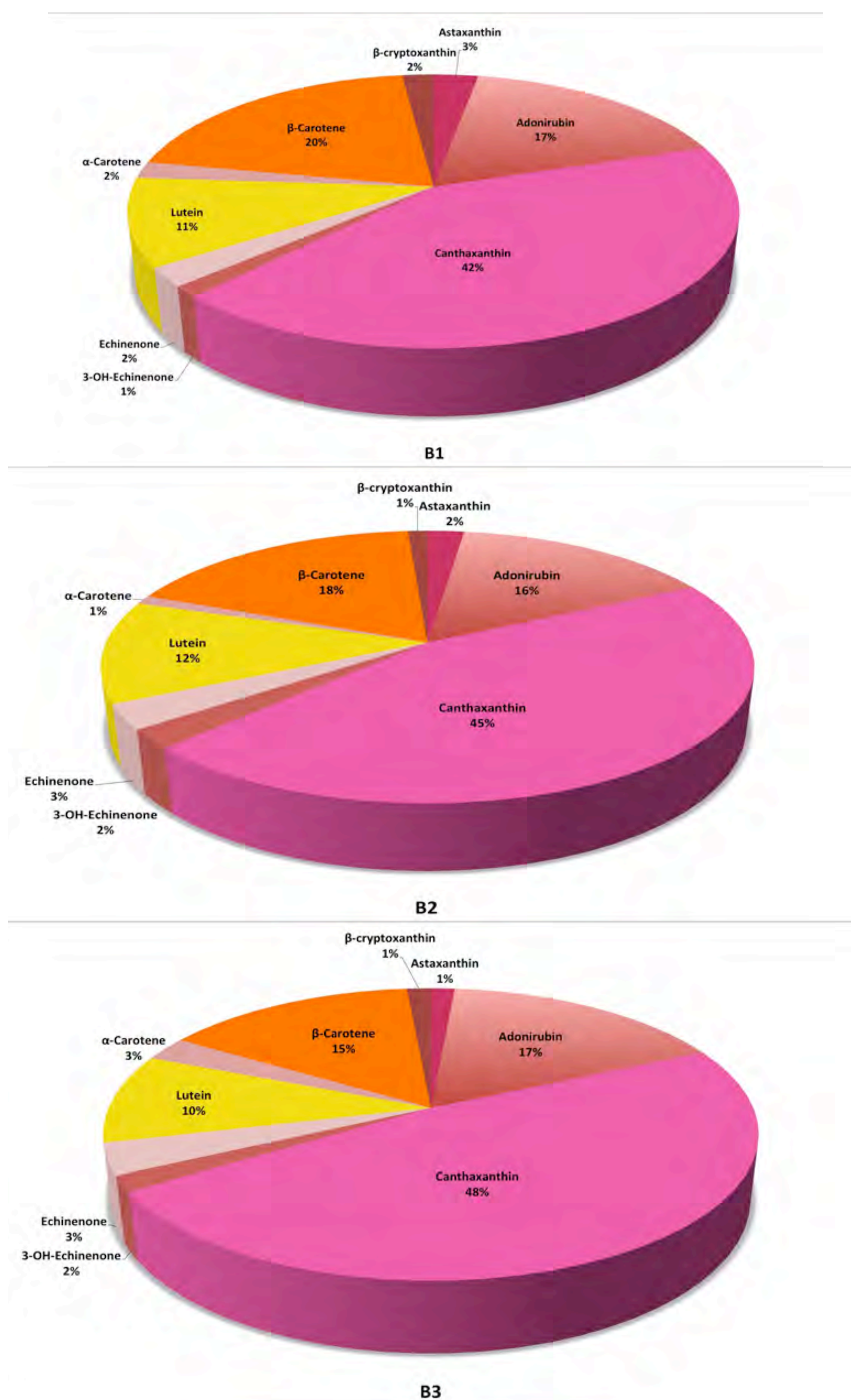

Figure 4 Distribution of carotenoids in transgenic rice endosperm by HPLC

The carotenoid profiles in the endosperm of transgenic lines B1, B2 and B3 were determined by UHPLC. Each molecule is represented as a proportion of the total and shown in a different color, averaged from six independent measurements

The diketocarotenoid canthaxanthin was the most abundant carotenoid species, accounting for nearly half the total carotenoids in all three lines (concentration 3–4 μg/g DW). Adonirubin, β-carotene and lutein were also abundant, each representing 10–20% of the total carotenoids and accumulating at levels up to 1 μg/g DW. In contrast, astaxanthin was a minor component, representing 2.9% of the total carotenoids in line B1 (concentration 0.20 μg/g DW).

Starting from steady-state carotenoid concentrations, Table 1b individually shows the activity values for the conversion of substrates and intermediates for the ketolation and hydroxylation reactions from β-carotene. These values for one-step and two-step ketolations and hydroxylations compare how effective these two reactions proceed in the rice lines. All three lines showed a similar high degree of ketolation compared to hydroxylation, as revealed by comparing the values for diketolation and dihydroxylation starting from β-carotene. In addition, high monodroxylation values and lower dihydroxylation values indicated a limitation of the second hydroxylation step. This is typical for low enzyme activity in a two-step reaction. The superior formation of keto products versus hydroxy products is also reflected by high canthaxanthin and adonirubin concentrations compared to astaxanthin (Table 1a). The data in Table 1 demonstrate that there is an excess of ketolase over hydroxylase activity in all three rice lines, limiting the accumulation of the dihydroxylated end product astaxanthin.

Table 1a Total carotenoid levels and composition in mature rice endosperm

Line	B1	B2	B3
Astaxanthin*§	0.2±0.04(2.9)	0.19±0.06(2.2)	0.09±0.01(1.5)
Adonirubin*§	1.22±0.24(17.6)	1.35±0.43(15.8)	1.07±0.07(17.3)
Canthaxanthin *	3.00±0.58(43.2)	3.96±1.28(46.5)	3.08±0.22(49.7)
β-cryptoxanthin§	0.14±0.02(2.0)	0.1±0.02(1.2)	0.09±0.01(1.5)
α-Carotene	0.14±0.03(2.0)	0.09±0.03(1.1)	0.17±0.02(2.7)
β-Carotene	1.42±0.28(20.4)	1.56±0.51(18.3)	0.95±0.07(15.3)
3-OH-Echinenone*§	0.09±0.01(1.3)	0.20±0.07(2.3)	0.11±0.01(1.8)
Lutein	0.74±0.15(10.6)	1.07±0.47(16.3)	0.64±0.05(10.3)
Echinenone*	0.16±0.03(2.3)	0.26±0.09(3.1)	0.22±0.02(3.5)
Total carotenoids	7.11±1.35	8.78±2.91	6.42±0.47
β/ε ratio	7.08	6.57	6.93
% ketocarotenoids	65.68	67.88	71.18

Data are means ± SD of three independent rice endosperm (at 40 DAP) lines and are expressed as µg/g DW. Values in parentheses represent the percentage of each carotenoid as a proportion of the total. *All ketolated β-carotene derivatives; §all hydroxylated β-carotene derivatives.

Table 1b Realtive ketolation and hydroxylation values calculated from the concentrations of β-carotene and ketolated products as well as β-carotene and hydroxylated products[a].

	Monoketolation Value	Diketolation Value
B1	4.1	72.6
B2	6.1	73.1
B3	6.0	76.8
	Monohydroxylation Value	**Dihydroxylation Value**
B1	47.2	6.5
B2	48.5	5.6
B3	55.0	3.9

[a]The diketolation value is calculated as the sum of astaxanthin, adonirubin plus canthaxanthin related to the sum of all ketocarotenoids plus β-carotene, the values of monoketolation as sum of echinenone plus 3-OH-echinenone related to the sum of all ketocarotenoids plus β-carotene. The dihydroxylation value is calculated as the sum of astaxanthin related to the sum of all hydroxycarotenoids plus β-carotene and the value of monohydroxylation is calculated as the sum of 3-OH-echinenone, adonirubin plus β-cryptoxanthin related to the sum of all hydroxycarotenoids plus β-carotene.

Endogenous β-carotene hydroxylase gene activity is upregulated in transgenic rice endosperm producing ketocarotenoids

Total RNA was extracted from the endosperm (25 DAP) of transgenic lines B1, B2, B3 and C, the latter a control line expressing the selectable marker alone. Each sample comprised RNA pooled from six siblings. The expression of *ZmPSY1*, *PaCRTI* and *sCrBKT* in the endosperm was confirmed by northern blot analysis in lines B1–B3 but not in the control line, as expected (Figure 5). Quantitative real-time RT-PCR analysis was then used to compare the expression levels of the endogenous β-carotene hydroxylase gene (*OsBCH*) in the endosperm of all four lines, normalized against the *actin* housekeeping gene. We found that *OsBCH* mRNA was 2.9–5.1 times more abundant in the endosperm of lines B1–B3 compared to the control line (Figure 6). This

indicated the presence of some form of feedback regulation in the lines producing ketocarotenoids, resulting in the induction of endogenous β-carotene hydrolase gene expression.

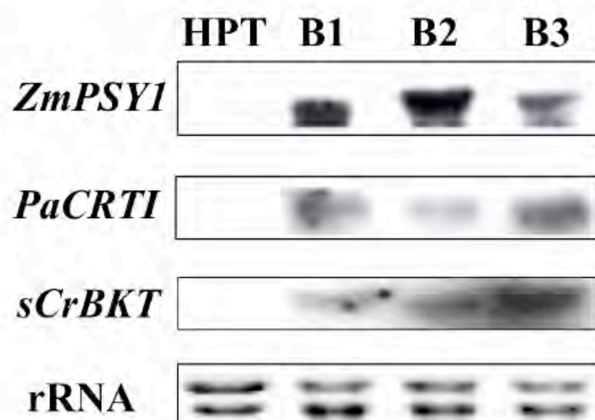

Figure 5 Transgene expression in rice endosperm

Northern blot analysis (25 μg of total RNA per lane) was used to monitor transgene expression in the endosperm (25 DAP) of transgenic lines B1, B2 and B3 and the *hpt* control. To ensure equal loading, rRNA was stained with ethidium bromide. Abbreviations: *ZmPSY1,* maize phytoene synthase 1 gene; *PaCRTI*, bacterial phytoene desaturase gene; *sCrBKT*, synthetic *Chlamydomonas reinhardtii* β-carotene ketolase gene; *hpt,* hygromycin phosphotransferase gene.

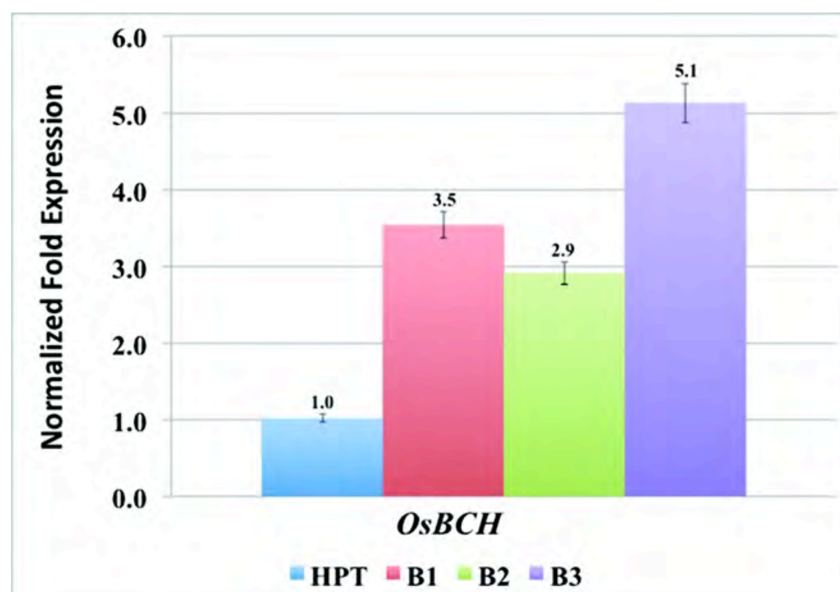

Figure 6 Relative abundance of the *OsBCH* mRNA in rice endosperm

The abundance of endogenous *OsBCH* mRNA was determined in rice endosperm (T3) at 25 DAP by quantitative real-time RT-PCR and presented as an average of three replicates ± SD. *OsBCH* expression was normalized against *actin*. Abbreviations: BCH, β-carotene hydroxylase; *hpt,* hygromycin phosphotransferase gene.

DISCUSSION

The visual screening of transgenic rice callus by color predicts the carotenoid profiles of the corresponding transgenic plants

An extended carotenoid pathway was reconstructed in rice endosperm by simultaneously expressing *ZmPSY1*, *PaCRTI* and a synthetic β-carotene ketolase gene (*sCrBKT*) under the control of three different endosperm-specific promoters. These constructs were introduced into 7-day-old mature zygotic rice embryos along with the selectable marker *hpt*, allowing transgenic plants to be regenerated on medium supplemented with hygromycin. Independent transgenic lines were recovered that produced pale pink grains, and these were shown by northern blot to express the three transgenes in addition to the selectable marker, as anticipated.

The callus tissue derived from embryos transformed with all three transgenes was also pink, and was also shown to express all three genes by northern blot (Bai *et al*., 2013). In contrast, colorless callus was shown to express *PaCRTI* and/or *sCrBKT* (Bai *et al*., 2013) and yellow callus was shown to express *ZmPSY1* and *PaCRTI* (Bai *et al*., 2013). Neither of these tissues accumulated ketocarotenoids (Bai *et al*., 2013) and transgenic plants derived from this callus tissue also lacked ketocarotenoids in the endosperm. These results confirmed that the screening of transgenic rice callus based its color phenotype can reliably predict the metabolite profiles of regenerated transgenic plants.

Reconstruction of the extended carotenoid pathway in rice endosperm produces canthaxanthin as a major product reflecting the limited availability of endogenous β-carotene hydroxylase for astaxanthin synthesis

In transgenic lines B1–B3, the major product was canthaxanthin (43.2–49.7% of total carotenoids) although there were also significant amounts of adonirubin (15.8–17.6%), β-carotene (15.3–20.4%) and lutein (10.3–16.3%) (Figure 4). Astaxanthin, echinenone, 3-hydroxyechinenone, β-cryptoxanthin and α-carotene were also present, albeit as minor components, each representing less than 4% of the total carotenoids. We were unable to detect phytoene or lycopene in the transgenic endosperm, which indicated that phytoene synthesis and subsequent desaturation reactions to all-*trans*-lycopene catalyzed by ZmPSY1 and PaCRTI were not rate-limiting steps for the accumulation of astaxanthin in rice endosperm.

The enzyme β-carotene hydroxylase can introduce hydroxyl groups at the 3 and 3' positions on the β-ionone ring whether or not there are already keto groups at the 4 and/or 4' positions (Fraser *et al.*, 1997). However, most β-carotene ketolases are less efficient if the β-ionone ring is already hydroxylated at the 3 or 3' position. Plants expressing such β-carotene ketolases generally accumulate small amounts of astaxanthin plus various intermediates including echinenone, 3'-hydroxyechinenone and adonixanthin (Misawa, 2009; Zhu *et al.*, 2009). We therefore used a chemically-synthesized version of the truncated *C. reinhardtii* β-carotene ketolase gene (*sCrBKT*) (Zhong *et al.*, 2011) optimized for maize codon usage, because the original truncated gene (*CrBKT*) encodes an enzyme that has proven superior to other ketolases for astaxanthin biosynthesis in *Arabidopsis thaliana*, tobacco and tomato (Huang *et al.*, 2012, 2013; Zhong *et al.*, 2011).

Accordingly, we found that the expression of *sCrBKT* in rice endosperm in addition to *ZmPSY1* and *PaCRTI* favored the accumulation of diketocarotenoids such canthaxanthin (Table 1a). All three lines showed evidence of a similar high degree of ketolation, given that canthaxanthin, adonirubin and astaxanthin together accounted for more than 70% of the total carotenoids (Table 1b). The ketocarotenoid profiles were similar among the three lines despite substantial differences in the abundance of the three transgene-derived mRNAs, suggesting that the ketolation reaction is not stringently regulated at the mRNA level (Figure 5).

In contrast, the dihydroxycarotenoid products in the three lines represented 3.9–6.5% of the total carotenoids and the monohydroxycarotenoid products represented ~50%. This indicates that endogenous β-carotene hydroxylase activity is rate-limiting for astaxanthin biosynthesis in the endosperm. The levels of endogenous *OsBCH* mRNA were induced 2.9–5.1-fold in the three transgenic lines compared to the *hpt* control (Figure 6). The observed upregulation of endogenous *OsBCH* in rice endosperm is similar to that previously reported in ketocarotenoid-producing carrot roots and canola seeds, where the expression levels of endogenous β-carotene hydroxylases also increased (Jayaraj *et al.*, 2008; Fujisawa *et al.*, 2009). The expression of *sCrBKT* in rice endosperm resulted in the accumulation of minor monoketocarotenoid intermediates such as echinenone and 3-hydroxyechinenone, but no detectable monoketocarotenoid intermediates with 3'-hydroxylated β-ionone rings such as 3'-hydroxyechinenone and adonixanthin (Figure 3). As mentioned above, this could reflect the greater preference of sCrBKT for carotenoids with hydroxylated β-ionone rings (Huang *et al.*, 2012, 2013; Zhong *et al.*, 2011).

Astaxanthin is the end product of the extended carotenoid biosynthesis pathway and a valuable target for metabolic engineering. Understanding the pathway and its regulatory

mechanisms will help to boost the production of astaxanthin in plants, and to engineer more effective pathways with alternative branch points leading to closely related but distinct chemical structures. We showed that it is possible to modulate the production of astaxanthin and canthaxanthin in rice endosperm by expressing a synthetic β-carotene ketolase gene (*sCrBKT*) in addition to maize phytoene synthase 1 (*ZmPSY1*) and bacterial phytoene desaturase (*PaCRTI*). These experiments showed that rice endosperm can be engineered to produce nutritionally-important ketocarotenoids but also identified the rate-limiting steps in the pathway that must be overcome in order to maximize flux towards target ketocarotenoid molecules.

CONCLUSIONS

Transgenic rice endosperm expressing phytoene synthase, phytoene desaturase and β-carotene ketolase accumulated large amounts of canthaxanthin and adonirubin, plus additional ketocarotenoids in smaller amounts, such as astaxanthin. The accumulation of such intermediates, particularly the abundance of canthaxanthin, provides evidence that the limited activity of endogenous β-carotene hydroxylases causes a bottleneck in the extended ketocarotenoid pathway despite evidence that they are upregulated in response to the accumulation of ketocarotenoids. This bottleneck must be removed in order to maximize the accumulation of the target end product, astaxanthin.

EXPERIMENTAL PROCEDURES

Growth and maintenance of rice plants

Wild type rice (*Oryza sativa* L. cv EYI-105) and transgenic rice plants were grown in the greenhouse and growth chambers at 28/20°C day/night temperature with a 10-h

photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21/18°C day/night temperature with a 16-h photoperiod thereafter.

Gene cloning and vector construction

The maize *PSY1* cDNA was cloned from inbred line B73 by RT-PCR using forward primer 5′-AGG ATC CAT GGC CAT CAT ACT CGT ACG AG-3′ and reverse primer 5′-AGA ATT CTA GGT CTG GCC ATT TCT CAA TG-3′, based on the maize *PSY1* sequence deposited in GenBank (accession number AY324431). The product was transferred to pGEM-T easy (Promega, Madison, WI, USA) for sequencing and then to plasmid p326 (Stoger *et al.*, 1999), containing the endosperm-specific wheat LWM glutenin promoter and *nos* terminator, to yield the final construct p326-ZmPSY1.

The *Pantoea ananatis* (formerly *Erwinia uredovora*) *CRTI* gene was fused in frame with the transit peptide sequence from the *P. vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) in plasmid pYPIET4 (Misawa *et al.*, 1994) and amplified by PCR using forward primer 5′-ATC TAG AAT GGC TTC TAT GAT ATC CTC TTC-3′ and reverse primer 5′-AGA ATT CTC AAA TCA GAT CCT CCA GCA TCA-3′, based on the *P. ananatis CRTI* gene sequence deposited in GenBank (accession number D90087). The product was transferred to pGEM-T easy for sequencing and then to pHorp-P (Sorensen *et al.*, 1996), containing the endosperm-specific barley D-hordein promoter and the rice ADPGPP terminator, to yield the final construct pHorP-PaCRTI.

The *Chlamydomonas reinhardtii* β-carotene ketolase gene was truncated according to previous publication (Zhong *et al.*, 2011) and codon optimized for maize (GenBank accession number JF304771). The chemically synthesized gene (*sCrBKT*) was fused

with the transit peptide sequence from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) and the 5′-untranslated region (5′UTR) of the rice alcohol dehydrogenase gene (Sugio *et al.*, 2008) and placed under the control of the maize γ-zein promoter (pGZ63-sCrBKT). The transit peptide sequence was also modified for optimal performance in monocotyledonous plants (GenBank accession number KF68948). The construct was verified by sequencing across all junctions.

Transformation of rice plants

Seven-day-old mature zygotic embryos were transferred on osmoticum medium (containing 4.4 g/l MS powder supplemented with 0.3 g/l casein hydrolysate, 0.5 g/l proline, 72.8 g/l mannitol and 30 g/l sucrose) four hours before bombarding and then bombarded with 10 mg gold particles coated with the carotenogenic constructs and the selectable maker *hpt* at a 3:3:3:1 ratio as previously described (Christou *et al.*, 1991, 1997). They were returned to osmoticum medium for 12 h before selection on MS medium (containing 4.4 g/l MS powder, 0.3 g/l casein hydrolysate and 0.5g/l proline and 30 g/l sucrose) supplemented with 50 mg/l hygromycin and 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark for 2–3 weeks (Farré *et al.*, 2012). Transgenic plantlets were regenerated and hardened off in soil.

Transgene expression analysis

Total RNA was extracted from rice endosperm at 25 DAP. We separated 25 µg of denatured total RNA by 1.2% (w/v) agarose-formaldehyde gel electrophoresis in 1x MOPS buffer and transferred the fractionated RNA to a membrane by capillary blotting (Sambrook *et al.*, 1989). The membrane was probed at 50°C overnight in DIG Easy

Hyb buffer with digoxigenin-labeled partial cDNAs prepared using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany). The forward and reverse primers for each transgene probe design are shown in Supplementary **Table S1**. The membrane was washed twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 20 min in 0.2x SSC, 0.1% SDS at 68°C, and finally twice for 10 min in 0.1x SSC, 0.1% SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemiluminescence generated by the CSPD substrate (Roche Diagnostics GmbH, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions.

Table S1 Oligonucleotide sequences of forward and reverse primers for probe design used for northern blot analysis

Transgenes	Primers sequence
***ZmPSY1*.F**	Forward 5´-GTGTAGGAGGACAGATGAGCTTGT-3´
***ZmPSY1*.R**	Reverse 5´-CATCTGCTAGCCTGTGAGAGCTCA-3´
***PaCRTI*.F**	Forward 5´-TGGAGAAGCGTTTACAGTAAGGT-3´
***PaCRTI*.R**	Reverse 5´-GCGTGCAGATAAAGTGAGAAGTC-3´
***sCrBKT*.F**	Forward 5´-GGATCCTCAGCCAGGAGCCAGTGCAGCGCCTCT-3´
***sCrBKT*.R**	Reverse 5´-GAATTCCATGGGGCCAGGCATTCAGCCCACTTCC-3´

Endogenous β-carotene hydroxylase gene expression analysis

Endogenous β-carotene hydroxylase (*OsBCH*) gene expression was monitored by quantitative real-time RT-PCR (qRT-PCR) using a Bio-Rad CFX96^{TM} system and 25-µl mixtures containing 10 ng of synthesized cDNA, 1x iQ SYBR green supermix (Bio-

Rad) and 0.2 μM forward and reverse primers. To calculate relative expression levels, serial dilutions (60–0.096 ng) were used to produce standard curves for each gene. Each sample was amplified in triplicate using 96-well optical reaction plates, starting with a heating step for 3 min at 95°C, followed by 40 cycles of 95°C for 15 s, 58.5°C for 1 min and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis in the temperature range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using CFX96™ system software. Values represent the mean of three replicates ± SD. Primers for endogenous rice carotenogenic genes and the internal control *actin* are listed in Supplementary **Table S2**.

Table S2 Oligonucleotide sequences of forward and reverse primers for quantitative real-time RT-PCR analysis

Endogenes	Primers sequence
***OsACTIN*.F**	Forward 5´-TGTCATGGTCGGAATGGGCCAG-3´
OsACTIN.R	Reverse 5´-CACTTCATGATGGAGTTGTA -3´
***OsBCH*.F**	Forward 5´-TCGAGAACGTGCCCTACTTCC-3´
***OsBCH*.R**	Reverse 5´-ACCCACCTCCTCCAACTCCTT-3´

Carotenoid extraction and quantification

Carotenoids were extracted from 50 mg freeze-dried mature seeds (40 DAP) in the dark using 50/50 (v/v) tetrahydrofuran and methanol at 60°C for 20 min and then partitioned into 30% ether in petrol. The upper carotenoid phase was collected and fractionated by isocratic high-performance liquid chromatography (HPLC) using a 15-cm Nucleosil 100

C18, 5µ column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase at 20°C, with a flow rate of 0.8 ml/min. Spectra were recorded online with a Kontron 440 photodiode array detector (Kontron, Straubenhard, Germany). Carotenoids were identified based on authentic standards (Sandmann, 2002) and quantified from rice grains representing six independently grown plants of each transgenic line and are given together with their standard deviations.

REFERENCES

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P. and Zhu C. (2013) An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. *Plant J.* doi: 10.1111/tpj.12384.

Bai, C., Twyman, R.M., Farré, G., Sanahuja, G., Christou, P., Capell, T. and Zhu, C. (2011) A golden era-pro-vitamin A enhancement in diverse crops. *In. Vitro Cell. Dev. Biol.–Plant* **47**, 205–221.

Chen, B.P. and Park, J.S. (2004) Carotenoid action on the immune response. *J. Nutr.* **134**, 257S–261S.

Chen, B.P., Park, J.S., Wong, M.W. and Wong, T.S. (1999) A comparision of the anticancer activities of dietary β-carotene, canthaxanthin and astaxanthin in mice *in vivo*. *Anticancer Res.* **19**, 1849–1853.

Christou, P. (1997) Rice transformation by bombardment. *Plant Mol. Biol.* **35**, 197–203.

Christou, P., Ford, T.L. and Kofron, M. (1991) Production of transgenic rice (*Oryza*

sativa L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Biotechnol.* **9**, 957–962.

Cunningham, F.X.Jr. and Gantt, E. (2005) A study in scarlet, enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*. *Plant J.* **41**, 478-492.

Farré, G., Naqvi, S., Sanahuja, G., Bai, C., Zorrilla-López, U., Rivera, S.M., Canela, R., Sandman, G., Twyman, R.M., Capell, T., Zhu, C. and Christou, P. (2012) Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. *Methods Mol. Biol.* **847**, 419–35.

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

Fraser, P.D., Miura, Y. and Misawa, N. (1997) *In vitro* characterization of astaxanthin biosynthetic enzymes. *J. Biol. Chem.* **272**, 6128–6135.

Fujisawa, M., Takita, E., Harada, H., Sakurai, N., Suzuki, H., Ohyama, K., Shibata, D. and Misawa, N. (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *J. Exp. Bot.* **60**, 1319–1332.

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

Huang, J.C., Zhong, Y., Sandmann, G., Liu, J. and Chen, F. (2012) Cloning and selection of carotenoid ketolase genes for the engineering of high-yield astaxanthin in plants. *Planta,* **236**, 691–699.

Huang, J.C., Zhong, Y.J., Liu, J., Sandmann, G. and Chen, F. (2013) Metabolic engineering of tomato for high-yield production of astaxanthin. *Metab. Eng.* **17**, 59–67.

Iwamoto, T., Hosoda, K., Hirano, R., Kurata, H., Matsumoto, A., Miki, W., Kamiyama, M., Itakura, H., Yamamoto, S. and Kondo, K. (2000) Inhibition of low-density lipoprotein oxidation by astaxanthin. *J. Atheroscler. Thromb*. **7**, 216–222.

Jayaraj, J., Devlin, R. and Punja, Z. (2008) Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic Res.* **17**, 489–501.

Jyonouchi, H., Sun, S., Tomita, Y. and Gross, M.D. (1995) Astaxanthin., a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. *J. Nutr*. **125**, 2483–2492.

Meyers, S.P. (1994) Developments in world aquaculture, feed formulations and role of carotenoids. *Pure Appl. Chem*. **66**, 1069–1076.

Misawa, N. (2009) Pathway engineering of plants towards astaxanthin production. *Plant Biotechnol.* **26**, 93–99.

Misawa, N., Yamano, S., Linden, H., de Felipe, M.R., Lucas, M., Ikenaga, H. and Sandmann, G. (1994) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–840.

Morris, W.L., Ducreux, L.J., Fraser, P.D., Millam, S. and Taylor, M.A. (2006) Engineering ketocarotenoid biosynthesis in potato tubers. *Metab. Eng*. **8**, 253–263.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**, 482–487.

Rivera, S.M., Vilaró, F., Zhu, C., Bai, C., Farré, G., Christou, P. and Canela, R. (2013) Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem.* **61**, 5279–5285.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press. 1, 63–70.

Sandmann, G. (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chem. BioChem*. **3**, 629–635.

Schaub, P., Al-Babili, S., Drake, R. and Beyer, P. (2005) Why is golden rice golden (yellow) instead of red? *Plant Physiol.* **138**, 441–450.

Schreier, P., Seftor, E., Schell, J. and Bohnert, H. (1985) The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J.* **4**, 25–32.

Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol. Gen. Genet*. **250**, 750–760.

Stoger, E., Williams, S., Keen, D. and Christou, P. (1999) Constitutive versus seed specific expression in transgenic wheat, temporal and spatial control. *Transgenic Res.* **8**, 73–82.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A. and Kato, K. (2008) The 5´-untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells. *J. Biosci. Bioeng.* **105**, 300–330.

Tanaka, T., Morishita, Y., Suzui, M., Kojima, T., Okumura, A. and Mori, H. (1994) Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. *Carcinogenesis,* **15**, 15–19.

Tatsuzawa, H., Maruyama, T., Misawa, N., Fujimori, K. and Nakano, M. (2000) Quenching of singlet oxygen by carotenoids produced in *Escherichia coli*-attenuation of singlet oxygen-mediated bacterial killing by carotenoids. *FEBS Lett*. **484**, 280–284.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**, 303–305.

Zhong, Y.J., Huang, J.C., Liu, J., Li, Y., Jiang, Y., Xu, Z.F., Sandmann, G. and Chen, F. (2011) Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic *Arabidopsis*. *J. Exp. Bot*. **62**, 3659–3669.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P. and Capell, T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl. Acad. Sci. USA* **105**, 18232–18237.

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

Zhu, C., Sanahuja, G., Yuan, D., Farré, G., Arjó, G., Berman, J., Zorrilla-López, U., Banakar, R., Bai, C., Pérez-Massot, E., Bassie, L., Capell, T. and Christou, P. (2013) Biofortification of plants with altered antioxidant content and composition, genetic engineering strategies. *Plant Biotechnol. J*. **11**, 129–141.

GENERAL CONCLUSIONS

1. An *in vitro* system was developed for the rapid functional characterization of carotenoid biosynthesis genes. I demonstrated the principle by identifying the functions of a well-characterized gene and two uncharacterized genes involved in carotenoid biosynthesis and sequestration.

2. I have demonstrated that the visual screening of transgenic rice callus based on its color phenotype allows the reliable prediction of metabolite profiles in regenerated transgenic plants. The rice callus platform therefore allows large numbers of constructs to be evaluated simultaneously in different combinations, so that complex multigene engineering strategies can be optimized before any transgenic plants are produced.

3. The platform can be used to investigate promoter activity, protein synthesis and metabolite production, thus facilitating a more quantitative approach to plant synthetic biology and allowing the development of more refined and sophisticated strategies for metabolic engineering.

4. The method is not dependent on a color phenotype and can be applied to any pathway and any gene product that can be analyzed using standard analytical approaches such as HPLC, mass spectrometry and NMR.

5. The novel ketocarotenoid 4-keto-α-carotene was identified as an unexpected byproduct in rice callus engineered with genes involved in ketocarotenoid biosynthesis.

6. I demonstrated that the insufficient supply of isoprenoid precursors derived from the MEP pathway, and also the absence of an effective carotenoid sequestration and deposition sink, are two key factors limiting carotenoid accumulation in rice endosperm.

7. I generated transgenic rice plants accumulating the ketocarotenoids astaxanthin and canthaxanthin in the endosperm tissue. This is the first successful example of astaxanthin engineering in rice. These experiments provided proof of concept that rice endosperm can be engineered to produce nutritionally-important ketocarotenoids and also identified the remaining rate-limiting steps in the pathway that must be overcome in order to maximize flux towards target ketocarotenoids.

8. The greater understanding of metabolic bottlenecks in the carotenoid pathway in rice endosperm offered by the work described in this thesis will allow the design and implementation of more targeted strategies for the creation of plants engineered to synthesize particular carotenoid profiles for diverse applications.

OUTPUTS

Participation in projects

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1. Caracterización de la respuesta a la infección viral de plantas de tomate y maíz tratadas con elicitores de resistencia sistémica adquirida e inducida. Programa Nacional de Recursos y Tecnologías Agroalimentarias (AGL). Ministerio de Ciencia e Innovación. Main researcher: Vicente Medina Piles AGL2010-15691. 2011-2013, Spain

2. BIOFORCE ERC Advanced Grand 2009 - 2014, UdL, Spain

3. Application of genetic engineering techniques to create a high nutritional quality of the new maize germplasm. Jilin Province Science and Technology Agency 2005.1.1-2007.12.31, China

4. Isolation and identification lutein biosynthesis gene and its promoter from *Gentiana lutea*. National Natural Science Foundation of Committee 2004.1.1-2006.12.1, China

Scientific publications

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1. **Bai C**, Rivera S, Medina V, Alves R, Vilaprinyo E, Sorribas A, Canela R, Capell T, Sandmann G, Christou P and Zhu C. (2013) An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. Plant J (DOI: 10.1111/tpj.12384).

2. Breitenbach J, **Bai C**, Rivera S, Canela R, Capell T, Christou P, Zhu C and Sandmann G. (2013) A novel carotenoid, 4-keto-α-carotene, as an unexpected by-product during genetic engineering of carotenogenesis into rice. Phytochemistry http://dx.doi.org/10.1016/j.phytochem.2013.12.008 (**Joint first author**).

3. **Bai C**, Twyman R, Farré G, Sanahuja G, Christou P, Capell T and Zhu C. (2011) A golden era-pro-vitamin A enhancement in diverse crops. In Vitro Cell Dev Biol- Plant 47: 205–221 (**Annex**).

4. Farré G, **Bai C,** Twyman R, Capell T, Christou P and Zhu C. (2011) Nutritious crops producing multiple carotenoids--a metabolic balancing act. Trends Plant Sci 16: 532–540 (**Annex**).

5. Zhu C, **Bai C**, Sanahuja G, Yuan D, Farré G, Naqvi S, Shi L, Capell T and Christou P. (2010) The regulation of carotenoid pigmentation in flowers. Arch Biochem Biophys 504: 132–141 (**Annex**).

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

7. Rivera S, Vilaró F, Zhu C, **Bai C**, Farré G, Christou P and Canela-Garayoa R. (2013) A Fast Quantitative Method for the Analysis of Carotenoids in Transgenic Maize. J Agric Food Chem 61: 5279–5285.

8. Zhu C, Yang Q, Ni X, **Bai C**, Sheng Y, Shi L, Capell T, Sandmann G and Christou P. (2013) Cloning and functional analysis of the promoters that upregulate carotenogenic gene expression during flower development in *Gentiana lutea*. Physiol Plant (doi:10.1111/ppl.12129).

9. Zorrilla-López U, Masip G, Arjó G, **Bai C**, Banakar R, Bassié L, Berman J, Farré G, Miralpeix B, Pérez-Massot E, Sabalza M, Sanahuja G, Vamvaka E, Twyman RM, Christou P, Zhu C and Capell T. (2013) Engineering metabolic pathways in plants by multigene transformation. Int J Dev Biol 57: 565–576.

10. Yang Q, Yuan D, Shi L, Capell T, **Bai C**, Wen N, Lu X, Sandmann G, Christou P and Zhu C. (2012) Functional characterization of the *Gentiana lutea* zeaxanthin

epoxidase (GlZEP) promoter in transgenic tomato plants. Transgenic Res 21: 1043–1056.

11. Li Q, Farré G, Naqvi S, Breitenbach J, Sanahuja G, **Bai C**, Sandmann G, Capell T, Christou P and Zhu C. (2010) Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. Transgenic Res 19: 1053–1068.

12. Yuan D, Bassie L, Sabalza M, Miralpeix B, Dashevskaya S, Farré G, Rivera S, Banakar R, **Bai C**, Sanahuja G, Arjo G, Avilla E, Zorrilla-Lopez U, Ugidos Damboriena N, Lopez A, Almacellas D, Zhu C, Capell T, Hahne G, Twyman R and Christou P. (2011) The potential impact of plant biotechnology on the Millennium Development Goals. Plant Cell Rep 30: 249–265.

13. Pérez-Massot E, Banakar R, Gómez-Galera S, Zorrilla-López U, Sanahuja G, Arjó G, Miralpeix B, Vamvaka E, Farré G, Rivera S, Dashevskaya S, Berman J, Sabalza M, Yuan D, **Bai C,** Bassie L, Twyman R, Capell T, Christou P and Zhu C. (2012) The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints. Genes Nutr 8: 29–41.

14. Zhu C, Sanahuja G, Yuan D, Farré G, Arjó G, Berman J, Zorrilla-López U, Banakar R, **Bai C**, Pérez-Massot E, Bassie L, Capell T and Christou P. (2012) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. Plant Biotechnol J 11: 129–141.

15. Berman J, Zhu C, Pérez-Massot E, Arjó G, Zorrilla-López U, Masip G, Banakar R, Sanahuja G, Farré G, Miralpeix B, **Bai C**, Vamvaka E, Sabalza M, Twyman RM, Bassié L, Capell T and Christou P. (2013) Can the world afford to ignore biotechnology solutions that address food insecurity? Plant Mol Biol 83: 5–19.

16. Qian R, Lu X D, **Bai C**, Yang QJ, Shi LX and Zhu C. (2007) Metabolic Engineering of Carotenoid Formation in carrot Roots. Mol Plant Breeding 5: 285–286.

Book chapters

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1. Farré G, Gómez-Galera S, Naqvi S, **Bai C**, Sanahuja G, Yuan D, Zorilla U, Tutusaus Codony L, Rojas E, Fibla M, Twyman RM, Capell T, Christou P and Zhu C. (2012) Nutritional improvement of crops using biotechnology. In: Meyers RA (ed) Encyclopedia of Sustainability Science and Technology. Springer NY, pp 1676–1723.

2. Farré G, Naqvi S, Sanahuja G, **Bai C**, Zorilla U, Rivera S, Canela R, Sandman G, Twyman RM, Capell T, Zhu C and Christou P. (2012) Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. Transgenic Plants: Methods and Protocols. Series: Methods in Molecular Biology. Dunwell, Jim M.; Wetten, Andy C. Springer, Berlin. 847: 419–435.

Invited articles/reports

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1. Gómez-Galera S, Naqvi S, Farré G, Sanahuja G, **Bai C,** Capell T, Zhu C and Christou P (2010) Feeding future populations with nutritionally complete crops. Information Systems for Biotechnology (ISB) News Rep 9-11.

Sequences submitted to GenBank

1. **Bai C,** Capell T, Christou P and Zhu C. (2013) Synthetic construct transit peptide sequence for Pea ribulose-1,5-bisphospate carboxylase (rbcS) small subunit gene. Accession Number: KF684948.

2. **Bai C,** Capell T, Christou P and Zhu C. (2012) *Zea mays* opaque-2 (O2) gene BankIt1517998 BankIt1517998 JQ723697.

3. **Bai C**, Capell T, Christou P and Zhu C. (2012) *Zea mays* prolamin box binding factor gene BankIt1518001 BankIt1518001 JQ723698.

4. Zhu C, Capell T, Farré G, **Bai C**, Sandmann G and Christou P. (2009) *Zea mays* carotene beta-ring hydroxylase mRNA, complete cds. Accession Number: GU130216.

5. Zhu C, Capell T, **Bai C**, Farré G, Sandmann G and Christou P. (2009) *Zea mays* carotene epsilon-ring hydroxylase mRNA, complete cds. Accession Number: GU130217.

6. **Bai C**, Li Q, Shi L, Yang Q, Qian R and Zhu C. (2006) *Gentiana lutea* mRNA for lycopene beta-cyclase2, complete cds. Accession Number: EF062505.

7. Zhu C, Yang Q, Shi L, Lu X, Li Q, **Bai C**, Wen N and Qian R. (2006) *Gentiana lutea* Lhca5 protein (Lhca5) mRNA, compete cds. Accession Number: EF069436.

Oral presentations

1. Bai C, Rivera S, Medina V, Canela R, Capell T, Sandmann G, Christou P and Zhu C. (2013) Carotenoid biofortification of rice endosperm through combinatorial transformation for multi-gene and multi-pathway engineering. A joint Meeting of

TERPNET2013, Cost Action "Plant Engine", Cost Action QualityFruit" and "EU-SmartCell" on "Biosynthesis, Function and Biotechnology of Isoprenoids in Terrestrial and Marine Organisms" held in Kolymvari, Crete, Greece, June 1-5.

2. **Bai C**, Rivera S, Medina V, Sandmann G, Canela R, Zhu C, Capell T and Christou P. (2011) An effective *in vitro* system for the functional characterization of carotenogenic genes in rice (*Oryza sativa*). The 16th International Symposium on Carotenoids held in Krakow, Poland, July 17-22.

3. **Bai C**, Rivera S, Medina V, Canela R, Sandmann G, Capell T, Christou P and Zhu C. (2010) Metabolic engineering of carotenoid accumulation in rice endosperm through combinatorial genetic transformation. In the 12th World Congress of the International Association for Plant Biotechnology (12th IAPB) and the 2010 In Vitro Biology Meeting of the Society for In Vitro Biology (SIVB) held in St. Louis, MO, USA, June 6-11.

Poster presentations

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1. **Bai C**, Rivera SM, Medina V, Sandmann G, Canela R, Zhu C, Capell T and Christou P. (2012) Rice callus and seeds as vehicles to understand carotenoid biosynthesis. The 10th International Congress on Plant Molecular Biology. October 21(Sun)-26(Fri), ICC, Jeju Island, Republic of Korea.

2. **Bai C**, Rivera SM, Medina V, Sandmann G, Canela R, Zhu C, Capell T and Christou P. (2011) An effective *in vitro* functional expression assay for carotenogenic genes in rice. TERPNET 2011, 10th International Meeting: Biosynthesis and function of isoprenoids in plants, microorganisms and parasites. May 22-May 26, 2011, Kalmar, Sweden. pp.141.

3. Zhu C, Farré G, Shi L, **Bai C**, Sanahuja G, Capell T, Sandmann G and Christou P (2011) Cloning and functional characterization of carotene hydroxylase genes from maize (*Zea mays. L.*) endosperm. TERPNET 2011, 10th International Meeting: Biosynthesis and function of isoprenoids in plants, microorganisms and parasites. May 22-May 26, 2011, Kalmar, Sweden. pp.143.

4. Christou P, Farré G, **Bai C**, Rivera SM, Sanahuja G, Capell T and Zhu C (2011) Multi-gene Constructs Invited Presentation. Meeting of the Society for In Vitro Biology Junes 4-8 Raileigh, North Carolina, USA.

5. Christou P, Farré G, **Bai C**, Snahuja G, Gomez-Galera S, Zorrilla Lopez U, Naqvi S, Capell T and Zhu C. (2010) Multi-step Pathway engineering in major staple cereal crops Invited lectura X reunion de Biología Molecular de Plantas RBMP palacio de Congresos 8-10 Julio, Valencia Spain.

6. **Bai C**, Maiam Rivera S, Medina V, Canela R, Sandmann G, Capell T, Christou P and Zhu C. (2010) Exploring the mechanism of carotenoid accumulation in rice (*Oryza sativa*) endosperm through multi-gene engineering. First Global Conference in Biofortification Washingtown DC USA, 8-11 November.

7. **Bai C**, Maiam Rivera S, Medina V, Canela R, Sandmann G, Capell T, Christou P and Zhu C. (2010) Carotenoid biofortification of rice endosperm through multiplex gene transformation. Gordon Research Conference in Carotenoids January 17-22 Ventura Beach Marriot, Ventura CA, USA.

8. Naqvi S, Farré G, Sanahuja G, **Bai C**, Capell T and Christou P. (2009) Simultaneous multi-pathway engineering in crop plants through combinatorial genetic transformation: creatin nutritionally biofortified cereal grains for food security. Event Planners Australia del 10/08 al 14/08 Tropical North Queensland (Australia).

9. **Bai C**, Zhu C, Capell T, Breitenbach J, Sandmann G and Christou P. (2009) Metabolic engineering of carotenoid accumulation in rice endosperm through combinatorial genetic transoformation. 14th European Meeting on Fat Soluble Vitamins. Potsdam (Germany).

10. Li Q, Yuan D, **Bai C**, Yang Q, Capell T, Sandmann G, Christou P and Zhu C. (2009) Cloning and characterization of a chromoplast-specific zeaxanthin epoxidase gene promoter from *Gentiana lutea*. XVIII-National Meeting of Spanish Society of Plant Physiology and XI-Congress Spanish-Portuguese of Plant Physiology. Zaragoza. Spain. pp. 138.

11. **Bai C**, Zhu C, Capell T, Breitenbach J, Sandmann G and Christou P. (2009) Exploring the mechanism of carotenoid accumulation in rice endosperm through multi-gene engineering. XVIII-National Meeting of Spanish Society of Plant Physiology and XI-Congress Spanish-Portuguese of Plant Physiology. Zaragoza. Spain. pp. 337.

12. Naqvi S, Farré G, Sanahuja G, Gomez-Galera S, **Bai C**, Capell T, Zhu C and Christou P. (2009) Simultaneous multi-pathway engineering in crop plants through combinatorial genetic transformation: Creating nutritionally BIOFORtified Cereal grains for food security. XVIII-National Meeting of Spanish Society of Plant Physiology and XI-Congress Spanish-Portuguese of Plant Physiology. Zaragoza. Spain. pp. 328.

ANNEX

An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation

Chao Bai[1], Sol M. Rivera[2], Vicente Medina[1], Rui Alves[3], Ester Vilaprinyo[3], Albert Sorribas[3], Ramon Canela[2], Teresa Capell[1], Gerhard Sandmann[4], Paul Christou[1, 5] and Changfu Zhu[1,*]

The Plant Journal (2014), 77: 464-475

*Corresponding author Changfu Zhu (email: zhu@pvcf.udl.cat)

the plant journal
SEB Society for Experimental Biology

The Plant Journal (2014) 77, 464–475 doi: 10.1111/tpj.12384

TECHNICAL ADVANCE

An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation

Chao Bai[1], Sol M. Rivera[2], Vicente Medina[1], Rui Alves[3], Ester Vilaprinyo[3], Albert Sorribas[3], Ramon Canela[2], Teresa Capell[1], Gerhard Sandmann[4], Paul Christou[1,5] and Changfu Zhu[1,]*

[1]*Department of Plant Production and Forestry Science, School of Agrifood and Forestry Science and Engineering (ETSEA), University of Lleida Agrotecnio Center, Avenida Alcalde Rovira Roure 191, 25198, Lleida, Spain,*
[2]*Department of Chemistry, School of Agrifood and Forestry Science and Engineering (ETSEA), University of Lleida Agrotecnio Center, Avenida Alcalde Rovira Roure 191, 25198 Lleida, Spain,*
[3]*Department Ciències Mèdiques Bàsiques, Universitat de Lleida & Institut de Recerca Biomédica de Lleida, Edifici Recerca Biomédica I, Avenida Alcalde Rovira Roure 80, 25198 Lleida, Spain,*
[4]*Institute of Molecular Bioscience, J.W. Goethe University Frankfurt, Max von Laue Straße 9, 60438 Frankfurt, Germany, and*
[5] *Institució Catalana de Recerca i Estudis Avancats, Passeig Lluís Companys 23, 08010 Barcelona, Spain*

Received 29 August 2013; revised 23 October 2013; accepted 11 November 2013; published online 23 November 2013.
*For correspondence (e-mail zhu@pvcf.udl.cat).

SUMMARY

We have developed an assay based on rice embryogenic callus for rapid functional characterization of metabolic genes. We validated the assay using a selection of well-characterized genes with known functions in the carotenoid biosynthesis pathway, allowing rapid visual screening of callus phenotypes based on tissue color. We then used the system to identify the functions of two uncharacterized genes: a chemically synthesized β–carotene ketolase gene optimized for maize codon usage, and a wild-type *Arabidopsis thaliana* ortholog of the cauliflower *Orange* gene. In contrast to previous reports (Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) *J. Exp. Bot.* 59, 213–223; Lu, S., Van Eck, J., Zhou, X., Lopez, A.B., O'Halloran, D.M., Cosman, K.M., Conlin, B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Küpper, H., Earle, E.D., Cao, J. and Li, L. (2006) *Plant Cell* 18, 3594–3605), we found that the wild-type *Orange* allele was sufficient to induce chromoplast differentiation. We also found that chromoplast differentiation was induced by increasing the availability of precursors and thus driving flux through the pathway, even in the absence of *Orange*. Remarkably, we found that diverse endosperm-specific promoters were highly active in rice callus despite their restricted activity in mature plants. Our callus system provides a unique opportunity to predict the effect of metabolic engineering in complex pathways, and provides a starting point for quantitative modeling and the rational design of engineering strategies using synthetic biology. We discuss the impact of our data on analysis and engineering of the carotenoid biosynthesis pathway.

Keywords: carotenoids, gene function, rice (*Oryza sativa*), 1–deoxy-D–xylulose 5–phosphate synthase, β-carotene ketolase, technical advance.

INTRODUCTION

The advent of large-scale DNA and RNA sequencing has provided unprecedented insights into the information content of plant genomes and transcriptomes (Ozsolak and Milos, 2011). However, the functions of many of the uncharacterized sequences remain unknown because annotations based on homology searching are only approximate, and the slow pace of conventional functional characterization experiments has created a major information bottleneck (Zhu and Zhao, 2007).

Several large-scale direct functional annotation approaches have been developed, but these typically focus on single genes. For example, plant genes may be characterized rapidly by large-scale insertional mutagenesis using either transposons or T–DNA insertions (Myouga *et al.*,

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2009), enhanced breeding approaches such as TILLING (Kurowska *et al.*, 2011), silencing approaches such as virus-induced gene silencing (VIGS; Purkayastha and Dasgupta, 2009) and RNA interference (RNAi; Purkayastha and Dasgupta, 2009), expression profiling using microarrays (Liu *et al.*, 2008), sequence census methods (RNA-seq; Ozsolak and Milos, 2011) or quantitative proteomics (Nikolov *et al.*, 2012), or by analysis of protein interactions using platforms such as the yeast two-hybrid system and its derivatives (Snyder and Gallagher, 2009). These approaches provide empirical functional data but the methods are indirect, i.e. function is inferred through expression profiles, loss-of-function phenotypes and associations with other gene products rather than direct biochemical analysis.

The drawbacks of *in silico* and empirical functional annotation have created significant challenges in characterization and engineering of plant metabolic pathways (Capell and Christou, 2004; Dafny-Yelin and Tzfira, 2007). Uncharacterized plant genes may be annotated on the basis of homology, but the assignments are often vague, e.g. a new sequence may be assigned as a cytochrome P450 mono-oxygenase, a glycosyltransferase or a methyltransferase, but this provides only a basic catalytic function without much information about substrate or product specificity. Similarly, a gene knockout or gene silencing experiment will show the overall impact of loss of function on plant metabolism and physiology, but there is no deeper insight into the role of the gene in metabolism. Yeast two-hybrid screens show potential interaction partners, but only if physical interactions occur. None of these methods, or any combination of them, completely joins the dots and shows the precise metabolic role of uncharacterized sequences, and how they fit into the surrounding context of known metabolic pathways.

We previously described a combinatorial gene transfer system that allows rapid analysis of metabolic genes in random combinations to provide insight into the roles of such genes, how they interact in the overall metabolic pathway, and the most optimum strategy to achieve synthesis of particular metabolic compounds (Zhu *et al.*, 2008; Farre *et al.*, 2013). This platform was established in maize (*Zea mays*) endosperm, which is most useful for assembling metabolic pathways that are relevant in seeds, such as those leading to the production of essential nutrients (Ramessar *et al.*, 2008; Zhu *et al.*, 2008). A more general platform for high-throughput functional analysis requires a less specific physiological structure and a shorter timescale than that provided by maize endosperm. Previous studies have shown that callus cultures in species such as maize, *Arabidopsis thaliana*, sweet potato (*Ipomoea batatas*), marigold (*Tagetes erecta*) and banana (*Musa acuminata*) may be used to test gene function (Paine *et al.*, 2005; Harada *et al.*, 2009; Kim *et al.*, 2011, 2013a,b; Vanegas-Espinoza *et al.*, 2011; Mlalazi *et al.*, 2012). However these studies have been limited to single genes driven by a constitutive promoter because complex and time-consuming strategies are required to construct cassettes carrying multiple transgenes. This makes them unsuitable for the analysis of multiple gene interactions in complex metabolic pathways.

We hypothesized that our combinatorial transformation platform may be used to investigate multiple gene functions in rice (*Oryza sativa*) callus tissue, which may be prepared, transformed and screened much more rapidly than transgenic maize plants. Gene expression and protein accumulation in rice callus involves the same mechanisms that occur in the mature plant. Therefore, rice callus derived from zygotic embryos may be used as a surrogate platform for rapid analysis and evaluation of multiple candidate transgenes in a metabolic pathway to predict their behavior in whole plants.

The approach was tested using well-characterized genes from the carotenoid biosynthesis pathway (Figure 1 and Figure S1; Zhu *et al.*, 2009, 2013; Ruiz-Sola and Rodriguez-Concepcion, 2012). Having confirmed that rice callus is suitable for the functional analysis of known genes, we then investigated the functions of a chemically synthesized uncharacterized β–carotene ketolase and an *Orange* ortholog, which could only be characterized by simultaneous expression of other genes from the carotenoid biosynthesis pathway in rice. The callus assay provides a rapid and inexpensive platform for functional characterization of uncharacterized genes by combinatorial expression, and may be combined with synthetic biology approaches for analysis of complex metabolic pathways and prediction of model-driven metabolic engineering strategies based on multigene expression (Zurbriggen *et al.*, 2012).

RESULTS

Combinatorial transformation of rice embryos with endosperm-specific carotenogenic transgenes generates rice callus lines that accumulate carotenoids

Embryo-derived rice callus is white in color and accumulates only minimal levels of carotenoids (Figures 2a and 3). Our analysis revealed the presence of small amounts of lutein and zeaxanthin in wild-type callus (Figure 3), which is similar to the carotenoid profile of white maize endosperm (Zhu *et al.*, 2008). This indicates that rice callus may likewise be used as a platform to test the combinatorial activity of multiple carotenogenic transgenes, but within a much shorter experimental time scale.

In pilot experiments to develop the platform, we transformed 7-day-old mature zygotic rice embryos with four constructs containing unlinked transgenes. These comprised the selectable marker *HPT* for hygromycin resistance, two carotenogenic transgenes with known functions in the committed carotenoid biosynthesis pathway, namely maize phytoene synthase 1 (*ZmPSY1*) and *Pantoea*

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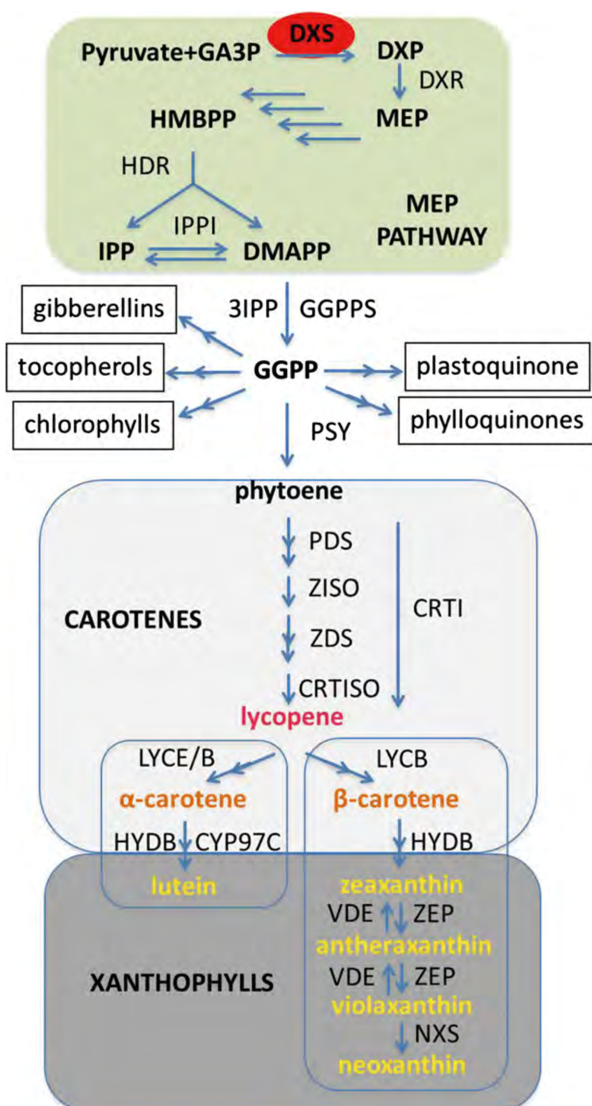

Figure 1. The carotenoid biosynthesis pathway (Farre *et al*., 2010, 2011).
All carotenoids are synthesized from the five-carbon monomeric building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Plastidial IPP is generated via the 2–C-methyl-D–erythritol 4–phosphate (MEP) pathway. The MEP pathway starts with glyceraldehyde-3–phosphate (GA3P) and pyruvate, which are converted into 1–deoxy-D–xylulose 5–phosphate (DXP) by DXP synthase (DXS). DXP reductoisomerase (DXR) converts DXP to MEP in the second step of the MEP pathway. MEP is then converted to 4–hydroxy-3–methylbut-2–enyl diphosphate (HMBPP) in four sequential reactions. Hydroxymethylbutenyl diphoshate reductase (HDR) simultaneously synthesizes IPP and DMAPP from HMBPP in the last step of the MEP pathway. Three IPP molecules are added to DMAPP to produce geranylgeranyl diphosphate (GGPP), which serves as the immediate precursor not only for carotenoids but also for biosynthesis of gibberellins and the side chain of chlorophylls, tocopherols, phylloquinones and plastoquinone. The first committed step in carotenoid biosynthesis is condensation of two molecules of GGPP by phytoene synthase (PSY) to produce phytoene. Phytoene is converted into all-*trans* lycopene by the action of two desaturases and two isomerases: phytoene desaturase (PDS), ζ–carotene isomerase (ZISO), ζ–carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). The bacterial phytoene desaturase (CRTI) performs all the desaturation and isomerization reactions performed by plant PDS, ZISO, ZDS and CRTISO. As CRTI has low homology with plant PDS and ZDS, and reduces the number of transgenes required, it is widely used in metabolic engineering. Lycopene represents a branch in the pathway, leading to either α- or β–carotenes. In the α–carotene branch, addition of one ε–ring and one β–ring to lycopene by lycopene ε–cyclase (LYCE) and lycopene β–cyclase (LYCB), respectively, produces α–carotene. In the β–carotene branch, lycopene is cyclized to produce the provitamin A carotenoid γ–carotene and then β–carotene by addition of β–rings to both ends of the linear lycopene molecule by LYCB. β- and α–carotene are redundantly hydroxylated by non-heme di-iron β–carotene hydroxylases (BCH1 and BCH2) and cytochrome P450-type β- and ε–hydroxylases (CYP97A, CYP97B and CYP97C). β–xanthophylls are epoxidated and de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively giving rise to the xanthophyll cycle.

ananatis phyoene desaturase (*PaCRTI*), and the *A. thaliana* 1–deoxy-D–xylulose 5–phosphate synthase (*AtDXS*) gene, a limiting enzyme of the 2–C-methyl-D–erythritol 4–phosphate (MEP) pathway that supplies carotenoid precursors. The *HPT* gene was expressed constitutively, and expression of the three carotenogenic genes was driven by endosperm-specific promoters.

Remarkably, we observed during selection that the transgenic rice callus ranged in color from white through various shades of yellow to orange, representing expression of various combinations of the three carotenogenic transgenes and thus different carotenoid profiles (Figure 2a). Analysis of steady-state mRNA levels showed that *ZmPSY1* was expressed in all the yellow and orange callus lines but not in white callus which did not express ZmPXY1 (even if the other carotenogenic transgenes were expressed), confirming that *ZmPSY1* is essential for carotenoid accumulation. Faint *ZmPSY1* hybridization bands in the white callus samples represented the endogenous *OsPSY1* gene, which is expressed at minimal levels in rice callus. The appearance of color in the callus tissue confirmed that the 'endosperm-specific' wheat (*Triticum aestivum*) low-molecular-weight glutenin, barley (*Hordeum vulgare*)_D–hordein and rice prolamin promoters were each also active in dedifferentiated rice tissue, as previously reported for the maize 27 kDa γ–zein promoter (Wu and Messing, 2009).

We found a precise correlation between the phenotypes and expressed transgenes at the mRNA level (Figure 2a,b). Rice callus expressing *ZmPSY1* alone was pale yellow in color, whereas callus expressing both *ZmPSY1* and *AtDXS* was darker yellow and callus expressing both *ZmPSY1* and *PaCRTI* was yellow/orange. Callus expressing all three carotenogenic transgenes was a darker orange color. Therefore, we were able to make accurate predictions of the transgenes expressed in each callus line by simple visual screening, allowing straightforward selection of callus pieces for further experiments or regeneration into transgenic plants.

Metabolic profiling and mathematical modeling of transgenic callus lines reveals a correlation between transgene expression and carotenoid accumulation

Analysis of carotenoid content and composition by UHPLC showed that the various color phenotypes reflected

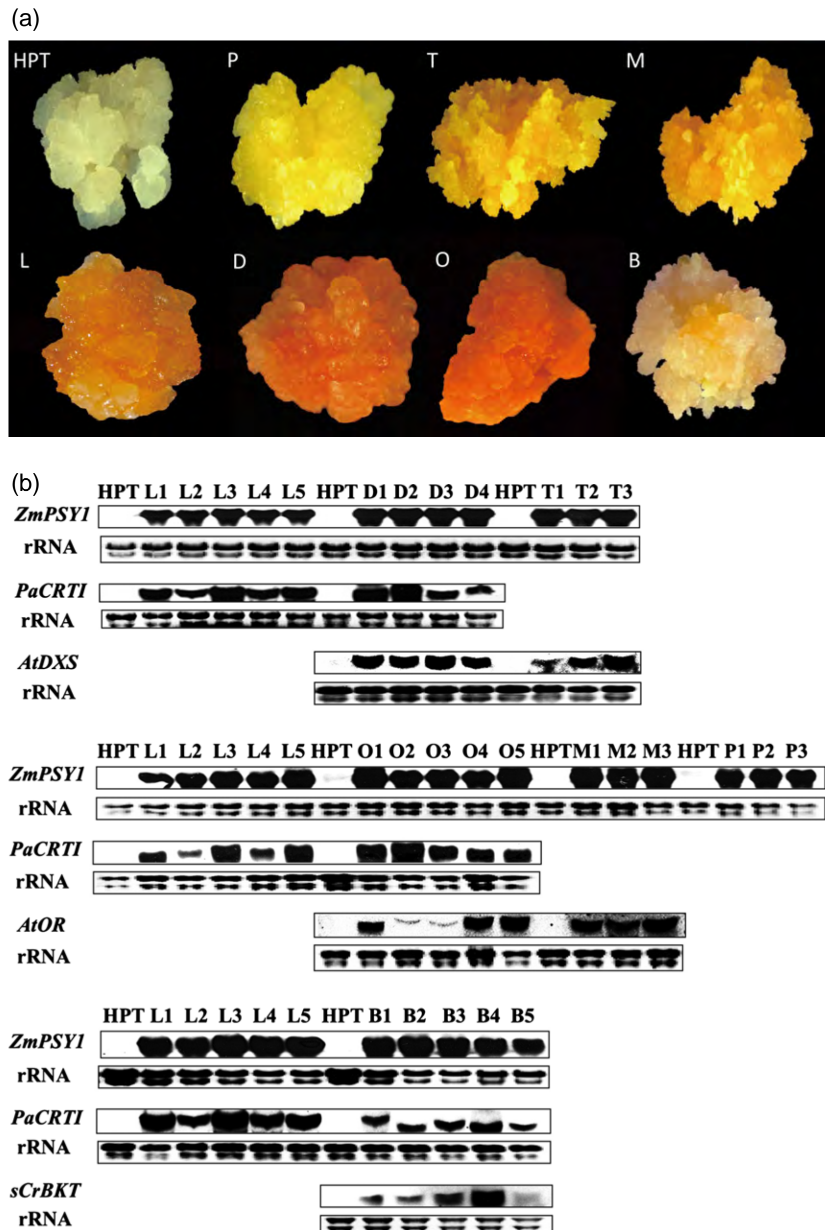

Figure 2. Phenotypes and genotypes of seven rice callus combinatorial transformants.
(a) Phenotypes of seven transgene combinations expressed in rice callus. HPT, callus expressing *HPT* (white color); P, expression of *ZmPSY1* alone results in a pale yellow color; expression of *ZmPSY1* and *AtDXS* in T, *ZmPSY1* and *AtOR* in M, and *ZmPSY1* and *PaCRTI* in L, result in a similar yellow color; expression of *AtDXS* in addition to *ZmPSY1* and *PaCRTI* in D, or in addition to *ZmPSY1*, *PaCRTI* and *AtOR* in O, results in a similar orange color; B, expression of *ZmPSY1*, *PaCRTI* and *sCrBKT* generates colors ranging from pink to red depending on the accumulation of ketocarotenoids.
(b) Analysis of mRNA transgenic rice callus (25 μg of total rRNA was loaded for each sample). Abbreviations: HPT, callus expressing *HPT*; L1–L5, transgenic callus expressing *ZmPSY1* and *PaCRTI*; D1–D4, transgenic callus expressing *AtDXS*, *ZmPSY1* and *PaCRTI*; T1–T3, transgenic callus expressing *ZmPSY1* and *AtDXS*; O1–O5, transgenic callus expressing *ZmPSY1*, *PaCRTI* and *AtOR*; M1–M3, transgenic callus expressing *ZmPSY1* and *AtOR*; P1–P3, transgenic callus expressing *ZmPSY1* alone; B1–B5, transgenic callus expressing *ZmPSY1*, *PaCRTI* and *sCrBKT*.

different metabolic profiles, confirming a direct correlation between the genotype and the metabolic phenotype of the callus lines. The white callus did not express *ZmPSY1* and contained similar levels of total carotenoids regardless of the additional transgene complement, i.e. control lines expressing *HPT* alone accumulated the same level of carotenoids as callus also expressing *PaCRTI* or *AtDXS* or both genes simultaneously. The yellow callus expressing *ZmPSY1* alone accumulated β–carotene and phytoene in similar amounts, as well as smaller amounts of α–carotene, lutein, violaxanthin and zeaxanthin (Figure 3, Figure S2 and Table S1). The darker yellow callus expressing *ZmPSY1* and *AtDXS* accumulated 1.8-fold more total carotenoids than callus expressing *ZmPSY1* alone, most of which was β–carotene, whereas the levels of phytoene and the minor carotenoids hardly changed. The yellow/orange callus expressing *ZmPSY1* and *PaCRTI* accumulated twice as much total carotenoids than callus expressing *ZmPSY1* alone, but, although most of this was again β–carotene, the next most abundant carotenoid was α–carotene, which was three times more abundant than phytoene. Finally, the orange callus expressing all three transgenes accumulated 3.5-fold more total carotenoids than callus expressing *ZmPSY1* alone, again mostly β–carotene, α–carotene and phytoene in similar proportions to the *ZmPSY1-PaCRTI* callus, but also with significant amounts of lutein (Figure 3, Figure S2 and Table S1). The presence of *AtDXS* in addition to *ZmPSY1* and *PaCRTI* therefore increased the

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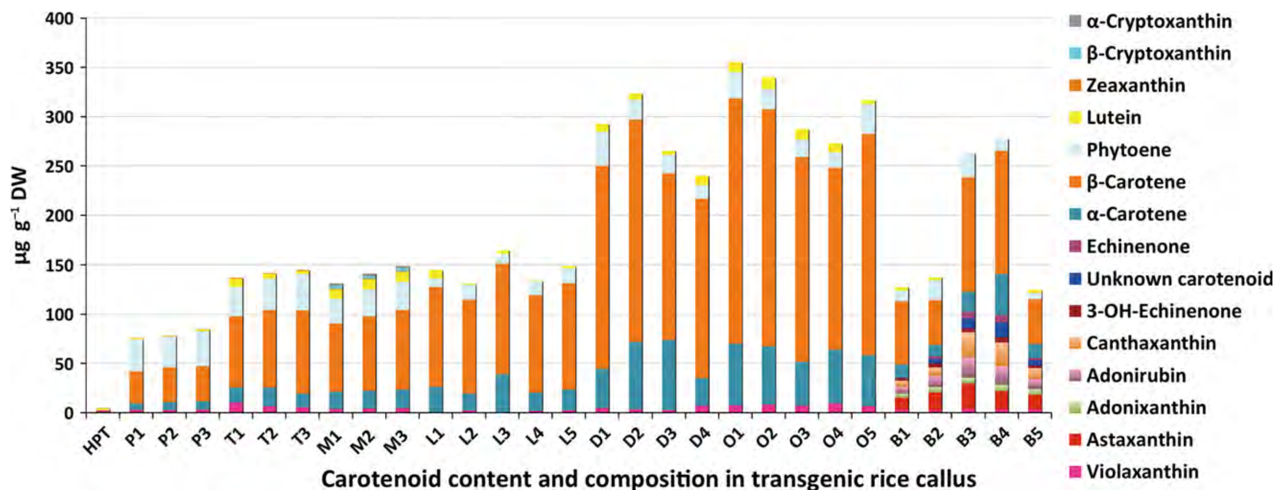

Figure 3. Carotenoid content and composition in transgenic rice callus. Carotenoid content and composition were analyzed by UHPLC. The various colored blocks represent different carotenoid compositions as indicated in the key. Values are means of three independent measurements. Column names are defined in the legend to Figure 2.

carotenoid content 1.9-fold, suggesting that the *A. thaliana DXS* gene boosts carotenoid biosynthesis by alleviating the limited supply of precursors into the pathway.

Although the transgene expression levels correlated with carotenoid accumulation (Figure 2a,b), this conclusion required the basic assumption that expression of each gene remained approximately constant among the various lines. In support of this assumption, quantification of mRNA in the Northern blots using ImageJ software (http://rsb.info.nih.gov/ij/) suggested that, for each transgene, the amount of expressed mRNA remained approximately constant in each transformed line (Figure 2b and Table S2). If we assume a constant rate of protein synthesis, the abundance of each protein should therefore be constant across the various callus lines also, and the metabolite concentrations should therefore allow us to model the behavior of carotenoid biosynthesis. We validated these assumptions experimentally as described below.

First, phytoene production appears to limit the production of downstream carotenoids. This may be inferred because transgenic callus expressing *ZmPSY1* increases the abundance of carotenoids 17-fold. Second, the quantity of phytoene precursors produced in the callus appears to limit the production of downstream carotenoids, even in the presence of ZmPSY1. This may be inferred because the *ZmPSY1-AtDXS* lines accumulate almost twice the level of total carotenoids present in the *ZmPSY1* lines. Third, endogenous carotene desaturase activity in rice appears to account for a significant proportion of carotenoid production from phytoene. This may be inferred because both the *ZmPSY1* and *ZmPSY1-AtDXS* lines produce 20–30-fold more carotenoids than control callus. However, this native activity may limit carotenoid production, because introducing the exogenous *CRTI* gene boosts carotene production and reduces the amount of phytoene compared to lines that do not express *CRTI*.

In order to validate these hypotheses, we created simplified mathematical models of the callus system, and investigated whether the dynamic behavior of the models was consistent with the experimental data (Table S3). Figure S3 shows the simplified pathway that was modeled. The modeling process is described in Experimental Procedures and Method S1. Table S4 summarizes the parameter values for the models. We found that the models quantitatively reproduced what was observed in the transgenic lines.

The first hypothesis cannot be tested by the modeling process, as it represents a founding assumption for the modeling process itself. Analysis of the model parameters revealed that introducing *AtDXS* generates approximately twice the amount of flux per unit time to produce phytoene than callus lines lacking this transgene. This is consistent with the second hypothesis. In addition, the production of carotenoids in lines that lack *CRTI* may be explained by endogenous flux channels. This activity accounts for a significant amount of α–carotene and β–carotene production even in lines expressing *CRTI*, and is consistent with the third hypothesis. A more detailed analysis is presented in Method S1.

Functional characterization of a chemically synthesized, codon-optimized *Chlamydomonas reinhardtii* β-carotene ketolase gene

Having established the suitability of the callus platform for analysis of carotenogenic genes with known functions, we analyzed an synthetic *C. reinhardtii* β–carotene ketolase gene (*sCrBKT*), codon-optimized for cereals, controlled by an endosperm-specific promoter, in this case the γ–zein gene promoter from maize (GZ63).

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We investigated the function of the chemically synthesized *sCrBKT* gene and the interaction of its product with other carotenogenic enzymes by transforming 7-day-old rice embryos with *ZmPSY1*, *PaCRTI* and *sCrBKT*. We recovered many independent callus lines under hygromycin selection, including a large proportion that were pink in color, suggesting accumulation of ketocarotenoids (Figure 2). This was confirmed by UHPLC analysis, which showed not only the presence of ketocarotenoids but also higher levels of β–carotene (Figure 3, Figure S2 and Table S1). The best-performing pink callus line contained 277.6 ± 0.6 μg g^{-1} dry weight of total carotenoids, including 18.5 ± 1.3 μg g^{-1} astaxanthin, 6.7 ± 0.2 μg g^{-1} adonixanthin, 18.6 ± 0.8 μg g^{-1} adonirubin, 24 ± 1.8 μg g^{-1} canthaxanthin, 5.3 ± 0.3 μg g^{-1} 3–OH-echinenone, 7.5 ± 1.2 μg g^{-1} echinenone, 15 ± 2.7 μg g^{-1} unknown molecule, 124.6 ± 3.8 μg g^{-1} β–carotene, 12.3 ± 1.6 μg g^{-1} phytoene, 41.6 ± 0.5 μg g^{-1} α–carotene and 3.3 ± 0.04 μg g^{-1} violaxanthin. These data show that ketolation of β–carotene in combination with hydroxylation by the endogenous rice hydroxylase occurred due to the presence of *sCrBKT*, which is functional in transgenic rice callus, and that the maize 'endosperm-specific' γ–zein gene promoter (GZ63) is active in callus tissue, like the other endosperm-specific promoters used in the preceding experiments.

Functional characterization of the *Arabidopsis thaliana Orange* gene

Another biological phenomenon that may be investigated using the callus platform is carotenoid accumulation in subcellular organelles. The cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* gene (*OR*) is involved in this process by regulating the development of chromoplasts, which store carotenoids and accumulate large amounts of β–carotene. The cauliflower *OR* gene was identified as a gain-of-function mutant allele that accelerates the formation of chromoplasts, creating a metabolic sink for carotenoid accumulation. This allele also functions in a heterologous background (Lu *et al.*, 2006; Li and Van Eck, 2007; Lopez *et al.*, 2008). Recently, a sweet potato ortholog of *OR* was shown to promote accumulation of carotenoids by inducing expression of carotenoid biosynthesis genes in transgenic sweet potato callus (Kim *et al.*, 2013b).

We identified the *A. thaliana OR* gene (*AtOR*) by sequence comparison with the cauliflower ortholog, and used this sequence to test our callus platform in more detail. We cloned *AtOR* from *A. thaliana* leaf tissue by RT–PCR, and then introduced it into a plant expression vector under the control of the wheat low-molecular-weight glutenin gene promoter. We transformed rice embryos with *ZmPSY1*, *PaCRTI*, *AtOR* and *HPT*, generating a number of callus lines that were orange or yellow in color, whereas the control callus remained white (Figure 2a). The callus expressing additional *AtOR* to the gene complement showed an increased carotenoid content (2.2-fold) compared with callus expressing *ZmPSY1* and *PaCRTI* (Table S1). Samples of the orange and yellow callus were analyzed by UHPLC. The best-performing orange callus was shown to express all the transgenes, and produced 355.3 ± 70.6 μg g^{-1} dry weight of total carotenoids, comprising 248.1 ± 46.4 μg g^{-1} β–carotene, 27.1 ± 6.8 μg g^{-1} phytoene, 62.8 ± 12.8 μg g^{-1} α–carotene, 10 ± 2.9 μg g^{-1} lutein and 7.3 ± 1.7 μg g^{-1} violaxanthin, confirming that the *AtOR* gene was functional and that was able to boost the accumulation of carotenoids in transgenic callus and particularly to enhance the accumulation of β–carotene (Figure 3, Figure S2 and Table S1).

Representative transgenic callus samples were examined by microscopy. Orange crystal-like structures were observed in the chromoplasts of orange callus samples expressing *OR* (Figure 4a), similar to those reported in transgenic plants expressing the cauliflower *OR* gene (Lu *et al.*, 2006; Lopez *et al.*, 2008). Interestingly, the same structures were observed in transgenic callus samples expressing *AtDXS* (Figure 4a). Transmission electron microscopy revealed numerous pigment-containing plastoglobuli that varied in size and electron density in both the *AtDXS* and *AtOR* transgenic lines (Figure 4b and Figure S4).

DISCUSSION

Rice callus provides a functional screening platform for carotenoid biosynthesis genes even if the genes are controlled by endosperm-specific promoters

We previously described a combinatorial gene transfer platform based on white maize endosperm for functional analysis of combinations of metabolic genes (Zhu *et al.*, 2008). The endosperm of M37W maize is white because the carotenoid biosynthesis pathway is blocked at the first committed step, providing a blank canvas for analysis of carotenogenic genes introduced by gene transfer, and further allowing rapid analysis of diverse genotypes by visual screening for endosperm color. The endosperm tissue may also be studied in more detail by UHPLC, allowing quantitative analysis of all carotenoids, and this means the platform may be extended to any other metabolic pathway that is also missing in endosperm tissue.

The usefulness of the maize platform in functional characterization of metabolic genes is constrained by the time needed to regenerate transgenic plants carrying combinations of transgenes. Other researchers have reported use of callus tissue from maize, *A. thaliana*, sweet potato, marigold and banana to characterize gene functions and expression levels, although only individual genes were tested in these cases (Paine *et al.*, 2005; Harada *et al.*, 2009; Kim *et al.*, 2011, 2013a,b; Vanegas-Espinoza *et al.*, 2011; Mlalazi *et al.*, 2012). We therefore adapted our combinatorial platform to work in rice callus, thus permitting

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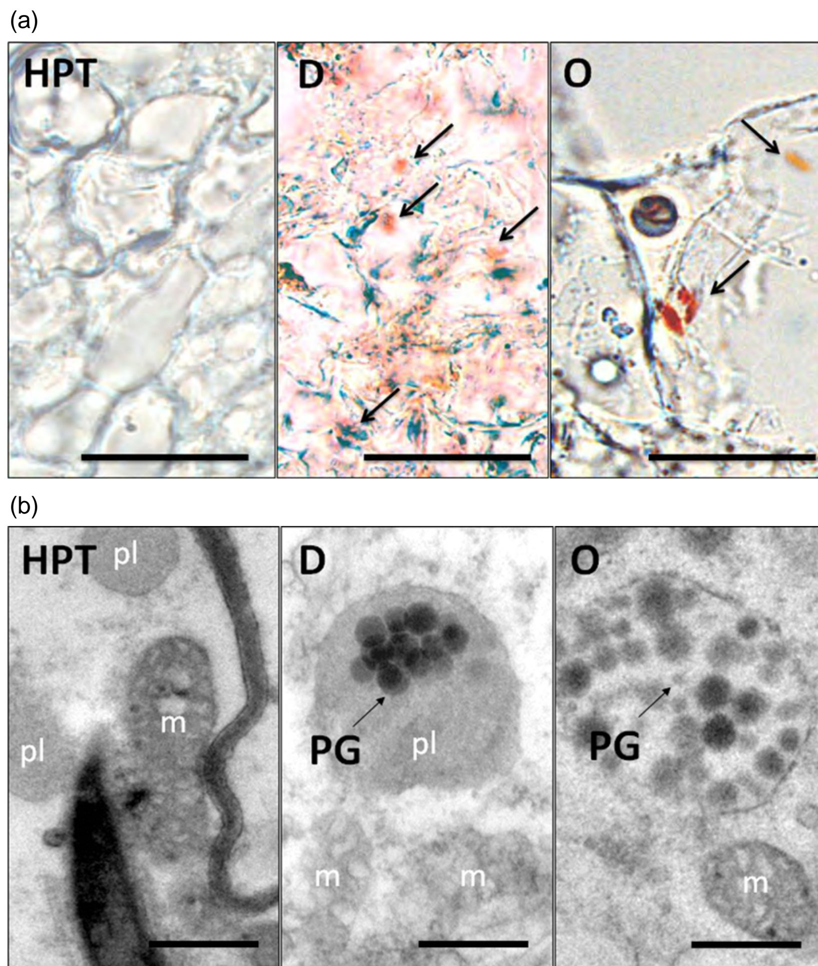

Figure 4. Microscopic cytological analysis of transgenic rice callus.
(a) Light micrographs of transgenic rice callus expressing *HPT* (HPT), transgenic rice callus expressing *ZmPSY1*, *PaCRTI* and *AtDXS* (D), and transgenic rice callus expressing *ZmPSY1*, *PaCRTI* and *AtOR* (O). Arrows indicate orange carotenoid crystal-like structures. Scale bars = 20 μm.
(b) Transmission electron micrographs of transgenic rice callus expressing *HPT* (HPT), transgenic rice callus expressing *ZmPSY1*, *PaCRTI* and *AtDXS* (D), and transgenic rice callus expressing *ZmPSY1*, *PaCRTI* and *AtOR* (O). Arrows indicate pigment-containing plastoglobuli (PG) in the chromoplast. Scale bars = 1 μm. pl, plastid; m, mitochondria.

analysis of multiple genes and their interactions over much shorter experimental time scales than possible in maize. Similar to white maize endosperm, rice callus is also white and does not accumulate significant levels of carotenoids. In addition, as a relatively undifferentiated tissue, it is likely that rice callus also lacks significant components of most of the complex secondary pathways found in plants, suggesting it may be used as a platform to test many aspects of secondary metabolism.

Although we anticipated the need to test transgenes using constitutive promoters suitable for undifferentiated tissues, we found that each of the endosperm-specific promoters that we used for the maize platform was also active in rice callus tissue, as revealed by growth of callus with a range of yellow, orange or pink hues depending on the transgene complement, showing that carotenoids were synthesized. The promiscuous activity of endosperm-specific promoters has been reported previously, e.g. Wu and Messing (2009) showed that a maize 27 kDa γ–zein promoter was sufficient to drive high-level expression of GFP in maize callus (the endogenous γ–zein protein also accumulated in the callus), and suggested that the tissue specificity of storage proteins was a more recent evolutionary event and that older storage protein genes were less restricted. By confirming the activity of four endosperm-specific promoters in the callus of a heterologous plant species, our data suggest that rice callus may be used as a platform to test transgene expression and activity in the endosperm without the need to generate mature, seed-bearing transgenic plants, therefore providing a rapid screening platform that avoids the labor-intensive process of regeneration and breeding.

The rice callus platform reveals multiple bottlenecks in the carotenoid biosynthesis pathway

One of the most important attributes of the combinatorial transformation system, either in seed endosperm or de-differentiated embryo-derived callus, is its ability to reveal multiple bottlenecks in metabolic pathways. Traditionally, metabolic engineering has involved a trial-and-error approach in which elimination of one bottleneck merely serves to reveal the next bottleneck, and many experiments are required to identify the most suitable engineering strategy. In contrast, combinatorial transformation

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generates a library of metabolic variants allowing the best strategy to be deduced in a single step if all necessary combinations of transgenes are represented in the population (Zhu *et al.*, 2008, 2013; Naqvi *et al.*, 2010; Bai *et al.*, 2011). Here, we established that rice callus expressing the *ZmPSY1* gene was pale yellow and accumulated 17-fold more total carotenoids than control callus, but addition of *AtDXS* boosted the accumulation of carotenoids by a further 1.8-fold, resulting in a darker yellow color compared to callus expressing *ZmPSY1* alone. Similarly, callus expressing both *ZmPSY1* and *PaCRTI* was yellow/orange in color, but addition of *AtDXS* generated orange callus that accumulated twice the amount of total carotenoids compared to callus expressing only the two transgenes.

These data suggest that DXS eliminates a bottleneck in the supply of precursors to the carotenoid pathway, increasing the overall flux. In previous studies, analysis of transgenic plants expressing DXS revealed higher levels of diverse isoprenoids, including chlorophylls, tocopherols, carotenoids, abscisic acid and gibberellic acid, showing that DXS is a rate-limiting enzyme in production of plastid-derived isopentenyl diphosphate (Estevez *et al.*, 2001). Expression of *A. thaliana* DXS in tomato fruits (*Solanum lycopersicum*) increased the carotenoid content 1.6-fold (Enfissi *et al.*, 2005), and expression of *Escherichia coli* DXS in potato tubers (*Solanum tuberosum*) increased the carotenoid content twofold (Morris *et al.*, 2006). The combined data from our callus experiments and the transgenic plants discussed above suggest that enough DXS is normally produced to provide 50% of the maximum potential flux into the carotenoid biosynthesis pathway, but expressing a DXS transgene boosts the flux to the maximum capacity of the carotenoid pathway in the absence of further augmentation, resulting in a doubling of total carotenoid levels in several species. Hence rice callus expressing *ZmPSY1* alone produces 84.9 ± 4.3 $\mu g\ g^{-1}$ dry weight total carotenoids, whereas expression of both *ZmPSY1* and *AtDXS* doubles the flux and the callus produces 144.8 ± 19.6 $\mu g\ g^{-1}$ dry weight total carotenoids.

Our mathematical model suggests the flux channel for phytoene production is approximately twice as strong when *AtDXS* is introduced, as may be seen by comparing the *k1* values for the callus line expressing *AtDXS* and *ZmPSY1* with the *k1* values for the callus line expressing only *ZmPSY1* (Table S4). This indicates that phytoene production is a limiting factor for carotenoid biosynthesis, and that AtDXS removes this bottleneck by increasing the availability of precursors.

The *CRTI* gene product catalyzes the entire desaturation sequence from 15–*cis*-phytoene to all-*trans*-lycopene, thus spanning the rice desaturation reactions. It has been previously shown that CRTI replaces the function of inhibited desaturases (Misawa *et al.*, 1993). When *PaCRTI* is introduced to the tobacco, the amounts of α- and β–carotene increase. Our parameter estimation results are consistent with this enzyme increasing the flux capacity of the callus desaturase system per unit time sixfold, as may be seen by comparing the *k2* + *k4* values in lines expressing *PaCRTI* with the *k2* values for callus lines lacking this gene (Table S4). The capacity for α–carotene synthesis is also increased by more than threefold, as may been seen by comparing the *k3* + *k5* values in the lines expressing *PaCRTI* with the k3 values for the callus lines lacking this gene (Table S4). The lines expressing *PaCRTI* are predicted to increase flux per unit time towards production of carotenoids. This may be seen by comparing the *k1* values in lines expressing and lacking *PaCRTI* (i.e. PSY versus PSY-CRTI and PSY-DXS versus PSY-CRTI-DXS). This result is consistent with a system in which conversion of phytoene into other carotenoid precursors limits production of those carotenoids.

The rice callus platform allows functional characterization of uncharacterized genes involved in carotenoid biosynthesis and accumulation

Having established the investigative value of the callus platform by testing it using a combinatorial panel of well-characterized carotenogenic genes, we next investigated the functions of genes that are absent in plants but may extend plant β-carotene and zeaxanthin biosynthesis pathways to astaxanthin biosynthesis pathway in rice, using a synthetic *C. reinhardtii* β–carotene ketolase (*sCrBKT*) gene optimized for maize codon usage. We co-transformed rice callus with *sCrBKT* plus *ZmPSY1* and *PaCRTI*, as these genes are required to establish the early part of the pathway and provide the necessary intermediates for ketolation. It was easy to identify the callus expressing *sCrBKT*, *ZmPSY1* and *PaCRTI* because accumulation of ketocarotenoids was indicated by a pink color. Ketocarotenoids represented 25–42% of total carotenoids in these lines, predominantly astaxanthin plus lower amounts of adonixanthin, adonirubin, canthaxanthin, 3–hydroxyechinenone and echinenone. Use of the callus system therefore confirmed that the optimized synthetic gene was expressed in plants, that the enzyme was active and cooperated with endogenous carotenogenic enzymes, and that the activity of *sCrBKT* depended on concurrent activity of *ZmPSY1* and *PaCRTI*, as expected.

In plants, carotenoids accumulate in specialized pigment-bearing structures known as plastoglobuli, within plastid-derived organelles called chromoplasts (Vothknecht and Soll, 2005; Brehelin *et al.*, 2007). The cauliflower *Orange* (*OR*) gene was discovered following analysis of a mutant cauliflower with an orange curd, and was shown to encode a chaperone-like protein that induced formation of chromoplasts and thus created a metabolic sink for carotenoids (Lu *et al.*, 2006; Li and Van Eck, 2007).

Orthologs of cauliflower wild-type *OR* have been identified in other species, but only the sweet potato ortholog

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has been shown to induce carotenoid accumulation (Kim *et al.*, 2013b). We therefore cloned the *A. thaliana OR* gene and found that the corresponding protein was 74.4% identical to the cauliflower wild-type ortholog and contained the DnaJ cysteine-rich domain, which is required for chaperone activity (Miernyk, 2001). Over-expression of *AtOR* increased the level of total carotenoids by twofold. Interestingly, the wild-type cauliflower *OR* allele did not increase carotenoid levels when it was expressed in potato, whereas the originally discovered mutant allele increased β–carotene levels in the tubers by sixfold, suggesting that the mutation caused a dominant gain of function (Lu *et al.*, 2006; Lopez *et al.*, 2008). It is unclear why the wild-type cauliflower *OR* allele was unable to increase carotenoid levels in potato tubers whereas the wild-type sweet potato gene was able to induce carotenoid accumulation in sweet potato callus (Kim *et al.*, 2013b) and the wild-type *A. thaliana* gene was able to increase carotenoid levels in rice callus (this study). The lack of carotenoid accumulation observed in transgenic potatoes may reflect the analysis of only four lines, which may have been subject to silencing (Lu *et al.*, 2006). Further experiments are required to identify and characterize proteins that associate with *OR*, in order to determine its precise role in chromoplast differentiation and carotenoid accumulation.

Carotenoid–lipoprotein structures may be induced either by *OR* gene expression or enhanced accumulation of carotenoids in the absence of *OR*

Chromoplasts are typically found in mature storage tissues, and are categorized as globular, tubular, reticulotubular, membranous or crystalline sub-types (Sitte *et al.*, 1980). For example, crystalline bodies have been observed in carrots (*Daucus carota*; Frey-Wyssling and Schwegler, 1965) and tomatoes (Harris and Spurr, 1969). We analyzed the structure and ultrastructure of rice callus expressing the *A. thaliana OR* gene, and observed one or two typical orange chromoplast structures per cell, as previously described in mutant cauliflower and transgenic potato tubers expressing the dominant cauliflower *OR* allele (Lu *et al.*, 2006; Lopez *et al.*, 2008). We assumed that ectopic chromoplasts were formed because the *OR* transgene induced precocious differentiation of these structures from immature plastids. However, we also observed chromoplast-like structures in callus expressing *AtDXS*, *ZmPSY1* and *PaCRTI*, but not in callus expressing *ZmPSY1* and *PaCRTI*. These data suggest that chromoplast differentiation may be triggered either by direct expression of a gene involved in the differentiation process (*OR*) or by increasing the flux through the carotenoid pathway to such an extent that the process of chromoplast differentiation is triggered by the abundance of carotenoids. This phenomenon has previously been observed in non-green *A. thaliana* tissues (callus and roots) expressing high levels of phytoene synthase (Maass *et al.*, 2009), suggesting that the chromoplast differentiation program may be a response to accumulation of carotenoids above a certain threshold unless it is triggered by *OR* before this threshold is reached (Maass *et al.*, 2009). There appears to be no relationship between these independent events, because the *OR* gene does not normally induce the activity of carotenogenic genes (Li *et al.*, 2001, 2006), thus suggesting that it may act at the level of the metabolome by shifting the chemical equilibrium in the cell towards carotenogenesis (Li *et al.*, 2001; Maass *et al.*, 2009). More recently, sweet potato *OR* was shown to induce carotenogenic gene expression, suggesting that *OR* may contribute towards increasing carotenoid levels to reach the threshold necessary to trigger chromoplast differentiation (Kim *et al.*, 2013b).

CONCLUSIONS

Our callus-based assay allows the rapid combinatorial testing of various expression constructs, making it an ideal platform for synthetic biology, which involves assembly of genetic circuits from components such as promoters, genes and protein targeting signals. The callus platform allows large numbers of constructs to be tested in parallel, in various combinations, so that ideal engineering strategies may be developed before any transgenic plants are produced. Similarly, the platform may be used to modulate promoter strength, protein synthesis and metabolite production, thus facilitating a more quantitative approach to synthetic biology and thus more refined and sophisticated strategies for metabolic engineering. The method is applicable to any pathway and any gene product that may be analyzed through standard analytical procedures such as HPLC, MS, NMR, etc. It is neither limited nor constrained by a color phenotype.

EXPERIMENTAL PROCEDURES

Gene cloning and vector construction

The *AtDXS* and *AtOR* cDNAs were cloned directly from *A. thaliana* mRNA by RT–PCR based on sequence data in GenBank (accession numbers NM203246 and U27099.1, respectively). The cDNAs were transferred to the pGEM-T Easy vector (Promega, www.promega.com), and the resulting plasmids pGEM-AtDXS and pGEM-AtOR were digested with *Eco*RI. *AtDXS* was introduced into vector pRP5 (Su *et al.*, 2001), containing the rice prolamin promoter and the ADPGPP terminator, whereas *AtOR* was introduced into vector p326 (Stoger *et al.*, 1999), containing the wheat low-molecular-weight glutenin gene promoter and the *nos* terminator.

A truncated β–carotene ketolase gene from *C. reinhardtii* (Zhong *et al.*, 2011) was chemically synthesized and optimized for maize codon usage. The modified gene (*sCrBKT*) was fused with the transit peptide sequence from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) and the 5′ UTR of the rice alcohol dehydrogenase gene (Sugio *et al.*, 2008) under the control of the maize γ–zein promoter. The transit peptide sequence and 5′ UTR were also optimized for maize codon usage.

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The maize *PSY1* cDNA was cloned from maize inbred line B73 by RT–PCR using forward primer 5′-AGGATCCATGGCCATCATAC TCGTACGAG-3′ and reverse primer 5′-AGAATTCTAGGTCTGGCCA TTTCTCAATG-3′ based on the *PSY1* sequence (GenBank accession number AY324431). The product was transferred to pGEM-T Easy (Promega) for sequencing, and then to the p326 vector containing the LWM glutenin promoter and *nos* terminator (Stoger *et al.*, 1999).

The *Pantoea ananatis* (formerly *Erwinia uredovora*) *CRTI* gene was fused in-frame with the transit peptide sequence from the *P. vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier *et al.*, 1985) in plasmid pYPIET4 (Misawa *et al.*, 1993), and amplified by PCR using forward primer 5′-ATCTAGAATGGCT TCTATGATATCCTCTTC-3′ and reverse primer 5′-AGAATTCTCAAA TCAGATCCTCCAGCATCA-3′. The product was transferred to pGEM-T Easy for sequencing, and then to pHorp–P (Sorensen *et al.*, 1996) containing the barley D–hordein promoter and the rice ADPGPP terminator. All transformation constructs were verified by sequencing.

Rice transformation

Seven-day-old mature rice zygotic embryos were bombarded with 10 mg gold particles coated with the carotenogenic constructs and the selectable maker *HPT* at a ratio 3:1 as previously described (Christou *et al.*, 1991). The embryos were returned to osmoticum medium contains MS medium supplemented with 0.3 g/l casein hydrolysate, 0.5g/l proline and 72.8 g/l mannitol for 12 h before selection on medium supplemented with 50 mg L^{-1} hygromycin and 2.5 mg L^{-1} 2,4–dichlorophenoxyacetic acid in the dark for 2–3 weeks (Christou, 1997). Callus was selected by visual screening for yellow, orange and pink coloring as appropriate, and was sub-cultured every 2 weeks to collect sufficient material for further analysis.

Analysis of mRNA

We separated 30 μg of denatured RNA by 1.2% w/v agarose/formaldehyde gel electrophoresis in 1× MOPS buffer, and transferred the fractionated RNA to a membrane by capillary blotting (Sambrook *et al.*, 1989). The procedures described by Zhu *et al.* (2008) were used. The forward and reverse primers for each transgene are shown in Table S5.

Carotenoid extraction and quantification

Carotenoids were extracted from 10 mg freeze-dried callus in the dark using 50/50 v/v tetrahydrofuran and methanol at 60°C for 20 min. The mixture was filtered, and the residue was re-extracted in acetone. Lutein and zeaxanthin were separated on a YMC C30 carotenoid HPLC column (particle size (μm): 3 μm; length (mm): 100mm; internal diameter (mm):2.0 mm; Waters, http://www.waters.com) using a mobile phase comprising solvent A (methanol:water, 80:20 v/v) and solvent B (100% *tert*–butylmethylether) at a flow rate of 0.30 ml min^{-1}. All other carotenoids were separated on a reversed-phase ACQUITY UPL BEH 300 Å C18 column (particle size (μm): 1.7 μm, length (mm): 150 mm, internal diameter (mm):2.1 mm; Waters) using a gradient system with the mobile phase consisting of solvent A (acetonitrile/methanol, 70:30 v/v) and solvent B (100% water) at a flow rate of 0.35 ml min^{-1}. The mixtures were analyzed using an ACQUITY Ultra Performance LC system (Waters) linked to a 2996 photo diode array detector (Waters). MassLynx software version 4.1 (Waters) was used to control the instruments, and for data acquisition and processing (Rivera *et al.*, 2013).

Microscopy

Rice callus pieces (0.5 × 2.0 mm) were fixed in 2.5% v/v glutaraldehyde and 2.0% v/v paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C for light and transmission electron microscopy. After three washes (10 min each) with 0.1 M sodium phosphate buffer (pH 7.2) at room temperature, they were sectioned on a Leica CM3050 (http://www.leica-microsystems.com) cryotome using CryoGel™ (High Viscosity Water Soluble Media for Frozen Sections, http://www.2spi.com/catalog/chem/cryogel.shtml) as the embedding medium for light microscopy. Thin sections (16 μm) were prepared with a diamond knife using a Reichert-Jung Ultramicrotome Ultracut E (Nova Scotia, http://www.leica-microsystems.com), and were mounted on glass slides for analysis under a Zeiss Axioplan light microscope (http://www.zeiss.com) coupled to a Leica DC 200 digital camera.

For transmission electron microscopy, the sections were washed three times in 0.1 M sodium phosphate buffer (pH 7.2), and post-fixed in 1% w/v osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h. They were then washed three times in re-distilled water and dehydrated in an alcohol series (30–100%) before embedding in epoxy resin Araldite® Embed 812 (Epon-812) from Aname Electron Microscopy Sciences, Madrid, Spain (URL: www.aname.es) and polymerizing at 60°C. Ultra-thin sections (80–90 nm) were prepared with a diamond knife using a Reichter Jung Ultramicrotome Ultracut E (Scotia), mounted on SPI-Chem™ Formvar®/carbon-coated copper grids, and stained with uranyl acetate and Reynold's lead citrate prior to examination using an EM 910 transmission electron microscope (Zeiss).

Mathematical modeling

Mathematica (Wolfram, 1999) was used to create and solve the mathematical models for carotenoid biosynthesis. It was also used to find the best-fit parameters for the models. Further details about the modeling process are provided in Method S1.

ACKNOWLEDGEMENTS

This study was supported by the Ministerio de Ciencia e Innovación, Spain (AGL2010-15691, BFU2010-17704 and BIO2011-22525), the Generalitat de Catalunya (2009SGR809), and a European Research Council Advanced Grant (BIOFORCE). We thank Veronica Teixido and Jorge Comas (Department Ciències Mèdiques Bàsiques, Universitat de Lleida & Institut de Recerca Biomèdica de Lleida Edifici Recerca Biomèdica I, Avenida Alcalde Rovira Roure 80, 25198 Lleida, Spain) for assistance with the ImageJ analysis.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Astaxanthin biosynthesis pathway.

Figure S2. Carotenoid profiles in transgenic rice callus.

Figure S3. Simplified representation of carotene biosynthesis in transgenic rice callus lines.

Figure S4. Electron micrographs of ultra-thin sections of rice callus expressing *ZmPSY1*, *PaCRTI* and *AtOR.*

Table S1. Carotenoid content and composition in transgenic rice callus expressing *ZmPSY1* and/or *PaCRTI* and *AtDXS/AtOR*, or *ZmPSY1*, *PaCRTI* and *sCrBKT.*

Table S2. Relative amounts of mRNA expressed in the transformed rice callus lines.

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Table S3. Quantification of metabolites in the various transgenic rice callus lines.

Table S4. Parameter estimation for the various rice callus lines.

Table S5. Oligonucleotide sequences of forward and reverse primers for mRNA blot analysis.

Method S1. Mathematical models for the callus lines transformed with *ZmPSY1* and/or *PaCRTI* and/or *AtDXS.*

REFERENCES

Bai, C., Twyman, R.M., Farre, G., Sanahuja, G., Christou, P., Capell, T. and Zhu, C. (2011) A golden era – pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol.* **47**, 205–221.

Brehelin, C., Kessler, F. and van Wijk, K.J. (2007) Plastoglobules: versatile lipoprotein particles in plastids. *Trends Plant Sci.* **12**, 260–266.

Capell, T. and Christou, P. (2004) Progress in plant metabolic engineering. *Curr. Opin. Biotechnol.* **15**, 148–154.

Christou, P. (1997) Rice transformation by bombardment. *Plant Mol. Biol.* **35**, 197–203.

Christou, P., Ford, T.L. and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Biotechnology*, **9**, 957–962.

Dafny-Yelin, M. and Tzfira, T. (2007) Delivery of multiple transgenes to plant cells. *Plant Physiol.* **145**, 1118–1128.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W. and Bramley, P.M. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol. J.* **3**, 17–27.

Estevez, J.M., Cantero, A., Reindl, A., Reichler, S. and Leon, P. (2001) 1–Deoxy-D–xylulose-5–phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* **276**, 22901–22909.

Farre, G., Sanahujaa, G., Naqvia, S., Bai, C., Capell, T., Zhu, C. and Christou, P. (2010) Travel advice on the road to carotenoids in plants. *Plant Sci.* **179**, 28–48.

Farre, G., Bai, C., Twyman, R.M., Capell, T., Christou, P. and Zhu, C. (2011) Nutritious crops producing multiple carotenoids – a metabolic balancing act. *Trends Plant Sci.* **16**, 532–540.

Farre, G., Rivera, S.M., Alves, R. *et al.* (2013) Targeted transcriptomic and metabolic profiling reveals temporal bottlenecks in the maize carotenoid pathway that can be addressed by multigene engineering. *Plant J.* **75**, 441–455.

Frey-Wyssling, A. and Schwegler, F. (1965) Ultrastructure of the chromoplasts in the carrot root. *J. Ultrastruct. Res.* **13**, 543–559.

Harada, H., Fujisawa, M., Teramoto, M., Sakurai, N., Suzuki, H., Shibata, D. and Misawa, N. (2009) Simple functional analysis of key genes involved in astaxanthin biosynthesis using Arabidopsis cultured cells. *Plant Biotechnol.* **26**, 81–92.

Harris, W.M. and Spurr, A.R. (1969) Chromoplasts of tomato fruits. II. The red tomato. *Am. J. Bot.* **56**, 380–389.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Lee, H.S. and Kwak, S.S. (2011) Down-regulation of β–carotene hydroxylase increases β–carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato. *Phytochemistry*, **74**, 69–78.

Kim, S.H., Kim, Y.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013a) Downregulation of the lycopene ε–cyclase gene increases carotenoid synthesis via the β–branch-specific pathway and enhances salt-stress tolerance in sweetpotato transgenic calli. *Physiol. Plant.* **147**, 432–442.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013b) Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol. Biochem.* **70**, 445–454.

Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I. and Maluszynski, M. (2011) TILLING – a shortcut in functional genomics. *J. Appl. Genet.* **52**, 371–390.

Li, L. and Van Eck, J. (2007) Metabolic engineering of carotenoid accumulation by creating a metabolic sink. *Transgenic Res.* **16**, 581–585.

Li, L., Paolillo, D.J., Parthasarathy, M.V., Dimuzio, E.M. and Garvin, D.F. (2001) A novel gene mutation that confers abnormal patterns of β–carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *Plant J.* **26**, 59–67.

Li, L., Lu, S., Cosman, K.M., Earle, E.D., Garvin, D.F. and O'Neill, J. (2006) β–carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. *Phytochemistry*, **67**, 1177–1184.

Liu, C.G., Calin, G.A., Volinia, S. and Croce, C.M. (2008) MicroRNA expression profiling using microarrays. *Nat. Protoc.* **3**, 563–578.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) Effect of the cauliflower *OR* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* **59**, 213–223.

Lu, S., Van Eck, J., Zhou, X. *et al.* (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β–carotene accumulation. *Plant Cell*, **18**, 3594–3605.

Maass, D., Arango, J., Wu, F., Beyer, P. and Welsch, R. (2009) Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS ONE*, **4**, e6373.

Miernyk, J.A. (2001) The J–domain proteins of *Arabidopsis thaliana*: an unexpectedly large and diverse family of chaperones. *Cell Stress Chaperones*, **6**, 209–218.

Misawa, N., Yamano, S., Linden, H., de Felipe, M.R., Lucas, M., Ikenaga, H. and Sandmann, G. (1993) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtl* in transgenic plants showing an increase of β–carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–840.

Mlalazi, B., Welsch, R., Namanya, P., Khanna, H., Geijskes, R.J., Harrison, M.D., Harding, R., Dale, J.L. and Bateson, M. (2012) Isolation and functional characterisation of banana *phytoene synthase* genes as potential cisgenes. *Planta*, **236**, 1585–1598.

Morris, W.L., Ducreux, L.J.M., Hedden, P., Millam, S. and Taylor, M.A. (2006) Overexpression of a bacterial 1–deoxy-D–xylulose 5–phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *J. Exp. Bot.* **57**, 3007–3018.

Myouga, F., Akiyama, K., Motohashi, R., Kuromori, T., Ito, T., Iizumi, H., Ryusui, R., Sakurai, T. and Shinozaki, K. (2009) The chloroplast function database: a large-scale collection of Arabidopsis Ds/Spm- or T-DNA--tagged homozygous lines for nuclear-encoded chloroplast proteins, and their systematic phenotype analysis. *Plant J.* **61**, 529–542.

Naqvi, S., Farre, G., Sanahuja, G., Capell, T., Zhu, C. and Christou, P. (2010) When more is better: multigene engineering in plants. *Trends Plant Sci.* **15**, 48–56.

Nikolov, M., Schmidt, C. and Urlaub, H. (2012) Quantitative mass spectrometry-based proteomics: an overview. *Methods Mol. Biol.* **893**, 85–100.

Ozsolak, F. and Milos, P.M. (2011) RNA sequencing: advances, challenges and opportunities. *Nat. Rev. Genet.* **12**, 87–98.

Paine, J.A., Shipton, C.A., Chaggar, S. *et al.* (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**, 482–487.

Purkayastha, A. and Dasgupta, I. (2009) Virus-induced gene silencing: a versatile tool for discovery of gene function in plants. *Plant Physiol. Biochem.* **47**, 967–976.

Ramessar, K., Sabalza, M., Capell, T. and Christou, P. (2008) Maize plants: an ideal production platform for effective and safe molecular pharming. *Plant Sci.* **174**, 409–419.

Rivera, S.M., Vilaro, F., Zhu, C., Bai, C., Farre, G., Christou, P. and Canela-Garayoa, R. (2013) Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem.* **61**, 5279–5285.

Ruiz-Sola, M.A. and Rodriguez-Concepcion, M. (2012) Carotenoid biosynthesis in Arabidopsis: a colorful pathway. *Arabidopsis Book*, **10**, e0158.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 63–70.

Schreier, P., Seftor, E., Schell, J. and Bohnert, H. (1985) The use of nuclear--encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J.* **4**, 25–32.

Sitte, P., Falk, H. and Liedvogel, B. (1980) *Chromoplasts*. Stuttgart and New York, NY: Gustav Fischer Verlag, pp. 117–148.

Snyder, M. and Gallagher, E.G. (2009) Systems biology from a yeast omics perspective. *FEBS Lett.* **583**, 3895–3899.

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Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol. Gen. Genet.* **250**, 750–760.

Stoger, E., Williams, S., Keen, D. and Christou, P. (1999) Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Res.* **8**, 73–82.

Su, P.H., Yu, S.M. and Chen, C.S. (2001) Spatial and temporal expression of a rice prolamin gene RP5 promoter in transgenic tobacco and rice. *J. Plant Physiol* **158**, 247–254.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A. and Kato, K. (2008) The 5′–untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells. *J. Biosci. Bioeng.* **105**, 300–330.

Vanegas-Espinoza, P.E., Ramos-Viveros, V., Jimenez-Aparicio, A.R., Lopez-Villegas, O., Heredia-Mira, F.J., Melendez-Martinez, A.J., Quintero-Gutierrez, A.G., Paredes-Lopez, O. and Del Villar-Martinez, A.A. (2011) Plastid analysis of pigmented undifferentiated cells of marigold *Tagetes erecta* L. by transmission electron microscopy. *In Vitro Cell. Dev. Biol.* **47**, 596–603.

Vothknecht, U.C. and Soll, J. (2005) Chloroplast membrane transport: interplay of prokaryotic and eukaryotic traits. *Gene*, **354**, 99–109.

Wolfram, S. (1999) *The Mathematica Book*, 4th edn. Cambridge, UK: Cambridge University Press.

Wu, Y. and Messing, J. (2009) Tissue-specificity of storage protein genes has evolved with younger gene copies. *Maydica*, **54**, 409–415.

Zhong, Y.J., Huang, J.C., Liu, J., Li, Y., Jiang, Y., Xu, Z.F., Sandmann, G. and Chen, F. (2011) Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic Arabidopsis. *J. Exp. Bot.* **62**, 3659–3669.

Zhu, M.J. and Zhao, S.H. (2007) Candidate gene identification approach: progress and challenges. *Int. J. Biol. Sci.* **3**, 420–427.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P. and Capell, T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl Acad. Sci. U S A* **105**, 18232–18237.

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

Zhu, C., Sanahuja, G., Yuan, D. *et al.* (2013) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J.* **11**, 129–141.

Zurbriggen, M.D., Moor, A. and Weber, W. (2012) Plant and bacterial systems biology as platform for plant synthetic bio(techno)logy. *J. Biotechnol.* **160**, 80–90.

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Phytochemistry (2014)

http://dx.doi.org/10.1016/j.phytochem.2013.12.008

* Corresponding author Gerhard Sandmann (email: sandman@bio.uni-frankfurt.de)

[1] J.B. and C.B. contributed equally to this work.

Phytochemistry xxx (2014) xxx–xxx

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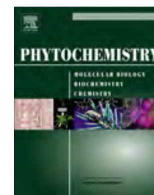

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[a] Molecular Biosciences, J.W. Goethe Universität Frankfurt, Max von Laue Str. 9, D-60438 Frankfurt am Main, Germany
[b] Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida-Agrotecnio Center, Avenida Alcalde Rovira Roure, 191, Lleida E-25198, Spain
[c] Departament de Química, Universitat de Lleida, Avenida Alcalde Rovira Roure, 191, Lleida E-25198, Spain
[d] Institucio Catalana de Recerca i Estudis Avancats, Barcelona, Spain

ARTICLE INFO

Article history:
Received 3 October 2013
Received in revised form 29 November 2013
Available online xxxx

Keywords:
Astaxanthin
Carotenoid biosynthesis
Genetic engineering
Rice endosperm
4-keto-α-carotene

ABSTRACT

Rice endosperm is devoid of carotenoids because the initial biosynthetic steps are absent. The early carotenogenesis reactions were constituted through co-transformation of endosperm-derived rice callus with phytoene synthase and phytoene desaturase transgenes. Subsequent steps in the pathway such as cyclization and hydroxylation reactions were catalyzed by endogenous rice enzymes in the endosperm. The carotenoid pathway was extended further by including a bacterial ketolase gene able to form astaxanthin, a high value carotenoid which is not a typical plant carotenoid. In addition to astaxanthin and precursors, a carotenoid accumulated in the transgenic callus which did not fit into the pathway to astaxanthin. This was subsequently identified as 4-keto-α-carotene by HPLC co-chromatography, chemical modification, mass spectrometry and the reconstruction of its biosynthesis pathway in *Escherichia coli*. We postulate that this keto carotenoid is formed from α-carotene which accumulates by combined reactions of the heterologous gene products and endogenous rice endosperm cyclization reactions.

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Introduction

Genetic engineering is a powerful tool to modulate existing biosynthesis pathway or establish novel routes in microbes and plants. Carotenogenesis is a key target for genetic engineering of staple crops due to the many nutritional and other health benefits of a number of such molecules for humans and animals (Fraser et al., 2009; Misawa, 2011). Successful examples are increase of the carotenoid yield, e.g., lycopene in tomato (Fraser et al., 2002), the accumulation of intermediates to higher levels, e.g., zeaxanthin in potato (Römer et al., 2002) or extension of an existing pathway to a novel end product, e.g., astaxanthin in maize (Zhu et al., 2008). In addition, maize was used to explore interactions between an induced transgenic carotenoid pathway and the endogenous pathway (Naqvi et al., 2011). On a number of occasions there have been examples of novel unexpected phenotypes due to unknown regulatory mechanisms and unpredicted enzyme interactions (see Sandmann et al. (2006) for review). Rice provides an interesting example of carotenogenesis by genetic engineering. In contrast to the endosperm of yellow maize which is pigmented due to the accumulation of lutein, zeaxanthin, and 5,6-epoxy derivatives Quackenbush et al. (1963)), rice endosperm is colourless. Nevertheless, rice endosperm possesses a hidden potential for carotenoid biosynthesis even though the initial steps in the carotenoid pathway are absent. The endogenous levels of phytoene synthase and phytoene desaturase in wild type endosperm are below the threshold level for carotenoid biosynthesis (Schaub et al., 2005). It has been demonstrated that the limitation of carotenogenesis can be overcome in rice endosperm by expressing genes encoding a phytoene synthase and a bacterial phytoene desaturase able to replace all plant desaturation and isomerisation reactions (Ye et al., 2000). The expected product of these reactions is lycopene. Interestingly biosynthesis proceeded beyond lycopene by cyclization to α- and β-carotene and the hydroxylation of both carotenes to lutein and zeaxanthin, respectively (Ye et al., 2000). Thus intrinsic rice cyclases and hydroxylases are expressed in the endosperm. A survey of phytoene synthase genes from different plant species indicated that the maize enzyme is the most effective in rice (Paine et al., 2005). Its use led to the generation of a rice line rich in α- and β-carotene which both exhibit provitamin A activity, in addition to lutein and zeaxanthin (structures shown in Fig. 1). In our current carotenogenesis engineering experiments we attempted to extend the pathway beyond carotenes to astaxanthin. Astaxanthin is a high priced carotenoid which is beneficial for human health

* Corresponding author.
E-mail address: sandman@bio.uni-frankfurt.de (G. Sandmann).
[1] J.B. and C.B. contributed equally to this work.

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http://dx.doi.org/10.1016/j.phytochem.2013.12.008

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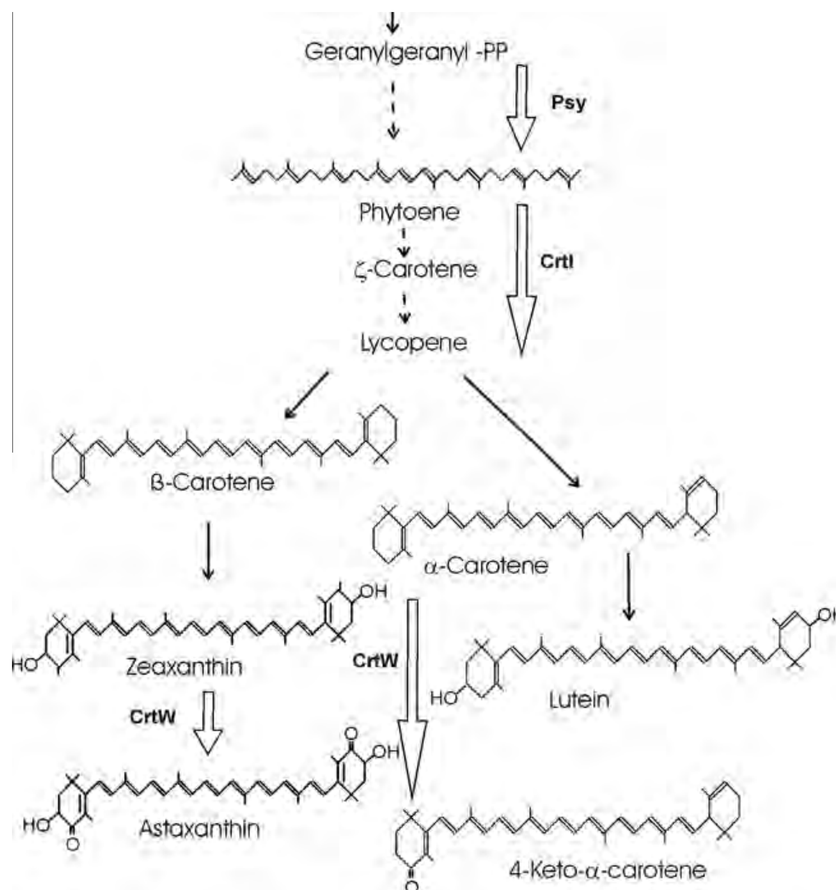

Fig. 1. The biosynthesis pathway of astaxanthin formation in transgenic endosperm-derived rice callus. Dotted arrows indicate pathway limitations in rice, open arrows indicate the reactions catalysed by the transgenes *psy* (phytoene synthase), *crtI* (phytoene desaturase) and *crtW* (carotene ketolase), solid arrows indicate maize-specific carotenogenic reactions.

(Guerin et al., 2003; Hussein et al., 2006) and is used as an essential feed additive in salmon farming (Bjerkeng, 2008).

Considerable effort has been invested to achieve sustainable and economic production of astaxanthin in microorganisms and plants (Zhu et al., 2009). Our strategy has been to engineer suitable plant material such as rice grains to synthesize astaxanthin for directly feeding or consumption (Sandmann, 2001). In previous work, we reported that transformation of maize with a ketolase and an additional hydroxylase gene resulted in the accumulation of astaxanthin (Zhu et al., 2008). However, the yields were not satisfactory. Therefore, we have chosen rice as an alternative crop for the engineering of carotenogenesis using combinatorial genetic transformation (Farre et al., 2012). The endosperm-based rice callus system allowed us to investigate the impact of genetic pathway modulation on carotenoid composition before the time consuming and labour intensive regeneration of intact plants. Transformation was carried out simultaneously with three genes, a maize phytoene synthase, a bacterial phytoene desaturase and a bacterial β-carotene ketolase gene which in combination with the endogenous endosperm expressed β-carotene hydroxylase should be able to synthesize astaxanthin.

Engineering of carotenoid biosynthesis in rice endosperm was successful in terms of engineering a ketolation pathway leading to astaxanthin as the end product. However in addition to the expected hydroxy and keto intermediates derived from β-carotene, we detected a novel carotenoid which did not fit directly into the pathway to astaxanthin. Its identification is the scope of this work.

Results and discussion

The initial synthesis of phytoene and its 4-step desaturation and isomerization to lycopene was engineered into rice callus in combination with a bacterial carotenoid ketolase (Fig. 1). Expression of carotenoid ketolase and all other transgenes has been shown previously (Bai et al.2013). Fig. 2A shows the HPLC carotenoid profile of a typical transgenic line. Two carotenes were synthesized by the interaction of phytoene synthase and desaturase and the endogenous lycopene cyclases, α-carotene (peak 5) and β-carotene (peak 6). The prominent carotenoid peak 1 at 6.7 min with the typical bell shaped optical absorbance spectrum with its maximum at 475 nm resembles astaxanthin. This is the end product of the ketolation pathway starting from β-carotene or zeaxanthin (Fig. 1) which was initiated by the transgenic carotenoid ketolase. This enzyme works by interaction with the endogenous β-carotene hydroxylase which is active and specific enough for astaxanthin synthesis. Two other peaks resemble intermediates of this pathway, 4-keto-zeaxanthin peak 2 and echinenone peak 4, both with an asymmetrical bell shaped optical absorbance spectrum with a maximum at 465 nm (Fig. 3B). The latter originated directly from ketolation of β-carotene, the other by ketolation of zeaxanthin.

One compound which does not fit into this pathway is the carotenoid represented by peak 3 (Fig. 2A). Its spectrum is shown in Fig. 3A. Although it has a very similar shape to the spectrum of echinenone (Fig. 3B) which may indicate a mono-keto carotenoid, its maximum of 452 nm is 14 nm lower than that of echinenone. The only known carotenoid with the same absorbance and

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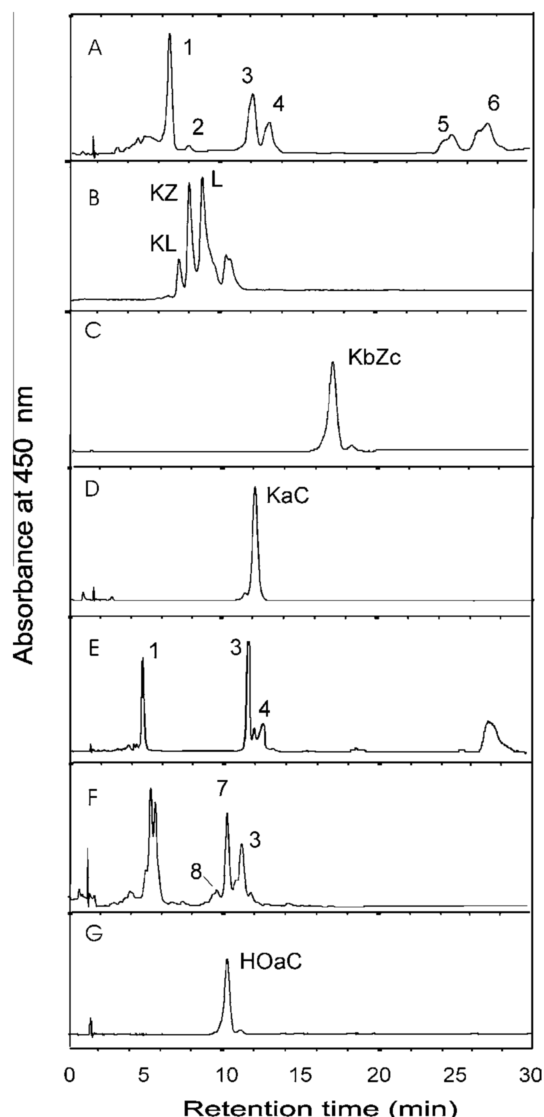

Fig. 2. HPLC separation of carotenoids from transgenic rice callus expressing a ketolase gene (A). (B) isolate with 4-keto-lutein, (C) 4-keto-β-zeacarotene (KbZc) standard, (D) 4-keto-α-carotene standard (KaC), (E) co-chromatography of rice extract with 4-keto-α-carotene standard, (F) carotenoids from transgenic rice callus after reduction with $NaBH_4$, (G) 4-hydroxy-α-carotene (HOaC) standard. Peak 1, astxanthin; 2, 4-keto-zeaxanthin; 3, novel unknown carotenoid; 4, echinenone; 5, α-carotene; 6, β-carotene; 7, 4-hydroxy-α-carotene; 8, 4-hydroxy echinenone. L, lutein; KL, 4-keto-lutein; KZ, 4-keto-zeaxanthin.

shape of the spectrum is 4-ketolutein (Fig. 3C). This was extracted from transgenic *Nicotiana tabaccum* nectary tissue transformed with a cyanobacterial β-carotene ketolase gene (Gerjets et al., 2007). However, it is more polar with a retention time of 7.5 min compared to 12.2 min for peak 3 (Fig. 2B). From the spectrum and the basic carotenoid pathway to α- and β-carotene related compounds, 4-keto-β-zeacarotene (7,8-dihydro-β,ψ-carotene-4-one) and 4-keto-α-carotene (β,ε-carotene-4-one) are the most likely candidates for the novel carotenoid molecule. Therefore, we generated these mono keto carotenoids which should exhibit not only a similar absorbance but also a similar polarity as reference compounds by combinatorial biosynthesis in *Escherichia coli* (Sandmann, 2002). In the case of 4-keto-β-zeacarotene the absorbance maximum was the same as that of the novel compound but the shape of the absorbance peak did not match (Fig. 3D). In addition, this carotenoid did not co-chromatograph with compound 3 due to its longer retention time (Fig. 2C). Only 4-keto-α-carotene matched exactly the spectrum of compound 3 (Fig. 3E), it exhibited the same retention time in HPLC (Fig. 2D) and co-chromatographied with peak 3 (Fig. 2E). For further confirmation of the structure of compound 3, the carotenoid extract from rice was reduced by sodium borohydride. This chemical modification of the keto group was also carried out with the 4-keto-α-carotene reference compound resulting in 4-HO-α-carotene. In each case, a more polar compound was formed (Fig. 2D and F). The spectrum of this hydroxy derivative shows the typical three maxima of non-ketolated carotenoids exhibiting a hypsochromic shift to 442 nm of the main central maximum (Fig. 3F) for reduction of a keto group conjugated with the polyene chain. The resulting spectrum resembles that of α-carotene (Britton et al., 2004).

For further structure elucidation of peak 3, a mass spectrum was determined by UHPLC-MS with atmospheric pressure chemical ionisation identifying the $[M+H]^+$ ions (Fig. 4A). The dominating mass peak in the spectrum is the ion at m/z 552. This may resemble the protonated molecular ion of compound 3 with a molecular mass of 551 g/mol. Several carotenoid masses fit this value including the one for echinenone with 550.86 g/mol (Rivera et al., 2011). Starting from $[M+H]^+$ ion of 552, transitions related to functional groups in the carotenoid structures were built. The ones shown in Fig. 4B were those with characteristic signals for identification. The ones to identify an echinenone-related structure are the transitions 551.6 > 203.1 resulting from the cleavage at C-10,11 of a 4-keto fragment as indicated in Fig. 4C (Van Breemen et al., 2012), transition 551.6 > 93 by in-chain elimination of toluene and transition 551.6 > 69 previously reported for echinenone (Enzell et al., 1969). One of the most intense transitions is 551.6 > 123.1. This transition is a specific indicator of the existence of an ε-ring in the structure of compound 3 (Enzell and Back, 1995). Taken together results from mass spectrometry suggest that compound 3 is a carotenoid combining the ketolated half of echinenone and the other half with the ε-ring of α-carotene (boxed in Fig. 4C). This is consistent with 4-keto-α-carotene which has the same molecular mass as echinenone.

HPLC co-chromatography, chemical modification and mass spectrometry all identified compound 3 as 4-keto-α-carotene. This is a unique carotenoid not reported thus far (Britton et al., 2004). A similar but different carotenoid is 2-keto-α-carotene from the stick insect *Ectatosoma tiaratum* (Kayser, 1981). Due to the position of the keto group at C-2, it does not contribute to the conjugated polyene chain which influences the optical absorbance spectrum with three pronounced peaks at 423, 446 and 475 nm, distinct from the spectrum in Fig. 3E. Formation of 4-keto-α-carotene is due to the relatively high α-carotene content in the transgenic rice callus. Obviously, the carotene ketolase from *Brevundimonas* sp. possesses broad substrate specificity. This enzyme typically ketolates both β-ionone rings in β-carotene (Nishida et al., 2005). In addition, it is also able to ketolate the β-ionone end of the α-carotene molecule. This broad substrate specificity in combination with the α-carotene concentrations as substrate for the ketolase is the primary reason for the formation of 4-keto-α-carotene in the genetically engineered rice callus.

Conclusion

Although genetic engineering of the carotenoid pathway to astaxanthin was successful, a specific side reaction occurred in rice callus. The expressed bacterial ketolase not only converted β-carotene and zeaxanthin to astaxanthin but also accepted the β-ionone ring of α-carotene as substrate for ketolation. The resulting product was identified as 4-keto-α-carotene. Its formation branches off the desired β-carotene to astaxanthin route. This loss for the

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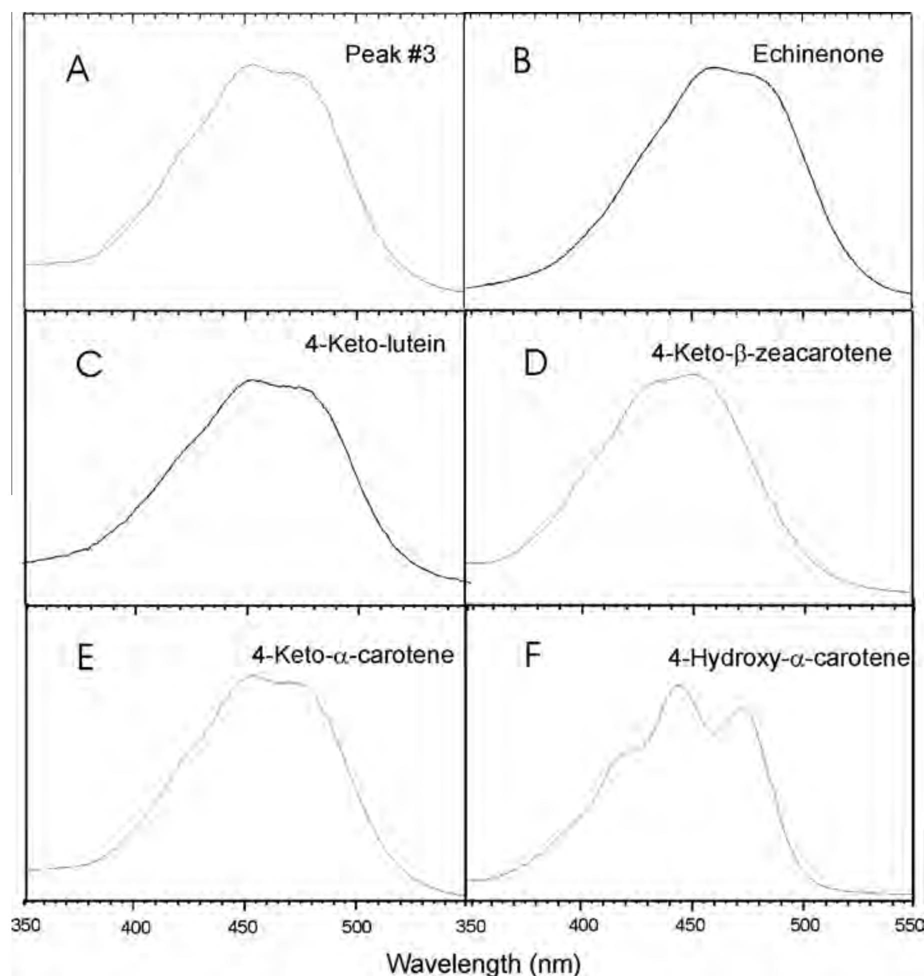

Fig. 3. Optical spectra of the novel unknown carotenoid (A), echinenone (=4-keto-β-carotene) (B), 4-keto-lutein (C), 4-keto-β-zeacarotene (D), 4-keto-α-carotene (E) and 4-hydroxy-α-carotene (F).

astaxanthin pathway may be avoided by further genetic engineering of carotenogenesis by antisense or RNAi down-regulation of the expression of rice specific lycopene ε-cyclase depleting the formation of α-carotene.

Experimental

Transgenic rice

The transgenic endosperm-derived rice callus was generated by combinatorial nuclear transformation which has been reported earlier (Zhu et al., 2008). The three plasmids contained phytoene synthase *Zmpsy1*, phytoene desaturase *Pacrtl* and β-carotene ketolase CrtW from *Brevundimonas* sp. Strain SD212 (Nishida et al., 2005). The latter gene was chemically synthesized according to the codon usage of *Brassica napus* and fused to the full-length rice alcohol dehydrogenase 5́-untranslated region (Sugio et al., 2008) and to the transit peptide sequence from pea ribulose-1, 5-bisphospate carboxylase small subunit (Schreier et al., 1985). This DNA fragment was inserted into plasmid GZ63 containing the maize γ-zein gene promoter and the *nos* terminator. The maize *psy1* cDNA (Buckner et al., 1996) was transferred into plasmid p326 containing the wheat LWM glutenin promoter (Stoger et al. 1999) and *nos* terminator. The *crtI* gene from *Pantoea ananatis* (formerly known as *Erwinia uredovora*) *was* fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase in plasmid pYPIET4 (Misawa et al., 1994), amplified by PCR and then transferred to pHorp-P containing the barley D-hordein promoter (Sørensen et al., 1996) and the rice ADPGPP terminator. All transformation constructs are based on pUC8 plasmids. Their maps are shown in Fig. 5. Transgenic coloured calli were selected and cultured on MS selection medium (Farre et al., 2012).

Combinatorial carotenoid synthesis in E. coli

For the synthesis of reference compounds the biosynthesis pathways for 4-keto-α-carotene and 4-keto-β-zeacarotene the following combinations of compatible plasmids were used to transform *E. coli* strain DH5α. Plasmid pACCAR16ΔcrtX (Misawa et al., 1995) in combination with pBBRK-ara-epsilon with the lycopene ε-cyclase from *Arabidopsis thaliana* (Cunningham et al., 1996) cloned into pBBR1-MCS1 with kanamycin as selection marker (Kovach et al., 1994) mediates the formation of α-carotene. This carotene is then ketolated at the β-ionone ring to 4-keto-α-carotene by expression of pPEU30crtO (Breitenbach et al., 2013). 4-Keto-β-zeacarotene is synthesized from neurosporene generated by pACCRT-EBI$_{RC}$ (Linden et al., 1993) which is cyclised by expression of plasmid pRK-crtY (Hausmann and Sandmann, 2000) and finally ketolated by expression of pCRBKT (Zhong et al., 2011). The *E. coli* transformant generating 4-keto-α-carotene was grown at 37 °C in the presence of ampicillin (100 μg/ml), chloramphenicol

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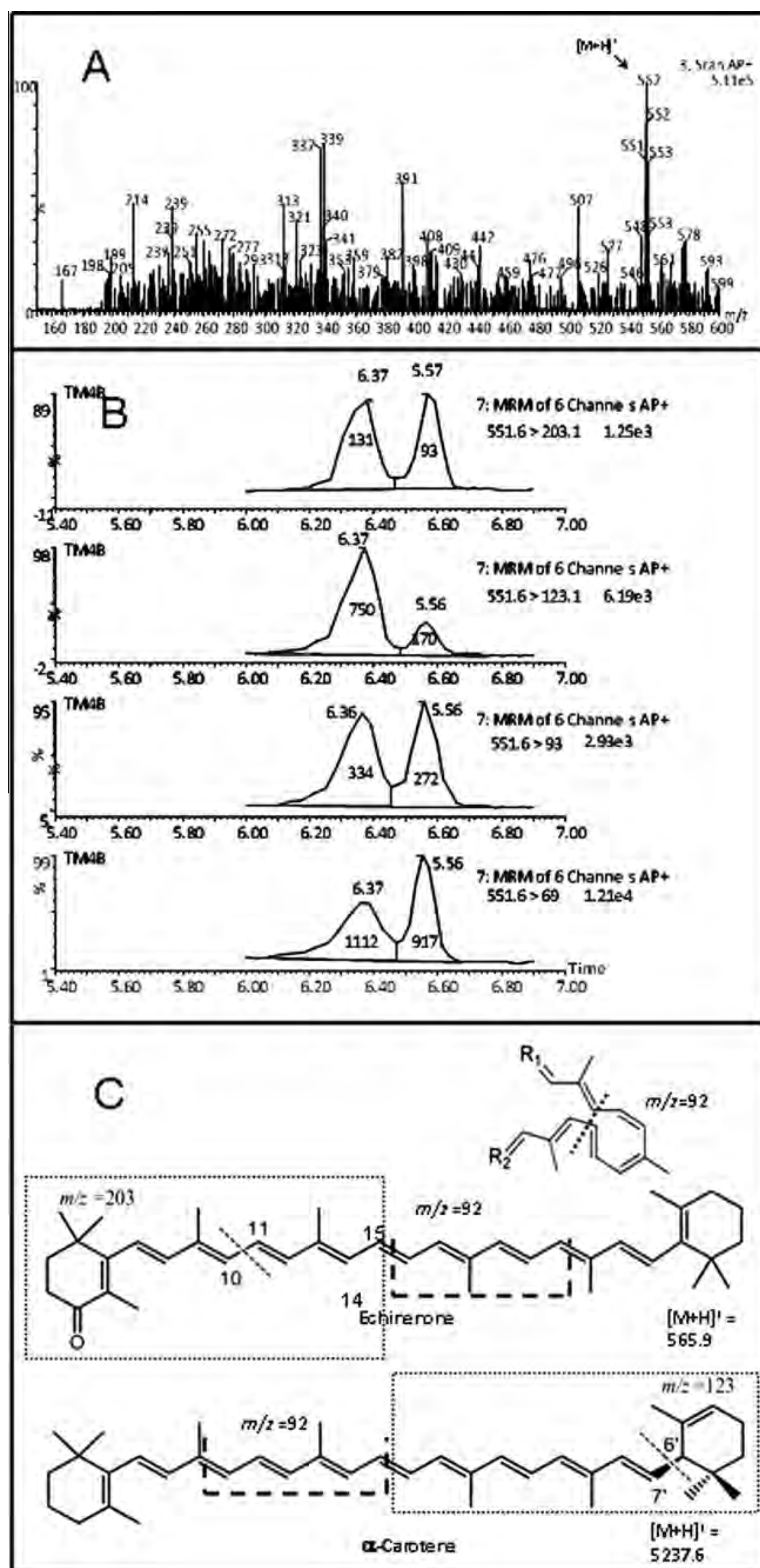

Fig. 4. Mass spectroscropy of novel unknown compound. (A) Atmospheric pressure chemical ionisation mass spectrum, (B) transitions from the $[M+H]^+$ ion of 552, (C) fragmentation pattern of echinenone and α-carotene. Boxes indicate the two halves of the identified molecule, 4-keto-α-carotene.

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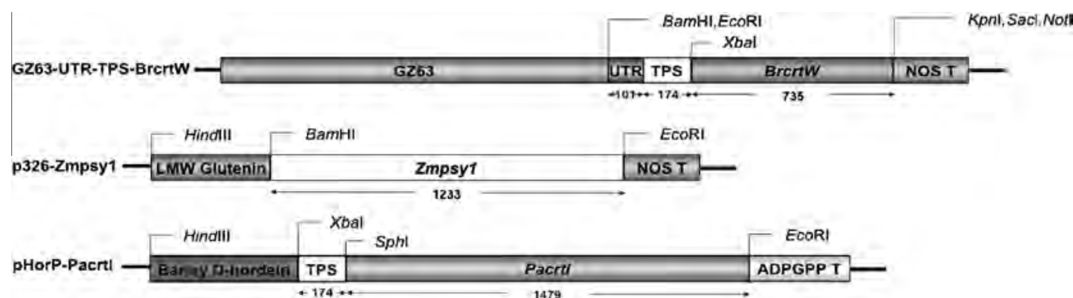

Fig. 5. pUC8-derived plasmid constructs used for rice transformation to express carotene ketolase (BrcrtW), phytoene synthase (Zmpsy1) and phytoene desaturase (PacrtI)). GZ63, maize γ-zein gene promoter; UTR, 5́-untranslated region from rice alcohol dehydrogenase; TPS, transit peptide from pea ribulose-1, 5-bisphospate carboxylase small subunit; NOS T, *nos* terminator; LMW Glutenin, LWM glutenin promoter; Barley D-hordein, barley D-hordein promoter; ADPGPP T, rice ADPGPP terminator.

(34 μg/ml), kanamycin (25 μg/ml) and IPTG (1 mM) in LB medium (Sambrook et al., 1989). The 4-keto-β-zeacarotene producing transformant was cultured at 28 °C in the same medium without IPTG replacing kanamycin with tetracyclin (25 μg/ml).

Carotenoid extraction and analysis

Carotenoids were extracted from freeze-dried rice callus, *E. coli* cells and nectary tissue from *N. tabaccum* (Gerjets et al., 2007) for 20 min with methanol at 60 °C and partitioning into 10% ether in petrol. In the case of tobacco nectary tissue, the 10% ether in petrol phase was diluted 3:1 with hexane, passed through a silica column and the adsorbed polar carotenoid fraction eluted with acetone (Steiger et al., 1999). Analytical HPLC was isocratic on a 15 cm Nucleosil C18, 3 μ column with acetonitrile/ methanol/2-propanol (85:10:5, v/v) at 10 °C and a flow 0.8 ml/min. Carotenoids were identified by their optical absorbance spectra and co-chromatography with standard carotenoids. In the case of the unknown carotenoid, its biosynthetic pathway was reconstructed in *E. coli*. This carotenoid was reduced to 4-hydroxy-α-carotene with sodium borohydride (Eugster, 1995). For mass analysis, UHPLC was carried out using an ACQUITY Ultra Performance LC TM system linked to an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx™ software version 4.1 (Waters, Milford, MA, USA) was used to control the instruments, and also for data acquisition and processing. Separation was on a reverse phase ACQUITY UPLC® C18 BEH 130 Å, 1.7 mm, 2.1 × 100 mm column (Waters, Milford, MA) and a gradient system with the mobile phase consisting of solvent A: acetonitrile:methanol (70:30, v/v) and solvent B: water 100% at a flow rate of 0.35 ml/min. (Rivera et al., 2013).

Acknowledgements

This study was funded by the European Union Framework 7 European Research Council IDEAS Advanced Grant (to PC) Program-BIOFORCE, RecerCaixa, Ministerio de Ciencia e Innovación, Spain (BIO02011-22525; PIM2010PKB-00746) and Plant KBBE CaroMaize project.

References

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorriba, A., Canela, R., Capell, T., Sandmann, G., Christou, P., Zhu, C., 2013. An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. Plant J.. http://dx.doi.org/10.1111/tpj.12384, 2013 Nov 23.

Bjerkeng, B., 2008. Carotenoids in aquaculture: fish and crustaceans. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.), Carotenoids, Natural Functions, vol. 4. Birkhäuser Verlag, Basel, pp. 237–254.

Breitenbach, J., Gerjets, T., Sandmann, G., 2013. Catalytic properties and reaction mechanism of the CrtO carotenoid ketolase from the cyanobacterium *Synechocystis* sp. PCC 6803. Arch. Biochem. Biophys. 529, 86–91.

Britton, G., Liaaen-Jensen, S., Pfander, H., 2004. Carotenoids Handbook. Birkhäuser Verlag, Basel.

Buckner, B., San Miguel, P., Janick-Buckner, D., Bennetzent, J.L., 1996. The *yl* gene of maize codes for phytoene synthase. Genetics 143, 479–488.

Cunningham, F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D., Gantt, E., 1996. Functional analysis of the β and lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8, 1613–1626.

Enzell, C.R., Back, S., 1995. Mass spectrometry. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.), Carotenoids, Spectroscopy, vol. 1B. Birkhäuser Verlag, Basel, pp. 261–320.

Enzell, C.R., Francis, G.W., Liaaen-Jensen, S., 1969. Mass spectrometric studies of carotenoids. 2. A survey of fragmentation reactions. Acta Chem. Scand. 23, 727–750.

Eugster, C.H., 1995. Chemical derivatization: microsomale tests for the presence of common functional groups in carotenoids. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.), Carotenoids, Isolation and Analysis, vol. 1A. Birkhäuser Verlag, Basel, pp. 71–80.

Farre, G., Naqvi, S., Sanahuja, G., Bai, C., Zorrilla-López, U., Rivera, S.M., Canela, R., Sandmann, G., Twyman, R.M., Capell, T., Zhu, C., Christou, P., 2012. Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. Methods Mol. Biol. 847, 419–435.

Fraser, P.D., Römer, S., Shipton, C.A., Mills, P.B., Kiano, J.W., Misawa, N., Darke, R.G., Schuch, W., Bramley, P.M., 2002. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. Proc. Natl. Acad. Sci. USA 99, 1092–1097.

Fraser, P.D., Enfissi, E.M., Bramley, P.M., 2009. Genetic engineering of carotenoid formation in tomato fruit and the potential application of systems and synthetic biology approaches. Arch. Biochem. Biophys. 483, 196–204.

Gerjets, T., Sandmann, M., Zhu, C., Sandmann, G., 2007. Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species. Biotechnol. J. 2, 1263–1269.

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

Hausmann, A., Sandmann, G., 2000. A single 5-step desaturase is involved in the carotenoid biosynthesis pathway to ß-carotene and torulene in *Neurospora crassa*. Fung. Gen. Biol. 30, 147–153.

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid in human health and nutrition. J. Nat. Prod. 69, 443–449.

Kayser, H., 1981. *Ectatosoma tiaratum*, isolation of β,ε-carotene-2-ol and β,ε-carotene-2-one. Z. Naturforsch. 36c, 755–764.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., Peterson, K.M., 1994. PBBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Linden, H., Vioque, A., Sandmann, G., 1993. Isolation of a carotenoid biosynthesis gene coding for a zeta-carotene desaturase from *Anabaena* PCC7120 by heterologous complementation. FEMS Microbiol. Lett. 106, 99–104.

Misawa, N., 2011. Pathway engineering for functional isoprenoids. Curr. Opin. Biotechnol. 22, 627–633.

Misawa, N., Masamoto, K., Hori, T., Ohtani, T., Böger, P., Sandmann, G., 1994. Expression of an *Erwinia* phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. Plant J. 6, 481–489.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., Miki, W., 1995. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J. Bacteriol. 177, 6575–6584.

Naqvi, S., Zhu, C., Farre, G., Sandmann, G., Capell, T., Christou, P., 2011. Synergistic metabolism in hybrid corn reveals bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. Plant Biotechnol. J. 9, 384–393.

Nishida, Y., Adachi, K., Kasai, H., Shizuri, Y., Shindo, K., Sawabe, A., Komemushi, S., Miki, W., Misawa, N., 2005. Elucidation of a carotenoid biosynthesis gene cluster encoding a novel enzyme, 2,2′-beta-hydroxylase, from *Brevundimonas* sp. strain SD212 and combinatorial biosynthesis of new or rare xanthophylls. Appl. Environ. Microbiol. 71, 4286–4296.

Please cite this article in press as: Breitenbach, J., et al. A novel carotenoid, 4-keto-α-carotene, as an unexpected by-product during genetic engineering of carotenogenesis in rice callus. Phytochemistry (2014), http://dx.doi.org/10.1016/j.phytochem.2013.12.008

Paine, J.A., Shipton, C.A., Chaggar, R.M., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L., Drake, R., 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. 23, 482–487.

Quackenbush, F.W., Firch, J.G., Brunson, A.M., House, L.R., 1963. Carotenoid, oil, and tocopherol content of corn inbreds. Cereal Chem. 40, 250–253.

Rivera, S., Vilaró, F., Canela, R., 2011. Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants. Analyt. Bioanalyt. Chem. 400, 1339–1346.

Rivera, S.M., Vilaró, F., Zhu, C., Bai, C., Farré, G., Christou, P., Canela-Garayoa, R., 2013. Fast quantitative method for the analysis of carotenoids in transgenic aize. J. Agric. Food Chem. 61, 5279–5285.

Römer, S., Lübeck, J., Kauder, F., Steiger, S., Adomat, C., Sandmann, G., 2002. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metabolic Engin. 4, 263–272.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manua, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandmann, G., 2001. Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. Trends Plant Sci. 6, 14–17.

Sandmann, G., 2002. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chem. Bio. Chem. 3, 629–635.

Sandmann, G., Römer, S., Fraser, P.D., 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metabolic Eng. 8, 291–302.

Schaub, P., Al-Babili, S., Drake, R., Beyer, P., 2005. Why is golden rice golden (yellow) instead of red? Plant Physiol. 138, 441–450.

Schreier, P., Seftor, E., Schell, J., Bohnert, H., 1985. The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. 4, 25–32.

Sørensen, M.B., Müller, M., Skerritt, J., Simpson, D., 1996. Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. Mol. Gen. Genet. 250, 750–760.

Steiger, S., Schäfer, L., Sandmann, G., 1999. High-light upregulation of carotenoids and their antioxidative properties in the cyanobacterium *Synechocystis* PCC 6803. J. Photochem. Photobiol., B 521, 14–18.

Stoger, E., Williams, S., Keen, D., Christou, P., 1999. Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. Transgenic Res. 8, 73–82.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A., Kato, K., 2008. The 5′-untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells. J. Biosci. Bioeng. 105, 300–330.

Van Breemen, R.B., Dong, L., Pajkovic, N.D., 2012. Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids. Int. J. of Mass Spectrometry 312, 163–172.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I., 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287, 303–305.

Zhong, Y.J., Huang, Y.C., Liu, J., Li, Y., Jiang, Y., Xu, Z.F., Sandmann, G., Chen, F., 2011. Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic *Arabidopsis*. J. Exp. Bot. 62, 3659–3669.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T., 2008. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc. Natl. Acad. Sci. USA 105, 18232–18237.

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

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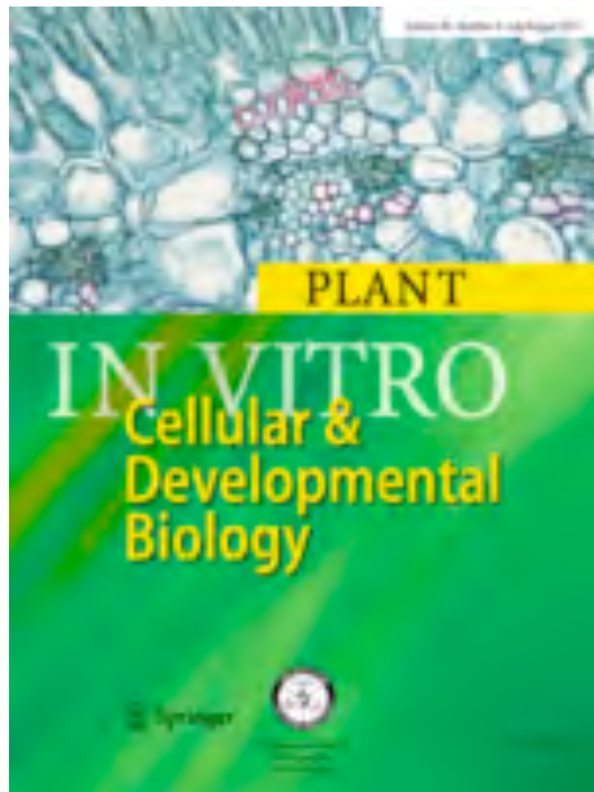
A golden era—pro-vitamin A enhancement in diverse crops

Chao Bai, Richard M. Twyman , Gemma Farré, Georgina Sanahuja, Paul Christou,

Teresa Capell, Changfu Zhu*



In Vitro Cell. Dev. Biol. –Plant (2011), 47: 205-221

*Corresponding author Changfu Zhu (email: zhu@pvcf.udl.cat)

Chao Bai and Richard M. Twyman contributed equally to this work.

In Vitro Cell.Dev.Biol.—Plant (2011) 47:205–221
DOI 10.1007/s11627-011-9363-6

INVITED REVIEW

A golden era—pro-vitamin A enhancement in diverse crops

Chao Bai · Richard M. Twyman · Gemma Farré · Georgina Sanahuja · Paul Christou · Teresa Capell · Changfu Zhu

Received: 28 December 2010 / Accepted: 18 February 2011 / Published online: 14 April 2011 / Editor: N. J. Taylor
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Abstract Numerous crops have been bred or engineered to increase carotenoid levels in an effort to develop novel strategies that address vitamin A deficiency in the developing world. The pioneering work in rice (not covered in this review) has been followed up in many additional crops, some of which are staples like rice whereas others are luxury products whose impact on food security is likely to be marginal. This review surveys the progress that has been made in carotenoid breeding and metabolic engineering, focusing on β-carotene enhancement in crops other than rice. We ask if these efforts have the potential to address vitamin A deficiency in developing countries by comparing bioavailable pro-vitamin A levels in wild type and enhanced crops to determine whether nutritional requirements can be met without the consumption of unrealistic amounts of food. The potential impact of carotenoid enhancement should therefore be judged against benchmarks that include the importance of particular crops in terms of global food security, the amount of bioavailable β-carotene, and the amount of food that must be consumed to achieve the reference daily intake of vitamin A.

Chao Bai and Richard M. Twyman contributed equally to this paper

C. Bai · G. Farré · G. Sanahuja · P. Christou · T. Capell · C. Zhu (✉)
Department of Plant Production and Forestry Science, ETSEA, University of Lleida,
Av. Alcalde Rovira Roure 191,
25198 Lleida, Spain
e-mail: zhu@pvcf.udl.es

R. M. Twyman
Department of Biological Sciences, University of Warwick, Warwick, Coventry CV4 7AL, UK

P. Christou
Institució Catalana de Reserca i Estudis Avançats,
Passeig Lluís Companys 23,
08010 Barcelona, Spain

Keywords Beta carotene · Metabolic engineering · Nutritional enhancement · Biofortification · Genetic engineering · Food crops

Dietary Vitamin A

Vitamin A is an essential nutrient in mammals that occurs in several forms known as retinoids. Each form is characterized by a common skeleton known as the retinyl group, comprising an unsubstituted β-ionone ring and an isoprenoid side chain (Fig. 1). The aldehyde form (retinal or retinaldehyde) is necessary for the production of rhodopsin in the eyes and for the maintenance of epithelial and immune cells, whereas the acidic form (retinoic acid) is a morphogen in development. Whereas many foods are regarded as good sources of vitamin A, they usually do not contain significant quantities of either retinal or retinoic acid (Olson 1994). Meat and dairy sources primarily contain retinyl esters that are converted into the alcohol form (retinol) in the small intestine, and from there into retinal or retinoic acid. In contrast, plant sources contain pro-vitamin A carotenoids, the most important of which is β-carotene. This can be converted into retinal by the enzyme β-carotene 15,15′-monooxygenase. Many carnivores lack this enzyme and cannot obtain vitamin A from plants, but herbivores and omnivores (including humans) can synthesize retinal from the abundant pro-vitamin A carotenoids present in dark green, yellow, and orange fruits and vegetables, such as oranges, broccoli, spinach, carrots, squash, sweet potatoes, and pumpkins (Harrison 2005).

The dietary reference intake (DRI) for vitamin A is best expressed as the retinol activity equivalent (RAE), which takes bioavailability into account. The recommended DRI for males is 900 RAE, for females it is normally 700 RAE

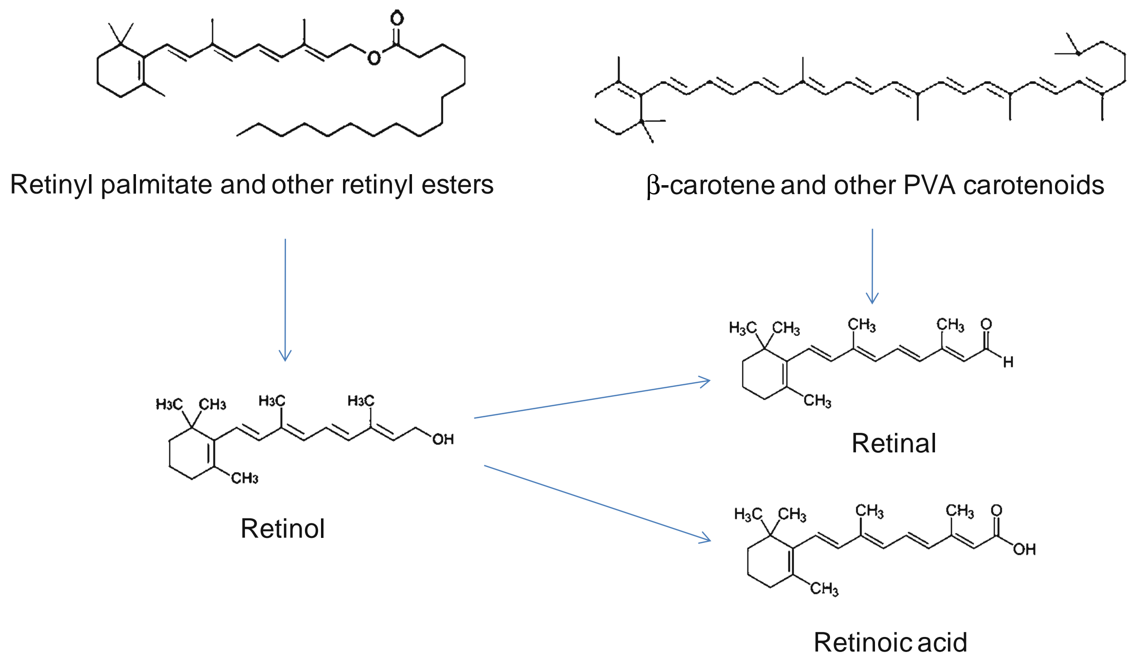


Figure 1. Vitamin A in humans consists of a group of molecules with a common retinyl group. The functional molecules are retinal and retinoic acid. Dietary sources are principally retinyl esters from meat and dairy products and pro-vitamin A (*PVA*) carotenoids from plants.

(770 RAE in pregnancy, and 1200–1300 RAE when lactating), and for children it is 400–500 RAE. One RAE is equivalent to 1 μg of retinol, 2 μg of β-carotene supplement dissolved in oil, or 12 μg of β-carotene in food (IOM 2001). A DRI of 900 RAE for males is therefore equivalent to 900 μg (3,000 IU) of retinol, 1,800 μg of β-carotene supplement or 1.08 mg of β-carotene in food (IOM 2001).

Most people in the developed world have diets of sufficient diversity to ensure they achieve the DRI for vitamin A. However, the situation in developing countries is very different, with many subsisting on a cereal-dominated diet with low levels of nutrients, including β-carotene. These individuals are severely at risk of vitamin A deficiency (VAD), which weakens the immune system, causes the deterioration of light-sensitive rod cells essential for low-light vision, and in extreme cases can lead to an irreversible form of blindness called xerophthalmia (UNICEF 2006). More than four million children worldwide exhibit signs of severe VAD (Fig. 2), including 250,000–500,000 per yr who become partially or totally blind. Women have a higher demand for vitamin A during pregnancy, and currently more than 20 million pregnant women in developing countries suffer from VAD. Approximately six million of these women show clinical signs of night blindness, with half the cases in India (http://www.harvestplus.org/content/vitamin). VAD in pregnancy causes nearly 600,000 deaths every yr (UNICEF 2006).

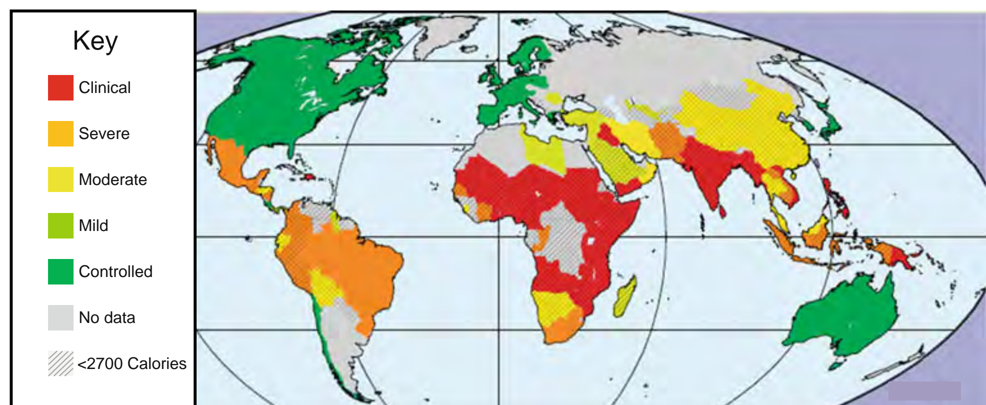


Figure 2. Impact of vitamin A deficiency around the world (World Health Organization data, 1996).

The Production of Pro-Vitamin A Carotenoids in Plants

Plants produce four pro-vitamin A carotenoids, distinguished by the possession of at least one retinyl group. Two of these molecules (α-carotene and β-carotene) accumulate in significant amounts whereas the others (γ-carotene and β-cryptoxanthin) are intermediates and tend to be converted rapidly into downstream products (Farre *et al*. 2010a, Zhu *et al*. 2010; Fig. 3).

Carotenoids are tetraterpenoids, *i.e.*, they are composed of eight condensed C5 isoprenoid precursors generating a C40 linear backbone. In plants, this condensation reaction involves the isomeric precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and occurs de novo within plastids (Chappell 1995). IPP and DMAPP are derived predominantly from the plastidial methylerythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat 2002), although the same precursors are formed by the cytosolic mevalonic acid pathway, and there is some evidence for the shuttling of intermediates between pathways (Rodriguez-Concepcion 2006). The condensation of three IPP molecules with one molecule of DMAPP produces the C20 intermediate geranylgeranyl diphosphate (GGPP), a reaction catalyzed by GGPP synthase (GGPPS). In bacteria, GGPPS is encoded by the *crt*E gene (Fig. 3).

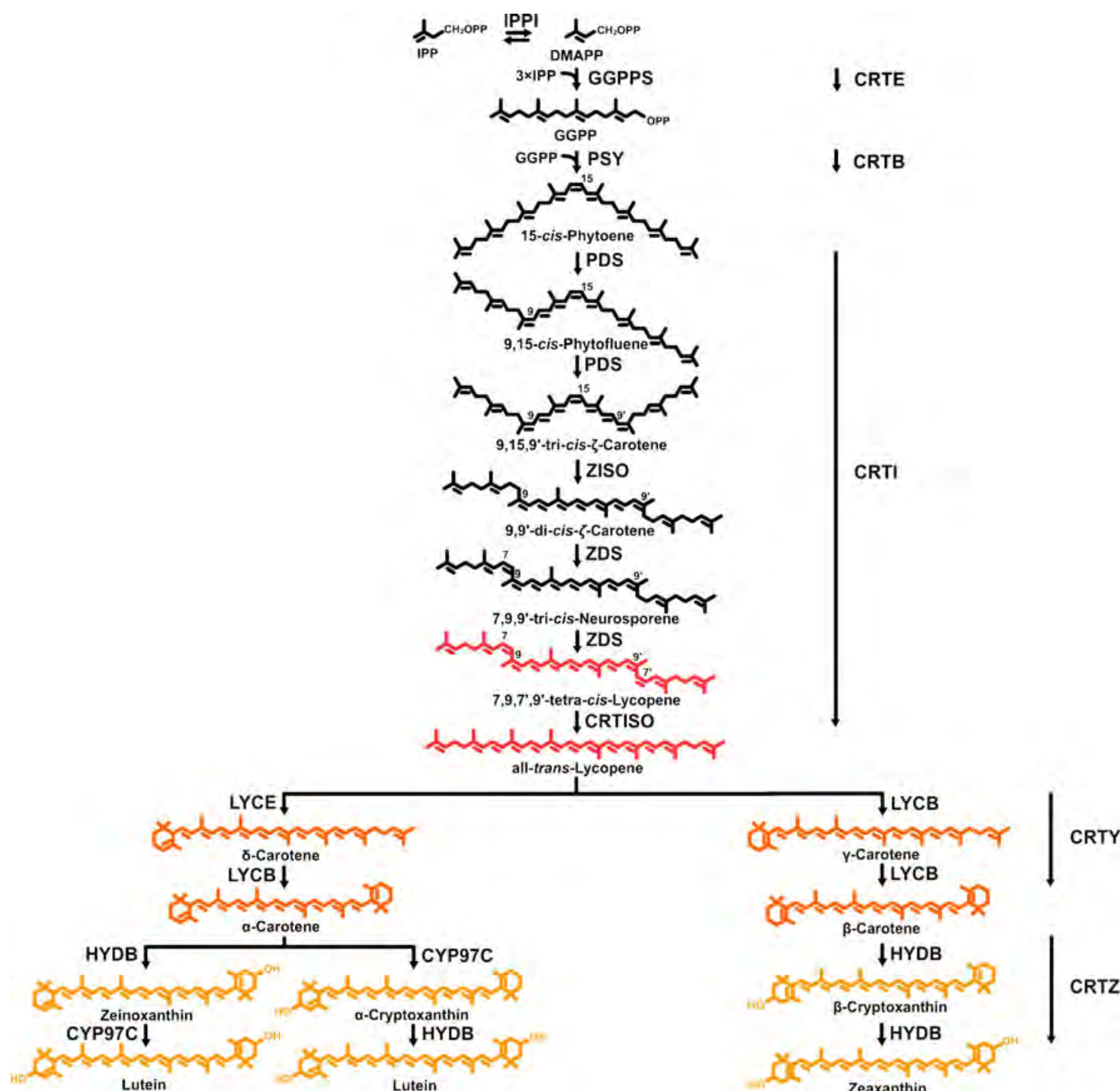


Figure 3. Carotenoid biosynthesis pathway in plants and equivalent steps in bacteria. *IPP* isopentenyl diphosphate; *IPPI* isopentenyl diphosphate isomerase; *DMAPP* dimethylallyl diphosphate; *GGPP* geranylgeranyl diphosphate; *GGPPS* GGPP synthase; *PSY* phytoene synthase; *PDS* phytoene desaturase; *Z-ISO* ζ-carotene isomerase; *ZDS* ζ-carotene desaturase; *CRTISO* carotenoid isomerase; *LYCB* lycopene β-cyclase; *LYCE* lycopene ε-cyclase; *CYP97C* carotene ε-ring hydroxylase; *HYDB* β-carotene hydroxylase; *CRTE* bacterial GGPP synthase; CRTB bacterial phytoene synthase; CRTI bacterial phytoene desaturase/isomerase; *CRTY* bacterial lycopene cyclase; *CRTZ* bacterial β-carotene hydroxylase.

The first committed step in plant carotenoid synthesis is the condensation of two GGPP molecules into 15-cis-phytoene by the enzyme phytoene synthase (PSY) (Misawa *et al*. 1994). The equivalent enzyme in bacteria is CrtB. A series of four desaturation reactions carried out in plants by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. The product of the first desaturation is 9,15,9′-tri-cis-ζ-carotene, which is isomerized by light and/or ζ-carotene isomerase (Z-ISO) to yield 9,9′-di-cis-ζ-carotene, the substrate of ZDS (Li *et al*. 2007; Chen *et al*. 2010). The end product of the desaturation reactions is converted to all-trans-lycopene by a carotenoid isomerase (CRTISO) in non-green tissue, and by light and chlorophyll (acting as a sensitizer) in green tissue (Breitenbach and Sandmann 2005; Isaacson *et al*. 2004; Li *et al*. 2010a). In bacteria, a single enzyme encoded by the *crt*I gene accomplishes all the above steps and produces all-trans-lycopene from 15-cis-phytoene directly (Fig. 3).

Lycopene is an important branch point in the carotenoid pathway, because it acts as the substrate for two competing enzymes, lycopene β-cyclase (LYCB), and lycopene ε-cyclase (LYCE; Cunningham *et al*. 1996). Both enzymes cyclize the linear backbone to generate terminal ionone rings, but the structures of these rings are distinct. The addition of one β-ring to lycopene by LYCB generates γ-carotene, and the addition of a second β-ring to the free end by the same enzyme produces β-carotene. The bacterial gene *crt*Y encodes LYCB. Both γ- and β-carotene therefore have retinyl groups and possess pro-vitamin A activity, but β-carotene is a better source because it possesses two such groups. Furthermore, as stated above, γ-carotene is rapidly converted into β-carotene and tends not to accumulate. Alternatively, the addition of one ε-ring to lycopene by LYCE generates δ-carotene (which has no pro-vitamin A activity). This is a poor substrate for LYCE so it is unusual for the second ε-cyclization to take place, but it is a good substrate for LYCB to add a β-ring to the free end generating α-carotene, which does have pro-vitamin A activity. In the presence of the enzyme β-carotene hydroxylase (BCH), both α-carotene and β-carotene can be converted into more complex downstream carotenoids that do not possess pro-vitamin A activity (Tian *et al*. 2003). In the case of α-carotene, this downstream product is lutein, and in the case of β-carotene the downstream product is zeaxanthin, although the reaction involves the intermediate β-cryptoxanthin, which also has pro-vitamin A activity. The bacterial gene *crt*Z encodes BCH. Whereas lutein represents the natural end point of the α-carotene branch, zeaxanthin enters the xanthophyll cycle and can be converted through a number of additional steps into the important plant hormone abscisic acid (Seo and Koshiba 2002).

Enhancing β-Carotene Levels in Plants

Addressing VAD in the developing world requires a robust strategy to increase access to foods rich in (pro)-vitamin A, and several approaches can be considered. Dietary supplements (vitamin tablets and suspensions) and fortification (artificially increasing vitamin levels by adding vitamins to processed food) campaigns have been highly successful in the developed world and have significantly reduced the incidence of deficiency diseases. Many processed foods, including bread, packaged cereals, milk, and soft drinks, are fortified with vitamins and minerals so that the average diet contains micronutrients well in excess of requirements. In developing countries, the less-robust and less-reliable food distribution infrastructure, poor governance, and the lack of funding renders such programs inefficient and unsustainable, especially when trying to reach remote areas (Darnton-Hill and Nalubola 2002). Vitamin A supplementation campaigns have enjoyed limited success, but most programs have failed to address VAD, especially at the local level when dealing with small and remote villages (Underwood 2003). An alternative approach to adding vitamin A directly to the diet is to enhance the accumulation of pro-vitamin A carotenoids in crops. There are both conventional and biotechnological approaches to biofortification, each of which has met with some success, as discussed below.

Conventional breeding. Conventional breeding is usually considered to include any form of breeding that does not involve the introduction of new genetic material using recombinant DNA technology, but it does not exclude accelerated mutation or forced hybridization methods that introgress genes from distant relatives, which similarly would not occur in nature. Conventional breeding also allows the use of molecular markers to facilitate the selection of productive crops.

The limitations of conventional breeding include its dependence on a compatible gene pool (*i.e.*, the collection of species with which the target crop can be forced to interbreed) and on the degree of available natural or inducible variation. If sufficient natural variation in vitamin A levels can either be identified within a breeding population or can be induced by mutation, then selection strategies can be used to produce nutritionally improved lines. Even so, programs to exploit such variation have met with only marginal success. For example, a recent attempt to use variation at the *lyc*E locus in maize to increase β-carotene levels achieved a fivefold increase (the best line contained 13.6 μg β-carotene per gram dry weight of endosperm; Harjes *et al*. 2008), whereas transgenic strategies have achieved >100-fold increases over a much shorter time-scale (the best line reported thus far contains 59.32 μg

β-carotene per gram dry weight of endosperm; Naqvi *et al*. 2009). The long time required to generate nutritionally improved lines, especially if a trait has to be introgressed into an elite local breeding line, is one of the greatest challenges in conventional breeding.

Conventional breeding can be accelerated by marker-assisted selection (MAS). The usefulness of molecular markers is particularly evident when dealing with quantitative traits such as nutrient levels, since these tend to reflect the activities of multiple genes contributing to the same phenotype. Breeding without markers can only identify the most productive combinations of alleles by chance, whereas MAS allows particular alleles, identified by the linked markers, to be stacked in the same line without any need for phenotypic analysis. MAS has allowed the construction of saturated linkage maps for many crops and has made it possible to map the quantitative trait loci (QTLs) that control them. Understanding how QTLs affect crop performance under different environmental conditions and in different genetic backgrounds can facilitate the development of enhanced crop varieties. Consequently, the mapping of QTLs for agronomic traits is an important component of conventional nutritional improvement programs (Dwivedi *et al*. 2007).

Conventional breeding can also be accelerated by mutagenesis, which generates new alleles more rapidly than would occur in nature. This can be achieved by irradiating seeds with X-rays or exposing them to chemical mutagens such as ethylmethanesulfonate, each of which causes random damage to DNA and usually generates point mutations. Any phenotypic effects of such mutations are observed in subsequent generations, depending on whether the effect is dominant or recessive, and must be mapped to identify the affected gene. An advanced method for identifying such point mutations is TILLING (Targeting Induced Local Lesions IN Genomes), a high-throughput method based on conformational electrophoresis for the detection of point mutations in large populations of plants (Comai and Henikoff 2006). TILLING can identify genetic variation in elite germplasm without the need to acquire variation from exotic cultivars, thus avoiding the introduction of agriculturally undesirable traits. Once a TILLING library is set up, it can be used for the analysis of many different gene targets. TILLING is a powerful reverse genetics approach that has the unique advantage of allowing the generation of an allelic series for any target gene, including essential genes (Slade and Knauf 2005). If a variety developed by TILLING has commercial potential, it is not subject to the same regulatory approval requirements for transgenic crops.

Even with methods such as TILLING facilitating the process of mapping novel mutations, it is still preferable to be able to identify mutated genes with a unique DNA signature. This is the benefit of insertional mutagenesis using unique DNA tags such as transposons or T-DNA, the former endogenous to plants, the latter introduced artificially. In this approach, randomly integrating DNA sequences disrupt genes and cause a loss of function. The identification of desirable mutant phenotypes is followed by DNA analysis using the insertional mutagen sequence as a probe, or as the basis for PCR primer design, allowing flanking gene sequences to be identified (Maes *et al*. 1999, Parinov and Sundaresan 2000).

Genetic engineering. Biofortification at the crop level can be achieved through the transfer of genes conferring enhanced nutritional traits directly into elite breeding lines, generating transgenic plants. Many crop species have been genetically engineered to produce higher levels of pro-vitamin A, and these are discussed in subsequent sections of this article. Compared to conventional breeding, transgenesis has the advantages of speed, direct engineering of breeding lines, simplicity, the potential for multiple simultaneous biofortification for different nutrients, and unrestricted access to genetic diversity (*i.e.*, genes from bacteria and animals, and even completely synthetic or artificially modified genes that do not exist in nature; Zhu *et al*. 2007).

Because pro-vitamin A carotenoids are synthesized de novo by plants, increasing the availability of these compounds must involve metabolic engineering with the focus on β-carotene, because it is the most important and potent of the four available pro-vitamin A carotenoids. However, intervention can take place at any point along the pathway, and multiple strategies are available (Capell and Christou 2004). General approaches include increasing flux through the entire carotenoid pathway by enhancing the production of GGPP, whereas more targeted approaches involve specifically boosting the production of β-carotene or reducing the amount of α-carotene, which can be regarded as a competitor because it shares a common precursor. As well as enhancing the synthesis of β-carotene, additional approaches include the inhibition of post-β-carotene steps to prevent conversion to zeaxanthin and other derivatives, and increasing the ability of plant cells to store β-carotene, thereby providing a metabolic sink and preventing feedback inhibition. Another useful approach is RNA interference (RNAi), in which short, double-stranded RNA molecules are used to induce a defense pathway that destroys homologous mRNAs and leads to potent post-transcriptional gene silencing (Lawrence and Pikaard 2003; Mansoor *et al*. 2006). RNAi has the advantage of being able to knock down the expression of multiple, related genes with one construct if they share a homologous core sequence, whereas conventional mutagenesis/TILLING and insertional mutagenesis only affect single genes. The ability to silence several genes

simultaneously using RNAi can speed up the creation of plant lines with novel or enhanced nutritional properties. All these methods have been attempted alone and in combination, with some remarkable achievements as discussed in more detail below.

Pro-Vitamin A Enhancement in Cereals

In addition to the pioneering work in rice (see Beyer 2010; Potrykus 2010, for reviews), carotenoid enhancement has been attempted by conventional breeding and/or genetic engineering in three major staple cereals: maize, wheat, and sorghum. Maize is a valuable model for carotenoid research because of its diverse gene pool, its amenability for genetic analysis, and the tendency for carotenoid variants to display distinct, color-based phenotypes. Maize kernels naturally accumulate lutein and zeaxanthin, and there is significant variation in their levels, suggesting that conventional breeding could be used to improve nutrition (Kurilich and Juvik 1999). A number of mutants have been identified with specific deficiencies in carotenoid metabolism. One of these is the *yellow 1* (*y1*) mutant, which maps to the *psy1* gene (there are three genes encoding PSY in maize, with overlapping functions; Li *et al*. 2008). The others (*vp2, vp5, vp7, vp9, w3,* and *y9*) combine two common mutant phenotypes—albinism (lack of color due to the absence of carotenoids) and viviparity (premature development due to the absence of abscisic acid; Wurtzel *et al*. 2001). These too have subsequently been mapped to genes encoding carotenogenic enzymes. Singh *et al*. (2003) identified an *Ac* element insertion named *pink scutellum1* (*ps1*), which maps to the same locus as *vp7* and represents an insertional disruption of the *lycb* gene.

Detailed QTL analysis for MAS in maize has been facilitated by the identification of molecular markers associated with the mutants listed above. For example, a simple sequence repeat marker associated with *y1* was linked to a major QTL that explains 6.6–27.2% of the phenotypic variation in carotenoid levels in maize and was eventually resolved to the *psy1* gene (Wong *et al*. 2004). A QTL associated with *y9* might also be useful for pyramiding favorable alleles controlling carotenoid levels in diverse germplasm (Chander *et al*. 2008). Harjes *et al*. (2008) described four polymorphisms in the maize *lyce* locus, and selection for low LYCE activity increased the β-carotene levels in seeds to 13.6 μg/g dry weight (a 30–40% improvement). Vallabhaneni *et al*. (2009a) characterized six carotene hydroxylase genes in genetically diverse maize germplasm collections, although only one appeared to affect carotenoid levels in endosperm. Three alleles of this *hyd3* gene explained 78% of the variation in the β-carotene/β-cryptoxanthin ratio (an 11-fold difference across varieties) and 36% of the variation in absolute β-carotene levels (a fourfold difference across varieties). These authors used a combination of bioinformatics and cloning to identify and map gene families encoding carotenogenic enzymes from maize and other grasses, and have identified those whose mRNA levels positively and negatively correlate with endosperm carotenoid levels (Vallabhaneni and Wurtzel 2009b).

Several groups have used biotechnology to increase carotenoid levels in maize, *e.g.*, Aluru *et al*. (2008) introduced the bacterial *crt*B and *crt*I genes under the control of an enhanced γ-zein promoter to provide strong endosperm-specific expression, increasing the total carotenoid content to 33.6 g/g dry weight. A significant advance was achieved by Zhu *et al*. (2008) with the development of a combinatorial nuclear transformation system designed to dissect and modify the carotenoid biosynthetic pathway in maize, using the white endosperm elite South African inbred M37W. The method involved transforming plants with multiple genes encoding the enzymes involved in carotenoid biosynthesis and then screening a library of random transformants for plants with appropriate metabolic profiles. The pilot study for this technique involved the introduction of five genes (maize *psy1*, *Gentiana lutea lycb* and *bch*, and bacterial *crt*I and *crt*W) under the control of endosperm-specific promoters. This recreates the entire pathway from GGPP to zeaxanthin (Fig. 3) and also adds the enzyme CrtW (β-carotene ketolase), which converts β-carotene into downstream ketocarotenoids. The M37W genetic background provided a blank template, because the endosperm in this variety has no PSY activity and therefore lacks all carotenoids. The recovery of plants carrying random combinations of genes resulted in a metabolically diverse library comprising plants with a range of carotenoid profiles, revealed by easily identifiable endosperm colors ranging from yellow to scarlet. The kernels contained high levels of β-carotene, lycopene, zeaxanthin, and lutein, as well as further commercially relevant ketocarotenoids such as astaxanthin and adonixanthin (Zhu *et al*. 2008).

Another recent breakthrough in this area was the development of transgenic maize plants transformed with multiple genes selected to simultaneously modulate three metabolic pathways in the endosperm, thereby increasing the levels of the three key nutrients β-carotene, ascorbate, and folate (Naqvi *et al*. 2009). The same M37W line was used as the basis for these experiments because, in addition to the absence of carotenoids, it also has very low ascorbate and folate levels. Kernels from the transgenic plants contained 169-fold more β-carotene than normal (60 μg/g dry weight) as well as six times the normal level of ascorbate (110 μg/g dry weight) and twice the normal level of folate (1.94 μg/g dry weight).

Hexaploid tritordeums produce more carotenoids than their respective wheat parents or hybrids derived from crosses between wild diploid barley and durum wheat (Alvarez *et al*. 1999). A double haploid wheat population, which was previously characterized for endosperm color (Clarke *et al*. 2006), was used to map the *psy1* and *psy2* genes against four QTLs affecting endosperm color, with one showing strong linkage (Pozniak *et al*. 2007). Carotenoid levels in elite wheat lines (*Triticum aestivum* EM12) have also been improved by genetic engineering. Transgenic wheat expressing maize *psy1* under the control of the endosperm-specific 1Dx5 promoter in combination with constitutive *crt*I produced yellow grains containing 10.8-fold the carotenoid levels of wild-type EM12 plants (Cong *et al*. 2009).

Natural variation in carotenoid levels in sorghum has been investigated by Kean *et al*. (2007), which determined the carotenoid profiles of eight selected yellow-endosperm cultivars in which zeaxanthin is the most abundant carotenoid. Salas Fernandez *et al*. (2008) detected several QTLs responsible for varying carotenoid levels in a recombinant inbred line population generated by crossing the yellow-endosperm variety KS115 with the white endosperm variety Macia. Among four QTLs for endosperm color and five for β-carotene levels, one was mapped to the *psy3* gene. This provides a starting point for the breeding of sorghum varieties with higher carotenoid levels.

Pro-vitamin A enhancement in Brassicas. Two members of the Brassica family have been the focus for carotenoid enhancement: canola (*Brassica napus*) and cauliflower (*Brassica oleracea*). Canola seeds contain high total carotenoid levels (up to 23 μg/g fresh weight, including 0.2 μg/g β-carotene). Therefore, canola oil is considered a valuable dietary source of pro-vitamin A and a good target for carotenoid enhancement (Fujisawa *et al*. 2009). Despite the existence of informative high-density genetic markers, no QTLs affecting carotenoid accumulation have yet been reported (Zamir 2001). In contrast, there has been impressive progress in carotenoid genetic engineering (Nesi *et al*. 2008). Shewmaker *et al*. (1999) expressed bacterial *crt*B using the seed-specific napin promoter and increased total carotenoid levels to more than 1 mg/g fresh weight, a 500-fold improvement, which included 401 μg/g fresh weight of β-carotene. This was bettered by Ravanello *et al*. (2003) using the same transgene (1.34 mg/g fresh weight total carotenoids including 739 μg/g fresh weight β-carotene). The combined expression of *crt*B and *crt*I in the same study boosted total carotenoid levels to 1.4 mg/g fresh weight with β-carotene levels reaching 857 μg/g fresh weight. However, the further addition of *crt*Y, which might be expected to increase total carotenoid and β-carotene levels even further, caused a marginal reduction in β-carotene levels (846 μg/g fresh weight) and a significant reduction in total carotenoids (1.23 mg/g fresh weight) presumably by increasing the flux to post-carotenoid steps in the pathway (Ravanello *et al*. 2003).

Fujisawa *et al*. (2009) introduced seven bacterial carotenogenic genes into canola. One of these genes (*ipi*) is from the MEP pathway, where its role is to increase the amount of the universal carotenoid precursor GGPP. Another four of the genes represented the linear part of the bacterial carotenoid synthesis pathway as shown in Fig. 3 (*crt*E, *crt*B, *crt*I, and *crt*Y), and the others were *crt*W (β-carotene ketolase) and *crt*Z (β-carotene hydroxylase) from the marine bacterium *Brevundimonas* SD212, which enable ketocarotenoid biosynthesis. Transgenic seeds expressing all seven transgenes accumulated up to 214 μg/g fresh weigh β-carotene, a 1,070-fold increase over wild type, and also produced ketocarotenoids rarely seen in the plant kingdom, such as echinenone, canthaxanthin, astaxanthin, and adonixanthin.

In a recent report, Wei *et al*. (2010) expressed the *Arabidopsis thaliana* microRNA gene *AtmiR156b* in canola either constitutively or under the control of the napin promoter. The function of this gene in Arabidopsis is to regulate a family of transcription factors, some of which control the transition from vegetative to reproductive growth. Constitutive expression of *AtmiR156b* in canola increased the amount of β-carotene in the seeds by up to 4.5-fold and also doubled the number of flowering shoots, whereas seed-specific *AtmiR156b* expression had no effect. *AtmiR156b* therefore appears to affect seed quality and shoot branching, and the transgenic lines showed significant variation in seed yield and weight. A single line produced apparently normal seeds with the higher carotenoid levels.

Although cauliflower seems an unusual target for carotenoid engineering, it has made a significant impact on the field through the discovery of the *Or* allele, the result of a spontaneous mutation that causes large quantities of carotenoids to accumulate in the edible curd, turning it orange. Homozygous *Or*/*Or* plants possess small but intensely orange curds, whereas heterozygous plants have less pigmentation but the curd is normal in size and therefore commercially viable (Crisp *et al*. 1975; Dickson *et al*. 1988). Li *et al*. (2001) investigated the metabolic profiles of *Or* mutants and found that the predominant carotenoid in curd tissue was β-carotene, at a concentration hundreds of times higher than in wild-type curds. This unique phenotype suggested that *Or* has a regulatory rather than enzymatic role in carotenogenesis. Investigating the phenomenon, Li *et al*. (2001) found that *Or* induces the assembly of large carotenoid-sequestering organelles by influencing the differentiation of proplastids into orange chromoplasts (Paolillo *et al*. 2004).

Li and Garvin (2003) identified ten amplified fragment length polymorphisms (AFLPs) closely linked to the *Or* locus, and resolved sequence-characterized amplified region (SCAR) markers to facilitate positional cloning of the gene. Lu *et al*. (2006) showed that *Or* encodes a plastid-associated protein containing a DnaJ cysteine-rich domain and that the dominant *Or* mutation is caused by the insertion of a long terminal repeat retrotransposon. The function of *Or* is related to the differentiation of proplastids or other non-colored plastids into chromoplasts. The dominant allele is a hypermorph, which induces abnormally large numbers of proplastids to differentiate into carotenoid-sequestering chromoplasts. The creation of a carotenoid sink may release the carotenoid pathway from feedback inhibition, allowing the production of higher amounts of β-carotene. A carotenoid-rich Chinese cabbage variety has recently been discovered whose orange leaves contain seven times the amount of carotenoids found in white cabbage (Feng *et al*. 2010). The locus has been mapped but, at the time of writing, the gene has not been cloned and its relationship to the cauliflower *Or* gene is unknown.

Pro-vitamin A enhancement in root vegetables. Four major root vegetable crops have been the focus of β-carotene enhancement strategies: carrot, potato, cassava, and sweet potato. Carrot root color is a result of various pigments that serve as intermediate products in the carotenoid pathway. The orange color of most modern cultivated carrots reflects the high levels of α- and β-carotene (Imam and Gabelman 1968), whereas white-colored roots have low levels of carotenoids (Buishand and Gableman 1979). The *rp* allele was the first recessive allele shown to cause pigment loss in carrot roots and is associated with a 96% reduction in carotenoid levels, predominantly α- and β-carotene (Goldman and Breitbach 1996).

QTL studies in carrots have been carried out using an intercross either between cultivated orange and wild-type lines or between specialized medium orange (Brasilia) and dark orange (HCM) lines (Santos and Simon 2002). Major QTLs were found that explain 4.7–8% of the total phenotypic variation in ζ-carotene, α-carotene, and β-carotene levels, and the positive correlation between root color and major carotenoid levels made selection straightforward. A later study involving wild white carrots identified PSY as the major bottleneck in carotenoid synthesis (Santos *et al*. 2005). The most recent study involved crosses between orange cultivated carrots and a wild white line, identifying two major interacting loci (*Y* and *Y2*, on linkage groups 2 and 5, respectively) that appear to control much of the variation in carotenoid levels. Positional candidates associated with these loci include the genes encoding zeaxanthin epoxidase, carotene β-hydroxylase, and carotenoid dioxygenase. The dominant allele of the QTL on linkage group 5 reduces all carotenes (but not xanthophylls), in contrast to the QTL on linkage group 2, of which the dominant allele reduces all carotenoids, both carotenes and xanthophylls (Just *et al*. 2009).

Genetic engineering for carotenoids in carrot roots has been reported by Maass *et al*. (2009). They expressed bacterial *crt*B, resulting in carrot roots with a 93-fold increase in carotenoid content. The increased flux through the early part of the pathway revealed bottlenecks further downstream. The overall balance between xanthophylls and β-carotene shifted in favor of the latter, and a number of carotene intermediates that are normally converted to downstream products were seen to accumulate in the transgenic roots.

The *Y* (*Yellow*) locus in potato controls tuber flesh color by influencing carotenoid accumulation, and there exists an allelic series of increasing dominance, beginning with the fully recessive *y* allele (white flesh, no carotenoids), then the *Y* allele (yellow flesh) and the fully dominant *Or* allele (orange flesh, reflecting the accumulation of zeaxanthin). The *Y* locus has been mapped to a region on chromosome 3 (Bonierbale *et al*. 1988) with two candidate genes, which encode PSY and BCH (Thorup *et al*. 2000), and possibly additional regulatory elements. It is therefore clear that the natural *Or* allele in potato is completely unrelated to the *Or* allele in cauliflower discussed above, and presumably represents an enzyme that has a greater activity than wild type or that is expressed at a higher level than wild type.

Three different approaches have been explored in potato to engineer increased carotenoid levels. The first, as in the other cases discussed above, involves the introduction of carotenogenic transgenes to promote the synthesis of carotenoids in tuber flesh. Transgenic potato plants have been produced expressing bacterial *crt*B, resulting in four- to sevenfold increases in carotenoid levels, including 11- and 19-fold increases in the amounts of β-carotene and lutein, respectively (Ducreux *et al*. 2005). Diretto *et al*. (2007a) introduced the bacterial *crt*B, *crt*I, and *crt*Y genes under the control of tuber-specific and constitutive promoters, increasing total carotenoid levels to 114 μg/g dry weight and β-carotene to 47 μg/g dry weight.

The second approach in potato inhibits competing enzyme activities to direct flux specifically towards the synthesis of β-carotene, preventing further conversion. Diretto *et al.* (2006) silenced the endogenous *lyce* gene, thereby eliminating competition at the branch point between the α- and β-carotene pathways. The total carotenoid content of transgenic tubers reached 9.9 μg/g dry weight (a 2.5-fold increase), while the β-carotene content was 0.043 μg/g dry weight (a 14-fold increase). Even higher levels were achieved by silencing the *bch* gene and thereby

preventing the further metabolism of β-carotene. Transgenic plants contained 14.5 μg/g dry weight total carotenoids (a 2.9-fold increase), including 0.085 μg/g dry weight β-carotene (a 38-fold increase; Diretto *et al.* 2007b). In a separate study, silencing the *bch* gene alone elevated β-carotene levels to 16.6 μg/g dry weight (Van Eck *et al.* 2007). Silencing the endogenous *zep* gene also increased total carotenoid levels to 60.8 μg/g dry weight (a 5.7-fold increase), predominantly represented by higher amounts of zeaxanthin, whereas violaxanthin levels were lower than normal (Romer *et al*. 2002).

The third approach involves the creation of a metabolic sink for carotenoids. This reflects the discovery of the cauliflower *Or* allele, as discussed above (Li *et al*. 2001). Expression of the cauliflower *Or* allele in potatoes under the control of the tuber-specific granule-bound starch synthase promoter resulted in the production of potato plants with orange-yellow tubers, and with carotenoid-sequestering organelles similar to those seen in the original cauliflower mutation, suggesting the regulation of proplastid differentiation is conserved (Lu *et al*. 2006). The total carotenoid levels in the *Or* transgenic lines reached 24 μg/g dry weight, a sixfold increase over wild-type tubers.

Cassava is a particularly important root crop in terms of carotenoid enhancement, because it is the preferred staple crop in some parts of Africa, but like cereals, it is generally a poor source of carotenoids. Cultivars with carotene-rich yellow roots are rare and most breeding populations have white roots (Ferreira *et al*. 2008; Nassar *et al*. 2009). Welsch *et al*. (2010) recently characterized the *psy2* locus in cassava and identified a polymorphism that increased carotenoid accumulation in cassava roots and also increased the rate of carotenoid synthesis when expressed in bacteria and yeast. The authors proposed that PSY is likely to be a significant bottleneck in cassava carotenoid synthesis and therefore introduced the bacterial *crt*B gene into cassava plants under the control of the *CP1* promoter. The resulting transgenic lines had deep orange roots containing up to 21.84 μg/g dry weight of β-carotene, compared to 6.67 μg/g in wild-type roots.

Sweet potatoes with yellow/orange flesh are rich in β-carotene, but in most developing countries the preferred sweet potato cultivars have cream or white flesh and a low carotenoid content. Wang *et al*. (2007) reported a mutant variety of sweet potato with orange flesh that contained up to 19.5 μg/g fresh weight of carotenoids, which is seven times more than wild-type plants. Genetic analysis has revealed eight QTLs affecting β-carotene content, three with very significant positive effects on the levels of β-carotene (Cervantes-Flores 2006).

Pro-vitamin A enhancement in fruits. Significant variation in β-carotene levels has been observed in mango, banana, pumpkin, melon, kiwifruit, and tomato. Because β-carotene accounts for more than half of the total carotenoid content in most mango cultivars, the fruit is a major source of pro-vitamin A in tropical and subtropical areas (Vasquez-Caicedo *et al*. 2005). The total β-carotene levels in nine Thai mango cultivars ranged from 8.92 to 112.49 μg/g dry mesocarp weight (Vasquez-Caicedo *et al*. 2005).

Bananas are also an important source of pro-vitamin A, and a number of yellow- and orange-flesh varieties are available that contain much higher levels of β-carotene than typical cream-flesh cultivars such as Williams (Englberger *et al*. 2006). Ten cultivars with yellow or yellow-orange flesh were compared to the commercially popular Williams variety, and the highest levels of β-carotene were found in Asupina, which contained 14.12 μg/g dry weight, 20 times more than Williams.

Murkovic *et al*. (2002) investigated the carotenoid composition of 22 different varieties of pumpkin from three species (*Cucurbita pepo*, *Cucurbita maxima,* and *Cucurbita moschata*, as well as a *C. maxima* × *C. moschata* hybrid) that are used mainly for food in Austria. The β-carotene content varied from 0.6 μg/g dry weight (variety Carneval di Venexia) to 74 μg/g (variety Imperial Elite) and the α-carotene content varied from undetectable (variety Gelber Zentner) to 75 μg/g (variety Flat White Boer). In most cases, β-carotene was the most abundant carotenoid. Similarly, Azevedo-Meleiro and Rodriguez-Amaya (2007) analyzed carotenoid profiles in five varieties of the same three pumpkin species currently marketed in Brazil, finding that *C. moschata* variety Menina Brasileira contained high levels of β-carotene (66.7 μg/g) and moderate levels of α-carotene (26.8 μg/g), whereas variety Goianinha had slightly lower levels of both molecules (56.7 and 23.8 μg/g, respectively).

Melons can also show significant variations in carotenoid levels, with β-carotene ranging from 9 to 25 μg/g fresh weight in mesocarp tissue, depending on the flesh color. Commercial melon varieties with orange flesh were shown to contain 9–18 μg/g fresh weight β-carotene (Navazio 1994; Cuevas *et al*. 2009) whereas the amount of β-carotene in mature fruits of recombinant inbred lines (RILs) ranged from 8.1 to 22.3 μg/g (California RIL) and 3.7 to 24.4 μg/g (Wisconsin RIL; Cuevas *et al*. 2008). These RILs were used to identify QTLs affecting β-carotene levels, and eight loci were identified, each explaining between 8% and 31% of the phenotypic variation. One QTL mapped to the same locus as a known melon gene encoding β-carotene hydroxylase (Cuevas *et al*. 2008). Six QTLs accounted for a significant amount of the observed variation in β-carotene levels, and are potentially useful for MAS (Cuevas *et al*. 2008). The nutritional value and yield of the US Western Shipping melon (USWS; *Cucumis melon* L.) could potentially be

improved by introgressing genes for early fruit maturation and mesocarp β-carotene content (Cuevas *et al*. 2009). Therefore, a set of 116 F3 families derived from the monoecious, early-maturing Chinese line Q 3-2-2 (no β-carotene, white mesocarp) and the andromonoecious, late-maturing USWS line Top Mark (β-carotene-rich, orange mesocarp) were examined for 2 yr in Wisconsin to identify QTLs associated with fruit maturation and β-carotene content. Three QTLs associated with β-carotene content were identified, accounting for much of the observed phenotypic variation. The locus of one QTL was uniformly aligned with a carotenoid-related gene (*Orange*), suggesting a relationship with the *white-flesh* (*wf*) gene and an important role in the control of β-carotene levels (Cuevas *et al*. 2009).

Kiwifruit also shows a significant variation in flesh color associated with carotenoid content. The common commercial varieties have green flesh (*Actinidia deliciosa*) or yellow flesh (*Actinidia chinesis*) and only moderate carotenoid levels, but several non-commercial varieties that accumulate high levels of anthocyanins and carotenoids have also been investigated (Ampomah-Dwamena *et al*. 2009). The major carotenoids in kiwifruit are β-carotene and lutein. Moderate levels of β-carotene were detected 150 d after full bloom in *A. chinesis* MP161 fruit (1.12 μg/g fresh weight), but much higher levels were found in three individual fruits from a hybrid *Actinidia macrosperma* × *Actinidia melanandra* population (7.07, 3.45, and 6.11 μg/g fresh weight, respectively) representing 43%, 39%, and 70% of total carotenoids, respectively. Recently, Kim *et al*. (2010) established an efficient genetic transformation procedure for kiwifruit using micro-cross sections of stems from the *A. deliciosa* variety Hayward. Transgenic leaves constitutively expressing mandarin (*Citrus unshiu*) GGPPS or PSY accumulated up to 1.3-fold the normal amounts of lutein or β-carotene (Kim *et al*. 2010). Although these represent only marginal improvements, this initial study paves the way for additional metabolic engineering studies to modulate β-carotene levels specifically in fruits.

Citrus fruits vary greatly in carotenoid content, depending on species, variety, and growing conditions (Gross 1987). Mandarin varieties such as Satsuma (*C. unshiu* Marc.) accumulate β-cryptoxanthin predominantly in the flavedo and juice sacs of mature fruit (Goodner *et al*. 2001; Ikoma *et al*. 2001), whereas mature sweet orange (*Citrus sinensis* Osbeck) accumulates violaxanthin isomers, principally 9-cis-violaxanthin (Molnar and Szabolcs 1980; Lee and Castle 2001). The most abundant carotenoids present in the peel and pulp of citrus fruits are β-xanthophylls, which do not have pro-vitamin A activity (Kato *et al*. 2004). Metabolic engineering in citrus fruits has therefore focused on increasing the β-carotene content, *e.g.*, by expressing the *PSY* gene from sweet orange (*C. sinensis* Osbeck) in Hong Kong kumquat (*Fortunella hindsii* Swingle). The transgenic fruits were found to contain up to 171.9 μg/g fresh weight of total carotenoids, twice the normal level (Zhang *et al*. 2009). The transgenic fruits contained twice the concentration of β-carotene and β-cryptoxanthin and three times the normal amounts of phytoene and lycopene.

Whereas moderate progress has been made in the fruit crops discussed above, much more extensive work has been done to engineer carotenoid levels in tomato (*Solanum lycopersicum*). Ripe tomato fruits contain up to 207 μg/g fresh weight total carotenoids, including large amounts of lycopene (70.5 μg/g), but lower amounts of β-carotene (36.8 μg/g) and lutein (6.4 μg/g) (Fraser *et al*. 1994). The *Beta* mutant, which has higher LYCB activity than wild-type fruits, accumulates 186 μg/g fresh weight of total carotenoids, slightly lower than the wild-type levels, but this includes 132 μg/g β-carotene and 38 μg/g lycopene, resulting in a characteristic orange fruit color (Rosati *et al*. 2000). Various transgenic strategies have been implemented to induce similar effects (Table 1). For example, the constitutive expression of bacterial *crt*I nearly doubled the amount of β-carotene but reduced overall carotenoid levels (Romer *et al*. 2000). Further investigation showed that endogenous lycopene β-cyclases were upregulated in the transgenic fruits, thus diverting flux towards β-carotene rather than lycopene, as had been predicted (Romer *et al*. 2000). A sevenfold increase in β-carotene was achieved expressing the native *lycb* gene using the fruit-specific tomato *pds* promoter (Rosati *et al*. 2000). The expression of bacterial *crt*B increased total carotenoid levels up to twofold, including a 2.5-fold increase in β-carotene levels (Fraser *et al*. 2002). Fruits of the Moneymaker variety expressing Arabidopsis *lycb* and pepper *bch* had 12 times the normal levels of β-carotene seen in the control lines (Dharmapuri *et al*. 2002). One of the most significant achievements is the HighCaro (HC) tomato variety, which expresses tomato *lycb* cDNA driven by the CaMV 35S promoter and produces orange fruits as a result of the complete conversion of lycopene to β-carotene (D'Ambrosio *et al*. 2004). The β-carotene content of HC tomato fruits is 205 μg/g fresh weight, 1.6-fold more than the *Beta* mutant described above. HC tomato has been evaluated extensively in greenhouse and field trials and has maintained its ability to convert lycopene to β-carotene into the T6 generation without negative effects on agronomic performance (Giorio *et al*. 2007). Transgenic fruits expressing plastid-targeted bacterial DXS produced 1.6-fold higher levels of carotenoids than wild-type fruits, including a 1.4-fold increase in β-carotene (Enfissi *et al*. 2005).

Like potato, tomato has also been engineered using the RNAi-mediated silencing approach. Silencing of the endogenous photomorphogenesis regulator gene *DE-ETIOLATED1* (*DET1*) resulted in fruits with 8.5-fold higher

Table 1. Total carotenoid and β-carotene levels in wild-type and transgenic plants

Species	Genes (origin)	Total carotenoid levels in wild type[z]	Total carotenoid levels (increase relative to wild type) in transgenic plants	β-Carotene levels in wild type[z]	β-Carotene levels (increase relative to wild type) in transgenic plants	References
Maize	*crt*B and *crt*I (*P. ananatis*)	0.99 μg/g DW	33.6 μg/g DW (34)	0.98 μg/g DW	9.8 μg/g DW (10)	Aluru *et al.* 2008
	psy1 (*Zea mays*; maize)	1.10 μg/g DW	146.7 μg/g DW (133)	0.14 μg/g DW	57.35 μg/g DW (410)	Zhu *et al.* 2008
	*crt*I (*Pantoea. ananatis*)					
	*crt*W (*Paracoccus* spp.)					
	lycb (*Gentiana lutea*)					
	psy1 (maize)	1.45 μg/g DW	163.2 μg/g DW (112)	0.35 μg/g DW	59.32 μg/g DW (169)	Naqvi *et al.* 2009
	*crt*I (*P. ananatis*)					
Wheat	*psy1* (maize)	0.46 μg/g DW	4.96 μg/g DW (10.8)	ND	ND	Cong *et al.* 2009
	*crt*I (*P. ananatis*)					
Potato	*ZEP* (*Arabidopsis thaliana:* Arabidopsis)	10.6 μg/g DW	60.8 μg/g DW (5.7)	0.7 μg/g DW	2.4 μg/g DW (3.4)	Romer *et al.* 2002
	*crt*B (*P. ananatis*)	5.6 μg/g DW	35.5 μg/g DW (6.3)	ND	10.3 μg/g DW	Ducreux *et al.* 2005
	Or (*Brassica oleracea*; cauliflower)	5.41 μg/g DW	28.22 μg/g DW (6)	ND	5.01 μg/g DW	Lopez *et al.* 2008
	Antisense *lyce* (*Solanum tuberosum*; potato)	4.6 μg/g DW	9.974 μg/g DW (2.5)	0.00317 μg/g DW	0.043 μg/g DW (14)	Diretto *et al.* 2006
	crtB, crtI and *crtY* (*P. ananatis*)	5.8 μg/g DW	114.4 μg/g DW (20)	0.013 μg/g DW	47.4 μg/g DW (3600)	Diretto *et al.* 2007a
	Antisense *bch* (potato)	4.88 μg/g DW	14.264 μg/g DW (2.9)	0.00225 μg/g DW	0.085 μg/g DW (38)	Diretto *et al.* 2007b
	Antisense *bch* (potato)	22.48 μg/g DW (75% water content)	23.52 μg/g DW (1.04) (75% water content)	0.04 μg/g DW (75% water content)	13.24 μg/g DW (331) (75% water content)	Van Eck *et al.* 2007
Cassava	*crt*B (*P. ananatis*)	0.65 μg/g DW	21.84 μg/g DW (33.6)	0.41 μg/g DW	6.67 μg/g DW (16)	Welsch *et al.* 2010
Carrot	*psy* (Arabidopsis)	5.5 μg/g DW	514.1 μg/g DW (93)	1.265 μg/g DW	214.627 μg/g DW (178)	Maass *et al.* 2009
Canola	*crt*B (*P. ananatis*)	36 μg/g FW	1055 μg/g FW (29.3)	5 μg/g FW	401 μg/g FW (80.2)	Shewmaker *et al.* 1999
	*crt*B (*P. ananatis*)	ND	1341 μg/g FW	ND	739 μg/g FW	Ravanello *et al.* 2003
	*crt*E and *crt*B (*P. ananatis*)		1023 μg/g FW		488 μg/g FW	
	*crt*B (*P. ananatis*) *crt*I (*P. ananatis*)		1412 μg/g FW		857 μg/g FW	
	*crt*B and *crt*Y (*P. ananatis*)		935 μg/g FW		459 μg/g FW	
	*crt*B (*P. ananatis*) and *lycb* (*Brassica napus;* canola)		985 μg/g FW		488 μg/g FW	
	*crt*B, *crt*I and *crt*Y (*P. ananatis*)		1229 μg/g FW		846 μg/g FW	
	lycopene ε-cyclase (Arabidopsis)	5.34 μg/g FW	227.78 μg/g FW (42.5)	0.49 μg/g FW	90.76 μg/g FW (185.2)	Yu *et al.* 2007
	RNAi to 5′ end					
	lycopene ε-cyclase (Arabidopsis)		94.09 μg/g FW (17.6)		27.02 μg/g FW (55)	
	RNAi to 3' end					
	*idi, crt*E, *crt*B, *crt*I and *crt*Y (*P. ananatis*)	21.7 μg/g FW	656.7 μg/g FW (30)	0.2 μg/g FW	214.2 μg/g FW (1070)	Fujisawa *et al.* 2009
	*crt*Z, *crt*W (*Brevundimonas* sp.)					
	microRNA miR156b (Arabidopsis)	3 μg/g FW (10% water content)	6.9 μg/g FW (2.45) (10% water content)	0.08 μg/g FW (10% water content)	0.38 μg/g FW (4.5) (10% water content)	Wei *et al.* 2010
Tomato[y]	*crt*I (*P. ananatis*)	285 μg/g FW	137.2 μg/g FW (0.5)	27.1 μg/g FW	52 μg/g FW (1.9)	Romer *et al.* 2000

Table 1. (continued)

Species	Genes (origin)	Total carotenoid levels in wild type[z]	Total carotenoid levels (increase relative to wild type) in transgenic plants	β-Carotene levels in wild type[z]	β-Carotene levels (increase relative to wild type) in transgenic plants	References
	lycb (*Solanum lycopersicum*; tomato)	66 μg/g FW	109 μg/g FW (1.7)	7 μg/g FW	57 μg/g FW (7.1)	Rosati *et al.* 2000
	lycb (Arabidopsis)	66.3 μg/g FW	100.7 μg/g FW (1.5)	5 μg/g FW	63 μg/g FW (12)	Dharmapuri *et al*. 2002
	bch (pepper; *Capsicum annuum*)					
	crtB (*P. ananatis*)	285.7 μg/g FW	591.8 μg/g FW (2.1)	33 μg/g FW	82.5 μg/g FW (2.5)	Fraser *et al.* 2002
	lycb (tomato)	94.5 μg/g FW	215.2 μg/g FW (2.3)	4.4 μg/g FW	205.0 μg/g FW (46.6)	D′Ambrosio *et al*. 2004
	dxs (*Escherichia coli*)	460 μg/g FW	720 μg/g FW (1.6)	50 μg/g FW	70.0 μg/g FW (1.4)	Enfissi *et al.* 2005
	Antisense *DET-1* (tomato)	36.4 μg/g FW	83.8 μg/g FW (2.3)	1.63 μg/g FW	13 μμg/g FW (8)	Davuluri *et al*. 2005
	CRY2 (tomato)	87.6 μg/g FW in ripe fruit pericarps	149 μg/g FW in ripe fruit pericarps (1.7)	7.8 μg/g FW in ripe fruit pericarps	10.1 μg/g FW in ripe fruit pericarps (1.3)	Giliberto *et al*. 2005
	psy1 (tomato)	181.20 μg/g FW	227.67 μg/g FW (1.25)	58.62 μg/g FW	81.93 μg/g FW (1.4)	Fraser *et al.* 2007
	fibrillin (pepper)	325 μg/g FW	650 μg/g FW (2.0)	90 μg/g FW	150 μg/g FW (1.6)	Simkin *et al.* 2007
	*crt*Y (*Erwinia herbicola*)	372.66 μg/g FW	323.71 μg/g FW (0.9)	6.91 μg/g FW	28.61 μg/g FW (4)	Wurbs *et al.* 2007
	lycb (*Narcissus pseudonarcissus*; daffodil)	76.67 μg/g FW	115 μg/g FW (1.5)	19 μg/g FW	95 μg/g FW (5)	Apel and Bock 2009
Kumquat	*psy* (*Citrus sinensis*; orange)	84.3 μg/g FW	131.9 μg/g FW (1.6)	0.70 μg/g FW	1.72 μg/g FW (2.5)	Zhang *et al.* 2009

DW dry weight, *FW* fresh weight, *ND* not determined

[z] Different wild-type carotenoid levels cited for each species reflect the different varieties used in each investigation

[y] We converted dry weight to fresh weight assuming the water content of tomato fruit is 90%

β-carotene levels than wild-type plants (Davuluri *et al.* 2005). The overexpression of tomato *CRY2*, a blue-light photoreceptor, increased carotenoid levels 1.7-fold, including a 1.3-fold increase in β-carotene (Giliberto *et al*. 2005). The overexpression of pepper fibrillin, which plays a role in the formation of lipoprotein carotenoid-storage structures, resulted in a 95% increase in total carotenoids, including a 64% increase in β-carotene and a 118% increase in lycopene (Simkin *et al*. 2007). Chloroplast transformation has also been used to increase carotenoid levels in tomato (Wurbs *et al*. 2007; Apel and Bock 2009). Bacterial *crt*Y driven by the *atp*I promoter was introduced into tomato plastids by particle bombardment and resulted in the conversion of lycopene to β-carotene, increasing the amount of β-carotene fourfold in transplastomic fruits, to 28.6 μg/g fresh weight (Wurbs *et al*. 2007). However, the expression of daffodil *lycb* in plastids under the control of the rRNA operon promoter increased β-carotene levels to 95 μg/g fresh weight in tomato fruits, together with a >50% increase in total carotenoids, suggesting that LYCB is normally a rate limiting step (Apel and Bock 2009).

Benchmarks for Nutritional Improvement

Nutritional improvement via conventional breeding and/or genetic engineering is one of the major objectives of applied plant biology, and the ultimate objective in such experiments is to provide an adequate source of nutrients for human consumption (Farre *et al*. 2010b). It is therefore frustrating to find that many articles reporting enhanced levels of various essential nutrients do so without addressing two major benchmarks: (1) how much of the nutrient is available when ingested, and (2) how the quantity of bioavailable nutrient in a reasonable portion of food compares to the DRI. In the context of VAD, we need to know how bioavailable are the additional carotenoids accumulating in these enhanced crops and how much food must be consumed to provide the DRI of vitamin A. Whereas animal sources of vitamin A are easily accessible, a wide range of factors has been identified that restricts the availability of pro-vitamin A carotenoids in plants. These are sometimes identified using the acronym SLAMENGHI, representing species of carotenoid, molecular linkage,

amount in the meal, food matrix properties, the presence of effectors, nutrient status, genotype, host specificity, and interactions between factors (Tanumihardjo 2002). Therefore, while average values provide a useful starting point, the actual bioavailability is likely to differ between crops and will need to be evaluated on a case by case basis.

The food matrix plays an important role in determining the bioavailability of β-carotene. The standard conversion factor averages at 1 RAE=12 μg of dietary β-carotene, but this varies by plant source, *e.g.*, 12:1 for fruit (de Pee *et al.* 1998; Khan *et al.* 2007), 13:1 for sweet potato (Haskell *et al.* 2004), 15:1 for carrots (Tang *et al.* 2005), and 10–28:1 for green vegetables (de Pee *et al.* 1998; Tang *et al.* 1999, 2005; Haskell *et al.* 2004; Khan *et al.* 2007). Tang *et al.* (2009) recently reported the potential nutritional effect of β-carotene in Golden Rice, indicating a conversion factor of 3.8:1 (Tang *et al.* 2009). Li *et al.* (2010b) determined that on average, 6.48 μg of the β-carotene in β-carotene-biofortified maize porridge and 2.34 μg of the β-carotene in the reference dose (a micro-crystalline suspension of β-carotene in corn oil) were each equivalent to 1 μg retinol (Li *et al.* 2010b).

Cooking can increase the extractability and bioavailability of carotenoids (Dietz *et al.* 1988; Hart and Scott 1995), which suggests that products that are often consumed raw (*e.g.*, carrots, tomatoes, and other fruits) may be less-suitable sources of pro-vitamin A than cereals, potatoes, and canola oil, which are generally cooked before consumption. Howard *et al.* (1999) reported that microwave cooking increases tissue degradation and increases the amount of β-carotene available for extraction. This may reflect the fact that carotenoids in plants are sequestered into protein complexes and that cooking helps to release them (Anderson *et al.* 1978; Braumann *et al.* 1982; Grimme and Brown 1984).

We have compared the best crops bred by conventional means and the best enhanced transgenic lines to estimate, in each case, how much of the product must be consumed to achieve the DRI of vitamin A for male adults: 900 RAE as recommended by the US Institute of Medicine (IOM 2001). Some key comparisons are presented below. The most striking examples are those in which conventional varieties contain so little β-carotene that the DRI is impossible to achieve without consuming unrealistic amounts of food (and excess calories), as is the case for many staple crops, whereas the enhanced varieties obtained by breeding or genetic engineering provide enough β-carotene to provide the DRI in a small portion. In this context, nearly 428 g of the best conventional maize kernels (13.6 μg/g dry weight; Harjes *et al.* 2008) need to be consumed per d to meet the DRI for vitamin A, but this falls to 99 g/d for the carotene-rich transgenic maize generated by Naqvi *et al.* (2009) (Table 1) if we assume 6.48 μg of the β-carotene-fortified maize porridge was equivalent to 1 μg retinol (Li *et al.* 2010b). Although a 428-g portion is not excessive, the variety described by Harjes *et al.* (2008) is not widely available. The white maize varieties preferred for human consumption in Africa contain so little β-carotene that nearly 2.6 kg is required to meet the DRI. The benefit of the genetic engineering approach is that carotenoid levels can be increased specifically in the white maize inbred lines that many in sub-Saharan Africa rely on as a staple. Similarly, it would be necessary to consume nearly 15 kg/d of the best conventional potato variety (0.7 μg/g dry weight; Romer *et al.* 2002) but only 230 g/d of the golden potatoes described by Direttto *et al.* (2007a), assuming a conversion factor of 12:1 (IOM 2001). It would be necessary to consume 292 g of normal commercial tomato fruits, 82 g of the β-carotene-rich *Beta* mutant (Rosati *et al.* 2000), but only 53 g of HC tomato (D'Ambrosio *et al.* 2004) assuming a conversion factor of 12:1 (de Pee *et al.* 1998; Khan *et al.* 2007). Interestingly, the DRI for vitamin A could be achieved by ingesting 12.6 g/d of oil from the enhanced canola varieties described by Ravanello *et al.* (2003), which suggests that a small amount of canola oil used in cooking would be sufficient, in contrast to the 1.35 kg of the best conventional canola oil (Li *et al.* 2001) that would be required to have the same effect assuming a conversion factor of 12:1 (IOM 2001).

Outlook and Future Research Directions

Vitamin A is an essential nutrient, which is generally provided by retinyl esters in meat and dairy products and pro-vitamin A carotenoids in plants. Although many crops are rich sources of pro-vitamin A, millions of people rely on staple diets of crops that have minimal levels of this nutrient (*e.g.*, rice, maize, wheat, potato, cassava), so there has been much interest in strategies to enhance pro-vitamin A carotenoid levels in such crops by conventional breeding and genetic engineering. Because of the limitations inherent in conventional breeding, genetic engineering is the only way to introduce enhanced nutritional traits directly into local elite cultivars without long-term breeding programs.

The carotenoid biosynthesis pathway is extremely complex, characterized by multiple branches, competition for intermediates, bottlenecks, and feedback loops that conspire to limit the synthesis of desirable molecules. Attempts to overcome these roadblocks in plants by breaking through them or going around them have met with varied success (Zhu *et al.* 2007). Although genetic engineering of pro-vitamin A accumulation has successfully increased pro-vitamin A content in crop plants as discussed above, knowledge of the underlying mechanisms controlling accumulation of pro-vitamin A in plants is relatively limited, and the subject is currently an area of active research. Regulation of the catalytic activity of

carotenoid biosynthesis is generally recognized to be important in controlling the final levels of carotenoid accumulation. The upstream pathways supplying pro-vitamin A precursors may also influence carotenoid accumulation (Rodriguez-Concepción 2010), and down-stream pathways that metabolize pro-vitamin A to non-pro-vitamin A carotenoids deplete the pro-vitamin A carotenoid pool (Diretto *et al*. 2006, 2007b; Yu *et al*. 2007; Harjes *et al*. 2008; Yan *et al*. 2010). The characterization of *Orange* (*Or*), a cauliflower mutation that causes proplastids and/or non-colored plastids to differentiate into chromoplasts, demonstrated that the creation of a metabolic sink to sequester carotenoids can also be used to promote pro-vitamin A carotenoid accumulation in plants (Lu *et al*. 2006; Lopez *et al*. 2008). Thus, the final content of pro-vitamin A carotenoids in plants is the net result of biosynthesis, degradation, and stable storage of synthesized products (Lu and Li 2008; Cazzonelli and Pogson 2010).

Pioneering work in rice to develop nutritionally enhanced varieties has been followed up in other staple crops, and these crops should be the focus of future efforts to increase the levels of bioavailable carotenoids in food. However, the practical impact of carotenoid enhancement should be emphasized, specifically by focusing on the amount of food that needs to be consumed in order to achieve the DRI of vitamin A in different target groups. In maize, the combinatorial transformation platform (Zhu *et al*. 2008; Naqvi *et al*. 2010) has provided a rapid way to assess metabolic variants, and the development of transgenic lines simultaneously enhanced for multiple vitamins (Naqvi *et al*. 2009) has provided the impetus to address nutrient deficiency as a holistic concept rather than a series of individual challenges. In the future, it should be possible to develop crops that address multiple nutrient deficiencies simultaneously, while ensuring that nutritional completeness can be achieved with a reasonable portion of food. Although dietary diversification should eventually become the normal way to achieve adequate nutrition, the provision of staple crops enhanced for essential nutrients should, in the short term, provide the most straightforward way to address micronutrient deficiencies in the developing world.

Acknowledgments Research in our laboratory is supported by the Ministry of Science and Innovation (MICINN) Grant BFU2007-61413; European Research Council Advanced Grant BIOFORCE; Center Consolider, MICINN, Spain; COST Action FA0804, Associated Unit CAVA; and the SmartCell, FP7 Integrated Project.

References

Aluru M.; Xu Y.; Guo R.; Wang Z.; Li S.; White W.; Wang K.; Rodermel S. Generation of transgenic maize with enhanced provitamin A content. *J. Exp. Bot.* 59: 3551–3562; 2008.

Alvarez J. B.; Martin L. M.; Martin A. Genetic variation for carotenoid pigment content in the amphiploid *Hordeum chilense*×*Triticum turgidum* conv. durum. *Plant Breed.* 118: 187–189; 1999.

Ampomah-Dwamena C.; McGhie T.; Wibisono R.; Montefiori M.; Hellens R. P.; Allan A. C. The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J. Exp. Bot.* 60: 3765–3779; 2009.

Anderson J. M.; Waldron J. C.; Thorne S. W. Chlorophyll-protein complexes of spinach and barley thylakoids. *FEBS Lett.* 92: 227–233; 1978.

Apel W.; Bock R. Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. *Plant Physiol.* 151: 59–66; 2009.

Azevedo-Meleiro C. H.; Rodriguez-Amaya D. B. Qualitative and quantitative differences in carotenoid composition among *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo*. *J. Agric. Food Chem.* 55: 4027–4033; 2007.

Beyer P. Golden Rice and 'Golden' crops for human nutrition. *New Biotechnol.* 27: 478–481; 2010.

Bonierbale M. W.; Plaisted R. L.; Tanksley S. D. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics* 120: 1095–1103; 1988.

Braumann T.; Weber G.; Grimme L. H. Carotenoid and chlorophyll composition of light harvesting and reaction centre proteins of the thylakoid membrane. *Photobiochem Photobiophys* 4: 1–8; 1982.

Breitenbach J.; Sandmann G. ζ-Carotene *cis* isomers as products and substrates in the plant poly-*cis* carotenoid biosynthetic pathway to lycopene. *Planta* 220: 785–793; 2005.

Buishand J. G.; Gableman W. H. Investigations of the inheritance of color and carotenoid content in phloem and xylem of carrot roots (*Daucus carota* L.). *Euphytica* 28: 611–632; 1979.

Capell T.; Christou P. Progress in plant metabolic engineering. *Curr. Opin. Biotechnol.* 15: 148–154; 2004.

Cazzonelli C. I.; Pogson B. J. Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci.* 15: 266–274; 2010.

Cervantes-Flores J. C. Development of a genetic linkage map and QTL analysis in sweet potato. *Mol. Breed.* 21: 511–532; 2006.

Chander S.; Guo Y. Q.; Yang X. H.; Zhang J.; Lu X. Q.; Yan J. B.; Song T. M.; Rocheford T. R.; Li J. S. Using molecular markers to identify two major loci controlling carotenoid contents in maize grain. *Theor. Appl. Genet.* 116: 223–233; 2008.

Chappell J. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. *Ann Rev Plant Physiol Plant Mol Biol* 46: 521–547; 1995.

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

Clarke F. R.; Clarke J. M.; McCaig T. N.; Knox R. E.; DePauw R. M. Inheritance of yellow pigment concentration in seven durum wheat crosses. *Canad J Plant Sci* 86: 133–141; 2006.

Comai L.; Henikoff S. TILLING: practical single-nucleotide mutation discovery. *Plant J.* 45: 684–694; 2006.

Cong L.; Wang C.; Chen L.; Liu H.; Yang G.; He G. Expression of phytoene synthase1 and carotene desaturase *crt*I genes result in an increase in the total carotenoids content in transgenic elite wheat (*Triticum aestivum* L.). *J. Agric. Food Chem.* 57: 8652–8660; 2009.

Crisp P.; Walkey D. G. A.; Bellman E.; Roberts E. A mutation affecting curd colour in cauliflower (*Brassica oleracea* L. var. *botrytis* DC). *Euphytica* 24: 173–176; 1975.

Cuevas H. E.; Staub J. E.; Simon P. W.; Zalapa J. E.; McCreight J. D. Mapping of genetic loci that regulate quantity of beta-carotene in fruit of US Western Shipping melon (*Cucumis melo* L.). *Theor. Appl. Genet.* 117: 1345–1359; 2008.

Cuevas H. E.; Staub J. E.; Simon P. W.; Zalapa J. E. A consensus linkage map identifies genomic regions controlling fruit maturity and beta-carotene-associated flesh color in melon (*Cucumis melon* L.). *Theor. Appl. Genet.* 119: 741–756; 2009.

Cunningham Jr. F. X.; Pogson B.; Sun Z.; McDonald K. A.; DellaPenna D.; Gantt E. Functional analysis of the β and ε lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* 8: 1613–1626; 1996.

D'Ambrosio C.; Giorio G.; Marino I.; Merendino A.; Petrozza A.; Salfi L.; Stigliani A. L.; Cellini F. Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato *lycopene β-cyclase* (*tlcy-b*) cDNA. *Plant Sci.* 166: 207–214; 2004.

Darnton-Hill I.; Nalubola R. Fortification strategies to meet micronutrient needs: successes and failures. *Proc. Nutr. Soc.* 61: 231–241; 2002.

Davuluri G. R.; Van Tuinen A.; Fraser P. D.; Manfredonia A.; Newman R.; Burgess D.; Brummell D. A.; King S. R.; Palys J.; Uhlig J.; Bramley P. M.; Pennings H. M. J.; Bowler C. Fruit-specific RNAi-mediated suppression of *DET1* enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* 23: 890–895; 2005.

de Pee S.; West C. E.; Permaesih D.; Martuti S.; Muhilal; Hautvast J. G. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and beta-carotene in schoolchildren in Indonesia. *Am. J. Clin. Nutr.* 68: 1058–1067; 1998.

Dharmapuri S.; Rosati C.; Pallara P.; Aquilani R.; Bouvier F.; Camara B.; Giuliano G. Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Lett.* 519: 30–34; 2002.

Dickson M. H.; Lee C. Y.; Blamble A. E. Orange-curd high carotene cauliflower inbreds. *HortScience* 23: 778–779; 1988.

Dietz J. M.; Sri K. S.; Erdman Jr. J. W. Reversed phase HPLC analysis of alpha- and beta-carotene from selected raw and cooked vegetables. *Plant Foods Hum. Nutr.* 38: 333–341; 1988.

Diretto G.; Al-Babili S.; Tavazza R.; Papacchioli V.; Beyer P.; Giuliano G. Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE* 2: e350; 2007a.

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

Diretto G.; Welsch R.; Tavazza R.; Mourgues F.; Pizzichini D.; Beyer P.; Giuliano G. Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. *BMC Plant Biol.* 7: 11; 2007b.

Ducreux L. J. M.; Morris W. L.; Hedley P. E.; Shepherd T.; Davies H. V.; Millam S.; Taylor M. A. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *J. Exp. Bot.* 56: 81–89; 2005.

Dwivedi S. L.; Crouch J. H.; Mackill D. J.; Xu Y.; Blair M. W.; Ragot M.; Upadhyaya H. D.; Ortiz R. The molecularization of public sector crop breeding: progress, problems, and prospects. *Adv. Agron.* 3: 163–319; 2007.

Enfissi E. M.; Fraser P. D.; Lois L. M.; Boronat A.; Schuch W.; Bramley P. M. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol. J.* 3: 17–27; 2005.

Englberger L.; Wills R. B.; Blades B.; Dufficy L.; Daniells J. W.; Coyne T. Carotenoid content and flesh color of selected banana cultivars growing in Australia. *Food Nutr. Bull.* 27: 281–291; 2006.

Farre G.; Sanahuja G.; Naqvi S.; Bai C.; Capell T.; Zhu C.; Christou P. Travel advice on the road to carotenoids in plants. *Plant Sci.* 179: 28–48; 2010a.

Farre G.; Twyman R. M.; Zhu C.; Capell T.; Christou P. Nutritionally enhanced crops and food security: Scientific achievements versus political expediency. *Curr. Opin. Biotechnol.* 22: 1–7; 2010b.

Feng H.; Li Y.; Liu Z.; Liu J. Mapping of or, a gene conferring orange color on the inner leaf of the Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). *Mol Breed*; 2010. doi:10.1007/s11032-010-9542-x.

Ferreira C. F.; Alves E.; Pestana K. N.; Junghans D. T.; Kobayashi A. K.; de Jesus Santos V.; Silva R. P.; Silva P. H.; Soares E.; Fukuda W. Molecular characterization of cassava (*Manihot esculenta Crantz*) with yellow-orange roots for beta-carotene improvement. *Crop Breed Appl Biotechnol* 8: 23–29; 2008.

Fraser P. D.; Enfissi E. M. A.; Halket J. M.; Truesdale M. R.; Yu D.; Gerrish C.; Bramley P. M. Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* 19: 3194–3211; 2007.

Fraser P. D.; Romer S.; Shipton C. A.; Mills P. B.; Kiano J. W.; Misawa N.; Drake R. G.; Schuch W.; Bramley P. M. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proc. Natl Acad. Sci. U.S.A.* 99: 1092–1097; 2002.

Fraser P. D.; Truesdale M. R.; Bird C. R.; Schuch W.; Bramley P. M. Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiol.* 105: 405–413; 1994.

Fujisawa M.; Takita E.; Harada H.; Sakurai N.; Suzuki H.; Ohyama K.; Shibata D.; Misawa N. Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *J. Exp. Bot.* 60: 1319–1332; 2009.

Giliberto L.; Perrotta G.; Pallara P.; Weller J. L.; Fraser P. D.; Bramley P. M.; Fiore A.; Tavazza M.; Giuliano G. Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.* 137: 199–208; 2005.

Giorio G.; Stigliani A. L.; D'Ambrosio C. Agronomic performance and transcriptional analysis of carotenoid biosynthesis in fruits of transgenic HighCaro and control tomato lines under field conditions. *Transgenic Res.* 16: 15–28; 2007.

Goldman I. L.; Breitbach D. N. Inheritance of a recessive character controlling reduced carotenoid pigmentation in carrot (*Daucus carota* L.). *J. Hered.* 87: 380–382; 1996.

Goodner K. L.; Rouseff R. L.; Hofsommer H. J. Orange, mandarin, and hybrid classification using multivariate statistics based on carotenoid profiles. *J. Agric. Food Chem.* 49: 1146–1150; 2001.

Grimme L. H.; Brown J. S. Functions of chlorophylls and carotenoids in thylakoid membranes. *Adv Photosyn Res* 2: 141–144; 1984.

Gross J. Carotenoids: pigments in Fruits. Academic, London; 1987.

Harjes C. E.; Rocheford T. R.; Bai L.; Brutnell T. P.; Kandianis C. B.; Sowinski S. G.; Stapleton A. E.; Vallabhaneni R.; Williams M.; Wurtzel E. T.; Yan J.; Buckler E. S. Natural genetic variation in *lycopene epsilon cyclase* tapped for maize biofortification. *Science* 319: 330–333; 2008.

Harrison E. H. Mechanisms of digestion and absorption of dietary vitamin A. *Ann Rev Nutr* 25: 87–103; 2005.

Hart D. J.; Scott K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.* 54: 101–111; 1995.

Haskell M. J.; Jamil K. M.; Hassan F.; Peerson J. M.; Hossain M. I.; Fuchs G. J.; Brown K. H. Daily consumption of Indian spinach (*Basella alba*) or sweet potatoes has a positive effect on total body vitamin A stores in Bangladeshi men. *Am. J. Clin. Nutr.* 80: 705–714; 2004.

Howard L. A.; Wong A. D.; Perry A. K.; Klein B. P. β-carotene and ascorbic acid retention in fresh and processed vegetables. *J. Food Sci.* 64: 929–936; 1999.

Ikoma Y.; Komatsu A.; Kita M.; Ogawa K.; Omura M.; Yano M.; Moriguchi T. Expression of a phytoene synthase gene and characteristic carotenoid accumulation during citrus fruit development. *Physiol. Plant.* 111: 232–238; 2001.

Imam M. K.; Gabelman W. H. Inheritance of carotenoids in carrots, *Daucus carota* L. *Proc Am Soc Hortic Sci* 93: 419–428; 1968.

IOM. Dietary reference intakes for vitamin a, vitamin k, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. National Academy Press, Washington, DC; 2001.

Isaacson T.; Ohad I.; Beyer P.; Hirschberg J. Analysis in vitro of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants. *Plant Physiol.* 136: 4246–4255; 2004.

Just B. J.; Santos C. A.; Yandell B. S.; Simon P. W. Major QTL for carrot color are positionally associated with carotenoid biosynthetic genes and interact epistatically in a domesticated x wild carrot cross. *Theor. Appl. Genet.* 119: 1155–1169; 2009.

Kato M.; Ikoma Y.; Matsumoto H.; Sugiura M.; Hyodo H.; Yano M. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiol.* 134: 824–837; 2004.

Kean E. G.; Ejeta G.; Hamaker B. R.; Ferruzzi M. G. Characterization of carotenoid pigments in mature and developing kernels of selected yellow-endosperm sorghum varieties. *J. Agric. Food Chem.* 55: 2619–2626; 2007.

Khan N. C.; West C. E.; de Pee S.; Bosch D.; Phuong H. D.; Hulshof P. J.; Khoi H. H.; Verhoef H.; Hautvast J. G. The contribution of plant foods to the vitamin A supply of lactating women in Vietnam: a randomized controlled trial. *Am. J. Clin. Nutr.* 85: 1112–1120; 2007.

Kim M.; Kim S. C.; Song K. J.; Kim H. B.; Kim I. J.; Song E. Y.; Chun S. J. Transformation of carotenoid biosynthetic genes using a micro-cross section method in kiwifruit (*Actinidia deliciosa cv. Hayward*). *Plant Cell Rep.* 29: 1339–1349; 2010.

Kurilich A. C.; Juvik J. A. Quantification of carotenoid and tocopherol antioxidants in *Zea mays*. *J. Agric. Food Chem.* 47: 1948–1955; 1999.

Lawrence R. J.; Pikaard C. S. Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *Plant J.* 36: 114–121; 2003.

Lee H. S.; Castle W. S. Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd Blood orange juices. *J. Agric. Food Chem.* 49: 877–882; 2001.

Li F.; Murillo C.; Wurtzel E. T. Maize *Y9* encodes a product essential for 15-cis-zeta-carotene isomerization. *Plant Physiol.* 144: 1181–1189; 2007.

Li F.; Vallabhaneni R.; Yu J.; Rocheford T.; Wurtzel E. T. The maize phytoene synthase gene family: overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. *Plant Physiol.* 146: 1334–1346; 2008.

Li L.; Garvin D. F. Molecular mapping of *Or*, a gene inducing β-carotene accumulation in cauliflower (*Brassica oleracea* L. var. *botrytis*). *Genome* 46: 588–594; 2003.

Li L.; Paolillo D. J.; Parthasarathy M. V.; Dimuzio E. M.; Garvin D. F. A novel gene mutation that confers abnormal patterns of beta-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *Plant J.* 26: 59–67; 2001.

Li Q.; Farre G.; Naqvi S.; Breitenbach J.; Sanahuja G.; Bai C.; Sandmann G.; Capell T.; Christou P.; Zhu C. Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. *Transgenic Res* 19: 1053–1068; 2010a.

Li S.; Nugroho A.; Rocherford T.; White W. S. Vitamin A equivalence of the β-carotene in β-carotene-biofortified maize porridge consumed by women. *Am. J. Clin. Nutr.* 92: 1105–1112; 2010b.

Lopez A. B.; Van Eck J.; Conlin B. J.; Paolillo D. J.; O'Neill J.; Li L. Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* 59: 213–223; 2008.

Lu S.; Li L. Carotenoid metabolism: biosynthesis, regulation, and beyond. *J. Integr. Plant Biol.* 50: 778–785; 2008.

Lu S.; Van Eck J.; Zhou X.; Lopez A. B.; O'Halloran D. M.; Cosman K. M.; Conlin B. J.; Paolillo D. J.; Garvin D. F.; Vrebalov J.; Kochian L. V.; Kupper H.; Earle E. D.; Cao J.; Li L. The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. *Plant Cell* 18: 3594–3605; 2006.

Maes T.; De Keukelerie P.; Gerats T. Plant tagnology. *Trends Plant Sci.* 4: 90–96; 1999.

Maass D.; Arango J.; Wust F.; Beyer P.; Welsch R. Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS ONE* 4: e6373; 2009.

Mansoor S.; Amin I.; Hussain M.; Zafar Y.; Briddon R. W. Engineering novel traits in plants through RNA interference. *Trends Plant Sci.* 11: 559–565; 2006.

Misawa N.; Truesdale M. R.; Sandmann G.; Fraser P. D.; Bird C.; Schuch W.; Bramley P. M. Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation in vivo and in vitro, and functional analysis of the various truncated gene products. *J. Biochem.* 116: 980–985; 1994.

Molnar P.; Szabolcs J. β-Citraurin epoxide, a new carotenoid from valencia orange peel. *Phytochemistry* 19: 633–637; 1980.

Murkovic M.; Mulleder U.; Neunteufl H. Carotenoid content in different varieties of pumpkins. *J Food Compos Anal* 15: 633–638; 2002.

Naqvi S.; Farre G.; Sanahuja G.; Capell T.; Zhu C.; Christou P. When more is better: multigene engineering in plants. *Trends Plant Sci.* 15: 48–56; 2010.

Naqvi S.; Zhu C.; Farre G.; Ramessar K.; Bassie L.; Breitenbach J.; Perez C. D.; Ros G.; Sandmann G.; Capell T.; Christou P. Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc. Natl Acad. Sci. U.S.A.* 106: 7762–7767; 2009.

Nassar N. M. A.; Fernandes P. C.; Melani R. D.; Pires Jr. O. R. Amarelinha do Amapa: a carotenoid-rich cassava cultivar. *Genet. Mol. Res.* 8: 1051–1055; 2009.

Navazio J. P. Utilization of high carotene cucumber germplasm for genetic improvement of nutritional quality. PhD thesis. University of Wisconsin- Madison; 1994.

Nesi N.; Delourme R.; Bregeon M.; Falentin C.; Renard M. Genetic and molecular approaches to improve nutritional value of *Brassica napus* L. seed. *C R Biol* 331: 763–771; 2008.

Olson J. A. Needs and sources of carotenoids and vitamin A. *Nutr. Rev.* 52: S67–73; 1994.

Paolillo D. J.; Garvin Jr. D. F.; Parthasarathy M. V. The chromoplasts of *Or* mutants of cauliflower (*Brassica oleracea* L. var. *botrytis*). *Protoplasma* 224: 245–253; 2004.

Parinov S.; Sundaresan V. Functional genomics in Arabidopsis: large-scale insertional mutagenesis complements the genome sequencing project. *Curr. Opin. Biotechnol.* 11: 157–161; 2000.

Potrykus I. Lessons from the 'Humanitarian Golden Rice' project: regulation prevents development of public good genetically engineered crop products. *New Biotechnol.* 27: 466–472; 2010.

Pozniak C. J.; Knox R. E.; Clarke F. R.; Clarke J. M. Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. *Theor. Appl. Genet.* 114: 525–537; 2007.

Ravanello M. P.; Ke D.; Alvarez J.; Huang B.; Shewmaker C. K. Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. *Metab. Eng.* 5: 255–263; 2003.

Rodriguez-Concepcion M. Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochem. Rev.* 5: 1–15; 2006.

Rodríguez-Concepción M. Supply of precursors for carotenoid biosynthesis in plants. *Arch. Biochem. Biophys.* 504: 118–122; 2010.

Rodriguez-Concepcion M.; Boronat A. Elucidation of the methylerthritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol.* 130: 1079–1089; 2002.

Romer S.; Fraser P. D.; Kiano J. W.; Shipton C. A.; Misawa N.; Schuch W.; Bramley P. M. Elevation of the provitamin A content of transgenic tomato plants. *Nat. Biotechnol.* 18: 666–669; 2000.

Romer S.; Lubeck J.; Kauder F.; Steiger S.; Adomat C.; Sandmann G. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metab. Eng.* 4: 263–272; 2002.

Rosati C.; Aquilani R.; Dharmapuri S.; Pallara P.; Marusic C.; Tavazza R.; Bouvier F.; Camara B.; Giuliano G. Metabolic engineering of β-carotene and lycopene content in tomato fruit. *Plant J.* 24: 413–419; 2000.

Salas Fernandez M. G.; Hamblin M. T.; Li L.; Rooney W. L.; Tuinstra M. R.; Kresovich S. Quantitative trait loci analysis of endosperm color and carotenoid content in sorghum grain. *Crop Sci.* 48: 1732–1743; 2008.

Santos C. A.; Senalik D.; Simon P. W. Path analysis suggests phytoene accumulation is the key step limiting the carotenoid pathway in white carrot roots. *Genet Mol Biol* 28: 287–293; 2005.

Santos C. A.; Simon P. W. QTL analyses reveal clustered loci for accumulation of major provitmain A carotenes and lycopene in carrot roots. *Mol. Genet. Genomics* 268: 122–129; 2002.

Seo M.; Koshiba T. Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci.* 7: 41–48; 2002.

Shewmaker C. K.; Sheehy J. A.; Daley M.; Colburn S.; Ke D. Y. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* 20: 401–412; 1999.

Simkin A. J.; Gaffe J.; Alcaraz J. P.; Carde J. P.; Bramley P. M.; Fraser P. D.; Kuntz M. Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Photochemestry* 68: 1545–1556; 2007.

Singh M.; Lewis P. E.; Hardeman K.; Bai L.; Rose J. K. C.; Mazourek M.; Chomet P.; Brutnell T. P. Activator mutagenesis of the *pink scutellum1/viviparous7* locus of maize. *Plant Cell* 15: 874–884; 2003.

Slade A. J.; Knauf V. C. TILLING moves beyond functional genomics into crop improvement. *Transgenic Res.* 14: 109–115; 2005.

Tang G.; Gu X.; Hu S.; Xu Q.; Qin J.; Dolnikowski G. G.; Fjeld C. R.; Gao X.; Russell R. M.; Yin S. Green and yellow vegetables can maintain body stores of vitamin A in Chinese children. *Am. J. Clin. Nutr.* 70: 1069–1076; 1999.

Tang G.; Qin J.; Dolnikowski G. G.; Russell R. M.; Grusak M. A. Spinach or carrot can supply significant amounts of vitamin A as assessed by feeding with intrinsically deuterium-labeled vegetables. *Am. J. Clin. Nutr.* 82: 821–828; 2005.

Tang G.; Qin J.; Dolinowski G. G.; Russell R. M.; Grusak M. A. Golden Rice is an effective source of vitamin A. *Am. J. Clin. Nutr.* 89: 1776–1783; 2009.

Tanumihardjo S. A. Factors influencing the conversion of carotenoids to retinol: bioavailability to bioconversion to bioefficacy. *Int. J. Vitam. Nutr. Res.* 72: 40–45; 2002.

Tian L.; Magallanes-Lundback M.; Musetti V.; DellaPenna D. Functional analysis of beta- and epsilon-ring carotenoid hydroxylases in Arabidopsis. *Plant Cell* 5: 1320–1332; 2003.

Thorup T. A.; Tanyolac B.; Livingstone K. D.; Popovsky S.; Paran I.; Jahn M. Candidate gene analysis of organ pigmentation loci in the Solanaceae. *Proc. Natl Acad. Sci. U.S.A.* 97: 11192–11197; 2000.

UNICEF. Vitamin A deficiency. UNICEF; 2006. http://www.childinfo.org/areas/vitamina/

Underwood B. A. Scientific research: essential, but is it enough to combat world food insecurities? *J. Nutr.* 133: 1434S–1437S; 2003.

Vallabhaneni R.; Gallagher C. E.; Licciardello N.; Cuttriss A. J.; Quinlan R. F.; Wurtzel E. T. Metabolite sorting of a germplasm collection reveals the *Hydroxylase3* locus as a new target for maize provitamin A biofortification. *Plant Physiol.* 150: 1635–1645; 2009.

Vallabhaneni R.; Wurtzel E. T. Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize. *Plant Physiol.* 150: 562–572; 2009.

Van Eck J.; Conlin B.; Garvin D. F.; Mason H.; Navarre D. A.; Brown C. R. Enhancing beta-carotene content in potato by RNAi-mediated silencing of the beta-carotene hydroxylase gene. *Am. J. Potato Res.* 84: 331–342; 2007.

Vasquez-Caicedo A. L.; Sruamsiri P.; Carle R.; Neidhart S. Accumulation of all-*trans*-beta-carotene and its 9-*cis* and 13-*cis* stereoisomers during postharvest ripening of nine Thai mango ciltivars. *J. Agric. Food Chem.* 53: 4827–4835; 2005.

Wang Y.; Wang F.; Zhai H.; Liu Q. Production of a useful mutant by chronic irradiation in sweetpotato. *Sci. Hortic.* 111: 173–178; 2007.

Wei S.; Yu B.; Gruber M. Y.; Khachatourians G. G.; Hegedus D. D.; Hannoufa A. Enhanced seed carotenoid levels and branching in transgenic *Brassica napus* expressing the *Arabidopsis* miR156b Gene. *J. Agric. Food Chem.* 58: 9572–9578; 2010.

Welsch R.; Arango J.; Bar C.; Salazar B.; Al-Babili S.; Beltran J.; Chavarriaga P.; Ceballos H.; Tohme J.; Beyer P. Provitamin A accumulation in cassava (*Manihot esculenta*) roots driven by a single nucleotide polymorphism in a *phytoene synthase* gene. *Plant Cell* 22: 3348–3356; 2010.

Wong J. C.; Lambert R. J.; Wurtzel E. T.; Rocheford T. R. QTL and candidate genes phytoene synthase and zeta-carotene desaturase associated with the accumulation of carotenoids in maize. *Theor. Appl. Genet.* 108: 349–359; 2004.

Wurbs D.; Ruf S.; Bock R. Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *Plant J.* 49: 276–288; 2007.

Wurtzel E. T.; Luo R.; Yatou O. A simple approach to identify the first rice mutants blocked in carotenoid biosynthesis. *J. Exp. Bot.* 52: 161–166; 2001.

Yan J.; Kandianis C. B.; Harjes C. E.; Bai L.; Kim E. H.; Yang X.; Skinner D. J.; Fu Z.; Mitchell S.; Li Q.; Fernandez M. G.; Zaharieva M.; Babu R.; Fu Y.; Palacios N.; Li J.; Dellapenna D.; Brutnell T.; Buckler E. S.; Warburton M. L.; Rocheford T. Rare genetic variation at Zea mays crtRB1 increases beta-carotene in maize grain. *Nat. Genet.* 42: 322–327; 2010.

Yu B.; Lydiate D. J.; Young L. W.; Schafer U. A.; Hannoufa A. Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase. *Transgenic Res.* 17: 573–585; 2007.

Zamir D. Improving plant breeding with exotic genetic libraries. *Nat. Rev. Genet.* 2: 983–989; 2001.

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

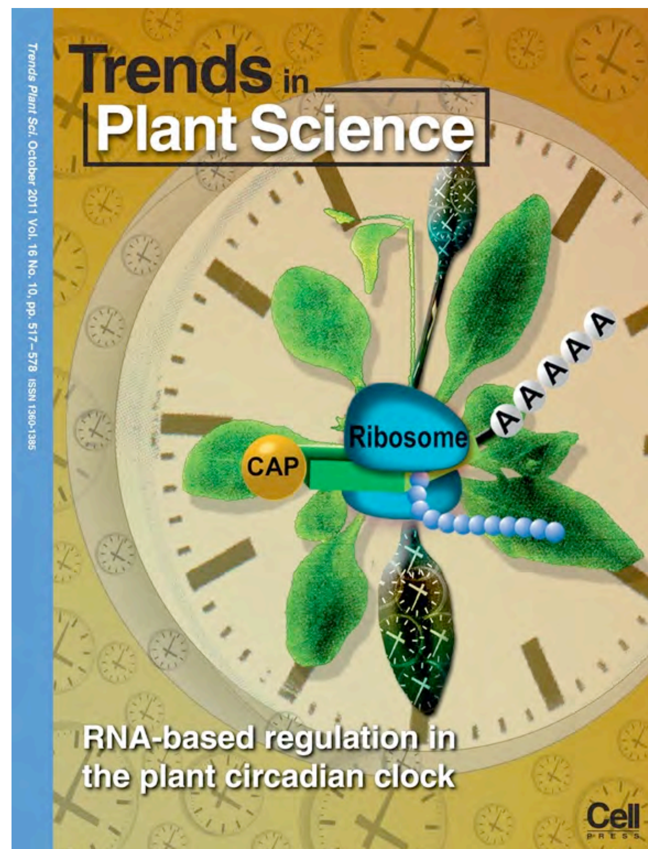
Zhu C.; Bai C.; Sanahuja G.; Yuan D.; Farre G.; Naqvi S.; Shi L.; Capell T.; Christou P. The regulation of carotenoid pigmentation in flowers. *Arch. Biochem. Biophys.* 504: 132–141; 2010.

Zhu C.; Naqvi S.; Breitenbach J.; Sandmann G.; Christou P.; Capell T. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl Acad. Sci. U.S.A.* 105: 18232–18237; 2008.

Zhu C.; Naqvi S.; Gomez-Galera S.; Pelacho A. M.; Capell T.; Christou P. Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* 12: 548–555; 2007.

Nutritious crops producing multiple carotenoids - a metabolic balancing act

Gemma Farré[1], Chao Bai[1], Richard M. Twyman[2], Teresa Capell[1], Paul Christou[1,3] and Changfu Zhu[1,]*



Trends in Plant Sci. (2011), 16: 532-540

*Corresponding author Changfu Zhu (email: zhu@pvcf.udl.cat)

Nutritious crops producing multiple carotenoids – a metabolic balancing act

Gemma Farré[1], Chao Bai[1], Richard M. Twyman[2], Teresa Capell[1], Paul Christou[1,3] and Changfu Zhu[1]

[1] Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida-CRA, Av. Alcalde Rovira Roure, 191, Lleida 25198, Spain
[2] Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
[3] Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Plants and microbes produce multiple carotenoid pigments with important nutritional roles in animals. By unraveling the basis of carotenoid biosynthesis it has become possible to modulate the key metabolic steps in plants and thus increase the nutritional value of staple crops, such as rice (*Oryza sativa*), maize (*Zea mays*) and potato (*Solanum tuberosum*). Multigene engineering has been used to modify three different metabolic pathways simultaneously, producing maize seeds with higher levels of carotenoids, folate and ascorbate. This strategy may allow the development of nutritionally enhanced staples providing adequate amounts of several unrelated nutrients. By focusing on different steps in the carotenoid biosynthesis pathway, it is also possible to generate plants with enhanced levels of several nutritionally-beneficial carotenoid molecules simultaneously.

The multiple nutritional roles of carotenoids

Several carotenoids have highly specific roles in human nutrition but most applied carotenoid research currently focuses on increasing the levels of β-carotene (pro-vitamin A) in grains, fruits and vegetables in an effort to tackle vitamin A deficiency (Box 1) [1,2]. It has been shown that plants can be engineered to produce multiple unrelated nutrients by targeting different metabolic pathways simultaneously [3], but attempting to replicate the same achievements with different nutritional molecules from the same pathway could run into difficulties if there is competition for enzymes and precursors. As an example, β-carotene lies downstream of a bifurcation in the carotenoid biosynthesis pathway, the alternative branch yielding α-carotene and ultimately lutein, while β-carotene itself is also further converted into zeaxanthin [1,2]. Both lutein and zeaxanthin have important nutritional roles in humans (Box 2) so it is possible that focusing too strongly on β-carotene as a target could draw attention away from competing carotenoids that are also essential nutrients. We found that transgenic maize plants engineered to accumulate higher levels of β-carotene are not generally deficient in other carotenoids, and indeed the increased flux towards β-carotene in many cases enhances the levels of lutein and zeaxanthin as well as other carotenoids [4–7]. We therefore analyzed the literature covering β-carotene enhancement in other transgenic and conventionally-bred plants to determine the impact on lutein and zeaxanthin and found a broadly similar picture. Even when specific steps are taken to avoid the synthesis of these other carotenoids, there is always some leakage which allows nutritionally adequate levels to accumulate.

Corresponding author: Zhu, C. (zhu@pvcf.udl.cat)

Carotenoid synthesis in plants

Carotenoids are tetraterpenoids whose synthesis in plants begins in the plastids with the condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to generate the C20 intermediate geranylgeranyl diphosphate (GGPP). This reaction is catalyzed by GGPP synthase (GGPPS) [8]. The first committed step is the condensation of two GGPP molecules into 15-*cis*-phytoene by the enzyme phytoene synthase (PSY, or CrtB in bacteria) [9]. A series of four desaturation reactions carried out in plants by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. In non-green tissue this is converted to all-*trans* lycopene by ζ-carotene isomerase (Z-ISO) [10] and carotenoid isomerase (CRTISO), whereas in green tissue the reaction occurs spontaneously in the presence of light and chlorophyll (acting as a sensitizer) [11,12]. In bacteria, all these steps are carried out by a single enzyme, CrtI.

All-*trans* lycopene represents a branch point in the pathway. This linear molecule can be cyclized at both ends by lycopene β-cyclase (LYCB, CrtY in bacteria) to generate the β-ionone end groups of β-carotene. Alternatively it can be cyclized at one end by lycopene ε-cyclase (LYCE) and at the other by LYCB to introduce the non-identical ε- and β-ionone end groups of α-carotene. Both these molecules can be converted into downstream products by carotene hydroxylases, such as the eponymous β-carotene hydroxylase (BCH). In the β-carotene pathway this yields β-cryptoxanthin, which is further converted by the same enzyme into zeaxanthin, whereas in the α-carotene branch the conversion yields lutein, the natural pathway endpoint [13]. Furthermore, zeaxanthin enters the xanthophyll cycle through the stepwise activities of zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE). These reactions are shown schematically in Figure 1.

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Box 1. Enhancing β-carotene levels to tackle vitamin A deficiency

Vitamin A is an essential nutrient in mammals that occurs in two important functional forms: retinal (required for low-light and color vision) and the regulatory morphogen retinoic acid. Both forms are derived from retinol in the diet, which is obtained from meat and dairy products in the form of esters such as retinyl palmitate. Humans and other herbivores/omnivores possess the enzyme β-carotene 15,15′-monooxygenase, which also allows the direct synthesis of retinal from pro-vitamin A carotenoids such as β-carotene produced in plants. In populations which lack access to animal-derived food, plants are therefore an important dietary source of vitamin A precursors.

The dietary reference intake (DRI) for vitamin A is expressed as the retinol activity equivalent (RAE), which takes bioavailability into account. The recommended DRI for males is 900 RAE, for females is 700 RAE (higher in pregnancy and when lactating), and for children it is 400–500 RAE. One RAE is equivalent to 1 μg of pure retinol, 2 μg of pure β-carotene dissolved in oil, or 12 μg of β-carotene in food [36]. Most people in the developed world have diets of sufficient diversity to ensure they achieve the DRI for vitamin A, but the situation in developing countries is very different. More than four million children, most from developing countries, exhibit clinical symptoms of severe vitamin A deficiency, including poor immunity, loss of vision in low light conditions (night blindness) and in extreme cases an irreversible form of blindness called xerophthalmia [37] (see also UNICEF 2006 report on vitamin A deficiency, http://www.childinfo.org/areas/vitamina/).

Several approaches can be considered to address vitamin A deficiency. Dietary supplements (vitamin tablets and suspensions) and fortification campaigns (artificially increasing vitamin levels by adding vitamins to processed food) have been highly successful in the developed world and have significantly reduced the incidence of deficiency diseases [38]. However, this strategy has little impact in remote areas of developing countries because of the incomplete food distribution network, poor governance and the lack of funding [39,40]. To address this, several attempts have been made to boost the levels of β-carotene in staple crops such as rice, maize and potato, either through conventional breeding or genetic engineering. Many poor people subsist on rice, which provides calories but has a very low nutrient content. In Golden Rice, the entire β-carotene biosynthesis pathway was reconstructed in the endosperm by expressing daffodil PSY and LYCB, as well as bacterial CrtI, increasing the endosperm carotenoid content to 1.6 μg/g dry weight [20]. This was not sufficient to provide the DRI of vitamin A in a reasonable portion of rice, so the more active maize PSY1 enzyme was used to replace its daffodil ortholog in ‘Golden Rice 2’, increasing the endosperm carotenoid content to 37 μg/g dry weight [23]. Golden Rice has been followed by similar programs in other staples, including Golden Potato and Multivitamin Maize (recently reviewed in [41]). Conventional breeding has also given rise to maize lines with high β-carotene levels, although the highest level achieved thus far relying on natural variation is 13.6 μg β-carotene per gram dry weight, which is by some considerable margin less than can be achieved by genetic engineer-

Increasing the flux towards β-carotene – the impact on lutein and zeaxanthin

Conventional breeding programs (Table 1) and genetic engineering strategies (Table 2) often aim to increase the levels of β-carotene in plants using the same approach, i.e. increasing flux through the pathway by increasing the activity of particular enzymes.

In conventional breeding, this is achieved by selecting plants that carry hypermorphic alleles, i.e. alleles encoding particularly active forms of carotenogenic enzymes or particularly active promoters that increase the quantity of the enzyme. A good example of such an allele is the *Beta* tomato (*Solanum lycopersicum*) mutant, which is orange in color and contains 45% more β-carotene than normal, corresponding to a hypermorphic variant of the enzyme LYCB [14]. Quantitative trait loci (QTLs) affecting β-carotene levels in this manner have been identified in many species, and have in a number of cases been traced to early enzymes in the carotenoid biosynthesis pathway [2]. For example, a QTL affecting β-carotene levels in sorghum has been mapped to the *psy3* gene [15]. In maize, a simple sequence repeat (SSR) marker has been identified [16] associated with *yellow1* (*y1*) that was linked to a major QTL explaining 6.6–27.2% of the phenotypic variation in carotenoid levels, and this was eventually resolved to the *psy1* gene. Similarly, another QTL was shown to be linked to *viviparous 9* (*vp9*), and this was found to encode ζ-carotene desaturase [16]. QTLs affecting β-carotene levels have also been identified in melon (*Cucumis melo*) fruits, which have flesh color ranging from green to orange because of differences in carotenoid levels. California and Wisconsin melon recombinant inbred lines were used to identify eight QTLs each accounting for 8–31% of phenotypic variation, one mapping to a gene encoding BCH [17].

In genetic engineering, effects similar to those of a hypermorphic endogenous allele can be achieved by expressing a heterologous enzyme (often a bacterial enzyme) under the control of a strong promoter. Targeting β-carotene in this manner tends to enhance other carotenoids simultaneously because the flux is distributed throughout the pathway, affecting all products to a greater or lesser degree. Differences in carotenoid profiles arise when further endogenous bottlenecks are revealed. As an example, the overexpression of PSY/CrtB often removes a significant bottleneck in the pathway and can increase the levels of all downstream carotenoids (e.g. [6,18]). In other cases, the immediate effect of PSY overexpression is only to reveal secondary restrictions further along the pathway so that the exact carotenoid composition depends on the relative activities of these later-acting enzymes. This is why transgenic maize and canola (*Brassica napus*) overexpressing PSY/CrtB accumulate different carotenoid end products, mirroring the situation in wild type seeds (where the bottlenecks similarly cause different carotenoids to accumulate) [6,19]. In contrast, transgenic rice seeds expressing PSY only accumulate the immediate downstream product of the PSY reaction (phytoene), because the subsequent enzyme is also expressed at vanishingly low levels in the endosperm [20].

Box 2. The nutritional importance of lutein and zeaxanthin

Lutein and zeaxanthin cannot be synthesized *de novo* in humans. There are many dietary sources of lutein, e.g. *Actinidia* spp. and maize seeds, but there are few good sources of zeaxanthin, e.g. some maize and *Capiscum annuum* varieties [42,43]. Lutein and zeaxanthin accumulate in the perifoveal and foveal regions of the retinal macula, respectively, and appear to protect these tissues from photodegradation [44]. There is a strong association between the dietary intake of lutein and zeaxanthin, and the degree of protection against age-related macular degeneration (ARMD) [45,46]. There is a lower incidence of this disease in people with a carotenoid-rich diet [47,48].

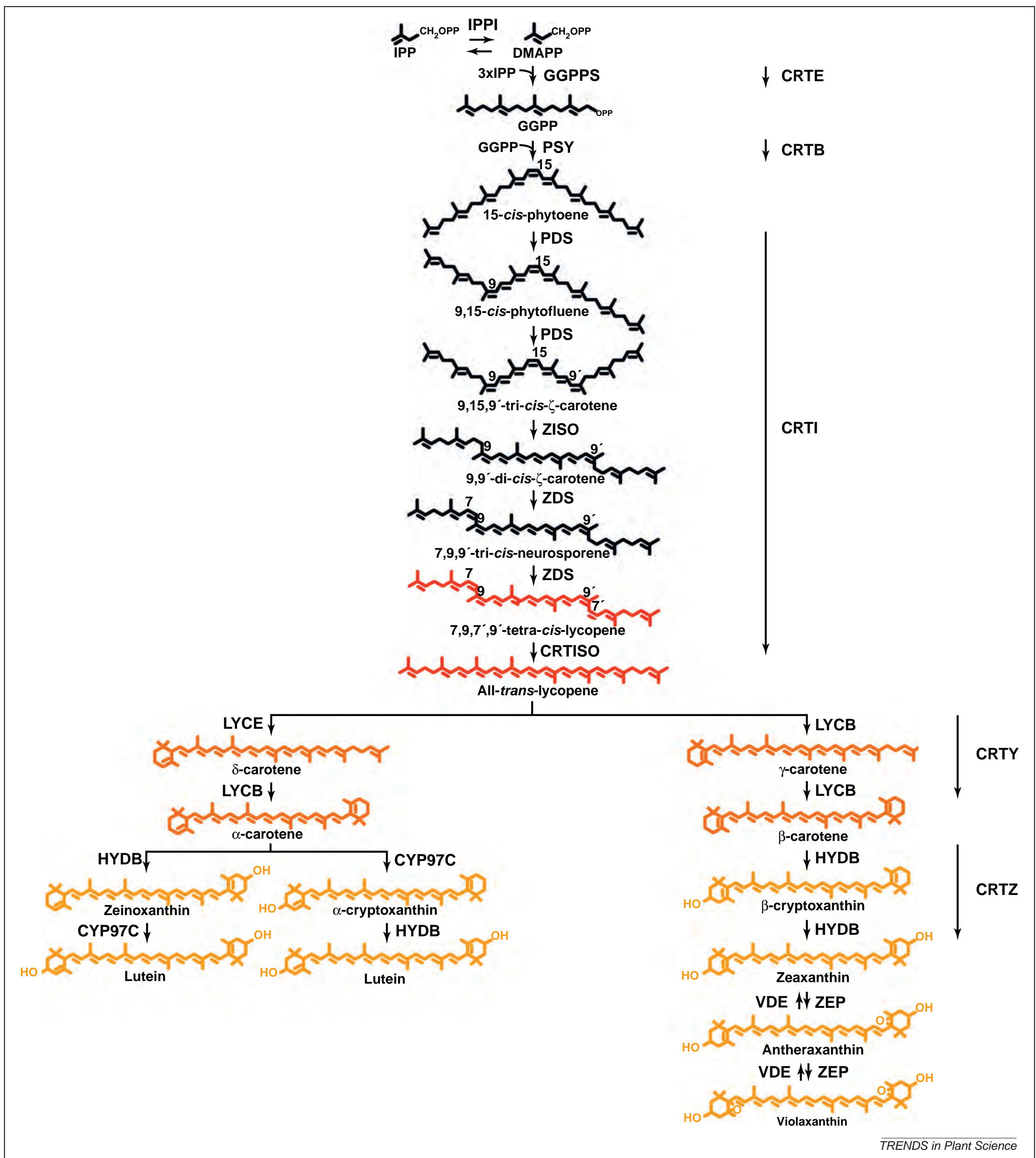

Figure 1. Carotenoid biosynthesis pathway in plants and equivalent steps in bacteria. CRTB, bacterial phytoene synthase; CRTE, bacterial geranylgeranyl diphosphate synthase; CRTI, bacterial phytoene desaturase/isomerase; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene cyclase; CRTZ, bacterial β-carotene hydroxylase; CYP97C, carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HYDB, β-carotene hydroxylase [non-heme di-iron hydroxylases, β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxylases, CYP97A and CYP97B]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase.

To pre-empt such secondary restrictions, it is becoming more common to express several different enzymes simultaneously in an effort to open up the carotenoid pathway to its full potential [5]. In addition to PSY/CrtB and CrtI from the linear part of the pathway, the next key target is LYCB/CrtY because the overexpression of this enzyme shifts the metabolic balance from the α to the β branch, and should therefore theoretically enhance β-carotene levels at the

Table 1. Total levels of carotenoids, β-carotene, lutein and zeaxanthin in different crop species[a]

Species	Total carotenoid levels in best line	β-Carotene levels in best line	Lutein levels in best line	Zeaxanthin levels in best line	Refs
Sorghum	190.18 μg/g DW	9.01 μg/g DW	101.49 μg/g DW	79.66 μg/g DW	[15]
	6.06–28.53 μg/TKDW	0.15–3.83 μg/TKDW	1.96–7.18 μg/TKDW	2.22–13.29 μg/TKDW	[49]
Canola	23 μg/g FW	0.2 μg/g FW	17.7 μg/g FW	ND	[19]
Tomato	207 μg/g FW	36.8 μg/g FW	6.4 μg/g FW	ND	[50]
Pumpkin		74 μg/g DW	170 μg/g DW	ND	[51]
Kiwifruit	16.34 μg/g FW	7.07 μg/g FW	6.20 μg/g FW	0.52 μg/g FW	[52]
Maize	29 μg/g DW	7 μg/g DW	20 μg/g DW	4 μg/g DW	[53]
	24.47 μg/g DW	13.63 μg/g DW	6.36 μg/g DW	2.77 μg/g DW	[32]
		4.7 μg/g DW	18.5 μg/g DW	24.5 μg/g DW	[25]

Abbreviations: DW, dry weight; FW fresh weight; ND, not determined; TKDW, thousand kernels dry weight.

[a]Recorded in the best-performing lines from conventional breeding programs.

expense of α-carotene and lutein, e.g. [21,22]. When LYCB is overexpressed, the levels of β-carotene do indeed increase, as seen in Golden Rice and Golden Rice II [20,23]. However, even with this bias towards the β-branch, there is enough upstream flux diverted into the α-branch to produce adequate amounts of lutein, and there is also enough leakage of flux past β-carotene to generate adequate amounts of zeaxanthin. For example, transgenic potato tubers expressing bacterial CrtB, CrtI and CrtY contained much more β-carotene than wild type tubers (up to 47.4 μg/g dry weight, a 3600-fold increase) but there were also significant increases in zeaxanthin (11 μg/g dry weight, a 5.8-fold increase) and lutein (23.1 μg/g dry weight, a 23-fold increase) [24]. Similarly, transgenic maize seeds expressing PSY1, CrtI and LYCB accumulated more carotenoids than wild type seeds, as would be expected from the general increased flux, and the β:α-carotene ratio increased from 1.21 to 3.51, showing that the additional LYCB activity skewed the competition for the common precursor lycopene and increased flux towards β-carotene [6]. Even so, there was also enhanced flux through the α-branch of the pathway, producing nearly 25-fold the normal levels of lutein (up to 13.12 μg/g dry weight). When the transgenic locus from this line was introgressed into a wild-type yellow-endosperm variety with a low β:α ratio (0.61), the hybrid offspring contained higher levels of both lutein (23.4 μg/g dry weight) and β-carotene (19.3 μg/g dry weight) compared with the parental plants [3].

These examples show that even when shifting the metabolic flux towards β-carotene, there is still enough flux through the α-branch of the pathway to produce more than enough lutein for human nutrition. The transgenic locus discussed above was also introgressed into a wild-type yellow endosperm variety with a high β:ε ratio (1.90). This gave rise to a novel hybrid line producing zeaxanthin at an unprecedented 56 μg/g dry weight. Even so, the seeds still contained higher levels of β-carotene than wild type seeds (15.24 μg/g dry weight) and lutein was also more abundant in the transgenic seeds (9.72 μg/g dry weight) [3]. Even yellow maize inbred lines with high levels of β-carotene (4.7 μg/g dry weight) also have higher levels of zeaxanthin (24.5 μg/g dry weight) and lutein (18.5 μg/g dry weight) [25].

Similar factors affect the balance between β-carotene and zeaxanthin when engineering downstream steps in the pathway. A transgenic maize line expressing PSY, CrtI, LYCB, BCH and CrtW (allowing the synthesis of ketocarotenoids that are rarely found naturally in plants) produced 25.78 μg/g dry weight β-carotene (a 184-fold increase over wild type seeds) but also 62-fold more zeaxanthin (16.78 μg/g dry weight) and 23-fold more lutein (12.27 μg/g dry weight) [6].

An alternative to multiple enzyme engineering is the modulation of transcriptional regulators that have multiple targets in the carotenoid pathway. *DE-ETIOLATED1* (*DET1*) is a regulatory gene encoding a transcription factor that represses several light-dependent signaling pathways, including those influencing carotenoid biosynthesis [26]. Loss-of-function mutations affecting the tomato ortholog of *DET1*, *high pigment-2* (*hp-2*), have more deeply-colored fruits that wild type plants when grown in the light because more flavonoids and carotenoids accumulate during fruit development [27]. Similarly, fruit-specific silencing of the tomato *hp-2* gene by RNAi gives rise to fruits with higher carotenoid and flavonoid levels than wild type fruits, but other quality attributes are unaffected [28]. In canola, silencing *DET1* by RNAi has been used as a deliberate strategy to increase carotenoid levels, generating seeds with higher levels of β-carotene (7 μg/g fresh weight, a 17.5-fold increase), zeaxanthin (0.8 μg/g fresh weight, a 4-fold increase) and lutein (13 μg/g fresh weight, a 2.1-fold increase) relative to wild type seeds [29]. The levels of these carotenoids were also enhanced, albeit to a lesser extent, when *DET1* silencing was seed-specific [29].

Diverting flux away from competing compounds – the impact on lutein and zeaxanthin

Increasing flux through the entire carotenoid biosynthesis pathway does not focus the benefits solely on β-carotene and therefore it is logical that other carotenoids are enhanced even if flux is diverted more towards the β-branch by overexpressing LYCB. More targeted approaches include the inhibition of enzymes that synthesize competing products, or the sequestration of β-carotene into subcellular compartments thus removing it from the active metabolic pool. These approaches might be expected to deplete competing carotenoids because specific enzymatic steps are inhibited or limited by nonproductive compartmentalization.

One example of the former approach is the deliberate inhibition of LYCE, to prevent the accumulation of lutein.

Table 2. Carotenoid engineering programs in diverse crops[a]

Species	Genes (origin)	Total carotenoid levels in wild type[a]	Total carotenoid levels (fold increase relative to wild type) in transgenic plants	β-Carotene levels in wild type[a]	β-Carotene levels (fold increase relative to wild type) in transgenic plants	Lutein levels in wild type[a]	Lutein (fold increase relative to wild type) in transgenic plants	Zeaxanthin levels in wild type[a]	Zeaxanthin (fold increase relative to wild type) in transgenic plants	Refs
Maize	*crt*B *and crt*I (*Pantoea ananatis*)	0.99 μg/g DW	33.6 μg/g DW (34)	0.98 μg/g DW	9.8 μg/g DW (10)	0.30 μg/g DW	6.68 μg/g DW (22)	0.68 μg/g DW	6.21 μg/g DW (9)	[54]
	PH7: *psy1* (*Zea mays*; maize) *crt*I (*P. ananatis*) *crt*W (*Paracoccus* spp.) *lycb* (*Gentiana lutea*) *bch* (*G. lutea*)	1.10 μg/g DW	102.1 μg/g DW (92.8)	0.14 μg/g DW	25.78 μg/g DW (184)	0.53 μg/g DW	12.27 μg/g DW (23)	0.27 μg/g DW	16.78 μg/g DW (62)	[6]
	PH4: *psy1* (maize) *crt*I (*P. ananatis*) *lycb* (*G. lutea*)	1.10 μg/g DW	148.78 μg/g DW (135)	0.14 μg/g DW	48.87 μg/g DW (349)	0.53 μg/g DW	13.12 μg/g DW (24.7)	0.27 μg/g DW	34.53 μg/g DW (127.8)	
	psy1 (maize) *crt*I (*P. ananatis*)	1.45 μg/g DW	163.2 μg/g DW (112)	0.35 μg/g DW	59.32 μg/g DW (169)	0.57 μg/g DW	14.68 μg/g DW (25)	0.32 μg/g DW	35.76 μg/g DW (111)	[4]
Potato	*ZEP* (*Arabidopsis thaliana*: *Arabidopsis*)	10.6 μg/g DW	60.8 μg/g DW (5.7)	0.7 μg/g DW	2.4 μg/g DW (3.4)	2.7 μg/g DW	5.2 μg/g DW (1.9)	0.3 μg/g DW	40.1 μg/g DW (133.7)	[55]
	*crt*B (*P. ananatis*)	5.6 μg/g DW	35.5 μg/g DW (6.3)	ND	10.3 μg/g DW	0.73 μg/g DW	11.01 μg/g DW (15)	0.11 μg/g DW	0.71 μg/g DW (6.5)	[56]
	Or (*Brassica oleracea*; cauliflower)	5.41 μg/g DW	28.22 μg/g DW (6)	ND	5.01 μg/g DW	3.42 μg/g DW	5.16 μg/g DW (1.5)	ND	ND	[57]
	Antisense *lyce* (*Solanum tuberosum*; potato)	4.6 μg/g DW	9.97 μg/g DW (2.5)	0.003 μg/g DW	0.04 μg/g DW (14)	0.588 μg/g DW (0.59)	1.004 μg/g DW (1.77)	0.26 μg/g DW	0.99 μg/g DW (3.8)	[30]
	*crt*B, *crt*I *and crt*Y (*P. ananatis*)	5.8 μg/g DW	114.4 μg/g DW (20)	0.013 μg/g DW	47.4 μg/g DW (3600)	1.0 μg/g DW	23.1 μg/g DW (23)	1.9 μg/g DW	11 μg/g DW (5.8)	[24]
	Antisense *bch* (potato)	4.88 μg/g DW	14.26 μg/g DW (2.9)	0.002 μg/g DW	0.085 μg/g DW (38)	0.43 μg/g DW	2.98 μg/g DW (7)	0.32 μg/g DW	0.04 μg/g DW	[58]
	Antisense *bch* (potato) (assumes 75% water content)	22.48 μg /g DW	23.52 μg/g DW (1.04)	0.04 μg /g DW	13.24 μg/g DW (331)	2.12 μg/g DW	5.48 μg/g DW (2.58)	23.72 μg/g DW	4.8 μg/g DW	[33]
Carrot	*psy* (*Arabidopsis*)	5.5 μg/g DW	514.1 μg/g DW (93)	1.26 μg/g DW	214.62 μg/g DW (178)	1.65 μg/g DW	5.14 μg/g DW (3)	ND	ND	[59]
Canola	*crt*B (*P. ananatis*)	36 μg/g FW	1055 μg/g FW (29.3)	5 μg/g FW	401 μg/g FW (80.2)	30 μg/g DW	72 μg/g DW (2.4)	ND	ND	[18]
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 5′ end	5.34 μg/g FW	227.78 μg/g FW (42.5)	0.49 μg/g FW	90.76 μg/g FW (185.2)	3.30 μg/g FW	76.22 μg/g FW (23)	ND	7.07 μg/g FW	[31]
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 3′ end		94.09 μg/g FW (17.6)		27.02 μg/g FW (55)	3.30 μg/g FW	37.64 μg/g FW (11.4)	ND	1.73 μg/g FW	

	idi, *crt*E, *crt*B, *crt*I and *crt*Y (*P. ananatis*) *crt*Z, *crt*W (*Brevundimonas* spp.)	21.7 µg/g FW	656.7 µg/g FW (30)	0.2 µg/g FW	214.2 µg/g FW (1070)	17.7 µg/g FW	27.6 µg/g FW (1.6)	ND	ND	[19]
	Antisense *DET1* (*Brassica napus*; canola), constitutive expression	6 µg/g FW	20 µg/g FW (3.3)	0.4 µg/g FW	7 µg/g FW (17.5)	6 µg/g FW	13 µg/g FW (2.1)	0.2 µg/g FW	0.8 µg/g FW (4)	[29]
	Antisense *DET1* (canola), seed expression	6 µg/g FW	14 µg/g FW (2.3)	0.4 µg/g FW	1.2 µg/g FW (3)	6 µg/g FW	12 µg/g FW (2)	ND	ND	
	microRNA *miR156b* (*Arabidopsis*)	3 µg/g FW (10% water content)	6.9 µg/g FW (2.3) (10% water content)	0.08 µg/g FW (10% water content)	0.38 µg/g FW (4.5) (10% water content)	2.7 µg/g FW (10% water content)	6.2 µg/g FW (2.3) (10% water content)	ND	ND	[60]
Tomato[b]	*crt*I (*P. ananatis*)	285 µg/g FW	137.2 µg/g FW (0.5)	27.1 µg/g FW	52 µg/g FW (1.9)	1.8 µg/g FW	4.1 µg/g FW (2.3)	ND	ND	[34]
	lycb (*Solanum lycopersicum*; tomato)	66 µg/g FW	109 µg/g FW (1.7)	7 µg/g FW	57 µg/g FW (7.1)	ND	ND	ND	ND	[21]
	lycb (*Arabidopsis*) *bch* (pepper; *Capsicum annuum*)	66.3 µg/g FW	100.7 µg/g FW (1.5)	5 µg/g FW	63 µg/g FW (12)	1.9 µg/g FW	1.8 µg/g FW	ND	13 µg/g FW	[61]
	*crt*B (*P. ananatis*)	285.7 µg/g FW	591.8 µg/g FW (2.1)	33 µg/g FW	82.5 µg/g FW (2.5)	ND	ND	ND	ND	[62]
	lycb (tomato)	94.5 µg/g FW	215.2 µg/g FW (2.3)	4.4 µg/g FW	205 µg/g FW (46.6)	ND	ND	ND	ND	[22]
	dxs (*Escherichia coli*)	460 µg/g FW	720 µg/g FW (1.6)	50 µg/g FW	70 µg/g FW (1.4)	ND	ND	ND	ND	[63]
	CRY2 (tomato)	87.6 µg/g FW in ripe fruit pericarps	149 µg/g FW in ripe fruit pericarps (1.7)	7.8 µg/g FW in ripe fruit pericarps	10.1 µg/g FW in ripe fruit pericarps (1.3)	2.3 µg/g FW	3.6 µg/g FW (1.6)	ND	ND	[64]
	psy1 (tomato)	181.20 µg/g FW	227.67 µg/g FW (1.25)	58.62 µg/g FW	81.93 µg/g FW (1.4)	9.96 µg/g FW	12.33 µg/g FW (1.2)	ND	ND	[65]
	fibrillin (pepper)	325 µg/g FW	650 µg/g FW (2.0)	90 µg/g FW	150 µg/g FW (1.6)	10 µg/g FW	16 µg/g FW (1.6)	ND	ND	[66]
	*crt*Y (*Erwinia herbicola*)	372.66 µg/g FW	323.71 µg/g FW (0.9)	6.91 µg/g FW	28.61 µg/g FW (4)	ND	ND	ND	ND	[67]
	lycb (*Narcissus pseudonarcissus*; daffodil)	76.67 µg/g FW	115 µg/g FW (1.5)	19 µg/g FW	95 µg/g FW (5)	ND	ND	ND	ND	[68]
Kumquat	*psy* (*Citrus sinensis*; orange)	84.3 µg/g FW	131.9 µg/g FW (1.6)	0.70 µg/g FW	1.72 µg/g FW (2.5)	5.6 µg/g FW	6.46 µg/g FW (1.5)	ND	ND	[69]

Abbreviations: DW, dry weight; FW, fresh weight; ND, not determined; TKDW, thousand kernels dry weight.

[a]Genes/enzymes involved in carotenoid engineering programs in different crops are shown as indicated and the absolute levels and relative improvement and/or reduction in total carotenoids, β-carotene, lutein and zeaxanthin. Where the original reports do not report zeaxanthin or lutein levels, they have been excluded from the table. Different wild-type carotenoid levels cited for each species reflect the different varieties used in each investigation.

[b]We converted dry weight to fresh weight assuming the water content of tomato fruits is 90%.

In transgenic plants, this has been achieved using antisense RNA and RNA interference (RNAi). The endogenous *lyce* gene was silenced in potato by expressing an antisense RNA construct, theoretically eliminating competition at the branch point between the α- and β-carotene pathways [30]. Antisense tubers contained up to 14-fold more β-carotene than wild type tubers, but there was no corresponding decrease in lutein levels because the total carotenoid level increased up to 2.5-fold in response to the general increase in flux resulting from the overexpression of upstream enzymes. These results suggest that LYCE is not rate-limiting for lutein accumulation in potato [30]. RNAi was also used to modulate carotenoid accumulation in canola seeds, again by targeting LYCE [31]. Transgenic seeds contained higher levels of β-carotene (90.76 μg/g fresh weight; 185-fold increase), zeaxanthin (7 μg/g fresh weight) and lutein (76.2 μg/g fresh weight) than wild type seeds, with the 23-fold increase in lutein demonstrating conclusively that LYCE is not a limiting step in canola either [31].

Similarly, conventional breeding programs can be used to select for plants with low levels of LYCE activity, by focusing on the selection of hypomorphic alleles and thus favoring the accumulation of β-carotene instead of lutein. Four polymorphisms have been described in the maize *lyce* locus, and conventional breeding for low LYCE activity increased the β-carotene levels in seeds to 13.6 μg/g dry weight (a 30–40% improvement) while lutein and zeaxanthin levels reached 6.36 and 2.77 μg/g dry weight, respectively [32].

Another common target of specific enzyme inhibition is the carotene hydroxylases, as this could prevent the conversion of β-carotene into zeaxanthin and therefore force the accumulation of β-carotene as an end-product (similarly, α-carotene would accumulate at the expense of lutein). However, this would prevent the formation of lutein and zeaxanthin only if the inhibition was 100% effective. The *bch* gene was silenced in potato, increasing β-carotene levels to 16.55 μg/g dry weight (a 331-fold improvement) and lutein to 6.85 μg/g dry weight a (2.5-fold improvement), while zeaxanthin levels were reduced from 29.65 to 6 μg/g dry weight, a fivefold reduction [33]. Therefore, even by targeting the particular enzymatic step leading to zeaxanthin, a significant amount of this carotenoid was still produced. In a converse example, the zeaxanthin content of potato tubers was enhanced by silencing the endogenous *zep* gene [34]. This increased the total carotenoid levels by 5.7-fold, β-carotene levels 3.4-fold and lutein levels 1.9-fold, while zeaxanthin was enhanced 133-fold.

Conventional breeding could also be used to increase zeaxanthin levels by selecting for hypomorphic versions of BCH. Although no studies with that specific aim have been reported, six hydroxylase genes were characterized in genetically diverse maize germplasm collections, one of which (*hyd3*) appeared to affect carotenoid levels in seeds [35]. Three *hyd3* alleles explained 78% of the variation in the ratio of β-carotene to β-cryptoxanthin (11-fold difference across varieties) and 36% of the variation in β-carotene absolute levels (4-fold difference across varieties).

There are additional potential strategies to increase carotenoid levels in plants, such as the inhibition of carotenoid cleavage dioxygenases. However, there are currently no data in the literature that report the improved β-carotene, zeaxanthin and lutein levels in plants following the application of these strategies so we do not discuss them further.

Conclusions

The above studies confirm that attempts to increase the availability of one particular nutrient in a metabolic pathway, in this case β-carotene in the carotenoid pathway, do not necessarily lead to deficiencies in upstream or downstream nutrients. There are several reasons for this phenomenon, which can be summarized as follows:

(i) The accumulation of specific carotenoids is a balance between the flux towards and away from a particular compound. As long as there is more flux towards a compound than away from it, that compound will accumulate.

(ii) Because the early steps in the carotenoid pathway are usually the rate-limiting steps, the overexpression of endogenous or heterologous enzymes (e.g. PSY/CrtB and CrtI) generally has a beneficial effect on all carotenoid molecules. The only intermediates that do not accumulate are those which are efficiently converted into the next downstream product, often as a result of multiple steps carried out by the same enzyme (e.g. β-carotene to β-crytoxanthin to zeaxanthin).

(iii) Targeting the branch point either positively (enhancing LYCB) or negatively (inhibiting LYCE) does not eliminate lutein accumulation. Indeed, enhancing LYCB increases the accumulation of lutein along with β-carotene. This indicates LYCE is not a limiting step and that the α-branch benefits from the increased pathway flux (because the α-branch requires LYCB activity too).

(iv) Targeting post β-carotene steps in the pathway to avoid depletion of the β-carotene pool is only partly effective. There are several carotene hydroxylases in plants, and therefore even RNAi knockdown of the principal enzyme, β-carotene hydroxylase, is insufficient to abolish the accumulation of lutein and zeaxanthin.

In conclusion, all the available evidence suggests that diverting flux specifically towards β-carotene by conventional and/or biotechnological means results in the collateral production of enough of the additional carotenoids lutein and zeaxanthin to satisfy human dietary requirements.

Acknowledgements

Research in our laboratory is supported by the Ministry of Science and Innovation (MICINN) Grant BFU2007-61413; European Research Council Advanced Grant BIOFORCE; Center Consolider, MICINN, Spain; COST Action FA0804, Associated Unit CAVA; and the SmartCell, FP7 Integrated Project.

References

1 Zhu, C. *et al.* (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* 12, 548–555
2 Farré, G. *et al.* (2010) Travel advice on the road to carotenoids in plants. *Plant Sci.* 179, 28–48

3 Naqvi, S. *et al.* (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7762–7767
4 Naqvi, S. *et al.* (2011) Synergistic metabolism in hybrid corn indicates bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. *Plant Biotechnol. J.* 9, 384–393
5 Naqvi, S. *et al.* (2010) When more is better: multigene engineering in plants. *Trends Plant Sci.* 15, 48–56
6 Zhu, C. *et al.* (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18232–18237
7 Zhu, C. *et al.* (2009) Metabolic engineering of ketocarotenoid biosynthesis in higher plants. *Arch. Biochem. Biophys.* 483, 182–190
8 Rodríguez-Concepción, M. (2006) Early steps in isoprenoid biosynthesis: Multilevel regulation of the supply of common precursors in plant cells. *Phytochem. Rev.* 5, 1–15
9 Misawa, N. *et al.* (1994) Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation *in vivo* and *in vitro*, and functional analysis of the various truncated gene products. *J. Biochem.* 116, 980–985
10 Chen, Y. *et al.* (2010) Isolation and characterization of the *Z-ISO* gene encoding a missing component of carotenoid biosynthesis in plants. *Plant Physiol.* 153, 66–79
11 Isaacson, T. *et al.* (2004) Analysis *in vitro* of the enzyme CRTISO establishes a poly-*cis*-carotenoid biosynthesis pathway in plants. *Plant Physiol.* 136, 4246–4255
12 Breitenbach, J. *et al.* (2005) ζ-Carotene *cis* isomers as products and substrates in the plant poly-*cis* carotenoid biosynthetic pathway to lycopene. *Planta* 220, 785–793
13 Zhu, C. *et al.* (2010) The regulation of carotenoid pigmentation in flowers. *Arch. Biochem. Biophys.* 504, 132–141
14 Ronen, G. *et al.* (2000) An alternative pathway to β-carotene formation in plant chromoplasts discovered by map-based cloning of *Beta* and *old-gold* color mutations in tomato. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11102–11107
15 Salas Fernandez, M.G. *et al.* (2008) Quantitative trait loci analysis of endosperm color and carotenoid content in sorghum grain. *Crop Sci.* 48, 1732–1743
16 Wong, J.C. *et al.* (2004) QTL and candidate genes phytoene synthase and ζ-carotene desaturase associated with the accumulation of carotenoids in maize. *Theor. Appl. Genet.* 108, 349–359
17 Cuevas, H.E. *et al.* (2008) Mapping of genetic loci that regulate quantity of β-carotene in fruit of US Western Shipping melon (*Cucumis melo* L.). *Theor. Appl. Genet.* 117, 1345–1359
18 Shewmaker, C.K. *et al.* (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* 20, 401–412
19 Fujisawa, M. *et al.* (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *J. Exp. Bot.* 60, 1319–1332
20 Ye, X. *et al.* (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303–305
21 Rosati, C. *et al.* (2000) Metabolic engineering of β-carotene and lycopene content in tomato fruit. *Plant J.* 24, 413–419
22 D'Ambrosio, C. *et al.* (2004) Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato lycopene β-cyclase (*tlcy-b*) cDNA. *Plant Sci.* 166, 207–214
23 Paine, J.A. *et al.* (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* 23, 482–487
24 Diretto, G. *et al.* (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE* 2, e350
25 Menkir, A. *et al.* (2008) Carotenoid diversity in tropical-adapted yellow maize inbred lines. *Food Chem.* 109, 521–529
26 Schafer, E. and Bowler, C. (2002) Phytochrome-mediated photoperception and signal transduction in higher plants. *EMBO Rep.* 3, 1042–1048
27 Mustilli, A.C. *et al.* (1999) Phenotype of the tomato *high pigment-2* mutant is caused by a mutation in the tomato homolog of *DE-ETIOLATED1*. *Plant Cell* 11, 145–157
28 Davuluri, G.R. *et al.* (2005) Fruit-specific RNAi-mediated suppression of *DET1* enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* 23, 890–895
29 Wei, S. *et al.* (2009) RNAi-mediated suppression of *DET1* alters the levels of carotenoids and sinapate esters in seeds of *Brassica napus*. *J. Agric. Food Chem.* 57, 5326–5333
30 Diretto, G. *et al.* (2006) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene ε-cyclase. *BMC Plant Biol.* 6, 13
31 Yu, B. *et al.* (2008) Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene ε-cyclase. *Transgenic Res.* 17, 573–585
32 Harjes, C.E. *et al.* (2008) Natural genetic variation in lycopene ε-cyclase tapped for maize biofortification. *Science* 319, 330–333
33 Van Eck, J. *et al.* (2007) Enhancing β-carotene content in potato by RNAi-mediated silencing of the β-carotene hydroxylase gene. *Am. J. Potato Res.* 84, 331–342
34 Romer, S. *et al.* (2000) Elevation of the provitamin A content of transgenic tomato plants. *Nat. Biotechnol.* 18, 666–669
35 Vallabhaneni, R. *et al.* (2009) Metabolite sorting of a germplasm collection reveals the *Hydroxylase3* locus as a new target for maize provitamin A biofortification. *Plant Physiol.* 150, 1635–1645
36 US Institute of Medicine (2001) *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc*, National Academy Press
37 Sommer, A. (2008) Vitamin A deficiency and clinical disease: an historical overview. *J. Nutr.* 138, 1835–1839
38 Farré, G. *et al.* (2010) The humanitarian impact of plant biotechnology: recent breakthroughs vs bottlenecks for adoption. *Curr. Opin. Plant Biol.* 13, 219–225
39 Darnton-Hill, I. and Nalubola, R. (2002) Fortification strategies to meet micronutrient needs: successes and failures. *Proc. Nutr. Soc.* 61, 231–241
40 Gomez-Galera, S. *et al.* (2010) Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Res.* 19, 165–180
41 Bai, C. *et al.* (2011) A golden era – pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol. Plants* 47, 205–221
42 Sommerburg, O. *et al.* (1998) Fruits and vegetables that are sources of lutein and zeaxanthin: the macular pigment in human eye. *Br. J. Ophthalmol.* 82, 907–910
43 Mínguez-Mosquera, M.I. and Hornero-Méndez, D. (1994) Comparative study of the effect of paprika processing on the carotenoids in peppers (*Capsicum annuum*) of the Bola and Agridulce varieties. *Food Chem.* 42, 1555–1560
44 Landrum, J.T. and Bone, R.A. (2001) Lutein, zeaxanthin, and the macular pigment. *Arch. Biochem. Biophys.* 385, 28–40
45 Botella-Pavia, P. and Rodriguez-Concepcion, M. (2006) Carotenoid biotechnology in plants for nutritionally improved foods. *Physiol. Plant.* 126, 369–381
46 Fraser, P.D. and Bramley, P.M. (2004) The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* 43, 228–265
47 Hammond, B.R. *et al.* (1997) Dietary modification of human macular pigment density. *Invest. Ophthalmol. Vis. Sci.* 38, 1795–1801
48 Landrum, J.T. *et al.* (1997) A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Exp. Eye Res.* 65, 57–62
49 Kean, E.G. *et al.* (2007) Characterization of carotenoid pigments in mature and developing kernels of selected yellow-endosperm sorghum varieties. *J. Agric. Food Chem.* 55, 2619–2626
50 Fraser, P.D. *et al.* (1994) Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiol.* 105, 405–413
51 Murkovic, M. *et al.* (2002) Carotenoid content in different varieties of pumpkins. *J. Food Comp. Anal.* 15, 633–638
52 Ampomah-Dwamena, C. *et al.* (2009) The kiwifruit lycopene β-cyclase plays a significant role in carotenoid accumulation in fruit. *J. Exp. Bot.* 60, 3765–3779
53 Kurilich, A.C. and Juvik, J.A. (1999) Quantification of carotenoid and tocopherol antioxidants in *Zea mays*. *J. Agric. Food Chem.* 47, 1948–1955
54 Aluru, M. *et al.* (2008) Generation of transgenic maize with enhanced provitamin A content. *J. Exp. Bot.* 59, 3551–3562

55 Romer, S. *et al.* (2002) Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metab. Eng.* 4, 263–272
56 Ducreux, L.J.M. *et al.* (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *J. Exp. Bot.* 56, 81–89
57 Lopez, A.B. *et al.* (2008) Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* 59, 213–223
58 Diretto, G. *et al.* (2007) Silencing of β-carotene hydroxylase increases total carotenoid and β-carotene levels in potato tubers. *BMC Plant Biol.* 7, 11
59 Maass, D. *et al.* (2009) Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PLoS ONE* 4, e6373
60 Wei, S. *et al.* (2010) Enhanced seed carotenoid levels and branching in transgenic *Brassica napus* expressing the *Arabidopsis miR156b* gene. *J. Agric. Food Chem.* 58, 9572–9578
61 Dharmapuri, S. *et al.* (2002) Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Lett.* 519, 30–34
62 Fraser, P.D. *et al.* (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1092–1097
63 Enfissi, E.M. *et al.* (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol. J.* 3, 17–27
64 Giliberto, L. *et al.* (2005) Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.* 137, 199–208
65 Fraser, P.D. *et al.* (2007) Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* 19, 3194–3211
66 Simkin, A.J. *et al.* (2007) Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry* 68, 1545–1556
67 Wurbs, D. *et al.* (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *Plant J.* 49, 276–288
68 Apel, W. and Bock, R. (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. *Plant Physiol.* 151, 59–66
69 Zhang, J. *et al.* (2009) Functional characterization of citrus *PSY* gene in Hongkong kumquat (*Fortunella hindsii* Swingle). *Plant Cell Rep.* 28, 1737–1746

The regulation of carotenoid pigmentation in flowers

Changfu Zhu [a,b], Chao Bai [a], Georgina Sanahuja [a], Dawei Yuan [a], Gemma Farré [a],

Shaista Naqvi [a], Lianxuan Shi [b], Teresa Capell [a], Paul Christou [a,c,*]

Arch. Biochem. Biophys. (2010), 504: 132–141

*Corresponding author Zhu Changfu (email: zhu@pvcf.udl.cat)

Archives of Biochemistry and Biophysics 504 (2010) 132–141

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review

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Changfu Zhu [a,b], Chao Bai [a], Georgina Sanahuja [a], Dawei Yuan [a], Gemma Farré [a], Shaista Naqvi [a], Lianxuan Shi [b], Teresa Capell [a], Paul Christou [a,c,*]

[a] Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida, Av. Alcalde Rovira Roure, 191, Lleida 25198, Spain
[b] School of Life Sciences, Northeast Normal University, Changchun 130024, China
[c] Institucio Catalana de Recerca i Estudis Avancats, Passeig Llúis Companys, 23, Barcelona 08010, Spain

ARTICLE INFO

Article history:
Available online 3 August 2010

Keywords:
Carotenoids
Ketocarotenoids
Regulation
Gene expression
Flower color
Chromoplast
Transgenic plants

ABSTRACT

Carotenoids fulfill many processes that are essential for normal growth and development in plants, but they are also responsible for the breathtaking variety of red-to-yellow colors we see in flowers and fruits. Although such visual diversity helps to attract pollinators and encourages herbivores to distribute seeds, humans also benefit from the aesthetic properties of flowers and an entire floriculture industry has developed on the basis that new and attractive varieties can be produced. Over the last decade, much has been learned about the impact of carotenoid metabolism on flower color development and the molecular basis of flower color. A number of different regulatory mechanisms have been described ranging from the transcriptional regulation of genes involved in carotenoid synthesis to the control of carotenoid storage in sink organs. This means we can now explain many of the natural colorful varieties we see around us and also engineer plants to produce flowers with novel and exciting varieties that are not provided by nature.

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Carotenoids are organic molecules comprising a C_{40} polyene backbone that is often cyclized to generate terminal ionone rings. This structure allows carotenoids to absorb short-wave visible light, and the wavelengths that are absorbed depend on the number and nature of the double bonds. Carotenoids are therefore pigments that range in color from yellow through orange to red. They are produced mainly by photosynthetic organisms, and in plants they are synthesized in both chloroplasts and chromoplasts, imparting color to photosynthetic tissues as well as fruits, storage organs and flowers [1].

In mature chloroplasts, carotenoids fulfill a number of functions essential in photosynthesis such as photosystem assembly, light harvesting and protection from photo-oxidation at high light intensities [2,3]. In contrast, carotenoids in chromoplasts appear to be synthesized primarily for the purpose of attracting other organisms, such as pollinating insects and seed-distributing herbivores [4]. Carotenoids are also important substrates for a class of carotenoid cleavage dioxygenases (CCDs)[1] that generate biologically active apocarotenoids such as pro-vitamin A [5], abscisic acid [6] and the recently-discovered hormone strigolactone [7,8]. Other apocarotenoids are well known as food colorings, cosmetics, flavors and fragrances, such as bixin, crocetin and β-ionone [9–12].

There is considerable interest in chromoplast-derived carotenoids because of their two direct benefits to humans – as antioxidants and pigments. When expressed in fruits and storage organs, carotenoids are primarily valued for their antioxidant properties, and we have discussed recent advances in the nutritional improvement of cereals, fruits and root vegetables by carotenoid metabolic engineering in other review articles [13–15]. Other excellent reviews have covered carotenoid metabolism and metabolic engineering [16–18], and the regulation of carotenoid biosynthesis using promoters, transcription factors, chromatin regulation and compartmentalization [19]. Here we focus on carotenoids as pigments in flowers. This has a strong impact in the cut flower industry purely for aesthetic reasons, but also in the areas of traditional medicines and nutrition because certain carotenoids are extracted

* Corresponding author at: Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida, Av. Alcalde Rovira Roure, 191, Lleida 25198, Spain. Fax: +34 973 238264.
E-mail address: christou@pvcf.udl.es (P. Christou).

[1] *Abbreviations used:* CCDs, carotenoid cleavage dioxygenases; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MEP, methylerythritol 4-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; ABA, abscisic acid; CCS, capsanthin–capsorubin synthase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase.

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doi:10.1016/j.abb.2010.07.028

Table 1
Carotenoid composition in petals of fully open flowers.

Species	Cultivars or mutants	Major carotenoids	Minor carotenoids	References
Solanum lycopersicum	Wild type	Violanxanthin and neoxanthin	Lutein	[44]
Solanum lycopersicum	*old-gold* (og)[a]	Violanxanthin and neoxanthin	Lutein and lycopene	[52]
Solanum lycopersicum	*tangerine*[3183b]	Neoxanthin, violaxanthin, phytoene and rubixanthin	Prolycopene, phytofluene, ζ-carotene, neurosporene, lycopene, γ-carotene, β-carotene, β-cryptoxanthin and lutein	[56]
Solanum lycopersicum	*tangerine*[micc]	Prolycopene, phytoene, neoxanthin, phytofluene and ζ-carotene	*cis*-Neurosporene, neurosporene, di-*cis*-lycopene, β-carotene and rubixanthin	[56]
Solanum lycopersicum	*white-flower* (*wf*)[d]	β-Carotene and neoxanthin,	Violaxanthin, lutein and phytoene	[44]
Solanum lycopersicum	*high-pigment 3* (*hp3*)[e]	Zeaxanthin	Antheraxanthin, neoxanthin, violaxanthin and lutein	[57]
Narcissus pseudonarcissus		Lutein	β-Carotene	[58]
Tagetes erecta	Lady	Lutein		[42]
Gentiana lutea		Lutein, violaxanthin, β-carotene and antheraxanthin	Neoxanthin, zeaxanthin and β-cryptoxanthin	[59]
Ipomoea sp.		β-Cryptoxanthin and zeaxanthin	Lutein, neoxanthin and violaxanthin	[60]
Ipomoea obscura			Zeaxanthin, lutein, neoxanthin and violaxanthin	[60]
Lilium spp.[f]	Connecticut King	Antheraxanthin, (9*Z*)-violaxanthin and *cis*-lutein	Violaxanthin, lutein and β-carotene	[61]
Lilium spp.[f]	Montreux	Violaxanthin, lutein and β-carotene	Antheraxanthin, (9*Z*)-violaxanthin and *cis*-lutein	[61]
Lilium spp.[f]	Saija	Capsanthin	Antheraxanthin	[61]
Chrysanthemum morifolium Ramat.	Yellow Paragon	Lutein		[62]
Cucumis sativus	var. Anguria	Lutein, β-carotene, flavoxanthin, auroxanthin and zeaxanthin	β-Crytoxanthin, α-carotene and violaxanthin	[63]
Nicotiana glauca		Lutein and β-carotene	Violaxanthin, antheraxanthin and β-cryptoxanthin	[64]
Lotus japonicus		Violaxanthin and antheraxanthin	Neoxanthin, lutein, zeaxanthin and β-carotene	[65]
Oncidium Gower Ramsey[g]		All-*trans* violaxanthin and 9-*cis*-violaxanthin	All-*trans* neoxanthin, 9-*cis*-neoxanthin and lutein	[66]
Crocus sativus[h]		Zeaxanthin, ζ-carotene and β-carotene	β-Cryptoxanthin, lycopene and phytofluene	[67]
Adonis aestivalis		Astaxanthin	3-Hydroxyechinenone, adonixanthin and adonirubin	[68,69]

[a] Accumulation of ~20% lycopene in the petals of *og* (null mutant of *CrtL-b2* encoding LYCB) compared with that of the wild type.
[b] Prolycopene (CRTISO substrate), phytofluene, ζ-carotene and neurosporene accumulate because *CRTISO* gene expression is impaired in *tangerine*[3183].
[c] Prolycopene, phytoene, phytofluene, ζ-carotene, *cis*-neurosporene and di-*cis*-lycopene accumulate due to loss of CRTISO function in *tangerine*[mic].
[d] The elimination of *CrtR-b2* (encoding BCH2) in *wf* petals results in an 80% reduction in total carotenoid concentration, possibly due to the inability of petals to store high concentration of carotenoids other than xanthophylls, and by degradation of β-carotene which accumulates as a result of the *wf* mutation.
[e] Petals of *hp3* accumulate large amounts of zeaxanthin, minute quantities of violaxanthin and neoxanthin compared to that of wild type since a substitution mutation in ZEP, which results in the reduction activity of ZEP in *hp3*.
[f] Fully developed tepals.
[g] Fully developed lips.
[h] Fully developed stigmata.

from dried flowers and used as food colorings and flavors. Although the leaves of most plants have a similar carotenoid composition, flowers offer distinct carotenoid profiles that depend on the species and variety, in the latter case often reflecting the effects of single-gene mutations (Table 1) [20]. In this review, we provide an overview of the molecular basis of carotenoid synthesis in flower chromoplasts and discuss recent advances in metabolic engineering that have been used to alter the carotenoid content of flowers.

Overview of carotenoid synthesis and metabolism in plants

In plants, carotenoids are synthesized *de novo* from the isomeric C_5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [21]. The reaction occurs in plastids and both precursors are derived primarily from the plastidial methylerythritol 4-phosphate (MEP) pathway [22,23] as shown in Fig. 1. The condensation of three IPP units and one molecule of DMAPP produces geranylgeranyl diphosphate (GGPP, C_{20}), a reaction catalyzed by the enzyme GGPP synthase (GGPPS). The first committed step in the pathway is the condensation of two molecules of GGPP into the colorless carotenoid precursor 15-*cis*-phytoene (C_{40}) by phytoene synthase (PSY) [24]. This is converted into bright red all-*trans* lycopene, the first pigmented carotenoid, in a series of four desaturation reactions catalyzed by phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). The product of the first desaturation is 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized by the recently characterized Z-ISO [25] and/or light to yield 9,9′-di-*cis*-ζ-carotene, the substrate for ZDS (Fig. 1) [26]. The end product of the desaturation reactions, 7,9,7′,9′-tetra-*cis*-lycopene (prolycopene), is converted to all-*trans* lycopene by CRTISO in non-green tissue, but by light in green tissue [26,27].

Lycopene represents the branch point of the carotenoid pathway because it acts as the substrate for two competing enzymes, lycopene β-cyclase (LYCB) and lycopene ε-cyclase (LYCE). Both enzymes cyclize the linear backbone to generate terminal ionone rings, but the structures of these rings are distinct. The addition of one β-ring to lycopene by LYCB generates γ-carotene, and the addition of a second β-ring to the free end by the same enzyme produces the orange pigment β-carotene, which is further converted to the yellow pigment zeaxanthin by the di-iron non-heme β-carotene hydroxylase BCH and/or the P450-type β-carotene hydroxylase CYP97A and CYP97B, in a two-step reaction via β-cryptoxanthin [28–33]. Alternatively, the addition of one ε-ring to lycopene by LYCE generates δ-carotene. This is a poor substrate for LYCE so it is unusual for the second ε-cyclization to take place, but it is a good substrate for LYCB which adds a β-ring to the free end generating the orange pigment α-carotene. In turn, α-carotene is converted into zeinoxanthin by BCH and/or CYP97A and CYP97B, and then into the yellow pigment lutein by the P450-type ε-hydroxylase, CYP97C [32–34] (Fig. 1). Whereas lutein represents the natural end point of the α-carotene branch, zeaxanthin enters the xanthophyll cycle [35] through the stepwise

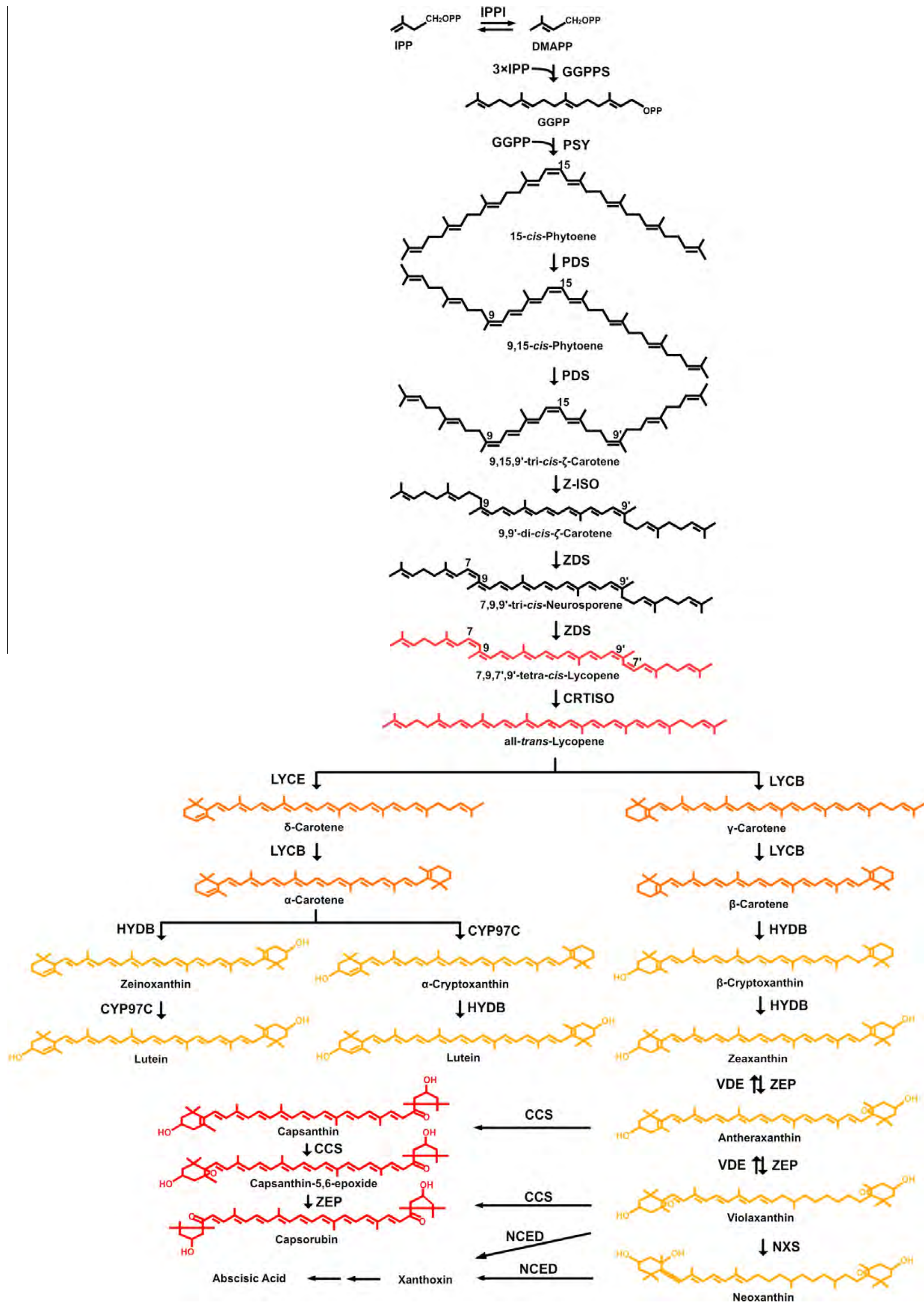

Fig. 1. Carotenoid biosynthetic pathway in higher plants. Abbreviations: IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CCS, capsanthin–capsorubin synthase; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene hydroxylase (BCH, CYP97A or CYP97B); ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

activities of zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE). The resulting yellow pigments antheraxanthin and violaxanthin are then converted to yellow neoxanthin by neoxanthin synthase (NXS) [36,37]. Finally, the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED, VP14) cleaves the 11,12(11′,12′) double bonds of the 9-*cis* isomers violaxanthin and neoxanthin to produce xanthoxin, the precursor of abscisic acid (ABA) (Fig. 1) [38,39].

In some plants, the biosynthesis pathway extends further reflecting an ability to synthesize specialized ketocarotenoids. One such example is the red fruits of chili peppers, in which the red ketocarotenoids capsanthin and capsorubin are synthesized from antheraxanthin and violaxanthin by capsanthin–capsorubin synthase (CCS) (Fig. 1) [40]. Another example, which we consider in more detail later, is the ornamental plant Summer pheasant's-eye (*Adonis aestivalis*) whose petals synthesize the red ketocarotenoid astaxanthin (Fig. 2), which is usually found only in microbes [41].

The quantity and diversity of carotenoids varies widely in the chromoplasts of different plants even within a single species (Table 1), and this reflects the different regulatory mechanisms affecting the carotenoid biosynthesis pathway. Many flowers with white petals contain few carotenoid molecules, whereas the dark orange petals of some marigold (*Tagetes erecta*) flowers contain up to 20-fold the carotenoid content of leaves [42]. Three major mechanisms are known to affect carotenoid accumulation in chromoplasts: (1) the transcriptional or post-transcriptional regulation of genes controlling carotenoid biosynthesis; (2) the transcriptional or post-transcriptional regulation of genes controlling carotenoid degradation; and (3) the regulation of lipoprotein-sequestering structures that act as carotenoid sinks. These mechanisms are discussed in more detail below using specific case studies.

Carotenoid biosynthesis and accumulation controlled by transcription of carotenogenic genes

The transcriptional regulation of carotenogenic genes is an important mechanism that contributes to the accumulation of specific carotenoids during flower development. The next section looks at specific case studies to illustrate how transcriptional regulation affects carotenoid accumulation in the flowers of different species.

Tomato

Tomato (*Solanum lycopersicum* L.) is widely regarded as the most important model plant species for studying carotenoid accumulation because of its diverse germplasm and the different carotenoid profiles in leaves, flowers and fruits [43]. The red color of ripe fruits reflects the accumulation of lycopene, whereas the intense yellow color of the flowers reflects accumulation of the xanthophylls violaxanthin and neoxanthin [44]. As in other species, the color of tomato flowers is thought to attract pollinating insects and that of the fruit is thought to attract herbivores for seed dispersal [45].

Carotenoid biosynthesis is controlled by different regulatory mechanisms in tomato tissues containing chloroplasts and chromoplasts [46]. Underlying this fact, at least four of the carotenogenic genes in tomato come in pairs, one set expressed preferentially in leaves (containing chloroplasts) and the other in flowers and fruit (containing chromoplasts) [44]. The enzymes GGPPS, PSY and BCH are thus represented by *GGPPS1*, *PSY2* and *CRTR-b1*, which are expressed preferentially in photosynthetic tissue, and by *GGPPS2*, *PSY1* and *CRTR-b2*, which are expressed more strongly in flowers and fruits [44,47–50]. In the case of LYCB, *CRTL-b1* is expressed in leaves and at very low levels in flowers whereas *CRTL-b2* (CYC-B) is expressed strongly in flowers and at very low levels in fruits [51,52]. The carotenoid content of tomato flowers increases approximately 10-fold during development and this is coincident with strong increases in the steady state levels of *PSY1* and *PDS* mRNAs [53]. This evidence indicates that carotenoid accumulation in tomato flowers is predominantly controlled at the level of transcription. Galpaz et al. [44] concluded that there has been strong selection pressure to maintain separate chromoplast isoforms of the carotenogenic enzymes to regulate the pigmentation of flowers and fruit. The regulation of carotenoid biosynthesis

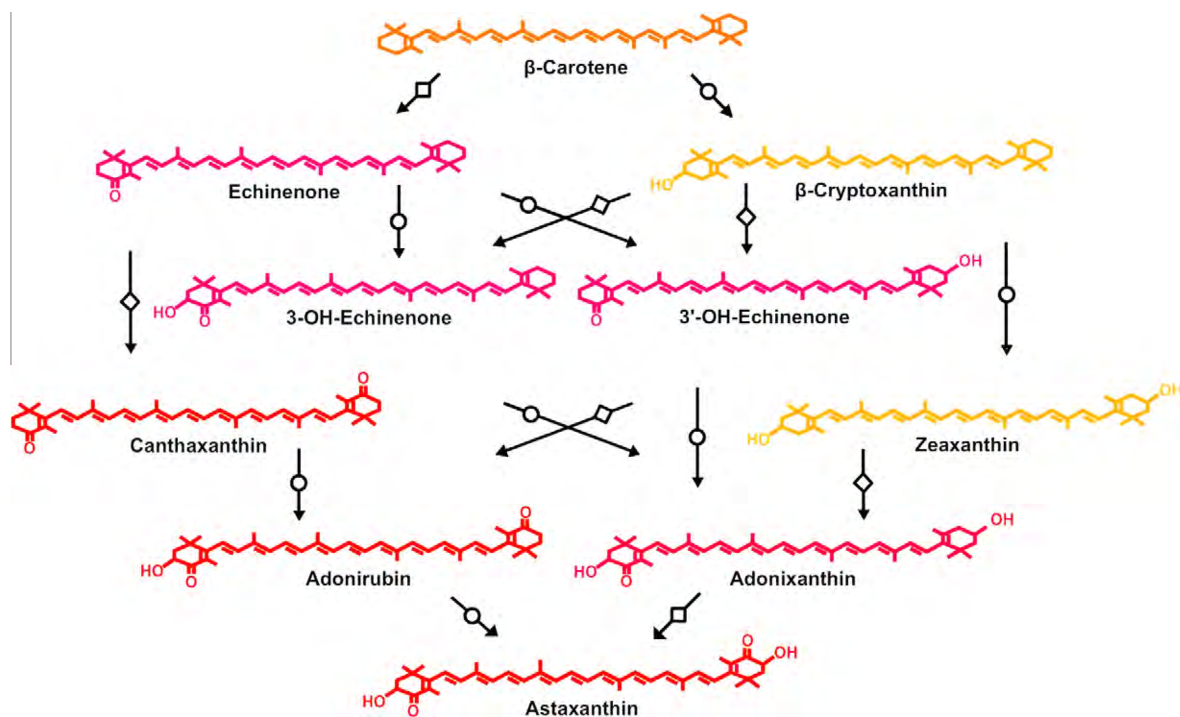

Fig. 2. Astaxanthin biosynthesis pathway from β-carotene. Arrow with inset square represents β-carotene ketolase – BKT, CRTW or CRTO. Arrow with inset circle represents β-carotene hydroxylase (HYDB) – BCH, CYP97A, CYP97B or CRTZ.

tomato flowers and fruits has been elucidated by functional analysis of the *PDS* and *CYC-B* promoters, both of which are preferentially expressed in chromoplast-containing tissues [54,55]. *PDS* is upregulated in the chromoplast-rich anthers of flowers, and in fruits at different stages of ripening [54]. The *CYC-B* promoter contains conserved *cis*-acting elements such as RAP2.2 and ERE (ethylene responsive element), which are responsible for a common regulation of carotenoid accumulation in flowers and fruits [55]. It will be interesting to characterize the signal transduction components and transcription factors that control *CYC-B* expression in order to provide further insight into carotenoid metabolism in tomato.

Marigold

Marigold flowers are a major commercial source of lutein, which is responsible for the orange to yellow hues of marigold petals. Carotenoid yields vary approximately 100-fold between white flowers and the most pigmented varieties [42], the latter of which are ground up and added to poultry feed [70] in order to improve the quality of egg yolks [71]. As in tomato, carotenoid accumulation in marigold appears to be regulated primarily at the level of transcription, but there is conflicting evidence as to which genes represent the primary bottlenecks in carotenoid biosynthesis.

Moehs et al. [42] cloned many of the relevant carotenogenic genes, i.e. those encoding PSY, PDS, LYCB, LYCE, BCH, and also the genes coding for 1-deoxy-D-xylulose 5-phosphate synthase (DXS) responsible for the first and rate-limiting step of the mevalonate-independent MEP pathway, isopentenyl diphosphate isomerase (IPP) and geranylgeranyl diphosphate (GGPP) synthase. They also cloned two genes involved in plastid division and replication (*MinD* and *FtsZ*). They surveyed mRNA levels in four marigold cultivars ranging in color from white to dark orange, with approximately equal carotenoid levels in green tissue. The clear finding was that the abundance of *PSY* mRNA correlated precisely with the carotenoid levels in flowers [42]. They also found that the mRNA for LYCE was expressed strongly whereas that for LYCB was not induced in the more pigmented flowers, indicating that the amount of lutein was determined predominantly by LYCE activity. In contrast to the above, Del Villar-Martinez et al. [72] found that the amount of *PSY* mRNA was not related to carotenoid content, e.g. there was more *PSY* mRNA in the yellow Alcosa variety than the deep orange Crackerjack variety. They also found that the mRNA for LYCB was barely detectable in the white variety Snowdrift, but was strongly expressed in Alcosa and Crackerjack [72]. It is possible that these differences are attributable to distinct regulatory mechanisms in different varieties with similar phenotypes, e.g. Moehs et al. [42] used the non-pigmented variety French Vanilla rather than the Snowdrift variety used by Del Villar-Martinez et al. [72].

Gentian

Gentian (*Gentiana lutea*) is a perennial plant with medicinal properties that has been adopted as model for carotenogenesis because of its bright yellow flowers, which are rich in β-carotene and xanthophylls [59]. Genes encoding all the major carotenogenic enzymes have been cloned from a yellow petal cDNA library, and the corresponding enzyme activities (i.e. GGPPS, PSY, PDS, ZDS, LYCB, LYCE, BCH and ZEP) have been verified by complementation in *Escherichia coli* [59,73].

Flower development involves the upregulation of carotenoid synthesis and a switch in emphasis from the ε-branch to the β-branch, with a corresponding shift in carotenoid profile from lutein to zeaxanthin, antheraxanthin and neoxanthin [59,73]. Underlying this, the *PSY* and *ZDS* mRNAs are strongly upregulated (6- to 7-fold) during development, coincident with a moderate upregulation of *LYCB*, *BCH* and *ZEP* (2-fold) and a fall in the level of *LYCE* mRNA to approximately 50% of its original level [59]. Interestingly, the level of *GGPPS* mRNA decreases over the same period, which suggests carotenoid synthesis utilizes an exclusive pool of GGPP that accumulates in the early stages of development [73].

Morning glory

The genus *Ipomoea* (morning glory) is renowned for its diverse flower colors including many species with carotenoid-rich yellow and orange petals. But the Japanese morning glory (*Ipomoea nil*) lacks a yellow cultivar and does not accumulate carotenoids. A recent study by Yamamizo et al. [60] compared Japanese morning glory with two yellow-flowered species in an attempt to determine the molecular basis of this phenomenon. During early flower development, all the species accumulated lutein, violaxanthin and β-carotene in the petals, the same carotenoids that accumulate in leaves. However, the yellow flowers switched to chromoplast-type carotenoid accumulation later in development (β-cryptoxanthin, zeaxanthin and β-carotene) and also contained esterified β-cryptoxanthin and zeaxanthin, as also seen in marigold and *Eustoma* [42,74]. The white flowers, however, did not accumulate any chromoplast-type carotenoids [60].

Genes encoding the enzymes involved in isoprenoid and carotenoid biosynthesis (*IPPI*, *GGPPS*, *PSY*, *PDS*, *ZDS*, *CRTISO*, *LYCB*, *LYCE* and *BCH*) and carotenoid cleavage (*ccd1* and *ccd4*) were isolated from immature petals [60] and it was noted that many of the genes were expressed at lower levels in the white petals compared to the yellow ones, particularly *BCH*. However, the expression of *CCD1* and *CCD4* did not correlate with the carotenoid level in petals [60]. These results suggest that the white color in petals is due to the lack of the ability to synthesize chromoplast-type carotenoids because of the transcriptional down-regulation of carotenogenic genes [60].

Lilies

The Asiatic hybrid lily (*Lilium* spp.) is another commercially valuable ornamental plant with flower colors ranging from red, orange and yellow (carotenoids), through pink (anthocyanins) to white. Carotenoid profiling has shown that most of the carotenoids in yellow petals are antheraxanthin, (9*Z*)-violaxanthin, *cis*-lutein and violaxanthin, whereas red petals are unusual in that they accumulate capsanthin (Table 1) [61,75]. The increase in carotenoid content in yellow cultivars was mirrored by the induction of *PSY*, *PDS*, *ZDS*, *CRTISO* and *BCH* mRNA expression, while *LYCB* expression remained constant and *LYCE* expression diminished, suggesting that transcriptional regulation is the predominant control mechanism [61]. However, the steady state levels of *PSY*, *PDS*, *ZDS*, *CRTISO* and *BCH* were similar in red and white petals, indicating another mechanism was probably responsible for the lack of carotenoids in white petals, perhaps the expression of CCDs although this remains to be demonstrated [61].

Carotenoid biosynthesis and accumulation controlled by carotenogenic enzyme levels

Post-transcriptional regulation of carotenogenic enzyme activity also plays a role in controlling carotenoid biosynthesis and accumulation in flowers. The yellow flowers of the wild daffodil *Narcissus pseudonarcissus* reflect the accumulation of large amounts of lutein and lower amounts of β-carotene and its derivatives [58]. Isolated chromoplasts can be used to synthesize β-carotene when fed with IPP, but upon disintegration they also yield significant amounts of α-carotene and ζ-carotene [76]. As is the case in tomato, there is evidence that carotenogenic genes in daffodil are transcriptionally upregulated during flower development [77] but several studies

have also shown that the corresponding enzymes are regulated post-transcriptionally by compartmentalization.

Daffodil flower development is characterized by the production of chromoplasts from chloroplast-like precursors, a process that involves the disassembly of thylakoids and the photosynthetic apparatus, the enhanced synthesis of membrane lipids and enhanced carotenoid biosynthesis [78]. Carotenoid accumulation is strongly induced during daffodil flower development and steady-state *PSY* transcript levels remain constant or even decrease from stage 1 to stage 4 [81]. In contrast to *PSY* expression there is an increase in PSY protein levels during flower stages 1 and 2 [81]. The level of PDS protein also increases to some extent during flower development [80]. However, the increases in PSY and PDS expression do not appear to be related to functionality, as the proteins detected include one soluble, complexed and inactive form, and a second, membrane-bound and active form [80,81]. The carotenogenic enzymes, which are all encoded by nuclear genes, are imported into the chromoplast and assembled into oligomeric complexes. The plastid molecular chaperones Cpn60 and Hsp70 are strongly upregulated during this process to facilitate the correct folding of the imported enzymes and the assembly of the multi-protein complex [79]. The carotenogenic complex is functional only when it is associated with the chromoplast membrane, but Al-Babili et al. [80] reported that the enzymes PSY and PDS could either be found associated with the membrane or as free, soluble proteins in the chromoplast milieu. The soluble forms of both PSY and PDS were non-functional, but could become activated by membrane association [80,81]. The PDS enzyme was localized correctly with respect to the membrane-bound lipophilic substrate and lipophilic co-factors such as quinones [80]. It has been speculated that the posttranslational mechanisms, a redox mechanism, in which PDS in conjunction with a membrane-localized redox chain employing quinones as intermediate electron acceptors [82] and oxygen as the final electron acceptor [76], may contribute to color formation, given the very low expression of PDS in carotenoid-overaccumulating chromoplasts [80].

Regulating carotenoid degradation

The abundance of CCDs

The enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) has been isolated from many different plants, the first being the corn *viviparous14* (*vp14*) gene product [6,83]. It is not yet clear whether this enzyme significantly affects carotenoid metabolism in flowers. For example, Zhu et al. [84] recently cloned two NCED cDNAs from gentian, one of which (*GlNCED1*) was expressed in flowers (strongly in the stamens, less strongly in the petals) but its impact on carotenoid accumulation is unknown. There are nine NCED homologs in Arabidopsis, with complex spatiotemporal expression patterns [85], and *AtNCED6* is the most closely related to *GlNCED1*. The first Arabidopsis NCED to be discovered (AtNCED1 or AtCCD1) has many orthologs including genes in petunia [11], tomato [12], crocus [9,86], grape [87] and rose [88]. The expression of *CCD1* is temporally associated with the emission of apocarotenoid volatiles in petunia corolla [11], grape berries [89] and tomato fruits [12]. The highest *CCD1* mRNA levels were detected in flower tissue, specifically in corollas, and *CCD1* regulation appears to fit with similar oscillations in the expression of *PDS* and *ZDS* indicating a circadian rhythm [11]. The examples below discuss specific case studies where CCDs are known to have a significant impact on carotenoid levels in flowers.

Regulating carotenoid degradation in crocus

Crocus (*Crocus sativus)* is a triploid sterile plant characterized by its long red stigmata, which when desiccated give rise to the expensive spice saffron [89]. The intense color of saffron is caused by apocarotenoids such as *cis*- and *trans*-crocins (crocetin digentiobiose ester), picrocrocin and its degradation product safranal, which represents 70% of total volatiles and gives saffron its distinct aroma [90]. These saffron apocarotenoids are generated by the cleavage and glucosylation of zeaxanthin [91]. The ability to synthesize these compounds is not common in plants. Crocin and picrocrocin are only found in crocus stigmata, in fruits of the genus *Gardenia* [92], in flowers of the genus *Buddleja* [93], and in two further species, *Jacquinia angustifolia* [94] and *Coleus forskolii* [95].

As is the case for other flowers, the accumulation of carotenoids in crocus appears to be controlled by the transcriptional regulation of corresponding carotenogenic genes during flower development, in this case the genes encoding PSY, LYCB and BCH [67]. Two different lycopene cyclase genes have been identified (*LYCB1* and *LYCB2a*), and the expression of *LYCB2a* in different crocus species indicates that transcriptional regulation of this gene affects both the carotenoid and apocarotenoid content in the stigmata [96]. The absence of *LYCB2a* orthologs in plants that do not accumulate high levels of carotenoids suggests that *LYCB2a* is a *LYCB1* paralog that has acquired a new expression pattern by sub-functionalization, allowing the tight control of carotenoids in very specific tissues. A positive correlation between zeaxanthin accumulation and *BCH* expression in fully developed stigmata suggests that zeaxanthin production is regulated at the transcriptional level, and that BCH enzyme activity could be the rate-limiting step in the formation of saffron apocarotenoids in the stigma [67].

The predominant apocarotenoid product in developing crocus stigmata is crocetin, although this is converted into its glucoside derivatives and picrocrocin as each stigma matures [97]. The volatiles profile undergoes significant changes during development, and in red stigmata the predominance of β-cyclocitral suggests that both β-carotene and zeaxanthin are involved in the synthesis of crocetin. As the stigmata mature, hydroxy-β-ionone and β-ionone are produced along with very low quantities of safranal [97]. These important volatiles are derived from carotenoids by the action of CCDs [98].

The crocus zeaxanthin 7,8(7′,8′) cleavage dioxygenase gene (*ZCD*) encodes a chromoplast enzyme that catalyses the synthesis of crocetin dialdehyde (C_{20}) and hydroxy-β-cyclocitral (C_{10}) from zeaxanthin [9]. More recently, Rubio et al. [86] identified four stigma-specific CCD genes (*CsCCD1a*, *CsCCD1b*, *CsCCD4a* and *CsCCD4b*), which belong to two different families, *CCD1* and *CCD4*. They have the same enzymatic activity even though they are localized in different compartments. It has been suggested that each enzyme class is responsible for carotenoid metabolism in a different subcellular compartment, during both normal development and in response to stress [86].

The subsequent step in apocarotenoid synthesis involves glucosylation of the crocetin and picrocrocetin cleavage products. Two crocetin GTase activities (UDP-glucose:crocetin 8,8′-O-glucosyltransferase and UDP-glucose:crocetinglucosylester 6′-O-glucosyltransferase) are thought to be present in saffron [99]. A glycosyltransferase gene (*UGTCs2*) encoding an enzyme that adds glucose to crocetin has also been isolated [100].

Regulating carotenoid degradation in chrysanthemum

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is a commercially valuable ornamental plant with bright yellow petals, mainly reflecting the accumulation of lutein [101]. Flower development involves a switch from lutein, β-carotene and violaxanthin synthesis to the predominant synthesis of lutein, which appears to involve the transcriptional upregulation of *LYCE* [102]. In contrast to the flowers, *LYCB* expression is higher in leaves, favoring the accumulation of β-carotene and its derivatives.

Interestingly, the yellow color of chrysanthemum is a recessive trait, with the dominant white trait apparently resulting from an allele that blocks carotenoid biosynthesis [103,104]. In this dominant white variety, the early accumulation of lutein, β-carotene and violaxanthin proceeds as normal, but the later accumulation of lutein is inhibited, resulting in the loss of pigmentation. In order to identify genes responsible for the loss of carotenoid accumulation in white flowers, Ohmiya et al. [62] cloned cDNAs that were differentially expressed in the yellow and white varieties, leading to the isolation of *CmCCD4a*, encoding a CCD homolog. The gene was petal-specific and was strongly expressed in white flowers but weakly expressed in yellow ones. RNAi constructs targeting the endogenous *CmCCD4a* mRNA were expressed in the white variety, resulting in a strong yellow color (Fig. 3a). This experiment confirmed that white chrysanthemums do synthesize carotenoids, but they are immediately degraded into colorless apocarotenoids.

'Jimba' is the most popular white-flowered chrysanthemum cultivar in Japan. A yellow-flowered cultivar with the same growth properties as 'Jimba' will benefit growers because both forms could be produced under the same conditions. Ohmiya et al. [105] recently succeeded in altering the petal color of 'Jimba' from white to yellow by introducing two separate *CmCCD4a* RNAi constructs. The transgenic 'Jimba' line with the deepest yellow flowers contained 102 μg/g FW carotenoids in the petals, and the expression level of *CmCCD4a* was 0.4% of the wild type level. Although the transformed plants were significantly smaller than the wild type, flower size was unchanged [105].

Extended ketocarotenoid synthesis in Adonis

Adonis (*A. aestivalis*), also known as Summer pheasant's-eye, has long petals which are orange for most of their length but have a deep red basal patch which gives the flower a striking, ocellar appearance. The color is caused by carotenoid accumulation, but unlike all other known flowering plants the pigments that accumulate are ketocarotenoids (Fig. 2) [68,69], predominantly astaxanthin (3,3′-dihydroxy-4,4′-diketo-β,β-carotene) with lesser amounts of 3-hydroxyechinenone (3-hydroxy-4-keto-β,β-carotene), adonirubin (3-hydroxy-4,4′-diketo-β,β-carotene) and adonixanthin (3,3′-dihydroxy-4-keto-β,β-carotene) [41]. The ability of *Adonis* flowers to synthesize and accumulate astaxanthin in such large quantities is therefore unique in the plant kingdom [106,107].

The ketocarotenoids are derived from zeaxanthin (3,3′-dihydroxy-β,β-carotene) which is present in the green tissues of most higher plants. Yu et al. [108] isolated the *CRTH1* (*BCH1*) gene, whose product was able to catalyze the formation of zeaxanthin and its intermediate precursor β-cryptoxanthin from β-carotene in functional assays carried out in *E. coli*. This gene is highly expressed in petals, roots and stems, with relatively low expression in leaves and developing seeds. The formation of astaxanthin from zeaxanthin requires only that a carbonyl group be introduced at the number 4 carbon of each β-ring, but it is thought this addition must occur prior to hydroxylation, i.e. β-carotene must be the substrate and echinenone (4-keto-β,β-carotene) and canthaxanthin (4,4′-diketo-β,β-carotene) would be the immediate products [109,110]. The genes for two β-carotene 4-ketolases required for this enzymatic step have been identified in *Adonis* and are named *Adketo1* and *Adketo2*. Sequence analysis suggested they were homologous to plant-type β-carotene 3-hydroxylases, but despite this similarity the enzymes demonstrated neither 4-ketolase nor 3-hydroxylase activity when presented with β-carotene as the substrate in *E. coli* [41]. Further investigation showed that they add carbonyl groups at the appropriate site via an indirect route that involves keto-enol tautomerization, i.e. desaturation at the 3,4 position and hydroxylation of the number 4 carbon.

Coupling carotenogenesis to chromoplast development and carotenoid storage

As stated above, Moehs et al. [42] cloned many of the carotenogenic genes from marigold to investigate their impact on flower color, and also found that the *MinD* and *FtsZ* genes were upregu-

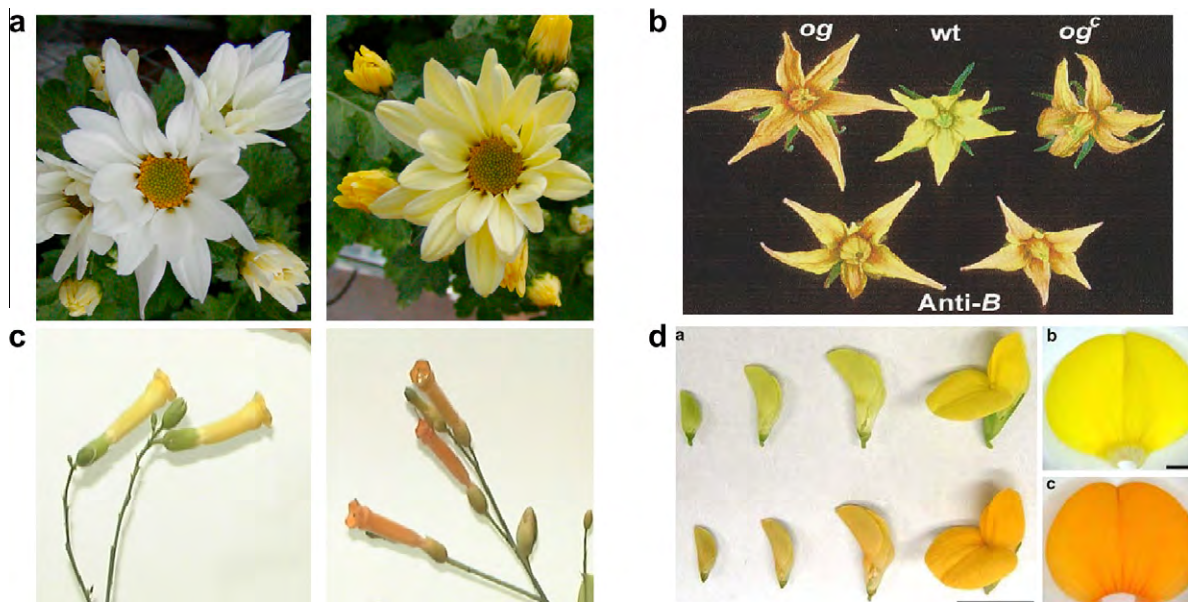

Fig. 3. (a) Suppression of endogenous carotenoid cleavage dioxygenase 4a (*CmCCD4a*) by RNAi in chrysanthemum, showing fully open wild type white flowers (left) and yellow flowers resulting from the inhibition of *CmCCD4a* (right) (from [62] with permission from Plant Physiology). (b) Wild type (wt) tomato flowers compared with mutants *old-gold* (*og*) and *old-gold crimson* (*og*^{c}) and with transgenic plant expressing antisense *CRTL-b2* (LYCB2) mRNA (from [52], Copyright 2000 National Academy of Sciences, USA, used with permission). (c) *Nicotiana glauca* flowers, wild type on left, transgenic plant expressing *Synechocystis* sp. PCC 6803 β-carotene ketolase (*CRTO*) on right (photo provided by C. Zhu and G. Sandmann). (d) The upper row shows a wild type *Lotus japonicus* flower, four developmental stages with sepals removed (bar = 2 mm) and a fully opened petal (bar = 10 mm). The bottom row shows the same stages for a transgenic flower expressing the *Agrobacterium aurantiacum* β-carotene ketolase gene (*CRTW*) (from [65] with permission from Plant Cell Reports).

lated in the most pigmented varieties during development while remaining at basal levels in paler varieties, suggesting a correlation between carotenogenesis and plastid replication. A full-length *FtsZ* cDNA has also been isolated from gentian petals but in this case the opposite expression profile was observed, with expression gradually declining during development [111]. There is only limited plastid replication during petal development in marigold [72] and gentian [111] because most chromoplasts arise from proplastids or pre-existing fully developed chloroplasts [112], so a link between plastid replication and carotenogenesis remains unlikely.

Although there is no definitive link between plastid replication and carotenoid accumulation, it is very likely that accumulation goes hand in hand with chromoplast differentiation. In support of this, a novel dominant mutation (*Orange*, *Or*) has been identified recently in cauliflower which causes the curd (edible inflorescence) to develop with a deep orange color [113]. The corresponding gene encodes a DnaJ homolog, not a carotenogenic enzyme, so it appears to be involved in protein folding and assembly. Further investigation has shown that the cellular phenotype of the mutation involves the arrest of plastid division and the differentiation of plastids into carotenoid-accumulating chromoplasts that act as a sink for carotenoid molecules [113]. Expression of an *Or* transgene in potato tubers did not affect the expression of endogenous carotenogenic genes, but nevertheless resulted in a 6-fold increase in carotenoid levels by increasing the capacity for carotenoid storage [114].

In chloroplasts, almost all carotenoid molecules are associated with functional light-harvesting complexes [115]. In contrast, chromoplasts have evolved as specialized structures whose major function is the storage and accumulation of carotenoids and other pigments [116,117]. Storage in certain chromoplast-types is achieved by carotenoid esterification which allows their association with specialized proteins known as fibrillins or plastid lipid associated proteins. Hydroxylation is an important process for the esterification of carotenoids, and the upregulation of BCH activity has been shown to accelerate carotenoid accumulation in many flowers, including morning glory [60], tomato [44] and lily [61].

The fibrillin-carotenoid conjugates are assembled into crystalline, membranous, fibrillar or tubular structures [116], or they may be packaged into plastoglobuli (lipid bodies), which consist of a layer of polar lipids and proteins covering the surface, with non-polar components (15–25% carotenoids) in the center. The fibrillar and tubular chromoplasts consist of equal parts proteins and lipids, and the non-polar compounds are mainly esterified xanthophylls [116]. The membranous chromoplasts are less well characterized. The crystalline chromoplasts contain pure carotenoid crystals [116].

Recent research has provided new information about specific fibrillins, which are also known as chromoplast proteins. CHRC (chromoplast protein C) is an abundant 35-kDa carotenoid-associated protein found in the corolla of cucumber flowers; it is not present in chloroplasts, and is therefore undetectable in cucumber leaves and fruits. Its abundance increases during flower development, peaking in mature flowers before rapidly disappearing [118]. Using an *in vitro* flower bud culture system that mimics flower development, Vainstein et al. [119] and Vishnevetsky et al. [120] showed that the accumulation of carotenoids, *CHRC* mRNA and CHRC protein was markedly enhanced when gibberellin (GA_3) was added to the medium, but was inhibited by ABA and ethylene. A similar developmental profile and hormone response was demonstrated for the 14-kDa CHRD (chromoplast protein D) [121]. *CHRC* promoter analysis revealed a GA response element whose deletion prevented GA_3 induction, and also led to the identification of MYBYS, a flower-specific MYB-like transcriptional activator with a similar expression profile to *CHRC* and *CHRD*. MYBYS activates the *CHRC* gene in flowers accumulating carotenoids and flavonoids [122,123]. Inhibiting carotenoid biosynthesis affects *CHRC* and *CHRD* expression at the post-transcriptional and translational levels, indicating some form of feedback circuit dependent on carotenoid abundance [117]. The inhibition of *CHRC* by RNAi in transgenic tomato plants reduced total carotenoid levels by 30%, indicating that carotenoid accumulation also depends on the presence of this protein [122,123]. A flower-specific *CHRC* ortholog was recently isolated from the hybrid orchid, *Oncidium* Gower Ramsey [124].

Carotenoid metabolic engineering to introduce new flower colors

Modulating the existing carotenoid pathway

Flowers evolved to attract pollinators but they are also aesthetically pleasing to humans, and breeding flowers to generate visually appealing color varieties has been an important aspect of floriculture for hundreds of years [20]. One of the obstacles faced by flower breeders is that, for many species, there are colors that cannot be accessed by conventional breeding because the pigments cannot be synthesized using available genotypes, e.g. vivid red petunias and delphiniums, and the elusive blue rose.

Metabolic engineering can overcome these limitations by allowing genes for the appropriate metabolic enzymes to be transferred to flowering plants under the control of a flower-specific promoter, thus altering the color phenotype of the engineered flower without affecting other traits. Until recently, research has focused on flavonoids rather than carotenoids, but the information now available about carotenogenesis in flowers means that carotenoid engineering is becoming a realistic prospect.

In the first report of carotenoid engineering in flowers, Bird et al. [125] generated transgenic tomato plants with an antisense TOM5 (*PSY1*) gene under the control of the constitutive CaMV 35S promoter. As anticipated, this reduced the total carotenoid content by 80%, resulting in yellow ripe fruits and pale yellow flowers. However, leaf carotenoid levels were not affected because (as stated earlier) *PSY1* is not involved in carotenoid synthesis in green tissues, a function fulfilled by *PSY2* [48,126]. Since LYCB enzyme activity in tomato is also represented by different genes for photosynthetic and non-photosynthetic tissues, an antisense fragment of the *CRTL-b2* gene expressed under the control of the CaMV 35S promoter had a similarly negligible impact on vegetative growth while causing marked changes in the flower phenotype [52]. Transgenic flowers displayed a tawny-orange color (Fig. 3b), typical of the *old-gold* mutant (a natural loss-of-function mutation affecting *CRTL-b2*; see Table 1) resulting from the accumulation of up to 12% lycopene in flowers. The fruit color was not affected significantly because the fruits normally accumulate large amounts of lycopene.

Extending the pathway to include ketocarotenoids

Most plants do not produce ketocarotenoids such as astaxanthin and canthaxanthin, so metabolic engineering that aims to achieve ketocarotenoid synthesis in flowers can introduce vivid red and scarlet colors that would otherwise be unachievable. As stated earlier, astaxanthin is derived from β-carotene by 3-hydroxylation and 4-ketolation at both ionone end groups, reactions catalyzed by β-carotene hydroxylase (HYDB) and β-carotene ketolase, respectively (Fig. 2). The hydroxylation reaction is widespread in higher plants but ketolation only occurs naturally in *Adonis* petals. Even so, the substrates for ketolation are abundant in both green and non-green tissue, such that ketocarotenoid production can be achieved simply by the introduction of a single heterologous enzyme.

When tobacco was transformed with the β-carotene ketolase (*BKT*) gene from the alga *Haematococcus pluvilis* under the control of the tomato *PDS* promoter [54], high levels of astaxanthin and other ketocarotenoids were produced in the nectary, the only tobacco tissue that contains chromoplasts [127]. These results were improved still further when Ralley et al. [128] simultaneously expressed *Paracoccus* sp. β-carotene ketolase (*CRTW*) and β-carotene hydroxylase (*CRTZ*) genes, increasing the ketocarotenoid content of the nectary tissue 9-fold. Constitutive expression of a *Synechocystis* sp. PCC 6803 ketolase gene (*CRTO*) shifted the pathway towards the formation of 3′-hydroxyechinenone, 3-hydroxyechinenone, 4-ketozeaxanthin (adonixanthin) esters, 4-ketolutein and 4′-ketolutein esters, representing more than 50% of the total carotenoid content [129].

Unlike cultivated tobacco, the wild tobacco species *Nicotiana glauca* has carotenoid-pigmented petals, sepals, pistils, ovaries and nectary tissues. The constitutive expression of a *Nostoc* sp. 73102 ketolase gene (*CRTW*) resulted in the accumulation of 4-ketozeaxanthin (adonixanthin) as the only detectable ketocarotenoid [129], whereas the expression of a *Synechocystis* sp. PCC 6803 *CRTO* gene resulted in a wider spectrum of ketocarotenoids comprising 4′-ketolutein, echinenone, 3′-hydroxyechinenone and 4-ketozeaxanthin [64]. The total ketocarotenoid content in petals was more than three times greater in the latter plants, due in part to the unexpected upregulation of the entire carotenoid pathway in leaves and even more so in flowers – consequently the color of flower petals changed from the original light yellow to deep orange (Fig. 3c). Likewise, the petals of *Lotus japonicus* plants overexpressing an *Agrobacterium aurantiacum CRTW* gene changed from yellow to deep yellow or orange (Fig. 3d) [65].

Outlook and conclusions

Over the last decade we have learned a great deal about the accumulation of carotenoid pigments in flowers, the genes and enzymes responsible for carotenoid synthesis and degradation, the cellular mechanisms responsible for carotenoid storage, and how all these processes are regulated during development and in response to external stimuli. The availability of efficient genetic transformation methods for commercially important cut flower varieties and flower-specific promoters [54,55,124] means that a full armory of molecular biology techniques can now be brought to bear, including the overexpression of heterologous enzymes and the abolition of endogenous enzyme activity using antisense suppression and RNAi, to modulate carotenoid metabolism in flowers.

The future therefore looks colorful, as we embark on an era in which it will become possible not only to dissect carotenoid metabolism using molecular biology techniques but also to engineer flowers expressing pigments ranging from pale yellow through to deep scarlet by modulating carotenoid metabolism. It may also be possible to use flowers as a source of nutritionally important carotenoid molecules, including those rarely produced in significant quantities by flowering plants.

Acknowledgments

We would like to thank Prof. G. Sandmann for permission to use an unpublished photograph in Fig. 3. This research was supported by the Ministry of Science and Innovation, Spain (BFU2007-61413 and BIO2007-30738-E) and European Research Council Advanced Grant (BIOFORCE) to P.C., C.Z. and L.S. are supported in part by grants from the National Natural Science Foundation of China (Nos. 30870222 and 30370123). S.N. and G.F. are supported by Ministry of Science and Innovation (Spain) Ph.D. fellowships. C.B. is supported by a fellowship from the University of Lleida, Spain.

References

[1] G. Britton, S. Liaaen-Jensen, H. Pfander, Carotenoids Handbook, Birkhäuser, Basel, Switzerland, 2004.
[2] H.A. Frank, R.J. Cogdell, Photochem. Photobiol. 63 (1996) 257–264.
[3] B. Demmig-Adams, W.W. Adams III, Science 298 (2002) 2149–2153.
[4] G.E. Bartley, P.A. Scolnik, Plant Cell 7 (1995) 1027–1038.
[5] J. Von Lintig, K. Vogt, J. Nutr. 134 (2004) 251S–256S.
[6] S.H. Schwartz, B.C. Tan, D.A. Gage, J.A. Zeevaart, D.R. McCarty, Science 276 (1997) 1872–1874.
[7] V. Gomez-Roldan, S. Fermas, P.B. Brewer, V. Puech-Pagès, E.A. Dun, J.P. Pillot, F. Letisse, R. Matusova, S. Danoun, J.C. Portais, H. Bouwmeester, G. Becard, C.A. Beveridge, C. Rameau, S.F. Rochange, Nature 455 (2008) 189–194.
[8] M. Umehara, A. Hanada, S. Yoshida, K. Akiyama, T. Arite, N. Takeda-Kamiya, H. Magome, Y. Kamiya, K. Shirasu, K. Yoneyama, J. Kyozuka, S. Yamaguchi, Nature 455 (2008) 195–200.
[9] F. Bouvier, C. Suire, J. Mutterer, B. Camara, Plant Cell 15 (2003) 47–62.
[10] F. Bouvier, O. Dogbo, B. Camara, Science 300 (2003) 2089–2091.
[11] A.J. Simkin, B.A. Underwood, M. Auldridge, H.M. Loucas, K. Shibuya, E. Schmelz, D.G. Clark, H.J. Klee, Plant Physiol. 136 (2004) 3504–3514.
[12] A.J. Simkin, S.H. Schwartz, M. Auldridge, M.G. Taylor, H.J. Klee, Plant J. 40 (2004) 882–892.
[13] G. Farré, G. Sanahuja, S. Naqvi, C. Bai, T. Capell, C. Zhu, P. Christou, Plant Sci. 179 (2010) 28–48.
[14] C. Zhu, S. Naqvi, S. Gomez-Galera, A.M. Pelacho, T. Capell, P. Christou, Trends Plant Sci. 12 (2007) 548–555.
[15] C. Zhu, S. Naqvi, T. Capell, P. Christou, Arch. Biochem. Biophys. 483 (2009) 182–190.
[16] G. Sandmann, S. Römer, P.D. Fraser, Metab. Eng. 8 (2006) 291–302.
[17] S. Lu, L. Li, J. Integr. Plant Biol. 50 (2008) 778–785.
[18] P.D. Fraser, E.M. Enfissi, P.M. Bramley, Arch. Biochem. Biophys. 483 (2009) 196–204.
[19] C.I. Cazzonelli, B.J. Pogson, Trends Plant Sci. 15 (2010) 266–274.
[20] Y. Tanaka, N. Sasaki, A. Ohmiya, Plant J. 54 (2008) 733–749.
[21] J. Chappell, Annu. Rev. Plant Physiol. Plant Mol. Biol. 46 (1995) 521–547.
[22] H.K. Lichtenthaler, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 47–65.
[23] W. Eisenreich, F. Rohdich, A. Bacher, Trends Plant Sci. 6 (2001) 78–84.
[24] N. Misawa, M.R. Truesdale, G. Sandmann, P.D. Fraser, C. Bird, W. Schuch, P.M. Bramley, J. Biochem. 116 (1994) 980–985.
[25] Y. Chen, F. Li, E.T. Wurtzel, Plant Physiol. 153 (2010) 66–79.
[26] J. Breitenbach, G. Sandmann, Planta 220 (2005) 785–793.
[27] T. Isaacson, I. Ohad, P. Beyer, J. Hirschberg, Plant Physiol. 136 (2004) 4246–4255.
[28] Z. Sun, E. Gantt, F.X. Cunningham Jr., J. Biol. Chem. 271 (1996) 24349–24352.
[29] F. Bouvier, Y. Keller, A. d'Harlingue, B. Camara, Biochim. Biophys. Acta 1391 (1998) 320–328.
[30] L. Tian, V. Musetti, J. Kim, M. Magallanes-Lundback, D. DellaPenna, Plant Cell 15 (2003) 1320–1332.
[31] R.F. Quinlan, T.T. Jaradat, E.T. Wurtzel, Arch. Biochem. Biophys. 458 (2007) 146–157.
[32] J. Kim, D. DellaPenna, Proc. Natl Acad. Sci. USA 103 (2006) 3474–3479.
[33] J.E. Kim, K.M. Cheng, N.E. Craft, B. Hamberger, C.J. Douglas, Phytochemistry 71 (2010) 168–178.
[34] L. Tian, D. DellaPenna, Plant Mol. Biol. 47 (2001) 379–388.
[35] B. Demmig-Adams, A.M. Gilmore, W.W. Adams III, FASEB J. 10 (1996) 403–412.
[36] F. Bouvier, A. d'harlingue, R.A. Backhaus, M.H. Kumagai, B. Camara, Eur. J. Biochem. 267 (2000) 6346–6352.
[37] S. Al-Babili, P. Hugueney, M. Schledz, R. Welsch, H. Frohnmeyer, O. Laule, P. Beyer, FEBS Lett. 485 (2000) 168–172.
[38] M. Seo, T. Koshiba, Trends Plant Sci. 7 (2002) 41–48.
[39] S.H. Schwartz, X. Qin, J.A. Zeevaart, Plant Physiol. 131 (2003) 1591–1601.
[40] F. Bouvier, P. Hugueney, A. d'Harlingue, M. Kuntz, B. Camara, Plant J. 6 (1994) 45–54.
[41] F.X. Cunningham Jr., E. Gantt, Plant J. 41 (2005) 478–492.
[42] C.P. Moehs, L. Tian, K.W. Osteryoung, D. DellaPenna, Plant Mol. Biol. 45 (2001) 281–293.
[43] P.M. Bramley, J. Exp. Bot. 53 (2002) 2107–2113.
[44] N. Galpaz, G. Ronen, Z. Khalfa, D. Zamir, J. Hirschberg, Plant Cell 18 (2006) 1947–1960.
[45] P.G. Kevan, in: C.E. Jones, R.J. Little (Eds.), Handbook of Experimental Pollination Biology, Van Nostrand Reinhold, New York, 1983, pp. 3–30.
[46] M. Thelander, J.O. Narita, W. Gruissem, Curr. Top. Plant Biochem. Physiol. 5 (1986) 128–141.
[47] G.E. Bartley, P.V. Viitanen, K.O. Bacot, P.A. Scolnik, J. Biol. Chem. 267 (1992) 5036–5039.
[48] G.E. Bartley, P.A. Scolnik, J. Biol. Chem. 268 (1993) 25718–25721.
[49] P.D. Fraser, M.R. Truesdale, C.R. Bird, W. Schuch, P.M. Bramley, Plant Physiol. 105 (1994) 405–413.
[50] P.D. Fraser, J.W. Kiano, M.R. Truesdale, W. Schuch, P.M. Bramley, Plant Mol. Biol. 40 (1999) 687–698.

[51] I. Pecker, R. Gabbay, F.X. Cunningham Jr., J. Hirschberg, Plant Mol. Biol. 30 (1996) 807–819.
[52] G. Ronen, L. Carmel-Goren, D. Zamir, J. Hirschberg, Proc. Natl Acad. Sci. USA 97 (2000) 11102–11107.
[53] G. Giuliano, G.E. Bartley, P.A. Scolnik, Plant Cell 5 (1993) 379–387.
[54] V. Corona, B. Aracri, G. Kosturkova, G.E. Bartley, L. Pitto, L. Giorgetti, P.A. Scolnik, G. Giuliano, Plant J. 9 (1996) 505–512.
[55] M. Dalal, V. Chinnusamy, K.C. Bansal, BMC Plant Biol. 10 (2010) 61.
[56] T. Isaacson, G. Ronen, D. Zamir, J. Hirschberg, Plant Cell 14 (2002) 333–342.
[57] N. Galpaz, Q. Wang, N. Menda, D. Zamir, J. Hirschberg, Plant J. 53 (2008) 717–730.
[58] L.R.G. Valadon, R.S. Mummery, Biochem. J. 106 (1968) 479.
[59] C. Zhu, S. Yamamura, M. Nishihara, H. Koiwa, G. Sandmann, Biochem. Biophys. Acta 1625 (2003) 305–308.
[60] C. Yamamizo, S. Kishimoto, A. Ohmiya, J. Exp. Bot. 61 (2010) 709–719.
[61] M. Yamagishi, S. Kishimoto, M. Nakayama, Plant Breed. 128 (2009) 172–177.
[62] A. Ohmiya, S. Kishimoto, R. Aida, S. Yoshioka, K. Sumitomo, Plant Physiol. 142 (2006) 1193–1201.
[63] G. Neamtu, Z. Nagy, C. Bilaus, T. László, in: Institutum Agronomicum "Dr. Petru Groza" Cluj-Napoca (Romania) Notulae Botanicle Horti Agrobotanici Cluj-Napoca, VIII, 1975/76, pp. 53–58.
[64] C. Zhu, T. Gerjets, G. Sandmann, Transgenic Res. 16 (2007) 813–821.
[65] S. Suzuki, M. Nishihara, T. Nakatsuka, N. Misawa, I. Ogiwara, S. Yamamura, Plant Cell Rep. 26 (2007) 951–959.
[66] A.D. Hieber, R.G. Mudalige-Jayawickrama, A.R. Kuehnle, Planta 223 (2006) 521–531.
[67] R. Castillo, J.A. Fernandez, L. Gomez-Gomez, Plant Physiol. 139 (2005) 674–689.
[68] K. Egger, Phytochemistry 4 (1965) 609–618.
[69] G. Neamtu, V. Tamas, C. Bodea, Rev. Roum. Biochem. 3 (1966) 305–310.
[70] F. Delgado-Vargas, O. Paredes-Lopez, J. Agri. Food Chem. 45 (1997) 1097–1102.
[71] M.I. Scott, I. Ascarelli, G. Olson, Poult. Sci. 47 (1968) 863.
[72] A.A. Del Villar-Martinez, P.A. Garcia-Saucedo, A. Carabez-Trejo, A. Cruz-Hernandez, O. Paredes-Lopez, J. Plant Physiol. 162 (2005) 1046–1056.
[73] C. Zhu, S. Yamamura, H. Koiwa, M. Nishihara, G. Sandmann, Plant Mol. Biol. 48 (2002) 277–285.
[74] M. Nakayama, M. Miyasaka, T. Maoka, M. Yagi, N. Fukuta, J. Jpn. Soc. Hort. Sci. 75 (2006) 161–165.
[75] J. Deli, P. Molnár, Z. Matus, G. Tóth, A. Steck, H. Pfander, Chromatographia 48 (1998) 27–31.
[76] P. Beyer, M. Mayer, H. Kleinig, Eur. J. Biochem. 184 (1989) 141–150.
[77] V. Nievelstein, J. Vandekerchove, M.H. Tadros, J.V. Lintig, W. Nitschke, P. Beyer, Eur. J. Biochem. 233 (1995) 864–872.
[78] B. Liedvogel, H. Kleinig, Planta 133 (1997) 249–253.
[79] M. Bonk, M. Tadros, J. Vandekerckhove, S. Al-Babili, P. Beyer, Plant Physiol. 111 (1996) 931–939.
[80] S. Al-Babili, J. von Lintig, H. Haubruck, P. Beyer, Plant J. 9 (1996) 601–612.
[81] M. Schledz, S. Al-Babili, J. Von Lintig, H. Haubruck, S. Rabbani, H. Kleinig, P. Beyer, Plant J. 10 (1996) 781–792.
[82] M.P. Mayer, P. Beyer, H. Kleinig, Eur. J. Biochem. 191 (1990) 359–363.
[83] B.C. Tan, S.H. Schwartz, J.A. Zeevaart, D.R. McCarty, Proc. Natl Acad. Sci. USA 94 (1997) 12235–12240.
[84] C. Zhu, K. Kauder, S. Römer, G. Sandmann, J. Plant Physiol. 164 (2007) 195–204.
[85] B.C. Tan, L.M. Joseph, W.T. Deng, L.L. Liu, Q.B. Li, K. Cline, D.R. McCarty, Plant J. 35 (2003) 44–56.
[86] A. Rubio, J.L. Rambla, M. Santaella, M.D. Gómez, D. Orzaez, A. Granell, L. Gómez-Gómez, J. Biol. Chem. 283 (2008) 24816–24825.
[87] S. Mathieu, N. Terrier, J. Procureur, F. Bigey, Z. Gunata, J. Exp. Bot. 56 (2005) 2721–2731.
[88] F.C. Huang, G. Horváth, P. Molnár, E. Turcsi, J. Deli, J. Schrader, G. Sandmann, H. Schmidt, W. Schwab, Phytochemistry 70 (2009) 457–464.
[89] G.M. Holloway, J.L. Gainer, J. Appl. Physiol. 65 (1988) 683–686.
[90] P.A. Tarantilis, M. Polissiou, M. Manfait, J. Chromatogr. 664 (1994) 55–61.
[91] H. Pfander, H. Schurtenberger, Phytochemistry 21 (1982) 1039–1042.
[92] S. Pfiter, P. Meyer, A. Steck, H. Pfander, J. Agric. Food Chem. 44 (1996) 2612–2615.
[93] Y.H. Liao, P.J. Houghton, J.R. Hoult, J. Nat. Prod. 62 (1999) 1241–1245.
[94] C.H. Eugster, H. Hurlimann, H.J. Leuenberger, Helv. Chim. Acta 52 (1969) 89–90.
[95] J.S. Tandon, S.B. Katti, P. Rüedi, C.H. Eugster, Helv. Chim. Acta 274 (1979) 2706–2707.
[96] O. Ahrazem, A. Rubio-Moraga, R.C. López, L. Gómez-Gómez, J. Exp. Bot. 61 (2010) 105–119.
[97] A. Rubio, J.L. Rambla, O. Ahrazem, A. Granell, L. Gómez-Gómez, Phytochemistry 70 (2009) 1009–1016.
[98] P. Winterhalter, R.L. Rouseff, Carotenoid-derived Aroma Compounds, American Chemical Society, Washington, DC, 2002.
[99] C. Dufresne, F. Cormier, S. Dorion, Planta Med. 63 (1997) 150–153.
[100] A. Rubio, P.F. Nohales, J.A. Pérez, L. Gómez-Gómez, Planta 219 (2004) 955–966.
[101] S. Kishimoto, T. Maoka, M. Nakayama, A. Ohmiya, Phytochemistry 65 (2004) 2781–2787.
[102] S. Kishimoto, A. Ohmiya, Physiol. Plant 128 (2006) 436–447.
[103] F.A. Langton, Euphytica 29 (1980) 807–812.
[104] K. Hattori, Jpn. J. Breed. 41 (1991) 1–9.
[105] A. Ohmiya, K. Sumitomo, R. Aida, J. Jpn. Soc. Hort. Sci. 78 (2009) 450–455.
[106] B. Czeczuga, Syst. Ecol. 15 (1987) 303–306.
[107] T.W. Goodwin, second ed., The Biochemistry of the Carotenoids, vol. 1, Chapman & Hall, London, 1980. pp. 377.
[108] B. Yu, D.J. Lydiate, U.A. Schäfer, A. Hannoufa, Planta 226 (2007) 181–192.
[109] P.D. Fraser, H. Shimada, N. Misawa, Eur. J. Biochem. 252 (1998) 229–236.
[110] T. Lotan, J. Hirschberg, FEBS Lett. 364 (1995) 125–128.
[111] Y. He, C. Zhu, D. Wang, D. Kong, J. Sun, Prog. Nat. Sci. 12 (2002) 592–597.
[112] M.R. Marano, E.C. Serra, E.G. Orellano, N. Carrillo, Plant Sci. 94 (1993) 1–17.
[113] S. Lu, J. Van Eck, X. Zhou, A.B. Lopez, D.M. O'Halloran, K.M. Cosman, B.J. Conlin, D.J. Paolillo, D.F. Garvin, J. Vrebalov, L.V. Kochian, H. Küpper, E.D. Earle, J. Cao, L. Li, Plant Cell 18 (2006) 3594–3605.
[114] A.B. Lopez, J. Van Eck, B.J. Conlin, D.J. Paolillo, J. O'Neill, L. Li, J. Exp. Bot. 59 (2008) 213–223.
[115] W. Kühlbrandt, D.N. Wang, Y. Fujiyoshi, Nature 367 (1994) 614–621.
[116] M. Vishnevetsky, M. Ovadis, A. Vainstein, Trends Plant Sci. 4 (1999) 232–235.
[117] M. Vishnevetsky, M. Ovadis, A. Zuker, A. Vainstein, Plant J. 20 (1999) 423–431.
[118] I. Smirra, A.H. Halevy, A. Vainstein, Plant Physiol. 102 (1993) 491–496.
[119] A. Vainstein, A.H. Halevy, I. Smirra, M. Vishnevetsky, Plant Physiol. 104 (1994) 321–326.
[120] M. Vishnevetsky, M. Ovadis, H. Itzhaki, A. Vainstein, J. Biol. Chem. 272 (1997) 24747–24750.
[121] Y. Libal-Weksler, M. Vishnevetsky, M. Ovadis, A. Vainstein, Plant Physiol. 113 (1997) 59–63.
[122] Y. Leitner-Dagan, M. Ovadis, E. Shklarman, Y. Elad, D. Rav David, A. Vainstein, Plant Physiol. 142 (2006) 233–244.
[123] Y. Leitner-Dagan, M. Ovadis, A. Zuker, E. Shklarman, I. Ohad, T. Tzfira, A. Vainstein, Planta 225 (2006) 89–102.
[124] C.Y. Chiou, K. Wu, W. Yeh, Biotechnol. Lett. 30 (2008) 1861–1866.
[125] C.R. Bird, J.A. Ray, J.D. Fletcher, J.M. Boniwell, A.S. Bird, C. Teulieres, I. Blain, P.M. Bramley, W. Schuch, Bio/Technology 9 (1991) 635–639.
[126] P. Bramley, C. Teulieres, I. Blain, C. Bird, W. Schuch, Plant J. 2 (1992) 343–349.
[127] V. Mann, M. Harker, I. Pecker, J. Hirschberg, Nat. Biotechnol. 18 (2000) 888–892.
[128] L. Ralley, E.M. Enfissi, N. Misawa, W. Schuch, P.M. Bramley, P.D. Fraser, Plant J. 39 (2004) 477–486.
[129] T. Gerjets, M. Sandmann, C. Zhu, G. Sandmann, Biotechnol. J. 2 (2007) 1263–1269.