Universitat de Lleida

Towards vitamin biofortification in staple cereal crops in a socio-political and food security context

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The photos of the cover

In front: Fruits and vegetables picture, taken from Vicki Zerbee, http://antiagingbydesign.com/what-are-carotenoids

In the spine a composition of different pictures from left to right and from up to down:

1. Peppers, taken from http://www.dynadis.com/html/carotenoids.html
2. Lettuce, taken from http://www.biofresh.liebherr.com/en-GB/129082.wfw/step-7
3. Almonds 1, taken from http://www.thedailygreen.com/healthy-eating/eat-safe/top-sources-vitamin-E-44111408
4. Tomatoes, taken from http://www.ecouterre.com/the-secret-to-a-healthier-tan-eat-more-fruits-and-veggies-says-study/
5. Broccoli, taken from http://ecoki.com/lutein-broccoli/
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9. Fruits, taken from http://www.naturalhomeandgarden.com/blogs/blog.aspx?blogid=2147483735&tag=carotenoids
10. Nuts, taken from http://vitamine.org.uk/

Behind: Fruits and vegetables picture, taken from http://www.freshplaza.com/news_detail.asp?id=94786

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Paul Christou i Changfu Zhu, professors del Departament de Producció Vegetal i Ciència Forestal de la Universitat de Lleida, i directors de la tesi realitzada per la senyora Gemma Farré Martinez, "Towards vitamin biofortification in staple cereal crops in a socio-political and food security context",

INFORMEM:

Que el treball d'investigació es considera ja finalitzat i que compleix les condicions exigibles a la legislació vigent per optar al grau de Doctor. Per tant autoritzem la seva presentació perquè pugui ser jutjada pel Tribunal corresponent a la Universitat de Lleida.

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Abstract

Malnutrition is a significant challenge, particularly in the developing world where measures that are commonplace in industrialized countries (varied diet, fortification schemes and dietary supplements) are largely absent. Vitamin deficiencies mostly affect impoverished people in developing countries because poor people cannot afford a diversified diet. Plants provide nearly all essentials vitamins required to maintain health and well-being in humans. Most staple food crops, particularly the major cereals maize and rice are deficient in key vitamins, amongst other micronutrients. Transgenic approaches hold great promise in alleviating, to a major extend such micronutrient deficiencies as exemplified by numerous reports in the literature over the past decade. However, there is a limited understanding of the regulatory mechanisms that control the accumulation of specific vitamins in plants. For this reason, I analyzed a maize population expressing combinations of the carotenogenic genes *Zmpsy1*, *Pacrt*I, *Gllycb*, *Glbch* and *Paracrt*W in an effort to unravel as yet unknown regulatory bottlenecks.

I also investigated in more detail the corn *bch* family. My results suggest that diverse regulatory strategies may be in operation to control the accumulation of carotenoids in endosperm tissue.

Generating cereal grains with a substantially enhanced content of vitamins E is an entirely feasible proposition. Such efforts are now possible as a result of our ability to clone all genes required for meaningful metabolic engineering, coupled with the development of effective multi-gene transfer methodology, applicable to the major target crops. I recovered multiple independent transgenic rice lines expressing *Arabidopsis thaliana* ρ-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the first committed step in vitamin E biosynthesis. My results revealed bottlenecks that act as metabolic tipping points in the pathway.

Many barriers prevent the deployment of genetically engineered crops, mainly reflecting the disharmonious regulations applied in different countries that depress international trade, political interference in an already overburdening and disproportionate regulatory process, and negative influences on the public perception of biotechnology despite its clear benefits.

Resum

La malnutrició és un problema molt important, sobretot als països en vies de desenvolupament on no es poden aplicar les mateixes mesures preses per afrontar-la que als països desenvolupats (dieta diversificada, sistemes de fortificació i suplements alimentaris). Les deficiències en vitamines afecten majoritàriament a la població pobra dels països en vies de desenvolupament al no poder-se permetre una dieta variada. Les plantes proporcionen la majoria de les vitamines essencials per mantenir una bona salut i aconseguir el benestar en les persones. La majoria dels cultius alimentaris, i en particular els cereals majoritaris com el blat de moro i l'arròs, són deficients en vitamines claus entre altres micronutrients. Les plantes transgèniques podrien ser una solució per mitigar les deficiències de micronutrientes, tal com s'ha descrit en la bibliografia durant la passada dècada. No obstant això, en les plantes son limitats els coneixements dels mecanismes que regulen l'acumulació de determinades vitamines. Per tal d'ampliar-los, he analitzat una població de blat de moro que expressa combinacions de gens involucrats en la ruta metabòlica dels carotenoids (*Zmpsy1*, *Pacrt*I, *Gllycb*, *Glbch* and *Paracrt*W) i al mateix temps n'he desxifrar les limitacions de la seva regulació.

També he investigat en detall la família dels gens beta-caroteno hidroxilasa (*bch*) en el blat de moro. Els meus resultats suggereixen que per controlar l'acumulació de carotenoids en l'endosperma pot ser que hi hagi en funcionament al mateix temps varies estratègies de regulació.

La generació de llavors de cereals amb un augment considerable de vitamina E és un objectiu totalment factible, basat en la nostra capacitat per clonar tots els gens necessaris per enginyar la ruta conjuntament amb la metodologia efectiva de transferència de múltiples gens que hem desenvolupat i aplicable a la majoria dels cultius. He regenerat diverses línies independents d'arròs transgènic que expressen l'*Arabidopsis thaliana* ρ-hidroxiphenilpiruvat dioxigenasa (HPPD), que és un enzim que catalitzà la primera reacció en la ruta de biosíntesis de la vitamina E. Els meus resultats demostren les limitacions que afecten determinats punts decisius en aquesta ruta metabòlica.

Actualment hi ha moltes traves que impedeixen el desenvolupament dels cultius genèticament modificats. Les regulacions contradictòries i diferents aplicades per països fan que es desestabilitzi el comerç internacional. Altres barreres són l'influencià política en l'estricte i desproporcionat procés de regulació, així com les influències negatives de la premsa que afecten la percepció pública sobre la biotecnologia, malgrat els seus beneficis evidents.

Resumen

La malnutrición es uno de los problemas más importantes que tiene que solucionar la sociedad actual, sobretodo en los países en vías de desarrollo, donde no se pueden aplicar las mismas medidas tomadas para afrontarla que en los países desarrollados (dieta diversificada, sistemas de fortificación y suplementos alimenticios). Las deficiencias vitamínicas se detectan mayoritariamente en la población pobre de los países en vías de desarrollo, al no poder permitirse una dieta variada. Las plantas proporcionan la mayoría de las vitaminas esenciales para mantener una buena salud y lograr el bienestar de las personas. La mayoría de los cultivos alimentarios, y en particular los principales cereales como el maíz y el arroz, son deficientes en las vitaminas básicas entre otros micronutrientes. Las plantas transgénicas pueden ser una solución muy prometedora para mitigar las deficiencias de micronutrientes, tal y como se ha descrito en la bibliografía durante la pasada década. Sin embargo, el conocimiento de los mecanismos que regulan la acumulación de determinadas vitaminas en las plantas es limitado. Para aumentar los conocimientos en este campo, he analizado una población de maíz que expresa combinaciones de genes involucrados en la ruta metabólica de los carotenoides (*Zmpsy1*, *Pacrt*I, *Gllycb*, *Glbch* y *Paracrt*W) en la cual he profundizado en el estudio de los pasos limitantes de su regulación.

También he investigado con más detalle la familia de genes beta-caroteno hidroxilasa (*bch*) en maíz. Mis resultados sugieren que varias estrategias de regulación trabajan conjuntamente para controlar la acumulación de carotenoides en el endospermo.

Aumentar el contenido de vitamina E en semillas de cereales es un objetivo que podemos alcanzar gracias a la capacidad que tenemos en el laboratorio para clonar todos los genes necesarios conjuntamente con el desarrollo de una metodología efectiva de transferencia de múltiples genes, aplicables a la mayoría de los cultivos. He regenerado varias líneas independientes de arroz transgénico que expresan *Arabidopsis thaliana ρ-hidroxiphenilpiruvata dioxigenasa (HPPD*), la enzima que cataliza la primera reacción en la ruta de biosíntesis de la vitamina E. Mis resultados aclaran algunas de las limitaciones que actúan en determinados puntos decisivos en la ruta metabólica.

Actualmente existen muchas barreras que impiden el desarrollo de los cultivos genéticamente modificados como las diferentes y contradictorias regulaciones aplicadas en los países que influencian el comercio internacional. Otra de las barreras es la presión política en el proceso de regulación de los cultivos genéticamente modificados siendo demasiado estricta y desproporcionada, sin olvidar el papel de los medios de comunicación en fomentar una percepción pública negativa de la biotecnología sin pensar en sus evidentes beneficios.

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List of Abbreviations

µg	Micrograms
µl	Microliters
µm	Micrometres
ABA	Abscisic acid
AIDS	Acquired Immunodeficiency Syndrome
Anti-DIG-AP	Anti-Digoxigenin-AP Fab fragments
BAC	Bacterial artificial chromosome
BCH	β-carotene hydroxylase
Bch	β-carotene hydroxylase
bch1	β-carotene hydroxylase 1
bch2	β-carotene hydroxylase 2
BKT	Bacterial β-carotene ketolase
Bt	Bacillus thuringiensis
cDNA	Complementary DNA
CI	Confidence interval
CoA	Coenzyme A
*crt*B	Bacteria phytoene synthase
*crt*I	Bacteria phytoene desaturase
CRTISO	Carotenoid isomerase
*crt*O	Bacterial β-carotene ketolase
*crt*W	Bacterial β-carotene ketolase
*crt*Y	Bacteria lycopene β-cyclase
CSPD	Disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricycle [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate

cv.	Cultivar
CYP97A	P450-type β-carotene hydroxylases
CYP97B	P450-type β-carotene hydroxylases
CYP97C	P450-type ε-hydroxylase
DAP	Days after pollination
DAP	Days after pollination
DFID	Department for International Development
DMAPP	Dimethylallyl diphosphate
DMGGBQ	2-dimethyl-6-geranylgeranylbenzoquinol
DMPBQ	3-dimethyl-5-phytyl-1,4-benzoquinone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRI	Dietary Reference Intake
dUMP/dTMP	Deoxyuridine monophosphate/ timidine monophosphate
DW	Dry weight
DXP	Deoxy-D-xylulose 5-phosphate
E.coli	Escherichia coli
EU	European Union
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FMN	Flavin mononucleotide
g	Grams
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GE	Genetic engineering
GGDP	Geranylgeranyldiphosphate
GGDR	Geranylgeranyl diphosphate reductase

GGH	Geranylgeranyldiphosphate hydratase
GGPP	Geranylgeranyl diphosphate
GGPPS	GGPP synthase
Gl	Gentiana lutea
HGA	Homogentisic acid
HGGT	Homogentisate geranylgeranyl transferase
HPLC	High-performance liquid chromatography
HPP	ρ-hydroxyphenylpyruvic acid
HPPD	ρ-hydroxyphenylpyruvate dioxygenase
hpt	Hygromycin phosphotransferase
HPT1	Homogentisate phytyltransferase
HYDB	b-carotene hydroxylase
IOM	US Institute of Medicine
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
kDa	KiloDalton
Lcyb	Lycopene β-cyclase
Lcye	Lycopene ε-cyclase
LKR/SDH	Lysine-ketoglutarate reductase/saccharophine dehydrogenase
Lut	Lutein
LYCB	Lycopene β-cyclase
LYCE	Lycopene ε-cyclase
MEP	Methylerythritol phosphate
mg	Milligrams
MGGBQ	2-methyl-6-geranylgeranylplastoquinol
MGGBQ-MT	MGGBQ methyltransferase

min	Minut
ml	Mililiter
mM	MiliMolar
MPBQ	2-methyl-6-phytyl benzoquinone
MPBQ-MT	MPBQ methyltransferase
mRNA	Messenger RNA
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADP	Phosphate nicotinamide adenine dinucleotide
ng	Nanograms
Or	Orange
Pa	Pantoea ananatis
Para	Paracoccus ssp.
PCR	Polymerase chain reaction
PDP	Phytyldiphosphate
PDS	Phytoene desaturase
Pds	Phytoene desaturase
PDA	Photodiode array
PHS	Pre-harvest sprouting
pI	Isoelectric point
PSY	Phytoene synthase
psy1	Phytoene synthase 1
psy2	Phytoene synthase 2
psy3	Phytoene synthase 3
QTLs	Quantitative trait loci
RAE	Retinol Activity Equivalent

RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
T3	Tocotrienols
TC	Tocopherol cyclase
Toc	Tocopherols
TYRA	Chorismate mutase-prephenate dehydrogenase
Ubi-1	Maize ubiquitin 1
UNICEF	The United Nations Children's Fund
US	United States
USDA	United States Department of Agriculture
v/v	Volume to volume ratio
VAD	Vitamin A deficiency
Viol	Violaxanthin
vp	Viviparous
w/v	Weight to volume ratio
WFP	The World and Food Programme
WHO	The World Health Organization
y1	Yellow 1
ZDS	ξ-carotene desaturase
Zeax	Zeaxanthin
ZEP	Zeaxanthin epoxidase
Z-ISO	z-carotene isomerase
Zm	Zea mays
α-Cry	α-cryptoxanthin
α-TTP	α-Tocopherol Transfer Protein

β-Car	β-carotene
β-Cry	β-cryptoxanthin
γ-TMT	γ-tocopherol methyltransferase

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General Introduction

1.1 Food insecurity, poverty and micronutrients malnutrition

Food insecurity is defined as the lack of access to sufficient amounts of safe and nutritious food to maintain an active and healthy life. The achievement of food security is currently one of the world's greatest challenges, but the situation is projected to deteriorate over the next decade in at least 70 developing countries according to the USDA Economic Research Service and the UN Food and Agriculture Organization (FAO/WFP, 2009). Food security is taken for granted in the industrialized world, which generally enjoys stable political and social structures. But the picture in the developing world could not be more different. Almost one billion people are chronically undernourished, regularly consuming less than 2000 calories per day (Figure 1.1; FAO, 2006). A further two billion people consume enough calories but nevertheless lack essential nutrients. This means that up to half the world's population at any one time may suffer from malnutrition (Graham et al., 2001).

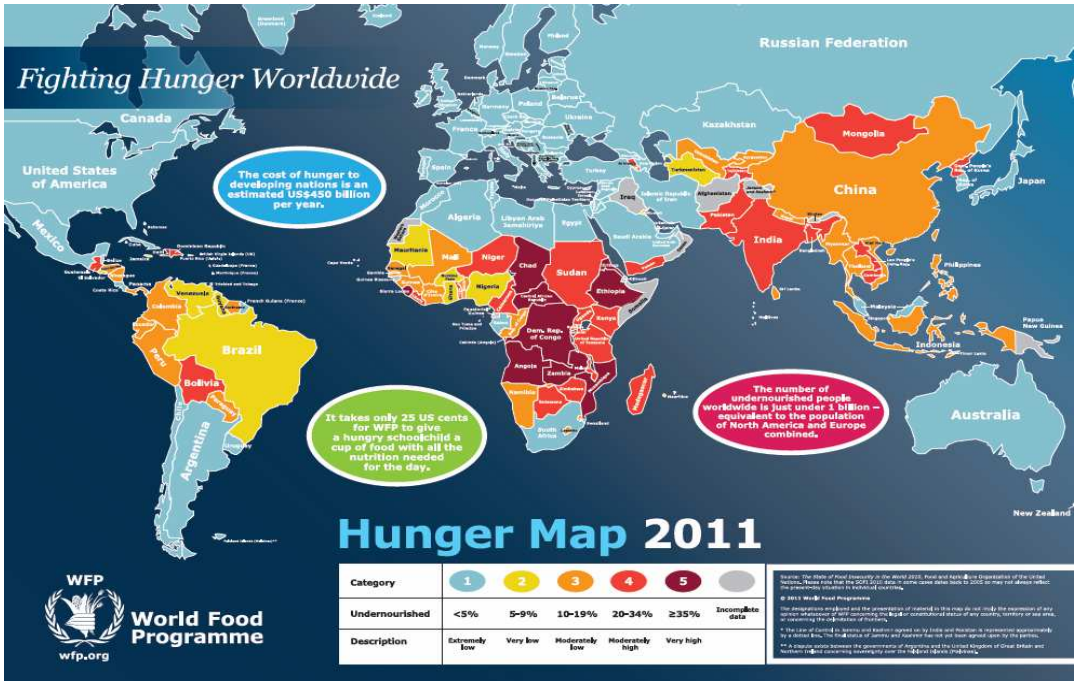

Figure 1.1 The 2011 hunger map (Source: World Food Programme 2011; see http://www.wfp.org/hunger/map).

One of the persistent myths concerning food insecurity is that a shortfall in food production is to blame. There is actually plenty of food to go around, at least at present. The reasons for food insecurity are complex, but one of the main factors is poverty. More than one billion people live on less than $US 1 per day with another two billion only marginally better off (World Bank, 2000) and most of these people are rural subsistence farmers in developing countries, where they can account for 70% or more of the population. In contrast, farmers account for only 1% of the US population.

Subsistence farmers have limited purchasing power, and generally cannot irrigate their crops or buy fertilizers, herbicides and pesticides. This leads to soil exhaustion and falling yields, and the crops become susceptible to pests, diseases and natural disasters such as drought (Ramessar et al., 2009). Many poor farmers are eventually forced to abandon their land and move to cities, adding to the growing problem of urban poverty and hunger (DFID, 2002). It is now thought that half the world's population lives in cities, so any disruption to agriculture could precipitate an urban food crisis in a matter of days. The markets can also increase the prevalence of food insecurity, i.e. when food prices increase but incomes are low or unemployment increases (FAO/WFP, 2009). Any long-term strategy to address food insecurity in the developing world must therefore tackle the underlying problem of poverty by increasing the level of rural employment-based income through increased agricultural productivity (Christou and Twyman, 2004).

Given projected population increases, fuel price hikes, falling reserves of fresh water and increased urbanization, the only solution to the above problem is to increase the yields of major food crops (particularly cereal grains) using the land that is currently available but less water, and to diversify the uses of crops to facilitate the creation of wealth. A variety of approaches can be envisaged, including the efficient use of organic and inorganic fertilizers, irrigation strategies, soil and water conservation, pest and disease management and the production of improved plant varieties with higher yields or novel products (Ramessar et al., 2009).

Food insecurity also increases malnutrition, which reflects the combined impact of poverty, poor access to food, inefficient food distribution infrastructure and an over-reliance on subsistence agriculture based on individual cereal crops that lack essential nutrients. Malnutrition is therefore endemic in developing countries where the lack of a

diverse diet means that many individuals are exposed to the risk of deficiency diseases (Pérez-Massot et al., 2012). Plant biotechnology provides a range of tools that can contribute significantly and sustainably to humanitarian efforts in developing countries where much of the economy is based on subsistence agriculture.

1.2 Vitamins – Sources, dietary requirements, functions in the human body and deficiency diseases

1.2.1 Carotneoids

The four pro-vitamin A carotenoids (β-carotene, α-carotene, γ-carotene and β-cryptoxanthin) are found in a diverse range of plants, and are required for the human body to synthesize vitamin A if there is no direct access to retinol from animal sources. All carotenoids are nutritionally valuable because they act as antioxidants (Bai et al 2011) but the pro-vitamin A carotenoids are the only ones classed as vitamins, because the reduced form of vitamin A (retinal) is required for the production of rhodopsin in the eyes as well as the maintenance of epithelial cells and a healthy immune system (Ross et al., 2010). The acidic form (retinoic acid) is a morphogen in development (Ross et al., 2010).

Carotenoids are the only natural tetraterpenes and they are synthesized *de novo* by photosynthetic organisms (including plants, algae and cyanobacteria) and some non-photosynthetic bacteria and fungi (Botella-Pavía and Rodríguez-Concepción, 2006). Aphids were also recently shown to synthesize carotenoids (Moran and Jarvik, 2010). Mammals cannot synthesize pro-vitamin A carotenoids because they lack a key enzyme, and need to acquire these essential nutrients in their diets (DellaPenna and Pogson, 2006).

Many foods are good sources of vitamin A, but these generally do not contain retinal itself but instead contain derivatives that can be converted into retinol and then into either retinal or retinoic acid (Harrison, 2005). Meat and dairy sources of vitamin A primarily contain an esterified form of retinol (retinyl palmitate) whereas plants produce pro-vitamin A carotenoids such as β-carotene that are cleaved to produce retinal directly. These are abundant in a wide variety of dark green, yellow and orange fruits

and vegetables, including oranges, broccoli, spinach, carrots, squash, sweet potatoes and pumpkins (Harrison, 2005).

Because both pro-vitamin A carotenoids and retinol can be converted into retinal, studies have been carried out to determine the amount of various carotenoids that must be consumed to provide an equivalent amount of retinol. In 2001, the US Institute of Medicine (IOM) recommended a new unit for the dietary reference intake (DRI) for vitamin A (the retinol activity equivalent or RAE) which takes bioavailability into account. One RAE corresponds to the activity of 1 μg of pure retinol, and this was found to be the same as 2 μg of pure β-carotene dissolved in oil, 12 μg of dietary β-carotene in a food matrix, or 24 μg of any combination of the three other pro-vitamin A carotenoids. The DRI for vitamin A is 900 RAE for males, 700 RAE for females (770 RAE in pregnancy and 1300 RAE when lactating) and 400–500 RAE for children (IOM, 2001).

Vitamin A deficiency (VAD) causes night blindness, i.e. the deterioration of light-sensitive cells (rods) essential for vision in low light intensity, and it can also damage the cornea resulting in a form of total blindness called xerophthalmia (Hammond et al., 1997). The lack of vitamin A also affects the immune system, leaving individuals susceptible to infections (FAO/WHO, 2002). VAD can also lead to diarrhea and respiratory diseases, which may sometimes be fatal. Low serum retinol concentrations (<0.70 μmol/l) affect more than 190 million pre-school children (95% CI, 178–202 million) and 19.1 million pregnant women (95% CI, 9.30–29.0 million) globally. This corresponds to 33.3% of the pre-school population and 15.3% of pregnant women. Night blindness affects 5.2 million pre-school children (95% CI, 2.0–8.4 million) and 9.8 million pregnant women (95% CI, 8.7–10.8 million), which corresponds to 0.9% and 7.8% of the population, respectively (WHO, 2009).

1.2.2 Vitamin E

Vitamin E is a group of eight fat-soluble compounds known as tocopherols and tocotrienols (collectively tocochromanols) the most active of which is α-tocopherol. The best sources of α-tocopherol are nuts, seeds and vegetable oils, but there are also high levels in green leafy vegetables and fortified cereals. Total tocopherol levels are

generally higher in seeds than in leaves with their oils containing 300–2000 mg tocochromanols per gram (McLaughlin and Weihrauch, 1979; Hess et al., 2001). Although seeds generally contain a low proportion of α-tocopherol and a higher proportion of α-tocopherol, seeds still represent the major source of naturally derived dietary α-tocopherol due to the large amount of vegetable oils consumed (DellaPenna, 2005).

The recommended daily intake (RDI) for vitamin E is quite low (15 mg/day) at ages over 14. All isomers can be absorbed equally during digestion (Traber, 1996) but the hepatic α-tocopherol transfer protein (α-TTP) shows a preferential retention for α-tocopherol (Traber and Arai, 1999), making it the most important form in terms of vitamin E activity in the human body.

Vitamin E acts as an antioxidant, preventing reactive oxygen species (ROS) from damaging lipid membranes. It protects blood vessels and their contents (especially blood lipids) as well as the nervous system from oxidative stress (Eggermont, 2006). Vitamin E deficiency symptoms are rare, but severe and prolonged insufficient intake results in anemia and neurological problems associated with nerve damage in the hands and feet (Farré et al., 2010a). Deficiency may be caused by malabsorption disorders, or the disruption of plasma lipoprotein transport or oxidative metabolism (Traber and Sies, 1996). High-dose supplements can achieve complete resolution of deficiency symptoms and avoid further clinical manifestations such as cardiac arrhythmia, blindness and dementia (Doria-Lamba et al., 2006). At low levels, α-tocotrienol and α-tocopherol have been shown to prevent neurodegeneration (Brigelius-Flohé and Traber, 1999) and suppresses the growth of human breast cancer cells (Nesaretman et al 1995).

1.2.3 Folate

Folate and folic acid (from Latin *folium*, for leaf) are collectively known as vitamin B9 and are essential for many metabolic processes because they are the source of a key metabolic intermediate, tetrahydrofolate. Good sources of folate include leafy vegetables (e.g. spinach and broccoli), lentils, beans and peas (US Department of Agriculture, Agricultural Research Service, 2003). Moderate amount are also found in fruits (e.g. citrus fruits and juices). The RDI for folate is 400 µg for adults (Table 1.1)

but it is very important for women in early pregnancy to increase their daily intake to 600 µg using fortified foods and/or supplements in addition to the folate from food, in order to reduce the risk of neural tube defects in the fetus (Shaw et al 1995). Folate deficiency is common and can be caused not only by inadequate consumption, but also by malabsoroption (e.g. celiac disease or certain drugs), the higher demand during pregnancy or faster excretion during renal dialysis. Folate deficiency is associated with some types of cancer and many other diseases, including megaloblastic anemia, neural tube defects, heart disease and several neurodegenerative disorders such as Alzheimer's disease (Ames, 1999; Green and Miller, 1999; Seshadri et al., 2002; Stanger, 2004; Choi and Friso, 2005). Low folate levels increase the cellular dUMP/dTMP ratio and promote the incorporation of dUTP into DNA, which generates point mutations, DNA breaks and chromosome damage (Lucock et al., 2003). Adequate dietary folate intake is critical between days 21 and 27 after conception, and the risk of neural tube defects in the fetus must therefore be reduced by consuming additional folate from the peri-conceptional phase until week 12 of gestation (Berry and Li, 2002).

1.2.4 Vitamin C

Vitamin C (ascorbate or ascorbic acid) is a potent antioxidant and also an essential cofactor in a number of key metabolic reactions. Plants are the most important sources of ascorbate in the human diet because they accumulate the molecule in chloroplasts at a concentration of up to 50 mM to ovoid oxidative stress (Smirnoff, 1996). Hydrated storage organs such as fruits and vegetables are better sources than dry seeds (Hancock and Viola, 2005). The best sources of ascorbate are acerola, rosehip and guava fruits, with 1300, 1000 and 23-300 mg of ascorbate per 100 g of fruit, respectively (Davey et al., 2000). Apple, pineapple and melon are also adequate sources (2–10, 12–25 and 10–35 mg per 100g, respectively) but Brussels sprouts are better (87–109 mg per 100g). The RDI for vitamin C is 90 mg for adults, although 46 mg/day is sufficient to prevent scurvy (Carr and Frei, 1999). A vitamin C intake of 120 mg/day can reduce the risk of chronic diseases such as cancer, cardiovascular disease, and cataracts, probably through antioxidant activity (Carr and Frei, 1999). Complete plasma saturation occurs with an intake of 1000 mg ascorbate and there is no clear benefit of exceeding this amount (Levine et al., 1996). The upper tolerable intake for vitamin C is 2000 mg/day and such

doses may acidify the urine, causing nausea and diarrhea, which interfere with the healthy antioxidant/pro-oxidant balance and promotes iron overload in patients with thalassemia or hemochromatosis.

Vitamin C is an essential nutrient because it acts as a cofactor for enzymes that synthesize collagen and the amino acid derivative carnitine, which is needed for the transport of fatty acids (Davidson et al., 1997; Rebouche, 1991). Insufficient vitamin C intake causes scurvy, whose symptoms include halitosis, gingivitis and ulceration (Steward and Guthrie, 1953; Bartholomew, 2002) reflecting the breakdown of connective tissues due to the lack of collagen (Myllyla et al 1978; Yu et al 1988). Scurvy is a usually a rare disease but it can surface in large populations that lack access to fresh fruits and vegetables (Frikke-Schmidt and Lykkesfekt, 2009).

1.2.5 Other vitamins

The vitamin B complex comprises eight distinct molecules (in addition to folic acid) with different properties and functions. Vitamin B_1 (thiamine) is a coenzyme in carbohydrate metabolism, with the triphosphate derivative particularly active in neurons. Deficiency causes beriberi, a disease with neurological and cardiovascular effects. Less severe deficiency can result in weight loss, various degrees of amnesia and psychosis (in its most severe form known as Korsakoff's syndrome), impaired perception, limb weakness, arrhythmia and swelling, possibly leading to heart failure and death (Harper, 2006; Fitzpatrick et al., 2012).

Vitamin B_2 (riboflavin) is the central component of the enzyme cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), whereas vitamin B_3 (niacin) is converted into the cofactors nicotinamide adenine dinucleotide (NAD) and its phosphate derivative NADP. Therefore both vitamins are required in many enzymes that take part in carbohydrate, fat and protein metabolism among other functions. Riboflavin deficiency causes ariboflavinosis, which is characterized by sensitivity to light, cracked lips, dermatitis and swelling of the tongue, pharyngeal and oral mucosa and genitals. It has also been associated with cancer, cardiovascular disease, anemia and neurological and developmental disorders (Powers, 2003). Niacin deficiency causes pellagra, which has varied symptoms including diarrhea, dermatitis, insomnia, fatigue and confusion,

leading in severe cases to dementia (Kohn, 1938; Lanska, 2010). Vitamin B_5 (pantothenic acid) is needed for the synthesis of coenzyme A (CoA), and is therefore a key requirements for the metabolism and synthesis of carbohydrates, proteins and fats (Webb et al., 2004), but deficiency is rare and complete deficiency has not been observed in humans. Vitamin B_6 (pyridoxal, pyridoxine or pyridoxamine) helps to balance sodium and potassium levels; it is also the precursor of pyridoxal phosphate, a cofactor required for the synthesis of heme and several important neurotransmitters. Deficiency may therefore lead to anemia due to the lack of heme, depression due to its impact on neurotransmitter production, high blood pressure and water retention due to the impact on electrolyte balance, and elevated levels of homocysteine, carpal tunnel syndrome and pellagra (Mooney and Hellmann, 2010). Vitamin B_7 (biotin) is a coenzyme in the metabolism of fatty acids and leucine, and it plays a role in gluconeogenesis. Deficiency may lead to stunted growth and neurological disorders in infants. Vitamin B_{12} (cobalamin) is involved in the regeneration of folate, which means that deficiency in many cases mimics folic acid deficiency and can be alleviated by adding folate to the diet. Therefore, even in the presence of adequate folate, cobalamin deficiency can result in the neurological symptoms associated with demyelination. Choline is a B vitamin that has three primary roles: structural integrity and signaling in cell membranes, cholinergic neurotransmission (acetylcholine synthesis) and the provision of methyl groups via its metabolite trimethylglycine (betaine) for the synthesis of S-adenosylmethionine. Choline deficiency is rare because, like vitamin D, humans can synthesize some choline although not always adequate amounts.

Vitamin D is required for normal calcium and phosphorus homeostasis. It is a ligand that, when bound to its receptor, acts as a transcription factor controlling genes that affect calcium and phosphorus absorption; it is therefore particularly important for bone growth and maintenance. Vitamin D deficiency causes impaired bone mineralization, leading to prevalent bone-softening diseases such as rickets (a childhood disease characterized by stunted growth and deformity of the long bones) and osteomalacia (an adult bone-thinning disorder characterized by proximal muscle weakness, bone fragility and chronic musculoskeletal pain). It has also been associated with lung disease, inflammation and immunodeficiency (Michael and Holick, 2007; Janssens et al., 2009).

Vitamin K is a group of fat-soluble vitamins derived from 2-methyl-1,4-naphthoquinone that are needed for γ-carboxylation, a form of protein post-translational modification. This is particularly important for the formation of the calcium-binding Gla domain, which is present in several blood-clotting proteins and osteocalcin. Vitamin K deficiency therefore affects blood coagulation as well as bone mineralization (Hathaway, 1993). Vitamin K1 (phylloquinone) needs to be sourced from the diet whereas vitamin K2 (menaquinone) is produced by bacteria in the large intestine and deficiency is rare except in maladsorption disorders or in patients with reduced gut flora (e.g. after treatment with broad-spectrum antibiotics). There are also several synthetic forms of this vitamin.

1.3 Strategies to address malnutrition

The most effective intervention to alleviate micronutrient malnutrition is a varied diet including fresh fruit, vegetables, fish and meat. This may be impractical in many developing countries because food is not widely available, but even where fresh food is abundant there can be compliance issues that result in persistent low level malnutrition. Where infrastructure allows, micronutrient nutrition can be improved using supplements (usually in tablet/sachet form) or conventional fortification, where micronutrients are added to processed foods, such as packaged cereals (Gómez-Galera et al., 2010). Unfortunately, such strategies have been largely unsuccessful in developing countries because of insufficient funding, poor governance and a poor distribution network (Farré et al., 2010b; Farré et al., 2011).

Biofortification is an alternative strategy in which the nutritional density of crops is increased at source, and this can be achieved by conventional breeding or genetic engineering (Farré et al., 2010c). Conventional breeding does not introduce new genetic material using recombinant DNA technology, but it encompasses accelerated mutation and forced hybridization methods that introgress genes from distant relatives, which similarly would not occur in nature (Bai et al., 2011). Molecular markers can then be used to select the most nutritious crops in conventional breeding programs. The limitations of conventional breeding include the comparatively long lead times before it has an appreciable effect and its dependence on a compatible gene pool. For example, a recent attempt to use variation at the *lyc*E locus in corn 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.

Biofortifed crops can also be generated by transferring genes directly into elite breeding lines resulting in transgenic plants with enhanced nutritional traits. Many crop species have been genetically engineered to produce higher levels of vitamins (see below). Compared to conventional breeding, transgenesis has the advantages of speed, direct engineering of breeding lines, simplicity, the potential for multiple simultaneous biofortification with different nutrients, and unrestricted access to genetic diversity including recombinant genes that do not occur in nature (Zhu et al., 2007). The benefits of conventional breeding and genetic engineering can also be combined to create synergic improvements. For example, the introgression of a recombinant carotenogenic mini-pathway into wild-type yellow-endosperm corn resulted in hybrids with higher levels of zeaxanthin (56 µg/g dry weight) and lutein (23.4 µg/g DW) compared with the parental plants (Naqvi et al., 2011).

Although genetically engineered crops with enhanced nutritional traits provide numerous potential socioeconomic benefits, they have been difficult to bring to the market and politicians need to do more to address the current bottlenecks. Politicians rely on immediate popular support and are unwilling to take politically-controversial decisions. In the short to medium term, nutritionally-enhanced crops would save millions of lives and in the long term would make a significant impact on the health, wellbeing and economic prosperity of the poorest people in the world (Farré et al., 2010b). More needs to be done to rationalize the regulatory framework, which is hampered by discordant international regulations relating to research, biosafety, and the trade and use of genetically engineered crops and their products (Farré et al., 2010b; Ramessar et al., 2010). The general public often receive inaccurate and incomplete information about genetic engineering and its applications, particularly from activists (Rubial-Mendieta and Lints, 1998).

1.3.1 Conventional breeding

Conventional breeding develops new plant varieties by selection over multiple generations and uses genetic material that is already present within a species or compatible species. Many plants show natural variation in vitamin levels due to the presence of major alleles and the additive impact of multiple quantitative trait loci (QTLs) (Farré et al., 2010c). Molecular markers linked to vitamin biosynthesis can be used to select for more nutritious crops in conventional breeding programs. QTLs or mutants with a positive impact on vitamin A, folate, vitamin E and vitamin C levels are therefore useful as research tools, either as a basis for complementation studies or as a starting point for further improvement using biotechnology. However, different nutritional traits (multiple vitamins cannot be enhanced simultaneously by conventional breeding because the resulting breeding programs would become too complex (Zhu et al., 2007; 2008).

1.3.1.1 Vitamin A

Several mutants in corn have been identified with specific deficiencies in carotenoid metabolism. For example, *yellow 1* (*y1*) maps to the *phytoene synthase 1* (*psy1*) gene (Li et al., 2008). Other viviparous (vp) mutants (*vp2, vp5* and *w3*) were mapped to the phytoene desaturase (PDS) gene (Li et al., 1996; Matthews et al., 2003; Hable et al., 1998), *vp9* to the ξ-carotene desaturase (ZDS) gene (Li et al., 2007; Matthews et al., 2003) and *vp7* to the lycopene β-cyclase (LYCB) gene (Singh et al., 2003). Viviparous mutants combine two common mutant phenotypes: albinism (loss of pigments) and viviparity (premature development due to the absence of ABA). Detailed QTL analysis for marker-assisted breeding in corn has been facilitated by the identification of molecular markers associated with the above mutants. For example, a simple sequence repeat (SSR) marker associated with *y1* was linked to a major QTL explaining 6.6–27.2% of the phenotypic variation in carotenoid levels, 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).

Four polymorphisms were found in the corn *lyce* locus. This encodes lycopene ε-cyclase (LYCE), an enzyme that competes with LYCB for lycopene and helps to determine the relative amounts of α- and β-carotenes (Harjes et al., 2008). Pre-harvest sprouting (PHS) mutants in rice (analogous to corn viviparous mutants) also show an albino phenotype because they lack carotenoids, and these have helped to identify the rice genes encoding PDS (*phs1*), ZDS (*phs2-1*, *phs2-2*) and CRTISO (*phs3-1*). Additional rice mutants *phs4-1* and *phs4-2*, encoding LYCB, have been shown to accumulate lycopene (Fang et al., 2008). Similar work has been carried out in other cereals such as wheat and sorghum. The yellow color of durum wheat semolina is due in part to the presence of carotenoid pigments found in the endosperm. Four QTLs affecting endosperm color, one exhibiting strong linkage to the expression of yellow endosperm color, were mapped to the *psy1* and *psy2* genes (Pozniak et al., 2007). The carotenoid profiles of eight selected yellow-endosperm sorghum cultivars have been determined, showing that zeaxanthin is the most abundant carotenoid (Kean et al., 2007).

Potato and tomato have been useful in breeding programs as they exhibit great variation in carotenoid levels. In potato, the *Y* (*Yellow*) locus controls tuber flesh color by influencing carotenoid accumulation (Bonierbale et al., 1988) and has been mapped to a chromosome region with two candidate genes encoding phytoene synthase (PSY) and β-carotene hydroxylase (BCH) as well as potential additional regulatory elements (Bonierbale et al., 1988). Many tomato mutants have been found that affect carotenoid profiles in tomato fruits. Low-carotenoid mutants include *white-flower* (*wf*), *yellow-flesh* (*r*) and *tangerine*, which represent the loss of function of BCH, PSY and CRTISO, respectively (Galpaz et al., 2006; Fray and Grierson, 1993; Isaacson et al., 2002). The tomato *delta* mutant accumulates δ-carotene instead of lycopene, reflecting the increased expression of lycopene ε-cyclase (Ronen et al., 1999). The dominant tomato mutant *Beta* has higher β-carotene levels in fruits, and the *old gold* mutant has lower β-carotene levels in fruits, both mutants representing opposite alleles at the *lycb* locus (Ronen et al., 2000). QTLs affecting fruit color have also been isolated. Liu et al. (2003) identified 16 loci in introgression lines, five of which cosegregated with candidate genes involved in carotenoid synthesis. Eight QTLs affecting lycopene levels in tomato fruits have been identified by crossing a lycopene-rich cultivar and a standard breeding

variety, one QTL accounting for 12% of the variation in lycopene content (Chen et al., 1999).

1.3.1.2 Vitamin E

There is natural variation among different corn breeding lines for tocochromanol levels (Rocheford et al., 2002). The two predominant isomers present in corn are γ-tocopherol and α-tocopherol. Most breeding lines naturally contain much more γ-tocopherol so it may be desirable to not only increase the levels of α-tocopherol in corn, but also the levels of γ-tocopherol (Rocheford et al., 2002). Conventional breeding to select progressively for QTLs that influence α-tocopherol levels is a slow and laborious process. Variants and mutants that affect tocochromanol levels and ratios are useful tools for vitamin E research, either as a basis for complementation studies or as a starting point for further improvement using biotechnology. In soybean seeds, four QTL were found to be associated with α-tocopherol, eight with γ-tocopherol, four with δ-tocopherol, and five with the total vitamin E content (Li et al., 2010).

To address the functions of tocopherols in plants, a series of tocopherol-deficient Arabidopsis mutants have been isolated and characterized. The disruption of the *VTE1* locus, which encodes tocopherol cyclase (TC), resulted in the replacement of tocopherols with the redox-active, lipid-soluble pathway intermediate DMPBQ, whereas the disruption of the *VTE2* locus, which encodes homogentisate phytyltransferase, eliminated tocopherols without causing the accumulation of pathway intermediates (Porfirova et al., 2002; Sattler et al., 2003; 2004). The *vte4-1* and *vte4-2* mutants accumulated high levels of γ-tocopherol at the expense of α-tocopherol (Bergmüller et al., 2003). The *vte4-1* mutant was found to be a null mutation of *VTE4*, which encodes γ-TMT gene. Further experiments showed α-tocopherol is replaced by γ-tocopherol in *vte4-1* mutants to protect the photosynthetic apparatus from oxidative stress caused by strong light, high temperature or cold (Van Eenennaam et al., 2003). Disruption of the *VTE-3* locus encoding 2-methyl-6-phytylbenzoquinol methyltransferase (MPBQ-MT) increased the accumulation of δ-tocopherol at the expense of γ-tocopherol (Van Eenennaam et al., 2003).

1.3.2 Genetic engineering

1.3.2.1 Vitamin A

The carotenoid biosynthesis pathway has two branches, and its regulation is complex, involving bottlenecks and competition for intermediates, conspuring to limit the synthesis of key target molecules (Bai et al., 2011). The amount of β-carotene produced by plants can be enhanced by several different engineering strategies (Table 1.1).

One strategy to enhance carotenoid levels is to increase flux through the pathway non-selectively by providing higher levels of precursors. For example, increasing the pool of available isopentenyldiphosphate (IPP) will increase flux generally towards terpenoid synthesis, including the carotenoids, by removing key bottlenecks in the plastidial MEP pathway. This has been achieved by overexpressing 1-deoxy-D-xylulose 5-phosphate (DXP) synthase to provide more DXP, an early pathway intermediate (Farré et al., 2010c). The drawback of this strategy is that the MEP pathway feeds several different downstream pathways, all of which draw on the larger pool of IPP. The first committed step in carotenoid synthesis is the conversion of GGPP into 15-*cis* phytoene by PSY making this enzyme a useful target to increase the flux in the carotenoid pathway alone. As an example, this strategy was applied in a corn line lacking endogenous PSY activity in the endosperm, removing the bottleneck, increasing the total carotene content up to 52-fold, and leading to the predominant accumulation of lutein and zeaxanthin (Zhu et al., 2008). Similarly, seed-specific expression of *crt*B in canola increased total carotenoid content by 50-fold, predominantly in the form of α- and β-carotene (Shewmaker et al., 1999).

Another strategy in addition to increasing the total carotenoid content is to shift metabolic flux to favor the production of specific carotenoid molecules, particularly those with commercial value or health benefits. For example, canola lines have been created expressing *crt*B, *crt*I and *crt*Y, and transgenic seeds expressing all three genes not only had a higher carotenoid content than wild type seeds as expected following the general increase in flux, but the β:α-carotene ratio increased from 2:1 to 3:1 showing that the additional lycopene β-cyclase activity provided by the bacterial *crt*Y gene skewed the competition for the common precursor lycopene and increased flux

specifically towards β-carotene (Ravanello et al., 2003). Wild-type tomato fruits accumulate lycopene rather than β-carotene, suggesting a lack of cyclase activity prevents the accumulation carotenes downstream of lycopene. Transgenic tomato fruits expressing *crt*I were therefore expected to accumulate more lycopene, since this would increase the flux to lycopene but not affect downstream enzyme activities including cyclization. Surprisingly, the resulting plants contained only 30% of the normal carotenoid content but the amount of β-carotene had tripled (Römer et al., 2000). This unexpected indicated that endogenous lycopene β-cyclase activity had been upregulated in the fruits, a hypothesis that was borne out by the analysis of steady state mRNA levels (Römer et al., 2000). The deliberate overexpression of lycopene β-cyclase in tomato fruits increased the β-carotene levels (Rosati et al., 2000; D'Ambrosio et al., 2004).

In some cases, rather than modulating an existing carotenoid pathway, the aim is to introduce new functionality, i.e. engineer carotenoid metabolism in plants that completely lack these molecules. The most significant example is rice endosperm, where the expression of PSY leads to the accumulation of phytoene but no other carotenoids, indicating the absence of downstream metabolic capability (Burkhardt et al., 1997). The original Golden Rice variety contained three transgenes: the daffodil gene for PSY together with bacterial *crt*I and *crt*Y. The grains accumulated up to 1.6 μg/g dry weight of β-carotene and also β-xanthophylls (Ye et al., 2000). This was not sufficient to provide the recommended RDI of vitamin A from a reasonable portion of rice, so the more active corn *psy1* gene was used to replace its daffodil ortholog, resulting in the Golden Rice 2 variety in which the total carotenoid content of the endosperm increased up to 37 μg/g DW and β-carotene levels reached 31 μg/g DW (Paine et al., 2005).

A final strategy to achieve carotenoid accumulation in plants is to modify the storage capacity, because β-carotene accumulates in specialized lipoprotein-sequestering structures. A spontaneous mutation in the cauliflower *Orange* (*Or*) gene resulted in deep orange cauliflower heads associated with the hyperaccumulation of carotenoids in chromoplasts (Li et al., 2001; Lu et al., 2006). The mutant allele has been cloned and expressed in potato tubers, where it increased the level of β-carotene up to 10-fold and turned the tuber flesh orange (Lopez et al., 2008).

1.3.2.2 Vitamin E

Vitamin E levels in plants can be increased by overexpressing genes involved in tocochromanol synthesis (Table 1.1). The two major strategies are to increase the total tocochromanol content or to skew tocochromanol synthesis towards the more potent vitamers, particularly α-tocopherol.

The first strategy has been achieved by expressing tocochromanol genes alone or in combination. For example, total vitamin E levels are increased by the overexpression of ρ-hydroxyphenylpyruvate dioxygenase (*HPPD*) but can be enhanced further by the simultaneous expression of chorismate mutase-prephenate dehydrogenase (*TYRA)*, the enzyme responsible for the synthesis of HPP from prephenate. *TYRA* expression has little effect on its own, but when combined with *HDDP* and *hpt1* in canola, tocochromanol levels in the leaves increased 3.7-fold (Rippert et al., 2004). The expression of *HPT1, HPPD* and *TYRA* increased the tocochromanol content of seeds up to 5-fold, and the further addition of geranylgeranyldiphosphate hydratase (GGH), which provides the precursor phytyldiphosphate, increased the total tocochromanol content by up to 15-fold (Karunanandaa et al 2005).

The second strategy has been achieved in lettuce by expressing the Arabidopsis *VTE4* gene, to increase the α:γ tocopherol ratio without influencing total tocopherol content (Cho et al., 2005). However, both the total tocochromanol content and the α:γ tocopherol ratio was increased by crossing lines individually expressing *HPT* and *VTE4* (Cho et al., 2005). In canola, total tocochromanol levels have been doubled by expressing genes encoding HPT, HPPD and TC (Raclaru et al., 2006). The simultaneous expression of Arabidopsis *HPPD* and *VTE3* in transgenic corn increased tocochromanol levels by threefold but γ-tocopherol was the only form present in the seeds, indicating that the conversion of γ- to α-tocopherol is limiting and that γ-TMT should be expressed in addition (Naqvi et al., 2010). Consistent with these findings, the expression of γ-TMT in transgenic soybean plants already expressing *VTE3* resulted in a dramatic increase in α-tocopherol content at the expense of γ-tocopherol, confirming the conversion of γ- to α-tocopherol (Van Eenennaam et al., 2003). The expression of *VTE4* in *Brassica juncea* resulted in transgenic seeds containing up to six times as much α-tocopherol as wild type seeds (Yusuf and Sarin, 2006). These data suggest that the expression of *VTE4* alone boosted the α-tocopherol level in seeds at the expenses of

others isomers. Recently, rice seeds expressing Arabidopsis *HPPD* resulted in a small increase in absolute tocotrienol synthesis (but no change in the relative abundance of the γ and α isoforms). In contrast, there was no change in the absolute tocopherol level, but a significant shift from the α to the γ isoform. These data confirm that HPPD is not rate limiting, and that increasing flux through the early pathway reveals downstream bottlenecks that act as metabolic tipping points (Farré et al., 2012).

1.4 Bioavailability

Although much progress has been made towards vitamin enhancement in staple crops, it is important to establish if the total vitamin content in food can be absorbed and used by the body. Sevetral *in vitro* and *in vivo* methods can be used to assess bioavailability (Lemmens et al., 2011; Granado et al., 2006). Typically, nutrient bioaccessibility is determined by *in vitro* methods, whereas nutrient bioavailability can be assessed both *in vitro* and *in vivo*. Nutrient bioaccessibility can be defined as the fraction of the ingested nutrients that is released from the food matrix and is available for intestinal absorption whereas nutrient bioavailability includes nutrient absorption, tissue distribution and metabolism (Lemmens et al., 2011). Therefore *in vitro* bioavailability tests that predict the absorption of phytochemicals by humans should be validated using *in vivo* systems (Granado et al., 2006).

Either animal or human models can be used for *in vivo* assessments, typically involving the isotopic labeling of vitamins in the food matrix with ^{14}C, ^{2}H or ^{3}H followed by the analysis of area under plasma/serum concentrations versus a time curve or urinary excretion as they provide more specificity and clarity (Gregory, 2001). *In vivo* assays in humans are preferred because there are well-documented differences in the intestinal absorption mechanisms among rats, chickens and humans (McNulty and Pentieva, 2004). The factors affecting bioavailability must also be taken into account for each particular vitamin, e.g. food processing techniques, meal components and preparation techniques can modify plant foods in ways that either promote or reduce the amount of bioavailable nutrients (Michaelsen and Friis, 1998).

1.4.1 Vitamin A

Carotenoids are solubilized into micelles in the intestinal lumen where they are absorbed into duodenal mucosal cells by passive diffusion (IOM, 2001). Several methods can be used to determine carotenoid bioavailability, including the balance method, the measurement of radioactive β-carotene in lymph, the use of uniformly labeled β-carotene, and the response in serum or lipoprotein fractions of carotenoids with standard doses of carotenoids (Castenmiller and West, 1998). Isotopic methods have shown that 9–22% of a single dose of β-carotene (45 μg to 39 mg) is absorbed from the intestine (Blomstrand and Werner, 1967; Goodman et al., 1966: Novotny et al., 1995) but that the efficiency of absorption declines with increasing dosage (Tang et al 2000). This low efficiency reflects a wide range of factors that restrict the availability of pro-vitamin A in plants, such as different types of food matrix, processing and interaction with other dietary compounds (Tanumihardjo, 2002; Bai et al., 2011).

Carotenoids in plants are sequestered into protein complexes and cooking has been shown to release them (Anderson et al., 1978; Braumann et al., 1982) because extractability and bioavailability increase in cooked foods (Dietz et al., 1988; Hart and Scott, 1995). This suggests that foods 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). Microwave cooking increases tissue degradation and the amount of β-carotene available for extraction (Howard et al., 1999).

Transgenic lines enhanced for vitamin A have been compared to commercial non-engineered varieties on the market in terms of the quantity of bioavailable β-carotene in a reasonable portion of food, and the relationship of these values to the DRI for vitamin A. Taking the requirements for adult males (900 RAE), the DRI would be achieved by consuming 171 g of a transgenic tomato with 63 μg β-carotene/g DW (Dharmapuri et al., 2002) but 2160 g of the commercial variety Moneymarker would be required, assuming a conversion factor of 12:1 (IOM, 2001). Similarly, 229g of the transgenic potato described by Diretto et al. (2007) would be required (47μg β-carotene/g DW). In cereals, the comparison is even more striking. More than 30 kg of the South African elite corn variety M37W would be required to achieve the DRI for vitamin A (0.013μg β-carotene/g DW), or 6.5 kg of the best conventionally bred corn (1.65 μg /g DW of β-

carotene) but just 229 g of a multivitamin transgenic corn developed by Naqvi et al. (2009a) assuming the same conversion factor of 12:1 (IOM, 2001).

1.4.2 Vitamin E

Like other fat-soluble vitamins, the absorption, transport and distribution of vitamin E within the body are linked to dietary fats. Intestinal absorption requires the presence of bile salts, pancreatic enzymes and adequate levels of fats (Kaydn and Traber, 1993). The food matrix therefore plays a critical role in vitamin E bioavailability (Jeanes et al., 2004). An increase of vitamin E bioavailability was reported in humans by regulating the amount of fat in the food ingested with the vitamin (Lodge, 2005). Vitamin E bioavailability was analyzed in two human experimental groups (Leonard et al., 2004). One group ingested d_2-α-tocopheryl acetate either encapsulated or in a fortified cereal at different meals. Vitamin E bioavailability was higher in the fortified breakfast cereal than in the encapsulated supplements. The adult DRI for vitamin E is 15 mg, which would be provided by 8.1g of transgenic canola seeds containing 1850 μg/g DW of vitamin E (Raclaru et al., 2006).

Table 1.1. Transgenic plants with enhanced levels of pro-vitamin A and vitamin E (updated from Farré et al. 2010a).

Species	**Genes (origin)**	**Promoters**	**Vitamin levels in transgenic plants**	**Reference**
VITAMIN A				
Rice (*Oryza sativa*)	*psy1* and *lycb* (daffodil) *crt*I (*Pantoea ananatis*)	Rice Gt1 (seed specific; *psy1* and *lycb*) and CaMV35S (constitutive; *crtI*)	1.6 μg/g dry weight (DW) total carotenoids	Ye et al 2000. Science 287:303-305
	psy1 (Zea mays) *crt*I (*Pantoea ananatis*)	Rice Gt1	37 μg/g DW total carotenoids	Paine et al 2005. Nat. Biotechnol. 23:482-487
Canola (*Brassica napus*)	*crt*B (*Pantoea ananatis*)	Napin (seed specific)	1617 μg/g fresh weight (FW) total carotenoids (50-fold) 949 μg/g FW β-carotene (316-fold)	Shewmaker et al 1999. Plant J. 20:401-412
	*crt*B (*Pantoea ananatis*)	Napin	1341 μg/g FW total carotenoids 739 μg/g FW β-carotene	Ravanello et al 2003. Metab. Eng. 5:255-263

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN A				
Canola (*Brassica napus*)	*crt*E and *crt*B (*Pantoea ananatis*)	Napin	1023 µg/g FW total carotenoids 488 µg/g FW β-carotene	Ravanello et al 2003. Metab. Eng. 5:255-263
	*crt*B and *crtI* (*Pantoea ananatis*)		1412 µg/g FW total carotenoids 857 µg/g FW β-carotene	
	*crt*B and *crt*Y (*Pantoea ananatis*)		935 µg/g FW total carotenoids 459 µg/g FW β-carotene	
	*crt*B (*Pantoea ananatis*) and *β-cyclase* (*B. napus*)		985 µg/g FW total carotenoids 488 µg/g FW β-carotene	
	*crt*B, *crt*Y and *crtI* (*Pantoea ananatis*)		1229 µg/g FW total carotenoids 846 µg/g FW β-carotene	
	lycopene β-cyclase (*B. napus*) RNAi to 5' end	CaMV35S	227.78 µg/g FW total carotenoids (42.6-fold) in seeds 90.76 µg/g FW β-carotene in seeds (185.2-fold)	Yu et al 2008. Transg. Res. 17:573-585
	lycopene β-cyclase (*B. napus*) RNAi to 3' end		94 µg/g FW total carotenoids in seeds (17.6-fold) 27 µg/g FW β-carotene in seeds (55-fold)	
	Idi, *crt*E, *crt*B, *crt*I, *crt*Y (*P. ananatis)* *crt*Z , *crt*W (*Brevundimonas* spp.)	CaMV35S (*crtE, crtI* and *crtY*), napin (*idi*,and *crtZ*) and Arabidopsis *FAE1(crtW* and *crtB)* (seed specific)	657 µg/g FW total carotenoids (30-fold) 214 µg/g FW β-carotene (1070-fold)	Fujisawa et al 2009. J. Exp. Bot. 60:1319-1332
	microRNA miR156b (*Arabidopsis thaliana*)	Napin	6.9 µg/g FW total carotenoids (2.45-fold) 0.38 µg/g FW β-carotene (6-fold) (10% water content)	Wei et al 2010. J. Agric. Food Chem. 57:5326-5333
Tomato (*Lycopersicon esculentum*)	*psy1* (tomato)	CaMV35S	1159 µg/g FW total carotenoids (1.14-fold) (assuming a water content of 90%)	Fray et al 1995. Plant J. 8:693-701
	*crt*I (*P. ananatis*)	CaMV35S	137.2 µg/g FW total carotenoids (0.5-fold) 52 µg/g FW β-carotene (1.9-fold)	Römer et al 2000. Nat. Biotechnol. 18:666-669
	LYCB (*Arabidopsis thaliana*)	Pds (fruit specific)	109 µg/g FW total carotenoids (1.7-fold) 57 µg/g FW β-carotene (7.1-fold) (assuming a water content of 90%)	Rosati et al 2000. Plant J. 24:413-419

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN A				
Tomato (*Lycopersicon esculentum*)	*LYCB* (*A. thaliana*) *b-chy* (pepper; *Capsicum annuum*)	Pds	100.7 µg/g FW total carotenoids (1.5-fold) 63 µg/g FW β-carotene (12-fold)	Dharmapuri et al 2002. FEBS Lett. 519:30-34
	*crt*B (*Pantoea ananatis*)	polygalacturonase (fruit specific)	591.8 µg/g FW total carotenoids (1.1-fold) 82.5 µg/g FW β-carotene (1.3-fold)	Fraser et al 2002. PNAS 99:1092-1097
	lycb (tomato)	CaMV35S	215.2 µg/g FW total carotenoids (2.3-fold) 205 µg/g FW β-carotene (46.6-fold) (assuming a water content of 90%)	D'Ambrosio et al 2004. Plant Sci. 166:207-214
	dxs (*Escherichia coli*)	fibrillin	720 µg/g FW total carotenoids (1.6-fold) 70.0 µg/g FW β-carotene (1.4-fold)	Enfissi et al 2005. Plant. Biotechnol. J. 3:17-27
	det-1 (tomato, antisense)	P119, 2A11 and TFM7 (fruit specific)	83.8 µg/g FW total carotenoids (2.3-fold) 13 µg/g FW β-carotene (8-fold) (assuming a water content of 90%)	Davuluri et al 2005. Nat. Biotechnol. 23:890-895
	CRY2 (tomato)	CaMV35S	149 µg/g FW total carotenoids (1.7-fold) 10.1 µg/g FW β-carotene (1.3-fold)	Giliberto et al 2005. Plant Physiol. 137:199-208
	*crt*Y (*P. ananatis*)	aptI	3237.1 µg/g FW total carotenoids (0.9-fold) 286.1µg/g FW β-carotene (4-fold)	Wurbs et al 2007. Plant J. 49:276-288
	Fibrillin (pepper)	fibrillin	650 µg/g FW total carotenoids (2.0-fold) 150 µg/g FW β-carotene (1.6-fold)	SimIkin et al 2007. Photochem. 68:1545-1556
	lycb (daffodil)	ribosomal RNA	115 µg/g FW total carotenoids (1.5-fold) 95 µg/g DW β-carotene (5-fold)	Apel and Bock 2009. Plant Physiol. 151:59-66
	psy1 (tomato)	CaMV35S	2276 µg/g FW total carotenoids (1.25-fold) 819 µg/g FW β-carotene (1.4-fold)	Fraser et al 2007. Plant Cell 19:3194-3211
Potato (*Solanum tuberosum*)	*ZEP* (Arabidopsis)	GBSS (tuber specific)	60.8 µg/g DW total carotenoids (5.7-fold) 2.4 µg/g DW β-carotene (3.4-fold)	Römer et al 2002. Metab. Eng. 4:263-272
	*crt*B (*P. ananatis*)	Patatin (tuber specific)	35 µg/g DW total carotenoids (6.3-fold) 10.3 µg/g DW β-carotene (10-fold)	Ducreux et al 2005. J. Exp. Bot. 56:81-89

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN A				
Potato (*Solanum tuberosum*)	*lyce* (potato, antisense)	Patatin	12.27 µg/g DW total carotenoids (2.6-fold) 0.043 µg/g DW β-carotene (14-fold)	Diretto et al 2006. BMC Plant. Biol. 6:13
	*crt*O (*Synechocystis* sp.)	CaMV35S	39.76 µg/g DW total carotenoids (2.1-fold)	Gerjets and Sandmann 2006. J. Exp. Bot. 57:3639-3645
	dxs (*E. coli*)	Patatin	7 µg/g DW total carotenoids (2-fold)	Morris et al 2006. J. Exp. Bot. 57:3007-3018
	*crt*B (*P. ananatis*) *bkt1* (*Haematococcus pluvialis*)	Patatin	5.2 µg/g DW total carotenoids 1.1 µg/g DW total ketocarotenoids	Morris et al 2006. Metab. Eng. 8:253-263
	bkt1 (*H. pluvialis*)		30.4 µg/g DW total carotenoids (4-fold) 19.8 µg/g DW total ketocarotenoids	
	or (cauliflower; *Brassica oleracea* var *botrytis*)	GBSS	24 µg/g DW total carotenoids (6-fold)	Lu et al 2006. Plant Cell 18:3594-3605
	bch (potato, antisense)	Patatin	21.7 µg/g DW total carotenoids (4.5-fold) 0.085 µg/g DW β-carotene (38-fold)	Diretto et al 2007. BMC Plant.Biol. 7:11
	crtB, *crtI* and *crtY* (*P. ananatis*)	Patatin	114 µg/g DW total carotenoids (20-fold) 47 µg/g DW β-carotene (3643-fold)	Diretto et al 2007. PLoS One 2 e350
	bch (potato, antisense)	CaMV35S	4.7 µg/g DW total carotenoids (1.04-fold) 2.64 µg/g DW β-carotene (331-fold) (assuming a water content of 80%)	Van Eck et al 2007. Am. J. Potato Res. 84:331-342
		GBSS	5.23 µg/g DW total carotenoids 2.36 µg/g DW β-carotene (assuming a water content of 80%)	
	or (cauliflower)	GBSS	31 µg/g DW total carotenoids (5.7-fold)	Lopez et al 2008. J. Exp. Bot. 59:213-223

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN A				
Corn (*Zea mays*)	*psy1* (*Z. mays*) *crt*I (*P. ananatis*) *crt*W (*Paracoccus* spp.) *lycb* (*Gentiana lutea*)	Wheat LMW glutelin, barley D-hordein, corn γ-zein, rice prolamin (all endospermspecific)	146.7 µg/g DW total carotenoids (133-fold) 57.35µg/g DW β-carotene (410-fold)	Zhu et al 2008. PNAS 105:8232-18237
	*crt*B *and crt*I (*P. ananatis*)	super γ-zein	33.6 µg/g DW total carotenoids (34-fold) 9.8 µg/g DW DW β-carotene (3.8-fold)	Aluru et al 2008. J. Exp. Bot. 59:3551-3562
	psy1 (*Z. mays*) *crt*I (*P. ananatis*)	Wheat LMW glutelin and barley D-hordein	163.2 µg/g DW total carotenoids (112-fold) 59.32 µg/g DW β-carotene (169-fold)	Naqvi et al 2009. PNAS 106:7762-7767
Lotus japonicus	*crt*W (*Agrobacterium aurantiacum*)	CaMV35S	387 µg/g FW total carotenoids (1.5-fold) 79.3 µg/g FW β-carotene (2.2-fold) 89.9 µg/g FW total ketocarotenoids	Suzuki et al 2007. Plant Cell Rep. 26:951-959
Kumquat	*psy (Citrus sinensis;* orange)	CaMV35S	131.9 µg/g FW total carotenoids (1.6-fold) 1.72 µg/g FW β-carotene (2.5-fold)	Zhang et al 2009. Plant Cell Rep. 28:1737–1746 .
Carrot	*bkt1* (*H. pluvialis*) *CHYB* (*Arabidopsis*)	CaMV35S and *Agrobacterium rhizogenes rol*D (root specific)	300 µg/g DW total carotenoids in root (assuming 87%water content)	Jayaraj et al 2008. Transg. Res. 17:489-501
	PSY (*Arabidopsis*)	CaMV35S	514.1 µg/g DW total carotenoids in roots (93-fold) 241.6 µg/g DW β-carotene (178-fold)	Maass et al 2009. PLoS One 4 e6373
Wheat	*psy1* (*Z. mays*) *crt*I (*P. ananatis*)	CaMV35S and 1Dx5 (constitutive)	4.96 µg/g DW total carotenoids (10.8-fold)	Cong et al 2009. J. Agric. Food Chem. 57:8652-8660
Cassava	*crt*B (*P. ananatis*)	CP1	21.84 µg/g DW total carotenoids (33.6-fold) 6.67 µg/g DW β-carotene (16-fold)	Welsch et al 2010. Plant Cell 22:3348-3356
VITAMIN E				
Corn	*hggt (*barley)	Embryo-specific	> 344.57 µg /g DW in seeds (6-fold)	Cahoon et al 2003. Nat. Biotech. 21:1082-1087

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN E				
Corn	*HPPD* and *VTE3* (*A.thaliana*)	Corn Ubi-1 (constitutive)	9.5 µg/g DW γ-tocopherol (3-fold)	Naqvi et al 2010a. Transg. Res. 20:177-181
Lettuce	*VTE4* (*A.thaliana*)	CaMV 35S	Improved α-/γ – tocopherol ratio up to 0.4 to 544 as compared to α/γ ratio in wild type, which is 0.6 to 1.2	Cho et al 2005. Mol. Cells 19:16-22
	VTE2 (Arabidopsis)	CaMV 35S	40.41 µg/g FW total tocopherol (5.7-fold) 0.46 α/γ ratio no changes	Li et al 2011. Biol Plantarum 55:453-460
	VTE4 (Arabidopsis)	CaMV 35S	12.44 µg/g FW total tocopherol (1.75-fold) 9.19 α/γ ratio (20-fold)	
	VTE2 and *VTE4* (Arabidopsis)	CaMV 35S	64.55 µg/g FW total tocopherol (9-fold) 8.34 α/γ ratio (18.5-fold)	
	Vte2 (Lettuce)	CaMV 35S	17.77 µg/g FW total tocopherol in leaves (2.6-fold of α- and γ-tocopherol) Tocotrienols and other tocopherols were negligible	Ren et al 2011. Afr J Biotech 10:14046-14051
Rice	*HPPD* (*A.thaliana*)	Corn Ubi-1	Significant shift from the γ to the α isoform	Farré et al 2012. Trang. Res. DOI: 10.1007/s11248-012-9601-7
Brassica juncea	*VTE4* (*A.thaliana*)	CaMV 35S	62.29 µg/g of α-tocopherol levels in seeds (6-fold)	Yusuf and Sarin 2006. Transg. Res.16:109-113
Canola	*HPPD* (*A.thaliana*)	DC3Ω	819 µg/g oil in seeds total tocochromanol (1.2-fold)	Raclaru et al 2006. Mol. Breed. 18:93-107
	HPPD, *HPT1*, *VTE1* (*A.thaliana*)	DC3Ω (*hppd*), napin (*hpt1*, *vte1)*	1850 µg/g oil in seeds total tocochromanol (2-fold)	
	tyrA (*E. uredovora*)	napin	540 µg/g of total tocochromanols in seeds (2-fold)	Karunanandaa et al 2005. Met. Eng. 7:384-400
	VTE1 (*A.thaliana*)	Napin	1018 µg/g of total tocochromanols in seeds (1.5-fold)	Kumar et al 2005. FEBS Lett. 579:1357-1364
	vte1 (*Zea mays*)		1159 µg/g of total tocochromanols in seeds (1.7-fold)	

Species	Genes (origin)	Promoters	Vitamin levels in transgenic plants	Reference
VITAMIN E				
Soybean	*tyrA* (*E. uredovora*), *HPPD*, *HPT1*, *GGH* (*A.thaliana)*	Napin CaMV 35S	4806 µg/g of total tocochromanols in seeds (15-fold)	Karunanandaa et al 2005. Met. Eng. 7:384-400
	VTE3 (*A.thaliana*)	7Sα	329 µg/g seed total tocopherol (γ-tocopherol represent 75 to 85%of total tocopherols)	Van Eenennaam et al 2003. Plant Cell 15:3007-3019
	VTE4 (*A.thaliana*)	7Sα	321 µg/g seed total tocopherol (100% of α- and γ-tocopherols in seeds)	
	VTE3 and *VTE4* (*A.thaliana*)	7Sα	320 µg/g seed total tocopherol (8-fold increase in α-tocopherol in seeds)	
	VTE4 (Perilla frutescens)	Vicilin	193 µg/g FW of α-tocopherol content in seeds (10.4-fold) 23.2 µg/g FW of β-tocopherol content in seed (12.8-fold)	Tavva et al 2007. Plant Cell Reports 26:61-70

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CHAPTER 2

AIM AND OBJECTIVES

Chapter 2. Aims and objectives

The focus of my research program has been on the cloning and characterization of a number of important genes involved in the carotenoid biosynthetic pathways and also the elucidation of the mechanism of their regulation in transgenic corn plants. I also explored the tocopherol biosynthetic pathway in rice by engineering a number of biosynthetic pathway genes into rice plants. A key outcome of my research is the effective modulation of the carotenoid and tocopherol biosynthetic pathways in corn and rice using combinatorial genetic transformation. In the process I developed an in depth mechanistic understanding of important factors contributing towards the regulation of the carotenoid pathway in maize and the tocopherol pathway in rice. Furthermore, I investigated socioeconomic and political aspects of plant biotechnology with emphasis on developing countries and identified and discussed critically barriers for the adoption of genetically enhanced staple crops in the developing world.

CHAPTER 3

TRANSCRIPTIONAL-METABOLIC NETWORKS IN CORN PLANTS EXPRESSING TRANSGENES OF THE CAROTENOID BIOSYNTHESIS PATHWAY

Chapter 3. Transcriptional-metabolic networks in corn plants expressing transgenes from the carotenoid biosynthesis pathway

3.1 Abstract

Carotenoids are a diverse group of pigments found in plants, fungi and bacteria. They play vital roles in plants and provide health benefits to mammals (indeed certain carotenoids are essential nutrients in humans). Unlike other cereal crops in the family *Poaceae* (grasses), corn accumulates significant amounts of carotenoids in the endosperm and is therefore a useful model to study carotenoid biosynthesis. The biofortification of major staple crops with carotenoids could help to alleviate malnutrition on a global scale. To enhance carotenoid levels and achieve predictive metabolic engineering in crops, it is necessary to understand the regulatory systems that control carotenoid accumulation in different tissues. We created transgenic corn plants overexpressing the carotenogenic genes *Zmpsy1*, *Pacrt*I, *Gllycb*, *Glbch* and *Paracrt*W. We had previously recovered and reported a diverse population of transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes that allowed us to identify and complement rate-limiting steps in the pathway. An increase in total carotenoid levels was observed in all transgenic lines, with the maximum level (approximately 97-fold increase 40 days after pollination) achieved in a line expressing *Zmpsy1*, *Pacrt*I, *Gllycb* and *Paracrt*W. We carried out an in-depth analysis at the transcript and metabolite levels in an attempt to determine the specific impact of five carotenogenic transgenes and eleven endogenous genes. We reconstructed the temporal profile of the corn carotenoid pathway at the mRNA and carotenoid levels (for total and individual carotenoids) thus increasing our understanding of the multiple bottlenecks in the pathway during endosperm development.

3.2 Introduction

Carotenoids are a group of about 800 lipid-soluble organic molecules whose spectral properties are responsible for the yellow, orange and red colors of the tissues in which they accumulate (Britton et al., 2004). Carotenoids are synthesized by all photosynthetic organisms and many non-photosynthetic bacteria and fungi. In plants, carotenoids fulfill

two essential functions during photosynthesis, i.e. light harvesting and protecting the photosynthetic apparatus from photo-oxidation (Demmig-Adams et al., 1996). They are also the precursors of signaling molecules that influence development and biotic/abiotic stress responses, thereby facilitating photomorphogenesis, non-photochemical quenching and lipid peroxidation, as well as attracting pollinators and seed-distributing herbivores (Park et al., 2002; Pogson et al., 1998; Franco et al., 2007; Havaux and Niyogi, 1999; McNulty et al., 2007; Calucci et al., 2004). Animals and humans are unable to synthesize carotenoids directly and must obtain them from their diets. In humans, carotenoids are required as metabolic precursors and also as anti-oxidants, which help to prevent certain types of cancer (Giovannucci, 2002), maintain the immune system (Chew and Park, 2004) and prevent blindness (Landrum and Bone, 2001; Fraser and Bramley, 2004).

In plants, the synthesis of carotenoids is initiated by the enzyme phytoene synthase (PSY), which condenses two molecules of genarylgeranyl diphosphate to produce the carotene 15-*cis*-phytoene (Misawa et al., 2003). The 15-*cis*-phytoene molecule undergoes four desaturation steps catalyzed by phytoene desaturase (PDS), zeta-carotene isomerase (Z-ISO) and ζ-carotene desaturase (ZDS) to generate the first colored carotene (pro-lycopene), which is converted to all-*trans*-lycopene by carotene isomerase (CRTISO) in non-green tissue and by light in green tissue (Chen et al., 2010; Zhu et al., 2008). In bacteria, a single enzyme encoded by the *crt*I gene accomplishes all the desaturation and isomerization steps and produces all-*trans*-lycopene from 15-*cis*-phytoene directly (Figure 3.1).

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) (Bai et al., 2011). Both enzymes cyclize the linear backbone to generate terminal ionone rings, but the structures of these rings are distinct. In one branch, the addition of one *ε*-ring to lycopene by LYCE generates α-carotene. This is a poor substrate for LYCE so it is unusual for the second epsilon-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 β-carotene. In turn, α-carotene is converted into zeinoxanthin by the di-iron non-heme β-carotene hydroxylase (BCH) and/or the P450-type β-carotene hydroxylases (CYP97A and CYP97B), and then into the yellow pigment lutein by the P450-type β-hydroxylase CYP97C (Bai et al., 2011). In the other branch, lycopene is

cyclized to produce the pro-vitamin A carotenoids α-carotene and β-carotene, which requires the addition of β-rings to both ends of the linear lycopene molecule by LYCB. Subsequent oxygenation of β-carotene results in the formation of β-cryptoxanthin and the non-provitamin A carotenoid zeaxanthin by BCH and/or CYP97A and CYP97B, in a two-step reaction via β-cryptoxanthin (Kim et al., 2010; Quinlan et al., 2007).

In both prokaryotes and eukaryotes, β-carotene can be converted into astaxanthin in alternative reactions catalyzed by β-carotene ketolase and β-carotene hydroxylase (HYDB) (Figure 3.1). There are two distinct pathways, one in which β-carotene is converted into zeaxanthin by HYDB and then into astaxanthin by β-carotene ketolase, and the other in which β-carotene is first converted into canthaxanthin by β-carotene ketolase and then into astaxanthin by HYDB.

Phytoene synthase (PSY), the first committed step in the carotenoid pathway, is the major rate-limiting step in corn endosperm (Wong et al., 2004; Li et al., 2008; Shewmaker et al., 1999; Cong et al., 2009; Zhu et al., 2008). Endosperm-specific expression of *psy1* in corn released this bottleneck, increasing the total carotene content by 52-fold and leading to the predominant accumulation of lutein and zeaxanthin (Zhu et al., 2008). The relative activity of LYCE and LYCB is another pressure point, as this determines the relative abundance of β/α branch carotenoids. The overexpression of LYCB shifts the balance towards the β branch, and tends to enhance β-carotenoids at the expense of α-carotene and lutein (e.g. Rosati et al., 2000; D'Ambrosio et al., 2004). Transgenic canola seeds expressing *crt*B, *crt*I and *crt*Y contained more carotenoids in total than wild-type seeds, and also the β:α ratio increased from 2:1 to 3:1 showing that the additional LYCB activity provided by the bacterial *crt*Y gene increased flux specifically towards β-carotene (Ravanello et al., 2003). Similarly, in transgenic corn seeds expressing *psy1*, *crt*I and *lycb*, the β:α ratio increased from 1.21 to 3.51 (Zhu et al., 2008). Nevertheless, there was also enhanced flux through the α-branch of the pathway, producing nearly 25-fold more lutein than normal (up to 13.12 μg/g dry weight). These examples show that even when shifting the metabolic flux towards β-carotene, there is still enough flux through the other branch of the pathway to produce more than enough lutein for human nutrition (Naqvi et al., 2011).

Another key step in the carotenoid pathway is the conversion of β-carotene to zeaxanthin by BCH, because this step can be targeted for inhibition to promote the

accumulation of β-carotene. Silencing the *bch* gene in potato tubers enhanced β-carotene levels 331-fold and lutein levels 2.5-fold at the expense of zeaxanthin (reduced from 29.65 to 6 μg/g dry weight) without abolishing it completely. In a converse example, the zeaxanthin content of potato tubers was enhanced by silencing the endogenous *zep* gene (Romer et al., 2002). This increased total carotenoid levels by 5.7-fold, β-carotene levels by 3.4-fold and lutein levels by 1.9-fold, while zeaxanthin levels increased 133-fold. The carotenoid content of crops can also be drained by the further conversion of zeaxanthin to violaxanthin, representing another target for intervention by metabolic engineering.

In order to shed more light on the above bottlenecks, we created a number of transgenic plants with particular transgene complements specifically designed to increase flux through the pressure points in the pathway. We carried out an in-depth analysis at the transcript and metabolite levels in an attempt to determine the specific impact of five carotenogenic transgenes and eleven endogenous genes. We reconstructed the temporal profile of the corn carotenoid pathway at the mRNA and carotenoid levels (for total and individual carotenoids) thus increasing our understanding of the multiple bottlenecks in the pathway during endosperm development.

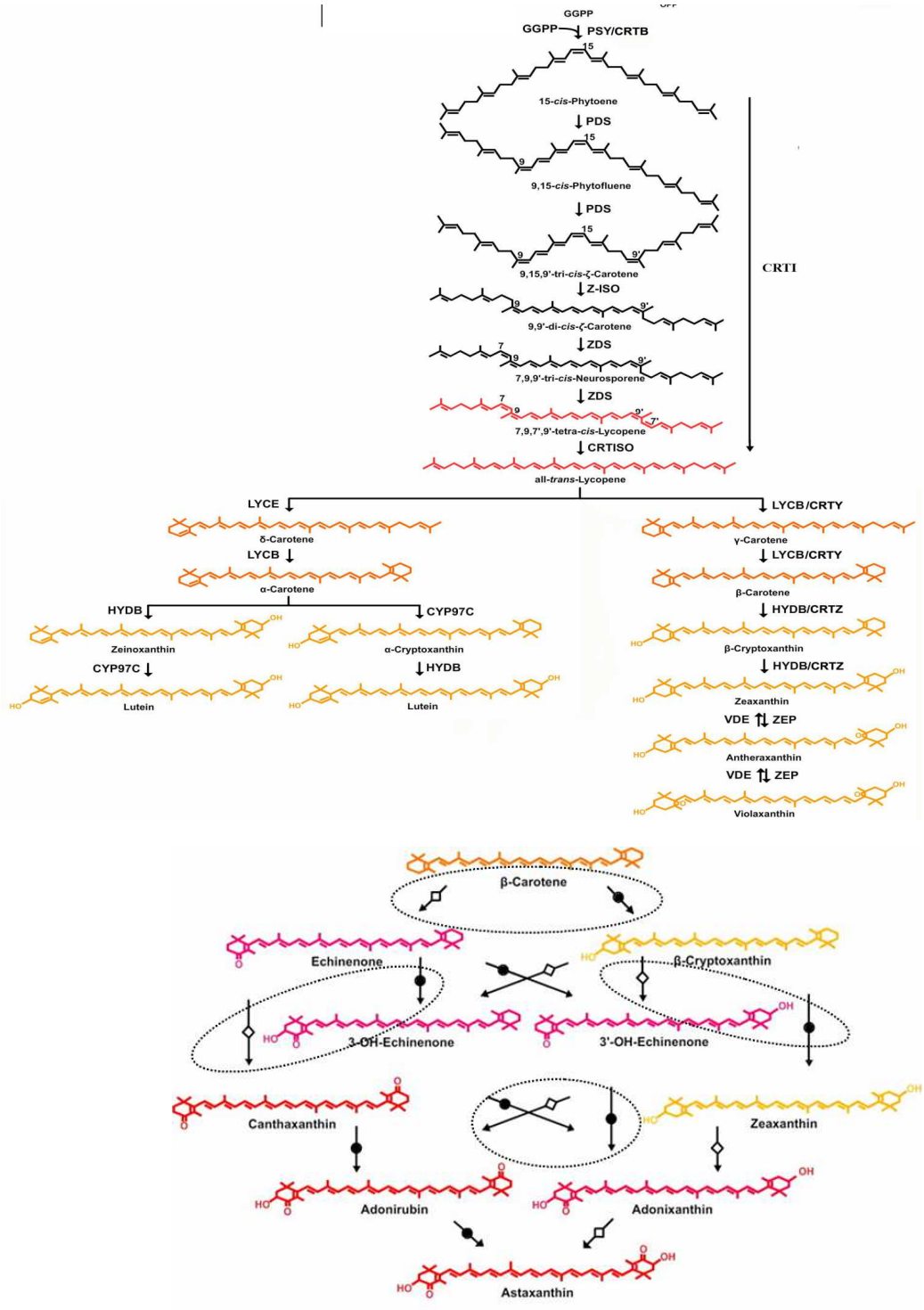


Figure 3.1. The carotenoid biosynthesis pathway in plants and equivalent steps in bacteria. Abbreviations: GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene

hydroxylase (BCH, CYP97A or CYP97B); arrow with square in the middle, β-carotene ketolase (BKT, CRTW or CRTO); arrow with black circle in the middle, β-carotene hydroxylase (BCH or CrtZ) (Bai et al., 2011).

3.3 Experimental procedures

3.3.1 Plant material

Six individual plants were grown for each transgenic corn line (Table 3.1) in the greenhouse at 28/20°C (day/night temperature) with a 10-h photoperiod and 60–90% humidity for the first 50 days, then at 21/18°C (day/night temperature) with a 16-h photoperiod thereafter. Endosperm samples were taken from immature seeds at 15, 20, 25, 30, 40, 50 and 60 days after pollination (DAP), frozen in liquid nitrogen and stored at –80°C prior to use.

Table 3.1. Transgenic corn lines. *Zmpsy1* (*Zea mays* phytoene synthase 1), *Pacrt*I (*Pantoea ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea* β-carotene hydroxylase) and *Paracrt*W (*Paracoccus* spp. β-carotene ketolase).

Line	Expressed genes
Line 1	*Zmpsy1*, *Pacrt*I
Line 2	*Zmpsy1*, *Pacrt*I, *Gllycb*
Line 3	*Zmpsy1*, *Pacrt*I, *Gllycb*, *Paracrt*W
Line 4	*Zmpsy1*, *Pacrt*I, *Gllycb*, *Paracrt*W, *Glbch*

3.3.2 Total RNA isolation

Total RNA was isolated from corn endosperm using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany). RNA was treated on-column for DNA contamination using RNase-free DNase (QIAGEN, Hilden, Germany). RNA concentrations were

determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

3.3.3 Quantitative real-time RT-PCR

We used 2 µg of total RNA for first-strand cDNA synthesis with the Omniscript® Reverse Transcription kit (QIAGEN). Quantitative real-time RT-PCR was carried out using a BioRad CFX96^{TM} system. Each 25-µl reaction comprised 5 ng of cDNA, 1x iQ SYBR green supermix (BioRad, Hercules, CA, USA), and 5 µM of the forward and reverse primers (Table 3.2). Relative expression levels were calculated on the basis of serial dilutions of cDNA (100–0.16 ng) which were used to generate standard curves for each gene. Triplicate amplifications were carried out in 96-well optical reaction plates by first heating to 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Amplification specificity was confirmed by product melt curve analysis over the temperature range 50–90°C with fluorescence acquired after every 0.5°C increase. The fluorescence threshold value and gene expression data were calculated with BioRad CFX96^{TM} software. Values represent the mean of three replicates ± standard deviation. Amplification efficiencies were compared by plotting the ΔCt values of different primer combinations of serial dilutions against the log of starting template concentrations using the CFX96^{TM} software. The Ct values were adjusted to the standard curves and were normalized against the levels of *actin* mRNA.

Table 3.2. Primer sequences for the amplification of corn *actin* and the endogenous and exogenous carotenogenic genes by quantitative real-time RT-PCR.

Gene	Forward primer	Reverse primer
Zmpsy1	5'-CATCTTCAAAGGGGTCGTCA-3'	5'-CAGGATCTGCCTGTACAACA-3'
Zmpsy2	5'-TCACCCATCTCGACTCTGCTA-3'	5'-GATGTGATCTACGGATGGTTCAT-3'
Zmpds	5'-TGTTTGTGCAACACCAGTCG-3'	5'-CTCCTGCTGAAAAGAAGGTGG-3'
Zmzds	5'-GAATGGAGGGAGTGGGAAATG-3'	5'-AGTCTGCATCCGCCGTGTAC-3'
Zmcrtiso	5'-GAATTATATGATTACGGTGTCAGG-3'	5'-TGAAGGGTATCTCAAAACAGAACT-3'

Zmlyce	5'-TTTACGTGCAAATGCAGTCAA-3'	5'-TGACTCTGAAGCTAGAGAAAG-3'
Zmlycb	5'-GACGCCATCGTAAGGTTCCTC-3'	5'-TCGAGGTCCAGCTTGAGCAG-3'
Zmbch1	5'-CCACGACCAGAACCTCCAGA-3'	5'-CATGGCACCAGACATCTCCA-3'
Zmbch2	5'-GCGTCCAGTTGTATGCGTTGT-3'	5'-CATCTATCGCCATCTTCCTTT-3'
ZmCYP97A	5'-CTGGAGCCATCTGAAAGTCA-3'	5'-GGACCAAATCCAAACGAGAT-3'
ZmCYP97B	5'-CTGAGGAGAAGGACTTGACGG-3'	5'-TCCACTGGTCTGTCTGCGAT-3'
ZmCYP97C	5'-GTTGACATTGGATGTGATTGG-3'	5'-AACCAACCTTCCAGTATGGC-3'
PacrtI	5'-ACCTCAACTGGCGAAACTGC-3'	5'-ACAGCGAGTGGAAAGAAAAC-3'
Gllycb	5'-GATTGGCGCGATTCACATCT-3'	5'-GCATGGCATAAAGAAAGGTGG-3'
Glbch	5'-CGGTGTTTGGAATGGCGTA-3'	5'-CGGAGTGATGAAGCGTGTGA-3'
ParacrtW	5'-GTGGCGCAAGATGATCGTCAAG-3'	5'-GCCAGAAGACCACGTACATCCA-3'
Zmactin	5'- CGATTGAGCATGGCATTGTCA-3'	5'- CCCACTAGCGTACAACGAA-3'

3.3.4 Chemicals

We obtained β-carotene, lycopene, lutein, β-cryptoxanthin and astaxanthin from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Zeaxanthin was acquired from Fluka (Buchs SG, Switzerland). Phytoene and antheraxanthin were obtained from Carotenature (Lupsingen, Switzerland). Methanol, ethyl acetate, ethyl ether, hexane, methyl *tert*-butyl ether, acetonitrile and acetone (HPLC grade) were acquired from J.T. Baker (Deventer, The Netherlands). Water was prepared using a Milli-Q reagent water system.

3.3.5 Carotenoid extraction from corn endosperm

Corn endosperm was excised by removing the seed coat and embryo. Samples were freeze-dried before extraction and were ground to a fine powder. Carotenoids in 50–100-mg samples were extracted in 15 ml methanol:ethyl acetate (6:4 v/v) at 60°C for 20 min. The mixture was filtered, transferred to a separation funnel, supplemented with 15 ml hexane:diethyl ether (9:1 v/v) and agitated gently for 1 min. We then added 15 ml of saturated NaCl, the aqueous phase was removed and the organic phase was washed twice with water. The samples were dried under N_2 at 37ºC, flushed with argon and stored at –80 ºC.

3.3.6 HPLC-MS and UHPLC-MS

HPLC (high performance liquid chromatography) and UHPLC (ultra high performance liquid chromatography) were carried out using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) combined with a 2996 photodiode array (PDA) detector (Waters, Milford, MA, USA) and 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 for data acquisition and processing.

A YMC C30 carotenoid 3-µm, 2.0 x 100 mm HPLC column (Waters, Milford, MA) was used for the separation of lutein and zeaxanthin, the mobile phase consisting of solvent A (methanol:water, 80:20 v/v) and solvent B (100% methyl *tert*-butyl ether) at a flow rate of 0.25 ml/min. The gradient program comprised an initial mixture of 97% A and 3% B for 6 min, with a linear gradient to 62% A and 38% B in 1 min, a hold for 8 min followed by a linear gradient to 32% A and 68% B in 1 min, a hold for 2 min followed by a linear gradient to 100% B in 1 min, a hold for 6 min, and a return to initial conditions in 5 min followed by equilibration for 5 min. The run-to-run time was 35 min. The injected volume was 10 µl. The column and sample temperatures were set at 25°C.

All other carotenoids were separated on an ACQUITY UPL® BEH 300 Å $C_1$8, 1.7 µm, 2.1 × 100 mm reversed phase column (Waters, Milford, MA) with a gradient system comprising solvent A (acetonitrile:methanol, 70:30 v/v) and solvent B (100% water) at a flow rate of 0.4 ml/min. The gradient program comprised an initial mixture of 80% A and 20% B for 2 min, with a linear gradient to 100% A in 1 min, a hold for 9 min, and a return to initial conditions in 0.1 min followed by equilibration for 2 min. The run-to-run time was 14 min. The injected volume was 5 µl. The column and the sample temperatures were set at 32°C and 25°C respectively. The extracts were dissolved in 300 µl acetonitrile:methanol:acetone (35:15:50 v/v).

3.3.7 Carotenoid identification and quantitation

Carotenoids were identified according to the following parameters: order of elution from the column, ultraviolet and visible spectra, the spectral fine structure (%III/II) (Britton et al., 2004), mass fragments based on literature data (Rivera et al., 2011) and

comparison to authentic standards. Those standards were also used for quantitation in combination with the extinction coefficients (Britton et al., 2004).

3.4 Results

3.4.1 Analysis of gene expression by quantitative real-time RT-PCR

3.4.1.1 Transgene expression profiles in developing corn endosperm

We determined the accuracy of quantitative real-time RT-PCR measurements by plotting the ΔC_t values of different primer combinations in serial dilutions against the log of the starting template concentrations using CFX96^{TM} software (BIO-RAD, Hercules, CA). The data presented in this chapter directly compare the expression levels of specific transgenes in four corn genetic backgrounds at different endosperm developmental stages from 10 to 30 days after pollination (dap) (Figure3.2).

The *Zmpsy1* transcript was not detected in wild-type M37W endosperm, in agreement with previous investigations (Zhu et al., 2008), but it was expressed in all four transgenic lines. Transcript levels peaked between 20 and 25 dap in lines 1, 3 and 4, but remained constant throughout the analysis period in line 2. The highest *Zmpsy1* expression levels were observed in line 1.

*Pacrt*I was expressed in all four transgenic lines, although at different levels, with line 3 showing the highest expression levels overall. The expression profile in line 3 peaked at 25 dap and then declined from 30 dap onwards.

Gllycb was expressed at similar levels in lines 2 and 3 and at higher levels in line 4 (Figure 3.2). The maximum expression was observed at 30 dap in all four lines. *Glbch* was only expressed in line 4, peaking at 20 dap. *Paracrt*W was expressed in lines 3 and 4. Expression was much higher in line 3 and peaked at 25 dap, whereas expression levels remained constant in line 4.

3.4.1.2 Endogenous gene expression profiles in developing corn endosperm

Eleven endogenous carotenogenic genes were analyzed during endosperm development in the four transgenic lines and wild-type plants, namely *Zmpsy2, Zmpds, Zmzds, Zmcrtiso, Zmlyce, Zmlycb, Zmbch1, Zmbch2, ZmCYP97A, ZmCYP97B* and *ZmCYP97C* (Figure 3.2). Quantitative real-time RT-PCR showed that *Zmpsy2* was expressed at low levels in all four lines (and wild-type plants) whereas *Zmpds* and *Zmzds* were expressed at higher levels in the transgenic plants. There was not much difference among the transgenic lines and the maximum expression level was reached at 30 dap. *Zmcrtiso* was also expressed at higher levels in the transgenic plants, although not to the degree seen with *Zmpds* and *Zmzds*. These genes were expressed more strongly in lines 2 and 3. *Zmlyce* was also induced in the transgenic plants and reached a maximum at 30 dap, but there were differences between lines earlier in development. In line 1, *Zmlyce* expression declined from 15 to 25 dap and then increased at 30 dap, whereas in line 2 it was expressed at a constant level from 15 to 25 dap followed by an increase at day 30. Lines 3 and 4 showed the same expression pattern, i.e. increasing expression up to day 20 followed by a plateau and then a peak at 30 dap. *Zmlycb* mRNA levels were similar in all transgenic and wild-type plants, and peaked at 25 dap.

Zmbch1 levels were similar to wild type in all lines, whereas *Zmbch2* levels were higher in the transgenic lines, especially in line 1. *Zmbch2* expression in all lines peaked at 30 dap except in line 3, where expression peaked at 25 dap. Lines 1 and 4 showed the same *Zmbch2* expression profile whereas the expression level in line 2 was lower. Line 1 showed the highest *Zmbch2* expression levels between all lines and it increased during development until 30 dap.

The expression of *ZmCYP97A, ZmCYP97B* and *ZmCYP97C* peaked at 30 dap in the transgenic lines but the overall levels were much lower than in wild-type endosperm.

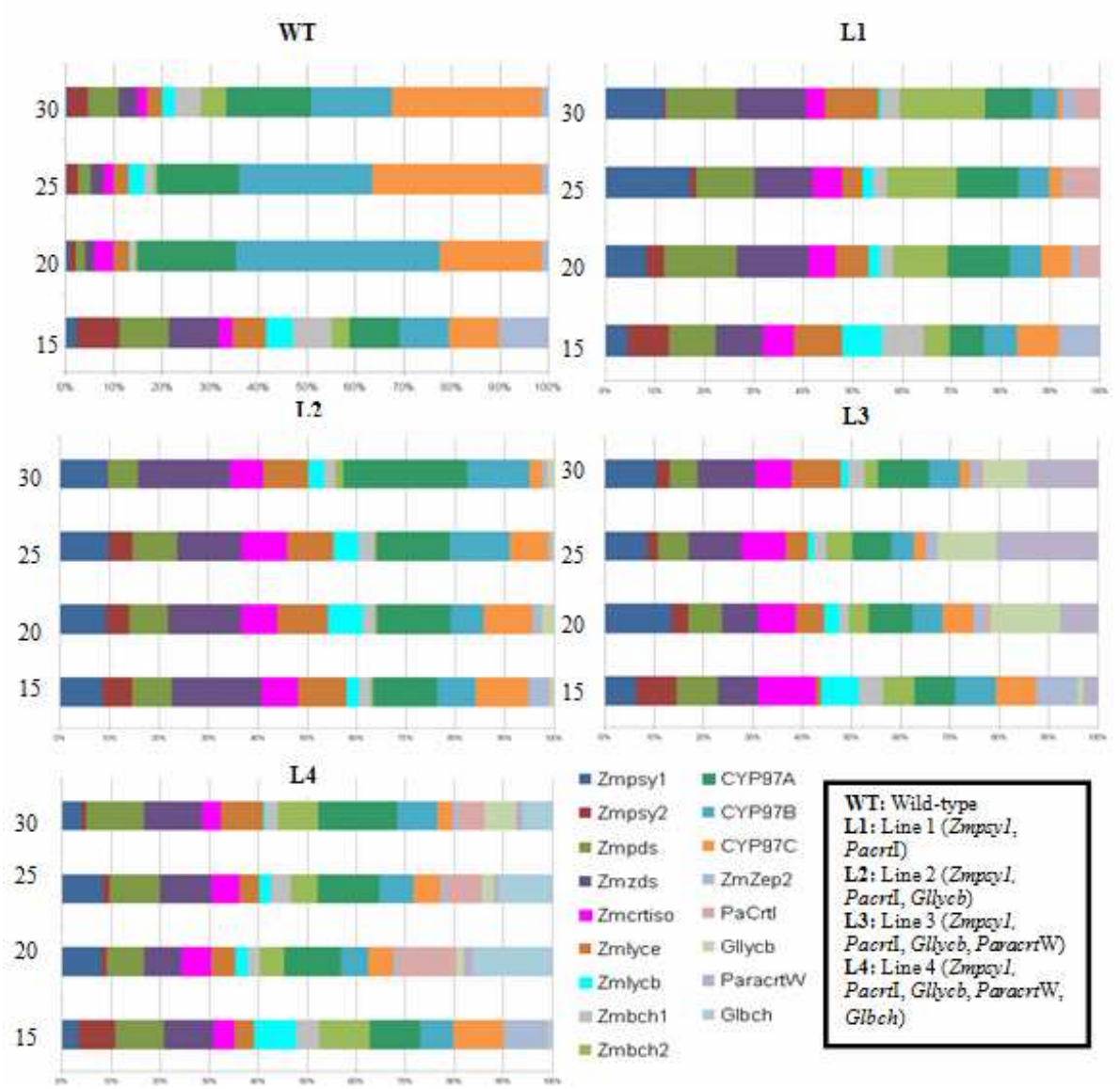


Figure 3.2. Real-time RT-PCR analysis showing relative mRNA levels for transgenes and endogenous carotenogenic genes in immature corn endosperm in the wild type (WT) and four transgenic lines (L1, L2, L3 and L4) at four different developmental stages (15, 20, 25 and 30 dap). Abbreviations: Zm, *Zea mays*; Pa, *Pantoea annatis*; Gl, *Gentanua lutea*; Para, *Paracoccus*; PSY1/2, phytoene synthase 1/2; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; BCH1/2, β-carotene hydroxylase 1/2. β-carotene hydroxylase; CYP97A, carotene ε-hydroxylase; CYP97C; ZEP2, zeaxanthin epoxidase 2.

3.4.2 Carotenoid measurement

Carotenoid levels were measured from 15 to 60 dap and we found that carotenoids are synthesized continuously in corn endosperm from 15 dap onwards. However, the concentration of most carotenoids peaked during development and then declined as the seeds matured, with only β-cryptoxanthin, α-cryptoxanthin and echinenone continuing to accumulate up to 60 dap (Table 3.3). The levels of some carotenoids declined marginally after peaking (e.g. lutein) whereas others declined significantly (e.g. antheraxanthin). This may reflect degradation caused by light or heat, the consumption of carotenoids in other metabolic pathways (such as carotenoid epoxides acting as precursors for abscisic acid (ABA) synthesis) or the dilution of carotenoids by accumulating starch. The individual carotenoid content and profile of each transgenic line was distinct, and different to that of wild-type plants.

Table 3.3. Maximum accumulation of different carotenoids presented as the number of days after pollination.

Carotenoid	**Line 1**	**Line 2**	**Line 3**	**Line 4**
Phytoene	*50*	40-50	50	40
Lycopene	*50*	0	40-50	-
β-Carotene	*30-50*	40	50	30-40
α-Carotene	-	-	50	30-50
β-Cryptoxanthin	*25-30*	40	40-60	25-60
α-Cryptoxanthin	*25-40*	40	40-60	50
Lutein	*30-40*	30-40	30-40	50
Zeaxanthin	*25-30*	*25*	*25-30*	*30-50*
Antheraxanthin	*20-25*	*25*	*20*	*25*
Echinenone	-	-	*40-50*	*30-60*
3-Hydroxyechinenone	-	-	*30-50*	*20*
Adonixanthin	-	-	*40*	*25*
Astaxanthin	-	-	*25*	-
Total	*30-50*	40	30-40	30-40

3.4.2.1 Carotene accumulation during endosperm development

Phytoene began to accumulate at 15 dap in line 3, and later in lines 1, 2 and 4, until it reached its maximum at 40–50 dap (Figure 3.3.D). The lowest phytoene concentration was found in line 4 (13.88 ± 0.67 μg/g DW) perhaps reflecting the weakest expression of the *Zmpsy1* transgene among the transgenic lines. Lycopene was only detected in lines 1 and 3, which expressed *Zmpsy1-Pacrt*I and *Zmpsy1-Pacrt*I-*Gllycb-Paracrt*W, respectively (Figure 3.3.I). Lycopene began to accumulate by 15–20 dap in both lines. The most significant increase in lycopene was observed between 20 and 25 dap in line 3, followed by a marginal increase to the maximum at 40–50 dap and a decline towards 60 dap, whereas in line 1 lycopene increased from 15 to 20 dap then reached a plateau between 20 and 25 dap, followed by another increase up to 30 dap and continued accumulation up to day 50. The highest concentration of lycopene was found in line 3, followed by line 1 (6.25 ± 0.22 and 3.72 ± 0.41 μg/g DW, respectively).

Line 4 contained the highest concentration of β-carotene, followed by lines 1, 2 and 3 (26.33 ± 0.15, 13.62 ± 0.45, 5.36 ± 0.14 and 5.34 ± 0.33 μg/g DW, respectively) (Figure 3.3.C). The accumulation of *β*-carotene began by 15 dap in line 1, but between 15 and 20 dap in lines 3 and 4 and after day 20 in line 2, reaching maximum levels between 30 and 60 dap depending on the line.

3.4.2.2 Xanthophyll accumulation during endosperm development

The profile of antheraxanthin accumulation was similar in all lines and the highest levels were found in line 2 (Figure 3.3.F). Antheraxanthin began to accumulate by 15 dap, peaked at 20–25 dap (depending on the line) and rapidly fell to undetectable levels thereafter, the most dramatic loss among all the carotenoids we tested. This behavior may indicate that antheraxanthin is more reactive than other carotenoids due to the presence of an epoxy group, or may reflect its status as a precursor of ABA.

The lutein profile was similar in all the transgenic lines (Figure 3.3.G), increasing steadily and peaking by 30–40 dap (lines 1, 2 and 3) or 50 dap (line 4) before declining slightly. The highest level of lutein was found in line 2, followed by lines 4, 3 and 1

(10.59 ± 0.17, 8.51 ± 0.02, 7.35 ± 0.17 and 6.70 ± 0.33 μg/g DW, respectively). Zeaxanthin was the major carotenoid found in all the four lines (39.92%, 49.65%, 32.95% and 28.18% of total carotenoid concentration at 30, 40, 40 and 30 dap, in lines 1, 2, 3 and 4, respectively) (Figure 3.3.B). Accumulation began by 15 dap and increased steadily until 25–30 dap in lines 1 and 3, 25 dap in line 2 and 30–50 dap in line 4. The accumulation of *β*-cryptoxanthin was similar in lines 2, 3 and 4 but the peak accumulation was higher and earlier in line 1 (Figure 3.3.H) and there was no decline from the peak in lines 3 and 4. The maximum concentration of α-cryptoxanthin was found by 25–40, 40, 40–60 and 50 dap in lines 1, 2, 3 and 4, respectively (Figure 3.3.J) followed by a decline in all the lines except line 3.

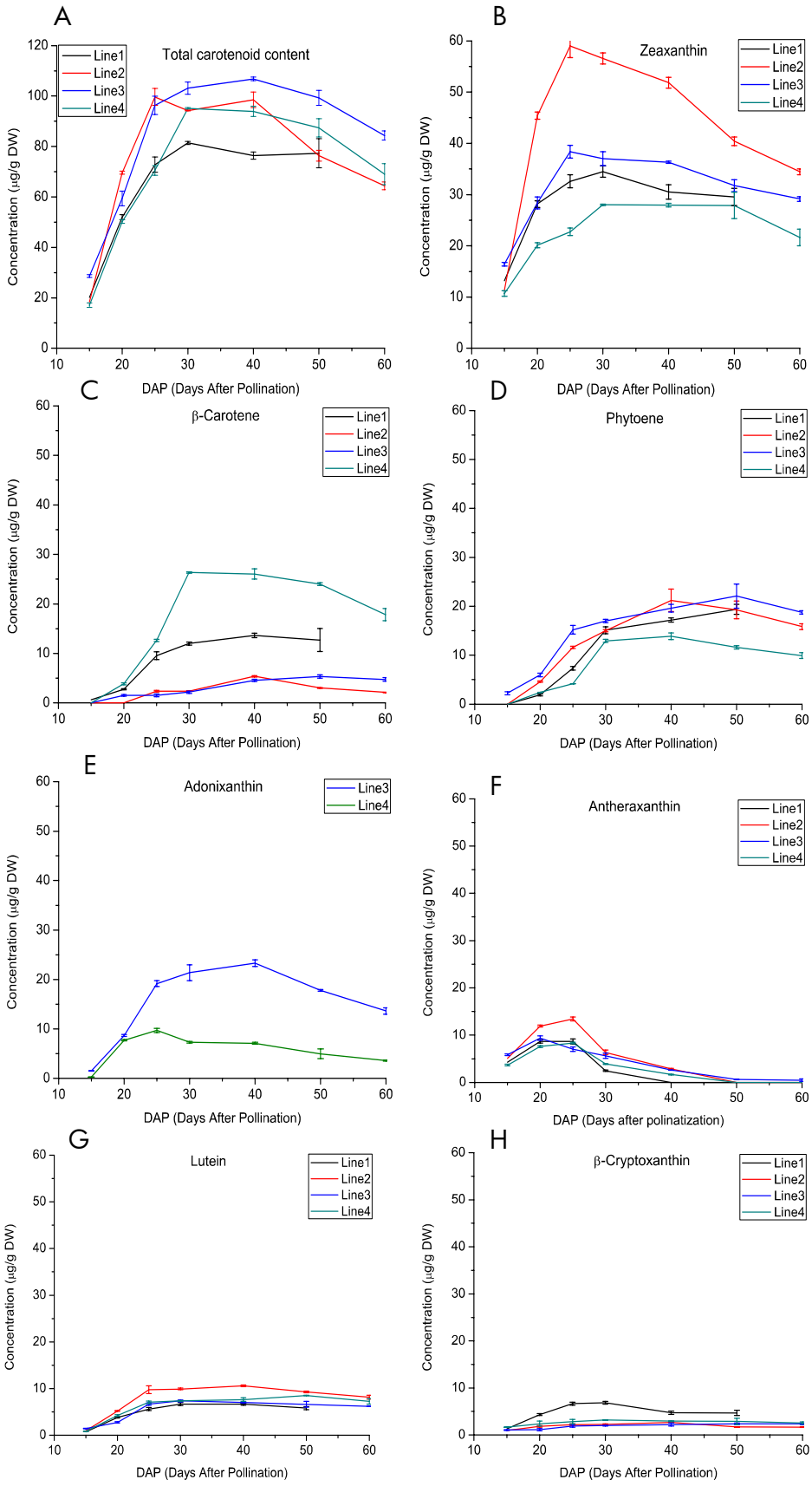
3.4.2.3 Individual and total ketocarotenoid accumulation during endosperm development

Ketocarotenoids were only detected in lines 3 and 4, reflecting the expression of *Paracrt*W in addition to *Zmpsy1-Pacrt*I-*Gllycb* (line 3) or *Zmpsy1-Pacrt*I-*Gllycb*-*Glbch* (line 4). The most abundant ketocarotenoid in both lines was adonixanthin (Figure 3.3.E). In line 3, adonixanthin levels increased significantly between 15 and 25 dap then more moderately until 40 dap before declining. In contrast, adonixanthin levels in line 4 increased significantly between 15 and 20 dap, peaking at 25 dap and then declining. We also detected 3-hydroechinenone and echinenone in lines 3 and 4 but at much lower concentrations than adonixanthin (Figure 3.3.K and 3.3.L). Echinenone accumulation began after 15 dap in line 4 but after 25 dap in the line 3, increasing to a maximum at 40–50 and 30–60 dap in lines 3 and 4, respectively, and declining only in line 3 (Figure 3.3.L). The maximum echinenone concentration was similar for both lines, whereas adonixanthin levels were higher in line 3 than line 4. Similarly, the highest levels of 3-hydroechinenone were found in line 3, where the concentration increased steadily and peaked at 30–50 dap compared to the earlier peak at 20 dap in line 4 (Figure 3.3.K). In both lines, the 3-hydroechinenone level declined after reaching its maximum concentration. Astaxanthin was only detected in line 3 at low levels, beginning after 15 dap and increasing between 20 and 25 dap before declining (Figure 3.3.M). Line 3 accumulated the highest total ketocarotenoid levels (up to 26 μg/g DW beginning by 15 dap; Figure 3.3.O). Total ketocarotenoids increased most significantly between 15 and

25 dap in line 3 and between 15 and 20 dap in line 4, then more moderately to a peak at 40 dap in line 3 and 25 dap in line 4 before declining.

3.4.2.4 Total carotenoid accumulation during endosperm development

Although individual carotenoid concentrations peaked on different days, the total carotenoid content peaked between 30 and 40 dap in all lines (Figure 3.3.A). The total carotenoid accumulation in lines 1, 2 and 4 was similar at 15 dap but there was a higher concentration in line 3. The most significant increase in total carotenoid levels took place between 15 and 25 dap in lines 1, 2 and 3 but between 15 and 30 dap in line 4. There was a further slight increase in lines 1, 3 and 4 until the maximum, whereas there was a small dip in concentration between 25 and 30 dap in line 2. The maximum total carotenoid content in line 1 was observed 30–50 dap (86.34 ± 0.65 μg/g DW), whereas in line 2 the maximum occurred at 40 dap (104.4 ± 3.08 μg/g DW) and in lines 3 and 4 at 30–40 dap (110.21 ± 0.72 and 99.42 ± 0.27 μg/g DW, respectively). Total carotenoids then declined in lines 2, 3 and 4. The highest overall total carotenoid content was observed in line 3, which expressed *Zmpsy1-Pacrt*I-*Gllycb-Paracrt*W (Figure 3.4).


A
Total carotenoid content
Line1
Line2
Line3
Line4
Concentration (μg/g DW)
DAP (Days After Pollination)
B
Zeaxanthin
C
β-Carotene
D
Phytoene
E
Adonixanthin
F
Antheraxanthin
DAP (Days after polinatization)
G
Lutein
H
β-Cryptoxanthin

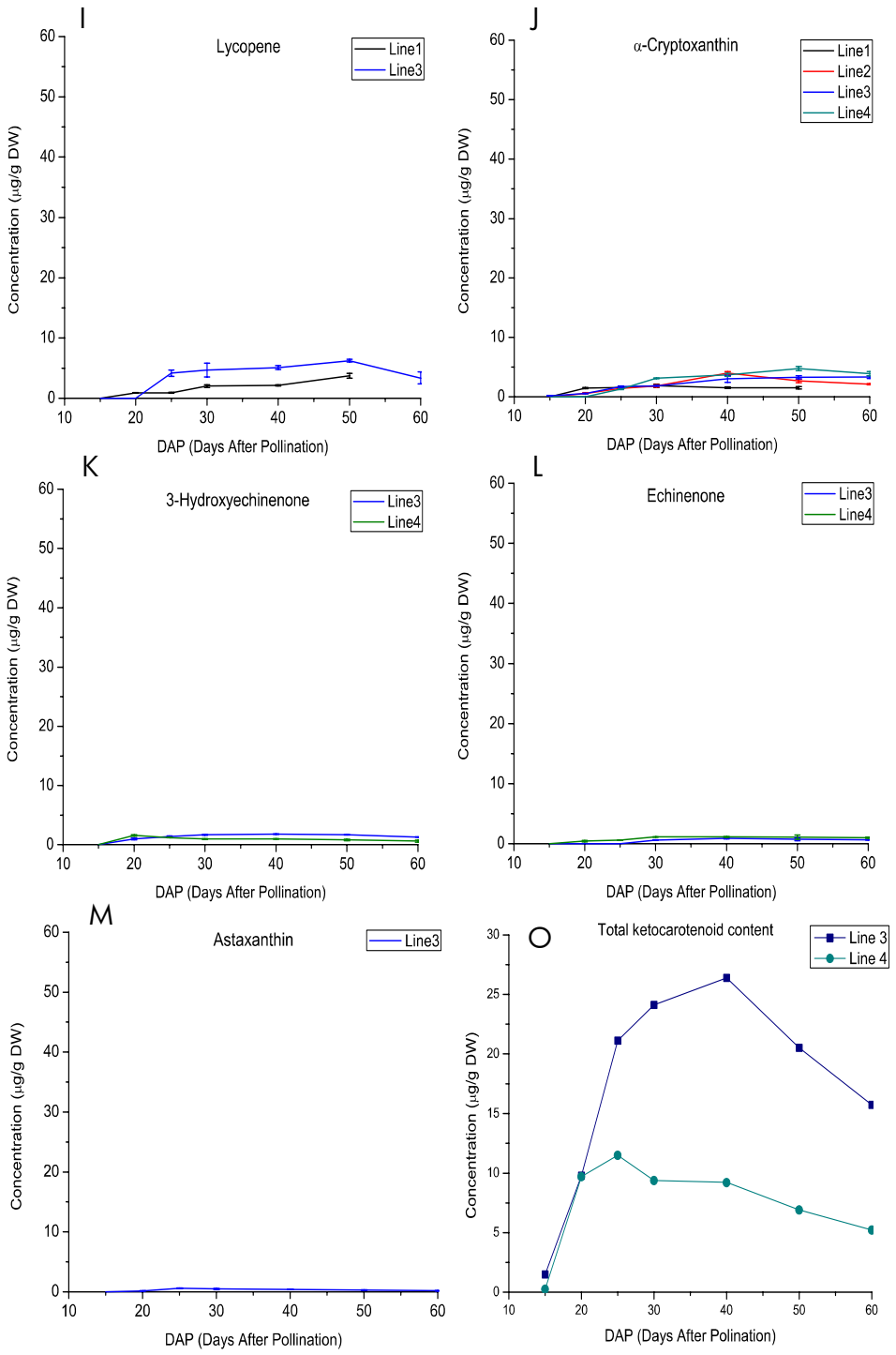


Figure 3.3. Total and individual carotenoid concentration profiles during corn endosperm development. (A) Total carotenoid content in μg/g dry weight (DW); carotenoids detected in the samples: (B) zeaxanthin, (C) β-carotene, (D) phytoene, (E) adonixanthin, (F) antheraxanthin, (G) lutein, (H) β-cryptoxanthin, (I) lycopene, (J) α-cryptoxanthin, (K) 3-hydroxyechinenone, (L) echinenone and (M) astaxanthin.

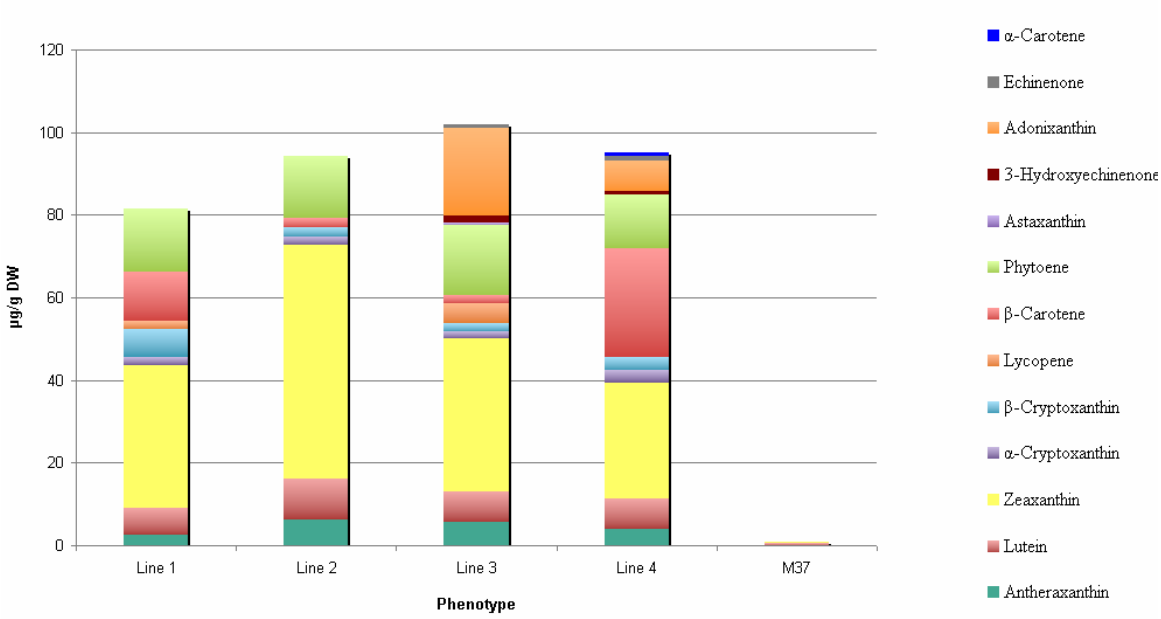


Figure 3.4. Carotenoid composition in the four transgenic lines.

3.5 Discussion

3.5.1 Regulation of carotenoid biosynthesis in corn endosperm

In plants, carotenoid biosynthesis occurs in the plastidial membranes. The enzymes required for carotenoid biosynthesis are encoded in the nucleus and targeted to the plastids (Bartley and Scolnik, 1994). Carotenoids accumulate in all types of plastids including those in fruits, flowers, roots and seeds, e.g. chloroplasts, chromoplasts and amyloplasts (Hirschberg, 2001). In starchy corn kernels, carotenoids accumulate predominantly in the endosperm tissue (Steenbock and Coward, 1927; Blessin et al., 1963) particularly in the amyloplasts, where they play an important role in ABA production and seed dormancy (Maluf et al., 1997). Their antioxidant activity also reduces membrane deterioration induced by free radicals (Calucci et al., 2004).

In plants, the genes encoding enzymes acting at nearly every step of the carotenoid pathway have been identified (Matthews and Wurtzel, 2007). Important advances have been made in order to characterize the function of carotenogenic genes in several different plant species. The regulation of carotenoid biosynthesis is complex and is

restricted to specific tissues (Lima et al., 2005). In order to understand carotenogenesis in plants fully, a comprehensive understanding of gene regulation, biochemical interactions among the enzymatic complexes catalyzing carotenoid biosynthesis, and the interconversion of different metabolites is necessary (Taylor and Ramsey, 2005). Carotenoid accumulation in fruits and flowers correlates with the abundance of transcripts representing key carotenoid genes (Cunningham, 2002). A positive correlation between higher *psy1* transcript levels and enzyme activity was observed in tomatoes, increasing the carotenoid content in the ripened fruit (Fraser et al., 1994). The accumulation of high levels of lycopene in tomato fruits suggests that a different regulatory mechanism could be involved in carotenogenesis in green tissues, which accumulate less lycopene (Fraser et al., 1994). Carotenoid biosynthesis in plastidial membranes might therefore be viewed as a distinct pathway (Wurtzel, 2004).

Phytoene synthesis

Phytoene synthase (PSY) is the first committed enzyme in carotenoid biosynthesis and it has been extensively studied in corn because it is rate-limiting for the production of carotenoids in the endosperm (Gallagher et al., 2004; Wong et al., 2004; Pozniak et al., 2007; Li et al., 2008). Corn has three *psy* paralogs and there are also multiple paralogs in other grasses, but in the endosperm only the abundance of the *psy1* transcript correlates with carotenoid levels (Buckner et al., 1996). Enzymes encoded by each of the three paralogs (*psy1*, *psy2* and *psy3*) are functional when expressed in bacteria (Gallagher et al., 2004). The three paralogous *psy* genes vary in tissue specificity in terms of expression in responses to abiotic stress (Li et al., 2008). The corn *psy2* transcript is mainly expressed in leaves and in lower levels in embryo (Gallagher et al., 2004). The corn *psy3* transcript is expressed predominately in the root and embryo, (Howitt and Pogson, 2006). These expression profiles suggest that the corn PSY genes may be functionally specific rather than redundant (Li et al., 2009).

The analysis of mRNA levels revealed that the *Zmpsy1* transcript is not present in M37W corn endosperm whereas all the transgenic lines expressed the transcript strongly. *Zmpsy2* expression as determined by quantitative real-time RT-PCR suggested that the residual carotenoid content of M37W endosperm may reflect the activity of

PSY2. Although *Zmpsy2* transcripts were detected in the M37W endosperm, the total carotenoid content remains low, confirming that PSY1 rather than PSY2 plays a crucial role in the accumulation of carotenoids in the endosperm.

Phytoene desaturation and isomerization

Phytoene undergoes four desaturation/isomerization steps to produce all-*trans*-lycopene. We found that phytoene desaturase (*Zmpds*), ζ-carotene desaturase (*Zmzds*) and carotene isomerase (*Zmcrtiso*) transcripts were present at similar levels in all lines, although *Zmpds* and *Zmzds* mRNAs were more abundant than *Zmcrtiso*. The expression of *Pacrt*I increased the lycopene content in lines 1 and 3. However, phytoene could still be detected in all lines, suggesting that the desaturation steps might be rate limiting. The *Pacrt*I and *Zmcrtiso* mRNAs were most abundant in line 3, which is consistent with the higher accumulation of lycopene in this line. Phytoene was not detected in wild-type M37W endosperm but it accumulated in all of the transgenic lines. This suggests that the conversion of phytoene to lycopene (catalyzed by both endogenous desaturases/isomerases and *Pacrt*I) is a rate-limiting step for carotenoid biosynthesis in the transgenic lines.

Lycopene cyclization

The cyclization of all-*trans*-lycopene is an important branch point in carotenoid biosynthesis. Symmetrical cyclization catalyzed by LYCB produces β-carotene, whereas LYCE adds one ε-ring and a second cyclization by LYCB produces α-carotene (Cunningham et al., 1996). Cyclases control the relative concentrations of lutein and zeaxanthin in the endosperm. Higher levels of lycopene accumulation in line 1 and 3, despite the expression of *Gllycb* in line 3, indicate that LYCB is also a rate-limiting enzyme in the M37W endosperm reflecting the insufficient conversion of lycopene into β-carotene. In contrast, no lycopene was detected in lines 2 and 4, confirming the efficient conversion of all-*trans*-lycopene into β-carotene. Lower levels of *Zmlyce* mRNA and lutein in lines 3 and 4 revealed an insufficient endogenous capacity for lycopene cyclization to produce α-carotene. Line 4 expressed *Gllycb* at the highest

levels and contained a higher β-carotene content. The ratio of *β*- to *ε*-ring derivatives was 5.0 in line 1 (*Zmpsy1+PacrtI*), 3.44 in line 2 (*Zmpsy1+PacrtI+Gllycb*), 4.47 in line 3 (*Zmpsy1+PacrtI+Gllycb+Pacrt*W) and 4.51 in line 4 (*Zmpsy1+PacrtI+Gllycb+Pacrt*W+*Glbch*) at 30 DAP. As observed in transgenic canola (Ravanello et al 2003) and rice (Ye et al 2000), the *β, β* branch of the pathway appears to be favored, perhaps implying the existence of a rate-limiting step in the *β, ε*-branch.

Hydroxylation

Hydroxylases convert pro-vitamin A carotenes to non-vitamin A xanthophylls, which typically have either a hydroxyl group at C3 or an epoxy group at the 5,6-position of the ionone ring (Howitt and Pogson, 2006). Hydroxylation of the β and ε rings is carried out by β-hydroxylases and ε-hydroxylase, respectively (Hirschberg, 2001; Pogson et al., 1996). Following gene duplication and divergence, many plants have multiple β-carotene hydroxylases, including Arabidopsis (Rissler and Pogson, 2001), tomato (Liu et al., 2003), saffron (Castillo et al., 2005) and corn (Zhu et al., personal communication). Hydroxylase levels play a key role in the regulation of pro-vitamin A carotenes in corn endosperm (Wurtzel, 2004). We observed low levels of *Zmbch1* in all lines and higher levels of *Zmbch2*, with particularly high levels in line 2 correlating with higher levels of zeaxanthin. Higher levels of hydroxylated products (such as lutein and zeaxanthin) and lower levels of β-carotene in lines 1, 2 and 3 indicated the efficient hydroxylation of α and β carotenes. In line 2, the low levels of β-carotenes and higher levels of zeaxanthin appear to reflect the high levels of *Zmbch2* and *ZmCYP97A* mRNA and the lower levels of *ZmCYP97C* mRNA.

Ketolation

The pathway can be extended to include ketocarotenoids such as astaxanthin by expressing *Paracrt*W. This was expressed together with *Zmpsy1, Pacrt*I and *Gllycb* in lines 3 and 4, the latter also expressing *Glbch*. This led to the synthesis of adonixanthin, echinenone (4-keto-β-carotene) and 3-hydroxy-echinenone in line 4, and these three carotenoids plus astaxanthin (3,3'-dihydroxy-4,4´-diketo-β-carotene) in line 3. Similarly,

line 4 has a genotype similar to line 2, with additional *Glbch* and *Paracrt*W genes, and it produced mainly β-carotene and zeaxanthin in addition to the ketocarotenoids.

Astaxanthin is formed from β-carotene by the addition of keto groups at the 4 and 4' positions and hydroxyl groups at the 3 and 3' positions of the β-ionone rings. These reactions are catalyzed by β-carotene ketolase and β-carotene hydroxylase, respectively. In the first step, each enzyme can carry out its reaction independently, but further events depend critically on which reaction occurs first (Fraser et al., 1997). *Paracoccus* β-carotene ketolase has a strong preference for carotenoids with at least one non-hydroxylated β-ionone ring, e.g. β-carotene, β-cryptoxanthin, echinenone and 3-hydroxy-echinenone. In contrast, 3-hydroxylated β-ionone rings like zeaxanthin, 3'-hydroxy-echinenone and adonixanthin are poor substrates for this enzyme (Fraser et al., 1998).

The product of the *Paracrt*W transgene utilizes the same substrate as β-carotene hydroxylase, an unsubstituted β-ionone ring. The hydroxylase and ketolase thus compete at four stages for different substrates in the extended carotenoid pathway: for β-carotene, the unsubstituted side of β-cryptoxanthin, echinenone and 3-hydroxyechinenone (Figure 3.1). In general, non-ketolated zeaxanthin, the mono-keto derivative 3'-hydroxy-echinenone and the monohydroxy-diketo carotenoid adonixanthin represent end products in the pathway to astaxanthin. Thus, astaxanthin in corn endosperm should be derived from echinenone via either 3-hydroxy-echinenone or canthaxanthin and then adonirubin.

Because the 3-hydroxylated β-ionone ring is poorly ketolated (Fraser et al., 1998), the ketolase has to overcome the hydroxylase twice – first during the ketolation of β-carotene, then during the ketolation of either echinenone or 3-hydroxyechinenone – or astaxanthin is not formed. Therefore, the accumulation of astaxanthin is determined by the abundance of the ketolase relative to the hydroxylase. Only plants expressing *Paracrt*W produce enough ketolase to ensure the formation of astaxanthin. Otherwise, adonixanthin is the final keto-hydroxy product of the pathway, as appears to be the case in line 4, where total concentrations of ketolated carotenoids are much lower than in line 3, and the pathway stops without the second ketolation at the level of adonixanthin. Line 4, expressing *Glbch* and *Paracrt*W, did not accumulate astaxanthin due to the competition between these two enzymes. Line 3 had the highest ketocarotenoid levels

and was the only line to synthesize astaxanthin, probably reflecting the relatively low hydroxylase levels (no GlBCH activity) and high ketolase levels (high CrtW activity). For many plants transformed with a ketolase gene such as *Paracrt*W, the conversion of adonixanthin to astaxanthin appears to be an important limiting step in astaxanthin biosynthesis. These data indicate that avoiding adonixanthin accumulation was crucial for astaxanthin production in transgenic corn endosperm.

3.5.2 Analysis of mRNA and carotenoid levels in different corn genetic backgrounds during seed development

All the transgenic lines showed similar transgene expression levels, which increased during endosperm development and peaked at 20–25 dap. However, *Gllycb* reached maximum expression levels at 30 dap and this may explain why β-carotene levels peaked 30–50 dap, depending on the line. Endosperm-specific promoters were used for all transgenes, and transgene expression had generally commenced by 15 dap. Endogenous gene expression varied considerably, with some genes expressed at constant levels throughout development (*ZmCYP97B*, *ZmCYP97C* and *Zmpsy2*), others increasing gradually up to a peak level at 30 dap (*Zmpds*, *Zmzds*, *Zmlyce*, *Zmbch2* and *ZmCYP97A*) and others with an earlier peak followed by a decline (*Zmlycb*, *Zmbch1* and *Zmcrtiso*).

Individual carotenoids peaked at different stages of seed developmental (Table 3). The early carotenoids (phytoene and lycopene) accumulated later (40–50 dap) suggesting that they are consumed during early development to produce downstream carotenoids. Similarly, the ε-branch end product lutein accumulated before its precursor α-cryptoxanthin, which in turn accumulated before *α*-carotene (Table 3). Zeaxanthin also accumulated before its precursors in all lines (Table 3). However, the accumulation of *β*-carotene and *β*-cryptoxanthin varied in the different lines. In lines 1 and 2, which did not express hydroxylase or ketolase transgenes, the end-products accumulated before the precursors as discussed above (zeaxeanthin followed by *β*-cryptoxanthin and finally *β*-carotene). However, line 3 accumulated astanxanthin, followed by adonixanthin and zeaxanthin, and then the precursors of those metabolites (3-hydroxyechinenone, echinenone, *β*-cryptoxanthin and *β*-carotene, respectively). The same behavior was

observed in line 4 although with no astanxanthin accumulation due to the lack of *Paracrt*W.

3.6 Conclusions

Important advances have been made in the characterization of many of the genes encoding enzymes in the carotenoid biosynthesis pathway. However, many of the regulatory mechanisms affecting carotenoid biosynthesis in corn endosperm are still unclear. A comparative investigation in different corn backgrounds focusing on targeted carotenoid transcript and metabolite analysis allowed the identification of several bottlenecks in the pathway. Quantitative real-time RT-PCR analysis revealed the absence of *Zmpsy1* transcripts in M37W corn endosperm whereas *Zmpsy2* transcripts were present in the endosperm suggesting that the residual carotenoid content probably reflects the activity of PSY2. This finding confirms that PSY1 rather than PSY2 plays the crucial role in carotenoid accumulation in corn endosperm. Another rate-limiting step in the pathway is the conversion of phytoene to lycopene, catalyzed by endogenous desaturases and isomerases. LYCB is also a rate-limiting enzyme in the M37W endosperm reflecting the insufficient conversion of lycopene into β-carotene. In many plants transformed with a ketolase gene such as *Paracrt*W, the conversion of adonixanthin to astaxanthin appears to be an important limiting step for astaxanthin biosynthesis. These results demonstrate that avoiding adonixanthin accumulation is crucial for the production of astaxanthin in transgenic corn endosperm. The accumulation profiles of individual carotenoids indicate that although carotenoid synthesis begins at the earliest stages of endosperm development, their accumulation depends on feedback. For example, the accumulation of zeaxanthin began 15 dap in all four lines whereas the accumulation of *β*-carotene (its precursor) began by 15 dap in line 1, after 15 dap in lines 3 and 4 and after 20 dap in line 2. The accumulation of *β*-carotene could not begin any earlier than zeaxanthin because the intermediate was initially consumed to make the end product. This behavior was observed for almost all the carotenoid precursors and end products. The identification of a number of rate-limiting steps will provide data for further investigations to ascertain in more detail the genetic and molecular factors that influence carotenoid content and composition in

different corn genetic backgrounds, and for future biofortification strategies aiming to increase certain carotenoids in corn endosperm.

3.7 References

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CHAPTER 4

CLONING AND FUNCTIONAL CHARACTERIZATION OF THE MAIZE *β-CAROTENE HYDROXYLASE* GENES AND THEIR REGULATION DURING ENDOSPERM MATURATION

Chapter 4. Cloning and functional characterization of the maize β-carotene hydroxylase genes and their regulation during endosperm maturation

4.1 Abstract

In order to gain further insight into the partly-characterized carotenoid biosynthetic pathway in corn (*Zea mays* L.), we cloned cDNAs encoding the enzyme β-carotene hydroxylase (BCH) using endosperm mRNA isolated from inbred line B73. Two distinct cDNAs were identified mapping to different chromosomes. The two *bch* cDNAs (*Zmbch1* and *Zmbch2*) mapped to unlinked genes each coding sequences containing five introns. ZmBCH1 was able to convert β-carotene into β-cryptoxanthin and zeaxanthin, but ZmBCH2 was able to form β-cryptoxanthin alone and had a lower overall activity than ZmBCH1. All two genes were expressed during endosperm development, with mRNA levels rising in line with carotenoid accumulation (especially zeaxanthin and lutein) until 25 days after pollination (DAP). Thereafter, expression declined for both genes. We discuss the impact of paralogs with different expression profiles and functions on the regulation of carotenoid synthesis in corn.

4.2 Introduction

Carotenoids are fat-soluble pigments synthesized by all plants and many microorganisms. In plants they are found on photosynthetic membranes where they participate in the light-harvesting reaction and protect the photosynthetic apparatus from photo-oxidation (reviewed by Bramley 2002). However, they are also precursors for the synthesis of abscisic acid (Creelman and Zeevart, 1984) and strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008), and they are the source of yellow, orange and red pigmentation in some flowers and fruits (Tanaka et al., 2008). In animals, carotenoids provide multiple health benefits (reviewed in Fraser and Bramley, 2004), prompting scientists to explore ways to improve carotenoid content and composition in staple crops (reviewed in Sandmann et al., 2006; Howitt and Pogson, 2006; Zhu et al., 2007; Giuliano et al., 2008; Fraser et al., 2009; Zhu et al., 2009).

Plant carotenoid synthesis begins with the conversion of geranylgeranyl pyrophosphate into 15-*cis* phytoene by the enzyme phytoene synthase (PSY) (Misawa et al., 1994) (Figure 4.1). A series of four desaturation reactions carried out by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. The product of the first desaturase is 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized by light (and perhaps an unknown enzyme; Li et al., 2007) to yield 9,9′-di-*cis*-ζ-carotene, the substrate of ZDS (Breitenbach and Sandmann, 2005). 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 (Isaacson et al., 2004; Breitenbach and Sandmann, 2005). All-*trans* lycopene is then cyclized by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) to introduce ε- and β-ionone end groups and produce α- and β-carotene, respectively. The introduction of hydroxyl moieties into the cyclic end groups by β-carotene hydroxylase (BCH) and carotene ε-hydroxylase results in the formation of zeaxanthin from β-carotene and lutein from α-carotene (Sun et al., 1996; Bouvier et al., 1998; Tian et al., 2003). In some plant tissues, zeaxanthin can be epoxidized to violaxanthin by zeaxanthin epoxidase (ZEP) (reviewed by Cunningham and Gantt, 1998).

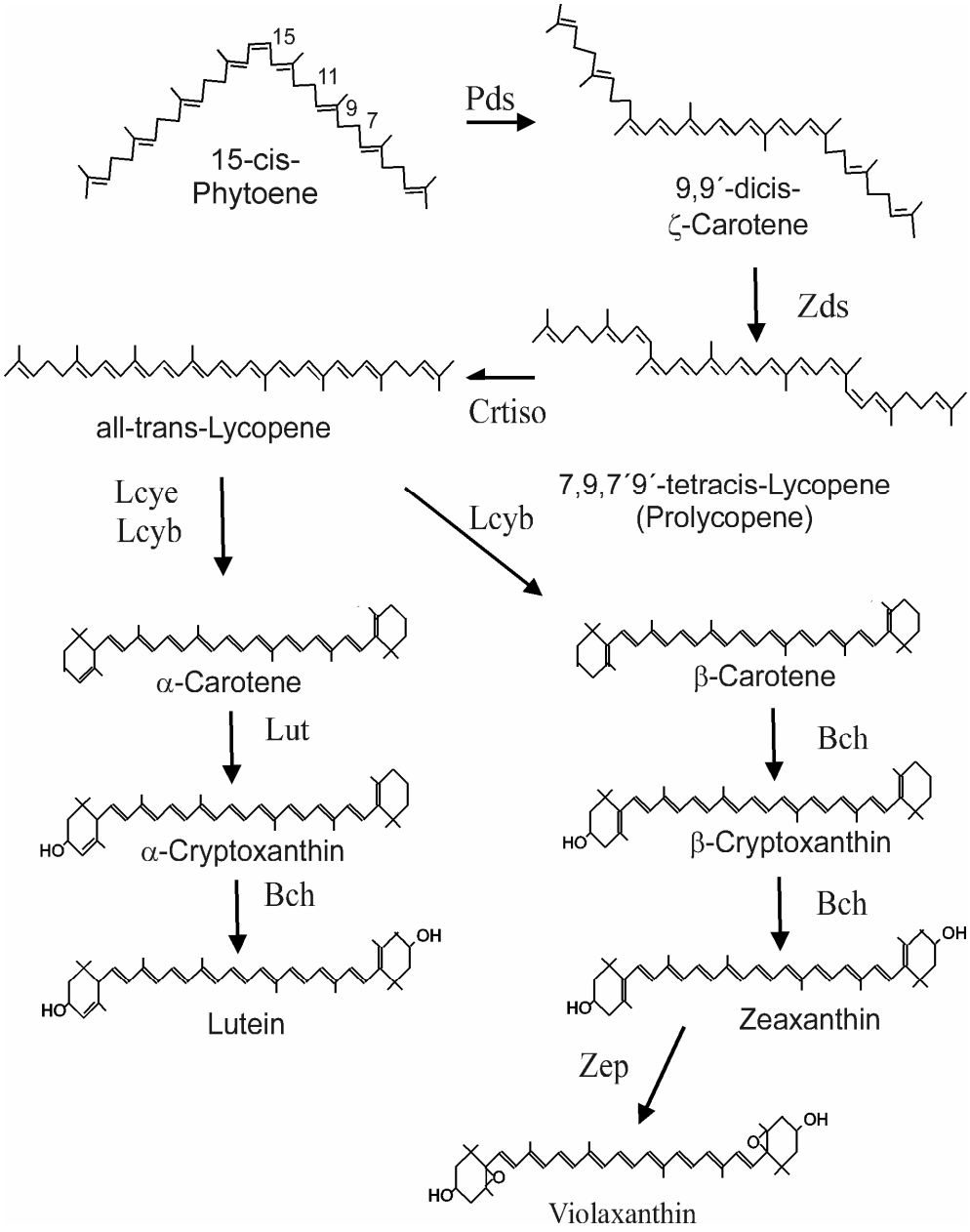

Figure 4.1. Carotenoid biosynthetic pathway in corn endosperm. Abbreviations: Pds, phytoene desaturase; Zds, ζ-carotene desaturase; Crtiso, carotenoid isomerase; Lcyb, lycopene β-cyclase; Lcye, lycopene ε-cyclase; Bch, β-carotene hydroxylase; Lut, carotene ε-ring hydroxylase and Zep, zeaxanthin epoxidase

The limited data concerning endogenous regulation of carotenogenic genes has made the precise engineering of crop plants to enhance carotenoid content and composition difficult (reviewed in Sandmann et al., 2006; Zhu et al., 2007; Fraser et al., 2009) despite recent progress in cereal crops, particularly corn (Harjes et al., 2008; Zhu et al.,

2008; Aluru et al., 2008; Naqvi et al., 2009). The corn genome contains three paralogous *psy* genes (Li et al., 2008a,b) and PSY1 is the key rate-limiting enzyme in endosperm carotenoid biosynthesis (Buckner et al., 1996; Palaisa et al., 2003; Li et al., 2008a). The endosperm-specific expression of a corn *psy1* transgene in white corn, which lacks endogenous PSY1 activity, increased the total endosperm carotenoid content >50-fold (Zhu et al., 2008). Identification of corn β-carotene hydroxylase (*bch*) gene family was recently reported (Vallabhaneni et al., 2009). In contrast, PDS, ZDS, LCYB and LCYE are encoded by single-copy genes in corn (Li et al., 1996; Matthews et al., 2003; Singh et al., 2003; Harjes et al., 2008), and *pds* and *zds* at least do not appear to regulate endosperm carotenoid accumulation since the corresponding transcript levels remain constant during endosperm development (Li et al., 1996; Matthews et al., 2003). Here we report the isolation and characterization of corn *bch* cDNAs, their developmental expression profiles and their functional characterization by complementation analysis in bacteria. The presence of small gene families for several carotenogenic genes in corn suggests that diverse regulatory strategies may be used to control the accumulation of carotenoids in endosperm tissue.

4.3 Materials and methods

4.3.1 Plant materials

Corn plants (*Zea mays* L.) representing lines B73, A632, EP42 (yellow corn) and M37W (white corn) were grown in the greenhouse and growth chamber at 28/20ºC day/night temperature with a 10-h photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21/18ºC day/night temperature with a 16-h photoperiod thereafter. Plants were self-pollinated to obtain seeds. Mature leaf and endosperm tissue were frozen rapidly in liquid nitrogen and stored at –80°C. Endosperm tissues were dissected at five developmental stages (10, 15, 20, 25 and 30 days after pollination (DAP)).

4.3.2 Nucleic acid isolation and cDNA synthesis

Genomic DNA was extracted from 5 g of leaf tissue as described by Sambrook et al., (1989). Total RNA was isolated using the RNeasy® Plant Mini Kit (QIAGEN, Valencia, CA, USA) and DNA was removed with DNase I (RNase-Free DNase Set, QIAGEN, Valencia, CA, USA). Total RNA was quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA), and 2 µg total RNA was used as template for first strand cDNA synthesis with Ominiscript Reverse Transcriptase in a 20 µl total reaction volume following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA).

4.3.3 Cloning and sequencing the corn bch cDNAs

Nested PCR was used to amplify the corn β-carotene hydroxylase 1 (*Zmbch1*) cDNA from endosperm tissue, based on the putative *Zmbch1* cDNA sequences already deposited in GenBank from an unknown corn cultivar (accession number AY844956). The PCR was carried out in a 50-µl reaction was carried out using the GoTaq® DNA Polymerase Kit (Promega, Madison, WI, USA) and synthetic degenerate oligonucleotide primers matching two conserved regions. Forward primer 5′- CAT GGC CGC CGG TCT GTC CGG CGC CGC GAT -3′ (BCH1F1) and reverse primer 5′- TGA GCT GGT GGT TCA TAA CAT GTC TCT AC-3′ (BCH1R1) in the first reaction and forward primer 5′-AGA ATT CCA TGG CCG CCG GTC TGT CCG-3′ (BCH1F2) (the terminal *Eco*RI restriction site and start codon are underlined) and 5′-AGG ATC CGG ACG AAT CCA TCA GAT GGT C-3′ (BCH1R2) (the terminal *Bam*HI restriction site is underlined) in the second reaction. The first reaction mix included 5% (v/v) dimethyl sulfoxide (DMSO), and 5 µl of the product was used to proceed the second reaction. Program used was 95°C for 3 min, followed by 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 90 s. After the last amplification cycle, samples were incubated at 72°C for 10 min. The Zm*bch1* product was purified from a 1.0% w/v agarose gel using the Geneclean® II Kit (BIO® 101 Systems, Solon, OH, USA) and cloned in the PCR® II TOPO® vector (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) for sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were used to query EST databases.

A similar nested PCR strategy was used to isolate the full-length *Zmbch2* cDNA using primer combinations based on the corresponding GenBank sequence (accession number AY844956). For this cDNA, the forward primers were 5′-GGA GAC TCG AGG CCA CTC TGC CTT-3′ (BCH2F1) and 5′-GAA TTC CAT GGC CGC CGC GAT GAC CAG-3′ (BCH2F2) (terminal *Eco*RI restriction site and start codon are underlined) and the reverse primers were 5′-GCT AGA ACT CAT TTG GCA CAC TCT G-3′ (BCH2R1) and 5′-GGA TCC TAG AAC TCA TTT GGC ACA CTC-3′ (BCH2R2) (terminal *Bam*HI restriction site is underlined). The PCR was carried out as above without DMSO in reaction mix, and the products were cloned and sequenced as described.

4.3.4 Bioinformatic analysis

The Maize Genetics and Genomic Database (MaizeGDB, *http://www.maizegdb.org/*), the GRAMENE database (*http://www.gramene.org/*) and GenBank (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) were searched for homologous sequences using BLAST, and multiple sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/clustalw/). Protein sequences were screened for chloroplast signal peptides using the ChloroP 1.1 Server at http://www.cbs.dtu.dk/services/ChloroP/ (Emanuelsson et al., 1999).

4.3.5 Construction of BCH expression vectors

pUC8-Zmbch1 and pUC8-Zmbch2 plasmids were constructed by digesting pCR-Zmbch1 and pCR-Zmbch2 with *Eco*RI and *Bam*HI simultaneously, and subcloned as in frame fusion into a pUC8 vector also digested with the same restriction enzymes. They were then utilized for functional analysis of the corresponding genes.

4.3.6 Functional characterization of bch cDNAs

Different carotenoid backgrounds were established in *Escherichia coli* for functional complementation experiments with the corn *bch* cDNAs using a selection of plasmids

containing carotenogenic genes. Plasmid pACCAR16ΔcrtX contained all the genes required to synthesize β-carotene (Misawa et al., 1990).

4.3.7 DNA and RNA blots

Leaf genomic DNA (20 µg) was digested separately with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I. The resulting fragments were separated by electrophoresis on a 0.8% (w/v) agarose gel and blotted onto a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were fixed by UV crosslinking. The transferred DNA fragments were hybridized with appropriate digoxigenin-labeled probes at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany). 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 then 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 disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich, USA) according to the manufacturer's instructions. The primer combinations used to generate *Zmbch1* probe (377 bp) were: 5′-TGG AAA AGG AGC TCG CGC GAA TCG-3′ and 5′-TGA GCT GGT GGT TCA TAA CAT GTC T-3′ for and 5′-GCT TGT TAG CAG TCC GGT GAG TGA A-3′ and 5′-GAA AGG AAG ATG GCG ATA GAT GTA-3′ for *Zmbch2* (251 bp).

Total RNA (30 µg) was fractionated on a denaturing 1.2% (w/v) agarose gel containing formaldehyde prior to blotting. The membrane was probed with digoxigenin-labeled partial cDNAs prepared as above using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), with hybridization carried out at 50°C overnight using DIG Easy Hyb. Washing and immunological detection and CSPD chemiluminescence were carried out as described above.

4.3.8 HPLC-MS and quantification

Carotenoids from freeze-dried *E. coli* co-transformants were extracted in darkness with acetone at 60°C for 20 min, partitioned into 10% ether in petrol (bp. 40–60°C) and analyzed by HPLC on a non-endcapped polymeric 3 µm C_{30} column (YMC Wilmington NC, USA) according to Sander et al. (1994). The mobile phases was methanol/methyl-tert-butyl ether/water (56:40:4, v/v/v) for 30 min followed by a solvent change to methanol/methyl-tert-butyl ether/water (56:40:4, v/v/v) at a flow rate of 1 ml/min (Breitenbach and Sandmann 2005). Spectra were recorded on-line with a Kontron 440 diode array detector. The sources of reference carotene isomers are described in previous publications (Breitenbach and Sandmann, 2005; Breitenbach et al., 2001). Carotenoids from freeze dried and ground endosperm tissue were extracted in a similar manner, although the acetone was replaced with tetrahydrofuran/methanol (50:50, v/v) followed by acetone re-extraction. The HPLC system used for the corn carotenoids was a C_{18} Vydac 218TP54 column with 1% water in methanol as the mobile phase.

4.3.9 Quantitative real time PCR

Real-time PCR was performed on a BIO-RAD CFX96^{TM} system using a 25-µl mixture containing 10 ng of synthesized cDNA, 1x iQ SYBR green supermix (BIO-RAD) and 0.2 mM forward and reverse primers for the target genes and the internal glyceraldehyde-3-phosphate dehydrogenase control (*Zmgapdh*) (Iskandar et al., 2004) as listed in Table 4.1. 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°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 CFX96^{TM} system software. Values represent the mean of three real time PCR replicates $\pm$ SD.

Table 4.1. Primer oligonucleotide sequences of corn β-carotene hydroxylase (*Zmbch*), and glyceraldehyde-3-phosphate dehydrogenase (*Zmgapdh*) genes for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
Zmbch1	5'-CCACGACCAGAACCTCCAGA-3'	5'-CATGGCACCAGACATCTCCA-3'
Zmbch2	5'-GCGTCCAGTTGTATGCGTTGT-3'	5'-CATCTATCGCCATCTTCCTTT-3'
Zmgapdh	5'-CTTCGGCATTGTTGAGGGTT-3'	5'-TCCAGTCCTTGGCTGAGGGT-3'

4.4 Results

4.4.1 Cloning and characterization of the corn bch genes

The sequence of the *Zmbch1* cDNA from B73 endosperm predicted a 309-amino-acid protein with a molecular weight of 33.6 kDa, a pI of 10.92 and a 68-amino-acid transit peptide, suggesting the 241-amino-acid mature protein has a molecular weight of 26.5 kDa and a pI of 9.10 (accession no. GQ131287). The cloned *Zmbch1* cDNA sequence has 98.9% identity at DNA level with β-carotene hydroxylase 4 cDNA (Vallabhaneni et al., 2009; accession number AY844956). The deduced amino acid sequence of ZmBCH1 shared 99.4% identity with hydroxylase 4. There is no mention of the corn cultivar about hydroxylase 4 they used (Vallabhaneni et al., 2009) consequently one cannot determine if our cloned gene (*Zmbch1*) is different from hydroxylase 4 because of a difference in cultivar used to clone the genes or different isozymes in B73. The sequence of the cloned *Zmbch2* cDNA is the same as β-carotene hydroxylase 3 (Vallabhaneni et al., 2009; accession number AY844958). It predicted a 319-amino-acid protein with a molecular weight of 34.6 kDa, a pI of 8.82 and a 69-residue transit peptide, yielding a mature 250-amino-acid protein with a molecular weight of 27.5 kDa and a pI of 6.67. The predicted amino acid sequences of ZmBCH1 and ZmBCH2 are aligned in Figure 4.2. The N-terminal sequences are highly conserved, despite the presence of four small gaps in ZmBCH2, whereas the C-terminal sequences differ considerably, not least because of the presence of 29 additional residues in the ZmBCH2 sequence (Figure 4.2). The screening of corn genomic resources localized

Zmbch1 to chromosome 2 (accession nos. AC196442) and *Zmbch2* to chromosome 10 (accession no. AC194430). A comparison of the cDNAs and corresponding BAC clones showed that both genes were present in their entirety in the BAC clones and both coding sequences contained five introns. The full-length amino acid sequences are 76.6% identical, both containing four predicted transmembrance helices and one highly conserved region described by Sun et al., (1996) as "Motif 1". The most striking features were the conserved histidine motifs, often found in iron-containing monooxygenases and fatty acid desaturases including β-carotene hydroxylases (Bouvier et al., 1998), which may act as iron-binding ligands (Shanklin et al., 1994). The four regions HX_4H, HX_2HH, HX_4H and HX_2HH are underlined in Figure 4.2.

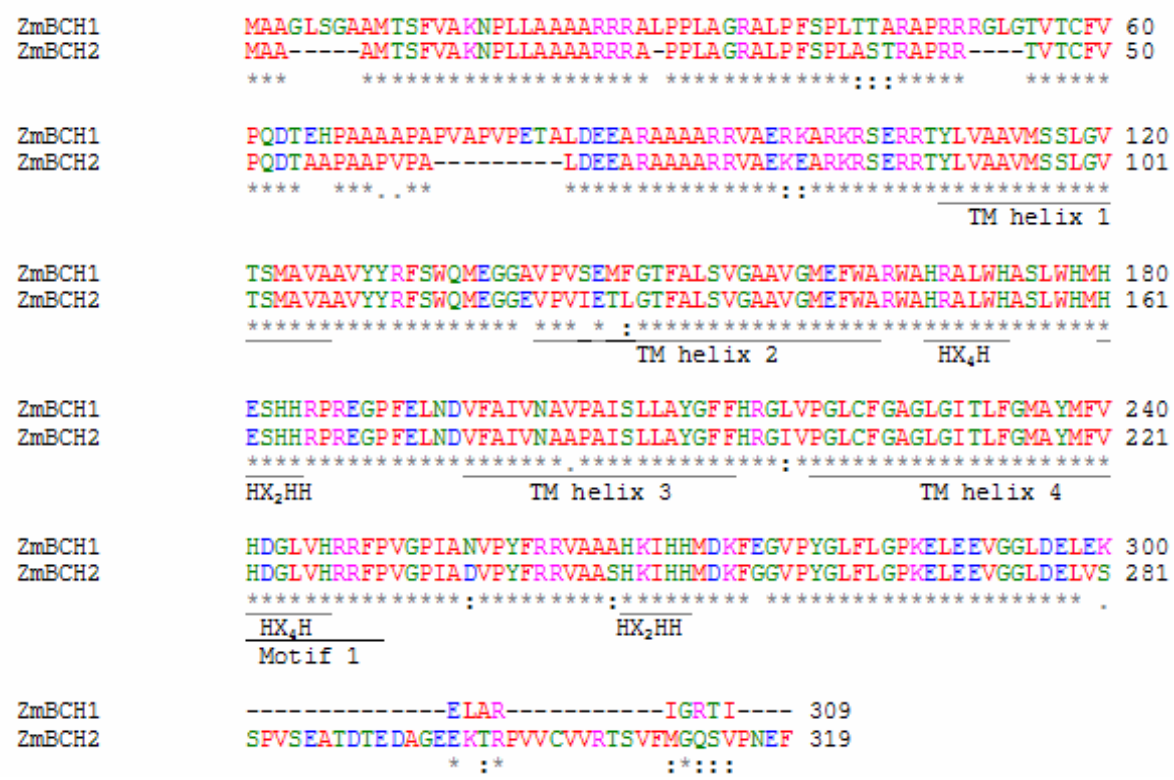

Figure 4.2. Alignment of deduced amino acid sequences of ZmBCH1 (*Zea mays* L., accession no. GQ131287) and ZmBCH2 (*Zea mays* L., accession no. AY844957). Four predicted transmembrance (TM) helices, one highly conserved region described by Sun et al. (1996) as "Motif 1" and the four regions HX_4H, HX_2HH, HX_4H and HX_2HH are underlined**.**

4.4.2 *bch* gene copy numbers

Southern blot analysis was carried out by digesting the corn genomic DNA with five different restriction enzymes, followed by hybridization with gene specific *Zmbch1* and *Zmbch2* probes. Detecting at least two bands per lane for *Zmbch1* and at least three for *Zmbch2* (Figure 4.3A and 4.3B). These results indicated that there are two or more copies of *Zmbch1* and three or more copies of *Zmbch2* in the corn genome bringing the total of *Zmbch* genes in corn to 5 or more in all. Our assumption was supported by the recent report (Vallabhaneni et al., 2009) that existence of six different non-heme di-iron monooxygenase β-carotene hydroxylase (*bch*) genes in the corn genome.

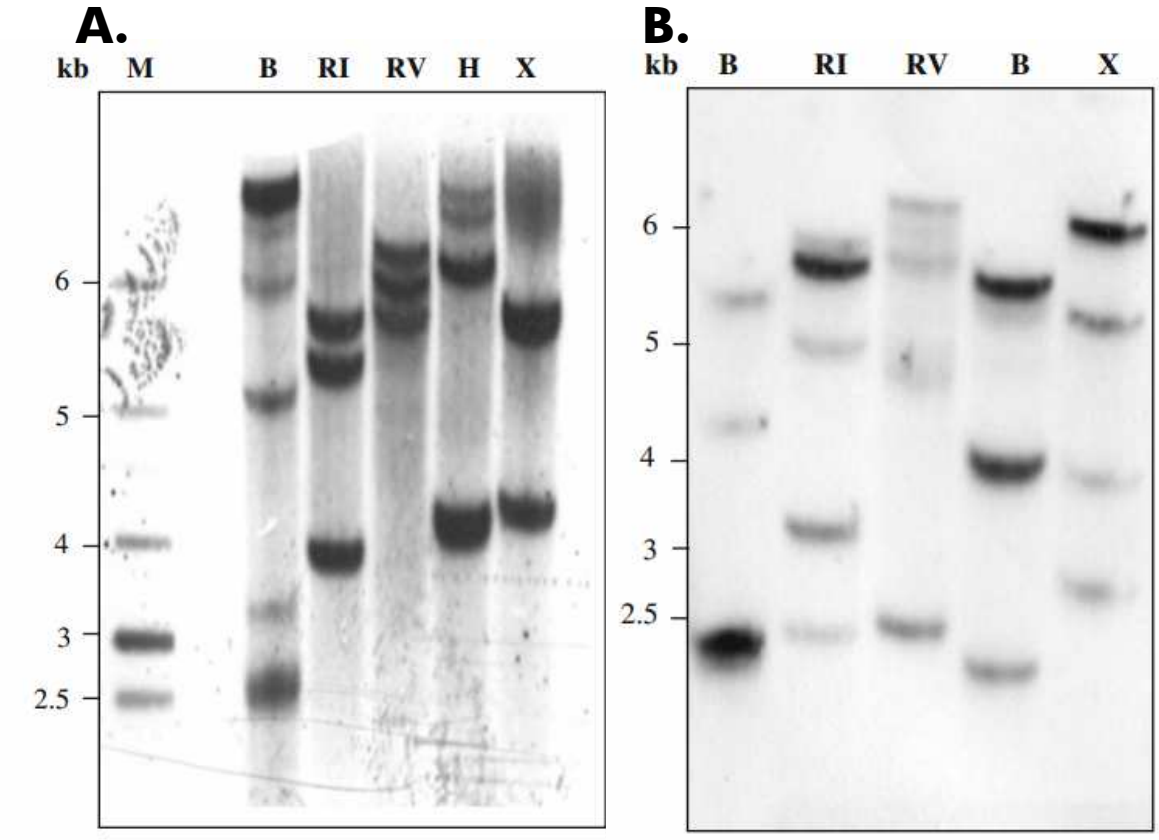

Figure 4.3. Southern blot analysis of *Zmbch* gene family in corn. Genomic DNA (20 µg) from mature leaves was separately digested with *Bam*HI (B), *Eco*RI (RI), *Eco*RV (RV), *Hin*dIII (H) and *Xba*I (X). Two different blots were hybridized with the *Zmbch1* (A) and *Zmbch2* (B) specific probes described in Materials and methods.

4.4.3 Functional analysis of corn bch

The *Zmbch1* and *Zmbch2* cDNAs were tested by complementation in *E. coli* strains accumulating β-carotene (Figure 4.4A**).** In strains cotransformed with *Zmbch1* cDNA, more than half of the β-carotene was converted into the monohydroxyl derivative β-cryptoxanthin (about 80% of the product) and the dihydroxyl derivative zeaxanthin (about 20% of the product) (Figure 4.4B). However, in strains cotransformed with *Zmbch2* cDNA, only 4.3 % of β-carotene was converted to β-cryptoxanthin and no zeaxanthin was formed (Figure 4.4C).

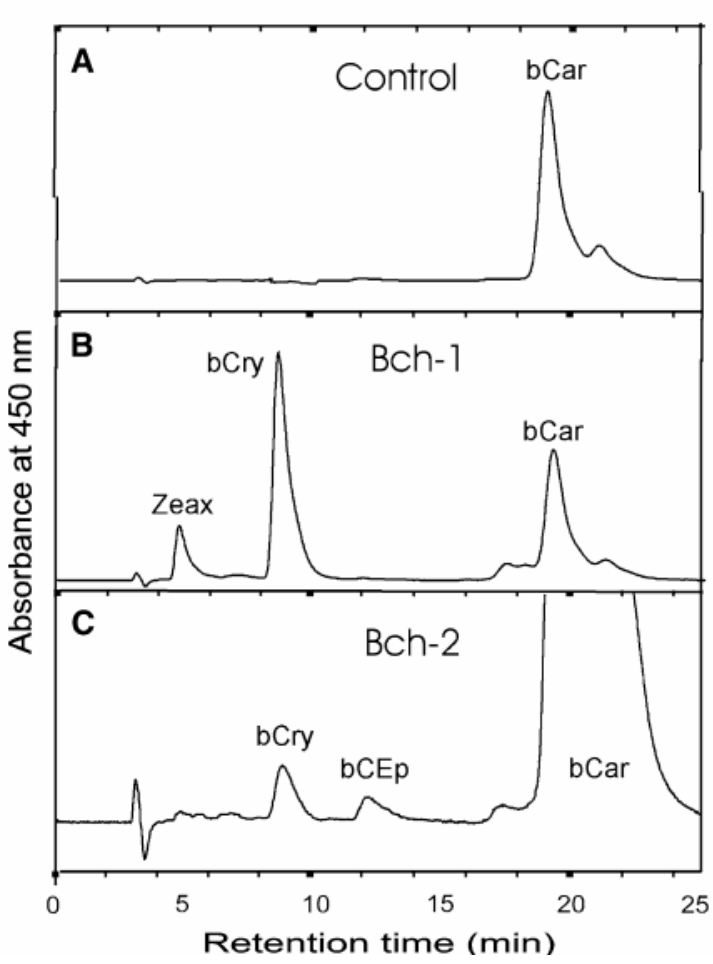

Figure 4.4. Functional analyses of *Zmbch1* and *Zmbch2*. **A**. *E. coli* expressing pACCAR16ΔcrtX. **B**. *E. coli* expressing pACCAR16ΔcrtX with additional plasmid pUC8-Zmbch1 (Bch1). **C**. *E. coli* expressing pACCAR16ΔcrtX with additional plasmid pUC8-Zmbch2 (Bch2). Abbreviations: bCar, β-carotene; Zeax, zeaxanthin; bCry, β-cryptoxanthin; bCEp, β-carotene epoxide.

4.4.4 Carotenoid accumulation and gene expression during endosperm development

The profile of carotenoid accumulation in developing B73 corn endosperm was assessed up to 30 DAP (Table 4.2). Yellow corn endosperm contains β-carotene, its monohydroxylated product β-cryptoxanthin and its dihydroxylated product zeaxanthin. Although α-carotene could not be detected, its hydroxylated products α-cryptoxanthin and lutein were present at detectable levels. A steady increase in total carotenoid content was observed during kernel maturation, although the levels of lutein, zeaxanthin and α-cryptoxanthin increased, β-carotene and β-cryptoxanthin levels decreased from 25–30 DAP.

Table 4.2. Carotenoids in B73 corn seed endosperm (µg/g dry weight) depending on the degree of maturation. Each value is the mean of three determinations from an extract of five individual seeds. Abbreviations: DAP, days after pollination; Viol, violaxanthin; Lut, lutein; Zeax, zeaxanthin; α-Cry, α-cryptoxanthin; β-Cry, β-cryptoxanthin; β-Car, β-carotene.

	Viol	Lut	Zeax	α-Cry	β-Cry	β-Car	Total
10 DAP	0.83±0.06	0.60±0.02	0.66±0.04	0.00±0.00	0.00±0.00	0.00±0.00	2.09
15 DAP	0.39±0.02	1.83±0.12	1.53±0.15	0.88±0.07	0.60±0.07	0.17±0.08	5.40
20 DAP	0.88±0.04	5.11±0.23	2.36±0.15	2.13±0.16	1.14±0.09	0.70±0.05	12.32
25 DAP	1.11±0.09	8.03±0.28	4.10±0.30	3.77±0.15	2.31±0.12	1.30±0.09	20.62
30 DAP	1.50±0.11	12.55±0.33	6.99±0.45	4.23±0.29	0.85±0.04	1.16±0.07	27.28

In order to understand how carotenoid accumulation is regulated during corn endosperm development, northern blots containing B73 endosperm mRNA from different developmental stages was hybridized with *Zmbch1* and *Zmbch2* gene-specific DNA probes. The signals for *Zmbch1* were below the detection threshold regardless of the probe used (data not shown). The steady-state levels of *Zmbch2* mRNA increased in the endosperm between 10 and 15 dap then remained constant until 25 dap, then declined at 30 dap, but no transcripts could be detected in leaves (Figure 4.5A). To increase

sensitivity, expression profiles were monitored by quantitative real time PCR (Figure 4.5B) which showed that the transcripts for all genes increased throughout endosperm development to 25 dap.

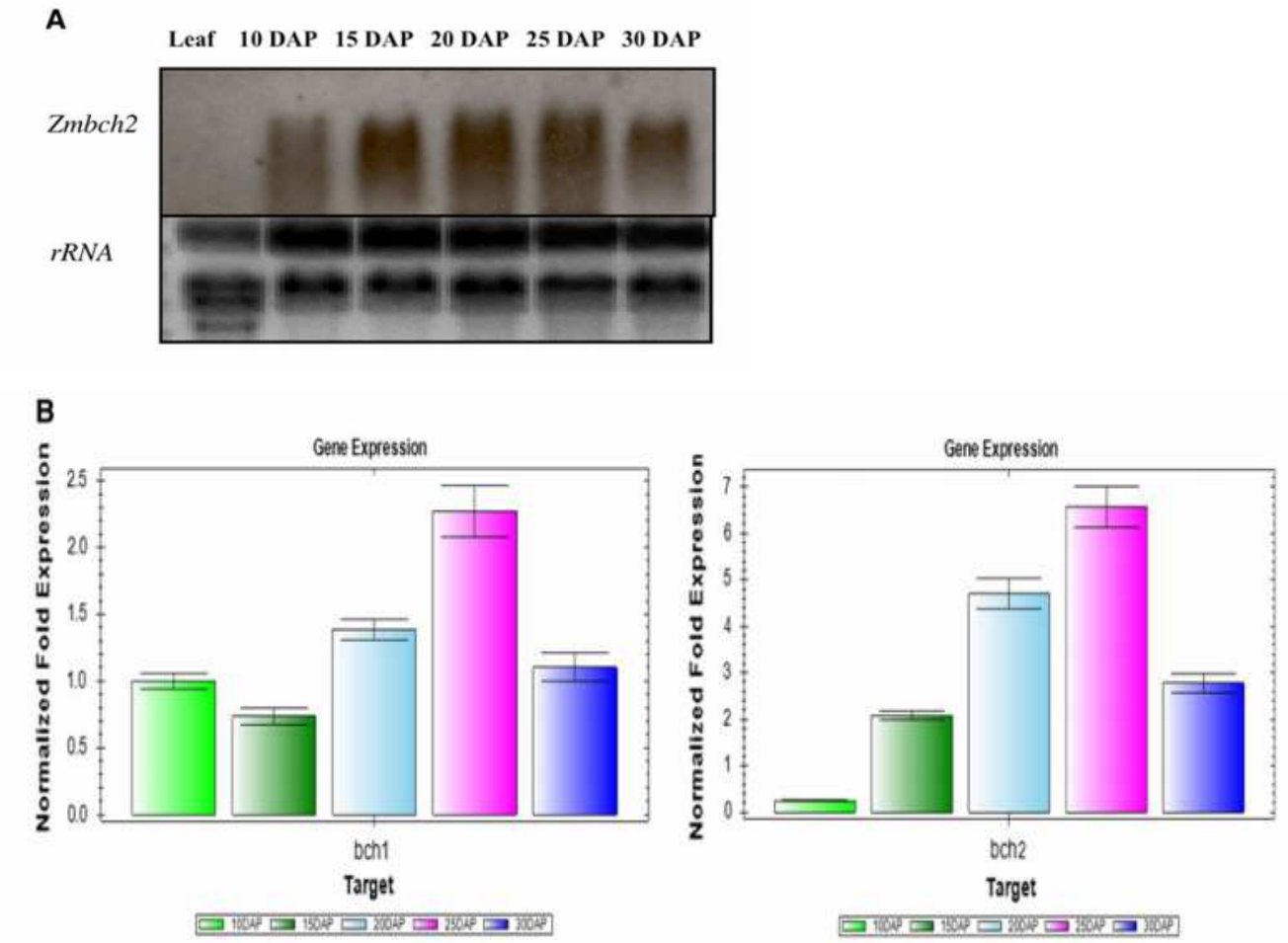

Figure 4.5. A. Northern analysis of *Zmbch2* transcript in leaves and corn endosperms. Each lane was loaded with 30 µg of total RNA. rRNA stained with ethidium bromide is shown as a control for loading of equal amounts of RNA. **B.** Transcript levels for *Zmbch1* and *Zmbch2*, from greenhouse grown B73 corn harvested at 10, 15, 20, 25 and 30 dap (days after pollination). Values are a mean of three quantitative real time PCR replicates with ±SD. Abbreviations: *Zm, Zea mays; bch1*, β-carotene hydroxylase 1 gene; *bch2*, β-carotene hydroxylase 2 gene.

4.5 Discussion

Carotenoids are nutritionally valuable compounds that provide a range of health benefits including protection against cancer and other chronic diseases (review by Fraser and Bramley, 2004). Humans cannot synthesize carotenoids and must obtain them from their diet, notably from fresh fruit and vegetables and seafood. However, since many people, particularly those in developing countries, subsist on a monotonous diet of staple cereal grains, there has been much interest in the development of strategies to improve carotenoid levels and composition in staple crops (Zhu et al., 2007; 2009). One significant hurdle to the improvement of carotenoid levels in cereal grains is the limited understanding of how carotenoid synthesis is normally regulated, particularly in the endosperm, which is the most nutritious component of the grain. A number of recent studies have shown the potential for carotenoid enhancement in corn endosperm, either through conventional breeding or transgenic strategies (Harjes et al., 2008; Zhu et al., 2008; Aluru et al., 2008; Naqvi et al., 2009). Despite this progress, much remains to be learned about the carotenoid synthesis pathway in corn endosperm and the regulatory mechanisms that control the accumulation of specific carotenoid molecules.

The first step towards understanding how carotenoids are synthesized is to identify the enzymes involved and isolate the corresponding genes. Several corn cDNAs encoding carotenogenic enzymes have already been cloned and identified including *psy1*, *psy2* and *psy3* (phytoene synthase), *pds* (phytoene desaturase), *zds* (ζ-carotene desaturase), *lcyb* (lycopene β-cyclase) and *lcye* (lycopene ε-cyclase) (Buckner et al., 1996; Gallagher et al., 2004; Li et al., 2008a; Li et al., 1996; Matthews et al., 2003; Singh et al., 2003; Bai et al., 2009). We have cloned two representing non-heme di-iron monooxygenase β-carotene hydroxylase (BCH).

The presence of multiple isoenzymes in plant metabolic pathways is a common phenomenon which often reflects the requirement for the same catalytic activity in different subcellular compartments, often the plastids and cytosol (Gottlieb, 1982). However, *bch* cDNAs appear to encode proteins with the transit peptide sequences, suggesting the isoenzymes are destined for the plastids. Another source of isoenzymes in diploid plants such as corn is the random duplication of chromosome segments followed by the functional diversification of the duplicated genes. We therefore sought

to determine whether the cDNAs encoding the enzyme were differentially expressed or whether the enzymes themselves were functionally distinct.

We looked at the expression of all two cDNAs during endosperm development using a combination of northern blot and quantitative real-time RT-PCR. The latter was necessary because only *Zmbch2* was expressed at a high enough level to be detected by mRNA blot. The two *bch* mRNAs were also differentially expressed, with *Zmbch2* alone detectable by mRNA blots. This showed that the mRNA was restricted to endosperm and below the limit of detection in leaves (Figure 4.5A), unlike other carotenogenic genes in corn which are thought to be expressed constitutively (Vallabhaneni and Wurtzel, 2009). The steady state *Zmbch2* mRNA levels in endosperm were significantly higher than in leaves (Figure 4.5A) which is different from the recent report that the similar mRNA levels existed in leaves and endosperm at 10 dap and 30 dap (Vallabhaneni et al., 2009). The expression of both *Zmbch1* and *Zmbch2* increased during endosperm development, suggesting that the expression profiles in the endosperm were concordant. The *Zmbch2* was preferentially expressed in amyloplasts-containing endosperm rather than chloroplasts-containing leaves (Figure 4.5A), similar to the situation in *Arabidopsis* where the *Atbch2* gene is induced rapidly and strongly during seed development. This suggests that AtBCH2 is preferentially involved in xanthophyll synthesis in seeds (Kim et al., 2009). One of the two *bch* genes in bell pepper, tomato and saffron is also preferentially expressed in flowers or during fruit development (Bouvier et al., 1998; Castillo et al., 2005; Galpaz et al., 2006). In tomato, a *bch2* mutant results in a colorless petal phenotype with no impact on xanthophyll synthesis in leaves (Galpaz et al., 2006), and the massive accumulation of xanthophylls during stigma maturation in saffron correlates with high expression of a single *bch* gene (Castillo et al., 2005). Although showing concordant expression profiles in endosperm, the activity of the two BCH isoenzymes is distinct. In bacteria producing β-carotene and expressing the *Zmbch1* cDNA, more than half of the β-carotene was converted into downstream products, approximately 80% β-cryptoxanthin and 20% zeaxanthin. In contrast, similar bacteria expressing *Zmbch2* cDNA were able to convert less than 5% of the available β-carotene and only β-cryptoxanthin was produced. This functional difference might indicate that the two genes are diverging to fulfill slightly different roles in carotenoid biosynthesis.

Six different corn paralogs encoding β-carotene hydroxylase (BCH) were recently identified (Vallabhaneni et al., 2009). Two *bch* gene functions (one is the same as *Zmbch2* gene in this paper) were confirmed that both encode β-carotene hydroxylase (Vallabhaneni et al., 2009). Two paralogs were pseudogenes, while the remaining two paralog functions were unknown (Vallabhaneni et al., 2009).

Any hypothesis addressing the roles of isoenzymes in carotenoid biosynthesis must look at the expression profiles and enzyme activities in the context of carotenogenesis during seed development. Previous studies have shown that total carotenoid levels increase steadily up to 30 DAP coincident with the upregulation of *psy1*, while *pds* and *zds* transcripts remain at a constant level (Li et al., 1996; Matthews et al., 2002; Li et al., 2008a; Vallabhaneni and Wurtzel, 2009). The coordinated upregulation of *Zmbch1* and *Zmbch2* until 25 DAP is consistent with the observed accumulation of carotenoids. The expression of *lcye* follows a similar profile (Vallabhaneni and Wurtzel, 2009). It is apparent that PSY1 is the rate-limiting enzyme of the carotenoid biosynthetic pathway (Li et al., 2008b), but the impact of increasing *Zmbch1* and *Zmbch2* expression over the same timescale is to gradually increase the availability of the β-branch of the pathway. The only remaining carotenogenic gene in corn lacking expression data is carotene ε-ring hydroxylase, catalyzing the 3-hydroxylation of the ε-ionone ring in the synthesis of lutein (Figure 4.1). When we compare carotenoid accumulation during seed development (Table 4.2) with *bch1* and *bch2* transcript kinetics (Figure 4.5B), we can predict that ε-ionone ring carotene hydroxylase will demonstrate similar transcript kinetics. This can be concluded from a proportional increase of zeaxanthin and lutein over the whole period of seed development and the equal participation of β-carotene hydroxylase and the ε-ionone ring carotene hydroxylase during formation of lutein from α-carotene (Figure 4.1).

In conclusion, we have cloned and characterized two cDNAs encoding corn carotenogenic enzymes, encoding BCH. The enzymes are highly conserved in sequence, expression and activity, but subtle differences in the expression and activities of the BCH enzymes hint at divergent roles in plant carotenoid biosynthesis that may be useful in the development of more refined strategies to engineer carotenoid synthesis and composition in staple crops.

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CHAPTER 5

TRANSGENIC RICE GRAINS EXPRESSING A HETEROLOGOUS ρ-HYDROXYPHENYLPYRUVATE DIOXYGENASE SHIFT TOCOPHEROL SYNTHESIS FROM γ THE TO α THE ISOFORM WITHOUT INCREASING ABSOLUTE TOCOPHEROL LEVELS

Chapter 5. Transgenic rice grains expressing a heterologous ρ-hydroxyphenylpyruvate dioxygenase shift tocopherol synthesis from the γ to the α isoform without increasing absolute tocopherol levels

5.1 Abstract

We generated transgenic rice plants overexpressing *Arabidopsis thaliana* ρ-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the first committed step in vitamin E biosynthesis. Transgenic grains accumulated marginally higher levels of total tocochromanols than controls, reflecting a small increase in absolute tocotrienol synthesis (but no change in the relative abundance of the α and γ isoforms). In contrast, there was no change in the absolute tocopherol level, but a significant shift from the γ to the α isoform. These data confirm HPPD is not rate limiting, and that increasing flux through the early pathway reveals downstream bottlenecks that act as metabolic tipping points.

5.2 Introduction

Vitamin E is an essential plant-derived nutrient in the human diet that comes in eight structurally-related forms known as tocochromanols (Figure 5.1). The chromanol head group can be joined to a saturated phytyl side chain to form tocopherol, or to an unsaturated geranylgeranyl side chain to form tocotrienol. The head group can then be methylated in different configurations, resulting in four alternative forms (α, β, γ and δ). Although all eight vitamers are absorbed in humans, the hepatic α-tocopherol transfer protein (α-TTP) preferentially retains α-tocopherol making this the most active form of vitamin E in the human diet (Traber and Arai, 1999).

Plants synthesize tocochromanols to protect the photosynthetic apparatus against reactive oxygen species and prevent lipid peroxidation, with γ-tocopherol playing a particularly important role in preventing the auto-oxidation of polyunsaturated fatty acids in seeds (Munné-Bosch and Alegre, 2002). Plants therefore provide an excellent dietary source of vitamin E, which is thought to reduce the risk of cancer, cardiovascular disease and neurodegenerative disorders in humans by scavenging reactive oxygen species and lipid-soluble oxidative stress by-products (Bramley et al., 2000).

There is considerable variation in both the absolute and relative tocochromanol levels in different plant species and tissues, suggesting the pathway can be modulated by genetic engineering to increase the nutritional value of plants with low levels of vitamin E. We generated transgenic rice plants constitutively expressing the *Arabidopsis thaliana PDS1* gene (encoding HPPD) to determine whether increasing flux through the common, early part of the pathway had any impact on overall tocochromanol levels and/or the relative levels of the different vitamers. We observed only a marginal increase in absolute tocochromanol levels in the transgenic grains, mainly due to the slightly higher tocotrienol content. However, the overall vitamin E activity of the grains increased significantly due to a marked shift from the γ to the α isoform in the tocopherol pathway. Our data indicate that even though HPPD might not be an appropriate target for vitamin E enhancement in rice, it is still a noteworthy target in vitamin E biofortification programs due to its capacity to enhance the absolute levels of α-tocopherol at the expense of the less nutritionally important γ isoform.

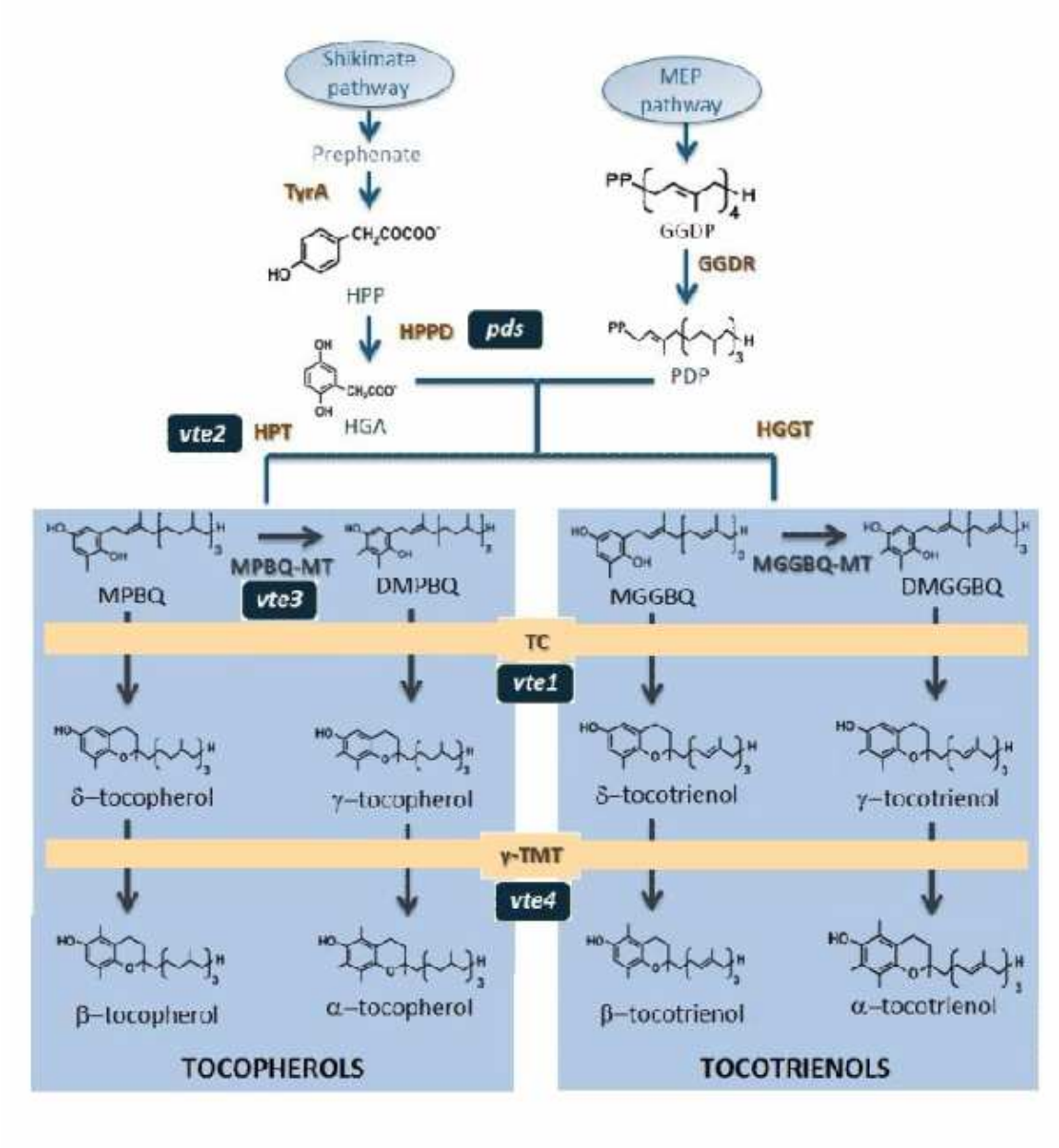

Figure 5.1. Vitamin E synthesis in plants. Tocochromanols are synthesized on the inner chloroplast membrane from precursors derived from the shikimate and methylerythritol phosphate (MEP) pathways. The shikimate pathway contributes the head-group precursor homogentisic acid (HGA), whereas the MEP pathway gives rise to the side-chain precursors phytyldiphosphate (PDP) and geranylgeranyldiphosphate (GGDP). The first committed step in the reaction is the cytosolic conversion of ρ-hydroxyphenylpyruvic acid (HPP) to HGA by ρ-hydroxyphenylpyruvic acid dioxygenase (HPPD). HGA is then prenylated with either PDP or GGDP to produce the intermediates 2-methyl-6-phytyl benzoquinone (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ). A second methyl group is added by MPBQ

methyltransferase (MPBQ-MT) in the tocopherol branch and MGGBQ methyltransferase (MGGBQ-MT) in the tocotrienol branch, producing the intermediates 3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ). All four of these intermediates are substrates for tocopherol cyclase (TC), which produces δ and γ tocopherols and tocotrienols. Finally, γ-tocopherol methyltransferase (γ-TMT) catalyzes a second ring methylation to yield α and β tocopherols and tocotrienols. Other abbreviations: HPT, homogentisate phytyltransferase; HGGT; homogentisate geranylgeranyl transferase; GGDR, geranylgeranyl diphosphate reductase.

5.3 Materials and methods

5.3.1 Cloning and vector construction

Arabidopsis thaliana PDS1 cDNA (encoding HPPD) was cloned by RT-PCR using forward primer 5'-AGG ATC CTC AAT GGG CCA CCA AAA CGC CGC CG-3' and reverse primer 5'-AAG CTT CAT CCC ACT AAC TGT TTG GCT TC-3' based on GenBank sequence data (accession numbers AF000228 and AF104220). The products were transferred to pGEM®-T (Promega, Madison, WI, USA) for sequencing and then to pAL76, containing the constitutive maize ubiquitin 1 (Ubi-1) promoter and first intron, and the nopaline synthase terminator, for expression in plants.

5.3.2 Rice transformation and verification of transgenic plants

Mature rice embryos (*Oryza sativa* L. *cv* EYI 105) were excised and cultured for 7 days and then transformed by particle bombardment as previously described (Sudhakar et al., 1998; Christou et al., 1991) using a 3:1 molar ratio of pAL76-PDS1 and a plasmid containing the hygromycin phosphotransferase (*hpt*) selectable marker (Sudhakar et al., 1998). Independent transgenic lines were recovered from callus regenerated on selection medium and were tested along with non-transformed control plants cultivated in parallel. Transgenic plants were verified by PCR using 100 ng leaf genomic DNA, the primers described above and 0.5 units of GoTaq® DNA polymerase (Promega, Madison,

WI). Reactions were heated to 95ºC for 3 min, followed by 30 cycles at 94 ºC for 45 s, 60 ºC for 45 s and 72 ºC for 90 s, and a final extension at 72 ºC for 10 min.

5.3.3 mRNA blot analysis

Total RNA and transgene expression analysis were carried out as described by Naqvi et al. (2011).

5.3.4 HPLC-MS

Total tocochromanols were analyzed as described by Naqvi et al. (2011).

5.4 Results and discussion

Plants are the major source of vitamin E in the human diet and the consumption of plants is preferable to synthetic supplements which tend to have much lower vitamin E activities (Naqvi et al., 2011; Farré et al., 2010). However, the vitamin E activity in different plant species and tissues varies widely because the absolute level of total tocochromanols and the relative levels of different vitamers can vary, reflecting the expression levels of enzymes involved in tocochromanol biosynthesis. This suggests the tocochromanol content and composition in plants could be modulated by genetic engineering.

We generated transgenic rice plants constitutively expressing the *A. thaliana PDS1* gene (encoding HPPD) by bombarding embryos and regenerating transgenic plants on medium containing hygromycin. There were no morphological or developmental differences between transgenic plants and controls. Transgene expression was confirmed by northern blot analysis and three representative lines (RVe1, RVe2 and RVe7) expressing *PDS1* at high levels (Figure 5.2) were selected for HPLC analysis to determine tocochromanol content and composition (Table 5.1).

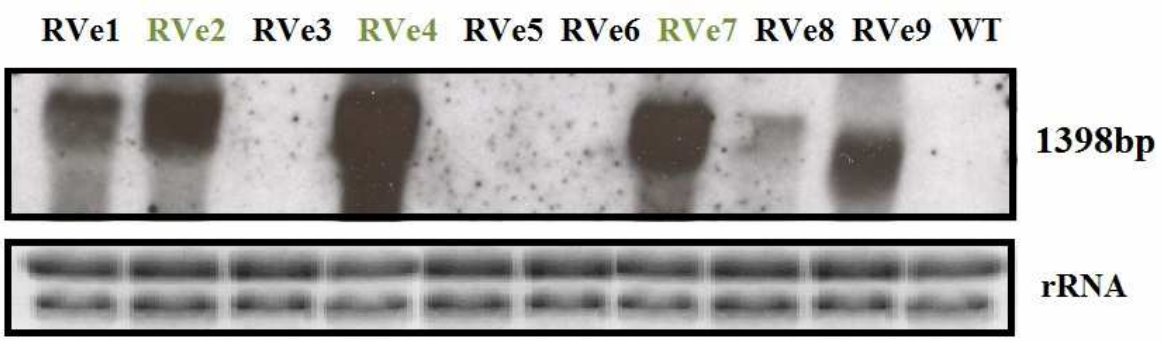

Figure 5.2. Northern blots showing *PDS1* expression in nine independent transgenic rice lines. Total RNA isolated using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) 30µg aliquots were fractionated on a denaturing 1.2% w/v agarose gels containing formaldehyde fractionated and transferred to a positively-charged nylon membrane, then probed with digoxigenin-labeled partial cDNA at 50ºC overnight in DIG Easy Hyb. After washing and immunological detection with anti-DIG-AP, the CSPD chemiluminescence substrate was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO). Probe was designed using the PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with forward primer (5'-AGGATCCTCAATGGGCCACCAAAACGCCGCCG-3') and reverse primer (5'-AAGCTTCATCCCACTAACTGTTTGGCTTC-3').

Table 5.1. The tocochromanol content of transgenic and control rice seeds (µg/g dry weight). T3 = tocotrienols, Toc = tocopherols. 2 samples with 2 determinations each.

Line	γ-T3	α-T3	Total T3	α/γ-T3	γ-Toc	α-Toc	Total Toc	α/γ-Toc	Total
RVe2	5.81±0.49	6.68±0.58	12.49	1.15	1.55±0.20	5.82±0.60	7.37	3.75	19.86
RVe4	3.11±0.39	5.21±0.56	8.32	1.68	1.51±0.22	6.00±0.59	7.51	3.97	15.83
RVe7	4.77±0.50	4.82±0.53	9.59	1.01	1.55±0.17	5.83±0.40	7.38	3.76	16.85
WT	3.79±0.42	4.45±0.47	8.24	1.17	2.24±0.39	5.27±0.48	7.51	2.35	15.75

Because HPPD catalyzes the first step in the pathway, we expected to see an overall increase in tocochromanol synthesis, which was not the case. Only a marginal increase was achieved and this was almost entirely due to a small rise in tocotrienol synthesis. There was no change in the α:γ ratio in the tocotrienol branch. In contrast, there was no change in the overall level of tocopherols, but the α:γ ratio increased significantly compared to control grains resulting in the amount of α-tocopherol increasing at the expense of γ-tocopherol and thereby increasing the vitamin E activity of the grains.

These data confirm that HPPD is not a rate-limiting enzyme in tocochromanol synthesis but that altering the flux disrupts the balance further along the pathway and changes the metabolic tipping points at pathway branches and bottlenecks. The first instance is the branch between the tocopherol and tocotrienol pathways, where almost all the additional flux appears to be diverted into the tocotrienol branch. The increased availability of HGA (the immediate product of HPPD) appears to influence the relative activities of HPT and HGGT, favoring the latter so that the additional flux is fed into tocotrienol synthesis. However, there is no compensatory depletion of tocopherol, which suggests that HPT is not a rate-limiting enzyme either. The second instance is the conversion between the γ and α isoforms of tocopherol (but not tocotrienol), where more flux is transferred to α-tocopherol in transgenic plants than controls. This suggests some form of positive feedback, in which the increased flux through the pathway influences the activity of γ-TMT. An interesting question is why this increased flux does not also affect the tocotrienol branch, where the α:γ ratio remains unchanged, since the same enzyme carries out the γ to α conversion in both branches. A possible explanation is that the effect is localized to enzyme complexes containing γ-TMT and HPT and/or MPBQ-MT, which are pathway specific, or that the phytyl side chain is an important factor in the enhanced γ-TMT activity.

Previous studies have shown that the overexpression of *PDS1* has either no impact on tocopherol levels, as in Arabidopsis (Tsegaye et al., 2002) and tobacco (Falk et al., 2005), or only a marginal effect, as in canola (Raclaru et al., 2006), indicating that it is not rate limiting in these plants. Naqvi et al. (2011) reported a three-fold increase in tocopherols in transgenic corn seeds by simultaneously expressing Arabidopsis HPPD and MPBQ-MT, and γ-tocopherol was the only vitamer detected. These data suggest that MPBQ-MT may be rate limiting, and that γ-TMT represents a significant bottleneck in a high-flux environment. This seems likely given the dramatic shift from γ

to α-tocopherol in soybean plants simultaneously expressing γ-TMT and MPBQ-MT (Van Eenennaam et al., 2003). Yusuf and Sarin (2006) expressed Arabidopsis γ-TMT in *Brassica juncea* plants and found a correlation between α-tocopherol levels and high γ-TMT expression, with the highest expressers producing six times as much α-tocopherol as wild type seeds (62.29 ng/mg).

Our study has shown that relatively simple alterations to a metabolic pathway can have complex effects by influencing downstream branch points and bottlenecks. Even though HPPD does not appear to be rate limiting it is still an interesting target for vitamin E engineering in rice because it has an indirect effect on the α-tocopherol content and therefore increases vitamin E activity significantly even when total tocochromanol levels increase only marginally. The biofortification of crops such as rice in this manner will offer a sustainable alternative to vitamin E supplementation, providing immense benefits to human health (Ajjawi and Shintani, 2004; Zhu et al., 2007).

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CHAPTER 6

Nutritionally enhanced crops and food security: scientific achievements versus political expediency

Chapter 6. Nutritionally enhanced crops and food security: scientific achievements versus political expediency

6.1 Abstract

Genetic engineering (GE) is one of a raft of strategies that can be used to tackle malnutrition. Recent scientific advances have shown that multiple deficiencies can be tackled simultaneously using engineered plant varieties containing high levels of different minerals and organic nutrients. However, the impact of this progress is being diluted by the unwillingness of politicians to see beyond immediate popular support, favoring political expediency over controversial but potentially lifesaving decisions based on rational scientific evidence.

6.2 Introduction

Food security depends not only on the availability of food but also its nutritional quality. Unfortunately, the poorest people in the world generally rely on a monotonous staple diet, and since most plants are deficient in certain vitamins, minerals, and essential amino acids, a diet restricted to one major staple will tend to be nutritionally incomplete (Christou and Twyman, 2004; Zhu et al., 2007). GE strategies have been used to tackle nutrient deficiency, with some remarkable advances in the past two years offering the prospect of nutritionally complete staple crops that could realistically address malnutrition on a global scale. Unfortunately, it is highly unlikely that such crops will be adopted in the short-to medium term because politicians in Europe (and developing countries in Europe's sphere of influence) often pander to hyperbolic arguments about perceived risks, while ignoring potential benefits. In this review we summarize some of the recent advances in the field and briefly discuss the political hurdles currently preventing the deployment of nutritionally enhanced crops, and how these might be overcome.

6.3 Nutritionally enhanced crops—recent achievements

Recent GE strategies to increase crop yields have been highly successful (Farré et al., 2009) but the most striking advances over the past two years have involved plants

engineered to produce missing nutrients or increase the level of nutrients that are already synthesized. An important trend is the move away from plants engineered to produce single nutritional compounds towards those simultaneously engineered to produce multiple nutrients, a development made possible by the increasing use of multigene engineering (Naqvi et al., 2010). Several recent reports have demonstrated how multigene metabolic engineering can increase the level of carotenoids in edible plant tissues, including the traditional target b-carotene (pro-vitamin A, whose absence in staple cereals is responsible for almost 500,000 cases of preventable blindness every year (UNICEF, 2010)) and other carotenoids with specific functions in the human body or generally beneficial antioxidant properties.

A combinatorial nuclear transformation method has been developed that allows the carotenoid synthesis pathway in corn to be dissected, and allows the production of diverse populations of transgenic plants containing different carotenoid profiles (Zhu et al., 2007). The system as originally reported involved the transformation of a white corn variety lacking endosperm carotenoids with five genes from the carotenoid pathway (Figure 6.1) each under the control of a different endosperm-specific promoter. The population of transgenic plants recovered in this approach contained random combinations of transgenes, thus each unique combination had a different metabolic potential and produced a distinct carotenoid profile. Normally, the random nature of transgene integration is considered disadvantageous because hundreds of lines may need to be screened to identify one with the correct genotype and phenotype. However, random transgene integration is an advantage in this new platform because it increases the diversity of the population, resulting in plants with high levels of carotenoids such as b-carotene, lutein, zeaxanthin, lycopene, and astaxanthin, alone or in combination. Recently, it was demonstrated that the engineered carotenoid pathway could be introgressed from a transgenic line with a high LYCB:LYCE (lycopene β-cyclase to lycopene ε-cyclase) ratio (thus favoring the bcarotene branch) into the genetic background of a wildtype yellow-endosperm corn variety also with a high LYCB:LYCE ratio, resulting in synergistic enhancement of the metabolic bias and creating hybrid lines producing unprecedented levels of zeaxanthin (56 µg/g DW) (Naqvi et al., 2010a). This novel strategy for combining GE and conventional breeding allows the development of ‘designer’ hybrid lines with specific carotenoid profiles, and is equally applicable to any staple crop where nutritional improvement would be beneficial.

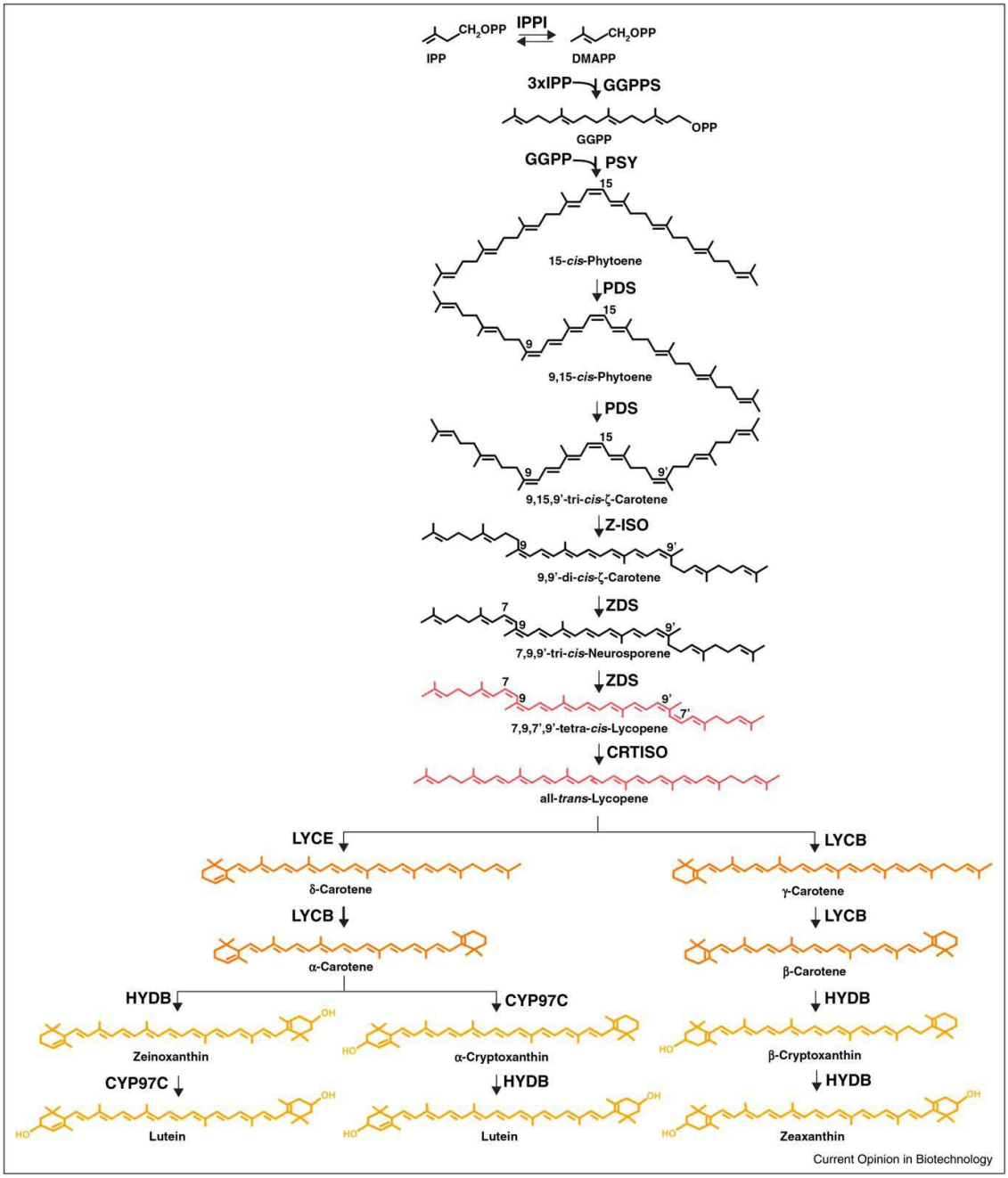

Figure 6.1. Carotenoid biosynthesis in corn endosperm. Abbreviations: IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, z-carotene isomerase; ZDS, z-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene b-cyclase; LYCE, lycopene e-cyclase; CYP97C, carotene e-ring hydroxylase; HYDB, b-carotene hydroxylase.

Diverse carotenoid profiles have also been reported in canola seeds from plants transformed with up to seven carotenogenic transgenes (Fujisawa et al., 2009). In this study, the authors aimed for seven-gene transformation and obtained their diversity as a byproduct, but the advantages are the same as those demonstrated in the combinatorial corn transformation platform. Most of the canola plants contained all seven genes and the total carotenoid content increased 30-fold, including a spectrum of novel ketocarotenoids that are not usually found in canola. Carotenoid levels can also be enhanced by increasing storage capacity, as seen in the cauliflower Or (orange) mutation that induces chromoplast differentiation and facilitates the hyperaccumulation of β-carotene resulting in a dark orange inflorescence (Lu et al., 2006). Expression of an *Or* transgene in potato tubers resulted in a 6-fold increase in carotenoid levels by increasing the capacity for carotenoid storage (López et al., 2008).

There has also been recent progress with other vitamins, such as the expression of a chicken *GTP cyclohydrolase I* gene in lettuce, which increased folate levels by nearly 9-fold (Nunes et al., 2009), the co-expression of Arabidopsis ρ-hydroxyphenylpyruvate dioxygenase and 2-methyl-6-phytylplastoquinol methyltransferase in corn, which increased γ-tocopherol levels by 3-fold at the expense of other, less potent tocopherols (Naqvi et al., 2010b), and the doubling of ascorbate levels in tobacco by encouraging recycling through the expression of phosphomannomutase or GDP-D-mannose pyrophosphorylase (Badejo et al., 2008; 2009). Whereas the enhancement of individual nutrients provides proof of principle, progress towards addressing micronutrient deficiencies in the real world will only be made once it is possible to target different nutrients at the same time. In this context, transgenic corn plants simultaneously enhanced for carotenes, folate and ascorbate provide the first example of a nutritionally enhanced crop targeting three entirely different metabolic pathways, going some way towards the goal of nutritionally complete staple crops (Naqvi et al., 2009). This was achieved by transferring four genes into the white corn variety described above, resulting in 407 times the normal level of b-carotene (57 µg/g DW), 6.1 times the normal level of ascorbate (106.94 µg/g DW) and twice the normal amount of folate (200 µg/g DW).

Whereas metabolic engineering can increase the levels of organic nutrients, minerals must instead be sequestered from the environment (Gómez-Galera et al., 2010). One notable recent report describes the hyperaccumulation of iron in rice plants transformed

with two genes, encoding nicotianamine synthase (required for iron transport through the vascular system) and ferritin (which increases the capacity for iron storage) (Wirth et al., 2009). Calcium levels in carrots are doubled when the transporter sCAX1 is expressed in the taproots, and feeding studies have shown that the extra calcium is bioavailable when carrots are fed to mice (Morris et al., 2008). Similarly, calcium levels increased in lettuce expressing the same transporter, without detectable changes to the organoleptic properties of lettuce leaves (Park et al., 2009).

Recent attempts to enhance the levels of essential amino acids and very long chain polyunsaturated fatty acids have also been successful, for example, the expression of an RNA interference construct to inhibit the key enzyme lysine-ketoglutarate reductase/saccharophine dehydrogenase (LKR/SDH) in order to increase lysine levels in corn (Frizzi et al., 2008), and the expression of a liverwort D6-desaturase, D6-elongase, and D5-desaturase to triple arachidonic acid levels and double eicosapentaenoic acid levels in transgenic tobacco plants (Kajikawa et al., 2008). Cheng et al. (2009) also produced high levels of eicosapentaenoic acid in canola.

6.4 Benefits and risks of deployment

6.4.1 Can nutritionally enhanced GE crops really improve food security?

Scientific advances are occasionally oversold in the pursuit of funding, patents or industry investment (Twyman et al., 2009), so it is fair to ask whether GE can realistically improve food security or whether the claims are exaggerated. It is clear that the world produces enough food for its current population, but poverty and poor health prevent access to adequate nourishment (DFID, 2010; Islam, 2008). These issues disproportionately affect the poorest, notably subsistence farmers in developing countries, often driving them to cities thus adding to the growing problem of urban poverty and hunger (FAO, 2009). Any long-term strategy to address food insecurity in the developing world must therefore tackle the underlying problem of poverty and poor health by increasing the level of rural employment-based income through increased agricultural productivity (Islam, 2008; FAO, 2009). The production of crops with higher nutritional value would add to the yield improvements made possible by GE and would

mean that a smaller proportion of each farmer's output would be needed for subsistence and more could be sold at market, and also there would be a lower burden of disease caused by malnutrition (Zhu et al., 2007). GE crops provide the only route to nutritional completeness and could be a valuable component of a wider strategy including conventional breeding and other forms of agricultural development to improve food security now and in the future, in combination with better governance, education and healthcare, and socioeconomic policies to improve the welfare of the rural poor in developing countries (FAO, 2009).

6.4.2 Are there risks to health and the environment?

Although there is little doubt that GE technology can improve the nutritional value of food, these benefits are offset by perceived risks to health and the environment. One of the main challenges is that many non-scientists have a very poor grasp of risk and often attach unrealistic likelihoods to risks that are infinitesimal in nature. The global area of GE crops has steadily increased over the past 14 years (James, 2009) despite much public distrust and political controversy, particularly in Europe. There is no evidence for any detriment to public health or the environment in those areas that have embraced GE agriculture. Other technologies, which do have quantifiable risks, are accepted with far less protest. For example, great significance has been attached to the near imperceptible risks of 'horizontal gene transfer' from transgenic plants containing antibiotic resistance genes to pathogenic bacteria in the human gut, whereas the much more quantifiable risks of pesticide exposure is routinely ignored. Given that nature teems with antibiotic-resistant bacteria and we consume billions of them every day without ill effects yet there is plenty of evidence of environmental damage and health problems caused by pesticide use, why is there so much controversy about the use of genes that are already abundant and harmless? Why was it necessary to invest so much in the development of politically expedient technology to remove them? An interesting case study from the European Union (EU) that provides insight into the reasons behind the negative perception of GE technology is discussed in Box 1.

Box 1 Only bad news sells.

Data from a field trial of Bt corn in Italy performed in 2005 as part of what was supposed to be a broad popular overview of GE in Italy were largely ignored. An analysis of the events surrounding this phenomenon leads to only one conclusion—the information was suppressed because it showed GE in a positive light (Marshall 2007). The outcomes were to be presented at a public meeting in 2006, but the full field trial data were never released. When it became clear that the Italian Ministry of Agriculture was not going to publish the trial data, a small group of determined researchers held a press conference in 2007 (Morandini and Defez 2007) in response to several months of intensive campaigning by a coalition of over 30 groups claiming to represent over 11 million Italians opposed to GE foods. The Italy/Europe Free of GMO (GEO) coalition, which encompassed several Italian farming unions, consumer associations, and environmental groups, such as Greenpeace and the Worldwide Fund for Nature, had organized nearly 2000 separate anti-GE events and in a mock referendum collected three million signatures calling for a complete ban on all GE foods in Italy. Fourteen of Italy's 20 regions had already declared themselves GE-free even though the field trial results showed that under field conditions, MON810 corn expressing Bt toxin can help maintain yield levels that are 28–43% higher than those of isogenic non-GE varieties (Marshall 2007; Morandini and Defez 2007). MON810 corn also outperformed conventional corn in terms of the levels of fumonisins, toxins that are produced by fungi able to infect plants through lesions caused by the corn borer. MON810 corn contained 60 or fewer parts per billion of fumonisin, whereas non-GE varieties contained over 6000 parts per billion, a level unsuitable for human consumption under Italian and European law. If it had been the MON810 varieties that contained high levels of fungal toxins, interest of the politicians, the media and the general public in the data would probably have been intense. But the response to these inconveniently positive field trial data was unreceptive at best.

Unable to find any direct evidence to prove that GE crops are inherently risky, protagonists often fall back on the claim that there are no long-term safety studies proving the absence of any harm to health from ingesting GE foods. It was in this vein that researchers at the University of Veterinary Medicine in Vienna launched a feeding study in mice to assess health over generations. The aim of the study was to assess the health effects of a variety of GE corn carrying two transgenes: cry1Ab from Bt and the Agrobacterium gene encoding 5-enolpyruvylshikimate-3-phosphate synthase, which confers tolerance to glyphosate herbicides. Although the authors stated that ‘‘no negative effects of GE corn varieties have been reported in peerreviewed publications’’, they wanted to assess health effects over several generations—something that has rarely been studied (Sinha 2009). The study was not published, nor was it peer reviewed. Rather, the results were announced at a press conference in 2008 (Velimirov 2008). Anti-GE groups like Greenpeace jumped on this ‘news’ and issued a press release stating: ‘‘Forget condoms – eat GE corn’’ and demanded a worldwide recall of all GE foods and crops, stating: ‘‘GE food appears to be acting as a birth control agent, potentially leading to infertility’’ (Greenpeace 2008).

The study was soon criticized to be flawed and was discredited in the scientific community (Sinha 2008). These errors make it unsuitable for risk assessment and/or regulatory purposes (Monsanto 2009). This study therefore served a political rather than a science-based agenda.

6.4.3 Political factors—the role of activists, the media, the public, and politicians

The political dimension to GE crops is best explained as a cycle of self-reinforced negative publicity (Figure 6.2). The media, politicians, and the public feed each other with (mis)information, becoming more risk averse and sensationalist with each cycle. The public are predominantly exposed to science through the mass media, which can be a good source of information on cutting edge technologies. However, depending on their financial and political influence, the media can also manipulate the public, causing scientific controversies that are rarely about science. Reporting biotechnology poses unique challenges because it is perceived as a controversial, evolving field, and it is difficult to produce quality news stories in an environment where science gets attention from activists and politicians. It is also clear that controversy itself attracts attention,

which makes biotechnology a tempting opportunity for journalists good and bad. Consumer acceptance depends directly on how much trust is placed on the available risk–benefit information. Some of the public carefully weigh potential benefits more heavily than risks, while others form their biotechnology attitudes solely on media sound bites.

As well as deliberate manipulation, even a benign media can provide misinformation in an attempt to provide 'balanced' coverage (i.e. presenting alternative views no matter how irrational) or by oversimplification for the target audience so that viewpoints are polarized into universal acceptance or rejection of a particular technology, with little room for reasoned discourse. Scientific data is often molded into a publication format that allows several different types of manipulation to take place, including suppression of positive data by omission (**Box 1**), publication of negative data without verification (and not publishing contrary evidence or retractions), or deliberately negative framing and labeling (e.g. 'killer corn'). This contrasts sharply with the description of novel recombinant cancer drugs as 'magic bullets' and 'wonder- drugs', and it is clear that medical stories are often presented positively. Where GE and medicine combine, as in transgenic plants producing pharmaceutical proteins, the media has been cautiously upbeat: 'Transgenic corn to cure HIV/AIDS' (GMO Africa: transgenic corn to cure HIV/AIDS (http://www.gmoafrica.org/2008/05/591.html).

It is often said that GE crops could solve Africa's hunger and poverty, but that, through inadequate investment, external lobbying, and stringent regulations, farmers are being deprived of the technology and prevented from achieving agricultural success (Paarlberg, 2008; Scoones and Glover, 2009). Many blame the European governments and non-governmental organizations for trying to foist their affluent values and precautionary sensibilities on Africa's poor. Politicians therefore play a key role in the eternal triangle because they listen to lobbies and respond with decisions that are handed down to regulators. If the public do not like GE they will lobby their politicians, who will in turn order the regulators to increase the regulatory burden. Even when the regulators consult independent advice, the politicians would rather fly in the face of this advice and impose 'solutions' to problems that do not exist, instead of evaluating the data properly. For this reason, the EU labors under such burdensome co-existence regulations in agriculture that is becomes virtually impossible for GE farmers to plant their crops without the risk of litigation (Ramessar et al., 2010). In a misguided attempt

to streamline the approval process for GM crops in Europe, the EU is considering allowing Member States to opt out of approvals, impose their own regulations, and create de facto GE-free zones in Europe, in direct contradiction of their aspirations to support innovation and develop a knowledge-based bioeconomy (Chipman, 2010).

6.4.4 What can be done to fix the negative cycle?

Where negative perception has a unique source it should be possible to correct the perception by providing education at the source, but in the self-sustaining loop shown in Figure 6.2 there is no single point of intervention that will work. What is required is a concerted and coordinated campaign to influence the media, politicians, and public about scientific realities and, in particular, the correct approach to risk evaluation (Box 2). Organizations such as Sense About Science, which provide a voice for scientists and a platform to address scientific misinformation about GE in the media (SAS, 2010), are a step in the right direction but are often placed in the position of a reactionary rather that a proactive force in the face of sometimes overwhelming media-promulgated ignorance and sensationalism. The negative cycle can only be broken by education, the exposure of myths and lies and something that is almost unheard of: holding the media to the same publication standards as the scientists they often criticize.

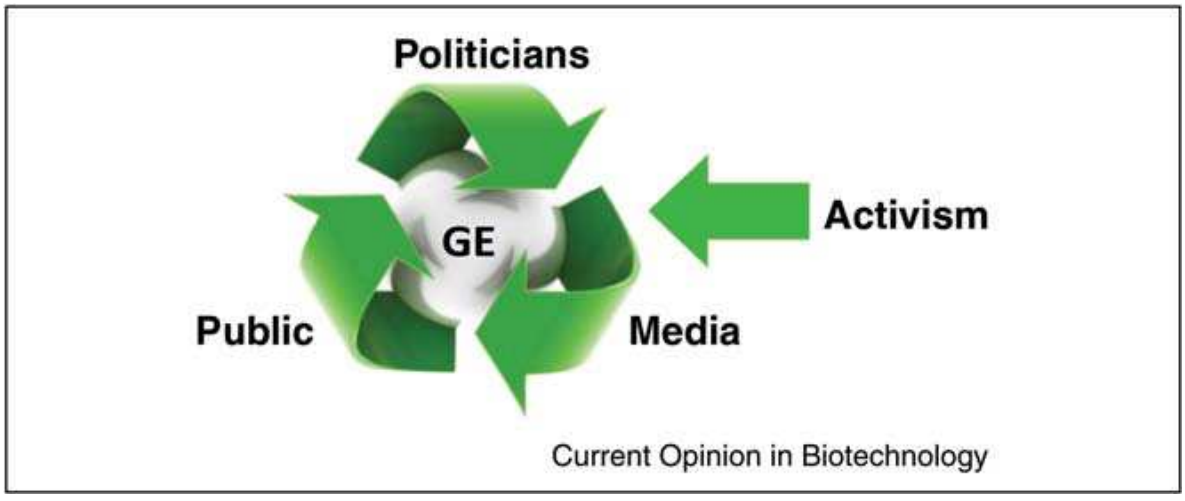

Figure 6.2. The eternal triangle of negative reinforcement. Media sensationalism, fed by propaganda from activists, misinforms the public about the risks of GE. The public vote for politicians to represent their views, so they pressure the regulators to treat GE more cautiously than the actual risk justifies. Activists and the media then use this as

evidence to support their claims that GE is risky. There is no room for scientists or rational debate in this cycle.

Box 2 Political issues and potential solutions

The political issues

- Politicians have more influence on the regulators than scientists.
- Public opinion drives political decisions more strongly than science.
- Public opinion is swayed by the media, which prefers sensationalist reporting.
- The media responds to sensationalist activist claims rather than rational scientific debate.
- Activists are not bound by the same rules of engagement as scientists.

Potential solutions

- Regulators should be protected from undue political pressure.
- Politicians should weigh up scientific evidence properly, for example, through independent bodies that have executive authority.
- There should be more effort to educate the public about science.
- The media should have a duty to report accurately and should hold activists to the same standard of evidence as scientists.
- The EU should enforce its own regulations and support farmers wishing to grow GE crops to the same extent as those growing conventional/organic crops.
- The regulation of GE crops should be handled in the same way as drugs—once safety has been confirmed a license should be given and marketing should be authorized throughout the EU, without provisions to permit Member States to interfere for reasons of political expediency.

6.5 Conclusions

GE strategies can be used to address micronutrient deficiency in both the developed and the developing world, as recent advances in the areas of metabolic engineering and mineral accumulation have demonstrated, particularly those studies simultaneously

tackling multiple nutrients. However, this can only be achieved with the support of the public, media, and politicians. Converting the current negative reinforcement cycle into a positive one will only be possible when there is less irrational hatred of GE, and this can only come about with a strenuous effort to educate the public, politicians, and the media about the realistic nature of risks, and the balance between risks and benefits in all areas of life.

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CHAPTER 7

GENERAL DISCUSSION

Chapter 7. General discussion

Food insecurity is defined as a lack of access to enough safe and nutritious food to maintain an active and healthy life. This is currently one of the world's greatest challenges. Food insecurity leads to malnutrition, which reflects the combined impact of poverty, poor access to food, inefficient food distribution infrastructure, and an over-reliance on subsistence agriculture based on individual cereal crops that lack essential nutrients. Malnutrition is therefore endemic in developing countries where the lack of a diverse diet means that many individuals are exposed to the risk of deficiency diseases (Pérez-Massot et al., 2012). The biofortification of staple crops by genetic engineering is a promising strategy to increase the vitamin content of cereal-based foods.

Several carotenoids (β-carotene, α-carotene, γ-carotene and β-cryptoxanthin) have pro-vitamin A activity and are also potent antioxidants that modulate the onset and progress of several chronic degenerative diseases. Vitamin A deficiency (VAD) is a serious health problem in developing countries where many people rely on cereals for most of their nutritional calorie intake and where fresh fruits and vegetables that are good sources of vitamin A are in short supply or prohibitively expensive. Meat and dairy sources of vitamin A primarily contain an esterified form of retinol (retinyl palmitate) whereas plants produce pro-vitamin A carotenoids such as β-carotene that are cleaved to produce retinal directly (Harrison, 2005).

Combinatorial genetic transformation is achieved by engineering plants with several genes simultaneously. Using this approach, a diverse population of transgenic plants was generated, providing a means to study very complex metabolic pathways and to modulate metabolic flux. This approach allows the modulation of independent metabolic pathways regardless of their complexity and it facilitates partial or total reconstruction using transgenes. The expression of multiple transgenes is usually stable over many generations. Combinatorial genetic transformation was previously used for the simultaneous expression of multiple carotenoid biosynthesis genes in a white corn inbred deficient for endosperm carotenoid synthesis, resulting in the creation of a combinatorial corn plant population with high levels of β-carotene and other carotenoids (Zhu et al., 2008). Five carotenogenic genes were introduced, namely *Zea mays* phytoene synthase 1 (Z*mpsy1*), *Pantoea ananatis* phytoene desaturase (*Pacrt*I),

Gentiana lutea β-carotene hydroxylase (*Glbch*), *G. lutea* lycopene β-cyclase (*Gllycb*) and *Paracoccus* spp. β-carotene ketolase (*Paracrt*W), all under the control of different endosperm-specific promoters.

One significant challenge relating to the biofortification of cereal grains with carotenoids is our limited understanding of how carotenoid biosynthesis is regulated, particularly in the endosperm, which is the most nutritious component of the grain. A number of recent studies have shown the potential for carotenoid fortification of corn endosperm, either through conventional breeding or transgenic strategies (Harjes et al., 2008; Zhu et al., 2008; Aluru et al., 2008; Naqvi et al., 2009). Despite this progress, much remains to be learned about carotenoid synthesis in the corn endosperm and the regulatory mechanisms that control the accumulation of specific carotenoids. The combinatorial population discussed above was used to identify and complement rate-limiting steps in the carotenoid biosynthesis pathway so that predictive metabolic engineering could be used to enhance carotenoid levels. The isolation, developmental profiling and functional characterization of corn *bch* cDNAs also suggested diverse regulatory strategies that could be used to control the accumulation of carotenoids in the endosperm.

Vitamin E comprises a group of eight organic compounds known as tocochromanols, namely the α, β, δ and γ isomers of tocopherol and tocotrienol. Plants and cyanobacteria can synthesize tocopherols, which probably act as antioxidants (Munné-Bosch, 2005). Recent studies have shown that α-tocopherol and also other forms of vitamin E play important roles in human health. The principal activity of tocopherols in humans is to scavenge and quench reactive oxygen species and lipid-soluble oxidative stress by-products (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Ricciarelli et al., 2002). Epidemiological data suggest that a high vitamin E intake (100-1000 IU per day) reduces the risk of certain types of cancer and cardiovascular disease (Bramley et al., 2000; Aggarwal et al., 2010; Colombo, 2010; Sen et al., 2010) and helps to maintain the immune system thus delaying the progression of degenerative diseases (Traber and Sies, 1996). More recently, consumer and media attention has focused on the role of vitamin E on cell membrane protection in the skin, and also the ability of α-tocopherol to reduce age-dependent increases in collagenase activity (Ricciarelli et al., 1999). The ability of vitamin E to prevent lipid peroxidation by free radicals and the anti-inflammatory

activity of γ-tocopherol have also been documented (Chiu and Kimball, 2003; Jiang and Ames, 2003; Jiang et al., 2000; 2001).

Plants provide the primary source of dietary vitamin E for humans but the tocochromanol content and composition varies between species and tissues. A high level of γ-tocopherol is present in oilseeds (Grusak and Dellapenna, 1999) but only low levels are found in cereal grains. The biofortification of important crop species such as rice offers a sustainable alternative to vitamin E supplementation, which in turn will be beneficial to human health (Ajjawi and Shintani, 2004).

The isolation of genes involved in tocochromanol biosynthesis has helped to characterize the pathway and to provide new insights into the regulatory relationships among the enzymes. Metabolic engineering has been used successfully to alter the levels of total and specific tocochromanols in agricultural crops, with a positive impact on nutrition and health (Farré et al., 2012).

Genetically engineered (GE) crops have a remarkable potential to tackle some of the world's most challenging socioeconomic problems, including hunger, malnutrition, disease and poverty. These are more prevalent, more entrenched and more intractable in the developing world than in the industrialized nations (Christou and Twyman, 2004). However, this potential will not be realized if the major barriers to adoption – which are political rather than technical – are not overcome. Regulatory harmonization would help to remove artificial trade barriers, accelerate the adoption of GE crops, foster technology transfer and protect developing countries from exploitation, instilling confidence and bringing the benefits of GE products to the consumer (Farré et al., 2010). Although the socioeconomic benefits of transgenic plants are quite clear, deployment is currently blocked by illogical regulatory frameworks because developing countries often base their regulations on models from the US and EU, which are fundamentally distinct (Ramessar et al., 2008). The EU regulatory system focuses on the production process rather than the end-product, i.e. exactly the same plants can be regulated differently according to how they are produced. In contrast, the US system focuses on the product and seeks to determine whether it is equivalent in terms of safety to its conventional counterpart. Another difference is that the EU system is based on the precautionary approach, which essentially means that a GE crop is not approved unless the absence of risk can be demonstrated. It is impossible to prove that a risk is zero (as opposed to vanishingly small) so this creates an effective moratorium on GE agriculture

in Europe. What is needed is a global, harmonized regulatory system that is flexible enough to adapt to regional differences as well as different platforms and products, while showing due respect for science-based risk assessment and the concerns of all stakeholders. The main problem is the politicization of biosafety assessments. Politicians interfere inappropriately in the EU approval process for GE crops which leads to protectionism and it makes difficult to release transgenic crops for humanitarian purposes (Herring, 2008). Consequently, many developing countries have followed the EU and banned GE products. This will definitely delay the deployment of nutritionally-improved GE crops in other developing countries, where they are needed the most (Ramaswami, 2007). All regulations delay the deployment of nutritionally enhanced GE varieties, not because regulation itself is undesirable but because the assessment process in the EU is influenced by political interference driven by uniformed public opinion and pressure from certain interest groups (Apel, 2010). Several reports provide evidence that the current regulatory systems are sufficient to detect and assess the safety of GE crops in terms of unanticipated effects where major metabolic pathways have been engineered (International Life Sciences Institute, 2004a; 2004b; 2008).

Negative public perception and media campaigns are another barrier to adoption. This negative and self-fulfilling cycle of oppressive regulation can only be broken by switching from risk assessment to risk-benefit analysis for the regulation of nutritionally-enhanced crops (Melo-Martín and Meghani, 2008; Ramessar et al., 2009). The regulatory framework needs to be built around solid scientific facts rather than what in some cases appear to be nothing but wild guesswork and scaremongering. This could be achieved by the establishment of a global foundation with the power to modify regulations based upon scientific principles and guidelines to address all sectors of the food chain in a way that would maximize the net benefits of the global food system.

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CHAPTER 8

CONCLUSIONS

Chapter 8. Conclusions

1. *Zmpsy1* transcripts are not present in wild type M37W corn endosperm but low levels of *Zmpsy2* mRNA were detected suggesting that the residual carotenoid content of the endosperm probably reflects the activity of PSY2. This confirms that PSY1 rather than PSY2 is the key enzyme responsible for carotenoid accumulation in corn endosperm.
2. A second rate-limiting step in the maize carotenoid pathway is the conversion of phytoene to lycopene, catalyzed by endogenous desaturases and isomerases. This step appears to be subject to feedback regulation because the endogenous genes are modulated by the introduction of transgenes acting later in the pathway.
3. LYCB is also a rate-limiting enzyme in the M37W endosperm reflecting the insufficient conversion of lycopene into β-carotene.
4. The conversion of adonixanthin to astaxanthin by *Paracrt*W is an important limiting step for astaxanthin biosynthesis. Avoiding adonixanthin accumulation by introducing *Paracrt*W is therefore crucial for the heterologous production of astaxanthin in transgenic corn endosperm.
5. The accumulation profiles of individual carotenoids in corn endosperm indicate that although carotenoid synthesis begins at the earliest stages of endosperm development and their levels depend on end product accumulation.
6. Two cloned *bch* cDNAs encoding the corn carotenogenic enzymes BCH1 and BCH2 were shown to be highly conserved in sequence, expression and activity, but there exhibited subtle differences hinting at their divergent roles in carotenoid biosynthesis that may be useful for the development of more refined strategies to biofortify staple crops.
7. Transgenic rice plants expressing the Arabidopsis *HPPD* gene confirmed that HPPD is not a rate-limiting enzyme in tocochromanol synthesis but that altering the flux disrupts the balance further along the pathway and changes the metabolic tipping points at pathway branches and bottlenecks.
8. GE strategies can be used to address micronutrient deficiency in both developed and developing countries, as recent advances in the areas of metabolic

engineering have demonstrated, particularly those tackling multiple nutrients simultaneously.

9. Deployment of GE crops can only be achieved with the support of the public, media and politicians. Changing the current negative reinforcement cycle into a positive one will only be possible when there is less irrational opposition to GE, and this can only come about with a strenuous effort to educate the public, politicians and the media about the realistic nature of risks, and the balance between risks and benefits in all areas of life.

OUTPUTS

1. Publications in peer reviewed journals

15. **Farré G**, Zorrilla U, Berman J, Zhu C, Christou P and Capell T (2012) Increasing the vitamin E content of food by in-plant production. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources (in press). (See Annex 1)

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12. **Farré G**, Twyman R, Zhu C, Capell T and Christou P. (2011) Nutritionally enhanced crops and food security: scientific achievements versus political expediency. Current Opinion in Biotechnology. Current Opinion in Biotechnology 22:245-251. (See Annex 4)

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1. Christou P, Zhu C, Naqvi S, **Farré G**, Capell T (2008) Combinatorial genetic transformation and the generation of plant mutant libraries for metabolic engineering. International Congress on Plant Genomics (PlantGEM7) Albena, Bulgaria, September 24-27, 2008. Invited Lecture.

ANNEX 1

Farré G, Zorrilla U, Berman J, Zhu C, Christou P and Capell T (2012) Increasing the vitamin E content of food by in-plant production. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources (in press)

Increasing the vitamin E content of food by in-plant production

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Abstract

Vitamin E is a group of eight, lipid-soluble molecules known as tocochromanols, featuring a conserved head group and an aliphatic side chain. They are produced by plants and some cyanobacteria, and act as free radical scavengers in mammals to prevent lipid oxidation. The different isomers of vitamin E can be classed as tocopherols or tocotrienols depending on the chemical conformation of the side chain. The natural isomer α-tocopherol (RRR-α-tocopherol) is more active than synthetic vitamin E, making it the most effective natural form of the vitamin. The composition of vitamin E differs widely among plant species and varieties, and many crops have been bred or engineered to increase their vitamin E levels. In the review, we discuss strategies to increase vitamin E levels and alter the vitamin E composition in plants, and present case studies that demonstrate the impact of these strategies in different crops.

Key words

Vitamin E; deficiency diseases; metabolic engineering; conventional breeding

1. Introduction

Vitamin E is a group of eight structurally-related molecules known as tocochromanols (or tocols) comprising four tocopherols and four tocotrienols [1]. These are amphipathic molecules, consisting of a chromanol head group with one, two or three methyl groups arranged in different configurations (forming the α, β, γ and δ forms) and an isoprenoid side chain. Tocopherols have a saturated phytyl side chain whereas tocotrienols have an unsaturated geranylgeranyl side chain (**Figure 1**). The eight tocochromanols vary in their vitamin E activity *in vivo*. All eight isomers (also known as vitamers) can be absorbed equally by the human gut [2] but the hepatic α-tocopherol transfer protein (α-TTP) preferentially retains α-tocopherol, making this the most nutritionally valuable form of vitamin E [3]. Natural α-tocopherol is 1.5 fold more active than synthetic α-tocopherol [4-7] because it is a single stereoisomer (RRR-α-tocopherol or d-α-tocopherol) whereas synthetic α-tocopherol is a racemic mixture of eight different stereoisomers, reflecting the presence of three stereocenters in the molecule [8]. The vitamin E activity of 1 mg of chemically synthesized all-racemic α-tocopherol is defined as 1 international unit (IU), which is equivalent to 0.45 mg of natural RRR-α-tocopherol. Humans and many animals are unable to synthesize tocochromanols de novo and must obtain them from their diet. The National Institutes of Health (NIH) have suggested a dietary reference intake (DRI) of 15 mg/day of natural α-tocopherol (http://www.iom.edu/Reports/2006/Dietary-Reference-Intakes-Essential-Guide-Nutrient-Requirements.aspx).

The nutritional value of vitamin E was first recognized in 1922 [9], and the principal activity of tocopherols in humans is to scavenge and quench reactive oxygen species and lipid-soluble oxidative stress by-products [5-6; 10]. Epidemiological data suggest that a high vitamin E intake (100-1000 IU per day) reduces the risk of certain types of cancer and cardiovascular disease [6], and helps to maintain the immune system thus delaying the progression of degenerative diseases [2]. More recently, consumer and media attention has focused on the role of vitamin E on skin cellular membrane protection. The protective role of vitamin E against lipid peroxidation by free radicals and the anti-inflammatory activity of γ-tocopherol have been documented [11-14]. α-Tocopherol protects against skin aging by reducing the age-dependent increase of collagenase activity [15] and tocotrienols play a role in the prevention of a number of diseases [16-18].

Tocochromanols are synthesized by plants and other oxygenic photosynthetic organisms. The presence of higher levels of α-tocopherols in photosynthetic tissues suggests it may protect the photosynthetic apparatus against oxidative stress and lipid peroxidation. The accumulation of γ-tocopherol in seeds is thought to help prevent the auto-oxidation of polyunsaturated fatty acids [19]. Other possible functions of tocochromanols in plants include the regulation of membrane fluidity and intracellular signaling [20, 21].

2. Vitamin E deficiency

Vegetable oils, nuts and green leafy vegetables are the major sources of vitamin E [22]. Vitamin E deficiency is uncommon in developed countries where most people have access to a varied diet, and tends to be caused either by fat malabsorption or, more rarely, a heritable defect of liver metabolism [23]. Vitamin E deficiency is much more common in the developing world where many people subsist on monotonous cereal-based diets and lack access to fresh fruits and vegetables [23].

The symptoms of vitamin E deficiency reflect the loss of its protective effects on fatty acids, low-density lipoproteins (LDLs) and other components of cell membranes [24]. This is particularly important in red blood cells and neurons because they are more vulnerable to free-radical damage, so severe vitamin E deficiency manifests as hemolytic anemia and neurological disorders. The degree of hemolysis caused by vitamin E deficiency in humans also depends on age, the availability of other antioxidants, oxidative stress levels and the abundance of polyunsaturated fatty acids (PUFAs) [24]. Progressive ataxia is more rapid in infants and young children with malabsorption from birth than in adults, suggesting that the normal development of the nervous system depends on an adequate supply of vitamin E [25,26]. The first symptom of vitamin E deficiency is usually peripheral neuropathy with degeneration of large-caliber axons in sensory neurons [26,27]. Vitamin E also influences T-cell signaling, which increases the percentage of old CD4+ cells that can form an effective immune synapse [28].

Vitamin E deficiency is diagnosed by measuring the ratio of plasma α-tocopherol to total plasma lipids, a low ratio suggesting inadequate intake. Treatment consists of oral vitamin E supplements. Early, high-dose supplements can achieve complete resolution

of deficiency symptoms and avoid further clinical manifestations such as cardiac arrhythmia, blindness and dementia [29].

3. Diversity of vitamin E composition in plants

The overall vitamin E content and vitamer composition differs widely among different plant species (Table 1). The predominant form of vitamin E in the green leaves of higher plants is α-tocopherol [30], whereas γ-tocopherol tends to be the major form found in the seeds of dicotyledonous plants [31,32] and tocotrienols are more abundant in the seeds of monocotyledonous plants such as rice, barley, wheat, palm and oat [33-37]. The variation in vitamin E composition in different plants reflects the expression, activity and substrate specificities of different enzymes in the tocochromanol biosynthesis pathway [32].

4. Tocochromanol biosynthesis in plants

The tocochromanol biosynthesis pathway in higher plants was characterized in the 1980s by carrying out a series of radiotracer studies [38]. Precursors are derived from the shikimate and methylerythritol phosphate (MEP) pathways. The shikimate pathway produces homogentisic acid (HGA) which contributes to the chromanol head group, whereas the MEP pathway contributes to the side chain [39]. The first committed step in tocochromanol biosynthesis is the conversion of ρ-hydroxyphenylpyruvic acid (HPP) to HGA by ρ-hydroxyphenylpyruvic acid dioxygenase (HPPD), a reaction that takes place in the cytosol (**Figure 2**). HGA is then imported into the plastids and prenylated with phytyldiphosphate (PDP) to produce the tocopherol intermediate 2-methyl-6-phytylbenzoquinone (MPBQ) or with geranylgeranyl diphosphate (GGDP) to produce the tocotrienol intermediate 2-methyl-6-geranylgeranylbenzoquinone (MGGBQ) [40].

Tocopherol biosynthesis requires the enzymes homogentisate phytyltransferase (HPT), MPBQ methyltransferase (MPBQ-MT), tocopherol cyclase (TC) and γ-tocopherol methyltransferase (γ-TMT), all of which are associated with the chloroplast envelope. The first reaction is the prenylation of HGA with PDP to generate MPBQ, which is catalyzed by HPT [21, 41]. MPBQ is a substrate for MPBQ-MT, which adds a second methyl group to form 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). MPBQ and DMPBQ are both substrates of TC, producing δ-tocopherol and γ-tocopherol,

respectively [38]. Both the δ and γ forms of tocopherol can be methylated by γ-TMT to produce β- and α-tocopherol, respectively [42, 43].

Analogous steps are required to synthesize tocotrienols. In this case HGA is prenylated with GGPP (catalyzed by homogentisate geranylgernalyltransferase, HGGT) and the resulting intermediate MGGBQ is a substrate for MGGBQ methyltransferase (MGGBQ-MT) to generate 2,3-dimethyl-5-gernaylgernalyl-1,4-benzoquinone (DMGGBQ). MGGBQ and DMGGBQ are substrates for TC, producing δ- and γ-tocotrienol, respectively [28], and these are methylated by γ-TMT to produce β- and α-tocotrienol, respectively [42, 43].

5. Enhancing vitamin E levels in plants

5.1 Conventional breeding and mutation analysis

As stated above, both the quantity and composition of tocochromanols differ among plant species and varieties, e.g. maize breeding lines naturally contain much more γ-tocopherol than α-tocopherol, whereas α-tocopherol is the most abundant vitamer in broccoli [44, 45]. Germplasm such as the IL2027-7 *sh2*, IL2022-11 *sh2* and IL451b *Su1* maize lines and the 'Pirate', 'Marathon' and 'Baccus' commercial broccoli lines can be used to enhance tocopherol levels [45].

Conventional breeding can be used for the progressive selection of quantitative trait loci (QTLs) that increase α-tocopherol levels, but this is a slow and laborious process. However, mutants and polymorphisms affecting vitamin E levels and composition are useful tools for vitamin E research, either as a basis for complementation studies or as a starting point for further improvement using biotechnology. As an example, soybean seeds have yielded four QTLs associated with α-tocopherol levels, eight associated with γ-tocopherol levels, four associated with δ-tocopherol levels and five influencing the total vitamin E content [46].

To address the functions of tocopherols in plants, a series of tocopherol-deficient Arabidopsis mutants has been isolated and characterized [20]. Disruption of the *VTE1* locus (encoding TC), resulted in the replacement of tocopherols with the intermediate DMPBQ, whereas disruption of the *VTE2* locus (encoding HPT) eliminated tocopherols without the accumulation of pathway intermediates [47, 48]. Mutations at the *VTE4* locus (encoding γ-TMT) accumulated high levels of γ-tocopherol at the expense of α-

tocopherol. In the null mutant *vte4-1*, α-tocopherol was replaced by γ-tocopherol but this was still able to protect the photosynthetic apparatus from oxidative stress [49]. Mutations at the *VTE3* locus (encoding MPBQ-MT) accumulated δ-tocopherol at the expense of γ-tocopherol in seeds [50].

5.2 Genetic engineering

Plants can be engineered to accumulate higher levels of vitamin E by introducing transgenes that encode enzymes involved in tocochromanol synthesis. Improvements can be achieved either by increasing the total tocochromanol content or skewing tocochromanol synthesis toward α-tocopherol.

The introduction of single or multiple enzymes can help to relieve bottlenecks and increase total vitamin E levels. For example, a two-fold increase in tocopherol levels was achieved in canola seeds by expressing the *Erwinia herbicola TYRA* gene, which encodes chorismate mutase-prephenate dehydrogenase and is responsible for the synthesis of HPP from prephenate [41]. In contrast, constitutive expression of the barley *hppd* gene in tobacco leaves has no effect on tocopherol levels [38]. Arabidopsis leaves expressing an Arabidopsis *HPT1* transgene accumulated 458.35 μg/g dry weight (DW) of total tocopherols, a 4.4-fold increase over wild-type levels [51]. The simultaneous expression of *Erwinia herbicola TYRA*, Arabidopsis *HPPD* and *Synechocystis* spp *VTE2* (HPT) in canola seeds resulted in a three-fold increase in total tocochromanols [41].

A number of different approaches have been used to modulate the vitamin E composition of transgenic plants. For example, the overexpression of MPBQ-MT diverts flux towards the α-branch at the expense of the δ-branch, because MPBQ-MT converts MPBQ to DMPBQ, which later can be converted to α-tocopherol via TC and γ-TMT. The impact of Arabidopsis *VTE3* and *VTE4* expression in soybean seeds, alone and in combination, has also been investigated [50]. The expression of *VTE3* alone (under the control of the napin promoter) increased the total tocopherol content only marginally, but it caused the preferential accumulation of γ-tocopherol (75–85% of total tocopherols) indicating that flux was diverted into the α-branch of the pathway and that the inefficient conversion of γ- to α-tocopherol by γ-TMT (*VTE4*) was the rate-limiting step [52]. In maize, the combination of HPPD and MPBQ-MT caused a three-fold increase in γ-tocopherol levels (to 9.5 μg/g DW) without changing the total tocopherol content, again showing that flux was directed into the α-branch but was blocked by low

γ-TMT activity, forcing the accumulation of γ-tocopherol [53]. In rice, expression of *AtHPPD* alone did not alter the α:γ ratio in the tocotrienol branch neither overall tocopherol levels; however the tocopherol α:γ ratio increased significantly, resulting in higher amounts of α-tocopherol in rice grains (1.4-fold at the expense of γ-tocopherol) [54].

The constitutive expression of γ-TMT (*VTE4*) alone resulted in the accumulation of 100% α- and β-tocopherol in soybean seeds [50], but expression under the control of the vicilin promoter resulted in a 41-fold increase in α-tocopherol levels in seeds [55]. Combining both *VTE3* and *VTE4* in soybean resulted in an eight-fold increase in α-tocopherol levels, and α-tocopherol was the main tocochromanol vitamer in the seeds [50].

By combining the above strategies, it is possible to increase overall tocopherol levels and skew the content towards the accumulation of α-tocopherol. In T_2 transgenic lettuce plants, a six-fold increase in the total tocopherol content was achieved (up to 42.67μg/g fresh weight) by the constitutive expression of Arabidopsis *HPT* and *VTE4*, also resulting in a six-fold increase in the α/γ ratio [56].

The success of the above approaches depended not only on the expression of functional enzymes, but also on factors such as promoter choice and transgene origin [57-59]. Most transgenic plants with enhanced vitamin E levels have been engineered with transgenes under the control of seed-specific promoters e.g. the canola napin promoter, the soybean 7Sα and vicilin promoters, and the *Daucus carota* DC3RΩ promoter, but constitutive promoters such as the *Cauliflower mosaic virus* 35S (CaMV 35S) and maize ubiquitin-1 (*Ubi1*) promoters have also been used. Appropriate promoter selection contributes to the higher vitamin E levels achieved in transgenic plants, e.g. the expression of Arabidopsis *HPPD* under the control of the DC3R promoter in Arabidopsis seeds achieved a 1.3-fold increase in the total tocopherol content (to 528 μg/g) compared to 1.1-fold (398 μg/g) using the CaMV 35S promoter [58]. Transgenic soybean lines expressing HGGT under seed-specific (rice globulin promoter) or constitutive (CaMV35S promoter) manner were generated. Seed-specific expression resulted in a 2-fold higher tocotrienol content, whereas constitutive expression resulted in a 20% higher γ-tocopherol content [59]. The transgene origin may have a significant impact on tocochromanol content because enzymes from different species have different

intrinsic levels of activity, and codon usage may also affect the efficiency of protein synthesis. For example, the maize *vte1* gene expressed in canola under the control of the napin promoter achieved a 1.55-fold increase in total tocochromanols (to 1159 μg/g seed oil) compared to the 1.36-fold increase achieved using the Arabidopsis ortholog *VTE1* (1018 μg/g) [60].

6. Conclusions and future prospects

Vitamin E is an essential nutrient for humans, and although many plants provide rich sources of vitamin E there is still widespread deficiency in developing countries because only low levels of tocochromanols are present in staple cereal grains. This has promoted research into the development of staple crops with enhanced vitamin E levels, a goal that can be achieved by both conventional breeding and genetic engineering or a combination of approaches. Over the last few years, our understanding of the genetic, molecular and biochemical aspects of tocopherol synthesis has increased significantly, resulting in targeted strategies to improve vitamin E levels in plants based on our knowledge of the corresponding metabolic pathway [40]. Although conventional breeding can accomplish moderate improvements over many generations, genetic engineering remains the only approach that can achieve substantial increases in vitamin E levels over a short timescale using local elite cultivars, and the only approach that allows nutritional crops engineered to accumulate different vitamins and minerals to be combined. More detailed studies are required to address vitamin E deficiency in developing countries, i.e. by seeking additional genetic variation for conventional breeding strategies, and novel sources of enzymes and promoters to increase metabolic flux towards the most potent vitamin E vitamers by genetic engineering. A combination of these approaches will help to provide sustainable nutrition for those most at risk of micronutrient deficiency, and will therefore help to improve global food security, health and welfare.

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Plant and organ	Total tocopherols (□g/g fresh weight)	Percent α-tocopherol	Percent others and major type (T = tocopherols, T3 = tocotrienols)
Potato (tuber)	0.7	90	10% γ, β-T
Rice (seed)	17	18	30% α-T3, 30% γ-T3, 18% γ-T
Lettuce (leaf)	7	55	45% γ-T
Spinach (leaf)	30	63	5% γ-T, 33% δ-T
Arabidopsis (leaf)	10-20	90	10% γ-T
Arabidopsis (seed)	200-300	0	95% γ-T, 5% δ-T
Sunflower (seed oil)	700	96	4% γ, β-T
Maize (seed oil)	1000	20	70% γ-T, 7% δ-T
Soybean (seed oil)	1200	7	70% γ-T, 22% δ-T
Broccoli (flower head)	1.7	78.5	19.3 % γ-T, 2.2 % δ-T
Palm oil	1200	23	26.6 % γ-T, 24% γ-T3, 11.6 α-T3
Barley (dry seed)	15.5 (dry weight)	11-19	42%–63% α-T3, 10 %–22% γ-T3, 7%–20% β-T3
Wheat (germ oil)	2700	47	25% β-T, 10% γ-T, 7% β-T3

Table 1: Tocopherol levels and compositions in selected plant tissues and oils [31, 36-37, 61-62].

Species	Genes (origin)	Promoter	Vitamin E levels and composition in transgenic plants	Reference
Corn	*Hggt* (barley)	Embryo-specific	> 344,57 μg /g DW in seeds (6-fold)	63
	HPPD and *VTE3* (Arabidopsis)	Corn Ubi-1	9.5 μg/g DW γ-tocopherol in seeds (3-fold)	53
Lettuce	*VTE4* (Arabidopsis)	CaMV 35S	Improved α/γ tocopherol ratio 0.4–544 (wild type = 0.6–1.2)	64
	VTE2 (Arabidopsis)	CaMV 35S	40.41 μg/g FW total tocopherol (5.7-fold) 0.46 α/γ ratio no changes	56
	VTE4 (Arabidopsis)	CaMV 35S	12.44 μg/g FW total tocopherol (1.75-fold) 9.19 α/γ ratio (20-fold)	
	VTE2 and *VTE4* (Arabidopsis)	CaMV 35S	64.55 μg/g FW total tocopherol (9-fold) 8.34 α/γ ratio (18.5-fold)	
	Vte2 (Lettuce)	CaMV 35S	17.77 μg/g FW total tocopherol in leaves (2.6-fold of α- and γ-tocopherol) in leaves Tocotrienols and other tocopherols were negligible	65
Mustard	*VTE4* (Arabidopsis)	CaMV 35S	609.7 μg/g total tocopherol in seeds (1.07-fold)	66

			367.6 μg/g α-tocopherol in seeds (6-fold) 79.08 μg/g β-tocopherol in seeds (1.63-fold) 211.5 μg/g γ-tocopherol in seeds (41% decrease) 31.3 μg/g δ-tocopherol in seeds (21% decrease)	
Canola	*HPPD* (Arabidopsis)	DC3Ω	819 μg/g total tocochromanol in seed oil (1.2-fold) 183 μg/g α-tocochromanol in seeds (1.07-fold) 606 μg/g γ-tocochromanol in seeds (1.24-fold) 16.7 μg/g δ-tocochromanol in seeds (1.67-fold)	67
	HPPD, *HPT1* and *VTE1* (Arabidopsis)	DC3Ω (*HPD*), napin (*HPT1, VTE1)*	1850 μg/g total tocochromanol in seed (2-fold) 610 μg/g α-tocopherol in seed (1.74-fold) 1010 μg/g γ-tocopherol in seed (2.5-fold) 163 μg/g δ-tocopherol in seed (14.8-fold)	
	TYRA (*E. herbicola*)	napin	540 μg/g total tocochromanols in seeds (2-fold)	41

	TYRA (*E. herbicola*), *HPPD* (Arabidopsis), *VTE2* (*Synechocystis* spp)	Arc (common bean arcelin-5), 7Sα	829 μg/g total tocochromanols in seeds (3-fold)	
	VTE1 (Arabidopsis)	Napin	1018 μg/g total tocochromanols in seed oil (1.36-fold) 436 μg/g α-tocochromanol in seed (1.4-fold) 510 μg/g γ-tocochromanol in seed (1.22-fold) 36 μg/g δ-tocochromanol in seed (3.6-fold)	60
	vte1 (corn)		1159 μg/g total tocochromanols in seed oil (1.55-fold) 386 μg/g α-tocochromanol in seed (1.24-fold) 720 μg/g γ-tocochromanol in seed (1.73-fold) 32 μg/g δ-tocochromanol in seed (3.2-fold)	
Soybean	*tyrA* (*E. herbicola*), *HPPD*, *VTE2* and *GGH* (Arabidopsis)	Arc, 7Sα	4806 μg/g total tocochromanols in seeds (15-fold) 94% tocotrienols slight reduction in total	50

			tocopherols	
	VTE3 (Arabidopsis)	7Sα	329 μg/g total tocopherols in seeds 10-20% α-tocopherol 0-1.9% β-tocopherol 75-85%% γ-tocopherol 11%δ-tocopherol	50
	VTE4 (Arabidopsis)	7Sα	321 μg/g total tocopherols in seeds 75% α-tocopherol 2-28% β-tocopherol 15-79% γ-tocopherol 0-28%δ-tocopherol	
	VTE3 and *VTE4* (Arabidopsis)	7Sα	320 μg/g total tocopherols in seeds 60-91% α-tocopherol (8-fold) no charge β-tocopherol, 4.3-26.9% γ-tocopherol 1-10% δ-tocopherol	
	Vte4 (*Perilla frutescens*)	Vicilin	193.61 μg/g FW α-tocopherol in seeds (10.4-fold) 23.96 μg/g FW β-tocopherol (14.9-fold) γ- and δ- tocopherol levels	68

			negligible	
	Vte4 (*P. frutescens*)	Vicilin	656 μg/g α-tocopherol in seed (41-fold) 208 μg/g β-tocopherol (1.23-fold) marginal change in γ- and α-tocopherol	55
	Hppt (Oryza sativa)	Rice globulin	184.4-217.1 μg/g DW total tocochromanol in seeds (1.3-fold) 177.5- 209.3 μg/g tocopherols 6.1-7.8 μg/g tocotrienols	59
		CaMV 35S	186.4-217.1 μg/g DW total tocochromanol in seeds (1.3-fold) 184.5- 215.5 μg/g tocopherols 1.5-1.9 μg/g tocotrienols	
Arabidopsis	*VTE4* (Arabidopsis)	*DC3*(carrot)	360.6 μg/g total tocopherol (no differences) 342 μg/g α-tocopherol in seeds (86-fold; 95.1% of total tocopherols)	52
			Reductions in γ-tocopherol (from 96.9% to 3.9%) and δ-tocopherol (from 2.18% to 0%)	

			(from 0% to 1%)	
	HPPD (Arabidopsis)	CaMV 35S	398 μg/g total tocopherol content in seeds (1.1-fold)	58
		DC3	528 μg/g total tocopherol in seeds (1.3-fold)	
	VTE2 (Arabidopsis)	CaMV35S	473 μg/g DW total tocopherol in leaves (4.4-fold; mainly α-tocopherols) 555.62 μg/g DW total tocopherol in seeds (40% increase; mainly γ-tocopherol)	51
	VTE2 (Arabidopsis)	Napin	926 μg/g total tocopherol in seeds (2-fold)	69
Tobacco	*TYRA* (*E. uredovora*)	Arabidopsis histone H4748	67 μg/g DW total tocotrienols in leaves (1.3-fold) 14.3 μg/g DW α-tocotrienol in leaves	70
	TYRA (*E. uredovora*) *HPPD* (Arabidopsis)	Arabidopsis histone H4748	551 μg/g DW total tocotrienols content in leaves (10-fold) 412.3 μg/g DW α-tocotrienol in leaves No change in tocopherol content	
	Hppd (barley)	CaMV 35S	58 μg/g FW γ-tocotrienol in	38

			seeds (2-fold) 50 μg/g FW γ-tocopherol in seeds (2-fold)	
Tomato	*Vte2* (apple)	CaMV 35S	4.5 μg/g FW α-tocopherol in fruits (1.7-fold)	71
Rice	*HPPD* (Arabidopsis)	Corn Ubi-1	α/γ tocopherol ratio increased 1.7-fold 6.00 μg/g DW α-tocopherol in seeds (1.4-fold)	54

Table 2: Vitamin E enhancement by genetic engineering. DW = dry weight; FW = fresh weight.

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ANNEX 2

Farré G, Sudhakar D, Naqvi S, Sandmann G, Christou P, Capell T, Zhu C (2012) Transgenic rice grains expressing a heterologous ρ-hydroxyphenylpyruvate dioxygenase shift tocopherol synthesis from the γ to the α isoform without increasing absolute tocopherol levels. Transgenic Research. DOI: 10.1007/s11248-012-9601-7.

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BRIEF COMMUNICATION

Transgenic rice grains expressing a heterologous ρ-hydroxyphenylpyruvate dioxygenase shift tocopherol synthesis from the γ to the α isoform without increasing absolute tocopherol levels

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Abstract We generated transgenic rice plants over-expressing *Arabidopsis thaliana* ρ-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the first committed step in vitamin E biosynthesis. Transgenic grains accumulated marginally higher levels of total tocochromanols than controls, reflecting a small increase in absolute tocotrienol synthesis (but no change in the relative abundance of the α and γ isoforms). In contrast, there was no change in the absolute tocopherol level, but a significant shift from the γ to the α isoform. These data confirm HPPD is not rate limiting, and that increasing flux through the early pathway reveals downstream bottlenecks that act as metabolic tipping points.

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Introduction

Vitamin E is an essential plant-derived nutrient in the human diet that comes in eight structurally-related forms known as tocochromanols (Fig. 1). The chromanol head group can be joined to a saturated phytyl side chain to form tocopherol, or to an unsaturated geranylgeranyl side chain to form tocotrienol. The head group can then be methylated in different configurations, resulting in four alternative forms (α, β, γ and δ). Although all eight vitamers are absorbed in humans, the hepatic α-tocopherol transfer protein (α-TTP) preferentially retains α-tocopherol making this the most active form of vitamin E in the human diet (Traber and Arai 1999).

Plants synthesize tocochromanols to protect the photosynthetic apparatus against reactive oxygen species and prevent lipid peroxidation, with γ-tocopherol playing a particularly important role in preventing the auto-oxidation of polyunsaturated fatty acids in seeds (Munné-Bosch and Alegre 2002). Plants therefore provide an excellent dietary source of vitamin E, which is thought to reduce the risk of cancer, cardiovascular disease and neurodegenerative disorders in humans by scavenging reactive oxygen species and lipid-soluble oxidative stress by-products (Bramley et al. 2000).

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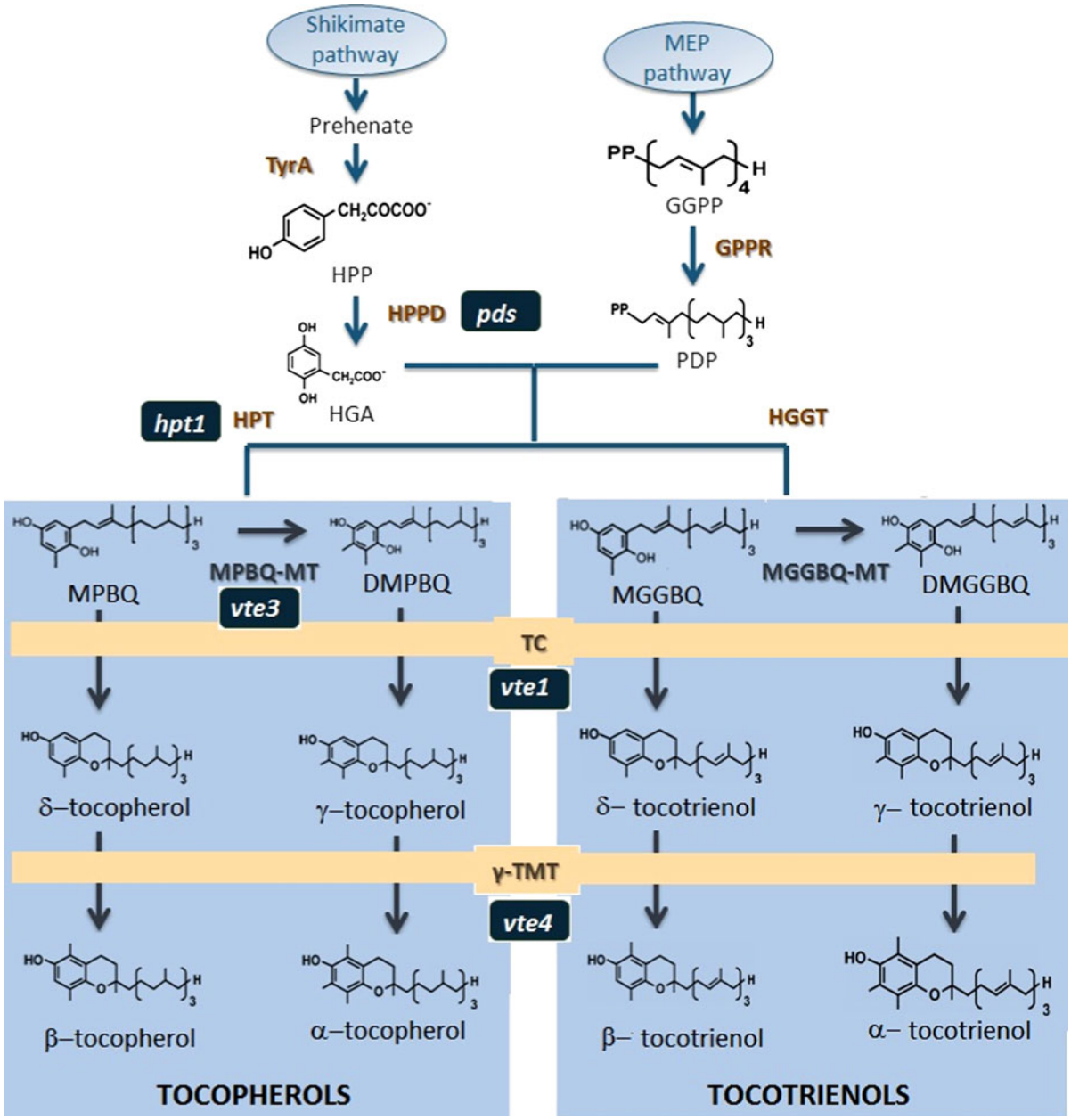

Fig. 1 Vitamin E synthesis in plants. Tocochromanols are synthesized on the inner chloroplast membrane from precursors derived from the shikimate and methylerythritol phosphate (MEP) pathways. The shikimate pathway contributes the head-group precursor homogentisic acid (HGA), whereas the MEP pathway gives rise to the side-chain precursors phytyldiphosphate (PDP) and geranylgeranyldiphosphate (GGDP). The first committed step in the reaction is the cytosolic conversion of ρ-hydroxyphenylpyruvic acid (HPP) to HGA by ρ-hydroxyphenylpyruvic acid dioxygenase (HPPD). HGA is then prenylated with either PDP or GGDP to produce the intermediates 2-methyl-6-phytyl benzoquinone (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ). A second methyl group is added by MPBQ methyltransferase (MPBQ-MT) in the tocopherol branch and MGGBQ methyltransferase (MGGBQ-MT) in the tocotrienol branch, producing the intermediates 3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ). All four of these intermediates are substrates for tocopherol cyclase (TC), which produces δ and γ tocopherols and tocotrienols. Finally, γ-tocopherol methyltransferase (γ-TMT) catalyzes a second ring methylation to yield α and β tocopherols and tocotrienols. Other abbreviations: *HPT* homogentisate phytyltransferase, *HGGT* homogentisate geranylgeranyl transferase, *GGDR* geranylgeranyl diphosphate reductase

There is considerable variation in both the absolute and relative tocochromanol levels in different plant species and tissues, suggesting the pathway can be modulated by genetic engineering to increase the nutritional value of plants with low levels of vitamin E. We generated transgenic rice plants constitutively expressing the *Arabidopsis thaliana PDS1* gene (encoding HPPD) to determine whether increasing flux through the common, early part of the pathway had any impact on overall tocochromanol levels and/or the relative levels of the different vitamers. We observed only a marginal increase in absolute tocochromanol levels in the transgenic grains, mainly due to the slightly higher tocotrienol content. However, the overall vitamin E activity of the grains increased significantly due to a marked shift from the γ to the α isoform in the tocopherol pathway. Our data indicate that even though HPPD might not be an appropriate target for vitamin E enhancement in rice, it is still a noteworthy target in vitamin E biofortification programs due to its capacity to enhance the absolute levels of α-tocopherol at the expense of the less nutritionally important γ isoform.

Materials and methods

Cloning and vector construction

Arabidopsis thaliana PDS1 cDNA (encoding HPPD) was cloned by RT-PCR using forward primer 5′-AGG ATC CTC AAT GGG CCA CCA AAA CGC CGC CG-3′ and reverse primer 5′-AAG CTT CAT CCC ACT AAC TGT TTG GCT TC-3′ based on GenBank sequence data (accession numbers AF000228 and AF104220). The products were transferred to pGEM®-T (Promega, Madison, WI, USA) for sequencing and then to pAL76, containing the constitutive maize ubiquitin 1 (Ubi-1) promoter and first intron, and the nopaline synthase terminator, for expression in plants.

Rice transformation and verification of transgenic plants

Mature rice embryos (*Oryza sativa* L. *cv* EYI 105) were excised and cultured for 7 days and then transformed by particle bombardment as previously described (Sudhakar et al. 1998; Christou et al. 1991) using a 3:1 molar ratio of pAL76-PDS1 and a plasmid containing the hygromycin phosphotransferase (*hpt*) selectable marker (Sudhakar et al. 1998). Independent transgenic lines were recovered from callus regenerated on selection medium and were tested along with non-transformed control plants cultivated in parallel. Transgenic plants were verified by PCR using 100 ng leaf genomic DNA, the primers described above and 0.5 units of GoTaq® DNA polymerase (Promega, Madison, WI). Reactions were heated to 95°C for 3 min, followed by 30 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 90 s, and a final extension at 72°C for 10 min.

mRNA blot analysis

Total RNA and transgene expression analysis were carried out as described by Naqvi et al. (2011).

HPLC analysis

Total tocochromanols were analyzed as described by Naqvi et al. (2011).

Results and discussion

Plants are the major source of vitamin E in the human diet and the consumption of plants is preferable to synthetic supplements which tend to have much lower vitamin E activities (Naqvi et al. 2011; Farré et al. 2010). However, the vitamin E activity in different plant species and tissues varies widely because the absolute level of total tocochromanols and the relative levels of different vitamers can vary, reflecting the expression levels of enzymes involved in tocochromanol biosynthesis. This suggests the tocochromanol content and composition in plants could be modulated by genetic engineering.

We generated transgenic rice plants constitutively expressing the *A. thaliana PDS1* gene (encoding HPPD) by bombarding embryos and regenerating transgenic plants on medium containing hygromycin. There were no morphological or developmental differences between transgenic plants and controls. Transgene expression was confirmed by northern blot analysis and three representative lines (RVe1, RVe2 and RVe7) expressing *PDS1* at high levels (Fig. 2)

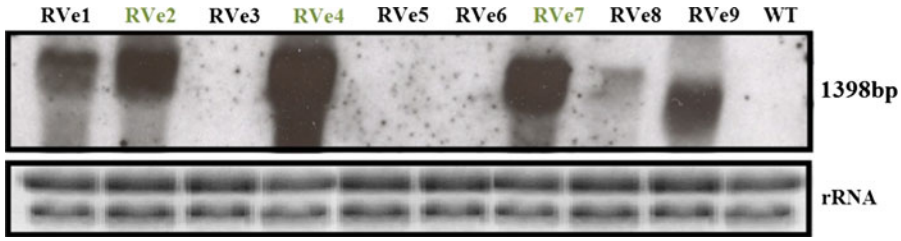

Fig. 2 Northern blots showing *PDS1* expression in nine independent transgenic rice lines. Total RNA isolated using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) 30 μg aliquots were fractionated on a denaturing 1.2% w/v agarose gels containing formaldehyde fractionated and transferred to a positively-charged nylon membrane, then probed with digoxigenin-labeled partial cDNA at 50°C overnight in DIG Easy Hyb. After washing and immunological detection with anti-DIG-AP, the CSPD chemiluminescence substrate was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO). Probe was designed using the PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with forward primer (5′-AGGATCCTCAATGGGCCACCAAAA CGCCGCCG-3′) and reverse primer)5′-AAGCTTCATCCCA CTAACTGTTTGGCTTC-3′)

Table 1 The tocochromanol content of transgenic and control rice seeds (μg/g dry weight)

Line	γ-T3	α-T3	Total T3	α/γ-T3	γ-Toc	α-Toc	Total Toc	α/γ-Toc	Total
RVe1	5.81 ± 0.49	6.68 ± 0.58	12.49	1.15	1.55 ± 0.20	5.82 ± 0.60	7.37	3.75	19.86
RVe2	3.11 ± 0.39	5.21 ± 0.56	8.32	1.68	1.51 ± 0.22	6.00 ± 0.59	7.51	3.97	15.83
RVe7	4.77 ± 0.50	4.82 ± 0.53	9.59	1.01	1.55 ± 0.17	5.83 ± 0.40	7.38	3.76	16.85
WT	3.79 ± 0.42	4.45 ± 0.47	8.24	1.17	2.24 ± 0.39	5.27 ± 0.48	7.51	2.35	15.75

T3 tocotrienols, *Toc* tocopherols. 2 samples with 2 determinations each

were selected for HPLC analysis to determine tocochromanol content and composition (Table 1).

Because HPPD catalyzes the first step in the pathway, we expected to see an overall increase in tocochromanol synthesis, which was not the case. Only a marginal increase was achieved and this was almost entirely due to a small rise in tocotrienol synthesis. There was no change in the α:γ ratio in the tocotrienol branch. In contrast, there was no change in the overall level of tocopherols, but the α:γ ratio increased significantly compared to control grains resulting in the amount of α-tocopherol increasing at the expense of γ-tocopherol and thereby increasing the vitamin E activity of the grains.

These data confirm that HPPD is not a rate-limiting enzyme in tocochromanol synthesis but that altering the flux disrupts the balance further along the pathway and changes the metabolic tipping points at pathway branches and bottlenecks. The first instance is the branch between the tocopherol and tocotrienol pathways, where almost all the additional flux appears to be diverted into the tocotrienol branch. The increased availability of HGA (the immediate product of HPPD) appears to influence the relative activities of HPT and HGGT, favoring the latter so that the additional flux is fed into tocotrienol synthesis. However, there is no compensatory depletion of tocopherol, which suggests that HPT is not a rate-limiting enzyme either. The second instance is the conversion between the γ and α isoforms of tocopherol (but not tocotrienol), where more flux is transferred to α-tocopherol in transgenic plants than controls. This suggests some form of positive feedback, in which the increased flux through the pathway influences the activity of γ-TMT. An interesting question is why this increased flux does not also affect the tocotrienol branch, where the α:γ ratio remains unchanged, since the same enzyme carries out the γ to α conversion in both branches. A possible explanation is that the effect is localized to enzyme complexes containing γ-TMT and HPT and/or MPBQ-MT, which are pathway specific, or that the phytyl side chain is an important factor in the enhanced γ-TMT activity.

Previous studies have shown that the overexpression of *PDS1* has either no impact on tocopherol levels, as in Arabidopsis (Tsegaye et al. 2002) and

tobacco (Falk et al. 2005), or only a marginal effect, as in canola (Raclaru et al. 2006), indicating that it is not rate limiting in these plants. Naqvi et al. (2011) reported a threefold increase in tocopherols in transgenic corn seeds by simultaneously expressing Arabidopsis HPPD and MPBQ-MT, and γ-tocopherol was the only vitamer detected. These data suggest that MPBQ-MT may be rate limiting, and that γ-TMT represents a significant bottleneck in a high-flux environment. This seems likely given the dramatic shift from γ to α-tocopherol in soybean plants simultaneously expressing γ-TMT and MPBQ-MT (Van Eenennaam et al. 2003). Yusuf and Sarin (2006) expressed Arabidopsis γ-TMT in *Brassica juncea* plants and found a correlation between α-tocopherol levels and high γ-TMT expression, with the highest expressers producing six times as much α-tocopherol as wild type seeds (62.29 ng/mg).

Our study has shown that relatively simple alterations to a metabolic pathway can have complex effects by influencing downstream branch points and bottlenecks. Even though HPPD does not appear to be rate limiting it is still an interesting target for vitamin E engineering in rice because it has an indirect effect on the α-tocopherol content and therefore increases vitamin E activity significantly even when total tocochromanol levels increase only marginally. The biofortification of crops such as rice in this manner will offer a sustainable alternative to vitamin E supplementation, providing immense benefits to human health (Ajjawi and Shintani 2004; Zhu et al. 2007).

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ANNEX 3

Farré G, Bai C, Twyman RM, Capell T, Christou P, Zhu C (2011) Nutritious crops producing multiple carotenoids- a metabolic balancing act. Trends in Plant Science 16: 532-540.

ANNEX 3

Farré G, Bai C, Twyman RM, Capell T, Christou P, Zhu C (2011) Nutritious crops producing multiple carotenoids- a metabolic balancing act. Trends in Plant Science 16: 532-540.

Review

Nutritious crops producing multiple carotenoids – a metabolic balancing act

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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.

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].

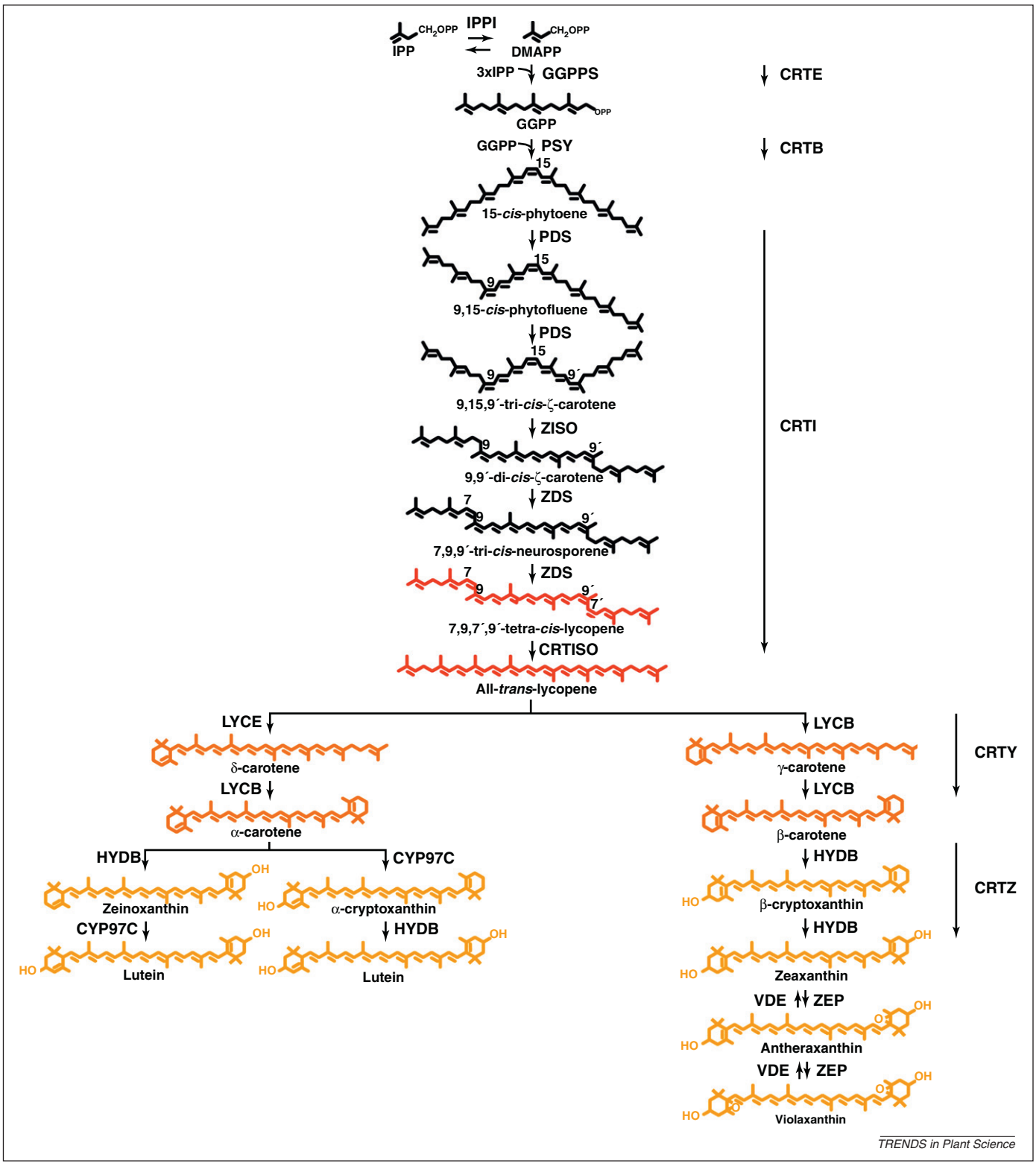

Figure 1. Carotenoid biosynthesis pathway in plants and equivalent steps in bacteria. CRTB, bacterial phytoene synthase; CRTE, bacterial geranylgeranyl diphosphate synthase; CRTI, bacterial phytoene desaturase/isomerase; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene cyclase; CRTZ, bacterial 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.

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ANNEX 4

Farré G, Twyman R, Zhu C, Capell T and Christou P. (2011) Nutritionally enhanced crops and food security: scientific achievements versus political expediency. Current Opinion in Biotechnology. Current Opinion in Biotechnology 22:245-251

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Nutritionally enhanced crops and food security: scientific achievements versus political expediency

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Genetic engineering (GE) is one of a raft of strategies that can be used to tackle malnutrition. Recent scientific advances have shown that multiple deficiencies can be tackled simultaneously using engineered plant varieties containing high levels of different minerals and organic nutrients. However, the impact of this progress is being diluted by the unwillingness of politicians to see beyond immediate popular support, favoring political expediency over controversial but potentially life-saving decisions based on rational scientific evidence.

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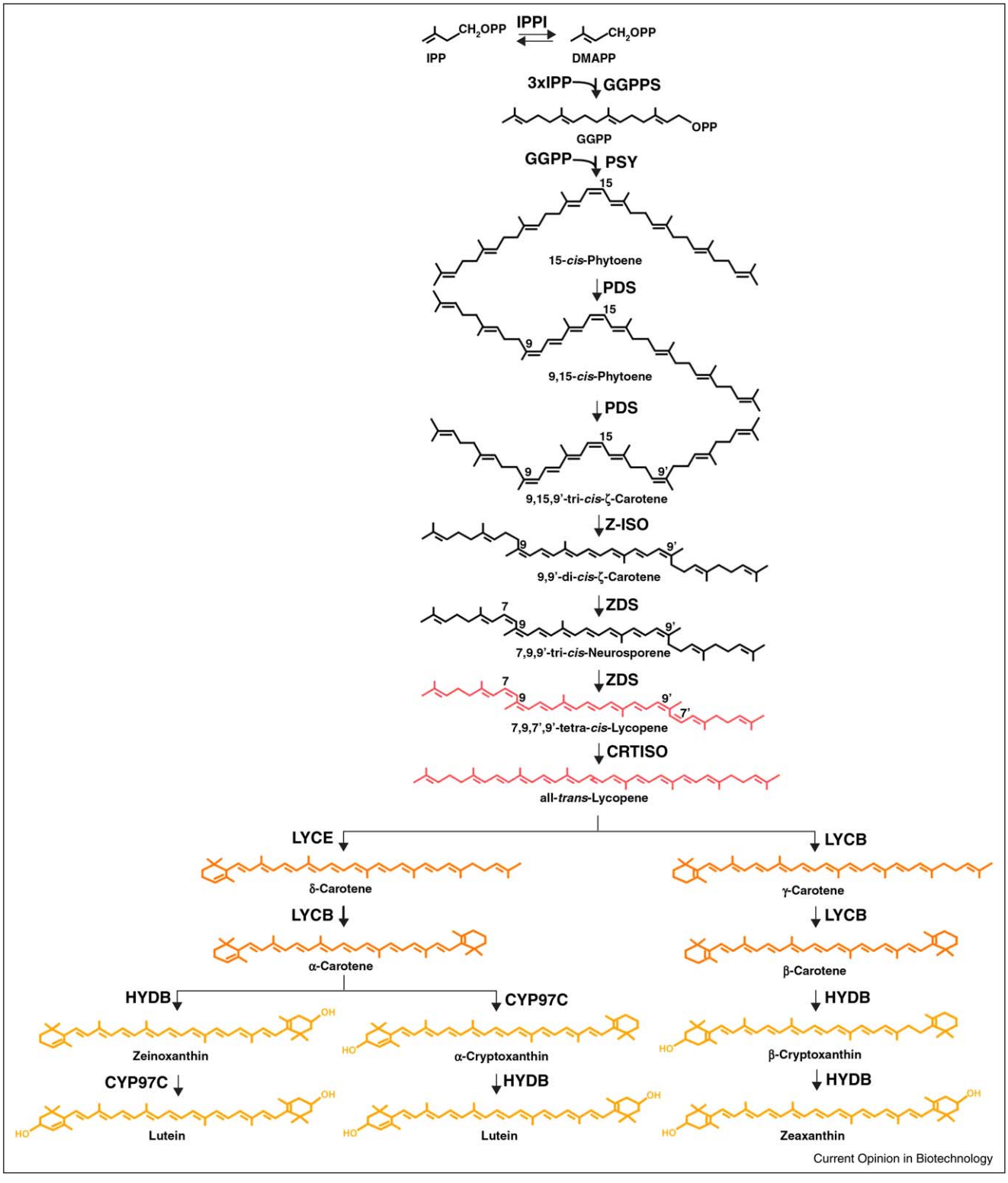
Introduction

Food security depends not only on the availability of food but also its nutritional quality. Unfortunately, the poorest people in the world generally rely on a monotonous staple diet, and since most plants are deficient in certain vitamins, minerals, and essential amino acids, a diet restricted to one major staple will tend to be nutritionally incomplete [1,2]. GE strategies have been used to tackle nutrient deficiency, with some remarkable advances in the past two years offering the prospect of nutritionally complete staple crops that could realistically address malnutrition on a global scale. Unfortunately, it is highly unlikely that such crops will be adopted in the short-to-medium term because politicians in Europe (and developing countries in Europe's sphere of influence) often pander to hyperbolic arguments about perceived risks, while ignoring potential benefits. In this review we summarize some of the recent advances in the field and briefly discuss the political hurdles currently preventing the deployment of nutritionally enhanced crops, and how these might be overcome.

Nutritionally enhanced crops—recent achievements

Recent GE strategies to increase crop yields have been highly successful [3] but the most striking advances over the past two years have involved plants engineered to produce missing nutrients or increase the level of nutrients that are already synthesized. An important trend is the move away from plants engineered to produce single nutritional compounds towards those simultaneously engineered to produce multiple nutrients, a development made possible by the increasing use of multigene engineering [4]. Several recent reports have demonstrated how multigene metabolic engineering can increase the level of carotenoids in edible plant tissues, including the traditional target β-carotene (pro-vitamin A, whose absence in staple cereals is responsible for almost 500,000 cases of preventable blindness every year [5]) and other carotenoids with specific functions in the human body or generally beneficial antioxidant properties. A combinatorial nuclear transformation method has been developed that allows the carotenoid synthesis pathway in corn to be dissected, and allows the production of diverse populations of transgenic plants containing different carotenoid profiles [6••]. The system as originally reported involved the transformation of a white corn variety lacking endosperm carotenoids with five genes from the carotenoid pathway (Figure 1) each under the control of a different endosperm-specific promoter. The population of transgenic plants recovered in this approach contained random combinations of transgenes, thus each unique combination had a different metabolic potential and produced a distinct carotenoid profile. Normally, the random nature of transgene integration is considered disadvantageous because hundreds of lines may need to be screened to identify one with the correct genotype and phenotype. However, random transgene integration is an advantage in this new platform because it increases the diversity of the population, resulting in plants with high levels of carotenoids such as β-carotene, lutein, zeaxanthin, lycopene, and astaxanthin, alone or in combination. Recently, it was demonstrated that the engineered carotenoid pathway could be introgressed from a transgenic line with a high LYCB:LYCE (lycopene β-cyclase to lycopene ε-cyclase) ratio (thus favoring the β-carotene branch) into the genetic background of a wild-type yellow-endosperm corn variety also with a high

Figure 1



Carotenoid biosynthesis in corn endosperm. 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; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene hydroxylase.

LYCB:LYCE ratio, resulting in synergistic enhancement of the metabolic bias and creating hybrid lines producing unprecedented levels of zeaxanthin (56 μg/g dry weight) [7•]. This novel strategy for combining GE and conventional breeding allows the development of 'designer' hybrid lines with specific carotenoid profiles, and is equally applicable to any staple crop where nutritional improvement would be beneficial.

Diverse carotenoid profiles have also been reported in canola seeds from plants transformed with up to seven carotenogenic transgenes [8•]. In this study, the authors aimed for seven-gene transformation and obtained their diversity as a byproduct, but the advantages are the same as those demonstrated in the combinatorial corn transformation platform. Most of the canola plants contained all seven genes and the total carotenoid content increased 30-fold, including a spectrum of novel ketocarotenoids that are not usually found in canola. Carotenoid levels can also be enhanced by increasing storage capacity, as seen in the cauliflower *Or* (orange) mutation that induces chromoplast differentiation and facilitates the hyperaccumulation of β-carotene resulting in a dark orange inflorescence [9]. Expression of an *Or* transgene in potato tubers resulted in a 6-fold increase in carotenoid levels by increasing the capacity for carotenoid storage [10•].

There has also been recent progress with other vitamins, such as the expression of a chicken GTP cyclohydrolase I gene in lettuce, which increased folate levels by nearly 9-fold [11], the co-expression of Arabidopsis ρ-hydroxyphenylpyruvate dioxygenase and 2-methyl-6-phytylplastoquinol methyltransferase in corn, which increased γ-tocopherol levels by 3-fold at the expense of other, less potent tocopherols [12], and the doubling of ascorbate levels in tobacco by encouraging recycling through the expression of phosphomannomutase or GDP-D-mannose pyrophosphorylase [13,14]. Whereas the enhancement of individual nutrients provides proof of principle, progress towards addressing micronutrient deficiencies in the real world will only be made once it is possible to target different nutrients at the same time. In this context, transgenic corn plants simultaneously enhanced for carotenes, folate and ascorbate provide the first example of a nutritionally enhanced crop targeting three entirely different metabolic pathways, going some way towards the goal of nutritionally complete staple crops [15••]. This was achieved by transferring four genes into the white maize variety described above, resulting in 407 times the normal level of β-carotene (57 μg/g dry weight), 6.1 times the normal level of ascorbate (106.94 μg/g dry weight) and twice the normal amount of folate (200 μg/g dry weight).

Whereas metabolic engineering can increase the levels of organic nutrients, minerals must instead be sequestered from the environment [16]. One notable recent report describes the hyperaccumulation of iron in rice plants transformed with two genes, encoding nicotianamine synthase (required for iron transport through the vascular system) and ferritin (which increases the capacity for iron storage) [17•]. Calcium levels in carrots are doubled when the transporter sCAX1 is expressed in the taproots, and feeding studies have shown that the extra calcium is bioavailable when carrots are fed to mice [18]. Similarly, calcium levels increased in lettuce expressing the same transporter, without detectable changes to the organoleptic properties of lettuce leaves [19].

Recent attempts to enhance the levels of essential amino acids and very long chain polyunsaturated fatty acids have also been successful, for example, the expression of an RNA interference construct to inhibit the key enzyme lysine-ketoglutarate reductase/saccharophine dehydrogenase (LKR/SDH) in order to increase lysine levels in corn [20], and the expression of a liverwort Δ6-desaturase, Δ6-elongase, and Δ5-desaturase to triple arachidonic acid levels and double eicosapentaenoic acid levels in transgenic tobacco plants [21]. Cheng *et al.* [22•] also produced high levels of eicosapentaenoic acid in canola.

Benefits and risks of deployment

Can nutritionally enhanced GE crops really improve food security?

Scientific advances are occasionally oversold in the pursuit of funding, patents or industry investment [23], so it is fair to ask whether GE can realistically improve food security or whether the claims are exaggerated. It is clear that the world produces enough food for its current population, but poverty and poor health prevent access to adequate nourishment [24,25]. These issues disproportionately affect the poorest, notably subsistence farmers in developing countries, often driving them to cities thus adding to the growing problem of urban poverty and hunger [26]. Any long-term strategy to address food insecurity in the developing world must therefore tackle the underlying problem of poverty and poor health by increasing the level of rural employment-based income through increased agricultural productivity [25,26]. The production of crops with higher nutritional value would add to the yield improvements made possible by GE and would mean that a smaller proportion of each farmer's output would be needed for subsistence and more could be sold at market, and also there would be a lower burden of disease caused by malnutrition [2]. GE crops provide the only route to nutritional completeness and could be a valuable component of a wider strategy including conventional breeding and other forms of agricultural development to improve food security now and in the future, in combination with better governance, education and healthcare, and socioeconomic policies to improve the welfare of the rural poor in developing countries [26].

Are there risks to health and the environment?

Although there is little doubt that GE technology can improve the nutritional value of food, these benefits are offset by perceived risks to health and the environment. One of the main challenges is that many non-scientists have a very poor grasp of risk and often attach unrealistic likelihoods to risks that are infinitesimal in nature. The global area of GE crops has steadily increased over the past 14 years [27] despite much public distrust and political controversy, particularly in Europe. There is no evidence for any detriment to public health or the environment in those areas that have embraced GE agriculture. Other technologies, which do have quantifiable risks, are accepted with far less protest. For example, great significance has been attached to the near imperceptible risks of 'horizontal gene transfer' from transgenic plants containing antibiotic resistance genes to pathogenic bacteria in the human gut, whereas the much more quantifiable risks of pesticide exposure is routinely ignored. Given that nature teems with antibiotic-resistant bacteria and we consume billions of them every day without ill effects yet there is plenty of evidence of environmental damage and health problems caused by pesticide use, why is there so much controversy about the use of genes that are already abundant and harmless? Why was it necessary to invest so much in the development of politically expedient technology to remove them? An interesting case study from the European Union (EU) that provides insight into the reasons behind the negative perception of GE technology is discussed in Box 1.

Political factors—the role of activists, the media, the public, and politicians

The political dimension to GE crops is best explained as a cycle of self-reinforced negative publicity (Figure 2). The media, politicians, and the public feed each other with (mis)information, becoming more risk averse and sensationalist with each cycle. The public are predominantly exposed to science through the mass media, which can be a good source of information on cutting edge technologies. However, depending on their financial and political influence, the media can also manipulate the public, causing scientific controversies that are rarely about science. Reporting biotechnology poses unique challenges because it is perceived as a controversial, evolving field, and it is difficult to produce quality news stories in an environment where science gets attention from activists and politicians. It is also clear that controversy itself attracts attention, which makes biotechnology a tempting opportunity for journalists good and bad. Consumer acceptance depends directly on how much trust is placed on the available risk–benefit information. Some of the public carefully weigh potential benefits more heavily than risks, while others form their biotechnology attitudes solely on media sound bites.

Box 1 Only bad news sells. . ..

Data from a field trial of Bt maize in Italy performed in 2005 as part of what was supposed to be a broad popular overview of GE in Italy were largely ignored. An analysis of the events surrounding this phenomenon leads to only one conclusion—the information was suppressed because it showed GE in a positive light [34]. The outcomes were to be presented at a public meeting in 2006, but the full field trial data were never released. When it became clear that the Italian Ministry of Agriculture was not going to publish the trial data, a small group of determined researchers held a press conference in 2007 [35] in response to several months of intensive campaigning by a coalition of over 30 groups claiming to represent over 11 million Italians opposed to GE foods. The Italy/Europe Free of GMO (GEO) coalition, which encompassed several Italian farming unions, consumer associations, and environmental groups, such as Greenpeace and the Worldwide Fund for Nature, had organized nearly 2000 separate anti-GE events and in a mock referendum collected three million signatures calling for a complete ban on all GE foods in Italy. Fourteen of Italy's 20 regions had already declared themselves GE-free even though the field trial results showed that under field conditions, MON810 maize expressing Bt toxin can help maintain yield levels that are 28–43% higher than those of isogenic non-GE varieties [34,35]. MON810 maize also outperformed conventional maize in terms of the levels of fumonisins, toxins that are produced by fungi able to infect plants through lesions caused by the maize borer. MON810 maize contained 60 or fewer parts per billion of fumonisin, whereas non-GE varieties contained over 6000 parts per billion, a level unsuitable for human consumption under Italian and European law. If it had been the MON810 varieties that contained high levels of fungal toxins, interest of the politicians, the media and the general public in the data would probably have been intense. But the response to these inconveniently positive field trial data was unreceptive at best.

Unable to find any direct evidence to prove that GE crops are inherently risky, protagonists often fall back on the claim that there are no long-term safety studies proving the absence of any harm to health from ingesting GE foods. It was in this vein that researchers at the University of Veterinary Medicine in Vienna launched a feeding study in mice to assess health over generations. The aim of the study was to assess the health effects of a variety of GE maize carrying two transgenes: *cry1Ab* from Bt and the *Agrobacterium* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase, which confers tolerance to glyphosate herbicides. Although the authors stated that ''no negative effects of GE maize varieties have been reported in peer-reviewed publications'', they wanted to assess health effects over several generations—something that has rarely been studied [36]. The study was not published, nor was it peer reviewed. Rather, the results were announced at a press conference in 2008 [37]. Anti-GE groups like Greenpeace jumped on this 'news' and issued a press release stating: ''Forget condoms – eat GE maize'' and demanded a worldwide recall of all GE foods and crops, stating: ''GE food appears to be acting as a birth control agent, potentially leading to infertility'' [38].

The study was soon criticized to be flawed and was discredited in the scientific community [36]. These errors make it unsuitable for risk assessment and/or regulatory purposes [39]. This study therefore served a political rather than a science-based agenda.

As well as deliberate manipulation, even a benign media can provide misinformation in an attempt to provide 'balanced' coverage (i.e. presenting alternative views no matter how irrational) or by oversimplification for the target audience so that viewpoints are polarized into

Box 2 Political issues and potential solutions

The political issues

- Politicians have more influence on the regulators than scientists.
- Public opinion drives political decisions more strongly than science.
- Public opinion is swayed by the media, which prefers sensationalist reporting.
- The media responds to sensationalist activist claims rather than rational scientific debate.
- Activists are not bound by the same rules of engagement as scientists.

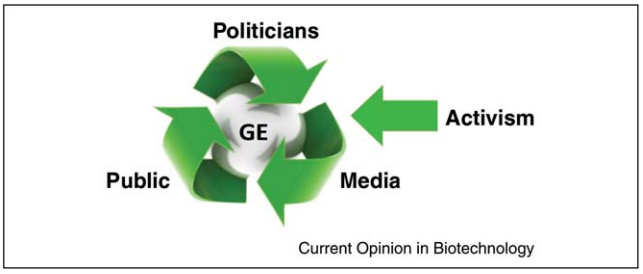
Potential solutions

- Regulators should be protected from undue political pressure.
- Politicians should weigh up scientific evidence properly, for example, through independent bodies that have executive authority.
- There should be more effort to educate the public about science.
- The media should have a duty to report accurately and should hold activists to the same standard of evidence as scientists.
- The EU should enforce its own regulations and support farmers wishing to grow GE crops to the same extent as those growing conventional/organic crops.
- The regulation of GE crops should be handled in the same way as drugs—once safety has been confirmed a license should be given and marketing should be authorized throughout the EU, without provisions to permit Member States to interfere for reasons of political expediency.

universal acceptance or rejection of a particular technology, with little room for reasoned discourse. Scientific data is often molded into a publication format that allows several different types of manipulation to take place, including suppression of positive data by omission (Box 1), publication of negative data without verification (and not publishing contrary evidence or retractions), or deliberately negative framing and labeling (e.g. 'killer corn'). This contrasts sharply with the description of novel recombinant cancer drugs as 'magic bullets' and 'wonder-drugs', and it is clear that medical stories are often presented positively. Where GE and medicine combine, as in transgenic plants producing pharmaceutical proteins, the media has been cautiously upbeat: 'Transgenic maize to cure HIV/AIDS' [28].

It is often said that GE crops could solve Africa's hunger and poverty, but that, through inadequate investment, external lobbying, and stringent regulations, farmers are being deprived of the technology and prevented from achieving agricultural success [29,30]. Many blame the European governments and non-governmental organizations for trying to foist their affluent values and precautionary sensibilities on Africa's poor. Politicians therefore play a key role in the eternal triangle because they listen to lobbies and respond with decisions that are handed down to regulators. If the public do not like GE they will

Figure 2



The eternal triangle of negative reinforcement. Media sensationalism, fed by propaganda from activists, misinforms the public about the risks of GE. The public vote for politicians to represent their views, so they pressure the regulators to treat GE more cautiously than the actual risk justifies. Activists and the media then use this as evidence to support their claims that GE is risky. There is no room for scientists or rational debate in this cycle.

lobby their politicians, who will in turn order the regulators to increase the regulatory burden. Even when the regulators consult independent advice, the politicians would rather fly in the face of this advice and impose 'solutions' to problems that do not exist, instead of evaluating the data properly. For this reason, the EU labors under such burdensome co-existence regulations in agriculture that is becomes virtually impossible for GE farmers to plant their crops without the risk of litigation [31]. In a misguided attempt to streamline the approval process for GM crops in Europe, the EU is considering allowing Member States to opt out of approvals, impose their own regulations, and create de facto GE-free zones in Europe, in direct contradiction of their aspirations to support innovation and develop a knowledge-based bioeconomy [32].

What can be done to fix the negative cycle?

Where negative perception has a unique source it should be possible to correct the perception by providing education at the source, but in the self-sustaining loop shown in Figure 2 there is no single point of intervention that will work. What is required is a concerted and coordinated campaign to influence the media, politicians, and public about scientific realities and, in particular, the correct approach to risk evaluation (Box 2). Organizations such as Sense About Science, which provide a voice for scientists and a platform to address scientific misinformation about GE in the media [33], are a step in the right direction but are often placed in the position of a reactionary rather that a proactive force in the face of sometimes overwhelming media-promulgated ignorance and sensationalism. The negative cycle can only be broken by education, the exposure of myths and lies and something that is almost unheard of: holding the media to the same publication standards as the scientists they often criticize.

Conclusions

GE strategies can be used to address micronutrient deficiency in both the developed and the developing world, as recent advances in the areas of metabolic engineering and mineral accumulation have demonstrated, particularly those studies simultaneously tackling multiple nutrients. However, this can only be achieved with the support of the public, media, and politicians. Converting the current negative reinforcement cycle into a positive one will only be possible when there is less irrational hatred of GE, and this can only come about with a strenuous effort to educate the public, politicians, and the media about the realistic nature of risks, and the balance between risks and benefits in all areas of life.

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References and recommended reading

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- • of special interest
- •• of outstanding interest

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The system presented in this paper could be applied to a range of metabolic pathways and provides a robust and versatile approach to studying metabolism as well as generating plants with specific metabolic phenotypes. In this instance, the authors were able to generate seven contrasting phenotypes accumulating high levels of different carotenoid compounds (as revealed by the different colored cobs) and also revealed that competition occurred between β-carotene hydroxylase and bacterial β-carotene ketolase for substrates in four sequential steps of the extended pathway.

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ANNEX 5

Li Q, **Farré G (joint first author)**, 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 *b*-carotene hydroxylase genes and their regulation during endosperm maturation. Transgenic Research 19:1053-1068.

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ORIGINAL PAPER

Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation

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Abstract In order to gain further insight into the partly-characterized carotenoid biosynthetic pathway in corn (*Zea mays* L.), we cloned cDNAs encoding the enzymes carotenoid isomerase (CRTISO) and β-carotene hydroxylase (BCH) using endosperm mRNA isolated from inbred line B73. For both enzymes, two distinct cDNAs were identified mapping to different chromosomes. The two *crtiso* cDNAs (*Zmcrtiso1* and *Zmcrtiso2*) mapped to unlinked genes each containing 12 introns, a feature conserved among all *crtiso* genes studied thus far. ZmCRTISO1 was able to convert tetra-*cis* prolycopene to all-*trans* lycopene but could not isomerize the 15-*cis* double bond of 9,15,9′-tri-*cis*-ζ-carotene. ZmCRTISO2 is inactivated by a premature termination codon in B73 corn, but importantly the mutation is absent in other corn cultivars and the active enzyme showed the same activity as ZmCRTISO1. The two *bch* cDNAs (*Zmbch1* and *Zmbch2*) mapped to unlinked genes each coding sequences containing five introns. ZmBCH1 was able to convert β-carotene into β-cryptoxanthin and zeaxanthin, but ZmBCH2 was able to form β-cryptoxanthin alone and had a lower overall activity than ZmBCH1. All four genes were expressed during endosperm development, with mRNA levels rising in line with carotenoid accumulation (especially zeaxanthin and lutein) until 25 DAP. Thereafter, expression declined for three of the genes, with only *Zmcrtiso2* mRNA levels maintained by 30 DAP. We discuss the impact of paralogs with different expression profiles and functions on the regulation of carotenoid synthesis in corn.

Keywords Corn · *Zea mays* L. Carotenoids · Carotenoid isomerase · β-Carotene hydroxylase · Gene family

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Introduction

Carotenoids are fat-soluble pigments synthesized by all plants and many microorganisms. In plants they are found on photosynthetic membranes where they participate in the light-harvesting reaction and protect

the photosynthetic apparatus from photo-oxidation (reviewed by Bramley 2002). However, they are also precursors for the synthesis of abscisic acid (Creelman and Zeevart 1984) and strigolactones (Gomez-Roldan et al. 2008; Umehara et al. 2008), and they are the source of yellow, orange and red pigmentation in some flowers and fruits (Tanaka et al. 2008). In animals, carotenoids provide multiple health benefits (reviewed in Fraser and Bramley 2004), prompting scientists to explore ways to improve carotenoid content and composition in staple crops (reviewed in Sandmann et al. 2006; Howitt and Pogson 2006; Zhu et al. 2007; Giuliano et al. 2008; Fraser et al. 2009; Zhu et al. 2009).

Plant carotenoid synthesis begins with the conversion of geranylgeranyl pyrophosphate into 15-*cis* phytoene by the enzyme phytoene synthase (PSY; Misawa et al. 1994; Fig. 1). A series of four desaturation reactions carried out by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. The product of the first

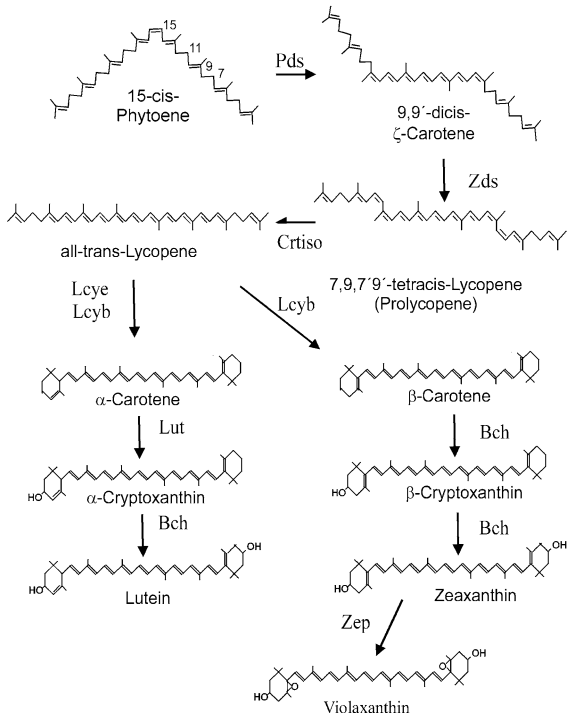

Fig. 1 Carotenoid biosynthetic pathway in corn endosperm. *Abbreviations*: Pds, phytoene desaturase; Zds, ζ-carotene desaturase; Crtiso, carotenoid isomerase; Lcyb, lycopene β-cyclase; Lcye, lycopene ε-cyclase; Bch, β-carotene hydroxylase; Lut, carotene ε-ring hydroxylase and Zep, zeaxanthin epoxidase

desaturase is 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized by light (and perhaps an unknown enzyme; Li et al. 2007) to yield 9,9′-di-*cis*-ζ-carotene, the substrate of ZDS (Breitenbach and Sandmann 2005). 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 (Isaacson et al. 2004; Breitenbach and Sandmann 2005). All-*trans* lycopene is then cyclized by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) to introduce ε- and β-ionone end groups and produce α- and β-carotene, respectively. The introduction of hydroxyl moieties into the cyclic end groups by β-carotene hydroxylase (BCH) and carotene ε-hydroxylase results in the formation of zeaxanthin from β-carotene and lutein from α-carotene (Sun et al. 1996; Bouvier et al. 1998; Tian et al. 2003). In some plant tissues, zeaxanthin can be epoxidized to violaxanthin by zeaxanthin epoxidase (ZEP) (reviewed by Cunningham and Gantt 1998).

The limited data concerning endogenous regulation of carotenogenic genes has made the precise engineering of crop plants to enhance carotenoid content and composition difficult (reviewed in Sandmann et al. 2006; Zhu et al. 2007; Fraser et al. 2009) despite recent progress in cereal crops, particularly corn (Harjes et al. 2008; Zhu et al. 2008; Aluru et al. 2008; Naqvi et al. 2009). The corn genome contains three paralogous *psy* genes (Li et al. 2008a, b) and PSY1 is the key rate-limiting enzyme in endosperm carotenoid biosynthesis (Buckner et al. 1996; Palaisa et al. 2003; Li et al. 2008a). The endosperm-specific expression of a corn *psy1* transgene in white corn, which lacks endogenous PSY1 activity, increased the total endosperm carotenoid content >50-fold (Zhu et al. 2008). Six different corn paralogs encoding β-carotene hydroxylase (BCH) were recently identified through bioinformatics analysis in corn (Vallabhaneni et al. 2009). Two *bch* genes were reported to encode β-carotene hydroxylase (Vallabhaneni et al. 2009); two paralogs were pseudogenes, while the remaining two paralog functions remain unknown (Vallabhaneni et al. 2009). In contrast, PDS, ZDS, LCYB and LCYE are encoded by single-copy genes in corn (Li et al. 1996; Matthews et al. 2003; Singh et al. 2003; Harjes et al. 2008), and *pds* and *zds* at least do not appear to regulate endosperm carotenoid accumulation since the corresponding transcript levels remain constant

during endosperm development (Li et al. 1996; Matthews et al. 2003). Here we report the isolation and characterization of corn *crtiso* and *bch* cDNAs, their developmental expression profiles and their functional characterization by complementation analysis in bacteria. The presence of small gene families for several carotenogenic genes in corn suggests that diverse regulatory strategies may be used to control the accumulation of carotenoids in endosperm tissue.

Materials and methods

Plant materials

Corn plants (*Zea mays* L.) representing lines B73, A632, EP42 (yellow corn) and M37W (white corn) 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 thereafter. Plants were self-pollinated to obtain seeds. Mature leaf and endosperm tissue were frozen rapidly in liquid nitrogen and stored at −80°C. Endosperm tissues were dissected at five developmental stages [10, 15, 20, 25 and 30 days after pollination (DAP)].

Nucleic acid isolation and cDNA synthesis

Genomic DNA was extracted from 5 g of leaf tissue as described by Sambrook et al. (1989). Total RNA was isolated using the RNeasy® Plant Mini Kit (QIAGEN, Valencia, CA, USA) and DNA was removed with DNase I (RNase-Free DNase Set, QIAGEN, Valencia, CA, USA). Total RNA was quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA), and 2 µg total RNA was used as template for first strand cDNA synthesis with Ominiscript Reverse Transcriptase in a 20 µl total reaction volume following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA).

Cloning and sequencing the corn *crtiso* and *bch* cDNAs

A partial corn *crtiso* cDNA was isolated by PCR using 1 µl cDNA prepared as above from 25 DAP corn endosperm tissue. The 50-µl reaction was carried out using the GoTaq® DNA Polymerase Kit (Promega, Madison, WI, USA) and synthetic degenerate oligonucleotide primers matching two conserved regions of the homologous *Arabidopsis* and tomato carotenoid isomerase (CRTISO) enzymes: CWKIFNS (forward primer 5′-TGY TGG AAR ATH TTY AAY IS-3′) and TYGPMPR (reverse primer 5′-CKI GGC ATI GGI CCR TAI GT-3′). The samples were heated to 95°C for 3 min, followed by 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 90 s. After the last amplification cycle, samples were incubated at 72°C for 10 min. The *crtiso* product was purified from a 1.0% w/v agarose gel using the Geneclean® II Kit (BIO® 101 Systems, Solon, OH, USA) and cloned in the PCR® II TOPO® vector (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) for sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were used to query EST databases and matches were used to design primers for full-length cDNA cloning. The EST sequences (DR812825 and EE176013) from B73 were found with high identities (100 and 98.7% in the overlapping part, respectively) to the 5′ and 3′-end of the partial *Zmcrtiso* sequence. *Zmcrtiso* was isolated using forward primer 5′-CAT GCC GCC GCT CGC CGC GCG CCT C-3′ based on 5′ EST sequence (accession number DR812825) and reverse primer 5′-CAG AAA GTT GAA GGG TAT CTC AA-3′ based on 3′ EST sequence (accession number EE176013). Utilizing the published maize genomic DNA, bacterial artificial chromosome (BAC) and expressed sequence tags (EST) sequences (MaizeGDB, http://www.maizegdb.org/), we identified a second and distinct *crtiso* gene (designated *Zmcrtiso2*) which has higher identity (86.1%) at the nucleotide level with the *crtiso* gene we report in this manuscript (designated *Zmcrtiso1*). The primer combination designed to amplify the full CRTISO protein coding *Zmcrtiso2* mRNA sequence was designed based on the EST sequences in the MaizeGDB database. The primers were: forward, 5′-CTC CCG AGT CCC AAT CCA AAC GGC TTC ACT C-3′ based on EST sequence with GenBank accession number EE045563; reverse, 5′-AAT TAC ACT GTT TGG CAT ACC ATG TAA CTT GT-3′ based on EST sequence with GenBank accession number FL449103. PCR, cloning and sequencing was carried out using the components

described above with initial heating to 95°C for 3 min followed by 35 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 3 min, followed by a final incubation at 72°C for 10 min.

Nested PCR was used to amplify the corn β-carotene hydroxylase 1 (*Zmbch1*) cDNA from endosperm tissue, based on the putative *Zmbch1* cDNA sequences already deposited in GenBank from an unknown corn cultivar (accession number AY844956). The PCR was carried out in a 50-µl reaction volume using the components described above, but substituting forward primer 5′-CAT GGC CGC CGG TCT GTC CGG CGC CGC GAT-3′ (BCH1F1) and reverse primer 5′-TGA GCT GGT GGT TCA TAA CAT GTC TCT AC-3′ (BCH1R1) in the first reaction and forward primer 5′-AGA ATT CCA TGG CCG CCG GTC TGT CCG-3′ (BCH1F2) (the terminal *Eco*RI restriction site and start codon are underlined) and 5′-AGG ATC CGG ACG AAT CCA TCA GAT GGT C-3′ (BCH1R2) (the terminal *Bam*HI restriction site is underlined) in the second reaction. The first reaction mix included 5% (v/v) dimethyl sulfoxide (DMSO), and 5 µl of the product was used to initiate the second reaction. Cloning and sequencing were carried out as above.

A similar nested PCR strategy was used to isolate the full-length *Zmbch2* cDNA using primer combinations based on the corresponding GenBank sequence (accession number AY844956). For this cDNA, the forward primers were 5′-GGA GAC TCG AGG CCA CTC TGC CTT-3′ (BCH2F1) and 5′-GAA TTC CAT GGC CGC CGC GAT GAC CAG-3′ (BCH2F2) (terminal *Eco*RI restriction site and start codon are underlined) and the reverse primers were 5′-GCT AGA ACT CAT TTG GCA CAC TCT G-3′ (BCH2R1) and 5′-GGA TCC TAG AAC TCA TTT GGC ACA CTC-3′ (BCH2R2) (terminal *Bam*HI restriction site is underlined). The PCR was carried out as above without DMSO in the reaction mix, and the products were cloned and sequenced as described.

Bioinformatic analysis

The Maize Genetics and Genomic Database (MaizeGDB, http://www.maizegdb.org/), the GRAMENE database (http://www.gramene.org/) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were searched for homologous sequences using BLAST, and multiple sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/clustalw/). Protein sequences were screened for chloroplast signal peptides using the ChloroP 1.1 Server at http://www.cbs.dtu.dk/services/ChloroP/ (Emanuelsson et al. 1999).

Construction of CRTISO and BCH expression vectors

Gene-specific primers, with terminal *Eco*RI and *Bam*HI restriction sites (forward primer 5′-CGA ATT CCA TGC CGC CGC TCG CCG CGC GCC TC-3′ and reverse primer 5′-GGA TCC CTA TGC AAG TGT TCT CAA CCA TCT GAG TAG-3′) were used to amplify the full-length *Zmcrtiso1* coding sequence, which was then inserted into the same sites in vector pUC8 to create pUC8-Zmcrtiso1. A similar strategy was used to create pUC8-Zmcrtiso2, with the primers in this case designed to incorporate terminal *Eco*RI and *Hin*dIII restriction sites (forward primer 5′-GAA TTC CAT GTT CGG CTT CTC CGA CAA G-3′ and reverse primer 5′-AAG CTT CTA TGC AAG TGT TCT CAG CCA T-3′). The stop codon in *Zmcrtiso2* was changed to serine using a recombinant PCR strategy (Higuchi 1990) with forward P1 primer 5′-GAA TTC CAT GCC GCC GCT CGC CGC GCG CCT CT-3′ (initiation codon underlined) incorporating an *Eco*RI site, reverse P2 primer 5′-CGC GAC CGC CAC CGC CGC CGC CTT CTC CGA-3′ (modified stop codon underlined), forward P3 primer 5′-GGC GGG TTC AGG AGA GGC GCG CTG GCA TCG-3′ (modified stop codon underlined) and reverse P4 primer 5′-AAG CTT CTA TGC AAG TGT TCT CAG CCA TCT GAG-3′ incorporating a *Hin*dIII site (stop codon underlined). The P1/P2 and P3/P4 reactions were performed separately by heating to 95°C for 3 min followed by 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 90 s, and a 10-min incubation at 72°C. The products were recovered and used as templates for a subsequent PCR with primers P1 and P4, wherein the samples were heated to 95°C for 3 min, followed by one cycle of 94°C for 45 s, 60°C for 45, 72°C for 10 min and 94°C for 3 min, then 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 2 min, followed by a final 10-min incubation at 72°C. The mutagenized *Zmcrtiso2* cDNA was cloned and sequenced as above to generate pUC8-Zmcrtiso2C.

pUC8-Zmbch1 and pUC8-Zmbch2 plasmids were constructed by digesting pCR-Zmbch1 and

pCR-Zmbch2 with *Eco*RI and *Bam*HI simultaneously, and subcloned as in frame fusion into a pUC8 vector also digested with the same restriction enzymes. They were then utilized for functional analysis of the corresponding genes.

Functional characterization of *crtiso* and *bch* cDNAs

Different carotenoid backgrounds were established in *Escherichia coli* for functional complementation experiments with the corn *crtiso* and *bch* cDNAs using a selection of plasmids containing carotenogenic genes. Plasmid pACCRT-EBP contained genes for geranylgeranyl pyrophosphate synthase (CRTE), phytoene synthase (CRTB) and a *pds* type phytoene desaturase from *Synechococcus*, allowing the formation of ζ-carotene (Linden et al. 1993). Plasmid pBBR1MCS2-zds contained a ζ-carotene desaturase gene (Breitenbach et al. 2001a). Plasmid pRKcrtY contained a lycopene cyclase gene (Schnurr et al. 1996). Plasmid pACCAR16ΔcrtX contained all the genes required to synthesize β-carotene (Misawa et al. 1990).

DNA and RNA blots

Leaf genomic DNA (20 µg) was digested separately with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I. The resulting fragments were separated by electrophoresis on a 0.8% (w/v) agarose gel and blotted onto a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were fixed by UV cross-linking. The transferred DNA fragments were hybridized with appropriate digoxigenin-labeled probes at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, 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[3.3.1.1^{3,7}] 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 primer combinations used to generate *Zmcrtiso* probe (394 bp) were: 5′-CAT GCC GCC GCT CGC CGC GCG CCT C-3′ and 5′-CTT GTC GGA GAA GCC GAA CAT GAC A-3′ using *Zmcrtiso1* cDNA as a template. This *Zmcrtiso1* DNA probe had 90.1% identity with *Zmcrtiso2*. The primer combinations for 3′ UTR specific probes were: 5′-GGA ATA CGA ATG GGT GTA CAG GTT-3′ and 5′-TGA AGG GTA TCT CAA AAC AGA ACT-3′ for *Zmcrtiso1* (212 bp), 5′-GAG GCT GGG CTA GCA GCA TGC GGT-3′ and 5′-AAT TAC ACT GTT TGG CAT ACC ATG-3′ for *Zmcrtiso2* (216 bp), 5′-TGG AAA AGG AGC TCG CGC GAA TCG-3′ and 5′-TGA GCT GGT GGT TCA TAA CAT GTC T-3′ for *Zmbch1* (377 bp), and 5′-GCT TGT TAG CAG TCC GGT GAG TGA A-3′ and 5′-GAA AGG AAG ATG GCG ATA GAT GTA-3′ for *Zmbch2* (251 bp).

Total RNA (30 µg) was fractionated on a denaturing 1.2% (w/v) agarose gel containing formaldehyde prior to blotting. The membrane was probed with digoxigenin-labeled partial cDNAs prepared as above using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), with hybridization carried out at 50°C overnight using DIG Easy Hyb. Washing and immunological detection and CSPD chemiluminescence were carried out as described above.

Carotenoid extraction and quantification

Carotenoids from freeze-dried *E. coli* co-transformants were extracted in darkness with acetone at 60°C for 20 min, partitioned into 10% ether in petrol (bp. 40–60°C) and analyzed by HPLC on a non-endcapped polymeric 3 µm C_{30} column (YMC Wilmington NC, USA) according to Sander et al. (1994). The mobile phase was methanol/methyl-tert-butyl ether/water (56:40:4, v/v/v) for 30 min followed by a change to 26:70:4 (v/v/v) at a flow rate of 1 ml/min (Breitenbach and Sandmann 2005). Spectra were recorded on-line with a Kontron 440 diode array detector. The sources of reference carotene isomers are described in previous publications (Breitenbach and Sandmann 2005; Breitenbach et al. 2001b). Carotenoids from freeze dried ground endosperm tissue were extracted in a similar manner, although the acetone was replaced with tetrahydrofuran/methanol (50:50, v/v) followed by acetone re-extraction. The HPLC system used for the corn carotenoids was

a C_{18} Vydac 218TP54 column with 1% water in methanol as the mobile phase.

Quantitative real time PCR

Real-time PCR was performed on a BIO-RAD CFX96^{TM} system using a 25-µl mixture containing 10 ng of synthesized cDNA, 1× iQ SYBR green supermix (BIO-RAD) and 0.2 mM forward and reverse primers for the target genes and the internal glyceraldehyde-3-phosphate dehydrogenase control (*Zmgapdh*) (Iskandar et al. 2004) as listed in Table 1. 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°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 CFX96^{TM} system software. Values represent the mean of three real time PCR replicates ±SD.

Results

Cloning and characterization of the corn *crtiso* genes

Two different cDNAs (*Zmcrtiso1* and *Zmcrtiso2*), encoding full-length carotenoid isomerase (CRTISO) enzymes, were amplified from 25-DAP corn endosperm mRNA (line B73) by RT-PCR. The sequences were deposited in GenBank with accession numbers FJ603466 and FJ765413, respectively. The full-length *Zmcrtiso1* cDNA encoded a 587-amino-acid protein with a molecular weight of 63.7 kDa, a pI of 8.24, and a putative 43-residue transit peptide for chloroplast targeting (Fig. 2). The ZmCRTISO1 amino acid sequences showed 80.7% similarity and 74.6% identity to *Arabidopsis* CRTISO (Park et al. 2002), 78.7% similarity and 71.5% identity to tomato CRTISO (Isaacson et al. 2002), and 73.2% similarity and 58.8% identity to a cyanobacterial CRTISO (Breitenbach et al. 2001a; Masamoto et al. 2001; Fig. 2).

The B73 *Zmcrtiso2* cDNA contained a C-to-A transversion at position +143 (the putative translation start codon of the *Zmcrtiso2* cDNA is referred to as +1) which was not present in homologous EST and cDNA sequences from other cultivars (accession nos. FL133727 and EU957482). The resulting nonsense mutation prematurely terminates protein synthesis and yields a truncated 127-amino-acid protein (Fig. 2). The presence of the mutation specifically in B73 corn genomic DNA was confirmed by PCR using the *Zmcrtiso2* gene-specific primers 5′-CAC CGT CCT CCG CCC TCC ACT GCA ACT AGC-3′ and 5′-CAG CCG CAG AGC AGA GTA AAC TGT AGA GTA-3′, based on the sequence of a *Zmcrtiso2* BAC clone (accession no. AC183901). Eight different clones were used to confirm the presence of the mutation specifically in B73 genomic DNA. Importantly, the mutation was not present in genomic DNA from M37W, A632 and EP42 corn (GenBank accession nos. GQ366381, GQ366382 and GQ366383, respectively; Fig. 2b). The truncated ZmCRTISO2 amino acid sequence showed 96.3% identity and 98.1% similarity to the corresponding region of ZmCRTISO1 (Fig. 2a).

The cDNA sequences were used to screen on-line corn genomic resources in order to identify the corresponding genes. *Zmcrtiso1* was localized to

Table 1 Primer oligonucleotide sequences of corn carotenoid isomerise (*Zmcrtiso*) and β-carotene hydroxylase (*Zmbch*), and glyceraldehyde-3-phosphate dehydrogenase (*Zmgapdh*) genes for quantitative real-time PCR analysis

Gene	Forward primer	Reverse primer
Zmcrtiso1	5′-GAATGGAGGGAGTGGGAAATG-3′	5′-TGAAGGGTATCTCAAAACAGAACT-3′
Zmcrtiso2	5′-TCAAATCGAGGCTGGGCTAG-3′	5′-CACCGTATCGTGTCAAGCAC-3′
Zmbch1	5′-CCACGACCAGAACCTCCAGA-3′	5′-CATGGCACCAGACATCTCCA-3′
Zmbch2	5′-GCGTCCAGTTGTATGCGTTGT-3′	5′-CATCTATCGCCATCTTCCTTT-3′
Zmgapdh	5′-CTTCGGCATTGTTGAGGGTT-3′	5′-TCCAGTCCTTGGCTGAGGGT-3′

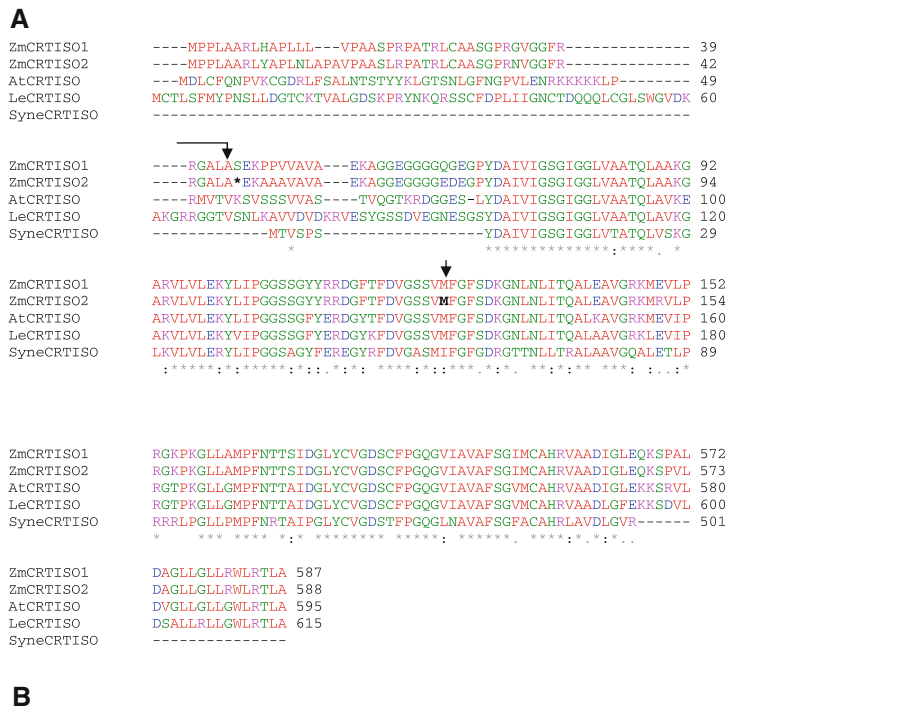

Fig. 2 **a** Alignment of deduced amino acid sequences of the N- and C-terminal region encoded by the *crtiso* genes. In B73 ZmCRTISO2, the 48th amino acid is interrupted by a stop codon as marked with the *asterisk*. Consequently, the predicted protein of *Zmcrtiso2* starts with the second methionine (M) marked in *black* as indicated by the *arrow*; it is truncated by 127 amino acid residues as compared to its M37W, A632 and EP42 paralogs. The putative ZmCRTISO1 protein possesses a 43-residue transit peptide for chloroplast targeting as marked by the *arrow*. ZmCRTISO1 (*Zea mays* L., accession no. FJ603 466); ZmCRTISO2 (*Zea mays* L., accession no. FJ765413); AtCRTISO (*Arabidopsis thaliana* L., accession no. AC01 1001); LeCRTISO (*Lycopersicon esculentum* cv. M82, accession no. AF416727) and SyneCRTISO (*Synechocystis* sp. PCC 6803, gene *sll0033*). **b** Comparison of the nucleotide sequence regions around position 143 (the putative translation start codon of the *Zmcrtiso2* cDNA is referred to as +1) of *Zmcrtiso2* in different corn cultivars

chromosome 4 (accession no. AC205563) and *Zmcrtiso2* to chromosome 2 (accession no. AC183901). Eleven introns were found in the available *Zmcrtiso1* gene sequence with the coding region located 383–3,826 bp downstream from the transcriptional start site. Since the BAC clone containing *Zmcrtiso1* did not overlap the first 392 bp of the cDNA, a pair of primers matching this sequence (5′-CAT GCC GCC GCT CGC CGC GCG CCT CCA-3′ and 5′-CAA CTG CTT CCA ATG CTT GTG TGA TCA-3′) was used to amplify the missing genomic DNA fragment. The 857-bp product was cloned and sequenced (accession no. FJ838766). The comparison of the amplified genomic DNA sequence, *Zmcrtiso1* cDNA and the BAC clone (accession no. AC205563) showed that the *Zmcrtiso1* gene contains 12 introns. The *Zmcrtiso2* BAC clone (accession no. AC183901) spanned the entire gene, which also contained 12 introns.

Cloning and characterization of the corn *bch* genes

The sequence of the *Zmbch1* cDNA from B73 endosperm predicted a 309-amino-acid protein with a molecular weight of 33.6 kDa, a pI of 10.92 and a 68-amino-acid transit peptide, suggesting the 241-amino-acid mature protein has a molecular weight of 26.5 kDa and a pI of 9.10 (accession no. GQ131287). The cloned *Zmbch1* cDNA sequence has 98.9% identity at DNA

level with β-carotene hydroxylase 4 cDNA (Vallabhaneni et al. 2009; accession number AY844 956). The deduced amino acid sequence of ZmBCH1 shared 99.4% identity with hydroxylase 4 as reported by Vallabhaneni et al. 2009. These authors do not report the specific corn cultivar used to identify hydroxylase 4 (Vallabhaneni et al. 2009) consequently one cannot determine if our cloned gene (*Zmbch1*) is different from hydroxylase 4 because of a difference in cultivar used to clone the genes or different isozymes in B73. The sequence of the cloned *Zmbch2* cDNA is the same as β-carotene hydroxylase 3 (Vallabhaneni et al. 2009; accession number AY844958). *Zmbch2* encodes a predicted 319-amino-acid protein with a molecular weight of 34.6 kDa, a pI of 8.82 and a 69-residue transit peptide, yielding a mature 250-amino-acid protein with a molecular weight of 27.5 kDa and a pI of 6.67. The predicted amino acid sequences of ZmBCH1 and ZmBCH2 are aligned in Fig. 3. The N-terminal sequences are highly conserved, despite the presence of four small gaps in ZmBCH2, whereas the C-terminal sequences differ considerably, not least because of the presence of 29 additional residues in the ZmBCH2 sequence (Fig. 3). The screening of corn genomic resources allowed us to localize *Zmbch1* to chromosome 2 (accession nos. AC196442) and *Zmbch2* to chromosome 10 (accession no. AC194430). A comparison of the cDNAs and corresponding BAC clones showed that both genes were present in their entirety in the BAC clones and both coding sequences contained five introns. The full-length amino acid sequences are 76.6% identical, both containing four predicted transmembrane helices and one highly conserved region described by Sun et al. (1996) as "Motif 1". The most striking features were the conserved histidine motifs, often found in iron-containing monooxygenases and fatty acid desaturases including β-carotene hydroxylases (Bouvier et al. 1998), which may act as iron-binding ligands (Shanklin et al. 1994). The four regions HX_4H, HX_2HH, HX_4H and HX_2HH are underlined in Fig. 3.

Crtiso and *bch* gene copy numbers

Most plants studied thus far appear to contain a single gene encoding CRTISO (Park et al. 2002; Isaacson et al. 2002) so the presence of a small gene family in corn warranted further investigation. Southern blot analysis was carried out by digesting the corn genomic DNA with five different restriction enzymes, followed by hybridization under high stringency with a 394-bp *Zmcrtiso1* probe lacking any of the enzyme sites. This *Zmcrtiso1* DNA probe had 90.1% identity with *Zmcrtiso2*. At least three hybridizing bands were present in each lane under high stringency conditions (Fig. 4a), suggesting that a further *crtiso* gene was present in addition to the two we had already cloned. Based on *Zmcrtiso1* and *Zmcrtiso2* cDNA and genomic DNA sequences, two gene specific probes without intron DNA fragments were prepared as described in

Fig. 3 Alignment of deduced amino acid sequences of ZmBCH1 (*Zea mays* L., accession no. GQ131287) and ZmBCH2 (*Zea mays* L., accession no. AY844957). Four predicted transmembrance (TM) helices, one highly conserved region described by Sun et al. (1996) as "Motif 1" and the four regions HX_4H, HX_2HH, HX_4H and HX_2HH are *underlined*

Materials and Methods. These two probes have just 43.3% identity at the nucleotide levels. There are two adjacent *Xba*I sites (791 bp) which cover *Zmcrtiso1* probe DNA fragment in *Zmcrtiso1* genomic DNA (accession number: AC205563). Thus, one 791 bp hybridizing band was detected in the *Xba*I digested genomic DNA for *Zmcrtiso1* gene specific DNA hybridization (Fig. 4b). There are two adjacent *Eco*RV sites (5,330 bp), two adjacent *Hin*dIII sites (6,202 bp) and two adjacent *Xba*I sites (867 bp), which cover *Zmcrtiso2* probe DNA fragment in *Zmcrtiso2* genomic DNA (accession number: AC183901). However, at least two hybridization bands were detected for *Eco*RV, *Hin*dIII and *Xba*I digested genomic DNA for *Zmcrtiso2* gene specific DNA hybridization (Fig. 4c). Thus we demonstrated that *Zmcrtiso1* probably exists as a single copy whereas there are two copies of *Zmcrtiso2* (Fig. 4b, c). A similar experiment using gene specific *Zmbch1* and *Zmbch2* probes detected at least two bands per lane for *Zmbch1* and at least three

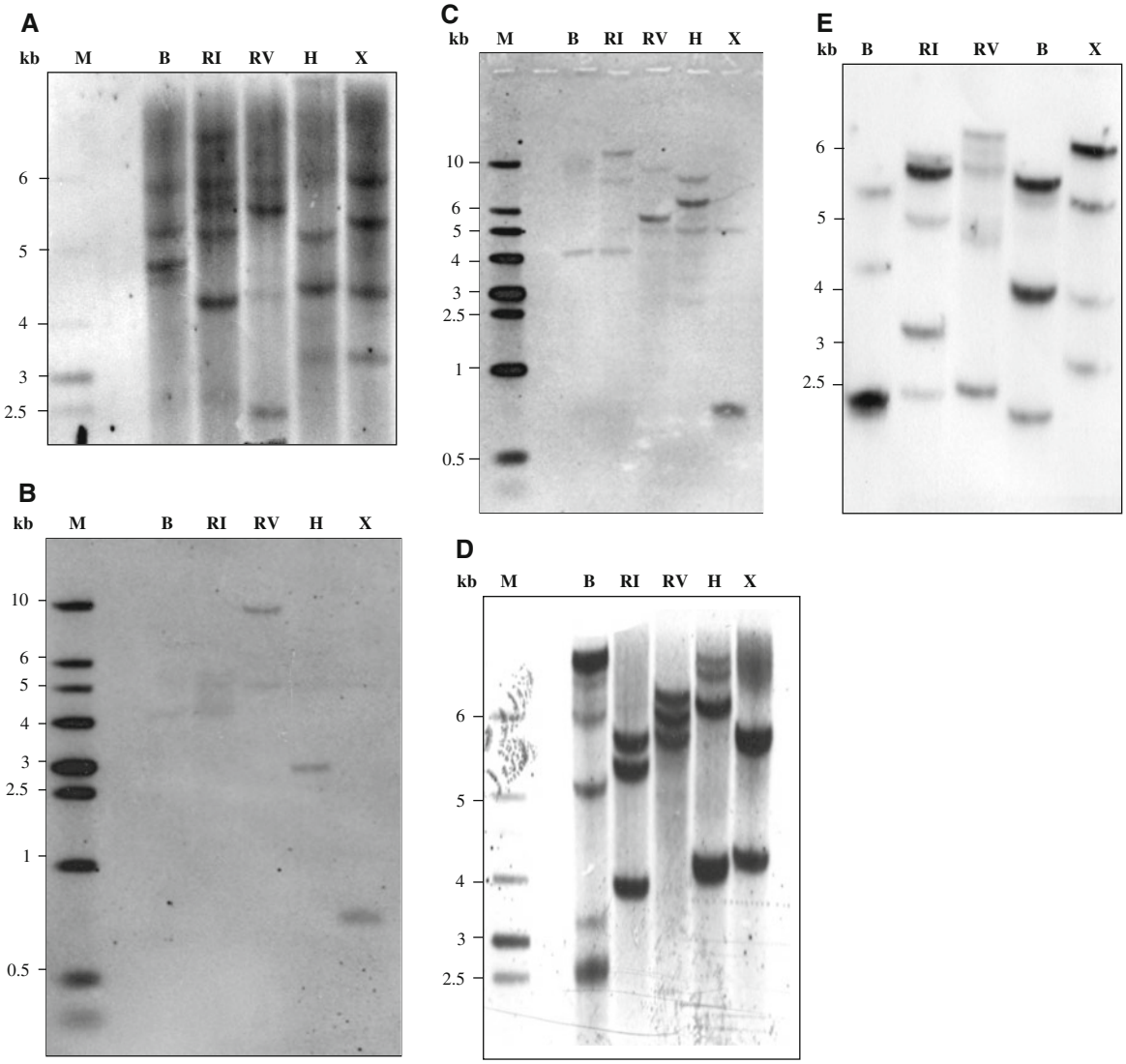

Fig. 4 DNA blot analysis of *Zmcrtiso* and *Zmbch* gene family in corn. Genomic DNA (20 μg) from mature leaves was separately digested with *Bam*HI (B), *Eco*RI (RI), *Eco*RV (RV), *Hin*dIII (H) and *Xba*I (X). Five different blots were hybridized with the *Zmcrtiso* probe (**a**), *Zmcrtiso1* (**b**), *Zmcrtiso2* (**c**), *Zmbch1* (**d**) and *Zmbch2* (**e**) specific probes described in "Materials and methods"

for *Zmbch2* (Fig. 4d, e), indicating there are two or more copies of *Zmbch1* and three or more copies of *Zmbch2* in the corn genome bringing the total of *Zmbch* genes in corn to 5 or more. The *Zmbch1* and *Zmbch2* probe nucleotide sequences were used to query Maize Genetics and Genomic Database (MaizeGDB, http://www.maizegdb.org/), respectively. Three different BAC clones (AC196442 located on chromosome 2, AC215681 on chromosome 4, and AC217349 on chromosome 6) in B73 matched the *Zmbch1* probe nucleotide sequence (96–100% identities), while four distinct BAC clones (AC194430 on chromosome 10, AC205313 on chromosome 1, AC197362 on chromosome 8, and AC201889 on chromosome 7) in B73 matched the *Zmbch2* probe nucleotide sequence (87–100% identities). Thus our data is consistent with the maize genome sequence.

Functional analysis of corn *crtiso* and *bch*

The activity of our cloned cDNAs was investigated by genetic complementation analysis in *E. coli*. For this purpose, *Zmcrtiso1*, *Zmcrtiso2* and *Zmcrtiso2C* cDNAs, the latter with the premature stop codon corrected, were expressed in *E. coli* strains cotransformed with plasmids pACCRT-EBP and pBBR1MCS2-zds, allowing the synthesis of prolycopene as the substrate for CRTISO (Fig. 5A). The composition of ζ-carotene isomers is shown in trace A′. When functional CRTISO1 is present, all-*trans* lycopene and 5-*cis* lycopene are produced at the expense of prolycopene (Fig. 5B). The *cis* ζ-carotene composition remains unchanged following the introduction of *Zmcrtiso1* (Fig. 5B′). Lycopene cyclase is unable to utilize prolycopene (Fig. 5C). However, in the presence of *Zmcrtiso1*, the correct all-*trans* lycopene isomer is formed, allowing cyclization (Fig. 5D). By complementation, we demonstrated that the B73 *Zmcrtiso2* is non-functional (data not shown). However, when the nonsense mutation is corrected, the functional enzyme was able to convert prolycopene into all-*trans* lycopene (Fig. 5E). This indicates that *Zmcrtiso2* encodes a functional isomerase in other corn cultivars. Figure 5F shows different lycopene isomers produced by CrtI phytoene desaturase.

The *Zmbch1* and *Zmbch2* cDNAs were tested by complementation in *E. coli* strains accumulating *β*-carotene (Fig. 6A). In strains cotransformed with *Zmbch1* cDNA, more than half of the *β*-carotene was converted into the monohydroxyl derivative *β*-cryptoxanthin (about 80% of the product) and the dihydroxyl derivative zeaxanthin (about 20% of the product; Fig. 6B). However, in strains cotransformed

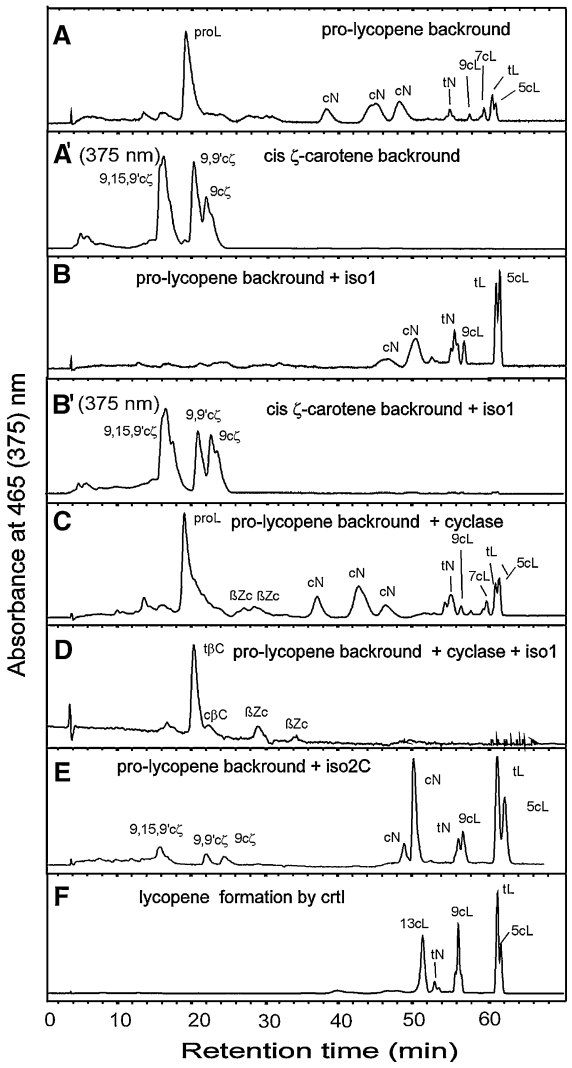

Fig. 5 Functional analyses of *Zmcrtiso* cDNAs. Formation of carotenes in *E. coli* transformed with different combinations of carotenogenic genes: *A* with plasmids pACCRT-EBP + pBB R1MCS2-zds establishing a *cis*-lycopene and a ζ-carotene (trace *A′*) background; *B* conditions as in *A* but with the B73 carotenoid isomerase cDNA *crtiso1* in pUC8-Zmcrtiso1 (iso1); *C* *cis*-lycopene background as in *A* with an additional lycopene cylase gene in pRK-crtY; *D* conditions as in *C* plus pUC8-Zmcrtiso1 (iso1); *E* conditions as in *A* but with the corn carotenoid isomerase gene *crtiso2C* in pUC8-Zmcrtiso2C (iso2c); Trace *F* shows several lycopene isomers as standards produced by plasmid pACCRT-EBI with the crtI-type phytoene desaturase. *Abbreviations*: proL, prolycopene; N, neurosporene; L, lycopene; ζ, ζ-carotene; *β*Zc, *β*-zeacarotene; *β*C, *β*-carotene; c, *cis*; t, *trans*

with *Zmbch2* cDNA, only 4.3% of β-carotene was converted to β-cryptoxanthin and no zeaxanthin was formed (Fig. 6C).

Carotenoid accumulation and gene expression during endosperm development

The profile of carotenoid accumulation in developing B73 corn endosperm was assessed up to 30 DAP (Table 2). Yellow corn endosperm contains β-carotene, its monohydroxylated product β-cryptoxanthin and its dihydroxylated product zeaxanthin. Although α-carotene could not be detected, its hydroxylated products α-cryptoxanthin and lutein were present at detectable levels. A steady increase in total carotenoid content was observed during kernel maturation, although the levels of lutein, zeaxanthin and α-cryptoxanthin increased, β-carotene and β-cryptoxanthin levels decreased from 25 to 30 DAP.

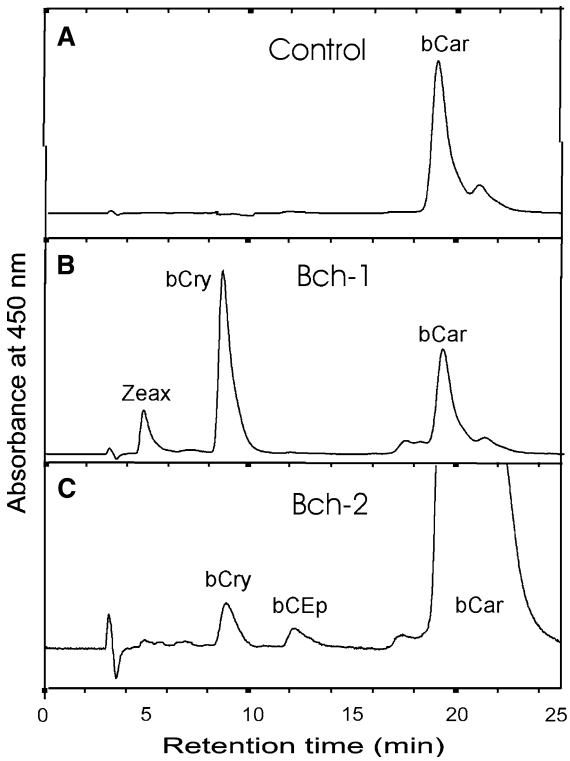

Fig. 6 Functional analyses of *Zmbch1* and *Zmbch2*. (*A*) *E. coli* expressing pACCAR16ΔcrtX. (*B*) *E. coli* expressing pACCAR16ΔcrtX with additional plasmid pUC8-Zmbch1 (Bch1). *C E. coli* expressing pACCAR16ΔcrtX with additional plasmid pUC8-Zmbch2 (Bch2). *Abbreviations*: bCar, β-carotene; Zeax, zeaxanthin; bCry, β-cryptoxanthin; bCEp, β-carotene epoxide

In order to understand how carotenoid accumulation is regulated during corn endosperm development, northern blots containing B73 endosperm mRNA from different developmental stages were hybridized with *Zmcrtiso1*, *Zmcrtiso2*, *Zmbch1* and *Zmbch2* gene-specific DNA probes. The signals for *Zmcrtiso1*, *Zmcrtiso2* and *Zmbch1* were below the detection threshold regardless of the probe used (data not shown). The steady-state levels of *Zmbch2* mRNA increased in the endosperm between 10 and 15 DAP then remained constant until 25 DAP, then declined at 30 DAP, but no transcripts could be detected in leaves (Fig. 7a). To increase sensitivity, expression profiles were monitored by quantitative real time PCR (Fig. 7b) which showed that the transcripts for all four genes increased throughout endosperm development to 25 DAP. Thereafter, the level of *Zmcrtiso2* mRNA remained constant until 30 DAP whereas the levels decreased for the other three genes.

Discussion

Carotenoids are nutritionally valuable compounds that provide a range of health benefits including protection against cancer and other chronic diseases (review by Fraser and Bramley 2004). Humans cannot synthesize carotenoids and must obtain them from their diet, notably from fresh fruit and vegetables and seafood. However, since many people, particularly those in developing countries, subsist on a monotonous diet of staple cereal grains, there has been much interest in the development of strategies to improve carotenoid levels and composition in staple crops (Zhu et al. 2007; 2009). One significant hurdle to the improvement of carotenoid levels in cereal grains is the limited understanding of how carotenoid synthesis is normally regulated, particularly in the endosperm, which is the most nutritious component of the grain. A number of recent studies have shown the potential for carotenoid enhancement in corn endosperm, either through conventional breeding or transgenic strategies (Harjes et al. 2008; Zhu et al. 2008; Aluru et al. 2008; Naqvi et al. 2009). Despite this progress, much remains to be learned about the carotenoid synthesis pathway in corn endosperm and the regulatory mechanisms that control the accumulation of specific carotenoid molecules.

The first step towards understanding how carotenoids are synthesized is to identify the enzymes

Table 2 Carotenoids in B73 corn seed endosperm (μg/g dry weight) depending on the degree of maturation

	Viol	Lut	Zeax	α-Cry	β-Cry	β-Car	Total
10 DAP	0.83 ± 0.06	0.60 ± 0.02	0.66 ± 0.04	0	0	0	2.09
15 DAP	0.39 ± 0.02	1.83 ± 0.12	1.53 ± 0.15	0.88 ± 0.07	0.60 ± 0.07	0.17 ± 0.08	5.40
20 DAP	0.88 ± 0.04	5.11 ± 0.23	2.36 ± 0.15	2.13 ± 0.16	1.14 ± 0.09	0.70 ± 0.05	12.32
25 DAP	1.11 ± 0.09	8.03 ± 028	4.10 ± 0.30	3.77 ± 0.15	2.31 ± 0.12	1.30 ± 0.09	20.62
30 DAP	1.50 ± 0.11	12.55 ± 0.33	6.99 ± 0.451	4.23 ± 0.29	0.85 ± 0.04	1.16 ± 0.07	27.28

Each value is the mean of three determinations from an extract of five individual seeds

Abbreviations: *DAP* Days after pollination, *Viol* violaxanthin, *Lut* lutein, *Zeax* zeaxanthin, *α-Cry* α-cryptoxanthin, *β-Cry* β-cryptoxanthin, *β-Car* β-carotene

involved and isolate the corresponding genes. Several corn cDNAs encoding carotenogenic enzymes have already been cloned and identified including *psy1*, *psy2* and *psy3* (phytoene synthase), *pds* (phytoene desaturase), *zds* (ζ-carotene desaturase), *lcyb* (lycopene β-cyclase) and *lcye* (lycopene ε-cyclase) (Buckner et al. 1996; Gallagher et al. 2004; Li et al. 2008a; Li et al. 1996; Matthews et al. 2003; Singh et al. 2003; Bai et al. 2009). We have cloned and characterized four additional cDNAs, two representing the enzyme carotenoid isomerase (CRTISO) and two representing non-heme di-iron monooxygenase β-carotene hydroxylase (BCH).

The two *crtiso* cDNAs (*Zmcrtiso1* and *Zmcrtiso2*) mapped to genes on different chromosomes and the predicted amino acid sequences showed >96% identity to each other, as well as >70% identity to the single CRTISO enzymes in *Arabidopsis* and tomato (Park et al. 2002; Isaacson et al. 2002). The corn genes, like the *Arabidopsis* and tomato genes, contained 12 introns, and a candidate *crtiso* gene with 12 introns was also identified in the rice genome (accession no. AC108871). Similarly, the two *bch* cDNAs (*Zmbch1* and *Zmbch2*) mapped to different chromosomes but encoded very similar proteins (76.6% identity, with highly conserved motifs typical for an iron-containing monooxygenase). However, in this case, the presence of two distinct genes reflected the situation in other plants such as *Arabidopsis* (Sun et al. 1996; Tian and DellaPenna 2001), pepper (Bouvier et al. 1998), citrus (Kim et al. 2001), saffron (Castillo et al. 2005) and tomato (Galpaz et al. 2006), where two *bch* genes are also present.

The presence of multiple isoenzymes in plant metabolic pathways is a common phenomenon which often reflects the requirement for the same catalytic activity in different subcellular compartments, often the plastids and cytosol (Gottlieb 1982). However, both the *crtiso* and *bch* cDNAs appear to encode proteins with the transit peptide sequences, suggesting the isoenzymes are destined for the plastids. Another source of isoenzymes in diploid plants such as corn is the random duplication of chromosome segments followed by the functional diversification of the duplicated genes. We therefore sought to determine whether the two cDNAs encoding each enzyme were differentially expressed or whether the enzymes themselves were functionally distinct.

We looked at the expression of all four cDNAs during endosperm development using a combination of mRNA blot and quantitative real-time RT–PCR. The latter was necessary because only *Zmbch2* was expressed at a high enough level to be detected by mRNA blot. Real-time PCR showed that the two *crtiso* mRNAs were differentially expressed. *Zmcrtiso1* increased during endosperm development to 25 DAP before dropping off, whereas *Zmcrtiso2* followed the same profile until 25 DAP but thereafter remained at a constant level. Although this suggests a distinct role for *Zmcrtiso2* in later endosperm development, the two enzymes appear to have identical activities when in their functional forms, both isomerizing prolycopene into all-*trans* lycopene and providing the correct isomer for the subsequent cyclization step forming either a β- or ε-ionone ring. Since the composition of ζ-carotene isomers is almost unchanged (Fig. 5A′, B′) when the CRTISO enzymes are expressed, additional ζ-carotene isomerase activity can be excluded, although this may be the function of the corn *y9* allele (Li et al. 2007). Interestingly, the *Zmcrtiso2* cDNA from inbred corn line B73 contained a nonsense mutation in the first

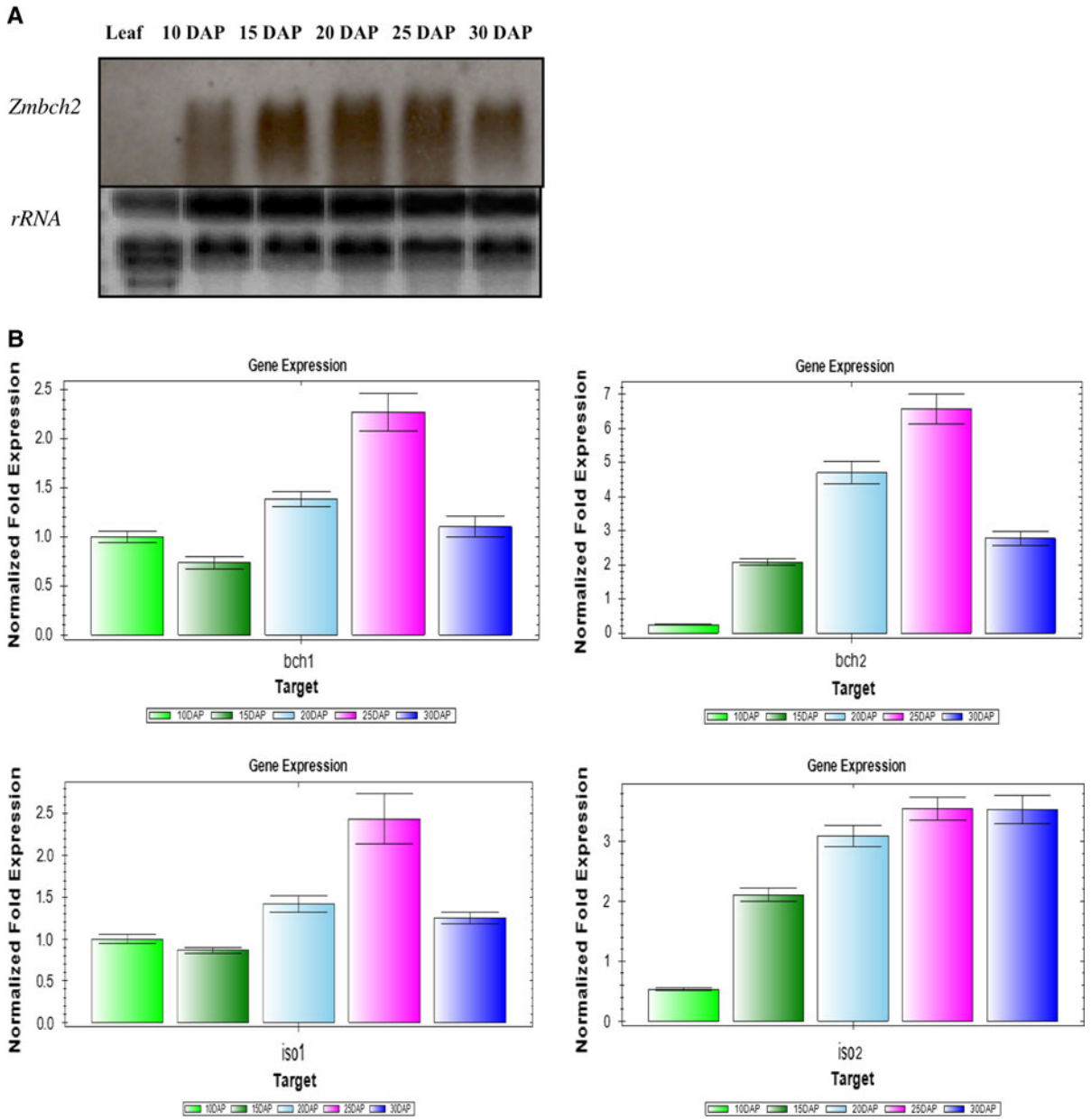

Fig. 7 **a** mRNA blot analysis of *Zmbch2* transcript in leaves and corn endosperms. *Each lane* was loaded with 30 μg of total RNA. rRNA stained with ethidium bromide is shown as a control for loading of equal amounts of RNA. **b** Transcript levels for *Zmcrtiso1*, *Zmcrtiso2*, *Zmbch1* and *Zmbch2*, from greenhouse grown B73 corn harvested at 10, 15, 20, 25 and 30 DAP (days after pollination). Values are a mean of three quantitative real time PCR replicates with ±SD. *Abbreviations*: *Zm*, *Zea mays*; *crtiso1*, carotenoid isomerase 1 gene; *crtiso2*, carotenoid isomerase 2 gene; *bch1*, *β*-carotene hydroxylase 1 gene; *bch2*, *β*-carotene hydroxylase 2 gene

exon converting it into a mutated gene which is not present in M37W (accession no. GQ366381), A632 (accession no. GQ366382), EP42 (GQ366383) and other unknown corn lines (accession nos. FL133727, EU957482). Translation can initiate from a subsequent methionine codon resulting in a truncated, non-functional protein. It also lacks a transit sequence for plastid targeting. Therefore, ZmCRTISO2 does not contribute to carotenogenesis in the inbred line B37, and from this we can conclude that

CRTISO is not a limiting step in carotenogenesis otherwise the pathway would be restricted at the level of prolycopene isomerization once the expression of ZmCRTISO1 dwindles during late endosperm development.

The two *bch* mRNAs were also differentially expressed, with *Zmbch2* alone detectable by mRNA blots. This showed that the mRNA was restricted to endosperm and below the limit of detection in leaves (Fig. 7a), unlike other carotenogenic genes in corn which are thought to be expressed constitutively (Vallabhaneni and Wurtzel 2009). The steady state *Zmbch2* mRNA levels in endosperm were significantly higher than in leaves (Fig. 7a), a finding which is in disagreement with the report by Vallabhaneni et al. 2009 claiming similar mRNA levels in leaves and endosperm at 10 and 30 DAP (Vallabhaneni et al. 2009). The expression of both *Zmbch1* and *Zmbch2* increased during endosperm development, suggesting that the expression profiles in the endosperm were concordant. The *Zmbch2* was preferentially expressed in amyloplast-containing endosperm rather than chloroplast-containing leaves (Fig. 7a), similar to the situation in *Arabidopsis* where the *Atbch2* gene is induced rapidly and strongly during seed development. This suggests that AtBCH2 is preferentially involved in xanthophyll synthesis in seeds (Kim et al. 2009). One of the two *bch* genes in bell pepper, tomato and saffron is also preferentially expressed in flowers or during fruit development (Bouvier et al. 1998; Castillo et al. 2005; Galpaz et al. 2006). In tomato, a *bch2* mutant results in a colorless petal phenotype with no impact on xanthophyll synthesis in leaves (Galpaz et al. 2006), and the massive accumulation of xanthophylls during stigma maturation in saffron correlates with high expression of a single *bch* gene (Castillo et al. 2005). Although showing concordant expression profiles in endosperm, the activity of the two BCH isoenzymes is distinct. In bacteria producing β-carotene and expressing the *Zmbch1* cDNA, more than half of the β-carotene was converted into downstream products, approximately 80% β-cryptoxanthin and 20% zeaxanthin. In contrast, similar bacteria expressing *Zmbch2* cDNA were able to convert less than 5% of the available β-carotene and only β-cryptoxanthin was produced. This functional difference might indicate that the two genes are diverging to fulfill slightly different roles in carotenoid biosynthesis.

Any hypothesis addressing the roles of isoenzymes in carotenoid biosynthesis must look at the expression profiles and enzyme activities in the context of carotenogenesis during seed development. Previous studies have shown that total carotenoid levels increase steadily up to 30 DAP coincident with the upregulation of *psy1*, while *pds* and *zds* transcripts remain at a constant level (Li et al. 1996; Matthews et al. 2003; Li et al. 2008a; Vallabhaneni and Wurtzel 2009). The coordinated upregulation of *Zmcrtiso1*, *Zmcrtiso2*, *Zmbch1* and *Zmbch2* until 25 DAP is consistent with the observed accumulation of carotenoids, although only *Zmcrtiso2* remains at high levels for the next 5 days while the carotenoid content continues to increase. The expression of *lcye* follows a similar profile (Vallabhaneni and Wurtzel 2009). It is apparent that PSY1 is the rate-limiting enzyme of the carotenoid biosynthetic pathway (Li et al. 2008b), but the impact of increasing *Zmcrtiso1*, *Zmcrtiso2*, *Zmbch1* and *Zmbch2* expression over the same timescale is to gradually increase the availability of the β-branch of the pathway. The only remaining carotenogenic gene in corn lacking expression data is carotene ε-ring hydroxylase, catalyzing the 3-hydroxylation of the ε-ionone ring in the synthesis of lutein (Fig. 1). When we compare carotenoid accumulation during seed development (Table 2) with *bch1* and *bch2* transcript kinetics (Fig. 7b), we can predict that ε-ionone ring carotene hydroxylase will demonstrate similar transcript kinetics. This can be concluded from a proportional increase of zeaxanthin and lutein over the whole period of seed development and the equal participation of β-carotene hydroxylase and the ε-ionone ring carotene hydroxylase during formation of lutein from α-carotene (Fig. 1).

In conclusion, we have cloned and characterized four cDNAs encoding corn carotenogenic enzymes, two encoding CRTISO and two encoding BCH. The enzymes are highly conserved in sequence, expression and activity, but subtle differences in the expression profiles of the CRTISO enzymes and the expression and activities of the BCH enzymes hint at divergent roles in plant carotenoid biosynthesis that may be useful in the development of more refined strategies to engineer carotenoid synthesis and composition in staple crops.

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ANNEX 6

Farré G, Ramessar K, Twyman RM, Capell T and Christou P (2010) The humanitarian impact of plant biotechnology: recent breakthroughs *vs* bottlenecks for adoption. Current Opinion in Plant Biology 13:219-225.

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The humanitarian impact of plant biotechnology: recent breakthroughs vs bottlenecks for adoption

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The deployment of genetically engineered (GE) crops in developing countries is regarded by some as a sinister manifestation of 'big business' in science. What is often overlooked, and sometimes even deliberately ignored by opponents of the technology, is that many researchers working in the field are not motivated by profits but by a desire to see such crops applied to humanitarian purposes. GE crops could help to address many of the world's most challenging, interrelated problems, including hunger, malnutrition, disease, and poverty. However, this potential will not be realized if the major barriers to adoption – which are political rather than technical – are not overcome.

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Introduction

Genetically engineered (GE) crops have a remarkable potential to tackle some of the world's most challenging socioeconomic problems, which are more prevalent, more entrenched, and more intractable in the developing world than in the industrialized nations [1]. In developing countries, nearly one billion people are chronically undernourished and up to three billion suffer from malnutrition due to micronutrient deficiency. Add to this the increased burden of disease and poverty resulting from the poor socioeconomic status and inadequate infrastructure of developing countries, and a vicious cycle is created where those most in need of food and medicine are least likely to receive them [1]. Although plant biotechnology is not a magic bullet, it could contribute significantly and sustainably to humanitarian efforts in developing countries where much of the economy is based on subsistence agriculture.

Recent breakthroughs

Pest and disease resistance

Pests and pathogens reduce crop yields worldwide by up to 30%, but in developing countries >80% losses can occur because the climate favors the proliferation of insect pests that consume and spoil food, and transmit diseases. In the developed world, herbicide tolerance and pest resistance are the most prevalent traits in commercial GE crops, and they are often stacked together in a single crop to offer maximum protection [2]. In July 2009, this culminated with the commercial release of Smartstax corn by Monsanto and Dow AgroSciences, a GE variety combining eight different herbicide and pest resistance traits [3•]. The pest resistance genes used in commercial pest-resistant GE crops are based on insecticidal toxins from *Bacillus thuringiensis* [2,4]. Although these plants have been extraordinarily successful, not all pests are targeted by Bt toxins and alternatives are required to avoid the evolution of resistance, so new strategies have been developed to tackle more recalcitrant pests.

One recently developed approach is the use of RNA interference (RNAi) for highly specific targeting of individual pests. Transgenic corn expressing dsRNA targeted against V-type ATPase of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) strongly suppressed corresponding mRNA levels in the insect and reduced feeding damage significantly compared to wild type plants [5•]. In a related approach, the expression of dsRNA targeted against the cotton bollworm (*Helicoverpa zea*) cytochrome P450 gene responsible for the catabolism of gossypol (a cotton secondary metabolite) increased the sensitivity of the insects to gossypol in transgenic tobacco and Arabidopsis plants [6•]. The technique also works against viruses, for example, dsRNA targeting the *Pns12* gene of Rice dwarf virus made rice plants highly resistant to infection [7].

Another novel strategy is the creation of GE plants that emit chemicals to reduce pest infestations. For example, corn plants transformed with an oregano gene encoding (E)-β-caryophyllene synthase constitutively exuded this sesquiterpene from the roots, attracting nematodes that feed on insect larvae, reducing root damage and halving the number of beetles associated with the roots [8]. Similarly, the insect-killing fungus *Metarhizium anisopliae*

was engineered to produce a scorpion toxin that is only activated in the presence of insect hemolymph [9].

Stress tolerance

Unfavorable environmental conditions such as drought, poor soil quality, and flooding also limit productivity in the developing world. Much progress has been made in determining the basis of stress responses in plants and the isolation of stress response genes that can be introduced into susceptible crops to increase their tolerance [1]. This can be achieved simply by expressing the enzymes or structural proteins that are involved in the direct response to stress, for example, the expression of enzymes for the biosynthesis of osmoprotectants, detoxifying enzymes and proteins involved in the maintenance of homeostasis [10]. A broader approach is to express the proteins involved in the regulation of stress pathways, such as receptors, intracellular signaling molecules and transcription factors, since these can activate multiple downstream effectors of stress tolerance [11••,12••,13,14].

Improved nutrition

The nutritional value of crops can be enhanced by increasing the overall yield (quantity) or increasing the nutritional density (quality) of food, especially by encouraging the accumulation of essential nutrients. A recent example of the former approach is the expression of the plasma membrane protein LRK1 in transgenic rice plants [15•]. This protein is normally expressed constitutively at low levels in leaves, young panicles, roots, and culms. Higher-level expression increased the number of panicles, the number of spikelets per panicle, and the grain weight, producing a 27% increase in yield overall per plant.

There have also been a number of recent breakthroughs in GE approaches to increasing nutritional density, particularly the enhancement of vitamin levels in staple crops. Several recent studies report new crop varieties with increased carotenoid levels, and a combinatorial nuclear transformation method has been developed to dissect and modify the carotenoid biosynthetic pathway in corn, resulting in the rapid production of a diverse population of multiplex-transgenic plants containing enhanced levels of specific carotenoids ([16••] and references therein). In the combinatorial system five carotenogenic genes (*Zmpsy1*, *PacrtI*, *Glbch*, *Gllycb*, and *ParacrtW*) under the control of endosperm-specific promoters were introduced into an elite white corn variety deficient for endosperm carotenoid synthesis. This resulted in a population of transgenic plants containing different combinations of transgenes and producing high levels of various carotenoids, including β-carotene, lutein, zeaxanthin, lycopene, and astaxanthin. Another study involved the transfer of seven carotenogenic transgenes into canola, increasing total carotenoid content 30-fold and producing key ketocarotenoids [17••]. A novel approach in potato was to express a cauliflower gene that promotes the differentiation of non-colored plastids into chromoplasts, thus generating a sink for carotenoid accumulation [18•].

Several recent studies report progress with other vitamins, such as folate, where the convergence of three metabolic pathways restricted to different cell compartments needs to be addressed, and ascorbate, which is recycled as well as synthesized *de novo* [19–21]. The recent report of transgenic corn plants simultaneously enhanced for carotenes, folate, and ascorbate is the first publication describing transgenic plants where three entirely different metabolic pathways have been targeted with the aim of nutritional improvement [22••]. Compared to normal corn, the seeds contained 407-fold more β-carotene (57 μg/g dry weight), 6.1-fold more ascorbate (106.94 μg/g dry weight) and twice the amount of folate (200 μg/g dry weight). Additionally, expression of the egoma (*Perilla frutescens*) γ-tocopherol methyltransferase gene increased the levels of α-tocopherol and β-tocopherol (vitamin E) in soybean seeds by 10.4-fold and 14.9-fold, respectively [23].

Attempts to enhance the levels of essential amino acids [24,25] and very long chain polyunsaturated fatty acids [26–28] have also been successful. For example, a recombinant heterotypical tRNA(Lys) has been developed that introduces lysine residues in place of other amino acids during protein synthesis [24], and RNAi has been used to inhibit the key enzyme lysine-ketoglutarate reductase/saccharophine dehydrogenase (LKR/SDH) in order to increase lysine levels in corn [25]. There has also been progress in the engineering of plants to increase the accumulation of mineral nutrients, through the expression of iron transporters, storage proteins or regulatory factors [29••,30] and calcium transporters [31,32].

Added value

Plants can be engineered to produce recombinant proteins and specific chemicals that significantly add to their value [33]. A number of vaccine candidates have been produced in transgenic plants, some progressing to clinical trials (reviewed in Ref. [34]), and one veterinary vaccine produced in tobacco cells has been approved by the USDA and has established a regulatory precedent (a vaccine against Newcastle disease in poultry, developed by Dow AgroSciences). More recent examples include a rice seed-based edible vaccine expressing T-cell epitope peptides derived from Japanese cedar major pollen allergens [35] and transgenic tomato plants expressing two recombinant viral proteins against foot-and-mouth disease [36]. Many other pharmaceutical products have been produced in plants, with recombinant antibodies leading the pack [37]. A significant recent development in this area with particular developing country impact is the successful production of full size HIV-neutralizing monoclonal antibodies in corn [38•,39•] as well as the algal proteins cyanovirin-N [40] and griffithsin [41•]. A fusion

protein comprising cyanovirin-N and the HIV-neutralizing antibody b12, which has greater potency than either of its components, has also been produced in tobacco [42]. In addition to pharmaceuticals, there have been recent developments in the creation of transgenic plants engineered for other added-value traits, such as cotton with increased fiber length and quality [43] and poplar with reduced lignin content [44].

Barriers to adoption

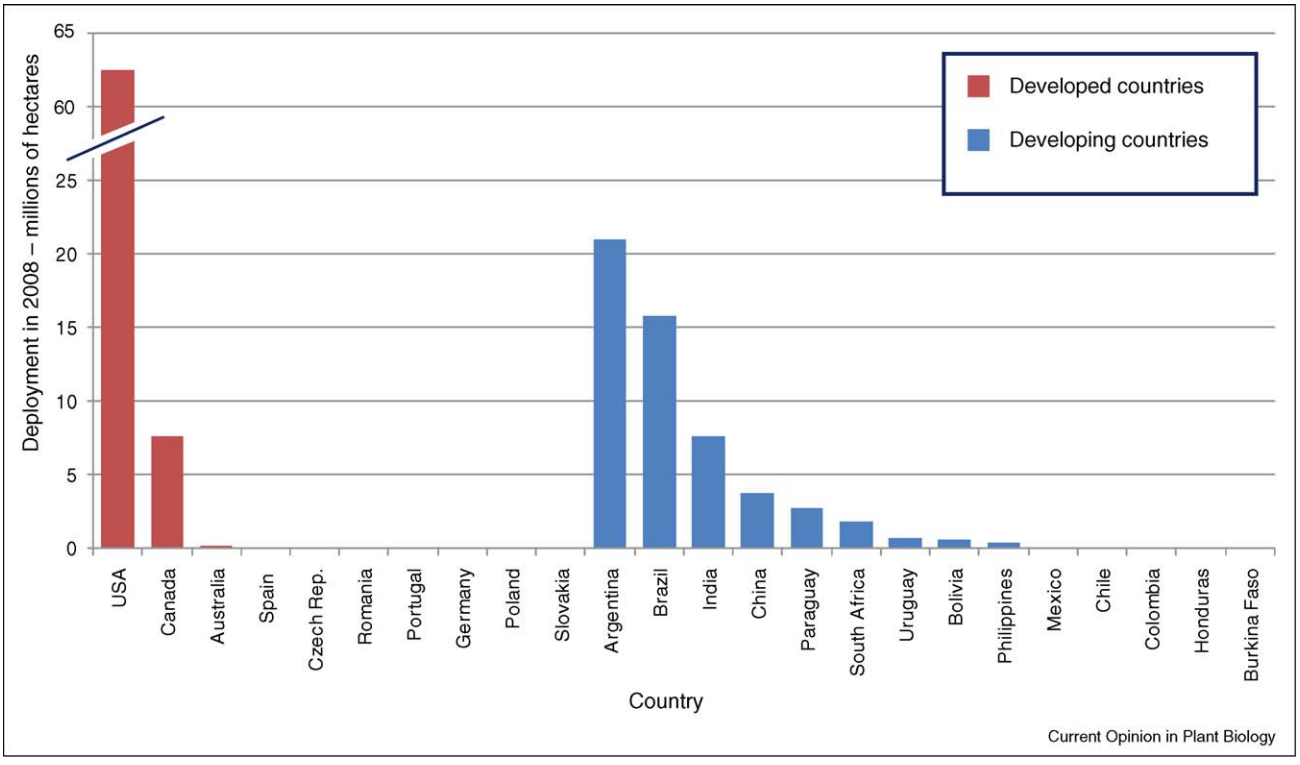
The advances described above provide a vignette of the potential of GE crops, and in an ideal world there would be rapid transfer of the most successful varieties from the laboratory to the field and then to deployment in areas where the benefit could be felt most. But two case studies, Golden Rice II [45] and virus-resistant papaya [46], show that even the best ideas in the laboratory struggle to reach their targets despite a long track record of safety and performance.

A major hurdle to the adoption of plant biotechnology is the widespread perception that only 'big business' benefits from it, with consumers generally failing to realize the indirect benefits to the environment. The benefits of plant biotechnology in developing countries would be more direct — addressing hunger and disease and increasing the wealth of the subsistence farmer who might be able to make a small profit from the extra yield, but take-up has nevertheless been slow (Figure 1) particularly in Africa where South Africa was the only participant in commercial GE agriculture until 2008 (Figure 2).

One prevalent feature of contemporary biotechnology is its proprietary nature, which means that inventions (products and processes) are almost always protected with patents. These are supposed to help promote the transformation of research into marketed products but many believe they obstruct research and development by blocking access to research tools, mandating the use of material transfer agreements and attracting litigation [47]. In developing countries, many key technologies for biotechnology products appear to be unprotected; problems could arise when crops developed with such technologies are exported, but their use for subsistence agriculture is legitimate. The donation of intellectual property for humanitarian purposes in developing countries is therefore a realistic prospect, as in the case of Golden Rice [48].

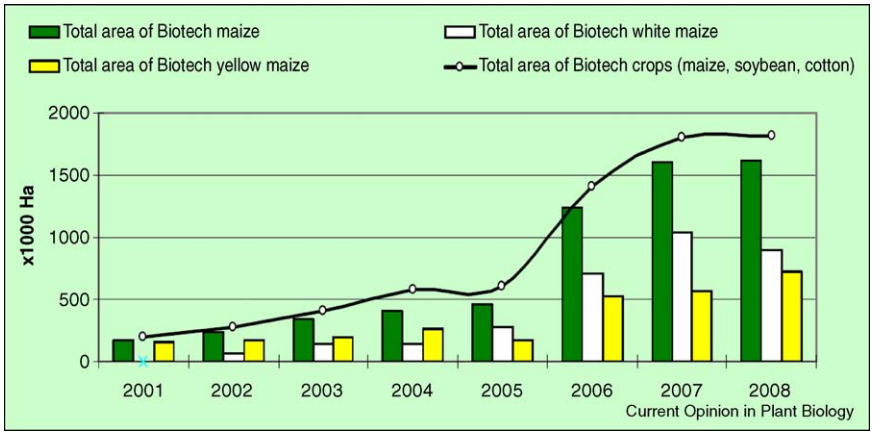
The media also plays a significant role in the likelihood of technology uptake, so how the public perceive GE technology depends greatly on how the information is packaged by the media. In developed countries, media

Figure 1



Adoption of genetically engineered crops in developed countries (red) and developing countries (blue) in millions of hectares per country. Notice the US remains by far the most enthusiastic adopter in the developed world, with Canada trailing a distant second and most other countries with fewer than 50 000 ha. Developing countries on three continents are pioneering the use of GE crops, with Argentina, Brazil, India, China, Paraguay, and South Africa prominent players, but the take-up is slower in other countries.Data from Ref. [2].

Figure 2

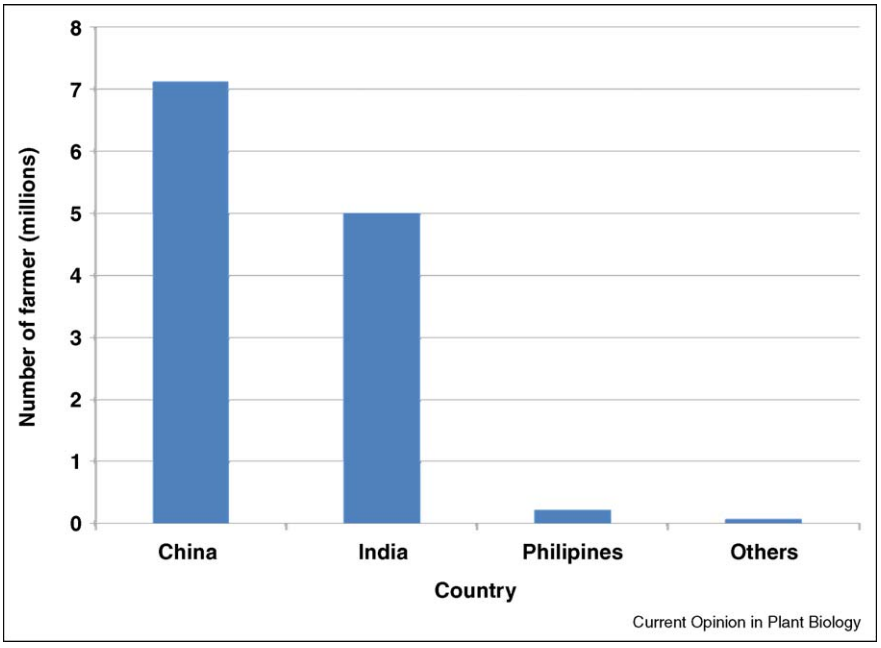


The adoption of genetically engineered maize in Africa, compared to other GE crops. South Africa remains by far the most enthusiastic adopter with increasing deployment of GE maize, soybean, and cotton. The trend is towards increasing production of GE white maize, which is used as food, over yellow maize, which is used as animal feed.Reproduced from Ref. [2].

access is taken for granted, but in many developing countries the news travels slowly and can be subject to political interference. Media involvement can also affect government decisions and policy (e.g., the 2002 GE food aid row that caused the Zambian government to ban GE aid from the US even though millions of its people faced starvation). It is often said that GE crops could help to address Africa's hunger and poverty, but that farmers are being deprived of the technology and prevented from achieving agricultural success [49]. Many blame the European governments and NGOs for trying to foist their affluent values and precautionary principles on Africa's poor [49].

Further development and adoption of biotechnology is hampered by discordant international regulations relating

Figure 3



Distribution of subsistence farmers in the developing world who have adopted genetically engineered crops. There were over 12 million small and resource-poor farmers from developing countries growing GE crops in 2008, over half of which (>7 million) were Chinese farmers growing Bt cotton, as well as 5 million Indian farmers growing the same crop. The remainder comprised approximately 200 000 farmers from The Philippines growing GE maize and thousands of farmers from South Africa and other developing countries growing GE maize, soybean, and cotton.Data from Ref. [2].

to research, biosafety, trade, and use of GE crops and products, particularly between the EU and US [50]. Despite admirable scientific progress in the development of GE crops that have the potential to address developing world challenges, the complex regulations applying in international markets generate overlapping and sometimes contradictory requirements that are a burden to the developing country farmer [51]. This reduces the likelihood that experimental GE crops will be developed into products, a problem exacerbated by the inexperience of public sector researchers in product development and the unwillingness of companies to be involved. Furthermore, trade becomes difficult when regulatory regimes vary so widely, particularly for developing countries that may lack the resources to comply with complex regimes or develop their own. International trade becomes limited as it is more difficult for producers in developing countries to maintain their supply contracts with distributors in the developed world. The end result is that developing countries may decide it is simply easier to avoid GE products (even if they are acknowledged to be safe), with some implementing bans on GE products that not only affect market access but also make it more difficult for them to gain financial support from industrialized nations, particularly in order to conduct research and build human capital for biotechnology activities.

Conclusions

Plant biotechnology can help to address many challenges in the developing world, but the political will to facilitate this process is weak, even though the benefits to small and resource-poor farmers have been demonstrated and the technology is becoming more popular (Figure 3). There have been many calls for the global harmonization of regulations, which would make the requirements compatible and consistent [50]. Eventually, the outcome of national regulations depends on public perceptions and public acceptance, as well as on cultural and institutional processes. Regulatory harmonization would help to remove artificial trade barriers, expedite the adoption of GE crops, foster technology transfer, and protect developing countries from exploitation, instilling confidence, and bringing the benefits of GE products to the consumer.

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- •• of outstanding interest

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ANNEX 7

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 Science 179:28-48.

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Review

Travel advice on the road to carotenoids in plants

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ABSTRACT

The carotenoids are a major class of organic pigments produced in plants and microbes. They fulfill many essential physiological and developmental processes in plants, and also have important roles in animal health and nutrition. As such they have been the focus of multidisciplinary research programs aiming to understand how they are synthesized in microbes and plants, and to clone genes encoding the corresponding enzymes and express them to modulate carotenoid production in recombinant microbial and plant systems. Our deeper understanding of carotenogenic gene regulation, in concert with the development of more effective multi-gene transfer systems for plants, has facilitated more ambitious strategies for the modulation of plant carotenoid biosynthesis not only in laboratory models but more importantly in staple food crops. Here we review the genetic and molecular tools and resources available for fundamental and applied carotenoid research, emphasizing recent achievements in carotenoid engineering and potential future objectives for carotenoid research in plants.

Contents

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1. Introduction

Carotenoids are organic pigments that are produced predominantly (but not exclusively) by photosynthetic organisms. In plants, their presence is revealed by the rich color of flowers, fruits and storage organs in the yellow-to-red part of the spectrum. This reflects the characteristic linear C_{40} molecular backbone containing up to 11 conjugated double bonds, the number and nature of which determines the excitation and emission maxima and resulting spectral properties [1]. Animals cannot synthesize carotenoids but may derive pigmentation from those in their diet, e.g. the yellow of egg yolk, and the pink of lobster shells, salmon flesh and flamingo feathers [2].

In plants carotenoids fulfill two essential functions during photosynthesis, i.e. light harvesting and protecting the photosynthetic apparatus from photo-oxidation [3]. They are also the precursors of signaling molecules that influence development and biotic/abiotic stress responses, thereby facilitating photomorphogenesis, non-photochemical quenching and lipid peroxidation, and attracting pollinators [4–9]. Four carotenoids (β-carotene, α-carotene, γ-carotene and β-cryptoxanthin) have vitamin A activity in humans, which means they can be converted into the visual pigment retinal and are classed as essential nutrients.

β-Carotene (pro-vitamin A) is a precursor of vitamin A in the human body. It is present in a wide variety of yellow-orange colored fruits and dark green and yellow vegetables such as broccoli, spinach, turnip greens, carrots, squash, sweet potatoes, and pumpkin [10]. Liver, milk, butter, cheese, and whole eggs are direct sources of vitamin A. Vitamin A plays an important role in the human body for normal growth and tissue repair. The visual and immune systems are particularly dependent on this vitamin for normal function [11].

Lycopene is the red pigment in many fruits and vegetables such as tomato, watermelon, pink grapefruit and guava [12] and it does not have pro-vitamin A activity; however, it is an excellent dietary antioxidant [13] and it plays a role in reducing the risk of a number of cancers and coronary heart disease [14].

Lutein and zeaxanthin are found in green, certain yellow/orange fruits and vegetables, for example corn, nectarines, oranges, papaya and squash. They constitute the major carotenoids of the yellow spot in the human retina [15] and they protect against age-related macular degeneration, the main cause of blindness in elderly people in the industrialized world [16,17].

These and other carotenoids also have general antioxidant activity and are considered important components of a healthy animal diet. In this context, they have been shown to protect humans from a range of chronic diseases [18]. Carotenoids are important substrates for a class of cleavage dioxygenases that are responsible for the synthesis of phytohormone apocarotenoids such as abscisic acid [19] and the recently discovered hormone strigolactone [20,21].

The importance of carotenoids in both plants and animals, and their many commercial applications in the fields of nutrition and health, has generated interest in the prospect of boosting carotenoid levels in food crops through both conventional breeding and genetic engineering [22,23]. Investigators have looked at carotenogenic pathways in microbes and plants and have isolated genes, enzymes and regulatory components from a range of organisms. In many cases, carotenogenic genes have been introduced into heterologous backgrounds for functional analysis or in an attempt to boost carotenoid accumulation.

Limited information concerning endogenous regulation of carotenogenic genes has hindered the engineering of crop plants to significantly enhance carotenoid content [23–24] although recent progress in cereal crops, particularly corn [25–27] has gone some way in addressing this shortcoming.

The bewildering array of available tools and resources makes it difficult to appreciate the best route to follow when embarking on carotenoid research. In this review, we provide a guide to the resources available to investigators and discuss the most effective strategies for carotenoid research in plants.

2. Carotenoid biosynthesis in plants

Carotenoids are tetraterpenoids, i.e. they comprise eight condensed C_5 isoprenoid precursors generating a C_{40} linear backbone. In plants, this condensation reaction involves the isomeric precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and occurs *de novo* within plastids [28,29]. IPP and DMAPP are derived predominantly from the plastidial methylerythritol 4-phosphate (MEP) pathway [30–32] although the same precursors are formed by the cytosolic mevalonic acid (MVA) pathway, and there is some evidence for the shuttling of intermediates [33,34]. The condensation of three IPP molecules with one molecule of DMAPP produces the C_{20} intermediate geranylgeranyl diphosphate (GGPP), a reaction catalyzed by GGPP synthase (GGPPS), which is encoded by the *crt*E gene (Fig. 1).

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), which is encoded by the *crt*B gene in bacteria [35]. A series of four desaturation reactions carried out in plants by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore (Fig. 1). The product of the first desaturation is 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized by light (and perhaps an unknown enzyme [36]) to yield 9,9′-di-*cis*-ζ-carotene, the substrate of ZDS [37]. 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 [37,38]. In bacteria, a single PDS encoded by the *crt*I gene fulfils all three enzymatic steps. All-*trans* lycopene is then cyclized at one end by lycopene β-cyclase (LYCB), and at the other end either by lycopene ε-cyclase (LYCE) or again by LYCB to introduce ε- and β-ionone end groups and produce α- and β-carotene, respectively. Bacterial LYCB is encoded by the *crt*Y gene.

The introduction of hydroxyl moieties into the cyclic end groups by β-carotene hydroxylase (BCH, encoded by *crt*Z in bacteria) and carotene ε-hydroxylase (CYP97C) results in the formation of zeaxanthin from β-carotene and lutein from α-carotene [39–41]. Two classes of structurally unrelated enzymes catalyze these ring hydroxylations: a pair of non-heme di-iron hydroxylase (BCH) [42–44] and three heme-containing cytochrome P450 hydroxylases (CYP97A, CYP97B and CYP97C) [45–48]. Zeaxanthin can be converted to antheraxanthin and then to violaxanthin by zeaxanthin epoxidase (ZEP) which catalyzes two epoxidation reactions [49]. Finally, antheraxanthin and violaxanthin are converted to neoxanthin by neoxanthin synthase (NXS) [50,51]. The C_{40} 9-*cis*-epoxycarotenoid precursors (9-*cis*-violaxanthin and 9′-*cis*-neoxanthin) are cleaved to xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) [52] and this is followed by a two-step conversion into abscisic acid (ABA), via ABA aldehyde [53].

Engineering metabolism constitutively has often major consequences on metabolism of other branches in the isoprenoid pathway (chlorophyll, GAs, volatile isoprenoids and others). Overexpression of *Psy-1* under a constitutive promoter in tomato or tobacco elevated the carotenoid content [54,55]. However, the expression resulted in altered chlorophyll content and a dwarf plant phenotype. This dwarf phenotype was due to the depletion of the endogenous precursor pool of GGPP leading to a shortage in gibberellins. Contrastingly in *Psy-1* antisense plants in tissues where carotenoids were reduced, gibberellins were elevated [54].

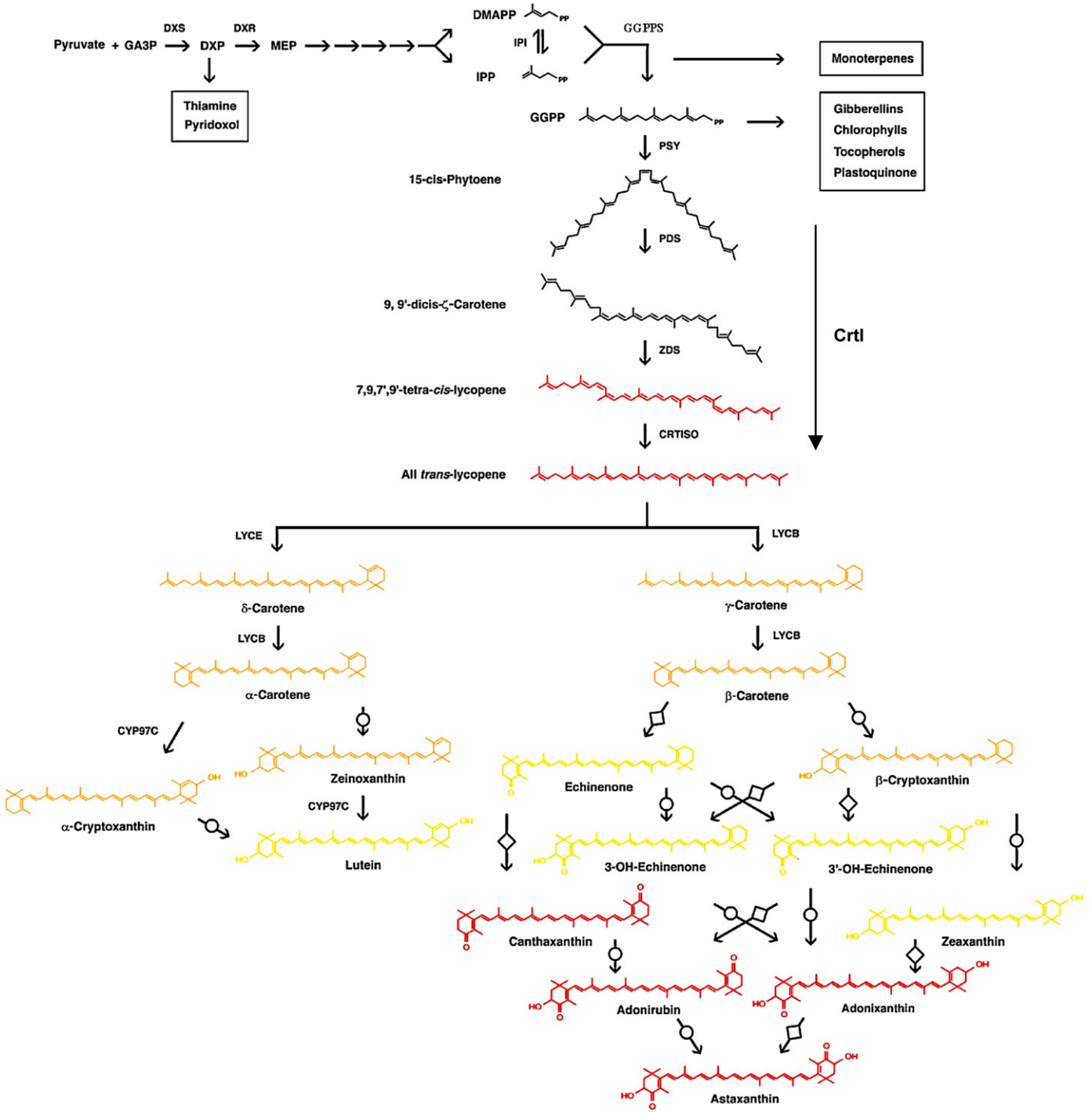

Fig. 1. The extended carotenoid biosynthetic pathway in plants. The precursor for the first committed step in the pathway is GGPP (geranylgeranyl pyrophosphate), which is converted into phytoene by phytoene synthase (PSY, CrtB). GGPP is formed by the condensation of IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate) which are derived predominantly from the plastidial MEP (methylerythritol 4-phosphate) pathway as depicted in the upper part of the figure. The pathway is linear until between phytoene and lycopene, and there are three steps that are catalyzed by separate enzymes in plants but by the single, multifunctional enzyme CrtI in bacteria. Lycopene is the branch point for the α- and β-carotene pathways, which usually end at lutein and zeaxanthin, respectively, through the expression of β-carotene hydroxylases (arrows with circles). An elaborated ketocarotenoid pathway can be introduced by expressing β-carotene ketolases (arrows with diamonds) since these compete for substrates with β-carotene hydroxylases and generate diverse products. Other abbreviations: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; IPI, IPP isomerase; GGPS, GGPP synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; CrtI, phytoene desaturase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; HydE, carotene ε-hydroxylase.

Specialized ketocarotenoid metabolism occurs in some plants, e.g. the synthesis of capsanthin and capsorubin in pepper fruits, catalyzed by capsanthin-capsorubin synthase (CCS) [56]. *Adonis aestivalis* (summer pheasant's eye) petals synthesize the ketocarotenoid astaxanthin, which is usually found only in marine microorganisms [57]. However, many bacteria also contain an extended ketocarotenoid pathway and the expression of bacterial genes such as *crt*Z/*crt*R/*crt*S (carotenoid hydroxylases), *crt*W/*crt*O (carotenoid ketolases) and *crt*X (zeaxanthin glucosylase) in different combinations in plants (Fig. 1) can vastly diversify the spectrum of carotenoids they synthesize, as discussed in more detail below.

3. Strategies to alter the carotenoid content and composition of plants

The full carotenoid biosynthesis pathway is extremely complex, characterized by multiple branches, competition for intermediates, bottlenecks and feedback loops which 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 [22,23].

One way in which carotenoid levels in plants can be enhanced is through increasing the flux non-selectively by providing higher levels of precursors. Increasing the pool of available IPP, for example, will increase flux generally towards terpenoid synthesis, including the carotenoids. This has been achieved by removing key bottlenecks in the plastidial MEP pathway, e.g. by overexpressing 1-deoxy-D-xylulose 5-phosphate (DXP) synthase to provide more DXP, an early pathway intermediate (Fig. 1). When this was carried out in Arabidopsis, the transgenic plants overexpressing DXP synthase showed elevated levels of many terpenoids including up to 1.5× the normal level of chlorophyll, twice the normal level of tocopherol, four times the normal level of ABA and approximately 1.5× the normal level of total carotenoids [58]. Similar results were achieved with regard to carotenoid levels in tomato [59].

One obvious disadvantage of the above is that the MEP pathway feeds several different downstream pathways, all of which draw on the larger pool of IPP. To concentrate the increased flux on the carotenoid pathway alone, it is necessary to modify a committed step. As stated above, the first committed step in carotenoid synthesis is the conversion of GGPP into 15-*cis* phytoene by PSY, so this enzyme is a useful target for upregulation. As an example, this strategy was applied in a corn line whose endosperm lacks endogenous PSY activity, effectively removing the bottleneck and increasing the total carotene content 52-fold, and leading to the predominant accumulation of lutein and zeaxanthin [26]. Similarly, the seed-specific expression of *crt*B in canola increased total carotenoid content by 50-fold, predominantly in the form of α- and β-carotene [60].

As well as increasing the total carotenoid content, it is often desirable to shift metabolic flux to favor the production of specific carotenoid molecules, particularly those with commercial value or health benefits. Removing a general bottleneck as with PSY overexpression above tends to reveal further bottlenecks in specific downstream branches of carotenoid metabolism, which results in certain plants favoring the accumulation of particular molecules over others. The exact carotenoid composition thus depends on the relative enzyme activities further down the pathway, hence the tendency for corn and canola overexpressing PSY to accumulate different end products, mirroring the situation in wild type plants where different carotenoids accumulate in different species. Further modulation with downstream enzymes can therefore shift the carotenoid profile in predictable directions. Canola lines have been created that express not only *crt*B as described above, but also *crt*I and *crt*Y. Transgenic seeds expressing all three genes not only had a higher carotenoid content than wild type seeds as would be expected following the general increase in flux, but the β- to α-carotene ratio increased from 2:1 to 3:1 showing that the additional lycopene β-cyclase activity provided by the bacterial *crt*Y gene skewed the competition for the common precursor lycopene and increased flux specifically towards β-carotene [61].

The outcome of such experiments is not always predictable. Tomato fruits accumulate lycopene rather than β-carotene suggesting that a lack of cyclase activity prevents the accumulation of α- and β-carotenes [62,63]. Transgenic tomato fruit expressing *crt*I were therefore expected to accumulate more lycopene, since this would increase flux up to lycopene but not affect downstream enzyme activities, specifically cyclization. Surprisingly, the resulting plants contained only 30% of the normal carotenoid content but the amount of β-carotene had tripled [64]. This unexpected result seemed to indicate that endogenous lycopene β-cyclase activity had been upregulated in the fruits, a hypothesis that was borne out by the analysis of steady state mRNA levels [64]. Modulating the carotenoid pathway by introducing new enzyme activities may therefore induce hitherto undiscovered feedback mechanisms with unpredictable results [65]. The deliberate overexpression of lycopene β-cyclase in tomato fruits has also resulted in (this time predictable) increases in β-carotene levels [66,67].

In some cases, rather than modulating an existing carotenoid pathway, the aim is to introduce new functionality, i.e. engineer carotenoid metabolism in plants that completely lack these molecules. The most significant example here is rice endosperm, where the expression of PSY leads to the accumulation of phytoene but no other carotenoids, indicating the absence of downstream metabolic capability [68]. The simultaneous expression of daffodil PSY and a bacterial *crt*I gene in rice endosperm induced the accumulation of β-carotene and β-xanthophylls, resulting in the first version of 'Golden Rice' [69]. Later, the corn gene encoding PSY proved more effective than the corresponding daffodil gene, resulting in a 17-fold increase in β-carotene in 'Golden Rice 2' [70]. The presence of cyclic carotenoids such as β-carotene in transgenic rice endosperm expressing corn PSY and bacterial *crt*I suggested that the endosperm tissue possessed a latent LYCB activity, which was subsequently confirmed by mRNA profiling [71]. Interestingly, the same experiments revealed the presence of endogenous transcripts encoding PDS, ZDS and CRTISO, which should provide carotenogenic potential even in the absence of bacterial *crt*I. The absence of other carotenoids in transgenic plants expressing PSY alone therefore indicated that the corresponding PDS, ZDS and/or CRTISO enzyme activity was likely to be very low.

Similar methodology to the above can be used to extend partial pathways and generate additional carotenoid products in plants with a limited repertoire. Most plants synthesize hydroxylated carotenoids but few (peppers and *Adonis aestivalis* being the major exceptions) can synthesize complex ketocarotenoids, although many carotenogenic microbes have this ability as stated above. Several strategies have been used to extend the carotenoid biosynthetic pathway in plants in order to produce nutritionally important ketocarotenoids. A transgenic potato line accumulating zeaxanthin due to the suppression of ZEP activity was re-transformed with the *Synechocystis* PCC 6803 *crt*O gene encoding β-carotene ketolase, resulting in the constitutive accumulation of echinenone, 3′-hydroxyechinenone and 4-ketozeaxanthin along with astaxanthin in the tubers [72]. The newly formed ketocarotenoids accounted for approximately 10–12% of total carotenoids. A Mayan Gold potato cultivar that naturally accumulates high levels of violaxanthin and lutein in tubers, and standard cultivar Desiree, which has low carotenoid levels, were transformed with a cyanobacterial β-carotene ketolase gene leading to the accumulation of ketolutein and astaxanthin [73]. Canola was transformed with *crt*Z (BCH) and *crt*W (β-carotene ketolase) from the marine bacterium *Brevundimonas* SD212, as well as the *Paracoccus* N81106 *ipi* gene and the general carotenogenic genes *crt*E, *crt*B, *crt*I and *crt*Y from *Pantoea ananatis*, and plants expressing all seven genes accumulated 18.6-fold more total carotenoids than wild type plants including ketocarotenoids such as echinenone, canthaxanthin, astaxanthin and adonixanthin, which are not found in wild type seeds [74]. More recently, the expression of corn *psy*, *Paracoccus crt*W and *crt*I, and the *lycb* and *bch* genes from *Gentiana lutea* resulted in the accumulation of ketocarotenoids such as adonixanthin, echinenone and astaxanthin in transgenic corn [26].

A final strategy to achieve carotenoid accumulation in plants is to modify their storage capacity. Carotenoids accumulate in chromoplasts [75], are often derived from fully developed chloroplasts

Table 1
Carotenogenic genes cloned from bacteria, cyanobacteria and fungi.

Gene (protein)	Species	Function	References
*crt*E (GGPP synthase)	Bacteria: *Pantoea ananatis*, *Erwinia herbicola Paracoccus* sp., *Rhodobacter capsulatus*	Converts IPP to GGPP	[84,86,168–170]
*crt*B (phytoene synthase)	Bacteria: *P. ananatis*, *E. herbicola*, *Paracoccus* sp., *Bradyrhizobium* sp. strain ORS278, *R. capsulatus*	Converts GGPP to phytoene	[84,86,132,168,170,171]
*crt*I (phytoene desaturase)	Bacteria: *P. ananatis*, *E. herbicola*, *Paracoccus* sp., *Deinococcus radiodurans*, *Bradyrhizobium* sp. strain ORS278	Converts phytoene to lycopene, equivalent to three enzymatic steps in plants	[84,86,132,171–173]
	Cyanobacteria: *Gloeobacter violaceus*		[174,175]
	Fungi: *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)		[88,176]
	Bacteria: *Rhodobacter sphaeroides*	Converts phytoene to neurosporene (three desaturation steps)	[177]
*crt*Y (lycopene β-cyclase)	Bacteria: *P. ananatis*, *E. herbicola*, *Paracoccus* sp., *Bradyrhizobium* sp. strain ORS278	Converts lycopene to β-carotene	[84,86,178,132,171]
*crt*YB	Fungi: *X. dendrorhous* (*P. rhodozyma*)	Bifunctional enzyme, equivalent to bacterial *Crt*B and *Crt*Y	[88,176]
*crt*Z (β-carotene hydroxylase)	Bacteria: *P. ananatis*, *E. herbicola*, *Paracoccus* sp. (incl N81106 and PC1) *Brevundimonas* sp. SD212	Converts β-carotene to zeaxanthin and can accept canthaxanthin as a substrate. Hydroxylates at C-3 on the β-ring of γ-carotene	[84,86,132,179,180]
	Cyanobacteria: *Haematococcus pluvialis*	Converts β-carotene to zeaxanthin. Diketolation at position 4 and 4′ to canthaxanthin; unable to convert zeaxanthin to astaxanthin	[181]
*crt*R (β-carotene hydroxylase)	Cyanobacteria: *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120	Converts β-carotene to zeaxanthin but is unable to accept canthaxanthin (i.e. the 4-ketolated β-ionone ring) as a substrate. *Anabaena* enzyme is poor in accepting either β-carotene or canthaxanthin as substrates Substrate for *Synechocystis* sp. PCC 6803: Deoxymyxol 2′-dimethylfucoside Substrate for *Anabaena sp*. PCC 7120: Deoxymyxol 2′-fucoside	[182]
*crt*X (zeaxanthin glucosylase)	Bacteria: *P. ananatis*, *E. herbicola*	Converts zeaxanthin to zeaxanthin-β-D-diglucoside	[84,183]
*crt*W (β-carotene ketolase)	Cyanobacteria: *G. violaceus*	Converts β-carotene to echinenone and a small amount of canthaxanthin	[174]
	Bacteria: *Paracoccus* sp., *Bradyrhizobium* sp. strain ORS278, *Brevundimonas* sp. SD212	Converts β-carotene to canthaxanthin. Introduction of keto group at the 4,4' position	[86,132,171,179,184]
	Cyanobacteria: *Nostoc punctiforme* PCC 73102; *Anabaena* sp. PCC 7120		[182,185]
*crt*O (β-carotene ketolase)	Bacteria: *Rhodococcus erythropolis* strain PR4; *D. radiodurans*	Converts β-carotene to canthaxanthin. Unable to accept 3-hydroxy-β-ionone ring as a substrate. Substrate: β-carotene	[157,184]
	Cyanobacteria: *Synechocystis* sp. PCC 6803		[184,186]
	Cyanobacteria: *H. pluvialis*	Bifunctional enzyme: synthesizes canthaxanthin via echinenone from β-carotene and 4-ketozeaxanthin (adonixanthin) with trace amounts of astaxanthin from zeaxanthin	[179,187]
	Cyanobacteria: *Chlorella zofingiensis*	Bifunctional enzyme: Converts β–carotene to canthaxanthin, and converts zeaxanthin to astaxanthin via adonixanthin	[89]
*crt*YE	Cyanobacteria: *Prochlorococcus marinus*	Bifunctional enzyme catalyzing the formation of ε- and β-ionone end groups	[188]
*crt*Yf and *crt*Ye (decaprenoxanthin synthase)	Bacteria: *Corynebacterium glutamicum*	Converts flavuxanthin to decaprenoxanthin	[189]
*crt*Eb (lycopene elongase)	Bacteria: *C. glutamicum*	Converts lycopene to cyclic C50 carotenoids	[189]
*crt*D (methochineurosporene desaturase)	Bacteria: *R. capsulatus*	Desaturase 1-hydroxy-neurosporene. Synthesizes demethylspheroidene	[190]
*crt*C (1-hydroxyneurosporene synthase)	Bacteria: *R. capsulatus*	Hydratase which adds water to the double bond at position 1,2 of the end group yielding a 1-hydroxy derivative. Synthesizes neurosporene and its isomers.	[190]
Astaxanthin synthase gene (cytochrome P450 monooxygenase)	Fungi: *X. dendrorhous* (*P. rhodozyma*)	Multifunctional enzyme catalyzing all steps from β-carotene to astaxanthin formation by oxygenation of C-3 and C-4	[191]

Table 1 (*Continued*)

Gene (protein)	Species	Function	References
*Crt*S (cytochrome-P450 hydroxylase)	Fungi: *X. dendrorhous* (*P. rhodozyma*)	Can hydroxylate canthaxanthin to phoenicoxanthin and finally to astaxanthin	[136]
P450 monooxygenase (CYP175A1)	Bacteria: *Thermus thermophilus* HB27	β-carotene hydroxylase. β-carotene-specific enzyme and does not accept canthaxanthin as a substrate	[192]
Gene s110033	Cyanobacteria: Synechocystis 6803	Carotene isomerase	[193]

during fruit ripening and flower development. However, they can also arise directly from proplastids in dividing tissues and from other non-photosynthetic plastids, such as leucoplasts and amyloplasts [76]. In all cases, chromoplasts accumulate large amounts of carotenoid compounds in specialized lipoprotein-sequestering structures [77]. A spontaneous mutation in the cauliflower *Orange* (*Or*) gene resulted in deep orange cauliflower heads associated with the hyperaccumulation of carotenoids in chromoplasts, increased carotenogenic activity and the appearance of sheet-like carotenoid-sequestering structures [78,79]. Expression of cauliflower *Or* in

Table 2
Carotenogenic genes cloned from plants, most of which have been characterized functionally by complementation in *E. coli*.

Gene (protein)	Species	Function	References
ggpps (GGPP synthase)	Arabidopsis (*Arabidopsis thaliana*), rubber tree (*Hevea brasiliensis*), pepper (*Capsicum annuum*), yellow gentian (*Gentiana lutea*)	Converts IPP to GGPP	[92,93,194–196]
psy (phytoene synthase)	Tomato (*Solanum esculentum*), yellow gentian	Converts GGPP to phytoene	[35,194]
	Corn (*Zea mays*; *psy1*, *psy2*), rice (*Oryza sativa*; *psy2*)	Two tissue-specific genes cloned from corn (from three present in the genome). Expression of *psy1* is in endosperm and is predominantly responsible for carotenoids in seed.	[90]
	Corn (*Zea mays*; *psy3*) and sorghum (*Sorghum bicolor*; *psy1* and *psy3* cDNAs)	*psy3* expression plays a role in controlling flux to carotenoids in roots in response to drought stress. Maize *psy3* is mainly expressed in root and embryo tissue	[95,96]
pds (phytoene desaturase)	Tomato, corn, pepper, yellow gentian, soybean (*Glycine max*)	Converts phytoene to ζ-carotene	[135,194,197–199]
zds (ζ-carotene desaturase)	Corn, yellow gentian	Converts ζ-carotene to pro-lycopene	[200,201]
lycb (lycopene β-cyclase)	Tomato, tobacco (*Nicotiana tabacum*), Arabidopsis, yellow gentian	Converts lycopene to β-carotene	[194,63,202]
	Papaya (*Carica papaya*)	Two papaya *lycb* genes: *lycb1* is downregulated during fruit ripening, and *lycb2* is chomoplast specific	[203,204]
lyce (lycopene ε-cyclase)	Arabidopsis, yellow gentian	Adds one ε-ionone ring to lycopene to δ-carotene	[201,202]
bch (β-carotene hydroxylase)	Arabidopsis, yellow gentian	Converts β-carotene to zeaxanthin	[39,201]
Zep (zeaxanthin epoxidase)	Yellow gentian	Converts zeaxanthin to antheraxanthin	[201]
HYD3 (nonheme diiron β-carotene hydroxylases)	Corn (*Zea mays*)	Encode carotene β-ring hydroxylases	[105]
HYD4 (nonheme diiron β-carotene hydroxylases)		Encode carotene β-ring hydroxylases	
cDNA encoding the enzyme β-carotene hydroxylase	Arabidopsis (*Arabidopsis thaliana*)	Adds hydroxyl groups to both β rings of the symmetrical β-carotene (β-β-carotene) to form zeaxanthin and converts the monocyclic β-zeacarotene to hydroxy- β-zeacarotene	[205]
P450 CYP97C2 (Clan C enzyme)	Rice (*Oryza sativa*)	ε-ring hydroxylase activity	[206]
P450 CYP97A4 (Clan A enzyme)	Rice (*Oryza sativa*)	β-ring carotene hydroxylase activity with some minor activity towards ε-rings	
CCD4 (*carotenoid cleavage dioxygenase protein*)	Apple (Malus × domestica)	Degrades β-carotene to yield β- ionone.	[207]
	Chrysanthemum (Chrysanthemum × morifolium)		
	Rose (Rosa × damascena)		
	Osmanthus (Osmanthus fragans)		
	Arabidopsis		
CCD1 (carotenoid cleavage dioxygenase)	Strawberry	Degradation of β-carotene in vivo	[208]
	Corn (*Zea mays*)	Cleaves carotenoids at the 9, 10 position	[209]
	Vitis vinifera	Cleaves zeaxanthin symmetrically yielding 3-hydroxy-β-ionone, a C_{13}-norisoprenoidic compound, and a C_{14}-dialdehyde.	[210]
CRTISO (*crtiso1*)	*Zea Mays*	Converts tetra-*cis* prolycopene to all-*trans* lycopene but could not isomerize the 15-*cis* double bond of 9,15,9′-tri-*cis*-ζ-carotene.	[211]
bch1 (β-carotene hydroxylase 1)		Convert β-carotene into β-cryptoxanthin and zeaxanthin	
bch2 (β-carotene hydroxylase 2)		Convert β-carotene into β-cryptoxanthin and had a lower overall activity than *ZmBCH1*	

Table 3
Carotenoid pathway mutants in higher plants.

Species	Mutant name	Phenotype	Gene/enzyme	Carotenoid profile	References
Tomato (*Solanum esculentum*)	*wf* (*white-flower*)	White to beige petals and pale anthers	BCH	Carotenoid analysis indicated a reduction of 80 to 84% in total carotenoids in petals of the various *wf* mutant alleles	[212]
	r (*yellow flesh*)	Yellow fruit color	PSY (*psy1*)	Low carotenoid content in fruit	[120]
	delta	Orange fruit color	LYCD	Accumulation of δ-carotene at the expense of lycopene	[62]
	tangerine	Orange fruit color	CRTISO	Accumulates pro-lycopene instead of all-trans-lycopene	[213]
	Beta	Orange fruit color	LYCE (chromoplasts)	*Beta* is a dominant mutation that results in a 5-10% increase in fruit β-carotene levels, reflecting increased LYCB activity, whereas *old gold* is a null allele at the same locus, which reduces the amount of β-carotene in fruit	[121]
	old-gold (*og*)	Tawny orange flowers			
	ghost mutant	Poorly colored petals compared with the yellow carotenoid-containing wild-type petals	plastid terminal oxidase (PTOX) gene	Accumulates phytoene in fruits instead of lycopene	[214]
Pepper (*Capsicum annuum*)	*y* (*yellow*)	Yellow ripening phenotype	CCS (capsanthin capsorubin synthase)	The CCS gene is not expressed in leaves or green fruits of pepper. The enzyme CCS was not found in yellow and green fruit mutants. Expression of CCS in transgenic tobacco and *Arabidopsis* leads to the accumulation of capsanthin	[215]
	c2	Yellow fruit color	PSY	Low level of carotenoids	[216]
Arabidopsis (*Arabidopsis thaliana*)	*lut1*	Single and double mutants showed no phenotype. The triple mutant was smaller and paler than wild type plants.	*LUTEIN1* (ε-hydroxylase)	80% reduction in lutein levels and accumulation of zeinoxanthin	[41]
	b1		CrtR-b1 (BCH, constitutive)	The *b1* mutation had a more significant impact on seed carotenoid composition than *b2*. The *b1* mutation decreased the level of total β-carotene–derived xanthophylls in seeds while in the *b2* mutation increased	
	b2		CrtR-b2 (BCH, flower-specific)		
	lut2	The rate of greening was wild type > *aba1* > *lut2aba1*	*LUTEIN2* (lycopene ξ-cyclase)	Reduction in lutein, compensatory increase in violaxanthin and antheraxanthin	[5]
	aba1		ZEP	Reduction in violaxanthin and neoxanthin, compensatory increase in zeaxanthin	
	ccr2	Disruption in pigment biosynthesis and aspects of plastid development	CRTISO	Accumulation of acyclic carotene isomers in the etioplast and a reduction of lutein in the chloroplast	[4]
Maize (*Zea mays*)	y1	Pale yellow ears	PSY (*psy1*)	Blocks endosperm carotenogenesis but does not interfere with leaf carotenogenesis	[95]
	vp2, vp5, w3	Albinism and viviparity	PDS	Accumulates phytoene	[197,200,217]
	vp9	Albinism and viviparity	ZDS	Accumulates of 9,9'-di-*cis*-ζ-carotene	[36,200]
	vp7	Albinism and viviparity	LYCB	Accumulates lycopene	[101,218]
	y9 (pale yellow 9)	*y9* homozygous mutants were non lethal recessives affecting only endosperm and leaves remained green	Isomerase activity upstream of CRTISO (putative Z-ISO)	9,15,9′-tri-cis-ζ-carotene was found to accumulate in dark-grown tissues of *y9* plants	[36]
Rice (*Oryza sativa*)	*phs1*	Albinism and viviparity	PDS	Accumulates phytoene in light	[107]
	phs2-1	Albinism and viviparity	ZDS	Minimal carotenoid content	
	phs2-2	Albinism and viviparity		Accumulates ζ-carotene in light	
	phs3	Albinism and viviparity	CRTISO	Reduction in lutein levels, increase in pro-lycopene	
	phs4-1, *phs4-2*	Albinism and viviparity	LYCB	Accumulates lycopene	
Sunflower (*Helianthus annuus*)	*nd-1*	Aberrant cotyledon development	ZDS	Minimal levels of β-carotene, lutein and violaxanthin	[219]

potato under the control of the granule-bound starch synthase (GBSS) promoter resulted in orange tuber flesh containing tenfold the normal level of β-carotene [80]. Whereas wild type amyloplasts in tuber cells contained starch granules of varying sizes, the amyloplasts in transgenic plants contained additional orange chromoplasts and derivative fragments [80].

CCD1 contributes to the formation of apocarotenoid volatiles in the fruits and flowers of several plant species. Reduction of *PhCCD1* transcript levels in transgenic petunias resulted in a significant decrease in β-ionone formation. The highest *PhCCD1* transcript levels were detected in flower tissue, specifically in corollas. Its regulation appears to fit with similar oscillations in the expression of phytoene desaturase and ξ-carotene desaturase (genes involved in the formation of β-carotene) indicating a circadian rhythm [81]. Kishimoto and Ohmiya [82] analyzed the carotenoid composition and content in petals and leaves of yellow- and white-flower chrysanthemum cultivars during development. Petals of the yellow-flower cultivar showed increased accumulation and drastic qualitative changes of carotenoids as they matured. Ohmiya et al. [83] searched for cDNAs that were differentially expressed in white and yellow petals, in order to identify factors that control carotenoid content in chrysanthemum petals. They identified a sequence for carotenoid cleavage dioxygenase (CCD; designated as CmCCD4a). CmCCD4a was highly expressed specifically in petals of white-flower chrysanthemum, while yellow-flower cultivars accumulated extremely low levels of CmCCD4a transcript. In order to determine the role of CmCCD4a gene product(s) in the formation of petal color, transgenic chrysanthemum plants were generated by introducing a CmCCD4a RNAi construct into the white-flower cultivar. Suppression of *CmCCD4a* expression thus resulted in a change of color in the petals from white to yellow color. This result suggests that normally white petals synthesize carotenoids but these immediately are degraded into colorless compounds, resulting in the white color [83]. The expression of a carotenoid cleavage dioxygenase CmCCD4a correlates inversely with the accumulation of carotenoids [83]. In white chrysanthemum petals carotenogenic genes were expressed suggesting that white petals are endowed with the capacity to synthesize carotenoids [82].

4. Resources for applied carotenoid research

4.1. Cloned genes and their corresponding enzymes

Perhaps the most important resource for carotenoid engineering in plants is the collection of genes encoding carotenogenic enzymes that has been isolated from bacteria, fungi, algae (Table 1) and higher plants (Table 2). Most of these genes have been cloned and expressed in *Escherichia coli*, which can be used for functional characterization by metabolic complementation (see below).

The microbial genes (Table 1) provide several important advantages over corresponding plant genes. First, their small size makes them easier to manipulate, and their isolation from bacteria is in many cases facilitated by their genomic clustering in metabolic islands or operons [84–87]. Another particular advantage of microbial genes is their multifunctional nature. The bacterial *crt*I gene combines three enzymatic functions that are represented by three separate enzymes in the endogenous plant pathways (PDS, ZDS and CRTISO, Fig. 1), which means fewer genes are needed for carotenoid engineering. A fungal gene has been isolated which combines the functions of *crt*B and *crt*Y (PSY and LYCB) [88] offering the tantalizing possibility that the entire pathway from GGPP to β-carotene could be provided by just two genes.

Microbial carotenogenic genes are also functionally very diverse, providing the sole source of many enzymes involved in the production of ketocarotenoids. Although these enzymes have broadly similar hydroxylase or ketolase activities, their precise substrate preferences and activities in different environments makes it possible to 'tweak' the metabolism of plants to produce highly specific carotenoid profiles. This reflects the complex metabolic pathway leading to astaxanthin, in which multiple enzymes can act on multiple intermediates, the resulting products depending on the balance of activities, substrate preferences and the order in which different reactions occur (Fig. 1). For example, genes encoding CrtW-type ketolases can synthesize canthaxanthin from β-carotene via echinenone and can synthesize astaxanthin from zeaxanthin via adonixanthin. In contrast, CrtO-type ketolases generally cannot synthesize astaxanthin from zeaxanthin, showing they are unable to accept the 3-hydroxy-β-ionone ring as a substrate. However, *Chlorella zofingiensis* CrtO, which is described as a β-carotene oxygenase, can convert zeaxanthin to astaxanthin via adonixanthin as well as β-carotene to canthaxanthin via echinenone [89].

Many plant carotenogenic genes have also been identified and cloned (Table 2). Although these lack the multifunctionality and diversity of their microbial counterparts, they are in some ways more suitable for use in transgenic plants because they are codon optimized, adapted for the intracellular environment *in planta* and endowed with the appropriate targeting signals to allow import into the correct subcellular compartment [90]. Plant genes also provide insight into the compartment-specific and tissue-specific aspects of metabolism which are irrelevant in bacteria, and functional differences arising from their unique origins. For example, Okada et al. [91] identified five different GGPPS cDNAs in Arabidopsis, each expressed in a different spatiotemporal profile. Their considerable sequence diversity suggests they have arisen by convergent evolution rather than the divergence of duplicated ancestors, and indicates the enzymes may have functional as well as structural differences [92,93].

An interesting and relevant example of this spatiotemporal and functional diversity is provided by corn PSY, which occurs as three isoenzymes encoded by the *psy1*, *psy2* and *psy3* genes. The specific roles of the three genes are not fully understood, but the *psy1* gene was first identified through the analysis of the *yellow 1* (*y1*) mutation, which confers a pale yellow kernel phenotype due to the loss of carotenoids [94], and the carotenoid content of endosperm correlates with the level of *psy1* mRNA (but not the other two paralogs) suggesting it has a specific role in endosperm carotenogenesis [95]. PSY1 is also required for carotenogenesis in the dark or under stress in photosynthetic tissue, while PSY2 is required for leaf carotenogenesis and PSY3 is associated with root carotenogenesis as well as the stress-dependent synthesis of ABA [96]. *PSY1* in white maize *y1-602C* is also photoregulated as is found for *PSY2* [97]. This has also been seen in rice *PSY1* and *PSY2* [98].

4.2. Germplasm (natural diversity and specific mutants)

Many plants show significant natural variation in carotenoid levels, in some cases reflecting the additive impact of alleles at multiple quantitative trait loci (QTLs) each with a minor individual effect, in other cases revealing the presence of a major gene in the carotenoid biosynthesis pathway that has a strong impact on its own, resulting in a striking phenotype that is transmitted as a Mendelian trait (Table 3). Conventional breeding to select progressively for QTLs with a desirable influence on carotenoid levels is a slow and laborious process, which is restricted to the available gene pool (and therefore to carotenoids that are already produced in the target plants). However, variants and mutants with interesting carotenogenic properties remain useful as tools in carotenoid research, either as a basis for complementation studies or as a starting point for further improvement using biotechnology.

4.2.1. Cereal crops

Corn 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 clear phenotypes. Corn kernels naturally accumulate lutein and zeaxanthin, and there is significant variation in their levels suggesting that conventional breeding could be used to improve nutrition [99]. A number of mutants have been identified with specific deficiencies in carotenoid metabolism. One of these is the *yellow 1* (*y1*) mutant already mentioned above, which maps to the *psy1* gene. The others (*vp2, vp5, vp7, vp9, w3* and *y9*) combine two common mutant phenotypes – albinism and viviparity, the latter referring to premature development due to the absence of ABA [100], and these too have subsequently been mapped to genes encoding carotenogenic enzymes (Table 3). Singh et al. [101] 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 marker-assisted breeding in corn has been facilitated by the identification of molecular markers associated with the above mutants. For example, a simple sequence repeat (SSR) marker associated with *y1* was linked to a major QTL explaining 6.6–27.2% of the phenotypic variation in carotenoid levels, and was eventually resolved to the *psy1* gene [102]. A QTL associated with *y9* might also be useful for pyramiding favorable alleles controlling carotenoid levels in diverse germplasm [103].

Harjes et al. [104] described four polymorphisms in the corn *lyce* locus which encodes lycopene ε-cyclase (LYCE), an enzyme that competes with LYCB for lycopene and helps to determine the relative amounts of α- and β-carotenes. Conventional breeding 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. [105] characterized six carotene hydroxylase genes in genetically diverse corn germplasm collections, although only one appeared to affect carotenoid levels in seeds. Three alleles of this *hyd3* gene explained 78% of the variation in the β-carotene/β-cryptoxanthin ratio (11-fold difference across varieties) and 36% of the variation in absolute β-carotene levels (four-fold difference across varieties). These authors have recently used a combination of bioinformatics and cloning to identify and map gene families encoding carotenogenic enzymes from corn and other grasses, and have identified those whose mRNA levels positively and negatively correlate with endosperm carotenoid levels [106].

Fig. 2. The carotenoid biosynthesis pathway in living color. *Escherichia coli* strain TOP10 was genetically engineered to accumulate different carotenoids as indicated [57].

Similar work has been carried out in other cereals, e.g. a subset of pre-harvest sprouting (PHS) mutants in rice (analogous to corn viviparous mutants) has been identified that also show an albino phenotype, and these have led to rice carotenogenic genes such as those encoding PDS (*phs1*), ZDS (*phs2-1*, *phs2-2*), CRTISO (*phs3-1*), all of which fail to accumulate carotenoids, and LYCB (*phs4-1*, *phs4-2*), which accumulates lycopene [107]. In wheat, hexaploid tritordeums produce more carotenoids than their respective wheat parents or hybrids derived from crosses between wild diploid barley and durum wheat [108]. One QTL (*carot1*) explaining 14.8% of the phenotypic variation in carotenoid levels is being considered for use in a marker-assisted breeding program [109]. A double haploid wheat population, which was previously characterized for endosperm color [110], was used to map the *psy1* and *psy2* genes against four QTLs affecting endosperm color, with one showing strong linkage [111]. In sorghum, Kean et al. [112] determined the carotenoid profiles of eight selected yellow-endosperm cultivars where zeaxanthin is the most abundant carotenoid. Salas Fernandez et al. [113] detected several QTLs responsible for varying carotenoid levels in a recombinant inbred line population, a cross between the yellow endosperm variety KS115 and a white endosperm variety Macia. Among four QTLs for endosperm color and five for β-carotene content, one was mapped to the *psy3* gene.

4.2.2. Root vegetables (potato and carrot)

Potatoes show great diversity in carotenoid content, and breeding programs using cultivars with red/purple tubers [114] and dark yellow tubers [115] have increased carotenoid levels to 8 μg/g fresh weight. 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 three with two candidate genes, encoding PSY and BCH, and possibly additional regulatory elements [116]. Note that the *Or* allele of the endogenous *Y* locus is not the same as the cauliflower *Or* gene (see above), which encodes a DnaJ homolog and has been introduced as a heterologous trait into potato to force β-carotene accumulation in amyloplasts [79]. QTL studies in carrots have been carried out using an intercross between cultivated orange and wild type lines, and between specialized medium orange (Brasilia) and dark orange (HCM) lines [117]. Major QTLs were found explaining 4.7–8% of the total phenotypic variation in ζ-carotene, α-carotene and β-carotene levels, and 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 [118]. The most recent study involved crosses between orange cultivated carrots and a wild white line, identifying QTLs in two linkage groups, one (*Y* locus) associated with total carotenoid levels and the other (*Y2* locus) associated with the accumulation of xanthophylls at the expense of other carotenoids [119].

4.2.3. Tomato and other fruit

Significant variation in carotenoid profiles is also found in tomato, where a number of mutations affecting the total content

Table 4
Recombinant *E. coli* strains used for the functional characterization of carotenogenic genes.

Genotype of recombinant strain (origin of genes)	Precursor	Source of test sequence	Major product(s)	Function of test sequence	References
*crt*E and *crt*B (*Pantoea annanatis*)	Phytoene	*P. annanatis*	Lycopene	*crt*I (phytoene desaturase)	[84]
crtE, *crtB* and *crtX* (*P. annanatis*)		*Xanthophyllomyces dendrorhous*	Lycopene	*crt*I (phytoene desaturase)	[220]
crtE, *crtB* and *crtI* (*P. annanatis*)	Lycopene	*P. annanatis*	β-Carotene	*crt*Y (lycopene cyclase)	[84]
crtE, *crtB* (*P. annanatis*) and *crtP* (*Synechocystis sp.*)	ζ-Carotene	*P. annanatis*	ζ-Carotene	*crt*Y (lycopene cyclase)	[221]
crtE, *crtB* and *crtI* (*Erwinia herbicola*)	Lycopene	*Arabidopsis thaliana*	β-Carotene	*crtL-b* (lycopene β-cyclase)	[202]
crtE, *crtB* and *crtI* (*E. herbicola*)		*A. thaliana*	ε,ψ-Carotene	*crtL-e* (lycopene ε-cyclase)	
crtE, *crtB* and *crtI* (*E. herbicola*)		*A. thaliana*	ε,ψ-Carotene, β-ε- Carotene	*crtL-b* (lycopene β-cyclase), *crtL-e* (lycopene ε-cyclase)	
crtE, *crtB* (*E. herbicola*) and *crtI* (*Rhodobacter capsulatus*)	Neurosporene	*A. thaliana*	β-Zeacarotene, neurosporene	*crtL-b* (lycopene β-cyclase)	
crtE, *crtB* (*E. herbicola*) and *crtI* (*R. capsulatus*)	Neurosporene	*A. thaliana*	Neurosporene, α-zeacarotene	*crtL-e* (lycopene ε-cyclase)	[202,222]
crtE, *crtB* (*P. annanatis*) and *crtI* (*R. capsulatus*)	Neurosporene	*P. annanatis*	Dihydro-β-carotene	*crt*Y (lycopene cyclase)	[221]
		Capsicum annuum		*crtL-b* (lycopene β-cyclase)	
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*P. annanatis*	Zeaxanthin	*crt*Z (β-carotene hydroxylase)	[84]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)		*Agrobacterium aurantiacum*			[132]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*Haematococcus pluviales*	Zeaxanthin, β -cryptoxanthin	*crt*Z (β-carotene hydroxylase)	[181]
crtE, *crtB*, *crtI*, *crtY* and *crtX* (*P. annanatis*)	β-carotene	*P. annanatis*	Zeaxanthin	*crt*Z (β-carotene hydroxylase)	[220]
CrtE, *crtB*, *crtI*, *crtY* (*P. annanatis*) and *crtC* (*R. shaeroides*)	β-carotene	*A. aurantiacum*	Zeaxanthin	*crt*Z (β-carotene hydroxylase)	[223]
CrtE, *crtB*, *crtI*, *crtY* and *crtZ* (*P. annanatis*)	Zeaxanthin	*P. annanatis*	Zeaxanthin-β-diglucoside	*crt*X (zeaxanthin glucosylase)	[84]
crtE, *crtB* (*P. annanatis*) and *crtI* (*R. capsulatus*)	Neurosporene	*P. annanatis*	Dihydrozeaxanthin dihydro- β -caroten-3,3'-ol, β -zeacaroten-3-ol	*crt*Z (β-carotene hydroxylase)	[221]
crtE, *crtB* (*P. annanatis*) and *crtI* (*R. capsulatus*)	Neurosporene	*P. annanatis*	7,8- dihydrozeaxanthin, 3-hydroxy- β -zeacarotene, 3/3'-hydroxy-7,8-dihydro- β -carotene	*crt*Z (β-carotene hydroxylase), *crtI* phytoene desaturase)	[190]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*Synechocystis sp*	Echinenone, canthaxanthin	*crt*O (β-carotene oxygenase)	[186]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*Haematococcus pluvialis*	Canthaxanthin	*bkt* (β-carotene oxygenase)	[224,222]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*Agrobacterium aurantiacum* or *Alcaligenes* PC-1	Canthaxanthin	*crt*W (β-carotene oxygenase)	[85]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene via echionenone	*A. aurantiacum*	Canthaxanthin	*crt*W (β-carotene oxygenase)	[132]
crtE, *crtB*, *crtI*, *crtY* (*P. annanatis*), *crtZ* and *bkt* (*H. pluvialis*)	β-carotene		Canthaxanthin, β-cryptoxanthin, zeaxanthin, adonixanthin, astaxanthin	*crtZ* (β-carotene hydroxylase), *bkt* (β-carotene oxygenase)	[181]
crtE, *crtB*, *crtI*, *crtY*, *crtZ* and *crtX* (*P. annanatis*)	β-carotene	*A. aurantiacum*	Astaxanthin-β-glucoside, Astaxanthin-β-D-glucoside	*crtW* (β-carotene oxygenase)	[133]
CrtE, *crtB*, *crtI*, crtY and crtX (*P. annanatis*)	β-carotene	*A. aurantiacum*	Astaxanthin, adonixanthin 3'- β-D-glucoside	*crtZ* (β-carotene hydroxylase), *crtW* (β-carotene oxygenase)	[133]
crtE, *crtB*, *crtI* and *crtY*	β-carotene	*P. annanatis*, *H. pluvialis*	Astaxanthin, canthanxanthin, zeaxanthin	*crtZ* (β-carotene hydroxylase), *bkt* (β-carotene oxygenase)	[226]
crtE, *crtB*, *crtI* and *crtY* (*P. annanatis*)	β-carotene	*P. annanatis*, *A. aurantiacum*)	Astaxanthin, phoenicoxanthin, adonixanthin, canthaxanthin	*crtZ* (β-carotene hydroxylase), *crtW* (β-carotene oxygenase)	[132]

Table 4 (*Continued*)

Genotype of recombinant strain (origin of genes)	Precursor	Source of test sequence	Major product(s)	Function of test sequence	References
crtE, crtB, crtI and *crtY* (*P. annanatis*)	β-carotene	*A. aurantiacum*	Adonixanthin, astaxanthin, canthaxanthin	*crtZ* (β-carotene hydroxylase), *crtW* (β-carotene oxygenase)	[132]
crtE, crtB and *crtI* (*P. annanatis*)	Lycopene	*R. capsulatus*	1,1′-dihydroxylycopene, 1-hydroxylycopene	*crtC* (hydroxyneurosporone synthase)	[190]
crtE, crtB (*P. annanatis*) and *crtI* (*R. capsulatus*)	Neurosporene	*R. capsulatus*	Hydroxyneurosporene	*crtC* (hydroxyneurosporone synthase)	[186,190]
crtE, crtB, crtI and *crtY* (*P. annanatis*)	β-carotene	*H. pluvialis*	Canthaxanthin	*bkt* (β-carotene oxygenase)	[181,226]
crtE, crtB (*P. annanatis*) and *crtI* (*R. capsulatus*)	Neurosporene	*R. capsulatus*	Demethylspheroidene	*crtC* (hydroxyneurosporone synthase), *crtD* (methoxyneurosporene desaturase)	[190]
crtE, crtB and *crtI* (*P. annanatis*)	Lycopene	*Corynebacterium glutamicum*	Flavuxanthin	*crtEb* (lycopene elongase)	[189]
crtE, crtB and *crtI* (*P. annanatis*)	Lycopene	*C. glutamicum*	Decaprenoxanthin	*crtEb* (lycopene elongase), *crtYe, crtYf* (decaprenoxanthin synthase)	[189]
ipi and *crtE* (*P. annanatis*)	Geranylgeranyl diphosphate	*P. annanatis*	Phytoene	*crtB* (phytoene synthase)	[222]
ipi, crtE and *crtB* (*P. annanatis*)	Phytoene	*Synechococcus* PCC7942	ζ-carotene	*crtP* (phytoene desaturase)	
ipi, crtE, crtB, crtI and *crtY* (*P. annanatis*)	β-carotene	*Synechococcus* PCC6803	Echienone, canthanxanthin, β-carotene	*crtW* (β-carotene ketolase)	
ipi, crtE, crtB, crtI, crtY and *crtZ* (*P. annanatis*)	β-carotene	*P. annanatis*	Zeaxanthin	*crtZ* (β-carotene hydroxylase)	
ipi, crtE, crtB, crtI, crtY (*P. annantis*)	β-carotene	*Synechocystis* PCC6803	Zeaxanthin	*crtR* (β-carotene hydroxylase)	[225]

and diversity of carotenoids have been identified. These include *r* (*yellow-flesh*), which is characterized by yellow fruit and has a loss-of-function mutation in PSY1 [120], and *delta*, which accumulates δ-carotene instead of lycopene, reflecting an increased expression of the gene encoding ε-cyclase [62]. The *tangerine* mutation, also named because of the color of the fruit, reflects a loss of CRTISO activity. Two mutations affecting LYCB activity have been identified, one named *Beta* (characterized by a 45% increase in β-carotene content compared to wild type, resulting in a characteristic orange fruit color) and another named *old-gold* (*og*) which lacks β-carotene but has higher than normal levels of lycopene [121]. Searches for QTLs affecting lycopene content in tomato fruit have been successful, with a cross between a lycopene-rich specialist cultivar and a standard breeding variety revealing eight QTLs, one accounting for 12% of the variation in lycopene content [122], and a more recent search for QTLs affecting fruit color in introgression lines identifying 16 loci, five of which cosegregated with candidate genes involved in carotenoid synthesis [123].

The deep red color of watermelon flesh reflects its carotenoid content and a comparative study of 50 commercial varieties has shown that total carotenoid levels in red-fleshed cultivars vary in the range 37–122 mg/kg fresh weight, with 84–97% of the content represented by lycopene and those with the highest lycopene levels also containing the highest levels of β-carotene [124]. Other culinary melons (*Cucumis melo*) have flesh ranging in color from green to orange, displaying a very diverse profile of carotenoids. California and Wisconsin melon recombinant inbred lines were used to identify QTLs affecting β-carotene levels, and eight loci were found each explaining between 8% and 31% of phenotypic variation, one mapping to a gene encoding BCH [125]. Carotenoid diversity in kiwifruit has also been investigated and it has been noted that the major products are β-carotene and lutein, both of which may be modulated by genetic variation at the *lycb* locus [126]. Significant variation has also been found in the sweet orange (*Citrus sinensis* L. Osbeck) with the identification of a mutant, 'Hong Anliu', which is deep red in color and contains over 1000-fold the levels of lycopene found in wild type fruits [127].

Red cultivars of *Capsicum* are worthy of special mention because they are one of the few examples of plants producing ketocarotenoids [128]. A genetic map was developed from an interspecific cross between *Capsicum annuum* (TF68, red) and *Capsicum chinese* (Habanero, orange). Several carotenogenic genes were mapped and served as candidate genes controlling carotenoid content and fruit color, including a gene for PSY that explained 53.4% of the variation [129]. Homozygous and heterozygous lines containing PSY alleles from the TF68 parent contained more than six-fold higher levels of carotenoids than fruits homozygous for the Habanero allele. A more recent study of 12 diverse pepper varieties identified a correlation between the levels of PSY, PDS and CCS activity and the carotenoid content [130].

4.3. Bacterial strains for complementation studies

Most of the carotenogenic genes described above and listed in Tables 1 and 2 have been functionally characterized through a combination of sequence analysis and complementation in *E. coli*, a non-carotenogenic bacterium. *E. coli* is well suited to this task because the absence of carotenoid synthesis means that recombinant strains can be created that partially recapitulate the pathway, or which are blocked at specific points along the pathway, allowing panels of cell lines accumulating different intermediates to be tested systematically with novel genes to determine their functions. The products synthesized in *E. coli* can then be identified by chromatography, although the colonies take on colors ranging from yellow to red which often provides an even quicker means of identification (Fig. 2) [75]. However, the GGPP pool in *E. coli* is insufficient

to drive robust carotenoid synthesis, so before this species can be used for complementation studies the amount of GGPP must be increased through the expression of geranylgeranyl diphosphate synthase (encoded by *crt*E), which catalyzes the addition of a C_5 isoprenoid unit onto Geranylgeranyl diphosphate (GGPP).

The addition of further carotenogenic genes then leads to the production of specific intermediates and downstream carotenoids, as summarized in Table 4. For example, the introduction of *crt*E, *crt*B, *crt*I and *crt*Y facilitates the *de novo* synthesis of lycopene, β-carotene and zeaxanthin [84,131] and the further addition of *crtZ* and *crtW* facilitates the synthesis of astaxanthin (representing 50% of total carotenoids) and various intermediates [132]. Adding *crt*X to the above facilitated the synthesis of two carotenoid glucosides, astaxanthin-β-D-diglucoside and adonixanthin 3′-β-D-glucoside [133].

Occasionally, other bacteria are used for functional analysis including *Zymomonas mobilis*, *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* [134,135] and the fungus *Mucor circinelloides* [136].

4.4. Transgenic plant lines with altered carotenoid profiles

The introduction of carotenogenic genes directly into plants provides a shortcut to the laborious breeding programs required to exploit natural diversity, and also allows genes to be introduced from beyond the natural gene pool. This second point is important because it remains the only strategy that can be used to introduce carotenogenesis *de novo* or to extend the carotenoid biosynthesis pathway beyond its natural endpoint, e.g. to produce ketocarotenoids in major staple crops.

There has been significant progress in the development of transgenic crop varieties producing higher levels of carotenoids, and more recently there have been a number of key achievements in the areas of branch point modulation (shifting flux towards particular molecules and away from others), *de novo* carotenogenesis (introducing the entire carotenogenic pathway into plant tissues lacking carotenoids) and pathway extension (Table 5). A number of noteworthy case studies are considered below.

4.4.1. Laboratory models

Although not of agronomic importance, laboratory model species such as Arabidopsis are amenable to genetic analysis and often provide breakthroughs that can be used as a springboard to launch more applied research in crop species. Transgenic Arabidopsis plants expressing a range of carotenogenic genes have been created and tested for carotenoid accumulation, including heterologous plant genes, bacterial genes and recombinant products such as the CrtZ-CrtW polyprotein [137]. Ralley et al. [138] achieved the production of ketocarotenoids in tobacco, which accumulated in leaves and in the nectary tissues of flowers at levels tenfold greater than normal, and included astaxanthin, canthaxanthin and 4-ketozeaxanthin, predominantly as esters. Recently, the overexpression of an Arabidopsis *PSY* gene in Arabidopsis and carrot has revealed a difference between photosynthetic and non-photosynthetic tissue in terms of carotenoid accumulation [139]. Seedlings were unaffected by the increased PSY levels but non-photosynthetic callus and root tissue accumulated up to 100-fold the level of carotenoids found in wild type tissues (up to 1.8 mg/g dry weight, predominantly β-carotene).

4.4.2. Golden rice

The 'Golden Rice' project was the first significant application of carotenoid engineering and was envisaged as a humanitarian mission to alleviate vitamin A deficiency, which results in millions of cases of preventable blindness every year in developing countries [140]. Large numbers of people subsist on monotonous diets of milled rice grains which contain little vitamin A, so a research project was conceived to introduce a partial carotenoid biosynthesis pathway into rice endosperm allowing the grains to accumulate β-carotene. The first Golden Rice line contained three transgenes: daffodil *psy1* and *lycb* genes together with bacterial *crt*I. The grains accumulated up to 1.6 μg/g dry weight of β-carotene [69]. This was not sufficient to provide the recommended daily intake of vitamin A from a reasonable rice meal, so the more active corn *psy1* gene was used to replace its daffodil ortholog, resulting in 'Golden Rice 2', in which the total carotenoid content of the endosperm increased to 37 μg/g dry weight [70] (Fig. 3a). The next scientific step in the deployment of Golden Rice, which has been under development for several years, is the introgression of the same traits into locally adapted varieties.

4.4.3. Amber potatoes and red carrots

As stated earlier, Lu et al. [79] isolated a clone corresponding to the *Or* allele from a mutant cauliflower variety with orange, carotenoid-rich heads. This clone was introduced into cauliflowers and replicated the effect, confirming that it was a dominant mutation (Fig. 3b). The same phenotype was observed in transgenic potatoes expressing *Or* [80] (Fig. 3c). Two further biotechnology approaches have been combined to improve carotenoid levels in potato tubers, one based on the introduction and expression of carotenogenic transgenes and the other based on the suppression of endogenous enzymes competing for common precursors (Fig. 3d). Diretto et al. [141,142] 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. Diretto et al. [142,143] also silenced the endogenous *lyce* and *bch* genes, thereby eliminating competition at the branch point between the α- and β-carotene pathways and preventing the further metabolism of β-carotene. In a separate study, silencing the *bch* gene alone elevated β-carotene levels to 3.31 μg/g dry weight [144]. Silencing the endogenous *zep* gene also increased total carotenoid levels, particularly zeaxanthin, whereas violaxanthin levels were reduced [145].

Although the roots of orange, cultivated carrot varieties are rich sources of α-carotene, β-carotene and lutein, they cannot produce ketocarotenoids. Recently, however, ketocarotenoid synthesis has been achieved in carrot roots by transforming them with an algal β-carotene ketolase gene fused to a plastid targeting sequence so the protein was successfully expressed in chloroplasts and chromoplasts [146]. This resulted in the conversion of up to 70% of the total carotenoid content into novel ketocarotenoids, which accumulated to a level of 2.4 mg/g root dry weight, and resulted in a significant color shift towards red (Fig. 3e). The experiments carried out by Maass et al. [139] in Arabidopsis and carrot (see above) increased the carotenoid levels in carrot roots to 858 μg/g dry weight.

4.4.4. Tomato and other fruits

Ripening tomatoes accumulate large quantities of red pigments including lycopene, but rather lower levels of β-carotene. Several investigators have attempted to overexpress either the endogenous *lycb* gene [67] or equivalent heterologous genes [66,147–149] in order to increase β-carotene, the immediate downstream product of LYCB (e.g. a 32-fold increase in the case of D'Ambrosio et al. [67], resulting in orange-colored tomato fruits; Fig. 3f). Another successful strategy was the suppression of the endogenous *DET1* gene, which regulates photomorphogenesis. The expression of a *det1* RNAi construct in tomato chromoplasts increased β-carotene levels 8-fold to 130 μg/g dry weight [150].

Some interesting work has also been carried out in citrus fruits. The *psy* gene from the Cara Cara navel orange (*Citrus sinensis* Osbeck) has been overexpressed in Hong Kong kumquat (*Fortunella hindsii* Swingle) [151], generating fruits with 2.5-fold higher

Table 5
Carotenoid enhancement in transgenic plants.

Species	Genes (origin)	Promoters	Carotenoid levels in transgenic plants	References
Rice (*Oryza sativa*)	*psy1* (daffodil)	CaMV35S (constitutive) Gt1 (seed specific)	0.3 μg/g dry weight (DW) phytoene in seeds 0.74 μg/g DW phytoene in seeds	[68]
	psy1 and *lycb* (daffodil) *crt*I (*Pantoea ananatis*)	Gt1 (*psy1* and *lycb*) and CaMV35S (*crt*I)	1.6 μg/g DW total carotenoids in endosperm	[69]
	psy1 (corn; *Zea mays*) *crt*I (*Pantoea ananatis*)	Gt1	37 μg/g DW total carotenoids in seeds	[70]
Canola (*Brassica napus*)	*crt*B (*P. ananatis*)	Napin (seed specific)	1617 μg/g fresh weight (FW) total carotenoids in seeds (50-fold)	[60]
	*crt*B (*P. ananatis*)	Napin	1341 μg/g FW total carotenoids in seeds	[61]
	*crt*E and *crt*B (*P. ananatis*)		1023 μg/g FW total carotenoids in seeds	
	*crt*B (*P. ananatis*) *crt*I *(P. ananatis)*		1412 μg/g FW total carotenoids in seeds	
	*crt*B and *crt*Y (*P. ananatis*)		935 μg/g FW total carotenoids in seeds	
	*crt*B and *β-cyclase* (*B. napus*)		985 μg/g FW total carotenoids in seeds	
	*crt*B and *crt*Y (*P. ananatis*) *crt*I *(P. ananatis)*		1229 μg/g FW total carotenoids in seeds	
	idi, *crt*E, *crt*B, *crt*I and *crt*Y (*P. ananatis*) *crtZ*, *crt*W (*Brevundimonas* sp.)	CaMV35S, napin and Arabidopsis *FAE1* (seed specific)	412–657 μg/g FW total carotenoids in seeds (30-fold) 60–190 μg/g FW total ketocarotenoids in seeds	[74]
Tomato (*Solanum lycopersicum*)	*psy1* (tomato)	CaMV35S	3615 μg/g DW total carotenoids in vegetative tissue (1.14-fold)	[227]
	psy1 (tomato)	CaMV35S	2276.7 μg/g DW total carotenoids in fruit (1.25-fold) 819 μg/g DW β-carotene in fruit (1.4-fold)	[228]
	*crt*I (*P. ananatis*)	CaMV35S	520 μg/g DW (1.9-fold) β-carotene in fruit	[64]
	lycb (Arabidopsis) *chyb* (pepper; *Capsicum annuum*)	pds	63 μg/g FW β-carotene in fruit (12-fold)	[147]
	*crt*B (*P. ananatis*)	Polygalacturonase (fruit specific)	825 μg/g DW β-carotene in ripe fruit (2.5-fold)	[229]
	dxs (*Escherichia coli*)	Fibrillin	7200 μg/g DW total carotenoids in fruit (1.6-fold)	[59]
	det-1 (tomato, antisense)	P119, 2A11 and TFM7 (fruit specific)	130 μg/g DW β-carotene (8-fold) in red-ripe fruit (assuming a water content of 90%)	[150]
	CRY2 (tomato)	CaMV35S	1490 μg/g DW total carotenoids ripe fruit pericarps (1.7-fold) 101 μg/g DW β-carotene ripe fruit pericarps (1.3-fold)	[154]
	chrd (cucumber; *Cucumis sativus*)	CaMV35S	Reduced carotenoid levels in flower	[230]
	*crt*Y(*P. ananatis*)	aptI	286 μg/g DW β-carotene in fruit (4-fold)	[148]
	Fibrillin (pepper)	Fibrillin	150 pg/g FW β-carotene in fruit	[231]
	lycb (Arabidopsis; *Arabidopsis thaliana*)	pds (fruit specific)	546 μg/g DW FW total carotenoids in fruit (7-fold) (assuming a water content of 90%)	[66]
	lycb (tomato)	CaMV35S	2050 μg/g DW total carotenoids in fruit (31.7-fold) (assuming a water content of 90%)	[67]
	lycb (daffodil)	Ribosomal RNA	950 μg/g DW β-carotene in fruit	[149]
Potato (*Solanum tuberosum*)	*ZEP* (Arabidopsis)	GBSS (tuber specific)	60.8 μg/g DW total carotenoids in tubers (5.7-fold)	[145]
	*crt*B (*P. ananatis*)	Patatin (tuber specific)	35 μg/g DW total carotenoids in tubers (6.3-fold) 11 μg/g DW β-carotene in tubers (19-fold)	[232]
	lyce (potato, antisense)	Patatin	9.9 μg/g DW total carotenoids in tubers (2.5-fold) 0.043 μg/g DW β-carotene in tubers (14-fold)	[143]
	*crt*O (*Synechocystis* sp.)	CaMV35S	39.76 μg/g DW total carotenoids in tubers Ketocarotenoids represented 10–12% of total carotenoids in tubers	[233]
	dxs (*E. coli*)	Patatin	7 μg/g DW total carotenoids in tubers (2-fold)	[234]
	*crt*B (*P. ananatis*) *bkt1* (*Haematococcus pluvialis*)	Patatin	5.2 μg/g DW total carotenoids in tubers 1.1 μg/g DW total ketocarotenoids in tubers	[73]
	bkt1 (*H. pluvialis*)		30.4 μg/g DW total carotenoids in tubers 19.8 μg/g DW total ketocarotenoids in tubers	
	or (cauliflower; *Brassica oleracea* var *botrytis*)	GBSS	25 μg/g DW total carotenoids (6-fold) in tubers	[79]

Table 5 (*Continued*)

Species	Genes (origin)	Promoters	Carotenoid levels in transgenic plants	References
	or (cauliflower)	GBSS	31 μg/g DW total carotenoids in tubers (5.7-fold)	[80]
	crtB, crtI and *crtY* (*P. ananatis*)	Patatin	114 μg/g DW total carotenoids in tubers (20-fold) 47 μg/g DW β-carotene in tubers (3600-fold)	[141]
	bch (potato, antisense)	Patatin	9.3 μg/g DW total carotenoids in tubers (4.5-fold) 0.085 μg/g DW β-carotene in tubers (38-fold)	[142]
	bch (potato, antisense)	GBSS and CaMV35S	3.31 μg/g DW β-carotene in tubers	[144]
Corn	*psy1* (*Z. mays*)	Wheat LMW glutelin, barley D-hordein, corn γ-zein, rice prolamin (all endosperm-specific)	146.7 μg/g DW total carotenoids in seeds	[26]
	*crt*I (*P. ananatis*) *crt*W (*Paracoccus* spp.) *lycb* (*Gentiana lutea*)		35.85 μg/g DW total ketocarotenoids in seeds	
	*crt*B *and crt*I (*P. ananatis*)	Super γ-zein	33.6 μg/g DW total carotenoids in seeds (34-fold)	[25]
	psy1 (*Z. mays*) *crt*I (*P. ananatis*)	Wheat LMW glutelin and barley D-hordein	163.2 μg/g DW total carotenoids in seeds (112-fold) 59.32 μg/g DW β-carotene in seeds (169-fold)	[27]
Lotus japonicus	*crt*W (*Agrobacterium aurantiacum*)	CaMV35S	387 μg/g FW total carotenoids in flower petals (1.5-fold) 89.9 μg/g FW total ketocarotenoids in flower petals (2.2-fold)	[235]
Carrot	*bkt1* (*H. pluvialis*) *chyB* (Arabidopsis)	CaMV35S and *Agrobacterium rhizogenes rol*D (root specific)	345.5 μg/g FW total carotenoids in root 2400 μg/g root DW novel ketocarotenoids	[146]
	psy (Arabidopsis)	CaMV35S	858.4 μg/g DW total carotenoids in roots	[139]
Tobacco	*crt*W and *crt*Z (*Paracoccus* sp.)	CaMV35S	1275 μg/g DW total carotenoids in leaves 64 μg/g FW total ketocarotenoids in leaves	[138]
	*crt*O (*Synechocystis* sp.) *crt*Z (*P. ananatis*)	CaMV35S	839 μg/g DW total carotenoids in leaves (2.5-fold) 342.4 μg/g DW total ketocarotenoid in leaves	[72]
	*crt*O (*Synechocystis* sp.)	CaMV35S	429 μg/g DW total carotenoids in leaves 156.1 μg/g DW total ketocarotenoid in leaves	[236]
	*crt*W and *crt*Z (*Brevundimonas* sp.)	*rrn*	7380 μg/g FW total carotenoids in leaves (2.1-fold) 7290 μg/g FW total ketocarotenoids in leaves	[137]
Wheat	*psy1* (*Z. mays*) *crt*I (*P. ananatis*)	CaMV35S and 1Dx5 (constitutive)	4.96 μg/g DW in seeds	[237]
Arabidopsis	*bkt1* (*H. pluvialis*)	Napin	4-keto-lutein, canthaxanthin and adonirubin seeds up to 13-fold	[238]
	bch (Arabidopsis)	CaMV35S	2274.8 nmol/g DW total carotenoids	[239]
	psy (Arabidopsis)	Napin	260 μg/g FW β-carotene in seeds	[240]
	psy (Arabidopsis)	CaMV35S	1600 μg/g DW (10-fold) in seed-derived calli and 500 μg/g DW (100-fold) of total carotenoids in roots	[139]
	chyB (Arabidopsis)	CaMV35S	285 mmol/chl a(mol) violaxanthin (2-fold) 728 mmol/chl a(mol) of total carotenoid	[241]
	AtB1 (Arabidopsis)	CaMV35S	38.2 μg/g β-carotene leaf tissue	
	CYP97A3 (Arabidopsis)	CaMV35S	41.7 μg/g β-carotene leaf tissue	
	CYP97B3 (Arabidopsis)	CaMV35S	36.7 μg/g β-carotene leaf tissue	
	CYP97C1 (Arabidopsis)	CaMV35S	41.3 μg/g β-carotene leaf tissue	

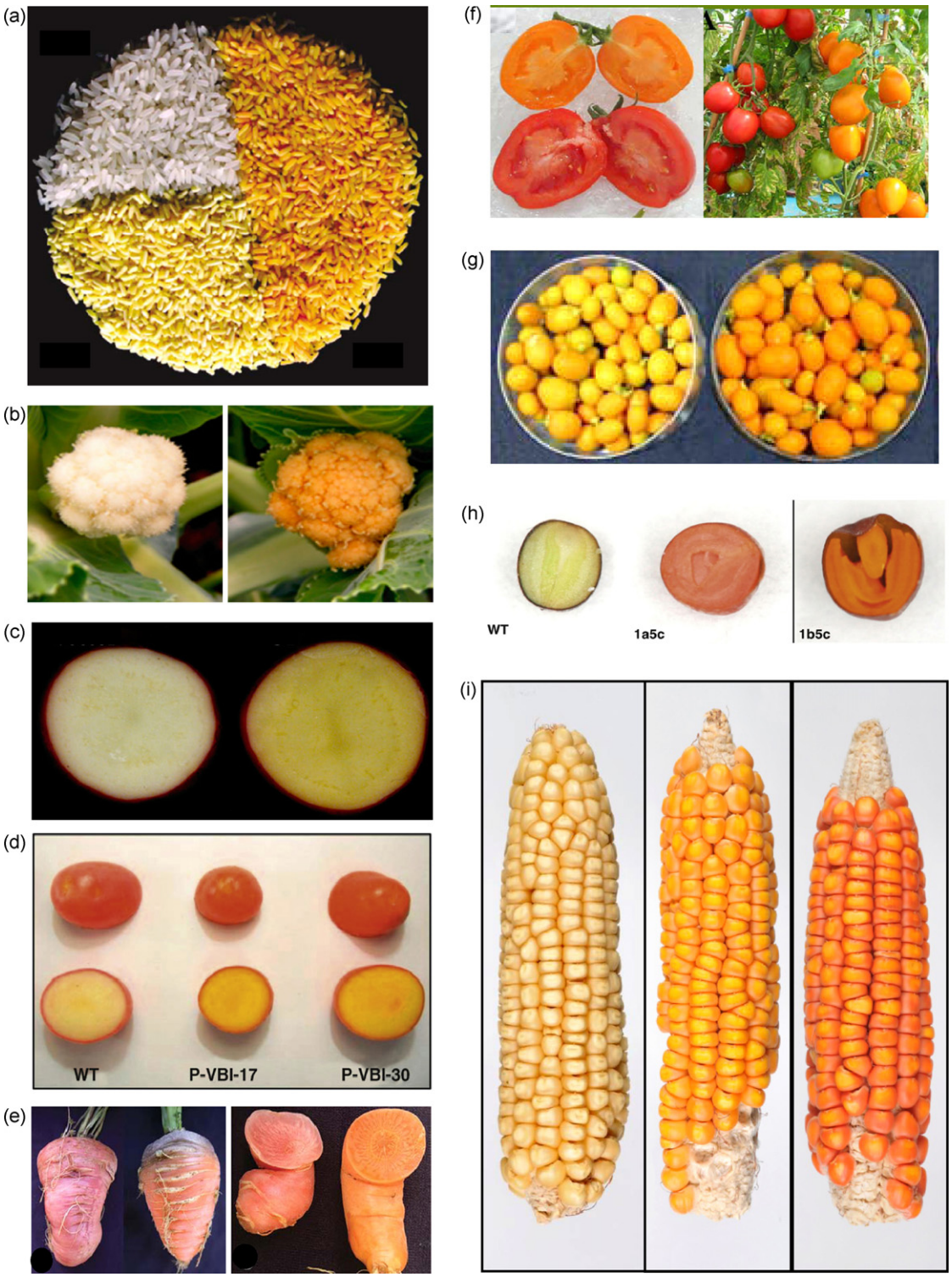

Fig. 3. Plants engineered to increase the levels of specific carotenoids. (a) Comparison of wild type rice grains (white, top left) with those of Golden Rice (bottom left) and Golden Rice 2 (right) [70]. (b) Wild type cauliflower heads (left) compared with a transgenic variety expressing the dominant *Or* allele [79]. (c) Wild type potato tuber (left) compared with a transgenic variety expressing the cauliflower *Or* transgene [80]. (d) Wild type potato tuber compared with two transgenic lines [highest carotenoid levels (>110 μg/g dry weight], expressing bacterial *crt*B, *crt*I and *crt*Y genes [141]. (e) Wild type carrot compared to transgenic red variety with a high ketocarotenoid content. Left panel shows uncut carrots, right panel shows same carrots cut transversely to show flesh. In each panel, the wild type variety is on the right and the transgenic variety is on the left [146]. (f) The panel shows wild type Red Setter tomato fruits (bottom) compared to an orange transgenic variety accumulating high levels of β-carotene (top). Right panel shows same fruits growing on the vine [67]. (g) Wild type Hong Kong kumquat (left) compared to transgenic fruit (right) expressing the *psy* gene from the Cara Cara navel orange, with higher levels of β-carotene [151]. (h) Wild type canola seed (left) compared to two transgenic varieties expressing seven carotenogenic transgenes and accumulating higher carotenoid levels [74]. (i) Wild type white endosperm corn M13W (left) compared with a transgenic line (middle) accumulating high levels of β-carotene (57 μg/g DW) [27], and a transgenic line (right) expressing five carotenogenic genes (corn *psy1*, *Paracoccus crt*W and *crt*I, and *Gentiana lutea lycb* and *bch*) and accumulating significant amounts of ketocarotenoids (35 μg/g DW) and β-carotene (34.81 μg/g DW) [28].(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

levels of phytoene (~71 μg/g fresh weight) and also higher levels of lycopene, β-carotene and β-cryptoxanthin, resulting in a significant shift from yellow to orange coloring (Fig. 3g). The levels of lutein and violaxanthin in the fruits remained largely unchanged.

4.4.5. Carotenoid-rich canola

Carotenoids are fat-soluble, so their consumption as a minor component of vegetable oil increases their bioavailability. Canola (*Brassica napus*) is an oil crop that produces large amounts of carotenoids (18–26 μg/g dry weight) and it is therefore considered a valuable dietary source and a good target for carotenoid engineering. Shewmaker et al. [60] increased the carotenoid content of canola to 1180 μg/g dry weight by expressing *crt*B, an achievement that was improved by Ravanello et al. [61] using the same gene (1341 μg/g dry weight). The combined expression of *crt*B and *crt*I boosted levels to 1412 μg/g dry weight, but the further addition of *crt*Y reduced total levels to 1229 μg/g dry weight although it increased the relative amount of β-carotene [61] (Fig. 3h). RNAi has been used to reduce the expression of LYCE in canola, increasing the levels of β-carotene, zeaxanthin and violaxanthin as expected, but also the levels of lutein suggesting that the endogenous *lyce* gene may represent a rate-limiting step [152]. As discussed earlier, Fujisawa et al. [74] introduced seven carotenogenic genes into canola including *crt*W and *crt*Z, which are involved in ketocarotenoid biosynthesis. The total amount of carotenoids in the seeds was 412–657 μg/g fresh weight, a 30-fold increase over wild type, including 60–190 μg/g of ketocarotenoids.

4.4.6. Combinatorial transformation in corn

Several groups have used biotechnology to increase carotenoid levels in corn, e.g. Aluru et al. [25] introduced the bacterial *crt*B and *crt*I genes under the control of a 'super γ–zein promoter' to provide strong endosperm-specific expression, increasing the total carotenoid content to 33.6 μg/g dry weight. A significant advance was achieved by Zhu et al. [26] with the development of a combinatorial nuclear transformation system designed to dissect and modify the carotenoid biosynthetic pathway in corn, using the white endosperm variety M37W. Essentially, the method involves 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 (the corn *psy1* gene, the *Gentiana lutea lycb* and *bch* genes and two bacterial genes *crt*I and *crt*W) under the control of endosperm-specific promoters. Using the M37W line as the genetic background provided a blank template because the endosperm in this variety lacks all carotenoids as it is blocked at the first stage of the pathway due to the complete absence of PSY activity. 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 (Fig. 3i). The plants contained high levels of β-carotene, lycopene, zeaxanthin, lutein, and additional commercially relevant ketocarotenoids such as astaxanthin and adonixanthin [26].

Another recent breakthrough in this area was the development of transgenic corn plants transformed with multiple genes enabling the simultaneous modulation of three metabolic pathways, increasing the levels of three key vitamins (β-carotene, ascorbate and folate) in the endosperm [27]. This was achieved by transferring four genes into the M37W corn variety described above, resulting in a 169-fold elevation of β-carotene levels (57 μg/g dry weight), a 6.1-fold increase in ascorbate (106.94 μg/g dry weight) and 2-fold increase in folate (200 μg/g dry weight).

5. Outlook

5.1. Outlook for fundamental research

Although the search for novel carotenogenic genes continues, the current status of carotenoid research is somewhat restricted by its reliance on the gene-by-gene approach to metabolic engineering. In other pathways, the focus has shifted away from individual genes or collections thereof and towards overarching regulatory mechanisms that may allow multiple genes in the pathway to be controlled simultaneously. One example of the above is the terpenoid indole alkaloid biosynthesis pathway, where many of the genes are under common transcriptional control through induction by methyl jasmonate. The recognition of this regulatory link led directly to the identification of a common transcription factor called ORCA2 that binds corresponding response elements in many of these genes' upstream promoters; the *ORCA2* gene is itself induced by jasmonate and its overexpression leads to coordinate upregulation of many of the enzymes in the pathway [153]. Few similar studies have been carried out with regard to carotenoid metabolism, although a number of candidate transcriptional regulators have been identified including CRY2, DDB1, HY5, DET1 and COP1 [150,154–156]. One promising approach, which has also been applied in the alkaloid metabolic pathway resulting in the identification of transcription factor ORCA3, is to use activation tagging and/or T-DNA mutagenesis in an effort to identify global regulators of carotenogenic genes. In such a strategy, random insertion lines containing mutagenic T-DNA sequences, or T-DNA sequences containing strong, outward-facing promoters to activate genes adjacent to the insertion site, would be tested to identify insertions that caused broad induction or repression of carotenogenesis.

Another key strategy for ongoing research into carotenoid metabolism is the identification of key residues in the ketocarotenoid-synthesizing enzymes that control substrate specificity. These enzymes are prime candidates for protein engineering since their precise affinity for different substrates and their kinetic properties play a predominant role in deciding the final spectrum of compounds that are produced. As an example, a CrtW-type β-carotene ketolase gene isolated from *Sphingomonas* sp. DC18 was subjected to localized random mutagenesis in order to increase its activity on hydroxylated carotenoids. As in other areas of carotenoid research, the ability to screen on the basis of color provided a handy and robust way to ascertain whether any of the mutations facilitated astaxanthin production. Six mutations showed improved astaxanthin production without affecting competitive reactions, but when two of these were combined in the same enzyme they had an additive effect and also reduced the production of canthaxanthin from β-carotene [157].

5.2. Outlook for applied research

The major application of carotenoid research is in health and nutrition, based on the numerous reports showing the health benefits of carotenoids, particularly those with vitamin A activity [18]. As well as the specific role of β-carotene, α-carotene, γ-carotene and β-cryptoxanthin in the production of retinal, most carotenoids have beneficial antioxidant activity, with lutein and zeaxanthin having a specific protective role in the macular region of the human retina. Astaxanthin, which is normally acquired from seafood, also has several essential protective functions including the prevention of lipid oxidation, UV damage and damage to the immune system [158]. The positive role of carotenoids in the diet is widely accepted and valued and foods rich in carotenoids (particularly fresh fruit, vegetables and seafood) are commonly regarded as essential components of a healthy diet [1,2].

Astaxanthin is the source of pink/red pigmentation in certain types of fish and seafood, and currently this molecule is extracted from the yeast *Xanthophyllomyces dendrorhous* or the green alga *Haematococcus pluvialis*, or is synthesized chemically [158]. It is added to feed so that aquaculture products (particularly salmon, rainbow trout and red sea bream) develop the appropriate quality characteristics demanded by consumers. This accounts for 25% of the total feed cost, and 12% of the overall cost of rearing fish. One likely output of carotenoid research in the near future is the provision of plant-based fish food incorporating astaxanthin and other carotenoids, as these will not only satisfy consumers but also contribute to fish health [2].

Many animals benefit from diets rich in carotenoids, and humans also benefit from the better quality food products. For example, the major carotenoids in hens' and quails' eggs are lutein and zeaxanthin, and these are concentrated in the yolk [159,160]. Feeding hens with corn enriched for carotenoids would contribute to a number of vital physiological and protective roles during embryonic development, growth and during the lifetime of the laying hens [161], while humans would benefit from the rich yolk color, which is an important quality trait [162], as well as the higher nutrient density and bioavailability (carotenoids are more bioavailable when consumed as egg yolk compared to most vegetable sources because of the lipid content [163]).

One further potential application of carotenoid engineering is for the extraction of specific carotenoid products for purification and use as antioxidants, pigments, food/feed additives, pharmaceuticals, nutraceuticals and cosmetics. The global carotenoid market is thought to be worth more than $US 2 billion, so the ability to produce higher levels of key carotenoid compounds, especially those with strong markets, would provide an enormous competitive advantage. Lycopene and β-carotene are both used as food additives to provide color, increase shelf life and improve nutrition. For example, margarine is naturally white and deteriorates rapidly due to oxidation, but the addition of β-carotene (extracted from carrots or canola) provides color, delays oxidation and also provides vitamin A in a lipophilic environment ready for adsorption. Lycopene, extracted from tomato juice, has recently been approved as a food additive in Europe [164]. Zeaxanthin is often extracted commercially from red marigold flowers which are also rich source of lutein. As discussed above, astaxanthin is extracted from specific yeast and algae or is synthesized chemically [158,164–167]. All these molecules could be extracted at a lower cost from transgenic plants, especially if the plants were engineered to produce multiple carotenoid molecules which could be extracted in a single step and then separated.

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