Towards vitamin biofortification in maize through multigene engineering

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TOWARDS VITAMIN BIOFORTIFICATION IN MAIZE THROUGH MULTIGENE ENGINEERING

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PhD Thesis

TOWARDS VITAMIN BIOFORTIFICATION IN MAIZE THROUGH MULTIGENE ENGINEERING

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SUMMARY

The major component of my research program focused on an in depth molecular and biochemical analysis of maize plants from different genetic backgrounds containing metabolic pathways introgressed from parents engineered with different transgenes representing the carotenoid, ketocarotenoid and vitamin E pathways. In the first instance I introgressed a carotenogenic mini-pathway into different yellow maize inbred lines having diverse carotenoid profiles. These experiments resulted in hybrids with higher amounts of carotenoids compared to their corresponding parents. Targeted transcriptomic and metabolite analysis revealed bottlenecks in sequential steps in the ε- and β-branches of the carotenoid pathway in the newly created hybrids. I discuss my results in terms of how plants I have generated might contribute towards a rapid and effective production of maize hybrids with high and diverse carotenoid content. I generated transgenic maize plants co-expressing *Arabidopsis thaliana PDS1*, *HPT1*, *VTE3* and *VTE4* genes in order to recreate the vitamin E biosynthetic pathway in maize. I then used a stacking strategy to introgress the vitamin E mini-pathway into a second transgenic maize line expressing *Zmpsy1* and *PacrtI* in an attempt to determine the specific impact on metabolite accumulation and interaction of the vitamin E and the carotenoid biosynthetic pathways. I also generated a novel maize line (ZWB line) co-expressing two β-carotene ketolases (*sBcrtW* and *sCrbkt*) and a β-carotene hydroxylase (*sBcrtZ*). This line accumulated astaxanthin as the only carotenoid, demonstrating total conversion of the carotenoid precursor pool in the wild type line used for the transformation experiment to this ketocarotenoid. Introgression of the ketocarotenoid biosynthetic pathway from ZWB into transgenic maize lines engineered previously with different carotenogenic genes and a wild type line accumulating high levels of β-carotene, resulted in hybrids which not only accumulated astaxanthin, but also other intermediate carotenoids and ketocarotenoids. My work demonstrates that choice of an appropriate genetic background containing a partial carotenoid pathway influences significantly carotenoid conversion to downstream molecules, including astaxanthin, in hybrids. I have also recovered and analyzed maize lines accumulating pABA and pterin, precursors involved in folate biosynthesis. The levels of these precursors in the transgenic lines I generated were not sufficient to enhance folate content in maize. I concluded that additional or alternative steps in the pathway and/or metabolism need to be engineered to achieve substantial folate accumulation in maize.

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RESUMEN

El componente principal de mi programa de investigación ha sido un análisis detallado, a nivel molécular y bioquímico de plantas de maíz provenientes de cruces con parentales con fondos genéticos diferentes. Los parentales estaban mejorados genéticamente con transgenes que codificaban para las rutas metabólicas de los carotenoides, cetocarotenoides y vitamina E. Inicié mi investigación introduciendo una mini-ruta metabólica carotenogénica en diferentes líneas puras de maíz amarillo que contenían diferentes perfiles de carotenoides. El resultado de este experimento fue la obtención de una colección de maíces híbridos con elevadas cantidades de carotenoides, al compararlos con sus parentales correspondientes. Los análisis transcriptómicos y metabólicos de los nuevos maíces híbridos nos mostraron limitaciones en pasos específicos de las ramificaciones ε y β de la ruta de los carotenoides. La conclusión más importante fue que las plantas que generadas pueden contribuir hacia una rápida y efectiva producción de híbridos con elevado y diverso contenido de carotenoides. Hice un segundo experimento de transformación de maíz donde co-introduje los genes *PDS1*, *HPT1*, *VTE3* y *VTE4* provenientes de *Arabidopsis thaliana* con la intención de recrear la ruta metabólica de la vitamina E. Para profundizar en el impacto específico de la acumulación e interacción de vitamina E con la ruta metabólica de los carotenoides, introduje la mini-ruta metabólica de la vitamina E en una línea de maíz transgénica que ya expresaba *Zmpsy1* y *PacrtI*. También generé una nueva línea de maíz (línea ZWB) co-expresando dos β-caroteno cetolasas (*sBcrtW* and *sCrbkt*) y una β-caroteno hidroxilasa (*sBcrtZ*). Esta línea acumuló astaxantina como único carotenoide, demostrando una total conversión de los carotenoides de la línea salvaje utilizada para la transformación en este experimento a este cetocarotenoide. La introgresión a una línea salvaje con alto contenidos de β-caroteno de la ruta metabólica de los cetocarotenoides proveniente de ZWB, resultó en híbridos que además de acumular astaxantina, también acumulaban otros carotenoides y cetocarotenoides intermediarios. Mi trabajo demuestra que la elección de un fondo genético que contenga una ruta metabólica parcial influye significativamente en la conversión de los carotenoides a moléculas de pasos posteriores en la ruta metabolica, incluyendo astaxantina, en los híbridos. Finalmente regenere y analice líneas de maíz que acumulaban pABA y pterina, precursores involucrados en la biosíntesis de folato. Los niveles de estos precursores en las líneas transgénicas que regeneré no fueron suficientes para el incremento de folato

en maíz. Mi conclusión fue que se requiere de un paso adicional o alternativo en la ruta metabólica y/o en el metabolismo para alcanzar una acumulación significativa de folato en maíz.

RESUM

El component principal del meu programa de investigació ha estat un anàlisi detallat, a nivell molecular i bioquímic de plantes de blat de moro que provenien de creuaments amb parentals de fons genètics diferents. Els parentals van ser millorats genèticament amb transgens que codificaven per a las rutes metabòliques dels carotenoids, cetocarotenoids i vitamina E. Vaig iniciar la meva recerca introduint una mini-ruta metabòlica carotenogènica en línies pures de blat de moro de color groc que contenien perfiles variats de carotenoids. El resultat va ser l'obtenció d'una col·lecció de blats de moro híbrids amb unes concentracions de carotenoids més elevades que els parentals originaris. Les anàlisis transcriptòmiques i metabòliques dels nous híbrids van mostrar limitacions en passos específics de les ramificacions ε i β de la ruta dels carotenoids. La conclusió més important va esser que aquestes plantes podrien contribuir cap una ràpida i efectiva producció d'híbrids amb un contingut divers i elevat de carotenoids. En un altre assaig de transformació vaig co-introduir els gens *PDS1*, *HPT1*, *VTE3* i *VTE4* d'*Arabidopsis thaliana* amb la intenció de recrear la ruta metabòlica de la vitamina E. Per a aprofundir en l'impacte específic de l'acumulació i interacció de la vitamina E amb la ruta metabòlica dels carotenoids, vaig introduir la mini-ruta metabòlica de la vitamina E en una línia de blat de moro transgènica que ja expressava *Zmpsy1* i *PacrtI*. També vaig generar una nova línia de blat de moro (línia ZWB) co-expressant dos β-carotè cetolases (*sBcrtW* i *sCrbkt*) i una β-carotè hidroxilasa (*sBcrtZ*). Aquesta línia només va acumular astaxantina, demostrant una conversió total dels carotenoids de la línia salvatge (utilitzada per a la transformació en aquest experiment) a cetocarotenoids. La introgressió de la ruta metabòlica dels cetocarotenoids de ZWB a una línia salvatge amb elevat contingut de β-carotè, va produir híbrids que, a més d'acumular astaxantina, també acumulessin altres carotenoids i cetocarotenoids intermediaris. Amb aquest treballs he demostrat que l'elecció d'un fons genètic que contingui una ruta metabòlica parcial influencia significativament la conversió dels carotenoids a molècules de passos posteriors en la ruta metabòlica, incloent l'astaxantina. Finalment vaig regenerar i analitzar línies de blat de moro que acumulaven pABA i pterina, precursors involucrats en la biosíntesis de folat. Els nivells d'aquest precursors en les línies obtingudes no van ser suficients per incrementar el contingut de folat en blat de moro. Vaig concloure que calia un pas addicional o

alternatiu en la ruta metabòlica i/o en el metabolisme per assolir una acumulació significativa de folat en blat de moro.

LIST OF ABBREVIATIONS

AFLPs	Amplified fragment length polymorphisms (AFLPs)
AtHPT	Homogentisate phytylpernyltransferase
AtPDS1	Phytoene desaturation mutant gene number 1
AtVTE2	Homogentisate phytylpernyltransferase
AtVTE3	2-Methyl-6-phytylplastoquinol methyltransferase
AtVTE4	γ-Tocopherol methyltransferase
BCH	Carotenoid β-hydroxylase
BKT	β-Carotene ketolase
CaMV 35S	Cauliflower Mosaic Virus 35S
cDNA	Complementary DNA
CRTB	Bacterial phytoene synthase
CRTI	Bacterial β-carotene desaturase
CRTISO	Carotenoid isomerase
CRTW	Bacterial β-carotene ketolase
CRTY	Bacterial lycopene cyclase
CRTZ	Bacterial β-carotene hydroxylase
CYP97	Carotene ε-ring hydroxylase
DAP	Days after pollination
DMAPP	Dimethylallyl diphosphate
DMGGBQ	2,3-Dimethyl-6-geranylgeranylbenzoquinol
DMPBQ	2,3-Dimethyl-5-phytyl-1,4-benzoquinol
DNA	Deoxyribonucleic acid
DW	Dry weight
DXP	1-Deoxy-D-xylulose-5-phosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
FW	Fresh weigh
γ–TMT	γ–Tocopherol methyltransferase
GGPP	Geranylgeranyl diphosphate
GGPPS	Geranylgeranyl diphosphate synthase
HDR	DXP reductoisomerase
HGA	Homogentisic acid
HGGT	Homogentisate geranylgeranyltransferase
HPP	Hydroxyphenylpyruvate
HPPD	Hydroxyphenylpyruvate dioxygenase
HPLC	High performance liquid chromatography
HPT	Hygromycin phosphotransferase
HYDB	β-Carotene hydroxylase
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
LYCB	Lycopene β-cyclase
LYCE	Lycopene ε-cyclase
MEP	2-C-Methyl-D-erythritol 4-phosphate pathway
MGGBQ	2-Methyl-6-geranylgeranylbenzoquinol
MPBQ	2-Methyl-6-phytylbenzoquinol
MPBQ-MT	2-Methyl-6-phytylbenzoquinol methyltransferase
mRNA	Messenger RNA
MVA	Mevalonic acid pathway

OR	Orange gene
p326/LMW	Wheat low molecular weight glutenin promoter
PCR	Polymerase chain reaction
PDP	Phytyldiphosphate
PDS	Phytoene desaturase
PSY	Phytoene synthase
QTL	Quantitative Trait Locus
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
TC	Tocopherol cyclase
VAD	Vitamin A deficiency
ZDS	ζ-Carotene desaturase
ZEP	Zeaxanthin epoxidase
ZISO	ζ-carotene isomerase
Zm	*Zea mays*

GENERAL INTRODUCTION

I.   Vitamins and antioxidants in human health

Vitamins as essential nutrients

Vitamins are defined as a group of complex organic compounds present in minute amounts in natural foodstuff that are essential to normal metabolism and lack of which in the diet causes deficiency diseases (McDowell, 2008). Vitamins consist of a mixed group of chemical compounds and are not related to each other as are proteins, carbohydrates or fats, and cannot be manufactured by the body in sufficient amounts in all circumstances and must therefore be obtained from the diet (Bender, 2003). Vitamins are required in trace amounts (micrograms or milligrams per day) in the diet for health, growth and reproduction. Omission of a single vitamin from the diet will produce deficiency symptoms (Basu & Dickerson 1996; Bender, 2003). Most vitamins are required because humans lack the metabolic capability to produce them, although vitamin D is an exception because it is produced in the skin during the exposure to UVB irradiation albeit not always in sufficient quantities to make dietary sources unnecessary (Basu & Dickerson, 1996). Vitamins are classified as either water- or fat-soluble. In the context of human health, there are four fat-soluble vitamins (A, D, E and K), which are transported through the body in fat globules and stored in the liver and other fatty tissues, and nine water-soluble vitamins (eight B vitamins and vitamin C) that are not stored in the body and must be replaced every day (Moreno & Salvadó, 2000). Vitamins are found primarily in plants and are present in animal tissue only as a consequence of consumption of plants, or because the animal harbours microorganisms that synthesize them (McDowell, 2008). Vitamin B_{12} is unique in that it occurs in plants as a result of microbial synthesis. Two of the four fat-soluble vitamins, A and D, differ from water-soluble B vitamins in that they occur in plants in a provitamin form (a precursor of the vitamin), which can be converted into a vitamin in the animal body. Vitamin and mineral deficiencies have been referred to as "hidden hunger"; such deficiencies occur on a population-wide basis when the diet lacks diversity or is overly dependent on a single staple crop, but in individual cases may reflect a genetic abnormality that prevents nutrient absorption or metabolism (Allen, 2003; FAO/WHO, 2002; Yuan et al., 2011).

Although all vitamins are equally important in supporting life, vitamin A is considered the most important vitamin from a practical standpoint (UNICEF, 2013). The reduced form of vitamin A (retinal) is required for the production of rhodopsin, which is essential for vision and also helps to maintain epithelial immune cells. The acidic form (retinoid acid) is a morphogen in development (Goodman & Huang, 1965). Humans can produce retinal and retinoic acid if provided with a source of retinol, one of its esters, or it can also be synthesized directly from β-carotene (also known as pro-vitamin A) (Goodman & Huang, 1965). A second fat-soluble vitamin, vitamin E, comprises eight related molecules known as tocochromanols (DellaPenna & Pogson, 2006). The tocochromanols are powerful antioxidants that protect fatty acids, low density lipoproteins (LDLs) and other components of cell membranes from oxidative stress (Ricciarelli et al., 2002). Vitamin B_9 (folate) is the source of tetrahydrofolate, an essential metabolite in many methylation reactions mediated by S-adenosyl methionine. Tetrahydrofolate is also essential for the synthesis of purines and thymidine and, therefore, for DNA and RNA synthesis (Reynolds, 2006). Vitamin B_9 together with vitamin, B_{12} is involved in the synthesis of methionine from homocysteine (Reynolds, 2006).

Antioxidants confer health benefits to humans and animals

Many non-essential molecules consumed in the diet are also antioxidants with health-promoting effects, and hence there is an overlap between essential nutrients and non-essential compounds (sometimes described as nutraceuticals) that act as antioxidants (Zhu et al., 2013). Antioxidants inhibit the oxidation of other molecules and thereby prevent them from causing oxidative damage, which is a major contributory factor to diseases associated with ageing and with ageing itself (Valko et al., 2007). Antioxidant molecules are generally either lipophilic or hydrophilic, but both types act through common molecular mechanisms for their antioxidant effects. Such mechanisms include hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT), sequential proton loss electron transfer (SPLET) and the formation of radical adducts (Zhu et al., 2013). Some antioxidants have a single active mechanism, for example vitamin C uses HAT alone, whereas others employ multiple mechanisms (Zhu et al., 2013).

The three major lipophilic antioxidant classes of molecules in mammals are carotenoids, tocochromanols and coenzyme Q_{10}, all of which are derived from terpenoids (Grassmann, 2005). Carotenoids and tocochromanols are obtained from the diet whereas coenzyme Q_{10} is synthesized de novo by a multistep pathway starting with acetyl-CoA (Zhu et al., 2011). All three classes act primarily by scavenging lipid peroxyl radicals (ROO·) and by disrupting free radical chain reactions in membranes (Zhu et al., 2013). The best known carotenoids are those with pro-vitamin A activity (particularly β-carotene) because these represent a major dietary source of vitamin A, but others with important antioxidant effects include xantophylls (e.g. zeaxanthin, lutein or lycopene) and ketocarotenoids (e.g. astaxanthin or canthaxanthin) (Guerin et al., 2003; Stahl & Sies, 2005). Carotenoids, being exceptionally efficient physical and chemical quenchers of $^1O2$ and other ROS, have garnered particular attention as potentially protective agents against ROS-mediated disorders (Zhu et al., 2013). A large body of data, mostly from experiments with β-carotene, lycopene, lutein and zeaxanthin, have been collected in a number of epidemiological, interventional and clinical studies, generally supporting the observation that the adequate intake of carotenoid-rich fruits and vegetables or carotenoid supplements may significantly reduce the risk of some chronic diseases (Böhm et al., 2012; Linnewiel-Hermoni et al., 2015; Stahl & Sies, 2012). Thus, the beneficial effects of carotenoid administration have been confirmed in the case of several types of cancer and cardiovascular and photosensitive disorders, as well as in eye-related diseases (Linnewiel-Hermoni et al., 2015). Clinical trials indicate that lutein and zeaxanthin have an important role in maintaining good vision. These two xanthophylls compose the macular pigment of the focal centre of the retina, where lutein is accumulated in the perifoeveal and zeaxanthin in the foveal region (Beatty et al., 2004; Landrum & Bone, 2001). The two molecules protect photoreceptor cells from free radicals and filter the high-energy wavelengths of blue light. Evidence suggests that a high intake of lutein and zeaxanthin in the diet protects against and prevents age-related macular degeneration (AMD) (Ma et al., 2012; Moeller et al., 2006). The prevalence of AMD is 15% among 65–74 year olds, 25% among 75–84 year olds, and in persons 85 years and older 30% (Klein et al., 2004). Nevertheless, due to the fact that some studies gave inconsistent results more data need to be generated before the carotenoid-ROS-mediated-disorder relationship is firmly established (Fiedor & Burda, 2014). Ketocarotenoids, such as astaxanthin, show a higher antioxidant activity than other carotenoids and also vitamin E (Yuan & Chen, 1999). Astaxanthin targets key

molecules in oncogenic signalling pathways, induces apoptosis and is a promising candidate for cancer prevention and therapy (Kavitha et al., 2013; Kowshik et al., 2014).

The nutritional value of vitamin E, which comprises tocopherols and tocotrienols, was first recognized in 1922 (Evans & Bishop, 1922). The most recognized function of tocopherols in humans is their ability to scavenge and quench reactive oxygen species and lipid-soluble oxidative stress by-products (Bramley et al., 2000; Brigelius-Flohe & Traber, 1999; Ricciarelli et al., 2002). Tocotrienols are more mobile within the biological membrane than tocopherols because of the presence of the unsaturated side-chain and hence penetrate tissues with saturated fatty layers, e.g. in brain and liver more efficiently. They have more recycling ability and are a better inhibitor of liver oxidation (Schaffer et al., 2005). Epidemiological data suggest that high vitamin E intake (100-1000 IU) correlates with a decreased risk of certain types of cancer and cardiovascular diseases (Bramley et al., 2000), improves the immune system and slows down the progression of human degenerative diseases (Traber & Sies, 1996). Due to its role as free radical scavenger, vitamin E is also believed to protect against degenerative processes, such as cancer and cardiovascular diseases (Burton & Traber, 1990; Kamal-Eldin & Appelqvist, 1996; Shewmaker et al., 1999). Recent data suggest the possible benefits of α-tocopherol and β-carotene supplementation on liver cancer and chronic liver disease (CLD), but long-term trial data are limited (Lai et al., 2014).

Folate has been shown to exhibit certain antioxidant properties. It is known to be an efficient in vitro scavenger of free radicals (Joshi et al., 2001) and antioxidant effects of folate have also been observed in patients with coronary artery disease (Doshi et al., 2001). Recent studies indicated that folate administration decreases oxidative status and blood pressure in postmenopausal women (Cagnacci et al., 2015); another study reports dietary folate-supplemented goat milk reduces both plasma transaminases levels, suggesting a hepatoprotective effect and has beneficial effects in situations of Fe-overload, improving the antioxidant enzymes activities and reducing lipid peroxidation (Alférez et al., 2015). However, further investigations are needed to better elucidate the antioxidant properties of folate.

Food insecurity in the 21ˢᵗ century

Food security is one of the pillars of health and well-being in society because humans rely on food not only to supply energy but also for essential nutrients that maintain the immune system and keep the body in a good state of repair. Adequate nutrition therefore correlates with lower morbidity and mortality from both infectious and non-infectious diseases and is particularly important in children and pregnant women where the lack of essential nutrients can lead to irreversible physical and mental damage during development (Hoddinott et al., 2008). Malnutrition is more prevalent in the developing world because it often reflects the lack of access to nutritious food. This in turn is frequently caused by poverty, which often occurs due to ill health and an inability to work, the typical consequences of malnutrition. Poverty, malnutrition and poor health therefore form a self-reinforcing cycle from which many people (and in some cases entire populations) find it impossible to escape (Pérez-Massot et al., 2013). Almost 50 % of the world's population is currently affected by malnutrition (Christou & Twyman, 2004). The majority are subsistence farmers, and their families, who depend entirely on staple cereal crops such as maize or rice for most, if not all their nutritional calories. Such monotonous diets are deficient in several essential nutrients (Zhu et al., 2007). Vitamin A deficiency (VAD) is one of the most prevalent deficiency diseases in developing countries affecting more than 4 million children each year, up to 500,000 of whom become partially or totally blind (Harrison, 2005). Folate and vitamin B12 deficiencies are derived from relatively small, local surveys, but these and national survey data from a number of countries suggest that deficiencies of both of these vitamins may be a public health problem that could affect many millions of people throughout the world (FAO/WHO, 2002).

The poverty-malnutrition-disease cycle needs to be broken by multipoint interventions that provide direct, effective and sustainable approaches to increase the economic welfare of the world's poorest people, including the provision of drugs and vaccines that tackle poor health, and adequate access to nutritious food (Serageldin et al., 2000) Although there are many global initiatives promoting short and mid-term strategies to tackle poverty, food insecurity/malnutrition and disease, sustainable solutions that provide the means for the world's poor to build their own healthy societies are needed as envisaged by the Millennium Development Goals (Yuan et al., 2011).

II.   Vitamins and antioxidants as industrial products

Nutraceuticals and nutricosmetics

For a long time, natural products obtained mainly from plants have been used as a major source of compounds for the prevention and treatment of diseases in humans and animals (Almada, 2008). At the present time, the relationship between food and drugs is converging. Thus, the term nutraceutical was firstly used 20 years ago to describe a union between nutrition and pharmaceuticals, both key contributors to human wellness (Haller, 2010). In the last 20 years, many publications were devoted to so-called "functional foods" and "nutraceuticals". Research into functional ingredients is promising for the use of such ingredients in food products, thereby creating added value for manufacturers and benefits for consumer health (Coppens et al., 2006). In the last 10 years, pharmacists, chemists, nutritionists, and physicians have been working together to develop new nutritional applications to satisfy people's needs and demands. More recently convergence between the cosmetics and food industries led to nutricosmetics, a blurry area unfamiliar to many consumers and sometimes even to food and cosmetics experts (Anunciato & da Rocha Filho, 2012). Among the ingredients used in nutricosmetics, antioxidants and vitamins represent critical components.

The best-known antioxidants are carotenoids (beta-carotene, lycopene, lutein, zeaxanthin and astaxanthin). While β-carotene accumulates for example in the skin of poultry providing a ''golden-yellow'' colour, lutein and zeaxanthin accumulate preferentially in the macula lutea, where they protect the retina against oxidative damage from UV light (Scarmo et al., 2010). Consequently, these two carotenoids have been studied extensively in the context of age-related macular degeneration (Biesalski & Tinz, 2008). Results from recent studies confirmed the role of lycopene against atherosclerosis and its potential in reducing LDL cholesterol similarly to statins in patients with slightly elevated cholesterol levels (Palozza et al., 2011; Ried & Fakler, 2011). Carotenoids present in the skin have an important role in photoprotection against UV radiation. Clinical studies have been conducted to assess their photoprotective capacity in the prevention of premature skin aging (Stahl & Sies, 2005, 2012). Carotenoid economic value in industry and market is reflected in the high number of patents register in The United States Patent and Trademark Office (USPTO). More than 50%

of these patents relate to human use applications, including nutraceuticals and cosmetics (Berman et al., in press).

Vitamin E has been touted as a panacea for age-related diseases, including cardiovascular disease and Alzheimer's disease. The demand has increased dramatically in recent years, in turn driving research to increase vitamin E production from plant sources (Ajjawi & Shintani, 2004). Vitamin E is also a useful product in skin treatment against UV radiation and other oxidative stresses such as ozone. Moreover, vitamin E plays different roles in the maintenance of skin physiological conditions (Nada et al., 2014). Nutraceutical formulations including vitamin E and folate have been patented with different applications, such as degenerative treatments or cosmetics (Kurfurst et al., 2013; Shea et al., 2012).

Animal feed

While metabolism needs are similar, dietary needs for vitamins differ among species. Some vitamins are metabolic essentials, but not dietary essentials for certain species, because they can be synthesized readily from other food or metabolic constituents (McDowell, 2000).

Poultry, swine and other monogastric animals are dependent on their diet for vitamins to a much greater degree than are ruminants (Aurousseau et al., 2006). Ruminants in which the rumen is fully functioning cannot suffer from a deficiency of B vitamins. It is generally assumed that ruminants can always satisfy their needs from the B vitamins naturally present in their feed, plus that synthesized by symbiotic microorganisms (Hill, 1997). However, under specific conditions relating to stress and high productivity, ruminants have more recently been shown to have requirements, particularly for the B vitamins, such as B_{12} (Spears & Weiss, 2014).

The rumen does not become functional with respect to vitamin synthesis for some time after birth. For the first few days of life, the young ruminant resembles a non-ruminant as it requires dietary sources of B vitamins (McDowell, 2000; Spears & Weiss, 2014). In monogastric animals, including humans, intestinal synthesis of many B vitamins is considerable, though not as extensive or as efficiently utilized as ruminants (McDowell, 2000).

Vitamin A is essential for animals as it is for humans, for normal vision, maintenance of healthy epithelial or surface tissues, and normal bone development (McDowell, 2012). Ruminants lacking vitamin A may be more susceptible to pink eye or other diseases related to the mucous membranes. In feed manufacturing, vitamin A is supplemented in different ways, such as part of a concentrate or liquid supplement, included with a free-choice mineral mixture, injectable product or in drinking water preparations (McDowell, 2012). Because of the lack of stability of vitamin A the feed industry has accepted the dry stabilized forms of the vitamin (Rodríduez-Huezo et al., 2006). In poultry feed, carotenoids such as xanthophylls gained economic interest in pigmenting broiler skin and the egg yolk. The intensity as well as the color can be controlled by the concentration and type of dietary xanthophylls (Breithaupt, 2008). In poultry ffeed, yellow xanthophylls (e.g. lutein) are the most important carotenoid additives. However, orange xanthophylls (e.g. zeaxanthin) are added in order to compensate for a specific final product color (Breithaupt, 2007). Vitamin E displays the greatest versatility of all vitamins in the range of deficiency signs which differ among species and even within the same species. Muscular dystrophy is the most common vitamin E deficiency in all species, while in poultry vitamin E deficiency has been related with subcutaneous edema, sterility and embryonic mortality (Julian, 2005; Xu, Wang & Wang, 2007). The amount of vitamin E required in diets can vary depending on such factors as levels of PUFAs, Se, antioxidants and sulphur amino acids in feed (McDowell, 2012).

III.   Crop biofortification

Fortification strategies

Fortification is the addition of essential micronutrients and other health-promoting compounds to food. It aims to reduce the number of people suffering from malnutrition (approximately 50% of the global population) and to increase general health and wellbeing (Zhu et al., 2011). In developing countries fortification programs are often unsustainable due to poor governance, inefficient food-distribution networks and the prevalence of subsistence agriculture in rural populations (Yuan et al., 2011; Zhu et al., 2011). The major deficiency diseases in developing countries that result from or that are exacerbated by the accumulation of oxidative damage to cells (including cancer, cardiovascular diseases and neurodegenerative

disorders) correspond to the low levels of essential nutrients (such as vitamins or minerals) present in milled cereal grains which act as antioxidants or promote the activity or availability of antioxidants (Zhu et al., 2013). The limited impact of conventional interventions in developing country settings has promoted the use of biofortification as a sustainable approach that is equally beneficial to subsistence farmers and consumers of processed foods (Pérez-Massot et al., 2013; Yuan et al., 2011).

Biofortification utilizes nutrients and other health-promoting compounds which are incorporated while the plant is still growing, and are therefore present in the harvested material and at all subsequent stages en route to the consumer (Saltzman et al., 2013). There are two general strategies for biofortification with any organic or inorganic nutrient, conventional breeding and genetic engineering. Both attempt to create plant lines carrying genes that favour the most efficient biosynthesis and/or accumulation of essential micronutrients and other health-promoting compounds (Zhu et al., 2011). Conventional breeding achieves this by crossing the best performing plants and selecting those with favourable traits over many generations, sometimes in combination with mutagenesis or marker assisted selection, whereas genetic engineering introduces the traits as recombinant DNA and allows the best-performing plants to be selected in a single generation (Cubero, 2003; Pérez-Massot et al., 2013) . Genetic engineering also permits nutritional traits to be targeted to specific organs (e.g. edible seeds) and multiple traits can be combined in the same plant without complex breeding programs (Naqvi et al., 2010; Pérez-Massot et al., 2013).

Molecular breeding and biotechnological strategies

The first step in the domestication of crop plants was a process that started 11,000 to 13,000 years ago with the cultivation and ultimate domestication of many wild plant species in various parts of the world (Allard, 1999). The second phase which established the foundations of contemporary plant breeding started early in the twentieth century as Darwinian and Mendelian principles became firmly established (Allard, 1999). Plant breeding develops new plant varieties by selection over multiple plant species and uses genetic material that is already present within the genetic pool of a species or a sexually compatible species (Cubero, 2003). In the late 20th and early 21st centuries, new and diverse techniques in plant

breeding were introduced. These dissociate and manipulate the components of the individual plant (explants, cells, molecular structures), thus paving the way to new possibilities in terms of sanitation, rapid propagation, cloning, mutagenesis, gene mapping, somatic hybridization and genetic transformation (Cubero, 2003).

Molecular markers linked to vitamin biosynthesis can be used to select for more nutritious crops in breeding programs. Biotechnology has developed tools in order to accelerate breeding programs such as marker assistant selection (MAS) (Dwivedi et al., 2007). Breeding without markers can only identify the most productive combinations of alleles by chance, whereas MAS allows particular alleles, identified by the linked markers, to be stacked in the same line without any need for phenotypic analysis (Dwivedi et al., 2007; Harjes et al., 2008). MAS has allowed the construction of saturated linkage maps for many crops and has made it possible to map the quantitative trait loci (QTLs) that control them. Understanding how QTLs affect crop performance under different environmental conditions and in different genetic backgrounds can facilitate the development of enhanced crop varieties (Collard et al., 2005). Consequently, the mapping of QTLs for agronomic traits is an important component of conventional nutritional improvement programs (Collard et al., 2005; Dwivedi et al., 2007). Breeding programs can also be accelerated by mutagenesis, which generates new alleles more rapidly than would occur in nature (Cubero, 2003). This can be achieved by irradiating seeds with X-rays or exposing them to chemical mutagens such as ethyl methanesulfonate (EMS), each of which causes random damage to DNA and usually generates point mutations (Chen et al., 2014). Any phenotypic effects of such mutations are observed in subsequent generations, depending on whether the effect is dominant or recessive, and must be mapped to identify the affected gene. An advanced method for identifying such point mutations is TILLING (Targeting Induced Local Lesions In Genomes), a high-throughput method based on conformational electrophoresis for the detection of point mutations in large populations of plants (Comai & Henikoff, 2006; Chen et al., 2014). TILLING can identify genetic variation in elite germplasm without the need to acquire variation from exotic cultivars, thus avoiding the introduction of agriculturally undesirable traits. Once a TILLING library is set up, it can be used for the analysis of many different gene targets. TILLING is a powerful reverse genetics approach that has the unique advantage of allowing the generation of an allelic series for any target gene, including essential genes (Slade et al., 2005). If a variety developed by TILLING has commercial potential, it is not

subject to the same regulatory approval requirements as transgenic crops. Even with methods such as TILLING making the process of mapping novel mutations easier, it is still preferable to be able to identify mutated genes with a unique DNA signature (Slade et al., 2005). This is the benefit of insertional mutagenesis using unique DNA tags such as transposons or T-DNA, the former endogenous to plants, the latter introduced artificially. In this approach, randomly integrating DNA sequences disrupt genes and cause loss of function. The identification of desirable mutant phenotypes is followed by DNA analysis using the insertional mutagen sequence as a probe, or as the basis for PCR primer design, allowing flanking gene sequences to be identified (Maes et al., 1999).

QTLs or mutants have been utilized in crop biofortification programs to increase vitamin A, folate, vitamin E and vitamin C content in edible organs or tissues. However, numerous studies have shown that so far only genetic engineering has provided the means to produce nutritionally enhanced crops that can meet RDI values (Sanahuja et al., 2013). Organic molecules such as amino acids, fatty acids, and vitamins are synthesized by the plant, and increasing the nutritional content therefore requires some form of metabolic engineering with the aim of increasing the amount of the desirable compound. To this end, methods had to be developed for the coordinated expression of one or more genes (Capell & Christou, 2004) to attain objectives, such as: (a) enhance the activity of enzymes at multiple rate-limiting steps in target pathways, e.g. by overexpression or expression of enzymes that are released from feedback inhibition; (b) increase the availability of upstream precursors to amplify the flux through the target pathway; (c) modulate pathway branch points to prevent the loss of flux; and (d) promote the development of sink compartments to store target compounds (Figure 1) (Zhu et al., 2013; Zorrilla-López et al., 2013).
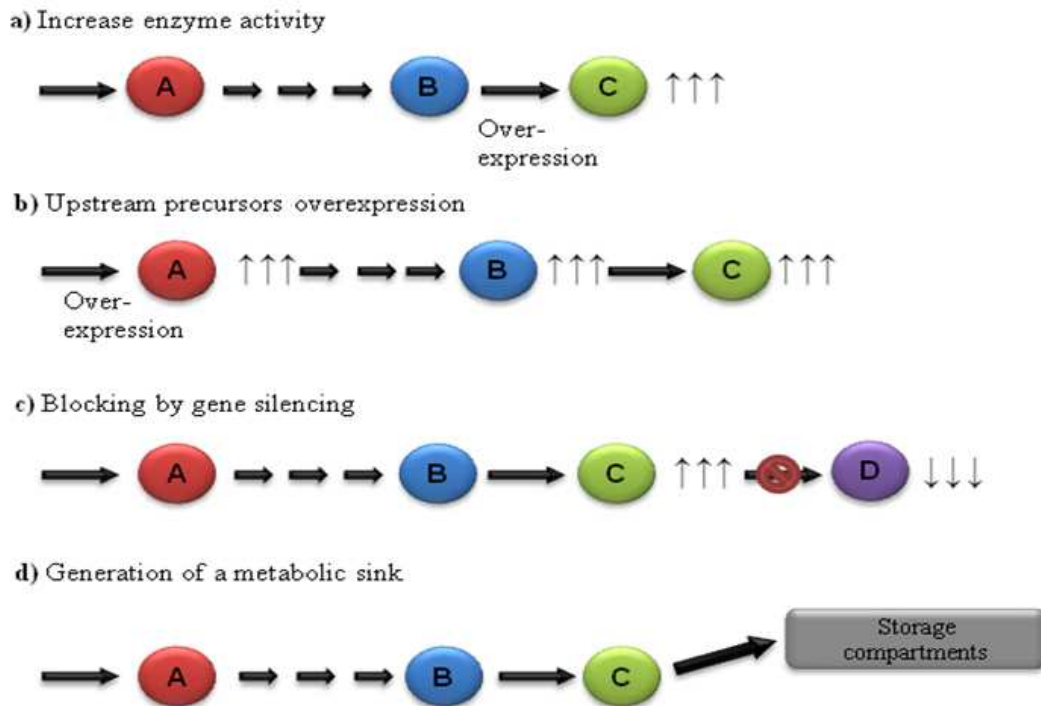
Figure 1: Strategies to modulate organic compound levels in plants. A and B are the precursors of C; C is the target product; D is the result of the target product conversion. (A) Modification of the activity of enzymes implicated in rate-limiting steps in the target pathway by modulation of one or two key enzymes, or multiple enzymes. (B) Upstream precursors enhancement by increasing flux through the pathway by overexpressing the enzyme(s) that catalyze(s) the first committed step of the pathway. (C) Blocked pathway branch points by RNA interference or antisense. (D) Enhanced accumulation of target metabolite by increasing sink compartments (Zorrilla-López et al., 2013).

Multigene Engineering strategies

Most agronomic traits in plants are controlled by multiple genes, as is also the case for the synthesis of complex organic compounds from primary and secondary metabolism, which often represent the outputs of long and convoluted metabolic pathways. Therefore, genetic engineering has seen a progressive change from single-gene intervention to multigene transformation to tackle increasingly ambitious objectives (Halpin, 2005). Examples of metabolic engineering in plants include primary metabolic pathways (carbohydrates, amino acids, and lipids) and secondary metabolic pathways (e.g. alkaloids, terpenoids, flavonoids,

lignins, quinones, and other benzoic acid derivatives) (Zhu et al., 2011). These pathways generate a large number of compounds that are useful to humans, including energy-rich foods, vitamins and many different pharmaceuticals. The introduction of multiple genes into plants was initially achieved using iterative processes, such as successive rounds of crosses between transgenic lines (Halpin, 2005) or sequential retransformation (Blancquaert et al., 2013). Both methods are labour intensive in terms of breeding, as the transgenes are unlinked and segregate independently in later generations (Zorrilla-López et al., 2013). The simultaneous introduction of two or more transgenes (cotransformation) via direct DNA transfer allows transgenic plants carrying multiple genes at one locus to be produced in a single generation, which can be achieved using genes in tandem on the same transformation plasmid or unlinked genes on different transformation plasmids. Each of these approaches is compatible with Agrobacterium -mediated and also direct DNA transfer (Naqvi et al., 2010). However, the linked-gene strategy becomes less efficient as more transgenes are added because of cloning difficulties, stability issues, and the declining efficiency of transformation of ever-larger DNA fragments. Occasional success has been achieved with the direct transfer of DNA fragments >100 kb in length (reviewed in Zorrilla-López et al., 2013) but the greatest success has resulted from modified high-capacity binary vectors based on bacterial artificial chromosomes (BIBACs) and bacteriophage P1-derived transformation-competent artificial chromosomes (TACs) (for reviews see Farré et al., 2014; Naqvi et al., 2010; Zorrilla-López et al., 2013). Cloning in high-capacity vectors has been simplified by the addition of Cre-lox P and Gateway site-specific recombination technology (Hubbard, 2014). A more recent approach is the plant minichromosome using either linear DNA or more stable circular maize minichromosomes (Birchler, 2015).

The next step for multigene metabolic engineering is synthetic biology. Synthetic biology involves the *de novo* assembly of genetic systems using pre-validated components (Haseloff & Ajioka, 2009). In the context of metabolic engineering in plants, a synthetic biology approach might utilize specific promoters, genes, and other regulatory elements to create ideal genetic circuits that will facilitate the accumulation of particular metabolites. Synthetic biology combines engineering principles and mathematical models to predict and validate the behavior of the resulting system, which can be considered as the next step in multigene metabolic engineering because it removes any dependence on naturally occurring sequences and allows the design of ideal functional genetic circuits from first principles

(Haseloff & Ajioka, 2009). Thus far, most work on synthetic biology has been carried out in microorganisms (Nikel et al., 2014; Cameron et al., 2014). Simple synthetic biology approaches have been described in plants, mostly in the context of signalling pathways and development, but also in the development of phytodetectors (Zurbriggen et al., 2012) and bio-fortified crops (Naqvi et al., 2009). The use of synthetic biology in development as well as metabolism is important because it not only controls the metabolic capacity of a cell, but also steps one level up in terms of organization and use of particular promoters and genes that control developmental processes to generate novel tissues, in which the cells have specialized biosynthetic or storage functions to accumulate target products in particular organs. This approach will facilitate the achievement of goals that are unattainable by conventional genetic engineering, such as the development of novel organisms with medical functions, the production of biofuels, and the removal of hazardous waste (Purnick & Weiss, 2009).

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AIMS&OBJECTIVES

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The overarching aim of my dissertation has been to modulate three complex metabolic pathways in maize seeds. A further aim was to develop a mechanistic understanding of molecular factors influencing the qualitative and quantitative profiles of metabolites in the transgenic seeds, and ascertain the influence of genetic background on ketocarotenoid biosynthesis in maize endosperm.

My specific objectives were to:

1. use a multigene engineering strategy to generate different transgenic maize lines with altered carotenoid, folate and vitamin E metabolism

2. investigate the interaction between the carotenoid and vitamin E biosynthetic pathways as they share common precursors

3. introgress a carotenogenic mini-pathway into a range of maize germplasm with different carotenoid profiles in order to understand molecular factors influencing flow of precursors into the two different branches of the pathway

4. ascertain the consequences of a one versus two gene strategy to modulate folate biosynthesis in maize seeds

5. determine the influence of genetic background on ketocarotenoid biosynthesis in maize endosperm.

CHAPTER 1

**FIELD-GROWN MAIZE HYBRIDS WITH DIVERSE CAROTENOID
PROFILES REVEAL THE COMPLEX INTERPLAY BETWEEN
SYNTHETIC AND ENDOGENOUS METABOLIC PATHWAYS IN
DIFFERENT GENETIC BACKGROUNDS**

1.1 Abstract

The two xanthophylls, lutein and zeaxanthin are important carotenoids for human health as they are implicated in the prevention of macular degeneration. While lutein is abundant in fruits and vegetables, zeaxanthin is only found in a few plants, e.g. in a number of yellow maize genotypes. Differences in the accumulation ratio of lutein and zeaxanthin are due to β:ε lycopene cyclase activity, which direct the carotenoid pathway to the β- and ε-branch, respectively. Hydroxylation of α- and β-carotene also plays a role in the formation of the two molecules. A transgenic maize line expressing *Zmpsy1* and *PacrtI* which accumulated high levels of carotenoids, including β-carotene had been generated previously in the laboratory. I report results from experiment in which I introgressed this transgenic mini-pathway into five different yellow maize inbred lines with different carotenoid profiles. Hybrids obtained in field experiments demonstrated that the yellow endosperm background can compensate in a number of cases limiting steps in the carotenoid pathway towards lutein and zeaxanthin. Other bottlenecks limiting lutein and/or zeaxanthin levels remained in the hybrids. Through combination of genetic engineering and conventional breeding we have been able to generate a novel hybrid with high amounts of lutein and zeaxanthin, up to ca: 27 and ca: 32 μg∕g DW respectively, in the field.

1.2 Introduction

1.2.1 Carotenoids

Carotenoids comprise a large isoprenoid family of mostly C40 tetraterpenoids derived from phytoene. They include well over 800 structures that provide fruits and flowers with distinctive red, orange and yellow colors (Britton et al., 2004). Carotenoids are divided into two groups: hydrocarbon carotenes such as β-carotene, α-carotene and lycopene and oxygenated xanthophylls such as lutein, zeaxanthin and violaxanthin (Zaripheh & Erdman, 2002). They are the only natural tetraterpenes, synthesised *de novo* by photosynthetic organisms (including plants, algae, and cyanobacteria) and some non-photosynthetic bacteria and fungi (Botella-Pavía & Rodríguez-Concepción, 2006). More recently aphids were shown to synthesize carotenoids, mostly γ-carotene, β-carotene, α-carotene, torulene and dehydro-γ,ψ-carotene (a carotenoid similar to torulene) (Moran & Jarvik, 2010). Lutein and

zeaxanthin belong to the subclass of non-provitamin A carotenoids known as xanthophylls, which are different from other carotenoids because they contain oxygenated substituents (Sander et al., 1994; Stahl & Sies, 2005). 2008).

Lutein and zeaxanthin accumulate in different amounts in food; lutein is most common in green leafy vegetables, while zeaxanthin is present in lower amounts in general and accumulates only in some yellow maize varieties (Perry et al., 2009). Although sweet-corn is a good source of zeaxanthin relative to other foods (Holden et al., 1999; Perry et al., 2009), the amount of zeaxanthin used in human supplements is substantially higher (2 mg/person/day) (Chew et al., 2013) and would require a daily consumption of 4–11 cobs of corn, 1.2 kg of kale, or 1–9 kg of eggs to achieve an equivalent concentration (O'Hare et al, 2015).

Animal farming practitioners are increasingly interested in enriching feed by adding xanthophylls, such as lutein and zeaxanthin, in order to pigment the skin of poultry and confer a commercially desirable color to egg yolks (Breithaupt 2007). In animals, xanthophylls are mainly found in meat, eggs, and in the skin of fish. Astaxanthin, canthaxanthin and lutein are the most important carotenoids in the feed industry (Alcaino et al., 2014; Breithaupt, 2007). Pigments, whether natural or synthetic, increase the production costs (Castaneda et al., 2005). In a recent study, feed rich in carotenoids was tested for its ability to prevent coccidiosis in poultry, a disease caused by protozoan parasites. Chickens were raised on a diet enriched with an engineered corn variety containing very high levels of four key carotenoids (β-carotene, lycopene, zeaxanthin and lutein) resulting in carotenoid accumulation in different body tissues and exhibiting normal growth after inoculation with *Eimeria tenella*, the causal agent of coccidiocis (Nogareda et al., 2015).

Maize is one of the most widely cultivated crops throughout the world in terms of harvested weight per year compared to other grains (IGC, 2012). Maize exhibits considerable natural variation for grain carotenoids because of variations in carotenogenic gene polymorphisms, and this is reflected as distinct color phenotypes, with some lines accumulating as much as 66 µg/g total carotenoids (Harjes et al., 2008). This natural variation can be exploited to increase β-carotene level by conventional breeding combining association analysis, linkage mapping, expression analysis or mutagenesis (Harjes et al., 2008; O'Hare et al., 2015). HarvestPlus, a consortium of international research institutions works to address

the challenge of micronutrient malnutrition, including enhancement of vitamin A in maize. Within the HarvestPlus program, the breeding target level of 18 μg of provitamin A per gram DW was established for maize to provide a 50% of the estimated average requirement (EAR) of vitamin A for non-pregnant, non-lactating women of reproductive age and for preschool children 4−6 years old (Saltzman et al., 2013; Gannon et al., 2014). The most recently developed lines have increased provitamin A through biofortified maize hybrids up to 7.5 to 10.3 μg/g DW (Mugode et al., 2014). In a recent breeding program in sweet corn, zeaxanthin and β-carotene concentrations were increased up to 87.1μg/g and 25.8μg/g FW at 60 days after pollination (DAP), respectively (O'Hare et al., 2015). The zeaxanthin concentration achieved at eating stage was 25μg/g FW, which appears to be significantly greater than that reported for sweet corn in the scientific literature, ranging from 0.02 to 6.8 μg/g FW (O'Hare et al., 2015).

Five carotenogenic genes (*Zmpsy1*, *PacrtI*, *Glbch*, *Gllycb*, and *ParacrtW*) under the control of endosperm-specific promoters were introduced into an elite white maize variety (M37W) deficient for endosperm carotenoid synthesis (Zhu et al., 2008). 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. One phenotype exhibited a substantial increase of ca: 57 μg/g DW of β-carotene, 169-fold the normal amount in comparison with wild type (Zhu et al., 2008). A transgenic mini-pathway comprising *Zmpsy1* and *PacrtI* was introgressed into two inbred lines, EP42 and A632. These lines were selected because they exhibit contrasting β:ε ratios of 0.61 and 1.90. These ratios reflect carbon flow favouring the ε or the β branch. Thus, metabolic synergy between the partial endogenous and induced heterologous pathways was used to enhance the levels of nutritionally important metabolites, compensating for the limiting steps of the native pathway in the two inbreds by the heterologous expression of *Zmpsy1* and *PacrtI* in the transgenic parent. Introgression of the mini-pathway from the transgenic line into A632 resulted in a hybrid in which zeaxanthin production was elevated to 56 μg/g DW and lutein to 9.7 μg/g DW. In hybrids between the transgenic line and EP42, zeaxanthin levels were ca: 23μg/g DW and lutein levels ca: 38 μg/g DW (Naqvi et al., 2011). The difference in β/ε ratios between the hybrids correlates with the levels of the endogenous *lycE* gene, the higher level in EP42 favouring higher lutein accumulation and the lower level in A632 favouring higher zeaxanthin accumulation (Naqvi et al., 2011).

Standard feed industry practices include vitamin A and other carotenoids as micronutrients not only in poultry but also in bovine and porcine production (McDowell, 2000). Thus, maize hybrids engineered with targeted carotenogenic mini-pathways may offer to the feed industry a valuable product where key carotenoids such as β-carotene, lutein and zeaxanthin are accumulated in higher amounts. Potential commercial value of these hybrids is enhanced as a result of cost savings and easier feed production because addition of colorants in the feed becomes unnecessary.

1.2.2 Carotenoid biosynthesis pathway

In plants, the biosynthesis of carotenoids occurs on chloroplast membranes, chromoplasts and amyloplasts, genetically identical plastids of very different internal membrane architecture. The plant enzymes, whose function is for the most part well understood, are encoded in the nucleus and targeted to the plastids (Gallagher et al., 2004). The tetraterpenoids are formed by eight condensed C5 isoprenoids precursors forming a C40 linear backbone (Fraser & Bramley, 2004). These reactions involve condensation of isopentenyl diphosphate (IPP) and dimethyllallyl diphosphate (DMAPP) (**Figure 1.1**). Plants synthesize IPP and DMAPP by two independent pathways: the mevalonic acid (MVA) pathway, which produces cytosolic IPP, and the plastidial methylerythritol phosphate (MEP) pathway (DMAPP). The addition of three molecules of IPP to one DMAPP unit catalyzed by the enzyme geranylgeranyl diphosphate synthase (GGDS), encoded by the crtE gene in bacteria, results in the formation of geranylgeranyl pyrophosphate (GGPP) (Botella-Pavía et al., 2004; Nishida et al., 2005). The condensation of two molecules of GGPP by phytoene synthase (CrtB in bacteria, PSY in plants) gives rise to 15-*cis*-phytoene, the first C40 hydrocarbon in the biosynthetic sequence in the carotenoid pathway (Giuliano et al., 2008).

Phytoene undergoes a series of four desaturation reactions that result in the formation of phytofluene and subsequently, zeta-carotene (ζ-carotene), neurosporene and lycopene (**Figure 1.1**). These desaturation reactions introduce a series of carbon–carbon double bonds that constitute the chromophore of carotenoid pigments, and they transform the colorless phytoene into pink-colored lycopene (Naik et al., 2003). The linear, symmetrical lycopene then undergoes cyclization to yield carotenes with two types of rings, β and ε, where the only difference between these two rings is the position of the double bond in one of the ionone moieties (Rivera & Canela-Garayoa, 2012). Then, two cyclases, lycopene β-cyclase (LYCB)

and lycopene ε-cyclase (LYCE) transform lycopene. LYCB can introduce two β-rings, one at each end of lycopene to obtain γ-carotene (monocyclic molecule) or β-carotene (β, β-carotene; dicyclic molecule). LYCE can only introduce one ε-ring at the end of lycopene to obtain σ-carotene (monocyclic compound) (Zhu et al., 2009). β-Carotene, with two β rings serves as a precursor of several other carotenoids in plants including the oxygenated xanthophylls. This occurs via hydroxylation and ketolation reactions. The introduction of hydroxyl moieties into β–ring carotenes is catalyzed by β-carotene hydroxylases (BCH) to generate zeaxanthin (Naik et al., 2003) (**Figure 1.1**). α-Carotene (also known as β, ε-carotene) is produced from lycopene by the addition of an ε-ring at one end and a β-ring at the other, reactions catalyzed by LYCE and LYCB, respectively (Zhu et al., 2009). Hydroxylation of α-carotene by ε-hydroxylase results in the formation of lutein (Pogson et al., 1996).
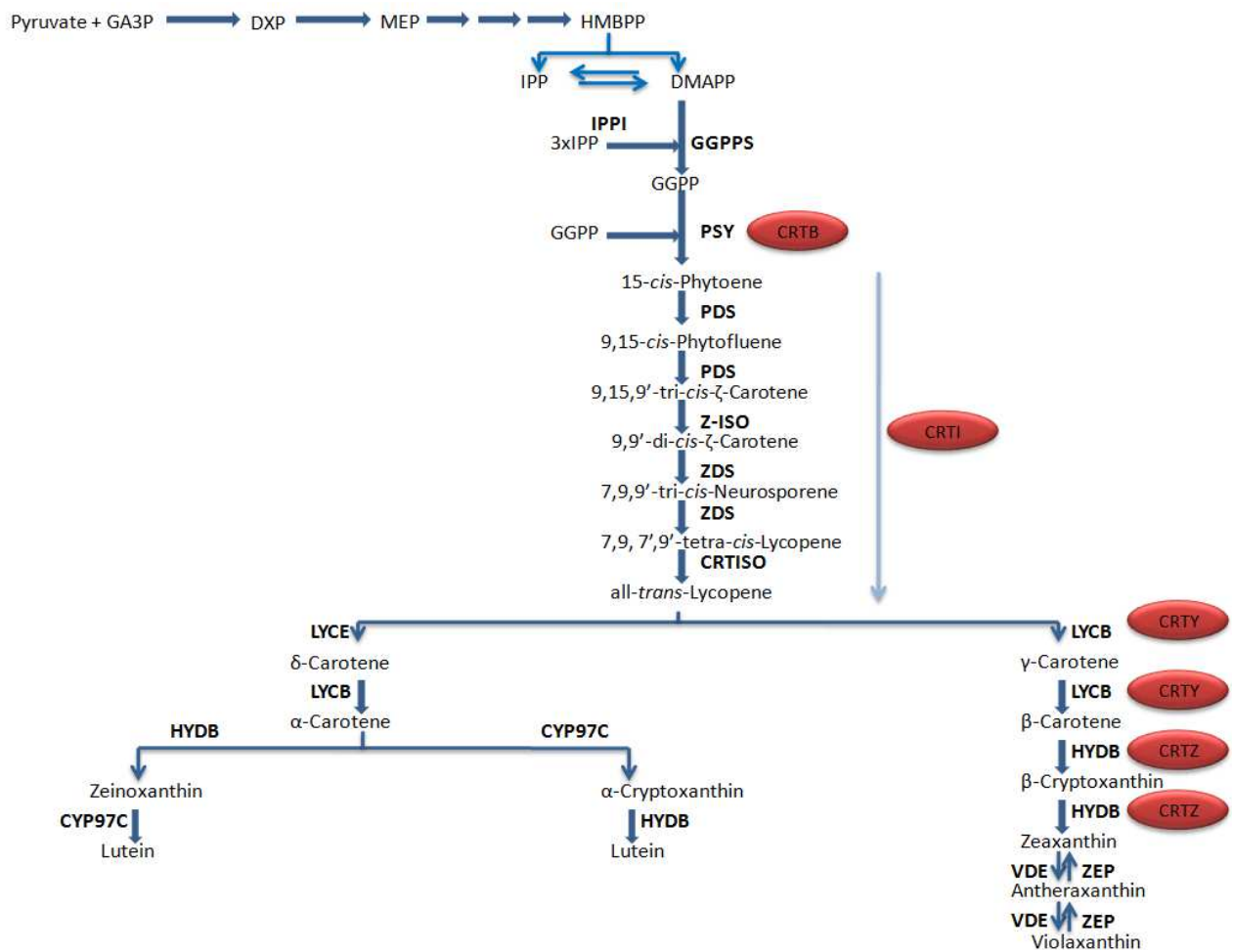
Figure 1. 1: Carotenoid biosynthetic pathway in plants and equivalent steps in bacteria.

Enzymes in the red ovals are from bacteria. Abbreviations: CRTB, bacterial phytoene synthase; CRTI, bacterial phytoene desaturase, which catalyze all desaturation and isomerization reaction from phytoene to lycopene; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene β-cyclase; CRTZ, bacterial β-carotene hydroxylase; CYP97C, heme-containing cytochrome P450 carotene e-ring hydroxylase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; GA3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HDR, HMBPP reductase; HMBPP, hydroxymethylbutenyl 4-diphosphate; HYDB, β-carotene hydroxylase [non-heme di-iron β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxyalses (CYP97A and CYP97B)]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; MEP, methylerythritol 4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin deepoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase (Farré et al., 2010; Farré et al., 2011).

1.2.2 Genetic engineering strategies for enhancing carotenoid content in plants

Several strategies have been used to increase the levels of carotenoids in plants. These include increasing flux through the carotenoid pathway by making more precursors available, modifying the activity of carotenogenic enzymes, blocking pathway branch points and creating sinks to store β-carotene and relieve feedback inhibition (**Table 1.1**).

The first committed step in carotenoid biosynthesis is the conversion of GGPP into phytoene by phytoene synthase (PSY) and this is recognized as a major pathway bottleneck. Therefore, increasing the activity of this enzyme by expressing a plant *psy* transgene or the bacterial equivalent *crt*B has increased total carotenoid levels in tomato, canola and maize by up to 50-fold, predominantly in the form of α- and β-carotene (Fraser et al., 2007; Shewmaker et al., 1999; Zhu et al., 2008). Phytoene is desaturated and isomerized in several steps to form lycopene, but one bacterial enzyme (CRTI) can accomplish all these reactions (Cong et al., 2009). Lycopene is then cyclized at each end by lycopene β-cyclase (LYCB, bacterial equivalent CRTY) to form β-carotene, or at one end by lycopene ε-cyclase (LYCE) and at the

other by LYCB to form α-carotene (Rosati et al., 2000). Several attempts have been made to increase the β-carotene content of plants by overexpressing LYCB or suppressing the activity of LYCE, thus shifting flux into the β-branch (for review see Farré et al., 2011). For example, in canola lines expressing *crt*B, *crt*I and *crt*Y, there was not only a higher total carotenoid content than wild type seeds (1229 μg/g fresh weight), but the β- to α-carotene ratio increased from 2:1 to 3:1 demonstrating that the additional LYCB activity skewed the competition for the common precursor lycopene and increased flux specifically towards β-carotene (Ravanello et al., 2003).

Cereal grains do not produce carotenoids or accumulate them at very small levels, and it is therefore necessary to devise strategies to remedy this deficiency. One of the most important examples is rice endosperm, where the expression of PSY leads to the accumulation of phytoene but no other carotenoids, thus the entire carotenoid pathway had to be imported to produce "Golden Rice" containing β-carotene (Burkhard et al., 1997). Similar methodology can be used to extent partial pathways and generate additional carotenoid products in plants, e.g. ketocarotenoids such as adonixanthin, echinenone and astaxanthin were obtained in transgenic maize by expressing maize *psy1, Paracoccus crt*W and *crt*I, and *Gentiana lutea lycb* and *bch* (Zhu et al., 2008).

Blocking the α-carotene branch to prevent competition for the common precursor lycopene can also direct flux towards β-carotene synthesis. This was achieved by using RNA interference (RNAi) to block LYCE expression in canola, increasing total carotenoids where ca: 42-fold to 227 μg/g fresh weight and increasing β-carotene levels ca: 185-fold to 90 μg/g fresh weight (Yu et al., 2008).

Carotenoids such as β-carotene accumulate in specialized lipoprotein-sequestering structures within chromoplasts, so a final strategy to enhance carotenoid accumulation in plants is to modify the storage capacity by increasing the number of storage compartments or encouraging chromoplast differentiation. The most notable example is the cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* (*Or*) gene that represents a rare dominant gene mutation. It encodes a DnaJ cysteine-rich domain-containing protein, which confers a high level of β-carotene accumulation in normally white tissues of the plant, turning them orange (Giuliano & Diretto, 2007; Lu et al., 2006). Rather than directly regulating carotenoid biosynthesis, the *Or* gene appears to mediate the differentiation of proplastids and/or non-

color plastids in apical shoot and inflorescence meristematic tissues of curd into chromoplasts (one or two per cell) for the associated carotenoid accumulation (Li et al., 2001; Li & Eck, 2007). This gene was expressed in potato tubers, resulting in an increase of 6-fold of total carotenoids (24 μg/g DW) (Lu et al., 2006). In another study, also in potato, an increase of 5.7-fold of total carotenoids (31μg/g DW) was reported but after storage for 6 month at 5ºC the total carotenoids in the transgenic tubers were increased 10-fold (López et al., 2008). More recently, *AtOR* has been expressed in rice endosperm together with *Zmpsy1* and *PacrtI*. Resulting grains accumulated ca: 25 μg/g DW total carotenoids and 10 μg/g DW β-carotene (no carotenoids could be measured in the wild type rice endosperm) (Bai et al., 2015).

Species	Genes (origin)	Promoters	Carotenoid levels in transgenic plants	Reference
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
	psy1 (Zea mays) *crt*I (*Pantoea ananatis*)	Rice Gt1	37 μg/g (DW) total carotenoids (23-fold)	Paine et al., 2005
	psy1 (Zea mays) *crt*I (*Pantoea ananatis*)	Endosperm-specific rice prolamin promoter	5.5 μg/g (DW) total carotenoids 2.15 μg/g (DW) β-carotene	Bai et al. 2015
	psy1 (Zea mays) *crt*I (*Pantoea ananatis*) OR (A. thaliana)		25.83 μg/g (DW) total carotenoids 10.52 μg/g (DW) β-carotene	
	psy1 (Zea mays) *crt*I (*Pantoea ananatis*) DXS (A. thaliana)		31.78 μg/g (DW) total carotenoids 16.6 μg/g (DW) β-carotene	
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
	*crt*B (*Pantoea ananatis*)	Napin	1341 μg/g FW total carotenoids 739 μg/g FW β-carotene	Ravanello et al., 2003
	*crt*E and *crt*B (*Pantoea ananatis*)		1023 μg/g FW total carotenoids 488 μg/g FW β-carotene	

	*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
	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
	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., 2009
Tomato (Lycopersicon esculentum)	*psy1* (tomato)	CaMV35S	1159 µg/g FW total carotenoids (1.14-fold) (assuming a water content of 90%)	Fray & Grierson, 1993
	*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., 2002
	LYCB (*Arabidopsis*	Pds (fruit specific)	109 µg/g FW total carotenoids (1.7-fold)	Rosati et al., 2000

	thaliana)		57 µg/g FW β-carotene (7.1-fold) (assuming a water content of 90%)	
	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
	*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., 2001
	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
	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
	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
	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
	*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
	Fibrillin (pepper)	fibrillin	650 µg/g FW total carotenoids (2.0-fold) 150 µg/g FW β-carotene (1.6-fold)	Simkin et al., 2007
	lycb (daffodil)	ribosomal RNA	115 µg/g FW total carotenoids (1.5-fold) 95 µg/g DW β-carotene (5-fold)	Apel & Bock, 2009
	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
Potato (*Solanum*	*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
m	*crt*B (*P. ananatis*)	Patatin (tuber specific)	35 µg/g DW total carotenoids (6.3-	Ducreux et al., 2005

tuberosum)			fold)
			10.3 µg/g DW β-carotene (10-fold)	
lyce (potato, antisense)	Patatin	12.27 µg/g DW total carotenoids (2.6-fold)	Diretto et al., 2006
		0.043 µg/g DW β-carotene (14-fold)	
*crt*O (*Synechocystis* sp.)	CaMV35S	39.76 µg/g DW total carotenoids (2.1-fold)	Gerjets & Sandmann, 2006
dxs (*E. coli*)	Patatin	7 µg/g DW total carotenoids (2-fold)	Morris et al., 2006a
*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., 2006b
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
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
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
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
	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
Maize (*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) 34.81µg/g DW β-carotene (248-fold)	Zhu et al, 2008
	*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
	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
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
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
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., 2007
	PSY (*Arabidopsis*)	CaMV35S	514.1 µg/g DW total carotenoids in roots (93-fold)	Maass et al., 2009

			241.6 µg/g DW β-carotene (178-fold)	
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
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

Table 1. 1: Carotenoid enhancement in crop plants by genetic engineering.

DW = dry weight; FW = fresh weight (Farré et al, 2011b).

In order to compensate for the limiting steps in the pathway and study carotenoid accumulation in maize endosperm we introgressed a transgenic mini-pathway, (*Zmpsy1* and *PacrtI*) into different maize inbred lines. The availability of different inbreds with diverse carotenoid profiles and β:ε ratios provides an opportunity to combine the transgenic mini-pathway in the transgenic maize with diverse backgrounds differing in the profiles of zeaxanthin and lutein to increase and study carotenoid accumulation. We carried out an in-depth analysis at the transcript and metabolite levels in an attempt to determine the specific impact of the introgressed mini-pathway in each inbred line.

1.2. Materials and methods

1.3.1 Plant material

Maize (*Zea mays*) varieties M37W (white endosperm), Mo17, EZ59, EZ6, B73 and A632 (all yellow endosperm), and transgenic line Carolight expressing maize *Zmpsy1* and *Pantoea annatis crtI* were planted in the experimental fields of the University of Lleida in May, 2013. Carolight was crossed with Mo17, EZ59, EZ6, B73 and A632 in August, 2013. Endosperm samples were harvested at 30DAP, frozen in liquid nitrogen and stored at -80ºC. The inbreds Mo17, EZ59, EZ6, B73 and A632 were provide by CSIC, Zaragoza, Spain, and M37W was obtained from CSIR, Pretoria, South Africa.

1.3.2 Total RNA isolation and mRNA analysis

Total RNA was isolated using RNeasy® Plant Min Kit (QIAGEN, Valencia, CA, USA) and 30µg aliquots were fractionated on a denaturing 1.2% w/v agarose gel containing formaldehyde. The RNA was transferred onto a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) via capillary transfer using standard methods (Sambrook et al., 1989). The membrane was probed with digoxygenin-labeled partial cDNAs at 50ºC, overnight using DIG Easy Hyb (Roche Diagnostics). The partial cDNAs were converted into probes using the DIG probe synthesis kit (Roche Diagnostics). After washing and immunological detection with anti-DIG-AP (fragment of sheep anti-digoxigenin antibody; Fab-Fragments, Diagnostics GMBH, Roche, Welwyn, UK) according to the manufacturer's instructions Chemiluminescence using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5-chloro)tricyclodecan}-4-yl)phenyl phosphate (CSPD) (Roche Diagnostics) was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO, USA). Primer sequences used for designing probes are shown in **Table 1.2**.

Transgene	Primer set
Zmpsy1	Forward: 5´-GTGTAGGAGGACAGATGAGCTTGT-3´
	Reverse: 5´-CATCTGCTAGCCTGTGAGAGCTCA-3´
PacrtI	Forward: 5´-TGGAGAAGCGTTTACAGTAAGGT-3´
	Reverse: 5´-GCGTGCAGATAAAGTGAGAAGTC-3´

Table 1. 2: Primer sequences used for transgene probe design.

1.3.3 Quantitative real time PCR

Real-time RT-PCR was performed on a BioRad CFX96^{TM} system using 25µl mixtures containing 10 ng of synthesized cDNA, 1x iQ SYBR green supermix (BioRad, Hercules, CA, USA) and 0.2 µM forward and reverse primers (**Table 1.3**). CYP97A and CYP97C primer information was obtained from Naqvi et al., 2011. Relative expression levels were calculated on the basis of serial dilutions of cDNA (125–0.2 ng) which were used to generate standard curves for each gene. PCR was performed in triplicate using 96 well optical reaction plates. Cycling conditions consisted of a single incubation step at 95ºC for 5 min followed by 44

cycles of 95ºC for 10 s, 58ºC for 35 s and 72 ºC for 15s. Specificity was confirmed by product melt curve analysis over the temperature range 50–90ºC with fluorescence acquired after every 0.5ºC increase, and the fluorescence threshold value and gene expression data were calculated with BioRad CFX96^{TM} software. Values represent the mean of three RT-PCR replicates ± SD. 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.

Gene	Forward primer	Reverse primer
Zmpsy1	5'-CATCTTCAAAGGGGTCGTCA-3'	5'-CAGGATCTGCCTGTACAACA-3'
Zmpsy2	5'-TCACCCATCTCGACTCTGCTA-3'	5'-GATGTGATCTACGGATGGTTCAT-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'-TCCACTGGTCTGTTCTGCGAT-3'
ZmCYP97C	5'-GTTGACATTGGATGTGATTGG-3'	5'-AACCAACCTTCCAGTATGGC-3'
Zmactin	5'- CGATTGAGCATGGCATTGTCA-3'	5'- CCCACTAGCGTACAACGAA-3'

Table 1. 3: Oligonucleotide sequences of maize actin and endogenous carotenogenic genes for quantitative Real-Time PCR analysis.

1.3.4 Carotenoid extraction from maize endosperm

Maize 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 50-100mg samples were extracted in 15 ml methanol:ethyl acetate (6:4 v/v) at 58ºC for 20 min. The mixture was filtered, transferred to a separatory funnel and 15 ml hexane:diethyl ether (9:1 v/v) were used and agitated gently for 1 min. Fifteen ml of saturated NaCl was added, 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 -80ºC.

1.3.5 HPLC-MS and UHPLC-MS

Maize 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 50-100mg samples were extracted in 15 ml methanol:ethyl acetate (6:4 v/v) at 58ºC for 20 min. The mixture was filtered, transferred to a separatory funnel, 15 ml hexane:diethyl ether (9:1 v/v) were added and agitated gently for 1 min. The organic phase was washed twice with saturated NaCl water and the aqueous phase was removed. The samples were dried under N_2 and stored at -80ºC until injection.

The extracts were dissolved in 210-600 µl injection solvent [ACN/MeOH 7:3, v/v]/acetone 3:2, v/v. UHPLC analysis was carried out at SCT-DATCEM, University of Lleida, using an Acquity Ultra Performance LC system linked to a PDA 2996 detector (Waters, Milford, USA). Mass detection was carried out using an Acquity TQD tandem-quadrupole MS equipped with a Zspray electrospray interface (Waters). MassLynx software version 4.1 (Waters) was used to control the instruments and also for data acquisition and processing. UHPLC separations were performed on a reversed-phase column Acquity UPLC C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm (Waters). The mobile phase consisted of solvent A, ACN/MeOH 7:3, v/v, and solvent B, water 100%. Carotenoids in samples were quantified using a PDA detector through the external standard method. Identification of carotenoids was carried out as previously described (Rivera et al. 2013). MS analyses were conducted by atmospheric pressure chemical ionization (APCI), and the conditions used are the same as those described by Rivera et al. 2011. Authentic standards used for quantification were β-carotene, lutein, β-cryptoxanthin and astaxanthin (Sigma), zeaxanthin (Fluka, Buchs SG, Switzerland), phytoene and antheraxanthin (Carotenature, Lupsingen, Switzerland).

1.3.6 Carotenoid identification and quantification

Carotenoids were identified according to the following: 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 quantification in combination with the extinction coefficients (Britton et al., 2004).

1.4 Results

1.4.1 Maize hybrids generated through breeding HC with yellow inbred lines

In order to gain further insights into the rate-limiting steps of carotenoid biosynthesis in maize endosperm, a carotenogenic mini-pathway was introgressed into a number of different yellow maize inbred lines. The hybrids were grown in the field as described in material and methods. The previously generated transgenic HC line, described in section 1.3.1, expressing *Zmpsy1* (encoding phytoene synthase 1) under the control of the wheat LMW glutenin promoter, and *PacrtI* (encoding phytoene desaturase) under the control of the barley D-hordein promoter in a white maize background (M37W) was crossed with Mo17, EZ6, EZ59, B73 and A632. mRNA blot analysis was carried out using 25 µg total RNA from 30 DAP endosperm of all yellow maize lines, M37W white maize parent, HC and corresponding hybrids (**Figure 1.2**). *Zmpsy1* transgene expression was measured in HC and in all hybrids along with very low levels of endogenous *psy1* transcripts in yellow maize inbreds, which was absent in M37W. *PacrtI* expression was also confirmed in transgenic HC and corresponding hybrids (**Figure 1.2**). Resulting hybrids exhibited visually distinct orange color phenotypes in the seeds (**Figure 1.3**).
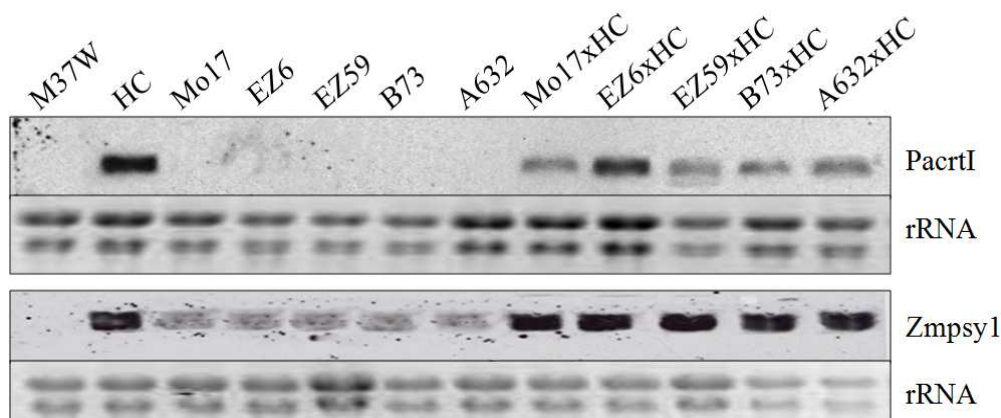
Figure 1.2: mRNA blot showing *Zmpsy* and *PacrtI* transcript accumulation in all parents and hybrids; 25µl of total RNA was loaded in each lane and rRNA (stained with ethidium bromide) was used as loading control.

1.4.2 Carotenoid composition and accumulation in wild-type and HC maize endosperm

Maize endosperm was excised 30 days after pollination (DAP) from field grown Mo17, EZ59, EZ6, B73, A632, HC and M37W plants. Carotenoid content was determined by HPLC (**Table 1.4**). This analysis confirmed that M37W endosperm contained only trace amounts of carotenoids (0.8 µg/g DW), whereas the yellow inbred lines Mo17, EZ59, EZ6, B73 and A632 accumulated up to 27.8, 13.5, 16.6, 17.8 and 9 µg/g DW, respectively. No, or very low accumulation of β-carotene, and α- and β-cryptoxanthin (the immediate precursors of lutein and zeaxanthin) were present in Mo17, EZ59, EZ6, B73 and A632 endosperm (**Table 1.4**). Lutein and zeaxanthin were the predominant carotenoids in yellow maize endosperm, but each line exhibited different β:ε ratios. I classified wild type yellow maize lines into three different groups: low β:ε ratio (less than 1), equal β:ε ratio (close to 1), or high β:ε ratio (2 or higher).

The first group with low β:ε ratio included Mo17 and B73 inbred lines exhibiting a ratio of ca: 0.4 and 0.15, respectively. Both lines had similar carotenoid profiles. The major carotenoids in Mo17 were β-carotene (2 µg/g DW), zeaxanthin (4.9 µg/g DW) and lutein (27.2 µg/g DW). Lutein was the most prevalent carotenoid (ca: 75% of the total; see **Figure 1.3** and **Table 1.4**). B73 accumulated ca: 12 µg/g DW of lutein (ca: 65% of the total amount of carotenoids). B73 also accumulated α-cryptoxanthin (ca: 3 µg/g DW), zeaxanthin (ca: 2 µg/g DW) and traces of antheraxanthin.

EZ6 had a β/ε ratio of ca: 1, accumulating near equal amounts of lutein and zeaxanthin, ca: 6 and 7 μg/g DW, respectively. Parental line M37W had a β/ε ratio of ca: 1 and also accumulated equal amounts of lutein and zeaxanthin, ca: 0.2 μg/g DW. EZ6 accumulated other carotenoids such as α-carotene or β-cryptoxanthin in lower amounts, and no β-carotene (**Figure 1.3** and **Table 1.4**). M37W accumulated only traces of zeaxanthin, lutein, antheraxanthin and violaxanthin.

EZ59 and A632 accumulated higher amounts of β-branch carotenoids. EZ59 was the only line accumulating phytoene (3.4 μg/g DW) (**Table 1.4**) suggesting a bottleneck in desaturation and isomeration steps of phytoene towards lycopene production. The most abundant carotenoid in EZ59 was zeaxanthin (ca: 8 μg/g DW), followed by lutein (4.5 μg/g DW). Only traces of antheraxanthin and β-cryptoxanthin were measured in this line. The higher accumulation of β-branch metabolites in EZ59 resulted in a β/ε ratio close to 2. A632 had the highest β/ε ratio, ca: 5, due to a higher accumulation of zeaxanthin versus lutein, ca: 6 μg/g DW and 1.5 μg/g DW, respectively. Traces of antheraxanthin and β-cryptoxanthin were also measured in A632 (**Figure 1.3** and **Table 1.4**).

Plant lines	Phytoene	β-caro	β-crypto	Zeaxant	Anthera	Violaxanth	α-caro	α-crypto	Lutein	Total	ratio β/ε
Mo17	0.0±0.0	2.0±0.1	0.7±0.1	4.9±0.1	0.3±0.0	0.0±0.0	0.0±0.0	0.0±0.0	19.9±0.6	27.8	0.38
Mo17xHC	7.0±0.9	4.6±0.4	3.3±0.5	32.1±6.5	0.0±0.0	0.0±0.0	2.5±0.1	10.3±0.5	27.2±5.2	87	1
EZ6	0.0±0.0	0.0±0.0	1.7±0.3	6.7±2.3	0.5±0.2	0.0±0.0	1.0±0.1	0.6±0.0	6.1±0.1	16.6	1.09
EZ6xHC	1.6±0.2	6.8±0.1	1.9±0.3	14.1±0.7	1.4±0.2	0.0±0.0	0.0±0.0	1.7±0.1	6.7±0.0	34.2	2.72
EZ59	3.4±0.1	0.0±0.0	0.6±0.0	7.9±0.4	0.4±0.0	0.0±0.0	0.0±0.0	0.0±0.0	4.5±0.3	16.8	1.88
EZ59xHC	14.7±2.6	9.2±1.9	3.7±0.3	21.5±7.5	1.4±0.4	0.0±0.0	3.6±0.8	3.0±0.6	10.8±2.8	67.9	1.98
B73	0.0±0.0	0.0±0.0	0.0±0.0	2.3±0.7	0.6±0.1	0.0±0.0	0.0±0.0	2.6±0.1	12.3±0.9	17.8	0.15
B73xHC	3.2±0.1	2.3±0.3	0.0±0.0	2.8±0.1	0.7±0.0	0.0±0.0	0.0±0.0	5.3±0.1	26.1±0.9	40.4	0.16
A632	0.0±0.0	0.0±0.0	0.8±0.0	6.4±1.0	0.3±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.5±0.1	9.0	4.9
A632xHC	2.7±0.2	7.3±0.6	3.6±0.1	26.4±3.5	1.6±0.8	0.0±0.0	0.0±0.0	0.0±0.0	4.1±0.7	45.7	9.07
HC	6.0±0.8	8.1±0.2	3.3±0.3	31.6±2.5	4.3±0.3	0.6±0.1	0.0±0.0	0.0±0.0	4.9±0.3	58.8	8.83
M37W	0.0±0.0	0.0±0.0	0.0±0.0	0.22±0.06	0.22±0.01	0.13±0.05	0.0±0.0	0.0±0.0	0.23±0.0	0.8	0.95

Table 1.4: Endosperm carotenoid content (presented as µg ∕ g dry weight (DW) ± SD (n = 3–5 mature seeds) of wild-type (M37W, Mo17, EZ6, EZ59, B73, A632), transgenic high carotenoid (HC), and hybrids (Mo17xHC, EZ6xHC, EZ59xHC, B73xHC and A632xHC) determined by HPLC. Abbreviations: β-caro, β-carotene; β-crypto, β-cryptoxanthin; zeaxant, zeaxanthin; anthera, antheraxanthin; violaxanth, violaxanthin; α-caro, α-carotene; α-crypto, α-cryptoxanthin.
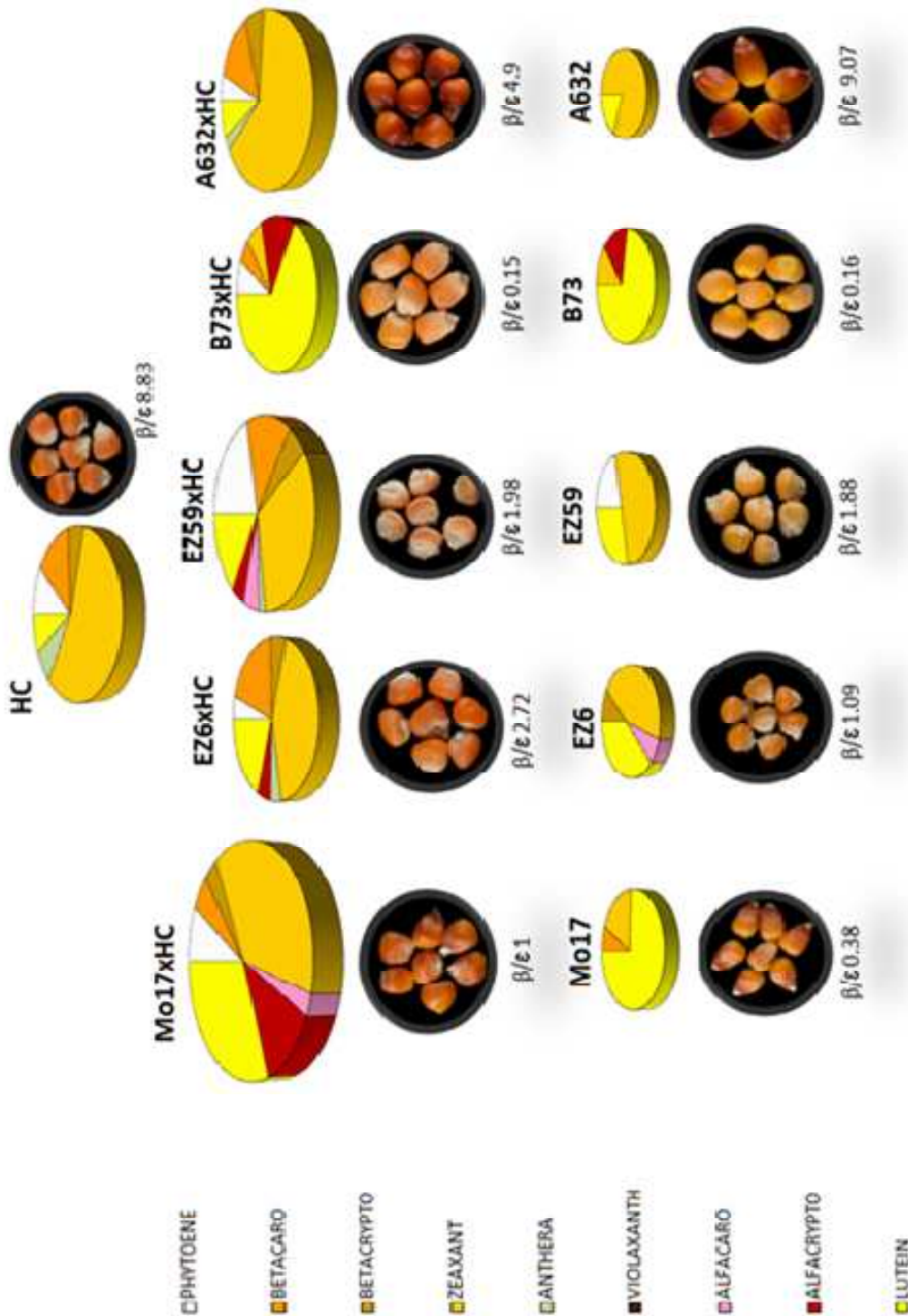
Figure 1.3: Carotenoid profiles in maize hybrids (Mo17xHC, EZ6xHC, EZ59xHC, B73xHC and A632xHC), transgenic line HC and wild-type inbreds (Mo17, EZ6, EZ59, B73 and A632). Color phenotypes reflect differences in carotenoid accumulation in the endosperm (represented as the surface area of pie charts corresponding to the total carotenoid content) and β/ε ratio for each phenotype. Abbreviations: betacaro, β-carotene; betacrypto, β-cryptoxanthin; zeaxant, zeaxanthin; anthera, antheraxanthin; violaxanth, violaxanthin; alfacaro, α-carotene; alfacrypto, α-cryptoxanthin.

1.4.3 Transcript levels of endogenous carotenogenic genes in wild-type maize endosperm

Transcript levels of endogenous genes in the carotenoid biosynthetic pathway were compared by real-time qRT-PCR. Relative expression levels of the endogenous carotenogenic genes were determined in the wild type inbreds (**Figure 1.4**). mRNA for *Zmpsy1* (encoding the endosperm-specific isoform of phytoene synthase) did not accumulate in the white M37W endosperm but did so in the yellow endosperm varieties. *Zmpsy2* mRNA (encoding the isoform of phytoene synthase that is preferentially accumulated in vegetative tissues) was present at lower levels in all inbred lines. *Zmpsy2* transcript also accumulated in white M37W endosperm, perhaps accounting for the trace levels of carotenoids in this line.

Levels of *ZmlycE* mRNA accumulation in Mo17 and B73 lines (low β/ε ratio lines), were higher than *ZmlycB*, (5- and almost 10-fold higher, respectively). *Zmbch2* mRNA accumulated at very low levels in both lines, whereas *Zmbch1* mRNA accumulated at considerably higher levels in B73. *ZmcypC* and *ZmcypB* transcript accumulation was similar in both lines. However, *ZmcypA* mRNA accumulation was almost double in Mo17 compared to B73 (**Figure 1.4**).

ZmlycE and *ZmlycB* transcripts accumulated at similar levels in EZ6 (β/ε ratio ca: 1). *Zmbch1* transcript accumulated at higher levels (50-fold higher) compared to *Zmbch2*. *ZmcypA*, *ZmcypB* and *ZmcypC* accumulated at similar levels in this line. *ZmlycB*, *Zmbch2* and *ZmcypA* mRNA accumulation was the highest in M37W (compared to all other lines; **Figure 1.4**).

ZmlycE mRNA accumulation was hardly measurable in EZ59 and A632 (β/ε ratio equal or higher than 2). *ZmlycB* mRNA accumulation was the highest in EZ59, (with the

exception of M37W). *ZmlycB* mRNA accumulation in A632 was ca: 2-fold lower than in EZ59 (**Figure 1.4**). *Zmbch2* mRNA accumulation was low in both lines, while *Zmbch1* mRNA accumulated at higher levels in A632 (ca: 7-fold higher than EZ59). A significantly higher accumulation of *ZmcypC* mRNA was measured in EZ59 (ca: 15-fold higher) compared to A632 and also all the other lines. Similarly, mRNA accumulation for *ZmcypB* doubled in EZ59 compared to A632 (**Figure 1.4**).
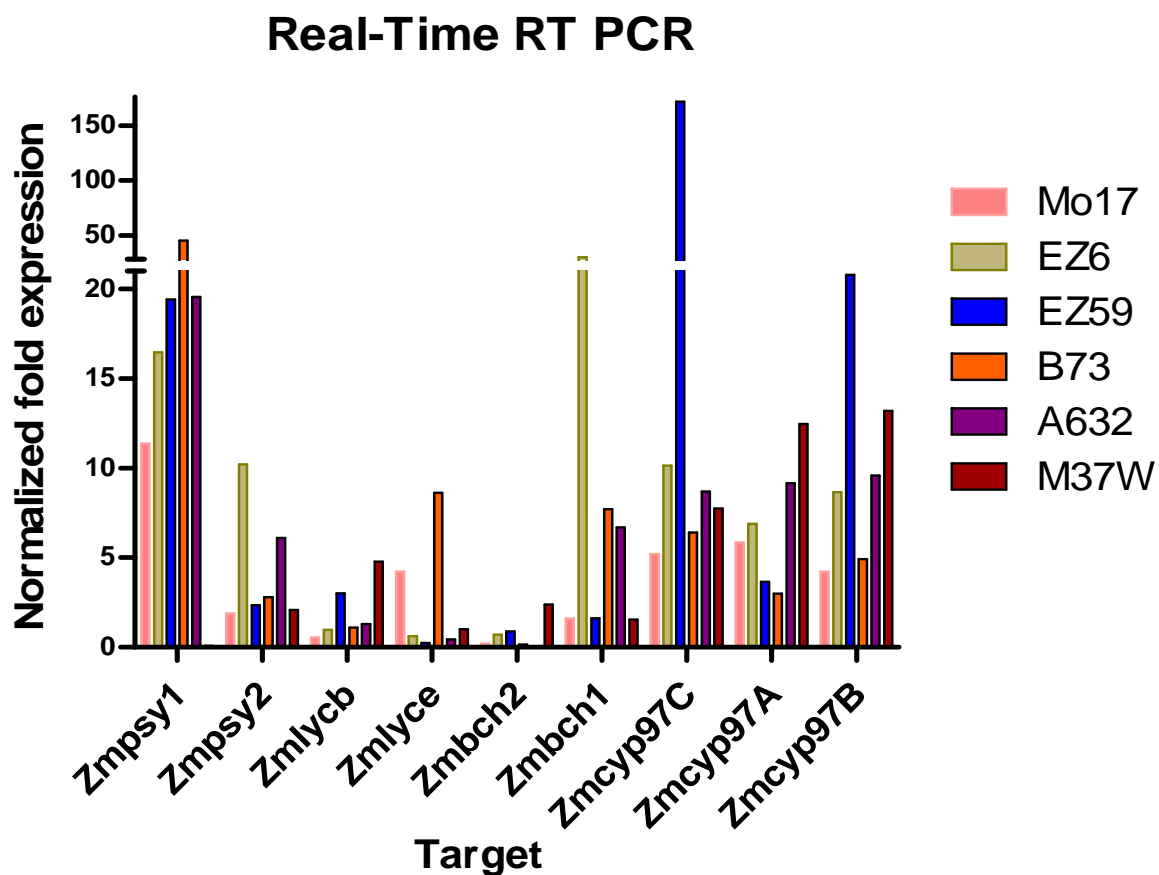
Figure 1.4: Quantitative Real Time RT-PCR analysis showing relative mRNA accumulation of endogenous carotenogenic genes in immature maize endosperm (30 DAP), normalized against actin mRNA and presented as the mean of three replicates ± SD. Abbreviations: PSY1/2, phytoene synthase 1/2; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; BCH1/2, β-carotene hydroxylase 1/2. CYP97A/B, β-carotene hydroxylase; CYP97C, carotene ε-hydroxylase.

1.4.4 Carotenoid profiles in hybrid lines

While M37W accumulates only traces of carotenoids (total content up to ca: 1 μg/g DW), *Zmpsy1* and *PacrtI* transcripts accumulated in HC and were responsible for the increased total carotenoid content up, to ca: 59 μg/g DW (**Table 1.4**). β-Carotene and zeaxanthin accumulated up to ca: 8 μg/g and 32 μg/g DW in HC, respectively. Additional β-branch carotenoids also accumulated. These included β-cryptoxanthin (ca: 3 μg/g DW), antheraxanthin (ca: 4 μg/g DW) and violaxanthin (traces). The only ε-branch carotenoid was lutein (ca: 5 μg/g DW). Due to this accumulation profile, the β/ε ratio increased up to 9-fold in HC over the wild type (**Table 1.4**). Phytoene also accumulated, in HC (up to 6 μg/g DW).

All hybrids exhibited different carotenoid accumulation profiles compared to their respective inbred parents and HC (**Table 1.4** and **Figure 1.3**). Thus, a number of the hybrids had a different β/ε ratio compared to their corresponding parents. Total carotenoid content increased in all hybrids compared to their corresponding yellow inbred parent. Higher levels of total carotenoids accumulated in Mo17xHC and EZ59xHC (ca: 87 μg/g DW and 68 μg/g DW, respectively) compared to HC (ca: 59 μg/g DW). Total carotenoid content in B73xHC, EZ6xHC and A632xHC increased versus their yellow maize parents (40 μg/g DW, 34 μg/g DW, 46 μg/g DW, respectively). However, total carotenoid levels in these three hybrids were lower compared to the HC transgenic parent (ca: 59 μg/g DW) (**Table 1.4**).

A 2.6-fold increase in the β/ε ratio was measured in Mo17xHC versus Mo17 due to an increase in β-branch carotenoids (**Table 1.4** and **Figure 1.3**). Zeaxanthin levels increased ca: 6-fold (up to 32 μg/g DW), β-cryptoxanthin 5-fold (up to 3 μg/g DW) and β-carotene levels more than doubled (ca: 5 μg/g DW) (**Table 1.4**). In the ε-branch, lutein levels were not increased significantly, while α-carotene and α-cryptoxanthin accumulated (ca: 2.5 μg/g DW and 10 μg/g DW, respectively). Phytoene accumulated up to 7 μg/g DW. The β/ε ratio in B73xHC did not change compared to B73; rather, the amounts of most carotenoids increased proportionally (**Table 1.4** and **Figure 1.3**). Lutein and α-cryptoxanthin accumulation doubled, while zeaxanthin and antheraxanthin levels did not change. β-Carotene was the only β-branch metabolite whose levels increased (ca: 2 μg/g DW). Phytoene also accumulated in this hybrid (ca: 3 μg/g DW).

EZ6xHC exhibited a β/ε ratio of ca: 2.7-fold versus EZ6 (**Table 1.4** and **Figure 1.3**). Accumulation of ε-branch carotenoids did not show significant variation, but in the β-branch,

zeaxanthin and antheraxanthin levels doubled. β-Carotene also accumulated (ca: 7 μg/g DW) and phytoene was detected (ca: 2 μg/g DW) in the hybrid.

EZ59xHC maintained a β/ε ratio of ca: 2 similarly to EZ59 (**Table 1.4** and **Figure 1.3**). Accumulation of different β-branch carotenoids increased in this hybrid. Zeaxanthin levels increased ca: 3-fold (21.5 μg/g DW), β-cryptoxanthin ca: 6-fold (3.7 μg/g DW), antheraxanthin 3.5-fold (1.4 μg/g DW), and β-carotene also accumulated (ca: 9 μg/g DW) (**Table 1.4**). Levels of ε-branch carotenoids also increased. Lutein levels doubled (ca: 10 μg/g DW), and α-carotene and α-cryptoxanthin also accumulated (ca: 4 μg/g DW and 3 μg/g DW, respectively). Phytoene accumulation increased ca: 4-fold (15 μg/g DW) (**Table 1.4**). The β/ε ratio in A632xHC increased up to ca: 9 compared to A632 (**Table 1.4** and **Figure 1.3**). This was due to a significant increase in zeaxanthin (ca: 4-fold, 26 μg/g DW), β-cryptoxanthin (ca: 4.5-fold, 4 μg/g DW) and antheraxanthin (ca: 5-fold, 1.5 μg/g DW) levels. β-Carotene also accumulated up to ca: 7 μg/g DW. Lutein was the only ε-branch carotenoid whose levels increased (ca: 4-fold, 4 μg/g DW). No other carotenoids were present, but phytoene accumulated up to ca: 3 μg/g DW in the hybrid (**Table 1.4**).

1.5 Discussion

1.5.1 Carotenoid profile in maize endosperm is controlled by the expression of endogenous carotenogenic genes

The biosynthesis and functions of carotenoids in plants have been extensively reviewed in recent years. However, despite significant progress in our understanding of carotenogenesis in plants, there are still a lot of questions about how carotenoid biosynthesis and accumulation regulating mechanisms operate. As compared to other staple cereals, maize possesses tremendous variability for endosperm carotenoids (Buckner, Kelson, & Robertson, 1990). In the current study mRNA analysis confirmed previous findings that *Zmpsy1* transcripts do not accumulate in white (M37W) maize endosperm; rather *Zmpsy2* transcripts accumulated as the predominant isoform moiety of this gene. This suggested that the residual carotenoid content in M37W endosperm was due to expression of *Zmpsy2*. Although *Zmpsy2* transcripts accumulated in M37W endosperm, the total carotenoid content remains extremely low, confirming that *Zmpsy1*, not *Zmpsy2*, plays a crucial role in the accumulation of carotenoids in endosperm in yellow maize, as reported in previous studies (Zhu et al, 2008).

Further supporting evidence for the key role of *Zmpsy1* in endosperm carotenogenesis is corroborated by the expression of the gene in diverse yellow maize germplasm such as Mo17, EZ6, EZ59, B73 and A632 inbred lines in which a positive correlation between the accumulation of *Zmpsy1* transcripts yellow endosperm phenotype, and carotenoid accumulation in the endosperm was measured. Phytoene undergoes four desaturation and subsequent isomerization steps to produce all-trans lycopene. The cyclization of all trans-lycopene is an important branch point in carotenoid biosynthesis. Alternatively, lycopene ε-cyclase (LYCE) adds one ε-ring at one end of all trans-lycopene and a second cyclization by LYCB produces α-carotene (Cunningham et al 1996). Cyclase enzymes play key roles in controlling relative concentrations of lutein and zeaxanthin in the endosperm. ZmlycE transcripts accumulated at higher levels in Mo17 and B73 inbred lines compared to EZ6, EZ59, A632 and HC (**Figure 1.5**). Precursor flow was thus directed towards lutein, and this was reflected in lower β/ε ratios (0.38 and 0.16, respectively for these two genotypes). On the other hand, *ZmlycB* mRNA accumulated at higher levels in EZ6, EZ59 and A632 inbred lines, and it correlated with higher amounts of zeaxanthin accumulation relative to lutein. The corresponding β/ε ratios in the three genotypes were ca: 1, 2 and 5, respectively.

1.5.2 Carotenoid accumulation in maize hybrids reveals the existence of bottlenecks towards zeaxanthin and lutein accumulation

The hybrid lines had a bright orange endosperm phenotype indicating the accumulation of higher amounts of carotenoids, and/or different carotenoid profiles compared to their corresponding parents (**Figure 1.4**). Introgression of a carotenogenic mini-pathway (comprising *Zmpsy1* and *PacrtI*) into different yellow maize genetic backgrounds increased significantly the total carotenoid content in each hybrid by eliminating early bottlenecks in the pathway. These bottlenecks constrain phytoene synthesis and its subsequent conversion to lycopene. The higher accumulation of total carotenoids in the hybrids indicated new bottlenecks in the carotenoid pathway in the different yellow maize inbreds: phytoene isomerization and desaturation, lycopene cyclization, and α- and β-carotene hydroxylation.

The first new bottleneck detected in the hybrids, phytoene isomerization and desaturation steps, have been shown earlier to limit carotenoid biosynthesis in tomato (Enfissi et al., 2005), potato (Morris et al., 2006), rice (Bai et al., 2015) and maize (Naqvi et al., 2011). Here we demonstrate that introducing *PacrtI*, which constrains phytoene isomerisation and

desaturation in HC, Mo17xHC, B73xHC, EZ6xHC, EZ59xHC and A632xHC increased phytoene levels in all of them compared to their corresponding parents. *Zmpsy1* transcript accumulation eliminated the first bottleneck in the pathway which impedes phytoene synthesis, but the high amounts of newly synthesized phytoene were not converted to lycopene efficiently by *PacrtI*.

No lycopene accumulated in any of the hybrids, indicating that *ZmlycE/B* converted all the lycopene to α- and β-carotene efficiently in HC, Mo17xHC, B73xHC, EZ6xHC, EZ59xHC and A632xHC. Previous reports confirmed that the β/ε ratio is determined by the relative levels of *ZmlycE* and *ZmlycB* transcripts in maize (Harjes et al., 2008; Naqvi et al., 2011). Similar results were reported in *Brassica napus* (Yu et al., 2008), tomato (Dharmapuri et al., 2002*; Rosa*ti et al., 2000; D'Ambrosio et al., 2004) and potato (Diretto et al., 2006).

Hydroxylation of α- and β-carotene is also critical in regulating relative amounts of lutein and zeaxanthin in maize endosperm (Wurtzel, 2004). We demonstrated that even in cases in which *Zmlycb* transcripts accumulated at higher amounts than the corresponding *Zmlyce* transcripts, α- and β-carotene hydroxylation was a determining factor for the accumulation of lutein and zeaxanthin in the endosperm. Each hybrid is discussed in detail bellow.

Mo17xHC hybrid

The main carotenoids in Mo17 are β-carotene (2 μg/g DW), zeaxanthin (ca: 5 μg/g DW) and lutein (ca: 27 μg/g DW). Lutein is the predominant carotenoid accounting for ca: 75% of total carotenoids (**Figure 1.4** and **Table 1.4**). This high accumulation of lutein was reflected in a β/ε ratio of 0.38. High accumulation of *ZmlycE* transcripts in Mo17 directed the pathway towards the ε-branch (**Figure 1.3** and **Figure 1.5**). Mo17xHC had a 3-fold increase in total carotenoid (87 μg/g DW) compared with Mo17 (**Table 1.4**). New carotenoids which were not present in Mo17 accumulated in the hybrid. These included phytoene, α-carotene and α-cryptoxanthin. Zeaxanthin accumulation in the hybrid increased significantly and this was also reflected in a β/ε ratio up to 1 in the hybrid (**Figure 1.4**). *ZmlycB* transcripts in the hybrid (residing in the genetic background of the transgenic parent-M37W) compensated the predominance of *ZmlycE* transcripts (from Mo17) generating a balance in the hybrid reflected as equal accumulation of total metabolites in the ε and β branches (**Figure 1.4** and **Figure 1.6**).

EZ6xHC hybrid

Lutein and zeaxanthin accumulated at near equal levels ca: 6 and 7 μg/g DW, respectively, in EZ6. Other carotenoids such as α-carotene or β-cryptoxanthin accumulated in lower amounts. EZ 6 contains no β-carotene (**Figure 1.4** and **Table 1.4**). A β/ε ratio of ca: 1 indicated that metabolite amounts in both branches were similar, as were the levels of *ZmlycE* and *ZmlycB* transcript accumulation (**Figure 1.3** and **Figure 1.5**). However, the β/ε ratio in EZ6xHC was more than double (ca: 3) compared with EZ6. Thus, zeaxanthin levels increased significantly whereas lutein amounts were similar as in EZ6 (**Figure 1.4** and **Table 1.4**). This increase in β/ε ratio was due to a higher accumulation of *ZmlycB* transcripts (from the M37W background of the transgenic parent) directing the pathway towards the β-branch (**Figure 1.3** and **Figure 1.6**). A bottleneck in the hydroxylation step from α-cryptoxanthin to lutein resulted in a higher accumulation of α-cryptoxanthin (more than 2-fold) in the hybrid, and no change in lutein accumulation (ca: 7 μg/g DW) (**Figure 1.3** and **Figure 1.6**). Other metabolites accumulated in the hybrid such as β-carotene (ca: 7 μg/g DW) and phytoene (ca: 2 μg/g DW), which were absent in EZ6 (**Figure 1.4** and **Table 1.4**).

EZ59xHC hybrid

EZ59 inbred line was the only wild type line accumulating phytoene (ca: 3 μg/g DW) (**Table 1.4**), indicating a bottleneck in desaturation and isomerization of phytoene towards lycopene. The same bottleneck was present in EZ59xHC. Phytoene levels increase ca: 5-fold (ca: 15 μg/g DW), more than double the amount than in the HC transgenic parent (ca: 6 μg/g DW). The β/ε ratio was similar between EZ59 and EZ59xHC, ca: 2. The total amount of carotenoids increased 4-fold in the hybrid compared to EZ59 (**Figure 1.4** and **Table 1.4**). Endogenous gene expression and metabolite accumulation profiles indicated that lack of *Zmpsy1* expression in the endosperm was the only bottleneck in EZ59 (**Figure 1.5** and **1.6**). Introgression of the carotenogenic mini-pathway into the hybrid increased the total amount of carotenoids and the accumulation of β-carotene (ca: 9 μg/g DW). β-carotene levels in the hybrid were similar to those in the HC parent (ca: 8 μg/g DW). Zeaxanthin and lutein amounts also increased proportionally, compared with EZ59 [(ca: 3- and 2-fold respectively) (**Figure 1.4** and **Table 1.4**)].

B73xHC hybrid

B73 has a proportionally similar carotenoid profile as that in Mo17. Lutein is the predominant carotenoid and the β/ε ratio indicates that the pathway is directed towards the ε branch (**Figure 1.4**). However, the β/ε ratio in B73xHC remained similar to the B73 parent (0.16 and 0.15, respectively). *ZmlycE* transcripts accumulated at higher levels compared with the other yellow endosperm lines and the HC transgenic parent. In this hybrid *ZmlycB* introgressed from the transgenic line did not compensate the pathway towards the β branch (**Figure 1.3**, **Figure 1.5** and **Figure 1.6**). The total amount of carotenoids was increased in the hybrid up to ca: 2-fold. The amount of a number of metabolites such as lutein and α-cryptoxanthin was increased. β-Carotene and phytoene absent in B73accumulated in the hybrid. Zeaxanthin accumulation was almost the same as in B73. In this hybrid low expression of *Zmbch1* and *Zmbch2* β-carotene hydroxylases generated a bottleneck in β-carotene hydroxylation towards zeaxanthin (**Figures 1.5** and **1.6**).

A632xHC hybrid

A632 has the lowest accumulation of total carotenoids (9 µg/g DW). The predominant carotenoids in this line are lutein and zeaxanthin (**Figure 1.4** and **Table 1.4**). The β/ε ratio in A632 (ca: 5) indicates that the carotenoid pathway in this line is directed toward the β-branch. In the A632xHC hybrid this ratio is almost double suggesting a synergistic effect in *ZmlycB* between A632 and HC (**Figure 1.5** and **1.6**). The amounts of metabolites in the β-branch of the carotenoid pathway such as β-cryptoxanthin, zeaxanthin and antheraxanthin increased up to ca: 4.5-, 4- and 5-fold, respectively, in the hybrid. β-carotene accumulated in the hybrid up to ca: 7 µg/g DW. In the case of the ε-branch, the only metabolite that accumulated was lutein, the levels of which increased ca: 3-fold in the hybrid. (**Table 1.4**).
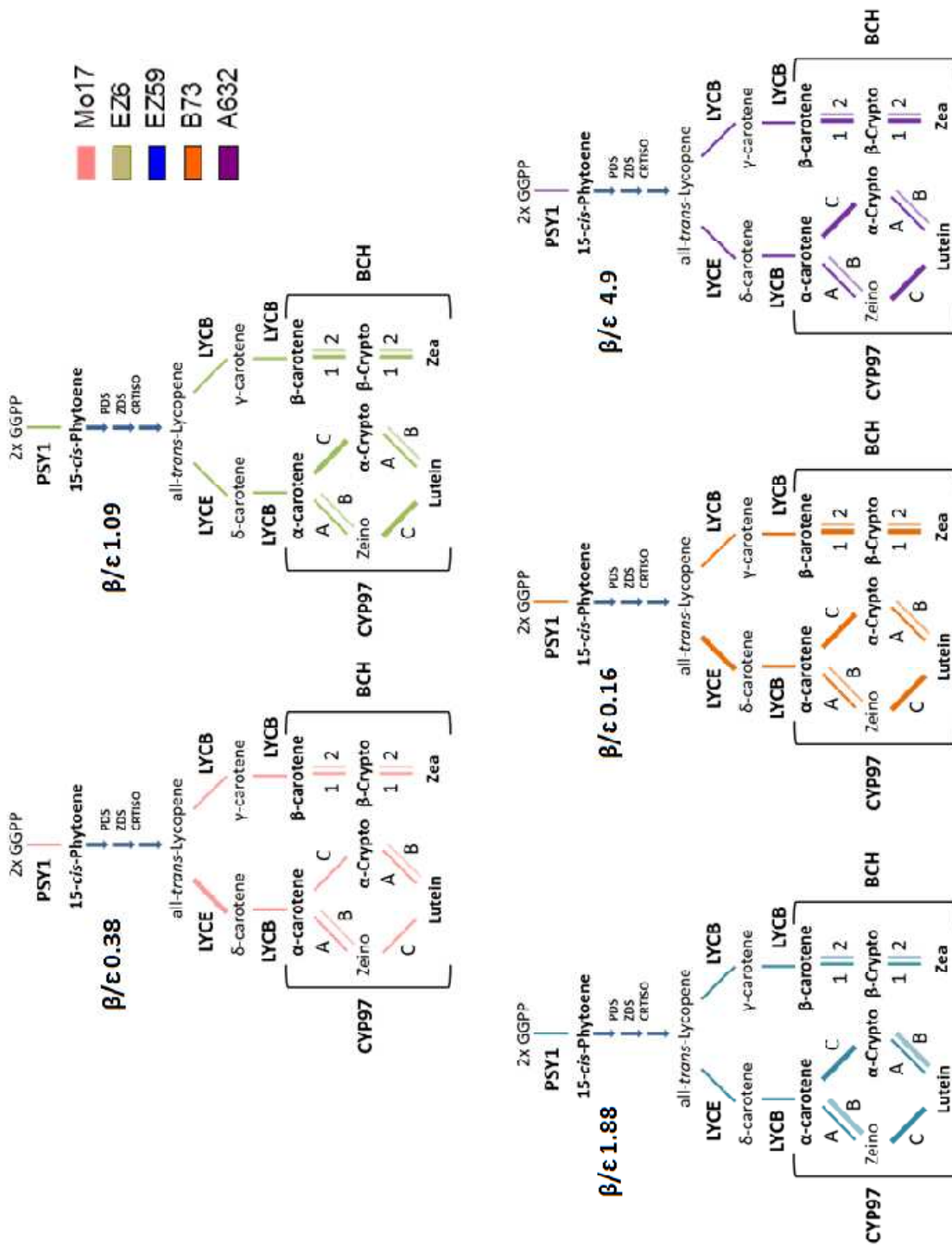
Figure 1.5: Gene expression in the wild type inbred parents compared with HC. Thicker lines represent a higher mRNA accumulation of the corresponding genes in each step compared with HC. β/ε ratio is also shown for each inbred line. Abbreviations: A, CYP97A; B, CYP97B (linked to a lighter line); C, CYP97C; 1, BCH1; 2, BCH2 (linked to a lighter line); CRTISO, carotenoid isomerase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase.
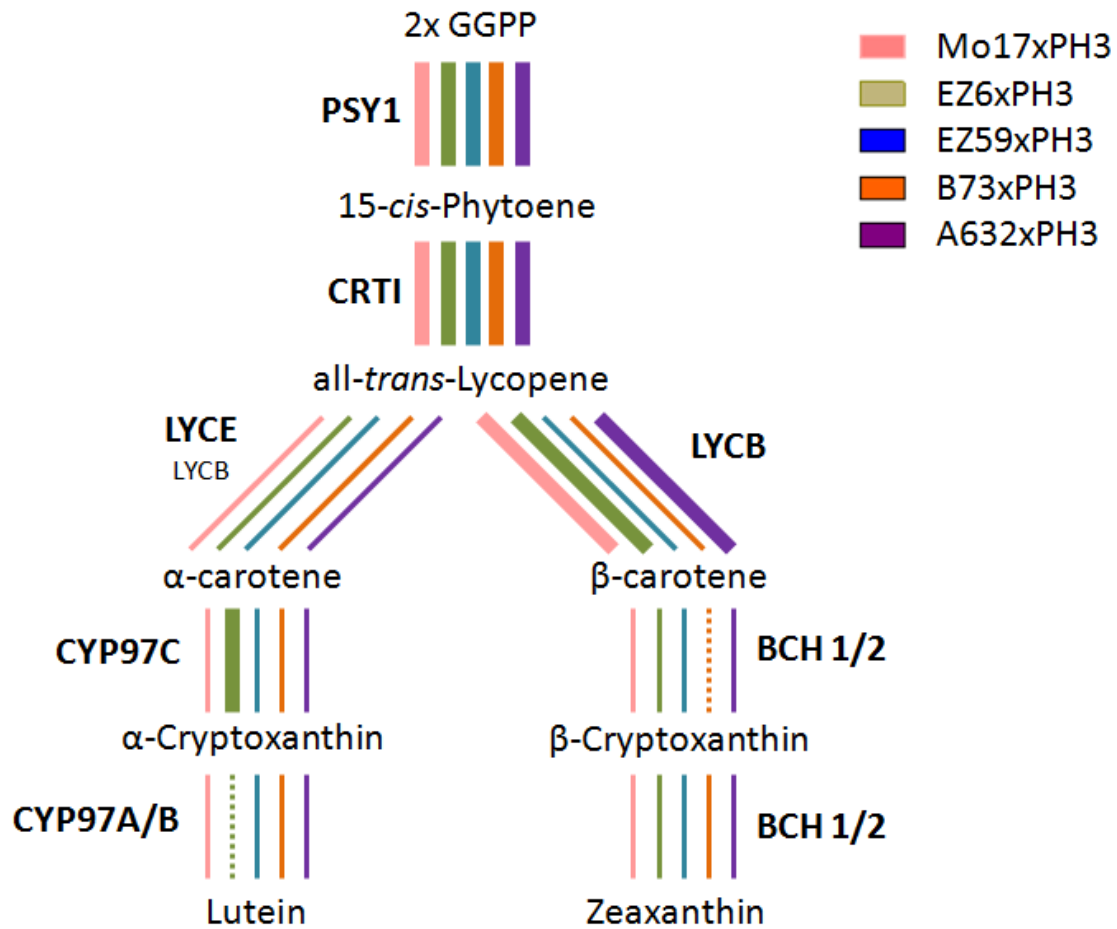
Figure 1.6: Representation of modulation of the carotenoid pathway given by HC in different inbred lines. Each color corresponds to a different hybrid; thicker lines represent reaction steps where introgression of HC background has eliminated a bottelneck; intermittent lines represent bottlenecks in the pathway that still remains in the hybrid; thinner lines represent no change in the reaction flux. Abbreviations: PSY1, phytoene synthase; CRTI, bacterial phytoene desaturase, which catalyze all desaturation and isomerization reaction from phytoene to lycopene; CYP97C, heme-containing cytochrome P450 carotene e-ring hydroxylase; BCH, β-carotene hydroxylase [non-heme di-iron β-carotene hydroxylase; CYP97A, CYP97B and CYP97C, heme-containing cytochrome P450 β-ring hydroxylases; LYCB, lycopene b-cyclase; LYCE, lycopene ε-cyclase.

1.6 Conclusions

A comparative investigation of several yellow endosperm maize inbred lines and the white endosperm M37W line, focusing on targeted carotenoid transcript and metabolite analysis allowed me to identify several bottlenecks in the pathway in these lines. mRNA analysis revealed the complete absence of *psy1* transcripts in M37W endosperm whereas I could measure *psy2* expression in the endosperm suggesting that the residual carotenoid content in white endosperm maize is most likely due to the activity of PSY2. This finding confirms that PSY1 (Y1), and not PSY2, plays a crucial role in the accumulation of carotenoids in corn endosperm.

Transcript levels for both lycopene ε-cyclase (*ZmlycE*) and β-cyclase (*ZmlycB*) varied among the different yellow endosperm lines and also M37W, generating different β/ε ratios. Transcript levels for α- and β-carotene hydroxylases (*Zmbch1/2* and *CYP97A/B/C*) were critical in lutein and zeaxanthin accumulation in the endosperm in all maize lines.

In order to develop further insights into the control of carotenoid accumulation in corn endosperm the impact of transgene expression on the regulation of carotenoid biosynthesis was compared between five yellow maize lines (Mo17, B73, EZ6, EZ59 and A632) and a transgenic high carotenoid line generated earlier (HC), itself originally derived from white maize M37W. I introgressed two transgenes (*Zmpsy1+PacrtI*) in the five yellow maize lines by crossing them with HC. Expression of the introgressed transgenes in Mo17xHC, B73xHC, EZ6xHC, EZ59xHC and A632xHC increased total carotenoid content up to ca: 3-, 2-, 2-, 4- and 5-fold, respectively, compared to the corresponding yellow endosperm parents (Mo17, B73, EZ6, EZ59 and A632). Maize hybrids accumulated different amounts of lutein and zeaxanthin, highlighting bottlenecks in α- and β-carotene hydroxylation in some of these hybrids. EZ6xHC exhibited a bottleneck in α-carotene hydroxylation towards lutein accumulation, while B73xHC exhibited a bottleneck in β-carotene hydroxylation towards zeaxanthin accumulation. In Mo17xHC endogenous carotenogenic genes from HC eliminated an existing bottleneck in Mo17 towards zeaxanthin accumulation. This fact allowed a high and equal accumulation of zeaxanthin and lutein in Mo17xHC. EZ59xHC increased proportionally all the carotenoids and A632xHC almost doubled the β/ε ratio.

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CHAPTER 2

SIMULTANEOUS RECONSTRUCTION OF THE CAROTENOID AND VITAMIN E BIOSYNTHETIC PATHWAYS IN MAIZE SEEDS

2.1 Abstract

Vitamin E is a group of four tocopherols and four tocotrienols collectively known as tocochromanols. The naturally occurring isomer α-tocopherol (RRR-α-tocopherol) is 1.5 fold more active than synthetic vitamin E. A heterologous vitamin E pathway comprising *AtPDS1*, *AtVTE2*, *AtVTE3* and *AtVTE4* in a transgenic line was introgressed into a second line expressing *Zmpsy1* and *PacrtI* (HC). The simultaneous reconstruction of the carotenoid and vitamin E biosynthetic pathways in the resulting hybrids caused a significant increase in α-tocopherol accumulation and a moderate decrease in carotenoid accumulation in maize seeds, demonstrating an interaction between the two pathways. Endosperm-specific carotenoid biosynthesis and constitutive tocopherol biosynthesis influenced core metabolic processes in the embryo and endosperm of the hybrids. This in turn resulted in an increase in zeaxanthin (ca: 3-fold) and α-tocopherol (ca: 8-fold) accumulation compared with HC, respectively, as the predominant metabolites in the two pathways, in the maize embryo. Even no α-tocopherol accumulation in the endosperm, total carotenoid amount decreased up to a ca: 28%. This decrease was primarily due to a reduction in the levels of zeaxanthin and phytoene accumulation.

2.2 Introduction

2.2.1 Vitamin E

Vitamin E is a group of four tocopherols and four tocotrienols, collectively known as tocochromanols or simply tocols (DellaPenna and Pogson 2006). They are amphipathic molecules consisting of a chromanol group with one, two or three methyl groups and an isoprenyl side chain. Tocopherols possess a saturated phytyl tail, while tocotrienols have an unsaturated geranylgeranyl side chain (**Figure 2.1**). Tocochromanols vary in their vitamin E activity *in vivo*. All isomers can be absorbed equally during digestion (Traber & Sies, 1996) but the hepatic α-tocopherol transfer protein (α-TTP) shows a preferential retention for α-tocopherol making it the most important form in terms of vitamin E activity in the human body (Traber & Arai, 1999).
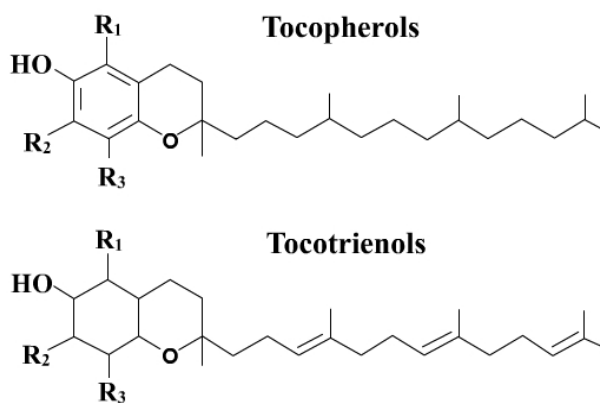
Tocochromanols	R_1	R_2	R_3
δ-	H	H	CH_3
β-	CH_3	H	CH_3
γ-	H	CH_3	CH_3
α-	CH_3	CH_3	CH_3

Figure 2.1: Chemical structures of tocopherols and tocotreinols. The table indicates the number and position of methyl groups in δ-, β-, γ-, α-tocopherols and tocotrienols (Sen et al., 2006).

Tocopherols are present in seeds, fruits, roots, tubers, cotyledons, hypocotyls, stems, leaves and flowers of higher plants (Munne-Bosch & Alegre, 2002). Tocochromanols are synthesized by plants and other photosynthetic organisms. In plants, the role of different vitamin E forms is still unclear. The presence of higher levels of α-tocopherol in photosynthetic tissues provides evidence of its role in the photosynthetic apparatus for protection against oxygen toxicity and lipid peroxidation. However, the accumulation of γ-tocopherol in seeds is thought to be involved in the prevention of auto-oxidation of polyunsaturated fatty acids (Munne-Bosch & Alegre, 2002). Other possible functions of tocochromanols in plants such as regulation of membrane fluidity and a role in intracellular signaling have received attention (Hofius & Sonnewald, 2003; Porfirova et al., 2002).

The nutritional value of vitamin E was first recognized in 1922 (Evans & Bishop, 1922). The most recognized function of tocopherols in humans is their ability to scavenge and quench reactive oxygen species and lipid-soluble oxidative stress by-products (Bramley et al., 2000; Brigelius-Flohe & Traber, 1999; Ricciarelli et al., 2002). Epidemiological data suggest that high vitamin E intake (100-1000 IU) correlates with a decreased risk of certain types of cancer and cardiovascular diseases (Bramley et al., 2000), improves the immune system and slows down the progression of human degenerative diseases (Traber & Sies, 1996). The human body cannot synthesize vitamin E and it must be provided in the diet. Recommended Daily Allowance (RDA) is 15 mg (35 µmol)/day of α-tocopherol (Institute of Medicine, 2000). Vitamin E deficiency can occur in three specific cases: a) people who either show incapacity to absorb dietary fat because they cannot secrete bile or suffer rare disorders of fat metabolism; b) people who suffer genetic abnormalities in the α-tocopherol transfer protein (α–TTP); and c) premature, very low weight birth infants [birth weights < 1500 grams (Office of dietary supplements, 2008)]. Vitamin E deficiency causes poor transmission of nerve impulses, muscle weakness, sclerosis of the gastrointestinal tract and degeneration of the retina that can cause blindness (Hanna, 1995; Tanyel & Mancano, 1997).

The composition of tocols varies widely among different plant species (**Table 2.1**). In green leaves of higher plants the predominant form of vitamin E is α-tocopherol, whereas generally in seeds γ-tocopherol is the major form (D DellaPenna & Last, 2006; Hess JL, 1983). Tocotrienols are the major form of vitamin E in seeds of monocots including rice, wheat, oat and maize (Barnes, 1983; Padley et al., 1994; Peterson & Qureshi, 1993). In dicots tocopherols are the principal vitamin E components in both leaves and seeds (Kamal-Eldin & Appelqvist, 1996; Padley et al., 1994). Tocol amount and composition in maize varies ca: from ca: 36µg/g to 44µg/g in whole seeds, where γ-tocopherol is the highest fraction (Weber, 1987). The major portion of the tocols in different maize genotypes is concentrated in the embryo (from 63% up to 91% in), while the endosperm contains from 9% up to 37%. Tocopherols are mostly concentrated in the maize embryo, while tocotrienols predominate in the endosperm (Weber, 1987). Much of the variation in plant tocochromanol content is due to the expression, activity and substrate specificities of different pathway enzymes (reviewed by DellaPenna and Last, 2006).

Plant and organ	Total tocopherols (ug/g fresh weight)	Percent α-tocopherol	Percent others and major type
Potato tubers	0.7	90	10% γ, β-T
Rice (grains)	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
Wheat germ oil	2700	47	25% β-T, 10% γ-T, 7% β-T3

Table 2.1: Tocopherol levels and composition in selected plant tissues and oils (Hess, 1983; Taylor & Barnes, 1981). α-T, β-T, γ-T and δ-T are α-, β-, γ- and δ-tocopherol, respectively. A-T3, β-T3, γ-T3 and δ-T3 are α-, β-, γ- and δ-tocotrienols, respectively.

1.1.2 Vitamin E biosynthetic pathway

The tocochromanol biosynthetic pathway in the plastids of higher plants was elucidated from radiotracer studies in the mid-1980s (Falk et al., 2003). Precursors are derived from the shikimate and methylerythritol phosphate (MEP) pathways. The shikimate pathway produces homogentisic acid (HGA) which contributes to the "head group" whereas the MEP pathway contributes to the side chain (Garcia et al., 1997) of tocochromanols. The first committed step is the conversion of ρ-hydroxyphenylpyruvic acid (HPP) to HGA by ρ-hydroxyphenylpyruvic acid dioxygenase that occurs in the cytosol (HPPD) (**Figure 2.2**). This enzyme participates in the catabolism of the aromatic amino acid tyrosine (Savidge et al., 2002). 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) (DellaPenna, 2005). Tocopherol biosynthesis takes place in the plastids and the enzymes homogentisate phyltransferase (HPT), MPBQ methyltransferase (MPBQ-MT), tocopherol cyclase (TC), γ–methyltransferase (γ–TMT)] are associated with the chloroplast

envelope. The first step in tocopherol biosynthesis is prenylation of HGA with PDP to generate MPBQ. This is a prenyl transfer reaction that is catalyzed by HPT (Hofius & Sonnewald, 2003; Karunanandaa et al., 2005). MPBQ is a substrate for MPBQ-MT, which adds a second methyl group to MPBQ to form 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). The initial step of tocotrienol biosynthesis is condensation of geranylgeranyl pyrophosphate (GGPP) with homogentisate (HGA), catalyzed by homogentisate geranylgeranyltransferase (HGGT), producing 2-methyl-6-geranylgeranylbenzoquinol (MGGBQ) (Cahoon et al., 2003). MGGBQ is the substract for MPBQ-MT, which adds a second methyl group to form 2,3-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ) (Matsuzuka et al, 2013).

All four intermediates (MPBQ, DMPBQ, MGGBQ and DMGGBQ) are substrates for tocopherol cyclase (TC), which produces δ and γ tocopherols and tocotrienols. Finally, γ-tocopherol methyltransferase (γ-TMT) catalyses a second ring methylation to yield α and β tocopherols and tocotrienols (Matsuzuka et al, 2013).
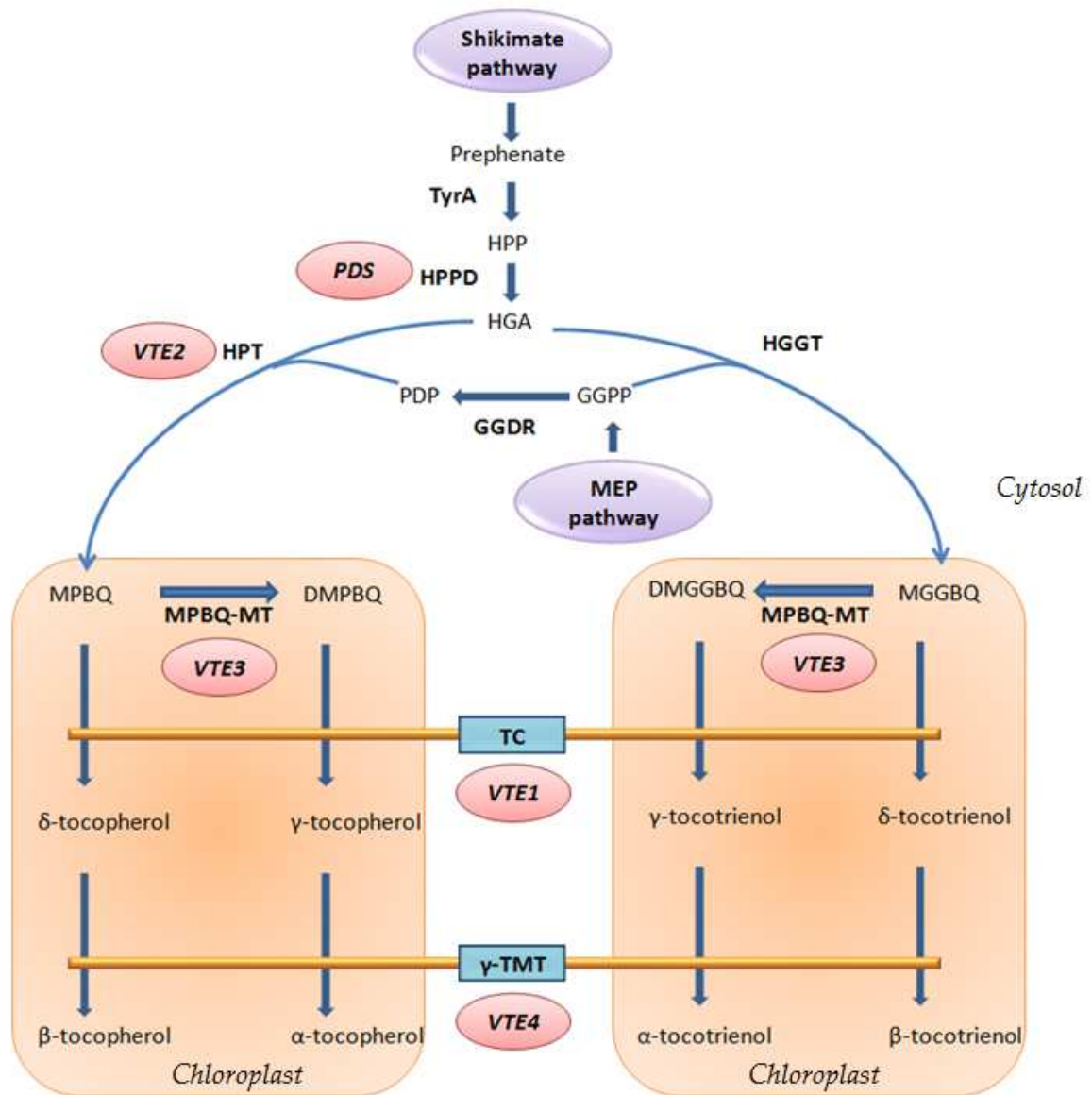
Figure 2.2: Vitamin E biosynthetic pathway in plants and transgenes used in the work described in this chapter. Enzymes in red ovals correspond to transgene product. Abbreviations: methylerythritol phosphate (MEP), ydroxyphenylpyruvate (HPP), hydroxyphenylpyruvate dioxygenase (HPPD), homogentisic acid (HGA), phytyldiphosphate (PDP), geranylgeranyl pyrophosphate (GGPP), homogentisate phytyltransferase (HPT), homogentisate geranylgeranyltransferase (HGGT), 2-methyl-6-phytylbenzoquinol (MPBQ), 2-methyl-6-phytylbenzoquinol methyltransferase (MPBQ-MT), 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ), 2-methyl-6-geranylgeranylbenzoquinol (MGGBQ), 2,3-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ), tocopherol cyclase (TC), γ–tocopherol methyltransferase (γ–TMT) (Adapted from Naqvi et al., 2011; Matsuzuka et al, 2013).

2.2.3 Genetic engineering strategies for enhancing vitamin E content in plants

Plants can be engineered to accumulate higher levels of vitamin E by introducing transgenes that encode enzymes involved in tocochromanol synthesis. Improvements in vitamin E accumulation and composition in plants can be achieved either by increasing the total tocochromanol/tocotrienol content or skewing tocochromanol synthesis toward α-tocopherol (**Table 2.2**).

The introduction of single or multiple limiting pathway 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 (Karunanandaa et al., 2005). In contrast, constitutive expression of the barley *hppd* gene in tobacco leaves had no effect on tocopherol levels (Falk et al., 2003). Arabidopsis leaves expressing Arabidopsis *HPT1* accumulated ca: 458 μg/g dry weight (DW) of total tocopherols, a 4.4-fold increase over wild-type levels (Collakova & DellaPenna, 2003). 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 (Karunanandaa et al., 2005).

Different approaches have been used to modulate vitamin E composition of plants. For example, the overexpression of MPBQ-MT diverts flux towards the α-branch of the pathway 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 or in combination, has also been investigated (Van Eenennaam et al., 2003). The expression of *VTE3* alone (under the control of the seed-specific napin promoter) increased the total tocopherol content only marginally. However, 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 in the pathway (Shintani & DellaPenna, 1998). In maize, the combination of HPPD and MPBQ-MT caused a three-fold increase in γ-tocopherol levels in whole seed (to 9.5 μg/g DW) without changing the total tocopherol content, again showing that flux was directed into the α-branch but was limited by low γ-TMT activity, forcing the accumulation of γ-tocopherol (Naqvi et al., 2011). In rice,

constitutive expression of HPPD did not change the α:γ ratio in the tocotrienol branch or the overall level of tocopherols in whole seed. However, the α:γ ratio increased significantly, resulting in an increased amount of α-tocopherol in rice seed (1.4-fold) at the expense of γ-tocopherol (Farré et al., 2012).

The constitutive expression of γ-TMT (*VTE4*) alone resulted in the accumulation of 100% α- and β-tocopherol in soybean seeds (Van Eenennaam et al., 2003), but expression under the control of the seed-specific vicilin promoter resulted in a 41-fold increase in α-tocopherol levels in seeds (Lee et al., 2011). Combining *VTE3* and *VTE4* in soybean resulted in an eight-fold increase in α-tocopherol levels; α-tocopherol was the main tocochromanol vitamer in the seeds (Van Eenennaam et al., 2003).

By combining the above strategies, it was 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 ca: 42μg/g fresh weight) by the constitutive expression of Arabidopsis *HPT* and *VTE4*, also resulting in a six-fold increase in the α/γ ratio (Li et al., 2011).

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 (Kumar et al., 2005; Tsegaye et al., 2002). 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 choice 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) when using the CaMV 35S promoter (Tsegaye et al., 2002). The transgene origin may have an 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 resulted in a 1.7-fold increase in total tocochromanols (to 1159 μg/g seed

oil) compared to the 1.5-fold increase achieved using the Arabidopsis ortholog *VTE1* (1018 µg/g) (Kumar et al., 2005).

Species	Genes (origin)	Promoter	Vitamin E levels and composition in transgenic plants	Reference
Maize	*hggt* (barley)	Corn oleosin (embryo-specific)	> 344,57 µg /g DW in seeds (6-fold)	Cahoon et al., 2003
	HPPD and *VTE3* (Arabidopsis)	Corn Ubi-1	9.5 µg/g DW γ-tocopherol in seeds (3-fold)	Naqvi et al, 2011
Lettuce	*VTE4* (Arabidopsis)	CaMV 35S	Improved α/γ tocopherol ratio 0.4-544 (wild type = 0.6-1.2)	Cho et al., 2005
	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
	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	Ren et al., 2011
	VTE1 (Arabidopsis)	rbcL	22 µg/g FW total tocopherol in leaves (1.3-fold)	Yabuta et al., 2013
Mustard	*VTE4* (Arabidopsis)	CaMV 35S	609.7 µg/g total tocopherol in seeds (1.07-fold) 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)	Yusuf & Sarin, 2007

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)	Raclaru et al., 2006
	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)	Karunananda a et al., 2005
	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)	Kumar et al., 2005
	vte1 (maize)		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 tocopherols	Karunananda a et al., 2005

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	Van Eenennaam et al., 2003
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 negligible	Tavva et al., 2007
	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	Lee et al., 2011
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) Reductions in γ-tocopherol (from 96.9% to 3.9%) and δ-tocopherol (from 2.18% to 0%) Increase in β-tocopherol (from 0% to 1%)	Shintani & DellaPenna, 1998
	HPPD (Arabidopsis)	CaMV 35S	398 µg/g total tocopherol content in seeds (1.1-fold)	Tsegaye et al., 2002

		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)	Collakova & DellaPenna, 2003
	VTE2 (Arabidopsis)	Napin	926 µg/g total tocopherol in seeds (2-fold)	Savidge et al., 2002
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	Rippert et al., 2004
	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 seeds (2-fold) 50 µg/g FW γ-tocopherol in seeds (2-fold)	Falk et al., 2003

	VTE1 (Arabidopsis)	rbcL	22µg/g FW total Toc in leaves (1.6-fold)	Yabuta et al., 2013
	VTE4 (Arabidopsis)	rbcL	No significant dfferences	
	VTE1 *VTE4* (Arabidopsis)	rbcL	27 µg/g FW total Toc (2.2-fold) in leaves 15 µg/g FW α-Toc(1.2-fold) in leaves	
Rice	*HPPD* (Arabidopsis)	Corn Ubi-1	Improved α/γ tocopherol ratio 3.97 (wild type = 2.35) 6.00 µg/g DW α-tocopherol in seeds (1.4-fold)	Farré et al., 2012
Tomato	vte2 (apple)	CaMV 35S	4.5 µg/g FW α-tocopherol in fruits (1.7-fold)	Seo et al., 2011

Table 2.2: Vitamin E enhancement by genetic engineering. DW = dry weight; FW = fresh weight (Farré et al., 2012).

Our experimental strategy for the enhancement of vitamin E in maize seeds involves the simultaneous expression of *Arabidopsis thaliana PDS1*, *HPT1*, *VTE3* and *VTE4* genes using a combinatorial transformation strategy (Zhu et al, 2008). A stacking strategy was used to introgress this vitamin E mini-pathway into a second transgenic maize line expressing *Zmpsy1* and *PacrtI*. We carried out an in-depth analysis at the transcript and metabolite levels in an attempt to determine the specific impact in metabolite accumulation and interaction of vitamin E and carotenoid biosynthetic pathways.

2.3 Materials and methods

2.3.1 Plant material

A transgenic maize line was generated by combinatorial nuclear transformation and was shown to express all four transgenes required for vitamin E biosynthesis in maize, *AtPDS1, AtVTE2, AtVTE3* and *AtVTE4*. This line was crossed with the Carolight[R] transgenic line expressing *Zmpsy1* and *PacrtI* (Chapter 1) generating a hybrid line expressing all six transgenes. Endosperm, embryo and seed samples were harvested at 30DAP for each parent, and corresponding cross, frozen in liquid nitrogen, and stored at -80ºC prior to use.

2.3.2 Cloning and vector construction

Genes involved in tocopherol biosynthesis (*phytoene desaturation* mutant gene number 1, *AtPDS1*; *homogentisate phytylpernyltransferase, AtHPT* or *AtVTE2*; *2-methyl-6-phytylplastoquinol methyltransferase, AtVTE3*; and *γ-tocopherol methyltransferase, AtVTE4*) were cloned from *Arabidopsis thaliana.* On the basis of gene information (GenBank accession numbers AF000228, AY089963, AB054257, AF104220, respectively) forward and reverse primers were designed and the full length cDNA of each gene was amplified by reverse transcriptase PCR. Genes were sub-cloned into the pGEM®-T easy vector (pGEM®-T Vector Cloning Kit, Promega, Madison, Wisconsin, USA) that was later digested with *Bam*HI and *Hin*dIII and was ligated into vector pAL76 containing the maize ubiquitin-1 promoter with its first intron and the *nos* terminator.

2.3.3 Combinatorial maize transformation

Maize plants *(Zea mays* L., cv. M37W, white endosperm maize) were grown in the greenhouse and/or growth room at 28/20ºC day/night temperature with a 10h 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. M37W immature zygotic embryos (IZEs) at 10-14 days after pollination were excised aseptically and cultured on N6 medium (Ramessar et al., 2008). After 5 days, the embryos were bombarded with 10 mg of DNA coated gold particles. Target tissues were incubated on N6 medium containing high osmoticum (0.2 M

mannitol and 0.2 M sorbitol) for 3 to 4 hours prior to and 16 hours after bombardment. The gold particles were coated at a molar ratio of 3:1 relatively to the gene of interest. The following amounts of plasmid was used to coat the gold particles: 0.0094 mg of PAL76-AtPDS1 (2245 ng/μl); 0.009 mg of PAL76-AtVTE2 (2100 ng/μl); 0.0088 mg of PAL76-AtVTE3 (2145 ng/μl); 0.0089 mg of PAL76-AtVTE4 (1220 ng/μl); and 0.018 selectable marker plasmid pTRAuxbar (1668ng/μl) derived from pAHC20 which contains the *bar* gene (Christensen & Quail, 1996) for selection (Christou et al., 1991). Bombarded callus was selected on phosphinothrin-supplemented medium (3mg/l) as described previously (Ramessar et al., 2008). The maize transformation process is illustrated in **Figure 2.3**. The media composition is listed in **Table 2.3**.

Compounds	N6	N6 osmoticum	N6/PPT	MR1/PPT (Shoot development)	MR2/PPT (Root development)
N6 macronutrients	50 ml	50 ml	50 ml	---	---
N6 micronutrients	5 ml	5 ml	5 ml	---	---
N6 Fe-EDTA source	5 ml	5 ml	5 ml	5 ml	5 ml
Sucrose	20 g	20 g	20 g	30 g	30 g
Casein hydrolase	0.1 g	0.1 g	0.1 g	---	---
L-proline	2.8 g	2.8 g	2.8 g	---	---
2,4-D	1 mg	1 mg	1 mg	0.025 mg	---
D-mannitol	---	36.4 g	---	---	---
D-sorbitol	---	36.4 g	---	---	---
Gelrite	4 g	---	4g	4 g	4 g
Agarose	---	4g	---	---	---
N6 vitamins	5 ml	5 ml	5 ml	5 ml	5 ml
AgNO$_3$	10 mg	10 mg	10 mg	0.85 mg	0.85 mg
PPT	---	---	3 mg	3 mg	3 mg
MS salts	---	---	---	4.4 g	4.4 g
Benzylaminopurine (BAP)	---	---	---	10 mg	---

Table 2.3: Media composition (amounts listed for 1l; pH was adjusted to 5.8 with1M KOH)
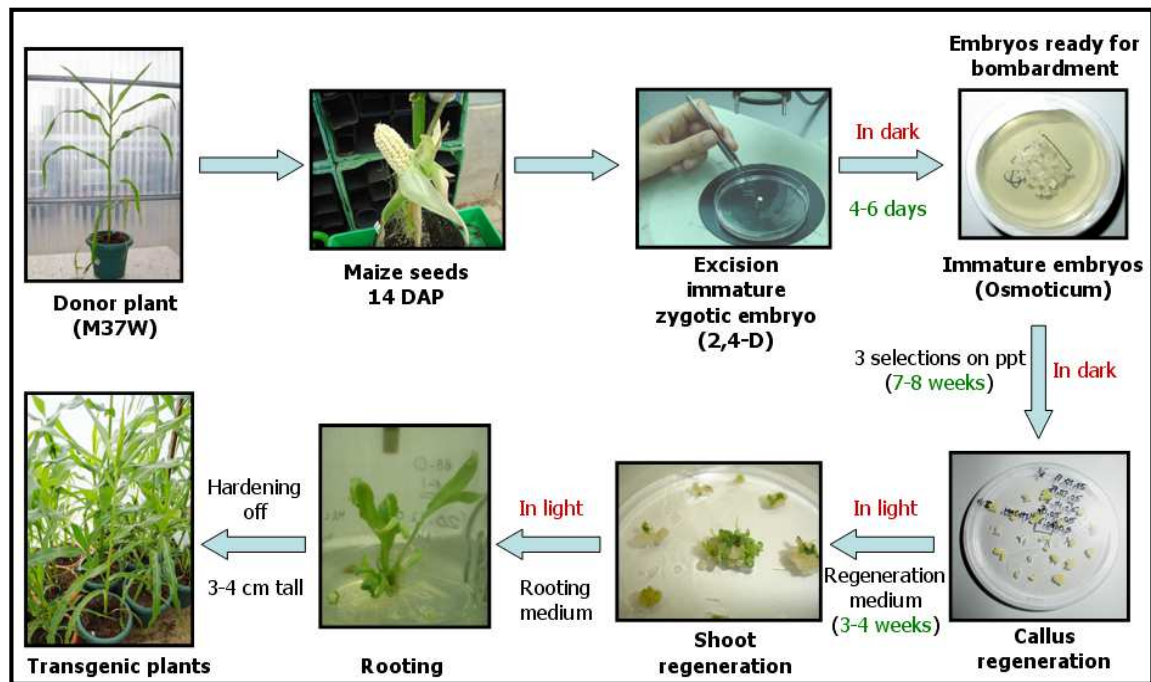
Figure 2.3: Maize transformation process.

2.3.4 Total RNA isolation and mRNA analysis

The protocol is described in detail in Chapter 1, section 1.3.2. Primer sequences used for designing probes are shown in Table 2.4.

Transgene	Primer set
AtPDS1	Forward: 5'-AGGATCCTCAATGGGCCACCAAAACGCCGCCG-3'
	Reverse: 5'-AAGCTTCATCCCACTAACTGTTTGGCTTC-3'
AtHPT1	Forward: 5'-AGGATCCATGGAGTCTCTGCTCTCTAGTTCTT-3'
	Reverse: 5'-GAAGCTTCACTTCAAAAAAGGTAACAGCAAG-3'
AtVTE3	Forward: 5'-AGGATCCATGGCCTCTTTGATGCTCAACGG-3'
	Reverse: 5'-CAAGCTTCAGATGGGTTGGTCTTTGGGAAC-3'
AtVTE4	Forward: 5'-AGGATCCTAAATGAAAGCAACTCTAGCAGCA-3'
	Reverse: 5'-GAAGCTTAGAGTGGCTTCTGGCAAGTGATG-3'

Table 2.4: Primer sequences used for probe design.

2.3.5 Quantitative real time PCR

The protocol and primer design for endogenous carotenogenic genes are described in detail in Chapter 1, section 1.3.3. Primer sequences used for designing forward and reverse primers for vitamin E endogenous genes are shown in **Table 2.5**.

2.3.6 Carotenoid and vitamin E analysis through HPLC

The protocol and primer design for endogenous carotenogenic genes are described in detail in Chapter 1, section 1.3.4. Total tocopherols and tocotrienols were extracted in 20 ml 50/50 (v/v) tetrahydrofuran (THF) and methanol at 60ºC for 15-20 min. Extracts were filtered into a funnel and the solid residues were re-extracted in acetone to ensure complete tocopherol extraction. The combined extracts were partitioned in petroleum ether 90/10 (v/v). THF and methanol residues were removed from the upper phase by washing twice with distilled water. For HPLC separation, the solvent was evaporated under a stream of N_2 at 37ºC and the residue was re-dissolved in 20 µl acetone and injected immediately. The samples were separated on a Nucleosil C18 3µ column (Macherey and Nagel, Dueren, Germany) with a mobile phase of 88% ethanol: 10% ethyl acetate: 1% triethylamine: 1% water, at a column temperature of 25°C. Samples were monitored with a Kontron DAD 440 photodiode array detector with on-line registration of the spectra. α-Tocopherol was identified by co-chromatography with an authentic reference compound and comparison of their spectra. This standard was also used for quantification in combination with the extinction coefficient (Davies 1976). These analyses were performed in Dr P. Fraser's laboratory at The Royal Holloway University of London, UK.

2.4 Results

2.4.1 Combinatorial transformation of vitamin E genes into maize endosperm and mRNA analysis of introduced transgenes

I transformed four-day-old immature zygotic embryos of South African elite white maize inbred M37W by bombarding them with gold particles coated with five constructs: four transgenes involved in vitamin E biosynthesis: *Arabidopsis thaliana PDS1*(encoding *ρ-hydroxyphenylpyruvate dioxygenase* or *HPPD*), *HPT1*(encoding *homogentisate phytyltransferase*), *VTE3* (encoding *2-methyl-6-phytylbenzoquinol methyltransferase* or *MPBQ-MT),* and *VTE4* (encoding *γ-tocopherol methyltransferase* or *γ-TMT*) and the selectable marker *bar*. The four genes (*ATPDS1*, *ATHPT1*, *ATVTE3* and *ATVTE4*) were individually ligated into separate pAL76 vectors containing the maize ubiquitin-1 promoter and its first intron. I recovered one transgenic line VE, which co-expressed all four input transgenes. This line was crossed with the high carotenoid content transgenic line (HC) described in Chapter 1 expressing *Zmpsy1* (encoding phytoene synthase 1) and *PacrtI* (encoding phytoene desaturase) also in the M37W genetic background. Transgene expression was confirmed by mRNA blot analysis (**Figure 2.4**).

ATPDS1, *ATHPT1*, *ATVTE3* and *ATVTE4* expression at the mRNA level were measured in VE, HC and in the corresponding cross VExHC. *Zmpsy1* and *PacrtI* expression was also confirmed in HC and in the corresponding cross VExHC (**Figure 2.4**).
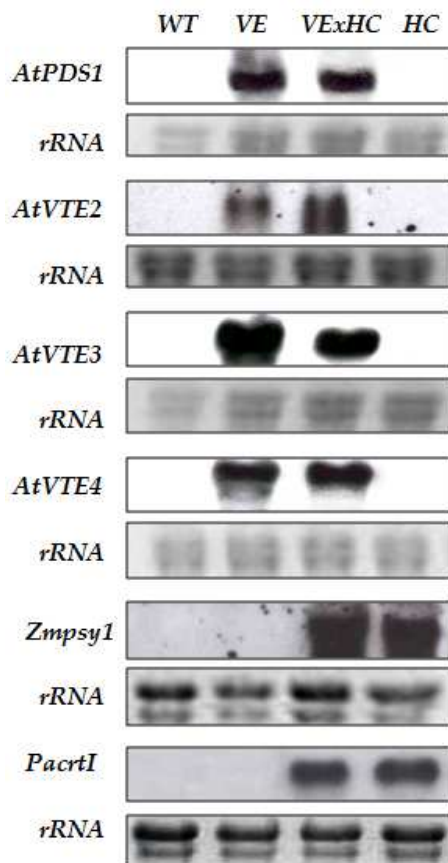
Figure 2.4: Transgene expression analyses in maize endosperm. mRNA blot analysis (25 μg of total RNA per line) was used to monitor transgene expression in the endosperm (30 DAP) of wild type M37W, and transgenic lines VE, HC and the corresponding cross VExHC.

3.4.2 UHPLC analysis of α-tocopherol and carotenoid accumulation in maize seeds

In order to elucidate the interaction of the tocopherol and the carotenoid biosynthetic pathways, VE and HC lines were crossed to generate VExHC. α-Tocopherol content of VE, HC, VExHC and M37W were analyzed by UHPLC separately in the embryo, endosperm and in the whole seed (**Table 2.5**). α-Tocopherol accumulation was in general higher in the embryo in all lines. VExHC, co-expressing all six transgenes (*ATPDS1*, *ATHPT1*, *ATVTE3*, *ATVTE4, Zmpsy1* and *PacrtI*), exhibited an increase of ca: 8-fold of α-tocopherol in the embryo compared to wild type, VE, and HC. α-Tocopherol also accumulated in whole seeds in VE and VExHC up to 0.02 and 0.05 μg/g DW, while only traces were detected in M37W and HC in the whole seed. Only traces of α-tocopherol were detected in the endosperm of all the lines.

PL	Tissue	α-tocopherol
HC	Embryo	0.09±0.01
	Endosperm	traces
	Seed	traces
VE	Embryo	0.06±0.01
	Endosperm	traces
	Seed	0.02±0.00
VExHC	Embryo	0.49±0.16
	Endosperm	traces
	Seed	0.05±0.03
WT	Embryo	0.06±0.00
	Endosperm	traces
	Seed	traces

Table 2.5: α-Tocopherol content of HC, VE, VExHC and WT (M37W), in the embryo, endosperm and whole seeds. Results are presented in μg/g DW± SD (n = 3–5 embryos, endosperm or seeds). Abbreviations: PL, plant lines.

Carotenoid content of VE, HC, VExHC and wild type M37W were analyzed by UHPLC in the endosperm and whole seeds (**Table 2.6**). VE did not show significant differences in carotenoid composition compared to wild type, which accumulate up to ca: 5μg/g DW total carotenoids in whole seeds and up to ca: 4 μg/g DW in the endosperm. HC had higher carotenoid content than wild type and VE in endosperm and seeds, accumulating ca: 79 μg/g DW total carotenoids in the seeds and ca: 86 μg/g DW in the endosperm. VExHC accumulated ca: 7% less total carotenoids in whole seeds and ca: 28% less in the endosperm, than HC.

Carotenoid accumulation in maize embryo varied between lines. VE and wild type accumulated zeaxanthin as the only carotenoid, in similar amounts (ca: 8 μg/g DW). However, HC and VExHC accumulated additional carotenoids, including β-carotene, antheraxanthin, and lutein, in addition to zeaxanthin. The most prevalent carotenoid in HC

and VExHC was zeaxanthin. Zeaxanthin accumulation in VExHC was 3-fold higher than in HC and 2-fold higher than in VE and wild type.

PL		Phyto	β-caro	β-cryp	Anthera	Zea	Lut	TC
WT	Seed	0.3±0.0	0.0±0.0	0.0±0.0	0.4±0.0	2.4±0.1	1.2±0.0	4.3
	Endos	0.3±0.0	0.0±0.0	0.0±0.0	0.3±0.0	2.0±0.1	1.1±0.0	3.7
	Embryo	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	7.8±0.5	0.0±0.0	7.8
VE	Seed	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.2	2.9±0.3	1.2±0.1	4.7
	Endos	0.3±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.1±0.0	1.1±0.0	3.6
	Embryo	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	7.5±0.3	0.0±0.0	7.5
VEx HC	Seed	14.2±0.1	12.4±1.0	6.2±0.4	5.2±0.9	25.5±2.0	9.9±0.5	73.4
	Endos	17.0±0.2	11.3±0.04	6.2±0.8	1.7±0.1	15.9±2.5	9.6±0.2	61.7
	Embryo	0.0±0.0	1.7±0.1	0.0±0.0	0.4±0.0	14.2±1.3	0.9±0.1	17.2
HC	Seed	19.7±0.9	14.5±0.8	6.7±0.5	2.3±0.4	25.4±1.0	9.9±0.4	78.6
	Endos	25.6±0.6	15.0±3.4	7.7±2.0	2.1±1.2	26.5±6.6	8.8±1.9	85.8
	Embryo	0.0±0.0	0.88±0.0	0.0±0.0	0.5±0.0	4.2±0.1	0.4±0.0	5.9

Table 2.6: Carotenoid content and composition in HC, VE, VExHC and WT (M37W) in the endosperm, embryo and whole seeds at 30 DAP. Results are presented in µg/g DW± SD (n = 3–5 endosperm and seeds). Abbreviations: Phyto, phytoene; β-cryp, β-cryptoxanthin; β-caro, β-carotene; Lut, lutein; Zea, zeaxanthin; Anthe, antheraxanthin; TC, total carotenoids; Endos, endosperm; PL, plant lines.

2.5 Discussion

Carotenoids and tocopherols are two important groups of antioxidants that are relatively abundant in maize (Egesel et al., 2003; Weber, 1987). Carotenoids and tocopherols typically found in maize include, β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin (Egesel et al., 2003; Weber, 1987) and α-, δ-, and γ -tocopherols (Weber, 1987), respectively. Higher levels of tocopherols are found in the embryo, while carotenoids are more commonly associated with the kernel endosperm (Weber, 1987). Several strategies have been developed to increase carotenoid levels in plants including maize (see Chapter 1). Different approaches have also been used to modulate α-tocopherol composition in plants.

Four transgenes (*AtPDS1*, *AtVTE2*, *AtVTE3* and *AtVTE4*) encoding rate limiting enzymes in the vitamin E pathway were co-expressed constitutively in maize in order to direct the pathway to the α-branch to relieve the γ-TMT limiting step and accumulate α-tocopherol in seeds. This vitamin E mini-pathway was introgressed into a High Carotenoid (HC) maize line co-expressing *Zmpsy1* and *PacrtI* in the endosperm (described in chapter 1) in order to generate maize accumulating α-tocopherol and also high amounts of carotenoids simultaneously in the same line, VExHC.

2.5.1 Introgression of the vitamin E biosynthetic pathway into HC resulted in a significant increase in α-tocopherol levels and a moderate decrease in carotenoid accumulation in maize seeds of resulting hybrids.

The enzymes that catalyze the later steps of the tocopherol pathway, MPBQ methyltrasferase (MBPQ MT), tocopherol cylcase (TC) and γ-TMT, are important in determining the composition of tocopherols in plants (Farré et al., 2012). The expression of *AtVTE3* (*MBPQ MT*) alone and also together with *AtVTE4* (*γ-TMT*) in soybean under the control of a seed specific promoter resulted in a significant increase in α- and γ-tocopherols in seeds, with a simultaneous decrease in the levels of the δ- and β- isomers, indicating a shift in the pathway from the δ- to the γ- branch (Van Eenennaam et al., 2003). On the basis of these results, the authors suggested that expression of *AtVTE3* under the control of a seed-specific promoter might be useful in reducing δ-tocopherol levels in favour of γ-tocopherol (Van Eenennaam et al., 2003).

Introduction of *AtVTE4* into transgenic soybean plants already expressing *AtVTE3*, resulted in dramatic increases in the levels of α-tocopherol, with simultaneous decreases in γ-tocopherol levels, demonstrating the conversion of γ- to α-tocopherols as a result of *AtVTE4* (*γ-TMT*) overexpression (Van Eenennaam et al., 2003). In earlier studies, M37W maize seeds co-expressing constitutively *AtPDS1* and *AtVT3* had increased amounts of γ-tocopherol, up to ca: 3-fold compared to wild type. However, in the case of maize co-expressing *AtPDS1* and *AtVTE3,* no α-tocopherol could be detected, as there was no γ-TMT activity in the maize seed (Naqvi et al., 2011). These results were in agreement with previous studies where γ-TMT was shown to be limiting in seeds of cereals such as maize (Shintani & DellaPenna, 1998).

In my experiments, VE maize line seeds overexpressing a vitamin E mini-pathway comprising (*AtPDS1*, *AtVTE2*, *AtVTE3* and *AtVTE4*), exhibited a redirection of the pathway towards the α-branch and also accumulated up to 0.02 µg/g DW of α-tocopherol in VE, and 0.05 µg/g DW in VExHC (overexpressing *AtPDS1*, *AtVTE2*, *AtVTE3*, *AtVTE4*, *Zmpsy1* and *Pacrtl*) (**Table 2.5**, Results section). This suggests that expression of *AtVTE4* facilitated conversion of γ- to α-tocopherol. γ-Tocopherol accumulation measurements are ongoing. In earlier studies in maize seeds co-expressing *AtPDS1* and *AtVTE3* in the same genetic background M37W, γ-tocopherol accumulated up to ca: 10 µg/g DW. In relation to carotenoid accumulation, VE and wild type differed in β-carotene accumulation (**Table 2.6**, Results section). β-Carotene, was not detected in M37W, whereas it accumulated in VE at ca: 1 µg/g DW. This suggested that the tocopherol mini-pathway influenced the carotenoid pathway resulting in β-carotene accumulation. In contrast, VExHC in which both pathways operate simultaneously exhibited a decrease in total endosperm carotenoids up to ca: 5% compared to HC. Phytoene and β-carotene accumulation decrease ca: 27 and 15%, respectively in VExHC. These results suggest that biosynthesis of carotenoid decreases in order to compensate the activation of the tocopherol biosynthetic pathway towards α-tocopherol.

2.5.2 Simultaneous modulation of carotenoid and tocopherol biosynthesis influences core metabolic processes in maize seeds

Tocopherol and carotenoid biosynthesis differ in maize embryo and endosperm. Most of the seed carotenoids accumulate in the endosperm (ca: 90%) with only a small amount (ca:

10%) in the embryo; the reverse is true for tocopherols which predominate in the embryo (Weber, 1987). In my experiments α-tocopherol was found exclusively in the embryo. This demonstrates a separation of α-tocopherol biosynthesis in endosperm and embryo. The embryo was the major site of carotenoid biosynthesis and accumulation compared to the endosperm (in M37W wild type and VE) (**Table 2.6**, Results section). In wild type and VE, carotenoid concentration was up to 2-fold higher in the embryo relatively to the endosperm. This corresponds to the accumulation of endogenous *Psy* transcripts in maize embryo (Singh et al., 2003). In contrast, in HC, carotenoid biosynthesis was engineered specifically in the endosperm. This not only enhanced carotenoid accumulation in the endosperm, making this tissue the major carotenogenic site in the seed but, in addition, it decreased carotenoid accumulation in the embryo (**Table 2.6**, Results section). These results corroborate an earlier in depth study in which metabolomic analysis in HC and wild type also confirmed a ca: 2-fold decrease in the embryos of HC compared with M37W (Decourcelle et al., 2015). In the same study sterols and γ-tocopherol were analysed, indicating a significant increase in sterols in HC endosperm and embryo, compared to wild type, but a decrease in γ-tocopherol accumulation, demonstrating that HC needs a higher flux through and out of the glycolytic pathway for the synthesis of carotenoids, sterols and fatty acids (Decourcelle et al., 2015).

The maximum accumulation of total carotenoids was in the embryo of VExHC, ca: 2-fold higher than in VE and wild type embryos, and ca: 3-fold higher than the embryo in HC. Carotenoid accumulation in the endosperm of VExHC decreased, ca: 26%, compared to HC. It thus appears that our results established the existence of a competition between the carotenoid pathways in the endosperm and the embryo. The prevalent carotenoid was zeaxanthin in both embryo and endosperm, whereas α-carotene, β-carotene and their hydroxyl-derivatives were below the limit of detection (in VE and wild type) or accumulated at very low levels (in HC and VExHC) (**Table 2.6**, Results section).

α-Tocopherol accumulation in the embryo of VExHC was ca: 8-fold higher than in the wild type. VE co-expressing all four transgenes (*AtPDS1*, *AtVTE2*, *AtVTE3* and *AtVTE4*) did not exhibit differences in α-tocopherol accumulation compared to wild type. The heterozygosity of VE could have diluted α-tocopherol accumulation as measured by UHPLC analysis. I have shown that modulation of carotenogenesis has collateral effects on other terpenoid or terpenoid-related pathways, particularly in non-target organs such as the embryo. In earlier studies, in which combined transcript, proteome and metabolite analysis through

and integrative model using the HC line, indicated that overexpression of a carotenogenic pathway resulted in pleotropic effects in core metabolism (Decourcelle et al., 2015). The model was supported by higher activities of fructokinase, glucose 6-phosphate isomerase, and fructose 1,6-*bis*phosphate aldolase indicating a higher flux through the glycolytic pathway for the synthesis of carotenoids, sterols and fatty acids. Although pyruvate and acetyl-CoA utilization was higher in the engineered line (HC), pyruvate kinase activity was lower indicating a down regulation of the citrate cycle (Decourcelle et al., 2015).

Further studies focusing on the modulation of carbohydrate metabolism in VExHC seeds will allow a deeper understanding of upstream utilization of precursors towards tocopherol and carotenoid accumulation and how the corresponding precursors are distributed in the endosperm and the embryo. Analysis of the remaining vitamin E forms, such as γ-tocopherol, will elucidate how *AtPDS1*, *AtVTE2*, *AtVTE3* and *AtVTE4* modulate the pathway in maize seeds.

2.6 Conclusions

I reconstructed the carotenoid and vitamin E biosynthetic pathways (*Zmpsy1* and *PacrtI*, and *AtPDS1*; *AtVTE2*, *AtVTE3* and *AtVTE4*) in a novel maize hybrid. I measured a significant increase in α-tocopherol accumulation and a moderate decrease in carotenoid accumulation in the seeds of the hybrid, demonstrating an interaction between these two pathways. I also demonstrated that endosperm-specific carotenoid biosynthesis and constitutive tocopherol biosynthesis influenced core metabolic processes in maize embryo and endosperm. This in turn resulted in the accumulation of zeaxanthin and α-tocopherol, respectively as the predominant metabolites in the two pathways, in the maize embryo. On the other hand, α-tocopherol was not detected in the endosperm, but total carotenoid decreased up to a ca: 28%, mostly because in the reduction in the levels of zeaxanthin and phytoene. Further studies focusing on the effects of the carotenoid and vitamin E pathways on core metabolites, such as sugars which are precursors for both pathways, and also other vitamin E forms, such as γ-tocopherol, will allow a deeper understanding of the interaction of these two pathways and their impact on core metabolism in maize seeds.

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CHAPTER 3

Underlying mechanisms for the conversion of carotenoids to astaxanthin in maize endosperm by expressing different combinations of heterologous β-carotene ketolase and hydroxylase genes in diverse genetic backgrounds

3.1 Abstract

Astaxanthin is a high-value ketocarotenoid rarely found in plants. It is derived from β-carotene by the 3-hydroxylation and 4-ketolation of both ionone end groups, in reactions catalyzed by β-carotene hydroxylase and β-carotene ketolase, respectively. We investigated the impact of introducing an extended carotenoid biosynthetic pathway into different maize endosperm backgrounds in order to optimize the production of astaxanthin. This allowed us to identify and resolve potential bottlenecks in the different genotypes that might facilitated the accumulation of this valuable compound in the endosperm. White maize endosperm does not accumulate high amounts of carotenoids because phytoene synthase, the enzyme responsible for the first committed step in the pathway, is not present in this tissuet. We therefore expressed a combination of ketocarotenogenic transgenes in maize endosperm, generating a maize line expressing two synthetic β-carotene ketolases and a synthetic β-carotene hydroxylase. This mini-pathway was introgressed into different maize backgrounds with different endosperm carotenoid profiles. The resulting lines predominantly accumulated astaxanthin at different levels, from ca: 9 μg/g up to 18 μg/g DW. We demonstrate that selection of an appropriate genetic background has significant effects on carotenoid conversion and astaxanthin accumulation.

3.2 Introduction

Ketocarotenoids are carotenoids that contain at least one keto group on the β-ionone ring(s). Many ketocarotenoids are synthetize by algae, fungi and bacteria and impart an attractive red color to the feathers and skin of many birds such as flamingo, scarlet ibis or roseate spoonbill (Hudon & Brush 1992). Astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione) is a commercially valuable ketocarotenoid (Margalith, 1999) because it is widely used in the aquaculture industry to produce pink flesh in fish and shellfish, such as salmon and lobster, respectively (Guerin et al., 2003). Demand for astaxanthin is increasing also because of applications in human dietary supplements and cosmetics (Berman et al., 2014. Partial chemical synthesis is used to manufacture astaxanthin (Nguyen, 2013; Hirschberg & Lotan, 2001). Although chemical synthesis produces a mixture of stereoisomers with limited applications, most of the commercial astaxanthin is produced in that way because it is more

economical than extraction or fermentation from natural sources, such as *Phaffia rhodozyma* or *Haematococcus pluvialis* (reviewed in Berman et al., 2014).

Astaxanthin is a strong antioxidant (Miki, 1991) protecting against a wide range of diseases (Guerin et al., 2003). It has anti-inflammatory properties and inhibits the oxidation of low-density lipoproteins in humans (Kishimoto et al., 2010). It also helps to prevent diabetic nephropathy in diabetic db/db mice (Naito et al., 2006), protects against cancer (Saino et al., 2000; Tanaka et al., 2012) and boosts the immune system (Chew & Park, 2004; Jyonouchi et al., 1991). The various functions of astaxanthin make it an attractive target for genetic engineering in plants where the objective is to increase its content (Giuliano et al., 2006; Naqvi et al., 2009; Sandmann et al., 2006; Zhu et al., 2008; Zhu et al., 2007a).

3.2.1 Astaxanthin biosynthetic pathway

Astaxanthin is synthesized from β-carotene by the introduction of keto and hydroxyl moieties at the 4,4′ and 3,3′ positions of the β-ionone rings (**Figure 3.1**). These reactions are catalyzed by a β-carotene ketolase and a β-carotene hydroxylase (Misawa et al. 1995; Zhu et al., 2009). Whereas the hydroxylation reaction is widespread in higher plants, ketolation is mostly restricted to bacteria, fungi, and some unicellular green algae (Misawa et al. 1995; Zhu et al., 2009). Therefore, whereas many higher plants can synthesize different hydroxylated carotenoids such as zeaxanthin, only a few species in the genus *Adonis* can synthesize ketocarotenoids, e.g. *Adonis aestivalis*, which accumulates ketocarotenoids in its flowers (Cunningham & Gantt, 2011). This is because β-carotene ketolase, which is responsible for the conversion of β-carotene to canthaxanthin and zeaxanthin to astaxanthin, is not usually expressed in plants.
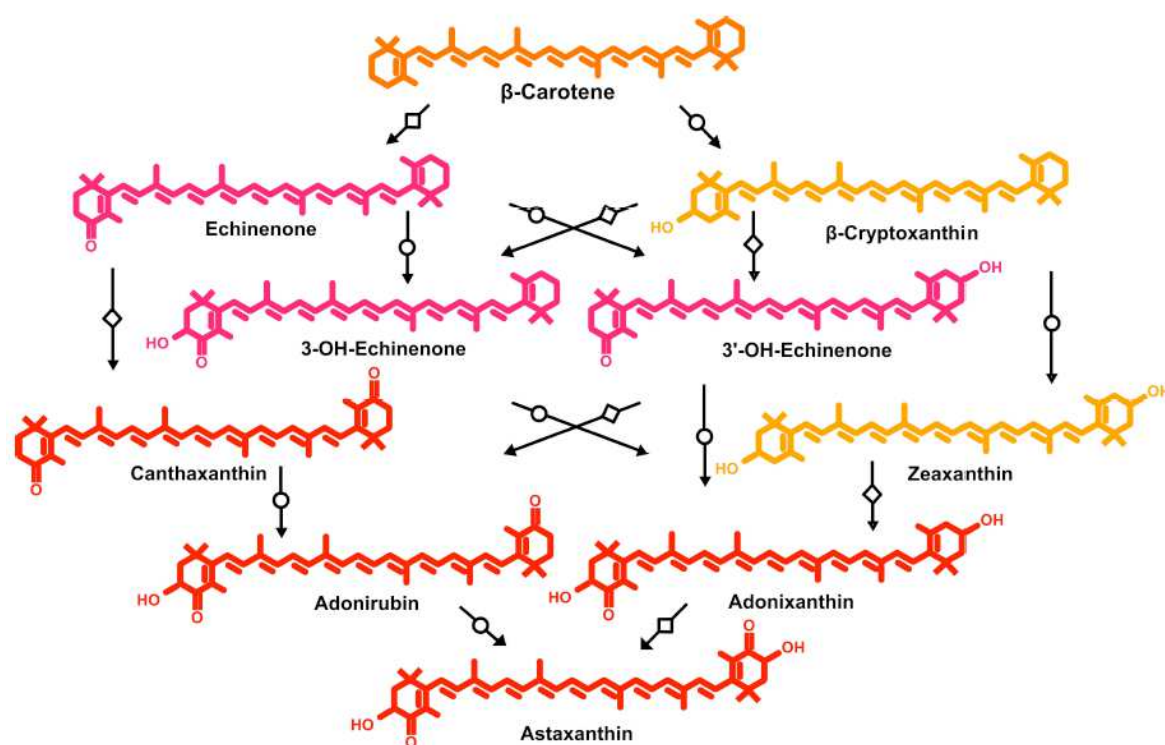
Figure 3.1: Astaxanthin biosynthesis pathway in bacteria and transgenic plants.

Catalytic function of β-carotene hydroxylase (arrow with circle) and β-carotene ketolase (arrow with square) (Zhu et al., 2009; Zhu et al., 2013).

3.2.2 Genetic engineering strategies for enhancing astaxanthin content in plants

Competition between β-carotene hydroxylase and β-carotene ketolase for substrates in the extended ketocarotenoid pathway provides a novel way for astaxanthin formation (**Figure 3.1**). A significant bottleneck limiting accumulation of high levels of astaxanthin in transgenic plants is the inefficient conversion of zeaxanthin to astaxanthin via adonixanthin, a reaction catalyzed by β-carotene ketolases (Zhu et al., 2009). There are three major classes of β-carotene ketolases, BKT, CRTO and CRTW (Mann et al., 2000; Ralley et al., 2004; Suzuki et al., 2007; Zhu et al, 2008). Previous literature reported that plants overexpressing the marine or bacterial CRTW can synthesize astaxanthin with lower efficiency (Mann et al., 2000; Morris et al., 2006; Ralley et al., 2004; Suzuki et al., 2007) (**Table 3.1**). Transgenic tobacco expressing *Brevundimonas* CRTW and CRTZ (β-carotene hydroxylase) accumulated more than 0.5% (dry weight) astaxanthin (ca: 70% of total carotenoids) in leaves, which developed a reddish brown color (Hasunuma et al., 2008). Transgenic *Arabidopsis thaliana* expressing

BKT from *Chlamydomonas reinhardtii* (*CrBKT*) exhibited orange pigmentation in leaves due to the accumulation of substantial amounts of astaxanthin (up to 2 mg/g dry weight with a 1.8-fold increase in total carotenoids) (Zhong et al., 2011). The efficiency of *CrBKT* for the accumulation of astaxanthin was therefore demonstrated to be much higher than other *BKT* genes, including *CzBKT* (*Chlorella zofingiensis BKT)* and *HpBKT3* (*Haematococcus pluvialis BKT3*) (Zhong et al., 2011). Expression of the two linked gene cassettes *Crbkt* and *Hpbch* in tomato resulted in the up-regulation of most intrinsic carotenogenic genes leading to massive accumulations of mostly free astaxanthin in leaves (ca: 3 mg/g) and esterified astaxanthin in fruits (ca: 16 mg/g). A 16-fold increase in total carotenoid accumulation was reported in these plants without any detrimental effects on plant growth and development (Huang et al., 2013) (**Table 3.1**).

Genes origin	Promoter	Plant species	Major ketocarotenoids (µg/g) / tissue/organ of accumulation	Reference
BKT (*H. pluvialis*)	Tomato PDS gene	Tobacco	Astaxanthin (23.5), adonirubin (17.1), adonixanthin (12), canthaxanthin (10.2), 3-OH echinenone (8.5), 3'-hydroxyechinenone (8.1)/nectaries FW	Mann et al., 2000
BKT (*H. pluvialis*)	Seed storage protein napA (oilseed rape)	Arabidopsis thaliana	4-keto-lutein, canthaxanthin and adonirubin/seeds	(Stålberg et al., 2003)
CRTW and CRTZ (*Paracoccus*	CaMV	Tobacco	Ketocarotenoids/leaves (800) DW and nectaries	Ralley et

spN81106)	35s		(64) DW	al., 2004
CRTO (*Synechocystis* spPCC6803)	CaMV 35s	Potato	Echinenone (7600), 3'-hydroxyechinenone (1500) and 4-ketozeaxanthin (2700)/ leaves DW; astaxanthin (1.8), 3'-hydroxyechinenone (0,4) and 4-ketozeaxanthin (8.5)/ tubers DW	Gerjets & Sandmann, 2006
BKT (*H. pluvialis*)	Patatin (potato)	Potato cv. Desiree	4-ketolutein (0.5) and astaxanthin (0.6)/desiree tubers(DW)	Morris et al., 2006
		Potato cv. Mayan Gold	4-Ketolutein(9.8) and astaxanthin(9.5)/ Mayan Gold tubers(DW)	
CRTW (*Paracoccus* spN81106)	CaMV 35S	*Lotus japonicus*	ketocarotenoids (89.8)/ flower petals(DW)	Suzuki et al., 2007
CRTO (*Synechocystis* spPCC6803), *CRTZ* (*Pananatis*)	CaMV 35S	Tobacco	Echinenone(8), 3´-Hydroxyechinenone(5.2), 4-keto-lutein(8.8)/leaves(DW); echinenone(11.6),	Gerjets et al., 2007

			4-keto-lutein(8.5) and 4-keto-zeaxanthin(12.9)/ nectaries(DW)	
CRTO (*Synechocystis spPCC6803)*	CaMV 35S	Tobacco	echinenone (2.3) and 4-keto-lutein (2.6)/ leaves, echinenone (83.), 4-keto-lutein (6.7) and 4-keto-zeaxanthin (8.3)/nectaries(DW)	Gerjets et al., 2007
CRTO (*Synechocystis* spPCC6803)	CaMV 35S	*Nicotiana glauca*	echinenone (3.8), 4-keto-zeaxanthin (7.8), ketolutein (9.9) and 3´-hydroxyechinenone (7.0)/petals(DW); echinenone (1.8), ketolutein (3.5) and 3´-hydroxyechinenone (4.4) /nectary(DW); echinenone (7.6), 4-keto-zeaxanthin (2.2), ketolutein (3.7) and 3´-hydroxyechinenone	Zhu et al., 2007b

			(11.4)/pistils(DW); echinenone (11.0) and 3´-hydroxyechinenone (14.5) /sepal(DW); echinenone (6.7) and 3´-hydroxyechinenone (12.9) /ovary(DW)	
CRTW and *CRTZ* (*Brevundimonas* spSD212)	Tobacco rrn	Tobacco	astaxanthin (5440), 4-ketoantherxanthin (370), adonirubin (140), adonixanthin (110), canthaxanthin (80) and 3´-hydroxyechinenone (30)/leaves(DW)	Hasunuma et al., 2008
BKT (*H. pluvialis*)	Double CaMV 35S	Carrot	astaxanthin (91.6), adonixanthin (15.9), adonirubin (57), canthaxanthin (50.1) and echinenone (21.4)/roots(DW); astaxanthin (34.7),	Jayaraj et al., 2007

			adonixanthin (5.0),	
			adonirubin (5.9),	
			canthaxanthin (4.0) and	
			echinenone	
			(2.7)/leaves(DW);	
			astaxanthin (12.4),	
			adonixanthin (3.2),	
			adonirubin (3.1),	
			canthaxanthin (5.8) and	
			echinenone	
			(5.6)/callus(DW)	
CRTW (*Paracoccus*spN81106)	λ-zein (maize)	Maize	astaxanthin (4.5), adonixanthin (22.4), 3'-hydroxyechinenone(3.8) and echinenone (5.1)/Maize endosperm(DW)	Zhu et al., 2008
Idi (*Paracoccus* spstrain N81106),	Pnos, Pnapin, from Bnapus,P	*Brassica napus*	echinenone (109.8), 3'-hydroxyechinenone (3.9), 3-hydroxyechinenone	Fujisawa et al., 2009

CRTW (*Brevundimonas* Spstrain SD212), *CRTZ*, *CRTE* (*Pantoea ananatis*, ATCC19321), *CRTB*, *CRTY*, *CRTI*	3 5S, CaMV 35S, PFAE, FAE1 seed-speci fic promoter from A. thaliana		(10.6), astaxanthin (0.6), adonixanthin (1.5), adonirubin (7.1) and canthaxanthin (51.1)/Bnapus seeds(DW)	
CrBKT (*Chlamydomonas reinhardtii*)	CaMV 35S	A. thaliana	echinenone (80), 3'-hydroxyechinenone (100), astaxanthin (1370), adonixanthin (390), adonirubin (370), canthaxanthin (240), and 4-ketoantheraxanthin (190)/*Arabidopsis* leaves(DW); echinenone (2.27), 3-hydroxyechinenone	Zhong et al., 2011

			(0.51), Astaxanthin (17.23), adonixanthin (16.64) and 4-Ketoantheraxanthin (0.1 4)/*Arabidopsis* seed(DW)	
HpBKT (*H. pluvialis*)	ibAGP1 promoter	Carrot	Astaxanthin (17.2), Adonirubin (5.6)/carrot root(DW)	Ahn et al., 2011
CrBKT (*Chlamydomonas reinhardtii*)	CaMV 35S	Tomato	Echinenone (60), 3'-hydroxyechinenone (190), 4-Ketoantheraxanthin (10), Astaxanthin (350), Adonixanthin (320), adonirubin (530) and canthaxanthin (1690)/tom ato leaves (DW), Echinenone (200), Astaxanthin (926), Adonirubin (828), canthaxanthin	Huang et al., 2013

			(2249)/tom ato fruits(DW)	
BKT (*Chlamydomonas reinhardtii*) and *HpBC* H (*Haematococcus pluvialis*)	CaMV 35S	Tomato	Echinenone (80), Astaxanthin (3120), Adonixanthin (910), adonirubin (230) and 4-Ketoantheraxanthin (250), Canthaxanthin (338)/toma to leaves(DW), Echinenone (562.7), Astaxanthin (16104.1), Adonixanthin (393), Adonirubin (197) and 4-Ketoantheraxanthin (117), Canthaxanthin (338)/tomato fruits (DW)	Huang et al., 2013
CRTZ and CRTW Brevundimonas sp. SD212,	CaMV 35s/ GBSS	Potato cv. Gold Mayan	Keto ester (16.3), Ketolutein (9.2), astaxanthin (7.1), other keto (2.4) tuber (DW)	Campbell et al., 2015

chemically synthesised/ OR (cauliflower)				
CRTZ and CRTW Brevundimonas sp. SD212, chemically synthesised	CaMV 35s	Potato cv. Gold Mayan	Keto ester (8.4), ketolutein (5.5), astaxanthin (4), other keto (1.5) tuber (DW)	Campbell et al., 2015
CRTZ and CRTW Brevundimonas sp. SD212, chemically synthesised/ OR (cauliflower)	CaMV 35s/ GBSS	Potato cv. 01H15	astaxanthin (53.2), keto ester (22), other keto (5.6) tuber (DW)	Campbell et al., 2015
CRTZ and CRTW Brevundimonas sp. SD212,	CaMV 35s	Potato cv. 01H15	astaxanthin (28), keto ester (18.8), other keto (2)/ tuber DW	Campbell et al., 2015

chemically synthesised				
CRTZ and CRTW Brevundimonas sp. SD212	rrn	Lettuce cv. Berkeley	Canthaxanthin (12.2), adonirubin (2.5), astaxanthin diester (113), astaxanthin monoester (41.8), astaxanthin (23), 4-ketoantheraxanthin (8)	Harada et al., 2014

Table 3.1: Ketocarotenoid levels in diverse transgenic plants. **(**DW = dry weight, FW = fresh weight) (Bai et al, 2011)

In order to increase astaxanthin accumulation in maize endosperm and get a better understanding of the roles of hydroxylase and ketolase enzymes in astaxanthin biosynthesis in maize endosperm, we expressed synthetic *Chlamydomonas reinhardtii BKT* and *Brevundimonas CRTW* (β-carotene ketolases) together with a synthetic *CRTZ* (β-carotene hydroxylase) in the white-endosperm elite South African maize inbred M37W which is deficient in carotenoid accumulation because of the absence of the enzyme phytoene synthase (PSY1). This line was crossed with four different transgenic maize lines which had been engineered previously with different carotenogenic genes (M37W genetic background) and a wild type yellow-endosperm maize line, accumulating higher levels of β-carotene. Targeted metabolomic analysis of each resulting hybrid allowed us to draw conclusions on the relative ability of ketolase and hydroxylase enzymes to generate diverse metabolite profiles. The mechanistic basis of the near quantitative conversion of carotenoids to keto-derivatives was elucidated, and this provides a framework for further more targeted interventions to create plants with specific ketocarotenoid profiles suited to particular commercial applications.

3.3. Materials and methods

3.3.1 Cloning and vector construction

Zea mays psy1 cDNA was cloned from maize inbred line B73 by RT-PCR based on the *Zmpsy1* sequence (GenBank: AY324431) and incorporating appropriate restriction sites

for vector construction. The product was inserted into pGEM®-T (Promega, Madison, WI, USA) for sequencing and then transferred to p326 containing the LMW glutenin promoter (Colot et al., 1987; Stoger et al., 1999) and nopaline synthase terminator.

The *Pantoea ananatis* (formerly known as *Erwinia uredovora*) *CrtI* gene fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (Schreier et al., 1985) in plasmid pYPIET4 (Misawa et al., 1993) was amplified by PCR based on the *PacrtI* sequence (GenBank: D90087). The product was inserted into pGEM®-T (Promega) for sequencing and then transferred to pHor-P containing the barley D-hordein promoter (Sørensen et al., 1996) and the rice ADPGPP terminator.

AtOR cDNA was cloned directly from *A. thaliana* mRNA by reverse transcriptase PCR based on sequence infromation in GenBank (accession number U27099.1). The cDNAs was transferred to the pGEM-T easy vector (Promega) and the recombinant vector was digested with *Eco*RI. *AtOR* was introduced into vector p326, containing the wheat low molecular weight (LMW) glutenin gene promoter and *nos* terminator.

The *Paracoccus* ssp. N81106 (formerly known as *Agrobacterium aurantiacum*) *CrtW* gene fused in frame with the *P. vulgaris* transit peptide signal described above (Schreier et al., 1985) in plasmid p35W2AZ (Ralley et al., 2004) was amplified by PCR using forward primer 5′-AGG ATC CAT GGC TTC TAT GAT ATC CTC TTC-3′ and reverse primer 5′-AGA ATTCTC ATG CGG TGT CCC CCT TGG TGC-3 incorporating appropriate restriction sites. The product was inserted into pGEM®-T (Promega) for sequencing and then transferred to vector pGZ63 containing the corn γ-zein promoter (Torrent et al., 1997) and nopaline synthase terminator.

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

β-carotene ketolase *sBcrtW* and hydroxylase *sBcrtZ* from *Brevundimonas* sp. Strain SD212 (Nishida et al., 2005) were chemically synthesized according to the codon usage of

Brassica napus (provided by Dr.Norihiko Misawa, Japan) and fused to the full-length rice alcohol dehydrogenase untranslated region (Sugio et al., 2008) and to the transit peptide sequence from the pea ribulose 1, 5-bisphospate carboxylase small subunit (Schreier et al., 1985). These DNA fragments were inserted into plasmid GZ63 containing the maize γ-zein gene promoter and the *nos* terminator.

3.3.2 Plant material

Four different transgenic lines were generated as described in Chapter 2, subsection 2.3.2; they are shown in Table 3.2. PSY, PCW, Or and a yellow endosperm maize inbred line C17 (obtained from Dr. Conchita Royo, IRTA, Lleida, Spain) were crossed with line ZWB co-expressing *sBcrtZ, sBcrtW* and *sCrbkt*. Endosperm samples from the resulting crosses were harvest at 30DAP, frozen in liquid nitrogen and stored at -80ºC prior to use.

Lines	Transgenes
PSY	*Zmpsy1*
PCW	*Zmpsy1+PacrtI+PacrtW*
OR	*AtOR*
ZWB	*sBcrtZ+sBcrtW+sCrbkt*

Table 3.1: Transgenic lines used in this study.

sBcrtZ and s*BcrtW* genes were chemically synthesised. *PcrtW* was cloned from *Paracoccus* sp.N81106.

3.3.3 DNA analysis of endogenous maize carotenoid pathway genes

Genomic DNA was isolated from maize leaves by phenol extraction (Edwards et al., 1991) and 20 µg aliquots were digested overnight with *EcoRI*. The DNA was fractionated by 0.8% agarose gel electrophoresis (Sambrook et al., 1989), transferred to a positively-charged nylon membrane (Roche) and fixed by UV cross-linking. DIG-labeled probes generated by PCR using the exon-specific primers listed in **Table 3.3** were purified using the QIAquick Gel Extraction Kit (Qiagen) and denatured at 95ºC for 10 min prior to hybridization overnight at 42ºC. Membranes were washed at high stringency (twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 30 min in 0.5 SSC, 0.1 SDS at 68ºC, once for 20

min in 0.2x SSC, 0.1% SDS at 68ºC, and once for 10 min in 0.1x SSC, 0.1% SDS at 68ºC) prior to chemiluminescent detection using the DIG Luminescent Detection Kit (Roche) according to the manufacturer's instructions. After washing, the membranes were incubated with CSPD chemiluminescent substrate (Roche) and exposed on BioMax light film (Kodak) at 37ºC for 2h at 37ºC (or overnight if the signal was weak).

3.3.4 Total RNA isolation and mRNA analysis

The protocol is described in detail in Chapter 1, section 1.3.2. Primer sequences used for designing probes are shown in **Table 3.3**.

Transgene	Primer set
Zmpsy1	Forward: 5´-GTGTAGGAGGACAGATGAGCTTGT-3´
	Reverse: 5´-CATCTGCTAGCCTGTGAGAGCTCA-3´
PacrtI	Forward: 5´-TGGAGAAGCGTTTACAGTAAGGT-3´
	Reverse: 5´-GCGTGCAGATAAAGTGAGAAGTC-3´
AtOR	Forward 5´-ATGTCATCTTTGGGTAGGATTTTGT-3´
	Reverse 5´-GGTTTTGGGCGGTGATAGAGA-3´
PacrtW	Forward: 5´-ATCGCGCATGACGCGATGCACGG-3´
	Reverse: 5´-GGTGCAGGTGGTGTTCGTGATGAT-3´
sCrbkt	Forward 5´- GGATCCTCAGCCAGGAGCCAGTGCAGCGCCTCT-3´
	Forward 5´-AATTCCATGGGGCCAGGCATTCAGCCCACTTCCG-3´
sBcrtZ	Forward 5´- ACGAATTCGAT GGCCTG GCT GACGT -3´
	Forward 5´- TAG AGGATCCTC AGG CGCCGCTGC TGG-3´
sBcrtW	Forward 5´- TACGAATTCGATGAGCGCCGCCGTCG -3´
	Forward 5´- TAGAGGATCCTCAAGACTCGCCGCGCCACAA -3´

Table 3.2: Primer sequences used for transgene probe design.

3.3.5 Quantitative real time PCR

The protocol is described in detail in Chapter 1, section 1.3.3.

3.3.6 Carotenoid extraction from maize endosperm

The protocol is described in detail in Chapter 1, section 1.3.4.

3.3.7 HPLC-MS and UHPLC-MS and carotenoid identification and quantification

The protocols are described in detail in Chapter 1, section 1.3.5 and section 1.3.6.

3.4 Results

3.4.1 Combinatorial transformation of carotenogenic genes into maize endosperm and mRNA analysis of introduced transgenes

I transformed four-day-old immature zygotic embryos of South African elite white maize inbred M37W by bombarding them with gold particles coated with four constructs: the selectable marker *bar* and three ketocarotenogenic genes: *sCrBKT* (*Chlamydomonas reinhardtii* truncated β-carotene ketolase), *sBcrtW* (modified β-carotene ketolase originally from *Brevundimonas* sp. Strain SD212), and *sBcrtZ* (modified β-carotene hydroxylase originally from *Brevundimonas* sp. Strain SD212). All transgenes were driven by the endosperm-specific maize γ-zein gene promoter. I selected two representative transgenic lines as discussed below. DNA blot analysis indicated that one line contained *sCrBKT, sBcrtW* and *sBcrtZ,* (line ZWB). Transgene expression was confirmed by mRNA blot analysis (**Figure 3.2**). In this chapter the abbreviation TC refers to total carotenoids, including ketocarotenoids, when present.

Line ZWB was crossed with different maize lines selected on the basis of their carotenoid composition:(a) wild type inbred line C17 was yellow in color and was selected because it accumulates naturally high levels of β-carotene; (b) transgenic line PSY, (M37W genetic background) expressing only *Zmpsy1* accumulates high levels of total carotenoids, mostly zeaxanthin, lutein and antheraxanthin; (c) transgenic line PCW (M37W genetic background) expressing *Zmpsy1*, *PacrtI* and *PacrtW*, which not only accumulated high amounts of total carotenoids (mostly zeaxanthin and β-carotene), but also accumulates the ketocarotenoid adonixanthin at ca: 5% of TC (d) transgenic line Or, expressing an *AtOR* transgene which generates a metabolic sink for carotenoids. These lines had been generated in the laboratory previously and are described in section 3.3.1. Transgene expression in all transgenic lines and corresponding crosses was confirmed by mRNA blot analysis (**Figure**

3.2). This analysis revealed that *Zmpsy1* mRNA accumulated only at detectable levels in lines PSY and PCW. Endogenous *Zmpsy1* expression was beyond the limit of detection in C17 by mRNA blot analysis, but qRT-PCR analysis confirmed accumulation of this transcript. *PacrtI* and *PacrtW* mRNA accumulated in PCW, and *AtOR* mRNA accumulated only in OR. *sCrBKT*,*sBcrtZ* and *sBcrtW* mRNA accumulated in transgenic line ZWB.
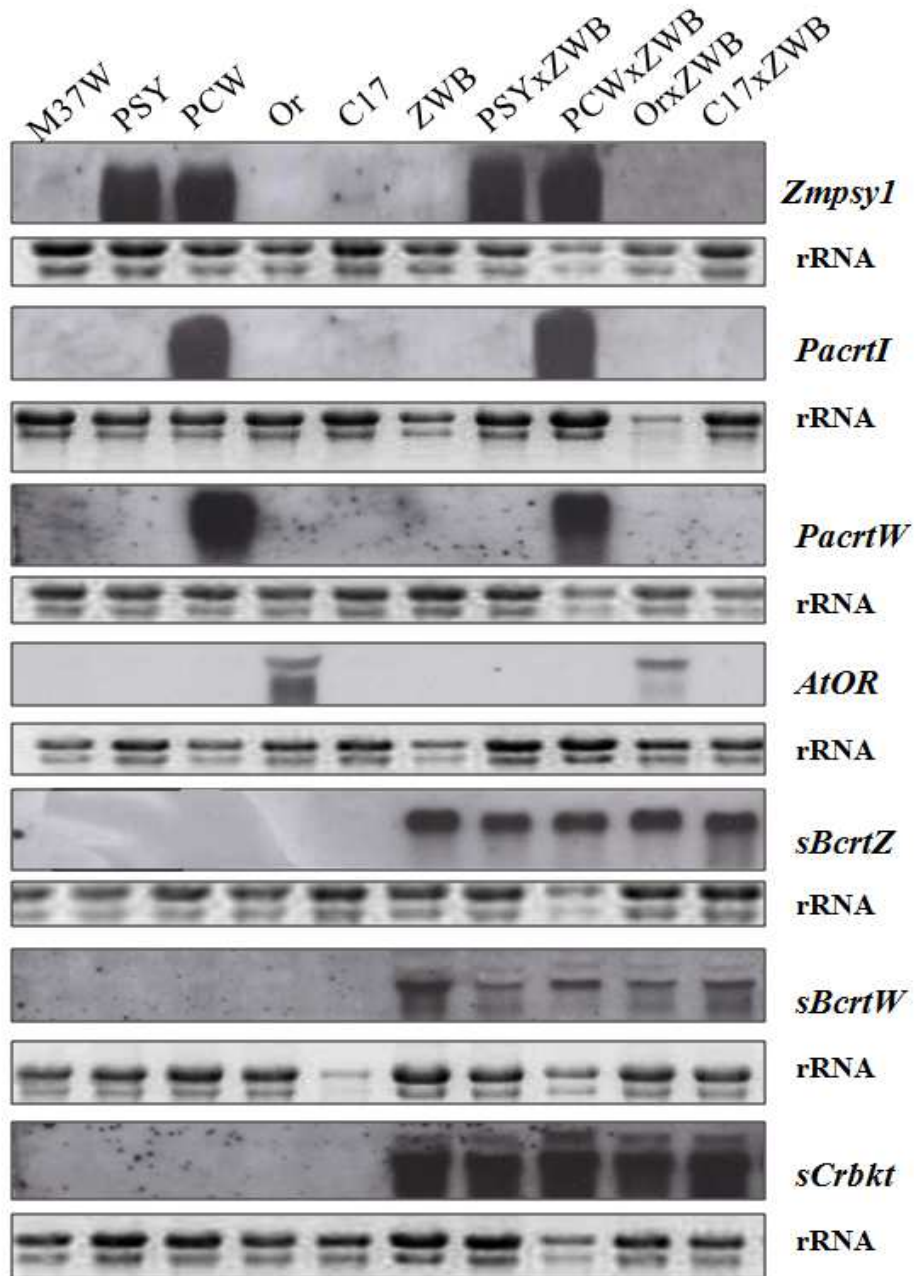
Figure 3.2: Transgene expression analyses in maize endosperm. mRNA blot analysis (25 μg of total RNA per lane) was used to monitor transgene expression in the endosperm (30 DAP,

T1 generation) of wild types M37W and C17, and transgenic lines PSY, PCW, OR and ZWB. Abbreviations: *Zmpsy1*, maize phytoene synthase 1 gene; *PacrtI*, bacterial phytoene desaturase gene; *PacrtW*, bacterial β-carotene ketolase; *AtOR*, *Arabidopsis thaliana* Orange gene; *sBcrtZ*, synthetic β-carotene hydroxylase gene; *sBcrtW*, synthetic β-carotene ketolase gene; *sCrbkt*, synthetic *C. reinhardtii* β-carotene ketolase gene.

3.4.2 Introgression of ketocarotenoid genes into diverse genetic backgrounds leads to different levels of astaxanthin accumulation in the resulting hybrids

In order to elucidate the mechanism of the conversion of carotenoids to astaxanthin in maize endosperm, ZWB was crossed with lines having different genetic backgrounds and carotenoid profiles: PSY, PCW, C17 and OR.

ZWB expressing *sBcrtZ*, *sBcrtW* and *sCrbkt* was crossed with PSY, PCW, OR and C17. PSYxZWB expressed four transgenes, *Zmpsy1*, *sBcrtZ*, *sBcrtW* and *sCrbkt* (**Figure 3.2**). PCWxZWB expressed six transgenes, *Zmpsy1*, PacrtI, *PacrtW*, *sBcrtZ*, *sBcrtW* and *sCrbkt*, [including three different β-carotene ketolases (**Figure 3.2**)]. ORxZWB expressed four different transgenes, *AtOR sBcrtZ*, *sBcrtW* and *sCrbkt* (**Figure 3.2**). C17xZWB expressed *sBcrtZ*, *sBcrtW* and *sCrbkt* (derived from ZWB) (**Figure 3.2**).

The new genotypes resulted in phenotypes with different carotenoid and ketocarotenoid profiles. The original ZWB transgenic line expressing *sBcrtZ*, *sBcrtW* and *sCrbkt* exhibited a light pink color seed phenotype (**Figure 3.3**). HPLC analysis confirmed that this phenotype was due to the complete conversion of the total carotenoids (0.7μg/g DW) to astaxanthin (0.4 μg/g DW) (**Table 3.4** and **Table 3.5)**. PSYxZWB, PCWxZWB, ORxZWB and C17xZWB had a red/orange endosperm phenotype suggesting the accumulation of astaxanthin (**Figure 3.3**).
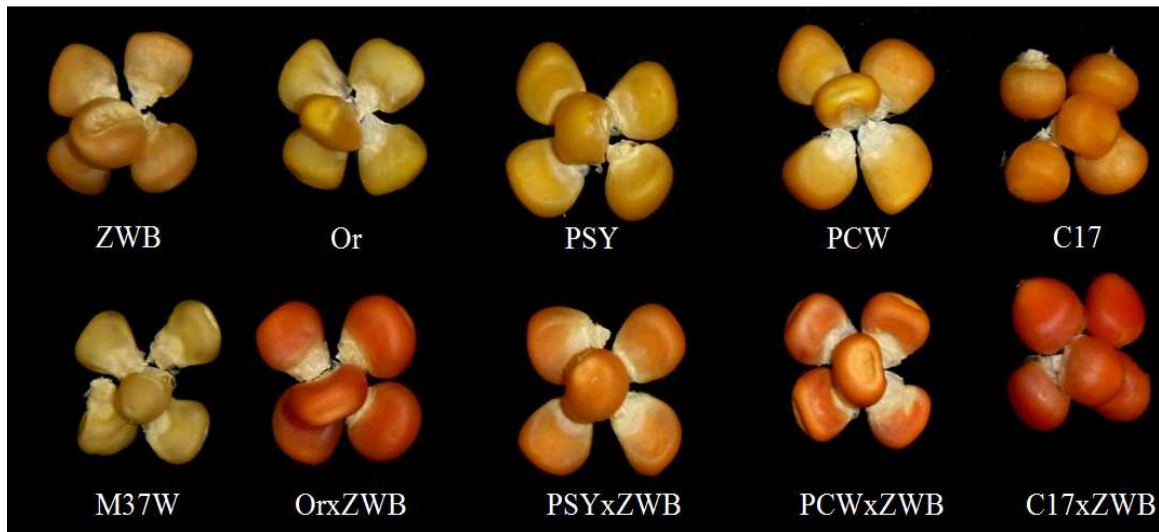
Figure 3.3: Phenotype of transgenic lines, wild type C17 (yellow), wild type M37W (white), and corresponding crosses with ZWB. ZWB exhibits a pale pink endosperm color, due to the accumulation of traces of astaxanthin; OR, PSY, PCW and C17 had yellow to orange endosperm due to the accumulation of carotenoids; ORxZWB, PSYxZWB, PCWxZWB and C17xZWB had orange-red endosperm due to different levels of astaxanthin.

PSYxZWB

The highest accumulation of TC in all parental lines reported in these experiments (ca: 66 µg/g DW) was measured in PSY, expressing only *Zmpsy1*. The predominant endosperm carotenoids were zeaxanthin (ca: 23 µg/g DW, 35% of TC), antheraxanthin (ca: 14 µg/g DW, 21% of TC), lutein (ca: 9 µg/g DW, 14% of TC and phytoene (ca: 8 µg/g DW, 12% of TC) (**Table 3.4**). Lycopene and β-carotene accumulated as minor components, ca: 3 µg/g DW (4-5% of TC) (**Table 3.4)**. The carotenoid accumulation profile changed dramatically in PSYxZWB (**Table 3.5**). TC increased up to ca: 79 µg/g DW, with phytoene and astaxanthin being the predominant metabolites, ca: 25 µg/g DW (32%) and 12 µg/g DW (15%) respectively. Unknown ketocarotenoids also accumulated up to 17% of TC (**Table 3.5)**. While different ketocarotenoids accumulated in the hybrid, the amount of zeaxanthin, antheraxanthin and lutein was reduced. They were accumulated only to 2-3% of TC. Lycopene and β-carotene levels remained similar to those in PSY (**Table 3.5**).

PCWxZWB

PCW accumulates high amounts of TC (ca: 62 μg/g DW), as well as adonixanthin, as the only ketocarotenoid (ca: 3 μg/g DW, 5% of TC). The main carotenoids in this line are zeaxanthin (ca: 31 μg/g DW, 51% of TC), β-carotene (ca: 8 μg/g DW, 13% TC) and phytoene (ca: 6 μg/g DW, 10% TC) (**Table 3.4**). PCWxZWB accumulated higher amounts of TC (ca: 80 μg/g DW), representing a ca: 60% conversion to ketocarotenoids. Astaxanthin was the predominant ketocarotenoid accumulated (ca: 17 μg/g DW, 22% TC) (**Table 3.5**). The conversion of carotenoids to ketocarotenoids consumed almost all carotenoids present in PCW. Phytoene and lycopene were the only carotenoids which accumulated in the hybrid. Phytoene levels increased up to ca: 17 μg/g DW and lycopene accumulated up to ca: 15 μg/g DW. Lycopene did not accumulate in PCW (**Table 3.5**).

ORxZWB

OR accumulated lower amounts of TC (ca: 22 μg/g DW) compared to PSY, PCW and C17 (**Table 3.4**). The main carotenoids in this line were zeaxanthin (ca: 7 μg/g DW, 32% TC), antheraxanthin (ca: 6 μg/g DW, 27% TC) and lutein (ca: ca: 6 μg/g DW, 25% TC). β-Carotene accumulation was very low (ca: 1 μg/g DW, 3% TC) and phytoene did not accumulate (**Table 3.4**). Introgression of the ketocarotenoid mini-pathway resident in ZWB into OR resulted in a 93% conversion of TC to ketocarotenoids. The only remaining carotenoids in the hybrid were lycopene (ca: 1 μg/g DW, 6 % TC) and traces of violaxanthin (**Table 3.5**). Astaxanthin was the most prevalent metabolite (ca: 8 μg/g DW, 52% TC), together with 3OH-echinenone (ca: 1 μg/g DW, 7% TC) and an unknown ketocarotenoid (36% of TC) (**Table 3.5**).

C17xZWB

Yellow maize C17 inbred line accumulates relatively higher amounts of β-carotene, compared to other wild type maize lines [ca: 11 μg/g DW (29% TC)], as well as lutein (ca: 12 μg/g DW, 31% TC). Only two additional carotenoids were detected, phytoene (ca: 9 μg/g DW, 22% TC) and zeaxanthin (ca: 6 μg/g DW, 16% TC) (**Table 3.4**). The TC in C17 and corresponding cross C17xZWB, were very similar, ca: 39 μg/g DW and 35 μg/g DW, respectively (**Table 3.4** and **Table 3.5**). However, C17xZWB had a different carotenoid profile with 100% of carotenoids converted to ketocarotenoids. Astaxanthin was the

predominant ketocarotenoid (ca: 12 µg/g DW, 35% TC). Canthaxanthin and adonixanthin also accumulated but in lower amounts, ca: 2 µg/g DW (5% TC) and 3 µg/g DW (9% TC), respectively. Slightly more than half of total carotenoids were unknown ketocarotenoids (**Table 3.5**).

Metabolite /plant line	**Phyto**	**Lyco**	**β-cryp**	**β-caro**	**ζ-beta + peroxi ζ-beta**	**Lut**	**Zea**	**Anthe**	**Viola**	**TC**
PCW	10	0	5	13	0	8	51	7	1	**61.8**
PCWx ZWB	22	19	0	0	0	0	0	0	0	**80.4**
Or	0	0	3	3	0	25	32	27	9	**22.2**
Orx ZWB	0	6	0	0	0	0	0	0	1	**16.4**
PSY	12	4	2	5	0	14	35	21	6	**65.7**
PSYx ZWB	32	4	0	4	2	2	3	2	1	**78.9**
C17	22	0	1	29	0	31	16	0	0	**38.9**
C17x ZWB	0	0	0	0	0	0	0	0	0	**35.1**
ZWB	0	0	0	0	0	0	0	0	0	**0.4**
M37W	0	0	0	0	0	29	57	0	14	**0.7**

Table 3.3: Carotenoid content of ZWB, PCW, Or, PSY and C17, and corresponding crosses PCWxZWB, OrxZWB, PSYxZWB and C17xZWB in the endosperm.

Results are presented as % of individual carotenoids and total carotenoids (TC) in µg/g dry weight (DW) ± SD (n = 3–5 mature seeds). These crosses were carried out in the spring of 2013. α-Cryptoxanthin and α-carotene did not accumulate. Abbreviations: Phyto, phytoene; Lyco, lycopene; β-cryp, β-cryptoxanthin; β-caro, β-carotene; ζ-beta + peroxi ζ-beta, ζ-betacarotene + peroxi ζ-betacarotene; Lut, lutein; Zea, zeaxanthin; Anthe, antheraxanthin; Viola, violaxanthin.

Samples/ Plant line	Asta	Cantha	Adoniru	Adonixan	3OH-echi	Other keto	% total KETO	TC
PCW	0	0	0	5	0	0	5	**61.8**
PCWx ZWB	22	4	4	1	8	21	59	**80.4**
Or	0	0	0	0	0	0	0	**22.2**
Orx ZWB	52	0	0	0	7	34	93	**16.4**
PSY	0	0	0	0	0	0	0	**65.7**
PSYx ZWB	15	6	6	0	8	17	51	**78.9**
C17	0	0	0	0	0	0	0	**38.9**
C17x ZWB	35	5	0	9	0	52	100	**35.1**
ZWB	100	0	0	0	0	0	100	**0.4**
M37W	0	0	0	0	0	0	0	**0.7**

Table 3.4: Ketocarotenoid content of ZWB, PCW, Or, PSY and C17, and corresponding crosses PCWxZWB, OrxZWB, PSYxZWB and C17xZWB in the endosperm.

Results are presented as % of individual and total carotenoids (TC) (TC in µg/g dry weight (DW) ± SD (n = 3–5 mature seeds)]. These crosses were carried out in the spring of 2013. Abbreviations: Asta, astaxanthin; Cantha, canthaxanthin; Adoniru, adonirubin; Adonixan, adonixanthin; 3OH-echi, 3OH-echinenone.

3.4.3 Endogenous carotenogenic gene expression profiles in hybrid lines

To further investigate whether endogenous carotenoid gene expression was influenced by the expression of the introduced transgenes in the hybrids described above, transcript levels of endogenous phytoene synthase 1 (*Zmpsy1*), lycopene β-cyclase (*ZmlycB*), lycopene ε-cyclase (*ZmlycE*), β-carotene hydroxylase 1 and 2 (*Zmbch1/2*) genes were monitored by quantitative real-time RT-PCR 30DAP in the endosperm of all parents (PSY, PCW, OR, C17, ZWB and M37W) and hybrids (PSYxZWB, PCWxZWB, ORxZWB, C17xZWB) (**Figure 3.5**).

Zmpsy1 transcripts accumulated at high levels in PCW, PSY, PCWxZWB and PSYxZWB consistent with the presence of the transgene in these lines (**Figure 3.5A**).

Endogenous *Zmpsy1* transcript accumulation was lower in C17, C17xZWB, OrxZWB, ZWB and M37W, compared to transgenic lines accumulating *Zmpsy1*. While no endogenous *Zmpsy1* transcript could be measured in OrxZWB, ZWB and M37W, it accumulated at moderate levels in C17 and C17xZWB (**Figure 3.5B**).

      ZmlycB transcript accumulation in C17 was the highest among all the lines, more than double compared to PCW, PSY, PCWxZWB, PSYxZWB, C17xZWB, OrxZWB, ZWB and M37W. However, *ZmlycB* transcript accumulation was reduced almost 3-fold in C17xZWB.

      ZmlycB transcript accumulation was also higher in PCWxZWB compared to the remaining lines (having the M37W background) (**Figure 3.5C**).

      ZmlycE expression exhibited the opposite trend in C17 compared to the other lines. This transcript did not accumulate in C17. However, *ZmlycE* transcript levels in C17xZWB were ca: 2- to 8-fold higher compared to the other lines. *ZmlycE* transcript levels were over 3-fold lower in OR and ORxZWB compared to PCWxZWB, PSYxZWB and M37W (**Figure 3.5D**).

      Relative accumulation of *Zmbch2* transcripts was in general low in all the lines. No accumulation of *Zmbch2* was measured in C17; however, qRT-PCR analysis confirmed the presence of this transcript in OR, ORxZWB and ZWB (**Figure 3.5E**). *Zmbch1* transcripts accumulated at very low levels in PCW, PSY, OR, PCWxZWB, PSYxZWB, C17xZWB, OrxZWB, ZWB and M37W, while *Zmbch1* transcript accumulated at higher levels in C17 and C17xZWB (ca: over 5-fold) compared to PCW, PSY, OR, PCWxZWB, PSYxZWB, C17xZWB, OrxZWB, ZWB and M37W (**Figure 3.5F**).
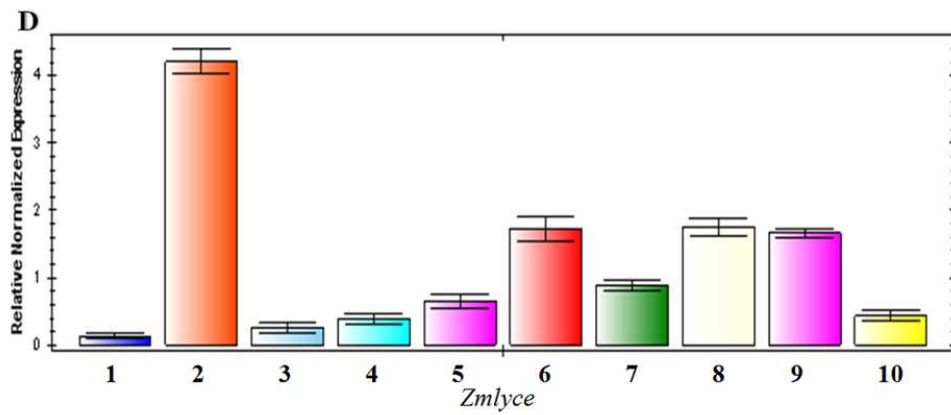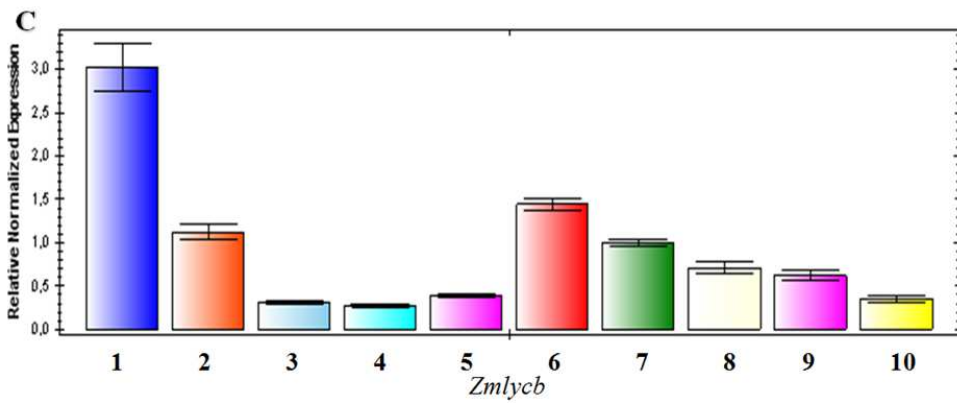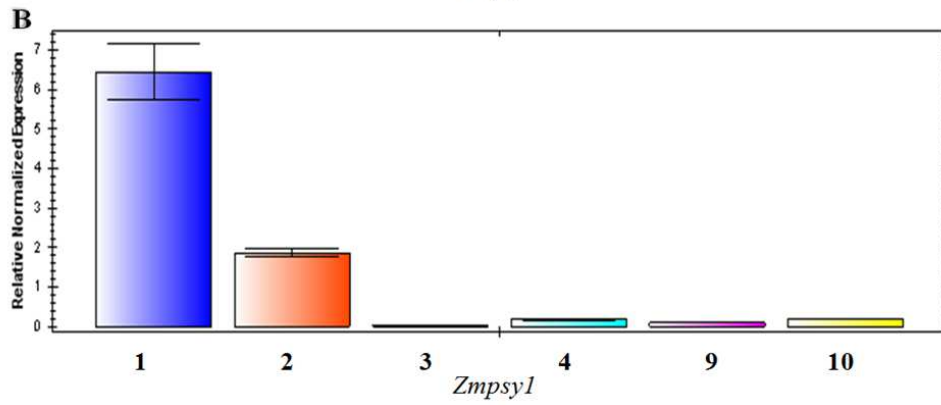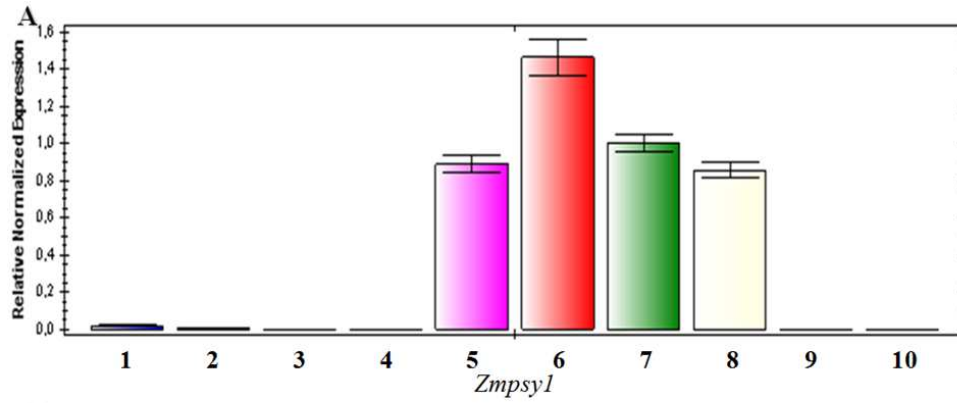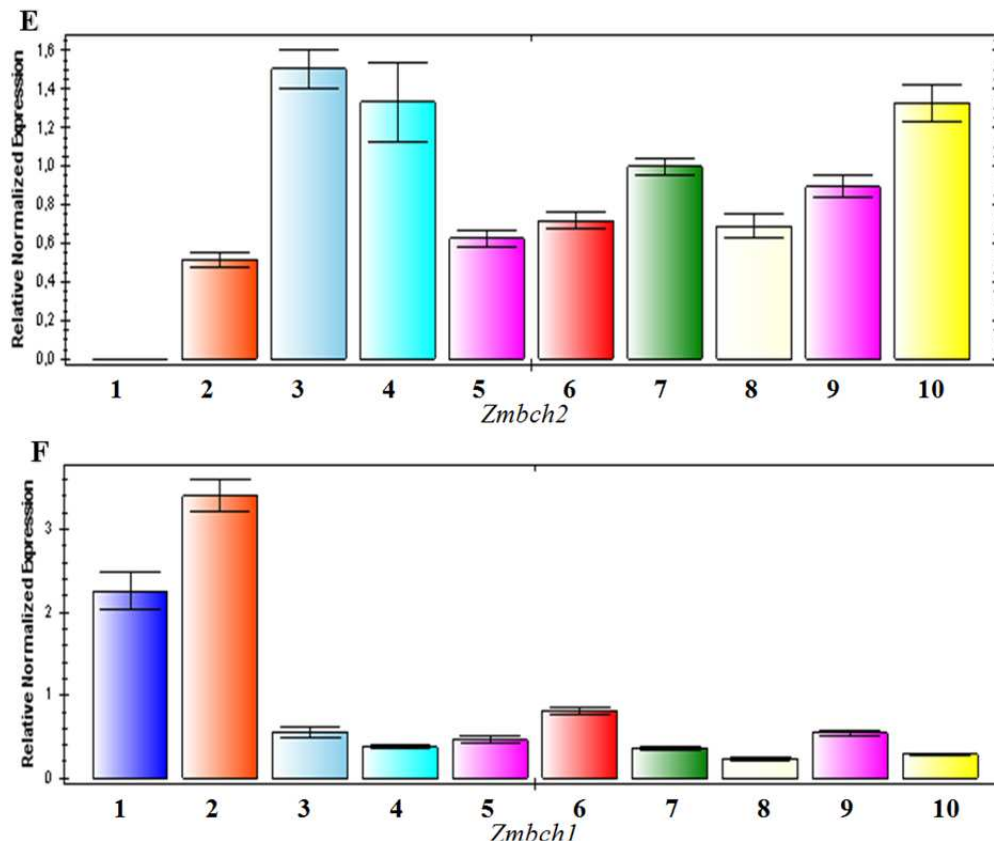
Figure 3.4: Expression analyses of endogenous carotenogenic genes in C17 (1), C17xZWB (2), OR (3), ORxZWB (4), PCW (5), PCWxZWB (6), PSY (7), PSYxZWB (8), M37W (9) and ZWB (10). Relative transcript levels of endogenous *Zmpsy1* (in all lines) (A), *Zmpsy1* (only wild type lines, Or and ZWB lines) (B), *ZmlycB* (C), *ZmlycE* (D), *Zmbch2* (E) and *Zmbch1* (F) genes in maize endosperm at 30 DAP. Values were normalized against actin mRNA and presented as the mean of three replicates ± SD.

3.5 Discussion

3.5.1 Competition between β-carotene hydroxylase and β-carotene ketolase for substrates in the extended ketocarotenoid pathway provides a novel strategy for astaxanthin production in maize endosperm

Metabolic engineering is an effective approach for the understanding and utilization of metabolic processes. Astaxanthin is a ketocarotenoid of commercial importance, and thus a suitable target for metabolic engineering in plants. Expression of β-carotene ketolase alone is not sufficient to convert carotenoids such as β-carotene or zeaxanthin to astaxanthin in maize, potato, tobacco or *Arabidopsis* (Zhong et al., 2011; Zhu et al., 2008; Gerjets & Sandmann, 2006; Gerjets et al., 2007; Zhu et al., 2007b). However, co-expression of β-carotene hydroxylase and β-carotene ketolase provides an effective strategy to convert β-carotene, zeaxanthin or β-cryptoxanthin to astaxanthin in plants (Campbell et al., 2015). We evaluated the impact of introgressing a ketocarotenoid mini-pathway resident in a transgenic line created earlier (line ZWB, with a M37W genetic background) into a range of maize genotypes specifically selected because of their diversity in their carotenoid profile, total carotenoid content and specifically β-carotene content in the endosperm. ZWB expressed one beta carotene hydroxylase (*sBcrtZ*) and two beta carotene ketolase (*sBcrtW* and *sCrbkt*) transgenes. Astaxanthin was the only carotenoid that accumulated in this line. Thus, the small amount of carotenoids in the M37W parent (lutein, zeaxanthin and violaxanthin) was quantitatively converted to astaxanthin (**Figure 3.5**).

In order to investigate the conversion of carotenoids to early pathway ketocarotenoid intermediates and astaxanthin, four transgenic maize lines (PSY, PCW, OR and C17) with different carotenoid profiles were crossed with ZWB. This resulted in different conversion efficiencies of carotenoids to astaxanthin and other ketocarotenoids in the resulting hybrids where the endogenous and induced carotenoid/ketocarotenoid pathways operated synergistically (**Figure 3.5**).

3.5.2 High carotenoid content is a necessary but not the only prerequisite for the subsequent efficient conversion of precursors to astaxanthin

Overexpression of *Zmpsy1* in PSY, PCB, PSYxZWB and PCBxZWB provides unequivocal confirmation that PSY1 is the key enzyme limiting carotenoid biosynthesis in

maize endosperm (Buckner et al., 1996; Palaisa et al., 2003; Zhu et al., 2008). Even when *Zmpsy1* transcripts accumulated at low levels as in C17 and C17xZWB total carotenoid levels in these two lines increased up to ca: 39 and 35 µg/g, respectively. Carotenoids accumulated in OR and ORxZWB, even in the absence of *Zmpsy1* expression, because of the generation of a carotenoid sink resulting from the expression of *AtOR* (Bai et al., 2014; Li et al., 2006). Accumulation of phytoene in PCW, PSY, C17, PSYxZWB and PCBxZWB indicates that conversion of phytoene to lycopene (catalyzed by endogenous desaturases and isomerases in PSY, C17 and PSYxZWB, and by *PacrtI* in addition to endogenous desaturases and isomerase in PCB, PCBxZWB and PCBxZWB) is a rate-limiting step for carotenoid biosynthesis in M37W (Naqvi et al., 2011; Zhu et al., 2008) and also in C17. However, accumulation of lycopene in PSY, PSYxZWB and PCWxZWB, is consistent with results reported in earlier studies, where co-expression of *Zmpsy1* and *PacrtI* and co-expression of *Zmpsy1*, *PacrtI* and *GllycE* in M37W resulted in the accumulation of lycopene (Zhu et al., 2008). In contrast to results obtained in rice expressing similar gene complements (Paine et al., 2005; Ye et al., 2000; Bai et al., 2015), transgenic canola seeds expressing bacterial *crtB* and *crtI*, or *crtB*, *crtI* and *crtY/Bnlycb* (Ravanello et al., 2003), and transgenic potato tubers expressing bacterial *crtB* and *crtI*, or *crtB*, *crtI* and *crtY* (Diretto et al 2007) accumulated lycopene. These results demonstrated that lycopene cyclization is a rate-limiting step in the conversion of lycopene to cyclic carotenes in maize endosperm. In contrast, C17 (yellow maize) did not accumulate lycopene because *ZmlycB* expression eliminated the bottleneck in the lycopene cyclization step by converting it to lutein.

Levels of astaxanthin accumulation in PSYxZWB and PCWxZWB endosperm are also influenced by the initial total carotenoid amount in PSY and PCW endosperm, both of them with the highest total carotenoid amount, accumulated more astaxanthin than ORxZWB and C17xZWB, with less total carotenoid amount. In previous studies in potato, a ketolase and a hydroxylase were co-expressed in two different varieties, where the potato variety 01H15.57 with higher levels of total carotenoids accumulated more astaxanthin than Mayan Gold (with less total carotenoids) (Campbell et al., 2015). In my experiments, co-expression of two beta carotene ketolases (*sBcrtW* and *sCbkt*) and a beta carotene hydroxylase (*sBcrtZ*) in PCWxZWB, PSYxZWB, ORxZWB and C17xZWB demonstrate that accumulation of astaxanthin in maize endosperm is determined by several factors, such as competition between these two enzymes for substrates in the extended maize ketocarotenoid pathway

towards astaxanthin biosynthesis. Co-expression of a β-carotene ketolase and a β-carotene hydroxylase has been one common strategy to convert carotenoids to astaxanthin (e.g., co-expression of CRTW and CRTZ). *Brevundimonas* sp. SD212, *Paracoccus* sp. N81106 or *Pananatis* have been used as source of these two genes. However, in previous studies chemically synthesized CRTW and CRTZ from *Brevundimonas* sp. SD212 were more effective for astaxanthin accumulation in potato (Campbell et al., 2015). The β-carotene ketolase BKT has also been widely used to accumulate astaxanthin in different plants, such as *HpBKT* in carrot and tobacco (Ahn et al., 2011; Jayaraj et al., 2007; Mann et al., 2000) and *CrBKT* in tomato (Huang et al., 2013) (**Table 3.1**). *Haematococcus pluvialis* or *Chlorella zofingiensis* have been the source for BKT, but is has been also demonstrated that synthetic BKT is more effective in terms of generating higher amounts of astaxanthin in plants compared to the corresponding gene from bacteria (Zhong et al., 2011).

Our experiments involving co-expression of two β-carotene ketolase and a β-carotene hydroxylase in PCWxZWB and PSYxZWB did not convert all carotenoids to astaxanthin or to other ketocarotenoids. PSYxZWB and PCWxZWB still accumulated several carotenoids such as zeaxanthin, β-carotene or lutein. Previous studies in potato reported similar results by co-expressing *sBcrtW* and *sBcrtZ* in a high total carotenoid background (Campbell et al., 2015). On the other hand, in PCWxZWB co-expression of three beta carotene ketolases (*PacrtW*, *sBcrtW* and *sCbkt*) and a beta carotene hydroxylase (*sBcrtZ*) converted most but not all the carotenoids in maize endosperm to ketocarotenoids; phytoene and lycopene still accumulated in the hybrids (Figure 3.6B). This fact supports earlier results in maize where lycopene cyclization was identified as a bottleneck in ketocarotenoid biosynthesis (Zhu et al., 2008). Canthaxanthin, adonirubin and echinenone accumulation in PSYxZWB and PCWxZWB indicates that overexpression of *sBcrtZ* is not sufficient to complete the hydroxylation steps towards astaxanthin. The same results were reported in other studies in tobacco, *Brassica napus* and lettuce, where by co-expressing CRTZ together with ketolases several ketocarotenoids accumulated, such as canthaxanthin, adonirubin and echinenone (Fujisawa et al., 2009; Harada et al., 2014; Hasunuma et al., 2008).

At the same time, effects of co-expression of *Or* with a ketocarotenoid pathway have been assessed in maize in this study (ORxZWB), as it has been also done in potato in a recent study (Campbell et al., 2015). A high percentage of carotenoids were converted to

ketocarotenoids in both maize and potato. However, astaxanthin was not the only ketocarotenoid accumulated, nor it was the only ketocarotenoid accumulated in PSYxZWB and PCWxZWB. However, C17xZWB had the most efficient conversion of carotenoids to ketocarotenoids. However, as for ORxZWB, PSYxZWB and PCWxZWB, several ketocarotenoid intermediates accumulated, in addition to astaxanthin. A high *Zmlyce* transcript accumulation in C17xZWB might indicate a pathway redirection towards ketolutein. Previous studies showed that plants overexpressing a ketocarotenoid pathway and accumulating lutein, such as potato, *Arabidopsis* and tobacco, were able to produce ketolutein (Gerjets et al., 2007; Morris et al., 2006b; Stålberg et al., 2003; Zhu et al., 2007b).
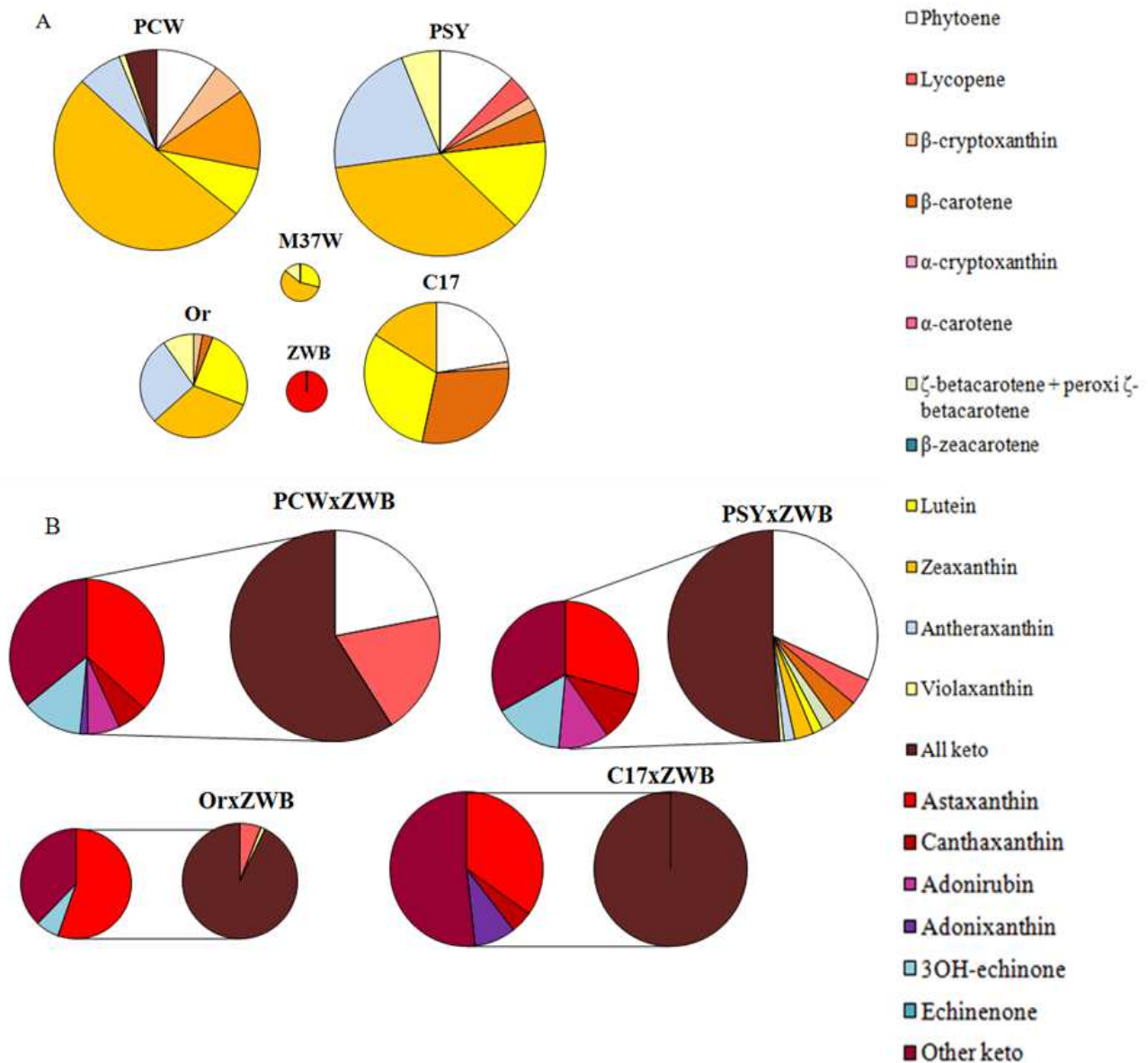
Figure 3.5: Carotenoid profiles in maize parents (A) and resulting hybrids (B) following introgression of the ketocarotenoid biosynthetic mini-pathway (*sBcrtZ*, *sBcrtW* and *sCrbkt*) originally present in line ZWB. Overexpressed transgenes in each parent and hybrid: PSY: *Zmpsy1*; PCW: *Zmpsy1*, *PacrtI*, *PacrtW*; OR: *AtOR*; ZWB: *sBcrtZ*, *sBcrtW* and *sCrbkt*; PSYxZWB: *Zmpsy1*, *sBcrtZ*, *sBcrtW* and *sCrbkt*; PCWxZWB: *Zmpsy1*, *PacrtI*, *PacrtW*, *sBcrtZ*, *sBcrtW* and *sCrbkt*; ORxZWB: *AtOR*, *sBcrtZ*, *sBcrtW* and *sCrbkt*; C17xZWB: *sBcrtZ*, *sBcrtW* and *sCrbkt*. Surface area of each pie chart represents total carotenoid content and each segment represents % accumulation of individual metabolites. Pie chart on the left hand side of each hybrid in B, shows the detailed composition of ketocarotenoids in each hybrid.

3.6 Conclusions

We explored factors limiting astaxanthin production in maize endosperm by overexpressing a combination of two β-carotene ketolases and a β-carotene hydroxylase in different maize lines with diverse carotenogenic backgrounds. Co-expression of *sBcrtW*, *sBcrtZ* and *sCrbkt* in a white endosperm background (M37W) allowed the conversion of almost all carotenoids to astaxanthin in the resulting transgenic line. However, the initial low amount of total carotenoids in M37W only permitted accumulation of traces of astaxanthin in the transgenic line. Introgression of the same transgene combination into maize lines accumulating higher carotenoid amounts proved to be a novel and effective strategy to accumulate astaxanthin at higher levels in maize endosperm. The accumulation of several intermediates, particularly high levels of canthaxanthin, provides evidence for the existence of different bottlenecks in the extended ketocarotenoid pathway in maize endosperm. These include lycopene cyclization, or hydroxylation of canthaxanthin, adonirubin or echinenone, among others. Introgeression of a ketocarotenoid mini-pathway in yellow endosperm maize backgrounds was the most effective strategy for the conversion of carotenoids to ketocarotenoids. Remaining bottlenecks towards astaxanthin accumulation must be removed in order to maximize its accumulation in maize endosperm.

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CHAPTER 4

TOWARDS FOLATE ACCUMULATION IN MAIZE THROUGH MODULATION OF PTERIN AND PARA-AMINOBENZOATE BIOSYNTHESIS

4.1 Abstract

Folates are important cofactors in one-carbon metabolism in all living organisms. Only plants and microorganisms are capable of biosynthesizing folates, therefore, humans depend entirely on their diet as a folate source. Given the low folate content of several staple crops, folate deficiency affects developed as well as developing countries. Folate biofortification of staple crops through enhancement of the folate precursors, pterin and para-aminobenzoate was successful in tomato and rice. However, the same strategy has not been sufficient to enhance folate levels in potato and *Arabidopsis thaliana*. In maize, the folate content was doubled by the enhancement of pterin levels. Our study shows that accumulation of these precursors individually or simultaneously was not sufficient to enhance folate content in maize. We conclude that additional or alternative steps in the pathway and/or metabolism need to be engineered to achieve substantial folate accumulation in maize endosperm. A better understanding of the folate biosynthetic pathway is required in order to determine optimal engineering strategies that can be generally applicable to most staple crops.

4.2 Introduction

4.2.1 Folate

Folic acid, from Latin *folium* (leaf) so called because it is especially abundant in green leaves of many plants (Williams et al, 1941). It is also known as folate, vitamin M, vitamin B_9, vitamin B_c (or folacin), pteroyl-L-glutamic acid, and pteroyl-L-glutamate. Folate is a generic term used to describe folic acid and related compounds that exhibit the biological activity of folic acid (Kamikawa et al, 2004). The term folate and folic acid are used interchangeably in this chapter.

Folate is the group name used to distinguish naturally occurring compounds of this class, the pure substance is designated pteroylmonoglutamic acid (Kamikawa et al., 2004). The chemical structure of folate (pteroylglutamic acid) is shown in figure 4.1. The molecule comprises three distinct units, glutamic acid, ρ-aminobenzoic acid (p-ABA), and a pteridine nucleus, the last two making up pteroic acid (Scott et al, 2000). Much of the folate in natural feedstuffs is conjugated with a varying number of extra glutamic acid molecules. Synthetic folate, however, is in the monoglutamate form. There are more biologically active forms of

folate than any other known vitamin (McDowell, 2008). Naturally occurring pteroylpolyglutamates constitute a large family of closely related compounds arising from modification of the three units of the parent compound pteroylglutamic acid. Changes in the state of reduction of the pteridine moiety, addition of various one-carbon substituents (C1-substituents) (such as CHO, CH_3, $-CH_2-$ and $=CH-$), and addition of glutamic acid residues lead to a wide array of compounds (**Figure 4.1**) (Scott et al., 2000). Folate is a tasteless and odourless yellowish-orange crystalline powder, which is insoluble in alcohol, ether, and other organic solvents (Oliphant, 1987). It is readily degraded by light and ultraviolet radiation. Cooking can considerably reduce food folate content (Stea et al, 2006; Dang et al, 2000).
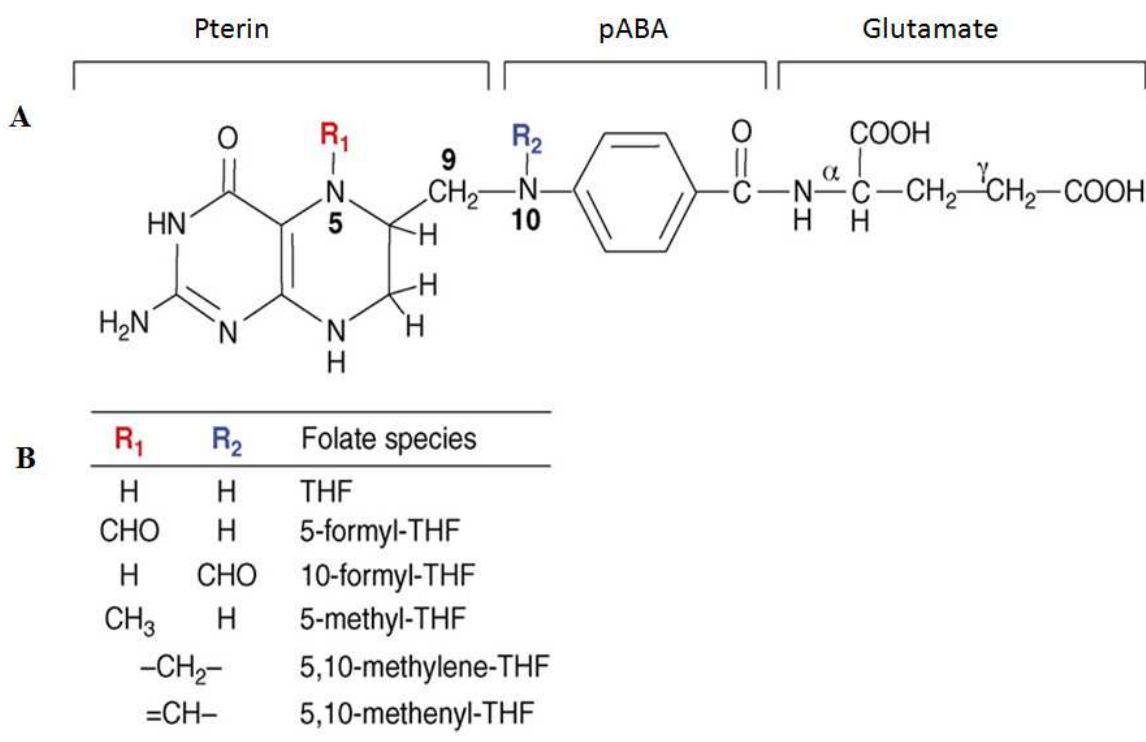
Figure 4.1: Chemical structures of folates. **A)** The folate molecule consists of pterin, p-ABA and glutamate moieties. The particular folate shown here is the monoglutamyl form of tetrahydrofolate (THF). Plant folates have γ-linked polyglutamyl tails of up to six residues attached to the first glutamate. **B)** One carbon (C_1) units at various degrees of oxidation (CHO, CH_3, $-CH_2-$ and $=CH-$) can be attached to two different positions (N5 and/or N10), as indicated by R_1 and R_2. The pteridine ring can exist as tetrahydro, dihydro, or fully oxidized forms (Lucock, 2000).

Folic acid, together with vitamin B_{12}, plays an important role in DNA metabolism (**Figure 4.2**) (Reynolds, 2006). It is required for the synthesis of dTMP (2′-deoxythymidine-5′-phosphate) from dUMP (2′-deoxyuridine-5′-phosphate). Under conditions of folic acid deficiency, dUMP accumulates and as a result uracil is incorporated into DNA instead of thymine (Fenech, 2001). There is good evidence suggesting that excessive incorporation of uracil in DNA not only leads to point mutations but may also result in the generation of single- and double-stranded DNA breaks, chromosome breakage and micronucleus formation (Blount et al., 1997; Fenech, 2001; Reynolds, 2006). The mutagenic effects of uracil are underscored by the observation that of eight known human glycosylases, four (UNG, TDG, hSMUG1, MBD4) are dedicated to the removal of uracil (Blount et al., 1997). Folic acid is also required for the synthesis of methionine and S-adenosyl methionine (SAM), the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (Niculescu & Zeisel, 2002; Whitehead et al., 1995). When the concentration of Vitamin B12 and methionine is low, SAM synthesis is reduced, methylation of DNA is reduced, inhibition by SAM of methylenetetrahydrofolate reductase (MTHFR) is minimised resulting in the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus, favouring an increase in the dUMP pool and uracil incorporation into DNA (Födinger et al, 2000). Deficiencies in folic acid, therefore, can lead to: (a) elevated DNA damage rate and altered methylation of DNA, both of which are important risk factors for cancer (Blount et al., 1997; Duthie, 2011); and (b) an increased level in homocysteine status, an important risk factor for cardiovascular disease (Jardine et al., 2012). These defects may also play an important role in developmental and neurological abnormalities (Frye et al, 2013; Mattson & Shea, 2003).

**Methionine synthesis
and SAM-mediated
methylations**

Thymidylate Synthesis

FOLIC ACID

DIHYDROFOLATE

dUMP

TS

METH

THF

SAM

MS
B12

5,10-METHYLENE THF

dTMP

SAH

MTHFR

HOMOCYS

5-METHYL THF

**DNA REPAIR
DNA SYNTHESIS**

DNA METHYLATION
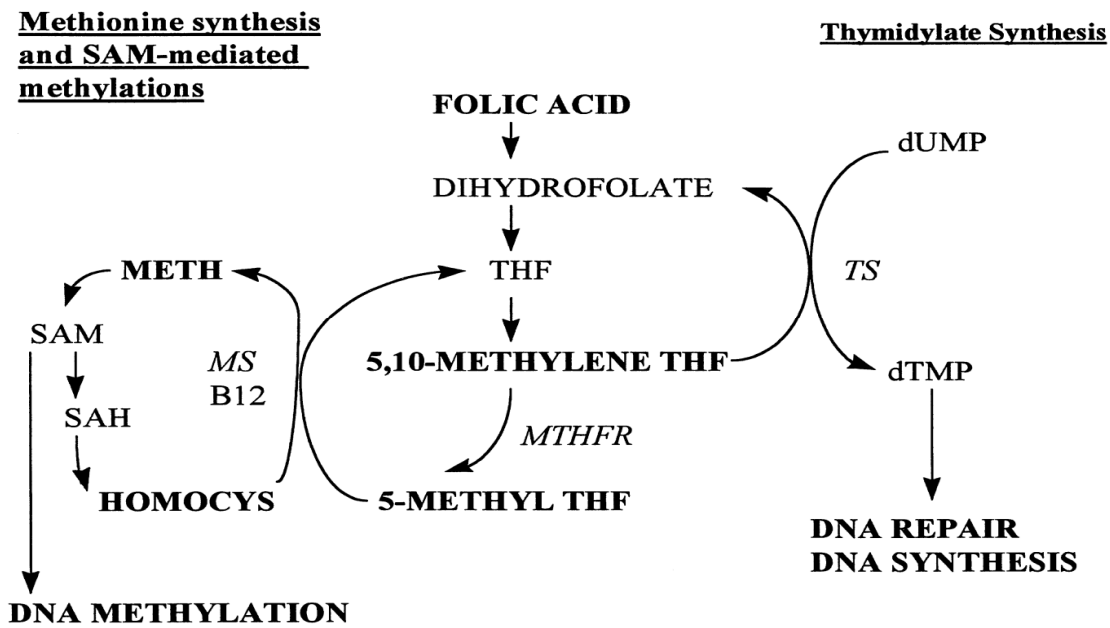
Figure 4.2: The main metabolic pathways in folate and homocysteine metabolism.

Abbreviations: B12, Vitamin B12; METH, methionine; THF, tetrahydrofolate; TS, thymidylate synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; dTMP, (2′-deoxythymidine-5′-phosphate); dUMP, (2′-deoxyuridine-5′-phosphate) (Fenech, 2001).

Intake recommendations for folate and other nutrients are provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (formerly National Academy of Sciences) (Alasfoor, 2013; Institute of Medicine, 1998). DRI is the general term for a set of reference values used for planning and assessing nutrient intakes of healthy people (National Institutes of Health, 2012). These values vary by age and gender (**Table 4.1**).

Folate (mg/day)			
Age	Female/Male	Pregnancy	Lactation
0-12 months	0.065-0.08	-	-
1-3 years	0.15	-	-
4-8 years	0.2	-	-
9-13 years	0.3	-	-
14 + years	0.4	0.6	0.5

Table 4.1: Dietary Reference Intakes (DRI) for Folate (National Institutes of Health, 2012).

Deficiency in adults causes macrocytic anemia and elevated levels of homocysteine (Clarke et al., 2003; Morris et al, 2007), but the impact in pregnant women is much more severe, leading to neural tube defects in the fetus. Spina bifida, in which bones of the spine do not completely enclose the spinal cord, is the most common congenital abnormality associated with folate deficiency (Au et al, 2010; Lucock, 2000). Based on the total number of neural tube defects, as reported in UNICEF's Global Damage Assessment Report of Vitamin and Mineral Deficiency (UNICEF, 2004), and the World Bank demographic databases on total population and birth rate (World Bank, 2011), the prevalence of Neural Tube Defects (NTDs) is calculated and provides an overview of the key areas of high NTD prevalence, defined as >10 NTDs per 10,000 births, reflecting folate deficiency.

A number of researchers have reported a high incidence of folate deficiency in pregnant women in both developed and developing countries (Imdad & Bhutta, 2012). It has been estimated that up to one-third of all pregnant women in the world may experience folate deficiency of varying severity (McGowan & McAuliffe, 2012). Megaloblastic anemia during pregnancy, resulting from low folate intake, is associated with poverty and poor diet (McNulty et al, 2012; Tripathi et al., 2012). While folate deficiency is extremely common in women 16 to 40 years of age because of the effects of pregnancy or lactation, it is rare in men younger than 60 years of age. After age 60, folate deficiency is equally high in both men and women (McDowell, 2008). Because of their rapid growth rate, cancer cells have an exceptionally high folate requirement (Lu & Low, 2012; Tedeschi et al., 2013). Therefore, drugs that inhibit folate-requiring enzymes are widely used in medicine for cancer

chemotherapy (Gonen & Assaraf, 2012; Lu & Low, 2012; Morris et al., 2014; Vergote et al, 2015).

Folate is found naturally in a wide variety of foods, including vegetables (especially dark green leafy vegetables), fruits and fruit juices, nuts, beans, peas, dairy products, poultry and meat, eggs, seafood, and grains. Spinach, liver, yeast, asparagus, and Brussels sprouts are among the foods with the highest levels of folate (National Institutes of Health, 2012).

4.2.2 Folate biosynthetic pathway

Folates are present throughout plant cells in the mitochondria, plastids, cytosol, and vacuoles (Akhtar et al., 2010; Chen et al, 1997; Goyer et al., 2005), but are synthesized only in mitochondria. The pterin and pABA precursors are synthesized in the cytosol and plastids, respectively (**Figure 4.3**). Pterin synthesis begins with the conversion of GTP to dihydroneopterin triphosphate, which is mediated by GTP cyclohydrolase I (GCHI) (Basset et al., 2002; McIntosh et al, 2008). GCHI is the first committed enzyme in pterin biosynthesis in plants, occurring in the cytosol and it is inhibited by GTP (Basset et al., 2002; McIntosh et al., 2008). The dihydroneopterin triphosphate product of GCHI is then dephosphorylated to dihydroneopterin in two steps. The first step in plants, as in bacteria, is the removal of pyrophosphate, which yields dihydroneopterin monophosphate (Klaus et al., 2005). Hydrolysis of dihydroneopterin monophosphate to dihydroneopterin may be carried out by a nonspecific phosphatase in plants, as in *Escherichia coli* (G. Basset et al., 2002; Roje, 2007; Suzuki & Brown, 1974), but there is as yet no biochemical or genetic evidence for this hypothesis in plants, so a specific enzyme remains a possibility. The side chain of dihydroneopterin is then cleaved to 6-hydroxymethyldihydropterin (HMDHP) and glycolaldehyde by dihydroneopterin aldolase. This enzyme also mediates the epimerization of dihydroneopterin to dihydromonopterin, which it likewise cleaved to yield HMDHP (Goyer et al., 2004). Dihydroneopterin and dihydromonapterin can be metabolized to β-D-glycosides, at least in tomato fruit engineered to overproduce pterins (Díaz de la Garza et al., 2004). Neither the sugar moiety nor the glycosyltransferase(s) involved have been identified, nor it is known whether the glycosides serve as a mobilizable reserve of pterins (Hanson and Gregory, 2011).

pABA is synthesized from chorismate, a product of the shikimate pathway, in two steps (**Figure 4.3**). Both steps are localized in the plastids, as is the entire shikimate pathway (Basset et al., 2004a; 2004b). In the first step chorismate is converted to aminodeoxychorismate by aminodeoxychorismate synthase (ADCS). Subsequently, aminodeoxychorismate lyase converts aminodeoxychorismate to pABA (Basset et al., 2004a). pABA can be esterified with glucose in a reversible reaction mediated by a cytosolic UDP-glucosyltransferase (Eudes et al., 2008; Quinlivan et al., 2003).

The HMDHP and pABA precursors are assembled into THF in the mitochondrion (**Figure 4.3**). HMDHP is first activated by pyrophosphorylation, and then coupled to pABA to yield dihydropteroate (Eisenhu et al, 2013; McIntosh & Henry, 2008). These reactions are catalyzed by HMDHP pyrophosphokinase and dihydropteroate synthase, respectively, two domains of a single bifunctional protein in plants (Bekaert et al., 2008). Subsequenlty, DHF synthase couples dihydropteroate to glutamate to yield DHF (Ravanel et al., 2001). Finally, DHF is reduced to THF by DHF reductase, which in plants is fused to thymidylate synthase for dTMP conversion from dUMP (see figure 4.2)(Schnell et al, 2004).

The polyglutamate tail is added to THF and its C1-substituted forms, one residue at a time, via the action of folylpolyglutamate synthase (FPGS). The folate polyglutamate tail is not a static entity but can be shortened or removed by γ-glutamyl hydrolase (GGH), which can have endo- and exopeptidase activities (Akhtar et al., 2008; Cossins, 2000; Orsomando et al., 2005). GGH is located in vacuoles (Orsomando et al., 2005).
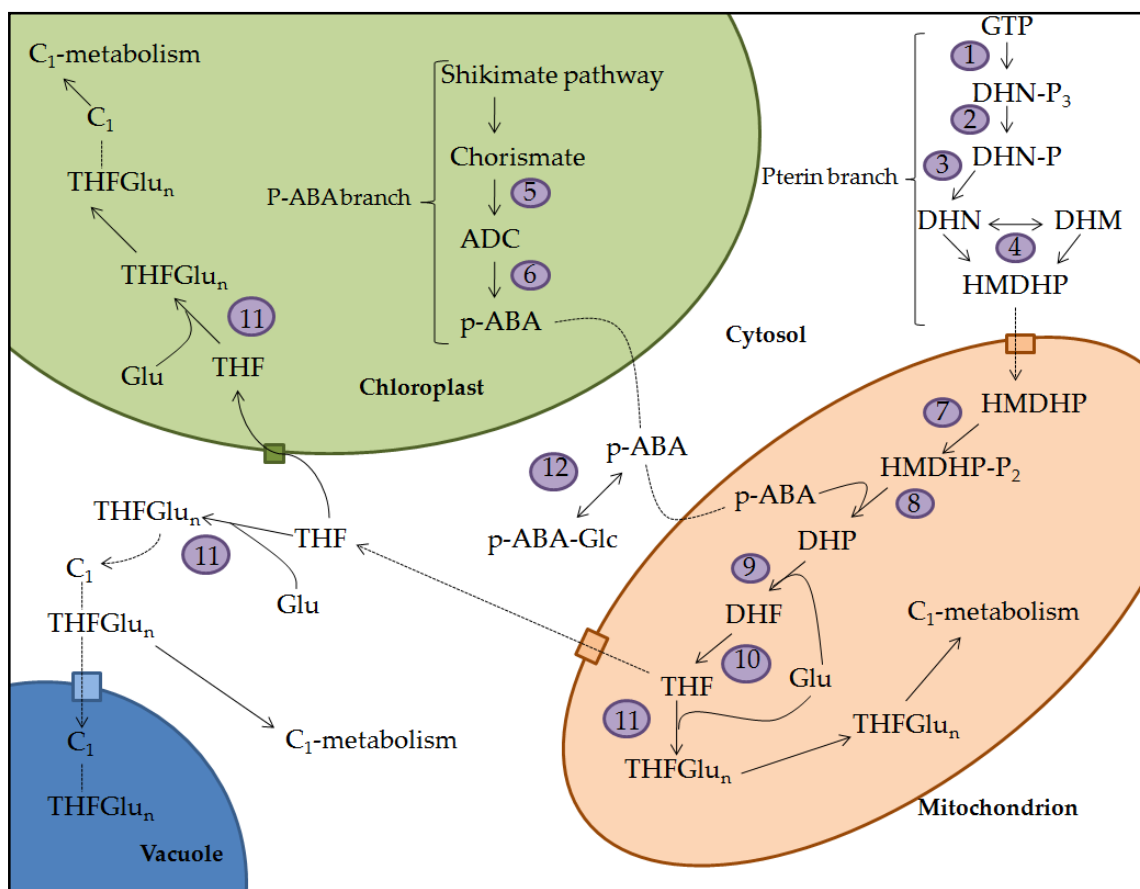
Figure 4.3: The folate biosynthesis pathway, its compartmentalization in plant cells, and carrier-mediated transport steps.

The two known folate carrier protein (plastidial) is shown in green. Hypothetical carriers are shown in blue and in orange, with dotted lines indicating hypothetical transport steps (the movement of p-ABA is most probably by diffusion). The hypothetical vacuolar folate carrier might transport polyglutamyl forms, unlike most other folate carriers. p-ABA occurs mainly as its glucose ester, which is formed in the cytosol via a reversible reaction with UDP-glucose. Compounds abbreviations: ADC, aminodeoxychorismate; DHF, dihydrofolate; DHM, dihydromonapterin; DHN, dihydroneopterin; DHP, diydropteroate; -Glc, glucose ester; -Glu_n, polyglutamate; HMDHP, hydroxymethyldihydropterin; -P, phosphate; -P_2, diphosphate; -P_3, triphosphate; THF, tetrahydrofolate. Enzymes: **1**, GTP cyclohydrolase I; **2**, DHN-P_3 pyrophosphatase; **3**, non-specific phosphatase; **4**, dihydroneopterin aldolase (which mediates the epimerization of DHN to DHM, and aldol cleavage of both); **5**, aminodeoxychorismate synthase; **6**, aminodeoxychorismate lyase; **7**, hydroxymethyldihydropterin pyrophosphokinase; **8**, dihydropteroate synthase; **9**,

dihydropholate synthase; **10**, dihydrofolate reductase; **11**, folypolyglutamate synthase; **12**, p-ABA glucosyltrasnferase (Adapted from Bekaert et al., 2008).

4.2.3 Genetic engineering strategies for enhancing folate content in plants

The biofortification of staple crops with folate through metabolic engineering offers a sustainable alternative to fight folate deficiency, especially for poor populations in rural remote areas. Thus far, metabolic engineering was applied solely through the overexpression of key folate biosynthesis genes, such *GTP cyclohydrolase I (gch1)* and *aminodeoxychorismate synthase (adcs)*. Over the past decade, engineering attempts were reported in Arabidopsis (Blancquaert et al., 2013; Hossain et al., 2004), tomato (Díaz de la Garza et al, 2004; 2007), rice (Storozhenko et al., 2007), lettuce (Nunes et al, 2009), maize (Naqvi et al., 2009), and potato (Blancquaert et al., 2013). These attempts can be divided into two groups: (i) the over-expression of GTP cyclohydrolase I (GTPCHI) (resulting in G lines), the first enzyme in the pterin branch of folate biosynthesis, and (ii) the combined overexpression of GTPCHI and aminodeoxychorismate synthase (ADCS), the first enzyme in the p-ABA branch (resulting in GA or G+A lines, depending on whether both genes were combined on the same T-DNA or separately transformed lines were crossed) (Table 4.3). Plants overexpressing GTPCHI alone exhibited a massive increase in pterin levels (up to 1250-fold) in Arabidopsis (Hossain et al., 2004), which coincided with a 2–8.5-fold increase in folate content (the highest increase being reported in lettuce (Nunes et al., 2009). In these lines, further folate enhancement was hampered due to a depletion of the p-ABA pool (Hossain et al., 2004; Nunes et al., 2009). Therefore, co-expression of GTPCHI and ADCS in tomato fruit (Díaz de la Garza et al., 2007) and rice seeds (Storozhenko et al., 2007) increased folate contents up to 25-fold in tomato and 100-fold in rice. Interestingly, p-ABA and pterin levels in these plants were also elevated compared with their respective wild types, indicating that an additional bottleneck is present against the higher accumulation of tetrahydrofolate. Attempts to biofortify Arabidopsis plants and potato tubers by enhancing both pterin and p-ABA levels did not result in high folate enhancement (Blancquaert et al., 2013). These data suggest that the two-gene strategy cannot be universally applied to biofortify crops with folate and that engineering strategies should be adapted in order to reach this goal. Recently, a further study has been reported in rice by overexpression separately in different rice plants two enzymes which perform the first and further additions of glutamate, dihydrofolate

synthase (DHFS) and folypolyglutamate synthase (FPGS) (**Figure 4.3**) resulting in a slight increase in seed folate content (**Table 4.3**) (Dong et al., 2014). GTPCHI and ADCS were also independently overexpressed in rice, resulting in up to a 6.1-fold and 1.8-fold increase of folate content, respectively (Dong et al., 2014).

Crop	Amount (fold increase over wild type)	Introduced transgenes	Reference
Tomato (*Solanum lycopersicum*)	6.18 μg /g FW total folate (14-fold)	*gch1* and *adcs1*	Waller et al., 2010
	1.02 μg /g FW total folate (2-fold)	*gch1*	Díaz de la Garza et al., 2004
	11.04 μg /g FW total folate (25-fold)	*gch1* and *adcs1*	Díaz de la Garza et al., 2007
Corn (*Zea mays*)	1.94 μg /g DW total folate (2-fold)	*gch1*	Naqvi et al., 2009
Rice (*Oryza sativa*)	16.9 μg /g (100-fold)	*gch1* and *adcs1*	Storozhenko et al., 2007
	1.15 μg /g of total folate (14.5–27.2 % increase)	*dhfs*	Dong et al., 2014
	1.2 μg /g of total folate (7.5 to 19.9 % increase)	*fpgs*	
	5.7 μg /g of total folate (6.1-fold)	*gch1*	
	1.4 μg /g of total folate (1.8-fold)	*adcs1*	
Lettuce (*Lactuca sativa*)	1.89 μg /g FW total folate (5.4-fold)	*gch1*	Nunes et al., 2009
Potato (*Solanum tuberosum*)	1.22 μg /g total folate (3-fold)	*gch1* and *adcs1*	Blancquaert et al., 2013

Table 4.2: Folate enhancement in plant crops by genetic engineering.

Our experimental strategy for the enhancement of folate in maize seeds involves the simultaneous expression of *AtADCS1* and *EcfolE* (equivalent to *gch1*) and also *EcfolE* alone, in maize endosperm. We carried out an in-depth analysis at the transcript and metabolite levels in an attempt to assess accumulation of folate, and its derivatives, in maize endosperm.

4.3 Material and Methods

4.3.1 Cloning and vector construction

The *Escherichia coli folE* gene was cloned from DH5α (non-pathogenic *E. coli* strain for laboratory use) by RT-PCR based on the *folE* sequence (GenBank: X63910) and incorporating appropriate restriction sites. The product was inserted into pGEM®-T (Promega) for sequencing and then transferred to pHor-P containing the barley D-hordein promoter (Sørensen et al., 1996) and the rice ADPGPP terminator.

AtADCS1 cDNA was cloned directly from *A. thaliana* mRNA by reverse transcriptase PCR based on sequence information in GenBank (accession number AT2G28880). The cDNAs was transferred to the pGEM-T easy vector (Promega) and the recombinant vector was digested with *Eco*RI. *AtADCS1* was introduced into vector p326, containing the wheat low molecular weight (LMW) glutenin gene promoter and *nos* terminator.

4.3.2 Plant material

Three different transgenic lines were generated as described in Chapter 2, subsection 2.3.2; they are shown in Table 4.4. Endosperm and aleurone samples were harvest at 40 DAP, frozen in liquid nitrogen and stored at -80ºC prior to use.

Lines	Transgenes
F1	*EcfolE*
F2	*EcfolE*
FA	*EcfolE+AtADCS1*

Table 4.3: Transgenic lines generated through combinatorial transformation.

4.3.3 Total RNA isolation and mRNA analysis

The protocol is described in detail in Chapter 1, section 1.3.2. Primer sequences used for designing probes are shown in Table 4.5.

Transgene	Primer set
EcfolE	Forward: 5´- ATGCCATCACTCAGTAAAGAAGCGG-3´
	Reverse: 5´- TAATCAGTTGTGATGACGCACAGCG-3´
AtADCS1	Forward: 5´-GTGGAATGCCAATTAATCGTCACAA-3´
	Reverse: 5´-CTTTGCTTCAAGCATTCCATTTCTGT-3´

Table 4.4: Primer sequences used for probe design.

4.3.4 Folate extraction and HPLC analysis from maize endosperm

Folates were extracted, purified and determined following the methodology described by Konings (1999), Pfeiffer et al. (1997) and Vahteristo et al. (1996). Briefly, folates contained in one g of sample were extracted using 50mM Ches/Hepes extraction buffer (pH 7.85) containing 2% ascorbic acid and 10mM 2-mercaptoethanol. An aliquot was incubated for 3 hr at 37° C with 1 ml of α-amylase from *Aspergillus oryzae* (Type X-A, Sigma), previously prepared to a concentration of 25 mg/ml in distilled water, and 1 ml of hog kidney conjugase prepared according to Gregory et al. (1984). The samples were then filtered through 0.45 μm pore size and 25 mm Ø nylon disposable filters (Whatman, Florham Park, NJ, USA) and purified in strong anion-exchange (SAX) cartridges connected to a vacuum manifold (Supelco, Bellefonte, PA, USA). The analysis were carried out on a HPLC-MS/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μ-wellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an electrospray (ESI) interface. 40-μl of each sample was injected onto a Supelco Discovery C18 HPLC column (5 μm, 10 × 2.1 mm), thermostatted at 40°C, and eluted at a flow rate of 200 μl/min. Mobile phase A, consisting of water + 0.1% formic acid, and mobile phase B, consisting of acetonitrile + 0.1% formic acid, were used for the chromatographic separation. Different control samples with known concentrations of folates were also run in

the same conditions. These analysis were performed by Dr Rubén Nicolás López at University on Murcia.

4.4 Results

4.4.1 Transgenic plants

Two different transgenic maize genotypes were generated. The first genotype expresses *EcfolE* alone. This gene is involved in pterin biosynthesis. The second genotype co-expresses *EcfolE* and *AtADCS1*. The later gene is involved in the pABA branch of the folate biosynthesis pathway. Two *EcfolE*-expressing plants and one plant co-expressing *EcfolE* and *AtADCS1* were studied in detail. Admittedly only a small number of transgenic plants were recovered and studied.

4.4.2 *EcfolE* and *AtADCS1* expression in maize endosperm

mRNA blot analysis was carried out to confirm expression of the transgenes in each line. The analysis confirmed the accumulation of full-length transcripts of the expected size for each of the transgenes (*EcfolE* = 971bp; *AtADCS1*=3,084bp) as shown in **Figure 4.4**. Transgenic line 3 expressed both genes and lines 1 and 2 express *EcfolE* alone.
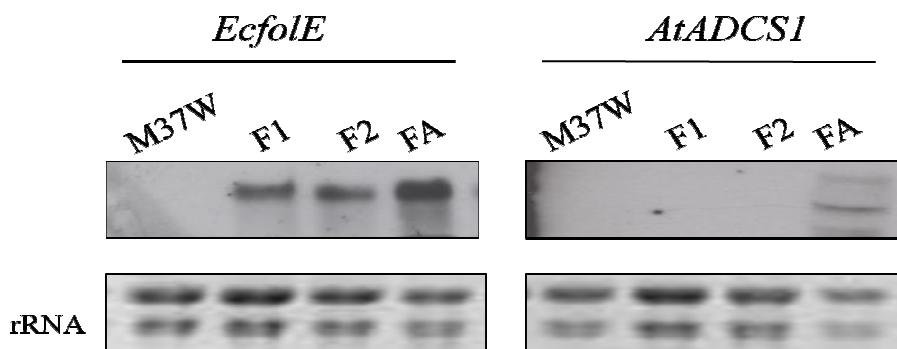

Figure 4.4: mRNA blot analysis showing endosperm-specific transgene expression in the different transgenic lines. Transgenic plants F1 and F2 expressed *EcfolE* and transgenic line FA expressing-expressed *EcfolE* and *AtADCS1*. The loading control was rRNA stained with ethidium bromide.

4.4.3 Pterin, para-aminobenzoate, and folate accumulation in maize endosperm

We analyzed folate, pterin and pABA content in maize endosperm samples in T2 seeds from transgenic lines and M37W wild type as negative control. The samples were analysed at 40 DAP, when seeds started to exhibit a yellow color, and at 60 DAP, in order to investigate accumulation of folate, pterin and pABA during seed maturation. Accumulation of folic acid (FA), dihydrofolate (DHF), tetrahydrofolate (THF), 5-methyl-tetrahydrofolate (5MTHF) and 5-formyl-tetrahydrofolate (5FTHF) is shown in **Figure 4.5**. No significant differences between wild type and transgenic lines were observed for any of the folic acid forms and accumulation decreased during seed maturation. pABA and pterin metabolite accumulation in transgenic lines was estimated by comparing HPLC peak areas of metabolites extracted from the transgenic lines and the M37W (**Figure 4.6**). HPLC peaks in M37W were designated as reference with a value of 100%, since standards were not available for pABA and pterin.
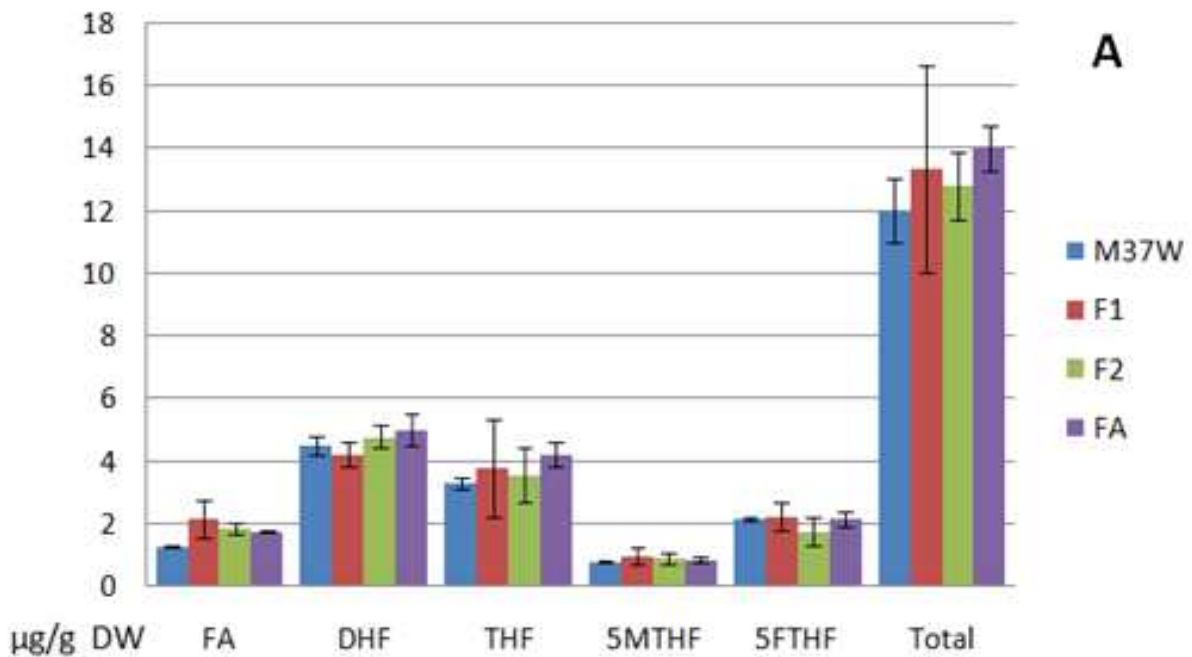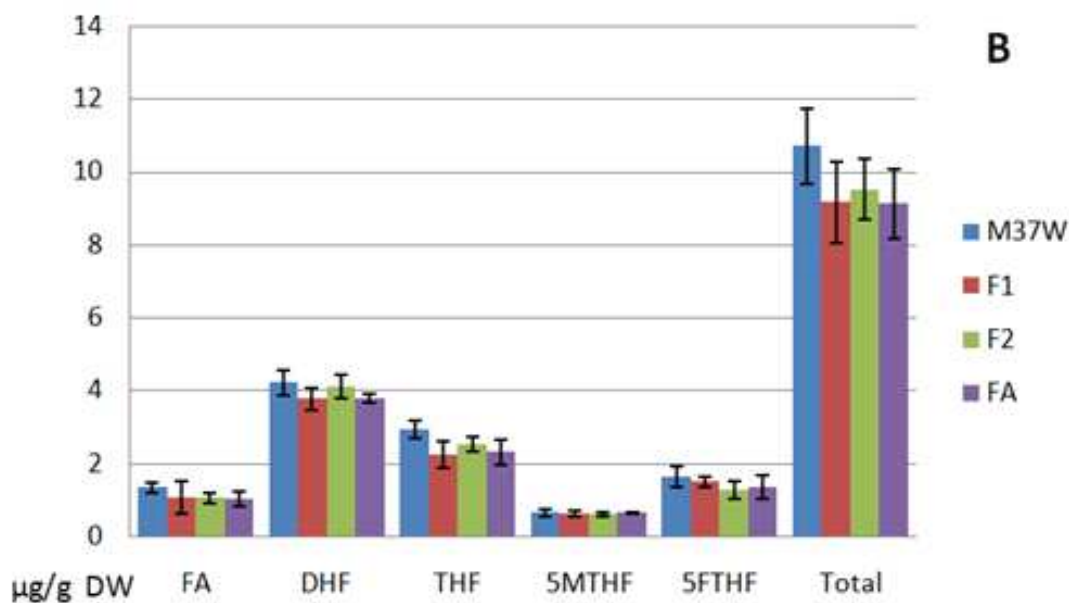
Figure 4.5: Folate content in maize endosperm in M37W, F1, F2 and FA transgenic lines at 40 DAP (A) and at 60 DAP (B). Abbreviations: FA, folic acid; DHF, dihydrofolate; THF, tetrahydrofolate; 5MTHF, 5-methyl-tetrahydrofolate; 5FTHF, 5-formyl-tetrahydrofolate.
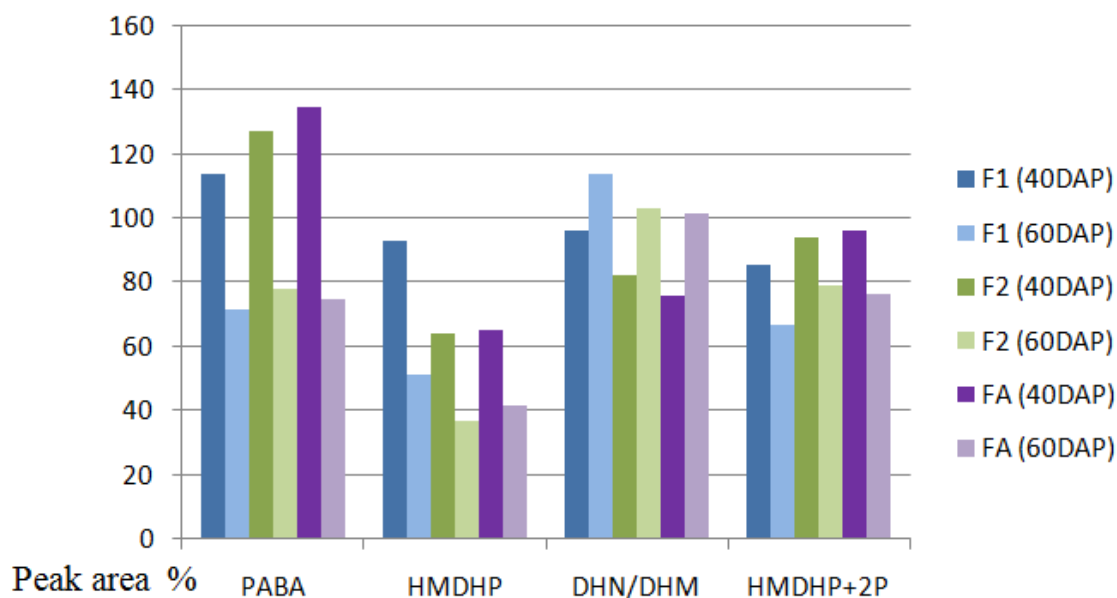
Figure 4.6: pABA and pterin accumulation in maize endosperm. Accumulation of these compounds in F1, F2 and FA lines at 40 and 60 DAP are represented in % of the area in the chromatogram result, taking as reference pABA and pterin accumulation in M37W (100%).

Abbreviations: PABA, p-aminobenzoic acid; HMDHP, 6-hydroxymethyldihydropterin; DHN, dihydroneopterin; DHM, dihydromonapterin; HMDHP+2P, hydroxymethyldihydropterin diphosphate.

4.5 Discussion

4.5.1 Overexpression of *GTP cyclohydrolase* or *GTP cyclohydrolase* together with *aminodeoxychorismate synthase* is not sufficient to enhanced folate levels in maize endosperm

Folates are important cofactors in one-carbon metabolism in all living organisms. Since only plants and microorganisms are capable of synthesizing folates, humans depend entirely on their diet as a folate source. Given the low folate content of several staple crops, folate deficiency affects regions all over the world (Blancquaert et al., 2014). Folate biofortification of staple crops through enhancement of pterin and para-aminobenzoate levels, precursors of the folate biosynthesis pathway, was reported to be successful in tomato and rice (Díaz de la Garza et al., 2004; Storozhenko et al., 2007).

In our folate engineering experiments white maize M37W inbred line was transformed with *E. coli FolE* encoding GTPCHI (lines F1 and F2) alone or in combination with *A. thaliana ADSCS1* (line FA). *EcfolE* had been overexpressed in an earlier study in the same genetic background where folate content was doubled (Naqvi et al., 2009). However, in the current study no significant differences were measured in folate accumulation between transgenic lines and wild type (**Figure 4.5**). The low number of transgenic lines I recovered does not permit a correlation between overexpressed transgenes and folate levels. More transgenic maize lines expressing both genes are needed in order to draw meaningful conclusions. In previous studies several lines were regenerated expressing GTPCHI alone or together with ADCS1 in potato and *Arabidopsis*, where even though both pterin and *p*-ABA levels were increased, this did not result in folate enhancement (Blancquaert et al., 2013). These data suggest that the two-gene strategy cannot be universally applied to biofortify crops with folate and that engineering strategies should be adapted in order to reach this goal. Although the new bottleneck in the flux toward folate enhancement has not been identified, several possibilities have been suggested in the literature, such as restricted import of pterin and *p*-ABA to mitochondria; limitation in activity of one or more tetrahydrofolate

biosynthetic enzymes in mitochondria; or a regulation of the enzyme activity implicated in these two branches (Blancquaert et al., 2013, 2014). Another important issue with respect to folate biofortification is folate stability, since obtaining high levels of this vitamin would be meaningless if they drop to basal levels upon food storage and processing. Indeed, folates are unstable compounds, susceptible to oxidative and photo-oxidative catabolism (Scott et al., 2000).

In the current experiments two different time points in seed development were analysed for folate content. Total amount of folate decreased over time in the wild type endosperm and also in the transgenic lines **Figure 4.7**. These data suggest a loss of total folate during seed maturation.
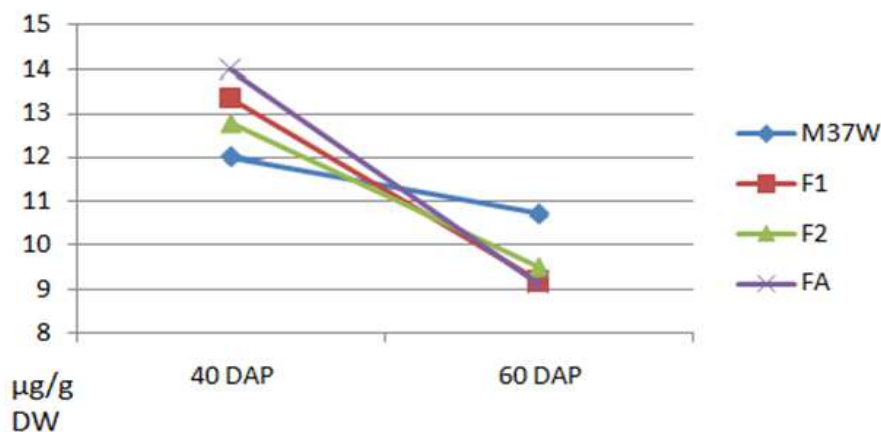
Figure 4.7: Total folate content in maize endosperm in three transgenic lines and a wild type control at two different seed maturity stages (40 DAP and 60DAP).

A number of approaches have been suggested to improve folate stability (Blancquaert et al., 2010): (i) engineering of a more stable compound; (ii) simultaneous accumulation of compounds with a protective mode of action (e.g. anti-oxidants such as ascorbate); (iii) engineering salvage and breakdown reactions; and (iv) association with folate binding proteins. In folate biofortified rice, 5-methyl THF is the most abundant folate form (Storozhenko et al., 2007) and at the same time it is the most stable naturally occurring folate form. However, in M37W the most accumulated folate form was dihydrofolate, and it also exhibits the same amount of dihydrofolate stability during seed maturity (**Figure 4.5,** Results section).

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4.5.2 Modulation in *p*-ABA and pterin metabolite accumulation reveals bottlenecks in folate biosynthesis in maize endosperm

In the current experiments *p*-ABA and pterin accumulation in maize endosperm showed different amounts in A.2, A.4 and FA lines compared to wild type (**Figure 4.6**). However, the accumulation profiles of these metabolites for both lines overexpressing GTPCHI alone (A.2 and A.4) did not differ from the line co-expressing GTPCHI and ADCS1 (FA). At the same time, two different seed development time points were analysed (40 and 60 DAP). Seeds at these two time points exhibited different metabolite accumulation profiles. *EcfolE* transcripts accumulated in all three transgenic lines (F1, F2 and FA), which accumulate dihydroneopterin triphosphate (DHN-P$_3$) (**Figure 4.3,** Results section). DHN-P$_3$ and DHN-P determinations were not carried out because of lack of standards, but the subsequent metabolites in the pathway, DHN/DHM were analysed. These two metabolites accumulated at lower levels in the transgenic lines compared to wild type at 40 DAP. DHN/DHM accumulation increased up to wild type levels at 60 DAP. Interestingly, the next metabolite in the pathway HMDHP showed significant differences with respect to the wild type. HMDHP levels decreased in transgenic lines to ca: 40% at 40DAP and ca: 60% at 60 DAP.

In order to obtain a better understanding of HMDHP accumulation differences in F1, F2, FA and wild type, HMDHP-P$_2$ (next metabolite in the pathway) was analysed, but no big differences were measured compared to wild type. Previous studies in *Arabidopsis* and potato suggested that the transport of pterin to mitochondria is restricted, generating a bottleneck (Blancquaert et al., 2013). In the current study HMDHP was decreased in transgenic maize lines suggesting that HMDHP was metabolized. However, HMDHP-P$_2$ and different folate forms did not increase their accumulation significantly (**Figure 4.5** and **4.6**). Also a cytosololic 7,8-dihydropteroate synthase (DHPS) was described, which converts HMDHP-P$_2$ into dihydropteroate (DHP) (Navarrete et al., 2012). The next steps of this cytosolic independent pathway are still unknown (**Figure 4.7**).
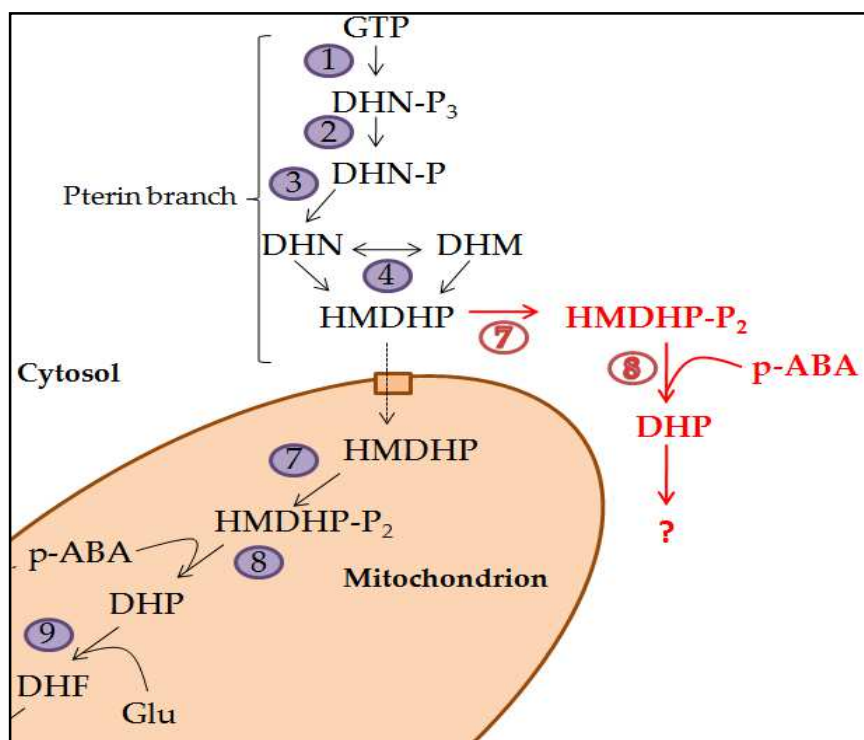
Figure 4.8: Pterin biosynthetic pathway towards folate biosynthesis with cytosolic hydroxymethyldihydropterin pyrophosphokinase (HPPK) (**7**) and cytosololic 7,8-dihydropteroate synthase (DHPS) (8) (in **red color**). Abbreviations: DHF, dihydrofolate; DHM, dihydromonapterin; DHN, dihydroneopterin; DHP, diydropteroate; -Glu, glutamate; HMDHP, hydroxymethyldihydropterin. Enzymes: **1**, GTP cyclohydrolase I; **2**, DHN-P_3 pyrophosphatase; **3**, non-specific phosphatase; **4**, dihydroneopterin aldolase (which mediates the epimerization of DHN to DHM, and aldol cleavage of both); **5**, aminodeoxychorismate synthase; **6**, aminodeoxychorismate lyase; **7**, hydroxymethyldihydropterin pyrophosphokinase; **8**, dihydropteroate synthase; **9**, dihydropholate synthase; (Adapted from Bekaert et al., 2008 and Navarrete et al., 2012).

Significant variability in *p*-ABA accumulation was measured in F1, F2 and FA compared to wild type (**Figure 4.6**). In the transgenic lines *p*-ABA levels increased by ca: 30% at 40 DAP with respect to wild type. *p*-ABA levels decreased at 60 DAP by ca: 30% compared to wild type. ADCS1 only expressed in FA line. However, no differences were measured between FA and the other two lines (F1 and F2 which only expressed *EcfolE*) in pABA and pterin accumulation, suggesting that differences in the accumulation of these metabolites in FA, F1 and F2 compared to wild type was only due to *EcfolE* expression. Results suggested that *p*-ABA accumulation was modulated by endogenous regulatory mechanisms. Different

studies have been carried out in order to unravel the regulation of folate biosynthesis in plants. These studies, however, did not identify regulatory bottlenecks which limit folate biosynthesis (Hanson & Gregory, 2011).

4.6 Conclusions

In the current study I demonstrated that modulation in *p*-ABA and pterin accumulation alone is not sufficient to enhance folate biosynthesis in maize endosperm through overexpression of *GTP cyclohydrolase* alone or in combination with *aminodeoxychorismate synthase* in the regenerated lines F1/2 and FA. However, due to the limited amount of lines I cannot reach a conclusion in general terms.

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GENERAL CONCLUSIONS

1. Multigene engineering was used to generate different transgenic maize lines with altered carotenoid or vitamin E profiles. I demonstrated that this strategy is useful to generate maize lines for in depth molecular and biochemical investigations in the seeds.

2. Introgressing a carotenogenic mini-pathway into a range of maize inbred lines with different carotenoid content and composition is an effective strategy to generate maize hybrids with high and diverse carotenoid content. Introgression of this mini-pathway allows the development of a better understanding of molecular factors influencing the flow of precursors into the two different branches of the carotenoid pathway.

3. I identified a number of bottlenecks that limit the production of lutein and zeaxanthin in maize endosperm by analyzing the above hybrids at the metabolite and mRNA level. Phytoene desaturation and isomerization towards lycopene accumulation influenced directly total carotenoid content in the endosperm.

4. Simultaneous reconstruction of the carotenoid and vitamin E biosynthetic pathways in maize hybrids resulted in a significant increase in α-tocopherol accumulation and a moderate decrease in carotenoid accumulation in maize seeds, demonstrating an interaction between these two pathways.

5. Endosperm-specific carotenoid biosynthesis and constitutive tocopherol biosynthesis influenced core metabolic processes in maize embryo and endosperm. This in turn resulted in the accumulation of zeaxanthin and α-tocopherol, respectively as the predominant metabolites in the two pathways, in the maize embryo. α-Tocopherol was not detected in the endosperm, but total carotenoid decreased up to a ca: 28%, mostly because of the reduction in the levels of zeaxanthin and phytoene.

6. Introgression of the ketocarotenoid biosynthetic pathway into maize lines with different carotenogenic backgrounds revealed bottlenecks in astaxanthin accumulation. Choice of an appropriate genetic background with a partial carotenoid pathway influences significantly carotenoid conversion to downstream molecules such as astaxanthin.

7. Co-expression of β-carotene ketolases and a β-carotene hydroxylase in maize endosperm resulted in the efficient conversion of carotenoids to astaxanthin, in the endosperm. A yellow endosperm genetic background was preferred for the total

conversion of carotenoid precursors to ketocarotenoids, in hybrids with the transgenic line expressing the ketolase and hydroxylase genes.

8. I recovered and analyzed maize lines with a modulated accumulation of pABA and pterin, precursors in folate biosynthesis. The levels of these precursors in the transgenic lines I generated were not sufficient to enhance folate content in maize. However, due to the limited amount of transgenic lines I cannot reach a conclusion in general terms.