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Claudia Stange *Editor*

Carotenoids in Nature

Biosynthesis, Regulation and Function

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Claudia Stange

Carotenoids in Nature

Biosynthesis, Regulation and Function

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Preface

Carotenoids are colored pigments widespread distributed in nature. These lipophilic molecules are synthesized in plants, algae, and some yeast and bacteria. In plants, carotenoids are synthesized in photosynthetic organs as well as in fruits, flowers, seeds, and reserve roots, providing attractive yellow, orange, and red colors. In plants and algae, carotenoids have important functional roles in photosynthesis, photomorphogenesis, and in photoprotection. They also give rise to apocarotenoids, such as the hormones abscisic acid and strigolactones, among other volatile terpenes. Additionally, they possess antioxidant properties acting as reactive oxygen species scavengers. In mammals, they act as provitamin A precursors and as powerful antioxidant molecules involved in the prevention of certain types of diseases. Carotenoid biosynthesis in plants is highly regulated, although all the processes involved are not completely known. During the past decades, huge knowledge has been published, and almost all carotenogenic genes have been identified and those functions dissected as a result of molecular, genetic, and biochemical approaches utilizing different plant, yeast, and algae model systems. The information has been used in genetic engineering for increasing abiotic stress tolerance, altering color and the nutritional value in plants, leading to the production of novel functional foods. In this book, an extensive and actual review of the main topics of carotenoid biosynthesis, regulation, and function in human health are brought together.

The first chapters provide an introduction to carotenoid biosynthesis in yeast, bacteria, and plants and a profound exposition on the structures of carotenoid molecules. The second part covers the function and regulations of carotenoids in photosynthesis as well as during plant, fruit, storage root, and alga development. We also included chapters that present an actual overview on plastids – accumulating carotenoids – on the epigenetic mechanisms that control carotenoid biosynthesis and on the oncoming topic regarding apocarotenoids. To finish, some chapters argue about the metabolic engineering of carotenoids in plants and seeds that point the way of carotenoid biotechnological application. Additionally, important topics on the effect of absorption mechanisms and carotenoids as antioxidants and vitamin A precursors for human nutrition were also included.

The chapters included in each section were prepared and reviewed by experts in the field. I would like to thank each of the authors for accepting to contribute to this book, for their dedicated effort providing carefully prepared manuscripts, and for their prompt attention to answer to requested information.

I wish to express my gratitude to the staff of Springer, in particular to Thijs van Vlijmen (Senior Publishing Editor) and to Sara Germans-Huisman (Springer Senior Editorial Assistant) for their kind assistance and patience.

It is the aim of the editor that this book will be of benefit and reference source to anyone researching the area on carotenoid synthesis and regulation.

Santiago, Chile Claudia Stange

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Part I
Biosynthesis of Carotenoids

Chapter 1
Carotenoid Distribution in Nature

Jennifer Alcaíno, Marcelo Baeza, and Víctor Cifuentes

Abstract Carotenoids are naturally occurring red, orange and yellow pigments that are synthesized by plants and some microorganisms and fulfill many important physiological functions. This chapter describes the distribution of carotenoid in microorganisms, including bacteria, archaea, microalgae, filamentous fungi and yeasts. We will also focus on their functional aspects and applications, such as their nutritional value, their benefits for human and animal health and their potential protection against free radicals. The central metabolic pathway leading to the synthesis of carotenoids is described as the three following principal steps: (i) the synthesis of isopentenyl pyrophosphate and the formation of dimethylallyl pyrophosphate, (ii) the synthesis of geranylgeranyl pyrophosphate and (iii) the synthesis of carotenoids *per se*, highlighting the differences that have been found in several carotenogenic organisms and providing an evolutionary perspective. Finally, as an example, the synthesis of the xanthophyll astaxanthin is discussed.

Keywords Carotenogenesis • Microbial carotenoids • Astaxanthin

1.1 Introduction

Carotenoids are red, orange and yellow natural pigments that are synthesized by plants and some microorganisms fulfilling important physiological functions. For example, in photosynthetic organisms, carotenoids are essential for photosynthesis and photoprotection, whereas in non-photosynthetic organisms; they participate in alleviating photooxidative damage. Considering their properties, carotenoids have various industrial applications as dyes, and due to their various beneficial effects for health, they have been exploited by the food and nutraceutical industries and recently, by the pharmacological industry, with an estimated annual production greater than 100 million tons (Fraser and Bramley 2004). For these reasons, there

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has been an increasing interest in finding new sources of carotenoids and optimizing the existing production systems.

Carotenoids are a large class of pigments of which more than 750 different chemical structures have been determined to date (Takaichi 2011). Carotenoids normally contain a hydrocarbon backbone of 40 carbon atoms, consisting of 8 isoprene units. The great diversity of these pigments generally derives from an acyclic $C_{40}H_{56}$ structure that has a long central light-absorbing conjugated polyene compound constituting the chromophoric system, which absorbs light from part of the visible region of the electromagnetic spectrum (400–500 nm). This central structure may have additional chemical modifications, such as variations in the level of desaturation, cyclization of one or both ends and the addition of oxygen-containing functional groups, including hydroxy, epoxy, and/or oxo groups (Britton 1995). According to the last type of modification, carotenoids are classified as carotenes or xanthophylls, with only the latter containing oxygen-containing functional groups (Bhosale and Bernstein 2005). The oxygen-containing functional groups affect the solubility of carotenoids, making xanthophylls more polar than carotenes, thus allowing their separation using many types of chromatography.

In plants, algae and phototrophic bacteria, carotenoids (mainly xanthophylls) are located in specific pigment-protein complexes, serving as accessory pigments to harvest light for photosynthesis and constituting the basic structural units of the photosynthetic apparatus. In this regard, the contribution of carotenoids to harvesting light is even greater in organisms that live in environments that have less available light (Britton 2008b). In addition to light harvesting, carotenoids also act as photoprotectors by quenching the energy excess under high-light stress and preventing the formation of the highly reactive singlet oxygen, which has damaging and potentially lethal effects. Additionally, the carotenoids of plants also play a vital ecological role, which is important for the survival and propagation of the species, because they contribute to the color of fruits and flowers, which is an important signal to attract insects and animals for pollination and seed dispersal (Lu and Li 2008).

In contrast, animals (with the exception of some aphids that naturally produce the carotenoid torulene (Moran and Jarvik 2010)) are unable to synthesize these important nutritional molecules *de novo* and thus rely on their diet to supply them (Goodwin 1984). In animals, these pigments accumulate in certain tissues, giving characteristic colors to many birds, marine invertebrates and fishes. Additionally, among marine invertebrates, it is common for carotenoids to form complexes with proteins (carotenoproteins), which extends the color range of carotenoids to green, purple and blue. A typical case is the color change from a bluish hue to red when lobsters are cooked. Under this condition, the color of the free xanthophyll (which is astaxanthin) is revealed as it is liberated from the carotenoid-protein complex during the denaturation of the carotenoprotein complex (Schiedt 1998). Moreover, carotenoids have essential functions and provide many important health benefits. For example, β-carotene, also known as pro-vitamin A, is the main source of vitamin A, a deficiency of which is one of the most damaging micronutrient deficiencies in the world. Vitamin A deficiency can lead to blindness, decreased immune function

and even death, affecting approximately 250 million preschool children according to the World Health Organization (World Health Organization 2014). It is not only vitamin A that is essential for ocular health, other carotenoids are also important. This is the case for the xanthophylls zeaxanthin and lutein, which are the pigmented components of the macula, a yellow spot at the center of the human retina. These xanthophylls provide protection against age-related macular degeneration, which can also cause blindness (Abdel-Aal et al. 2013). In addition, given the antioxidant properties of carotenoids, a higher ingestion of carotenoids is related to a lower risk of chronic diseases, including cardiovascular diseases, cataract development and some types of cancer. Among the carotenoids, the xanthophyll astaxanthin stands out due to its strong antioxidant properties, which have been proven to be stronger than those of other recognized antioxidants, such as β-carotene or even α-tocopherol (Miki 1991).

Carotenoids are also important membrane components because they are lipid-soluble pigments that are generally located deep within the hydrophobic lipid core and are oriented parallel to the membrane surface (in the case of nonpolar carotenoids such as β-carotene) or span the membrane bilayer (in the case of polar carotenoids such as astaxanthin) (Britton 2008b). In this last case, the oxygen-containing functional groups on the ionone-rings contact the polar head of the membrane phospholipids, whereas the chromophore is located within the hydrophobic core of the membrane. Thus, in addition to providing photoprotection to prevent membrane damage, carotenoids also have effects on the structure and dynamics of membranes. For example, polar carotenoids restrict the molecular motion of lipids and increase the rigidity of the membrane in its liquid crystalline state; thus, they modulate the membrane fluidity (Gruszecki and Strzalka 2005). Moreover, the electron-rich polyene chain of carotenoids is susceptible to enzymatic or non-enzymatic oxidative cleavage and the generated products, known as apocarotenoids, also have important functions. Indeed, vitamin A is an example of an apocarotenoid because it is the product of the symmetrical oxidative cleavage of β-carotene (Britton 2008a). In plants, apocarotenoids are involved in development, serve as antifungals and contribute to the flavor and aroma of fruits and flowers (Lu and Li 2008). Among the plant apocarotenoids, abscisic acid is a well-known phytohormone that is involved in a wide range of biological processes, including plant development and growth, integrating various stress signals and regulating stress responses (Tuteja 2007).

1.2 Carotenoid Distribution in Microorganisms

Microorganisms that produce and accumulate carotenoid pigments are widely found in the three domains of life. As in plants and animals, it is proposed that pigments play important physiological roles in microbial cells, mainly those associated with the stress response. Nevertheless, studies of the cellular functions of pigments in microbes are scarce, mainly because the objectives of the research

in this field are focused on the use of microorganisms as sources of economically important pigments. Our knowledge of the effects of culture conditions on microbial pigment production comes mainly from studies related to improving pigment productivity under controlled conditions, because microorganisms in their natural environments generally provide insufficient yields for commercialization. Several criteria for industrial carotenoid production by microbes have been evaluated, including the ability to use a wide range of (preferably inexpensive) carbon and nitrogen sources, tolerance to pH, temperature requirement, necessary mineral concentration, tolerated oxygenation level and required light conditions. The interest in discovering new microorganisms that produce a particular pigment or that produce undescribed pigments with novel characteristics applicable in commercial areas has increased significantly during the last few decades. Below are several examples of microorganisms that produce carotenoid pigments, with an emphasis on the novel compounds, and some examples of the cellular functions of carotenoids are reviewed.

1.2.1 Carotenoids in Bacteria, Microalgae and Archaea

Astaxanthin is a red-orange carotenoid that is one of the most commercially important carotenoids. The global market for astaxanthin is the third largest of the total global market for carotenoids (after the markets for lutein and β-carotene, which hold the second and first place, respectively), which in 2010 was estimated at nearly US$225 million and is expected to exceed US$250 million by 2018 (BCC-Research 2011). Astaxanthin is best known for its use in aquaculture for salmon-flesh pigmentation, which has a considerable economic impact on this industry. This xanthophyll is also used in chicken feed to enhance the pigmentation of the yolk and flesh. Furthermore, astaxanthin has strong antioxidant properties and there is growing interest in this compound due to recent findings of its beneficial roles in degenerative diseases and other potential benefits for human health (Wang et al. 2000; Higuera-Ciapara et al. 2006; Park et al. 2010; Yasui et al. 2011). This interest has led to a considerable increase in the effort to find new sources of this ketocarotenoid; however, there are few reports of microbial astaxanthin production to date.

One of the most promising biosources of astaxanthin is the microalgae *Haematococcus pluvialis*, which has been reviewed elsewhere (Guerin et al. 2003; Lemoine and Schoefs 2010). In recent studies, it was found that the microalgal species *Dunaliella salina*, *Tetraselmis suecica*, *Isochrysis galbana* and *Pavlova salina*, which were isolated from coastal or brackish water in Australia, produced 4.7–6.9 mg/g dry weight of carotenoids. In the same report, it was stated that *T. suecica* and *Nannochloropsis* sp. BR2 produced astaxanthin, which represented 39 % (2,261 mg/g dry weight) and 16 % (321 mg/g dry weight) of the total carotenoids, respectively (Ahmed et al. 2014). The production of carotenoids by the microalgal specie *Chlorella protothecoides* is induced under nutritional, luminosity

and salinity stresses, as demonstrated by the shift in coloration from green to orange-red. However, a minor amount of free astaxanthin is produced, with 7 % of the total carotenoid content (0.8 % w/w) being canthaxanthin (23 %) and echinenone (15 %), the main carotenoids produced (Campenni' et al. 2013). In the case of the mixotrophic microalgae *Chromochloris zofingiensis*, an increase in astaxanthin production was observed as a response to increased concentrations of iron in the culture media. The production of astaxanthin was doubled at 0.2 mM Fe^{2+}, an effect that was not observed when different concentrations of Mn^{2+} and Mg^{2+} were tested (Wang et al. 2013). Regarding bacteria, two species belonging to the *Paracoccus* genus have been reported to produce astaxanthin, including *P. haeundaensis* sp. nov., isolated from the Haeundae Coast of Korea (Lee et al. 2004), and *P. carotinifacien*, which was reported to be effective for coloring the flesh of Coho salmon, Atlantic salmon and rainbow trout (Bories et al. 2007). A flagellated Gram-negative strictly aerobic bacterium that developed reddish-orange colonies on marine agar was isolated from coastal surface seawater near the Taichung harbor of Taiwan, which based on molecular and chemotaxonomic characterization, was proposed to be a novel species within the *Sphingomicrobium* genus. The isolate was named *Sphingomicrobium astaxanthinifaciens* sp. nov. and the type strain CC-AMO-30BT synthesized approximately 40 mg/g dry weight of astaxanthin (Shahina et al. 2013). In addition, some marine bacterial strains produce astaxanthin derivatives such as (3S,3′S)-astaxanthin-beta-D-glucoside and (3S,3′R)-adonixanthin-beta-D-glucoside, which are produced by *Paracoccus* sp. (formerly *Agrobacterium aurantiacum*) (Yokoyama et al. 1995) and 2-hydroxyastaxanthin, which is synthesized by *Brevundimonas* sp. (Yokoyama et al. 1996).

It has long been known that prokaryotic microorganisms produce other carotenoid pigments; at present, it is estimated that 180 prokaryotic microorganisms produce potential carotenoids (Prokaryotic Carotenoid DataBase 2014). A method for the isolation and purification of the purple pigment spirilloxanthin from *Spirillum rubrum* Esmarch was published in 1935; later the name of bacterium was changed to *Rhodospirillum rubrum*, and in 1958, the spirilloxanthin synthesis pathway was described (Jensen et al. 1958). In studies of spheroidenone and spirilloxanthin in sediments from Little Round Lake, Ontario Canada, it was proposed that *Rhodopseudomonas* bacterial species were responsible for these *fossil pigments* (Brown 1968). Currently, the phototrophic purple non-sulfur bacterium *R. rubrum* is known to produce as many as eighteen different carotenoids (including phytoene, lycopene and neurosporene), isoprenoid-quinones, bacteriopheophytin and different phosphatidylglycerol species during microaerophilic growth (Bona-Lovasz et al. 2013). The xanthophyll zeaxanthin is commonly found as the major carotenoid produced by several members of the *Flavobacteriaceae* family, such as *Zeaxanthinibacter enoshimensis* (Asker et al. 2007a), *Flavobacterium multivorum* (Bhosale and Bernstein 2004), *Muricauda lutaonensis* (Hameed et al. 2011) and *Mesoflavibacter zeaxanthinifaciens* (Asker et al. 2007b). Two probable novel species of the genus *Muricauda* isolated from sandy beaches of the southwestern coast of India accumulated high amounts of zeaxanthin (1.2–1.5 mg/g) in media supplemented with glutamic acid, and when compared to commercial zeaxanthin,

the bacterial zeaxanthin showed higher *in vitro* antioxidant activity (Prabhu et al. 2013). Zeaxanthin is also the main carotenoid produced by a strain of the microalga *Chlorella saccharophila* isolated from the marine waters of New Zealand. The yield of zeaxanthin reached 11 mg/g when the microalga was cultured in media containing glycerol as the carbon source and this organism also produced 5 mg/g of β-carotene (Singh et al. 2013). Another example of a microorganism that produces several carotenoid derivatives is the thermophilic bacterium *Thermus filiformis*, which produces zeaxanthin and zeaxanthin derivatives, including zeaxanthin monoglucoside, thermozeaxanthins and thermobiszeaxanthins (Mandelli et al. 2012).

β-carotene is another carotenoid with commercial appeal because it is used as a food colorant and by the pharmaceutical and cosmetic industries. There are few reports of natural β-carotene-producing bacteria; two examples of such organisms are the carotenoid-accumulating *Sphingomonas* sp. that produce 1.7 mg/g dry weight of total carotenoids (29 % β-carotene and 36 % nostoxanthin) (Silva et al. 2004) and *Serratia marcescens*, which produces 2.5 μg/ml of β-carotene (Wang et al. 2012). The extremely halophilic archaeon *Halorubrum* sp., which was isolated from Urmia Lake, produces 11,280 μg/l of total carotenoids, with bacterioruberin being the most predominant compound, followed by lycopene and β-carotene (Naziri et al. 2014). A promising natural source of β-carotene is the green unicellular algae *Dunaliella salina*, in which β-carotene can comprise up to 10 % of its dry weight under stressful conditions, such as high salinity, nutrient deprivation, extreme temperatures and high light intensity (Lamers et al. 2010, 2012; Wichuk et al. 2014). Other microalgae that produce β-carotene are *Eustigmatos magnus, Eustigmatos polyphem, Eustigmatos vischeri, Vischeria helvetica, Vischeria punctata* and *Vischeria stellate*. All of these eustigmatophytes produce β-carotene as more than 50 % of their total carotenoids when cultured in a bubble-column photobioreactor, with *V. stellata* being the major producer of β-carotene, which can account for up to 5.9 % of its dry weight (Li et al. 2012).

Many groups have sought novel bioactive molecules with antioxidant, anticancer, antiproliferative and apoptosis-inducing properties. In this regard, a halophilic archaea that forms red-orange colonies, most likely *Halobacterium halobium*, was isolated from the brine of a local crystallizer pond of a solar saltern in Tunisia. Its carotenoid content was as high as 7.6 mg/l, consisting mainly of a bacterioruberin-like carotenoid, and the carotenoid extract exhibited significant antiproliferative activity against HepG2 human cancer cell lines (Abbes et al. 2013). Shindo and Misawa (2014) evaluated a collection of rare and novel marine bacteria that produce orange or red pigments as sources of new carotenoids. They reported the production of diapolycopenedioic acid xylosylesters A–C by *Rubritalea squalenifaciens*, methyl 5-glucosyl-5,6-dihydro-apo-4,4′-lycopenoate by *Planococcus maritimus* and (3R)-saproxanthin and (3R,2'S)-myxol, which had strong antioxidant properties, by novel species of the family *Flavobacteriaceae* (Shindo and Misawa 2014). Based on assays of lipid peroxidation in rat brain homogenates, the rare carotenoid saproxanthin showed high antioxidant activity, which was even stronger than those of β-carotene and zeaxanthin (Shindo et al. 2007). A

yellow-orange bacterium that produces the saproxanthin derivative (3R, 2'S)-2'-isopentenylsaproxanthin was isolated from a seaweed collected in Nabeta Bay in Shizuoka, Japan. The carotenoid production rate of this bacterium, most likely *Jejuia pallidilutea* according to 16S rDNA analysis, was high in an alkaline medium (pH 9.2), reaching 1.1 mg/g of cell dry weight or 1.2 μg/ml (Takatani et al. 2014). Another ketocarotenoid with a strong ability to scavenge reactive oxygen species is deinoxanthin, which is the main pigment produced by species of the *Deinococcus* genus. This xanthophyll may contribute to the high resistance to gamma and UV radiation shown by members of this genus, because *D. radiodurans* was isolated from irradiated canned meat and was classified as the second most radiation-resistant organism after *Thermococcus gammaloteran* (Lemee et al. 1997). The genome of a *Deinococcus xibeiensis* strain isolated from radiation-contaminated soils was assembled and several carotenogenic genes were found. These findings will facilitate the understanding of the biosynthetic pathway of deinoxanthin and the eventual identification of key genes as modification targets to increase the pigment yield (Hu et al. 2013). Another pigment identified as a main factor that protects cells against the lethal effects of oxidative DNA-damaging agents is the red pigment bacterioruberin, found in *Halobacterium salinarium* (Shahmohammadi et al. 1998). Bacterioruberin produced by the extremely halophilic archaeon *Haloarcula japonica* showed much higher antioxidant capacity than that of β-carotene (Yatsunami et al. 2014). The halobacterium *Haloferax mediterranei* requires 20–25 % NaCl for optimal growth and lyses at salt concentrations below 10 %, and it increased the production of carotenoids, including bacterioruberin, in response to stress caused by a low concentration of NaCl (<20 %) (D'Souza et al. 1997). Bacterial survival in low-temperature environments is also enhanced by the presence of bacterioruberin, which plays important roles in regulating membrane fluidity, which affects the water-barrier property of membranes and the permeability of oxygen and other molecules (Fong et al. 2001).

From our anthropocentric point of view, the production of carotenoids by microorganisms is an adaptive response to certain environmental and stressor conditions. In this regard, the production of carotenoids in microbial strains isolated from Antarctic environments would be pivotal for resistance to freeze-thaw cycles and solar radiation. For example, carotenoid-pigmented bacterial strains showed higher survival rates compared with those of non-pigmented isolates when exposed to solar radiation, 61 % versus 0.01 % (Dieser et al. 2010). Additionally, the response to solar radiation has been exploited for the adaptive laboratory-based evolution of species of the microalga *Dunaliella* to select economically valuable traits; in *D. salina*, an enhancement of lutein and β-carotene accumulation was achieved in the presence of blue light (Wichuk et al. 2014).

Unlike most eukaryotes and bacteria, the response of the archaea *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* to DNA-damaging agents in nature do not involve the induction of DNA-repair proteins. Instead, the up-regulated expression of β-carotene biosynthetic enzymes, which have UV-protective properties, was observed (Gotz et al. 2007). In addition, carotenoids play an important role in bacterial aggregation and biofilm formation because a correlation between the

accumulation of carotenoids and aggregation/biofilm formation in *Rhodococcus* sp. SD-74 was recently described (Zheng et al. 2013). This analysis was performed using label-free Raman microspectroscopy, a noninvasive technique that allows for measurement of the intracellular concentration of carotenoids. It has been proposed that using carotenoids as biomarkers in conjunction with resonance Raman spectroscopy will facilitate the exploration for life beyond earth (Marshall et al. 2007).

1.2.2 Carotenoids in Fungi

Although fungi (filamentous fungi and yeasts) are a broad group of organisms that are widely distributed in nature, it is estimated that few species synthesize carotenoids mainly because, in contrast to plants, they are non-photosynthetic organisms. Fungi produce a large variety of carotenoids; however, the most predominant of these are carotenes, and xanthophylls are produced to a lesser extent. In the 1970s, a possible taxonomic relationship between fungi and carotenoids was proposed, suggesting that some of these pigments could be used as good taxonomic markers (Valadon 1976). In this regard, the presence of carotenoids and balistoconidias in basidiomycetes was used as a criterion to classify species in a particular genus. For this purpose, the carotenoid production was related to the 18S rRNA molecular-marker data, but the results of these studies did not support the phylogenetic relationship among basidiomycetous yeasts, indicating that carotenoids were a less significant taxonomic criterion than was originally believed (Nakase et al. 1993).

With respect to carotenes, in 1972, Goodwin reported that the most abundant carotenoid in nature, β-carotene, was present in a significant number of ascomycete fungi, among them *Calycella citrina*, *Heliotum citrinus*, *Mitrula paludosa*, *Spathularia flavida* and others from the Helotiales order and *Aleuria rhenana*, *Leucoscypha rutilans*, *Pithya vulgaris*, *Pulvinula constellatio*, *Sowerbyella radiculata* and others from the Pezizales order (Goodwin 1972). Other ascomycetes, such as *Protomyces pachydermus* and *P. inouyei*, produce β- and γ-carotene, as well as lycopene (Valadon 1964). Moreover, these carotenoids are also distributed among the basidiomycetes, such as *Ustilago scabiosae*, *Gymnosporangium juniperi-virginianae*, *Calocera viscosa*, *Dacrymyces ellisii*, *Cantharellus cibarius*, *Phyllotopsis nidulans*, *Cryptococcus flavescens*, *C. laurentii*, *C. luteolus*, *Rhodotorula aurea*, *R. flava* and *R. penaus*. Among the zygomycetes, specifically those in the Mucorales order, which comprises approximately 205 species, three species stand out for their ability to produce significant amounts of β-carotene as the major pigment, including *Phycomyces blakesleeanus*, *Mucor circinelloides* and *Blakeslea trispora*. Carotenogenesis in these organisms has been extensively studied due to its biological functions and potential biotechnological applications in the industrial production of natural β-carotene (Tisch and Schmoll 2010; Avalos and Limón 2014). Other β-carotene-producing species within the Mucorales order have been

reported, including *Mucor flavus*, *M. hiemalis*, *Choanephora circinans*, *Mortierella remanniana*, *Pilaria anomala* and *Pilobolus crystallinus*, among others.

The production of γ-carotene has been identified in a variety of fungi, such as the zygomycetes *Cladochytrium replicatum*, *Rhizophlyctis rosea*, *Allomyces arbuscula*, *A. macrogynus* and *A. moniliformis*, among others (Goodwin 1972) and the ascomycetes *Penicillium multicolor*, *Microglossum olivaceum*, *Aleuria aurantia*, *Cheilymenia crucipila*, *Scutellinia ampulosa*, *S. arenosa*, *S. scutellata*, *S. superba*, *S. umbrarum* and *S. trechispora*, the latter of which also produces β-zeacarotene. Some basidiomycetes have been reported produce to γ-carotene, including *Ustilago scabiosae*, *Clitocybe venustissimus* and *Sphaerobolus stellatus*, among others; to produce torulene, such as *Rhodotorula gracilis*, *R. infirmominata*, *R. pallida*, *R. sannieri*; and to produce torularhodine, such as the species *Rhodotorula mucilaginosa*, *R. minuta*, *R. rubra*, *Sporidiobolus johnsonii*, *Sporobolomyces roseus*, *S. ruberrimus* and *S. salmonicolor*. In 2002, Echavarri-Erasun and Johnson described the most representative carotenoids produced by different phylogenetic groups of fungi. For example, 3,4-didehydrolycopene, neurosporaxanthin, γ-carotene and β-carotene are produced by Myxomycetes; γ-carotene, lycopene and β-carotene by Chytridiomycetes; β-carotene by Zygomycetes; γ-carotene, lycopene, β-carotene, torulene, lycoxanthin and neurosporaxanthin by Pyrenomycetes and β-carotene, γ-carotene, 3,4-didehydrolycopene, torulene, phillipsiaxanthin, aleuriaxanthin and plectaniaxanthin by Discomycetes, being both Ascomycota; β-carotene, HDCO, echinenone, astaxanthin by the Basidiomycetous yeast *X. dendrorhous* (Echavarri-Erasun and Johnson 2002). Among fungi, members belonging to genus *Rhodotorula*, *Sporobolomyces* and *Xanthophyllomyces* may be prominent biotechnological sources of a variety of carotenoids (Mata-Gomez et al. 2014). As in many microorganisms, the carotenoid production and composition varies according to the culture conditions and carbon source and among strains belonging to different species within the *Rhodototula* genus (Frengova and Beshkova 2009). In order to use yeast as commercial sources of pigments, studies to identify inexpensive carbon sources and culture media and to determine suitable physiological and environmental conditions to increase the production are required. Several factors, including the carbon source, light, temperature, oxygen, salts, metals and chemicals, such as ethanol, among others, affect carotenoid production in yeasts, which has recently been reviewed (Mata-Gomez et al. 2014).

Another fungal xanthophylls are neurosporaxanthin, which was identified in the ascomycete fungus *Neurospora crassa* (Zalokar 1957), cordyxanthin, found in the mushroom *Cordyceps militaris* (Dong et al. 2013; Yang et al. 2014), canthaxanthin found in *Cantharellus cinnabarinus* (Haxo 1950) and astaxanthin, which is produced by the yeast *X. dendrorhous* (Miller et al. 1976; Andrewes and Starr 1976). Moreover, three basidiomycete carotenogenic yeast species, including *Dioszegia* sp., *Rhodotorula mucilaginosa* and *R. laryngis*, have been isolated from ice samples obtained from an Italian Alpine glacier. Of these, *Dioszegia* sp. produces a still unidentified xanthophyll that contains two hydroxyl groups (Amaretti et al. 2014).

Some fungal species have been used as model microorganisms in carotenogenesis studies. Among them are the zygomycetes *Phycomyces blakesleeanus*, *Blakeslea trispora* and *Mucor circinelloides* (Avalos et al. 2014), in which each step of the carotenoid biosynthetic pathway, the genes encoding the enzymes involved and their regulation are known. Regarding the above, the expression of the carotenogenic genes *carB* and *carRA* in *B. trispora* are induced during the sexual reproductive phase; that is, the transcription of these genes is mating-dependent (Schmidt et al. 2005). Moreover, β-carotene was reported to play an important role in the biology of the fungus in *P. blakesleeanus*, specifically in its sexual reproduction because its pheromones are derived from β-carotene. This process is controlled by the *carS* gene, which in addition to producing a gene product that cleaves β-carotene, regulates the carotenoid biosynthesis and sexual reproduction of this fungus (Tagua et al. 2012). The genes controlling the biosynthesis of carotenoids in *N. crassa* were identified using classical genetic techniques and after, relevant regulatory mechanisms were ellucidated. The *al*-1, *al*-2, *al*-3 and *ylo*-1 genes of *N. crassa* were isolated and their involvement in the neurosporaxanthin biosynthetic pathway was studied (Schmidhauser et al. 1990, 1994; Nelson et al. 1989; Estrada et al. 2008). The biosynthesis of carotenoids in this organism is induced by light with the participation of the White Collar photosystem (Nelson et al. 1989).

The basidiomycete yeast *X. dendrorhous* produces astaxanthin, a xanthophyll that is widely used in salmon farming. The carotenogenic genes from this organism have been isolated and characterized at the molecular level, and metabolic engineering approaches have been applied for the heterologous production of astaxanthin in *S. cerevisiae*, albeit at a minimal level (Ukibe et al. 2009). In general, the results indicated that the latter yeast was able to produce only β-carotene when the currently described carotenogenic genes of *X. dendrorhous* are introduced, suggesting that other genetic elements of *X. dendrorhous* are required to successfully transform β-carotene into astaxanthin in *S. cerevisiae*. In addition, comparative studies have been conducted for the biotechnological production of astaxanthin using various natural sources of this xanthophyll and chemical synthetic processes, focusing on the advantages of the yeast *X. dendrorhous* as a model producing organism and its potential industrial use (Schmidt et al. 2011).

1.3 Carotenoid Biosynthesis

Carotenoid pigments are one of the most representative groups of isoprenoids, and isoprenoids constitute one of the largest families of natural compounds produced by members of the three domains of life. Carotenoids are formed using the same C_5 building block, the isoprene (C_5H_8) unit, from which isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) are produced. In isoprenoid synthesis, three molecules of IPP are sequentially added to DMAPP by prenyl transferase enzymes to yield geranylgeranyl-pyrophosphate (GGPP, C_{20}). The later condensation of two molecules of GGPP yields phytoene (C_{40}), which is

the first carotenoid to be synthesized. Phytoene is a symmetrical colorless linear carotene with only three conjugated double bonds; the great diversity of carotenoids is derived through chemical modifications of this molecule (Ajikumar et al. 2008). Thus, it is possible to divide the synthetic pathway of carotenoids into three major stages as follows:

(i) The synthesis of IPP and the formation of DMAPP
(ii) The synthesis of GGPP
(iii) The synthesis of carotenoids *per se*

In different carotenoid-producing organisms, several downstream modifications of the carotenoid backbone, such as desaturation, cyclization, oxidization and further modifications, such as glycosylation and oxidative cleavage, may be performed. All of these modifications contribute to the enormous diversity of this type of compound in nature. As an example, the synthesis of astaxanthin is discussed.

1.3.1 The Synthesis of IPP and DMAPP

The carotenoid precursors IPP and DMAPP are essential metabolites and the production of which varies among organisms and cellular compartments. IPP and DMAPP can be synthesized via the following two independent non-homologous metabolic pathways: the mevalonate (MVA) pathway (Miziorko 2011) and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate (DXP) or the non-MVA pathway (Lichtenthaler 2000). The MEP pathway [KEGG: M00096] (Kanehisa and Goto 2000; Kanehisa et al. 2014) begins with the condensation of D-glyceraldehyde 3-phosphate and pyruvate by DXP synthase, which releases CO_2 and produces DXP (Fig. 1.1). DXP is then reduced into MEP by DXP isomeroreductase, which is subsequently converted into IPP via five catalytic steps that sequentially involve the following enzymes: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, 4-(Cytidine 5'-pyrophospho)-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate synthase, 4-hydroxy-3-methylbut-2-enyl-pyrophosphate synthase and 4-hydroxy-3-methylbut-2-enyl pyrophosphate reductase. Interesting, the last enzyme of this pathway is also known as IPP:DMAPP synthase because it is able to produce IPP and DMAPP simultaneously in an 85:15 IPP/DMAPP ratio (Tritsch et al. 2010). Thus, DMAPP can be synthesized by this enzyme via the MEP pathway and by an IPP-isomerase (see below). In the MVA pathway [KEGG: M00095] (Kanehisa and Goto 2000; Kanehisa et al. 2014), the synthesis of IPP involves 6 steps, starting with the condensation of two molecules of acetyl-coenzyme A (CoA) by acetoacetyl-CoA thiolase to give acetoacetyl-CoA (Fig. 1.1). Next, acetoacetyl-CoA is converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG synthase and afterward, HMG-CoA is reduced to MVA by HMG-CoA reductase. Then, two phosphorylation steps at the C-5 of MVA and a decarboxylation reaction by MVA kinase, phospho-MVA (MVA-P) kinase

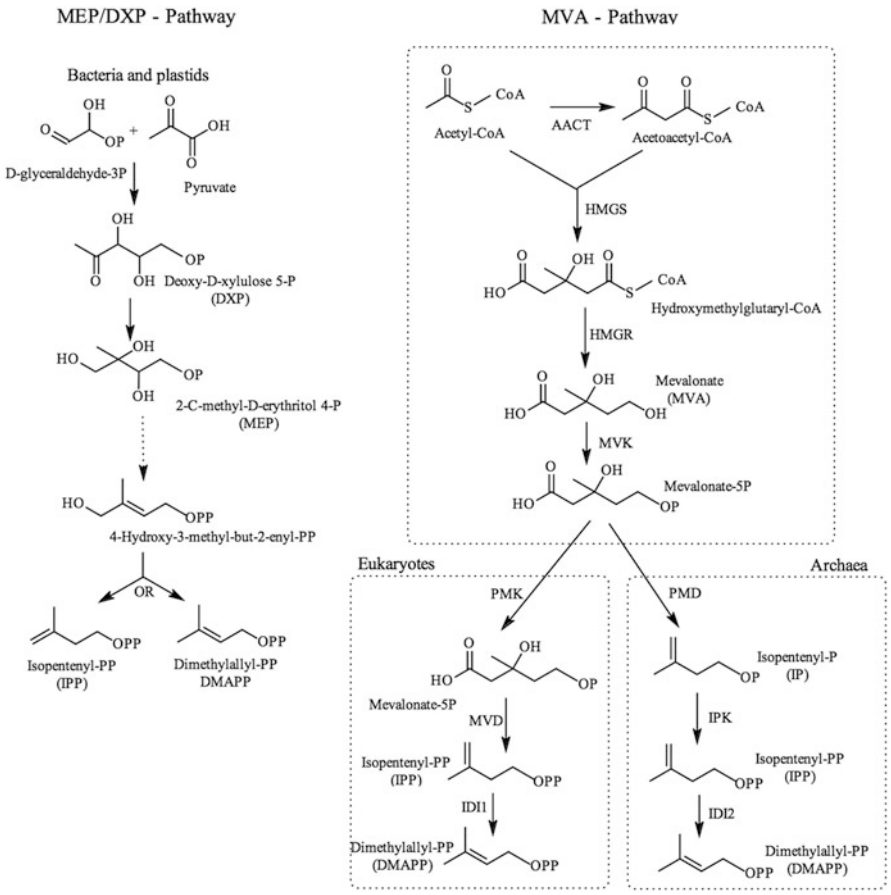

Fig. 1.1 Biosynthesis of isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Schematic representations of the synthesis of IPP and DMAPP through the MEP/DXP pathway in bacteria and in plant plastids (on the left, adapted from [KEGG: M00096] (Kanehisa and Goto 2000; Kanehisa et al. 2014)) and through the MVA pathway in eukaryotes and archaea (on the *right*, adapted from (Lombard and Moreira 2011)). The MVA pathways of eukaryotes and archaea are somewhat different; they share the only first four steps (*boxed*). Abbreviations: *MEP* (2-C-methyl-D-erythritol-4-P), *DXP* (Deoxy-D-xylulose-5-P), *MVA* (mevalonate), *IPP* (isopentenyl pyrophosphate), *DMAPP* (dimethylallyl pyrophosphate), *AACT* (acetoacetyl-CoA thiolase), *HMGS* (hydroxymethylglutaryl-CoA synthase), *HMGR* (hydroxymethylglutaryl-CoA reductase), *MVK* (mevalonate kinase), *PMK* (phosphomevalonate kinase), *MVD* (mevalonate pyrophosphate decarboxylase), *PMD* (phosphomevalonate decarboxylase), *IPK* (isopentenyl phosphate kinase), *IDI1* (isopentenyl pyrophosphate isomerase type 1) and *IDI2* (isopentenyl pyrophosphate isomerase type 2)

and MVA-PP decarboxylase, respectively, produce IPP. Finally, the interconversion between IPP and DMAPP is catalyzed by an IPP-isomerase.

With the exception of plants, most organisms only have one pathway for the synthesis of IPP and DMAPP. Most bacteria and cyanobacteria only have the MEP pathway, whereas in most eukaryotes and archaea; only the MVA pathway is present. Although plants have both pathways, the pathways have different cellular localizations; IPP is synthesized in the cytoplasm via the MVA pathway, whereas this metabolite is synthesized via the MEP pathway in plastids (Vranova et al. 2013; Misawa 2010). This strict compartmentalization of two alternative pathways for IPP synthesis in plants is consistent with the prokaryotic origin of chloroplasts, which are presumed to have derived from a cyanobacteria-like endosymbiont (Lange et al. 2000). Interestingly, the isoprenoids of the green algae *Chlorella fucsa*, *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* are synthesized via the MEP pathway (Dobrowolska 2006) and no MVA-pathway encoding genes were found in the *C. reinhartdtii* genome (Vranova et al. 2013). These observations indicate that the green algae ancestor most likely never had the MVA pathway or that this metabolic route was lost in these organisms during evolution. In either case, efficient mechanisms for exporting isoprenoids and their intermediates from plastids to the cytoplasm for the synthesis of cytosolic isoprenoids, such as sterols, have been developed in green algae (Vranova et al. 2013). In contrast, the MVA pathway has been found in only a few bacteria, which was attributed to acquisition via horizontal gene transfer from eukaryotes or archaea (Lombard and Moreira 2011).

Even though the presence of the MVA pathway is characteristic of eukaryotes and archaea, there are differences between the MVA pathways of the two domains. Analyses of archaeal genomes revealed that some species lack the genes encoding the last enzymes of the MVA pathway including those encoding MVA-P kinase, MVA-PP decarboxylase and IPP isomerase (Lombard and Moreira 2011). To explain this situation, it has been proposed that non-homologous replacements of these genes occurred in archaea (Smit and Mushegian 2000). In support of this hypothesis, an enzyme that can phosphorylate isopentenyl phosphate (IP) to produce IPP (the isopentenyl phosphate kinase, IPK) has been found in some archaea (Grochowski et al. 2006), suggesting that the order of the last phosphorylation and decarboxylation steps in the MVA pathway in archaea might be switched. Related to this idea, a phosphomevalonate decarboxylase (PMD) was recently identified and characterized together with an IPK, both from *Haloferax volcanii*, clarifying the existence of an alternate mevalonate pathway in archaea and proving that the archaeon decarboxylation step occurs prior to the phosphorylation step (Vannice et al. 2014). In addition, an IPP isomerase that is non-homologous to the eukaryotic enzyme was first described in *Streptomyces* sp. (Kaneda et al. 2001) and later was found to be encoded by several archaea genomes (Barkley et al. 2004) (see below). Considering these observations, recent phylogenomic analyses of the available data for the bacterial, eukaryotic and archaeal MVA-pathway genes, strongly supported the hypothesis that the MVA pathway could have been an ancestral metabolic pathway in the three domains of life, so it was likely present in the last common ancestor of all organisms and was lost and replaced by the MEP pathway in

a variety of bacterial phyla (Lombard and Moreira 2011). Additionally, the last MVA-pathway genes in most archaeal species could have been modified by non-homologous gene replacement, excluding Sulfolobales because they have MVA-P kinase- and MVA-PP decarboxylase-encoding genes (Lombard and Moreira 2011).

IPP isomerases are present in the living organisms all of kingdoms, and considering that the MEP pathway produces both IPP and DMAPP, whereas the MVA pathway yields only IPP, IPP isomerase is essential in organisms that possess only the MVA pathway. Based on their amino acid sequences and coenzyme requirements, IPP isomerases have been classified into two subfamilies, type 1 (IDI1) and type 2 (IDI2), which based on their gene sequence are most likely evolutionally independent. These two types of IPP-isomerases may be found in organisms that have either the MEP and/or MVA pathway (Laupitz et al. 2004). IDI1 has a wide distribution in nature, covering a large variety of organisms among eukaryotes, prokaryotes and archaea, and it was first discovered in crude extracts of *S. cerevisiae* in the late 1950s (Berthelot et al. 2012). This enzyme functions as a monomer in the presence of a divalent metal cation, such as Mg^{+2} or Mn^{+2}. In contrast, IDI2 was more recently discovered (Kaneda et al. 2001) and functions as a homotetramer which, in addition requiring to a divalent metal cation for activity, requires FMN and NAD(P)H (Kuzuyama et al. 2010). IDI2 is found mainly in Gram-positive bacteria, proteobacteria, cyanobacteria and a few Achaea, with an intriguing prevalence in thermophilic species (Berthelot et al. 2012).

1.3.2 The Synthesis of GGPP

As mentioned before, the biosynthesis of isoprenoids originates from a basic C_5 isoprene unit as its active forms IPP and DMAPP. First, IPP is isomerized to DMAPP by IPP-isomerase and then, isoprenyl pyrophosphate synthases successively add three molecules of IPP to allylic pyrophosphate molecules in a head-to-tail manner, beginning with DMAPP, resulting in geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}) and finally, GGPP [KEGG: hsa_M00367] (Fig. 1.2, Kanehisa and Goto 2000; Kanehisa et al. 2014). These three last molecules are the precursors of most of the isoprenoid compounds. For example, the condensation of two units of FPP yields squalene, which is the precursor of sterols, whereas the union of two molecules of GGPP yields phytoene, the first carotenoid that is synthesized in carotenogenesis. Prenyltransferases are a class of enzymes that transfer allylic prenyl groups to acceptor molecules, including DMAPP, aromatic intermediates of quinones and specific proteins (Brandt et al. 2009). Based on the length of the final product and the stereochemistry of the double bonds that are formed during product elongation, prenyltransferases are classified as *trans*- or (*E*)-prenyltransferases and *cis*- or (*Z*)-prenyltransferases (Vandermoten et al. 2009). Short-chain prenyltransferase enzymes are *trans*-prenyltransferases, and GPP-synthase, FPP-synthase and GGPP-synthase, are included in this group.

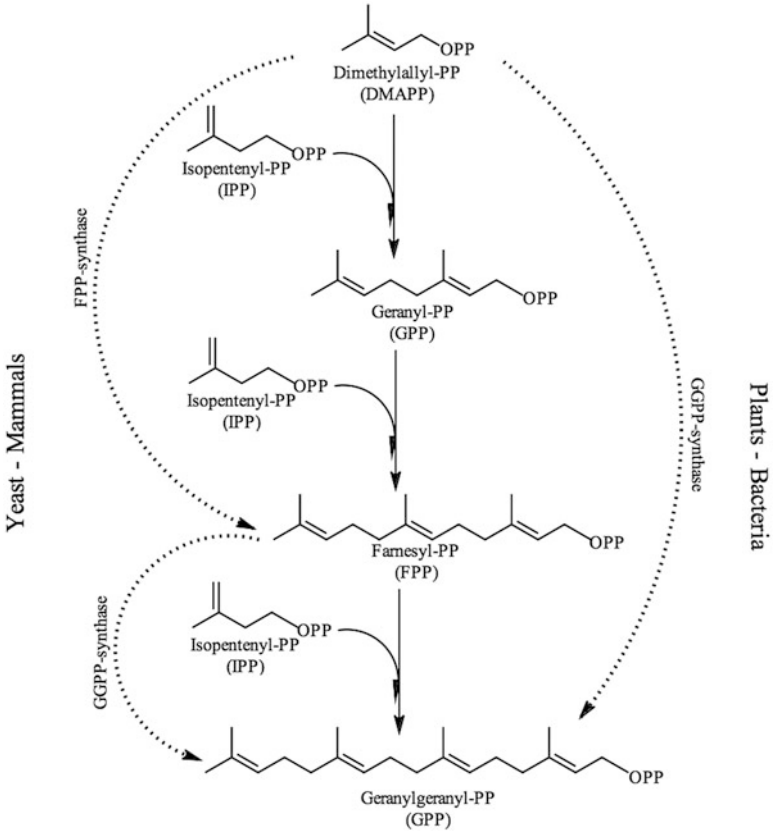

Fig. 1.2 Biosynthesis of geranylgeranyl pyrophosphate. Geranylgeranyl pyrophosphate can be synthesized from isopentenyl pyrophosphate through two prenyl transferases, FPP-synthase and GGPP-synthase (*left*) or through one prenyl transferase, GGPP-synthase (*right*). Abbreviations: *DMAPP* (dimethylallyl pyrophosphate), *IPP* (isopentenyl pyrophosphate), *GPP* (geranyl pyrophosphate), *FPP* (farnesyl pyrophosphate) and *GGPP* (geranylgeranyl pyrophosphate)

The short-chain prenyltransferases of various organisms share many features, suggesting that they are derived from a common ancestor, and currently, seven conserved regions (I to VII) are recognized. Among these conserved regions, II and VI contain the First and the Second Aspartic acid-Rich Motifs (DDxxD), known as FARM and SARM, respectively. Mutagenesis studies showed that the aspartic residues within the FARM motif are involved in catalysis and the binding of the allylic substrate (Vandermoten et al. 2009). Additionally, a Chain-Length Determination (CLD) region involved in the determination of the product chain length was identified, localized upstream of the FARM motif (Ohnuma et al. 1997). The presence of at least one aromatic residue at the fourth and fifth positions before the FARM motif is conserved among the FPP synthases, whereas smaller residues are found at these positions in longer chain-producing homologs (Gao et al. 2012).

GPP-synthase catalyzes the condensation of DMAPP and IPP to produce GPP, the precursor of mono (C_{10})-terpene biosynthesis (Fig. 1.2). Monoterpenes are the main constituents of the essential oils that are produced by plants and also function as aggregation and dispersion pheromones in insects, but only a few GPP-synthase genes have been described (Vandermoten et al. 2009). In contrast, much more is known about FPP- and GGPP-synthases. FPP-synthase catalyzes the condensation of IPP and DMAPP to produce GPP, which then receives a second molecule of IPP, producing FPP. FPP is the precursor of sesqui (C_{15})- and tri (C_{30})-terpenes, including sterols. GGPP-synthase produces GGPP, the precursor of di (C_{20})-terpenes and of phytoene (C_{40}), which in turn is the precursor of most carotenoids. In plants and bacteria, GGPP-synthase sequentially adds IPP to the allylic co-substrates DMAPP, GPP and FPP to finally produce GGPP; whereas in yeast and mammals, GGPP-synthase uses only FPP as a co-substrate to produce GGPP (Vandermoten et al. 2009). Thus, GGPP may be synthesized from IPP and DMAPP via different systems involving one or two enzymes, an FPP-synthase and/or a GGPP-synthase. In the first system, which is common in plants and bacteria, only a GGPP-synthase that is able to produce GGPP from IPP and DMAPP is involved. In the second system, which is common in mammals and yeasts, two enzymes are involved; first FPP-synthase produces FPP from IPP and DMAPP in two condensation steps and then, GGPP-synthase produces GGPP from FPP and IPP (Alcaino et al. 2014). Considering that FPP is essential, it is interesting that even though their growth rate was lower than that of wild-type strains, *E. coli* mutants lacking the FPP-synthase gene were still viable and exhibited a low level of FPP-synthase activity (Saito et al. 2007), suggesting that there is a third system that combines the other two systems to simultaneously yield GGPP.

1.3.3 The Synthesis of Carotenoids per se

A tree-like hierarchical structure has been proposed for the carotenoid biosynthetic pathways, with the trunk being the assembly of the carotenoid backbone from which several variable branches and sub-branches arise, generating the vast diversity of carotenoids that are produced in nature (Umeno et al. 2005). Several downstream modifications to the carotenoid backbone contribute to the broad diversity of carotenoids, including the introduction of additional conjugated double bonds, cyclization of one or both ends of the polyene carotenoid backbone, introduction of oxygen-containing functional groups and further modifications, such as glycosylation and oxidative cleavage.

Carotenoid synthases catalyze complex reactions similar to those of squalene synthase, which is the first enzyme involved in sterol biosynthesis. It is likely that these enzymes have a common evolutionary origin because they share certain conserved domains (Umeno et al. 2005). In general, the synthesis of carotenoids begins with the head-to-head condensation of two molecules of GGPP by the enzyme phytoene synthase, which yields phytoene (C_{40}), the first carotenoid to be

synthesized (Fig. 1.3). However, some bacteria produce unusual C_{30} carotenoids that derive from diapophytoene, which is formed by the condensation of two molecules of FPP, instead of GGPP, by diapophytoene synthase (CrtM) (Misawa 2010). In both cases, the catalysis is performed in two steps. In first step, the pyrophosphate group is removed from one prenyl donor, which then undergoes condensation with a prenyl acceptor to form a stable cyclopropyl intermediate. Next, the cyclopropyl intermediate is rearranged, releasing the second pyrophosphate group (Umeno et al. 2005).

Phytoene synthase is encoded by the *crtB* gene in bacteria and by the *PSY* gene in plants, algae and cyanobacteria (Sieiro et al. 2003), but analyses of their sequences suggest that these genes share a common ancestor (Takaichi 2013). A bifunctional enzyme has been described in fungi, which in addition to having phytoene synthase activity, also has lycopene-cyclase activity to produce β-carotene from lycopene. For this reason, the fungal enzyme was named phytoene β-carotene synthase (PBS, encoded by the *crtYB* gene), in which the phytoene synthase activity resides in the C-terminus, whereas the lycopene cyclase activity is restricted to the N-terminus. This peculiarity was first demonstrated by heterologous complementation studies of the "supposed" lycopene cyclase-encoding gene of the carotenogenic basidiomycete yeast *X. dendrorhous* in recombinant *E. coli* strains bearing bacterial carotenogenic gene clusters (Verdoes et al. 1999). Although the deduced amino acid sequence of the *crtYB* product resembled that of bacterial and plant phytoene synthases, the fungal phytoene synthase domain has an approximately 300-residue extension at its C-terminus (Verdoes et al. 1999). Interesting, similar genes encoding this unique enzyme have been reported in other fungi, such as the ascomycete *N. crassa* (Schmidhauser et al. 1994) and the zygomycetes *M. circinelloides* (Velayos et al. 2000) and *P. blakesleeanus* (Arrach et al. 2001). Its wide distribution throughout the fungal kingdom suggests that this bifunctional enzyme was acquired early in the evolution of fungi (Krubasik and Sandmann 2000).

In the next stage of carotenogenesis, phytoene is typically converted into lycopene through four desaturation reactions, sequentially giving phytofluene, ζ-carotene, neurosporene and lycopene (Fig. 1.3). In non-photosynthetic carotenogenic organisms, such as fungi and eubacteria, the formation of the four double bonds in phytoene that leads to lycopene is performed by only one phytoene desaturase enzyme encoded by the *crtI* gene. Nevertheless, the bacterial phytoene desaturase of *Rhodobacter capsulatus* produces neurosporene, which is synthesized from phytoene in three desaturation steps (Sandmann 2009). In addition, in the C_{30}-carotenogenic pathway, a diapophytoene desaturase that is encoded by the *crtN* gene, which is homologous to *crtI*, catalyzes a three-step desaturation (Misawa 2010). However, the C_{40} four-step desaturases appear to be most closely related to the three-step C_{40} desaturases than to the C_{30} CrtN desaturases (Sandmann 2009). In contrast, in photosynthetic organisms, such as plants, algae and cyanobacteria, two closely related isomerases are involved in this stage. First, two double bonds are sequentially introduced into phytoene, giving ζ-carotene. This process is performed by a phytoene desaturase, which is encoded by the *PDS* gene in plants and algae and by the *crtP* gene in cyanobacteria (Sieiro et al. 2003). Then, a ζ-carotene desaturase, which is encoded by the *ZDS* gene in plants and algae and by the *crtQ* gene in

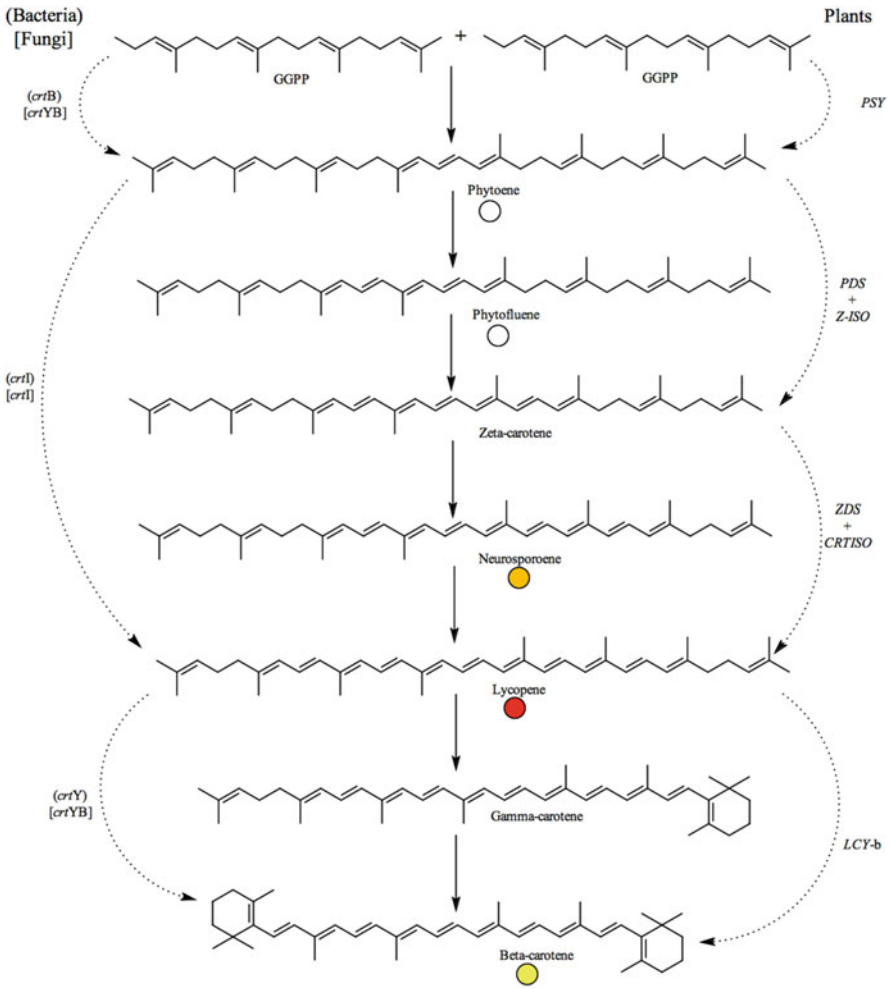

Fig. 1.3 Biosynthesis of β-carotene (beta-carotene). Schematic representation of the synthesis of β-carotene in bacteria and fungi (on the left) and in plants (on the right). β-carotene is synthesized from two molecules of geranylgeranyl pyrophosphate (GGPP) through phytoene, phytofluene, ζ-carotene (zeta-carotene), neurosporene, lycopene and γ-carotene (gamma-carotene). The relevant bacterial genes (in parentheses) are *crtB* (phytoene synthase), *crtI* (phytoene desaturase) and *crtY* (lycopene cyclase); the relevant fungal genes (in square brackets) are *crtYB* (phytoene β-carotene synthase) and *crtI* (phytoene desaturase) and the relevant plant genes are *PSY* (phytoene synthase), *PDS* (phytoene desaturase), *Z-ISO* (ζ-carotene isomerase), *ZDS* (ζ-carotene desaturase), *CRTISO* (carotene isomerase) and *LCY*-b (lycopene β-cyclase). The metabolic pathways were adapted from [KEGG: M00097] (Kanehisa and Goto 2000; Kanehisa et al. 2014) and the carotenoid structures were based on those of (Britton et al. 2004)

cyanobacteria, introduces two additional double bonds to ζ-carotene, producing lycopene (Sieiro et al. 2003). Interesting, *Gloeobacter violaceus*, a primitive cyanobacterium, has a CrtI-type phytoene desaturase instead of a plant-related

one (Steiger et al. 2005). This situation suggests that in photosynthetic organisms, the desaturation steps at this stage of carotenogenesis evolved from a simpler one-enzyme process into a more complex process (Sandmann 2009). Moreover, most carotenoids exist in an all-*trans* configuration, but some are produced in *cis* configuration. In plants, two isomerases (a ζ-carotene isomerase or Z-ISO (Chen et al. 2010) and a carotene isomerase or CrtISO (Isaacson et al. 2004)), participate in producing the final all-*trans* form of lycopene. These isomerases are phylogenetically related to the CrtI-type phytoene desaturase, which appears to also have the property of catalyzing the *trans* conversion of carotenes (Sandmann 2009).

The cyclization of lycopene by lycopene cyclases is a frequent step in the biosynthesis of carotenoids, which can occur at one or both of its ends, generating mono- and bi-cyclic carotenoids, respectively. There are two major types of rings, the β-ionone and the α-ionone rings (β- and ε-rings, respectively) (Britton 1998). The β-ring is the most common form, and it is found in the carotenoids of the majority of photosynthetic organisms, including cyanobacteria, algae, and higher plants, as well as in non-photosynthetic bacteria, yeasts, and fungi. In contrast, the distribution of the ε-ring form is more restricted, and it is found in plants and cyanobacteria. Carotenoids generally have two β-rings, a ε-ring and a β-ring, or have only one β-ring. The introduction of a β-ring at both ends of lycopene gives β-carotene, through γ-carotene, which is one of the best-known carotenoids (Fig. 1.3). The introduction of a ε-ring followed by the formation of a β-ring in the other end of the carotene gives α-carotene through δ-carotene in higher plants (Misawa 2010).

The first lycopene β-cyclase-encoding gene, which was isolated from *Pantoea ananas* (formerly *Erwinia uredovora*) and was named *crtY* (Misawa 2010) is distributed among the eubacteria. In plants and cyanobacteria, the *crtL*-b (or *LCY*-b) gene encodes lycopene β-cyclase, whereas lycopene ε-cyclase is encoded by the *crtL*-e (or *LCY*-e) gene. Additionally, lycopene β-monocyclase-encoding genes (*crtYm*, *crtLm*) have been described in eubacteria. These cyclases, which have five conserved regions and contain an NAD(P)/FAD-binding motif, have been further divided in two families, the CrtY family and the CrtL family (Takaichi 2013). Other unrelated classes of lycopene cyclases have been described. For example, the actinomycete bacterium *Brevibacterium linens* has a heterodimeric lycopene cyclase that is responsible for the conversion of lycopene to β-carotene, which is formed by two polypeptides encoded by the *crtYc* and *crtYd* genes (Krubasik and Sandmann 2000). As mentioned above, a bifunctional phytoene synthase and lycopene cyclase (*crtYB* gene) has been identified in fungi, and the fungal lycopene cyclase domain appears to be related to the *crtYc* and *crtYd* gene products of *B. linens*. Thus, the bifunctional enzyme in fungi most likely developed through the recombination of these two genes and a phytoene synthase gene (Krubasik and Sandmann 2000). In addition, a third class of lycopene cyclase (*cruA* gene) was recently identified in the green sulfur bacterium *Chlorobium tepidum* through heterologous complementation in a lycopene-producing *E. coli* strain (Maresca et al. 2007). Two homologous genes (denoted *cruA* and *cruP*), the products of which showed lycopene cyclase activity, were found in the cyanobacterium *Synechococcus* sp. PCC 7002 and in other cyanobacterial genomes, demonstrating their wide

distribution among cyanobacteria (Maresca et al. 2007). However, even though the *Anabaena* sp. strain PCC 7120 and the *Synechocystis* sp. strain PCC 6803 contain *cruA* orthologous genes in their genomes, no lycopene cyclase activity related to these genes has been detected (Mochimaru et al. 2008). Considering that these organisms produce β-carotene and that in addition, no lycopene cyclase-encoding genes related to *crtY* or to *crtL* have been found in their available and complete sequenced genomes, the existence of another additional type of lycopene β-cyclase is suspected (Takaichi 2013).

The post-phytoene acyclic, monocyclic and dicyclic carotenes may be subjected to a series of downstream enzymatic modifications. In this regard, the wide diversity of xanthophylls results from the incorporation of oxygen-containing functional groups, such as hydroxy-, epoxy- and keto- groups, into carotenes, mainly α- and β-carotene. As mentioned before, one of the most studied xanthophylls is astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), largely due to its numerous commercial applications. Currently, commercial astaxanthin is mainly produced via chemical synthesis and only a few organisms produce this xanthophyll naturally. However, astaxanthin is of special interest because its biosynthesis does not proceed in the same way in all of the astaxanthin-producing organisms; thus, these pathways represent good examples of the evolution of carotenogenesis.

1.3.4 The Synthesis of Astaxanthin

To date, few organisms that naturally synthesize astaxanthin are known, which include the microalga *H. pluvialis* (Lemoine and Schoefs 2010), some marine bacteria, such as *Paracoccus haeundaensis* (Lee and Kim 2006) and *Brevudimonas* sp. (Yokoyama and Miki 1995), the basidiomycete yeast *X. dendrorhous* (Andrewes and Starr 1976) and the plant *Adonis aestivalis*, in which this pigment accumulates in the petals of the flowers (Renstrøm et al. 1981). However, the mechanisms involved in the biosynthesis of astaxanthin in these organisms are different. In general, astaxanthin is produced by the introduction of a hydroxyl- and a keto-group to the carbons at positions 3 and 4, respectively, in each β-ionone ring of β-carotene via eight possible intermediate xanthophylls (Fig. 1.4, echinenone,

Fig. 1.4 (continued) Biosynthesis of astaxanthin. Schematic representation of the synthesis of astaxanthin from β-carotene, showing the genes that control each step, in the yeast *X. dendrorhous* (in square brackets, obtained from (Ojima et al. 2006)), the microalgae *H. pluvialis* (in parentheses, obtained from (Huang et al. 2012)), bacteria (no special indicators, obtained from (Tao et al. 2006)) and in the plant *A. aestivalis* (enclosed in an oval on the right, obtained from (Cunningham and Gantt 2011)). The astaxanthin biosynthetic pathway was summarized from [KEGG: map00906] (Kanehisa and Goto 2000; Kanehisa et al. 2014) and the carotenoid structures were based on those of (Britton et al. 2004). The corresponding gene products are astaxanthin synthase (*crtS*), bacterial β-carotene ketolase (*crtW*), bacterial β-carotene hydroxylase (*crtZ*), β-carotene ketolase (*bkt*), carotenoid hydroxylase (*chy*), carotenoid β-ring 4-dehydrogenase (*cbfd*) and hydroxy-β-ring 4-dehydrogenase (*hbfd*)

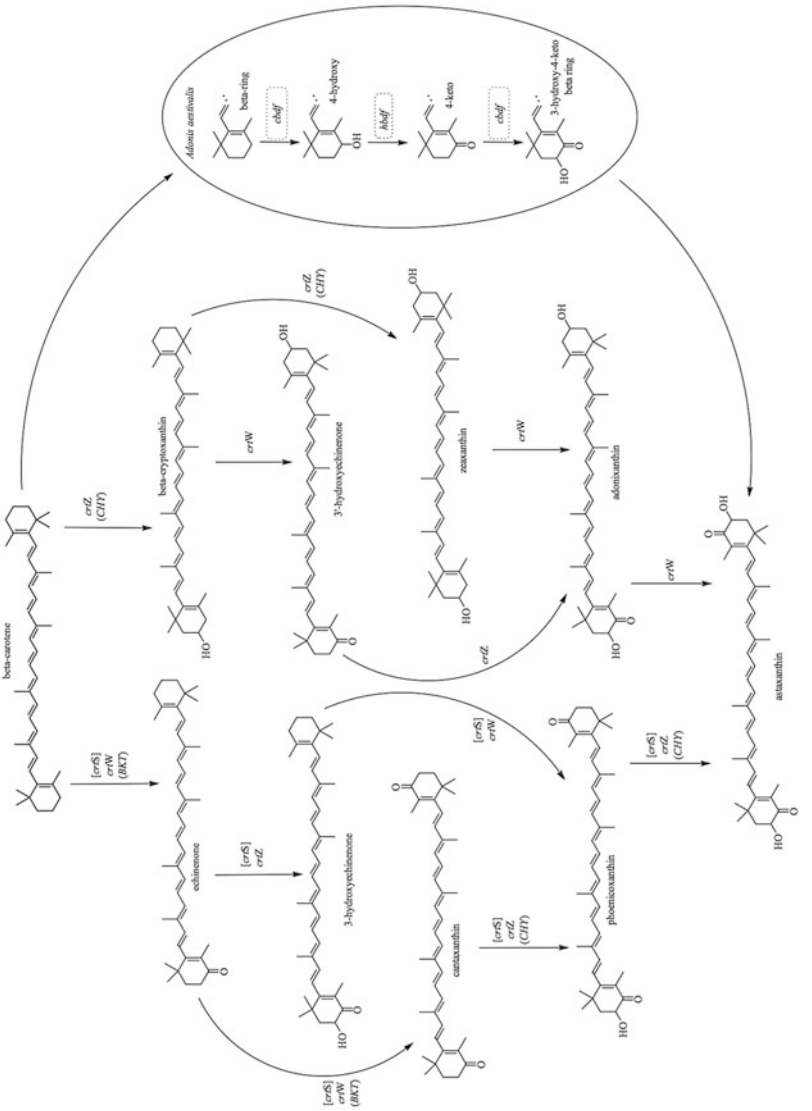

Fig. 1.4 (continued)

3-hydroxyechinenone or 3′-hydroxyechinenone, β-cryptoxanthin, canthaxanthin, zeaxanthin, phoenicoxanthin and adonixanthin), which vary depending on the producing organism.

In most astaxanthin-producing organisms, the oxidation of β-carotene is performed by ketolases and hydroxylases (Fig. 1.4). There are several organisms that produce keto-xanthophylls other than astaxanthin but, in general, plants do not produce ß-carotene-derived ketocarotenoids (Zhu et al. 2007). However, *Adonis* plants are able to produce the ketocarotenoid astaxanthin from β-carotene through three steps that are catalyzed by two enzymes, carotenoid-β-ring-4-dehydrogenase (CBFD) and 4-hydroxy-β-ring-4-dehydrogenase (HBFD). A first hydroxyl group is introduced to the carbon at position 4 of the β-ring by CBFD, which then is further dehydrogenated by HBFD to form a keto group at this position. Next, CBFD introduces another hydroxyl group to the carbon at position 3 to yield a 3-hydroxy-4-keto-β-ring (Cunningham and Gantt 2011).

A different mechanism has been proposed in *H. pluvialis*, which produces astaxanthin only under stressful conditions (Vidhyavathi et al. 2008). During its green vegetative phase, *H. pluvialis* produces zeaxanthin by the incorporation of a hydroxyl group at position 3 of both β-ionone rings via a hydroxylase enzyme (CHY). However, under stressful conditions, a keto group is introduced at position 4 of both β-ionone rings by a β-carotene ketolase (BKT), which is encoded by two paralogous genes, *bkt1* and *bkt2*. Nevertheless, it is unlikely that the β-carotene in *H. pluvialis* is converted into astaxanthin through an initial hydroxylation step because it has been shown that BKT can use only a non-substituted β-ionone ring as a substrate (Lemoine and Schoefs 2010). Thus, in *H. pluvialis*, astaxanthin should be produced from β-carotene primarily via echinenone, canthaxanthin and phoenicoxanthin, with β-carotene and echinenone being the substrates of BKT and canthaxanthin and phoenicoxanthin being substrates of CHY. In addition, the hydroxyl groups of astaxanthin are further esterified in *H. pluvialis*, leading to the production of mono- and di-esterified astaxanthins (Lemoine and Schoefs 2010). In contrast, bacterial ketolases (encoded by the *crtW* gene) can convert a non-substituted β-ionone ring as well as 3-hydroxylated β-ionone rings into their respective 4-keto forms. In the same manner, bacterial β-carotene hydroxylases (encoded by the *crtZ* gene) can use non-substituted and 4-ketolated β-ionone rings as substrates to produce their respective 3-hydroxylated forms (Misawa et al. 1995).

There are two main types of β-carotene hydroxylases, the non-heme di-iron (NH-di-iron) hydroxylases and the cytochrome P450 monooxygenases (P450s) (Martin et al. 2008). The first types are related to fatty-acid desaturases and require molecular oxygen, iron, ferredoxin, and ferredoxin oxido-reductase for their functions (Tian and DellaPenna 2004). In contrast, the P450s comprise a large superfamily of heme-containing monooxygenases that are distributed in organisms from all domains of life (Estabrook 2003; McLean et al. 2005). Cytochrome P450s are involved in the oxidative metabolism of a wide range of exogenous and endogenous substrates (Degtyarenko and Archakov 1993) and act as a terminal electron acceptor in multicomponent P450-dependent monooxygenation systems, which lead to the reductive activation of molecular oxygen followed by the insertion

of one oxygen atom into the substrate molecule and the reduction of the other to water (van den Brink et al. 1998). Two electrons are required for cytochrome P450 catalysis, which are transferred primarily from NADPH via a redox partner (van den Brink et al. 1998). Recently, the involvement of cytochrome P450s in carotene hydroxylation has been demonstrated (Inoue 2004), which was first described in the thermophilic bacterium *Thermus thermophiles* (CYP175A1) (Blasco et al. 2004) and in *Arabidopsis thaliana* (CYP97C1) (Tian et al. 2004). It was shown that CYP175A1 could introduce hydroxyl groups into both β-rings of β-carotene to produce zeaxanthin in a recombinant *E. coli* strain that carried carotenoid biosynthetic genes (Blasco et al. 2004). In contrast, in *A. thaliana*, CYP97C1 was shown to be involved in the synthesis of lutein, which is an α-xanthophyll. The synthesis of lutein from α-carotene (β, *ε*-carotene) requires the hydroxylation of one β- and one *ε*-ring, and it was demonstrated that CYP97C1 could achieve *ε*-ring hydroxylation as well as also contribute to β-carotene hydroxylase activity *in vivo* (Tian et al. 2004). However, it was later demonstrated that another P450 (CYP97A3) was also involved in the synthesis of lutein in *Arabidopsis* and in this case, the enzyme displayed β-carotene-hydroxylase activity (Kim and DellaPenna 2006). Thus, the synthesis of lutein from α-carotene in *Arabidopsis* involves two P450s that act sequentially, whereas in the synthesis of β-xanthophylls from β-carotene that requires two β-ring-hydroxylation reactions, two non-heme di-iron-type β-carotene hydroxylases are involved (Kim et al. 2009).

In *X. dendrorhous*, a single gene (*crtS*) controls the synthesis of astaxanthin from β-carotene. For this reason, the gene product was named astaxanthin synthase. The astaxanthin-synthase enzyme catalyzes the hydroxylation and ketolation of β-carotene to produce astaxanthin and interesting, this enzyme also belongs to the cytochrome P450 protein family (Alvarez et al. 2006; Ojima et al. 2006). Although two different functional groups are incorporated through the oxygenation of β-carotene to form astaxanthin, these are products of hydroxylations performed by astaxanthin synthase. The proposed synthetic mechanism (Ojima et al. 2006) involves a series of hydroxylations of allylic carbons, starting at the C4 of the β-ionone ring of β-carotene, which is hydroxylated twice, followed by the spontaneous elimination of a water molecule to form the keto group at this position. Next, the C3 in the C4-keto intermediate β-ring is hydroxylated. In this manner, the synthesis of astaxanthin proceeds from β-carotene through echinenone to phoenicoxanthin via canthaxanthin or hydroxy-echinenone, to yield astaxanthin.

To date, a P450 system involved in the synthesis of astaxanthin from β-carotene has been reported only in *X. dendrorhous*, suggesting that a unique P450 system evolved independently in this yeast and specialized in the synthesis of astaxanthin through a mechanism different from that employed in other astaxanthin-producing organisms. Furthermore, in the eukaryotic microsomal class II P450 systems, the P450 redox partner is generally the flavoprotein cytochrome P450 reductase (CPR) (McLean et al. 2005; van den Brink et al. 1998), and a *X. dendrorhous* mutant strain lacking the wild-type CPR-encoding gene accumulates β-carotene because it is unable to synthesize astaxanthin (Alcaino et al. 2008). In addition, in a recombinant *S. cerevisiae* strain that carried the *X. dendrorhous* carotenogenic genes, astaxanthin

production, although in a very small proportion, was achieved only in the presence of the *X. dendrorhous* CPR enzyme even though *S. cerevisiae* has an endogenous CPR and could heterologously express several functional cytochrome P450s (Ukibe et al. 2009). Furthermore, based on protein modeling and molecular dynamic simulations, the larger interfacial area of interaction and the higher number of hydrogen bonds and saline bridges formed at the interaction surface indicated that astaxanthin synthase preferentially interacts with the cognate CPR rather than with the CPR of *S. cerevisiae* (Alcaino et al. 2012). These observations suggested that astaxanthin synthase is a unique P450 enzyme that has a high specificity for its own CPR.

1.4 Concluding Remarks

Carotenoids are a large group of natural compounds that are broadly distributed in nature and that are responsible for the color of many animals, plants and microorganisms. These metabolites play essential and important biological roles as accessory light-harvesting pigments of photosynthetic systems, signals for pollination and seed dispersal, photoprotective antioxidants, regulators of membrane fluidity and contributors to ocular health and to the health of other animal and human bodily systems, among others. Due to their numerous properties and applications, carotenoids have also received particular attention from commercial enterprises. In this regard, the global market for commercially applied carotenoids was estimated to be approximately US$1.2 billion in 2010 and it is expected to grow to US$1.4 billion in 2018 through a compound annual growth rate of 2.3 % (BCC-Research 2011).

Carotenoids are found in many fungi and bacteria; however, not all of these microorganisms produce these pigments and no carotenogenic genes are found in their genomes. Interestingly, carotenoids are not essential for all of the carotenoid-producing organisms. Whereas carotenoids are essential for photosynthetic organisms, several mutant microorganisms unable to produce these pigments do not exhibit any special phenotypic modifications under laboratory-controlled conditions, except the evident deficiency of pigmentation. However, albino mutants obtained from several carotenoid-producing species have been shown to be less tolerant to oxidative or light stress. These findings suggest that even though carotenoids are not essential for the survival of some species, without doubt, the ability to produce these pigments provide a protective advantage to microorganisms.

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Chapter 2
Biosynthesis of Carotenoids in Plants: Enzymes and Color

Carolina Rosas-Saavedra and Claudia Stange

Abstract Carotenoids are the most important biocolor isoprenoids responsible for yellow, orange and red colors found in nature. In plants, they are synthesized in plastids of photosynthetic and sink organs and are essential molecules for photosynthesis, photo-oxidative damage protection and phytohormone synthesis. Carotenoids also play important roles in human health and nutrition acting as vitamin A precursors and antioxidants. Biochemical and biophysical approaches in different plants models have provided significant advances in understanding the structural and functional roles of carotenoids in plants as well as the key points of regulation in their biosynthesis. To date, different plant models have been used to characterize the key genes and their regulation, which has increased the knowledge of the carotenoid metabolic pathway in plants. In this chapter a description of each step in the carotenoid synthesis pathway is presented and discussed.

Keywords Carotenoid plant models • Synthesis regulation • Carotenoid gene characterization • Multienzymatic complex • Key enzymes

2.1 Introduction

In nature, colors obtained from fruits, flowers, roots, vegetables and microalgae are called biocolors because of their biological origin (Pattnaik et al. 1997). Biocolors are mainly attributed to chemically distinct pigments such as carotenoids, flavonoids, betalains, and chlorophylls, among others, with carotenoids being mainly responsible for yellow, orange and red colors (Grotewold 2006). Carotenoids are synthesized in plastids of all photosynthetic organisms (Cuttriss et al. 2011) as well as in fruits, flowers, seeds and reserve roots (Ruiz-Sola and Rodríguez-

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Concepción 2012). Some bacteria and fungi are also capable of producing carotenoids in response to growth and environmental conditions (Velayos et al. 2000; Iniesta et al. 2008). Other organisms, such as animals do not synthesize carotenoids and must acquire them through dietary intake as these pigments, especially α-carotene, β-carotene and β-cryptoxanthine, are precursors of vitamine A, retinal and retinoic acid, which play essential roles in nutrition, vision and cellular differentiation, respectively (Krinsky et al. 1994). Some curious exceptions exist in nature such as the synthesis of carotenoids in human protist parasites and aphids (Tonhosolo et al. 2009; Moran and Jarvik 2010), which are explained by the existence of a remnant plastid, known as an apicoplast, and as a result of lateral transfer of carotenogenic genes from a fungus respectively. This makes aphids the only known animal to date capable of synthesizing their own carotenoids.

In animals, the regular ingestion of carotenoids also delays aging due to their antioxidant properties (Bartley and Scolnik 1995; Snodderly 1995; Mayne 1996; Giuliano 2000; Delcourt et al. 2006; Rao and Rao 2007; Zu et al. 2014). Birds, fish and crustaceans utilize carotenoids for pigmentation and nutritional purposes too. For example, the cetocarotenoid astaxanthin is responsible for the orange color of salmon meat and lobster shells (Grotewold 2006, see Chap. 3). Carotenoids also have commercial relevance in the food and cosmetic industries (Klaui 1982; Bjerkeng 2000) as well as in the animal feed industry where they are mainly manufactured as poultry and fish feed additives (Bjerkeng 2000).

From a chemical point of view, carotenoids are the second most abundant naturally occurring pigments on earth, with more than 750 structurally different compounds (Nisar et al. 2015). Also, carotenoids are undoubtedly the most important natural polyunsaturated isoprenoids, widely distributed in the world (Britton et al. 2004). These molecules typically consist of a C-40 hydrocarbon backbone, composed of eight isoprene units joined in a head-to-tail fashion except the central unit, which has a reverse connection (Álvarez et al. 2013, see Chap. 3). This means that carotenoids are polyisoprenoid compounds and can be divided into two main groups:

(i) Carotenes or hydrocarbon carotenoids, which are composed of carbon and hydrogen atoms
(ii) Xanthophylls that are oxygenated hydrocarbon derivatives with cyclic and acyclic structures that contain at least one oxygen function such as hydroxyl, keto, epoxy, methoxy, or carboxylic acid groups (Armstrong 1994).

Several prefixes, preceded by the number of the carbon atom(s) that contain the modification, are used for the systematic nomenclature of modified carotenoids. For example, the *apo* prefix designates the shortening of the skeleton by the formal removal of fragments from one or both ends of the carotenoid molecule, *epoxy* is used to indicate oxygen bridges and *hydro* or *dehydro* designates the addition or removal of hydrogens, and applies to the elimination of CH3, CH2, or CH groups (Álvarez et al. 2013).

In plants, carotenoids are synthesized in both light- and dark-grown tissues, such as leaves, endosperm, and roots. The carotenoid biosynthetic pathway occurs

in plastids were they are also stored and accumulated in envelope/thylakoid membranes and plastoglobuli (Jeffrey et al. 1974; Siefermann-Harms 1978; Austin et al. 2006). It has been suggested that the differential distribution of carotenoids between etioplasts and chloroplasts might also require differential localization of their biosynthetic enzymes (Shumskaya et al. 2012, see Chap. 9).

2.2 Biological Functions of Carotenoids

Carotenoids play an important role in human health and nutrition. Vertebrates do not synthesize carotenoids and depend on dietary carotenoid sources for making their retinoids, such as retinal (the main visual pigment), retinol (vitamin A) and retinoic acid (a substance controlling morphogenesis) (Fraser and Bramley 2004; Krinsky and Johnson 2005, see Chap. 14). The main precursor of retinoids is β-carotene, also called provitamin A, which contains two unsubstituted beta-ionone rings at the two ends of the molecule (Grune et al. 2010). β-carotene deficiency in human diet causes symptoms ranging from night-blindness to those of xerophthalmia and keratomalacia, leading to total blindness (Berson 1982; Sommer and Vyas 2012). Furthermore, vitamin A deficiency exacerbates afflictions like diarrhea, respiratory diseases and childhood diseases such as measles (Mayo-Wilson et al. 2011). On the other hand, certain carotenoids, such as α- and β-carotene, are good antioxidants and are necessary for human health. Supplementation of the human diet with the carotenoids lycopene or β-carotene has been shown to reduce the risk for the incidence of a number of diseases such as macular degeneration (Delcourt et al. 2006), certain types of cancer (van Poppel and Goldbohm 1995; Zu et al. 2014), neurodegenerative disease (Lu et al. 2010; Obulesu et al. 2011) and coronary heart diseases (Mayne 1996; Karppi et al. 2013).

In plants, carotenoids and their derivatives have an indispensable and prominent role. These molecules endow flowers and fruits with distinct colors and fragrance. Indeed flower color is considered one of the major attractants to pollinators in insect pollinated plants by offering a visual signal (Kevan and Baker 1983). In addition, volatile apocarotenoids mediate plant–animal interactions for pollination or seed dispersal and enhance the flavor characteristics of food crops. Certain carotenoids in the endosperm tissue of some food crops provide nutritional value and have been targets for improvement, especially in cereal crops of the grass family (Kevan and Baker 1983; Harjes et al. 2008; Vallabhaneni and Wurtzel 2009; Yan et al. 2010; Wurtzel et al. 2012, see Chap. 13). Furthermore, carotenoids play essential and multiple roles in photoprotection against photooxidative damage and heat stress to plant cells via energy dissipation and free radical detoxification, which limits damage to membranes and proteins (DellaPenna and Pogson 2006). Therefore, carotenoid antioxidants increase heat and light stress tolerance by protecting membranes from reactive oxygen species and lipid peroxidation (Davison et al. 2002; Havaux et al. 2007; Johnson et al. 2007; Li et al. 2008; Havaux 2014). For example, two molecules of β-carotene in the reaction centre of photosystem II (PSII) participate in singlet oxygen quenching and in the cyclic electron flow along

with Cyt b-559 (Miyake et al. 2002; Telfer et al. 2003, see Chap. 4). In addition, plants with reduced zeaxanthin levels exhibit increased sensitivity to light stress, as zeaxanthin prevents the oxidative damage of the thylakoid membranes (Havaux and Niyogi 1999; Verhoeven et al. 2001). Carotenoids are also involved in photosystem assembly where, from the antenna complex, they harvest light in a broader range of wavelengths in the blue region of the visible light spectrum and subsequently transfer the energy to chlorophyll (Grotewold 2006; Dall'Osto et al. 2007; Walter and Strack 2011).

Carotenoids play a key role as precursors to fitohormones such as ABA and strigolactones which influence processes as diverse as morphogenesis, seed dormancy, and environmental adaptation of plants (Auldridge et al. 2006; Paszkowski 2006; Gomez-Roldan et al. 2008; Messing et al. 2010; Walter et al. 2010; Walter and Strack 2011, see Chap. 12). Particularly, ABA is a C_{15} apocarotenoid derived from the cleavage of xanthophylls (Nambara and Marion-Poll 2005) and it has been linked to several physiological processes in plants, among them the regulation of seed dormancy and germination, and the response to abiotic stress (temperature, light and drought) (Raghavendra et al. 2010). On the other hand, the biosynthetic pathway to strigolactones has recently been shown to involve carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8, to convert β-carotene to a lactone, given the name carlactone (Alder et al. 2012, see Chap. 12). In addition to their important role as rhizosphere signaling molecules (Akiyama et al. 2005; Bouwmeester et al. 2007), it has been demonstrated that strigolactones also act as a new hormone class that inhibits shoot branching in plants and hence regulates above-ground plant architecture (Gomez-Roldan et al. 2008; Umehara et al. 2008, see Chap. 12).

2.3 Vegetal Models as a Tool Box to Study Plant Carotenogenesis

Biochemical and biophysical approaches based on *in vitro* and *in vivo* testing have provided significant advances in our understanding of the structural and functional roles of carotenoids in plants. The use of molecular genetics has been critical in the study of carotenoid synthesis and function by using genetically disruption or over expression of key components of the pathway *in planta.* The identification, characterization and utilization of mutant plant lines, in which the carotenoid composition has been predictably manipulated at the genetic level, has allowed insights into the *in vivo* role of carotenoids and carotenogenic genes in plants. Today, the ongoing research in this field has allowed the study of carotenoid biosynthesis and function in different carotenoid-enriched plants used as models, including *Arabidopsis* (*Arabidopsis thaliana*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), daffodil (*Narcissus sp.*), citrus (*Citrus sp.*), tobacco (*Nicotiana tabacum*), carrots (*Daucus carota*) among others (Dogbo et al. 1988; Beyer et al. 1989; Cunningham and Gantt 1998; Hirschberg 2001; Fraser and Bramley

2004; Stange et al. 2008; Fuentes et al. 2012; Kachanovsky et al. 2012; Ruiz-Sola and Rodríguez-Concepción 2012). Particularly, extensive study of carotenoid biosynthesis in *Zea mays* (maize), a major food crop, has benefitted from the diverse germplasm collection, phenotypic mutants, genetic and physical mapping, and quantitative trait loci (QTL) affecting carotenoid biosynthesis (Wurtzel et al. 2012). Other interesting models for understanding carotenogenesis include saffron (Frusciante et al. 2014) and kiwifruit (Ampomah-Dwamena et al. 2009). However, much remains to be understood about the regulation of carotenoid biosynthesis in the context of the manifold roles of carotenoids in plants.

To date, the knowledge generated has helped to define and characterize the key enzymes for carotenoids production in plants (Farré et al. 2011; Quinlan et al. 2012; Ruiz-Sola and Rodríguez-Concepción 2012) and provided insight into the integration of carotenoid synthesis with components of other metabolic pathways.

2.4 Carotenoid Biosynthetic Pathway: Genes, Proteins and Products

Carotenoids, as well as other isoprenoids, are built from the 5-carbon (5C) compound isopentenyl pyrophosphate (IPP). In plants, there are two sources of IPP: The cytosolic mevalonic acid pathway (MVA, see Chap. 1, Fig. 1.1) and the plastid methylerythritol 4-phosphate (MEP, Chap. 1, Fig. 1.1) pathway (Lichtenthaler et al. 1997; Rodríguez-Concepción and Boronat 2002; Eisenreich et al. 2004). In the cytosolic pathway, IPP is formed from three molecules of acetyl-CoA via 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA), mevalonic acid (MVA), mevalonic acid 5-phosphate (MVAP) and mevalonic acid 5-diphosphate (MVAPP). Hence, this pathway is called the mevalonic acid pathway (MVA). Before chain elongation begins IPP isomerase (IDI) catalyzes IPP conversion into its allylic isomer, dimethylallyl pyrophosphate (DMAPP), which is the initial, activated substrate for the formation of sesquiterpenes (C15) and triterpenes (C30) such as sterols (Laule et al. 2003; Hsieh and Goodman 2005). DMAPP condenses with a molecule of IPP to give the C10 compound, geranyl pyrophosphate.

In contrast to the MVA pathway, the MEP pathway is localized in plastids and is the major source for plant isoprenoids biosynthesis (Bramley 2002, Lichtenthaler 1999). The IPP and DMAPP used for carotenoid biosynthesis in plants are derived from the MEP pathway (Rodríguez-Concepción and Boronat 2002; Eisenreich et al. 2004). In addition, a diverse range of compounds including monoterpenes, tocopherols, phylloquinone, gibberellins (GA), chlorophylls, and plastoquinone are also derived from IPP and DMAPP (Rodríguez-Concepción 2010).

Along the pathway, carotenoids can be cleaved into apocarotenoids by enzymes of the carotenoid cleavage dioxygenase (CCD) family (Auldridge et al. 2006, see Chap. 12). Apocarotenoids are a class of terpenoid compounds, which include some important volatile compounds such as β-ionone, geranyl acetone, pseudoionone,

α-ionone and 3-hydroxy-β-io-none (Simkin et al. 2004). The *Arabidopsis* CCD family has nine members, including four CCDs (CCD 1, 4, 7 and 8) and five 9-cis-epoxycarotenoid dioxygenases (NCED 2, 3, 5, 6 and 9, see Chap. 11). NCEDs specifically catalyze the first step of the ABA biosynthesis pathway and use 9-cis-viola-xanthin and 9-cis-neoxanthin as substrates (Nambara and Marion-Poll 2005).

2.4.1 Plastid Localization of the Carotenogenic Pathway

The core of the carotenoid biosynthetic pathway consists of around 10 enzymes. In plants, the enzymatic formation of carotenoids occurs on plastid membranes and is mediated by nuclear-encoded enzymes (DellaPenna and Pogson 2006; Cuttriss et al. 2011). However, the location of the biosynthetic pathway as a complete entity for controlling the unique spatial distribution of carotenoids remains unclear, even though biochemical studies made clear that carotenoid enzyme location is critical for activity. This suggests that carotenoids destiny may be different on the plastid envelope (e.g. conversion to apocarotenoids involved in mediating signaling) as compared to carotenoids on thylakoid membranes, where these pigments function as structural components for photosynthesis or photoprotection. According to Cunningham and Gantt (Cunningham and Gantt 1998) the carotenoid biosynthetic enzymes are likely to be organized in protein complexes in plants. Evidence of *in vivo* channeling (De la Guardia et al. 1971; Candau et al. 1991), and detection of high molecular weight complexes containing biosynthetic enzymes (Al-Babili et al. 1996; Lopez et al. 2008) support the fact for the limited detection of pathway intermediates (Wurtzel 2004; Quinlan et al. 2012).

Mass spectrometry studies of the chromoplast proteome found individual carotenoid enzymes mostly in plastoglobuli (Ytterberg et al. 2006) or in membrane fractions (Wang et al. 2013). Plastoglobulies attached to thylakoid membrane of chloroplasts contain PSY (with the exception of the “yellow endosperm” allele for maize PSY1), which would suggest that PSY is physically separated from the rest of the enzymes of the pathway (Shumskaya et al. 2012). The envelope localization of carotenoid enzymes can be explained by the need to supply carotenoids for abscisic acid (ABA) synthesis, which occurs outside of the plastid (Cutler et al. 2010). In particular, in recent proteomic studies on *Arabidopsis thaliana* chloroplasts (Joyard et al. 2009; Ferro et al. 2010), many, but not all carotenoid biosynthetic enzymes are found in envelope membranes, while only a few carotenoid enzymes are identified in thylakoids (Ytterberg et al. 2006; Joyard et al. 2009; Ruiz-Sola and Rodríguez-Concepción 2012). These enzymes include xanthophyll cycle enzymes and phytoene desaturase (PDS). In pepper (*Capsicum annuum*) fruit chromoplasts, most carotenoid enzymes are localized to plastoglobuli. In maize (*Zea mays*), through proteomic studies, PDS and ZDS were the only carotenoid enzymes detected in membrane fractions of bundle sheath and mesophyll cells, respectively (Friso et al. 2010). Although, carotenoids are found in both cell types, no other carotenoid biosynthetic enzymes were detected. In maize PSY, isozymes differ in

chloroplast suborganellar localization and can be found either in plastoglobuli or in the stroma and thylakoid membranes (Shumskaya et al. 2012). Even though all of the pathway enzymes are known today and most OMIC approaches have reported the location of individual enzymes, the three-dimensional structure of the "pathway" and a putative metabolomic organization is not yet understood (Shumskaya and Wurtzel 2013).

2.4.2 MEP Pathway to GGPP

The plastid-localized MEP pathway combines glyceraldehyde-3-phosphate and pyruvate to form deoxy-D-xylulose 5-phosphate (DXP), a reaction catalysed by DXP synthase (DXS) (Fig. 2.1). MEP is subsequently formed via an intramolecular rearrangement and reduction of DXP by the enzyme DXP reductoisomerase (DXR). Both DXS and DXR are important in carotenoid flux regulation. In Arabidopsis, both enzymes are encoded by single genes and appear to be rate-determining enzymes. Indeed, the Arabidopsis *Cla1* mutant, in which the DXS gene of the MEP pathway is disrupted, present photo bleaching due to the absence of protective carotenoids (Araki et al. 2000; Estévez et al. 2000). In contrast, over expression of DXS and DXR in *Arabidopsis* seedlings increases carotenoid production (Estévez et al. 2001; Carretero-Paulet et al. 2006) and the expression of *PSY* resulted in increased carotenoid accumulation and a concomitant accumulation of the DXS enzyme (Rodríguez-Villalón et al. 2009). Moreover, Recent work has also suggested the post-transcriptional regulation of DXS activity mediated by the involvement of J-protein (J20) and heat shock protein 70 (Hsp70) chaperones. Indeed, mutants defective in J20 activity exhibit reduced DXS enzyme activity and accumulate DXS protein only in an inactive form (Pulido et al. 2013). It is suggested that plastidial J20 protein appears to assist Hsp70 chaperone in the proper folding and assembly of DXS and participate in the regulation of MEP-derived isoprenoid biosynthesis (Pulido et al. 2013). In poplar, a feedback inhibition of DXS by DMAPP reveals an important regulatory mechanism of the MEP pathway and thus carotenoid biosynthesis (Ghirardo et al. 2014). The enzymes of the MEP and carotenoid pathways are preferred targets for herbicides (Withers and Keasling 2007) because these pathways are not present in animals and the blockage of any of the steps preceding the formation of lycopene inhibits carotenoid synthesis. The herbicide clomazone inhibits DXS (Müller et al. 2000, Fig. 2.1) by an indirect mechanism once it is absorbed into the plant. Clomazone is oxidized by cytochrome P450 monooxygenases (P450s) to form the putative active herbicidal form (Müller et al. 2000), ketoclomazone. This requirement for metabolic activation was shown in plants treated with P450s inhibitors such as phorate, where the herbicidal effect of clomazone was prevented (Ferhatoglu et al. 2005; Ferhatoglu and Barrett 2006). Although the antibiotic fosmidomycin is not used as an herbicide, this compound inhibits DXR (Singh et al. 2007) (Fig. 2.1), the second step of the MEP pathway (Rohmer 1998; Rohmer et al. 2004). The potential benefits of developing new

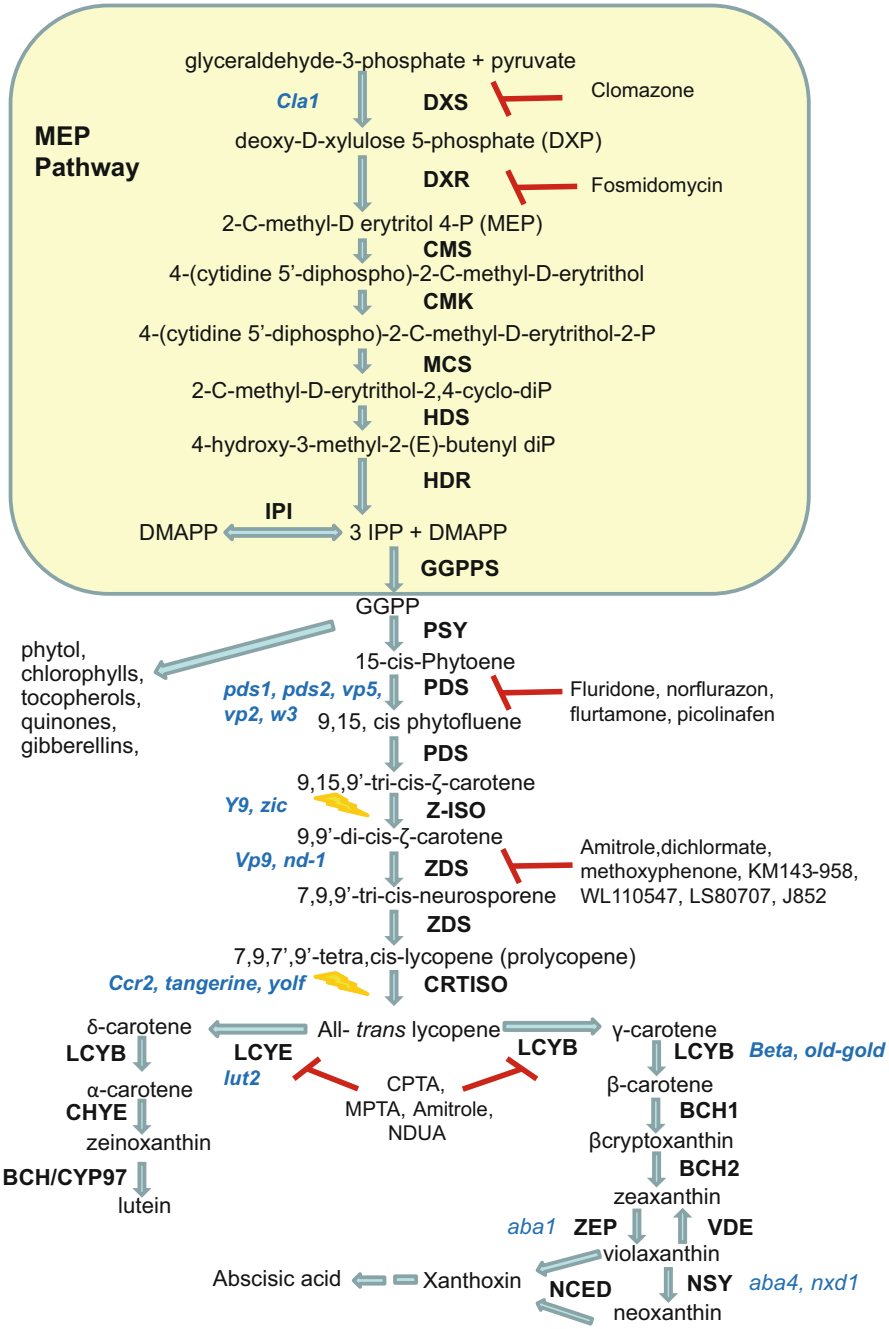

Fig. 2.1 (continued)

herbicides which interfere with pathways leading to carotenoids, such as the MEP and isoprenoid pathways, has been addressed recently (Hale et al. 2012; Corniani et al. 2014) taking into account the emergence of resistance to herbicides as an increasing problem facing agriculture (Service 2007, 2013). A number of steps are then required in the condensation between IPP and its allylic isomer DMAPP (Fig. 2.1), to form the C10 compound, geranyl pyrophosphate (GPP). The further addition of two IPP units results in the formation of C20 geranylgeranyl pyrophosphate (GGPP) the precursor of carotenoid biosynthesis (Bramley 2002; Cunningham 2002; Fraser and Bramley 2004). The sequential addition of three IPP molecules to a DMAPP molecule is catalyzed by GGPP synthase (GGPPS).

2.4.3 GGPP to Phytoene: Phytoene Synthase

Phytoene synthase (PSY) is the first dedicated enzyme of the carotenoid biosynthesis pathway and catalyzes the condensation of two GGPP molecules into phytoene as a 15-*cis* isomer by introducing a central 15-Z double bond during hydrogen elimination (Cunningham and Gantt 1998). Due to the fact that PSY is the enzyme that catalyzes the first committed step in the plant carotenoid pathway, it has been postulated to be rate-limiting, regulating the reaction flux. In fact, dosage effect of the maize *Y1* allele upon seed carotenoid content was noted as early as the 1940s (Randolph and Hand 1940), where three copies of the dominant *Y1* allele in the triploid endosperm conditioned the most yellow seeds (endosperm) in contrast to homozygous white *y1* seeds. Cloning and subsequent sequence analyses identified the *Y1* gene as the gene coding for *PSY1* (Buckner et al. 1996). Considering PSY as rate-limiting, it has been a preferred target for genetic manipulation. Fray et al. (1995) were the first to report ectopic quantitative and qualitative changes in

Fig. 2.1 (continued) Isoprenoid and Carotenoid pathway in plants. Schematic representation of the plastidial MEP (2-C-methyl-D-erythritol-4-P) pathway: DXS: deoxyxylulose 5-phosphate synthase, DXR: deoxyxylulose 5-phosphate reductoisomerase, DXP: Deoxy-D-xylulose-5-P, CMS: 2C-methyl-D-erythritol 4-phosphate cytidyltransferase, CMK: 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol kinase, MCS: 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, IPP (isopentenyl pyrophosphate), GGPPS: geranylgeranyl pyrophosphate synthase, DMAPP: dimethylallyl pyrophosphate, IPI: isopentenyl pyrophosphate isomerase, GGPP: geranylgeranyl pyrophosphate. The relevant genes for carotenoid synthesis are: PSY: phytoene synthase, PDS: phytoene desaturase, Z-ISO: ζ-carotene isomerase, ZDS: ζ-carotene desaturase, CRTISO: carotene isomerase, LCYB: lycopene β-cyclase, LCYE: lycopene ε-cyclase, BCH: carotenoid β hydroxylase, CHYE: carotenoid ε-hydroxylase (CYP97C1, CYP97A3), ZEP: zeaxanthin epoxidase, VDE: violaxanthin deepoxidase, NSY: neoxanthin synthase, NCED: 9-cis-epoxycarotenoid dioxygenase. Reported mutants in isoprenoid and carotenogenic genes are written in blue. Chemical inhibitors of some enzymes are included with a red T symbol. Light, referred as a yellow ray can replace Z-ISO and CRTISO activity in photosynthetic organs

carotenoids through fruit-specific expression of a phytoene synthase cDNA under a CaMV 35S promoter in tomato. Interestingly, the overexpression lines present a dwarf phenotype with a 30-fold reduction in gibberellin A1 (GA1) and chlorophyll levels. The dwarf character was inherited with an inverse relationship between plant height and expression of *PSY*, which redirects GGPP to phytoene and diverts this intermediate away from the gibberellin (GA) and phytol biosynthetic pathways. On the other hand, the overexpression of an exogenous daffodil *PSY* in Japonica rice model variety Taipei 309 leads to phytoene accumulation in endosperm, which was the first instance of carotenoid engineering in rice (Burkhardt et al. 1997). The regulation and rate-limiting role of PSY was also evident in *Brassica napus* expressing an additional *PSY* gene from the bacterium *Erwinia uredovora*, crtB (Shewmaker et al. 1999). The transgenic plants resulted in a 50-fold increase in total carotenoid content, predominantly α-carotene, β-carotene and phytoene. A similar study was reported in transgenic tomato carrying the additional *E. uredovora crtB* gene under the control of fruit-specific polygalacturonase promoter (Fraser et al. 2002). The PSY activity in these transgenic plants was increased five to ten-fold. This was accompanied by a two- to four-fold increase in the total carotenoid content of primary transformants whereas phytoene was increased 2.4 fold. In another study, *E. uredovora* crtB was overexpressed in potato (Ducreux et al. 2005, see Chap. 13). In addition, constitutive *PSY* overexpression has enhanced total carotenoid contents and substantially increased synthesis of β-carotene in a variety of many other vegetal species such as linseed flax, carrots, maize, and cassava roots (Fujisawa et al. 2008; Maass et al. 2009; Naqvi et al. 2009; Welsch et al. 2010, see Chap. 13). It is important to take into account that most of those results obtained after over expression of the *PSY* gene in carotenoid-producing fruits may result in a shift in the regulatory control point within the pathway. Furthermore, the bigger increase in the carotenoid content in those cases of using a plant *PSY* instead of the bacterial *crtB* could be due to the less effective protein-protein interaction in the multienzymatic complexes produced with CRTB compared with those formed with plant *PSY*s.

Recently, a phytoene synthase-RNAi construct was delivered into the *Oncidium* hybrid orchid as the first report using RNAi approaches to break down carotenogenesis in this plant (Liu et al. 2014). Transgenic lines displayed a semi-dwarf phenotype and brilliant green leaves as a consequence of changes in the carotenoid, gibberellin, abscisic acid and chlorophyll biosynthetic pathway (Liu et al. 2014).

The localization of PSY within the chloroplast is another highlight finding reported recently (Shumskaya et al. 2012; Shumskaya and Wurtzel 2013). While the majority of phytoene synthases, such as maize (PSY2 and PSY3), rice and Arabidopsis PSYs, are found to localize to plastoglobuli, maize PSY1 isozymes are localized to distinct chloroplast suborganellar sites (e.g. globular or fibrillar) based on their allelic variation, suggesting that PSY1 sequence variation can affect suborganellar localization of carotenoid storage and bioavailability.

2.4.4 *Lycopene Synthesis: Teamwork of Desaturases and Isomerases*

In plants and cyanobacteria, phytoene undergoes four successive dehydrogenations and isomerizations to produce lycopene. The first two desaturation steps are catalyzed by phytoene desaturase (PDS), while the latter two reactions are catalyzed by (zeta) ζ-carotene desaturase (ZDS). These desaturation reactions introduce a series of carbon–carbon double bonds that constitute the chromophore in carotenoid pigments, and they transform the colorless phytoene into the red-colored lycopene. These reactions lead to a poly-cis pathway in contrast to the all-trans pathway carried out by CRTI in bacteria and fungi (Sandmann 1994; Armstrong 1997, see Chap. 1). Therefore, in concert to these steps, ζ-carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO) catalyze *cis–trans* reactions to isomerize the four cis-bonds introduced by the desaturases (Fig. 2.1). Z-ISO activity occurs upstream of CRTISO and catalyzes the *cis* to *trans* conversion of the product of PDS to form the substrate of ZDS (Li et al. 2007) whereas CRTISO is involved in the conversion of poly-*cis* lycopene to *trans*-lycopene (Figs. 2.1 and 1.3. Isaacson et al. 2002; Park et al. 2002). Reactions performed by Z-ISO and PDS precede ZDS which requires substrates with desaturated 11 and 11′ bonds (Albrecht et al. 1996) and 15-15′ in *trans* (Breitenbach and Sandmann 2005). The isomerization reaction carried out by CRTISO is the last reaction since it requires substrates to have adjacent 7- and 9-*cis* bonds (Isaacson et al. 2004). It is not understood why bacteria and fungi accomplish these reactions with a single enzyme, CRTI. The most likely explanation refers to possible roles of the *cis*-intermediates in regulatory signaling pathways (Kachanovsky et al. 2012, see Chap. 1)

2.4.4.1 Desaturases

Two sequential desaturations of phytoene, catalyzed by PDS, result in the formation of phytofluene, followed by the formation of ζ-carotene. Additional desaturations, catalyzed by Z-ISO and ZDS, give rise to 7,9,9′-*cis*-neurosporene. The deduced peptide sequences of PDS and ZDS show a high degree of similarity between different plant species. Nevertheless, PDS shows 33% to 35% identity to ZDS, they can be grouped together in a phylogenetic tree, indicating a common ancestor for both proteins (Beyer et al. 1989; Albrecht et al. 1995; Hirschberg et al. 1997). The desaturases require FAD and the association with a membrane to be active (Nievelstein et al. 1995; Bonk et al. 1997). The FAD appears to feed an electron transport chain involving quinones and the plastid terminal oxidase (PTOX). Particularly, quinones act as electron acceptors for PDS and ZDS desaturation reactions, as demonstrated in daffodil and Arabidopsis (Beyer 1989; Mayer et al. 1990; Norris et al. 1995), in which the impaired plastoquinone biosynthesis in *pds1* and *pds2* mutants resulted in albino phenotypes and phytoene accumulation (Norris et al. 1995; Norris et al. 1998). The cloning of the *IMMUTANS* gene encoding PTOX also

revealed a link between phytoene desaturation and redox poise of the chloroplast (Al-Babili et al. 1996; Josse et al. 2000; Carol and Kuntz 2001). The disruption of the *IMMUTANS* gene prevents oxidation during chlorophyll biosynthesis and results in the accumulation of phytoene (Carol et al. 1999; Wu et al. 1999; Aluru et al. 2001).

Phytoene desaturase is also a rate-limiting enzyme in carotenoid synthesis (Chamovitz et al. 1993). Maize mutants viviparous5 (*vp5*), *vp2* and *white3* (*w3*) are associated with the function of PDS by the accumulation of phytoene both in leaf and seed (Treharne et al. 1966; Neill et al. 1986; Hable et al. 1998) The *vp5* locus was established as the structural gene for PDS. A soybean cDNA encoding PDS was cloned using a heterologous probe derived from the *Synechococcus PDS* gene (Bartley et al. 1991). The tomato *PDS* cDNA was cloned by a similar approach and its identity was confirmed by expression in *E. coli*, which resulted in the formation of ζ-carotene (Pecker et al. 1992). The pepper *PDS* coding sequence, isolated using antibodies raised against the purified protein, showed high homology to soybean and tomato *PDS* genes and active PDS recombinant enzyme was obtained in *E. coli* (Hugueney et al. 1992). Transgenic tobacco plants overexpressing *PDS* had no phenotypic or metabolic effects (Busch et al. 2002; D'Haeze 2002). However, *PDS* down-regulation led to a dramatic accumulation of phytoene (Busch et al. 2002; D'Haeze 2002), a lethal phenotype in homozygous seedlings (Busch et al. 2002) and albinism (Travella et al. 2006; Kirigia et al. 2014). Transgenic tobacco plants showing different degrees of albinism caused by *PDS* silencing presented a decrease in total carotenoid, chlorophyll and PSII efficiency (Wang et al. 2009).

The protective function of carotenoids depends on the complete conjugated double-bond system. As a consequence, any step in carotene biosynthesis up to lycopene formation is a potential target for a herbicidal inhibitor (Böger and Sandmann 1998). PDS is the molecular target site for several herbicides such as fluridone, norflurazon, flurtamone and picolinafen (Dayan and Duke 2003). These herbicides compete for the binding site of plastoquinone (Breitenbach et al. 2001) as an essential cofactor for this enzyme (Norris et al. 1995). ZDS is essential for cell growth, stress responses and carotenoid biosynthesis, providing a precursor for cyclization reactions (Bartley et al. 1999). Coding sequences for *ZDS* have been isolated and characterized in maize (Matthews et al. 2003), pepper (Breitenbach et al. 1999), common fig (Araya-Garay et al. 2012) and goji berry plants (Li et al. 2015). Maize ZDS did not function efficiently in a heterologous complementation system where PDS did (Matthews et al. 2003). Suggested reasons for the low activity of carotenoid desaturases include: End product inhibition of ZDS (Sandmann and Böger 1989; Breitenbach et al. 1999), failure to reconstitute the interactions of an assembly complex responsible for substrate channeling (Breitenbach et al. 1999), the absence of a carotene isomerase (Bartley et al. 1999), among others.

Mutation of the *ZDS* gene from Arabidopsis resulted in impaired carotenoid biosynthesis and subsequent spontaneous cell death from the increased content of superoxide (Dong et al. 2007).

Moreover, it has been shown that loss of function of the ζ -carotene desaturase encoded by *ZDS*/CLB5/SPC1/PDE181 arrests chloroplast biogenesis at a very early

stage of development and Arabidopsis plants also exhibit dramatic alterations in leaf morphology and in the expression of a variety of chloroplast proteins and genes required for leaf development. These phenotypes were not observed in mutants deficient in the closely related desaturase *PDS* (Avendaño-Vázquez et al. 2014). The *vp9* maize *ZDS* mutant has reduced or suppressed ABA and carotenoid accumulation in both endosperm and vegetative tissues, even when it accumulates ζ-carotene (Matthews et al. 2003), while the non dormant-1 (*nd*-1) mutant of sunflower (*Helianthus annuus* L.) was characterized by an albino and viviparous phenotype (Conti et al. 2004).

Compared to inhibitors of phytoene desaturation, fewer compounds interfere with the ZDS reaction. The following compounds have been reported to accumulate ζ-carotene in whole plants: Amitrole, Dichlormate (Burns et al. 1971), Methoxyphenone (NK049) (Fujii et al. 1977), KM 143-958 (Chollet et al. 1990), WL 110547 (Sandmann et al. 1996; Kerr and Whitaker 1987), LS 80707 (Sandmann et al. 1985), J852 (Chollet et al. 1990).

The modes of action of J852 and LS 80707 (including related compounds) in carotene biosynthesis are well defined. Inhibitors treatment causes ζ-carotene accumulation and the concurrent bleaching of plants (Sandmann and Böger 1989; Chollet et al. 1990). Pepper leaves treated with the herbicide J852 showed an accumulation of phytoene and ζ-carotene. Unexpectedly, none of the genes coding for PSY, PDS, ZDS or the Terminal Oxidase associated with phytoene desaturation were induced upon herbicide treatment in pepper leaves or seedlings (Simkin et al. 2000).

2.4.4.2 Isomerases

Plants do not only require desaturases to convert 15-*cis*-phytoene into all-*trans* lycopene. In fact, while PDS is active it prefers 15-*cis* substrates (Breitenbach and Sandmann 2005). ZDS is only active against substrates with a 15-*trans* conformation, (Breitenbach and Sandmann 2005) indicating the requirement of two types of isomerase enzymes. Particularly 15-*cis* phytoene is transformed by PDS into 15,9′-di-*cis*-phytofluene, and eventually 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized at the 15-*cis*-double bond to form the substrate of ZDS, 9,9′-di-*cis*-ζ-carotene (Bartley et al. 1999; Matthews et al. 2003; Breitenbach and Sandmann 2005). The isomerization step of the 15-*cis* bond of ζ-carotene to *trans*, can be mediated either by light or by the 15-*cis*-ζ-carotene isomerase (Z-ISO) occurring in the chloroplasts of photosynthetic tissues or in other plastid types, respectively (Breitenbach and Sandmann 2005; Li et al. 2007, Fig. 2.1). However, the loss-of-function phenotypes detected in mutants of maize and Arabidopsis strongly suggest that Z-ISO activity is required even in the presence of light. Plants with insufficient Z-ISO also grow poorly under the stress of fluctuating temperature. Z-ISO is predicted to be an integral membrane protein with several membrane-spanning domains, but there is no available information about its sub organellar localization, showing whether the protein is found in the envelope or the thylakoid membranes

(Janick-Buckner et al. 2001, Ruiz-Sola and Rodríguez-Concepción 2012; Beltrán et al 2015). Because climatic variations alter the need for photosynthetic and non-photosynthetic carotenoids, Z-ISO facilitates plant adaptation to environmental stress, a major factor affecting crop yield. It was also described that Z-ISO exists in a high-molecular-weight protein complex of about 480 kDa (Shumskaya and Wurtzel 2013), similarly to other carotenoid enzymes (Zybailov et al. 2008; Ishikawa et al. 2009). Z-ISO homologues can be found in plants and cyanobacteria. A mutant in the Z-ISO gene of maize, referred to as pale yellow 9 (*y9*), in addition to the isolation of Arabidopsis *zic* mutant, showed 9,15,9′-tri-*cis*-ζ-carotene accumulation in the dark, leading to the identification of 15-*cis*-ζ-carotene isomerase (Z-ISO). Expression of the *Z-ISO* transcript is highly correlated with the expression of other carotenogenic genes. Single copy genes appear to encode *Z-ISO* in most plants (reviewed in Ruiz-Sola and Rodríguez-Concepción 2012), but no evidence of alternate transcripts was found in plants such as maize or rice, except for Arabidopsis (Chen et al. 2010). The single Arabidopsis gene encoding Z-ISO (At1g10830) produces two alternate transcripts, *Z-ISO*1.1, which is highly expressed and encodes a functional enzyme, and a shorter *Z-ISO*1.2, which encodes a truncated protein that lacks the predicted C-terminal transmembrane domain and shows no Z-ISO activity. Therefore, the effect of the deletion suggests that the C-terminal TM domain is important for the function of Z-ISO, either for activity or proper folding (Chen et al. 2010). Screening for impaired carotenoid formation in dark germinated seeds of maize (*Zea mays*) and Arabidopsis mutants were performed to identify the gene by functional complementation assays in *E. coli*, expressing bacterial *GGPPS* and *PSY*. In maize Z-ISO is an enzyme related to nitrite and nitric oxide reductase U (NnrU), which is normally present in denitrifying bacteria. Considering that the bacterial denitrification pathway produces nitrogen oxides as alternate electron acceptors for anaerobic growth, it has been suggested that plant carotenogenesis evolved by recruitment of genes from noncarotenogenic bacteria (Li et al. 2007; Chen et al. 2010).

Based on the central location of the double bond in the substrate of Z-ISO, it is predicted that this enzyme recognizes the carotenoid molecule differently in comparison to the mechanism proposed for CRTI, PDS, ZDS or CRTISO, which act on one half of the molecule. The lack of sequence homology of Z-ISO to any known carotenogenic enzyme also suggests a different mechanism of reaction. Recently, it was shown that Z-ISO is a bona fide enzyme, which independently catalyzes the *cis-trans* isomerization of the 15–15′ carbon-carbon double bond in 9,15,9′-*cis*-ζ-carotene through a unique mechanism requiring a redox-regulated heme β cofactor that undergoes redox-regulated ligand switching between the heme iron and alternate Z-ISO amino acid residues (Beltrán et al. 2015). Reduction of the heme iron switches coordination of the heme to *bis*-histidine and exposes the active site for substrate binding. These results let to the proposal that Z-ISO as a metalloprotein, representing a new prototype for heme β proteins that potentially uses a new chemical mechanism.

Following the production of 9,9′-di-*cis*-ζ-carotene by Z-ISO, the enzyme ZDS carries out the stereospecific abstraction of protons from 7 and 7′, which results in

the formation of the 7 and 7′ *cis* bonds in 7,9,9′-tri-*cis*-neurosporene (Breitenbach and Sandmann 2005; Sandmann 2009) followed by the synthesis of 7,9,9′,7′-tetra-*cis*-lycopene (also called pro-lycopene) with very low efficiency (Bartley et al. 1999). Because 7,9,7′,9′-tetra-*cis*-lycopene is not a substrate for β or ε-cyclases (Schnurr et al. 1996; Yu et al. 2011), the formation of the poly-*cis* intermediates requires the recruitment of an additional isomerase to generate the all-trans-lycopene, a suitable substrate for β and ε-cyclases (Breitenbach and Sandmann 2005). In chloroplasts, the isomerization of pro-lycopene to all-*trans*-lycopene can occur non-enzymatically in the presence of light whereas the activity of CRTISO (Fig. 2.1) fulfills a critical role in converting the *cis*-double bonds introduced by PDS and ZDS to all-*trans* in tissues receiving no light exposure and in non-photosynthetic tissues (Isaacson et al. 2002; Park et al. 2002). Therefore, CRTISO-deficient plants can still synthesize carotenoids but at slower rate and only in specific tissues. The isomerase activity of CRTISO requires the presence of membranes and the flavin adenine dinucleotide (FAD) binding motif, which bonds to the reduced form of the cofactor (FADred) to catalyze a reaction without net redox changes (Isaacson et al 2004; Yu et al. 2011). The regional specificity and the kinetics of the isomerization reaction were recently determined (Yu et al. 2011).

CRTISO is thought to be evolved from the bacterial-type desaturase (CRTI) considering the high sequence homology between both proteins (Isaacson et al. 2002; Park et al. 2002; Isaacson et al. 2004; Chen et al. 2010). Nevertheless, in terms of mechanisms of reaction related to the reduced flavin requeriment for its activity, it is especially interesting that CRTISO seems to be more related to CRTY (bacterial lycopene cyclase) than to its possible ancestor CRTI (Mialoundama et al. 2010; Yu et al. 2010; Yu et al. 2011).

The Arabidopsis, tomato, and melon mutants of *CRTISO* (*ccr2*, *tangerine*, and *yolf*, respectively) accumulate *cis*-carotenes in etioplasts of seedlings or chromoplasts of fruits (Isaacson et al. 2002; Park et al. 2002; Galpaz et al. 2013). However, it is interesting to note that the CRTISO activity can partially be substituted by exposure to light in green tissues via photoisomerization (Isaacson et al. 2002; Park et al. 2002). Photoisomerization of the *cis* bonds facilitates carotene synthesis in the chloroplasts of the *ccr2* mutant, but delays greening and chlorophyll accumulation during photomorphogenesis. Similarly, loss of CRTISO causes partial inhibition of lutein biosynthesis in light-grown tissues and varying degrees of chlorosis in newly developed leaves of tomato and rice (Isaacson et al. 2002; Masamoto et al. 2004; Fang et al. 2008; Wei et al. 2010; Chai et al. 2011). In contrast to green tissues, CRTISO activity cannot be substituted by light in non-photosynthetic tissues. The etiolated tissues of *ccr2*, *tangerine*, and *yolf* fruits exhibit an orange color due to the accumulation of *cis*-lycopene (Isaacson et al. 2002; Park et al. 2002; Galpaz et al. 2013). Such accumulation is associated with a metabolite-dependent feedback regulation of early carotenoid synthesis genes. The feedback regulation of early tomato carotenoid genes (*PSY1*, *PDS*, and *ZDS*) observed in *tangerine* elucidates the recently discovered epistasis effect of *tangerine* over the *r* mutation in *PSY*, which partially restores *PSY1* expression by *cis*-carotene accumulation in *tangerine* (Kachanovsky et al. 2012).

Even though most carotenoids found in nature are primarily in the more stable all-*trans* configurations (Britton 1995), a small proportion of *cis* isomers are encountered. Interestingly, they have different biological potency than their *trans* counterparts (e.g. lower pro vitamin A activity); they are constituents of the light harvesting complex (LHC) such as 9-*cis*-neoxanthin (Liu et al. 2004) and supply substrates for the biogenesis of plant hormone, ABA such as 9-*cis*-epoxyxanthophylls (Schwartz et al. 1997).

2.4.5 Lycopene to Cyclic Carotenes: Cyclases

Lycopene is the starting compound of various end group modifications that produces a large variety of carotenoids with different physical properties. In higher plants two enzymes, lycopene β-cyclase (LYCB) (Pecker et al. 1996) and lycopene ε-cyclase (LYCE) (Cunningham and Gantt 2001, Fig. 2.1) compete for lycopene as substrate, leading to carotenoids with either β or ε-ionone rings. Therefore, cyclization of lycopene represents the first branching point in the carotenogenic pathway (Fig. 2.1).

The amino acid sequence of lycopene cyclases from plants and cyanobacteria are polypeptides with around 400 amino acids and have a molecular mass of 43 kDa (Schnurr et al. 1996). The enzymes from plants also have an additional N-terminal transit sequence of 100 amino acids (Krubasik and Sandmann 2000).

In addition, the N terminus of plant LCYB and CCS contains dinucleotide-binding motifs characteristic of an N-terminal Rossmann fold of FAD-dependent oxidoreductases (Rossmann et al. 1974; Cunningham et al. 1994; Hugueney et al. 1995). Indeed, it has been reported that CCS-bound FAD is required for enzyme activity in the presence of NADPH, which functions as a reductant of FAD (Cunningham et al. 1994). *Erwinia uredovora* LCYB, which cyclizes lycopene into β-carotene, strictly requires NADPH but proceeds without any net redox change (Schnurr et al. 1996; Hornero-Méndez and Britton 2002).

LCYB and LCYE share significant similarities in their amino acid sequences, suggesting that they have originated from a common ancestor through gene duplication (Sandmann 2002). There are also two other related carotenoid cyclase enzymes: the capsanthin-capsorubin synthase (CCS) of pepper (Cunningham et al. 1996) and the neoxanthin synthase (NSY) of tomato (Bouvier et al. 2000) and potato (Al-Babili et al. 2000). CCS catalyzes the formation of the unusual five-carbon k ring (Bouvier et al. 1994), converting antheraxanthin or violaxanthin into the k-cyclic carotenoids capsanthin or capsorubin, respectively, by a similar mechanism to lycopene cyclization (Bouvier et al. 1997). Interestingly, CCS exhibits LCYB activity when lycopene is provided as a substrate (Hugueney et al. 1995).

The LCYB enzyme catalyzes the cyclization of one end of the linear lycopene molecule to produce γ-carotene. Subsequently, the enzyme forms a second β-ring at the other extreme to yield β-carotene (Fig. 2.1). LCYE introduces a single ε-ring into lycopene to produce δ-carotene and a subsequent cyclization of the ψ- acyclic

end of δ-carotene by LCYB generates α-carotene. Carotenoids containing two ε-rings are rarely found in plants and algae (Goodwin 1980; Cunningham et al. 1996) with the notable exception of *Lactuca sativa* (lettuce), in which a unique LCYE can cyclize both ψ-ends of lycopene to generate ε,ε-carotene and the dihydroxy derivative lactucaxanthin (Siefermann-Harms et al. 1981; Phillip and Young 1995; Cunningham and Gantt 2001). Several specific domains and amino acid residues have been identified which determine whether LCYE introduces one or two ε-ionone rings (Cunningham and Gantt 2001).

Lycopene cyclases, LCYB and LCYE, are involved in determining carotenoid content and composition in different plants. Some plants fine-tune carotenoid content using tissue-specific isoforms of lycopene cyclases such as those chromoplast-specific *LCYB* genes expressed in the fruits and flowers of tomato, papaya and saffron that correlate with the accumulation of β-carotene and/or downstream xanthophylls (Ronen et al. 2000; Ahrazem et al. 2010; Devitt et al. 2010). Two color mutations in tomato, *Beta* and *old-gold* (og) affect the fruit and flower-specific *LCYB* which is encoded by the B gene (Ronen et al. 2000). *Beta* is a partially dominant, single-locus mutation that causes an orange color in the fully ripened fruit because of the accumulation of β-carotene at the expense of lycopene due to an important increase in the transcription of the B gene. By contrast, the *old-gold* (og) mutant carries a null allele in the locus B resulting in an elevated concentration of lycopene and a reduction of β-carotene (Ronen et al. 2000).

A mutation in the papaya *LCYB2* dramatically reduces its expression and is responsible for the difference between red- and yellow-fleshed fruits as consequence of lycopene accumulation (Blas et al. 2010; Devitt et al. 2010).

Transcript analysis of homo- and heterozygous Arabidopsis *lut2* mutants demonstrates that *lut2* is semidominant and their biochemical phenotype is consistent with a disruption of epsilon ring cyclization (Pogson et al. 1996). In particular, *lut2* mutation has significantly higher levels of the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) as well as β-carotene. In tomato, expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant Delta where a single dominant gene, Del, changes the fruit colour to orange as a result of accumulation of delta-carotene at the expense of lycopene (Ronen et al. 1999). Metabolic engineering to manipulate the lycopene cyclase expression and enzyme activity can bias the flux toward one or the other branch of the carotenogenic pathway (Yu and Beyer 2012; Giorio et al. 2013). Enhanced levels of β-carotene were observed in *LCYE* tuber-specific silencing of potato (Diretto et al. 2006). Similarly, *LCYE* downregulation in canola results in the enhanced accumulation of β-carotene, zeaxanthin, violaxanthin, and lutein (Yu et al. 2008). Suppression of *LCYE* in sweet potato and tobacco showed increased synthesis of a β-branch–specific pathway and enhanced tolerance to abiotic stress (Kim et al. 2013; Shi et al. 2014). Maize endosperm tissues lacking LCYB activity accumulate ε,ε-carotene, δ-carotene, and ε-carotene produced by a dual mono- or bicyclic LCYE enzyme activity (Bai et al. 2009).

The expression levels of *LCYB* and *LCYE* in tomato, rice, and Arabidopsis affect the relative cyclase activities as well as the synthesis of cyclic carotenoids playing

a critical role in determining the β-carotene/α-carotene ratio (Yu and Beyer 2012; Giorio et al. 2013).

Inhibition of these enzymes produces growth abnormalities as well as herbicidal effects. The only specific non-competitive inhibitors of lycopene cyclases are N,N-diethyl-N-[2-(4-chlorophenylthio)ethyl]amine (CPTA) and N,N-diethyl-N-[2-(4-methylphenoxy)ethyl]amine (MPTA) (Yokoyama et al. 1977; Spurgeon, 1983; Schnurr et al. 1998). CPTA exhibited preferential inhibition of the LCYB over LCYE, resulting in an accumulation of the monocyclic carotenoid precursor δ-carotene as well as lycopene (at the expense of β-carotene and xanthophylls) (La Rocca et al. 2007). MPTA belongs to a group of substituted triethylamines that have a number of effects including inhibition of lycopene cyclases (Phillip and Young 2006; Liu and Puckhaber 2011). Another inhibitor of carotenoid biosynthesis on the step of cyclization of lycopene is the bleaching herbicide amitrole (AM) (Agnolucci et al. 1996; La Rocca et al. 1998). It has been reported that amitrole (AM) treatment decreased salt tolerance of *Salicornia Europaea* by inhibiting lycopene cyclization (Chen et al. 2011). However amitrole also has other effects in plants such as inhibition of geranylgeraniol hydrogenation to phytol in chlorophyll synthesis of wheat seedlings that have been transferred from darkness to light (Rüdiger and Benz 1979).

The bleaching herbicidal compound N,N-diethyl-N-(2-undecynyl) amine (NDUA) was also identified as an inhibitor of lycopene cyclase in *Lepidium sativum* plants. Plants under NDUA treatment showed both lycopene accumulation and chlorotic effects similar to CPTA and MPTA treatments (Fedtke et al. 2001).

2.4.6 Cyclic Carotenes to Xanthophylls Cycle: Dynamic Role of Hydroxylases, Epoxidases and More

From a chemical point of view, xanthophylls are oxygenated hydrocarbon derivatives of carotenoids that contain at least one oxygen function, such as carboxylic acid, epoxy, hydroxyl, keto or methoxy groups. Xanthophylls are among the main carotenoid pigments in the photosystems of plants. The oxygen-containing xanthophylls are produced from either α- or β-carotene and require ring-specific hydroxylation reactions. β–Hydroxylase catalyzes two hydroxylation reactions, converting β-carotene to zeaxanthin via β-cryptoxanthin whereas α-carotene is twice hydroxylated by two different enzymatic reactions catalyzed by the ε- and β–hydroxylases. In *Arabidopsis thaliana*, these reactions are catalyzed by a set of four enzymes (Kim et al. 2009). Two non-heme di-iron enzymes (β-carotene hydroxylase-1 (BCH1) and -2 (BCH2)) are primarily responsible for β-ring hydroxylation of β-carotene and produce zeaxanthin, while two heme-containing cytochrome P450 enzymes (CYP97A3 and CYP97C1) preferentially hydroxylate the ε- and β-ionone rings of α-carotene, yielding lutein (Fig. 2.1). In carrot,

unusually high levels of α- and β-carotene are accumulated in leaves due to the presence of a defective carotene hydroxylase CYP97A3 (Arango et al. 2014).

Zeaxanthin is further epoxidized by the enzyme zeaxanthin epoxidase (ZEP), leading to antheraxanthin, violaxanthin and neoxanthin, a reversible reaction carried out by violaxanthin de-epoxidase (VDE), representing the xanthophyll cycle (Niyogi et al. 1998). Six types of xanthophyll cycles have been described. Four of them are based on β-xanthophylls and two on α-xanthophylls (Garcia-Plazaola et al. 2007). The common factor in all xanthophyll cycles is the light-dependent transformation of epoxidized xanthophylls to de-epoxidized ones, which facilitates the dissipation of excitation energy, and their reversion to epoxidized xanthophylls in low light (Muller et al. 2001; Latowski et al. 2004). Two of the xanthophyll cycles have been described for land plants: The violaxanthin cycle, in which violaxanthin is reversibly converted to zeaxanthin via antheraxanthin (Sapozhnikov et al. 1957; Yamamoto et al. 1962; Jahns et al. 2009) and the lutein epoxide cycle, in which lutein epoxide is reversibly converted into lutein (Bungard et al. 1999; Garcia-Plazaola et al. 2007). Both cycles are involved in the light-regulated switching of PSII from a light-harvesting state (with epoxidized xanthophylls, violaxanthin and lutein epoxide present in low light or darkness) to an energy dissipating state (with de-epoxidized xanthophylls, antheraxanthin, zeaxanthin and lutein, present in high light). Therefore, these cycles facilitate the short- and long-term acclimation of plants to varying light conditions. Although the violaxanthin cycle is present in all land plants, the lutein epoxide cycle is restricted to some species only (Esteban et al. 2009). The most commonly occurring type of xanthophyll cycles in plants and the most intensively studied is the violaxanthin cycle, also called the xanthophyll cycle, where the main product of strong light-stimulated de-epoxidation is zeaxanthin. The violaxanthin or xanthophyll cycle, first described in lettuce by Yamamoto et al. (1962), consists of the light-driven de-epoxidation of the diepoxide violaxanthin through the intermediate monoepoxide antheraxanthin into zeaxanthin, and the dark epoxidation of zeaxanthin via antheraxanthin into violaxanthin. The cycle is catalyzed by VDE and ZEP that act in two successive steps. These enzymes are differentially located in chloroplast thylakoids; VDE is located in the thylakoid lumen and ZEP is on the stromal side (Hager 1980; Pfundel et al. 1994). Low pH and ascorbate are required by VDE that converts violaxanthin into zeaxanthin via the intermediate antheraxanthin (Pfundel et al. 1994; Bratt et al. 1995; Kramer et al. 1999). Under low light and relatively alkaline conditions (optimum pH of 7.5), ZEP hydroxylates β-rings of zeaxanthin in two consecutive steps to yield antheraxanthin and then violaxanthin, thus forming an integrated cycle. This cycle is present in all higher plants studied to date, as well as in ferns, mosses, lichens, and some algae (Phaeophyta, Chlorophyta and Rhodophyta).

Finally violaxanthin is converted to neoxanthin by neoxanthin synthase (NSY), which also represents the final step in the core carotenoid biosynthetic pathway.

The lutein epoxide cycle was reported in green tomato fruits (Rabinowitch et al. 1975) and was later found in the photosynthetic stems of the parasitic plant *Cuscuta reflexa* Roxb. (Bungard et al. 1999). It involves the de-epoxidation of lutein epoxide (monoepoxide) to lutein and the epoxidation of lutein to lutein epoxide.

For the lutein epoxide cycle, two different types have been described:

(i) A complete lutein epoxide cycle, in which lutein Lut is reconverted to lutein epoxide overnight
(ii) A truncated lutein epoxide cycle, in which lutein Lut reconversion is not observed overnight (Garcia-Plazaola et al. 2007).

Essential photoprotective functions have been assigned to lutein and the xanthophyll cycle pigments antheraxanthin and zeaxanthin, particularly related to the heat dissipation of excess light energy (Non-Photochemical Quenching).

The xanthophyll precursor pool plays an important role in the biosynthesis of the phytohormone abscisic acid (ABA) (Fig. 2.1, Li and Walton 1990; Seo and Koshiba 2002; Wasilewska et al. 2008). *De novo* synthesis of ABA requires ZEP-catalyzed epoxidation of zeaxanthin to violaxanthin. Subsequently, the violaxanthin-derivatives neoxanthin and xanthoxin are converted into ABA through a series of isomerization and dehydrogenation reactions (Milborrow 2001). In the ABA-deficient mutant *aba1* (an allele of *npq2*), ZEP is not functional and causes accumulation of zeaxanthin in parallel with the decrease in the epoxy-xanthophylls antheraxanthin, violaxanthin and neoxanthin (Duckham et al. 1991; Marin et al. 1996). In the xanthophyll cycle, VDE requires ascorbate as reductant to convert violaxanthin to zeaxanthin (Bratt et al. 1995). As a result, reduced levels of ascorbate in the *Arabidopsis vtc1* (vitamin C1) mutant stimulate ABA production (Pastori et al. 2003). In contrast, enhanced VDE activity can reduce ascorbate levels and antagonize ABA synthesis (Pastori et al. 2003). Therefore, the regulation of the xanthophyll cycle allows ABA levels to be modified.

2.4.7 Neoxanthin Synthase

The final step of the β,β-branch in the classic carotenoid pathway is the conversion of yellow-colored violaxanthin into a xanthophyll carrying an allenic double bond, named neoxanthin which represents the classical final step in plant xanthophyll formation (Li and Walton 1990; Parry and Horgan 1991). Neoxanthin together with violaxanthin can further be used for the production of the apocarotenoid hormone, ABA. Interestingly, in tomato violaxanthin is a sufficient precursor for ABA production *in vivo* (Neuman et al. 2014).

Even though, neoxanthin is synthesized from violaxanthin in plants (Li and Walton 1990; Parry and Horgan 1991; Rock and Zeevaart 1991; Galpaz et al. 2008; Qin et al. 2008), the identity of the gene coding for neoxanthin synthase (NSY) and the mechanism of formation of this xanthophyll, are still not known. Two previous reports have suggested that NSY is encoded by a gene of the lycopene cyclase gene family (Al-Babili et al. 2000; Bouvier et al. 2000). Indeed NSY enzyme was practically identical to the chromoplast-targeted lycopene β-cyclase from tomato (LCYB2/CYC-B isoform) (Ronen et al. 2000). However, the ortholog of this gene in pepper codes for capsanthin–capsorubin synthase,

(CCS) an enzyme involved in the production of ketocarotenoids in pepper fruit chromoplasts (Bouvier et al. 1994). Genetic and molecular analyses of two alleles of *nxd1*, a recessive neoxanthin-deficient mutation in tomato, identified a gene of unknown function, which is necessary for neoxanthin synthesis in tomato (Neuman et al. 2014). Two unrelated mutations in the gene *NXD1* were identified in tomato and a loss-of-function mutation in the orthologous *NXD1* gene of Arabidopsis showed an identical phenotype of lack of neoxanthin in leaves, but the study was unable to demonstrate any neoxanthin synthesis activity by NXD1 in a functional expression assay in *E. coli* (Neuman et al. 2014). Although, no NSY homologous gene has been identified in the genome of Arabidopsis, the ABA4 (At1g67080) gene of Arabidopsis (North et al. 2007) led to increased accumulation of trans-neoxanthin when overexpressed, whereas the loss-of-function in aba4 mutant was defective in this xanthophyll, indicating that the ABA4 protein has a direct role in neoxanthin synthesis. However, in vitro activity of the cloned gene has never been demonstrated.

Considering the results obtained at present, the identification of a functional NSY has several hindrances in view of that this reaction is not readily accessible by classical methods of protein purification, since it has not been possible until now to perform the reaction *in vitro* starting from exogenous synthetic violaxanthin. The high degree of lipophilicity of the substrate and product, suggest that the enzymatic activity could be dependent on membrane structures. Also the incapability of transformed *E. coli* to synthesize epoxidated xanthophylls *in vivo* and the very high spectral similarity of violaxanthin and neoxanthin (Marin et al. 1996) rule out color complementation methods in *Escherichia coli* even though this strategy have proven to be successful with other carotenoid biosynthetic enzymes. Therefore, the identification of a mutant defective in this biosynthetic step is expected to give better insights on the identification and characterization of NSY.

2.5 Concluding Remarks

The updated information provided in this chapter aims to confirm that until now the plant carotenoid pathway and the characterization of each enzymatic step is ongoing. Several efforts have been done to understand the metabolic flux in different plant models. Although most knowledge has been obtained from *Arabidopsis thaliana* and mutants obtained thereof, we may conclude that each plant is a whole and unique specie for carotenoid synthesis regulation.

At present most of the genes and enzymes have been identified and characterized, which has been very useful and amenable to continuously improve the carotenogenic metabolism of many different vegetal species by genetic engineering.

This obviously provides opportunities for the improvement of the nutritional value of various food plants and it also opens up the possibility for the metabolic engineering of compounds further downstream in the carotenoid pathway (Chap. 13), for which naturally accumulating mutants or genotypes are not currently

available. Those of more than 750 structurally different yellow, orange, and red colored molecules found in both eukaryotes and prokaryotes that were classified in simplified terms in this chapter comprised an estimated global market totaled $1.5 billion in 2014. This market is expected to reach nearly $1.8 billion in 2019, with a compound annual growth rate (CAGR) of 3.9% (According to "The Global Market for Carotenoids" from PR Newswire Association LLC). This projected market size reflects the multiple functions and uses of carotenoids as cells protectors against photooxidative damage involving important applications in disease control, environment, food and nutrition, and as potent antimicrobial agents.

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Chapter 3
Structures and Analysis of Carotenoid Molecules

Delia B. Rodriguez-Amaya

Abstract Modifications of the usual C_{40} linear and symmetrical carotenoid skeleton give rise to a wide array of structures of carotenes and xanthophylls in plant tissues. These include acyclic, monocyclic and dicyclic carotenoids, along with hydroxy and epoxy xanthophylls and apocarotenoids. Carotenols can be unesterified or esterified (monoester) in one or two (diester) hydroxyl groups with fatty acids. *E-Z* isomerization increases the array of possible plant carotenoids even further. Screening and especially quantitative analysis are being carried out worldwide. Visible absorption spectrometry and near infrared reflectance spectroscopy have been used for the initial estimation of the total carotenoid content or the principal carotenoid content when large numbers of samples needed to be analyzed within a short time, as would be the case in breeding programs. Although inherently difficult, quantitative analysis of the individual carotenoids is essential. Knowledge of the sources of errors and means to avoid them has led to a large body of reliable quantitative compositional data on carotenoids. Reverse-phase HPLC with a photodiode array detector has been the preferred analytical technique, but UHPLC is increasingly employed. HPLC-MS has been used mainly for identification and NMR has been useful in unequivocally identifying geometric isomers.

Keywords Carotenoids • Structures • Carotenes • Xanthophylls • E-Z isomers • Screening • Quantitative analysis • HPLC methods • UHPLC methods • HPLC-DAD-MS • NMR

3.1 Introduction

Carotenoids are widely distributed, lipophilic, naturally occurring yellow, orange, or red pigments. They are noted for their structural diversity and multiple functions and actions. It is estimated that about 100 million tons of these compounds are produced annually in nature (Isler et al. 1967). About 750 carotenoids, exclusive

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of *E* (*trans*) and *Z* (*cis*) isomers, have been isolated and characterized from natural sources (Britton et al. 2004). Most of these carotenoids come from higher plants, algae, bacteria and fungi.

3.2 Nomenclature

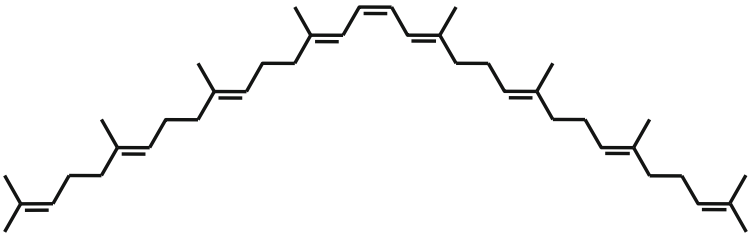
A semisystematic nomenclature for carotenoids that conveys structural information, including stereochemistry/three-dimensional structure, was devised by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC/IUB 1975; Weedon and Moss 1995). The names are based on the stem name "carotene," preceded by Greek-letter prefixes (β, ε, ψ, κ), denoting the two end groups. The numbering for the carotenoid skeleton is shown for lycopene and β-carotene in Figs. 3.1 and 3.2. Changes in hydrogenation and the presence of oxygen-containing substituents are indicated by standard prefixes and suffixes used in organic chemistry. The absolute stereochemistry of chiral, optically active carotenoids is indicated by the R/S designation.

Carotenoids have trivial names, usually derived from the biological sources from which they were first isolated. For the sake of simplicity, these short and familiar trivial names will be used throughout this chapter. The trivial names are accompanied by the semi-systematic names in Figs. 3.1, 3.2, 3.3, and 3.4. The *E/Z* designation is now preferred to indicate the configuration of the double bonds and will be used in this chapter instead of the still widely used *trans*/*cis* terminology.

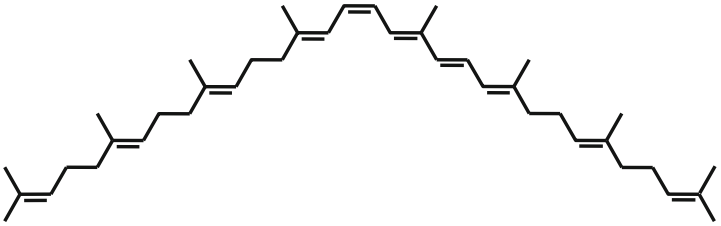
3.3 Structures

Carotenoids in plants are mostly C_{40} tetraterpenes/tetraterpenoids formed from eight C_5 isoprenoid units joined head-to-tail, except at the center where a tail-to-tail linkage reverses the order (Fig. 3.1). The basic linear and symmetrical skeleton has lateral methyl groups separated by six C-atoms at the center and the others by five C-atoms. The most distinctive structural feature is a centrally located, long system of alternating double and single bonds, in which the π-electrons are effectively delocalized throughout the polyene chain, although electron density appears to be greater at or towards the end of the chain. This conjugated double-bond system constitutes the light-absorbing chromophore that gives carotenoids their attractive color and is mainly responsible for their special properties and many functions. On the other hand, it renders the molecule susceptible to geometric isomerization and oxidative degradation.

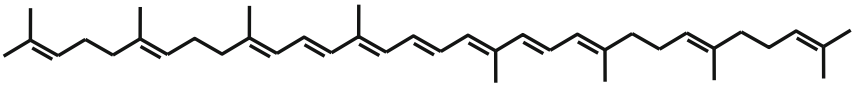
The basic structure is modified in many ways, including cyclization, hydrogenation, dehydrogenation, introduction of oxygen-containing groups, migration of double bonds, rearrangement, chain shortening or elongation, or combinations thereof, resulting in an immense array of structures.



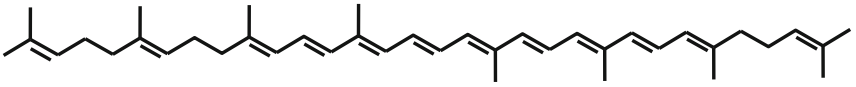
Phytoene 7,8,11,12,7′,8′,11′12′-octahydro-ψ,ψ-carotene



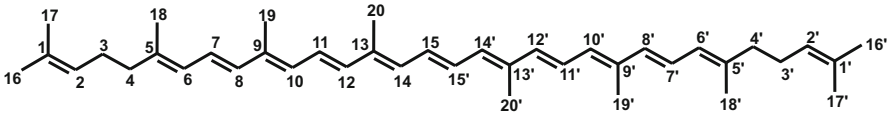
Phytofluene 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene



ζ-Carotene 7,8,7′,8′-tetrahydro-ψ,ψ-carotene

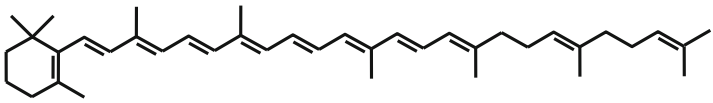


Neurosporene 7,8-dihydro-ψ,ψ-carotene

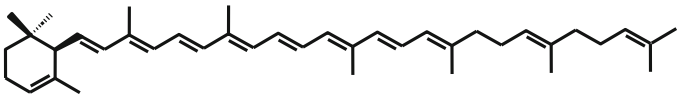


Lycopene ψ,ψ-carotene

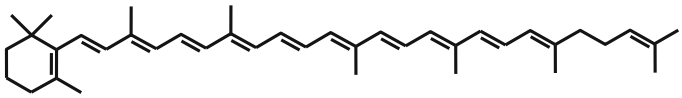
Fig. 3.1 Structures of acyclic plant carotenes



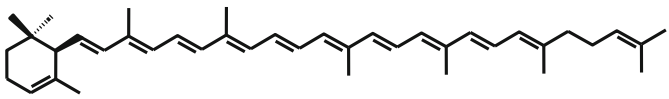
β-Zeacarotene 7′,8′-dihydro-β,ψ-carotene



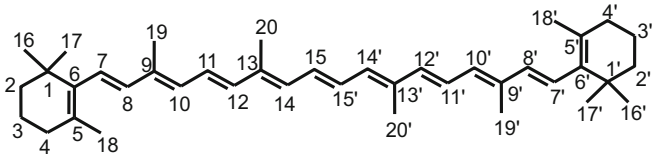
α-Zeacarotene (6R)-7′,8′-dihydro-ε,ψ-carotene



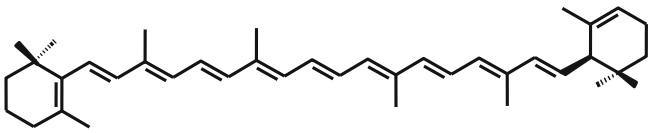
γ-Carotene β,ψ-carotene



δ-Carotene (6R)-ε,ψ-carotene



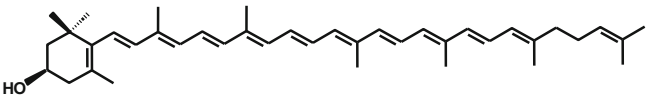
β-Carotene β,β-carotene



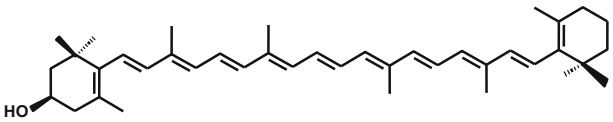
α-Carotene (6′R)-β,ε-carotene

Fig. 3.2 Structures of monocyclic and dicyclic plant carotenes

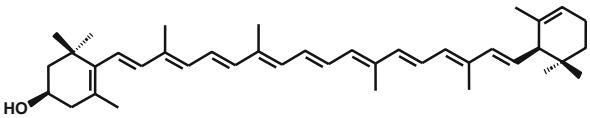
Hydrocarbon carotenoids (e.g. β-carotene, lycopene) are collectively called carotenes. Derivatives containing oxygen functions are termed xanthophylls. Common oxygen-containing substituents are hydroxyl- (as in β-cryptoxanthin), keto- (as in canthaxanthin), epoxy- (as in violaxanthin), and aldehyde (as in β-citraurin, (3R)-3-hydroxy-8′-apo-β-caroten-8′-al).



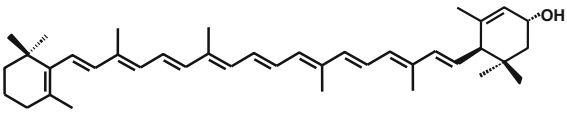
Rubixanthin (3R)-β,ψ-caroten-3-ol



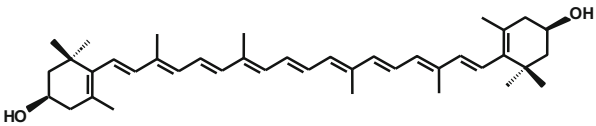
β-Cryptoxanthin (3R)-β,β-caroten-3-ol



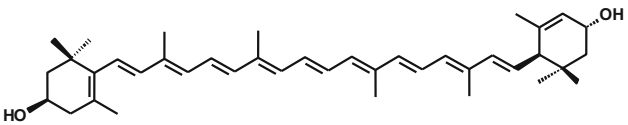
Zeinoxanthin (3R,6′R)-β,ε-carotene-3-ol



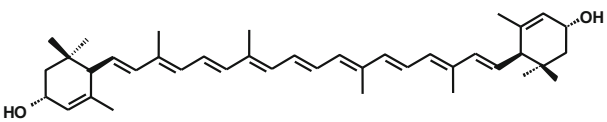
α-Cryptoxanthin (3′R,6′R)-β,ε-caroten-3′-ol



Zeaxanthin (3R,3′R)-β,β-carotene-3,3′-diol



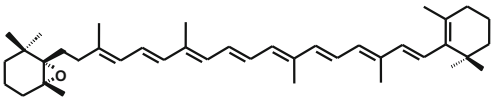
Lutein (3R,3′R,6′R)-β,ε-carotene-3,3′-diol



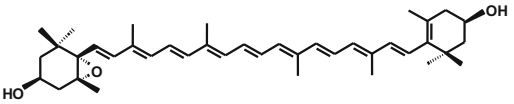
Lactucaxanthin (3R,6R,3′R,6′R)-ε,ε-carotene-3,3′-diol

Fig. 3.3 Structures of plant carotenols (hydroxy carotenoids)

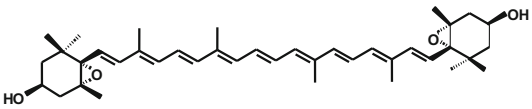
Fig. 3.4 Structures of plant epoxy carotenoids



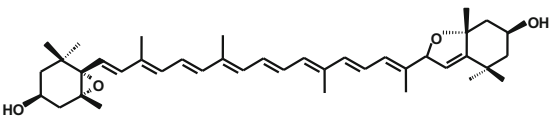
β-Carotene-5,6-epoxide (5R,6S)-5,6-epoxy-5,6-dihydro-β,β-carotene



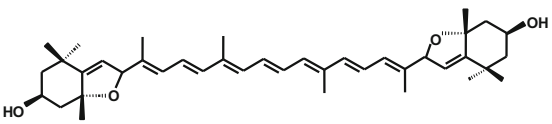
Antheraxanthin (3S,5R,6S,3′R)-5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol



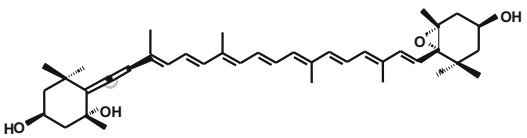
Violaxanthin (3S,5R,6S,3′S,5′R,6′S)-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol



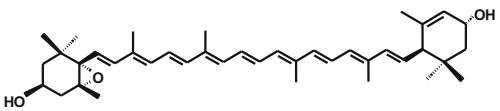
Luteoxanthin 5,6,5′,8′-diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol



Auroxanthin (3S,5R,8RS,3′S,5′R,8′RS)-5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene-3,3′-diol



Neoxanthin (3S,5R,6R,3′S,5′R,6′S)-5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol



Lutein-5,6-epoxide (3S,5R,6S,3′R,6′R)-5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol

Carotenoids may be acyclic (e.g. lycopene, ζ-carotene) or may have a six-membered ring at one end (e.g. γ-carotene, δ-carotene) or at both ends (e.g. β-carotene, α-carotene) of the molecule. Exceptionally, capsanthin and capsorubin have five-membered rings.

Because plants are able to synthesize carotenoids *de novo*, along with the principal carotenoids, low levels of their biosynthetic precursors and derivatives are also found. The carotenoid composition is variable and often complex.

3.3.1 Carotenes

Of the uncyclized carotenes (Fig. 3.1), lycopene and ζ-carotene are the most common. Lycopene is a $C_{40}H_{56}$ carotene with 11 conjugated and 2 non-conjugated double bonds. ζ-carotene, with a molecular formula of $C_{40}H_{60}$, has seven conjugated double bonds and four isolated double bonds. The colorless phytoene ($C_{40}H_{64}$) and phytofluene ($C_{40}H_{62}$) contain, respectively, three and five conjugated double bonds and six and five isolated double bonds. Neurosporene ($C_{40}H_{58}$) has nine conjugated and three unconjugated double bonds.

The monocyclic β-zeacarotene ($C_{40}H_{58}$) and γ-carotene ($C_{40}H_{56}$) contain 9 and 11 conjugated double bonds, respectively. Both have one end cyclized into a β-ring in which one of the conjugated double bonds is situated; the other end has an isolated double bond. α-Zeacarotene ($C_{40}H_{58}$) and δ-carotene ($C_{40}H_{56}$), with 8 and 10 double bonds, respectively, possess an *ε*-ring in which the double bond is out of conjugation.

β-carotene is a ($C_{40}H_{56}$) carotene with both ends of the molecule cyclized into β-rings (Fig. 2.2). It has 11 conjugated double bonds, two of which are located in the β-rings. The ring double bonds are not coplanar with those of the polyene chain. Also bicyclic, α-carotene ($C_{40}H_{56}$) has a β-ring, an *ε*-ring and 10 conjugated double bonds.

3.3.2 Xanthophylls

A diversity of xanthophylls is found in plants. Figure 3.3 shows the structures of common carotenols, with the hydroxyl groups located at the C-3 and C-3′ positions. Rubixanthin and β-cryptoxanthin (both $C_{40}H_{56}O$, 11 conjugated double bonds) are monohydroxy derivatives of γ-carotene and β-carotene, respectively, with the hydroxyl group at C-3 of the β-ring (Fig. 3.3).

The hydroxylation of α-carotene produces two monohydroxylated derivatives (both $C_{40}H_{56}O$, 10 conjugated double bonds): zeinoxanthin with the hydroxy group in the β-ring and α-cryptoxanthin with the allylic hydroxyl in the *ε*-ring (Fig. 3.3).

Lutein and zeaxanthin are dihydroxy, dicyclic carotenoids derived from α- and β-carotene, respectively, both having a molecular formula of $C_{40}H_{56}O_2$ (Fig. 3.3).

Both have hydroxyl groups at the 3 and 3′ positions. They differ only in the location of a single double bond, resulting in lutein having a β-ring and an ε-ring and zeaxanthin having two β-rings. Lutein has 10 conjugated double bonds, one of which is in the β-ring; an isolated double bond is in the ε-ring. Zeaxanthin contains 11 conjugated double bonds, two of which are located in β-rings.

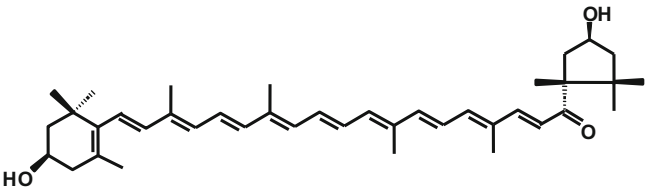
Carotenoids with both ends cyclized into ε-rings are rare. The dihydroxy derivative of ε-carotene ($C_{40}H_{56}$, (6R, 6′R)-ε,ε-carotene), lactucaxanthin ($C_{40}H_{56}O_2$, 9 conjugated double bonds, 2 non-conjugated double bonds in ε-rings), is a major carotenoid of lettuce, but has not been encountered in other leaves.

Carotenols in green leaves (Kobori and Rodriguez-Amaya 2008) are not esterified and those of corn (Rodriguez-Amaya and Kimura 2004; de Oliveira and Rodriguez-Amaya 2007) are also mostly unesterified. Carotenols in ripe fruits are generally esterified with fatty acids. Exceptions are the carotenols of a few fruits, particularly those that remain green when ripe such as kiwi (Gross 1987), which undergo limited or no esterification. Lutein, the main carotenoid, occurs free or esterified in one (monoester) or both (diester) hydroxyl groups in the edible nasturtium (Niizu and Rodriguez-Amaya 2005) and marigold (Breithaupt et al. 2002) flowers, with the esters predominating. Esterification, which occurs progressively during maturation, appears to be of physiological importance. Acylation increases the lipophilic character of the xanthophylls, facilitating their accumulation in the chromoplasts (Gross 1987).

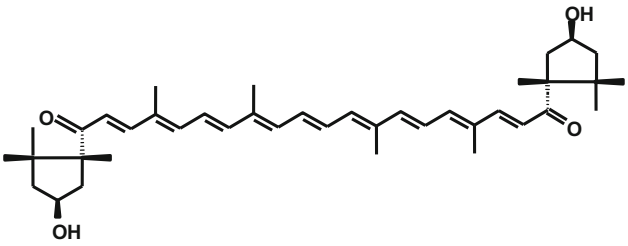
Epoxy carotenoids comprise a large group of plant xanthophylls (Fig. 3.4). The zeaxanthin-derived dihydroxy epoxides, antheraxanthin ($C_{40}H_{56}O_3$, 10 conjugated double bonds), violaxanthin ($C_{40}H_{56}O_4$, 9 conjugated double bonds), luteoxanthin ($C_{40}H_{56}O_4$, 8 double bonds), auroxanthin ($C_{40}H_{56}O_4$, 7 conjugated double bonds) and neoxanthin ($C_{40}H_{56}O_4$), are widely distributed. While antheraxanthin has one 5,6-epoxy substituent, violaxanthin has both 5,6- and 5′,6′-epoxides. Luteoxanthin contains epoxy groups in the 5,6 and 5′,8′ (often referred to as furanoid) positions; auroxanthin has two furanoid groups. Neoxanthin has a more complex structure, having an allene and a 5′,6′-epoxy groups, with hydroxyls at positions 3, 5 and 3′. The 5,8-epoxy derivative of neoxanthin, neochrome ($C_{40}H_{56}O_4$), is occasionally detected, this is also the case with the 5,6-epoxide of lutein ($C_{40}H_{56}O_3$).

The epoxy carotenoids derived from β-carotene, β-carotene-5,6-epoxide, β-carotene-5,8-epoxide (mutatochrome), β-carotene-5,6, 5′,6′-diepoxide, β-carotene-5,6, 5′,8′-diepoxide (luteochrome) and β-carotene-5,8, 5′,8′-diepoxide (aurochrome), and those of β-cryptoxanthin, especially β-cryptoxanthin-5,6-epoxide and β-cryptoxanthin-5,8-epoxide (cryptoflavin), are also encountered in plant tissues.

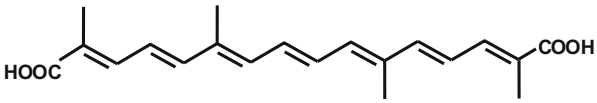
Except for violaxanthin and neoxanthin, epoxy carotenoids are usually detected in trace levels. In spite of their wide distribution, their natural occurrence is often questioned because they can be generated during analysis.



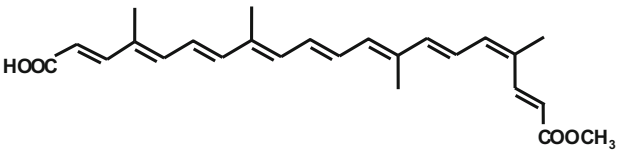
Capsanthin (3R,3′S,5′R)-3,3′-dihydroxy-β,κ-caroten-6′-one



Capsorubin (3R,5R,3′S,5′R)-3,3′-dihydroxy-κ,κ-carotene-6,6′-dione



Crocetin 8,8′-diapocarotene-8,8′-dioic acid



Bixin methyl hydrogen (9′*Z*)-6,6′-diapocarotene-6,6′-dioate

Fig. 3.5 Structures of major carotenoids in food colorants

Species-specific carotenoids (Fig. 3.5) also occur. The most prominent examples are capsanthin ($C_{40}H_{56}O_3$) and capsorubin ($C_{40}H_{56}O_4$), the predominant pigments of red pepper. Capsanthin has one end cyclized into a β-ring and the other end cyclized into a five-membered trimethylcyclopentyl κ-ring (Fig. 3.5). It has two hydroxyls attached at the 3 and 3′ positions and a carbonyl substituent at the 6′ position. With the carbonyl double bond, the total number of conjugated double bonds is 11. Capsorubin has κ-rings at both ends, 2 carbonyl groups, 11 conjugated double bonds.

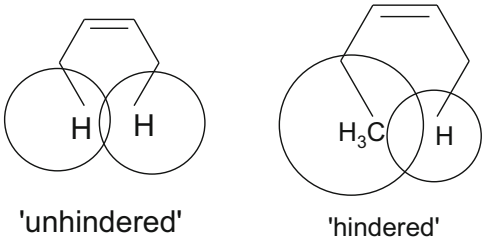
3.3.3 Apocarotenoids

Some carotenoids have their carbon skeletons shortened by the removal of fragments from one (apocarotenoids) or both (diapocarotenoid) ends of the usual C_{40} molecules (Weedon and Moss 1995). Natural examples are bixin, the major pigment of the food colorant annatto, and crocetin, the main yellow coloring component of saffron. Bixin ($C_{25}H_{30}O_4$) is the monomethyl ester of a dicarboxylic *Z*-diapocarotenoid, having a total of 11 conjugated double bonds, 9 carbon-carbon and two carbon-oxygen double bonds (Fig. 3.5). It blends with turmeric or paprika oleoresin, giving rise to yellow to reddish-orange color range in the food. Crocetin, which has a molecular formula of $C_{20}H_{24}O_4$, is a symmetrical diapocarotenoid with seven carbon-carbon double bonds and carboxylic groups at both ends.

3.3.4 Z-Isomers

In nature, carotenoids occur primarily in the generally more thermodynamically stable all-*E* form. Notable exceptions are the first two carotenoids formed biosynthetically, phytoene and phytoflene, which have the 15-*Z* configuration in most natural sources. Another exception is bixin, the principal pigment of the colorant annatto, which occurs naturally in the *Z* form. With the greatly improved efficiency of chromatographic separations, small amounts of *Z* isomers have been detected in plant foods, which increase appreciably during thermal processing and light exposure.

In principle, each carbon-carbon double bond in the polyene chain of carotenoids can isomerize from the *E* to the *Z* form. Some double bonds, however, are prevented from undergoing such isomerization because the *Z* configuration is sterically hindered. This is the case with the C-7,8, C-11,12, C-7′,8′ and C-11′,12′ double bonds, in which steric hindrance can be observed between a hydrogen atom and a methyl group. As illustrated below, this prevents the *Z* configuration for this double bond (Zechmeister et al. 1941; Weedon and Moss 1995; Liaan-Jensen 2004). The *Z* isomers of symmetrical β-carotene (Fig. 3.5) (Lessin et al. 1997; Marx et al. 2000; Dachtler et al. 2001) and zeaxanthin (Dachtler 2001; Humpries and Khachik 2003; Updike and Schwartz 2003; Aman et al. 2005) that are commonly found in foods are the 9-*Z*-, 13-*Z*- and the 15-*Z*-isomers (Fig. 3.6), the formation of which possess relatively little hindrance as it comes from two hydrogen atoms.



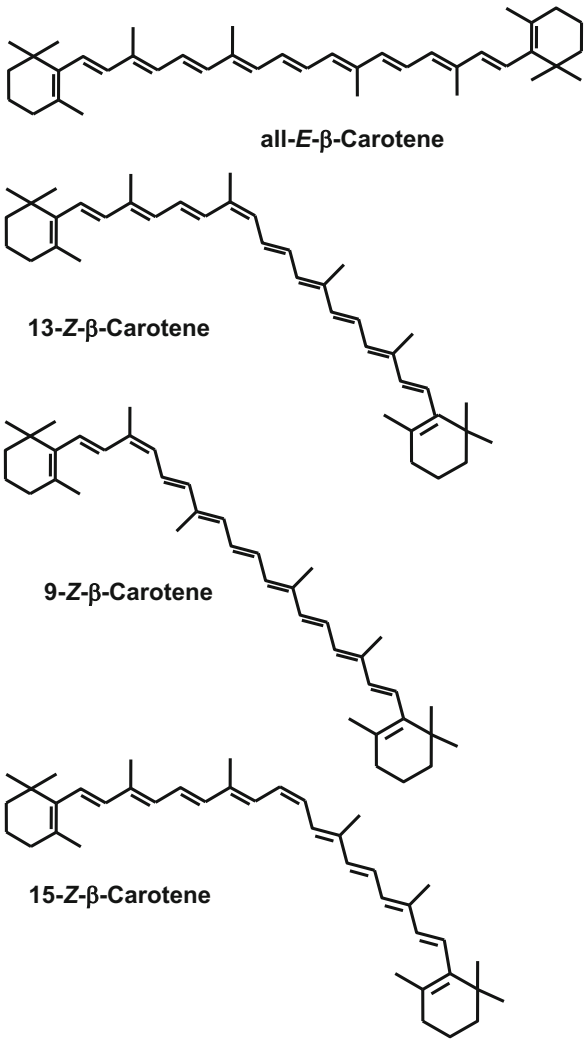


Fig. 3.6 Common geometric isomers of β-carotene

Carbon-carbon double bonds located in the cyclic part of the carotenoid structure, as the C-5,6 double bond in β-carotene, are also sterically hindered and are not isomerized. However, this double bond in the acyclic lycopene is unhindered and 5-*cis*-lycopene is increasingly detected in tomato and tomato products, along with the 9-*Z*-, 13-*Z*-, and 15-*Z*-isomers (Fig. 3.7) (Tiziani et al. 2006; Li et al. 2012; Stinco et al. 2013).

The unsymmetrical all-*E*-α-carotene (Lessin et al. 1997), all-*E*-β-cryptoxanthin (Lessin et al. 1997) and all-*E*-lutein (Fig. 3.8) (Dachtler et al. 2001; Humphries and Khachick 2003; Updike and Schwartz 2003; Aman et al. 2005; Achir et al. 2010) give rise to 13′-*Z*- and 9′-*Z*-isomers in addition to 13-*Z*-, 9-*Z*-, 15-Z-isomers (Fig. 3.8).

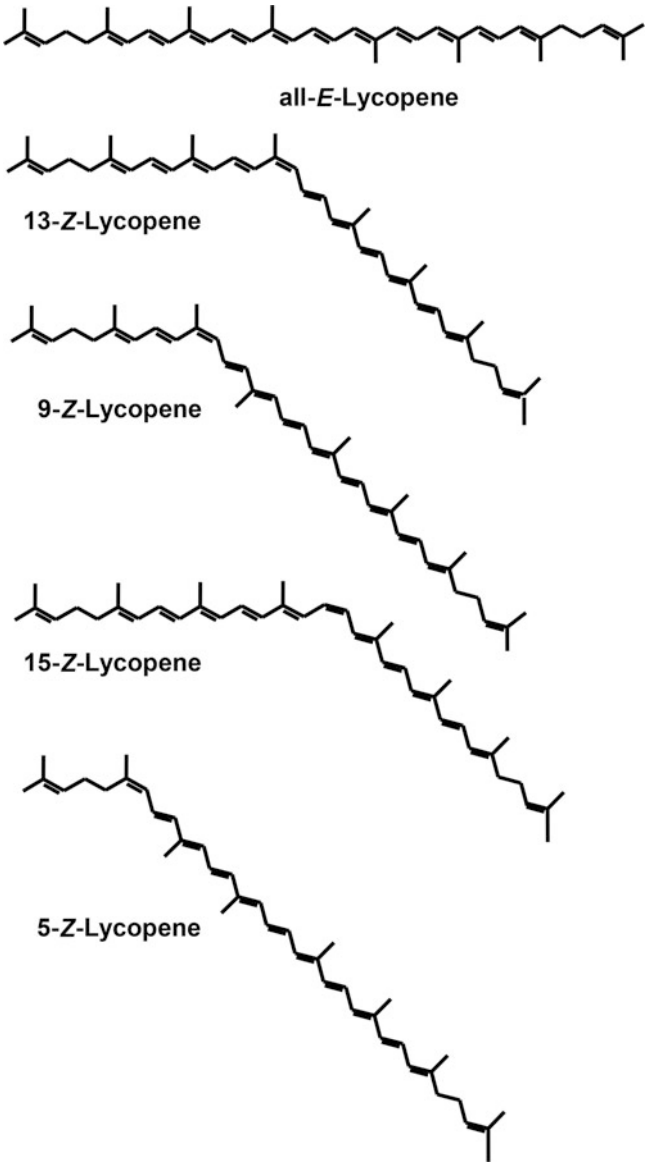


Fig. 3.7 Common geometric isomers of lycopene

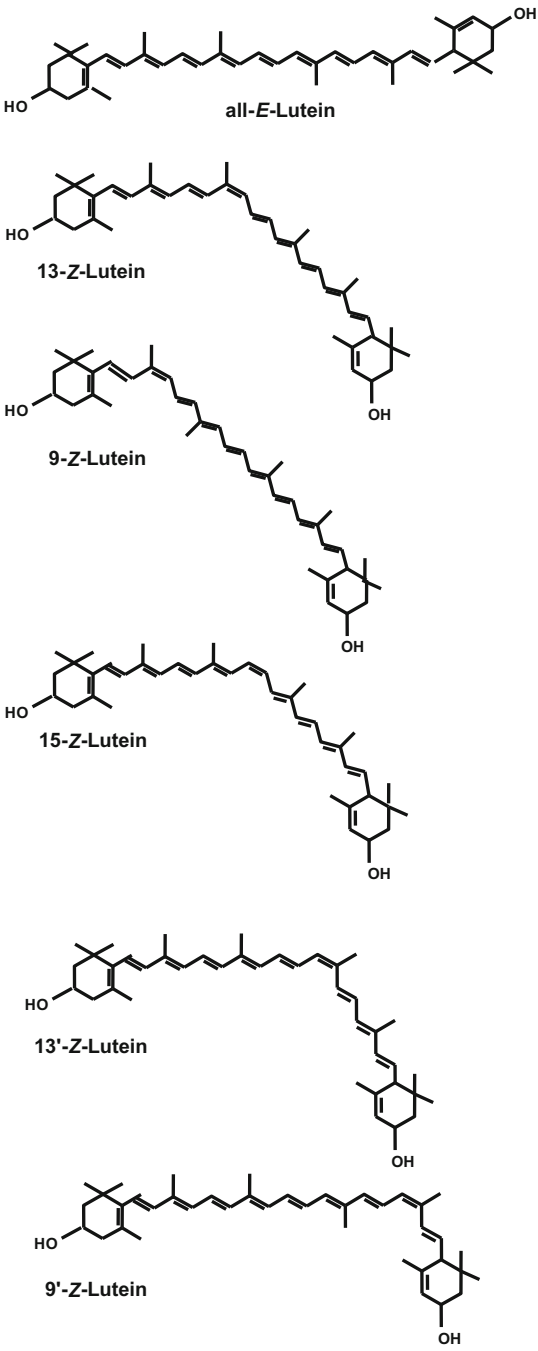


Fig. 3.8 Common geometric isomers of lutein

3.4 Carotenoid Analysis

The development or adoption of methods is generally guided not only by the methods' performance but also by the intended use of the analytical data. Trends in carotenoid analysis have reflected advances and refinements in analytical methodology and instrumentation, commensurate with the evolving knowledge on the actions and functions of these important compounds.

3.4.1 Raman Mapping

Raman mapping provides detailed information on the relative contents and spatial distribution of carotenoids in carrot roots of different colors (Baranska et al. 2006a, b). Near-infrared Fourier transform (NIR-FT) Raman spectroscopy measures β-carotene, lycopene and α-carotene/lutein (with strong bands at 1520, 1510 and 1527 cm^{-1}, respectively) *in situ* without preliminary sample preparation. The Raman mapping technique revealed that β-carotene in the secondary phloem increased gradually from the periderm towards the core, but declined in cells close to the vascular cambium. α-Carotene was deposited in younger cells at a higher rate than β-carotene, while lycopene in red carrots accumulated throughout the whole secondary phloem at the same level. The same research group utilized NIR-FT-Raman spectroscopy in the *in situ* analysis of a variety of raw plant tissues of various species: vegetables (yellow and orange carrots, red tomato, green French bean pods, red pepper), fruits (nectarine, apricot, watermelon), flowers (marigold, chamomile), leaves (ivory, begonia, *Euonymus fortune*) and saffron stigmas (Schulz et al. 2005). The results showed the usefulness of Raman spectroscopy in evaluating the distribution of individual carotenoids in intact plant tissues and in investigating the *E-Z* isomerization of carotenoids during processing.

Raman contour mappings of carrot root cross-sections were also obtained by Gonzalvez et al. (2014) over the spectral range of 500 to 2500 cm^{-1} as a function of the root radius. The Raman intensity signal was very low throughout the xylem tissue, but was high, indicating high carotene concentration, in the secondary phloem and periderm.

3.4.2 Screening

In certain cases (e.g. breeding experiments), a large number of samples has to be analyzed within a short time. Estimating the total carotenoid content is no longer considered adequate for most carotenoid research, but can be employed for this initial screening (Rodriguez-Amaya and Kimura 2004; Kimura et al. 2007). Briefly, the carotenoids are extracted with acetone, transferred to petroleum ether and the

visible absorption spectrum is taken. The total carotenoid content is calculated using the absorbance at the wavelength of maximum absorption and the absorption coefficient of the major carotenoid in the solvent used.

In recent years, near infrared reflectance spectroscopy (NIRS) has been used as a fast, simple, safe and nondestructive technique for the estimation of total carotenoid and/or principal carotenoid contents, principally to assist breeding programs. Sample treatment is minimal, involving only drying and grinding of the sample. NIRS has been employed in carotenoid content estimation in maize (Brenna and Berardo 2004), tritordeum (Atienza et al. 2005), banana (Davey et al. 2009b), potato (Bonierbale et al. 2009), cassava (Sánchez et al. 2014), and summer squash (Martínez-Valdivieso et al. 2014), mostly for germplasm characterization and breeding projects.

Visible spectrometric determination of the total carotenoid content has the disadvantages of being comparatively more lengthy and laborious than NIRS measurement, and it uses organic solvents for extraction. However, the equipment required is usually available in analytical laboratories.

Several analytical techniques and approaches relying on photothermal phenomena have been shown to be applicable for pretreatment-free and rapid screening of carotenoids in foods (Bicanic et al. 2010; Bicanic 2011). Laser photoacoustic spectroscopy (PAS) was used for estimating the total carotenoid content of corn and sweetpotato flours, using UV-visible spectrometry as reference method (Luterotti et al. 2011).

3.4.3 Quantitative Analysis

Since carotenoids differ in their properties (e.g. polarity, stability, bioavailability, antioxidant activity) and health-promoting actions and efficacy, accurate identification and quantification of individual carotenoids are essential. Because of the varied nature of plant matrices and the qualitative and quantitative variation in the carotenoid composition, a single analytical method/procedure is not likely to be appropriate for different plant samples (Rodriguez-Amaya and Kimura 2004; Rodriguez-Amaya 1999; Kimura et al. 2007). Method optimization (e.g de Sá and Rodriguez-Amaya 2004), development/validation (e.g. Hart and Scott 1995; Kimura and Rodriguez-Amaya 2002; Kimura et al. 2007; Akhtar and Bryan 2008) and the evaluation of performance in the analyst's laboratory, even when using a validated method (e.g. Dias et al. 2008), should precede data generation.

Quantitative carotenoid analysis generally consists of (1) sampling, (2) preparation of the analytical sample, (3) extraction, (4) partition to a solvent compatible with the subsequent chromatographic step, (5) saponification and washing (optional), (6) concentration or evaporation of solvent, (7) chromatographic separation, (7) identification, (8) quantification, and (9) data processing and interpretation.

A detailed description of the procedures for quantitative carotenoid analysis is beyond the scope of this chapter. Instead, a brief discussion of the different steps,

sources of errors and precautions to avoid errors will be presented. The readers are referred to Rodriguez-Amaya (1999) and Rodriguez-Amaya and Kimura (2004) for detailed procedures (including weights, volumes and calculations). Quality assurance for carotenoid analysis is discussed in Rodriguez-Amaya (2010).

3.4.3.1 Inherent Difficulties

Several factors make quantitative carotenoid analysis of plant samples challenging (Rodriguez-Amaya 1999; Rodriguez-Amaya and Kimura 2004):

- There is a large number of naturally occurring carotenoids. Conclusive identification is a pre-requisite to accurate quantification.
- The carotenoid composition varies substantially, qualitatively and quantitatively. The analytical procedure should be adapted to the carotenoid composition of the plant sample under investigation.
- The carotenoid concentrations in a given sample vary over a wide range. Preparation of standard solutions and construction of standard curves are done at widely differing levels.
- The plant matrices are diverse and complex. The analytical procedure, especially the preparation of the analytical sample and extraction, should be optimized for each type of matrix.
- The distribution of carotenoids between samples and in a fruit, leaf, seed or root is not uniform. Thus, statistically sound sampling and preparation of the analytical sample should be established.
- The highly unsaturated carotenoid molecules are prone to isomerization and oxidation during analysis and during storage of samples and standards.
- Carotenoids occur at low levels (μg/g), together with much higher concentrations of other compounds that can interfere in the analysis or at least make complete extraction difficult.

The inherent difficulty in carrying out quantitative carotenoid analysis was evident in an interlaboratory study among 26 US and European laboratories, using a baby food composite (Sharpless et al. 1999). The relative expanded uncertainties were higher than those generally expected for certified values. Certified concentrations were provided for some carotenoids, but only reference values could be given for other carotenoids. For lycopene, for example, only reference values were provided because of greater variation in the values obtained, which was attributed to degradation of this analyte in some participating laboratories.

That the difficulties vary with the sample matrix is shown in the results of a European study, involving 14 laboratories. β-carotene and its *cis*-isomers were determined in commercially processed foods (margarine, vitamin drink, pudding powder, natural mixed vegetable), which were chosen according to their type of matrix, their range of concentration and their availability in food stores (Schüep and Schierle 1997). The best repeatability and reproducibility were obtained with the supplemented drink and the worst results with the pudding powder.

Isomerization and oxidation of carotenoids are major causes of analytical errors. Irrespective of the analytical method chosen, precautionary measures to avoid formation of artifacts and quantitative losses should be taken. These include (Schiedt and Liaaen-Jensen 1995; Rodriguez-Amaya 1999): completion of the analysis within the shortest possible time, exclusion of oxygen, protection from light, avoiding high temperature and contact with acid, use of solvents free from damaging impurities (e.g. peroxides), addition of antioxidants (e.g. butylated hydroxytoluene, pyrogallol and ascorbyl palmitate) and neutralizing agents (e.g. MgCO3) (Schiedt and Liaaen-Jensen 1995; Rodriguez-Amaya 1999) and adequate storage conditions or execution of analysis immediately after sample collection (Rodriguez-Amaya 1999).

Loss or change of color at any time during the analysis is an indication of degradation or structural modification.

Oxygen, especially in combination with light and heat, is highly destructive. The presence of even traces of oxygen in stored samples (even at deep freeze temperatures) and of peroxides in solvents (e.g. diethyl ether and tetrahydrofuran) or of any oxidizing agent in extracts of carotenoids can rapidly lead to degradation and the formation of artifacts, such as epoxy carotenoids and apocarotenals (Britton 1991). Oxygen can be excluded at several steps during analysis and during storage with the use of vacuum and a nitrogen or argon atmosphere. Antioxidants may also be used, especially when the analysis is prolonged. They can be added during sample disintegration and saponification or added to solvents and isolates.

Carotenoid analysis from the extraction step must be done under subdued light. The speed of manipulation and the protection from light, especially direct sunlight and ultraviolet light, are particularly important in extracts containing chlorophylls (e.g. extracts of green leafy and non-leafy vegetables) or other potential sensitizers. In the presence of these sensitizers, photodegradation and isomerization can occur very rapidly, even with brief exposure to light. Gold lighting, used by some laboratories to filter out visible light, was shown by O'Neil and Schwartz (1995) to only slow down, not to prevent, sensitized photoisomerization.

3.4.3.2 Storage of Samples and Standards

Ideally, samples should be analyzed as soon as they are collected because it is difficult to avoid changes in the carotenoid composition during storage, even at very low temperature. Moreover, because carotenoid concentration is expressed per unit weight of sample, changes in the sample's weight (e.g. due to loss or gain of water during storage) also affect the final result.

When storage is unavoidable, raw plant samples are better stored intact. Tissue disintegration should be postponed until after storage and then carried out immediately before or simultaneously with extraction to avoid enzymatic oxidation of the carotenoids (Rodriguez-Amaya 1999). Loss of moisture and volatile compounds is also greater with disintegrated tissues, concentrating the carotenoids and leading to overestimation of the concentrations, if corrections are not made. Storage of extracts

should also be avoided or limited to a very short period. Once extracted, carotenoids lose the natural protection of the plant's cellular structure and degrade rapidly, even at low temperature. In carotenoid extracts from banana, for example, a breakdown rate of around 5 % per day was observed even in the dark at −20 °C and in the presence of antioxidants (Davey et al. 2009a).

Lyophilization has been considered the appropriate means of preserving samples that need to be stored before analysis. Davey et al. (2006, 2009a) reported no significant difference in the recoveries of total provitamin A carotenoids from lyophilized and fresh banana, and no further significant loss during storage of lyophilized tissue in the dark at −20 °C, whereas variable losses occurred in frozen pulp tissues. On the other hand, degradation of carotenoids, especially of lycopene, does occur during lyophilization (Craft et al. 1993), and it increases sample porosity and consequently the exposure to oxygen during storage. Moreover, to transform the carotenoid concentrations obtained with lyophilized samples to those of the foods as purchased or as consumed, the preferred form in databases, the moisture content is used. The determination of moisture is simple but it is a proximate analysis; the error from this macrocomponent analysis can be carried over and appreciably affect the microcomponent concentration.

Fractions or isolates should be kept dry under nitrogen or argon or dissolved in a hydrocarbon solvent such as petroleum ether or hexane, and kept at −20 °C or lower when not in use. Leaving carotenoids in solvents such as cyclohexane, dichloromethane, diethyl ether (Craft and Soares 1992) and acetone can lead to substantial degradation. Carotenoids extracted with acetone should be immediately transferred to petroleum ether.

Standard carotenoid crystals should be sealed in ampoules under nitrogen or argon and stored at −20 °C or preferably at −70 °C until use. Stock and working solutions, even when kept at low temperature, have limited validity. The analyst should know when degradation commences under his laboratory's conditions.

3.4.3.3 Sampling

The analytical data can only be reliable and truly useful if the sample taken for analysis is representative of the lot under investigation and is adequately prepared for analysis. Errors introduced in these initial steps cannot be corrected or compensated for in the subsequent steps. Natural variation should be clearly distinguished from analytical errors and compositional data should be accompanied by pertinent information such as the variety, stage of maturity, geographical origin, season, and part of the plant analyzed.

Carotenoids are highly affected by genetic and environmental factors (Rodriguez-Amaya et al. 2008). Aside from the considerable variation between plant materials, in a given fruit, vegetable or other material, compositional variation also occurs due to factors such as cultivar/variety, maturity at harvest, climate/season/geographic site of production, part of the plant utilized, farming practices, harvesting and post-harvest handling, processing and storage conditions.

Sampling is therefore not an easy task (Rodriguez-Amaya 1999; Rodriguez-Amaya and Kimura 2004). All factors that can potentially influence the composition should be accounted for in the sampling plan. Several sample lots are individually submitted to analysis and mean values are reported, accompanied by a statement of uncertainty, usually expressed in terms of the standard deviation.

The more heterogeneous the plant material is, the greater the difficulty and effort needed to obtain the representative composition. Representativity can be enhanced by increasing the sample size, the weight of the subsample submitted to analysis (analytical sample), the degree of comminuting and the number of analytical runs. Because of the heterogeneity of plant samples, a large number of samples should ideally be analyzed. In practice, however, the sampling and sample preparation procedures adopted are usually a compromise between heterogeneity considerations and operational costs. Moreover, the current initiative to apply green, environmentally friendly analytical chemistry recommends minimal sample size and a minimal number of samples (Gałuszka et al. 2013)

The number of analytical runs needed per plant material depends on the compositional variability. In tomato fruit, Darrigues et al. (2008) observed that between-plot field variation accounted for 50 and 52 % of the total variation for lycopene and β-carotene, respectively. The corresponding contributions of within-plot variation were 7 % and 3 %, and of uncontrolled error 43 % and 45 %. There was no significant variance due to replicated extraction or replicated HPLC injection for either carotenoid. Also working with tomato, Dias et al. (2008) concluded from the results of 12 samples harvested in the same region that analyzing five primary samples would be sufficient to estimate the population mean value with a level of confidence of 95 %.

In African bananas and plantains (Davey et al. 2007), analysis of the between-plant, within-plant, within-hand and within-fruit variations of *Musa* varieties cultivated under standardized field conditions demonstrated that the provitamin A carotenoid content varied significantly across all sample groups. It was necessary to collect fruits from hands at the proximal end, the middle and the distal end of the bunch to obtain representative values.

3.4.3.4 Preparation of the Analytical Sample

The sample that is collected and brought to the laboratory is usually too large for analysis, both in bulk and particle size. A small homogeneous, representative subsample should be obtained for analysis. The procedure should be adapted to the nature of the sample, the analyte, the analytical method, and the distribution of the analyte in the sample. Sample preparation and extraction are the most time-consuming and error-prone steps in the analytical process.

Variation in the carotenoid concentration along the longitudinal and across the transversal axes of the cassava root was shown by Chávez et al. (2008). The

carotenoid content was higher in the part of the root closest to its attachment to the stem (proximal section), gradually decreasing towards the opposite end (distal section). Across the root, carotenoid content was higher in the core, lower towards the periphery. Quartering roots and fruits longitudinally is therefore the appropriate manner of reducing the sample's volume to obtain the analytical sample.

Subsampling and homogenization may be done simultaneously. Physical operations, such as chopping, cutting into pieces, mixing, milling, blending and sieving, are carried out, along with bulk reduction, for example, by quartering and riffling. The process can be done manually or through commercially available mills, blenders, grinders, riffle cutters, etc.

Once homogenized, the analytical sample should be weighed and extraction should immediately follow, because tissue disruption releases enzymes (e.g. lipoxygenase) that catalyze carotenoid oxidation and acids that promote geometric isomerization. Usually, sample maceration, homogenization and extraction with an organic solvent are carried out simultaneously.

Currently, the major errors in carotenoid analysis appear to be due to the sampling/sample preparation schemes. In many papers, the sampling plan and reduction of the gross sample to the analytical sample are not described at all or if they are, only superficially. Errors from these initial steps are not observed in intralaboratory and interlaboratory evaluations in which the same homogenized samples are analyzed by the participating analysts/laboratories.

3.4.3.5 Extraction

For efficient extraction, the solvent should be able to penetrate the food matrix and efficiently dissolve the range of carotenoids in the sample, without altering or degrading them. The solvent should not pose toxic effects to the analyst. Because carotenoids are found in a variety of plant materials, the extraction procedure should be adapted to suit the sample being analyzed. Plant samples generally contain large amounts of water, thus water-miscible organic solvents, such as acetone, methanol, ethanol or mixtures thereof are used as extracting solvents to allow good solvent penetration. Dried materials can be extracted with water-immiscible solvents, but extraction is usually more efficient if the samples are rehydrated prior to extraction with water-miscible solvents.

Extraction should be carried out under a fume hood to protect the analyst from inhaling solvent vapor. The sample is generally homogenized with celite (or Hyflosupercel) and the solvent in a suitable mechanical blender for 1–2 min or with a mortar and pestle. Celite facilitates both tissue disintegration and the subsequent filtration. A Waring blender is fast and efficient for mechanical disruption and homogenization of soft fruits and juice. The Polytron homogenizer is widely used because it provides rapid and uniform homogenization. Vortexing has also been employed, but it should only be used for finely ground and easy to extract samples. For samples such as fresh leaves, the simple mortar and pestle is better because small pieces of leaves, which can escape the homogenizer's blades, can be

ground well. Leaves and other difficult-to-extract matrices may also need previous soaking in the extracting solvent (about 15 min for leaves) to soften the cell wall. Prolonged soaking should be avoided to prevent isomerization and degradation of the carotenoids.

Acetone has been the traditional solvent for carotenoid extraction. It has three advantages:

– It dissolves both carotenes and xanthophylls efficiently
– It penetrates the plant matrix well
– Subsequent partitioning to an apolar solvent occurs more easily.

Our HPLC method (Fig. 3.9), which makes use of acetone as extracting solvent, has been validated in our laboratory using a certified reference material (Kimura et al. 2007) and in an international interlaboratory study (Rodriguez-Amaya et al. 2012).

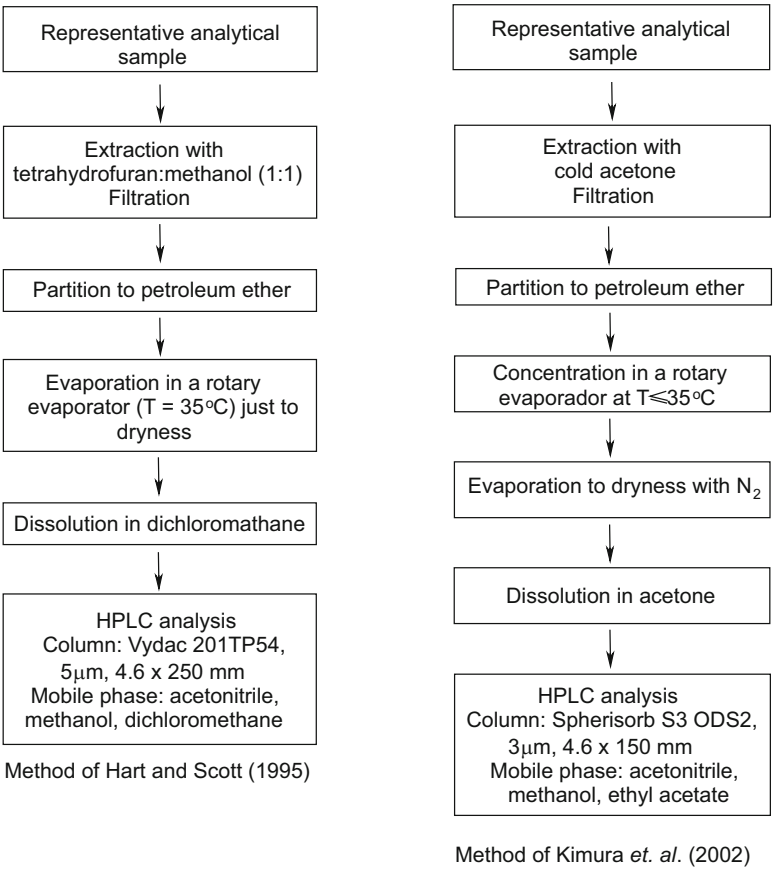


Fig. 3.9 Schematic diagrams of two widely used, validated HPLC methods for the quantitative analysis of carotenoids

Tetrahydrofuran (THF) became a popular extracting solvent when HPLC was introduced. Solubility of both β-carotene and lutein in this solvent was shown to be excellent (Craft and Soares 1992). The widely used method of Hart and Scott (1995) employs THF:methanol (Fig. 3.9). This method was validated in the European interlaboratory study of Scott et al. (1996).

Other solvents, such as hexane, petroleum ether, methanol and ethanol have also been used. The first two solvents readily dissolve carotenes but not the xanthophylls; on the other hand, methanol and ethanol dissolve the xanthophylls efficiently but not the carotenes. Thus, mixtures of solvents such as hexane:ethanol (Taungbodhitham et al. 1998; Lin and Chen 2003; Cortés et al. 2004), hexane:acetone:ethanol (Periago et al. 2004; Barba et al. 2006) and hexane:ethanol:acetone:toluene (Chen et al. 2004) have been employed.

Carotenoids may decompose, dehydrate, or isomerize in the presence of acids. 5,6-Epoxy carotenoids, such as violaxanthin and neoxanthin, readily undergo rearrangement to the 5,8-epoxides. A neutralizing agent (e.g., calcium carbonate, magnesium carbonate, or sodium bicarbonate) may be added during extraction to neutralize acids liberated from the sample itself. Extracting solvents such as chloroform, which has traces of hydrochloric acid, should be avoided. Strong acids and acidic reagents should not be used in rooms where carotenoids are handled. Most carotenoids are stable towards alkali.

In our experience, using cold acetone (left in the refrigerator for a short time before use) and doing the extraction immediately or simultaneously with sample maceration, not only prevents enzymatic oxidation but also makes the addition of neutralizing agents unnecessary.

Filtration can be done with a sintered glass funnel or with a Buchner funnel. The solid residue is returned to the homogenizer and re-extracted with fresh solvent; extraction and filtration are repeated until the residue is colorless (three extractions are usually sufficient).

Using the Placket-Burman experimental design, Periago et al. (2007) examined 15 factors that affect the extraction and quantification of lycopene from tomato and tomato products: sample weight; volume of extraction solution; antioxidant (BHT) concentration; neutralizing agent ($MgCO_3$) concentration; light presence during extraction; homogenization velocity and time; agitation time; temperature during the extraction process; water volume for separation of polar/nonpolar phases; presence of inert atmosphere throughout the process; time, temperature and light presence during separation of phases and time delay for reading. The sample weight, neutralizing agent concentration and water volume for separation of polar/nonpolar phases could be considered the key factors for raw and processed tomato. For tomato sauce, sample weight and volume of extraction solution had the greatest impact on the results.

A European study, with 17 participating laboratories, investigated various possible problem areas, including the chromatographic systems, standardization of carotenoid stock solutions, extraction procedures and data handling, using a lyophilized vegetable mix (Scott et al. 1996). The effect of the chromatographic

system did not appear to be the major variable. In the more experienced laboratory, variation in the standardization of the carotenoid solution was also not thought to be a significant problem. The results suggested that the preparation of the extract might account for about 13 % of the overall variance of around 23 %. Similarly, in an international interlaboratory study involving 19 Asian, African, European, Latin American and US laboratories with widely differing laboratory conditions and experience in carotenoid analysis, using sweetpotato, cassava and corn flours as test materials, incomplete extraction was found to be a major problem (Rodriguez-Amaya et al 2012).

3.4.3.6 Partition

The extract usually contains a substantial amount of water, which can be removed by the partition to hexane, petroleum ether, diethyl ether, or dichloromethane or mixtures of these solvents. This partition also serves as a clean-up step. Diethyl ether or a mixture of ether with hexane or petroleum ether is preferred for extracts with large amounts of xanthophylls, part of which is lost with the washing water during the partition to pure hexane or petroleum ether (Kobori and Rodriguez-Amaya 2013).

A procedure, which has been used for years, still seems to be the best way of carrying out the partition. Small portions of the extract are added to petroleum ether or another appropriate solvent in a separatory funnel (Rodriguez-Amaya 1999). After each addition, water is added gently to avoid formation of an emulsion, preferably by allowing the water to flow along the walls of the funnel. The two layers are allowed to separate, without agitation, and the lower aqueous phase (with the water miscible extracting solvent) is discarded. When the entire extract has been added, the petroleum ether phase is washed four or five times with water to remove residual extracting solvent. In our experience, this procedure is efficient and emulsions are less likely to form.

Alternatively, the acetone extract can be added to petroleum ether in the separatory funnel all at once, followed by addition of water. Some workers then agitate the mixture, but this practice leads to the formation of emulsion, which is difficult to break and results in loss of carotenoids to the aqueous phase. After separation of phases, the lower layer is drawn off and re-extracted with fresh petroleum ether. The combined petroleum ether solution is then washed 4 or 5 times with water.

The solvents used in the extraction or partition is subsequently removed or at least reduced by evaporation, thus, solvents with low boiling points are chosen to avoid prolonged heating. The lower-boiling fractions of petroleum ether (b.p. 30-60 °C or 40-60 °C) is used instead of the higher-boiling fractions. Dichloromethane (b.p. 42 °C) is preferred instead of chloroform (b.p. 61 °C).

3.4.3.7 Saponification

Saponification removes chlorophyll and unwanted lipids; it also hydrolyzes the carotenol esters, simplifying the chromatographic separation. However, it extends the analysis time and is error prone. Considerable losses during this step and the subsequent washing have been reported through the years, especially of lutein, zeaxanthin, violaxanthin and other dihydroxy, trihydroxy and epoxycarotenoids (Khachik et al. 1986; Rodriguez-Amaya et al. 1988; Kimura et al. 1990; Marsili and Callahan 1993; Riso and Porrini 1997; Yue et al. 2006), although provitamin A carotenoids (α-carotene, β-carotene, γ-carotene, β-cryptoxanthin) appeared stable (Rodriguez-Amaya et al. 1988; Kimura et al. 1990). The extent of carotenoid degradation depends on the conditions used, being greater with higher concentration of alkali and hot saponification (Kimura et al. 1990).

Saponification should therefore be included in the analytical procedure only when indispensable. It is unnecessary, for example, in the analysis of leafy vegetables, tomato and carrot, all of which are low-lipid materials and essentially free of carotenol esters. The chlorophylls co-extracted with carotenoids from leaves can be separated during chromatography. For the high-lipid dry corn, saponification is dispensable when gradient elution is used (Rodriguez-Amaya and Kimura 2004).

If required, this step should be thoroughly evaluated and optimized, and the subsequent washing carefully done to avoid losing carotenoids with the discarded aqueous phase (de Sá and Rodriguez-Amaya 2004). Complete hydrolysis of carotenol esters to the free carotenoids should also be verified.

Losses of lutein during saponification and the subsequent washing are frequently underestimated, as in leafy vegetables. Except in lettuce, if the lutein content is almost equal to or lower than that of β-carotene in a leafy vegetable, as is sometimes seen in the literature, lutein loss during analysis is strongly indicated.

Concern about the negative effects of saponification has led analyst to shorten the duration of ambient temperature saponification (e.g., to 1 or 2 h). In our experience, however, longer saponification times are required for complete hydrolysis of carotenol esters.

Saponification is best done after partition of the carotenoids to petroleum ether or hexane, by adding an equal volume of 10 % methanolic potassium hydroxide. The mixture is left overnight at room temperature in the dark, after which the carotenoid solution is washed about 5 times with water to remove the alkali. To avoid losing carotenoids into the washing water, especially the more polar xanthophylls, this step should be done in the same manner as in the partition, described above. When apocarotenals (e.g., β-citraurin in citrus) are present in the sample, all traces of acetone must be removed before saponification to avoid aldol condensation between the apocarotenals and acetone.

3.4.3.8 Concentration or Evaporation of the Solvent

The carotenoid solution, after partitioning for unsaponified sample or after washing for saponified samples, is dried with anhydrous sodium sulfate and concentrated in a rotary evaporator at reduced pressure and a temperature $\leq$35 °C. If complete removal of the solvent is desired, concentration in the rotary evaporator is done first, and evaporation to dryness is accomplished with a stream of nitrogen or argon. Bringing the carotenoid solution to complete dryness in the rotary evaporator increases the possibility of degradation, especially of lycopene (Tonucci et al. 1995), and may leave the carotenoids tightly adhered to the glass walls, making quantitative removal from the flask difficult.

3.4.3.9 Chromatographic Separation

Reversed-phase high performance liquid chromatography (HPLC) has been the chromatographic technique most widely used for quantitative carotenoid analysis for over two decades. Compilations of HPLC methods for carotenoids can be found in review articles (Su et al. 2002; Rivera and Canela-Garayoa 2012; Amorim-Carrilho et al. 2014). Two examples of widely used, validated HPLC methods are shown in Fig. 3.9.

Two developments in HPLC advanced quantitative carotenoid analysis considerably:

- Columns that provide baseline separation of the range of carotenoids in a given food, up to the separation of geometric isomers
- The photodiode array detector that provides the visible absorption spectra of the separated carotenoids on-line.

The most widely used column for carotenoid separation for some time was the polymeric C_{18} Vydac 201TP54 (250 x 4.6 mm, 5 μm) column. It has been surpassed by the polymeric C_{30} YMC (250 x 4.6 mm, 5 μm) column. Especially designed for the separation of carotenoid isomers (Sander et al. 1994), the C_{30} stationary phase has gained wide application in food analysis in general (Sander et al. 2000).

Carotenoid separation has been carried out with 5 μm spherical particles packed in a 250 × 4.6 mm column. The C18 columns with 5 μm particles have often been found not to provide adequate separation of food carotenoids, especially the geometric isomers. Shorter columns with smaller particles have demonstrated greater separation efficiency and requires much less mobile phase. The C18 Spherisorb ODS 2 (150 x 4.6 mm, 3 μm) column used in our laboratory, for example, has provided excellent chromatograms for the carotenoids of different foods, including those with complex carotenoid composition, such as leafy vegetables (Kimura and Rodriguez-Amaya 2002; Kobori and Rodriguez-Amaya 2008), squashes and pumpkins (Azevedo-Meleiro and Rodriguez-Amaya 2007), pepper (Azevedo-Meleiro and Rodriguez-Amaya 2009) and the tropical fruit *Eugenia uniflora* L (Azevedo-Meleiro and Rodriguez-Amaya 2004).

As mobile phases for carotenoids, the primary solvents have been acetonitrile and methanol. In all our work using the 3 μm C_{18} column and in most of those employing the Vydac column, the mobile phase has these two solvents. Ammonium acetate improves the recovery of carotenoids from the column when added to acetonitrile-based solvents. The addition of triethylamine to the mobile phase, which contains ammonium acetate, further increases recovery, from around 60 % to over 90 % (Hart and Scott 1995).

For the C_{30} column, a combination of methanol and methyl-*tert*-butyl ether has been used in majority of the studies.

Since geometric isomers of carotenoids have different bioavailability and antioxidant activity, when measurable amounts of the *Z*-isomers are present, their separation and quantification should be carried out, especially in health-oriented studies. Examples of foods in which quantification of the *Z*-isomers is important are thermally prepared or processed foods (Lessin et al. 1997), such as tomato and tomato products (Tavares and Rodriguez-Amaya 1994), prepared/processed corn (Aman et al. 2005; de Oliveira and Rodriguez-Amaya 2007), carrot juice (Marx et al. 2000) and cooked spinach (Glaser et al. 2003). Fresh foods generally have negligible amounts of *Z*-isomers (Godoy and Rodriguez-Amaya 1994, 1998). An exception is cassava root in which appreciable levels of (9-*Z*)-β-carotene and (13-*Z*)-β-carotene accompany the principal carotenoid, (all-*E*)-β-carotene (Kimura et al. 2007). The two columns cited above can be used for this separation: the 3 μm C18 column and the C30 column. Identification is easier in the former because the *Z*-isomers elute close to the corresponding all-*E*-carotenoid, but separation of the isomers is better in the latter. Equivalent results were obtained for (all-*E*)-, (9-*Z*-)- and 13-*Z*-β-carotene of cassava when these two columns were used (Kimura et al. 2007).

Gradient elution should be used only when the analysis cannot be done isocratically. Isocratic separation is rapid and results in stable baseline and more reproducible retention times. Gradient elution, on the other hand, has the advantages of greater resolving power, improved sensitivity and elution of strongly retained compounds. It is more likely to resolve the whole range of carotenoids found in a given food. However, it has several disadvantages:

- Increased complexity
- Need for column re-equilibration between runs
- Greater differential detector's response (i.e., different detector's signals for the same concentration of different compounds)
- Often poor reproducibility

Good solvent miscibility is required to prevent baseline disturbance resulting from outgassing and refractive index effects (Craft et al. 1992).

In the 1990s, lycopene had drawn analytical concern because of reported low recoveries from the HPLC column (Konings and Roomans 1997), high intralaboratory (Hart and Scott 1995) and interlaboratory (Scott et al. 1996) coefficients of variation and low range of linearity (Riso and Porrini 1997). Each laboratory should verify if their analytical procedure and/or chromatographic system lead to loss of this carotenoid.

3.4.3.10 Identification

Since inconclusive or even erroneous identifications could be found in the literature, Schiedt and Liaaen-Jensen (1995) recommended the following minimum criteria for the identification of carotenoids:

1. The visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested
2. Chromatographic properties must be identical in two systems, preferably TLC (R_F) and HPLC (t_R), and co-chromatography with an authentic sample should be demonstrated
3. A mass spectrum should be obtained, which allows at least the confirmation of the molecular mass.

The chromatographic behavior and the ultraviolet and visible absorption spectrum provide the first clues for the identification of carotenoids. Both the position of the absorption maxima (λmax) and the shape (fine structure) of the spectrum reflect the chromophore. Tables giving the λmax values for carotenoids are available in many reviews and books published through the years (e.g. Britton 1995; Rodriguez-Amaya 1999). Identification of carotenoids based solely on the retention times/co-chromatography with standards or the absorption spectra may lead to erroneous conclusions. Different carotenoids can have the same retention time in a given chromatographic system, and different carotenoids may have the same chromophore, thus presenting the same absorption spectrum. However, carotenoids with well-known structure can be conclusively identified by the combined and judicious use of chromatographic behavior, UV-visible absorption spectra and, for xanthophylls, specific group chemical reactions (Azevedo-Meleiro and Rodriguez-Amaya 2004). In the absence of mass spectra, the chemical tests can confirm the type, location and number of functional groups (Eugster 1995; Rodriguez-Amaya 1999).

The absorption spectra of carotenoids are markedly solvent dependent. This has to be remembered in HPLC when spectra are taken by the photodiode array detector in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution. The λmax values recorded in hexane, petroleum ether, diethyl ether, methanol, ethanol and acetonitrile are practically the same, but higher by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane and 18–24 nm in toluene (Britton 1995).

Many laboratories around the world now have access to HPLC-MS. Aside from the molecular formula, important characteristic fragment ions facilitate the identification of carotenoids. Enzell and Back (1995) tabulated data for 170 different carotenoid end groups.

Several ionization methods have been used for the MS analysis of carotenoids: electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) atmospheric pressure photoionization (APPI), and atmospheric pressure solids analysis probe (ASAP) (Enzell and Back 1995; van Breemen 1995, 1997; Dachtler et al. 2001; Aman et al. 2005).

EI was the most common ionization method. Carotenoids generally give good molecular ions and many fragmentations diagnostic of particular structural features had been identified (Enzell and Back 1995).

APCI has rapidly become the most widely used ionization technique. APCI produces molecular ions and/or protonated or deprotonated molecules, depending upon the mobile phase conditions and abundant fragment ions, especially for xanthophylls (van Breemen 1997). The main advantage of APCI is its high linearity of detector response over a carotenoid concentration range of at least three orders of magnitude, suggesting that APCI LC/MS might be the preferred mass spectrometric technique for carotenoid quantification. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations because it tends to reduce the abundance of the molecular ions.

Fruits and flowers have a complicated mixture of esterified carotenoids, which are difficult to identify in the ester form. HPLC-APCI/MS was used for the identification of carotenoid esters in mango (Pott et al. 2003), red orange essential oils (Dugo et al. 2008), marigold flowers, several fruits (Breithaupt et al. 2002), tritordeum grains (Mellado-Ortega and Hornero-Méndez 2012), wolfberries, Chinese lanterns, orange pepper and sea buckthorn (Weller and Breithaupt 2003).

Tandem mass spectrometry (MS/MS) offers many advantages for the analysis of carotenoids. Analyzing zeaxanthin and its oxidation products in biological samples, Prasain et al. (2005) affirmed that tandem mass spectrometric analysis offers added selectivity and specificity and requires minimal sample clean-up, leading to high sample throughput. It reduces interference by impurities in the extract and allows the following (Rivera and Canela-Garayoa 2012):

- Minimal sample clean-up
- Distinguishing between carotenoids that coelute
- Information about structural isomers
- A decrease in overall analysis time.

A limitation of MS for carotenoids is its inability to distinguish between all-*E*- and *Z*-isomers and between 5,6- and 5,8-epoxy carotenoids. HPLC-NMR (Dachtler et al. 2001; Glaser et al. 2003; Aman et al. 2005) and off-line NMR (Tiziani et al. 2006) have been utilized particularly for the unambiguous identification of geometric isomers. One- and two-dimensional NMR successfully determined the carotenoid profile of typical tomato juice, including the Z-isomers, with minimal purification procedures (Tiziani et al. 2006).

Xanthophylls undergo functional group chemical reactions that can serve as simple and rapid tests for the identification of carotenoids (Eugster 1995; Rodriguez-Amaya 1999). Primary and secondary hydroxyl groups are acetylated by acetic anhydride in pyridine. Allylic hydroxyls, isolated or allylic to the chromophore, are methylated with acidic methanol. Both the acetylated and methylated products have unchanged chromophores, thus unaltered UV-visible spectra, but are less polar than the original carotenoids.

Epoxy groups in the 5,6 or 5,6,5′,6′ positions are easily detected by their facile conversion to the furanoid derivatives in the presence of an acid catalyst, reflected by a hypsochromic shift of 20–25 nm or 40–50 nm, respectively.

Apocarotenals undergo reduction with LiAlH4 or NaBH4, manifested by the appearance of the three-maxima spectra of the resulting hydroxyl carotenoid, in lieu of the single broad band of the original apocarotenal.

Iodine-catalyzed *cis-trans* isomerization, which can be done directly in the spectrometer cuvette, results in a 3–5 nm shift of the λmaxs of all-*E*-carotenoids to lower wavelengths, whereas those of *cis* carotenoids (such as 15-*cis* and 13-*cis*-β-carotene) will shift to longer wavelengths after 1–5 min exposure to light.

3.4.3.11 Quantification

HPLC quantification is usually carried out by external calibration with the respective standards, although internal standardization has also been used. Because carotenoids absorb maximally at different wavelengths and have different coefficients of absorption, a carotenoid cannot be quantified accurately using another carotenoid as standard, as is sometimes seen in the literature.

In the calibration process, for each carotenoid being quantified, the analyst has to prepare standard solutions of varying concentrations (usually 5 concentrations), inject each solution usually in triplicate, and construct the standard curve (Rodriguez-Amaya and Kimura 2004). The standard curve should be linear, pass through the origin, and should bracket the expected concentrations of the food samples.

The concentration of the standard solution is determined by visible absorption spectrometry, using the absorption coefficient of the carotenoid being quantified (Rodriguez-Amaya and Kimura 2004). Absorption coefficients of the major all-*E*-carotenoids in foods in specific solvents are available (Britton 1995; Rodriguez-Amaya 1999). For *Z*-carotenoids, the absorption coefficients of the corresponding all-*E*-carotenoid is used. Since the coefficients for *Z*- isomers are generally lower, this practice underestimates the *Z*-isomer contents (Liaaen-Jensen 2004).

Since quantification is based on the detector's response for the analyte compared to the response for standard solutions of known concentrations, errors introduced in the preparation of the standard solutions, determination of the concentrations and construction of the calibration curves will be reflected in the analytical results. The purity of the standards should be verified and the concentrations of the standard solutions corrected accordingly. If necessary (i.e., low purity), the standards should be repurified.

Carotenoid standards are costly, unstable and often unavailable. Open column chromatography can be used to separate and isolate standards with purity comparable or even better than that of commercial standards (Kimura and Rodriguez-Amaya 2002; Rodriguez-Amaya and Kimura 2004; Kimura et al. 2007). This procedure has been used in other laboratories (e.g. Morris et al. 2004; Gama and de Sylos 2007; Giuffrida et al. 2007; Griffiths et al. 2007).

Even when HPLC-MS is used for identification, quantification has been carried out mostly with the DAD detector. As exceptions, Frenich et al. (2005), Matsumoto et al. (2007), and Slavin et al. (2009) quantified with HPLC-MS by selective ion monitoring (SIM). Weller and Breithaupt also quantified zeaxanthin esters by HPLC-MS.

3.4.3.12 UHPLC-DAD Methods

HPLC-DAD has provided a large body of reliable and detailed data on carotenoid composition of foods. However, because of environmental and economic concerns, the search for faster methods that use less organic solvents has continued.

Developments in column technology and instrumentation have led to ultra-high-performance liquid chromatography (UHPLC). This technique, in comparison with HPLC, uses narrow bore, shorter columns (2.1×50 mm vs. 4.6×200 mm); less run time (10 min vs. 30 min); lower flow rate (0.3 mL/min vs. 1 mL/min); lower injection volume (1.4 μL vs. 20 μL) and solvent volume/sample (4.0 mL vs. 27 mL); smaller particle size (2 μm vs. 2–5 μm); much higher back pressure (up to 103.5 MPa vs. 35–40 MPa) (Boboyo-Gil et al. 2012; Rivera and Canela-Garayoa 2012). High strength silica (HSS) C18 and T3 and ethylene bridged hybrid (BEH) C18 stationary phases have been successfully used to separate carotenoids.

Several advantages have been cited for UHPLC over conventional HPLC, including: (a) faster analyses due to shorter retention times; (b) narrower peaks, giving increased signal-to-noise ratio, and (c) higher resolution and sensitivity (Rivera and Canela-Garayoa 2012). Moreover, it is estimated that UHPLC typically saves at least 80 % of mobile phase compared to HPLC (Chen and Kord 2009); much lower solvent consumption is in accordance with green analytical chemistry.

Nevertheless, as with the HPLC methods, UHPLC methods have to undergo rigorous method standardization, intralaboratory and interlaboratory validation to guarantee data reliability. Special attention should be given to sampling and sample preparation because the smaller the analytical sample, the more difficult it is to guarantee representativity. There is also a tendency to transform the sample into dried or lyophilized, ground fine powder (Li et al 2012, 2013; Rivera and Canela 2012; Kaulmann et al. 2014), considered to be more amenable to small-scale analysis. Both drying and grinding can lead to substantial losses of carotenoids (Rodriguez-Amaya 1999, 2010).

UHPLC has been used for the quantification of carotenoids in corn (Rivera and Canela 2012; Rivera et al. 2013), Brassica vegetables (Guzman et al. 2012), durum wheat (Hung and Hatcher 2011), milk (Chauveau-Duriot et al. 2010), tomato (Li et al. 2012, 2013; van Meulebroek et al. 2012), honeybee pollen, pumpkin, and nectarine (Boboyo-Gil 2012), buckthorn berries and leaves (Pop et al. 2014), *Brassica oleraceae* and plum varieties (Kaulmann et al. 2014). In milk, the UHPLC method gave similar concentration of (all-*E*)-β-carotene to that obtained by an HPLC method (Chauveau-Duriot et al. 2010). UHPLC was compared to HPLC, using seven carotenoid standards, and the quantified concentrations were statistically indistinguishable (Boboyo-Gil et al. 2012)

3.4.3.13 Other Techniques and Methods

Carotenoid extraction with organic solvents generates large amounts of waste, the disposal of which is an environmental problem. An environmentally friendly alternative is supercritical fluid extraction with CO_2, which also has the advantages of inertness, low toxicity and reactivity. This technique has been applied to the extraction of carotenoids from food samples without a modifier (Gómez-Prieto et al. 2003) or with ethanol or methanol as modifier (Barth et al. 1995; Mathiasson et al. 2002). Several vegetable samples obtained with a supercritical CO_2 procedure with ethanol as modifier, performed in approximately 30 min, averaged 23 % higher than those of ethanol-pentane extraction, performed in 90 min (Marsili and Callahan 1993).

Other environmentally friendly extraction methods have been introduced, such as microwave-assisted extraction (Xiao et al. 2012; Hiranvarachat et al. 2013), accelerated solvent extraction (Breithaupt 2004; Sun et al. 2012), ultrasound-assisted extraction (Sun et al. 2006; Yue et al. 2006; Lianfu and Liu Zelong 2008; Li et al. 2013). Microwave-assisted extraction, which shortens the extraction time and improves the yield of the extraction, is simple and is not limited by solvent selectivity (Kiss et al. 2000). Ultrasound assisted extraction was used in quantifying lutein from chicken liver by HPLC (Sun et al. 2006). With three samples, the concentrations of lutein in the ultrasound assisted solvent extracted samples had higher levels (6.0, 10.4 and 5.5 μg/g) than those of saponified solvent extracted samples (2.9, 4.5, 2.5 μg/g).

Matrix solid-phase dispersion has been proposed for the rapid, mild (without artifact formation), complete and reproducible extraction of carotenoid *Z/E* isomers as shown with raw and cooked spinach samples (Glaser et al. 2003). It was also used for carotenoids in corn flour and kiwi (Gentili and Caretti 2011). In comparison to liquid-liquid extraction (LLE) and solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD) is easy to handle because the solid or viscous biological tissue can be directly mixed with the sorbent material; it combines the advantages of being time-saving and requiring less solvent (Dachtler et al. 2001). As the carotenoids elute in a small fraction, the evaporation of large amounts of solvent, as is usual in liquid-liquid extraction, is not necessary. With MSPD, the carotenoids are protected against oxidation and isomerization during the quick and gentle extraction process.

Isocratic separation and determination of carotenoids in vegetables by capillary electrochromatography was found to effectively resolve β-carotene, lycopene and lutein (Herrero-Martínez et al. 2006). The concentrations of β-carotene and lutein in carrot and spinach, and of lycopene in tomato obtained with this technique were within the ranges reported in the literature. Recently, ultra-high performance supercritical fluid chromatogaphy (UHPSFC) was applied to carotenoids in paprika oleoresin (Berger and Berger 2013).

Attenuated total reflectance infrared spectroscopy (ATR-IR) (Baranska et al. 2006b; de Nardo et al. 2009), Fourier transform infrared spectroscopy (FTIR) (Rubio-Diaz et al. 2011), Raman spectroscopy (Bhosale et al. 2004; Darwin et al. 2007) and NIRS (Pedro and Ferreira 2005; Clément et al. 2008) have been proposed for the determination of carotenoids. Values obtained by NIRS and

HPLC were found to be in good agreement (Berardo et al. 2004; Brenna and Berardo 2004), indicating the potential of NIRS as a quantitative method. However, in a comparison of Fourier transform-Raman (FT-Raman), ATR-IR, and NIRS for measuring lycopene and β-carotene, ATR-IR showed the best statistics. The prediction quality of Raman was poorer, and NIRS had the worst prediction potential (Baranska et al. 2006b).

It was also shown that X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) could be used for direct measurement of the major carotenoid in the annatto seed (Felicissimo et al. 2004).

As with the HPLC methods, these alternative procedures or methodologies should undergo standardization and validation, including interlaboratory studies, before they can be adopted for wider use.

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Part II
Regulation of Carotenoids Biosynthesis

Chapter 4
Carotenoids and Photosynthesis

Hideki Hashimoto, Chiasa Uragami, and Richard J. Cogdell

Abstract Carotenoids are ubiquitous and essential pigments in photosynthesis. They absorb in the blue-green region of the solar spectrum and transfer the absorbed energy to (bacterio-)chlorophylls, and so expand the wavelength range of light that is able to drive photosynthesis. This is an example of singlet–singlet energy transfer, and so carotenoids serve to enhance the overall efficiency of photosynthetic light reactions. Carotenoids also act to protect photosynthetic organisms from the harmful effects of excess exposure to light. Triplet–triplet energy transfer from chlorophylls to carotenoids plays a key role in this photoprotective reaction. In the light-harvesting pigment–protein complexes from purple photosynthetic bacteria and chlorophytes, carotenoids have an additional role of structural stabilization of those complexes. In this article we review what is currently known about how carotenoids discharge these functions. The molecular architecture of photosynthetic systems will be outlined first to provide a basis from which to describe carotenoid photochemistry, which underlies most of their important functions in photosynthesis.

Keywords Photosynthesis • Light-harvesting systems • Lutein • Neoxhantin • Purple photosynthetic bacteria

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4.1 Introduction

Most carotenoids found on earth are synthesized by photosynthetic organisms (Britton et al. 1998). The scale of the manufacture of carotenoids is enormous. The amount of chlorophyll synthesized each year has been determined by observation from the SeaWiFS satellite. Each year it has been estimated that about one billion tons of chlorophyll is produced (Jeffrey et al. 2005). Assuming that the production of carotenoids is about one-tenth of that of chlorophyll, then the annual amount of carotenoid produced by photosynthetic organisms can be estimated to be around 1000 million tons.

Chlorophylls that play a major role in photosynthesis cannot absorb much light in the 450–550 nm region where the solar radiation profile (spectrum) at the surface of earth has its maximum intensity. This is precisely the region where carotenoids absorb light strongly. They are able to transfer this excitation energy to the chlorophylls, making it available to power photosynthesis (Green and Parson 2003; Fromme 2008). This energy-transfer reaction allows the carotenoids to function as accessory light-harvesting pigments, broadening the spectral range over which light can support photosynthesis. This role of carotenoids is particularly significant in the cases of purple photosynthetic bacteria, heterokontophyta (*e.g.*, diatoms and brown algae), and dinoflagellates, all of which tend to occupy environmental niches where light intensity is usually limiting for growth, and where, especially in the case of dinoflagellates, most of the available solar energy is in the 450–550 nm region (Green and Parson 2003). However, the reason that carotenoids are essential for photosynthesis is not because of their light-harvesting role but because of their ability to prevent photo-damage that occurs under conditions of too much light (Young and Britton 1993; Frank and Cogdell 1996; Frank et al. 1999; Green and Parson 2003). The functions of carotenoids in photosynthesis can only really be understood in the context of the structure of the pigment-protein complexes in which most of them are organized. In the next section a brief account of these structures is provided.

4.2 Molecular Architecture of Photosynthetic Systems

Photosynthesis is not only found in plants and algae but also in a wide range of other types of organisms, even including anaerobic photosynthetic bacteria. Though these organisms can be strikingly different, their basic pattern of light reactions is quite similar. They all contain light-harvesting systems that are coupled to reaction centres (RCs). The majority of the light-harvesting pigments (mainly chlorophylls (Chls), carotenoids, and phycobilins) are organized into light-harvesting complexes. A small proportion of the Chls and carotenoids come together to form the RCs where the absorbed light energy is initially converted into chemical energy. Whereas the structures of the RCs are all rather similar, those of the light-harvesting complexes

are bewilderingly variable. Once the RCs have used the absorbed solar energy to drive the initial electron transfer reactions, subsequent electron transfer takes place within the photosynthetic membranes that finally result in the production of ATP and reduced NADP. These two chemicals are then consumed in the no-light CO_2 fixation reactions where carbohydrates are produced (Clayton 1980; Blankenship et al. 1995; Hunter et al. 2009). The light reactions typically take place in and on the photosynthetic membranes, while the dark reactions take place in the aqueous phase, either in the stroma of the chloroplast or the cytoplasm of the photosynthetic cells.

Figure 4.1 shows the schematic illustration of the structure and pigment composition of the major types of photosynthetic antenna pigment-protein complexes. It should be pointed out that although most antenna complexes involve proteins as well as pigments, this is not the case for chlorosomes, which are just highly organized pigment only complexes.

As shown in Fig. 4.1 anoxygenic photosynthetic organisms only contain one type of RC. The green bacteria typically contain so-called type I RCs that have iron-sulfur complexes as their terminal electron acceptors. The purple bacteria contain type II RCs that have quinones as their terminal electron acceptors. Oxygenic photosynthetic organisms have both types of RC, in this case usually called PS I and PS II. The light-harvesting complexes are arranged around the RCs. Often these light-harvesting complexes can be thought of as consisting of two basic types, the core and the peripheral light-harvesting complexes. Usually all RCs have core light-harvesting complexes associated with them that form their minimum light-harvesting compliment. Surrounding these photosystems, the peripheral light-harvesting complexes are arranged. The type and the amount of these peripheral light-harvesting complexes are controlled by a variety of environmental factors such as light intensity and light quality. Typically, the lower the light intensity is, the larger the size of the peripheral light-harvesting systems will be.

X-ray crystallography has been a powerful tool with which to determine the structure of the photosynthetic pigment protein complexes. The first structure of a Chl containing protein was that of the water-soluble Fenna-Matthews-Olson (FMO) protein (Fenna and Matthews 1975). However, since most of the important photosynthetic pigment-protein complexes containing Chls and carotenoids are membrane proteins, there was a long delay between the description of the structure of FMO and the determination of the purple bacterial RC. The first example of the X-ray crystallography of the RC was reported in 1985 (Deisenhofer et al. 1985). This pioneering work, carried out by three German researchers, Michael, Deisenhofer, and Hüber, was recognized by the award of the Nobel Prize in chemistry in 1988. This achievement broke the logjam so that we now have high resolution structures of PS I (Fromme et al. 2001) and PS II (Umena et al. 2011; Suga et al. 2014) from cyanobacteria, LHCII from higher plants (Liu et al. 2004) and LH2 (McDermott et al. 1995) and RC-LH1 core complexes (Roszak et al. 2003; Qian et al. 2013; Niwa et al. 2014) from purple bacteria. These structures are all illustrated in Fig. 4.2. Also, remarkably, in recent years high-resolution transmission electron microscopy (TEM) and atomic force microscopy (AFM) have been used

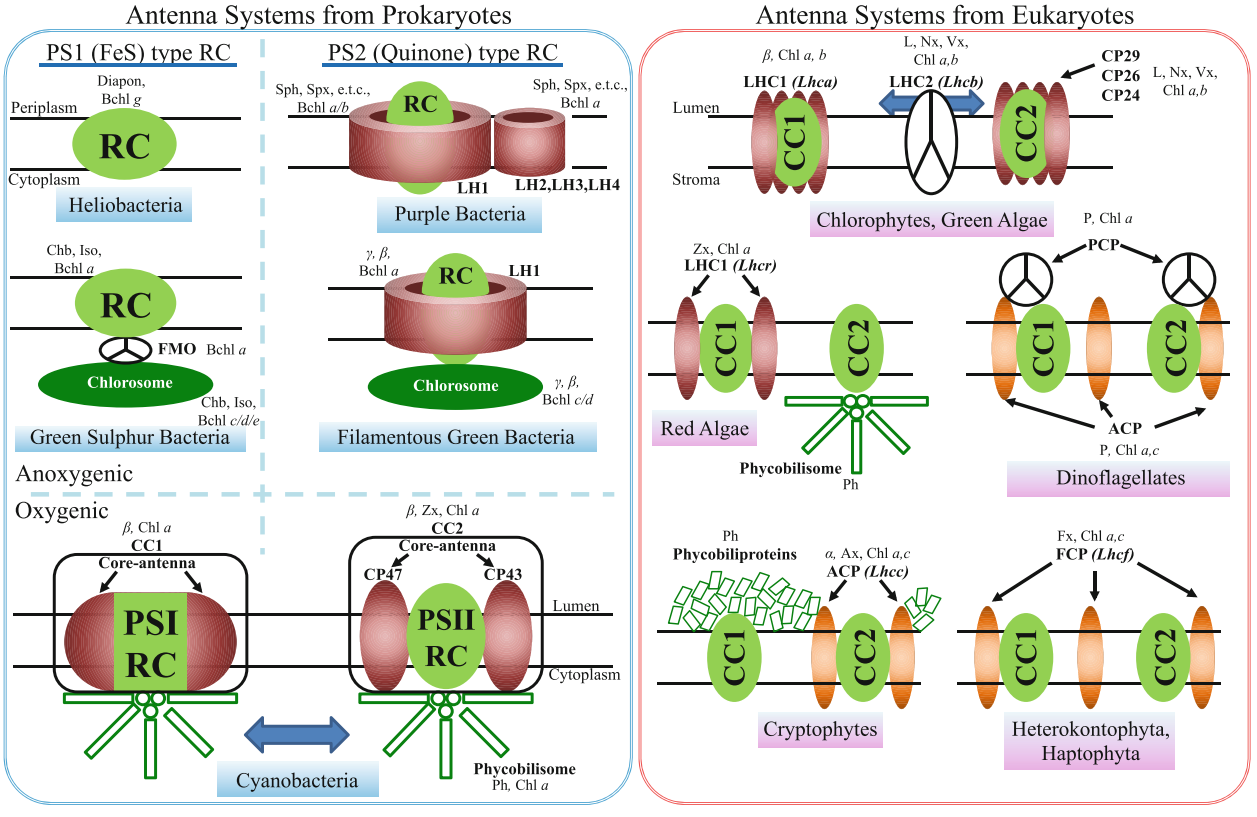

Fig. 4.1 (continued)

to visualize the organization of these complexes within fully native photosynthetic membranes (Siebert et al. 2004; Scheuring and Sturgis 2009; Aartsma and Matysik 2008). As a result of these, we now have both a detailed structural picture of the individual complexes and well-defined maps of how these complexes interact with each other within the photosynthetic membranes.

Carotenoids are found in all of the pigment-protein complexes shown in Fig. 4.1, both in the reaction centers and in the light-harvesting complexes (Green and Parson 2003; Fromme 2008). Table 4.1 describes the carotenoid composition of some of the different RCs and the light-harvesting complexes. In oxygenic photosynthetic organisms the RCs and core complexes mainly contain β-carotene, whereas the carotenoid composition of the peripheral light-harvesting complexes is more varied. For example, the higher plant LHCII complexes mainly contain a mixture of lutein, neoxanthin, zeaxanthin, and violaxanthin. The peripheral antenna complexes found in heterokontophyta, which includes diatoms and brown algae, contain carotenoids such as fucoxanthin, which functions as much more efficient accessory light-harvesting pigment. This is only to be expected since this group of photosynthetic organisms essentially obtains all of their light energy from that absorbed by carotenoids. A similar situation is found in dinoflagellates, where their major light-harvesting complex, the peridinin-Chl *a* complex, also uses another unique carotenoid that is highly efficient at transferring energy to the Chls. In this case, the peridinin-Chl *a* complex is water-soluble and its structure has been determined by X-ray crystallography (Hofmann et al. 1996). It is rather unusual, as it contains more carotenoids than Chls. The red algae and cryptophytes also contain water-soluble antenna complexes. These are called phycobiliproteins and contain neither chlorophylls nor carotenoids. Their light absorbing pigments are the group of open chain tetrapyrroles called the phycobilins. Interestingly these are the only examples of the light-harvesting pigments that are covalently attached to the pigment binding proteins. The phycobiliproteins are typically organized into large multi-subunit structures called the phycobilisomes. The final type of light-harvesting system is seen in the green bacteria. These bacteria contain chlorosomes that are bags of bacteriochlorophyll (Bchl) *c*, *d*, and *e*, surrounded by a lipid monolayer. These Bchls form strongly exciton-coupled aggregates that absorb soar energy and transfer it *via*

Fig. 4.1 (continued) Schematic illustrations of antenna systems from prokaryotes and eukaryotes. The ellipses display the protein of LHC superfamily, and their genes are written in parentheses. The complexes that function with trimer unit are described with parting line in the circle. The complexes that proceed the state transition due to the light condition is described with two-way arrows. Abbreviations stand for as follows. α : α-Carotene; β: β-Carotene; γ: γ-Carotene; Ax: Alloxanthin; Bchl: Bacteriochlorophyll; Chb; Chlorobactene; Chl: Chlorophyll; Diapon: Diaponeurosporene; Fx: Fucoxanthin; Iso: Isorenieratene; L: Lutein; Nx: Neoxanthin; P: Peridinin; Ph: Phycobiline; Sph: Spheroidene; Spx: Spirilloxanthin; Vx: Violaxanthin; Zx: Zeaxanthin; CC1,CC2: Core Complex 1,2; RC: Reaction Centre; PSI, II: Photosystem I, II; PCP: Peridinin-Chlorophyll a binding Protein; ACP: Chlorophyll a/c Protein; FCP: Fucoxanthin- Chlorophyll a/c binding Protein; LH1, LH2, LH3, LH4: Light Harvesting Antenna 1,2,3,4 (Modified from Green and Parson 2003)

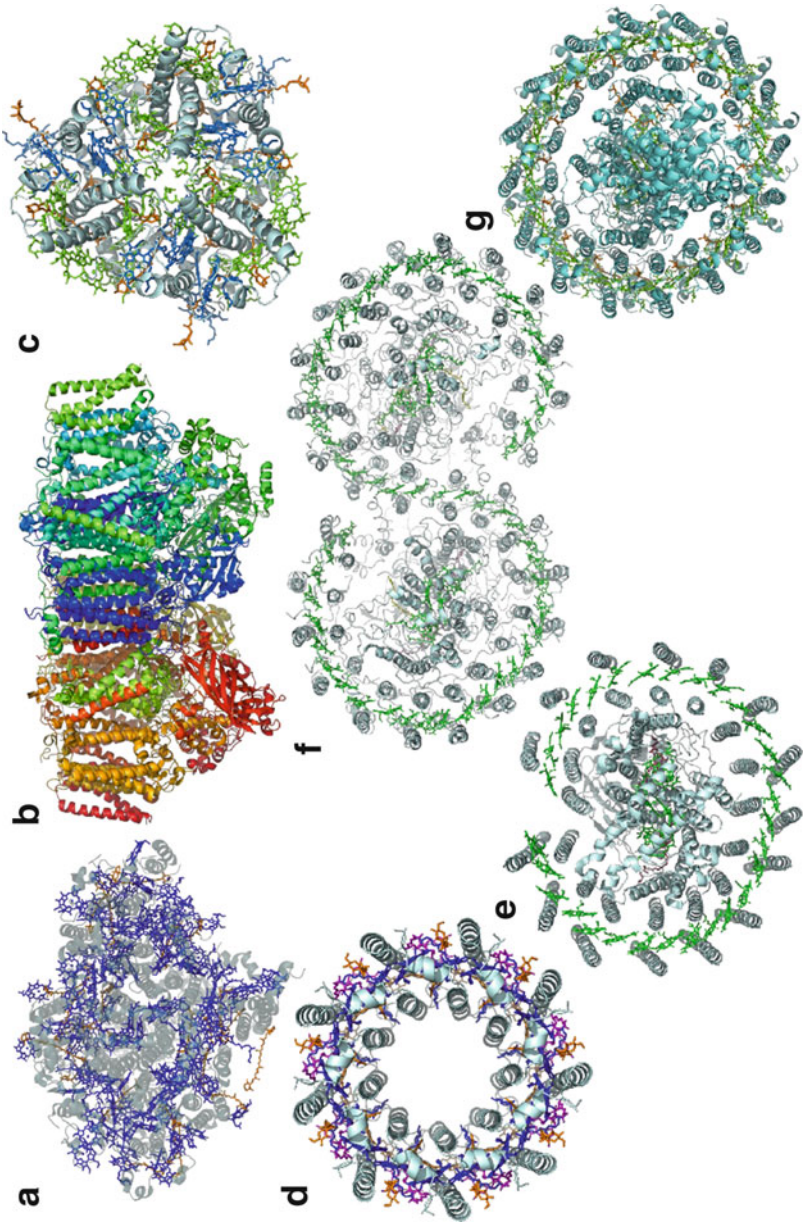

Fig. 4.2 (continued)

Table 4.1 Carotenoid composition of some of the different RCs and light-harvesting complexes

Antenna systems from prokaryotes			
Name	Type of RC	Oxygenic or anoxygenic	Carotenoids
Heliobacteira	Type I (FeS)	Anoxygenic	Diaponeurosporene
Green Sulphur Bacteria	Type I (FeS)	Anoxygenic	Isorenieratene
Purple Bacteria	Type II (Quinone)	Anoxygenic	Spheroidene, Spirilloxanthin, etc.
Filamentous Green Bacteria	Type II (Quinone)	Anoxygenic	γ-Carotene, β-Carotene
Cyanobacteria	PS I (FeS)/ PS II (Quinone)	Oxygenic	β-carotene, Zeaxanthin

Antenna systems from eukaryotes	
Name	Carotenoids
Chlorophytes, Green Algae	β-Carotene, Lutein, Neoxanthin, Violaxanthin
Red Algae	Zeaxanthin
Dinoflagellates	Peridinin
Cryptophytes	α-Carotene, Alloxanthin
Heterokontophyta, Haptophyta	Fucoxanthin

a base plate structure onto the membrane bound RCs. In the green sulfur bacteria FMO mediates the energy transfer from the base plate to the RCs.

4.3 Purple Photosynthetic Bacteria as a Model System

The purple photosynthetic bacterium has been used as a model to understand the overall energy and electron transfer reactions that occur in the early stages of photosynthesis. In this section, the basic concepts of energy and electron flow in the primary process of photosynthesis are explained by referencing to the example of purple photosynthetic bacteria (Hunter et al. 2009). The light reactions in purple photosynthetic bacteria take place in and on an extensive system of intracytoplasmic membranes. In bacteria like *Rhodobacter* (*Rba.*) *sphaeroides* and *Rhodospirillum* (*Rsp.*) *rubrum* these membranes are vesicular (individual vesicles are

Fig. 4.2 (continued) High resolution crystallographic structures of (**a**) PS I (PDB: 1JB0) and (**b**) PS II (3WU2) from cyanobacteria, (**c**) LHCII (1RWT) from higher plants, (**d**) LH2 (1KZU) from a purple photosynthetic bacterium Rps. acidophila 10050, and RC-LH1 core complexes from (**e**) Rps. palustris (1PYH), (**f**) Rba. sphaeroides (4V9G), and (**g**) Thermochromatium tepidum (4V8K), which are drawn using PyMol software with data from protein databank (PDB)

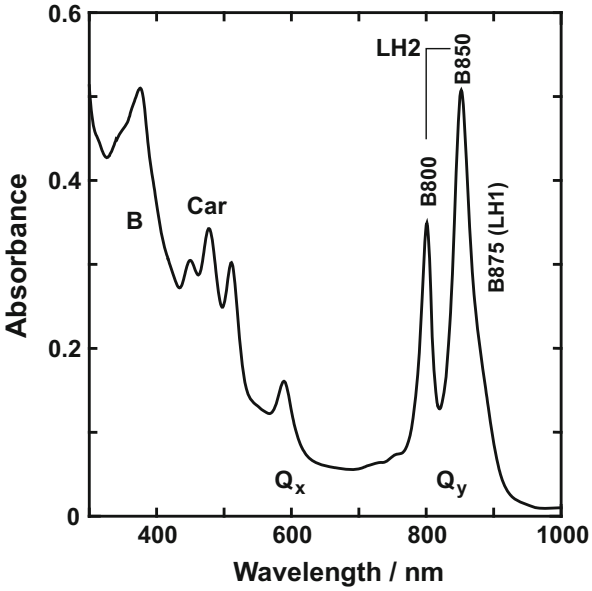

Fig. 4.3 Absorption spectrum of chromatophores (photosynthetic membranes) from a purple photosynthetic bacterium Rba. sphaeroides 2.4.1 recorded at room temperature in Tris-HCl (pH = 8.0) buffer solution

called chromatophores), while in other species, such as *Rhodopseudomonas* (*Rps.*) *acidophila*, *Rps. palustris*, and *Rps. viridis* (now called *Blastochloris* (*Blc.*) *viridis*), they are lamella. Figure 4.3 shows the absorption spectrum of the chromatophores of *Rba. sphaeroides* strain 2.4.1 recorded at room temperature. Structured absorption bands spread over a wide spectral range, including the ultraviolet, visible, and near infrared regions. They are all due to two types of photosynthetic pigments, carotenoid and Bchl *a*. Figure 4.4 shows the chemical structures of the carotenoid, spheroidene, and Bchl *a*, which are bound to the *Rba. sphaeroides* strain 2.4.1 complexes. In Fig. 4.2 the absorption band noted with Car is due to spheroidene, and those noted with B, Q_x, and Q_y are due to Bchl *a*. Carotenoids are hydrocarbons with a linearly conjugated polyene chain (see Fig. 4.4a). The positions of their absorption bands depend on the length of conjugation. Since in purple bacteria the major Bchl *a* Q_y absorption bands lie outside the color range seen by our eyes, the differences in the conjugation length of the carotenoids are the reason for the difference of colors of purple photosynthetic bacteria (see Fig. 4.8, *vide infra*). Figure 4.4b shows the chemical structure of Bchl *a*. All of its optical and photophysical properties reside in the conjugated bonds seen running around the bacteriochlorin ring. The phytol chain is chemically and photochemically inert. The position of the Q_y absorption band of Bchl *a* is sensitive to how the pigment is bound into the light-harvesting apoproteins. Three distinct Q_y absorption bands of Bchl *a* are observed at 800, 850 and 880 nm. These pigment molecules are bound to the light-harvesting apoproteins. In general, most purple bacteria have three major pigment-protein complexes, the peripheral LH2 antenna complexes, core LH1 antenna complexes, and RCs. The antenna pigment-protein complexes capture light energy and transfer it to the RC,

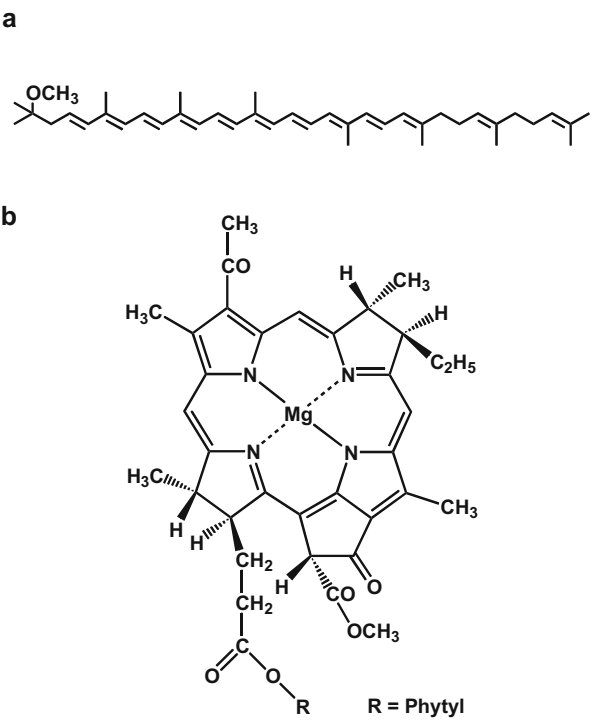

Fig. 4.4 Chemical structures of (**a**) spheroidene and (**b**) bacteriochlorophyll *a*

in the form of excitation energy. The energy flows from LH2 → LH1 → RC (see Fig. 4.6, *vide infra*). In the RC, charge separation takes place using the transferred excitation energy, and electron transfer reactions follow as a consequence.

Figure 4.5 shows the pigment arrangement determined by X-ray crystallography of the RC from *Rba. sphaeroides* (Roszak et al. 2004). Here P is the special pair Bchl *a* dimer, B is a Bchl *a* monomer, H is bacteriopheophytin, and Q is ubiquinone. These pigments are arranged with pseudo two-fold symmetry in the RC. The excitation energy is transferred from LH1 complexes to the Bchl *a* dimer (P). Then the electron transfer reactions follow. Although the pigments are arranged into two arms that show pseudo two-fold symmetry, electron transfer only takes place down the pathway that is denoted with subscript "A" (A-branch). An electron leaves P and is transferred *via* B and H arriving at Q_A within 200 ps after charge separation. One carotenoid molecule is also bound to the RC and has a 15-*cis* conformation. This carotenoid breaks the two-fold symmetry and lies beside the inactive B-branch and it protects the RC from destructive photo-damage when excess amounts light energy are present (photoprotective function) (Fraser et al. 2001). The details of this photoprotective reaction will be described below.

The electron on Q_A is eventually transferred to Q_B. Q_B is capable of accepting two electrons. $Q_B{}^{2-}$ then binds with two protons (H^+) and generates Q_BH_2. Finally, Q_BH_2 is released from the RC. As illustrated in Fig. 4.6, Q_BH_2 released

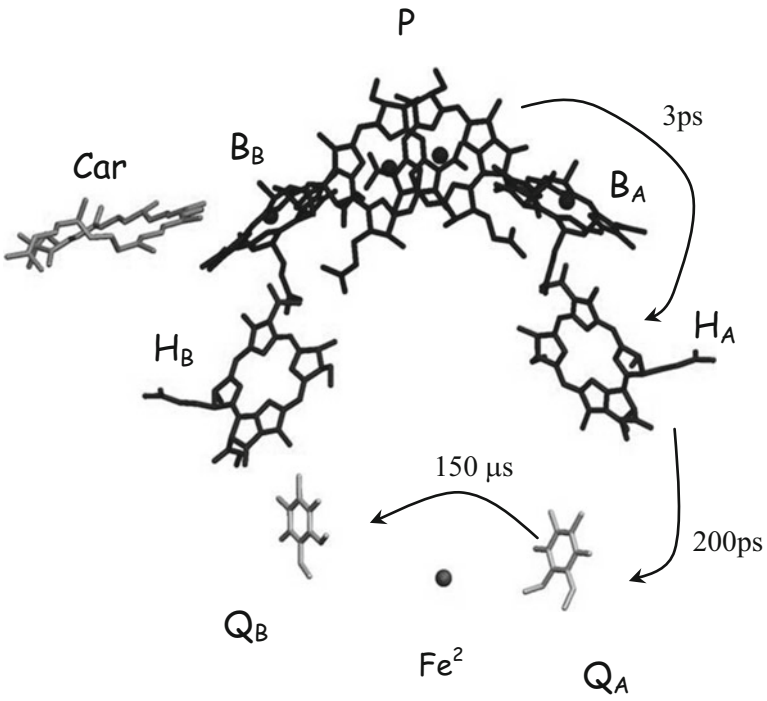

Fig. 4.5 Pigment arrangement and the electron transfer pathway in the reaction centre from a purple photosynthetic bacterium Rba. sphaeroides, which is drawn with PDB data (1RGN) using VMD software

from the RC is transferred into the quinone pool that exists in the photosynthetic membranes. The fully reduced quinone passes its electrons to the cytochrome bc_1 complex. From the cytochrome bc_1 complex the electron is passed to cytochrome c_2. This cytochrome c_2 carries the electron back to the RC and re-reduces P^+ to P. During this process of cyclic electron transport, protons (H^+) are pumped across the membrane generating a proton motive force (ΔpH). This proton gradient then acts on the ATP synthase to generate ATP (adenosine tri-phosphate) by the classical chemiosmotic mechanism. X-ray crystallographic structures have now been determined for cytochrome bc_1 (Xia et al. 1997), cytochrome c_2 (Benning et al. 1991), and ATPase (Stock et al. 1999).

The first report of the X-ray crystallographic analysis of a membrane bound antenna pigment-protein complex was presented after 10 years of that of the RC (McDermott et al. 1995). Figure 4.7 (right-hand side) shows the structure of the LH2

Fig. 4.6 (continued) Schematic illustration that shows the flows of excitation energy, electron, and proton (H) in the photosynthetic membrane from purple photosynthetic bacteria

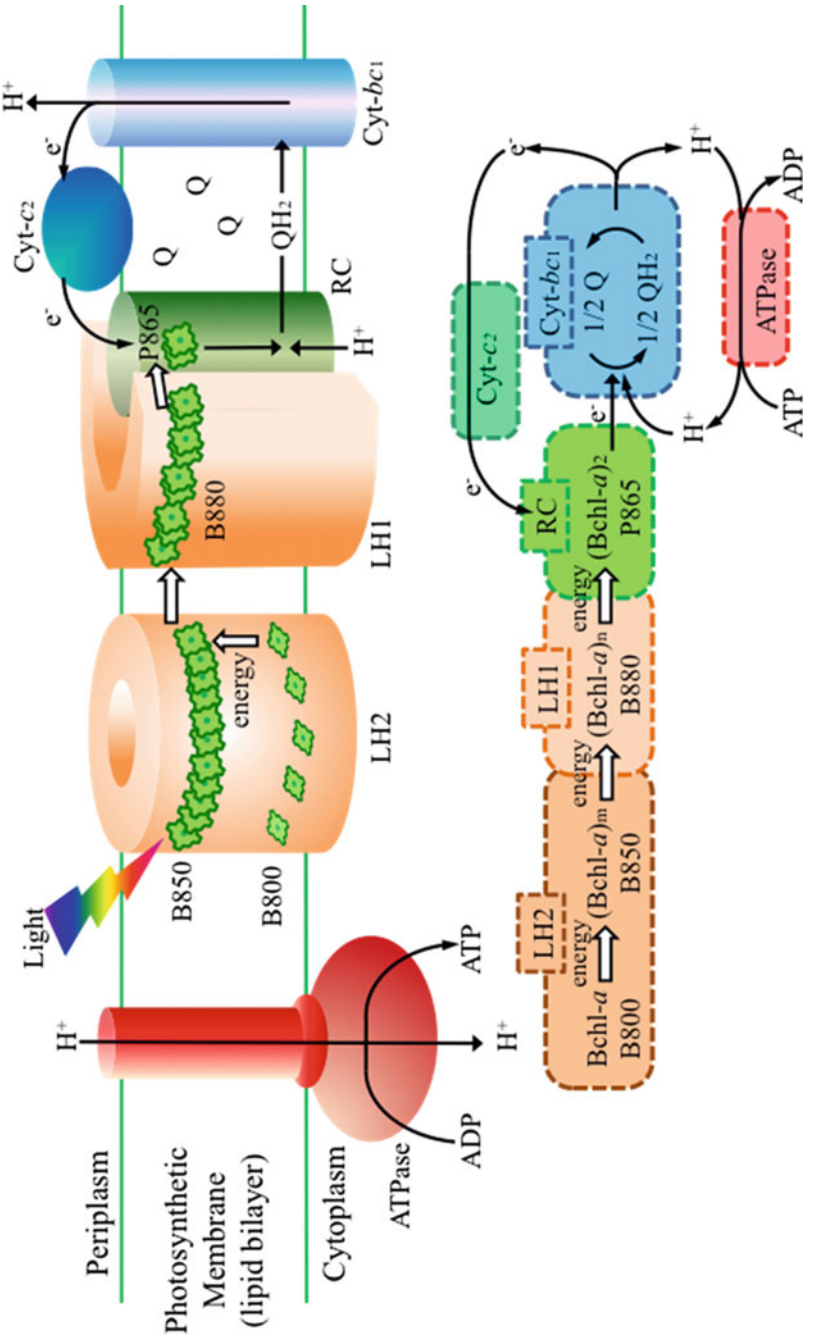

Fig. 4.6 (continued)

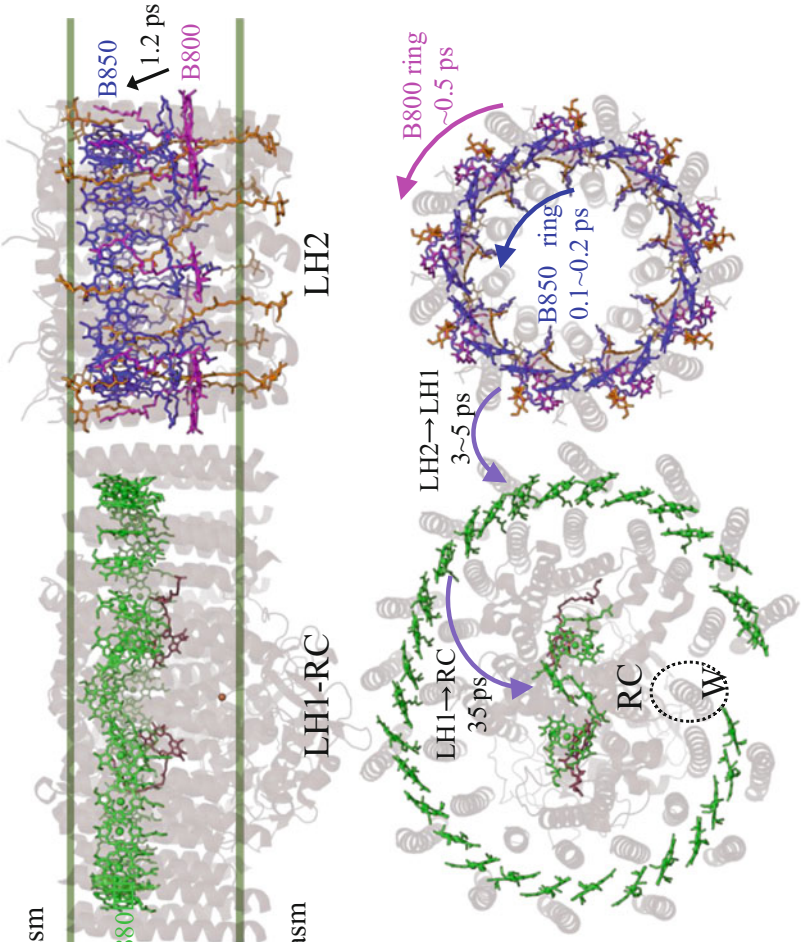

Fig. 4.7 (continued)

complex from the purple photosynthetic bacterium *Rps. acidophila* strain 10050. The LH2 complex is an oligomer of a monomer unit that is composed of a pair of α-helical apoproteins (α-polypeptide (inner) and β-polypeptide (outer)) in which one Bchl *a* monomer (B800 Bchl *a*), one Bchl *a* dimer (B850 Bchl *a*'s), and one carotenoid (rhodopsin glucoside) are non-covalently bound. Nine of these monomer units aggregate to form a beautiful ring structure with 9-fold symmetry. As shown in Figs. 4.3 and 4.8, there are two populations of Bchl *a* that have Q_y absorption at 800 and 850 nm, which are present in a single LH2 complex. The monomeric Bchl *a* gives rise to the 800 nm absorption band, and the dimeric Bchl *a*'s give rise to the 850 nm absorption band. Dimerization of the Bchl *a* molecules stabilizes the excited state energy by excitonic interaction, and then the $Q_y$absorption maximum shows a 50 nm red-shift compared to that of monomer. The carotenoid that is bound to LH2 is in *van der Waals* interaction with both the B800 and B850 Bchls. The carotenoid absorbs the blue-green region of sun light where Bchl *a* has scant absorption, and transfers the captured energy to both B800 and B850 Bchls (light-harvesting function of carotenoids). The detailed mechanisms involved in these energy transfer reactions will be described later in this review. The carotenoid molecule binds together neighboring monomer units of LH2 and stabilizes its structure (Fraser et al. 2001). In many cases, if the carotenoid is not present then the LH2 complexes will fail to assemble.

Following the determination of the structure of LH2 a low-resolution projection map of LH1 (a reconstituted sample from *Rsp. rubrum*) showed that it was a larger ring structure (a 16-mer) (Karrasch et al. 1995). The first low resolution X-ray crystal structure of an LH1-RC core complex from a purple bacterium was described in 2003 (Roszak et al. 2003). This complex was obtained from *Rps. palustris* and the structure was determined at a resolution of 4.8 Å. This structure is illustrated in Fig. 4.7 (left hand side). The LH1 complex is composed of 15 pairs of transmembrane α-helical α- and β-polypeptides and one transmembrane α-helical polypeptide (denoted W in Fig. 4.7). The LH1 ellipse surrounds the RC. The W polypeptide is thought to be a pufX homologue and to prevent the closure of the LH1 ellipse, thereby providing a gap through which the Q_BH_2 released from the RC can go through the LH1-RC complex, and connect with the other components of the cyclic electron transport pathway. More recently the X-ray crystallography of two other types of LH1-RC complexes have been reported (Qian et al. 2013; Niwa et al. 2014). The LH1-RC complex from *Rba. sphaeroides* is a dimer and the complex from *Chromatium tepidum* is a monomer without a pufX like protein in the LH1 ring.

Fig. 4.7 (continued) Structures of LH2 complex (*right-hand side*) from a purple photosynthetic bacterium Rps. acidophila 10050 (PDB: 1KZU) and those of RCLH1 core complex (*left-hand side*) from Rps. palustris (PDB: 1PYH), which are drawn using PyMol software. The *upper panel* shows a viewgraph from horizontal direction of the photosynthetic membrane, and the *lower panel* shows that from the cytoplasmic side

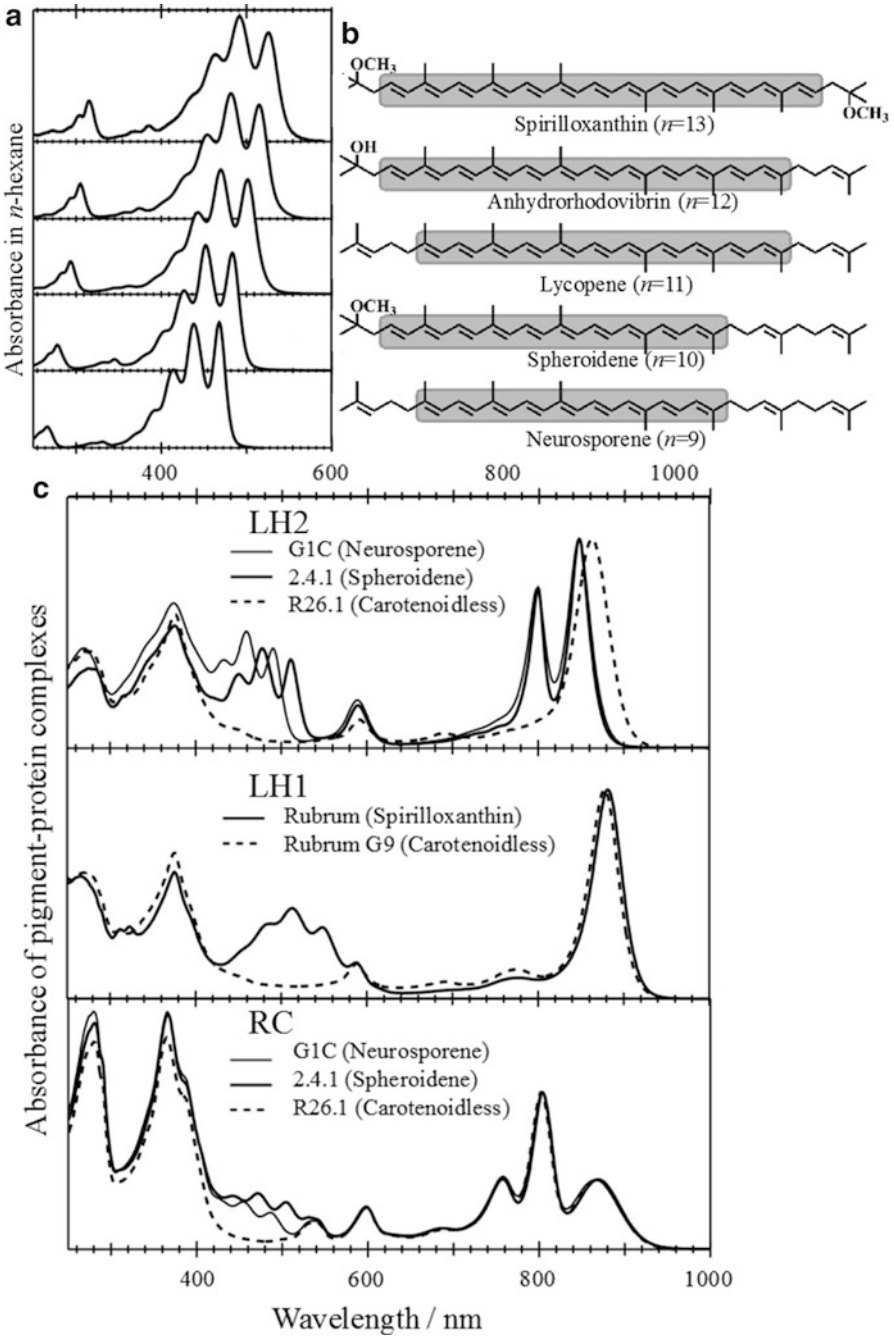

Fig. 4.8 (**a**) Absorption spectra in *n*-hexane solutions and (**b**) chemical structures of carotenoids bound to purple photosynthetic bacteria. Absorption spectra of pigment-protein complexes in buffer solutions are shown in (**c**)

The development of ultrafast laser spectroscopies has allowed real time observation of the excitation energy transfer (EET) in light-harvesting complexes to become possible (Sundström et al. 1999). In Fig. 4.7 typical rate constants of the EET in a purple bacterial photosynthetic unit have been summarized. The rates of EET are very fast. This is essential since a typical lifetime for a Bchl *a* first excited singlet state is about one nanosecond (10^{-9} s). If any EET process is going to be efficient, then its rate must be much faster than one nanosecond. The rate of EET from B800 to B850 in LH2 is 0.9 ps at room temperature. It then takes 3–5 ps for the energy to be transferred from B850 in LH2 to B875 in LH1. The slowest step in the EET pathway to the RC is from B875 to the special in the RC. This step takes about 35 ps. The slowness is due to the longer distance involved in this final EET step. It should be pointed out though that the total transfer time from B800 in LH2 to the special in the RC only takes about 40 ps and that this beats the one nanosecond target by a long way and so ensures high efficiency.

4.4 An Outline of Ultrafast Relaxation Processes of Carotenoids Following Photoexcitation

Carotenoids have strong absorption of visible light in the blue and green region of the spectrum. Most of the carotenoids found in photosynthetic organisms have characteristic yellow, orange, and red colors. The lowest excited singlet-state in most pigment molecules represents the lowest energy, optically allowed one-photon transition from the ground state. The energy of this state controls the color of that pigment molecule. However, carotenoids have a non-standard pattern of excited states. The lowest energy optically allowed excited singlet-state is not the lowest energy singlet-state. The lowest singlet excited energy state is formally a one photon forbidden state. This unusual photophysical pattern is a result of symmetry rules. The ground electronic state of the carotenoid has the $^1A_g^-$ symmetry based on the assumption that it has a C_{2h} point symmetry in its polyene backbone. The lowest singlet excited state, S_1 has $2^1A_g^-$ symmetry and a one photon induced transition is therefore optically forbidden. The lowest optically allowed state is the $1^1B_u^+$ (S_2) state. When the $1^1B_u^+$ state is induced by a short excitation pulse it decays into the $2^1A_g^-$ state within 100–300 fs. The S_1 state typically decays back to the ground state in a few picoseconds (Frank 2001). The exact rate constants of these processes depends factors such as the number of conjugated double bonds. This however is probably an over simplification. Theoretical studies based primarily on symmetrical polyenes have predicted other possible excited singlet states such as $3^1A_g^-$ and $1^1B_u^-$ (Tavan and Schulten 1986, 1987). The possibility of these multiple excited states and indeed others has made understanding carotenoid photophysics extremely complicated (Sashima et al. 2000).

In the following discussion of the light-harvesting role of carotenoids in pigment–protein complexes from purple bacteria we will initially present a

simplified view and then introduce some of the complicationess and try to interpret where this field is currently. Probably the simplest way to begin this discussion is to describe the experiments of Macpherson et al. (2001). These authors compared the excited state kinetics of a carotenoid in organic solvent with the same carotenoid when it was bound into its LH2 complex. The idea was that any carotenoid singlet excited state that was capable of transferring energy to Bchl *a* in LH2 would be expected to have a shorter excited state lifetime in the antenna complex than in the organic solvent, since the energy transfer pathway would open another decay channel thereby accelerating the overall rate of decay of that excited singlet state. Moreover it would be expected that the decay of the donor carotenoid excited singlet state should match the rate of the energy arriving at the Bchl *a* molecule. In the case of the carotenoid rhodopin glucoside the decay of the S_2 state in the LH2 complex from *Rps. acidophila* was faster than that of the carotenoid in organic solvent (56 fs in LH2 and 133 fs in benzyl alcohol). The rise time of the arrival of the energy at both B800 and B850 matched decay time of rhodopin glucoside's S_2 state. The decay rate of the S_1 state of rhodopin glucoside was the same in the LH2 complex as in organic solvent. In this case the S_1 state is not active in energy transfer to Bchl *a*. However, in LH2 complexes from *Rba. sphaeroides* which contain carotenoids with fewer conjugated double bonds the S_1 state is able to transfer energy to the Bchl a molecules and its decay is accelerated in LH2 compared to be in organic solvent (Rondonuwu et al. 2004).

Since these experiments where the excitation pulses were relatively long it has become possible to use much shorter femtosecond pulses. When this was done by Cerullo *et al.* the data showed the presence of an intermediate state between S_2 and S_1 (Cerullo et al. 2002). This state was formed as S_2 decayed and gave rise to S_1 as it decayed. However with these extremely fast reactions it was not possible to be sure that this intermediate state was another pure excited singlet state such as $1^1B_u^-$. Since this time there have been many studies that have been suggested to require such an intermediate state to fully explain the experimental data. Therefore appearance of the nonlinear optical effect was suspected (Kosumi et al. 2005). But even this is not a complete picture. There have been yet other studies that have not seen or required the presence of such extra intermediate state to fit the data (Hashimoto et al. 2004). This has lead a lot of confusion. Most recently broadband two-dimensional (2D) electronic spectroscopy measurements on light-harvesting proteins from purple bacteria and isolated carotenoids were performed in order to characterize in more detail the excited-state manifold of carotenoids, which channel energy to Bchl molecules. The data revealed a well-resolved signal (cross peak) consistent with a previously postulated carotenoid dark state, the presence of which was confirmed by global kinetic analysis. The results point to this state's role in mediating energy flow from carotenoid to Bchl (Ostroumov et al. 2013).

Another type of intermediate excited state, termed as S* has been found with carotenoids both free in solution and bound to light- harvesting complexes, and so things become even more complicated (Gradinaru et al. 2001;

Papagiannakis et al. 2003a; Papagiannakis et al. 2003b; Wohlleben et al. 2004; Kosumi et al. 2012b). At the higher-energy side of the $S_1 \rightarrow S_n$ transition, a new transient absorption band was detected by means of pump–probe time-resolved absorption spectroscopy and subsequent spectral analysis using SVD (singular value decomposition) and global fitting. This newly identified absorption band was assigned to the S* state. The lifetime of this particular state was determined to be between 5 and 12 ps depending on both the species of carotenoid and on whether it was in or out of the light-harvesting complexes. The S* state decayed into the triplet state when the carotenoid was bound to the LH2 complex. However, when the carotenoid was free in organic solvent the S* state decayed to the ground state without generating the triplet state. Applying a pump–dump and transient absorption technique for β-carotene, lycopene, and zeaxanthin, Wohlleben et al. re-examined the origin of the S* state with the carotenoid free in solution (S^*_{sol}) (Wohlleben et al. 2004). They suggested that the S^*_{sol} state is a vibrational excited ground-state ($S^*_{sol} = \text{hot } S_0$), which is populated by a combination of impulsive Raman scattering of the pump pulse and $S_1 \rightarrow S_0$ internal conversion. They also found the S* state of the protein-bound carotenoid and re-designated it as S^*_T. These ideas have recently been supported by these authors' groups for spirilloxanthin both free in solution and bound to light-harvesting complexes (Kosumi et al. 2012b).

Involvement of vibrationally excited states in the relaxation process of carotenoids after photoexcitation was initially detected by time-resolved absorption spectroscopy (Andersson and Gillbro 1995; Polívka et al. 2001; Billsten et al. 2002), and has also been studied by time-resolved stimulated Raman spectroscopy (Bautista et al. 1999; Frank et al. 2000; Yoshizawa et al. 2001, 2002; McCamant et al. 2002).

For carotenoids that contain carbonyl groups there is the possibility of forming intramolecular charge-transfer states (S_{ICT}). These states have been well documented in the case of carotenoids such as peridinin and fucoxanthin (see Fig. 4.8 for chemical structures of these molecules) (Bautista et al. 1999; Frank et al. 2000; Zigmantas et al. 2001, 2002; Kosumi et al. 2009, 2011a, b, 2012a, 2014a, b). The importance of these charge transfer state seem to be that they allow carotenoid to chlorophyll energy transfer to be highly efficient. However, discussion of these charge transfer states is beyond the scope of this review. Readers who are interested in more details about this state should consult the excellent review by Polívka and Sundström (Polívka and Sundström 2004).

Figure 4.9 shows a schematic illustration of the relative energies of the carotenoid excited singlet states discussed above together with the proposed relaxation pathways from the S_2 state as well as the energy-transfer pathways between carotenoid and Bchl. Since the relaxation from the S_2 state is very fast, ultrafast vibrational spectroscopies are going to be important to clarify the structure–function relationship of the above singlet excited-states (Hashimoto et al. 2015). Such experiments remain to be done.

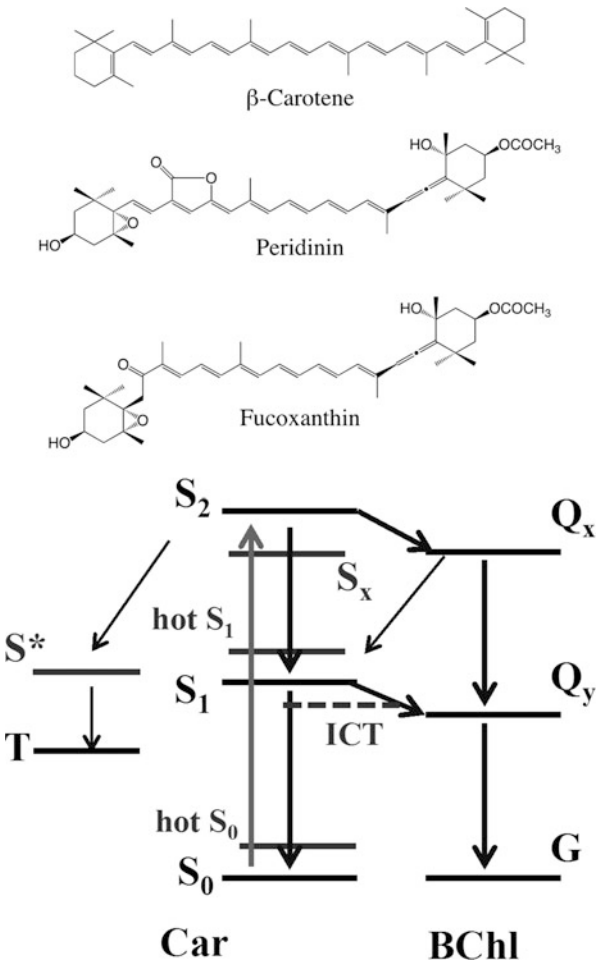

Fig. 4.9 Chemical structures of β-carotene, peridinin, and fucoxanthin, and a schematic description of energy diagrams together with relaxation and energy-transfer pathways of carotenoids following photoexcitation up to the S_2 state

4.5 Physiological Functions of Carotenoids in Photosynthesis

4.5.1 Are Carotenoids Absolutely Necessary in Photosynthesis?

There are no wild type photosynthetic organisms that survive without carotenoids. Why should this be? It is possible to make carotenoid-less mutant strains in some purple photosynthetic bacteria? For example, *Rba. sphaeroides* strain R26 and R26.1, and *Rsp. rubrum* strain G9+ are carotenoid-less mutants. These strains can be grown photosynthetically but only so long as all oxygen is removed from the media before the cells are exposed to light. They can grow photosynthetically under these conditions because these strains can produce carotenoid-less LH1-RC core complexes, and these can still function to convert light energy into chemical energy

in the normal way. Interestingly, *Rba. sphaeroides* strain R26.1 can also produce altered LH2 complexes without any carotenoids. These 'strange' LH2 complexes lack B800 Bchl *a*'s. It is also known that the presence or absence of carotenoids in the RC does not affect the rate of the primary electron transport reactions. So under laboratory conditions some photosynthetic organisms can be maintained in the absence of carotenoids. However, these carotenoid-less strains are very sensitive to light in the presence of oxygen. Under growth conditions in the wild, even though these bacteria only grow photosynthetically under anaerobic conditions, there is always chance that anaerobic layers in ponds and streams etc. will be disturbed, and the bacteria will find themselves in the presence of both light and oxygen. Under these conditions they will be rapidly killed by a mechanism that is described below. As a result of these, no carotenoid-free strains survive in the wild.

The following example serves to illustrate this point. Takaichi *et al.* have created a set of carotenoid deletion strains from the purple photosynthetic bacterium *Rubrivivax gelatinosus* (Harada et al. 2001; Pinta et al. 2003; Takaichi 2006). These strains either contained no carotenoids or carotenoids with different numbers of conjugated C = C double bonds, from three to thirteen. A mixture of these different mutant strains was grown in the light under semi-aerobic conditions. They then looked to see how the different strains both survived and competed with each other. The results were quite dramatic. Strains with either no carotenoids or ones with carotenoids where the conjugation length was three (phytoene) or five (phytofluene) all died out very quickly. Those strains with carotenoids that had seven or more conjugated bonds were able to survive under these growth conditions. Unfortunately, this study did not continue long enough to see whether those strains with the carotenoids that had longer conjugation length had a selective advantage over those with the shorter conjugation length. Another interesting finding from this study was the suggestion that the carbonyl containing carotenoids provided more protection than the non-carbonyl containing ones.

Interestingly, there are reports of carotenoid-free mutants of oxygenic photosynthetic organisms surviving photosynthetic growth conditions. Indeed, herbicides that act by inhibiting carotenoid biosynthesis in plants illustrate this point very clearly (Duke 1990).

4.5.2 Are Carotenoids Required for the Structural Integrity of Photosynthetic Pigment-Protein Complexes?

The answer to this question is in some cases yes and in some cases no. Looking first at purple photosynthetic bacteria, it is a general finding that LH1 complexes and RCs can be stably assembled in the absence of carotenoids (Ghosh et al. 1988; Roszak et al. 2004). However, in most cases, the LH2 complexes cannot be stably assembled in the absence of carotenoids (Frank 1999), but there exceptions to this. As discussed above the carotenoid-less strain R26.1 of *Rba. sphaeroides* can

synthesize an unusual variant of LH2 that lacks the B800 Bchl *a*. However, if a carotenoid-less mutant of *wild type Rba. sphaeroides* is made, then usually no LH2 can be stably assembled. It is also worth noting that when carotenoids are added back into the carotenoid-less LH2 from strain R26.1 it has not proved possible to re-incorporate extra Bchl *a* to recover the B800 absorption band. In some *Chromatium* species, such as *Chromatium vinosum*, LH2 complexes can be stably assembled in the absence of carotenoids. The reasons for the difference between the different types of LH2 complexes and their requirements for carotenoids are not clear. Carotenoid-less LH1 complexes have proved to be very useful experimentally. It is possible to reconstitute a wide range of different carotenoids into LH1 complexes. This has been exploited in the case of the LH1 complex from *Rsp. rubrum* so that the effect of incorporating carotenoids with different numbers of conjugated double bonds on functions such as light-harvesting could be systematically studied.

Higher plant LHCII complexes can be reconstituted from mixing overexpressed apo-proteins with Chls and carotenoids. Since the types of carotenoids used in these reconstitution experiments can be varied, it has been possible to investigate whether any specific carotenoids are absolutely required to produce intact native LHCII complexes. It has been clearly demonstrated that two lutein molecules are absolutely required to reconstitute native LHCII complexes. In most of the other carotenoid binding sites within the LHCII complexes there is much less specificity shown and a variety of different carotenoids can be successfully incorporated. In the case of LHCII complexes from chlorophytes, carotenoids are also important for the maintenance of the trimeric structure of these antenna complexes. In this case, it has been reported that the presence of neoxanthin is required for the formation of this trimer. This key neoxanthin has been shown to be a 9'-*cis* isomer. Normal functioning of LHCII requires the presence of trimers and 9'-*cis* neoxanthin is important *in vivo* (Takaichi and Mimuro 1998).

4.6 Light-Harvesting Function of Carotenoids

In the case of higher plants, carotenoids do act as accessory light-harvesting pigments but they do not contribute very much to the overall light-harvesting capacity of the plants. Plants typically intercept solar energy directly and so are often subjected to too much light rather than too little light. Many other photosynthetic organisms live in ecological niches where this is not true. Purple photosynthetic bacteria for example typically live in the anaerobic layers in ponds where oxygenic photosynthetic organisms that live above them have intercepted the incident solar radiation first. This means that the purple bacteria receive less solar energy but also that the wavelength of light that are strongly absorbed by Chl are removed from the spectrum of light that these bacteria receive. The purple bacteria must make their living by absorbing green light that is not used by the oxygenic photosynthetic organisms above them or light of wavelengths in the red spectrum where Chl stops absorbing. Carotenoids absorb the transmitted blue-green light

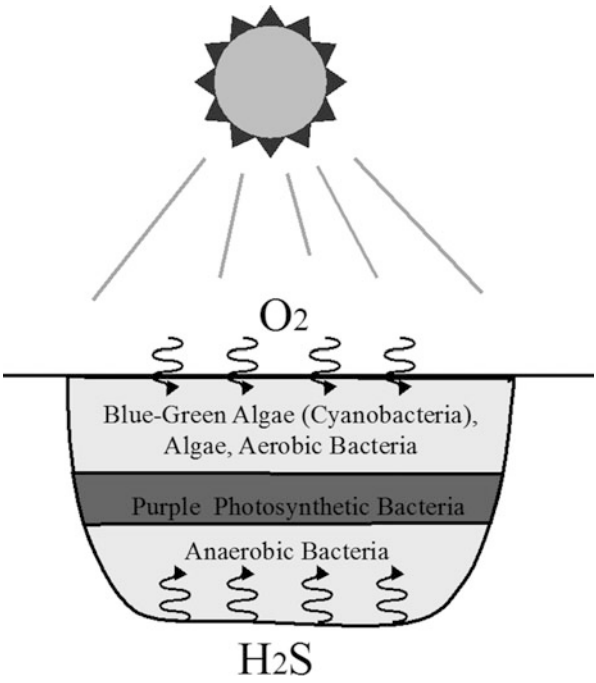

Fig. 4.10 Schematic illustration of ecological niches for phototrophs in water

and are therefore very important for purple bacterial photosynthesis. As described above, the absorption spectrum of carotenoids depends on the number of conjugated double bonds. The purple bacteria have selected carotenoids with between nine and thirteen conjugated double bonds which absorb exactly in that green window left in the incident solar spectrum after the light has passed through the upper layer of oxygenic phototrophs. The consequence of the dependence of the spectrum of light available to these bacteria in their ecological niches is illustrated in Fig. 4.10.

4.7 Photoprotective Function of Carotenoids

4.7.1 Photoprotection by Quenching Chlorophyll Triplet States

In the presence of light, there is always the possibility that Chl and Bchl triplet states will be made. These triplet states typically last for tens of microseconds or milliseconds and can therefore react with molecular oxygen to generate singlet oxygen. Singlet oxygen is a very powerful destructive oxidizing agent. It can destroy Chls, lipids, proteins, and DNA. Indeed any cell that is subjected to singlet oxygen is usually killed. This is called photo-oxidative killing and is the reason why there are no *wild type* photosynthetic organisms that are carotenoid-less. Carotenoids are able to quench Chl triplets and therefore prevent the harmful generation of singlet

oxygen. This quenching reaction is a triplet-triplet exchange reaction. This reaction requires that the carotenoid is packaged closely to the Chls and the carotenoids triplet energy level lies lower than both that of triplet excited Chl and singlet oxygen. Most light-harvesting complexes and reaction centres contain carotenoids that are in van der Waals contact with the Chl pigments. The energy level requirements means that carotenoids have to have greater than eight conjugated double bonds to be able strongly photoprotect. Typically, carotenoid triplets are formed from Chl triplets in a few nanoseconds or faster. This beats the time taken for the bi-molecular collision of Chl triplets with molecular oxygen by several orders of magnitude. When the triplet excited states of carotenoids relax to the ground state, they release the energy harmlessly as heat.

4.7.2 Protection of Living Organisms (Chemical Reaction Process)

As well as preventing the formation of singlet oxygen, carotenoids can also act as scavengers of both singlet oxygen itself and other harmful radicals. Typically, when carotenoids scavenge radicals this process involves the π-electrons in their conjugated polyene chain. In this process, carotenoids are often converted into epoxides or additives of lipids. They then often decompose and therefore this process is essentially irreversible. Carotenoids have also been suggested to play a similar anti-oxidant role in humans.

4.8 The Involvement of Carotenoids in the Regulation of Photosynthesis

Carotenoids are active participants in the regulation of photosynthesis in two main ways. They can act as secondary electron donors in photosystem II and they play a key role in non-photochemical quenching (Frank and Brudvig 2004). In PS II RCs, when P680 gets over-oxidized it is very dangerous. $P680^+$ is so highly oxidizing that it is capable of destructive oxidation of amino acid side chains. β-Carotene bound in the PS II RCs is able to transfer an electron to $P680^+$ when it is over-oxidized and so help to prevent the harmful oxidation reactions that would otherwise damage PS II. The rate of photosynthesis of many plants saturates at quite low light levels. This means that often there is far more light available than the light reactions can use. Over-excitation with light is potentially very dangerous. Many oxygenic photosynthetic organisms have evolved a way to overcome this problem by down regulating the efficiency of the light-harvesting system. This down regulation has been called non-photochemical quenching (NPQ) (Ruban and Horton 1995; Müller et al. 2001; Rohacek et al. 2007; van Grondelle 2011). It is thought that over-excitation results in a conformational change in the light-

Table 4.2 The classification of regulation mechanisms of photosynthesis

Name of regulation	Time scale	Outline	Distribution
Xanthophyll cycle	Few minutes	Reversible structural change of carotenoids inside the antenna with enzymatic reactions in order to optimize the quenching of chlorophyll *a* fluorescence (singlet excited state).	Higher plants and Green algae
			Analogous reactions are found for diatoms, brown algae, dinoflagellates, and euglenoids.
State transition	Few minutes	Reorganization of the antenna proteins to optimize the light-harvesting function.	Higher plants, Green algae, Red algae, and Cyanobacteria
Optimization of the amount ratio of PS I/PS II	Few hours – few days	Optimization of the gene expression.	Higher plants, Green algae, Red algae, and Cyanobacteria
Optimization of the amount ratio of peripheral antenna/RC	Few hours?	Optimization of the gene expression.	Purple bacteria and Green bacteria

harvesting system, which results in rapid de-excitation, resulting in the harmless dissipation of the excess light energy as heat. Carotenoids appear to be involved in maintaining the light-harvesting system in the protected, quenched state. The functions of carotenoids in NPQ are summarized in Table 4.2. Readers who wish to learn more about NPQ should consult recent book edited by Demmig-Adams *et al.* (Demmig-Adams et al. 2014).

4.9 Structure and Function Relationship of Carotenoids (Future Perspectives)

Carotenoids are fascinating molecules. They have remarkable photophysical properties (Frank et al. 1999). Their functions can be tuned and regulated by proteins. They participate in a wide variety of reactions in photosynthesis that exploit their chemical and photophysical potentials. Though a lot of detail is known about how carotenoids participate in these functions, there is still a lot more detail to be uncovered. Now is a particularly exciting time to be involved in carotenoid research. It has been particularly notable how, as physical methods have evolved and have been applied to studying carotenoids, the knowledge of the way in which carotenoids function in photosynthesis has advanced. We expect this trend

to continue. One can highlight areas where we expect these developments to really help understand the molecular mechanisms by which carotenoids discharge their photosynthetic functions. Examples are the further application of advanced 2D coherent time-resolved spectroscopies (Cho 2008, 2009) and time-resolved stimulated Raman spectroscopy (Yoshizawa and Kurosawa 1999; Kukura et al. 2007; Frontiera and Mathies 2011; Challa et al. 2012). These methods should be able to help resolve the ongoing problems of understanding the pattern of carotenoid excited singlet states and their involvement in light-harvesting. Further application of X-ray crystallography (Tomita et al. 2009) with hopefully the provision of structure information of LHCII in both the quenched and non-quenched states will reveal the role carotenoids have in NPQ. New intense X-ray sources such as those produced by free electron lasers open the door to both time-resolved structural studies and ultimately the ability to determine the structures of individual molecules (Behrens et al. 2014; Hirata et al. 2014; Arnlund et al. 2014; Suga et al. 2014). This will then allow such processes as carotenoid isomerization to be visualized, which has been a long held dream of workers in this area.

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Chapter 5
Regulation of Carotenoid Biosynthesis in Photosynthetic Organs

Briardo Llorente

Abstract A substantial proportion of the dazzling diversity of colors displayed by living organisms throughout the tree of life is determined by the presence of carotenoids, which most often provide distinctive yellow, orange and red hues. These metabolites play fundamental roles in nature that extend far beyond their importance as pigments. In photosynthetic lineages, carotenoids are essential to sustain life, since they have been exploited to maximize light harvesting and protect the photosynthetic machinery from photooxidative stress. Consequently, photosynthetic organisms have evolved several mechanisms that adjust the carotenoid metabolism to efficiently cope with constantly fluctuating light environments. This chapter will focus on the current knowledge concerning the regulation of the carotenoid biosynthetic pathway in leaves, which are the primary photosynthetic organs of most land plants.

Keywords Carotenoids • Carotenoid biosynthesis • Metabolic regulation • Photosynthesis • Land plants

5.1 A Brief Introduction to Carotenoids

Carotenoids represent a large and diverse class of biological compounds indispensable to photosynthetic organisms, and hence essential to sustain our current biosphere (Croce and van Amerongen 2014; Polivka and Frank 2010; Sundstrom 2008; Blankenship 2010). They are widely distributed in nature, and occur in all three domains of life (archaea, bacteria and eukarya) (Sieiro et al. 2003; Moise et al. 2014). Carotenoids are commonly regarded as pigments because they selectively absorb certain wavelengths of the visible light spectrum and transmit or reflect other wavelengths that, in combination, most often appear yellow, orange or red

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(Armstrong 1994, Chap. 4). Formally, carotenoids are a subgroup of isoprenoid metabolites, that is, molecules built up of isoprene units (C_5H_8). Two main classes of carotenoids can be distinguished. If strictly composed of hydrocarbons, they are referred to as carotenes. If they are oxygenated molecules, they are called xanthophylls. The enzymatic cleavage of carotenoids produces apocarotenoids, a diverse class of compounds with signaling properties as colors, flavors, and hormones.

At the structural level, carotenoids are typically tetraterpene (C_{40}) molecules. Certain bacteria, however, are capable of synthesizing carotenoids with shorter (C_{30}) and longer (C_{45} and C_{50}) backbones (Pelz et al. 2005; Armstrong 1997; Krubasik et al. 2001, Chap. 3). Each double bond in the polyene chain can exist in the *trans* or *cis* geometrical configuration, although most carotenoids in nature occur predominantly in the all-*trans* isoform (Armstrong 1997). The structure of the conjugated polyene chain determines the shape, and the chemical and light absorption properties of the carotenoid molecule. In general, the carotenoid polyene chain needs to have at least seven conjugated double bonds to impart color (Armstrong 1997). This is because conjugated systems with fewer double bonds absorb energy in the ultraviolet region of the electromagnetic spectrum (below 390 nm) without altering the range of wavelengths that conform the visible light (about 390–700 nm), and hence look colorless to the human eye. As the number of conjugated double bonds increases, the polyene chain absorbs photons of longer wavelengths, resulting in the transmission or reflection of wavelengths that impart color in the yellow to red range (Armstrong 1994).

With very few exceptions (e.g. apocarotenoids) (Frusciante et al. 2014), carotenoids are lipophilic and usually occur associated with specific membranes and proteins. Because carotenoid molecules typically have the suitable length to match the thickness of membrane lipid bilayers, positioning their rigid polyene backbone spanning the hydrophobic lipid core of the bilayer, it is believed that they were initially selected to function as membrane stabilizers in ancient organisms (Vershinin 1999; Rohmer et al. 1979; Rottem and Markowitz 1979). Notwithstanding, the physicochemical properties derived from their characteristic chemical structure has allowed carotenoids to spread throughout the tree of life, where they have been co-opted to play a multitude of functions. Carotenoids are synthesized by all photosynthetic organisms (algae, land plants and every known photosynthetic bacterium), as well as by some non-photosynthetic bacteria, archaea, fungi, and the noted (apparent) exception of a few animals (e.g. aphids, spider mites and gall midges) (Altincicek et al. 2012; Moran and Jarvik 2010; Cobbs et al. 2013; Blankenship 2010; Raymond et al. 2002, Chaps. 1 and 2). Other organisms, like the vast majority of animals, do not produce carotenoids *de novo*, but incorporate them through their diet as a source of ornamental pigments and precursors of retinoids and vitamin A (Bartley and Scolnik 1995; Bollag 1996; Fraser and Bramley 2004).

In the particular case of photosynthetic organisms, carotenoids carry out specialized functions in the photosynthetic reaction center and the associated light-harvesting antennae (Chap. 4). Algae, land plants and cyanobacteria use chlorophylls as their primary light harvesting photosynthetic pigment. Other

photosynthetic bacteria use closely related molecules, called bacteriochlorophylls, for the same purpose (Croce and van Amerongen 2014; Polivka and Frank 2010). Together with (bacterio)chlorophylls, carotenoids are bound to specific proteins in photosynthetic membranes, where they maximize light harvesting by extending the spectral range of light that can be utilized in photosynthesis. As functional components of these pigment-protein complexes, carotenoids collect energy in the absorption gap of (bacterio)chlorophyll, the blue-green region (450–570 nm) of the light spectrum (the spectral region in which the sun irradiates maximally), and transfer the absorbed energy to neighboring (bacterio) chlorophyll molecules (Croce and van Amerongen 2014; Polivka and Frank 2010). Thus, the photosynthetic reactions are driven by the light energy that is collectively absorbed by carotenoids and chlorophylls.

Besides their function as light harvesting molecules, carotenoids play a different and more important role in photosynthesis, they protect the photosynthetic machinery from harmful oxidative photodamage caused by intense light (Chap. 4). This latter function is crucial, since photosynthetic organisms are subjected to photooxidative stress derived from the formation of highly reactive byproducts in the photosynthetic apparatus when the absorbed light energy exceeds that used in photosynthesis. Carotenoids dissipate the excess of light energy, scavenge radicals and quench potentially harmful chlorophyll triplets and singlet oxygen molecules ($^1O_2$) (Holt et al. 2005; Kim and DellaPenna 2006; Demmig-Adams et al. 1996; Niyogi et al. 1997; Pogson et al. 1998; Baroli and Niyogi 2000; Dall'Osto et al. 2007; Kim et al. 2009). The protective effects of carotenoids are evident in carotenoid-deficient photosynthetic organisms, which suffer extensive photodamage (Cazzaniga et al. 2012; Havaux et al. 2000; Sagar and Briggs 1990).

Probably as an evolutionary consequence of their importance in the photosynthetic process, algae and land plants invariably synthesize carotenoids in the very same plastidial organelle where photosynthesis takes place, the chloroplast. Land plants also synthesize carotenoids in other plastid types that are non-photosynthetic, such as fruit and flower chromoplasts, where they frequently act as an advertisement for animals that contribute to pollination or seed dispersal (Cazzonelli 2011; Seymour et al. 2013, Chap. 6). Despite plastids having their own independent genome (the plastome), all of the genes coding for carotenoid biosynthetic enzymes are located in the nucleus. Therefore, their protein products are translated in the cytosol and subsequently imported into the plastid compartment.

Carotenoid-derived metabolites play many other functions in plants. As indicated above, carotenoids can be enzymatically cleaved to form apocarotenoids, which can act as hormones, signaling compounds, chromophores and aroma constituents. They can also be oxidized in a non-enzymatic fashion to produce carotenoid derivatives that promote acclimation to stress conditions (Havaux 2014; Ramel et al. 2012, 2013; Giuliano et al. 2003; Walter et al. 2010, Chap. 9). While extremely interesting, these later topics lie beyond the scope of this chapter, which will focus mainly on the mechanisms underlying the regulation of the carotenoid biosynthetic pathway in the primary photosynthetic organs of most land plants, the leaves.

5.2 Carotenoid Biosynthesis

All carotenoids derive from successive condensations of the five-carbon molecule isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Ruzicka 1953). These common carotenoid precursors can be derived from two independent non-homologous metabolic routes, the methylerythritol 4-phosphate (MEP) pathway or the mevalonate (MVA) pathway. The MEP pathway occurs in bacteria and photosynthetic eukaryotes (i.e. algae and land plants), while the MVA pathway is found in eukaryotes, archaea and few bacteria. Photosynthetic eukaryotes possess both the MVA and the MEP pathway, although they synthesize carotenoids exclusively through the MEP pathway; one interesting exception being the unicellular alga *Euglena gracilis* that is apparently capable of synthesizing carotenoids from both the MVA and MEP pathways (Kim et al. 2004; Lange et al. 2000; Lichtenthaler et al. 1997; Lombard and Moreira 2011, Chaps. 1 and 2).

In algae and land plants, the MEP pathway enzymes are entirely localized in the plastid, while the enzymes of the MVA pathway are distributed in different subcellular compartments. The main rate-determining enzyme of the MVA pathway, hydroxymethylglutaryl CoA reductase (HMGR), is anchored to the membrane of the endoplasmic reticulum, with its catalytic domain exposed towards the cytosol. The other enzymes of the pathway have been found to localize in the cytosol and peroxisomes (Sapir-Mir et al. 2008; Simkin et al. 2011). Although the MVA and MEP pathways are generally considered to operate independently in their corresponding subcellular locations, multiple studies have established that there is some exchange of metabolites between the two pathways (Rodriguez-Concepcion et al. 2004; Schuhr et al. 2003). Whether this metabolic crosstalk has a biological relevance, remains to be established. However, since both the genetic and pharmacological inhibition of either the MVA or the MEP pathway results in lethal phenotypes, it is clear that the inactivation of either of the two pathways cannot be compensated for by the activity of the remaining pathway. A detailed and recently revised map of the MVA and MEP pathways in plants is available (Pulido et al. 2012).

The condensation of one molecule of DMAPP with three molecules of IPP generates geranylgeranyl diphosphate (GGPP), the ubiquitous isoprenoid precursor of carotenoids and several other groups of metabolites, including chlorophylls, gibberellins, ubiquinones and tocopherols (Pulido et al. 2012, Chaps. 1 and 2). The subsequent condensation of two GGPP molecules to form phytoene is considered the first committed step of the carotenoid pathway. This step is catalyzed by phytoene synthase (PSY), which is the main rate-determining enzyme of the carotenoid pathway, and has a close evolutionary relationship in archaea, bacteria and eukaryotes (Sandmann 2002). Many of the enzymes that catalyze further modification reactions on carotenoid molecules are evolutionary related and some diverged through gene duplications that led to enzymes with different catalytic functions (Klassen 2010; Sandmann 2002; Raisig and Sandmann 2001; Moise et al. 2005). Others are the product of convergent evolution of different types

of enzymes, which are evolutionary unrelated but nevertheless catalyze the same reaction (Klassen 2010; Krubasik and Sandmann 2000; Sandmann 2002, 2009; Villanueva et al. 2014). In general, the core carotenoid pathway is conserved among related species. However, particular species synthesize specific carotenoids via unique biosynthetic branches, generating the tremendous diversity of carotenoid molecules observed in nature (Maresca et al. 2008).

From phytoene, the biosynthesis of carotenoids continues with a series of desaturation and isomerization reactions that sequentially convert it into phytofluene, then ζ-carotene, then neurosporene, then pro-lycopene, and finally lycopene (Ruiz-Sola and Rodriguez-Concepcion 2012). Algae, land plants, cyanobacteria and green sulfur bacteria require at least four enzymes (phytoene desaturase, PDS; zeta-carotene desaturase, ZDS; zeta-carotene isomerase, Z-ISO; and carotenoid isomerase, CRTISO) to carry out these reactions (Moise et al. 2014; Frigaard et al. 2004). Lycopene is then used for the biosynthesis of most carotenoids. In plants, the carotenoid pathway bifurcates after lycopene to give beta-carotenoids (carotenoids with two β-rings, including β-carotene and derived β,β-xanthophylls) and epsilon-carotenoids (carotenoids with one β-ring and one ε-ring, including α-carotene and derived β,ε-xanthophylls) (Cazzonelli 2011; Cazzonelli and Pogson 2010; Moise et al. 2014). The only difference between the two types of carotenoid cyclic end groups is that the double bond of the β-rings is in conjugation with the polyene chain, whereas the double bond of the ε-rings is not conjugated. As a result, β-rings only have one conformation and ε-rings have a relatively free rotation (Kim and DellaPenna 2006). Carotenoids with two ε-rings are uncommon in plants. An exception is lactucaxanthin, which is a major xanthophyll in a few species (e.g. *Lactuca sativa*) (Phillip and Young 1995). Interestingly, while carotenoids with β-rings are ubiquitous in nature, carotenoids with ε-rings appear to be restricted to cyanobacteria, algae, and land plants. It is therefore possible that the enzymes that participate in the formation and modification of the ε-ring evolved first in cyanobacteria, and were then inherited by algae and land plants through the cyanobacterial ancestor of the plastid (Kim and DellaPenna 2006; Timmis et al. 2004).

In the branch leading to beta-carotenoids, a single enzyme (lycopene β-cyclase, LCY-B) introduces β-rings at both ends of lycopene, catalyzing the initial reactions to form β-carotene (Pecker et al. 1996; Cunningham et al. 1996, Chap. 2). Two subsequent hydroxylation reactions on β-carotene, catalyzed by carotenoid β-hydroxylase (CHY-B), give β-cryptoxanthin, and then zeaxanthin, which can be epoxidized to violaxanthin, with antheraxanthin as an intermediate product by zeaxanthin epoxidase (ZEP). Violaxanthin can be then de-epoxidated back into zeaxanthin by violaxanthin de-epoxidase (VDE), or converted to neoxanthin by neoxanthin synthase (NSY) (Cazzonelli 2011; Cazzonelli and Pogson 2010; Moise et al. 2014). The branch that leads to epsilon-carotenoids initially requires the sequential activity of two cyclase enzymes (lycopene ε-cyclase, LCY-E; and LCY-B) to introduce an ε-ring and a β-ring at each end of the lycopene molecule, forming α-carotene (Cunningham and Gantt 2001; Cunningham et al. 1996). Subsequent

hydroxylations catalyzed by CHY-B and carotene ε-hydroxylase (CHY-E) first form zeinoxanthin and then lutein, respectively (Cazzonelli 2011; Cazzonelli and Pogson 2010; Moise et al. 2014).

Most of the enzymes involved in carotenoid biosynthesis are associated to membranes, and are thought to work as an "assembly line" of dynamic multiprotein complexes (i.e. metabolons) that consecutively channel substrates and intermediates between themselves (Shumskaya and Wurtzel 2013; Cunningham and Gantt 1998; Ruiz-Sola and Rodriguez-Concepcion 2012). Although the existence of carotenoid metabolons still awaits experimental confirmation, this idea is supported by the limited detection of pathway intermediates, evidence of *in vivo* channeling, and the existence of high molecular weight complexes containing carotenoid-related enzymes (Al-Babili et al. 1996; Bonk et al. 1997; Camara et al. 1982; Fraser et al. 2000; Kreuz et al. 1982; Lopez et al. 2008; Maudinas et al. 1977; Candau et al. 1991; De la Guardia et al. 1971; Quinlan et al. 2012).

5.3 Regulation of Carotenoid Biosynthesis

Given the paramount importance of carotenoids to the photosynthetic process, it should not be surprising that photosynthetic organisms would have evolved regulatory mechanisms allowing the fine-tune adjustment of the carotenoid metabolism. In fact, there is compelling evidence indicating that carotenoid biosynthesis is regulated at multiple levels, often coordinated to related metabolic pathways and tightly linked to developmental and environmental cues (Cazzonelli and Pogson 2010; Fraser and Bramley 2004; Hirschberg 2001; Pizarro and Stange 2009; Sun et al. 2010; Bauer et al. 2003; Ghassemian et al. 2006; Meier et al. 2011; Wille et al. 2004). Remarkably, and unlike chromoplasts, which are largely heterogeneous in their carotenoid profiles depending on the species, the chloroplasts of land plants present a highly constant carotenoid composition (Ruiz-Sola and Rodriguez-Concepcion 2012). That being said, we only grasp a limited part of the molecular regulation underlying carotenoid biosynthesis, and this is an area of research that will likely remain a hot topic for the next years.

5.4 Regulation at the Level of Gene Expression

Arguably, transcriptional control of genes coding for carotenoid biosynthetic enzymes in response to light is the most studied and best understood level of regulation. Actually, all photosynthetic organisms are known to modulate the expression of carotenoid biosynthetic genes when encountering changing light conditions (Bouvier et al. 2005; Fernandez-Gonzalez et al. 1998; Bohne and Linden 2002; Kianianmomeni 2014; Steinbrenner and Linden 2003; Ghassemian et al. 2006; Meier et al. 2011; Woitsch and Romer 2003; Bugos et al. 1999; Rossel

et al. 2002; Simkin et al. 2003b; Simkin et al. 2003a). This is also true for non-photosynthetic carotenogenic bacteria and fungi, which induce the transcription of genes of the carotenoid pathway when exposed to light, likely not for energetic purposes, but probably as a protective mechanism against harmful solar radiation (Tisch and Schmoll 2010; Fontes et al. 2003; Takano et al. 2006).

Turning now to land plants, the expression of carotenoid genes is low when seeds germinate under soil and in the dark. Once etiolated seedlings emerge from the soil surface, light triggers a transcriptional program that promotes photomorphogenic development. This results in the differentiation of etioplasts (pale non-photosynthetic plastids) into chloroplasts, and a concomitant burst in the synthesis of carotenoids and chlorophylls (Romer and Fraser 2005; Philippar et al. 2007; Meier et al. 2011; Rodriguez-Villalon et al. 2009; Toledo-Ortiz et al. 2010; Welsch et al. 2000; Frosch and Mohr 1980; Albrecht and Sandmann 1994). Carotenoid accumulation enhances energy absorption and provides protection against photo-oxidative stress to the photosynthetic machinery, hence facilitating the transition from heterotrophic to photoautotrophic growth (Park et al. 2002; Rodriguez-Villalon et al. 2009). Notably, while several genes of the carotenoid pathway are upregulated during the de-etiolation process, the same occurs with many genes of the MEP pathway (Ghassemian et al. 2006; Meier et al. 2011; Rodríguez-Concepción 2006). The coordinated expression of genes from both pathways makes sense when considering that the MEP pathway supplies precursors to the carotenoid pathway. This concerted transcriptional regulation is also apparent when blocking the MEP pathway, which leads to a downregulation in the abundance of *PSY* transcripts (Laule et al. 2003). Congruently, overexpression of genes coding for MEP enzymes enhance total carotenoid content, whereas silencing them has the opposite effect (Estevez et al. 2001; Carretero-Paulet et al. 2006; Botella-Pavia et al. 2004).

Plants perceive light through different photoreceptors that sense light of particular wavelengths. Phyotochromes (PHYs) function in the red (660 nm) and far-red (730 nm) range, while cryptochromes (CRYs), phototropins and Zeitlupe family members operate in the blue (390–500 nm) and ultraviolet-A (320–390 nm) region of the spectrum (Briggs and Olney 2001; Franklin et al. 2005; Moglich et al. 2010, Chap. 4). Ultraviolet-B (280–315 nm) perception is mediated by the UVR8 photoreceptor (Rizzini et al. 2011). Photoreceptors orchestrate light-dependent transcriptional regulation, with PHYs as the most well characterized in land plants. Phytochromes exist in a dynamic photoequilibrium between an inactive and an active form (Neff et al. 2000). Upon photoactivation, PHYs translocate from the cytoplasm to the nucleus, where they interact with transcription factors bound to conserved promoter motifs to finally tune the expression of target genes (Bae and Choi 2008; Leivar and Monte 2014). The light-induced up-regulation of transcripts for carotenoid biosynthetic enzymes during photomorphogenesis, together with the coordinated expression of genes involved in the production of chlorophylls and carotenoid-bearing proteins is mainly mediated by PHYs (Fig. 5.1, Welsch et al. 2000; Welsch et al. 2008; von Lintig et al. 1997; Toledo-Ortiz et al. 2010; Meier et al. 2011; Wille et al. 2004; Woitsch and Romer 2003; Stange and Flores 2012). The induction of transcripts coding for carotenoid enzymes under light conditions,

including the main flux-controlling PSY and many genes of the MEP pathway, further supports the implication of PHY receptors in the regulation of carotenoid biosynthesis (Hsieh and Goodman 2005; Carretero-Paulet et al. 2002; Cordoba et al. 2009; Thompson et al. 2000; Facella et al. 2008).

Current data indicate that the PHY-mediated signal transduction process propagates to the transcriptional network via direct interaction of light-activated photoreceptors with bHLH (basic helix-loop-helix) Phytochrome-Interacting transcription Factors (PIFs), causing their inactivation or proteasome-mediated degradation (Castillon et al. 2007; Duek and Fankhauser 2005; Leivar and Monte 2014; Leivar and Quail 2011; Bae and Choi 2008). Analyzes of mutants defective in PIFs have shown that these transcription factors generally act as negative regulators of genes involved in the biosynthesis of chlorophylls and carotenoids (Leivar et al. 2009; Shin et al. 2009; Toledo-Ortiz et al. 2010; Stephenson et al. 2009). Recent studies in *Arabidopsis thaliana* have demonstrated that PIFs, particularly PIF1, bind to a G-box motif (CACGTG) in the promoter of the *PSY* gene to directly repress its expression (Toledo-Ortiz et al. 2010). Furthermore, the bZIP (basic leucine zipper) transcription factor Long Hypocotyl 5 (HY5) has been shown to activate *PSY* gene expression and antagonize PIFs by co-localizing to the same G-box motif of the *PSY* promoter (Toledo-Ortiz et al. 2014). In addition to PHYs, although far less studied, carotenoid biosynthesis also appears to be regulated by CRY photoreceptors (Li et al. 2008; Giliberto et al. 2005).

Another, recently identified, level of regulation for carotenoid biosynthesis is determined by epigenetic changes in the genomic landscape (Fig. 5.1). In Arabidopsis, the expression of the gene coding for the CRTISO enzyme is in part controlled by the chromatin methylation status of its promoter, which is under the regulation of a chromatin-modifying histone methyltransferase (Set Domain Group 8, SDG8). In SDG8-defective mutants, the methylation of the chromatin associated with the *CRTISO* gene is altered, thereby reducing gene expression and impairing lutein biosynthesis (Cazzonelli et al. 2009, 2010). To date, there are only two other studies showing epigenetic regulation of carotenoid biosynthesis; both of them in tomato (*Solanum lycopersicum*) fruit. One study showed that the inhibition of the ripening-related carotenoid biosynthesis in *Cnr* (colorless nonripening gene) epimutants was likely attributed to cytosine hypermethylation of the *Cnr* promoter (Manning et al. 2006). The other study showed that cytosine methylation has a regulatory role in the transcription of carotenoid-related genes during fruit ripening (Zhong et al. 2013).

Photosynthesis and metabolism have long been known to be coordinated by the circadian clock, which is a cellular time-keeper mechanism that regulates biological rhythms and allows organisms to anticipate changes associated with the Earth's day-night cycles (Harmer 2009; Yakir et al. 2007). In *wild type* and in short- and long-period clock mutants, leaves contain more chlorophyll when the oscillator period matches that of the light-dark environment. This enables the optimal use of energy by timing metabolism with diurnal solar oscillation and therefore provides a competitive advantage to plants (Dodd et al. 2005). Although it is yet not clear whether carotenogenesis is regulated by the clock (Fig. 5.1), several transcripts involved in carotenoid biosynthesis, including *PSY* and most MEP pathway genes,

have been shown to oscillate in a diurnal or circadian manner (Cordoba et al. 2009; Facella et al. 2008; Fukushima et al. 2009; Hsieh and Goodman 2005; Thompson et al. 2000; Woitsch and Romer 2003; Pan et al. 2009; Covington et al. 2008). Furthermore, it is worth mentioning that the accumulation of some MEP-derived plastidial isoprenoids, such as the gibberellins hormones and the carotenoid-derived abscisic acid (ABA), do present diurnal oscillations in many plant species (Barta and Loreto 2006; Aharoni et al. 2003; Dudareva et al. 2005; Hedden and Kamiya 1997).

Lastly, while changes in the reduction/oxidation (redox) state of signaling molecules is an important regulatory mechanism controlling photosynthetic gene expression, there is evidence indicating that the expression of some carotenoid biosynthetic genes might also be under redox control (Fig. 5.1, Pfannschmidt et al. 2001; Woitsch and Romer 2003). Particularly, experiments performed in tobacco (*Nicotiana tabacum*) have indicated that *CHY-B* and *ZEP* transcript levels are redox controlled by the plastoquinone pool (Woitsch and Romer 2003). Regulatory mechanisms controlling carotenoid biosynthesis at the level of gene expression in photosynthetic organs are shown in Fig. 5.1.

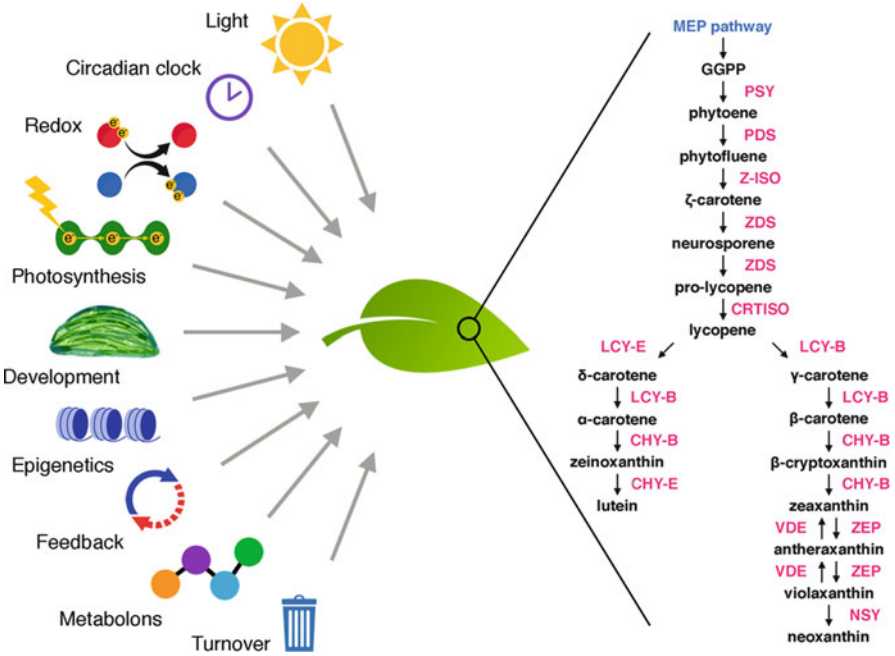

Fig. 5.1 Regulation of carotenoid biosynthesis in leaves. Known and presumed regulatory mechanisms controlling carotenoid biosynthesis are shown on the *left*. The core of the carotenoid biosynthetic pathway is shown on the *right*. Metabolites and enzymes are shown in *black* and fuchsia, respectively. *MEP* methylerythritol 4-phosphate, *GGPP* geranylgeranyl diphosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *Z-ISO* zeta-carotene isomerase, *ZDS* zeta-carotene desaturase, *CRTISO* carotenoid isomerase, *LCY-E* lycopene ε-cyclase, *LCY-B* lycopene β-cyclase, *CHY-B* carotenoid β-hydroxylase, *CHY-E* carotene ε-hydroxylase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NSY* neoxanthin synthase

5.5 Regulation at the Level of Enzyme Activity

Having looked at the gene expression control of carotenoid biosynthesis, let us move now to mechanisms that regulate the activity of the carotenoid pathway at other levels. One such mechanism involves feedback regulation at the level of protein accumulation of MEP enzymes by PSY activity (Rodriguez-Villalon et al. 2009). The molecular mechanism underlying this feedback signaling is presently unknown, although it can be speculated that this regulatory control could be important in adjusting the flow of intermediates through the MEP pathway in order to cope with the demand of isoprenoid precursors imposed by the carotenoid pathway.

Redox and photosynthetic-related processes also exert control on the biosynthesis of carotenoids at a post-translational level (Fig. 5.1). The ferredoxin/thioredoxin system of oxygenic photosynthesis, which links light to the regulation of photosynthetic enzymes, also seems to regulate the activities of many enzymes of the MEP pathway through the reduction of specific disulfide groups (Balmer et al. 2003; Schurmann and Jacquot 2000; Buchanan et al. 2002). On the other hand, several carotenoid biosynthetic enzymes such as PDS, ZDS, CRTISO, LCY-B, LCY-E, and ZEP might be under redox control since they all contain a conserved motif for binding to the redox cofactor flavin adenine dinucleotide (FAD) (Busch et al. 2002; Hugueney et al. 1992; Isaacson et al. 2004; Marin et al. 1996; Mialoundama et al. 2010; Schnurr et al. 1996; Yu et al. 2011; Yu et al. 2010). The PDS and ZDS enzymes use plastoquinone, a molecule involved in the electron transport chain in the light-dependent reactions of photosynthesis, as a hydrogen acceptor. Thus, PDS and ZDS activities are directly associated to the redox state of the photosynthetic electron transport chain (Carol and Kuntz 2001).

The activities of the ZEP and VDE enzymes are additionally regulated by the photosynthetic state of the plant in what is known as the xanthophyll cycle. Under intense light conditions, the carotenoid composition of leaves adjusts to protect the photosynthetic apparatus by converting violaxanthin into zeaxanthin, which is a more efficient molecule in dissipating excess light energy. Low light conditions have the opposite effect and promote the transformation of zeaxanthin back into violaxanthin (Demmig-Adams et al. 1996; Hieber et al. 2000; Yamamoto 2006; Li et al. 2009). Although the regulation of the xanthophyll cycle is in part controlled at the level of gene expression, it appears that the major determinants modulating the activities of ZEP and VDE enzymes are the changes in the luminal pH linked to the activity of the photosynthetic proton pump and the ascorbate content (Hirschberg 2001; Rossel et al. 2002; Woitsch and Romer 2003; Bugos and Yamamoto 1996; Rockholm and Yamamoto 1996; Yamamoto et al. 1972; Demmig-Adams et al. 1996; Hieber et al. 2000; Li et al. 2009; Yamamoto 2006). To a lesser extent, non-enzymatic light-driven processes also catalyze isomerization reactions that are equivalent to those catalyzed by both Z-ISO and CRTISO enzymes (Breitenbach and Sandmann 2005; Isaacson et al. 2002; Li et al. 2007; Park et al. 2002; Sandmann 2009).

As explained before, the enzymes of the carotenoid pathway are thought to operate as dynamic metabolons that are capable of channeling metabolic intermediates (Shumskaya and Wurtzel 2013). Channeling would improve metabolic efficiency and protect intermediates from being diluted in the plastid or diverted to competing reactions catalyzed by enzymes of other metabolic pathways. Theoretically, particular combinations of enzymes may influence the metabolon activity and/or localization, enabling them to address unique physiological needs (Shumskaya and Wurtzel 2013). Actually, the intrinsic dynamic nature of metabolons raises the hypothesis that such a system may also allow sequential transient interactions with different enzymes, hence enabling the swift re-direction of metabolic intermediates to synthesize multiple products (Fig. 5.1). We can evoke a mental picture of this proposed molecular mechanism by making an analogy with a flashlight having a rotating color filter wheel, where different filters and color beams would represent different enzymes and corresponding metabolic products, respectively.

The accumulation of carotenoids within plastids is another important regulatory mechanism (Cuttriss et al. 2007; Lu et al. 2006; Tzvetkova-Chevolleau et al. 2007). The assembly of photosynthetic complexes, thylakoid membranes and plastoglobules occurring during the differentiation of etioplasts into chloroplasts enhances the carotenoid storage capacity of the plastid (Ghassemian et al. 2006; von Lintig et al. 1997; Welsch et al. 2000). Defects in targeting of light-harvesting proteins and chloroplast development hence result in a reduced total carotenoid content (Tzvetkova-Chevolleau et al. 2007; Flores-Perez et al. 2008; Sauret-Gueto et al. 2006). Conversely, the activity of the carotenoid pathway can affect plastid development. Whereas mutants with affected carotenoid biosynthesis have defects in chloroplast development, overexpression of *PSY* can induce the development of chromoplast-like plastids that accumulate high amounts of carotenoids (Dong et al. 2007; Fraser et al. 2007; Maass et al. 2009; Park et al. 2002; Qin et al. 2007).

One final feature affecting carotenoid accumulation in photosynthetic organs is their metabolic turnover, which helps maintain the balance between biosynthesis and degradation. Based on recent data, indicating that the continuous synthesis of carotenoids in leaves occurs at much greater rates than previously estimated, it has been inferred that there must be active degradation to maintain steady-state carotenoid levels (Beisel et al. 2010). Although the processes controlling carotenoid degradation remain poorly characterized, known mechanisms include enzymatic oxidation and non-enzymatic photooxidation. Peroxidases and lipoxygenases can non-specifically oxidize carotenoids, but there is also a group of enzymes referred to as carotenoid cleavage dioxygenases (CCDs) that carry out oxidative cleavage on specific carotenoid molecules (Carail and Caris-Veyrat 2006; Walter and Strack 2011). Besides their involvement in carotenoid catabolism, CCD enzymes are responsible for the production of apocarotenoids, which fulfill multiple biological functions (Havaux 2014; Ramel et al. 2012; Giuliano et al. 2003; Ramel et al. 2013; Walter et al. 2010). Regulatory mechanisms controlling carotenoid biosynthesis at the level of enzyme activity in photosynthetic organs are shown in Fig. 5.1.

5.6 Concluding Remarks

Photosynthetic organs function as natural solar power stations that depend largely on carotenoids for their correct function. Thus, complex mechanisms have evolved to regulate the biosynthesis of carotenoids. Extensive research has been done over the last years on describing the carotenoid metabolism and we now have a rather good picture of the operation of the carotenoid biosynthetic pathway, mostly at the level of components involved in its transcriptional regulation. One key endeavor will be the elucidation of a much largely unexplored topic, the events shaping post-translational regulatory networks and synchronized dynamic protein interactions governing carotenoid metabolism. We can envision that increasing our understanding of how carotenoid biosynthesis is regulated in photosynthetic organs will lead to an expansion in our ability to breed crops with improved stress tolerance and better health-promoting properties. Furthermore, engineering the carotenoid pathway to produce synthetic carotenoid molecules with superior photoprotection or light-harvesting properties could lead to increased photosynthesis, thereby enhancing crop yield or providing extra energy and carbon building blocks to synthesize high value bioproducts designed from first principles.

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Chapter 6
Regulation of Carotenoid Biosynthesis During Fruit Development

Joanna Lado, Lorenzo Zacarías, and María Jesús Rodrigo

Abstract Carotenoids are recognized as the main pigments in most fruit crops, providing colours that range from yellow and pink to deep orange and red. Moreover, the edible portion of widely consumed fruits or their derived products represent a major dietary source of carotenoids for animals and humans. Therefore, these pigments are crucial compounds contributing to fruit aesthetic and nutritional quality but may also have protecting and ecophysiological functions in coloured fruits. Among plant organs, fruits display one of the most heterogeneous carotenoids patterns in terms of diversity and abundance. In this chapter a comprehensive list of the carotenoid content and profile in the most commonly cultivated fleshy fruits is reported. The proposed fruit classification systems attending to carotenoid composition are revised and discussed. The regulation of carotenoids in fruits can be rather complex due to the dramatic changes in content and composition during ripening, which are also dependent on the fruit tissue and the developmental stage. In addition, carotenoid accumulation is a dynamic process, associated with the development of chromoplasts during ripening. As a general rule, carotenoid accumulation is highly controlled at the transcriptional level of the structural and accessory proteins of the biosynthetic and degradation pathways, but other mechanisms such as post-transcriptional modifications or the development of sink structures have been recently revealed as crucial factors in determining the levels and stability of these pigments. In this chapter common key metabolic reactions regulating carotenoid composition in fruit tissues are described in addition to others that are restricted to certain species and generate unique carotenoids patterns. The existence of fruit-specific isoforms for key steps such as the phytoene synthase,

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lycopene β-cyclases or catabolic carotenoid cleavage dioxygenases has allowed an independent regulation of the pathway in fruit tissues and a source of variability to create novel activities or different catalytic properties. Besides key genes of the carotenoid pathway, changes in carotenoid accumulation could be also directly influenced by differences in gene expression or protein activity in the pathway of carotenoid precursors and some relevant examples are discussed. The objective of this chapter is to provide an updated review of the main carotenoid profiles in fleshy fruits, their pattern of changes during ripening and our current understanding of the different regulatory levels responsible for the diversity of carotenoid accumulation in fruit tissues.

Keywords Carotenoids in fruits • *Citrus paradisi* • Fruit ripening • Pinalate • Carotenoid catabolism in fruits • Chromoplasts

6.1 Diversity of Carotenoids Content and Composition in Fleshy Fruits

Earlier investigations in fruit carotenoids were primarily devoted to the chemical characterization of carotenoid composition in commonly consumed fruits and their variations during ripening (reviewed in Goodwin 1980 and Gross 1987). In the last two decades, analytical techniques and experimental procedures in carotenoid research have experienced a notable development (Amorim-Carrilho et al. 2014) allowing an expansion of the knowledge on carotenoid composition in fruits and the effect of genetic factors or environmental conditions on their content. Moreover, the interest in fruit-carotenoid research has been rapidly amplified due to the health promoting and nutritional benefits of carotenoids and their use as nutraceuticals (Berman et al. 2014).

As in other plant organs, carotenoid content in fruit is directly influenced by the stage of developmental and their environmental conditions. At the green stage, carotenoid composition is the characteristic of chloroplastic tissues, lutein being the most representative, followed by β-carotene, violaxanthin and neoxanthin, and other minor carotenoids as zeaxanthin and antheraxanthin (Gross 1987; Bramley 2013). This carotenoid profiling is similar in fruits of different species and colour when the carotenoids are masked by chlorophylls. Contrastingly, as fruit ripening progresses, remarkable differences in carotenoid content and composition are detected among species. A characteristic of carotenoids in fruits is the differential accumulation in peel/skin and pulp/flesh tissues, with larger amounts of carotenoids in the peel/skin than in the pulp/flesh. For example, carotenoid rich citrus fruits accumulate between 5 to10-times more carotenoids in the peel than in the pulp (Alquézar et al. 2008a) and in red- and white-fleshed loquat varieties, the content is between 5 and 70 times higher in the peel than in the pulp, respectively (Fu et al. 2012, 2014). A similar situation also occurs in *Cucurbita pepo* varieties with contrasting colouration (Obrero et al. 2013). This feature is also present in low-

carotenoid accumulating fruits, like commercial apple varieties, such as Granny Smith and Gala, which accumulate between 3 and 5-times more carotenoids in the peel than in the pulp (Ampomah-Dwamena et al. 2012). Moreover, the qualitative composition of carotenoids can also be variable between fruit tissues, suggesting divergent mechanisms of regulation of carotenoid biosynthesis and accumulation in peel and pulp, and remarking the need to investigate carotenoid profiling and their regulatory mechanisms in fruit tissues separately. Table 6.1 summarizes the total carotenoid content and the main carotenoids present in the most common edible fruits consumed worldwide, illustrating the high variability in content and composition in the different fruit species, varieties of the same species or even in different tissues of the same fruit.

6.1.1 Classification Systems

When considering total carotenoid content, fruits can be classified in four different groups according to the criteria proposed by Britton and Khachik (2009):

- Low (between 0 and 1 $\mu g\ g^{-1}$ f.w.)
- Moderate (between 1 and 5 $\mu g\ g^{-1}$ f.w.)
- High (5–20 $\mu g\ g^{-1}$ f.w.)
- Very high (more than 20 $\mu g\ g^{-1}$ f.w.)

However, the concentration of total carotenoid is not a unique criteria used to classify fruits since a great variability in the carotenoid profile has been found. Goodwin (1980), and later revised by Gross (1987) and Bramley (2013), established a classification of fruits into eight groups attending the carotenoid composition at ripen stage:

- Group I includes fruits with insignificant amounts of carotenoids
- Group II fruits with a chloroplastic-type carotenoids pattern, mainly lutein, β-carotene, violaxanthin and neoxanthin
- Group III clusters fruits with large amounts of lycopene accompanied by partly saturated acyclic polyenes such as phytoene, phytofluene or ζ-carotene
- Group IV fruits containing large amounts of β-carotene and its hydroxyl-derivatives, β-cryptoxanthin and zeaxanthin
- Group V, fruits with moderate to large amounts of epoxides as violaxanthin, anteraxanthin or luteoxanthin
- Group VI, fruits containing unique carotenoids such as capsanthin; group VII, poly-*cis* carotenoids
- Group VIII fruits with apocarotenoids such as β-citraurin or 8-β-apocarotenal.

Attending to this categorization, the pulp of banana is a good example of group I with negligent amounts of carotenoids, representatives of group II are kiwi, grape or melon, from group III tomato, red watermelon or guava, from group IV and V are loquat and apricot, from group VI red pepper, from group VII tangerine tomato

Table 6.1 Total carotenoid content and main carotenoids in commonly consumed fleshy fruits

Fruit		Total carotenoid content	Main carotenoids[a]	References
Apple (*Malus domestica*)	Skin	10-25 f.w.	**Lutein**, Violaxanthin, Luteoxanthin, Neoxanthin	Gross (1987), Ampomah-Dwamena et al. 2012, and Delgado-Pelayo et al. (2014)
		17-151 d.w.		
	Flesh	<2–29 f.w.	Lutein,Violaxanthin, Neoxanthin	
		5–30 d.w		
Avocado (*Persea americana*)		12 f.w.	**Lutein,** Neoxanthin**,** β-Carotene	Gross (1987)
Apricot (Orange) (*Prunus armeniaca*)		5–40 f.w.	**β-Carotene,** Phytoene**,** Phytofluene	Gross (1987), Marty et al. (2005), and Dragovic-Uzelac et al. (2007)
Banana (*Musa paradisiaca*)	Peel	6 f.w.	**Lutein,** α-Carotene**,** β-Carotene	Gross (1987)
	Pulp	1–30 f.w	**β-Carotene, α-Carotene,** Lutein	Gross (1987) and Harding et al. (2012)
Cherry (*Prunus avium*)		<4 f.w.	Lutein, β-Carotene	Gross (1987), and McCune et al. (2011)
Grape (*Vitis vinifera*)		1–3 f.w.	**Lutein, β-Carotene,** Violaxanthin	Mendes-Pinto et al. (2005) and Young et al. (2012)
Grapefruit (Red and pink) (*Citrus paradisi*)	Peel	7.5–62 f.w.	Phytoene, Phytofluene, Lycopene, Violaxanthin	Alquézar et al. (2008a) and Rodrigo et al. (2013a)
	Pulp	1–53 f.w.	Phytoene, Lycopene, β-carotene	Xu et al. (2006), Alquézar et al. (2008a)
Grapefruit (White) (*Citrus paradisi*)	Peel	0.9–3.8 f.w.	Phytoene, Phytofluene, Violaxanthin	Xu et al. (2006) and Alquézar et al. (2008a)
	Pulp	>2 f.w.	Phytoene,Violaxanthin, Zeaxanthin	Xu et al. (2006), Fanciullino et al. (2006), and Alquézar et al. (2013)
Guava (Pink) (*Psidium guajava*)		56–62 f.w.	Lycopene, β-Carotene	Gross (1987) and Mercadante et al. (1998)
Kiwifruit (*Actinidia chinensis*)		1–19 f.w.	**β-Carotene,** Lutein**,** violaxanthin	Ampomah-Dwamena et al. (2009)
Lemon (*Citrus limon*)	Peel	77 f.w.	Phytoene	Kato et al. (2004)

Loquat (*Eriobotyra japonica*)	Skin	13–120 f.w.	β-Carotene, β-Cryptoxanthin	Gross (1987) and Fu et al. (2012, 2014)
	Flesh	0.2–22 f.w.	β-Carotene, β-Cryptoxanthin	Gross (1987) and Fu et al. (2012, 2014)
Mandarin (*Citrus reticulata, Citrus clementina, Citrus unshiu*)	Peel	50–300 f.w.	**β-Cryptoxanthin, 9-*cis*-Violaxanthin,** Phytoene**,** C_{30}-Apocarotenoids**,** 9-*cis*-Antheraxanthin**,** Zeaxanthin	Kato (2012) and Rodrigo et al. (2013a)
	Pulp	20–34 f.w.	β-Cryptoxanthin, Violaxanthin	Fanciullino et al. (2006), Kato (2012), and Alquézar et al. (2008a)
Mango (*Mangifera indica*)		12–100 f.w.	**Violaxanthin,** β-Carotene, Luteoxanthin**,** Auroxanthin	Gross (1987) and Mercadante and Rodríguez-Amaya (1998)
Melon (orange-fleshed) (*Cucumis melo*)		12–50 f.w.	**β-Carotene,** ζ-Carotene	Ibdah et al. (2006) Fleshman et al. (2011)
Melon (white and green-fleshed) (*Cucumis melo*)		0–10 f.w.	**Lutein,** Violaxanthin, Luteoxanthin**,** β-Carotene	Gross (1987), Ibdah et al. (2006)
Oil palm fruit (*Elaeis guineensis*)		718 d.w.	β-Carotene, 9-*cis*-β-Carotene, α-carotene	Mortersen (2005) and Tranbarger et al. (2011)
Papaya (Yellow) (*Carica papaya*)		8–33 f.w.	β-Cryptoxanthin, β-Carotene, ζ-Carotene	Chandrika et al. (2003), Wall (2006), Schweiggert et al. (2011a),
		32 d.w.		
Papaya (Red) (*Carica papaya*)		60 f.w.	Lycopene, β-Cryptoxanthin	Wall (2006), and Schweiggert et al. (2011a, b)
		34 d.w.		
Peach (White) (*Prunus persica*)		<2 f.w.	Zeaxanthin, Lutein	Brandi et al. (2011)
Peach (Yellow) (*Prunus persica*)		5–11 f.w	Anteraxanthin, Zeaxanthin, Luteoxanthin, Mutatoxanthin	Gross (1987) and Brandi et al. (2011)
Pear (Green) (*Pyrus communis*)	Skin	<1 f.w.	**Lutein,** β-Carotene	Gross (1987)
Pepper (Red) (*Capsicum annuum*)		100–850 f.w.	**Capsanthin,** Violaxanthin**,** Zeaxanthin**,** β-Carotene**,** Capsorubin	Curl (1962), Davies et al. (1970), Marín et al. (2004), Ha et al. (2007), Minguez-Mosquera and Hornero-Méndez (1994), and Guzman et al. (2010)
		12000−200 d.w.		

(continued)

Table 6.1 (continued)

Fruit		Total carotenoid content	Main carotenoids[a]	References
Pepper (Yellow) (*Capsicum annuum*)		10–22 f.w.	**Violaxanthin,** Lutein	Ha et al. (2007), Davies et al. (1970), and Guzman et al. (2010)
		300 d.w.		
Pepper (Green) (*Capsicum annuum*)		227 d.w.	**Lutein,** β-Carotene**,** Violaxanthin	Gross (1987) and Ha et al. (2007)
Persimmon (*Diospyros kaki*)	Skin	12–491 f.w.	**β-Cryptoxanthin, β-Carotene,** Lycopene	Ebert and Gross (1985) and Veberic et al. (2010)
	Flesh	0.5–15 f.w	**β-Cryptoxanthin,** Zeaxanthin**,** β-Carotene**,** Lycopene	Zhao et al. (2011) and Zhou et al. (2011)
Pineapple (*Ananas comusus*)		<1 f.w.	**Violaxanthin,** Neoxanthin**,** β-Carotene	Gross (1987) and Sian and Ishak (1991)
		22 d.w.		
Plum (*Prunus domestica*)		0.9–25 f.w.	**β-Carotene,** Lutein**,** Violaxanthin	Gross (1987) and Fanning et al. (2014)
Pumpkin (*Cucurbita maxima*)		50–75 f.w.	Violaxanthin, Lutein, β-Carotene, Zeaxanthin	Kreck et al. (2006), Azevedo-Meleiro and Rodriguez-Amaya (2007), and Nakkanong et al. (2012)
		17–570 d.w.		
Strawberry (*Fragaria ananassa*)		<0.5 f.w.	Lutein, β-Carotene	García-Limones et al. (2008) and Zhu et al. (2015)
Sweet orange (*Citrus sinensis*)	Peel	40–120 f.w.	**9-*cis*-Violaxanthin,** C_{30}-Apocarotenoids**,** Phytoene**;** 9-*cis*-Antheraxanthin**;** β-Cryptoxanthin	Rodrigo et al. (2013a)

	Pulp	4–30 f.w.	**9-*cis*-Violaxanthin,** 9-*cis*-Antheraxanthin**;** β-Cryptoxanthin	Kato et al. (2004), Alquézar et al. (2008a), and Meléndez-Martínez et al. (2008)
Tomato (*Solanum lycopersicum*)		50–135 f.w.	**Lycopene,** β-Carotene**,** Phytoene**,** Phytofluene	Fraser et al. (1994), Abushita et al. (2000), Aherne et al. (2009), and Guil-Guerrero and Rebolloso-Fuentes (2009)
		133–583 d.w.		
Watermelon (Red) (*Citrullus lanatus*)		35–112 f.w.	**Lycopene,** β-Carotene**,** Phytoene**,** Phytofluene	Perkins-Veazie et al. (2006), Grassi et al. (2013), and Lv et al. (2015)
Watermelon (Yellow-Orange) (*Citrullus lanatus*)		3–60 f.w.	**Violaxanthin,** Lutein; Prolycopene**,** **β-carotene,** Phytoene**,** ζ-Carotene	Perkins-Veazie et al. (2006) and Lv et al. (2015)

Fruits are listed in alphabetical order and in some cases cultivars with contrasting carotenoid content are indicated. Unless indicated carotenoid content is in the edible portion of the fruit

[a]When a carotenoid is predominant is indicated in *bold letter*

f.w.: mg g^{-1} *f.w. (fresh weight); d.w.: mg* g^{-1} *d.w. (dry weight)*

mutant or Pinalate sweet orange, and from group VIII mature peel of mandarins or red pepper (Table 6.1). These carotenoid patterns can be overlapped or merged into each other and a simplified classification can be suggested in view of the profiling and content of carotenoids: type I encloses fruits with moderate to high carotenoid content with a complex pattern, while in type II fruits carotenoid profile is restricted to one or two principal components. On the other hand, fruits accumulating low to moderate carotenoids with a complex pattern can be grouped as type III while those with a profile relatively simple would be type IV. Finally, fruit with low carotenoid content with a profile usually very simple, mainly lutein, zeaxanthin and β-carotene can be categorized as type V.

6.1.2 Dynamics in Carotenoids Profile During Fruit Development and Ripening

The extraordinary diversity in carotenoid content and composition in fruits also correlates with different patterns of carotenoid evolution from immature to fully ripened. A representative fruit with a low carotenoid content is strawberry (*Fragaria ananassa*) with a total content below 1 $\mu g\ g^{-1}$ f.w. (Table 6.1), which notably decrease during ripening, showing a simple pattern consisting mainly of lutein, β-carotene, zeaxanthin and β-cryptoxanthin (García-Limones et al. 2008; Zhu et al. 2015). Another example of low carotenoid content is white grapefruit (*Citrus paradisi*) but different tendencies in the pattern may be distinguished between pulp and peel during fruit development and ripening (Fig. 6.1a). The peel of immature fruit contains the typical chloroplastic-type profile with a moderate to high carotenoid content, however, as the fruit matures, the content is reduced and at advanced stages, only minute amounts of colourless carotenes and β,β-xanthophylls are detected. The pulp (juice sacs) of immature fruits contains very low amounts of carotenoids and remains almost constant during ripening (Alquézar et al. 2013). Grape berries (*Vitis vinifera*) also contain low amounts of carotenoids at ripen stage (1–4 $\mu g\ g^{-1}$ f.w.) and show a maximum (1–5 $\mu g\ g^{-1}$ f.w.) after *veraison* and decreasing afterwards. β-carotene and lutein are the main carotenoids identified in the whole berry, as well as lower amounts of violaxanthin, zeaxanthin and neoxanthin (Crupi et al. 2010; Young et al. 2012).

Fruits representative of low to moderate carotenoid content are peach (*Prunus persica*), apricot (*Persea armeniaca*), apple (*Malus domestica*), persimmon (*Diospyrus kaki*) or loquat (*Eriobotyra japonica*) with minimum levels between 1 to 5 $\mu g\ g^{-1}$ f.w. and reaching values up to 30 $\mu g\ g^{-1}$ f.w., usually at full ripen stage. However, remarkable differences can be observed among the varieties of each species, and also between the skin and the flesh. In apple, the carotenoid concentration can vary between 3 and 10 $\mu g\ g^{-1}$ f.w. in the skin and between and 0.89 $\mu g\ g^{-1}$ f.w. in the flesh (Table 6.1) (Ampomah-Dwamena et al. 2012; Delgado-Pelayo et al. 2014). In a recent study, the carotenoid content and composition in

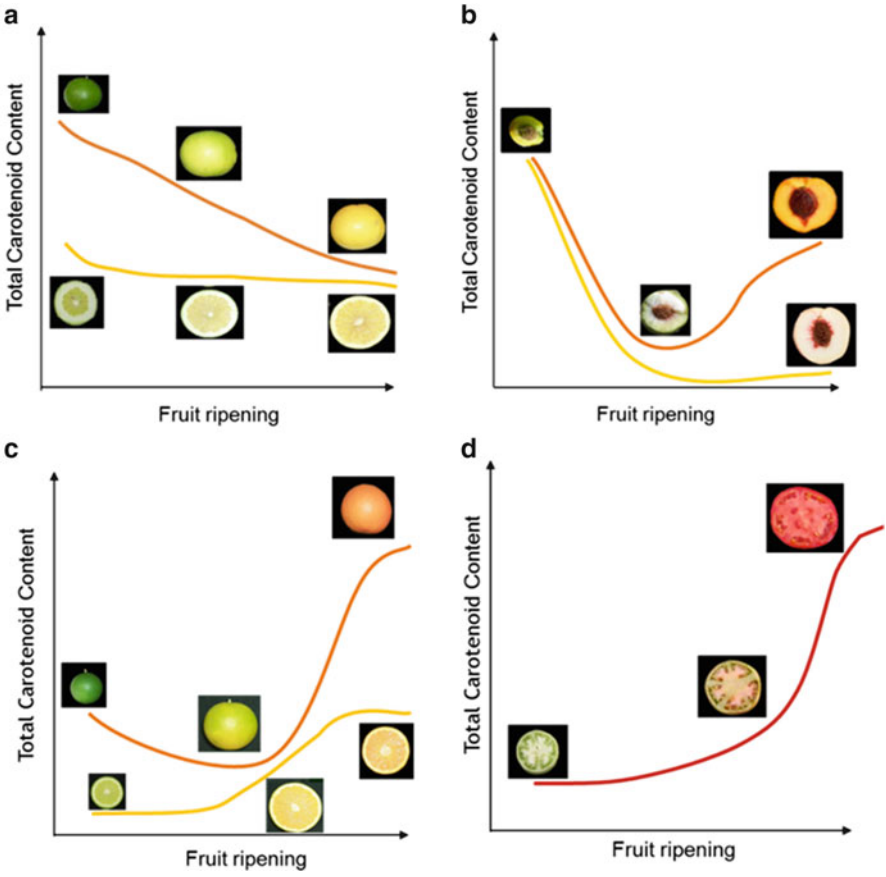

Fig. 6.1 Evolution of total carotenoid content in different fruit tissues and species during ripening. Changes in concentration of total carotenoids in peel (*orange line*) and pulp (*yellow line*) of white grapefruit (**a**), orange (*orange line*) and white-flesh (*yellow line*) peach (**b**), in peel (*orange line*) and pulp (*yellow line*) of sweet orange (**c**), and in the pericarp of tomato (**d**). Note that scale is not the same for the different panels

the flesh and skin of thirteen apple cultivars showed that, as a general rule, mature yellow-skinned varieties contain similar carotenoid levels in the skin and flesh, while green and red-skinned contain much lower levels in the flesh than in the peel (Delgado-Pelayo et al. 2014). The flesh from immature apple usually contains moderated amounts of chlorophylls and chloroplastic-type carotenoids, with lutein being the most predominant, but as fruit ripen, these pigments disappear and β,β-xanthophylls, mainly neoxanthin and violaxanthin mono- and diesterified with fatty acids, are accumulated from low to moderate levels (Ampomah-Dwamena et al. 2012; Delgado-Pelayo et al. 2014). Interestingly, wild or ancestral apple varieties like Aotea, an orange-coloured fleshy apple, is able to reach concentrations as high as 30 $\mu g\ g^{-1}$ f.w. in both skin and flesh (Ampomah-Dwamena et al. 2012).

Therefore, it seems that the ability to accumulate carotenoids in apple fruit has been negatively selected during breeding (Ampomah-Dwamena et al. 2012). Yellow-fleshed peach fruit can also be categorized as having a moderate carotenoid content, but, in contrast to apple, the composition in mature fruits can be very complex and more than 45 carotenoids have been identified in a profile dominated by esterified xanthophylls (Gross 1987). In immature peach fruit, carotenoid content is relatively high (20 to 30 $\mu g\ g^{-1}$ f.w.) (Ma et al. 2013a), with lutein being the most predominant, followed by β-carotene and low amounts of β-cryptoxanthin and violaxanthin (Brandi et al. 2011) (Fig. 6.1b). As fruit ripen, yellow flesh varieties accumulate different proportions of anteraxanthin, luteoxanthin, zeaxanthin, β-cryptoxanthin, β-carotene, violaxanthin, phytofluene, lutein and neoxanthin together with other minor carotenoids, reaching values close to 10 to 15 $\mu g\ g^{-1}$ f.w, while in white varieties they decrease to almost zero (<1 $\mu g\ g^{-1}$ f.w.) (Gross 1987; Brandi et al. 2011; Ma et al. 2013a) (Fig. 6.1). Apricot can be also classified as fruit with low to moderate carotenoid content but with a more simple carotenoid profile compared to peach. Orange-fleshed varieties of apricot contain mainly β-carotene (up to 20 $\mu g\ g^{-1}$ f.w.), minor amounts of colourless carotenes and other xanthophylls (Marty et al. 2005; Dragovic-Uzelac et al. 2007), while white varieties accumulate the colourless carotenes phytoene and phytofluene (Marty et al. 2005). The evolution of total carotenoid content observed in apricot flesh is similar to that described for peach. Loquat fruits also display a low to moderate carotenoid content in the flesh, ranging between 5 to 20 $\mu g\ g^{-1}$ f.w. in orange varieties, so called red-fleshed, while the concentration is below 1 $\mu g\ g^{-1}$ f.w. in white varieties (Fu et al. 2012, 2014). The skin of loquat contains a significantly higher concentration of carotenoids than the flesh, reaching values 20-times higher in red-orange cultivars, and up to 100-times higher in the skin than in the pulp. Main carotenoids in ripen loquat are β-cryptoxanthin, β-carotene and lutein, although phytoene and other β,β-xanthophylls are also detected (Fu et al. 2012, 2014).

Examples of fruit with moderate to high carotenoid content are the pulp of sweet oranges (*Citrus sinensis*), mandarins (*Citrus reticulata*, *C. clementina* and *C. unshiu*) and mandarin hybrids, the peel and pulp of red grapefruits and orange-fleshed melons (*Cucumis melo*). In mature orange-fleshed melon varieties, the carotenoid concentrations range between 12–50 $\mu g\ g^{-1}$ f.w. with a simple profile mainly composed by β-carotene, accounting for 80–95 % of the total (Ibdah et al. 2006; Fleshman et al. 2011). By contrast, the pulp of sweet oranges and mandarins show one of the most complex carotenoids pattern described in fruits, with values between 4–34 $\mu g\ g^{-1}$ f.w. The immature pulp of sweet orange and mandarins contain negligible levels of carotenoids and just before the breaker stage, a burst in carotenoid biosynthesis occurs, resulting in the massive accumulation of β,β-xanthophylls, with β-cryptoxanthin being the predominant species in mandarins and violaxanthin (mainly 9-*cis*-isomer) in oranges (Fig. 6.1) (Kato 2012). As in other carotenogenic fruits or in chromoplastic tissues, xanthophylls are predominantly mono- or diesterified with fatty acids such as myristic and palmitic acid (Dhuique-Mayer et al. 2007; Giuffrida et al. 2010). In most red grapefruit cultivars, the total

carotenoid content is similar in the peel and in pulp, ranging between 10–20 $\mu g\ g^{-1}$ f.w., with lycopene and the colourless phytoene and phytofluene being the most predominant carotenoids with minor amounts of β-carotene and β,β-xanthophylls. These equivalent levels of carotenoids between peel and pulp in some red grapefruit are very unusual in fruits, and particularly in the *Citrus* genus (Alquézar et al. 2013).

The last category corresponds to fruits considered as carotenoid-rich or with high carotenoid content. This category can be divided in two groups based on the complexity of the carotenoid pattern. The first cluster consists of fruits with a simple pattern composed of two or three carotenoids, such as tomato (*Solanum lycopersicum*), the peel of lemon (*Citrus limon*), watermelon (*Citrullus lanatus*) and pumpkins (*Cucurbita maxima*), while the second includes fruits with a very complex carotenoid profile such as the peel of coloured citrus fruits (oranges, mandarins and their hybrids) or red peppers (*Capsicum annuum*). Tomato has been considered as a model fruit to investigate carotenogenesis, and total carotenoid concentrations ranges between 50 and 135 $\mu g\ g^{-1}$ f.w. in commercial varieties (Table 6.1), but these values appear to be underestimated, since colourless carotenes are not considered (Fraser et al. 1994; Meléndez-Martínez et al. 2015; Aherne et al. 2009). Carotenoid profiling in mature tomato fruits is relatively simple, with lycopene accounting for up to 90 % of the total, moderate amounts of phytoene and phytofluene and a minor fraction of β-carotene (Fraser et al. 1994; Fantini et al. 2013). In the immature stage, the carotenoid content is low with a chloroplastic-type profile but after breaker, lycopene accumulates and there is an increase in the content of colourless carotenes and a decrease in that of xanthophylls and β-carotene (Fraser et al. 1994, 1999) (Fig. 6.1). Similarly to tomato, red papaya (*Carica papaya*) and watermelon fruits can accumulate up to 100 $\mu g\ g^{-1}$ f.w. of total carotenoids and profiling is also relatively simple, predominantly lycopene, which accounts approximately 50 % in papaya and more than 90 % in watermelon (Tadmor et al. 2005; Kang et al. 2010; Schweiggert et al. 2011a, b). In addition to lycopene, red papaya also contains β-cryptoxanthin (30 %) and β-carotene (4 %), while the watermelon pattern is complemented with phytoene, phytofluene and β-carotene (in total up to 30 %). In general, yellow and orange varieties of payaya and watermelon do not only have an altered carotenoid profile but also display a reduction in the total carotenoids. The pattern of carotenoids in yellow papaya is similar to that of the red varieties, with the exception of the absence of lycopene, which is replaced by β-cryptoxanthin (60 %) and β-carotene (7 %) (Schweiggert et al. 2011a, b). White-yellow and orange watermelon cultivars show relevant differences in carotenoid composition, where the total content can vary between 3 and 60 $\mu g\ g^{-1}$ f.w. In orange-fleshed varieties, pro-lycopene (8.0 $\mu g\ g^{-1}$ f.w.) is the most dominant, followed by phytoene (5.4 $\mu g\ g^{-1}$ f.w.) and ζ-carotene (4.6 $\mu g\ g^{-1}$ f.w.), while in some yellow genotypes the xanthophylls lutein and violaxanthin and the most prominent, with only traces of β-carotene (Tadmor et al. 2005; Perkins-Veazie and Collins 2006) or (Lv et al. 2015). Therefore, changes in the visual colouration in watermelon and papaya are mainly due to differences in lycopene accumulation.

Cucurbita species, as zucchini, winter squash and pumpkin (*C. peppo, C. moschata* and *C. maxima*) are also recognized as carotenoid-rich fruits, but with notable variations in skin and flesh and between varieties. In *C. moschata*, the carotenoid profile is composed of β- and α-carotene, with minor proportions of lutein, violaxanthin and neoxanthin, with a total content of around 25 $\mu g\ g^{-1}$ f.w. (Nakkanong et al. 2012; Zhang et al. 2014). *C. maxima* fruits accumulate as major carotenoids violaxanthin, lutein and β-carotene, reaching concentrations between 50–75 $\mu g\ g^{-1}$ f.w. (Kreck et al. 2006; Azevedo-Meleiro and Rodriguez-Amaya 2007; Nakkanong et al. 2012). *C. pepo* is one of the species among the genus with the largest diversity in skin and flesh coloration (Obrero et al. 2013). Interestingly, mature green skinned varieties contain very high levels of carotenoids (650 $\mu g\ g^{-1}$ f.w.) with a profile basically composed of lutein and β-carotene, while the skin of yellow-orange varieties account for 80–100 $\mu g\ g^{-1}$ f.w., mostly due to the presence of lutein. Contrastingly, a higher carotenoid content can be found in the flesh of yellow-orange varieties (22 $\mu g\ g^{-1}$ f.w.) than in that of white and green varieties (Obrero et al. 2013).

Red peppers are recognized to contain the highest carotenoid concentration among commonly consumed fruits, reaching levels of nearly 900 $\mu g\ g^{-1}$ f.w. (Table 6.1), they also have a complex carotenoid profile (Hornero-Méndez et al. 2000). The main carotenoid in red pepper fruit is the ketoxanthophyll capsanthin, accounting for more than 80 % of the total content and responsible for its characteristic colouration. Ketocarotenoids contain a keto group at the central chain and a cyclopentanol ring (κ) at one or both ends. Among ketocarotenoids, capsanthin is the more abundant but capsorubin, with κ-rings at both ends, and capsanthin epoxides are also present in peppers. The massive accumulation of ketoxanthophylls during pepper ripening is almost exclusive of the genus *Capsicum*, due to the presence of the capsanthin-capsorubin synthase activity (Bouvier et al. 1994; Hugueney et al. 1995). At the immature stage, pepper fruit accumulates chloroplastic-type carotenoids but once ripening begins, this carotenoid complement is replaced by a massive *de novo* synthesis of β,β-xanthophylls (zeaxanthin, antheraxanthin and violaxanthin) and their corresponding ketoxanthophylls derivatives (Hornero-Méndez et al. 2000). Interestingly, the presence of ketocarotenoids in the tropical fruits red Mamey and Chinese Maracuya has recently been reported, although the biosynthetic origin in these fruits remains to be elucidated (Murillo et al. 2013). Pepper also displays a great diversity in fruit colouration (Thorup et al. 2000). In yellow cultivars, total carotenoid content remains almost constant during ripening (10–20 $\mu g\ g^{-1}$ f.w.), with lutein being the most abundant carotenoid (41–67 %), but α-carotene is synthetized *de novo* and β-carotene is gradually transformed into β-cryptoxanthin and zeaxanthin (Ha et al. 2007). The orange colour of *Capsicum* varieties is the result of different carotenoid patterns. Some orange varieties show a deficiency in the biosynthesis of ketoxanthophylls and only accumulate orange and yellow carotenoids, while others display a carotenoid profile similar to red varieties, but considerably reduced levels (Lang et al. 2004; Guzman et al. 2010; Rodriguez-Uribe et al. 2012).

Another fruit tissue which is highly enriched in carotenoids is the flavedo (outer coloured portion of the peel) of ripeed orange-colored citrus fruits, which in some cultivars reaches concentrations of close to 300 and 150 $\mu g\ g^{-1}$ f.w. (Rodrigo et al. 2013a). The diversity in carotenoids is one of the highest reported, with up to 110 different carotenes and xanthophylls identified (Gross 1987), including different geometric isomers, and the presence of specific C_{30} apocarotenoids, responsible for the intense deep-orange colouration of some varieties (Ma et al. 2013b; Rodrigo et al. 2013b). In the flavedo of mandarins and oranges, the carotenoid content and composition change dramatically during fruit development and ripening (Fig. 6.1). The flavedo of immature green fruit contains high levels of chloroplastic carotenoids (lutein, β and α-carotene, zeaxanthin and *trans*-violaxanthin), but as ripening progresses, carotenoid concentration decreases to a minimum at the breaker stage. After this stage, the concentration of carotenoids in the flavedo markedly increases, with being 9-*cis*-violaxanthin as the predominant in oranges (up to 80 %) (Kato et al. 2004; Rodrigo et al. 2003, 2004) and with similar proportions of β-cryptoxanthin and 9-*cis*-violaxanthin in mandarins (Kato et al. 2004). In addition, different geometric isomers of these carotenoids and other β,β-xanthophylls, such as antheraxanthin, zeaxanthin, luteoxanthin, mutatoxanthin and lutein, are often found, and as described for pulp tissues, an important fraction of the mono- and polyhydroxylated xanthophylls are esterified with fatty acids. This complex pattern of carotenoids in mature orange and mandarins peel also includes the presence of β-carotene and important amounts of colourless carotenes (phytoene and phytofluene).

6.2 Carotenoids Function in Fruits

The function of carotenoids in green tissues has been largely described, because they are essential for photosynthesis. Carotenoids functions in chloroplast are centralized in the thylakoidal membranes, a site of intense carotenoid biosynthesis and accumulation (Vishnevetsky et al. 1999; Nogueira et al. 2013). Peel or skin cells of unripe green fruits contain active photosynthetic chloroplasts and the presence of carotenoids is necessary to integrate the light harvesting complex, regulating the efficacy of photosystem II, acting as accessory antennae pigments and as photo-protectors (Ma et al. 2003; Holleboom et al. 2013). Nevertheless, the role of photosynthesis in tomato fruit has recently been re-evaluated and it appears not to be crucial for fruit ripening (Lytovchenko et al. 2011).

The functions of Carotenoids in coloured (non-green) fruits has been investigated to a lesser extent but they have an ecophysiological role, as signalling molecules attracting seed-dispersing herbivores (Bartley and Scolnik 1995; Blount and McGraw 2008; Britton 2008). Moreover, consumer preferences have led to the selection of novel varieties with brighter coloured fruits usually associated with higher carotenoid content or/and substantial changes in composition by breeders. Carotenoids in fruits may also perform other functions, such as protecting fruits against harmful environmental conditions. This function, although less known and

explored, could be accomplished by the stabilization of membrane lipids, since their chemical structures determine fluidity depending on environmental conditions (Britton 1995, 2008; Vishnevetsky et al. 1999). In this sense, β-carotene localization inside membranes could favour a higher fluidity during low temperature stress, protecting fruits against cold stress, while oxygenated carotenoids like xanthophylls, orientated almost perpendicular to the membrane surface, may contribute to a lower fluidity and higher stability under light stress or high temperatures (Havaux 1998). Carotenoids may also act as powerful antioxidants, protecting fruit tissues from different stresses (Whitaker 1994; Bouvier et al. 1998a; Lado et al. 2015a). The content of lycopene, a carotene displaying strong antioxidant activity (Krinsky 1989; Aizawa et al. 2011) in tomato fruit has been linked to reduced damage during cold storage (Whitaker 1994). Chilling stress may also reduce lycopene content in this fruit (Rugkong et al. 2011), establishing a possible relationship between both factors. Similarly, cold stress in citrus fruit has been associated with an oxidative burst (Sala 1998; Sala and Lafuente 2000) and an accumulation of lycopene in the peel of grapefruit has been shown to exert a protective role during cold storage, preventing the appearance of peel pitting and maintaining cell and chromoplasts integrity (Lado et al. 2015a). Moreover, lycopene-induced chilling tolerance in grapefruits appears to be directly related to an enhancement of the singlet oxygen scavenging capacity (Lado et al. 2016).

6.3 Carotenoid Biosynthesis in Fruits: Metabolic Sequence and Levels of Regulation

Carotenoid regulation in fruits can be rather complex due to the dramatic changes in content and composition during ripening, which are also dependent on the fruit tissue and the developmental stage. The concurrency of different regulatory levels has been described, such as transcriptional control and post-transcriptional modifications of structural enzymes and accessory proteins of the biosynthetic and catabolic pathways. The sequestration of carotenoids in sink structures during the massive accumulation that takes place during ripening may be also an additional factor. An additional feature is the existence of fruit-specific isoforms for key steps of the pathway, which allow an autonomous regulation of the flux and, from an evolutionary point of view, a potential source of variability to generate novel enzymatic activities with different catalytic properties.

The main structural genes of the biosynthetic and, more recently, catabolic pathways have been identified (Fig. 6.2) in a large variety of fruit species, with tomato as the model fruit to establish the fundamental regulatory mechanisms of carotenoid accumulation (Bramley 2002, 2013; Liu et al. 2015). Nevertheless, although most of the knowledge on the regulation of carotenoid biosynthesis has been obtained from tomato and can be inferred to other fleshly fruits, novel enzymatic activities or specific regulatory mechanisms have been identified as

responsible for the large diversity in carotenoid structures and accumulation patterns in the different fruit species (Table 6.1; Fig. 6.2). In this section common regulatory mechanisms recognized in fruits are described as well as others which are restricted to certain species with unique carotenoid patterns.

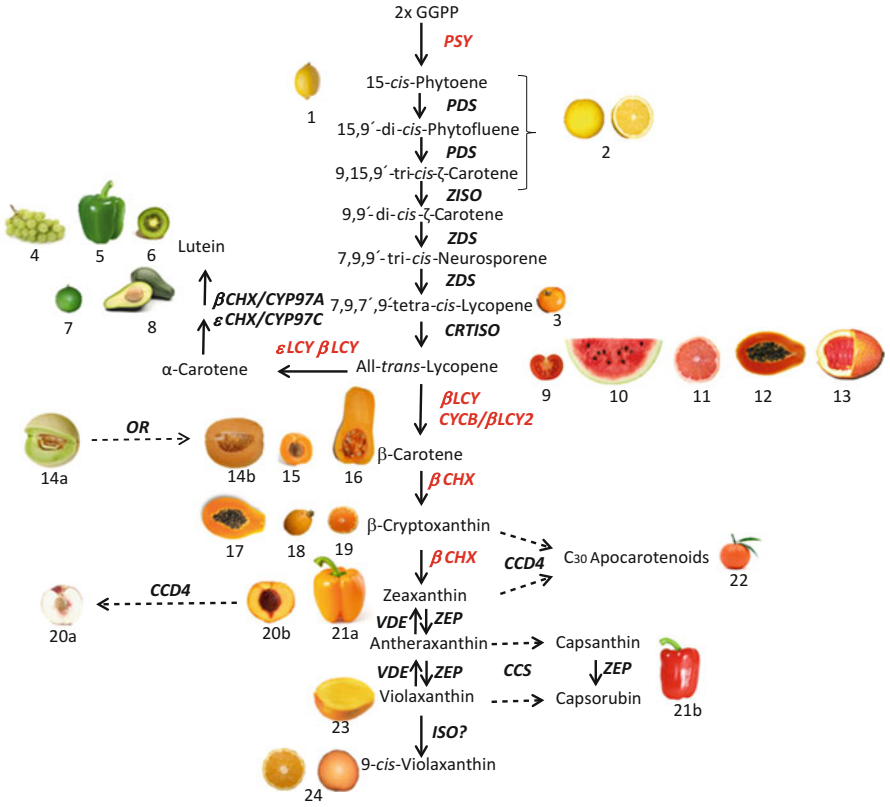

Fig. 6.2 Representative scheme of the carotenoid pathway in fruits. Each fruit is positioned in the pathway at the level of the predominant carotenoid responsible for its colouration (see also Table 6.1). Name of enzymes in red indicates main control points in the pathway and discontinuous arrows indicate steps that are restricted to a specific species or genus. Fruit codes: *1*, lemon; *2*, sweet orange mutant Pinalate (Rodrigo et al. 2003); *3*, tomato tangerine mutant (Isaacson et al. 2002); *4*, grape; *5*, immature green pepper; *6*, kiwifruit; *7*, peel of immature mandarin; *8*, avocado; *9*, tomato; *10*, red watermelon; *11*, pulp of red grapefruit; *12*, red papaya; *13*, gac; *14a*, white-flesh melon; *14b*, orange-flesh melon; *15*, orange-flesh apricot; *16*, orange-flesh pumpkin; *17*, yellow papaya; *18*, loquat; *19*, pulp of mandarin; *20a*, white-flesh peach; *20b*, yellow-flesh peach; *21a*, orange pepper; *21b*, red pepper; *22*, peel of mandarin; *23*, mango; *24*, sweet orange. *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZISO* ζ-carotene isomerase, *ZDS* ζ-carotene desaturase, *CRTISO* lycopene isomerase, *ε-LCY* lycopene ε-cyclase, *CYP97A* and *CYP97C* heme-containing cytochrome P450 hydroxylases, *β-LCY*1 lycopene β-cyclase1, *CYCB/LCYβ2* chromoplasts lycopene β-cyclase2, βCHX non-heme β-carotene hydroxylase, *CCS* capsanthin capsorubin synthase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *OR* orange protein, *CCD4* carotenoid cleavage dioxygenase type 4

6.3.1 Transcriptional Changes in the Biosynthetic Pathway Regulating Carotenoids Accumulation in Fruits

The biosynthesis of phytoene, the first colourless and linear carotenoid in the pathway, catalysed by the phytoene synthase (PSY), is generally accepted as the most important regulatory step in the biosynthesis of carotenoid in plants and it is tightly regulated by different environmental cues and endogenous factors (reviewed in Cazzonelli and Pogson 2010; Bramley 2013). In tomato, the increase in carotenoid content during fruit ripening correlates with changes in the expression of carotenoid biosynthetic genes, and PSY exerts the greatest contribution to the carotenoid pathway flux (Fraser et al. 2001, 2007). During tomato ripening, there is a notable increment in the expression of the fruit-specific isoform *PSY1*, accompanied by a down-regulation of lycopene cyclases (*LCYβ* and *LCYε*), which determines the massive accumulation of the red carotene lycopene (Fraser et al. 1994; Ronen et al. 1999, 2000). Intensive research using tomato mutants with altered fruit pigmentation and transgenic approaches has been carried out to understand the role of PSY in carotenoid regulation. Transgenic tomato plants transformed with an antisense *PSY1* were impaired in carotenoid accumulation in ripe fruit while, no effects were detected in the leaves (Bramley et al. 1992). Similarly, in the pleiotropic *Cnr* tomato mutant, carotenoid biosynthesis was almost abolished, resulting in mature fruit with white pericarp (Thompson et al. 1999). The pale-yellow phenotype described in *Cnr* mutants was common to the *r*-mutant (*yellow-flesh*), defective in the *PSY1* gene (Fray and Grierson 1993) and virus-induced silencing of the *PSY1* gene caused a complete disappearance of linear carotenoids and only trace amounts of xanthophylls and β-carotene (Fantini et al. 2013).

Different transcription factors also appeared to be related to the regulation of fruit ripening, affecting different metabolic steps of the pathway (Liu et al. 2015). The tomato gene *MADS1/RIN* was proposed as a negative regulator of fruit ripening since the expression of *PSY1* and carotenoid content were enhanced in RNAi transgenic lines, this also occurred with genes related to ethylene biosynthesis and response (Dong et al. 2013). Overall, these works demonstrate that the induction of *PSY1* during fruit ripening is determinant for tomato colouration and carotenoid content. Similarly, a relationship between the enhancement of *PSY* gene transcription and the increase in total carotenoid content during ripening has been described in a number of fruits, such as citrus (Kato 2012; Alquézar et al. 2008a; Peng et al. 2013), pepper (Rodriguez-Uribe et al. 2012), persimmon (Zhao et al. 2011), loquat (Fu et al. 2014), watermelon (Lv et al. 2015) or banana (Harding et al. 2012). However, in ripened fruits with low carotenoid content, such as strawberry, apple, white-fleshed peach, white grapefruit or grape, the role of PSY is less obvious, since transcript levels during ripening do not parallel carotenoid concentration, suggesting the involvement of other post-transcriptional events controlling activity or other unidentified regulatory steps downstream in the pathway.

Downstream in the carotenoid biosynthetic pathway, the isomerase ZISO catalyses the isomerization of tri-*cis* ζ-carotene to di-*cis* ζ-carotene (Li et al. 2007; Chen et al. 2010). In tomato fruit, *ZISO* is highly induced during ripening (Fantini et al. 2013) while in apple, a fruit with low carotenoid content, a reduction in gene expression in both skin and flesh occurred during ripening, which may limit the flux through the pathway and prevent carotenoid accumulation (Ampomah-Dwamena et al. 2012). A second isomerase, CtrISO, that converts pro-lycopene into *all-trans*-lycopene was initially reported in tomato (Isaacson et al. 2002). Expression of *CtrISO* is highly enhanced during tomato ripening and inactivation of the *CtrISO* gene in the *tangerine* (*tg*) mutant leads to the accumulation of prolycopene, resulting in a pink-orange phenotype (Isaacson et al. 2002) (Fig. 6.2). Moreover, the *tg* mutant is epistatic to the *yellow-flesh* (with absence of *PSY1* transcripts), leading to the accumulation of phytoene in the *yellow-flesh* mutants in a *tangerine* genetic background. Several evidences suggest that the mechanism underlying this effect is a positive feedback regulation of *cis*-neurosporene and pro-lycopene on *PSY1* (Kachanovsky et al. 2012). In citrus fruits, the expression of *CtrISO* in peel and pulp during ripening has also been evaluated and in the species investigated the level of transcripts decreased after the green stage, suggesting a minor contribution to the massive β,β-xanthophyll accumulation (Kato et al. 2004).

A recent study in tomato has examined the carotenoid complement in fruit in which different steps of the carotenoid biosynthetic pathway were individually silenced. The results suggest the existence of three functional units comprising of *PSY1*, *PDS/ZISO* and *ZDS/CRTISO* genes, which are responsible for the synthesis of 15-*cis*-phytoene, 9,9-di-*cis*-ζ-carotene and *all-trans*-lycopene, respectively. Moreover, silencing the desaturase (*PDS* or *ZDS*) resulted in the induction of the isomerase in the same functional unit (*ZISO* or *CRTISO*, respectively) (Fantini et al. 2013).

It is interesting to mention that high accumulation of phytoene and phytofluene (>20 $\mu g\ g^{-1}$ f.w.) is restricted to a limited number of fruit species such as some cultivars of tomato, watermelon, apricot and citrus (Meléndez-Martínez et al. 2015). The peel of ripe lemon fruit is characterized by an extremely high level of phytoene and a low concentration of β,β-xanthophylls (Table 6.1 and Fig. 6.2) (Kato et al. 2004; Fanciullino et al. 2006; Matsumoto et al. 2007). This could primarily be explained by the high expression of *PSY* and the reduced levels of the desaturase *PDS* (Kato et al. 2004). Pinalate, a sweet orange mutant with yellow-coloured fruit (Fig. 6.2), contains in its flavedo one of the highest concentration of phytoene reported in fruits (Lado et al. 2015b), together with significant amounts of phytofluene and ζ-carotene isomers, suggesting a partial blockage at the ζ-carotene desaturation of the carotenoid pathway (Rodrigo et al. 2003).

The cyclization of lycopene is located at the branching point of the pathway (Fig. 6.2) and enzymes catalysing these reactions, lycopene β-cyclases and ε-cyclases, modulate carotenoid composition because their transcriptional changes during the ripening process. This shifts the flow of the pathway from the β,ε- to β,β-branch (Hirschberg 2001; Bramley 2013). Moreover, lycopene is the predominant carotenoid in different fruit crops (Table 6.1) and in many cases the content of this

carotene appears to be closely related to the levels of lycopene ε- or β-cyclases transcripts. Pioneering works studying several tomato mutants demonstrated that the up-regulation of *LCYβ* in *Beta* mutant (Ronen et al. 2000) or a higher expression of *LCYε* in *delta* mutants (Ronen et al. 1999) during fruit ripening lead to the accumulation of β-carotene or δ-carotene, respectively, at expenses of lycopene content, conducting finally to orange phenotypes. It is interesting to note that the repression of a second chromoplasts-specific *LCYβ* gene, so called *CYCB* or *LCYβ2*, in the *old-gold* mutant increases lycopene content but abolishes β-carotene. The *CYCB* gene is transiently expressed in tomato at the breaker stage and is responsible for *de novo* synthesis of β-carotene in this fruit (Ronen et al. 2000). The *CYCB* gene was probably originated from a duplication of the *LCYβ* acquiring a predominant role in the composition of carotenoids in chromoplastic tissues, without altering the balance in photosynthetic organs (Ronen et al. 2000). Apart from tomato, other fruits also present the ability to accumulate lycopene and in most of them is linked to diverse alterations in *LCYβ2*, pointing to a crucial role of this gene in the control of the carotenoid flux towards the β,β-branch. Papaya is a tropical fruit with varieties of yellow- and red-fleshed colouration. β-Carotene and β-cryptoxanthin are the predominant in the yellow-fleshed and lycopene and β-cryptoxanthin in the red-fleshed type (Fig. 6.2) (Schweiggert et al. 2011b). A mutation in the *LCYβ2* gene identified in red fruit has been associated with a loss of protein functionality, provoking also a lower gene expression in the lycopene-accumulating variety (Blas et al. 2010; Devitt et al. 2010). No differences in *LCYβ1* gene sequence nor in transcript levels were detected between yellow and red papaya during ripening, ruling out the role of this gene as regulator of lycopene cyclization in coloured tissues (Skelton et al. 2006). The same mechanism was proposed to be behind the massive lycopene accumulation in red watermelon varieties (Fig. 6.2), since a triple single nucleotide polymorphism in the coding region of *LCYβ* gene was directly linked to the red phenotype and a drastic reduction in *LCYβ* and *βCHX* occurred during lycopene accumulation in red varieties (Bang et al. 2007; Kang et al. 2010). Moreover, the levels of *LCYβ* were permanently low and constant during development and ripening in red-fleshed fruits (Grassi et al. 2013). Among other fruits accumulating lycopene, arils from gac (*Momordica cochinchinensis*), a *Cucurbitaceae* tropical fruit indigenous the Southeast Asia (Fig. 6.2), are described as one of the richest sources of lycopene and β-carotene, reaching values as high as 350 and 100 μg g^{-1} f.w., respectively (Vuong et al. 2006). A global transcriptomic analysis during fruit ripening has revealed that the repression of *LCYε* gene in combination with the up-regulation of *PSY* and other upstream genes of the pathway are the main mechanisms responsible for this particular carotenoid composition (Kyung et al. 2012).

In fruits with a predominant composition of β-carotene, β,β- or β,ε-xanthophylls (Fig. 6.2) transcriptional regulation of lycopene cyclases during ripening has also a remarkable influence in carotenoid profile. In the peel of immature citrus fruits (Fig. 6.2), the expression level of *LCYε* is high and decreased during maturation, consistent with the predominance of β,ε-xanthophylls (mainly lutein) and cyclic carotenes. At the breaker stage, the concomitant induction of *LCYβ1* gene, shifting

the flux of the pathway from the β,ε-branch to the β,β-branch (Kato 2012; Rodrigo et al. 2013a). Additionally, as occurs in other carotenogenic fruits, the expression of a chromoplast-specific *LCYβ2* is stimulated in the peel and pulp of sweet oranges and mandarins, playing a key role redirecting the flux of carotenes into the β,β-branch (Alquézar et al. 2009; Zhang et al. 2012). Interestingly, red grapefruit varieties which accumulate lycopene, showed a reduced *LCYβ2* transcript level in comparison to orange-coloured and white grapefruit (Alquézar et al. 2009, 2013; Mendes et al. 2011; Zhang et al. 2012) and, moreover, pulp of red grapefruit expressed predominantly a *LCYβ2* allele with very low cyclase activity (Alquézar et al. 2009). In loquat fruits, the high lutein content at green stages appears to be correlated with an marked and transient increase in the expression of *LCYε* gene in both red and white varieties that decreased as fruit ripen as well as occurred with lutein content (Fu et al. 2012). Concomitantly, a relevant increase in β-carotene content occurred during fruit maturation, which accounts for more than 60 % of total carotenoids in ripe fruit, which was correlated with an increment in the expression of *LCYβ2* after the breaker stage, suggesting a key role of this gene in carotenoid accumulation in loquat (Fu et al. 2012). Moreover, red-fleshed varieties showed higher *PSY* and *LCYβ2* transcript levels during ripening and three times higher carotenoid content compared with white-fleshed (Fu et al. 2012). Similarly, a potential bottleneck role was suggested for *LCYβ* gene in kiwifruit to explain the differences on β-carotene accumulation in different species and varieties (Ampomah-Dwamena et al. 2009). Total carotenoid content in the peel and flesh of summer squash varieties increased concomitantly with ripening, being lutein and β-carotene the main carotenoids. Changes in *LCYε* expression were correlated with the increment in carotenoids in green and yellow-orange varieties during ripening (Obrero et al. 2013). In low-carotenoid containing fruit, such as apple, a key role of *LCYε* has been suggested as potential rate-limiting step, since differences in gene expression among cultivars drive to important modifications in carotenoid accumulation (Ampomah-Dwamena et al. 2012).

In fruits with a predominant content of β,β-xanthophylls or other downstream products (e.g. C_{30} apocarotenoids or ketoxanthophylls) a pivotal role for *βCHX* has been proposed. The most representative fruits of this group are sweet oranges, mandarins and pepper, showing a significant transcriptional up-regulation of *βCHX* during fruit ripening (Ha et al. 2007; Kato 2012; Rodrigo et al. 2013a). Interestingly, only few fruits accumulate significant amounts of β-cryptoxanthin which is the intermediate product of two sequential hydroxylations on the β-carotene rings to render zeaxanthin. In peel and pulp of mandarin or mandarin hybrids, accumulation of β-cryptoxanthin and derived apocarotenoids occurs in a significant proportion, while sweet oranges accumulate a only minor content of β-cryptoxanthin in favour to 9-*cis*-violaxanthin (Kato et al. 2004). The deduced protein sequence of sweet orange and mandarin *βCHX* is identical, however, transcript abundance was lower in mandarin while the expression of upstream genes was higher than in orange (Kato et al. 2004). Therefore, it has been hypothesized that higher β-carotene substrate and lower activity of *βCHX* in mandarin fruit tissues may favour first hydroxylation of β-carotene and, consequently, β-cryptoxanthin accumulation (Kato 2012).

Of particular interest is the transcriptional biosynthetic regulation of the ketoxanthophylls capsanthin and capsorubin in pepper fruit (Fig. 6.2). The singular composition of carotenoids in red pepper is due to the exclusive presence of the enzyme capsanthin capsorubin synthase (CCS) in *Capsicum annuum* (Hugueney et al. 1995). This enzyme is highly related to the chromoplast-specific *LCYβ2*/*CYCB* and *in vitro* is able to function as lycopene β-cyclase (Hugueney et al. 1995). A strong induction of upstream carotenoid biosynthetic genes as well as *βCHX* and *CCS* occurrs during colour change in red pepper cultivars, leading to the synthesis and accumulation of ketoxanthophylls (Bouvier et al. 1994, 1998b; Martínez-López et al. 2014). Contrastingly, in yellow and orange pepper varieties (Fig. 6.2), that do not accumulate ketoxanthophylls, the changes in carotenogenic genes expression detected during ripening were mainly associated with the absence of *CCS* transcripts (*CCS* gene deletion) or the presence of structural mutations in *CCS* gene, resulting in a non-functional enzyme (Popovsky and Paran 2000; Ha et al. 2007; Rodriguez-Uribe et al. 2012). Orange pepper phenotype can be also originated by a mutation in a second chromoplast-specific *βCHX* gene, resulting in fruits with higher β-carotene content (Borovsky et al. 2013). By contrast, white varieties contain only minute amounts of carotenoids and showed almost no expression of carotenogenic genes at ripe stages (Ha et al. 2007). Virus-induced silencing of *PSY*, *LCYβ, βCHX* and *CCS* similarly affected capsanthin accumulation in red peppers whereas the synthesis of other carotenoids was less affected, suggesting that silencing of any key gene would influence either direct or indirectly the synthesis of capsanthin (Tian et al. 2014). Interestingly, the silencing of the four genes together caused a green-reddish phenotype with a 4-times lower carotenoid concentration compared to wild-type (Tian et al. 2014).

It is generally observed the existence of different isoforms for key enzymes of the carotenoid biosynthetic pathway. The isoforms are generated by gene duplication and differentially regulated in the plant tissues, acquiring then specialized functions in each organ or tissue. Therefore, the recruitment of isoforms for a tissue-specific expression appears to be a common mechanism sustaining carotenoid differences among fruits without affecting composition in other organs such as leaves, flower or roots. This tissue-specialization was firstly confirmed in tomato fruit where the absence of *PSY1* transcripts in green tissues was concomitant with a relevant enzymatic activity, suggesting the existence of different *PSY* isoforms regulating carotenoid biosynthesis in green and coloured tissues (Fray and Grierson 1993; Fraser et al. 1994). Tomato mutants with impaired *PSY1* gene expression showed a substantial reduction in carotenoid content in coloured tissues without affecting pigment composition in leaves (Bramley et al. 1992; Thompson et al. 1999). The second *PSY i*soform was preferentially expressed in vegetative tissues and was not related with changes in carotenoid composition in coloured tissues (Fraser et al. 1999). A third isoform, *PSY3*, was described to be upregulated in roots under stress conditions (Walter et al. 2015). Furthermore, virus-induced silencing of *PSY2* and *PSY3* isoforms in tomato fruit only caused minor changes in carotenoid complement compared to the great differences obtained with *PSY1* (Fantini et al. 2013). This organ specificity was also described for PSY isoforms in melon, where

PSY1 was expressed in all plant tissues but at higher levels in fruits, flowers and leaves, whereas *PSY2* was preferably expressed in the roots (Qin et al. 2011). The differential expression of isoforms has been also proposed as a regulatory mechanism governing differences in carotenoids between fruit skin and flesh tissues. In loquat fruit, phytoene biosynthesis is governed by *PSY1* in the peel and by *PSY2* in the flesh. Interestingly, a mutation in the C-terminal region of the *PSY2A* isoform is responsible for the lack of carotenoids in the flesh of white varieties. Further on, a third isoform (*PSY2B*) appears to be responsible for carotenoid biosynthesis in loquat leaves (Fu et al. 2014), demonstrating a highly specialized tissue-specific carotenoid regulation. In citrus fruits, usually displaying marked differences in carotenoid content between peel and pulp, isoform specialization has been also described for *PSY*. Three different isoforms of *PSY* have been identified in the mandarin and sweet orange genome (Peng et al. 2013). The *PSY1* isoform is preferably expressed in fruit tissues and markedly upregulated in different species during ripening (Kato et al. 2004, 2012; Rodrigo et al. 2004; Zhang et al. 2012; Alquézar et al. 2013). This gene is also expressed in other vegetative tissues such leaf or flower suggesting that is not a fruit-specific isoform (Peng et al. 2013). Interestingly, *PSY1* transcripts levels correlated with the differential accumulation of carotenoid content in the peel but not in the pulp in different citrus species (Peng et al. 2013). The existence of a third isoform, *PSY3*, was also described, however, its expression was not detected neither in fruit nor in leaves or flowers (Peng et al. 2013). Recently, four different *PSY* isoforms have been isolated and functionally characterized in apple and their involvement in carotenoid content was investigated. Based on their sequence only isoforms *PSY1, PSY2* and *PSY4* were predicted to be functional and *PSY2* was the predominant isoform in fruit and vegetative tissues. However, transcript levels of the *PSY* isoforms expressed in fruit tissues do not correlate with carotenoid content suggesting the existence of other levels of regulation (Ampomah-Dwamena et al. 2015).

A similar isoform-specialization mechanism has been described for the step catalysed by the lycopene β-cyclase in different fruits such as citrus, tomato, or papaya, illustrating the tissue-compartmentalization in carotenoid biosynthesis. In green tissues (leaves or immature green fruits) this reaction is controlled by the *LCYβ1* gene whereas in fruit tissues this activity is regulated during ripening by the chromoplasts-specific isoform CYCB or LCYβ2 (Ronen et al. 2000; Alquézar et al. 2009; Blas et al. 2010; Devitt et al. 2010; Zhang et al. 2012). The existence of two different isoforms of *βCHX* (non-heme β-carotene hydroxylases) has been also reported in plants (Kim et al. 2009). In two fruit crops, tomato and pepper, only one isoform, *CRT-b2* in tomato and *CRT-b1* in pepper, are induced in flowers and fruit, respectively, indicating a specialized role regulating accumulation of β,β-xanthophylls in chromoplastic organs (Galpaz et al. 2006). It remains to be investigated the existence of a second βCHX isoform in other species and its potential function in fruits where a massive accumulation of β,β-xanthophylls occurs during fruit ripening.

Besides key genes of the carotenoid biosynthetic pathway, changes in carotenoid accumulation could be also directly influenced by differences in gene expression or

protein activity in the pathway of precursors for carotenoid biosynthesis. The 2-C-methylerythritol 4-phosphate (MEP) pathway is localized in plastids and changes in the main key steps could be determining differences in carotenoid accumulation in fruits (Lois et al. 2000). In tomato fruit, DXS and HDR enzymes have been described as key control regulatory steps in the MEP pathway (Lois et al. 2000; Botella-Pavía et al. 2004) being the type I DXS isoform the responsible to supply precursor during fruit ripening (Lois et al. 2000). In citrus higher *DXS, HDS* and *HDR* genes expression was described in the pulp of the red CaraCara oranges during ripening that preferably accumulate phytoene and lycopene, compared with the orange-coloured pulp of the parental variety, pointing to an enhancement of the precursors supply as an important factor determining the carotenoid differences (Alquézar et al. 2008b). In a study using fruits of different citrus species and cultivars, a good correlation between *DSX1* (type I) gene expression and carotenoid content in the pulp was obtained (Peng et al. 2013). Similarly, the study of different DXS isoforms in melon (*Cucumis melo*) revealed the existence of three isoforms and, interestingly, only the type II isoforms, *CmDXS2a* and *CmDXS2b* (type II *DXS*s), were induced during ripening in orange-pulp varieties but not in white ones (Saladie et al. 2014).

Carotenoid biosynthesis regulation in fruit has been also associated to oxidative stress and the role of reactive oxygen species (ROS) as secondary messengers stimulating the expression of carotenoid biosynthetic genes was demonstrated in pepper (Bouvier et al. 1998a). Similarly, application of a ROS-inductor in juice sacs of the red orange mutant Hong Anliu notably boosted lycopene accumulation as well as stimulated the expression of *CsPSY, CsZDS, CsCRTISO, Csβ CHX* and *CsZEP* genes while *CsPDS* and *CsLCYε* were downregulated (Pan et al. 2009). It has been proposed that ROS/redox status and sugars/carbon status are integrated factors that account for most effects of the major environmental factors affecting carotenoid biosynthesis in fruits (Fanciullino et al. 2014). Based on this model, it has proposed that the activation of the redox-sensitive systems directly up-regulates the transcription of carotenogenic genes and the activity of enzymes which demand redox state of the plastoquinone pool, or indirectly stimulates carotenoid accumulation by promoting fruit ripening and the conversion of chloroplasts into chromoplasts (Fanciullino et al. 2014). Other factors, such as redox status and electron chain reactions in plastids have been proposed as carotenoid regulators since an Orr^{DS} tomato mutant (deficient in a plastidial NADH dehydrogenase complex involved in electron fluxes), showed lower carotenoid content as well as a reduced expression of *PSY1* gene. Strikingly, phytoene, phytofluene, ζ-carotene and lycopene content were notably reduced in the mutant, while β-carotene and lutein increased only in chromoplasts-containing coloured tissues and not in chloroplast-containing green tissues (Nashilevitz et al. 2010). Therefore, alterations in the plastid redox status as well as in the plastid type dominating in the tissue may be also influence carotenoid biosynthesis and accumulation in tomato fruits (Nashilevitz et al. 2010).

Light has been also proposed as a key environmental factor regulating carotenoid biosynthesis in plants (Pizarro and Stange 2009). In tomato fruit, light stimulates

carotenoid biosynthesis (Alba et al. 2000) and a direct impact on carotenoid content has been reported by modifying light signalling (Azari et al. 2010). In tomato fruit, the red to far-red ratio increases in pericarp tissue during development and ripening and short red-light treatments in mature-green fruit stimulated lycopene accumulation by 2.3-times, establishing that light-induced accumulation of lycopene in tomato is regulated by fruit-localized phytochromes (Alba et al. 2000). Moreover, in tomato fruit disks exposed to different pulses of red and far-red light and darkness treatments, more than 50 % increase in carotenoids was only observed in fruits under red-light/darkness treatment which was associated with higher PSY activity but not gene transcription (Schofield and Paliyath 2005). In citrus fruits, a higher carotenoid content (x1.5–3.0) has been described in Clementine mandarin fruit growing outside the tree canopy (high light irradiation) compared to inside fruits (Cronje et al. 2013) which corroborates the positive effect of light on citrus peel carotenoid accumulation. However, in red grapefruit, which accumulates preferentially linear carotenes, absence of light during fruit ripening stimulated accumulation of phytoene and lycopene in comparison to light-exposed fruits but this effect could not be correlated with expression of carotenogenic genes (Lado et al. 2015c). This apparent contradictory effect of light on carotenoid content in the peel of diverse citrus species may be related to the marked differences in carotenoid profile and transcriptional control of carotenogenesis in these species.

Cryptochromes are blue-light photoreceptors and overexpression of *CRY1* in tomato plants resulted in an increase of lycopene content in the fruit mediated by a decrease of *LCYβ* expression (Giliberto et al. 2005). Moreover, the modulating effect of light on carotenoids accumulation has been demonstrated in high pigmented *hp1 (*homolog of Arabidopsis *DDB1)* and *hp2* (homolog of Arabidopsis *DET12*) or *COP1-like* tomato mutants which are negative regulators of light signalling, resulting in a higher carotenoid accumulation in the fruit (Thompson et al. 1999; Davuluri et al. 2005; Kolotilin et al. 2007). The loss of function in *hp1* and *hp2* tomato mutants stimulated a chromoplast-specific associated protein which may facilitate accumulation of carotenoids due to a sink effect (Kilambi et al. 2013)

6.3.2 *Development of Sink Structures for Carotenoid Sequestration in Fruits*

Fruits the ripening is accompanied in most species by the conversion of chloroplast into chromoplast (Egea et al. 2010, 2011; Li and Yuan 2013). Chloroplasts from unripen green fruit tissues contain similar substructures than those described in chloroplast from vegetative photosynthetic tissues, with stacked grana, stroma thylakoids, starch granules and plastoglobuli, however, chlorophyll and photosynthetic activity per unit area is much lower in fruits (Gross 1987). The Fig. 6.3a shows a representative picture of a chloroplast from the peel of an immature sweet orange fruit showing the characteristic substructures. It is interesting to note that mature

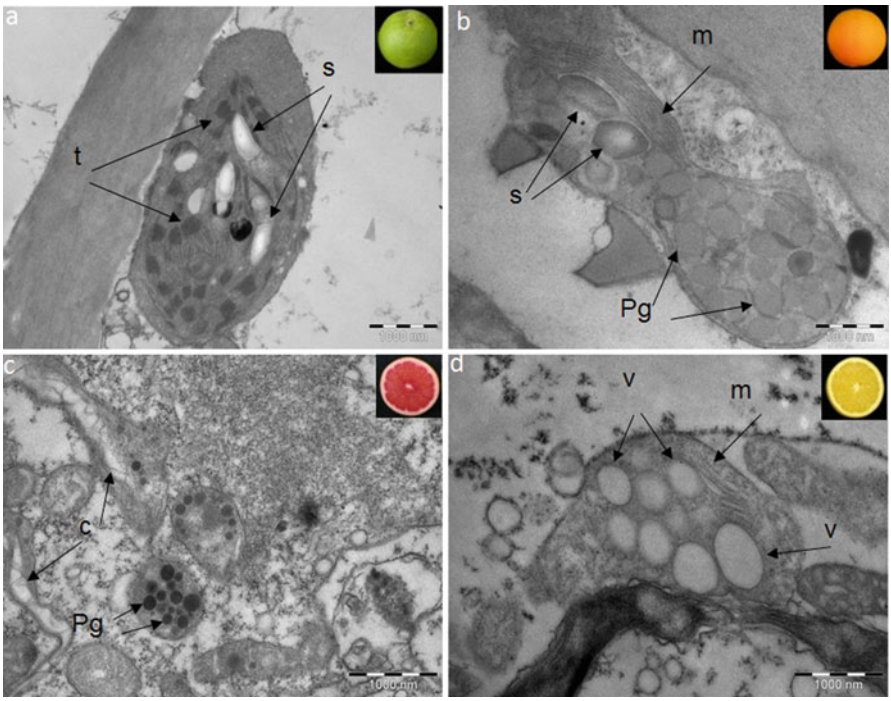

Fig. 6.3 Transmission electronic microscopy images showing the plastids ultrastructure of the peel of orange fruit at immature-green (**a**) and full colour (**b**) stages, and juice sacs of red grapefruit (**c**) and Pinalate mutant fruit (**d**). *c* lycopene crystals with membrane remnants inside, *m* achlorophyllous membranes, *Pg* plastoglobuli, *s* starch grains, *v* even and round vesicles only detected in Pinalate mutant. After osmium fixation the crystalloids are lost during the dehydration procedure and their expanded envelopes are shrunken into an undulating shape

fruits of green colour, such as the pulp of avocado, green-fleshed kiwifruit or melon, or the skin of certain cultivars of apple, contain chloroplasts, although structures of the mesocarp plastids are variable and not always well-structured as those from peel or skin tissues (Gross 1987). Chromoplasts differentiation coincides with an increment in the biosynthesis of carotenoids, making these organelles the major sink for storage of these pigments. In the flesh of some fruits, however, the process involves the differentiation of proplastids or leucoplastids into chromoplasts, which determines variable structures for carotenogenic enzymes anchorage and different efficiency in synthetizing and sequestering carotenoids (Schweiggert et al. 2011a; Hempel et al. 2014; Lado et al. 2015b). The presence of diverse types and number of plastids in the different tissues may determine important changes in the capacity to sequester carotenoid which is usually higher in the peel than in the pulp (Gross 1987).

Inside the chromoplast, plastoglobuli are lipid bodies containing different carotenoid biosynthetic enzymes, constitute a key structure where carotenoids are synthetized and stored (Bréhélin and Kessler 2008; Egea et al. 2010; Li and Yuan

2013). Plastoglobuli in the chromoplasts have been reported in numerous different carotenoid-containing fruits (Vasquez-Caicedo et al. 2006; Schweiggert et al. 2011a; Nogueira et al. 2013; Lado et al. 2015b), and increase in size and number during ripening (Ljubesic et al. 1991). Besides plastoglobuli, other carotenoid-storage structures have been described in the chromoplast, leading to the classification as globular, tubular, membranous and crystalloid (Sitte et al. 1980; Egea et al. 2010). It is interesting to remark that more than one type of chromoplasts is usually found in fruit tissue cells (Lado et al. 2015c). In globular chromoplasts, plastoglobuli are the predominant structures in fruit tissues with high content of esterified xanthophylls such as sweet orange, mango or orange pepper (Vasquez-Caicedo et al. 2006; Li and Yuan 2013; Lado et al. 2015c). In Fig. 6.3b a characteristic globular chromoplast of peel cells from ripe sweet orange is shown containing non-phytosynthetic membranes, most likely derived from the disorganization of the lamellar system, a high number of plastoglobuli and starch granules. In pepper fruit the presence of electron-dense plastoglobuli was also observed as ripening progressed, which fusion resulted in the appearance of less osmiophilic tubules and fibrils in chromoplast (Liu 2013). Fibrils were also reported in ripe pepper fruit where nearly 95 % of total carotenoids are accumulated (Deruère et al. 1994). Fibril assembly in pepper (Deruère et al. 1994) or the plastoglobuli conformation in tomato (Nogueira et al. 2013) have been described as a common process to target the excess of membrane lipids into sinks structures to avoid possible destabilising or harmful effects.

The study of subchromoplast structures revealed a direct link between the properties of carotenoids and the main sink developed for sequestration. Moreover, perturbations in carotenoid composition are strongly associated with changes in the type of plastid and with chromoplast-like structures arising prematurely during fruit development (Fraser et al. 2007). The massive presence of phytoene and lycopene in tomato (Egea et al. 2010; Nogueira et al. 2013) or in red grapefruit (Lado et al. 2015c) was associated to the development of round plastoglobuli for phytoene accumulation (*15-cis* isomer) as well as crystalloid structures accumulating *all-trans*-lycopene (Fig. 6.3c). The same crystals are present in red papaya fruit, where *all-trans*-lycopene is the main carotenoid (Schweiggert et al. 2011a). Contrastingly, in yellow-orange papaya β-carotene and β-cryptoxanthin are accumulated in globular and tubular structures whereas in mango β-carotene could be accumulated in both plastoglobuli or crystals depending on the *cis-trans* configuration (Vasquez-Caicedo et al. 2006). Interestingly, plastids of fruits with very low carotenoid content display a lack of defined substructures (Fu et al. 2012; Lado et al. 2015b), while fruits with substantial amounts of rare or uncommon carotenoids developed plastids with special structures. A good example is the accumulation of *cis*-isomers of γ-carotene and lycopene in the mesocarp of peach palm (*Bactris gasipaes*), which determinate the development of a novel globular structures where carotenes are lipid-dissolved (Hempel et al. 2014). Similarly, a special type of chromoplasts (so called vesicular chromoplast) have been recently identified in the pulp of Pinalate orange, a yellow-coloured mutant, which accumulates high levels of 15-*cis*-phytoene and diverse isomers of phytofluene and α-carotene (Lado et al. 2015b) (Fig. 6.3d). From a nutritional or functional perspective the type of structure

developed for carotenoid accumulation is anticipated to affect their bioaccesibility or bioavailability, because carotenoids lipid-dissolved are expected to be more bioavailable than those accumulated in crystalloid aggregates (Jeffery et al. 2012a, b; Schweiggert et al. 2014).

Changes in plastid number or size have revealed a direct link between plastid development or differentiation and total carotenoid content. In tomato high pigmented (*hp*) mutants the increased plastid number or plastid compartment size was directly related to the higher (x2) carotenoid accumulation in these mutants (Yen et al. 1997; Galpaz et al. 2008), however, no changes in carotenoid pattern were detected (Bramley 1997). These results suggest that key steps of the carotenoid pathway appears not been affected in *hp* mutants and the differences may be mainly explained by an earlier differentiation and higher number of biosynthetic and sink organelles (Yen et al. 1997; Kolotilin et al. 2007; Galpaz et al. 2008). An early differentiation of chromoplast structures under dark conditions allowed a massive and early accumulation of phytoene and lycopene in the peel of the red grapefruit during ripening that apparently was not related to parallel changes in the expression of carotenoid biosynthetic genes (Lado et al. 2015b).

As discussed above, plastid structure is tightly related to fruit carotenoid accumulation (Cazzonelli and Pogson 2010; Li and Yuan 2013). A reduced number of genes have been described to be directly involved in carotenoid sequestering in fruits, being fibrillin and Or the best characterized examples. Fibrillin (also called PAP, plastid-associated protein) is a plastid-localized protein associated with carotenoid storing structures in chromoplast and one of the most abundant proteins in pepper chromoplast (Deruère et al. 1994; Wang et al. 2013). Pepper fibrillin overexpression in tomato plants has resulted in increased content of carotenoids but associated with slower thylakoids degradation, suggesting a protecting role for these proteins (Simkin et al. 2007). A recent work carried out with different pepper cultivars with high variability in carotenoid content and composition, showed that fibrillin transcript levels were differentially correlated with specific carotenoids, but negatively with capsanthin content (Kilcrease et al. 2015). Therefore, further research is needed to understand the specific role of this protein in fruit carotenoid accumulation. The *Or* gene encodes for a DnaJ cysteine-rich domain-containing protein and expression of a mutated allele from *Brassica olareace* in different non-phytosyntetic tissues leads to the accumulation of carotenoids (Li et al. 2001; Lu et al. 2006; Lu and Li 2008). Further studies suggest that Or function is related to the stimulation of chromoplasts formation from proplastids in non-coloured tissues, creating a metabolic sink for carotenoid accumulation (Li et al. 2012). Interestingly, the *Or* gene has been identified as a major regulator of carotenoids accumulation in melon fruit (Tzuri et al. 2015). Two main haplotypes were identified for the *O*r gene in green, white and yellow-fleshed melons and one amino acid change in the *Or* sequence resulted in a protein that induce β-carotene accumulation (Fig. 6.2) (Tzuri et al. 2015). The precise mechanism by which Or enhances carotenoid accumulation in melon flesh is not known but functional studies in Arabidopsis *calli* indicate that Or triggers chromoplast biogenesis to sustain carotenoid production (Tzuri et al. 2015; Yuan et al. 2015). Other gene related to plastid differentiation in fruits is the

APRR2-like gene. This gene is upregulated at breaker stage in tomato and sweet pepper fruit while its overexpression lead to an increase in plastid number, area and carotenoid accumulation without affecting carotenoid *PSY1* gene expression (Pan et al. 2013) as occurred in tomato *hp* mutants (Enfissi et al. 2010). Similarly, a higher expression of the chaperone small heat shock proteins in tomato and red grapefruit peel was associated with a faster chloro- to chromoplast differentiation, as well as to an earlier red-colour development and carotenoid accumulation (Neta-Sharir et al. 2005; Lado et al. 2015a).

6.4 The Role of Carotenoid Catabolism in Fruit Pigmentation

Structural genes and enzymes of carotenoid biosynthetic pathway in fruits have been intensively studied for more than 25 years, but the role of the catabolism in carotenoid accumulation has only been addressed more recently, shedding light on the mechanisms governing pigmentation in important fruit crops. Carotenoid fragmentation is catalysed by a group of enzymes called carotenoid cleavage dioxygenases (CCD) and generically divided in five subfamilies, according to the cleavage position and/or the substrate preference: NCED (nine-*cis* epoxy-carotenoid dioxygenases), CCD1, CCD4, CCD7 and CCD8 (Walter and Strack 2011). In fruit, expression of *CCD* genes is restricted to members of the NCED, CCD1 and CCD4 subfamilies, but only some of them have a direct influence on carotenoid content. During grape berries ripening there is a steady decline in carotenoid content after *veraison* which appears to be related to chloroplast disappearance and to the formation of carotenoid-derived norisoprenoid volatiles (β-ionone and β-damascenone) (Oliveira et al. 2004; Crupi et al. 2010; Young et al. 2012). Two other processes occurs concomitantly: down-regulation of *PSY1*, *PDS1, LCYβ1* and *βCHX2* genes through ripening and a dramatic increase in transcript levels of *CCD* genes (*CCD1.1, CCD1.2, CCD4a* and *CCD4b*), suggesting that this two mechanisms may explain the reduction of carotenoids in promotion of the norisoprenoid volatiles (Young et al. 2012). In strawberry, carotenoid content also decreased nearly 50 % during ripening and the pattern of biosynthetic genes only partially correlated to the accumulation of carotenoids (Zhu et al. 2015). Interestingly, the marked reduction in lutein content has been associated with an induction of the *CCD1* during strawberry ripening (García-Limones et al. 2008). A differential regulation *CCD* gene in different fruit cultivars has been also described as the mechanism responsible for the variability in carotenoid composition. In three colour-contrasting summer squash (*Cucurbita pepo*) varieties no relevant differences in the expression of carotenoid biosynthetic genes among white, green and yellow-orange varieties explained the differences on carotenoid accumulation (Obrero et al. 2013). However, the transcriptional analysis of *CCD* genes showed that the expression of *CCD4a* and *CCD4b* were inversely correlated with carotenoid accumulation in the peel and

flesh and, therefore, are good candidates to regulate fruit colour (González-Verdejo et al. 2015). One of the best characterized examples of the influence of CCDs on fruit pigmentation is peach (*Prunus persica*). Peaches can be classified in white- and yellow-fleshed varieties, and carotenoid content is at least 10-times lower in the white than in yellow (Brandi et al. 2011). Analysis of the expression of main carotenoid biosynthetic genes did not reveal significant differences between both types of cultivars that may account for the contrasting pigmentation and carotenoid content (Brandi et al. 2011). Higher norisoprenoid carotenoid-derived levels in the white-fleshed peach suggested a higher carotenoid degradation (CCD) activity (Brandi et al. 2011). Independent works have recently demonstrated that three different mutational mechanism on *CCD4* gene are responsible for the accumulation of carotenoids in the yellow varieties (Adami et al. 2013; Falchi et al. 2013; Fukamatsu et al. 2013; Ma et al. 2013b). In all yellow peach cultivars a significant reduction in *CCD4* transcript levels and/or generation of a truncated CCD4 protein prevented carotenoid degradation in fruit mesocarp during ripening. No significant differences in the expression of *PSY1*, *PDS* and *ZDS* genes have been observed in white and orange-flesh cultivars of apricot (*Prunus armeniaca)*, despite of the 5-times higher carotenoid content in the orange-flesh fruits (Marty et al. 2005). Since apricot is a very close species to peach, it is tempting to suggest that a mechanism similar to that reported in peach may be responsible of carotenoid accumulation in apricot and an alteration in *CCD4* gene may lead to orange-fleshed apricots.

An active carotenoid catabolism in fruits or other chromoplastic tissues has been characteristically associated to the absence of colour. However, in some species of the *Citrus* genus fragmentation of C_{40} carotenoid results in a significant enhancement of fruit peel pigmentation (Ma et al. 2013b; Rodrigo et al. 2013b). It has been reported that peel of mandarins and oranges accumulate during ripening significant amounts of C_{30} apocarotenoids, mainly β-citraurin and 8-β-apocarotenal, which provide an intense and attractive orange-reddish colour to the peel (Farin et al. 1983; Gross 1987; Oberholster et al. 2001). The identification of CCD4-like family in the sweet orange and mandarin genome revealed the presence of a novel member, *CCD4b*, which transcript level was highly correlated with the pattern of accumulation of C_{30} apocarotenoids in mandarins and sweet orange during ripening (Ma et al. 2013b; Rodrigo et al. 2013b). The *CCD4b* gene encodes a novel enzyme activity cleaving the β,β-xanthophylls β-crytoxanthin and zeaxanthin to generate C_{30} apocarotenoids, which is induced during fruit ripening and promoted fruit colouration (Ma et al. 2013b; Rodrigo et al. 2013b).

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Chapter 7
Carotenoid Biosynthesis in *Daucus carota*

Kevin Simpson, Ariel Cerda, and Claudia Stange

Abstract Carrot (*Daucus carota*) is one of the most important vegetable cultivated worldwide and the main source of dietary provitamin A. Contrary to other plants, almost all carrot varieties accumulate massive amounts of carotenoids in the root, resulting in a wide variety of colors, including those with purple, yellow, white, red and orange roots. During the first weeks of development the root, grown in darkness, is thin and pale and devoid of carotenoids. At the second month, the thickening of the root and the accumulation of carotenoids begins, and it reaches its highest level at 3 months of development. This normal root thickening and carotenoid accumulation can be completely altered when roots are grown in light, in which chromoplasts differentiation is redirected to chloroplasts development in accordance with an altered carotenoid profile. Here we discuss the current evidence on the biosynthesis of carotenoid in carrot roots in response to environmental cues that has contributed to our understanding of the mechanism that regulates the accumulation of carotenoids, as well as the carotenogenic gene expression and root development in *D. carota*.

Keywords Carrot • β-carotene • Root development • Gene expression • Plastid development

7.1 Introduction

Carrot (*Daucus carota L.*) is a bienal plant that belongs to the botanic group Umbelliferae or Apiaceae. Within this diverse and complex family of plants we can find a large number of other vegetables, like fennel (*Foeniculum vulgare*), celery (*Apium graveolens*), parsley (*Petroselinum crispum*), cilantro (*Coriandrum sativum*) and dill (*Anethum graveolens*). *D. carota* varieties include domesticated cultivars of carrots commonly cultured for human consumption and wild varieties of carrot that are found throughout West Asia, Europe, Africa and America. These

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wild carrots are usually referred as subspecies or varieties of *D. carota*, and they generally grow close to the ocean. The primary root of these wild carrots is pale and small, has a bitter taste and a fibrous texture (Vaughan and Geissler 2009). Wild carrots and the early form of cultivated carrots were used as spices and medicinal herbs due to its aromatic seeds (Prohens-Tomás and Nuez 2007). On the other hand, the domesticated carrot has a bigger primary root, usually full of pigments, with a sweet taste and a crunchy texture. Although the carrot is a plant that grows in a predominantly temperate climate, it can be cultivated in tropical and subtropical regions, especially in high grounds. It is believed that the domesticated carrot first emerged in the Near East before the tenth century, in the region between Afghanistan and Turkey (Mackevic 1929). From that point, the carrot began to expand both west and east, reaching Asia minor in the tenth/eleventh century, Spain in the twelfth century, continental northwestern Europe in the fourteenth century, England in the fifteenth century, China in the sixteenth century and Japan in the seventeenth century (Vaughan and Geissler 2009). All the grown carrots in northwestern Europe were purple or yellow until the seventeenth century, and the yellow carrot was preferred over the purple one, because the last dyed the soup or food. The selective breeding of yellow carrots led to the appearance of the orange carrot in the seventeenth century in The Netherlands. This orange carrot would be the progenitor of the modern variety of currently grown carrot (International Plant Genetic Resources and Badra 1998; Vaughan and Geissler 2009). Thus, according to the pigmentation of the roots, the carrot can by subdivided in two groups: the eastern-type that includes the yellow and purple carrots and the western-type consisting of yellow, orange and red carrots (Heiwood et al. 1983).

Through a transcriptomic analysis of different carrot varieties, Rong et al. concluded that western carrots may have originated from the eastern ones and that domestication may have been offset by introgression from the wild carrot (Rong et al. 2014).

The consumption of carrots and its derived products has increased steadily in the last decades, principally due to the recognition of the carrot as an important source of natural antioxidants. This has had a direct impact in the production of carrots, becoming one of the most economically important grown vegetables worldwide, both in the production area and in the market value (FAOSTAT Database on Agriculture 2014). At the present, Asia produces approximately the 61.6 % of the total of carrots and turnips produced in the world, followed by Europe with a 22.6 % and America with a 9.4 % of the production (FAOSTAT Database on Agriculture 2014).

7.2 Nutritional Importance of Carrots

Although the storage root is the commonly consumed part of carrots, leaves can also be consumed. In China and Japan, the young leaves of carrots are occasionally used in salads or as a stir-fried herb (Rubatzky et al. 1999). The nutritional importance

of carrots lies in many characteristics, such as the nutritional content and the low caloric value, providing 41 kcal/100 g of raw carrot (U.S. Department of Agriculture 2014). Even though the content of nutrients in raw carrots can vary significantly between cultivars, during the processing and storage of carrots and even due to different methods of analysis, diverse studies have determined that the storage root of carrots is made up of approximately 86.0–88.8 % water, 6.0–10.6 % carbohydrates, 1.2–6.44 % fiber, 0.7–0.93 % protein and 0.2–0.5 % fat (Gopalan et al. 1989; Holland et al. 1991; Li et al. 2002; Da Silva et al. 2007; U.S. Department of Agriculture 2014). The carbohydrates present in carrot are almost exclusively simple sugars, predominantly sucrose, glucose, fructose and starch (Li et al. 2002; U.S. Department of Agriculture 2014). Cellulose and hemicellulose constitute the greatest portion of the total dietary fiber in fresh carrot (50 to 92 %) (Kochar and Sharma 1992; Marlett 1992). Specifically, the dietary fiber is composed of 80.94 % cellulose, 9.14 % hemicellulose and in a small amount of pectin (7.41 %) and lignin (2.84 %) (Nawirska and Kwaśniewska 2005).

Carrots are also a good source of important minerals for the human diet, like potassium, calcium, phosphorus, iron and magnesium. Although the content of minerals present in the storage root of carrots can vary depending on the cultivar, it has been reported that the most important mineral in carrot is potassium (Nicolle et al. 2004). The amount of calcium in the root of carrots seems not to be influenced by the cultivar, but the concentration of other minerals like iron or sodium varies widely among different cultivars.

However, the main nutritional importance of carrots in human diet relies on the large amount of phytochemicals present in the storage root, these include carotenoids, anthocyanins and other phenolic compounds (Arscott and Tanumihardjo 2010), which makes the carrot a functional food (Hager and Howard 2006; Hasler and Brown 2009). Moreover, different studies have proven that the carotenoids in the storage root of carrots are bioavailable for animals and humans (Brown et al. 1989; Muller et al. 1999; Horvitz et al. 2004; Tanumihardjo et al. 2009).

7.2.1 Carotenoid Composition in Diverse Carrot Varieties

At present diverse carrot varieties with different pigment compositions in the storage root exist. Carrot varieties currently grown in the world possess storage roots which vary from white, yellow, orange, to red and there are even varieties of carrot with purple storage roots (Surles et al. 2004; Arscott and Tanumihardjo 2010). The main reason for this diversity of colors is the ability of carrot to synthesize and accumulate a large amount of different types of carotenoid and antocyanins (only for purple varieties) in the root (Surles et al. 2004; Montilla et al. 2011). The amount of pigments in carrot is also affected by the growing season, soil type and different genetic factors (Heinonen 1990; Hart and Scott 1995; Rosenfeld et al. 1997; Nicolle et al. 2004; Grassmann et al. 2007). In addition, the profile and amount

of carotenoids in the storage root also vary dramatically during their development and between different growing conditions (Stange et al. 2008; Fuentes et al. 2012). Among the different varieties of carrot, the white cultivars only accumulate traces of lutein and other carotenoids. The color observed in the root of yellow cultivars is mainly due to the lutein accumulation, though they are also able to synthesize α- and β-carotene to a lesser extent. The red color in some cultivars is caused by the presence of large amounts of lycopene, ranging from 6000 to 10000 μg/100 g of fresh weight. The concentration of lycopene present in the red cultivars of carrot is even greater than those in tomatoes, one of the most common sources of lycopene (3000–5000 μg/100 g of fresh weight) (Dumas et al. 2003). Orange cultivars of carrot get their coloration due to the large amount of β-carotene and α-carotene, whereas they have little lutein and are unable to accumulate lycopene (Surles et al. 2004). The purple cultivars accumulate not only carotenoids, but they are the only variety able to accumulate anthocyanins, which give them their particular coloration (Alasalvar et al. 2001; Grassmann et al. 2007; Montilla et al. 2011). Within the purple varieties, some of them have a dark purple root, usually known as black carrots, and other varieties have a purple phloem but a white, yellow or orange xylem (Arscott et al. 2010).

The backbone of carotenoids can be cleaved by region-specific oxidative enzymes to form different compound. These are called norisoprenoids and in plants this oxidative cleavage is mainly catalyzed by carotenoid cleavage dioxygenases (see Chap. 11). Norisoprenoids contribute significantly to the aroma and flavor of many plants, including carrots (Auldridge et al. 2006; Walter et al. 2010; Walter and Strack 2011). Although the norisoprenoids biosynthetic pathway has been poorly studied in carrot, Yahyaa et al. (2013) found different norisoprenoids in orange and purple carrots, but not in the yellow and white varieties. Furthermore, the level of norisoprenoids derived from the cleavage of α- and β-carotene is more abundant in the purple and orange varieties, which correlates with the amount of β-carotene present in these cultivars. These results suggest that the carotenoid composition present in different carrot cultivars with different profiles of carotenoids accounts for the norisoprenoids content (Yahyaa et al. 2013).

7.3 Carrot Development and Carotenoid Accumulation

Carotenoid accumulation in plants is known to be the result of biosynthesis, degradation and storage (Cazzonelli and Pogson 2010). In contrast with other plants, almost all carrot cultivars can accumulate a large amount of pigments in their storage root. Under normal growth conditions (underground in darkness), the small root of a 4 week-old carrot is thin and pale with almost no carotenoids. After the first month of development the storage root begins to swell and to accumulate carotenoids, which is clearly visible at 8 weeks of growth (Fig. 7.1). A dramatic growth of the root and acceleration in carotenoid accumulation is observed generally at the 3th month of development, just before secondary growth is complete (Fig. 7.1) (Clotault et al. 2008; Stange et al. 2008; Fuentes et al. 2012).

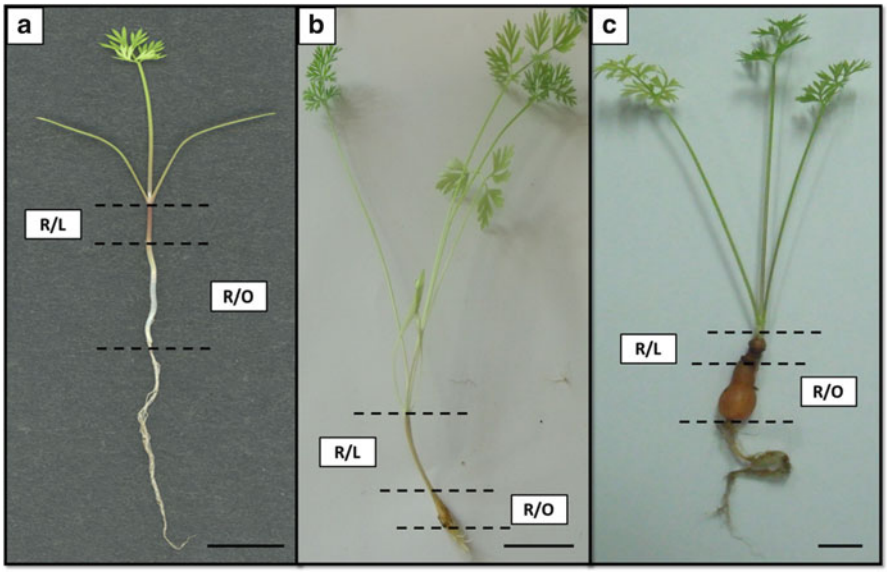

Fig. 7.1 Carrotroot development and pigmentation. (**a**) A 4 weeks old carrot plantlet, showing a pale and thin root, (**b**) A 8 weeks old carrot has a thin root that begins to swell and to accumulate carotenoids, (**c**) A 3 months old mature carrot reaches the highest carotenoid accumulation and root growth. *R/D*: Segment of the carrot root grown in darkness (normal growth conditions). *R/L*: Segment of the carrot root grown under photoperiod

The principal function of carotenoids in photosynthetic tissues is to contribute to light-harvesting during photosynthesis (see Chap. 3) and to protect the vegetal cell against photo-oxidative damage through the dissipation of any excess of light energy and detoxification of reactive oxygen species. Besides, carotenoids bring color to non-photosynthetic organs as flowers and fruits acting as attractants to pollinators and seed dispersal agents (Davison et al. 2002; Grotewold 2006; Lu and Li 2008).

Although all plastids can synthesize carotenoids, the amount of carotenoid produced can vary significantly, depending on the nature of the plastid and the tissue where they are generated. In carrots, the principal carotenoids that are accumulated in leaves are lutein, β-carotene and α-carotene (Fig. 7.2, Surles et al. 2004; Clotault et al. 2008; Fuentes et al. 2012). These pigments are present in the inner membranes of chloroplasts, usually directly associated to lipoproteins and membrane lipids to form part of the light-harvesting complex and the reaction center of Photosystem II. Due to the important function of carotenoids in photosynthetic organs, the amount of these pigments remains relatively constant during development, reaching around 400 μg/gdw in total carotenoids, 100 μg/gdw in β-carotene, 50 μg/gdw in α-carotene and 100 μg/gdw in lutein from 4 weeks to 12 weeks of culture (Fig. 7.2, Fuentes et al. 2012). However, the expression pattern of genes that codify for key enzymes involved in the synthesis of these compounds is not in a direct relationship with carotenoid abundance, perhaps due to post translational regulation of the enzymes (Fuentes et al. 2012).

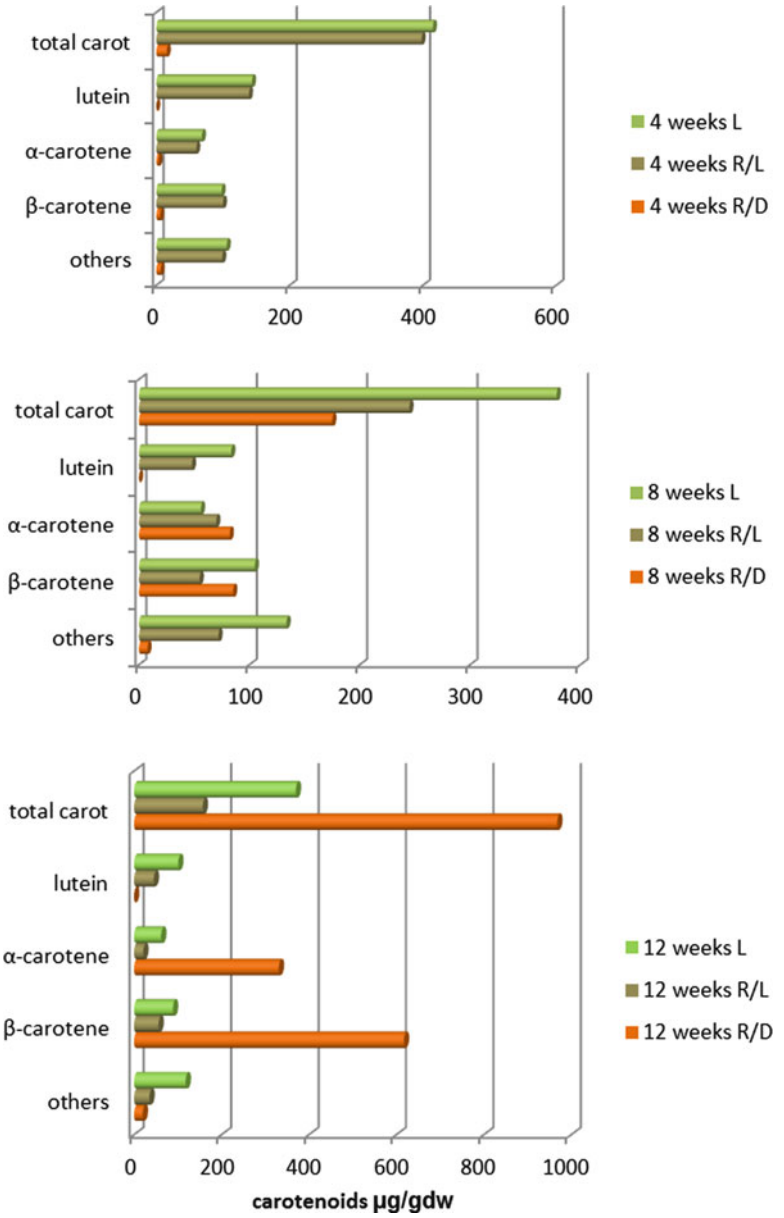

Fig. 7.2 Carotenoid profile in leaves and roots of carrots grown under normal development and exposed to light. Specific carotenoids were determined by HPLC in carrot roots and leaves of 4, 8 and 12 weeks-old plants. R/D: Roots grown in dark under normal development conditions. R/L: Roots grown under 16 h photoperiod treatment, L: Leaves. Carotenoid concentration are indicated in µg/g dry weight (µg/gdw)

However, carotenoids that accumulate in the storage root of carrots seem to occur as a result of the accumulation of mutations in genes related with the synthesis and storage of carotenoids during the process of carrot domestication. In the storage root of orange carrots, β-carotene and α-carotene are the most abundant carotenoids synthesized and are accumulated in large crystals inside chromoplasts (Baranska et al. 2006; Maass et al. 2009; Kim et al. 2010). Chromoplasts differentiate from proplastids during normal storage root development in the dark (Ben-Shaul and Shimon 1965). In the storage root, a direct relationship of carotenoid gene expression levels and carotenoid composition is observed (Fuentes et al. 2012). When the carrot storage root develops, an increase in total carotenoids, β-carotene and α-carotene is observed (Fig. 7.2), reaching 980 μg/gdw in total carotenoids, 600 μg/gdw of β-carotene and 320 ug/gdw of α-carotene at 12 weeks of culture. The increase in carotenoid content correlates with a significant increase in the expression of almost all carotenogenic genes, preferably *PSY*2, *ZDS2*, *PDS*, *LCYB1*, *LCYE, ZEP* and *NCED1* (Fig. 7.3a). In the carrot storage root grown in darkness, a direct correlation of carotenoid accumulation and gene transcription can be observed as carotenoid turnover is slower than in leaves, because they are protected from light and oxidative stress damage.

7.4 Gene Regulation During Carotenoid Biosynthesis in Carrot

Most knowledge about the enzyme coding genes of the carotenoid biosynthesis pathway in carrot comes from the gene isolation and sequencing from other plant genomes. The available information has allowed the design of primers for heterologous PCR experiments and full-length transcript sequences were obtained by RACE-PCR and submitted to the NCBI Genebank (Just et al. 2007a, b). However, to date no enzyme-coding sequences for ζ-carotene isomerase (ZISO) and neoxanthin synthase (NSY) have been described, while for violaxanthin epoxidase (VDE), carotene β-hydroxylase 3 (CHYB3) and carotenoid cleavage dioxygenases (CCD2/3) only partial sequences have been found (Rodriguez-Concepcion and Stange 2013). A recent work found that DXS1, one of the three carrot genes encoding DXS, was the only gene in the MEP pathway whose transcript levels positively correlated with the carotenoid content of carrot accessions displaying differentially pigmented roots (Iorizzo et al. 2016). Different studies, including metabolic control analysis, have shown that DXS is the main rate-determining enzyme of the MEP pathway (Wright et al. 2014; Rodriguez-Concepcion and Boronat 2015). We determined that a higher expression of DXS, but not DXR, in fact results in an enhanced accumulation of carotenoids in dark-grown storage roots, as well as an increment in carotenoids and chlorophylls in leaves of transgenic carrot plants (Simpson et al. 2016 under revision). The increased DXS activity produces an increment in the expression of PSY-encoding genes which might contribute to the

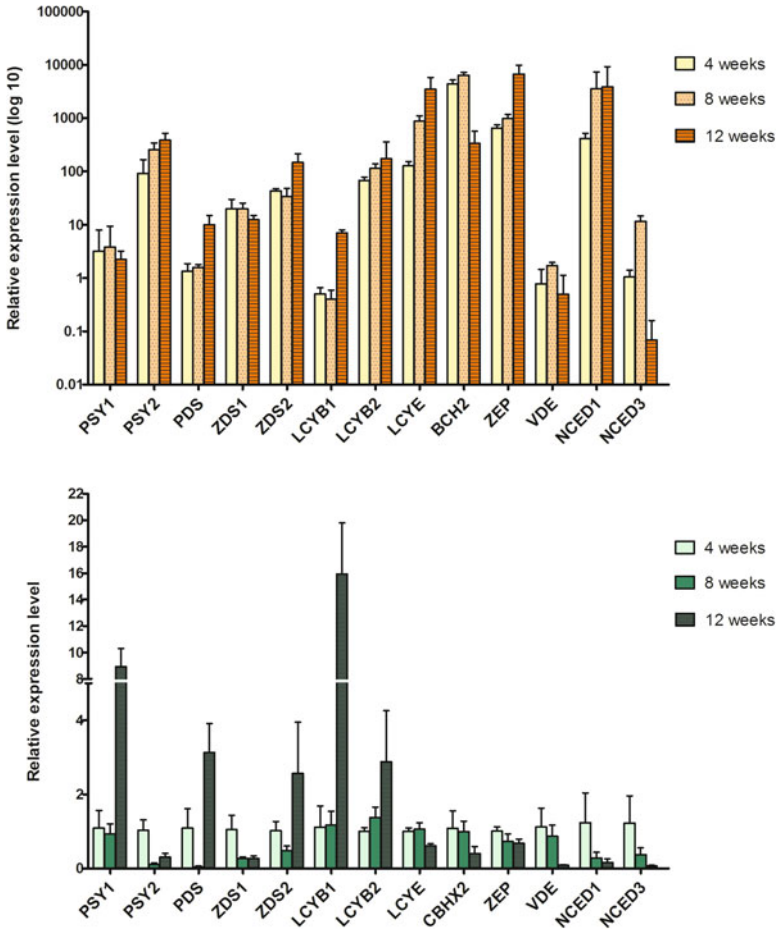

Fig. 7.3 Expression pattern of carotenogenic genes in leaves and roots during carrot development. Relative expression of *PSY1*, *PSY2*, *PDS*, *ZDS1*, *ZDS2*, *LCYB1*, *LCYB2*, *LCYE*, *CHXB*, *ZEP*, *VDE*, *NCED1* and *NCED3* genes that codify for enzymes acting in the synthesis of carotenoid and abscisic acid was measured in (**a**) roots and (**b**) leaves of 4, 8 and 12 weeks-old carrot plants. Ubiquitin was used as normalizer in qRT-PCR measurements.Columns and bars represent the means and SE (n = 6). In storage root, data were presented in log10 and for leaves, they were presented in linear scale

increased accumulation of carotenoids detected in these lines. This let us to propose that DXS has a central regulatory role for the production of MEP-derived precursors in carrot leaves and dark-grown roots (Simpson et al. 2016 under revision).

Consistent with an increase in total carotenoid content in the storage root, the expression of most genes, especially those involved in α- and β-carotene biosynthesis are induced during carrot development (Fig. 7.3a, Fuentes et al. 2012). Interestingly, *LCYE* and *LCYB1* genes, coding for enzymes directly involved in

the synthesis of α- and β-carotene, have an increased expression in the root of carrots, which is also accompanied by an increase in the expression of the *ZEP* gene. ZEP participates in the synthesis of violaxanthin, which is required as a precursor for ABA and the syntheses of other xanthophylls. The decreased expression of violaxanthin de-epoxidase (VDE), which can transform violaxanthin back into zeaxanthin, may support sufficient precursors for ABA, as neither zeaxanthin nor violaxanthin are accumulated in the storage root. In the case of leaves, a significant boost in the transcript level of *PSY1*, *PDS* and *LCYB1* is observed in mature carrot plants (Fig. 7.3b) in accordance to α-carotene, β-carotene and lutein accumulation (Fig. 7.2). Despite such correlations, the finding that not all genes are expressed in a direct relationship with the carotenoid accumulation in leaves suggests that there is an involvement of mechanisms such as post-transcriptional and/or post-translational regulation, among others (Ruiz-Sola and Rodriguez-Concepción 2014), that may regulate carotenoid biosynthesis and turnover. On the contrary, in the carrot storage root grown in darkness a mostly direct correlation of carotenoid accumulation and gene transcription can be observed as carotenoid turnover is slower that in leaves, because they are protected from light and oxidative stress damage. However, post-transcriptional and/or post-translational regulation in the carotenoid pathway may also be important in both R/L and R/D segments to explain their carotenoid profiles (Figs. 7.2 and 7.3a).

In carrot, at least 2 isoforms for *PSY*, *ZDS*,*LCYB* and *NCED* have been found (Just et al. 2007a, b; Rodriguez-Concepcion and Stange 2013), which have different expression levels in leaves and in the storage root during carrot development (Fuentes et al. 2012). In plants, the presence of more than one isoform of carotenogenic enzymes generally leads to the specialization of the paralogs, allowing tissue specific carotenoid biosynthesis (Ronen et al. 2000; Welsch et al. 2008). In the case of carrots, preferential carotenoid production and root development have been correlated with gene expression of particular paralog genes. The pattern of *PSY2*, and *LCYB2* expression suggest that these genes could mainly be involved in carotenoid biosynthesis in chromoplasts, while *PSY1* and *LCYB12* may contribute preferentially to the carotenoid production in chloroplasts (Fig. 7.3a, b, Fuentes et al. 2012). In addition, in wild white (Ws) and orange inbred lines, the expression of *PSY* correlates with carotenoid accumulation in roots but not in leaves, showing a higher expression for *PSY2* than *PSY1* (Wang et al. 2014). The production of phytoene from GGPP by PSY activity is limiting in carrot roots (Santos et al. 2005; Maass et al. 2009). Based on the described modest three-fold upregulation of *PSY2* transcripts in maturing storage carrot root, it is likely that transcript levels might not be the main determinant of PSY activity in carrot chromoplasts. Current studies on *PSY1* and *PSY2* genes indicate that both codify for functional enzymes. The expression of *PSY2* in a heterologous plant increases total carotenoid and chlorophyll levels and enhances salt stress tolerance (Acevedo et al. in preparation). This result is in agreement with those reported by Ruiz-Sola et al. where a *GFP* reporter protein directed by the *AtPSY* promoter was higher in the root after salt treatment and that *PSY* transcript expression correlated with an increase in ABA levels in response to salt stress in *A. thaliana* (Ruiz-Sola et al. 2014).

Even though the carotenoid accumulation during root development shows a correlation with the expression profile of *PSY2*, *PDS*, *ZDS2*, *LCYB1*, *LCYE, ZEP* and *NCED1* (Fig. 7.3a, Fuentes et al. 2012), the expression level is not always directly correlated with function and enzyme activity and some isoforms could also participate in carotenoid biosynthesis in both chloroplast and chromoplast enriched organs. In this respect, the over expression of *LCYB1* in carrots boost total carotenoids and β-carotene not only in leaves, but also in the storage root (Moreno et al. 2013) and the down regulation of this gene in carrots results in a decrease in carotenoid levels in both organs, suggesting that although its expression is higher in leaves, the enzyme is required for carotenoid synthesis in the whole plant (Moreno et al. 2013). The down expression of both *LCYB1* and *LCYB2* produces a dwarf phenotype with necrotic leaf damage (not published), demonstrating that both genes are required for normal plant development. Moreover, carrot *LCYB1* produces an enhancement in plant fitness when expressed in *Nicotiana tabacum* as determined by biomass, photosynthetic efficiency and carotenoid/chlorophyll composition through a positive feedback on the expression of the key carotenogenic genes, *NtPSY1, NtPSY2* and *NtLCYB*, as well as those involved in the synthesis of chlorophyll (*NtCHL*), gibberellin (*NtGA20ox*, *NtCPS* and *NtKS*) and isoprenoid precursors (*NtDXS2* and *NtGGPPS*), accompanied by an increment in carotenoids, chlorophyll and active gibberellins (GA4) in leaves (Moreno et al. 2016).

Leaves of orange carrots unusually accumulate high levels of α- and β-carotene, attributed to a defect in Carotene hydroxylase CYP97A3 (Arango et al. 2014). The expression of AthCYP97A3 reduces α-carotene and total carotenoids in leaves and the amount of PSY protein, which explains the reduced carotenoid accumulation (Arango et al. 2014).

In addition, a cDNA encoding a carotenoid cleavage dioxygenase, *DcCCD1*, was identified in carrots and its functionality was proven by over expression in *E. coli*, suggesting that this enzyme is involved in the biosynthesis of α- and β-ionone flavor molecules (Yahyaa et al. 2013). Experimental investigation of the metabolic flow in this pathway and the demonstration of the enzymatic activity of most of the enzymes are required to complement these evidences.

Even though functional studies have been limited in carrot, the advent of massive sequencing technologies and a web-based genomic and transcriptomic database for *D. carota* (CarrotDB) (Xu et al. 2014) as well as the de novo high quality genome assembly of a orange carrot (Iorizzo et al. 2016) will be useful in the identification and characterization of genes and metabolic pathways in *D. carota*.

7.4.1 Control Mechanisms of Carotenoid Synthesis in Carrots: Root as a Study Model

Because of its importance in plants and humans, the regulation of carotenoid biosynthesis has been studied extensively during the last 45 years, especially in

conventional plant models (Naik et al. 2003; Giorio et al. 2007; Farre et al. 2011). Although, the understanding how plant cells are able to regulate the synthesis and accumulation of carotenoids in plastids remains limited. It is becoming clear however, that the regulation of carotenoid biosynthesis is related to other processes in which these pigments are essential, indicating that carotenoid metabolism is probably regulated at multiple levels (Fraser and Bramley 2004; Ruiz-Sola and Rodriguez-Concepcion 2012). Plants have developed different mechanisms to control the synthesis and accumulation of carotenoids, allowing differential distribution of carotenoids throughout plant development and in different tissues (Lu and Li 2008). Thus, carotenoid biosynthesis regulation can occur

1. At the genetic level where different tissue-specific enzyme isoforms are present in the plant (Ronen et al. 2000; Li et al. 2008; Welsch et al. 2008)
2. By its sequestration and accumulation in different types of plastids (Deruere et al. 1994; Vishnevetsky et al. 1999; Li and Van Eck 2007)
3. At the post-translational level (Welsch et al. 2000)
4. At the epigenetic level (Cazzonelli et al. 2009)

However, one of the most important mechanisms is the regulation at the transcriptional level, in which the expression of genes directly involved in carotenoid synthesis occur in a spatiotemporal context (Cazzonelli and Pogson 2010). These mechanisms appear to be closely coordinated through the plant life cycle, by internal signals triggered through development as well as external environmental stimuli, in which light has a prominent role (Cazzonelli and Pogson 2010; Fuentes et al. 2012).

Despite the relevance of carotenoids, the understanding of carotenoid biosynthesis and accumulation in roots is still scarce. However, due to the ability to accumulate large amounts of carotenoids in their modified root, carrots have emerged as an interesting plant model to study the regulation of the accumulation of these pigments in roots. In carrots, early studies were focused on the understanding of the mechanisms that account for the high levels of pigmentation in modified roots through genetic approaches. These studies have provided information regarding the genomic context of candidate genes and major QTLs that explain the carotenoid content (and therefore the associated color), and contributed to an estimation of the number of genes, the limiting steps and the heritability for carotenoid production in storage roots (Santos and Simon 2006; Just et al. 2007; Just et al. 2009). In particular, the analysis of populations originating from the cross between wild white carrots and domesticated orange varieties have provided proof that the inheritance of β-carotene and total carotenoid content may be associated with at least to two major loci, *Y* and *Y*2, acquiring successive mutations during the 1000 years since the separation of the white carrots from orange ones, respectively (Just et al. 2009). These two major loci associated with the members of the carotenoid biosynthetic gene families of zeaxanthin epoxidase, carotene hydroxylase and carotenoid dioxygenase would have played an important role in the domestication of the carrot (Just et al. 2009). Recently, a fine mapping identified a 75kb region in the same chromosome of the Y gene in which the DCAR 032551 gene appears to be the only among eight that segregates with high carotenoid pigmentation.

This gene co-expresses with COP1 and HY5 suggesting that DCAR 032551 may be a regulatory gene involved in repressing photomorphogenesis (Iorizzo et al. 2016). Other genetic approaches suggest that upstream genes like *IPI, PDS* and *CRTISO* present more polymorphism than those located downstream of lycopene (Clotault et al. 2012). Additionally, pair wise HKA (Hudson-Kreitman-Aguade) test showed significant divergence of *PDS* with all other genes when compared those belonging to white and orange carrots, suggesting a positive selection of *PDS* in this species (Clotault et al. 2012). Recent studies performed by Jourdan et al. are in agreement with these findings (Jourdan et al. 2015). An association analysis with an unstructured population on *Daucus carota* L. revealed that *ZEP* and *PDS* are candidate genes involved in total carotenoid, β-carotene, phytoene and phytofluene content in carrot roots. Authors concluded that an impaired function of the zeaxanthin epoxydase enzyme may result in an accumulation of β-carotene (Jourdan et al. 2015). However, results reported by Arango et al. suggest that the lack of functionality of the carotenoid β-hydroxylase enzyme (CHYB) would be responsible for the high levels of α- and β-carotene present in the storage root of *D. carota* (Arango et al. 2014).

Interestingly, further studies confirmed that the expression of heterologous *PSY* was sufficient to increase the production of β-carotene and the total carotenoid levels in white carrots (Maass et al. 2009). These reports suggest that the accumulation of mutations in early genes of the carotenoid biosynthetic pathway through the domestication of the carrot could have led to an increase in the flux of precursors toward the synthesis of carotenoid, resulting in the high levels found today in the storage root of carrots.

The importance of transcriptional regulation on genes of the carotenoid biosynthesis pathway gives rise to different opportunities to analyze the different carrot varieties. To understand the molecular basis underlying the carotenoid accumulation in carrot roots, one of the first studies aimed to establish a correlation between the transcriptional regulation of carotenoid biosynthesis genes and the carotenoid accumulation during the carrot root development of white, yellow, orange, and red cultivars. This analysis showed that yellow and red cultivars have high level expression of *LCYE* and *ZDS* genes, respectively, which could explain the high level of lutein and lycopene found in these cultivars, respectively (Clotault et al. 2008). However, considering that almost all the carotenogenic genes analyzed are expressed in the white cultivar, which does not contain carotenoids, and that the high levels of carotenoids in orange varieties can hardly be explained by the moderate increase of carotenogenic gene expression during the development of the root, accumulation of lutein and lycopene in the yellow and red cultivars might only partially be explained by the transcriptional level of genes directing the carotenoid biosynthesis pathway, suggesting that other mechanisms are also relevant in determining the final carotenoid content (Clotault et al. 2008). One possible mechanism to explain this apparent incongruence is the existence of non-functional alleles, which is supported by the carotenoid accumulation observed in the white QAL carrot cultivar after a heterologous expression of the Arabidopsis *PSY* gene (Maass et al. 2009).

Other studies in the orange cultivar Nantaise have established that light can negatively regulate the development of carrot storage root in a reversible form, inhibiting the root thickening, preventing chromoplast development and repressing the expression of genes involved in the synthesis of carotenoids (Stange et al. 2008). The analysis of carotenoid content in the roots of carrots grown either in light or darkness showed that they have a completely different profile of carotenoid, with higher levels of carotenoids in those segments exposed to darkness (R/D) and a decreased level in illuminated root (R/L) sections (Fig. 7.2). Interestingly, R/L accumulate lutein, β-carotene and α-carotene in a similar amount than is seen in photosynthetic leaves. With some exceptions, a related pattern was observed in genes directly involved in carotenoid biosynthesis, particularly an induction in *LCYB1* and *LCYE* expression and a repression of *CHYB2* is correlated with α- and β-carotene content in later stages of carrot root development (Fuentes et al. 2012). The profile of carotenoids found in dark-grown and light-grown carrot root can be partially correlated with the expression level of carotenogenic genes and it appears that the identity of plastids is associated directly with the development of carrot root with the environmental signals playing a major role in the definition of carotenoid profile and content in *D. carota* (Fuentes et al. 2012). It has been described that the large accumulation of carotenoids in chromoplasts is due to their ability to sequester carotenoids in lipoprotein structures (Vishnevetsky et al. 1999; Egea et al. 2010). The differentiation of root proplastids to chloroplasts during the development of carrot would explain the carotenoid profile found in root sections exposed to light, which is comparable to chloroplasts containing organs, such as leaves (Fig. 7.2). Furthermore, carrot root segment grown in darkness, presents a direct correlation between the differentiation of chromoplast during secondary growth of the storage root and an increased levels of α-carotene, β-carotene and total carotenoids accumulated as crystals (Fuentes et al. 2012; Rodriguez-Concepcion and Stange 2013). Together, these results suggest the existence of a complex feedback mechanism that coordinates the expression of carotenogenic genes during development of the root in *D. carota*. This idea is supported by recent work that showed that the overexpression or silencing of *D. carota LCYB1* is able to induce or repress, respectively, the expression of key endogenous carotenogenic genes, mainly *PSY1*, *PSY2* and *LCYB2*, in leaves and storage roots of carrots (Moreno et al. 2013), as well as in tobacco (Moreno et al. 2016). These changes in gene expression were accompanied by a modulation in chlorophyll and total carotenoids content, suggesting that a change in the expression of carotenogenic genes like *LCYB1* not only alters the expression of genes in the same pathway, but also in pathways which are closely related to carotenoids (Moreno et al. 2013, 2016).

7.5 Projections and Future Research

All studies done to date suggest that the carotenoid profile in carrot roots is determined by environmental factors during development and the expression of carotenogenic genes, plastid differentiation and enzyme activity. Master genes

involved in photo morphogenesis inhibition in carrot avoiding the root to sense the absence of light is another hypothesis. However, the molecular factors involved in this control have not yet been identified. Given the key role of light in regulating carotenogenesis in carrots, the first step in the identification of molecular factors could be the use of high throughput technologies and analysis of the transcriptomic profile of carrot roots during development in the presence of light. At present, work on these experimental approaches is under way in our research group. Nevertheless, light does not only have a role in the expression of carotenogenic genes and plastid differentiation, it also affects the thickness and normal storage root development (Stange et al. 2008; Fuentes et al. 2012), suggesting that the level or activity of growth regulators (eg hormone) could also be modulated by light, as has been seen in other plant models (Egea et al. 2010; Klee and Giovannoni 2011). Abscisic acid, which is able to influence the number and volume of chromoplasts in tomato fruits and also regulates the expression of key genes in the carotenoid biosynthesis at the root of many plants (Li et al. 2008; Welsch et al. 2008; Arango et al. 2010; Meier et al. 2011), could be involved in a similar process in carrot. Moreover, auxin, usually involved in root development, is also an interesting candidate to be studied in *D. carota* and represents an attractive challenge for future studies.

As one of the most important sources of provitamin A in the human diet, *D. carota* has become a significant target for the development of genetically modified cultivars. Different studies have reported the generation of transgenic carrots resistant to commercial herbicides (Aviv et al. 2002; Chen and Punja 2002), with increased tolerance to salt (Kumar et al. 2004) or pathogens (Punja and Raharjo 1996; Melchers and Stuiver 2000; Chen and Punja 2002; Imani et al. 2006) and with increased calcium levels (Park et al. 2004). In 2008 Jayaraman and Punja reported the production of different types of ketocarotenoids in carrot plants expressing an algal β-carotene ketolase gene. These ketocarotenoids were absent in *wild type* carrots. Leaves of transgenic carrots accumulated up to 56 μg/g of fresh weight of total ketocarotenoids, with higher levels of β-carotene and lower levels of α-carotene and lutein. Although the photosynthetic capacity was not significantly altered, transgenic carrots grew better than the control plants when they were exposed to UV-B irradiation. Furthermore, leaf tissue of the β-carotene ketolase expressing carrots exposed to oxidative stress showed less injury and retained higher levels of chlorophylls (Jayaraj and Punja 2008).

The constitutive expression of carrot the *LCYB1* gene produced an increase of 1.6–1.8-fold in total carotenoids and 2.6–2.8-fold in β-carotene in the leaves of transgenic carrot lines compared to the control plants, whereas in the storage root of transgenic lines, total carotenoids and β-carotene levels increased 1.1–1.8-fold and 1.2–2-fold, respectively (Moreno et al. 2013).

Carrots are widely consumed because of its pleasant taste which is mainly due to the presence of volatile isoprenoids and sugars (Simon et al. 1980; Alasalvar et al. 2001), and although carotenoids have no major influence on this attribute, they contribute to root color, which is also a desirable character by consumers (Surles et al. 2004). The importance of carotenoids also lies in its important role as phytonutrients that contribute to human health (Fraser and Bramley 2004; Hey 2010). In particular,

the orange carrot varieties represent an important source of pro-vitamin A in the Western diet, so future studies focused on the identification of transcription factors and hormonal regulators of the synthesis of carotenoids in carrots could eventually provide additional tools to improve the visual characteristics and nutritional content of this edible plant.

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Chapter 8
Carotenoids in Microalgae

Vitalia Henríquez, Carolina Escobar, Janeth Galarza, and Javier Gimpel

Abstract Carotenoids are a class of isoprenoids synthesized by all photosynthetic organisms as well as by some non-photosynthetic bacteria and fungi with broad applications in food, feed and cosmetics, and also in the nutraceutical and pharmaceutical industries. Microalgae represent an important source of high-value products, which include carotenoids, among others. Carotenoids play key roles in light harvesting and energy transfer during photosynthesis and in the protection of the photosynthetic apparatus against photooxidative damage. Carotenoids are generally divided into carotenes and xanthophyls, but accumulation in microalgae can also be classified as primary (essential for survival) and secondary (by exposure to specific stimuli).

In this chapter, we outline the high value carotenoids produced by commercially important microalgae, their production pathways, the improved production rates that can be achieved by genetic engineering as well as their biotechnological applications.

Keywords *Haematococcus pluvialis* • *Dunaliella salina* • Algae • Metabolic engineering • Cetocarotenoids • Astaxanthin

8.1 Introduction

Microscopic algae are thought to be among the oldest forms of life on earth. Microalgae comprise one of the most diverse kinds of plant life and they are the world largest group of photosynthetic organism which is capable of growing using CO_2 as the sole carbon source and light as energy (Gong et al. 2011; Leu and Boussiba 2014).

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Microalgae occupy the bottom of the food chain in aquatic ecosystems (Guedes et al. 2011). They contribute to approximately 50 % of the global photosynthetic activity, despite the fact that their photosynthetic biomass represents only about 0.2 % of that available on land (Parker et al. 2008). Microalgae are ubiquitous in nature. They have colonized nearly all of Earth's existing ecosystems, from the polar regions to deserts and hot springs (Cadoret et al. 2008). The estimated number of microalgae species that are known to exist ranges between 40,000 and 60,000, but estimations of the number of undescribed species range from hundreds of thousands to millions of species spread across the globe (Cadoret et al. 2012). Only a few thousands strains are kept in collections, a few hundred are investigated for chemical content and only a handful are currently of commercial significance (Olaizola 2003).

Microalgae have attained particular attention in recent years since they are an enormous biological resource, representing one of the most promising sources for new products and applications for commercialization (Pulz and Gross 2004). The application of microalgal biomass and/or its metabolites is an interesting and innovative approach for the development of healthier food products (Batista et al. 2011). Furthermore, some species of microalgae such as *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus*, and *Schizochytrium* have been classified as food sources, falling into the GRAS (Generally Regarded as Safe) category by the U.S. Food and Drug Administration (Chacón-Lee and González-Mariño 2010). The potential of microalgal photosynthesis for the production of high-value compounds or for energetic use is well recognized because of their more efficient utilization of sunlight energy when compared with higher plants. Currently, algae are the main sustainable source of commercial carotenoids for the aquatic food chain (Dufosse et al. 2005; Borowitzka 2013). Microalgae are extremely efficient solar energy converters and they can generate a great variety of metabolites that can be harnessed for commercial use (Gong et al. 2011). Many microalgal species can naturally synthesize a wide range of metabolites, such as proteins, lipids, carbohydrates, carotenoids or vitamins for health, food and feed additives, cosmetics, pharmaceutical industries and for energy production (Chacón-Lee and González-Mariño 2010; Priyadarshani and Biswajit 2012).

Undoubtedly, carotenoids have received increased attention during the last decade due to their intrinsic antioxidant activity and potential function in preventing adverse health conditions in humans. Carotenoids are accessory pigments in the photosynthetic apparatus with a primary role in light harvesting. Carotenoids are accumulated up to 8–14 % of biomass in microalgae and are divided into carotenes, which are true hydrocarbons, and xanthophylls, which also contain oxygen atoms (Priyadarshani et al. 2012; Mulders et al. 2014). Carotenoids accumulated in microalgae can be classified as primary and secondary carotenoids. Primary carotenoids are essential for survival, acting as structural and functional components of the cellular photosynthetic apparatus. The secondary carotenes are only accumulated after exposure to specific environmental stimuli via carotenogenesis (Guedes et al. 2011). The carotenes include β-carotene and lycopene and the xanthophyll carotenoids include lutein and astaxanthin, among others (Fassett and Coombes 2011). In most green algae, carotenoids are synthesized within plastids

and accumulate therein only. But in other microalgae, such as *Haematococcus pluvialis sp*., the secondary xanthophyll astaxanthin accumulates in lipid vesicles in the cytoplasm (Guedes et al. 2011). The most important source of carotenoids belongs to the Chlorophyceae family, which includes *Chlorella*, *Chlamydomonas*, *Dunaliella*, *Muriellopsis* and *Haematococcus* spp. (Pulz and Gross 2004). They have the biggest diversity of pigments and are the only group that overproduces secondary carotenoids in response to adverse growth conditions, when they have photoprotective roles (Mulders et al. 2014). Only a few microalgae strains are currently being commercially exploited for the production of β-carotene, astaxanthin or lutein, but research is seeking to optimize the production of these compounds as well as other commercially important carotenoids. Natural sources of these compounds studied to date are mainly achieved by *Dunaliella salina* (orange pigment β-carotene), *Haematococcus pluvialis* (red pigment astaxanthin) and *Scenedesmus sp.* and *Chlorella sp*. (lutein pigment). Additionally, some species of microalgae can be efficiently transformed, which makes it possible to enhance the productivity of natural compounds through metabolic engineering (Leon et al. 2007; Cordero et al. 2011). This chapter focuses on the high value carotenoids produced by these commercially important microalgae, their production pathways, improvement of production by genetic engineering and the biotechnological applications of these compounds.

8.2 Carotenogenesis Pathways Among Algae

The large groups of secondary metabolites called carotenoids are synthesized within the chloroplast but are catalyzed by enzymes encoded by nuclear genes. These enzymes are synthesized as precursor polypeptides with a transit peptide at the amino-terminus that targets them to the chloroplast (Leon et al. 2007). The most abundant naturally occurring carotenoids are hydrophobic tetraterpenoids that contain a C40 methyl branched hydrocarbon backbone. The polyene chains of carotenoids, consisting of conjugated double bonds, are responsible for the pigmentation of carotenoids and their ability to absorb photons in visible wavelengths (Baroli and Niyogi 2000; Leon et al. 2007). Carotenoids are synthesized by all photosynthetic organisms as well as by many non-photosynthetic bacteria and fungi. There are two main classes of naturally occurring carotenoids: carotenes, which are hydrocarbons that are either linear or cyclized at one or both ends of the molecule, such as β-carotene, and xanthophylls, that are oxygenated derivatives of carotenes such as astaxanthin (Fig. 8.1). All xanthophylls produced by higher plants, for example violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein, are also synthesized by green microalgae. However, in contrast to land plants, specific green algae species possess additional xanthophylls such as loroxanthin (Baroli and Niyogi 2000), astaxanthin and canthaxanthin (Grünewald et al. 2001). In addition, diatoxanthin, diadinoxanthin, and fucoxanthin are produced by brown algae or diatoms (Lohr and Wilhelm 1999; Lohr and Wilhelm 2001).

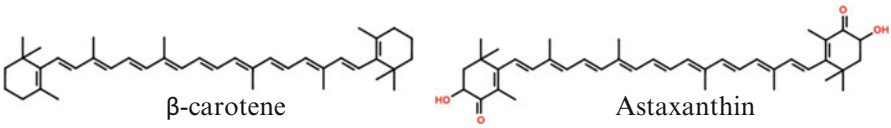

Fig. 8.1 Chemical structures of β-carotene and astaxanthin found in microalgae

Carotenoids play major roles in oxygenic photosynthesis, where they function in light harvesting and protect the photosynthetic apparatus from excess light by energy dissipation (Frank and Cogdell 1996; Baroli and Niyogi 2000; Telfer 2005; Leon et al. 2007). Carotenoids that fulfill these processes are commonly referred as primary carotenoids and are structural and functional components of the photosynthetic apparatus of the cell and therefore essential for cellular survival. In contrast, secondary carotenoids are defined functionally as carotenoids that are not obligatory for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. Nevertheless, in some green algae, secondary carotenoids are accumulated outside the plastid in cytoplasmic lipid vesicles (Krishna and Mohanty 1998; Lemoine and Schoefs 2010) in large quantities as a response to stresses (carotenogenesis) like nutrient starvation, high salinity, high light, etc. (Lemoine et al. 2008; Moulin et al. 2010).

Actually, a large number of green microalgae and their relatives display the capacity to accumulate secondary carotenoids under stress (Lemoine and Schoefs 2010), despite this, only algae, such as *Haematococcus pluvialis* (Camacho et al. 2013; Nunes et al. 2013; Chekanov et al. 2014; Giannelli et al. 2015), *Chlorella zofingiensis* (Wang et al. 2009; Li et al. 2009; Liu et al. 2014), *Chlorella photothecoides* (Wei et al. 2008; Li et al. 2013). *Dunaliella salina* (Coesel et al. 2008a; Chen et al. 2011; Prieto et al. 2011) and *Scenedesmus almeriensis* (Cerón et al. 2008; Sanchez et al. 2008; Macias-Sanchez et al. 2010) have been extensively studied. This is because they are used in the commercial production of astaxanthin and/or β-carotene and lutein in medium- and large scale cultures (for reviews, see García-González et al. 2005; Sun et al. 2014; Lamers et al. 2008; Ye et al. 2008; Guedes et al. 2011; Lopez et al. 2013; Han et al. 2013; Leu and Boussiba 2014; Ranga Rao et al. 2014; Wan et al. 2014; Hong et al. 2015).

Recently, many genes responsible for carotenoid production, particularly related to astaxanthin biosynthesis, have been cloned and characterized in *H. pluvialis* (Cui et al. 2012; Gao et al. 2012; Kathiresan et al. 2015), *Chlorella zofingiensis* (Huang et al. 2008; Cordero et al. 2010; Liu et al. 2010, 2014) and *Chlamydomonas reinhardtii* (Cordero et al. 2011; Couso et al. 2011; Liu et al. 2013; Zheng et al. 2014). This has provided the opportunity to study the pathways and regulation of carotenoid biosynthesis and to further understand the biological role of astaxanthin in the stress response.

Isopentenyl pyrophosphate (IPP) is the precursor for carotenoid synthesis (Lichtenthaler 1999). Two distinct pathways for IPP biosynthesis have been found in higher plants: the mevalonate pathway in the cytosol and the non-mevalonate

1-deoxy-D-xylulose-5-phosphate pathway in the chloroplast (DOXP pathway or MEP pathway) (Lichtenthaler et al. 1997). In unicellular green microalgae such as *H. pluvialis* and *Chlamydomonas reinhardtii*, IPP is thought to be synthesized solely from the non-mevalonate DOXP pathway (Disch et al. 1998). Subsequently, the isopentenyl pyrophosphate isomerase (IPI) catalyzes the isomerization of IPP to dimethylallyl diphosphate (Lichtenthaler 1999; Rohdich et al. 2003; Rohmer 2007). In the unicellular green microalgae *H. pluvialis* and *Chlamydomonas reinhardtii*, IPP and its allylic isomer dimethylallylpyrophosphate (DMAPP) are synthesized along the non-mevalonate pathway in plastids (Disch et al. 1998; Ladygin 2000; Rohmer 2010). Two cDNAs of IPI, *IPI1* and *IPI2* have been cloned and characterized in *H. pluvialis* (Sun et al. 1998). Transcripts of both *IPI* genes increased in response to oxidative stress, but only *IPI2* was up-regulated at the translational level. Moreover, only the IPI2 protein was detected in the mature red cysts in which astaxanthin was accumulated, suggesting that *IPI2* is responsible for synthesis of the secondary carotenoids, whereas the *IPI1* is responsible for primary carotenoid synthesis in the chloroplast of *H. pluvialis* (Sun et al. 1998).

Phytoene synthase (PSY) catalyzes the first committed step for carotenoid biosynthesis through condensation of two 20-carbon geranylgeranyl pyrophosphate (GGPP) molecules to form a 40-carbon phytoene, the precursor for all other carotenoids (Cunningham and Gantt 1998). Two classes of PSYs were found in certain green algae like *Ostrecoccus* and *Micromonas*, while some other green algae like *C. reinhardtii* and *C. vulgaris* only possess one class of PSYs (Tran et al. 2009). One copy of the *PSY* gene has been cloned and characterized from a number of microorganisms including *H. pluvialis* (Steinbrenner and Linden 2001) and *C. zofingiensis* (Cordero et al. 2011).

As shown in Fig. 8.2, the successive desaturation reactions of phytoene synthase (PSY) are catalyzed by two structurally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), converting the colorless phytoene into red lycopene. Specifically, PDS catalyzes the first two dehydrogenation reactions to form phytofluene and ζ-carotene, whereas ZDS catalyzes two further reactions converting ζ-carotene to neurosporene and lycopene (Cunningham and Gantt 1998). These dehydrogenation reactions extend the conjugated carbon-carbon double bonds to form the chromophore of carotenoids (Lemoine and Schoefs 2010).

These FAD-containing enzymes require PTOX (Plastid Terminal Oxidase) and plastoquinone (PQ) as electron acceptors (Wu et al. 1999; Houille-Vernes et al. 2011). One *pds* and two *ptox* genes (i.e., *ptox1* and *ptox2*) have been cloned and characterized in *H. pluvialis* (Grünewald and Hagen 2000; Wang et al. 2009; Li et al. 2010). High light illumination and nitrogen deprivation increase the number of transcripts of *PDS* and *PTOX* simultaneously in *H. pluvialis*, suggesting that PDS and PTOX may act in concert to dehydrogenate phytoene and remove excess electrons under stress, thereby preventing over-reduction of the photosynthetic electron transport chain and the formation of excess ROS (Reactive Oxygen Species) (Grünewald and Hagen 2000; Wang et al. 2009; Li et al. 2010).

The cyclization of lycopene catalyzed by lycopene β-cyclase (LCY-b) and lycopene ε-cyclase (LCY-e) is a branching point in carotenoid biosynthesis

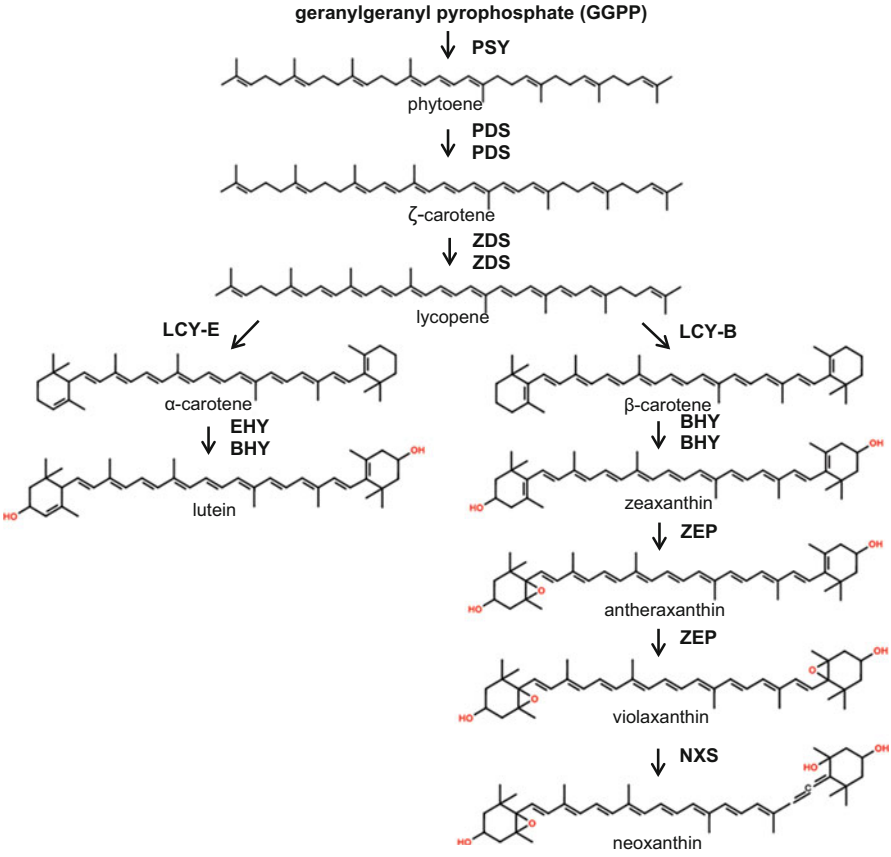

Fig. 8.2 A hypothetical pathway for carotenogenesis in microalgae. Enzymes for the relative conversions are in *bold*. *PSY* Phytoene Synthase, *PDS* Phytoene Desaturase, *ZDS* ζ-carotene Desaturase, *LYC-E* Lycopene ε-cyclase, *LYC-B* lycopene β-cyclase, *EHY* ε-carotene Hydroxylase, *BHY* β-carotene Hydroxylase, *ZEP* Zeaxanthin Epoxydase, *NXS* Neoxanthin Synthase (Modified from Ye et al. 2008)

(Cunningham and Gantt 1998). Lycopene is cyclized on both ends by the enzyme lycopene β-cyclase (LCY-b) to form β-carotene. The two beta rings of β-carotene are subjected to identical hydroxylation reactions to yield zeaxanthin, which in turn is epoxidated once to form antheraxanthin and twice to form violaxanthin. Neoxanthin is derived from violaxanthin by an additional rearrangement (Hirschberg 2001; Naik et al. 2003; Ye et al. 2008; Sandmann 2009). Higher plants and green algae have additional carotenoids, α-carotene derivatives (β, ε-carotene), which are also derived from lycopene by the action of LCY-b and LCY-e, for example hydroxylation of the β-ring and ε-ring of α-carotene forms lutein (Pogson et al. 1996, 1998). During xanthophyll formation (Fig. 8.2) the carotenoid structures are modified such that the end product pigments are often species-specific (Grossman et al. 2004; Sandmann

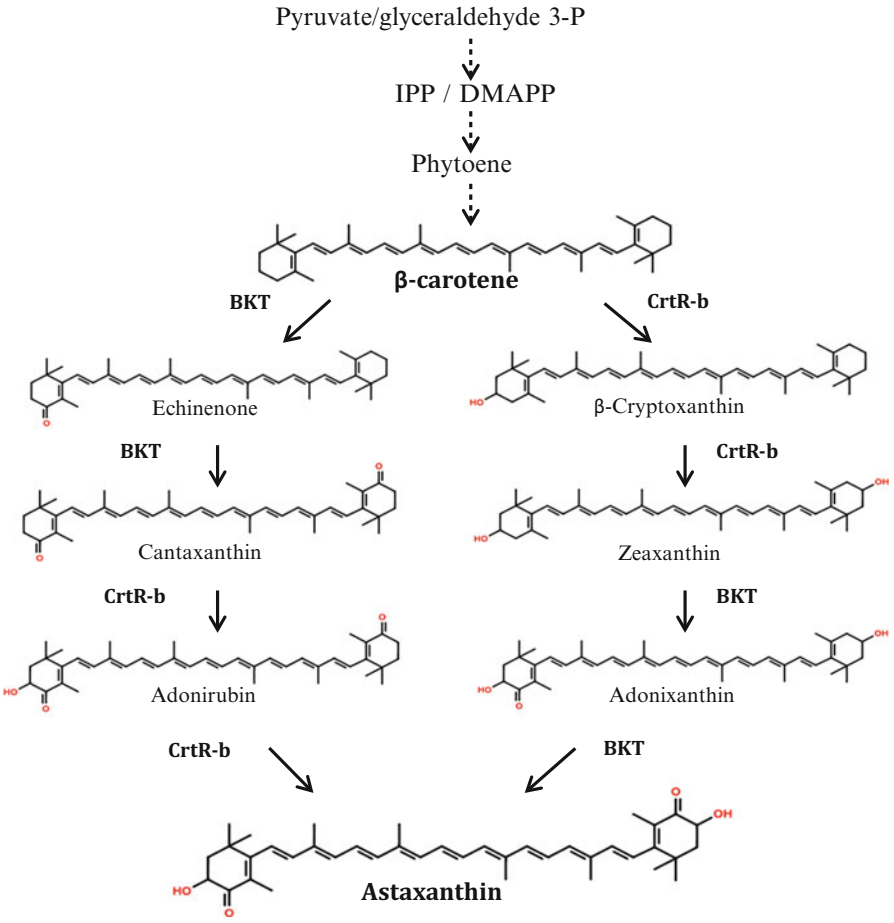

Fig. 8.3 *H. pluvialis* pathway of secondary carotenoid synthesis. The enzymes catalyzing the late enzymatic steps, namely β-carotene oxygenase (*CRTO, β*-carotene ketolase, BKT) and the β-carotene hydroxylase (*CrtR-B*) are indicated (Modified from Grünewald et al. 2001)

et al. 2006; Giuliano et al. 2008; Vidhyavathi et al. 2008; Ye et al. 2008; Lemoine and Schoefs 2010; Couso et al. 2011; Camacho et al. 2013). In *Haematococcus*, astaxanthin accumulation occurs in extra-plastidic lipid globules as a secondary carotenoid (Grünewald et al. 2001). Figure 8.3 shown the formation of astaxanthin at the expense of β-carotene, which requires the introduction of two hydroxyl groups at C3 and C3′ by the β-carotene hydroxylase, CrtR-b (synonymous Chy/CrtZ) gene product (3,3′-hydroxylase) and two keto groups at C4 and C4′ by β-carotene oxigenase, the CrtO gene product (synonymous bkt) (4,4′-ketolase), (Grünewald et al. 2001; Vidhyavathi et al. 2008; Lemoine and Schoefs 2010).

In contrast to *H. pluvialis*, *C. zofingiensis* may synthesize astaxanthin through the zeaxanthin pathway (Huang et al. 2006; Li et al. 2008). This xanthophyll is formed

through the catalytic action of violaxanthin deepoxidation (Moulin et al. 2010). Recently, the genes involved in the biosynthesis of astaxanthin in this species have been cloned and characterized, including *PSY* (Cordero et al. 2011), *PDS* (Huang et al. 2008), *LCY-b* (Cordero et al. 2010), *LCY-e* (Cordero et al. 2012), *BKT* (*CrtO*) (Huang et al. 2006), *CrtR-b* (*Chy-b*) (Li et al. 2008). Under high light conditions, the *PSY*, *PDS*, *BKT*, *CrtR-b* genes were up-regulated, whereas the mRNA levels of *LCY-b* and *LCY-e* remained constant, leading to formation of secondary carotenoids (Li et al. 2009; Cordero et al. 2012). Functional analysis of *C. zofingiensis* BKT demonstrated that this enzyme did not only convert β-carotene to canthaxanthin via echinenone, but it also exhibited high enzymatic activity in converting zeaxanthin to astaxanthin via adonixanthin (Huang et al. 2006).

Another carotenoid in high demand around the world is β-carotene (Ye et al. 2008). Some species of *Dunaliella*, especially *D. salina* and *D. bardawil*, have the ability to accumulate large amounts of carotenoids when exposed to specific extreme environmental conditions, such as high light intensity, high salinity, extreme temperatures and/or nutrient deprivation. *D. salina* can accumulate up to ∼10 % of dry algal biomass as β-carotene (García-González et al. 2005; Lamers et al. 2008).

Although the biochemistry and physiology of *Dunaliella* has been well investigated, molecular elucidation of the carotenogenic pathway of *Dunaliella* has only been conducted in recent years, and only some of the carotenogenic enzymes have been isolated (Yan et al. 2005; Sun et al. 2008a, b; Ramos et al. 2009). The carotenogenic pathway of *Dunaliella* is in accordance with that of higher plants (Ye and Jiang 2010). The first two enzymes specifically used in the carotenoid biosynthetic pathway are PSY and PDS, which together convert two geranylgeranyl diphosphate molecules into phytoene and ζ-carotene, respectively. These genes (*psy* and *pds*) are under transcriptional control in response to environmental stimuli and are considered to play a key role in the regulation of carotenogenesis (Rabbani et al. 1998; Sanchez-Estudillo et al. 2006; Coesel et al. 2008a, b). The pathway of carotenogenesis from geranylgeranyl pyrophosphate (GGPP) to β-carotene in *Dunaliella* is shown in Fig. 8.2.

Several microalgae have been proposed as potential producers of lutein, such as *Chlorella protothecoides* (Shi et al. 2002; Li et al. 2013) and *Scenedesmus almeriensis* (Sanchez et al. 2007, 2008). Lutein is one of the main photosynthetic pigments in the xanthophyllic family, and contains a large conjugated carbon system attached with hydroxyl or carbonyl groups (Ho et al. 2014). Lutein biosynthesis follows the general pathway of carotenoids, with PDS being the key enzyme involved in biosynthesis (Li et al. 2013). As shown in Fig. 8.2, in one branch the concentrated action of β- and ε-cyclases results in the formation of α-carotene, hydroxylation of which leads to the formation of lutein (Pogson et al. 1996, 1998; Li et al. 2013).

The most important factors that affect lutein content in microalgae are temperature, light, pH, the availability and source of nitrogen, salinity (or ionic strength) and the presence of oxidizing substances (or redox potential); however, specific growth rate also plays a crucial role in the biosynthesis of lutein (Wei et al. 2008; Guedes et al. 2011; Campenni et al. 2013) .

8.3 Metabolic Engineering of Carotenoid Biosynthesis in Eukaryotic Microalgae

Despite the high value of carotenoids and the advantages of microalgal platforms, there are few efforts towards the optimization of carotenoid production through metabolic engineering of these organisms. Since most of the carotenogenic pathway occurs in the chloroplast of green algae, two genetic strategies can be undertaken for this purpose. Expression of inserted genes from the chloroplast genome ensures that the proteins will be localized in the right place, and it is generally regarded as the best strategy for high levels of protein accumulation (Barrera et al. 2014). On the other hand, nuclear expression requires that the proteins should be translocated to the chloroplast (as happens for all the endogenous carotenogenic enzymes). Expression from the nucleus is usually associated with lower expression levels due to silencing and position effects, but it is the only location where one can express proteins that require eukaryotic post-translational modifications for their function (Kempinski et al. 2015).

Chlamydomonas reinhardtii has served as the main model organism for studying the effects of genetic engineering in carotenoid accumulation. In the first report of this type, Fukusaki et al. (2003) successfully expressed an archeal gene for a heat-stable version of the geranylgeranyl-pyrophosphate (GGPP) synthase enzyme involved in the early steps of carotenoid biosynthesis in the chloroplast of *C. reinhardtii*. Unfortunately, there were no measurable effects on the isoprenoid profile of the algae. Three years later, another group attempted to produce keto-carotenoids (e.g. astaxanthin) in *C. reinhardtii* by nuclear overexpression of the β-carotene ketolases from *H. pluvialis* (*bkt3*) and *C. reinhardtii* itself (*CRBKT*). Following several efforts using different expression vectors, no keto-carotenoids could be detected (Wong 2006). In parallel, Leon et al. (2007) used an analogous approach, but using the *bkt1* gene from *H. pluvialis* instead. In this case a small peak of 4-keto-lutein could be detected, which was not present in the parental strain. Unfortunately, no peak for astaxanthin was seen. RNA interference technology has also been used for altering the carotenoid profile of *C. reinhardtii*. By targeting the *pds* gene coding for the enzyme responsible for second step of carotenoid biosynthesis, its mRNA was reduced up to 93 % percent. Nonetheless, the carotenoid content of the algae did not change significantly, pointing towards the existence of additional rate-limiting processes (Vila et al. 2008). Additionally, the *psy* gene, which encodes the first step enzyme for carotenoid synthesis, has been transformed into the *C. reinhardtii* nucleus, causing an increase in carotenoid accumulation. Transformed strains overexpressing *psy* from *Dunaliella salina* and *Chlorella zofingiensis* stored 2.6 and 2.2-fold more lutein than the wild-type algae, respectively (Cordero et al. 2011; Couso et al. 2011). Recently, the *C. reinhardtii* nucleus has been transformed with a point mutant version of its endogenous *pds* gene. The mutant enzyme had a 27 % increase in its desaturase activity *in vitro*. The algae became resistant to the herbicide norflurazon and accumulated more lutein, β-carotene, zeaxanthin, and violaxanthin *in vivo* (Liu et al. 2013).

H. pluvialis, *D. salina* and *Chlorella* are also highly desirable candidates for carotenoid metabolic engineering given their commercial relevance. In the first attempt of stable nuclear transformation, *H. pluvialis* was engineered with a mutated *pds* gene that conferred resistance to norflurazon. Transgenic strains accumulated up to 26 % more astaxanthin than the *wild-type* control after 48 h of high light induction (Steinbrenner and Sandmann 2006). RNA interference constructs have been used to reduce the mRNA accumulation of the *D. salina pds* gene by up to 72 %. Intriguingly, the carotenoid content of these strains was not reported (Sun et al. 2008a, b). The most recent metabolic engineering effort involves the development of a nuclear transformation method for *Chlorella zofingiensis*. A mutant version of the endogenous *pds* gene was transformed, conferring resistance to norflurazon. The mutant PDS enzyme had a 33 % higher *in vitro* desaturation activity. Transformed *C. zofingiensis* strains accumulated up to 32.1 % more total carotenoids and 54.1 % more *in vivo* astaxanthin (Liu et al. 2014).

Finally, it is worth mentioning that the efforts related to carotenoid metabolic engineering in eukaryotic microalgae will probably increase in the short-term, given the great amount of newly sequenced genomes, and the increasing efforts to develop efficient and stable transformation techniques for commercially relevant strains. Furthermore, terpenoids from microalgae are now also regarded as a highly desirable feedstock, as a basis for biofuels and for the production of specialty chemicals, which will further broaden the interest for research towards optimizing the production of these compounds (Davies et al. 2014; Heider et al. 2014).

8.4 Biological Functions and Applications

Nowadays, there is a growing interest in the production of secondary carotenoids by microalgae, because they are molecules of high commercial value, specifically for the pharmaceutical industry and nutritional applications. These compounds have pigmentation properties, which have extensive application in the food and feed industry (Lorenz and Cysewski 2000). They play key role in high-grade animal nutrition, from aquaculture to farm animals (García-Chavarría and Lara-Flores 2013) as well as providing protection due to their antioxidant activity in many organisms, including in human health (Guerin et al. 2003). The powerful anti-oxidative properties make carotenoids an important class of nutrients in health promotion. In aquaculture and animal farming they have positive effects on adequate growth and reproduction of commercially valuable species (Del Campo et al. 2007). Appropriate levels of intake prevent or delay chronic diseases in humans (Onogi et al. 1998; Ciccone et al. 2013; Fernández-Sevilla et al. 2010). The carotenoid market has grown exponentially in the last few years and this trend is projected to continue. According to the update of the BCC Research report FOD025C, The Global Market for Carotenoids published in 2008 (http://www.reportlinker.com), the worldwide market value of the commercial application of carotenoids was estimated at nearly $1.2 billion in 2010, with a chance to grow to $1.4 billion in 2018 with a compound

annual growth rate of 2.3 %. Growth of this market has been led by Europe followed by North America. The U.S. carotenoid market not only dominates North America but also the global market, but the Asia-Pacific region is projected to be the fastest-growing carotenoid market for the period under consideration (Carotenoids Market by Type, Source, Application, & by Region - Global Trends & Forecasts to 2019, http://www.reportlinker.com). The major carotenoids with commercial interest are β-carotene followed by the xanthophylls - astaxanthin and lutein. The β-carotene from *Dunaliella salina* was the first carotenoid from algae to be commercialized. *Dunaliella salin*a is a unicellular, bi-flagellate and naked green alga with no cell wall. This halotolerant microalga is the richest natural source of the carotenoid β-carotene when exposed to stress conditions such as high light intensity or nutrient starvation (over 10 %) (Emeish 2012). Like all other carotenoids, β-carotene is an antioxidant, protects the body from damaging free radicals and is a source of photosynthetic dye pigments (Oren 2005). β-Carotene is a provitamin that is converted to vitamin A, which is needed to form rhodopsin in the outer segment of eye rod cells. Vitamin A is required for good vision and eye health, for a strong immune system, and for healthy skin and mucous membranes (Perusek and Maeda 2013). β-Carotene is also used as a food coloring (the yellow color in margarine), as a food additive to enhance the color of the flesh of fish and the yolk of eggs, and to improve the health and fertility of grain-fed cattle (Borowitzka and Borowitzka 1987). Research studies by the National Cancer Institute have shown that β-carotene is anti-carcinogenic; other studies have found that is effective in controlling cholesterol and in reducing the risk of heart disease as well (Priyadarshani and Biswajit 2012). Moreover, β-carotene is a valuable nutraceutical, used as a vitamin C supplement. Lastly, *D. salina* is commercially produced in several countries, including Israel, Australia and USA (Borowitzka 2013).

Haematococcus pluvialis is a ubiquitous freshwater green microalga of major economic interest, known to synthesize and accumulate astaxanthin under specific natural and artificial culture conditions (Boussiba 2000). Astaxanthin has received extensive attention because it is a strong antioxidant and a natural colorant with high market value (Leu and Boussiba 2014). It can be found in the encysted cells of *Haematococcus* as a symmetric ketocarotenoid (3.3 % dihydroxy-b, β-carotene 4.4 % dione) responsible for the red-pink color of many freshwater and marine fish as well as other aquatic organisms. Astaxanthin is one of the most abundant carotenoids in nature (Breithaupt 2007). Since the color of the muscle is an important quality parameter, xanthophylls are used for pigmentation in the aquaculture industry, also offering strong antioxidant and provitamin A activity to fish (Matsuno 2001; Miki 1991). Astaxanthin has terminal carbonyl groups that are conjugated to a polyene backbone and it is a more potent antioxidant and scavenger of free radicals than carotenoids such as β-carotene (Fassett and Coombes 2011). Thus, dietary supplementation with astaxanthin could potentially provide antioxidant protection of cells, including protection of the skin from the effects of UV radiation, amelioration of macular degeneration, protection against chemically induced cancers, atherosclerotic cardiovascular disease and enhancement of immune system (Lorenz and Cysewski

2000; Ranga Rao et al. 2014). Astaxanthin is a high value ($15,000/Kg pigment) nutraceutical antioxidant, which has been extensively studied and produced worldwide by several companies in Chile, Israel and China (Leu and Boussiba 2014). Indeed, the unicellular green algae *Haematococcus pluvialis* accumulates astaxanthin esters mainly in cytoplasmic lipid vesicles at up to 5 % of its total cellular dry weight when exposed to unfavorable growth conditions (Leu and Boussiba 2014).

At present, no commercial production of lutein is obtained from microalgae, although several species have been viewed as a promising alternative feedstock for production of the xanthophyll lutein (3R,30R,60R-β-carotene-3,30-diol). The most natural source of commercial lutein is from marigold flowers but this lutein is esterified with half of the weight corresponding to fatty acids and thus, chemical saponification is needed for purification (Lin et al. 2014). In this regard, microalgae such as *Muriellopsis sp*., *Chlorella zofingensis*, *Scenedesmus sp.* and *Chlorella protothecoides* appear to be potential lutein producers capable of accumulating a much higher content: 0.5–1.2 % dry weight (Sun et al. 2014; Chana et al. 2013). Pilot scale outdoor production has already been set up for lutein-rich cells of strains of *Muriellopsis sp*. and *Scenedesmus sp*. (Yaakob et al. 2014).

Lutein is often consumed as an additive used for flavor and color in foods, drugs and cosmetics (Yaakob et al. 2014). Furthermore it is employed as feed additive to brighten the colors of poultry feathers and deepen the yellow of egg yolk (Lin et al. 2014). Moreover, lutein is considered as an effective functional nutrient, providing beneficial properties to human health by ameliorating cardiovascular diseases (Dwyer et al. 2001), cancers (Ho et al. 2014), and preventing the development of cataracts and also preventing blindness or decrease in vision caused by age-related macular degeneration (Yaakob et al. 2014; Chiu and Taylor 2007).

8.5 Concluding Remarks

As described in this chapter, microalgae have attained particular attention since they are an enormous potential biological resource of a wide range of metabolites, especially the carotenoids, which represent an important group of structurally diverse terpenoid pigments with broad application in the feed, food, nutraceutical and pharmaceutical industries. Carotenoids also have intrinsic antioxidant activity and a potential role in preventing degenerative diseases and health conditions in humans. To significantly improve the carotenoid production, future research should be focused on metabolic engineering strategies in combination with cultivation optimization.

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Chapter 9
Apocarotenoids: A New Carotenoid-Derived Pathway

Juan Camilo Moreno Beltran and Claudia Stange

Abstract Carotenoids are precursors of carotenoid derived molecules termed apocarotenoids, which include isoprenoids with important functions in plant-environment interactions such as the attraction of pollinators and the defense against pathogens and herbivores. Apocarotenoids also include volatile aromatic compounds that act as repellents, chemoattractants, growth simulators and inhibitors, as well as the phytohormones abscisic acid and strigolactones. In plants, apocarotenoids can be found in several types of plastids (etioplast, leucoplast and chromoplast) and among different plant tissues such as flowers and roots. The structural similarity of some flower and spice isoprenoid volatile organic compounds (β-ionone and safranal) to carotenoids has led to the recent discovery of carotenoid-specific cleavage oxygenases, including carotenoid cleavage dioxygenases and 9-cis-epoxydioxygenases, which tailor and transform carotenoids into apocarotenoids. The great diversity of apocarotenoids is a consequence of the huge amount of carotenoid precursors, the variations in specific cleavage sites and the modifications after cleavage. Lycopene, β-carotene and zeaxanthin are the precursors of the main apocarotenoids described to date, which include bixin, crocin, picrocrocin, abscisic acid, strigolactone and mycorradicin.

The current chapter will give rise to an overview of the biosynthesis and function of the most important apocarotenoids in plants, as well as the current knowledge about the carotenoid cleavage oxygenase enzymes involved in these biosynthetic pathways.

Keywords Carotenoid cleavage dioxygenases • 9-cis carotenoid cleavage dioxygenases • Strigolactone • Carlactone • SL synthesis • Branching

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9.1 Introduction

Apocarotenoids are a class of terpenoid compounds generated through the oxidative cleavage of carotenoids. The great variety of apocarotenoids result from the large number of carotenoid precursors (more than 750 have been identified to date), variations in specific cleavage sites and modifications after cleavage (Schwartz et al. 2001). Depending on the size of the chromophore, apocarotenoids can absorb visible light and are therefore useful as color pigments in the attraction of pollinators and as seed dispersal agents. In plants, apocarotenoids can be found in the etioplast, leucoplast and chromoplast and among different plant tissues such as flowers and roots. The conversion from carotenoids to apocarotenoids is performed by regiospecific carotenoid cleavage oxygenases (CCDs), which target different double bonds of the carotenoid polyene chain (Fig. 9.1). The precursors of the main important apocarotenoids described, are lycopene, β-carotene and zeaxanthin.

Two of the most important apocarotenoids, due to their great commercial value, are bixin and crocin. Bixin is a polyene derived from the central part of a C_{40} lycopene after enzymatic cleavage of the 5-6 and 5′-6′ double bonds. This cleavage is carried out by lycopene cleavage dioxygenase (LCD) and bixin aldehyde dehydrogenase (BiADH) and gives rise to the C_{24} dicarboxilic acid, norbixin. Norbixin methyltransferase (nBMT) catalyzes the reaction between norbixin and S-adenosyl L-methionine to produce bixin, which is mostly obtained from the seeds of *Bixa orellana* (annatto bush native to Central and South America). Crocin is the glycosylated form of crocetin, which accumulates in large amounts (up to 8 % by dry weight) in saffron stigmas. Both pigments are responsible for the red pigmentation of the saffron stigmas, while picrocrocin is responsible for the bitter flavor and saffranal is responsible for the aroma of saffron (Fig. 9.1) (Caballero-Ortega et al. 2007). The proposed biosynthetic pathway starts with a symmetric cleavage of zeaxanthin at the 7,8/7′,8′ positions by a nonheme iron carotenoid cleavage dioxygenase (CCD2). The two cleavage products, 3-OH-β-cyclocitral and crocetin dialdehyde, are dehydrogenated by ALDH and glycosylated by UGT to yield picrocrocin and crocin, respectively (Fig. 9.1) (Bouvier et al. 2003)

Fig. 9.1 (continued) Biosynthetic pathways of main plant apocarotenoids. The classical carotenoid pathway with the respective enzymes and products is shown in a dashed-square. Apocarotenoid biogenesis starts from lycopene, β-carotene and zeaxanthin precursors leading to different end products including plant hormones (abscisic acid and strigolactones), aroma, flavor and scent compounds (crocin, picrocrocin), pigments (bixin) and signal molecules (mycorradicin). Cleavage enzymes are marked by scissors (CCDs and NCED). *LCD* lycopene cleavage dioxygenase, *BiADH* bixin aldehyde dehydrogenase, *nBMT* norbixin methyltransferase, *D27* β-carotene isomerase, *CCD (1), (2), (7), (8)* carotenoid cleavage dioxygenase, *MAX1* cytochrome P450, *CBHx*Carotene β-hydroxylase, *ALDH* aldehyde dehydrogenase, *UGTs* UDPG-glucosyltransferases, *ZEP* zeaxanthin epoxidase, *NSY* neoxanthin synthase, *NCED* 9-cis epoxy-carotenoid cleavage dioxygenases, *ABA2* xanthoxin dehydrogenase 2, *AAO3* abscisic aldehyde oxidase 3

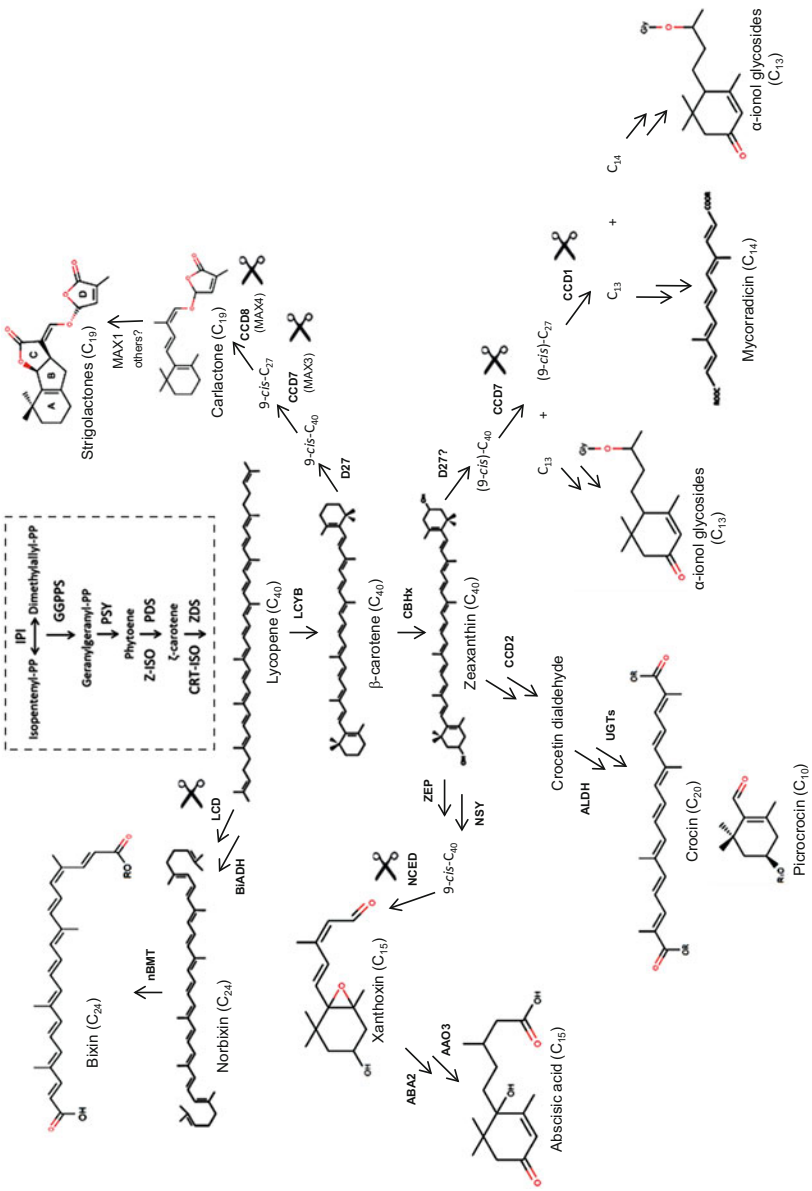

Fig. 9.1 (continued)

and transported to the vacuoles (Grilli-Caiola and Canini 2004). Interestingly, the expression of CsCCD2 presents a daily rhythmic regulation as well as an up-induction by β-cyclocitral suggesting that this apocarotenoid may act as plastid-to-nucleus signalling molecule for crocetin production in saffron stigmas (Ahrazem et al. 2016). In addition, tissues that do not accumulate crocetin present intron retention in CsCCD2 transcripts relative to saffron stigma tissue (Ahrazem et al. 2016) suggesting an organ specific regulation of CsCCD2.

Other apocarotenoids are well known phytohormones involved in the regulation of plant architecture and growth. One of these is abscisic acid (ABA), which plays an important role in the regulation of seed development, drought resistance and sugar sensing (Schwartz et al. 2003; Taylor et al. 2005). The first step that is specific to the ABA biosynthesis is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which takes place in the plastid (Fig 9.1). This step is catalyzed by a zeaxanthin epoxidase (ZEP), whose molecular identity was first revealed in tobacco (Marin et al. 1996). After a series of structural modifications, violaxanthin is converted to 9-*cis*-epoxycarotenoid. Oxidative cleavage of the major epoxycarotenoid 9-*cis*-neoxanthin by the 9-*cis*-epoxycarotenoid dioxygenase (NCED) yields a C_{15} intermediate, which is called xanthoxin (Fig. 9.1) (Schwartz et al. 1997). This step is considered the first committed step in the ABA biosynthetic pathway. The product xanthoxin is then exported to the cytosol, where it is converted to ABA through a two-step reaction. A short-chain alcohol dehydrogenase/reductase (SDR), encoded by the *AtABA2* gene (Rook et al. 2001; Cheng et al. 2002; Gonzalez-Guzman et al. 2002), catalyzes the first step of this reaction and generates ABA aldehyde. ABA aldehyde oxidase (AAO) then catalyzes the last step in the biosynthesis pathway (Fig. 9.1).

The recently discovered plant phytohormone strigolactone (SL) is involved in many physiological processes, such as shoot branching, plant development and the coordination of plant growth and root architecture in accordance with phosphate availability (Brewer et al. 2013; Rasmussen et al. 2013; Ruyter-Spira et al. 2013). The main tissue of strigolactone synthesis is the root and begins with the reversible isomerization of β-carotene, which is catalyzed by the D27 protein, producing 9-*cis* β-carotene (9-*cis* C_{40}) as is shown in Fig. 9.1. The carotenoid cleavage dioxygenase CCD7 then cleaves 9-*cis* β-carotene, generating the 9-*cis*-β-apo-10′-Carotenal (9-*cis* C_{27}) plus β-Ionone. The 9-*cis* C_{27} compound is then cleaved by the carotenoid cleavage dioxygenase CCD8 to produce carlactone (CL), which is subsequently converted into strigolactone (SL), a structure composed of the tricyclic lactone (ABC ring), by the action of MAX1 and probably other enzymes that are yet to be identified (Fig. 9.1).

Two other apocarotenoids, C_{13} α-ionol (formerly called cyclohexenone) derivatives (Maier et al. 1995) and mycorradicin (a C_{14} linear polyenic dicarboxylic acid) (Klingner et al. 1995) (Fig. 9.1), both promote the arbuscular mycorrhizal (AM) symbiosis (Maier et al. 1995; Recorbet et al. 2013). These compounds are produced in roots upon specific colonization by AM fungi, but not in pathogenic interactions. Both the C_{13} and the C_{14} compounds are generated from the concomitant activity of D27, CCD7 and CCD1 (Fig. 9.1) and can accumulate to fairly high levels in

later stages of the AM symbiosis. The C_{14} compound is yellow and imparts a macroscopically visible yellow coloration known as the "yellow pigment" (Klingner et al. 1995) to roots of some species (e.g. maize) upon dense colonization by AM fungi. Both compounds are either glycosylated (C_{13}) or esterified (C_{14}) to unknown compounds (Fester et al. 2002).

9.2 CCDs and NCEDs: Carotenoid Tailoring Enzymes

Two types of carotenoid oxygenases have been identified in plants: carotenoid cleavage dioxygenases (CCDs) and the *cis*9- carotenoid cleavage dioxygenases (NCEDs). The CCDs are structurally conserved and belong to a multienzyme family of nine members in *Arabidopsis thaliana* (Tan et al. 2003; Sui et al. 2013). Members of this family include the five 9-*cis*-epoxydioxygenases (NCEDs, NCED2, NCED3, NCED5, NCED6 and NCED9) involved in the biosynthesis of the plant hormone ABA (Schwartz et al. 1997), the CCD1s that cleave a broad range of carotenoids to form volatile aromatic compounds (Simkin et al. 2004a; Auldridge et al. 2006b), the CCD4s that catalyze the cleavage of carotenoids forming aromatic and pigment compounds (Bouvier et al. 2003; Ohmiya et al. 2006; Huang et al. 2009), and the CCD7s and CCD8s that catalyze the sequential cleavage of carotenoids to form SLs. The CCDs have low sequence similarity to the NCEDs and the substrate specificity and enzyme activity of the encoded products also differs from those of the NCEDs (Tan et al. 2003; Ohmiya 2009). The majority of the CCDs have been shown to be located in plastids, where their substrates are also localized. The only exception is CCD1, which acts in the cytoplasm or in association with the outer membrane of the plastid (Vidi et al. 2006; Ytterberg et al. 2006; Floss and Walter 2009).

These CCDs can act via a single cleavage of a C_{40} carotenoid substrate, such as lycopene or β-carotene, or via several sequential cleavage events. Due to this mechanism of action a great number of apocarotenoids with a high variety of functions, like drought tolerance, attraction of pollinators, as well as growth and developmental regulation, can be obtained by CCD-mediated cleavage (Bouvier et al. 2003; Bouvier et al. 2005). Other plant carotenoid cleavage products include pigments, aromatic and scent compounds, as well as regulatory molecules with yet unknown functions (Giuliano et al. 2003; Bouvier et al. 2005; Auldridge et al. 2006a; Walter et al. 2007; Yamamizo et al. 2010).

9.2.1 Carotenoid Cleavage Dioxygenases: Mode of Action

To date, the crystal structure of a plant CCD has not been resolved, but the three-dimensional structure of a related family member, apocarotenoid 15,15′-oxygenase (ACO) from the cyanobacterium *Synechocystis* was determined by Kloer et al. (Kloer et al. 2005). The high degree of similarity shared between the amino acid

sequences of ACO and plant CCDs in important structural regions of the proteins (Kloer and Schulz 2006) has allowed for the proposal of a mode of action for the CCDs. *In vitro*, ACO cleaves the 15,15′ double bond and its preferred substrates are apocarotenols of C_{27}-C_{30} chain length with hydroxylated ionone rings. ACO has a proposed seven-bladed β-propeller tertiary structure, with four characteristic histidine side chains holding the catalytic ferrous iron in the propeller axis. A hydrophobic patch on the surface of ACO, which sits at the entrance of the active site tunnel, has been proposed to be important for regulation and substrate channeling or availability (Auldridge et al. 2006a). ACO has two monomers which are present within the crystal forms. Those associate at this site to form a combined hydrophobic patch, which might be utilized for membrane localization and extraction of non-polar substrates (Kloer et al. 2005).

9.2.2 Role of Carotenoid Cleavage Dioxygenases in Plants

As was mentioned before, CCDs have low sequence homology to the NCEDs and therefore their activities and substrate specificities also differ from each other. CCD1, which is the only CCD located in the cytosol, is involved in the formation of aroma and scent compounds, as well as in carotenoid turnover. The contribution of CCD1 enzymes to the generation of important apocarotenoid volatile compounds (β-ionone, β-cyclocitral, geranylacetone and pseudoionone) in fruit and flowers has been demonstrated in a vast number of different plant species (Schwartz et al. 1997; Simkin et al. 2004a; Simkin et al. 2004b; Mathieu et al. 2005; Garcia-Limones et al. 2008; Simkin et al. 2008; Vogel et al. 2008; Huang et al. 2009). Due to the subcellular location of CCD1, this enzyme does not have direct access to the carotenoids located in the plastids (Auldridge et al. 2006a; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011). Therefore, it is speculated that plant CCD1s convert the plastid-released apocarotenoids that have arisen through either non-enzymatic oxidative cleavage processes or enzymatic cleavage by other CCDs (CCD4 and/or CCD7). This scenario might explain the multiple cleavage sites and the wide substrate specificity displayed by CCD1 enzymes (Ilg et al. 2010). CCD1 enzymes are involved in the cleavage of the 5,6 (5′,6′) (Vogel et al. 2008), 7,8 (7′,8′) (Ilg et al. 2009) and 9,10 (9′,10′) (Schwartz et al. 2001) double bonds to produce a variety of volatiles. Studies done in Arabidopsis, tomato and petunia, through *CCD1* mutants or gene silencing, have raised doubts as to whether the generation of C_{13} apocarotenoids by CCD1 is the exclusive role for these enzymes (Simkin et al. 2004a; Simkin et al. 2004b; Auldridge et al. 2006a). RNA interference (RNAi)-mediated repression of a *Medicago truncatula CCD1* gene in hairy roots results in a differential reduction of C_{13} and C_{14} apocarotenoids (Floss and Walter 2009). This result is in conflict with the hypothesis of a symmetrical cleavage action of CCD1 *in planta*. A prominent color change to yellow-orange was observed in the mycorrhizal RNAi roots as consequence of C_{27} accumulation, which suggests that

C_{27} derivatives are the main substrates for CCD1 in mycorrhizal roots and not C_{40} carotenoids as previously thought (Floss et al. 2008; Floss and Walter 2009).

Since CCD1 enzymes do not have direct access to the carotenoids located in the plastids (Auldridge et al. 2006a; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011), it is thought that CCD1 enzymes cleave C_{27} and C_{40} intermediates that are transported from the chloroplast to the cytoplasm. However, during senescence, when the chloroplast membranes disintegrate, CCD1s will have access to its substrates. C_{27} apocarotenoids have rarely been found in nature, perhaps due to the activity of CCD1s in plant tissues (Walter et al. 2010) and it is speculated that plant CCD1s also convert the plastid-released C_{27} apocarotenoids that have arisen through the non-enzymatic oxidative cleavage processes (Ilg et al. 2010). Recently, a novel plant CCD enzyme from saffron, *Cs*CCD2, which catalyzes the cleavage step leading to crocetin biosynthesis starting from the precursor, zeaxanthin, was reported (Frusciante et al. 2014).

Similar to CCD1, CCD4 is involved in the formation of pigment and aroma compounds and carotenoid turnover. From *in vitro* studies, it was found that both CCD1 and CCD4 enzymes cleave carotenoids at the same C_9,C_{10} (C_9',C_{10}') double bond position and that they have a key role in the formation of β-ionone and other fruit and flower apocarotenoids. According to Huang et al. (Huang et al. 2009), plants generally produce two different forms of the CCD4 enzyme, which seem to be more substrate specific than CCD1 and have a broader substrate tolerance, producing numerous C_{13} apocarotenoid products (Rubio et al. 2008).

CCD4 enzymes are located in the plastid, which means these enzymes have direct access to carotenoid substrates, implicating their role in carotenoid degradation and apocarotenoid synthesis within the plastid (Rubio et al. 2008; Brandi et al. 2011). CCD4 enzymes from *A. thaliana* (Vidi et al. 2006; Ytterberg et al. 2006) and *C. sativus* (Rubio et al. 2008) have been identified in the plastoglobule proteome (*At*CCD4 and *Cs*CCD4). Plastoglobules are structures associated with protein-lipid membranes of thylakoids in chloroplasts, which are involved in the optimization of photosynthesis, light acclimation and repair (Lundquist et al. 2012), as well as to protect the thylakoid membranes against oxidative stress (Brehelin and Kessler 2008). RNA interference studies have revealed that suppression of *CmCCD4a* expression contributed to yellow color formation in chrysanthemum petals. This suggested that the white color is a result of the degradation of carotenoids into colorless compounds by *Cm*CCD4a. *Cm*CCD4a cleaves β-carotene at the 9,10 (9′,10′) double bond positions, resulting in the formation of white petals (Ohmiya et al. 2006; Huang et al. 2009). In *Ipomoea* plants, CCD4 is not involved in the degradation of chromoplast-type carotenoids, but is involved in the degradation of chloroplast-type carotenoids (Tai and Chen 2000; Kishimoto et al. 2005). The carotenoid content in the petals of *Ipomoea* plants is not related to carotenoid degradation activity or to the sink capacity of carotenoids, but it is linked to the transcriptional down-regulation of the carotenogenic gene *CHYB*, which encodes a β-carotene hydroxylase (Yamamizo et al. 2010). Potato tubers from the white-fleshed cultivar showed higher transcript levels of a *CCD4* gene compared to the

yellow-fleshed tubers. Stably transformed RNAi lines of the white-fleshed cultivar with down-regulated *CCD4* expression produced tubers with 2- to 5-fold higher carotenoid content. The carotenoid content was also raised in the petals of the RNAi lines, but not in the leaves, stem or roots (Campbell et al. 2010).

Citrus *CCD4a* and *CCD4b* showed opposite expression patterns (Pan et al. 2012) consistent with a difference in substrates specificity. *CCD4b*, but not *CCD4a*, was down-regulated in citrus (*cv. cara cara*), suggesting that CCD4b may play a role in lycopene accumulation rather than CCD4a. Validating the functions of CCD4 enzymes, clarifying their substrate specificities and investigating their expression patterns will shed light on their roles in color and aroma formation in citrus fruits and flowers (Pan et al. 2012).

The steady state levels of carotenoids are expected to be dependent on the storage capacity of plastids, but also on the balance between biosynthesis and degradation (Ruiz-Sola and Rodriguez-Concepcion 2012). Thus, there is continuous turnover of carotenoids and chlorophyll in photosynthetic tissues (Lu and Li 2008), suggesting that CCD4 may be involved in those processes.

CCD7 and CCD8 are special carotenoid cleavage dioxygenases implicated in the synthesis of the plant hormone strigolactone (SL). CCD7 and CCD8 were first characterized as the two remaining members of the *Arabidopsis* NCED/CCD family. AtCCD7 exhibited specific C_9,C_{10} (C_9',C_{10}') cleavage activity *in vitro* by converting β-carotene to the C_{27} compound β-*apo*-10′-carotenal and the C_{13} compound β-ionone. When *AtCCD7* was co-expressed in *E. coli* with *AtCCD8*, β-*apo*-13-carotenal was obtained as an additional cleavage product. This product was not identified when *AtCCD8* was expressed on its own. The conclusion was that β-*apo*-13-carotenal was formed by a secondary cleavage of the C_{27} compound β-*apo*-10′-carotenal, formed by AtCCD7, at the C_{13},C_{14} (C_{13}',C_{14}') double bond position (Schwartz et al. 2004; Walter et al. 2010). *In vivo* studies have showed the formation of β-apo-13-carotenone from all-*trans*-β-apo-10′-carotenal by CCD8 enzymes from several plant species (Alder et al. 2008). The structure of β-apo-13-carotenone has very little similarities with that of SLs and therefore it was questionable whether β-carotene was the initial substrate in the pathway for SL biosynthesis. Moreover, when a rice *ccd8* mutant was treated with β-apo-13-carotenone, the wild type phenotype was not restored (Alder et al. 2012).

Alder et al. (2012) demonstrated that D27 is a β-carotene isomerase that converts the all-*trans*-β-carotene into a 9-*cis*-β-carotene, which is subsequently cleaved by CCD7 into a 9-*cis*-configured aldehyde. CCD8 then incorporates three oxygen molecules into 9-*cis*-β-apo-10′-carotenal and rearranges it molecularly to form CL (Figs. 9.1 and 9.3). Additional shoot branching was observed in *d27*, *ccd7* and *ccd8* mutants in the absence of CL. With the addition of *in vitro* produced CL to rice mutants *d27*, *ccd7* (*htd-1*) and *ccd8* (*d10*), the *wild type* phenotype was restored, indicating that CL is probably an SL precursor (Alder et al. 2012). SLs are a fairly new class of plant hormones which are involved in several aspects of plant growth and development. They were originally identified as germination stimulants for root parasitic plant seeds of the family *Orobancheaceae*, such as *Striga, Phelipanche* and *Orobanche* spp. (Bouwmeester et al. 2003). SLs are exuded by plant roots and

act as host detection signals for symbiotic interactions with arbuscular mycorrhizal fungi (AM), stimulating their metabolism and hyphal branching (Akiyama et al. 2005; Parniske 2008). The most extensively characterized role of SLs is their involvement in the inhibition of shoots branching (Domagalska and Leyser 2011; Dun et al. 2012; Waters et al. 2012). SL-response and SL-deficient mutants have illustrated several additional roles, namely enhanced lateral (Arite et al. 2007; Kapulnik et al. 2011b; Kapulnik et al. 2011a; Stirnberg et al. 2012) and adventitious root development (Rasmussen et al. 2012a; Rasmussen et al. 2012b), and reduced stature and suppression of cambium ring development in the main stem (secondary development) (Agusti et al. 2011).

Experiments using the synthetic SL, GR24, have demonstrated that this hormone (or a derived compound) could partially restore the *wild type* branching phenotypes and bud outgrowth in *ccd7* and *ccd8* mutants of pea, rice and Arabidopsis (Gomez-Roldan et al. 2008; Umehara et al. 2008). Studies using Flouridone, an inhibitor of carotenoid biosynthesis, have illustrated that carotenoid biosynthesis is necessary for normal levels of SLs (Ito et al. 2010).

Arabidopsis and petunia (*Petunia hybrid*) are important plant models in which axillary branching have been studied. In *P. hybrid*, a mutation of the *PhCCD7* gene was shown to have a less severe branching phenotype than a mutation of the *PhCCD8* gene. Analysis of expression of *PhCCD7* and *PhCCD8* in the *wild type*, mutants and grafted petunia suggested that *PhCCD7* and *PhCCD8* are coordinately regulated (Drummond et al. 2009). On the other hand, recombinant *At*CCD7 cleaves multiple carotenoid substrates (Booker et al. 2004; Schwartz et al. 2004). Expression analysis of *AtCCD7* showed that the transcripts were more abundant in seeds than other organs. The robust expression of *AtCCD7* in mature seeds is opposite to previous findings, which illustrated its highest expression to be in roots (Liang et al. 2011).

9.2.3 Role of Carotenoid Cleavage Dioxygenases NCED in Plants

The first enzyme found to be specifically involved in the cleavage of carotenoids, viviparous14 (VP14), was identified by the analysis of a viviparous ABA-deficient mutant in maize (Schwartz et al. 1997). Enzyme activity assays of VP14 showed that it cleaves the 11,12 (11′,12′) double bonds of the 9-*cis* isomers of violaxanthin and neoxanthin to produce xanthoxin (C_{15} apocarotenoid), which is the precursor of ABA. The initial work on VP14 made the discovery of related enzymes easier in different plant species and other organisms (Tan et al. 2003). According to their substrate specificity, VP14 and its orthologues have been named 9-*cis* epoxycarotenoid cleavage dioxygenases (NCEDs) (Auldridge et al. 2006b).

The NCED sub-family is involved in the synthesis of one of the most studied plant apocarotenoids, the phytohormone ABA. NCEDs are plastid-localized, and are therefore co-localized with carotenoids (Tan et al. 2003; Floss and Walter 2009). NCEDs are unique among other CCDs in that they accept only *cis*-isomers of their substrates (Tan et al. 2003; Walter and Strack 2011). ABA levels rise under stress conditions as well as during seed and bud dehydration. The cleavage reaction of *9-cis*-violaxanthin and *9-cis*-neoxanthin is the rate-limiting step in the biosynthesis of ABA (Cutler and Krochko 1999). Mutations of *NCED* genes in maize (*Zea mays*) resulted in droopy phenotypes with reduced ABA levels (Tan et al. 1997). *NCED*-encoding genes have been isolated from numerous species including maize (*Zea mays*) (Tan et al. 1997), tomato (*Solanum lycopersicum*) (Burbidge et al. 1999), bean (*Phaseolus vulgaris*) (Qin and Zeevaart 1999), avocado (*Persea americana*) (Chernys and Zeevaart 2000), cowpea (*Vigna unguiculata*) (Iuchi et al. 2000), *Arabidopsis thaliana* (Iuchi et al. 2001), potato (*Solanum tuberosum*) (Destefano-Beltran et al. 2006), orange (*Citrus sinensis*) (Rodrigo et al. 2006) and grape (*V. vinifera*) (Zhang et al. 2009). In some plant species, *NCED*-like genes were identified, comprising a small multi-gene family, with only a subgroup involved in stress responses and regulation of ABA biosynthesis (Chernys and Zeevaart 2000; Tan et al. 2003; Rodrigo et al. 2006; Qin et al. 2008). Five members of the *Arabidopsis* NCED family were identified, namely *At*NCED2, *At*NCED3, *At*NCED5, *At*NCED6 and *At*NCED9. All five *At*NCEDs are plastid-localized, although they differ in their binding activity to the thylakoid membrane (Tan et al. 2001). *At*NCED2, *At*NCED3 and *At*NCED6 are found in both the stroma and thylakoid membrane-bound compartments. *At*NCED5 is bound to thylakoids and *At*NCED9 is soluble in the stroma (Tan et al. 2003). Expression analysis of the *NCEDs* in avocado (Chernys and Zeevaart 2000); tomato (Thompson et al. 2000); *Arabidopsis* (Tan et al. 2003) and in other higher plants (Han et al. 2004) has suggested a key role for NCED in the response of vegetative tissues to water stress and has suggested an associations between NCED expression and ABA accumulation.

Transgenic Arabidopsis plants overexpressing *AtNCED3* and a peanut *NCED* displayed enhanced water stress resistance and increased ABA levels (Iuchi et al. 2001). These results were also found in tobacco expressing *LeNCED1 (Tung et al.* 2008) and *PvNCED1* (Qin and Zeevaart 2002). *NCED* knock-outs showed weakened ability for ABA biosynthesis in stressed leaves (Burbidge et al. 1999; Iuchi et al. 2001). In peach (*Prunus persica*) and grapes *(V. vinifera)*, *PpNCED1* and *VvNCED1* genes were expressed only at the initial stages of fruit ripening according with the highest ABA level (Zhang et al. 2009), clearly indicating that *NCEDs* are transcriptionally regulated.

It has been proposed that the mechanism for ABA biosynthesis by the dioxygenase is through the cleavage of the *9-cis*-carotenoid bond (Schwartz et al. 2003), but the structure and nature of the determinants of specificity of the dioxygenase remains unknown (Messing et al. 2010). The structure of VP14 was used to identify amino acid residues that played a role in determining which bond was cleaved

(Messing et al. 2010). These residues were contrasted and compared to the plant CCD family. As was suggested by Kloer & Schultz (Kloer and Schulz 2006), the β-propeller portion of the structure was a conserved characteristic throughout the CCD enzyme family, as it was present in both the prokaryotic apocarotenoid 15,15′-oxygenase (ACO) and the eukaryotic VP14. The helical domain may be the structural feature that differentiates plant CCDs from the rest of the members of the CCD family (Messing et al. 2010). Messing et al. identified the remarkable level of sequence identity between VP14, NCEDs and the CCDs family in plants. This made VP14 a suitable prototype to be used as a template to construct a model of *Zea mays* CCD1 (*Zm*CCD1). Comparisons between the two models showed that the differences in substrate specificity were due to three crucial regions in the structures. Mutational studies done on *Zm*CCD1 validated the use of VP14 as a template for mapping important residues in the substrate tunnels of plant CCDs and provided a basis for understanding their substrate specificity (Messing et al. 2010).

9.3 Apocarotenoid Functions: More Than Flavor Compounds

In the past decade, more and more apocarotenoids and their functions have been revealed. Among them, are the volatiles C_{13} β-ionone and β-damascenone, which are responsible for the characteristic aroma of rose scent (Huang et al. 2009) and the C_{13} norisoprenoids that contribute to the floral and fruity characteristics of wine in grapevine (*Vitis vinifera* L.) berries (Mendes-Pinto 2009). Other examples have illustrated roles for apocarotenoids as repellents, chemo-attractants, growth simulators and inhibitors (Bouvier et al. 2003; Bouvier et al. 2005). Apocarotenoid volatiles are emitted by many flowers or vegetative tissues to promote plant-insect interactions (McQuate and Peck 2001; Azuma et al. 2002). Additional to the well-studied apocarotenoid related to aroma, flavor and scent, new and interesting functions have come to light. Well known examples are the hormones involved in the regulation of plant architecture and growth, as ABA and SLs (Fig. 9.1).

9.3.1 Strigolactones

Strigolactones (SLs) are carotenoid-derived compounds involved in plant signaling. They have two main functions:

- Endogenous hormones to control plant development
- Components of root exudates to promote symbiotic interactions between plants and soil microbes.

SLs were discovered in root exudates due to their ability to stimulate the germination of seeds of the parasitic plant *Striga*, the "witchweed". Since the discovery of the first natural SL, strigol, as a germination stimulant of *Striga lutea* (Al-Babili and Bouwmeester 2015), other SLs have been identified in exudates of different plant species and have been shown to stimulate seed germination in root parasitic plants of the genera *Striga*, *Orobanche*, *Alectra*, and *Phelipanche* (Yoneyama et al. 2013). The discovery that SLs mediate the establishment of beneficial symbiosis with arbuscular mycorrhizal fungi by stimulating the branching of their hyphae (Akiyama et al. 2005) explained why plants release SLs. Approximately 80 % of land plants engage in symbioses with these fungi, in which fixed carbon from the plant is exchanged for minerals absorbed by the fungus through an extensive network of hyphae (Gutjahr and Parniske 2013). This is extremely important for nutrients with low mobility in soil, particularly phosphate (Gutjahr and Parniske 2013). Consistent with this role, SL synthesis and secretion into the soil is upregulated in response to phosphate deficiency (Umehara et al. 2008; Kohlen et al. 2011; Yoneyama et al. 2013). However, another important function has been assigned to SLs, they were also shown as an endogenous signaling molecule involved in a growing list of processes, including lateral root formation, root hair elongation, root growth, adventitious rooting, secondary growth, stem elongation, leaf senescence, leaf expansion, drought and salinity responses, and even more noticeable, shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008; Agusti et al. 2011; Kapulnik et al. 2011b; de Saint Germain et al. 2013). Experiments performed to study the role of SL in shoot-branching control were essential in the characterization of the SL biosynthesis pathway. Moreover, genetic screens for shoot-branching mutants in several higher plant species led to the isolation of key genes for SL synthesis and signaling (Waldie et al. 2014). Even though the SL biosynthesis pathway is almost completely characterized, efforts are still needed to clarify SL perception and transduction.

9.3.2 Strigolactone Biosynthesis and Its Key Players

The SLs from all plants in which it has been identified to date share a common tricyclic backbone which is composed of three different "ABC" rings (Fig. 9.2). This backbone is connected to a D-ring butenolide group via an enol-ether bridge (Fig. 9.2), which is the unmodified part in all natural active SLs (Zwanenburg et al. 2009; Zwanenburg et al. 2013). Moreover, the AB ring could be modified by further hydroxylations, epoxidations/oxidations, methylation or ketolation and give rise to new SLs (Fig. 9.2). To date, more than 20 naturally occurring SLs have been described and characterized from root exudates of various land plants (Yoneyama et al. 2010; Kisugi et al. 2013). Due to the structure of the BCD rings, SLs can be grouped into two different families (Fig. 9.2):

- BCD rings that have the same stereochemistry as (+)-strigol
- BCD rings that have the same stereochemistry as (−)-orobanchol.

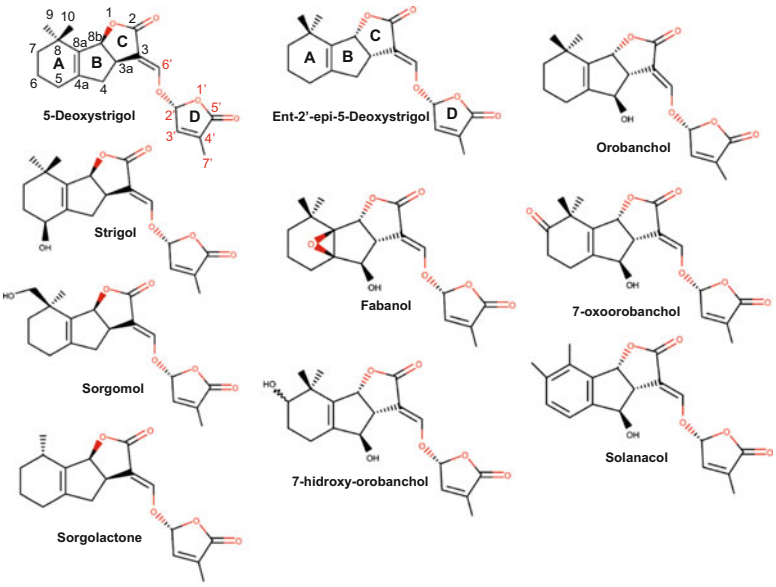

Fig. 9.2 Structures of naturally occurring SLs synthesized in plants. Structure and stereochemistry of the simple SL 5-deoxystrigol and its derivatives Strigol, Sorgomol and Sorgolactone which conserve the ABC ring backbone besides methylation and hydroxylation in the A ring. D ring presents no changes through all SL that derived from 5-deoxystrigol. Structure and stereochemistry of Ent-2′-epi-5-deoxystrigol which is the precursor of orobanchol which produces different SLs after oxidation, hydroxylation and methylation (Fabanol, 7-oxoorobanchol, 7-hidroxy-orobanchol and Solanacol). The ABC ring is modified through the addition of different chemical groups in ring A and B. D ring remained unmodified in all the SLs derived from orobanchol

Strigol and orobanchol differ in the stereochemistry of the B-C ring junction (Fig. 9.2) (Ueno et al. 2011). All the strigol-like SLs (derived from 5-deoxystrigol) possess a β configuration in their C-ring (Fig. 9.2; up; 8bS configuration) whereas all the orobanchol-like SLs (derived from Ent-2′-epi-5-deoxystrigol) are in an α configuration (Fig. 9.2; down; 8bR configuration). As was mentioned before, the addition of methyl, acetyl or other groups can influence the structure, but also the function of the SLs and therefore the *in planta* responses. The bioactiphore for the germination of parasitic weed seeds was found to reside in the CD part (Zwanenburg et al. 2013). Essential structural features within the CD part for AM fungi appear to be similar, but important SL structural variations could affect its bioactivity, such as the presence of the ABC-rings with different modifications. The ABC ring was reported as essential for AM fungi, but not for parasitic seeds and for branching (Akiyama et al. 2010; Cohen et al. 2013). More specifically, the presence of hydroxyl groups is generally associated with enhanced germination activity compared with acetyl-containing SLs (Sato et al. 2005; Yoneyama et al. 2008). In contrast, hydrophobic or acetyl-containing SLs have stronger bud inhibition activity compared with hydroxyl-containing SLs in pea (Boyer et al. 2012). The findings

mentioned above support the idea that different components of the SL structure influence not only in the final function and the response by the plant to SLs but also in its perception in a variety of ways.

With the major finding of the structure of CL, an intermediate in the formation of 5-deoxystrigol via putative cytochrome P450 enzyme(s), possibly More Axillary Branching 1 (MAX1) (Fig. 9.3), the gap between carotenoids and common SLs containing a tricyclic lactone core (ABC ring) was closed. After this finding, a different division of the SLs family was proposed. The first group of SLs shared the ABC ring-containing strigol-like or orobanchol-like compounds and a second group which lacks the tricyclic lactone, such as CL, which contains a β-ionone ring instead (Figs. 9.2 and 9.3). The study of *d17* and *d10* mutants from rice, deficient in CCD7 and CCD8, respectively, and the *ccd8/rms1* mutant of pea (Gomez-Roldan et al. 2008; Umehara et al. 2008) that present deficiency in SLs production, provided strong evidence of the biosynthetic pathway of SLs. Besides these two CCDs, a cytochrome P450, MAX1, was shown to function in SL biosynthesis (Crawford et al. 2010). Furthermore, D27, a novel class of Fe-containing protein, was also found as a SL biosynthetic component (Lin et al. 2009).

There is wide analytical evidence that plants synthesize SLs in their roots (Ruyter-Spira et al. 2011). However there are other plant tissues that also produce this hormone but in less amounts. Studies in wild type branching phenotypes of wild type scions grafted onto rootstocks of Arabidopsis and pea SL biosynthetic mutants suggested that SL are also produced in shoots (Morris et al., 2001; Turnbul et al., 2002). Moreover, transcripts of genes involved in SL biosynthesis were detected in several plants in aerial tissues and around axillary buds. However, in some cases there was no correlation between high transcript levels and high SL accumulation. For instance, transcript abundance of *CCD7* (*MAX3*) in Arabidopsis is very high in roots (Waters et al. 2012), and also in the aerial tissues of rice and in the immature green fruits of tomato, (Vogel et al. 2010). The explanation of this behavior is not yet known, however it is believed that SL biosynthetic genes are involved in other processes. For instance, CCD7 participates in the production of mycorradicin and cyclohexenone pigments in mycorrhizal roots (Fig. 9.1) (Vogel et al. 2010).

Thus far, most of the genes involved in SL biosynthesis have been characterized in many different plant species. In the past few years, the first three steps and the enzymes involved in these reactions were confirmed using all-trans-β-carotene as substrate in the presence of the enzymes D27, CCD7 and CCD8 obtaining CL *in vitro* (Alder et al. 2012). D27 catalyzes the reversible isomerization of all-trans-β-carotene at C9 position to produce 9-cis-β-carotene. Early classification of this enzyme placed it as a small iron-binding protein without any conserved domain or homology to any known enzyme (Lin et al. 2009). However, the fact that CCD7 has preference for 9-cis-configured β-carotene led to the hypothesis that D27 may encode a 9-cis/all-trans-β-carotene isomerase that provides the substrate in the right configuration. *In vitro* experiments incubating a D27 enzyme from rice with 9-*cis*- and all-*trans*-β-carotene demonstrated that the all-*trans*- to 9-*cis*-β-carotene isomerase activity mediates the interconversion of both isomers (Alder et al. 2012). Since the branching phenotype of *d27* mutant is not as

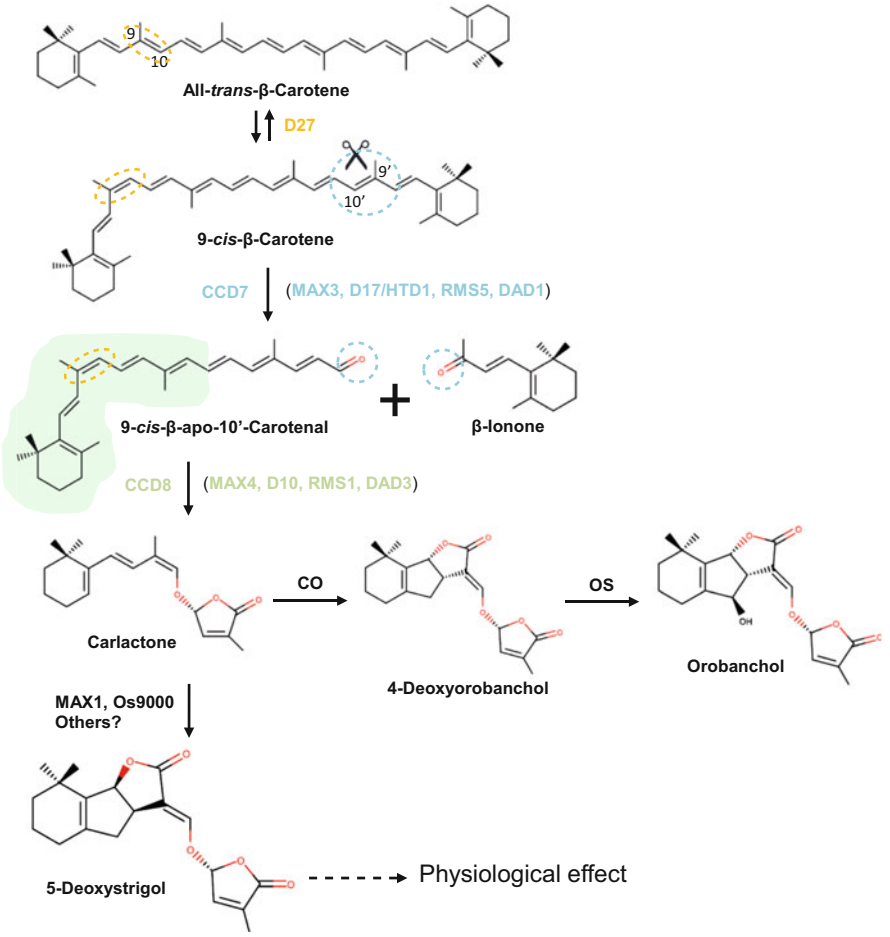

Fig. 9.3 Strigolactone (SL) biosynthesis pathway. The first step is the reversible isomerization of all- trans-β-carotene by the D27 enzyme (*yellow boldfaced*). Then CCD7 (*light blue boldfaced*) enzyme (MAX3, D17/HTD1, RMS5 or DAD1) mediates the stereospecific cleavage of the C9′-C10′ double bond (*light blue doted circle*) from the 9-*cis*-β-carotene yielding the products 9-*cis*-β-apo-10′-carotenal plus β-ionone. In one step CCD8 (*green boldfaced*) enzyme (MAX4, D10, RMS1, DAD3) converts 9-*cis*-β-apo-10′-carotenal (in *green*) into Carlactone (CL) and an unidentified second product (not shown). CL is thought to derive from the green shaded part of the 9-*cis*-β-apo-10′-carotenal. Afterwards MAX1 (*black boldfaced*) and probably other enzymes convert CL into 5-deoxystrigol. In rice, CL is the substrate of carlactone oxidase (CO) to produce 4-Deoxyorobanchol, which is then converted to orobanchol by the action of orobanchol synthase (OS). Physiological effect is achieved after SLs perception

severe as mutations downstream in *ccd8* (Waters et al. 2012), the spontaneous isomerization to interconvert 9-*cis*-β-carotene/all-*trans*-β-carotene is postulated as an alternative mechanisms The next step is then catalyzed by CCD7 enzyme which transform the 9-*cis*-β-carotene into 9-*cis*-β-apo-10′-carotenal by cleavage

at the C9′-C10′ position (Fig. 9.3). Afterwards, CCD8 catalyzed the conversion of 9-*cis*-β-apo-10′-carotenal into CL forming the A and D ring and the enol-ether bridge (Alder et al. 2012). Characterization of CCD7 and CCD8 enzymes were done due to the availability of several branching and dwarf mutants in *A. thaliana*, pea (*Pisum sativum*), rice (*Oryza sativa*) and petunia (*Petunia hybrida*) affected in SL biosynthesis or perception (Stirnberg et al. 2002; Sorefan et al. 2003; Ishikawa et al. 2005; Simons et al. 2007; Drummond et al. 2009). Four *A. thaliana max* (*more axillary branching*) mutants and eight *d* (*dwarf, cell elongation diminuto*) or *htd* (*high tillering dwarf*) rice mutants have been characterized at the molecular level as well as pea *rms* (*ramosus*) and petunia *dad* (*decrease apical dominance*) mutants. CCD cleavage activity is frequently investigated *in vivo* or *in vitro*, using either *E. coli* strains engineered to accumulate specific carotenoids or adding the initial precursors and required enzymes in the plant. *E. coli* strains created to accumulate carotenoids generally produce all-*trans*-configured carotenoids. However, carotenoids and apocarotenoids are exposed to photo- and thermo-isomerization in *E. coli* cells, which might cover the stereospecificity of the introduced cleavage enzymes (Al-Babili and Bouwmeester 2015). For this reason, activity of CCD7 enzymes was revisited, using *in vitro* assays adding the pure stereoisomer substrates (Alder et al. 2012). The results showed that CCD7 enzyme cleaves exclusively 9-*cis*-β-carotene (and no other kind of isomers) to produce 9-*cis*-configured-β-apo-10′-carotenal (Alder et al. 2012). The configuration has recently been confirmed by NMR analysis (Bruno et al. 2014). The same experimental approach permits to conclude that CCD8 enzymes converted the 9-*cis* isomer into noncarbonyl, trioxygenated C_{19} compound identical to known SLs in the number of C atoms and the presence of a butenolide ring, which is the invariable part of the SLs backbone (D-ring), coupled with an enol ether bridge (Alder et al. 2012). This new product, called CL, was a major finding to successfully continue the research on SLs. This discovery demonstrated that CCD8 catalyzes two types of reactions: the typical carotenoid cleavage reaction with the all-*trans* substrate and a preferred, complex combination of reactions that includes the addition of three oxygen molecules and rearranging the backbone to form the A-ring, the characteristic D-ring and the enol-ether bridge (Alder et al. 2012). The exact mechanism in which CCD8 produces CL is not well understood; however it was proven that its skeleton is entirely derived from 9-*cis*-β-apo-10′-carotenal (Fig. 9.3) and the oxygen molecules were taken from the atmosphere (Alder et al. 2012). With these results a mechanism explaining CL formation was proposed where CCD8 catalyzes a combination of different reactions including cis/trans isomerization, repeated oxygenation and Baeyer-Villiger-like rearrangements. After the cleavage of all-*trans* and 9-*cis* substrates a second unidentified product should be produced with C_9 structure (all-*trans*-β-apo-10′-carotenal C_{27} minus β-apo-13-carotenone C_{18}) and C_8 product (9-*cis*-β-apo-10′-carotenal C_{27} minus carlactone C_{19}), respectively (Al-Babili and Bouwmeester 2015). Nevertheless, the high instability showed by both products could be the reason why they have not been so far detected. CL was proposed as an intermediate that acts as a bridge between carotenoid and

the classical SLs with the ABC-D structure. Adler *et al*. (2012) reported that CL suppressed the shoot branching phenotype of rice in the SL biosynthetic mutant *d10* and *d27*, as well as stimulated the germination of *Striga hermonthica* seeds, suggesting that CL is a biosynthetic precursor for SLs (Alder et al. 2012). Moreover, a recent report showed the conversion of ^{13}C-labeled CL to accordingly labeled, tricyclic lactone-containing SLs in rice (Seto et al. 2014). In addition, endogenous CL was detected in rice and Arabidopsis root extracts and shown to occur only in the 11R configuration (Seto et al. 2014), consistent with the C-2′ configuration in tricyclic lactone-containing SLs. In the same work, Seto et al. (2014) reported the identification of CL in rice by using liquid chromatography-quadruple/time-of-flight tandem mass spectrometry (LC-MS/MS) and its stereochemistry which was consistent with the stereochemistry present in all known SLs. With all these data CL was confirmed as the precursor of the SLs in rice and Arabidopsis. Afterwards, the cytochrome P450 (CYP) MAX1, takes carlactone to redirect the synthesis to the SL pathway (Fig 9.3). Grafting experiments in which a *max1* rootstock fully rescued the *d27* branching phenotype proved that D27 acts upstream of MAX1 (Waters et al. 2012). CYPs are heme-containing monooxygenases that catalyze a wide variety of reactions including epoxidation, alkylation, oxygenation and C- hydroxylation among others (Isin and Guengerich 2007). MAX1 is a member of the CYP711 family from Arabidopsis and contains an endoplasmic reticulum membrane anchor, suggesting a cytosolic localization in contrast of the plastid-localized D27, CCD7 and CCD8 enzymes (Al-Babili and Bouwmeester 2015). First results showing the implication of MAX1 in conversion of CL into SL came from reciprocal grafting experiments, where *max1* rootstocks grafted either to *d27*, *max3*, or *max4* scions have wild type levels of branching, whereas reciprocal grafts are highly branched (Booker et al. 2004; Waters et al. 2012). These findings support the idea of a mobile SL intermediate located downstream of the D27 and carotenoid cleavage dioxygenases. *MAX1* is expressed in the cambial region and xylem-associated parenchyma while *MAX3* and *MAX4* expression is highest in root tips, cortex, and hypocotyls (Drummond et al. 2009) (Booker et al. 2004). This may suggest that *MAX1* may function during loading and unloading of the mobile SL precursor from the xylem. Due to the evidence that CL over accumulates in *max1* mutant and is unable to rescue *max1* shoot phenotype it was proposed that CL is the prime candidate for the mobile precursor acting between *MAX4* and *MAX1* (Scaffidi et al. 2013; Seto et al. 2014). Considering the CL structure (Fig. 9.3), just few steps are necessary, including further oxidations at positions C_{19} and C_7, to convert it into a simple SL (Alder et al. 2012). Due to the absence of SL biosynthetic candidate genes, *MAX1* has been proposed to catalyze these steps. However, it is still possible that unidentified enzymes are involved in this reaction and in further steps to give rise to the whole gamma of SLs (Ruyter-Spira et al. 2011; Seto et al. 2012). The detection of some SLs such as Orobanchol and orobanchylacetate was reported in Arabidopsis (Kohlen et al. 2011); however, Seto et al. could not detect these SLs using LC-MS/MS, probably due to the low abundance of these compounds (Seto et al. 2014). Instead, another SL-like compound called SL-LIKE1 was found in

Arabidopsis root extracts (Seto et al. 2014). Interestingly, feeding experiments of ^{13}C-CL using SL-deficient *max4* and *max1/max4* Arabidopsis mutants showed that CL is converted into SL-LIKE1 in a reaction in which *MAX1* is involved. With these results the participation of MAX1 in the conversion of CL to SL was confirmed. Moreover, high levels of CL were detected in the *max1* mutant, suggesting that CL is a substrate for MAX1 (Seto et al. 2014). Although *max1* mutant accumulates high levels of CL, it shows the branching phenotypes as do *max3* and *max4* mutants, and SL treatment can restore their phenotype (Seto and Yamaguchi 2014). The evidence mentioned above in addition with the fact that exogenously applied CL did not rescue the branching phenotype of *max1* but did rescue that from *max4* (Scaffidi et al. 2013), indicate that CL is a biological inactive precursor for SLs shoot branching inhibition.

Interestingly, in rice there are 5 homologs to MAX1, including a non-functional mutated version (Challis et al. 2013). Expression of *OsD27*, *OsCCD7* (D17) and *OsCCD8* (D10) resulted in the accumulation of only 11*R*-configured CL (Zhang et al. 2014). When the *MAX1* homologs, *Os900*, were co-expressed with the genes involved in CL production, the content of the CL product decreased dramatically accompanied by the formation of 4-deoxyorobanchol (Fig. 9.3). In addition, experiments performed in microsomes isolated from a yeast strain expressing *Os900* showed the same results. With this evidence *Os900* was confirmed as a carlactone oxidase that produces the parent SL of the Orobanchol-like SL subfamily by catalyzing the stereospecific formation of the B-C moiety (Fig. 9.3). The discovery of the carlactone oxidase completes the elucidation of the biosynthesis of a parent ABC-ring-containing SL from β-carotene.

However, the residual amounts of SLs in different SLs biosynthetic mutants, suggested the possibility of alternative route(s) for the production of this hormone. The roots of *max1* and *max4*/*ccd8* mutants have between three- to five-fold less orobanchol than the roots in the wild type (Kohlen et al. 2011). Same pattern is observed in *rms1*/*ccd8* mutants where orobanchol and orobanchyl acetate are not detected, although detectable levels of fabacyl acetate are present in root exudates (Foo and Davies 2011). Moreover, in rice, *d17*/*ccd7* and *d10*/*ccd8* mutants have nearly undetectable 2′-epi-orobanchol levels but one of the *d10* alleles has detectable levels of 2′-epi-orobanchol or its isomer (Umehara et al. 2008; Lin et al. 2009). The behavior of these mutants suggests that they could produce, at some extend, some SL species. It is unlikely that this amounts of SLs produced in the mutants is due to the leakiness of the mutations, because mutants carrying a full deletion of *CCD8* gene are still capable of produce detectable levels of SL strigol in mosses (Proust et al. 2011).

The overexpression of *MAX2* in Arabidopsis partially reduced the branching phenotype in *max1*, *max3* and *max4* mutants (Stirnberg et al. 2007). This could be due to a ligand-independent signaling or to a MAX3/MAX4/MAX1-independent source of SL, consistent with the detection of orobanchol in some of these mutants (Kohlen et al. 2011). Interestingly, an endogenous butenolide signal, mimicked by smoke-derived karrikin compounds, and closely related to SLs was

proposed to function in a MAX3/MAX4/MAX1-independent manner (Fig. 9.4) (Nelson et al. 2011; Waters et al. 2012; Scaffidi et al. 2013). Recently, evidence showing different SL-related and non-SL compounds with germination stimulation activity has been shown. For instance, non-SL sesquiterpene lactone compounds with Orobanche germination stimulation activity were reported in *Helianthus annus* (sunflower) (Raupp and Spring 2013).

With the findings mentioned above enough information to understand the biosynthesis of SLs was obtained; however, there are still some enzymes from the last step in SL conversion to be uncover.

9.3.3 Regulation of Strigolactone Biosynthesis: Factors Affecting SL Biosynthesis and Spatial Distribution

As is usual for other phytohormones and biosynthetic pathways, the activity and biosynthesis of SLs is regulated not only by a complex network of interactions with other hormones but also by nutrient availability and abiotic stress. Auxin regulates SL biosynthesis and is involved in most of the SL-regulated developmental processes (Al-Babili and Bouwmeester 2015) and several lines of evidence also suggest a link between ABA and SLs. Moreover, adaptation of the SL biosynthetic capacity is an important element in the response of plants to environmental changes. Therefore, it is important to know how plants are responding to different SL signals and how plants are regulating this pathway to achieve a better performance when faced with changes in the environment.

9.3.4 SL Transport

Grafting experiments in Arabidopsis, petunia and pea, have shown that SLs can be transported exclusively from the root to the shoot (Beveridge et al. 1996; Napoli 1996; Booker et al. 2004; Simons et al. 2007); however, this process still remains largely unknown. Recently, SLs have been detected in the xylem sap of tomato and Arabidopsis, which is consistent with the unidirectional shoot ward movement (Kohlen et al. 2011). In petunia, SL exudation into the soil is known to require the Pleiotropic Drug Resistance 1 (PDR1) protein (Fig. 9.4) (Kretzschmar et al. 2012). This is the only case reported to date of a protein with SL transport function, supported by mutant and over expresser lines, that possess altered SL exudation. PDR1 is a component of the ATP Binding Cassette (ABC) family of transporters that have been implicated in the transport of several plant hormones such as ABA and auxin (Petrasek and Friml 2009; Kang et al. 2010; Kuromori et al. 2010; Foo and Davies 2011). *PDR1* expression pattern has the highest peak in the root tip, where SL biosynthesis gene expression is also highest, and in passage cells

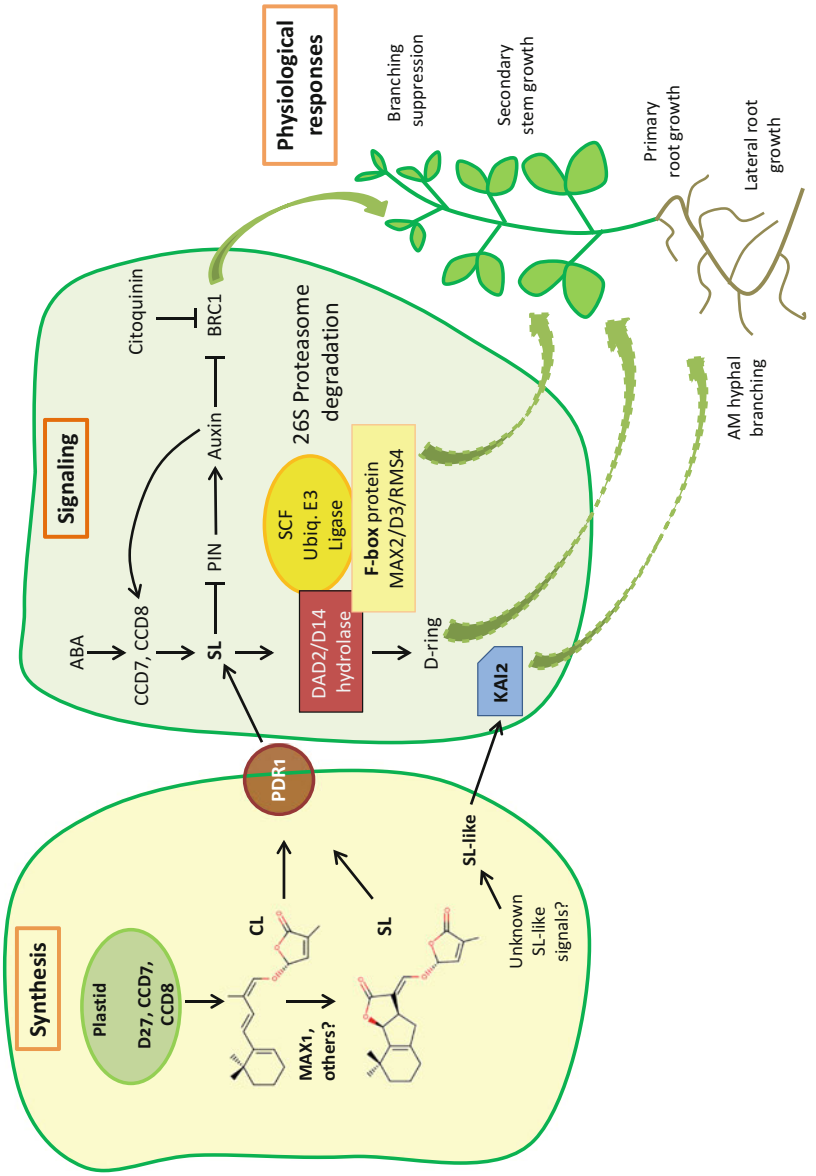

Fig. 9.4 (continued)

further up the root, where AM hyphal entry usually occurs (Sharda and Koide 2008). Surprisingly, no ortholog for PDR1 was found in Arabidopsis and the closest homologue is the ABA transporter ABCG40 (Kang et al. 2010). The lacking of a SL transporter, in contrast with the presence of synthesis and signaling components in Arabidopsis, should be related with the absence of mycorrhization. The fact that Arabidopsis exudates contain relatively low levels of SL compared with other species (Kohlen et al. 2011) supports the idea exposed above. However, the scenario for SLs transport in shoots is not as clear as in roots. In petunia, PDR1 is expressed in the nodes and stems, but not in dormant buds (Waldie et al. 2014) and it has increased bud outgrowth but only at basal nodes. However, the high expression levels of *PDR1* near the axillary buds suggest that SLs have a direct role in inhibiting their outgrowth. Whether this requires SLs production in the root with subsequent transport to the shoot or local SL production (or both) remains unclear.

9.3.5 SL Perception

The complete signaling cascade of the SL transduction process has not been completely elucidated so far, but there is some important progress in this topic. Possible perception signaling components have been identified from the characterization of SL-insensitive mutants. With this information, the internal perception of SLs by plant receptor complexes was characterized to quite some detail (Bennett and Leyser 2014; Waldie et al. 2014). The core perception component of the Skp1, Cullin, F-box (SCF)-containing receptor complex is an F-box domain protein represented by MAX2 of Arabidopsis. Orthologues of *At*MAX2 were also found in rice, named Dwarf 3 (D3), and in pea, as the Ramosus 4 (RMS4). *MAX2*/*D3*/*RMS4* encodes an F-box protein (Fig. 9.3), which is known to be the substrate recognition subunit of the SCF ubiquitin E3 ligase for proteasome-mediated proteolysis (Ishikawa et al.

Fig. 9.4 (continued) Schematic representation of SLs biosynthesis, signal transduction and responses in the plant. At the left, the major pathway for SL synthesis is depicted with three plastid-localized enzymes D27, CCD7 and CCD8, which are required for CL synthesis. MAX1 and possibly other enzymes are involved in the conversion of CL to the simplest SL (5-deoxystrigol). The presence of an alternative biosynthetic pathway may contribute to the SLs pool. Unknown SL-signals might be present in plants. PDR1, the SL transporter, is the intermediary between signal and transduction. In the middle, signal transduction process is represented. In the nucleus, MAX2 and D14 are involved in SL perception and signaling transduction. There is also a proposed MAX2-independent signaling possibly via KAI2. Interaction of D14 with MAX2 lead to SCF Ubiquitin E3 ligase recruitment and 26S proteasome degradation. SL also reduces PIN1 levels in xylem parenchyma cells, accompanied by a reduction in polar auxin transport BRC1 up regulation. Auxin and ABA induce SL synthesis through the induction of *CCD7* and *CCD8* genes. Citoquinin promotes SL effect by inhibiting BRC1. The transduction of the signals trigger shoot branching inhibition, secondary stem growth, leaf senescence, lateral and primary root growth, AM hyphal branching, hypocotyl elongation and seed germination

2005; Stirnberg et al. 2007). Moreover, none of these mutants can be complemented by exogenous addition of SLs, suggesting that all of them function in a similar way. The F-box is a leucine-rich repeat protein, which was suggested to act locally at the node in branching suppression (Stirnberg et al. 2007). The key step in SLs perception process seems to be the hydrolysis of SLs by a member of the α/β hydroxylase superfamily represented by D14 protein of rice and Decreased Apical Dominance 2 (DAD2) of petunia (Fig. 9.3). Map-based cloning confirmed that the *D14* gene encodes a protein that belongs to the α/β-hydrolase family (Arite et al. 2009). D14 hydrolase and their orthologs cleave off the D-ring from the full size SL molecule and this D-ring does indeed seem to be the signal-bearing part of the SL molecule, as D-ring-like molecules alone can set off, at least in part, SLs responses (Walter et al. 2015). SL binding and cleavage by DAD2 is a precondition for physical interaction of DAD2 with MAX2, initiating an SCF-mediated signal transduction cascade (Hamiaux et al. 2012).

The protein D14 was proposed as a possible activating enzyme to transform SL into a bioactive molecule. Moreover, characterization of the DAD2 from petunia revealed that the α/β-hydrolase showed both enzymatic and receptor activities, and suggested a novel mechanism of action of SL on its target protein (Hamiaux et al. 2012). Interestingly, the gibberellin receptor GID1 also belongs to the same protein family (Ueguchi-Tanaka et al. 2005) supporting the latest findings by Hamiaux et al. which proposed the D14 protein as a SL receptor. Crystallographic analysis showed that D14 and its orthologs of petunia (DAD2) and Arabidopsis (AtD14) present a structurally conserved catalytic triad (Ser, His, Asp), which is necessary for the hydrolysis (Hamiaux et al. 2012; Kagiyama et al. 2013; Nakamura et al. 2013). In addition, biochemical analysis of these recombinant proteins showed that they can catalyze the hydrolysis of a synthetic SL analog called GR24 (Hamiaux et al. 2012; Nakamura et al. 2013). However, yeast-two-hybrid experiments showed that DAD2 interacts with *Ph*MAX2 from petunia in a GR24-independent manner (Hamiaux et al. 2012).

It has been proposed that MAX2/D3/RMS4-mediated protein degradation and/or ubiquitination is integral to SL perception and signaling. Like D14, mutations in *MAX2/D3/RMS4* results in a highly branched phenotype, which cannot be suppressed by exogenous SL addition or by grafting to wild type roots (Beveridge et al. 1996; Ishikawa et al. 2005; Gomez-Roldan et al. 2008; Drummond et al. 2009), and indeed clonal analysis in Arabidopsis suggests that MAX2 functions in a largely cell-autonomous manner (Stirnberg et al. 2007). Unlike *d14*, *max2/d3/rms4* mutants have a range of additional phenotypes, such as an elongated hypocotyl and reduced germination, which have been attributed to its role in KAI2-dependent signaling (Fig. 9.4) (Stirnberg et al. 2002; Nelson et al. 2011; Waters et al. 2012; Scaffidi et al. 2013). KAI2 is supposed to be the receptor for an SL-like compound and to have been co-opted as the karrikin receptor in the fire-following species (Fig. 9.4).

As it was mentioned before, MAX2 encodes a member of the F-box protein family, (Woo et al. 2001; Stirnberg et al. 2002). Commonly, protein ubiquitination results in protein degradation by the *26S* proteasome, but it is also possible that this cause a modification in protein activity or changes in protein localization (Vierstra

2012). MAX2 has been shown to be part of the SCF complex, and mutations in the F-box domain results in MAX2 lost of function causing a weak dominant negative phenotype (Stirnberg et al. 2007). There are over 700 F-box proteins encoded by the Arabidopsis genome, suggesting that regulated ubiquitination is a common signaling mechanism in plants (Waldie et al. 2014). To date, the same signaling mechanism for other plant hormones has been reported. For instance, GID1 receptor, which interacts with gibberellin (GA) leading binding with members of the DELLA family of transcriptional regulators, which in turn leads to the interaction of this complex with $SCF^{GID2/SLY1}$, resulting in the ubiquitination and degradation of the DELLASs and induction of GA-induced genes (Ueguchi-Tanaka et al. 2005; Waldie et al. 2014). Therefore, some speculations about SLs signaling occurring in a very similar manner have been proposed. Recently, evidence for this hypothesis, have been collected in rice and *Petunia*. It was shown that the synthetic SL GR24 promotes the interaction of DAD2/D14 with D3 and *Ph*MAX2 (one of the two MAX2 orthologues in *Petunia*) in yeast-two-hybrid and *in vitro* pulldown assays (Hamiaux et al. 2012; Jiang et al. 2013; Zhou et al. 2013). These two genes have similar expression patterns and they are generally highly expressed in xylem associated cells in the leaf vasculature (Ishikawa et al. 2005). MAX2 is primarily nuclear localized (Fig. 9.4) (Stirnberg et al. 2007; Dong et al. 2013) and the generation and analysis of D14-GFP reporter lines showed both nuclear and cytoplasmic signals in Arabidopsis and rice protoplasts, suggesting that the interaction between D14-MAX2 is possible *in planta* (Fig. 9.4) (Nakamura et al. 2013; Zhou et al. 2013); however, Wang et al. (Wang et al. 2013) reported that there is no interaction between MAX2 and D14 in Arabidopsis.

9.3.6 Strigolactones and Other Plant Hormones: "An Intricate Signaling Network"

SLs are part of a phytohormone network controlling plant development. In this process SLs are involved in hormonal crosstalk during root development. When plants are facing detrimental conditions, SLs repress lateral root formation (Kapulnik et al. 2011b; Ruyter-Spira et al. 2011) and promote root hair elongation (Kapulnik et al. 2011b). Auxin plays an important role in lateral root formation as a key regulator and its distribution determines lateral root positioning, initiation and elongation (De Smet 2012). SLs could affect lateral root formation via changes in auxin efflux in the root. Polar auxin transport (PAT) determines local auxin concentrations and gradients, which also determines its activity. This is a dynamic and specialyzed system that controls the cell-to-cell movement of auxin. The presence and direction of PAT are controlled by the tissue-specific and polarly distributed Pin-Formed (PIN) efflux carrier proteins (Al-Babili and Bouwmeester 2015). In vascular cells, SLs decreased PAT by removing PIN1 from the plasma membrane (Fig. 9.4) (Shinohara et al. 2013), reducing axillary bud outgrowth as a consequence of

inhibition of auxin export from the axillary buds (Crawford et al. 2010). In addition, when SLs are exogenously supplemented into transgenic PIN1-GFP plants, they interfere with PIN auxin-efflux carriers (Koltai et al. 2010), and reduce PIN1-GFP intensity in lateral root primordia. Therefore, SLs might alter the auxin optimum needed for lateral root formation (Ruyter-Spira et al. 2011). However, the inhibitory effect of SLs can be also explained by the induction of transcription factor-encoding genes such as Arabidopsis Branched 1 (BRC1)/Teosinte Branched 1-Like1 (Fig. 9.4), whose expression negatively correlates with bud outgrowth (Aguilar-Martinez et al. 2007; Mashiguchi et al. 2009). Based on the analysis of mutants lacking proper auxin and SLs signaling, it was demonstrated that auxin signaling acts downstream of SLs in roots (Kapulnik et al. 2011b; Mayzlish-Gati et al. 2012). Auxin is also a major regulator of SL biosynthesis, maintaining the basal transcript levels of *CCD7* and *CCD8* (Al-Babili and Bouwmeester 2015). In pea, when auxin sources are removed, either by decapitation or by detaching young upper leaves, in combination with the application of auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA), a considerable decrease in *RMS5 (CCD7)* and particularly *RMS1(CCD8)* transcript levels is observed in the upper part of the stem, which could be rescued by supplying auxin to the decapitated plants (Foo et al. 2005; Johnson et al. 2006).

Similarly, cytokinins positively regulate axilary bud outgrowth and act antagonistically to SLs by supressing *Ps*BRC1 transcription (Dun et al. 2012). Moreover, they can negatively affect lateral root formation, possibly due to interference with auxin transport in lateral root primordia (Bishopp et al. 2011). SLs and cytokinins also act as supressors of the adventitious formation of roots from non-root tissues (Li et al. 2009; Rasmussen et al. 2012a). SLs mutants of pea and Arabidopsis with decreased cytokinin levels in the xylem (Beveridge et al. 1997; Foo et al. 2007), present higher level of adventitious root formation. However, recent evidence on response to hormone analysis in mutant plants, showed that SLs and cytokinins act independently on adventitious rooting (Rasmussen et al. 2012a). ABA, has been also reported to affect SLs levels (Schwartz et al. 1997; Burbidge et al. 1999). SLs and ABA derived from 9-*cis*-configured carotenoids (Fig. 9.1) and therefore is tempting to speculate that they interact with each other at the biosynthetic level. However, recent evidence testing *in vitro* activity for OsD27 and *At*CCD7/*Ps*CCD7 did not provide any information about isomerization of violaxanthin and neoxanthin by *Os*D27 (M. Bruno & Al-Babili, unpublished data), or about the cleavage of 9-*cis*-violaxanthin by CCD7 from Arabidopsis and pea (Bruno et al. 2014). This data support the idea that SL biosynthetic enzymes do not interfere with ABA biosynthesis, and up to date, no evidence for the cleavage of the SL precursor 9-*cis*-β-carotene by NCEDs have been reported. Nevertheless, there are reports on disruption of ABA biosynthesis that showed a decrease in SL production. For example, *vp14* mutants from maize and tomato (*notabilis*) that are deficient in ABA synthesis, have also a lower SL content (Schwartz et al. 1997; Burbidge et al. 1999; Matusova et al. 2005; Lopez-Raez et al. 2010). The decrease in SL content observed in these mutants is probably due to lower transcript levels of the SL biosynthetic

genes *CCD7* and *CCD8* (Lopez-Raez et al. 2010), suggesting that SL biosynthesis is regulated by ABA. This was supported with experimental data where application of exogenous ABA led to a clear increase after one hour of *CCD7* and *CCD8* in Arabidopsis (Ha et al. 2014). On the other hand, SLs can also have an effect on ABA synthesis. Heat stress experiments, with temperatures ranging from 32 to 34 °C, inhibit the germination of Arabidopsis seeds owing to an ABA-dependent biosynthesis induction and a represion of GA biosynthesis and signaling (Toh et al. 2008). These seeds showed a decrease in ABA content (by lowering the induction of the ABA biosynthetic gene *NCED9*) and increased GA4 content (Toh et al. 2012). Exogenous application of GR24 to the seeds, alleviated this phenomenom. Similar results were obtained after GR24 application to imbibed *Striga hermonthica* seeds reducing ABA/GA ratio and causing a pronounced increase in cytokinin *trans*-zeatin content (Toh et al. 2012). When this experiment was performed in *max1* and *max2* mutants the thermoinhibition was much more pronounced (Toh et al. 2012).

9.4 Conclusions

Carotenoid cleavage molecules, named as apocarotenoids, are mainly derived from lycopene, β-carotene and zeaxanthin. Most of them present biological important features in plants, such as aroma, repellents, chemoattractants, hormones and pigments. In the last few years big progress has been made in our understanding of apocarotenoid biosynthesis, signal transduction, and its biological functions. Among them, SL has been one of the main research focusses. The recent discovery of MAX1, involved in 4-deoxyorobanchol synthesis, completes the biosynthetic pathway of this hormone. It is also possible to categorize the SLs more accurately, with one SLs group sharing the ABC ring-containing strigol-like or orobanchol-like compounds and a second group containing a β-ionone instead the tricyclic lactone, such as carlactone (CL). The identification of the SL intermediate CL was a major breakthrough in the apocarotenoid biosynthesis pathway and this finding contributed to the further elucidation of the enzymes involved in the last steps of the SL production. The main question to be resolved is to determine the biological function of CCD7 and CCD8, especially if the synthesis of β-*apo*-13-carotenal also occurs *in planta* and the natural SL molecule that triggers the *in vivo* response. It is important to note that SLs functions are extremely related with other hormones, such as auxin, citokinin and ABA, suggesting a main regulatory network between plant hormones in the shoot and root branching mechanisms and in plant architecture.

Without no doubt, new mutants will permit the identification of other factors involved in apocarotenoid perception, biosynthesis and signaling in plants.

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Chapter 10
Plastids and Carotenoid Accumulation

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Abstract Plastids are ubiquitously present in plants and are the organelles for carotenoid biosynthesis and storage. Based on their morphology and function, plastids are classified into various types, i.e. proplastids, etioplasts, chloroplasts, amyloplasts, and chromoplasts. All plastids, except proplastids, can synthesize carotenoids. However, plastid types have a profound effect on carotenoid accumulation and stability. In this chapter, we discuss carotenoid biosynthesis and regulation in various plastids with a focus on carotenoids in chromoplasts. Plastid transition related to carotenoid biosynthesis and the different capacity of various plastids to sequester carotenoids and the associated effect on carotenoid stability are described in light of carotenoid accumulation in plants.

Keywords Etioplasts • Chloroplasts • Amyloplasts • Chromoplasts • Carotenoid stability

10.1 Introduction

Plastids are ubiquitously found in higher plants. They are essential organelles that are believed to have evolved from the endosymbiosis of an ancestral cyanobacterial progenitor. During evolution, most of the ancestor genes are transferred to the nucleus and only a small number was left in the plastid genome. Although plastids of a plant possess the same plastid genome, they vary greatly in their morphology and function within the plant following the import of many nucleus-encoded proteins. Based on their morphology and function, plastids are classified into a number of subtypes, i.e. proplastids, etioplasts, chloroplasts, leucoplasts, and chromoplasts (Lopez-Juez and Pyke 2005).

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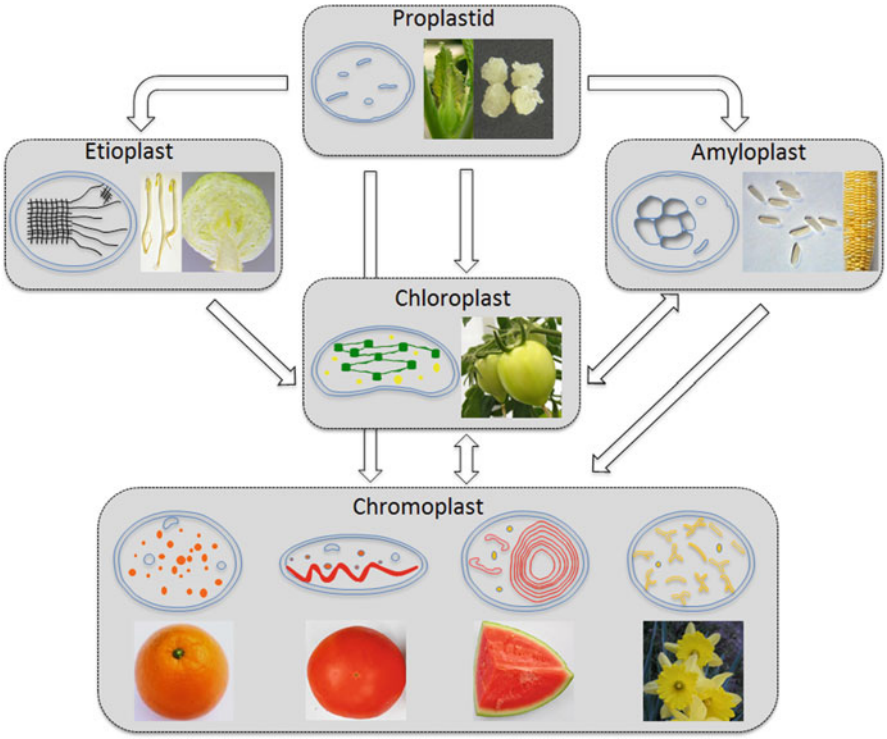

Fig. 10.1 Major plastid types and their interconversion in plant. Proplastid found in meristematic tissues can differentiate into all kinds of other plastids. Proplastid is small, undifferentiated plastid and does not have the capacity to synthesize carotenoids. Etioplast occurs during dark growth of plants and is regarded as a transient stage in chloroplast formation (Fig. 10.1). Etioplast is characterized with membranous pro-lamellar and synthesizes low level of carotenoids. Amyloplast is filled with starch in storage tissues and of great agricultural importance. Amyloplast can be converted into chloroplast or chromoplast, and synthesizes low to mediate level of carotenoids. Chloroplast is the most prominent plastid in green tissue and has distinctive thylakoid discs. Chloroplast can interconvert into amyloplast or chromoplast during ripening and synthesizes high level of carotenoids coordinately with chlorophyll synthesis. Chromoplast is loaded with carotenoid pigments. Chromoplast is classified into globular, crystalline, membranous and tubular types based on the carotenoid-lipoprotein sequestering structures. Chromoplast possesses unique mechanisms to synthesize and accumulate massive amounts of carotenoids, giving the vivid orange, red and yellow color in many flowers, fruits and vegetables (Fig. 10.1)

Various plastids are enriched in different tissues with unique properties. Proplastids are small, colorless, and undifferentiated plastids abundant in cells of meristematic tissues of apical shoot, root tips, and developing young fruits as well as of reproductive tissues. All plastids are derived from proplastids. Despite of their importance, not much is known about proplastid biology due to the difficulty in isolating them. Electron microscopy analyses provide an estimate of 10–20 proplastids per meristematic cell (Lopez-Juez and Pyke 2005). Etioplasts

occur during dark growth of plants and are regarded as an intermediary stage in the chloroplast formation from proplastids. Etioplasts are characterized by the presence of a unique inner membrane network of the prolamellar body and by the absence of chlorophylls. While the etioplast stage is frequently transient under natural conditions, etioplasts are believed to be the plastids in some special tissues such as cabbage heads (Solymosi and Schoefs 2010). Chloroplasts are the plastids that define plants. Chloroplasts contain the green pigment chlorophylls and have distinctive internal thylakoid discs. They are found in green leaves and other green tissues. Chloroplasts are the best characterized plastids due to their importance to the overall plant metabolism and plant survival. Leucoplasts are the general term for a group of differentiated and colorless plastids, which include amyloplasts, elaioplasts, and proteoplasts. Amyloplasts are of great economic and agricultural importance as they accumulated starch, which provides the major human energy. Amyloplasts are enriched in many storage tissues, such as in the endosperm of many seeds particularly of cereal seeds, in tubers, in root vegetables, and in some fruits. Amyloplasts generally arise from proplastids but also can be derived from other plastid types (Lopez-Juez and Pyke 2005). Elaioplasts and proteoplasts store lipids and proteins, respectively. Elaioplasts are filled with numerous globules or plastoglobules and enriched in oil seeds. Proteoplasts harbor crystalline, fibrillary, or amorphous masses of proteins. Both elaioplasts and proteoplasts are much less studied plastids in plants. Chromoplasts are characterized with high level accumulation of carotenoid pigments. The vivid red, orange and yellow colors in many flowers, fruits, and vegetables own their hue to the presence of carotenoids in chromoplasts. Chromoplasts are often derived from chloroplasts during green fruit ripening and also arise directly from proplastids or other non-colored plastids during fruit development and root growth (Li and Yuan 2013; Yuan et al. 2015).

Plastids fulfil various important roles in plant metabolism. Apart from their well-known functions, such as photosynthesis in chloroplasts, carotenoid metabolism in chromoplasts, and starch biosynthesis in amyloplasts, plastids are also the major sites for synthesis of many primary and secondary metabolites, and assimilation of nitrogen and sulfur (Kuntz and Rolland 2012; Lopez-Juez 2007; Neuhaus and Emes 2000). Proplastids as undifferentiated plastids in meristematic tissues are able to synthesize amino acids for protein synthesis, fatty acids for membrane lipid proliferation, and nucleotide precursors for DNA and RNA synthesis (Brautigam and Weber 2009). Although etioplasts are characterized by the absence of chlorophylls, etioplasts synthesize chlorophyll precursor (pchlide), carotenoids, and metabolites to be ready for the establishment of photosynthesis once illumination with light (Solymosi and Schoefs 2010). Chloroplasts serve not only the site of fixation of CO_2 for photosynthesis, but are also responsible for the synthesis of amino acids, fatty acids, and many secondary metabolites, as well as for the storage of starch and oil compounds (Jensen and Leister 2014; Linka and Weber 2012). Similarly, chromoplasts are found to contain the enzymes involved in the synthesis and metabolism of many plastid metabolites such as amino acids and fatty acids in add to carotenoid biosynthesis (Egea et al. 2010; Li and Yuan 2013). The same is true for amyloplasts although they are primarily responsible for the synthesis and storage

of starch (Dupont 2008). While the photosynthetic chloroplasts are autotrophic, the other non-photosynthetic plastids are heterotrophic. All plastids are surrounded by double envelope membranes and retain a semi-autonomous character. In addition, plastid types are inter-convertible in response to developmental or environmental cues.

Plastids are the site for carotenoid biosynthesis and storage. Carotenoids as a group of widely distributed pigments are *de novo* synthesized in all types of plastids except proplastids (Howitt and Pogson 2006; Shumskaya and Wurtzel 2013). As carotenoid biosynthetic enzymes are either membrane associated or membrane proteins, carotenoid biosynthesis is believed to be carried out in plastid membranes (Cazzonelli and Pogson 2010; Li and Yuan 2013; Nisar et al. 2015; Ruiz-Sola and Rodriguez-Concepcion 2012; Yuan et al. 2015). Proteomics research reveals that plastoglobules isolated from chromoplasts also contain carotenoid biosynthesis enzymes (Ytterberg et al. 2006). These studies suggest ability of plastoglobules for carotenoid biosynthesis in addition to their functions in carotenoid sequestration and storage.

Different types of plastids possess different capacity to synthesize and sequester carotenoids. Thus, the type of plastids exerts profound effect on carotenoid accumulation and stability (Li and Yuan 2013; Ruiz-Sola and Rodriguez-Concepcion 2012). Due to the negative regulation of phytoene synthase (PSY) gene expression by phytochrome-interacting factors abundant in the etiolated tissues (Toledo-Ortiz et al. 2010), etioplasts in the etiolated tissues exhibit low biosynthetic activity and contain very low levels of carotenoids. The majority of the carotenoids synthesized are lutein and violaxanthin (Rodriguez-Villalon et al. 2009; Welsch et al. 2000), which give yellowish color of etioplasts and the etiolated tissues. Many storage tissues of important crops, such as wheat, maize, potato, and cassava synthesize and accumulate low to mediate levels of carotenoids in amyloplasts of seeds and roots (Howitt and Pogson 2006). In many cases, xanthophylls (i.e. lutein, zeaxanthin, and violaxanthin) are the major accumulated carotenoids to yield yellow color. Carotenoids are found in the amyloplast envelope membranes (Lopez et al. 2008). The lack of appropriate lipoprotein sequestering substructures in the amyloplasts may restrict their capacity to significantly accumulate and stably store carotenoids (Li et al. 2012). Carotenoids are essential components of photosynthetic complexes in chloroplasts of leaves and other green tissues. Carotenoid biosynthesis is believed to occur in the envelope and the thylakoid membranes in chloroplasts (Cazzonelli and Pogson 2010; Ruiz-Sola and Rodriguez-Concepcion 2012). The formation of chlorophyll and carotenoid lipoprotein complex in thylakoid membranes enables to sequester and store the synthesized carotenoids for high level of accumulation in chloroplasts. Chromoplasts as carotenoid accumulating plastids develop unique mechanisms to synthesize and accumulate massive amounts of carotenoids. The carotenoid lipoprotein sequestering substructures and/or plastoglobules within chromoplasts promotes continuous carotenoid biosynthesis as well as sequestration and stable storage of synthesized carotenoids in chromoplasts (Li and Yuan 2013; Yuan et al. 2015). These unique features of chromoplasts enable massive accumulation of carotenoids found in many flowers, fruits and vegetables.

This chapter will describe carotenoid biosynthesis and storage in various plastids with a focus on carotenoids in chromoplasts. The plastid transition and carotenoid stability in different plastids will be discussed in light of carotenoid accumulation for crop nutritional quality improvement.

10.2 Carotenoids in Etioplasts

Etioplasts are considered as an intermediary stage in chloroplast formation under natural condition although most of the studies are carried out in dark-grown seedlings (Solymosi and Schoefs 2010). Etioplasts in the etiolated tissues *de novo* synthesize a set of carotenoids similar to that of green tissues with lutein and violaxanthin as the predominant carotenoids (Park et al. 2002; Welsch et al. 2000). These carotenoids accumulated in etioplasts are believed to be critical for the dark grown seedlings to optimize transition to photosynthetic development upon illumination and contribute to the adaptation of soil-emerging seedlings to sunlight (Rodriguez-Villalon et al. 2009). This is supported by the observation of Arabidopsis *ccr2* mutations, which cause delayed greening during photomorphogenesis (Park et al. 2002). The dark-grown *ccr2* mutants lack lutein and violaxanthin and do not produce the prolamellar bodies that define etioplasts (Cuttriss et al. 2007; Park et al. 2002). The findings also establish the specific role of these xanthophylls in prolamellar body assembly in etioplasts.

Carotenoid biosynthetic genes and enzymes are expressed in etioplasts in dark grown plants. However, they are regulated differently in etiolated tissues to control carotenoid biosynthesis. Phytoene synthase (PSY) is the key enzyme in the carotenoid biosynthetic pathway and the major determent of carotenoid level in plants (Cazzonelli and Pogson 2010; Nisar et al. 2015; Ruiz-Sola and Rodriguez-Concepcion 2012; Yuan et al. 2015). In etiolated tissues, PSY transcript level is low. Examination of its expression in responses to different light shows that *PSY* expression is mediated by phytochrome (von Lintig et al. 1997). More recent work reveals that it is phytochrome-interacting factors that negatively suppress *PSY* gene expression in dark-grown seedlings (Toledo-Ortiz et al. 2010). The phytochrome-interacting factors directly binds to the promoter of the *PSY* gene, resulting in suppressing *PSY* expression. In addition to transcriptional regulation under phytochome control, the topological localization of PSY protein also regulates its capacity in controlling carotenoid biosynthesis at enzymatic activity level. In etiolated seedlings, most of PSY protein are located within the prolamellar bodies and exhibit low enzymatic activity because of lacking competent membranes (Welsch et al. 2000). In contrast to PSY, the expression of phytoene desaturase (PDS), the next enzyme in the pathway, is not regulated by phytochrome and the PDS enzyme activity does not show topological redistribution at protein level. The transcript level of PDS along with geranylgeranyl pyrophosphate synthase (GGPS) is consistent in response to various light treatment (von Lintig et al. 1997). PDS

protein abundance remains relatively constant in the membrane even following expose to light (Welsch et al. 2000). As PSY is the first and rate-limiting enzyme in the carotenoid biosynthetic pathway, the low transcript level plus its topological localization within the prolamellar bodies are responsible for the low level of carotenoid accumulation in etioplasts.

Etioplasts are differentiated into chloroplasts during de-etiolation when dark-grown seedlings are exposed to light. During the transition, a strong increase in carotenoid content along with chlorophyll content leads to the formation of functional photosynthetic apparatus. The increased carotenoid accumulation is associated with up-regulation of *PSY* gene expression, protein level, and enzyme activity (Botella-Pavia et al. 2004; von Lintig et al. 1997; Welsch et al. 2000). The light induced *PSY* expression is uncovered to be due to a rapid de-repression of *PSY* gene expression following light-triggered degradation of the negative regulators phytochrome-interacting factors (Toledo-Ortiz et al. 2010). In addition, PSY activity is localized to the thylakoids membrane of chloroplasts following the establishment of photosynthetic apparatus (Welsch et al. 2000), which greatly enhances its capacity for carotenoid biosynthesis. Unlike PSY, the other carotenoid biosynthetic enzymes, i.e. GGPS and PDS, are constitutively expressed even carotenoid and chlorophyll contents increase greatly during the transition (von Lintig et al. 1997; Welsch et al. 2000). They are not regulated by the phytochrome-interacting factors (Toledo-Ortiz et al. 2010). However, a strong and coordinated increase in carotenoid production and the expression of *DXS* and *HDR*, two genes in the upstream of carotenoid biosynthetic pathway, are observed during deetiolation from etioplasts to chloroplasts (Botella-Pavia et al. 2004).

10.3 Carotenoids in Chloroplasts

Chloroplasts along with all other plastids are surrounded by two envelope membranes. In addition to these membranes, chloroplasts are unique in containing thylakoids, which are the sites for the light reaction of photosynthesis. Carotenoids serve as light-harvesting pigments and are ubiquitous protective agent in the membranes of chloroplasts. Carotenoid composition in photosynthetic leaf tissue of plants is remarkably conserved with lutein, β-carotene, violaxanthin, and neoxanthin occurring in decreased order (Pogson et al. 1998). The majority of carotenoids in photosynthetic tissues are localized in the thylakoid membranes with a small fraction of non-protein bound carotenoids acting as antioxidants in photosynthetic apparatus (Domonkos et al. 2013; Havaux et al. 2004). While β-carotene is part of the reaction center subunits of photosystem I and II, the xanthophylls are accessory pigments and structural units of light-harvesting complexes (Nelson and Ben-Shem 2004). In addition to serve as the essential structural components of photosynthetic complexes, carotenoids are critical for photoprotection of chloroplasts and for the assembly and stabilization of protein complexes in the thylakoid membranes. The formation of lipoprotein pigment complexes and the build-up of thylakoid

membranes along with the development of plastoglobuli in chloroplasts promote sequestration and storage of the synthesized carotenoids, resulting in high level of accumulation in chloroplasts.

While the majority of carotenoids are localized in thylakoids, the chloroplast envelope membranes are evidenced by chloroplast proteomics analysis to be the major site for carotenoid biosynthesis and metabolism, and the thylakoids appear to be restricted to xanthophyll cycle (Joyard et al. 2009). The suborganelle localization of carotenoid enzyme proteins is supported by data from biochemical, genetic and other studies (Ruiz-Sola and Rodriguez-Concepcion 2012). However, how carotenoids synthesized in the chloroplast envelope membranes are transported into the thylakoids remains unknown. Carotenoid biosynthesis in chloroplasts of green tissues occurs in a coordinated manner with other cellular processes for assembly of the photosynthetic apparatus. Thus, it is not surprising to find that defects in chlorophyll biosynthesis and chloroplast development result in reduced levels of carotenoid accumulation observed in many cases (Casanova-Sáez et al. 2014; Fambrini et al. 2004; Yu et al. 2012; Zhang et al. 2016). It is well established that carotenoid steady-state regulatory mechanisms are pronounced in green leaf tissues for maintaining optimal photosynthesis and photoprotection due to that the carotenoid combination is the most functionally adaptive (Dall'Osto et al. 2007; Kim et al. 2009; Pogson et al. 1996; Tian et al. 2003). As a result, overexpression of PSY, the rate-limiting enzyme in the carotenoid biosynthetic pathway, generally does not significantly alter carotenoid content in leaves (Maass et al. 2009). The increased phytoene production by the enhanced PSY activity is likely to be rapidly converted into downstream carotenoids and then apocarotenoids (Zhou et al. 2015). Indeed, data from $^{14}CO_2$ pulse-chase labeling experiments demonstrate continuous turnover of carotenoids in Arabidopsis leaves, much high than expected (Beisel et al. 2010). The regulation of continuous biosynthesis and turnover of pigments takes place as essential part of the maintenance and formation of photosynthetic membranes (Beisel et al. 2010). Constitutive overexpression of *PSY* in some cases is observed to cause severe dwarf phenotype and change carotenoid content and composition due to the perturbance of gibberellin and other pathways (Busch et al. 2002; Fray et al. 1995). Because of the pronounced regulation of carotenoid biosynthesis in chloroplasts, overexpression of other carotenoid biosynthetic genes, such as *PDS*, also exerts little effect on carotenoid accumulation and the expression of other carotenogenic genes (Busch et al. 2002).

Studies of carotenoid mutants in chloroplasts contribute greatly to our understanding of carotenoid metabolism and the functional roles of carotenogenic genes and enzymes in plants. Lutein is the most abundant carotenoid found in leaf tissue of plants. Characterization of *lut1*, *lut2*, and *lut5* mutants defective in lutein biosynthesis in Arabidopsis reveals novel carotenoid hydroxylases and the preferred pathway for lutein synthesis in plants (Kim and DellaPenna 2006; Pogson et al. 1996; Tian and DellaPenna 2001). *ccr2* disrupts carotenoid biosynthesis and causes a reduction in lutein under light due to the defective in carotenoid isomerase (*CRITISO*) (Park et al. 2002). A defect in *CRTISO* is also found to be responsible for the orange head Chinese cabbage (Zhang et al. 2015). Investigation of the *ccr2* mutant along with

the *tangerine* mutant of tomato reveals light-induced isomerization in chloroplasts of green tissues, but not in chromoplasts of fruit and flowers (Isaacson et al. 2002; Park et al. 2002). Further studies of *ccr1* mutant that causes reduced *CRTISO* expression shows an epigenetic mechanism that contributes to the regulation of carotenoid isomerization (Cazzonelli et al. 2009; Cazzonelli et al. 2010). Zeaxanthin and violaxanthin form xanthophyll cycle to dissipate excess light energy in the light-harvesting complexes and protect the photosynthetic apparatus from high light intensity (Niyogi 1999). The Arabidopsis *npq1* mutant unable to convert violaxanthin to zeaxanthin shows nonphotochemical quenching (NPQ) reduction, establishing a central role of xanthophyll cycle in the regulation of photosynthetic energy conversion (Niyogi et al. 1998). Neoxanthin is essential components of photosynthetic complexes in chloroplasts. The Arabidopsis *aba4* mutant affects the conversion of violaxanthin to neoxanthin and shows more sensitive to oxidative stress (North et al. 2007). Furthermore, the *aba4* mutant contains reduced level of ABA, demonstrating that neoxanthin serves as precursor for ABA biosynthesis. In addition to produce plant hormones ABA and strigolactones, carotenoid-derived apocarotenoids are shown to function as signaling molecules in regulating early chloroplast and leaf development through investigation of ζ-carotene desaturase mutants in Arabidopsis (Avendano-Vazquez et al. 2014), and as stress signal in inducing defense mechanisms against reactive oxygen species induced by high light in chloroplasts (Havaux 2014; Ramel et al. 2012).

Some recent studies in Arabidopsis plants provide additional new insights into the posttranscriptional regulation of carotenoid metabolism in plants. The upstream deoxy-D-xylulose 5-phosphate synthase (DXS) is important for carotenoid flux control. Its activity is found to be posttranscriptionally regulated by J20 and Hsp70 chaperones in Arabidopsis leaves, as mutant defective in plastidial J20 protein results in reduced DXS enzyme activity with increased level of enzymatically inactive DXS protein (Pulido et al. 2013). PSY protein level and enzyme activity are discovered to be posttranscriptionally regulated by the OR family proteins in a positive manner (Zhou et al. 2015). High level of the OR proteins increases PSY protein expression, and low expression reduces PSY protein level, concomitant with altered carotenoid levels.

Chloroplasts are differentiated into chromoplasts as often seen the color change from green to red during green fruit ripening, such as in tomato and pepper. This process is characterized by complete degradation of chlorophyll and breakdown of thylakoid structures of chloroplasts, accompanied with remodeling of internal membrane systems and dramatic accumulation of carotenoids (Bian et al. 2011; Egea et al. 2010; Li and Yuan 2013). As a result, carotenoids become localized in plastoglobuli and/or various carotenoid lipoprotein substructures within chromoplasts. During the transition, photosynthesis-related genes are pronouncedly down-regulated (Kahlau and Bock 2008). Accordingly, strong decrease in the abundance of proteins involved in photosynthesis, Calvin cycle and photorespiration along with carbohydrate metabolism is observed (Barsan et al. 2012). Plastids gradually acquire new biosynthetic capacities, particularly the ability to massively synthesize and accumulate carotenoids. The expression of upstream genes of

carotenoid metabolites accumulated is enhanced, while that of downstream genes is suppressed. For example, during fruit ripening from chloroplasts to chromoplasts, the accumulation of lycopene in tomato is accompanied with increased transcript levels of *DXS*, *PSY*, and *PDS*, and decreased expression of *Lcye* and *Lcyb* (Lois et al. 2000; Ronen et al. 1999). The accumulation of capsanthin and capsorubin in pepper is paralleled by up-regulation of *DXS*, *GGPS*, *BCH* and CCS during plastid transition (Bouvier and Camara 2007; Bouvier et al. 1994). Similarly, the accumulation of β-cryptoxanthin, zeaxanthin, and violaxanthin in some citrus fruits is accompanied with a simultaneous increase in the expression of upstream genes of *PSY*, *PDS*, *ZDX*, *LycB*, *BCH*, and *ZEP* (Kato et al. 2004). Apart from the increased capacity of biosynthesis, the remodeling of the internal membrane systems during the transition from chloroplasts to chromoplasts distinguishes their capacities in sequestrating and accumulating newly synthesized carotenoid end products. This is demonstrated in tobacco where expression of a β-carotene ketolase under control of tomato *PDS* promoter results in only trace amounts of ketocarotenoid production in chloroplasts in leaves, but produces high levels of them in chromoplasts of nectaries of the transgenic plants (Mann et al. 2000).

10.4 Carotenoids in Amyloplasts

Amyloplasts as starch enriched organelles are agronomically important because they are abundance in cereal seeds of major staple crops (e.g. rice, maize, wheat, and sorghum), roots (e.g. cassava), and tubers (e.g. potato). A general low to mediate levels of carotenoids are synthesized and accumulated in amyloplasts in majority of these staple crops (Wurtzel et al. 2012). Unlike carotenoid constituents in chloroplasts of green tissues, carotenoid composition and content found in amyloplasts of storage organs vary to give colors ranging from white to yellow with lutein or zeaxanthin as the major carotenoid (Howitt and Pogson 2006). None of natural rice germplasm contains carotenoids in endosperm due to lack of functional endosperm specific PSY (Chaudhary et al. 2010; Welsch et al. 2008). Thus, rice endosperm with carotenoid accumulation such as Golden Rice could be only accomplished with biotechnology approaches. Maize exhibits significant variation in carotenoid content and composition (Harjes et al. 2008). While the yellow varieties contain mainly lutein and/or zeaxanthin, there are orange varieties with relative abundance of β-carotene (Berardo et al. 2009; Yan et al. 2010). Limited natural variation exists in wheat endosperm and a general low level of carotenoids is observed with lutein as the main carotenoid (Howitt et al. 2009). Yellow sorghum varieties contain zeaxanthin as the most abundant carotenoid followed by lutein with low level of β-carotene, but their carotenoid contents are significantly lower than yellow maize (Kean et al. 2007). Similarly, cassava roots are generally white and the yellow-rooted cultivars contain low level of β-carotene (Adewusi and Bradbury 1993; Kimura et al. 2007). Potato tuber flesh colors range from white to dark yellow. While the common cultivated varieties contain low level of carotenoids with lutein

and/or β-cryptoxanthin as the main carotenoid, the dark yellow ones accumulate zeaxanthin as the primary carotenoid (Brown et al. 1993; Morris et al. 2004; Zhou et al. 2011). The roles of low levels of carotenoids in these storage organs are suggested to be associated with ABA production for dormancy and they contribute to the antioxidant system to protect membranes (Howitt and Pogson 2006).

Studies of carotenoid accumulation in amyloplasts also reveal new information for our understanding of carotenoid metabolism in plants. In the grass family such as maize, rice, and sorghum, multiple PSYs share tissue-specific regulation of carotenoid metabolic flux (Li et al. 2008b). PSY1 encoded by the *yellow1* (*y1*) locus is required for carotenoid accumulation in maize endosperm (Li et al. 2008a). *PSY1* transcript levels are shown to exist statistically significant correlation with total endosperm carotenoid content in a maize germplasm diversity collection (Li et al. 2008a). In contrast, *PSY3* influences root carotenoid biosynthesis and is induced in response to abiotic stress to interfere with stress-induced ABA accumulation in both maize and rice (Li et al. 2008b; Welsch et al. 2008). Interestingly, the three maize PSY enzyme proteins exist in distinctive plastid compartments when they are transiently expressed in leaves. While PSY2 and PSY3 are localized to plastoglobules of chloroplasts, PSY1 shows altered localization depending on its allelic variant (Shumskaya et al. 2012). All maize varieties with yellow endosperm have the same specific amino acid variant of PSY1, which appears to be a soluble protein in plastids. This variant happens to be the one used for the production of Golden Rice 2 (Shumskaya and Wurtzel 2013). In contrast, the mutated variant of PSY1 with a specific amino acid replacement promotes the formation of fibrils, characteristic for high levels of carotenoid accumulation in some chromoplasts (Shumskaya et al. 2012). It has been suggested that the different suborganellar localization of PSY1 isozyme could affect carotenoid biosynthesis and stability (Shumskaya and Wurtzel 2013). In addition, in cassava a single nucleotide polymorphism of PSY is observed in yellow-rooted cultivars; the resulting change of a single amino acid in a highly conserved region of PSY has an increased catalytic activity and enhanced carotenoid production (Welsch et al. 2010). Investigation of the maize *y9* locus identifies a previously unknown pathway enzyme, ζ-carotene isomerase (Z-ISO), for carotenoid biosynthesis. Z-ISO isomerizes the 15-*cis* double bond in 9,15,9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene. It is required for carotenoid biosynthesis in seed endosperm and suggested to play an important role in adaption of environmental stress (Chen et al. 2010).

Multiple control points have been identified to regulate carotenoid amount and composition in amyloplasts. PSY as a key regulator of carotenoid biosynthesis plays an important role for carotenoid accumulation in staple crops, such as in maize kernel (Li et al. 2008a), wheat endosperm (Howitt et al. 2009), and cassava root (Welsch et al. 2010). In maize, *PSY1* accounts for up to 27.2 % of phenotypic variation in seed carotenoid content in a QTL analysis (Chander et al. 2008). β-carotene hydroxylase (BCH) is found to be responsible for potato tuber flesh color (Brown et al. 2006; Kloosterman et al. 2010; Zhou et al. 2011). The high level of zeaxanthin accumulation in potato tubers is controlled by a combination of the dominant *BCH2* allele and homozygous recessive *ZEP* allele (Wolters et al. 2010)

and associated with low expression of *CCD4* (Campbell et al. 2010). BCH also likely represents another control point for β-carotene level in crops. *Hydoxylase3* locus was found to contribute to β-carotene level in maize (Vallabhaneni et al. 2009). Lycopene ε-cylase (LcyE) is critical in directing metabolic flux from one branch to another branch within the carotenoid biosynthetic pathway. It is a main regulator in determination of the β-carotene/α-carotene ratio and underlies a major β-carotene QTL in maize kernel (Bai et al. 2009; Harjes et al. 2008; Yan et al. 2010).

Carotenoid biosynthesis in the major staple crops is believed to occur in the membranes of amyloplasts. At least some of the carotenoid biosynthetic pathway enzyme proteins are found to be associated with amyloplast envelope membranes (Wurtzel et al. 2012), such as PSY1 is localized in the amyloplast membranes in maize endosperm (Li et al. 2008a). Because amyloplasts are lack of carotenoid lipoprotein sequestering substructures within the plastids (Lopez et al. 2008), carotenoids synthesized in amyloplasts are known to be more vulnerable for degradation during both plant growth and postharvest storage (Li and Yuan 2013). Carotenoid content increases during early maize endosperm development, but decreases before seed mature (Farre et al. 2013). A general loss of carotenoids has been observed during storage of many staple crops (http://www.harvestplus.org/). A range between 30–56 % of provitamin A carotenoids are lost during storage of a number of commercial high-carotenoid yellow maize (Dua et al. 1965). Similarly, between 20 and 48 % of total carotenoids in yellow wheat are lost during storage at 20 °C (Hidalgo and Brandolini 2008).

Amyloplasts are shown to be able to directly differentiate into chromoplasts. The color in saffron deep-red stigmas of flowers is due to high level of carotenoid accumulation in chromoplasts. The deep-red stigmas start from the colorless ovary along the style up. Electron microscope analysis of plastid development in the pistil reveals that the saffron chromoplasts in the deep-red stigma originate from amyloplasts since amyloplasts are the only plastids existing in the colorless basal portion of style and the plastid transition is via a unique amylo-chromoplast plastid type (Grilli Caiola and Canini 2004). Similarly, in developing tobacco floral nectaries as they change to orange color, amyloplasts are converted into chromoplasts via amylo-chromoplasts, resulting in the accumulation of carotenoids with bright orange nectary (Horner et al. 2007). The conversion of amyloplasts into chromoplasts leads to enhanced capacity to massively synthesize and accumulate carotenoids.

10.5 Carotenoids in Chromoplasts

Chromoplasts as carotenoid enriched plastids are mostly found in maturing fruits and some vegetables. Chromoplasts can be differentiated or converted from a number of plastid types. As discussed above, chromoplasts are derived from fully developed chloroplasts as seen during fruit ripening from green to red or yellow fruits. In the fruit peel of tomato, pepper, mango, and sweet orange, chloroplasts

differentiate into photosynthetically inactive chromoplasts; this conversion process emerges in early ripening stages and accompanies with markedly increased accumulation of carotenoids in colored chromoplasts in subsequent fruit maturation (Frey-Wyssling and Kreutzer 1958; Rosso 1968; Vísquez-Caicedo et al. 2006). Chromoplasts also arise from non-photosynthetic plastids. Amyloplasts are shown to be differentiate into chromoplasts in saffron red-stigmas of flowers (Grilli Caiola and Canini 2004) and in developing tobacco floral nectary as it changes to orange color (Horner et al. 2007). Similarly, colorless proplastids and leucoplasts can also directly differentiate into chromoplasts as the case in carrot root (Kim et al. 2010), in orange curd cauliflower mutant (Lu et al. 2006), and in papaya and berries (Knoth et al. 1986; Schweiggert et al. 2011). While most studies on chromoplasts have been focused on the biosynthesis of carotenoids (Egea et al. 2010; Bian et al. 2011; Li and Yuan 2013), little is known about the molecular mechanisms underlying chromoplast biogenesis (Li and Yuan 2013; Yuan et al. 2015). The *Or* gene isolated from cauliflower orange curd mutant represents the only known gene that acts as a *bona fide* molecular switch to trigger chromoplast biogenesis (Giuliano and Diretto 2007; Lu et al. 2006). However, how *Or* initiates chromoplast differentiation remains an enigma.

Chromoplasts exist with various sequestering substructures such as crystalline, globular, tubular, and membranous structures, and are classified based on these morphologies (Camara et al. 1995; Egea et al. 2010; Li and Yuan 2013). Generally, there are more than one pigment-bearing substructures within a chromoplast or in a crop species. For example, crystalline bodies are observed in chromoplasts of tomato, but globular- and membrane-shaped structures also coexist (Harris and Spurr 1969). The typical saffron stigma chromoplasts have reticulo-tubular structure, contain a mix of tubules, vesicles and plastoglobules (Grilli Caiola and Canini 2004). A more recent study of chromoplast structures in nine carotenoid-rich fruits and vegetables also reveals the coexistence of more than one substructure within a chromoplast (Jeffery et al. 2012). However, some crops appear to be predominant with chromoplasts containing a particular type of pigment-bearing substructures. For example, carrot, tomato, and red papaya are enriched with crystal chromoplasts; mango contains high concentration of plastoglobuli in chromoplasts; and watermelon, mango and butternut are prominent with membranous chromoplasts (Jeffery et al. 2012).

Unlike other type of plastids, chromoplasts synthesize and accumulate diverse carotenoid compounds in different fruits and vegetables. In some cases, the specific carotenoids accumulated appear to be associated with the formation of specific carotenoid sequestering substructures within chromoplasts. By examining diverse chili pepper fruits with various carotenoid chemical composition, it establishes a linkage between the unique carotenoids accumulated with various specific chromoplast architecture as determined by chromatographic methods (Kilcrease et al. 2013). Crystalline bodies observed in chromoplasts of carrots, papaya, and tomato are found to predominantly consist of β-carotene or lycopene (Harris and Spurr 1969; Schweiggert et al. 2011). Globular and/or tubular-globular chromoplasts described for yellow fruits such as from kiwi and yellow papaya contained lutein or β-cryptoxanthin as major carotenoid (Montefiori et al. 2009;

Schweiggert et al. 2011). The highly heterogeneous nature of the substructures in chromoplasts contributes to the various profiles of carotenoid accumulation found in fruits, vegetables, and roots.

The specific feature of chromoplasts with various carotenoid-lipoprotein sequestering substructures is responsible for massive amount of carotenoid accumulation in chromoplasts. Indeed, a strong association between proliferation of carotenoid-lipoprotein sequestering substructures and increase of carotenoid accumulation is observed (Al Babili et al. 1996; Li et al. 2012; Rabbani et al. 1998). The increased carotenoid content during long term storage of transgenic *OR* potato tubers directly correlates with increased amount of pigment-bearing substructures released from chromoplasts (Li et al. 2012). Additionally, the development of chromoplasts with high level of carotenoid accumulation during tomato fruit ripening is accompanied with large amounts and considerably enlargement of plastoglobules (Bian et al. 2011). Plastoglobules are well-known for their function in carotenoid sequestration and storage. Carotenoids are highly stable in plastoglobules. These carotenoid-lipoprotein sequestering substructures are believed to serve two roles for the high capacity of chromoplasts in promoting carotenoid biosynthesis and accumulation. One is to facilitate the sequestration of newly synthesized carotenoids to avoid the overloading of end products at the site of carotenoid biosynthesis in chromoplast membranes for continuous biosynthesis; and the other one is to serve as deposition sink for stable storage of carotenoids (Li and Van Eck 2007; Li and Yuan 2013; Vishnevetsky et al. 1999).

It is thus not surprising that biogenesis of chromoplasts exerts strong effect on carotenoid content in crops. The cauliflower *Or* causes the normal white curd tissue to accumulate high levels of β-carotene, producing a striking orange curd mutant phenotype. The *Or* gene, which encodes a protein containing DnaJ cysteine-rich zinc finger domain, is found to induce carotenoid accumulation by triggering the differentiation of non-colored plastids into chromoplasts (Li et al. 2001; Lu et al. 2006). Ectopic expression of the *Or* transgene in both white cauliflower and potato tubers leads to the formation of chromoplasts with enhanced carotenoid content (Lopez et al. 2008; Lu et al. 2006). Similarly, increase of chromoplast compartment size and number also results in enhanced carotenoid levels. In the tomato *high pigment* mutants as well as in the tomato *flacca* and *sitiens* mutants, the high levels of carotenoids are found to be directly linked to an increased plastid number and/or compartment size (Galpaz et al. 2008; Kolotilin et al. 2007; Liu et al. 2004).

Since the prominent role of chromoplasts is involved in synthesis and accumulation of carotenoid pigments, carotenoid metabolism and regulation are best investigated in chromoplasts (Egea et al. 2010; Li and Yuan 2013; Yuan et al. 2015). Although the carotenoid biosynthetic enzyme protein capsanthin/capsorubin synthase represents the most abundant protein in pepper chromoplasts (Siddique et al. 2006; Wang et al. 2013), the other core enzyme proteins are generally present at low abundance in plants. Expression of carotenoid biosynthetic genes has been subjected to extensive studies in many chromoplast-containing tissues. Transcriptional regulation of carotenoid biosynthesis prevails in chromoplasts during fruit ripening from green to red or yellow fruits in tomato and pepper as described

above. Similar transcriptional regulation also occurs in red-flesh orange mutant of "Hong Anliu" when compared to wide type "Anliu" (Liu et al. 2007). However, the transcriptional control has been found not to be always the primary regulatory mechanism in some other cases, especially in chromoplasts derived from white tissues (Yuan et al. 2015). No correlation between carotenogenic gene expression and carotenoid accumulation is observed in orange cauliflower mutant, squash, and carrot root (Clotault et al. 2008; Li et al. 2001; Zhang et al. 2014). Thus, carotenoid biosynthesis and accumulation in chromoplasts is under complex regulation.

Because chromoplasts accumulate high levels of carotenoids that are essential for nutritional and sensory quality of agricultural products, chromoplast proteomes from a number of carotenoid enriched crops have been investigated to provide insights into the general metabolism and function of chromoplasts. A large number of chromoplast proteins from various crop species such as watermelon, tomato, carrot, orange cauliflower, red papaya, citrus, and red bell pepper have been identified (Barsan et al. 2010; Siddique et al. 2006; Wang et al. 2013; Zeng et al. 2011). Examination of these chromoplast proteomes identifies relative abundance of early core carotenoid pathway proteins, which may suggest an important role of them for metabolic flux into carotenoid biosynthetic pathway (Wang et al. 2013). In addition, a number of key metabolic and cellular processes appear to be crucial for chromoplast biogenesis and development, which include lipid metabolism for plastid membrane proliferation, carbohydrate metabolism to provide precursors for the biosynthesis of carotenoids and many other metabolites within chromoplasts, and chaperones for protein modification and translocation, as well as redox system and reactive oxygen species, and energy production and import into chromoplasts (Egea et al. 2010; Li and Yuan 2013).

While carotenoids in amyloplasts of staple crops are more vulnerable for degradation, carotenoids in chromoplasts appear to be more stable due to sequestering in carotenoid lipoprotein substructures including plastoglobules. In transgenic potato tubers, the induction of chromoplast formation by the *Or* transgene has been found to not only help retain carotenoids, but also stimulate continuous carotenoid accumulation during long term storage (Li et al. 2012). The enhanced carotenoid stability and accumulation are closely associated with amount of colored carotenoid lipoprotein sequestering substructures formed. Carotenoids in chromoplasts of some other fruits and vegetable are also known to be more stable. Carrot retains carotene content and continuous accumulation during storage (Booth 1951; Imsic et al. 2010; Kopas-Lane and Warthesen 1995). Similarly, storage of butternut leads to continuously increased carotenoid content. The increased accumulation has been shown to be associated with the transition of amylochromoplasts into chromoplasts (Zhang et al. 2014).

Chromoplasts provide a plastid-localized sink, which not only facilitates active carotenoid biosynthesis, but also enhances stable storage of the synthesized products with increased carotenoid stability. The specific characteristics of chromoplasts provide potential for high capacity of carotenoid biosynthesis and stable accumulation. Vitamin A plays vital role in human health and its deficiency leads to eye damage, growth retardation, and reduced immune responses. Worldwide,

some 250 million preschool children suffer from vitamin A deficient, which is caused primarily by simple diets of staple crops with low levels of provitamin A carotenoids. Many important staple crops such as wheat, rice, barley, maize, potato, and cassava synthesize and store low levels of carotenoids in the membranes of amyloplasts in the edible seeds or roots. In addition to low content, retention of these carotenoids in staple crops has been a major concern during post-harvest storage and over 50 % of provitamin A carotenoids can be lost during storage. Because of the ability and capacity of chromoplasts to actively synthesize and effectively sequester carotenoids, induction of chromoplast formation or conversion of some amyloplasts into chromoplasts in the edible organs likely facilitates carotenoid synthesis and stable storage in these major staple crops with improved nutritional quality.

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Chapter 11
Evidence of Epigenetic Mechanisms Affecting Carotenoids

Jacobo Arango, Jesús Beltrán, Jonathan Nuñez, and Paul Chavarriaga

Abstract Epigenetic mechanisms are able to regulate plant development by generating non-Mendelian allelic interactions. An example of these are the responses to environmenal stimuli that result in phenotypic variability and transgression amongst important crop traits. The need to predict phenotypes from genotypes to understand the molecular basis of the genotype-by-environment interaction is a research priority. Today, with the recent discoveries in the field of epigenetics, this challenge goes beyond analyzing how DNA sequences change. Here we review examples of epigenetic regulation of genes involved in carotenoid synthesis and degradation, cases in which histone- and/or DNA-methylation, and RNA silencing at the posttranscriptional level affect carotenoids in plants.

Keywords Epigenetic • Genotype-environment • Histone methylation • SDG8 • microRNA

11.1 Introduction

Carotenoids are involved in multiple biological processes vital for plants, such as photosynthesis, photomorphogenesis, photoprotection and development. As recently elucidated, they also serve as precursors to a diverse set of apocarotenoids and to the important hormones abscisic acid (ABA) and strigolactones (Alder et al. 2012; Nisar et al. 2015). Carotenoids influence a variety of phenotypes ranging from resistance to abiotic stresses (Ruiz-Sola et al. 2014), flowering (Cazzonelli et al. 2010) and fruit ripening (Zhong et al. 2013). It has been shown that in some of the biological phenomena in which carotenoids are essential components, epigenetic regulation plays a crucial role (Grimanelli and Roudier 2013). As defined by Rapp and Wendel (2005) epigenetics constitute *"... the alteration of phenotype,*

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morphological or molecular, without change in either the coding sequence of a gene or the upstream promoter region . . . ". These phenotypic alterations are stably inherited, although reversible in some cases, and can be mediated by chromatin remodeling; methylation or acetylation of DNA's histones, or methylation of genes' regulatory sequences (promoter methylation) to up- or down-regulate transcription, sometimes mediated by small RNA molecules and by RNA-mediated down-regulation of genes by shutting down mRNA production and translation (Offermann and Peterhansel 2014). In the last decade it has been shown that epigenetic mechanisms are operating during crucial moments of plant development generating allelic interactions that are difficult to explain by conventional Mendelian genetics. In fact, today, transgressive segregation (complementation and epistasis) is better understood through epigenetics (Shivaprasad et al. 2012). Some examples of how carotenoids are affected by epigenetics are described below.

11.2 Epimutation: Methylation

DNA hyper methylation is one of the most studied mechanisms by which the transcription of genes is epigenetically modulated. Methylation occurs in CpG, CpCpG, CpHpHp, and CpNpG motifs in the DNA. Arabidopsis' enzymes like CHROMOMETHYLASE-3 (CMT3), DOMAINS REARRANGED METHYLASE 2 (DRM2) and METHYLTRANSFERASE 1 (MET1) transfer methyl groups to Cytosines in these "CpG islands" resulting in gene silencing (Reviewed by Rapp and Wendel 2005). Epigenetic regulation has been reported in plants under adverse abiotic conditions such as water shortage where the hormone ABA triggers different physiological responses. Violaxanthin and neoxanthin carotenoids (carotenoids carrying oxygen groups known as xanthophylls) are substrates for ABA biosynthesis. The enzymatic reaction mediated by NCED3 (9-cis epoxycarotenoid dioxigenase) is a rate limiting step for ABA formation in the cleavage of 9-cis epoxycarotenoids (Cai et al. 2015). The function of the *NCED3* gene is affected in *ATX1* mutants, which are deficient in producing trithorax proteins, a family of enzymes responsible for trimethylating the histone H3, specifically at the lysine 4 (H3K4me3) position. This H3 methylation could be the consequence of a water stress signal, and takes place at the nucleosome where *NCED3* gene is located, which correlates with the decrease of *NCED3*'s expression level, so defining an epigenetic control (Ding et al. 2011). The trithorax proteins are also associated with the epigenetic regulation of flowering in rice and Arabidopsis as a response to environmental signals (Choi et al. 2014, Pien et al. 2008). This group of proteins has become important recently with the discovery of their role in the variability of cellular processes that are influenced by biotic and abiotic factors. An interesting feature of the epigenetic regulation on ABA synthesis is that, in its functional state, this hormone stimulates epigenetic regulation of metabolic cascades mediated by silencing through siRNA, hypermethylation of gene regulatory regions and the modification of histones that regulate processes such as seed ripening, dormancy and germination (Chinnusamy

et al. 2008). Additionally, ABA can influence the expression of the *PSY* gene suggesting an ABA-dependent mechanism to induce carotenogenesis in the roots of Arabidopsis (Ruiz-Sola et al. 2014). These examples demonstrate the dynamic nature of the epigenetic mechanisms that affect physiological processes where carotenoids play a role.

Different researchers have studied the epigenetic factors involved in tomato ripening (Zhong et al. 2013; Manning et al. 2006). The treatment with methylation inhibitors (5-azacytidine) showed that fruits treated with this chemical present a variegated ripening pattern of red and green coloring. High levels of methylation were found at the promoter region of the *Cnr* (colorless non-ripening) gene promoter in the fruit green zones, while in the red colored areas this methylation pattern was not found. Also, phytoene synthase (*PSY1*) gene expression, the rate limiting step in carotenoid synthesis, was affected. More examples of methylation-mediated control of carotenoid synthesis will be given later on in this chapter. The new generation sequencing technologies, together with global methylation profiles (methylome) obtained by bisulfite sequencing, allows the acquisition of large amounts of information that facilitates the global analysis of the dynamics of epigenetic regulation on genomes. For example, a detailed analysis of the methylome of the tomato fruit showed a substantial epigenetic resetting at the fruit development stage (Zhong et al. 2013; Table 11.1; see also Osorio et al. 2013). The conclusions on the epigenetic control of tomato fruit ripening, anticipated by Osorio et al. (2013), are worth repeating here: 1) methylation of the promoter regions of ripening-related genes in wild type fruits decreases while the ripening proceeds, and 2) this does not occur in mutants with impaired fruit ripening (*Cnr* and *Rin*) where the percentage of methylated cytosines is higher, revealing that organ development in plants is under epigenetic regulation.

Another example of epigenetic mutations in the *Cnr* locus was characterized by Manning et al. (2006) in tomato. They found that the *Cnr* locus is a member of the SPL family of genes. The gene that they reported, called *LeSpn-cnr*, was found hyper-methylated in the promoter region (2,4 kb upstream of *Cnr*'s coding sequence), having at least 18 more methylated cytosines in DNA of mutant fruits than the wild type. The methylation status of the *Cnr* locus was determined by bisulfite sequencing to demonstrate that hypermethylation of the *Cnr* promoter, in the DNA of fruits and leaves, varied between cultivars and resulted in lower expression of *LeSpn-cnr*, rendering tomato fruits unable to ripen normally. Thus, mature fruits remained yellow, not red, with occasional revertant sectors that became red spontaneously, indicating that this epimutation was reversible in the fruit. We must remember that the orthologue of *LeSpn-cnr* in Arabidopsis is At*Spl*3, which is epigenetically regulated by *miR156 (*Jones-Rhoades et al. 2006; Seymour et al. 2008), which pinpoints at least two levels of epigenetic regulation of *Cnr* that affect carotenoid content in tomato fruits: promoter methylation and microRNA-mediated silencing. In fact, *cnr* mutants are deeply affected in gene expression of *PSY* (Eriksson et al. 2004), which explains the absence of red color (lycopene) in *LeSpn-cnr.*

Table 11.1 Genes involved in epigenetic mechanisms affecting carotenoid composition in plants

Role	Gene name	Function	Epigenetic mechanism	Most probable carotenoid gene(s) down/up regulated	Carotenoid phenotype	References
Plant development	*miR156*	Post-transcriptional gene silencing of several genes	miRNA-mediated degradation of SPL15 mRNA	Down regulation of *Ccd*s and *Nced*s	Enhanced carotenoid levels in seeds; Enhanced branching	Wei et al. (2012) and Emami-Meybodi (2013)
Tomato fruits ripening	*Spn-cnr* (Arabidopsis orthologue At*Spl*3)	Transcription factor of the type Squamosa Promoter-Binding-Protein-Like (SPL) family.	Methylated in the promoter region	Lower expression level of *Psy*	Tomato mature fruits don't ripen remaining yellow	Manning et al. (2006) and Zhong et al. (2013)
Color flower in *Osmanthus fragrans*	*Ccd4*	Cleavage of carotenoids	Methylated in the promoter region	Lower expression level of *Ccd*	Yellow flowers	Han et al. (2014)
Photo-and antioxidant protection	*Vte3*	Synthesis of γ- and α-tocopherol	Natural epiallele. Methylated in the promoter region	Nr	Increase of lycopene and α-carotene content	Quadrana et al. (2014)
Response to environmental stimuli	*Sdg8*	Methyltransferase activity. Histone methylation	Methylation of histone 3 lysine (H3K4)	Lower expression level of *CrtISO*	Decreased levels of lutein and increased levels of xanthophylls early in development.	Cazzonelli et al. (2009a, b)
Response to environmental stimuli. Response to abiotic stress	*Atx1*	Methyltransferase activity. Histone methylation	Triple-Methylation of histone 3 lysine (H3K4me3)	Lower expression level of *NCED3*	Reduced carotenoid derivative ABA.	Ding et al. (2011)

Nr not reported

The identification of genes and mechanisms that impact the nutritional value of crops is of great interest for plant breeders. Tocopherols and Carotenoids (vitamins E and A respectively) are the two groups of antioxidants located in the chloroplast (DellaPenna and Pogson 2006) and therefore their synthesis and accumulation in plant organs influences the nutritive content of crops. These metabolites share a GGPP (Geranylgeranyl pyrophosphate) molecule as precursor, connecting the two pathways. Recent reports have provided hints on the epigenetic regulation of vitamin E synthesis in tomato fruits. What is interesting is that gene activity of vitamin E metabolic pathways can potentially influence the accumulation of some carotenoids. The study published by Quadrana et al. (2014), in which wild and domesticated species of tomato were analyzed to explain the epigenetic variation of tocopherol (vitamin E) accumulation, determined that it was defined by the presence of "epialleles", or epigenetic variants with an effect on the phenotype, inherited in a stable manner. A genomic region from *Sollanum pennelli* (wild species) introgressed into *Solanum lycopersicum* (commercial species), explained close to 50% of vitamin E variability. Fine mapping allowed researchers to identify in this region a sequence of the *VTE3* gene that encoded a 2-methyl-6- phytylquinol methyl transferase enzyme, a rate limiting step in the synthesis of α- and β-tocopherol. In terms of *in silico* protein primary structure analysis of VTE3 from *S. pennelli* and *S. lycopersicum*, there was a difference in one amino acid localized in the transit peptide which should not affect enzyme functionality, but this gene was expressed at low levels in *S. lycopersicum* which also accumulated lower levels of vitamin E in the fruits when compared to *S. pennelli*. The phenotype was explained by an insertion of a transposable element of the SINE type in the 5′UTR region of the *VTE3* gene. This insertion was absent in the same 5'UTR of the *S. pennelli*'s *VTE3* gene. Based on this fact, the authors suggested that the transposon insertion influences methylation patterns in this region impacting gene expression and therefore lowering accumulation of tocopherol in *S. lycopersicum*. The methylomes of Arabidopsis and tomato are consistent in revealing that regions associated with transposons are methylated, and this characteristic remains even when physiological changes occur in the plant such as fruit ripening for example (Lister et al. 2009; Zhong et al. 2013). Similar mechanisms are likely to be affecting the biosynthesis and accumulation of carotenoids in different species and organs. As a matter of fact, the analysis of tissues where the *VTE3* is silenced shows an increment of key carotenoid compounds for nutrition such as lycopene and α-carotene. Moreover, the antioxidant capacity of these tissues is increased, suggesting that it could be a strategy of the cell to maintain its redox balance by compensating for the lack of tocopherols with higher amounts of carotenoids.

Methylation of promoter regions of genes involved in carotenoid degradation, like *CCD*, has been reported to modify flower color in *Osmanthus fragrans* (Han et al. 2014). 70% of CNG islands (CpNpG, where N = A, C, G or T) were methylated upstream of the 5′ end of *CCD4* in Chenghong Dangui cultivar, whose flowers were much yellower than those of cultivar Zi Yingui in which only 40% of CNG islands were methylated. In the orchid *Oncidium* a similar situation was reported for the promoter region of Og*CCD1* (Chiou et al. 2010) whose hypermethylated

pattern probably gave rise to down-regulated Og*CCD1* transcripts producing yellow flowers in the cultivar Gower Ramsey. In the white-flowered cultivar White Jade, *CCD1* transcripts didn't appear down-regulated during most floral stages (Chiou et al. 2010).

There is an increasing interest in discovering epialleles due to their drastic effect on crop phenotypes. If the methylation of DNA influences the epimutations that can be passed to the next generation without changes in the DNA sequence, the phenotypic variants induced by environmental factors through the methylation of epialleles, can actually influence the adaptability of plants and species to their surrounding environment (Weigel and Colot 2012; Verhoeven et al. 2010).

Epigenetic regulation seems to play a role in shaping phenotypes for example color in aphids, which actually contain the complete biosynthetic machinery to synthesize carotenoids as a result of a fascinating evolutionary process that includes lateral transference from fungi to aphids (Moran and Jarvik 2010). Pasquier and collaborators (2014), report that in these insects the carotenoid synthesis is dependent upon the environment (as in some plant organs as discussed above); under ideal external conditions such as temperature, carotenoid biosynthesis is favored and aphids display an orange coloration while in cold conditions, green individuals are favored. On the other hand, high population density relates to carotenoid inhibition evidenced by the presence of white individuals. The aphid's genome methylation profiles showed clear differences in the epigenetic labels among orange and white individuals, supporting the idea that strong epigenetic changes are related with observed phenotypic variations (Pasquier et al. 2014). What is interesting is that white and orange aphids can produce progenies of any of the above mentioned colors, showing that epigenetics can regulate the adaptive response of the species to environmental stimuli.

11.3 Epigenetic Regulation of Crtiso Expression Alters Plant Carotenoid Composition

In plants, carotenoid biosynthesis is regulated by environmental stresses and developmental cues (Cuttriss et al. 2011). In many cases, an increase in carotenoid content correlates with transcript abundance of the rate-limiting pathway genes and/or with allelic variation (Vallabhaneni and Wurtzel 2009; Welsch et al. 2010). However, little is known about direct epigenetic mechanisms regulating carotenoid composition during development and/or under stress conditions.

One of the best examples of aphysiological response mediated by epigenetic mechanisms is the vernalization process, where prolonged exposure to low temperatures induces early flowering in plants (Jaenisch and Bird 2003; Sheldon et al. 2000). Interestingly, a chromatin modifying protein with histone methyltransferase activity (SET DOMAIN GROUP 8; SDG8), known for mediating vernalization responses, has recently been linked to carotenoid biosynthesis and regulation

(Cazzonelli et al. 2009a, b). SDG is a protein family of lysine methyltransferases that modify chromatin by methylation at lysine residues on the tails of histone proteins (Cazzonelli et al. 2009b; Cazzonelli 2011; Thorstensen et al. 2011). These chromatin modifications serve to recruit additional regulatory factors that affect DNA accessibility by the RNA polymerase II transcriptional complex (Cazzonelli et al. 2009b; Thorstensen et al. 2011; Cazzonelli 2011). Therefore, these modifications cause profound effects on gene mRNA transcript abundance.

SDG8 appears to modulate the synthesis of lutein, which is the most abundant carotenoid in plants and has well-established roles in photosynthesis (Cazzonelli et al. 2009a). This regulation affects the branching point of the pathway, where α- or β-carotene are produced and where CRTISO is a regulatory enzyme affecting the flux of carotenoid intermediaries and precursors of plant hormones such as strigolactones, which control plant architecture (Cazzonelli et al. 2009a; Gomez-Roldan et al. 2008; Alder et al. 2012).

The identification of SDG8 as an epigenetic modulator of carotenoid biosynthesis began with early investigations on lutein biosynthesis, leading to the identification of an Arabidopsis mutant (*ccr1*; carotenoid and chloroplast regulation). *ccr1* plants have decreased levels of lutein and increased levels of xanthophylls early in development (Park et al. 2002). The authors also found that *ccr1* etiolated tissues accumulated *cis*-isomers. Thus, the carotenoid profile of the *c cr1* plants was similar to that of the *ccr2* mutant which harboured a lesion in the *CrtISO* gene. However, the exact mechanism affecting carotenoid composition in *ccr1* plants was not completely clear; *ccr1* did not appear to be the consequence of a disruption in a carotenoid biosynthetic gene and was epistatic to *ccr2* (Park et al. 2002). In addition, these plants exhibited a set of developmental phenotypes such as increased shoot branching, reduced fertility, increased cauline node branching, pale green leaf color and early flowering in short days.

Further investigation by Cazzonelli and collaborators (2009a) into *ccr1* plants led to the identification of the *CCR1* gene which encodes an SDG8 and is allelic to EARLY FLOWERING IN SHORT DAYS (EFS). Additionally, SDG8 is necessary to maintain *CrtISO* expression throughout chromatin-level regulation. This mechanism was evident as plants having mutations in *SDG8* showed reduced trimethylate histone 3 lysine (H3K4) in chromatin surrounding the translation start site of *CrtISO*. This change in chromatin configuration resulted in a significant reduction (>90 %) of *CrtISO* mRNA levels (as determined by real time PCR), therefore, explaining the changes in carotenoid composition present in *ccr1* plants. In the same study, a genome-wide transcript analysis using microarrays was carried out to check which other genes were altered in *ccr1* plants. These experiments revealed that the disruption of *SDG8* also altered the expression of a small set of genes (113) of which 75 % were down-regulated. Among these down-regulated genes were *CrtISO*, FLOWERING LOCUS C (*FLC*) and genes associated with signaling and developmental processes, but no other genes of the carotenoid pathway with the exception of *LCYEε* gene whose expression was slightly down-regulated in roots. The authors suggested that down-regulation of *LCYE* was likely due to pleiotropic effects by CRTISO although further investigation would be valuable to clarify this

point. The absence of SDG8 activity appeared to affect genes neighboring *CRTISO*, supporting the observation that *SDG8* controls *CRTISO* in a specific manner in the same way that it controls *FLC* (Kim et al. 2005; Cazzonelli et al. 2009a). The authors suggested that blockage of the carotenoid pathway in *ccr1* might have caused, at least in part, the branching phenotype due to reduction of carotenoid precursors for hormones like strigolactones.

A further study by Cazzonelli and collaborators (2010) was carried out to understand how both *CRTISO* and *SDG8* gene promoters are regulated during plant development. This revealed additional aspects of the epigenetic mechanism controlling carotenoid composition. Using several reporter-fusion constructs, the pattern of expression of *CRTISO* and *SDG8* was mapped in different tissues and developmental stages in Arabidopsis. These experiments revealed that *CRTISO* and *SDG8* displayed unusual variability in their expression patterns in a large number of transgenic lines. Regarding tissue specificity, *CRTISO* and *SDG8* promoters were highly overlapped in their expression patterns both were produced in the hypocotyls, leaf vasculature, shoot apical meristem and pollen, as determined by GUS staining assays. Notably, the ability of the *CRTISO* promoter to drive GUS expression in the *ccr1* background was considerably lower than in the WT background, further demonstrating that *SDG8* is required to maintain active expression of *CRTISO*. Interestingly, the same study showed that sequences of both the *CRTISO* reading frame and its promoter were necessary for *SDG8* to control the expression of *CRTISO*. Expressing the *CRTISO* gene under its promoter in the *ccr1* background did not restore wild-type *CRTISO* mRNA levels as expected. The molecular nature by which *SDG8* is targeted to *CRTISO* and *FLC* genes remains to be elucidated (Cazzonelli 2011).

The discovery of an epigenetic mechanism controlling plant carotenoid composition opens the path for further investigation into more detailed mechanisms of chromatin-level regulation of gene expression. More insight is needed to elucidate new molecular mechanisms regulating carotenoid biosynthesis in plants, beyond enzyme function and localization.

11.4 micro RNAS That Affect Carotenoid Content and Branching Patterns

Micro RNA's (miRNA) are tiny RNA molecules, 21–24 nt in length, distributed all over the eukaryotic kingdom that influence gene expression in plant development, for example, during abaxial/adaxial leaf determination, organ ontogeny for flowering and heterochronic shifts (Rapp and Wendel 2005; Kidner and Martienssen 2005). They also influence several biochemical/developmental pathways including that of carotenoids (see below). The role of miRNAs in plant development has been reviewed in Jones-Rhoades et al. (2006). A miRNA named miR156 is conserved among plants including Arabidopsis, rice and cottonwood. In Arabidopsis alone miR156 comprises a family of at least 12 genes (Reinhart et al. 2002) that target transcription factors of the type Squamosa Promoter-Binding-Protein-Like

(SPL) family. *SPL15* is a member of the *SPL* family that contains the SBP Box in the promoter region (Cardon et al. 1999), a 5′-NNGTACR-3′ core sequence where usually N = C and R = AGTAC (Wei et al. 2012). SPLs in general promote vegetative phase change, the transition from juvenile to mature phase in plants. For example, SPL15 and SPL9 control shoot maturation in Arabidopsis (Schwarz et al. 2008) and in the juvenile state SPL protein expression is repressed by miR156 and miR157 (Poethig 2013).

In an attempt to demonstrate the role of *miR156* in carotenoid metabolism, Wei and collaborators (2012) enhanced the expression of *miR156b* in Arabidopsis by inserting a T-DNA with four CaMV35S enhancers (Robinson et al. 2009) in a region containing at least six loci, among them *miR156b*. The activation-tagged mutant obtained, named *sk156*, had elevated levels of lutein, α-carotene, violaxanthin and zeaxanthin in mature seeds and an increased number of reproductive branches; all of which are phenotypes assigned to the suppression of SPL15 mediated by the overexpression of *miR156b*, whose transcript level was >90 times higher than in wild type. *Sk156* also showed an increased numbers of rosette leaves, the appearance of trichomes on sepals and shoot tips, delayed bolting, substantial stunting of cauline stems, and decreased flower and silique sizes. But, what was the relationship between *SPL15* gene, *miR156b* and the carotenoid pathway? First, the authors showed that the sequences of *SPL15* mRNA and *miR156b* were complementary. In the former there were three cleavage sites, one of which was very frequently used to cleave *SPL15* mRNA. Therefore, *miR156b* could base-pair with *SPL15* mRNA to promote its cleavage and so down-regulate *SPL15* expression in an epigenetic manner. Rhoades et al. (2002) had predicted the existence of this target site in *SPL* genes before. Second, they also provided evidence that wild type carotenoid levels could be restored in dry seeds of transgenic *SK156* by overexpressing a mutated version of *SPL15* called *SPL15m*, which had 11 nucleotides mutated in its DNA sequence, specifically those interacting with *miR156b*, yet preserving the amino acid sequence of native SPL15 (a change in the nucleotide sequence of *SPL15m* changes the ability of *miR156b* to target it for down regulation. It doesn't however change the codons that codify for the SPL15 protein, and therefore it can be expressed as in the wild type plant). *SPL15m* was insensitive to the inhibitory action of *miR156b* and therefore plants that overexpressed it, had wild type-like carotenoid content and branching patterns. Lastly, the evidence suggested that SPL15 itself might regulate the expression of *miR156b* by directly binding to its promoter region. In fact, the *miR156b* promoter contained at least seven 5′-NNGTACR-3′ repeats (Wei et al. 2012). The authors couldn't however discard the possibility that the altered carotenoid content and morphological patterns observed in *SK156* were the result of *miR156b*-mediated *SPL15* suppression alone. Most probably other SPL transcription factors were also suppressed and/or SPL15 did interact with several other *SPL* gene products.

The work of Wei et al. (2012) demonstrated at the molecular level what was previously observed in *Arabidopsis thaliana* (Schwab et al. 2005), *Brassica napus* (Wei et al. 2010), *Panicum virgatum* (Fu et al. 2012) and *Oryza sativa* (Xie et al. 2012), which was the epigenetic effect that the overexpression of the micro-RNA

miR156b had on plants: it altered branching pattern and, in some cases, increased carotenoid content in specific organs. What was missing in the Wei et al. (2012) study was a direct molecular link between *SPL15* down-regulation (mediated by *miR156b*) and genes of the carotenoid pathway. Was it the down/up regulation of one or several genes responsible for the phenotypes observed? The fact that the phenotype of *SK156* resembled that of the *MAX3* and *MAX4* mutants (more-axillary-growth genes; Sorefan et al. 2003; Booker et al. 2004, 2005), plants with defective biosynthesis of strigolactones, prompted Emami-Meybodi (2013) to search for tighter molecular links between *miR156*, *SPL15* and carotenoid-degrading genes. None of the carotenogenic genes analyzed by Wei et al. (2012) had sequences complementary to the mature *miR156b*, and they were not up-regulated, suggesting that the increase in carotenoid content and branching was probably due to reduced carotenoid catabolism, which in turn resulted in lower levels of strigolactone –the hormone that regulates branching in plants- (Umehara et al. 2008; Gomez-Roldan et al. 2008). The roles of CCDs and NCEDs in producing strigolactone were therefore investigated in relation to the overexpression of *miR156b*. The results showed that, in roots of 10 day old *SK156* seedlings, the transcript levels of *CCD4*, 7 and 8, and *NCED*2, 3, 6 and 9 were significantly reduced; only the transcript levels of *NCED*6 were augmented in roots of the same plants. Meanwhile, in 10 days post siliques anthesis the transcript levels of *CCD7* and *NCED*2, 3, 5 and 9 were lower than those of wild type-plants, while *NCED*6 transcripts where higher. *CCD*1 was neither up nor down-regulated and, coincidentally, it has no GTAC motifs in its promoter (Emami-Meybodi 2013), while all other *CCDs* and *NCED*s analyzed have between 2 and 7 GTAC motifs to which SPL15 bound specifically (quantified by ChIP-qPCR). In summary, the evidence presented suggested that the microRNA *miR156b* epigenetically regulated the expression of *SPL15* by modulating its mRNA production. Thus, the conclusion of these experiments may be that sufficient production of *SPL15* mRNA results in the transcription of *CCD* and *NCED* genes to whose promoters bind SPL15 through their GTAC motifs. This ultimately results in carotenoid degradation and strigolactone production.

11.5 Concluding Remarks

Scientists are now aware of how epigenetics influences many key processes in nature ranging from human diseases to carotenoid biosynthesis in aphids. Therefore, it is not enough to decode and study the DNA sequence, but also the mechanisms that, without changing the DNA code, are able to switch genes on and off through processes such as those described in this chapter. What is even more thrilling is that these mechanisms, now called epigenetic factors, are passed to the next generations, are reversible and are in many cases influenced by external conditions such as temperature, chemicals and light, among others.

Carotenoid biosynthesis is of course not exempt from being affected by epigenetic factors as supported by the abundant scientific evidence now emerging

It is almost certain that with this accumulated knowledge, scientists will be able to better understand carotenoid biosynthesis, degradation and accumulation in different organs of different plant species, thus allowing identification of novel ways to modulate carotenoid production for the benefit of humans. The fact that epigenetic mechanisms regulate the abundance of carotenoids in diverse plant structures, highlight a new factor regulating how these metabolites accumulate or degrade in addition to the well described mechanisms such as transcriptional and post-transcriptional regulation.

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Part III
Carotenoids for Human Health

Chapter 12
Manipulation of Carotenoid Content in Plants to Improve Human Health

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Abstract Carotenoids are essential components for human nutrition and health, mainly due to their antioxidant and pro-vitamin A activity. Foods with enhanced carotenoid content and composition are essential to ensure carotenoid feasibility in malnourished population of many countries around the world, which is critical to alleviate vitamin A deficiency and other health-related disorders. The pathway of carotenoid biosynthesis is currently well understood, key steps of the pathways in different plant species have been characterized and the corresponding genes identified, as well as other regulatory elements. This enables the manipulation and improvement of carotenoid content and composition in order to control the nutritional value of a number of agronomical important staple crops. Biotechnological and genetic engineering-based strategies to manipulate carotenoid metabolism have been successfully implemented in many crops, with *Golden rice* as the most relevant example of β-carotene improvement in one of the more widely consumed foods. Conventional breeding strategies have been also adopted in the bio-fortification of carotenoid in staple foods that are highly consumed in developing countries, including maize, cassava and sweet potatoes, to alleviate nutrition-related problems. The objective of the chapter is to summarize major breakthroughs and advances in the enhancement of carotenoid content and composition in agronomical and nutritional important crops, with special emphasis to their potential impact and benefits in human nutrition and health.

Keywords Human nutrition • Antioxidants • Metabolic engineering • Nutritional important crops • Carotenoid improvement

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12.1 Introduction

12.1.1 Importance of Carotenoids for Human Nutrition and Health

Carotenoids are plants pigments that play essential and multiple roles in plants, in addition to provide coloration to flowers, fruits and seeds in many species. They are important during photosynthesis, as part of the light-harvesting complex and in the protection from photo-oxidation. Carotenoids are precursors of some volatiles, play a role in the attraction of pollinators and are also precursors of the plant hormones abscisic acid (ABA) and strigolactones (Krinsky 1989; Havaux 1998; Niyogi 1999; de Saint Germain et al. 2013). Besides these functions in plant physiology and development, carotenoids are key compounds for human nutrition and health. Four carotenoids (α-, β- and γ-carotene, and β-cryptoxanthin) have provitamin A activity, since they are precursors for the synthesis of retinoid, retinol (vitamin A), retinal (main visual pigment), and retinoic acid (Fraser and Bramley 2004; Krinsky and Johnson 2005). β-carotene has the greatest vitamin A activity while β-cryptoxanthin and α-carotene have approximately half the vitamin A activity of β-carotene. Vitamin A deficiency is still a cause of childhood blindness and is associated with reduced immune function and increased risk of mortality from gastrointestinal disease and measles (Maida et al. 2008) but in specific populations of developing countries, dietary carotenoids do not provide yet the required daily vitamin A intake (Olson 1994). On the other hand, zeaxanthin and lutein are also important for maintaining eye health since these two carotenoids are macular pigments that can prevent age-related macular degeneration (Carpentier et al. 2009). Furthermore, carotenoids have antioxidant properties, serving as antioxidant scavengers and inhibitors of pro-inflammatory and pro-thrombotic factors, hence they may provide potential benefits in the prevention of cardiovascular and other chronical diseases (Fassett and Coombes 2012; Mordente et al. 2011). Moreover, higher levels of lycopene in serum are inversely correlated with the incidence of prostate cancer (Etminan et al. 2004). Other studies on the mechanisms of action of this carotenoid revealed that lycopene and its derivatives (metabolites or oxidation products) are antagonists of nuclear receptors such as transacting antioxidant response elements (Zaripheh et al. 2006). Therefore, increasing carotenoid content in crop plants to improve their nutritional value and the benefits for human nutrition, preventing the risk of several food-related diseases, have been a major goal and the objective of many research programs worldwide. Over the last three decades significant progress have been done in the manipulation of carotenoid content and composition in a large number of crops by either genetic engineering or conventional breeding, which have been the subject of exhaustive reviews (Giuliano et al. 2008, 2014; Fraser et al. 2009; Rosati et al. 2009; Farre et al. 2010, 2011; Ye and Bhatia 2012). The aim of this chapter is to summarize major breakthroughs and advances in the enhancement of

carotenoid content and composition in agronomical and nutritional important crops, with special emphasis to their potential impact and the benefits for human nutrition and health.

Carotenoids have multiple functions in human nutrition and thus in order to provide food crops with a specific benefit or a particular health-promoting effect, the enhancement of total carotenoid content is not necessarily the best strategy. Rather, strategies should be designed to manipulate specific steps of the carotenoid biosynthetic pathway to enhance when possible the content of the carotenoid of interest, trying to avoid metabolic interferences which may originate negative consequences in the concentration of other metabolically-related carotenoids (Farre et al. 2010). Moreover, carotenoid biosynthetic pathway is complex and each plant species have adopted particular regulatory mechanisms to accumulate a specific carotenoid compliment and that may be also tissue-specific and dependent of the stage of development (Nisar et al. 2015). Then, experimental strategies to enhance carotenoid content and composition in the different crops should be based in a precise understanding of the pathway for each plant species and tissue, identifying key regulatory genes, limiting steps and the mechanism governing the synthesis and degradation of the carotenoid that should be modified. Examples of efficient enhancement of total carotenoids and/or to some particularly useful carotenoids have been obtained in agronomical and nutritional relevant crops, such as rice (Paine et al. 2005), tomato (D'Ambrosio et al. 2014), potato (Diretto et al. 2006), carrot (Jayaraj et al. 2008) or canola (Shewmaker et al. 1999; Ravanello et al. 2003).

12.1.2 Carotenoid Biosynthesis in Plants and Bacteria

Carotenoids are hydrophobic tetraterpenoids (C40 isoprenoids) that are mostly synthesized in the plastids of photosynthetic organisms such as plants, algae, bacteria and fungi, and also in chromoplasts. In contrast, animals cannot synthesize carotenoids and due to their vital importance for nutrition and health, they must obtain them through the diet. Some animals display visible amounts of carotenoids which are obtained by their feeding, e.g. flamingo feathers, lobster shells or salmon flesh among others (Olson 1994). Exceptionally, the red pea aphid (*Acyrthosiphon pisum*) and the two spotted spider mite (*Tetranychus urticae*) have adquired the mechanisms to produce carotenoids by horizontal gene transfer from fungi (Moran and Jarvick 2010; Altinciceck et al. 2012).

Carotenoid biosynthetic pathway is complex and have been elucidated in many organism (Chaps. 2 and 3). Alternatively, synthesis of carotenoids in bacteria is different and simpler than in plants, and has been used for the generation of transgenicplants (Table 12.1, Chap. 3). Condensation of two molecules of GGPP

Table 12.1 Examples of genetically modified plants with enhanced carotenoid levels

Plant species	Organ with enhanced carotenoid content	Gene	Carotenoid profile	References
Rice (*Oryza sativa*)	Seeds	*PSY* (*Narcissus pseudonarcissus*)	Phytoene in the endosperm	Burkhardt et al. (1997)
Golden rice 1	Seeds	*PSY* and *LCYB* (*Narcissus pseudonarcissus*) and *crtI* (*Pantoea ananatis*)	Accumulation of carotenoids in the endosperm	Ye et al. (2000)
Golden rice 2	Seeds	*PSY* (*Zea mays*) and *crtI* (*Pantoea ananatis*)	23-fold increase in total carotenoids in seeds compared to Golden Rice 1	Paine et al. (2005)
White corn (*Zea mays*)	Seeds	*PSY* (*Zea mays*)	Zeaxanthin accumulation	Zhu et al. (2008)
	Seeds	*crtI* (*Pantoea ananatis*),	Slight increase in total carotenoids	Zhu et al. (2008)
	Seeds	*PSY* (*Zea mays*) *crtI* (*Pantoea ananatis*)	Accumulation of lycopene	Zhu et al. (2008)
	Seeds	*PSY* (*Zea mays*), *crtI* (*Pantoea ananatis*), *Lycb* (gentiana lutea),	Accumulation of β-carotene	Zhu et al. (2008)
	Seeds	*PSY* (*Zea mays*), *crtI* (*Pantoea ananatis*), *CBHX and crtW (Paracoccus spp), LYCB (Gentiana lutea)*	Enhanced carotenoid accumulation and presence of ketocarotenoids	Zhu et al. (2008)
Hi-II corn (*Zea mays*)	Seeds	*crtB* and *crtI* (*Pantoea ananatis*)	A 34-fold increase in total carotenoids	Aluru et al. (2008)
Wheat (*Triticum aestivum*)	Seeds	*PSY* (*Zea mays*) and *crtI* (*Pantoea ananatis*)	11-fold increase in carotenoid content	Cong et al. (2009)
	Seeds	*crtB* (*Pantoea ananatis*)	Slightly increase carotenoid content	Wang et al. (2014)
	Seeds	*crtI* (*Pantoea ananatis*)	Slightly increase carotenoid content	Wang et al. (2014)
	Seeds	*crtB* and *crtI* (*Pantoea ananatis*)	A 65-fold increase in provitamin A content	Wang et al. (2014)

Sorghum (*Sorghum bicolor*)	Seeds	*PSY* (*Zea mays*) and *crtI* (*Pantoea ananatis*)	A three to fourfold increase in carotenoid content	Lipkie et al. (2013)
Soybean (*Glycine max*)	Seeds	*PSY* (*Capsicum*) and *crtI* (*Pantoea ananatis*)	A 62-fold increase in carotenoid content (especially β-carotene)	Kim et al. (2012)
	Seeds	*crtB* (*Pantoea ananatis*)	1500-fold increase of β-carotene accumulation	Schmidt et al. (2014)
Tomato (*Solanum lycopersicum*)	Fruit	*crtI* (*Pantoea ananatis*)	A threefold increase in β-carotene	Römer et al. (2000)
	Fruit	*lycb* (*Arabidopsis*), *CBHX* (*Capsicum annuum*)	A significant increase in β-carotene, β -cryptoxanthin and zeaxanthin	Dharmapuri et al. (2012)
	Fruit	*crtB* (*Pantoea ananatis*)	A 2.5-fold increase in β-carotene	Fraser et al. (2002)
	Fruit	*DXS* (*Escherichia coli*)	A 1.6-fold increase in total carotenoids	Enfissi et al. (2005)
	Fruit	*DET*-1 (*Solanum lycopersicum* antisense)	A eightfold increase in β-carotene	Davuluri et al. (2005)
	Fruit	*CRY-2* (*Solanum lycopersicum*)	A 1.7-fold increase in total carotenoids	Giliberto et al. (2005)
	fruit	*PSY*-1 (*Solanum lycopersicum*)	Up to a 1.4-fold increase in total carotenoids	Fraser et al. (2007)
	Fruit	*ctrY* (*Pantoea ananatis*)	A fourfold increase in β-carotene	Wurbs et al. (2007)
	Fruit	*Fibrillin* (*Capsicum annuum*)	A twofold increase in β-carotene	Simikin et al. (2007)
	Fruit	*LYCB* (daffodil)	A 1.6-fold increase in β-carotene	Apel and Bock (2009)
	Fruit	*crtR-b2* (*Solanum lycopersicum*)	Accumulation of xanthophylls	D'Ambrosio et al. (2011)

(continued)

Table 12.1 (continued)

Plant species	Organ with enhanced carotenoid content	Gene	Carotenoid profile	References
	Fruit	RNAi silencing of *SlNCED1*	Up to a 1.5-fold increase in total carotenoids	Sun et al. (2012)
	Fruit	*BZR1-1D* (Arabidopsis)	Up to a twofold increase in total carotenoids in peel and pericarp of mature fruit	Liu et al. (2014)
Potato (*Solanum tuberosum*)	Tuber	*zep* (*Arabidopsis*)	Up to a 130-fold increase in zeaxanthin and 5.7-fold increase in total carotenoids	Römer et al. (2002)
	Tuber	*crtB* (*Pantoea ananatis*)	A 6.3-fold increase in total carotenoids	Ducreux et al. (2005)
	Tuber	*LYCE* (potato antisense)	A 2.5-fold increase in total carotenoids	Diretto et al. (2006)
	Tuber	*crtO* (*Synechocystis* sp)	Ketocarotenoids account for 12 % of total carotenoids	Gerjets and Sandmann (2006)
	Tuber	*DXS* (*Escherichia coli*)	A twofold increase in total carotenoids	Morris et al. (2006)
	Tuber	*crtB* (*Pantoea ananatis*) and *BKT1* (*Hamaetococus pluvialis*)	Accumulation of astaxanthin and ketolutein	Morris et al. (2006)
	Tubes	*Or* (cauliflower, *Brassica oleracea*)	A sixfold increase in total carotenoids. Tubers accumulate β-carotene	Lu et al. (2006) and Li et al. (2012)
	Tubes	*crtB*, *crtI* and crtY (*Pantoea ananatis*)	A 20-fold increase in total carotenoids	Diretto et al. (2007a, b)
	Tuber	*CBH* X (potato antisense)	A 4.5-fold increase in total carotenoids	Diretto et al. (2007)
	Tuber	*CBH* X (RNAi silencing)	Increase in β-carotene content (3 μg/g DW)	Van Eck et al. (2007)
	Tuber	*Or* (cauliflower, *Brassica oleracea*)	A tenfold increase in total carotenoids after long term cold storage	Lopez et al. (2008)
	Tuber	*IbOr*	A 2.7-fold increase in total carotenoids	Goo et al. (2015)

Potato (*Solanum phureja*)	Tuber	*crtB* (*Pantoea ananatis*)	A threefold increase in total carotenoids	Ducreux et al. (2005)
Sweet potato (*Ipomoea batatus*)	Tuber	*IbOr-Ins*	Up to a sevenfold increase in total carotenoids	Park et al. (2015)
Carrot (*Daucus carota*)	Root	BKT1 (*Hamaetoccoccus pluvialis*) and chy (Arabidopsis)	Accumulation of novel ketocarotenoids	Jayaraj et al. (2008)
	Root	*LCYB1* (*Daucus carota*)	Up to a 1.8-fold increase in total carotenoids	Moreno et al. (2013)
Cassava (*Manihot esculenta*)	Root	*crtB* (*Pantoea ananatis*), *dxs* (*Arabidopsis*)	Up to a 14-fold increase in total carotenoids	Failla et al. (2012)
Lettuce (*Lactuca sativa*)	Leave	*crtW*, *crtZ* (*Brevundimonas*) and *ipi* (*Paracoccus* sp.)	Accumulation of ketocarotenoids, accounting for 94.9 % of total carotenoids	Harada et al. (2014)
Orange (*Citrus sinensis*)	Fruit	*Csβ-CHX* silencing and *CsFT* overexpression	A 36-fold increase in β-carotene in the pulp of mature fruit	Pons et al. (2014)

to form 15-*cis*-phytoene is catalyzed by the *crt*B gene, and all desaturations and isomerization are fulfilled by the single *crt*I gene.

Furthermore, the ketocarotenoid astaxanthin is derived from β-carotene by 3-hydroxylation and 4-ketolation at both ionone groups by the action of β-carotene hydroxylase and β-carotene ketolase, respectively. These reactions are catalyzed by two types of enzymes, a non-hemo hydroxylase (CBHX) and three heme-containing cytochrome 450-hydroxylases (Nisar et al. 2015, Chap. 3).

Although some plant species may perform ketolation of carotenoid, as pepper fruits, ketocarotenoids are mainly found in marine organisms, and also many bacteria contain a ketocarotenoid pathway (Bouvier et al. 1994; reviewed in Zhu et al. 2009). However, many ketocatotenoids have important commercial interest. Astaxanthin and canthaxanthin represent more than 55 % of the global market for carotenoids, since they are extensively used as animal feeding additive to improve coloration. Other evidences also support the importance of ketocarotenoids in human health, since astaxanthin has potential health-promoting effects in the prevention of many diseases, such as cancers, metabolic syndrome, and cardiovascular, gastrointestinal, liver and neurodegenerative diseases (reviewed in Giuliano et al. 2008; Yuan et al. 2011). Therefore, there are several plants that have been genetically engineered to produce ketocarotenoids using the ketocarotenoid pathway genes form bacteria and algae (Gerjets and Sandmann 2006; Morris et al. 2006; Jayaraj et al. 2008; Zhu et al. 2008; Fujisawa et al. 2009; Harada et al. 2014, Chap. 8).

12.2 Metabolic Engineering to Enhance Carotenoid Content in Crop Plants

Different experimental strategies have been used to generate food crops with enhanced carotenoid content and composition. Conventional plant breeding and genetic modifications (also referred as genetic engineering) are the two basic approaches addressed in most crops. The rapid development of many biotechnological techniques and procedures, as transformation strategies, introgression lines, new mutant collections and genome sequencing, among others, with no doubt have facilitated and assisted both strategies. Conventional breeding or marker-assisted breeding have produced some progress in the selection of carotenoid improved lines, but the process is slow, time-consuming and restricted to a limited number of species. Collection of genetic variants and mutants with altered carotenoid content and composition are especially useful. Vitamin A biofortification in maize, sweet potato and cassava are excellent examples in which conventional and assisted breeding have allowed substantial enhancement of β-carotene to improve the nutritional and health-related benefits of the population of developing countries (Hotz et al. 2012; Ceballos et al. 2013; Pixley et al. 2013). Genetic manipulation of carotenoid content provides many advantages and has been extensively used

in many species (Fig. 12.1), even tough has also disadvantages over conventional techniques. Targeting of specific genes in a controlled manner to increase or to suppress their expression is a direct approach allowing modification of the concentration of the specific carotenoid of interest. Moreover, the short time required for transformation and regeneration, and the increasing availability of genes from the same species have leaded to a rapid an efficient selection of crops with enhanced carotenoid composition over conventional techniques. However, public concerns in some countries and that the technical requirements and background necessary for genetic manipulation is not available in many developing countries, have limited their expansion.

Significant progresses have been done in the generation of genetic modified crops with altered carotenoid content and composition (Fig. 12.1). The rapid development of these technologies and their application in many crops have allowed not only the enhancement of different carotenoids which are deficient in such a crops but also the modulation and extension of the pathway to produce new carotenoids, such as ketocarotenoids (Farre et al. 2010). These strategies have been shown feasible and efficient to enhance carotenoid content in many plant species, from the model plant Arabidopsis or tomato to staple foods, such rice, maize, or sweet potato, that are the basis of the diet of malnourished population in many developing countries. A revised list of important carotenoid-enhanced crops which are especially relevant for their health-promoting properties are summarized in Table 12.1.

12.2.1 Fleshy Fruits

12.2.1.1 Tomato as the Model Fruit-System

Tomato fruits have become the favorite model plant for research in many aspects of fruit development. The large collection of mutant and more recently isogenic or near-isogenic lines, relatively rapid plant cycle, genetic background, well established genetic transformation protocols, well characterized physiological and biochemical changes during fruit ripening, have turned tomato as a favorite model plant for fleshy fruit studies. Tomato fruits display a characteristic ripening behavior in which the transformation from chloroplast to chromoplast and the concomitant accumulation of carotenoids is controlled by the autocatalytic rise in ethylene production (Seymour et al. 2013). Mature tomato fruits accumulate significant amounts of the linear red-carotene lycopene, but only trace amounts of xanthophylls. Regulation of carotenoid biosynthesis and accumulation during tomato fruit ripening is probably one of the most extensively studied (Fraser et al. 1994; Liu et al. 2015). Evidences from these studies indicate that stimulation of *PSY* gene expression at the onset of ripening is the key regulatory factor challenging the flux of carotenoid trough the pathway, and the down regulation of *LCYB* and *LCYE* genes facilitate the characteristic accumulation of lycopene during ripening. The

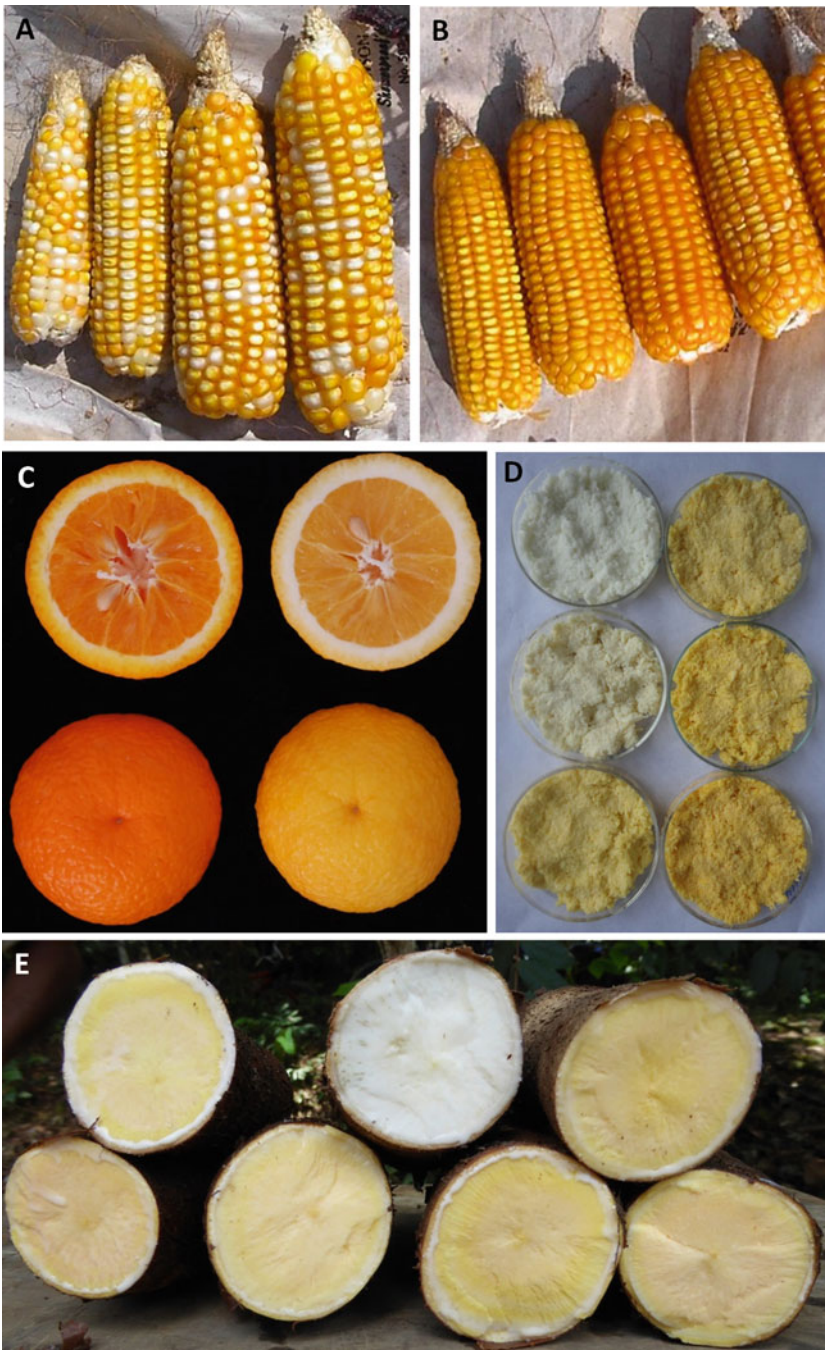

Fig. 12.1 Plants with enhanced carotenoid content and composition. Bio-fortified maize from the HarvestPlus program showing (**a**) cobs with *white* (null) and *pale-yellow* (moderately-enriched) grains of segregating populations and, (**b**) highly β-carotene-enriched *deep-orange* grains.

knowledge of regulatory steps of the pathway and the availability of genes have enabled the generation of many transgenic plants with modified fruit carotenoid content, that in addition have revealed the interaction with other metabolic pathway (Fraser et al. 2007).

The first tomato line transformed with the endogenous *PSY* gene presented detrimental phenotypic effects associated with the presence of the transgenes. Overexpression of the gene restored synthesis of the carotenoid lycopene in the *yellow-flesh* mutant fruits but produced also pigment accumulation in other cell types, and some lines also exhibited inhibition of carotenoid production by the phenomenon of co-suppression (Fray and Grierson 1993). Moreover, the transformation of tomato plants with the *PSY1* gene and the CaMV 35S promoter induced the ectopic accumulation of carotenoids, but generated dwarf plants with reduced chlorophyll contents. Interestingly, the dwarf phenotype was due by an important reduction of gibberellins concentration (Fray et al. 1995). These results indicate a competition of two pathways for the common substrate GGPP, in a way that increasing PSY activity could redirect the channeling of GGPP to the carotenoid pathway at expenses of GA synthesis and the phytyl chain of chlorophylls. Similarly, a feedback repression of the endogenous *PSY* genes was also observed in transgenic plants expressing the bacterial *CrtI* genes, encoding the enzyme converting phytoene into lycopene. Fruit of transformed plants did not display an increase in total carotenoids although the content of β-carotene content increased near 3-times (Römer et al. 2000). The use of fruit specific promoters and chloroplast transit peptides greatly overcome the undesirable side effects produced by the ectopic expression of carotenoid biosynthetic genes. Thus, up to fourfold increase in total carotenoid content was obtained in tomato fruits overexpression of *crtB* gene from *Pantoea ananatis* (formerly *Erwinia uredovora*) under the tomato polygalacturonase promoter, and the tomato phytoene synthase-1 transit sequence (Fraser et al. 2002; Moise et al. 2013).

Modification of carotenoid biosynthetic by alteration of upstream metabolic pathways has been also attempted in tomato fruits. Synthesis of IPP was studied by transformation of tomato with the *1-deoxy-d-xylulose-5-phosphate synthase* (*DXS)* and *3-hydroxymethylglutaryl CoA* (*HMGR-1*) genes from the methylerythritol-4-phosphate (MEP) and the mevalonic acid (MVA) pathways, respectively (Rodriguez-Concepción 2010). Fruits from plants containing *DXS* targeted to the plastid showed a 1.6-fold increase in carotenoid content while those

Fig. 12.1 (continued) (**c**) Internal and external appearance of mature pineapple (wilt-type) sweet orange fruits (*left side*) and transgenic fruits in which the β-carotene hydroxylase (*CsbCHX*) gene was silencing by iRNA, showing the 'Golden Orange' phenotype (*right side*). (**d**) Variation in color intensity of dried root powder from bio-fortified β-carotene-enriched cassava genotypes. (**e**) Transversal sections of roots from bio-fortified β-carotene-enriched cassava genotypes showing variations in color by differential accumulation of β-carotene. Pictures are courtesy of Dra. Natalia Palacios (CIMMYT/Harvest Plus, Mexico) (**a** and **b**); Dra. Elsa Pons and Dr. Leandro Peña (IBMCP, Spain and Fundecitrus, Brasil) (**c**); and Dr. Hernan Ceballos (CIAT, Colombia) (**d** and **e**)

containing an additional *HMGR-1* did not show any change in carotenoids (Enfissi et al. 2005). Hence, it appears that the IPP required for carotenoid biosynthesis was solely or at least mainly derived from the MEP pathway. In addition, the manipulation of the last steps of the pathway e.g. silencing by iRNA the *SlNCED* gene, also turned out to be efficient enhancing carotenoid accumulation, indicating that at least in tomato, such deficiency in the end-product of the pathway doesn't results in altered carotenoid profiling (Sun et al. 2012).

Since mature tomato fruits accumulate negligible amounts of β-carotene, attempts have been done to increase the concentration of this carotenoid with significant provitamin A activity. Orange-colored fruits with a 2–4 times increase in β-carotene was obtained with a constitutive overexpression of the *crtI* from *Erwinia*. Other xanthophylls downstream β-carotene were also enhanced but the content of lycopene was reduced. These metabolic effects appear to be due to the stimulation of the endogenous *LCY* genes (Römer et al. 2000). The use of other transformation procedures with the *Arabidopsis LCYB* gene or the *CtrY* from *Erwinia* enabled the enhancement of β-carotene without substantial reduction of lycopene (Rosati et al. 2000; Wurbs et al. 2007). Another more complex strategy generated tomato plants overexpressing the *LCYB* from Arabidopsis and the pepper *CHYB* genes, and the corresponding fruits contained high levels of β-carotene and the xanthophylls β-cryptoxanthin and zeaxanthin, that were virtually absent in wild type fruits (Dharmapuri et al. 2012). A recent study also reported an elevation of β-carotene in tomato fruits overexpressing the endogenous *β-carotene hydroxylase 2* gene (*CrtR-b2*). However, immature fruits were yellow by the reduced content of chlorophyll and mature fruits accumulated violaxanthin and large amount of esterifies xanthophylls (D'Ambrosio et al. 2011). Lycopene levels in tomato fruit may be also increased by the silencing of both *lycopene cyclase* and *β-carotene hydroxylase* genes but carotenoid content in vegetative tissue was unaffected by using fruit-specie promoters (Rosati et al. 2000). Collectively, these evidences indicate the feasibility of manipulating tomato fruit carotenoid content and composition, but a complex balance/equilibrium appears to be operative during fruit development and ripening, and that alteration in enzyme activity in one step or co-suppression of one gene may lead to an unbalanced pathway and to unexpected carotenoid profiling (Fraser et al. 2009; Fantini et al. 2013).

Enhancement of carotenoid content in tomato fruit has been also accomplished by modification of genes involved in other metabolic process accessory to carotenogenesis. Particularly relevant are genes implicated in photomorphogenesis and light perception, which illustrate the complexity of signals involved in the regulation of carotenoid biosynthesis that direct or indirectly may modulate the carotenoid profiling. Indeed, silencing the expression of the morphogenic repressors *de-etiolated homolog 1* (*DET1*) and *Le COP1LIKE* produce transgenic tomato fruits with enhanced carotenoid and flavonoid contents, indicating common regulatory nodes and the significant improvement of the nutritional value of the fruits (Davuluri et al. 2005). Similarly, overexpression of the blue light receptor *cryptochrome 2* (*CRY2*) resulted in the overproduction of flavonoids and lycopene in fruits (Giliberto et al. 2005). Silencing of other genes involved in light signaling (*LeHY5*) also reduced

lycopene content (Liu et al. 2004). Thus, all these evidences demonstrated the feasibility of signals that may be manipulated in order to enhance carotenoid content and composition and open new avenues and technological strategies to improve the health-related benefits of many other food crops. Other endogenous signals and regulators such as brassinosteroids (BR), has been also showed to influence carotenoid content. Transgenic tomato lines overexpressing the mutant *BZR1-1D* gene from Arabidopsis resulted in an up-regulation of carotenoid biosynthetic genes and increasing lycopene content (Liu et al. 2013). Fibrillins are proteins involved in the formation of lipoprotein structures, such as plastoglobules and in pepper chromoplasts have been implicated in the over-production of pigments due to a sink effect. When the pepper fibrillin gene was expressed in tomato fruits, fibrils were not observed but a two-fold increase in lycopene, total carotenoids and carotenoid-derived flavor volatiles were observed (Simikin et al. 2007).

Transplastomic plants are genetically modified plants in which the new gene is inserted into the chloroplastic DNA. The major advantage of this technology is that in many plant species plastid DNA is not transmitted through pollen, therefore avoiding gene flow from the genetically modified plant to the neighboring plants. Engineering of the plastids genome for the nutritional enhancement of tomatoes has been developed (Wurbs et al. 2007; Apel and Bock 2009). Plastid expression of a bacterial *crtY* gene triggered the conversion of lycopene to β-carotene and produced fruits that contained fourfold more provitamin A than the wild type (Wurbs et al. 2007). Similarly, transplastomic tomatoes with an inserted *LCYB* from daffodil (*Narcissus pseudonarcissus*) efficiently converted lycopene into provitamin A (β-carotene) and showed a 50 % increase in total carotenoids (Apel and Bock 2009).

The production of ketocarotenoids in tomato fruits has been also achieved by the co-expression of the β-carotene ketolase gene from the algal *Chlamydomonas reinhardtii* and β-carotene hydroxylase from *Haematococcus pluvialis* . Transgenic tomato plants up-regulated most carotenogenic genes and increased the carbon flux into carotenoids, resulting in the massive accumulation of free astaxanthin in leaves (3.12 mg/g) and esterified astaxanthin in fruits (16.1 mg/g, Huang et al. 2013), which are carotenoids that are not usually produced in tomato.

12.2.1.2 Citrus

Citrus fruits are hesperidium berries, characterized by having a leathery peel surrounding the edible and juicy portion of the pulp. Citrus peel and pulp are a complex source of carotenoids, and more than 100 different carotenes, xanthophylls and derivate have been described. Particular accumulation of carotenoids and xanthophylls originate the high color diversity among fruits of the different species and varieties, from the yellow of lemon and grapefruits, to the red pulp of some grapefruits and orange mutants, through the characteristic bright-orange of mandarins and oranges (Gross 1987; Rodrigo et al. 2004; Alos et al. 2006, 2008). Citrus fruits are widely recognized by the high consumption as both juice and fresh fruits, and therefore constitute an important source of carotenoids for the

human diet. Carotenoid content is much higher in the peel than in the pulp, being violaxanthin the primary xanthophyll in mature orange fruits, and violaxanthin and β-cryptoxanthin predominate in the pulp of mandarins and hybrids (Rodrigo et al. 2013). The potential provitamin A activity in *Citrus* fruits is, however, moderated, since β-cryptoxanthin is the only xanthophyll with this activity in the pulp of mandarin fruits but its presence in orange is negligible (Alquezar et al. 2008). Thus, increasing the provitamin A activity in *Citrus* would be of much interest and has been addressed. The *CsPSY* gene from CaraCara orange was overexpressed in kumquat, a *Citrus* relative family developing small fruits (*Fortunella hindsii*). Fruits of the transgenic plants showed a deeper orange color and contained a significant increase in phytoene, lycopene, β-carotene, and β,β-cryptoxanthin concentrations while lutein and violaxanthin contents remained almost unchanged, indicating an enhancement of the flux to the β,β-branch of the xanthophylls pathway (Zhang et al. 2009).

Engineering of β-carotene content in orange fruits has been successfully accomplished in a recent study. The rational of that work was to silence the endogenous *β-carotene hydroxylase gene* (*Csβ-CHX*) that is involved in the conversion of β-carotene into xanthophylls, under the expression of the regulatory gene *FLOWERING LOCUS T* from sweet orange that accelerates flowering and reduces the fruiting time. As expected, β-carotene content was enhanced by more than 36-times and the content of downstream xanthophylls were reduced, producing fruits of yellow color both in the peel and pulp which were then termed as "Golden Orange" (Fig. 12.1c). The enhanced nutritional capacity of the juice of the β-carotene-enriched oranges was assessed in the model nematode *Caenorhabditis elegans*. The antioxidant effect of the golden-orange juice against an *in vivo* oxidative stress was a 20 % higher than that of the conventional orange juice, providing a direct evidence of the enhanced health-related benefits of biotechnological modified orange fruits (Pons et al. 2014).

12.2.2 Cereals

Cereals are considered an important source of nutrients, as carbohydrates, proteins or vitamins, and major contributors to the diary food consumption of the population of many under-developed and developing countries. The content of carotenoids in cereal grains is relatively low compared to the majority of fruits and vegetables and, therefore, their fortification may have an enormous impact on the nutrition and health benefits of the consumers (Mellado-Ortega and Hornero-Mendez 2015). Enhancement of carotenoid content and composition in cereal crops, such as rice, wheat, sorghum and maize have received much attention in past decades, and international effort have been developed (Harvestplus programs; http://www.harvestplus.org) to provided enriched-carotenoid cereal products to malnourished populations. Following is a summary of the main contributions to the enhancement of carotenoid content in selected cereal crops.

12.2.2.1 Rice

Rice is probably the staple food more widely distributed and consumed, and constitutes the basis of the diet of large populations in many countries. Unfortunately, rice is rich in carbohydrates but the endosperm doesn't contain carotenoids and the deficiency in vitamin A may results in millions of people suffering from blindness and several other diseases in developing countries. In the early 1990s and trying to alleviate vitamin A deficiencies, research projects initiated in Europe leaded to one of the major biotechnological breakthroughs in food breeding. Rice lines of the japonica and indica background with enhanced β-carotene content were obtained, and international effort has been done to overcome restrictions from public and private patents, allowing application of conventional breeding to introduce the enhanced β-carotene phenotype in local varieties (http://www.goldenrice.org/).

Transformation of rice with the daffodil (*Narcissus pseudonarcissus*) phytoene synthase gene (*PSY*) was the first attempt to increase carotenoid content but only produced accumulation of phytoene in rice endosperm but no other downstream carotenoids (Burkhardt et al. 1997). The first generation of β-carotene-enhanced rice endosperm was obtained by the expression of a mini-pathway, containing the *PSY* and *LCYB* genes from daffodil and the bacterial desaturase/isomerase *crtI* (*Pantoea ananatis*) under the control of different promotors. Transformed grains contained up to 1.6 μg/g dry weight of β-carotene, and also lutein, zeaxanthin and α-carotene (Ye et al. 2000), and because the yellow color of the endosperm it was termed as "Golden Rice". This increment in β-carotene in rice endosperm was still insufficient for the recommended daily vitamin A intake from a usual rice meal and a second generation of Golden Rice was generated. The rational for the new strategy was incorporating a more active *PSY* gene from maize which increased up to 23-times the concentration of β-carotene (37 μg/g dry weight) in the endosperm of the so-called Golden Rice2 (Paine et al. 2005) and fulfill the daily requirement of vitamin A. Thereafter, stability of the transgenes and their introgression into locally adapted and consumed rice cultivars have been developed (Datta et al. 2006, 2007) and it is expected that new β-carotene-enriched rice cultivars would be available in a near future (http://www.goldenrice.org/).

12.2.2.2 Maize

Maize is an important staple crop with a world production over 1000 million Tm and an harvested area of 184 million Ha in 2013 (http://faostat.fao.org). In the population of many underdeveloped countries where maize is the most important food crop, the incidence of child and maternal vitamin A deficiency is very high (West 2002). Although traditional corn varieties have provitamin A, the concentration in seeds is too low to cover vitamin A deficiencies. Therefore, the nutritional enhancement of provitamin A in maize is of paramount relevance for their impact in the amelioration of the diet and the nutritional status of malnourished populations.

The first biotechnological attempt to obtain vitamin A-enriched maize grains was based on the successful experiments of the Golden Rice 2 project. Transgenic plants of Hi-II corn expressed the bacterial *crtB* and *crtI* genes in an endosperm-specific manner, using a modified and highly active β-zein promoter fused to transit peptide of the small subunit of the pea Rubisco (rbcS). Interestingly, the seeds of the transgenic Hi-II corn contained up to 34-fold increase in total carotenoids with a significant accumulation of β-carotene which seemed to be due to the upregulation of the endogenous *LCYB* (Aluru et al. 2008). Then, a highly ambitious strategy was designed to introduce a 'mini-pathway' of five transgenes into the elite variety M37W, in order to increase simultaneously the concentration of three vitamins; A, C and B9 (folate). The successful approach produced new corn transgenic lines accumulating 60 μg/g DW β-carotene, 23 μg/g lycopene, and 36 μg/g zeaxanthin in the endosperm, which represent concentration substantially higher than those found in previous studies (Naqvi et al. 2009). Moreover, when *PSY* (*Zea mays*), *crtI* (*Pantoea ananatis*) and *LCYB* (*Gentiana lutea*) were introgressed into yellow corn varieties, zeaxanthin production was enhanced and reached 56 μg/g dry weight, suggesting the existence of metabolic synergy between endogenous and heterologous pathways (Naqvi et al. 2011). Furthermore, the insertion of five carotenogenic genes (*PSY*, from *Zea mays; crtI* from *Pantoea ananatis, CHBX and crtW* from *Paracoccus spp* and *LCYB* from *Gentiana lutea*) under the control of different endosperm-specific promoters into a white maize variety, generated plants with significantly higher levels of β-carotene and other carotenoids, including complex mixtures of hydroxycarotenoids and ketocarotenoids (Zhu et al. 2008). These examples illustrate the potential of biotechnological transformation strategies to enhance not only carotenoid content and composition, but also other nutrients to deliver new superior maize cultivars. There are, however, vast germplasm collections of maize cultivars available worldwide, and significant progress have been done by conventional and marker-assistant breeding in the search of favorable alleles and to fortify carotenoids in new maize lines (Pixley et al. 2013; Mellado-Ortega and Hornero-Mendez 2015) that are discussed below.

12.2.2.3 Wheat

Wheat is also a staple food and its grain provides a fifth of the calories and the protein for the world's population (Shiferaw et al. 2013). Although wheat breeding research has been intensively developed, numerous features of wheat grains remain to be ameliorated as for example seed nutritional content. Lutein is the main carotenoid found in the wheat endosperm and is, in most cases, accompanied by lower amounts of zeaxanthin, β-cryptoxanthin and β-carotene that may be also heavily esterified (Kaneko et al. 1995; Hentschel et al. 2002; Rodríguez-Suárez et al. 2014; Mellado-Ortega and Hornero-Mendez 2015). Hence, the provitamin A content in wheat seeds is quite low and consequently a number of strategies through

conventional breeding and transgenic techniques have been carried out for the last decades. In the hexaploid wheat variety EM12 cotransformed with the *PSY* (*Zea mays*) and *crtI* (*Pantoea ananatis*) genes under the control of an endosperm-specific promoter (1Dx5) or the constitutive CaMV 35S, total carotenoid content increased around 11-fold (4.96 μg/g dry weight). Moreover, the yellow color of the transgenic wheat lines was not due to the upregulation of the endogenous wheat carotenoid biosynthetic pathway (Schaub et al. 2005; Cong et al. 2009). Another approach to increase the carotenoid content of wheat seeds by biotechnological applications was the insertion of *crtB* and *crtI* genes from *Pantoea ananatis*, which produced a similar enhancement of total carotenoids content (4.76 μg/g dry weights). Importantly, β-carotene was the predominant carotenoid, accounting for 67 % of the total. On the other hand, transformation with either *crtB* or *crtI* alone was not sufficient to enhance the accumulation of carotenoids in the seeds, indicating a reduced activity of the endogenous pathway and that introgression of at least these two enzymes is necessary to obtain carotenoid-enriched seeds by biotechnological modification (Wang et al. 2014). A comprehensive review summarizes current genetic and genomic resources and achievements in wheat in relation to carotenoid metabolism (Wurtzel et al. 2012). Other attempts to get superior wheat lines with high carotenoid concentration in the endosperm have been carried out by conventional breeding and are discussed below in the chapter (see Sect. 12.3).

12.2.2.4 Sorghum

Sorghum and millet are two key components of the Africa Sub-Saharan diets. Consumption of sorghum in African countries is very relevant among cereals, with an average of 20 million Tm per year, which is one third of the total world production. Sorghum seeds are rich in carbohydrates but poor in vitamins and other essential nutrients. Hence, biofortified sorghum lines are being developed in order to reduce the malnutrition associated to the seed-based diets. The primary target of the Biofortified Sorghum project is to provide seeds with increased bioavailable vitamin A, lysine, and iron and zinc (Lipkie et al. 2013; http://www.biosorghum.com). For an efficient malnutrition control not only a sufficient amount of provitamin A in the food should be provided but also appropriate bioaccessibility is required for the assimilation of the compounds. Biotechnological modified sorghum with enhanced levels of total carotenoids and β-carotene has been obtained and the bioaccessibility of carotenoids was assessed, as well that of conventionally breed lines, using porridge preparations. Bioaccessible β-carotene in transgenic sorghum (Homo188-A) was near 5-times higher than that non-transformed. Bioavailability and bioconversion of provitamin A in these sorghum grains should be confirmed *in vivo*, but results provide direct evidences supporting the beneficial nutritional effects of the β-carotene-enriched sorghum lines to enhance total and bioaccessible provitamin A carotenoid levels (Lipkie et al. 2013).

12.2.3 Roots

12.2.3.1 Carrot

Carrot (*Daucus carota*) storage roots accumulate massive amounts of carotenoid pigments, including β-carotene and, to a lower extent, α-carotene. Carrot germplasm display a variety of color (purple, red, yellow, white) envisaging a complex regulation of carotenoid biosynthesis and accumulation. Moreover, carotenoid accumulation and chromoplast formation in carrot is accomplished under dark, contrary to the situation in aerial-growing tissues (Stange et al. 2008; Fuentes et al. 2012; Rodriguez-Concepcion and Stange 2013, Chap. 7). Overexpression of the *Arabidopsis thaliana PSY* gene in carrot resulted in roots of intense color, enhanced total carotenoids and the formation of crystals. The content of β-carotene was substantially increased but also other upstream carotenoids, such as phytoene, phytofluene and ζ-carotene, suggesting that carotene desaturation may be a rate-limiting step (Maass et al. 2009). Moreno et al. (2013) described that the overexpression of *Daucus carota LCYB* (*DcLLCYB1*) correlated with the increase in carotenoid levels in roots and leaves. Moreover, transgenic carrot with higher or reduced levels of *DcLCYB1* displayed increased or decreased, respectively, content of total carotenoids and β-carotene in leaves and in storage roots. Alteration of the transgene *DcLcyb1* was accompanied by a modulation in the expression of endogenous carotenogenic genes, indicating a tight regulation of carotenoid biosynthesis in carrot root in which the cyclization of lycopene may be a essential step (Moreno et al. 2013). On the other hand, transgenic carrot plants have been experimentally tested for ketocaronoid production and accumulation. The ketocarotenoid pathway was introduced by the insertion of the *β-carotene ketolase* gene *Haematococcus pluvialis*() under three different promoters. All the transformed lines presented high root expression, and the expression of the endogenous β-carotene hydroxylase genes was also enhanced in transgenic leaves and roots. Interestingly, up to 70 % of the total carotenoids were converted to novel ketocarotenoids (2400 mg/g root dry weight). The most predominant carotenoids were astaxanthin, adonirubin, and canthaxanthin, and therefore demonstrated that high potential of carrots as biopharming for the production of ketocarotenoid (Jayaraj et al. 2008).

12.2.3.2 Cassava

Cassava, in the Sub-Saharan Africa, is the second most important food crop after maize in terms of total energy consumption and the first in land area planted (www.faostat.fao.org). It is worth noting that cassava is more drought tolerant than maize and their roots can be banked in the soil for up to 3 years with little loss due to herbivory (Ihemere and Sayre 2008). Thus, cassava provides increased food security in comparison to other major crops in Africa (Sayre et al. 2011a, b).

The BioCassava Plus program (BC+) was one of the projects funded by Bill and Melinda Gatess Foundation to alleviate malnutrition in sub-Saharan African

populations. In general, the aims of the project were to improve several limiting nutrients for biofortification in cassava including protein, β-carotene iron and zinc as well as value-added traits (low cyanogens, virus resistance, and increased shelf life) (Njoku et al. 2011). Biofortification through conventional breeding in cassava have generated several lines with increasing β-carotene and intensive studies have demonstrated that the provitamin A activity is substantially higher to fulfill daily requirements and is retained after processing (Montagnac et al. 2009; Ceballos et al. 2012, 2013). Representative pictures of other carotenoid-enriched cassava roots obtained by conventional breeding are shown in Fig. 12.1d, e, and discussed below.

Biotechnological transformation of cassava roots to enrich β-carotene accumulation has been also addressed. Failla et al. (2012) reported the generation of transgenic lines overexpressing two transgenes, the *crtB* from *Pantoea*, and the Arabidopsis *DXS* under the control of a potato root-specific promoter. Carotenoid content was between 15–34-fold greater in the new lines that in wild-type roots, and β-carotene was the primary carotenoid, accounting for about 92 % of the total. These results illustrate the potential for biotechnological enrichment of β-carotene and indicate that cassava roots have the metabolic capability to drive the carotenoid flux to the formation of β-carotene if enough precursors are provided. This assumption is consistent with previous findings demonstrating that a single nucleotide polymorphism in one of the *PSY* genes is able to enhanced catalytic activity, leading to carotenoid accumulation and yellow-colored cassava root (Welsch et al. 2010). Then, similarly to rice PSY appears to be the rate-limiting for carotenoid biosynthesis in cassava roots.

In addition to the concentration of β-carotene in a food matrix, as cassava roots, the type of processing may affect its stability and the absorption by intestinal cells. Analysis of the β-carotene bioavailability in cassava roots using a *in vitro* digestion system and absorption in Caco-2 cells demonstrated that the amount of β-carotene transformed is proportional to the concentration in the roots and then the quantity provide by an enriched-cassava is higher that the corresponding wild-type (Thakkar et al. 2007, 2009). Although all the methods of root processing decreased to different extend the content and stability of β-carotene, the *in vitro* digestion system indicated that the β-carotene-enriched cassava still provide greater amounts of bioaccessible provitamin A to the consumers than conventional roots (Failla et al. 2012).

12.2.4 Tubers

12.2.4.1 Potato

Potato is the most widely consumed vegetable in the world and then a primary food source for population of many countries. Potato tubers are rich in carbohydrates, micronutrients and vitamin C, but unfortunately very poor in carotenoids and provitamin A. Despite potato shows a great diversity in germplasm, β-carotene is not available in commercial and wild species of potato although many tubers varieties

may contain also anthocyanins (Morris et al. 2004; Brown et al. 2005). Because of the relevance of potato for a large world population, intense effort has been done in last decade first to decipher regulation of carotenoid biosynthesis (Taylor and Ramsay 2005; Zhou et al. 2011) and second to enrich the tuber in nutritionally important carotenoids, primary β-carotene to reduce vitamin A deficiency.

Because the negligible amount of early carotenes in potato tubers, a biotechnological strategy to increase carotenoid content was to enhance the flux of precursors by the introgression of early biosynthetic genes. Overexpression of the bacterial *DXS* increased phytoene and total carotenoid content, but also shifted the flux of the pathway to the accumulation of the cytokinine *trans*-zeatine riboside and produced an early sprouting (Morris et al. 2006). An important increment (sixfold) in β-carotene was obtained by the overexpression of the bacterial *CrtB* under the control of the tissue-specific patatin promotor (Ducreux et al. 2005). Similar approaches overexpressing the *β-carotene ketolase* (*CrtO*) (Gerjets and Sandmann 2006) from several organisms, as *Synech ocistis*, or green algae (Morris et al. 2006) or a combination of the *crtW* and *crtZ* forma bacteria and the *Or* from cauliflower (Campbell et al. 2015) substantially enhanced the concentration of astaxanthin and also total ketocarotenoids, up to 12 % of total carotenoids.

The spontaneous cauliflower orange mutant *Or*, allowed the identification of a gene, named as *Or*, that when mutated produces accumulation of β-carotene in chromoplasts. The function of the gene is not yet defined by interacts with plastid-associated proteins that modified the capability to sequesters carotenoids in specialized structures originating accumulation of β-carotene head crystals (Li et al. 2001; Lu et al. 2006). Overexpression of the *Or* gene in potato produced the same phenotype as in cauliflower and tubers accumulated more than tenfold the normal levels of β-carotene. Moreover, *Or*-overexpressing potatoes also accumulated phytoene and phytofluene, and the amyloplast also accumulated β-carotene crystals that were stable under cold (Lopez et al. 2008).

A major enhancement of carotenoid content in potato was accomplished by Diretto et al. (2007a) overexpressing a mini-pathway from *Erwinia* composed by the *CrtB*, phytoene synthase *CrtI* and lycopene βcyclase *CrtY*. Constitutive expression of the genes interfered with the endogenous expression of the genes in leaves and produced unusual accumulation of carotenoids. By contrast, coordinated expression of the three genes under a tuber-specific promoter originated a massive accumulation of total carotenoids (20-fold increment) and reached levels of β-carotene as high as 47 μ g/g dry weight. These tubers displayed a characteristic deep-yellow color (*Golden* phenotype) and accumulated also xanthophylls.

Other rational to enhance carotenoid content in potato was the silencing of genes of both branch of lycopene cyclization. Decreasing the expression of *LCYE* increased the levels of upstream carotenoids but it interfered with the endogenous expression of carotenoid biosynthetic genes (Diretto et al. 2006). A more severe phenotype was obtained by silencing *LCYE*, which eliminates the competence from the α-carotene pathway, and also *CHY* in a tuber-specify manner, generating tubers with a 38-fold increment in β-carotene and 4.5-fold in total carotenoids (Diretto et al. 2007b). However, and as expected, a partial reduction in downstream

xanthophylls was also detected, probably by modification of the endogenous pathway. A similar strategy was adopted by Van Eck et al. (2007) in other potato cultivars in which silencing only the *CHBY* gene generated potatoes enriched in β-carotene. Finally, silencing a downstream gene in the pathway, as *ZEP*, increased zeaxanthin content without affecting β-carotene and violaxanthin, but unexpectedly there was also an increment in the concentration of tocopherol (Römer et al. 2002). All these results illustrate the feasibility to modify carotenoid content in potato tuber, and particularly, β-carotene, by different biotechnological strategies and it is expected that may be the basis for further improvement of the nutritional value of this crop.

12.2.4.2 Sweet Potato

Sweet potato (*Ipomoea batatas* L.) is also a nutritionally important staple food for the population of many locations of Asia and Africa countries. Sweet potato exhibit a large diversity in flesh coloration by the presence of different proportion of the two pigments carotenoids or anthocyanins, and large germplasm collections can be found in many sweet potato-producing countries. This tuber is a good example of intensive international effort in biofortification by conventional genetic breeding a many new orange-fleshed varieties have been obtained (Tunmegamire et al. 2014). However, molecular studies of the regulation of carotenoid biosynthesis and its manipulation are scarce. Park et al. (2015) overexpressed the homologues of the *Or* gene in sweet potato and found an elevation of the carotenoid that are present in non-transformed control, α- and β-carotene, lutein, zeaxanthin and β-cryptoxanthin. Moreover, the enriched-carotenoid sweet potato was more resistant to salt and oxidative stress, indicating an enhanced antioxidant capacity. In other recent study, genetic transformation of sweet potato with transcription factors of anthocyanin biosynthesis resulted in tuber with an altered carotenoid biosynthesis (Goo et al. 2015). Highly anthocyanin-pigmented tubers contained less carotenoid and vice versa, indicating that both biosynthetic pathways may be connected. If this relationship is by a directly metabolic connection or by an indirect mechanism, as the antioxidant status remains to be elucidated.

12.2.5 Other Crops

12.2.5.1 Canola

Genetic engineering of canola for the production of carotenoids in seeds was one of the pioneering works addressing the biotechnological enrichment of carotenoid content in foods (Chap. 13). Earlier studies expressing the *Crtb* gene under a seed-specific promoter were very efficient increasing carotenoid content in canola seeds. Total carotenoids were enhanced by more than 50-times compared with

un-transformed plants, increasing primary β-carotene and α-carotene in a ratio 2:1 (Shewmaker et al. 1999). Thereafter, new strategies were developed to obtain new canola transgenic lines with improved carotenoid content and composition. Hence, the expression of additional bacterial genes for the enzymes *geranylgeranyl diphosphate synthase* (*crtE*), *phytoene desaturase* (*crtI*), *crtY* and the plant *LCYB* from *Brassica napus* were engineered in transgenic canola seed in combination with phytoene synthase (*crtB*). From the different gene combination assayed, the insertion of *crtB*, *crtI* and *crtY* from *Pantoea ananatis* was the only one increasing significantly the ratio β- to α-carotene, from 2:1 to 3:1 (Ravanello et al. 2003). Silencing of the *LCYE* gene using RNAi in canola, revealed that this step may be rate-limiting in carotenoid biosynthesis, because total carotenoid content as well as β-carotene, zeaxanthin, violaxanthin and unpredictably lutein concentration increased in the transgenic seeds (Yu et al. 2013).

Canola plants have been also successfully used to understand the role of novel regulators of carotenoid biosynthesis initially identified in the model plant *Arabidopsis thaliana*. Up to 41-fold increase in β-carotene content was detected in seeds of canola plants transformed with the chloroplast signal recognition particle 54 kDa subunit gene (*cpSRP54*, Arabidopsis) under the *napin* promoter (Yu et al. 2012). Moreover, constitutive expression of *AtmiR156b* in *Brassica napus* resulted in enhanced levels of lutein and β-carotene in the seed (Wei et al. 2010). These results illustrated that additional genes and regulatory factors of unknown function may be relevant in the regulation of carotenoid biosynthesis and they modification would result in altered pigment content. Manipulation of canola to produce ketocarotenoids has been also achieved by the insertion of seven key genes involved in ketocarotenoid formation (Chap. 13), namely: *IDI* (*Paracoccus* sp.), *crtE*, *crtB*, *crtI* and *crtY* (*Pantoea ananatis*) and *crtZ* and *crtW* (*Brevundimonas* sp.). The total carotenoid content increased 19-30-fold and, in addition, the total amount of ketocarotenoids ranged from 60–190 μg/g fresh weight (Fujisawa et al. 2009).

12.2.5.2 Soybean

Soybean contain an unusually complete aminoacid composition, and a diversity of minerals, vitamins, isoflavones and a highly polyunsaturated fatty acid content that makes soy- derived products convenient for a healthy diet (Erdman and Fordyce 1989). Because of the importance of soybean in the total world production, researches and public companies pay attention to the generation of nutritional-enhanced soybean early than in other crops. Despite insect and herbicide resistant soybean were created several decades ago (Hinchee et al. 1988), carotenoid-enhanced soybean phenotypes were more recently generated. Insertion of two carotenoid biosynthetic genes, *PSY* from *Capsicum* and *crtI* from *Pantoea* in Korean soybean, increased total carotenoid contents up to 62-fold, being 77 % β-carotene (Kim et al. 2012). Remarkably, the seed-specific overexpression of the *Pantoea ananatis crtB* gene targeted to plastids resulted in the accumulation of

high levels of β-carotene (over 1500-fold compared with un-transformed lines). In addition, the transgenic seeds also displayed two collateral traits: elevated de-saturated oil and increased protein content which are important to enhance the nutritional value of the crop (Schmidt et al. 2014).

12.2.5.3 Lettuce

Lettuce is a leafy vegetable highly consumed in the world, but unfortunately it has a low nutritional value (Mou 2009). Therefore, even small increases in the concentrations of dietary constituents in lettuce could have widespread effects. Metabolic engineering has been applied to generate lettuce plants enriched in vitamin E and folate (Lee et al. 2007; Nunes et al. 2009; Yabuta et al. 2013). In addition, the red pigment astaxanthin which has diverse clinical benefits against age-related diseases, and muscle or eye fatigue (Guerin et al. 2003; Kidd 2011), was also synthesized in transformed lettuce plants. The plastid genome of lettuce has been site-specifically modified with the insertion of three transgenes from marine bacterium, *crtZ*, *crtW* (*Brevundimonas* sp.) and *ipi* (*Paracoccus*). Astaxanthin is naturally produced by a unicellular green alga *Haematococcus pluvialis*. This carotenoid extracted from the green algae comprised a fatty acid diester (28 %), a monoester (70 %), and the free form (2 %) (Okada et al. 2009). Astaxanthin has been produced in the edible organs of several crops such as maize (Zhu et al. 2008), potato (Morris et al. 2006; Gerjets and Sandmann 2006), tomato (Huang et al. 2013), carrot (Jayaraj et al. 2008), and rapeseed (Fujisawa et al. 2009). In general, the free form of astaxanthin may accumulate in all the crops with the exception of tomato fruits (Huang et al. 2013). Transformed lettuce leaves produced astaxanthin fatty acid (myristate or palmitate) diester (49.2 % of total carotenoids), astaxanthin monoester (18.2 %), and the free form of astaxanthin (10.0 %) and other ketocarotenoids (17.5 %). These results indicated that artificial ketocarotenoids account for up to 95 % of total carotenoids (230 μg/g fresh weight) while the native carotenoids lactucaxanthin and lutein represented only the 3.8 and 1.3 %, respectively (Harada et al. 2014).

12.3 Bio-fortification and Quantitative Trait Loci Related to High Carotenoid Contents in Plants

As discussed in previous sections, bio-fortification is a conventional breeding strategy to enhance the content in micronutrients, minerals, pigments or any other element in a staple food to improve its nutritional value and its health-beneficial effect for malnourish population. This genetic improvement of a food crops is particularly suitable for rural-based population and in the case of carotenoid has been very successful and has allowed the alleviation of vitamin A deficiency that is

one of the major nutrition-related problems in many underdeveloped and developing countries (Bouis et al 2011; Dwivedi et al 2012). Major achievement has been done in selected crops by international networking strategies and collaborative projects, in which the HarvestPlus (http://www.harvestplus.org) and other institutions have been essential and played determinant roles. Staple food with enhanced β-carotene content, such as maize (Pixley et al. 2013), cassava (Ceballos et al. 2012) or sweet potato (Tunmegamire et al. 2014) have been obtained and locally adapted and spread in Asian and Africa countries.

Other genetic analysis, as quantitative trait loci (QTL) has been applied to the study of the DNA regions of a plant genome related to carotenoid accumulation. QTL studies have improved the knowledge on DNA fragments involved in the variation of carotenoid content and have provided information on the genetic architecture of this trait in several plant species. Moreover, QTLs have led to the identification of candidate genes that could participate in the regulation of carotenoid concentration. A recombinant inbred (RI) population with 233 RI maize lines derived from a cross between By804 and B73 was used to detect QTL revealing that much of the phenotypic variation in carotenoids contents may be explained by two loci (*y1* and *y9*). A gene targeted marker (Y1ssr) in the candidate gene *phytoene synthase 1* (*PSY1*) tightly linked to a major QTL explained up to 27 % of phenotypic variation for carotenoids content (Chander et al. 2007). Other experiment showed that a 378-bp InDel upstream of the transcription start site and a SNP in the fifth exon resulting in a Thr to Asn substitution may be functional sites associated with total carotenoid levels in maize grain (Fu et al. 2013). PSY has also been studied in wheat and the allelic variation at *PSY1*-A1 was associated with the yellow pigment trait (YP). Hence, the *PSY1*-A1o allele was associated with elevated pigment in a validation population comprising 93 diverse cultivars and breeding lines (Singh et al. 2009).

Major QTLs for carrot color are positionally associated with carotenoid biosynthetic genes and interact epistatically in a domesticated x wild carrot cross. Based on the progeny of a wild white carrot (QAL), which doesn't accumulate pigments crossed with domesticated orange carrot (B493), one of the richest sources of carotenoid pigments-mainly provitamin A β- and α- carotenes, two major interacting loci, *Y* and *Y(2)*, associated with the carotenoid biosynthetic genes *zeaxanthin epoxidase* and *carotene hydroxylase*, and *carotenoid dioxygenase* gene family members, were found to control much variation for carotenoid accumulation in carrot roots (Just et al. 2009).

Two QTLs for increased fruit lycopene content, inherited from a high-lycopene *S. pimpinellifolium* accession, were detected in tomato chromosomes 7 and 12 using a *S. lycopersicum* × *S. pimpinellifolium* RIL population, and were identified as potential targets for marker-assisted selection and positional cloning. Statistical analyses revealed that while lyc7.1 did not significantly increase lycopene content in the heterozygous condition, individuals harboring lyc12.1 (localized to tomato chromosome 12) in the heterozygous condition contained 70.3 % higher lycopene than the recurrent parent. The derived sub-NILs could be used for transferring

lyc12.1 to other tomato varieties (Kinkade and Foolad 2013). A major QTL for pigment content in pepper fruit, pc8.1, is associated with variation in plastid compartment size. Quantitative variation in pigment content was studied in a cross between a dark-green *Capsicum annuum* pepper and a light-green *C. chinense* pepper. QTL pc8.1, affected carotenoid content in the ripe fruit and found that the QTL exerts its effect via increasing chloroplast compartment size in the dark-green genotypes (Brand et al. 2012).

Other genetic strategies based on genome-wide association (GWAS) are now being used to identify allelic variation for genes controlling carotenoid content and composition. This technique requires wide allelic diversity and a high-density genotyping, that are now only available for selected plant species, as maize (Owens et al. 2014; Suwarno et al. 2015) but it may provide the basis to address similar objectives in other agronomical important plants in the future.

12.4 Concluding Remarks

Biotechnological manipulation of carotenoid content and composition to enhance the nutritional value and health related benefits of many foods have been successfully addressed in past decades. Strategies have been based on the silencing of particular genes to increase the concentration of upstream carotenoids of the pathway or to overexpress key biosynthetic genes to challenge the pathway to increase metabolic precursors. Evidences accumulate indicate that each plant species and tissue may have particular regulatory and rate-limiting steps but, in general, three points appears to be key in the pathway: first, early step regulating the flux of entrance (phytoene synthase); second, the branching point diverging from lycopene, particularly lycopene β-cyclase; and third, β-carotene hydroxylase, that depletes the level of carotenes with provitamin A activity. Moreover, it becomes evident that other accessory or side-associated steps of the pathway may be also important regulating carotenoid accumulation and are potential targets to enhance their content. Application of genetic engineering strategies has also strong limitations, by scientific and technical reasons in many plants species and by public concerns in many countries. Conventional breeding has becoming successful enriching carotenoid content in important staple foods, and β-carotene-biofortified maize, cassava and sweet potato varieties have been obtained and are spread in countries with malnourished population affected of vitamin A deficiency. The rapid development of new "omic" technologies, transformation of recalcitrant plants and a better understanding of carotenoid biosynthesis and catabolism in different plants species will open new avenues to enrich foods with carotenoid not only to alleviate deficiencies but also to provide a better antioxidant balance and other health-related benefits.

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Chapter 13
Modern Breeding and Biotechnological Approaches to Enhance Carotenoid Accumulation in Seeds

M. L. Federico and M. A. Schmidt

Abstract There is an increasing demand for carotenoids, which are fundamental components of the human diet, for example as precursors of vitamin A. Carotenoids are also potent antioxidants and their health benefits are becoming increasingly evident. Protective effects against prostate cancer and age-related macular degeneration have been proposed for lycopene and lutein/zeaxanthin, respectively. Additionally, β-carotene, astaxanthin and canthaxanthin are high-value carotenoids used by the food industry as feed supplements and colorants. The production and consumption of these carotenoids from natural sources, especially from seeds, constitutes an important step towards fortifying the diet of malnourished people in developing nations. Therefore, attempts to metabolically manipulate β-carotene production in plants have received global attention, especially after the generation of Golden Rice (*Oryza sativa*). The endosperms of Golden Rice seeds synthesize and accumulate large quantities of β-carotene (provitamin A), yielding a characteristic yellow color in the polished grains. Classical breeding efforts have also focused in the development of cultivars with elevated seed carotenoid content, with maize and other cereals leading the way. In this communication we will summarize transgenic efforts and modern breeding strategies to fortify various crop seeds with nutraceutical carotenoids.

Keywords Carotenoids in Seeds • *Brassica napus* • Golden rice • Metabolic engineering • Crop fortification

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13.1 Introduction

Consumers are becoming increasingly aware and interested in healthy foods. In the human diet, carotenoids have been shown to have antioxidant activity which may help to prevent certain kinds of cancers, arthritis and atherosclerosis (Stahl and Sies 2003). Free radicals are by-products of many metabolic reactions in the human body. They can have damaging effects on DNA, proteins and cell membranes and as such have been linked to numerous diseases/aliments. Carotenoids are potent anti-oxidants and might help prevent aliments due to oxidative damage. Although there are over 700 carotenoids identified in nature (Britton et al. 2004; Delgado-Vargas et al. 2000), only the consumption of six of them, α-carotene, β-carotene, lutein, lycopene, zeaxanthin and astaxanthin, have been shown to provide health benefits (Johnson 2002). β-carotene might be the most known of the nutraceutical carotenoids since it is pro-vitamin A and after its consumption there is a conversion of it to its active vitamin A (retinol) form in the intestine (Olson 1989). β-carotene has been shown to alleviate deficiencies leading to night blindness and other related nutritional insufficiencies (Haskell et al. 2005) and may improve gut and immune health (Chew 1993). Lutein and zeaxanthin have been implicated as protective agents against acquired ocular diseases, such as cataracts and age related macular degeneration (Tan et al. 2008). Astaxanthin has also gained attention as a potent antioxidant (Chap. 1). The addition of both ketone and hydroxyl groups to β-carotene by the enzyme β-carotene ketolase produces the red hue nutraceutical carotenoid astaxanthin. Most higher plants do not possess the ability to synthesize it. Thus, manipulation of ketocarotenoid synthesis requires the addition of metabolic steps not usually present in the organs of crop plants.

13.2 Seed Carotenoids

Seeds are global commodities as much of our food/feed, fuel and fiber are derived from them. As the vehicle to propagate a plant's next generation, plants sequester energy reserves in the form of carbohydrates, oils and proteins to aid the seed in germination and to ensure propagation of the species. Seeds are high in needed nutrients. They are dry individual entities that can easily be stored or shipped long distance without alteration in the seed content or viability. This represents an advantage over fruits and vegetables which are excellent sources of carotenoids but have shorter shelf lives and may require refrigeration. Undoubtedly, the fortification of staple seed crops is a more cost effective, and thus feasible venue, to cope with malnutrition in developing countries.

Seed carotenoid composition can vary widely depending on the crop species, cultivar, developmental stage and growing conditions. As seen in Table 13.1, seeds of different crop species primarily accumulate lutein, zeaxanthin and β-carotene (Aluru et al. 2008; Kim et al. 2012; Rodriguez-Suarez et al. 2014; Salas Fernandez

Table 13.1 Seed carotenoid content in different crop species

	Cultivar/Genotype	Total carotenoid content (μg/g)	Carotenoid composition (μg/g)	References
Canola	DH12075	5.34	3.3 (lutein) 0.49 (b-carotene)	Yu et al (2007)
Durum wheat	Simeto, Claudio, Don Pedro, Kofa, UC1113	3.22	2.92 (lutein) 0.24 (zeaxanthin) 0.05 (b-carotene)	Rodriguez-Suarez et al (2014)
Maize	B73	33	16.74 (lutein) 6.33 (zeaxanthin) 4.5 (zeinoxanthin)	Aluru et al (2008)
Sorghum	KS115	168.5	89.5 (lutein) 71.5 (zeaxanthin) 7.4 (b-carotene)	Salas Fernandez et al (2008)
Soybean	Kwangan	2.36	2.28 (lutein) 0.06 (b-carotene)	Kim et al (2012)
Tritordeum	HT621, HT240, HT335, HT609, HT630	9.14	9.06 (lutein) 0.08 (b-carotene)	Rodriguez-Suarez et al (2014)

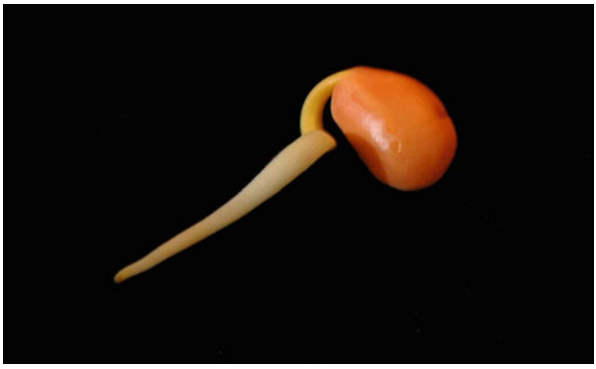

Fig. 13.1 Phytoene synthase transgenic soybean seed accumulating enhanced levels of β-carotene (Schmidt et al. 2015) display no difficultly in germination

et al. 2008 , Yu et al. 2007). However, provitamin A carotenoids are present at low percentages and biofortification of staple crops is an important breeding objective. The role that carotenoids play in seed tissues is less clear than in other plant organs but is emerging (Howitt and Pogson 2006). Interestingly, carotenoid synthesis in seeds is related to abscisic acid (ABA) synthesis which in turn is required for seed dormancy (Maluf et al. 1997). In addition, carotenoids act as antioxidants preventing seed ageing and contributing to seed viability and successful germination (Fig. 13.1) (Havaux et al. 1991; Pinzino et al. 1999).

Understanding the biochemical steps of carotenoid synthesis have not resulted yet in the predictable control of the biosynthesis in plants through either breeding or transgenic approaches. Duplication of key biosynthetic genes, pathway bottlenecks, enzyme sub-organelle localization, metabolon assembly and activity are only

partially known, species specific and can have a direct effect on seed carotenoid accumulation (Shumskaya et al. 2012; Shumskaya and Wurtzel 2013; Giuliano 2014).

13.3 Regulation of Carotenoid Biosynthesis and Accumulation in Seeds

At least three major mechanisms involved in the regulation of carotenoid biosynthesis and accumulation in higher plants have been described to date. These mechanisms include (i) transcriptional regulation of key carotenoid biosynthetic genes, (ii) the existence of specialized carotenoid-sequestering structures, and (iii) the extent of carotenoid degradation (catabolism).

Several reports indicate that transcript levels of key biosynthetic genes correlate with increased levels of carotenoid content in plants (Chap. 2, Harjes et al. 2008; Vallabhaneni and Wurtzel 2009; Vallabhaneni et al. 2009; Yan et al. 2010; Rodriguez-Suarez et al. 2014). A positive correlation of *PSY* gene expression and total carotenoid seed content has been the best described example (Li et al. 2008a, b; Vallabhaneni and Wurtzel 2009; da Silva Messias et al. 2014). In *Arabidopsis thaliana*, PSY is encoded by a single copy gene (Scolnik and Bartley 1994) but most plant species contain a *PSY* gene family composed of at least two or three homologous genes (Bartley et al. 1992; Bartley and Scolnik 1993; Busch et al. 2002; Gallagher et al. 2004; Li et al. 2008a; Arango et al. 2010; Cardenas et al. 2012). Therefore, functional characterization of *PSY* homologues could be essential to understanding carotenoid accumulation in seed tissues (Lopez Emparan et al. 2014). The same is true for other gene families involved in carotenoid biosynthesis, underpinning the importance of genome complexity of individual plant species. In maize, the *y1* gene encodes for *PSY1*, one of the three *PSY* homologues present in this crop genome and several alleles for this gene have been described (Buckner et al. 1996; Gallagher et al. 2004). *PSY1* transcript accumulation, but not that of *PSY2* and *PSY3*, positively correlates with carotenoid accumulation in maize endosperm (Li et al. 2008b; Vallabhaneni and Wurtzel 2009). In addition, *PSY1* alleles exhibit expression differences that correlate with endosperm carotenoid levels (Buckner et al. 1996). Elevated seed carotenoid content is also the result of lower expression rates of specific biosynthetic genes. Natural genetic variants of low expression/activity of lycopene ε cyclase (*LCYE*) in maize are correlated with up to threefold differences in β-carotene (Harjes et al. 2008). Similarly, screening and characterization of genetically diverse maize germplasm revealed that elevated β-carotene levels correlate with low transcription of genes encoding for β-carotene hydroxylases (Vallabhaneni et al. 2009; Yan et al. 2010). These elevated β-carotene levels in the kernels are thought to be explained by the reduced conversion of β-carotene to downstream xanthophylls, such as zeaxanthin.

Carotenoid accumulation is influenced by the presence of structures capable of storing carotenoids (Cazzonelli and Pogson 2010). Depending on the plant organ,

carotenoids will be stored in different plastids (Chap. 10). Although carotenoids can be stored in all plastid types, the stability in each of them may vary. For example, carotenoids accumulated in chromoplast plastoglobuli exhibit much higher light stability than carotenoids in chloroplast membranes (Merzlyak and Solovchenko 2002). The Orange (*Or*) gene mutation in cauliflower (*Brassica oleracea var botrytis*) produces the accumulation of high levels of β-carotene in tissues that normally do not contain carotenoids by triggering the differentiation of proplastids or non-colored plastids into chromoplasts (Chap. 10, Lu et al. 2006; Li et al. 2001). In seeds, elaioplasts are better seed carotenoid-sequestering structures than amyloplasts (DellaPenna and Pogson 2006). Recently, the study of sub-organelle localization in maize cells, rice and Arabidopsis *PSY* allelic variants revealed that different PSY1 isozymes localize to distinct plastid compartments, highlighting the importance of enzyme and metabolome localization (Shumskaya et al. 2012). Interestingly, transient expression studies revealed that maize PSY2 and PSY3, rice and Arabidopsis PSYs localize to plastoglobuli, which are mostly attached to thylakoid membranes, while maize PSY1 exhibited a dual localization and was also found in the stroma (Shumskaya et al. 2012). A deep study of maize and other grasses PSY1 coding sequences discovered that 99 % of 79 varieties with yellow endosperm carry a threonine residue at position 257 (T_{257}) of the PSY1 protein. Most other PSY1s from white endosperm varieties and two species of Teosinte (the maize wild ancestor), carried either proline or serine at this position. Different structural variants of PSY were thus described and it was suggested that the combination of an insertion in the PSY1 promoter region (providing endosperm expression to the yellow allele) and a unique structural variation of the PSY1 protein, asparagine (N_{168}) and threonine (T_{257}), resulted in the ideal for amyloplast carotenogenesis (Shumskaya et al. 2012).

Carotenoid catabolism has been shown to be another important aspect of carotenoid content regulation. For example, loss of function mutants of the carotenoid cleavage dioxygenase 1 (CCD1) gene in *Arabidopsis* exhibit 40 % increased levels of total carotenoids in seeds (Auldridge et al. 2006). This is consistent with a functional characterization of a recombinant maize CCD1 that showed that provitamin A carotenoids in the grains could be cleaved by this enzyme (Sun et al. 2008). In addition, both CCD1 copy number variation and CCD1 transcript accumulation during grain development negatively correlated with seed carotenoid content in maize (Vallabhaneni et al. 2010, da Silva Messias et al. 2014). Expression of *CCD1* during grain development varied widely among maize landraces, with a white variety exhibiting the highest *CCD1* expression levels (da Silva Messias et al. 2014). Similarly, loss of function of the carotenoid cleavage dioxygenase 4 (CCD4) gene in *Arabidopsis* greatly reduced carotenoid degradation during seed desiccation, resulting in an 8.4-fold increase of β-carotene levels (Gonzalez-Jorge et al. 2013). Clearly, further characterization of seed crops germplasm is required to fully understand *CCD1* and *CCD4* gene expression in other crop species to better recognize the implications of carotenoid degradation in breeding for enhanced carotenoid content in seeds.

13.4 Biotechnological Efforts to Enhance Seed Carotenoid Content

13.4.1 β-Carotene Increment in Seeds

Due to the high incidence of vitamin A deficiency in developing nations, many crops have been biofortified with β-carotene. With the success of 'Golden Rice' and 'Golden Rice 2' (Paine et al. 2005; Enserink 2008) pioneering the way, numerous crops have now been reported to have engineered levels of β-carotene predominantly by the over expression of the phytoene synthase gene from *Erwinia uredovora*. The use of PSY alone or in combination with other downstream carotenoid enzymes indicates the effectiveness of shuttling the initial substrate in a successful metabolic engineering effort (Schmidt et al. 2015; Fig. 13.1). Emphasizing the importance of *PSY* gene, emerges the evidence that the *Narcise pseudonarcise* (daffodil) *PSY* was a limiting step in the initial enriched Golden Rice (Ye et al. 2000). Subsequently, through systematic testing of different *PSY* genes, authors selected those belonging to maize and coupled it with the original *E.uredovora* carotene desaturase (*CRTI*) gene that resulted in a 23-fold increase in β-carotene in rice grains, giving rise to 'Golden Rice 2' (Paine et al. 2005). This, *PSY* was overexpressed and resulted in elevated β-carotene levels in other seed crops, such as canola (*Brassica napus*) (Fig. 13.2) (Shewmaker et al. 1999), maize (*Zea mays*) (Aluru et al. 2008), flaxseed (*Linum usitatissimum*) (Fujisawa et al. 2008), wheat (*Triticum aestivum*) (Wang et al. 2014) and soybean (*Glycine max*) (Schmidt et al. 2015; Kim et al. 2012). Efforts to further increase β-carotene

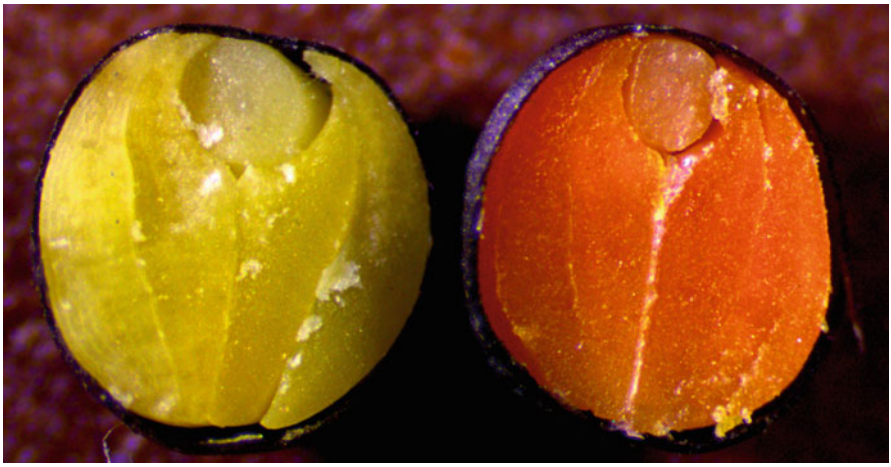

Fig. 13.2 Seed cross section of genetically modified canola with enhanced carotenoid content (*right*). A wild type seed (*left*) is shown for comparison

in rice endosperm focused on either increasing the up-stream isoprenoid carotenoid precursor or creating a carotenoid storage sink. Firstly, investigators reproduced the 'Golden Rice 2' genotype by introducing the same two carotenoid biosynthesis steps in an endosperm-specific manner in rice and then over expressing the 1-deoxy-D-xylose-5-phosphate synthase (*DXS) Arabidopsis thaliana* gene increasing through this strategy the pool of geranalygeranyl diphosphate (GGPP) carotenoid precursor. This DXS enzyme catalyzes the first, and likely rate-limiting, step of the MEP pathway (2-C-methyl-D-erythritol 4-phosphate) resulting in the synthesis of isoprenoids, and hence carotenoid biosynthesis metabolites. Bai et al. (2016) found that transgenic rice with the overexpressed *DXS* together with *ZmPSY* and *EuCRTI*' transgenes had a 2.7–5.8-fold increase in total carotenoids, with β-carotene being the most abundant, compared to seeds expressing only the two 'Golden Rice 2' gene cassettes. A sink of the carotenoid metabolites was produced by also introducing the *A. thaliana* ORANGE (*OR*) gene. The *OR* gene was originally discovered as a naturally occurring dominant mutant in cauliflower and investigations have shown it results in enhanced β-carotene accumulation through the creation of a storage sink for carotenoids (Li et al. 2001; Lu et al. 2006; Lopez et al. 2008). Bai et al. (2016) overexpressed *OR* gene in rice endosperm with/without the two transgene cassettes composed by *ZmPSY* and *EuCRTI* and found a 2.1–4.7-fold increase in the total carotenoid content, due mostly to β-carotene and lutein over accumulation. Carotenoid enhancement was only detected in the transgenic seeds expressing the 'Golden Rice 2' transgenes and the newly introduced seed-specific OR cassette. In contrast to cauliflowers, the overexpression of *OR* itself is insufficient to result in carotenoid accumulation and chromoplast differentiation in rice seeds. It is likely the presence of the OR protein may enhance chromoplast production only in an environment of sufficient carotenoid metabolites (Bai et al. 2016). It is interesting to note that in 'Golden Rice 2' among 75–84 % of total carotenoids corresponds to β-carotene, while Bai et al. (2016) using the same strategy, obtained only 25–39 % of β-carotene in transgenic rice endosperm. In most biofortified transgenic species, variations in carotenoid accumulation using similar carotenoid engineering strategies may be due to variation in the production of transgenics.

Other approaches to elevate β-carotene include a suppression strategy targeting lycopene ε cyclase to exclusively shuttle the conversion of lycopene to β-carotene. This strategy was used successfully in both *Brassica* seeds (Yu et al. 2007) and potato tubers (Diretto et al. 2007). In an effort to produce high carotenoid and low anti-nutritional *Brassica* seeds, investigators suppressed the negative regulatory gene of light mediated responses DET1 (DE-ETIOLATED1) both constitutively and seed-specifically (Wei et al. 2009). Sinapate esters, a type of phenolypropanoid metabolites, that produces off flavor and taste in cruciferous seeds, were reduced in both sets of transgenic plants, while total carotenoids, lutein, β-carotene and zeaxanthin, were elevated especially in the constitutively suppressed DET1 lines which exhibited increments of 1.5-fold lutein, 3.9-fold zeaxanthin and 12-fold of β-carotene. Likewise, branching, and hence seed yield, has been associated to seed carotenoid content by the discovery that strigolactones, metabolites derived

from carotenoids (Chap. 9), inhibit plant branching (Gomez-Roldan et al. 2008). Specifically the microRNA, *AtmiR156b*, has been shown to suppress the expression of *SPL* (SQUAMOSA PROMOTER BINDING PROTEIN LIKE) transcription factor, that regulate leaf primordium initiation and transition from vegetative to reproductive stages (Wang et al. 2009). Wei et al. 2010 overexpressed *AtmiR156b* both constitutively and seed-specifically in *Brassica napus*. In addition to the increased flower number, plants also present an increase in β-carotene in the seeds (up to fourfold) only in the constitutive overexpressing transgenic plants. This suggests a complicated and interconnected regulatory network of source / sink components to determine seed carotenoid content. These findings suggest the importance of photosynthate resources availability to determine carotenoid seed composition.

The most important aim in seed fortification is to enhance the health and wellbeing of consumers, humans or animals. The benefits of β-carotene enhanced food crops to children's eye-health has been well documented (Rao and Rao 2007) and the testing of β-carotene feed on animal health is starting to emerge. Fully oxidized β-carotene may confer anti-inflammatory properties in cattle with respiratory tract disease, such as pneumonia (Duquette et al. 2014). Nogareda et al. (2015), noted a positive impact of a high-carotenoid corn diet on broiler chickens and their resistance to the protozoan parasitic coccidiosis disease. Findings on both cattle and chicken, suggest carotenoids interact beneficially with vaccinations, indicating carotenoids could be used as a complementary strategy to boost disease resistance.

In addition to carotenoid themselves having additional, unexpected, beneficial health impacts, the enhanced accumulation of carotenoids themselves might render the seeds more nutritious. Oil composition analysis of β-carotene fortification efforts in certain seed crops, *Brassica* and soybean, have shown alterations in fatty acids profiles containing less unsaturated fatty acids (Shewmaker et al. 1999; Schmidt et al. 2015). Oils high in unsaturated fatty acids when used during baking or frying result in the production of heart unhealthy trans fats. Such oil seed crops engineered to have a healthy fatty acid composition will contribute to the reduction of the incidence of coronary heart disease, currently the leading cause of death for Americans (Astrup et al. 2011). Also, engineered oil containing both enhanced β-carotene and decreased levels of unsaturated fatty acids should rival red palm oil's health and cooking benefits without the destruction of tropical environments and animal habitats (Azhar et al. 2014).

13.4.2 Zeaxanthin Increments in Seeds

In photosynthetic organisms, zeaxanthin protects cells against photooxidation and membranes against lipid peroxidation by quenching reactive radicals that have been

created as toxic byproducts during photosynthesis reactions. The fortification of crops with zeaxanthin gained momentum after reports on the correlation of this carotenoid and lutein in the prevention of age related macular degeneration (AMD) (Tan et al. 2008; Gale et al. 2003). Age-related Macular Degeneration (AMD) is the leading cause of irreversible vision loss in adults age 55 years and older and is currently estimated to affect ten million Americans (Friedman et al. 2004). Due to a combination of an increase in life expectancy and aging 'Baby Boomers', studies indicate that there will be 71 million Americans over the age of 65 in 2030 compared to 12 million in 1990. It has been estimated that a 6–10 mg daily intake of zeaxanthin and lutein in Americans would have a $2.5 billion net savings to Medicare system over a 5 years period (Lewin 2009).

Zeaxanthin got its name from the yellow corn, *Zea mays* L., as it is the principal yellow pigment in corn. It can be found in many fruits and vegetables however its levels in most foodstuff is measured in μg and it needs to be ingested at ~10 mg levels / day to be beneficial to eye health – a magnitude of 100–1000 fold too low to be biologically relevant. Zeaxanthin-enriched *Brassica napus* seeds were attempted to be produced by silencing the lycopene ε cyclase enzyme via RNAi technology. Lycopene is a branched point in the carotenoid biosynthesis pathway, suppressing this cyclase enzyme production should inhibit the formation of the ε cycle downstream carotenoids, namely lutein and α carotene, and simultaneously allowing predominantly β rings to form from the other branch pathway giving rise to enhanced levels of β-carotene, zeaxanthin and violaxanthin. The constitutive suppression of the ε cyclase resulted in a notable increase in the desired carotenoids, plus inexplicably lutein, yet levels of zeaxanthin accumulated were very low in maturing seeds (0.26 μg/g) and undetectable in mature and dry seeds. Zeaxanthin levels have been successfully enhanced in non-seed tissues (Romer et al. 2002; Dharmapuri et al. 2002; Wolters et al. 2010) so it might be that zeaxanthin as part of the xanthophyll cycle and a precursor to the phytohormone abscisic acid (ABA) presents unique hurdles to stably accumulate in seeds.

Researchers took advantage of the inherent variation in carotenoid content and composition in corn kernels to produce a zeaxanthin-enriched variety. Naqvi et al. (2011) strategy was to combine an already successful engineering approach, to increase carotenoids and breed the transgenes into a genetic background primed for β ring carotenoid production. They too targeted the splitting of lycopene into either the β:β ring structures or β:ε ring structures by the action of lycopene β cyclase and lycopene ε cylase, respectively. Two cultivars that varied presumably in their lycopene ε cyclase activity that exhibit 0.61 and 1.90 ratios of β:ε carotenoids were chosen to cross with their previously engineered enhanced carotenoid line having a 3.51 β:ε ratio as a result of the endosperm-specific expression of three transgenes: maize phytoene synthase, bacterial phytoene desaturase and *Genetiana lutea* lycopene β-cyclase (Zhu et al. 2008). The result was the β:ε ratio of the 1.90 cultivar increased to 6.80, translating to 56 μg zeaxanthin/ g dry kernel.

13.4.3 Astaxanthin Increase in Seeds

Astaxanthin has also gained attention from the plant biotechnology community as it is a dietary antioxidant and colorant in aquaculture industries (Chap. 1). The first attempt to engineer astaxanthin into a plant was the expression of β-carotene ketolase from the algae *H. pluvialis* under the regulatory control of tomato phytoene sythase promoter (Mann et al. 2000). It was the first demonstration that this complex keto carotenoid could successfully be produced and accumulated in plants. Although there were non-detectable levels of astaxanthin in the leaf tissue, it did constitute 23 % of the carotenoids found in the nectaries of the transgenic tobacco plants. Transgenic canola (*Brassica napus*) seeds were engineered with seven carotenoid genes in an effort to produce astaxanthin and other ketocarotenoids with the best line accumulating 0.2 μg astaxanthin/g of dry weight. Ralley et al. (2004) transformed tobacco with two carotenoid genes from the marine bacteria *Paracoccus sp.*, β-carotene ketolase (*BKT*) and β-carotene hydroxylase (*BHY*) under constitutive regulatory control in tobacco with the result of the nectar carotenoid containing 5 % astaxanthin, up to nearly 64 μg astaxanthin/g. Huang et al. 2013 also obtained high amounts of astaxanthin not only in vegetative leaves but also in the fruit of a tomato variety with high synthesis capacity for β-carotene through the expression of the algal *BKT* and *BHY* . Moreover, the fruit accumulated fivefold more astaxanthin than the leaves, reaching 16.1 mg/g cell dry weight, similar to *Haematoccocus pluvialis*. Recently, seed-specific accumulation of up to 7 μg/g astaxanthin and 52 μg/g canthaxanthin has been achieved in soybean seeds by chloroplast targeted *PSY* gene from *Pantoea ananatis* and *BKT* gene from Brevundimonas (Pierce et al. 2015).

Researchers speculate that the enzyme to convert zeaxanthin into astaxanthin itself varies greatly in its efficiency and that this is largely the rate-limiting step in producing astaxanthin. Zhong et al. 2011 reported the *in vivo* conversion rate of zeaxanthin to astaxanthin by β-carotene ketolase isolated from three algae sources, namely *Chlamydomonas reinhardtii*, *Chlorella zofingiensis* and *H. pluvialis*, as 85 %, 38 % and 1 %, respectively. They then constituatively expressed these *BKT* genes individually in a chloroplast-targeted manner in *Arabidopsis* and also found that the levels of astaxanthin in dry leaf tissue varied depending on the source of the gene – high levels when the *Chlamydomonas* gene was used (2 mg/g), moderate levels when the *Chlorella* gene was used (0.24 mg/g) and non-detectable levels of astaxanthin when the *Haematococcus* gene was used. This finding indicates that astaxanthin can be made efficiently if the correct β-carotene ketolase enzyme is used. Zhu et al. (2008) also stressed the importance of the ketolase enzyme in the production of astaxanthin. Their work using a combination of up to five transgenes involved in carotenoid biosynthesis into the white maize kernel naturally mutant in phytoene synthase, demonstrated the competition between β−carotene ketolase and hydroxylase for β-carotene as a substrate. A mechanism to streamline the production of astaxanthin might be the use of a multifunctional enzyme, such as astaxanthin

synthase from *Xanthophyllomyces dendrohous*, what seems to be able to convert β-carotene directly to astaxanthin (Ojima et al. 2006).

Genetic enhancement of carotenoids in crop seeds either through conventional breeding or transgenic approaches has already made significant impacts on human and animal health. The field of research will only continue to grow and move forward as scientific breakthroughs on plant genomes, gene regulation and novel transgenic approaches are honed.

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Chapter 14
Carotenoids as a Source of Antioxidants in the Diet

Ana Augusta Odorissi Xavier and Antonio Pérez-Gálvez

Abstract Carotenoids, widely distributed fat-soluble pigments, are responsible for the attractive colorations of several fruits and vegetables commonly present in our daily diet. They are particularly abundant in yellow-orange fruits (carrots, tomatoes, pumpkins, peppers, among others) and, although masked by chlorophylls, in dark green leafy vegetables. Several health benefits have been attributed to carotenoids or to foods rich in these pigments, by means of different mechanisms-of-action, including the role as provitamin A of almost 50 different carotenoids and the antioxidant activity that protects cells and tissues from damage of free radicals and singlet oxygen, providing enhancement of the immune function, protection from sunburn reactions and delaying the onset of certain types of cancer. Common food sources and the efficiency of the absorption of carotenoids, analytical approaches used for measurement of their antioxidant effect and an overview of some epidemiological studies that have been performed to assess the beneficial impact of carotenoids in human health are outlined in this chapter.

Keywords Health benefits • Antioxidant capacity • Antioxidant activity • Carotenoid dietary source • Cancer markers

14.1 Introduction

The definition of an *antioxidant* is commonly subject to the intended aim or use of the antioxidant substance, the nature of the radical species, how and where they are generated and what target of damage is measured. A working definition of antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge 2007). With this definition several natural compounds families could be defined as antioxidants and the carotenoids are one of them. Carotenoids are fat-soluble natural pigments widely distributed in nature with

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a polyenic skeleton of 40 carbon atoms that presents different structural changes, such as cyclization of one or both ends, hydroxylation, and / or the introduction of oxygenated functions. Carotenoids can be classified in two groups: carotenes which are strictly hydrocarbons, and xanthophylls, which are derived from the former and contain oxygenated functions. The presence of the long, extensive system of conjugated double bonds is responsible for one of the most distinctive characteristics of the carotenoids: light absorption. The main biological function of carotenoids in photosynthetic organisms is energy transfer in photosynthesis and photoprotection (Krinsky 1994). In mammals, the only biological function of some carotenoids is their role as precursors of vitamin A, necessary for vision, growth, cell differentiation, and other physiological processes (Olson 1996). Not all carotenoids present the structural requirements for conversion to vitamin A. Only those with at least one type β-ring without any oxygen function and with a polyenic chain containing at least 11 carbon atoms are potential precursors of vitamin A. With these structural requirements only the 10 % of the 700 carotenoids described so far shows activity of provitamin A. The most relevant provitamin A carotenoids, either for their high activity and wide distribution in food, are α- and β-carotene, some xanthophylls such as β-cryptoxanthin and some *apo*-carotenoids (Mínguez-Mosquera and Hornero-Méndez 1997). From all of them, β-carotene presents the highest provitamin A activity since each carotene molecule produces two retinal molecules that are reduced to vitamin A (retinol). Table 14.1 contains the most representative carotenoids with provitamin A activity and their efficiency compared to β-carotene.

One of the first biological actions described for carotenoids was their characterization as very effective *quenchers* of singlet oxygen (Foote and Denny 1968). The quenching mechanism is based on a physical process by which the excess energy of the oxygen molecule is absorbed by the carotenoid getting

Table 14.1 Provitamin A activity of carotenoids relative to β-carotene

Carotenoid	Activity (%)
all-*trans*-β-carotene	100
9-*cis*-β-carotene	38
13-*cis*-β-carotene	53
all-*trans*-α-carotene	53
9-*cis*-α-carotene	13
13-*cis*-α-carotene	16
all-*trans*-β-cryptoxanthin	57
9-*cis*-β-cryptoxanthin	27
15-*cis*-β-cryptoxanthin	42
β-carotene-5,6-epoxide	21
muthatocrome	50
γ-carotene	42–50
β-zeacarotene	20–40

Data according to Bauernfeind (1972) and Zechmeister (1949)

back the oxygen molecule to its ground energy state. Then, the excess energy of the carotenoid is dispersed through the environment without causing damage to neighboring molecules. The continuous reiteration of this process will finally affect the chemical structure of the carotenoid by reactions involving the addition of singlet oxygen to the polyenic chain yielding carotenoid endoperoxides and carbonyl derivatives. Although a single molecule of β-carotene can quench 1000 singlet oxygen molecules before oxidation, the chemical irreversible reaction takes place, giving an end to the quenching process (Liebler 1993). However, the positive effect of the action is beyond any doubt. Indeed there is a great interest in the development of synthetic chemical derivatives combining the excellent efficiency of carotenoids as quenchers with the ability of flavonoids in stabilizing the radical intermediates formed in the quenching process. Thus, it is possible to synthesize compounds resulting from the fusion of these two groups of phytochemicals like the carotenylflavonoids, with a high quenching capacity and greater stability to the chemical oxidation processes that may have potential applications in sunscreens (Beutner et al. 2007).

However, any other biological action of carotenoids causes greater interest and controversy than the antioxidant action. A helpful notation to understand the relevant features of the antioxidant action is to distinguish *antioxidant action* and *antioxidant activity* concepts, which are different although they are commonly used without distinction. While the antioxidant activity is associated with oxidative processes in vitro, antioxidant capacity is associated with oxidative processes in vivo and really involves a biological action.

14.2 Antioxidant Activity

The term antioxidant activity is defined as the constant rate of the reaction between an antioxidant and radical species. This concept correlates with the chemical reaction process of the antioxidant/radical pair, structure of both substances and the reaction mechanism(s) that take place. Antioxidants may react with radical species mainly by different mechanisms: electron transfer to produce radical cations, reduction, radical adducts formation, and hydrogen atom abstraction. These mechanisms may happen at once but the dominant process depends on the structure of the antioxidant and the characteristics of both the reaction environment and the radical species. Some methods for determining the antioxidant activity may be developed to measure the progress of these mechanisms, while other methodologies measure the progress of one of them. Carotenoids are lipophilic antioxidants and display activity towards reactive oxygen species produced in biological systems. Some of them are the superoxide anions produced within mitochondria, hydroxyl radicals with a very short lifetime, acting close to their site of formation, perhydroxyl radical responsible for initiation of lipid autoxidation. Additionally nitrogen derived radicals may be the target molecules for carotenoid antioxidant activity.

Electron transfer mechanism that yields a carotenoid radical cation takes place when the radical species possess high redox potential (Jomova et al. 2009) while reduction of the carotenoid molecule is a consequence of reduction reactions yielding the corresponding radical carotenoid anion (Bobrowski and Das 1985). These radical intermediates may react with biological substrates and produce oxidative damage (Everett et al. 1996; Miller et al. 1996). The mechanisms reviewed by Britton (1995) and Edge et al. (1997) produce neutral carotenoid radicals. The hydrogen abstraction mechanism only affects those hydrogens in allylic positions to a double bond, which are prone to react with peroxyl radicals. This process generates a resonance-stabilized radical that may continue radical propagation chain. The addition process to the polyenic chain involves the oxidation of the carotenoid via radical addition and the generation of carotenoid-peroxyl adducts that can undergo heterolytic or homolytic breakdown (Liebler 1993). The former process does not continue the radical propagation chain while the latter produces two new reactive species. If the addition reaction takes place at the double bond position of the ring, the product arising is a radical species and the autoxidation process is continued. Figure 14.1 reproduces the reaction mechanisms discussed above and denotes that the production of new radical species during development of the antioxidant process will increase the oxidative potential of the environment

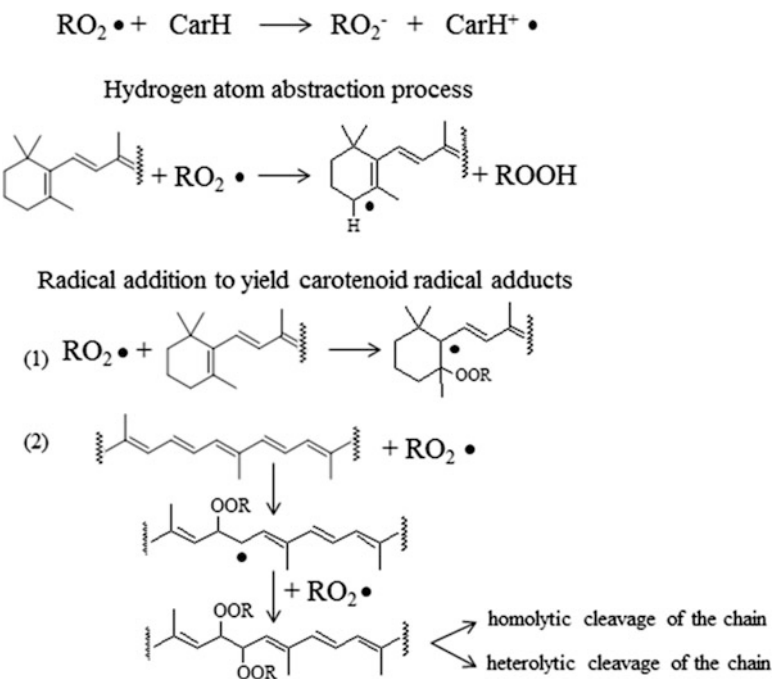

Fig. 14.1 Reaction mechanisms between carotenoids and radical species. (1) Addition to the ring. (2) Addition ot the polyenic chain

where the reaction takes place. When the generation of radical species exceeds the rate at which the antioxidant removes them, the autoxidation process is observed.

Although these reaction mechanisms are the general pattern of the antioxidant activity of carotenoids, the rate of the process depends on the presence of functional groups, so that the structural characteristics of each carotenoid modulate its oxidation and consequently its antioxidant activity. Particularly, the intermediate peroxyl radical is essential in determining the progress of the reaction mechanism because its stabilization by electron delocalization is different depending on the functional groups located at the ends of the polyenic chain. Furthermore, these groups may prevent oxidation progress through one of the reaction mechanisms discussed above and, therefore, reduce the tendency to autoxidation of the carotenoid (Liebler 1993; Pérez-Gálvez and Mínguez-Mosquera 2002).

Two decades ago there was a methodologies span devoted to measurement of antioxidant activity of different natural, synthetic substances, food extracts, and even complete food systems (Cao et al. 1993, 1995; Frankel and Meyer 2000; Karadag et al. 2009; Ou et al. 2001; Prior et al. 2003). The chemistry behind these methods has been detailed in the reviews of Huang et al. 2005 and Prior et al. 2005. Some of the commonly used methods are the oxygen radical absorbance capacity, total radical trapping antioxidant parameter, trolox equivalent antioxidant capacity, total oxyradical scavenging capacity and peroxyl radical scavenging capacity methods (note that the word capacity is used to name these methods). In the case of carotenoids, the methods for determining antioxidant activity generally applied are the one described by Terao (1989) and the TRAP method (Bartosz et al. 1998), methodologies based on the reactions chain of the lipid autoxidation process. Results obtained from the application of these and other chemical assays of the antioxidant activity are worthy for comparison of the activity of different substances and to gain information regarding the influence of the chemical structure for a family of compounds, but they are not good models to determine the behavior of the antioxidant in biological systems.

14.3 Antioxidant Capacity

Antioxidant capacity is defined as the amount of radical species which is removed or neutralized from the reaction environment by the action of a certain amount of antioxidant. This concept is applied to the antioxidant action in tissues and biological samples such as plasma and cell cultures. Measurement methods that are applied to determine the antioxidant capacity of carotenoids are the TEAC method (Re et al. 1999), the DPPH method (Brand-Williams et al. 1995) and the method of ex vivo oxidation of LDLs (Carpenter et al. 1997). These methods are an approximation (more or less realistic) to ascertain the antioxidant capacity of carotenoids. In biological systems, lipids constitute most of the cell membranes and other cellular structures, and lipid oxidation has a negative impact on the functionality of the membrane and its integrity. Since carotenoids are accommodated in such structures,

they are able to delay radical propagation chain generated by lipid oxidation, so that these compounds together with tocopherols are known as membrane antioxidants. Thus, the antioxidant effect of carotenoids in vivo is observed in LDLs and cellular membranes where they exert their ability through the reaction mechanisms described above (Ringer et al. 1991). The fact that carotenoids exercise this capacity in biological systems is one of the main supports to the oxidative stress theory and the promise that a high intake of foods rich in carotenoids will lower the risk of developing degenerative processes. However, the results obtained from analytical approaches designed for estimation of the antioxidant capacity introduce diverse uncertainties about the efficiency of the antioxidant action in such biological systems. Particularly, the ex vivo oxidation of LDLs method evaluates the antioxidant action of carotenoids when they are added to plasma or isolated LDLs, or introduced into them by enriching the diet with supplements or with fruit and vegetables. Most studies show that carotenoids including β-carotene, zeaxanthin, lutein and lycopene are effective antioxidants (Dugas et al. 1998; Panasenko et al. 2000) but some publications report no protection of LDLs to oxidation although they increased their carotenoid content (Carroll et al. 2000; Chopra et al. 1996; Krinsky 1994; Rock et al 1996). This controversy claims to an acute screening of the factors that might affect LDLs proneness to oxidation, not only carotenoid content but also characteristics of the study population, design of the intervention trial, the selected carotenoid source, the efficiency of assimilation of antioxidants present in the diet, i.e. what proportion of the ingested amount is absorbed and therefore bioavailable, possible biotransformation into other compounds with lower antioxidant efficiency, or the generation of new free radicals, as indicated in the description of the antioxidant activity concept, the amount deposited in the tissue where it will develop its action, the nature of the oxidative stress biomarker used to determine the correlation, and the participation of other antioxidants naturally present in the food source (Wright et al. 2002).

14.4 Antioxidant and Non-Antioxidant Actions Related to Modification of Carcinogenesis Markers

One research line regarding the antioxidant action of carotenoids that has generated a huge amount of relevant evidences is based on the link among the initiation and progression of cancerous processes with the presence of free radicals. This association point to the addition of antioxidants, such as carotenoids, to our body as a strategy to positively contribute to risk reduction of cancer. There are evidences about the involvement of free radicals in the initiation and progression of carcinogenesis. Reactive oxygen species act directly as mutagens to oxidize DNA bases, and are also able to activate pre-carcinogens making them reactive substances that modify DNA (Marnett 1987). The continuous decrease of antioxidant defense

levels caused by the activity of reactive oxygen species is another evidence of their involvement in carcinogenesis, as this effect is also produced by the activity of genuine carcinogens (Cerutti 1985; Kensler and Taffe 1986). Consequently, if carotenoids as antioxidants delay the activity of reactive oxygen species, their action may decrease the risk of developing cancer (Malone 1991; Byers and Perry 1992; Voorrips et al. 2000; Holick et al. 2002).

Another beneficial effect derived from the antioxidant capacity of carotenoids is the enhancement of the immune system. UV light produces an increase in the concentration of free radicals that damage the immune system, so that the activity of natural antioxidants limits the negative effect and delays the onset of the reduction of sensitive markers of the immune system. The studies of Fuller et al. (1992) and Herraiz et al (1998) show the positive effect of the intake of β-carotene supplementation on the immune system on volunteers subjected to UV radiation. Nevertheless, carotenoids may change the function of the immune system by non-antioxidant mechanisms which also has a positive significance in the risk of developing cancer, due to the essential role that the immune system in preventing this degenerative process. It has been shown that intake of β-carotene supplementation (50 mg on alternate days for 10–12 years) increased NK activity, natural-killers type cells, which reduce the likelihood of tumor formation (Santos et al. 1996). In this case the mechanism by which β-carotene modulates cellular activity is not known. It has also been suggested that the regulation of the biosynthesis of prostaglandin E2, a recognized immunosuppressant, is due to the intervention of β-carotene in enhancing the immune system (Halevy and Sklan 1987). It can be seen that most of the studies conducted focused exclusively on β-carotene. Experimentation with other carotenoids and their effect on immune function are very infrequent or has remained inconclusive or positive (Hughes et al. 2000).

Improvement of the intercellular communication is an inversely correlated marker with cancer progression. In this case the mechanism of action of carotenoids is not based on the antioxidant action since in most studies focused on the inhibitory effect of carotenoids in the proliferation of neoplastic cells, any correlation among the effect and the antioxidant capacity was found. In fact, the addition of other membrane antioxidants as α-tocopherol did not show inhibitory effect what means the presence of a different mechanism of action. In this case the mechanism of action is based on the ability of carotenoids to increase or re-establishing the intercellular communication that takes place through channels or pores called connexons. Through these various pore cells signaling molecules are exchanged including antiproliferative agents.

Intercellular communication is interrupted in cancer cells because the gene expression of the protein structure forming part of these pores or channels of communication, connexin 43, is decreased. As a consequence communication between cancer cells to normal cells that surround them fails resulting in loss of the control of proliferation of transformed cells (Loewenstein 1979). Conversely, if the intercellular communication between normal and cancer cells is restored, there is a

possibility to delay the proliferation. Several studies demonstrated that carotenoids increase expression of the gene encoding connexin 43, increasing intercellular communication with healthy tissue and decreases proliferation of transformed cells. The mechanism of regulation of gene expression by means of carotenoids is unknown. The prevailing hypothesis is that carotenoids, and particularly some of their metabolic products, are able to activate a series of nuclear receptors (RAR-α and RXR-α) that activate gene expression.

14.5 In vivo Antioxidant Action of Carotenoids. Epidemiological and Intervention Studies

Several prospective an retrospective studies of diet and some types of cancer suggest that dark green and yellow orange vegetables rich in carotenoids reduce cancer risk, particularly linking this correlation with an increased consumption of β-carotene food sources and with increased plasma levels in this carotene. The study of Stähelin et al. (1991) associated bronchus and stomach cancers with a low mean plasma carotene level. Population-based cohort studies have found inverse associations of plasma carotenoids with risk of chronic diseases. The studies of Eichholzer et al. 1996 and Yuan et al. 2001 showed that increased levels of carotenoids other than β-carotene obtained through a high intake of fruit and vegetables are associated with a lower incidence of lung cancer. Association seems to be clear in the case of carotenoids and lung cancer but less consistent results are obtained in the case of other types of cancer and other carotenoids. Indeed, when the positive correlation observed from observational epidemiologic studies among carotenoid intake and cancer risk has been tested with intervention trials, no association or even unexpected negative and harmful effects have been obtained. Three large intervention trials were conducted in 1980s providing harmful effects of high dose (20 mg) β-carotene supplementation (Heinonen and Albanes 1994; Omenn et al. 1996) or no effect (Hennekens et al. 1996). Several questions arose from the results of these intervention trials including possible differences between supplemental and dietary β-carotene, health status of the population receiving the supplementation, high dose effects and different metabolism of β-carotene derivatives in smokers, etc (Upritchard et al. 2003). It has been suggested that excluding of other dietary antioxidants (vitamin C) from intervention trials could have an impact in the results even they could be the source(s) of the cancer prevention potential of fruits and vegetables (Zhang and Omaye 2001). Results of literature survey are definitively confusing and either positive or negative association must be considered with caution. Sufficient evaluation of antioxidant supplements should be provided but this should not point diets rich in fruit and vegetables as harmful. They must be considered as a source of nutritional antioxidants with many health benefits.

14.6 Dietary Sources of Carotenoids

Although more than 700 carotenoids have been described in nature, not all natural sources of them are present in our normal diet. It is estimated that we only have access to about 40 carotenoids that can be absorbed, metabolised, and/or used in our bodies. That number is reduced to six if we consider the carotenoid profile that is usually detected in human blood plasma. This group includes α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin and lutein, which are regularly present in the foods listed in Table 14.2 (for structures see Fig. 14.2, Chaps. 1, 2 and 3). The carotenoid content of the foods listed in this and other tables can be found in databases that have been developed for this purpose. The database of Mangels et al. (1993) only included fruits and vegetables as carotenoid-containing foods. Later on, a new database was developed, published by Holden et al. (1999),

Table 14.2 Common fresh fruits and vegetables contributing to the major carotenoids described in human tissues

Lutein/zeaxanthin	Lycopene	α-carotene	β-carotene	β-cryptoxanthin
			Apricot	
Beans			Beans	
Beet			Beet	
			Blueberry	
Broccoli			Broccoli	
Brussel sprouts			Brussel sprouts	
Carrots		Carrots	Carrots	
Celery			Celery	
Coleslaw		Coleslaw	Coleslaw	
Courgette			Courgette	
Cucumber			Cucumber	
Kiwi				
Leeks				
Lettuce			Lettuce	
			Mango	
				Oranges
Parsley			Parsley	
Peas			Peas	
Pepper			Pepper	Pepper
			Plum	
Pumpkin			Pumpkin	
			Spinach	
Sweetcorn				
	Tomatoes			
			Watermelon	

Minimum content of the carotenoid in the food item is 0.1 mg/100 g of food
For a complete compilation of carotenoid content in food sources see O'Neill et al. (2001)

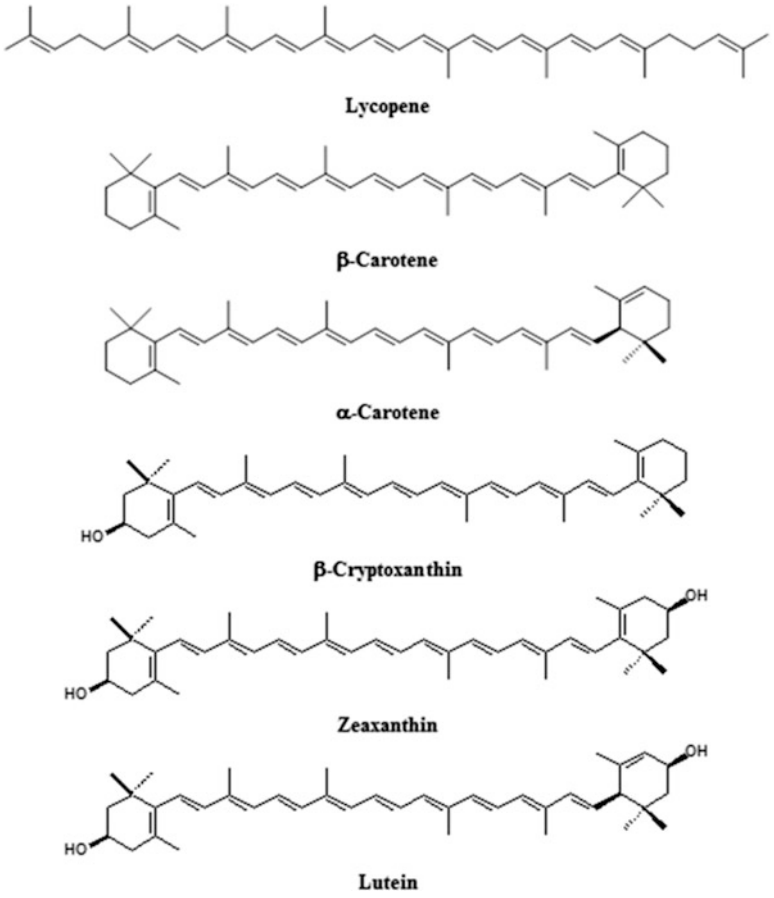

Fig. 14.2 Structure of the main carotenoids described in human tissues

that also included other foods such as vegetable oils, butter, eggs, cheese, and other products made of vegetables (pizzas, salads, etc). This database evaluated up to 200 references on the carotenoid content of 215 foods, tabulating the average content and standard deviation, as well as the number of studies conducted for each food. It is available at the following web site: http://www.ars.usda.gov/Services/docs.htm?docid=8964 (last accessed 22/12/2014). One of the main uses of this database is the estimation of the provitamin A contribution of a given dietary intake. Another database on the average content of carotenoids in foods is offered by the Linus Pauling Institute Micronutrient Information Center (http://lpi.oregonstate.edu/infocenter/phytochemicals/carotenoids/index.html, last accessed 22/12/2014) including information on α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin and lutein. Another complete database is published in the work of O'Neill et al. (2001) with information on the five main dietary carotenoids content in various food

sources consumed in five European countries. Despite the correlation between high carotenoid content in plasma, which comes exclusively from the intake of foods rich in carotenoids, and lower risk of developing severe degenerative processes, adequate intake levels of these components have not been established since the positive health effects may be due to other constituents that are ingested along with carotenoids. Neither the health-promoting biological actions that these compounds may have in our bodies (antioxidant capacity, immune enhancement, increased intercellular communication) or the fact that some of them exhibit provitamin A activity have been, at the moment, reasons to establish a recommendation on the appropriate amount of carotenoid intake. However, by using data from epidemiological studies on the consumption of fruits and vegetables and their effect on health, normal values may be set for carotenoid intake, which may be associated with a lower risk of developing degenerative diseases (cancer, cardiovascular disease, etc). Even so, there are discrepancies on mean intake values in the consulted references. There is currently no recommendation for daily intake of carotenoids, even though a reference value of 6 mg/day has been proposed, based on the contribution of carotenoids with provitamin A activity. Mammals rely on diet to incorporate those carotenoids that develop provitamin A activity as they are metabolized to retinol. It has been shown that 14 μg of β-carotene are necessary to produce 1 μg of retinol, that is, 1 retinol activity equivalent. FAO and WHO recommendations for vitamin A intake are 700–900 μg of retinol per day what should mean a daily intake of 10–13 mg of β-carotene (if this carotenoid would be the only vitamin A source). The importance of other physiological functions (antioxidant and non-antioxidant activities) demands further study of them and of the dose-effect correlation in order to set daily intake values for these compounds.

Thus, individuals who eat a diet rich in fruits and vegetables ingest about 6 mg/day according to studies by Lachance (1997) and published guidelines from Health Canada (1997). However, the study published by the WCRF/AICR (1997) raises the average intake value to 9–18 mg/day. For intervention studies conducted with controlled dietary carotenoid content, it is suggested that an intake of 3–6 mg/day of carotenoids is sufficient to maintain plasma levels of these components (Micozzi et al. 1992; Yong et al. 1994; Zino et al. 1997).

In particular, the Mediterranean diet offers perhaps the most diversity and amount of carotenoid intake due to its high content of fruits and vegetables (fresh and/or processed) and vegetable oils. Bearing in mind the six most representative carotenoids mentioned above, Table 14.2 shows the amounts found in foods of the Mediterranean diet. All green vegetables contain a considerable amount of lutein, β-carotene, and β-cryptoxanthin, with the concentration varying greatly from one source to another. The best sources of α-carotene are carrots and pumpkins, while β-carotene is found more widely in fruits and vegetables such as carrots, red bell peppers, oranges, potatoes, broccoli, and green vegetables. β-Cryptoxanthin is found in minor concentration in some vegetables (Table 14.2), although in ripe red peppers and tropical fruits like papaya in one of the major pigments. Tomato and its derived products (pasta and sauces), together with watermelon and pink grapefruit, are the main sources of lycopene. Rich sources of lutein include green

vegetables such as spinach, Brussels sprouts, broccoli, and peas, while zeaxanthin is found in high concentrations in egg yolks and corn. In the last decades an increased interest has been focused on the inclusion of information concerning nutritional content of foodstuffs in the food labeling. Legislation concerning this issue has aimed food producers to offer valuable information, from which the consumer can adapt the selection of food items to cover requirements for key nutrients and to maintain health. The consideration of recommended daily intake values theoretically should promote an increase in nutritional quality of food because manufacturers have to analyze in detail the nutritional composition of their products, assuring that key nutrients are present in the levels indicated on the label and controlling the effect of processing on those compounds in order to restore adequate levels if necessary. Similarly, introducing the term "bioaccessibility" would theoretically promote further changes. Bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract, and thus becomes available for intestinal absorption (i.e. enters the blood stream). Studies concerning health benefits based on functionality of nutrient or bioactive compounds (carotenoids) would be significant if the required amount to achieve the in vitro bioactivity is compared with the bioaccessible amount that can be reached from some natural sources or food formula where the bioactive compound is present. In vitro digestion procedures have been applied to carotenoid-rich meals or formulations with different experimental conditions. Garrett et al. (1999) developed a complete in vitro digestion procedure in combination with Caco-2 cell-culture model to assess bioaccessibility of carotenoids from meals. Effect of various factors on incorporation of carotenoids to mixed micelles was studied (impact of gastric phase, presence or absence of bile extract and pancreatin, and lipid composition). Any source of interaction at the micellization process will reduce efficiency of the digestion step and affects final bioaccessibility. Carotenoids as lipophilic compounds need to be incorporated to mixed micelles to reach epithelial cells so that, the carotenoid amount incorporated to the micellar fraction gives an estimation of the efficiency of the digestion step, and it is usually expressed in terms of percentage with respect to the total initial carotenoid amount. The method of Garret et al. (1999) has been applied to assess bioaccessibility of different test meals and supplements in other studies (Ferruzzi et al. 2001; Garrett et al. 2000). Other procedures just apply the in vitro digestion model and did make use of the assimilation stage with a cell culture-based model. The experimental approach developed by Granado-Lorencio et al. (2007) is an optimization of the validated method of Oomen et al. (2003) and includes a complete in vitro digestion procedure (simulation of mastication and saliva solution, gastric and intestinal phases) with the addition of human pancreatic lipase and other specific enzymes (cholesterol esterase, phospholipase A2) to achieve more physiological conditions. The method applied by Hedrén et al. (2002) applies an in vitro digestion method to estimate the maximum amount of carotenoids released from food matrix without isolation of micellar fraction and determination of cellular uptake. The latter studies and others (Breithaupt et al. 2002; Fernández-García et al. 2007, 2008; Serrano et al. 2005) have been performed with the aim of establishing in detail the factors involved

on the micellization process and its efficiency, including the effect of composition of the lipid environment and the activity of pancreatic lipases on hydrolysis of xanthophylls esters. Strength of the in vitro protocols arises from their consistency with the results obtained from in vivo studies. Thus, most of the carotenoid bioaccessibility values obtained through in vitro assessment and predictions of changes in bioaccessibility due to dietary factors or physiological modifications, outlined from those procedures, are similar to the in vivo observations. However it must be stressed that an in vitro vs. in vivo validation process should be carried out to delimitate reliability of the in vitro models.

14.7 Conclusions

One of the factors setting the cause and progress of some chronic diseases like cancer is the imbalance in tissues between free radical species and antioxidants of either endogenous or diet origin. The wide array of free radicals structures, their different sites of formation and activity and the reactions chain they initiate are clearly compensated by the high diversity of antioxidants found in common dietary sources from which fruits and vegetables are of outstanding interest. Epidemiological and case–control trials clearly point to protective effects regarding the onset of degenerative diseases. In vitro studies of the antioxidant activity with the use of different analytical approaches obtain valuable comparative information about influence of structures features and characteristics of reaction environment. A more complicated picture is obtained from the application of ex vivo experiments as different factors might affect results although a close approximation to the antioxidant action of the substance in biological systems is obtained. Controversial results appear when in vivo measurements of the effect of dietary antioxidants are measured through intervention trials or case–control studies. In these cases conclusions either positive or negative must be considered with caution as several aspects deserve thoughts when dissection of the results is made. Carotenoids do not escape from these considerations. It has been demonstrated that they are good antioxidants through a wide arrange of methodologies suitable to their lipophilic nature, and the application of ex vivo tools of measurement also pinpoint this family of pigments as compounds with potential benefits to health. These features joined to their wide distribution in fruits and vegetables consumed in our diet make carotenoids as one group of possible dietary chemopreventive agents. However with data obtained so far it is not advisable to recognize one single family of compounds as responsible of health benefits of dietary compounds but the complex and cooperative cocktail of phytochemicals that fruits and vegetables contribute to our body.

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Chapter 15
Carotenoids in Adipose Tissue Biology and Obesity

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Abstract Cell, animal and human studies dealing with carotenoids and carotenoid derivatives as nutritional regulators of adipose tissue biology with implications for the etiology and management of obesity and obesity-related metabolic diseases are reviewed. Most studied carotenoids in this context are β-carotene, cryptoxanthin, astaxanthin and fucoxanthin, together with β-carotene-derived retinoids and some other apocarotenoids. Studies indicate an impact of these compounds on essential aspects of adipose tissue biology including the control of adipocyte differentiation (adipogenesis), adipocyte metabolism, oxidative stress and the production of adipose tissue-derived regulatory signals and inflammatory mediators. Specific carotenoids and carotenoid derivatives restrain adipogenesis and adipocyte hypertrophy while enhancing fat oxidation and energy dissipation in brown and white adipocytes, and counteract obesity in animal models. Intake, blood levels and adipocyte content of carotenoids are reduced in human obesity. Specifically designed human intervention studies in the field, though still sparse, indicate a beneficial effect of carotenoid supplementation in the accrual of abdominal adiposity. In summary, studies support a role of specific carotenoids and carotenoid derivatives in the prevention of excess adiposity, and suggest that carotenoid requirements may be dependent on body composition.

Keywords Carotenoids • Apocarotenoids • Retinoids • Vitamin A metabolism • Obesity • Adiposity • Energy metabolism • White adipose tissue *browning* • Human epidemiological studies

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Abbreviations

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AMPK	AMP-dependent protein kinase
atRA	all trans retinoic acid
BAT	brown adipose tissue
BC	β-carotene
BCO1	β-carotene-15,15′-oxygenase
BCO2	β-carotene-9',10′-oxygenase
BMI	body mass index
bw	body weight
C/EBP	CCAAT-enhancer binding protein
CD36	cluster of differentiation 36
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
FABP	fatty acid binding protein
ISX	intestine-specific homeobox
LRAT	lecithin: retinol acyltransferase
LDLr	low density lipoprotein receptor
LPL	lipoprotein lipase
NF-κB	nuclear factor κB
Nrf2	nuclear factor erythroid 2-related factor 2
PPAR	peroxisome proliferator activated receptor
Rald	retinaldehyde
RAR	retinoic acid receptor
RBP (or RBP4)	retinol binding protein
RBPR2	RBP receptor 2
RDH	retinol dehydrogenase
REH	retinyl ester hydrolase
ROS	reactive oxygen species
RXR	retinoid X receptor
SR-B1	scavenger receptor class B, member 1
STRA6	stimulated retinoic acid 6
UCP1	uncoupling protein 1
WAT	white adipose.

15.1 Introduction

Carotenoids are liposoluble C-40-based isoprenoid pigments usually red, orange or yellow in color produced by plants and certain photosynthetic microorganisms, in which they function as structural and functional accessories of the photosynthetic

apparatus and serve other specific functions. Carotenoids obtained through the diet (mainly fruits and vegetables) also serve specific functions in humans and other mammals. The one best known is to serve as precursors of vitamin A (retinol) and related retinoids such as retinal and retinoic acid that play important roles in the visual cycle and in gene regulation linked to many developmental and physiologic processes, respectively (Grune et al. 2010). The main provitamin A carotenoid in the human diet is β-carotene (BC), followed by α-carotene and β-cryptoxanthin. Non-provitamin A carotenoids – such as lycopene, lutein, and zeaxanthin, which are abundant in human diet and human serum – and the provitamin A carotenoids may serve other functions whose relevance for human health is still uncertain, such as acting as antioxidants and blue light filters for photoprotection (Fiedor and Burda 2014; Johnson 2014). The spectrum of processes impacted by the retinoids in mammals through genomic and non-genomic mechanisms is expanding (Brun et al. 2013). Additionally, novel biological activities of intact carotenoids and carotenoid-derived products other than the retinoids are emerging, which relate to their interaction with mammalian cell-signaling pathways and nuclear receptors transcription factors, sometimes similarly and sometimes quite differently to retinoids (Elliott 2005; Eroglu and Harrison 2013).

In recent years, a novel perspective of the function of carotenoids and carotenoid-derived products is emerging that connects these compounds to the control of adipocyte biology, lipid metabolism and body fat accumulation, with possible implications for the etiology and management of obesity and obesity-related metabolic diseases such as insulin resistance, diabetes and cardiovascular disease (Bonet et al. 2003, 2012; Tourniaire et al. 2009; Landrier et al. 2012; Bonet et al. 2015). This evidence is presented in this chapter.

15.2 Overview of Carotenoid Metabolism and Its Relationship with Vitamin A Metabolism

Dietary carotenoids are absorbed as part of mixed micelles consisting of lipids and bile components (Harrison 2012; von Lintig 2012; Reboul 2013; Shete and Quadro 2013). The intestinal absorption occurs via passive diffusion or through facilitated transport via scavenger receptor class B, member 1 (SR-BI) and perhaps other lipid transporters, such as CD36. In the enterocyte, BC undergoes central cleavage by β-carotene-15,15′-oxygenase (BCO1) to yield two molecules of retinal (also called retinaldehyde, Rald). BCO1 is a cytosolic enzyme specific for provitamin A carotenoids containing at least one nonsubstituted β -ionone ring (such as BC, α-carotene and β-cryptoxanthin) and is the key enzyme for retinoid production from BC, as evidenced from studies in knockout mouse models (Chapter 16, Hessel et al. 2007; Amengual et al. 2013). Enterocytes and many other cell types express a second carotenoid cleavage enzyme, β-carotene-9',10′-oxygenase (BCO2), which locates to mitochondria and cleaves BC asymmetrically to generate

diverse β-apocarotenals and β-apocarotenones. BCO2 has broad substrate specificity and it is also active on other carotenoids such as the acyclic carotene lycopene and oxygenated carotenoids (i.e. xanthophylls, such as lutein and zeaxanthin) (reviewed in von Lintig 2012). Some BCO2 cleavage products, in particular β-apo-10′-carotenol, can be converted into retinal, with the participation of BCO1 (Amengual et al. 2013).

Within the enterocyte, BC-derived retinal is converted to retinol which, together with preformed retinol in the diet, undergoes esterification to long-chain fatty acids and is packaged as retinyl ester in chylomicrons, for distribution to tissues. The efficiency of intestinal BC cleavage varies greatly across species. Adult rats and mice are efficient cleavers since they convert most absorbed BC to retinol; however, even following mild acute or chronic oral BC supplementation (20–35 mg/kg body weight (bw)/day), some BC is absorbed intact in mice, as revealed by accumulation in tissues including white adipose tissue (WAT) (Lobo et al. 2010a; Amengual et al. 2011a). In humans and other mammals, such as horses and ferrets, substantial amounts of absorbed BC (about 17–45 % of the ingested BC) escape intestinal cleavage and, together with other dietary carotenoids, is incorporated into chylomicrons and found associated with circulating lipoproteins. Intestinal BC absorption and conversion into retinoids are dependent on the animal's vitamin A status through a feedback mechanism that involves the retinoic acid-dependent induction of an intestine-specific transcription factor, ISX, that negatively regulates the expression of both SR-BI and BCO1 (Lobo et al. 2010b).

Circulating carotenoids and retinyl esters in lipoproteins are taken up by tissues through the action of lipoprotein-specific receptors, such as SR-BI (which is the receptor for HDL), low-density lipoprotein receptor (LDLr), LDLr related protein-1 or lipoprotein lipase (LPL) (Shete and Quadro 2013), or the action of CD36, the latter implicated in particular in the cellular uptake of lycopene in adipocytes (Moussa et al. 2011). Liver, followed by adipose tissue, kidney, skin and lung are important sites of accumulation of BC and other carotenoids (Shete and Quadro 2013). The intracellular metabolism of carotenoids is not well known. Importantly, BCO1 and BCO2 are broadly expressed in peripheral tissues besides the intestinal mucosa; such widespread expression, together with the wide distribution of carotenoids within the body, has suggested that local tissue-specific conversion of carotenoids may contribute to the *in situ* generation of retinoids and other apocarotenoids capable of impacting tissue metabolism (von Lintig 2012). Additionally, BCO2 activity in mitochondria, the site of its subcellular localization, appears to be required to avoid mitochondrial dysfunction due to carotenoid accumulation in the organelle (Lindqvist and Andersson 2002; Amengual et al. 2011b; Lobo et al. 2012).

Vitamin A in the body is mainly stored as retinyl esters in liver stellate cells. Mobilization of vitamin A from hepatic stores depends on the 21-kDa retinol-binding protein (RBP, also known as RBP4) produced in hepatocytes and secreted into the circulation in a retinol-dependent manner. Extrahepatic tissues take retinol

from the retinol-RBP (holo-RBP) complexes (see Fig. 15.1). The plasma membrane protein STRA6 (stimulated retinoic acid 6) is a high affinity RBP receptor and has been implicated in retinol uptake from holo-RBP complexes in tissues such as the eye (Kawaguchi et al. 2007; Berry et al. 2013), as well as in mediating cellular responses to holo-RBP related to the activation of the JAK-STAT pathway (Berry et al. 2011, 2013). A STRA6 paralog, RBPR2 (for RBP receptor 2) is characteristically expressed in liver and intestine and strongly induced in adipose tissue of obese mice (Alapatt et al. 2013). Retinol in cells can be esterified to fatty acids catalyzed by lecithin:retinol acyltransferase (LRAT) and possibly other acyltransferases or be reversibly metabolized to Rald via alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs). Rald can also be obtained from intracellular BC. Retinoic acid is produced from Rald, through irreversible oxidation catalyzed by aldehyde dehydrogenase-1 family of enzymes (Aldh1, also known as Raldh). Intracellular metabolism of retinoids takes place with them bound to specific binding proteins, which may represent an important mechanism to direct the various retinoids to specific metabolizing enzymes and targets. These intracellular binding proteins include cellular retinol-binding proteins (CRBPs), which chaperone retinyl ester biosynthesis catalyzed by LRAT, and cellular retinoic acid binding proteins (CRABPs) and fatty acid binding protein 5 (FABP5), which are involved in delivering retinoic acid to the nucleus and to specific nuclear receptors within it.

15.3 Molecular Bases of the Biological Activity of Carotenoids and Carotenoid Derivatives in Adipose Tissue Biology and Obesity

Carotenoids and carotenoid conversion products impact gene expression and cell function through multiple mechanisms, notably by interacting with transcription factors of the nuclear receptor superfamily. Gene expression control by retinoic acid has been most studied (Bastien and Rochette-Egly 2004). Retinoic acid isomers are agonist ligands of two subfamilies of nuclear receptors, the retinoic acid receptors (RARs; 3 isoforms, α, β and γ) and the retinoid X receptors (RXRs; 3 isoforms: α, β and γ): all-trans retinoic acid (atRA) binds the RARs, whereas 9-cis retinoic acid binds both the RARs and the RXRs. Heterodimers of RAR with RXR control the expression of typical retinoid-target genes by binding to defined retinoic acid response elements in the gene promoter and modulating transcription in a manner that depends on atRA binding to the RAR moiety and subsequent recruitment of cofactor complexes (Bastien and Rochette-Egly 2004). Several genes encoding proteins in energy and lipid metabolism are up-regulated at the transcriptional level by RAR-dependent pathways, among them genes for uncoupling proteins one and three (UCP1 and UCP3, involved in inefficient substrate (fat) oxidation), medium-chain acyl-CoA dehydrogenase (involved in mitochondrial β-oxidation) and hormone

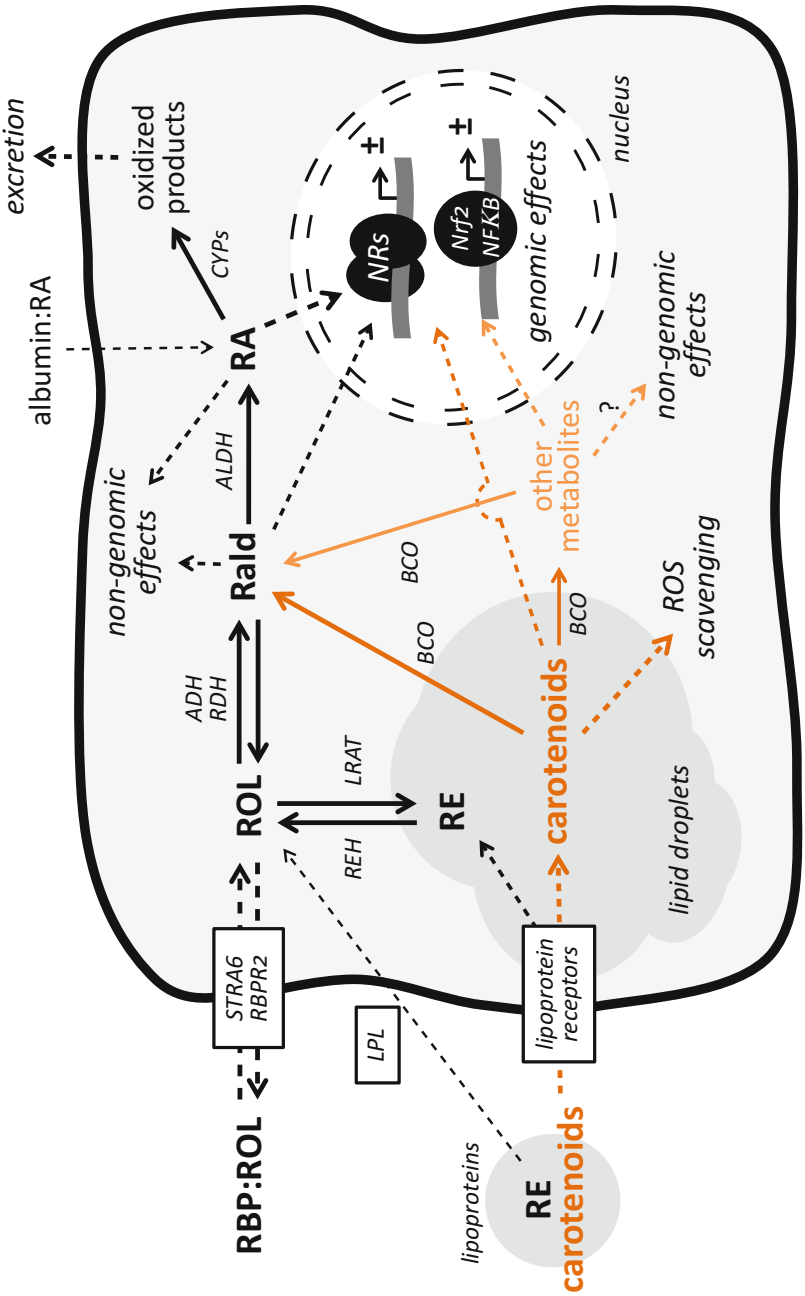

Fig. 15.1 (continued)

sensitive lipase (involved in adipocyte lipolysis) (reviewed in (Bonet et al. 2012). RXRs are obligate dimerization partners for many other nuclear receptors besides the RARs, including the peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR), farnesoid X receptor, pregnane X receptor, thyroid hormone receptor and vitamin D receptor. Some RXR heterodimers (so-called permissive) respond to ligands of either partner and are synergistically activated when both ligands are bound, providing a mechanism for widespread effects of retinoids on gene expression (Aranda and Pascual 2001). Notably, LXRs and PPARs, both of which are deeply involved in the control of different aspects of lipid metabolism, act on (at least some) target genes as permissive heterodimers with RXR (Willy et al. 1995; Mukherjee et al. 1997). Moreover, besides activating the canonical RARs, atRA (but not 9-cis retinoic acid) can behave as an activating agonist ligand of PPARβ/δ (but not the other PPAR isoforms) (Shaw et al. 2003), although there are results conflicting (Rieck et al. 2008). PPARβ/δ activation increases lipid catabolism in skeletal muscle and WAT, prevents the development of obesity and improves insulin sensitivity in obesity-prone mouse models (Luquet et al. 2005; Wang et al. 2003; Wang et al. 2004; Lee et al. 2006). The partitioning of atRA between RARs and PPARβ/δ in cells may depend on the relative expression levels of CRABP-II and FABP5, which appear to deliver atRA to RARs and PPARβ/δ, respectively (Schug et al. 2007).

Besides retinoic acid, other retinoids and apocarotenoids different from retinoids interact with nuclear receptor transcription factors to either antagonize or promote their action on target genes in cells. In particular studies have suggested that, besides

Fig. 15.1 (continued) Overview of cellular carotenoid and retinoid metabolism. Circulating retinol (ROL) bound to retinol binding protein (RBP) is internalized in cells through the action of specific surface receptors (STRA6, RBPR2) or by diffusion across the plasma membrane. Efficient ROL uptake depends on the activity of lecithin:retinol acyltransferase (LRAT) in esterifying ROL to fatty acids to form retinyl esters (RE), which can be stored in lipid droplets. RE associated with circulating lipoproteins can be hydrolyzed to ROL by lipoprotein lipase (LPL) and taken up by the cells. Alternatively, circulating lipoproteins containing RE and carotenoids can be internalized in cells whole by endocytosis, through the action of lipoprotein receptors. Within the cell, RE is hydrolyzed by RE hydrolases (REH) to release ROL, which can be reversibly oxidized to retinaldehyde (Rald) by several dehydrogenases (of the RDH and ADH families). Rald is irreversibly oxidized to retinoic acid (RA) by the action of aldehyde dehydrogenases (ALDH). RA can also be taken up from the circulation where it is found bound to albumin, and is catabolized by the cytochrome enzymes (CYP) to oxidized products that are excreted from cells. Carotenoids within the cell can be cleaved by β-carotene oxygenases (BCO) to give rise to Rald (formed mainly upon cleavage of β-carotene and other provitamin A carotenoids by BCO1) and other metabolites (apocarotenals and apocarotenones). Retinoids (RA, Rald) and other carotenoid metabolites exert genomic effects by modulating the activity of distinct nuclear receptors (NR) and other transcription factors such as nuclear factor κB (NF-κB) and the nuclear factor erythroid 2-related factor 2 (Nrf2), through direct and indirect manners, and they exert as well non-genomic effects including reactive oxygen species (ROS) scavenging. Not shown in the Figure are specific cellular retinol and retinoic acid binding proteins which help solubilizing these retinoids and chaperoning their intracellular interactions

atRA, Rald (the precursor of atRA and direct product of BCO1) (Kiefer et al. 2012), apo-10′-lycopenoic acid (a product of eccentric cleavage of lycopene) (Gouranton et al. 2011) and intact β-cryptoxanthin (Shirakura et al. 2011) can behave as RAR agonist ligands. On the other hand, some β-apocarotenoids resulting from eccentric cleavage of BC, such as β-apo-14′-carotenal, β-apo-14′-carotenoic acid and β-apo-13-carotenone appear to behave as RAR-antagonists, at least in cells of hepatic origin (Eroglu et al. 2012). Because some of these compounds are contained in foods and found in human plasma, it has been proposed they may act as naturally occurring retinoid antagonists (reviewed in (Eroglu and Harrison 2013). Studies have also suggested PPARγ antagonism by Rald (Ziouzenkova et al. 2007a), β-apo-14′-carotenal (a product of eccentric cleavage of BC) (Ziouzenkova et al. 2007b) and intact astaxanthin (Inoue et al. 2012).

Carotenoids and carotenoid derivatives impact mammalian biology by additional mechanisms, besides their physical interaction with nuclear receptors (Breitman and Takahashi 1996; Blomhoff and Blomhoff 2006; Bonet et al. 2012; Al Tanoury et al. 2013; Eroglu and Harrison 2013; Kaulmann and Bohn 2014). For instance, liganded RAR (i.e., atRA-bound RAR) can interfere with the activity of other transcription factors such as activator protein-1 or CCAAT-enhancer binding proteins (C/EBPs). In fact, interference with C/EBPs by liganded RAR is an important contributor to the inhibitory effect of atRA on adipocyte differentiation (adipogenesis) (Schwarz et al. 1997). Additionally, carotenoids modulate signaling pathways such as the nuclear factor κB (NF-κB) and the nuclear factor erythroid 2-related factor 2 (Nrf2) pathways, which are associated with inflammatory and oxidative stress responses, respectively. Finally, carotenoids and carotenoid derivatives may impact cell physiology and metabolism through extragenomic actions including scavenging of reactive oxygen species (ROS), retinoylation (acylation by retinoic acid) of proteins and activation of protein kinase cascades.

15.4 Aspects of Adipose Tissue Biology Affected by Carotenoids and Carotenoid Derivatives

Adipose tissue is an important site of carotenoid (Kaplan et al. 1990; Parker 1989) and retinol storage/accumulation (Tsutsumi et al. 1992). It has been estimated that 15–20 % of the total body retinol in rats is stored in WAT, in particular in the adipocytes (rather than in the stromal vascular cells) and mostly in the form of non-esterified retinol (as opposed to preferential storage of vitamin A as retinyl ester in the liver) (Tsutsumi et al. 1992). Carotenoids are found in adipocytes mainly with triacylglycerol in the lipid droplet, and also in association to cell membranes (Gouranton et al. 2008). In humans, carotenoid concentrations in abdominal fat depots show a strong association with both dietary carotenoid intake and plasma carotenoid concentrations (Kohlmeier and Kohlmeier 1995; Chung et al. 2009; El-Sohemy et al. 2002).

Pro-vitamin A carotenoids and retinol in adipocytes may serve to regulate systemic vitamin A homeostasis (in fact, WAT produces RBP and adipose retinol/retinyl esters stores are readily mobilized under conditions of dietary vitamin A deficiency (Frey and Vogel 2011)) and may also serve specific functions in adipose tissue and within the mature adipocytes. Different lines of evidence sustain a role for carotenoids and retinoids in adipose tissue biology. WAT expresses all intracellular binding proteins and enzymes involved in retinol and retinoic acid production and metabolism, including BCO1 and BCO2 (Hessel et al. 2007; Tourniaire et al. 2009; Landrier et al. 2012). Besides retinol, other retinoids including Rald and atRA have been detected in WAT (Kane et al. 2008a; Kane et al. 2008b). Studies suggest crosstalk of intracellular retinoid metabolism and lipid droplet dynamics, with an association of enzymes of retinoid metabolism with the lipid droplet coat which appears to be dependent on active acyl ester biosynthesis (Jiang and Napoli 2012, 2013). Animal (Sima et al. 2011) and human (Gerhard et al. 2014; Kiefer et al. 2012; Perez-Perez et al. 2009; Peinado et al. 2010) studies – including omic studies – have revealed a differential expression of genes for carotenoid/retinoid metabolizing enzymes in visceral and subcutaneous adipose tissue, which display important differences regarding developmental origin, metabolism, endocrinology, capacity for adipogenesis and the health risk they entail (Hamdy et al. 2006; Lee et al. 2013). Genetic ablation of different carotenoid/retinoid-metabolising enzymes and transport proteins – such as BCO1, Aldh1a1, Rdh1, retinol saturase and CRBPs I and III – results in alterations of adiposity in mice (Hessel et al. 2007; Ziouzenkova et al. 2007a; Zhang et al. 2007; Schupp et al. 2009; Zizola et al. 2008, 2010; Kiefer et al. 2012) (reviewed in (Frey and Vogel 2011)). Finally, treatment/supplementation experimental studies indicate that specific carotenoids and carotenoid derivatives impact essential aspects of adipose tissue biology including the control of adipogenesis, adipocyte metabolism (relative capacities for fat storage and oxidation), oxidative stress and the production of regulatory signals and inflammatory mediators. These aspects are briefly introduced next.

15.4.1 Adipogenesis

Adipose tissue can greatly expand to facilitate energy storage through both increased lipid filling within existing mature adipocytes to increase adipocyte size (hypertrophic adipose tissue growth) and increased differentiation of adipocyte precursor cells to increase adipocyte number (hyperplastic adipose tissue growth or adipogenesis). The latter process is triggered by nutritional and hormonal signals that activate a cascade of transcription factors in preadipocytes including the C/EBPs and PPARγ (reviewed in (Farmer 2006)). PPARγ is considered the master regulator of adipogenesis and it is also required to maintain the adipocyte phenotype (Tamori et al. 2002) and to mediate high fat diet-induced adipocyte hypertrophy

(Kubota et al. 1999), as it transactivates genes for proteins that facilitate uptake, cytosolic binding and activation of fatty acids for triacylglycerol synthesis and lipid droplet formation and maintenance (Dalen et al. 2004) and references therein)]. Even in (human) adult adipose tissue, about 10 % of adipocytes turn over every year (Spalding et al. 2008) and, both in rodents (Klyde and Hirsch 1979) and humans (Tchoukalova et al. 2010), adipogenesis can be induced by environmental cues such as consumption of a high fat diet. Proper adipogenesis is critical for maintaining health: defects in adipogenesis in the face of a positive energy balance (i.e. more energy ingested than expended per day) may result in ectopic fat deposition and lipotoxicity, leading to insulin resistance, diabetes and vascular complications (Virtue and Vidal-Puig 2010). However, many obese humans have more than the average number of adipocytes and weight loss decreases the volume of adipocytes but not adipocyte number, which may facilitate body weight regain (Arner and Spalding 2010). In fact, studies in rodents have suggested that increased adipocyte number might *per se* lead to obesity (Naaz et al. 2004). In this context, the control of adipogenesis emerges as a potential co-adjuvant therapeutic target in obesity when coupled to strategies enhancing a negative energy balance, and agents capable of tipping the adipocyte birth-death balance in favor of reducing the number of fat cells become of interest (Arner and Spalding 2010). Remarkably, accumulating evidence links carotenoids and carotenoid conversion products to the inhibition of adipogenesis and fat storage capacity of mature adipocytes through suppression of PPARγ, either by acting as direct PPARγ antagonists or by repressing PPARγ secondarily to RAR activation.

15.4.2 Adipocyte Energy Metabolism

Not all adipocytes in mammals have equal metabolic properties. Brown adipocytes in typical brown adipose tissue (BAT) depots, on the one hand, are rich in mitochondria, have a high oxidative capacity and are specialized in the regulated production of heat (nonshivering adaptive thermogenesis) through oxidation of fatty acids and other fuels linked to the subsequent dissipation of the proton electrochemical gradient generated by the respiratory chain via uncoupling protein 1 (UCP1), an inner mitochondrial membrane protein that behaves functionally as a proton transporter (Cannon and Nedergaard 2004). Typical white adipocytes in WAT depots, on the other hand, are poor in mitochondria, have a low oxidative capacity, do not express UCP1 and are specialized in the storage and release of energy, according to biological needs (besides their endocrine function). A third type are the so-called “brite” (from brown-in-white) or “beige” adipocytes: these are brown-like, UCP1-expressing adipose cells which can be induced in WAT depots in response to cold and a variety of nutritional and pharmacological factors, in a process known as *browning* or WAT-to-BAT remodeling (Bonet et al. 2013; Giralt and Villarroya 2013). Until recently, BAT was believed to play a negligible role in

the adult human and thought to be only present in neonates. However, it has gained substantial interest since active BAT has been shown to be present in adults and BAT activity is negatively associated with increasing BMI in humans (Saito 2013). Increasing BAT activity and promoting the induction of beige adipocytes in WAT both represent attractive strategies to counteract obesity (Palou et al. 2013; Palou and Bonet 2013). Moreover, pharmacological and nutrient-dependent stimulation of mitochondrial oxidative capacity and fatty oxidation in WAT independent of UCP1 has been documented which may also contribute to a lean phenotype (Flachs et al. 2013). Carotenoids such as fucoxanthin and astaxanthin and retinoids such as atRA and Rald have been shown to impact on BAT thermogenic function and on WAT oxidative capacity and WAT browning to stimulate these processes/capacities, thus favoring energy consumption in adipose tissues (see below). Furthermore, feeding studies in rodents indicate that the thermogenic capacity of BAT is dependent on the animal's vitamin A status, being reduced following a vitamin A-deficient diet and increased following a vitamin A (retinyl ester)-supplemented diet (Kumar et al. 1999; Bonet et al. 2000; Felipe et al. 2003).

15.4.3 Adipose Tissue Secretory Function and Inflammation

Besides storing energy, WAT has an important secretory function. Thus, WAT actively participates in the systemic control of energy balance, glucose homeostasis, insulin sensitivity, inflammation, vascular haemostasis and other processes through the secretion of signaling molecules, among them many signaling proteins (collectively named adipokines) produced by the adipocytes and/or cells of the stromal-vascular fraction, often in concert. The two roles of WAT are closely related to one another. Thus, the production of many of these signals, including endocrine signals and immunomodulatory factors, is altered in obesity, which is nowadays recognized as a state of chronic, low-grade local (WAT) and systemic inflammation, which links central obesity to metabolic disturbances characteristic of the metabolic syndrome (Wisse 2004; Wellen and Hotamisligil 2005). Both hypertrophied adipocytes and infiltrating macrophages in WAT are a source of inflammatory mediators in obesity. Anti-inflammatory properties of BC and non-provitamin A carotenoids (such as lycopene, astaxanthin or fucoxanthin, among other) have been demonstrated in different contexts and tissues/cell types and are thought to arise from the ability of these compounds to activate Nrf2, thus reducing oxidative stress, and to suppress NF-κB activation, thus inhibiting the downstream production of inflammatory cytokines (Kaulmann and Bohn 2014). Carotenoids and their conversion products can affect the inflammatory and secretory profile of adipose tissue by actively interacting with these pathways in adipocytes and adipose tissue macrophages, by impacting the expression of specific adipokines through effects on other specific transcription factors, and/or in a passive manner, i.e. secondary to their effects on adipocyte lipid content and body fat. Remarkably,

cross-sectional and prospective studies in humans fairly consistently show that a higher intake and status of carotenoids is associated with lower levels of low-grade inflammation in relation to overweight, obesity and the metabolic syndrome (Calder et al. 2011).

15.4.4 Oxidative Stress in Adipocytes

Oxidative stress generated by adipose tissue in the context of obesity is an important pathogenic mechanism of obesity-associated metabolic syndrome (Furukawa et al. 2004; Le Lay et al. 2014) tightly linked to adipose tissue inflammation and secretion of systemic inflammatory mediators, since ROS activate inflammatory pathways. Cell and *in vivo* studies have suggested that carotenoids have antioxidant properties that stand both from their ability to scavenge several ROS and to interact with and potentiate the Nrf2 pathway, enhancing Nrf2 translocation to the nucleus and the subsequent activation of the expression of a collection of Nrf2 target genes encoding antioxidant and cytoprotective enzymes (Kaulmann and Bohn 2014). However, carotenoids may act as prooxidants depending on their concentration in cells, the cell oxidative environment and other factors (Palozza 1998; Rodriguez et al. 2005; Fuster et al. 2008; van Helden et al. 2009). In fact, studies suggest that, as in other cell types and tissues, carotenoids in adipocytes/adipose tissue may protect against oxidative stress or contribute to it, depending on the dose and cell/tissue factors.

15.5 Carotenoids as Modulators of Adiposity and Obesity: Cell an Animal Studies

15.5.1 Beta Carotene and Beta Carotene Derivatives

15.5.1.1 Cell Studies

BC (50 μM) inhibited the adipose conversion of murine 3 T3-L1 preadipocytes, the prototypical model for studies of adipogenesis (Kawada et al. 2000). This is most likely due to BC conversion into atRA, whose inhibitory effect on adipogenesis when applied at relatively high doses (0.1–10 μM) at defined, early stages of the process is well known (Murray and Russell 1980; Kuri-Harcuch 1982; Xue et al. 1996). Inhibition of adipogenesis by atRA is explained by several non-mutually exclusive mechanisms which translate into repression of PPARγ, the master transcription factor for adipogenesis. One is the interference of liganded RAR with the activity of the early transcription factor C/EBPβ on its downstream target genes in the adipogenic program (Schwarz et al. 1997; Marchildon et al. 2010). Additionally, atRA works upstream C/EBPβ in preadipocytes by inducing (at the transcriptional level, through an RAR pathway) specific proteins that inhibit

adipogenesis (such as Pref-1, Sox9 and KLF2) (Berry et al. 2012). Retinoylation of regulatory proteins resulting in reduced PPARγ availability for transcription regulation also appears to play a role, this one independent of RAR and RXR (Dave et al. 2014). Promotion of apoptosis of primary rat preadipocytes and clonal preadipocytes by atRA has also been reported (Kim et al. 2000; Chawla and Lazar 1994).

Remarkably, atRA effects on adipogenesis are concentration-dependent. Thus, whereas relatively high doses of atRA suppress adipogenesis, atRA induces the opposite effect, i.e. it promotes adipogenesis, when applied at low doses (1 pM to 10 nM range) (Safonova et al. 1994). Moreover, stem cell commitment into the adipocyte lineage requires a time-defined treatment with atRA (Dani et al. 1997; Bost et al. 2002), and endogenous RA production appears to be required for efficient adipogenesis of 3 T3-L1 fibroblast cell lines (Reichert et al. 2011).

BC metabolites other than atRA have been shown to repress adipogenesis of preadipocyte cell lines. This is the case of Rald (Kawada et al. 2000; Ziouzenkova et al. 2007a) and certain β-apo-carotenals resulting from asymmetric cleavage of BC, such as β-apo-8′-carotenal (Kawada et al. 2000) and, especially, β-apo-14′-carotenal (Ziouzenkova et al. 2007b). Rald and β-apo-14′-carotenal behave as weak RAR agonists, but their inhibitory effect on adipogenesis has been traced to their ability to suppress PPARγ- and RXR-mediated responses trough RAR- independent mechanisms and possibly following their direct physical binding to PPARγ and RXR (Ziouzenkova et al. 2007a, b).

Besides impacting adipogenesis, carotenoids and carotenoid derivatives impact metabolism of mature adipocytes. In particular, exposure to BC (2 μM) boosted the production of atRA and reduced lipid content and the expression of PPARγ and PPARγ target genes in mature 3 T3-L1 adipocytes (Lobo et al. 2010a). These effects were BCO1- and RAR-dependent, as they were abrogated by both a BCO1 inhibitor and a pan-RAR antagonist (Lobo et al. 2010a). Importantly, BC effects in 3 T3-L1 adipocytes were not reproduced upon incubation of the cells with vitamin A (retinol), suggesting that BC is a key precursor for retinoid production in mature adipocytes (Lobo et al. 2010a). Remarkably, while BCO1-derived products repress PPARγ, BCO1 itself is encoded in a PPARγ target gene (Boulanger et al. 2003) that is induced during adipocyte differentiation (Lobo et al. 2010a), which is suggestive of a negative feed-back loop to keep control of PPARγ.

Early work showed that BC and other provitamin A carotenoids can induce UCP1 expression in primary brown adipocytes (Serra et al. 1999), possibly reflecting local conversion to atRA, which is a potent signal for UCP1 gene transcription (Alvarez et al. 1995; Puigserver et al. 1996; del Mar Gonzalez-Barroso et al. 2000). The retinoid responsiveness of the UCP1 gene is explained by the presence of both a noncanonical retinoic acid response element and PPAR response element in its promoter (Larose et al. 1996; Rabelo et al. 1996; Sears et al. 1996), and is mediated by RARs and RXRs (Alvarez et al. 2000) and also by p38 mitogen-activated protein kinase (p38 MAPK) (Teruel et al. 2003).

Besides inducing UCP1 in brown adipocytes, retinoids stimulate the acquisition of brown adipocyte features in white adipocytes. Thus, exposure to atRA increased

basal lipolysis and fatty acid oxidation rate, triggered changes in gene expression consistent with increased fatty acid mobilization, oxidation and turnover and, upon prolonged treatment, reduced intracellular lipid content in mature 3 T3-L1 adipocytes (Mercader et al. 2007). More recently, atRA has been shown to induce oxidative phosphorylation and mitochondria biogenesis in adipocytes (Tourniaire et al. 2015). Even if atRA treatment failed to induce UCP1 in 3 T3-L1 adipocytes (Mercader et al. 2010; Murholm et al. 2013), it potently induced UCP1 in other murine models of white adipocytes, such as adipocytes derived from primary mouse embryo fibroblasts (MEFs) and mature C3H10T1/2 adipocytes (Mercader et al. 2010; Murholm et al. 2013), in a p38 MAPK-dependent (Mercader et al. 2010), RAR-dependent (Murholm et al. 2013) and PPARγ coactivator 1α -independent manner (Mercader et al. 2010; Murholm et al. 2013). However, atRA failed to induce UCP1 expression in human adipocyte cell lines and primary human white adipocytes (Murholm et al. 2013). Rald was also capable of inducing UCP1 expression in murine MEFs-derived adipocytes, though at higher effective doses than atRA (10 μM vs 1 μM for atRA), as it could be expected for a Rald effect following its intracellular conversion to atRA (Mercader et al. 2010). Subsequently, evidence was presented that Rald can induce UCP1 expression in cultured white adipocytes independently of its conversion to atRA, following binding to and activation of RAR (Kiefer et al. 2012).

Cell-autonomous effects of carotenoids and carotenoid derivatives on the secretory function of mature adipocytes have been demonstrated. Elevated levels of the adipokines resistin (Steppan et al. 2001; Stofkova 2010), leptin (Huang et al. 2004; Stofkova 2009) and RBP4 (Yang et al. 2005; Esteve et al. 2009) have been associated with insulin resistance and inflammation in rodents and humans. We and others have shown that exposure to atRA suppresses adipocyte production of leptin (Bonet et al. 2000; Menendez et al. 2001; Hollung et al. 2004), resistin (Felipe et al. 2004) and RBP4 (Mercader et al. 2008) through transcriptional mechanisms that involve both RAR- and RXR-dependent pathways (Felipe et al. 2004; Felipe et al. 2005; Mercader et al. 2008). Additionally, exposure to atRA limited pro-inflammatory cytokine (interleukins 6 and 1β) expression in TNFα-treated 3 T3-L1 adipocytes (Gouranton et al. 2011). Adiponectin is an adipokine quite specific of adipocytes that is down-regulated in obesity and has well-established insulin-sensitizing, anti-inflammatory and anti-atherogenic effects (Turer and Scherer 2012). In our hands, atRA treatment did not affect adiponectin expression in 3 T3-L1 adipocytes (or atRA-treated mice) (Bonet, Ribot and Palou, unpublished results); however, exposure to BC (20 μM, from days 4 to 8 of differentiation) induced adiponectin expression in differentiating 3 T3-L1 adipocytes (Kameji et al. 2010). All in all, the scenario is suggestive of a provitamin A-independent effect of BC on adiponectin production in adipocytes.

Reports dealing specifically with carotenoids activity on oxidative stress in adipocytes are scarce and suggest that, as in other cell types, carotenoids might be antioxidant or prooxidant in fat cells. For instance, whereas pre-treatment with BC (10 μM) suppressed TNFα-induced ROS production in 3 T3-L1 adipocytes, suggesting that intracellular BC accumulation enables elimination of ROS in these

cells (Kameji et al. 2010), exposure to BC (10–30 μM) led to mitochondrial dysfunction in immortalized Chub-S7 human preadipocytes (Sliwa et al. 2012), likely linked to increased oxidative stress.

15.5.1.2 Animal Studies

Animal studies linking dietary BC supplementation, vitamin A status and retinoid treatment to changes in body adiposity and responses to a high fat diet are addressed in this section.

Dietary BC supplementation (~35 mg/kg bw/day, 14 weeks) was shown to reduce body fat content without affecting body weight in wild-type mice (Amengual et al. 2011a). The anti-adiposity effect of BC is linked to the suppression of PPARγ and PPARγ target genes in WAT, as indicated by both transcriptomic and targeted gene and protein expression analyses (Amengual et al. 2011a; van Helden et al. 2011), and is dependent on BC conversion to retinoids via BCO1, as it was not seen in BCO1-deficient ($BCO1^{-/-}$) mice, despite these mutants accumulated in WAT depots large amounts of BC and of the BCO2 product β-10´-apocarotenol (derived from BC) upon supplementation (Amengual et al. 2011a). Importantly, following dietary BC supplementation, BC accumulation was detected in serum and WAT of wild-type mice, supporting the notion that a BCO1-dependent, local production of retinoids from BC may modulate fat storage capacity in adipocytes (Amengual et al. 2011a). In ferrets, which absorb intact BC efficiently but are not as efficient as humans and rodents in converting BC to vitamin A (Lederman et al. 1998; Lee et al. 1999), 6-month BC supplementation (3.2 mg/kg bw/day) did not reduce body fat (Murano et al. 2005; Sanchez et al. 2009), whereas treatment with atRA did (Sanchez et al. 2009). Whether dietary BC can effectively counteract diet-induced obesity in animal models has not been studied, to our knowledge.

Chronic feeding of a diet restricted in retinol (vitamin A-deficient diet) led to increased adiposity and PPARγ expression in WAT depots in rodents (Ribot et al. 2001; Esteban-Pretel et al. 2010), and diets low in carotenoids and vitamin A are used traditionally to favor the development of adipose tissue and the formation of intramuscular fat in the cattle (Kawada et al. 1996; Gorocica-Buenfil et al. 2007), the so-called bovine marbling. Reciprocally, chronic dietary vitamin A supplementation (as retinol or retinyl palmitate, at 40- to 50-fold the control dose) led to reduced adiposity in lean rats (Kumar et al. 1999) and genetically obese WNIN/Ob rats (Jeyakumar et al. 2006, 2008; Sakamuri et al. 2011). However, vitamin A supplementation had only a modest effect counterbalancing the development of diet-induced obesity in obesity-prone mice (Felipe et al. 2003), and did not affect body weight and adiposity gain in response to a cafeteria diet in rats (Bairras et al. 2005). Moreover, there are studies pointing to a pro-obesogenic effect of excess intake of preformed vitamin A (retinol) in critical periods in early life, through mechanisms that may relate to changes in WAT development (Redonnet et al. 2008; Granados et al. 2013) (see also section 7).

In keeping with an anti-adiposity action of retinoids, atRA treatment reduces adiposity and enhances glucose tolerance and insulin sensitivity in both lean and obese rodents (Puigserver et al. 1996; Bonet et al. 2000; Ribot et al. 2001; Felipe et al. 2004, 2005; Mercader et al. 2006, 2008; Strom et al. 2009; Berry and Noy 2009; Amengual et al. 2010; Manolescu et al. 2010). These effects have been evidenced with different atRA dosages (from 0.25 to 100 μg/g bw/day), routes of administration and treatment durations (reviewed in (Bonet et al. 2012)). The slimming action of atRA is not due to reduced food intake, and it has been traced to increased oxidative metabolism and energy expenditure in tissues including BAT, WAT, skeletal muscle and the liver, and to reduced PPARγ expression and activity in WAT depots. atRA treatment in rodents activates BAT with induction of UCP1 (Puigserver et al. 1996; Kumar and Scarpace 1998; Bonet et al. 2000) and in WAT it increases the expression of genes linked to mitochondria biogenesis and function, thermogenis and fatty acid oxidation – including UCP1 protein in the subcutaneous (inguinal) WAT depot – and the appearance of adipocytes with a multilocular distribution of intracellular fat, which are paramount features of WAT browning (Mercader et al. 2006; Tourniaire et al. 2015). Enhancement of lipid catabolism in skeletal muscle (Felipe et al. 2003; Amengual et al. 2008; Berry and Noy 2009) and liver cells (Amengual et al. 2010, 2012) also contribute to the slimming effect of atRA treatment in mice. Most effects of atRA on adiposity in rodents appear to be dependent on RAR and PPARβ/δ activation (Amengual et al. 2008; Berry and Noy 2009; Bonet et al. 2012). Acute atRA treatment also reduced adiposity and increased multilocularity and UCP1 content in the retroperitoneal adipose depot of ferrets (Sanchez et al. 2009). Importantly, simultaneous chronic treatment with atRA has been shown to suppress high fat diet-induced adipogenesis and adipocyte hypertrophy, thus counteracting the development of diet -induced obesity in obesity-prone (C57BL/6) mice (Berry et al. 2012; Noy 2013).

The immediate precursor of atRA, Rald, also appears to have an anti-adiposity action *per se in vivo*. Mice lacking Aldh1a1 – which is the main aldehyde dehydrogenase involved in atRA production from Rald in adipocytes/ adipose tissue (Reichert et al. 2011; Sima et al. 2011) – had increased Rald levels in WAT and resisted diet-induced obesity due to hypermetabolism (Ziouzenkova et al. 2007a). Moreover, the *in vivo* knockdown of Aldh1a1 in WAT (through antisense oligonucleotide) limited the development of diet-induced obesity in obesity-prone mice (Kiefer et al. 2012) and administrating Rald or a Aldh1a1 inhibitor reduced subcutaneous fat mass in obese ob/ob mice (Ziouzenkova et al. 2007a). Similar to atRA treatment, the anti-adiposity effect of Aldh1a1 knockout/ knockdown has been related to WAT browning, since lack of Aldh1a1 enhanced cold-induced thermogenesis and induced a BAT-like transcriptional program in visceral WAT of the knockout mice, without changes in BAT (Kiefer et al. 2012), and WAT-selective Aldh1a1 knockdown in adult obese mice triggered WAT browning (Kiefer et al. 2012). It appears, therefore, that atRA and Rald have somewhat redundant effects on adipose tissue metabolism, likely owing to their shared ability to activate RARs.

In good concordance with results in adipocyte cell models, treatment studies in rodents have revealed a down-regulatory effect of atRA treatment on the adipose

production of leptin (Kumar and Scarpace 1998; Bonet et al. 2000; Menendez et al. 2001; Hollung et al. 2004), resistin (Felipe et al. 2004) and RBP4 (Mercader et al. 2008). Elevated levels of these three adipokines associate with inflammation and insulin resistance in humans and rodents (Steppan et al. 2001; Stofkova 2010; Huang et al. 2004; Stofkova 2009; Yang et al. 2005; Esteve et al. 2009), and their down-regulation was paralleled by improved insulin sensitivity in the atRA-treated mice (Felipe et al. 2004; Mercader et al. 2008). atRA-induced down-regulation has been demonstrated in humans besides rodents for leptin (Menendez et al. 2001; Hollung et al. 2004). Remarkably, down-regulation of RBP4 by atRA is adipocyte-specific, as hepatic RBP4 expression was unaffected by atRA treatment (Mercader et al. 2008); this is of interest, since it is specifically RBP4 of adipose origin which has been related to inflammation and insulin resistance (although any physical difference between hepatic and adipose RBP4 remains to be established, to our knowledge). Similar to atRA treatment, dietary vitamin A supplementation to rodents resulted in reduced adipose expression and circulating levels of resistin and leptin to an extent that largely exceeded the reduction of adipose mass (Felipe et al. 2004; Kumar et al. 1999; Felipe et al. 2005), and opposite changes of leptin (i.e., up-regulation) were demonstrated in mice fed a vitamin A deficient-diet (Bonet et al. 2000). Moreover, transcriptome analysis revealed leptin, RBP4 and resistin among the top fifty down-regulated genes in inguinal WAT of BC-supplemented wild-type mice, and these effects were provitamin A-dependent, as they were absent in BC-supplemented $BCO1^{-/-}$ mice (Amengual et al. 2011a).

15.5.2 Cryptoxanthin

β-cryptoxanthin is a provitamin A carotenoid which displays both structural and functional similarities to BC. Oral supplementation with β-cryptoxanthin (0.8 mg/kg bw/day contained in 400 mg of a powder derived from Satsuma mandarins, *Citrus unshiu* Marc., 8 weeks) reduced body weight, visceral adipose tissue mass, adipocyte hyperthorphy and serum lipid concentrations in a genetic obese mouse model (Tsumura Suzuki obese diabetic), independent of changes in food intake (Takayanagi et al. 2011). Anti-obesity effects have also been reported for mango (*Mangifera indica* L.) pulp (10 % w/w) – which is a rich source of β-cryptoxanthin, violaxanthin and BC (Cano and de Ancos 1994); supplementation with mango reduced body fat gain and improved glucose tolerance and insulin sensitivity in mice on a high fat diet (Lucas et al. 2011).

The anti-adiposity effect of β-cryptoxanthin in rodents might be in keeping with studies in differentiating 3 T3-L1 adipocytes showing reduction of lipid accumulation following exposure of the cells to β-cryptoxanthin (1–10 μM) (Shirakura et al. 2011; Goto et al. 2013), although there are results conflicting (Okada et al. 2008). β-cryptoxanthin effects on adipogenesis entail RAR activation and subsequent PPARγ down-regulation (Shirakura et al. 2011). Results from *in vitro* nuclear receptor

binding assays indicated that β-cryptoxanthin can efficiently bind RARs (but not PPARγ), which raises the possibility that β-cryptoxanthin acts *per se* as a RAR agonist to down-regulate PPARγ in adipocytes (Shirakura et al. 2011).

15.5.3 Astaxanthin

Astaxanthin is a natural antioxidant, non-provitamin A carotenoid, abundant in marine animals. Astaxanthin (6–30 mg/kg bw/day) prevented visceral fat accumulation, metabolic syndrome, and insulin resistance induced by high fat diet feeding in mice and rats (Ikeuchi et al. 2007; Arunkumar et al. 2012), and reduced oxidative stress markers in adipose tissue and skeletal muscle of the high fat diet-fed mice (Arunkumar et al. 2012). The anti-adiposity action of astaxanthin was not due to changes in food intake and was traced to enhanced systemic fatty acid utilization, as indicated by reduced respiratory quotient in indirect calorimetry tests (Ikeuchi et al. 2007). Whether this enhanced fat catabolism occurred in adipose tissue depots was not addressed. Astaxanthin has also been shown to inhibit rosiglitazone (a PPARγ ligand)-induced adipogenesis of 3 T3-L1 cells by antagonizing PPARγ transcriptional activity, possibly upon direct binding, since results of *in vitro* assays (CoA-BAP) indicated that astaxanthin is able to selecetively bind PPARγ (but not PPARα or PPARβ/δ) (Inoue et al. 2012).

15.5.4 Fucoxanthin

Fucoxanthin is an orange colored carotenoid present in edible brown seaweeds, such as *Undaria pinnatifida* (Wakame), *Hijikia fusiformis* (Hijiki), *Laminaria japonica* (Ma-Kombu) and *Sargassum fulvellum.* It is a non-provitamin A xanthophyll whose distinct structure includes an unusual allenic bond (reviewed in (Miyashita et al. 2011)). Fucoxanthin or fucoxanthin-rich seaweed extract, alone or as part of mixtures with other selected agents, counteracts the development of dietary obesity in susceptible mice (when added to the obesogenic diet at 0.05–2 %, w/w) and reduces abdominal WAT in genetically obese KK-A^y mice (when added to the control diet at 0.2 % but not in lean mice. Fucoxanthin reduces WAT mass in obese animals by favoring fatty acid oxidation, UCP1 induction and heat production in abdominal WAT (Maeda et al. 2005, 2007; Jeon et al. 2010; Okada et al. 2011; Hu et al. 2012). Notably, fucoxanthin intake promotes WAT browning at doses at which it does not affect UCP1 expression in BAT, suggesting a WAT selective effect (Maeda et al. 2005). The actual mammalian targets for interaction with fucoxanthin metabolites in mature white fat cells remain to be identified, but enhancement of the sensitivity of adipocytes to sympathetic (SNS) nerve stimulation (which favors thermogenic activation) (Maeda et al. 2009) and

activation of AMP-dependent protein kinase (AMPK) (Kang et al. 2012) appear to contribute to the fucoxanthin effects in WAT. Obese (KK-A^y) mice (but not lean mice) fed a fucoxanthin-supplemented (0.2 %) diet had reduced expression levels of pro-inflammatory factors such as monocyte chemoattractant protein-1 and tumor necrosis factor α (TNFα), and a reduced infiltration of macrophages in visceral WAT compared with control animals (Hosokawa et al. 2010). Likewise, treatment with fucoxanthin reduced the expression of pro-inflammatory factors, oxidative stress markers (maleic dialdehyde) and macrophage infiltration in the mammary gland of mice on a high fat diet, although analysis of classical WAT depots was not included in this report (Tan and Hou 2014). Overall, animal studies sustain anti-adiposity and anti-inflammatory actions of fucoxanthin in WAT of obese animals and animals under obesogenic diets.

Interaction of fucoxanthin with adipogenesis of preadipose cells has been described that could contribute to its anti-adiposity action. Fucoxanthin and its metabolite, fucoxanthinol (which is found in WAT of treated animals) suppressed adipogenesis of 3 T3-L1 preadipocytes by down-regulating PPARγ when applied at intermediate and late stages of the adipogenic process (Maeda et al. 2006), although it enhanced adipogenesis when applied at an early stage (coincident with preadipocyte clonal expansion) (Kang et al. 2011). More recently, inhibition of 3 T3-L1 preadipocyte differentiation by Xanthigen™ (a source of fucoxanthin plus pomegranate seed oil extract rich in the conjugated linolenic acid punicic acid) has been traced to down-regulation of PPARγ and C/EBPs, up-regulation of Sirtuin 1, activation of AMPK and modulation of FoxO pathways (Lai et al. 2012). Fucoxanthinol attenuated the TNFα-induced expression of pro-inflammatory cytokines and chemokines in differentiating 3 T3-F442A adipocytes (Hosokawa et al. 2010) and the response of RAW 264.7 macrophages (a macrophage cell line) to pro-inflammatory agents such as lipopolysaccharide or palmitic acid (Kim et al. 2010; Hosokawa et al. 2010). This is of interest because in the obese WAT there is a vicious cycle between adipocytes and macrophages contributing to inflammation. Inhibition of the NF-κB pathway and of mitogen-activated protein kinase pathways (such as the JNK pathway) underlies the anti-inflammatory action of fucoxanthin in adipocytes and macropohages (Kim et al. 2010). In mature adipocytes, exposure to fucoxanthin (up to 10 μM) did not affect basal lipolysis but reduced glucose uptake (Kang et al. 2011).

15.5.5 Other Carotenoids

Animal studies indicate an anti-adiposity action for some other carotenoids. For instance, crocetin (50 mg/ kg bw/day) – which is a natural antioxidant carotenoid abundant in saffron, *Crocus sativus* Linn – prevented visceral fat accumulation, metabolic syndrome, and insulin resistance induced by high fat diet feeding in mice and rats, without affecting food intake (Sheng et al. 2008). The effects were traced to enhanced hepatic fatty acid oxidation for crocetin (Sheng et al. 2008). In

another report, supplementation with violaxanthin-rich crude thylakoids prepared from spinach leaves reduced body weight and body fat gain in mice on a high fat diet without affecting energy intake, suggestive of the involvement of a metabolic mechanism (Emek et al. 2010).

The acyclic carotenoid lycopene commonly found in tomatoes is well known for its antioxidant properties and evidence is increasing that lycopene or tomato preparations can decrease inflammatory markers, and may improve diseases with chronic inflammatory backgrounds such as obesity (Ghavipour et al. 2013). Lycopene and its metabolite, apo-10´-lycopenoic acid, have been reported to display anti-inflammatory effects in adipocytes (Gouranton et al. 2010; Gouranton et al. 2011) and macrophage-like cells (RAW 264.7) (Marcotorchino et al. 2012) through inhibition of the NF-κB pathway (Gouranton et al. 2010). A supplementation study in animals revealed an anti-inflammatory effect of dietary lycopene on the inflamed obese WAT independent of effects on adiposity (Luvizotto Rde et al. 2013). Thus, in obese Wistar rats lycopene supplementation (10 mg/kg bw/day during 6 weeks) did not affect body weight or adiposity, but decreased leptin, resistin, interleukin 6 and monocyte chemoattractant protein-1 gene expression in gonadal adipose tissue and plasma concentrations of the former three proteins (Luvizotto Rde et al. 2013). Cell studies showed no effect of lycopene and apo-10´-lycopenoic acid on adipogenesis, even if apo-10´-lycopenoic acid was able to activate the RAR and impact the transcription of certain RAR target genes in adipocytes (Gouranton et al. 2011). Overall, results indicate that lycopene does not exert anti-adiposity action but, as in other cell types, has anti-inflammatory properties in adipocytes and obese WAT tissue.

15.6 Carotenoids as Modulators of Adiposity and Obesity: Human Studies

15.6.1 Human Epidemiological Studies

Serum levels of carotenoids including BC are reduced in overweight and obese individuals, both adults and children/adolescents (*e.g.* (Moor de Burgos et al. 1992; Decsi et al. 1997; Yeum et al. 1998; Sarni et al. 2005; Burrows et al. 2009)). Furthermore, several large population-based, cross-sectional epidemiological studies indicate an inverse association between carotenoid concentrations in blood and BMI and other measures of obesity including adiposity, in some cases even when adjusted for other factors associated with carotenoid concentration, such as intake of fruit and vegetables, fat, fibre, alcohol, supplement use, smoking, gender and lipid concentrations (Brady et al. 1996; Strauss 1999; Neuhouser et al. 2001; Wallstrom et al. 2001; Ford et al. 2002; Kimmons et al. 2006; Suzuki et al. 2006; Andersen et al. 2006; de Souza Valente da Silva et al. 2007; Wang et al. 2008; Gunanti et al. 2014). Cross-sectional studies have also reported lower serum carotenoid concentrations in

adults and children with the metabolic syndrome (Beydoun et al. 2012; Beydoun et al. 2011). In fact, independent associations of low serum carotenoids with risk factors/biomarkers of the metabolic syndrome including increased insulin resistance index (HOMA-IR), fasting insulinemia, oxidized LDL, glycosylated hemoglobin, and circulating levels of inflammatory markers such as C-reactive protein have been reported, that persisted after adjusting for confounders including BMI or other obesity-related measures (Ford et al. 1999; Kritchevsky et al. 2000; Erlinger et al. 2001; Ford et al. 2003; van Herpen-Broekmans et al. 2004; Sugiura et al. 2006; Hozawa et al. 2007; Beck et al. 2008; Wang et al. 2008). These latter associations are generally considered to be related to antioxidant and anti-inflammatory activities of carotenoids.

Several arguments are generally provided to explain the inverse association between carotenoid concentrations in blood and BMI or adiposity. First, BMI and serum carotenoids may be correlated because of dietary and other lifestyle factors that affect them both. Second, since serum carotenoids are partially fat soluble, adipose tissue may act as a sink for them, so that relatively fewer are located in the blood (Brady et al. 1996; Wallstrom et al. 2001). However, the concentration of carotenoids in adipose tissue and isolated adipocytes is also lower in obese people (Chung et al. 2009; Virtanen et al. 1996; Kabagambe et al. 2005; Osth et al. 2014). A recent report showed that isolated adipocytes from obese subjects contain 50 % lower concentrations of BC than cells from lean or non-obese subjects (Osth et al. 2014). Third, adipose tissue in obesity generates oxidative stress (Higdon and Frei 2003), and the carotenoids may be reduced because of defending against this stress and being consumed upon their action as antioxidants (see (Andersen et al. 2006) and references therein), leading to reduced carotenoids levels in both blood and adipose tissue in obesity.

Notwithstanding the aforementioned explanations, we believe a mechanistic link cannot be ruled out in view of data indicating that, in adipocytes, BC rather than retinol may function as the precursor for the local synthesis of retinoids capable of exerting an anti-adiposity action (Lobo et al. 2010a). Reduced BC per adipocyte in obesity could reflect increased BC consumption in antioxidant reactions needed to neutralize increased reactive species, and perhaps also an attempt to counteract adipocyte hypertrophy by increasing retinoid production from BC. Whatever its origin, if not corrected through dietary consumption, once established reduced BC per adipose cell could contribute to the maintenance and further development of pathological obesity by limiting BC-derived anti-adiposity retinoid signaling (Fig. 15.2). Moreover, obese subjects may have a lowered capability to convert BC to retinoids, since an inverse association of BMI with BC conversion efficiency as assessed by a stable-isotope reference method was demonstrated in humans (Tang et al. 2003) and another study found a slower rate of BC decline in serum after cessation of dietary supplementation in individuals with the highest BMI (Wise et al. 2009). Reduced efficiency of BC conversion to retinoids in the obese subjects could further contribute to their obese state.

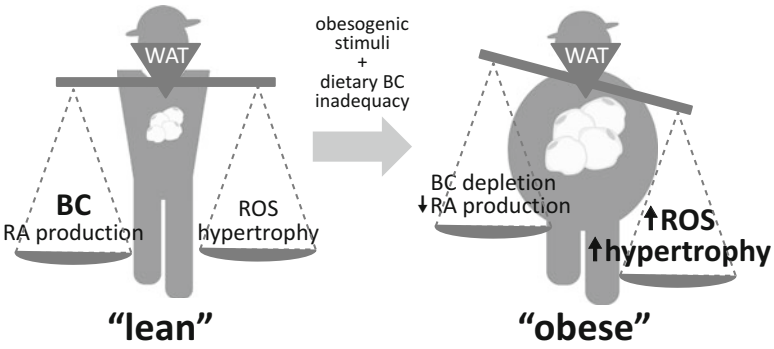

Fig. 15.2 Proposed involvement of β-carotene adipose stores in counteracting adiposopathy. β-Carotene (BC) and possibly other provitamin A carotenoids in adipocytes may serve to scavenge reactive oxygen species (ROS) and to produce retinoids such as retinoic acid (RA) capable of repressing adipocyte hyperthrophy (by suppressing PPARγ and enhancing energy utilization, see text). These activities may help counteracting adipose tissue pathological expansion and keeping the individual lean. However, BC as such is consumed in the course of these activities. An inadequate intake of dietary carotenoids on the top of an obesogenic diet and lifestyle would led to depletion of adipose BC stores and suppression of local RA production, this contributing to increased ROS, adipocyte hyperthrophy, and hence the development of pathological obesity

An association of vitamin A status (as serum retinol levels) with human obesity is less clear. Some of the studies reporting an inverse association of carotenoids in blood and obesity found instead serum retinol to be constant across BMI values or between obese and non-obese groups (Decsi et al. 1997; Neuhouser et al. 2001; Sarni et al. 2005; de Souza Valente da Silva et al. 2007), likely reflecting homeostatic regulation of circulating retinol through controlled storage in and release from the liver. A recent study reported higher concentrations of serum retinol (and lower of serum carotenoids) to be associated with increased probability of overweight and obesity in children (Gunanti et al. 2014). Other studies, in overweight and obese (adult) subjects, found the opposite, i.e. lower serum retinol to be associated with increased BMI (Viroonudomphol et al. 2003; Botella-Carretero et al. 2010).

Studies have also investigated possible associations of dietary intake of vitamin A or carotenoids as evaluated through food frequency questionnaires with measurements of adiposity. Early studies highlighted vitamin A as one of few micronutrients with a frequently inadequate intake among population samples with a high prevalence of obesity (Wolfe and Sanjur 1988; Vaughan et al. 1997). Accordingly, an inverse association between preformed vitamin A intake and several measurements of adiposity – such as body weight, BMI, waist circumference and waist-to-hip ratio – after adjusting for total energy intake has been reported in healthy young adults (Zulet et al. 2008). Higher total carotenoid intakes, mainly those of BC and lycopene, were found to be associated with lower waist circumferences and visceral and subcutaneous fat mass and lower prevalence of metabolic syndrome in a cross-sectional study involving middle-aged and elderly

men (n = 374) (Sluijs et al. 2009). A high intake of carotenoids derived from a high consumption of vegetables and fruit, as in the Mediterranean diet, was shown to associate with lower development of metabolic syndrome traits including increased waist circumference in a prospective study involving 3232 subjects (Kesse-Guyot et al. 2013). Altogether, the results of these studies point to an association between higher dietary intakes of vitamin A and carotenoids and reduced adiposity.

15.6.2 Human Intervention Studies

Randomized controlled intervention studies specifically designed to test the impact of dietary carotenoid supplementation on adiposity are scarce, and have been conducted mainly for BC, β-cryptoxanthin and fucoxanthin. Two small pilot double blind placebo-controlled studies in overweight and obese children using similar doses of BC (3–4 mg/day as part of supplements) reported significant changes in the 6 month rate of accrual in abdominal adiposity (Canas et al. 2012, 2014). In the first of these two studies, lean and overweight children underwent daily supplementation with an encapsulated supplement of fruit and vegetable juice concentrate (providing approximately 3.75 mg of BC, 117 mg of vitamin C, 22.5 IU of vitamin E, 210 mg of folate and 30 mg of calcium per day) or placebo in the presence of nutritional counseling; the supplement led to increased serum BC levels and resulted in a reduction in abdominal fat mass in conjunction with an improvement in insulin resistance in the overweight children (Canas et al. 2012). In the second study, obese children completed a 2-week intense lifestyle intervention program followed by 6 months of supplementation with Jarrow Formulas CaroteneAll® complex (providing daily 5000 IU of β- and α-carotene, 20 mg of lutein, 4 mg of zeaxanthin, 20 mg of lycopene, 1 mg of astaxanthin and 20 mg of vitamin E) or placebo; in the treatment group, reductions in both subcutaneous and visceral adiposity relative to baseline values were reported together with concomitant increases in serum adiponectin, while these parameters changed in the opposite, unwanted, direction in the placebo group (Canas et al. 2014).

The administration of β-cryptoxanthin extracted from Satsuma mandarin (0.5 mg/day as part of test drink) to moderately obese Japanese males resulted in increased levels of β-cryptoxanthin in serum and led to reductions in body weight, visceral fat and waist circumference (Tsuchida et al. 2008; Takayanagi and Mukai 2014). Another 3-week long study in 17 postmenopausal obese women supplemented with a beverage containing β-cryptoxanthin (4.7 mg/day) reported no differences in body weight or BMI but a fourfold increase in adiponectin serum levels after the treatment, suggestive of a reduction in adiposity (data on body fat mass were not include in this report) (Iwamoto et al. 2012).

Regarding fucoxanthin, in a small randomized double blinded placebo-controlled trial involving adult non-seaweed consuming subjects in Ecuador with at least

one symptom of metabolic syndrome, researchers showed that 6 grams per day of dietary brown seaweed containing fucoxanthin consumed for 2 months resulted in decreased waist circumference in women and improved systolic blood pressure (Teas et al. 2009). Another 16 week study investigating the effects of the fucoxanthin-containing product Xanthigen^{TM} reported statistically significant reductions in body weight, waist circumference, body fat content and serum triglycerides in obese, non-diabetic female volunteers with non-alcoholic fatty liver disease and normal liver fat content compared to baseline (Abidov et al. 2010).

15.7 Programming Effects of Vitamin A on Adipose Tissue Expandability

Nutritional factors at critical stages in early life can condition the susceptibility to obesity and metabolic alterations later in life (reviewed in (Pico and Palou 2013)). In this sense, the net impact of vitamin A supplements on body adiposity may be developmental stage-dependent. We have shown that treatment with a moderate, threefold excess vitamin A (as retinyl ester) during the suckling period – which is a critical period in the development of adipose tissue in the rat (Cryer and Jones 1979) – favors the accumulation of small, immature adipocytes, with a reduced expression of PPARγ and an increased expression of proliferating cell nuclear antigen, a classical marker of proliferative status (Granados et al. 2013), which might be in line with known anti-adipogenic action of retinoids. Nevertheless, these changes appeared to favor the hyperplasic component of fat expansion upon an obesogenic stimulus, since vitamin A-treated animals gained more adiposity – but not body weight – than their controls on a high fat diet owing mainly to a higher increase in WAT cellularity (DNA content) (Granados et al. 2013). A previous report already evidenced a synergic effect of exposure to a fourfold excess vitamin A (retinol) and high fat diet on WAT expansion in young (3-week-old) rats that paralleled a higher proliferation competence of precursor cells isolated from the fat depots of excess vitamin A fed animals' (Redonnet et al. 2008). These studies point, therefore, to a pro-obesogenic effect of excess vitamin A intake (even if moderate) in early life, likely by influencing the proliferative status of adipocytes.

Interestingly, different from preformed vitamin A intake, a threefold excess vitamin A intake as BC during the suckling period did not elicit changes in the developing WAT of rats at weaning, even though BC was readily absorbed and partially metabolized by the suckling rats, as indicated by BC accumulation in serum and liver and enhanced atRA-dependent transcriptional responses in intestine and liver (Musinovic et al. 2014). This latter work establishes a new potential model in studies of BC action, and suggests that BC supplementation may serve to replenish liver retinol stores in infants/children while avoiding eventual unwanted effects of early-life supplementation with pre-formed vitamin A on the susceptibility to obesity later in life (Musinovic et al. 2014).

15.8 Summary and Concluding RemarkS

Carotenoids and carotenoids conversion products seem to play a substantial role in the control of key aspects of adipose tissue biology including the production of novel adipocytes from precursor cells (adipogenesis) and the metabolic and secretory capacities of mature adipocytes, such as those for hyperthrophy, browning and endocrine and pro-inflammatory signal production. Mechanisms of action are emerging and notably include physical and/or functional interaction with transcription factors of the nuclear receptor superfamily and with pro-inflammatory and antioxidant signaling pathways in adipocytes and cells of the stromal-vascular fraction. Studies support an anti-adiposity and anti-inflammatory action of specific carotenoids and carotenoid conversion products in obesity. Interestingly, some of these compounds – such as fucoxanthin, astaxanthin and the BC-derived retinoids atRA and Rald– exert both suppressive effects on PPARγ activity and adipogenesis and activating effects on lipid oxidation and thermogenesis in mature brown and white adipocytes and other cell types. These compounds might, therefore, help moderating the formation of new adipocytes under obesogenic conditions and resetting adipocyte cell number in obese subjects while simultaneously favoring the dissipation of excess energy. Moreover, adipose tissue is an important site of carotenoid accumulation and results suggest that local production of retinoids from BC may modulate fat storage capacity in adipocytes. Human epidemiological studies reveal a low intake and status of carotenoids among obese subjects, including obese children/adolescents, which needs to be addressed. So far, there have been limited human intervention studies with carotenoid-rich supplements or extracts to reduce adiposity or obesity-related co-morbidities; even if these studies are insufficient to prove a cause-effect relationship, the scenario they depict is encouraging, since both in children and adults the interventions had beneficial effects on the accrual of body fat, abdominal fat and related risk parameters. Importantly, beneficial effects of BC supplements on adiposity in humans have been achieved under mild BC supplementation, at doses quite lower than the ones that caused concern and controversy in large intervention trials in the past (The Alpha-Tocopherol BCCPSG 1994; Omenn et al. 1996; Gallicchio et al. 2008). In summary, the literature reviewed herein supports a role of specific carotenoids and carotenoid derivatives in the prevention of excess adiposity, and suggests that carotenoids requirements may be dependent on body composition, among other factors.

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Chapter 16
Absorption of Carotenoids and Mechanisms Involved in Their Health-Related Properties

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Abstract Carotenoids participate in the normal metabolism and function of the human body. They are involved in the prevention of several diseases, especially those related to the inflammation syndrome. Their main mechanisms of action are associated to their potent antioxidant activity and capacity to regulate the expression of specific genes and proteins. Recent findings suggest that carotenoid metabolites may explain several processes where the participation of their parent carotenoids was unclear. The health benefits of carotenoids strongly depend on their absorption and transformation during gastrointestinal digestion. The estimation of the 'bioaccessibility' of carotenoids through *in vitro* models have made possible the evaluation of the effect of a large number of factors on key stages of carotenoid digestion and intestinal absorption. The bioaccessibility of these compounds allows us to have a clear idea of their potential bioavailability, a term that implicitly involves the biological activity of these compounds.

Keywords Bioactivity • Bioaccessibility • Absorption • Food matrix

16.1 Carotenoids and Their Health Protective Effects

Carotenoids are hydrophobic pigments constituted by eight isoprene units. They can be acyclic or contain rings in one or both terminal groups. They have been classified in xanthophylls, oxygen containing structures, and carotenes, which only contain hydrogen and carbon atoms in their structure (Chaps. 1, 2, and 3). The characteristic double-bond system in the carotenoid structure is responsible of their color and reactivity. Up to date, approximately 700 carotenoids have been identified from natural sources, but only 100 have been found in the typical human diet (Khachik et al. 1991). In plant foods, they are typically esterified with fatty acids, with

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exception of green leafy vegetables and some fruits like kiwi (Chap. 14, Rodriguez-Amaya and Kimura 2004). Epidemiological studies have related the consume of carotenoid-rich foods with a high content of carotenoids in plasma and a lower incidence of some forms of cancer (such as lung and stomach), age-related macular degeneration, obesity and cardiovascular diseases (Chaps. 14 and 15, Kohlmeier et al. 1997; Seddon et al. 1994; van Poppel and Goldbohm 1995). However, the involvement of carotenoids in these effects is unclear.

16.1.1 Provitamin A Activity

The vitamin A deficiency is a public health problem in some developing countries. This deficiency can lead to xerophthalmia, anemia and weakening of the immune system, increasing the risk of infections. It has been estimated that carotenoids from fruits and vegetables provide from 30 to 85 % of the daily vitamin A requirements in developed and developing countries, respectively (Chap. 14, Bramley et al. 2000). Only the carotenoids with at least one β-ionone ring (without oxygenated groups) and a polyene side-chain of at least 11 carbons can be metabolized to vitamin A (Furr and Clark 1997). Carotenoids may be cleavage to yield retinoids through two pathways, the first of them involves the cleavage of the central double bond (15,15′) by the cytosolic enzyme β,β-carotene 15–15′-monooxygenase (BCO1) to yield one, from β-cryptoxanthin and α-carotene, or two, from β-carotene, molecules of retinal, which can be reduced to retinol or oxidized to retinoic acid. The second pathway, involves the eccentric cleavage of carotenoids to produce β-apo-carotenals and minor fragments. Some β-apo-carotenals can be converted to β-apo-carotenoic acids and serve as precursors of retinoic acid. Others may be transported to the liver and oxidized, through a stepwise procedure, to retinal molecules (Furr and Clark 1997; Harrison 2012; Failla and Chitchumroonchokchai 2005; Ornelas-Paz et al. 2010). It is commonly accepted that the eccentric cleavage, also known as random cleavage, can be performed by a non-enzymatic auto-oxidation of carotenoids. However, Kiefer et al. (2001) reported the presence of a carotene dioxygenase in mouse responsible for the asymmetric oxidative cleavage of β-carotene (BCO2) at the 9′,10′ double bound. The central cleavage is the main pathway of vitamin A formation from carotenoids. Nagao et al. (1996) reported that 94–100 % of the consumed β-carotene was converted in retinal. Accordingly, Barua and Olson (2000) reported that β-apo-carotenals accounted in less than 5 % of the total retinoids formed from β-carotene in intestines of rats. These bioconversion reactions mainly occur in the enterocytes and liver (Harrison 2012; Parker 1996). However, Lindqvist and Andersson (2004) reported the expression of BCO1 in epithelial cells from the gastrointestinal tract, hepatic parenchymal cells, pancreas, kidney, adrenal gland, skin, among other organs in humans. The bioconversion efficiency is strongly affected by the vitamin A status of the individual; the maximum molar proportion (1:2) for the conversion of β-carotene in retinal can be almost achieved only in deficient organisms supplemented with low doses of β-carotene (Ornelas-Paz et al. 2010; van Lieshout et al. 2001; Yahia and Ornelas-Paz 2010). Other factors, such

as the bioavailability of carotenoids, the activity of β-carotene 15:15′ dioxygenase, and the reducing activity of intestinal cells also affect the bioconversion efficiency (Ornelas-Paz et al. 2010; van Lieshout et al. 2001). The retinoic acid acts as transcriptional regulator of hundreds of genes, exert chemopreventive activities, promotes the growth, reproduction, cell differentiation, and a well-functioning immune system (McGrane 2007; Yahia and Ornelas-Paz 2010).

16.1.2 Health Properties of Carotenoids Mediated by the Regulation of the Expression of Genes and Proteins

Some epidemiological studies have reported an inverse relationship between the high consumption of carotenoid-rich fruits and vegetables and a low incidence of some forms of cancer (Eliassen et al. 2012; Giovannucci 1999; Slattery et al. 2000). This evidence for the protective effects of carotenoids against cancer is unclear since the supplementation with pure carotenoids seems to increase the risk of cancer in humans (Omenn et al. 1996). However, some mechanistic studies support a protective role of these pigments on cancer risk through several mechanisms, mainly by the modulation of grown factor signaling, cell cycle progression, cell differentiation and apoptosis (Niranjana et al. 2014; Tanaka et al. 2012). Karas et al. (2000) demonstrated that the growth stimulation of mammary cancer cells by IGF-1 was inhibited by lycopene. The inverse association between the frequency of consumption of cooked tomatoes (rich in lycopene) and the circulating levels of IGF-1 and IGF-1/IGFBP-3 has also been reported in epidemiological studies (Mucci et al. 2001). The effect of carotenoids on the cell cycle arrest has been well documented in many *in vitro* studies with human cancer cell lines such as leukemia, colon, liver, breast, skin, prostate, and lung. The involvement of carotenoids on the growth of tumor cells seems occur at different phases of the cell cycle, according to findings obtained with β-carotene, lycopene, fucoxanthin, lutein, neoxanthin and β-cryptoxanthin (Niranjana et al. 2014). Interestingly, some metabolites of lycopene (apo-10′-lycopenoic acid and apo-12′-lycopenal) also inhibited the normal growth cell cycle in cancer cells from lung and prostate (Ford et al. 2011; Lian et al. 2007). Recently, Kaulmann and Bohn (2014) suggested that the reported activity of lycopene in the activation of the Nrf2 in different cancer cell lines (Ben-Dor et al. 2005) might be explained by the presence of apo-lycopenals, which are more polar compounds. This conjecture explains the contradiction between the lycopene hydrophobicity and the cytoplasmic location of the Nrf2. The Nrf2 increases the expression of different cytoprotective genes, protecting cells from toxicants and carcinogens (Jaramillo and Zhang 2013). On the other hand, several *in vitro* studies have also demonstrated the proapoptotic activity of carotenoids in different human cancer cell lines (leukemia, colon, lung, prostate, melanoma, liver, among others) (Niranjana et al. 2014). The induction of apoptosis by carotenoids

is mainly mediated by the reduction of the mitochondrial membrane permeability, the release of mitochondrial cytochrome c, activating different caspase forms, and increasing and reducing the Bax and Bcl-2 genes expression, respectively (Hantz et al. 2005; Palozza et al. 2003; Rokkaku et al. 2013).

Carotenoids also increase the gap junctional intercellular communication (GJIC) through mechanisms independent of their provitamin A and antioxidant activity. This communication changes the cancerous phenotype of cells. In C3H10T1/2 cells, β-carotene, cantaxanthin and lycopene (10–5 M) enhanced the number of junctional channels through the up-regulation of Conexin 43 (Cx43) mRNA and protein (Zhang et al. 1992). Lycopene and β-carotene (3–7 μM) also increased the expression of Cx43 RNA and protein in cancer cells from the oral cavity, but lycopene up-regulated GJIC more effectively than β-carotene (Livny et al. 2002). More recently, it has been demonstrated that fucoxanthin (1–20 μM) increased the expression of Cx43 and Cx42 in cancer cells from the liver (SK-Hep-1) and enhanced the GJIC (Liu et al. 2009). The presence of residues of the Cx43 phosphorylation competes with the gap junctional communication. Junctional communication regulates the cell growth and carcinogenesis (Zhang et al. 1991; Zhang et al. 1992).

The involvement of carotenoids in the immune system is mainly based on their provitamin A activity. The deficiency of vitamin A and the consequent increasing risk of infections have been well documented. However, the carotenoids themselves also exert an important activity in the strengthening of the immune system. Elliot (2005) suggested, according to studies in animals, that the effects of carotenoids on immune system might be explained by their capacity to stimulating the killing activity of blood neutrophils, increasing the mitogen-induced proliferation of lymphocytes, enhancing the antibody responses and increasing the cytochrome oxidase and peroxidase activities in macrophages. The supplementation with β-carotene (30–60 mg/d/2 months) increased the circulating T-helper cells, cells with IL-2 receptors and natural killer cells in healthy humans (Watson et al. 1991). Lycopene inhibited the maturation of dendritic cells and reduced their T-cell stimulatory capacity (Kim et al. 2004). Dendritic cells induce the expression of inflammatory markers such as cytokines and TNF-α (Kim et al. 2004). Lutein increased the excretion of MMP9 (matrix metalloproteinase 9) in murine macrophages (RAW264.7) and human monocytes (U937), increasing their phagocytic ability (Lo et al. 2013).

16.1.3 Protective Effects of Carotenoids Related with Their Antioxidant Activity

Reactive molecules (free radicals and singlet oxygen) are generated in the body under normal conditions or as a consequence of external factors. These radicals are highly reactive chemical species that contain one or more unpaired electrons. When the amount of these reactive species exceeds the normal levels in the body, they may

exert harmful effects, damaging important molecules, such as proteins, DNA, lipids, and carbohydrates, causing an abnormal cell operation and various pathologies (Bramley et al. 2000; Stahl and Sies 2003). The antioxidant activity of carotenoids has been extensively studied and associated with the conjugate double bonds in the carotenoid structure since it constitutes a reactive electron-rich system susceptible to react with electrophilic compounds (van den Berg et al. 2000). In animal systems, the carotenoid capacity to quench singlet oxygen and peroxyl radicals, deactivate electronically excited sensitizer molecules and filter blue light are of major importance. The three primary chemical reactions to scavenge oxidizing free radicals by carotenoids are electron transfer ($CAR + ROO^{\bullet} \rightarrow ROO^{-} + CAR^{\bullet+}$ or $ROO^{+} + CAR^{\bullet-}$), adduct formation ($CAR + ROO^{\bullet} \rightarrow ROOCAR^{\bullet}$) and hydrogen atom transfer ($CAR + ROO^{\bullet} \rightarrow ROOH + CAR^{\bullet}$) (Böhm et al. 2012; Edge and Truscott, 2010; van den Berg et al. 2000). The physical capacity of carotenoids to inactivate singlet oxygen depends on the number of double bonds in their backbone because of at triplet energy level they are able to receive the excitation energy from the singlet oxygen and then dissipating the energy as heat to the surrounding media, returning to their ground state. The β-carotene, zeaxanthin, cryptoxanthin, and α-carotene are highly active quenchers of singlet oxygen (Edge and Truscott 2010; Stahl and Sies 2003). Carotenoids can interrupt the production of peroxyl radicals generated during the lipid oxidation. Some harmful carotenoid radicals may be generated as intermediates in peroxidation systems; however, the involved chemical mechanism depends, among other factors, on the reactivity of peroxyl radicals (Edge and Truscott 2010; El-Agamey et al. 2004; van den Berg et al. 2000). The scavenging of peroxyl radicals protects cellular membranes and lipoproteins from oxidative damage (Stahl and Sies 2003). The carotenoids protect the long-chain polyunsaturated fatty acids of the retina from the reactive oxygen species generated by the high-energy short wavelength visible light and reduce the formation of lipofuscin (Ma and Lin 2010). The carotenoids can be oxidized under some conditions, such as oxidative stress, deficiency of antioxidants or high levels of carotenoids. The β-carotene autooxidation produces epoxy-carotenoids, β-apo-carotenones and β-apo-carotenals, with some of them being precursors of vitamin A (Stahl and Sies 2003; van den Berg et al. 2000).

Lutein and zeaxanthin are accumulated in the retina and lens within the eye (Ma and Lin 2010). They act as filters for the blue light, attenuating in about 40 % the light that reaches photoreceptors, retinal pigment epithelium and choriocapillaris, reducing their damage (Krinsky et al. 2003). Additionally, zeaxanthin and lutein provide protection against photooxidation. High amounts of reactive oxygen species are generated in the retina by the simultaneous exposure to both light and oxygen. The high content of long-chain polyunsaturated fatty acids in the retina increases their vulnerability to oxidative damage. Thus, xanthophylls inhibit the peroxidation of membrane phospholipids and reduce the photooxidation of lipofuscin fluorophores (Schalch et al 2010; Ma and Lin 2010). The lipofuscin is a potent photoinducible generator of reactive oxygen species that has been highly related to the pathogenesis of AMD (Age-related Macula Degeneration). Epidemiological studies have associated the high consumption of lutein and zeaxanthin with reduced

risk of AMD (Seddon et al. 1994; Snellen et al. 2002). The intake of lutein has also been associated with a reduced risk for cataracts (Lyle et al. 1999). Gale et al. (2001) reported that the plasma concentration of α-carotene and β-carotene was negatively correlated with the risk of nuclear cataract, whereas high plasma concentrations of lycopene and lutein reduced the risk of cortical and subcapsular cataract, respectively.

Epidemiological studies have shown that the consumption of carotenoid-rich fruits and vegetables is associated with a lower risk of cardiovascular diseases; however, other dietary components from fruits and vegetables, like vitamin C, might be responsible of this protective effect (Koh et al. 2011; Ito et al. 2006; Sesso et al. 2004). The main action mechanism of carotenoids in this regard has been associated with their antioxidant activity. Carotenoids scavenge reactive oxygen species (ROS), protecting the low-density lipoproteins from oxidation, a key process in the pathogenesis of atherosclerosis. After the daily consumption of tomato juice (40 mg lycopene), carrot juice (22.3 mg β-carotene) or a liquid spinach powder preparation (11.3 mg lutein) for two weeks, only the tomato juice reduced the lipid peroxidation in LDL of healthy men (Bub et al. 2000). Contrarily, in another study the oxidation of LDL was inhibited by β-carotene (15 mg) but not by lycopene supplementation (34 mg) (Dugas et al. 1999). Further work is required to evaluate the effect of carotenoids in cardiovascular diseases.

The etiology of rheumatoid arthritis has been strongly associated with a chronic inflammation state, in which the active function of macrophages, monocytes and granulocytes induce the formation of free radicals, which has been found in synovial fluids of patients with rheumatoid arthritis (Cerhan et al. 2003; Costenbader et al. 2010; Merry et al. 1989). Thus, dietary antioxidants have been considered in the prevention and treatment of this disease. An epidemiological study reported an inverse relationship between the high intake of β-cryptoxanthin and the risk of rheumatoid arthritis, but any relation was found for other carotenoids such as β-carotene, lycopene or lutein/zeaxanthin (Cerhan et al. 2003). Other studies neither could relate the consumption of carotenoids with the prevention of rheumatoid arthritis (Costenbader et al. 2010; Heliövaara et al. 1994).

The pathogenesis of Alzheimer Disease (AD) has been related with oxidative stress. The brain is particularly susceptible to the oxidation due to its high metabolic activity and demand of oxygen and because it contains abundant amounts of polyunsaturated fatty acids (Mecocci et al. 2002). Jiménez-Jimenéz et al. (1999) reported that AD patients showed low levels of β-carotene and vitamin A. Mecocci et al. (2002) also reported high plasmatic levels of the oxidative indicator 8-hydroxy-2′-deoxyguanosine and low levels of antioxidants (zeaxanthin, β-cryptoxanthin, lycopene, and α-carotene and β-carotene) in AD patients. High levels of phospholipid hydroperoxides and amyloid β-peptide (Aβ) have been reported in the red blood cells of AD patients (Nakagawa et al. 2011). Some *in vitro* and *in vivo* studies have demonstrated that lutein, astaxanthin and β-carotene decreased the interaction between erythrocytes and Aβ in cells from human and mice (Nakagawa et al. 2011). The retinoic acid may regulate genes involved in the Aβ expression, such as the β-secretase enzyme, AbPP, and presenilin (Obulesu et al. 2011).

16.2 Digestion, Absorption and Metabolism

The bioactivity of carotenoids in the body depends in a first instance of their absorption. Only the carotenoids released from the food matrix may be available for absorption by the intestinal epithelium. Of course, the carotenoid liberation from the food in the gastrointestinal tract is incomplete. Then, the carotenoids must be incorporated into the lipid phase of the emulsified gastrointestinal contents and then to micelles. Only this reduced fraction of carotenoids can potentially reach the enterocytes and perform a biological action. These key steps of the carotenoid absorption process can be favored or hampered by many factors.

The majority of the studies about the health properties of carotenoids have been carried out in cancer cell lines or animals. These studies have showed that carotenoids are capable to regulate genes or proteins associated with the cancer cell growth, apoptosis, cell signaling, and phagocytic activity of cells from immune system. They have generated invaluable clues. However, the carotenoid concentration that typically is assayed (up to 50 μM) does not reflect the physiological concentrations of these pigments, even those reported in plasma after acute supplementation with carotenoids (Table 16.1). Additionally, the carotenoids added to cell cultures are dissolved in organic solvents, which may affect the normal cell function or inhibit the carotenoid absorption in the plasmatic membrane. The incorporation mechanisms are also different to those occurring under normal conditions. On the other hand, many efforts have been done to increase the absorption of carotenoids but, up to date, there is not a clear evidence of that an increased absorption rate could increase the biological action of carotenoids.

16.2.1 Digestion Process

The carotenoids must be released from the food matrix through the physical and chemical rupture of vegetable cells and intracellular compartments (Fig. 16.1). The physical disruption of the food occurs during mastication and as a consequence of the peristaltic movements of gastrointestinal tract. The chemical disruption of the food is driven by digestive enzymes (amylase, gastric lipase, pepsine, etc.) and the hydrochloric acid secreted in the stomach. These processes result in the partial liberation of carotenoids from the food matrix. Then, the carotenoids should be incorporated into lipid droplets dispersed in the gastrointestinal contents. Carotenoids distribute within the lipid droplet accordingly to their solubility. Carotenes and xanthophylls occupy the core and surface of these droplets, respectively (Furr and Clark 1997). Basically, their distribution depends on their polarity (Furr and Clark 1997). At the duodenum, the size of fat droplets is reduced by the emulsifying action of bile salts, facilitating the action of lipolytic enzymes and the formation of micelles, which are constituted by bile salts, phospholipids, and digested lipids (free fatty acids, monoglycerides, carotenoids, lipophilic vitamins,

Table 16.1 Overview of recent *in vivo* studies evaluating bioaccessibility of carotenoids

Model	Carotenoid source	Studied carotenoids	[a]Administered dose	Tested factors	[b]Bioaccessibility		References
					Plasma or serum	Other tissues	
Humans	Tomato juice	LUT, *cis*-LUT, αC, βC, LYC, *cis*-LYC	141 mg/750 g/70 kg BW	With or without oil	0.04–2.28 mg/L		Arranz et al. (2015)
Humans	Cabbage	LUT, βC	1.6 mg/300 g/day	Varieties (black and red cabbage)	~0.23–5.47 mg/L		Bacchetti et al. (2014)
Humans	Algae Lutein-fortified fermented milk	LUT	4–120 mg/100 mL/day	Dose levels (low and high)	0.12–0.22 μmol/L (2.1–2.5 %)		Granado-Lorencio et al. (2010)
Humans	Fruit juice	LUT, ZEA, βC, βCX	280–575 μg/2 × 250 mL/day	Absorption modifiers (milk and iron)	0.04–0.43 μmol/L (18–75 %)		Granado-Lorencio et al. (2009)
Humans	Broccoli	LUT, βC	7.6–12.6 mg/200 g/day	Modified atmosphere packaging	0.01–0.051 μmol		Granado-Lorencio et al. (2008)
Humans	Tomato, carrot, spinach, lettuce, and wolfberry	LUT, ZEA, αC, βC, LYC, αCX, βCX	>0.75 mg/day	Serving size (one large meal or two small meals)	0.004–0.038 μmol/L		Goltz et al. (2013)
Humans	Tomato, carrot, spinach, lettuce, and wolfberry	LUT, ZEA, αC, βC, LYC	25 mg/serving	Amount and source of triacylglycerols	0.0013–0.04 μmol/L		Goltz et al. (2012)
Rats	Leafy Vegetables (onion, lettuce, spinach, radish, broccoli, etc.)	LUT, ZEA	2.69 mg/kg diet	Food matrix and oil type	4–25 pmol/mL	20–70 pmol/g	Lakshminarayana et al. (2007)

Humans	Curly kale	LUT, βC, LYC, *cis*-LYC	0.003–2.13 mg/3 mL/day	Two oily carotenoid formulations	0.04–0.28 μg/mL	0.14–0.18 a.u.	Meinke et al. (2010)
Rats	Microalgal biomass	LUT, βC, AST	200 μmol/rat/day	Algae varieties (*S. platensis, H. pluvialis and B. braunii*)	∼0.26–0.48 μmol/mL	0.4–0.9 μmol/g	Rao et al. (2013)
Rats	Algae	LUT, βC, AST	200 μmol/rat/day	Algae varieties (*S. platensis, H. pluvialis and B. braunii*)	∼0–0.13 μmol/mL	∼0.02–0.28 μmol/g	Rao et al. (2010)
Humans	Carrot, tomato and papaya	βC, LYC	2.3–16.4 mg	Food matrix	0.058–0.61 μmol/L		Schweiggert et al. (2014)
Humans	Carrots	αC, βC	23.2–24.2 mg/200 g FW	Cultivation (organic and conventional)	∼0.05–0.82 μmol/L		Stracke et al. (2009)
Rats	Carrot puree and natural yoghurt	βC	0.03–0.044 mg/kg	Food matrix	0.0067–0.057 μmol/L	0.2–11.5 nmol/g	Sy et al. (2013)
Rats	Carrots, tomatoes, spinach and salmon	βC, LYC, LUT, AST	0.16–0.33 mg/kg/day	Food matrix	0.0029–0.014 μmol/L	0.1–2.5 nmol/L	Sy et al. (2012)

Note:
BW body weight, *FW* fresh weight, *LUT* lutein, *cis*-LUT lutein isomers, *AST* astaxanthin, *αC* α-carotene, *βC* β-carotene, *cis*-βC β-carotene isomers, *LYC* lycopene, *cis*-LYC lycopene isomers, *αCX* α-cryptoxanthin, *βCX* β-cryptoxanthin

[a] Administered dose is the sum of individual carotenoids

[b] Bioaccessibility corresponds to the minimum and maximum values of individual carotenoids in different treatments

[c] Values in the parenthesis indicate the estimated bioaccessibility (%)

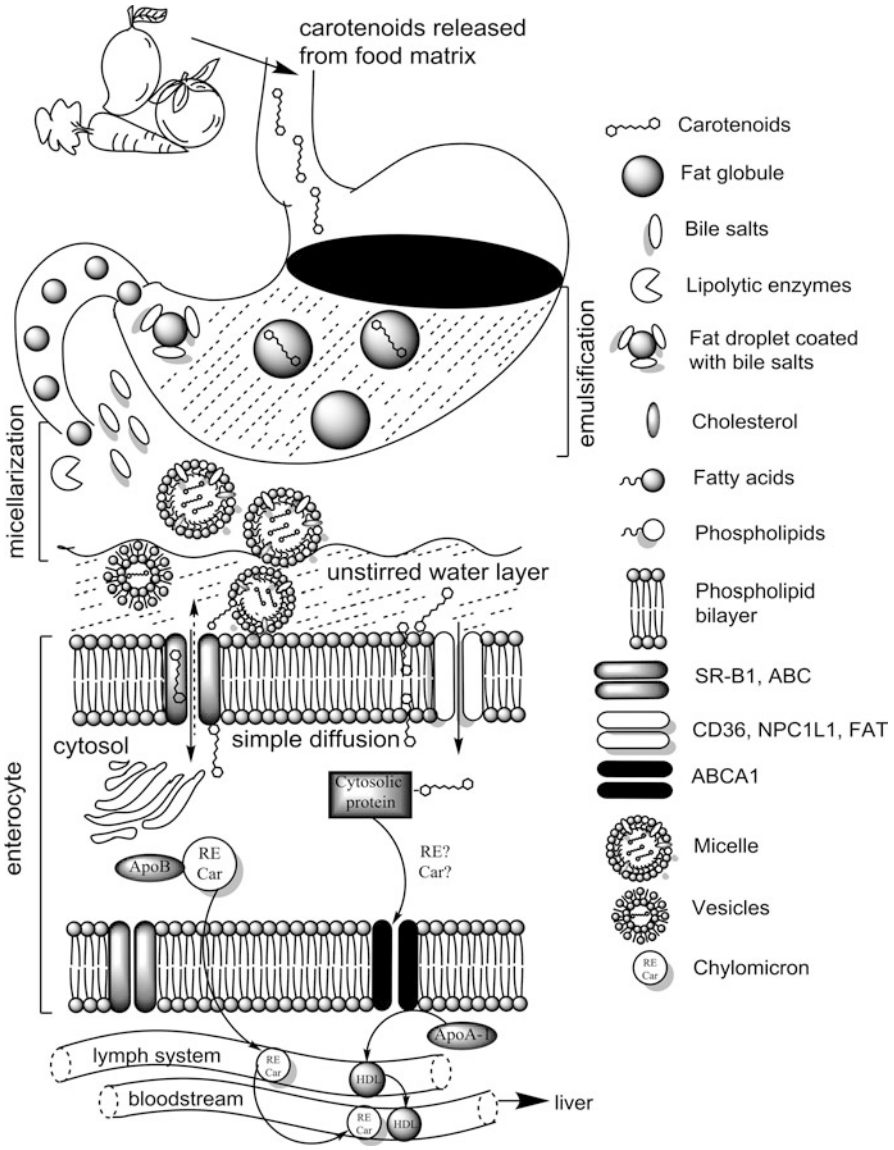

Fig. 16.1 Digestion and absorption processes of carotenoids

and cholesterol) (Guyton and Hall 2001). There is evidence of that some esterified xanthophylls are hydrolyzed by lipolytic enzymes mainly by the carboxyl ester lipase. The lipase and colipase have a minor participation in this regard (Breithaupt et al. 2002; Chitchumroonchokchai and Failla 2006). The micelles size ranges from 4 to 60 nm (Parker 1996; Yonekura and Nagao 2007). The micelles transport

carotenoids through the aqueous intestinal medium and across the unstirred water layer adjacent to the brush border of enterocytes. This layer has an acidic nature, favoring the protonation of fatty acids and release from the micelle. This facilitate the liberation of other lipophilic molecules, such as carotenoids, due to the micelle dissociation (Guyton and Hall 2001; Reboul 2013). Carotenoids may also be transported through the gastrointestinal aqueous media within unilamellar or multilamellar vesicles of phospholipids (Reboul and Borel 2011).

16.2.2 Absorption Process

The first studies about the absorption of carotenoids suggested that they cross the membrane of enterocytes through a passive diffusion process, dependent on the carotenoid concentration (Fig. 16.1) (Hollander et al. 1978; Moore et al. 1996). However, recent studies have suggested that cholesterol transporters are involved in the carotenoid absorption by enterocytes (Borel 2012). Some studies on rats and Caco-2 cells reported that the lipid transporter SR-BI, regulated the absorption of β-carotene, lycopene and lutein (Moussa et al. 2008; Reboul et al. 2005; van Bennekum et al. 2005). This transporter protein also was associated with the preferred absorption of xanthophylls in comparison with β-carotene in human retinal pigment epithelial cells (ARPE-19 cells) (During et al. 2008). Additionally, it has been demonstrated that retinoic acid can repress the expression of the SR-BI transporter through the induction of the intestinal transcription factor ISX (Lobo et al. 2010). Other membrane proteins of intestinal cells, such as CD36 (cluster determinant 36), FAT (fatty acid translocase), NPC1L (Nieman Pick C1-like 1), and the ABCG5/G8 from the ABC transporters superfamily have been associated with the absorption of carotenoids; however, there is not strong evidence about their role in carotenoid transport (Borel 2012; Herron et al. 2006; Reboul 2013; van Bennekum et al. 2005).

Once within enterocytes, the carotenoids are transported to the Golgi apparatus and assembled in nascent chylomicrons, which are secreted into the lymphatic system for their transport in the bloodstream (Parker 1996). The non-provitamin A carotenoids are transported through cytosol of enterocytes by some transporter proteins (Furr and Clark 1997). The CD36 has also been detected in the Golgi apparatus (Reboul and Borel 2011). Other proteins such as NPC1L1, SR-BI, or FABPs (fatty acid-binding proteins) are potentially responsible for the intracellular transport of carotenoids due to they are able to transport different fat-soluble nutrients as well as by their strategic location in endosomes, lysosomes, mitochondria, cytoplasmatic lipid droplets and tubulovesicular membranes (Borel 2012; Reboul 2013). However, further genetic studies are needed to confirm their participation in the transport of carotenoids within enterocytes.

The carotenoids that were not assembled in chylomicrons eventually may return to intestinal lumen (Parker 1996). Transmembrane proteins (SR-BI and ABC

transporters) may also act in the efflux of carotenoids from enterocytes (Reboul 2013). In addition to chylomicrons, it has been suggested that HDL may participate in the transport of carotenoids and retinoids from enterocytes to the lymph. This flux seems to be mediated by an ABCA1 transporter (Reboul 2013; Reboul and Borel 2011). Additionally, it has been reported that the expression of genes involved in lipid absorption, metabolism and transport may be responsible of the inter-individual variation in the content of carotenoids in plasma (Borel et al. 2007; Borel 2012).

Up to date, it is unclear if an exclusive or many proteins participate in the carotenoid absorption in the intestine and other tissues; however, their involvement in the carotenoid absorption process might explain the inter-individual variations in the efficiency of carotenoid absorption, the saturable absorption, the selective absorption and the competition for absorption between different carotenoids (Reboul, 2013). Borel (2012) suggested that passive diffusion might occur with high levels of carotenoids while their protein-mediated transport might take place at dietary doses.

16.2.3 Metabolism

The metabolic fate of carotenoids depends on their chemical structure and nutritional status of individuals. Once within enterocytes, the provitamin A carotenoids are immediately transformed in retinal, mainly by the action of BCO1. The level of bioconversion to vitamin A depends on the nutritional status of the subject. The supplementation with β-carotene may induce carotenoderma without alter the retinol levels in plasma in individual with normal levels of vitamin A (Faulks et al. 1998). Pro- and non-pro-vitamin A carotenoids may be eccentrically cleavage by BCO2 enzymes or by auto-oxidation processes, resulting in the formation of volatile and non-volatile oxygenated cleavage products of carotenoids (Caris-Veyrat 2010). Kiefer et al. (2001) identified the β,β-carotene-9′,10′-oxygenase (BCO2), which produce β-apo-10′-carotenal and β-ionone. The apo-carotenals may be converted in retinaldehyde or β-apo-carotenoic acids. The β-carotenoic acids may be precursors of retinoic acid (Failla and Chitchumroonchokchai 2005; Wang 2012). Ho et al. (2007) found β-apo-8′-carotenals in plasma of a healthy man after the supplementation with *all-trans*[10,10′,11,11′-^{14}C]-β-carotene. Traces amounts of β-apo-carotenals (8′,10′,12′,14′) have been reported in rat intestines (Barua and Olson 2000). Some eccentric cleavage products from lycopene have been found in rats supplemented with this carotene. Gajic et al. (2006) identified apo-8′-lycopenal and apo-12′-lycopenal as metabolites of lycopene in rat livers. Also, apo-10′-lycopenol was reported in lungs from ferrets after the consumption of high lycopene diets (Hu et al. 2006). More recently, Kopec et al. (2010) identified apo-6′, 8′, 10′, 12′, and 14′-lycopenals in plasma of humans after eight weeks of daily consumption of tomato juice; however, they could not differentiate between metabolized and

ingested lycopenals. Other possible metabolites derived from carotenoid oxidation have been identified in human plasma. Khachik et al. (1992) suggested that the presence of ketocarotenoids in human plasma could be produced by the oxidation, reduction and double bond migration of lutein and zeaxanthin. In another study, it was suggested that the presence of capsanthone in plasma from subjects who had been ingested paprika juice was a consequence of the oxidation of capsanthin (Etoh et al. 2000). The preferential rupture of non-provitamin A carotenoids by BCO2 enzymes may be explained by variations in the intracellular localization of BCO1 (cytosolic) and BCO2 (inner mitochondrial membrane) and the specific carotenoids storage sites within cells (Palczewski et al. 2014).

The metabolism of carotenoids in the body is unclear and subject of debate since these molecules are highly susceptible to oxidation. The oxidation products of carotenoids can be generated during the extraction processes, during gastrointestinal digestion or be natural components of foods. Regardless the source, the non-volatile apo-carotenoids and apo-lycopenoids and the volatile β-ionone products have shown chemopreventive activities such as the inhibition of cell growth, stimulation of cell differentiation, activation of nuclear receptors, antagonize nuclear receptor activation and induction of apoptosis (Wang 2012).

16.3 Bioaccessibility

Given the positive associations between the high consumption of fruits and vegetables and the low incidence of different diseases, many researchers have tried to explain the protective effects of carotenoids from dietary sources. Only the carotenoids delivered into the target organ can exert biological actions. This fraction of carotenoids has been estimated, with limitations, by the measurement of their bioaccessibility. The bioavailability of carotenoids is defined as the fraction of consumed carotenoids that can be absorbed, transported, stored and/or employed in the normal biological functions. This is a complex measurement. The term 'bioaccessibility' refers to the fraction of dietary carotenoids that is liberated from the food matrix during digestion, transferred into mixed micelles and/or absorbed by enterocytes and delivered in the blood stream. Only this carotenoid fraction is available to be used or stored by the body (Fernández-García et al. 2012). Several *in vivo* and *in vitro* methods have been used to estimate the bioaccessibility and bioavailability of carotenoids. The *isotope method* consists in the administration of physiologic doses of carotenoids labeled with stable isotopes (^{2}H, ^{13}C, ^{14}C). This method provides the most reliable estimations about the absorption and metabolism of carotenoids, allowing the discrimination of newly absorbed and existing carotenoids and carotenoid metabolites (Failla and Chitchumroonchokchai 2005; van den Berg et al. 2000; van Lieshout et al. 2003). This method has been employed to estimate the absorption of β-carotene, α-carotene and β-cryptoxanthin from yellow and green leafy vegetables, and has been very useful to estimate

the bioconversion efficiency of β-carotene into vitamin A (Ribaya-Mercado et al. 2007; Tang et al. 2003; van Lieshout et al. 2003). However, this method is expensive. The *metabolic balance* technique involves the quantification of carotenoids in the ingested meal and excreted faeces, assuming that the difference represent the amount of absorbed carotenoids (Failla and Chitchumroonchokchai 2005; van Lieshout et al. 2003). Some variants of this method are the g*astrointestinal lavage* and the *ileostomy balance* methods (van den Berg et al. 2000). The *ileostomy balance* involves the recuperation of digesta from ileostomic patients. This method offers advantages over the other balance methods since in such method the carotenoids are not exposed to the degradative colonic microflora. Additionally, the absorption results are comparable to those obtained measuring the plasma response of carotenoids (Failla and Chitchumroonchokchai 2005; van den Berg et al. 2000; van Lieshout et al. 2003). The balance methods have been used to evaluate the effects of food processing (Livny et al 2003; Unlu et al 2007) and dietary fat on carotenoid absorption (Faulks et al 1997; van Loo-Bouwman et al 2010). The quantitative *plasma response* of carotenoids after feeding carotenoid-rich foods is other method that has been used to estimate the bioavailability and bioaccessibility of carotenoids. In this method, the plasmatic carotenoids are commonly monitored during periods that vary from 12 h to weeks (Failla and Chitchumroonchokchai 2005; van den Berg et al. 2000). This technique does not allow the discrimination of newly absorbed carotenoids from those already existing in the blood stream. It assumes that different carotenoids have similar and static rates of clearance from plasma (Failla and Chitchumroonchokchai 2005). This is the most common method employed to estimate the carotenoid bioavailabity and bioaccessibility (Table 16.1).

The bioaccessibility of carotenoids may be estimated by *in vitro* methods. These techniques include the simulation of the gastrointestinal digestion and their further uptake by Caco-2 cells. The *in vitro* methods are particularly used to determine the transference efficiency of carotenoids to micelles. Only micellarized carotenoids can be absorbed (Guyton and Hall, 2001; Failla and Chitchumroonchokchai 2005; Tyssandier et al. 2003). Reboul et al. (2006) reported significant correlations between the *in vitro* bioaccessibility of carotenoids, measured as their micellarization efficiency, and their plasma response in humans. Most of the observed trends of carotenoid micellarization as a function of different factors coincide with those observed under *in vivo* conditions. This is true for factors like the isomeric form of carotenoids, polarity differences, chromoplast morphology in the food matrix and dietary sources (Failla et al. 2014; Goltz et al. 2012; Reboul et al. 2006; Schweiggert et al. 2012, 2014). Thus, the estimation of the micellarization by *in vitro* methods has been considered as valid method to estimate the potential absorption of carotenoids.

The bioaccessibility of carotenoids depends on many factors involved in key stages of their digestion and intestinal absorption, including their liberation from the food matrix, the emulsification of lipids (lipolysis, viscosity, lipid droplet size), micellarization, absorption by enterocytes and secretion into the lymph. The bioaccessibility of carotenoids from common foods is highly variable (Tables 16.1 and 16.2) and depends on many factors.

Table 16.2 Overview of recent *in vitro* studies about bioaccessibility of carotenoids

Model	Carotenoid source	Studied carotenoids	[a]CCTF (Amount of food in digestion)	Tested factors	[b]Bioaccessibility		References
					Micellarization	Uptake (Caco-2)	
Simulated GI digestion	Tomato	βC, LUT, LYC	1.8–8.4 mg/100 g (2.0 g)	Geographical origin and tomato type	0.1–127 %		Aherne et al. (2009)
Simulated GI digestion	Orange and juice	αC, βC, LUT, βCX	0.18–0.27 mg/100 g FW (5 g)	Processing (Orange: chopped and homogenized. Juice: fresh, flash-pasteurized and pasteurized)	7–48 %		Aschoff et al. (2015)
Simulated GI digestion and Caco-2 cells	Spinach	βC, LUT	33.7 μmol/L (4.0 g)	Effect of divalent ions (Ca, Mg, Zn, Fe)	∼0.01–68 %	∼1.3–44.5 pmol/mg cell	Biehler et al. (2011)
Simulated GI digestion	Orange-fleshed sweet potato	βC, *cis*-βC	30.5–34.4 mg/100 g DW (3.0 g)	Thermal processing (fresh, boiling, homogenization and cooking) and fat	16–70 %		Bengtsson et al. (2010)
Simulated GI digestion	Orange-fleshed sweet potato	βC, *cis*-βC	6.1–9.7 mg/100 g DW (3.0 g)	Mechanical (cylinders and slices) and thermal (boiling, steaming, microwave heating) processing and fat	0.5–56 %		Bengtsson et al. (2009)
Simulated GI digestion	Yellow potatoes	LUT, ZEA	0.33–1.35 mg/100 g FW (0.5 g)	Different accessions	33–71 %		Burgos et al. (2013)
Simulated GI digestion	Watermelon and guava	βC, LYC, LUT	3.9–4.9 mg/100 g FW (0.5 g)	Food matrix	21–73 %		Chandrika et al. (2009)

(continued)

Table 16.2 Overview of recent *in vitro* studies about bioaccessibility of carotenoids

Model	Carotenoid source	Studied carotenoids	[a]CCTF (Amount of food in digestion)	Tested factors	[b]Bioaccessibility		References
					Micellarization	Uptake (Caco-2)	
Simulated GI digestion	Fruit juices	ZNX, βC, NEO + *cis*-VIO, ZEA, LUT, βCX	0.024–0.22 mg/100 mL (80 g)	Processing (thermal and high pressure) of whole, skimmed, and soy milk beverages	4–149 %		Cilla et al. (2012)
Simulated GI digestion	Tomato pulp	LYC	(5.0 g)	Processing and fat type (coconut, olive and fish oil)	2–18 %		Colle et al. (2013)
Simulated GI digestion	Tomato pulp	LYC	4.0–5.4 mg/100 g (5.0 g)	Fat type (cocoa butter, coconut, palm, olive, sunflower, and fish oils)	1–5 %		Colle et al. (2012)
Simulated GI digestion	Tomato pulp	LYC, *cis*-LYC	94.8–130.0 mg/100 g DW (5.0 g)	Thermal processing at different temperatures	∼12–36 %		Colle et al. (2010)
Simulated GI digestion	Basil, coriander, dill, mint, parsley, rosemary, sage, tarragon	βC, βCX, ZEA + LUT	2.0–25.8 mg/100 g (2.0 g)	Food matrix	0–27 %		Daly et al. (2010)
Simulated GI digestion, synthetic micelles and Caco-2 cells	Citrus juices (orange, mandarin and lemon juices)	βC, βCX, βCX-esters	0.12–1.64 mg/100 mL (20 g)	Food matrix	∼15–40 %	0.6–21 %	Dhuique-Mayer et al. (2007)
Simulated GI digestion	Bananas and plantains	αC, βC, *cis*-βC	0.13–22 mg/kg (5.0 g)	Boiled fruits and dishes from plantain and banana	0–34 %		Ekesa et al. (2012)

Simulated GI digestion and Caco-2 cells	Salad (tomato, carrot, spinach, lettuce, wolfberry)	αC, βC, LYC, LUT, ZEA	9.1 mg/100 g (2.4 g)	Fat type (butter, olive, canola, and soybean oils)	0–57 %	1.5–35.0 pmol/mg protein	Failla et al. (2014)
Simulated GI digestion and Caco-2 cells	Melon	βC	1.3–2.3 mg/100 g FW	Orange-fleshed honeydew melon	3.2 %	11.6 %	Fleshman et al. (2011)
Simulated GI digestion	Yellow-fleshed cassava roots	βC	1.1–2.4 mg/100 g DW	Cooking process (boiling and frying)	5–14 %		Gomes et al. (2013)
Simulated GI digestion	Lutein-fortified milk	LUT	4–8.2 mg/100 mL (5.0 mL)	Dose levels (low and high)	∼4–12 %		Granado-Lorencio et al. (2010)
Simulated GI digestion	Fruit juice	LUT, ZEA, βCX, βC	0.11–0.23 mg/100 mL	Absorption modifier (milk and iron)	18–75 %		Granado-Lorencio et al. (2009)
Simulated GI digestion	Peppers (Arbol, Chipotle, Guajillo and Morita)	βC, βCX, ZEA	87.6–373.3 mg/100 g DW (0.3 g)	Varieties	20–49 %		Hervert-Hernández et al. (2010)
Simulated GI digestion	Carrots	βC	196.6 mg/100 g DW	Drying temperature (70, 80, and 90 °C)	13–73 %		Hiranvarachat et al. (2012)
Simulated GI digestion	Carrots	αC and βC	(5.0 g)	Cooking and addition of oil at different levels	29–80 %		Hornero-Méndez and Mínguez-Mosquera (2007)
Simulated GI digestion	Squash, carrot grapefruit, mango, melon papaya, sweet potato, tomato, watermelon	αC, βC, LYC, LUT, VIO, PE	1.33–45.1 mg/100 g FW (5 mL)	Food matrix	0–96 %		Jeffery et al. (2012)

(continued)

Table 16.2 Overview of recent *in vitro* studies about bioaccessibility of carotenoids

Model	Carotenoid source	Studied carotenoids	[a]CCTF (Amount of food in digestion)	Tested factors	[b]Bioaccessibility		References
					Micellarization	Uptake (Caco-2)	
Simulated GI digestion	Carrots	βC and *cis*-βC	126–158 mg/100 g DW (5.0 g)	Thermal and mechanical processing (high pressure homogenization, thermal or high pressure pasteurization) and addition of olive oil	~10–36 %		Knockaert et al. (2012a)
Simulated GI digestion	Tomatoes	LYC	(5.0 g)	Thermal and high pressure processing	~50–97 %		Knockaert et al. (2012b)
Simulated GI digestion	Carrots	βC		Cooking (gently and intensely cooked) and mechanical processing (cell breakage and separation)	~0–19 %		Lemmens et al. (2010)
Simulated GI digestion	Spinach, komatsuna, pumpkin, and carrot	βC, LUT	(1.5 g)	Food matrix and individual fats and oils	~6–80 %		Nagao et al. (2013)
Simulated GI digestion	Orange, kiwi, red grapefruit, honeydew melon, spinach, broccoli, red pepper, sweet potato	βC, LYC, LUT, βCX, ZEA	(2.0 g)	Food matrix	2–109 %		O'Connell et al. (2007)
Simulated GI digestion and Caco-2 cells	Mango	βC	1.1–3.9 mg/100 g (1.5 g)	Ripening stage, dietary fat and pectin concentration	4.5–40 %	13.8–19.6 pmol/mg cell	Ornelas-Paz et al. (2008)

Simulated GI digestion and Caco-2 cells	Bell and chili peppers	βC, LUT, βCX, ZEA	1.98–44.5 mg/100 g (∼2.0 g)	Ripening stages and varieties	6–113 %	1–32 %	O'Sullivan et al. (2010)
Simulated GI digestion	Orange carrots, red carrots, red tomatoes and atomic red carrots	βC, LYC	0.8–8.5 mg/100 g	Matrix and thermal processing (water bath 65–95 °C; oil bath 95–125 °C)	1–38 %		Palmero et al. (2014)
Simulated GI digestion	Red, orange and yellow tomatoes	LYC, ζC, LUT	0.07–4.6 mg/100 g (5.0 g)	Food matrix and high pressure homogenization (20, 50, 100 MPa)	7–96 %		Panozzo et al. (2013)
Simulated GI digestion	A juice with orange, kiwi, pineapple, and mango	αC, βC, *cis*-VIO+ NEO, *cis*-ANT, ANT, LUT, αCX, βCX	0.031–0.64 mg/100 mL (200 mL)	Beverage formulation (with or without milk)	8–27 %		Rodríguez-Roque et al. (2014)
Simulated GI digestion	A juice with orange, kiwi, and pineapple	αC, βC, *cis*-VIO+ NEO, *cis*-ANT, ANT, LUT, αCX, βCX	0.54 mg/100 mL (200 mL)	Beverage formulation	8–17 %		Rodríguez-Roque et al. (2013)
Simulated GI digestion and Caco-2 cells	Courgette, red pepper and tomato	βC, LYC, LUT, βCX	(2.0 g)	Cooking procedures (boiling, grilling, microwave-cooking, and steaming) and food matrix	2–106 %	∼7–35 %	Ryan et al. (2008)

(continued)

Table 16.2 Overview of recent *in vitro* studies about bioaccessibility of carotenoids

Model	Carotenoid source	Studied carotenoids	[a]CCTF (Amount of food in digestion)	Tested factors	[b]Bioaccessibility		References
					Micellarization	Uptake (Caco-2)	
Simulated GI digestion	Carrot, mango, papaya, and tomato	αC, βC, LYC, LUT, βCX, βCX-esters	0.3–4.44 mg/100 g (10 g)	Food matrix and dietary fat (with or without oil)	~0.4–15 %		Schweiggert et al. (2012)
Simulated GI digestion and Caco-2 cells	Marine spore-forming *Bacillus* sp., carrots, purified βC	βC	30–44.2 μg/digestion	Food matrix (purified βC, carrots and bacteria)	13–60 %	6–11 %	Sy et al. (2013)
Simulated GI digestion and Caco-2 cells	Purified carotenoids, carrots, tomatoes, spinach and salmon	βC, LYC, LUT, AST	30–215 μg/digestion	Food matrix	1–50 %	7–11 %	Sy et al. (2012)
GI digestion simulated	Cassava	βC, *cis*-βC	2.8–4.09 mg/100 g DW (0.3 g)	Style of processing (raw, boiled, Gari and Fufu)	~12–30 %		Thakkar et al. (2009)
GI digestion simulated	Jalapeño peppers	NEO, NCR, VIO, LTX, CAP-epox, αC, βC, CAP, CAP-esters, βCX, βCX-esters, ZEA-esters	1.4–22.6 mg/100 g DW (2.0 g)	Processing style (boiling and grilling), ripening stage (green and red), and fat type (saturated and unsaturated)	0–308 %		Victoria-Campos et al. (2013b)

GI digestion simulated	Jalapeño peppers	NEO, NCR, VIO, LTX, CAP-epox, αC, βC, CAP, CAP-esters, βCX, βCX-esters, ZEA-esters	5.4–22.5 mg/100 g FW (2.0 g)	Processing style ripening stage and fat type	2–349 %		Victoria-Campos et al. (2013a)
Simulated GI digestion and Caco-2 cells	Purified carotenoids	βC, LUT	200 nmol/L (15 mg)	Fiber type (alginate, apple and citrus pectin)	41–99 %	0.18–0.41 pmol/μg protein	Yonekura and Nagao (2009)

FW fresh weight, *DW* dry weight, *LUT* lutein, *VIO* violaxanthin, *cis*-VIO violaxanthin isomers, *ANT* antheraxanthin, *cis*-ANT antheraxanthin isomers, *AST*astaxanthin, *NEO* neoxanthin;, *ZNX* zeinoxanthin, *αC* α-carotene, *βC* β-carotene, *cis*-βC β-carotene isomers, *ζC* ζ-carotene, *LYC* lycopene, *cis*-LYC lycopene isomers, *PE* phytoene, *PF* phytofluene, *NCR* neochrome, *LTX* luteoxanthin, *αCX* α-cryptoxanthin, *βCX* β-cryptoxanthin, *βCX-esters* β-cryptoxanthin-esters, *CAP* capsanthin, *CAP-epox* capsanthin 5,6-epoxide, *CAP-esters* capsanthin-esters, *ZEA* zeaxanthin, *ZEA-esters* zeaxanthin-esters

[a] *CCTF* Carotenoid content in tested foods, estimated as the sum of individual carotenoids

[b] Bioaccessibility corresponds to the minimum and maximum values of individual carotenoids in different treatments

16.3.1 Food Matrix

The effect of the food matrix includes the combined effects of all factors from a food that simultaneously promote or reduce the bioavailability/bioaccessibility of carotenoids (Ornelas-Paz et al. 2008). The food matrix effect includes differences in the composition and storage sites of carotenoids as well as changes in the food by ripening and processing.

16.3.1.1 Chromoplast Morphology

The bioaccessibility of carotenoids from fruits is significantly higher than that of vegetables (de Pee et al. 1998). This effect has been associated to the differential physical disposition of carotenoids within chromoplasts. Typically, carotenoids are stored as a lipid solution in globular and tubular chromoplasts of mature fruits; however, they can also be accumulated as crystalline structures. They may be complexed with proteins in chloroplasts of green vegetables (Schweiggert et al. 2012; Vásquez-Caicedo et al. 2006). During digestion, lipid bodies rich in carotenoids from fruits may easily interact with the lipidic phase of the gastrointestinal content, making them more bioaccessible and bioavailable (West and Castenmiller 1998). In contrast, crystalline forms are not completely dissolved during their transit through the gastrointestinal tract (de Pee et al. 1998). The *in vitro* and *in vivo* bioaccessibility of β-carotene from different sources followed the order of mango > papaya > tomato > carrot and this order was explained in terms of differences in the chromoplast morphology and accumulation forms of carotenoids in fruits and vegetables (Schweiggert et al. 2012, 2014). The difference in β-carotene bioaccessibility from mango and papaya could be consequence of differences in the presence of carotenoids in liquid-crystalline stores in the chromoplasts of these foods. The results for tomato could not be explained in these terms. Ornelas-Paz et al. (2010) also demonstrated in rats that β-carotene from mango was two times more bioavailable than from carrots. Carrillo-Lopez et al. (2010) reported that the levels of hepatic retinol in rats depended on the source of β-carotene, following the order of mango > carrot > spinach > parsley.

16.3.1.2 Ripening

Ripening modifies the amount and type of carotenoids in fruits and vegetables. The chloroplasts of green vegetables and immature fruits mainly contain lutein, β-carotene, violaxanthin and neoxanthin. During ripening, the chloroplasts are transformed in chromoplasts with an increased biosynthesis of carotenoids (Rodriguez-Amaya and Kimura 2004; Yahia and Ornelas-Paz 2010). In some cases, the carotenoids of chloroplast serve as precursor of other carotenoids during ripening (Cervantes-Paz et al. 2012, 2014). The increase of total carotenoids during

the ripening has been reported for different fruits and vegetables. The fully ripe mango, endive and lettuce have from 2.5 to 4 times more total carotenoids than the slightly ripe fruits or young leaves (Azevedo-Meleiro and Rodriguez-Amaya 2005b; Ornelas-Paz et al. 2008). The carotenoid content in red peppers (Jalapeño, Agridulce, Bola, Szentesi Kosszarvú Paprika) is 11 to 85 times higher than in green peppers (Cervantes-Paz et al. 2012; Deli et al. 1996; Mínguez-Mosquera and Hornero-Méndez 1994b). Of course, this behavior is not observed in all vegetable foods, as occur in for young and mature leaves of kale or spinach (Azevedo-Meleiro and Rodriguez-Amaya 2005a, b). These qualitative and quantitative changes may influence the carotenoid bioaccessibility. Thakkar et al. (2007) reported a positive correlation between the content of β-carotene in cassava and its efficiency of micellarization and uptake by Caco-2 cells.

The esterification of xanthophylls during ripening has been reported in peppers, sea buckthorn berries, bananas, kiwis, among others (Andersson et al. 2009; Cervantes-Paz et al. 2012, 2014; Mínguez-Mosquera and Hornero-Méndez, 1994a; Montefiori et al. 2009). The esterification of carotenoids reduces their polarity and bioaccessibility. After *in vitro* digestions of citrus juices, the micellarization of free β-cryptoxanthin was three times higher than that of the monoesterified forms (Dhuique-Mayer et al. 2007). The *in vitro* micellarization of free zeaxanthin was about two and seven times higher than that of mono and diesterified forms in wolfberry, orange pepper, red pepper and squash (Chitchumroonchokchai and Failla 2006). These tendencies were also seen for the uptake by Caco-2 cells in both studies. Victoria-Campos et al. (2013a, b) reported that the micellarization of free and esterified forms of capsanthin, antheraxanthin, mutatoxanthin, and zeaxanthin also followed the order of free > monoesterified > diesterified forms after *in vitro* digestions of raw or heat-processed red peppers. The study of the *in vitro* bioaccessibility of different monoesterified forms of capsanthin and β-cryptoxanthin suggests that their micellarization is influenced by the polarity provided by the fatty acid bounded to the carotenoid backbone, following an order of micellarization efficiency of laurate > myristate > palmitate (Dhuique-Mayer et al. 2007; Victoria-Campos et al. 2013a, b). However, Breithaupt et al. (2003) demonstrated that free and esterified forms of β-cryptoxanthin showed similar *in vivo* bioaccessibility. The absence of esterified carotenoids in human plasma after the consumption of fruits rich in carotenoid esters suggests that only free forms are absorbed or that some esterases cleavage carotenoid esters (Granado et al. 1998; Wingerath et al. 1995). Further studies about the digestion, absorption and metabolism of esterified carotenoids are needed.

Ripening also cause fruit softening, which involve the solubilization, depolymerization and demethylation of pectins from cell walls (de Roeck et al. 2008; Gross and Sams 1984; Redgwell et al. 1997). Pectins and other fibers could alter the emulsification of lipids in the gastrointestinal medium and their further hydrolysis (Pasquier et al. 1996). These fibers are also able to interact with bile salts, disturbing the micellarization processes (Dongowski et al. 1996; Pasquier et al. 1996). Ornelas-Paz et al. (2008) demonstrated that the bioaccessibility of β-carotene was significantly enhanced by the ripening of mango. This effect was

associated with the quantitative and qualitative ripening-related changes of mango pectin. Victoria-Campos et al. (2013a, b) reported that ripening of peppers did not affect the micellarization of free carotenoids; however, the ripening stage of fruits determined the number and micellarization efficiency of esterified xanthophylls. The information about the effect of ripening on the bioaccessibility of carotenoids is scarce; however, some studies suggest that qualitative and quantitative changes of the intrinsic pectin substances during fruit ripening play an important role. Cell wall composition and metabolism vary widely between plant foods.

16.3.1.3 Heat Processing

Heat processing reduces the negative effects of food matrix on carotenoid bioaccessibility and bioavailability (Ornelas-Paz et al. 2008; Yahia & Ornelas-Paz 2010). The heat processing causes the disruption of food matrix, loss of cellular integrity and breaking of protein-carotenoid complexes. These effects may increase the carotenoid extractability during digestion and their further bioaccessibility and bioavailability (Yahia and Ornelas-Paz 2010). The softening of fruits by the heat processing has been associated with the solubilization, depolymerization and demethylation of pectins (de Roeck et al 2008; Ramos-Aguilar et al. 2015; Sila et al. 2006). These heat processing mediated effects in the food matrix may vary as a function of time, intensity and type of processing. The bioaccessibility of lycopene from tomato pulp increased as the heat processing temperature rose from 60 to 140 °C, with the bioaccessibility of *cis*- and *trans*-lycopene being almost 2 times higher in puree treated at 140 °C, as compared with raw samples (Colle et al. 2010). The bioaccessibility of carotenes from carrots was 80 and 57 % greater after cooking (100 °C, 10 min) and blanching (80 °C, 10 min), respectively, as compared with raw samples (Netzel et al. 2011). Lemmens et al. (2009) reported that the bioaccessibility of β-carotene from carrots increased as the duration and temperature of heat processing increased from 0 to 50 min and from 90 to 110 °C, respectively. The heat-processing style also alters the carotenoid bioaccessibility. Bengtsston et al. (2009) reported that the bioaccessibility of β-carotene from carrots was almost 50 % lower after microwave heating in comparison with boiling and steaming. Ryan et al. (2008) demonstrated that the bioaccessibility of β-carotene from boiled courgette, red pepper and tomato was higher than that of the raw, grilled, microwaved and steamed foods. Similar tendencies were observed for lutein. The bioaccessibility of lycopene from courgette was increased by grilling and microwaving. Contrarily, these treatments hindered the bioaccessibility of β-cryptoxanthin from all evaluated fruits (Ryan et al. 2008). Victoria-Campos et al. (2013b) reported that heat processing decreased the bioaccessibility of many free and esterified carotenoids from red peppers. These studies suggest that the effect of heat processing on carotenoid bioaccessibility depends on carotenoid type and plant food.

The heat processing also alters the qualitative and quantitative profile of carotenoids. These pigments are highly thermolabile. Heat processing may

induce the *trans* to *cis* isomerization, epoxidation and degradation of carotenoids (Cervantes-Paz et al. 2012, 2014; Rodriguez-Amaya 1999). The micellarization of 13-*cis* and 9-*cis* isomers of β-carotene is higher than that of the form all-*trans* (Bengtsson et al. 2009; Bechoff et al. 2009; Ekesa et al. 2012; Tyssandier et al. 2003; Victoria-Campos et al. 2013b). The micellarization and uptake of *cis*-lycopene by Caco-2 cells was also higher than that of all-*trans*-lycopene (Failla et al. 2008). Accordingly, the greater bioaccessibility of *cis*-lycopene in comparison with the all-*trans* isomer has also been reported *in vivo* (Boileau et al. 1999; Cooperstone et al. 2015; Stahl and Sies 1992). This phenomenon might be explained in terms of the higher solubility of the *cis* isomers of carotenoids due to the bent backbone. This might favor their transference to the micelles (Yahia and Ornelas-Paz 2010). There is scarce information about the bioaccessibility of carotenoid epoxides. It has been suggested that they are not absorbed in humans (Stinco et al. 2012). Recently, Victoria-Campos et al. (2013a, b) reported the formation of capsanthin 5,6-epoxide in Jalapeño peppers as a consequence of heat processing and demonstrated that this compound was efficiently micellarized. However, Asai et al. (2008) reported that although some epoxyxanthophylls (neoxanthin and fucoxanthin) are efficiently micellarizaced, their concentration in plasma (about 1 nmol/L) does not increase after the consumption of foods containing these carotenoids.

16.3.1.4 Mechanical Processing

The release of carotenoids from the food matrix is directly enhanced by the rupture of cells and cellular compartments before the consumption of a plant food. van het Hof et al. (2000) reported that the concentration of lycopene in the triglyceride-rich lipoprotein fraction of plasma increased 32 and 62 % after the ingestion of mildly and severely homogenized tomato products, respectively, as compared with the consumption of non-homogenized tomatoes. The concentration of β-carotene in this plasma fraction increased 5.6 and 8.2 times as a consequence of these homogenization levels. Livny et al. (2003) also demonstrated the β-carotene bioaccessibility from carrots puree is 50 % greater than that of chopped carrots. Castenmiller et al. (1999) reported that the estimated relative bioavailability of β-carotene increased 86 % after the consumption of liquefied spinach in comparison to whole leaf in healthy subjects. Recently, Aschoff et al. (2015) found that the *in vitro* micellarization of lutein, β-cryptoxanthin, α-carotene, and β-carotene increased from 1.3 to 3.5 times when the orange segments were replaced by orange juice in the digestive reactions. These studies collectively indicate that homogenization style alters the bioaccessibility of carotenoids. The new homogenization technologies such as high-pressure homogenization did not represent an advantage on the bioaccessibility of lycopene from tomato pulp (Colle et al. 2010). Apparently, this technology induces a fiber network that entraps lycopene.

16.3.2 Dietary Fat

Dietary fat is the main effector of carotenoid bioaccessibility because it can mediate all processes involved in carotenoid absorption. Dietary fat promotes the carotenoid diffusion from the food matrix to the emulsified content of the gastrointestinal tract. It stimulates the secretion of acid in the stomach and of bile salts and pancreatic enzymes at the duodenum, facilitating the micelle formation. Hydrolyzed lipids may compete with carotenoids for the transporter proteins during absorption. Dietary fat promotes the chylomicron secretion and consequently the bioaccessibility of carotenoids (Guyton and Hall 2001; Yahia and Ornelas 2010). Recently, Failla et al. (2014) reported that the secretion of carotenoids (lutein and β-carotene) into the basolateral medium was positively associated with the apical concentration of fatty acids (0.5–2.0 mmol/L) in Caco-2 cell cultures. The required levels of dietary fat to enhance the micellarization of carotenoids differ for different fat types, polarity of carotenoids and food matrix.

To date, there is not an exact recommendation about the amount of fat that is required to obtain a good carotenoid absorption. *In vivo* studies have demonstrated that low levels (6–12 g) of dietary fat in a meal are enough to enhance the carotenoid concentration in plasma (Brown et al. 2004; Unlu et al. 2005). Fat contents (avocado, canola oil, soybean oil or butter) of 12 and 28 g in a meal seem to be optimal to get the highest possible plasma levels of carotenoids (α- and β-carotene, lutein, zeaxanthin, and lycopene) when these came from raw salads (Brown et al 2004; Goltz et al. 2012; Unlu et al. 2005). Roodenburg et al. (2000) did not find differences in the plasma concentration of α-carotene and β-carotene after the consumption of a hot meal with 3 and 36 g of dietary fat, but lutein in plasma increased with the highest level of fat, which could occur because of the meal contained esterified lutein. The optimal amount of dietary fat for the maximal absorption of carotenoids likely depends on the food matrix and type of carotenoid in a meal. This might explain the variability in results from different studies.

Results from *in vitro* studies suggest that a percentage of fat close to 10 is enough to get a good carotenoid micellarization. Failla et al. (2014) demonstrated that the micellarization efficiency of zeaxanthin, α-carotene, β-carotene and lycopene from a salad puree was higher when the amount of soybean oil was increased from 1–3 to 8 % (Failla et al. 2014). The micellarization of β-carotene from carrots increased slightly (∼6–17 %) when the digestion reaction contained 5 % of olive oil, as compared with digestions without fat; however, this variable dramatically increased (54–117 %) when the amount of fat was increased to 10 % (Honero-Méndez and Mínguez-Mosquera 2007).

There is an effect of fat type on carotenoid bioaccessibility and bioavailability, but it is not completely understood (Colle et al. 2012). Some studies have demonstrated that monounsaturated and polyunsaturated fatty acids promote the carotenoid bioaccessibility in comparison with saturated fatty acids. Accordingly, Goltz et al. (2012) observed higher levels of carotenoids (lutein, zeaxanthin, α-carotene, β-carotene and lycopene) in human plasma after the consumption of a carotenoid-rich

meal with canola oil (rich in C18:1) and soybean oil (rich in C18:2) than with butter (rich in C16:0 and C14:0). Failla et al. (2014) also reported that the micellarization, uptake by Caco-2 cells, and basolateral secretion of lutein, zexanthin, α-carotene, β-carotene and lycopene were enhanced by the addition of soybean, canola and olive oils in comparison with butter. However, highly unsaturated fatty acids (C18:3, C20:4) may decrease the bioaccessibility of carotenoids (Nagao et al. 2013).

Additionally, the fatty acid chain length may also influence carotenoid micellarization. Huo et al. (2007) found that the micellarization of α-carotene and β-carotene from a salad puree was positively associated with the chain length of fatty acids in the order of C18:1 > C8:0 > C4:0. Nagao et al. (2013) also reported higher micellarization of β-carotene from spinach with oleic acid (C18:1) than fatty acids with medium-chain lengths (C6-C10). On the other hand, the increase of the carotenoid bioaccessibility as a function of fat type is clear for carotenes, lycopene and esterified xanthophylls but not for free xanthophylls (Failla et al. 2014; Gleize et al. 2013; Huo et al. 2007; Roodenburg et al. 2000; Victoria-Campos et al. 2013a, b).

From these studies the positive effect of dietary fat on the bioaccessibility of carotenoids is evident. Apparently, mono- and di-unsaturated fatty acids with long-chain (C:18) induce greater favorable effects than saturated fatty acids with medium-chain lengths. However, further studies are needed to explain the mechanisms of dietary fat on the micellarization and absorption processes for different carotenoids.

16.3.3 Dietary Fiber

The dietary fiber plays an important role in the bioaccessibility of carotenoids. Different fibers (pectin, guar gum, alginate, cellulose or wheat bran) are able to reduce (33–47 %) the plasma concentration of dietary carotenoids (β-carotene, lycopene, lutein), with major decreases being observed with water-soluble fibers (Riedl et al. 1999). Among dietary fibers, pectin is one the most abundant polysaccharide in plant cell walls. It's chemical properties have been strongly associated with textural and firmness changes of different fruits (Gross and Sams 1984; Ramos-Aguilar et al. 2015; Van Buren 1979). Pectin may affect the viscosity of the gastrointestinal content, the lipid droplet size, the availability of bile salts and the enzymatic lipolysis of triglycerides (Pasquier et al. 1996). Rock and Swendseid (1992) reported that citrus pectin reduced the plasma response of β-carotene in more than 50 % as compared with the consumption of the test meal without pectin. In another study, the bioaccessibility of pure β-carotene was significantly reduced by the addition of pectin from mangoes, with the largest decrease being found with pectins from slightly ripe mangoes in comparison with pectins from fully ripe fruits (Ornelas-Paz et al. 2008). The impact of pectin on carotenoid bioaccessibility can be regulated by the physicochemical characteristics of pectin, which naturally vary between different fruits and during the fruit ripening (Ramos-Aguilar et al., 2015).

In general, a detrimental effect of pectin on carotenoid bioaccessibility has been established (Aschoff et al. 2015; Ornelas-Paz et al. 2008; Riedl et al. 1999; Rock and Swendseid 1992). However, this effect has quantitative and qualitative connotations. The amount of pectin has a clear negative effect of carotenoids bioaccessibility. Verrijssen et al. (2013) demonstrated that the bioaccessibility of β-carotene from carrots was higher (20–30 %) with low amounts (1–3 %) of citrus pectin than high concentrations of it (3.5–5 %). However, the structural characteristics of these polysaccharides seem to have the highest effect on carotenoid bioaccessibility. Verrijssen et al. (2014) found that citrus pectins with low esterification degree (14 %) reduced the bioaccessibility of β-carotene in 40 %, as compared with pectins with a high esterification degree (66–99 %). Dongowski (1995) found the interaction between pectins and bile salts increased as the concentration, esterification degree and molecular weight of pectins also increased; however, the degree of acetylation and amidation of pectins was inversely related with their association with bile salts. The structural characteristics and botanical source of pectins can change their negative effect on carotenoid bioaccessibility. Pectins also may act as emulsifiers and fat emulsification is a key step on carotenoid bioaccessibility (Leroux et al. 2003). Beet pectin seems to have higher emulsifying properties than citrus pectin. Acetylated citrus pectin showed a higher emulsifying property than the non-acetylated counterpart (Leroux et al. 2003). Citrus and beet pectins also reduced the interfacial tension between the water and oil phases. Bonnet et al. (2005) demonstrated that some pectins (high methoxylated pectins) make more stable the oil-in-water emulsions under acidic conditions. The particle size in emulsions is reduced by pectins at intermediate pH values (pH = 5.5). These authors suggested that the pectins form a network that connects the oil droplets. In addition, low esterification pectins enhanced the stability of emulsions in comparison with highly esterified pectins (Kovacova et al. 2009). These studies collectively suggest that pectin might increase the carotenoid bioaccessibility under some conditions.

16.3.4 Interaction Between Carotenoids

Many carotenoids typically coexist in a single food. Some studies have reported the competition of carotenoids to be micellarized or absorbed. Tyssandier et al. (2002) found that the chylomicron concentration of lycopene was significantly higher after the consumption of tomato puree than when tomato puree was accompanied with chopped spinach. They also observed that the response of lutein from spinach was reduced by the ingestion of a lycopene pill or tomato puree. In another study in healthy men, the supplementation with β-carotene decreased the plasma concentration of lutein in comparison with placebo, suggesting a possible interaction between these carotenoids (Micozzi et al. 1992). During et al. (2002) demonstrated that β-carotene reduced, in a dose-dependent fashion, the uptake of α-carotene and lycopene by Caco-2 cell layers and their subsequent basolateral secretion. However, they (During et al. 2002) did not observe an association between

the uptake or transport of lutein and β-carotene. Reboul et al. (2005) reported that mixed micelles with both lutein and β-carotene decrease the uptake of lutein (20 %) in Caco-2 cells in comparison with micelles containing only lutein.

16.3.5 Interaction Between Different Factors

Some studies have demonstrated the existence of complex interactions between all factors involved on carotenoid bioaccessibility and bioavailability. Mathematically, an interaction means that two or more factors play a role together, which is different to the effect of individual factors (Castenmiller and West 1998). However, this type of studies is scarce. The effect of the interaction between heat processing and dietary fat is one of the most studied. In many cases, the positive effect of cooking in the micellarization of carotenoids is potentiated by the presence of dietary fat (Colle et al. 2013; Hornero-Méndez and Mínguez-Mosquera 2007). Hornero-Méndez and Mínguez-Mosquera (2007) reported that the micellarization of carotenes increased from ~ 29 %, with raw carrots, to ~ 51 % with cooked carrots, and up to ~ 80 % with cooked carrots plus 10 % of olive oil. Similarly, the heat treatment (120 °C for 20 min) increased the bioaccessibility of lycopene from tomato pulp, but greater increments of bioaccessibility were observed when different oils (coconut, olive or fish) were added (Colle et al. 2013). Cilla et al. (2012) reported that the high-pressure processing (HPP) and thermal treatment (TT) decreased the bioaccessibility of carotenoids from fruit juice-milk beverages, but the addition of skimmed milk and whole milk induce the lowest decrease in the bioaccessibility of total carotenoids.

Dietary fat may also increase the benefits of mechanical processing of plant foods. Colle et al. (2013) reported that the combination of high pressure homogenization and the addition of coconut, olive or fish oil increased the bioaccessibility of lycopene from tomato pulp in comparison with non-treated samples and treated samples without lipids. Lemmens et al (2010) reported an interaction between the severity of food matrix disruption and heat processing. They found that the bioaccessibility of β-carotene was higher with small particles (<160 μm) of carrots that had been cooked for 3 min than with particles of raw carrots or carrots cooked for 25 min. However, with the largest carrot particles (161–6300 μm) the cooking for 25 min increased the bioaccessibility of β-carotene, as compared with particles of raw carrots or carrots cooked for 3 min.

The effect of the interaction of ripening, heat processing style and dietary fat type in the bioaccessibility of carotenoids from Jalapeño peppers was recently studied. Victoria-Campos et al. (2013a, b) demonstrated that the effect of dietary fat type influence more the bioaccessibility of carotenoids from ripe peppers, presumably as consequence of their highest content of esterified carotenoids, than those of immature fruits, where the content of esterified carotenoids is minimal. They (Victoria-Campos et al. (2013a, b) also demonstrated that the global impact of the heat processing was negative for fruits at early ripening stages, with this negative

effect being less notable with fruits at the most advanced stages of ripening. This could be due to the major thermostability of carotenoids from ripe peppers or by a compensation effect of dietary fat, which ameliorate the negative effects of the heat processing.

16.4 Concluding Remarks and Future Trends

In the last years, the health protective effects of carotenoids have been associated more to their ability to regulate the expression of genes than with their antioxidant activity. This is of importance given the high incidence of chronic diseases as cancer, cardiovascular diseases, and type 2 diabetes. However, *in vivo* evidence is still needed.

Carotenoid metabolites such as apo-carotenoids seem exert important biological activities; however, their identification is a major challenge due to their high instability. Thus, future works should propose new analytical methods, and strategies to know the proportion of bioaccessible or bioavailable metabolites that are naturally present in the fruit or generated during gastrointestinal digestion.

The digestive process of carotenoids is more or less known; however, there are still some gaps of knowledge needing to be clarified, such as the digestion, hydrolysis and absorption of esterified xanthophylls as well as the carotenoid distribution in micelles and/or vesicles in the aqueous phase.

It has been suggested that both passive diffusion and facilitate transport of carotenoids determine their bioavailability. However, the participation of each transport mechanism seems to be dependent on the intestinal carotenoid concentration and specificity of the transporters. Genetic studies about the transmembrane and intracellular proteins might clarify the mechanisms that regulate the carotenoid transport in cells.

Finally, plant foods contain a wide variety of carotenoids (free and esterified especies). The bioaccessibility and bioavailability of carotenoids from the same matrix are differentially affected by the intrinsic properties of the food (fruit matrix, chromoplast morphology, ripening stage, etc.), processing type (mechanical and thermal), presence of some dietary components (dietary fat, phytosterols, fiber), etc. These factors do not actuate alone but also they interact each other. The interaction between factors should be studied in a deeper way.

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