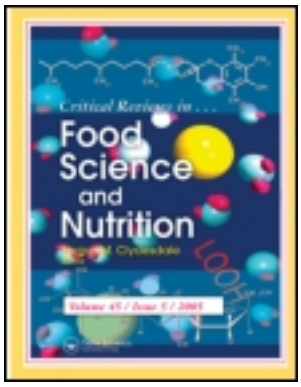

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Chemistry and Biotechnology of Carotenoids

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Chemistry and Biotechnology of Carotenoids

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Carotenoids are one of the most widespread groups of pigments in nature and more than 600 of these have been identified. Beside provitamin A activity, carotenoids are important as antioxidants and protective agents against various diseases. They are isoprenoids with a long polyene chain containing 3 to 15 conjugated double bonds, which determines their absorption spectrum. Cyclization at one or both ends occurs in hydrocarbon carotene, while xanthophylls are formed by the introduction of oxygen. In addition, modifications involving chain elongation, isomerization, or degradation are also found. The composition of carotenoids in food may vary depending upon production practices, post-harvest handling, processing, and storage. In higher plants they are synthesized in the plastid. Both mevalonate dependent and independent pathway for the formation of isopentenyl diphosphate are known. Isopentenyl diphosphate undergoes a series of addition and condensation reactions to form phytoene, which gets converted to lycopene. Cyclization of lycopene either leads to the formation of β-carotene and its derivative xanthophylls, β-cryptoxanthin, zeaxanthin, antheraxanthin, and violaxanthin or α-carotene and lutein. Even though most of the carotenoid biosynthetic genes have been cloned and identified, some aspects of carotenoid formation and manipulation in higher plants especially remain poorly understood. In order to enhance the carotenoid content of crop plants to a level that will be required for the prevention of diseases, there is a need for research in both the basic and the applied aspects.

Keywords Carotenoid biosynthesis, isomerization, lycopene, β-carotene, metabolic engineering

INTRODUCTION

Carotenoids, the colored pigments ranging from light yellow through orange to deep red, are biosynthesized by all photosynthetic bacteria, cyanobacteria, algae, higher plants and also by some non-photosynthetic bacteria, fungi, and yeasts. The characteristic colors of many birds, insects, and marine invertebrates are also due to the presence of carotenoids, which originate from the diet. Animals are not able to synthesize carotenoids *denovo* and rely upon the diet as a source of these compounds. More than 600 carotenoids are characterized structurally and the list is increasing continuously as newer compounds are being discovered. Commercially, carotenoids are used as colorants for human food and nutritional supplements, as feed additives to enhance the pigmentation of fish and eggs, as pharmaceutical products, and in the agriculture and cosmetic industry (Bramley, 2003).

The major function of these isoprenoid molecules in plants is in photosynthesis wherein they protect the photosynthetic apparatus from excess light. They are also intermediates in the biosynthesis of abscissic acid and other apocarotenoids. In recent years there has been considerable interest in the dietary carotenoids due to their provitamin A activity (Olson and Hayaishi, 1965; Nagao et al., 1997), high antioxidant potential (Sies and Stahl, 2003), and their ability to prevent the onset of certain cancers (Giovannuci, 1999; Gann et al., 1999) as well as age-related macular degeneration (Landrum and Bone, 2001).

The beneficial role of carotenoids in maintaining human health, their important role in plant photo protection, their versatile usage as food and feed supplements, and their applications in cosmetic and pharmaceutical industries make them potential candidates for enhancement and manipulation. Over the past three decades advances in molecular genetics and biotechnological approaches have led to the understanding of carotenoid biosynthesis and its manipulation in microorganisms and higher plants. Even though the structural genes of carotenoid biosynthesis have been identified and cloned, the regulation of carotenoid biosynthesis pathway is poorly understood. Therefore, the type and amount of carotenoids to be accumulated by transformation is still unpredicted. The current paper reviews the advances made in carotenoid biosynthesis and its regulation. It also gives information about the metabolic engineering

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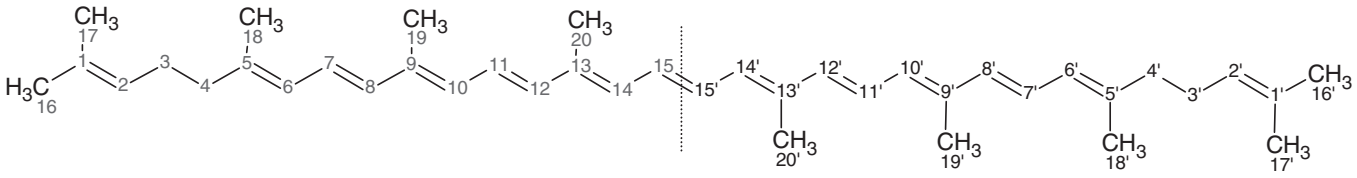

Figure 1 Structure of carotenoid lycopene with common numbering system.

attempted in various microbes and higher plants with future research directions.

CAROTENOID STRUCTURE AND CLASSIFICATION

Carotenoids are a class of hydrocarbons consisting of eight isoprenoid units (ip), joined in a head-to-tail pattern, except at the center, to give symmetry to the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship (Fig. 1). Based on this structure a semisystematic numbering system is used and carotenoids are named as a derivative of parent compound (Fig. 1 and 2). Greek letters are used to describe the end groups of the structure in IUPAC system. The position of hydrogenation and group substitution is indicated by prefixes and suffixes. The structures of a few carotenoids with this nomenclature are shown in Fig. 3.

The majority of carotenoids are derived from a 40-carbon polyene chain, which could be considered as the backbone of the molecule (Fig. 1). This chain may be terminated by cyclic end-groups (rings) and may be complemented with oxygen-containing functional groups. Based on their chemical structure, carotenoids are classified into two groups—hydrocarbons commonly known as carotenes and xanthophylls, the oxygenated derivatives of these hydrocarbons. The other classification system (Table 1) is based on their biological functions, which ultimately depend on the structure of a carotenoid. The characteristic pattern of alternating single and double bonds in the polyene backbone of carotenoids allow them to absorb excess energy from other molecules, which may account for their antioxidant properties in vivo. The nature of the specific end groups in carotenoids may influence their polarity, which may explain the differences in the way in which individual carotenoids interact with biological membranes (Britton, 1995). Some of the characteristics of carotenoids such as their semi-systematic name, number of conjugated double bonds, absorption maxima in different solvents, and color are listed in Table 2.

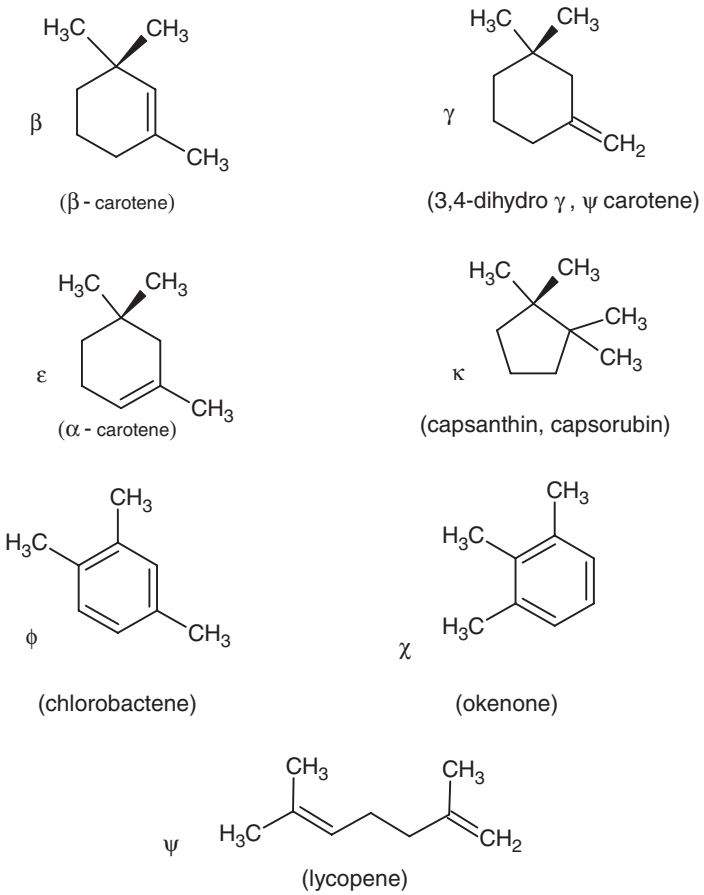

Figure 2 Characteristic end groups of carotenoids (names of carotenoids written in bracket are examples of carotenoids in food circle based on these end groups).

Distribution

Carotenoids are widely distributed in nature. Different colors produced by this class of pigments viz., brilliant red, pink, orange, yellow are found in every form of life. In higher plants, carotenoids are found in plastids. They are found in chloroplasts of photosynthetic tissues and in chromoplasts in fruits and flowers. The carotenoid composition is almost similar in leaves of all species with lutein (45% of total), β-carotene (25–30%), violaxanthin (15%), neoxanthin (10%), and small amounts of α-carotene, β-cryptoxanthin, zeaxanthin, and antheraxanthin. They occur as free form in leaves and are esterified in other tissues. Some carotenoids like astaxanthin from *Haematococcus pluvialis* is extraplastidial in nature. Different sources of carotenoids and their uses have been listed in Table 3.

Dietary Sources

Carotenoids in human diet are primarily derived from crop plants where they are naturally present in edible leaves,

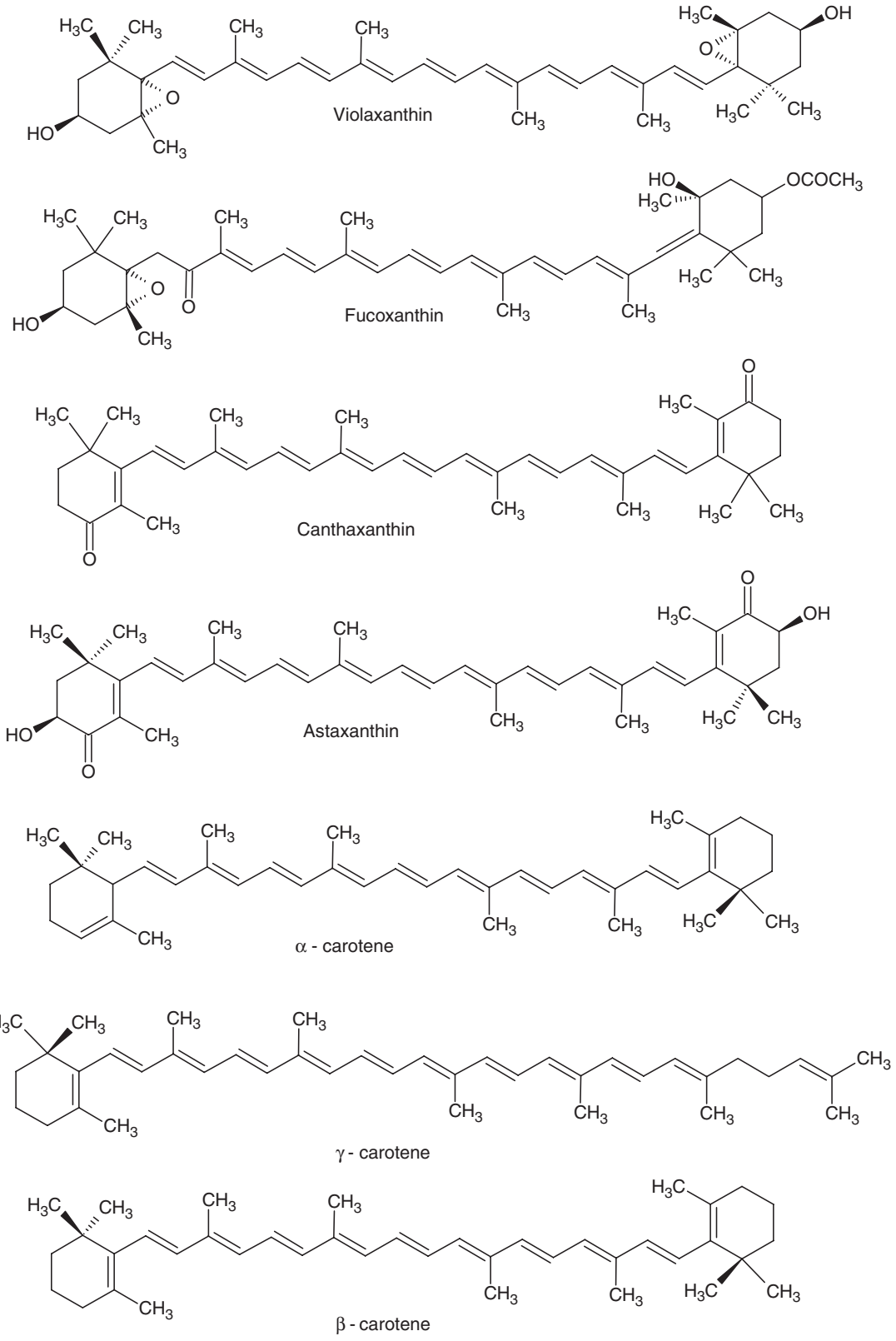

Figure 3 Structures of some common carotenoids.

Table 1 Classification of carotenoids

Basis	Sub-group	Characteristics	Examples
Structure	Carotenes	Constituting carbon and hydrogen	α-Carotene, β-Carotene, β-cryptoxanthin
	Xanthophylls	Constituting carbon, hydrogen, and oxygen	Lutein, zeaxanthin, violaxanthin, fucoxanthin
Cyclization	Acyclic	End group not closed	Lycopene
	Alicyclic		
	a. Monocyclic	One end group open, one closed	γ-Carotene
	b. Bicyclic	Both closed	β-Carotene
Structural alteration	Allenic	Continuous double bond	Neoxanthin
	Acetylenic	Presence of a triple bond	Dehydro apocarotenoid
	Apocarotenoid	Less than 40 Carbon atoms	Bixin
	Higher carotenoid	More than 40 Carbon atoms	Crocetin
Function	Primary	Required for photosynthetic process	β-Carotene, zeaxanthin, Lutein, violaxanthin, antheraxanthin, neoxanthin
	Secondary	Presence not directly related to plant survival	α-Carotene, capsanthin, bixin, lycopene, astaxanthin (carotenoids localized in fruits and flowers)

Source: Delgado-Vargas and Paredes-Lopez, 2003; Schwartz et al., 2008

flowers, and fruits. In general, increasing the intensity of color in a plant organ indicates a higher level of carotenoids. Hydrocarbon carotenes such as β-carotene and lycopene are typically present in free form, which is entrapped within chloroplast and chromoplast bodies. Xanthophylls, such as lutein and zeaxanthin, are abundant in a number of yellow or orange fruits and vegetables such as peaches, mango, papaya, prunes, squash, and oranges. The carotenoids content in various vegetables and fruits are given in Table 4. Food sources of lutein and zeaxanthin include corn, egg yolk, fruits, and green vegetables such as broccoli, green beans, green peas, brussel sprouts, cabbage, kale, collard greens, spinach, and lettuce (Lakshminarayana et al., 2005; Raju et al., 2007). Lutein and zeaxanthin are also found in algae and the petals of many yellow flowers. Lycopene found in raw (unprocessed) tomatoes is contained within a matrix. The richest sources of β-carotene are yellow, orange, and red fruits and vegetables such as carrots, spinach, lettuce, tomatoes, sweet potatoes, water melon, broccoli, and winter squash (Zeb and Mehmood, 2004; Kim et al., 2007; Raju et al., 2007; Kandlakunta et al., 2008). Generally, the free forms of carotenoids are present in the chloroplasts of green plants such as alfalfa, spinach, kale, and leafy green plant materials and they provide better adsorption when consumed in foods or as a supplement.

BIOAVAILABILITY OF CAROTENOIDS

Bioavailability is defined as the fraction of an ingested nutrient that becomes available to the body for utilization in physiological functions or for storage (Jackson, 1997). The bioavailability of carotenoids is influenced by many dietary and physiological factors which include; species of carotenoid, molecular linkage, amount consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and interactions (Castenmiller and West, 1998). Since carotenoids are lipid soluble, their absorption from the intestine is better from a fatty diet, although the amount of fat required is low (Van het Hof et al., 2000) and recent studies have proved that absorption of carotenoids increases when they are ingested with dietary lipids (Brown et al., 2004; Unlu et al., 2007) (Table 5). The mechanism of fat-stimulated absorption may involve enhanced incorporation into mixed micelles. With respect to the food matrix, it has been shown that lutein is more bioavailable from spinach and following disruption of the plant cell wall, the bioavailability of β-carotene was enhanced from whole leaf and minced spinach (Castenmiller et al., 1999). The uptake of β-carotene from vegetables is low (14% for mixed vegetables) compared with purified β-carotene added to a simple matrix (Van het Hof et al., 2000). *Cis* isomers of carotenoids are reported to be more bioavailable than the all-*trans* forms (Boileau et al., 1999). Intestinal absorption of dietary carotenoids with respect to the food matrix, dietary components, and carotenoid species has been recently reviewed by Yonekura and Nagao (2007).

The bioavailability of lycopene from tomatoes has been found to increase with processing. Lycopene uptake in humans was found to be higher (2-fold increase) from processed tomato juice than from unprocessed juice. This is attributed to the isomerization of *trans* to *cis* form of lycopene on heating and its release from ruptured cells due to thermal processing (Stahl and Sies, 1992; Boileau et al., 2002). Unlu et al. (2007) found that *cis* isomers of lycopene are better absorbed and are more bioavailable than their *trans* counterparts. Similarly, a higher absorption of *trans* β-carotene in humans fed with cooked carrot ($65.1 \pm 7.4\%$) as compared to raw carrots ($41.4 \pm 7.4\%$) was reported (Livny et al., 2003). Lakshminarayana et al. (2007) found that carotenoid uptake is dependent on lipolysis or hydrolysis of triglycerides and release of fatty acids in the intestinal tract by lipase. Based on experiments on aged lutein deficient rats, they concluded that olive oil might enhance the intestinal accessibility of lutein.

Table 2 Chemistry of Carotenoids

Common name	Semisystematic name	No. of conjugated double bonds	λ_{max} (nm)	Absorption Co-efficient	Color	Principal Source of carotenoid
Antheraxanthin	5,6-epoxy-5, 6-dihydro-β, β-carotene-3, 3'-diol	10	423, 444, 473[a] 421, 443, 473[b] 424, 448, 475[c]	2350 at 446 nm	Yellow	Green leaves
Astaxanthin	3,3' –dihydroxy- β, β-carotene-4,4' –dione	13	478[b,g]	2100 at 470 nm	Red	*Haematococcus* sp.
Bixin	methyl hydrogen 9'- *cis*- 6,6'- diapocarotene- 6,6'- dioate	9	432, 456, 490[a] 439, 470, 503[d]	4200 at 456 nm	Red	Annatto
Canthaxanthin	β ,β-carotene-4, 4'-dione	11	466[a],477[b]	2200 at 466 nm	Red	Green leaves
Capsanthin	3, 3'-dihydroxy- β, κ-caroten-6'-one	11	450, 475, 505[a] 468, 483, 518[f]	2072 at 483nm	Red	Red pepper
Capsorubin	3, 3'-dihydroxy- β, κ-carotene-6, 6'-dione	11	444, 474, 506[a] 460, 489, 523[f]	2200 at 489 nm	Red	Red pepper
$\alpha-$carotene	β, ε-carotene	10	422, 444, 473[a] 424, 448, 476[e] 421, 445, 473[g]	2800 at 444 nm	Yellow	Carrot
$\beta-$carotene	β, β-carotene	11	425, 449, 476[a] 427, 454, 480[e] 432, 454, 480[c]	2592 at 449 nm	Yellow	Carrot
$\gamma-$carotene	β, ψ-carotene	11	437, 462, 494[a] 435, 461, 490[g]	2760 at 462 nm	Bright orange	Tomato, Peach palm, rose hips
$\zeta-$carotene	7, 8, 7', 8'-tetrahydro- ψ, ψ-carotene	7	378, 400, 425[a] 415, 440, 468[g]	2555 at 400 nm	Pale yellow	Brazilian Passion fruit
Crocetin	8, 8'- diapo- 8, 8' – dioic acid	7	400, 422, 450[a] 401, 423, 447[b]	4320 at 450 nm	Yellow	Saffron
$\beta-$Cryptoxanthin	β, β-caroten-3-ol	11	425, 449, 476[a,c] 428, 450, 476[b]	2386 at 449 nm	Yellow/orange	Papaya
Fucoxanthin	3'-acetoxy-5, 6-epoxy-3, 5'-dihydroxy-6', 7'-didehydro-5, 6, 7, 8, 5', 6'-hexahydro-β-carotene-8-one	9	435, 446, 473[a] 437, 450, 478[g]	1100 at 585 nm	Yellow	Fungi
Lutein	β, ε-carotene-3, 3'-diol	10	421, 445, 474[a] 422, 445, 474[b] 426, 447, 474[c]	2550 at 445 nm	Yellow	Leaves, green vegetables
Lycopene	ψ, ψ -carotene	11	444, 470, 502[a] 446, 472, 503[b] 447, 473, 505[c]	3450 at 470 nm	Pink/red	Tomato
Neoxanthin	5', 6'-epoxy-6, 7-didehydro-5, 6, 5', 6'-tetrahydro- β, β-carotene-3, 5, 3'-triol	8	416, 438, 467[a] 415, 439, 467[b] 414, 437, 465[c]	2243 at 439 nm	Yellow	Leaves
Norbixin	2E, 4E, 6E, 8E, 10E, 12E, 14E, 16E, 18E- 4,8,13,17-tetramethylicosa-2,4,6,8,10,12,14,16, 18-nonaenedioic acid	9	442, 474, 509[d]	2850 at 456 nm	Red	Annatto
Phytoene	7, 8, 11, 12, 7', 8', 11', 12'-octahydro- ψ, ψ -carotene	3	276, 286, 297[a,g]	1250 at 286 nm	Colorless	green vegetables
Phytofluene	7, 8, 11, 12, 7', 8' -hexahydro- ψ, ψ -carotene	5	331, 348, 367[a,g]	1350 at 348 nm	Colorless	green vegetables
Violaxanthin	5, 6, 5', 6'-diepoxy-5, 6, 5', 6'-tetrahydro- β, β-carotene-3, 3'-diol	9	416, 440, 465[a] 419, 440, 470[b] 417, 440, 470[c]	2250 at 440 nm	Yellow	Leaves
Zeaxanthin	β, β-carotene-3, 3'-diol	11	424, 449, 476[a] 428, 450, 478[b] 432, 454, 480[c]	2348 at 449 nm 2540 at 450 nm	Orange	Yellow corn

a = light petroleum; b = ethanol; c = acetonitrile/ethylacetate, d = chloroform; e = acetone; f = benzene; g = hexane
Source: Cuttriss and Pogson, 2004; Delgado-Vargas and Paredes-Lopez, 2003; Rodriguez-Amaya, 2001

Table 3 Dietary sources of carotenoids and their use

Source	Main carotenoids	Uses
Annatto (*Bixa orellana*)	Bixin and norbixin	Coloring foods, cosmetics and Textiles
Dunaliella sp.	β-carotene	Feed and food additive; dietary supplement
Haematococcus sp.	Astaxanthin	Feed additive; nutraceutical agent
Marigold (*Tagetes erecta*) and green leafy vegetables	Lutein and Zeaxanthin	Poultry and fishery feed additive; purified oleoresin as food additive
Paprika (*Capsicum annum*)	Capsanthin and capsorubin	Used as spice in food to add color and flavor
Saffron (*Crocus sativus*)	Crocetin and crocin	Foods and pharmaceutical products
Tomato, red grapes, watermelon, pink Grapefruit, papaya and apricots.	Lycopene and β-carotene	Nutraceutical and food colorant
Vegetables (carrots, pumpkins, sweet potatoes) and vegetable oils	β-carotene	Food and Feed additive

Source: Delgado-Vargas and Paredes-Lopez, 2003; Rodriguez-Amaya, 2001

EFFECT OF STORAGE AND PROCESSING ON CAROTENOIDS

In a given food, the composition of carotenoids may vary due to many factors such as cultivar, maturity state, growing season, production practices, post-harvest handling, processing, and storage of food. The major cause of carotenoid destruction during the storage and processing of food is enzymatic as well as non-enzymatic oxidation as they are highly unsaturated compounds and are prone to isomerization and oxidation. Reducing processing time and temperature and the time lag between peeling, cutting, and processing can substantially increase the retention of carotenoids (Zanoni et al., 1998). Oxygen content in the medium is most crucial for carotene stability (Goldman et al., 1983), and reduced water activity of the medium has a protective role (Minguez and Galan, 1995). The occurrence and extent of oxidation depends on the presence of oxygen, metals, enzymes, unsaturated lipids, pro-oxidants, antioxidants, exposure to light, type and physical state of the carotenoids present, severity and duration of processing, packaging material, and storage conditions (Rodriguez-Amaya, 2003). Freezing and addition of antioxidant generally preserves the carotenoids, but drying, juicing, and peeling results in substantial losses. During thermal processing, isomerization of *trans*-carotenoids to *cis*-forms occurs due to light, heat, and acid, which changes its bioavailability and biological activity (Dugave and Demange, 2003). A brief description of the storage and processing techniques used for different vegetables and the changes in their carotenoid content is given in Table 6.

A reduction in the carotene content of fresh vegetables irrespective of storage conditions has been reported by Leth (1986). Unfavorable relative humidity and temperature has been shown to hasten the loss of carotenes during storage of fresh produce, wherein spinach lost almost 63.5% of the original carotenoids after wilting (Akpapunam, 1984). Negi and Roy (2003) reported up to 85.0% losses in β-carotene in fresh green leaves (amaranth) depending on duration and storage conditions with packaged samples retaining higher β-carotene than their open counterparts. During storage of fresh carrots, a steady decrease (Negi and Roy, 2000b) and a slight increase

Table 4 Carotenoid content in vegetables and fruits

	Carotenoids (μg/100g)					
Source	Total	β-carotene	α-carotene	Lutein	Zeaxanthin	Lycopene
Apricot	2196	1766	37	101	31	—
Broccoli	2533	919	—	1614	—	—
Bitter gourd	967	84	—	—	—	—
Bottle gourd	186	50	—	—	—	—
Cabbage	226	26	—	—	—	—
Capsicum	719	157	—	—	—	—
Carrot	9460–15870	4650–9020	3060–4890	360–56	—	—
Green chilli	2410	1020	—	-	—	—
Lettuce	8480	1290	40	2950	—	—
Orange	211	14	—	64	50	—
Papaya	3440	59–220	—	38	21	2070
Pink Grapefriut	3500	2340	—	—	—	2770
Spinach	17300	3250	900	9540	350	—
Sweet corn	1978	59	60	522	437	—
Tomato	30900	40–2200	—	10–200		2000–5000
Yellow Pumpkin	2120	1180	—	—	—	—

Source: Fraser and Bramley, 2004; Kandlakunta et al., 2008

Table 5 Bioavailability of carotenoids

Carotenoid	Dietary lipid used in the study	Increase in bioavailability	Source
Lycopene, β-carotene, α-carotene, lutein, zeaxanthin	Canola oil (0 g-no fat 6 g-reduced fat 28 g-full fat)	Absorption was better from salads with full fat than with reduced or no fat salads	Brown et al., 2004
β-carotene	Processed chicken as a source of exogenous fat	Significant increase in the β-carotene in micelle fraction was observed as fruit ripened and when chicken was mixed with mango before digestion	Ornelas-Paz et al., 2008
β-carotene	3, 6, 12% fat	Enhanced bioavailability of β-carotene was observed as evidenced by higher liver VA storage and efficient conversion of β-carotene to VA in gerbils fed with higher fat	Mills et al., 2009

followed by decrease (Lee, 1986) in β-carotene content have been reported.

The short-time heat treatment such as blanching may cause some loss of carotenoids, but the inactivation of oxidative enzymes can prevent loss before, during, and after thermal processing and during storage. Contradictory reports indicating substantial loss (Khachik et al., 1986; Chandler and Schwartz, 1988; Speek et al., 1988; Nagra and Khan, 1988; Rahman et al., 1990, Negi and Roy, 2000a; 2000b) to little or no change (Gody and Rodriguez-Amaya, 1987; Khachik et al., 1992) and an increase in the amount of β-carotene levels in the processed foods (Gomez, 1981) due to blanching are available in the literature. Shyu et al. (1998) reported that blanched carrot slices fried under vacuum at 100°C for 25 min retained 76 and 83% of α- and β-carotene, respectively.

Cooking tomatoes at 88°C increases the *trans* lycopene content of tomatoes (Dewanto et al., 2002). After pressure cooking and normal boiling the retention of β-carotene was substantially greater in carrots than pumpkin (Gayathri et al., 2004). Pressure cooking helped to retain more β-carotene in carrots, while in pumpkin loss of β-carotene was higher during pressure cooking. Cooking also reduces β-carotene of Swamp cabbage, Chinese cabbage, Bai kaprao, and Water mimosa (Wasantwisut et al., 1995).

Carotenes are susceptible to oxidation when exposed to light (Saguy et al., 1985), oxygen, and enzymes (Gregory, 1996). Dehydrated vegetables lose color due to the oxidation of highly unsaturated molecules upon exposure to air during storage. The degradation of β-carotene is associated with the development of an off flavor in dehydrated carrots (Ayer et al., 1964). Sun drying resulted in loss of carotenoids, but losses were lesser compared to sun drying followed by cooling (Speek et al., 1988). Blanching and sulfiting are effective in reducing the loss of carotenoids in dehydrated carrots (Baloch, 1987; Mohamed and Hussein, 1994; Zhao and Chang, 1995; Negi and Roy, 2000b), savoy beet, amaranth, and fenugreek (Negi and Roy, 2000a). Sulfite treatment retards carotenoid breakdown, inhibits lipid oxidation, and decreases discoloration of dehydrated carrots (Baloch et al., 1981).

A rapid degradation of β-carotene during air-drying of carrots was observed (Park, 1987), but low temperature drying (30 ± 2°C) resulted in better retention of β-carotene as compared to sun, shade, solar, and cabinet drying of savoy beet, amaranth, fenugreek (Negi and Roy, 2000a), and carrots (Negi and Roy, 2000b). Preparation of semi-dried tomato resulted in less than 10% reduction of lycopene (Toor and Savage, 2006). Maeda and Salunkhe (1981) and Park (1987) reported a significant reduction in β-carotene of air-dried vegetables. Onayemi and Okeibuno Badifu (1987) and Negi and Roy (2001b) found higher carotene content in cabinet dried vegetables as compared to their solar dried counterparts. Jayaraman et al. (1991) also observed 49% loss of β-carotene in direct sun-dried carrots as compared to 30% and 16% loss in solar-dried and cabinet-dried carrots. A continuous decrease in β-carotene during long-term storage was observed in dried carrots (Zhao and Chang, 1995; Negi and Roy 2000b; 2001a) and dehydrated green leaves (Negi and Roy, 2001a, 2001b).

Air drying of tomatoes does not cause a significant reduction in lycopene (Zanoni et al., 1998; Chang and Liu, 2007); however, during hot air drying a slight decrease (Zanoni et al., 1998; Shi et al., 1999; Lavelli et al., 1999; Leoni et al., 2001) as well as an increase in lycopene content has been reported (Stahl and Sies, 1992; Shi and Le Maguer, 2000; Dewanto et al., 2002). During preparation of tomato powder a moderate loss of lycopene takes place due to isomerization and oxidation (Cabassi et al., 2001). Takeoka et al. (2001) found lycopene losses to range from 9% to 28% during paste manufacture at 90°C. Re et al. (2002) reported higher lycopene in juice prepared by hot break process at 95°C in contrast to higher lycopene content observed in the cold break process (Tamburini et al., 1999), but the hot break process was found to preserve lycopene for longer storage duration as compared to the cold break process. Reduction of about 35% and 50% of lycopene were reported in tomato juice after sterilization at 121°C for 2 min or pasteurization at 80°C for 20 min followed by 50 min cooking in tomato soup, respectively (Seybold et al., 2004). Wang et al. (1996) observed that heat-processed tomato juice had much higher lycopene content than fresh tomatoes and thermal processing enhances the nutritional value of tomato by increasing the lycopene content (Dewanto et al., 2002).

Bao and Chang (1994) observed that β-carotene in carrot juice was sensitive to processing operations and losses of β-carotene were more than α-carotene. Zhang (1996) developed a

Table 6 Effect of storage and processing techniques on carotenoid content in foods

Food Source	Storage and Processing	Loss of carotenoids	Source
Spinach	Fresh storage	63.5% total carotenoids	Akapapunam, 1984
Savoy beet	Fresh storage (2 days)	84–94% β-carotene	Negi and Roy, 2000c
	Blanching	50.3–54.8% β-carotene	Negi and Roy, 2000a
	Drying	60.3–71.6% β-carotene	
	Storage of dried product: 3 months	22.5–39.7% β-carotene	Negi and Roy, 2001b
	6 months	26–53.3% β-carotene	
	9 months	39.4–59.7% β-carotene	
Amaranth	Fresh storage (2 days)	46.5–84.8% β-carotene	Negi and Roy, 2003
	Blanching	1.5–7.6% β-carotene	Negi and Roy, 2000a
	Drying	44.1–81.8% β -carotene	
	Storage of dried product: 3 months	15.9–57.7% β-carotene	Negi and Roy, 2001b
	6 months	39.5–70.9% β-carotene	
	9 months	48–83.7% β-carotene	
Fenugreek	Fresh storage (2 days)	24.2–72.2% β -carotene	Negi and Roy, 2003
	Blanching	8.5–24.5% β -carotene	Negi and Roy, 2000a
	Drying	14.9–58.7% β -carotene	
	Storage of dried product: 3 months	21.3–24.4% β -carotene	Negi and Roy, 2001c
	6 months	28.9–43% β -carotene	
	9 months	33.1–73.5% β -carotene	
Carrot	Fresh storage (8 days)	45.6–78.6% β -carotene	Negi and Roy, 2000b
	Blanching	11.7–33.1% β -carotene	Negi and Roy, 2000b
	Drying	21.8–43.2% β-carotene	
	Storage of dried product: 3 months	8–32.7% β -carotene	Negi and Roy, 2001a
	6 months	31.5–39% β -carotene	
	9 months	59.8–71.8% β -carotene	
Carrot	Frying under vacuum at 100°C for 25 min	76% α-carotene, 83% β -carotene	Shyu et al., 1998
Carrot	Drying-Sun drying	49% β -carotene	Jayaraman et al., 1991
	Solar drying	30% β -carotene	
	Cabinet drying	16% β -carotene	
Swamp cabbage	Blanching at 98°C, 5min	11% β -carotene	Wasantwisut et al., 1995
	Stir frying at 178°C, 3.5 min	18% β -carotene	
Water mimosa	Boiling at 97°C, 5min	3% β -carotene	
	Stir frying at 178°C, 2 min	42% β -carotene	
Bai kaprao	Blanching at 98°C, 5min	5% β -carotene	
	Boiling at 97°C, 2 min	20% β -carotene	
	Stir frying at 178°C, 3 min	28% β -carotene	
Tomato	Drying at 110°C	10% β-carotene	Zannoni et al., 1999
Tomato	Drying at 80°C	2% β-carotene	Lavelli et al., 1999
Tomato pulp	Drying at 70°C	0.7% β-carotene	
Tomato	Paste manufacture at 90°C	9–28% lycopene	Takeoka et al., 2001
Tomato	Sterilization 121°C, 2 min followed by 50 min cooking	35% lycopene	Seybold et al., 2004
	Pasteurization 80°C, 20 min followed by 50 min cooking	50% lycopene	
Pure lycopene	Drying: 100°C, 60 min	50% lycopene	Mayeaux et al., 2006
	125°C, 20 min		
	150°C <10 min		
Tomato slurry	Baking: 177°C, 15 min	34.9% lycopene	Mayeaux et al., 2006
	218°C, 15 min	48.5% lycopene	
	177°C, 45 min	62.7% lycopene	
	218°C, 45 min	74.9% lycopene	
	High power microwave cooking for 1 min	35.6% lycopene	
	Frying: 145°C, 1 min	63.4% lycopene	
	165°C, 1 min	64.5% lycopene	
Sweet bell pepper, Sweet potato, Tomato	Stir-frying, ½-3 min	2–27% β-carotene	Kidmose et al., 2006
Orange juice	High intensity pulse electric field processing: 25, 30, 40 kv/cm for 30–340 μs Pasteurization 90°C, 20 sec	9.6%, 6.3%, 7.8% β -carotene 12.6% total carotenoids	Cortes et al., 2006
Chilli	Heat treatment 80–100°C, 5–10 min	25–34% total carotenoids	Ute et al., 2007

(Continued on next page)

Table 6 Effect of storage and processing techniques on carotenoid content in foods *(Continued)*

Food Source	Storage and Processing	Loss of carotenoids	Source
Paprika	Heat treatment 80–100°C, 5–10 min	20–53% total carotenoids	
Chilli powder	Storage at ambient temperature in light	16.7% total carotenoids	
	Storage at ambient temperature in dark	9.6% total carotenoids	
Paprika powder	Storage at ambient temperature in light	39.7% total carotenoids	
	Storage at ambient temperature in dark	38.8% total carotenoids	
Orange-fleshed sweet potato roots	Boiling: 20 min	22% β-carotene	Bengtsson et al., 2008
	Steaming: 30 min	23% β-carotene	
	Deep frying: 10 min	22% β-carotene	
	Drying: 57°C in forced air-oven, 10 h	12% β-carotene	
	Solar drying	9% β-carotene	
	Open air drying	16% β-carotene	

combination of peeling, blanching, vacuuming, and sterilizing treatments to retain carotenoids in juice. Carotenoid content in milk beverages made from apple, lemon, pear, strawberry, kiwifruit, pineapple, and banana were low in carotenoids, whereas orange, apricot, mango, and peach were found to increase β-cryptoxanthin and β-carotene concentrations of beverage. Passion fruit provides ζ-carotene and the levels of α-carotene and β-carotene increase in the presence of carrot. Mixing of fruit juices with milk can reduce provitamin A activity due to conversion of all *trans* β-carotene and β-cryptoxanthin to their *cis* isomers, α-carotene, and β-cryptoxanthin (Zulueta et al., 2007).

Heat treatment resulted in significat losses of carotenoids in chilli (upto 34%) and paprika (upto 53%) depending on the severity of the treatment with mono- and diesters carotenoids being more stable than non-esterified carotenoids (Ute et al., 2007). Fast drying of fresh pepper for the production of paprika leads to the destruction of carotenoids, while slow drying was found to enhance the level of carotenoids in some varieties of pepper. Illumination was found to induce the synthesis of capsanthin, capsorubin, and capsolutein in pepper (Minguez-Mosquera and Hornero-Mendez, 1994).

Chen et al. (1995) studied the effect of processing on carotenoid content in carrots and found that canning resulted in the highest destruction of carotenoids, followed by HTST (high temperature short time) treatment and acidification. 13-*cis*-β-carotene was formed in largest amount during heating, followed by 13-*cis*-lutein and 15-*cis*-α-carotene. Canning prevents oxidation during ambient storage, but due to severe heat treatment it can reduce the carotenoid content of carrots. Despite the loss of carotenes during processing, its higher retention during storage was reported because of removal of oxygen (Kalpalathica et al., 1988). Carrot juice color changes from orange to yellow with intensive heat treatment (Desorby et al., 1998). The predominant isomer found in carrot samples subjected to heating was found to be 13-*cis*-β-carotene (Hiranvarachat et al., 2008).

In sweet potato, blanching, peeling, and pureeing increased the carotene content while baking, microwave cooking, dehydration, and canning resulted in the loss of carotenoids (Chandler and Schwartz, 1988). Increase in carotene content was attributed to an enhanced extraction efficiency of heat-treated samples. Heat processing induces the formation of 13-*cis*-β-carotene isomer, but a significant decrease in lycopene content of tomato was observed after baking, microwave cooking, and frying as lycopene is not stable when exposed to cooking temperatures above 100°C (Mayeaux et al., 2006). The lower losses in lycopene during microwave cooking were attributed to shorter exposure and lower temperature treatment than baking or frying. Further, high frying temperatures could cause the oil to produce hydroperoxide free radicals and accelerate the degradation of lycopene, which was evident from the very high losses observed in lycopene during frying as compared to microwave cooking and baking of tomato slurry.

Freezing is the most frequent way used to preserve carrots and prevent loss of carotenes for long-term storage, although some cell disruption may occur during freezing which causes softening (Prestamo et al., 1998). Freezing was found to be an efficient method in preserving carotenoid in frozen mangoes (Cano and de Ancos, 1994) and Kiwi fruits (Cano and Marin, 1992). After blanching and freezing, negligible losses in β-carotene content occurred in first 6 months of frozen storage (Puupponen-Pimia et al., 2003). During freeze-drying, changes in the total carotenoids were not observed in carrots (Schadle et al., 1983); however, Bao and Chang (1994) reported higher losses of carotenoids during freeze drying. Kaminski et al. (1986) also observed higher carotene degradation during freeze-drying and reported that air-drying was more efficient for carotene preservation when stored at room temperature.

Lycopene is stable to temperature used for tomato concentration and also during storage of processed tomato product; although stability goes down if the cell wall damage occurs during processing (Leoni et al., 2001). Lycopene is readily degraded by exposure to air, especially in the presence of cationic pro-oxidant metals such as copper. Lycopene retention can be improved by storage at low temperature, low light, and low water content (Shi and le Maguer, 2000). The heating temperature and time are important for lycopene retention and contradictory reports exist indicating both increase as well as decrease in lycopene content of tomato during storage, semidrying, paste, and juice processing (Anguelova and Warthesen, 2000; Takeoka et al., 2001; Dewanto et al., 2002; Hackett et al., 2004; Seybold et al., 2004; Goula et al., 2006; Toor and Savage, 2006). The increase

in lycopene content can be attributed to the temperature (below 80°C) used in those tomato processing methods which increases free lycopene by disrupting cell walls or hydrolyzing lycopene derivatives rather than degrading the lycopene (Thompson et al., 2000). The exposure of oxygen, high temperature, and low water activity can enhance the degradation of lycopene. Lycopene can be lost as a result of thermal degradation and oxidation. During thermal processing, the naturally occurring *trans*-isomer can be converted to the *cis*-isomer and the degree of isomerization is directly correlated to the degree of thermal treatment (Shi and le Maguer, 2000).

The effect of different dehydration processes on the lycopene stability in tomatoes has shown that osmotic treatment does not affect the isomeric profile of lycopene and this was attributed to the sugar deposition on the surface layer which may prevent lycopene oxidation (Shi et al., 1991). Chen and Tang (1998) observed that the total amount of all *trans* and *cis* forms of lutein, α- and β-carotenes in the spray dried carotenoid powder obtained from the carrot decreased with increasing storage time and temperature, and a high correlation was observed between color changes and carotenoid content.

Degradation of β-carotene in different drying conditions (hot air, vacuum, and low pressure super heated steam) showed higher losses in hot air drying, probably due to an increased rate of isomerization (Cui et al., 2004). The lower loss of β-carotene in vacuum and low pressure superheated steam was attributed to partial conversion of all trans β-carotene into 13-*cis*-β-carotene (Hiranvarachat et al., 2008). Pasteurization and sterilization were also found to cause thermal degradation of carrot juice by *cis-trans* isomerization (Chen et al., 1995; Marx et al., 2003). The loss of orange color in the irradiated carrots was attributed to the oxidation of carotenes (Chervin and Boisseau, 1994) as irradiation creates free radicals, which are known to alter pigments. High-pressure treatment and storage up to 21 days at 4°C did not cause any significant difference in carotene content of blend of orange-lemon-carrot juice (Garcia et al., 2001).

FUNCTIONS OF CAROTENOIDS

The Role of Carotenoids in Photosynthesis

Carotenoids are present in all photosynthetic organisms, where they play vital and crucial role in photosynthesis. They are involved in photosystem assembly, light harvesting, and provide protection from excess light through energy dissipation and free radical detoxification, which helps in limiting membrane damage.

Xanthophylls are bound to antenna of photosystem II (PSII) where they are responsible for folding and stability of antenna proteins (Plumley and Schmidt, 1987). The light harvesting antenna is composed of major and minor light harvesting complex (LHC) apoproteins (Jansson, 1999). Based on a combination of structural studies on in vitro reconstitution of recombinant proteins and analysis of pigment binding to native LHC's four distinct carotenoid binding sites in LHC's- L1, L2, N1, and V1 have been identified (Bassi and Caffari, 2000). Lutein binds to L1 and L2 and N1 is specific to neoxanthin, and they play an important role in harvesting of light energy by absorbing in a region of visible spectrum and transferring it to chlorophyll molecules. The V1 site is proposed to bind to violaxanthin under high light stress conditions. The pigments bound to V1 have been shown to be involved in "Xanthophyll cycle" activity induced by high light stress, thus acting as a regulator in light harvesting process (Caffarri et al., 2001).

Lutein is also required for the correct assembly of LHC holocomplex and loss of LHC trimers in the absence of lutein has been reported (Lokstein et al., 2002; Rissler and Pogson, 2000). Lutein, violaxanthin, and neoxanthin also bind to minor LHC antennae proteins (Bassi and Caffari, 2000; Ruban et al., 1999; Bassi et al., 1999). β-carotene is the only carotene found in the core complex of PS II along with small amounts of lutein (Alfonso et al., 1994). β-carotene plays a dual role at the reaction center either by providing incoming photons to the reaction center (Hanley et al., 1999; Vrettos et al., 1999) or quenching singlet oxygen for repair of PS II (Anderson and Chow, 2002; Tefler, 2002).

The role of carotenoids in plastid development and differentiation were studied by Joyard et al. (1998) and Park et al. (2002). Xanthophylls lutein and zeaxanthin have been shown to prevent lipid peroxidation in thylakoid membranes thus maintaining its fluidity and themostability.

Carotenoids also protect plants against photo-oxidative damage by dissipating excess energy via nonphotochemical quenching (NPQ) mediated by xanthophylls as well as quenching of triplet chlorophyll by carotenoids. Lutein plays an important role in quenching chlorophyll triplet state and singlet state oxygen during maximum daylight when the reaction center is saturated. Zeaxanthin carries out three functions during high light conditions, namely protection against photo-oxidation due to physical quenching of oxygen singlet energy, absorption of chlorophyll triplet energy, and transfer of photons to neighboring chlorophyll molecules, thereby increasing the overall absorption spectrum of the photosytem (Niyogi, 1999).

Health Benefits of Carotenoids

Carotenoids have a broad range of functions, especially in relation to human health and their role as biological antioxidants. The mechanism of disease prevention by carotenoids with regard to eye health, cancer, and cardiovascular disease is presented in Table 7.

Provitamin A Activity

This is the best-established function of carotenoids and almost 50 carotenoids with β-ionone end groups, such as β-carotene, α-carotene, and β-cryptoxanthin have provitamin A

Table 7 Mechanism of disease prevention of carotenoids

Disease	Mechanism of action	Carotenoid associated	Source
Eye health xerophthalmia	quenching active oxygen species	Lutein Zeaxanthin	Brown et al., 1999
Cataract and macular degeneration	protect the macula from blue light-induced damage and scavenge free radicals	Lutein Zeaxanthin	Brown et al., 1999
CVD	Reduction of LDL oxidation Reduction of oxidative stress at plaque side Reduction of lipoprotein sensitivity to oxidative damage	β-carotene, lycopene and combination of various carotenoids	Kohlmeier and Hastings, 1995; Hadley et al., 2003; Rissanen et al., 2002
Lung cancer	1. Inhibits cell proliferation, transformation, and micronucleus formation 2. Modulates expression of certain genes leading to tumor formation	β-carotene wide range of carotenoid β-carotene	Wright et al., 2003
Breast cancer	3. Inhibition of cell cycle progression at G1 phase	lycopene	Dorgan et al., 1998; Nahum et al., 2001
Prostate cancer		Lycopene	Giovannucci et al., 1995; Van Breeman et al., 2002
Colorectal cancer		β-carotene, lutein	Potter et al., 1993; Slattery et al., 2000
Light-induced erythema	filtering of blue light and scavenging reactive intermediates	β-carotene, lycopene, lutein	Gollnick et al., 1996; Stahl and Sies, 2002; Eichler et al., 2002

activity. After ingestion, carotenoids are cleaved by an intestinal 15–15′-dioxygenase and monooxygenase to form retinal (Olson and Hayaishi, 1965; Nagao et al., 1997).

The efficiency of the bioconversion of pro-vitamin A to vitamin A has recently been re-evaluated to establish the amount of carotenoids required in the diet to prevent vitamin A deficiency. Earlier it was reported that 6 mg of β-carotene in oil or 12 mg in mixed foods has the same vitamin A activity as 1 mg retinal (FAO food balance sheets and the FAO/WHO conversion rates), but the recent studies indicate that, instead of 12 mg, 21 mg of β-carotene have the same vitamin A activity as 1 mg of retinal suggesting that effective vitamin A intake is lower than recommended (West et al., 2002). Therefore, to combat vitamin A deficiency only vitamin A supplementation is not sufficient, and food-based approaches, including food fortification, and possibly the introduction of new cultivars of plants with enhanced provitamin A activity must be adapted.

Antioxidant Activity

Carotenoids are considered to be most potent quenchers of singlet oxygen (Boileau et al., 1999; Paiva and Russel, 1999) and can react with any of the radical species likely to be encountered in the biological system such as hydrogen peroxide, singlet oxygen, nitrogen oxides, super oxide anion, and other reactive oxygen species (Fig. 4). In a majority of these reactions, carotenoids break down to biologically active degradation products (Krinsky and Yeum, 2003).

The polyene chain length of carotenoids is chemically responsible for quenching the singlet oxygen. Other factors which contribute to antioxidant activity include its isolated double bond, open chain, and lack of oxygen substituents. It is also postulated that the number of conjugated double bonds are an effective parameter, and antioxidant activity increases as the number of conjugated double bonds increase in the carotenoids molecule (Kobayashi and Sakamoto, 1999). Lycopene with eleven conjugated and two non-conjugated double bonds is the most efficient singlet oxygen quencher of the natural carotenoids (Krinsky, 1998). Alternative mechanisms of oxidative damage protection include β-carotene as a chain-breaking antioxidant to terminate lipid oxidation and β-carotene and lutein decrease the cellular release of lactate dehydrogenase to protect cells from lipid peroxidation and membrane damage (Martin et al., 1996).

Protection against Photo Oxidative Damage

Cataract and macular degeneration, the leading cause of irreversible blindness in aged persons, are caused by blue-light mediated free radical damage to the retina and are common across the world. The carotenoids lutein and zeaxanthin present within the entire retina and macula are responsible for the yellowish cast in the macular eye region. Lutein, the eye-protective

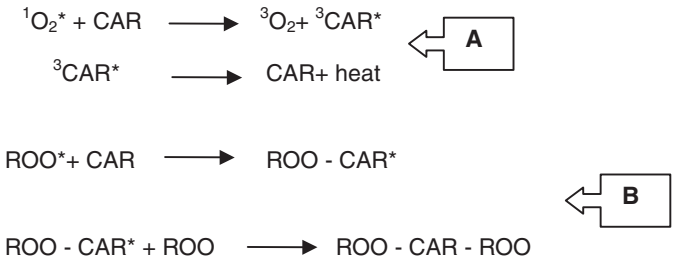

Figure 4 Reaction of carotenoid with singlet oxygen (A) and peroxy radical (B).

nutrient has been shown to be inversly associated with cataracts and age-related macular degeneration by Khachik et al. (2002). Snodderly (1995) has shown that the intake of vegetables rich in lutein and zeaxanthin gives protection against age-related macular degeneration.

Carotenoids having vitamin A activity such as α-carotene, β-carotene, and β-cryptoxanthin are good for vision health. Due to their photoprotective abilities, carotenoids are used in therapies for photosensitive diseases (Ziegler, 1993) and β-carotene is used along with vitamin E for beneficial effects. Lycopene, lutein, and zeaxanthin, even though they do not contribute to vitamin A activity, are known to be excellent antioxidants and are used in oral sun protectants that protect the exposed tissues against light-induced damages. β-carotene, lycopene, and lutein were found to reduce UV-induced lipid peroxidation of human skin fibroblast cells (Eichler et al., 2002). Sies and Stahl (2003) have attributed the mechanisms of these photoprotectants to filtering of blue light and scavenging reactive intermediates generated during photo-oxidation.

Protection against Cancer

Any alteration in the gap junction communication leads to unrestrained cell proliferation (Lila, 2004). One of the possible mechanisms of cancer preventive activity of carotenoids has been the stimulation of gap junctional communication. Carotenoids help in upregulating gap junctions by increasing the expression of connexin-43, a protein subunit present in cell membranes, thereby enhancing cell-to-cell communication (Yamasaki, 1990). Natural carotenoids are found to be more efficient in inducing gap junctional communication than synthetic ones (Stahl et al., 1997). There has also been increasing evidence that oxidation products of carotenoids, the retinoic acid analogs, also play a role in cancer preventive activity (Sies and Stahl, 2003). Carotenoids are associated with inhibition of several cancers like cervical, lung, prostate, colorectal, stomach, pancreatic, and esophagus.

Dietary intake of tomato and its products, which are rich in lycopene, has been associated with decrease in the risk of chronic diseases such as cancer and cardiovascular disease (Rao and Rao, 2007). This evidence is based on epidemiological, tissue culture, and animal studies and clinical trials. Giovannuci (1999) has demonstrated that an increased intake of lycopene and increase in the levels of serum and tissue lycopene are inversely related to various cancers such as prostrate, breast, cervical, ovarian, liver, and other organs and the risk of cancers was found to reduce significantly. Tissue culture studies with human cancer cell lines in the presence of lycopene have shown inhibition in cell growth (Prakash et al., 2001; Karas et al., 2000). Animal studies have also revealed an inverse association between dietary lycopene and growth of spontaneous and transplanted tumors (Sharoni et al., 1997; Nagasawa et al., 1995). Moreno et al. (2002) have reported that supplementation of β-carotene and vitamin-A inhibited cell proliferation in carcinogenesis. Kotake-Nara et al. (2001) demonstrated the potential of carotenoids in inhibiting the proliferation of human prostate cancer cell lines. The intake of lutein and zeaxanthin has also been linked to reduced risk of prostate cancer (Cohen et al., 2000).

Capsanthin diester and capsorbin diester from *Capsicum annuum* have been shown to possess potent in vitro anti-tumor-promoting activity with inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Further, capsanthin and related compounds also exhibited potent anti-tumor-promoting activity in an in vivo mouse skin two-stage carcinogenesis assay using 7, 12-dimethylbenz[a]anthracene as an initiator, and TPA as a promoter (Maoka et al., 2001).

Protection against Cardiovascular Disease

Cholesterol levels in the serum and oxidation of low-density lipoprotein (LDL) are thought to play a key role in the development of atherosclerosis leading to heart attack and ischemic strokes (Witztum, 1994; Parthasarathy et al., 1992). The role of lycopene in the adipose tissue and acute myocardial infraction has been evaluated by various studies (Kohlmeier et al., 1997; Kohlmeier and Hastings, 1995). In subjects consuming tomato paste, tomato juice, and lycopene oleoresin capsules, lycopene significantly reduced the levels of oxidized LDL (Agarwal and Rao, 1998). Fuhramn et al. (1997) have shown that antioxidant mechanism is not the only mechanism responsible for the ability of β-carotene or lycopene to reduce the LDL cholesterol levels, but a feedback mechanism to inhibit HMG-CoA reductase also occurs. The work has also shown that 10 μM concentration of either β-carotene or lycopene lowers the risk of cardiovascular disease (CVD) by inhibiting cholesterol synthesis in macrophage cell lines.

Protection against Other Diseases

Carotenoids also play an important role in boosting the immune response. Hughes et al. (1997) have shown the influence of β-carotene on the immune response by monocytes, TNF-α, and T-helper cells. Several studies also report the other mechanisms by which carotenoids bolster the immune system such as their roles in increasing lymphocytes response to mitogens, increasing natural killer cell activity in aging cells, increasing total white blood cells, and CD4/CD8 ratio in HIV infected persons (Boileau et al., 1999).

Epidemiological studies have shown that oxidative stress is associated with osteoporosis and that antioxidants may counteract this effect. The stimulatory effects of lycopene on the cell proliferation as well as inhibitory effects on osteoclasts formation and resorption give an evidence of lycopene involvement in bone health (Ishimi et al., 1999; Rao et al., 2003; Kim et al., 2003). A direct relation between serum lycopene and decrease

in the risk of osteoporosis among postmenopausal women has also been reported (Rao et al., 2007).

Various investigations on the role of lycopene in other human diseases such as decreasing the hypertension (Paran, 2006), neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and vascular dementia (Foy et al., 1999) has also been reported. Significant reduction in the levels of lycopene was observed in these types of pathological conditions indicating an increased free-radical activity.

CAROTENOID BIOSYNTHESIS

The biosynthetic pathways involved in carotenoids formation were elucidated in the middle of the last century using various classical biochemical and mutational studies (Hirschberg, 2001). Various modern molecular and biochemical techniques have facilitated functional complementation of genes leading to the creation of transgenic plants. These studies have enhanced the knowledge of carotenoid biosynthesis, its regulation, and the enzymes involved in the pathway (Cunningham and Gantt, 1998).

Formation of Carotenoids

In higher plants carotenoids are synthesized and localized in plastid and are biosynthetically linked to other isoprenoids such as tocopherol, phylloquinones, gibberellins, chlorophylls, and abscisic acid via the five carbon compound isopentenyl diphosphate (IPP) (Fig. 5) (Fraser and Bramley, 2004). Originally it was believed that all isoprenoids were produced by using mevalonate (MVA) as a precursor of IPP, which is synthesized from acetyl-CoA via mevalonic acid (McGarvey and Croteau, 1995), but later, the MVA-independent pathway for the formation of IPP was also discovered, which involves 1-deoxy-D-xylulose-5-phosphate (DXP) (Eisenreich et al., 2001). Eukaryotes, with the exception of the photosynthetic eukaryotes, use the MVA pathway for the isoprenoid synthesis (Delgado-Vargas and Paredes-Lopez, 2003). The mevalonate-independent Methyl erythritol 4-phosphate (MEP) pathway is now well established in the plants as well as many bacteria (Rodriguez-Concepcion and Boronat, 2002). MVA is operational in cytoplasm, whereas the MEP pathway is chloroplastidic in nature. Recent studies reveal that plastid isoprenoids at some developmental stages can arise partially from the

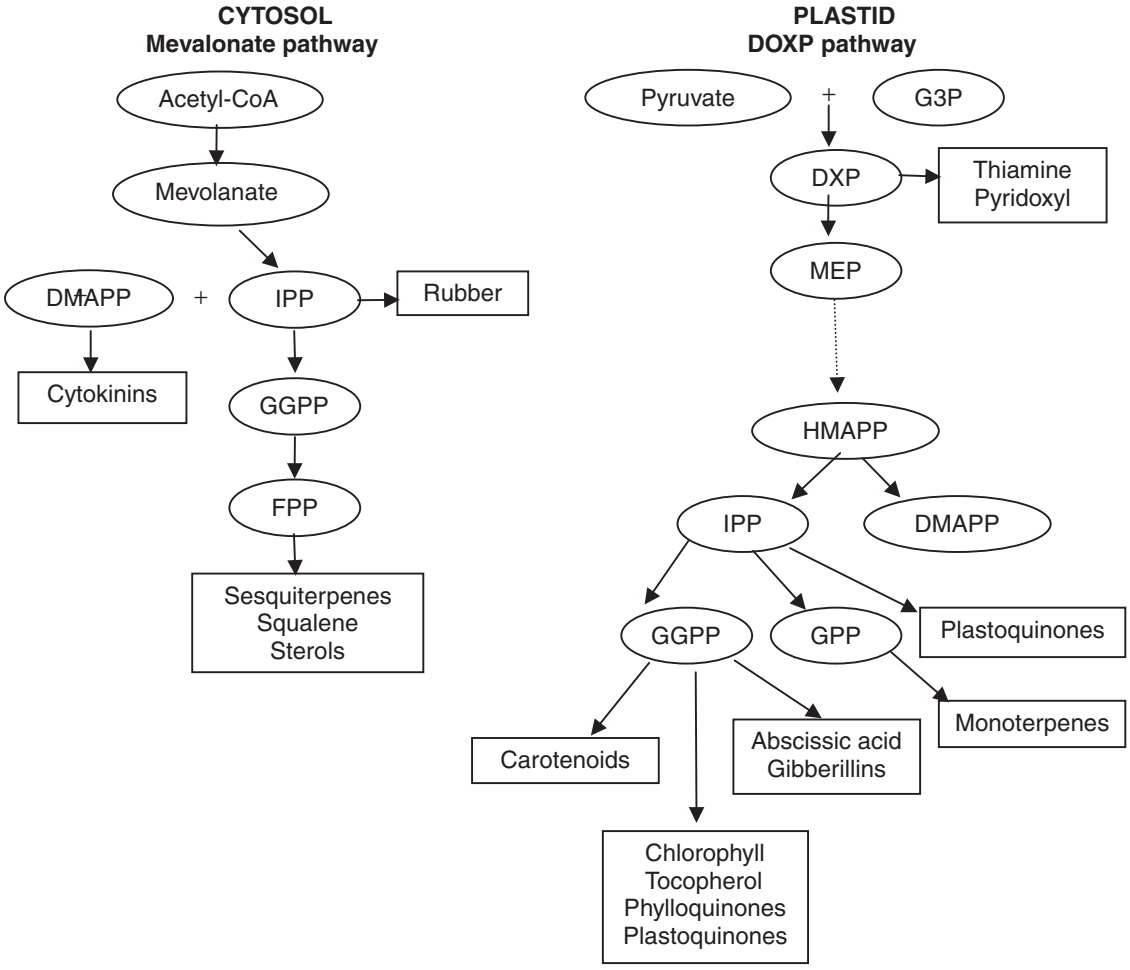

Figure 5 Biosynthesis of isoprenoid compounds in higher plants.

MVA pathway also (Kasahara et al., 2002, Fraser and Bramley, 2004).

The first reaction of the MEP pathway is transketolase condensation of pyruvate with glyceraldehyde-3-Phosphate (G3P) to yield 1-deoxy-D-xylulose 5-phosphate in the presence of 1-deoxy-D-xylulose 5-phosphate synthase (DXPS). This enzyme preferentially functions at an early stage of leaf development (Araki et al., 2000). DXP, a precursor of various vitamins (Rohmer, 1999), is subsequently reduced to form Methylerythritol 4-phosphate (MEP) (Arigoni et al., 1997). MEP is considered the first committed precursor of the pathway. Carotenoids are mostly produced from MEP derived precursors in light-grown plants (Rodriguez-Concepcion and Boronat, 2002; Eisenreich et al., 2001; Lichtenthaler, 1999). Hydroxymethylbutenyl diphosphate (HMBPP) is formed from MEP by the enzyme HMBPP synthase (HDS). HMBPP is finally converted by the enzyme HMBPP reductase (HDR) into a 5:1 mixture of IPP and dimethyl allyl pyrophosphate (DMAPP) (Botella-Pavia et al., 2004). These prenyl-diphosphate units can also be formed from each other in a reversible reaction catalyzed by the enzyme IPP isomerase (IPI) (Fraser and Bramley, 2004). The remaining enzymes are localized in plastids and encoded by nuclear gene (Botella-Pavia and Rodriguez-Concepcion, 2006; Eisenreich et al., 2004). In plants GGPP represents a major branching point to the formation of different isoprenoid products, and it could be expected that some of the plastid-localized GGPP synthase (GGPS) enzymes which catalyze the production of GGPP from IPP and DMAPP, might have carotenoid-specific isoforms.

Geranylgeranyl diphosphate (GGPP), a common precursor for several groups of plastid isoprenoids, is formed by combining three IPP molecules with one DMAPP unit (Poulter and Rilling, 1981). Condensation of IPP with DMAPP in the presence of IPP isomerase in a head to tail manner makes the C-10 compound, Geranyl Pyrophosphate (GPP), and the addition of IPP to GPP results in Farnesyl pyrophosphate (FPP, C-15 compound). The addition of one more IPP yields GGPP (C-20 compound) catalyzed by GGPP synthase (characterized in *Capsicum*, *Arabidopsis, Catharanthus,* and *Lupius*). Two GGPP molecules not only make a 40-carbon isoprenoid polyene chain, the backbone of most of the plant carotenoids, but also determine their physical and biological properties (Britton, 1995). The most important step in the carotenoid biosynthesis pathway is the head-to-head condensation of two GGPP molecules to form phytoene (Fig. 6) through an intermediate pre-phytoene pyrophosphate (Cunningham and Gantt, 1998; Hirschberg, 2001). This two-step reaction is catalyzed by phytoene synthase (PSY) which is membrane bound. Phytoene (C-40 compound), a colorless symmetrical hydrocarbon accumulates in all carotenogenic plants as the 15-*cis* geometric isomer (Than et al., 1972). Biochemical characterization of phytoene synthase has been exploited in maize (Buckner et al., 1996), daffodil (Schledz et al., 1996), and melon (Karvouni et al., 1995). In tomato, there are two genes for PSY—*Psy-1*, which encodes a fruit- and flower-specific isoform, and *Psy-2*, which encodes an isoform that predominates in green tissues (Bartley and Scolnik, 1993; Fraser et al., 2000). PSY is a rate-limiting enzyme of carotenoid biosynthesis in ripening tomato fruits (Bramley et al., 1992; Fraser et al., 1994), in canola seeds (Shewmaker et al., 1999), and in marigold flowers (Moehs et al., 2001). This rate-limiting feature makes PSY suitable to be a key regulator of carotenogenesis.

Phytoene undergoes a series of four desaturation reactions to form phytofluene, ζ-carotene, neurosporene, and finally lycopene. These desaturation reactions serve to lengthen and increase the conjugated series of carbon-carbon double bonds that constitutes the chromophore in carotenoid pigments, and thereby transform the colorless phytoene into the pink-colored lycopene. The four sequential desaturations are catalyzed by two related enzymes in plants; phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). In contrast, bacteria and fungi achieve the same result with a single gene product (Sandmann, 1994). PDS has conserved the dinucleotide binding site domain at amino terminus which was confirmed after being identified from tomato (Pecker et al., 1992), pepper (Hugueney et al., 1992), and daffodil (Fraser and Bramley, 2004). ZDS isolated from pepper (Albrecht et al., 1995), maize (Luo and Wurtzel, 1999), and *Arabidopsis* (Scolnik and Bartley, 1996) catalyze ζ-carotene into lycopene via neurosporene. PDS and ZDS both have amino acid sequence signatures that are conserved (33-35%) in pyridine nucleotide-disulphide oxidoreductases (Fraser and Bramley, 2004), and an isolated pepper chromoplast PDS was shown to bind added FAD (Hugueney et al., 1992). The desaturases are found to be membrane-associated in plants (Bramley, 1985). A carotenoid isomerase (CRTISO) activity is additionally required in Arabidopsis etioplasts and tomato (*Solanum lycopersicum*) chromoplasts to transform the poly *cis* lycopene (pro-lycopene), a product of PDS and ZDS activities to the all-*trans* isomer (lycopene) found in plant cells (Issacson et al., 2004). The bacterial phytoene desaturase (*Crt I*) however, produces all *trans*-lycopene (Bartley et al., 1999).

A branching point in the plant carotenoid pathway is marked by the cyclization of lycopene. Lycopene β-cyclase (LCYB/ CRTL-B) catalyzes to produce β-carotene in a two-step reaction that creates one β-ionone ring at each end of the lycopene molecule. In the other branch, δ-carotene is produced by the addition of one ε-ring to lycopene in the presence of lycopene ε-cyclase (LCYE/ CRTL-E). The addition of a β-ring to the other end of δ-carotene catalyzed by LCYB leads to the production of α-carotene, whereas LCYE produces γ-carotene by the addition of another ε-ring. In tomato, there are two lycopene β-cyclase enzymes, LCY-B (CRTL-B) (Pecker et al., 1996) and CYC-B (chromoplast-specific lycopene cyclase) (Ronen et al., 2000), whose amino-acid sequences are 53% identical. LCY-B is active in green tissues, whereas CYC-B functions only in chromoplast-containing tissues (Ronen et al., 2000). As compared to LCY-B, tomato CYC-B is more similar (86.1% identical in amino acid sequence) to capsanthin-capsorubin synthase (CCS) from pepper, an enzyme that converts antheraxanthin and violaxanthin to the red xanthophylls capsanthin and capsorubin, respectively (Bouvier et al., 1994). A comprehensive study with daffodil plastid suggests that all-*trans* lycopene is

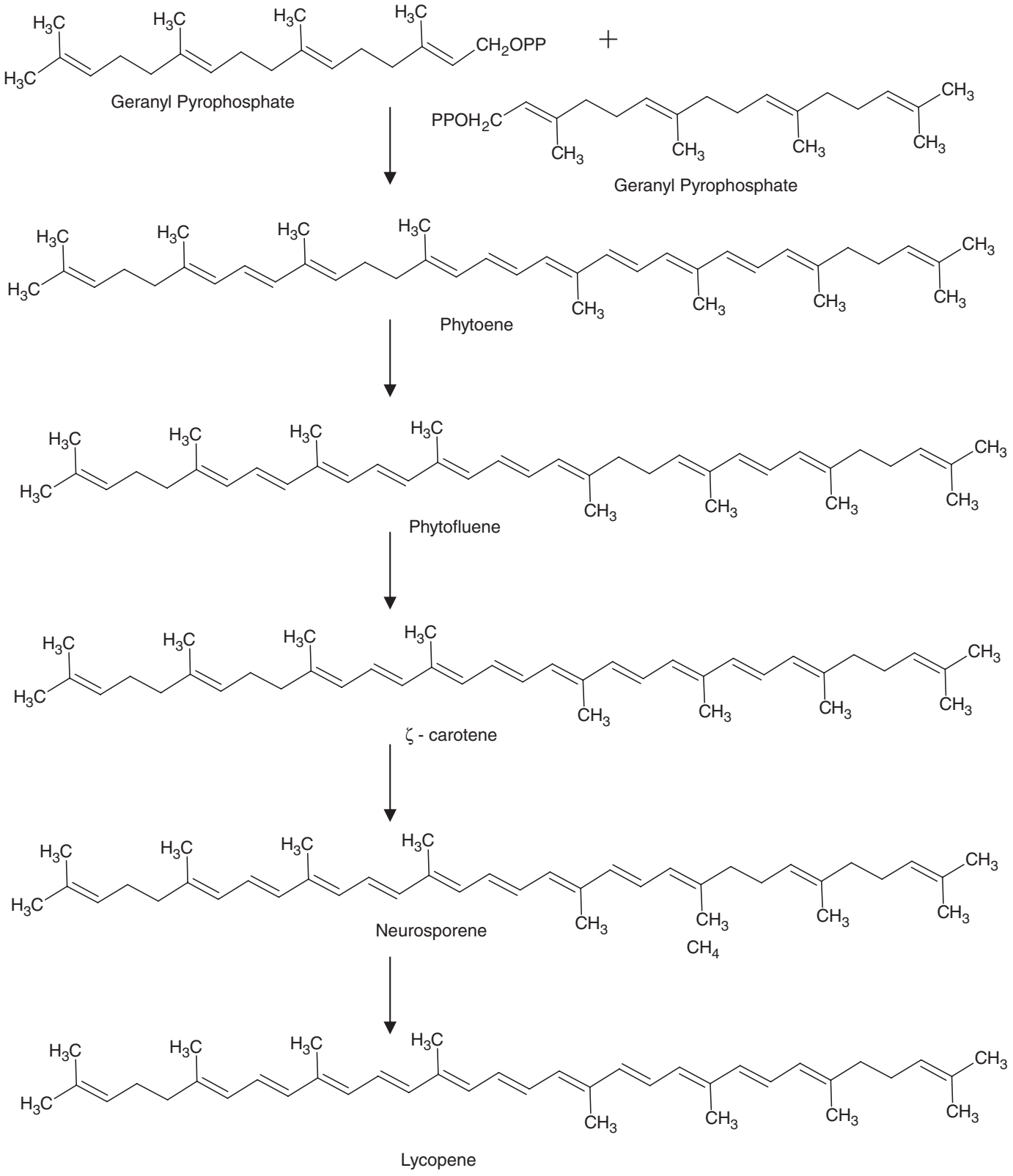

Figure 6 Lycopene synthesis from two molecules of geranylgeranyl pyrophosphate.

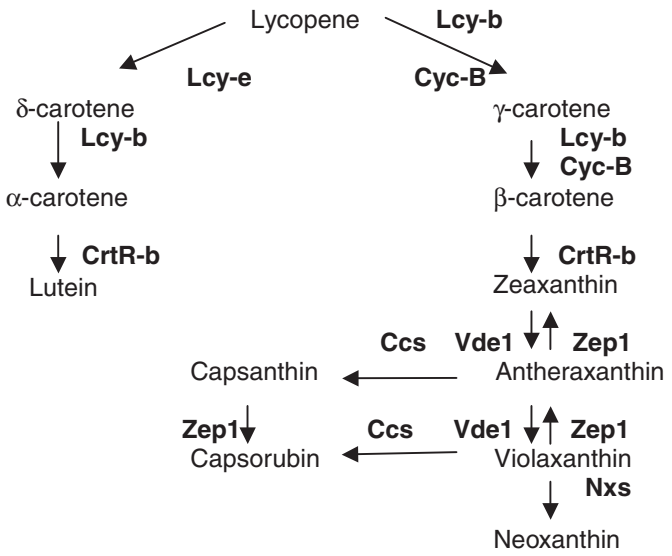

Figure 7 Carotenoid biosynthetic pathway from lycopene.

substrate for cyclization with NADPH as cofactor (Beyer et al., 1991), while cell free cyclase activity has also been observed in tomato (Fraser et al., 2000) and pepper chromoplast (Camara and Dogbo, 1986). Both LCYB and LCYE from *Arabidopsis* and tomato have significant resemblance of 30% at the amino acid level. The lettuce LCYE enzyme, a close homologue of the *Arabidopsis* and tomato proteins, produces the bicyclic ε, ε-carotene (lactucaxanthin) rather than δ-carotene from lycopene (Cunningham and Gantt, 2001).

Zeaxanthin and lutein, the well-known xanthophylls, are produced by hydroxylation at the C-3 position of each ring of β-carotene and α-carotene via α-cryptoxanthin and β-cryptoxanthin. Introduction of hydroxyl moieties into β-ring carotenes is catalyzed by hydroxylases (Fraser and Bramley, 2004; Botella-Pavia and Rodriguez-Concepcion, 2006). Carotenoid β-ring hydroxylases (CHYB) found in pepper (Bouvier et al., 1998) and tomato (Hirschberg, 1998) have invariably been shown to be non-heme di-iron monooxygenases, whereas the ε-ring hydroxylase (CHYE) recently identified in *Arabidopsis* is a cytochrome P450-type monooxygenase (Tian et al., 2004).

Zeaxanthin is readily converted to violaxanthin via antheraxanthin (Fig. 7) by the introduction of 5, 6- epoxy groups into the 3-hydroxy β-rings, a reaction catalyzed by the enzyme zeaxanthin epoxidase (ZEP) that requires reduced ferredoxin (Bouvier et al., 1996). In leaves exposed to strong light, violaxanthin deepoxidase (VDE) catalyzes the two-step de-epoxidation reaction that transforms violaxanthin back to zeaxanthin (Pfundel et al., 1994). This is reversible reaction and under low-light conditions transformation of zeaxanthin into violaxanthin can occur. This interconversion of zeaxanthin and violaxanthin (xanthophyll cycle), is a key for plant adaptation to changing environmental conditions (Demmig-Adams et al., 1996). In the last step of the carotenoid biosynthesis pathway, violaxanthin is transformed into neoxanthin by the activity of neoxanthin synthase (NSY) (Bouvier et al., 2000). Genes encoding NSY have been reported in potato (Al-Babili et al., 2000) and tomato (Bouvier et al., 2000).

Capsanthin and Capsorubin, two characteristic ketoxanthophylls with an unusual cyclopentane κ-ring, are unique to ripened fruits of pepper (*Capsicum annuum*). The pepper chromoplast associated enzyme capsanthin–capsorubin synthase (CCS), transforms antheraxanthin and violaxanthin into capsanthin and capsorubin, respectively (Bouvier et al., 1994). CCS is similar to tomato CYCB and posseses β-cyclase activity (Lefebvre et al., 1998).

Degradation of Carotenoids

The amount of carotenoids found in a plant tissue represents a steady-state value determined by biosynthesis and metabolization. Control of the degradation processes may assist in maintaining high carotenoid concentrations. In addition to non-enzymatic degradation especially by the invasive effect of light, defined enzymatic cleavage reactions can cause the degradation of carotenoids. Some of these degradation products occur as phytohormones, defence compounds, and volatile aromas (Sandmann et al., 2006). Violaxanthin and neoxanthin are identified as a precursor of abscisic acid (Schwartz et al., 2004) and *cis*- xanthoxin in maize (Schwartz et al., 1997). An oxidative cleavage product of lycopene, bixin dialdehyde, is used for the production of bixin, a red-colored apocarotenoid, widely used in the food and cosmetic industry. The cDNA coding the enzyme lycopene dioxygenase has been isolated from *Bixa orellana* seeds (Bouvier et al., 2003a). Oxidative cleavage of zeaxanthin leads to the formation of crocetin in *Crocus sativus* (Bouvier et al., 2003b). One of the cleavage products, bluemenin, is found to possess biological activity (Fester et al., 1999) and some may also act as signalling molecules (Sorefan et al., 2003).

Carotenoid Biosynthetic Pathway Regulation

The important role played by carotenoids and their metabolites in photosynthesis, plant development and adaptation, and the nutritional and sensory quality of plant storage organs is well known (Britton, 1995; Sandmann et al., 2006), but many more studies are required to understand the regulation of carotenoid biosynthesis at the gene and enzyme level in plants. The identification of *Or* gene in cauliflower (Li et al., 2001) and the apricot (*Ap*) gene in a tomato mutant (Jenkins and MacKinney, 1995) look promising in unraveling few of the regulatory mechanisms. As the pathway contains many steps, and the isoprenoid precursors of carotenoid biosynthesis are shared with a large range of other non-carotenoid compounds which serve important functions, carotenoid regulation is likely to occur at several levels and may involve both transcriptional and post-transcriptional events.

Transcriptional and Post-Transcriptional Regulation

1-Deoxyxylulose-5-phosphate serves not only as a precursor for IPP synthesis, but also for thiamine and pyridoxyl, which are essential for the function of plastids (Julliard, 1992; Julliard and Douce, 1991). Therefore the formation of ME4P from DX5P has been considered as the first regulatory step in IPP formation (Lange and Croteau, 1999; Takahasi et al., 1998). The DXS mRNA accumulation is observed when there is a change in fruit color from mature green to orange, indicating that DXS is induced at the onset of ripening. Based on this strong correlation of DXS expression pattern and accumulation of carotenoids, Lois et al. (2000) contemplated that DXS activity may be the rate-limiting step in carotenoid biosynthesis during ripening of tomato.

Romer et al. (2000) observed that as the β-carotene content increases, the total carotenoids in tomato decrease suggesting a feedback inhibition in the pathway. The higher β-carotene production observed in the transgenic tomato plants due to reduced PSY enzyme activity and corresponding reduction in PSY gene expression suggested that the regulation of pathway occurs at PSY level (Romer et al., 2000). A similar observation was also made by Fraser et al. (1994) in tomato and concluded that PSY is a rate-limiting enzyme. PSY1 is considered as a regulatory enzyme in fruit carotenoid biosynthesis (Bartley and Scolnik, 1995). In red ripe fruits of tomato, DXS and PSY1 show a similar expression pattern suggesting a coordinated role of these enzymes in carotenoid biosynthesis during ripening (Lois et al., 2000). However, the kinetics of DXS and PSY1 transcript accumulation shows a different pattern during transition from the growing to the ripening stage. The mRNA levels of PSY1 begin to increase in mature green fruits before the onset of first color change (Giuliano et al., 1993; Ronen et al., 1999; Lois et al., 2000). The induction of PSY1 before DXS expression and the changes in carotenoid intermediates and/or end products due to PSY activity might be involved in controlling the expression of DXS during ripening of fruit (Lois et al., 2000). Thus the induction of PSY1 transcript accumulation by DXS feeding and the modulation of DXS expression by PSY1 activity during the last stage of fruit development suggest a significant cross talk between PSY1 and DXS activities that may contribute to the fine regulation of carotenoid accumulation in tomato fruit. Based on these observations, Lois et al. (2000) proposed that DXS rather than PSY1 would control the start of massive carotenoid accumulation when tomato fruit enters the ripening phase. In the young fruits, the coordinated activities of these two enzymes result in the production of carotenoids for photosynthesis related processes, but in mature green fruits, the DXS transcript level does not significantly change despite the higher level of PSY1 activity and no significant increase in carotenoids occur probably because DXS activity does not provide enough DX5P (the substrate of PSY) to be channeled to GGPP. In ripe fruit the high increase in both DXS and PSY1 expression correlates to massive accumulation of carotenoids.

In tomatoes higher levels of expression of genes in the central isoprenoid pathway during early fruit development has been reported (Lois et al., 2000; Josse et al., 2000). At the "breaker" stage of ripening, the color changes from green to orange because of the accumulation of lycopene, due to increased synthesis of carotenoid enzymes. The mRNA levels for the enzymes that produce lycopene (PSY and PDS) increase 10–20-fold at the breaker stage of ripening, but the mRNAs of both lycopene cyclases, *Lcy-b* (*CrtL-b*) and *Lcy-e* (*CrtL-e*) disappear (Pecker et al., 1992; Giuliano et al., 1993; Pecker et al., 1996; Bramley, 1997; Ronen et al., 1999). It has been established that the increase in *Psy* and *Pds* expression during the breaker stage is controlled by transcriptional regulation (Corona et al., 1996). Namitha et al. (Unpublished data) also reported differential expression of carotenoid biosynthetic pathway genes in ripening tomato fruits. The hypothesis that differential gene expression is the major reason for the accumulation of lycopene is further corroborated by the accumulation of δ-carotene in the fruits of the *Delta* tomato mutant, which results from increased transcription of *CrtL-e* (*Lcy-e*) (Ronen et al., 1999), and by the synthesis of β-carotene in fruits of the *Beta* tomato mutant, which is caused by the upregulation of a second lycopene β-cyclase gene, *Cyc-b* (Ronen et al., 2000). A similar upregulation of carotenoid gene expression during fruit development has been found in bell pepper (Kuntz et al., 1992; Bouvier et al., 1996; Bouvier et al., 1998), melon (Aggelis et al., 1997), and *Satsuma mandarin* (*Citrus unshiu* Marc) (Ikoma et al., 2001). Transcriptional upregulation of carotenoid biosynthesis genes also appears to be the major regulatory mechanism in carotenogenesis that takes place in tomato (Pecker et al., 1996; Ronen et al., 2000; Giuliano et al., 1993; Bramley, 1997; Ronen et al., 1999), tobacco (Mann et al., 2000), daffodil (Schledz et al., 1996), and marigold (Moehs et al., 2001). Regulation at the enzymatic level is predicted to account for the higher concentration of lycopene in fruits of the tomato mutants *old-gold* (Ronen et al., 2000) and *old-gold-crimson* (Bramley, 2003).

Light Regulation

Light intensity alters carotenoid formation by increasing the expression of certain carotenoid biosynthetic genes (Grumbach and Lichtenthaler, 1982). Also, under high light conditions carotenoid degradation in leaves may exceed the capacity of biosynthesis (Simkin et al., 2003a). Control of carotenogenesis by light is predominately regulated at the transcriptional level. The dark-light grown leaves show only trace amounts of the mRNA for Psy and Pds compared to illuminated leaves in pepper whereas the later carotenogenic genes like Zds or lycopene cyclase were down-regulated to a much lesser extent by light intensity, confirming that the down-regulation of Psy is an influential step in regulating carotenoid biosynthesis (Simkin et al., 2003b). A good correlation between the amounts of Pds mRNA and the enzyme level was observed in tomato after a prolonged dark period. Enhanced levels of Psy mRNA in the light have

been reported for tomato leaves (Giuliano et al., 1993), *Sinapis alba* and *A. thaliana* (Von Lintig et al., 1997), which may operate through a phytochrome-mediated process (Sandmann et al., 2006). In addition, light-induced membrane association is believed to regulate phytoene synthase activity during photomorphogenesis in plants (Welsch et al., 2000). Changes in Lcy-b and Lcy-e mRNAs have also been reported due to changes in light intensity (Ronen et al., 1999). The breaker tomato discs exposed to red light showed 50% higher accumulation of carotenoids over dark and Red /Far Red treated discs after 14 days of incubation period. Irrespective of treatment, mRNA transcript levels of Psy1 and DXS increased 2–4-fold respectively, indicating that phytochrome regulation of carotenogenesis is not through transcriptional regulation of Psy1 or DXS (Schofield and Paliyath, 2005). Ninu et al. (1999) also suggested that although carotenoid biosynthesis is necessary for the development of chloroplasts, the expression of carotenoid genes collectively is neither light-dependant nor controlled by cryptochrome 1 genes.

The light regulation of *Haematococcus* carotenoid biosynthesis and gene expression of lycopene cyclase, phytoene synthase, phytoene desaturase, and carotenoid hydroxylase was analyzed in green flagellate cells by Steinbrenner and Linden (2003). All four genes revealed higher transcript levels in response to increased illumination. Not only was the induction of astaxanthin biosynthesis but also carotenoid gene expression found to be correlated with the redox state of the photosynthetic electron transport. Accordingly, increased transcript levels for carotenoid biosynthesis genes were detected under both blue and red light conditions suggesting that in *Haematococcus* not only the specific astaxanthin pathway but also general carotenoid biosynthesis is subjected to photosynthetic redox control. A similar pattern of up-regulation of phytoene synthase and phytoene desaturase genes in response to light was observed, due to transcriptional control in unicellular green alga *Chlamydomonas reinhardtii* (Bohne and Linden, 2002). The transcript levels of both the genes were upregulated only on illumination with blue light but not with red light.

The cyanobacterium *Synechococcus* also showed an up-regulation of carotenoid biosynthesis in the presence of strong light. Realtime PCR studies demonstrated that regulation of genes encoding phytoene synthase; phytoene desaturase, ζ-carotene desaturase, and β-carotene hydroxylase are at transcriptional level. The mRNA levels of the first three follow a similar kinetics, whereas the mRNA level of β-carotene hydroxylase increased much faster upon transfer to high light. The promoter activity of the phytoene desaturase/synthase operon was higher under strong light conditions (Schaffer et al., 2006).

Other Regulatory Factors

Expression of carotenogenic genes in pepper fruits is enhanced by oxidative stress, which facilitates carotenoid synthesis during chromoplast differentiation (Hugueney et al., 1996; Bouvier et al., 1998). Various stresses like nutrient depletion (Grunewald et al., 2000); reactive oxygen species, which act as a secondary messenger in the induction and control of chromoplast specific gene expression in pepper (Hugueney et al., 1996); and salt stress and high-light intensities (Steinbrenner and Linden, 2001) in alga *Haematococcus pluviaris* are also reported to enhance carotenoid content.

The sequestration of carotenoids within the cell is a form of regulation, which also facilitates higher accumulation of carotenoids. In chloroplasts, end-product carotenoids are associated with light-harvesting complexes (Kuhlbrandt, 1994). The unbound carotenoids are likely to associate with specific proteins as small plastoglobules (Milicua et al., 1991; Liu et al., 2004). In chromoplasts of pepper, end-product carotenoids are typically esterified and associated with the fibrillin protein as fibrils (Deruere et al., 1994) and in tomato chromoplasts lycopene appears as crystals (Waters et al., 2004). In transgenic canola (Shewmaker et al., 1999), tobacco (Ray et al., 2000), tomato (Romer et al., 2000), and potato (Ducreux et al., 2005) plastid ultrastructure were altered in response to higher accumulation of carotenoids. Similarly, esterification of carotenoids has been reported to enhance the carotenoid levels in tobacco (Mann et al., 2000), daffodil (Schledz et al., 1996), marigold (Moehs et al., 2001), annatto (Bouvier et al., 2003a), and *Haematococcus pluvialis* (Grunewald et al., 2001).

METABOLIC ENGINEERING OF CAROTENOIDS IN MICROORGANISMS

A number of carotenogenic genes have been cloned from microorganisms as well as plants and expressed in *E. coli* thereby allowing the recombinant biosynthesis of various acyclic, cyclic, and oxygenated carotenoids. By combining genes from various organisms, different carotenoids can be produced in recombinant microorganisms (Misawa and Shimada, 1998; Sandmann et al., 1999). Metabolic engineering and molecular reshuffling of carotenoid biosynthetic genes have resulted in the production of novel carotenoids (Schmidt-Dannert et al., 2000; Umeno et al., 2005).

Most of the carotenogenic genes employed in the recombinants are either from *Erwinia* or *Rhodobacter* species (Sandmann, 1994; Armstrong, 1997). The studies on purple non-sulphur anaerobic photosynthetic bacteria (*Rhodobacter capsulatus* and *Rhodobacter sphaeroides*), non-photosynthetic bacteria (*Erwinia herbicola* and *Erwinia uredovora*), and cyanobacteria (*Synechococcus* sp., *Synechocystis* sp., and *Anabaena* sp.) have contributed immensely to the understanding of carotenoid biosynthesis at genetic and molecular level. The identification of nucleotide sequences of carotenoid genes in *Rhodobacter capsulatus* (Armstrong et al., 1989), *Erwinia herbicola* (Hundle et al., 1993), *Erwinia uredovora* (Misawa et al., 1990), and *Myxococcus xanthus* (Fontes et al., 1993) have given an in-depth knowledge of the biosynthetic pathway, pigments accumulation, and the gene functions.

E. coli

E. coli is a convenient host for carotenoids production as it has simple genetic makeup and is able to make a variety of carotenoids such as lycopene, β-carotene, canthaxanthin, and astaxanthin. Many of the carotenoid biosynthetic genes from bacteria, fungi, and higher plants are expressed in *E. coli* to study their functionality (Cunnigham et al., 1993; Ruther et al., 1997). The availability of plasmids belonging to different incompatibility groups harboring different antibiotic markers can be introduced into *E. coli*, allowing different gene combinations (Sandmann, 2000). *E. coli* as a potential candidate for carotenoids production has been reviewed by Sandmann et al. (1999). By combining genes of organisms having different carotenoid biosynthetic branches, novel carotenoids have been synthesized (Umeno et al., 2005). High titer of carotenoids such as lycopene, β-carotene, and zeaxanthin have been achieved in *E. coli* by metabolic engineering techniques (Alper et al., 2006; Yoon et al., 2006, 2007; Yuan et al., 2006).

Increase in carotenoid production in *E. coli* has been achieved by using various strategies like metabolic engineering of MEP pathway, introducing foreign MVA pathway to increase the supply of IPP for carotenoid production, metabolic engineering of prenyl diphosphate pathway, and central metabolic pathway (Das et al., 2007).

Random shuffling of bacterial phytoene desaturase (*Crt I*) and β-cyclase (*Crt Y*) genes allowed the production of variety of colored compounds including highly desaturated compound 3, 4, 3′, 4′- tetrahydro lycopene. A monocyclic terpenoid torulene was produced for the first time in *E. coli* by a new metabolic route, unknown in nature (Schmidt-Dannert et al., 2000). Using similar strategies, new bioactive compounds and carotenoids such as 1-OH-3′, 4′- didehydrolycopene, diapotorulene have been synthesized in *E. coli*, which were either inefficient to synthesize or extract in natural condition (Albrecht et al., 2000; Lee et al., 2003; Wang et al., 2000).

Yuan et al. (2006) have shown that overexpressing rate-limiting genes incorporated in the chromosome for increased IPP synthesis gives a better yield than overexpressing genes on a multi-copy plasmid. Replacing the native promoters of isoprenoid pathway genes with a strong bacteriophage T5 promoter, β-carotene as high as 6 mg/g DCW (dry cell weight) was produced in *E. coli* harboring *crtEBIY* operon. Yuan et al. (2006) also introduced a foreign MVA pathway in *E. coli* to produce increased amount of carotenoids in them. Introduction of bottom MVA pathway genes from *S. pneumoniae* produced 3-fold increase in lycopene content. Similarly β-carotene producing recombinant *E. coli* harboring MVA bottom pathway and *dxs* gene of MEP pathway produced 503 mg/L of β-carotene, which is the highest level of β-carotene in *E. coli* (Yoon et al., 2007).

Alper et al. (2005b) used a systematic computational search to increase the lycopene production in *E. coli* and obtained several single and multiple gene knockout strains based on stoitiometric analysis. A triple gene knockout strain ($\Delta gdhA\ \Delta aceE\ \Delta fdhF$) produced 6.6 mg lycopene /g DCW over its parental strain without any gene knockout. By using systematic and combinatorial gene knockout methods Alper et al. (2005a) generated an efficient triple knockout strain ($\Delta gdhA\ \Delta aceE\ \Delta pyjiD$) which produced 18 mg lycopene /g DCW. Production of zeaxanthin was increased in *E.coli* when an operon composed of zeaxanthin synthesis genes, assembled in *Bacillus subtilis* was introduced into them (Nishizaki et al., 2007).

Candida and Saccharomyces

The non-carotenogenic food yeast, *Saccharomyces cerevisiae* and *Candida utilis*, has also been successfully used as a host for the production of lycopene and β-carotene through genetic modification. The bacterial carotenoid biosynthetic genes (*crtE, crtB*, and *crtI* genes from *E. uredovora*) were expressed in them under the control of constitutive promoter from the host (Misawa and Shimada, 1998). The transformed yeasts produced phytoene and lycopene in the stationary phase.

METABOLIC ENGINEERING IN MODEL SYSTEMS

A gene encoding for an endogenous phytoene synthase was cloned and coupled to a seed-specific promoter in the seed of *Arabidopsis* to examine the effects of its overexpression on carotenoid content (Lindgren et al., 2003). The resulting transgenic plants produced darker seeds and had a 43-fold increase in β-carotene with significant increase in lutein, violaxanthin, and chlorophyll levels in seed extracts as compared to their non-transgenic counterparts. In addition, substantial levels of lycopene and α-carotene were produced in the seeds, whereas only trace amounts were found in control plants. Transformation of *Arabidopsis* with an algal β-carotene oxygenase also leads to the accumulation of ketocarotenoids (Stalberg et al., 2003). The major ketocarotenoids were 4-keto-lutein, adonirubin, and canthaxanthin.

Metabolic engineering of tobacco plants to produce astaxanthin has been achieved by Mann et al. (2000) by the expression of the *crtO* gene (β-carotene ketolase) from *Haematococcus pluvialis*. This enzyme catalyses the conversion of β-carotene to canthaxanthin via echinenone (Lotan and Hirschberg, 1995). Even though only trace amounts of astaxanthin were produced in chloroplasts of transgenic tobacco plants expressing *crtO* in a regulated manner, high concentration of ketocarotenoids was found to accumulate in chromoplasts of nectary tissue of flowers. The majority of ketocarotenoids, astaxanthin along with adonixanthin and adonirubin, were esterified and accounted for more than 20% of total carotenoids. Total nectary carotenoids in the transgenic plants were increased by 170% as compared to controls. The astaxanthin produced in the transgenic plants had the same chirality ($3S3'S$) as the natural astaxanthin found in marine organisms.

Ralley et al. (2004) introduced two astaxanthin biosynthetic genes (ketolase and hydroxylase) from bacterium *Paracoccus* simultaneously to tobacco and tomato. In transgenic tomato and tobacco leaves the principal ketocarotenoid was echinenone which accounted up to 900 μg/g DW in the latter. In the nectary tissues of tobacco flowers a quantitative increase of 10-fold was observed with the presence of astaxanthin, canthaxanthin, and 4-ketozeaxanthin, which predominantly occured as esters.

Introduction of a cyanobacterial ketolase gene into *Nicotiana glauca*, a tobacco species with carotenoid-containing yellow pigmented flower petals, resulted in transformed plants which accumulated 4-ketozeaxantin (adinoxanthin), the first ketocarotenoid synthesized in flower petals by genetic modification. By expression of a ketolase gene, it was possible to engineer the biosynthetic pathway towards the formation of 3′-hydroxyechinenone, 3-hydroxyechinenone, 4-ketozeaxanthin, 4-ketozeaxanthin esters, 4-ketolutein, and 4-ketolutein esters. Some of these ketocarotenoids were also formed in the leaves of the trangenic plants. The total carotenoids in nectaries increased by more than 2.5- fold when ketolase gene was co-expressed in combination with a hydroxylase gene under a ubiquitous promoter. In the transformed nectaries, more than 50% of the accumulating carotenoids were keto derivatives in free form rather than the usual esterified form (Gerjets et al., 2007).

METABOLIC ENGINEERING IN CROP PLANTS

Even though conventional plant breeding approaches have increased the productivity successfully, the advantages of genetic engineering over this method include the ability to transfer genes in a faster and targeted manner. Carotenoid profiling of the target crop will help in selecting the gene(s) for metabolic engineering. Metabolite/precursor pool sizes, enzyme activities and location, gene expression profiles, carotenoid catabolism, interaction with other isoprenoid pathways, and regulatory mechanisms influence the choice and combination of genes and promoters necessary to manipulate the pathway. Currently amplification of the enzyme with the highest flux control coefficient or the "rate-limiting" enzyme is the principal target for carotenoid manipulation. Increasing flux through the pathway (quantitative engineering) seems to be promising for increasing the end product. Changing the composition of carotenoids or creating a new carotenoid in the tissue of interest (qualitative engineering) is the other objective of metabolic engineering. A new trend of altering the carotenoid content as a result of manipulations to another pathway or biological process (pleiotrophic engineering) is also being attempted to enhance valuable carotenoids for commercial purposes (Mann et al., 2000).

Phytoene synthase catalyses the first committed step in the carotenoid pathway and has been manipulated in a number of crop speices. Its overexpression altered the level of carotenoids significantly in carrots (Hauptmann et al., 1997), tomato fruit (Fraser et al., 2002), and canola seeds (Shewmaker et al., 1999). The extent of increase varied between 1.6-fold in tomato to 50-fold in canola seeds. However, the constitutive expression of the cDNA of Psy in transgenic tomato plants led to dwarfism, due to redirection of GGPP from the gibberellin pathway into carotenoid synthesis (Fray et al., 1995). In contrast, a two-fold increase in total fruit carotenoids was achieved in tomato plants that expressed the bacterial phytoene synthase gene *Crt B* from *E. uredovora* in a fruit-specific manner (Fraser et al., 2002). The differences in origin of genes may be responsible for the differential effects of psy overexpression in both the cases. Further, bacterial and plant encoded proteins differ in their ability to form protein-protein complexes leading to different balances of the product, which may result in decrease in the flux control coefficient of *Psy* on overexpression. This may cause other steps in the pathway to become rate limiting (Fraser et al., 2002). The metabolic bottleneck thus created may not be the same in different plant species and their organs, leading to the accumulation of different carotenoids. Therefore it becomes imperative to have a specific crop and gene based approach for metabolic engineering of carotenoids. A summary of metabolic engineering for carotenoid enhancement in crop plants is presented in Table 8.

Rice

Rice endosperm lacks provitamin A and other carotenoids, but express several genes involved in carotenoid formation (Burkhardt et al., 1997). Introduction and expression of three heterologus genes namely phytoene synthase (*Psy*) and lycopene β-cyclase (*Lcy-b*) from *Narcissus pseudonarcissus* under the control of endosperm specific promoter of the glutelin gene and phytoene desaturase (*Crt I*) from *E.uredovora* under the control of the cauliflower mosaic virus 35S promoter allowed the production of lutein, zeaxanthin, and α and β-carotene in varying proportions in rice grains (japonica rice cultivar variety) (Ye et al., 2000). The maximum level of β-carotene in the endosperm was 1.6 μg/g. A second generation of golden rice has been produced using maize phytoene synthase, which in conjunction with a larger population of transgenics enabled the elevation of carotenoids up to 23-fold (37 μg/g) of which β-carotene accounts to 31 μg/g. The increase in the total carotenoid content brought about by the highly effective maize *Psy* gene was due to the preferential increase in the β-carotene rather than proportional increase in all carotenoids (Paine et al., 2005).

Canola

Wild type, mature canola (*Brassica napus*) embryos contain low levels of lutein but spectacular increases in carotenoid levels resulting from metabolic engineering have been achieved in it. A 50-fold increase in the carotenoids content in the mature seeds of canola was observed when the bacterial phytoene synthase (*Crt B*) gene was overexpressed in a seed-specific manner

Table 8 Metabolic Engineering of carotenoids in plants

Target Plant	Gene used	Promoter	Phenotype	Source
Arabidopsis seed	Endogenous *Psy* overexpression	Seed specific *nap* promoter	43-fold increase in β-carotene, significant increase in lutein and violaxanthin; darker seeds; delayed germination correlated to increase in chlorophyll, abscisic acid and carotenoids.	Lindgreen et al., 2003
Arabidopsis seed	*Crt O* (β-carotene oxygenase) from *H. pluvialis*	*nap A* promoter	Free and esterified ketocarotenoids in transgenic plants with red seeds; major ketocarotenoids: 4-ketolutein, adinorubin and canthaxanthin	Stalberg et al., 2003
Canola seeds	*Crt B* overexpression	*napin* promoter	50-fold increase in total carotenoid content; orange colored transgenic embryos with β- and α-carotene in the ratio 2:1; reduction in chlorophyll and tocopherol levels	Shewmaker et al., 1999
Canola seeds	*Crt B*, *Crt I* from bacteria; *Lcy E* overexpression	*napin* promoter	50% increase in the the β- to α-carotene ratio from 2:1 to 3:1 in transgenic seeds	Ravenello et al., 2003
Canola seeds	ε-*CYC* silencing	*35 S CaMV* promoter	Increased levels of β-carotene, zeaxanthin, violaxanthin and lutein in transgenic seeds	Yu et al., 2007
Carrot	β-carotene ketolase from *H. pluvialis*	double *CaMV 35S*, *Arabidopsis*-ubiquitin, and *RolD* from *Agrobacterium rhizogenes*	70% of total carotenoids were converted to ketocarotenoids, with accumulation up to 2,400 μg/g DW in root. Most prevalent ketocarotenoids were astaxanthin, adonirubin, and canthaxanthin followed by echinenone, adonixanthin and β-cryptoxanthin	Jayaraj et al., 2008
Lotus japonicus	*Crt W* from *Agrobacterium auranticum*	*CaMV 35S* promoter	The color of flower petals in transgenic plants changed from light yellow to deep yellow to orange; leaves and petals accumulated ketocarotenoids astaxanthin, adonixanthin, canthaxanthin, and echinenone	Suzuki et al., 2007
Flaxseed	*Crt B* from *P. ananatis*	*CaMV 35S* and *FAE 1* seed-specific promoter	Orange seeds in transformed plants; increase in lutein and accumulation of phytoene, α-carotene and β-carotene; 7.8–18.6 fold increase in total carotenoids	Fujisawa et al., 2008
Maize	*Crt B* and *Crt I* from *E. herbicola*	γ-*zein* promoter	34-fold increase in total carotenoids; preferential β-carotene accumulation in endosperm	Aluru et al., 2008
Potato tuber	*Zep* silencing	*GBSS* promoter	Zeaxanthin accumulation; 5–7 fold increase in tuber carotenoid content; 2–3-fold increase in α-tocopherol content	Romer et al., 2002
Potato tuber	*Crt B* from *E. uredovora*	*patatin* promoter	7-fold increase in total tuber carotenoids, mainly lutein and β-carotene	Ducreux et al., 2005
Potato tuber	*Crt O* from *Synechocystis*	*CaMV* promoter	Ketocarotenoids in leaves and tubers, mainly echinenone, 3'-hydroxyechinenone, 4-ketozeaxanthin, astaxanthin	Gerjets and Sandmann, 2006
Potato tuber	β-carotene ketolase from *H. pluvialis*	*patatin* promoter	ketocarotenoids ketolutein, and astaxanthin in potato tubers	Morris et al., 2006a
Potato tuber	*DXS* overexpression	*patatin* promoter	Enhanced zeatin levels and early sprouting	Morris et al., 2006b
Potato tuber	*Lcy E* silencing	*patatin* promoter	Enhanced β-carotene (14-fold) and total carotenoids (2.5-fold)	Diretto et al., 2006
Potato tuber	*CHY* silencing	*patatin* promoter	Increase in total carotenoids upto 4.5-fold and β-carotene 38-fold	Diretto et al., 2007a
Potato tuber	*Crt B*, *Crt I* and *crt Y* from *E. herbicola*	*35S CaMV/patatin1/ patatin 2* promoter	20-fold increase in total carotenoids with 3600-fold enhancement in β-carotene content	Diretto et al., 2007b
Rice	*Psy* and *Lcy-b* from *N. pseudonarcissus* and *Crt I* from *E. uredovora*	*glutelin/CaMV 35 S* promoter	Production of lutein, zeaxanthin, α- and β-carotene in rice grains	Ye et al., 2000
Rice	*Psy* from maize	Maize polyubiquitin (*Ubi 1*) promoter	23-fold elevation in total carotenoids with 31 μg/g β-carotene	Paine et al., 2005
Tobacco	*Crt O* from *H. pluvialis*	Tomato *PDS* promoter	Change in the color of nectary flowers from yellow to red; ketocarotenoids accumulation in leaves and nectaries mainly astaxanthin, adonixanthin, and adonirubin	Mann et al., 2000

(Continued on next page)

Table 8 Metabolic Engineering of carotenoids in plants *(Continued)*

Target Plant	Gene used	Promoter	Phenotype	Source
Tobacco and Tomato	*Crt W* and *Crt Z* from *Paracoccus* sps.	*CaMV 35 S* promoter	Ketocarotenoids in leaves and fruits of tomato; 9-fold in crease in carotenoid content in nectary tissues of tobacco	Ralley et al., 2004
Tobacco	Ketolase gene from *Synechocystis*	Ubiquitous promoter	2.5-fold increase in total carotenoids in nectaries	Gerjets et al., 2007
Tobacco	cyanobacterial *crt O* ketolase gene	*35S CaMV* promoter	Ketocarotenoids in both leaves and flowers such as 4'-ketolutein, echinenone, 3'-hydroxyechinenone and 4-ketozeaxanthin	Zhu et al., 2007
Tomato	Endogenous *psy* overexpression	*35S CaMV* promoter	Dwarfism and reduced gibberellin content	Fray et al., 1995
Tomato	*Crt B* from *E. uredovora*	Tomato *polygalacturonase* promoter	2–4-fold increase in total carotenoids with enhanced levels of phytoene, lycopene, β-carotene, and lutein	Fraser et al., 2002
Tomato	*Lcy-e* from *A. thaliana*	*Pds* promoter	orange colored fruits with 5-fold increase in β-carotene	Rosati et al., 2000
Tomato	*Crt I* from *E. uredovora*	*CaMV 35 S* promoter	orange colored fruits with 2–4-fold increase in β-carotene	Romer et al., 2000
Tomato	Endogenous *Lcy-B* overexpression	*CaMV 35 S* promoter	Complete conversion of lycopene to β-carotene with greater fruit productivity	D'Ambrosio et al., 2004
Tomato	*Dxs* from *E. coli* and *Hmgr-1* from *A. thaliana*	*CaMV 35S* promoter	Transgenic tomato plants with *hmgr-1* had enhanced phytosterols upto 2.4-fold; *Dxs* transgenic plants showed 1.6-fold increase in carotenoids with 2.4- and 2.2-fold increase in phytoene and β-carotene levels respectively	Enfissi et al., 2005
Tomato	*Lcy-B* from *A. thaliana* and *Chy-B* from Pepper	*Pds* promoter	Increased levels of β-carotene, β-cryptoxanthin and zeaxanthin	Dharmapuri et al., 2002
Wheat	Maize *y*1 gene and *E. uredovora crt I* gene	*1Dx5* and *CaMV 35S* promoter	Increase in total carotenoids up to 10.8-fold	Cong et al., 2009
Hongkong kumquat	*Psy* from citrus	*CaMV 35S* promoter	2-fold increase in total carotenoids with enhanced levels of phytoene, lycopene, β-carotene, and β-cryptoxanthin	Zhang et al., 2009

(Shewmaker et al., 1999). The transgenic embryos were orange in color and contained β- and α-carotene in the ratio 2:1 along with a significant amount of phytoene. Lutein, the predominant carotenoid in the control seeds, was not increased. Other effects observed were reduction in tocopherol and chlorophyll levels in transgenic seeds, while fatty acid composition and cellular ultrastructure were altered.

Ravenello et al. (2003) showed an increase in the levels of total carotenoid in transgenic canola seeds expressing double constructs viz. phytoene synthase (*Crt B)* and phytoene desaturase (*Crt I*); *Crt B* and plant lycopene β-cyclase (*Lcy-b*) similar to the one when *Crt B* was expressed alone with minimal changes in β- to α-carotene ratio. However, expression of a triple construct consisting of bacterial phytoene synthase, phytoene desaturase, and lycopene β-cyclase in transgenic seeds showed a 50% increase in the the β- to α- ratio from 2:1 to 3:1. The levels of phytoene and lycopene were found to decrease significantly in these seeds.

The higher level of carotenoids accumulated in canola than rice is possibly because the carotenogenesis takes place in canola seeds inherently (Ravenello et al., 2003) and the transgenic phytoene synthase simply released a bottleneck in the pathway, whereas in rice seeds, the whole pathway is inactive.

Tomato

Tomato fruit shows similar carotenoid composition to leaf until mature green stage. At breaker stage the fruit color starts to change from green through pink to red due to tremendous increase in the carotenoid content (400-fold) with lycopene accounting 90% of the total. This is due to the upregulation of *Psy, Pds,* and *Zds*, and downregulation of *Lcy b* gene (Fraser et al., 1994; Ronen et al., 1999). It has been demonstrated that the increase in *Psy* and *Pds* expression during the breaker stage is controlled by transcriptional regulation (Corona et al., 1996) and the differential gene expression is the major reason for the accumulation of lycopene (Ronen et al., 1999).

Phytoene synthase exhibits the highest flux control coefficient among the enzymes of the pathway (Fraser et al., 2002), and it exerts the greatest control over flux through the pathway. Therefore, it has been the principal target for amplification with the objective of increasing the lycopene and β-carotene content in fruit. The first report on the constitutive expression of tomato phytoene synthase-1 (*Psy-1*) in transgenic tomato resulted in reduced levels in ripe tomato fruit due to gene silencing with endogenous gene; dwarfism and marked reduction in gibberellin content due to redirection of GGPP from the gibberellin

pathway. There was an abnormal carotenoid production in a variety of organs. Though the fruits produced lycopene earlier than the wild type, the final lycopene concentrations were low (Fray et al., 1995). The use of the endogenous *Psy-1* in combination with a constitutive promoter may have caused silencing (co-suppression) of the endogenous *Psy-1* (Carthew, 2001). In order to overcome the detrimental effects experienced with *Psy-1* expression, a tissue-specific promoter (tomato polygalacturonase promoter) and non-homologous phytoene synthase (bacterial phytoene synthase (*Crt B*) from *Erwinia uredovora*) were employed to prevent gene silencing. As a bacterial gene has only 35% homology with the tomato gene, it can be assumed that this might have prevented gene silencing. Transgenic plants (primary transformants) expressing *Crt B* in a fruit specific manner exhibited a 2–4 fold increase in total carotenoids when compared to control, which were inherited by progeny. The levels of phytoene, lycopene, β-carotene, and lutein were elevated by 2.4-, 1.8-, 2.2-, and 1.6-folds respectively (Fraser et al., 2002). Expression of the additional phytoene synthase, however, reduced the influence of this step over flux through the pathway, suggesting that the endogenous pathway can compensate for fluctuations in precursor/product equilibrium and redistribute the balance of control within the pathway.

D'Ambrosio et al. (2004) reported that the transgenic tomato plants are capable of converting all lycopene into β-carotene under optimal conditions when transformed with tomato lycopene β-cyclase cDNA under the control of CaMV 35S promoter. The engineered plants also showed a greater fruit productivity than control under both optimal and reduced water supply conditions.

Another step that has been subjected to genetic manipulation in tomato is the phytoene desaturation. Transgenic tomato (CV. Ailsa Craig) plants expressing the bacterial phytoene desaturase (*Crt I*) from *E. uredovora* with the CaMV 35S promoter had orange-colored fruit and carotenoid analysis showed increase in β-carotene, neoxanthin, antheraxanthin, lutein, zeaxanthin, and tocopherols, and decrease in carotenoids prior to β-carotene. In ripe fruit, β-carotene increased by 2–4 fold, but no elevation in the lycopene level was observed despite increase in phytoene desaturase activity (Romer et al., 2000). The levels of gene expression and enzyme activities showed crisscross within the pathway, whereby the presence of *Crt I* induced endogenous lycopene cyclization and reduced the content of compounds prior to phytoene desaturation.

Transgenic tomato plants (var. Moneymaker), expressing the *Arabidopsis thaliana* lycopene β-cyclase (β-Lcy) under the control of the tomato phytoene desaturase (*Pds*) promoter yielded orange-colored ripe fruit with a 5-fold increase in β-carotene (Rosati et al., 2000). The amounts of other carotenoids in these fruits were unaffected, and no major changes in endogenous carotenoid gene expression or gene silencing were reported. Contrastingly, when the endogenous tomato *β-Lcy* was placed in an antisense orientation under *Pds* promoter control, transformants showed up to 50% down-regulation of *β-Lcy* expression in ripe fruit and lycopene content of the fruit was slightly increased. Similar compositional changes were found when the *B*-gene, encoding a tomato fruit ripening specific lycopene β-cyclase, was expressed in tomato (cv VF36) in both sense and antisense orientations under its own promoter (Ronen et al., 2000).

The ripe tomato fruits accumulate significant amounts of lycopene and only trace amounts of oxygenated carotenoids. An attempt was made to increase the xanthophyll content of tomatoes by Dharmapuri et al. (2002) by expressing the lycopene β-cyclase (*b-Lcy*) gene from *Arabidopsis thaliana* and β-carotene hydroxylase (β- *Chy*) gene from pepper under the control of fruit specific Pds promoter. The transformed fruits showed a significant increase in β-carotene, β-cryptoxanthin, and the zeaxanthin content. High levels of hydroxylated β-carotene derivatives were coincided with β-chy protein. β-hydroxylase activity was found only when *β-chy* is expressed in conjugation with *LcyB*. The expression of endogenous carotenoid biosynthetic genes was unaltered in transformed lines suggesting that the production of xanthophylls was the result of the expression of introduced transgenes rather than the deregulation of endogenous genes.

Conversion of β-carotene to zeaxanthin was attempted with cDNA's encoding hydroxylases from both plants and bacteria in tomatoes. The orange-colored ripe fruit from transgenic tomatoes expressing the *Arabidopsis thaliana* lycopene β-cyclase under the control of tomato pds produced 5-fold increase in β-carotene, but no zeaxanthin was produced despite the production of an active hydroxylase (Rosati et al., 2000). Recently, zeaxanthin formation has been successfully engineered into tomato (var. Moneymaker) fruit by co-transformation with separate constructs containing the *Arabidopsis b-Lcy* and *Capsicum* β-carotene hydroxylase (*b-Chy*), both under the tomato Pds promoter control (Dharmapuri et al., 2002). Ripe fruit from these transformants contained both zeaxanthin and β-cryptoxanthin. These data suggest that in order to obtain the desired product the precursor pool must be accessible for subsequent metabolism.

To achieve a desired result by metabolic engineering targetting a specific step or steps in the biosynthetic pathway has been followed, but recently Mehta et al. (2002) described transgenic tomato plants (var. Alisa Craig), which showed an increase in lycopene content, while aiming to alter polyamine levels in order to enhance vine life and juice quality. Apparently, as a consequence of extended vine longevity, lycopene levels were elevated. These results showed the existence of cross talk not only within a biosynthetic pathway, but also among different pathways.

Potato

Compared to other fruits and vegetables, the carotenoid content in potato is low and it produces only xanthophylls such as lutein and violaxanthin. The endogenous zeaxanthin epoxidase cDNA, under a tuber specific promoter control, was used in the antisense and sense direction for the conversion of zeaxanthin to violaxanthin. Downregulation of zeaxanthin epoxidase

in potato tubers led to a dramatic increase in zeaxanthin content in some transgenic lines and the tuber carotenoid content increased upto 5–7 fold. This also resulted in elevated transcript levels of phytoene synthase and β-*chy* and a 2–3 fold increase in α-tocopherol content (Romer et al., 2002). An inverse correlation between the zep transcript and tuber carotenoids was also observed by Morris et al. (2004).

An enhancement in the level of tuber carotenoids mainly lutein and β-carotene compared to control was observed by Ducreux et al. (2005) when *E. uredovora* phytoene synthase (*Crt B*) gene was expressed in potato tubers. The total carotenoids were increased upto 7-fold in transgenic potatoes. This also led to the increased transcript levels for a protein fibrillin, which functions in carotenoids storage.

To enhance the ketocarotenoid production, a transgenic potato line accumulating zeaxanthin (inactivated zeaxanthin epoxidase) was co-transformed with the *Crt O* (β-carotene ketolase) gene from the cyanobacterium *Synechocystis* under 35 S CaMV promoter. The transformed plants expressing this gene, was found to accumulate echinenone, 3′-hydroxyechinenone and 4-ketozeaxanthin in leaves, as well as 3′-hydroxyechinenone, 4-ketozeaxanthin together with astaxanthin in the tuber. The amount of ketocarotenoids accounted for 10–12% of total carotenoids in leaves and tubers (Gerjets and Sandmann, 2006). Morris et al. (2006a) produced *Solanum tuberosum* and *Solanum phureja* transgenic lines that expressed an algal bkt1 gene, encoding a β-ketolase, and accumulated ketocarotenoids. Two major ketocarotenoids, ketolutein and astaxanthin, were accumulated in both the transgenic lines.

To enhance the β-carotene content, potato was transformed with a mini-pathway of bacterial genes, encoding phytoene synthase (*Crt B*), phytoene desaturase (*Crt I*), and lycopene β-cyclase (*Crt Y*) from *Erwinia*, under tuber specific or constitutive promoter control (Diretto et al., 2007b). Expression of all three genes, under the control of a tuber specific promoter resulted in tubers with a deep yellow ("golden") phenotype without any change in leaf carotenoids. These golden tubers accumulated β-carotene to the level of 47 mg/g dry weight (more than 3600-fold increase compared to parental genotype) with simultaneous increase in total carotenoids accounting to 110 mg/g dry weight (approximately 20-fold increase compared to the parental genotype). This is reported to be the highest carotenoid and β-carotene content for bio-fortified potato as well as for any of the four major staple foods.

Carrot

A 2–5-fold increase in β-carotene content in the root of transgenic carrot was observed when *Erwinia herbicola Crt B* genes were introduced under the control of organ specific *mas* promoter along with RUBISCO transit peptide sequence. The transformed roots exhibited yellow/orange color throughout the cross sections (Ausich et al., 1997; Hauptmann et al., 1997).

An attempt was made to engineer the ketocarotenoid biosynthetic pathway in carrot tissues by introducing a β-carotene ketolase gene from the alga *Haematococcus pluvialis*. Three promoters (double CaMV 35S, *Arabidopsis*-ubiquitin, and RolD from *Agrobacterium rhizogenes*) were used to make gene constructs and the enzyme was targeted to plastids in leaf and root tissues. All three promoters provided strong root expression, with double CaMV 35S and ubiquitin promoters exhibited strong leaf expression also. The recombinant ketolase protein was successfully targeted to the chloroplasts and chromoplasts. Endogenous expression of carrot β-carotene hydroxylases was up-regulated in transgenic leaves and roots, and up to 70% of total carotenoids were converted to ketocarotenoids, which accumulated up to 2.4 mg/g root dry weight. The most prevalent carotenoids in root were astaxanthin, adonirubin, and canthaxanthin followed by echinenone, adonixanthin, and β-cryptoxanthin (Jayaraj et al., 2008).

Flaxseed

Linseed flax is an important industrial oilcrop and linseed oil is an excellent source of α-linolenic acid and lignan. Transgenic studies to increase the carotenoid content in seeds were carried out by Fujisawa et al. (2008). Phytoene synthase (*Crt B*) from *P. ananatis* was expressed under the control of *CaMV* promoter/*FAE 1* seed-specific promoter. The transformed flax plants produced orange seeds with increased amount of lutein and accumulation of phytoene, α-carotene, and β-carotene, while non-transformed flax plants produced light-yellow seeds wherein only lutein was detected. The total carotenoids in transformed seeds were 65.4–165.3 μg/g FW corresponding to 7.8–18.6-fold increase than untransformed controls.

Maize

Traditional yellow varieties of maize, an important staple crop contain low amounts of β-carotene (0.25–2.5 μg/g DW), while white varieties do not have pro-vitamin A content as they possess *Psy* allele (Gallagher et al., 2004). Transgenic maize with enhanced pro-vitamin A in kernels were generated by over-expression of phytoene synthase (*Crt B*) and phytoene desaturase (*Crt I*) from *E. herbicola* under the control of γ-*zein* promoter. A 34-fold increase in total carotenoids was observed with preferential accumulation of β-carotene in endosperm (Aluru et al., 2008).

Wheat

Cong et al. (2009) carried out studies to increase the total carotenoid content in elite wheat (*Triticum aestivum* L.) EM 12 variety. Transgenic wheat was generated by expressing maize *y*1 gene encoding phytoene synthase under endosperm specific *1Dx5* promoter along with phytoene desaturase (*Crt I*) gene

from *E. uredovora* under constitutive *CaMV 35S* promoter. The transgenic wheat showed light yellow colored endosperm and a 10-fold increase in the total carotenoid content was observed as compared to nontransgenic EM 12 variety.

Hongkong Kumquat Fruit

Phytoene synthase (*Psy*) gene from cara cara navel orange (*Citrus sinensis* Osbeck) was transformed into Hongkong kumquat (*Fortunella hindsii* Swingle) under the control of *35S CaMV* promoter. The transgenic plants showed varying fruit colors from deep yellow to orange over the wild plants which produced yellow colored fruits. The transformed fruits were found to contain 171.9 μg/g FW total carotenoids which were 2-fold higher than the untransformed ones. The phytoene and lycopene content of the transgenic lines also showed 1.7–3.0- and 2.2–2.9-fold increase over nontransformed controls. The less abundant Hongkong kumquat carotenoids such as β-carotene and β-cryptoxanthin were also elevated by 2.0- and 2.3-fold, respectively (Zhang et al., 2009).

CONCLUSIONS

The fundamental reaction sequences involved and the genes responsible for carotenoid biosynthesis have been isolated and characterized in several laboratories (Hirschberg, 2001; Moehs et al., 2001; Tian et al., 2004; Giuliano et al., 2008). Successful enhancement in the carotenoid content has been achieved by various research groups by increasing plastid number/size (Cookson et al., 2003; Liu et al., 2004), extraplastidial biosynthesis (Grunewald et al., 2001), modification of intracellular storage (Shewmaker et al., 1999; Ray et al., 2000; Ducreux et al., 2005), modification of carotenoids synthesized like esterification (Bouvier et al., 2003a; Mann et al., 2000; Grunewald et al., 2001), synthesis in unconventional plants/ plant parts (Mann et al., 2000; Ye et al., 2000), and the removal of feed back inhibition (Romer et al., 2000).

The enhanced accumulation of provitamin A content may provide, at least partially, a solution to overcome vitamin A deficiency and related health problems like blindness, xeropthalmia, and growth retardation in developing countries, especially among children. The problem of delivering vitamin A orally in high risk countries where VAD is prevalent may be resolved by supplementing their staple food such as rice with biofortified vegetables such as potato and tomato. Assuming daily requirement of vitamin A as 300 μg retinol equivalents/day for children and 800 μg for adults, and a conversion ratio of β-carotene into retinol of 6:1 used in human supplementation studies (West et al., 2002), 100% of vitamin A requirement for children and 38% for adults can be met by approximately 5 g/day transgenic canola oil (Shewmaker et al., 1999), 60 g/day golden rice (Paine et al., 2005) and 150 g/day golden potatoes (Diretto et al., 2007b).

Inspite of the significant progress in elucidation of carotenoid biosynthesis in plants, its various aspects, especially metabolic fluxes leading to their accumulation, are yet to be understood to improve our capability to manipulate carotenoids in crop plants. The challenges faced in increasing the carotenoid concentration to higher levels is due to our limited knowledge regarding mechanisms and signals controlling plant carotenogenesis (Taylor and Ramsay, 2005; Fraser and Bramley, 2004; Hirschberg, 2001) and cross-talk between metabolic pathways (Rodriguez-Concepcion 2006; Wille et al., 2004), which makes it difficult to predict the outcome of biotechnological approaches. Development of isoprenoid profiling methodologies and microarray approaches, and studies on mutants with respect to regulatory mechanisms that control carotenoid accumulation in chromoplasts of non-photosynthetic systems will provide additional insight into these mechanisms. Mapping analysis of quantitative trait loci (QTL) to identify genes responsible for carotenoid accumulation (Liu et al., 2003; Santos and Simon, 2002) and transcript profiling of gene expression in carotenoid-accumulating tissues and organs (Alba et al., 2004; Ducreux et al., 2005; Fei et al., 2004) might throw an additional light in identifying the potentiality of regulatory genes. All this knowledge will be helpful in designing and developing transgenic crop plants with carotenoid content tailored to the needs of a specific end group.

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