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Progress in Lipid Research 43 (2004) 228–265

Progress in Lipid Research

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Review

The biosynthesis and nutritional uses of carotenoids

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Abstract

Carotenoids are isoprenoid molecules that are widespread in nature and are typically seen as pigments in fruits, flowers, birds and crustacea. Animals are unable to synthesise carotenoids de novo, and rely upon the diet as a source of these compounds. Over recent years there has been considerable interest in dietary carotenoids with respect to their potential in alleviating age-related diseases in humans. This attention has been mirrored by significant advances in cloning most of the carotenoid genes and in the genetic manipulation of crop plants with the intention of increasing levels in the diet. The aim of this article is to review our current understanding of carotenoid formation, to explain the perceived benefits of carotenoids in the diet and review the efforts that have been made to increase carotenoids in certain crop plants.

Contents

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doi:10.1016/j.plipres.2003.10.002

1. Introduction

The carotenoids are the most widespread group of pigments in nature, with over 600 characterised structurally and an estimated yield of 100 million tonnes per annum. They are present in all photosynthetic organisms and responsible for most of the yellow to red colours of fruits and flowers. The characteristic colours of many birds, insects and marine invertebrates are also due to the presence of carotenoids, which have originated in the diet. Commercially, carotenoids are used as food colourants and in nutritional supplements [1], with an estimated global market of some $935 million by 2005. Since animals are unable to synthesise carotenoids de novo, they rely upon the diet as the source of these compounds. There is considerable interest in dietary carotenoids with respect to their antioxidant properties and ability to alleviate chronic diseases. The large amount of research in this topic has been paralleled by the significant

advances in cloning most of the genes involved in carotenogenesis and our increased understanding of factors that regulate carotenoid formation and deposition in plants. Taken together, it is these advances in knowledge that now open the way to enhancing carotenoid levels in crop plants and foods.

1.1. Structures and nomenclature

Carotenoids are isoprenoids and generally consist of eight isoprene units joined together so that the linking of units is reversed at the centre of the molecule to give methyl groups 20 and 20′ with a 1,6 positional relationship, whereas the remaining methyl groups are 1,5. The most obvious feature of the carotenoid molecule is the long polyene chain, which may extend from 3 to 15 conjugated double bonds. The length of the chromophore determines the absorption spectrum of the molecule and hence its colour to the eye. All are based upon seven different end groups of which only four (β,ε,κ,ψ) are found in higher plant carotenoids [2].

Cyclisation of the carbon skeleton occurs at one or both ends of the molecule, whilst xanthophylls are formed from the hydrocarbon carotenes by the introduction of oxygen functions. In addition, modifications involving chain elongation or degradation can occur. Many of the commonly used trivial names of carotenoids relate to the original source from which they were isolated, e.g. β-carotene from carrots. There is a systematic nomenclature [2]. In this article, however, the trivial names will be used.

1.2. Dietary sources of carotenoids

Carotenoids found in the human diet are primarily derived from crop plants, where the carotenoids are located in roots, leaves, shoots, seeds, fruit and flowers. Around 60 different carotenoids have been identified in fruits and vegetables consumed by humans [3–5]. To a lesser extent, carotenoids can also be ingested in eggs, poultry and fish, where typically plant or algal products have been included in the feed of poultry or fish itself, e.g. zeaxanthin from maize in poultry feed. Typical amounts of carotenoids in crop plants are shown in Table 1.

More recently, a carotenoid database for European fruits and vegetables has been published [6], whilst those in vegetable oils are listed by Ong and Tee [7]. In contrast to most dietary carotenoids, sources of lycopene are limited, with at least 85% of our dietary lycopene coming from tomato fruit and tomato-based products, with the remainder being obtained from watermelon, pink grapefruit, guava and papaya (Table 2). Of the tomato products, juice, ketchup, soup and pizza and spaghetti sauces are the major contributors in the diet. A survey of carotenoid intake across Europe revealed considerable variation in the major dietary sources of carotenoids between countries [6].

1.3. Bioavailability from the diet and tissue distribution

Bioavailability is defined as the fraction of an ingested nutrient that becomes available to the body for utilisation in physiological functions or for storage [8]. There are at least 9 factors that influence the bioavailability of carotenoids: species of carotenoid, molecular linkage, amount consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and

Table 1
Carotenoid content of raw leafy green vegetables, fruits, roots and seeds[a]

Species	Carotenoid (μg/g fresh weight)					
	Total	Zea	Lutein	α-Carotene	β-Carotene	Lycopene
Brussel sprout	1163	–	610	–	553	–
Green bean	940	–	494	70	376	–
Broad bean	767	–	506	–	261	–
Broccoli	2533	–	1614	–	919	–
Green cabbage	139	–	80	–	59	–
Lettuce	201	–	110	–	91	–
Parsley	10,335	–	5812	–	4523	–
Pea	2091	–	1633	–	458	–
Spinach	9890	–	5869	–	4021	–
Watercress	16,632	–	10,713	–	5919	–
Apricot	2196	31	101	37	1766	–
Banana	126	4	33	50	39	–
Carrot (May)	11,427	–	170	2660	8597	–
Carrot (Sept)	14,693	–	283	3610	10,800	–
Orange	211	50	64	Nd	14	–
Pepper	2784	1608	503	167	416	–
Peach	309	42	78	Tr	103	–
Sweet corn	1978	437	522	60	59	–
Tomato	3454	–	78	–	439	2937

[a] Taken from [5]. Values include *cis* and *trans* isomers. Nd = not detected; Tr = trace; α-car = α-carotene; β-car = β-carotene; zea = zeaxanthin.

Table 2
Lycopene content of fruit and tomato products[a]

Fruit or tomato product	Lycopene content (μg/g wet weight)
Fresh tomato	8.8–42.0
Watermelon	23.0–72.0
Pink guava	54.0
Pink grapefruit	33.6
Papaya	20.0–53.0
Tomato sauce	62.0
Tomato paste	54.0–1500.0
Tomato juice	50.0–116.0
Tomato ketchup	99.0–134.4
Pizza sauce	127.1

[a] Data taken from [5].

bioconversion, nutrient status of the host, genetic factors, host-related factors and interactions. These are often summarised in the mnemonic SLAMENGHI [9–11]. Since carotenoids are lipid-soluble they are taken up from the intestine far better from a fatty diet, although the amount of fat required is low, at about 3–5 g per meal [12]. 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 following disruption of the plant cell wall [13]. The uptake of β-carotene from vegetables is low (14% for mixed vegetables) compared with purified β-carotene added to a simple matrix [12]. *Cis* isomers of carotenoids appear to be more bioavailable than the all-*trans* forms [14], perhaps because they are more soluble in bile acid micelles and so preferentially incorporated into chylomicrons. It has been suggested that individual carotenoids antagonise absorption of each other, e.g. canthaxanthin inhibits lycopene uptake [15] and It is likely that uptake by intestinal cells is a facilitated process [16]. The use of isotopic tracer techniques to study bioavailability will improve the accuracy of such measurements [17, 18].

Once ingested, carotenoids appear in plasma, initially in the VLDL and chylomicron fractions and later in LDL and HDL. The highest levels are found in LDL. Serum concentrations, however, vary enormously, e.g., lycopene levels are from 50 to 900 nM, with large interperson variations. Studies of β-carotene uptake and plasma clearance, using human ileostomy volunteers showed that absorbed β-carotene is rapidly cleared from the plasma to an unobservable pool at a rate similar to that of chylomicron triacylglycerols [19].

Lycopene is found in most human tissues, but is not deposited uniformly (Table 3). These differences suggest that there are specific mechanisms for the preferential deposition of lycopene, particularly in the adrenals and testes.

2. Roles of carotenoids in animals

Carotenoids have a broad range of functions, especially in relation to human health and their role as biological antioxidants.

Table 3
Lycopene levels in human tissues[a]

Tissue	Lycopene (nmol/g wet weight)
Adipose	0.2–1.3
Adrenal	1.9–21.6
Brainstem	Not detectable
Breast	0.8
Colon	0.3
Liver	1.3–5.7
Lung	0.2–0.6
Ovary	0.3
Prostate	0.8
Skin	0.4
Stomach	0.2
Testis	4.3–21.4

[a] Taken from [20–22].

2.1. Provitamin A activity

This is the best-established function of carotenoids. It is restricted to some 50 carotenoids with β-ring end groups, such as β-carotene, zeaxanthin and β-cryptoxanthin. These carotenoids, when ingested in the diet, were reported to be cleaved by an intestinal 15-15′ -dioxygenase to form retinal almost 40 years ago [23]. However, it is only in the last few years that the mammalian gene has been cloned [24] and biochemical studies carried out [25]. It is now established that the central cleavage of β-carotene is, in fact, a mono-oxygenase type reaction. The recombinant human enzyme, called BCO, has been characterised and is highly expressed in the digestive system and the liver [26]. It is, however, also expressed in non-digestive tissues where it may convert pro-vitamin A carotenoids obtained from plasma into retinal locally [26]. As well as the symmetric cleavage of such β-ring carotenoids, they can also be cleaved asymmetrically to produce a range of apocarotenoids, e.g. by cleavage of the 9′, 10′ double bond to yield β-ionone and β-apo-10′-carotenal, which may be a precursor of retinoic acid. Interestingly, this 9′, 10′-dioxygenase can also cleave non- ring carotenoids such as lycopene [27].

The efficiency of the bioconversion of pro-vitamin A to vitamin A (bioefficiency) has recently been re-evaluated in order to proved recommendations for the amount of such carotenoids needed in the diet to prevent vitamin A deficiency. In developed countries, 6 μg of β-carotene in oil or 12 μg in mixed foods has the same vitamin A activity as 1 μg retinol. Based on existing FAO food balance sheets and the FAO/WHO conversion rates, all populations should be able to meet their vitamin A requirements from existing dietary sources. However, using the new US Institute of Medicine conversion rates, populations in developing countries cannot achieve adequacy. Additionally, field studies suggest that, instead of 12 μg, 21 μg of β-carotene have the same vitamin A activity as 1 μg of retinol, which implies that effective vitamin A intake is even lower [28]. Therefore, controlling vitamin A deficiency in developing countries requires not only vitamin A supplementation but also food-based approaches, including food fortification, and possibly the introduction of new strains of plants with enhanced provitamin A activity.

2.2. Roles in the prevention of diseases

The first correlation between a high intake of carotenoids and health benefits appeared in the literature in the 1970s. Diets high in fruits and vegetables were associated with reduced rates of cancer and coronary heart disease, and the converse was also found to be true. As a consequence, the population has been encouraged to eat at least 5 portions of fruit and vegetables per day, which contain about 6 mg of carotenoids. Later research has focused on individual carotenoids in the diet, and especially β-carotene, lycopene, lutein and zeaxanthin. In particular, their roles as biological antioxidants and as regulators of the immune system have been the subject of intense scrutiny. There are several comprehensive reviews on this subject [29–32]. Many of these studies have speculated that the antioxidant activities of carotenoids are a key factor in reducing the incidence of many diseases [e.g. 33], but it should be also noted that in certain high risks groups (e.g. smokers) there can be adverse effects of high doses of carotenoids, perhaps because of their pro-oxidant properties [34]. It has also been reported that carotenoids induce apoptosis in T-lymphocyte cell lines [35] and can protect genome stability [36].

2.2.1. Carotenoids and eye health

Vitamin A deficiency leads to xerophthalmia. As discussed in Section 2.1 above, provitamin A carotenoids in the diet can prevent such ocular consequences [37]. Age-related diseases of the eye such as cataract and macular degeneration (AMD) are common problems across the world. In both cases there is evidence to show that particular dietary carotenoids can reduce the incidence of these diseases, probably by quenching active oxygen species.

In vitro studies on human lens epithelial cells (HLEC) have shown that addition of lycopene to cell cultures significantly prevents vacuolization in HLEC [38]. Prospective epidemiological data over 8 years showed a 19% lower risk of cataract in men taking high levels of lutein and zeaxanthin [39]. A randomized trial with β-carotene and cataract in US physicians over 12 years showed no benefit in healthy men, but did attenuate the excess risk of cataract in smokers by about one quarter [40].

The macula of the eye contains two carotenoids: lutein and zeaxanthin, although it is not known why these particular compounds but not other carotenoids accumulate in the macula [41]. Similar pigments are found in other primates [42] and oil droplets rich in carotenoids are found in birds, reptiles, fish and amphibians [43]. It is thought that these carotenoids protect the macula from light-induced damage and scavenge free radicals formed in the photoreceptors. The dietary intake of antioxidants and its correlation to AMD was investigated in the NIH Eye Disease Case-Control Study [44]. There was a statistically significant and linear trend for reduction of risk with increasing amounts of carotenoids, with lutein and zeaxanthin showing the strongest association with reduced risk of AMD. Evidence for the protective effect of carotenoids and antioxidant vitamins has been reviewed by Snodderly [45].

2.2.2. Cardiovascular disease (CVD)

Early epidemiological studies on the role of carotenoids (especially β-carotene) in the development of CVD support the hypothesis that carotenoids have preventative potential [46]. Reductions of LDL oxidation and of oxidative stress at the plaque formation are common suggestions for their effect. The consumption of processed tomato products does reduce lipoprotein sensitivity to oxidative damage [47] and low serum lycopene is associated with an increased risk of atherosclerotic vascular events in middle-aged men [48]. However, the first intervention trial with β-carotene (α-Tocopherol, β-Carotene Cancer Prevention Study), conducted on Finnish male smokers showed that lung cancer was elevated and β-carotene did not reduce CVD mortality [49]. Other studies have also shown little or no effect of β-carotene in reducing mortality from heart disease or strokes [reviewed by 29]. Indeed, one common conclusion is that a combination of carotenoids and other antioxidants from fruit and vegetables is more effective than dietary supplements of single components.

2.2.3. Cancer

In vitro cell culture experiments have shown that carotenoids inhibit cell proliferation, transformation and micronucleus formation as well as modulating expression of certain genes. These properties are all consistent with a protective effect against carcinogenesis [reviewed by 36]. One of the earliest discoveries in this respect was that certain carotenoids, in vitro, increased gap junctional communication in a dose-dependent manner [50]. Since this early study, there has been a wealth of investigations on individual carotenoids and their effect of cancers.

2.2.3.1. Lung cancer. Observational epidemiological studies in the 1960s and 1970s showed a consistent inverse association between fruit and vegetable intake and the risk of lung cancer and other cancers [51]. Further studies suggested that β-carotene may be the compound responsible for this association, although they did not provide direct evidence that this was the case. Therefore, several β-carotene intervention trials were started in the 1980s and 1990s to test this hypothesis [e.g. 52,53]. These trials showed that β-carotene did not reduce the risk of lung cancer, and in the trial using Finnish male heavy smokers there was an increase in lung cancer perhaps because β-carotene was acting as a pro-oxidant rather than an antioxidant in these circumstances [54]. More recently, Wright et al. [55] have reported that the consumption of a wide variety of vegetables has a greater bearing on lung cancer than intake of any specific carotenoid or total carotenoids.

2.2.3.2. Breast cancer. A review of the epidemiologic literature in 1997 [56] showed that β-carotene was considered in 16 case-control studies. Higher β-carotene consumption was associated with a lower risk of breast cancer in 11 studies, with significant results in four and the remaining five showing no association. More recent studies, reviewed by Cooper et al. [29], show a mixed picture, although high lycopene levels were associated with decreased risk in one study [57]. The mechanism of action of lycopene in the inhibition of breast cancer is associated with inhibition of cell cycle progression at the G_1 phase [58]. In contrast, a particularly large study on Canadian women in 2002 showed no association between dietary intake of carotenoids and breast cancer risk [59].

2.2.3.3. Prostate cancer. The first interest in the hypothesis that dietary lycopene protects against prostate cancer was in 1995, when Giovannucci and co-workers reported that high-lycopene foods were associated with a reduced risk of prostate cancer, in the Health Professionals Follow-Up Study. Intake of lycopene in a lipid diet, e.g. tomato sauce or pizza was particularly effective [60]. It is known that supplementation of the diet with tomato sauce leads to a substantial increase in total lycopene in serum and prostate [61]. There have been several follow updates since this first report and Giovannucci has reviewed some 72 such studies [62]. There have been about 57 reported inverse associations between tomato intake or blood lycopene level and risk of cancer at a defined anatomic site, with 35 being statistically significant. At the present time, the accumulated data suggest that intake of tomato or tomato products may be associated with a lower risk of prostate cancer [63], but it is not certain that lycopene is the only compound in the tomato that contributes to this effect. Other carotenoids and phytochemicals in the fruit may contribute also [64, 65]. Interestingly, it has also been shown that a high tomato diet can reduce leukocyte oxidative DNA damage and prostate tissue oxidative damage in patients already diagnosed with prostate cancer [66], thus suggesting that such foods can be used in the treatment of prostate cancer as well as its prevention. In vitro studies with prostate LNCaP cancer cells have shown that lycopene in the growth medium reduces their proliferation [67].

2.2.3.4. Colorectal cancer. β-Carotene intake has been inconsistently associated with colorectal cancer [68], but the effects of other carotenoids have only been studied more recently. Slattery and co-workers [69] used dietary data to show that lutein was inversely associated with colon cancer in both men and women, whilst other carotenoids such as lycopene, zeaxanthin and β-cryptoxanthin

were unremarkable. In contrast, a more recent study concluded that there was no association between dietary intake of carotenoids and colorectal cancer risk [70].

2.2.4. Light-induced erythema

β-Carotene supplements are widely used as 'oral sun protectants'. However, there have been few controlled studies on their protective effect against UV irradiation. In the mid 1990s two studies produced contradictory conclusions; Garmyn et al. [71], found no protective effect of β-carotene, whereas Gollnick and co-workers concluded that the development of erythema was lower on a β-carotene supplement [72]. A later investigation, using a combination of dietary β-carotene and vitamin E, concluded that this mixture was more effective than β-carotene alone, and suggested that this is due to the antioxidant properties of these compounds [73]. Apparently, the duration of supplementation is more important than dosage. A later study by the same group used tomato paste over 10 weeks and found that erythema was significantly lowered [74], whilst UV-induced lipid peroxidation of human skin fibroblast cells in vitro was lowered by addition of β-carotene, lycopene or lutein [75].

3. The biosynthesis of carotenoids

The biosynthetic pathways involved in carotenoid formation were elucidated in the 1950s and 1960s using classical biochemical approaches, using specific inhibitors and mutants blocked at certain steps in the pathway. In the early 1970s in vitro systems were developed [76] enabling the study of biosynthetic enzymes. Unfortunately, the practical difficulties associated with these enzymes prevented purification in most cases. The advent of modern molecular genetic techniques has facilitated gene isolation, leading to functional complementation in vivo, characterisation of recombinant enzymes and the creation of transgenic plants. These studies have furthered our knowledge of carotenoid biosynthesis, its regulation and the enzymes involved in the process. This section focuses upon the reaction sequences responsible for carotenoid formation in higher plants. However, where alternative reactions performed by other organisms have been exploited to enhance nutritional carotenoid content in crop plants, comparisons will be made.

3.1. Formation of isopentenyl diphosphate

In higher plants carotenoids are synthesised and localised in the plastid. They are biosynthetically linked to other isoprenoids such as gibberellins, tocopherols, chlorophylls and phylloquinones via the five-carbon (C_5) compound isopentenyl diphosphate (IPP). Since the discovery of the mevalonic (MVA) pathway in the 1950s it was assumed that IPP was synthesised from acetyl-CoA via mevalonic acid [77]. Experimental data, however, consistently indicated that the MVA pathway could not account exclusively for the formation of plastid-derived isoprenoids. In the early 1990s retro-biosynthetic studies established the presence of an alternative, MVA-independent pathway for the formation of IPP and dimethylallyl diphosphate (DMAPP) [78]. The use of functional genomics has now led to the elucidation of the MVA-independent pathway in plants as well as many bacteria [79]. Recent studies have indicated, however, that the formation of plastid isoprenoids at some developmental stages can arise partly from the MVA pathway [80].

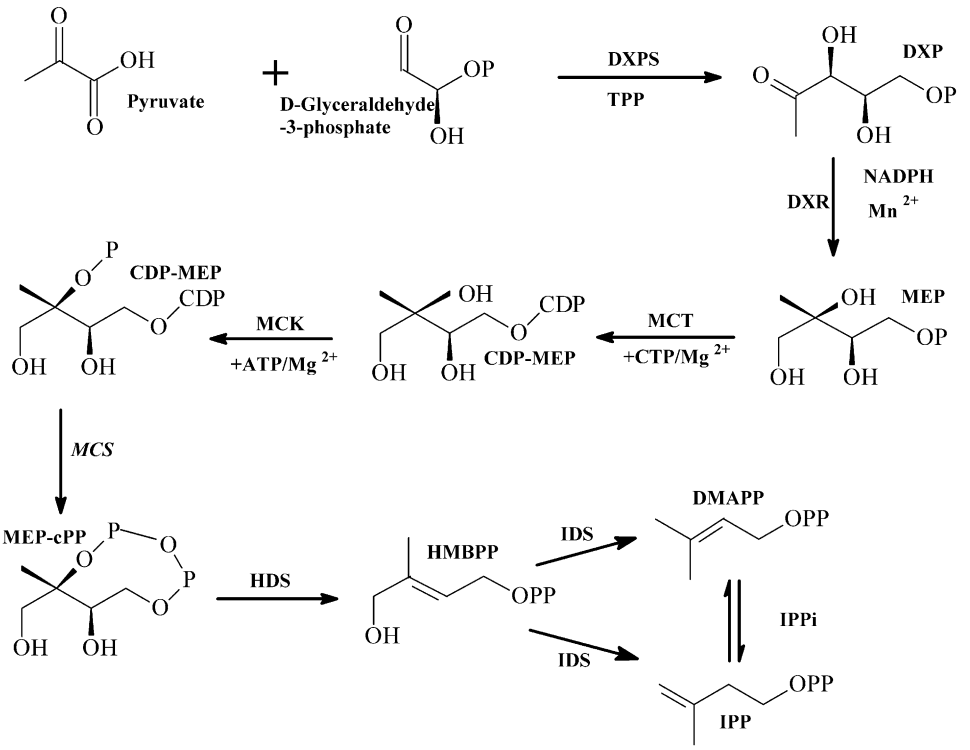

Fig. 1. Formation of isopentenyl diphosphate by the MVA-independent pathway. Abbreviations: TPP, thiamine pyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; CDP-MEP, 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; MEP-cPP, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXPS (EC 4.1.3.37),1-deoxy-D-xylulose 5-phosphate synthase; DXR (EC 1.1.1.267), 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT (EC 2.7.7.60), 2-*C*-methyl-D-erythritol 4-phosphate cytidyl transferase; CMK (EC 2.7.1.148), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase; MCS (EC 4.6.1.12), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate synthase and IPPi, isopentenyl diphosphate isomerase.

The first reaction of the MVA-independent pathway involves the head to head condensation of (hydroxyethyl) thiamine, derived from pyruvate, with the C1 aldehyde group of D-glyceraldehyde–3-phosphate (G3P) to yield 1-deoxy-D-xylulose 5-phosphate (DXP). This transketolase reaction is catalysed by 1-deoxy-D -xylulose 5-phosphate synthase (DXPS, EC 4.1.3.37; Fig. 1). *Dxps* genes have been isolated from a variety of higher plants and functionality demonstrated either by complementation and/or in vitro activity (Table 4). The sequencing of the *Arabidopsis* genome has revealed the presence of three *Dxps* orthologs, although the functionality has only been shown for *Dxps*-2 to date [79,91].

Besides the MVA-independent pathway, DXP also serves as a precursor for the synthesis of vitamins B1 (thiamine) and B6 (pyridoxal) [81]. The first unique reaction of the MVA-independent pathway results from an intramolecular rearrangement and subsequent reduction of DXP to form 2-*C*-methyl-D-erythritol 4-phosphate (MEP) [82]. The enzyme responsible for this conversion, DXP reductoisomerase (DXR, EC 1.1.1.267), requires NADPH and Mn^{2+} as cofactors.

Table 4
Encoding genes and enzyme properties for the formation of IPP from pyruvate and glyceraldehyde-3-phosphate

Step/enzyme	Gene/species	Assignment	Enzyme characteristics Purity/activity	MW/kDa	cofactors	K_m (μM)	Ref.
(1) 1-Deoxy-D-xylulose 5-phosphate synthase (DXS)	*DXPS, Capsicum annuum*	Function	*E. coli* expression, –	70[a]	–	pyruvate– 500 G3P–750	[83]
	DXPS, Lycopersicon esculentum	Function	*E. coli* expression	–	–	–	[84]
	DXPS Mentha pierita	Function	–	–	–	–	[85]
				–	–	–	[79,91]
	DXPS (1), *Arabidopsis thaliana*	Homology	–	–	–	–	[86]
	DXPS (2), *Arabidopsis thaliana*			–	–	–	[79,91]
	DXPS (3), *Arabidopsis thaliana*	Function	–				
	DXPS, Tagetes erecta L.	Homology Function					[102]
(2) 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)	*DXR, Lycopersicon esculentum*	Function	–	45[a]	–	–	[87]
	DXR, Mentha pierita	Function	*E. coli* expression	43.5[b]	NADPH,	–	[88]
	DXR, Arabidopsis thaliana	Function	*E. coli* expression	–	Mn^{2+}/NADPH,	–	[89]
(3) 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (MCT)	*MCT, Arabidopsis thaliana*	Function	*E. coli* expression	29[b]	CTP	CTP–114?M	[90]
					Mg^{2+}	MEP–500 ?M	
(4) 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythtitol kinase (CMK)	*CMK, Arabidopsis thaliana*	Homology	–	–	–	–	[91]
	CMK, Lycopersicon esculentum	Function	*E. coli* expression,	36.4[b]	ATP, Mg^{2+}		[92]
	CMK, Mentha pierita	Function	*E. coli* expression,	31[b]	–	–	[93]
(5) 2-C-methyl D-erythritol 2,4-cyclodiphosphate synthase (MCS)	*MECPS, Arabidopsis thaliana*						
		Homology					
			–	–	–	–	[79,91]
(6)Hydroxymethylbutenyl 4-diphosphate synthase (HDS)	*GcpE, Arabidopsis thaliana*	Function	–	–	–	–	
	[94]						
(7) Isopenttenyl diphosphate/ dimethylallyl diphosphate (IDS)	*LytB, Arabidopsis thaliana*	Homology	–	–	–	–	[91]

[a] Determined by immunoblot.
[b] Determined following expression in *E. coli*.

MEP is converted to IPP and DMAPP via 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-MEP), 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate (HMBPP). The enzymes responsible for these reactions are 2-*C*-methyl-D-erythritol 4-phosphate cytidyl transferase (MCT, EC 2.7.7.60), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK, EC 2.7.1.148), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MCS, EC 4.6.1.12) and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate synthase (HDS). Although not functionally characterised in plants, comparison with bacterial genes/enzymes suggests that one enzyme IPP/DMAPP synthase (IDS) is responsible for the conversion of HMBPP to a 5:1 mixture of IPP and DMAPP. The properties of the genes and enzymes involved in the MVA-independent pathway are summarised in Table 4.

3.2. Formation of phytoene from IPP

Geranylgeranyl pyrophosphate (GGPP) is the immediate precursor of carotenoids. It is a C_{20} compound derived from four C_5 isoprene units. In order to initiate chain elongation IPP (C_5) is isomerised to its allylic isomer, DMAPP. Condensation of IPP and DMAPP in a head to tail fashion generates the C_{10} molecule geranyl pyrophosphate (GPP). Addition of IPP to GPP results in farnesyl pyrophosphate (FPP, C_{15}) formation and a further IPP molecule yields GGPP (Fig. 2). These condensation reactions involve the attack of a carbonium ion to the electron rich C-3,4 double bond of the IPP molecule with the concurrent loss of inorganic phosphate [95]. Two molecules of GGPP are condensed in a head to head manner to form the intermediate pre-phytoene pyrophosphate. Subsequent elimination of the diphosphate group and stereospecific proton abstraction results in phytoene (C_{40}) formation. Phytoene (7,8,11,12,7′, 8′, 11′, 12′-octahydro-ψ-ψ-carotene) is a colourless, symmetrical hydrocarbon containing three conjugated double bonds. Its C_{40} skeleton forms the basis from which all carotenoids are derived. In plants and most carotenogenic organisms phytoene typically accumulates as the 15-*cis* geometric isomer [96].

In order to synthesise phytoene from IPP three enzyme-catalysed reactions are required. The first enzyme, IPP isomerase (EC 5.3.3.2), catalyzes the reversible isomerisation of IPP and its allylic isomer DMAPP. Plastidic forms of IPP isomerase have been purified from tomato [97,98], pepper [99] and daffodil [100]. The enzymes require a divalent ion, typically Mg^{2+} or Mn^{2+}, for optimum activity. Their other properties are summarised in Table 5. IPP/DMAPP isomerase genes encoding plastidic enzymes have been identified from *Clarkia brewer* (*Ippi* 2) [101], marigold [102] and *Arabidopsis* (*Ippi* 2) [91], by homology and the presence of an N-terminal extension. This indicates plastid targeting and thus differentiates between them and the IPP activity found in the cytoplasm.

GGPP synthase is the enzyme responsible for the conversion of IPP/DMAPP to GGPP. It has been isolated as a soluble and functional homodimer (74kDa) from the chromoplasts of pepper [99]. The corresponding cDNA has been identified and sequenced [103]. Partial purification has also been achieved from tomato chromoplasts and chloroplasts [97,98]. In all cases, the enzyme requires a divalent ion (Mg^{2+}) for activity (Table 5). The tomato cDNA has also been isolated [104]. The *Arabidopsis* genome contains a *Ggpps* multigene family with 12 members. Two of these have been shown to encode plastidial isoenzymes [105], with 7 others believed to encode plastidic enzyme on the basis of putative plastid targeting sequences [91]. *Ggpps* genes have also been

isolated and identified from *Catharanthus roseus* (Ac no. X92893), sunflower [106], marigold [102] and *Lupinus albus* [107]. Whether the gene products are involved in the formation of carotenoids is not known. Recently, a plastid localised GPP synthase (*Gpps*) has been identified which converts IPP and DMAPP into GPP [108]. It is believed that this enzyme is involved in monoterpene biosynthesis, but not carotenoid formation.

Phytoene synthase catalyses the first committed step in the formation of carotenoids. Genes encoding phytoene synthase have been identified and isolated from *Arabidopsis* [91], maize [110], melon [111], carrot (AB032797), *Citrus paradisi (AF152892)* and *unshiu* (AF220218), daffodil [112], rice (D48697), pepper [103], marigold [102] and soybean (Ac no. BF067862). Two phytoene synthase genes have been found in tobacco [113] and tomato [114,115], while *Gentiana lutea* contains four phytoene synthases (E15680-1, 2, 3).

Detailed biochemical characterisation of phytoene synthases has been carried out with pepper [116] and tomato chromoplasts (PSY-1) and tomato chloroplasts (PSY-2) [97]. In addition, the daffodil PSY has been studied following expression in insect cells [117]. All of these phytoene synthases are membrane associated, but not integral membrane proteins. The pepper and tomato (PSY-1and 2) enzymes are active in vitro as soluble proteins, after release by high ionic strength buffers. The daffodil PSY exists in two distinct topological forms, which require galactolipid replenishment for activity. Plant phytoene synthases utilise all-*trans* GGPP and yield

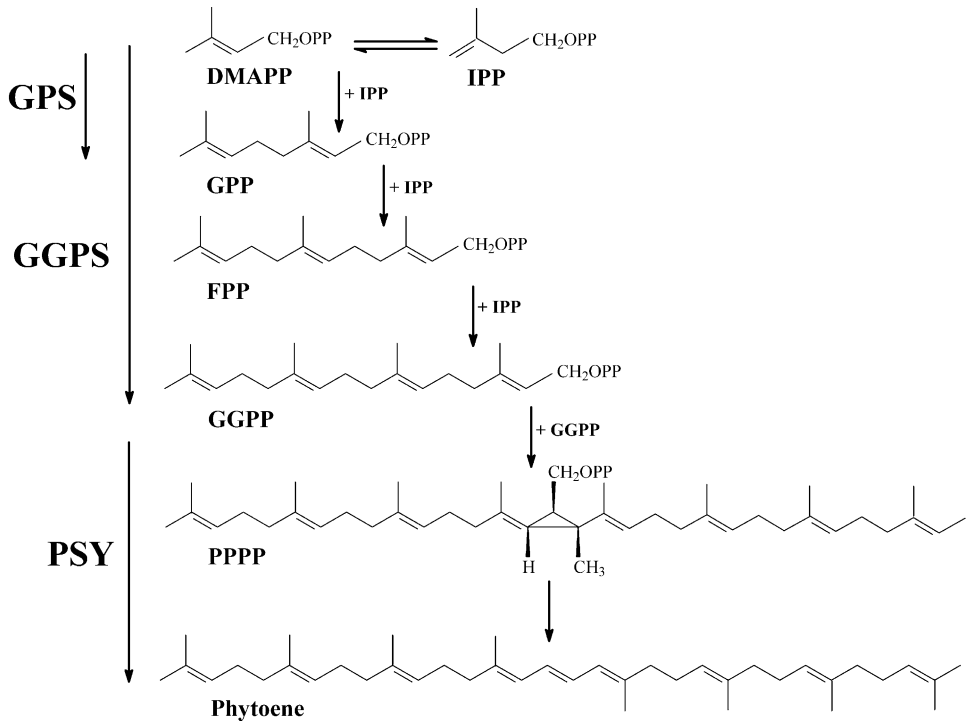

Fig. 2. Phytoene synthesis from IPP and DMAPP. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; PPPP, prephytoene diphosphate; GPS, geranyl diphosphate synthase; GGPS, geranylgeranyl diphosphate synthase and PSY, phytoene synthase.

Table 5
Properties of higher plant enzymes involved in the formation of phytoene from IPP, desaturation and isomerisation of acyclic carotenes, cyclisation of lycopene and xanthophyll formation

Source	MW (kDa)	No of isoforms	Km (μM)	Cofactor	pH optimum	Purity	Ref.
IPP isomerase							
Pepper fruit	33.5	1	6	Mg^{2+}	7–8	Homogenous	[99]
Daffodil flowers	–	1	7.6	Mn^{2+}	6–8	Partial	[100]
Tomato green and ripe fruit	34 (>200)*	1	5.7	Mn^{2+}/Mg^{2+}	8.2	Partial	[97] [98]
GGPPS							
Tomato	>200[a]	–	–	Mg^{2+}/Mn^{2+}	6.5 – 7.5	Partial	[97] [98]
Sinapis alba	38 (74)	–	33-IPP, 2-DMAPP	Mn^{2+}/Mg^{2+}	6.5 – 7.5	Homogenous	[109]
Pepper fruit	37 (74)[b]	1	3- IPP, 0.95- DMAPP, 1-GPP, 1.2– FPP.	Mn^{2+}	6.5 –7.5	Partial	[99]
PSY							
Tomato ripe fruit	37 (>200)[a]	1	10-GGPP	Detergent/ATP/ Mn^{2+}/Mg^{2+}	7.5	Partial	[97]
Tomato green fruit	43 (>200)[a]	1	5-GGPP	Detergent/ATP/ Mn^{2+}/Mg^{2+}	6.5	Partial	[97]
Daffodil flowers	42	1		Galactolipid/Mn^{2+}		Heterologous expression-crude cell free	[117]
Pepper ripe fruit	47.5	1	0.3 GGPP 0.27 PPPP	Mn^{2+}	7.6	Homogenous	[116]
PDS							
Pepper	57	1	–	FAD	7.5	Homogenous	[123]
Daffodil	56 (300)*	1		FAD/quinones	–	Crude cell free prep	[124]
Tomato	–	–	–	–	–	Crude cell free prep.	[97]
ZDS							
Pepper	53	1	–	O_2/quinones	–	Homogenous following heterologous expression	[132]
Daffodil flowers	–	–	–	O_2/quinones	–	Crude cell free prep.	[124]
LCY							
Tomato	–	–	–	Detergent	–	Crude cell-free prep.	[97]

(*continued on next page*)

Table 5 (*continued*)

Source	MW (kDa)	No of isoforms	Km (μM)	Cofactor	pH optimum	Purity	Ref.
Daffodil flowers	–	–	–	NADPH	–	Crude cell-free prep.	[138]
Pepper	–	–	–	Detergent	–	Crude cell-free prep.	[139]
Hydroxylases							
Pepper CRTR-b1and2	25	2	–	O_2/NADPH ferredoxin/ferredoxin oxidoreductase	–	Homogenous following heterologous expression	[148]
Epoxidase							
Pepper ZEP	65	1	–	NADPH, oxygen, reduced ferredoxin	–	Homogenous following heterologous expression	[152]
De-epoxidase							
Lettuce VDE	43	1	–	Ascorbate, MGDG	5.2	Homogenous	[153]
Spinach VDE	43	1	–	Ascorbate, MGDG	5.2	Partial	[154]
Capsanthin/capsorbin synthase	50	1	–	FAD	–	Homogenous	[159]
Neoxanthin synthase	56	1	–	–	–		[158]

[a] Evidence for protein complex existence.
[b] Exists as a dimer in the native form.

predominantly 15-*cis* phytoene in vitro. The pepper phytoene synthase requires Mn^{2+} solely for activity, as does the daffodil enzyme. The tomato phytoene synthases require ATP as well as a divalent ion. PSY-2 favours Mn^{2+} and ATP, while PSY-1 has a greater tolerance for Mn^{2+} or Mn^{2+}. Further properties of the enzyme are shown in Table 5.

3.3. Desaturation reactions

A series of conjugated carbon–carbon double bonds constitute the characteristic chromophore of carotenoid pigments. These are introduced into phytoene by a series of desaturation reactions (Fig. 3). Four desaturations form, sequentially, phytofluene (7,811,12,7′, 8′-hexahydro-ψ,ψ-carotene), ζ-carotene (7,811,12,7′,8′-terahydro-ψ,ψ-carotene), neurosporene (7,8-dihydro-ψ,ψ-carotene) and finally lycopene (ψ,ψ-carotene). The extended chromophore converts colourless phytoene into red-coloured lycopene.

The enzyme responsible for the desaturation of phytoene to ζ-carotene is phytoene desaturase (PDS). Phytoene desaturase genes have been identified and isolated from pepper [118], *Arabidopsis* [91], tomato [119], maize [120], daffodil [121], rice (Ac no. D23378), soybean [122] and tobacco [113]. All contain a conserved dinucleotide (FAD/NADP) binding site domain at the

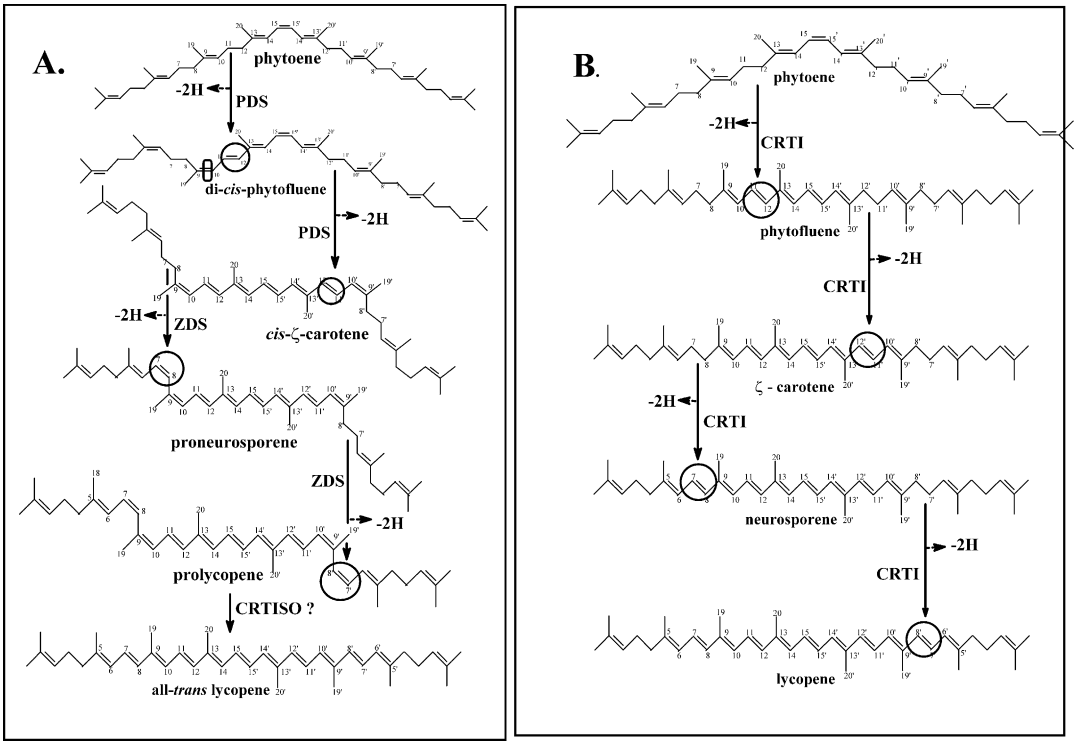

Fig. 3. Desaturation and isomerisation of phytoene. A. Desaturation and isomerisation reaction sequence occurring in higher plants. PDS, phytoene desaturase; ZDS, ζ-carotene desaturase and CRTISO, carotene isomerase. Circles indicate the position of newly formed double bonds. B. Reaction sequence utilised by bacterial and fungal (CRTI type) phytoene desaturases.

amino terminus. In vitro desaturase activity has been detected in plastid preparations from tomato [97], pepper [123] and daffodil [124]. In all cases the activity is predominantly membrane bound. The only report of a purified phytoene desaturase is from pepper [118]. Following heterologous expression, the daffodil PDS has been studied and found to require FAD and oxidised quinones [121]. Further properties of the desaturases are provided in Table 5.

The removal of two hydrogen atoms during each desaturation step suggests the involvement of an electron transport chain for regeneration of reductants. Components of this electron transport chain have been elucidated via characterisation of *Arabidopsis* [125] and tomato (ghost) [126] mutants that accumulate phytoene. This phenotype arises as a consequence of a non-functional alternative oxidase in the redox chain that inhibits desaturation. In addition, mutants of *Arabidopsis* containing a non-functional *p*-hydroxyphenylpyruvate dioxygenase (HPPD) accumulated phytoene [127]. HPPD is necessary for the formation of tocopherols and plastoquinones. Hence phytoene accumulation occurs, as desaturation cannot proceed without plastoquinone as a reductant. This phenotype is mimicked by treatment with cyclohexanedione bleaching herbicides, a class of herbicides inhibiting HPPD [128].

ζ-Carotene desaturase catalyses the desaturation of ζ-carotene into lycopene, via neurosporene. Encoding genes have been identified from pepper [129], *Arabidopsis* [130], maize (AF047490), soybean (Ac no. AI495658), daffodil (Ac no. AJ224683) and tomato [131]. All genes contain a dinucleotide (FAD/NADP) binding site domain at the amino-terminus. Comparison of *Pds* and *Zds* gene sequences shows 33 to 35% similarity. To date there is only one report of a purified ZDS from pepper following expression in *E. coli* [132]. The enzyme shows a broad substrate specificity of geometric isomers and requires oxygen and plastoquinones (Table 5).

3.4. Isomerisation

In higher plants phytoene exists predominantly as the 15-*cis* isomer, while the predominant geometric isomer of lycopene is all-*trans*, suggesting isomerisation must occur at some stage. In the early 1980s NMR characterisation of the pigments present in the tomato *tangerine* mutant revealed the presence of 7,9,7′,9′-tetra-*cis* lycopene (prolycopene) and traces of its poly-*cis* precursors [133]. Combinations of PDS and ZDS complemented in *E. coli* [134] as well as in vitro studies with daffodil chromoplasts detected the formation of prolycopene, unless light was present during incubation [135]. These two independent lines of evidence have led to suggestions that in higher plants an isomerase activity exists to mediate *cis* to *trans* conversion.

Map-based/positional cloning of the gene responsible for the *tangerine* tomato fruit phenotype resulted in the isolation of *Crtiso* [136]. When co-expressed in *E. coli* with *Pds* and *Zds* a greater proportion of all-*trans* lycopene was observed. Simultaneously the *Arabidopsis Crtiso* homolog termed *Ccr*-2 was isolated [137].

Phenotypically, *Ccr*-2 *Arabidopsis* mutants contained reduced levels of lutein and accumulated poly-*cis* carotene precursors in the dark. Interestingly, green tissues of *Ccr*-2 *Arabidopsis* or *tangerine* show no phenotypic difference. These data suggest that light and chlorophyll (or chlorophyll precursors) seem to facilitate photoisomerisation and alleviate the necessity for *cis*-*trans* isomerase activity. Both the *Arabidopsis* and the tomato *Crtiso* genes like *Pds* and *Zds* contain a dinucleotide (FAD/NADP) binding domain. The genes encoding *Crtiso* show more identity to bacterial phytoene desaturases (*Crt*I) than the other plant desaturases (*Pds* and *Zds*).

Interestingly, the bacterial desaturases convert 15-*cis* phytoene to all-*trans* lycopene or all-*trans* neurosporene in *Erwinia* and *Rhodobacter* species, respectively. Based on the current data it appears that the desaturation sequence proceeds from 15-*cis* phytoene with the introduction of *trans* double bonds at the 11, 11′ positions by PDS to form 9,9′-di-*cis* ζ-carotene. ZDS then introduces *cis* bonds to form 7,9,9′,7′-tetra- *cis* lycopene. From precursor/product profiles of *Arabidopsis Ccr* and the *tangerine* mutant it appears that CRTISO acts on prolycopene to form all-*trans* lycopene that subsequently enables cyclisation. Further biochemical studies on this aspect of the pathway are required.

3.5. Cyclisation

Carotenoid cyclisation is limited to the formation of a six-membered ring at one or both ends of the acyclic precursor. Typically, lycopene is the precursor for cyclisation. Formation of cyclic end groups is initiated by proton attack at the C-2 of the terminal C-1,2 double bond which is isolated from the polyene chain. Two types of cyclic end groups occur, α and β-ionone rings. These end groups only differ by the position of a double bond within the cyclohexane ring (Fig. 4). The type of end group depends on the nature of the cyclase enzyme. Unfortunately very few in vitro studies on cyclisation have been reported. The most comprehensive, with daffodil chromoplasts, suggests that all-*trans* lycopene is the substrate for cyclisation and NADPH is the cofactor required [138]. Cell-free cyclase activity has also been determined in tomato [97] and pepper chromoplast membranes [139] (Table 5).

Isolation and functional complementation in *E. coli* has revealed the existence of two major cyclase enzymes in higher plants. These are lycopene-β-cyclase (LCY-b), which introduces β-rings [140] and the lycopene-ε-cyclase (LCY-e) that introduces ε-rings [141]. Interestingly the LCY-b

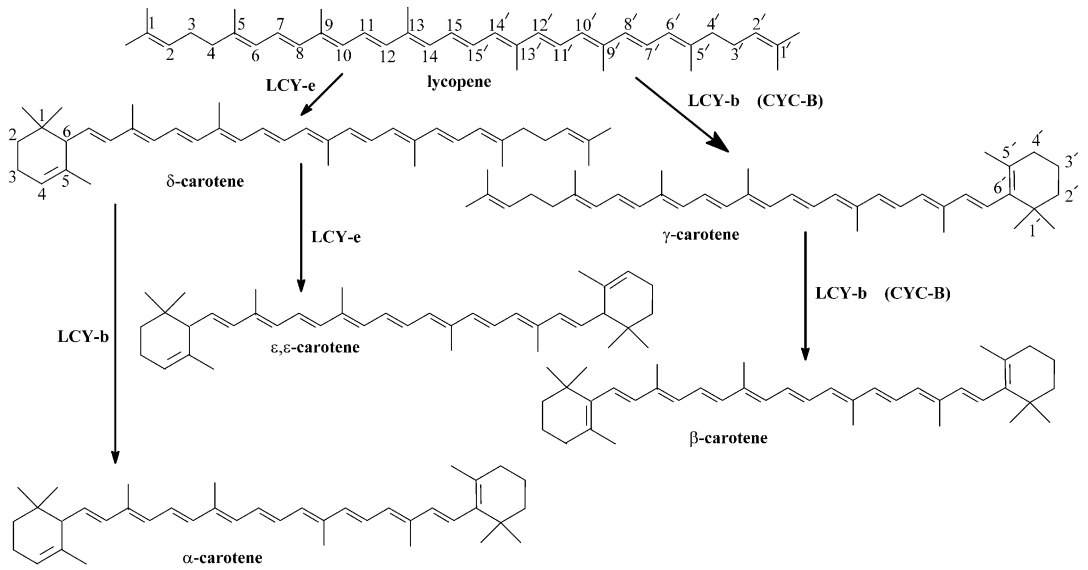

Fig. 4. Cyclisation of lycopene. Abbreviations: LCY-e, ε-cyclase; LCY-b / CYC-B, β-cyclase. * Introduction of two ε-rings only shown to occur in lettuce.

can catalyse the introduction of two β-rings into lycopene to form β-carotene via δ-carotene but the LCY-e can only incorporate one ε-ring forming δ-carotene. In order to form α-carotene both LCY-e and b must act. It has been suggested that the inability of ε-cyclase to add more than one ε-ring provides a mechanism for controlling cyclic carotenoid formation [140].

Lcy-b genes have been identified from *Arabidopsis* [142], daffodil (Ac no. X98796), tobacco (Ac no. F13561) and pepper [143]. In tomato two lycopene-β-cyclases have been isolated, one which shows chromoplast-specific expression (*Cyc-b*). The protein has an amino acid sequence that is 53% identical to the LCY-b [144]. *Lcy*-e genes have been isolated from *Arabidopsis* [141] and tomato [145]. Both LCY-e and b show significant resemblance at the amino acid level, with 30% identity. The two types of cyclase also possess characteristic dinucleotide (FAD/NADP)-binding sequence motif at the amino terminus.

Certain species of *Lactuca sativa* form the carotenoid lactucaxanthin (ε,ε-carotene-3,3′-diol). The LCY-e from lettuce shows 80% identity at the amino acid level to the *Arabidopsis* homolog. However, functional complementation in *E. coli* has shown that this gene product can introduce two ε-rings unlike its *Arabidopsis* counterpart [140].

3.6. Xanthophyll formation

3.6.1. Hydroxylation

In higher plants xanthophylls are enzymically formed oxidation products of α and β-carotene. Hydroxylation of the C-3 and C-3′ positions of α-carotene and β-carotene result in the formation of lutein and zeaxanthin via α-cryptoxanthin and β-cryptoxanthin, respectively (Fig. 5). The introduction of hydroxyl moieties into β-ring carotenes is catalysed by hydroxylases. In *Arabidopsis* two β-carotene hydroxylases have been identified [91,146] and homologs have been found in tomato [147] and pepper [148]. Heterologous expression has shown that the pepper hydroxylases are ferredoxin dependent and require iron (Table 5). The deduced hydroxylase amino acid sequences indicate the presence of transmembrane helices, suggesting an integral membrane location in vivo. In addition, conserved histidine clusters are observed in a similar manner to oxygen-dependant di-iron containing oxygenases. This motif has also been reported in the bacterial β-carotene oxygenase and hydroxylases [149]. To date, no hydroxylase capable of introducing a hydroxyl group in the 3 position of ε-rings has been identified. This reaction is necessary for the formation of lutein.

3.6.2. Epoxidation and de-epoxidation

Violaxanthin is formed from zeaxanthin through the introduction of 5,6-epoxygroups into the 3-hydroxy-β-rings. This reaction proceeds via the mono-epoxidated intermediate antheraxanthin. The reaction sequence is reversible and de-epoxidation can convert violaxanthin back to zeaxanthin (Fig. 5). These reactions are collectively known as the violaxanthin cycle [150]. Zeaxanthin epoxidase (*Zep*1) gene sequences have been identified in *Arabidopsis* [91], apricot (Ac no. AF159948), tomato (Ac no. Z83835), tobacco [151] and pepper [152]. Functionality following heterologous expression has been shown with the pepper gene product where the reaction was dependent on reduced ferredoxin [152] (Table 5). Violaxanthin de-epoxidase (VDE) have been isolated and purified from lettuce [153] and spinach [154]. The enzyme has a MW of 39 kDa, an optimal pH of 5 and requires ascorbate for activity [153]. *Vde* genes have been identified from

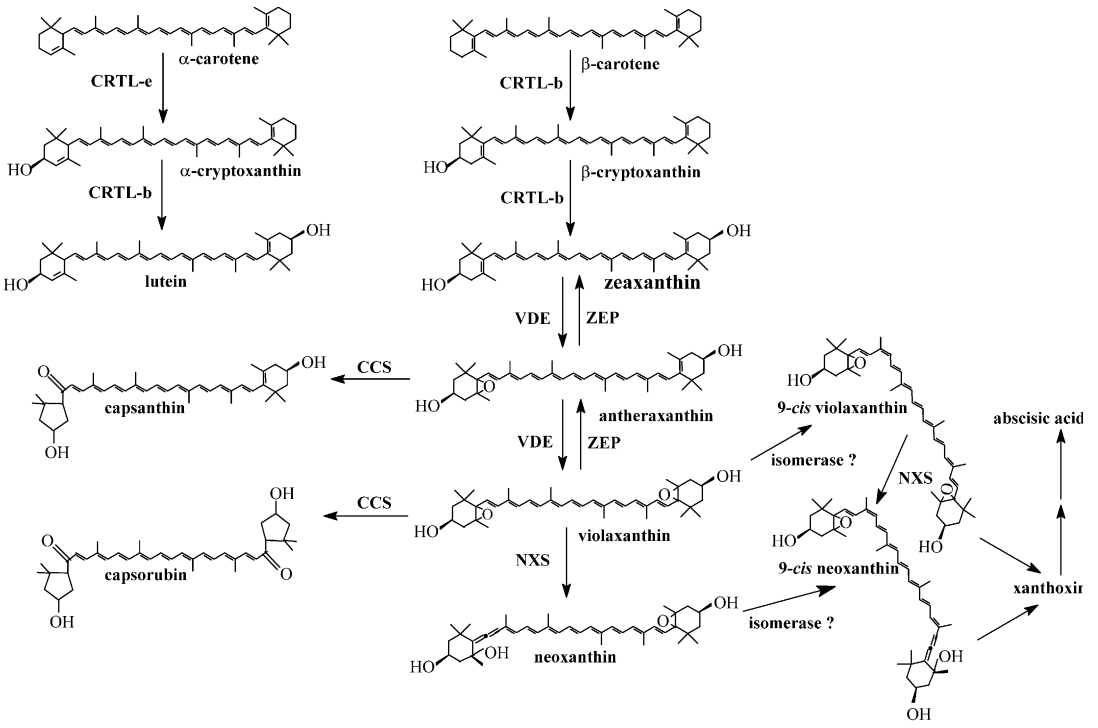

Fig. 5. Formation of xanthophylls. Abbreviations: CRTL-e, ε-ring hydroxylase; CRTL-b, β-ring hydroxylase; ZEP-1, zeaxanthin epoxidase; NXS, neoxanthin synthase; VDE, violaxanthin de-epoxidase and CCS, capsanthin /capsorubin synthase.

lettuce [155], tobacco (U34817), spinach (AJ250433) and *Arabidopsis* [91]. Both *Zep* and *Vde* deduced amino acid sequences indicate that they are members of a group of proteins known as lipocalins that bind and transport small hydrophobic molecules [156].

3.6.3. Neoxanthin synthesis

Neoxanthin is formed from violaxanthin and contains a single allenic end group. It is assumed that this end group originates from proton abstraction from C-7 which is adjacent to the 5,6-epoxy-5,6-dihydro-β-rings of violaxanthin. Rearrangement of the epoxy group to 5-hydroxy then arises (Fig. 5). Isolation and functionality for the potato [157] and tomato [158] gene products has been achieved (Table 5). Interestingly, no *Nxs* have been identified in the *Arabidopsis* genome. In addition the deduced amino acid sequence of tomato NXS is identical to CYC-B. This has lead to speculation that the enzyme is bi-functional under certain conditions.

3.6.4. Capsanthin and capsorubin synthesis

Capsanthin and capsorubin are not found in chlorophyll containing tissues of higher plants and are relatively unique to ripe fruits of peppers. These ketolated carotenoids contain an unusual cyclopentane ring (κ-ring) which is formed from the 3-hydroxy-5,6-epoxy β-rings found in violaxanthin and antheraxanthin (Fig. 5). Capsanthin/capsorubin synthase has been purified from pepper chromoplasts [159]. Its functional properties are summarised in Table 5. The *ccs* gene

sequence possesses an amino terminal dinucleotide (FAD/NADP) binding motif and shows close resemblance to the *Lcy*-b [159]. This has led to the demonstration of cyclase enzyme activity [143].

3.7. Catabolism and degradation of carotenoids

The isolation of ABA deficient mutants and subsequent gene identification has revealed the role of carotenoids, especially violaxanthin and neoxanthin as ABA precursors. The epoxycarotenoid cleavage (NC) enzyme in maize (VIP14 gene) has now been shown to cleave 9-*cis* isomers of violaxanthin and neoxanthin to yield *cis*-xanthoxin [160] (Fig. 5). Within the *Arabidopsis* genome a family of seven NC genes has been identified on the basis of homology. Tomato cleavage enzymes have also been identified [91]. The substrate specificity of these cleavage enzymes appears from functional complementation and in vitro assays to be broad and not reflective of the in vivo situation. Genuine in vivo products and their biological function await further investigation. Recently, the genes involved in oxidative cleavage of zeaxanthin to crocetin have been isolated from *Crocus sativus* and functionality characterised [161].

4. Regulation of carotenoid biosynthesis in higher plants

The regulation of carotenoid biosynthesis at the gene and enzyme level is poorly understood. No regulatory genes involved in carotenoid formation have been isolated, although the *Or* gene identified in cauliflower [162] and the apricot (*Ap*) tomato mutant look promising in this respect. The central role of carotenoids in plant development and adaptation suggest that their synthesis is coordinated with other developmental processes such as plastid formation, flowering and fruit development.

The practical difficulties of working with carotenoids and their biosynthetic enzymes contribute to our partial knowledge of pathway regulation. A branched pathway such as that of carotenoid formation from isoprenoid precursors is unlikely to be controlled by a sole regulatory process. Instead, control points are likely to be at each branch point and probably involve both transcriptional and post transcriptional events.

4.1. Transcriptional regulation

Tomato and pepper fruit ripening, flower development in daffodil, tomato and marigold (*Tagetes erecta*) are all associated with plastid differentiation, the development of chromoplasts and de novo carotenoid formation. Biogenesis of plastids is associated with an increase and deposition of carotenoid pigments. Both up- and down-regulation of transcription of carotenoid genes have been found. For example, during tomato fruit ripening expression of a ripening-enhanced phytoene synthase (*Psy*-1) and *Pds* are increased [163,145]. Concurrently both *Lcy*-b and *Lcy*-e mRNAs are reduced [145]. Determinations of enzyme activities in ripe fruit have extrapolated these changes in gene expression to the enzyme level [163]. Calculation of flux control coefficients has also enabled assessment of the control exerted by each step in the pathway [164]. These data indicate that phytoene synthase-1 exerts the greatest control over pathway flux. Collectively, the data show that large quantities of lycopene accumulate in ripe tomato fruit by increasing the acyclic carotenes of the pathway and blocking the formation of end products at the

cyclisation step. Tomato colour mutants and transgenic varieties have been valuable in elucidating regulatory mechanisms operating during fruit ripening [145,115]. Pigment analysis of the *crt*I transgenic tomato variety along with *Lcy*-b mutant *Og* , *Ogc* and high-beta, suggest that feedback inhibition could operate within the pathway either from β-carotene or a product of β-carotene. This evidence is supported by the increased expression of *Pds* and *Psy*-1that resulted from the chemical inhibition of lycopene cyclisation in tomato leaves [165]. However, in contrast no feedback inhibition of *Pds* gene expression was observed in the phytoene-accumulating *immutans* mutant [166]. Recently, it has been proposed that the MVA-independent pathway metabolite DX up-regulates downstream carotenoid biosynthetic genes such as *Psy*-1 [84].

Transcriptional regulation resulting in increased enzyme activities have also been illustrated in pepper fruit ripening [104]. In addition, oxidative stress, which facilitates carotenoid synthesis during chromoplast differentiation, has also been shown to up-regulate expression of carotenoid genes [167]. Up-regulation of carotenoid gene expression is reported during flower development of daffodil [117,121] and marigold [102] as well as melon [111] and citrus fruit [168] development and ripening.

Light intensity appears to alter carotenoid formation by increasing the expression of certain carotenoid biosynthetic genes. For example, in developing seedlings of mustard (*Sinapis alba*) *Psy* mRNA increases in the light while *Pds* and *Ggpps* remain constant. This effect appears to operate through a phytochrome-mediated process [169]. Changes in *Lcy*-b and *Lcy*-e mRNAs are also apparent when changes in light intensity are experienced [170]. Structural and functional characterisation of the phytoene synthase promoter from *Arabidopsis* has identified regions that are responsible for modulating different light qualities [171]. Although carotenoid biosynthesis is necessary for the development of chloroplasts, expression of carotenoid genes collectively is neither light dependant nor controlled by cryptochrome 1 genes [172].

4.2. Post-transcriptional regulation

During the formation of isoprenoids a key regulatory issue is what mechanisms control the partitioning of precursors into the branches of the pathway. The concept of metabolic channelling between each branch of the pathway has been postulated. The existence of multigene families has added support to this theory and experimental evidence of associated enzymes has been obtained in tomato [97], pepper [99] and daffodil [100]. In addition, the relatively small changes of isoprenoids in transgenic plants, despite increased enzyme activities support the concept that such branches are relatively independent of each other. In contrast, however, the dwarfing that results from constitutive *Psy*-1 expression in tomato suggests that the GGPP pool can be diverted from one branch (Gas) to another (carotenoids) at this stage of plant development and metabolic cross talk does exist [173].

In chromoplasts of daffodil membrane association appears to be a post-transcriptional means of regulating activation of PSY and PDS. It is believed such a mechanism is linked to the redox state of the membrane-bound electron acceptors in the case of PDS. In addition, light-induced membrane association is believed to regulate phytoene synthase activity during photomorphogenesis [174].

Substrate specificity of the LCY enzymes is suggested as a mechanism for regulating the partitioning of β and ε-ring carotenoids [141]. Direct inhibition of enzyme activity by carotenoid end products has been demonstrated to a certain extent [97].

4.3. Carotenoid sequestration

The sequestration of carotenoids within the cell is a form of regulation. It would appear that chloroplasts and chromoplasts differ considerably in the way they sequestrate end-product carotenoids. In chloroplasts end-products carotenoids are associated with light-harvesting complexes [175]. The unbound carotenoids are likely to associate with specific proteins as small plastoglobules. In chromoplasts of pepper end-product carotenoids are typically esterified and associated with the fibrillin protein as fibrils [176]. Tomato chromoplasts appear to sequestrate lycopene as crystals. In transgenic Canola [177] and tomato [178] plastid ultrastructure has been altered in response to changes in carotenoid content. Esterification of carotenoids would appear to be an effective mechanism used by many flowers (e.g. sunflower, daffodil and marigold) to produce high levels.

5. Strategies for enhancing carotenoid levels in crop plants

Before embarking on an experimental programme to enhance nutritionally important carotenoids in crop plants several pre-requisites should be considered. Confronting these issues at an early conceptual stage will enable achievable aims and objectives to be devised as well as placing proof of concept approaches on sound foundations for subsequent exploitation.

One of the first questions to be considered is what are the disease states to be addressed by dietary intake? Secondly, is there sound experimental evidence supporting the health benefits claimed? For example, as detailed in Section 2.1, vitamin A deficiency is a clear contributor to childhood blindness in Southeast Asia, while strong epidemiological evidence indicates that high dietary intake of lycopene (in the form of tomato products) reduces the onset of prostate cancer in western societies. These two examples also show how geographical and social-economic factors must be taken into account when devising an effective strategy, which can be tailored to enhance a specific carotenoid. However, the health benefits of carotenoid mixtures and their synergy with other phytochemicals in fruits and vegetables must not be overlooked.

These factors also have a bearing on the choice of crop. Ideally, the crop plant should be amenable to conventional breeding approaches and/or genetic manipulation, contain an endogenous carotenoid pathway and a known basal profile of carotenoids. It is advantageous, but not essential, for the crop plant to be a staple dietary consistent. Alternatively, production of carotenoids in non-food crops followed by introduction into the food chain as supplements may represent a means of supplying the consumer with desired product, whilst alleviating concerns over consumption of genetically modified (GM) foods.

Once the particular carotenoid(s) and crop plants have been targeted there are two strategies available for the enhancement of carotenoids in plants: (1), conventional plant breeding/varietal differences and (2), genetic engineering (often termed metabolic engineering or genetic manipulation).

5.1. Conventional plant breeding

Over the past 50 years modern plant breeding has successfully increased productivity by pursuing higher yields and better adaptation to biotic and abiotic stress. However, breeding programmes

Table 6
Tomato mutants with altered carotenoid contents

Genotype	Phenotype	Mutation/activity	Chromosome	Ref
R; yellow flesh	No carotenoids	PSY-1/phytoene synthesis	3–29	[115]
hp-1; high pigment-1	Increased carotenoids	Light signalling	12	[181]
hp-2; high pigment-2	Increased carotenoids	DET1/light signalling	12	[182]
t; tangerine	Increased poly- *cis* lycopene	CRTISO/carotene isomerase	10–24	[136]
cr; old gold crimson	Increased lycopene, lower β-carotene	CYC-B/lycopene cyclisation	6–106	[144]
B; Beta	Increased β-carotene, very low lycopene	CYC-B/lycopene cyclisation	?	[144]
Del; Delta	Increased δ-carotene, reduced lycopene	LCY-e/lycopene cyclisation	?	[145]
at; apricot	Reduced carotenoids	NI[a]	5	[183]
sh; sherry	No carotenoids	NI	10L	[183]

[a] NI-not identified.

aimed at improving health-promoting phytochemicals (such as carotenoids) have been largely overlooked. Tomato is a case where the effect of genotypic variation on carotenoid content has been studied. Of the nine related species of the genus *Lycopersicon* six have green coloured fruits with a carotenoid and chlorophyll profile similar to that found in leaf tissue. The two species *L. cheesmanni* and *L. pimpinellifolium* yield ripe fruit containing no chlorophyll but β-carotene and lycopene, respectively. These species are the phylogenetically closest to *L. esculentum*, which has with ripe fruit containing both lycopene and β-carotene [179]. Tomato breeding has resulted in a wide range of varieties with different carotenoid profiles (Table 6) many of which await full nutritional exploitation. Pepper (*Capsicum*) varieties also exist with a diverse range of carotenoid profiles [180]. Recently a variety of cauliflower (*Brassica oleracea* var. botrytis) with an intense orange-coloured curd, contrasting the white/cream of the wild type has been reported [162].

5.2. Genetic engineering of carotenoid biosynthesis in crop plants

The advantages of genetic engineering over conventional breeding methods include the ability to transfer gene(s) in a faster and targeted manner. In addition to the transfer of genes and cDNAs from the same species, modern recombinant technologies facilitate the introduction of genetic material from diverse *planta* and unrelated species such as microorganisms. The main disadvantage of second-generation GM crops at present resides with the lack of consumer acceptance of novel foods within the market place.

A good starting point prior to performing plant transformations is to determine the profile and levels of carotenoids present in the target crop and/or tissue of interest and compare these with amount of carotenoid required. The latter may be extrapolated from RDAs or related to the amount consumed in an average meal. Such data will help define the engineering approach required. For example, if more of the end product carotenoid is required then a strategy aimed at increasing flux through the pathway is appropriate (e.g. quantitative engineering of the pathway). Typically, amplification of the 'rate-limiting' enzyme or more correctly the enzyme with the

highest flux control coefficient is the principal target for manipulation. Alternatively, it may be desirable to change the composition of carotenoids or create a new carotenoid pathway in the tissue of interest (e.g. qualitative engineering). Another approach that has been successful, but is difficult to design rationally, is pleiotrophic engineering. In this instance the carotenoid content is altered as a result of manipulations to another pathway or biological process from which crosstalk with carotenoid formation (often normally silent) operates.

Metabolite/precursor pool sizes, enzyme activities and location, gene expression profiles, carotenoid catabolism, interaction with other isoprenoid pathways and regulatory mechanisms are needed to influence the choice and combination of genes and promoters necessary to manipulate the pathway. As described in Section 3 most of the carotenoid biosynthetic genes have now been isolated and characterised from bacteria, fungi, algae and higher plants. Several genes encoding retinoic acid (vitamin A) have also been isolated from animals [184]. Only one gene, fibrillin has been isolated that is associated with carotenoid sequestration [176] and no regulatory genes influencing carotenoid formation have been isolated from plants. A comprehensive selection of biosynthetic genes or cDNAs with diverse homologies but functional identity is advantages to prevent genetic effects such as co-suppression (sense suppression and/or gene silencing). If bacterial or fungal genes are used targeting to the plastid is achieved by creating a chimeric gene, containing a leader sequence fused to the 5′ end of the transgene.

The choice of promoter is very important, as its properties control the dynamics, level and tissue specificity of expression of gene of interest. From a commercial viewpoint, it is important to be aware of intellectual and tangible property (IP/TP) rights. Being aware of ownership and material transfer agreements (MTA) can save time in the long-term and impact on downstream commercial development [185].

Since the first successful plant transformation in 1988 there have been reports of transformation systems for virtually all crop plants [186]. However, it is important to ensure that the system is applicable to varieties within a genus and will create stable lines. Finally, molecular, biochemical and nutritional methodologies must be in place not only to assist in plant regeneration but direct further rational design of metabolic engineering, assess nutritional potential and implement safety requirements.

5.3. Examples of genetically modified crops with altered carotenoid levels

In Section 4.2 the pre-requisites that should be considered when attempting to alter carotenoid levels in crop plants were described. The present section provides examples of main crops that have been subjected to metabolic engineering (Table 7). The approaches used will be illustrated, together with any difficulties encountered and how they may be overcome.

5.3.1. Tomato

Tomato fruit and its products represent the principal dietary source of lycopene and an important source of β-carotene among western diets. It is not surprising, therefore, that it has been the most intensely studied crop in terms of carotenoid manipulation.

During tomato fruit ripening there is a massive (400-fold) deposition of carotenoids of which lycopene represents some 90% of the total [163]. Lycopene accumulation occurs as a result of down-regulating lycopene cyclisation and up regulating the expression of a fruit ripening-enhanced phytoene synthase (*Psy*-1). Phytoene synthase exhibits the highest flux control

Table 7
Examples of the metabolic engineering of carotenoid biosynthesis in crop plants

Inserted gene/cDNA	Promoter	Transformation	Variety	Phenotype (carotenoid μg/g FW), (proVitA RDA/ portion)	Zygosity/inherited phenotype	Ref.
Rice						
Psy cDNA from Daffodil	CaMV 35S	Microprojectile bombardment	Japonica, taipei 309	Phytoene (0.3 μg/g FW) accumulation in endosperm	Ho,T_2 inherited	[201]
Psy cDNA from Daffodil	Glutelin	Microprojectile bombardment	Japonica, taipei 309	Phytoene (0.6 μg/g FW) accumulation in endosperm	Ho,T_2 inherited	[201]
crtI (*E. uredovora*) + *Psy* + *Lcy*-b (Daffodil).	CaMV 35S Glutelin	*Agrobacterium, co-transformation*	Japonica, taipei 309	β-carotene (1.6 μg/g FW) accumulation in endosperm, 8% RDA/300 g portion.	ND, ND	[202]
crtI + Psy + *Lcy*-b	CaMV 35S Glutelin	*Agrobacterium, co-transformation*	Indica, varieties	β-carotene (1.6 μg/g FW) accumulation in endosperm, ca. 8% RDA/300 g portion	ND, T_2 inherited	[203]
Tomato						
tomato *Psy*-1 antisense	CaMV 35S	*Agrobacterium*	*Ailsa Craig*, Wild type	100-fold reduction in carotenoids, increased gibberellins 3-5-fold	Ho, T_6 inherited	[188]
*E.uredovora, crt*I	CaMV 35S	*Agrobacterium*	*Ailsa Craig*, Wild type	2–4-fold increased β-carotene, (20–45 μg/g FW), ca. 60% RDA/fruit, decreased lycopene.	Ho, T_4 inherited	[178]
*E.uredovora, crt*B	PG	*Agrobacterium*	*Ailsa Craig*, Wild type	2–3-fold increases in phytoene, lycopene, β-carotene.	Ho,T_3 inherited	[164]
Tomato *Psy*-1 sense	CaMV 35S	*Agrobacterium*	*Ailsa Craig*, Wild type	Sense suppression, premature lycopene accumulation, dwarf plants (decreased gibberellins and increased ABA)	Ho, T_3 inherited	[187]
Tomato CYC-B antisense	CaMV 35S	*Agrobacterium*	CV VF36	Decreased proportion of β-carotene	ND, ND	[144]
Tomato CYC-B sense				Increased proportion of β-carotene		
Yeast *ySAMdc*	CaMV 35S	*Agrobacterium*	CV VF36	3-fold increase lycopene (100 μg)	ND, ND	[144]
Tomato *Lcy-b sense*	*E8*	*Agrobacterium*	Ailsa Craig, Wild type	Up to 7-fold increase in β-carotene, 70% RDA / fruit	Ho, T_2 inherited	[195]
Tomato *Lcy*-b antisense	*Pds*	*Agrobacterium*	Moneymaker		ND, ND	[193]
Tomato *Lcy*-b + pepper	*Pds*	*Agrobacterium*	Moneymaker	Up to 30% increase in lycopene β-cryptoxanthin 5 μg/g FW, zeaxanthin produced 13 μg/g FW	ND, ND	[193]

(*continued on next page*)

Table 7 (*continued*)

Inserted gene/cDNA	Promoter	Transformation	Variety	Phenotype (carotenoid μg/g FW), (proVitA RDA/ portion)	Zygosity/inherited phenotype	Ref.
CrtR-b	*Pds*	*Agrobacterium*	Moneymaker		ND, ND	[194]
Canola						
*E. uredovora, crt*B	*Napin*	*Agrobacterium*	Cultivar 212/86 Quantum	50-fold increase in carotenoid content (Lutein), 1400 μg/g FW, 12% RDA/g FW.	Ho, T_4 and Field trial inherited	[177]
Carrot						
E. herbicola, crt genes	CaMV 35S	*Agrobacterium*	–	2-5 fold increase in carotenoid content	Ho, T_2	[197]
Potato						
Tobacco *Zep*, antisense and sense.	*GBSS*	*Agrobacterium*	Freya	130-fold increase in zeaxanthin (4μg.gFW), 5.7-fold increase in total carotenoid (6μg.gFW), 1% RDA/potato	ND, ND	[198]
			Baltica			

coefficient among the enzymes of the pathway [164]. These data suggest that phytoene synthase possess the greatest control over flux through the pathway. Consequently, it has been the principal target for amplification with the objective of increasing the lycopene and β-carotene content in fruit. However, the first report of such a transgenic tomato line (CV. Ailsa Craig), generated with the tomato *Psy*-1 under the control of the constitutive cauliflower mosaic virus (Ca MV) 35S promoter, exhibited detrimental pleiotropic effects. Firstly, the majority of plants showed a dwarf phenotype [173,187]. Dwarfism correlated with increased *Psy*-1 expression, elevated carotenoid and abscisic acid (ABA) content, but reduced gibberellin (GA) levels. The data suggest that dwarfism was a consequence of metabolic competition for the common carotenoid and GA precursor GGPP, channelling it into carotenoids and away from GA synthesis. A fine balance in the equilibrium of these compounds would appear to be necessary during fruit development. Secondly, the plants showed intense pigmentation in tissues typically associated with low carotenoid contents, i.e. seeds and roots [173,187]. Transformants surviving the effects of dwarfism showed pre-mature lycopene accumulation in developing fruits. Ripe fruit possessed varying degrees of reduced carotenoid content and in some cases virtual absence of carotenoids [173,187], similar to *Psy*-1 antisense plants [188,189]. Apparently, the use of the endogenous *Psy*-1 in combination with a constitutive promoter caused gene silencing (co-suppression) of the endogenous *Psy*-1 [190]. From a metabolic engineering perspective gene silencing is either a problem, as shown with the *Psy*-1 lines, or an opportunity if reduction of a particular metabolite is required.

In order to overcome the detrimental effects experienced with *Psy*-1 expression, a tissue-specific promoter and non-homologous phytoene synthase were employed in later experiments [164]. The tomato polygalacturonase (PG) promoter was used to express the bacterial phytoene synthase (*crt*B) from *Erwinia uredovora* in a fruit ripening specific manner. This approach avoided effects due to perturbations of GGPP levels in developing fruit, as in ripe fruit the pool of GGPP is greater than in green tissues and GA synthesis is no longer required [191]. The use of a bacterial gene that was functionally identical but possessed low homology at the nucleotide level (ca. 33% identity) prevents gene silencing. An alternative approach, using the tomato *Psy*-1 that had been chemically changed in every third codon to create a synthetic gene with homology below 60% identity, was also successful [192].

Transgenic plants expressing *crt*B in a fruit specific manner exhibited a 2–3-fold increase in carotenoids. Phytoene, lycopene and β-carotene levels were all increased. Biochemical analysis showed that active CRTB protein was produced and targeted to the plastid [164]. 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.

Phytoene desaturation is another step that has been subjected to amplification. Transgenic tomato (CV. Ailsa Craig) plants expressing the bacterial phytoene desaturase (*crt*I) from *Erwinia uredovora* with the CaMV 35S promoter have been generated [178]. The CRTI protein was targeted to the plastid with the plastid transit sequence from the pea small sub unit (SSU) of RuBisCo. Transformants had orange coloured fruit. Carotenoid analysis showed compositional changes in vegetative tissues, with the proportion of β-carotene and its metabolites increased. In ripe fruit, β-carotene levels increased 2–4 fold, thus providing half the RDA of provitamin A per fruit. Besides β-carotene the content of neoxanthin, antheraxanthin, lutein, zeaxanthin and

biosynthetically related compounds such as tocopherols were increased [178]. In contrast, the levels of carotenoids prior to β-carotene were all reduced. As a consequence, the total amount of carotenoids present was lower than that in the wild type. Although phytoene desaturase activity was increased in these transgenic plants, no elevation of lycopene was found. Levels of gene expression and enzyme activities alluded to crosstalk within the pathway, whereby the presence of *crt*I induced endogenous lycopene cyclisation and reduced the content of compounds prior to phytoene desaturation. It is noticeable that although the bacterial and plant desaturase(s) differ in their preference of carotene isomers as substrates, no alteration in the composition carotenoid isomers was found among the *crt*I transformants.

The accumulation of lycopene in ripe tomato fruit provides a large pool of precursor for cyclisation to β-carotene. Transgenic tomato plants (var. Moneymaker), expressing the *Arabidopsis thaliana* lycopene β-cyclase (β-*Lcy*) under the control of the tomato phytoene desaturase (*Pds*) promoter have been produced [193]. Transformants with orange coloured ripe fruit were generated, with a 5-fold increase in β-carotene. The amounts of other carotenoids in these fruit were unaffected, as were those in leaf tissue. 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. The lycopene content of the fruit was slightly increased and leaf carotenoids were unaffected. 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 [144].

One of the limitations of ripe tomato fruit as a source of health-related carotenoids is the absence of significant levels of xanthophylls such as lutein or zeaxanthin. High β-carotene tomato varieties exist (Table 6). Metabolic engineering to metabolise β-carotene to zeaxanthin was attempted with cDNA's encoding hydroxylases from both plants and bacteria in these varieties. Despite the production of an active hydroxylase no zeaxanthin was produced [193]. Recently, zeaxanthin formation has been successfully engineered into tomato (var. Moneymaker) fruit by co-transformation with separate constructs containing the *Arabidopsis* β-*Lcy* and *Capsicum* β-carotene hydroxylase (β-*Chy*), both under tomato *Pds* promoter control [193]. 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.

The examples of genetic engineering described above have targeted a specific step or steps in the biosynthetic pathway. Recently Mehta et al. [192] described transgenic tomato plants (var. Ailsa Craig) with increased lycopene content. The objective of the study was 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.

An important caveat in generating new tomato varieties by genetic engineering is inheritance of the phenotype. Gene silencing can arise as progeny are generated. To date data have only been reported for the *Psy*-1 [188,173], *crt*B [164] and *crt*I [178] transgenic plants in the T_2, T_3 and T_4 generations, respectively. In all cases stable homozygous lines were obtained from primary transformants with single gene inserts. Segregation of the transgene with the phenotype occurred in an approximate Mendelian manner.

5.3.2. *Carrot* (Daucus carota)

Amoco Bioproducts were the first group to report the successful genetic manipulation of carotenoid biosynthesis in a crop plant. The *Erwinia herbicola crt* genes were introduced into carrot, resulting in a 2-5-fold increase in β-carotene in the root [196,197].

5.3.3. *Potato* (Solanum tuberosum)

Basal carotenoid levels in potato are low in comparison to most fruits and vegetables. Potato contains carotenoids typically found in chloroplastidic tissues e.g. xanthophylls such as lutein and violaxanthin. Transgenic varieties have been produced with increased zeaxanthin (5–130-fold) and total carotenoids [198]. In order to enhance the carotenoid profile of potato in such a manner both antisense and sense suppression approaches have been used. Firstly, varietal differences were exploited to select a high carotenoid yielding genotype. The endogenous zeaxanthin epoxidase cDNA, under tuber specific promoter control, was then used to antisense and sense suppress the conversion of zeaxanthin to violaxanthin. As a result zeaxanthin accumulated. In addition, the whole carotenoid biosynthetic pathway was affected as total carotenoid levels were elevated. Interestingly, despite violaxanthin being an important intermediate in the synthesis of ABA no effects due to phytohormone imbalance were observed.

5.3.4. *Canola* (Brassica napus)

To date the most spectacular increases in carotenoid levels resulting from metabolic engineering have been achieved in canola. Wild type, mature canola embryos contain low levels of lutein. A single gene transformation with the *Erwinia uredovora* phytoene synthase (*crt*B), under control of the seed-specific napin promoter from *Brassica rapa* [199], resulted in a 50-fold increase in carotenoids [177]. The transgenic embryos were visibly orange in colour, containing α and β-carotene predominantly. Phytoene was also detected. In addition, chlorophyll and tocopherol levels were decreased, while fatty acid acyl composition and cellular ultrastructure were altered. Homozygous lines were generated and the phenotype stably inherited into the T_4 generation. Filed trials also demonstrated this phenotype in two varieties, *Brassica napus* L. Cultivar 212/86 and Quantum.

5.3.5. *Rice* (Oryza sativa)

The potential humanitarian benefits in alleviating vitamin A deficiency offered by the generation of transgenic rice with β-carotene containing endosperm have received massive publicity and led to the term ''Golden Rice''. Rice endosperm lacks provitamin A and other carotenoids. However, expression of some genes involved in carotenoid formation was detected [200]. Biochemical studies indicated that GGPP was formed in the endosperm [201]. Therefore, in order to enable metabolism of GGPP to β-carotene cDNAs of three biosynthetic genes were co-transformed via two vectors. The daffodil (*Narcissus pseudonarcissus*) phytoene synthase and lycopene β-cyclase cDNAs were placed under endosperm-specific control (glutelin promoter) and *E. uredovora* phytoene desaturase (*crt*I) under constitutive control (CaMV35S). The endosperm of resulting transformants contained lutein, zeaxanthin, β-carotene and α-carotene in varying proportions [202]. It was also observed that, akin to the effect found with tomato [178], the presence of just *crt*I and *Psy* yielded β-carotene and derived xanthophylls. This is presumably due to induction of the lycopene cyclase gene in response to lycopene formation. Inheritance of the

phenotype and segregation with transgenes to generate homozygous lines has not yet been reported.

The level of carotenoid formed in the transgenic endosperm has been estimated at 1.6 μg/g endosperm. It is estimated that in order to supply the RDA of provitamin A in an average rice meal (300 g), 2.0 μg of β-carotene must be present per g endosperm. These levels would seem to be attainable. However, the calculation does not take into account differential provitamin A activities of lutein, zeaxanthin and α-carotene compared to β-carotene. The non-oily food matrix of rice is also likely to reduce carotenoid bioavailability and reports suggest that in malnourished individuals induction of the retinol (vitamin A) forming enzymes in the gut are repressed [204].

5.3.6. Model plant systems

The qualitative and quantitative diversity of carotenoids in non-chloroplast containing tissues suggests that in comparison to the highly regulated carotenoid content of chloroplasts greater fluctuations can be tolerated. Typically non-chloroplast containing tissues of crop plants are important dietary components. Transformation and regeneration of these crop plants are often time-consuming and laborious. In order to provide an indication of the effects that manipulating a specific step or steps in a pathway will have, an efficient reliable model system would be useful. Both *Arabidopsis thaliana* and tobacco (*Nicotiana*) are routinely used as model systems, although they possess predominantly chloroplastidic tissues.

Numerous examples exist to show the usefulness of these two plants as model systems from which engineering outcomes can be extrapolated to crop plants. In the case of the *E. uredovora* phytoene desaturase (*crt*I) when expressed in tobacco, higher levels of β-carotene and its metabolites were found [205]. Such data correlate well with those from tomato [178] and rice [202]. Useful information can also be taken from transgenic model systems expressing β-carotene hydroxylases, phytoene synthases and desaturases [206,207].

Modern viral vectors are being used to produce valuable proteins (e.g. vaccine proteins) in plants [208]. The potential of this approach in altering carotenoid content has been demonstrated [209]. In this case an RNA viral vector containing capsanthin-capsorubin synthase (*Ccs*) was used to transfect *Nicotiana benthamiana* leaves. Of the total carotenoids produced, 36% was capsanthin. It is feasible that such an approach could be used to produce carotenoids destined for nutritional supplements.

6. Future prospects

The application of molecular genetics to the biotechnological exploitation of carotenoid formation has facilitated rapid advancements in our understanding of carotenoid biosynthesis and its manipulation in higher plants. The fundamental reaction sequences involved in the biosynthesis of carotenoids are now known and all encoding genes, with the exception of the α-hydroxylase, have now been isolated and identified. Quantitative and qualitative manipulations of the pathway have been reported. Despite these significant advances, however, some aspects of carotenoid formation and manipulation in higher plants remain poorly understood. In order to make further progress and attain a state of expertise that will alter the carotenoid content of crop plants at a level that impacts on the prevention of human disease states, research must proceed

concurrently in fundamental and applied directions. Diverse scientific disciplines as well as emerging technologies need to be used.

Fig. 6 illustrates some of the areas of current and future work that will have an important bearing on our understanding and manipulation of the pathway. The manipulation of carotenoid biosynthesis has facilitated our understanding of the pathway and its regualtion. It is important to maintain the generation and full characterisation of varieties (either transgenic, natural mutant or from biodiversity) in which carotenoid levels are altered.

Typically studies have tended to focus on a specific step in the biosynthetic pathway, either at a gene or enzyme level. Such studies remain valuable, but there is a growing need to perform a systems approach to the pathway by determining changes occurring within the whole pathway in response to perturbations arising in response to biological processes and/or manipulation. The advances in targeted metabolite profiling, global metabolomics and mathematical assignment of fluxes will greatly assist in furthering our appreciation of the pathway and its relationship with cellular metabolism and function. Hopefully, identification of limiting substrates and cellular compartments for accumulation will occur. At the level of gene expression transcriptome analysis will provide a valuable insight into regulatory aspects of the pathway and target possible sites for pleiotropic engineering, whilst proteomic studies can assess changes of the protein components

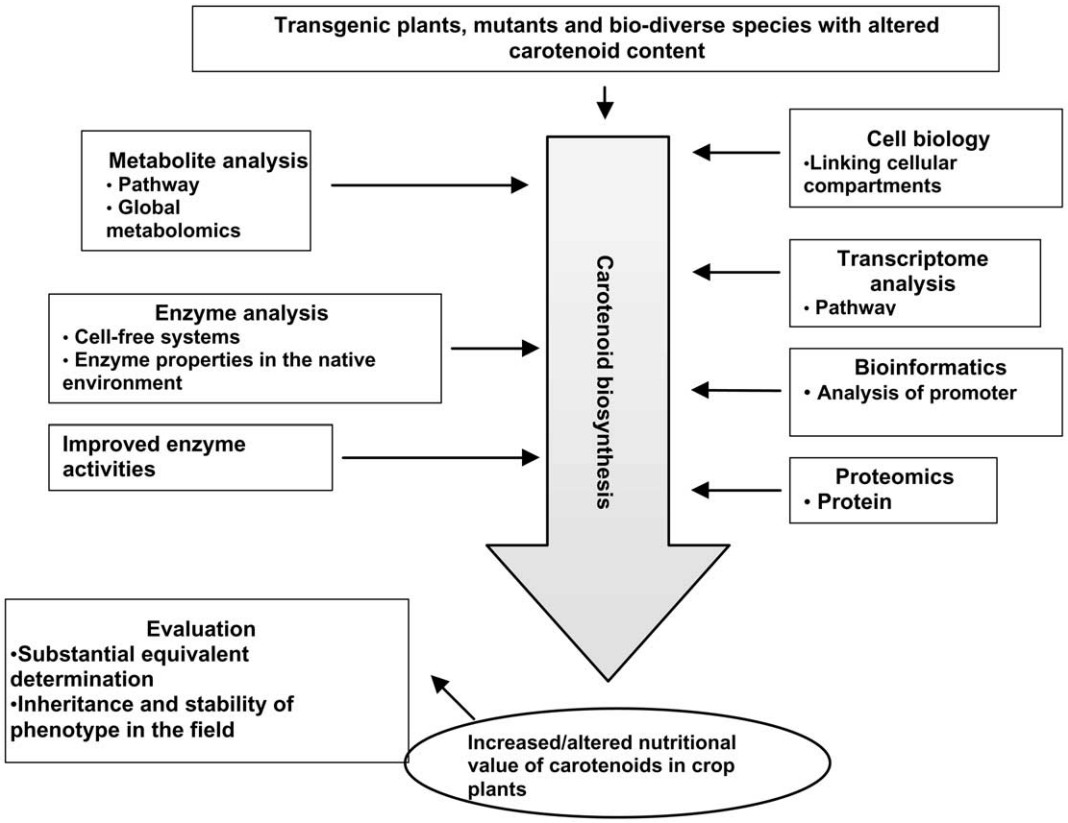

Fig. 6. Areas of research that contribute to the generation of crop plants with improved nutritional carotenoid content.

that result from alterations to carotenoid content in higher plants. In addition, protein in biosynthetic complexes could be identified providing an insight into the mechanisms of metabolite channelling, a regulatory process that appears to limit the effectiveness of metabolic engineering.

A common feature of many studies attempting to manipulate carotenoid biosynthesis by amplifying a single enzyme, judged to rate-limiting, has been the relative ineffectiveness of the approach, in part due to the ability of other pathway components to compensate for the fluctuations. These findings have led to suggestions for multiple gene manipulations or the use of transcription factors to facilitate the co-ordinate expression of the pathway. Such an approach has been used successfully with flavonoid formation. To date, no transcription factor influencing carotenoid formation has been identified, but hopefully techniques such as activation tagging and the *Arabidopsis* ORF knockout collection (SIGnal collection) will enable putative transcription factors influencing carotenoid formation to be identified. In addition, comparative bioinformatic studies on promoter regions of carotenoid genes may also elucidate common binding motifs involved in carotenoid formation.

Although this review has focused on carotenoid biosynthesis and manipulation in higher plants, it is important not to lose sight of carotenoid formation in microorganisms and the metabolic diversity that exists among bacteria and fungi. Gene shuffling and direct evolution with genes from these sources could provide valuable products with improved catalytic activities and/or altered regulatory properties. Finally, the consumer perception of GM crops means that it is important to show conclusively that alterations to carotenoid content in plants have no adverse effects and represent a substantial equivalent.

With regard to the perceived health benefits of dietary carotenoids, there are many epidemiological and human supplementation studies underway that should clarify the rather confusing evidence that has been published to date. Similarly, investigations on the relative benefits of pure carotenoid supplements compared to high carotenoid foods are in progress. In all of these cases, extrapolations from animal studies to humans need to be viewed cautiously. Finally, post genomic technologies will allow studiers of human biomarkers that will help to elucidate the precise modes of action of these compounds.

Acknowledgements

The authors would like to thank past and present members of the laboratory for their valuable contributions to our studies quoted in this review. We also acknowledge with gratitude funding from the BBSRC, European Union, Royal Society, MAFF and Syngenta.

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