Carotenoids
Volume 5: Nutrition and Health

Chapter 6

Genetic Manipulation of Carotenoid Content and Composition in Crop Plants

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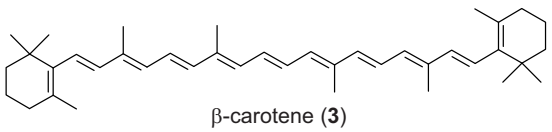
A. Introduction

Over the past 50 years, modern plant breeding has focused on improved productivity, through increased yield and adaptation to biotic and abiotic stress. In comparison, the enhancement of quality traits such as improved nutritional content and aesthetic colour has been neglected. Now, however, consumers increasingly demand improved food quality and safety and, as a consequence, plant breeding has been forced to address these issues. One example of this is to enhance the levels and types of carotenoids in fruits and vegetables, not only for aesthetic purposes, but also because of the increasing evidence that fruit and vegetables containing high levels of dietary carotenoids are associated with health benefits [1]; such crops are sometimes categorized as 'functional foods' [2].

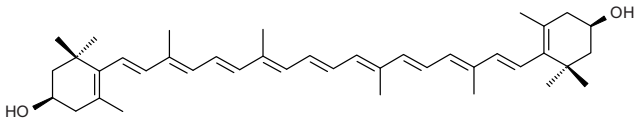


β-carotene (**3**)

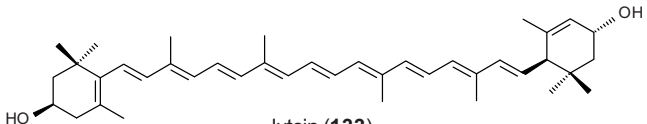
The value of carotenoids to human health is supported by a significant body of evidence, as discussed in later chapters in this *Volume*, much of it based on associations between dietary carotenoids and risk of the onset of chronic disease states. β-Carotene (**3**) is the most potent precursor of vitamin A, deficiency of which will cause blindness and eventually death [3].

The xanthophylls zeaxanthin (**119**) and lutein (**133**) have been associated with reduced risk of macular degeneration [4], whilst lycopene (**31**) is associated with the reduction of certain cancers such as prostate cancer [5]. Astaxanthin (**404-406**) has more recently received attention as a carotenoid that may confer preventative effects against cardiovascular disease [6]. The most advantageous effects of carotenoids on health occur when they are eaten in a fruit or vegetable matrix [7], presumably because of synergistic effects with the other health-promoting phytochemicals present in the food. These findings have had a big impact on national health policies of most Western countries, resulting in the recommendation that individuals should consume large quantities of fruits and vegetables ('five a day'), which contain health-promoting phytochemicals, such as carotenoids [8].

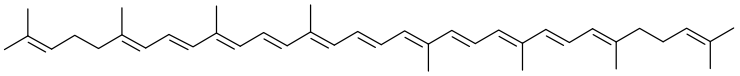
Commercially, carotenoids are used in the food, feed, pharmaceutical and cosmetic industries. Although chemical synthesis is the method most often used to produce carotenoids industrially, natural production of carotenoids from plants can offer a more cost-effective and environmentally favourable option.



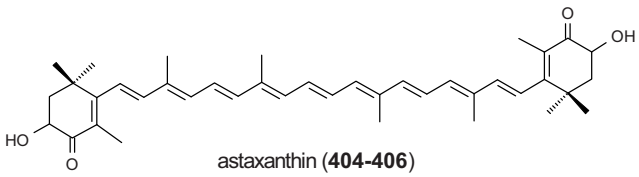
(3*R*,3'*R*)-zeaxanthin (**119**)



lutein (**133**)



lycopene (**31**)



astaxanthin (**404-406**)

B. Strategies for Enhancing Carotenoids in Crop Plants

1. General considerations

The predominant aim of enhancing carotenoids in crop plants is to provide tangible benefits to the quality of human and animal life. In order to achieve this goal there are several prerequisites that should be considered. Addressing these issues at an early conceptual stage will place 'proof of concept' approaches on sound foundations for subsequent scientific developments. The disease state to be addressed through dietary intake needs to be considered, as well as the strength of the experimental and medical evidence supporting the perceived health benefits [8,9]. From a commercial viewpoint, the market needs to be evaluated and the most suitable crop chosen for the countries in which the crop will be used. For example, as the case of the high β-carotene 'Golden Rice' has shown, a local variety of the crop should be used [10]. These factors influence the choice of crop and the target carotenoid(s) within the crop. Synergy with other health-promoting phytochemicals must also be considered. Although not essential, it is advantageous if the crop plant is a staple dietary component, ideally with an established endogenous carotenoid pathway and a known basal carotenoid profile. The generation of genetically modified (GM) crops, especially those possessing traits such as improved nutritional quality, has been restricted by public concerns. The time it may take for these attitudes to change is an important factor that must be considered and has an important bearing on proof of concept, intellectual property and development. Production of carotenoids in non-food crops, followed by bio-fortification of the food chain with supplements, is an alternative means of supplying the consumer with enhanced carotenoid intake [11].

2. Experimental strategies

There are two basic approaches available to generate crop plants with enhanced carotenoid compositions, namely conventional plant breeding, and genetic modification (GM, also termed metabolic or genetic engineering, or genetic manipulation). Over recent years, there have been significant scientific advances in both approaches, due to the development of new technologies. For example, the development of introgression populations and genome sequencing has facilitated efficient molecular marker-assisted breeding [12,13], whilst more efficient transformation vectors and plastid transformation protocols are now widely used. Ideally, the crop plant to be utilized should be amenable to both breeding approaches.

Conventional breeding of tomato has resulted in a wide range of varieties with different carotenoid profiles. These include the high pigment mutants *hp-1* and *hp-2* which have elevated levels of carotenoids, but have weak stems and poor vigour, thus making them unsuitable for commercial exploitation [14]. More recently, a concerted effort to screen the genetic diversity of the tomato has been undertaken, leading to collections of saturated mutant

libraries [15] and introgression lines [16] for which metabolic profiles for carotenoid levels can be determined.

The advantage of genetic engineering over conventional plant breeding is the ability to target and transfer gene(s) in a controlled manner and, therefore, in a much shorter time. In addition, the genes can be transferred from unrelated species, including bacteria. There are many reports of the successful elevation of carotenoid levels in crop plants, by use of a variety of genes or cDNAs and promoters. Examples of these are described in section C, and are summarized in Table 1.

Table 1. Examples of genetically modified crops with altered levels of carotenoids.

Inserted gene/cDNA	Promoter	Variety	Carotenoid phenotype	Ref.
Rice				
Psy cDNA from daffodil	CaMV 35S	Japonica taipei 309	Phytoene (0.3 μg/gFW) accumulation in endosperm	[17]
Psy cDNA from daffodil	Glutelin	Japonica taipei 309	Phytoene (0.6 μg/gFW) accumulation in endosperm	[17]
crtI (*E. uredovora*) + *Psy* + *Lcy-b* (daffodil)	CaMV 35S Glutelin	Japonica taipei 309	β-Carotene (1.6 μg/gFW) accumulation in endosperm	[18]
crtI + *Psy* + *Lcy-b*	CaMV 35S Glutelin	Indica varieties	β-Carotene (1.6 μg/gFW) accumulation in endosperm	[19]
crtI + *Psy*	Glutelin	Indica	β-Carotene (6.8 μg/gFW) accumulation	[20]
Tomato				
Tomato *Psy-1* antisense	CaMV 35S	Ailsa Craig	100-fold reduction in carotenoids, 3 to 5-fold increase in gibberellins	[21]
E. uredovora, *crtI*	CaMV 35S	Ailsa Craig	2 to 4-fold increase in β-carotene (20-45 μg/gFW), decreased lycopene	[22]
E. uredovora, *crtB*	CaMV 35S	Ailsa Craig	2 to 3-fold increases in phytoene, lycopene and β-carotene	[23]
Tomato *Psy-1* sense	PG	Ailsa Craig	Sense suppression, premature lycopene accumulation, dwarf plants (decreased gibberellins and increased ABA)	[24]

Table 1, continued

Inserted gene/cDNA	Promoter	Variety	Carotenoid phenotype	Ref.
Tomato				
Yeast *ySAMdc*	E8	VF 36	Increased proportion of β-carotene, 3-fold increase in lycopene	[25]
Tomato *Lcy-b* sense and antisense	Pds	Moneymaker	Up to 7-fold increase in β-carotene	[26]
Tomato *Lcy-b* + Pepper *CrtR-b*	Pds	Moneymaker	Up to 30% increase in lycopene; β-cryptoxanthin: 5 μg/gFW; zeaxanthin 13μg/gFW	[27]
Paracoccus crtW + *crtZ*	CaMV 35S	Ailsa Craig	Ketocarotenoids in leaf	[28]
Tomato *Cry-2*	CaMV 35S	Moneymaker	Hp phenotype	[29]
E. coli Dxps	CaMV 35S and fibrillin	Ailsa Craig	Increase in carotenoids	[30]
Tomato *Det-1*	RNAi + fruit specific promoters	Moneymaker	Increase in carotenoids and flavonoids	[31]
Canola				
E. uredovora, *crtB*	Napin	Cv 212/86 Quantum	50-fold increase in carotenoids (lutein): 1400 μg/gFW	[32]
Carrot				
E. herbicola, *crt* genes	CaMV 35S	-	2 to 5-fold increase in carotenoids	[33]
Potato				
Tobacco *Zep*, antisense and sense	GBSS	Freya and Baltica	130-fold increase in zeaxanthin (4 μg/g FW); 5.7-fold increase in total carotenoid (6 μg/gFW)	[34]

Table 1, continued

Inserted gene/cDNA	Promoter	Variety	Carotenoid phenotype	Ref.
Potato				
E. uredovora, *crtB*	Patatin	Desiree Mayan Gold	7-fold increase in carotenoids 4-fold increase in carotenoids	[35]
E. coli Dxps	Patatin	Desiree	2-fold increase in carotenoids	[36]
Potato *e-Lcy*, antisense	Patatin	Desiree	14-fold increase in β-carotene; 2.5-fold increase in total carotenoids	[37]
Algal *Bkt-1*	Patatin	*S. tuberosum*, *S. phureja*	Accumulation of ketocarotenoids	[38]
Synechocystis crtO + antisense *crtZ*	CaMV 35S	Desiree	Accumulation of ketocarotenoids, *e.g.* astaxanthin	[39]
Erwinia crtB, *crtI* and *crtY*	CaMV 35S or Patatin	Desiree	20-fold increase in β-carotene	[40]
Antisense *Chy-1* and *Chy-2*	Patatin	Desiree	38-fold increase in β-carotene	[41]

All transformations were carried out through *Agrobacterium*-mediated protocols, apart from those in [17], which used microprojectile bombardment. Examples of the transformation of tobacco can be found in [42].
Abbreviations: FW, fresh weight: *Psy*, phytoene synthase; *Pds*, phytoene desaturase; *crtI*, phytoene desaturase; *crtB*, phytoene synthase; *crtO*, carotenoid 4-oxygenase (‘ketolase’); *crtW*, β-carotene 4-oxygenase (‘ ketolase’); *crtY*, lycopene β-cyclase; *crtZ*, β-carotene hydroxylase; β-*Lcy*, lycopene β-cyclase; ε-*Lcy*, lycopene ε-cyclase; *Chy 1 and 2*, β-carotene hydroxylases; *Det-1*, de-etiolated 1; *Zep,* zeaxanthin epoxidase; y*SAM*dc, yeast S-adenosyl methionine decarboxylase; *Dxps*, 1-deoxy-D-xylulose 5-phosphate synthase; *Bkt*-1, β-carotene 4-oxygenase (‘ketolase’); CaMV 35S, cauliflower mosaic virus promoter; PG, polygalacturonase; GBSS, granule-bound starch synthase.

3. Optimizing conditions

In order to optimize and control the changes in carotenoid content and composition in crop plants, several prerequisites should be addressed, including the location and activities of enzymes, flux control coefficients, gene expression profiles, carotenoid catabolism, interaction with the biosynthesis of other isoprenoids, regulatory and end-product sequestration mechanisms. A general framework [43] and one addressed more specifically to carotenoid biosynthesis [44] have been described.

a) Choice of crop

In the early studies on genetic modification, the choice of crop was often limited to those that could be transformed efficiently by *Agrobacterium*-based protocols. It is now the case, however, that most crops can be modified effectively in this way, so the choice of crop and species relates to the carotenoids in the wild type, and the species used in the diet in a particular country. In addition, the utility of a plastid transformation system to alter tomato fruit carotenoid content has been demonstrated [45]. As shown in Table 1, the most popular crops used are tomato, rice and potato.

b) Choice of biosynthetic step(s) to target

It is well established that the regulation of carotenogenesis involves the coordinated flux of isoprenoid units into the C_{40} carotenoids and other isoprenoids such as sterols, gibberellins, phytol and terpenoid quinones [46]. An understanding of the complexities of regulation of the pathway is desirable to guide attempts to introduce changes in plant carotenoids by genetic manipulation. However, our understanding of the regulation of the carotenoid pathway is incomplete, so the choice of which step to target cannot be based solely on the current scientific evidence.

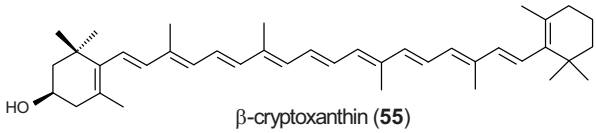
Levels of specific carotenoids can also be increased by up-regulation and down-regulation of carotenogenic genes. Qualitative engineering approaches focus primarily on altering the carotenoid composition of a crop. Typically, this is done by utilizing an existing precursor pool in the plant or tissue and redirecting this precursor into the formation of carotenoids. These carotenoids may not be endogenous to the crop undergoing manipulation (*e.g.* astaxanthin in potato [39]). Where no endogenous carotenoids are present, *e.g.* in rice endosperm [18], qualitative and quantitative engineering is required and more than one biosynthesis enzyme must be amplified. To facilitate these approaches, appropriate vectors must be available for multi-gene constructs, for example, vectors that generate self-cleavable poly-proteins [28]. Alternatively, co-transformation or crossing of individual transgenic lines can be used. Carotenoid levels in a crop can also be elevated as a consequence of altering an enzyme or a structural or regulatory protein, in a pathway or biological process which is not directly involved in carotenoid biosynthesis but which nevertheless influences carotenoid formation [31]. In order to achieve down-regulation, antisense and RNAi technology must be feasible in the wild-type crop, as shown recently with tomato [31].

Of the crops that have been manipulated genetically with respect to carotenogenesis (Table 1), probably the most extensively studied is the tomato fruit. Phytoene synthase is significantly up-regulated in ripening tomato fruit [47]. The fruit-specific isoform PSY-1 exhibits the highest flux control coefficient of the enzymes in the carotenoid pathway [23] and has, therefore, often been the target for transformation (Table 1). However, upon introduction of an extra phytoene synthase (*CrtB* from *Erwinia uredovora*), the flux control coefficient for this step decreases, suggesting that control is altered following perturbations of the pathway

itself [23]. The expression of the *E. coli* 1-deoxy-D-xylulose 5-phosphate synthase (*Dxps*) in tomato has also been reported. This is thought to be the rate-limiting enzyme in the methylerythritol phosphate (MEP) pathway [48,49], so its up-regulation should increase the formation of the end-product carotenoids. This was indeed the case, albeit with only a modest (1.6-fold) increase in carotenoids in ripe fruit [30].

Phytoene desaturation has also been chosen as a target for genetic engineering. For example, transformation with the *CrtI* gene from *Erwinia* resulted in fruit showing an orange phenotype due to an increase in β-carotene [22].

Transgenic tomatoes have been produced that contain carotenoids not normally present in the fruit. These include ones that contain zeaxanthin (**119**) and β-cryptoxanthin (**55**), through the expression of two cDNAs: the *Arabidopsis β-Lcy* and *Capsicum* β-carotene hydroxylase (*β-Chy*), both with the tomato *Pds* promoter [27]. Tomato has been transformed with two genes from *Paracoccus*, namely the carotene 4,4′-oxygenase (*crtW*) and 3,3′-hydroxylase (*crtZ*), in an attempt to produce ketocarotenoids such as astaxanthin in fruit. Although some ketocarotenoids were found in leaf tissue, none were detected in ripe fruit [28].



β-cryptoxanthin (**55**)

c) Choice of promoter and gene/cDNA

Most of the carotenoid biosynthesis genes have now been isolated from bacteria, fungi, algae and higher plants, and characterized. To have such a collection of biosynthetic genes, displaying functional similarity but differing homologies at the nucleotide level, is advantageous, because technical problems associated with co-suppression (sense suppression and/or gene silencing) can be alleviated. In addition, it is postulated (or known in some cases), that heterologously expressed enzymes are less susceptible to the regulatory controls, such as allosteric regulation, protein modification or association, that are found with the endogenous system. Only two genes involved in carotenoid sequestration are known (fibrillin and the *Or* genes) and no carotenoid-specific regulatory genes have been isolated from plants.

Various promoters have been used, as outlined in Table 1. These range from constitutive promoters such as CaMV 35S to fruit-ripening specific promoters such as polygalacturonase (PG) and fibrillin. In early studies, the importance of the temporal and spatial expression of the transgene with respect to pleiotropic effects was perhaps overlooked. Experience now suggests that the use of a constitutive promoter usually causes pleiotropic effects that can be detrimental to plant vigour, whilst more specific promoters such as PG, Pds and fibrillin allow

metabolic changes to be limited to the fruit itself. An example of this phenomenon was found when the tomato *Psy-1* cDNA was used with the CaMV 35S constitutive promoter, which caused virtually complete absence of fruit carotenoids in some of the transgenic lines [24]. In these cases, the phenotype was very similar to that found with an antisense construct of the same cDNA [21]. Those lines that did not exhibit co-suppression had pleiotropic phenotypes of dwarfism and premature fruit pigmentation, the former being caused by a significant reduction in gibberellins [50]. Co-suppression was successfully avoided by using a synthetic cDNA with low homology to the endogenous gene (<60%) [51], or by using the bacterial homologue of phytoene synthase from *Erwinia uredovora* [23]. Both strategies resulted in increased carotenoid levels in ripe fruit. Probably the most effective promoters with respect to enhancing carotenoid levels in fruit, without detrimental effects, are those involved in very early fruit ripening [31].

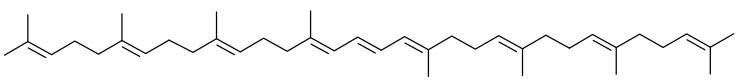
d) Targeting of the transgenic protein

When bacterial genes are used for genetic engineering of carotenoids in plants, the transformation vectors must include a plastid transit sequence upstream of the gene of interest. This allows specific targeting of the transgenic protein to the plastid and the subsequent import of the protein in an enzymically active form. Several sequences have been used successfully, including the small subunit of Rubisco (SSU) [22,28], and a modified sequence from *Psy-1* of tomato [24].

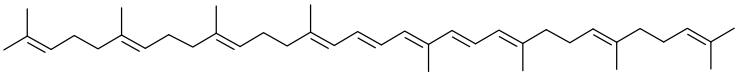
C. Examples of the Application of Metabolic Engineering to Carotenoid Formation in Crop Plants

1. Tomato

Developing tomato fruit at first contain chloroplasts and the associated carotenoids but then, as ripening proceeds, chromoplasts develop within the cells and a massively increased (400-fold) accumulation of lycopene occurs. Other acyclic carotenes such as phytoene (**44**) and phytofluene (**42**) also accumulate [47].



phytoene (**44**)



phytofluene (**42**)

Expression studies have revealed that a number of carotenogenic genes are up-regulated during fruit ripening, *e.g*. phytoene synthase-1 (*Psy-1*) [47], carotene isomerase (*CRTISO*) [52], and lycopene β-cyclase [26]. Thus, evidence from gene expression, enzyme activity, flux control values and metabolite levels suggests that phytoene synthase, the first committed step in the formation of carotenoids, exerts the greatest control of flux throughout the pathway [47]. In contrast to the up-regulation of phytoene formation, the down-regulation of lycopene cyclization is also an important factor in facilitating lycopene accumulation in ripe fruit [24].

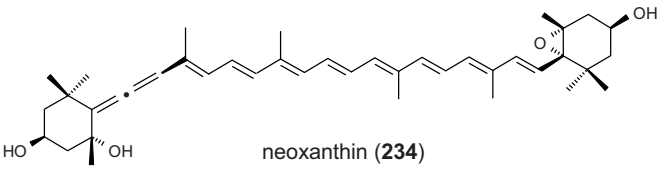
This knowledge has enabled two strategies to be developed for the quantitative engineering of lycopene content in tomato, namely up-regulation of phytoene synthase and down-regulation of lycopene β-cyclase. Use of an endogenous copy of *Psy-1* and the CaMV 35S promoter resulted in transgenic progeny with detrimental pleiotropic effects [24], especially a dwarf phenotype due to the elevation of carotenoids and abscisic acid (ABA), but reduced gibberellin (GA) levels. In contrast, both ABA and GA levels were reduced in the *Psy-1* antisense fruit [21]. Collectively, these data suggest that the equilibrium of the pool of geranylgeranyl diphosphate (GGDP) is perturbed and more GGDP is channelled into the carotenoid pathway, thus redirecting it from the GA pathway. More recent metabolomic studies have illustrated that effects also extend to intermediary metabolism [53].

Transgenic plants expressing the *E. uredovora* phytoene synthase (*crtB*) showed no pleiotropic effects and ripe fruit contained 2-3 fold increases in carotenoids [23]. These lines prove that amplification of the step in the pathway that has the highest flux control coefficient results in a quantitative increase once co-suppression has been avoided. Characterization of these crtB transgenic plants indicated that the endogenous pathway can compensate for the increased enzyme activity and fluctuations in precursor/product equilibrium by redistributing the balance of control within the pathway. In this case, it appeared, from the accumulation of phytoene, that the subsequent desaturase had become the limiting step [23]. The depletion of prenyl diphosphates and subsequent reduction in GA levels suggests that, in developing fruit, these precursors (or specifically GGDP) are limiting. However, transgenic tomato plants over-expressing the *Erwinia* GGDP synthase (*crtE*) showed no significant increase in end-product carotenoids.

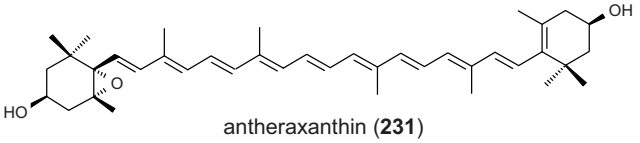
A feed-forward regulatory mechanism associated with *Psy-1* gene expression has been suggested after up-regulation of *Psy-1* with exogenously supplied deoxy-D-xylulose 5-phosphate [49]. On the basis of these findings, transgenic plants over-expressing the *E. coli* deoxy-D-xylulose 5-phosphate synthase (*Dxps*) under constitutive and fruit-ripening enhanced promoters showed moderate increases in end-product carotenoids, but 2-3 fold increases in phytoene levels, suggesting a shift in the equilibrium between precursors and products of the pathway and in the point of control.

The *Erwinia* phytoene desaturase (*crtI*) can convert phytoene into (all-*E*)-lycopene directly. Transgenic tomato plants expressing the *crtI* under constitutive control yielded orange-coloured fruit due to 2 to 4-fold increased β-carotene levels, thus providing 50-100% of the RDA for provitamin A per ripe fruit. Levels of lutein (**133**), zeaxanthin (**119**), neoxanthin

(**234**), antheraxanthin (**231**) and tocopherols were also increased [22] whilst carotenoid intermediates in the pathway to lycopene were all decreased. Gene expression analyses showed a reduction in *Psy-1*, but elevation in the two lycopene β-cyclase genes. Therefore the *crtI* gene product induces subsequent steps in the pathway in a feed-forward manner, but the resulting metabolites appear to be involved in a feedback-inhibition mechanism. Elevations in the β-carotene content of tomato fruit without reduction of lycopene have also been achieved through the expression of lycopene β-cyclase genes by use of either a plastid-based [45] or nuclear-based [26] transformation procedure. Lycopene levels in ripe fruit are also increased (2-fold) by down-regulating *β-Lcy* and *CYC-B* expression through anti-sense technology with the *Pds* and *CYC-B* promoters, respectively [26]. In both cases the carotenoid composition of vegetative tissues was unaffected.



neoxanthin (**234**)



antheraxanthin (**231**)

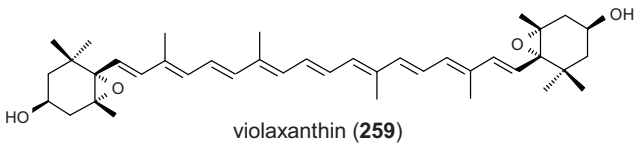
It is clear from the examples given above and from Table 1 that the manipulation of a specific step or steps in the biosynthetic pathway has been the principal focus of efforts to engineer genetically carotenoid formation in tomato. However, examples of pleiotropic engineering have been reported. For example, high-lycopene transgenic tomato plants resulting from alteration of polyamine levels have been described [25]. The objective of the study was to extend vine longevity, which in turn elevated lycopene levels. More recently, the manipulation of components operating in the light signal transduction pathway and photoreceptors has been reported [29,31]. These studies have shown that the levels of several health-promoting phytochemicals can be elevated. The mode of action of the transgenes in generating such phenotypes is unknown but it appears that increased plastid area during early fruit development is a key factor [29]. This, in turn, suggests that the sequestration and storage within the cell is influential in the accumulation of carotenoids. In order to increase the carotenoid storage potential in plants, cDNAs encoding gene products responsible for plastid division have been expressed in tomato [54]. Despite dramatic effects on plastid morphology to create larger plastids, no increase in carotenoids was observed. The *Capsicum*

fibrillin gene product has been shown to facilitate carotenoid sequestration. Expression in tomato, however, resulted only in a moderate elevation of carotenoids [55].

As an alternative to elevating the synthesis of carotenoids, transgenic plants in which the cleavage dioxygenases are down-regulated have been generated [56]. Although the lines showed reduced levels of apocarotenoids, the carotenoid content of the fruit was not altered significantly.

2. Potato

Potato is the most widely consumed vegetable and thus a staple food source for many populations. Potato tubers have a low carotenoid content, with most varieties containing violaxanthin (**259**), antheraxanthin and lutein. Expression of the *Erwinia* phytoene synthase (*crtB*) in a tuber-specific manner led to 6-fold higher carotenoid levels in the flesh of tubers, including a compositional change that resulted in the accumulation of nutritionally significant levels of β-carotene compared to trace levels in the controls [35]. Total carotenoid content has also been increased in potato tubers by down-regulating the endogenous zeaxanthin epoxidase both by sense and antisense suppression [34]. Astaxanthin (**406**) and other ketocarotenoids have been produced in potato tubers through the expression of the β-carotene 4,4′-oxygenase (*Bkt*) from *Haematococcus* [38]. Despite this manipulation of levels of β-ring xanthophylls, which act as ABA precursors, no pleiotropic effects have been reported in potato tubers. Potato tubers heterologously expressing a bacterial *Dxps*, however, showed altered tuber morphology and early tuber sprouting. This phenotype is attributed to increased levels of cytokinins, which are derived biosynthetically from plastid-derived IDP/DMADP [36]. The overall carotenoid content of these potato lines was increased 2-fold, with phytoene being elevated 6-7 fold. Recently potato tubers producing lycopene and very high β-carotene contents have been generated through the silencing of a lycopene ε-cyclase [37] and heterologous expression of a mini-pathway [40].



violaxanthin (**259**)

3. Carrot

Carrot roots are a significant source of provitamin A carotenes in the diet. The first report of successful genetic manipulation of carotenoid biosynthesis in a crop plant was reported in carrot, where the *crt* genes from *E. herbicola* were introduced, resulting in 2 to 5-fold increases in root β-carotene content [33]. Apart from this, the study of carotenogenesis in carrot surprisingly seems to have been neglected.

4. Rice

Vitamin A deficiency is the most common dietary problem affecting children worldwide, and is responsible for 2 million deaths annually (see *Chapter 9*). Rice is the staple foodstuff in many regions, especially Asia, but rice endosperm does not contain carotenoids. The quantitative and qualitative engineering of rice endosperm to produce β-carotene at an appropriate level that could alleviate vitamin A deficiency has been achieved [18] and is now at the development stage [10]. To reach this stage, it was first determined that GGDP was formed and could be utilized [17]. In order to metabolize GGDP to β-carotene, three biosynthetic cDNAs were co-transformed with two vectors. The daffodil (*Narcissus pseudonarcissus*) phytoene synthase and lycopene β-cyclase cDNAs were placed under endosperm-specific control (glutelin promoter) and *Erwinia* phytoene desaturase (*crtI*) under constitutive control. Transformants contained lutein, zeaxanthin, β-carotene and α-carotene (**7**) in their endosperm, with the total carotenoid content being about 1.6 μg/g endosperm. The variety of rice produced was termed 'Golden Rice' [18]. Through the systematic evaluation of different phytoene synthase(s), the carotenoid content of the Golden Rice has now reached 16-26 μg/g endosperm. This variety has been designated 'Golden Rice II' [57] and the levels of β-carotene in the endosperm should provide the RDA of provitamin A in an average rice meal (300 g). The carotenoid phenotypes have also been bred into local cultivars and nutritional and risk assessments are under way.

5. Canola (rape seed)

Canola is not a direct dietary source of carotenoids, but canola vegetable oils are used to prepare many foodstuffs. There have been few basic studies on carotenogenesis in wild-type canola embryos, presumably because of the low carotenoid content. The carotenoid present is typically lutein. The carotenoid content of canola embryos was elevated dramatically (50-fold) by transformation with the *Erwinia* phytoene synthase (*crtB*), expressed in a seed-specific manner [32]. Transgenic canola has also been generated with multiple steps in the biosynthetic pathway amplified. There were qualitative changes in carotenoid composition and additional products were seen following this amplification. Manipulation of phytoene synthase had the greatest influence. The effect of manipulation of additional or multiple gene products did not surpass the effect of the bacterial phytoene synthase alone [58].

D. Conclusions and Perspectives

Over the past 10-15 years, a considerable amount of knowledge has been acquired that facilitates the metabolic engineering of carotenoids in agricultural crops, creating the potential to improve human health through nutritional enhancement. In several crops such as tomato,

potato, rice and recently maize [59], the feasibility or 'proof of concept' investigations have been performed successfully. It is now important to carry out safety evaluations, ascertain agronomical properties and assess the nutritional impact of these phenotypes. Transfer of the traits to elite and geographically important varieties can then be carried out. The development of Golden Rice is a very important barometer to the acceptance not only of carotenoid-enhanced crops, but also the feasibility of GM approaches to future developments. The advances in molecular breeding offer an alternative approach if the consumer continues to reject GM crops.

Plant biology is undergoing a period of rapid innovation encompassing interdisciplinary approaches such as the 'omic' technologies which require considerable data handling skills. It is important that such technologies are utilized to evaluate fully metabolic engineering experiments, identify traits in modern breeding programmes and assist nutritional and safety assessments for the enhancement not only of carotenoids, but also of other important nutrients.

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