Review

Nutritious crops producing multiple carotenoids – a metabolic balancing act

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Plants and microbes produce multiple carotenoid pigments with important nutritional roles in animals. By unraveling the basis of carotenoid biosynthesis it has become possible to modulate the key metabolic steps in plants and thus increase the nutritional value of staple crops, such as rice (*Oryza sativa*), maize (*Zea mays*) and potato (*Solanum tuberosum*). Multigene engineering has been used to modify three different metabolic pathways simultaneously, producing maize seeds with higher levels of carotenoids, folate and ascorbate. This strategy may allow the development of nutritionally enhanced staples providing adequate amounts of several unrelated nutrients. By focusing on different steps in the carotenoid biosynthesis pathway, it is also possible to generate plants with enhanced levels of several nutritionally-beneficial carotenoid molecules simultaneously.

The multiple nutritional roles of carotenoids

Several carotenoids have highly specific roles in human nutrition but most applied carotenoid research currently focuses on increasing the levels of β-carotene (pro-vitamin A) in grains, fruits and vegetables in an effort to tackle vitamin A deficiency (Box 1) [1,2]. It has been shown that plants can be engineered to produce multiple unrelated nutrients by targeting different metabolic pathways simultaneously [3], but attempting to replicate the same achievements with different nutritional molecules from the same pathway could run into difficulties if there is competition for enzymes and precursors. As an example, β-carotene lies downstream of a bifurcation in the carotenoid biosynthesis pathway, the alternative branch yielding α-carotene and ultimately lutein, while β-carotene itself is also further converted into zeaxanthin [1,2]. Both lutein and zeaxanthin have important nutritional roles in humans (Box 2) so it is possible that focusing too strongly on β-carotene as a target could draw attention away from competing carotenoids that are also essential nutrients. We found that transgenic maize plants engineered to accumulate higher levels of β-carotene are not generally deficient in other carotenoids, and indeed the increased flux towards β-carotene in many cases enhances the levels of lutein and zeaxanthin as well as other carotenoids [4–7]. We therefore analyzed the literature covering β-carotene enhancement in other transgenic and conventionally-bred plants to determine the impact on lutein and zeaxanthin and found a broadly similar picture. Even when specific steps are taken to avoid the synthesis of these other carotenoids, there is always some leakage which allows nutritionally adequate levels to accumulate.

Carotenoid synthesis in plants

Carotenoids are tetraterpenoids whose synthesis in plants begins in the plastids with the condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to generate the C20 intermediate geranylgeranyl diphosphate (GGPP). This reaction is catalyzed by GGPP synthase (GGPPS) [8]. The first committed step is the condensation of two GGPP molecules into 15-*cis*-phytoene by the enzyme phytoene synthase (PSY, or CrtB in bacteria) [9]. A series of four desaturation reactions carried out in plants by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) then generates the carotenoid chromophore. In non-green tissue this is converted to all-*trans* lycopene by ζ-carotene isomerase (Z-ISO) [10] and carotenoid isomerase (CRTISO), whereas in green tissue the reaction occurs spontaneously in the presence of light and chlorophyll (acting as a sensitizer) [11,12]. In bacteria, all these steps are carried out by a single enzyme, CrtI.

All-*trans* lycopene represents a branch point in the pathway. This linear molecule can be cyclized at both ends by lycopene β-cyclase (LYCB, CrtY in bacteria) to generate the β-ionone end groups of β-carotene. Alternatively it can be cyclized at one end by lycopene ε-cyclase (LYCE) and at the other by LYCB to introduce the non-identical ε- and β-ionone end groups of α-carotene. Both these molecules can be converted into downstream products by carotene hydroxylases, such as the eponymous β-carotene hydroxylase (BCH). In the β-carotene pathway this yields β-cryptoxanthin, which is further converted by the same enzyme into zeaxanthin, whereas in the α-carotene branch the conversion yields lutein, the natural pathway endpoint [13]. Furthermore, zeaxanthin enters the xanthophyll cycle through the stepwise activities of zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE). These reactions are shown schematically in Figure 1.

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Box 1. Enhancing β-carotene levels to tackle vitamin A deficiency

Vitamin A is an essential nutrient in mammals that occurs in two important functional forms: retinal (required for low-light and color vision) and the regulatory morphogen retinoic acid. Both forms are derived from retinol in the diet, which is obtained from meat and dairy products in the form of esters such as retinyl palmitate. Humans and other herbivores/omnivores possess the enzyme β-carotene 15,15′-monooxygenase, which also allows the direct synthesis of retinal from pro-vitamin A carotenoids such as β-carotene produced in plants. In populations which lack access to animal-derived food, plants are therefore an important dietary source of vitamin A precursors.

The dietary reference intake (DRI) for vitamin A is expressed as the retinol activity equivalent (RAE), which takes bioavailability into account. The recommended DRI for males is 900 RAE, for females is 700 RAE (higher in pregnancy and when lactating), and for children it is 400–500 RAE. One RAE is equivalent to 1 μg of pure retinol, 2 μg of pure β-carotene dissolved in oil, or 12 μg of β-carotene in food [36]. Most people in the developed world have diets of sufficient diversity to ensure they achieve the DRI for vitamin A, but the situation in developing countries is very different. More than four million children, most from developing countries, exhibit clinical symptoms of severe vitamin A deficiency, including poor immunity, loss of vision in low light conditions (night blindness) and in extreme cases an irreversible form of blindness called xerophthalmia [37] (see also UNICEF 2006 report on vitamin A deficiency, http://www.childinfo.org/areas/vitamina/).

Several approaches can be considered to address vitamin A deficiency. Dietary supplements (vitamin tablets and suspensions) and fortification campaigns (artificially increasing vitamin levels by adding vitamins to processed food) have been highly successful in the developed world and have significantly reduced the incidence of deficiency diseases [38]. However, this strategy has little impact in remote areas of developing countries because of the incomplete food distribution network, poor governance and the lack of funding [39,40]. To address this, several attempts have been made to boost the levels of β-carotene in staple crops such as rice, maize and potato, either through conventional breeding or genetic engineering. Many poor people subsist on rice, which provides calories but has a very low nutrient content. In Golden Rice, the entire β-carotene biosynthesis pathway was reconstructed in the endosperm by expressing daffodil PSY and LYCB, as well as bacterial CrtI, increasing the endosperm carotenoid content to 1.6 μg/g dry weight [20]. This was not sufficient to provide the DRI of vitamin A in a reasonable portion of rice, so the more active maize PSY1 enzyme was used to replace its daffodil ortholog in 'Golden Rice 2', increasing the endosperm carotenoid content to 37 μg/g dry weight [23]. Golden Rice has been followed by similar programs in other staples, including Golden Potato and Multivitamin Maize (recently reviewed in [41]). Conventional breeding has also given rise to maize lines with high β-carotene levels, although the highest level achieved thus far relying on natural variation is 13.6 μg β-carotene per gram dry weight, which is by some considerable margin less than can be achieved by genetic engineer-

Increasing the flux towards β-carotene – the impact on lutein and zeaxanthin

Conventional breeding programs (Table 1) and genetic engineering strategies (Table 2) often aim to increase the levels of β-carotene in plants using the same approach, i.e. increasing flux through the pathway by increasing the activity of particular enzymes.

In conventional breeding, this is achieved by selecting plants that carry hypermorphic alleles, i.e. alleles encoding particularly active forms of carotenogenic enzymes or particularly active promoters that increase the quantity of the enzyme. A good example of such an allele is the *Beta* tomato (*Solanum lycopersicum*) mutant, which is orange in color and contains 45% more β-carotene than normal, corresponding to a hypermorphic variant of the enzyme LYCB [14]. Quantitative trait loci (QTLs) affecting β-carotene levels in this manner have been identified in many species, and have in a number of cases been traced to early enzymes in the carotenoid biosynthesis pathway [2]. For example, a QTL affecting β-carotene levels in sorghum has been mapped to the *psy3* gene [15]. In maize, a simple sequence repeat (SSR) marker has been identified [16] associated with *yellow1* (*y1*) that was linked to a major QTL explaining 6.6–27.2% of the phenotypic variation in carotenoid levels, and this was eventually resolved to the *psy1* gene. Similarly, another QTL was shown to be linked to *viviparous 9* (*vp9*), and this was found to encode ζ-carotene desaturase [16]. QTLs affecting β-carotene levels have also been identified in melon (*Cucumis melo*) fruits, which have flesh color ranging from green to orange because of differences in carotenoid levels. California and Wisconsin melon recombinant inbred lines were used to identify eight QTLs each accounting for 8–31% of phenotypic variation, one mapping to a gene encoding BCH [17].

In genetic engineering, effects similar to those of a hypermorphic endogenous allele can be achieved by expressing a heterologous enzyme (often a bacterial enzyme) under the control of a strong promoter. Targeting β-carotene in this manner tends to enhance other carotenoids simultaneously because the flux is distributed throughout the pathway, affecting all products to a greater or lesser degree. Differences in carotenoid profiles arise when further endogenous bottlenecks are revealed. As an example, the overexpression of PSY/CrtB often removes a significant bottleneck in the pathway and can increase the levels of all downstream carotenoids (e.g. [6,18]). In other cases, the immediate effect of PSY overexpression is only to reveal secondary restrictions further along the pathway so that the exact carotenoid composition depends on the relative activities of these later-acting enzymes. This is why transgenic maize and canola (*Brassica napus*) overexpressing PSY/CrtB accumulate different carotenoid end products, mirroring the situation in wild type seeds (where the bottlenecks similarly cause different carotenoids to accumulate) [6,19]. In contrast, transgenic rice seeds expressing PSY only accumulate the immediate downstream product of the PSY reaction (phytoene), because the subsequent enzyme is also expressed at vanishingly low levels in the endosperm [20].

Box 2. The nutritional importance of lutein and zeaxanthin

Lutein and zeaxanthin cannot be synthesized *de novo* in humans. There are many dietary sources of lutein, e.g. *Actinidia* spp. and maize seeds, but there are few good sources of zeaxanthin, e.g. some maize and *Capiscum annuum* varieties [42,43]. Lutein and zeaxanthin accumulate in the perifoveal and foveal regions of the retinal macula, respectively, and appear to protect these tissues from photodegradation [44]. There is a strong association between the dietary intake of lutein and zeaxanthin, and the degree of protection against age-related macular degeneration (ARMD) [45,46]. There is a lower incidence of this disease in people with a carotenoid-rich diet [47,48].

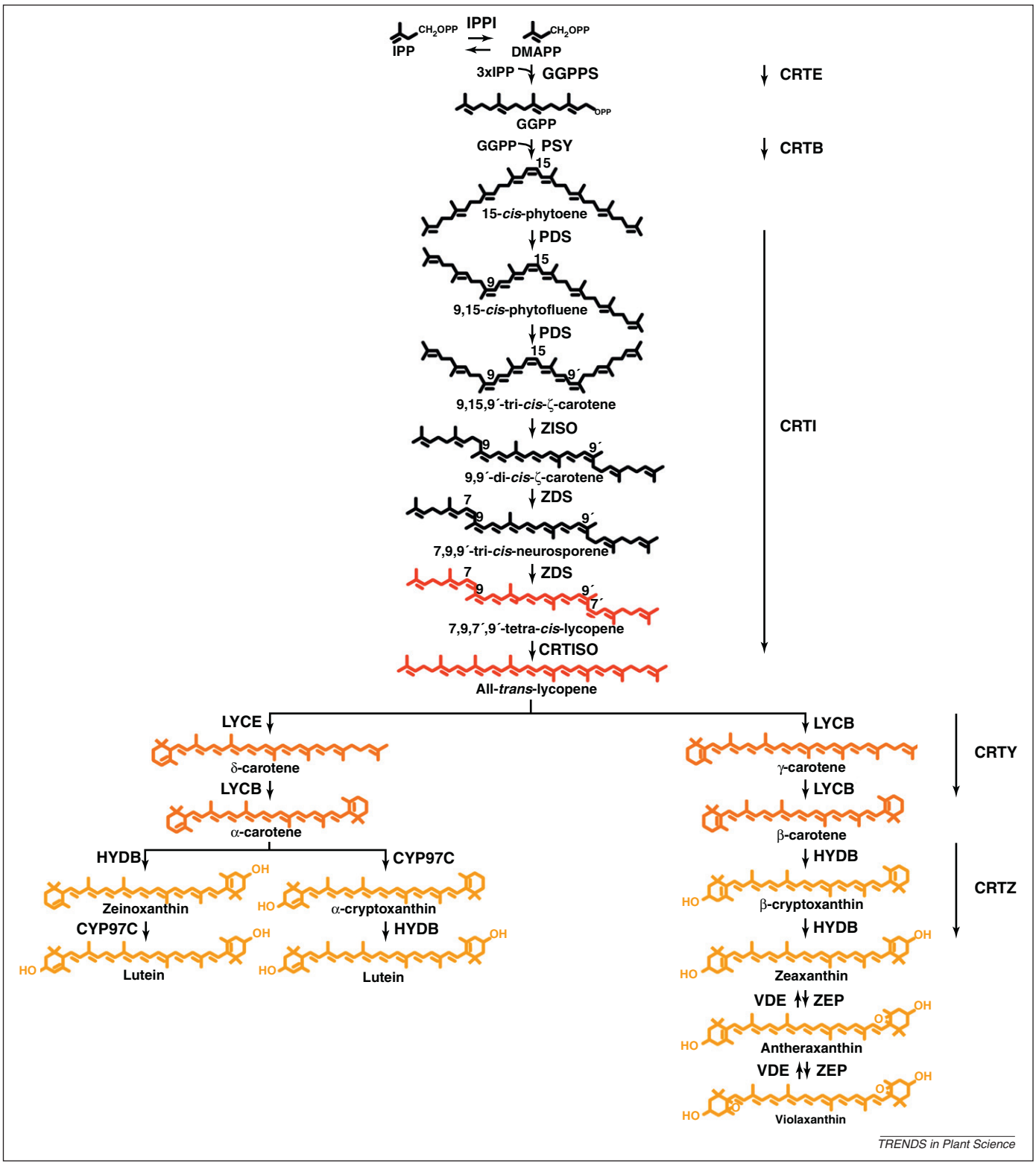

Figure 1. Carotenoid biosynthesis pathway in plants and equivalent steps in bacteria. CRTB, bacterial phytoene synthase; CRTE, bacterial geranylgeranyl diphosphate synthase; CRTI, bacterial phytoene desaturase/isomerase; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene cyclase; CRTZ, bacterial β-carotene hydroxylase; CYP97C, carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HYDB, β-carotene hydroxylase [non-heme di-iron hydroxylases, β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxylases, CYP97A and CYP97B]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase.

To pre-empt such secondary restrictions, it is becoming more common to express several different enzymes simultaneously in an effort to open up the carotenoid pathway to its full potential [5]. In addition to PSY/CrtB and CrtI from the linear part of the pathway, the next key target is LYCB/CrtY because the overexpression of this enzyme shifts the metabolic balance from the α to the β branch, and should therefore theoretically enhance β-carotene levels at the

Table 1. Total levels of carotenoids, β-carotene, lutein and zeaxanthin in different crop species[a]

Species	Total carotenoid levels in best line	β-Carotene levels in best line	Lutein levels in best line	Zeaxanthin levels in best line	Refs
Sorghum	190.18 μg/g DW	9.01 μg/g DW	101.49 μg/g DW	79.66 μg/g DW	[15]
	6.06–28.53 μg/TKDW	0.15–3.83 μg/TKDW	1.96–7.18 μg/TKDW	2.22–13.29 μg/TKDW	[49]
Canola	23 μg/g FW	0.2 μg/g FW	17.7 μg/g FW	ND	[19]
Tomato	207 μg/g FW	36.8 μg/g FW	6.4 μg/g FW	ND	[50]
Pumpkin		74 μg/g DW	170 μg/g DW	ND	[51]
Kiwifruit	16.34 μg/g FW	7.07 μg/g FW	6.20 μg/g FW	0.52 μg/g FW	[52]
Maize	29 μg/g DW	7 μg/g DW	20 μg/g DW	4 μg/g DW	[53]
	24.47 μg/g DW	13.63 μg/g DW	6.36 μg/g DW	2.77 μg/g DW	[32]
		4.7 μg/g DW	18.5 μg/g DW	24.5 μg/g DW	[25]

Abbreviations: DW, dry weight; FW fresh weight; ND, not determined; TKDW, thousand kernels dry weight.

[a]Recorded in the best-performing lines from conventional breeding programs.

expense of α-carotene and lutein, e.g. [21,22]. When LYCB is overexpressed, the levels of β-carotene do indeed increase, as seen in Golden Rice and Golden Rice II [20,23]. However, even with this bias towards the β-branch, there is enough upstream flux diverted into the α-branch to produce adequate amounts of lutein, and there is also enough leakage of flux past β-carotene to generate adequate amounts of zeaxanthin. For example, transgenic potato tubers expressing bacterial CrtB, CrtI and CrtY contained much more β-carotene than wild type tubers (up to 47.4 μg/g dry weight, a 3600-fold increase) but there were also significant increases in zeaxanthin (11 μg/g dry weight, a 5.8-fold increase) and lutein (23.1 μg/g dry weight, a 23-fold increase) [24]. Similarly, transgenic maize seeds expressing PSY1, CrtI and LYCB accumulated more carotenoids than wild type seeds, as would be expected from the general increased flux, and the β:α-carotene ratio increased from 1.21 to 3.51, showing that the additional LYCB activity skewed the competition for the common precursor lycopene and increased flux towards β-carotene [6]. Even so, there was also enhanced flux through the α-branch of the pathway, producing nearly 25-fold the normal levels of lutein (up to 13.12 μg/g dry weight). When the transgenic locus from this line was introgressed into a wild-type yellow-endosperm variety with a low β:α ratio (0.61), the hybrid offspring contained higher levels of both lutein (23.4 μg/g dry weight) and β-carotene (19.3 μg/g dry weight) compared with the parental plants [3].

These examples show that even when shifting the metabolic flux towards β-carotene, there is still enough flux through the α-branch of the pathway to produce more than enough lutein for human nutrition. The transgenic locus discussed above was also introgressed into a wild-type yellow endosperm variety with a high β:ε ratio (1.90). This gave rise to a novel hybrid line producing zeaxanthin at an unprecedented 56 μg/g dry weight. Even so, the seeds still contained higher levels of β-carotene than wild type seeds (15.24 μg/g dry weight) and lutein was also more abundant in the transgenic seeds (9.72 μg/g dry weight) [3]. Even yellow maize inbred lines with high levels of β-carotene (4.7 μg/g dry weight) also have higher levels of zeaxanthin (24.5 μg/g dry weight) and lutein (18.5 μg/g dry weight) [25].

Similar factors affect the balance between β-carotene and zeaxanthin when engineering downstream steps in the pathway. A transgenic maize line expressing PSY, CrtI, LYCB, BCH and CrtW (allowing the synthesis of ketocarotenoids that are rarely found naturally in plants) produced 25.78 μg/g dry weight β-carotene (a 184-fold increase over wild type seeds) but also 62-fold more zeaxanthin (16.78 μg/g dry weight) and 23-fold more lutein (12.27 μg/g dry weight) [6].

An alternative to multiple enzyme engineering is the modulation of transcriptional regulators that have multiple targets in the carotenoid pathway. *DE-ETIOLATED1* (*DET1*) is a regulatory gene encoding a transcription factor that represses several light-dependent signaling pathways, including those influencing carotenoid biosynthesis [26]. Loss-of-function mutations affecting the tomato ortholog of *DET1*, *high pigment-2* (*hp-2*), have more deeply-colored fruits that wild type plants when grown in the light because more flavonoids and carotenoids accumulate during fruit development [27]. Similarly, fruit-specific silencing of the tomato *hp-2* gene by RNAi gives rise to fruits with higher carotenoid and flavonoid levels than wild type fruits, but other quality attributes are unaffected [28]. In canola, silencing *DET1* by RNAi has been used as a deliberate strategy to increase carotenoid levels, generating seeds with higher levels of β-carotene (7 μg/g fresh weight, a 17.5-fold increase), zeaxanthin (0.8 μg/g fresh weight, a 4-fold increase) and lutein (13 μg/g fresh weight, a 2.1-fold increase) relative to wild type seeds [29]. The levels of these carotenoids were also enhanced, albeit to a lesser extent, when *DET1* silencing was seed-specific [29].

Diverting flux away from competing compounds – the impact on lutein and zeaxanthin

Increasing flux through the entire carotenoid biosynthesis pathway does not focus the benefits solely on β-carotene and therefore it is logical that other carotenoids are enhanced even if flux is diverted more towards the β-branch by overexpressing LYCB. More targeted approaches include the inhibition of enzymes that synthesize competing products, or the sequestration of β-carotene into subcellular compartments thus removing it from the active metabolic pool. These approaches might be expected to deplete competing carotenoids because specific enzymatic steps are inhibited or limited by nonproductive compartmentalization.

One example of the former approach is the deliberate inhibition of LYCE, to prevent the accumulation of lutein.

Table 2. Carotenoid engineering programs in diverse crops[a]

Species	Genes (origin)	Total carotenoid levels in wild type[a]	Total carotenoid levels (fold increase relative to wild type) in transgenic plants	β-Carotene levels in wild type[a]	β-Carotene levels (fold increase relative to wild type) in transgenic plants	Lutein levels in wild type[a]	Lutein (fold increase relative to wild type) in transgenic plants	Zeaxanthin levels in wild type[a]	Zeaxanthin (fold increase relative to wild type) in transgenic plants	Refs
Maize	*crt*B *and crt*I (*Pantoea ananatis*)	0.99 μg/g DW	33.6 μg/g DW (34)	0.98 μg/g DW	9.8 μg/g DW (10)	0.30 μg/g DW	6.68 μg/g DW (22)	0.68 μg/g DW	6.21 μg/g DW (9)	[54]
	PH7: *psy1* (*Zea mays*; maize) *crt*I (*P. ananatis*) *crt*W (*Paracoccus* spp.) *lycb* (*Gentiana lutea*) *bch* (*G. lutea*)	1.10 μg/g DW	102.1 μg/g DW (92.8)	0.14 μg/g DW	25.78 μg/g DW (184)	0.53 μg/g DW	12.27 μg/g DW (23)	0.27 μg/g DW	16.78 μg/g DW (62)	[6]
	PH4: *psy1* (maize) *crt*I (*P. ananatis*) *lycb* (*G. lutea*)	1.10 μg/g DW	148.78 μg/g DW (135)	0.14 μg/g DW	48.87 μg/g DW (349)	0.53 μg/g DW	13.12 μg/g DW (24.7)	0.27 μg/g DW	34.53 μg/g DW (127.8)	
	psy1 (maize) *crt*I (*P. ananatis*)	1.45 μg/g DW	163.2 μg/g DW (112)	0.35 μg/g DW	59.32 μg/g DW (169)	0.57 μg/g DW	14.68 μg/g DW (25)	0.32 μg/g DW	35.76 μg/g DW (111)	[4]
Potato	*ZEP* (*Arabidopsis thaliana*: *Arabidopsis*)	10.6 μg/g DW	60.8 μg/g DW (5.7)	0.7 μg/g DW	2.4 μg/g DW (3.4)	2.7 μg/g DW	5.2 μg/g DW (1.9)	0.3 μg/g DW	40.1 μg/g DW (133.7)	[55]
	*crt*B (*P. ananatis*)	5.6 μg/g DW	35.5 μg/g DW (6.3)	ND	10.3 μg/g DW	0.73 μg/g DW	11.01 μg/g DW (15)	0.11 μg/g DW	0.71 μg/g DW (6.5)	[56]
	Or (*Brassica oleracea*; cauliflower)	5.41 μg/g DW	28.22 μg/g DW (6)	ND	5.01 μg/g DW	3.42 μg/g DW	5.16 μg/g DW (1.5)	ND	ND	[57]
	Antisense *lyce* (*Solanum tuberosum*; potato)	4.6 μg/g DW	9.97 μg/g DW (2.5)	0.003 μg/g DW	0.04 μg/g DW (14)	0.588 μg/g DW (0.59)	1.004 μg/g DW (1.77)	0.26 μg/g DW	0.99 μg/g DW (3.8)	[30]
	*crt*B, *crt*I *and crt*Y (*P. ananatis*)	5.8 μg/g DW	114.4 μg/g DW (20)	0.013 μg/g DW	47.4 μg/g DW (3600)	1.0 μg/g DW	23.1 μg/g DW (23)	1.9 μg/g DW	11 μg/g DW (5.8)	[24]
	Antisense *bch* (potato)	4.88 μg/g DW	14.26 μg/g DW (2.9)	0.002 μg/g DW	0.085 μg/g DW (38)	0.43 μg/g DW	2.98 μg/g DW (7)	0.32 μg/g DW	0.04 μg/g DW	[58]
	Antisense *bch* (potato) (assumes 75% water content)	22.48 μg /g DW	23.52 μg/g DW (1.04)	0.04 μg /g DW	13.24 μg/g DW (331)	2.12 μg/g DW	5.48 μg/g DW (2.58)	23.72 μg/g DW	4.8 μg/g DW	[33]
Carrot	*psy* (*Arabidopsis*)	5.5 μg/g DW	514.1 μg/g DW (93)	1.26 μg/g DW	214.62 μg/g DW (178)	1.65 μg/g DW	5.14 μg/g DW (3)	ND	ND	[59]
Canola	*crt*B (*P. ananatis*)	36 μg/g FW	1055 μg/g FW (29.3)	5 μg/g FW	401 μg/g FW (80.2)	30 μg/g DW	72 μg/g DW (2.4)	ND	ND	[18]
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 5′ end	5.34 μg/g FW	227.78 μg/g FW (42.5)	0.49 μg/g FW	90.76 μg/g FW (185.2)	3.30 μg/g FW	76.22 μg/g FW (23)	ND	7.07 μg/g FW	[31]
	lycopene ε-cyclase (*Arabidopsis*) RNAi to 3′ end		94.09 μg/g FW (17.6)		27.02 μg/g FW (55)	3.30 μg/g FW	37.64 μg/g FW (11.4)	ND	1.73 μg/g FW	

	idi, *crt*E, *crt*B, *crt*I and *crt*Y (*P. ananatis*) *crt*Z, *crt*W (*Brevundimonas* spp.)	21.7 μg/g FW	656.7 μg/g FW (30)	0.2 μg/g FW	214.2 μg/g FW (1070)	17.7 μg/g FW	27.6 μg/g FW (1.6)	ND	ND	[19]
	Antisense *DET1* (*Brassica napus*; canola), constitutive expression	6 μg/g FW	20 μg/g FW (3.3)	0.4 μg/g FW	7 μg/g FW (17.5)	6 μg/g FW	13 μg/g FW (2.1)	0.2 μg/g FW	0.8 μg/g FW (4)	[29]
	Antisense *DET1* (canola), seed expression	6 μg/g FW	14 μg/g FW (2.3)	0.4 μg/g FW	1.2 μg/g FW (3)	6 μg/g FW	12 μg/g FW (2)	ND	ND	
	microRNA *miR156b* (*Arabidopsis*)	3 μg/g FW (10% water content)	6.9 μg/g FW (2.3) (10% water content)	0.08 μg/g FW (10% water content)	0.38 μg/g FW (4.5) (10% water content)	2.7 μg/g FW (10% water content)	6.2 μg/g FW (2.3) (10% water content)	ND	ND	[60]
Tomato[b]	*crt*I (*P. ananatis*)	285 μg/g FW	137.2 μg/g FW (0.5)	27.1 μg/g FW	52 μg/g FW (1.9)	1.8 μg/g FW	4.1 μg/g FW (2.3)	ND	ND	[34]
	lycb (*Solanum lycopersicum*; tomato)	66 μg/g FW	109 μg/g FW (1.7)	7 μg/g FW	57 μg/g FW (7.1)	ND	ND	ND	ND	[21]
	lycb (*Arabidopsis*) *bch* (pepper; *Capsicum annuum*)	66.3 μg/g FW	100.7 μg/g FW (1.5)	5 μg/g FW	63 μg/g FW (12)	1.9 μg/g FW	1.8 μg/g FW	ND	13 μg/g FW	[61]
	*crt*B (*P. ananatis*)	285.7 μg/g FW	591.8 μg/g FW (2.1)	33 μg/g FW	82.5 μg/g FW (2.5)	ND	ND	ND	ND	[62]
	lycb (tomato)	94.5 μg/g FW	215.2 μg/g FW (2.3)	4.4 μg/g FW	205 μg/g FW (46.6)	ND	ND	ND	ND	[22]
	dxs (*Escherichia coli*)	460 μg/g FW	720 μg/g FW (1.6)	50 μg/g FW	70 μg/g FW (1.4)	ND	ND	ND	ND	[63]
	CRY2 (tomato)	87.6 μg/g FW in ripe fruit pericarps	149 μg/g FW in ripe fruit pericarps (1.7)	7.8 μg/g FW in ripe fruit pericarps	10.1 μg/g FW in ripe fruit pericarps (1.3)	2.3 μg/g FW	3.6 μg/g FW (1.6)	ND	ND	[64]
	psy1 (tomato)	181.20 μg/g FW	227.67 μg/g FW (1.25)	58.62 μg/g FW	81.93 μg/g FW (1.4)	9.96 μg/g FW	12.33 μg/g FW (1.2)	ND	ND	[65]
	fibrillin (pepper)	325 μg/g FW	650 μg/g FW (2.0)	90 μg/g FW	150 μg/g FW (1.6)	10 μg/g FW	16 μg/g FW (1.6)	ND	ND	[66]
	*crt*Y (*Erwinia herbicola*)	372.66 μg/g FW	323.71 μg/g FW (0.9)	6.91 μg/g FW	28.61 μg/g FW (4)	ND	ND	ND	ND	[67]
	lycb (*Narcissus pseudonarcissus*; daffodil)	76.67 μg/g FW	115 μg/g FW (1.5)	19 μg/g FW	95 μg/g FW (5)	ND	ND	ND	ND	[68]
Kumquat	*psy* (*Citrus sinensis*; orange)	84.3 μg/g FW	131.9 μg/g FW (1.6)	0.70 μg/g FW	1.72 μg/g FW (2.5)	5.6 μg/g FW	6.46 μg/g FW (1.5)	ND	ND	[69]

Abbreviations: DW, dry weight; FW, fresh weight; ND, not determined; TKDW, thousand kernels dry weight.

[a]Genes/enzymes involved in carotenoid engineering programs in different crops are shown as indicated and the absolute levels and relative improvement and/or reduction in total carotenoids, β-carotene, lutein and zeaxanthin. Where the original reports do not report zeaxanthin or lutein levels, they have been excluded from the table. Different wild-type carotenoid levels cited for each species reflect the different varieties used in each investigation.

[b]We converted dry weight to fresh weight assuming the water content of tomato fruits is 90%.

In transgenic plants, this has been achieved using antisense RNA and RNA interference (RNAi). The endogenous *lyce* gene was silenced in potato by expressing an antisense RNA construct, theoretically eliminating competition at the branch point between the α- and β-carotene pathways [30]. Antisense tubers contained up to 14-fold more β-carotene than wild type tubers, but there was no corresponding decrease in lutein levels because the total carotenoid level increased up to 2.5-fold in response to the general increase in flux resulting from the overexpression of upstream enzymes. These results suggest that LYCE is not rate-limiting for lutein accumulation in potato [30]. RNAi was also used to modulate carotenoid accumulation in canola seeds, again by targeting LYCE [31]. Transgenic seeds contained higher levels of β-carotene (90.76 μg/g fresh weight; 185-fold increase), zeaxanthin (7 μg/g fresh weight) and lutein (76.2 μg/g fresh weight) than wild type seeds, with the 23-fold increase in lutein demonstrating conclusively that LYCE is not a limiting step in canola either [31].

Similarly, conventional breeding programs can be used to select for plants with low levels of LYCE activity, by focusing on the selection of hypomorphic alleles and thus favoring the accumulation of β-carotene instead of lutein. Four polymorphisms have been described in the maize *lyce* locus, and conventional breeding for low LYCE activity increased the β-carotene levels in seeds to 13.6 μg/g dry weight (a 30–40% improvement) while lutein and zeaxanthin levels reached 6.36 and 2.77 μg/g dry weight, respectively [32].

Another common target of specific enzyme inhibition is the carotene hydroxylases, as this could prevent the conversion of β-carotene into zeaxanthin and therefore force the accumulation of β-carotene as an end-product (similarly, α-carotene would accumulate at the expense of lutein). However, this would prevent the formation of lutein and zeaxanthin only if the inhibition was 100% effective. The *bch* gene was silenced in potato, increasing β-carotene levels to 16.55 μg/g dry weight (a 331-fold improvement) and lutein to 6.85 μg/g dry weight a (2.5-fold improvement), while zeaxanthin levels were reduced from 29.65 to 6 μg/g dry weight, a fivefold reduction [33]. Therefore, even by targeting the particular enzymatic step leading to zeaxanthin, a significant amount of this carotenoid was still produced. In a converse example, the zeaxanthin content of potato tubers was enhanced by silencing the endogenous *zep* gene [34]. This increased the total carotenoid levels by 5.7-fold, β-carotene levels 3.4-fold and lutein levels 1.9-fold, while zeaxanthin was enhanced 133-fold.

Conventional breeding could also be used to increase zeaxanthin levels by selecting for hypomorphic versions of BCH. Although no studies with that specific aim have been reported, six hydroxylase genes were characterized in genetically diverse maize germplasm collections, one of which (*hyd3*) appeared to affect carotenoid levels in seeds [35]. Three *hyd3* alleles explained 78% of the variation in the ratio of β-carotene to β-cryptoxanthin (11-fold difference across varieties) and 36% of the variation in β-carotene absolute levels (4-fold difference across varieties).

There are additional potential strategies to increase carotenoid levels in plants, such as the inhibition of carotenoid cleavage dioxygenases. However, there are currently no data in the literature that report the improved β-carotene, zeaxanthin and lutein levels in plants following the application of these strategies so we do not discuss them further.

Conclusions

The above studies confirm that attempts to increase the availability of one particular nutrient in a metabolic pathway, in this case β-carotene in the carotenoid pathway, do not necessarily lead to deficiencies in upstream or downstream nutrients. There are several reasons for this phenomenon, which can be summarized as follows:

(i) The accumulation of specific carotenoids is a balance between the flux towards and away from a particular compound. As long as there is more flux towards a compound than away from it, that compound will accumulate.

(ii) Because the early steps in the carotenoid pathway are usually the rate-limiting steps, the overexpression of endogenous or heterologous enzymes (e.g. PSY/CrtB and CrtI) generally has a beneficial effect on all carotenoid molecules. The only intermediates that do not accumulate are those which are efficiently converted into the next downstream product, often as a result of multiple steps carried out by the same enzyme (e.g. β-carotene to β-crytoxanthin to zeaxanthin).

(iii) Targeting the branch point either positively (enhancing LYCB) or negatively (inhibiting LYCE) does not eliminate lutein accumulation. Indeed, enhancing LYCB increases the accumulation of lutein along with β-carotene. This indicates LYCE is not a limiting step and that the α-branch benefits from the increased pathway flux (because the α-branch requires LYCB activity too).

(iv) Targeting post β-carotene steps in the pathway to avoid depletion of the β-carotene pool is only partly effective. There are several carotene hydroxylases in plants, and therefore even RNAi knockdown of the principal enzyme, β-carotene hydroxylase, is insufficient to abolish the accumulation of lutein and zeaxanthin.

In conclusion, all the available evidence suggests that diverting flux specifically towards β-carotene by conventional and/or biotechnological means results in the collateral production of enough of the additional carotenoids lutein and zeaxanthin to satisfy human dietary requirements.

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