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Provitamin A biofortification of crop plants: a gold rush with many miners

Giovanni Giuliano

Carotenoids are synthesized *de novo* by plants, where they play fundamental physiological roles as photosynthetic pigments and precursors for signaling molecules. They are also essential components of a healthy diet, as dietary antioxidants and vitamin A precursors. Vitamin A deficiency is a public health problem in developing countries, which has prompted a series of efforts toward the biofortification of plant-derived foods with provitamin A carotenoids (mainly β-carotene), giving rise to 'golden' crops. Since the 'golden rice' exploit, a number of biofortified crops have been generated, using transgenic approaches as well as conventional breeding. Bioavailability studies have demonstrated the efficacy of several 'golden' crops in maintaining vitamin A status. This review presents the state of the art and the areas that need further experimentation.

Address
Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Casaccia Research Center, Via Anguillarese 301, Roma 00123, Italy

Corresponding author: Giuliano, Giovanni (giovanni.giuliano@enea.it)

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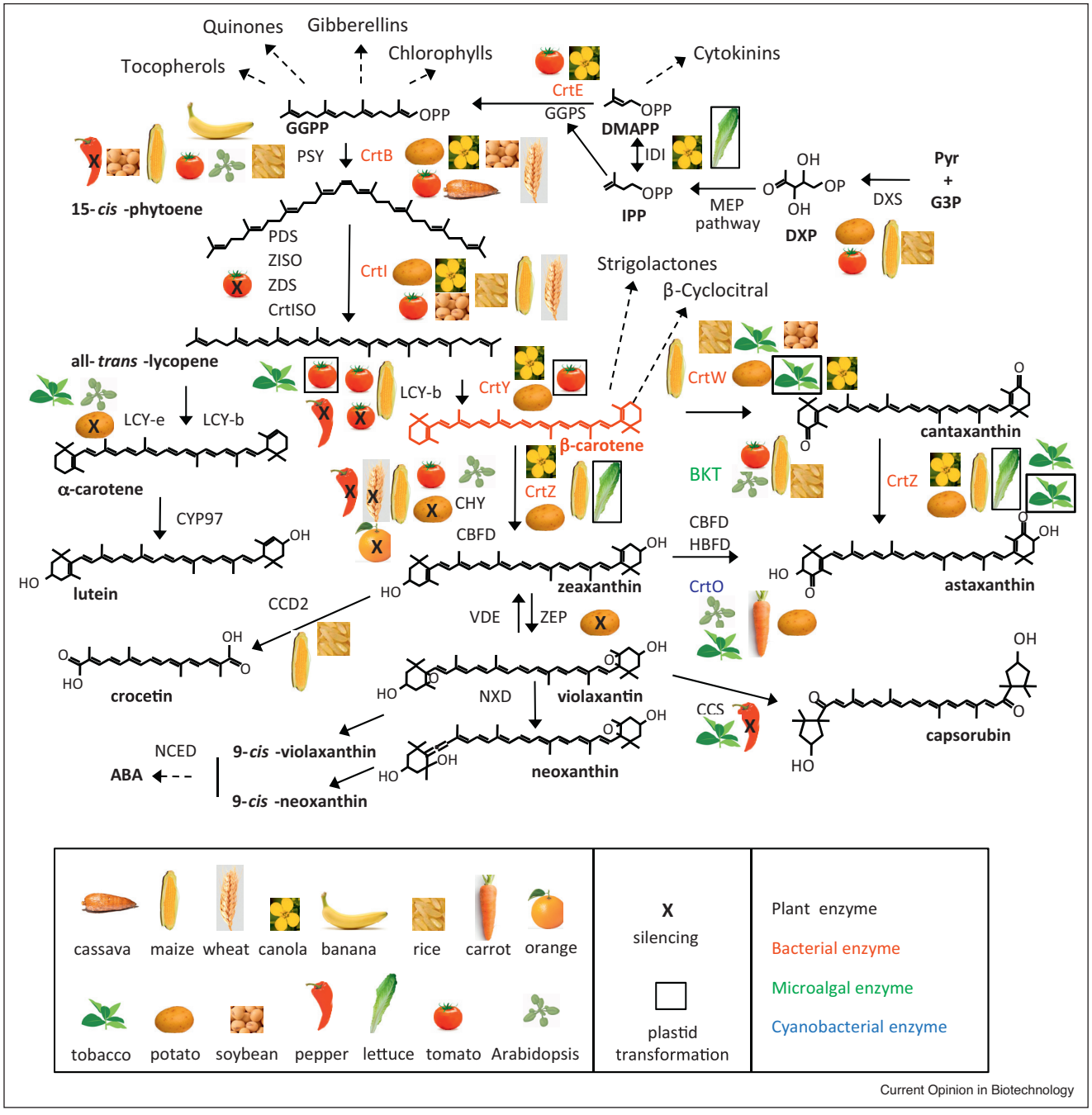
Carotenoid metabolism in plants

Carotenoids are C40 isoprenoid pigments. In plants, they are synthesized through the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (reviewed in Ref. [1]) (Figure 1). The C5 prenyl phosphates, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are converted into each other by isopentenyl diphosphate isomerase (IDI) [2•] and then condensed into geranylgeranyl pyrophosphate (GGPP), the precursor for a series of isoprenoid molecules, including carotenoids, by geranylgeranyl pyrophosphate synthase (GGPS). GGPP is the precursor of a series of isoprenoid molecules, like gibberellins, quinones, and the isoprenoid moieties of chlorophylls and tocopherols (Figure 1).

The condensation of two GGPP molecules by phytoene synthase (PSY) yields 15-*cis*-phytoene, and is the first dedicated step in carotenoid biosynthesis. 15-*cis*-phytoene is converted into all-*trans*-lycopene by a poly-*cis* pathway involving two desaturases (phytoene desaturase or PDS and ζ-carotene desaturase or ZDS) and two isomerases (ζ-carotene isomerase or ZISO and prolycopene isomerase or CrtISO). The *ZISO* gene was cloned recently and, unlike other known carotenoid isomerases, was shown to encode an unusual heme-containing enzyme [3••]. A bacterial desaturase/isomerase (CrtI) converts directly 15-*cis* phytoene into all *trans*-lycopene, substituting for the four plant enzymes, and for this reason is often used in metabolic engineering. Lycopene is the substrate for two competing cyclases: lycopene β-cyclase (LCY-b) and lycopene ε-cyclase (LCY-ε), introducing, respectively β-rings (double bond at the 5,6 position) and ε-rings (double bond at the 4,5 position). Only carotenoids containing non-substituted β-rings are vitamin A precursors. The two cyclases start two competing branches: the α-branch proceeds through α-carotene (ε-β-carotene) to its 3,3′ diol, lutein, while the β-branch proceeds through β-carotene (β-β-carotene) to its 3,3′ diol, zeaxanthin. In most plants, LCY-ε is monofunctional, so the third possible branch (proceeding through ε-ε-carotene) to its 3,3′ diol, lactucaxanthin, is present only in a handful of plants, including lettuce. Two non-heme carotene hydroxylases (CHY1 and CHY2) and two heme hydroxylases (CYP97A and CYP97C) introduce hydroxyl groups at the 3 and 3′ positions of the rings. In the β-branch, epoxidation by zeaxanthin epoxidase (ZEP) and de-epoxidation by violaxanthin de-epoxidase (VDE) generates the xanthophyll cycle in leaves (Figure 1).

Several species-specific carotenoid/apocarotenoid biosynthetic pathways are known: isomerization of antheraxanthin/violaxanthin by capsanthin capsorubin synthase (CCS), an LCY-b homolog, leads to the production of the κ-ring xanthophylls capsanthin and capsorubin in pepper fruits [4]; hydroxylation/ketolation of β-carotene by carotenoid β-ring 4-dehydrogenase (CBFD) and 4-hydroxy-β-ring 4-dehydrogenase (HBFD) leads to astaxanthin production in *Adonis* flowers [5]; cleavage of zeaxanthin or β-cryptoxanthin by carotenoid cleavage dioxygenase 4 (CCD4) leads to citraurin production in citrus peel [6•]; and cleavage of zeaxanthin by CCD2 leads to crocetin production in saffron stigmas [7•].

Figure 1



Carotenoid pathway engineering in crop plants. All depicted steps occur in the plastid. Plant enzyme names are in black letters, bacterial in red, cyanobacterial in blue, microalgal in green. Next to each enzymatic step are shown the species in which it has been overexpressed (plain image) or silenced (crossed image). Boxed images represent species in which plastid transformation was performed. For details, see Supplementary Table S1.

DXS: 1-deoxy-D-xylulose 5-phosphate synthase; IDI: isopentenyl diphosphate isomerase; GGPS: geranylgeranyl pyrophosphate synthase; PSY: phytoene synthase; PDS: phytoene desaturase; ZISO: 15-*cis*-zeta-carotene isomerase; ZDS: zeta-carotene desaturase; CrtISO: prolycopene isomerase; LCY-b: lycopene beta-cyclase; LCY-e: lycopene epsilon-cyclase; CYP97: heme hydroxylase; CHY: non-heme hydroxylase; CBFD: carotenoid β-ring 4-dehydrogenase; HBFD: 4-hydroxy-β-ring 4-dehydrogenase; ZEP: zeaxanthin epoxidase; VDE: violaxanthin de-epoxidase; NXD: neoxanthin deficient; CCD: carotenoid cleavage dioxygenase; CrtE: bacterial geranylgeranyl pyrophosphate synthase; CrtB: bacterial phytoene synthase; CrtI: bacterial phytoene desaturase/isomerase; CrtO: cyanobacterial ketolase; CrtZ: bacterial hydroxylase; CrtW: bacterial ketolase; BKT: ketolase from green algae; Pyr: pyruvate; G3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; ABA: abscisic acid.

Different mechanisms contribute to the maintenance of carotenoid steady state levels in plastids. In chromoplasts, carotenoids accumulate often in esterified form. The role of esterification in enhancing carotenoid stability is exemplified by the *pale yellow petal* (*pyp1*) mutant, which disrupts a xanthophyll esterase and decreases xanthophyll accumulation in tomato flowers [8•]. In chloroplasts, where maintenance of a rigorous stoichiometry between carotenoids, chlorophylls and pigment binding proteins is of essence for the proper functioning of the photosynthetic apparatus, excess carotenoids generated by PSY overexpression are not esterified, but are cleaved into apocarotenals and then glycosylated into apocarotenoid glycosides, thus maintaining carotenoid homeostasis [9•]. In chromoplasts [10] and chloroplasts [11] carotenoids are also bound by different classes of pigment binding proteins, contributing to their stability.

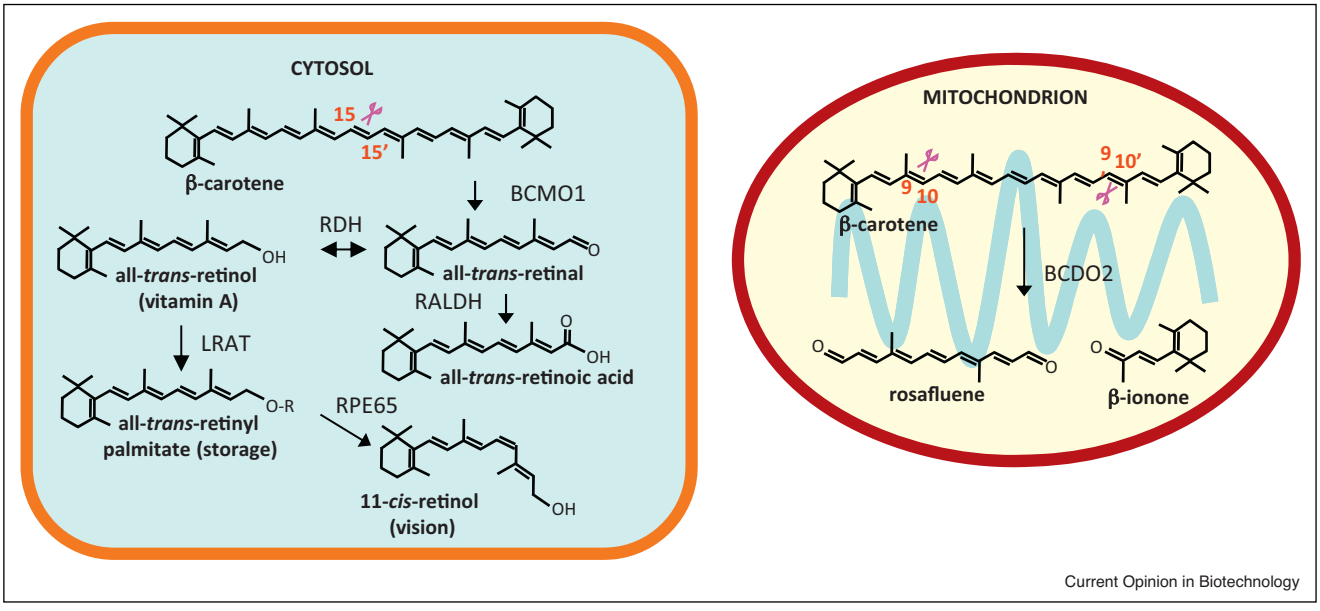
Carotenoid and vitamin A metabolism in vertebrates and their health effects

Vertebrates – including humans – have an absolute requirement for vitamin A their diet. Vitamin A deficiency affects over 125 million preschool-aged children and 7 million pregnant women in low-income countries. It is the leading cause of preventable pediatric blindness and of over 650 000 early childhood deaths due to diarrhea, measles, malaria, and other infections each year [12]. Vitamin A (retinol) can be either absorbed by consuming animal-derived foods, or can be synthesized in the body from provitamin A carotenoids, containing at least one non-substituted β-ring [13,14] (Figure 2). The main provitamin A carotenoid, β-carotene, is found in very different concentrations in different plant-derived foods (Table 1).

Intestinal absorption of carotenoids and several other lipids is mediated by an intestinal scavenger receptor class B type I (SR-BI) [15]. Two main reactions involving dietary carotenoids occur in vertebrate tissues (Figure 2): (i) the 15/15′ cleavage of provitamin A carotenoids by β-carotene 15,15′-monooxygenase (BCMO1), which occurs in the intestine and is the first dedicated step in retinoid biosynthesis, and (ii) the 9/10, 9′/10′ cleavage of a series of other dietary carotenoids by β-carotene-9′,10′-dioxygenase (BCDO2), controlling carotenoid homeostasis and accumulation in the bloodstream and target tissues, including bird retina, plumage and skin, primate retina, and adipose tissue. In the primate retina, lutein and zeaxanthin accumulate in the macula lutea, where they act as photoprotectants.

The intestine-specific homeobox (ISX) transcription factor controls the intestinal expression of both *SR-BI* and *BCMO1*, thus acting as a gatekeeper of provitamin A absorption and retinoid formation [16••,17••]. Given this mechanism, while absorption of excess vitamin A can lead to hypervitaminosis and teratogenesis, the consumption of provitamin A-rich foods is highly unlikely to lead to excess vitamin A production. On the other hand, diets rich in vitamin A are likely to affect the absorption and metabolism of non-provitamin A carotenoids and other lipids, mediated by SR-BI.

Figure 2



Carotenoid and retinoid metabolism in vertebrates.
BCMO1: β-carotene 15,15′-monooxygenase; BCDO2: β-carotene-9′,10′-dioxygenase; RDH: retinol deydrogenase; RALDH: retinal dehydrogenase; LRAT: lecithin retinol acyltransferase; RPE65: retinal pigment epithelium-specific 65 kDa protein.

Table 1

β-carotene content of plant-derived foods from common, transgenic (T) and mutant (M) genotypes. The last two columns indicate the quantities required to reach 25% of the vitamin A recommended daily allowance (RDA) at different equivalency ratios (ER)

Food	β-carotene μg/100 g	Source	Grams for 25% RDA (ER 2.5:1)[b]	Grams for 25% RDA (ER 8:1)[b]
Apricots	1094	USDA[a]	46	146
Bananas, Cavendish	26	USDA[a]	1923	6154
Golden bananas (T)	200	[54•]	250	800
Papuan bananas	2594	[108]	19	62
Broccoli	361	USDA[a]	139	443
Cauliflower	7	USDA[a]	7143	22 857
OR cauliflower (M)	800	[86•]	63	200
Canola oil	600[c]a	[45•]	83	267
Golden canola oil (T)	150 000[c]	[45•]	0.3	1
Carrots	8225	USDA[a]	6	19
Cassava	2.5	[50•]	6250	20 000
Yellow cassava (M)	800	[50•]	63	200
Golden cassava (T)	1800	[51•]	28	89
Chicory greens	3430	USDA[a]	15	47
Kale	5927	USDA[a]	8	27
Margarine	610	USDA[a]	82	262
Melon, honeydew	30	USDA[a]	1667	5333
$OR^{Arg>His}$ Melon (M)	3000		17	53
Palm oil, red	4700	[109]	11	34
Peas, green	449	USDA[a]	111	356
Potatoes	5	USDA[a]	10 000	32 000
Golden potatoes I (T)	180	[110]	278	889
Golden potatoes II (T)	810	[48•]	62	198
Pumpkin	3100	USDA[a]	16	52
Rice	1	USDA[a]	50 000	160 000
Golden Rice I (T)	200	[46••]	250	800
Golden Rice II (T)	3100	[47•]	16	52
Sorghum	50	[53•]	1000	3200
Golden sorghum (T)	1230	[53•]	41	130
Spinach	5626	USDA[a]	9	28
Sweet corn, yellow	47	USDA[a]	1064	3404
Multivitamin corn (T)	5900	[111••]	8	27
Biofortified corn	1500	[37•]	33	107
Sweet potatoes	8500	USDA[a]	6	19
Tomatoes	450	USDA[a]	111	356
Golden Tomatoes I (T)	5500	[112,113]	9	29
Golden Tomatoes II (T)	20 000	[114]	3	8
Wheat	5	USDA[a]	10 000	32 000
Golden wheat (T)	500	[60•]	100	320

[a] USDA Food Composition Databases (https://ndb.nal.usda.gov/ndb/).
[b] Assuming a retinol RDA = 800 μg.
[c] Assuming a concentration in oil twice than that reported in whole seeds.

Besides their role as vitamin A precursors, dietary carotenoids have been implicated in the prevention of several chronic diseases. High dietary intake of zeaxanthin and lutein is associated with a decreased risk of late age-related macular degeneration (ARMD) [18]. The role of dietary lycopene and its metabolites in cancer prevention remains controversial [19–21]. β-carotene does not have an evident role in cancer prevention and, if assumed at pharmacological doses (20–30 mg/day) it is associated with an increased risk for some types of cancer in smokers and asbestos workers [22•].

Bioavailability, bioaccessibility and vitamin A equivalence

Bioavailability defines the fraction of an ingested carotenoid that is available for utilization in normal physiological functions and/or for storage, while bioaccessibility defines the amount of a carotenoid released from the food matrix and accessible for absorption [23•]. Bioaccessibility is determined using *in vitro* methods, like *in vitro* digestion and/or the Caco-2 cell line, while to determine bioavailability animal models such as mice, rats, chickens, Mongolian gerbils, or human volunteers are used [23•]. Bioavailability/bioaccessibility of β-carotene from different food matrixes varies greatly, from 2% to 70%, being generally higher in fat-rich, cooked matrixes [23•]. Vitamin A equivalency ratio (ER) is defined as the amount of dietary β-carotene that is equivalent to a unit amount of retinol (vitamin A) and is determined using animal models mimicking human retinoid metabolism, or human volunteers. This ratio varies from 3.8:1 to 28:1, with simple matrixes showing lower ER (and thus higher conversion

rates) than complex ones [24]. The amounts of plant-derived foods needed to provide 25% of the vitamin A RDA, a dose sufficient to prevent avitaminosis A, assuming different ERs, are shown in Table 1. As can be seen, none of the five major staple crops (wheat, corn, rice, potato, cassava) is able to provide a significant portion of the RDA, even at the lowest ER.

Several studies have addressed the bioaccessibility, bioavailability, and in some cases the ER of β-carotene from biofortified plant matrixes. *In vitro* bioaccessibility studies conducted on biofortified cassava [25,26], sweet potato [27], melon [28], sorghum [29] and potato [30] concluded that, with the exception of sweet potato, biofortified crops show good β-carotene bioaccessibility which is comparable to their non-transgenic counterparts, and that processing and cooking are the major factors influencing bioaccessibility. Carotenoids from transgenic biofortified corn have been shown to be highly bioavailable when fed to poultry, accumulating in different organs, including skin and egg yolk and protecting them from coccidiosis [31•,32]. Bioavailability studies on Mongolian gerbils (whose provitamin A metabolism is similar to that of humans) found β-carotene to retinol conversion ratios of 3:1 for biofortified maize [33•] 3.5–6.5:1 for sweet potato [34] and of 3.7:1 for cassava [35]. Finally, studies involving human volunteers have found vitamin A equivalency ratios of 3.8–6.5:1 in non-transgenic β-carotene-fortified maize [36•,37•], of 2.3:1 for transgenic Golden Rice [38••] and of 4.2:1 for non-transgenic biofortified cassava [39•]. As for *in vitro* studies, different cooking styles and fat contents affected bioavailability, which increased with increasing fat content [34,35,40].

One crucial aspect that needs further experimentation is whether β-carotene-fortified crops can improve vitamin A status in the main targets of the biofortification efforts, that is, malnourished adults and children. In a recent study, β-carotene-fortified maize was able to improve serum β-carotene levels, but not retinol levels in marginally nourished Zambian children [41•].

Metabolic engineering of plant carotenoids

Since 1999, over 90 papers have been published on carotenoid metabolic engineering in plants (Supplementary Table S1). The main enzymatic steps engineered and the relative crops are depicted in Figure 1. The different strategies used have been the subject of several reviews [42–44] and can be schematically divided into several classes:

i) 'Push' strategies, enhancing the metabolic flux upstream of a target compound through the overexpression of one or more biosynthetic enzymes. Well-known examples include β-carotene-fortified 'golden' canola [45•], rice [46••,47•], potatoes [48•], maize [49••], cassava [50•,51•], wheat [52], sorghum [29,53•] and bananas [54•]. Overexpression of biosynthetic enzymes may lead to the depletion of biosynthetic precursors, thus limiting the accumulation of the desired product. This is exemplified by the overexpression of *PSY* and *CrtI* in rice callus and endosperm. The simultaneous overexpression of the first dedicated enzyme in the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*), leads to increased total carotenoids and β-carotene contents, indicating that supply of isoprenoid precursors is rate-limiting [55•,56]. These have proven by far the most efficient strategies, resulting in 100- to 1000-fold increases of carotenoid content.

ii) 'Block' strategies, decreasing the metabolic flux downstream of a target compound or on a competing metabolic branch through gene silencing; examples include potatoes fortified with zeaxanthin or β-carotene through silencing of *ZEP* [57], or *LCY-e* [58] and oranges fortified with β-carotene through silencing of *CHY* [59]. These strategies have resulted in total carotenoid increases of up to 10-fold. Enhanced and arrested metabolic flux strategies can be combined in a single event, such as in the case of *CrtB* overexpression combined with *CHY* silencing in wheat endosperm, which results in a large increase in β-carotene levels [60•] or *Psy1/CrtZ/Bkt* overexpression combined with *LCY-e* silencing in maize endosperm, which results in accumulation of astaxanthin and keto-zeaxanthin [61].

iii) Strategies aimed at increasing the size of the 'metabolic sink', that is, of the compartment where carotenoids are accumulated; This concept was first used to describe the β-carotene accumulation induced by overexpression of the *OR* gene product, which is able to induce chromoplast differentiation and carotenoid accumulation [62]. Genetic interventions on the light perception/signal transduction pathway [63–65] or the ABA pathway [66] are also able to increase plastid size or number and have a positive effect on lycopene accumulation in tomato fruits. The overexpression of a carotenoid binding protein from pepper chromoplasts, fibrillin, in tomato fruits resulted in a minor increase in carotenoid content, but also in other unexpected phenotypes, such as retarded breakdown of thylakoid membranes and an increase of carotenoid-derived volatiles [67]. Crossing of an astaxanthin-accumulating transgenic maize line into a high oil–maize genotype in order to increase the storage capacity for lipophilic astaxanthin resulted instead in a marginal increase in astaxanthin and in an actual reduction of total carotenoids [61]. In general, strategies aimed at stabilizing carotenoids or increasing the metabolic sink, with the exception of *OR* overexpression – whose inclusion in this category appears doubtful (see below) – result at best in a twofold increase in total carotenoid content.

iv) Strategies aimed at increasing post-harvest carotenoid stability. Some engineered crops, like 'golden potatoes', actually increase their carotenoid content during cold storage [68] (G. Diretto and G. Giuliano, unpublished results). In contrast, carotenoids in cereal grains are prone to oxygen- and lipoxygenase-mediated degradation [69,53•]. Deletion of lipoxygenase-encoding loci, or metabolic engineering for increased vitamin E content have been shown to increase carotenoid stability during storage and/or processing [69,53•], and hold promise for further improving the nutritional impact of biofortified cereals such as 'golden rice'.

'Natural' genetic variation for carotenoid biofortification

Several crop plants exhibit a large phenotypic variation in the quantity and types of carotenoids accumulated. Often, the genetic basis underlying this variation is simple and involves differential expression of structural genes in the carotenoid pathway. The *Beta* and *Delta* genes of tomato represent dominant *CYC-b* (an *LCY-b* paralog) and *LCY-e* alleles, which are overexpressed during fruit ripening and thus result in the accumulation of β- and δ-carotene, respectively, in ripe fruits [70,71•]. In maize endosperm, major quantitative trait loci (QTLs) map to the *PSY1*, *LCY-e*, *ZDS* and *CHY1* genes, and minor ones to the *DXS2*, *CYP97A*, *GGPS1*, *GGPS2*, *CHY3*, *CCD1* and *ZEP* genes [72,73••,74•,75•,76,77]. *LCY-e* alleles resulting in increased β-carotene accumulation are recessive, indicating loss-of-function. Different *LCY-e* alleles show either reduction of transcript expression or changes in the amino acid sequence [73••]. *CHY1* alleles resulting in increased β-carotene accumulation are also recessive and show reduction of *CHY1* expression [75•]. In potato, recessive *ZEP* alleles, carrying a retrotransposon insertion in the first intron show reduced *ZEP* expression and increased content of total carotenoids (but not of β-carotene), while alleles resulting in *CHY2* overexpression result in the selective accumulation of zeaxanthin [78] (M. Sulli at al, unpublished). In wheat, QTLs resulting in high lutein content map to *LCY-e* (amino acid substitution) and *PSYA1* (alternative splicing) [79]. In cassava roots, accumulation of β-carotene in yellow genotypes (Table 1) is due to an Ala > Asp mutation in the *PSY2* gene product [50•]. In sweet potato, crosses between a low β-carotene African variety and a high β-carotene variety revealed at least eight loci controlling β-carotene content as well as transgressive segregation, suggesting that further improvement of this trait is possible [80]. One challenge in cassava breeding is to combine high β-carotene content with high starch content, since these traits show a negative correlation [80].

Orange carrots have an unusual carotenoid composition, combining extremely high levels of β- and α-carotene. They were introduced in Europe, probably in the Netherlands, in the 17th century, substituting the previously used, purple-colored carrots, high in anthocyanin but low in carotenoids [81]. The orange color of carrot roots is controlled by two major loci, *Y* and Y_2, whose dominant, wild type alleles repress carotenoid accumulation. White roots are *YY* Y_2Y_2, yellow roots, containing mainly xanthophylls, are *yy* Y_2Y_2, and orange roots, containing high levels of β- and α-carotene, are *yy* y_2y_2 [82•]. The *Y* locus encodes a putative repressor of photomorphogenesis, and loss-of function mutations at this locus are hypothesized to trigger carotenogenesis in the root even in the absence of light [83••]. The identity of the Y_2 locus is less clear: orange carrot genotypes contain a loss-of-function mutation in the *CYP97A3* gene, mediating the hydroxylation of the β-ring of α-carotene, and accordingly accumulate α-carotene in both leaves and tap roots [84•]. Complementation with the Arabidopsis *CYP97A3* gene causes reduction of α-carotene in both leaves and roots and simultaneous reduction of β-carotene in roots. The recessive nature of the mutation and its association with α- and β-carotene accumulation make it a good candidate for the Y_2 locus. However, *CYP97A3* maps on carrot chromosome 7 [83••], while Y_2 maps on chromosome 2 [82•]. Additional candidate genes controlling carrot root pigmentation have been identified [82•,85], and one of them may represent the Y_2 locus.

The β-carotene contents of several crops (cauliflower, cassava, corn, melon) biofortified through conventional breeding are shown in Table 1. In the majority of cases, classical breeding approaches are still sufficient to provide significant provitamin A activity (Table 1). Only in some crops, like rice, potato and wheat, natural variation in β-carotene content is not sufficient to reach significant levels of provitamin A activity, making transgenic approaches necessary.

The OR conundrum

ORANGE (*OR*) is a semi-dominant mutant inducing β-carotene accumulation in cauliflower curd in heterozygous state (Table 1), while in homozygous state produces a semi-lethal phenotype [86•]. Cloning of the gene showed it contains a Cys-rich zinc finger domain found in DnaJ co-chaperones, and overexpression of the gene in potato tubers induced chromoplast differentiation and β-carotene accumulation without affecting transcript levels of endogenous carotenoid genes [87••,88]. Initially, it was proposed that, rather than regulating the metabolic flux in the carotenoid pathway, OR was an activator of chromoplast differentiation, thus creating a metabolic sink for β-carotene accumulation [62]. Recently, analysis of a population of melon segregating for β-carotene content showed that the high β-carotene phenotype co-segregated with a dominant *OR* allele carrying an Arg > His mutation. This allele was able to induce β-carotene accumulation when overexpressed in Arabidopsis transgenic calli, while the wild-type allele did not [89••

,90•]. Importantly, the Arg > His mutation results in β-carotene accumulation in the context of different OR proteins, both from monocots and from dicots. OR seems to control β-carotene accumulation through at least two different molecular mechanisms: (i) the wild-type isoform stabilizes the enzyme mediating the first dedicated step in carotenoid biosynthesis, PSY, enhancing its activity [91••,92•] and (ii) the Arg > His isoform prevents β-carotene turnover [92•]. Contrasting data have been reported on the effect of OR overexpression on endogenous carotenoid gene expression: no effect has been reported by some groups [93,89••,90•,91••,92•], while other groups reported significant changes in the expression of several carotenoid pathway genes [55•,94,56]. A further complication is that OR induces differentiation of at least two different types of chromoplasts: crystal-type (where β-carotene accumulates in crystalloids) by the cauliflower *OR* mutant and membrane-type (where it accumulates in membrane structures) by the Arg > His isoform (R Welsch and L Li, personal communication). The most parsimonious explanation of these different results is that OR action is predominantly exerted at the posttranscriptional level, that it shows a threshold effect depending both on the OR isoform used and the recipient plant/tissue, and that the alteration in the expression of endogenous carotenoid genes is an indirect result of altered carotenogenesis induced by OR, rather than a direct effect of OR on endogenous carotenoid gene expression. Whatever the case, it seems increasingly clear that in the case of *OR* overexpression, chromoplast differentiation is the result of increased β-carotene accumulation, rather than the opposite, as suggested by the fact that differentiation of chromoplasts containing β-carotene crystals in Arabidopsis roots can be induced also by *PSY* overexpression [95•].

Phenotypic alterations and perturbations of non-carotenoid metabolism

Several isoprenoids playing important roles in photosynthesis (tocopherols, plastoquinone, and the phytol moiety of chlorophylls) are synthesized from the same precursor utilized by carotenoids, GGPP (Figure 1). Additionally, several plant hormones or signaling molecules are synthesized from DMAPP (cytokinins), GGPP (gibberellins), β-carotene (strigolactones, β-cyclocitral) or 9-*cis*-epoxyxanthophylls (abscisic acid or ABA) (Figure 1). Carotenoids play also essential photosynthetic functions in leaves. Therefore, it is not surprising that carotenoid metabolic engineering results, in several cases, in altered developmental phenotypes and perturbations of metabolism, especially when strong, constitutive promoters such as cauliflower mosaic virus *35S* are used. One such example is *35S:PSY* overexpression in tomato, causing a dwarf phenotype due to gibberellin depletion [96]. Another example is *35S:CrtI* overexpression in potato, resulting in chlorophyll discoloration in leaves, possibly due to depletion of the phytol pool [48•] (unpublished results). The use of promoters restricting expression of the transgene to the target tissue may alleviate this problem: the potato leaf discoloration phenotype is very reduced, or absent, when the *CrtI* transgene is driven by a tuber-specific promoter, which also results in much higher levels of tuber β-carotene [48•]. Other pleiotropic phenotypes observed in engineered plants include cytokinin overproduction/early sprouting in *DXS*-overexpressing potato tubers [97]; increased tolerance to light, drought or salt stress in several species overexpressing *CHY*, *BKT* or *OR* genes [98–101]; and increased gibberellin content, plant biomass and early flowering in *LCY-b* - overexpressing tobacco [102].

Carotenoid metabolic engineering also induces perturbations in isoprenoid metabolism: *PSY1* overexpression reduces GGPP and chlorophyll content in tomato fruits, while tocopherols are increased [103•]; similar effects are observed in canola seeds overexpressing the *CrtB* bacterial gene [45•]; furthermore, in *PSY1*-overexpressing tomato fruits, intermediate metabolism undergoes changes that can be explained in terms of accelerated fruit ripening [103•]. Expression of endogenous carotenoid genes is altered in engineered tomato and potato, suggesting that mechanisms are in place, able to sense carotenoid levels and regulate carotenoid gene expression accordingly [103•,104]. Maize seeds engineered for enhanced carotenoid content contain increased levels of sterols and fatty acids, of sucrose, proline and aspartic acid, as well as altered levels of transcripts for intermediate metabolism [105]. An intriguing hypothesis is that all these different and apparently unrelated transcriptional and metabolic changes, are due to the altered abundance, in engineered plants, of signaling apocarotenoid molecules. The involvement of signaling molecules is further suggested by the fact that the metabolic changes are observed not only in the organ targeted by metabolic engineering (eg maize endosperm) but also in adjacent organs (eg the embryo) [105]. These metabolic perturbations are within the range of natural variation of the maize crop [61] and have no major effects on agronomic performance or grain yield [106•].

Concluding remarks

Since 1999, biofortification with both provitamin A and non-provitamin A carotenoids has been obtained in a series of crops. Initially, transgenic 'push' strategies and, more recently, strategies based on *OR* gene overexpression and traditional breeding have resulted in large increases in carotenoid levels in several crops, with large potential nutritional impacts (Table 1). Conversion rates of β-carotene from biofortified Golden Rice, maize and cassava into vitamin A are high, suggesting that these staple crops can have a large nutritional impact. Actually, some of the most extreme cases (Golden canola, Golden tomatoes II) contain extremely high doses of β-carotene

(Table 1), suggesting that they should be treated as food supplements, rather than as foods *per se*.

A major problem with biofortified transgenic crops is their public acceptance and the lengthy regulatory process needed before they get clearance for cultivation and human consumption. On the other hand, the long and complicated breeding cycles, or the lack of high β-carotene germplasm, in some crops (bananas, potatoes, wheat, rice), make conventional breeding approaches impractical. The development of new plant breeding techniques like cisgenesis or intragenesis can accelerate the transfer of 'golden' traits from sexually compatible germplasm to elite varieties in crops, like banana or cassava, for which traditional breeding is time consuming. Knock-out mutations blocking β-carotene hydroxylation, or lycopene ε-cyclization, which are able to increase β-carotene content in several different crops, can be induced by gene editing technology [107]. Finally, the developments of such technology will, in the foreseeable future, make possible the introduction in elite varieties of point mutations able to increase β-carotene content, like the *OR* Arg > His or the *PSY* Ala > Asp mutations.

Areas requiring further research are the post-harvest stability of carotenoids in some crops (such as cereals), the metabolism of the accumulated carotenoids in plant tissues, potentially leading to the synthesis of bioactive apocarotenoids [19], and the bioconversion of β-carotene into vitamin A in marginally nourished or undernourished segments of the population, which are the obvious beneficiaries of 'golden' crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.copbio.2017.02.001.

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