Carotenoid biofortification in crop plants: *citius*, *altius*, *fortius*

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Abstract

Carotenoids are indispensable for human health, required as precursors of vitamin A and efficient antioxidants. However, these plant pigments that play a vital role in photosynthesis are represented at insufficient levels in edible parts of several crops, which creates a need for increasing their content or optimizing their composition through biofortification. In particular, vitamin A deficiency, a severe health problem affecting the lives of millions in developing

countries, has triggered the development of a series of high-provitamin A crops, including Golden Rice as the best-known example. Further carotenoid-biofortified crops have been generated by using genetic engineering approaches or through classical breeding. In this review, we depict carotenoid metabolism in plants and provide an update on the development of carotenoid-biofortified plants and their potential to meet needs and expectations. Furthermore, we discuss the possibility of using natural variation for carotenoid biofortification and the potential of gene editing tools.

Key word: Carotenoids, Apocarotenoids, Vitamin A, Biofortification, Genetic engineering, Breeding, Gene editing

1. Introduction

Carotenoids are lipophilic isoprenoids synthesized by all photosynthetic organisms, including plants, algae and cyanobacteria, as well as by several non-photosynthetic fungi and prokaryotes [1-5]. Plant carotenoids consist of a common C₄₀ backbone equipped with a conjugated system containing 3 to 11 double bonds that can assume different stereo-configurations. Carotenoids are divided into the oxygen-free carotenes and the oxygen-containing xanthophylls, and are further distinguished by the structure of their end groups. For non-biologists, carotenoids are best known for their bright colors that accumulate in many flowers and fruits to attract animals for pollination and seed dispersal, and also in many birds, fish and crustacean species [1, 6-9]. However, the essential need of plants and other photosynthetic organisms for carotenoids is explained by the vital role of these pigments in protecting the photosynthesis apparatus from photo-oxidation, which is brought about by quenching triplet state chlorophylls that can trigger the formation of singlet oxygen, a powerful Reacting Oxygen Species (ROS) that can oxidize

and destroy chlorophylls and other cellular components. Besides preventing its formation, carotenoids scavenge this ROS by forming instable oxygen-containing epoxides and structures that also include other types of lipids [10]. In addition to photo protection, carotenoids contribute to photosynthesis as accessory light-harvesting pigments absorbing light in blue-green region and transferring the energy to chlorophylls [10]. Carotenoids are also the precursors of important plant hormones, i.e. abscisic acid [11] and strigolactones [12, 13], and of different signaling molecules [14, 15], such as β -cyclocitral [16, 17], zaxinone [18] and anchorene [19]. All of these carotenoid-derivatives are formed by oxidative cleavage of carotenoids, generally catalyzed by carotenoid cleavage dioxygenases (CCDs), a ubiquitous family of non-heme iron enzymes, found in bacteria, fungi, plants and animals [20-23]. Oxidative cleavage of carotenoids can be also mediated by lipoxygenases that use them as co-substrate [24, 25], or triggered non-enzymatically by ROS [16, 26]. For instance, chemical quenching of singlet oxygen in chloroplasts results in oxidative breakdown of β -carotene and the formation of the volatile signaling molecule and growth regulator β -cyclocitral [16, 17]. Besides biosynthetic and storage capacities, degradation processes are a further determinant of carotenoid levels in plant tissues [5].

In general, animals do not synthesize carotenoids *de novo*, but obtain them through their diet. In mammals, the enzyme β -carotene cleavage oxygenase 1 (BCO1), a member of the CCD enzyme family, catalyzes the first step in vitamin A (retinol) synthesis by forming retinal through the cleavage of the central C15-C15' double bond in β -carotene and other carotenoids containing a non-substituted β -ionone ring [27, 28]. The contribution of carotenoids to vitamin A supply is particularly important if animal derived food is absent. In addition to their role as vitamin A precursors, carotenoids are required for human health as important antioxidants and ROS

scavengers, preventing cancer, cardiovascular diseases and neurodegenerative disorders [29-33]. The non-provitamin A carotenoids zeaxanthin and lutein that accumulate in the macula are thought to alleviate and decrease the risk of age-related macular degeneration [32]. Moreover, lutein is the predominant carotenoid in the human brain tissue, and recent studies indicate a role of this carotenoid in brain development and cognitive performance throughout the lifespan [31, 34]. For instance, it has been shown that post-mortem brain lutein concentrations correlate with pre-mortem cognitive function [35]. Randomized supplementation clinical trial also suggested a positive impact of lutein on brain function and cognitive performance [36]. Though still disputed, the acyclic carotene lycopene has been implicated in preventing prostate cancer [33]. Several health benefits, including decreasing the risk of certain cancers and arteriosclerosis, have been suggested for the colorless, acyclic carotenes phytoene and phytofluene, which can be used as nutricosmetics to protect the skin from damage caused by free radicals [37-39]. Astaxanthin, a ketocarotenoid synthesized by algae and cyanobacteria, is an efficient antioxidant and a high-value feed additive used in aquaculture to obtain red/pink flesh color and enhance the immunity of farmed fish [40, 41]. Considering their various health benefits and attracting colors, carotenoids, mainly obtained through chemical synthesis, are used commercially as food and feed additives, as well as in the cosmetic industry [4, 42].

Vitamin A deficiency (VAD) is still one of the most severe global health problems. Thus, lack of this micronutrient is impacting over 125 million preschool-aged children and 7 million pregnant women in developing countries [43]. VAD is the major reason for childhood mortality, causing annually over 650 000 early childhood deaths and preventable pediatric blindness [43]. Considering the severity of VAD and its fatal consequences, there have been large efforts to biofortify different crops with provitamin A carotenoids, mainly β -carotene, as a sustainable

strategy to compensate the scarcity of animal-derived diet and combat VAD. These efforts led to the generation of Golden Rice, a milestone in plant metabolic/genetic engineering, and to many other “Golden Crops” with enhanced provitamin A content [44]. The demand for natural carotenoids as food and feed additives, and their versatile health benefits has also prompted the engineering of further carotenoid-biofortified crops, either by generally increasing carotenoid content (quantitative engineering) or by installing specific pathways from other organisms to produce new high-value carotenoid metabolites, e.g. astaxanthin and crocin, (qualitative engineering).

In this review, we provide an update on plant carotenoid metabolism and underlying regulatory processes, summarize the progress in generating and assessing the utility of carotenoid-biofortified crop plants, and discuss currently employed strategies. Finally, we touch on the potential of knowledge-based multiple gene engineering and gene editing in carotenoid biofortification.

2. Carotenoid metabolism in plants

Understanding biosynthetic pathways and the underlying regulation mechanisms is an essential prerequisite for efficient harnessing of plants metabolic capacities for biofortification. The plant carotenoid biosynthesis pathway has been almost completely elucidated, thanks to the efforts of many scientists in the field. However, our knowledge about the regulation of carotenogenesis is still limited.

2.1 Biosynthesis of the isoprenoid building block

Like other isoprenoids, carotenoids are formed from the universal C₅ building block isopentenyl diphosphate (IPP) through condensation with its isomer dimethylallyl diphosphate (DMAPP)

(**Fig. 1**). Higher plants and some algae are unique in having two different pathways for IPP biosynthesis [45]. The first one, the mevalonate pathway, provides the precursor for larger cytosolic isoprenoids, including sesquiterpenes, phytosterols, dolichols, triterpenes, and also isoprenoids for protein modification. The mevalonate pathway involves six enzymes and starts with the condensation of two acetyl-CoA molecules into acetoacetyl-CoA, which is catalyzed by the enzyme acetoacetyl-CoA thiolase (AACT) (**Fig. 1**). In the next step, the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) catalyzes a second condensation reaction with one acetyl-CoA unit, yielding HMG-CoA. The latter is then reduced by the enzyme HMG-CoA reductase (HMGR) to mevalonate (MVA), the substrate of the enzyme mevalonate kinase (MVK) that forms phosphomevalonate. A second phosphorylation reaction, catalyzed by the phosphomevalonate kinase (PMK), yields mevalonate 5-diphosphate (MVAPP). Finally, the diphosphomevalonate decarboxylase (MVD) mediates a reductive decarboxylation of MVAPP to form IPP. The enzyme isopentenyl diphosphate isomerase converts IPP into its isomer DMAPP. The mevalonate pathway is responsible for IPP formation in animals, fungi, archaebacteria and some gram-positive bacteria, such as enterococci and staphylococci, and is the main source for IPP in the cytosol of plants [45]. The second IPP synthesis route, the methylerythritol 4-phosphate (MEP) pathway, is common in plastids of higher plants and some algae, and is the sole source of IPP in most gram-negative bacteria, cyanobacteria and green algae [45]. The MEP pathway starts with the condensation of the glycolytic glyceraldehyde 3-phosphate (GA-3-P) and pyruvate (**Fig. 1**). This initial reaction yields 1-deoxy-*D*-xylulose 5-phosphate (DXP) and is catalyzed by the enzyme 1-deoxy-*D*-xylulose 5-phosphate synthase (DXS). In the next step, the enzyme 1-deoxy-*D*-xylulose 5-phosphate reductoisomerase (DXR) mediates the reversible rearrangement of DXP into 2-C-methyl-*D*-erythritol 4-phosphate (MEP). The introduction of a

cytidyl moiety, linked through a diphospho bridge, converts MEP into 4-(cytidine-diphospho)-2-C-methyl-*D*-erythritol (CDP-ME). CDP-ME is then phosphorylated by the enzyme 4-(cytidine 5'-diphospho)-2-C-methyl-*D*-erythritol kinase (CMK) to produce CDP-ME 2-phosphate (CDP-MEP). The latter reactions are catalyzed by the enzyme 2-C-methyl-*D*-erythritol 2, 4-cyclodiphosphate synthase (MDS) and yield 2-C-methyl-*D*-erythritol 2,4-cyclodiphosphate (MEcPP). Finally, IPP and DMAPP are formed through two sequential reduction reactions mediated by the enzymes (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and reductase (HDR) [2, 46, 47].

Figure 1. The MVA and MEP pathways in plants. Dashed arrows mark multiple steps of each pathways. Open arrows represent possible transport of IPP and DMAPP between plastids and cytoplasm. GA-3-P, glyceraldehyde 3-phosphate; DXP, 1-deoxy- *D*-xylulose 5'-phosphate; MEP, 2-C-methyl-*D*-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5-diphospho)-2-C-methyl-*D*-erythritol; CDP-MEP, CDP-ME 2-phosphate; MEcPP,

2-C-methyl-*D*-erythritol 2,4-cyclodiphosphate; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, di-methylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate. HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; MVA, mevalonic acid; MVP, 5-phosphomevalonate; MVAPP, 5-diphosphomevalonate. Enzymes of MEP pathway are indicated in bold: DXS, 1-Deoxy-*D*-xylulose 5-phosphate synthase; DXR, 1-Deoxy-*D*-xylulose 5-phosphate reductoisomerase; MCT, 2-C-methyl-*D*-erythritol 4-phosphate cytidyltransferase; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-*D*-erythritol kinase; MDS, 2-C-methyl-*D*-erythritol 2,4-cyclodiphosphate synthase; HDS, HMBPP synthase; HDR, HMBPP reductase; IDI, isopentenyl diphosphate isomerase; GGDS, GGPP synthase; PSY, phytoene synthase. Enzymes of MVA pathway: AACT, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MVK, MVA kinase; PMK, MVP kinase; MVD, MVPP decarboxylase;

Several lines of evidence suggest an exchange of isoprene building units (IPP and DMAPP) between plastids and cytoplasm (for review, see [48]). This exchange has been demonstrated by using specific inhibitors that target either the MEP or the MVA pathway [49, 50], by feeding with labeled precursors [51] and by analyzing genetic blocks in one of the two pathways [52]. However, this exchange is limited; and it can be assumed that the mevalonate pathway does not significantly contribute to carotenoid biosynthesis, as shown by the albino phenotype of mutants disrupted in the MEP pathway or treated with the DXR inhibitor fosmidomycin [48].

All enzymes of the MEP pathway are regulated at transcriptional level. In addition, it has been shown that the activity of the first enzyme in the pathway, DXS, which shows highest flux control coefficient in ^{13}C labeling experiments [53], and of DXR and HDS is also regulated at the translational and post-translational levels (reviewed in: [54, 55]). Besides phosphorylation, the post-translational regulation of DXS activity includes reactivation or Clp-protease dependent degradation of misfolded enzyme, depending on the growth conditions [56-58]. The Clp protease system also regulates the activity of other enzymes in the MEP pathway [58, 59]. In addition, MEP pathway enzymes undergo redox regulation [54], possibly through the action of thioredoxin [60]. The MEP pathway is also regulated by the end products IPP and DMAPP that repress the

activity of the key enzyme DXS [61, 62]. The decrease in the levels of these end products leads to increased DXS protein levels [47].

2.2 Biosynthesis of carotenes and xanthophylls

The next step towards carotenoid biosynthesis is mediated by the enzyme geranylgeranyl diphosphate synthase (GGPS) that catalyzes the stepwise 1'-4 head-to-tail condensation of three IPP units and one DMAPP molecule to form the C₂₀ metabolite geranylgeranyl diphosphate (GGPP; C₂₀) via the intermediates GPP and farnesyl diphosphate (FPP) (**Fig. 1**). In addition to carotenoids, GGPP is the precursor of tocopherols, of the side chain of chlorophylls, phytol, of plastoquinones, and of the phytohormones gibberellins. Therefore, increasing carotenoid content may cause a shortage in GGPP supply, which negatively impacts the growth and development of the plant (s. below).

The first committed step in carotenogenesis is the head to head condensation of two C₂₀ geranylgeranyl diphosphate (GGPP), which yields the colorless carotene 15-*cis*-phytoene (C₄₀) (**Fig. 2**). This reaction is catalyzed by the enzyme phytoene synthase (PSY), which is considered as the key enzyme in plant carotenoid biosynthesis and assumed to control the carotenoid metabolic pool size [63, 64]. Transcript, protein abundance and activity change of PSY can dramatically affect metabolism flux to carotenoid; thus, it is an important target for elucidating the regulation of carotenoid biosynthesis and for increasing carotenoid content by metabolic engineering. A series of desaturation and isomerization reactions extends the carotene polyene from three to eleven double bonds, converting the colorless 15-*cis*-phytoene into the red all-*trans*-lycopene (**Fig. 2**). In plants and cyanobacteria, the formation of all-*trans*-lycopene through the poly-*cis* pathway requires four enzymes. The first one, phytoene desaturase (PDS), introduces, coupled with *cis/trans*-isomerization, two double bonds, leading to 9, 15,

9'-tri-*cis*- ζ -carotene via 9, 15-di-*cis*-phytofluene. 9, 15, 9'-tri-*cis*- ζ -carotene underlies an isomerization reaction catalyzed by the ζ -carotene isomerase (Z-ISO) that changes the configuration of the central double bond to yield 9,9'-di-*cis*- ζ -carotene (**Fig. 2**). The following two dehydrogenation reactions are catalyzed by the ζ -carotene desaturase (ZDS) that forms 7, 9, 9', 7'-tetra-*cis*-lycopene (prolycopene) via 7, 9, 9'-tri-*cis*-neurosporene. The fourth enzyme, the carotene isomerase CRTISO converts prolycopene into all-*trans*-lycopene [65-68]. Remarkably, most of carotenogenic bacteria, with the exception of cyanobacteria, require only a single enzyme, CrtI, which carries out four desaturations and the required *cis/trans*-isomerizations, to form all-*trans*-lycopene from 15-*cis*-phytoene [69].

The reason why plants and cyanobacteria rely on the complex poly-*cis* pathway for all-*trans*-lycopene synthesis is still elusive. It is assumed that *cis*-configured lycopene precursors are a source of signaling molecules that regulate several aspects of plant development and the carotenoid biosynthesis itself [5, 70]. The capability of CrtI in replacing four plant enzymes has made it an excellent tool for carotenoid biofortification. Thus, most of crops engineered for high carotenoid content contain bacterial *CrtI*, mainly from *Pantoea agglomerans* (formerly *Erwinia herbicola*) or *Pantoea ananatis* (formerly *Erwinia uredovora*), as a transgene.

Figure 2. General carotenoid metabolic pathway in plant. Plant enzyme names are in black letters. GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ -carotene isomerase; ZDS, ζ -carotene desaturase; CRTISO, carotenoid isomerase; LCYe, lycopene ϵ -cyclase; LCYb, lycopene β -cyclase; BCH, β -carotene hydroxylase; CYP97A, cytochrome P450-type monooxygenase 97A (carotene β -hydroxylase); CYP97C, cytochrome P450-type monooxygenase 97C (carotene ϵ -hydroxylase); ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase. NXS, neoxanthin synthase. CCS, capsanthin-capsorubin synthase; D27, iron-containing carotenoid isomerase; CCD7, carotenoid cleavage dioxygenase 7; and CCD8, carotenoid cleavage dioxygenase 8. CCD4, carotenoid cleavage dioxygenase 4; CCD2, carotenoid cleavage dioxygenase 2; NCED, 9-cis-epoxycarotenoid dioxygenase. CCS, capsanthin-capsorubin synthase; CBFD, carotenoid β -ring 4-dehydrogenase; HBFD,

4-hydroxy- β -ring 4-dehydrogenase. Bacterial enzyme names are in red letters, microalgal in green, cyanobacterial in blue. CrtB, bacterial phytoene synthase; CrtI, bacterial phytoene desaturase/isomerase; CrtW, bacterial ketolase; CrtZ, bacterial hydroxylase; BKT, ketolase from green algae; CHY, β -carotene hydroxylase from green algae; CrtO, cyanobacterial ketolase; ABA, abscisic acid; SLs, strigolactone.

Plant carotenoid biosynthesis bifurcates at the level of lycopene into two branches defined based on the type of introduced ionone ring (**Fig. 2**). The β -branch starts with the lycopene β -cyclase (LCYb) catalyzed reaction that converts the linear ends of lycopene into β -ionone rings, forming β -carotene. The first carotene in the second branch is α -carotene that carries one β - and one ϵ -ionone ring, which are formed by LCYb and lycopene ϵ -cyclase (LCYe) [71, 72]. Both cyclic carotenes are subjected to ring-specific hydroxylation reactions that convert β -carotene into zeaxanthin via the mono-hydroxylated β -cryptoxanthin, and α -carotene into lutein (**Fig. 2**). The hydroxylation of β -carotene is mainly catalyzed by ferredoxin-dependent non-heme β -ring hydroxylases (BCH1 and BCH2 in Arabidopsis), which contain an iron-coordinating histidine cluster essential for activity [73-75]. In contrast, lutein biosynthesis requires two distinct hydroxylation reactions of the β - and ϵ -rings, which are catalyzed by the cytochrome P450-type β -ring hydroxylase (CYP97A3) and ϵ -ring hydroxylase (CYP97C1) [76-78]. Zeaxanthin is the substrate for the zeaxanthin epoxidase (ZEP) that introduces an epoxy group in each of the β -ionone rings, forming violaxanthin via the monoepoxydated antheraxanthin (**Fig. 2**). The epoxidation reactions are reversible and constitute, together with the violaxanthin de-epoxidase (VDE) catalyzed de-epoxidation steps, the xanthophyll cycle that determines - depending on light intensity - the content of the photoprotective zeaxanthin [79]. The final step in the core biosynthetic pathway of plant carotenoids is the introduction of an allenic double bound in violaxanthin, which is catalyzed by a presumed neoxanthin synthase (NXS) and leads to

neoxanthin [5]. However, the identity of the neoxanthin synthesizing enzyme remains elusive, as neoxanthin-deficient mutants reported so far, i.e. the *Arabidopsis aba4* and the tomato *nxd1*, are affected in genes that are required but not sufficient for neoxanthin production [80, 81].

In addition to the above depicted common plant carotenoid pathway, there are several species-specific carotenoid biosynthesis pathways, which occur in certain organs and expand the structural diversity of carotenoids. A well-known example is the formation of the β -ring xanthophylls capsanthin and capsorubin from antheraxanthin and violaxanthin, respectively (**Fig. 2**). The enzyme, capsanthin-capsorubin synthase (CCS), catalyzing this reaction is a homolog of the lycopene cyclizing enzyme LCYb [6, 82]. Flowers of the ornamental and medicinal plant *Adonis aestivalis*, accumulate astaxanthin that is usually present in algae and some cyanobacteria. The synthesis of this ketocarotenoid (3,3'-dihydroxy- β,β -carotene-4,4'-dione) likely proceeds via 4-hydroxy- β -ionone ring intermediates and is catalyzed by the sequential action of the two enzymes carotenoid β -ring 4-dehydrogenase (CBFD) and 4-hydroxy- β -ring 4-dehydrogenase (HBFD) (**Fig. 2**) [83]. The first step of astaxanthin biosynthesis in green algae, such as *Haematococcus pluvialis* the richest natural source of this carotenoid, is catalyzed by the β -carotene ketolase (BKT) that introduces a keto group at the C4 and C4' position, forming canthaxanthin via the mono-keto carotenoid echinenone, followed by the hydroxylase (CHY) mediated hydroxylation at C3 and C3' position [84]. In marine bacteria, 4, 4' carotenoid oxygenase (CrtW) can introduce keto groups at the C4 and C4' position of the carotenoid β -ionone rings with and without hydroxylation at the 3, 3' position [85]. CrtO is a cyanobacterial ketolase which acts asymmetrically to introduce a 4-keto group in the carotenoid β -ionone rings [2].

2.3 Carotenoid storage, sequestration and further metabolism

Plants contain different types of plastids that determine the level and pattern of carotenoids in a plant cell [64, 86]. Photosynthetically active plastids, i.e. chloroplasts, have defined carotenoid composition that enables proper running of photosynthesis. In these plastids, carotenoids are composed of around 45% lutein, 25-30% β -carotene, and 10–15% each of violaxanthin and neoxanthin. Other types of plastids show large variations in the amount and pattern of accumulated carotenoids. Leucoplasts, for instance, contain only small amounts of carotenoids with significantly higher ratio of β -ionone ring containing xanthophylls [87, 88]. To confer intense colors to flowers, fruits and other organs, plants develop a specialized type of plastids, the chromoplasts, which accumulate high levels of certain carotenoids, such as lutein in daffodil flowers or lycopene in tomato fruits. Chromoplasts can originate from proplastids, chloroplasts or the starch storing amyloplasts, and are characterized by an intensive biosynthesis of carotenoids and other structural components, such as multi-layer membrane structures, lipoproteins or fibrils, which stabilize and accommodate high carotenoid concentrations [89]. These structures sequester newly synthesized carotenoids, preventing overloading the site of synthesis with end products and enabling stable storage [89]. Therefore, triggering the formation of chromoplasts may be a very fruitful approach for carotenoid-biofortification. However, our knowledge about the genetic control of chromoplast biogenesis is limited. The *ORANGE (Or)* gene is able to induce chromoplast differentiation in different plant species, including cauliflower, melon fruit and potato tuber [90-92]. The *Or* gene encodes a protein with Cys-rich zinc finger domain found in DnaJ co-chaperones and was originally discovered in a semi-dominant cauliflower mutant that accumulates β -carotene in its curds in heterozygous state without showing an increase in the transcript levels of carotenoid biosynthesis enzymes [90, 93, 94]. It is likely that *Or* acts through at least two different mechanisms to increase carotenoid content:

Wild-type *Or* isoform enhances the stability of PSY, the key enzyme in carotenogenesis, leading to an increase in its activity and, hence, in total carotenoid content, while the Arg > His isoform reduces β -carotene degradation [92, 95, 96]. *Or* plays also a crucial role in chromoplast biogenesis, as shown by genetic analysis of the *or* cauliflower and orange flesh melon mutants and by the ability of ‘Golden SNP’ containing *Or* in triggering chromoplast formation [90-92]. This role is further supported by inducing chromoplast formation in *Arabidopsis* callus and potato tubers upon ectopic expression of the *Or* gene [97, 98].

Overexpression of plant or bacterial phytoene synthase genes is able to trigger the formation of chromoplast-like structures accumulating large amounts of intra-plastidial carotenoids [88, 99, 100]. Transcriptome analyses indicate that this process closely resembles chloroplast to chromoplast transition in tomato fruits [100]. The application of Occam’s razor suggests that chromoplast differentiation triggered by *Or* and *PSY* overexpression are mechanistically related, and that they are triggered by the high carotenoid levels accumulated within the plastid.

The presence of an extended, conjugated double bond system in carotenoids makes them prone to degradation caused by oxidative cleavage. This process takes continuously place in chloroplasts, particularly under high illumination rate that increases ROS formation, leading to a wide range of oxidation products that are subjected to hydroxylation and further modified to apocarotenoid glycosides [101-103]. Some products of this oxidative degradation are perceived, with or without modification, in the cell as signaling molecules modulating gene transcription, as shown for β -cyclocitral that also acts as a root growth regulator [16, 17]. It can be assumed that carotenoid degradation is compensated by an active biosynthesis pathway that maintains the rigorous stoichiometry between chlorophylls, carotenoids and structural proteins, which is required for smooth functioning of photosynthesis and ensures sufficient supply for the synthesis

of carotenoid-derived signaling molecules and hormones. Conversion of carotenoids into apocarotenoid glycosides is also used to maintain carotenoid homeostasis in chloroplasts of *PSY* overexpressing plants [103].

Xanthophyll esterification with fatty acids is a widespread mechanism for carotenoid sequestration that allows accumulation of carotenoids and improves their stability. In tomato, map-based positional cloning identified a carotenoid-modifying gene *PYP1* (*Pale Yellow Petal 1*) that is required for violaxanthin and neoxanthin esterification in flowers. *PYP1* encodes an acyltransferase-related protein and mutations of the gene result in loss of xanthophyll esters, decreased xanthophyll accumulation and altered chromoplast development [104]. The enzymatic function of *PYP1* has not been demonstrated. However, expression of a pepper homolog of *PYP1* correlates with accumulation of esterified xanthophylls in pepper fruits [105].

In bread wheat, xanthophylls are esterified, and a major locus called *Lute*, located on the D genome, controls xanthophyll esterification. *Lute* encodes a GDSL-like lipase able to catalyze transesterification of xanthophylls. Interestingly, the protein shows an apoplastic, rather than plastid localization, prompting speculation that it acts on xanthophylls released from the plastid during seed maturation [106-108]. Very little is known about the mechanisms (if any) through which carotenoids are transported within and between cells. It is notable that some CCDs have a cytoplasmic localization, therefore it can be assumed that their carotenoid and/or apocarotenoid substrates must leave the plastid, or at least be exposed on its surface, to become accessible. Much more is known about the mechanisms mediating the inter- and intra-cellular transport of apocarotenoids. ABA transport and vacuolar sequestration is mediated by ATP-Binding Cassette (ABC) and Multidrug and Toxic compound Extrusion (MATE) transporters [109-112].

Strigolactone translocation in petunia and vacuolar sequestration of crocins in saffron are mediated by ABC transporters [113, 114].

2.4 Carotenoid cleavage

Beside non-enzymatic degradation processes [16, 115], plant carotenoids are subjected to enzymatic cleavage reactions that play a significant role in determining carotenoid content and initiate anabolic pathways forming various hormones/signaling molecules, novel pigments, and volatile/flavor compounds. Lipxygenases (LOXs), enzymes catalyzing the peroxidation of polyunsaturated fatty acids, such as linoleic, linolenic and arachidonic acid, form fatty acid peroxy radical intermediates that can attack and degrade carotenoids *in vitro* [116]. Fortification of wheat flour with lipxygenase-containing soybean flour increases protein content, improves the rheological properties of the resulting dough, and bleaches carotenoids for obtaining white bread [24, 25]. A deletion at the Lpx-B1 locus of durum wheat is associated with greater carotenoid stability during pasta making, while RNAi-mediated down-regulation of lipxygenase activity improves carotenoid stability during storage of Golden Rice [117, 118]. Recently, a comparative genomic study has provided genetic evidence for the role of tomato LoxC in producing the carotene-derived volatiles β -cyclocitral and β -ionone [119].

The enzymology and biology of CCDs have been extensively studied in the last years. These enzymes constitute a ubiquitous family present in bacteria, fungi, plants and animals [21, 23, 120]. Vertebrates contain three members of the CCD family: (i): the β -carotene 15,15'-monooxygenase (BCO1) [121, 122], which plays a key role in retinol (vitamin A) synthesis, by catalyzing symmetrical cleavage of carotenoids at the central 15,15' double bond, and (ii) the (BCO2) that catalyzes the asymmetrical cleavage of carotenoids and is thought to determine carotenoid homeostasis [121, 122], and (iii) the Retinal pigment epithelium-specific

65 kDa protein (RPE65) a retinoid isomerohydrolase required for the vision cycle [123]. Plants contain diverse types of CCDs. For instance, there are nine *CCD* genes in the model plant *Arabidopsis thaliana*. Five of them encode 9-*cis*-epoxycarotenoid dioxygenases (NCEDs: NCED2, NCED3, NCED5, NCED6 and NCED9) that form the abscisic acid precursor xanthoxin [11-13, 124]. The other four encodes enzymes designated as CCD1, CCD4, CCD7 and CCD8, which have different cleavage activities and biological functions [13, 14]. *Arabidopsis* CCD1, as well as its orthologs from other plant species, are characterized by relaxed substrate and double bond specificity. *In vitro* enzymatic studies have shown that CCD1 enzymes cleave several carotenoids and apocarotenoid substrates at different double bonds in carotenoids backbone [125-128]. This activity gives rise to many volatile compounds that contribute to the aroma and flavor of fruits and flowers, and to dialdehydes with different chain lengths [125, 126]. The role of CCD1 in the formation of carotenoid-derived volatiles has been confirmed using mutants and RNAi approaches [129-131]. CCD2, a close CCD1 relative common in *Crocus* species cleaves the (C7-C8) and (C7'-C8') double bounds, converting zeaxanthin into crocetin dialdehyde and 3-hydroxy- β -cyclocitral (**Fig. 2**) [132, 133]. Crocetin dialdehyde is the precursor of crocins and 3-hydroxy- β -cyclocitral is the indirect precursor of picrocrocin which are responsible for the color and flavor of saffron, respectively [134, 135].

CCD4 is a further CCD that contributes to the production of volatile compounds. The *Arabidopsis* CCD4 cleaves the (C9-C10) double bond in bicyclic carotenoids, yielding β -apo-10'-carotenoids and C₁₃-volatiles (**Fig. 2**) [136]. This cleavage activity leads to the conversion of carotenoids into color-less metabolites [137] and has been also reported for the potato CCD4 [138]. However, there is a second type of CCD4 enzymes, which cleaves the (C7-C8) double bond in bicyclic carotenoids, including β -carotene, β -cryptoxanthin and

zeaxanthin, as shown for Citrus CCD4b that forms the citrus pigments β -citraurin and β -citraurinene (**Fig. 2**) [137, 139-142]. Other forms of CCD4 enzymes catalyze double cleavage of carotenoids. The *Buddleja davidii* CCD4 enzymes BdCCD4.1 and BdCCD4.3 cleave zeaxanthin at the C7-C8 and C7'-C8' double bonds, producing crocetin dialdehyde and 3-hydroxy- β -cyclocitral [143]. CCD7 and CCD8, are strigolactone biosynthesis enzymes that act sequentially to form the SL precursor carlactone (**Fig. 2**) [144]. The stereospecific CCD7 cleaves 9-*cis*- β -carotene provided by the carotene isomerase DWARF27 at the (C9'-C10') double bond to produce 9-*cis*- β -apo-10'-carotenal and β -ionone [144-147]. In the next step, 9-*cis*- β -apo-10'-carotenal is converted by CCD8 into carlactone [144]. It is assumed that CCD8 catalyzes a combination of different reactions, including isomerization, intramolecular rearrangement and repeated oxygenation, when exposed to the 9-*cis*- β -apo-10'-carotenal [148]. Remarkably, CCD8 enzymes mediate a common double bond cleavage reaction with all-*trans*- β -apo-10'-carotenal, which leads to the ketone β -apo-13-carotenone [144, 149]. Conversion of carlactone by CYP450 enzymes (711A1 clade) gives rise to different SLs [150, 151].

There are CCD types other than those present in Arabidopsis. In addition to the aforementioned CCD2, Zaxinone synthase (ZAS) represents a common CCD subfamily that has been recently unraveled by analyzing CCD genes from 69 plant species [14, 15, 18]. The rice zaxinone synthase (ZAS) cleaves the apocarotenoid all-*trans*-3-OH-apo-10'-carotenal (all-*trans*-apo-10'-zeaxanthinal) at the C13-C14 double bond, forming the C₁₈-ketone all-*trans*-apo-13-zeaxanthinone that was called for simplicity zaxinone [18]. Analysis of the rice *zas* mutant and exogenous application of zaxinone suggest that this apocarotenoid is a novel growth regulator required for normal rice growth and a plant hormone candidate [18].

The negative impact of CCD activity on carotenoid content has been demonstrated for CCD4 enzymes in different tissues, including potato tubers, chrysanthemum and brassica species flowers, peach fruits, Arabidopsis seeds [152-158], and very recently in Japanese morning glory (*Ipomoea nil*) petals [159]. A recent study suggests that the Arabidopsis CCD4 is degraded by a plastoglobule-localized metallopeptidase PGM48, and that PGM48 overexpression leads to a dramatically reduced level of CCD4 protein [160]. However, it remains to be answered whether PGM48 is a positive regulator of carotenoid content. CCD1 enzymes seem to have less impact on carotenoid content [154] and are thought to cleave already destructed carotenoids, i.e. apocarotenoids, rather than carotenoids *in planta* [125, 161]. Indeed, overexpression of rice CCD1 in Golden Rice endosperm did not significantly affect carotenoid content [161]. Considering that the products of NCEDs and CCD7/CCD8 are hormone precursors occurring at low concentrations, it can be assumed that the activity of these enzymes does not play a major role in determining total carotenoid content. Nevertheless, the upregulation of *PSY3* transcript levels in response to stimuli increasing ABA level in cereal roots indicates that precursor availability is a limiting factor in ABA biosynthesis and points to NCED activity as a determinant of carotenoid content in this tissue [162, 163].

2.5 Regulation of carotenogenesis

Plant carotenoid biosynthesis is regulated at different levels, including transcriptional, post-translational and end product mediated feedback regulation [5, 89, 164]. In general, factors regulating transcript levels differ between green and non-photosynthetic tissues [165]. In photosynthetic tissues, the Arabidopsis Phytochrome Interacting Factor 1 (PIF1) and bZIP transcription factor Long Hypocotyl 5 (HY5) are perhaps the best understood transcriptional regulators of the carotenoid pathway. Both HY5 (activator) and PIFs (repressors) can directly

interact with same G-box motif located in the *PSY* promoter, which constitutes a dynamic activation-repression module that responds to temperature and light cues [166]. The Arabidopsis RAP2.2 was shown to bind the *PSY* and *PDS* promoters *in vitro* [167], however, its precise role in regulating carotenoid biosynthesis remains elusive.

Several known transcriptional regulators have been shown to control carotenoid biosynthesis in fruits, especially in the model species tomato. For example, some MADS-box proteins, such as tomato Ripening Inhibitor (RIN), AGAMOUS-LIKE1 (TAGL1), FRUITFULL1 (FUL1), and FUL2, have been shown to positively regulate the transcript levels of *PSY*, *ZDS*, *Z-ISO*, *CRTISO*, and *BCH* genes, while repressing the transcripts of lycopene cyclases, i.e *LCYb*, *CYCB* (fruit-specific *LCYb*) and *LCYe* [165]. During tomato fruit development, carotenoid biosynthesis is also regulated by factors involved in hormone biogenesis and light signaling. For example, the NAC4 transcription factor involved in the regulation of ethylene biosynthesis acts also as a positive regulator of *PSY1* and a suppressor of the expression of *LCYb*, *CYCB* and *LCYe* [168]. NAC1, a further NAC family member, showed the opposite effect [169, 170]. Several regulatory factors exert their effect by direct interaction with carotenoid gene promoters: the MADS box transcription factor RIN binds directly to the promoter of *PSY1*, *Z-ISO* and *CrtISO* of tomato, inducing the transcription of the downstream genes [171]. The tomato B-box protein BBX20 binds a G-box in the *PSY1* promoter to induce its expression [172], while the kiwifruit transcription factor AdMYB7 can directly bind to the *AdLCYb* promoter *in vitro* [173]. Overexpression of *AdMYB7* in tobacco leaves enhanced the expression of *NbPSY*, *NbPDS*, *NbZDS*, *NbLCYb*, and *NbLCYe* genes [173]. The citrus MADS6 transcription factor is a positive regulator of carotenoid biosynthesis, which directly interacts with promoter of *CsPSY*, *CsPDS* and *CsLCYb1*, thus providing a target for multigene manipulation [174]. *PSY* and *PDS*

expression has been shown to be regulated by a feedback loop mediated by end products and/or cis-intermediates of the carotenoid pathway in carrot and tomato [70, 175, 176].

Several lines of evidence suggest that the expression of carotenoid biosynthesis genes is also regulated at epigenetic level. For example, it has been shown that a histone methyltransferase SDG8 (chromatin-modifying enzyme SET DOMAIN GROUP 8) is responsible for maintaining permissive histone methylation surrounding the promoter of *CRTISO* gene, and affects the level of the corresponding transcript in Arabidopsis [177]. Accordingly, disruption the *SDG8* gene led a reduction in *CRTISO* mRNA level [177]. In addition to transcriptional regulation, post-translational control of enzyme activity and abundance is a further factor that determines carotenogenesis. As indicated above, the Or chaperone protein, the major post-translational regulator of PSY, has been shown to trigger carotenoid biosynthesis by increasing the stability of PSY [92, 95]. In a recent study, it was demonstrated that PSY protein homeostasis is maintained through the opposite effects of the Clp-protease (CLP) degrading activity and the Or protein that increases the stability of this enzyme, which represents a new post-translational regulatory mechanism of plant carotenoid biosynthesis [178].

3. Exploiting natural variation for carotenoid biofortification

Forward genetics allows the identification of novel genetic factors underlying the phenotype of interest and opens up possibilities for developing new biofortified varieties, by using modern molecular breeding tools, such as Marker Assisted Selection (MAS) and Targeting Induced Local Lesions in Genomes (TILLING). In addition, this approach provides novel targets for metabolic engineering [179-181]. There is a large natural variation in carotenoid content and composition in several crop plants, which is caused by the presence of various alleles in structural carotenoid biosynthesis genes following simple Mendelian inheritance (major quantitative trait loci (QTLs),

or by multiple minor QTLs. Maize endosperm carotenoid content is determined by major and minor QTLs. The first group maps to carotenoid biosynthesis genes *PSY1*, *LCYe*, *ZDS* and *CHY1* [182-184]. *LCYe* and *BCH* alleles causing increased β -carotene content are recessive, which can be explained by loss-of-function of the corresponding genes [182, 183]. The negative impact of *LCYe* and *CHY1* (β -carotene hydroxylase 1) on β -carotene content is consistent with their enzymatic activity: *LCYe* diverts the precursor lycopene into the competing α -branch while *CHY1* converts β -carotene into xanthophylls. The two QTLs corresponding to *LCYe* and *CHY1* were used for breeding of provitamin A biofortified maize hybrids through MAS [185]. In cassava, the allelic variation (Ala > Asp mutation) in *PSY* leads to enhanced enzymatic activity and, hence, to increased carotenoid accumulation in yellow-rooted cassava varieties with increased provitamin A content [186]. Recently, examination of two tomato *PSY* isoforms (*PSY1* and *PSY2*) identified a neighboring aromatic-aromatic combination in their core structure, which is conserved in *PSY* enzymes from different species but absent in the fruit specific and enzymatically less efficient *PSY1* [187]. Activity studies and site directed mutagenesis confirmed the role of this structural element [187]. Additional amino acid differences were found to be responsible for large differences in the activities of the daffodil and maize *PSY* enzymes, used to engineer “Golden rice” plants with different provitamin A contents [187, 188]. The identification of highly-efficient *PSY* alleles paves the way for generating them in other tissues or plant species by employing modern gene editing tools, such as CRISPR mediated targeted base editing [189].

The commonly grown orange carrots are characterized by extremely high levels of β - and α -carotene [190]. Two major recessive loci, *Y1* and *Y2*, are responsible for the color of orange carrot genotypes that are *yy y2y2* [191]. The *Y1* locus has been shown to encode a putative

repressor of photomorphogenesis. The corresponding recessive, loss-of-function allele (*y1*) is supposed to allow root carotenoid production even in the absence of light, while the identity of *Y2* is still elusive [192]. In addition, orange carrot genotypes are disrupted in the *CYP97A3* gene responsible for β -ring hydroxylation in α -carotene, which results in high accumulation of α -carotene in both leaves and roots [175]. Complementation of orange carrot with Arabidopsis *CYP97A3* gene led to a decrease of α -carotene content in leaves and roots, accompanied by reduction of root β -carotene and total carotenoid level, which confirms the contribution of *CYP97A3* to carrot color [175]. Recently, association analysis of domesticated and wild carrot accessions showed that a nonsynonymous mutation in *Or* gene is associated with carotenoid presence in domesticated carrots, indicating that the *Or* gene may play a role in the emergence of carotenoid-accumulating carrot roots [193]. Genetic variation in *ZEP* is also coupled with variation in total carotenoid content. Retrotransposon insertion in the first potato *ZEP* intron reduces *ZEP* expression and causes an enhancement in total carotenoid content [194]. Recently, it was also shown that *ZEP* is a major genetic determinant of carotenoid content and composition in Arabidopsis seeds and that mutants disrupted in *ZEP* have a 6-fold higher carotenoid content, suggesting that epoxidation of carotenoids makes them more accessible to degradation [195].

Variations in *CCD* gene loci contribute to differences in carotenoid and apocarotenoid content among varieties of the same species, as shown for petals in different *Brassica* species [156, 157]. The role of *CCD4* determining carotenoid content has been also demonstrated for Arabidopsis seeds, peach fruit flesh and potato tubers [152-154]. Consistently, CRISPR/Cas9-mediated disruption of *CCD4* resulted in a remarkable increase of carotenoids and flower color in *Ipomoea nil* [159]. In contrast to these examples, citrus *CCD4*, which forms red C_{30} apocarotenoid pigments by cleaving the C7-C8 double instead of the C9-C10 double bond targeted by

Arabidopsis CCD4 [136, 137], is a positive regulator on citrus peel color [139, 140, 142]. Very recently, mQTL analysis of C₃₀ apocarotenoid pigments showed that natural variation in CCD4b *cis*-regulatory region is a major genetic determinant of the content of β -citraurin and β -citraurinene, C₃₀-apocarotenoid pigments, in different citrus varieties [141].

The Arg > His mutation found in the melon *Or* gene, designated as “Golden” SNP has been shown to be responsible for high β -carotene level and orange phenotype of fruits [91]. Precise gene editing tools will allow the introduction of the “Golden” SNP in other crops, which may lead to a set of novel biofortified plants. Very recently, CRISPR/Cas9-mediated modification of rice *Or*, which mimics the cauliflower mutation, resulted in producing orange-colored rice callus with β -carotene hyper-accumulation [196].

In plants showing little genetic variation for β -carotene content, induced mutations can be a viable alternative to transgenic approaches. Recently, a TILLING approach has been used to knock down the expression of the two *LCYe* homeologous genes in durum wheat [197]. In spite of a slight increase of CHY expression, the levels of two of provitamin A carotenoids (β -carotene and β -cryptoxanthin) showed a slight increase in grains, while α -branch carotenoids (α -carotene and lutein) disappeared completely.

4. Metabolic engineering strategies for carotenoid biofortification in crop plants

The occurrence of accessions and varieties with diverse carotenoid content and compositions in crops, such as cauliflower, cassava, and corn, allows the employment of the biofortification concept by using conventional breeding. However, breeding is still a slow and laborious process, and not applicable if natural variation in carotenoid content is insufficient or, as it is the case with

rice endosperm, even not present. Therefore, there has been a need for alternative genetic engineering approaches for carotenoid biofortification. Indeed, metabolic engineering has been in the last decades widely and successfully employed to generate crops with increased or modified carotenoid content (**Table 1**). **Figure. 2** depicts the pathway steps that have been engineered by using plant and/or non-plant enzymes and the target crops. Metabolic engineering of carotenoids has been pursuing following strategies: a) increasing carotenoid production by overexpressing one or more biosynthetic enzymes; b) reducing further metabolization of the target carotenoid or the metabolic flux into a competing metabolic branch through gene silencing; c) increasing the “carotenoid sink” by providing additional sequestering capacities for carotenoid storage and d) enhancing the stability of formed carotenoids.

The strategy of enhancing the metabolic flux into carotenoid pathway by over-expressing genes encoding “rate-limiting” enzymes, in particular PSY, has been frequently used to produce high-carotenoid crops. Indeed, there are several examples for increasing the content of β -carotene and/or other carotenoids in various plant tissues, such as rice endosperm, tomato and banana fruits by using the “simple” approach of constitutive overexpression of a *PSY* gene from the same or different plant species [198-200]. Constitutive overexpression of Arabidopsis *PDS* gene also effectively enhanced carotenoids accumulation in tomato fruit [201]. However, constitutive overexpression of carotenogenic enzymes can cause unwanted pleiotropic developmental effects, such as dwarfism, loss of vigor and senescence [202-205]. These developmental phenotypes are likely the result of competition between metabolic routes and of alterations in the content of plant hormones, particularly gibberellins, which derive from carotenoids or their isoprenoid precursors. Negative pleiotropic effects coupled with overexpressing carotenoid biosynthesis enzymes may be avoided by employing tissue specific

promoters, e.g. fruit-, tuber- or endosperm-specific promoters. It is also recommended to use genes from a different plant species or bacterial ones to circumvent co-suppression. For instance, overexpression of the bacterial phytoene synthase *CrtB* driven by a fruit-specific promoter increased fruit total carotenoid content without significant alteration of other related isoprenoid pathways [63], in contrast to constitutive expression of the tomato fruit specific *PSY1* [199].

Carotenoid engineering can also have positive pleiotropic effects. Constitutive overexpression of *CHY* in transgenic *Arabidopsis* and tobacco results in increased the tolerance to light and/or drought stress [206, 207]. Constitutive overexpression of *LCYb* in tomato results in virtually complete lycopene conversion into β -carotene in the fruit and in higher fruit productivity per plant [208]. Fruit-specific overexpression of the same gene results in increased content of β -carotene and of its downstream metabolite, ABA, in decreased ethylene production, and in increased cuticle thickness and fruit shelf-life [209]. Shelf life extension has been also observed in cassava roots with increased carotenoid content, which showed reduced post-harvest deterioration [210].

Seed-specific overexpression of bacterial *CrtB* in Canola (*Brassica napus*) seeds, using the napin promoter, resulted in 50-fold increase of carotenoids, predominantly β - and α -carotene, which was, however, accompanied by a significant decrease in tocopherol levels and alterations in fatty acid composition [211]. Very recently, ‘Golden’ cotton seeds with around 6-fold increase in oil β -carotene content were generated by expressing a cotton phytoene synthase gene driven by a seed-specific promoter [212]. It has been also shown that elevating the plastid isoprenoid production upstream of the carotenoid pathway can be sufficient to enhance carotenoid content. For example, the overexpression of a bacterial *DXS*, a key enzyme in plastid IPP biosynthesis, results in modest increase in the carotenoid content of tomato fruits and potato tubers [213, 214].

Often, overexpression of a single biosynthetic gene is not sufficient to achieve the desired accumulation of β -carotene or increase in total carotenoid content, particularly if the target tissue is deficient in endogenous downstream enzymatic activities. The role of endogenous enzymatic activities in determining the outcome of engineering carotenoid biosynthesis is exemplified by the β -carotene (pro-vitamin A)-fortified ‘Golden Rice’ that have been generated through overexpression of a plant *PSY* and the bacterial phytoene desaturase gene *CrtI* in rice endosperm [215-217]. Endosperm-specific overexpression of *PSY* as a single gene resulted in the accumulation of phytoene as the sole carotenoid, suggesting lack of phytoene desaturase activity [200]. Combining *PSY* with *CrtI* did not result in the expected accumulation of lycopene, the enzymatic product of *CrtI*, but led to yellow grains containing β - and α -carotene, zeaxanthin and lutein [215], due to the presence of endogenous lycopene cyclase and hydroxylase activity in rice endosperm [218]. Similarly, the combined expression of the bacterial *CrtB* and *CrtI* in Canola seeds led to higher increase in carotenoid content, compared to *CrtB* alone [219]. This combination gave also rise to “Golden Wheat” [220], while the generation of “Golden Potato” required the introduction of a bacterial mini-pathway consisting of the three transgenes *CrtB*, *CrtI* and *CrtY* (bacterial *LYCB*) [221]. For the production of ketocarotenoids in canola, seven bacterial genes, including 5 *Pantoea ananatis* genes that enable zeaxanthin production from IPP/DMAPP, a *Paracoccus* *IPP/DMAPP* isomerase and a *Brevundimonas* ketolase gene, which led to up to around 1000-fold increase in β -carotene content and to the accumulation of high amounts of ketocarotenoids, particularly canthaxanthin [222]. More recently, a ketocarotenoid mini-pathway, which consists of four genes including an algal ketolase, was introduced in rice under the control of endosperm-specific promoters. The obtained grains accumulated canthaxanthin and astaxanthin and showed a striking orange-red color [223]. The above

described examples of introducing complete pathways relied on *Agrobacterium* mediated transformation of multigene constructs. Alternatively, multi-gene engineering can be achieved using biolistic combinatorial transformation of independent gene constructs. An example for this strategy is the generation of maize lines with different endosperm carotenoid compositions by combining 5 genes [224]. Polycistronic expression different genes coupled with a viral peptidase in a single transcript is a further approach for introducing complete pathways. This strategy ensures coordinated expression of the different enzymes and has been successfully used in carotenoid metabolic engineering [225-227].

Enzyme fusions are a powerful synthetic biology tool that decreases diffusion of intermediates - or losing them to a different metabolic route - and ensures simultaneous presence of the fused enzymes. This approach has been recently tested through generation of GGPS11-PSY and CrtZ-CrtW (bacterial β -carotene hydroxylase-ketolase) fusions. Transgenic *Arabidopsis* cotyledons and transgenic callus lines expressing the *GGPS11-PSY* fusion showed higher carotenoid content, compared to single *PSY*- or *GGPS11*-overexpressing lines [228]; the chimeric CrtZ-CrtW metabolon increased the efficiency of astaxanthin production *E. coli* and reduced the amounts of ketocarotenoid intermediates upon transient introduction in *Nicotiana benthamiana* [85]. A BCH-BKT (fusion of *Capsicum* β -carotene hydroxylase and *Haematococcus* β -carotene ketolase) has been successfully employed for stepwise metabolic engineering of astaxanthin in rice endosperm [229]. Most of carotenoid-biofortified crops were produced by directing the transgene(s) into the nucleus. However, plastid transformation has been used to modify the carotenoid pattern in tobacco and lettuce [230, 231], and to insert the entire cytosolic mevalonate pathway into tobacco chloroplast genome [232]. The recently reported successful usage of chitosan-complexed single-walled carbon nanotube carriers for

transgene delivery to chloroplasts is a promising approach that may make the transgenic strategy applicable to wide range of crops and facilitate carotenoid biofortification [233].

The second strategy for carotenoid bio-fortification targets precursor-metabolizing or downstream enzymatic activities, with the aim of reducing the metabolic flux into competing metabolic routes or apocarotenoid formation. This so called “block” strategy utilizes RNA interference-mediated repression- gene silencing- and will benefit from CRISPR gene editing tools to reduce the activity of/disrupt genes that negatively impacts the accumulation of desired carotenoid(s). Gene silencing has been successfully used to increase the carotenoid content in different plant tissues, including petals, tubers and fruits [153, 155, 234-236]. For instance, *ZEP*, *LCYe* and β -Carotene hydroxylase (*CHY* or *BCH*) have been shown to be negative regulators of total carotenoids accumulation in different plant tissues and species [175, 183, 195, 237]. Indeed, silencing of *ZEP* and *LCYe* in potato tubers resulted in significant increase in β -carotene content [238, 239]. A further example is the generation of orange fruits biofortified with β -carotene through silencing of *BCH* [240]. The combination of the “block strategy”, i.e. endosperm-specific silencing of the *BCH* gene, and the aforementioned “push strategy”, i.e. increasing precursor flux by overexpression of *CrtB*, led to up to 31-fold enhancement of levels of β -carotene levels in wheat [241]. A similar approach by silencing *LCYe* and overexpressing *PSY*, *CHY* and *ketolase* was followed to produce astaxanthin in maize endosperm [242].

The third strategy for enhancing carotenoid production is to increase the storage capacity, i.e. the “carotenoid sink”, of the target tissue, [44, 243]. One of the most promising “hypothetical” approaches to increase the carotenoid sink is to trigger the differentiation of chromoplasts in the tissue of interest [244]. The discovery of the *Or* gene has made this approach in several cases feasible and opened up the possibility of generating chromoplasts from other plastid types (see

above; [89, 90, 92, 98, 243, 245]). Ectopic expression of the Arabidopsis *Or* in tomato increases also the size of plastids, exerting an additional positive effect on carotenoid accumulation [246]. Size and number of plastids, and, hence, fruit lycopene content, are also increased in the ABA-deficient *high pigment 3* mutant and upon fruit-specific, RNAi-mediated suppressing of the photomorphogenesis regulatory gene, *DET1* [247-249].

The fourth strategy for carotenoid biofortification aims at increasing the post-harvest stability of carotenoids. Genetic evidence suggests a role of lipoxygenases in degrading wheat carotenoids during storage [118]. Indeed, down-regulation of lipoxygenase activity in Golden Rice increased postharvest carotenoid stability [117]. In potato, it has been shown that the transformation of the cauliflower *Or* gene also stimulates carotenoid accumulation during cold storage, providing a further evidence for the importance of this gene in determining carotenoid content [244]. Esterification of carotenoids can promote their sequestration and improve their storage stability in wheat and might also reduce carotenoid degradation in citrus fruits [250-253]. Post-harvest carotenoid stability can be also improved by enhancing the antioxidant capacity of the target tissue. For instance, increasing vitamin E content in transgenic sorghum by co-expressing *homogentisate geranylgeranyl transferase (HGGT)* with carotenoid biosynthesis genes, resulted in increased β -carotene accumulation and storage stability [254]. The simultaneous introduction of vitamin E and β -carotene biosynthetic genes is a promising strategy for improving the nutritional value of other carotenoid-biofortified cereals.

5. Are carotenoids from biofortified crops useful?

Apart from being present in significant concentrations, carotenoids accumulated in biofortified crops need to be released, absorbed and metabolized efficiently to fulfill their function as antioxidants or provitamin A. This complex process is defined by different terms.

Bioaccessibility refers to the portion of carotenoid that can be released from the food matrix and become accessible for the digestive apparatus and is usually measured *in vitro* using simulated digestion systems or Caco-2 cells [255, 256]. Bioavailability defines the portion of diet carotenoids, which is taken up and becomes available for utilization in normal physiological functions, metabolism, and storage and is usually determined by using human volunteers or animal models such as mice, chickens, or Mongolian gerbils. In general, the bioavailability of β -carotene from plant sources is low (with a maximum of 65%), due to the presence of cell walls, fibers and carotene-protein complexes, which hamper digestion and degradation [255], and is increased by cooking and consuming fat-rich food matrix [256]. The terms bioconversion and bioefficacy are more specific to provitamin A carotenoids [256]. Bioconversion (bioactivity) refers to the fraction of the absorbed provitamin A carotenoids that is metabolized into vitamin A (retinol), while bioefficacy defines the efficiency of the absorption of provitamin A carotenoids and their conversion into retinol, and is expressed as retinol equivalents (RE). One RE is defined as the biological activity associated with 1 μ g of all-*trans* retinol ([255]; <https://www.nrv.gov.au/nutrients/vitamin-a>).

Retinol equivalency as well as bioavailability of β -carotene are affected by several diet-related factors, e.g. fat, fiber, total carotenoid and β -carotene content, as well as by genetic polymorphisms related to β -carotene metabolism, vitamin A status and other features of the target population [257]. Retinol equivalency of β -carotene from plant sources shows large variation, ranging from 3.8:1 to 28:1 [257]. The term Recommended Daily Allowance (RDA) defines the average daily level of intake that is sufficient to meet the nutrient requirements of nearly all (97%-98%) healthy people (NIH; https://ods.od.nih.gov/Health_Information/Dietary_Reference_Intakes.aspx). None of the five

major staple crops (wheat, corn, rice, potato, cassava) can provide 25% of the RDA, which is sufficient to prevent vitamin A deficiency [44]. There are several studies on the bioavailability, bioaccessibility and retinol equivalency of carotenoids from biofortified crops (reviewed in: [44, 256-258]. The bioaccessibility of β -carotene from biofortified cassava, melon, sorghum and potato is comparable with that from the corresponding original varieties [44]. Studies on the bioavailability of β -carotene from biofortified crops showed RE ratios of 3:1 (transgenic maize), 3.5-6.5:1 (sweet potato), 3.7:1 (cassava), 3.8-6.5:1 (non-transgenic, biofortified maize), 4.2:1 (non-transgenic biofortified cassava) and 2.3:1 (Golden Rice) which are significantly lower than those of vegetables [257]. Several *in vivo* studies demonstrated that consuming provitamin A carotenoids (e.g. β -carotene) from biofortified crops help increases vitamin A intake and status. For example, consumption of biofortified orange sweet potato improved total-body vitamin A stores across different age groups [255, 259, 260]. Similarly, intake of biofortified orange maize resulted in a significant improvement in vitamin A status, indicated by an increase in pupillary responsiveness, of marginally vitamin A deficient children [255, 261]. Finally, Golden Rice has the potential to fulfill up to 89–113% and 57–99% of the estimated average requirement for vitamin A for preschool children in Bangladesh and the Philippines, respectively [258]. Fortunately, Bangladesh, a country where vitamin A deficiency affects about 21% of children, is expected to approve Golden Rice for planting soon [223].

Golden Rice has been already documented as safe for human consumption in several countries, including United States, Australia, Canada and New Zealand [224]. Very recently, Golden Rice has been also approved “as safe as conventional rice” by the Department of Agriculture in the Philippines, which paves the way for its employment in combating VAD (<https://www.newscientist.com/article/2228793-gm-golden-rice-gets-landmark-safety-approval-i>

n-the-philippines/).

Biofortified crops have been also shown to an efficient source of carotenoids for animals. For example, feeding poultry with carotenoid biofortified maize enhanced the accumulation of carotenoids in muscles, skin and egg yolk, and increased liver vitamin A content under lab and farm conditions [262]. In addition, carotenoids from biofortified maize has been shown to protect broilers against coccidiosis without the need for artificial carotenoid additives [263]. Astaxanthin is the most expensive fish food ingredient added to obtain a desirable pink flesh color of salmon and trout. Biological sources of this ketocarotenoid are not sufficient to meet the global astaxanthin demand [262]. Recently, astaxanthin-biofortified transgenic maize and tomato fruit have been shown to be more efficient in conferring the desired color to egg yolk and salmon flesh, respectively, compared to synthetic astaxanthin additives [41, 263]

6. Future Perspectives

Conventional breeding is a classical approach that has been used to increase carotenoid content in crops, such as maize and carrot (**Fig. 3**). However, classical breeding is a relatively slow and laborious process and can be deployed only in species in which natural variation in carotenoid content is high. This is true for some staple crops, such as maize, sweet potato and cassava, while in the case of rice, wheat, and potato, the use of different approaches to increase genetic variation, such as radiation or chemical mutagenesis, gene editing, cis- and/or transgenesis, is essential [44]. In the last decades, genetic engineering of carotenoid biosynthesis has relied mainly on transgenesis, and has generated several carotenoid biofortified crops that hold promise for combating vitamin A deficiency and increasing the nutritional value of plant-derived diets for a better health (**Table 1 and Fig. 3**). However, transgenic crops have to achieve a broad public acceptance in some areas of the world, and to fulfill stringent legal and regulatory requirements,

which make their deployment costly and time-consuming. Gene editing tools, such as the CRISPR/Cas system, allow the production of biofortified, non-transgenic crops that do not contain foreign genes [264-269]. Such an approach may find a better public acceptance and accelerate the regulatory approval process, thus bringing biotech crops faster to market [205, 268]. There are already examples for employing the CRISPR/Cas9 technology to alter carotenoid content in flower, callus and fruit [159, 196, 270]. Obvious targets for gene editing are the *BCH*, *LCYe* and *ZEP* genes that have a negative impact on β -carotene content. In addition, the development of CRISPR mediated targeted 'base editing' tools [189], allows precise manipulation of structural or regulatory genes, such as the introduction of the 'Golden SNP' into the *Or* gene [196]. Gene editing may also accelerate the introduction of entire metabolic pathways and stacking of multiple genes [271], which is required to produce carotenoids in tissues such as rice endosperm. The combination of transgenic approaches with conventional breeding (crossing or introgression) is a further direction for integrated carotenoid biotechnology, which has been recently used in carotenoid biofortification in important crops such as tomato and rice [229, 272]. In countries where gene editing is regulated like transgenesis, more imprecise and laborious techniques like TILLING may find application.

Areas requiring further research are genetic variation of carotenoid content in crop plants, the mechanisms regulating the pathway, including those triggered by end products and intermediates, and the mechanisms governing carotenoid stability and sequestration. Advancement of knowledge in these key areas will not only accelerate modern breeding, but also provide new engineering targets for carotenoid biofortification in crop plants. The combination of genomics-assisted breeding and knowledge-based transgenic and/or gene editing approaches holds promise for the successful biofortification of further crop plants, thus reducing the global

burden of vitamin A deficiency.

Figure 3. Summary of representative Carotenoids biofortified crop plants developed by transgenic and breeding approaches. Phytoene biofortified crop plants: Tomato, rice and cassava; Lycopene biofortified crop: Tomato; β-carotene biofortified crops: Tomato, rice, canola (*Brassica napus*), potato, wheat, banana, flaxseed (*Linum usitatissimum*), pepper, orange, cotton,

maize, sorghum, cassava, cauliflower and melon. Zeaxanthin biofortified crops; Tomato, maize, potato and rice. Capsanthin biofortified crop: Rice; Astaxanthin biofortified crops; Potato, carrot, maize, rice, soybean, tobacco, canola and lettuce. The detail of metabolic engineering of plant carotenoids is shown in Table 1.

Table 1. Metabolic engineering for carotenoids biofortification in crop plants.

Plant species	Strategy	Phenotypic changes in carotenoids	Reference(s)
Tomato	Nuclear transformation		
	<i>DXS</i> overexpression	Enhanced fruit carotenoids	[214]
	<i>Or</i> Overexpression	Increase of total carotenoids	[246]
	<i>FIBRILLIN</i> overexpression	Enhanced carotenoids, carotenoid-derived volatiles	[273]
	<i>PSY1</i> overexpression	Enhanced phytoene, β -carotene	[199]
	<i>PDS</i> overexpression	Enhancement of lycopene, β -carotene	[201]
	<i>BKT</i> , <i>CHY</i> overexpression	Astaxanthin accumulation in leaves, esterified astaxanthin accumulation in fruits	[274]
	<i>CRY2</i> overexpression	Enhanced carotenoids	[275]
	<i>DET1</i> , <i>DDB1</i> , <i>COP1-like</i> silencing	Enhanced carotenoids	[247, 248, 276]
	<i>CrtB</i> overexpression	Enhanced lycopene in fruit	[226]
	<i>CYCb</i> , <i>LCYb</i> silencing	Enhanced lycopene in fruit	[71, 237]
	<i>CrtI</i> overexpression	β -carotene accumulation in fruit	[277]
	<i>LCYb</i> and <i>CHY</i> overexpression	β -carotene, zeaxanthin accumulation	[237, 278, 279]
	<i>CrtB</i> , <i>CrtE</i> and <i>CrtI</i> overexpression	Accumulation of different carotenoids in different sub-chromoplast compartments	[280]
	RNAi of <i>SGR1</i> , CRISPR mediated gene knock-out of <i>SGR1</i>	Enhancement of lycopene, β -carotene	[270, 281]
	<i>CrtZ</i> , <i>W</i> polyprotein overexpression	Ketocarotenoid accumulation in fruit and leaves	[41, 226]
	<i>CrtZ</i> and <i>W</i> overexpression	Overproduction of lycopene, accumulation of astaxanthin	[282]
	<i>BZR1-1D</i> overexpression	Enhancement of carotenoid accumulation	[283]
	<i>MADS6</i> overexpression	Enhancement of carotenoid accumulation	[174]
	<i>BBX20</i> overexpression	Enhancement of carotenoid accumulation	[172]
Rice	<i>PSY</i> , <i>CrtI</i> overexpression	β -carotene, zeaxanthin accumulation in endosperm	[188, 200, 215, 227]
	Combinatorial overexpression of <i>Psy1</i> , <i>DXS</i> , <i>CrtI</i> , <i>OR</i> , <i>BKT</i>	Accumulation of different carotenoids in endosperm	[284]
	Combinatorial Overexpression of <i>Psy1</i> , <i>CrtI</i> , <i>BKT</i>	Ketocarotenoid accumulation in endosperm	[285]
	<i>PSY1</i> , <i>CrtI</i> , <i>BKT</i> and <i>BCH</i> overexpression	Ketocarotenoid accumulation in endosperm	[223]
	<i>PSY-CrtI</i> polyprotein and <i>BCH-BKT</i> polyprotein overexpression	Accumulation of zeaxanthin, astaxanthin and capsanthin in endosperm	[229]
Potato	<i>CrtO</i> overexpression	Ketocarotenoid accumulation in leaves, tubers	[286, 287]
	<i>CrtW</i> , <i>CrtZ</i> <i>Or</i> overexpression	Astaxanthin accumulation in tubers	[288]
	<i>CrtB</i> , <i>I</i> , <i>Y</i> overexpression	α -, β -carotene accumulation in tubers	[221, 289, 290]
	<i>DXS</i> overexpression	Enhanced total carotenoids	[213]
	<i>ZEP</i> silencing	Zeaxanthin accumulation in tubers	[238]
	<i>LCYe</i> silencing	Enhanced β -carotene, total carotenoids in tubers	[239]
	<i>CHY</i> silencing	Enhanced β -carotene in tubers	[291, 292]
	<i>Or</i> overexpression	β -carotene, total carotenoid accumulation in tubers	[90, 293]

	RNAi of <i>CCD4</i>	Increased carotenoid content in tubers and flowers	[153]
Maize	Combinatorial overexpression of <i>Psy1</i> , <i>CrtI</i> , <i>Chy</i> , <i>LCYb</i> , <i>CrtW</i>	Increase of total carotenoids	[224]
	Combined overexpression of <i>Psy1</i> , <i>CrtI</i> , <i>Dhar</i> , <i>FolE</i>	Increase of β -carotene content	[294]
	<i>Psy1</i> , <i>CrtZ</i> , <i>Bkt</i> overexpression, <i>LCYe</i> silencing	Accumulation of astaxanthin, ketozeaxanthin	[295]
			[242]
Wheat	<i>CrtB</i> , <i>CrtI</i> overexpression	Increase of carotenoid and β -carotene in endosperm	[220]
	<i>CrtB</i> overexpression, <i>CHY</i> silencing	Enhanced β -carotene accumulation in endosperm	[241]
Banana	<i>PSY</i> overexpression	Enhanced β -carotene accumulation in fruit	[198]
Sorghum	<i>Psy1</i> , <i>CrtI</i> , <i>HGGT</i> overexpression	β -carotene, vitamin E accumulation in endosperm	[254, 296]
Soybean	<i>Psy1-CrtI</i> polypeptide overexpression	β -carotene accumulation in seed	[297]
	<i>CrtB</i> , <i>CrtW</i> , <i>BKT</i> overexpression	Ketocarotenoid accumulation in seed	[298]
Cotton	<i>Psy</i> overexpression in seed	Enhanced β -carotene accumulation in seed	[212]
Brassica napus	<i>CrtB</i> , <i>I</i> , <i>Y</i> overexpression	α -, β -carotene accumulation in seed	[211, 219]
	<i>IDI</i> , <i>CrtE</i> , <i>CrtB</i> , <i>CrtI</i> , <i>CrtY</i> , <i>CrtW</i> , <i>CrtZ</i> overexpression	β -carotene, ketocarotenoid accumulation in seed	[222]
Carrot	<i>CrtO</i> overexpression	Ketocarotenoid accumulation in leaves and roots	[299]
Cassava	<i>CrtB</i> overexpression in root	Accumulation of phytoene, phytofluene, β -carotene in root.	[186]
<i>Lilium</i>	<i>IDI</i> , <i>CrtE</i> , <i>CrtB</i> , <i>CrtI</i> , <i>CrtY</i> , <i>CrtW</i> , <i>CrtZ</i> overexpression	Ketocarotenoid accumulation in leaves.	[300]
<i>Lotus japonicus</i>	<i>CrtW</i> overexpression	Ketocarotenoid accumulation in flower	[301]
Orange	<i>CHY</i> silencing	β -carotene accumulation in fruit pulp	[240]
Sweet potato	<i>Or</i> overexpression	Increase in total carotenoids in root	[302]
Nicotiana plant	<i>CrtZ</i> , <i>W</i> polypeptide overexpression	Ketocarotenoid accumulation in leaves and nectary (flower)	[226]
	<i>CrtZ</i> overexpression	Enhanced zeaxanthin biosynthesis under high light, enhanced UV tolerance	[303]
	<i>CrtO</i> overexpression	Ketocarotenoid accumulation in leaves, nectary, petals	[304-306]
	<i>CrtZ</i> and <i>W</i> overexpression		[307]
Tomato fruit	Plastid transformation		
	<i>LCYb</i> , <i>CrtY</i> overexpression	β -carotene accumulation, increase of total carotenoid	[308, 309]

Lettuce leaves	<i>CrtZ</i> , <i>CrtW</i> , overexpression	<i>IDI</i>	Astaxanthin ester accumulation	[231]
Tobacco leaves	<i>CrtZ</i> , <i>CrtW</i> overexpression	Astaxanthin, accumulation	4-ketoantheraxanthin	[230, 310]

Declaration of Competing Interest

The Authors declare no conflict of interest related to this work.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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