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Open avenues for carotenoid biofortification of plant tissues

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ABSTRACT

Plant carotenoids are plastidial isoprenoids that function as photoprotectants, pigments, and precursors of apocarotenoids such as the hormones abscisic acid and strigolactones. Humans do not produce carotenoids but need to obtain them from their diet as precursors of retinoids, including vitamin A. Carotenoids also provide numerous other health benefits. Multiple attempts to improve the carotenoid profile of different crops have been carried out by manipulating carotenoid biosynthesis, degradation, and/or storage. Here, we will focus on open questions and emerging subjects related to the use of biotechnology for carotenoid biofortification. After impressive achievements, new efforts should be directed to extend the use of genome-editing technologies to overcome regulatory constraints and improve consumer acceptance of the carotenoid-enriched products. Another challenge is to prevent off-target effects like those resulting from altered hormone levels and metabolic homeostasis. Research on biofortification of green tissues should also look for new ways to deal with the negative impact that altered carotenoid contents may have on photosynthesis. Once a carotenoid-enriched product has been obtained, additional effort should be devoted to confirming that carotenoid intake from the engineered food is also improved. This work involves ensuring post-harvest stability and assessing bioaccessibility of the biofortified product to confirm that release of carotenoids from the food matrix has not been negatively affected. Successfully addressing these challenges will ensure new milestones in carotenoid biotechnology and biofortification.

Key words: biofortification, biotechnology, carotenoids, plastidial isoprenoids, vitamin A

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INTRODUCTION

Carotenoids are lipophilic isoprenoids synthesized by all photosynthetic organisms (including plants, algae, and cyanobacteria) and by some non-photosynthetic life forms such as prokaryotes and fungi. In photosynthetic systems, carotenoids participate in light harvesting and are essential for photoprotection, whereas in non-photosynthetic tissues and organisms, they play a role as pigments that provide yellow to red colors (Lichtenthaler, 2012; Rodriguez-Concepcion et al., 2018). Plant carotenoids are responsible for the autumn colors of some leaves (in which their presence becomes obvious when chlorophyll is degraded) and for the pigmentation of many fruits and flowers. Carotenoids provide the orange color of oranges and pumpkins, the red color of tomatoes and watermelon, and the yellow color of corn kernels and daffodil petals. Although they are present at very low levels in the roots of most plants, carotenoids are responsible for the orange color of carrots (from which they take their name). Cleavage of carotenoids produces compounds with biological activity such as the hormones abscisic acid (ABA) and strigolactones (SLs) (Lichtenthaler, 2012; Ruiz-Sola and Rodriguez-Concepcion, 2012; Rodriguez-Concepcion et al., 2018). With only a few exceptions (Moran and Jarvik, 2010; Cruz et al., 2013; Rodriguez-Concepcion et al., 2018), animals cannot synthesize their own carotenoids but instead obtain them from their diet.

Like all isoprenoids, carotenoids are synthesized from the universal C5 isoprenoid precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In plants, these compounds derive from two independent pathways: the mevalonate pathway in the cytosol and the methylerythritol 4-phosphate pathway in the plastids (Rodríguez-Concepción and

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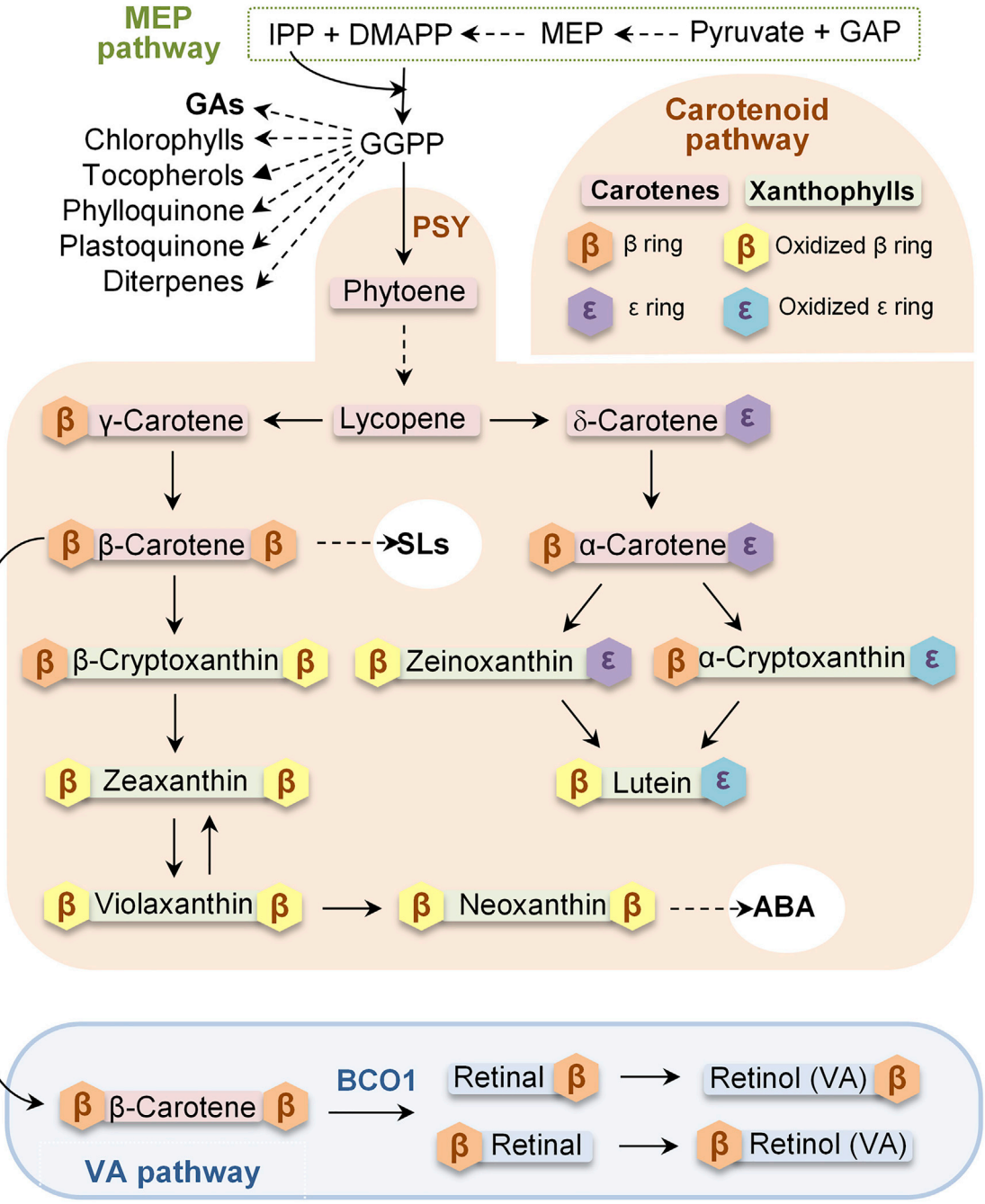

Figure 1. Carotenoid biosynthesis and related pathways.
Dashed arrows represent multiple steps. The plastidial methylerythritol 4-phosphate (MEP) pathway provides the metabolic precursors for carotenoids and other isoprenoids in plant cells. The first committed step of the carotenoid pathway is catalyzed by phytoene synthase (PSY). Carotenoid cleavage in plants produces apocarotenoids such as the hormones strigolactones (SLs) and abscisic acid (ABA). In animal cells (in blue), cleavage of carotenes with an unmodified β ring by the enzyme β-carotene 15,15′–monooxygenase 1 (BCO1) leads to production of vitamin A (VA). The main pro-vitamin A carotenoid is β-carotene, as it is the only one with two β rings, and one β-carotene molecule therefore produces two vitamin A molecules. GAP, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GAs, gibberellins.

Boronat, 2002). In plants, carotenoids are formed from methylerythritol 4-phosphate-derived IPP and DMAPP in plastids (Figure 1). Condensation of three IPP and one DMAPP molecule forms C20 geranylgeranyl diphosphate, the direct precursor for carotenoids and other plastidial isoprenoids such as diterpenes, gibberellins (GAs), chlorophylls, tocopherols, plastoquinones, and phylloquinones (Figure 1). The first committed step of the carotenoid pathway is the formation of C40 phytoene from two molecules of geranylgeranyl diphosphate catalyzed by the enzyme phytoene synthase (PSY). Colorless phytoene is then converted through sequential desaturation and isomerization reactions to lycopene, a red pigment. Cyclization of the two

ends of the linear lycopene molecule is the first branching step in the pathway (Figure 1). Introduction of two β rings generates β-carotene, whereas addition of one ε and one β ring leads to formation of α-carotene. The presence of two ε rings is very rare in plant carotenoids, although there are some crop examples, such as lactucaxanthin in lettuce (Britton, 1995; Phillip and Young, 1995). Oxidation of the rings by hydroxylation and epoxidation reactions generates oxygenated carotenoids, better known as xanthophylls (Figure 1). In the β,β branch, hydroxylation of β-carotene produces zeaxanthin, which is subsequently transformed into violaxanthin and neoxanthin, whereas the ε,β branch eventually produces lutein. Carotenoids are the precursors of plant hormones such as ABA and SLs as well as other products collectively known as apocarotenoids, which have roles in plant developmental regulation and environmental interactions (Rodriguez-Concepcion et al., 2018; Moreno et al., 2021).

Accumulation of carotenoids in plastids depends on their biosynthesis and degradation rates but also on the availability of suitable structures for their storage and sequestration (Sun et al., 2018; Sadali et al., 2019; Torres-Montilla and Rodriguez-Concepcion, 2021; Morelli et al., 2022). The two plastid types that accumulate the highest levels of carotenoids are chloroplasts and chromoplasts. The carotenoid composition of chloroplasts is similar in most plant species, and the most abundant carotenoids are lutein (45% of the total), β-carotene (25%–30%), violaxanthin (10%–15%), and neoxanthin (10%–15%) (Esteban et al., 2015). Most of these carotenoids are associated with proteins in different complexes of the photosynthetic apparatus located in the thylakoid membranes. Light-harvesting complexes contain mostly xanthophylls, whereas β-carotene is associated with both photosystems (PSI and PSII) and the cytochrome b6f complex. Carotenoids are also found at lower levels in the envelope membranes (Pascal et al., 2005; Lichtenthaler, 2012; Domonkos et al., 2013). Unlike chloroplasts, chromoplasts are very diverse in carotenoid composition and ultrastructure, and their only common feature is their specialization for the production and storage of carotenoids. Chromoplasts are present in non-photosynthetic tissues such as carrot roots, marigold flowers, and tomato fruit, where they differentiate from different types of plastids, including leucoplasts, amyloplasts, and, most frequently, chloroplasts (Sun et al., 2018; Sadali et al., 2019). Recent results have demonstrated that any plastid type can be converted into a chromoplast provided that (i) photosynthesis is absent or reduced below a certain threshold and (ii) carotenoid biosynthesis is activated (Llorente et al., 2020; Torres-Montilla and Rodriguez-Concepcion, 2021). Although there is not a genetically determined developmental program per se involved in chromoplast differentiation, formation of chromoplasts is linked to the development of internal structures that can sequester and store carotenoids. These subplastidial structures serve to classify chromoplasts as crystalline, globular, tubular, or membranous, although more than one of these pigment-bearing structures can be present in the same chromoplast and different types of chromoplasts can co-exist in the same plant species (Sun et al., 2018; Sadali et al., 2019). Crystalline bodies are observed, for example, in tomato chromoplasts, which accumulate lycopene crystals. However, tomato chromoplasts also accumulate β-carotene in plastoglobules, which are structures typical of globular chromoplasts like those present in mango and citrus fruits, whereas carrot chromoplasts accumulate β-carotene in crystalline bodies. Our current understanding is that the diversity of storage structures in chromoplasts is determined by the carotenoid profiles (type and concentration) of different tissues and plant species.

CAROTENOIDS AND HEALTH

One of the main roles of carotenoids in plants is protection against photooxidative damage. Carotenoids dissipate excess light energy as heat in a process called non-photochemical quenching, but they are also powerful antioxidants that promote free radical detoxification by quenching excited triplet chlorophyll and singlet oxygen (Domonkos et al., 2013). Oxidative stress also occurs in humans, triggering cellular and tissue-wide damage and activating transcription factors that can affect the expression of hundreds of genes related to growth factors, production of molecules involved in cell-cycle regulation, or inflammatory cytokines, eventually resulting in chronic inflammation and the development of several diseases such as cancer, diabetes, and cardiovascular pathologies (Meléndez-Martínez et al., 2021). Some of the health benefits of a carotenoid-rich diet have been associated with the antioxidant properties of carotenoids, mainly related to the presence of conjugated double bonds in their polyene chain (Müller et al., 2011; Niranjana et al., 2015). However, the main contribution of carotenoids to human health is their role as precursors of retinoids, including vitamin A (VA) (Eggersdorfer and Wyss, 2018; Rodriguez-Concepcion et al., 2018). VA deficiency is a major health problem in many developing countries that mostly affects preschool children and leads to blindness, growth problems, and even death. The major sources of VA in the human diet are animal products such as dairy, liver, and fish, which contain preformed VA, also called retinol (Figure 1). Instead of VA, plant products contain pro-VA carotenoids (PACs), which are those with unsubstituted β rings. The only carotenoid with two unsubstituted β rings, and hence the main PAC, is β-carotene. Other PACs are β-cryptoxanthin, α-carotene, and additional carotenoids that are rarely present in human diets, such as α-cryptoxanthin and γ-carotene (Figure 1) (Sommer, 2008; Zheng et al., 2020). Although excess VA in the diet can lead to hypervitaminosis, PAC conversion into VA is metabolically regulated, and excess PAC is excreted without posing any health issue. The first step in VA synthesis is the conversion of PAC into retinal catalyzed by β-carotene 15,15′–monooxygenase 1, an oxygenase that cleaves PAC at the central double bond to yield one or, in the case of β-carotene, two molecules of retinal (Figure 1). Retinal can then be oxidized irreversibly into retinoic acid or reduced reversibly into retinol (VA). Retinal-based proteins, including mammalian rhodopsin, are light-sensitive chromoproteins present in photoreceptors and spread across diverse organisms, including archaea, eubacteria, fungi, algae, and animals. Retinoic acid acts as a major regulator of gene expression by interacting with a nuclear receptor superfamily of proteins (Rodriguez-Concepcion et al., 2018; Meléndez-Martínez, 2019).

Carotenoids other than retinoid precursors are also important for human vision. Lutein and zeaxanthin are found in the human retina, where they are concentrated in the macula. Macular

pigments absorb high-energy light and protect the retina from photochemical injury by locally neutralizing reactive oxygen species and protecting against UV-induced peroxidation. Age-related macular degeneration, one of the most common types of vision loss among elderly people, can actually be delayed by supplementing the diet with lutein and zeaxanthin (Ma and Lin, 2010; Eggersdorfer and Wyss, 2018). Apart from their role in vision, lutein and other carotenoids have been reported to affect cognitive function by eliminating free radicals, which are considered a modifiable risk factor for cognitive decline leading to problems such as dementia or Alzheimer's disease. Carotenoids delay the progression of neurodegenerative diseases through several pathways, for example by suppressing proinflammatory cytokines, triggering A-peptide production, and reducing oxidative stress. Because they bind to Alzheimer's disease-associated receptors, carotenoids such as β-carotene also have the potential to act as Alzheimer's disease antagonists. Carotenoids have also been demonstrated to control the inflammation, dyslipidaemia, and thrombosis implicated in the development of cardiovascular diseases. The capacity of these compounds to reduce oxidative stress and control lipid and energy metabolism has been reported to provide benefits for other pathologies such as UV-related skin damage, osteoporosis, diabetes, obesity, and some forms of cancer (Eggersdorfer and Wyss, 2018; Rodriguez-Concepcion et al., 2018; Meléndez-Martínez, 2019). In many cases, however, it remains unknown whether the reported health benefits are caused by native carotenoids or by products derived from their metabolism within the human body.

CAROTENOID BIOFORTIFICATION OF CROPS

Micronutrient (including VA) deficiency, also known as hidden hunger, is a major problem in countries of sub-Saharan Africa, the Caribbean, and East, Southeast, and Western Asia. Food supplementation and fortification strategies (i.e., the incorporation of required micronutrients as dietary supplements or as ingredients added to a processed food product, respectively) have been implemented, but they remain unaffordable for rural communities (Fitzpatrick et al., 2012). A possible solution is crop biofortification, i.e., the development of plant varieties enriched in micronutrients. Approaches for biofortification include agronomic management, conventional breeding, and recombinant DNA technology (biotechnology). Agronomic approaches aim at improving micronutrient contents in plant tissues by altering plant growth conditions (light, temperature, salinity) or using treatments such as fertilizers or foliar sprays. Because light is a major regulator of carotenoid production and storage (Llorente et al., 2017), treatments with different light quantities and qualities (including UV) can substantially change the carotenoid composition of plant products. Conventional breeding involves finding accessions that contain a naturally high concentration of target micronutrients and crossing them over time with elite cultivars until the desired concentrations are reached. The occurrence of accessions and varieties with diverse carotenoid contents and compositions in crops such as cauliflower, cassava, and corn makes conventional breeding a valid strategy for biofortification (Giuliano, 2017; Siwela et al., 2020). However, breeding is still a slow and labor-intensive process that is not applicable if natural variation in carotenoid content is insufficient or simply unavailable. Lastly, biotechnology enables modification of target micronutrient amounts by insertion, removal, activation, inactivation, or editing of relevant genes (Zhu et al., 2019). Biotechnology enables us to (1) "push" the metabolic flux into and through a target pathway by overexpressing rate-limiting enzymes and/or eliminating metabolic bottlenecks, (2) "block" the accumulation of particular metabolites by suppressing downstream, side, competing, and degradation pathways, or (3) "pull" metabolites into sequestering structures that improve their storage and increase the sink capacity of the tissue (Figure 2). For rational biotechnological designs, however, it is imperative to achieve a good understanding of how plant cells make, degrade, and store the target metabolites in different tissues and the regulatory networks that control their accumulation. In the case of carotenoids, our understanding of these topics is far from complete, but the available information and molecular tools have enabled a large number of "push," "block," and "pull" approaches (either individually or in combination) for highly refined metabolic engineering of carotenoids and biofortification of multiple plant-derived foods. This subject has already been extensively covered by a number of reviews (Botella-Pavía and Rodríguez-Concepción, 2006; DellaPenna and Pogson, 2006; Yuan et al., 2015; Giuliano, 2017; Rodriguez-Concepcion et al., 2018; Feder et al., 2019; Watkins and Pogson, 2020; Zheng et al., 2020). Here, we will focus on providing forward-looking perspectives on open questions and emerging subjects related to carotenoid biofortification (Figure 2).

GMOs AND GENOME EDITING

Carotenoid biotechnology has delivered astonishing examples of biofortification, including the widely known Golden Rice (Ye et al., 2000; Paine et al., 2005). Golden Rice accumulates β-carotene in the seed endosperm, thereby contributing to fighting VA deficiency in developing countries where rice is a staple crop. Despite continuous development to further increase carotenoid content and introgress this trait into local rice varieties (Welsch and Li, 2022), the products are still genetically modified organisms (GMOs) that must overcome multiple constraints associated with long and costly regulatory processes and low public acceptance. Indeed, it took more than two decades for Golden Rice to be recently approved for planting in the Philippines (Welsch and Li, 2022). CRISPR–Cas-mediated genome editing may be a solution to this problem. Although decisions on its regulatory status differ among countries even within the same region, many countries are exempting the products of genome editing from existing (and highly restrictive) GMO regulation, and others are debating this issue (Sprink et al., 2022). The capacity of CRISPR–Cas to introduce targeted and precise nucleotide changes into plant genomes has been pivotal for the development of agricultural traits of interest, including enhanced carotenoid contents (Zhu et al., 2020; Zheng et al., 2021). CRISPR–Cas has recently been used to introduce a Golden Rice gene cassette into a rice genomic safe harbor, i.e., a chromosomal region that can accommodate transgenes without adverse effects on the host organism (Dong et al., 2020). CRISPR–Cas was also used to develop β-carotene-enriched bananas through use of a "block" strategy based on knocking out the gene for lycopene ε-cyclase to divert the metabolic flux towards the β,β branch (Figure 1)

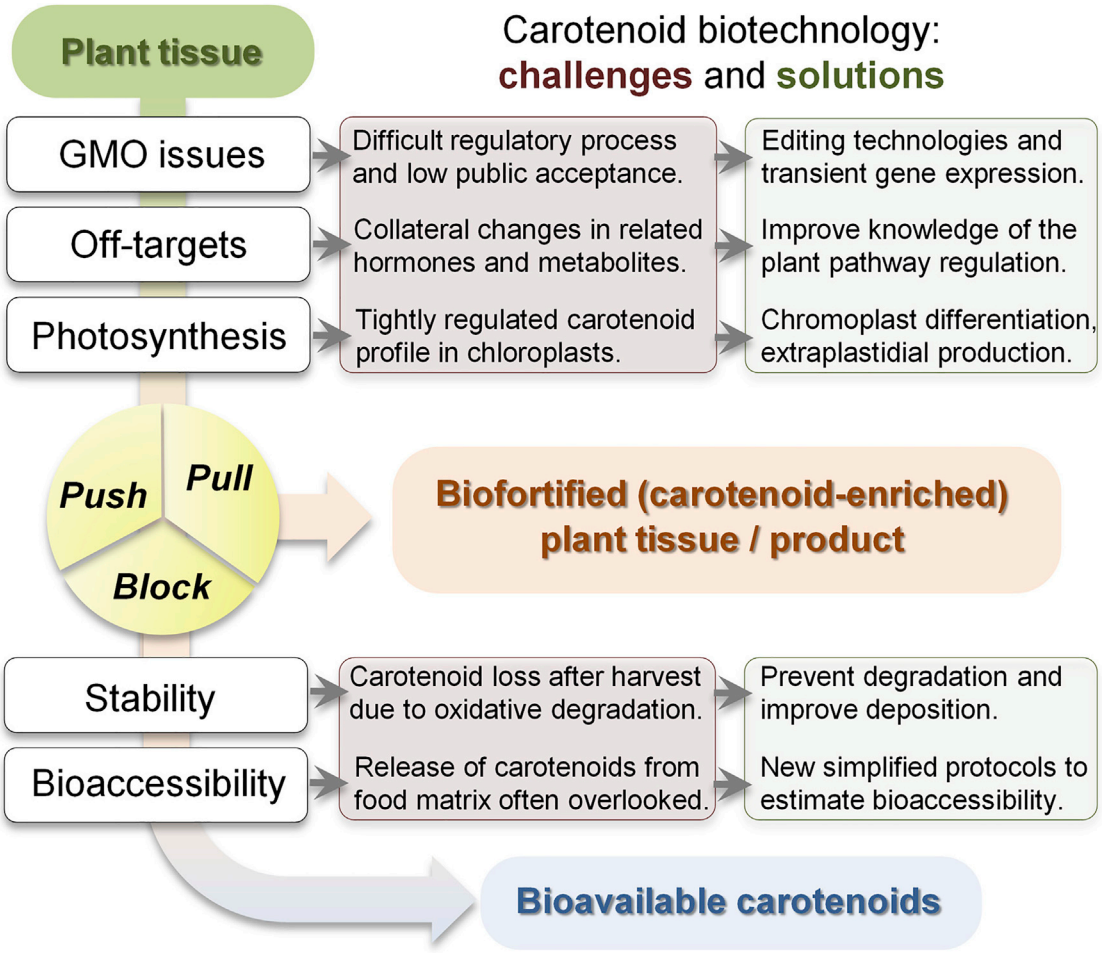

Figure 2. The biofortification pathway.
Successful design of biotechnological approaches for carotenoid biofortification of plant tissues should consider two major phases, each of which involves several challenges and possible solutions. The first phase is the manipulation of tissue carotenoid contents. Challenges in this phase include regulatory constraints that the final product must overcome to reach the market, as well as possible effects of carotenoid manipulation on related metabolites (including hormones) and photosynthesis (when targeting green tissues such as leaves). Once these and other technical considerations are implemented in "push," "block," and/or "pull" approaches and the carotenoid-enriched product is generated, a second phase that is often overlooked is the assessment of shelf-life and bioaccessibility of the carotenoids in the product. These factors ensure high intake and proper delivery of carotenoids to target tissues in order to provide health benefits.

(Kaur et al., 2020). Interestingly, the level of β-carotene in the ripe banana fruit tissue was comparable to that in Golden Rice. In tomato, CRISPR–Cas9-mediated disruption of carotenoid biosynthetic enzymes and regulators of plastidial metabolism caused a significant increase in fruit carotenoid content (lycopene and β-carotene) (Li et al., 2018; Gianoglio et al., 2022). Biofortified bananas and tomatoes can be consumed raw, avoiding the problems associated with carotenoid degradation during processing and cooking. Another advantage compared with Golden Rice is that foreign DNA associated with stable plant transformation can be removed by segregation of the CRISPR–Cas cassette in subsequent generations. Recent reports have demonstrated the suitability of delivering the CRISPR–Cas reaction components (typically a Cas nuclease and one or more guide RNAs) to plant tissues by transient expression systems (Rodriguez-Concepcion and Daros, 2022). Transiently expressed Cas nucleases and/or mobile guide RNAs have been shown to reach germline cells and result in heritable genome editing (Rodriguez-Concepcion and Daros, 2022). These and other advances should open the door to faster and more efficient CRISPR–Cas-based approaches, preventing the use of potentially integrative DNA elements.

PLEIOTROPIC EFFECTS

Carotenoids share precursors with other important metabolites involved in photosynthesis (chlorophylls, phylloquinone, plastoquinone), photoprotection (tocopherols), regulation of plant development (GAs), and environmental interactions (diterpenes) (Figure 1). Carotenoid-derived products also play roles in the regulation of plant development and environmental interactions (ABA, SLs, and other apocarotenoids). Therefore, interfering with the carotenoid pathway can produce undesired collateral effects. In an early work, constitutive overexpression of *PSY* in tomato resulted in higher carotenoid levels in fruit, but it also caused a dwarf plant phenotype due to a reduction in GA

production (Fray et al., 1995). More recently, overexpression of a lycopene β-cyclase exerted a "push" effect to successfully increase β-carotene levels, but it also resulted in enhanced levels of downstream ABA (Figure 1), which contributed to a diverse array of ripening phenotypes such as delayed softening, thicker cuticle, and extended shelf life (Diretto et al., 2020). Preventing conversion of β-carotene into downstream carotenoids should facilitate β-carotene accumulation and, at the same time, minimize the negative effect of higher ABA production on seed germination, as recently illustrated for *Arabidopsis* (Sun et al., 2021). Although some phenotypes derived from the manipulation of β-carotene contents at the whole-plant level may be positive, including enhanced abiotic stress tolerance or fruit yield and shelf life (Diretto et al., 2020; Mi et al., 2022), the overwhelming and often unpredicted consequences of interfering with associated metabolic and hormonal networks (Figure 1) are undesirable for a rational biofortification approach. A more detailed understanding of plant carotenoid pathway regulation in the context of global metabolism and developmental control networks will be necessary to alleviate this important problem.

THE CHALLENGE OF GREEN TISSUES

Leafy vegetables still present a challenge for biofortification. Edible plants such as lettuce, spinach, and cabbage grow faster than fruit crops such as tomato, mango, and citrus, and they already have relatively high starting levels of carotenoids in their chloroplasts. Increasing these levels or changing set carotenoid profiles, however, often involves interference with photosynthesis and therefore has a global effect on plant fitness, physiology, and growth. In chloroplasts, carotenoid levels are finely balanced with chlorophyll levels for the efficient assembly and functionality of photosynthetic complexes (Pascal et al., 2005; Lichtenthaler, 2012; Domonkos et al., 2013; Esteban et al., 2015). Changes in carotenoid:chlorophyll ratios result in conformational changes of the photosynthetic apparatus and disruption of the capacity of chloroplasts to efficiently harness light energy. Changes in the carotenoid profile can also disturb photoprotection and the plant capacity to efficiently dissipate excess light energy as heat. To prevent a negative impact on photosynthesis and photoprotection, the carotenoid pathway is so tightly regulated in green tissues that strategies that successfully enhance carotenoid accumulation in non-photosynthetic tissues such as ripe fruit or calli have not worked to increase the carotenoid content of leaves (Maass et al., 2009; Yazdani et al., 2019). Despite these limitations, successful cases of carotenoid biofortification of plant leaves have been reported. The transplastomic transformation of lettuce and tobacco plants with exogenous pathways for production of astaxanthin and other bacterial ketocarotenoids (which are highly appreciated pigments in feed supplements) resulted in red plants in which endogenous carotenoids were barely detectable (Harada et al., 2014; Bock, 2022). Interestingly, transplastomic plants lost their typical chloroplast features (including thylakoid membranes and grana) and developed large plastoglobule-like osmophilic particles (Lu et al., 2017). A similar phenotype of chromoplast-like plastids developing in leaves was observed after transient expression of a bacterial PSY-encoding gene, *crtB* (Llorente et al., 2020; Morelli et al., 2022). The high production of phytoene by plastid-localized crtB was proposed to interfere with photosynthetic functionality, eventually making chloroplasts competent for chromoplast differentiation as extra phytoene was steadily converted into downstream carotenoids by endogenous plant enzymes (Llorente et al., 2020). The proliferation of plastoglobules and other storage structures in crtB-triggered artificial chromoplasts also stimulated accumulation of other isoprenoid compounds such as tocopherols (vitamin E) and phylloquinone (vitamin K) (Morelli et al., 2022). This strategy works to biofortify leaves and other green tissues with carotenoids and lipophilic isoprenoid vitamins in multiple plant systems, including edible crops such as lettuce and zucchini (Llorente et al., 2020; Morelli and Rodriguez-Concepcion, 2021; Torres-Montilla and Rodriguez-Concepcion, 2021; Houhou et al., 2022). The loss of photosynthetic activity associated with the transition from chloroplasts to chromoplasts, however, makes this approach suitable for plant biofortification only when photosynthesis is dispensable (e.g., immediately before harvest).

An alternative way to enrich green tissues with carotenoids without interfering with photosynthesis is to engineer production of these phytonutrients outside the chloroplast. Following initial attempts using viral vectors in tobacco (Majer et al., 2017), a more recent work was able to produce lycopene crystals in the cytosol of agroinfiltrated leaf cells by upregulating the endogenous mevalonate pathway and redirecting the enhanced supply of IPP and DMAPP to the production of lycopene using bacterial genes (Andersen et al., 2021). In addition to preventing direct interference with photosynthesis, targeting the cytosol as a new cell site for carotenoid production and storage offers multiple advantages. For example, carotenoid intermediates that are known to promote health but are produced at low levels in plants (such as phytoene) can be synthesized at high levels without being converted into downstream products, as plant enzymes involved in carotenoid metabolism are found only in plastids.

POST-HARVEST STABILITY

Evaluations of biotechnological "push," "block," or "pull" approaches rarely go beyond measurement of carotenoid contents in freshly collected tissues from the engineered plants. However, this is only the first step in the biofortification pathway (Figure 2). Two subsequent critical steps are often overlooked: assessments of post-harvest stability and carotenoid bioaccessibility in the engineered product. In this section, we will focus on several strategies for preventing carotenoid loss after harvest and increasing shelf-life of the biofortified product. With only some exceptions, free carotenoids are not stable compounds, and they rapidly decay, mainly through oxidative degradation. Production and deposition of target carotenoids in cell compartments such as the cytosol that reduce exposure and reactions with oxygen or oxygen radicals (Andersen et al., 2021) may therefore increase carotenoid half-life. Moreover, the way in which carotenoids are sequestered makes a significant contribution to their stability and capacity for oxidation. Lycopene, for example, is less likely to be oxidized when it reaches the crystal form (Boon et al., 2008). Carotenoid stability can also be enhanced by increasing the antioxidant capacity of the target tissue. This can result not only in improved shelf-life but also in higher nutritional quality of the product. For example, tocopherols are powerful antioxidants that have vitamin E activity and are metabolically

related to carotenoids (Figure 1) (DellaPenna and Pogson, 2006). Co-expression of genes for tocopherol and carotenoid biosynthetic enzymes has been shown to increase β-carotene accumulation and stability in sorghum and *Arabidopsis* seeds (Che et al., 2016; Sun et al., 2021). The presence of high tocopherol levels together with specialized storage compartments such as plastoglobules in artificial chromoplasts of crtB-expressing leaves is thus expected to increase the stability of carotenoids in general and β-carotene in particular (Morelli et al., 2022). Among the few other molecular tools available to promote formation of chromoplasts, the ORANGE (OR) chaperone is the most extensively studied (Chayut et al., 2017; Sun et al., 2018; Feder et al., 2019; Torres-Montilla and Rodriguez-Concepcion, 2021). The OR protein promotes PSY activity and accumulation (by preventing its degradation), but it also facilitates β-carotene accumulation by preventing its metabolism by unknown mechanisms (Chayut et al., 2017; Feder et al., 2019). In potato, overexpression of OR stimulates carotenoid accumulation during cold storage (Li et al., 2012), illustrating the potential of this gene for improving post-harvest carotenoid content.

Carotenoid degradation can be performed by specific carotenoid-cleavage dioxygenases, a family of non-heme-iron-dependent enzymes that carry out the oxidative cleavage of double bonds in the carotenoid molecule. Carotenoid-cleavage dioxygenases are responsible for carotenoid losses in many crops, and modulation of their activity has been shown to be an important factor determining the carotenoid content of plant organs such as flower petals, fruits, and seeds (Watkins and Pogson, 2020). Carotenoids can also be co-oxidized by products of other enzymes such as lipoxygenase-derived hyperperoxides (Gao et al., 2019). Downregulation of lipoxygenase activity in Golden Rice increased post-harvest carotenoid stability (Gayen et al., 2015). Other enzymes that affect carotenoid stability are those involved in the modification of free carotenoids. Carotenoids can be associated with sugars (e.g., glucose) or lipids (e.g., fatty acids), and this has an impact in their physicochemical properties. Whereas glycosylation makes them more hydrophilic, esterification with fatty acids increases their lipophilicity and stability. Enzymes that catalyze these modifications are known in plants, but they remain to be exploited for carotenoid biofortification (Watkins and Pogson, 2020; Torres-Montilla and Rodriguez-Concepcion, 2021).

BIOACCESSIBILITY AND BEYOND

One factor that is often overlooked in the development of a carotenoid biofortification strategy is the dietary intake of the accumulated compounds (La Frano et al., 2014; Nogareda et al., 2016; Watkins and Pogson, 2020; Zheng et al., 2020). Enriched carotenoid contents in a given food do not necessarily enable more carotenoids to reach their targets in our bodies because these lipophilic isoprenoids must first be released from the food matrix and then incorporated into lipid-containing water-miscible intestinal micelles. Bioaccessibility is defined as the fraction of a nutrient (e.g., a carotenoid) that is released from the food matrix and incorporated into micelles to be accessible for absorption, whereas bioavailability describes the next step, as it considers the fraction of the nutrient that reaches the target cells and becomes available for utilization in normal physiological functions and/or for storage (Giuliano, 2017; Rodriguez-Concepcion et al., 2018; Meléndez-Martínez et al., 2021). Biofortification strategies should ideally complement compositional data with information on bioaccessibility and/or bioavailability. Assessment of bioavailability requires *in vivo* assays, but bioaccessibility can be determined using *in vitro* methods that simulate the chemical environment and reactions that occur in the human gastrointestinal tract during digestion. Recent efforts to simplify bioaccessibility determinations and adapt the protocols to the expertise and equipment typically available in molecular biology laboratories like those involved in carotenoid biotechnology are expected to help make bioaccessibility data a routine part of biofortification reports (Morelli and Rodriguez-Concepcion, 2021).

Bioaccessibility is highly dependent on the matrix in which carotenoids are found in plant cells, and different physicochemical contexts and subcellular environments thus impact bioaccessibility (Watkins and Pogson, 2020; Zheng et al., 2020). In general, crystalline or aggregated carotenoids are less bioaccessible, whereas those associated with lipids (e.g., in plastoglobules) show increased bioaccessibility because of their easier micellization. Unfortunately, it is often difficult to find a good compromise between high bioaccessibility and high stability because enhanced stability implies that the pigment is less prone to be accessible to external factors such as those that promote its degradation but also its release from the food matrix. Nonetheless, some of the strategies described above show that it is possible to improve production, storage (i.e., stability), and bioaccessibility. For example, the bioaccessibility of phytoene was higher when it was produced in the cytosol rather than the chloroplasts of leaf cells (Andersen et al., 2021), and β-carotene was more bioaccessible when acquired from crtB-promoted chromoplasts than from chloroplasts of lettuce leaves (Morelli and Rodriguez-Concepcion, 2021).

CONCLUDING REMARKS

Most efforts for the biofortification of crops have been directed towards biotechnological strategies for improving carotenoid contents by manipulating carotenoid biosynthesis, degradation, and/or storage (including modification of plastid ultrastructure or testing of non-natural sites such as the plant cell cytosol). Future focus in this area should be directed towards overcoming regulatory constraints and improving consumer acceptance (e.g., by using genome-editing technology) and also towards preventing off-target effects (e.g., those that result from altered hormone levels and metabolic homeostasis) and negative effects on photosynthesis when not dispensable (e.g., in leaves). Furthermore, more effort should be devoted to confirming that enrichment of carotenoid contents in plant-derived foods eventually results in improved carotenoid intake. This involves ensuring post-harvest stability and assessing bioaccessibility in the engineered product to make sure that carotenoid release from the food matrix is not negatively affected. Successfully meeting these goals will ensure a new golden age of carotenoid biotechnology and biofortification.

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AUTHOR CONTRIBUTIONS

Both co-authors conceived the review structure, discussed the main issues to cover, and wrote the manuscript.

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