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Transient expression systems to rewire plant carotenoid metabolism

Manuel Rodriguez-Concepcion and José-Antonio Daròs

Abstract

Enrichment of foodstuffs with health-promoting metabolites such as carotenoids is a powerful tool to fight against unhealthy eating habits. Dietary carotenoids are vitamin A precursors and reduce risk of several chronical diseases. Additionally, carotenoids and their cleavage products (apocarotenoids) are used as natural pigments and flavors by the agrofood industry. In the last few years, major advances have been made in our understanding of how plants make and store carotenoids in their natural compartments, the plastids. In part, this knowledge has been acquired by using transient expression systems, notably agroinfiltration and viral vectors. These techniques allow profound changes in the carotenoid profile of plant tissues at the desired developmental stage, hence preventing interference with normal plant growth and development. Here we review how transient expression approaches have contributed to learn about the structure and regulation of plant carotenoid biosynthesis and to rewire carotenoid metabolism and storage for efficient biofortification of plant tissues.

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Agroinfiltration, Biofortification, Biotechnology, Carotenoid, Chloroplast, Chromoplast, Leaf, *Nicotiana benthamiana*, Viral vector.

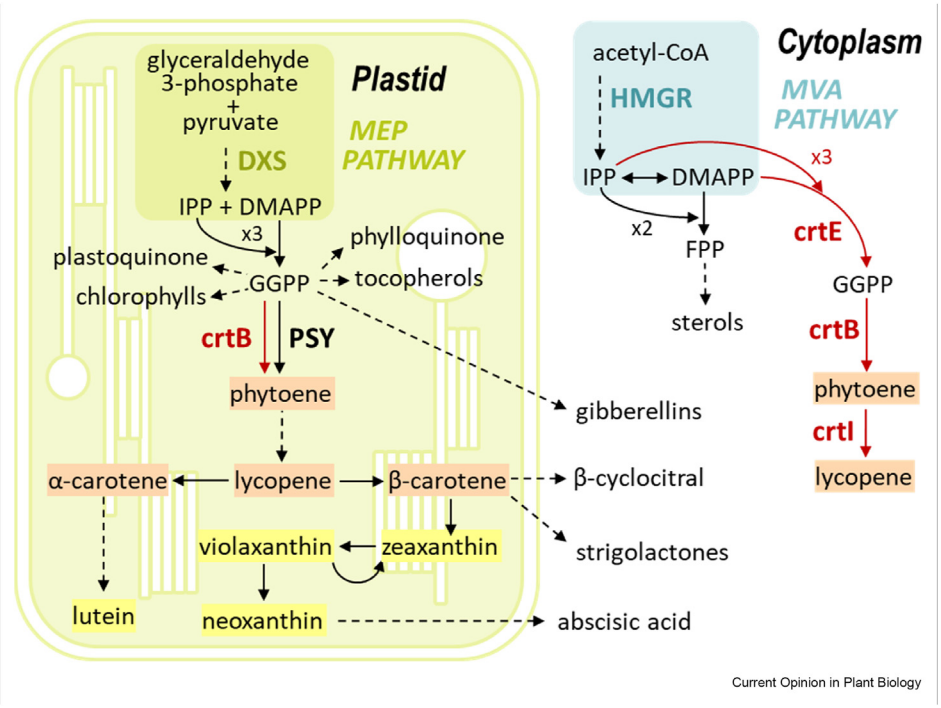
Introduction

Several of the UN Sustainable Development Goals (SDG) are directly related to nutrition, including SDG-2 (Zero Hunger) and SDG-3 (Good Health and Well Being). Without regular and nutritious food, humans cannot live, learn, fend off diseases or lead productive lives. Malnutrition goes beyond low food intake. More than two billion adults, adolescents and children are now obese or overweight according to the Food and Agriculture Organization, FAO (http://www.fao.org/sustainable-development-goals/goals/goal-2/en/). The consequences are severe for public health and for individuals' and communities' quality of life. Biofortified foods are expected to become a powerful tool to fight against unhealthy eating habits.

Plant isoprenoids include a wide diversity of metabolites that humans cannot produce but need to acquire from food sources. They derive from metabolic precursors produced by the mevalonic acid (MVA) pathway in the cytosol and the methylerythritol 4-phosphate (MEP) pathway in plastids [1,2] (Figure 1). The vast majority of isoprenoids are secondary (i.e. specialized) metabolites that participate in the interaction of plants with the environment. But there are isoprenoids with primary (i.e. essential) roles for plant physiology, such as MVA-derived sterols (regulators of plasma membrane architecture) and MEP-derived carotenoids and tocopherols (powerful antioxidants and photoprotectants), that also play important functions as phytonutrients in animals. In particular, carotenoids are required for photosynthesis and photoprotection in leaves [3] but function as communication signals in flower petals and ripe fruit, e.g. as colors and flavors that attract pollinators and seed-dispersing animals [4]. Furthermore, oxidative cleavage of carotenoids can generate biologically active molecules in plants and animals. In plants they include hormones such as abscisic acid and strigolactones, and retrograde signals such as beta-cyclocitral. In animals, they include retinoids such as vitamin A. Additional health-related properties associated with carotenoid-rich diets include a reduced risk of diseases such as age-related macular degeneration, cognitive malfunctioning, type-2 diabetes, obesity, cardiovascular diseases, and some types of cancer [5,6]. While the properties of carotenoids and their cleavage products as natural pigments and aromas make them important economic targets of cosmetic, pharma and agrofood industries, their main interest for humans is their nutritional value.

Biofortification of plant-derived foods with carotenoids is a major strategic goal to achieve optimal nutrition and

Figure 1



Endogenous and engineered pathways for carotenoid biosynthesis in plant cells. The endogenous carotenoid pathway is found in plastids and fed by precursors supplied by the MEP pathway. The first and main rate-determining enzymes of the MEP and carotenoid pathways are deoxyxylulose 5-phosphate synthase (DXS) and phytoene synthase (PSY), respectively. Carotenes and xanthophylls produced by the carotenoid pathway are boxed in orange and yellow, respectively. Extraplastidial production of carotenoids can be achieved using MVA-derived cytosolic precursors by engineering the production of the indicated bacterial enzymes (in red). MVA pathway flux is mainly controlled by hydroxymethylglutaryl-coenzyme A reductase (HMGR). When located in plastids, the bacterial crtB enzyme promotes the production of MEP-derived endogenous carotenoids and eventually the differentiation of chloroplasts into chromoplasts.

a healthier diet for two main reasons. Firstly, combinations of carotenoids with other antioxidants and phytonutrients present in the food matrix are known to be much more effective than taking dietary supplements of single components. And secondly, dietary carotenoids are mainly obtained from plants (fruits and vegetables). Most biotechnological strategies aimed to enrich plant tissues with carotenoids, including Golden Rice, have been focused on manipulating their biosynthesis, degradation and/or storage in transgenic lines generated by stable transformation techniques [7–10]. However, little attention has been given to the manipulation of carotenoid levels by transient expression systems, an alternative that is particularly interesting for photosynthetic (green) tissues. These techniques allow profound and persistent changes in the carotenoid profile of plant tissues without the need of the lengthy and often challenging process of plant stable transformation. They have the added advantage of restricting the changes to a particular stage or time, e.g. just before harvest, hence allowing normal plant growth and development up to that time. Here we will review recent advances in the use of transient expression systems to manipulate the carotenoid content of plant tissues. The biological context will also be covered by briefly providing basic information on plant carotenoid metabolism (i.e. biosynthesis, storage and degradation).

Carotenoid biosynthesis, storage and degradation

Plants synthesize carotenoids in plastids using isoprenoid precursors supplied by the MEP pathway [5]. MEP-derived isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the five-carbon (C5) precursors used to produce C20 geranylgeranyl diphosphate (GGPP), a common precursor of C40 carotenoids and other phytonutrients such as tocopherols (vitamin E) and phylloquinone (vitamin K), photosynthesis-related metabolites (including chlorophylls and plastoquinone) and hormones such as gibberellins (Figure 1). Bacterial GGPP synthases are referred to as crtE. The enzyme phytoene synthase (PSY) transforms GGPP into phytoene in the first committed step of the carotenoid pathway (Figure 1). Direct interaction of plant GGPP synthases and PSY enzymes facilitates channeling of GGPP into the carotenoid pathway [11]. Because PSY is the main rate-

determining enzyme of the pathway [12], isoforms from plants and bacteria (named crtB) have been often used to boost the metabolic flux to carotenoids [7,9]. The non-colored phytoene is converted into the red-colored lycopene by sequential desaturation and isomerization reactions catalyzed by four enzymes in plants but only one (crtI) in bacteria [5]. The ends of the linear lycopene molecule can then be cyclized to form β- or ε-ionone rings. Two β rings yield β-carotene (β,β branch) whereas one β and one ε ring make α-carotene (β,ε branch). Introduction of two ε rings is rare in plants. Ring hydroxylation and epoxidation transforms these oxygen-lacking carotenoids (named carotenes) into oxygenated carotenoids (named xanthophylls) such as lutein (β,ε branch) or zeaxanthin, violaxanthin and neoxanthin (β,β branch) (Figure 1).

Free carotenoids are hydrophobic metabolites that accumulate in lipid-rich environments, sometimes as aggregates and crystals. But they can also be associated to proteins (e.g., in photosynthetic complexes), sugars (e.g., glucose) or lipids (e.g. fatty acids). Carotenoid glycosylation increases their hydrophilicity whereas esterification with fatty acids renders them more lipophilic. Plant enzymes involved in these modifications have been identified but are yet to be exploited for carotenoid biofortification [8,10]. Carotenoid storage is also dependent on the presence of appropriate subplastidial structures for their sequestering and accumulation. In chloroplasts, most carotenoids are associated with proteins of the photosynthetic apparatus and their levels are tightly balanced with those of chlorophylls to maintain proper photosynthesis and photoprotection [3]. But the most efficient carotenoid-sequestering structures are found in chromoplasts, which are plastids specialized in the production and accumulation of carotenoids [13,14]. Different types of chromoplasts provide color to non-photosynthetic tissues such as carrot roots, daffodil flowers and tomato fruits. Depending on their carotenoid composition, they develop sequestering structures such as lipid vesicles (e.g., plastoglobules), lipoprotein complexes (e.g., fibrils), membrane systems or crystals, which classify them as globular, fibrillar, membranous, or crystalline, among other classes.

Carotenoid degradation is usually mediated by carotenoid cleavage dioxygenases (CCDs) that catalyze the oxidative breakdown of the electron-rich polyene backbone of carotenoids in different double bond positions. The resulting oxidative breakdown products are often referred to as apocarotenoids and some of them have biological roles as pigments, aromas, hormones or stress signals [15]. CCD activity is negatively correlated with carotenoid levels in some plants and tissues, but there are non-enzymatic processes that may also contribute to carotenoid loss. They include oxidation by reactive oxygen species (ROS) and cooxidation by lipoxygenase-derived hyperperoxides [16,17].

Agroinfiltration

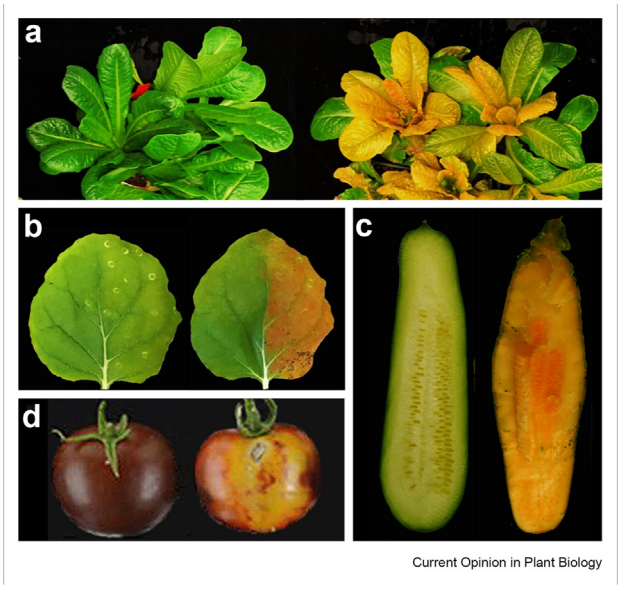
Several systems have been developed for transient expression of exogenous sequences in plant cells with the aim of either overexpressing or silence target genes. In this review we will focus on the two main systems: agroinfiltration and viral vectors. The delivery of DNA sequences to plant cells using *Agrobacterium tumefaciens* (agrobacterium) strains carrying appropriate transfer DNA (T-DNA) constructs is a powerful tool for transient gene expression. Such constructs can be easily transferred to plants by direct infiltration of leaves with agrobacterium cultures in a technique commonly referred to as agroinfiltration or agroinjection. Co-infiltration of two or more cultures, each harboring a different set of genes, is a common practice. This is a major advantage of the agroinfiltration technique as it allows to readily test different combinations of multi-step pathways. Agroinfiltration was initially optimized for *Nicotiana benthamiana* leaves, and has resulted in industrial-scale manufacturing processes for producing recombinant proteins, biopharmaceuticals, vaccines and biomaterials in plant biofactories [18]. However, the technique can also be adapted to economically important crop plants [19].

Agroinfiltration of *N. benthamiana* leaves is now a widespread general technique to test gene function. In the carotenoid field, it has allowed to confirm the identity of genes involved in carotenoid biosynthesis [20], in their enzymatic conversion into apocarotenoids [21], and in the metabolism of such apocarotenoids [22–25]. It has also been used to test the functionality of chimeric fusion enzymes [26] and the role of transcription factors controlling carotenoid gene expression [27,28]. Most interestingly, agroinfiltration of *N. benthamiana* leaves has been the central approach leading to the discovery of two new ways to create extra sites for the production and accumulation of carotenoids in plant cells: the transformation of chloroplasts into carotenoid-overaccumulating plastids, i.e. chromoplasts [29], and the extraplastidial production of carotenoids [30]. These two recent accomplishments will be summarized below.

Because carotenoids are central for photosynthesis and photoprotection, manipulating their levels in photosynthetic tissues is challenged by the requirement to maintain the functionality of chloroplasts. The carotenoid composition of chloroplasts is similar in most plant species, with lutein and β-carotene being the most abundant followed by β,β xanthophylls [3,5]. Leaves are virtually devoid of phytoene and lycopene, which are intermediates with a high interest from a nutritional point of view [5,30]. Engineering their extraplastidial

synthesis and accumulation appeared as a solution to enrich the leaf carotenoid content without compromising the balance of particular carotenoids (and chlorophylls) required for photosynthesis. A first step in this direction was the cytosolic production of phytoene and lycopene in tobacco (*Nicotiana tabacum*) using an RNA virus vector derived from the potyvirus tobacco etch virus (TEV) carrying genes from the plant pathogenic bacterium *Pantoea ananatis* (formerly known as *Erwinia uredovora*) [31]. Besides reaching the goal of producing carotenoid intermediates out of their natural compartments in plant cells (see next paragraph below), this work led to an unexpected discovery. It was observed that the sole expression of the *crtB* gene encoding the bacterial phytoene synthase (Figure 1) was sufficient to change the leaf color from green to yellow (Figure 2a). This was surprising as phytoene lacks color and it is not expected to be converted into downstream carotenoid pigments when produced out of plastids. A more recent work using agroinfiltrated crtB constructs showed that the color change is due to the irreversible transformation of chloroplasts into plastids of chromoplast features that accumulate high levels of carotenoids [29]. It was demonstrated that the presence of a cryptic plastid-targeting sequence in the N-terminal region of the crtB protein allowed its partial import into plastids, where it produced phytoene from MEP-derived GGPP [29,30]. Such crtB-mediated production of phytoene was so high that it impaired photosynthesis. Reduced photosynthesis was found to be the first step in the chromoplastogenesis process, making chloroplasts competent to be differentiated into chromoplasts. The second step in the process involves the conversion of phytoene into downstream carotenoids [29]. The resulting two-step model also applies to natural systems, in which developmental signals must weaken chloroplast identity in the first phase of the process and upregulate carotenoid biosynthetic genes in the second step [29]. In non-photosynthetic tissues, the first phase is not required and promoting a high enough production of carotenoids is all that it takes for chromoplasts to differentiate [29].

Figure 2



Examples of carotenoid biofortification using transient expression systems. **(a)** Lettuce plants either untreated (left) or infected with the crtB-carrying virus LMV-crtB (right) as described [29]. **(b)** *N. benthamiana* leaves agroinfiltrated with an empty vector control (left) or with constructs harboring bacterial genes for the extraplastidial production of lycopene (right) as described [30]; only the right half of the leaves were agroinfiltrated. (c) Zucchini fruits from plants either untreated (left) or infected with the crtB-carrying virus ZYMV-crtB (right) as described [38]. (d) Tomato RD fruits either untreated (left) or infected with a TRV-DR-PDS vector for VIGS (right) as described [41].

The first attempts to produce carotenoids in extraplastidial locations of plant cells were done using viral vectors carrying the *P. ananatis* genes for enzymes converting IPP and DMAPP into GGPP (crtE), GGPP into phytoene (crtB) and phytoene into lycopene (crtI) (Figure 1). They resulted in only transient and relatively minor accumulation of lycopene in the cytosol of *N. tabacum* leaf cells [31]. Delivering constructs to *N. benthamiana* leaf cells by agroinfiltration allowed major improvements [30], including the possibility of readily testing different construct combinations. IPP and DMAPP are produced in the cytosol by the activity of the MVA pathway (Figure 1) and then used for the production of sterols and other isoprenoids [1,2]. Adding a truncated (i.e. deregulated) version of hydroxymethylglutaryl-coenzyme A reductase (HMGR), the main rate-determining enzyme of the MVA pathway [1], boosted cytosolic IPP and DMAPP production and substantially improved crtE-mediated GGPP supply [30]. The use of a version of crtB with a N-terminal GFP tag that disrupted the cryptic plastid-targeting sequence and hence retained the fusion protein in the cytosol led to an efficient conversion of MVA-derived GGPP into cytosolic phytoene while preventing chromoplast differentiation. As a consequence, agroinfiltrated cells accumulated levels of cytosolic phytoene (and lycopene when crtI was included in the combination) that were similar to those of total endogenous (i.e. plastidial) β-carotene and xanthophylls [30]. In particular, lycopene accumulation was so high that it resulted in a reddish leaf color (Figure 2b) and a deposition as extraplastidial crystals that were very similar to those found in the chromoplasts of ripe tomato fruit. These extraplastidial carotenoids were retained in the leaf tissue for much longer than when using viral vectors. However, the phenotype was not as stable as the crtB-triggered carotenoid overaccumulation in artificial chromoplasts, which were maintained for weaks after agroinfiltration and never dedifferentiated to chloroplasts [29]. Both artificial chromoplastogenesis and extraplastidial carotenoid accumulation are new biofortification strategies that were demonstrated to also work in edible

leaves such as lettuce [29,30] (Figure 2a). Most interestingly, carotenoid bioaccessibility (i.e. the proportion released from the food matrix during digestion and incorporated into micelles in the gastrointestinal tract for eventual absorption) was higher in leaves engineered to overproduce these phytonutrients in artificial chromoplasts [32] or in extraplastidial locations [30], hence demonstrating the multiple benefits of using these agroinfiltration-based strategies for food biofortification.

Viral vectors

Plant virus-derived vectors offer some advantages to transient gene expression of T-DNA constructs. Chief among them are to boost expression as a consequence of viral genome amplification and to spread systemically the genes of interest throughout the whole plant as a result of the virus capacity to move cell-to-cell and long-distance. However, agroinfiltration and the use of viral vectors are not mutually exclusive techniques, since viral vectors are frequently arranged in agrobacterium T-DNAs and inoculated into the host plants by a particular case of agroinfiltration that is usually known as agroinoculation [33]. In addition, a continuity with other transient expression strategies exist, because some viral vectors consist of deleted versions of plant viruses that lost movement capacity or just individual viral elements, such as promoters, translation enhancers or RNA silencing suppressors, that are incorporated into the T-DNAs to compose the expression cassettes [34]. A drawback of viral vectors is a limited cargo capacity. However, a complete carotenoid pathway was successfully engineered in the cytosol of *N. tabacum* cells, as described above [31]. The coding regions of the three enzymes composed an RNA insert of circa 3.4 kb, which is a limiting cargo size for a plus-strand RNA virus able to replicate and move systemically. Stability of this particular viral vector harboring such a large insert was achieved by transferring a 1.5-kb viral gene, namely NIb, coding for the RNA-dependent RNA polymerase, to the plant genome as a transgene [35]. This same viral vector and strategy to accommodate large cargoes was used to co-express *P. ananatis* crtB with a saffron (*Crocus sativus*) CCD to engineer apocarotenoid production in *N. benthamiana*. This system represents the first heterologous production of highly appreciated apocarotenoids responsible for saffron color (crocins) and aroma (picrocrocin) outside the species and tissues in which they naturally accumulate [36]. Levels of 0.35% and 0.8% leaf dry weight of crocins and picrocrocin, respectively, were achieved in only two weeks. The *P. ananatis* crtB enzyme alone has been expressed in different viral vectors to trigger the accumulation of carotenoids that, together with a reduction in chlorophylls, results in a bright yellow pigmentation of infected tissues due to the conversion of chloroplast into carotenoid-rich chromoplasts [29,31]. This result was repeatedly observed in leaves from different host plants with vectors derived from viruses from different families and genera, such as lettuce mosaic virus (LMV) (Fig. 2a), tobacco mosaic virus (TMV), potato virus X (PVX) or zucchini yellow mosaic virus (ZYMV), hence allowing to set up a visual yellow reporter to track plant virus infection [29,31]. Virus-based expression of *P. ananatis* crtB and consequent induction of yellow pigmentation in infected tissues was shown to improve visual infection diagnosis of a mild isolate of watermelon mosaic virus in different cucurbit accessions [37]. Most interestingly, the crtB protein can be used as a tool for biofortification of green tissues other than leaves. For example, inoculation of selected leaves of adult zucchini plants that nurture mature flowers with a ZYMV vector that expresses *P. ananatis crtB* induces production of biofortified yellow-orange zucchinis (Fig. 2c) in which accumulation of healthy carotenoids such as β-carotene (vitamin A) lutein and phytoene was significantly increased in both rind and flesh [38].

In addition to overexpression of genes of interest, viral vectors can be used to knock-down expression of particular genes. This strategy, known as virus-induced gene silencing (VIGS), is typically used to interrogate the function of specific genes or to block particular pathways in biotechnological designs. VIGS originates in the small RNA-based antiviral response that the host plants deploy after infection, and can be triggered by simple insertion of a fragment of the gene of interest in the VIGS vector. Most effective VIGS vectors are derived from mild viruses, strains and mutants, in which the viral suppressors of RNA silencing are unable to dismantle the host counter-attack to virus infection. Most popular VIGS vectors are based on tobacco rattle virus (TRV) that infect some model plants such as *N. benthamiana* or *Arabidopsis thaliana* [39]. However, TRV displays a particular host range. Fortunately, researchers have been able to set up more than fifty VIGS systems that collectively are able to target almost all botanical families and important crop plants. A comprehensive list of VIGS vectors and target plant species is available [40]. Development of VIGS systems is inherently related to carotenoid metabolism because phytoene desaturase (PDS), the enzyme catalyzing the second step of the biosynthetic pathway, is often chosen as visual reporter to track VIGS progress in different plant systems, including tomato fruit [41] (Figure 2d). In the carotenoid pathway, VIGS was used to dissect the role of several candidate genes in lycopene biosynthesis in tomato fruits [42] and for the functional analysis of PSY isoforms in tobacco [43] and carotenoid isomerase in Chinese kale [44]. VIGS analyses also enlightened the role of regulatory factors of carotenoid accumulation in tomato fruits, such as *SlNAP7* involved in chloroplast development and lycopene accumulation [45], *Tomato Agamous-like 1* (*TAGL1*) that regulates fruit ripening and nutrient accumulation [46], or *Arancio* involved in carotenoid accumulation [47].

Future perspectives

Biotechnology faces a moment of neat enthusiasm due to amazing recent advances in genome editing based on the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nucleases from archaea and bacteria [48], and plant biotechnology and crop improvement are not alien to this enthusiasm [49,50]. Advances in CRISPR-Cas-based genome editing, epigenetic control and gene expression regulation will improve our knowledge about the carotenoid pathway and will impulse the design of edited crops with carotenoid-enriched phenotypes. Most common CRISPR-Cas arrangements in plants involve expression of the reaction components, typically a Cas nuclease and one or more guide RNAs, after stable transformation of plant tissues. However, recent reports have shown the suitability of viral vectors to express mobile guide RNAs that reach the germline cells and induce heritable genome editing [51,52] or the possibility to transiently express not only the guide RNAs but also the Cas nucleases [53,54]. These advances open the path to rapid and efficient CRISPR-Cas-based synthetic biology in plants, avoiding time-consuming and labor-intensive tissue culture processes and circumventing the use of potentially integrative DNA elements, which frequently raises concerns in regulatory agencies. The use of viral vectors in CRISPR-Cas-based synthetic biology in plants is expected to keep improved performance in view of recent discoveries of compact Cas nucleases [55–57], whose viral expression should be easier. Another exciting perspective is to directly deliver the guide RNAs and the Cas nucleases into plant tissues using nanotechnology [58]. Advances in synthetic biology should permit to deploy complex gene circuits in plants that may be activated after sensing chemical or physical cues to produce specialized carotenoids at the appropriate developmental stage.

All these technologies may be used to produce biofortified food and feed, but also diverse carotenoid products for the pharmaceutical or industrial sectors. Whether or not such developments will eventually reach the market or will stay in laboratories is strongly dependent on the evolution of societal and political concerns towards genetic manipulation [59]. RNA virus-based vectors could be used to manipulate carotenoid metabolism with no DNA involved and negligible risk of unintended insertion of foreign genes [38]. Nonetheless, the use of (disarmed) plant pathogens or elements thereof is not free of controversy and regulatory issues [60]. Concerns over consumption of plants loaded with bioengineered bacterial or/and viruses together with the extra hurdles associated with the need to infect the plants to deliver the selected constructs currently appear as important limitations of available transient expression systems. Future developments for the efficient delivery of DNA/RNA sequences and proteins into plant tissues without the need of bacterial or viral vectors [61] may solve at least some of these limitations. Regardless of whether these expected improvements will make economically viable the use of transient expression systems to improve the carotenoid content of plants for nutritional or industrial purposes, currently available agroinfiltration and viral vectors are expected to remain powerful tools to test functionality of genes and engineered circuits. In the end, public acceptance of biotechnology is expected to remain the main bottleneck for the advances obtained in the laboratory to eventually reach the market (e.g., as new edited cultivars) and have a positive impact in our society.

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 article.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Rodriguez-Concepcion M, Boronat A: **Breaking new ground in the regulation of the early steps of plant isoprenoid biosynthesis**. *Curr Opin Plant Biol* 2015, **25**:17–22.
2. Hemmerlin A, Harwood JL, Bach TJ: **A raison d'etre for two distinct pathways in the early steps of plant isoprenoid biosynthesis?** *Prog Lipid Res* 2012, **51**:95–148.
3. Domonkos I, Kis M, Gombos Z, Ughy B: **Carotenoids, versatile components of oxygenic photosynthesis**. *Prog Lipid Res* 2013, **52**:539–561.
4. Llorente B, D'Andrea L, Rodríguez-Concepción M: **Evolutionary recycling of light signaling components in fleshy fruits: new insights on the role of pigments to monitor ripening**. *Front Plant Sci* 2016, **7**:263.
5. Rodriguez-Concepcion M, Avalos J, Bonet ML, Boronat A, Gomez-Gomez L, Hornero-Mendez D, Limon MC, Melendez-Martinez AJ, Olmedilla-Alonso B, Palou A, *et al.*: **A global perspective on carotenoids: metabolism, biotechnology, and benefits for nutrition and health**. *Prog Lipid Res* 2018, **70**:62–93.
6. Amengual J: **Bioactive properties of carotenoids in human health**. *Nutrients* 2019, **11**:2388.
7. Giuliano G: **Provitamin A biofortification of crop plants: a gold rush with many miners**. *Curr Opin Biotechnol* 2017, **44**:169–180.
8. Watkins JL, Pogson BJ: **Prospects for carotenoid biofortification targeting retention and catabolism**. *Trends Plant Sci* 2020, **25**:501–512.
9. Zheng X, Giuliano G, Al-Babili S: **Carotenoid biofortification in crop plants: citius, altius, fortius**. *Biochim Biophys Acta Mol Cell Biol Lipids* 2020, **1865**:158664.

10. Torres-Montilla S, Rodriguez-Concepcion M: **Making extra room for carotenoids in plant cells: new opportunities for biofortification**. *Prog Lipid Res* 2021, **84**:101128.

11. Barja MV, Rodriguez-Concepción M: **Plant geranylgeranyl diphosphate synthases: every (gene) family has a story**. *aBIOTECH* 2021, **2982**:289.

12. Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM: **Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner**. *Proc Natl Acad Sci U S A* 2002, **99**: 1092–1097.

13. Sadali NM, Sowden RG, Ling Q, Jarvis RP: **Differentiation of chromoplasts and other plastids in plants**. *Plant Cell Rep* 2019, **38**:803–818.

14. Sun T, Yuan H, Cao H, Yazdani M, Tadmor Y, Li L: **Carotenoid metabolism in plants: the role of plastids**. *Mol Plant* 2018, **11**: 58–74.

15. Moreno JC, Mi J, Alagoz Y, Al-Babili S: **Plant apocarotenoids: from retrograde signaling to interspecific communication**. *Plant J* 2021, **105**:351–375.

16. Gao L, Gonda I, Sun H, Ma Q, Bao K, Tieman DM, Burzynski-Chang EA, Fish TL, Stromberg KA, Sacks GL, *et al.*: **The tomato pan-genome uncovers new genes and a rare allele regulating fruit flavor**. *Nat Genet* 2019, **51**:1044–1051.

17. Havaux M: **Carotenoid oxidation products as stress signals in plants**. *Plant J* 2014, **79**:597–606.

18. Bally J, Jung H, Mortimer C, Naim F, Philips JG, Hellens R, Bombarely A, Goodin MM, Waterhouse PM: **The rise and rise of Nicotiana benthamiana: a plant for all reasons**. *Annu Rev Phytopathol* 2018, **56**:405–426.

19. Torti S, Schlesier R, Thummler A, Bartels D, Romer P, Koch B, Werner S, Panwar V, Kanyuka K, Wiren NV, *et al.*: **Transient reprogramming of crop plants for agronomic performance**. *Native Plants* 2021, **7**:159–171.

20. Leonelli L, Erickson E, Lyska D, Niyogi KK: **Transient expression in Nicotiana benthamiana for rapid functional analysis of genes involved in non-photochemical quenching and carotenoid biosynthesis**. *Plant J* 2016, **88**:375–386.

21. Rubio-Moraga A, Rambla JL, Fernandez-de-Carmen A, Trapero-Mozos A, Ahrazem O, Orzaez D, Granell A, Gomez-Gomez L: **New target carotenoids for CCD4 enzymes are revealed with the characterization of a novel stress-induced carotenoid cleavage dioxygenase gene from Crocus sativus**. *Plant Mol Biol* 2014, **86**:555–569.

22. Diretto G, Ahrazem O, Rubio-Moraga A, Fiore A, Sevi F, Argandona J, Gomez-Gomez L: **UGT709G1: a novel uridine diphosphate glycosyltransferase involved in the biosynthesis of picrocrocin, the precursor of safranal in saffron (Crocus sativus)**. *New Phytol* 2019, **224**:725–740.

23. Lopez-Jimenez AJ, Frusciante S, Niza E, Ahrazem O, Rubio-Moraga A, Diretto G, Gomez-Gomez L: **A new glycosyltransferase enzyme from family 91, UGT91P3, is responsible for the final glucosylation step of crocins in saffron (crocus sativus L.)**. *Int J Mol Sci* 2021, **22**:8815.

24. Sun G, Putkaradze N, Bohnacker S, Jonczyk R, Fida T, Hoffmann T, Bernhardt R, Hartl K, Schwab W: **Six uridine-diphosphate glycosyltransferases catalyze the glycosylation of bioactive C13-apocarotenols**. *Plant Physiol* 2020, **184**: 1744–1761.

25. Demurtas OC, de Brito Francisco R, Diretto G, Ferrante P, Frusciante S, Pietrella M, Aprea G, Borghi L, Feeney M, Frigerio L, *et al.*: **ABCC transporters mediate the vacuolar accumulation of crocins in saffron stigmas**. *Plant Cell* 2019, **31**:2789–2804.

26. * Nogueira M, Enfissi EMA, Welsch R, Beyer P, Zurbriggen MD, Fraser PD: **Construction of a fusion enzyme for astaxanthin formation and its characterisation in microbial and plant hosts: a new tool for engineering ketocarotenoids**. *Metab Eng* 2019, **52**:243–252.

A good example of how transient expression system can be used to test functionality of synthetic enzymes in a fast and efficient way.

27. Ampomah-Dwamena C, Thrimawithana AH, Dejnoprat S, Lewis D, Espley RV, Allan AC: **A kiwifruit (Actinidia deliciosa) R2R3-MYB transcription factor modulates chlorophyll and carotenoid accumulation**. *New Phytol* 2019, **221**:309–325.

28. Zhu F, Luo T, Liu C, Wang Y, Yang H, Yang W, Zheng L, Xiao X, Zhang M, Xu R, *et al.*: **An R2R3-MYB transcription factor represses the transformation of alpha- and beta-branch carotenoids by negatively regulating expression of CrBCH2 and CrNCED5 in flavedo of Citrus reticulate**. *New Phytol* 2017, **216**: 178–192.

29. ** Llorente B, Torres-Montilla S, Morelli L, Florez-Sarasa I, Matus JT, Ezquerro M, D'Andrea L, Houhou F, Majer E, Pico B, *et al.*: **Synthetic conversion of leaf chloroplasts into carotenoid-rich plastids reveals mechanistic basis of natural chromoplast development**. *Proc Natl Acad Sci U S A* 2020, **117**: 21796–21803.

A recent example of the power of transient expression systems to understand long-standing basic biological questions (i.e. how chromoplasts are made).

30. * Andersen TB, Llorente B, Morelli L, Torres-Montilla S, Bordanaba-Florit G, Espinosa FA, Rodriguez-Goberna MR, Campos N, Olmedilla-Alonso B, Llansola-Portoles MJ, *et al.*: **An engineered extraplastidial pathway for carotenoid biofortification of leaves**. *Plant Biotechnol J* 2021, **19**:1008–1021.

Pioneer work demonstrating that producing carotenoids in the cytosol improves their bioaccessibility.

31. Majer E, Llorente B, Rodriguez-Concepcion M, Daros JA: **Rewiring carotenoid biosynthesis in plants using a viral vector**. *Sci Rep* 2017, **7**:41645.

32. Morelli L, Rodriguez-Concepcion M: **A fast and simplified method to estimate bioaccessibility of carotenoids from plant tissues**. *Methods Enzymol* 2021, https://doi.org/10.1016/bs.mie.2021.10.007.

33. * Pasin F, Menzel W, Daros JA: **Harnessed viruses in the age of metagenomics and synthetic biology: an update on infectious clone assembly and biotechnologies of plant viruses**. *Plant Biotechnol J* 2019, **17**:1010–1026.

Revision of new technologies and current achievements to harness plant virus towards synthetic biology.

34. Wang M, Gao S, Zeng W, Yang Y, Ma J, Wang Y: **Plant virology delivers diverse toolsets for biotechnology**. *Viruses* 2020, **12**: 1338.

35. Daros JA: **Use of potyvirus vectors to produce carotenoids in plants**. *Methods Mol Biol* 2020, **2083**:303–312.

36. * Marti M, Diretto G, Aragones V, Frusciante S, Ahrazem O, Gomez-Gomez L, Daros JA: **Efficient production of saffron crocins and picrocrocin in Nicotiana benthamiana using a virus-driven system**. *Metab Eng* 2020, **61**:238–250.

First heterologous production of highly appreciated saffron apocarotenoids outside the species and tissues in which they naturally accumulate.

37. Aragones V, Pérez de Castro AM, Cordero T, Cebolla J, López del Rincón C, Pico MB, Daros JA: **A Watermelon mosaic virus clone tagged with the yellow visual maker phytoene synthase facilitates scoring infectivity in melon breeding programs**. *Eur J Plant Pathol* 2019, **153**:317–323.

38. Houhou F, Cordero T, Aragonés V, Martí MC, Cebolla-Cornejo J, Pérez de Castro A, Rodriguez-Concepcion M, Picó B, Daròs JA: **Carotenoid and tocopherol fortification of zucchini fruits using a viral RNA vector**. *bioRxiv* 2021, https://doi.org/10.1101/2021.02.18.431807.

39. Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP: **Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus**. *Plant J* 2002, **30**: 415–429.

40. * Dommes AB, Gross T, Herbert DB, Kivivirta KI, Becker A: **Virus-induced gene silencing: empowering genetics in non-model organisms**. *J Exp Bot* 2019, **70**:757–770.
Systematic revision of VIGS systems to apply to different botanical families.

41. Orzaez D, Medina A, Torre S, Fernández-Moreno JP, Rambla JL, Fernández-Del-Carmen A, Butelli E, Martin C, Granell A: **A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation**. *Plant Physiol* 2009, **150**:1122–1134.

42. Fantini E, Falcone G, Frusciante S, Giliberto L, Giuliano G: **Dissection of tomato lycopene biosynthesis through virus-induced gene silencing**. *Plant Physiol* 2013, **163**:986–998.

43. Wang Z, Zhang L, Dong C, Guo J, Jin L, Wei P, Li F, Zhang X, Wang R: **Characterization and functional analysis of phytoene synthase gene family in tobacco**. *BMC Plant Biol* 2021, **21**:32.

44. Jiang M, Zhang F, Yuan Q, Lin P, Zheng H, Liang S, Jian Y, Miao H, Li H, Wang Q, *et al.*: **Characterization of BoaCRTISO reveals its role in carotenoid biosynthesis in Chinese kale**. *Front Plant Sci* 2021, **12**:662684.

45. Fu DQ, Meng LH, Zhu BZ, Zhu HL, Yan HX, Luo YB: **Silencing of the SlNAP7 gene influences plastid development and lycopene accumulation in tomato**. *Sci Rep* 2016, **6**:38664.

46. Zhao X, Yuan X, Chen S, Meng L, Fu D: **Role of the tomato TAGL1 gene in regulating fruit metabolites elucidated using RNA sequence and metabolomics analyses**. *PLoS One* 2018, **13**:e0199083.

47. D'Amelia V, Raiola A, Carputo D, Filippone E, Barone A, Rigano MM: **A basic Helix-Loop-Helix (SlARANCIO), identified from a Solanum pennellii introgression line, affects carotenoid accumulation in tomato fruits**. *Sci Rep* 2019, **9**:3699.

48. Shivram H, Cress BF, Knott GJ, Doudna JA: **Controlling and enhancing CRISPR systems**. *Nat Chem Biol* 2021, **17**:10–19.

49. Ronspies M, Dorn A, Schindele P, Puchta H: **CRISPR-Cas-mediated chromosome engineering for crop improvement and synthetic biology**. *Native Plants* 2021, **7**:566–573.

50. Zhu H, Li C, Gao C: **Applications of CRISPR-Cas in agriculture and plant biotechnology**. *Nat Rev Mol Cell Biol* 2020, **21**:661–677.

51. ** Ellison EE, Nagalakshmi U, Gamo ME, Huang PJ, Dinesh-Kumar S, Voytas DF: **Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs**. *Native Plants* 2020, **6**:620–624.
First description of transformation-free heritable gene editing using viral vectors.

52. Uranga M, Aragones V, Selma S, Vazquez-Vilar M, Orzaez D, Daros JA: **Efficient Cas9 multiplex editing using unspaced sgRNA arrays engineering in a Potato virus X vector**. *Plant J* 2021, **106**:555–565.

53. Ma X, Zhang X, Liu H, Li Z: **Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9**. *Native Plants* 2020, **6**:773–779.

54. Uranga M, Vazquez-Vilar M, Orzáez D, Daròs JA: **CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors**. *CRISPR J* 2021, **4**:761–769.

55. Pausch P, Al-Shayeb B, Bisom-Rapp E, Tsuchida CA, Li Z, Cress BF, Knott GJ, Jacobsen SE, Banfield JF, Doudna JA: **CRISPR-CasPhi from huge phages is a hypercompact genome editor**. *Science* 2020, **369**:333–337.

56. Xu X, Chemparathy A, Zeng L, Kempton HR, Shang S, Nakamura M, Qi LS: **Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing**. *Mol Cell* 2021, **81**:4333–4345.

57. Kannan S, Altae-Tran H, Jin X, Madigan VJ, Oshiro R, Makarova KS, Koonin EV, Zhang F: **Compact RNA editors with small Cas13 proteins**. *Nat Biotechnol* 2021, https://doi.org/10.1038/s41587-021-01030-2.

58. Demirer GS, Silva TN, Jackson CT, Thomas JB, We D, Rhee SY, Mortimer JC, Landry MP: **Nanotechnology to advance CRISPR-Cas genetic engineering of plants**. *Nat Nanotechnol* 2021, **16**:243–250.

59. Smyth SJ, McHughen A, Entine J, Kershen D, Ramage C, Parrott W: **Removing politics from innovations that improve food security**. *Transgenic Res* 2021, **30**:1–12.

60. Brewer HC, Hird DL, Bailey AM, Seal SE, Foster GD: **A guide to the contained use of plant virus infectious clones**. *Plant Biotechnol J* 2018, **16**:832–843.

61. Wang JW, Cunningham FJ, Goh NS, Boozarpour NN, Pham M, Landry MP: **Nanoparticles for protein delivery in planta**. *Curr Opin Plant Biol* 2021, **60**:102052.