Engineering synthetic pathways for β-carotene production in *Nicotiana benthamiana* leaves.

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Author: Diego Reig López

Tutor: Manuel Rodríguez Concepción

UPV Tutor: María Pilar López Gresa

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Tutor: Manuel Rodríguez Concepción

UPV Tutor: María Pilar López Gresa

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Summary: Carotenoids are plastidial pigments of nutritional interest as vitamin A precursors and health-promoting phytonutrients and antioxidants. Several strategies have been developed to boost carotenoid production in green leaves. Namely, the overexpression of crtB, a bacterial gene encoding a phytoene synthase, triggers the differentiation of leaf chloroplasts into chromoplast-like plastids that accumulate high levels of β-carotene, the main pro-vitamin A carotenoid. Bacterial genes can also be used to build an entire synthetic pathway to produce β-carotene in the cytosol of leaf cells. The first aim of this work was to analyze strategies to further improve the production of β-carotene in the artificial chromoplasts that develop in crtB-expressing leaves. For that end, enzymes known to enhance the supply of their natural precursors were co-expressed with crtB in *Nicotiana benthamiana* leaves by agroinfiltration. Then, this strategy was combined with the synthetic cytosolic pathway to boost overall β-carotene production and capacity.

Key words: *Nicotiana benthamiana*, Carotenoids, β-carotene, antioxidant, biofortification.

Resumen: Los carotenoides son pigmentos plastídicos de interés nutricional como precursores de la vitamina A y como antioxidantes y fitonutrientes saludables. Se han desarrollado diversos métodos para incrementar la producción de carotenoides en hojas verdes. Concretamente, la sobreexpresión de crtB, un gen bacteriano que codifica una fitoeno sintasa, desencadena la diferenciación de los cloroplastos de las hojas en estructuras similares a cromoplastos que acumulan altos niveles de β-caroteno, el carotenoide de la provitamina A. Por otro lado, genes bacterianos también se pueden usar para la construcción de una ruta sintética entera del β-caroteno en el citosol de células foliares. El primer objetivo de este trabajo fue analizar estrategias para mejorar la producción de β-caroteno en los cromoplastos artificiales que se obtienen de las hojas que expresan crtB. Para ello, enzimas aumentan el suministro de sus precursores se coexpresaron junto con crtB en hojas de *Nicotiana benthamiana* mediante agroinfiltración. Después se combinó esta estrategia con la ruta citosólica sintética para aumentar la producción general de β-caroteno y la capacidad antioxidante.

Palabras clave: *Nicotiana benthamiana*, Carotenoides, β-caroteno, antioxidante, bioenriquecimiento.

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1. Introduction.

The sessile nature of plants has conducted their evolution into developing sophisticated biochemical machinery to produce a wide array of metabolites that help them perform their vital functions as well as all their communication functions. The evolution of animals alongside plants has made them reliant on many of these plant compounds which they are unable to synthesize. The growing understanding of plant metabolism and physiology has also allowed humans to utilize for their own benefit some of these compounds in many different industries such as agriculture, manufacturing, or pharmaceuticals, so they hold a great deal of value.

1.1. Role of isoprenoids in plants.

Also known as terpenoids, isoprenoids are one of the largest families of plant metabolites, with nutritional and industrial interest. They show a wide variety of chemical structures (Vickers et al., 2014). Because of this diversity, they occupy many different niches in their functions within plants. Some of them are considered primary metabolites and are present in almost all plant species because they have an essential role on vital processes such as photosynthesis, and respiration (Rodríguez-Concepción, 2014). They include: chlorophylls (pigments responsible for light harvesting), phylloquinones and plastoquinones (prenylquinones participating in the electron transport chain system), carotenoids (pigments contribute to light harvesting and photoprotection), tocopherols (tocochromanols that protect membranes from oxidative stress), ubiquinone (a prenylquinone that participates in the electron transport chain in mitochondria, synthesized is the cytosol), phytosterols (compounds involved in membrane fluidity and stability) and many plant hormones, like gibberellins, abscisic acid, strigolactones, brassinosteroids and cytokinins.. Other isoprenoids are considered secondary metabolites and are mainly involved in plant-environment interaction. They are usually not found in all organs of the plant or in every plant and exhibit a high degree of specialization. (Rodríguez-Concepción & Boronat, 2015; Tholl, 2015). Carotenoids serve a double role being involved in primary functions such as light harvesting and photosystem protection but also participating in fruit and flower coloration, helping the plant attract its pollinators or seed dispersers (Rodríguez-Concepción & Boronat, 2015).

1.2. Role of isoprenoids and β-carotene in human health.

Besides having a vital role in plants, isoprenoids also have a great impact on animal and human health and nutrition. Most animals cannot produce isoprenoids, so they rely on the ones produced by photosynthetic organisms and assimilate them through the diet. These compounds and their derivatives have been reported to have anti-inflammatory, anti-oxidative, antiaggregatory, anti-coagulative effects, anti-tumor, sedative, and analgesic activities and thus have a very important health related function (Zhao et al., 2016). Most importantly, many isoprenoids are essential nutrients or precursors to vitamins A, K, E, coenzyme Q10 and many others. The main biological function of some carotenoids in mammals resides, for example, in their role as precursors of vitamin A, which is fundamental for vision, growth, cell differentiation, and other physiological processes (Olson, 1996; Rodriguez-Concepcion et al., 2018). Not all the carotenoids present the suitable structure for conversion to vitamin A. In fact, only carotenoids with at least one β-type ring without any oxygen and with a polyenic chain at least 11 carbon atoms long are potential precursors for this compound. Thus, the most relevant provitamin A carotenoids, either because of their high activity or food distribution are α- and β-carotene, some xanthophylls, and some apocarotenoids. In particular, β-carotene is the one with higher provitamin A activity because each molecule has two β-rings and produces two retinal molecules that are reduced to vitamin A (Retinol) (Goodman & Huang, 1965).

Retinol or vitamin A is of great importance to the correct development of sight because of its key role in the visual cycle: all-trans-retinol is converted into 11-cis-retinol and then into 11-cis-retinal

by retinol dehydrogenases. This compound binds to an opsin in the photoreceptors to form rhodopsin. The isomerization of this 11-cis retinal to all-cis-retinal by the rhodopsin reaction to light exposure is what sends the visual signal. In order to have a constant pool of usable 11-cis-retinal, all-cis-retinal is cleaved from the protein, converted by different retinol dehydrogenases into all-trans-retinol and this molecule is transported to the retinal pigment epithelium to start the cycle all over again. (Parker & Crouch, 2010). Other carotenoids such as lutein and zeaxanthin also have an important role to play in the retina and lens, where they function as light filters, reducing the amount of blue light that reaches the photoreceptors by as much as 40%, protecting them from excessive photooxidation. (Ma & Lin, 2010).

Carotenoids are also highly considered because of their antioxidant properties. In fact, carotenoids are stored in lipidic membranes and prevent and reverse their oxidation by binding to ROS and to lipid peroxyl radicals. This function is shared with other isoprenoids such as phylloquinones and especially tocopherols. Studies have shown that a carotenoid, phylloquinone and tocopherol-rich diet reduces the risk of cardiovascular disease and cancer. However, this is only the case when taken as part of a food matrix not in supplements. (Niranjana et al., 2015).

1.3. Carotenoid biosynthesis.

All isoprenoids are synthesized from two common precursors, the 5-carbon isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). In plants, these compounds can be derived from two separate metabolic pathways which occur in different cellular compartments: the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol 4-phosphate (MEP) pathway (Rodríguez-Concepción & Boronat, 2002) (Figure 1).

The first step in the cytosolic MVA pathway is the reversible condensation of two acetyl-CoA molecules to form acetylacetyl-CoA. Another acetyl-CoA molecule is added to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This molecule is then reduced in two steps by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) using NADPH, converting it into mevalonic acid (MVA). In two subsequent reactions, MVA is doubly phosphorylated, producing MVA 5-diphosphate. The last step in IPP production consists in an ATP-dependent decarboxylation. For further isoprenoid production, IPP is isomerized to DMAPP, which is the substrate for the production of downstream isoprenoids such as ubiquinone and sterols. This pathway is shared by a great number of organisms such as bacteria, archaea, yeasts, and animals. The MEP pathway is also not unique to plants, as it is found in bacteria and algae. However, higher plants and some algae are unique in retaining and using both pathways for isoprenoid synthesis (Vranová et al., 2013). IPP and DMAPP produced in the cytosol can be imported into the plastid and vice versa, although this exchange takes place at a very low rate. The identity of the transporters remains unknown (Pick & Weber, 2014).

The MEP pathway starts with the condensation of D-glyceraldehyde 3-phosphate (GAP) and pyruvate, producing a 1-deoxy-D-xylulose 5-phosphate (DXP) and a CO_2 molecule. This step is catalyzed by (DXS) and commits carbon to the pathway. In the next step, DXP reductoisomerase (DXR) rearranges and reduces DXP into MEP, which is then converted into 4-(cytidine 5'-diphospho)-2-methyl-D-erythritol 4-phosphate with the addition of a CDP group. This molecule is then phosphorylated and cyclized, losing a CMP group. The resulting product is called 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP). MEcPP is then reduced two times with the help of NADPH to produce IPP and DMAPP in a ratio between 4:1 and 5:1 (Rodríguez-concepción, 2010; Vranová et al., 2013). MEP-derived can isomerize to one another thanks to plastidial isoforms of IPP isomerase (IPPI), creating an equilibrium. This pathway is generally used in plants to produce monoterpenes, diterpenes and photosynthesis-related isoprenoids such as chlorophylls, phylloquinones, plastoquinones, tocopherols and carotenoids.

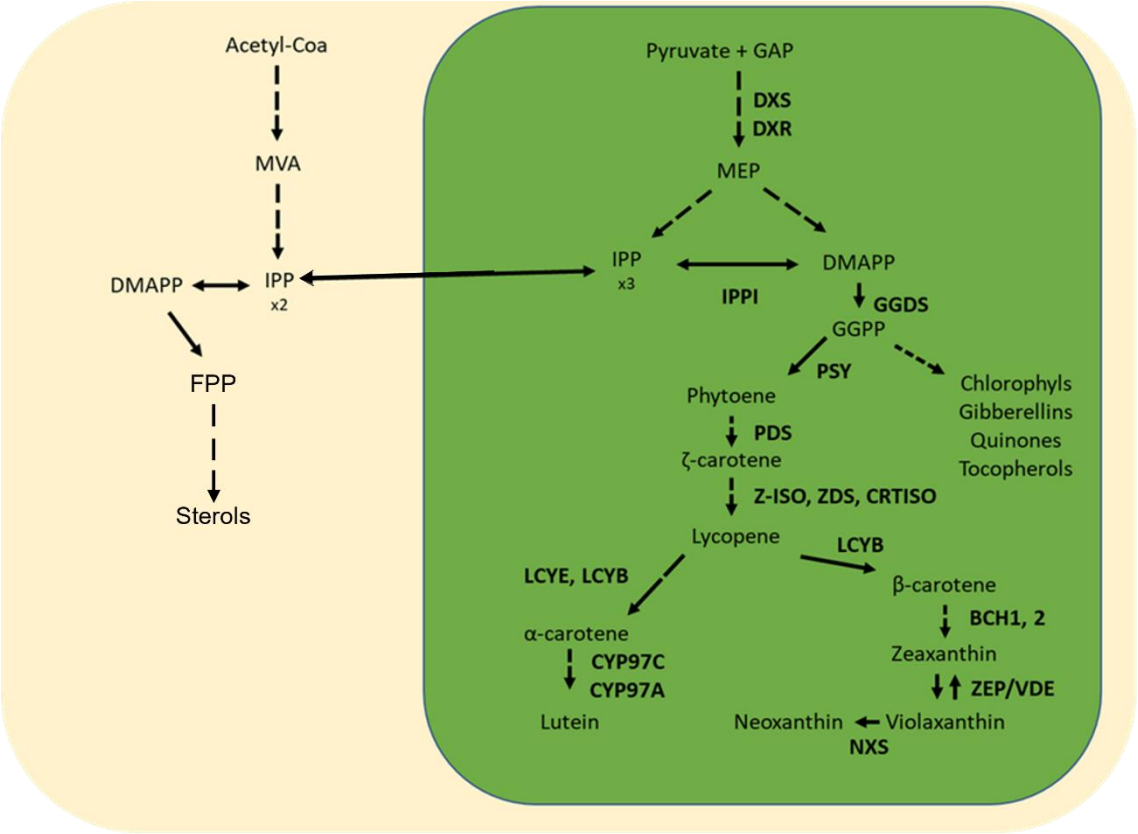

Figure 1: Simplified schematic representation of isoprenoid biosynthesis in plants. MVA pathway is presented in a tan box representing the cytosol and the MEP pathway is in a green box representing the plastid. Dashed lines symbolize multiple reaction steps. Enzyme names are in bold font. MVA mevalonate, IPP isopentenyl diphosphate, DMAPP dimethylallyl pyrophosphate, GGPP geranylgeranyl diphosphate, FPP farnesyl diphosphate, GAP D-glyceraldehyde 3-phosphate, MEP methylerythritol 4-phosphate, DXS 1-deoxy-D-xylulose 5-phosphate synthase, DXR 1-deoxy-D-xylulose 5-phosphate reductoisomerase, IPPI IPP isomerase, GGDS GGPP desaturase, PSY phytoene synthase, PDS phytoene desaturase, Z-ISO ζ-carotene isomerase, ZDS ζ-carotene desaturase, CRTISO carotenoid isomerase, LCYB lycopene β-cyclase, LCYE lycopene ε-cyclase, CYP97A cytochrome P450 carotene β-hydroxylase, CYP97C cytochrome P450 carotene ε-hydroxylase, BCH β-carotene hydrolase, ZEP zeaxanthin epoxidase, VDE violaxanthin de-epoxidase, NXS neoxanthin synthase.

The 5C units IPP and DMAPP are condensed to form 10C geranyl diphosphate (GPP) by GPP synthase in plastids as a precursor for monoterpenes. One more IPP molecule can be added by farnesyl diphosphate (FPP) synthase to produce FPP in the cytosol and mitochondria as a precursor of sesquiterpenes and triterpenes (including sterols). If one more IPP unit is added, geranylgeranyl diphosphate (GGPP) is produced. This step is catalyzed by GGPP synthase (GGPPS) isoforms located in different cell compartments. In the plastid, GGPP acts as a precursor for the synthesis of gibberellins and other diterpenoids, chlorophylls, phylloquinone, plastoquinone, plastochromanols, tocopherols and carotenoids.(Vranová et al., 2013).

Focusing on carotenoids, they are only synthesized in plastids (Sun et al., 2017). GGPP is the direct precursor to carotenoid synthesis. GGPPS can associate itself with different enzymes to dedicate the GGPP produced to different biosynthetic pathways. For instance, GGPPS can associate itself with phytoene synthase (PSY), the first dedicated enzyme in carotenoid production, to direct metabolic flux towards that pathway (Camagna et al., 2019; Ruiz-Sola et al., 2016). The first step in said pathway is the condensation of two GGPP molecules to produce the first 40C carotenoid, 15-cis-phytoene. This reaction is the main rate-limiting step in carotenoid synthesis.

From this point, 5 reactions and 4 different enzymes are needed in plants to produce all-trans-lycopene: first, phytoene desaturase (PDS) performs two consecutive desaturations to create 9,15-di-cisphytofluene and then 9,15,90-tri-cis-ζ-carotene, then ζ-carotene isomerase (Z-ISO) converts it into 9,90-di-cis-ζ-carotene, which is then desaturated via ζ-carotene desaturase (ZDS) to introduce two additional double bonds and form 7,9,9',7'-tetracis-lycopene (prolycopene). Finally, prolycopene is isomerized into all-trans-lycopene by carotenoid isomerase (CRTISO). For PDS and ZDS to work they must be associated with plastidial terminal oxidase (PTOX) and oxidized plastoquinone.

The lycopene produced is then cyclized, and this presents a branching point in the carotenoid biosynthetic pathway depending on how those rings are formed (Figure 1). Two enzymes participate in the formation of those rings, lycopene ε-cyclase (LCYE), which produces ε-rings, and lycopene β-cyclase (LCYB) which produces β-rings. When LCYB makes two β-rings (one in each of the two ends of the linear lycopene molecule, β-carotene is produced, and when LCYB and LCYE produce one ring each, an α-carotene molecule is obtained.

Carotenoid biosynthesis can further continue, to form the xanthophylls. Starting from α-carotene, two heme-containing cytochrome P450 type hydroxylases (CYP97C and CYP97A) hydroxylate the two rings to produce lutein, the most abundant xanthophyll in plants and the endpoint of this pathway branch. On the other side, starting from β-carotene, two nonheme β-ring hydroxylases (BCH1 and BCH2) sequentially hydroxylate both rings to produce zeaxanthin. This compound is then epoxidized by zeaxanthin epoxidase (ZEP) to sequentially produce antheraxanthin and then violaxanthin. Violaxanthin can be reversed back to zeaxanthin by violaxanthin de-epoxidase (VDE) as part of the xanthophyll cycle. The final step in the core xanthophyll biosynthetic pathway is the transformation of violaxanthin into neoxanthin by neoxanthin synthase (NXS) (Sun et al., 2020).

Carotenogenesis is not a process unique to photosynthetic organisms such as plants, as it is also present in non-photosynthetic fungi and bacteria. Bacteria however tend to have fewer genes for the same pathway, as their enzymes can catalyze reactions that take multiple steps in plants. For the production of β-carotene, bacteria use IPP and DMAPP derived either from the MVA pathway or from the MEP pathway and only need a GGPP synthase (crtE), a phytoene synthase (crtB), a phytoene desaturase (crtI) and a lycopene β-cyclase (crtY), a process that would involve 7 enzymes in plants (Sun et al., 2020).

1.4. Storage of carotenoids in plants.

Carotenoids are not only produced but also stored in plastids. Plastids are ubiquitous and essential organelles in plants. A according to endosymbiotic theory, they are descendant from a common cyanobacterial ancestor and as such contain their own genome. Plastids, unlike mitochondria, are very malleable and adaptative, displaying a wide array of morphologies and chemistries depending on the physiological state of the plant or the plant's need. Based on their morphology and functions plastids are classified in distinct subtypes.

Proplastids are small, colorless, and undifferentiated plastids from which all other plastids derive. They are mostly present in meristematic tissues. Etioplasts are intermediate plastids between proplastids and chloroplasts. They occur when plants are etiolated or grown in darkness and lack any chlorophyll, but contain small amounts of carotenoids. Chloroplasts are green plastids where photosynthesis takes place. They have fully developed thylakoids, are the energetic machinery of the plants and are debited to several signaling and developmental functions. Leucoplasts are colorless storage plastids that can be separated in different categories depending on which compound they store: amyloplasts if they accumulate starch, elaioplasts if they accumulate lipids and proteoplasts if they store proteins. These plastids are usually present in storage tissues such as tubers, roots, and seeds. Chromoplasts are plastids specialized in the accumulation of

carotenoids whose color ranges from yellow to red. They have internal structures that accumulate carotenoids and are usually present in ripe fruits and flowers but only occasionally in leaves. They can derive from proplastids but usually differentiate from chloroplasts whose internal membranes disaggregate and lose photosynthetic competence. Finally, gerontoplasts are the product of chloroplast degradation during senescence. They have a disorganized internal structure and present a large number of big plastoglobuli that typically store chlorophyl degradation products, unlike the plastoglobuli found in chromoplasts which accumulate carotenoids (Sadali et al., 2019).

Almost all types of plastids produce and store carotenoids to some extent. However, chromoplasts are the most suited to their accumulation. Chloroplasts naturally transform into chromoplasts during the development of many flowers and the ripening of many fruits in a process called chromoplastogenesis. This process is coupled with the loss of photosynthetic competence, as thylakoid membranes disintegrate, an overaccumulation of carotenoid pigments and tocopherols and an overall increase in sink strength (Egea et al., 2010).

1.5. Strategies for β-carotene biofortification.

The growing understanding of the carotenoid biosynthetic pathway and of the health benefits of β-carotene and other carotenoids have created the opportunity to engineer biofortified plant products to use as nutraceuticals.

There have been many approaches to increase β-carotene or carotenoid content in plants. Perhaps the most notable example is the Golden Rice initiative: a genetically engineered rice which has the genes necessary for β-carotene production introduced into its genome, as rice does not naturally express those genes in the endosperm. In the first version of Golden Rice a *PSY* gene from daffodil under an endosperm-specific glutelin promotor was introduced via *Agrobacterium*-mediated transformation, as well as the *crtI* gene from *Pantoea ananatis* under the constitutive CaMV (cauliflower mosaic virus) 35S promoter and a LCYB gene from daffodil under the glutelin promotor. This strategy produced at least one line with 1.6 μg/g of carotenoid content in the endosperm, most of that being β-carotene (Ye & Beyer, 2000). The second version of the product saw a major improvement in pro-vitamin A content after many plant *PSY* genes were tested and the daffodil *PSY* gene was replaced with a maize one. This strategy was able to produce in one line 37 μg/g (Paine et al., 2005).

Other strategies have included engineering transgenic cassava, a major subsistence crop in sub-Saharan Africa to express the *DXS* gene from *Arabidopsis thaliana,* and the bacterial *crtB* gene from *Pantoea ananatis,* resulting in root carotenoid levels of ≤50 μg/g, a 15 to 20-fold increase compared to non-transgenic plants. This also granted the roots an increased shelf life (also demonstrated in tomato (Diretto et al., 2020)), but lowered by half the dry matter content, showcasing the relationship between β-carotene biofortification and starch metabolism in starch-rich organs (Beyene et al., 2018). A similar strategy to the Golden Rice project was proved in banana using a *PSY* gene from Fe'i banana, yielding up to 55μg/g of β-carotene (Paul et al., 2017) A greater effect was demonstrated in soybean, where the transgenic expression of *Pantoea ananatis crtB* gene resulted in a β-carotene content of 845μg/g (Schmidt et al., 2015). Other efforts have been focused on vitamin E biofortification, supplemental to β-carotene biofortification, as it prevents the oxidation and therefore loss of active β-carotene, a major hurdle in some projects. This was achieved in sorghum (Che et al., 2016). All these strategies have in common the utilization on a phytoene synthase gene, as it is believed to be the main rate-limiting factor in carotenogenesis. But perhaps most importantly they showcase the importance of applying this technology to major staple foods in the places where vitamin A deficiency is most predominant.

A different approach is based on silencing the first step in the ε-β branch, LCYE, specifically in the potato tuber, to redirect the metabolic flux towards the production of β-carotene, achieving up to a 14-fold increase compared to wild type, although not a very high yield in absolute terms

(Diretto et al., 2006). Silencing the expression of the two non-heme β-carotene hydroxylases, BCH1 and BCH2, achieved a fold change of up to 38 (Diretto et al., 2007). LCYE-silencing β-carotene biofortification was also attempted in canola seeds with some success, but β-carotene levels dropped as the seed matured (Yu et al., 2008).

All these strategies have focused on carotenoid accumulation in non-green edible parts of the plant. However, another approach shows that crtB can be transiently overexpressed in leaves, achieving almost double the amount of carotenoids compared to a normal leaf (Llorente et al., 2020). More than that, this experimental system (which is one of the founding grounds of the present TFG work) helped to propose a mechanistic model for the differentiation of chloroplasts into chromoplasts. Overexpressing crtB to overcome PSY's endogenous regulation produces a considerable amount of phytoene, which in turn interferes with the photosynthetic capacity of chloroplasts, acting as a metabolic threshold switch mechanism that weakens chloroplast identity. This step makes chloroplast competent for the second required step, increase in downstream carotenoid levels, which pushes the chloroplast to transition into a chromoplast-like structure, suitable for carotenoid storage. This strategy has been demonstrated to double total carotenoid leaf contents in lettuce, zucchini, *Arabidopsis thaliana* and *Nicotiana benthamiana.* When applied in an inducible manner, it should allow to turn fully grown green parts of plants into carotenoid-rich food and unusable parts of plants such as the aerial parts of potatoes into biofortified feed after potato harvest.

Another approach that will also be used in this TFG work is the construction of a synthetic carotenoid synthesis pathway to accumulate carotenoids in the cytosol. This was achieved first in *Nicotiana tabacum* leaves by using a viral vector expressing the *Pantoea ananatis* genes encoding crtE, crtB and crtI to produce lycopene (Majer et al., 2017). An optimized strategy was developed in *Nicotiana benthamiana* leaves by transiently overexpressing the same genes together with a truncated version of HMGR (tHMGR) to enhance the supply of MVA-derived IPP and DMAPP precursors. As a result, engineered leaves accumulated phytoene and lycopene in the cytosol at levels that were similar to those of endogenous chloroplast carotenoids and resembled those found in chromoplasts such as those of tomato ripe fruit (Andersen et al., 2021).

2. Objectives.

The main objective of this work was to increase the carotenoid content in *Nicotiana benthamiana* leaves beyond that achieved in the host lab by using crtB to trigger chromoplastogenesis and a cytosolic pathway to produce carotenoids in extraplastidial locations. In particular, the work was focused on the biofortification of leaves in β-carotene, the main pro-vitamin A carotenoid.

The first specific objective was the upregulation of the MEP pathway flux to improve the supply of IPP and DMAPP in chloroplasts by overexpressing, in addition to crtB, a DXS protein, (push strategy). An important part of this objective was to determine a suitable DXS candidate for this purpose.

A second specific goal was to combine the previous approach with a synthetic cytosolic β-carotene production pathway for the simultaneous production of this carotenoid in plastids (i.e. crtB-triggered chromoplasts) and the cytosol (from MVA pathway precursors) As a complementary activity, the antioxidant capacity of extracts of leaves resulting from these experiments was evaluated as it is an interesting aspect of β-carotene fortified plants to consider.

3. Materials and methods.

3.1. Plant material and growth conditions.

Nicotiana benthamiana used for the transient expression assays were grown in a greenhouse under standard long-day conditions (LD, 14 h light at 26 ± 1°C and 10 h dark at 21 ± 1°C).

3.2. Gene constructs.

Constructions used in this study were available in the host laboratory (Table 1). Constructions for the overexpression of the different isoforms of DXS (35S:SlDXS1:myc, 35S:SlDXS2:RFP and 35S:AtDXS:GFP) were a kindly provided by Xueni Di (IBMCP, Valencia, Spain).

Table 1: Gene constructs used on the transient expression assays.

Construct name	Vector	Promoter	Gene	Tag	Organism
HcPro	pGWB702	35S	*HCPRO*	-	Watermelon mosaic virus
CrtB	pGWB405	35S	*crtB*	GFP	*Pantoea ananatis*
DXS1	pGWB21	35S	*dxs1*	10xMyc	*Solanum lycopersicum*
DXS2	pGWB454	35S	*dxs2*	RFP	*Solanum lycopersicum*
AtDXS	pGWB405	35S	*DXS*	GFP	*Arabidopsis thaliana*
(t)HMGR*	pEAQ-USER	35S	*HMGR1*	-	*Arabidopsis thaliana*
CrtE	pGWB405	35S	*crtE*	GFP	*Pantoea ananatis*
CrtI	pGWB405	35S	*crtI*	GFP	*Pantoea ananatis*
(p)CrtI*	pCAMBIA1304	35S	*crtI*	GFP	*Pantoea ananatis*
CrtY	pGWB405	35S	*crtY*	-	*Pantoea ananatis*

*(p) stands for transit peptide, (t) for truncated (Cankar et al., 2015).

3.3. Transient expression assays.

For agroinfiltration assays, the second or the third youngest leaves of 4-5-week-old *N. benthamiana* plants were infiltrated in the abaxial part of leaves. *A. tumefaciens* GV3101 strains were transformed with constructs of interest and grown on Luria-Bertani (LB) agar plates with the corresponding antibiotics at 28°C for 3 days. A single colony per construct was inoculated in 5 mL antibiotic-complemented LB media and incubated overnight at 28 °C in 300 rpm continuous agitation. 200µL of the grown culture were then inoculated in 15 mL of LB media and incubated overnight at 28 °C in 300 rpm continuous agitation the day before performing agroinfiltration. OD600 of each liquid culture was spectrophotometrically measured and then cultures were centrifuged at 4400 rpm for 10 min. Bacterial pellets were resuspended in infiltration buffer (10 mM MES pH 5.5-6, 10 mM MgCl2, 150µM acetosyringone). Cultures were mixed in identical proportions for the various combinations to reach a final OD600 of 0.5. Gene silencing was prevented by co-agroinfiltration with an agrobacterium strain EHA101 carrying the helper component protease (HcPro) of the watermelon mosaic virus (WMV) in plasmid HcProWMV-pGWB702 (kindly provided by Juan José López-Moya and Maria Luisa Domingo-Calap (CRAG-Barcelona, Spain)).

3.4. Metabolite analysis.

Leaf carotenoids, were extracted in 2mL Eppendorf tubes from 4mg of freeze-dried leaf tissue, using 375µl of methanol as extraction solvent and 25µl of 10 % (w/v) solution of canthaxanthin in chloroform (Sigma) as internal standard. After vortexing for 10 s and lysing the tissue with 4 mm glass beads for 1 min at 30 Hz in a TissueLyser II (QIAGEN), 400µL of Tris-NaCl pH 7.5 were added followed by 1 min of TissueLyser. After this, samples were added with 800µl of

chloroform and processed again with the TissueLyser for 1 min. Samples were then centrifuged for 5 min at 13,000 rpm and 4 °C. Organic phase (lower) was transferred in a new tube and evaporated for 1 h using a SpeedVac system (Eppendorf Concentrator plus). Extracted metabolites were then completely re-dissolved in 200µL of acetone by sonicating them for 15 seconds and filtered with 0.2µm filters into amber-colored 2mL glass vials. Separation and detection of isolated compounds was performed from 10µL of prepared samples using Waters 2695 series HPLC system (Waters corporation) with a YMC C30 Carotenoid 250 x 4,6mm 3µm column. Working conditions are a flow of 1mL/min, 25ºC and solvent composition is established on Table 2. The HPLC equipment was coupled to a Photometric Diode Array (PDA) detector (Waters 996), allowing the detection of the full UV-visible absorption spectra of the different metabolites. Peak areas of carotenoids at 472 nm (lycopene, lutein, β-carotene, violaxanthin, neoxanthin, canthaxanthin) or 290 nm (phytoene) were determined using the Waters Empower 3 software.

The quantification of the compounds of interest was done by using a concentration curve built with a commercial standard (Sigma).

Table 2: flow gradient composition during HPLC analysis. Solvent A: methanol, solvent B: H_2O:Methanol:ammonium acetate (80%/20%/0,1%), solvent C: tert-butyl methyl ether.

Time (min)	Solvent A%	Solvent B%	Solvent C%
0	90	0	10
16	90	0	10
20	80,8	4,2	15
38	33,5	1,5	65
38,1	0	0	100
41	0	0	100
44	90	0	10
50	90	0	10

3.5. Antioxidant capacity assay.

Metabolite extracts prepared as described above were diluted in 400 µL of diethyl-ether and saponified by adding 100 µL of 10%(w/v) KOH in methanol to avoid interference from chlorophylls in the assay. Samples were left shaking for 30 min at 4 °C in darkness and then diluted with 400 µL of milliQ water before centrifugation for 5 min at 13 000 rpm and 4 °C. The upper phase was collected, dried in a SpeedVac, and resuspended in 200 µL of acetone. ABTS assay was performed by preparing an $ABTS^{o+}$ solution by adding to a 7mM ABTS solution (SIGMA), ammonium persulfate (APS) to a final concentration of 2,45mM, letting the oxidation reaction occur overnight in darkness. The solution was then diluted with water to reach an absorbance of 0.700±0.020. 100µLs of sample were added to 1 ml of ABTSo+ and absorbance at 734 nm was measured after 4 minutes. The obtained values were plotted against a standard curve made by substituting the sample with increasing 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) concentrations: 11µM, 22µM, 44µM, 88µM and 176µM; to calculate the antioxidant activity as µM of Trolox equivalents.

3.6. Photosynthetic measurements.

Photosynthetic efficiencies for lettuce plants were assessed by measuring chlorophyll fluorescence with a Handy-GFP fluorcam (Photon system instruments (PSI)) fluorometer by using an actinic light (AL) corresponding to 21 photosynthetic active radiation (PAR) as it is the last value able to generate a response in the crtB-infiltrated areas before having a null photosynthetic activity. The effective quantum yield of PSII (ϕPSII, ΔF/Fm') was measured as (Fm'−Fs)/Fm'.

4. Results and discussion.

4.1. DXS increases phytoene but not downstream carotenoid content.

Twelve *Nicotiana benthamiana* leaves were agroinfiltrated as previously described in materials and methods to assess the effect of DXS overexpression in carotenoid production. In the first experiment, we used the tomato DXS1 isoform. Half of the leaves was agroinfiltrated with constructs harboring a GFP control on their left half and crtB on their right half, while the other three leaves received DXS1 alone on their left half and a combination of DXS1 and crtB on their right, as shown in Figure 2A. Samples were collected 4 days post-infiltration (dpi) for HPLC analysis.

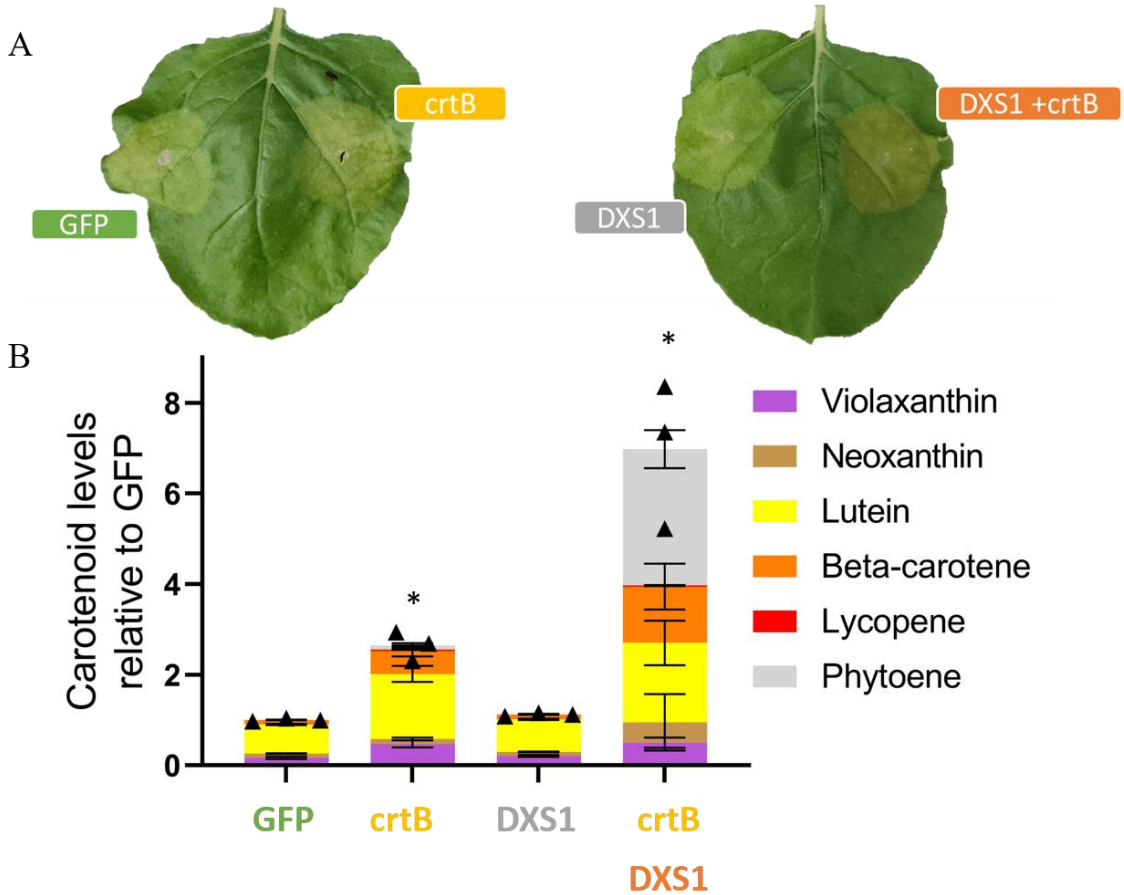

Figure 2: A) *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and samples were collected at 4 dpi for HPLC analysis. B) Carotenoid levels of leaves expressing either GFP, crtB, DXS1 or DXS1 and crtB. Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$). Triangles mark the total carotenoid values of individual replicates.

DXS1 on its own did not have a significant effect on carotenoid content (Figure 2B), likely because increasing the precursor supply could not be translated into phytoene and downstream carotenoids by a tightly regulated endogenous PSY, which is still the rate limiting factor of the pathway (Sun et al., 2020). Combining DXS1 and crtB yielded 55% more downstream carotenoids than just crtB (a statistically significant difference) (Figure 2B). However, this result, as shown in subsequent experiments, is highly variable. By contrast, a striking and highly reproducible overaccumulation of phytoene was detected in all experiments containing DXS1 and crtB. Phytoene levels in this particular experiment with DXS1 and crtB was 31,5 times higher than the amount found in crtB samples, reaching levels similar to the rest of carotenoids combined (Figure 2B). This suggests a limiting capacity of the natural carotenogenic enzymes to convert phytoene into downstream carotenoids. It also confirms the correct functioning of the overexpressed DXS1, as presumably the greater level of precursor synthesis is what allows crtB

to produce enough phytoene to saturate the next enzyme on the pathway, phytoene desaturase (Figure 1).

4.2. Tomato DXS1 performs better than other homologs.

Once the feasibility of expressing a DXS was established, tomato DXS1 was compared to tomato DXS2. Unlike DXS1, considered to be a house keeping enzyme whose expression is highest is ripening fruits, DXS2 is expressed mostly in trichomes and responds to environmental stress (Paetzold et al., 2010). The DXS ortholog from *Arabidopsis thaliana* (*AtDXS*), which is the only one present in this plant species, was also tested. Due to logistical reasons, DXS1 and DXS2 were compared to a crtB control in the same experiment while AtDXS was tested at a different time and compared to its own crtB control. Twelve *Nicotiana benthamiana* leaves were agroinfiltrated. A third of them expressed a crtB control on their left half and DXS1 on their right, another third expressed a crtB control on their left half and DXS2 on their right and the remaining third expressed a crtB control on their left half and AtDXS on their right as shown in Figure 3A. Samples were collected at 4 dpi for HPLC analysis.

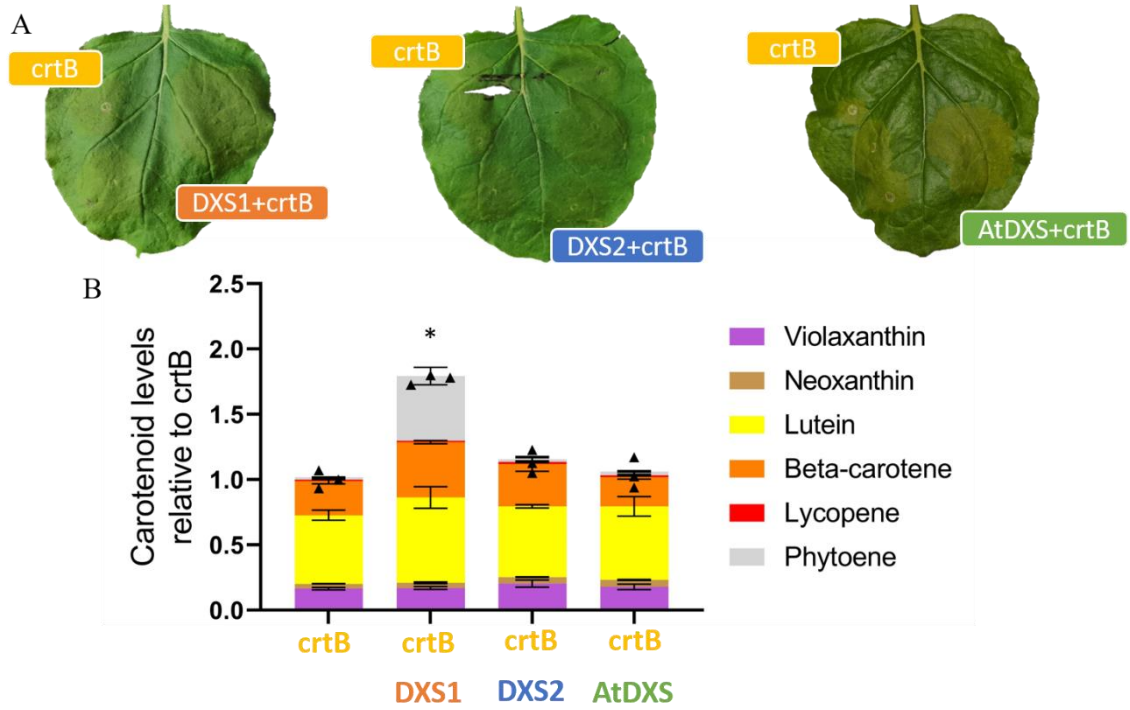

Figure 3: A) *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and samples were collected at 4 dpi for HPLC analysis. B) Carotenoid levels of leaves expressing either crtB, DXS1, DXS2 or AtDXS. Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$). Triangles mark the total carotenoid values of individual replicates.

Only DXS1 produced a statistically significant difference in carotenoid levels compared to crtB. In this experiment DXS1+crtB yielded 29% more carotenoids and 27 times more phytoene than just crtB Again, the increase of downstream carotenoids was limited but a massive increase in the phytoene levels contributed to double the content of total carotenoids compared to crtB alone (Figure 3B).

To follow the crtB-dependent chromoplastogenesis process, the effective quantum yield of PSII (ϕPSII) was measured for each type of sample in 3 different agroinfiltrated areas of three different leaves over the course of the 4 days at 24h. The results support the hypothesis that the presence of high phytoene levels destabilizes the PSII complex and causes the loss of photosynthetic activity (Llorente et al., 2020), since the leaves that accumulated the most phytoene (i.e, those with DXS1) seemed to have a more accentuated loss of photosynthetic capacity when compared

to crtB, especially in the early stages of the process (Figure 4).This hypothesis also proposes that the mechanism for chromoplastogenesis requires a second phase of massive increase in the production of downstream carotenoids. Based on (ϕPSII) values, it appears that chromoplastogenesis in DXS1+crtB-infiltrated leaves was not faster after the initial drop of photosynthetic competence detected at 24 hpi.

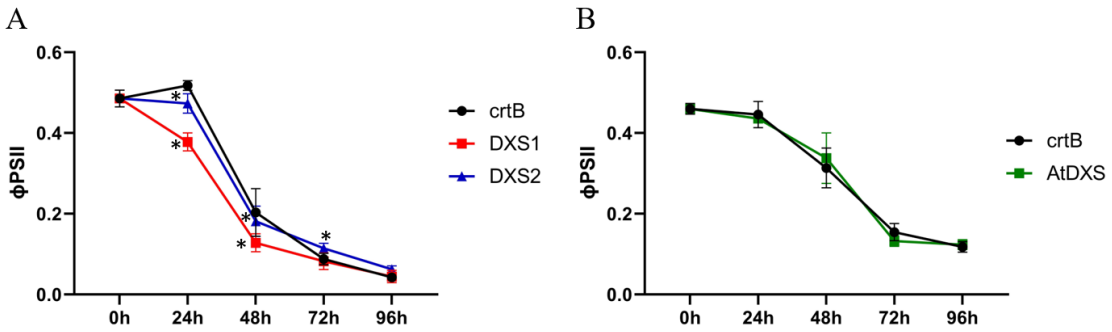

Figure 4: *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and ϕPSII was measured over the course of four days. A) ϕPSII levels in leaves overexpressing DXS1 and DXS2 relative to its crtB control. B) ϕPSII levels in leaves overexpressing AtDXS relative to its crtB control. Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$).

4.3. Introducing (p)crtI does not convert excess phytoene into downstream carotenoids.

Considering that the endogenous enzymes were unable to efficiently convert excess phytoene into other downstream products we decided to test the possibility to unlock the pathway by using a plastid-targeted version of the bacterial crtI protein, known to transform phytoene into lycopene. This version was provided with a plastid transit peptide at its N-terminal end that is expectedly cleaved after plastid import, hence resulting in a mature active enzyme. Twelve *Nicotiana benthamiana* leaves were agroinfiltrated as previously described. Half of them were agroinfiltrated with constructs harboring a GFP control on their left half and crtB on their right half and the other half received DXS1+crtB on their left half and a combination of DXS1, crtB and (p)crtI (named DBI) as shown in Figure 5A. Samples were collected at 4 dpi for HPLC analysis.

Although all crtB-containing combinations significantly increased downstream carotenoid content when compared to the GFP control, neither DXS1+crtB nor DBI showed any statistically significative difference when compared to the sole crtB (Figure 5B). This shows once again how variable the effect of DXS1 can be. Phytoene levels were once again elevated in all combinations containing DXS1 compared to crtB, indicating that DXS1 was correctly overexpressed (Figure 5B). Strikingly, no statistically significant differences in the levels of phytoene were found between DXS1+crtB and DBI, indicating that the incorporation of (p)crtI did not unlock the bottleneck in phytoene conversion. Therefore, further usage of (p)crtI was discarded as it was not deemed productive. Unpublished data from the host group shows that phytoene produced by crtB in green leaves is predominantly located in plastoglobuli. By contrast, crtI is mainly associated to envelope membranes, at least when expressed in tomato chromoplasts (Nogueira et al., 2013), so it may not be able to access the pool of phytoene and convert it into lycopene. Also, a different mode of action of crtI on green leaf chloroplast compared to what observed in tomato chromoplasts cannot be excluded.

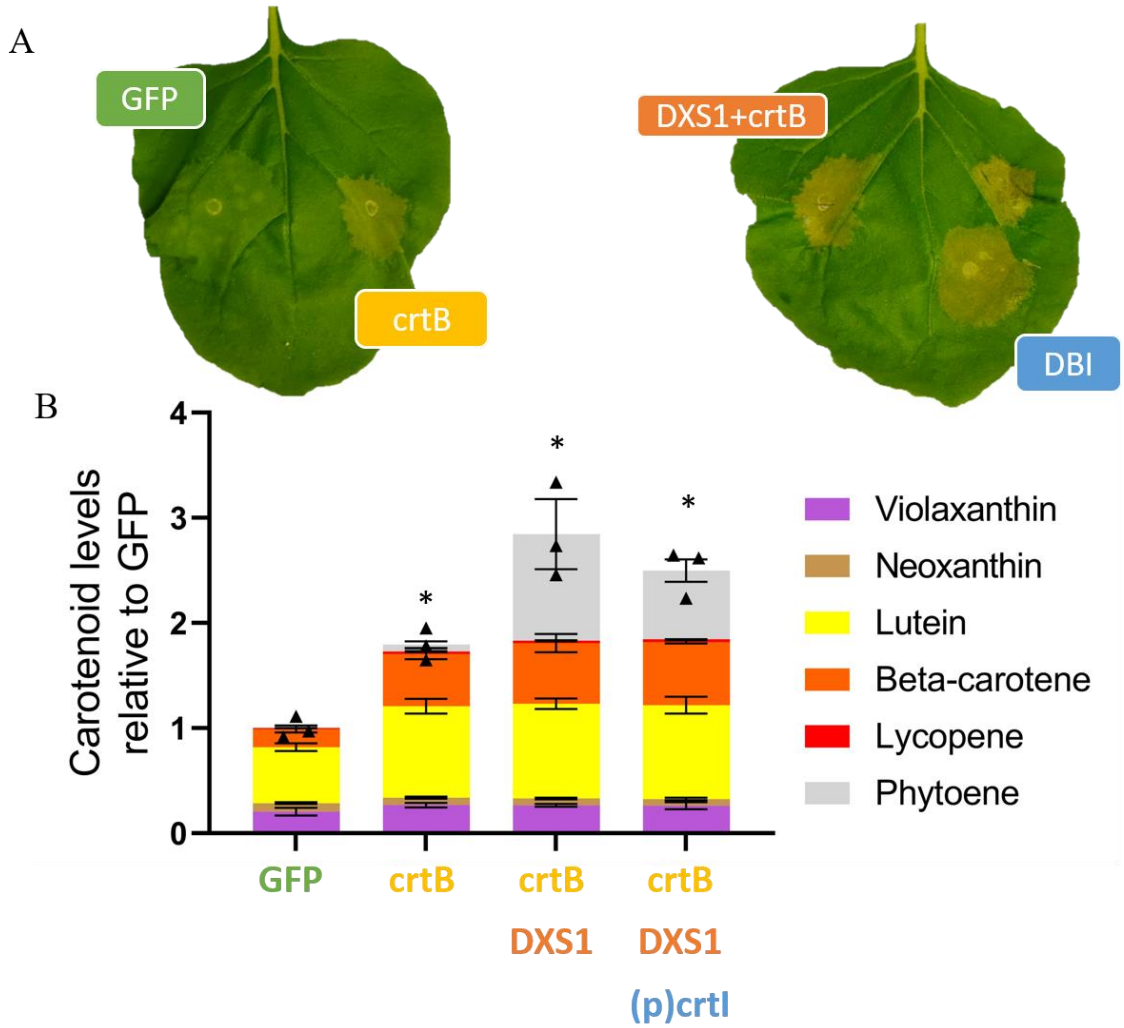

Figure 5: A) *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and samples were collected at 4 dpi for HPLC analysis. B) Carotenoid levels of leaves expressing the indicated combinations. Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$). Triangles mark the total carotenoid values of individual replicates.

4.4. Construction of a cytosolic β-carotene biosynthetic pathway greatly increases β-carotene content and enhances antioxidant activity.

Synthetic cytosolic pathways for the production of specific carotenoid intermediates had been previously described for lycopene and phytoene (Andersen et al., 2021; Majer et al., 2017). This TFG work introduced two changes relative to the one found to be most effective (Andersen et al., 2021). The first one was the addition of an extra gene to produce β-carotene from lycopene. The second one was the use of an unmodified crtB protein able to localize both in the cytosol and the plastid, which poses the advantage of accessing a different isoprenoid precursor pool through the MVA pathway while allowing synthetic chromoplastogenesis to occur. The constructions introduced were (t)HMGR (represented by the letter H), a truncated version of HMGR to produce MVA; crtE (represented by the letter E), to produce GGPP, crtB to produce phytoene both in the cytosol and plastid; crtI (represented by the letter I) to produce lycopene and crtY (represented by the letter Y) to produce β-carotene. The combination of these constructions comprises the cytosolic pathway (named crtB + HEIY) (Figure 6). 28 *Nicotiana benthamiana* leaves were agroinfiltrated as previously described. Four of them received a GFP control on their left half and crtB on their right half, four of them received DXS1 on their left half, ten of them received the crtB + HEIY combination on their left half and the remaining ten received a combination of crtB, DXS1 and HEIY on their left half as shown in Figure 7A. Samples were collected at 6 dpi for

HPLC analysis. The higher amount of leaves used for the last two combinations, as well as the longer time before harvesting the leaves are due to it being reported (Andersen et al., 2021) that the phenotype for leaves infiltrated with the cytosolic combination takes longer to develop and because of this, necrosis can appear and so a higher number of leaves ensures enough plant material will be in good condition for analysis.

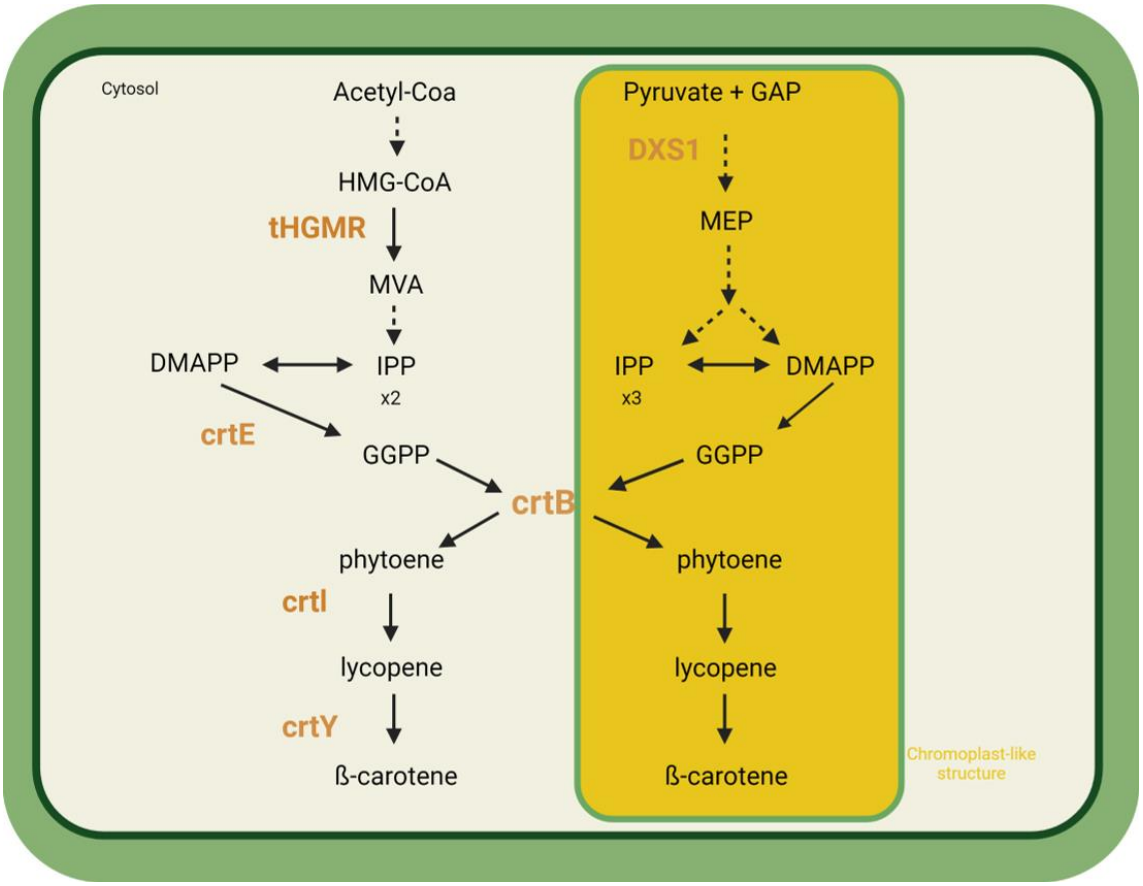

Figure 6: Schematic representation of the pathways and enzymes related to this work. The tan box represents the cytosol and the orange box chromoplast-like structures. HMG-CoA, hydroxymethylglutaryl coenzyme-A; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GAP, glyceraldehyde 3-phosphate; MEP, methylerythritol 4-phosphate. Enzymes expressed in the system are shown in brown: tHMGR, truncated HMG-CoA reductase; crtE, bacterial GGPP synthase; crtB, bacterial phytoene synthase; crtI, bacterial desaturase/isomerase; crtY, bacterial lycopene β-cyclase and DXS1, 1-deoxy-D-xylulose-5-phosphate synthase.

Both crtB + HEIY and DXS1+ crtB + HEIY-infiltrated leaves showed a significant increase in carotenoid content compared to crtB and DXS1+crtB leaves. crtB + HEIY and DXS1+ crtB + HEIY -infiltrated leaves yielded a 42% and 52% increase respectively in carotenoid levels when compared to crtB and a 101% and 121% increase respectively when compared to the GFP control (Figure 7C). However, they did not show a statistically significant different amount of carotenoids downstream of phytoene amongst themselves. Differences in phytoene content were not statistically significant between crtB and crtB + HEIY leaves. However, there were differences in phytoene levels between DXS1+crtB and DXS1+ crtB + HEIY leaves which were 39 and 22 times higher than the crtB control, respectively. This difference could be because protein overexpression is limited when many constructs are expressed at once or because the presence of the cytosolic pathway recruits more crtB into the cytoplasm, letting less of it into the plastid for phytoene production. However, whether proteins in the crt family physically interact to produce carotenoids in the cytosol or not is yet unknown. β-carotene levels did not significantly differ between crtB and DXS1+crtB but it was higher in crtB + HEIY and DXS1+ crtB + HEIY leaves,

which showed 4,5 and 4,9 times the amount of β-carotene than GFP respectively and 2,2 and 2,3 times as much as just crtB (Figure 7B).

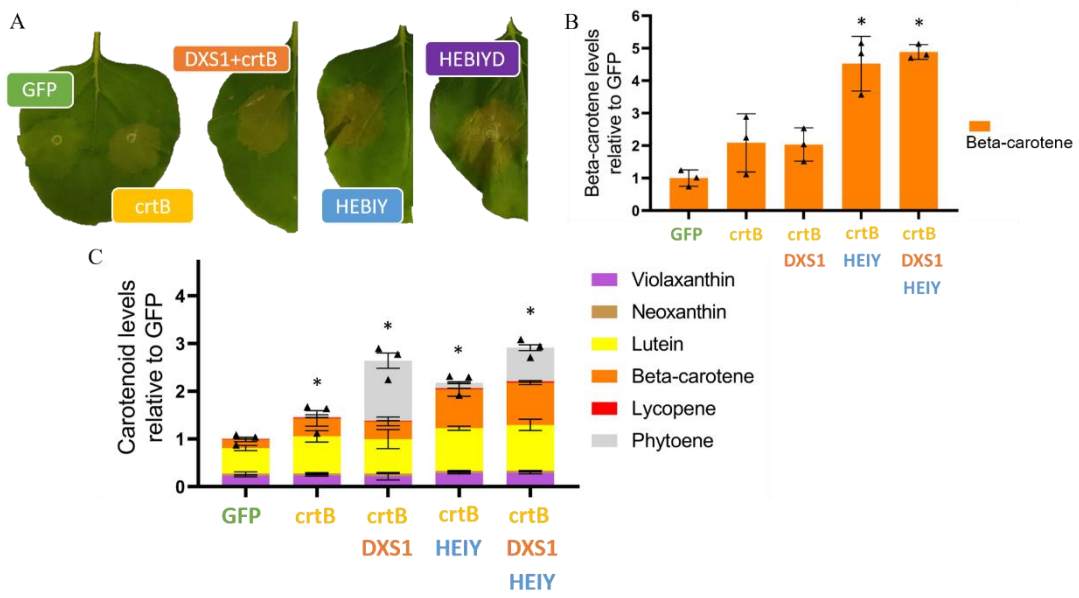

Figure 7: A) *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and samples were collected at 6 dpi for HPLC analysis. B) β-carotene levels of leaves expressing the indicated combinations. C) Carotenoid levels of leaves expressing the indicated combinations. Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$). Triangles mark the total carotenoid or βcarotene values of individual replicates.

To test whether altered levels of phytoene and/or β-carotene had an impact on the antioxidant capacity of leaves, ABTS antioxidant capacity assay (αTEAC) was also performed, in the aforementioned samples. Samples from crtB + HEIY and DXS1 + crtB + HEIY leaves had similar antioxidant capacities, higher than that of crtB and DXS1 + crtB leaves. They were all significantly higher than the GFP control (Figure 8A). However, it is important to note that carotenoids are not the only compounds affecting antioxidant capacity, as tocopherols, for instance, also play an important role. But more importantly, each carotenoid contributes differently to the total antioxidant capacity because of their different chemical properties (Müller et al., 2011). The carotenoid mainly participating in antioxidant activity in crtB and GFP samples was lutein, while in DXS1+crtB we observed an increased contribution from phytoene despite its relatively low antioxidant potential. In crtB + HEIY and DXS1 + crtB + HEIY leaf extracts β-carotene was the carotenoid with the main contribution to the antioxidant activity (Figure 8B).

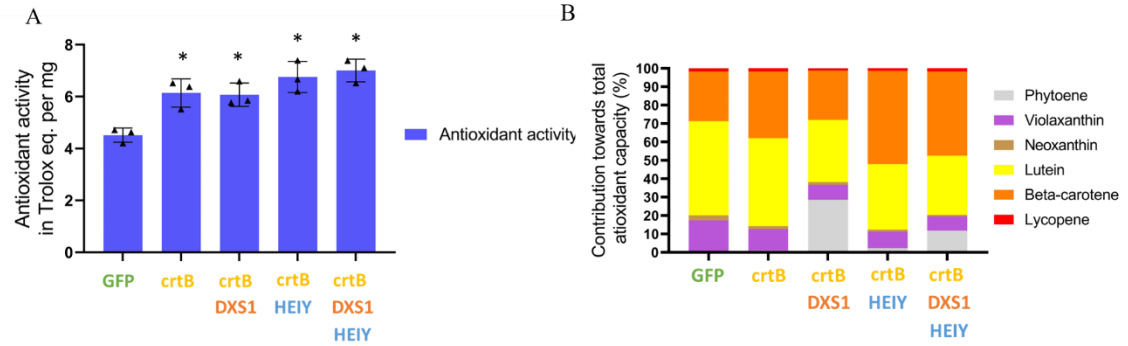

Figure 8: *N. benthamiana* leaves were agroinfiltrated with the indicated constructs and samples were collected at 6 dpi for ABTS antioxidant capacity assay. A) Antioxidant capacity in Trolox equivalents of samples expressing the indicated combinations. B) Individual contribution to antioxidant capacity for each tested carotenoid of samples expressing the indicated combinations as estimated basing on Muller et al.

Asterisks mark statistically significant changes relative to control samples (t-test, $P < 0.05$). Triangles mark the total antioxidant activity values of individual replicates.

This strategy has yielded promising results, but it has some drawbacks. Mainly, crtB + HEIY and DXS1 + crtB + HEIY leaves need at least 5 or 6 days to correctly develop the phenotype (Andersen et al., 2021), but at this time necrosis also starts to appear probably because the cell doesn't have dedicated structures to store these lipophilic carotenoids synthetically produced in the cytosol and they interfere with the cell's membranes normal functions. It also shows the limitation of not being able to massively increase the production of downstream carotenoids in plastids unless the entire pathway is replaced, as endogenous enzymes are subject to a tight regulation.

In the future, a combination of this push strategy with a pull one to further increase the storage capacity of carotenoids should be attempted. This could be done by subjecting the plants to a high light treatment before agroinfiltration, at it has been shown to increase the number and size of plastoglobuli, structures where carotenoids are mostly stored, in chromoplasts (Van Wijk & Kessler, 2017).

5. Conclusions.

In conclusion, orthogonal expression systems, which bypass endogenous regulation thanks to being too evolutionarily distant with its host, represent a great tool to engineer plant metabolism and produce compounds of interest in an efficient and sustainable manner. The implementation of one of such systems in this work has proved the feasibility of converting a nutritionally poor part of a plants such as the leaves into a carotenoid-rich tissue. Constructing a cytosolic pathway with *P. ananatis* enzymes to produce β-carotene has proved very effective compared to just the chromoplastogenic effect of overexpressing crtB. Even if DXS1 does not lead to a major increase in downstream plastidial carotenoids because of the tight regulation of the rest of the pathway, the great amounts of phytoene produced because of the increased precursor supply provide a valuable addition to the nutritional value and shed light on the regulation mechanisms that can affect this pathway. This strategy has proven effective at creating a β-carotene-rich tissue even if there are still open possibilities in order to maximize β-carotene production and to further implement it into actual crops.

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