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ORIGINAL PAPER

Carotenoid Pathway Engineering in Tobacco Chloroplast Using a Synthetic Operon

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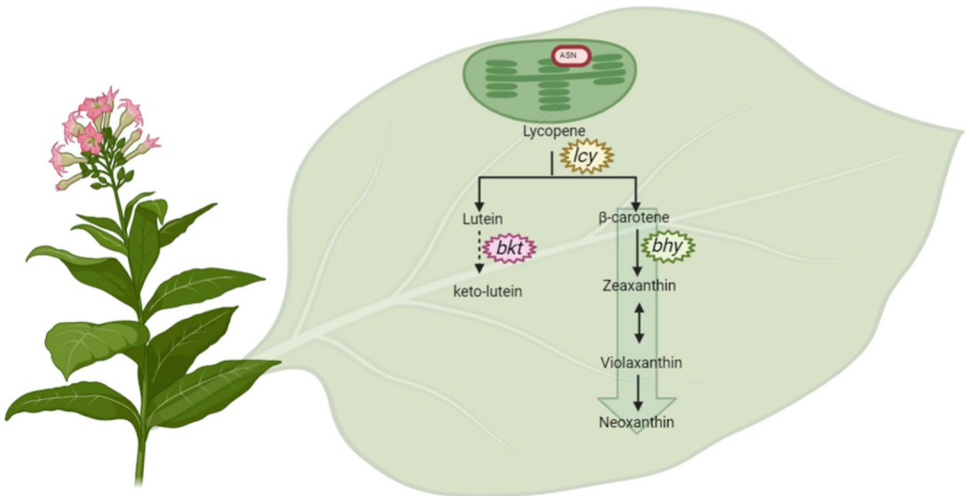
Abstract

The carotenoid pathway in plants has been altered through metabolic engineering to enhance their nutritional value and generate keto-carotenoids, which are widely sought after in the food, feed, and human health industries. In this study, the aim was to produce keto-carotenoids by manipulating the native carotenoid pathway in tobacco plants through chloroplast engineering. Transplastomic tobacco plants were generated that express a synthetic multigene operon composed of three heterologous genes, with Intercistronic Expression Elements (IEEs) for effective mRNA splicing. The metabolic changes observed in the transplastomic plants showed a significant shift towards the xanthophyll cycle, with only a minor production of keto-lutein. The use of a ketolase gene in combination with the lycopene cyclase and hydroxylase genes was a novel approach and demonstrated a successful redirection of the carotenoid pathway towards the xanthophyll cycle and the production of keto-lutein. This study presents a scalable molecular genetic platform for the development of novel keto-carotenoids in tobacco using the Design–Build–Test–Learn (DBTL) approach.

Graphical Abstract

This study corroborates chloroplast metabolic engineering using a synthetic biology approach for producing novel metabolites belonging to carotenoid class in industrially important tobacco plant. The synthetic multigene construct resulted in producing a novel metabolite, keto-lutein with high accumulation of xanthophyll metabolites. This figure was drawn using BioRender (https://www.biorender.com).



Keywords Metabolic engineering · Chloroplast transformation · Synthetic operon · Carotenoids · Xanthophyll cycle

Abbreviations

IEE	Intercistronic expression element
aadA	Spectinomycin
CrtW/bkt	β-Carotene ketolase
CrtZ/bhy	β-Carotene hydroxylase

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lcy	β-Lycopene cyclase
CrtE	GGPP synthase
CrtB	Phytoene synthase
CrtI	Phytoene synthase
CrtY	Lycopene cyclase
LB	Luria–Bertani media
SBMSN	Super broth with ammonium and sucrose medium
2YT	Yeast extract and tryptone media
TB	Tryptone broth
MS	Murashige and Skoog medium

Introduction

Carotenoids are colour-producing isoprenoid molecules found in all photosynthetic and some non-photosynthetic species of bacteria and fungi [1]. Their chromogenic property arises due to the conjugated double bonds present in the polyene chain. Plants contain tetraterpenes derived from 40 carbon isoprenoid phytoene. The colour of carotenoids ranges from yellow to red, with some variations showing hues of brown and purple [2]. Ingested carotenoids not only contribute to body colours of many birds, fishes and invertebrates but also act as important nutritional components for humans [3]. These pigments are not synthesized by animals and are obtained via dietary intake for essential nutrient sources, like retinoids. Carotenoids belong to important antioxidant compounds that have been reported to enhance the immune system and disease protection. Provitamin A which belongs to this category of molecules is converted to vitamin A in the human body and is known for its crucial role in maintaining eye health [4]. Carotenoids are principally categorized as carotenes (hydrocarbons that can be cyclized at one or both ends of the molecule) and xanthophylls (oxygenated derivatives of carotenes) (Fig. 1). Xanthophylls are major carotenoids and are well known for their health promoting and therapeutic activity [5]. Xanthophylls such as lutein and zeaxanthin play crucial roles in protecting plants through oxidative damage, as plants are prone to photo-oxidative damage due to light exposure. Astaxanthin also belongs to the same category of molecules but is rarely found in plants and is known for its extremely high antioxidant activity [6]. Multiple reports have been published describing genetic modifications to enable the production of astaxanthin in plants through extending the carotenoid pathway through both nuclear and plastidial routes as described below [7].

Chloroplast genome engineering in higher plants is an efficient method in multiple aspects compared to nuclear genome modifications as it enables targeted gene insertion, gene containment, prevents gene silencing with no epigenetic modifications and has a high foreign gene expression capacity [8]. Moreover, targeting the carotenoid biosynthetic pathway in chloroplasts offers the big advantage of substrate availability to the expressed enzymes as this pathway is predominantly operative in chloroplast stroma [9]. This technology has been well established in tobacco when compared to other plant species. Metabolic engineering of the carotenoid biosynthetic pathway for astaxanthin production has been attempted through chloroplast transformation in tobacco [10] and lettuce [11] where CrtW (β-carotene ketolase) and CrtZ (β carotene hydroxylase) genes from a marine bacterium *Brevundimonas* sp., strain SD212 were heterologously expressed, which resulted in the production of 3120.0-µg/g and 70.0-µg/g astaxanthin, respectively, along with other related pathway keto-carotenoids. Other studies conducted by Mann et al. [2] and Jayaraj et al. [12] in tobacco and carrot through nuclear transformation demonstrated production of astaxanthin in lower amounts of 23.5 µg/g and 91.6 µg/g, respectively, by expressing only a ketolase gene from *Haematococcus pluvialis* and did not supplement this enzyme with any other hydroxylase enzyme. Supplementing more enzymes to the targeted pathway could help in achieving a higher substrate ratio for achieving higher amounts of the desired product. Also, different combinations of hydroxylase and ketolase genes from varied sources have been tried for keto-carotenoids and astaxanthin production due to high industrial value of astaxanthin and its related keto-carotenoids in aquaculture and pharmaceuticals. Synthetic multigene operons have also been tried in chloroplast genome engineering by making use of IEE (Intercistronic Expression Elements) which is known for promoting simultaneous and stronger gene expression of multigenes [13, 14]. Expression of the lycopene β cyclase gene from daffodils has been shown to result in high accumulation of β-carotene in tomato [15], the β-carotene hydroxylase gene from *H. pluvialis* has been shown to strongly hydroxylate β-rings containing substrates instead of ε-rings [16] and the β-carotene ketolase gene was used in the study and was selected due to its high ketolation activity shown in both the tobacco and carrot plant system as mentioned above. In the current study, these specific gene combinations were examined together for the first time in a higher plant system through a chloroplast synthetic biology approach through stable genetic transformation.

Since the protocol of chloroplast transformation has been well established in tobacco as compared to other plants as an industrial crop species [17], we have conducted this study using a tobacco plant system. The present study demonstrates the effect of three genes from heterologous sources when used as an operon organization to extend the carotenoid pathway in tobacco for the production of keto-lutein rather than astaxanthin. Additionally,

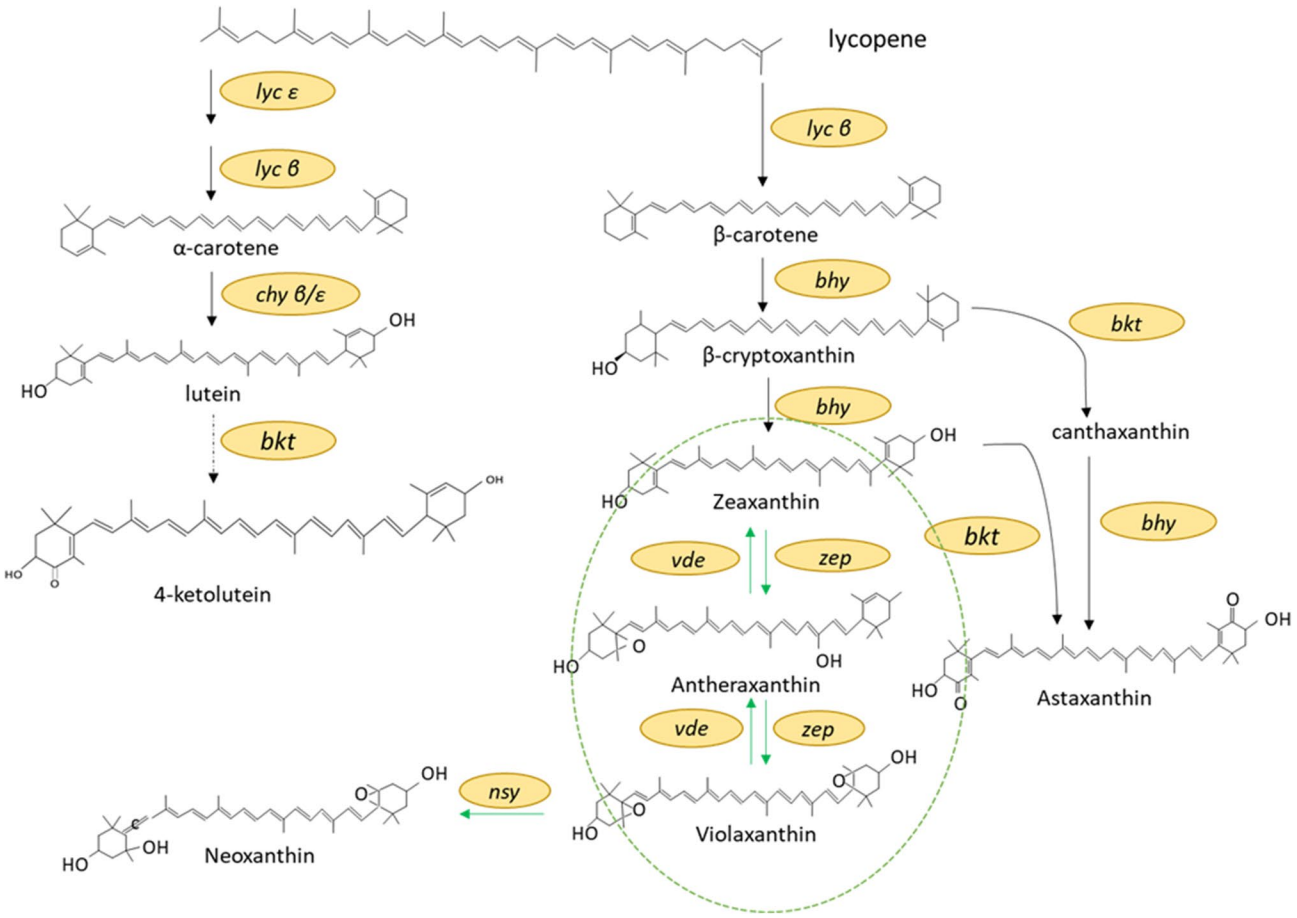

Fig. 1 Proposed scheme of carotenoid biosynthetic pathway in transplastomic tobacco plant. Hydroxylated and/or ketolated metabolites resulting from the introduction of *bhy* and *bkt* genes from heterologous sources in the tobacco plant plastome are highlighted in bold typeface. Enzymes are represented as their gene assignment symbols. Metabolites indicated with dashed arrow represents the proposed keto-carotenoid found in transplastomic tobacco. *lycε* lycopene ε-cyclase, *lycβ* lycopene β-cyclase, *chyβ/ε* β/ε carotene hydroxylase, *bhy* beta-carotene hydroxylase, *bkt* beta-carotene ketolase, *zep* zeaxanthin epoxidase, *vde* violaxanthin de-epoxidase, *nsy* neoxanthin synthase

the gene combination resulted in accumulating high levels of zeaxanthin and other metabolites produced *via* xanthophyll cycle by the plant.

Materials and Methods

Synthetic Construct for Chloroplast Transformation

The polycistronic synthetic operon (we named it as ASN) consisting three genes separated by IEE were cloned in to the chloroplast transformation vector pRB94 (EMBL accession no. AJ312392), a plasmid pBluescript derivative, as described in Ruf et al. [18] (a gift from Dr. R. Bock) shown in Fig. 2. The insertion site of synthetic operon is between intergenic regions trnfM and trnG (Fig. 2), using *Sac*II and *Spe*I restriction sites. The synthetic gene construct was designed with three foreign genes using 'SnapGene' software (http://www.snapgene.com) with total size of 4.2 kb. Genes used for preparing the construct are Lycopene β-cyclase (*lcy*) gene taken from Daffodil (NCBI accession number GQ327929), β-carotene hydroxylase (*bhy*) gene (NCBI accession number KP866869.1) and β-carotene ketolase (*bkt*) gene (NCBI accession number X86782) from *H. pluvialis*. The ASN insert along with IEE was custom synthesized as a single operon from Thermo Fisher Scientific, USA. The synthetic DNA was dissolved in Tris–EDTA (TE) buffer and PCR amplified for cloning into pRB94.

The plastid transformation vector pRB94 [18] consists of chimeric *aadA* selectable marker gene driven by rRNA operon promoter that confers resistance against spectinomycin and streptomycin antibiotics to the transformed plant/chloroplasts. The transgenes present in the vector are located between inverted repeat spacer regions of the plastid genome having high transcriptional activity, which were reported for

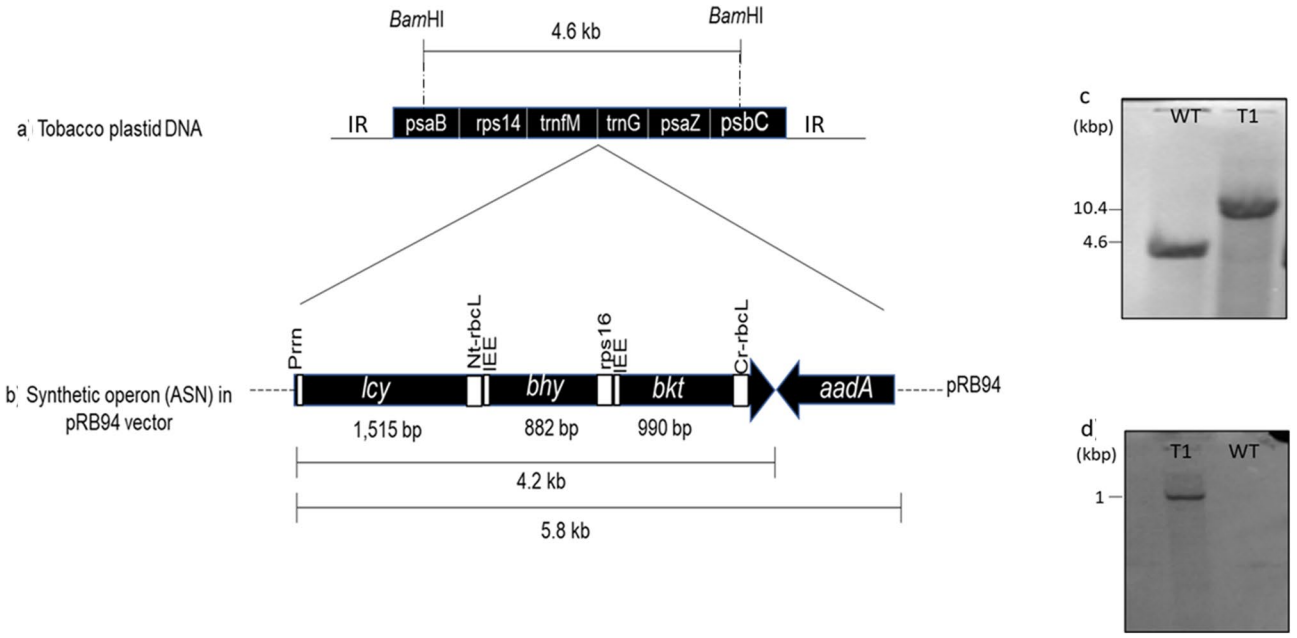

Fig. 2 **a** Tobacco plastid DNA region showing targeting region of transgene integration flanked with *Bam*HI restriction sites, with a fragment size of 4.6 kb in WT or spontaneous mutant (mutant explant shoots that would grow under spectinomycin selection pressure and do not contain operon gene construct). **b** Physical map of the synthetic transgene construct (ASN) consisting three transgenes (*lyc* lycopene cyclase, *bhy* beta-carotene hydroxylase, *bkt* beta-carotene ketolase cloned in pRB94 vector giving rise to 4.2-kb fragment and including *aadA* fragment present in the vector backbone, making a 5.8-kb fragment. **c** Southern blot analysis of T1 plastid transformants; total DNA was digested with *Bam*HI and probed with *psaB* (probe size 250 bp) and **d** *bkt* gene (probe size 250 bp). Single band at 10.4 kb probed with *psaB* represents gene integration of the synthetic operon at the desired location and complete elimination of wild-type plastomes as compared to the WT (wild-type) plant. *IR* inverted repeat sequence

higher protein accumulation in plants [19] and reported to be widely used region for transgene integration in chloroplast genome recently [20].

Plant Material and Growth Conditions

Aseptically grown plants were obtained from surface-sterilized tobacco (*Nicotiana tabacum* cv. Petite Havana) seeds, using 800-µL ethanol, 1 drop of Tween 20 and 800-µL 4% (v/v) sodium hypochlorite solution for 90–100 tobacco seeds in a 2-mL Eppendorf tube. The seeds were vigorously shaken for 2–4 min followed by washing with sterile distilled water for at least six times. Seeds were then placed in sterile jar containers (130 mm × 70.80 mm × 63 mm) of MS medium for germination. The germinated tobacco seedlings were transferred to sterile containers of MS medium and kept in culture room for ~ 4–7 weeks till the plant reaches 5–7 leaf stage. Plants were grown in a plant growth chamber at 22–25 °C under 16-h light/8-h dark cycle with fluorescent lamps providing ~ 1900 lx light intensity. 3–4 young leaves of tobacco were used for transformation experiments. Homoplasmy of T1 plants was tested by keeping seeds of self-pollinated plants under spectinomycin (500 mg/L) selection.

For regeneration of explants, plant regeneration media (PRM) was used; complete MS media (for 1 L media) with 20-g sucrose along with the plant hormones 0.2 mL of 1 mg/mL of Indole 3-acetic acid (IAA):1 mg/mL (in 0.1-M NaOH), 3 mL of N^6-benzylamino purine (BAP):1 mg/mL (in 0.1-M HCl), and 100-mg myo-inositol, pH 5.8 (0.2-M KOH). After addition of 6-g/L agar, the medium is sterilized by autoclaving and cooled to approximately 60 °C, at which point antibiotics (spectinomycin and/or streptomycin 500 µg/mL) were added [21].

Gold Particle Preparation and DNA Coating

Preparation of gold particle stock: 50 mg of gold particles (0.6 µm, Bio-Rad) were added to a 1.5-mL microfuge tube along with 1 mL of molecular grade 100% ethanol. The tube was vortexed for 2 min and centrifuged at 10,000 × *g* for 3 min. The supernatant was discarded and gold particles were resuspended in 1 mL of 70% (v/v) ethanol by vortexing for 1 min. This was incubated at room temperature (24 ± 2 °C) for 15 min and mixed intermittently by gentle tapping. Gold particles were pelleted by centrifuging at 5000 × *g* for 2 min and supernatant was discarded. Gold particles were then washed three to four times after resuspension in 1-mL sterile dH_2O and then left undisturbed for 1 min at room

temperature followed by centrifugation at 5000 × *g* for 2 min. After the final centrifugation step, gold particles were resuspended in 1 mL of sterile 50% (v/v) glycerol and stored at − 20 °C until further use for DNA coating [22].

Coating gold particles with DNA: 50 µL of gold particles (for 5 bombardment shots) were taken from the resuspended stock to a 1.5-mL microcentrifuge tube. Whilst vortexing, 5 µg of plasmid DNA was added followed by 50 µL of 2.5-M $CaCl_2$ and 20 µL of 0.1-M spermidine. Vortexing was continued for 20 min at 4 °C, followed by centrifugation at 10,000 × *g* for 1 min. The supernatant was removed and pellet was washed with 200 µL of 70% (v/v) ethanol, followed by an additional wash with 100% ethanol. The DNA-coated pellet was resuspended in 50 µL of 100% ethanol and DNA-coated gold particles were stored on ice for particle bombardment [22].

Chloroplast Transformation and Selection of Stable Transplastomic Lines

Young leaves with seven to ten days of age were used for transformation, which were grown under aseptic conditions in MS medium. Leaves were placed in a Petri dish on top of a thin layer of PRM without antibiotics. Bombardment was done on the abaxial side of the leaves with the plasmid DNA-coated gold particles. For the Bio-Rad PDS-1000/He biolistic gun, settings for the transformation (using the Mono Adaptor setup of the Bio-Rad PDS-1000/He particle gun) were as follows: Helium pressure at the tank regulator: 1300–1400 psi, Rupture discs: 1100 psi (Bio-Rad), macrocarrier (flying disc) assembly: level one from the top, Petri dish holder: level two from the bottom and vacuum (at the time of the shot): 27–28 Hg.

After 2 days of incubation in the dark the bombarded leaves were segmented using sterile scalpel in ~ 5-mm^2 segments. The bombarded sides were kept in full contact with the regeneration media containing spectinomycin antibiotic (500 mg/L). Regeneration of putative tobacco shoots was observed within 4–8 weeks of bombardment. Bombarded leaf segments were kept under low light conditions, at approximately 7 µE/m^2 s (16-h light/8-h dark cycle). The potential of spontaneous mutation due to 16S rRNA point mutations in the chloroplast gene was tested by placing the spectinomycin-resistant shoots on double selection of streptomycin and spectinomycin (500 µg/mL, each) [23]. After the first cycle of selection, 4–5 selection cycles were given to the resistant shoots for developing homoplasmy, a condition where all genomic copies of chloroplast contain the synthetic ASN cassette. Finally, regenerated shoots were grown on regeneration media and placed in phytohormone free MS medium for root induction. Plants were then transplanted to sterile soilrite™ (Gardenesia, India) and later transferred to pots containing soil in green house for maturation.

Isolation of Nucleic Acids, Quantitative Gene Expression Analysis and Hybridization Procedures

Total plant DNA was isolated through the CTAB (cetyltrimethylammonium bromide) method [24] and plant DNA (30 µg) was digested with *Bam*HI, separated on a 0.8% agarose gel and then transferred onto a Hybond XL nylon membrane Merck SA, Germany by capillary blotting. Preparation of specific probe, hybridization and washings were carried out with DIG high-prime DNA labelling and detection kit (Roche starter kit 1, Indianapolis, IN, Cat. No. 11745832910), following the manufacturer's guidelines.

A 550-bp PCR product (probe) generated by amplification of *psaB* region using *Psab* 5′-CCCAGAAAGAGGCTG GCCC-3′/5′-CCCAAGGGGCGGGAACTGC-3′ primers and 246-bp probe of *bkt* gene with primers bkt 5′-ATCAAACCA GGCGTACAAGG-3′/5′-CAAATCAAGCTTCCGACCTC-3′ was used for confirming transgene integration in plastome.

Total plant RNA was extracted from tobacco leaves using HiPurA™ plant and fungal RNA miniprep purification kit (HiMedia, India). For cDNA synthesis, RNA was reverse transcribed by revertaid first-strand cDNA synthesis kit with random primers (Thermo Scientific™ USA). Real-time expression analysis was done on QuantStudio 3 real-time PCR system, Thermo Fisher. Primers used for real-time analysis are listed in Supplemental Table 1.

Pigment Extraction from Transformed Tobacco

Pigment extraction from tobacco was carried out using dried (lyophilized) leaf and flower samples weighing 10 g were ground to make a fine powder. The powder was stirred overnight in a mixture of CH_2Cl_2:MeOH (1:1) 300 mL each twice in an opaque glass bottle. The extracts were combined and concentrated in rota vaporizer at 40 °C. For complete drying, samples were kept under stream of N_2. Extracted pigments were stored at − 20 °C. The extract was resuspended in a solvent with mixture of acetonitrile:methanol (7:3) and acetone (6.7 + 3.3) followed by vortexing for 15 min and centrifugation at 10000 rpm for 10 min at room temperature followed by filtration of supernatant through 0.2-µm syringe filter (Millipore, Germany).

Spectrophotometric Determination of Total Carotenoids, Chlorophyll *a* and Chlorophyll *b*

Extracts of dry leaf powder (1 mg/mL) were prepared in 100% methanol and spectroscopic analysis of both carotenoids and chlorophylls was done according to the protocol

by Lichtenthaler and Buschmann [25]. Total carotenoids content in the extract was estimated by spectrophotometric reading using the following equation (Lichtenthaler 1987):

Chlorophyll a, $C_a = 11.24A661.5 - 2.04A645$,

Chlorophyll b, $C_b = 20.13A645 - 4.19A661.5$,

Total carotenoids, $C_x + c = (1000A470) - \left[\left(1.9C_a\right) + \left(63.14C_b\right)\right]V \times \mathrm{DF}/214$,

where V is the Total volume of the extract and DF is the dilution factor.

Metabolite Analysis Using LC–MS

For metabolic analysis of tobacco samples Nexera™ ultra-high performance liquid chromatography (UHPLC) system (Shimadzu), coupled online via electrospray ionization source (ESI) with a 5600 triple ToF mass spectrometer (AB Sciex), was employed for global metabolomics profiling with 4-µL sample injection volume. UHPLC chromatographic separations were performed on a reversed-phase column ACQUITY UPLC® BEH 130 Å C_{18}, 1.7 µm, 2.1 × 150 mm, Waters, Milford, MA) and a gradient system with the mobile phase consisting of solvent A: H_2O 100% and solvent B: acetonitrile:methanol (70:30, v/v) containing 0.1% formic acid. The flow rate was adjusted at 0.3 mL/min with a linear gradient elution over 25 min. From the start to 2 min, solvent B was held at 85%, linearly increased to 100% till 3 min and then 100% during next 8 min and then to 98% in 15 min. Subsequently, solvent B was linearly decreased to 85% (initial conditions) till 25 min. The temperature of column oven was set at 40 °C and the sample manager was maintained at 8 °C.

Mass Spectrometry Optimisation and Data Analysis

The data were acquired using a data-dependent acquisition (DDA) method on Triple-TOF 5600+ instrument (SCIEX, Framingham, MA) interfaced with Shimadzu Ultra Performance-Liquid Chromatography (UPLC) system Nexera™ LC-30 AD (Shimadzu, Singapore). The instrument was operated under positive ion mode to acquire data in the *m/z* range of 50–1000 Da. 4-µL sample was analysed on reverse-phase chromatography using ACQUITY UPLC® BEH 130 Å C_{18}, 1.7 µm, 2.1 × 150 mm (Waters, Milford, MA) and a gradient system with the mobile phase consisting of solvent A: H_2O 100% and solvent B: acetonitrile:methanol (70:30, v/v) containing 0.1% formic acid. DDA MS/MS scans were acquired for the top 12 highest intensity ions at 15,000 resolutions with a cycle time of 1.0203 and the collision energy 30 eV. Identification of metabolites was carried out by searching the available databases such as KEGG, Massbank and GNPS using exact mass and MS/MS fragmentation patterns.

Results

Analysis of Stable Chloroplast Transformation in Tobacco

Plastid transformation vector (*pRB94*) used in the study is designed to integrate the transgene cassette into the plastome through homologous recombination. In this case, *psaB* and *psbC* are the region of ASN construct integration (Fig. 2). Bombardment experiments conducted with young tobacco (cv. Petite Havana) leaves resulted in production of five independent transplastomic lines. Shoot development under high spectinomycin (500 µg/mL) selection aided in preliminary screening of the developing shoots. Five successive cycles of selection were then conducted to develop homoplasmic lines. The obtained putative transgenic shoots were given a final selection cycle with spectinomycin and streptomycin (500 µg/mL each) as described by Ruf and Bock [21]. For developing stable T1 lines, these lines (T0) were selfed and the obtained seeds were selected on spectinomycin (500 µg/mL each) MS media (Fig. 3a, b) for use in subsequent analysis.

Phenotypically no difference was observed in wild-type and transplastomic tobacco plant leaf, flower petals or nectary (Fig. 3b–d). However, total chlorophyll content of the transplastomic plants was found to be lower as compared to wild-type plants (Table 1). Photosynthesis is affected by assimilation of chlorophyll and carotenoid pigments and carotenoids transfer absorbed energy to chlorophyll and protect chlorophyll from excessive light damage as well [26]. Similar studies have also found that an increase in the concentration of carotenoids has caused a decrease in the total chlorophyll content in plants [10].

Molecular and Expression Analysis of Transplastomic Tobacco Plants for Carotenogenic Gene Analysis

Southern hybridization of five transplastomic tobacco lines taken forward confirmed the presence of synthetic transgene operon (ASN) in the chloroplast genome (Fig. 2c, d; Fig. S1). Expression analysis of upstream isoprenoid pathway genes revealed upregulation of individual genes by 2- to 7-fold in the mature lines that resulted in increased production of β-carotene in the transplastomic

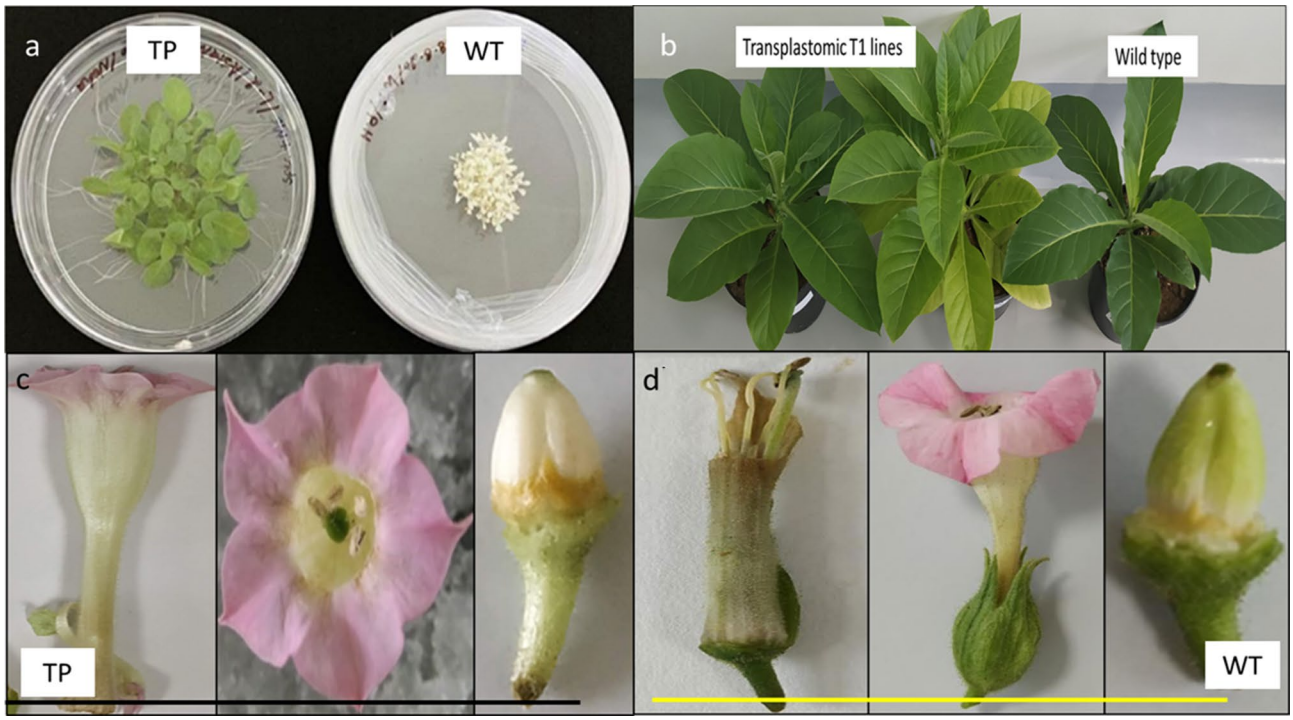

Fig. 3 Phenotype of T1 transplastomic tobacco plant (**b**) and flower (**c**) w.r.t. wild-type plant (**b**) and flower (**d**); *TP* transplastomic plant, *WT* wild-type; the total chlorophyll content of TP plants was found to be less as compared to the WT plants. **a** Seed selection of wild-type and transplastomic seeds under spectinomycin selection MS media for selection of stable transplastomic lines (since the seeds from T0 line consists of spectinomycin resistance gene, *aadA* due to maternal inheritance in the plastome, their colour did not bleach out in the presence of spectinomycin as in the case of WT seeds

Table 1 Photosynthetic pigment concentration in wild-type vs. transplastomic tobacco plant (mg/g dw)

Plant type	Chlorophyll *a*	Chlorophyll *b*	Xanthophylls + carotenes
Wild-type	4.939 ± 0.52	1.975 ± 0.06	0.842 ± 0.03
Transplastomic tobacco	2.803 ± 0.05	0.231 ± 0.21	1.121 ± 0.61

± Standard error

plant (Fig. 4). The highest levels of transcripts were found for the DXS gene (deoxy-xylulose 5-phosphate synthase) being the gateway and limiting enzyme of plastidic isoprenoid pathway, followed by CRTL (lycopene cyclase) which converts lycopene to β-carotene as this gene was supplemented by one additional copy of lycopene cyclase from the ASN operon. Enhanced expression levels were also found in PDS (phytoene desaturase) and PSY (phytoene synthase), genes which are known for initiating carotenogenesis. Moreover, it was observed that both endogenous and exogenous carotenoid pathway genes were found to be showing higher expression levels in mature leaves (15–20 days old) as compared to young leaves (5–7 days old). In transplastomic plants (TP) highest transcript copies were found in β-carotene ketolase gene (*bkt*) followed by β-carotene hydroxylase (*bhy*) and lycopene cyclase (*lcy*) gene from the operon construct. Even though the accumulation of the ketolase gene was found to be the highest amongst all, it did not result in production of any ketolated carotenoid, instead high accumulation of zeaxanthin was found which could be due to result of efficient hydroxylase enzyme activity from *H. pluvialis* (Table 1).

Carotenoid Analysis of Transplastomic Tobacco Plants

The main carotenoids found in transplastomic tobacco leaf were neoxanthin, violaxanthin and zeaxanthin, with higher peak intensity and area under the curve compared to other carotenoids and wild-type plant carotenoids, as listed in Table 2. A minor new peak with HPLC–MS mass spectra at *m/z* 583.416 [M+H] was observed in addition to other carotenoids at absorption spectra of keto-carotenoids with retention time of about 4.17 min. This peak was predicted to be representing either 4-keto-lutein or its isomer

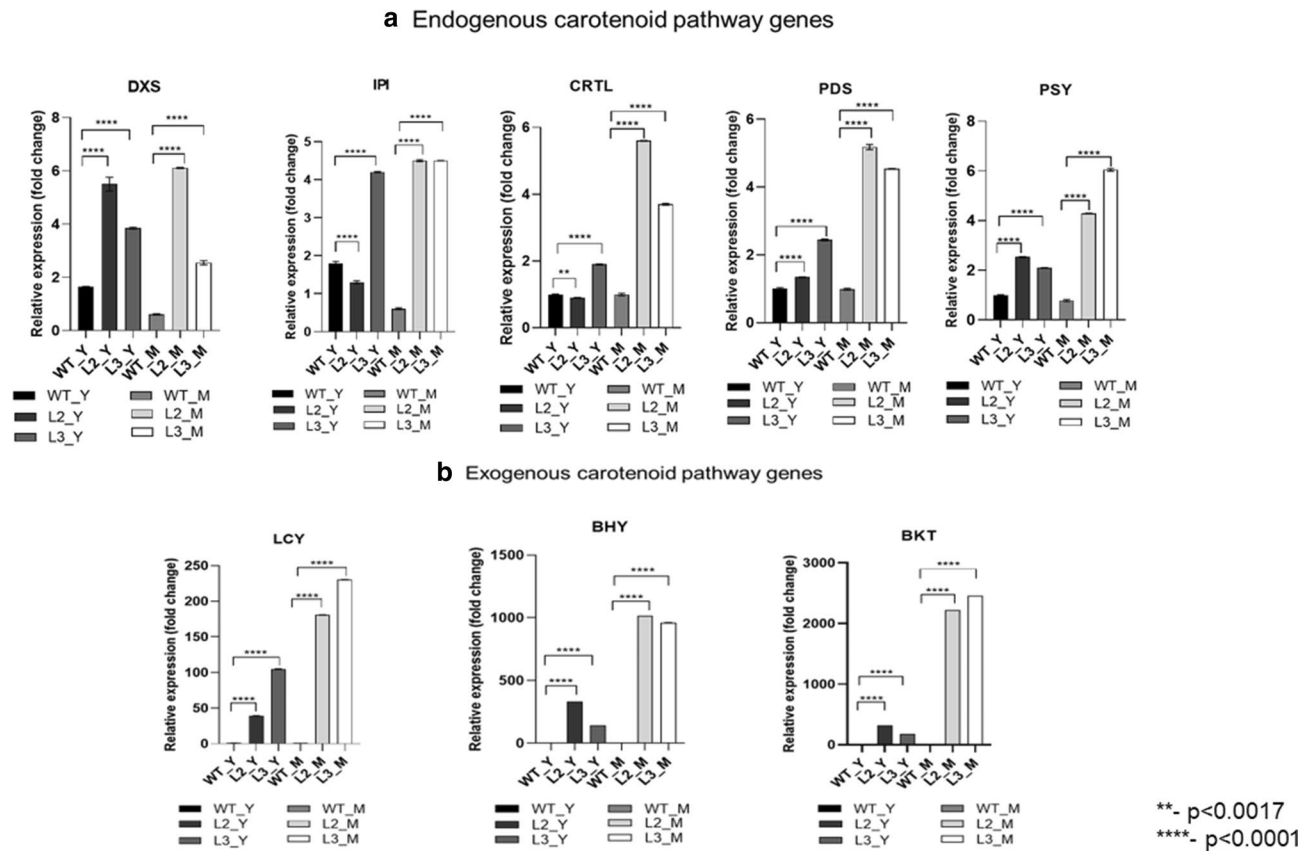

Fig. 4 Relative expression levels of plant's endogenous carotenogenic genes (*dxs, ipi, crtL, pds, psy*) and exogenous genes from the operon (*lcy, bhy* and *bkt*) in stable transplastomic tobacco lines and wild-type plants determined by RT-qPCR ($n=3$). Comparative expression analysis of all the genes were carried out in both young and mature leaves. *WTY/M* Wild-type Young/Mature, *L2Y/M* Line 2 Young/Mature, *L3Y/M* Line 3 Young/Mature, *bkt* β-carotene ketolase, *bhy* β-carotene hydroxylase, *lcy* β-lycopene cyclase, *dxs* deoxy-xylulose 5-phosphate synthase, *ipi* IPP isomerase, *crtL* lycopene cyclase, *pds* phytoene desturase, *psy* phytoene synthase

Table 2 List of predicted metabolites found in leaf and flower samples of transplastomic and wild-type tobacco plants

Names of predicted carotenoids	Molecular formula	Theoretical mass *m/z*	Measured mass *m/z*	Accuracy (ppm)	RT_ median	Area_SA1	Area_SA2	Area_SA3	Area_SA4
Phytoene 1,2-epoxide	$C_{40}H_{64}O$	560.495	561.506	−1.011	3.07	5677.270	30,901.193	160,7489.898	529,507.884
β-Carotene	$C_{40}H_{56}$	536.872	537.459	−0.587	20.55	1623.218	1973.812	623.112	821.456
β-Cryptoxanthin	$C_{40}H_{56}O$	552.887	553.471	−0.584	8.05	456.685	5403.221	17,413.906	45,461.490
Zeaxanthin	$C_{40}H_{56}O_2$	568.428	568.425	0.003	6.72	1767.380	206,509.607	20,752.185	27,697.250
Lutein	$C_{40}H_{56}O_2$	568.428	569.441	−1.013	6.71	32,848.735	31,058.096	0	0
Antheraxanthin	$C_{40}H_{56}O_3$	584.422	584.446	−0.024	4.56	456.685	3564.352	5741.665	6244.032
Violaxanthin	$C_{40}H_{56}O_4$	600.417	601.427	−1.01	4.52	1978.380	496,509.607	20,773.147	308,527.710
Neoxanthin	$C_{40}H_{56}O_4$	600.884	600.418	0.466	3.68	827,996.521	1,215,562.211	17,548.673	239,232.16
Keto-zeaxanthin/4-keto-lutein	$C_{40}H_{54}O_3$	582.407	583.416	−1.009	4.17	0	9602.063	0	0

SA1 Wild-type tobacco leaf, *SA2* Transplastomic tobacco leaf, *SA3* Wild-type tobacco flower, *SA4* Transplastomic tobacco flower

4-keto-zeaxanthin in transplastomic tobacco plants leaf samples only. No other keto-carotenoid was observed in the transplastomic tobacco plants.

Discussion

Heterologous astaxanthin production study has been conducted in tobacco [2], tomato [27], potato [28], carrot [12], maize [29], soybean [30] and canola [31] through nuclear transformation routes with highest astaxanthin levels of 16,000.0 µg/g dry weight, reported in tomato fruit. Production in tobacco [10] and lettuce [11] have also been attempted through plastid transformation, with very high levels reported in tobacco (3120.0 µg/g fresh weight). Amongst all the studies conducted previously, the combination of these three genes (*lcy, bhy* and *bkt*) targeting the carotenoid pathway has not been conducted before. Moreover, it has been observed that previous studies used the ketolase gene from *H. pluvialis* individually without supplementing it with other pathway enzyme genes, like hydroxylase gene. The goal of the present study was to opt best possible genes that have been reported earlier to give rise to a better synthetic construct for enhanced production of astaxanthin through chloroplast transformation.

This study demonstrated unexpected interaction of exogenous carotenogenic genes that have been incorporated into the plastome of tobacco in an operon form. The synthetic operon (ASN) was designed by integrating genes belonging from carotenoid biosynthetic pathway for enhancing the precursor metabolites that can be effectively used by algal ketolase gene (*HpBKT*). The ketolase gene used in the study shares 74.50% nucleotide similarity with *Chlamydomonas reinhardtii* ketolase gene (*CrBKT*) that resulted in highest accumulation of astaxanthin ever reported in higher plant species.

Expression analysis of isoprenoid and carotenoid biosynthetic pathway in tobacco revealed high transcript production of mRNA transcripts from multigene operon which was found to influence upstream isoprenoid pathway genes by increasing their transcript levels due to which higher precursor substrates became available for successive enzymes to act upon. The expression analysis showed 2–7-fold increase in all the tested isoprenoid enzymatic genes.

Carotenoid metabolite analysis in tobacco plant system resulted in minor accumulation of keto-lutein in tobacco leaves. Similar results were observed in the study done in *C. reinhardtii* [32] where expression of *bkt* gene (used in this study) did not produce any keto-carotenoid. Since no traces of intermediate metabolites like canthaxanthin, adonixanthin or adonirubin were found, it is very unlikely that this ketolase enzyme would have oxygenated both the β-ione rings of zeaxanthin to give rise to 4-keto-zeaxanthin. Therefore, the peak observed is most likely to represent 4-keto-lutein. Moreover, we predict that even if the concentration of zeaxanthin was higher as compared to lutein, this ketolase enzyme chooses to oxygenate the single β-ione ring of lutein, irrespective to the substrate availability. This is in contrast to the observation by Leon et al. (2007) in *C. reinhardtii*, where high 4-keto-lutein formation was predicted to be due to high substrate availability of lutein [31].

This study also found that the carotenoid pathway of transplastomic plants showed a higher influx towards xanthophyll cycle of the plant by showing higher accumulation of metabolites like zeaxanthin, antheraxanthin, violaxanthin and neoxanthin instead of producing astaxanthin and other pathway keto-carotenoids. This suggested more effective functionality of beta-carotene hydroxylase (*bhy*) enzyme as compared to the ketolase enzyme. However, the expression analysis suggested higher accumulation of *bkt* mRNA as compared to *bhy* or *lcy*. This data suggests the heterologous ketolase enzyme lacking or having very poor oxygenating activity.

However, other studies done with the same ketolase gene in tobacco [2] and carrot [12] using nuclear genome engineering showed opposing results by producing astaxanthin with other intermediate keto-carotenoids in flower nectary of tobacco and carrot tissues. A previous study with transgenic tobacco showed about 50% keto-carotenoids accumulation of the total carotenoid content in the transgenic tobacco nectary and no accumulation in the leaf sample. Similarly, work done in carrot reported approximately 70% keto-carotenoid accumulation of the total carotenoid content in carrot tissues. Keeping these studies in consideration we designed the synthetic ASN construct in the form of an operon for promoting much better product formation by delivering higher substrate levels of zeaxanthin. Overall, we are able to postulate that zeaxanthin is not a substrate for this algal ketolase enzyme. Poor metabolic conversion could have several proposed reasons like limited substrate availability over low enzyme activity [33], lack of substrate accessibility in chromoplast or chloroplast [34], presence of plant carotenogenic enzymes in multi-subunit form with integration in the plastid membrane for decreasing substrate escape [35], carotenes are preferentially ligated to the reaction centres of photosystems and xanthophylls are found in light-harvesting complexes making them better available than carotenes to the carotenogenic enzymes [36, 37]. In

the present study exogenous genes are expressed inside the chloroplast itself which negate some of the above reasons of substrate accessibility and availability. Also, we predict that marine bacterial ketolases (crtW) are found to result in higher accumulation of keto-carotenoids in higher plants as compared to algal ketolases, such as *HpBKT* (from *Haematococcus*) and *CrBKT* (from *Chlamydomonas*) [10, 18, 30, 38]. In addition, we propose that hydroxylated xanthophyll like zeaxanthin is not a good substrate for *bkt* used in the synthetic operon construct thereby forming very low levels of 4-keto-lutein in transplastomic leaf sample. Moreover, the algal hydroxylase gene used in this study worked very efficiently and resulted in higher production of Zeaxanthin leading to accumulate xanthophyll cycle metabolites.

Conclusion

This study has shown transgene expression of carotenogenic genes assembled as a synthetic operon is possible through stable chloroplast transformation in tobacco, resulting in the production of 4-keto-lutein. ASN operon functionality in tobacco plant gave rise to different metabolites, which could be due to availability and accessibility of different precursors in the plant. 4-keto-lutein could have been formed due to the presence of α-carotene or lutein in the plant system as both consist of single β-ionone ring in their structure and it would be comparatively easier for the enzyme to catalyse one ring instead of two. The gene combinations of lycopene cyclase (*lcy*), β-carotene hydroxylase (*bhy*) and β-carotene ketolase (*bkt*) used in this study is used for the first time in this combination in any higher plant system. Moreover, this work concludes that enzyme activity is more important than its accessibility to its substrate in case of close chloroplast compartments of higher plants, as high accumulation of available zeaxanthin precursor metabolite could not lead to production of astaxanthin.

Importantly, these results clearly show that algal ketolase (*bkt*) have certain limitations and should not be used in the future for producing higher amounts of keto-carotenoids in plants. Although, the study revealed some unexpected results but it confirmed important enzymological aspects and functionality of the heterologous ketolase gene used in the plant system. Further Design–Built–Test–Learn (DBTL) approach is needed to find out the best gene combination for heterologous astaxanthin production using the synthetic operon approach for chloroplast metabolic engineering. The molecular genetic tool developed in this study will facilitate the above DBTL approach.

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Author Contributions NT conducted experiments, analysed data and drafted the MS. JER and DMC participated in drafting and data analysis and contributed reagents/chemicals. SKL participated in drafting and data analysis, contributed reagents/chemicals and designed experiments. All the authors read and approved the final MS.

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Data availability Datasets generated from this study are provided in the main article and as supplemental files.

Declarations

Conflict of interest The authors declare no competing financial interest or any conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors. Further, the authors have considered Committee on Publication Ethics (COPE) guidelines for preparing this article.

References

1. Ruiz-Sola, M. Á., & Rodríguez-Concepción, M. (2012). Carotenoid biosynthesis in *Arabidopsis*: A colorful pathway. *Arabidopsis Book, 10*, e0158.
2. Mann, V., Harker, M., Pecker, I., & Hirschberg, J. (2000). Metabolic engineering of astaxanthin production in tobacco flowers. *Nature Biotechnology, 18*(8), 888–892.
3. Kim, S. H., Ahn, Y. O., Ahn, M.-J., Lee, H.-S., & Kwak, S.-S. (2012). Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweet potato. *Phytochemistry, 74*, 69–78.
4. Swapnil, P., Meena, M., Singh, S. K., Dhuldhaj, U. P., Harish, & Marwal, A. (2021). Vital roles of carotenoids in plants and humans to deteriorate stress with its structure, biosynthesis, metabolic engineering and functional aspects. *Current Plant Biology, 26*, 100203.
5. Aziz, E., Batool, R., Akhtar, W., Rehman, S., Shahzad, T., Malik, A., Shariati, M. A., Laishevtcev, A., Plygun, S., Heydari, M., Rauf, A., & Ahmed Arif, S. (2020). Xanthophyll: Health benefits and therapeutic insights. *Life Sciences, 240*, 117104.
6. Naguib, Y. M. (2000). Antioxidant activities of astaxanthin and related carotenoids. *Journal of Agricultural and Food Chemistry, 48*(4), 1150–1154.
7. Misawa, N. (2009). Pathway engineering of plants toward astaxanthin production. *Plant Biotechnology, 26*(1), 93–99.

8. Jin, S., & Daniell, H. (2015). The engineered chloroplast genome just got smarter. *Trends in Plant Science, 20*(10), 622–640.
9. Llorente, B., Torres-Montilla, S., Morelli, L., Florez-Sarasa, I., Matus, J. T., Ezquerro, M., D'andrea, L., Houhou, F., Majer, E., & Picó, B. (2020). Synthetic conversion of leaf chloroplasts into carotenoid-rich plastids reveals mechanistic basis of natural chromoplast development. *Proceedings of the National Academy of Sciences of USA, 117*(35), 21796–21803.
10. Hasunuma, T., Miyazawa, S. I., Yoshimura, S., Shinzaki, Y., Tomizawa, K. I., Shindo, K., Choi, S. K., Misawa, N., & Miyake, C. (2008). Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. *The Plant Journal, 55*(5), 857–868.
11. Harada, H., Maoka, T., Osawa, A., Hattan, J.-I., Kanamoto, H., Shindo, K., Otomatsu, T., & Misawa, N. (2014). Construction of transplastomic lettuce (*Lactuca sativa*) dominantly producing astaxanthin fatty acid esters and detailed chemical analysis of generated carotenoids. *Transgenic Research, 23*(2), 303–315.
12. Jayaraj, J., Devlin, R., & Punja, Z. (2008). Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic Research, 17*(4), 489–501.
13. Lu, Y., Rijzaani, H., Karcher, D., Ruf, S., & Bock, R. (2013). Efficient metabolic pathway engineering in transgenic tobacco and tomato plastids with synthetic multigene operons. *Proceedings of the National Academy of Sciences of USA, 110*(8), E623–E632.
14. Zhou, F., Karcher, D., & Bock, R. (2007). Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. *The Plant Journal, 52*(5), 961–972.
15. Apel, W., & Bock, R. (2009). Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. *Plant Physiology, 151*(1), 59–66.
16. Zhou, P., Ye, L., Xie, W., Lv, X., & Yu, H. (2015). Highly efficient biosynthesis of astaxanthin in *Saccharomyces cerevisiae* by integration and tuning of algal crtZ and bkt. *Applied Microbiology and Biotechnology, 99*(20), 8419–8428.
17. Maliga, P., Tungsuchat-Huang, T., & Lutz, K. A. (2021). Transformation of the plastid genome in tobacco: The model system for chloroplast genome engineering. *Methods in Molecular Biology, 2317*, 135–153.
18. Ruf, S., Hermann, M., Berger, I. J., Carrer, H., & Bock, R. (2001). Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nature Biotechnology, 19*(9), 870.
19. McBride, K. E., Svab, Z., Schaaf, D. J., Hogan, P. S., Stalker, D. M., & Maliga, P. (1995). Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Bio/Technology, 13*(4), 362–365.
20. Daniell, H., Jin, S., Zhu, X. G., Gitzendanner, M. A., Soltis, D. E., & Soltis, P. S. (2021). Green giant—A tiny chloroplast genome with mighty power to produce high-value proteins: History and phylogeny. *Plant Biotechnology Journal, 19*(3), 430–447.
21. Ruf, S., & Bock, R. (2014). Plastid transformation in tomato. In *Chloroplast biotechnology* (pp. 265–276). Springer.
22. Verma, D., Samson, N. P., Koya, V., & Daniell, H. (2008). A protocol for expression of foreign genes in chloroplasts. *Nature Protocols, 3*(4), 739–758.
23. Day, A., & Goldschmidt-Clermont, M. (2011). The chloroplast transformation toolbox: Selectable markers and marker removal. *Plant Biotechnology Journal, 9*(5), 540–553.
24. Doyle, J. (1991). DNA protocols for plants. In *Molecular techniques in taxonomy* (pp. 283–293). Springer.
25. Lichtenthaler, H. K., & Buschmann, C. (2001). Chlorophylls and carotenoids: Measurement and characterization by UV–Vis spectroscopy. *Current Protocols in Food Analytical Chemistry, 1*(1), F4.3.1-F4.3.8.
26. Zielewicz, W., Wróbel, B., & Niedbała, G. (2020). Quantification of chlorophyll and carotene pigments content in Mountain Melick (*Melica nutans* L.) in relation to edaphic variables. *Forests, 11*(11), 1197.
27. Huang, J.-C., Zhong, Y.-J., Liu, J., Sandmann, G., & Chen, F. (2013). Metabolic engineering of tomato for high-yield production of astaxanthin. *Metabolic Engineering, 17*, 59–67.
28. Campbell, R., Morris, W. L., Mortimer, C. L., Misawa, N., Ducreux, L. J., Morris, J. A., Hedley, P. E., Fraser, P. D., & Taylor, M. A. (2015). Optimising ketocarotenoid production in potato tubers: Effect of genetic background, transgene combinations and environment. *Plant Science, 234*, 27–37.
29. Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., & Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proceedings of the National Academy of Sciences of USA, 105*(47), 18232–18237.
30. Pierce, E. C., LaFayette, P. R., Ortega, M. A., Joyce, B. L., Kopsell, D. A., & Parrott, W. A. (2015). Ketocarotenoid production in soybean seeds through metabolic engineering. *PLoS ONE, 10*(9), e0138196.
31. Fujisawa, M., Takita, E., Harada, H., Sakurai, N., Suzuki, H., Ohyama, K., Shibata, D., & Misawa, N. (2009). Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *Journal of Experimental Botany, 60*(4), 1319–1332.
32. León, R., Couso, I., & Fernández, E. (2007). Metabolic engineering of ketocarotenoids biosynthesis in the unicellular microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology, 130*(2), 143–152.
33. Stålberg, K., Lindgren, O., Ek, B., & Höglund, A. S. (2003). Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. *The Plant Journal, 36*(6), 771–779.
34. Ralley, L., Enfissi, E. M., Misawa, N., Schuch, W., Bramley, P. M., & Fraser, P. D. (2004). Metabolic engineering of ketocarotenoid formation in higher plants. *The Plant Journal, 39*(4), 477–486.
35. Zhang, Y., & Fernie, A. R. (2021). Metabolons, enzyme–enzyme assemblies that mediate substrate channeling, and their roles in plant metabolism. *Plant Communications, 2*(1), 100081.
36. Sun, T., Rao, S., Zhou, X., & Li, L. (2022). Plant carotenoids: Recent advances and future perspectives. *Molecular Horticulture, 2*(1), 3.
37. Grossman, A. R., Harris, E. E., Hauser, C., Lefebvre, P. A., Martinez, D., Rokhsar, D., Shrager, J., Silflow, C. D., Stern, D., & Vallon, O. (2003). *Chlamydomonas reinhardtii* at the crossroads of genomics. *Eukaryotic Cell, 2*(6), 1137–1150.
38. Allen, Q. M., Febres, V. J., Rathinasabapathi, B., & Chaparro, J. X. (2022). Engineering a plant-derived astaxanthin synthetic pathway into *Nicotiana benthamiana*. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2021.831785

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