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Metabolic engineering of tomato for high-yield production of astaxanthin

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ABSTRACT

Dietary carotenoids have been shown to be beneficial to health by decreasing the risk of many diseases. Attempts to enhance carotenoids in food crops have been successful although higher plants appear to resist big changes of carotenoid biosynthesis by metabolic engineering. Here we report the generation of a more nutritious tomato by modifying the intrinsic carotenes to astaxanthin, a high-value ketocarotenoid rarely found in plants. This was achieved by co-expression of the algal β-carotene ketolase from *Chlamydomonas reinhardtii* and β-carotene hydroxylase from *Haematococcus pluvialis*, a unique pair of enzymes identified to co-operate perfectly in converting β-carotene to astaxanthin by functional complementation in *Escherichia coli*. Expression of the two enzymes in tomato up-regulated most intrinsic carotenogenic genes, and efficiently directed carbon flux into carotenoids, leading to massive accumulations of mostly free astaxanthin in leaves (3.12 mg/g) but esterified astaxanthin in fruits (16.1 mg/g) and a 16-fold increase of total carotenoid capacity therein without affecting the plant normal growth and development. This study opened up the possibility of employing crop plants as green factories for economical production of astaxanthin.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) is a unique red carotenoid responsible for the pigmentation of many marine animals which acquire the red pigment from their diets not only for coloring but also for improving survival and growth of juveniles (Chien and Shiau, 2005). Growing evidence also shows that astaxanthin provides beneficial effects on human health, including the enhancement of general well-being and the immune system, protection against lipid-membrane peroxidation and DNA damage (Yuan et al., 2011). While chemically synthetic astaxanthin is restricted in use as a feed additive for salmon and trout farming, the demand for natural astaxanthin is expected to grow rapidly in the nutraceutical, cosmeceutical and pharmaceutical markets. However, the bioresources of astaxanthin are limited and thus expensive, which has attracted scientists to develop heterologous biosynthesis hosts for the sustainable production of natural astaxanthin (Misawa, 2009; Zhu et al., 2009).

Typically the biosynthesis of astaxanthin from β-carotene requires a ketolase (BKT) and a hydroxylase (BHY) to add a carbonyl and a hydroxyl at positions 4 and 3 of each terminal β-ionone ring, respectively (Fig. 1). BHYs are ubiquitous in all plants and organisms with oxygenic photosynthesis. In contrast, BKTs (assigned as *CrtW* in prokaryotes) are present only in some bacteria and a few green algae (Kajiwara et al., 1995; Lotan and Hirschberg, 1995; Misawa et al., 1995; Fernández-González et al., 1997). These genes have been used for genetic engineering of astaxanthin synthesis in plants (Mann et al., 2000; Hasunuma et al., 2008; Jayaraj et al., 2008). The highest astaxanthin content achieved so far is 5.44 mg/g (dry weight) by transplastomic tobacco (Hasunuma et al., 2008), which is about one tenth of that in the green microalga *Haematococcus pluvialis* (Boussiba et al., 1999). In contrast to *H. pluvialis* which possesses an efficient mechanism for sequestrating astaxanthin in esterified forms, plant vegetative leaves, bacteria and yeasts lack a storage mechanism for depositing astaxanthin in the same way as the alga. Certain plant tissues, such as fruits or flowers, do have the ability to store carotenoids (e.g. xanthophylls) in ester forms at very high levels (Piccaglia et al., 1998), suggesting the possibility of the tissues to accumulate much higher amounts of astaxanthin than that in vegetative leaves.

Tomato can serve as an excellent producer for astaxanthin because sufficient precursors (lycopene and β-carotene) are available and the storage capacity for lipophilic carotenoids is high (Fraser et al., 2007). Some successes in enhancing the contents of endogenous carotenoids, especially β-carotene (pre-vitamin A) in tomato fruit have been achieved (Fraser et al., 2009). However, tomato seems to resist engineered changes of intrinsic carotenes to non-native astaxanthin as demonstrated by transgenic tomato co-expressing the *crtW* and *crtZ* from *Parococcus* (Ralley et al., 2004). This might be due to the poor co-operation of the bacterial

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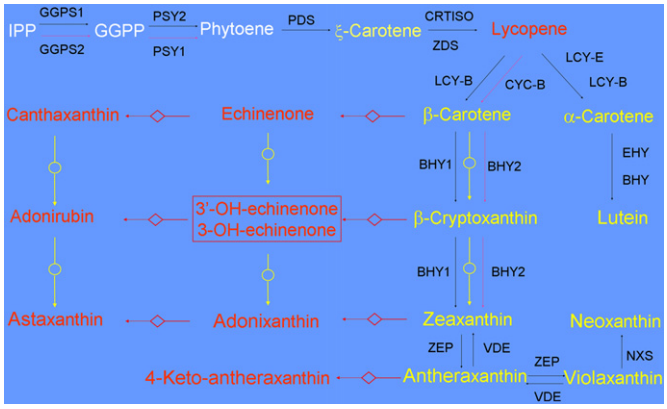

Fig. 1. Carotenoid biosynthetic pathways in tomato. Pink arrows indicate carotenogenesis in chromoplasts. Arrows with square or cycle in middle indicate ketolating or hydroxylating reactions conferred by transgenes. GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCY-B, lycopene β-cyclase; CYC-B, an isoenzyme of LCY-B responsible for the high β-carotene accumulation in B-type tomato fruit; LCY-E, lycopene ε-cyclase; BHY, β-ring hydroxylase; EHY, ε-ring hydroxylase; NXS, neoxanthin synthase; VDE, violaxanthin de-epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; ZEP, zeaxanthin epoxidase. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

crtW with plant carotenogenic enzymes. We have recently demonstrated that the *BKT* from *Chlamydomonas reinhardtii* confers efficient astaxanthin biosynthesis to the model plants Arabidopsis (Zhong et al., 2011) and tobacco (Huang et al., 2012). This special algal ketolase suits to efficiently convert tomato carotenes to astaxanthin, a xanthophyll that could possibly be sequestrated in esterified forms at high levels in the fruit.

The present study reported the engineering of tomato for high-yield production of astaxanthin by expressing a specific pair of algal *BKT* and *BHY* genes that were identified as the best combination for astaxanthin production from β-carotene in an *Escherichia coli* system. The resulting tomato accumulated high levels of non-native astaxanthin not only in vegetative leaves but also in the fruit of a tomato variety with high synthesis capacity for β-carotene. Moreover, the fruit accumulated 5-fold more astaxanthin than the leaves, reaching attractive levels for commercial production of natural astaxanthin.

2. Materials and methods

2.1. Constructs for E. coli transformation

The coding sequence of tomato *BHY*1 (Ronen et al., 2000) was cloned between the *Hind*III and *Xba*I sites of the plasmid pUC19. Similarly, the *BHY* coding sequences from *C. zofingiensis*, *H. pluvialis*, *Pantoea ananatis*, and Arabidopsis were cloned between the same sites of pUC19. For co-expression of *CrBKT* with different *BHYs* in *E. coli*, polycistronic plasmids were constructed by adding a Shine–Dalgarno (SD) sequence (AGGAGGACAGCC) upstream of the first ATG of *CrBKT* (Zhong et al., 2011) which was then inserted between the *Xba*I and *Sac*I sites of the *BHY*-harboring PUC19 plasmids resulting in polycistronic plasmids. *E. coli* JM109 carrying pACCAR16ΔcrtX was used as the host for functional analysis of the cooperation of *CrBKT* with various *BHYs* and tomato *BHY*1, respectively.

2.2. Constructs for plant transformation

pBI-SlTpCrBKT was derived from pBI121-CRBKT (Zhong et al., 2011) by replacing the transit peptide sequence of Arabidopsis *RBCS* with tomato one (GenBank No. M15236) using PCR-based cloning techniques. The *CrBKT* of pBI-SlTpCrBKT was replaced by *HpBHY* coding sequence (GenBank No. BD250390) to create pBI-SlTpHpBHY. The *CaMV35S*::*SlTpHpBHY*::*nos* cassette was amplified and inserted in the pBI121-SlTpCRBKT at the *Cla*I site resulting in pBI-SlTpHpBHY-SlTpCrBKT.

2.3. Tomato transformation

The generation of transgenic tomato plants was performed according to the protocol as described (van Roekel et al., 1993). Wild-type (cv. UC82B) and *Beta* mutant LA0316 tomato (*Solanum lycopersicum* Mill.) seeds were provided by TGRC (http://www.tgrc.ucdavis.edu/index.aspx). The presence and expression of the transgenes was verified by RT-PCR using *CrBKT* and *HpBHY* specific primers. The kanamycin-resistant putative transformed plants were grown in a green house.

2.4. Pigment extraction and analysis

Caroenoids were extracted and analyzed as described (Huang et al., 2006; Zhong et al., 2011). Pigments were identified on the basis of absorption spectra and retention times relative to standard compounds. Pigments were finally quantified by integrating peak areas that were converted to concentrations by comparison with authentic standards. Saponification of astaxanthin esters were performed according to Yuan and Chen (1999) and allotments of the extract solutions were analyzed by HPLC. Pigments were identified by comparing retention times and spectra against known standards and were finally quantified by integrating peak areas and converted them to concentrations by comparison with authentic standards that purchased from Sigma and Wako. Astaxanthin was measured at 480 nm and other carotenoids were measured at 450 nm.

2.5. Gene expression analysis

Total RNA samples were extracted from young tomato leaves or fruit harvested at the break stage. Real-time quantitative RT-PCR of endogenous carotenogenic genes was performed according to Zhong et al. (2011). Primers used in this study are listed in Table 1. PCR was run in a BIO-RAD iCycler IQ Multi-Color RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta Ct}$ method (Blois, 1958) using *actin* gene for normalization.

2.6. Photosynthesis analysis

The maximum quantum yield of PSII photochemistry was measured in dark-adapted samples by $(F_m-F_0)/F_m=F_v/F_m$ ratio, where F_0 is the dark-adapted initial fluorescence level and F_m is the maximum fluorescence level. CO_2 uptake (gas exchange) was measured using an LI-6400 portable photosynthesis system (LI-COR, http://www.licor.com). The leaf cuvette enclosed 6 cm^2 of leaf and was equipped with a thermostat to control leaf temperature. Light was provided by a Li-Cor light-emitting diode (LED). Leaves were measured the rate of CO_2 uptake (μmol CO_2 $m^{-2}s^{-1}$) in light photons of 0–1600 μmol photons $m^{-2}s^{-1}$ until the CO_2 rate is not increasing.

2.7. Antioxidant activity

Homogenized tomato samples (1 g of fresh full ripe fruit) were each extracted twice with acetone. DPPH (1.1-diphenyl-2-picrylhydrazyl) radical scavenging activity was determined according to the method of Livak and Schmittgen (2001). Equal volume (1 ml) of tomato extract and DPPH solution (0.2 mM in ethanol) was kept at room temperature for 30 min before the decrease of the solution

Table 1
Primers used for vector construction and transcriptional detection of carotenogenic genes.

Primer	Sequence	GenBank accession No.
CRBKTL F	5′–GAG AAG CTT CAT GGG CCC TGG GGA TAC A-3′	AY860820
CRBKTL R	5′–GCG TCT AGA TCA GGC CAG GGC TGC GCC GCG-3′	
LE-RBSCTPF	5′–GCG TCG ACC CGG GGA ACC AAA AAA AGA GAG AAG-3′	M15236
LE-RBSCTPR	5′–CCC AAG CTT GGC ATG CAG CTA ACT CTT CCA C-3′	
HPBHY F	5′–GAG AAG CTT AAT TAC CAC GAT GCT G-3′	AY187011
HPBHYR	5′–GCG TCT AGA CAT CTA GTA ACA TAG A-3′	
BHYBKT F	5′–CCA TCG ATA AGC TTG CAT GCC TGC-3′	
BHYBKTR	5′–CCA TCG ATG AAT TCC ATC TAG TAA CAT AGA-3′	
Cr-BKT F	5′–CCG CCT TCC GCC TGT TCT ACT A-3′	AY860820
Cr-BKT R	5′–GGC GGC ACT TGG GCA GCT-3′	
LE-actin F	5′–GGT CGT ACA ACT GGT ATT GTG TTG-3′	BT013524
LE-actin R	5′–TTC CCG TTC AGC AGT GGT GGT-3′	
HP-CRTZ F	5′–CGC AAA CGG GAG CAG CTG TCA TA-3′	AY187011
HP-CRTZ R	5′–CGC GCC ACC AAC CAC CAA GA-3′	
LE-DXS1 F	5′–GTT GGT CAT CAG TCT TAT CCT CAC-3′	FN424051
LE-DXS1 R	5′–TGG CAC CAT CAC CTA TTA CGG-3′	
LE-GGPS2 F	5′–GTA CCT CGC TAC CGC TAC AAC-3′	DQ267903
LE-GGPS2 R	5′–TAA TCC CAC ATT AGG GTT ACC AG-3′	
LE-PSY1 F	5′–GGC AAT ATA TGT ATG GTG CAG AAG-3′	EF650010
LE-PSY1 R	5′–CGC CCA TTG AAA ACA TCT TCT A-3′	
LE-PSY2 F	5′–GTT GAT ATT CAG CCA TTC AGA GAT-3′	TOMPSY2A
LE-PSY2 R	5′–TTC AGG TGC AAT GCC CAT AA-3′	
LE-PDS F	5′–GCC GCT CCA GTG GAT ATT TTC-3′	EF650011
LE-PDS R	5′–GCA GTG AGC TTC TGC TGA AGA GC-3′	
LE-ZDS F	5′–CCA TGT CAA AGG CCA CTC AGA-3′	EF650012
LE-ZDS R	5′–TAC GGT AAC AAC AGG CAC TCC-3′	
LE-CRTL-B F	5′–AGG TGA TTC ATG AGG AAT C-3′	EF650013
LE-CRTL-B R	5′–AAG AAC CTC TTG TAG CAG-3′	
LE-B F	5′–TTG GTG GGA ATT CAG GGA TAG T-3′	AF254793
LE-B R	5′–AGG CCA CAA ACC ATT CCA AAC T-3′	
LE-CRTR-B1 F	5′–TTG GTG CTG CTG TAG GAA TG-3′	Y14809
LE-CRTR-B1 R	5′–GCA ATG AGG CCT TTA TGG AA-3′	
LE-CRTR-B2 F	5′–GGC GCT GCC GTA GGA ATG G-3′	DQ864755
LE-CRTR-B2 R	5′–CCG AAA CAG AGG CCA GGG AC-3′	

absorbance was measured at 517 nm. The percentage of inhibition was calculated by % inhibition=[1–(absorbance of the solution with extracts/ absorbance of the solution without extracts)] × 100%. Lipid peroxidation was determined by fluorescence according to the method of Kang et al. (2006). Inhibiting rate (%) is calculated as [fluorescence intensity (395ex/460em) of BSA adding with fatty acid—flurescence intensity (395ex/460em) of BSA with fatty acid and extract]/ fluorescence intensity (395ex/460em) of BSA adding with fatty acid × 100.

2.8. Data analysis

Carotenoid contents were analyzed by one-way ANOVA which was carried out by a post hoc Tukey's Honestly Significant Difference (HSD) test for more than three group means or Paired-Samples *T* test for two group means by software SPSS. Quantitative data of real-time PCR, growth and photosynthesis were analyzed by Paired-Samples *T* test.

3. Results

3.1. Selection of specific β-carotene ketolase and hydroxylase for efficient synthesis of astaxanthin

Tomato genome harbors two BHY genes, which are involved in zeaxanthin synthesis of leaf chloroplasts (*BHY1*) and of flower chromoplast (*BHY2*), respectively (Galpaz et al., 2006). Therefore, to trigger tomato to accumulate astaxanthin in fruit, besides a suitable BKT, an additional BHY is also required. We used a β-carotene-producing *E. coli* system to screen well-cooperating BKT and BHY for astaxanthin formation. The *C. reinhardtii BKT* (*CrBKT*) was selected because it is superior to other sources of the enzymes for astaxanthin biosynthesis in plants (Huang et al., 2012; Zhong et al., 2011). Several *BHY*s from various organisms were co-expressed with *CrBKT* in β-carotene-producing *E. coli*. The *BHY* from *H. pluvialis* (*HpBHY*) was found to be the best one coordinating with CrBKT for astaxanthin formation in terms of astaxanthin conversion rate and proportion (Fig. 2). Therefore *HpBHY* was selected to reinforce carotenoid hydroxylase activity in tomato.

3.2. Generation of astaxanthin-producing tomato plants

We prepared two binary vectors containing *CrBKT* or this gene with *HpBHY* (Fig. 3A). The strong constitutive CaMV *35S* promoter was used to drive gene expression in two tomato lines, a wild-type (WT) accumulating lycopene and a mutant B-type (BT) which synthesizes mainly β-carotene together with some lycopene, This ubiquitous promoter was chosen to compare the differential astaxanthin formations between leaf chloroplasts and fruit chromoplasts where different carotenoid pathways and carotenoid storage occur (Ronen et al., 2000).

Twenty to thirty independent lines were generated for each of the two expression constructs, among them four primary transformants, designated as WT-bkt9, WT-bktbhy20, BT-bkt10, and BT-bktbhy8, were investigated in detail. The presence and expression of the transgenes were confirmed by RT-PCR analysis (Fig. 3B). A marked change of the color phenotype was observed in all transgenic plants which showed brown leaves and red flowers instead of the normal green and yellow (Fig. 3C and D).

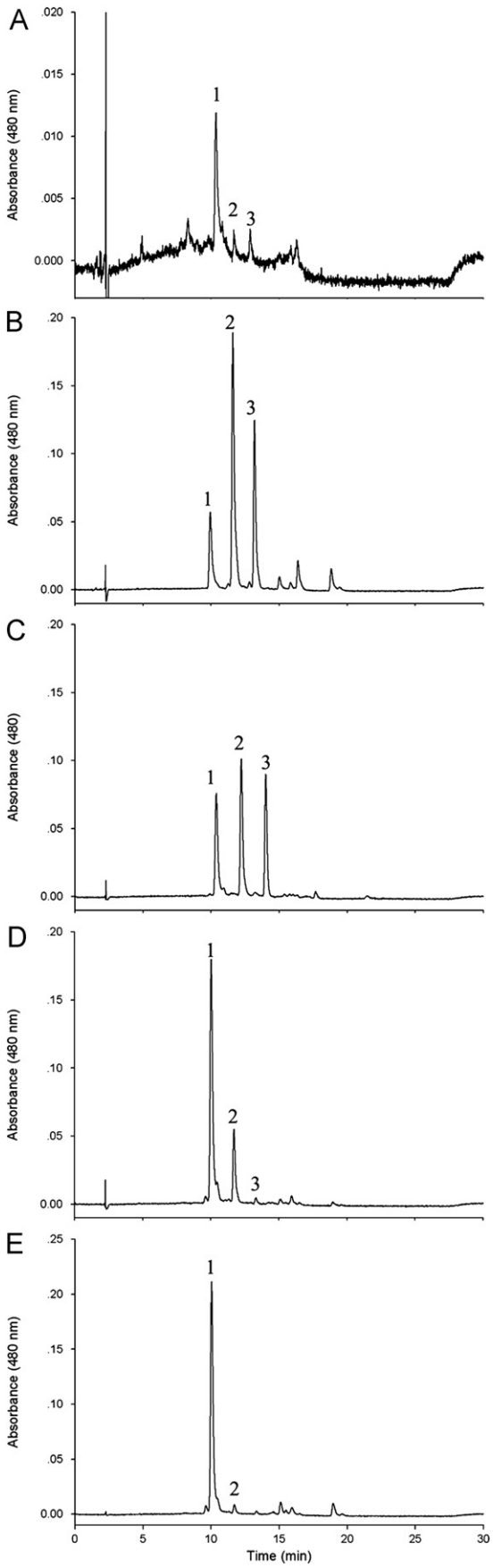

Fig. 2. HPLC elution profiles of carotenoids produced in a β-carotene-accumulating *E. coli* strain in the presence of cDNAs encoding *Chlamydomonas* BKT with *Erwinia* CrtZ (A), *C. zofingiensis* BHY (B), tomato BHY1 (C), Arabidopsis BHY1(D) or *H. pluvialis* BHY (E). 1, astaxanthin; 2, adonixanthin; 3, zeaxanthin.

Transgenic fruits developed normally except that the fruits of B-type transformants showed a much more intense red pigmentation compared to the more orange pigmentation in controls (Fig. 3D and F).

3.3. Carotenoid contents and compositions in leaves and fruits

The leaves and fruits of transgenic and control lines were subjected to carotenoid extraction and high performance liquid chromatography (HPLC) to determine their carotenoid profiles (Tables 2 and 3). Leaves of WT and B-type share a similar carotenoid profile with lutein, violaxanthin, β-carotene, and neoxanthin as the major carotenoids. Transgenic lines overexpressing *CrBKT* (WT-bkt9, BT-bkt10) predominantly accumulated non-native

Fig. 3. Phenotypes of T1 generation tomatoes over-expressing the *CrBKT* or *HpBHY-CrBKT* genes. (A) Schematic diagram of the T-DNAs in the two plasmids used to transform tomato. (i) The T-DNA consisted of a selectable marker (*nptⅡ*) cassette (Kan^r), the 35S promoter (35S) from cauliflower mosaic virus, *C. reinhardtii bkt* coding sequence (*CrBKT*) functionally fused to the tomato RUBISCO chloroplast transit peptide (TP), with a *nos* terminator. (ii) This T-DNA resembles construct (i) with an extension of an additional cassette comprising the *H. pluvialis BHY* coding sequence fused to the tomato TP. (B) RT-PCR detection of *CrBKT* and *HpBHY* expression in transgenic plants. (C) B-type tomato plant (left) and its CrBKT transformant (BT-bkt10, center) and its CrBKT-HpBHY transformant (BT-bktbhy8, right) showing different color in leaves. (D) Phenotypes of B-type flowers (left), BT-bkt10 (right) and cross-section of ripe B-type (left) and BT-bktbhy8 (right) fruit; (E,F) fruits at different ripening stages of B-type (E) and transgenic plant TB-bktbhy8 (F).

Table 2
Carotenoid content and composition in leaves of wild-type (WT), B-type (BT) and their transgenic tomato plants that express *Crbkts* (WT-bkt9, BT-bkt10) or *Crbkts* and *Hpbhy* (WT-bktbhy20, BT-bktbhy8).

	Carotenoid content (mg/g DW) and composition (%)											
	WT		WT-bkt9		WT-bktbhy20		BT		BT-bkt10		BT-bktbhy8	
	mg/g DW	%	mg/g DW	%	mg/g DW	%	mg/g DW	%	mg/g DW	%	mg/g DW	%
β-carotene	0.46 ± 0.01	14.1	0.08 ± 0.00	2.1	0.24 ± 0.01	3.92	0.48 ± 0.02	15.4	0.17 ± 0.02	4.5	0.08 ± 0.00	1.5
zeaxanthin	0	0	0	0	0	0	0.10 ± 0.03	3.2	0	0	0	0
Antheraxanthin	0.09 ± 0.00	2.8	0	0	0	0	0.07 ± 0.01	2.2	0	0	0	0
Violaxanthin	0.59 ± 0.02	18	0.05 ± 0.01	1.3	0.04 ± 0.00	0.7	0.35 ± 0.02	11.3	0.02 ± 0.00	0.5	0.02 ± 0.00	0.4
Neoxanthin	0.32 ± 0.02	9.8	0.02 ± 0.00	0.5	0.02 ± 0.00	0.4	0.29 ± 0.04	9.3	0.03 ± 0.01	0.8	0.03 ± 0.00	0.6
Lutein	1.81 ± 0.10	55.3	0.36 ± 0.03	9.5	0.79 ± 0.04	12.9	1.84 ± 0.07	59.2	0.46 ± 0.06	12.0	0.34 ± 0.01	6.4
Echinenone	0	0	0.04 ± 0.00	1.1	0.06 ± 0.00	0.9	0	0	0.06 ± 0.01	1.6	0.08 ± 0.01	1.5
3′-OHechinenone	0	0	0.28 ± 0.01	7.4	0	0	0	0	0.19 ± 0.03	5.0	0.04 ± 0.00	0.8
4-ketoantheraxanthin	0	0	0	0	0.29 ± 0.01	4.7	0	0	0.01 ± 0.00	0.3	0.25 ± 0.01	4.7
Canthaxanthin	0	0	1.59 ± 0.18	42.0	0	0	0	0	1.69 ± 0.30	44.2	0.14 ± 0.00	2.6
Adonirubin	0	0	0.69 ± 0.04	18.2	0.39 ± 0.03	6.4	0	0	0.53 ± 0.04	13.9	0.23 ± 0.06	4.3
Adonixanthin	0	0	0.28 ± 0.01	7.4	1.95 ± 0.34	31.8	0	0	0.32 ± 0.06	8.4	0.91 ± 0.10	17.2
Astaxanthin	0	0	0.40 ± 0.06	10.6 (27.3)	2.36 ± 0.10	38.5 (10.5)	0	0	0.35 ± 0.03(51)	9.2	3.12 ± 0.17	59.0 (10.3)
Total ketocarotenoid	0	0	3.29 ± 0.11	86.7	5.05 ± 0.52	82.3	0	0	3.15 ± 0.37	82.5	4.77 ± 0.17	90.2
Total carotenoid	3.27 ± 0.16	100	3.79 ± 0.13	100	6.13 ± 0.43	100	3.11 ± 0.18	100	3.82 ± 0.44	100	5.29 ± 0.24	100

The data represent average values from measurements of three individual tomato plants, ± SEM. Values in parentheses indicate astaxanthin esters (as percentage of total astaxanthin). DW, dry weight of tissue.

Table 3
Carotenoid content and composition in ripe fruits of wild-type (WT), B-type (BT) and their transgenic tomato plants that express (WT-bkt9, BT-bkt10) or *Crbkts* and *Hpbhy* (WT-bktbhy20, BT-bktbhy8).

	Carotenoid content (mg/g DW) and composition (%)											
	WT		WT-bkt9		WT-bktbhy20		BT		BT-bkt10		BT-bktbhy8	
	μg/g DW	%	μg/g DW	%	μg/g DW	%	μg/g DW	%	μg/g DW	%	μg/g DW	%
Phytoene	102.1 ± 12.3	9.65	97.6 ± 10.4	6.1	164.0 ± 18.6	5.3	19.9 ± 6.2	1.7	134.8 ± 23.6	2.1	0	0
Lycopene	881.2 ± 35.3	83.3	1363.4 ± 54.7	84.9	2853.4 ± 198.0	92.1	495.3 ± 43.2	43.1	542.2 ± 82.3	9.3	685.1 ± 32.7	3.6
β-carotene	38.5 ± 2.1	3.64	53.7 ± 7.4	3.3	28.7 ± 0.8	0.9	579.8 ± 53.8	50.5	931.5 ± 208.3	16.0	655.7 ± 60.4	3.4
Lutein	44.5 ± 1.4	4.2	31.6 ± 0.8	2.0	31.5 ± 0.4	1.0	72.3 ± 3.7	6.3	0	0	0	0
Echinenone	0	0	29.2 ± 1.4	1.8	0	0	0	0	200.3 ± 25.5	3.4	562.7 ± 10.5	2.9
4-ketoan-therxanthin	0	0	0	0	0	0	0	0	0	0	117.0 ± 9.1	0.6
Canthaxanthin	0	0	0	0	0	0	0	0	2249.7 ± 429.9	38.7	338.4 ± 6.5	1.8
Adonirubin	0	0	35.5 ± 1.0	2.2	0	0	0	0	828.6 ± 24.1	14.2	197.3 ± 14.5	1.0
Adonixanthin	0	0	55.2 ± 9.2	3.4	1.1 ± 0.5	0	0	0	0	0	393.4 ± 66.1	2.1
Astaxanthin	0	0	174.8 ± 22.0	10.9 (50.0)	19.9 ± 1.1	0.6 (58.4)	0	0	926.1 ± 91.2	15.9 (66.7)	16104.7 ± 308.1	84.5 (81.3)
Total ketocarotenoid	0	0	294.7 ± 32.3	18.3	20.8 ± 0.8	0.6	0	0	4204.6 ± 570.6	72.3	17713.5 ± 264.4	93.0
Total carotenoid	1058.0 ± 35.5	100	1606.5 ± 50.9	100	3098.4 ± 210.0	100	1148.2 ± 106.1	100	5813.1 ± 884.8	100	19054.4 ± 174.0	100

The data represent average values from measurements of three individual tomato plants, ± SEM. Values in parentheses indicate astaxanthin esters (as percentage of total astaxanthin). DW, dry weight of tissue.

canthaxanthin and other ketocarotenoids including astaxanthin (up to 86.7% of total carotenoids) at the expense of endogenous carotenoids. The low contents of astaxanthin (ca. 10% of total carotenoids) in the leaves of the transgenic lines were associated with the poor enzymatic activity of the endogenous BHY1 (also known as *CrtR-b*1) in converting canthaxanthin to astaxanthin (data not shown). This is further supported by the transformants (WT-bktbhy20 and BT-bktbhy8) co-expressing *CrBKT* and *HpBHY* to reinforce hydroxylase activity, leading to 6- to 9-fold increases of astaxanthin and a 1.8-fold increase of total carotenoids. B-bktbhy8 accumulated 3.12 mg/g (dry weight, DW) of astaxanthin in the leaves, accounting for 59% of total carotenoids with 90% in free form and 10% in esterified forms. Although the strong accumulation of ketocarotenoids in transgenic leaves was accompanied by decrease of chlorophyll contents (up to 30%), the efficiency of photosynthetic CO_2 assimilation was not affected nor showed the transgenic plants a significantly difference in heights (Fig. 4A and B).

Different carotenoid profiles were found in the ripe fruits of WT, BT and their transformants (Table 3). The most striking differences were the content of β-carotene and ketocarotenoids. WT-bkt9 and WT-bktbhy20 accumulated 1.5-fold and 2.9-fold higher amounts of total carotenoids in WT, respectively, with lycopene as the major carotenoid and ketocarotenoids as the minor ones due to the limitation of β-carotene. In contrast, the expression of *CrBKT* in B-type fruits resulted in a 5.1-fold increase of total carotenoids with ketocarotenoids as the predominant carotenoids including moderate increases of the endogenous carotenoids. Unexpectedly, BT-bktbhy8 contained a 16-fold higher concentration of the wild type total carotenoids, and an amount of astaxanthin (16.1 mg/g cell dry weight) similar to *H. pluvialis*.

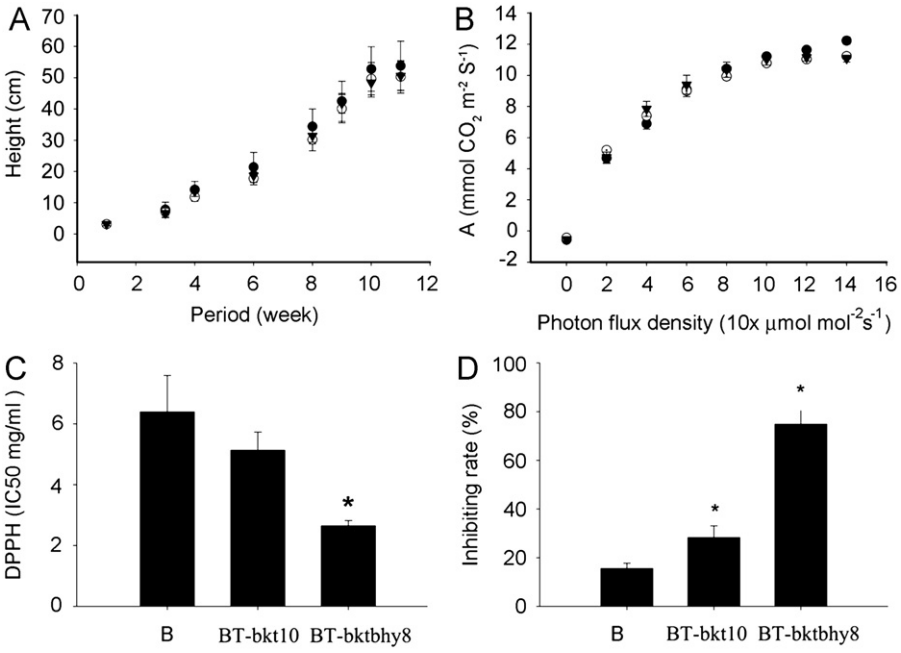

Fig. 4. Plant growth, photosynthesis and fruit antioxidant activity. (A) Changes in height of B-type (●), BT-bkt10 (◦) and BT-bktbhy8 (▾) plants grown under ambient CO_2 and 300 μmol photons m^{-2} s^{-1} light conditions. (B) Light-response curves of CO_2 assimilation rates in the leaves of B-type (●), BT-bkt10 (◦) and BT-bktbhy8 (▾) plants under 1000 μmol photons m^{-2} s^{-1} light conditions. (C,D), analysis of lipophilic antioxidant activity in ripe tomato fruit from B-type and its transgenic lines BT-bkt10 and BT-bktbhy8 determined by scavenging of 1.1-diphenyl-2-picrylhydrazyl (DPPH) (C) or by inhibiting rate of production of lipofuscin-like fluorescent proteins formed during lipid peroxidation (D). Data represent mean values ± SE and are derived from at least seven plants or three fruits per plant. * represents significant difference from controls ($p < 0.05$).

Moreover, BT-bktbhy8 accumulated mostly esterified astaxanthin (Fig. 5) like the alga.

Besides the vast accumulation of carotenoids, BT-bktbhy8 fruits even accumulated 2.2- and 2.4-fold of the normal vitamin C and reduced sugars, respectively (Table 4). In addition, BT-bktbhy8 fruit showed up to 3- and 5-fold higher antioxidant activity than controls based on its radical scavenging activity and fatty acid peroxidation inhibiting activity (Fig. 4C and D).

3.4. Expression of isoprenoid and carotenoid biosynthesis genes

The enhanced accumulation of carotenoids in the fruit was associated with the up-regulation of most carotenoid biosynthetic genes (Fig. 6). In all transformants, the transcripts of carotenogenic genes responsible for the formation of lycopene increased between 2- and 9-fold. Highest levels were found for the *DXS* gene encoding deoxyxylulose 5-phosphate synthase, the gateway enzyme for the terpenoid pathway, *GGPPS* encoding geranylgeranyl pyrophosphate synthase which provides the direct precursor for carotenoid formation, and *PSY1* (fruit-specific phytoene synthase gene) in combination with *PDS* (phytoene desaturase gene) which together initiate carotenogenesis. In transgenic B-type plants (B-bkt10 and B-bktbhy8) *cycB* was also strongly up-regulated (Fig. 2B). In contrast, the transcripts of *BHY1* and *BHY2* kept at low levels in fruits of both transgenic lines and controls, correlate to the high accumulation of canthaxanthin instead of astaxanthin in the transgenic B-type lines expressing only *CrBKT*.

4. Discussion

In this study, we successfully demonstrated that a specific pair of algal enzymes directed the tomato carotenoid biosynthesis pathway into non-native astaxanthin and consequently resulted in a 16-fold increase of total carotenoids in the fruit.

Carotenoid production in an organism depends on precursor supply, pathway capability, and carotenoid sequestration. Plants are superior to non-carotenogenic microorganisms including *E. coli* and yeast strains for metabolic engineering of astaxanthin production because plants contain all the enzymes except one, a ketolase that is responsible for catalyzing the final-step synthesis in astaxanthin formation. Furthermore, plants possess the potential to sequester secondary carotenoids in chromoplasts of specific organs at very high levels (Vishnevetsky et al., 1999). To date, the highest astaxanthin concentration obtained in transgenic microorganisms is 1.4 mg/g (dry weight) by a modified *E. coli* (Lemuth et al., 2011). Thus, pathway capability could be the limiting factor for metabolic engineering of astaxanthin production in plants. The conversion of β-carotene into astaxanthin is catalyzed by BKT and BHY which can compete for the same precursors, leading to the generation of various intermediates (Fig. 1). Therefore pathway engineering for high-yield production of astaxanthin in plants requires distinct control of the enzymes. Chloroplasts exhibit high BHY activity and therefore are rich in hydroxyl carotenoids. Since typical BKTs poorly convert hydroxyl carotenoids to astaxanthin, plants expressing such a BKT generally synthesized small amounts of astaxanthin with various intermediates (Misawa, 2009; Zhu et al., 2009). The most promising attempt to increase astaxanthin production in plants so far was the transformation of *crtW* and *crtZ* (an orthologous gene of algal and plant *BHY*) from a marine bacterium into the tobacco chloroplast genome (Hasunuma et al., 2008). As a result, the transplastomic tobacco yielded astaxanthin at 5.5 mg/g in leaves. However, plastid transformation is applicable only for a few model plants, making it difficult to copy the efficient pathway to plant tissues rich in chromoplasts and carotenoids for economical production of astaxanthin. Recently, two novel genes involved in astaxanthin formation in *Adonis* species were isolated and characterized (Cunningham and Gantt, 2011). The potential of the genes for modifying crop plants for astaxanthin production remains to be investigated.

Previously, transgenic tomato co-expressing the *crtW* and *crtZ* from *Paracoccus* sp. produced only trace amounts of ketocarotenoids

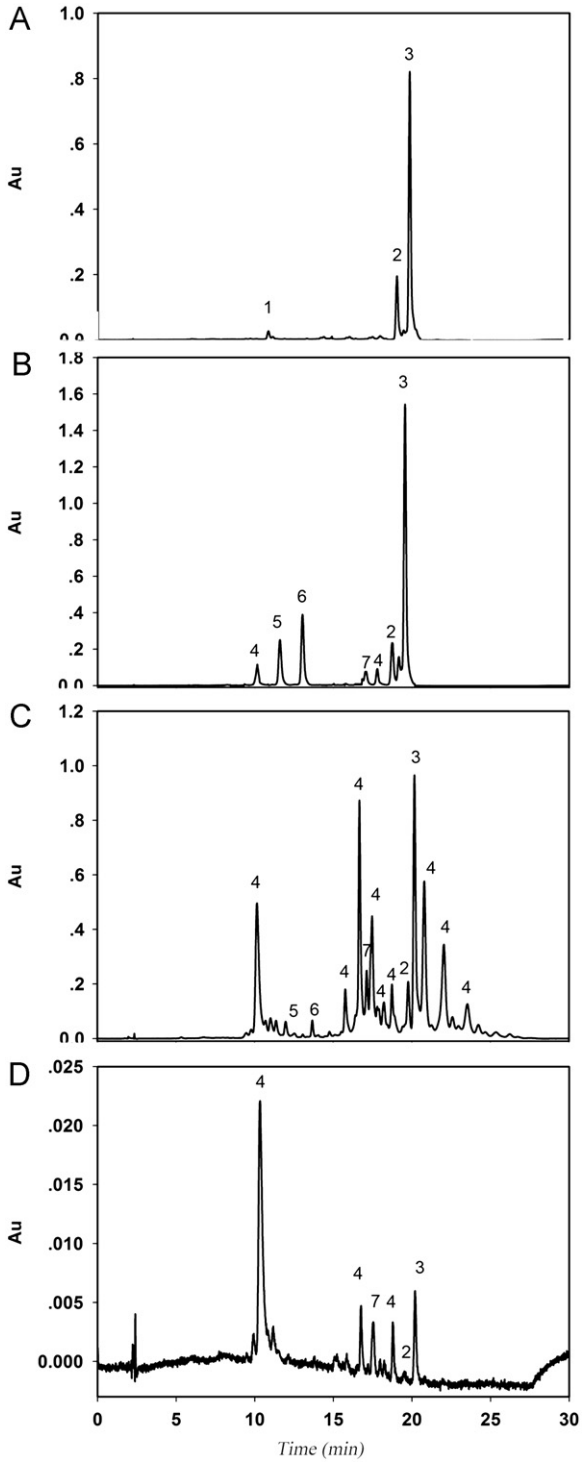

Fig. 5. HPLC chromatogram of carotenoids extracted from full ripening fruits of B type tomato (A) and its transgenic line BT-bkt10 (B), BT-bktbhy8 before (C) and after (D) saponification. Peak 1-violaxanthin; 2-lycopene; 3-β-carotene; 4-astaxanthin in free form (at retention time 10 min) or in ester forms (at other retention times); 5, adonirubin; 6, canthaxanthin; 7, echinenone.

in the leaf and fruit (Ralley et al., 2004). Most likely, the bacterial transgene products poorly co-operate with tomato carotenogenic enzymes. We recently showed that high zeaxanthin ketolating efficiency was essential for plants to accumulate astaxanthin at high levels (Zhong et al., 2011). Arabidopsis and tobacco expressing the function-enhanced BKT from *C. reinhardtii* accumulated high amounts of astaxanthin in the leaves (Huang et al., 2012; Zhong et al., 2011). This particular ketolase was used in this study to

Table 4
Vitamin C, reducing sugar contents of B-type and its transgenic tomato plants.

	BT	BT-bkt10	BT-bktbhy8
Vitamin C[a]	34.80 ± 1.18	63.39 ± 7.76*	76.36 ± 7.98*
Reducing sugars[b]	22.61 ± 1.11	35.84 ± 3.46*	54.18 ± 7.64*

Each value represents the mean ± SE ($n=3$).

[a] Total Vitamin C content was expressed as mg per 100 g fresh weight.

[b] Total reducing sugar content was expressed as mg g^{-1} fresh weight.

* means significant difference from control by student *T* test ($p<0.05$).

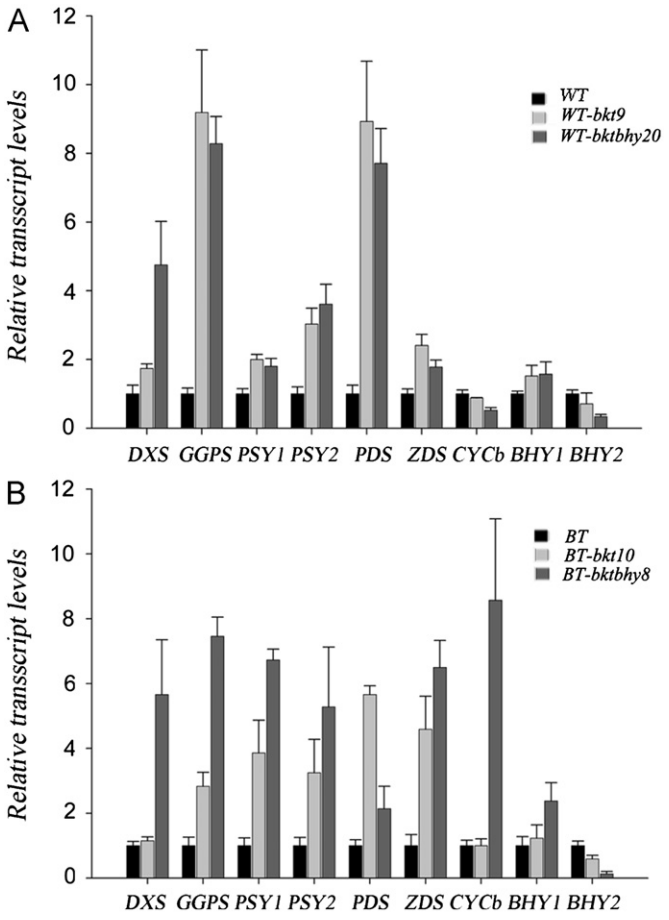

Fig. 6. Relative transcript levels of isoprenoid and carotenoid biosynthetic genes in tomato fruits at break stage. qPCR analysis of the expression of *DXS*, *GGPS*, *PSY1*, *PSY2*, *PDS*, *ZDS*, *CYCb*, *BHY1* and *BHY2* in the fruits of WT, its transformants WT-bkt9 and WT-bktbhy20 (A) and in the fruits of B-type and its transformants BT-bkt10 and BT-bktbhy8 (B). The transcript levels of the genes are normalized to actin transcript levels and expressed relative to the WT or B-type which is set as 1. In all cases, means ± SD ($n=3$) are represented.

investigate the biosynthesis, regulation, and storage of astaxanthin in leaf chloroplasts and fruit chromoplasts. Transformants expressing only the ketolase accumulate canthaxanthin as a major ketocarotenoid in both leaves with high native BHY activity and fruits with low native BHY activity. In contrast, transgenic tomato co-expressing the BKT and BHY from *H. pluvialis* triggered high accumulation of astaxanthin in both leaves and fruits. The astaxanthin content in the transgenic tomato leaves was in the same range as in transplastomic tobacco leaves (Hasunuma et al., 2008) and represents the highest in leaves that have been achieved so far by nuclear transformation. Moreover, much higher amounts of astaxanthin were reached in the transgenic B-type tomato fruits with a β-carotene background, which are 5-fold and 16-fold higher than that in the transplastomic tobacco leaves and in transgenic

carrot roots (Jayaraj et al., 2008), respectively. Typically, the astaxanthin contents in various lines of transgenic BT tomato expressing the BKT and BHY range from 9.8 mg/g to 16.1 mg/g in their full ripe fruits (data not shown). The variation could result from the inserted sites or/and number of the transgenes. Thus, both higher and lower contents of astaxanthin may be accumulated in next generations of the transformants. This provides the possibility of obtaining transgenic lines with higher concentrations of astaxanthin by screening more transformants and their subsequence generations. The extremely high content of astaxanthin in the tomato fruits could be attributed to a very high proportion of astaxanthin (84.5%) and its sequestration in esterified form (81.3%) as compared with a low proportion of astaxanthin in transgenic carrot roots and the production of astaxanthin in free form in both transplastomic tobacco leaves and nuclear transgenic carrot roots (Hasunuma et al., 2008; Jayaraj et al., 2008).

Pathway de-regulations are frequent during metabolic engineering or other manipulations (Fraser et al., 2009). It was first shown by inhibition of phytoene desaturase with bleaching herbicides (Simkin et al., 2000). Examples for regulatory effects in genetically modified carotenoid pathways are potato tuber in which zeaxanthin accumulated by antisense inactivation of zeaxanthin expoxidase accompanied by an up-regulation of the phytoene synthase transcript (Römer et al., 2002) and tomato transformed with a lycopene cyclase gene for β-carotene formation in which the synthesis of total carotenoids was substantially increased after segregation of the primary transformants (D'Ambrosio et al., 2004). A common explanation for these cases is a negative control by some carotenoids on the formation of key carotenogenic enzymes. In our case, the accumulation of astaxanthin at the expense of β-carotene may impair a feedback mechanism that regulates carotenoid biosynthesis (Mann et al., 2000). As a result, the metabolic fluxes in the transgenic tomato fruit were redirected to carotenoid biosynthesis mediated by the up-regulation of carotenogenic genes (Fig. 6). Alternatively, lutein may serve as a negative regulator which controls the expression of key carotenogenic enzymes and carotenoid formation because it is the only carotenoid that decreases upon genetic manipulation in leaves and in fruit (Tables 2 and 3). Details of the regulation of carotenogenesis are poorly understood (Sandmann et al., 2006). Lutein may play the same role as the global down regulators of carotenogenesis like *det1* (Davuluri et al., 2005) or the *or* gene (Lu et al., 2006).

We have successfully modified tomato carotenes to the xanthophyll astaxanthin that could be sequestrated in the esterified form and accumulated at much higher levels than ever achieved. Our transgenic plants show normal viability and growth as well as a photosynthesis activity compared to their non-transgenic lines (Fig. 4A and B). Since plants (e.g., marigold) can accumulate carotenoids up to 5% of cell dry weight (Piccaglia et al., 1998), the astaxanthin production in B-bktbhy8 may be still below its theoretical maximum and could be further enhanced through an increase in precursor availability (Fraser et al., 2002) or through regulatory mechanisms (Davuluri et al., 2005; Lu et al., 2006). Compared to the microalga *H. pluvialis* which needs a well controlled environment (e.g. growth in an enclosed photobioreactor) for pure culture, tomato is a food crop cultivated cost-efficiently worldwide with very high yields. Therefore, our astaxanthin-producing tomato can make a potent source for commercial production of natural astaxanthin.

In summary, the present study demonstrated a drastic alteration of the carotenoid compositions and contents in an important crop plant mediated by two specific algal enzymes which efficiently convert β-carotenes to astaxanthin. Currently carotenoids produced by chemical synthesis dominate the global carotenoids market (ca. $1 billion, www.strategyr.com) because biological sources are still limited and extremely expensive. Rising health concerns and a global growing aging population have driven increasing demand for natural carotenoids serving as potent antioxidants to protect against various diseases. Our study opens the possibility of making crop plants as green factories for the economical production of high-value carotenoids as animal feed and for human consumption.

Author contributions

J.C. H. planned, designed the project, constructed transformantion vectors and contributed to the planning and writing of the paper. Y.J. Z. undertook the transformation experiments, performed all of the pigment, molecular, and other analysis. J.L. constructed vectors. G. S. and F. C. designed the project and contributed to the writing of the manuscript.

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