Fruit-specific expression of *crtB, HpBHY, CrBKT* and *SlLCYB* triggers hyper-production of carotenoids in tomato fruit

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Abstract

Background Tomato is a major source of dietary carotenoids that play an important role in human health. The biosynthesis of carotenoids involved in a number of catalytic steps, among which the phytoene synthase (crtB) is the first committed enzyme for carotenoid biosynthesis, and the lycopene β-cyclase (LCYB) catalyzes the synthesis of β-carotene, β-carotene hydroxylase (BHY) or β-carotene ketolase (BKT) plays a vital role in synthesizing xanthophylls. Although there are some studies about improving the carotenoids of tomato fruit, it is still challenged to engineer tomato landraces with good traits for hyper-production of carotenoids, and the effects of vital carotenogenic genes working together remain unclear. The aim of this study is to improve the production of carotenoids in a tomato landrace by fruit-specific expression of four carotenogenic genes.

Results A plant expression cassette containing the tomato E8 promotor to drive the four genes (crtB , HpBHY , CrBKT , and SlLCYB) in a polycistronic structure was constructed and introduced into a tomato landrace " Huang Song ". Three independent lines were confirmed to be putative transformants, which showed similar phenotypes to wild-type (WT) control excepting for the fruit colors. The transgenic fruit exhibited deep red color due to the accumulation of novel ketocarotenoids including astaxanthin, canthaxanthin, and ketolutein and much more lycopene. The contents of total carotenoids in transgenic fruit were up to 3.1~6.6 mg/g dry weight (DW), 20~44-fold of that in WT fruit which only accumulated low levels of lutein, lycopene and β-carotene. Furthermore, various generations of the transgenic plants (T0, T1, and T2) exhibited similar growing status and carotenoid profiles, indicating that the transformants were genetically stable. Moreover, most of the endogenous carotenogenic genes were up-regulated in transgenic fruits at mature stage, and more plastoglobules were found in chromoplast, suggested the causes of the enhanced accumulation of carotenoids and antioxidant activity in transgenic plants.

Conclusions Fruit-specific expression of crtB , HpBHY , CrBKT and SlLCYB triggers hyper-production of carotenoids and the synthesis of ketocarotenoids in tomato fruit. This study provides insights into metabolic engineering of tomato for enhanced production of value-added carotenoids.

Background

Carotenoids are biosynthesized in photosynthetic organisms where they play important roles in photosynthesis and photoprotection [1, 2]. These yellow to red pigments are also of benefit to human health, including improvement of the immune capability, prevention of chronic diseases, cardiovascular diseases, certain types of cancers, and age-related degeneration [3–5]. Canthaxanthin and astaxanthin are the most competitive and demanding nutritional compounds in market due to their health-promoting effects [6, 7], which has attracted great attentions to extend the carotenoid pathways in higher plants to produce ketocarotenoids by metabolic engineering [8].

Serving as a major source of carotenoids and vitamin C in human diets, tomato (Solanum lycopersicum) is one of the most important and favorite crops, and the fruit nutrition directly influences its market competitiveness. To meet the ever-increasing competitive market, improving the carotenoid profiles of tomato fruit has become a primary aspect in tomato breeding, and among these, germplasms especially landraces or traditional varieties play important roles in the improvement of agricultural lines to both environmental stresses and health-promoting traits [9, 10]. Although the Tomato Genetics Resource Center has been conserved over 20,000 tomato accessions, there are still lots of excellent resources not recorded around the world. The "Huang Song" tomato is a landrace cultivated in Yunnan China for hundreds of years, it exhibits vigorous growth and good fruit yields. However, it produces the yellowish fruits which contain much less carotenoids than common lines of tomato, hence with less nutrition, and less welcomed.

The biosynthesis of typical carotenoids has been well characterized (Fig. 1) [11]. Generally, the biosynthesis of carotenoids is mainly regulated by the expression of carotenogenic genes, especially certain rate-limiting genes, though reducing carotenoid degradation and improving carotenoid storage capacity also can elevate carotenoid contents [12]. For examples, as the first committed enzyme for carotenoid biosynthesis, increased expression of phytoene synthase (PSY/CrtB) generally elevated carotenoid production remarkably [13–16]. In contrast, over-expression of a lycopene β cyclase gene (LCYB) did not change total carotenoids significantly but converted lycopene to β-carotene effectively [17]. In some microalgae, β-carotene and zeaxanthin can be further converted into the ketocarotenoids canthaxanthin and astaxanthin by a β-carotene ketolase (BKT) [18]. Overexpressing a specific pair of BKT and β-carotene hydroxylase (BHY) triggered the hyper-accumulation of non-native ketocarotenoids in the leave and fruit of a Beta-mutant tomato [19].

Previous studies commonly used a strong constitutive promoter to drive no more than two targeted genes. While the involvement of constitutive promoters commonly resulted in the interference of endogenous metabolic network [20, 21]. This problem could be overcome by using a tissue specific promoter, e.g. the tomato E8 promoter [22, 23]. The biosynthesis of most metabolites including carotenoids involves in a number of catalytic steps. Thus, to effectively improve the production of a metabolite, it is sometimes necessary to express more than two key enzymes. Multigene transformation is still a challenge of plant genetic engineering, which usually depends on a large construct or co-transformation of multiple constructs [24, 25]. 2A peptide sequence of the foot-and-mouth disease virus could govern self-processing of polyproteins, which was successfully used in multigene co-transformation [26].

In this study, a 2A-linked polycistronic expression cassette consisting of an endogenous E8 promotor to drive four rate-limiting carotenoid pathway genes encoding for phytoene synthase (PSY/crtB), β-carotene hydroxylase (BHY), β-carotene ketolase (BKT), and Lycopene β-cyclase (LCYB) was constructed and introduced into the tomato landrace "Huang Song". Via driving four genes by one fruit specific promoter, this study alters the

carotenoid profiles of a tomato landrace in fruit with not only enhanced production of native carotenoids but also produced the high-value ketocarotenoids (canthaxanthin, astaxanthin, and ketolutein), leading to up to 44-fold increase of total carotenoids and the higher antioxidant activity.

Results

2.1 Generation of transgenic tomato lines with increased carotenoids

To improve the production of carotenoids in "*Huang Song*" tomato fruit, an expression cassette containing the E8 promoter and four rate-limiting genes linked by three 2A sequences was constructed (Figure 2A) and introduced into the landrace using the method described in materials and methods. Confirmed by PCR and RT-PCR analysis (Figure 2B, 2C), three independent lines designated as BBBB1, 2, 3 were achieved as putative transformants (T_0). E8 promoter is a fruit specific promoter, which directs the gene expression starting from the fruit become ripening [27]. The expression pattern of the exogenous genes coordinated with the E8 expression pattern in that *crtB, HpBHY and CrBKT* expressed markedly in the ripening stages but not in leaf, flower, and mature fruit (Figure 2C). The transformants shared the same phenotype as their mother line (Figure 2D) except for the color of the fruits (Figure 2E). In contrast to the yellowish fruit of controls, the transgenic lines bear deep red fruit, indicating that different carotenoid profiles might exist between the fruits.

2.2 Analysis of the carotenoids in transgenic tomato fruit

Since the transgenic tomato fruit exhibited different color to controls, we detected the compositions and contents of carotenoids in the fruit using the Ultra-performance liquid chromatography (UPLC) technique.

In contrast to the WT that consisted of mainly lutein, β-carotene and lycopene in fruit, the transgenic tomato accumulated large amounts of lycopene together with some non-native ketocarotenoids (canthaxanthin, ketolutein, astaxanthin) (Figure 3). Moreover, the contents of each carotenoid from WT and transgenic fruits showed significant difference (Table 1). WT fruit mainly accumulated lutein (56 μg/g DW), lycopene (41 μg/g DW) and β-carotene (38 μg/g DW) with a total carotenoid content of 150 μg/g DW. In contrast, the transgenic plants synthesized much higher amounts of lycopene (2561-6345 μg/g DW) and β-carotene (98-243 μg/g DW) in addition to significant amounts of non-native ketocarotenoids, leading to 21~44-fold increase of total carotenoids compared to the WT. Interestingly, the pool of lutein between WT and transgenic lines (lutein + ketolutein) seems to be constant, but unexpectedly, the transgenic plants only produced small amounts of ketocarotenoids, which might result from the poor conversion from lycopene to β-carotene, as well as canthaxanthin to astaxanthin based on the carotenoid profiles (Table 1).

To investigate genetic stability of the three transgenic lines, we investigated two more generations of the plants and found no difference in plant phenotypes among the samples. As to carotenoid profiles, similar data (Table 1) were found among the different generations though some variations occurred, possibly resulted from the different planting time. Thus, the transgenic lines could inherit stably with normal growth and stable carotenoids feature.

2.3 Expression of the carotenogenic genes

To correlate the dynamic changes of carotenoids with the expression of endogenous genes involved in carotenoid biosynthesis, we measured the contents of carotenoids and the transcriptional levels of carotenogenic genes in BBBB3 line at four stages of fruit development and ripening: mature green (MG), breaker (B), pink (P) and over red (OR) stages. Obviously, at MG stage, both transgenic plant and WT shared similar carotenoid profiles (Table 2). However, starting from B stage, the BBBB3 rapidly accumulated lycopene as well as other carotenoids including the ketocarotenoids, leading to a 40-fold increase of total carotenoids as compared with WT (Table 2). In contrast to other carotenoids which showed steady increases during the fruit development, the α-branch carotenoids (lutein and ketolutein) in BBBB3 fruit seemed almost not change (Table 2).

Table 1 Carotenoid contents and compositions in ripe fruits of WT and transgenic lines.

	Carotenoid content (μg/g DW)											
	T0				T1				T2			
	WT	BBBB1	BBBB2	BBBB3	WT	BBBB1	BBBB2	BBBB3	WT	BBBB1	BBBB2	BBBB3
Phytoene	n/d	19.03±1.74	51.43±22.89	41.12±26.62	n/d	75.02±5.91	62.33±3.75	84±9.51	n/d	83.82±6.77	75.02±5.91	129.55±14.71
Lycopene	41.36±5.7	2,899.76±129.01***	6,345.49±866.29***	5,470.08±241.02***	29.29±3.34	2,561.48±185.65***	4,344.41±622.12***	3,808.73±247.47***	40.46±6.38	4,316.84±129.42***	5,763.14±244.11***	5,545.04±334.25***
β-carotene	38.75±4.5	98.2±29.24*	112.64±47.63**	129.44±22.52**	39.12±6.42	159.16±5.81**	187.31±36.49**	243.18±9.83**	46.28±1.25	181.89±6.89**	159.16±5.81**	146.59±6.79**
Lutein	56.07±7.15	20.97±3.35*	21.5±5.23*	21.13±6.77*	51.59±5.65	22.16±0.97**	11.84±3.53***	12.02±1.22***	56.82±7.77	25.82±1.26**	22.16±0.97**	32.9±1.09*
Violaxanthin	14.62±1.85	n/d	n/d	n/d	14.95±1.62	n/d	n/d	n/d	21.4±3	n/d	n/d	n/d
ketolutein	n/d	31.45±5.03	32.24±7.85	49.3±15.79	n/d	33.24±1.46	17.76±5.3	28.05±2.85	n/d	38.73±1.89	33.24±1.46	20.11±2.54
Canthaxanthin	n/d	35.31±2.89	38.2±8.38	38.4±5.03	n/d	31.86±0.99	32.06±3.99	35.05±1.38	n/d	26.13±1.24	31.86±0.99	84.62±3.07
Astaxanthin (easters	n/d	42.57±11.08	40.87±10.45	64.55±4.95 (17.77)	n/d	69.52±1.95 (15.28)	83.07±11.85	124.72±5.16	n/d	58.91±4.9 (14.19)	69.52±1.95 (15.19)	92.46±7.42 (16.47)
%)		(10.50)	(14.57)				(19.86)	(22.91)				
Total ketocarotenoids	n/d	109.34±18.49	111.32±19.51	152.25±21.83	n/d	134.62±4.39	132.89±8.34	187.83±6.63	n/d	123.78±3.93	134.62±4.39	197.19±12.97
Total carotenoids	150.80±9.31	3,147.29±132.41***	6,642.37±953.88***	5,814.02±296.81***	134.94±13.76	2,952.45±182.25***	4,738.78±670.26***	4,335.75±248.44***	164.96±5.95	4,732.14±144.37***	6,154.1±247.77***	6,051.27±369.72***

The data represent average values from measurements of three individual tomato, ± SD. Values in parentheses indicate astaxanthin esters (as percentage of total astaxanthin). DW, dry weight. n/d = Not detected. Significant differences were determined by Student's *t*-tests. The *P*-Values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

Table 2 Carotenoid contents and compositions at various fruit development stages.

	Carotenoid content (μg/g DW)							
	MG		T		P		OR	
	WT	BBBB3	WT	BBBB3	WT	BBBB3	WT	BBBB3
Phytoene	n/d	n/d	n/d	n/d	n/d	67.23±9.36	n/d	129.55±14.71
Lycopene	n/d	n/d	13.12±1.69	190.67±17.42***	39.84±2.52	4,266.67±267.73***	40.46±6.38	5,545.04±334.25***
β-carotene	16.84±4.47	20.2±1.54	16.01±2.67	47.32±3.25*	40.7±5.35	157.84±11**	46.28±1.25	146.59±6.79**
Lutein	75.54±5.97	88.21±5.19	72.98±5.94	48.34±3.8*	61.54±8.9	34.78±1.4*	56.82±7.77	32.9±1.09*
Violaxanthin	17.82±2.88	15.36±0.49	26.35±6.3	n/d	26.66±1.21	n/d	21.4±3	n/d
Neoxanthin	3.36±0.85	5.57±0.21	n/d	n/d	n/d	n/d	n/d	n/d
ketolutein	n/d	n/d	n/d	27.51±1.2	n/d	24.49±3.27	n/d	20.11±2.54
Canthaxanthin	n/d	n/d	n/d	40.62±0.99	n/d	77.33±3.24	n/d	84.62±3.07
Astaxanthin	n/d	n/d	n/d	25.22±1.71	n/d	72.86±5.9	n/d	92.46±7.42
Total ketocarotenoids	n/d	n/d	n/d	93.35±3.71	n/d	174.68±5.92	n/d	197.19±12.97
Total carotenoids	113.56±8.73	129.34±3.09	128.46±2.76	379.68±18.56**	168.74±4.33	4,701.20±242.97***	164.96±5.95	6,051.27±369.72***

The data represent average values from measurements of three individual tomato, ± SD. DW, dry weight. n/d = Not detected. MG, mature green stage; B, breaker stage; P, pink stage; OR, over red stage. Significant differences were determined by Student's *t*-tests. The *P*-Values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

Transcript levels of carotenoid pathway genes were quantified by qRT-PCR. The statistically significant changes were observed from MG stage to OR stage. At MG stage, geranylgeranyl pyrophosphate synthase (*GGPPS2*), *PSY1*, *PDS* were up-regulated compared to control, and the relative expression of lycopene β-cyclase (*CYCB*), β-carotene hydroxylase (*CrtR-b2*) were 3-fold more than control, but the lycopene ε-cyclase (*LCY-E*) was down-regulated by 84%. In contrast, P450 carotenoid β- and ε-hydroxylases (*CYP97A29* and *CYP97C11*) were up-regulated by 2-fold at B stage. At P and OR stage, significant increases were observed in 1-deoxy-D-xylulose-5-phosphate synthase (*DXS1*) that might direct more carbon flux into carotenoids. In contrast, at OR stage, *CrtR-b2*, *CYP97A29* and *CYP97C11* were down-regulated by approximately 61% and 65% compared with control (Table 3).

2.4 Transgenic lines showed higher antioxidant activity

To investigate if the enhanced production of carotenoids in transgenic tomato fruits leads to a change of antioxidative activity, we measured the antioxidant activity of BBBB fruits based on DPPH(1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity [28]. All the 3 lines exhibited significantly higher antioxidant activity than that of control, with a tendency of higher levels to higher contents of carotenoids among the transgenic lines (Figure 4A). In addition, we measured the contents of vitamin C and reduced sugars in the fruit, which showed that transgenic plants produced much more vitamin C. In contrast, the contents of reduced sugars remained unchanged (Figure 4B).

Table 3 Transcript levels of carotenoid pathway genes in BBBB3 fruit at various fruit development stages relative to the WT control.

	Transcript level relative to control			
gene	MG	B	P	OR
GGPPS2	3.2±0.97**	1.26±0.11	1.14±0.3	1.13±0.02
DXS1	0.4±0.09*	0.72±0.05	1.73±0.15*	1.61±0.16*
PSY1	4.92±0.71***	1.13±0.06	0.85±0.12	0.69±0.08
PSY2	0.89±0.23	0.66±0.07	1.42±0.16	0.82±0.1
PDS	1.72±0.41*	0.93±0.07	1.16±0.15	0.69±0.07
ZDS	1.06±0.31	0.98±0.03	0.71±0.05	0.67±0.06
CYCB	3.29±0.55**	0.75±0.07	2.34±0.17*	0.39±0.06*
CrtR-b1	1.33±0.17	1.19±0.02	1.13±0.21	0.75±0.06
CrtR-b2	3.45±0.41**	0.73±0.07	0.71±0.11	0.37±0.04*
LCY-E	0.16±0.04***	2.2±0.29*	0.68±0.1	0.92±0.12
CYP97A29	1.23±0.07	2.37±0.36*	1.22±0.16	0.39±0.02*
CYP97C11	0.73±0.04	2.85±0.62**	1.1±0.21	0.35±0.04*

qRT-PCR was performed with gene-specific primers (Supplement Table 1), the transcript levels of genes were normalized to the expression level of *Actin*. Statistical determinations are shown as means ± SD (n = 3). Significant differences were determined by Students *t* -tests and the *P*-Values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001. MG, mature green stage; B, breaker stage; P, pink stage; OR, over red stage.

Discussion

In this study, we manipulated a high-yielding but low carotenoid-containing tomato landrace by the fruit-specific expression of four rate-limiting enzymes which triggered the great enhanced production of native carotenoids as well as significant amounts of ketocarotenoids, leading to up to 44-

fold increase of total carotenoids in the transgenic fruit as compared with the mother line, then the enhanced antioxidant activity.

Generally, the constitutive promoter CaMV35S was used to drive gene expression in a variety of plant tissues and developmental stage [29], however, its constitutive characteristics might interfere endogenous metabolic network, normal plant development, and fruit ripening [20, 21, 30]. In addition, other unexpected results, e.g., susceptibility to environmental influences, ununiform accumulation of target product, were found for transgenic plants using the 35S promoter [31, 32]. In this regard, tissue specific promoters could overcome these problems [13, 33]. Using the fruit specific promoter E8 to drive four rate-limiting step genes, we generated stable tomato lines that showed normal growth and accumulated ~ 6 mg/g lycopene together with significant amounts of extra canthaxanthin, ketolutein, and astaxanthin, leading to 3-fold increase of total carotenoids in the transgenic fruit as compared with that of common cultivated lines[34]. Furthermore, the involvement of E8 promoter might guarantee the stable growth, development, and carotenoid profiles of the transgenic plants when their progenies were planted in different seasons though small variation of carotenoid contents existed (Table 1). The small variation of carotenoids among different generations of the transgenic plants could result from such environmental factors as temperature and light that had been shown to influence carotenoid production in tomato fruit [35–37], since T_1 plants were planted in winter-spring seasons, whereas T_2 in spring-summer seasons.

The expression of transgenes led to enhanced production of endogenous carotenoids and keto-carotenoids production during fruit ripening (Table 2). This coincided with the upregulation of most of the endogenous carotenogenic genes (GGPPS, PSY1, PDS, CYCB) at mature stage and sustained upregulation of DXS1 during late stages of fruit ripening (Table 3). Coincidently, ultrastructure of the tomato fruit cells revealed that transgenic cells consisted of much more lycopene crystals and plastoglobules than controls (Supplement Fig. 1). This result supports that besides the synthetic pathway, carotenoid storage also plays a role in carotenoid production.

It is unexpected that the fruit-specific expression of the four transgenes led to the accumulation of lycopene up to 6 mg/g but much less production of ketocarotenoids in the fruit. This could result from uneven translation of the four genes linked by the 2A sequence. 2A-polycistronic system is a useful tool for multigene transformation [38–41], but the cleavage efficiency and relative expression of the linked genes may not be consistent and therefore could result in unexpected transgene function [42, 43]. Possibly, the upstream position of crtB resulted in more efficient conversion of GGPP into phytoene which could be further converted to lycopene by the endogenous downstream genes.

It seemed that the conversion of lycopene to β-carotene remained to be a limiting step in the transgenic lines because lycopene accounted for ~ 90% of total carotenoids. And interestingly, there were no violaxanthin or zeaxanthin in BBBB lines except at MG stage (Table 2), which could result from the low expression of both endogenous hydroxylase (CrtR-b2, CYP97A29 and CYP97C11) (Table 3) and transgenic HpBHY. Thus, the low production of ketocarotenoids could result from the poor availability of their precursors (β-carotene and zeaxanthin) and poor conversion from canthaxanthin to astaxanthin.

Carotenoid accumulation depends on not only the biosynthetic and metabolic rates but also the sequestration capacity [44, 45]. The proportion of esterified astaxanthin are correlate to the final content as esterified carotenoids are more stable and easier to store in plastids [46, 47]. Previously we had used 35S promoter to drive overexpression of HpBHY and CrBKT in a Beta mutant of tomato which accumulated much higher amounts of astaxanthin with esterified astaxanthin accounting for 81.3% of total astaxanthin [19]. In contrast, only 22.91% of total astaxanthin was astaxanthin-esters in this study (Table 1). Two main factors differed between the previous and present studies: the promoters and tomato lines used. Thus, to further enhance ketocarotenoid production, stronger fruit specific promoters and β-carotene-rich tomato lines have to be used. Furthermore, the activity of lycopene β-cyclase and β-carotene hydroxylase need to be enhanced. One alternative choice is to cross the transgenic lines with a Beta mutant line which can provide a high level of β-carotene and possibly higher activity of astaxanthin esterification, since similar approaches have been shown to greatly increase ketocarotenoids in tomato fruit [30, 31].

Conclusions

In summary, we have successfully manipulated a tomato landrace for high-yield production of carotenoids including the high-value ketocarotenoids specifically in the fruit. The transformants can serve as starting materials for further enhancement of astaxanthin production in tomato. Our study provides insights into metabolic engineering of tomato for enhanced production of value-added carotenoids.

Methods

5.1 Construct for tomato transformation

A pBI121-based binary vector with replacing the 35S promoter with the tomato E8 promoter to drive the *crtB* of *Erwinia uredovora* (GenBank: BAA14128), *BHY* of *Haematococcus pluvialis* (GenBank: AEA35045), *BKT* of *Chlamydomonas reinhardtii* (GenBank: Q9SPK6), and *LCYB* of *Solanum lycopersicum* (GenBank: 544104) was constructed (Figure 2A). The transit peptide sequence of tomato RBCS (GenBank: M15236) was fused to the crtB, BKT, and BHY according to Huang et al [19], while the 2A peptide sequence of foot-and-mouth disease virus [26] was used to link the four genes so as to make them co-express. The DNA sequence of the recombinant genes (BBBB:TP-crtB-2A-TP-BHY-2A-TP-BKT-2A-LCYB) was chemically synthesized by Tsingke (Kunming, China). "*Huang Song*" tomato (*Solanum lycopersicum*) landrace seeds were obtained from Yunnan Agricultural

University. Agrobacterium-mediated tomato transformation was performed according to the methods described in Huang et al [19]. Kanamycin resistant plants were detected by PCR analysis with specific primers of *crtB*, *HpBHY* and *CrBKT*.

5.2 Plant growth conditions and sample collection

Transgenic seeds were screened on MS (Murashige and Skoog) media with 75 mg/L kanamycin, and seedlings confirmed by PCR analysis of *CrBKT* were transplanted into soil. T1 plants were grown at winter-spring seasons, T2 plants were grown at spring-summer seasons. According to days after anthesis (DAA) and fruit color, we divided the ripening stages into 7 stages: 20 d (IG, immature green), 35 d (MG, mature green), 38 d (B, breaker), 40 d (T, turning), 42 d (P, pink), 43 d (HR, harvest red), and 45 d (OR, over red) [48]. We harvested nine fruits divided into three groups as three replication samples at each stage, all the samples were stored at -80 ℃ refrigerator until further use.

5.3 Extraction and analysis of carotenoids

Carotenoids were extracted and analyzed as described by Huang [19] and Ye [49]. 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 purchased from Sigma-Aldrich.

5.4 Gene expression analysis

Total RNA was isolated from leaf, flower, and fruit using Plant RNA Kit (Omega). cDNA was synthesized from 2 μg total RNA using Prime Script II 1st Strand cDNA Synthesis kit (TaKaRa Biotech Co., Ltd., Dalian, China) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) with a 20 μL reaction volume: 0.25 mM of each primer (Supplement Table 1), 10 μL of iTaq SYBR Green Super-mix (Bio-Rad), 1 μL of template cDNA and add ddH_2O up to 20 μL. Standard cycling condition was 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 15 s,72 °C for 15 s followed by 0.5 °C increment at 5 seconds/step from 65 °C to 95 °C for Melt Curve analysis. Tomato *Actin* gene was used as an internal control, relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method and data were analyzed using CFX Manager™ Software v3.1 (Bio-Rad).

5.5 Analysis of antioxidant activity, reducing sugar and vitamin C

Fruit antioxidant activity analysis was conducted by DPPH radical scavenging activity [28]. Comparing with the standard curve constructed from Trolox, we quantified the antioxidant activity as Trolox equivalent antioxidant capacity (TEAC) in μmol Trolox equivalent per gram of fresh fruit. Total ascorbic acid (vitamin C) content was determined by the 2, 4-dinitrophenylhydrazine method [50]. The total ascorbic acid content was calculated by comparison with ascorbic acid standard curve and absorbance. Reduced sugars concentration was determined using dinitrosalicylic acid assay according to Miller [51].

5.6 Statistical analysis

At least three biological replications and two measurement replicates were applied to all the experiments. Data were shown as means ± SD (Standard Deviation) and analyzed using IBM SPSS Statistics 25 (IBM, Armonk, NY, USA). The significant differences between transgenic lines and WT were examined by Student's *t*-test.

Supplementary Information

Supplementary data associated with this article can be found in the online version. Supplementary Table 1, primers used in this study. Supplementary Figure 1, transmission electron micrographs of plastids from WT and BBBB3 fruit pericarp.

List Of Abbreviations

2A: 2A peptide; B: breaker stage; BHY: β-carotene hydroxylase; BKT: β-carotene ketolase; CaMV35S: Constitutive cauliflower mosaic virus 35S promoter; CRTISO: carotene isomerase; *CrBKT*: *BKT* of *Chlamydomonas reinhardtii*; CYP97A29: Cyt P450-type monooxygenase 97A29; CYP97C11: Cyt P450-type monooxygenase 97C11; DAA: days after anthesis; DPPH: 1:1-diphenyl-2-picrylhydrazyl; DW: dry weight; E8: E8 promoter of *Solanum lycopersicum*; F: flower; FW: fresh weight; GGPPS: geranylgeranyl pyrophosphate synthase; *HpBHY*: *BHY* of *Haematococcus pluvialis*; HR: harvest red stage; IG: immature green stage; IPI: isopentenyl diphosphate isomerase; L: leaf; LB: left bord; LCYB: lycopene β-cyclase; LCY-E: lycopene ε-cyclase; MG: mature green stage; NOS: nos terminator; NXS: neoxanthin synthase; OR: over red stage; P: pink stage; PDS: phytoene desaturase; PSY (crtB): phytoene synthase; RB: right bord; *SlLCYB*: *LCYB* of *Solanum lycopersicum*; T: turning stage; TEAC: Trolox equivalent antioxidant capacity; TP: a tomato RUBISCO chloroplast transit peptide; UPLC: Ultra-performance liquid chromatography; VDE: violaxanthin de-epoxidase; WT: "*Huang Song*" tomato; ZDS: ζ-carotene desaturase; ZEP: zeaxanthin epoxidase; Z-ISO: ζ- carotene isomerase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files. All plant materials are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

J. C. H. designed the experiments. Y. Y. L. performed the experiments and analyzed the data. Y. Y. L. and J. C. H. wrote the manuscript and all authors have read and approved the manuscript.

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Figures

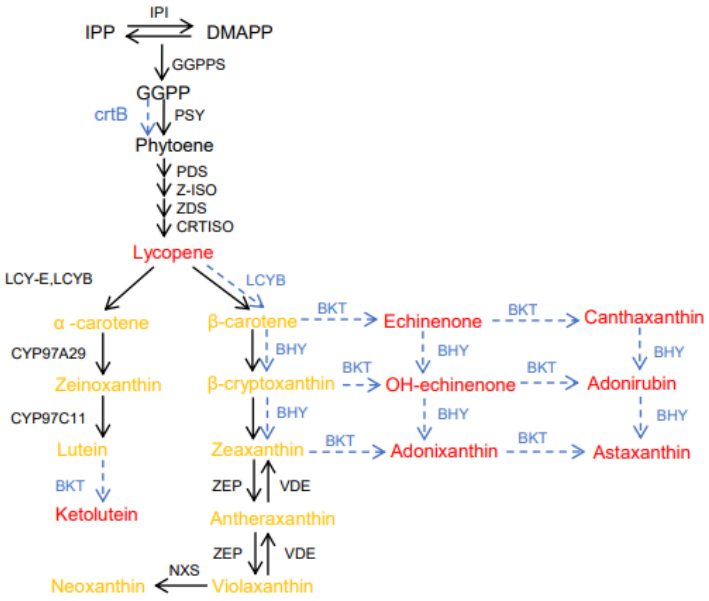

Figure 1

Schematic carotenoid biosynthesis pathway. IPI, isopentenyl diphosphate isomerase; GGPPS, geranylgeranyl pyrophosphate synthase; PSY (crtB), phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ- carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; CYP97A29, Cyt P450-type monooxygenase 97A29; CYP97C11, Cyt P450-type monooxygenase 97C11; BHY, β-carotene hydroxylase; BKT, β-carotene ketolase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase. Enzymes in blue and dash arrows indicate transgenic steps in this study.

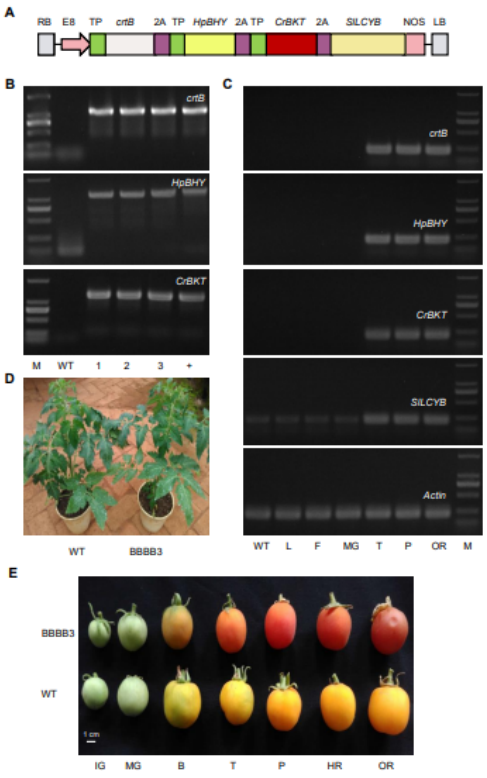

Figure 2

Expression construct, PCR-based detection and phenotypes of transgenic lines. (A) Schematic diagram of plasmid used for the transformation of “Huang Song”, the binary construct containing the four functional genes (crtB, HpBHY, CrBKT and SlLCYB) driven by E8 promoter. (B) PCR analysis of exogenous genes in BBBB lines. (C) RT-PCR analysis of crtB, HpBHY, CrBKT and SlLCYB in BBBB3. (D) Phenotype of WT and BBBB3 plants. (E) The fruits of WT and BBBB3 plants at various development stages. Full-length gels of (B) and (C) were presented in Supplementary material (Figure 2B-original and 2C-original). TP, a tomato RUBISCO chloroplast transit peptide; 2A, 2A peptide; WT, “Huang Song” tomato; 1-3, transgenic line BBBB1-3; +, positive control with pBI121-E8BBBB plasmid; M, DL2000 DNA Marker (from top to bottom: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); L, leaf; F, flower; IG, immature green stage; MG, mature green stage; B, breaker stage; T, turning stage; P, pink stage; HR, harvest red stage; OR, over red stage.

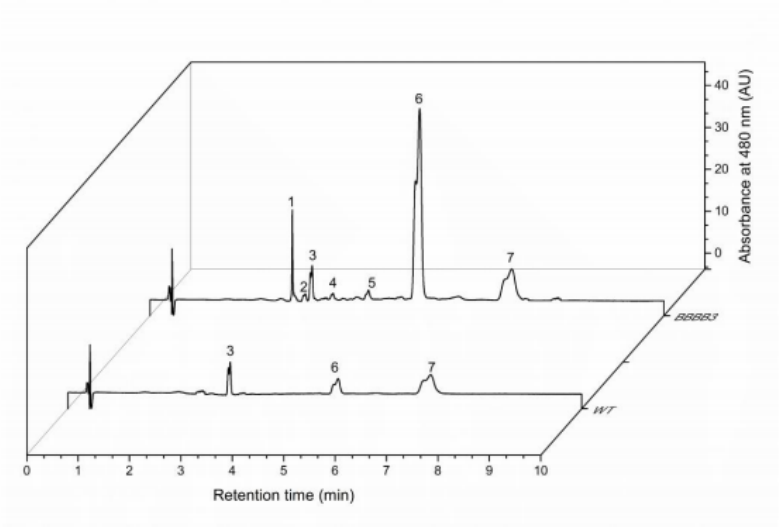

Figure 3

UPLC analysis of carotenoids in ripe fruits of WT and BBBB3. 1, astaxanthin; 2, ketolutein; 3, lutein; 4, canthaxanthin; 5, cis-lycopene; 6, trans-lycopene; 7, β-carotene.

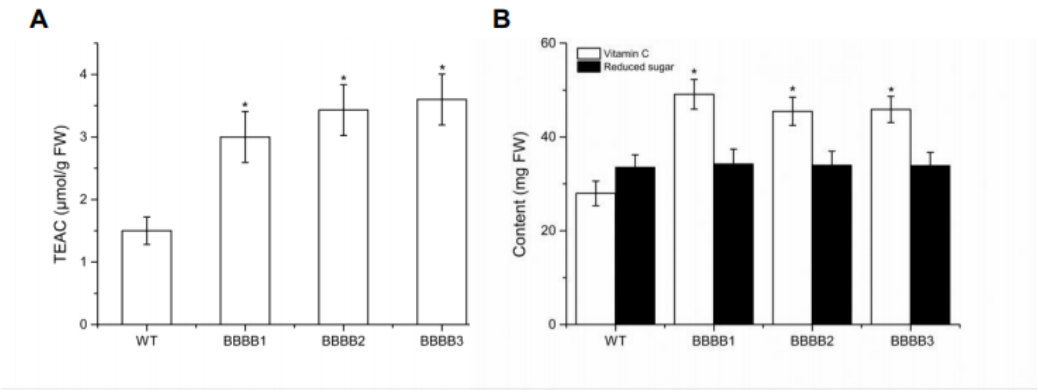

Figure 4

The antioxidant activity, Vitamin C and reduced sugar analysis of ripe fruits. (A) The antioxidant activity analysis of ripe fruits. The antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC) in μmol Trolox equivalent per gram of fresh weight (FW). (B) Vitamin C and reduced sugar analysis of ripe fruits. Vitamin C content was expressed as mg per 100 g FW; Total reduced sugar content was expressed as mg/g FW. Data represent mean values ± SD and are derived from at least three fruits per plant. * represents significant difference from controls ($P<0.05$).

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