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Enrichment of health-promoting lutein and zeaxanthin in tomato fruit through metabolic engineering

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ABSTRACT

Carotenoids constitute a large group of natural pigments widely distributed in nature. These compounds not only provide fruits and flowers with distinctive colors, but also have significant health benefits for humans. Lutein and zeaxanthin, both oxygen-containing carotenoids, are considered to play vital roles in promoting ocular development and maintaining eye health. However, humans and mammals cannot synthesize these carotenoid derivatives, which can only be taken from certain fruits or vegetables. Here, by introducing four endogenous synthetic genes, *SlLCYE, SlLCYB, SlHYDB,* and *SlHYDE* under fruit-specific promoters, we report the metabolic engineering of lutein/zeaxanthin biosynthesis in tomato fruit. Transgenic lines overexpression of one (*SlLCYE*), two (*SlLCYE* and *SlLCYB*; *SlLCYB* and *SlHYDB*), and all these four synthetic genes re-established the lutein/zeaxanthin biosynthetic pathways in the ripe tomato fruit and thus resulted in various types of carotenoid riched lines. Metabolic analyses of these engineered tomato fruits showed the strategy involved expression of *SlLCYE* tends to produce α-carotene and lutein, as well as a higher content of β-carotene and zeaxanthin was detected in lines overexpressing *SlLCYB*. In addition, the different combinations of engineered tomatoes with riched carotenoids showed higher antioxidant capacity and were associated with a significantly extended shelf life during postharvest storage. This work provides a successful example of accurate metabolic engineering in tomato fruit, suggesting the potential utility for synthetic biology to improve agronomic traits in crops. These biofortified tomato fruits could be also exploited as new research subjects for studying the health benefits of carotenoid derivatives.

1. Introduction

In nature, carotenoids constitute a large group with more than 700 derivatives that are widely distributed in various species. The biochemical nature of carotenoids are C_{40} tetraterpenoids derived from phytoene. These compounds are lipid-soluble antioxidants produced in chloroplasts [1,2]. For plants, carotenoids provide distinctive colors for fruits, vegetables, and flowers [1]. Certain oxygen-containing carotenoids, such as lutein and zeaxanthin, are accessory components of the light-harvesting complexes that abundant accumulation in the photosynthetic tissues in plants, and exhibit the distinctive roles of photosystem assembly, and photoprotection [3].

Besides their indispensable function in plant growth, carotenoids also constitute the pigmentation tissues of many birds, fish, and crustaceans [4,5]. In addition, some specific carotenoids have significant health benefits. For example, β-carotene is an essential precursor of mammalian for vitamin A synthesis [1]. Unlike plants, humans and animals do not synthesize carotenoids by themselves, and can only take carotenoids through diet.

Lutein and zeaxanthin, the oxygen-containing carotenoids that are

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collectively referred to as the macular pigment, have been reported to have important physiological features and clinical benefits. They are the only carotenoids containing active ingredients in the primate retina of the eye [3,6], where they can effectively counteract the negative actions of UV light and protection against oxidative stress, thus delaying the onset of age-related macular degeneration (AMD) [7,8]. Apart from their active properties in the prevention of cataracts, glaucoma, and diabetic retinopathy [3,6,9], increasing medical evidence suggests that lutein/or zeaxanthin can also reduce the risk of many chronic diseases, such as protection against the development of early atherosclerosis [10], exhibit neuroprotective and anti-inflammatory function in microvascular complications of diabetes [11], increases antioxidant status for cardiovascular protection [12], and against cancer development [13].

The consumption of lutein/zeaxanthin-enriched food/products has increased steadily in the past ten years. Today, the petals of marigold flowers (*Tagetes erecta*) are the main plant source of lutein compounds. As the production of marigold flowers is a labor-intensive and land-demanding process, more efficient production approaches are demanded [14]. In these circumstances, some microalgae have been considered potential sources of lutein production [14,15]. The advantage of microalgae sources compared to marigold petals, such as higher lutein content, use quite less labor, regardless of land and weather conditions, making it an alternative solution for lutein and other carotenoid derivatives production. On the other hand, specific compounds usually demand certain strains of microalgae, a precisely designed process, and extensive technology for extraction and purification, allowing for the higher cost of microalgal biomass compared to marigold petals [14]. To accomplish a balance between cost savings and increased yield that meets the requirements of metabolic engineering has intensified interest in lutein and zeaxanthin biosynthesis in industrial crops and its further commercial exploitation.

Recently, in addition to traditional microbial chassis, using plants as hosts to produce high-value metabolites has become a new trend [16]. Tomato (*Solanum lycopersicum*) is an ideal chassis for plant engineering of carotenoids due to the abundance of isoprenoid precursors in the red fruit tissues [17,18]. The degradation of chlorophyll as well as the accumulation of carotenoids, including lycopene and β-carotene, determine the characteristic red coloration of ripening tomato fruits [19]. After lycopene, the plant carotenoid biosynthetic pathway has two different cyclization branches: one is lycopene ε-cyclase enzyme (LCYE) introduced α-carotene branch, and the other one is lycopene β-cyclase enzyme (LCYB) introduced β-carotene branch, yielding lutein and zeaxanthin in the following steps, respectively [20,21]. Furthermore, α-carotene is preferentially hydroxylated by two heme-containing cytochrome P450 enzymes, such as carotenoid β-ring hydroxylase (HYDB) and carotenoid ε-ring hydroxylase (HYDE), introduce the ε- and β-ionone rings to yield lutein. Additionally, β-carotene is hydroxylated by β-ring hydroxylase (HYDB) with two β-ionone end groups and produces zeaxanthin [21,22] (Fig. 1A). However, due to a tightly regulated physiological mechanism, the transcript level of rate-limited genes catalyze lycopene cyclization reactions of *SlLCYE* and *SlLCYB* in tomato fruit is hampered by a tightly regulated physiological mechanismaround the breaker stage and decreased to an undetectable level at the subsequent ripening stages, resulting in the low content of lutein and zeaxanthin in ripe tomato fruit [23,24].

The engineering of the metabolic pathway in tomato fruit is a promising strategy to overcome the physiological block of lutein and zeaxanthin biosynthesis, as has been performed in other plants [25–27]. Our previous study has shown that overexpression of *SlLCYE* alone can significantly increase lutein production in tomato fruit [17]. In this study, by using a combinatorial approach in the GoldenBraid 2.0 assembly system, we designed another three binary constructs containing different combinations of four endogenous synthetic genes of lutein and zeaxanthin pathway, encoding *SlLCYE*, *SlLCYB*, *SlHYDB*, and *SlHYDE* under the control of fruit specific promoters *pE8* or *p2A11* respectively, for generating lutein or zeaxanthin enriched tomato fruits. The engineered tomato fruits showed not only higher content of total carotenoids but also the accumulation of target carotenoid derivatives, including lutein and zeaxanthin. Additionally, these apocarotenoid-enriched tomatoes showed higher antioxidant capacity, as well as extended shelf life during postharvest storage.

2. Materials and methods

2.1. Plant materials and chemical standards

Tomato (*Solanum lycopersicum* cv. MicroTom) seeds were purchased from PanAmerican Seed. The seedlings were grown in a greenhouse under environmentally controlled conditions at a temperature of 24 ± 1 °C, with a 16/8-h light/dark daily cycle, with a relative humidity of 65%. Fruit tissues from different combinations of plants at Br+3 and Br+10 stages were collected.

Carotenoid standards were purchased from Sigma (https://www.sigmaaldrich.cn/CN/zh), including lycopene (SMB00706), α-carotene (40395), β-carotene (C4582), γ-carotene (54765), lutein (07168) and zeaxanthin (14681). The uHPLC-grade methanol and acetone were purchased from ThermoFisher (https://www.thermofisher.cn/cn/zh/home.html).

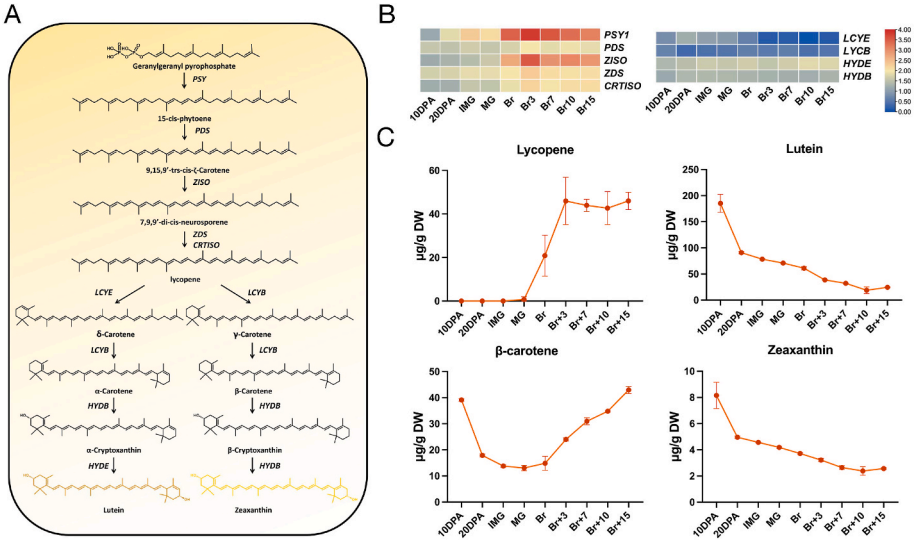

Fig. 1. The carotenoids biosynthesis pathway in tomato. (A) The representative scheme of the carotenoid pathway in tomato. (B) The expression pattern of carotenoid biosynthetic genes in tomato fruit at different developmental stages (data were standardized by log10 (TPM + 1) per gene). (C) The contents of several representative carotenoids in the fruits of WT. Fruit samples were harvested at 10 days post-anthesis (10 DPA), 20 DPA, immature green (IMG), mature green (MG), breaker (Br), 3 days post breaker stage (Br+3), Br+7, Br+10, and Br+15. Data in (B) and (C) are presented as means ± SD (n = 3).

2.2. Plasmid construction and transformation of tomato plants

The GoldenBraid 2.0 kit was purchased from Addgene (Addgene kit # 1000000076) [28,29]. The complete coding sequence of *SlLCYE* (*Solyc12g008980*), *SlLCYB* (*Solyc06g074240*), *SlHYDB* (*Solyc04g051190*), and *SlHYDE* (*Solyc10g083790*) were obtained by PCR amplication, gene-specific primers were listed in Table S1, respectively. These genes were then subcloned into a *pUPD2* donor vector after synonymous mutation of the potential sites of *Bsa*I and *Bsm*BI type IIS restriction enzymes. According to a standard combinatorial approach in the GoldenBraid 2.0 assembly system [28,29], three recombinant binary vectors for lutein synthesis and one vector for zeaxanthin synthesis were constructed. The different combinations of plasmids were then introduced into EHA105 *Agrobacterium tumefaciens* strain and then transformed into tomato according to the previously reported method [30]. Positive transgenic tomato plants were selected by kanamycin in MS medium and verified by genomic DNA PCR for the presence of corresponding promoter and relative gene linkage.

2.3. RNA extraction and quantitative PCR analyses

The total RNA of tomato fruit at 5 days after the breaker (Br+5) stage was extracted using a Plant RNA Purification Reagent Kit (Takara Bio, Kusatsu, Japan, AHF1820A). The first strand cDNA was synthesized using the PrimeScripttm RT reagent Kit (Takara Bio, Kusatsu, Japan, AK4201). The quantitative real-time reverse transcriptase PCR (qRT-PCR) experiments were operated by the Bio-Rad CFX384 Real-Time System, with tomato *Ubiquitin* (*Solyc01g056940*) as the internal reference. The primers used for qRT-PCR were designed by qPCR Primer Database (https://biodb.swu.edu.cn/qprimerdb/) [31] and listed in Table S1.

2.4. Protoplast extraction and microscopic observation

Protoplast extraction was performed using the flesh of fruit, according to the previous published method [32]. Briefly, transgenic and wild-type (WT) tomato fruits were harvested at 10 days after the breaker (Br+10) stage, deprived of seeds and peel. Cut the pulp into about 1 mm wide filaments with a sharp blade, and immediately transfer it into 5 mL of enzymatic hydrolysis solution (0.5% Macerozyme R10, 1.5% Cellulase R10, 0.1% BSA, 0.5 M Mannitol, 10 mM MES, 10 mM $CaCl_2$, 20 mM KCl, adjust pH to 5.7 with KOH). The enzyme solution was vacuumed for 20 min, and then kept in the incubator at 23 °C for at least 2 h. After digestion, an equal volume of W5 solution (5 mM KCl, 125 mM $CaCl_2$, 154 mM NaCl, 2 mM MES, adjusted to pH 5.7 with 1 M NaOH) was added to dilute the enzyme/protoplast solution, then the protoplasts were released by shaking the solution slightly and horizontally for 10 s before filtration. The enzyme solution containing protoplasts was filtered into a 50 mL round-bottomed tube using a 75-μm nylon membrane. The flow-through was centrifuged horizontally at 100*g* for 3 min to pellet the protoplasts and the supernatant was discarded. Add the suitable volume of W5 solution to resuspend the sediment and repeat the previous step. Finally, add 5 mL of W5 solution to resuspend the protoplast pellet and put it on ice for the test. The protoplasts were observed and photographed with Nikon DS-Ri2 (connected with NIS-Elements D 5.01.00 software).

2.5. Extraction and determination of carotenoids

The carotenoids were extracted and determined as described previously [17]. Briefly, both WT and different combinations of transgenic tomato fruits were harvested at the Br+10 stage, when deprived of seeds, the pericarp tissues were then frozen in liquid nitrogen immediately. After lyophilized, the fresh tomato pericarp tissues were ground into a fine powder, and 100 mg of dried powder was extracted with n-hexane: acetone: ethanol (1:1:2, V/V/V) mixed solution until the powder became colorless. The extracts were then evaporated to dryness and dissolved with a mixed solution of methanol: methyl *t*-butyl ether (3:1, V/V). After centrifuge (12,000 $g \times 10$ min), the supernatant was then filtered through a 0.22 μm membrane filter before LC-MS/MS analysis. All the above operations shall be conducted away from strong light. The carotenoid metabolites were analyzed based on the Sciex TRIPLE QUADTM 5500 LC-MS/MS platform and controlled by Analyst 1.6.3 software (AB Sciex). The LC analysis was using a YMC C30 carotenoid column (ACCUCORE C30 chromatographic column, Penn Eagle Industrial Park, Bellefonte, PA16823, USA). Mass spectrometry was performed using an atmospheric pressure chemical ionization (APCI) source operating with a negative ion mode. The individual carotenoids were identified by using co-migration with standards and quantified by integrating the peak areas.

2.6. Antioxidant activity tests

WT and transgenic tomato fruits from three independent biological samples were harvested at the Br+10 stage, and the pericarp tissues of each line were lyophilized and ground into a fine powder. For hydrophilic extraction, 10 mg of dried sample were extracted with 700 μL water under agitation for 20 min at room temperature, the solution was centrifuged at 8000 g for 10 min, and collect the supernatant, extraction was repeated with 300 μL of water, and supernatants were combined. For lipophilic extraction, the dried powder was first extracted by the addition of 700 μL of acetone and repeated extraction with 300 μL of acetone. Two independent extracts were immediately analyzed for their antioxidant capacity.

2.6.1. Oxygen radical antioxidant capacity (ORAC) analysis

The ORAC assay was performed using tomato pericarp tissues according to the previously reported method [33]. Briefly, a fluorescein solution was prepared by mixing 40 μL of the above extracts (5 mg/mL), Trolox (50, 100, 200, 300, 400, and 500 μM dissolved in ddH_2O or acetone), ddH_2O or acetone with 40 μL of a 0.01 M PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, adjust pH to 7.4 with NaOH) solution containing 50 μM fluorescein. AAPH (100 mM in PBS, pH = 7.4) was added after 10 min of incubation at 37 °C, and the fluorescence was recorded every 2 min for 150 min at 485 nm excitation and 538 nm emission wavelengths using a *Synergy*™ H1 hybrid multimode microplate reader (BioTek, UK). According to the linear relationship between the area under the fluorescence attenuation curve (AUC) and ORAC values, the linear regression equation was calculated with Trolox standards. Then the ORAC value of the samples was calculated with the linear regression equation and presented in μmol Trolox equivalents per gram dry weight (μmol TE/g DW).

2.6.2. ABTS radical scavenging assay

The ability of the samples to scavenge the ABTS radical was measured by the Trolox equivalent antioxidant capacity (TEAC) assay [33]. In PBS solution (0.01 M,pH = 7.4), 7 mM ABTS was reacted with 2.45 mM potassium persulfate at room temperature in the dark for 12 h to produce the ABTS radical solution. After dilution with PBS, the radical solution was diluted to an absorbance of 0.7 ± 0.02 at 734 nm. Tomato extracts (100 μg/mL) or Trolox (6.25, 12.5, 25, 37.5, 50 μM) were mixed with the diluted ABTS solution (1:4, V/V), then incubated at 25 °C for 3 min, and measured A_{734} with a microplate reader. The linear regression equation was also calculated with Trolox standards. Then the ability of the samples to scavenge the ABTS radical was calculated with the linear regression equation and presented in TEAC values (μmol TE/g DW).

2.7. Malondialdehyde content and catalase activity analysis

Both WT and transgenic fruits from three independent biological samples were plucked at 10 d after the breaker stages. The tomato fruits were lyophilized using a vacuum freeze-dryer (Scientz-100F) after being

ground into a fine powder, and then weighed 10 mg of each sample for further extraction. The malondialdehyde (MDA) content of tomato fruits was detected with an MDA kit (Sangon Biotech, CHN, D799762-0100), according to the manufacturer's instructions. The absorbance of each sample at A_{450}, A_{532} and A_{600} was measured using a *Synergy*™ H1 hybrid multimode microplate reader (BioTek, UK). Then the formula in the kit was used to calculate the MDA content of each sample. The CAT activity was detected using Catalase Activity Assay Kit (Sangon Biotech, CHN, D799598-0100), according to the manufacturer's instructions. The absorbance of each sample at A_{240} was recorded using a Biospectrometer basic (Eppendorf, GER). The CAT activity was calculated according to the equation shown in the kit.

2.8. Postharvest storage tests

Tomato fruits from each sample were harvested at the Br+10 stage. At least 9 fruits from 3 independent plants of each genotype were used for postharvest storage tests. All of the samples were stored in a clean can and kept at 25 °C. The fruit weight was conducted as water loss and measured every 3 days after harvest, the fruit dimensions were recorded every 10 days and continued for 60 days.

2.9. Statistical analysis

All experiments were performed using at least three biological replicates with three technical replicates. The data are presented as means ± SD (n = 3) unless specifically addressed. To compare group differences, paired or unpaired, two-tailed one-way ANOVA was used, and *P* values less than 0.05 were considered significant. All data were analyzed and presented by using GraphPad Prism version 8.

3. Results

3.1. Lutein and zeaxanthin are deficient in ripe WT tomato fruit

As a climacteric fruit, the color change constitutes the most obvious visual characteristic of tomato fruit ripening. This process is associated with chlorophyll degradation in green fruit and carotenoid accumulation in ripe fruit [34,35]. Lycopene, in turn, undergoes a series of cyclization reactions and marks a branching point in the lutein and zeaxanthin biosynthesis: lycopene ε-cyclase (LCYE) creates one ε-ring to give δ-carotene to produce lutein, lycopene β-cyclase (LCYB) catalyzes a two-step reaction that creates β-carotene and its derivative zeaxanthin (Fig. 1A) [36,37]. According to the MicroTom Metabolic Network (MMN database) of our previous study [38], the gene encoding *phytoene synthase* 1 (*PSY1*), known to catalyze the first committed step in the carotenoid pathway, was highly expressed throughout the developmental stages of tomato fruit, thus is accompanied by lycopene and β-carotene accumulation (Fig. 1B and C). However, the transcript level of *LCYE* and *LCYB* were decreased at the young fruit stage and to an undetectable level at the subsequent ripening stages (Fig. 1B), resulting in the low content of lutein and zeaxanthin in ripe wildtype tomato fruit (Fig. 1C).

3.2. Engineering transgenic tomato with different carotenoid derivatives

To obtain the transgenic tomato lines with high enrichment of lutein and zeaxanthin in fruits, two different fruit-specific promoters (*pE8* and *p2A11*) were selected and evaluated for cassette gene expression. Four binary vectors containing different combined endogenous synthetic genes were created and used for the tomato transformations (Fig. 2A). The four recombinant binary vectors were named as follows:

LE: pDGB_α-[*pE8:SlLCYE:T35S-pNos:Hyg:Tnos*] [17]; ***LE/LB***: pDGB_α-[*pE8:SlLCYE:T35S-p2A11:SlLCYB:Tnos-pNos:Hyg:Tnos*]; ***LB/HB***: pDGB_α-[*pE8:SlLCYB:T35S-pE8:SlHYDB:Tnos-pNos:Hyg:Tnos*]; ***LE/LB/HB/HE***: pDGB_Ω-[*pE8:SlLCYE:T35S- p2A11:SlLCYB:Tnos-pE8:SlHYDB:Tnos-p2A11:SlHYDE:Tnos-pNos:Hyg:Tnos*].

The widely used tomato variety MicroTom was selected for *Agrobacterium*-mediated transformation, and the positive transgenic lines were selected by kanamycin at T_0 generation. Transgenic lines were obtained for all combination constructs, including *LE* + *LB* (16 lines), *LB* + *HB* (17 lines), and *LE* + *LB* + *HB* + *HE* (11 lines) (Fig. S2), then the individual lines showed the representative pure yellow-orange color phenotype, as well as high expression level of relative biosynthetic genes, were selected for further analyses at T_1 generation (Fig. S3).

The transgenic plants overexpressing *SlLCYE* alone driven by the *E8* promoter were derived from our previous study [17]. The expression levels of the introduced lutein/zeaxanthin synthetic genes were further confirmed by RT-qPCR analysis (Fig. 2B), and there were no significant phenotypic differences between WT and transgenic seedlings at the vegetative growth stage (Fig. S4). These carotenoid enrichment tomato fruits developed normally in morphology except that the fruits of all the constructs showed a striking yellow or similar orange color phenotype, which could be also observed in their internal cross-section (Fig. 2C). Interestingly, depart from the MicroTom, all generations of the transgenic tomato fruits produced chromoplasts with a yellow-orange color

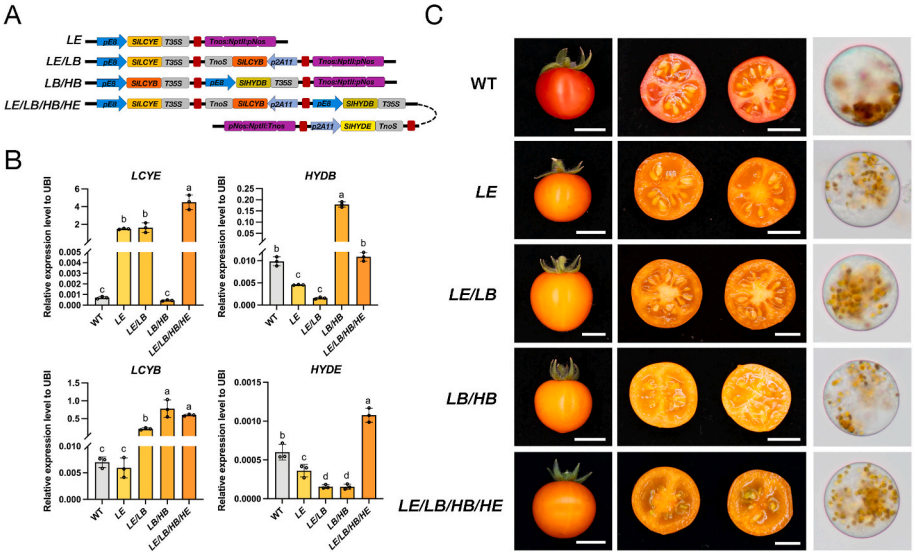

Fig. 2. Phenotypes of tomato fruits from WT and different combinations of transgenic plants. (A) Schematic diagrams of vectors with different gene combinations for tomato transformation. (B) Relative expression levels of *SlLCYE*, *SlLCYB*, *SlHYDB,* and *SlHYDE* in WT and transgenic tomato fruits at the 5 days post breaker stage (Br+5), as determined by RT-qPCR. Values are means ± SD of three biological replicates. Different letters indicate the significant difference according to one-way ANOVA ($p < 0.05$). (C) Representative phenotype of single fruit and the corresponding protoplast from WT and different transgenic lines at Br+10 stage. Scale bar = 1 cm.

similar to the peel (Fig. 2C), this transformation in cytoplasmic coloring is primarily due to the synthesis of carotenoids in the plastid driven by the *E8* or *2A11* fruit-specific promoters [39].

3.3. Carotenoid compounds are enriched in transgenic tomato fruit

To further investigate the internal causes of color differences observed between WT and transgenic tomato fruits, the carotenoid profile was investigated in various tomato fruit at the Br+10 stage. Consistent with the phenotypic changes in transgenic tomato fruits, the dried powder and acetone extraction solution also presented distinctive color characteristics (Fig. 3A). In addition, lycopene, α-carotene, β-carotene, γ-carotene, lutein, and zeaxanthin were identified as the predominant carotenoids in these transgenic tomato fruits and were quantified using targeted metabolic quantification by LC-MS/MS. Compared to WT fruit, the total contents of carotenoids were significantly increased in all transgenic lines. In the *LE/LB/HB/HE* line, the total carotenoid content reached almost three times as the WT (Fig. 3B). Notably, the contents of lycopene were decreased in all transgenic lines, as its downstream pathways had been activated (Fig. 3B and Fig. S5). We detected significant enrichment of α-carotene and lutein in lines overexpressing *LCYE* (*LE, LE/LB, LE/LB/HB/HE*). On the other hand, significant enrichment of β-carotene and zeaxanthin were detected in lines overexpressing *LCYB* (*LE/LB, LB/HB, LE/LB/HB/HE*) (Fig. 3C, Table S2). These results are consistent with the previous studies that high expression levels of *LCYE* or *LCYB* genes guide the metabolic flux of the pathway to the α- or β-carotene branch, then convert to lutein and zeaxanthin in two steps, respectively (Fig. 1A) [40,41].

3.4. Accumulation of carotenoids in tomato fruit enhances antioxidant activity

The health benefits of carotenoids are often assigned to their high antioxidant activities [42], and antioxidant assays have been widely used in plant species or tissue extracts to screen for potential biological activity [43,44]. For this reason, we measured the Trolox equivalent antioxidant capacity (TEAC) levels and oxygen radical antioxidant capacity (ORAC) values of the metabolic components to determine the antioxidant activity of those carotenoid-riched tomato fruits. It was shown that the antioxidant properties of both water extract and acetone extract of tomato fruits, presented by the tested TEAC or ORAC values of the fruits of those transgenic lines were significantly higher, indicating the significantly higher antioxidant activities of transgenic tomato fruits than that of the MicroTom (Fig. 4A–D).

To investigate the oxidative stress of carotenoid-rich tomato fruit, the selected parameters of malondialdehyde (MDA) and catalase (CAT) activity were determined to assess the effect of lipid damage on the antioxidant system [45]. Physiological measurements of MDA content revealed that the transgenic tomato fruit was significantly lower than the WT (Fig. 4E), as well as the significantly increased CAT activity in the transgenic tomato fruit (Fig. 4F). The formation of MDA is generally described as an index of lipid peroxidation induced by reactive oxygen species (ROS) [46], this, together with the activity of antioxidant enzymes, indicates an enhanced oxidative stress resistance of carotenoid enrichment tomato fruit.

3.5. Enrichment of carotenoids in tomato fruit extends shelf life

Previous studies indicate that increased antioxidant capacity can significantly extend the shelf life of transgenic tomato fruit [47,48]. To further examine the physiological function of carotenoid-rich transgenic

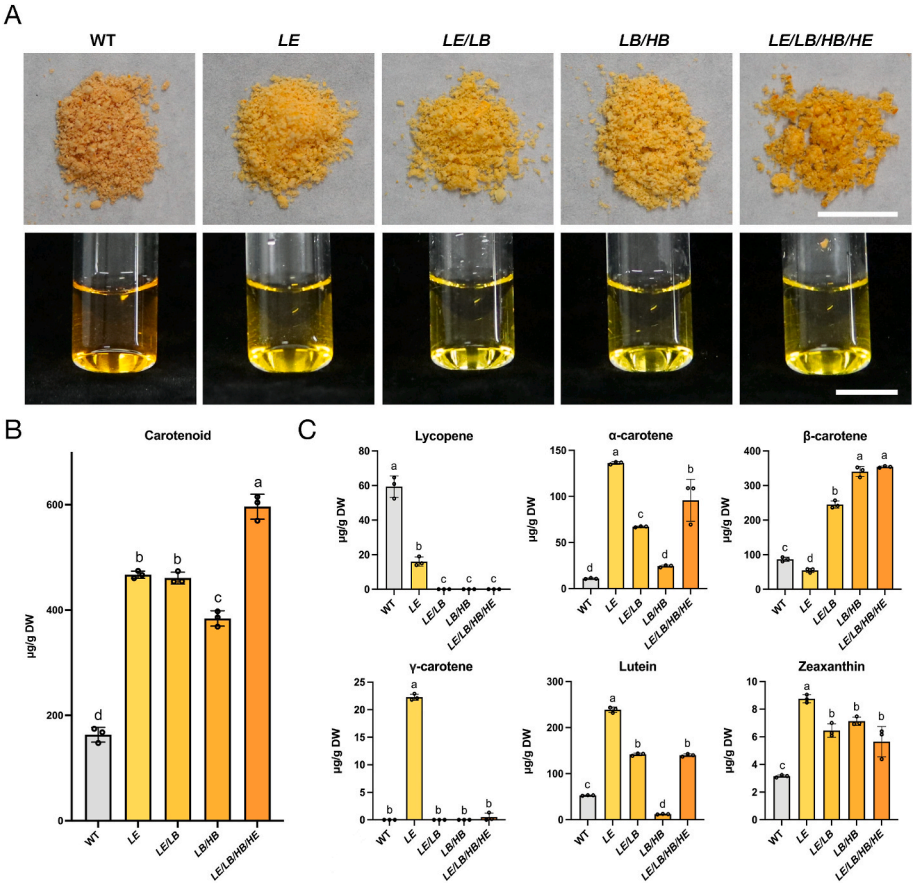

Fig. 3. Characterization of lutein and zeaxanthin enriched tomatoes. (A) Phenotypes of the lyophilized powder and acetone extract from WT and transgenic tomato fruits. Scale bars = 1 cm. (B) The contents of total carotenoids in the fruits of WT and different combinations of transgenic plants at the Br+10 stage. (C) The contents of major carotenoids in the fruits of WT and different combinations of transgenic plants at the Br+10 stage. Data are means ± SD (n = 3). Statistical significance was calculated by one-way ANOVA. Different letters at (B) and (C) indicate significant differences at the $P < 0.05$ levels.

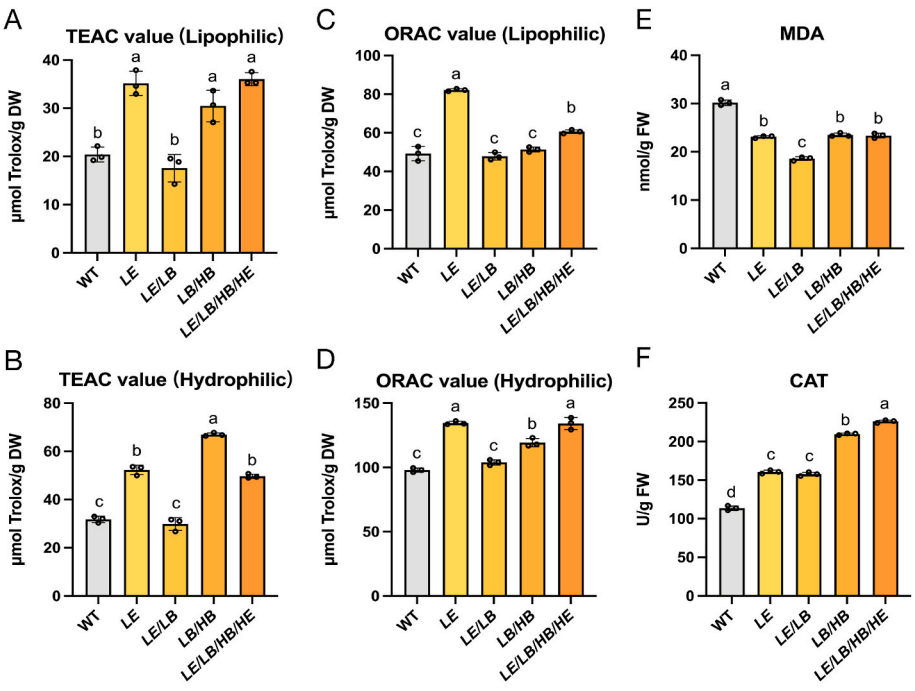

Fig. 4. In vitro antioxidant activities of WT and different transgenic tomatoes at Br+10 stage. (A) and (B) ABTS radical scavenging activities expressed as TEAC values (A, ABTS scavenging activity of lipophilic; B, ABTS scavenging activity of hydrophilic). (C) and (D) Antioxidant capacities expressed as ORAC values. (C, hydrophilic ORAC; D, lipophilic ORAC). (E) The MDA content. (F) The CAT activity. Data are means ± SD of three biological replicates. Different letters indicate significant differences according to one-way ANOVA ($P < 0.05$).

lines in postharvest deterioration, the tomato fruits (n = 9) were harvested at the Br+10 stage and stored in a clean container at room temperature for up to 60 days. During the storage time, the WT tomato fruit displayed extensive tissue collapse and desiccation in the peel from 10 days after harvest. In contrast, the transgenic tomato fruit exhibited delayed postharvest ripening phenotypes than the equivalent red ones of MicroTom (Fig. 5A). We also detected a major difference in reduction in weight, which was taken as an indication of water loss (Fig. 5C, Table S3), and firmness, defined here as the change of the initial area of intact fruit at natural compression (Fig. 5B). Taken together, these results suggest that the accumulation of carotenoids in tomato fruits is efficient to delay over-ripening and extend shelf life during postharvest storage.

4. Discussion

Rising health concerns and a global growing aging population have driven increasing demands for high nutritious crops to protect against various diseases. Breeding crops with health-promoting compounds has become one of the major goals for plant breeding [49]. Carotenoids not only provide distinctive colors for fruits, vegetables, and flowers [1], but also are accessory components of the light-harvesting complexes in the photosynthetic tissues in plants [3]. In addition, carotenoids have been reported to have important physiological features and clinical benefits [3,6]. All these merits make carotenoids vital targets for plant metabolic engineering.

Tomatoes include a sink tissue that contains an organelle predisposed to carotenoid accumulation exists, as well as the highly activated carotenoid pathway, where revealed to be a good platform for engineering high-value apocarotenoid metabolites [17,50]. Lycopene, a major component of carotenoids in ripe WT tomato fruit, confers the attractive red color phenotype and most valuable quality to the fruit [51]. LCYE and LCYB catalyze the cyclization of lycopene to introduce ε- or β-ionone into all-*trans* lycopene to produce α-carotene or β-carotene, respectively. They independently control the rate-limiting step to the metabolic flux diversion towards the apocarotenoids pathway. In tomato fruit, however, the expression levels of *LCYE* and *LCYB* decline significantly during the ripening process, leading to the low contents of lutein and zeaxanthin in ripe tomato fruit (Fig. 1) [23,34,52]. Therefore, using conventional breeding or metabolic engineering to develop

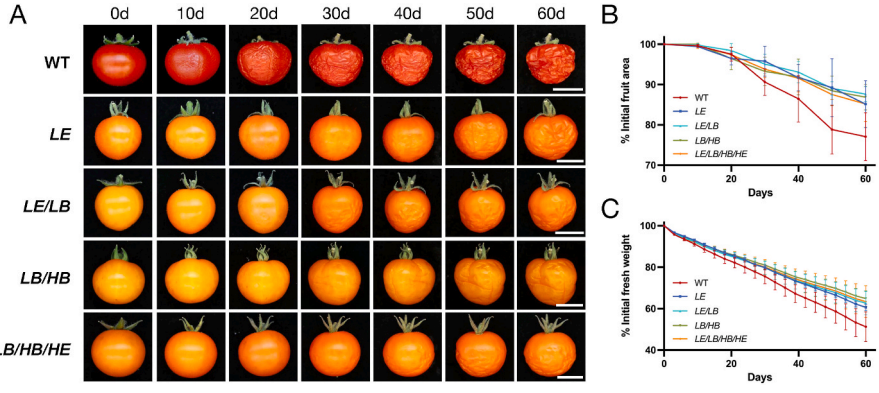

Fig. 5. Accumulation of lutein or zeaxanthin in tomato fruit delays over-ripening and extends shelf life. (A) WT and transgenic tomato fruits were harvested at the Br+10 stage and stored at room temperature, the photos were taken every ten days after picking to record the phenotypic changes. Scale bar = 1 cm. (B) and (C) The change of initial area (multiply fruit length by diameter) and weight of fruit harvested at Br+10 stage and stored for up to 60 days.

tomato fruit with enrichment of lutein or zeaxanthin compounds is a vital breeding target.

An effective multigene assembly system for overexpressing explicit target genes of metabolic pathways is crucial for the success of accurate metabolic engineering [27,50]. In this study, we selected a GoldenBraid 2.0 multigene assembly strategy using four endogenous synthetic genes (*LCYE*, *LCYB*, *HYDB*, *HYDE*) driven by fruit-specific *pE8* or *p2A11* promoter in tomato fruit for lutein and zeaxanthin metabolic engineering (Fig. 2). Compounds of lutein or zeaxanthin were significantly increased in all the selected transgenic tomato fruits (Fig. 3). However, in the *LE/LB/HB/HE* transgenic line, the expression of the *HYDB* gene, which is also driven by the *p2A11* promoter, was not significantly increased. This might be due to the transcriptional silencing caused by repeated use of the same promoter in a shorter interval, which reminds us that using different specific promoters to drive the expression of their respective genes in the future study may improve this situation [27,53].

All generations of these transgenic lines produced tomato fruits with a yellow-orange color phenotype (Fig. 2C), which is largely due to lutein/zeaxanthin accumulation and lycopene reduction in ripe tomato fruits (Fig. 3C). The engineered tomato fruit showed not only higher antioxidant properties of water and acetone extracts but was also associated with lower MDA content and higher CAT activity (Fig. 4). The increase of carotenoids can significantly improve the antioxidant levels. However, the different carotenoid derivatives have distinct antioxidant activities [42], therefore, the variations in the contents of carotenoids may lead to various antioxidant properties of different transgenic lines (Fig. 4A–D). In addition, this consequence of carotenoid enrichment tomato fruit showed longer shelf life is the comprehensive function associated with lutein or zeaxanthin accumulation, due to slower over-ripening and reduced susceptibility to oxidative damage (Fig. 5) [35,47,48].

Tomato fruits have successfully been used to engineer a variety of health-promoting compounds, such as flavonoids [54,55], astaxanthin [56,57], and crocins [50]. Notably, in a pilot test, the cancer-susceptible *Trp53*$^{-/-}$ mice showed a significant extension of life span after feeding a diet supplemented with high-anthocyanin tomatoes [54]. As well as the crocins-riched tomatoes showed higher antioxidant capacity and were able to protect against neurological disorders in a *Caenorhabditis elegans* model of Alzheimer's disease [50]. Saying that overproduction of endogenous metabolites or accumulation of newly formed ectopic products in tomato fruits can still maintain their biological activity, are key aspects for successful prototype development. Amid the COVID-19 crisis, the global carotenoids market is estimated at $1.5 billion in the year 2020 and is projected to reach $2.2 billion by 2027 (Global industry analysts, Inc., USA. www.strategyr.com). However, due to the biological sources being still limited and extremely expensive, this huge global carotenoids market is now firmly dominated by chemical synthesis. Although enrichment of various carotenoid derivatives has been achieved in tomato breeding or genetic improvement [6,58], there are few reports on metabolic engineering of specific metabolic pathways or precise metabolites based on tomato chassis. In this study, by using a combinatorial approach in GoldenBraid 2.0 assembly system, we produced a series of transgenic lines with tomato fruits enrichment in lutein or zeaxanthin compounds and improvement of multiple physiological traits, which provides a successful example for the accurate metabolic engineering in plant synthetic biology, and opens the possibility of making tomato fruits as green factories for the economical production of all three classes of secondary metabolites [59]. These biofortified tomato fruits could be also exploited as excellent research materials for comparing the health benefits of carotenoid derivatives in different research model.

CRediT authorship contribution statement

Yanjun Wu: contributed equally to the work, Visualization, partial writing, performed experiments and analyzed data. **Yong Yuan:** contributed equally to the work, Formal analysis, performed experiments and analyzed data, drafted the manuscript and writing review. **Wenqian Jiang:** helped to performe the experiments. **Xin Zhang:** helped to analyze the experimental data. **Siyan Ren:** helped to performe the experiments. **Hsihua Wang:** assistance with sample preparation and carotenoids metabolic analysis. **Xiaolan Zhag:** Supervision, Resources. **Yang Zhang:** Supervision, Resources. Administration, Funding acquisition, critically revised the manuscript and writing review & editing.

Declaration of competing interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.08.005.

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