Engineering an oilseed crop for hyper-accumulation of carotenoids in the seeds without using a traditional marker gene

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Abstract

Key message Ketocarotenoids were synthesized successfully in *Camelina sativa* seeds by genetic modification without using a traditional selection marker genes. This method provided an interesting tool for metabolic engineering of seed crops.

Abstract *Camelina sativa* (L.) Crantz is an important oil crop with many excellent agronomic traits. This model oil plant has been exploited to accumulate value-added bioproducts using genetic manipulation that depends on antibiotic- or herbicide-based selection marker genes (SMG), one of the major concerns for genetically modified foods. Here we reported metabolic engineering of *C. sativa* to synthesize red ketocarotenoids that could serve as a reporter to visualize transgenic events without using a traditional SMG. Overexpression of a non-native β-carotene ketolase gene coupled with three other carotenogenous genes (phytoene synthase, β-carotene hydroxylase, and Orange) in *C. sativa* resulted in production of red seeds that were visibly distinguishable from the normal yellow ones. Constitutive expression of the transgenes led to delayed plant development and seed germination. In contrast, seed-specific transformants demonstrated normal growth and seed germination despite the accumulation of up to 70-fold the level of carotenoids in the seeds compared to the controls, including significant amounts of astaxanthin and keto-lutein. As a result, the transgenic seed oils exhibited much higher antioxidant activity. No significant changes were found in the profiles of fatty acids between transgenic and control seeds. This study provided an interesting tool for metabolic engineering of seed crops without using a disputed SMG.

Keywords *Camelina sativa* · Carotenoid · β-Carotene · Astaxanthin · Marker gene

Introduction

*Camelina sativa* (L.) is an important oilseed crop with excellent agronomic and food traits, including a relatively short life cycle, high tolerance to biotic and abiotic stresses and high α-linolenic acid contents (C18:3 28–40%) (Budin et al. 1995). With a simple and efficient transgenic system, *C. sativa* has served as an ideal model oilseed for testing novel metabolic engineering strategies (Yuan and Li 2020). For example, *C. sativa* has been engineered to contain docosapentaenoic and eicosapentaenoic acids in the seeds at the levels of fish oil (Ruiz-Lopez et al. 2014). Increasing interest has focused on developing *C. sativa* oils with much higher nutrition value (Lu and Kang 2008). However, little has been done concerning enhanced production of carotenoids in the seeds, especially astaxanthin with high value and health benefits for humans.

Astaxanthin is a red ketocarotenoid that is only synthesized in a limited number of organisms (Orosa et al. 2000;
Wang et al. 2003; Cunningham and Gantt 2011). This lipophilic carotenoid has a number of benefits to human health, including improvement of the immune system and prevention of chronic diseases, cardiovascular diseases, certain types of cancers and age-related degeneration (Cooper 2004; Milani et al. 2017). Over 700 carotenoids with colors from yellow to red have been identified in nature (Delgado-Vargas et al. 2000). Carotenoid biosynthesis has been extensively studied (Sandmann 2001). In higher plants, the initial precursors for carotenoid formation are generated in plastids via the 2-C-methyl-d-erythritol 4-phosphate pathway (Zhu et al. 2009). Geranylgeranyl diphosphate (GGPP) is the direct precursor for carotenoid biosynthesis, with two GGPP molecules condensed by phytoene synthase (PSY for plants, CrtB for bacteria) to form the colorless phytoene which is converted to the red lycopene via phytoene desaturase (PDS), ζ-carotene (ZDS) desaturase and carotene isomerases. Lycopene undergoes cyclization to form β- or α-carotenes, followed by hydroxylation to form xanthophylls such as zeaxanthin and lutein (Liu et al. 2015). Astaxanthin is biosynthesized from β-carotene catalyzed by β-carotene ketolase (BKT) and β-carotene hydroxylase (CHYb) (Higuera-Ciapara et al. 2006) (Fig. 1). Generally, higher plants do not synthesize astaxanthin due to the lack of a BKT (Cunningham and Gantt 2005; Zhu et al. 2009). We previously found that expression of a heterologous BKT from microalgae in higher plants could extend the endogenous carotenoid pathway to the red astaxanthin, leading to changed color of plant tissues or organs (Zhong et al. 2011; Huang et al. 2012, 2013). Most transgenic research uses antibiotic resistant genes as markers for easy selection of transformants. The existence of such a marker in genetically modified (GM) foods has long been criticized, due to concerns about the safety of both foods and the environment. To overcome this problem, complicated and time-consuming approaches have been applied to generate marker-free transgenic plants (Tuteja et al. 2012; Wang et al. 2017; Liu et al. 2020).

In this study, we developed a novel strategy to engineer C. sativa for hyper-accumulation of value-added carotenoids in the seeds without using a disputed selectable marker. This was achieved by extending the endogenous carotenoid pathway to red ketocarotenoids via overexpression of a polycistronic gene cluster containing genes CrtB, HpBHY, CrBKT and Orange (OR). We found that transgenic C. sativa expressing these genes generated red seeds that could be easily discriminated via the naked eye from untransformed yellow seeds. Thus, no additional selectable marker genes were required for screening of putative transformants. We achieved seed-specific expressing transformants which accumulated carotenoids up to 70-fold of control levels, including the high value ketocarotenoids astaxanthin and keto-lutein. Our study provided an additional tool suitable for plant biotechnology, and was suitable for making safer GM foods.

**Materials and methods**

**Expression constructs for C. sativa**

We selected the four genes, the phytoene synthase gene from Pantoea ananatis (CrtB) (GenBank: CP001875.2), β-carotene hydroxylase gene from Haematococcus pluvialis (HpCHYB) (GenBank:KP866868.1), algal β-carotene ketolase gene from Chlamydomonas reinhardtii (CrBKT) (GenBank:AY860820.1) and OR from Cauliflower (GenBank: DQ482460), for enhanced production of carotenoids, which were reported in previous studies (Shewmaker et al. 1999, Li et al. 2001, Lu et al. 2006, Lopez et al. 2008, Zhong et al. 2011, Huang et al. 2013). Genes CrtB, HpCHYB and CrBKT were fused with the plastid transit peptide sequence of Arabidopsis thaliana rubisco small subunit (GenBank: BAH57175.1) at the N-terminal of each gene respectively, and the four genes were linked by three 2A peptide-coding sequences from foot and mouth disease virus (Halpin et al. 1999; Halpin 2005; Wang et al. 2021). This chimeric gene sequence was codon-optimized using CodonOpt (https://sg.idtdna.com/
Codon Opt) according to the codon usage of C. sativa, and chemically synthesized. The chemically synthesized cassette was inserted into SmaI and SacI cloning sites of the pBI121 vector which was downstream of CaMV35S and a seed-specific promoter (Napin) to generate the constructs SBBBO and NBBBO (Fig. 2).

Agrobacterium strain, plasmid and bacterial growth

The constructs SBBBO and NBBBO were introduced into Agrobacterium tumefaciens strain LBA4404 by electroporation according to a previously described method (Wang et al. 2021). Positive cells were selected by PCR detection and used for transformation. Bacterial growth was commenced 2–3 days prior to plant transformation. A single colony was inoculated into a culture tube containing 3 mL of YEP medium (10 g/L yeast extract, 10 g/L peptone and 5 g/L NaCl) supplemented with 100 mg/L streptomycin and kanamycin. Overnight culture of the 3 mL of bacteria was then transferred into a 500-mL flask containing 200 mL of LB medium and growth continued for 12 h at 28 °C. Bacteria were harvested by centrifugation at 6000×g for 10 min and the pellet was suspended in the infiltration medium, consisting of half strength MS salts, 30 g/L sucrose and 0.1% (v/v) Silwet L77.

C. sativa transformation and regeneration

The C. sativa cultivar ‘SNC104’ was used for the genetic manipulation. For transformation, seeds were germinated in 9-cm Petri plates with 1% agar for 7 days, then transferred to pots filled with soil and kept in the greenhouse of Kunming Institute of Botany, CAS. Growth conditions in the greenhouse were 20/16 °C (day/night) with natural lighting. The relative humidity was maintained at 60%. The plants were used for Agrobacterium-mediated transformation according to a published method (Lu and Kang 2008) with some modifications. The pots with plants at the early flowering stage were placed upside down in a vacuum desiccator and the inflorescences were immersed into the inoculum in a 300-mL beaker. Vacuum was applied and held for 5 min at a pressure of 85 kPa. The treated plants were covered by black plastic bags for 24 h before returning to the greenhouse. This procedure was repeated again a week later. Seeds were harvested and screened by naked eye for putative transformants using seed color (Figs. 3 and 4). Transformants were verified using PCR analysis with specific primers for amplifying the transgenes as listed in Supplement Table 1.

Carotenoid extraction and analysis

Carotenoid extraction and analysis were performed according to previously described methods (Ye et al. 2019; Ye and Huang 2020; Wang et al. 2021) with some adjustments. Briefly, mature C. sativa seeds were ground in liquid nitrogen and extracted with acetone until they were almost colorless. The extracts were filtrated through 0.2-μm filter and used for ultra-performance liquid chromatography (UPLC) analysis. The UPLC was conducted using an Agilent Technologies 1290, with the column ZORBAX Eclipse (1.8 μm, 3.0 × 50 mm, Agilent, Santa Clara, CA, USA) for carotenoid separation and analysis. Carotenoids were measured at 480/280 nm wavelength. Carotenoids were identified by authentic standards purchased from Sigma and the UV absorption spectral properties of carotenoids. The carotenoid concentrations were calculated according to standard curves.

Fatty acid extraction and analysis

The fatty acid methyl esters (FAMEs) were prepared by heating 0.2 g of seeds at 80 °C in 1 mL of 2% H₂SO₄ (v/v) in methanol for 60 min, and extracted with 2 mL of trichloromethane and 1.5 mL of 0.75% NaCl (w/v), and analyzed using a gas chromatography–mass spectrometry machine (Agilent Technologies 7890/5975) equipped with a DB-5 (30 m × 250 μm × 0.25 μm) column (Agilent). Helium was used as carrier gas (constant flow: 1.2 mL/min), the injector was kept at 250 °C with an injection volume of 1 μL under...
a split ratio of 20:1, and column temperature was first set at 80 °C for 1 min and then increased at 10 °C/min to 250 °C, the rate was then changed to 8 °C/min until 280 °C was reached and maintained for 5 min. Identification of individual FAMEs was based upon comparing the mass to charge ratio with that of standard FAME mixtures and the NIST database (Ye et al. 2019).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from 28-DAP (day after pollination) seeds of *C. sativa* using a Plant RNA Mini Kit (OMEGA). RNA was used to synthesize cDNA using Prime Script II 1st Strand cDNA Synthesis kit (TaKaRa Biotech Co., Ltd., Dalian, China); quantitative RT-PCR
(RT-qPCR) was performed on a CFX Connect Real-Time System (Bio-Rad, USA) using an Evagreen 2 × qPCR Master Mix Kit (ABM). Reactions were carried out under the following conditions: 95 °C/30 s (one cycle); 95 °C/5 s, 60 °C/15 s, 72 °C/15 s (40 cycles) and followed by 0.5 °C increments at 5 s/step from 65 °C to 95 °C for the melt curve analysis. The *C. sativa Actin* gene was used as a reference, primers used for RT-qPCR are listed in Supplement Table 1.

**Transmission electron microscopy**

Transmission electron microscopy (TEM) was performed according to a previously described protocol (Luo et al. 2013) with some modifications. Briefly, *C. sativa* seed sections (1–3 mm) of 45-DAP were fixed in 2.5% (v/v) glutaraldehyde and stored at 4 °C overnight. The samples were washed three times with phosphate buffer solution for 10–15 min and fixed in 1% osmic acid for 14–18 h, and then the samples were washed three times with phosphate buffer solution for 10–15 min. After dehydration through an ethanol series, samples were embedded in Epon812 (SPI Supplies Division of Structure Probe, West Chester, USA) and polymerization was conducted at room temperature for 24 h and then at 60 °C for 24 h. Specimens were sectioned to a thickness of 1–2 mm with glass knives on a Leica EM UC7 microtome. Ultrathin sections (70–90 nm) were prepared using a diamond knife on a Leica EM UC7 ultramicrotome and pieces taken with 100-mesh copper mesh, and stained with uranyl acetate and lead citrate. Sections were examined and photographed using a JEOL TEM1200EXII transmission electron microscope under 80 kV acceleration voltage.

**Total antioxidant capacity analysis**

The Trolox equivalent antioxidant capacity (TEAC) levels indicate the total antioxidant activities. Using the oxygen radical absorption capacity (ORAC) method (Liu et al. 2016a, b; Zhu et al. 2018), the antioxidant activity of wild-type (WT) and transgenic mature seeds were measured. The *C. sativa* seed samples (~ 0.1 g) were ground into powder, extracted with 1 mL of extraction solution with ice bath homogenate and centrifuged at 12,000× g for 15 min at 25 °C. The supernatants were filtered through a Millipore filter (0.45 mm diameter) and the extracts used for analysis by ORAC method.

**Statistical analysis**

All experiments had three biological replications. Data were expressed as means ± standard deviation (SD) and analyzed using IBM SPSS Statistics 25 (IBM, Armonk, NY, USA).

| LINE          | WT       | SBBBO-3       | NBBBO-5     | NBBBO-(NPTII)-1 |
|---------------|----------|---------------|-------------|-----------------|
| Carotenoid content μg/g FW (% composition) | Neoxanthin | ND | 4.31 ± 0.95 (1.03%) | ND | 108.61 ± 2.14 (11.12%) |
|               | Ketolutein | ND | 32.55 ± 0.42 (7.81%) | 163.3 ± 7.01 (17.34%) | 42.72 ± 0.71 (4.37%) |
|               | Astaxanthin | ND | 13.32 ± 3.07 (3.19%) | 15.23 ± 0.73 (1.62%) | 42.72 ± 0.71 (4.37%) |
|               | Lutein | 13.21 ± 2.05 (100%) | 16.99 ± 0.43 (4.07%) | 26.78 ± 5.91” (2.84%) | 27.69 ± 1.50” (2.83%) |
|               | β-cryptoxanthin | ND | 2.26 ± 0.02 (0.54%) | 3.27 ± 0.04 (0.35%) | 8.73 ± 1.14 (0.89%) |
|               | Echinone | ND | 2.21 ± 0.14 (0.53%) | 5.46 ± 0.82 (0.58%) | 6.00 ± 0.56 (0.61%) |
|               | Lycopene | ND | 38.09 ± 2.57 (9.13%) | 226.6 ± 6.40 (24.06%) | 18.36 ± 0.43 (1.88%) |
|               | α-carotene | ND | 56.74 ± 1.60 (13.61%) | 112.7 ± 2.54 (11.96%) | 128.90 ± 4.28 (13.19%) |
|               | β-carotene | ND | 201.14 ± 5.95 (48.24%) | 235.0 ± 3.01 (24.95%) | 470.62 ± 4.07 (48.17%) |
|               | phytoene | ND | 32.24 ± 7.85 (7.73%) | 56.07 ± 7.15 (5.95%) | 64.55 ± 4.95 (6.61%) |
|               | Others | ND | 17.67 ± 2.14 (4.24%) | 95.26 ± 1.42 (10.11%) | 81.19 ± 1.59 (8.31%) |
|               | Total | 13.21 ± 2.05 (100%) | 442.42 ± 7.76” (100%) | 942.0 ± 11.08” (100%) | 977.54 ± 11.20” (100%) |
| Fatty acid content mg/g FW (% composition) | C18:1 | 8.44 ± 0.32 (6.43%) | 6.85 ± 0.65 (5.58%) | 7.40 ± 0.55 (5.78%) | 6.42 ± 0.12 (4.94%) |
|               | C18:2 | 38.98 ± 1.45 (29.69%) | 36.64 ± 2.12 (30.94%) | 36.35 ± 4.27 (28.39%) | 36.50 ± 0.21 (28.07%) |
|               | C18:3 | 40.59 ± 2.93 (34.28%) | 41.71 ± 5.10 (32.57%) | 39.94 ± 1.71 (30.71%) | 47.18 ± 0.83 (36.28%) |
|               | Others | 45.17 ± 2.15 (34.40%) | 34.34 ± 4.17 (29.00%) | 42.6 ± 6.08 (33.27%) | 47.18 ± 0.83 (36.28%) |
|               | Total | 131.31 ± 7.23 (100%) | 118.42 ± 98.62 (100%) | 128.06 ± 4.63 (100%) | 130.04 ± 12.15 (100%) |

The data represent average values of three individual measurements, ”significance, as determined by Student’s t test, **P < 0.01. ND not detected.
Results and discussion

Constructs for enhanced production of carotenoids in *C. sativa*

Due to its high content of the polyunsaturated fatty acid (PUFA) α-linolenic acid (C18:3 28–40%), *C. sativa* is an emerging oilseed crop. In contrast, less than 15 μg/g dry weight of carotenoids (mainly lutein) was found in *C. sativa* seeds (Table 1). Carotenoids are potent antioxidants that may protect PUFA’s from oxidation. To enhance the production of carotenoids in *C. sativa* seeds, we constructed three expression cassettes (Fig. 2) for *C. sativa* transformation. Phytoene synthase (PSY /CrtB) is the committed enzyme for carotenoid formation (Shewmaker et al. 1999; Fraser et al. 2002). Overexpression of this enzyme in *Brassica napus* led to a 50-fold increase of total carotenoids (Shewmaker et al. 1999). We previously showed that extending the carotenoid pathway to astaxanthin greatly increased carotenoid production in tomato (Huang et al. 2013). Thus, we united four genes associated with carotenoid biosynthesis as a polycistronic open reading frame mediated by three 2A sequences of the food and mouth virus (Szymczak et al. 2004; Ha et al. 2010). The OR gene is involved in chromoplast initiation (Li et al. 2001, Lu et al. 2006, Lopez et al. 2008). Two promoters (constitutive CaMV 35S and seed-specific napin promoter), were used to drive the gene cluster with (Fig. 2A and B) or without the NPT II marker (Fig. 2C) to detect the effect of promoters on gene expression and if the accumulation of red ketocarotenoids in the seeds could be used to screen putative transformants.

Generation of transgenic *C. sativa* with enhanced carotenoid production

Transformation of *C. sativa* with the constructs shown in Fig. 2 was performed according to the procedures of Lu et al. (Lu and Kang 2008) with some modifications as described in the Materials and methods. Generally, of 1000 seeds collected from plants treated with *Agrobacterium* harboring each of the three constructs (Fig. 2), 10–15 seeds were orange in color rather than the normal yellow (Fig. 3A and B). These red seeds could result from the co-expression of the transgenes, leading to the production of red ketocarotenoids, as well as enhanced production of endogenous carotenoids. The putative transgenic red seeds were germinated. Six constitutively transgenic lines (T0: SBBBO 1–6) and 12 seed-specific transformants (T0: eight with and four without NPTII marker) were regenerated and selected for detailed study. Insertion of the transgenes in the possible transformants (plants from red seeds) was confirmed by PCR analysis (Fig. S1). Accordingly, the transformation rates were calculated to be 1.2%, comparable to previous reports (Beranova et al. 2008).

Constitutive expression of the transgenes in *C. sativa* resulted in the hyper-production of carotenoids in both vegetative and reproductive tissues, hence, SBBBO lines demonstrated different phenotypes from WT control in that the transgenic plants had increased axillary branching, and some lines even showed dwarfing and stunting (Fig. 3C; Fig. S2). Furthermore, the transgenic seeds had delayed germination and a lower germination rate (Fig. 3D and E). Similar results were observed in a number of previous studies with constitutive overexpression of carotenogenic genes in *Arabidopsis thaliana* (Lindgren et al. 2003), *Nicotiana plumbaginifolia* (Frey et al. 1999; Qin and Zeevaart 2002) and tomato (Thompson et al. 2000). The CaMV 35S promoter is a constitutive promoter that can drive gene expression in a variety of plant tissues and developmental stages, which might interfere with the endogenous metabolic network and plant development (Benfey and Chua 1990). Constitutive expression of a phytene synthase in tomato resulted in dwarfism caused by redirecting the GGPP from gibberellin formation (Fray et al. 1995).

To overcome these problems, we replaced the CaMV 35S promoter with a seed-specific promoter (napin) (Fig. 2B and C). All the transgenic lines demonstrated normal phenotypes of plant growth and development except for the seed color (Fig. 4). Germination of the seeds showed no difference from that of WT (Fig. 4). To evaluate the carotenoid contents and compositions of the transformants, we analyzed the carotenoid profiles in the seeds with HPLC approach. The results are shown in Fig. 5 and Table 1. Seeds of WT only accumulated trace amounts of lutein. In contrast, overexpression of the transgenes greatly promoted the production of carotenoids, including β-carotene, lutein and their ketolat- ing products, such as keto-lutein and astaxanthin (Fig. 5). The chemical structures and UV–VIS spectra of identified carotenoids are shown in Figs. S3 and S4.

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. Mostly higher plants do not produce ketocarotenoids for the β-carotene ketolases exist only in some species of microalgae, bacteria, and yeast, and not in the majority of higher plants (Cunningham and Gantt 2005; Zhu et al. 2009). While BKTs (also known as CrtW or CrtO) from various sources demonstrate functional diversity in ketolating hydroxylated carotenoids to astaxanthin.

Of all the transformants, the transgenic lines of NBBBO(-NPTII)-1 produced the highest total carotenoid content of about 70-fold of the control (Table 1). Previously overexpression of *CrtB* in *B. napus* was reported to increase carotenoid content up to 50-fold in the seeds (Shewmaker et al. 1999). In our study, in addition to *CrtB*, the other three
transgenes, especially the OR gene, might also contribute
to direct the carbon source to carotenoid production as
illustrated by a number of studies (Li et al. 2001; Lu et al.
2006; Lopez et al. 2008). In addition, the co-expression of
CrBKT and HpBHY could trigger ketocarotenoid accumu-
lation, therefore enhancing total carotenoid production as
compared with other corresponding approaches for non-
ketocarotenoids (Stalberg et al. 2003; Ralley et al. 2004;
Morris et al. 2006).

To locate the storage site of carotenoids in the seeds,
TEM was performed for seeds of WT and NBBBO
(-NPTII)-1 line T4 (Fig. 6). Many more plastoglobulins
(PGs) occurred in NBBBO (-NPTII)-1 gerontoplasts than
in WT (Fig. 7). In C. sativa seeds, chloroplasts degenerate
to gerontoplasts with the disappearance of thylakoids and
an increase in PGs that contain a variety of compounds
including carotenoids. This result indicated that carot-
enoids including astaxanthin might be stored in PGs as
we previously showed (Zhong et al. 2011). Previously, we
had shown that Arabidopsis accumulated high amounts
of esterized astaxanthin in PGs of old leaves (Zhong
et al. 2011). In contrast, transplastomic tobacco was able
to accumulate high amounts of free astaxanthin in chloro-
plasts of the leaves (Hasunuma et al. 2008). Thus, the
non-native ketocarotenoids production in transgenic plants
could be associated with efficiency of synthetic pathway, ketocarotenoid storage in specific tissues.

Regulation of carotenoid biosynthesis in the transgenic plants

Transgenic C. sativa plants exhibited improved production of carotenoids (Fig. 5, Table 1). Generally, carotenoid biosynthesis is mainly regulated at the transcriptional level (Huang et al. 2013; Zhu et al. 2018). To expand our understanding of the regulation of carotenoids in C. sativa, we further investigated transcription of the key enzymes of the pathway using RT-qPCR. The RNA samples prepared from C. sativa seeds at 28-DAP in the homozygous T4 generation of NBBBO-NPTII-1 and WT were used and the relative expression of 10 genes selected was measured by RT-qPCR. We selected these genes due to their roles in carotenoid biosynthesis as reported in a number of plants (Huang et al. 2013; Enfissi et al. 2019; Lin et al. 2021; Lin and Huang 2021; Wang et al. 2021). Except for CYCb and CYP79, the other eight genes were significantly upregulated in the transgenic plants compared with WT control, with up to 1.8–58-fold increased transcripts (Fig. 7). The LYCe had the greatest upregulated gene, followed by ZDS, LCYb, GGPPS, PDS, and PSY. This result indicated that seed-specific expression of the heterologous BBBO genes led to upregulation of the genes involved in the endogenous carotenoid pathway, triggering the synthesis of lutein and β-carotene (Table 1). The β-carotene is the direct precursor for astaxanthin biosynthesis. Lutein is the dominant carotenoid in WT seeds, in contrast, the transgenic seeds contain both α-pathway (α-carotene and lutein) and β-pathway (β-carotene, echinenone, and astaxanthin) carotenoids. Thus, the heterologous expression of BBBO redirected the biosynthesis of carotenoids to the β-pathway, leading to the high yield of β-carotene and the novel ketocarotenoids. Because β-carotene accounted for almost one-half of the total carotenoids in the transgenic seeds, the enzymatic activities of BKT and BHY might be the limiting steps for astaxanthin yield.

Strategies for metabolic engineering of carotenoids in crops have been focused on over-expressing key pathway genes to increase the biosynthetic activity (Huang et al. 2013; Li and Yuan 2013; Sun et al. 2018, 2021). PSY/CrtB is a key enzyme in defining carotenoid pool size in plants (Sun and Li 2020). OR is the major regulator of PSY for carotenogenesis as well as promoting chromoplast biogenesis and development (Sun et al. 2020). Seed-specific expression of PSY increased total carotenoids in various plants (Shewmaker et al. 1999; Ducreux et al. 2005; Park et al. 2017; Yao et al. 2018; Sun et al. 2021), but the increased carotenoids were prone to degradation (Sun et al. 2021). Co-expression of PSY and OR could overcome the problems as recently demonstrated by Sun et al. (2021). The OR protein was shown to be a post-translational regulator of PSY, enhancing PSY stability and enzymatic activity (Zhou et al. 2015; Sun et al. 2018). Therefore, OR has been served as a genetic tool for enhancing carotenoid accumulation in potato, melon, carrot and sweet potato (Lu et al. 2006; Tzuri et al. 2015; Ellison et al. 2018; Gemenet et al. 2020).

Fig. 7: Relative expression of 10 endogenous carotenogenic genes (GGPPS, DXS, PSY, PDS, ZDS, LCYb, LCYe, CYCb, CRTZ, and CYP79) for C. sativa transformation. The data represent average values of four individual measurements. *, **statistical significance, determined by student’s t test, *P < 0.05; **P < 0.01.
Fatty acid profiles and antioxidant activity of transgenic *C. sativa* seeds

To reveal if the transgene expression could result in changes of fatty acid profile in *C. sativa*, mature seeds of three representative transformants were analyzed for their fatty acid contents. No significant changes in fatty acid compositions and contents were found in the mature seeds of the transgenic lines and WT control although all transgenic seeds accumulated much higher amounts of carotenoids (Table 1). Carotenoids have been shown to exhibit powerful antioxidant activity (Hussein et al. 2006; Liu et al. 2016a, b). To investigate if the hyper-accumulation of carotenoids in the transgenic seeds led to their higher antioxidant activity, we measured and compared the antioxidant activity of the T3 transgenic red seeds and the WT yellow seeds. The transgenic red seeds exhibited up to 3–fivefold higher antioxidant activity than WT based on the free-radical scavenging activity (Fig. S5). Thus, our transgenic plants should have great potential in a range of industry applications because they can produce seeds with much higher amounts of high-value carotenoids and thus have higher antioxidant activity, which may increase the stability of PUFAs in the seeds.

In summary, we succeeded in generating transgenic *C. sativa* plants with high contents of carotenoids without using a disputed selectable marker. This achievement may promote consumer acceptance of GM foods and facilitate the development of *C. sativa* with high nutritional value.

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Declarations  

Conflict of interest  The authors declare no conflicts of interest.

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