Construction of astaxanthin metabolic pathway in the green microalga

*Dunaliella viridis*

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**A B S T R A C T**

*Dunaliella viridis* is a green microalga containing β-carotene, which is the precursor of astaxanthin, the most active antioxidant in *Haematococcus pluvialis*. Two key enzymes of *H. pluvialis*, β-carotene hydroxylase (CRTR-B) and β-carotenoid ketolase (BKT), are required for converting β-carotene to astaxanthin in *D. viridis* via the astaxanthin biosynthetic pathway. Considering the location of β-carotene in the chloroplast of *D. viridis*, the two modified genes encoding BKT and CRTR-B in *H. pluvialis* were integrated via homologous recombination into the chloroplast genome, in this study. In the chloroplast, the homologous recombination vector pHD-bkt-crtR (16S-TrnA-atpA-bkt-crtR-B-rbcL-psbA-bar-TrnI-23S), bkt and crtr-B were regulated by the ap4A promoter in a polycistron. The presence of astaxanthin in the mutant expressing BKT and CRTR-B was verified using high performance liquid chromatography (HPLC), and the maximum content of total astaxanthin and canthaxanthin after high light induction were 77.5 ± 7.7 and 50.1 ± 0.8 μg g⁻¹ in dry weight, respectively. Our results indicate that *D. viridis* can be used as a cell factory for astaxanthin production.

1. Introduction

Astaxanthin is a high-value carotenoid with strong antioxidative activity and significant application potential. The sources of astaxanthin are limited to a few bacteria, yeast, certain microalgae, shrimp, crab, salmon, trout, and other organisms that feed on microalgae [1]. The green alga *Haematococcus pluvialis* can over-accumulate astaxanthin and astaxanthin esters up to 4% g⁻¹ dry weight, which makes it the optimal and main source of astaxanthin [2]. In plants and green algae, carotenoid biosynthesis begins in the chloroplast with the synthesis of phytoene, followed by multiple reduction and isomerization steps leading to lycopene, and additional cyclization reactions resulting in production of β-carotene. The details of the subsequent synthesis steps leading to astaxanthin are shown in Fig. 1. The metabolism of carotene is divided into two pathways catalyzed by β-carotene hydroxylase (CRTR-B) and β-carotenoid ketolase (BKT) (Fig. 1). In one pathway, carotene is first transported to the cytoplasm, after which BKT catalyzes the conversion of β-carotene to echinenone, which is then converted to canthaxanthin. Finally, canthaxanthin is converted to astaxanthin by BKT. In the other pathway, β-carotene is first converted to zeaxanthin by BKT in the chloroplast stroma. Zeaxanthin is transported to the cytoplasm, converted to astaxanthin by BKT [3], and stored as lipid droplets in the cytoplasm [4]. Many higher plants and microalgae do not contain astaxanthin, while the hydroxy derivatives of carotenoids are abundant in their chloroplast, e.g., β-carotene, canthaxanthin, and zeaxanthin. Therefore, expression of the bkt gene is preferred for astaxanthin production in most transgenic systems [5]. Furthermore, CRTR-B is also required to obtain higher productivity of astaxanthin [6–8].

Despite the high concentration of astaxanthin in *H. pluvialis*, largescale cultivation of this organism for astaxanthin production still faces many challenges. First, *H. pluvialis* is susceptible to contamination by other microalgae and microorganisms during growth. Although a completely enclosed photobioreactor system can avoid contamination...
with other organisms [9], it cannot be used widely owing to the associated capital expense. Second, growth and astaxanthin accumulation cannot be regulated simultaneously in H. pluvialis culture, which considerably affects the yield of astaxanthin [1]. Third, the cell wall of H. pluvialis affects the extraction efficiency of astaxanthin. There are multiple methods for extracting astaxanthin, such as chemical, ultrasonic, supercritical CO2 extraction methods, and so on [10,11]. However, these methods are generally expensive and cause environmental pollution, and hence are not suitable for commercial production. Owing to these factors, a less expensive source that can yield large quantities of astaxanthin is desirable for further industrial development, and strategies based on microbe-based metabolic engineering are needed for enhanced astaxanthin production.

The halophilic green alga Dunaliella viridis is a potential chassis cell for astaxanthin production with several advantages: (1) D. viridis is a single-celled eukaryote without a cell wall, and methods for its genetic transformation and product extraction are relatively simple; (2) D. viridis contains β-carotene, the precursor of astaxanthin, at the edge of the chloroplast stroma [12]; (3) transformation technologies have been established for Dunaliella species, which provide the basis for metabolic engineering [13–15]. Georgianna, et al. [16] reported that exogenous recombinant enzymes (xylosidase, α-galactosidase, phytase, phosphate anhydrolase, and β-mannanase) can be synthesized in Dunaliella tertiolecta via direct chloroplast transformation.

In this study, the bkt and crtR-B genes of H. pluvialis were transferred into the D. viridis chloroplast genome to extend the carotenoid synthesis pathway to astaxanthin. Furthermore, the selected D. viridis mutant was verified by assessing astaxanthin and canthaxanthin levels, which indicated that the complete and effective astaxanthin synthetic pathway was functional in the D. viridis mutant. This is a preliminary study on D. viridis metabolic engineering for the production of astaxanthin and other beneficial carotenoids, which will provide a new biosynthetic strategy.

2. Materials and methods

2.1. Media and strains

D. viridis strain FACHB-435 was purchased from the Institute of Aquatic Biology, Chinese Academy of Sciences. This strain is analogous to UTEX LB 200 and CCAP 19/3. D. viridis was incubated in modified artificial seawater medium (MASM, https://www.ccap.ac.uk/media/documents/MASM.pdf) at 25 ± 1 °C with 60–70 μmol m⁻² s⁻¹ light (light cycle: 12 h light: 12 h dark). The growth was monitored using a hemocytometer under a light microscope (BM-1000, Jiangnan Company, China) for 14 days. The initial cell concentration was 5.0 × 10⁵ cells ml⁻¹. For high light induction, D. viridis was cultured in MASM at 25 ± 1 °C with 260–290 μmol m⁻² s⁻¹ light (light cycle: 12 h light: 12 h dark) for four days.

H. pluvialis was cultured in BG11 medium [17] at 20 ± 1 °C with 40–50 μmol m⁻² s⁻¹ light (light cycle: 12 h light: 12 h dark) [18]. Escherichia coli (strain: TOP10) was cultivated in Luria Bertani (LB) medium at 37 °C with shaking at 150 rpm [19].

2.2. Vector construction

D. viridis and H. pluvialis at logarithmic phase were harvested via centrifugation at 8000 × g for 10 min, and D. viridis genomic DNA was extracted using a plant genomic DNA kit (Omega Bio-Tek, America). The total RNA of H. pluvialis was isolated using the plant RNA kit (Omega Bio-Tek, America) and was immediately converted to cDNA using the Strand cDNA synthesis kit (Takara, China). The chloroplast transformation vector of D. viridis was designed to harbor the foreign genes between the homologous fragments 16S-trnl (NCBI accession No. KX530454.1) and trnA-23S (NCBI accession No. GQ250046.1) [20]. Five endogenous fragments, including the 16S-trnl, trnA-23S, and aprA promoter (NCBI accession No. MK560872), the rbcL terminator (NCBI accession No. MK560874), and the psaA promoter (NCBI accession No. MK560873), were amplified using the D. viridis genome as the template. The cDNA of bkt (NCBI accession No. AY603347.1) and crtR-B (NCBI accession No. KP866868.1) were amplified using the H. pluvialis total cDNA as the template. The bar gene from the pPSB [20] vector was used for selection. All the primers were designed using Primer Premier 5 and are listed in Table 1. The amplification conditions were as follows: 95 °C for 120 s; 35 cycles of 95 °C, 60 s; 60 °C, 60 s; 72 °C, 90 s; final extension at 72 °C for 10 min. The amplified fragments were assembled into a pMD18-T vector (Takara, China) using the ClonExpress Ultra one-step cloning kit (Vazyme Biotech, China). All sequences and vectors were sequenced to verify their accuracy (Ruibo biotechnology, China).

2.3. Genetic transformation using microparticle bombardment

D. viridis cultured to the late logarithmic phase was centrifuged at 6500 × g for 10 min, adjusted to 1 × 10⁹ cell ml⁻¹ with fresh culture medium, and then evenly coated on the center of the solid medium as a circle of about 2 cm diameter. The plasmid constructed was purified from E. coli TOP10 using a TIANprep mini plasmid kit (Tiangen Biotech,
Table 1  
Primer sequences for pMD-bkt-crt vector construction. Underlined tagging sequence is His-tag; CCTCCCTC is Rbs sequence; Lower case is the homologous sequence used to construct the vector.

| Primer | Primer sequences (5’–3’) |
|--------|--------------------------|
| Vector construction primer | 16S-trnl  
F: cagatatgaacattatggactTTACCAGGGTTGCACTGCTAGAA  
R: ggatccGGCAAGCTTGCAAGACGTCGAG  
trnA-23s  
F: cagatatgaacattatggactTTACCAGGGTTGCACTGCTAGAA  
R: ggatccGGCAAGCTTGCAAGACGTCGAG  
atpA promoter  
F: tatagcccaATCCGCGTAGAGTAATAGG  
R: acgacggccagtgccaagcttTTCAGCTGTTTCGTTTTTAGAAAACT  
rbcl terminator  
F: gatgaGGGAATGTAGCTCAGTTGGTAGAGC  
R: aacccgcactatctgatgcgcatctcttccctcctTCCTCCTCACTGCTAGAA |
| Vector detection primer |  crtr-b  
F: atgcattacatctatatattCTATCGTTGACGGGAACGTCGAG  
R: aacctaaagacgactagCTACGCTGCAAAGACGGTCGAG  
pshA promoter  
F: tatagcccaacatgatgcgcatctcttccctcctTCCTCCTCACTGCTAGAA  
R: aacctaaagacgactagCTACGCTGCAAAGACGGTCGAG  
bar  
F: tagcctttgggcacgtATGAGCCCAGAACGACGCC  
R: acgacggccagtgccaagcttTTCAGCTGTTTCGTTTTTAGAAAACT |

China). Microparticle bombardment using the GJ-10000 system (Scientz, China) was performed according to the method of Yulin, et al. [20]. A shooting distance of 8 cm was used with helium pressure at 450 psi for introduction of the prepared microparticles into D. viridis. The bombarded plates were placed in the dark for 8 h, and then transferred to liquid medium for normal culture for 3 days before adding 15 μg ml⁻¹ phosphothiolin. Two weeks later, the selected cultures were moved to solid medium containing 10 μg ml⁻¹ phosphothiolin. Then, the surviving single colonies were selected further in liquid medium and on solid medium containing 5 μg ml⁻¹ phosphothiolin for two months. D. viridis bombarded with black particles (without the plasmid DNA) were used as the negative control. All the above experiments were conducted in triplicate.

2.4. Selection of transformed D. viridis

After 3 months of selection, the resistant colonies were analyzed further. First, bar, bkt, crtr-R, and the homologous fragments were amplified using polymerase chain reaction (PCR) with the total DNA of the resistant D. viridis colonies as the template. Primers 1-10 are listed in Table 1. Primer 1 and primer 10 were outside the insertion cassette. The colonies with positive PCR results were then identified using Southern blotting with the bkt, crtr-R, and bar genes as probes using the DIG High Prime DNA labeling and detection starter kit 1 (Roche, Germany). For Southern blotting, 30 μg genomic DNA for each sample was digested randomly using two sets of restriction endonucleases: Xhol and EcoRi; BamHI and HindIII.

To determine the mRNA levels, the total RNA of the resistant D. viridis colonies was isolated and immediately converted to cDNA. Three sets of primers were designed for the bar, bkt and crtr-B transcripts in D. viridis: bar-F and bar-R; BKT-F and BKT-R; CRTR- F and CRTR-R (Table 1).

For western blotting, cultures of the resistant D. viridis colonies in the late logarithmic phase (50 ml for each sample, 5.0 × 10⁵ cells ml⁻¹) were collected via centrifugation and resuspended in 1 ml phosphate buffered saline (PBS). The alga was then ultrasonicated using a 5-min cycle (work 5 s, interval 5 s) at 240 W in an ice bath, and centrifuged at 12,000 × g for 20 min. The supernatant was purified and concentrated using Ni Sepharose, and eluted using 200 mM imidazole (dissolved in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4). Western blotting was performed following the method described by Georgianna, et al. [16] with mouse anti-His IgG and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma, USA).

2.5. Carotenoid profiling

The cultures of wild type D. viridis and the mutant in the late logarithmic phase were induced by high light for 7 days and the carotenoids were profiled using HPLC (Agilent Technologies 1200 Series, Agilent Technologies, America). To extract free astaxanthin, 10 mg algal powder of each sample was ground in liquid nitrogen, followed by addition of 1 ml methanol, thorough mixing, and static incubation for 1 h in the darkroom. Following the method of Hideo, et al. [21], the total astaxanthin content in the mutant was determined using cholesteryl esterase, which catalyzed the astaxanthin ester in the sample.

HPLC was performed per the method described by Anila, et al. [3]. A photodiode array detector [22] and Acclaim 120 C18 column (Thermo, USA) were used, and the HPLC peaks were integrated using the Agilent ChemStation software (Agilent Technologies, America) at 450 nm to astaxanthin. The standard all-trans-carotenoids, astaxanthin, canthaxanthin, and lutein were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The species of astaxanthin stereoisomers in the mutant were determined using HPLC analysis with the Chiralpak IC column (Daicel, Japan), following the method described by Wang, et al. [23]. The HPLC peaks were integrated at 470 nm to astaxanthan. The standard all-trans-astaxanthin and 13-cis-astaxanthin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Stability of D. viridis -bkt-crt

D. viridis-bkt-crt was inoculated into the new medium (phosphothiolin-free) and cultured to the logarithmic growth phase. Then, the mutant was continuously cultured for generations, and foreign genes were detected using PCR with bar-F/bar-R, BKT-F/ BKT-R, and CRTR-F/CRTR-R primer pairs (Table 1).

2.7. Statistical analysis

All experiments were performed using biological triplicates to ensure reproducibility. Values are presented as means ± SD. Statistical analyses were performed using the SPSS statistical package (http://www-01.ibm.com/software/analytics/spss/). The paired-samples t test was applied. Differences were considered statistically significant at P values < 0.05.

3. Results

3.1. D. viridis mutant expressing the BKT and CRTR-B enzymes in the chloroplast can synthesize astaxanthin

The chloroplast homologous recombination vector pMD-bkt-crt constructed in this study is shown in Fig. 2. The homologous fragments
in this vector were from the 16S-trnI (901 bp) and trnA-23S (731 bp) regions of the D. viridis chloroplast genome. bkt (978 bp size, NCBI accession No. AY603347.1) and crtR-B (882 bp size, NCBI accession No. KP866868.1) were placed in a single polycistron and both were regulated by the endogenous atpA promoter (943 bp) and rbcl terminator (329 bp). The bar selection marker was driven by the psbA promoter (511 bp).

The control died after 10 weeks of selection and three algal colonies survived in the plate containing the herbicide Basta. A set of primers were designed based on the pMD-bkt-crt vector to test the three colonies (Fig. 3a). PCR and sequencing analysis showed that one D. viridis colony harbored bar, bkt, and crtR-B between the two homologous fragments (Fig. 3b), which was named D. viridis -bkt-crt. The PCR product generated using primers 1 and 10 showed two amplified fragments when D. viridis -bkt-crt was used as the template and one fragment when the wild type was used (Fig. 3c), which indicated that D. viridis -bkt-crt was still heteroplasmic. However, no bands were detected in the wild type samples using Southern blotting, whereas bands corresponding to bkt and crtR-B (1800 bp in group 1 DNA and 4000 bp in group 2 DNA) and bar (1300 bp in group 1 DNA, and 1000 bp in group 2 DNA) were detected for D. viridis -bkt-crt (Fig. 4). These results showed that the fragment was correctly inserted into the chloroplast genome in the mutant D. viridis -bkt-crt.

The reverse transcription-PCR products of the bkt, crtR-B, and bar genes obtained using D. viridis -bkt-crt cDNA as template (Fig. 3d) demonstrated that the three genes were successfully transcribed in the mutant. The western blot of the BKT and CRTR-B enzymes with anti-β-His-tag antibody showed bands for two proteins: β-carotene hydroxylase (39.85 kDa) and β-carotenoic ketolase (32.85 kDa) (Fig. 5).

In addition to other carotenoids, HPLC analysis showed the presence of astaxanthin and canthaxanthin in the mutant D. viridis -bkt-crt (Fig. 6). Peak 3 detected using the photodiode array detector of the HPLC system showed the characteristic absorption spectra of astaxanthin (Supplementary Fig. 1). Lutein and β-carotene were the major carotenoids in the wild type and transformed D. viridis. HPLC analysis of the standard sample showed that the retention time of lutein was similar to that of the zeaxanthin using the method followed in this study. The concentrations of total astaxanthin, free astaxanthin, and canthaxanthin are shown in Table 2. It is noteworthy that the free astaxanthin content accounts for 48.9% of the total astaxanthin content. HPLC analysis showed that (3S,3S)-all-trans-astaxanthin was the main astaxanthin in the mutant (Fig. 7).

The growth curve obtained after 14 days of culture with the same initial concentration of wild type D. viridis and D. viridis-bkt-crt are shown in Fig. 8. The final concentration of the wild type strain was 45.00 ± 4.27 × 10^5 ml g^-1, which was higher than that of the mutant (33.33 ± 5.35 × 10^5 ml g^-1). These results showed that the growth rate of D. viridis-bkt-crt was slower than that of the wild type.

3.2. Astaxanthin and canthaxanthin were induced by stress in D. viridis -bkt-crt

The β-carotene, astaxanthin, and canthaxanthin content increased in D. viridis-bkt-crt under stress treatments, and the highest concentrations of β-carotene (3078.3 ± 23.1 µg g^-1 dry weight), free astaxanthin (32.6 ± 0.6 µg g^-1 dry weight), canthaxanthin (50.1 ± 0.8 µg g^-1 dry weight), and total astaxanthin (77.5 ± 7.7 µg g^-1 dry weight) were obtained in the mutant after 7 days’ treatment with high light (Table 2). Compared to the wild type strain, the pigment content of the mutant changed to some extent; however, the most significant change was the decrease in the content of neoxanthin, violaxanthin, and chlorophyll a, even after light induction (Table 2).

3.3. Genetic stability of D. viridis- bkt-crt

After subculture, the genetic stability of D. viridis -bkt-crt was determined using PCR (Fig. 9). The first and second generations of D. viridis-bkt-crt contained bar, bkt, and crtR-B, although these foreign genes could not be sub-cultured steadily in the third and fourth generation mutants.
4. Discussion

In this study, the carotenoid biosynthetic pathway of D. viridis was extended to astaxanthin by expressing the bkt and crtR-B genes from H. pluvialis. H. pluvialis harbors three isoenzymes of BKT, namely, BKT1, BKT2, and BKT3. All the three BKT isoenzymes can catalyze the conversion of β-carotene and zeaxanthin, and showed higher affinity for β-carotene than for zeaxanthin [24,25]. Among the three isoenzymes, BKT3, a β-carotene ketolase is the most efficient [26], while BKT2 showed the highest activity as zeaxanthin ketolase. BKT2 is preferred for astaxanthin production, as it can catalyze zeaxanthin to astaxanthin; however, the limited astaxanthin production might be due to the low content of zeaxanthin (< 1%) in D. salina [5]. BKT3 was used in this study to completely utilize the β-carotene in D. viridis, and the most active CRTR-B in H. pluvialis was utilized to extend the metabolic pathway to produce astaxanthin [7].

Astaxanthin exists in several isomeric forms (cis and trans) depending on the biological sources used for its isolation [23]. Three stereoisomers are known for astaxanthin: a pair of enantiomers (3R,3′R- and 3S,3′S-astaxanthin) and an optically inactive mesoform (3R,3′S-astaxanthin). 3S,3′S-astaxanthin is the most abundant isomer and has the strongest antioxidant activity. In this study, the astaxanthin in the mutant was identified to be (3S,3′S)-all-trans-astaxanthin, which is identical to the astaxanthin species found in H. pluvialis.

The carotenoid composition of the mutant D. viridis-bkt-crt was
similar to that of *H. pluvialis*; however, astaxanthin was shown to mostly exist in the monoester form (90%) in *H. pluvialis* [27], whereas the free form (about 42% of total astaxanthin) was detected in the mutant. This is beneficial for the extraction of astaxanthin from the mutant, as the high content of astaxanthin esters in wild type *H. pluvialis* usually requires complex saponification or enzymolysis to obtain free astaxanthin [28]. The complex process of separation and the inevitable degradation of astaxanthin during the purification of astaxanthin. As the mutant *D. viridis*-bkt-crt contains high levels of free astaxanthin, the extra separation step is avoided, which is advantageous for the extraction of astaxanthin.

In the present study, the total carotenoid content of the wild type and mutant *D. viridis*-bkt-crt were almost similar; a similar observation was also made in a previous study on *D. salina* [5,29]. However, the carotenoid constituents and proportions differed significantly between the *D. viridis*-bkt-crt and the wild type strain. In terms of carotenoid constituents, the most significant changes in *D. viridis*-bkt-crt were the production of canthaxanthin and astaxanthin, with decrease in the content of violaxanthin, neoxanthin, and chlorophyll a. Interestingly, as violanthin and neoxanthin are the metabolites of zeaxanthin cyclization [30], it is suggested that the ketonization of zeaxanthin by BKT possibly weakened its cyclization in *D. viridis*-bkt-crt.
Previous studies have shown that due to the genetic relationship between Dunaliella species and H. pluvialis, many genes of D. salina can be successfully expressed in H. pluvialis [31]. Anila et al. [5] reported that the mutant strain of D. salina harboring bkt accumulated 3.5 μg/g astaxanthin. Zheng et al. [6] reported that the astaxanthin content increased successfully to 1.6 mg/g when bkt and crtR-B were expressed in Chlamydomonas reinhardtii CC-849, accumulating 34% more astaxanthin than the wild type strain. β-Carotene exists in the chloroplasts in Dunaliella species [32]. To take advantage of these large amounts of β-carotene, the bkt and crtR-B genes were inserted into the chloroplast genome via homologous recombination, as BKT and CRTR-B can directly catalyze the conversion of β-carotene to astaxanthin in the chloroplast without transportation of pigments or enzymes between the chloroplast membranes. In this study, the two genes were placed in one polycistron with a ribosome binding site (RBS) [33–35], and the native strong promoters in D. viridis were used to regulate the expression of foreign genes, for example, the atrA promoter for the bkt and crtR-B genes, and the psbA promoter for the bar selection marker. However, astaxanthin production was not high, and the extended astaxanthin synthetic pathway might perturb the balance of the primary metabolic pathway in D. viridis, leading to slow growth rate and low astaxanthin

Fig. 7. HPLC separation of astaxanthin stereoisomeric. a, standard of 9-cis-astaxanthin; b, standard of all-trans-astaxanthin; c, carotenoids of D. viridis-bkt-crt. 1, (3S, 3′S)-trans-astaxanthin; 2, (3S, 3′R)-trans-astaxanthin; 3, (3R, 3′R)-trans-astaxanthin; 4, 9-cis-astaxanthin.

Fig. 8. Growth curve of wild and mutant strains of D. viridis. wt, wild D. viridis; tf, mutant D. viridis-bkt-crt. Data are shown as mean ± SD, n = 3.
synthesis by *D. viridis*-bkt-ctrt. The lack of codon optimization of *bkt* and *crtR-B* can be another reason for the low astaxanthin production in the mutant [20]. Furthermore, the lower β-carotene and higher lutein content in *D. viridis* FACHB-435 were also responsible for the limited production of astaxanthin. In addition, the stability of foreign genes also limits the applicability of the mutants. Future investigations will focus on homogeneous screening, the role of total metabolic networks and intermediate products, such as acetyl-CoA and pyruvic acid, and the use of inducible promoters [36,37] to separate microalgae growth and intermediate products, such as astaxanthin and canthaxanthin.

5. Conclusion

Using chloroplast transformation, a *D. viridis* mutant harboring *bkt* and *crtR-B* of *H. pluvialis* was constructed, and the mutant *D. viridis*-bkt-ctrt could synthesize astaxanthin and canthaxanthin. After high light treatment, the mutant *D. viridis*-bkt-ctrt accumulated more astaxanthin and canthaxanthin than the wild type strain. After high light induction for 7 days, the total astaxanthin and free astaxanthin content in *D. viridis*-bkt-ctrt reached 77.5 ± 7.7 and 32.6 ± 0.6 μg g⁻¹ dry weight, respectively. Thus, this study lays the foundation for efficient utilization of *D. viridis* toward production of valuable carotenoids using a chloroplast transformation system.

**Statement of informed consent**

Informed consent and ethical approval for human or animal experiments are not applicable for this study.

**Author contributions**

Chunxiang Meng and Song Qin designed the experiments. Bin Lin and Yulin Cui analysed the data and wrote the manuscript. Bin Lin, Mingyan Yan, Yinchu Wang and Zhengquan Gao performed all experiments.

**Declaration of Competing Interest**

The authors have no competing interests.

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