# Chapter 17

# Ketocarotenoid Biosynthesis in Transgenic Microalgae Expressing a Foreign β-C-4-carotene Oxygenase Gene

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## Abstract

Ketocarotenoids are obtained by the action of the β-carotene ketolase, which catalyses the addition of a keto-group at the C4 position of carotenoids β-ion-rings. Most microalgae and higher plants do not posses the carotene ketolase activity and consequently do not synthesize ketocarotenoids, which are highly demanded as feed supplements and as nutraceutical for human nutrition. Here we propose the use of the unicellular microalga *Chlamydomonas reinhardtii* to express the *Bkt* (β-carotene ketolase) gene from *Haematococcus pluvialis* and synthesize a new ketocarotenoid that the vegetative cells of *Chlamydomonas* do not synthesize in the natural way. The methodology needed to successfully achieve metabolic engineering of ketocarotenoids synthesis in *Chlamydomonas* is described in this chapter, including the construction of a vector containing the *Bkt* gene, transformation of *Chlamydomonas*, selection of transformants, and carotenoids analysis.

**Key words:** *Chlamydomonas reinhardtii*, *Haematococcus pluvialis*, β-Carotene ketolase, Ketocarotenoids, Transgenic microalgae

## 1. Introduction

Ketocarotenoids are xanthophylls obtained by oxygenation of carotenes or their hydroxylated derivatives by the action of a group of β-carotene ketolases, which catalyse the addition of a keto-group in the C4 position of carotenoids β-ione-rings (see Fig. 1). There is a huge worldwide market for ketocarotenoids, such as astaxanthin and cantaxanthin, which are highly demanded as feed supplements for fish aquaculture (1) and as nutraceutical for human nutrition (2). Astaxanthin is an essential feed additive to provide their characteristic pigmentation in aquaculture-growth salmon, trout, or shrimp (1).

Several classes of carotenoid ketolases have been reported. Those encoded by *CrtW* genes, found in bacteria (3, 4), which are

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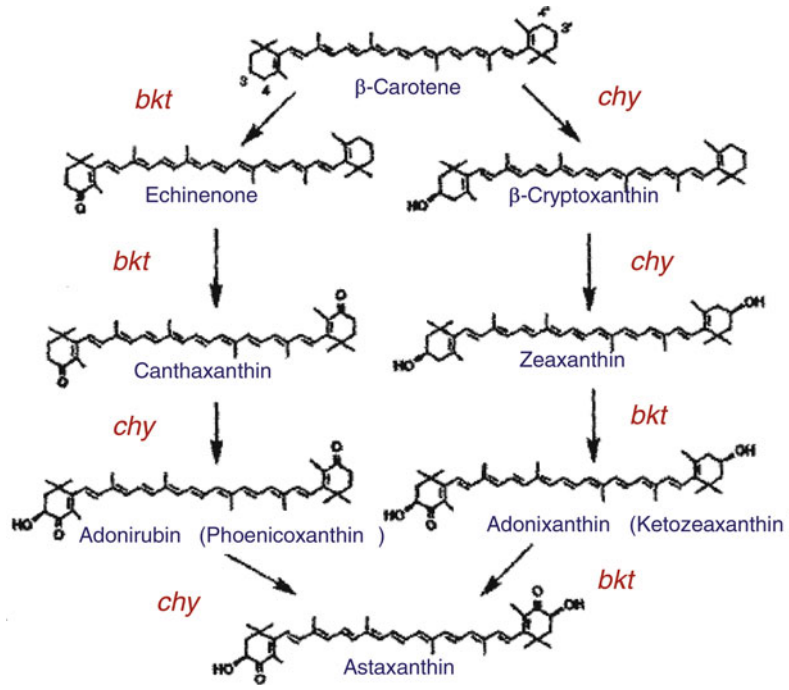


Fig. 1. Metabolic pathways for the synthesis of cantaxanthin and astaxanthin in plant cells. *Chy* β-carotene hydroxylase, *bkt* β-carotene ketolase.

very similar in functionality and sequence to the *Bkt* genes found in some algae and plants, and catalyze the oxygenation of both ione-rings of β-carotene. And the *CrtO* type, found in cyanobacteria (5), which asymmetrically add one keto-group to β-carotene to form echinenone, that seems to have arisen independently in evolution as they share very little sequence similarity with the *CrtW* type. *CrtW* ketolases are related to bacterial (*CrtZ*) and plant (*Bhy*) beta-ring hydroxylases; all members of a large class of membrane-integral, di-iron oxygenase enzymes (6).

Most microalgae and higher plants do no posses the carotene oxygenase activity and consequently do not synthesize ketocarotenoids. One important exception is the green microalga *Haematococcus pluvialis*, which is able to accumulate around 4% of its dry weight of astaxanthin under stressing conditions and has become the first biotechnological source of natural ketocarotenoids (1), but its culture at industrial scale is not free of problems. In vegetative cells of *Chlamydomonas reinhardtii* no ketocarotenoids have been found, although recent studies have demonstrated the existence of a putative *Bkt* gene in its genome and the presence of ketocarotenoids in mature zygospores of this microalga (7).

In vivo production of astaxanthin and other ketocarotenoids by organisms that do not synthesize them in the natural way has been

achieved by metabolic engineering in *E. coli* (8, 9), cyanobacteria (10), and several higher plants (11–15). Here we propose the use of the unicellular microalga *C. reinhardtii* to express the *Bkt* gene from *H. pluvialis*, inserted in an adequate construction, to synthesize a new ketocarotenoid that the vegetative cells of *Chlamydomonas* do not synthesize in the natural way. The methodology needed to successfully achieve metabolic engineering of ketocarotenoids synthesis in *Chlamydomonas* is developed in the next subheadings of this chapter and includes: (1) total RNA extraction from *H. pluvialis*. (2) Synthesis of *Bkt* cDNA from *H. pluvialis*. (3) Integration of the obtained *Bkt* gene in pS104-PLK-tp plasmid. (4) Transformation of *C. reinhardtii* with the obtained construction. (5) Selection of positive transformants.

## 2. Materials

### 2.1. Isolation of RNA

1. *H. pluvialis* SAG 192-80 (16).
2. Hutner traces solution: Solve 10 g of EDTA (free acid) in 250 mL of $H_2O$. In a different recipient, solve in 500 mL of water at 100°C and in the following order 2.28 g of $BO_3H_3$, 4.40 g of $ZnSO_4{\cdot}7H_2O$, 1.02 g of $MnCl_2{\cdot}4H_2O$, 1 g of $FeSO_4{\cdot}7H_2O$, 0.32 g of $CoCl_2{\cdot}6H_2O$, 0.32 g of $CuSO_4{\cdot}5H_2O$, and 0.22 g of $(NH_4)_6MoO_{24}{\cdot}4H_2O$. Mix both solutions and heat the mixture again to 100°C. Let the solution to cool until 80–90°C and adjust to pH 6.8 with 20% KOH. Complete the final solution to 1 L total volume and store it in the dark for 2 days before use (17).
3. Liquid medium for *H. pluvialis*: 0.72 g/L of $KPO_4H_2$, 1.44 g/L of $K_2PO_4H$, 0.25 mM $MgSO_4{\cdot}7H_2O$, 0.13 mM $CaCl_2{\cdot}2H_2O$, 0.5 g/L of $NH_4Cl$, and 5 mL/L of Hutner traces solution. For nitrogen-free medium no ammonium was added.
4. Lysis RNA solution: 50 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 5 mM EDTA, and 2% SDS, prepared in DEPC treated water (see Note 1).
5. PCI: phenol–chloroform–isoamyl alcohol 25:24:1, saturated with 10 mM Tris buffer pH 8.0 (see Note 2).
6. Chloroform saturated with 10 mM Tris buffer pH 8.
7. 0.15 M sodium acetate.

### 2.2. Synthesis of Bkt cDNA from H. pluvialis

1. Oligo dT (Invitrogen, Carlsbad, CA, USA).
2. SuperScript II (Invitrogen, Carlsbad, CA, USA).
3. RNaseH (Invitrogen, Carlsbad, CA, USA).

4. dNTP mix 10 mM: For 2 mL add 200 µL of 100 mM dATP, 200 µL of 100 mM dCTP, 200 µL of 100 mM dGTP, 200 µL of 100 mM dTTP, and 1,200 µL of milli-Q water.
5. Forward primer A: 5′-TGCCGCTCGAGAGCCTCAAATAA-3′. XhoI site is underlined.
6. Reverse primer B: 5′-CACTCCTGCAGACGCAAGACATC-3′. PstI site is underlined.
7. Agarose (BMA, Rockland, ME, USA).
8. TAE buffer stock 50× solution: 242 g of Tris, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA pH 8.
9. Ethidium bromide 5 mg/mL in water (see Note 3).
10. UV-transilluminator.
11. Quiaquick gel extraction kit (Qiagen, Hilden, Germany).
12. pGemT plasmid (Promega, Madison, WI, USA).

### 2.3. Integration of the Obtained Bkt Gene in the Constructed Plasmid

1. pSI103 (18) (see Note 4).
2. pSI104-tpPLK (19) (see Note 5).
3. LB (Luria Bertani): Add 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl into 1 L of deionized water. Autoclave at 121°C for 15 min.
4. LB-agar: LB and 20 g/L agar. Autoclave at 121°C for 15 min.
5. TCM: 10 mM Tris, 10 mM $CaCl_2$, and 10 mM $MgCl_2$, pH 7.
6. Solution I: 50 mM Glucose, 25 mM Tris–HCl pH 8, and 10 mM EDTA.
7. Solution II: 0.2 M NaOH and 1% SDS.
8. Solution III: 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of water.
9. T4 DNA ligase.
10. Ligase buffer 10×: 300 mM Tris–HCl (pH 7.8 at 25°C), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP.
11. *E. coli* strain DH5α (20).
12. 50 mM $CaCl_2$.
13. Ampicillin (Sigma, St. Louis, MO, USA).

### 2.4. Transformation of Chlamydomonas reinhardtii with the Obtained Construction

1. *Chlamydomonas reinhardtii* 704 strain (Cw15, Arg7, mt +) (21).
2. Solution Tris: Tris 121 g/L.
3. Solution A: 5 g/L of $CaCl_2 \cdot 2H_2O$, 10 g/L of $MgSO_4 \cdot 7H_2O$, and 40 g/L of $NH_4Cl$.
4. Solution B: 115 g/L of $K_2HPO_4$, and 6 g/L of $KH_2PO_4$.
5. TAP: For 1 L of TAP medium add 20 mL of solution Tris 1 M, 10 mL of solution A, 1 mL of solution B, 1 mL of Hutner

traces solution, and 0.95 mL of glacial acetic acid, and adjust to pH 7. Autoclave at 121°C for 15 min.

6. TAP-agar: TAP and 20 g/L agar. Autoclave at 121°C for 15 min.
7. Microalgal transformation mix: 0.3 g of sterile glass beads (0.4–0.6 mm ∅) and 0.2 mL of 20% polyethylene glycol 8000.
8. Paromomycin (Sigma, St. Louis, MO, USA).

***2.5. Selection of Positive Transformants***

1. Lysis buffer for genomic DNA: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 3% SDS.
2. TE buffer: 10 mM Tris–HCl pH 8.0, and 1 mM EDTA.
3. TE buffer for genomic DNA: 10 mM Tris–HCl, pH 8 and 0.1 mM EDTA.
4. 5 mM Tris–HCl, pH 8.
5. Forward primer C: 5′-AGCGGTGCCCTCCTGATAAAC-3′.
6. Reverse primer D: 5′-TTCCGGTAAGCTGCTCCAACAT-3′.
7. Nylon syringe filters (Fisher Scientific, Hudson, NH, USA).
8. 3 M sodium acetate, pH 5.2.
9. HPLC apparatus equipped with UV-Vis detector and RP-18 column.
10. Mobile phase: Solvent A (ethyl acetate) and solvent B (acetonitrile/water) 9:1 (v/v).

## 3. Methods

***3.1. Total RNA Extraction from H. pluvialis***

1. Transfer a well-grown *H. pluvialis* culture to high light *conditions* at 25°C for 3 days (see Note 6).
2. Harvest 200 mL of the *H. pluvialis* cells by centrifugation at 4,000 × *g* for 5 min.
3. Resuspend the pellet in 3 mL of the buffer lysis solution.
4. Vortex for 20 min and incubate on ice for 5 min (see Note 7).
5. Add an equal volume of PCI, mix vigorously, and centrifuge at 4,000 × *g* for 5 min at room temperature.
6. Transfer the upper aqueous phase to another tube and repeat the extraction with PCI until the interface is clear.
7. Add the same volume of chloroform, mix, and centrifuge at 4,000 × *g* for 10 min at room temperature. Take the aqueous phase.
8. Add 2.4 volumes of ethanol absolute and incubate at –20°C for at least 4 h.
9. Centrifuge at 4°C and 4,000 × *g* during 10 min.

10. Discard flow-through, resuspend the pellet in 0.5 mL of water-DEPC, and add the same volume of LiCl 8 M.
11. Incubate a 0–4°C overnight.
12. Centrifuge at 4,000 × *g* for 30 min at 4°C.
13. Wash the pellet with ethanol 70% two times.
14. Dry the pellet and resuspend it in water-DEPC.
15. Add 2.5 volumes of 0.15 M sodium acetate and incubate a –20°C overnight.
16. Centrifuge and wash the pellet with ethanol 70%.
17. Dry the pellet and resuspend in water-DEPC.
18. Store at –80°C.

### 3.2. Synthesis of Bkt cDNA from H. pluvialis

1. To synthesize single-strand *Bkt* cDNA, add the following components to a nuclease-free microcentrifuge tube: 1 µL of Oligo dT (500 µg/mL), 1 µL of the total RNA previously obtained, 1 µL dNTP mix (10 mM each), and 9 µL of sterile distilled water.
2. Heat the mixture to 65°C for 5 min and quickly chill on ice.
3. Add 4 µL of the 5× first strand buffer supplied with the SuperScript II RNaseH-reverse transcriptase, mix contents of the tube gently, and incubate at 42°C for 2 min.
4. Add 1 µL of the reverse transcriptase (SuperScript II RNaseH 200 units) and mix very gently again.
5. Incubate at 42°C for 50 min.
6. Inactivate the reaction by heating at 70°C for 15 min.
7. Add 1 µL of RNAase H and incubate at 37°C for 20 min to remove the RNA complementary to the cDNA.
8. Using 2 µL of the retrotranscriptase reaction mixture, and the specific forward primer A and reverse primer B for *HpBkt*, carry out the PCR reaction.
9. To carry out the PCR amplification add in a total volume of 50 µL: 2 µL of the RT reaction mixture, 20 pmol of each primer, 0.2 mM dNTPs, 1 U pfu *Taq* DNA polymerase, 5 µL of specific 10× buffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). Run the following PCR program: 0.5 min at 96°C, 0.5 min at 42°C, and 1.5 min at 72°C for 30 cycles.
10. To purify the PCR product, run an electrophoresis in a 1% agarose gel.
11. The 1% agarose gel is prepared by solving 1 g of agarose in 100 mL of TAE buffer 1× and melting it in a microwave. Add 5 mL of ethidium bromide solution and gently pour the gel in the tray (try to avoid air bubbles) and place a separation comb

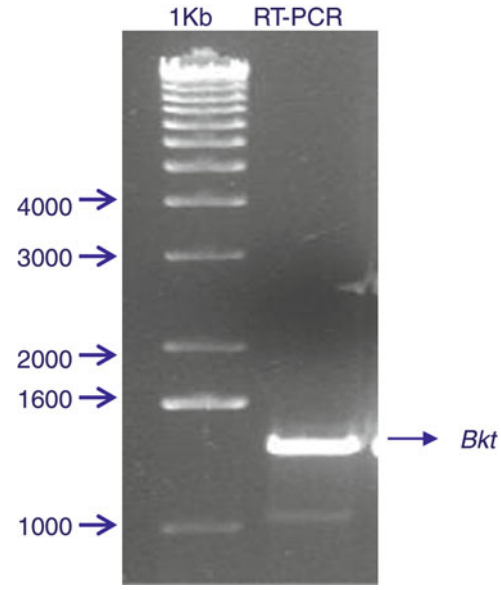


Fig. 2. DNA fragment corresponding to the *HpBkt* cDNA obtained by RT-PCR from total RNA and visualized in an EtBr-stained agarose 1% gel. The obtained band has a size of 1.4 kb.

into the tray. Allow the gel to completely solidify. The gel is ready to be loaded and run.

12. Visualize the band on a UV-transilluminator by staining with ethidium bromide (see Note 3). The expected band has a size of 1.4 kb (see Fig. 2).
13. Isolate the DNA containing the *HpBkt* cDNA fragment from the agarose band using the Quiaquick gel extraction kit (see Note 8).
14. Store frozen the obtained cDNA until its utilization or clone it in pGemT plasmid (see Note 9).

### *3.3. Integration of the Obtained Bkt Gene in the pSI104-tpPLK Plasmid*

1. Inoculate the bacteria which contain plasmid pSI104-tpPLK and plasmid pGemT-*HpBkt* in different tubes with 3 mL of LB medium. Incubate overnight at 37°C with agitation (200 rpm) and isolate the plasmid following the alkaline lysis protocol (22) as described below.
2. Harvest 1.5 mL of the culture by centrifugation at 12,000 × *g* in a bench microcentrifuge for 30 s. Remove the medium, leaving the bacterial pellet as dry as possible.
3. Resuspend the pellet in 0.2 mL of cold solution I by vigorous vortexing.
4. Add 0.2 mL of cold freshly prepared solution II, and mix by inverting the tube several times. Do not vortex. Leave the tube in ice for 5 min.

5. Add 0.2 mL of cold solution III (60 mL of potassium acetate 5 M, 11.5 mL of glacial acetic acid and 28.5 mL of water) and mix gently by inverting the tube 6 or 8 times. Leave the tube in ice between 5 and 10 min.
6. Centrifuge at 12,000 × *g* for 5 min in a microfuge. Transfer the supernatant to a fresh tube.
7. Add 600 mL of PCI (see Note 2), mix by vortexing, and centrifuge at 12,000 × *g* for 3 min.
8. Transfer the aqueous upper phase to a fresh tube and repeat PCI extraction until the interphase is clear.
9. Precipitate the plasmidic DNA with 2 volumes of ethanol at −20°C for 10–20 min. Centrifuge at maximum speed for 5 min at 4°C in a microfuge and remove the supernatant.
10. Rinse the pellet with 1 mL of 70% ethanol, remove the supernatant, and allow the plasmidic pellet to dry in the air for 10 min.
11. Digest pSI104-tpPLK and pGemT-*HpBkt* with the restriction enzymes *XhoI* and *PstI* (see Note 10).
12. Run electrophoresis in 1% agarose gel (see Subheading 2.2).
13. Cut the bands corresponding to the digested pSI104PLK (about 3.9 kb) and *HpBkt* cDNA (about 1.3 kb).
14. Purify the DNA using the Quiaquick gel extraction kit (see Note 8).
15. To ligate both fragments with T4 ligase, add the following components to a nuclease-free microcentrifuge tube: vector DNA, insert DNA, ligase 10× buffer, T4 DNA ligase, and nuclease-free water to final volume of 10 μL. Use a 1:3 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. Incubate the reaction at 16°C overnight.
16. Transform *E. coli* competent cells with the obtained pSI104-tpPLK-*Bkt* plasmid (see Notes 11 and 12).
17. Select 2 or 3 of the colonies that have appeared and grow them in liquid LB with ampicillin (see Note 13).
18. Isolate the plasmid from the chosen colonies by alkaline lysis method (see steps 2–10) and check by digestion or sequencing that the isolated plasmids are pSI104PLK-tp-*HpBkt*.
19. Use the new plasmid pSI104PLK-tp-*HpBkt* to transform *C. reinhardtii* cells.

### 3.4. Transformation of C. reinhardtii with pSI104PLK-tp-HpBkt Plasmid

Transformation is carried out according to the glass-beads method of Kindle (23) with minor modifications.

1. Grow a culture of *C. reinhardtii* in TAP medium at 25°C to a cell density of about $10^7$ cells/mL (see Note 14).

2. Harvest 600 mL of *C. reinhardtii* cells and resuspend them in 0.6 mL fresh TAP medium to obtain a 100-fold concentrated cell suspension.
3. Add the concentrated cell suspension (0.6 mL) to a conical tube containing the transformation mix and about 500 ng of the plasmid pSI104PLK-tp-*HpBbkt*, obtained as described in Subheading 3.3, and 500 ng of plasmid pSI103 which carries resistance to the antibiotic paromomycin.
4. Vortex for 8 s, add 5 mL of TAP medium, centrifuge 3 min at 6,000 × *g*, and discard supernatant.
5. Resuspend the cellular pellet in a small volume of TAP medium and transfer to a new 50-mL tube. Complete with fresh sterile TAP until 50 mL and incubate at 25°C and standard light overnight.
6. After this incubation in the absence of antibiotic, pellet the cells and spread them onto TAP Petri dishes with 30 μg/mL paromomycin (see Note 13).
7. Incubate at 25°C. Transformed colonies will be visible after 4 or 5 days.

### 3.5. Selection of Positive Transformants

The next step is to check which of the paromomycin-resistant transformants of *C. reinhardtii* has the plasmid inserted in their genome.

1. To isolate the genomic DNA from the transformants, scrap one loop of each *Chlamydomonas* transformant from the plate, resuspend it in 10 μL of lysis buffer and incubate at room temperature for 15 min.
2. Add 500 μL of TE buffer and 60 μL of 3 M sodium acetate, pH 5.2.
3. Extract the DNA with PCI and precipitate with isopropanol.
4. Wash the pellet with 70% ethanol, dry, and resuspend in 5 mM Tris–HCl, pH 8 (see Note 15).
5. Use 1 μL of the genomic DNA solution to carry out a PCR reaction using the forward primer C and the reverse primer D, which anneal with *RbcS2* promoter and *HpBkt* gene amplifying a 547-bp fragment. It is expected that about 30–50% of the transformants obtained with two plasmids acquired both of them.
6. Take several of the positive transformants in which insertion of the *HpBkt* gene has been shown to analyse their pigment composition.
7. Grow a culture (3 mL) of each transformant in TAP medium at 25°C.
8. Take 1 mL of a well grown culture, heat at 100°C for 5 min, and add 4 mL of acetone.

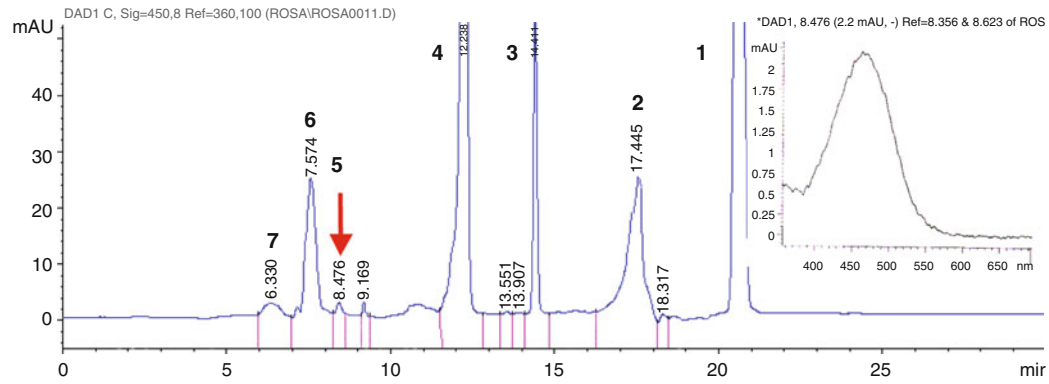


Fig. 3. HPLC profile of carotenoids present in acetone extracts from transgenic *Chlamydomonas* cells containing the *H. pluvialis Bkt* gen. The *arrow* indicates the new carotenoid present in the transformed cells. The absortion spectra corresponding to the new peak is also shown. Peaks identification: 1-β-carotene, 2-chlorophyll a, 3-chlorophyll b, 4-lutein, 5-keto-lutein, 6-violaxanthin, 7-neoxanthin.

9. Vortex vigorously and centrifuge at 6,000 × *g* for 10 min to eliminate the cell debris.
10. Filter the supernatant through nylon syringe filters and inject a fraction of the filtered supernatant in an HPLC equipped with UV-Vis detector and RP-18 column.
11. Apply the mobile phase at a flow rate of 1 mL/min with the following gradient programme: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100% A. Injection volume 100 µL. Carry out pigments detection at 450 nm (24). In Fig. 3 it is shown an example of the chromatographic pigment analysis of one of the transgenic *Chlamydomonas* strains expressing the *Bkt* gene from *H. pluvialis* showing the presence of the new ketocarotenoid, keto-lutein.

## 4. Notes

1. Diethylpyrocarbonate (DEPC) is used to inactivate the RNase enzymes from water and other laboratory utensils. Solve 0.1% (v/v) DEPC in the water or solution to be treated and stir with a magnetic bar for at least 1 h at room temperature. Then autoclave to transform traces of DEPC, into $CO_2$, $H_2O$, and EtOH that will evaporate. DEPC cannot be used with Tris buffer or HEPES since their amino groups will inactivate DEPC by reacting with it.
2. To make PCI 25:24:1. Add to 50 mL of phenol, 50 µL of dihydroxiquinoline dihydrate and 50 mL of Tris 1 M and mix with a magnetic bar. Add 450 mL of water and mix again. Wait until two phases appear. Control the pH of the upper aqueous phase. It must be between 7.8 and 8. Discard most of the aqueous

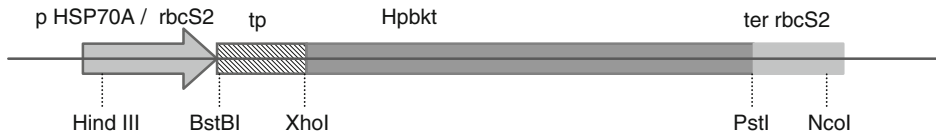


Fig. 4. Schematic representation of the construction used to express the *HpBkt* gene from *H. pluvialis* in *Chlamydomonas*. *prbcS2* ribulose 1,5 bisphosphate carboxylase small subunit promoter; *ter rbcS2* ribulose 1,5 bisphosphate carboxylase small subunit terminator region; *pHSP70A* heat shock protein 70A promoter; *tp* transit peptide; *Bkt* β-carotene ketolase. The whole construction has 2,550 bp.

phase (leave only 1 or 2 cm) and add 24 parts of chloroform and 1 part of isoamyl alcohol. Keep the solution at 4°C preserved from the light. All the process should be carried out with gloves and in a fume hood due to high toxicity of phenol.

3. Ethidium bromide (EtBr) is a very dangerous mutagenic agent. It must be handled with gloves and with extreme caution. Solutions containing EtBr and solids contaminated with EtBr should be discarded as toxic chemical waste.
4. Plasmid pSI103 (18) contains the *AphVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3′ phosphotransferase that confers resistance to the antibiotic paromomycin, under the control of the strong constitutive promoters *RbcS2* and *Hsp70A* and terminated by the 3′ untranslated region of *RbcS2*.
5. Plasmid pSI104-tpPLK contains a polylinker region under the control of the strong constitutive promoters *RbcS2* and *Hsp70A* and terminated by the 3′ untranslated region of *RbcS2*. It also has the RBCS2 transit peptide encoding sequence immediately after the promoter and before the polylinker to ensure transport to the chloroplast of the desired protein (see Fig. 4). This plasmid has been obtained in our lab after modification of plasmid pSI103 (19).
6. Cultures are grown in mineral liquid medium at 25°C and bubbled with air containing 3% (v/v) $CO_2$. Use a light intensity of 500 $\mu E/m^2/s^1$.
7. All tips, tubes, and solutions used for manipulation of DNA should be treated in autoclave and handled with gloves to avoid degradation of DNA by nucleases.
8. Gel extraction kits are commercialized by many companies, such as Quiagen or Eppendorf. These kits allow the purification of DNA fragments up to 10 kb from agarose gels based on silica-membrane columns. Buffers for binding, washing, and elution of DNA are provided with the kit.
9. Direct digestion of PCR products can be difficult, especially when restriction sites are near the ends of the fragment. Cloning the fragment into a plasmid allows easy conservation and propagation of the obtained DNA and easy digestion and

isolation of the desired fragment. pGEMT is one of the most popular plasmids used for cloning PCR fragments. This vector is provided linearized and with 3′ terminal thymidine overhangs (3′-T) which prevent recircularization of the vector and a compatible overhang for ligation of PCR products generated by certain thermostable polymerases which often add a single deoxyadenosine to the 3′ ends of amplified fragments. The plasmid is provided in a kit with 2× rapid ligation buffer which allows reactions to be completed in 1 h at room temperature.

10. To carry out plasmid digestion incubate the DNA (up to 1 μg) with 1 unit of the desired restriction enzyme and the corresponding buffer at 37°C for 2 or 3 h.
11. Competent bacterial cells are bacteria that have gained the ability to take up extracellular DNA and can be obtained by different methods. The method that we use in our lab consists on:
    (a) Inoculate 3 mL of LB medium with *E. coli* DH5α and incubate overnight at 37°C with shaking at 200 rpm. Transfer 60-μL aliquots of the culture into new tubes with 3 mL of LB media and incubate for 2–3 h at 37°C with shaking at 200 rpm until $DO_{580}$ near to 0.5 (it is important that the optical density is not higher than 0.5).
    (b) Transfer the 6 mL (two tubes) of the cells to a sterile 15-mL polypropylene centrifuge tube, and collect the cells by centrifugation at 6,000 × *g* for 5 min. After centrifugation, decant the supernatant and resuspend gently the cell pellet in one-half of the original volume (3 mL) of cold, sterile 50 mM $CaCl_2$. Incubate in ice for 20–30 min, and centrifuge at 4°C at 6,000 × *g* for 5 min.
    (c) Decant the supernatant and gently resuspend the cell pellet in one-tenth the original volume (0.6 μL) of cold, sterile 50 mM $CaCl_2$. Keep the competents cells obtained at –80°C until their use.
12. For the transformation of competent cells, transfer 200 μL of competent cell to a nuclease-free microcentrifuge tube, and add 100 μL TCM buffer and DNA (no more than 50 ng in a volume of 10 μL). Mix the contents and incubate in ice for 20–30 min. Incubate at 43°C for 90 s. Do not shake the tubes. Add 0.7 mL of LB medium and incubate at 37°C for 30–40 min with shaking at 200 rpm. Transfer the transformed competent cells (1 mL) onto agar LB medium plates containing 100 μg/mL ampicillin. Incubate overnight at 37°C. All the process should be carried out in sterile conditions.
13. Antibiotics (ampicillin and paromomycin) are solved in water, sterilized by filtration through 0.2 μm filters, and stored at –20°C.
14. Standard cultures are grown in mineral liquid medium at 25°C, bubbled with air containing 3% (v/v) $CO_2$, and continuously

illuminated with cool white and daylight from fluorescent lamps (100 $\mu E/m^2/s^1$).

15. Additional information can be seen at http://www.chlamy.org/methods/dna.html.

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