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Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*

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Abstract

Most higher plants and microalgae are not able to synthesize ketocarotenoids. In this study the unicellular chlorophyte *Chlamydomonas reinhardtii* has been genetically engineered with the β-carotene ketolase cDNA from *Haematococcus pluvialis*, *bkt1* (GeneBank accession no. X86782), involved in the synthesis of astaxanthin, to obtain a transgenic microalga able to synthesize ketocarotenoids. The expression of *bkt1* was driven by the *Chlamydomonas* constitutive promoter of the rubisco small subunit (*RbcS2*) and the resulting protein was directed to the chloroplast by the *Chlamydomonas* transit peptide sequences of Rubisco small subunit (*RbcS2*) or Ferredoxin (*Fd*). In all transformants containing the *bkt1* gene fused to the *RbcS2* or the *Fd* transit peptides a new pigment with the typical ketocarotenoid spectrum was detected. Surprisingly this ketocarotenoid was not astaxanthin nor canthaxanthin. The ketocarotenoid was identified on the basis of its mass spectrum as 3,3′-dihydroxy-β,ε-carotene-4-one (4-keto-lutein) or its isomer ketozeaxanthin.

Keywords: Carotenoids; Food additives; *Chlamydomonas reinhardtii*; β-Carotene ketolase; Microalgae transformation; Ketocarotenoids

1. Introduction

Carotenoids are a wide group of isoprenoids present in all photosynthetic organisms and in some non-photosynthetic ones. They bind to integral proteins of the thylakoid membranes where they participate in light harvesting processes and prevent photoxidative damage of the photosynthetic apparatus (Demming-Adams et al., 1996; Baroli and Niyogi, 2000).

The synthesis of α- and β-carotene from the first uncoloured carotenoid phytoene is a universal pathway in higher plants and green algae (Fig. 1) that involves phytoene desaturase (*pds*), ζ-carotene desaturase (*zds*), carotenoid isomerase (*crtiso*) and lycopene cyclases (*lcy-ε* and *lcy-β*). The hydroxylated derivates lutein and zeaxanthin and the epoxidated violaxanthin are also found in most green tissues (Cunningham and Gantt, 1998; Botella-Pavía and Rodríguez-Concepción, 2006). Not so the keto-carotenoids, such as astaxanthin and canthaxanthin, which are only synthesized in a number of bacteria (Misawa et al., 1990, 1995), cyanobacteria (Fernández-González et al., 1997) or fungi (Johnson, 2003; Visser et al., 2003); in certain microalgae such as the chlorophytes *Haematococcus pluvialis* and *Chlorella zofigiensis* (Boussiba, 2000; Huang, 2005); and in some species of the higher plant genus *Adonis*, such as *Adonis aestibalis* (Cunningham and Gantt, 2005).

Carotenoids have been industrially exploited for a long time as natural pigments and provitamin factors. Animals and other organisms that not synthesize carotenoids *de novo* must include then in their diet to acquire their characteristic colours, this is the case of salmons, shrimps and many birds. Carotenoids are also precursors for essential compounds such as vitamin A or the visual pigment retinal. The intake of carotenoids has proved to offer protection against macular degeneration, UV-induced skin damage and some age-related degenerative diseases (Guerin et al., 2003). Recent studies suggest that their antioxidant properties and other unexpected biological functions related to gene regulation or junctional communication could provide additional health benefits, such as tumour suppressing activity (Bertram,

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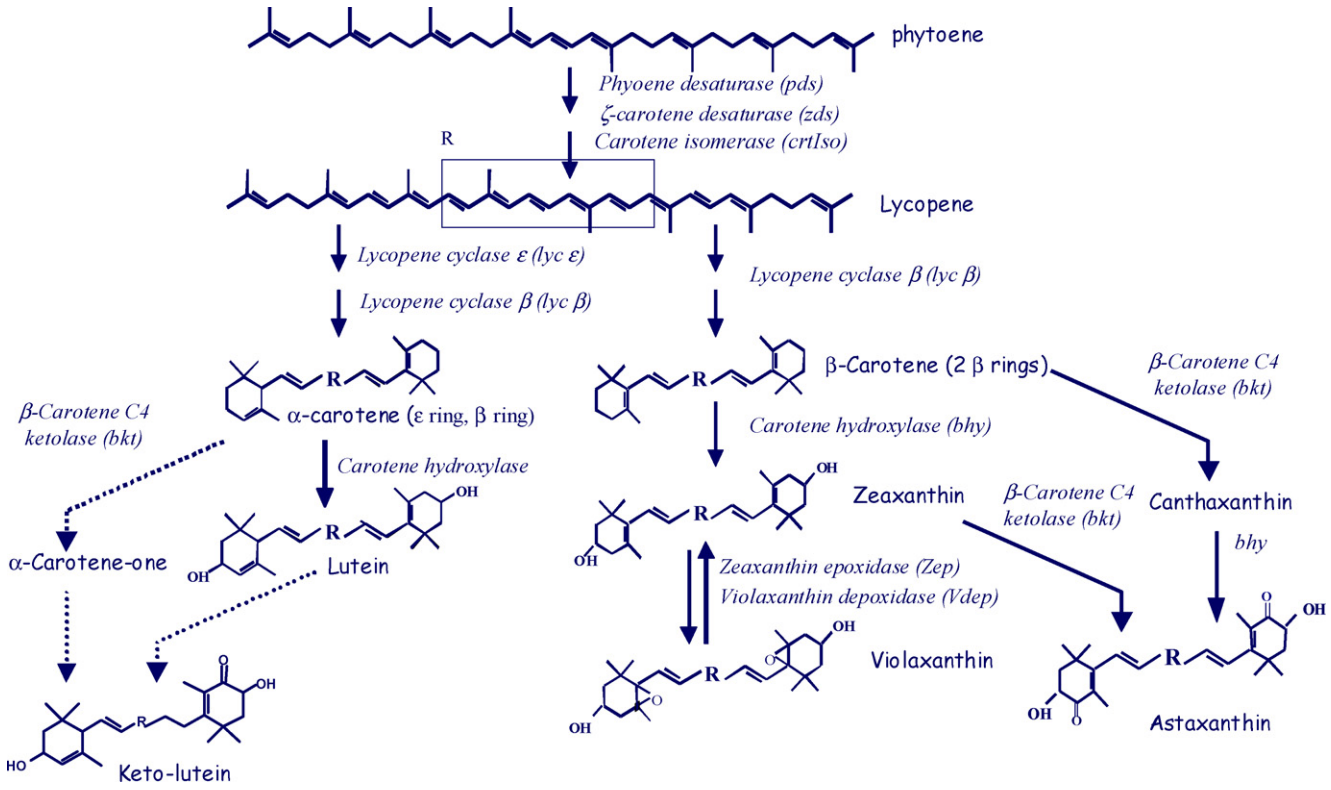

Fig. 1. Metabolic pathway for the synthesis of carotenoids and ketocarotenoids in higher plants and microalgae. *psy* (phytoene synthase); *pds* (phytoene desaturase); *Crtiso* (carotenoid isomerase); *lyc-β* (lycopene cyclase *β*); *lyc-ε* (lycopene cyclase *ε*); *bhy* (β-carotene hydroxylase); Z *ep* (Zeaxanthin epoxidase); *Vdep* (Violaxanthin deepoxidase); *bkt* (β-carotene ketolase). Dashed arrows indicate possible reactions for the synthesis of ketolutein.

1999; Demming-Adams and Adams, 2002; Stahl and Sies, 2005).

All these reasons have stimulated research to improve the nutritional value of several crop plants by the genetic manipulation of the carotenongenic pathway (Botella-Pavía and Rodríguez-Concepción, 2006; Sandmann, 2001; Giuliano et al., 2000; Sandmann et al., 2006). The genetic manipulation of the carotenogenic route in plants has not been free of difficulties but many of them have been solved thanks to creativity and hard work of different researchers. For example the low productivity of β-carotene in the endosperm of original golden rice (Ye et al., 2000) has been partially overcome by substituting daffodil by maize *psy* in the initial gene construction, this has increased total carotenoid amount in the rice endosperm from 1.6 to 37 $\mu g\ g^{-1}$ DW (Paine et al., 2005). Or the undesirable collateral effects observed in transgenic tomato overexpressing the *psy* gene (Fray et al., 1995) – low size and reduced chlorophyll content due to the alterations in the synthesis of abscisic acid and phytol from its precursor GGPP – have been circumvented by targeting the *psy* to the fruit chromoplast fusing it to the specific transient sequence (Fraser et al., 2002). Carotenoid synthesis has been also achieved in transgenic bacteria, cyanobacteria and yeast (Sandmann, 2001; Schmidt-Dannert, 2000), but the genetic manipulation of this pathway in eukaryotic microalgae has not been accomplished yet; excepting the very recent report of Steinbrenner and Sandmann (2006), who have expressed a modified *Haematococcus pds* gene with higher resistance to the herbicide norflurazon using *Haematococcus* itself as host. They propose that this modified *pds* gene can be used as reporter gene for transformants isolation in the presence of norflurazon.

Microalgae combine the fast and easy growth of bacteria and other unicellular microorganisms with an active isoprenoid metabolism that ensures enough precursors for carotenogenic pathway and adequate storage capacity. For these reasons they can be adequate hosts for heterologous genes encoding carotenoid biosynthetic enzymes that allow them to overproduce typical carotenoids or produce new ones not usually synthesized by microalgae. In addition many microalgal strains are considered GRAS (generally regarded as safe) organisms, which is useful for the production of carotenoids or carotenoids-enriched microalgae for pharmaceutical and feed applications (León et al., 2001). Unfortunately the nuclear transformation of microalgae is still limited to a small number of species (León-Bañares et al., 2004).

In most chlorophytes the levels of carotenoids and their composition is very similar to that of higher plants, but there are important exceptions, such as the halophilic green microalga *Dunaliella salina* and the freshwater one *H. pluvialis*, which are the main source of natural β-carotene and astaxanthin, respectively. In normal conditions, the carotenoids concentration in these two strains is similar to that of the rest of the photosynthetic tissues, but it can be extraordinarily increased when they are subjected to stressing conditions.

The production of the ketocarotenoid astaxanthin by *Haematococcus* is an unusual exception because most microalgae and higher plants do no posses the β-carotene ketolase (*bkt*)

activity. Ketocarotenoids, such as astaxanthin and cantaxanthin are highly demanded as feed supplements for fish aquaculture (Lorenz and Cysewski, 2000) and as nutraceutical for human nutrition (Guerin et al., 2003). Their production by *Haematococcus* at industrial scale is not free of problems—*Haematococcus* grows slowly and can be easily contaminated (Lee and Zhang, 1999); it is difficult to obtain high cell density cultures of this microalga and astaxanthin production is linked to the development of red inert aplanospores (hematocysts) (Lorenz and Cysewski, 2000). In vivo production of astaxanthin and other ketocarotenoids by organisms that do not synthesize it in a natural way has been achieved by metabolic engineering in *Escherichia coli* (Breitenbach et al., 1996; Lotan and Hirschberg, 1995), cyanobacteria (Harker and Hirschberg, 1997) and several higher plants (Ralley and Fraser, 2004; Stalberg et al., 2003; Mann et al., 2000; Gerjets and Sandmann, 2006; Morris et al., 2006). All these transgenic hosts expressed *bkt* genes from *H. pluvialis*, *crtW* genes from several strains of bacteria or CrtO gene from cyanobacteria. Expression of the enzyme β-carotene ketolase in *Dunaliella*, which can reach intracellular β-carotene concentrations of about 10% its dry weigh, should be very interesting; but despite several reports (Sun et al., 2005; Tan et al., 2005; Geng et al., 2003) its stable genetic transformation is far from being well stated.

The unicellular microalgae *Chlamydomonas reinhardtii* is the first and best studied transformed chlorophyte, grows at high rates under photoautotrophic, heterotrophic or mixotrophic conditions and its nuclear genetic manipulation is easy and well established. A great variety of transformation methods and constructions have been designed for this microalga that counts with other interesting molecular and genomic tools, such as a commercial microarray with about 10 000 unique array elements covering around 87% of known transcriptome (Eberjard et al., 2006), an EST database (http://www.chlamy.org), and a draft of the complete genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) (Harris, 2001; Grossman et al., 2003). All these reasons make of *C. reinhardtii* a good candidate to express foreign carotenogenic genes and synthesize new carotenoids, for both carrying out basic metabolic and regulatory studies of the pathway and for the biotechnological production of interesting carotenoids.

In the present work we have isolated the *bkt* gene from *H. pluvialis*, inserted it in adequate constructions including the promoter and terminator regions and chloroplast transit peptides sequences, and expressed it in *C. reihardtii*. This is the first example of manipulation of the carotenogenic pathway in eukaryotic microalgae, which can be an interesting tool to develop basic studies on the synthesis of new carotenoids in transgenic plant cells and a promising approach for the massive production of ketocarotenoids in transgenic microalgae.

2. Materials and methods

2.1. Microorganisms and standard culture conditions

H. pluvialis (SAG 192-80) was kindly provided by the culture service of the Plant Biochemistry and Photosynthesis Institute (CSIC, Seville). Standard cultures were 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 μE m^{-2} s^{-1}), unless other conditions are indicated. The composition of the liquid medium was the described by Seuoka et al. (1967) for *C. reihardtii*. *C. reinhardtii* cell-wall deficient strain 704 was kindly provided by Loppes et al. (1999) and cultured photomixotrophically in liquid or agar solidified TAP medium (Gorman and Levine, 1965) at 25 °C under continuous white light irradiation (100 μE m^{-2} s^{-1}). The *E. coli* strain used for in vivo amplification of DNA and functional complementation experiments was DH5α, cultured in LB medium as previously described (Sambrook et al., 1989) supplemented with either ampicillin (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}) or both of them.

2.2. Complementation experiments

Functional analysis of the isolated *bkt1* transcripts was carried out in a β-carotene accumulating strain of *E. coli* This bacteria produces β-carotene due to the plasmid pACCAR16ΔcrtX (Misawa et al., 1995) that contains the soil bacteria *Erwinia* sp. carotenoid biosynthetic genes, *crtE*, *crtB*, *crtI* and *crtY*, which are responsible for the conversion of farnesyl pyrophospate (FPP) to β-carotene via phytoene and lycopene and harbours the Cm^r gene that confers resistance to chloramphenicol.

2.3. RNA extraction and reverse transcription-PCR

H. pluvialis cells were subjected to nitrogen starvation and high light intensity (400 μE m^{-2} s^{-1}) to induce the synthesis of astaxanthin. Cell cultures (200 ml) were harvested by centrifugation and resuspended in 3 ml of a buffered solution containing 50 mM Tris–HCl, pH 8, 0.3 M NaCl, 5 mM EDTA and 2% SDS. Isolation of total RNA was performed by phenol–chloroform–isoamyl alcohol (50:48:2) extraction and selective precipitation with 4 M LiCl, according to previously described protocols (Schloss et al., 1984; Sambrook et al., 1989). All solutions were DEPC treated. Single strand *bkt1* cDNA was synthesized from total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen) and used as substrate for PCR reactions.

The PCR amplification was performed from 2 μl of the RT reaction mixture in a total volume of 50 μl containing 20 pmol of each primer, 0.2 mM dNTPs, 1 U pfu *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 5 μl of specific 10× buffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96 °C, 0.5 min at 42 °C, and 1.5 min at 72 °C for 30 cycles.

2.4. Genomic DNA preparation for PCR screening of transformants

One loopful of *Chlamydomonas* cells was scrapped from a plate, resuspended in 10 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3% SDS) and incubated at room temperature for 15 min. After the incubation, 500 μl of TE (10 mM Tris–HCl,

pH 8.0, 1 mM EDTA) and 60 μl of 3 M sodium acetate, pH 5.2, are added. DNA is extracted with phenol/chloroform and precipitated with isopropanol. The pellet is washed with ethanol 70%, dried and resuspended in 5 mM Tris–HCl, pH 8. The genomic DNA solution (1 μl) is used to carry out the PCR reaction as described before (see http://www.chlamy.org/methods/dna.html for more details).

2.5. Construction of expression vectors for Chlamydomonas

Plasmid pSI104PLK was obtained from plasmid pSI103 prepared by Sizova et al. (2001) that contains the *AphVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3′-phosphotransferase that confers resistance to the antibiotic paramomycin, under the control of the strong constitutive promoters *RbcS2* and *Hsp70A* and terminated by the 3′-untranslated region of RbcS2. PSI103 was cut with *Not*I and religated to eliminate unwanted restriction sites, then the *AphVIII* gene was cut with *Bst*BI and *Bam*HI and substituted by a polylinker region obtained by PCR amplification from pBluescript II KS+ (Promega). We called the obtained plasmid pSI104PLK.

The β-carotene oxygenase cDNA (*bkt1*) was amplified by RT-PCR based on the sequence (GeneBank accession no. X86782) published by Harker and Hirschberg (1997). The obtained PCR product (1.4 kb) was ligated to pGEM-T vector (Promega), sequenced and subcloned between the *Xho*I and *Pst*I restriction sites of the *Chlamydomonas* expression vector pSI104PLK.

DNA fragments encoding the desired transit peptides were synthesized by Genescript Co. (NJ, USA) and cloned in the *Eco*RV site of the multiple cloning region of the plasmid pUC57, from where they were cut, purified and fused to the *bkt1* gene to drive the encoded protein into chloroplast, as explained in Section 3.

2.6. Nuclear transformation of C. reinhardtii

Transformation was carried out using the glass-bead method of Kindle (1990) with minor modifications. *C. reihardtii* cells grown to a cell density of about 10^7 cells/ml, were harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100 fold concentrated cell suspension. The concentrated cell suspension (0.6 ml) was added to a conical tube containing 0.3 g of sterile glass beads (Ø0.4–0.6 mm), 0.2 ml of 20% polyethylene glycol 8000 and about 1 μg of the desired plasmid. Cells were vortexed for 8 s and resuspended in 50 ml of fresh sterile TAP medium where they were incubated in the dark overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP medium with paramomycin (30 μg ml^{-1}). Transformed colonies were visible after 4 or 5 days.

2.7. Pigments extraction and analysis

Carotenoids and chlorophylls were extracted with 80% acetone as previously described (León et al., 2005). The separation and chromatographic analysis of pigments was preformed in a Merck Hitachi HPLC equipped with a UV–vis detector as described by Young et al. (1997), using a RP-18 column and a flow rate of 1 ml min^{-1}. The mobile phase consisted on: solvent A, ethyl acetate; solvent B acetonitrile/water (9:1, v/v) and the gradient programme applied was: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100%. Injection volume 100 μl. Pigments detection was carried out at 450 nm. Pigments standards were supplied by SIGMA or DHI (Hoersholm, Denmark). The Ketocarotenoid was identified using an Agilent 1100 series chromatograph equipped with a diode-array and a mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA).

2.8. Dry weight determination

Dry weight was determined by filtering an exact volume of microalgae culture (30 ml) on pre-targeted glass-fiber filters (1 μm pore size). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts and dried at 100 °C for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference.

2.9. DNA analysis

Sequences were analysed using the Lasergene program (DNASTAR, Inc.) and the NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results and discussion

3.1. Isolation and functionality of β-carotene ketolase (bkt1) gene from H. pluvialis

It has been reported the existence of several classes of carotenoid ketolases. Those ketolases encoded by *CrtW* genes, found in bacteria (Misawa et al., 1990; Misawa et al., 1995) that catalyse the oxygenation of both ione-rings of beta-carotene and are very similar in functionality and sequence to the *bkt* genes found in *Haematococcus*. And *CrtO* type found in the cyanobacteria *Synechocystis* (Fernández-González et al., 1997), which asymmetrically adds one keto-group to β-carotene to form echinenone, that seems to have arisen independently as they share very little sequence similarity with the *CrtW* type. CrtW type ketolases are related to the plant (*bhy*) and bacteria (*CrtZ*) beta-ring hydroxylases. They all are members of a large class of membrane-integral, di-iron oxygenase enzymes (Cunningham and Gantt, 1998). Recently a gen coding a multifunctional enzyme with both 4-ketolase and 3-hydroxylase activities has been cloned in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) (Ojima et al., 2006), confirming the relation between these two genes.

Originally two different carotene β-ione ring ketolase cDNAs were isolated from two different strains of *H. pluvialis* grown in different induction conditions, *bkt1* (GeneBank accession no. X86782), originally named as *CrtO* (Lotan and Hirschberg, 1995; Harker and Hirschberg, 1997) and *bkt2* (GeneBank accession no. D45881) (Kajiwara et al., 1995). The genomic sequences DQ086233, that generates the *bkt1* ARNm, and

Table 1
Comparison of codon usage of several beta-carotene ketolases encoded by genes from different sources with the usual codon usage in *Chlamydomonas reinhardtii*

Gene (GeneBank accession no.)	Organism	Reference	Difference (%)
Bkt1 (X86782)	*Haematococcus pluvialis*	Harker and Hirschberg (1997)	11.09
Bkt2 (D45881)	*H. pluvialis*	Kajiwara et al. (1995)	15.95
Bkt3 (AY603347)	*H. pluvialis*	Huang et al. (2006)	16.08
Bkt4 (DQ257290)	*H. pluvialis*		16.13
CrtW (D58420)	*Agrobacterium aurantiacum* (*Paracoccus* sp. *N81106*)	Misawa et al. (1995)	18.48
CrtW (D58422)	*Alcaligenenis PCI* (*Paracoccus PC1*)	Misawa et al. (1995)	17.45
CrtO (BA000022)	*Synechocystis PCC 6803*	Fernández-González et al. (1997)	26.14

The percentage corresponds to the mean difference between codon usages of the indicated gene and the genome of *C. reinhardtii*. Codon usage comparison was performed using Graphical codon usage analyser v. 2.0 (http://gcua.schoedl.de/index.html). *Chlamydomonas* codon usage was calculated from 602 genes.

AF534876 which transcripted sequence is very similar to *bkt2*, were isolated and deposited in the NCBI databank in 2005 and 2002, respectively. Other versions of *bkt* cDNA deposited in the gene bank database since then are highly homologous to bkt2 (GeneBank accession nos. AY603347; DQ257290). A Recent study (Huang et al., 2006) demonstrates the presence of at least three of the *bkt* transcripts in the same *H. pluvialis* strain. They propose the existence of multiple *bkt* genes in *H. pluvialis*, that are up-regulated by different stress conditions. Different *bkt* genes can also have different degrees of affinity for different substrates, such as β-carotene and zeaxanthin.

We chose *bkt1* gene from *H. pluvialis* to express β-carotene ketolase activity in *C. reinhardtii* due to the close phylogenetic relationship between both green microalgae and after analysing the codon usage of several *bkt*/*crtW* genes. The mean difference between the codons present in *bkt1* gene and in *Chlamydomonas* genome was only 11.09%, this difference was increased to 18.48% for the beta-carotene ketolase of *Agrobacterium aurantiacum* and to 26.14% in the case of beta-carotene ketolase from *Synechocystis* (Table 1). Codon usage comparison was performed using Graphical codon usage analyser v. 2.0 (http://gcua.schoedl.de/index.html). *C. reinhardtii* codon usage was calculated from 602 genes.

A 1.4 kb cDNA fragment was obtained by RT-PCR from total mRNA of *H. pluvialis* (SAG-192-80). This mRNA was isolated from cells grown during 48 h without nitrogen source and at high light intensity (400 $\mu E\,m^{-2}\,s^{-1}$), conditions that have been reported to stimulate the synthesis of astaxanthin (Boussiba et al., 1999; Boussiba, 2000) and to induce the expression of carotenogenic genes (Eom et al., 2005; Steinbrenner and Linden, 2003). The forward (5′-TGCCGCTCGAGAGCCTCAAATAA-3′) and reverse primers (5′-CACTCCTGCAGACGCAAGACATC-3′) were based on the sequence published by Harker and Hirschberg (1997) and were designed to contain *Xho*I and *Pst*I restriction sites, respectively (underlined). Sequencing of the obtained product confirmed that it was *bkt1* cDNA (GeneBank accession no. X86782), following the nomenclature proposed by Huang et al. (2006). Functionality of the isolated cDNA was checked by complementation experiments in a β-carotene accumulating bacterium. This *bkt1* cDNA was subcloned into a bacterial expression vector pQE80 (Qiagen), introduced in *E. coli* cells that carried the plasmid pACCAR16ΔcrtX (Misawa et al., 1995) responsible for the synthesis of β-carotene and its expression induced with IPTG (1 mM, 12 h). Expression of BKT1 protein in β-carotene accumulating *E. coli* resulted in the production of canthaxanthin with trace amounts of the monoketolated intermediate echinenone (Fig. 2A) confirming the functionality of the obtained cDNA.

3.2. Fusion of the β-carotene ketolase (bkt1) gene with DNA sequences encoding different transit peptides

The obtained PCR product was then subcloned between the *Xho*I and *Pst*I restriction sites of the *Chlamydomonas* expression vector pSI104PLK. This plasmid, obtained as described in Section 2.5, contains the HSP70A and RbcS2 promoters and the RbcS2 3′-untranslated terminator region of *Chlamydomonas* (Schroda et al., 2000). *C. reinhardtii* was co-transformed by this new construction, that we called pSI104PLK-bkt1, and the plasmid pSI103 prepared by Sizova et al. (2001) that contains the *AphVIII* gene from *S. rimosus*, coding for an aminoglycoside 3′-phosphotransferase that confers resistance to the antibiotic paramomycin. Transformants were first selected on the basis of paramomycin resistance and submitted to a screening with genomic DNA PCR to test the insertion of *bkt1* cDNA in the algal genome. About 40% of the transformants contained both *aphVIII* and *bkt1* genes. But none of the transformants that had the *btk1* gene adequately inserted in its genome was able to produce ketocarotenoids.

Carotenoids synthesis occurs within the chloroplast and is catalysed by enzymes encoded by nuclear genes. These enzymes are thus synthesized in the cytoplasm as precursor polypeptides with an amino-terminal extension, the transit peptide (tp), that targets them to their final location in the chloroplast. Rubisco small subunit (RbcS2) and Ferredoxin (Fd) are targeted to the stroma and the interthylakoid lumen of the chloroplasts, respectively. The transit sequences of the *Chlamydomonas* Rubisco and of the intra-thylakoid lumen protein ferredoxin are peptides with 32 amino acids, well known at both primary sequence and structural levels (Roesler and Ogren, 1990; Krimm et al., 1999). Detailed sequences of each transit peptide are shown in Fig. 3.

The DNA sequences encoding these two transit peptides flanked by the *Bst*BI and *Xho*I restriction sites (Fig. 3) were synthesized and fused in the same reading frame to *bkt1*, previously inserted in the polylinker region of the plasmid pSI104PLK. The structure of this construction carrying the *bkt1* gene and the corresponding transit peptides are detailed in Fig. 3. *C. reinhardtii* cells were cotransformed with plasmid pSI104PLK-tpbkt1 that

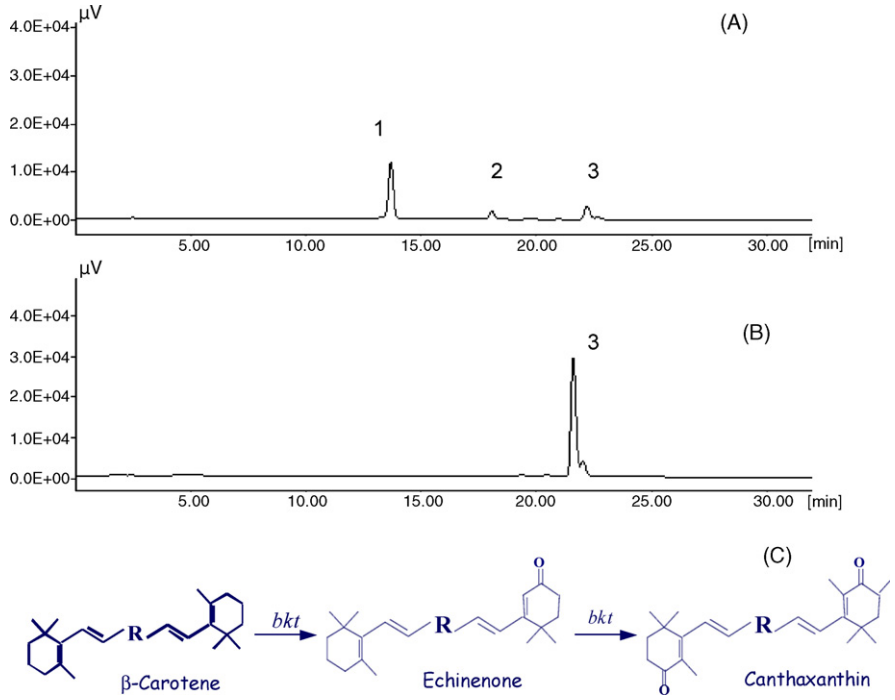

Fig. 2. Chromatographic analysis of pigment extracts from *Escherichia coli* transformed with the plasmid pACCAR16ΔcrtX (responsible for the accumulation of β-carotene) (B) and cotransformed with the plasmids pACCAR16ΔcrtX + pQE80-bkt1 (A) *E. coli* cells transformed with the indicated plasmids were isolated in the presence or chloramphenicol + ampicillin (A) or chloramphenicol (B), grown in LB liquid medium until a OD_{600} of 0.5–0.7 and incubated for 12 h in the presence of IPTG (1 mM). The cells were then harvested by centrifugation and their pigments extracted with acetone and analysed by HPLC. Peaks identification: (1) canthaxanthin; (2) echinenone; (3) β-carotene. β-Carotene ketolase catalyses the transformation of β-carotene to canthaxanthin via the intermediate echinenone, as shown in panel (C).

harbours the described construction and the plasmid pSI103 (Sizova et al., 2001). Transformants were newly selected by their resistance to paramomycin and by testing the insertion of *bkt1* cDNA sequence in their genomes by PCR, as detailed in Section 2.4.

3.3. Screening of bkt1 transformed C. reinhardtii

One hundred colonies (50 for each of the two transit peptides used) obtained after cotransformation with the plasmids pSI104-tp *bkt1* and pSI103 that showed resistance to paramomycine were screened by PCR to confirm integration in their genome of the *bkt1* gene fused to *rbcS2* and *Fd* transit peptides, respectively (Fig. 4). About 40% of the microalgae transformed with the construction that contained the *rbcS2* transit peptide and 31% of those transformed with the construction that contained the *Fd* one had integrated the foreign construction in their genomes. The primers designed for PCR analysis of the *rbcS2*:*tp*:*bkt1* constructions were: AGCGGTGCCCTCCTGATAAAC (forward) and TTCCGGTAAGCTGCTCCAACAT (reverse) that anneal with the *RbcS2* promoter and the *bkt1* gene, respectively, and amplify a fragment of 547 bp (Fig. 4A).

The positive co-transformants selected by PCR were grown in TAP liquid medium transferred to nitrogen-free medium where they were incubated during 48 h and analysed for their pigments content by HPLC. In all the transformants bearing the *bkt1*

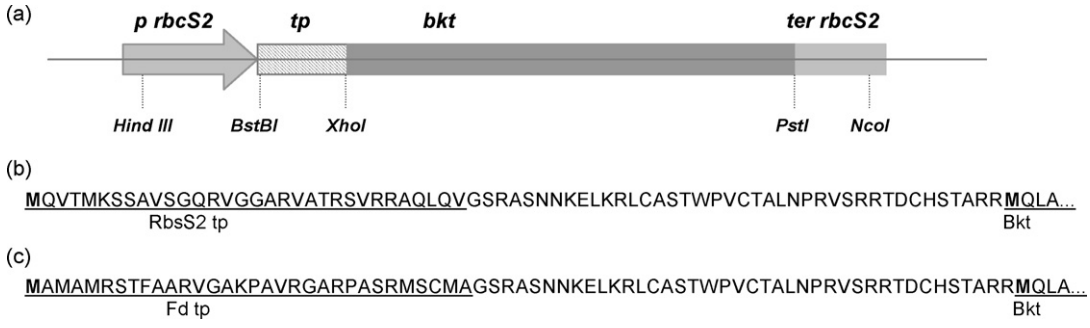

Fig. 3. (a) Schematic representation of the construction used to express the *bkt1* gene in *Chlamydomonas*. *p rbcS2*, ribulose 1,5 biphosphate carboxylase small subunit promoter; *ter rbcS2*, ribulose 1,5 biphosphate carboxylase small subunit terminator region; *pHSP70A*, heat shock protein 70A promoter; *tp*, transit peptide; *bkt*, β-carotene ketolase. Detailed sequence of the constructions containing rubisco (b) and ferredoxin (c) transit peptides are also shown. The sequences corresponding to the transit peptides and to the beginning of the *bkt1* product are underlined. The whole construction has 2550 bp.

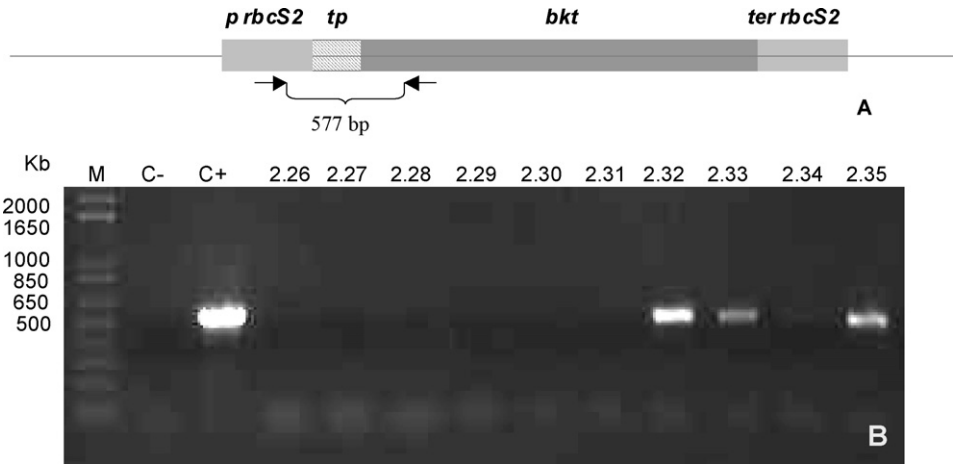

Fig. 4. PCR to screen the insertion of the constructions rbcS2:tp:bkt1 in the genome of *C. reihardtii*. PCR primers were specifically designed to detect rbcS2:tp:bkt1 constructions (indicated with arrows in panel A), being the transit peptide either RbcS2 or Fd corresponding transit peptides. In panel B an example of screening over 10 paramomycin resistant colonies isolated after cotransformation with pSI104PLK:Fdtp:bkt1 and pSI103 is shown. The M lane is the1Kb plus DNA ladder. Transformants 2.32, 2.33 and 2.34 showed a band of 577 bp and correspond to positive transformants with the desired construction inserted in their genomes.

cDNA fused to either r *bcS2* or *Fd* transit peptides, the presence of a new pigment with the typical ketocarotenoid spectrum was detected. Both *RbcS2* and *Fd* transit peptides seemed to be equally effective to target *bkt1* gene to its final location in the chloroplast. *Bkt1* preceded by either of these tp are probably directed to the chloroplast and finally integrated into the thylakoid membrane due to its own structure in which several intramembrane helices are present (Cunningham and Gantt, 1998). There are many cases where it has been shown that the ultimate location of proteins produced by exogenous genes is determined by its own structural information (Misawa et al., 1993).

3.4. Carotenoids analysis of parental and bkt1-transformant strains of Chlamydomonas

The main carotenoids found in *C. reinhardtii* cells subjected to nitrogen starvation during 48 h were lutein and β-carotene followed by violaxanthin (Fig. 5). The carotenoid profile of parental and transgenic *Chlamydomonas* were very similar. The most significant difference is the presence of a small new peak, with the typical ketocarotenoid absorption spectrum and a retention time of about 8.4 min, in the extracts of bkt1-transformats (Fig. 5). This retention time is lower than that of the pigments canthaxathin (RT ≈ 13.2 min) or astaxanthin (RT ≈ 12.7 min). HPLC-MS analysis of the pigment extracts showed a mass spectra for the new ketocarotenoid with a major peak at *m/z* 583.4 (*M* + H). This means that the ketocarotenoid formed in transgenic *Chlamydomonas* is 4-keto-lutein or its isomer 4-keto-zeaxanthin. Lutein is the most abundant carotenoid in *Chlamydomonas* and has only one β-type ring susceptible of being oxygenated by the ketolase, while zeaxanthin is only present in minor quantities, and in the case it were substrate for the ketolase it would be expected that its both β-ione rings were oxygenated to yield astaxanthin, so this new peak correspond very probably to the unusual ketocarotenoid 4-keto-lutein. The content of the ketocarotenoid was about 0.75% of the lutein content of the cells, this means about 20 μg g^{-1} DW. No *esterified* forms of the ketocaotenoid were found.

There are not many data about the ability of 4-β-ketolases to oxygenate the β-ione-ring of α-carotene or lutein to produce 4-ketolutein, also called α-doradexanthin. It has been found as a minor carotenoid in the red tilefish *Branchiostegus japonicus* (Tsushima and Matsuno, 1998), and in transgenic *Arabidopsis thaliana* expressing the *bkt gene of H. pluvialis* (Stalberg et al., 2003). Here we have also shown that *C. reinhardtii* cells transformed by *bkt1* cDNA from *H. pluvialis* synthesize ketolutein or its isomer keto-zeaxanthin and no astaxanthin or cantaxanthin. The levels of ketolutein in transformant seeds of *Arabidopsis* (Stalberg et al., 2003) were, as we have observed in *Chlamydomonas*, very low. Only in plants obtained by crossings *bkt* transformants with phytoene synthase overexpressing plants, considerable amounts of ketocarotenoids were observed. The content of total ketocarotenoids obtained in transgenic tobacco or tomato leafs (Ralley and Fraser, 2004) was also very low, not higher than 2% of the total carotenoids. On the other hand, in transgenic tobacco flowers expressing *H. pluvialis bkt1* gene targeted to the chloroplast by the transit peptide of tomato *pds* (Mann et al., 2000) about 50% of the total carotenoids were ketocarotenoids, which means a much more efficient transformation of carotenoids to the corresponding keto-carotenoids by the recombinant ketolase.

Stalberg et al. (2003) proposed that limitations on the availability of substrates more than low activity of the enzyme could be the cause for such low quantities of ketocarotenoids obtained in transgenic seeds of *Arabidopsis*. Ralley and Fraser (2004) also propose lack of substrate accessibility in chromoplasts and chloroplasts of certain plant tissues as the possible cause for poor metabolic conversion of carotenoids by ketolases genes. Cunningham and Gantt (1998) have postulated the existence of multisubunit enzyme aggregates associated with the plastid membranes of plant cells where the carotenogenic enzymes should be integrated. Phytoene, the product of soluble phytoene synthase should be the initial substrate for these complexes,

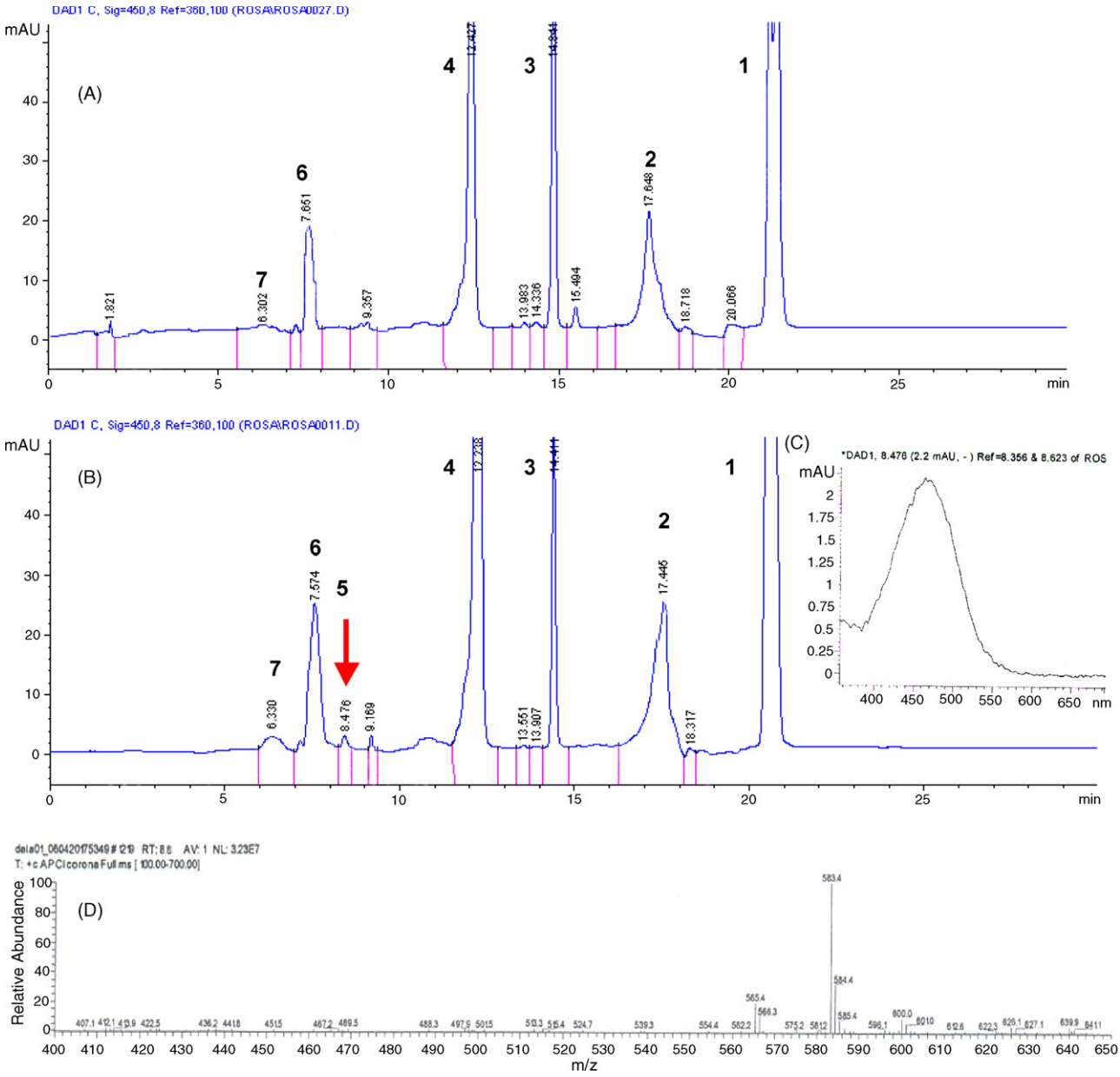

Fig. 5. HPLC profiles of carotenoids present in acetone extracts from control non-transformed (A) and from transgenic *Chlamydomonas* cells (transformant 2.35) containing the *H. pluvialis bkt1* cDNA fused to ferredoxin transit peptide (B). The arrow indicates the new pigment present in the transformed cells. The absorption spectra (C) and the mass spectra (D) corresponding to the new peak were obtained by a diode array detector and a mass spectrometer detector, respectively. Peaks identification: (1) β-carotene; (2) chlorophyll *a*; (3) chlorophyll *b*; (4) lutein; (5) keto-lutein; (6) violaxanthin; (7) neoxanthin.

through which the rest of the carotenoids would be channeled. This postulate was previously proposed to operate in fungus (Candau et al., 1991) and could explain the low activity observed for exogenous carotenogenic genes introduced by genetic engineering in plant cells. If the foreign ketolase enzyme is expressed and directed to the thylakoid membrane, but is not integrated in the carotenogenic complexes, it will have poor access to its substrates. This could explain higher levels of ketocarotenoides observed by Mann et al. (2000) in transgenic tobacco flowers were the *bkt* enzyme was directed to the chloroplast by the transit peptide of other carotenogenic enzyme, phytoene desaturase (*pds*), and presumably integrated in these carotenogenic complexes. Substrates for carotenogenic enzymes are also integrated in functional ordered structures, the photosystems, which contain hundreds of chlorophyll and carotenoid molecules bounded to integral membrane proteins. Several reports propose that in most photosynthetic tissues carotenes are preferentially ligated to the reaction centres of photosystems, while xanthophylls are more usually found in the light harvesting complexes and consequently are more accessible than carotenes (Grossman et al., 2004), nevertheless hydroxylated xanthophylls seem to be not as good substrates to *bkt1* as carotenes (Lotan and Hirschberg, 1995). This could explain the low levels of keto derivates observed in many transformants systems.

The present work corroborates that carotenoid ketolases can act in vivo over other substrates than β-carotene, such

as the one-β-ione-ring lutein or α-carotene, to synthesize new ketocarotenoids such as keto-lutein. Conversion yields are nevertheless very low in most cases. The rigid organization of carotenoids and carotenogenic enzymes in the thylakoid membrane can influence the low conversions achieved by exogenous carotenogenic enzymes produced by recombinant genes. Strategies such as overexpressing endogenous enzymes of the initial steps of the route or directing the *bkt1* gene to the chloroplast by transit peptides of carotenogenic enzymes, such as phytoene desaturase, have allowed to increase the yield of ketocarotenoids production in *Arabidopsis* seeds (Stalberg et al., 2003) and tobacco (Mann et al., 2000). It is interesting to know whether these strategies might also work in *Chlamydomonas* and other plant species. This is the first example of a microalga expressing an exogenous carotenogenic enzyme.

Although the presence of ketocarotenoids in *Chlamydomonas* has never been reported, a gene coding a protein with high homology to the ketolases from *H. pluvialis* has been surprisingly found in a comparative genome study of chlorophyll and carotenoid biosynthetic metabolic pathways in *Chlamydomonas* (Lohr et al., 2005). Lohr et al. (2005) did not detect ketocarotenoids neither in control nor in nutrient limited cultures of *Chlamydomonas*. Our attempts to detect ketocarotenoids in wild *Chlamydomonas* pigment extracts also corroborate these observations. The possible function of this gene in a non-ketocarotenoid-producing microalga is not clear. Grossman et al. (2004) propose the possibility that this gene product had acquired a different catalytic function.

4. Conclusions

We have shown that the expression of exogenous carotenogenic genes in *C. reinhardtii* and the production of new carotenoids not synthesized by the parental strains is feasible. Nevertheless there are limits to obtain high yield productions. Not only in microalgae, also in higher plants, our limited knowledge of the mechanisms and signals that control carotenoids biosynthesis, modification and storage is the main challenge to obtain higher levels of carotenoids through metabolic engineering of the carotenoid biosynthetic pathway.

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