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**Short Communication**

# Cloning and Characterization of β-Carotene Ketolase Gene Promoter in *Haematococcus pluvialis*

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**Abstract** The unicellular green alga *Haematococcus pluvialis* accumulates a highly valuable ketocarotenoid, astaxanthin, under various environmental stresses. β-carotene ketolase (BKT) plays a key role in astaxanthin biosynthesis in *H. pluvialis*. In this paper, an approximate 700 bp 5′-flanking region of the *bkt* gene containing a putative promoter was cloned through walking upstream. The results of the sequence analysis showed that this *bkt* 5′-flanking region might have *cis*-acting elements such as sterol regulatory element (SRE-1)-like motifs, the C-repeat/dehydration responsive element (DRE) and *al-3* proximal element (APE)-like motifs, except for typical TATA and CCAAT boxes. The results of the β-galactosidase assay and the transient expression of *lac*Z driven by a series of sequential deletions revealed that a minimal promoter-like region might exist from –630 to –408 bp, and the highest promoter activity was observed to span the positions from –630 to –308 bp. The results of the site-directed mutagenesis of a C-repeat/DRE and two APE-like motifs in a promoter-like region (–630 to –308 bp) suggested that two APE-like motifs might be essential for transcriptional control of the *bkt* gene.

**Key words** *Haematococcus pluvialis*; astaxanthin; β-carotene ketolase gene (*bkt*); *cis*-acting element; promoter

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), a type of red ketocarotenoid, is of great commercial interest [1] because of its high price (approximately US$2500/kg) [2]. There is an expanding market for astaxanthin. It is not only used as feed supplement in marine fish aquaculture and as a pigment source for egg yolks, but it also has potential clinical applications in human health because of its higher antioxidant capacity compared to β-carotene and vitamin E [3,4]. The unicellular freshwater green alga *Haematococcus pluvialis* reveals the highest astaxanthin accumulation (up to 4% by dry weight) when exposed to unfavorable growth conditions such as high light intensity, nitrogen and phosphate limitations and salt stress, and it seems to be the most suitable microbial source for the commercial production of natural astaxanthin [5–7].

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The genes involved in astaxanthin biosynthesis in *H. pluvialis* have been well documented [8–12]. β-carotene ketolase (BKT), which is responsible for the conversion from β-carotene to canthaxanthin and from zeaxanthin to astaxanthin, plays a key role in astaxanthin biosynthesis in *H. pluvialis*. Previous studies have revealed that the regulation of *bkt* expression is at a transcriptional level, at least partially [12,13]. However, much is still unknown about the regulatory mechanisms of *bkt* gene expression.

This paper reports on the characterization of a promoter-like region (–630 to –308 bp) by cloning, sequence analysis and functional verification of the *bkt* 5′-flanking region of about 700 bp.

## Experimental Procedures

The *bkt* 5′-flanking region was cloned as follows. A

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Universal GenomeWalker kit (Clontech, California, USA) was used. According to the manufacturer's instructions, the total genomic DNA of *H. Pluvialis*, which was extracted using a Plant genomic DNA mini kit from Huashun Company (Shanghai, China), was separately digested with the restriction endonucleases *Dra*I, *Eco*RV, *Pvu*II and *Stu*I, and the products were ligated to a GenomeWalker adaptor respectively before being used as the templates for nested polymerase chain reaction (PCR). The adaptor primer AP1 (5′-GTA-ATACGACTCACTATAGGGC-3′) in the kit and one *bkt* gene-specific primer GSP1 (5′-CTTTAGCGCAGG-ACGAGCTGCGTCTGA-3′) that was designed based on the data from GenBank No. D45881 were used in the primary PCR amplification. Furthermore, the other adaptor primer AP2 (5′-ACTATAGGGCACGCGTGGT-3′) and the other nested *bkt* gene-specific primer GSP2 (5′-AGGCAGGCCCCGACGCGAGATTTGGCA-3′) that was designed based on the same data as GSP1 were used in the secondary PCR amplification. The nested PCR product was cloned into a pMD 18-T vector (TaKaRa, Tokyo, Japan) and sequenced by CA Sarray Company Limited (Shanghai, China). Moreover, computer softwares (BLAST 2.0 and CLUSTAL 1.8) [14] and Internet tool (http://oberon.fvms.ugent.be:8080/PlantCARE/) [15] were used to perform the sequence analysis, including the prediction of possible *cis*-acting elements.

After sequence analysis, a series of deletion constructs, termed as p*bkt-5* (–630 to –1 bp), p*bkt-4* (–630 to –111 bp), p*bkt-3* (–630 to –308 bp), p*bkt-2* (–630 to –408 bp) and p*bkt-1* (–630 to –508 bp), were separately generated by inserting unidirectional cloning of PCR fragments into the *Xho*I and *Hind*III sites of the pβgal-Basic vector (Clontech, California, USA), which lacks the promoter region needed to drive the expression of the *lacZ* (encoding β-galactosidase) reporter gene. The *H. pluvialis* genomic DNA was used as the template. Moreover, a C-repeat/dehydration responsive element (DRE) site (–566 to –562 bp, named Mutation-1) and two *al-3* proximal element (APE)-like sites (–561 to –551 bp, named Mutation-2, and –450 to –441 bp, named Mutation-3) were mutated according to the instructions provided in a TaKaRa MutanBest kit (TaKaRa, Tokyo, Japan). The mutation nucleotides were indicated in lowercase as follows: 5′-AAATGTaatAC-3′ (Mutation-1), 5′-aagGTGTTGTA-3′ (Mutation-2); and 3′-CGaggGCATTGC-5′ (Mutation-3). All of the generated constructs were confirmed by sequencing.

The *H. pluvialis* (strain 712) was kindly provided by Prof. Jianguo LIU from the Institute of Oceanology, Chinese Academy of Sciences (Qingdao, China). For the transient β-galactosidase assay, $5\times10^6$ motile *H. pluvialis* cells were plated on sterile filter paper in a 60-mm-diameter dish and transformed with the recombinant constructs using the micro-particle bombardment method [16]. Forty-eight hours after transformation, the expression product, that is, β-galactosidase, was detected by *in situ* histochemical staining using X-gal (5-bromo-4-chloro-3-indolyl-β-*D*-galactoside) (Sangon, Shanghai, China) as the substrate, which turns blue in the presence of β-galactosidase. At the same time, the quantitative β-galactosidase assay was performed using a β-galactosidase enzyme assay system (Promega, California, USA) according to the manufacturer's instructions. The cells that were not bombarded served as the blank control, and the cells that were bombarded using the empty pβgal-Basic vector served as the negative control. Furthermore, the cells that were bombarded using the pβgal-Control vector (Clontech, California, USA) with an SV40 promoter-enhancer to drive *lacZ* expression served as the positive control. The relative value of the β-galactosidase activity was calculated by setting the value of the negative control as 1.0. All of the transformation experiments were carried out in triplicate.

## Results and Discussion

Using the genome-walking method, an approximate 700 bp fragment (GenBank accession No. AY334016) was shown to be part of the *bkt* 5′-flanking region (**Fig. 1**), according to the results of BLAST. In addition, a 160 bp fragment (–272 to –113 bp) was firstly reported in our research according to the results of alignment between two 5′-end sequences from GenBank No. AY334016 (strain 712) and No. D45881 (strain NIES-144) because of the genetic varieties among experimental algae (**Fig. 2**). Furthermore, this *bkt* 5′-flanking region has neither typical TATA nor CCAAT boxes, but has potential regulatory elements, such as four SRE-1-like motifs at –609 bp, –573 bp, –369 bp and –355 bp, respectively, a C-repeat/DRE motif at –562 bp, and two APE-1-like motifs at –551 bp and –441 bp, respectively (**Fig. 1**). Previous studies have reported that the SRE-1 motif, characterized by a short C-enriched sequence 5′-CACCCCAC-3′, is a type of *cis*-acting element associated with the carotene biosynthesis related gene *cbr* in *Dunaliella bardawilin* [17]. The DRE 5′-TACCGACAT-3′ was firstly identified in the promoter of the drought-responsive gene *rd29A* from *Arabidopsis* [18]. Similarly, a *cis*-element with a similar

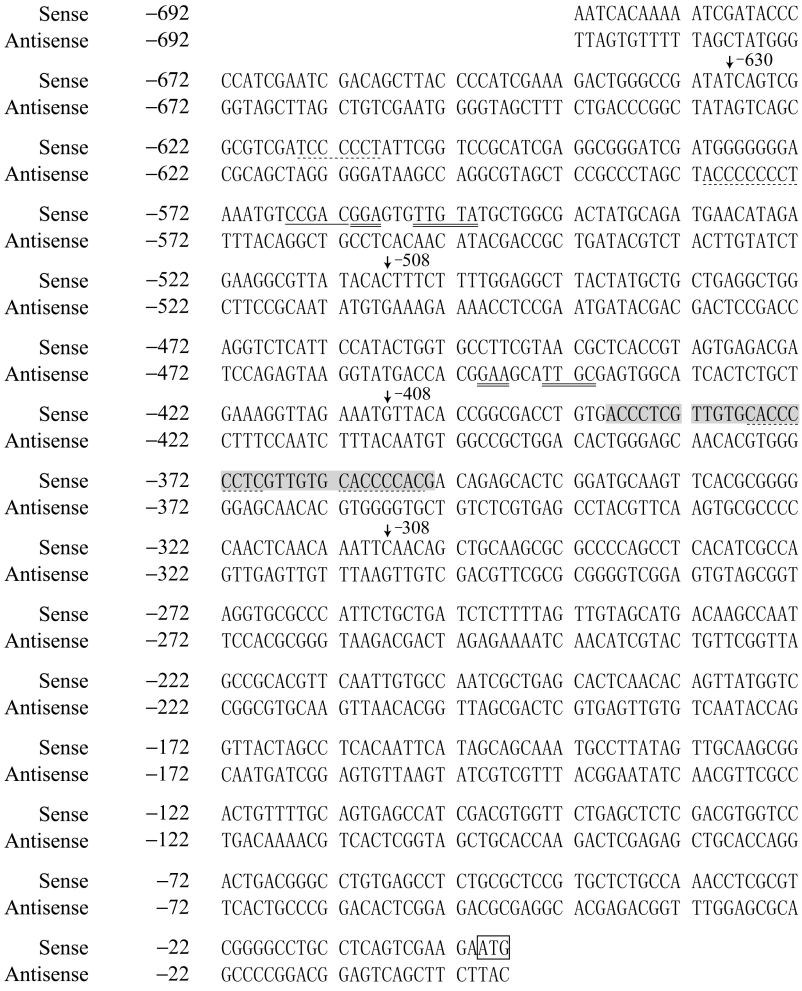


**Fig. 1 Nucleotide sequence of part of the *bkt* 5′-flanking region**

The putative C-repeat/DRE is marked with a single underline, APE-like motifs are marked with double-underlines and the putative C-enriched SRE-1-like motifs are marked with dotted underlines. The shaded sequences are short repeats and the arrows indicate the deletion mutant regions. The start codon ATG is boxed, and the information about the ATG start codon is derived from the data in GenBank No. D45881.

sequence to the DRE, the C-repeat/DRE 5′-TGGCCGAC-3′ (containing the core 5′-CCGAC-3′), was identified in the promoter of the cold-inducible gene *cor15a* from *Arabidopsis* and proved to play a role in its response to low temperature [19]. However, there has been little research on the role of the C-repeat/DRE in other algae. The APE was firstly revealed to be located in the promoter of the *albino-3* gene encoding geranylgeranyl diphosphate synthase (GGPP) in *Neurospora crassa*, and proved to be up-regulated by blue light [20]. Results of recent studies have also shown that several APE-like motifs participate in the blue light regulation of the expression of *carB* (phytoene dehydrogenase) and *carG* (geranyl pyrophosphate synthase) in *Mucor circinelloides* [21,22]. It has also been reported that only illumination with blue light, and not red light, is effective in elevating the transcript levels of the phytoene synthase gene *psy* and the phytoene desaturase gene *pds* in the unicellular green alga *Chlamydomonas reinhardtii* [23]. Most importantly, increased transcript levels for the genes of carotenoid biosynthesis related enzymes (lycopene cyclase, phytoene synthase, phytoene desaturase and carotenoid hydroxylase) have been detected under both blue and red light conditions in *H. pluvialis* [24].

To identify the important regions regulating the *bkt* transcription, sequential deletions were generated in the *bkt* 5′-flanking region from –630 to –1 bp, and then fused to the pβgal-Basic vector accordingly. From our previous studies, the β-galactosidase activity in transformed *H. pluvialis* motile cells reached a climax at 48 h post-trans-

```
seq1                                                        CGGGG
seq2                                                        CGGGG
con                                                         *****

seq1  CAACTCAACA AATTCAACAG CTGCAAGCGC GCCCCAGCCT CACATCGCCA
seq2  CAACTCAAGA AATTCAACAG CTGCAAGCGC GCCCCAGCCT CACAGCGCCA
con   ******** * ********** ********** ********** **** *****
                ↓-272
seq1  AGGTGCGCCC ATTCTGCTGA TCTCTTTTAG TTGTAGCATG ACAAGCCAAT
seq2  ---------- ---------- ---------- ---------- ----------
con

seq1  GCCGCACGTT CAATTGTGCC AATCGCTGAG CACTCAACAC AGTTATGGTC
seq2  ---------- ---------- ---------- ---------- ----------
con

seq1  GTTACTAGCC TCACAATTCA TAGCAGCAAA TGCCTTATAG TTGCAAGCGG
seq2  ---------- ---------- ---------- ---------- ----------
con
                ↓-113
seq1  ACTGTTTTGC AGTGAGCCAT CGACGTGGTT GTGAGCGCTC GACGTGGTCC
seq2  ---------- AGTGAGCTAT CGACGTGGTT CTGAGCTCTC GACGTGGTCC
con             ******* ** **********  ***** *** **********

seq1  ACTGACGGGC CTGTGAGCCT CTGCGCTCCG TGCTCTGCCA AATCTCGCGT
seq2  ACTGACGGGC CTGTGAGCCT CTGCGCTCCG TCCTCTGCCA AACCTCGCGT
con   ********** ********** ********** * ******** ** *******

seq1  CGGGGCCTGC CTAAGTCGAA GAATG
seq2  CGGGGCCTGC CTCAGTCGAA GAATG
con   ********** ** ******** *****
```

**Fig. 2 Alignment of partial of the 5′-end sequence between two different strains using CLUSTAL 1.8**

"seq1" represents partial of the data based on GenBank accession No. AY334016 (strain 712) while "seq2" represents partial of the data based on GenBank accession No. D45881 (strain NIES-144). The arrows indicate the fragment (–272 to –113 bp).

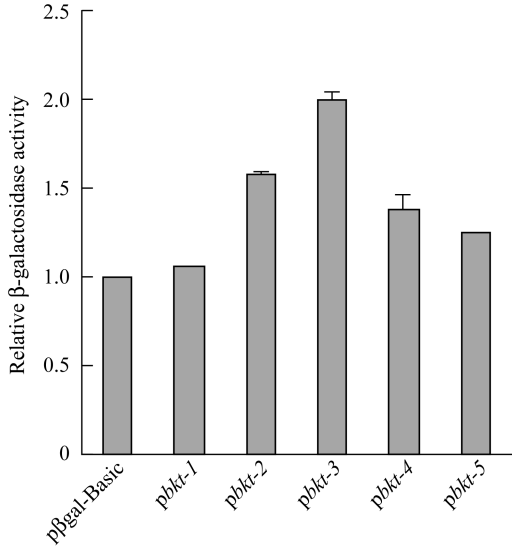


**Fig. 3 Deletion mutants of the *bkt* 5′-flanking region**

The sequential deletions were generated using PCR. *H. pluvialis* motile cells were transformed with the different reporter gene constructs together with the pβgal-Basic vector, which served as a negative control. The β-galactosidase activity of each sample was subsequently compared with the mean value of the empty pβgal-Basic vector to acquire the fold induction of each construct. The data are shown as mean±SD ($n$=3).

formation (data not shown). Therefore, these deleted constructs were transformed into *H. pluvialis* motile cells and their β-galactosidase activities were determined respectively 48 h after transformation.

**Fig. 3** shows that, among the various deletion constructs, the highest β-galactosidase activity was observed in p*bkt-3* (–630 to –308 bp) transformed cells, which was double that of the negative control, whereas the p*bkt-2* (–630 to –408 bp) transformation resulted in a β-galactosidase activity 1.5 times that of the negative control. Consequently, the short repeats in the shaded region in **Fig. 1**, including two C-enriched sequences CACCCC–(C)A(T)C from –408 to –308 bp, were supposed to enhance the promoter activity to a certain extent. However, the p*bkt-1* (–630 to –508 bp) transformation expressed nearly the same β-galactosidase activity as that of the negative control, suggesting that a minimal promoter-like region might lie somewhere from –630 to –408 bp. Furthermore, the p*bkt-4* (–630 to –111 bp) and p*bkt-5* (–630 to –1 bp) transformations resulted in a β-galactosidase activity that was 1.25 times that of the negative control, suggesting that some negative elements might exist in the region from –308 to –1 bp.

The results of the histo-chemical staining showed that the p*bkt-2*, p*bkt-3*, p*bkt-4* and p*bkt-5* transformations all generated blue cells in *H. pluvialis* (not all data are shown), whereas no positive results were found in either the blank or negative controls [**Fig. 4(C)**]. **Fig. 4(A)** shows a blue cell with the expression product (β-galactosidase) of the *lacZ* gene driven by a promoter-like region (–630 to –308 bp) of *bkt* in the p*bkt-3* construct and **Fig. 4(B)** shows a blue cell with the expression product (β-galactosidase) of the *lac*Z gene driven by an SV40 promoter-enhancer in the positive control. The bombardments performed by using those deleted constructs mentioned above and the pβgal-Control vector have been shown to display equal and stable transformational efficiencies by trial and error (data not shown).

The results of the transient transformation of mutants and β-galactosidase assays showed that transformation with Mutation-1 (–566 to –562 bp) resulted in a similar level of β-galactosidase activity as that of p*bkt-2* (–630 to –408 bp), with the β-galactosidase activity being nearly 1.5 times that of the negative control, whereas transfor-

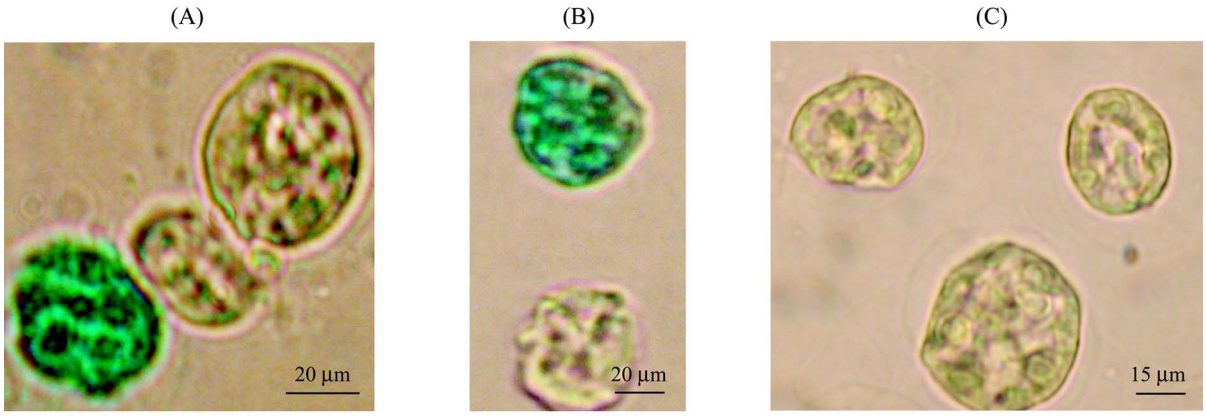


**Fig. 4 Transient expression of *lacZ* in *H. pluvialis***

(A) Expression of *lacZ* driven by a promoter-like region (–630 to –308 bp) of *bkt* in *H. pluvialis.* (B) Expression of *lacZ* driven by an SV40 promoter-enhancer in *H. pluvialis.* (C) No expression of *lacZ* in either the negative control or blank control in *H. pluvialis.*

mation with Mutation-2 (–561 to –551 bp) or Mutation-3 (–450 to –441 bp) resulted in no β-galactosidase activity (**Fig. 5**). These results suggest that the two APE-like motifs in the promoter-like region (–630 to –308 bp) might be directly involved in *bkt* gene expression in *H. pluvialis*.

The *cis*-acting elements mentioned above are thought to direct expression in response to some physiological conditions, such as low temperature or blue light. However, further studies are necessary to explore the interactions between these elements and their relevant environmental factors.

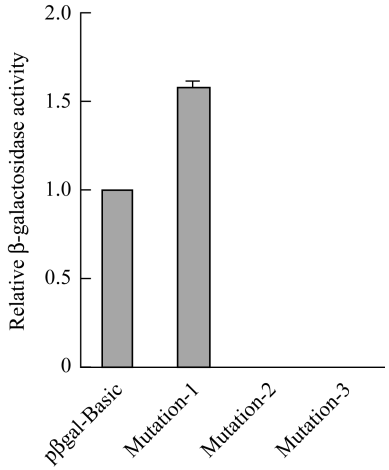


**Fig. 5 Site-directed mutation in a promoter-like region (–630 to –308 bp) of *bkt***

Mutation-1 (–566 to –562 bp), Mutation-2 (–561 to –551 bp) and Mutation-3 (–450 to –441 bp) were used to transform *H. pluvialis* motile cells. The β-galactosidase activity of each sample was subsequently compared with the mean value of the empty pβgal-Basic vector to acquire the fold induction of each construct. The data are shown as mean±SD (*n*=3).

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