FUNCTIONAL ANALYSIS OF THE PROMOTER OF *BKT* ENCODING BETA-CAROTENE KETOLASE IN *HAEMATOCOCCUS PLUVIALIS*

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Abstract: The unicellular green alga *Haematococcus pluvialis* accumulates a high-valuable astaxanthin under stress conditions. Beta-carotene ketolase (BKT), a key enzyme in astaxanthin biosynthesis in *H. pluvialis*, catalyzes the conversion of β-carotene to canthaxanthin and zeaxanthin to astaxanthin. Electrophoresis mobility shift assay (EMSA) was used in *H. pluvialis* to identify transcription factor binding sites within a 309 bp promoter region (−617/−309) of beta-carotene ketolase gene and a 59 bp sequence between −396 and −338 bp was found to have a specific binding activity to the nuclear protein. Sequence analysis revealed that this important functional region contains neither TATA nor CAAT box but a G-box involved in the responsiveness of light, anaerobiosis, p-coumaric acid and hormone.

Key words: EMSA; *Haematococcus plwialis; bkt;* Promoter; Transcription factor binding sites
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Promoters are responsible for modulating gene expression via different interaction with transcription factors. The binding of transcription factors to regulatory elements imparts a direct effect upon transcription. Therefore, these regulatory elements and DNA binding proteins serve to provide essential termini for cellular signal cascades that allow a cell to adjust to changing developmental or environmental conditions[1].

The unicellular fresh-water green alga *Haematococcus pulvialis* accumulates large amounts of astaxanthin when exposed to unfavorable growth conditions or following different environmental stresses such as phosphate or nitrogen starvation, high concentration of salt in the growth medium or high light intensity[2]. The genes involved in astaxanthin biosynthesis have been characterized in *H. pluwialis*[3−8]. Beta-carotene ketolase (BKT), responsible for the conversion from beta-carotene to canthaxanthin and from zeaxanthin to astaxanthin, plays a key role in astaxanthin biosynthesis in *H. pluvialis*. Former studies have reported that high light intensities and nitrogen limitation led to increase of *bkt* mRNA[7]. Two different *bkt* 5′-flanking regions (1.5 kb and 2 kb) were cloned and a promoter-like region (−617/−309) was characterized through transient expression and deletion analysis[9]. However, there is still no experimental evidence concerning the interaction of regulatory elements in the promoter region with trans-acting factors. In present study, we describe the use of EMSA to identify smaller and more specific fragments responsible for transcriptional regulation within this 309bp promoter region and their significance as cis-acting elements is further discussed.

1 Materials and methods

1.1 Preparation of nuclei

It was performed from *H. pluwialis* cells using Plant Nuclei Isolation / Extraction Kit (Sigma), according to the manufacturer's instructions. Experiments were carried out at 4 ℃. *H. pluwialis* cells (1 g) were powdered in liquid nitrogen with a mortar and pestle and homogenized lx NIB buffer. The lysis was then filtered through a filter mesh, and centrifuged at 1260 g for 10 min in a swinging bucket rotor. The pellet was resuspended in lx NIB buffer adding

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10% Triton X-100 to 0.3% final concentration. Apply the lysate on top of 0.8ml sucrose 2.3M cushion in 1.5ml tubes (approximately 0.6ml of lysate in each tube). Centrifuge at 12,000 g for 10 min. Aspirate the upper green phase and the sucrose cushion layer without disturbing the pellet of nuclei. Wash the pellet twice by resuspending in 1 mL of NIBA and centrifuging 5 min at 12,000 g. At this stage the nuclei can either be stored without further manipulations or extracted for nuclear proteins.

1.2 Preparation of nuclear protein extracts

Thoroughly resuspend the nuclei pellet with the extraction buffer (2/3 of the pellet volume). Mount the tube on a vortex and agitate at medium-high speed for 15 to 30 min at 4 ℃. Centrifuge for 10 min at 12,000 g. Transfer the supernatant to a clean chilled test tube. The nuclear protein extracts are aliquoted, frozen in liquid nitrogen, and stored at – 80 ℃.

1.3 DNA probes

The 309 bp (– 617/– 309), 172 bp (– 480/– 309), 100 bp (– 480/– 381) and 122 bp (– 430/– 309) fragments of the *bkt* 5′ flanking region were prepared by PCR with the Genomic DNA as template (Fig. 1 and Fig. 2). Two DNA fragments [W59 (– 396/– 338), W55 (– 363/– 309)] were synthesized and annealed manually to double-stranded DNA probes. All probes were end-labeled with DIG-11-ddUTP using a DIG Gel Shift Kit (Roche) according to the manufacturer's instructions.

– 617GATCCCCCTATTCGGTCCGCATCGAGGCGGGATCGATGGGGGGGAAA
– 570ATGTCCGACGGAGTGTTGTATGCTGGCGACTATGCAGATGAACATAG
– 523AGAAGGCGTTATACACTTTCTTTTGGAGGCTTACTATGCTGCTGAGGC
– 475TGGAGGTCTCATTCCATACTGGTGCCTTCGTAACGCTCACCGTAGTGA
– 427GACGAGAAAGGTTAGAAATGTTACACCGGCGACCTGTGACCCTCGTT
– 380GTGCACCCCCTCGTTGTGCACCCCACGACAGAGCACTCGGATGCAAG
– 333TTCACGGGGGCAACTCAACAAATTC

Fig. 1 Sequence of 309 bp 5′ upstream region and 172 bp probe (underlined) Nucleotide number is relative to the first codon nucleotide from the initiating methionine

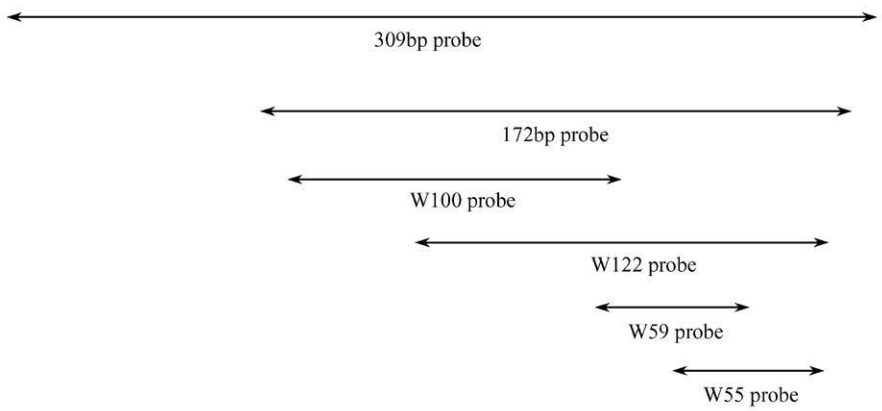

Fig. 2 Probes used in EMSA

1.4 Electrophoresis mobility shift assay (EMSA)

About 1—3μl of nuclear extracts corresponding to 2—6μg of proteins were incubated for 15 min at room temperature with the labeled oligonucleotide probe in a buffer consisting of 100 mM Hepes (pH 7.6), 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% (w/v) Tween 20, 150 mM KCl. In all binding assays, 1 μg of double-stranded poly-(dI-dC) was added as a nonspecific competitor. In the competition reactions, unlabeled specific competitors (at 25 or 50-fold excess) were used with probes to distinguish sequence-specific interactions. After incubation, the complexes were resolved by electrophoresis in 6% gel native polyacrylamide gel. The protein. DNA complexes were subsequently transferred to a nylon

membrane by electroblotting and detected by using the DIG-detection kit.

1.5 Sequence analysis

Internet tool (http://oberon.rug.ac.be:8080/PlantCARE/index.html)[10] was used to perform sequence analysis (using the data from the Genebank No. AY334016) including prediction of transcriptional elements.

2 Results

EMSA has been widely devised to detect the interaction of nuclear proteins with promoters of high plants[11]. A *H. plwialis* promoter fragment (309 bp, −617 to −309 bp from the translation start site) was used in these experiments because (a) it containsed several consensus domains homologous to light dependent boxes found in other photoregulated plant gene promoters and (b) it was previously shown to direct the transient expression of the reporter gene *lacZ* in *H. plwialis* cells[11]. The 309 bp probe was labeled by digoxigenin-11-ddUTP and incubated with nuclear protein extracts. One specific retarded band was observed in EMSA (Fig. 3 lane 2). Pretreatment of the protein extracts with proteinase K prior to electrophoresis caused the disappearance of the retarded bands establishing that the shift resulted from protein-DNA interactions (Fig. 3 lane 3).

To further identify the protein binding sequences on the promoter DNA, five subfragments covering most of the 309 bp probe of the *bkt* promoter were prepared (Fig. 2) and used in EMSA. Retarded bands with nuclear protein extracts were seen with the 172 bp(−480/−309), 122 bp (−430/−309) and W59 (−396/−338) fragments. In a competition experiment, a 50-fold molar excess of the unlabeled probes caused the retarded band to decrease and almost be non-detectable when higher concentrations of the competitor were used (Fig. 4, 5, 6). Thus, this DNA-protein complex, as detected in the EMSA, was resulted from a specific interaction.

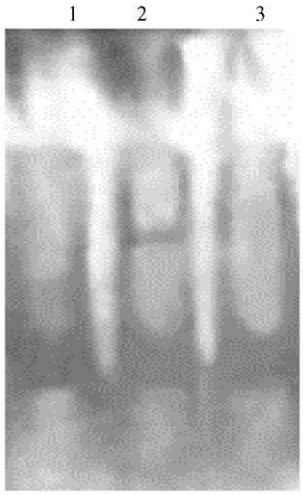

Fig. 3 EMSA with 309 bp probe. Lane1: free probe. Lane2: probe with nuclear protein. Lane3: 50-fold molar excess of the unlabeled probe

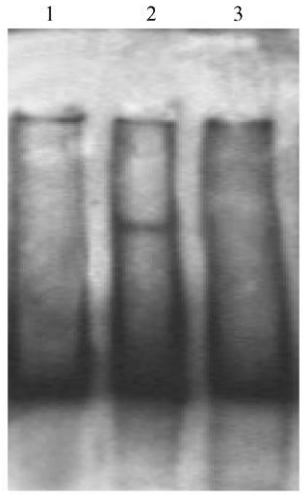

Fig. 4 EMSA with 172 bp probe. Lane1: free probe. Lane2: probe with nuclear protein. Lane3: 50-fold molar excess of the unlabeled probe

3 Discussion

Results of computer search using PlantCARE for potential cis-acting elements revealed that the cis-acting element contributing to the binding of nuclear proteins in the 59 bp fragments (−396/−338) is probably neither TATA nor CAAT box but a G-box which plays a key role in the responsiveness of plant promoters to light, anaerobiosis, p-coumaric acid and hormones such as abscisic acid, ethylene and methyl jasmonate.

The G-box is a hexameric motif found in many diverse plant genes. This sequence functions as a cis-acting promoter element, and was first characterized in early studies on the 5′ noncoding region of the light-regulated ribulose 1, 5-bisphosphate small subunit (RBCS) genes. Protein known as G-box factors (GBF) binds to G-boxes in a context-specific manner, mediating a wide variety of gene expression patterns. In Arabidopsis, An 8-base pair

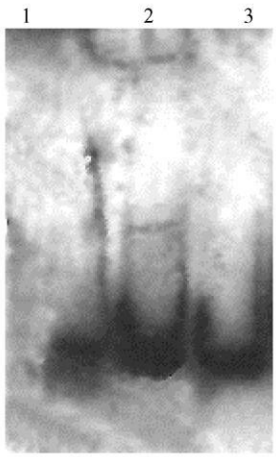

Fig. 5 EMSA with 122 bp probe. Lane1: free probe. Lane2: probe with nuclear protein. Lane3: 50-fold molar excess of the unlabeled probe

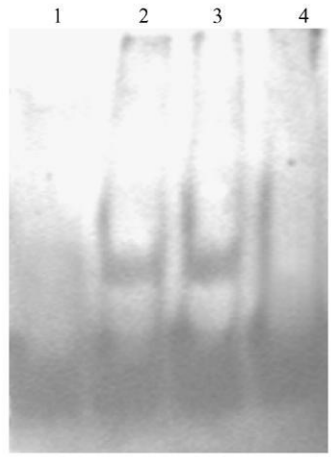

Fig. 6 EMSA with W59 bp probe. Lane1: free probe. Lane2, 3: probe with nuclear protein. Lane4: 50-fold molar excess of the unlabeled probe

G-box element is associated with light-induced expression of *RbcS* and *chalcone synthase*[12]. Site-specific mutations of G-box in the promoter of *adh* gene induced by hypoxic stress result in greater than 60% reduction in activity and disrupt G-box factor binding in vitro[13]. Within the bean *chsl*5 gene promoter, H-box (consensus CCTACC(N)$_7$CT) and the G-box (CACGTG) are required for stimulation of the *chsl*5 promoter by 4-CA (p-coumaric acid)[14]. In soybean, *vspB* expression is stimulated by methyl jasmonate (MeJA) and sugars. The MeJA-responsive domain identified in promoter of *vspB* contains a G-box motif and a G-rich sequence[15]

Former studies have reported that high light intensities and nitrogen limitation led to increase of *bkt* mRNA[7]. Therefore, there must be some regulatory elements in the promoter region to induce the expression of *bkt* by binding to different trans-acting factors. According to the result of EMSA, G-box in the 59 bp fragments may be one of these cis-acting elements for the responsiveness of light or nitrogen limitation. Further experiments are needed to find the trans-acting factors and the exact binding sites in this fragment.

The work shown in this paper is a detailed description of a specific part of 5′ flanking region of *bkt* in *Haematococcus pluvialis*. The method of EMSA was used to detect the possible transcription factor binding sites. The prediction of cis-elements and discovery of transcriptional factor binding region will provide base for the future study of *bkt* transcriptional regulation.

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雨生红球藻β-胡萝卜素酮化酶(*bkt*)启动子功能分析

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摘要: 单细胞绿藻——雨生红球藻在逆境条件下积累大量的虾青素。β-胡萝卜素酮化酶(*bkt*)催化在β-胡萝卜素和玉米黄素的β-紫罗酮环C-4位引入酮基的反应, 是虾青素合成过程中的关键酶。我们利用凝胶阻滞的方法研究雨生红球藻中*bkt*基因309 bp(−617/−309)启动子区域的转录因子结合位点并发现在−396/−338的59 bp探针存在特异的核蛋白结合位点。通过序列分析, 发现此59 bp区域并不包含TATA或者CAAT-box, 而是存在对光、缺氧、p-香豆酸及激素反应的G-box。

关键词: 凝胶阻滞; 雨生红球藻; *bkt*; 启动子; 转录因子结合位点