Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*

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ABSTRACT

The unicellular green alga *Haematococcus pluvialis* accumulates large amounts of ketocarotenoid astaxanthin under a variety of environmental stresses. Here we report the identification and expression of three different β-carotene ketolase genes (*bkt*) that are involved in the biosynthesis of astaxanthin in a single strain of the alga. *Bkt1* and *bkt2* proved to be the *crtO* and *bkt* previously isolated from two different strains of *H. pluvialis*. *Bkt3* is a novel third one which shared 95% identical nucleotide sequence with *bkt2*. Nitrogen deficiency alone could not induce the alga cells to produce astaxanthin in three days even though it enhances the expression of the *bkt* genes to 3 times of that in normal growing cells within 16 h. High light irradiation (125 μmol m^{-2} s^{-1}) or 45 mM sodium acetate greatly increased the expression of *bkt* genes to 18 or 52 times of that in normal growing cells, resulting in an accumulation of substantial astaxanthin (about 6 mg/g dry biomass) in three days. It is suggested that the existence of the multiple *bkt* genes and their strong up-regulation by different stress conditions is one of the reasons that *H. pluvialis* accumulates large amounts of astaxanthin in an instant response to stress environments.

Key-words: astaxanthin; β-carotene ketolase; *Haematococcus pluvialis*; real-time PCR

1. Introduction

Haematococcus pluvialis accumulates large amounts of ketocarotenoid astaxanthin in response to high light irradiation, nitrogen limitation and salt stress (Yong and Lee, 1991; Kobayashi et al., 1992; Boussiba, 2000). Diverse functions of astaxantin in *H. pluvialis* related to its antioxidative properties have been proposed (Hagen et al., 1993; Kobayashi et al., 1997). This green microalga is the main natural source of astaxanthin as feed supplements in the farming of trout and salmon (Meyers, 1994; Johnson and Schroeder, 1996). It is also an ideal organism to study the regulation of ketocarotenoid synthesis (Sun et al., 1998; Linden, 1999; Boussiba, 2000; Grünewald et al., 2000; Grünewald et al., 2001; Steinbrenner and Linden, 2001, 2003). Higher plants and green algae share the same carotenoid biosynthetic pathway to β-carotene (Cunningham and Gantt, 1998). In *H. pluvialis* the specific steps leading to astaxanthin are further catalyzed by β-carotene ketolase (Kajiwara et al., 1995; Lotan and Hirschberg, 1995) and β-carotene hydroxylase (Linden, 1999). The β-carotene ketolase is the only enzyme that exclusively participates in the secondary carotenoid pathway to astaxanthin in *Haematococcus*. This enzyme plays an essential role in stress-dependent initiation of astaxanthin synthesis. Two different β-carotene ketolase cDNAs *bkt1** (formerly *crtO* (Lotan and Hirschberg, 1995)) and *bkt2* (formerly *bkt* (Kajiwara et al., 1995)) were isolated from two different strains of *H. pluvialis* on different induced conditions. It is still unknown whether this indicates the existence of multiple carotene ketolases in *Haematococcu*s (Sun et al., 1998), or only reflects strain differences (Grünewald et al., 2000). Therefore it is important to know about the nature of the ketolase genes and the relationship between the regulation of these genes and the accumulation of astaxanthin in *Haematococcus* when the algal cells are undergone different stress conditions.

In the present study, we focus on the nature of *bkt* genes, their differential expression and astaxanthin accumulation in *H. pluvialis* Flotow NIES-144 in response to different stress conditions.

*Foot note: All β-carotene ketolase genes were designated *bkt* as introduced in Misawa et al. (1995b). It uses 3 letters indicating their function according to the nomenclature of carotenogenic genes from eukaryotes. Numbers were given in the order of publication.

2. Materials and methods

2.1. Haematococcus pluvialis and growth conditions

H. pluvialis Flotow NIES-144 was obtained from the National Institute for Environmental Studies (Tsukuba, Japan). *H. pluvialis* was grown in 250 ml Erlenmeyer flasks containing a medium as described by Kobayashi *et al*. (Kobayashi et al., 1993). For control samples, sodium acetate (14.8 mM) was removed from the medium. Cells were grown at 20 °C under a dark/light cycle of 12 h of illumination at 20 μmol m^{-2} s^{-1} and 12 h of darkness for 5 days. For induction of astaxanthin biosynthesis, sodium acetate or/and $FeSO_4$ were used at final concentrations of 45 mM and 450 μM, respectively, and sodium chloride at 100 mM. For nitrogen deficiency experiment, cells grown mixotrophically were harvested, washed with sterile deionized water and resuspended in a TAP medium (Gorman and Levine, 1966) without nitrogen source and sodium acetate. For high light treatment, cultures of *H. pluvialis* were illuminated at 125 μmol m^{-2} s^{-1}.

2.2. DNA and RNA extraction

DNA and RNA techniques were followed according to the standard methods (Sambrook et al., 1989). DNA was extracted using a modified CTAB method (Stewart and Via, 1993). RNA was isolated from aliquots of about 10^7 cells harvested after 5 d of growth and 16 h of varying inductions using TRI REAGENT® (Molecular Research Center, Ohio) according to the manufacturer's instructions. The concentration of total DNA and RNA was determined spectrophotometrically at 260 nm.

2.3. PCR, RT-PCR, and 3'RACE

The sequences of *bkt1* (Genbank accession **X86782**) and *bkt2* (Genbank accession **D45881**) were aligned with Clustal X. The divergent 3' ends of the cDNA were selected as targets of PCR amplification. Primers were designed with the computer software Genetool. Primer sets and PCR product characteristics are listed in Table 1. Detection of *bkt* genes in *H. pluvialis* was performed by conventional PCR (35 cycles of 94 °C for 20 s, 61 °C for 20 s, 72 °C for 30 s). RT-PCR was performed with a one-step RT-PCR kit (Invitrogen) following the manufacturer's procedure. Forty ng of total RNA was used in a 25µl reaction volume. As an internal control for the relative amount of RNA, a fragment of *actin* gene was amplified with primers (forward: 5'-AGCGGGAGATAGTGCGGGACA; reverse: 5'-ATGCCCACCGCCTCCATGC) designed from a cloned fragment of the *actin* gene from *H. pluvialis* (unpublished data). cDNA was synthesized at 55 °C for 15 min followed with PCR amplification (32 cycles (for *bkt* genes) or 22 cycles (for *actin* gene) of 94 °C for 20 s, 61 °C for 20 s, 72 °C for 1 min). 3' RACE of *bkt* was performed using a 5'/3' RACE kit (Roche molecular biochemicals, Germany). Primers F1, F2, and F3 (Table 1) were used for PCR amplification of dA-tailed cDNA and a nested PCR. The final PCR

amplification product of 3'RACE was purified and cloned into pTZ57R/T vector (MBI Fermentas).

2.4. Southern blot

Eight micrograms of genomic DNA was digested with *Sac*I or *Kpn*I and *Sal*I, three restriction enzymes without cutting site in the probed regions of the ketolase genes. The digested DNA was separated with a 0.8% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim/Roche), and hybridized with Dig-labeled DNA probes in the presence of 50% (v/v) formamide at 47 °C for 16 hs. DNA probes were prepared by amplifying a 1 kb fragment from plasmid containing *bkt*2 gene with a pair of primers (Table 1, Fp and Rp) (30 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 1 min). Probe labeling and hybridization were carried out according to the instructions in the DIG Nonradioative Nucleic Acid Labeling and Detection System (Boehringer Mannheim/Roche). After hybridization, the membrane was washed twice with 0.1 × SSC containing 0.1% SDS at 68 ˚C for 15 min.

2.5. DNA sequencing

The nucleotide sequences of *bkt* genes derived from 3'RACE and RT-PCR were determined for both strands using the ABI prism Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems).

2.6. Functional analysis of bkt3 in E. coli

The open reading frame of *bkt3* was PCR amplified and cloned into the vector pBluescript II KS as an in-frame fusion to the *lacZ* gene resulting in plasmid pHPb3. *E. coli* strain JM109 was used as a host for complementation experiments by co-transformation of pHPb3 with plasmids either pACCAR16ΔcrtX or pACCAR25ΔcrtX that harbor the carotenoid biosynthesis genes for

producing β-carotene or zeaxanthin, respectively (Misawa et al., 1990). Carotenoids in the transformants were extracted and analyzed by HPLC according to Yuan and Chen (1998).

2.7. Reverse Transcription and Real-Time PCR

Reverse transcription was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instruction. Real-time PCR was performed using a Rotor-Gene-2072 cycler (Corbett Research, Australia). Amplification was performed in Platimum®SYBR® Green qPCR Supermix UDG (Invitrogen), specific primers at 0.5 μM and cDNA of 36 ng total RNA in a reaction volume of 20 μl. Plasmids containing the cDNA of *bkt1*, or *bkt3* were serially diluted (10^{-1} – 10^{-7} fmol) to generate a standard curve for each gene. The house-keeping gene *actin* was used as an internal standard for normalization of target RNA and RT reaction. The thermocycling program consisted of: hold at 50 ^{o}C for 5 min, denaturation at 94 ^{o}C for 2 min followed by 40 cycles of PCR (94 ^{o}C for 15 s, 58 ^{o}C for 15 s, 68 ^{o}C for 30 s).

2.8. Carotenoid extraction and HPLC analysis

Carotenoids were extracted and analyzed according to Yuan and Chen (1998). Algal and *E. coli* cells were collected by centrifugation and freeze-dried. Extraction was carried out with a mixture of dichloromethane and methanol (25:75, v/v) until the cell debris was almost colorless. The combined extracts were evaporated to dryness and separated on a 5 μm ODS2 4.6 x 250 mm analytical column (Waters Spherisorb®) with a Waters high-performance liquid chromatograph. Individual carotenoids were identified by absorption spectra and their typical retention times compared to standard samples of pure carotenoids. Quantification of astaxanthin from *H. pluvialis* was carried out by area calibration with an authentic standard.

3. Results

3.1. Detection of bkt genes in H. pluvialis

To detect whether *H. pluvialis* NISE 144 contains both *bkt*1 and *bkt*2, genomic PCR was performed. The forward primer (F1, see Table 1) could base pair with both *bkt*1 and *bkt*2, while the reverse primer R1 is specific to *bkt*1 and R2 specific to *bkt*2. According to the sequences of *bkt*2, PCR with primers F1 and R2 and genomic DNA of *H. pluvialis* NISE 144 would generate a 348 bp fragment. If the alga also contains *bkt*1, an about 500 bp fragment would be amplified with primers F1 and R1. PCR amplification with the specific primers F1 + R1/R2 did generate both 348 and 500 bp fragments, indicating the possibility that *H. pluvialis* NISE 144 contains both *bkt*1 and *bkt*2 (Fig. 1). Furthermore this alga may contain one more *bkt* gene as indicated by the presence of an additional fragment (about 370 bp) in the genomic PCR product of *bkt*2 (Fig. 1, lane 2). Therefore we performed Southern blot to reveal the number of *bkt* gene in the genome of the alga.

H. pluvialis genomic DNA was digested with different restriction enzymes and subjected to Southern blot analysis. Using a 1 kb fragment of *bkt*2 as a probe, the homologous fragments showed strong hybridization signals (Fig. 2). Each of the two separate digests showed three bands, suggesting the presence of three *bkt* gene in the haploid genome of *H. pluvialis*. To verify this result and isolate the third *bkt* gene, 3' RACE of the *bkt* genes was carried out.

3.2. 3'RACE of bkt genes in H. pluvialis

Three forward primers (Table 1, F1, F2, and F3) were used for 3'RACE. F2 and F3 anneal to the last two exons of *bkt*1 and *bkt*2 separated by the last intron of the genes. Coupled with 3'RACE PCR anchor primer, F2 and F3 were used for the first round of 3'RACE. Both *bkt*1 and *bkt*2 share the same sequence of primer F1. A nested PCR with F1 and 3'RACE PCR anchor primer generated fragments of about 700 bp in length that may contain the 3'portions of individual *bkt* genes. The fragments were subcloned into pTZ57R/T vectors. Clones with different inserts were detected by PCR with primers F1 + R1/R2. Of 10 clones analyzed, 5 generated the 500 bp fragment, 4 generated the 348 bp fragment, and 1 generated the 370 bp fragment. The inserts were sequenced, which proved to be the 3' termini of *bkt1* (bases from 1060 to 1761 in **X86782**), *bkt2* (bases from 1034 to 1662 in **D45881**), and a similar *bkt*2 (94 % identity) assigned as *bkt3*. Figure 3 shows the alignment of the three sequences. Although highly similar, *bkt2* and *bkt3* represent two distinct genes because in addition to 48 bp differences (out of 635 bp), *bkt3* was 22 bp longer than *bkt2*. The 370 bp band in Fig. 1 (lane 2) is derived from this *bkt*3.

3.3. Cloning of the full-length *bkt*3

Since *bkt*2 and *bkt*3 share highly homologous 3' terminal sequence, we predict they also share highly homologous 5' terminal sequences. Thus RT-PCR with the reverse primer Rb2 (priming to both *bkt*2 and *bkt*3; see Fig. 3) and the forward primer Fb2 (priming to *bkt*2; see Table 1) may amplify both *bkt*2 and *bkt*3 cDNA. RT-PCR with the primers generated 1.7 kb products. These products were cloned into pTZ57R/T vector. Clones containing *bkt*3 were detected by PCR with primers F1 and R2, which generated 370 bp rather than 348 bp fragments. Positive clones were isolated and sequenced. Sequence data reveals the existence of a full-length *bkt3* (GenBank accession no. **AY603347**). The *bkt*3 was highly homologous to *bkt2* with only 22

nucleotide differences in their coding region resulting in only two amino acid differences from that of *bkt*2.

3.4. Functional analysis of bkt3 in E. coli

In order to find out whether the *bkt3* encodes a functional β-carotene ketolase, the plasmid pHPb3 (described in Materials and methods) was co-transformed with pACCAR16ΔcrtX or pACCAR25ΔcrtX respectively into JM109. HPLC analysis of carotenoids from the transformants is shown in Fig. 4. Compared with pigments extracted from *E. coli* harboring only the pACCAR16ΔcrtX which accumulates β-carotene (Fig. 4B), the *E. coli* containing the pHPb3 and pACCAR16ΔcrtX accumulated canthaxanthin (peak 1) and echinenone (peak 2) in addition to β-carotene (peak 3). These results indicate that similar to *bkt 1* and *bkt2*, *bkt*3 encodes an enzyme with activity of converting β-carotene to echinenone and canthanxanthin (Kajiwara et al., 1995; Lotan and Hirschberg, 1995; Breitenbach et al., 1996). Concordant with the result from Lotan and Hirschberg (1995), no astaxanthin was found in the pigments from *E. coli* carrying the pHPb3 and pACCAR25ΔcrtX (data not shown).

3.5. Assays of bkt expression by multiple RT-PCR

The expression of *bkt* genes under high light intensity (125 μmol photons m^{-2} s^{-1}), nitrogen limitation, and salt stress was monitored using multiple RT-PCR. The forward primers (F2, F3) used anneal to sequences in the last two exons on both sides of the last intron. Thus potential contaminating genomic DNA did not interrupt the results. RT-PCR generated three expected fragments correspondent to the 3 *bkt* cDNAs in correct sizes (Fig. 5), which further supports the existence of 3 *bkt* genes. Differential expression of the *bkt* genes was observed under the stress conditions (Fig. 5). Basal expression of the *bkt* genes was detected from green flagellate cells

cultured in the medium without acetate (Fig. 5 lane 1). Low concentration of sodium acetate (14.8 mM) enhanced the expression of *bkt2* and *bkt3* (lane2). Nitrogen deficiency and high light irradiation preferentially induced the expression of *bkt1* (lane 3, 4), while high concentration of NaCl (100 mM) or sodium acetate (45 mM) greatly stimulated the expression of all the three *bkt* genes (lane 5, 6). $FeSO_4$ seemed to have a minor effect on the expression of the *bkt* genes (lane 7).

3.6. Assays of bkt expression by real-time RT-PCR

In order to confirm the results obtained by multiplex RT-PCR, we performed real-time quantitative RT-PCR that allows quantifying the absolute level of the *bkt* transcripts. Because *bkt2* and *bkt3* share highly homologous sequence, it is difficult to design absolutely specific primers to each of the genes for real-time quantitative PCR with SYBR® Green detection method. Multiple RT-PCR revealed that the expression patterns of *bkt2* and *bkt3* are very similar. Therefore the expression of *bkt2* and *bkt3* was only quantified in combination with one pair of primers annealing to the coding region (Table 1. Fq2+3, Rq2+3). PCR of cDNA reversely transcribed from total RNA with the primers (Table 1. Fq1, Rq1; Fq2+3, Rq2+3) gave a single product of the expected size determined by agarose gel electrophoresis and melting curve analysis in each case (data not shown). Serial concentrations of plasmid standards resulted in a reproducible standard curve that was linear from 10^{-7} -10^{0} fmol with R value higher than 0.99 (data not shown). The concentrations of *bkt* mRNA under different stress conditions are shown in Figure 6. The basal expression levels of *bkt2* and *bkt3* are higher than that of *bkt1*. A low concentration of acetate (14.8 mM) could stimulate *bkt1-3* expression by about two times. Nitrogen deficiency stimulates *bkt1-3* expression by 3 times of that in normal growing cells. High light irradiation (125 μmol photons m^{-2} s^{-1}) or high sodium acetate concentration (45 mM)

strongly increased the expression of all the three *bkt* genes (Fig. 6). Simultaneous application of Fe^{2+} to high sodium acetate alleviated the increase of the expressions of all the three ketolase genes. No astaxanthin was detectable in the cells treated with 14.8 mM sodium acetate or nitrogen deficiency although the *bkt* transcripts in these cells increase to 2 to 4 times of that in non-induced cells (Fig. 7). High light irradiation (125 μmol m^{-2} s^{-1}) increased the expression of *bkt* genes to 18 times resulting in an accumulation of substantial astaxanthin (about 7 mgg^{-1} dry biomass) in three days (Fig. 7). A higher concentration of sodium acetate (45 mM) greatly increased the *bkt* transcripts (about 52 fold of basic level) accompanied with an accumulation of about 6 mg astaxanthin per g dry weight (Fig. 7). This result indicated that apart from the *bkt* transcripts, other factors mediated by high light were important for the yield of astaxanthin.

4. Discussion

Green cells of *H. pluvialis* normally generate chloroplast-typical non-keto carotenoids (Boussiba, 2000). Upon stress when green cells are transformed to red cysts, total carotenoids in the cells increased and ketocarotenoids are preferentially formed. This is accompanied by the up-regulation of β-carotene hydroxylase and β-carotene ketolase genes (Grünewald et al., 2000; Grünewald et al., 2001; Steinbrenner and Linden, 2001). For β-carotene ketolase, two cDNAs were isolated separately from two different strains of *H. pluvialis* (Kajiwara et al., 1995; Lotan and Hirschberg, 1995). The predicted amino acid sequences of the cDNAs shared about 80% identity and highly similarity to the bacterial ketolase enzymes (Kajiwara et al., 1995; Lotan and Hirschberg, 1995; Misawa et al., 1995a, b). There is a discrepancy of the existence of *bkt* genes in *H. pluvialis* (Sun et al., 1998; Grünewald et al., 2001). In this study, we demonstrated the co-existence of *bkt1* and *bkt2* in a single strain of *H. pluvialis* (Fig. 1). In addition, a third ketolase

gene, *bkt3*, was found in the genome of *H. pluvialis* (Fig. 1, 2, 3, 5). By heterologous expression in *E. coli*, *bkt3* encoded an enzyme with activity similar to *bkt1* and *bkt2* (Kajiwara et al., 1995; Lotan and Hirschberg, 1995). These results open the question on the functional significance of multiple *bkt* genes in *H. pluvialis.*

For the first time, quantitative real-time RT-PCR made it possible to determine the steady-state mRNA levels for the three β-carotene ketolases in response to stress. By checking the sequences of the primers used in a previous study (Sun et al., 1998), we found that the primers designed according to the sequence of *bkt2* (Kajiwara et al., 1995) could amplified the combined transcripts of *bkt2* and *bkt3* simultaneously. While in another study the primers could only recognize the *bkt1* mRNA (Grünewald et al., 2000). Our results revealed basic expression of the three ketolase genes in green un-induced cells (Fig. 5). Sun et al. (1998) also detected the presence of *bkt* mRNA in green cells in a different strain of *H. pluvialis*. The transcript levels of the *bkt* genes in normal growing cells and cells cultured with 14.8 mM sodium acetate or induced by nitrogen deficiency were below a threshold value necessary for astaxanthin biosynthesis. As a result, no astaxanthin was detected in the cells under the cultured conditions. When the transcript levels of the *bkt* genes reached 1.6×10^{-6} fmol/ng total RNA, substantial amounts of astaxanthin were produced as in the case of the alga cells treated with high light irradiation or high salt stress (Fig. 7).

Astaxanthin is located in cytoplasmic lipid globules (Johnson and An, 1991) while its direct precursor β-carotene is synthesized in chloroplast (Grünewald et al., 2000). There is no clear indication of a transit sequence in *bkt1*, *bkt2* and *bkt3*. The ketolase was detected in both cytoplasm and chloroplast (Grünewald et al., 2000). It is not possible to gain information about

the spatial distribution of the three ketolase proteins at present. The major difference of the individual *bkt* genes was revealed in their response to stress. The expression of all the *bkt* genes were up-regulated under stress but to different levels. The expression level of *bkt1* was even higher than that of the combined amount of *bkt2* and *bkt3* (Fig. 6) when the alga cells were treated with high light irradiation or sodium acetate stress. Fe^{2+} (450 μM) alleviated the expression of the *bkt* genes and the yield of astaxanthin induced by sodium acetate (45 mM). This negative effect was confirmed at the messenger level of the β-carotene hydroxylase and the formation of astaxanthin in the same alga (Steinbrenner and Linden, 2001). Sodium chloride had a similar effect as sodium acetate in inducing the expression of the three genes (Fig. 5), supporting the interpretation that sodium acetate initiates a salt stress as sodium chloride (Boussiba, 2000; Steinbrenner and Linden, 2001).

In conclusion, *H. pluvialis* contains three different *bkt* genes which are differential regulated by a variety of environmental factors. The existence of multiple *bkt* genes in *H. pluvialis* and their high expression upon stress conditions make it easy to reach a threshold of total *bkt* mRNA that is essential for the biosynthesis of large amounts of astaxanthin.

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TABLE 1.Primer sets and PCR product characteristics

Aim		Oligonucleotide sequence 5'-3'	Product size (bp)	Genbank correspondence
Detection of *bkt*	R1	CACCACCCTCCCGCACCT	497	1060-1556 X86782
	F1	GACCTGCACTGGGAGCACCAC		
	R2	GACGTTGGCCACCGCTACTGA	348	1028-1375 D45881
Multiplex RT-PCR	F2	GCTACCACTTCGACCTGCACTG	507	1050-1556 X86782
	R1			
	F3	GCTACCACTTTGACCTGCACTG	358	1018-1375 D45881
	R2			
Probe	Fp	CATGACGCAATGCATGGCACCA	1052	518-1569
	Rp	GGTCAAAGTGGTAGCATGTCAG		AF534876
Isolation of *bkt3*	Fb2	CGGGGCAACTCAAGAAATTC	1662	1-1662 D45881
	Rb2	AGACAGAGTCATTCATTACAG		
Real-time PCR	Fq1	CGCTTGTTCTACTTTGGCACG	302	916-1218
	Rq1	TCACCCAGTCCTGCCACAAC		X86782
	Fq2+3	CACGTCGCATCGGCACTAA	208	171-379
	Rq2+3	GCGGTCCAGGTGCCAATGAT		AY603347

F = Forward; R = reverse;

Figure legends

Fig. 1. PCR detection of *bkt* genes in *H. pluvialis* NISE 144. The *bkt* specific PCR product was separated on a 1.5% agarose gel. PCR was performed using primers specific to *bkt2* (1, 2) or *bkt1* (3, 4) and DNA templates of plasmid containing the *bkt2* (1), *bkt1* (3), or *H. pluvialis* genomic DNA (2, 4). M, 100 bp DNA ladder plus (Fermentas).

Fig. 2. Southern analysis of genomic DNA from *H. pluvialis* NISE 144. Genomic DNA (8 μg) was digested with *Sac*I (lane 2), or *Kpn*I + *Sal*I (lane 3). Plasmids (400 pg) containing the *bkt*2 gene cut by *Sac*I (lane 1) or *Eco*RI (releasing the *bkt*2, lane 4) were used as positive controls.

Fig. 3. Nucleotide sequence alignment of 3' termini of *bkt* genes from *H. pluvialis* NISE 144. The sequences are aligned by using the program CLUSTAL X. Bold nt sequences correspond to forward primers, bold and italic nt sequences correspond to reverse primers used in the detection of *bkt* genes.

Fig. 4. HPLC analysis of carotenoid pigments extracted from *E. coli* cells carrying plasmid pACCAR16ΔcrtX and pHPb3 (A) or pACCAR16ΔcrtX (B). 1-canthaxanthin; 2-echinenone; 3-ß-carotene.

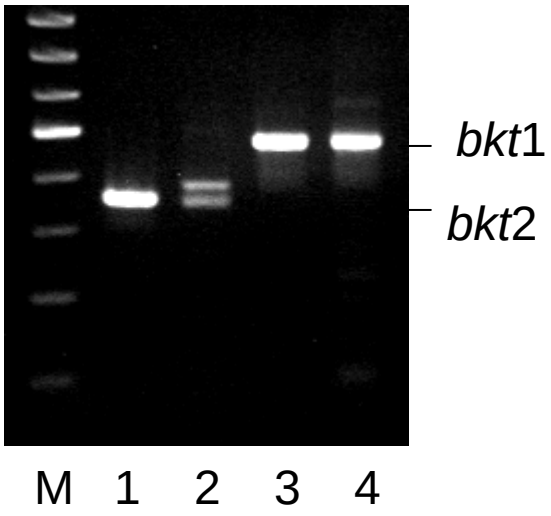
Fig. 5. Analysis of the differential expression of *bkt* gene in *H. pluvialis* cells using RT-PCR. RT-PCR was performed using RNAs from cells grown in a medium without sodium acetate (1), with

14.8 mM sodium acetate (2), nitrogen deficiency (3), under high light irradiation (4), with 100 mM NaCl (5), with 45 mM sodium acetate (6), or with 450 μM $FeSO_4$ (7) for 16 h. The *bkt* specific PCR product was separated on a 2.5% agarose gel (A), along with internal controls (actin) amplification (B). M, 100 bp DNA ladder plus (Fermentas).

Fig. 6. Expression of *bkt1* (□) and *bkt2+3* (■) detected by quantitative real-time PCR. Real-time PCR was carried out with cDNA from total RNA isolated from *H. pluvialis* cells induced by 14.8 mM sodium acetate (Lac), nitrogen deficiency (ND), high light irradiation (HL), 45 mM sodium acetate (Hac), and 45 mM sodium acetate with 450 μM Fe^{2+} for 16 h. AcDe represents samples from normal growing cells cultured with no sodium acetate. Each value is the mean ± SE (n = 2).

Fig. 7. Expression of bkt1-3 (A) and astaxanthin accumulation (B) after growth under various stress condition. Gene expression was quantified by real-time PCR after 16 h of induction. Astaxanthin was quantified after 72 h of induction.

Fig. 1.



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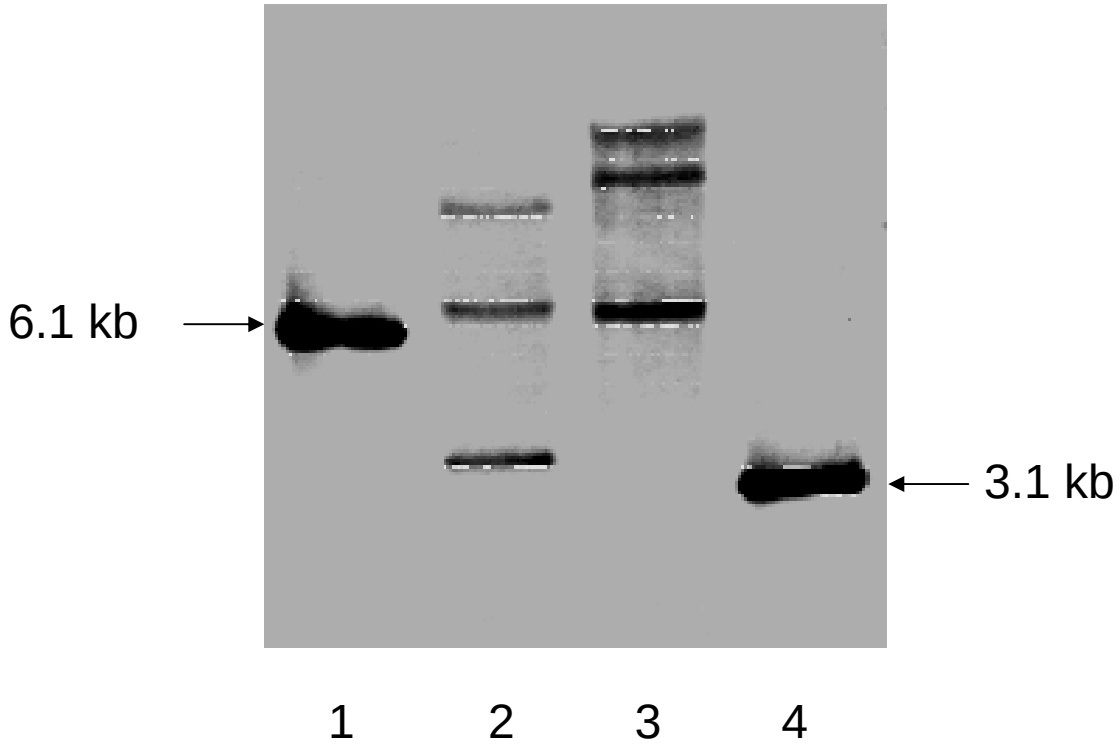

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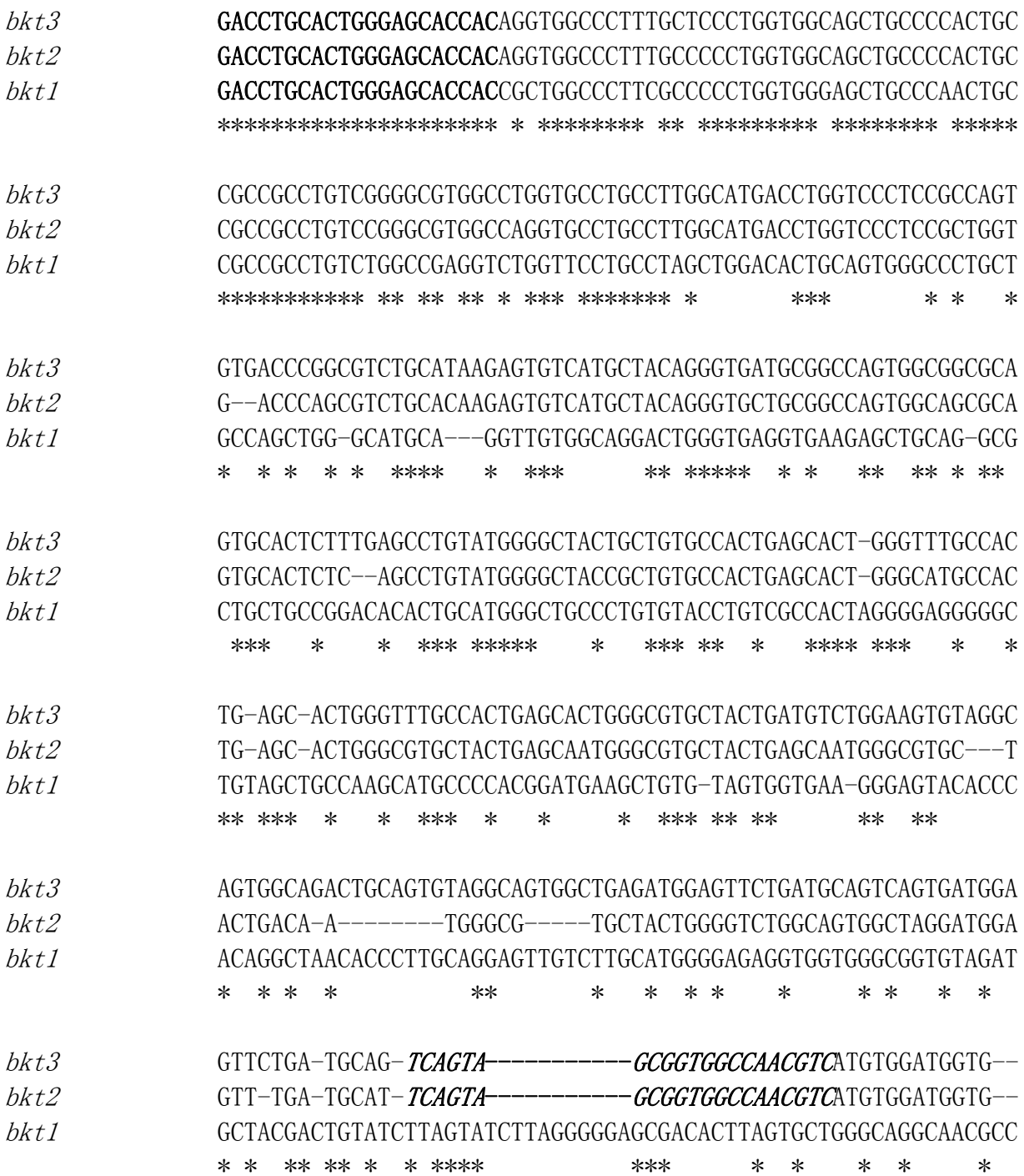
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bkt1 GACCTGCACTGGGAGCACCACCGCTGGCCCTTCGCCCCCTGGTGGGAGCTGCCCAACTGC
bkt3 CGCCGCCTGTCGGGGCGTGGCCTGGTGCCTGCCTTGGCATGACCTGGTCCCTCCGCCAGT
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bkt1 CGCCGCCTGTCTGGCCGAGGTCTGGTTCCTGCCTAGCTGGACACTGCAGTGGGCCCTGCT
bkt3 GTGACCCGGCGTCTGCATAAGAGTGTCATGCTACAGGGTGATGCGGCCAGTGGCGGCGCA
bkt2 G--ACCCAGCGTCTGCACAAGAGTGTCATGCTACAGGGTGCTGCGGCCAGTGGCAGCGCA
bkt1 GCCAGCTGG-GCATGCA---GGTTGTGGCAGGACTGGGTGAGGTGAAGAGCTGCAG-GCG
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bkt2 GTGCACTCTC--AGCCTGTATGGGGCTACCGCTGTGCCACTGAGCACT-GGGCATGCCAC
bkt1 CTGCTGCCGGACACACTGCATGGGCTGCCCTGTGTACCTGTCGCCACTAGGGGAGGGGGC
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bkt2 TG-AGC-ACTGGGCGTGCTACTGAGCAATGGGCGTGCTACTGAGCAATGGGCGTGC---T
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bkt2 ACTGACA-A--------TGGGCG-----TGCTACTGGGGTCTGGCAGTGGCTAGGATGGA
bkt1 ACAGGCTAACACCCTTGCAGGAGTTGTCTTGCATGGGGAGAGGTGGTGGGCGGTGTAGAT
bkt3 GTTCTGA-TGCAG-TCAGTA----------GCGGTGGCCAACGTCATGTGGATGGTG--
bkt2 GTT-TGA-TGCAT-TCAGTA----------GCGGTGGCCAACGTCATGTGGATGGTG--
bkt1 GCTACGACTGTATCTTAGTATCTTAGGGGGAGCGACACTTAGTGCTGGGCAGGCAACGCC

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bkt2        ----GAAGTGCTGAGGGGT-TTAGGCAGCCGGCATTTGAGAGGGCTAAGTTATAAATCGC
bkt1        CTGCAAGGTGCCGACACAAGCTAGG-AGCTGACGTGGACGAGGAGTCAGTGGCAGGCAGA
                  **** **       **** *** * * *    ****  * ***   *    *

bkt3        --ATGCTGCTCATGCGCACATATCTGCACACAGCCAGGGGAATCC--CTTCGA-GAGTGA
bkt2        --ATGCTGCCCATGCGCACATATCTGCACACAGCCAGGGAAATCCT-CTTCGA-GAGTGA
bkt1        TGAAGAGGTGCGGGAGGGTGGTGCCACACCCACTGGGCAAGACCATGCTGCAATGCCTGG
              * *  *  *  * *       *  *** **    *    * *   ** * * *  **

bkt3        TTATGGGACACTTGTAT-----TGGTT--TCGTGCTATTGTTTTATTCAGCACCAGCACT
bkt2        TTATGGGACACTTGTAT-----TGGTT--TCGTGCTATTGTTTTATTCAGCACCAGCACT
bkt1        CGGTGTGGCAGTGAGAGCTGCGTGATTAACTGGGCTATGGATTGTTTGAGCAGTCTCACT
               ** * ** *   *      ** **    * ***** * **  ** ****    ****

bkt3        TAGT----GAGGGTGAGAGCAGGGTGGTGAGAGTGGAGTGAGTGAGCATGAACCTGGTCA
bkt2        TAGT----GAGGGTGAGAGCAGGGTGGTGAGAGTGGAGTGAGTGAGCATGAACCTGGTCA
bkt1        TATTCTTTGATATAGACACTGGTCAGGCAGGTCAGGAGAGAGTGAGTATGAACAAGTTGA
            ** *    **    ** *   *   **   *   **** ******* ******  * * *

bkt3        GCAA-GGTGAAAAGTCTGTA-ATGAATGACTCTGTCT--------------
bkt2        GCGA-GGT-AACAGCCTGTA-ATGAATGACTCTGTCT--------------
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            *    ***     * *  *      ****  ****
```

Fig. 4

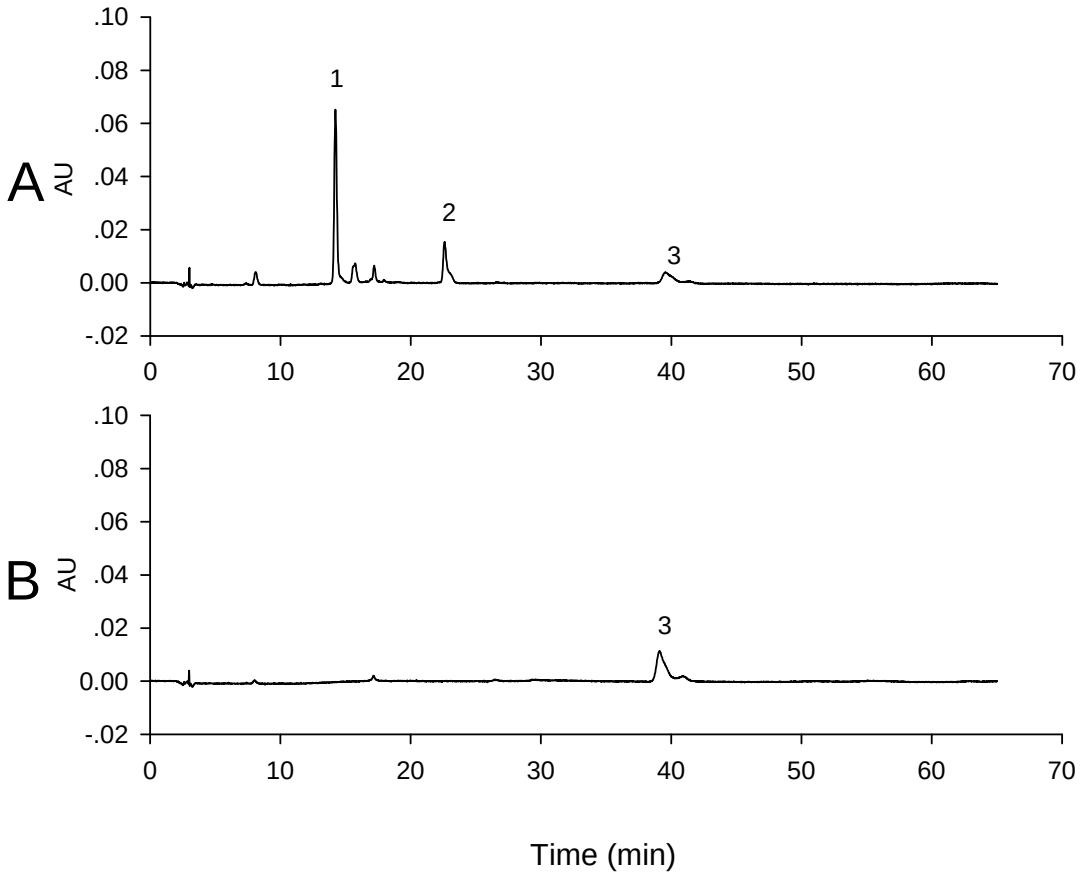
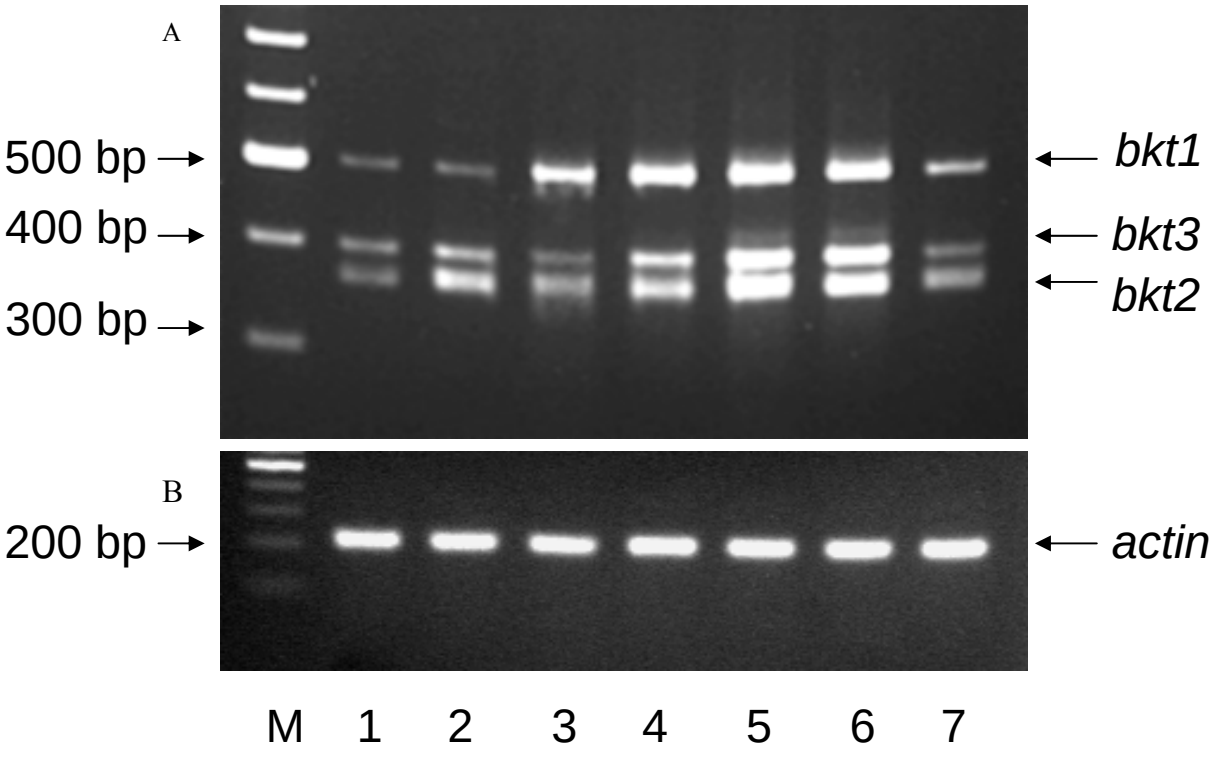


Fig. 5.



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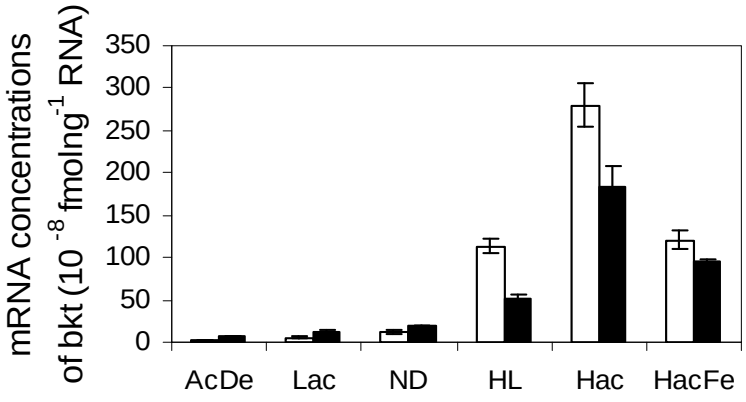
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of bkt (10 -8 fmolng-1 RNA)
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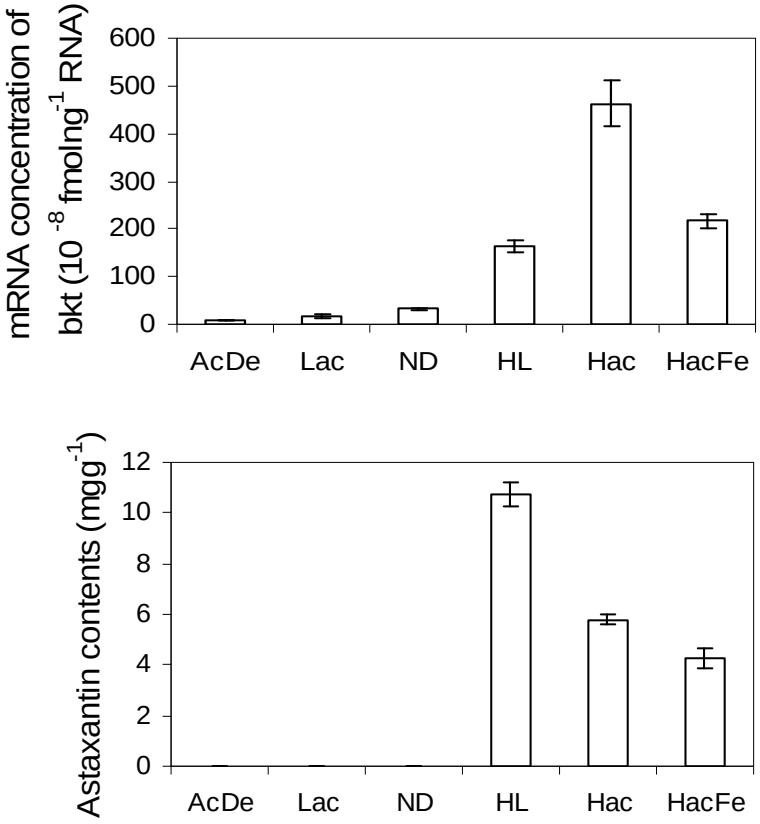
250
200
150
100
50
0
AcDe
Lac
ND
HL
Hac
HacFe

Fig. 7.


mRNA concentration of bkt (10 -8 fmolng-1 RNA)
600
500
400
300
200
100
0
AcDe
Lac
ND
HL
Hac
HacFe
Astaxantin contents (mgg-1)
12
10
8
6
4
2
0
AcDe
Lac
ND
HL
Hac
HacFe