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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* is used as a biological production system for astaxanthin. It accumulates large amounts of this commercially interesting ketocarotenoid 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 gene, which shared 95% identical nucleotide sequence with *bkt2*. Nitrogen deficiency alone could not induce the alga cells to produce astaxanthin in 3 days even though it enhances the expression of the *bkt* genes to three times of that in normal growing cells within 16 h. High light irradiation (125 $\mu mol\, m^{-2}\, s^{-1}$) 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 $mg\, g^{-1}$ dry biomass) in 3 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.

Keywords: Astaxanthin; β-Carotene ketolase; *Haematococcus pluvialis*; Physiological conditions; Real-time PCR

1. Introduction

Haematococcus pluvialis accumulates large amounts of ketocarotenoid astaxanthin in response to high light irradiation, nitrogen limitation and salt

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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, 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*[1] (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 more 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. A better understanding of the regulatory mechanism at a molecular level will be helpful for the optimization of astaxanthin production in *Haematococcu*s.

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.

[1] All β-carotene ketolase genes were designated *bkt* as introduced in Misawa et al. (1995b). It uses three 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. H. 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. (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. The initial cell density started from about 20,000 cells/ml reaching a value of about 350,000 cells/ml after 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 days of growth and 16 h of varying inductions using TRI REAGENT® (Molecular Research Center, OH) 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