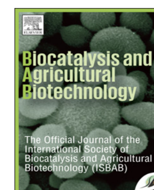
Biocatalysis and Agricultural Biotechnology 16 (2018) 378–384

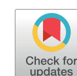
Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab



Transformation of *Dunaliella parva* with PSY gene: Carotenoids show enhanced antioxidant activity under polyethylene glycol and calcium treatments



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ARTICLE INFO

Keywords:
Antioxidant activity
Carotenogenesis
Expression
Food
Reactive oxygen species (ROS)
Stress

ABSTRACT

Carotenoids are antioxidant compounds have been used for human benefits and in many industrial applications. The halophilic microalga *Dunaliella parva* is rich with natural carotenoids such as β-carotene. To enhance carotenogenesis, an external phytoene synthase gene (*PSY*) from *Synechocystis* sp. strain PCC 6803 was successfully cloned into *D. parva* cells (*D-PSY*). *D-PSY* showed higher carotenoids value (3.8 mg g^{-1} DW) compared to the wild-type (WT) strain (2.5 mg g^{-1} DW). For further assessment, both WT and *D-PSY* strains were subjected to two different stresses (polyethylene glycol (PEG) and $CaCl_2$). Application of $CaCl_2$ enhanced the chlorophylls (Chl a, Chl b) and carotenoids contents of both strains compared to PEG. The maximum carotenoids contents were obtained by treatment of *D-PSY* strain with 30 ppm PEG (7.0 mg g^{-1} DW) and 60 ppm $CaCl_2$ (6.0 mg g^{-1} DW). The soluble carbohydrates contents of *D-PSY* cells recorded high value (83.5 mg g^{-1} DW) compared to that of WT strain (52 mg g^{-1} DW). While 60 ppm $CaCl_2$ significantly stimulated the carbohydrate contents of both WT and *D-PSY* (58.4 and 117 mg g^{-1} DW, respectively), 30 ppm PEG showed an inhibitory effect (50.4 and 58.5 mg g^{-1} DW of WT and *D-PSY*, respectively).

The antioxidant activities of the carotenoids samples were analyzed through five different assays. Generally, the antioxidant activities of *D-PSY* carotenoids were superior to that of WT. Moreover, stress applications (30 ppm PEG and 60 ppm $CaCl_2$) enhanced the antioxidant activities of the carotenoids samples of both strains.

1. Introduction

Carotenoids compounds reported to be tetraterpenes which have eight unconjugated double bonds and are made up of 40 carbon atoms. They are present naturally in living organisms ranging from prokaryotes (e.g. bacteria) to eukaryotes as algae and higher plants (Basu et al., 2001). Carotenoids have been extensively used as a natural nontoxic food colorant (Mortensen, 2006), nutrient supplement (Bramley, 2003), feed additives (Bhosale and Bernstein, 2005), and as components in many other industrial purposes such as nutraceuticals, cosmetic, and pharmaceutical applications (Srinivasan et al., 2017).

Foods rich in carotenoids have been considered as possible protectors against some chronic diseases including cancer, cardiovascular disease, cataracts and other age-related diseases (Basu et al., 2001). Moreover, previous studies reported the antioxidative power of carotenoids which reduce the harmful effects imposed on living organisms as a result of oxidative stress (Tian et al., 2007; Yeum et al., 2009).

The high economic value of carotenoids encouraged researchers to seek available natural sources rich with carotenoids and to increase carotenoid biosynthesis through different techniques as exposure to abiotic stress and genetic engineering tools (Ben-Amotz and Avron, 1983; Tian et al., 2007). One of the best commercial sources of carotenoids was the green microalga *Dunaliella* spp. They are motile halophilic unicellular microalgae, characterized by the enormous accumulation of carotenoids (especially β-carotene) under stress conditions e.g. high temperature, light, and salt concentration (Lv et al., 2016; Srinivasan et al., 2017; Shang et al., 2018).

Interestingly, salinity and osmotic stresses are widespread environmental problem that affect algae and other living organisms. Specifically, calcium ($CaCl_2$) considered as an essential nutrient that regulates the growth, pigment and development of algae and improves their productivity. It also plays significant role in membranes stabilization, signal transduction and control of enzyme activity (Srivastava et al., 2013). However, like other salts, the increased concentration

Abbreviations: PEG, Polyethylene glycol; *PSY*, Phytoene synthase gene; WT, Wild type strain; *D-PSY*, Transgenic strain

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https://doi.org/10.1016/j.bcab.2018.09.011
Received 19 March 2018; Received in revised form 6 September 2018; Accepted 11 September 2018
Available online 13 September 2018
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generates an oxidative stress which disturbs the homeostasis of the algal cells (López-Lefebre et al., 2001).

Moreover, polyethylene glycol (PEG) is a water soluble polymer widely applied in experimental research to mimic drought and induce an osmotic stress condition by decreasing the water potential of the algal cell (Zagallai et al., 2005). The salinity and osmotic stresses have a critical induction effect on carotenogenesis in algae. The accumulation of carotenoids was supposed as a protecting mechanism for the algal cells against different stresses, which accounts for their wide distribution in diverse habitats (Ben-Amotz and Avron, 1983; Singh et al., 2016; Shang et al., 2018). However, the prolonged exposure to stresses may results in high mortality of the algal cells and serious reduction of carotenoids contents and photosynthetic efficiency (Ben-Amotz and Avron, 1983; Benavente-Valdés et al., 2016).

The biosynthetic process of carotenoids was located in plastids and achieved through several steps including desaturation, cyclization, hydroxylation, and epoxidation (Wang et al., 2014). One of the major limiting enzymes in carotenoids biosynthesis is the phytoene synthase (PSY). PSY irreversibly directed the dimerization (condensation) of two molecules of geranylgeranyl pyrophosphate (GGPP) to phytoene (a 40-carbon intermediate in the carotenoids biosynthesis) (Srinivasan et al., 2017). Previous studies showed the increased carotenogenesis by the insertion of *PSY* e.g. in *C. reinhardtii* (Cordero et al., 2011), marine diatom *Phaeodactylum tricornutum* (Kadono et al., 2015), rice (Nandakumar et al., 2008) and carrot plant (*Daucus carota*; Wang et al., 2014). Moreover, Liang et al. (2017) investigated the involvement of salt and light-regulated elements which may responsible for the up-regulation of *PSY* in *D. bardawil* under stress. However, Shang et al. (2018) reported the independent *PSY* regulation under stress conditions.

The present study aimed to 1) maximize the carotenoid production of *D. parva* by assessment of overexpression of additional *PSY* (from the cyanobacterial *Synechocystis* sp. strain PCC 6803); 2) to investigate the production and antioxidant properties of the carotenoids under two stress factors; polyethylene glycol and calcium treatments.

2. Materials and methods

2.1. Algal strain, medium and culturing condition

Dunaliella parva strain was obtained from the Phycology Lab, Faculty of Science, Zagazig University, Egypt. The culturing of axenic *D. parva* was done according to (Borowitzka, 1988) on modified Johnsons medium (pH 7.5) contained 1.5 M NaCl. The culture media (98 ml each) were distributed in Erlenmeyer flasks (capacity of 250 ml) and autoclaved. After cooling, the flasks were inoculated aseptically with 2 ml of *D. parava* mid-log culture-inoculum (50×10^3 cells ml^{-1}), and then incubated under light: dark cycle (16:8 h) with cool white fluorescent light (50 μmol photons $m^{-2} s^{-1}$) at 25 ± 2 °C. The cultures were hand-shaken twice daily.

2.2. Isolation, amplification and cloning of PSY gene

The *PSY* gene (GenBank accession No. X69172.1) was isolated from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (kindly obtained from Department of Botany and Microbiology, Faculty of Science, Helwan University, Ain Helwan, Egypt) and maintained on BG-11 culture medium as recommended previously (Stanier et al., 1971). Firstly, *PSY* of *S. elongatus* was extracted from mid-log culture (8 days old) following the protocol of Murray and Thompson (1980). Secondly, the *PSY*-DNA sequence was amplified by PCR amplification protocol. Briefly, in a 25-μl reaction mixture, 2 μl of extracted *S. elongatus* DNA was added to 0.5 μl of each of specific forward (Psy-Fw*Nco I*, 5`-ACG TCCATGGCTAACGGTCAATTTCT-3`) and reverse (Psy-Rev*Xba I*, 5`-ACTATCTAGATTAAAGCACCTGGCCCGCA-3`) (SibEnzyme, Estonia) primers (10 μM; engineered to have *Nco I* and *Xba I* recognition sequences, as underlined respectively), 2.5 μl PCR buffer (10×), 0.5 μl dNTPs (100 μM), 0.25 μl (1 U) *Taq* DNA polymerase (Thermoscientific, USA), and made up to total volume (25 μl) by sterile ribonuclease-free water. PCR amplification was then applied by Master Cycler Gradient PCR (Techne Genius, USA) following the PCR thermal cycle: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, followed by 1 cycle of 72 °C for 5 min. After gel-verification, the final PCR product (1030 bp) was purified by Spin-PCR purification kit (Thermo Scientific, USA) and cloned into the expression vector pTRA-K-TL-cTP (7820 bp, pre-digested by *Nco I* and *Xba I*; Fig. S1A) using T4-DNA ligase (Thermo Scientific, USA). The cloned pTRA-K-TL-PSY vector (Fig. S1B) was then transformed into *E. coli* strain DH5α for propagation screened LB containing 25 μg ml^{-1} kanamycin. However, due to the resistance of the algal cells to high concentrations of kanamycin (which reached 600 μg/ml), it was not suitable as a selective marker for discriminating the transgenic algal cells, therefore was replaced by phosphoinotricin marker (BASTA herbicide). For this purpose, the pTRA-K-TL-PSY vector was firstly extracted (from the DH5α cells), purified, and then digested with *PmeI* and *AscI* (done singly). A new expression vector (pTRA-PT-GlcF; 8691 bp; Fig. S1C) containing BASTA- marker was digested by the same *AscI* and *PmeI* enzymes. Finally, a ligation reaction was done between the *PSY* and the pre-digested vector (by T4-DNA ligase) to have the recombinant pTRA-PT-PSY (Fig. S1D) vector suitable for algal transformation.

The vectors used in this study (pTRA-K-TL-cTP and pTRA-PT-GlcF) were kindly obtained from Prof. Rashad Kebeish (Institute for Biology I, RWTH- Aachen University, Aachen, Germany). All handling procedures of the DNA were done following the standard methods of Sambrook et al. (1989).

2.3. Transformation of D. parva by PSY gene

The recombinant pTRA-PT-PSY (Fig. S1D) vector was then transformed into DH5α cells for propagation (screened on LB containing ampicillin 100 μg ml^{-1}), extracted and finally transformed into *Agrobacterium tumefaciens* strain GV 3101 (pMP90RK Gm^R, Km^R), Rif^R (Koncz and Schell, 1986) cells following the procedure of Höfgen and Willmitzer (1988). The transformed *A. tumefaciens* cells were screened on LB containing three different antibiotic markers, i.e. ampicillin, rifampicin (300 μg ml^{-1} for each), and kanamycin (25 μg ml^{-1}). The cloned pTRA-PT-PSY vector was verified by PCR reaction.

The transformed *A. tumefaciens* was used for the transformation of *D. parva* cells by *PSY* gene following the method of Anila et al. (2011). The transformed algal cells were suspended in liquid TAP and plated onto solid TAP containing Basta (Fig. S2A). The Basta-resistant cells of *D. parva* (grown on selection TAP medium) were transferred every 2 days into liquid TAP medium with a progressive increase in the concentration of NaCl, up to the standard 1.5 M, in order to adapt the cells successively (Fig. S2B). Finally, the cells were grown as usual in the standard *D. parva* growth medium. The transgenic *D. parva* cells were checked for validity and stability via PCR analysis using the specified gene primers (Fig. S3).

2.4. Treatment of D. Parva with polyethylene glycol (PEG) and $CaCL_2$

To investigate the effect of PEG as well as $CaCl_2$ on the transgenic *D. parva* (*D-PSY*), with reference to the wild-type (WT) culture, different concentrations of polyethylene glycol (PEG-6000; 30, 60, 90 and 120 ppm) and $CaCl_2$ (60, 90 and 120 ppm) were added separately to Erlenmeyer flasks containing 98 ml *D. parva* culture medium for each treatment of the wild and *D-PSY* cells. The flasks were autoclaved, cooled, and inoculated with 2 ml of inoculums (8 days-old culture, 50×10^3 cells ml^{-1}) under aseptic conditions, and then incubated at 25 ± 1 °C. The experimental design included three replicates for each of the examined stress concentration.

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