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Enhanced biomass production by *Phaeodactylum tricornutum* overexpressing phosphoenolpyruvate carboxylase

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ABSTRACT

Phaeodactylum tricornutum is an extensively studied model diatom and a promising candidate for biochemical and biotechnological engineering aimed at increasing biomass production. Phosphoenolpyruvate carboxylase (PEPCase), which converts phosphoenolpyruvate (PEP) to oxaloacetate (OAA) using bicarbonate, is a key metabolic enzyme of the tricarboxylic acid (TCA) cycle and the C4 photosynthetic pathway. Two transgenic *P. tricornutum* lines overexpressing PtPEPCase1 (PtPEPC1, JGI protein ID: 27976) were constructed and their photosynthetic productivity, cell growth, and biomass were characterized. The levels of PtPEPC1 mRNAs in the two transgenic lines were increased by 2.3 and 11.2-fold and the amounts of the PEPCase protein by 1.3 and 2.3-fold, respectively. PtPEPC1 was targeted to mitochondria. Addition of bicarbonate to the *P. tricornutum* culture increased biomass of the transformants by about 12% compared to wild type, and their maximum specific growth rate in exponential phase was about 10% greater than that of wild type. The transformants also exhibited higher photosynthetic productivity. We conclude that overexpression of mitochondrial PtPEPC1 enhanced both photosynthetic productivity and TCA cycle activity in *P. tricornutum*, thereby enhancing biomass production in the presence of dissolved inorganic carbon. Our findings suggest that *P. tricornutum* overexpressing PtPEPC1 can be used to mitigate rising atmospheric CO_2 levels or for sustainable microalgal biomass production.

1. Introduction

To develop a source of renewable and clean energy to replace fossil fuel, worldwide scientific efforts have focused on biomass energy that is produced through photosynthesis by autotrophic plants and microalgae [1–4]. Biomass is produced mainly by terrestrial and marine primary organisms, with approximately equal contributions [5]. Marine diatoms are responsible for 40% of the oceanic primary production of organic carbon [6]. Therefore, biomass produced by marine diatoms accounts for about 20% of the planet's primary production [7]. Diatoms are distributed widely throughout temperate oceans and even the Polar regions, and have developed photosynthetic CO_2 fixation adapted to a wide range of inorganic carbon concentrations [8,9]. They usually contain about 25% carbohydrate and 20% lipid under normal growth conditions [10,11]. Therefore, diatoms are promising candidates for biochemical and biotechnological engineering aimed at improving biomass and lipid production [12–14].

Phaeodactylum tricornutum is an extensively studied model marine diatom, and its full genome has been sequenced [15]. Over the last 20 years, genetic engineering tools for *P. tricornutum* were developed [16–18] and applied to enhance microalgal biomass or lipid production [19].

When microalgae fix inorganic carbon to synthesize organic compounds, phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31) converts phosphoenolpyruvate (PEP) to the four-carbon organic acid oxaloacetate (OAA) using bicarbonate. PEPCase has a higher affinity for CO_2 than Rubisco [20]. The Michaelis constant (K_m value) means the substrate concentration at which the enzyme reaction rate is a half maximum. The lower K_m value of PEPCase than that of Rubisco reflects a higher CO_2 fixation rate [21]. PEPCase also plays a crucial role in providing and replenishing the intermediates, OAA and/or malate, of the tricarboxylic acid (TCA) cycle which is a key metabolic pathway in bacteria, plants, and microalgae [22]. *Phaeodactylum tricornutum* has two PEPCases, similar to another diatom, *Thalassiosira pseudonana*, the green alga *Chlamydomonas reinhardtii*, and higher plants [23,24]. OAA produced by PEPCase is a main component of TCA cycle. The increase of OAA by the enhanced transcription of PEPCase could encourage abundant flow of TCA cycle and positive effects of metabolic control for the TCA cycle [25,26]. Thus, PEPCase could be an important enzyme in the production of biomass and in CO_2 mitigation [23].

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Our previous study described *P. tricornutum* PEPCase 1 (PtPEPC1), which has a higher sequence similarity with bacterial-type PEPCases of green algae and higher plants than with plant-type PEPCases [23]. PtPEPC1 expressed in and purified from an *E. coli* expression system was enzymatically active *in vitro*. To investigate the *in vivo* role of PtPEPC1 in *P. tricornutum*, in the present study we have generated transgenic *P. tricornutum* lines overexpressing PtPEPC1 and characterized their phenotypes. We confirmed PEPCase expression in *P. tricornutum*; the enzyme was located in the mitochondria. Overexpressed PEPCase increased cell growth and biomass accumulation upon bicarbonate addition or CO_2 aeration. These results suggest a promising engineering strategy for producing a high-biomass diatom strain that could be used for the production of biofuel and biological CO_2 mitigation technology.

2. Materials and methods

2.1. Strains and growth conditions

Axenic cultures of *P. tricornutum* Bohlin (CCMP632) were purchased from the National Center for Marine Algae and Microbiota (NCMA) at the Bigelow Laboratory for Ocean Sciences (East Boothbay, ME, USA). The cells were grown in a shaking incubator (130 rpm) in f/2 + Si medium (ASW) with 40 mM Tris-HCl, pH 7.4 at 20 °C under a 12L:12D cycle; light was provided by fluorescent lamps (~50 μmol m^{-2} s^{-1}). Normally, cells were grown without addition of CO_2 or bicarbonate. For the experiments, 10 mM bicarbonate or bubbling with air containing 2% CO_2 (flow rate: 80 mL/min) was added to the medium. The pH of the medium with or without bicarbonate was 7.4–7.5.

2.2. Vector construction

Plasmid PhaT-BP-PtPEPC1 was constructed from the previously designed plasmid pPhaT-BP-Luc [18]. To remove the fragment encoding luciferase, pPhaT-BP-Luc was digested with *Xba*I and *Spe*I. The full-length cDNA of the open reading frame encoding PtPEPC1 (XM_002180991) was amplified with specific primers (forward (Fw), 5′-ACTAGTATGTTGTCGTCTTCCTGCCG-3′; reverse (Rv), 5′-ACTAGTTTAACCAGTGTTGCCCATTC-3′; the *Spe*I sites are underlined) and cloned into pPhaT-BP-Luc, whose luciferase gene was removed. For tagging with enhanced yellow fluorescence protein (EYFP), the *eyfp* gene was amplified with the primers Fw (5′-TCTAGATGAGCAAGGGCGAGGAGCTG-3′; the *Xba*I site is underlined) and Rv (5′-ACTAGTTTACTTGTACAGCTCGTCCAT-3′; the *Spe*I site is underlined). The *eyfp* fragment was excised with *Xba*I and *Spe*I and cloned into the pPhat-BT-PtPEPC1 digested with *Spe*I, resulting in pPhaT-BP-PtPEPC1:EYFP.

2.3. Transformation and screening of P. tricornutum for PtPEPC1 overexpression

M17 (1.1 μm in diameter) tungsten particles were coated with plasmids linearized with *Psi*I and *Apa*I; 5×10^7 *P. tricornutum* cells in exponential phase were plated in the center of f/2-Si 1.2% agar plates. Particle bombardment was performed using a Biolistic Particle Delivery System PDS-1000/He (Bio-Rad Laboratories, CA, USA) fitted with 1550 psi rupture discs as recommended by the manufacturer. Gene transfer was performed essentially as described by Falciatore et al. [17]. Cells were spread on an f/2 medium agar plate containing 100 μg mL^{-1} Zeocin, and resistant colonies were selected for 2–3 weeks. Colonies were picked onto the new plates, and transformants that grew well were screened by colony PCR, which was performed as follows. Cells were picked from agar, suspended in distilled water, and used as a template for PCR with rTaq 5× PCR Master Mix (ELPIS-Biotech, Daejeon, Korea). Primers used for detection of exogenous fcpB::PtPEPC1 were 5′-CCCATGCACGACGTTGTAAAACGA-3′ (Fw) and 5′-GTTTGGTCAGCGGCTTGCTTCG-3′ (Rv). Internal transcribed spacer (ITS) was amplified as a positive control with the primers 5′-TCCGTAGGTGAACCTGCGG-3′ (Fw) and 5′-TCCTCCGCTTATTGATATGC-3′ (Rv). Quantitative real-time PCR (qRT-PCR) was performed as described by Seo et al. [18]. Total RNAs were purified at 5 h after light exposure for qRT-PCR analysis and the relative expression levels of *PtPEPC1* gene were obtained from wild type and two transformants. All values are presented as the means of two technical replicates for each of independently prepared biological samples ($n = 3$) with standard deviation. Primers used for qRT-PCR for the *PtPEPC1* gene were 5′-GTCAACTTCACATGGCGACG-3′ (Fw) and 5′-TGGCAACCCCTGTAATGGTC-3′ (Rv).

2.4. Southern blot analysis

Genomic DNA of *P. tricornutum* was extracted according to Murray and Thompson [27]. PCR was performed using genomic DNA from wild-type and transgenic *P. tricornutum* as a template. Primers were the same as for colony PCR. Genomic DNA (5 μg) digested with *Hin*dIII, *Spe*I, or *Eco*RV was separated on a 0.8% agarose gel and transferred onto a Hybond-N^+ membrane (GE Healthcare, NJ, USA). Partial *PtPEPC1* sequence was used as a probe, which was synthesized by PCR using the primers Fw (5′-CGAAAGATGCCGGTCGAATG-3′) and Rv (5′-TTAACCAGTGTTGCCCATTCCATT-3′); the probe was labeled and hybridized using Amersham AlkPhos Direct Labelling Reagents (GE Healthcare). For detection, Amersham CDP-Star Detection Reagents (GE Healthcare) were used according to the manufacturer's instructions.

2.5. Immunoblotting

Cultures were harvested by centrifugation at 2000 × *g* for 15 min. Pellets were suspended in extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% SDS), and 1× protease inhibitor cocktail (Thermo Scientific, IL, USA). The cells were lysed by sonication (4 × 30 s). Extracted total protein was measured by using Pierce BCA protein assay kit (Thermo Scientific) and loaded (20 μg per lane) on a 10% SDS-PAGE gel. The gel was stained with Coomassie Blue to ensure equal loading. Alternatively, separated proteins were electro-transferred to a PVDF membrane for immunoblotting. Primary antibody against PtPEPC1 (Abfrontier, Seoul, Korea) was used at a dilution of 1:1000. The antibody was raised in a rabbit injected with a mixture of two PtPEPC1 peptides (NH_2-KTWRDQGAGRDPSTKQAADQ-COOH and NH_2-QELAILQKRRSGDKSASG-COOH). Band intensities were quantified by using Image J software and expressed as arbitrary units.

2.6. Growth and mass analysis

Batches were inoculated with 10^6 cells mL^{-1} in exponential growth phase. Growth was determined by measuring the optical density value at 750 nm (OD_{750}) with a spectrophotometer. The values were converted into the cell number calculated with correlation equation between the cell number and optical density. The cell number was estimated by cell counting with a Neubauer chamber.

Cultures were harvested with 1.2 μm Isopore membrane filters (RTTP; Merck Millipore, Cork, IRL). Before filtration, empty filters were weighed. In exponential, transition (exponential to stationary), and stationary phases (3, 5, and 7 days of culture, respectively), 10 mL of cultures were filtered. The filters were fully dried in a 65 °C chamber for 24 h and weighed, and then cell weight was calculated.

Growth rate (μ) per culture day was estimated using the equation [28]

$$\mu = \frac{1}{t_2 - t_1} \cdot \left[\ln\left(\frac{C_2}{C_0}\right) - \ln\left(\frac{C_1}{C_0}\right) \right]$$

where 1 and 2 refer to an earlier and later days of culture, and C is biomass (cell mass) concentration at any time, t. calculated from optical