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Identification of a putative patatin-like phospholipase domain-containing protein 3 (PNPLA3) ortholog involved in lipid metabolism in microalga *Phaeodactylum tricornutum*

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ABSTRACT

Patatin-like phospholipase domain-containing protein 3 (PNPLA3) has been associated with nonalcoholic fatty liver disease which promoted hepatic lipid synthesis. With high lipid content, microalgae are found to be a potential source of biofuel. *Phaeodactylum tricornutum*, a fast-growing and oleaginous microalga, is a promising target for enhancing the biofuel by means of metabolic engineering. Here a putative PtPNPLA3 gene from *P. tricornutum* was cloned and characterized in transgenic *P. tricornutum* for the first time. Amino acid sequence analysis showed a high homology between PNPLA3 from *P. tricornutum* and other organisms. Overexpression of PtPNPLA3 increased the transcript level of PtPNPLA3 by 70% in transgenic microalgae compared to wild type. The neutral lipid content in transgenic microalgae was significantly increased by 70%, representing a notable enhancement of the lipid productivity in the transgenic microalgae. The fatty acid profile was also altered as determined by GC–MS analysis, with a significant increase of C20:4 in the transgenic microalgae compared to wild type. This work identified a microalgal PNPLA3 and proved it to be an important node in regulating lipid accumulation in microalgae, also demonstrating an efficient way to improve lipid productivity in microalgae by metabolic engineering.

1. Introduction

The global warming, reducing global oil reserves and incessant consumption of fossil fuels have provoked remarkable interest in using renewable biological energy as alternative fuel source. Microalgae are considered as the potential source of biodiesel due to its rich biomass and high lipid content. These microalgae synthesize triacylglycerol and have the capability to generate high amount of lipids by means of photoautotropism than oil crops [1]. *Phaeodactylum tricornutum* is a unicellular marine diatom drawing a significant attention of the researchers for the biofuel production because of its oleaginous nature, fast growth, and available sequenced genome. This diatom can accumulate the potential renewable energy resources and can be cultured in the simple medium with low quality water that do not encroach farmlands. As microalgae are considered to afford the raw material for future biofuel requirement, there is a need for the extensive research in microalgae model systems for the better understanding of triacylglycerol (TAG) production that leads to the commercialization of microalgae based biofuel [2].

Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is a membrane-bound protein and exists in the liver which causes the fatty liver disease with triacylglycerol accumulation and storage [3,4]. PNPLA3 accumulated on lipid droplets and caused hepatic steatosis in Pnpla3I148M knockin mice [3]. There are very few reports on the PNPLA3 protein expression and its role in the lipid metabolism other than animals. PNPLA3 protein expressed in yeast *Pichia pastoris* showed high triglyceride hydrolase activity with low lysophosphatidic acid acyl transferase activity (LPAAT) [5]. PNPLA3 purified from *Escherichia coli* had a higher triglyceride synthase activity with lysophosphatidic acid acyl transferase activity (LPAAT) [6]. So far, no study has been reported on algal PNPLA3.

Metabolic engineering is a promising tool to genetically improve the microalgal strains with increased TAG accumulation by expressing certain key genes [7,8]. As an ideal biological resource of renewable energy, *P. tricornutum* is a good candidate to be genetically improved. Still modifications in terms of enhancing the lipid accumulation capability of *P. tricornutum* will represent a major progress in the field of microalgal biofuels. In this study, we identified a putative PNPLA3 in microalgae for the first time and successfully demonstrated the higher lipid accumulation in transgenic diatom by using PNPLA3 as a target.

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2. Materials and methods

2.1. Strain and culture conditions

Phaeodactylum tricornutum Bohlin was obtained from the Freshwater Algae Culture Collection, Institute of Hydrobiology, CAS, China (No: FACHB-863). The microalgae were grown as batch cultures in f/2 medium without $Na_2SiO_3 \cdot 9H_2O$. The seawater with f/2 medium was filter-sterilized through a 0.22 μm membrane. *P. tricornutum* was grown at 21 ± 1 °C in an artificial climate incubator provided with cool-white fluorescence light of 200 μmol photons $m^{-2} \cdot s^{-1}$ under 12/12 h light/dark. Growth curve was determined by counting with Brightline hemocytometer using microscope every day.

2.2. Cloning, vector construction and algae transformation

The amino acid sequence of putative PNPLA3 was predicted by BLAST at NCBI against PNPLA3 of *Homo sapiens*. Amino acid sequence similarity and alignment among species was determined by ClustalW and the phylogenetic tree of PtPNPLA3 was established by using MEGA5. The subcellular localization was predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP/). Total RNA of *P. tricornutum* was extracted using Plant RNA kit (Omega, USA) and employed for cDNA synthesis using ReverTra Ace -α- kit (Toyobo, Japan). The 1041-bp length cDNA of PtPNPLA3 gene was amplified by PCR with primers Pt801 and Pt802 designed from PtPNPLA3 sequence (Table 1).

The full-length PtPNPLA3 ORF was cloned into a previously constructed vector [8] by homologous recombination using ClonExpress II One Step Cloning kit (Vazyme, China). The recombined expression vector pHY18–PtPNPLA3 was cloned under the control of fcpC promoter and fcpA terminator from fucoxanthin chlorophyll a/c binding protein genes of *P. tricornutum*. *P. tricornutum* was transformed with the expression vector by electroporation with a Bio-Rad GenePulser Xcell apparatus (Bio-Rad, USA) as per the protocol reported [8].

The transformed algal cells were transferred into liquid medium and cultured at shaker in dark for 24 h. After 24 h the cells were transferred into the solid selection medium supplemented with chloramphenicol (250 μg/mL). The growing transformed cells in the selection medium were picked and grown in a liquid medium with chloramphenicol and subcultured every week.

2.3. Detection of transgenic microalgae by molecular approaches

The integration of the gene into transgenic cells was confirmed by PCR. Genomic DNA was extracted from transgenic diatom cells using HP Plant DNA kit (Omega, USA) and used as the template for PCR to detect the integration of *CAT* gene present in the pHY18–PtPNPLA3 using *CAT* gene primers.

The expression of PtPNPLA3 mRNA was quantified by quantitative real-time PCR (qPCR) using SYBR Green qPCR SuperMix (Invitrogen, USA) on ABI PRISM® 7500 Sequence Detection System (ABI, USA). Total RNA was prepared from transformed cells and wild type cells using RNAiso plus kit (Takara, Japan), first strand cDNA was prepared using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan).

Table 1
List of primers used in this study.

Primer	Sequence (5′ → 3′)
Pt801	ACAATTACAATCCAGTGGTACCATGGTGGCTCTACAGGCT
Pt802	GAGTTTTTGTTCCAGGTGTGTTTGCACCACATTTGTACTTAT
Pt366	ATGGAGAAAAAAATCACTGGATATACC
Pt367	TTACGCCCCGCCCTGCCACT
PNPLA3-qf	GTCAGCAACAGCATTTCACG
PNPLA3-qr	GAGCGGAATCACCTTGTCTT
ACT1-f	AGGCAAAGCGTGGTGTTCTTA
ACT1-r	TCTGGGGAGCCTCAGTCAATA

The reactions were performed in 8 strip PCR tubes in a reaction volume of 20 μL following the manufacturer's instruction (ABI, USA). The native β-actin gene was used as the reference gene with a forward primer (ACT1-f) and reverse primer (ACT1-r). The threshold cycle (Ct) value for PtPNPLA3 in both transgenic and wild type cells was then normalized by using the corresponding reference gene. Primer sequences used in this study were listed in Table 1.

To confirm the expression of PtPNPLA3 protein in transgenic microalgae, Western blot analysis was carried out. About 200 mL of the stationary phase microalgae cells (subculture about 7 days, 8×10^6 cells/mL) were harvested at 4400 rpm for 10 min at 4 °C. Total protein was extracted from the harvested cells using total DNA/RNA/Protein kit (Omega, USA), and protein concentration was detected by BCA protein determination method. β-actin from *P. tricornutum* was used as reference in Western blot. Protein fraction was subjected to electrophoresis on 10% SDS-PAGE and separated proteins were transferred to PVDF membrane for Western blotting. Thereafter, membrane was blocked with non-fat milk for 2 h at 4 °C. The membrane was treated with primary anti-Myc antibody (1:3000, Sigma-Aldrich, USA). HRP-conjugated goat anti-rabbit antibody (CST) at a dilution of 1:5000 was used as secondary antibody. Membrane development was performed using chemiluminescent system.

2.4. Neutral lipid analysis

The neutral lipid content of *P. tricornutum* was determined by Nile red staining method as per the protocol reported by Yang et al. [9]. To 300 μL of algal culture, 3 μL of Nile red (0.1 mg/mL, in acetone; Sigma Aldrich, USA) was added, then mixed by rapid inversion and incubated in dark at room temperature for 20 min. The stained cell culture, unstained cell culture and stained seawater medium were transferred to 96-well plate and the fluorescence intensity was determined by a microplate reader (Bio-Tek, USA) with a wavelength of 530 nm for excitation and a 592 nm for emission. The intensity of relative fluorescence gave a contrast of neutral lipid content between transgenic and wild type cells. The percentage (%) of neutral lipid content in *P. tricornutum* dry weight was estimated according to the protocol reported by Huang et al. [10]. To 20 mg of lyophilized cells, 2 mL of chloroform, 2 mL of methanol and 1 mL of 5% sodium chloride were added and subjected to vortex for 2 min. The content was centrifuged at 8000 × *g* for 4 min and chloroform layer was collected and this step was repeated thrice. The collected samples were evaporated to dry under nitrogen gas and further dried in oven at 60 °C for at least 10 h. Finally, the dry weight was weighed in electronic balance.

2.5. Fatty acid composition analysis

Lipids were extracted from transgenic and wild type cells. Fatty acid composition was analyzed as FAMEs by Gas Chromatography–Mass Spectrometry (GC–MS) according to Yang et al. [9]. Fatty acids were analyzed using the equipped NBS spectrum library. The integrated peak areas were determined and normalized to obtain the relative content of fatty acid composition.

2.6. Morphological observation of P. tricornutum

To observe the oil bodies in *P. tricornutum*, cells were stained with Nile red fluorescence dye and observed under a fluorescence microscope. One milliliter microalgae cells were stained with 10 μL of Nile red (0.1 mg/mL in acetone) and incubated in darkness for 20 min. A laser-scanning confocal microscope Zeiss LSM 510meta (Zeiss, Germany) with excitation wavelength of 488 nm and emission wavelength of 505–550 nm was used to observe the morphology of the stained cells.