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Glucose-6-phosphate dehydrogenase as a target for highly efficient fatty acid biosynthesis in microalgae by enhancing NADPH supply

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ABSTRACT

Oleaginous microalgae have great prospects in the fields of feed, nutrition, biofuel, etc. However, biomass and lipid productivity in microalgae remain a major economic and technological bottleneck. Here we present a novel regulatory target, glucose-6-phosphate dehydrogenase (G6PD) from the pentose phosphate pathway (PPP), in boosting microalgal lipid accumulation. G6PD, involved in the formation of NADPH demanded in fatty acid biosynthesis as reducing power, was characterized in oleaginous microalga *Phaeodactylum tricornutum*. In G6PD overexpressing microalgae, transcript abundance of G6PD increased by 4.4-fold, and G6PD enzyme activity increased by more than 3.1-fold with enhanced NADPH production. Consequently, the lipid content increased by 2.7-fold and reached up to 55.7% of dry weight, while cell growth was not apparently affected. The fatty acid composition exhibited significant changes, including a remarkable increase in monounsaturated fatty acids C16:1 and C18:1 concomitant with a decrease in polyunsaturated fatty acids C20:5 and C22:6. G6PD was localized to the chloroplast and its overexpression stimulated an increase in the number and size of oil bodies. Proteomic and metabolomic analyzes revealed that G6PD play a key role in regulating pentose phosphate pathway and subsequently upregulating NADPH consuming pathways such as fatty acid synthesis, thus eventually leading to lipid accumulation. Our findings show the critical role of G6PD in microalgal lipid accumulation by enhancing NADPH supply and demonstrate that G6PD is a promising target for metabolic engineering.

1. Introduction

Incessant consumption of depleting fossil fuel resources and global climate change have stimulated the resurgence of alternative renewable biofuel research. Oil crops, oleaginous fungi and microalgae could be potential candidates for biofuel production. Lipid content and maximum lipid productivity of several oleaginous microalgae and fungi were listed in Table 1. Microalgae offer great potential as feedstock for a wide range of high-value products, including food and feed supplements, biofuels, bioactive compounds, etc. Oleaginous microalgae have garnered considerable research attention over terrestrial crops and oleaginous fungi, as these sunlight driven cell factories possess high growth rate, capability to grow in wide range of waters (fresh, marine or brackish) and accumulate high lipid content (Wijffels and Barbosa, 2010). Moreover, microalgal oil can be easily converted by transesterification to biodiesel. Thus, the exploitation of oleaginous microalgae as feedstock is a cornerstone of the burgeoning field of economic viable biofuel production. Recently, the unicellular diatom *Phaeodactylum tricornutum* has emerged as a model system for studying the molecular mechanisms underlying lipid metabolism (Yang et al., 2013). Functional genomic analysis of *P. tricornutum* revealed that it possesses special metabolic circuits such as the occurrence of plastidial triacylglycerol synthesis (Balamurugan et al., 2017).

With the availability of sequenced genomes of several microalgal species and the establishment of genetic transformation systems for few model microalgal species including *P. tricornutum* (Li et al., 2012, 2016; Xue et al., 2015), a critical issue is to identify key metabolic pathways and target genes for algal strain improvement. As lipids are highly reduced metabolites, *de novo* biosynthesis requires constant supply of NADPH as sole source of reducing power for reduction of acetyl groups (CH_3-CO-) into growing acyl chain of fatty acid (-CH_2-CH_2-) (Ratledge, 2014). Many genetic and biochemical aspects of fatty acid biosynthesis and its driving factor in eukaryotic oleaginous microalgae remain unclear. The pentose phosphate pathway (PPP) can generate NADPH

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Table 1
Lipid content and maximum oil production in some oleaginous microorganisms.

Oleaginous microorganism	Lipid content (%, w/w)	Oil productivity (mg L^{-1} day^{-1})	Reference
Phaeodactylum tricornutum	18–57	45	Rodolfi et al. (2009)
Nannochloropsis sp.	21.3–37.8	4.59–20.0	Huerlimann et al. (2010)
Chlorella ellipsoidea YSR03	32 ± 5.9	22.38	Abou-Shanab et al. (2011)
Pavlova salina CS49	30.9	49.4	Rodolfi et al. (2009)
Nannochloropsis oculata	22.7–41.2	84–151	Chiu et al. (2009)
Chlorella sorokiniana	19–22	45	Cuaresma et al. (2009)
Cunninghamella echinulata	57.7	562	Fakas et al. (2009)
Lipomyces starkeyi	47	1012	Huang et al. (2014)
Trichosporon coremiiforme	37.8	962	Huang et al. (2013)
Yarrowia lipolytica Po1g	48.02	1032	Tsigie et al. (2012)
Mortierella isabellina	65.5	633	Fakas et al. (2009)
Trichosporon capitatum	43.1	1100	Wu et al. (2011)
Umbelopsis isabellina	70	1895	Liu et al. (2017)

and pentose phosphates, thus providing 50–75% of the indispensable reducing equivalents for fatty acid synthesis in liver (Salati and Amir-Ahmady, 2001). It plays a critical role in maintaining the NADPH biosynthetic capability and redox homeostasis (Salati and Amir-Ahmady, 2001; He et al., 2014). Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the primary reaction in PPP and it has been identified in many organisms including animals, yeast, plants, as well as human (Legan et al., 2008; Lin et al., 2013; Loiudice et al., 2001; Zhang et al., 2008). Overexpression of G6PD may elevate the G6PD enzymatic activity, leading to the enhancement of NADPH biosynthetic capability, which can extend the life span of *Drosophila melanogaster* (Legan et al., 2008). In contrast, G6PD knockdown by small interfering RNA reduced intracellular lipid accumulation and attenuated adipocyte marker gene expression (Park et al., 2005). In addition, a decrease in serum lipoprotein concentrations and lipogenic rate was found in G6PD-deficient patients (Dessi et al., 1992). These reports propose the possibility of G6PD in promoting fatty acid synthesis; however, so far G6PD has yet to be characterized in algae. In this report we identified and characterized G6PD in algae, and demonstrated the potential role of G6PD in elevating lipid accumulation via lipidomic, proteomic and metabolomic strategies.

2. Materials and methods

2.1. Microalgal strain and growth conditions

Microalgal strain *Phaeodactylum tricornutum* was obtained from the National Center for Marine algae and Microbiota (NCMA, formerly the CCMP), USA (CCMP2561). Microalgae were grown as batch cultures in flasks containing f/2 medium without $Na_2SiO_3 \cdot 9H_2O$ (Yang et al., 2013). Cultures in liquid medium or on the plate were grown at 21 ± 1 °C in an artificial climate incubator, under a 15:9 h light/dark photoperiod provided by cool white fluorescence light with 200 μmol photons m^{-2} s^{-1} irradiance (Ningbo, China). For liquid culture, 100 mL culture was incubated in 250 mL conical flasks and no air or CO_2 was pumped for optimization of culture conditions in lab scale.

2.2. Gene cloning and analysis of G6PD

The 1434-bp full-length coding region of predicted G6PD of *P. tricornutum* (GenBank: XM_002183678.1) was amplified by reverse transcription PCR with primers: forward 5′-ACCATGATAATTTGCAGTCTCACTTTTTGC-3′ and reverse 5′-GTAAGTGCAGACGGAGGAGG-3′. The amplified cDNA was cloned into TA vector and confirmed by sequencing analysis. The resultant G6PD amplicon was cloned into a *P. tricornutum* expression vector pHY11 (Xue et al., 2015) by In-Fusion method (Clontech, CA, USA). Amino acid sequence similarity among species were examined using BLAST on NCBI (http://blast.ncbi.nlm.nih.gov/Blast/), then phylogenetic tree of protein clusters from species was constructed by neighbor-joining (NJ) method using software MEGA 5 (Tamura et al., 2007). Conserved domains of G6PD were predicted by using Conserved Domain Architecture Retrieval Tool (CDART) (Geer et al., 2002). The subcellular localization of G6PD was predicted by using online tools including Target P (http://www.cbs.dtu.dk/services/TargetP/), PSORT II (http://psort.hgc.jp/form2.html), and Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/).

2.3. Expression of G6PD in engineered microalgae

The recombinant expression vector pHY-G6PD was electroporated into microalgae using a GenePulser Xcell apparatus (Bio-Rad, USA) following the previously reported protocol (Xue et al., 2015). The transformed algal cells were cultured in f/2 liquid medium in darkness for 24 h and thereafter, the cells were harvested and cultured into the solid selection medium supplemented with chloramphenicol (250 μg/mL). The surviving colonies were picked up and grown in liquid medium with chloramphenicol and subcultured every week. To preclude the impact of chloramphenicol in engineered microalgae, cells were cultured in f/2 medium without chloramphenicol for 3 culture cycles prior to biochemical and molecular analyses. At least 3 replicate algal cultures were used for quantitative assays.

In order to detect the integration of the *G6PD* gene into transformed microalgae, genomic PCR was performed with genomic DNA extracted from transformants as the template. Sequences spanning 5'-G6PD and that in the transformation vector were chosen for PCR, with the forward (5'-ATGGAGAAAAAAATCACTG-3') and the reverse (5'-TAAGCATTCTGCCGACAT-3').

The relative abundance of *G6PD* transcripts in the log phase was quantified by quantitative real-time PCR (qPCR). Total RNA was extracted from microalage and reverse-transcribed with random hexamer primers using an Omniscript reverse transcription kit (Qiagen). The reactions were performed in 96-well plates with a final volume of 20 μL using SYBR Green Kit (Takara) and 7300 Sequence Detection System (Applied Biosystems) following the manufacturer's instructions. Gene specific primers were designed for G6PD (Forward 5'-TGACCGCTACGGCATCATAC-3' and reverse 5'-GCACATTCC TCCACGTCTCA-3'). The predicted β-actin which was annotated as actin like protein (ACT1, Phatrdraft_51157) in *P. tricornutum*, was used as internal reference with primers: forward 5'-AGGCAAAGC GTGGTGTTCTTA-3' and reverse: 5'-TCTGGGGAGCCTCAGTCAATA-3'. Each sample was assayed in triplicates and a control without template was performed with every assay. The threshold cycle (Ct) values for *G6PD* in both engineered and wild type (WT) cells were then normalized to the corresponding reference gene.

To examine the expression of G6PD protein in the engineered lines, western blot analysis was performed. The anti-Myc antibody was used to detect the recombinant G6PD proteins carrying Myc-tag at the C-