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# Manipulation of a glycolytic regulator alters growth and carbon partitioning in the marine diatom *Thalassiosira pseudonana*

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ABSTRACT

The regulation of carbon partitioning in diatoms is key to understanding mechanisms related to their high productivity, and the mechanisms controlling carbon flux towards different metabolic fates are currently not well understood. A unique variant of 6-phosphofructo-2-kinase/fructose 2,6 bisphosphatase (PFK2/F2BP), a regulator of glycolytic flux previously uncharacterized in single-cell photosynthetic eukaryotes, was identified in diatom genomes and overexpressed in *Thalassiosira pseudonana*. Overexpression resulted in increased activity of the glycolytic rate-determining enzyme phosphofructokinase (PFK) and altered carbon partitioning among intracellular carbohydrate, lipid, and protein levels. Higher PFK activity in transformant lines correlated with reduced growth rate and an extension of the G1 phase of the cell cycle, demonstrating a link between carbon metabolism and the control of cell cycle processes. While the relationship between growth and carbon flux under environmental stress (such as nutrient limitation) is well documented in diatoms, our data demonstrate that manipulation of carbon metabolism, independent of an environmental trigger (such as nutrient limitation), is sufficient to delay cell cycle progression. The inverse relationship between growth and PFK activity (as an indicator of glycolytic flux) differs from those described for other unicellular eukaryotes and supports an alternate organization and regulation of central carbon metabolism in diatoms, including a prominent regulatory role for PFK2/F2BP.

## 1. Introduction

Unicellular algae are among the most highly productive organisms in terms of converting $CO_2$ to biomass. In particular, marine diatoms contribute ~40% of the total primary productivity in the modern oceans [1], and thus are major players in the global carbon cycle. Diatoms are attractive candidates for use in biotechnology because they produce valuable carbon-based biopolymers such as lipid suitable for fuel production or other commercial applications [2]. In contrast to green algae, diatoms have a distinct evolutionary history involving a secondary endosymbiosis with a red alga [3] that resulted in increased genetic diversity [4,5] and a distinctive organization of carbon metabolism [6]. This includes unique compartmentation of glycolysis/gluconeogenesis, pyruvate metabolism, and carbohydrate biosynthesis [6,7]. For example, diatoms store carbohydrates as chrysolaminarin (a soluble β-1,3-linked glucan) in a cytoplasmic vacuole (Fig. 1), instead of storing starch in the plastid. In addition, diatom mitochondria contain a conserved glycolytic pathway for the production of energy and pyruvate (Fig. 1), a distinctive feature found only in diatoms and non-photosynthetic oomycetes [6–8]. These unique organizational differences have metabolic and energetic implications that ultimately relate to the productivity of diatoms.

Informed genetic engineering of diatom metabolism requires knowledge of the regulation of carbon flux and partitioning within cells. Nutrient limitation causes shifts in carbon partitioning in diatoms, during which growth slows and biomolecules accumulate as trade-offs between carbohydrate, lipid, and amino acid metabolism occur [9–17]. In *Skeletonema costatum*, short-term nitrogen limitation mobilizes storage carbohydrate to produce amino acids in the dark [17]. In *Phaeodactylum tricornutum*, more severe nitrogen limitation triggers triacylglycerol (TAG) accumulation [11,18], derived in part from carbon repurposed from amino acids [14,15]. Silicon starvation also stimulates TAG accumulation in most diatom species [13,16,18,19]. In *Cyclotella cryptica*, silicon starvation-induced lipid accumulation is due to both *de novo* lipid synthesis and redistribution of carbon from non-lipid compounds, such as carbohydrate, into lipids [16]. Although the effects of nutrient limitation on growth and carbon metabolism are well documented, the mechanisms involved have not been elucidated. A potentially fruitful approach would be to genetically manipulate carbon flux independent of nutrient limitation, which would enable an independent

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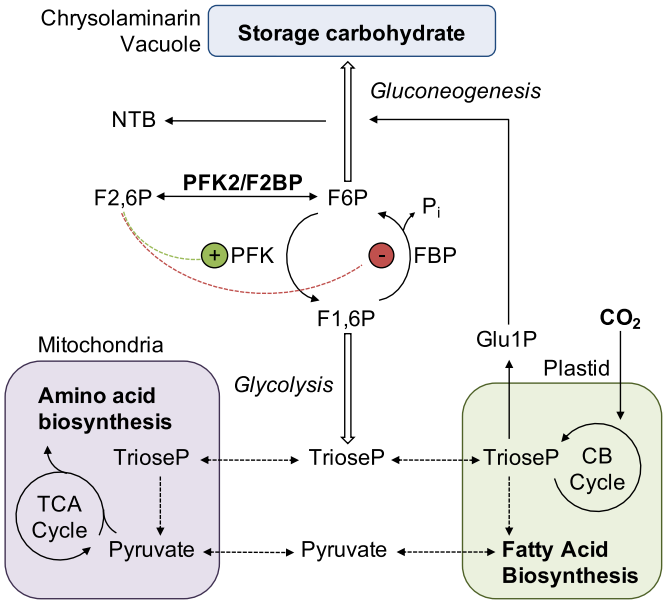


**Fig. 1.** Model for the regulatory influence of phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/F2BP) on carbon flux in diatoms. Key: F1,6P = fructose 1,6-bisphosphate; F2,6P = fructose 2,6-bisphosphate; F6P = fructose 6-phosphate; FBP = fructose-1,6-bisphosphatase; PFK = phosphofructokinase; Glu1P = glucose-1-phosphate; NTB = nucleotide biosynthesis. PFK2 activity produces more F2,6P, which allosterically activates PFK (promoting glycolysis) and inhibits FBP (reducing gluconeogenesis).

evaluation of the effect of carbon flux on growth.

In eukaryotic cells, a primary decision point in central carbon metabolism is the second bypass of glycolysis, where carbon is unidirectionally shunted towards either glycolysis or gluconeogenesis (Fig. 1). Enzymes that regulate this metabolic checkpoint are allosterically controlled by fructose-2,6-bisphosphate (F2,6P), which plays a key role in the control of carbohydrate metabolism in eukaryotes [20] by affecting phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBP) (Fig. 1). The reciprocal regulation of these pathways prevents futile cycling of glycolytic intermediates and exerts control over carbon storage, cellular energetics and cell cycle progression [21–23]. This important allosteric regulator is phosphorylated and dephosphorylated by the bifunctional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/F2BP) [24]. The N-terminal kinase domain of the PFK2/F2BP protein phosphorylates fructose 6-phosphate (F6P) to produce F2,6P, while the C-terminal phosphatase domain performs the reverse reaction.

The modulation of carbon flux at the second bypass can affect carbon partitioning into biosynthetic pathways (including carbohydrate, protein, and lipid synthesis), with larger consequences for growth and productivity. Manipulation of F2,6P levels in model eukaryotic systems affects carbon partitioning and cell proliferation. In mammals, overexpression of PFK2 increased glycolytic flux into lipids [25,26], and elevated F2,6P levels may maintain high glycolytic rates characteristic of cancer cells [23,27,28]. In plants, PFK2/F2BP coordinates the rate of sucrose synthesis in the cytosol with carbon fixation and plastidic starch production [29–31]. Elevated F2,6P levels increased partitioning of carbon into starch synthesis, while reduced levels increased partitioning into sucrose [31–33]. Overall the role of PFK2/F2BP in multicellular organisms relates to controlling the rate of glycolysis to support cell proliferation. The situation is more complicated in heterotrophic unicellular eukaryotes such as yeast, where changes in F2,6P had little phenotypic effect or resulted defective cell division [34,35]. The functional roles of these important metabolic regulators have not been explored in microalgae.

Despite the demonstrated importance of PFK2/F2BP in controlling carbon partitioning and productivity in other organisms, microalgal PFK2/F2BP has not yet been characterized. Previous analysis suggested that diatoms possess two functionally distinct forms of PFK2/F2BP, with one form containing a critical amino acid substitution (Fig. S1) that suggests it functions solely as a kinase to produce fructose-2,6-bisphosphate and promote glycolysis [7]. Given the lack of previous investigation in microalgae, and the demonstrated vital role for these enzymes in central carbon flux in other eukaryotes, we chose to further investigate the effect of PFK2/F2BP on the major carbon storage pools and growth in marine diatom *Thalassiosira pseudonana*. We demonstrate for the first time in a microalgal system that PFK2 influences glycolysis, with consequences for growth and productivity in terms of carbohydrate, protein, and lipid storage.

## 2. Materials and methods

### 2.1. Sequence analysis and phylogeny

*T. pseudonana* PFK2/F2BP sequences were obtained from the JGI genome browser (http://genome.jgi.-pdf.org/cgi-bin/searchGM?db=Thaps3) and gene models were manually adjusted based on RNAseq coverage (Fig. S2). The models were used to search the NCBI database and a custom database containing diatom transcriptomes from the Marine Microbial Eukaryote Transcriptome Sequencing Project for PFK2/F2BP sequences. Truncated sequences containing only one of two conserved domains were excluded. Fifty-one amino acid sequences were manually curated and aligned using MAFFT with default parameters [36] using Geneious [37]. MEGA5 was used to determine the best-fit substitution model (WAG + G) and to estimate the maximum likelihood tree using 1000 bootstrap replicates. FigTree software was used to generate the final graphical representation. Sequences analyzed are included in Supplemental File 1.

### 2.2. Vector construction and diatom transformation

Destination and expression vectors were constructed using MultiSite Gateway Technology (Life Technologies) as described previously [38]. The PFK2-2 overexpression construct (Fig. S3) was generated by cloning the manually adjusted Thaps3_109629 open reading frame upstream of *eGFP* in a modified version of the constitutive expression vector pTpfcpGFP [39]. Expression vectors were cotransformed with pMHL_9, a vector expressing the *nat1* gene under the control of the acetyl coenzyme A carboxylase promoter. The construct pMHL_17 containing *eGFP* under the control of the fcp promoter was used as a control for effect of *eGFP* expression (Fig. S3). Vectors were introduced into *T. pseudonana* cells using the Biolistic DS-1000/He particle delivery system as described in [40].

### 2.3. Cultivation and sampling

Axenic cultures of *T. pseudonana* (CCMP 1335) were grown in ASW media [41] under constant illumination at 150 μmol $m^2$ $s^{-1}$. Initial screening of 50 ml cultures was done in 125 ml flasks grown at 20 °C on a rotary shaker. Larger scale experiments for biochemical characterization were done in 2 L volume at 22 °C with magnetic stirring and aeration with sterile air. For synchrony experiments, exponential phase cultures were harvested by centrifugation (4000 × *g* for 7 min), rinsed once in silicon-free medium, and resuspended in silicon-free ASW medium at a density of approximately $1 \times 10^6$ cells $ml^{-1}$. Cell counts were performed using a Muse® Cell Analyzer (Millipore Corp., Billerica MA, USA).

### 2.4. Fluorescence microscopy

Diatom transformants expressing eGFP were imaged with a Zeiss Axio Observer Z1 inverted microscope equipped with an ApoTome and