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Silencing UDP-glucose pyrophosphorylase gene in *Phaeodactylum tricornutum* affects carbon allocation

Research Paper

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The effects of the suppression of UDP-glucose pyrophosphorylase (UGPase) on chrysolaminaran biosynthesis and carbon allocation were investigated in *Phaeodactylum tricornutum*. The 69% decrease in UGPase activity was accompanied by a 4.89 fold reduction in *Ugp* transcript abundance. Inactivation of UGPase in *P. tricornutum* led to a significant decrease in chrysolaminaran content and an increase in lipid synthesis. These findings suggest that UGPase is a rate-limiting enzyme and may play an important role in chrysolaminarin biosynthesis and carbon allocation. Our results support a theoretical deduction that *Ugp* is a good candidate for improving lipid synthesis in diatoms.

Introduction

Diatoms are unicellular, photosynthetic and eukaryotic microalgae, which are responsible for around 40% of the total carbon fixation in oceans [1]. Despite the important roles of diatoms in aquatic ecosystems and in the global cycling of carbon, relatively little is known about chrysolaminarin pathways in these algae. The fixed carbon is stored mainly as lipids and as the β-1,3-glucan known as chrysolaminaran in these species. To date, the biosynthetic pathway of chrysolaminaran has not been fully elucidated and no direct genetic evidence regarding the functionalities of related genes predicted to be involved in this pathway has been reported [2], although these functionalities were indicated by transcriptome analysis in *Phaeodactylum tricornutum* [3].

Chrysolaminaran is a water soluble polysaccharide that accumulates in cytosolic vacuoles [4] and UDP-glucose (UDP-Glc) serves as the substrate for chrysolaminaran synthesis [5,6]. In higher plants, UDP-glucose pyrophosphorylase (UGPase) is a key enzyme in carbohydrate metabolism, catalyzing the reversible production of UDP-Glc and pyrophosphate (PPi) from glucose-1-phosphate (Glc 1-P) and UTP. For instance, Park *et al*. [7] found that UGPase is functionally redundant in *Arabidopsis thaliana* for carbohydrate metabolism in the vegetative and reproductive phases but plays a critical role in both vegetative growth and pollen development. However, Meng *et al*. [8] showed that UGPase is not rate limiting for sucrose/starch or cell wall synthesis, but is essential in *A. thaliana*. Thus, it can be seen that the function of UGPase in plants is still ill-defined despite the fact its central role in carbohydrate metabolism has been widely accepted.

Based upon its strategic positioning at the crossroads of several pathways for carbohydrate synthesis, UGPase might also play an important role in carbon partitioning. As indicated by Coleman *et al*. [9], carbon partitioning to starch and cellulose could be altered due to over-expression of UGPase under the control of a ubiquitous promoter in hybrid poplar. Wang *et al*. [10] also reported that a functional UGPase gene from cotton (*GhUGP*) might play a crucial role in the allocation of carbon, especially in cellulose biosynthesis. However, little is known about the effects of UGPase genes from diatoms on chrysolaminaran synthesis and carbon allocation.

P. tricornutum is one of the most widely used model systems for studying the ecology, physiology, biochemistry, and molecular biology of diatoms. This diatom can be genetically transformed [11,12] and has a fully sequenced genome [13] so that, together

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with the feasibility of exogenously triggered RNA-mediated silencing [14,15], with constructs containing either anti-sense or inverted repeat sequences of selected target genes, it is an appealing model system for further study to explore the functions of genes.

In order to study *in vivo* the functionality of UGPase in synthesizing chrysolaminaran and regulating carbon partitioning, we targeted the gene encoding the UGPase of *P. tricornutum* using exogenous constructs that express antisense or inverted-repeat-containing RNAs. Our results suggest that suppressing UGPase gene reduces the synthesis of chrysolaminaran and facilitates total lipid accumulation. These findings indicate that UGPase may play an important role in partitioning carbon into lipid synthesis. To the best of our knowledge, this is the first report of genetic manipulation of UGPase gene in the model diatom *P. tricornutum*.

Materials and methods

Strains and growth conditions

The wild-type *P. tricornutum* Bohlin (Laboratory of Applied Microalgae Biology, Ocean University of China) and transformants were incubated in sterile 50% seawater f/2 medium [16] at 20 ± 1°C and illuminated with 75 μmol photons m^{-2} s^{-1} provided by white fluorescent tubes (Philips, TLD 36W/765-54) in a 12:12 h dark-light (D:L) cycle. In order to monitor the growth of the wild type and transformants, three cultures (300 mL each treatment) were cultivated axenically. The optical density (at 750 nm) was measured every 2 days at 2 h after the illumination segment of each D:L cycle using a UV-3310 spectrophotometer (Hitachi, Tokyo, Japan) [17]. A sample of 50% seawater f/2 medium was used as a control. The specific growth rate (μ, d^{-1}) was determined from the measurements of optical density at 750 nm using the following equation [18]: $\mu = (\ln N_t - \ln N_0)/(t_1 - t_0)$, where N_t and N_0 are OD_{750} at time t_1 and t_0, respectively.

Construction of antisense and inverted-repeat vectors

According to the methods described by De Riso *et al*. [14] and Lavaud *et al*. [15], all fragments used for silencing vector construction were amplified by PCR from UDP-glucose pyrophosphorylase gene (*Ugp*) and then inserted into Pha-T1 [19], which allows transformants to be selected using Zeocin™ (Invitrogen, Carlsbad, CA, USA). To construct two pUGP-AS (antisense) plasmids of different lengths, a 516-bp antisense fragment was amplified using the primers *UgpAsf* (containing an *Eco*RI site) and *UgpAsr1* (containing an *Xba*I site) and a 237-bp product was obtained with *UgpAsf* and *UgpAsr2* (containing an *Xba*I site) (see Table 1). These two fragments, which have the first 237 bp in common, were inserted in antisense orientation into pPha-T1 at multiple cloning sites, resulting in pUGP-AS516 and pUGP-AS237 (Fig. 1). To generate an inverted-repeat construct, a longer fragment (508 bp) was amplified with the primers *UgpIRf1* (containing an *Eco*RI site) and *UgpIRr1* (containing an *Xba*I site) and subsequently cloned in sense orientation into pPha-T1. The resulting vector was termed pUGP-S508. In the following steps, a shorter fragment (228 bp) was generated using the primers *UgpIRf2* (containing a *Hind*III site) and *UgpIRr2* (containing an *Xba*I site) and ligated in antisense orientation with pUGP-S508, yielding pUGP-IR (Fig. 1).

TABLE 1

Primers used for the construction of transformation vectors, for LAPCR and for qPCR measurements. (Underlining in primer sequences indicates the digested nucleotide sequences of the restriction enzyme.)

Primer name	Primer sequence (5′–3′)
UgpAsf	CGTCTAGAGGGAATCTGCTATGTGC
UgpAsr1	CGGAATTCGACCAAACCCTTGATAGTC
UgpAsr2	GCGAATTCCATAGCGGTTTCCAACT
UgpIRf1	CGGAATTCTGCTATGTGCGCTGAA
UgpIRr1	CCCTCTAGACCAAACCCTTGATAGTC
UgpIRf2	GGAAGCTTGCTATGTGCGCTGAA
UgpIRr2	CCGTCTAGATAGCGGTTTCCAACT
Sh ble fw	CACGGTTGCCAGATGTCAAGATGGCCAAG
Sh ble rv	GGTTCAGTCCTGCTCCTCGGCCACGAAGTG
LAf1	TGCCAGATGTCAAGATGGCCAAGTTGACCAGT
LAf2	CCAGATGTCAAGATGGCCAAGTTGACCAGT
RT-UGP-*fw*	ATTGCGGGACAAAAGCCTGGT
RT-UGP-*rv*	CGCCGTTAGCAACACCCATCT
RT-H4-*fw*	GTGGTAAAGGAGGCAAGGGTC
RT-H4-*rv*	GGCGTGCTCGGTATAGGTGA

Biolistic transformation

The silencing vectors were introduced into *P. tricornutum* using a Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA) [19] fitted with 1550 psi rupture discs. Tungsten particles were made according to the manufacturer's protocol. Bombarded cells were illuminated for 24 h before suspension in 0.5 mL of sterile 50% seawater f/2 medium. For the selection of transformants, 200 μL of this suspension were plated onto 50% fresh seawater agar plates (1% agar) supplemented with 100 μg/mL Zeocin™. After about 3 weeks of incubation under constant illumination (75 μmol photons m^{-2} s^{-1}) at 20 ± 1°C, individual resistant colonies were inoculated into sterile 50% seawater f/2 medium with 100 μg/mL Zeocin™.

DNA genomic extraction and LA-PCR

Cells in the late-exponential phase were harvested and the total genomic DNA was isolated as described by Watanabe *et al*. [20]. The silenced transformants were screened by checking the integration of *Sh ble* gene with the gene-specific primers *Sh ble fw* and *Sh ble rv* (Table 1).

To further verify the integration of *Sh ble* gene, genomic DNA was digested with *Eco* RI, *Xba* I and *Sau 3AI* (MBI Fermentas, Fast Digest, USA). According to the manufacturer's instructions for the LA PCR™ *in vitro* Cloning Kit (cat. #RR015, TaKaRa, Japan), the digested fragments were precipitated by ethanol and then ligated to cassettes provided in the kit at 16°C for 30 min. After ethanol precipitation, 1 μL of ligation product was suspended in 33.5 μL of sterilized distilled water and heated at 94°C for 10 min; this mixture was then added to the reaction reagents to perform the first PCR reaction with the primers *S1* and *C1* (Table 1). In the second reaction, the first PCR product was diluted 100-fold and 1 μL of the diluted solution was used as the template to specifically amplify the long unknown region with primers *S2* and *C2*