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The inhibition of TOR in the model diatom *Phaeodactylum tricornutum* promotes a get-fat growth regime

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ABSTRACT

The target of rapamycin (TOR) signaling pathway regulates fundamental intracellular functions critical for cell viability and proliferation. Manipulation of TOR in high lipid-producing microalgae may help overcome the trade-off between biomass production and lipid yield that still impairs the viable production of biofuel from microalgae. In this study, we inhibited the TOR kinase in the model diatom *Phaeodactylum tricornutum* using the selective TOR inhibitor AZD-8055, and analyzed cell proliferation, chlorophyll content, lipid synthesis and carbon metabolism. AZD-8055 inhibits cell proliferation in a dose-dependent manner compared to N deprivation which stops growth. Microscopy, flow cytometry, and quantitative analyses of lipids also demonstrated that AZD-8055 treatment strongly promotes triacylglycerol (TAG) accumulation while decreasing the quantity of sterols. The TAG productivity of AZD-8055 treated cultures was significantly higher than for N deprived cultures. Measurement of the activities of the key metabolic enzymes glyceraldehyde phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH) revealed opposite effects for AZD-8055 treatment and N-starvation on the activity of the glycolytic enzyme GAPDH. This suggests that TOR inhibition and N starvation may have distinct impacts on general metabolism and lipid accumulation. Our main finding is that treating cultures with AZD-8055 results in higher TAG productivity than N starvation in *P. tricornutum*. The chemical or genetic manipulation of the TOR signaling pathway in *P. tricornutum* and other diatoms may lead to the development of strains or approaches suitable for the enhanced production of TAGs for biofuel.

1. Introduction

Since the second half of the XIX century the use of natural resources, like fossil fuels, gas and coal for sustaining human activities has been experiencing exponential growth. Although this historical phenomenon, known as the Industrial Revolution, had beneficial effects on human society, it has also led to long-term catastrophic effects on the environment with consequences for human health. Combustion of fossil fuels is indeed one of the main factors responsible for greenhouse gases emissions (CO_2) and atmospheric release of toxic compounds, which in turn are involved in global warming, ocean acidification and various types of diseases [1,2]. The search for sustainable and less polluting energy alternatives has now become a mission of paramount importance. Third-generation biofuels from microalgae reconcile the high demand for liquid fuels with many of the technical problems encountered in other oil-producing organisms like crops [3]. While unicellular photosynthetic organisms represent only about 0.2% of the global biomass, they account for almost 50% of the global net primary production [4] and the energy that they can store in the form of lipids represents > 20% of dry weight under nutrient-sufficient growth conditions [5]. This percentage can rise to 70–80% in some species of eukaryotic microalgae under nutrient limitation [3].

Diatoms (*Bacillariophyceae*) are the predominant class of eukaryotic microalgae in the oceans, where they account for about 40% of net

Abbreviations: AZ, TOR inhibitor AZD-8055; DAG, diacylglycerol; FFA, free fatty acids; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde phosphate dehydrogenase; GC–MS, gas chromatography coupled to mass spectrometry; MAG, monoacylglycerol; MDH, malate dehydrogenase; TAG, triacylglycerol; TLC-FID, thin layer chromatography coupled to flame ionization detection; TOR, target of rapamycin

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primary production (the remainder accounted by other eukaryotic organisms and cyanobacteria; [6]), and possess qualities (high photosynthetic efficiency, fast growth and the capacity to store energy and carbon in the form of neutral lipids) that make them one of the most suitable groups for biofuel production [7,8]. The advanced genetic engineering tools developed in the diatom *Phaeodactylum tricornutum*, whose genome is known [9–13], make it one of the preferred model microalgae for biotechnology studies. In spite of this, biofuel production from microalgae is still a challenging process as the highest lipid accumulation per cell is achieved when the algae are subject to stress conditions like nitrogen starvation [14], which ultimately limits the overall biomass and thus lipid yield. The study and manipulation of signaling pathways regulating cell growth and metabolism, for instance, the target of rapamycin (TOR) pathway, may help to combine high lipid yields with the maintenance of cell proliferation. TOR manipulation for biofuel production was first proposed in a study showing that TOR repression in *Arabidopsis thaliana* artificial microRNA (amiR) lines affects carbon metabolism and results in starch and TAG accumulation associated with growth repression [15]. Further studies showed that TOR inhibitors could induce TAG accumulation in the red alga *Cyanidioschyzon merolae* and the green alga *Chlamydomonas reinhardtii* [16,17]. However, TAGs were measured only at TOR inhibitor concentrations that stop proliferation and TAG productivity was not studied. A recent study on *Euglena gracilis*, a distant alga belonging to the Excavata, reported that the TOR inhibitor rapamycin has little effect on proliferation, yet could induce a 1.4-fold increase in neutral lipids per cell [18]. This suggests that TOR inhibition could be a route to increasing TAG productivity in some algae. The TOR signaling network plays a key role in the regulation of cell growth integrating the responses to a variety of signals, like nutrient levels and stress, and transmitting them to the metabolic machinery (reviewed in [19,20]). TOR is a large protein kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family that includes the checkpoint kinases ataxia telangiectasia mutated (ATM) and ATM- and RAD3-related protein (ATR), which are master controllers of cell cycle signaling pathways [21]. TOR can be found in two multiprotein complexes, TORC1 and TORC2 [19]. While TORC2, involved in cytoskeleton organization in animals and yeast, is not conserved in Viridiplantae and diatoms, TORC1 is found in all eukaryotes, with the exception of intracellular parasites, thus indicating a fundamental role for TORC1 in eukaryotes [22]. TORC1 is stimulated by nutrients and inhibited by stress-related signals [23]. The conserved function of active TORC1 is to activate protein synthesis, cell-cycle progression, and energy metabolism, while inhibiting stress responsive genes and autophagy. In yeast and animal cell lines, TOR inhibition blocks cell division, increases autophagy resulting in altered cell physiology, fate and morphology in manner reminiscent of a nutrient-starvation response [24]. In addition to a potential application in biofuel production, the study of TOR signaling in diatoms is of fundamental importance as the evolution of the TOR pathway in algae with secondary plastids might have been influenced by the multiple endosymbiosis events that characterized their evolution [9].

In this study, we used a synthetic and highly specific ATP-competitive inhibitor of the TOR kinase known as AZD-8055 [28] to test how modulating TOR activity affects cell proliferation, TAG accumulation and productivity, and primary metabolism in *P. tricornutum*. Two other TOR inhibitors, rapamycin and WYE-132, were also used to evaluate the specificity of this response towards TOR inhibition.

2. Materials and methods

2.1. Algal strain and AZD-8055 treatment

P. tricornutum Pt1_8.6 (RCC 2967) was grown for eight days at 18 °C in F/2 medium [29] supplemented with silica (F/2 + Si medium) in an incubator equipped with a shaking plate (Innova 4230, New Brunswick Scientific, Edison NJ, USA). Irradiance was kept at 80 mol photons $m^{-2} s^{-1}$ for 14 h day^{-1}. For the AZD-8055 treatments, a culture of *P. tricornutum* from an early exponential phase was diluted to $OD_{750} \approx 0.003$ in a 5-l Erlenmeyer flask containing 2.5 l of fresh F/2 + Si medium. The OD_{750} was measured daily until it reached ≈ 0.03 (after 72 h), a value corresponding to the beginning of the exponential phase for this strain; the culture was then split into nine 1-l Erlenmeyer flasks containing 250 ml of medium each. The TOR inhibitor AZD-8055 (Chemdea, Ridgewood, NJ, USA; [28]) was then added to a final concentration of 0.001, 0.01, 0.1, 1, 2, 4 and 10 mol l^{-1}. The inhibitor was dissolved in dimethyl sulfoxide (DMSO), whose final concentration was adjusted to 0.1% for each of the different cultures and the control. The ninth part of the initial culture was subjected to nitrogen starvation. For N starvation, cells were centrifuged at 3500*g* for 15 min, the supernatant discarded and the pellet washed three times in N-free F/2 + Si medium; the pellet was then resuspended in 250 ml of fresh N-free F/2 + Si medium. Three independent experiments were conducted for each culture condition. Another set of experiments was performed to compare the effect of high concentrations of AZD-8055 and other TOR inhibitors on cell proliferation and neutral lipid content in *P. tricornutum*. Cultures were grown as described above. The TOR inhibitors AZD-8055 (10 and 40 mol l^{-1}), rapamycin (10 mol l^{-1}; LC Laboratories, Woburn, MA, USA) and WYE-132 (5 mol l^{-1}; Chemdea, Ridgewood, NJ, USA) were added to early exponential phase cells as described above.

2.2. Cell fixation and Nile red staining

Each day a volume of *P. tricornutum* culture, varying from 5 to 200 ml depending on biomass production, was harvested by centrifugation at 3500 g for 15 min. The pellet was resuspended in 0.5 ml of a fixing solution containing HEPES (0.1 mol l^{-1}, pH 7), $CaCl_2$ (0.01 mmol l^{-1}), $MgCl_2$ (0.01 mmol l^{-1}) and glutaraldehyde 2% (v/v). After one hour incubation at 4 °C in the dark, the fixing solution was removed and the cells resuspended in 1 ml of HEPES (0.1 mol l^{-1}, pH 7). The samples were stored at 4 °C in the dark.

For visualization of neutral lipids, the fixed cells were incubated with a Nile red solution (FluoProbes, Interchim; 488–530/575–580 nm excitation/emission; [30]) and analyzed by fluorescence microscopy and analytical flow cytometry. A 0.25 mg ml^{-1} Nile red solution in DMSO was added to the fixed cell suspensions to a final concentration of 1 g ml^{-1} [31]. The mixtures were incubated at 4 °C for 5 min prior to analysis.

2.3. Flow cytometry

A benchtop flow cytometer (BD Accuri C6, BD Biosciences, Ann Arbor, MI, USA) was used to assess *P. tricornutum* cell abundance during the whole course of the experiments. The instrument is equipped with two excitation laser beams emitting at 488 and 640 nm. The flow cytometer measures chlorophyll red fluorescence (CHL, > 675 nm; channel FL3), orange fluorescence (PE, 585 ± 20 nm; channel FL2), green fluorescence (530 ± 15 nm; channel FL1), side scatter (SSC, light scattered by particles at 90° to the direction of the laser beam) and forward light scatter (FSC, light scattered by particles at narrow angles in the same direction as the laser beam). Their combination allows detecting different wavelength emission ranges [32]. Diatom density was estimated by the cytogram forward scatter (FSC) versus channel FL3, whereas side scatter (SSC) combined with the FL3 channel was used to estimate chlorophyll auto-fluorescence. The specific growth rate for each culture condition was calculated on the exponential portion of the growth curve, according to the equation of Monod [33]. It has been demonstrated that lipid fluorescence in microalgae estimated through the combination of Nile red staining and flow cytometry shows a positive correlation with the content of lipids quantified by thin layer chromatography [34]. We then performed flow cytometry analyses on