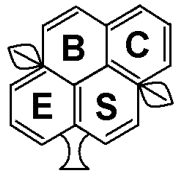

Journal

EFFECTS OF *VACUOLAR TRANSPORTER CHAPERONE 1* DISRUPTION ON PRODUCTION OF TRIACYLGLYCEROLS QUALITY AND QUANTITY IN *CHLAMYDOMONAS REINHARDTII* UNDER PHOSPHORUS DEPRIVATION

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ABSTRACT

Microalgal lipid biosynthesis has been extensively studied under nitrogen depleted conditions where triacylglycerols (TAGs) rapidly accumulate while cell growth is arrested. However, less is known about TAG production under phosphorus depleted conditions. Also, available knowledge about polyphosphate metabolism in microalgae remains limited. In this study, the molecular mechanisms of polyphosphate biosynthesis and its link with the lipid biosynthesis pathway are investigated in *Chlamydomonas reinhardtii* using a mutant defective in the *vacuolar transporter chaperone 1* gene (*VTC1*). The presence of polyphosphate was abundant in the *C. reinhardtii VTC1* rescue strain when phosphorus was copious, whereas polyphosphate was degraded when phosphorus was depleted in the medium. In addition, deletion of the *VTC1* gene enhanced TAGs production under phosphorus depleted conditions, resulting in a 9.6-fold increase in TAGs concentration at day 21. A defect in the *VTC1* gene also affected the fatty acid composition of

TAGs, promoting accumulation of mono- and poly-unsaturated fatty acids at the expense of saturated fatty acids. These data suggest a negative relationship between polyphosphate biosynthesis and TAG biosynthesis in microalgae, and provide a new strategy to improve the quality and yield of algal biofuels through manipulation of polyphosphate biosynthesis.

Key words: *Chlamydomonas reinhardtii*, vacuolar transporter chaperone 1,phosphorus deprivation, polyphosphate, triacylglycerols

INTRODUCTION

Microalgae have gained extensive attention for their ability to generate lipid feedstocks for production of biofuels and bioproducts **(Hu *et al.*, 2008; Sivakumar *et al.*, 2012; Banerjee *et al.*, 2016 and Goncalves *et al.*, 2016)**. The unicellular green alga *Chlamydomonas reinhardtii* is a model organism for many physiological and biochemical processes **(Harris, 2001; Aksoy *et al.*, 2014; Baltz *et al.*, 2014 and Zalutskaya *et al.*, 2018)**. Having a simple life cycle, easy isolation of mutants, full genome sequence and a growing array of tools as well as techniques for molecular biology studies helped *C. reinhardtii* to be a robust model to study many biological processes **(Wykoff *et al.*, 1998 and Nagy *et al.*, 2018)**. However, biosynthesis of large amounts of storage lipid triacylglycerols (TAGs) in microalgae, which are ideal feedstocks for production of biodiesel, can only be stimulated under stress conditions, e.g. nitrogen (N) deprivation or high irradiance **(Hu *et al.*, 2008; Fields *et al.*, 2014; Goncalves *et al.*, 2016 and Lenka *et al.*, 2016)**. As a result, the low biomass productivity is still a primary bottleneck for algal biofuels production **(DOE, 2010)**.

Phosphorus (P) is nonrenewable and often a limiting nutrient for growth of microalgae **(Grossman and Aksoy, 2015)**. It is an essential element for many fundamental processes that sustain life, including nucleic acid synthesis, membrane synthesis, energy metabolism, signaling, redox reactions, and modification of protein activities.

A boost of bioavailability of P in aqueous environments usually results in a massive bloom of microalgae **(Sharma *et al.*, 2013)**.

Polyphosphate (poly P), an inorganic chain polymer consisting of a few to hundreds of orthophosphate (P_i) residues conjugated with phosphoanhydride bonds, is a storage molecule for cellular phosphates and ubiquitous from bacteria to plant and animals **(Kornberg *et al.*, 1999)**. It has wide varieties of biological functions beside phosphate

storage, including cation homeostasis, pH homeostasis, osmoregulation, gene expression regulation, post-translation modifications and signal transduction **(Kornberg *et al.*, 1999; Gray *et al.*, 2003; Luo *et al.*, 2005 and Azevedo *et al.*, 2015)**. PolyP metabolism has been extensively studied in bacteria and yeast. Novel gene family members, *Vacuolar Transporter Chaperone1* (*VTC1*), *VTC2*, *VTC3* and *VTC4* genes, were identified to be involved in polyP biosynthesis in *Saccharomyces cerevisiae* **(Cohen *et al.*, 1999 and Ogawa *et al.*, 2000)**.

By analyzing sequences deposited in microalgal databases, **Aksoy *et al.* (2014)** revealed the protein sequences and the domain architecture similar to *S. cerevisiae* VTC1 protein (ScVTC1) present in *C. reinhardtii*, *Volvox carteri* and *Chlorella variabilis* as well as a putative VTC4 in *C. reinhardtii*. They also reported deletion of *VTC1* gene impairs polyP accumulation in *C. reinhardtii***.**

The presence of poly P granules in microalgae has been known for decades **(Seufferheld and Curzi, 2010)**, but the cellular functions of polyP molecules have yet to be clarified. Moreover, the molecular interactions of polyP biosynthesis with other cellular pathways, lipid biosynthesis in particular, have not been investigated.

In this study, a Δ*vtc1* knockout mutant strain deficient in polyP and a *VTC1* rescued strain of *C. reinhardtii* were comparatively characterized in order to study the effects of *VTC1* disruption on P uptake as well as the quantity and fatty acid composition of TAGs. Also, the effects of N and P deprivation on TAG biosynthesis in the Δ*vtc1* background were discussed.

MATERIALS AND METHODS

This study was executed from Dec, 2015 to Oct, 2017 in Li's Lab at Institute of Marine and Environmental Technology (IMET), University of Maryland Center for Environmental Science (UMCES), Baltimore, MD, USA.

1. Strains and growth conditions

C. reinhardtii vtc1 mutant strain CC5165 (Δ*vtc1*) and the *VTC1* rescue strain CC5166 (*ars76::VTC1*) **(Aksoy *et al.*, 2014)** were obtained from the Chlamydomonas Resource Center. Both strains were maintained in Tris-acetate-phosphate (TAP) medium containing 5 to 15 μg/ml paromomycin. As shown in **Table (1),** TAP, P-free TAP (TAP-P) and N-free TAP (TAP-N) media were used for nutrient replete, P-depleted, and

N-depleted conditions, respectively, as previously described by **Li *et al.* (2010b)**.

Table (1) Chemical composition (g) per one liter TAP, TAP-P and TAP-N media

Component	TAP	TAP-P	TAP-N
Tris base	2.42	2.42	2.42
Acetic acid	1ml	1ml	1ml
K_2HPO_4	0.108	-	0.108
KH_2PO_4	0.056	-	0.056
KCl	-	0.112	-
KOH	0.017	0.017	0.017
NH_4Cl	0.4	0.4	-
$MgSO_4.7H_2O$	0.1	0.1	0.1
$CaCl_2.2H_2O$	0.05	0.05	0.05
Na_2EDTA	0.05	0.05	0.05
$ZnSO_4.7H_2O$	0.022	0.022	0.022
H_3BO_3	0.0114	0.0114	0.0114
$MnCl_2.4H_2O$	0.00506	0.00506	0.00506
$CoCl_2.6H_2O$	0.00161	0.00161	0.00161
$CuSO_4.5H_2O$	0.00157	0.00157	0.00157
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0011	0.0011	0.0011
$FeSO_4.7H_2O$	0.00499	0.00499	0.00499
Final pH is adjusted by acetic acid to	7	7	7

TAP-P medium was prepared by replacing potassium phosphate with 1.5 mM KCl. TAP-N medium was prepared by omitting NH_4Cl from TAP. Seed cultures were grown in TAP medium until late exponential or early stationary phases and then inoculated into fresh medium with 1 x 10^6 cells/ml initial cell density. Cell density was measured using a hemocytometer (Hausser Scientific). To impose nutrient deprivation, the inoculum was washed with correspondent nutrient depleted medium twice before inoculation. All batch cultures were grown at room temperature under continuous light illumination at 60 µmol photons m^{-2} s^{-1} and shaking at 150 rpm.

2. Total P and polyP assays :

In order to measure the amount of cellular total P and polyP, the whole cell lysate and poly P extraction from *C. reinhardtii* cells were prepared.

Preparation of the whole cell lysate for total P measurement and polyP extraction from microalgae was the same as described in **Ota *et al.* (2016)**. Two x 10^7 cells were harvested for each assay and washed twice with 1 ml distilled water by centrifugation at 3,000 g for 5 min at room temperature. For total P measurement, the cell pellet was resuspended in 1 ml distilled water, followed by vigorously vortexing with glass beads (425-600 μm) for 15 min at 4^{o}C to prepare the whole cell lysate. For the preparation of the polyP fraction, the cell pellet was resuspended in 1 ml 5% sodium hypochlorite and followed by vortexing with glass beads (425-600 μm) for 15 min at 4^{o}C. After the disruption, the pellet was collected by centrifugation at 14,000 g for 3 min at 4^{o}C, and washed twice with 1 ml 5% sodium hypochlorite. The pellet was then resuspended in 100 μl distilled water and incubated for 5 min at room temperature. The pellet and the supernatant were separated by centrifugation at 14,000 g for 3 min at room temperature, and the supernatant was collected. The resuspension and incubation of the pellet in 100 μl distilled water were repeated, and the supernatant (total volume of 200 μl) was combined. Ethanol (90% as final concentration) was added to the collected supernatant, and the mixture was incubated at -20^{o}C overnight to precipitate polyP. The poly P pellet was collected by centrifugation at 14,000 g for 15 min at 4^{o}C and washed with 70% ethanol then separated by centrifugation at 14,000 g for 15 min at 4^{o}C. The supernatant was decanted, and the dry pellet was dissolved in distilled water (50 μl). Samples from total P or polyP fractions were hydrolyzed to P_i by incubating in 1 M perchloric acid (final concentration) at 90^{o}C for 30 min **(Lonetti *et al.*, 2011)**. After P_i hydrolysis, samples were subjected to molybdenum/malachite-green based phosphate assay in a microtiter plate as described by **Hothorn *et al.* (2009)**. Each reaction contained 10 μl sample (in 1M perchloric acid), 90 μl phosphate assay buffer [20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, and 100 mM ammonium acetate], 86 μl of 28 mM ammonium heptamolybdate in 2.1 M H_2SO_4 and 64 μl 0.76 mM malachite-green in 0.35% polyvinyl alcohol. The reaction was incubated for 20 min at room temperature, and the absorbance at 595 nm (A_{595}) was measured using SpectraMax M5 microplate reader (Molecular Devices). Zero to 500 μM KH_2PO_4 was used as the standard.

3. Fluorescence staining and microscopy :

Nile red was used to stain lipid bodies in live cells according to **Greenspan *et al.* (1985)** with modifications. Prior to staining, cells were washed briefly. Afterwards, they were mixed with 200 µl 0.1 µg/ml Nile red solution (diluted in McIlaine buffer from a 1 mg/ml stock solution prepared in acetone) and incubated for 5 min in the dark, followed by washing out the excess dye.

Fifty to hundred µl of stained cell suspension was placed on a cover slip and left undisturbed for 30 min in the dark. The stained specimen was dried and mounted with Slow Fade Diamond (Invitrogen) and imaged using wide-field fluorescence microscopy with a Zeiss Axio Observer Z1 microscope and Hamamatsu Orca-R2 charge-coupled device (CCD) camera. Zeiss filter sets10 (beam pass excitation 450-490 nm; beam pass emission 515-565 nm) and 15 (beam pass excitation 540-552 nm; long pass emission 590 nm) were used to detect Nile red staining (the green fluorescence) and chlorophyll autofluorescence (the red fluorescence), respectively. The images were analyzed with Volocity® 6.2.1 software (PerkinElmer). The fluorescence channels were deconvolved using the restoration function of the Volocity® software.

4. Lipid extraction, quantification and fatty acid analysis :

Total lipids from *C. reinhardtii* were extracted using chloroform: methanol (2:1, v/v) as previously described be **Yoon *et al.* (2012)**. The lipid extracts were dried under a gentle stream of nitrogen gas and re-dissolved in chloroform. Total lipids were resolved by thin-layer chromatography (TLC) on a silica gel 60 F_{254} plates (EMD Millipore) using a hexane: t-butyl methyl ether: acetic acid (80:20:2, v/v/v) solvent mixture as a mobile phase to develop TAG. For visualization, the developed TLC plates were sprayed with 8% (w/v) H_3PO_4 solution containing 10% (w/v) copper (II) sulfate pentahydrate, and then charred at 180°C for 3 min. Fatty acid analysis was done by gas chromatography-mass spectrometry (GC-MS) as previously described by **Li *et al.* (2010a) and Wang *et al.* (2017)**. Briefly, TAG spots on a developed TLC plate were visualized by I_2 vapor and then dissolved in hexane. Fatty acids of the isolated TAG were converted to fatty acid methyl esters (FAMEs) with 1% H_2SO_4 in methanol at 85^{o}C for 1.5 h and then profiled using TSQ 8000 Triple GC-MS System (Thermo Scientific). FAME standards (Sigma) and heptadecanoic acid (C17:0) (Sigma) were used as the external and internal standards, respectively, for fatty acid analysis. The quantification for TAG on the developed TLC plates can be achieved by

the standard curves of a TAG standard (C18:1/C18:1/C18:1; Sigma) using densitometry and the ImageJ software (NIH) or by the calculation of fatty acid concentration based on GC-MS analysis.

RESULTS AND DISCUSSION

1. Δ*vtc1* mutation affects growth and P accumulation in *C. reinhardtii*

To assess the effect of *VTC1* deletion (Δ*vtc1*) on cell growth, the growth phenotype of the Δ*vtc1* mutant (CC5165) and the *VTC1* rescue strain (CC5166) was analyzed under TAP and TAP-P conditions. When inoculated into fresh TAP medium, the Δ*vtc1* strain grew faster than the *VTC1* strain and reached the stationary phase earlier than the rescue strain (solid lines in **Figure 1**). When grown in TAP-P medium, the growth of both strains was comparably compromised by P deprivation (dash lines in **Figure 1**).

As expected, P deprivation altered accumulation of cellular P level. When grown in fresh TAP medium, *VTC1* cells rapidly accumulated P resulting in a peak and a 4-fold increase of total P within one day; total P increased from 21 to 77 μg in 10^7 cells from day 0 to day 1. After day 1, the level of total P gradually leveled off (red solid line in **Figure 2a**). By contrast, the level of total P in *VTC1* cells decreased when the cells were shifted to P-depleted conditions (red dash line in **Figure 2a**). Interestingly, Δ*vtc1* cells had very low levels of total P regardless the availability of P in the growth medium (blue lines in **Figure 2a**), suggesting that Δ*vtc1* mutation impairs P uptake in *C. reinhardtii*.

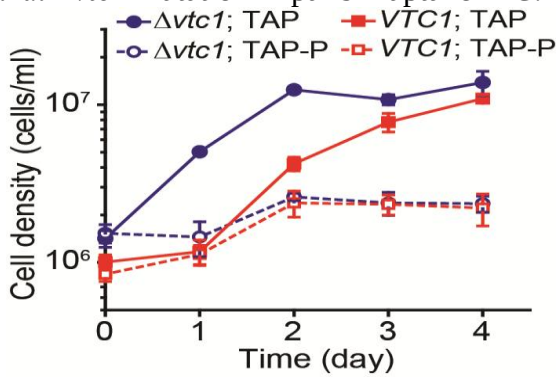

Figure (1) Growth curves of batch cultures of *C. reinhardtii* Δ*vtc1* and *VTC1* rescue strains in TAP or TAP-P medium. Values are mean ± standard deviation (SD) of three biological repeats.

The Δ*vtc1* mutation has been suggested to impair response to sulfur deprivation and polyP accumulation in *C. reinhardtii* **(Aksoy *et al.*, 2014)**. To further test this, the polyP level in the cells of Δ*vtc1* mutant was quantitatively measured and evaluated whether it was correlated to the availability of P in the medium.

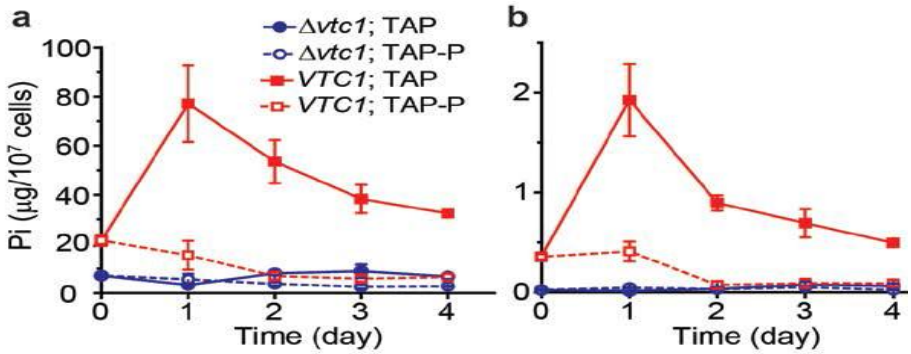

Figure (2) Kinetic of P and polyP accumulation (μg Pi/10^7 cells) in *C. reinhardtii* Δ*vtc1* and *VTC1* cells total P (a) and poly P (b) grown in batch cultures under TAP or TAP-P conditions. Data represent mean ± standard deviation (SD) of three biological repeats.

Similar to the kinetics of total P, the amount of polyP in *VTC1* cells had a rapid increase from 0.4 μg in 10^7 cells after inoculated into fresh TAP medium to a peak with 1.9 μg polyP in 10^7 cells after one day, then a sharp decline afterwards (red solid line in **Figure 2b**). However, when *VTC1* cells initially inoculated to P-depleted conditions, the level of polyP (0.4 μg in 10^7 cells) stayed constant for one day and then decreased to a non-detectable level after day 2; < 0.1 μg in 10^7 cells (red dash line in **Figure 2b**). The proportion of polyP in total P was 1.5% - 2.5% throughout the 4-day experimental period under P-replete conditions. Under P-depleted conditions, the proportion of polyP was 1.1% - 1.7% from day 0 to day 2. On the other hand, the level of polyP in Δ*vtc1* cells was undetectable whether in TAP or TAP-P medium (blue lines in **Figure 2b**), suggesting this strain is unable to accumulate any polyP.

It is generally believed that microalgae produce and accumulate polyP when P is abundant and break down this polyP reserve upon P deprivation, which triggers substitution of phospholipids by non-P-

containing lipids such as sulfolipids and betaine lipids in microalgae **(Martin *et al.*, 2014 and Abida *et al.*, 2015)**.

Since VTC1 protein, the subunit 1 of the vacuolar transporter chaperone complex, is a small membrane protein containing three transmembrane domains docking the complex to the vacuolar or ER membrane **(Ogawa *et al.*, 2000)** and VTC4 protein contains the catalytic domain of the polyP polymerase **(Hothorn *et al.*, 2009)**. Deletion of *VTC1* or *VTC4* gene impairs polyP accumulation in yeast, indicating VTC1 and VTC4 are essential for polyP biosynthesis **(Ogawa *et al.*, 2000)**. Here, the presented data agreed with these points and showed in *C. reinhardtii*, the presence of polyP is abundant when P is copious, whereas polyP is degraded when P is depleted in the environment. Also, polyP is only present in the *VTC1* rescue strain, but absent in the Δ*vtc1* strain.

2. Δ*vtc1* mutation enhances TAG accumulation in *C. reinhardtii*

Interestingly, a link between polyP metabolism and lipid biosynthesis was discovered in microalgae. A defect in the *VTC1* gene drastically increased TAG content and TAG yield in *C. reinhardtii* under P deprivation. This held true even for an extended cultivation period (up to 21-day), validating its biotechnological importance for improved TAG production.

It was reported that P deprivation induced TAG accumulation and lipid body biogenesis in *C. reinhardtii* while maintaining moderate cell growth **(Iwai *et al.*, 2014)**. To understand whether the Δ*vtc1* mutation affected lipid biosynthesis under P deprivation, formation of lipid bodies and TAG concentrations were surveyed in Δ*vtc1* and *VTC1* cells. When grown in P-replete medium, both Δ*vtc1* and *VTC1* cells contained a few small lipid bodies, indicating a small amount of TAG accumulation. By contrast, when grown in P-depleted medium, the lipid bodies were present in larger size and number in both strains, indicating an increased amount of TAG accumulation **(Figure 3a)**. Consistent with microscopic analysis, biochemical assay showed that the TAG concentrations were low when cells were grown in P-replete medium. However, when cells underwent P deprivation, TAG concentration increased over time **(Figure 3b)**. Interestingly, the increase of the TAG concentration was more drastic in Δ*vtc1* cells than in *VTC1* cells, and the level of the TAG concentration in Δ*vtc1* cells reached 5-fold higher than that in *VTC1* cells after 48 h following P deprivation **(Figure 3b)**.

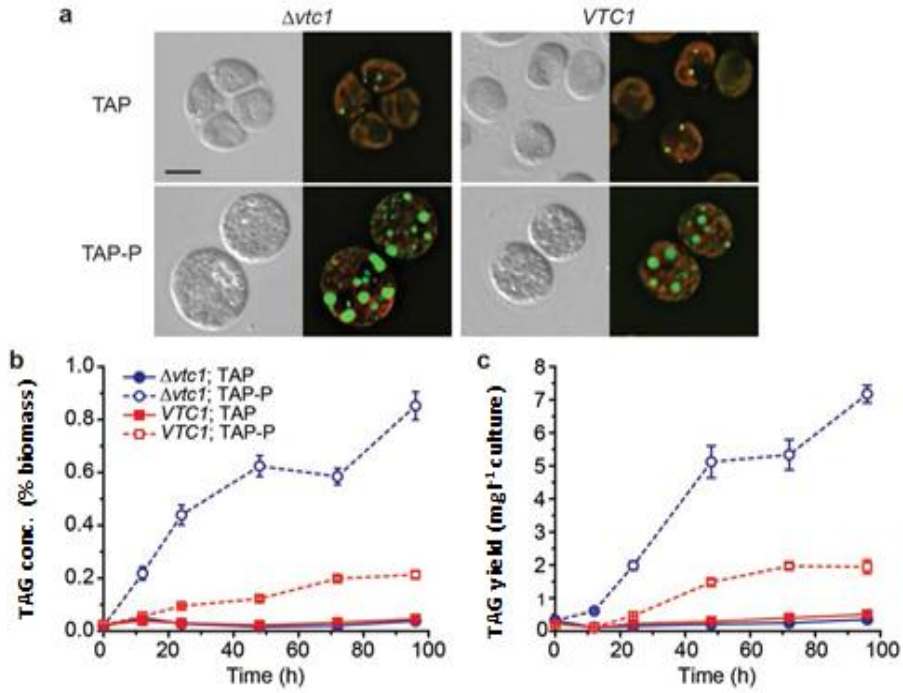

Figure (3) TAG accumulation in *C. reinhardtii* Δ*vtc1* and *VTC1* cells (a) Detection of lipid bodies in cells by Nile red staining. *C. reinhardtii* Δ*vtc1* and *VTC1* cells were cultured in TAP or TAP-P medium for 4 days. DIC and fluorescent images of Nile red-stained cells are shown. Fluorescent images were shown in two channels: green fluorescence, lipid bodies; red fluorescence, chlorophyll autofluorescence. Bar = 5 µm. (b) TAG concentration (% biomass) and (c) TAG yield (mgl^{-1}) batch cultures under TAP and TAP-P conditions were measured. Data are mean ± standard deviation (SD) of three biological repeats.

TAG productivity was measured taking in the account TAG content and biomass yield. TAG productivity in P-depleted cultures increased greatly after 12 h, and it reached a plateau (2 mgl^{-1}) in *VTC1* cells after 72 h. However, the TAG productivity in the P-depleted Δ*vtc1* cells continued to increase reaching 7.2 mg per liter culture (3.7-fold higher than that of the *VTC1*) after 96 h **(Figure 3c)**. The results demonstrate deletion of the *VTC1* gene enhances TAG accumulation in *C. reinhardtii* under P deprivation.

To assess whether or not the increase in TAG production in the Δ*vtc1* mutant is constant, the TAG concentration and the TAG yield were compared between the two strains grown under P-depleted conditions for 21 days. The resulted data showed the enhancement of TAG production in the Δ*vtc1* strain remained true in 7-, 14- and 21-day cultures **(Figure 4)**. The TAG concentration and the TAG yield in Δ*vtc1* cultures continued to increase under P-depleted conditions up to 14 days before plateau; by contrast, the level of TAG in *VTC1* cultures increased until day14 and then decreased afterwards. At day 21, the TAG concentration and TAG yield in the Δ*vtc1* mutant were 9.6-fold and 6.7-fold higher, respectively, than in the *VTC1* strain **(Figure 4)**.

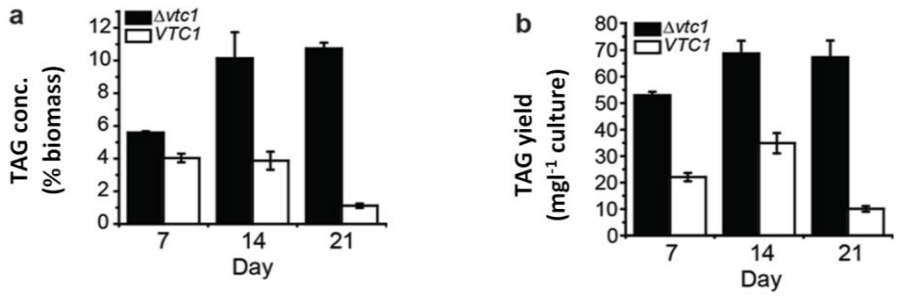

Figure (4) TAG accumulation in *C. reinhardtii* Δ*vtc1* and *VTC1* cells under prolong P-starvation (a) TAG concentration (% biomass) and (b) TAG yield (mgl^{-1}) batch cultures under TAP-P for 7, 14 and 21 days were measured. Data are mean ± standard deviation (SD) of three biological repeats.

3. Δ*vtc1* mutation affects the fatty acid composition in TAG :

Next, the effect of the Δ*vtc1* mutation on the fatty acid profile of TAGs was evaluated under different conditions. Seven major fatty acid species (C16:0, C18:0, C16:1, C18:1, C16:4, C18:2 and C18:3) were identified in TAG by analyzing TAG-derived fatty acid methyl esters (FAMEs). In the Δ*vtc1* and *VTC1* strains, saturated fatty acids (SFAs) (C16:0 and C18:0) were the most abundant (60 - 88% of total fatty acids, TFAs) fatty acids in TAG. After 48 h, C16:0 and C18:0 accounted for 48 - 70% and 11 -18% of the TFAs of TAG, respectively **(Figure 5)**. Compared with the *VTC1* strain, significant decreases (*t*-test, $P < 0.05$) of SFAs and significant increases (*t*-test, $P < 0.05$) of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) were found in the Δ*vtc1* mutant grown under P deprivation. After 48 h under P

deprivation, the concentrations of C16:0 (47.9 and 67.5% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively) and C18:0 (10.7 and 16.8% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively) were decreased by 30 and 37%, respectively **(Figure 5)**. As a result, a 31% decrease of TAG-SFA was found in Δ*vtc1* cells compared with *VTC1* cells **(Figure 5)**. Meanwhile, the concentrations of C18:1 (25.8 and 7.5% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively), C16:4 (1.9 and 1.0% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively), C18:2 (4.2 and 1.8% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively), and C18:3 (7.3 and 3.2% of TAG-TFA in the Δ*vtc1* and *VTC1* strains, respectively) were increased by 245, 96, 131 and 133%, respectively, resulting in 185 and 127% increases of TAG-MUFA and TAG-PUFA, respectively, in Δ*vtc1* cells, compared with *VTC1* cells **(Figure 5)**. In summary, the Δ*vtc1* mutation significantly (*t*-test, $P < 0.05$) increased MUFA (C18:1) and PUFA concentration of TAG at the expense of SFAs under P deprivation conditions **(Figure 5)**.

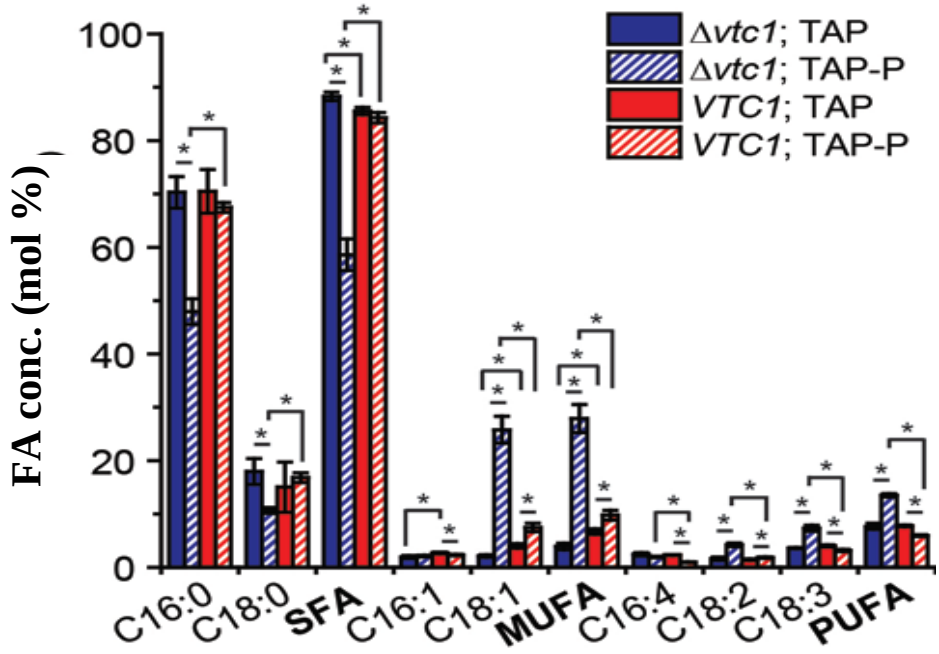

Figure (5) Fatty acid composition of TAG accumulated in *C. reinhardtii* Δ*vtc1* and *VTC1* cells. Batch cultures were grown for 48 h under TAP or TAP-P conditions. Data are mean ± standard deviation (SD) of three biological repeats. An asterisk indicates significance by Student's *t*-test (*p* value ≤ 0.05).

All in all, deletion of the *VTC1* gene altered fatty acid composition of TAGs. Phosphorus deprivation has been shown to affect fatty acid compositions in microalgal TAG. In some marine microalgae, the relative concentrations of C16:0 and C18:1 are increased and that of some PUFAs are decreased in TAG under P deprivation **(Reitan *et al.*, 1994)**. In this study, the results showed a similar effect of P deprivation in *C. reinhardtii* with increases of C18:1 concentrations and a decrease of the concentrations of PUFAs in TAG accumulated in *VTC1* cells **(Figure 5)**. The Δ*vtc1* mutation increased MUFA concentration (C18:1) of TAG at the expense of SFA, such as C16:0, under P deprivation conditions. Algae feedstocks with high MUFA concentration are ideal for biodiesel production, as higher MUFA level in algal lipids improves fuel density, lubricity, and iodine value of the final biofuel products **(Stansell *et al.*, 2012)**. Impaired polyP biosynthesis increases both TAG yield and TAG-MUFA concentration in *C. reinhardtii*, suggesting a new strategy to improve both fuel quality and yield through manipulation of the polyP biosynthesis pathway.

Phosphorus metabolism is important to chloroplast since P is a major component of structural lipids of thylakoid membranes (e.g. phospholipids). P deprivation leads to a decrease of phospholipids (e.g. phosphatidyl glycerol, PG) and an increase of sulfolipids (e.g. Sulfoquinovosyl diacylglycerol, SQDG), and the sum of PG and SQDG remains unchanged **(Sato *et al.*, 2000)**. Hence, it is expected that defects of *VTC1* and polyP biosynthesis will impact the polar lipid metabolism, chloroplast remodeling, and ultimately photosynthesis and growth. In agreement, the *VTC1*-deficient mutant has a lower chlorophyll fluorescence level than in the rescued strain **(Figure 6c)**.

4. Δ*vtc1* mutation affects growth and TAG accumulation under N or P deprivation

Recent advance in studying algal lipid metabolism has helped develop rational genetic engineering approaches to increase algal TAG production while maintaining cell growth, e.g. through inhibition of starch biosynthesis **(Li *et al.*, 2010b; Work *et al.*, 2010 and Breuer *et al.*, 2014)**, inhibition of lipid catabolism **(Trentacoste *et al.*, 2013)**, down-regulation of putative plastid TAG biosynthesis pathway **(Liu *et al.*, 2016 and Xin *et al.*, 2017)**, or reducing antenna size in the photosynthetic machinery **(Polle *et al.*, 2003 and De Mooij *et al.*, 2015)**. However, limited success has been achieved and none of the engineered strains has been validated under outdoor production conditions.

N deprivation is the most commonly used stressor to induce TAG production in microalgae **(Hu *et al.*, 2008)**. Under N deprivation, however, algal cell growth is almost completely arrested and TAG production is compromised. By contrast, P deprivation is found to promote TAG production while maintaining moderate cell growth, leading to higher TAG yield **(Iwai *et al.*, 2014 and 2015)**.

To evaluate the effect of Δ*vtc1* mutation on cellular response to different nutrient regimes, the effect of N or P deprivation was compared on the growth, lipid body biogenesis, and TAG accumulation in Δ*vtc1* and *VTC1* cells. The maximum density of *VTC1* and Δ*vtc1* cells increased 4.5- and 2.2-fold, respectively, under P-depleted conditions. By contrast, the growth of both strains was arrested and the cell density barely changed under N-depleted conditions **(Figure 6a)**. The color of the batch culture (after 6 days of inoculation) under each condition reflected the cell density of the culture **(Figure 6b)**.

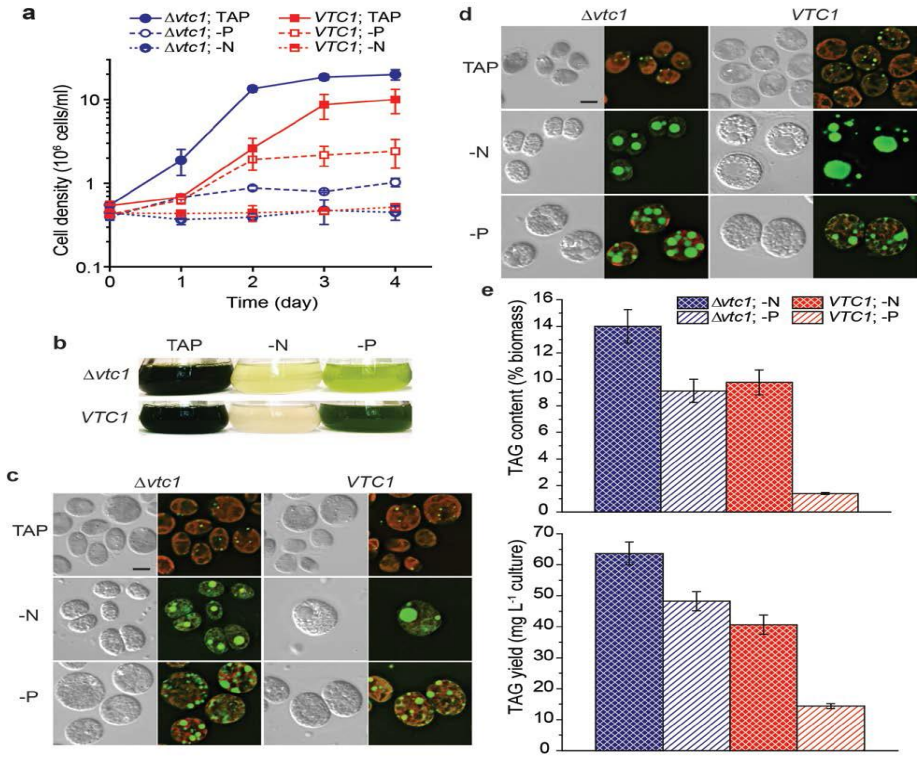

Figure (6) Effects of P-starvation and N-starvation on the growth and TAG accumulation in *C. reinhardtii* Δ*vtc1* and *VTC1* cells (a) Growth curves of *C. reinhardtii* Δ*vtc1* and *VTC1* rescue strains grown in TAP, TAP-N or TAP-P medium. Data are mean ± standard deviation (SD) of three biological repeats. (b) Batch cultures in TAP, TAP-N or TAP-P medium for 6 days. (c, d) Detection of lipid bodies in cells by Nile red staining. *C. reinhardtii* Δ*vtc1* or *VTC1* cells were cultured in TAP, TAP-N or TAP-P medium for 4 days (c) or 8 days (d). DIC and fluorescent images of Nile red-stained cells are shown. Fluorescent images were shown in two channels: green fluorescence, lipid bodies; red fluorescence, chlorophyll autofluorescence. Bar = 5 µm. (e, f) In the cultures of *C. reinhardtii* Δ*vtc1* and *VTC1* rescue strains under P-deplete or N-deplete after 14 days, TAG concentration (e) and TAG yield (f).

Microscopic analysis showed that, when grown under N- and P-replete conditions, Δ*vtc1* and *VTC1* cells appeared to have strong chlorophyll autofluorescence but only few small lipid bodies **(Figure 6c,**

d). When grown in N-depleted medium, Δ*vtc1* and *VTC1* cells had some large lipid bodies that took up a large portion of each cell, and the signal of chlorophyll autofluorescence was low at day 4 (**Figure 6c**) or undetectable at day 8 (**Figure 6d**). When grown in P-depleted medium, multiple lipid bodies occupying a large portion of a cell were observed **(Figure 6c, d)**, particularly in the Δ*vtc1* cells **(Figure 6d)**. However, unlike under N-depleted conditions, considerable amounts of chlorophyll autofluorescence signals remained visible in Δ*vtc1* and *VTC1* cells under P-depleted conditions **(Figure 6c, d)**. These microscopic images indicate that although both N and P deprivation are able to induce TAG accumulation in *C. reinhardtii*, cells undergo more severe breakdown of chlorophyll and photosystems under N-depleted conditions. Under P-depleted conditions, cells are able to retain some chloroplast integrity, in line with the growth kinetics of these strains **(Figure 6a, b)**. It is also noteworthy that the size of the Δ*vtc1* cells was larger under P-depleted conditions than under P-replete, N-replete, and N-depleted conditions **(Figure 6c, d)**. This result is consistent with a previous report showing that *C. reinhardtii* cells were larger under P-depleted conditions than under N-depleted conditions **(Iwai *et al.*, 2014)**.

TAG concentrations in Δ*vtc1* and *VTC1* cells after 14-day exposure to N or P deprivation was quantified. In the same strain background, TAG concentrations were higher in cells grown under N deprivation than under P deprivation. For the Δ*vtc1* strain, the TAG concentration was 1.5-fold higher in cells grown under N deprivation than in cells grown under P deprivation (14.0% vs 9.1% dry weight; **Figure 6e**). For the *VTC1* strain, the TAG concentration was 6.9-fold higher in cells grown under N deprivation than in cells grown under P deprivation (9.8% *vs* 1.4%; **Figure 6e**). When assessing the effect of the Δ*vtc1* mutation, Δ*vtc1* cells produced higher level of TAGs than *VTC1* cells under both N depleted or P depleted conditions. The Δ*vtc1* mutation resulted in a more drastic increase in TAG under P-depleted conditions; the TAG concentration in Δ*vtc1* cells under N and P-depleted conditions were 1.5-fold and 6.9-fold higher, respectively, than those of *VTC1* cells (**Figure 6e**). The same trend was observed for TAG yield, i.e. in the same strain background, TAG yield was higher from cultures grown under N deprivation than under P deprivation (**Figure 6e**). Moreover, the Δ*vtc1* mutation resulted in a more drastic increase in TAG yield under P deprivation (a 2.8-fold increase) than under N deprivation conditions (a 1.3-fold increase) (**Figure 6f**).

In conclusion, N deprivation is often used to induce TAG in microalgae, while cell response to P deprivation has been less explored for TAG production **(Hu *et al.*, 2008; Fields *et al.*, 2014 and Goncalves *et al.*, 2016)**. The effect of *VTC1* defect on cell response to N or P deprivation was comparatively studied. The results showed while N deprivation completely halted the cell growth, P deprivation still allowed considerable cellular growth during TAG accumulation, in line with previous reports that P deprivation promotes TAG production while maintaining moderate cell growth **(Iwai *et al.*, 2014)**. Algal biomass productivity is thought to be a key bottleneck for algae biofuel production **(DOE, 2010)**. As such, several genetic engineering strategies have been designed to increase algal photosynthetic efficiency and biomass productivity under N deprivation **(Lenka *et al.*, 2016)**. Manipulation of polyP biosynthesis under P-depleted conditions may be a promising alternative to N deprivation-based method, allowing simultaneous cell growth and TAG production.

CONCLUSION

In this study, the physiological and molecular mechanisms of polyphosphate biosynthesis and its link with lipid biosynthesis in *C. reinhardtii* were investigated using the *Δvtc1* strain. It is generally believed that microalgae produce polyP when P is abundant and break down polyP reserve upon P deprivation. *VTC1* deficiency affects the P_i uptake in *C. reinhardtii*. The Δ*vtc1* mutant is more sensitive to P deficit.

Interestingly, a link between polyP metabolism and lipid biosynthesis in microalgae is discovered. A defect in the *VTC1* gene drastically increased TAG concentration and TAG yield in *C. reinhardtii* under P deprivation. This held true even for an extended cultivation period (up to 21-day), validating its biotechnological importance for improved TAG production. Moreover, deletion of the *VTC1* gene altered fatty acid composition of TAGs. The Δ*vtc1* mutation increased MUFA content (C18:1) of TAG at the expense of SFA under P deprivation conditions, suggesting a new strategy to improve both fuel quality and yield through manipulation of the polyP biosynthesis pathway.

By comparing the effects of *VTC1* deletion on cell response to N or P deprivation, the results showed that while N deprivation completely halted the cell growth, P deprivation still allowed considerable cellular growth during TAG accumulation. Thus, manipulation of polyP biosynthesis under P-depleted conditions may be a promising alternative to N deprivation-based method, allowing simultaneous cell growth and TAG production.

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تأثيرات تعطيل جين الناقل الفجوي المرافق 1 (*VTC1*) على إنتاج الجلسريدات الثلاثية كما و نوعا في طحلب الكلاميدوموناس رينهاردتي تحت ظروف الحرمان من الفوسفور

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تم دراسه عمليات تخليق الدهون الطحلبية بكثافة تحت ظروف الحرمان من النيتروجين، حيث يتسارع معدل تراكم الجلسريدات الثلاثية و يتضاءل النمو الخلوي. و على العكس من ذلك، فما زال الكثير مجهولا عن عمليات إنتاج الجلسريدات الثلاثية تحت ظروف الحرمان من الفوسفور، و ما زالت المعلومات محدودة عن عمليات بناء مركبات عديدات الفوسفات و هدمها. لذا فقد حاولت هذه الدراسة أن تتعرف أكثر على كيفية تخليق عديدات الفوسفات على المستوى الجزيئي و الرابط بينها و بين عمليات تخليق الدهون في طحلب الكلاميدوموناس رينهاردتي مستخدمين سلالة منزوعة جين الناقل الفجوي المرافق 1 (*ΔVTC1*). و كانت أهم النتائج المتحصل عليها: زيادة تواجد عديدات الفوسفات في السلالة المُكمَّلة في ظل توافر الفوسفور، و تكسّرت عند الحرمان من الفوسفور. و لقد تضاعف إنتاج الجلسريدات الثلاثية 9.6 ضعفا في السلالة المطفرة المحرومة من الفوسفور بعد 21 يوما من الحرمان، كما تغير محتوى الأحماض الدهنية فيها؛ حيث زادت نسب الأحماض الدهنية غير المشبعة الأحادية و العديدة على حساب المشبعة. هذه النتائج تبرز وجود علاقة سلبية بين تخليق عديدات الفوسفات و تخليق الجلسريدات الثلاثية في الطحالب الدقيقة، كما تقترح استراتيجية جديدة لتحسين جودة و كمية الوقود الطحلبي الحيوي المنتج عن طريق تعديل مسارات تخليق عديدات الفوسفات.