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EFFECTS OF *VACUOLAR TRANSPORTER CHAPERONE 1* DISRUPTION ON PHOSPHORUS STRESS RESPONSE NETWORK IN *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

Phosphorus (P) stress response network and transport has been studied in microalgae. However, less is known about the interactions between polyphosphate (polyP) biosynthesis and P stress response network. Also, knowledge about polyP metabolism in microalgae remains limited. In this study, the molecular mechanisms of polyP biosynthesis and its link with P stress response regulation and phosphate (Pi) transport were investigated in *Chlamydomonas reinhardtii* using a mutant defective in the *vacuolar transporter chaperone 1* gene (*VTC1*). The presence of polyP was abundant in the *C. reinhardtii VTC1* rescue strains when Pi was copious, whereas polyP was degraded when P was depleted in the medium. Also, deletion of *VTC1* severely repressed the expression (20-fold compared to the expression in *VTC1* rescue) of *Phosphorus Stress Regulator 1* (*PSR1*) as well as *Phosphate Transporter Type B2* (*PTB2*) and *PTB4*, suggesting that defects of *VTC1* may perturb the P stress response through repression of *PSR1* expression. Moreover, differentiated expressions of type A (acidic phosphate transporters) and type B (alkaline phosphate transporters) are observed in response to P deprivation. These

data suggest that PTBs in *C. reinhardtii*, grown in neutral to slight basic medium pH conditions, may be the primary P_i transporters under P deprivation and they are controlled by *PSR1* which is may be regulated by polyP molecules. Thus, manipulation of polyP biosynthesis under P stress condition may be a promising strategy for agriculture in alkaline soil to face and tolerate P deprivation stress.

Key words: *Chlamydomonas reinhardtii*, vacuolar transporter chaperone 1, polyphosphate, phosphorus stress response regulators, phosphate transporters, alkaline soils

INTRODUCTION

While phosphorus (P) is an abundant element in the earth's crust, its availability can limit the growth of organisms present in both aquatic and terrestrial environments **(Grossman and Aksoy, 2015)**. P is essential 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)**. The major form of P readily assimilated and utilized by most organisms is the phosphate anion (P_i). While available P_i (soluble P_i) is generally present at concentrations of <10 μM, most organisms require cellular P_i concentrations in the millimolar range **(Moseley *et al.*, 2006)**. To cope with this limited availability, plants and microalgae have evolved adaptive mechanisms to facilitate improved acquisition and conservation of P and to allow survival under P starvation conditions **(Moseley *et al.*, 2006 and Rouached *et al.*, 2010)**

The unicellular green alga *Chlamydomonas reinhardtii* is a model organism for many physiological and biochemical processes. Having a simple life cycle, easy isolation of mutants, full genome sequence and a growing array of tools and techniques for molecular biology studies helped *C. reinhardtii* to be a robust model to study many physiological processes **(Harris, 2001; Aksoy *et al.*, 2014; Baltz *et al.*, 2014 and Zalutskaya *et al.*, 2018)**. In *C. reinhardtii*, P limitation triggers the expression of a series of genes, homologous to yeast *PHOSPHATE* (*PHO*) regulon, involved in phosphate uptake and transport to rapidly respond to the P limitation stress **(Moseley *et al.*, 2006)**.

Polyphosphate (polyP), 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, signal transduction and post-translation modifications **(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. **Cohen *et al.* (1999) and Ogawa *et al.* (2000)** identified novel gene family members, *Vacuolar Transporter Chaperone1* (*VTC1), VTC2*, *VTC3* and *VTC4* genes, to be involved in polyP biosynthesis in *Saccharomyces cerevisiae*. **Ogawa *et al.* (2000)** reported that VTC1 protein, the subunit 1 of the vacuolar transporter chaperone, is a small membrane protein containing three transmembrane domains docking the complex to the vacuolar or ER membrane. Deletion of *VTC1* gene impairs polyP accumulation in yeast, indicating VTC1 is essential for polyP biosynthesis.

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 polyP 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, phosphorus stress response regulation network, have not been investigated yet.

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 interactions of polyP biosynthesis with phosphorus stress response network.

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 and P-free TAP media were used for nutrient replete and P-depleted conditions, respectively, as previously described by **Li *et al.* (2010)**.

Table (1) Chemical composition (g) per one liter TAP or TAP-P medium

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

TAP-P medium was prepared by replacing potassium phosphate with 1.5 mM KCl. 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. Medium phosphate determination

For medium phosphate assay, 1 ml of culture's supernatant was used according to the method of **Chen *et al.* (1956)**.

3. Total P and polyP assays

In order to measure the amount of cellular total P and polyP, the whole cell lysate and polyP 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 for 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 as 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 polyP 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 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.

4. RNA isolation and quantitative real-time PCR

Total RNA was isolated from *C. reinhardtii* cells using Trizol reagent (Invitrogen) and quantified using NanoDrop 2000c spectrophotometer (Thermo Scientific). One µg RNA was converted to cDNA using Protoscript II First Strand cDNA synthesis kit (NEB). The expression of *vtc1* was determined by quantitative real-time PCR (qRT-PCR) using Applied Biosystems 7500. Each qRT-PCR reaction contains 25 ng cDNA, 400 nM of each primer pair and 10 µl of SYBR Green PCR Master Mix (Invitrogen) to a final volume of 20 µl. PCR cycling conditions consisted of an initial polymerase activation step at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The expression of 18s rRNA was used as the reference. The relative expression level was measured using the $2^{-\Delta\Delta CT}$ method. The experiment was performed twice, with three biological replicates and four technical replicates for each sample. The primer sequences used in qRT-PCR are listed in **Table (2)**.

Table (2) Primer sequences used in this study

Primer name	Sequence (5' to 3')
CrVTC1F	TAAGTCATATTTCGCCAACGAG
CrVTC1R	TTGTACGCTTTGAGATGAGGTG
CrPSR1F	CTTCTGACTTTATGCAAACGGGC
CrPSR1R	CAGCATGTACTCTGAGGACTGC
CrPTB2F	CTCGTGCATTCTCTTCCTGATTG
CrPTB2R	GAGTCTTCTTGGCACCCTTG
CrPTB4F	GCGTCTGCCATCATCTTCTG
CrPTB4R	GCCTTGTTCGAGTCCCAGT
CrPTA1F	AGGTGACGCTGCTTACAATG
CrPTA1R	GAAGTAAGCCTCGCAGAACATTC
CrPTA3F	GGAAGGTCGAGTACAAGAACTGC
CrPTA3R	GCGACACTGCCCCATTTG
CrPTA4F	GGAAGGTCGAGTACAAGAACTG
CrPTA4R	TGACACTGCCCCATTTGC
Cr18SrRNA_f	ACGAGACCTCAGCCTGCTAAAT
Cr18SrRNA_r	TTATCGCCTCATACTTCCATTGG

5. Fluorescence staining and microscopy

High concentration 4'-6-diamino-2-phenylindole (DAPI), a fluorophore usually used to detect nucleus, was used for *in situ* staining of polyP granules in cells. The interaction of DAPI and polyP resulted in a yellow fluorescence emission (peak wavelength at 526 nm), instead of blue fluorescence emission (peak wavelength at 456 nm) from the interaction with DNA **(Tijssen *et al.*, 1982)**. Eight million *C. reinhardtii* cells from a batch culture grown in TAP or TAP-P medium were harvested and washed with McIlaine buffer (20 mM citric acid, 160 mM Na_2HPO_4, and pH 7.0). The cells were fixed with 200 µl 4% formaldehyde (prepared in McIlaine buffer) and incubated at room temperature for 30 min with gentle rocking and then washed with McIlaine buffer for 3 times to remove the fixative. They were then treated with 200 µl 0.3% (v/v) Triton X-100 (prepared in McIlaine buffer) for 5 min to enhance the permeability of the cell wall followed by washing with McIlaine buffer. Next, the cells were mixed with 200 µl 20 µg/ml DAPI solutions (prepared in McIlaine buffer) and incubated for 30 min at room temperature in the dark. The stained cells were washed with McIlaine buffer to remove the excess dye and resuspended in 200 µl McIlaine buffer.

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. Chroma 89006 ECFP/EYFP/mCherry filter set (beam pass excitation 420-443 nm; beam pass emission 522-549 nm) and Zeiss filter set 49 (excitation peak 365 nm; beam pass emission 420-475 nm) were used to observe polyP granule (in the yellow fluorescent channel) and nucleus (in the blue fluorescent channel), respectively, stained by DAPI. The images were analyzed with Volocity® 6.2.1 software (PerkinElmer). The fluorescence channels were deconvolved using the restoration function of the Volocity® software.

RESULTS AND DISCUSSION

1. Mutation stability

RT-PCR reactions for both mutant and rescue strains grown under TAP and TAP-P media in different time points, in **Figure (1),** emphasized absence of *VTC1* transcripts in the mutant and presence of *VTC1*

transcripts in the rescue strain under all tested conditions. This was an initial step to ensure the stability of *Δvtc1* mutation and the purity of the mutant strain.

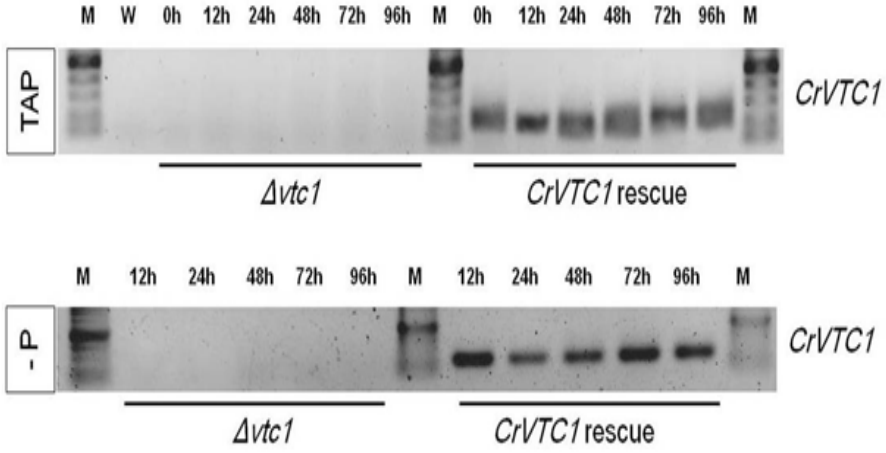

Figure (1) Stability of *ΔVTC1* Agarose ethidium bromide stained gel is showing amplified expression of *VTC1* in the mutant (expression is absent) and rescue strain grown under P deprivation compared to *VTC1* expression of mutant (expression is absent) and rescued strain grown in TAP medium after 30 cycles of PCR amplification. W is a negative control.

2. 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 phenotypes of the Δ*vtc1* mutant (CC5165) and the *VTC1* rescue strain (*VTC1,* CC5166) under P-repleted (TAP) and P-depleted conditions (TAP-P) were analyzed. 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 2**). This phenotype may be interpreted by the negative relationship between polyP and the activity of topoisomerase I, DNA super-coiled relaxing enzyme **(Azevedo *et al.*, 2015);** when polyP is absent, topoisomerase I is active to relax DNA super-coiling as a step to enhance DNA replication and cell division as well as minimize generation time. However, grown in TAP-P medium, the growth of both strains was comparably compromised by P deprivation (dash lines in **Figure 2**).

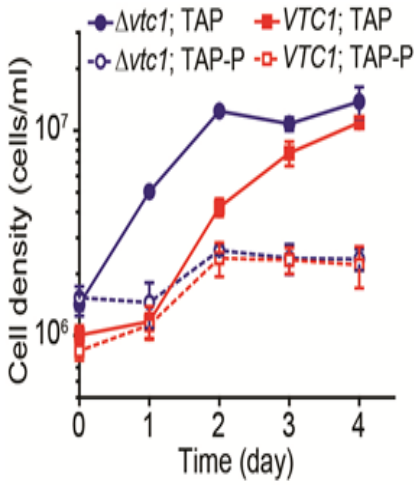

Figure (2) Growth curves of batch cultures of *C. reinhardtii* Δ*vtc1* and *VTC1* rescue strains in full TAP (TAP) or P-free TAP (TAP-P) medium. Values are mean ± standard deviation (SD) of three biological repeats.

Also, a significant difference of Pi uptake is observed between Δ*vtc1* and rescue strains grown in TAP after two days from inoculation till the end of the experiment **(Figure 3)**. The concentrations of P in the culture of Δ*vtc1* and rescue strains were 32.1 and 31.3 ppm respectively at zero time. After 24, 48, 72 and 96 hours from inoculation, the concentrations of P in medium of Δ*vtc1* were 29.4, 25.7, 25.6 and 26.2 mgl^{-1} respectively, while P concentrations in medium of rescue strain were 30.6, 18.8, 14.7 and 14 mgl^{-1} respectively at the same time points. These observations are aligned with growth rate records of rescue strain not Δ*vtc1* strain **(Figure 2)**. This may be a result of disruption in Pi transport system at the level of plasma membrane or vacuolar membrane by absence of VTC1. It is stated that both Pi uptake from the soil and its translocation in *Planta* (between organs and between subcellular compartments) require the coordination of a set of proteins with Pi transport activities **(Gu *et al.*, 2016)**. Also, a comparative transcriptome analysis revealed that Pi application to Pi- starved arbuscular mycorrhizal (AM) fungi hyphae induced the expression of the genes encoding Pi symporters, P- type ATPases and VTC complex **(Kikuchi *et al.*, 2014)**. Interestingly, the vacuolar endopolyphosphatase (PPN) responsible for polyP hydrolysis **(Kumble and Kornberg, 1996)** and a V- type Pi

exporter (PHO91) that releases Pi from the vacuoles **(Hürlimann *et al.*, 2007)** were also up- regulated **(Kikuchi *et al.*, 2014)**, supporting the idea that polyP is dynamically turning over during the accumulation and translocation **(Ezawa *et al.*, 2001)**.

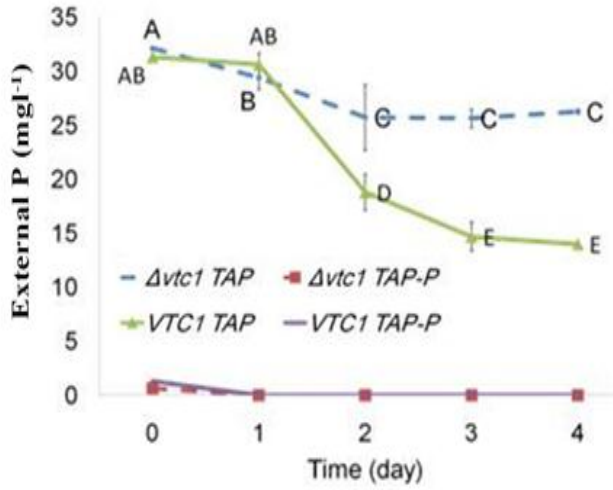

Figure (3) External phosphorus concentration (mgl^{-1}) in cultures of *C. reinhardtii* Δ*vtc1* (dash lines) and *VTC1* (solid lines) rescue strains in full TAP (TAP) or P-free TAP (TAP-P) medium. Values are mean ± standard deviation (SD) of three biological repeats.

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 4a**). 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 4a**). Interestingly, Δ*vtc1* cells had very low levels of total P regardless the availability of P in the growth medium (blue lines in **Figure 4a**), this is also suggesting that Δ*vtc1* mutation impairs P uptake in *C. reinhardtii*.

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, polyP accumulation in the Δ*vtc1* mutant was measured and evaluated whether it was correlated to the availability of P in the medium. *In situ* microscopic study showed no polyP signal was visible in the Δ*vtc1* mutant, whereas yellow fluorescence from polyP

granules in *VTC1* cells was obvious only under P-replete conditions **(Figure 5)**. Next, the dynamic of polyP accumulation was determined in *VTC1* cells under P-replete and P-depleted conditions. In this study, staining with DAPI showed that the signal of polyP granules increased under P-replete conditions on day 1, and remained visible throughout the four-day cultivation period; by contrast, under P-depleted conditions, polyP granules became undetectable after day 2 **(Figure 4d)**.

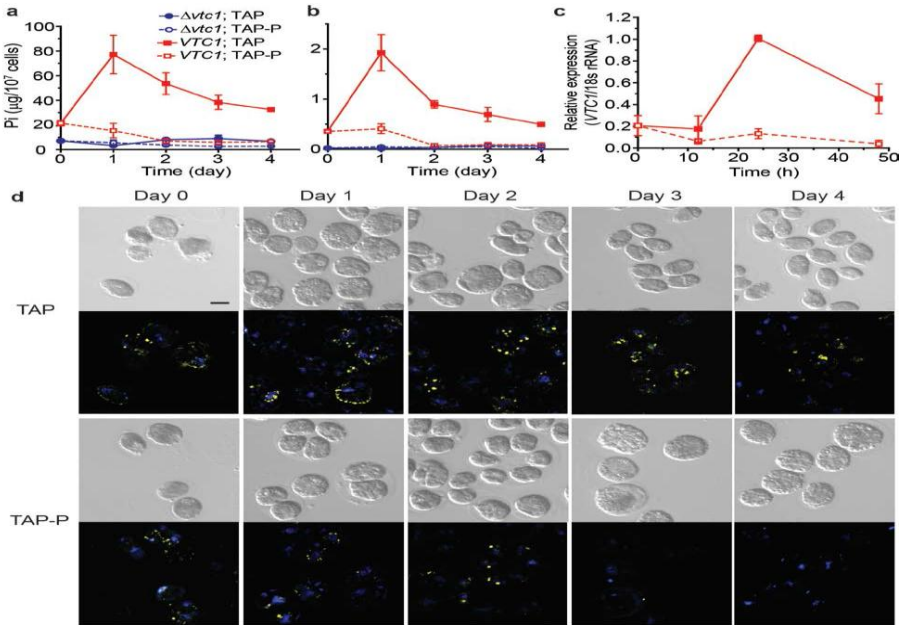

Figure (4) P starvation and Δ*vtc1* mutation affect P accumulation and the *VTC1* gene expression in *C. reinhardtii*. (a, b) Kinetic of P accumulation in *C. reinhardtii* Δ*vtc1* and *VTC1* cells. Total P (a) and poly P (b) in batch cultures under P-replete (TAP) or P-deplete (TAP-P) conditions were measured. Data represents mean ± standard deviation (SD) of three biological repeats. (c) Expression of the *VTC1* gene in *C. reinhardtii VTC1* cells. mRNA levels in batch cultures under P-replete and P-deplete conditions were measured by RT-qPCR. The relative expression level of the *VTC1* gene was normalized to that of 18s rRNA. Data represents mean ± standard deviation (SD) of three biological repeats. (d) *In situ* detection of poly P granules in *C. reinhardtiiVTC1* cells under P-replete or P-deplete conditions for 0 - 4 days. Differential interference contrast (DIC) and fluorescent images of DAPI-stained cells are shown. Fluorescent images were shown in two channels: yellow fluorescence, poly P granules; blue fluorescence, nucleus. Bar = 5 µm.**

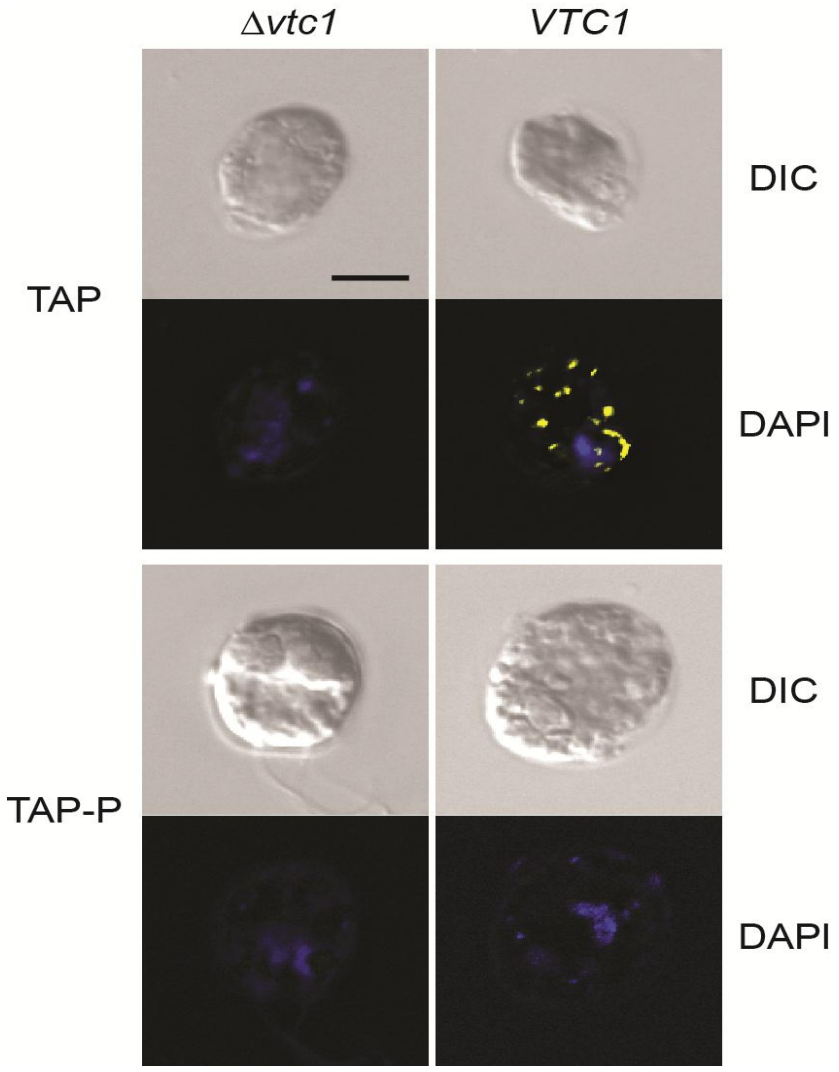

Figure (5) Microscopic detection of poly P granules stained by DAPI. Differential interference contrast (DIC) and fluorescent (DAPI) images of DAPI-stained *C. reinhardtii* Δ*vtc1* and *VTC1* cells are shown. Fluorescent images were shown in two channels: yellow fluorescence, poly P granules; blue fluorescence, nucleus. Bar = 5 µm.

Moreover, the polyP level in the cells was quantitatively measured. 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, a peak (with 1.9 µg polyP in 10^7 cells) at day 1 and a sharp decline afterwards (red solid line in **Figure 4b**). When shifted to P-

depleted conditions, the level of polyP in *VTC1* cells (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 4b**). 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 content of polyP was 1.1% - 1.7% from day 0 to day 2. The level of polyP in Δ*vtc1* cells was undetectable under these experimental conditions (blue lines in **Figure 4b**), suggesting this strain is unable to accumulate any polyP.

It is generally believed that microalgae produce 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 **(Abida *et al.*, 2015 and Martin *et al.*, 2014)**. PolyP is believed to serve as an energy reserve in bacteria **(Kornberg *et al.*, 1999 and Rao *et al.*, 2009)**, but its biological function and role in microalgae remains largely unknown. Data in this study 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* complemented strain, but absent in the Δ*vtc1* strain.

3. Expression of *VTC1* gene corresponds to polyP accumulation

To evaluate whether the expression of the *VTC1* gene is in line with the dynamic of polyP levels in *C. reinhardtii*, the relative abundance of the *VTC1* mRNA was measured. Under P-replete conditions, the level of the *VTC1* expression remained constant during 0-12 h and then showed a rapid increase to reach a peak at 24 h. The level of expression at 24 h was 5.7-fold higher than that at 0 h (solid line in **Figure 4c**). When *VTC1* cells were switched to P-depleted conditions, the level of the *VTC1* expression gradually decreased (dash line in **Figure 4c**). The trend and the peak of *VTC1* expression follows the same fashion as the kinetics of polyP abundance in *VTC1* cells upon P repletion and P deprivation **(Figure 4b and c)**.

In the *VTC1* complemented strain, the level of total cellular P_i and polyP, implying the uptake of phosphate and polyP biosynthesis, rapidly increased four-fold and reached a peak at 24 h under P-replete conditions **(Figure 4b)**. The level of polyP decreased when P was depleted **(Figure 4b)**. The intracellular levels of total P in Δ*vtc1* cells, even under P replete conditions, were very low, suggesting *VTC1* deficiency affects the P_i uptake in *C. reinhardtii*. The Δ*vtc1* mutant is also more sensitive to P deficit as it showed a lower growth rate compared with the rescue strain

(Figure 2); likely because of its inability to assimilate P and its lower cellular P sink. Similar effect has been reported in VTC-deficient yeast strains. The ability of P_i uptake was severely interrupted in the Δ*vtc1* or Δ*vtc4* single mutants and Δ*vtc2*/Δ*vtc3* double mutant *S. cerevisiae* **(Ogawa *et al.*, 2000)**, confirming P_i uptake required functional VTC complex.

4. Δ*vtc1* mutation affects the expression of genes involved in the phosphate stress response and phosphate uptake in *C. reinhardtii*

The Δ*vtc1* mutation impairs phosphate uptake, thus it is likely that the expression of P_i transporters may also be affected by the Δ*vtc1* mutation. Since the P_i transporters are controlled by, the transcriptional regulator, Phosphorus Stress Regulator 1 (PSR1) during P stress response **(Moseley *et al.*, 2006 and Bajhaiya *et al.*, 2016)**, we further hypothesize Δ*vtc1* mutation disturbs the expression of PSR1 and subsequently that of P_i transporter genes downstream. To test this hypothesis, the expression of *PSR1* and the previously reported *PSR1*-responsive P_i transporter genes (*PTB2*, *PTB4*, *PTA1*, *PTA3* and *PTA4*) **(Moseley *et al.*, 2006 and Bajhaiya *et al.*, 2016)** was measured in Δ*vtc1* and *VTC1* strains.

In **(Figure 6)**, results showed in the *VTC1* strain, the expression of PSR1 was triggered by P deficiency. The relative level of PSR1 expression of *VTC1* cells was 10.7-fold higher under P deprivation than P-replete conditions. The expression of *PTB2* and *PTB4* in the *VTC1* strain was also elevated (by 2.3-fold and 1.3-fold, respectively) under P deprivation. As expected, Δ*vtc1* mutation strongly repressed *PSR1* expression as well as *PTB2* and *PTB4* expression under both P-replete and P-depleted conditions. *PSR1* expression showed a 20-fold decrease in Δ*vtc1* cells during P deprivation compared with that in *VTC1* cells. Similarly, *PTB2* and *PTB4* expression showed 5-fold and 6-fold decreases, respectively, under P-replete conditions by the Δ*vtc1* mutation, and 4-fold and 20-fold decreases, respectively, under P deprivation. The expression of the *PTA1*, *PTA3* and *PTA4* genes was low or undetectable under P-depleted conditions. However, under P-replete conditions, *PTAs* expression also showed Δ*vtc1*-dependent manners: the expression of *PTA3* and *PTA4* were suppressed by the Δ*vtc1* mutation while the expression of *PTA1* was increased. The expression levels of both *PTB*s, however, were much higher (up to 50-fold) than the *PTA*s in both strains. This is in line with previous reports suggesting that the PTB-type transporters are the predominant P_i transporters in *C. reinhardtii* **(Moseley *et al.*, 2006 and Bajhaiya *et al.*, 2016)**.

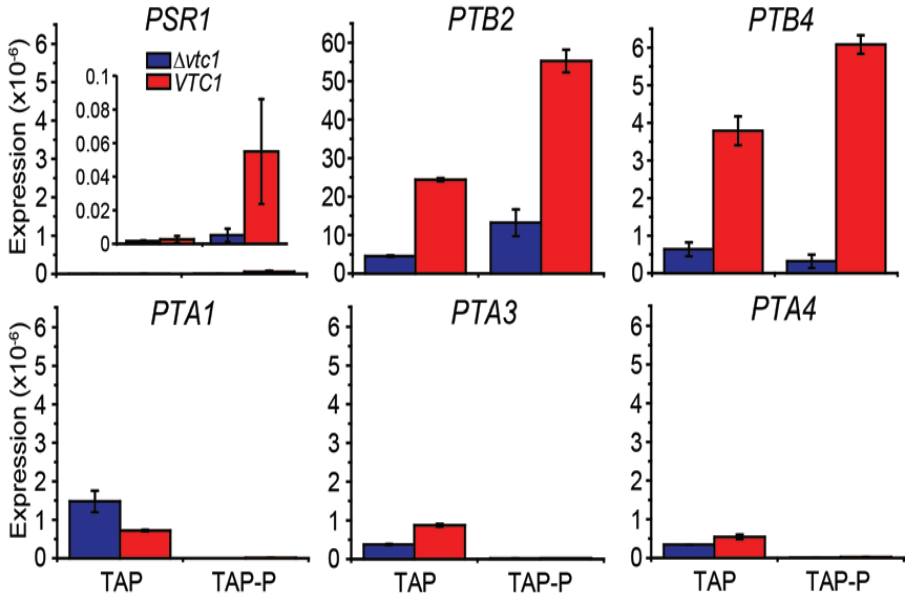

Figure (6) Expression of phosphate response and transporter genes in *C. reinhardtii* Δ*vtc1* and *VTC1* cells. mRNA levels in batch cultures under 24 h of P-replete (TAP) and P-deplete (TAP-P) conditions were measured by RT-qPCR. The relative expression levels of the selected gene (*PSR1*, *PTB2*, *PTB4*, *PTA1*, *PTA3* and *PTA4*) was normalized to that of 18s rRNA. Data represents mean ± standard deviation (SD) of three biological repeats.

The P limitation stress response pathway in *C. reinhardtii* is under the control of MYB-like transcriptional regulator PSR1 **(Shimogawara *et al.*, 1999; Wykoff *et al.*, 1999 and Moseley *et al.*, 2006)**. Microarray and qPCR analyses demonstrated that P deprivation, possibly through *PSR1*, up-regulated the expression of an alkaline phosphatase encoded by the *PHOX* gene, type B P_i transporters (alkaline P_i/Na^+ symporters) encoded by *PTB* genes (homologs of *PHO89* in *S. cerevisiae*) *PTB2*, *PTB3*, *PTB4* and *PTB5*, repressed the expression of the *PTA1*, and little to moderate up-regulated the expression of type A P_i transporters (acidic P_i/H^+ transporter) encoded by *PTA* genes (homologs of *PHO84* in *S. cerevisiae*) the *PTA2* and *PTA3* genes **(Moseley *et al.*, 2006 and Bajhaiya *et al.*, 2016)**.

In agreement, the results of this study showed the deletion of *VTC1* severely repressed the expression of *PSR1*, suggesting that defects of *VTC1* may perturb the P stress response in *C. reinhardtii* through

repression of *PSR1* expression. Also, differentiated expressions of type A and type B P_i transporters in *C. reinhardtii* were observed in response to P deprivation. The expression of type B P_i transporter genes (*PTB*s) was increased under P deprivation, correlated with that of *PSR1*; by contrast, the expression of *PTA*s was repressed under P deprivation **(Figure 6)**. In corroboration, the growth of *C. reinhardtii* is promoted in neutral to slight basic medium pH conditions, suggesting PTBs (the alkaline P_i transporters) may be the primary P_i transporters of *C. reinhardtii* grown under P deprivation. Conversely, *S. cerevisiae* growth usually promotes lower medium pH and acidic conditions. In line with that, VTC-mediated polyP biosynthesis has been reported to be correlated with the expression of genes in *PHO* pathway (e.g. *PHO4*, *PHO5* and *PHO84*), which encode homologs of PTA-type transporters **(Desfougeres *et al.*, 2016)**. By taking in the account of all these data, a promising strategy for agriculture in alkaline soil to face and tolerate P deprivation stress can be suggested. This strategy may depend on enhancing the expression of *VTC1* under Pi scarce alkaline environments to up regulate all of *PSR1*, PTB2 and PTB4 in order to increase P influx inside the grown plants.

CONCLUSION

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. The Δ*vtc1* mutant is more sensitive to P deficit.

As expected, a link among polyP metabolism, *PSR1* and PTBs in microalgae is observed; the deletion of *VTC1* severely repressed the expression of *PSR1*, suggesting that defects of *VTC1* may perturb the P stress response in *C. reinhardtii* through repression of *PSR1* expression. Also differentiated expression of type A and type B P_i transporters is observed in response to P deprivation. The expression of *PTB*s is positively correlated with that of *PSR1* under P deprivation; by contrast, the expression of *PTA*s is negatively correlated. In corroboration, the growth of *C. reinhardtii* is promoted in neutral to slight basic medium pH conditions, suggesting PTBs (the alkaline P_i transporters) may be the primary P_i transporters of *C. reinhardtii* grown under P deprivation. Thus, manipulation of polyP biosynthesis under P stress condition may be a promising strategy for agriculture in alkaline soil to face and tolerate P deprivation stress. This strategy may depend on enhancing the expression of *VTC1* to up regulate all of *PSR1*, PTB2 and PTB4 in order to increase P influx inside the grown plants.

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تأثيرات تعطيل جين الناقل الفجوي المرافق 1 (*VTC1*) على شبكة استجابات طحلب الكلاميدوموناس رينهاردتي للإجهاد الناتج عن الحرمان من الفوسفور

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لقد دُرست مجموعة الاستجابات للإجهاد الناتج عن الحرمان من الفوسفور في الطحالب الدقيقة من قبل و كذلك ميكانيكيات انتقال الفوسفور، و لكن المعلومات المتاحة عن علاقة عملية تخليق مركبات عديدات الفوسفات و مجموعة الاستجابات الناتجة عن الحرمان من الفوسفور ما زالت قليلة. بل إن المعلومات عن عمليات البناء و الهدم لعديدات الفوسفات نفسها ما زالت محدودة. لذا، عملت هذه الدراسة على استكشاف الرابط بين الميكانيكيات الجزيئية لتخليق عديدات الفوسفات من جهة و تداول الفوسفات و تنظيم الاستجابة للحرمان من الفوسفور من جهة أخرى في طحلب الكلاميدوموناس رينهاردتي. و استخدمت الدراسة لهذا الغرض سلالة منزوعة جين الناقل الفجوي المرافق 1 (*ΔVTC1*). و اتضح من الدراسة أن وفرة تواجد عديدات الفوسفات كان في السلالة المكمّلة بجين الناقل الفجوي المرافق 1 في ظل توافر الفوسفور، بينما تكسرت عديدات الفوسفات عند ندرة الفوسفورفي بيئة النمو. كما أوضحت الدراسة أن حذف هذا الجين أدى إلى تثبيط تعبير جين تنظيم الإجهاد الفسفوري 1 (*PSR1*) 20 مرة، وكذلك تثبيط تعبير جينَي نواقل الفوسفات الثاني و الرابع من الطراز B (*PTB2* and *PTB4*). لذا تقترح نتائج الدراسة أن حذف أو تعطيل جين الناقل الفجوي المرافق 1 قد يعيق الاستجابة الواقية من الإجهاد الناتج عن الحرمان من الفوسفور عن طريق تثبيط تعبير جين الإجهاد الفسفوري 1. كما اتضح من خلال الدراسة التباين بين استجابات نواقل الفوسفات في الوسط الحمضي من النوع A و نواقل الفوسفات في الوسط القلوي من النوع B تحت ظروف الحرمان من الفوسفور و حذف جين الناقل الفجوي المرافق 1. و من النتائج السابقة برز اقتراح بأن النواقل القلوية من النوع B هي نواقل الفوسفات الرئيسة في طحلب الكلاميدوموناس النامي في البيئات المتعادلة و المائلة للظروف القلوية الخفيفة، و ينظم عمل هذه النواقل جين تنظيم الإجهاد الفسفوري 1 و تتحكم في نشاطه عديدات الفوسفات. و هذا يسهم في اقتراح استراتيجية زراعية جديدة لمواجهة و تحمل الإجهاد الناتج عن نقص الإمداد بالفوسفور في الأراضي القلوية عن طريق التحكم في تخليق عديدات الفوسفات.