Transcriptional responses of the marine diatom Chaetoceros tenuissimus to phosphate deficiency

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1. Introduction

Marine diatoms play crucial roles in primary production and global carbon fluxes (Nelson et al., 1995). Among marine planktonic diatoms, *Chaetoceros tenuissimus* is a picoplanktonic diatom and widely distributed throughout the coastal waters of Japan (Tomaru et al., 2018), the Narragansett Bay (Rines, 1988), the Mediterranean Sea (Kooistra et al., 2010; Montresor et al., 2013), North Sea (Grzebyk et al., 2022), and the San Matias Gulf (Sar et al., 2002), in addition, metabarcoding data record the appearance of *C. tenuissimus* on the African, Asian, and New Zealand coasts (De Luca et al., 2019). In the coastal waters of Japan, *C. tenuissimus* frequently forms blooms during warm seasons (Tomaru et al., 2011) due to its great growth potential. According to laboratory data, *C. tenuissimus* clones (NIES-3714 and -3715) undergo over three divisions/day at high temperatures of 25°C−30°C (Tomaru et al., 2014). Furthermore, growth rates of six divisions/day have been recorded under semicontinuous culture with strong photoirradiation (Tomaru et al., 2021). These ecophysiological characteristics may allow *C. tenuissimus* to behave as a cosmopolitan species and form blooms at euphotic layers in warm coastal waters.

Diatom growth is controlled by physical, chemical, and biological factors. Generally, dissolved inorganic nitrogen and phosphorus are essential elements for microorganisms in oceanic waters. Orthophosphate (herein PO_4^{3-}), is a typical algal-available phosphorus (P) source (Paytan and McLaughlin, 2007). In coastal surface waters, however, phosphate concentrations are sometimes deficient and limit primary production (Duhamel et al., 2021; Matsubara et al., 2023). In particular, when water bodies are stratified during warm seasons, lower layers fail to supply euphotic layers with phosphate (Yamaguchi and Sai, 2015). Physiological responses of *C. tenuissimus* to P stress should play an important role in bloom dynamics in warm coastal waters.

Under phosphate-deficient conditions, *C. tenuissimus* NIES-3715 cultures appear to induce two enzymatic activities: phosphomonoesterase (synonym alkaline phosphatase; APase) and phosphodiesterase (PDEase) (Yamaguchi et al., 2014). Both enzymes drive hydrolysis of phosphate ester compounds that sometimes appear in equivalent amounts to orthophosphate in coastal waters,

as suggested by Suzumura et al. (1998) and Monbet et al. (2009). Although P-repleted cells of *C. tenuissimus* prefer orthophosphate as a primary P source (Yamaguchi et al., 2022b), they can access various types of phosphorus compounds, such as phospholipids and nucleic acids, as a sole phosphorus source (Yamaguchi et al., 2022b, 2014). Therefore, *C. tenuissimus* can endure P-limited conditions in nature. Molecular responses in P-stressed cells have been previously examined using a microarray analysis approach, whereby alkaline phosphatase and phosphate transporter were significantly upregulated (Yamaguchi et al., 2022a). Studies on gene responses to P deficiency were performed in several diatoms, namely *Thalassiosira pseudonana* (Dyhrman et al., 2012), *Thalassiosira weissflogii* (Wang et al., 2014), *Phaeodactylum tricornutum* (Cruz de Carvalho et al., 2016; Feng et al., 2015; Yang et al., 2014), *Skeletonema costatum* (Zhang et al., 2016), and *Chaetoceros affinis* (Shih et al., 2015). Among those studies, APases and phosphate transporters were highly expressed in addition to genes related to modified cell membranes containing sulfolipids. Indeed, cell membranes of phytoplankton, including *T. pseudonana*, were replaced by phospholipids with nonphosphorus membrane lipids, such as sulphoquinovosyl diacylglycerol (SQDG) and betain, maintained under P-deficient conditions (Martin et al., 2011; Van Mooy et al., 2009). These responses are directly related to increasing uptake of inorganic phosphate (Pi) and scavenging of P from dissolved organic phosphate (DOP) or intracellular P sources. Other gene responses for P-deficient conditions were observed in the glycolytic pathway for *T. pseudonana* (Alipanah et al., 2018; Dyhrman et al., 2012) and *P. tricornutum* regulates the transcription factor that regulates P deficiency-induced genes (PtPSR) (Kumar Sharma et al., 2020). In addition, P-deficient conditions influence cell proliferation and photosynthetic efficiency (Feng et al., 2015; Shih et al., 2015; Wang et al., 2014; Yang et al., 2014; Zhang et al., 2016).

The previously conducted the microarray analysis was not sufficient to detect the gene response in detail, similar to the aforementioned diatoms, owing to the incomplete reference genome sequence used for the microarray probes. However, in this study, we examined the transcriptome and metabolite profile of P-stressed *C. tenuissimus* NIES-3715 using the latest genome information

(Hongo et al., 2021), and explored its molecular and physiological responses to further understand its survival strategies in nature.

2. Materials and methods

2.1. Culture conditions

Algal cultures were grown in modified SWM-3 medium enriched with 2 nM Na_2SeO_3(Imai et al., 1996) under a 12/12-h light-dark cycle at 20°C. Light irradiance was 850 µmol m^{-2} s^{-1} using white LED illumination. The final concentration of additive phosphate (NaH_2PO_4 H_2O) for SWM-3 is 100 µM (Pi-replete SWM-3 media). We also prepared a phosphate deficiency SWM-3 medium with an additional final phosphate concentration of 3.3 µM (Pi-depleted SWM-3 media).

Exponentially growing cultures of *C. tenuissimus* NIES-3715 (1.1×10^6 cells/mL) were inoculated into eight 250 mL Pi-replete and Pi-depleted SWM-3 media (total 16 flasks) (0.36% v/v) at a final cell concentration of 4.0×10^3 cells/mL, respectively. Cells were counted with an image-based cytometric analysis with the Tali image-based cytometer (Thermo Fisher Scientific Ltd., Waltham, MA, USA) using the red channel (excitation filter, 543/22 nm; longpass emission filter, 585 nm). Cell size range in a bright field, red fluorescent threshold, circularity, and sensitivity were set at 3–20 µm, 1200, 8, and 9, respectively. A 25-µL aliquot of a *C. tenuissimus* culture was placed in disposable counting slides (Thermo Fisher Scientific Ltd., Waltham, MA, USA) and cells were counted according to supplier instructions after standing for 10 min in the dark at room temperature (Tomaru and Kimura, 2016). Four of the eight flasks after two days of incubation in Pi-replete and Pi-depleted conditions were used for metabolite analysis of exponential growth phase conditions of *C. tenuissimus*, while the other four flasks were sampled after seven days for metabolite and mRNA analysis of stationary phase conditions.

2.2. Measuring of alkaline phosphatase activity

Alkaline phosphatase activity in cell suspension was measured in triplicates, following the fluorescence methods of Hoppe (2003) and Yamaguchi et al. (2005). Methylumbelliferyl phosphate (Nacalai tesque, Inc., Kyoto, Japan) was used as the substrate. The time duration of methylumbelliferone released from the substrate was measured using a fluorescence plate reader (Wallac 1420 ARVOsx, PerkinElmer). Bulk activity was divided by cell density to obtain activity per diatom cell (mol $cell^{-1}$ $time^{-1}$).

2.3. Metabolite analysis

Pretreatment methodology for metabolomics followed our previous protocol (Hano and Tomaru, 2019) with slight modification. Briefly, an aliquot of each diatom culture (approx. 4×10^7 cells) were retained onto a 0.4 μm polycarbonate membrane filter (PC MB 47 mm; GE Healthcare Japan, Tokyo, Japan), then the filter retaining the diatom cells was embedded in a glass Petri dish on ice and treated with 750 μL of methanol (HPLC-analytical grade, FUJIFILM Wako Chemicals, Osaka, Japan) and 75 μL of internal standard (IS) solution (ribitol 100 mg/L in ultrapure water, FUJIFILM Wako Chemicals). A volume of 660 μL of the mixture solution was mixed with 240 μL of ultrapure water and 240 μL of chloroform (pesticide-analytical grade, Kanto Kagaku Chemical, Tokyo, Japan). After vortexing for 10 s, samples were shaken for 30 min at 37°C and centrifuged for 5 min at 16,000 × *g* and 4°C. Mixtures were then mixed with 400 μL of ultrapure water and centrifuged again for 5 min. The 800 μL supernatant (water layer) was mixed with 50 μL of d_{27}-myristic acid solution (Sigma-Aldrich, 200 mg/L in methanol), after which samples were dried completely in a vacuum centrifuge drier and metabolites were derivatized in two steps, oximation and silylation (Hano et al., 2018).

We performed gas chromatographic separation of metabolites using the same GC-MS system as described previously (Hano and Tomaru, 2019). The Agilent Fiehn Retention Time Locking Library was used to identify metabolites and generate semiquantitative relative response factors, which were rationed against IS. Significant differences between groups were identified using *t*-tests for

parametric data and Welch's *t*-tests for nonparametric data after false discovery rate (FDR) adjustment at a significance threshold of 0.05 ($q < 0.05$).

2.4. RNA extraction and sequencing

Cells in 30 mL of the sample were retained onto 0.4 μm polycarbonate membrane filters (GE Healthcare), then put into 1.5-mL tubes and rapidly frozen with liquid nitrogen. Samples were then stored at −80°C until analysis, after which we extracted total RNA with the RNeasyPlus Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA concentration and quality were measured using Nanodrop 2000 (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer, respectively. The TruSeq Stranded mRNA Sample Prep Kit (Illumina) was used for cDNA library construction according to the manufacturer's instructions and the libraries were sequenced by 100 bp paired-end reads using the Illumina HiSeq 2500 platform.

2.5. Differential expression gene (DEG) analysis

Sequence reads with any adapter sequences, low-quality ends (<QV30), and unpaired reads were removed from sequences using Trimmomatic (Bolger et al., 2014). The remaining paired-end reads generated from transcripts for each condition were mapped to genome sequences of *C. tenuissimus* NIES-3715 (accession no. GCA_021927905.1) using HISAT2 (Kim et al., 2019). The mapped reads within coded genes were counted as transcription levels by *samtools* (Li et al., 2009) and *htseq-count* (Putri et al., 2022). Transcription levels were then normalized among libraries using the trimmed mean of M-value method (Robinson and Oshlack, 2010) and statistically compared with the *edgeR* package (Robinson et al., 2010) in R software ver. 3.6.0. Significant differences (*P*-values) in the transcription levels were adjusted by false discovery rate (FDR, Benjamini and Hochberg, 1995) and the FDR less than 5% was chosen as the threshold for differentially-expressed genes. Normalized fragments per kilobase of exon per million mapped reads (FPKM) were calculated in *edgeR* as the transcription levels.

2.6. Kegg pathway analysis

The Kegg ortholog (KO) number for *C. tenuissimus* NIES-3715 was assigned using BlastKOALA (Kanehisa et al., 2016) at the Kegg website (https://www.kegg.jp/blastkoala/), which conducts BLAST searching against a nonredundant dataset generated from the KEGG GENES database and assigns a KO number (Kanehisa et al., 2016). Genes related to metabolic pathways were retrieved using the KO numbers from the kegg pathway: glycolysis/gluconeogenesis (00010), pentose phosphate pathway (00030), carbon fixation in photosynthetic organisms (00710), glycerolipid metabolism (00561), glycerophospholipid metabolism (00564), Inositol phosphate metabolism (00562), Pentose and glucuronate interconversions (00040), and TCA cycles (00020).

2.7. Prediction of protein localization

Cellular localization of all proteins coded in *C. tenuissimus* NIES-3715 genome were predicted using SignalP (Teufel et al., 2022), TargetP (Almagro Armenteros et al., 2019), and TMHMM (Möller et al., 2001) tools locally. If protein localization could not be determined using these analysis tools, the website version of DeepLoc (Thumuluri et al., 2022) on the website (https://services.healthtech.dtu.dk/service.php?DeepLoc-2.0) was used.

2.8. Phylogenetic relationship of alkaline phosphatase

Amino acid sequences of APase for several organisms were retrieved from Lin et al. (2013). These sequences are homologous to six APases of *C. tenuissimus* NIES-3715 (CTEN210_11224, CTEN210_01177, CTEN210_18438, CTEN210_14915, CTEN210_11546, and CTEN210_12547) were aligned using MAFFT ver. 7.427 (Katoh et al., 2002) and gaps were automatically trimmed by trimAl (Capella-Gutiérrez et al., 2009) using the "-automated1" command option and default settings for all other options. We identified the best fit evolutionary model for the alignment by ModelFinder (Kalyaanamoorthy et al., 2017) using the Akaike Information Criterion. We then generated an

unrooted maximum likelihood (ML) tree using IQ-TREE (Minh et al., 2020) with 1000 bootstrap replicates.

3. Results

3.1. Physiological response under culture conditions

Cell concentrations of both Pi-replete and Pi-depleted cultures logarithmically increased from zero to three days of culture (log phases), then maintained until seven days of culture (stationary phases, Fig. 1a). In the stationary phase, cell concentration of Pi-depleted cultures was half that of Pi-replete cultures (Fig. 1a). Consequently, the color of Pi-depleted culture at seven days was paler than Pi-replete culture (Fig. 1b) and photosynthetic activity of Pi-depleted culture at seven days was also significantly lower than Pi-replete culture (*t-test* < 0.05, Fig. 1c). In both growth phases, APase activity in Pi-depleted culture was significantly higher than the Pi-replete culture (*t-test* < 0.05, Fig. 1d).

We detected 44 metabolites in this study and the relative abundance of these metabolites in the stationary growth cells is shown in supplementary figure 1. Using principal component analysis, we separated four clusters from these metabolites characterized by growth and nutritional conditions (Fig. 1e). The first principal component (PC1) explained 54.8% of variance and was related to growth condition, while the second principal component (PC2) explained an additional 17.3% of variance and was related to nutritional condition.

3.2. Specific response genes for phosphorus deficiency

We yielded an average of 11.3 million paired-end reads from 8 cDNA libraries, which corresponded to four replications under Pi-replete and Pi-depleted cultures during the stationary phase. These sequence reads were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession numbers DRR255165–DRR255168 and DRR255173–DRR255176. Comparing

transcription levels of culture conditions, 4,740 and 4,602 genes were up- and downregulated in Pi-depleted culture, respectively (supplementary figure 2).

In the Pi-depleted culture, two transcripts (CTEN210_01177 and CTEN210_12547) out of six genes encoding APases, three transcripts (CTEN210_06843, CTEN210_06844, and CTEN210_06846) out of four genes encoding phosphodiesterase (PDEase), of which the protein possessed the exonuclease–endonuclease–phosphatase domain and was predicted as a PDEase, all transcripts (CTEN210_08228 and CTEN210_08217) out of two genes encoding phytase, and three transcripts (CTEN210_07924, CTEN210_07925, and CTEN210_10191) out of five genes encoding sodium-dependent phosphate transporter (Pi-transporter) were significantly upregulated (Table 1 and Fig. 2), while two APases (CTEN210_14915 and CTEN210_11546) and one Pi-transporter (CTEN210_01094) were significantly downregulated (Table 1 and Fig. 2). One transcript (CTEN210_12441), which was homologous to PtPSR, was upregulated 4,230-fold (Table 1 and Fig. 2).

3.4. Alkaline phosphatase

The ML tree showed that APase was classified into four groups (Fig. 3). Group 1 was formed as a typical APase, while groups 2–4 were formed as atypical APases. CTEN210_01177 was grouped with typical APases [bootstrap probability (BP) = 98% in Fig. 3]. Five APases (CTEN210_18438, CTEN210_14915, CTEN210_11546, CTEN210_11224, and CTEN210_12547) were clustered with alkaline phosphatase D (PhoD) of several organisms, which were classified as atypical APases (BP = 95% in Fig. 3).

3.5. DEGs for the Calvin cycle and pentose phosphate pathway

Almost all transcripts for the Calvin cycle were down- or not regulated in Pi-depleted conditions (Fig. 4 and supplementary table). Ribose-5-phosphate isomerases (EC:5.3.1.6) commonly function in the Calvin cycle and the pentose phosphate pathway, but their localizations vary. Localization of two

genes for ribose-5-phosphate isomerase (CTEN210_15943 and CTEN210_16554) was predicted as mitochondria by the signal peptide. However, these protein sequences were homologous to *T. pseudonana* (XP_002287305.1) with 87% identity, which was predicted to be chloroplast location.

Glucose-6-phosphate 1-dehydrogenase (EC:1.1.1.49, CTEN210_03330) and 6-phosphogluconate dehydrogenase (EC:1.1.1.44, CTEN210_10904) in the pentose phosphate pathway were upregulated 2.1- and 1.6-fold (Fig. 4 and supplementary table), respectively. Although two transcriptions of ribulose-phosphate 3-epimerase (EC:5.1.3.1) were not regulated under Pi-depleted conditions, phosphoketolase (EC:4.1.2.9, CTEN210_05835) was upregulated 83-fold (Fig. 4 and supplementary table). Two out of four transaldolase transcripts (EC:2.2.1.2, CTEN210_04683, and CTEN210_09483) were downregulated 1.8- and 2.5-fold, while one (CTEN210_16426) was upregulated 1.5-fold (Fig. 4 and supplementary table).

3.6. DEGs for carbohydrate metabolism

In the cytoplasm, transcripts of phosphofructokinase (EC:2.7.1.11, CTEN210_04188), diphosphate-dependent phosphofructokinase (EC:2.7.1.90, CTEN210_03919), phosphoglycerate kinase (EC:2.7.2.3, CTEN210_16935), and triosephosphate isomerase (EC:5.3.1.1, CTEN210_02718) were upregulated 4.5-, 2.4-, 2.3-, and 6.8-fold, respectively (Fig. 5 and supplementary table), while 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (EC:5.4.2.11, CTEN210_01774) was downregulated the most at 10-fold (Fig. 5 and supplementary table). Although functioning genes in mitochondria were not upregulated in Pi-depleted conditions, transcripts of phosphoenolpyruvate synthase (EC:2.7.9.2, CTEN210_14381) and glucose-6-phosphate isomerase (EC:5.3.1.9, CTEN210_09656) functioning in chloroplasts were upregulated 1,417- and 17-fold, respectively (Fig. 5 and supplementary table).

3.7. DEGs for membrane lipid metabolism

For phospholipid degradation, four of six genes of phospholipase A (EC:3.1.1.4, PLA), which hydrolyzes phosphatidic acid (PA), were upregulated from 10- to 1.4-fold, while other genes were downregulated by 3.2- and 3.8-fold (Fig. 5 and supplementary table). Two genes of phospholipase B (EC:3.1.1.5, PLB), which also hydrolyze PA, were not significantly regulated (Fig. 5 and supplementary table). Two of three phospholipase C genes (EC:3.1.4.3, PLC, CTEN210_07879 and CTEN210_12599), which were homologous to phosphoinositide-specific phospholipase C (PI-PLC) and hydrolyzes PA to diacylglycerol, were upregulated 6.4- and 1.9-fold, respectively, while another gene (CTEN210_14474) homologous to nonspecific phospholipase C was not significantly regulated (Fig. 5 and supplementary table). Two phospholipase D genes (EC:3.1.4.4, PLD), which hydrolyzes phosphatidyl esters and produces PA, was upregulated 3.9- and 2.2-fold (Fig. 5 and supplementary table). Three genes of glycerophosphoryldiester phosphodiesterases (GDPEs, EC:3.1.4.46) that hydrolyze glycerophosphodiester to glycerol 3-phosphate were upregulated 5.8-, 97-, and 104-fold, respectively (Fig. 5 and supplementary table). Glycerol 3-phosphate is also synthesized from glyceraldehyde 3-phosphate. Phosphoketolase's transcript (EC:4.1.2.9), which converts xylulose 5-phosphate into glyceraldehyde 3-phosphate, was upregulated 83-fold (Fig. 5 and supplementary table). Following the reaction to glyceraldehyde 3-phosphate, cytosolic triosephosphate isomerase (EC:5.3.1.1, CTEN210_02718) and glycerol-3-phosphate dehydrogenase (EC:1.1.5.3, CTEN210_03022), which react to glycerol 3-phosphate, were also upregulated 6.8- and 2.5-fold, respectively (Fig. 5 and supplementary table). The resulting glycerol 3-phosphate was then converted to diacylglycerol 3-phosphate via two enzymes, glycerol-3-phosphate O-acyltransferase (EC:2.3.1.15) and 1-acylglycerol-3-phosphate O-acyltransferase (EC:2.3.1.51), of which isoforms were regulated (Fig. 5 and supplementary table). Although phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin the major components of mitochondrial membrane lipids, are produced from diacylglycerol 3-phosphate, genes related to each of their production were downregulated (Fig. 5 and supplementary table). Moreover, PI-PLC degrades PI to diacylglycerol and myo-inositol 1-phosphate. The transcripts related to synthesis of UDP-glucose from

myo-inositol 1-phosphate were down- or not significantly regulated, respectively (Fig. 5 and supplementary table), although the gene responsible for part of this synthesis, glucuronokinase (EC:2.7.1.43), was not identified in the genome (Fig. 5). Phosphatidate phosphatase (EC:3.1.3.4) hydrolyzes diacylglycerol 3-phosphate to diacylglycerol, and the transcript was slightly upregulated 1.5-fold (Fig. 5 and supplementary table). For synthesis of phosphatidylethanolamine (PE), one of the two genes of ethanolaminephosphotransferase (EC:2.7.8.1) that produces PE, was slightly downregulated 2.3-fold, while the other gene was not significantly regulated (supplementary table). However, phosphatidylserine decarboxylase (EC:4.1.1.65), which produces PE from phosphatidyl-L-serine, was upregulated by 17-fold (Fig. 5 and supplementary table).

Galactolipid synthesis, such as monogalactosyldiacylglycerol synthase (EC: 2.4.1.46) related to MGDG synthesis, digalactosyldiacylglycerol synthase (EC:2.4.1.241) related to DGDG synthesis, and UDP-sulfoquinovose synthase (EC:3.13.1.1) and sulfoquinovosyltransferase (EC:2.4.1.-) related to SQDG synthesis were upregulated from 1.3- to 4.2-fold (Fig. 5 and supplementary table).

4. Discussion

4.1. Genes related to phosphate acquisition

Under Pi-depleted conditions, cell densities were different after culture day 3, which entered the stationary growth phase (Fig. 1a). After seven days, cell pigment of Pi-depleted culture was noticeably paler (Fig. 1b) and photosynthetic activity of Pi-depleted culture was lower than Pi-replete culture (Fig. 1c). Furthermore, metabolites of *C. tenuissimus* formed clearly four delimited clusters corresponding to their growth and Pi-depleted conditions, as shown by the PCA analysis (Fig. 1e). In particular, the metabolites changed in the stationary phase rather than the log phase (Fig. 1e). Therefore, the transcripts were expected to change clearly among Pi-replete and Pi-depleted conditions during the stationary phase.

C. tenuissimus highly expressed APase, PDEase, phytase, and Pi-transporter to complement P acquisition during limitation (Fig. 2). In addition, the transcription factor, which is homologous to

PtPSR, was also highly transcribed (Fig. 2) and appeared to control P-deficient genes. As a corollary, APase activity of the Pi-depleted culture was higher than the Pi-replete culture (Fig. 1d). This phenomenon is commonly observed in several diatoms (Shih et al., 2015; Yamaguchi et al., 2014; Zhang et al., 2016). Although *C. tenuissimus* NIES-3715 codes six APases in the genome, two APases responded with significantly high transcription under P deficiency (Fig. 2). Further, APase (CTEN210_01177) was grouped with the APase of *C. affinis* and *P. tricornutum*, which belong to typical APases (Fig. 3, BP = 100% and 94%). When surrounding P is deficient, APase of *C. affinis* is highly transcribed (Shih et al., 2015) and *P. tricornutum* secretes APase to the outside of the cell (Lin et al., 2013). Because N-terminal amino acid sequences (1–17 aa.) in CTEN210_01177 were predicted as a signal peptide (Table 1), it was considered that *C. tenuissimus* secretes CTEN210_01177 like *P. tricornutum* and retrieves P using Pi-transporter under Pi-depleted conditions. The other APase, CTEN210_12547, was grouped with *Nitzschia inconspicua* and *Fistulifera solaris*, which belong to the PhoD of atypical APase (Fig. 3, BP = 100%). In general, typical APases are activated by Zn^{2+}, but PhoD is activated by Ca^{2+} and this replacement of Zn^{2+} with Ca^{2+} could be essential when selecting PhoD over typical APases in the ocean (Luo et al., 2009). Recently, PhoA and PhoD in *P. tricornutum* were identified and the functional differentiation between these was revealed by CRISPR/Cas9-based mutagenesis (Zhang et al., 2022). The secretion type of PhoA contributes the majority of total AP activity whereas the intracellular type of PhoD (Phatr3_J45757) contributes a minor fraction of total AP activity, and the expression of each gene compensates for the other after they are disrupted (Zhang et al., 2022). Therefore, it is possible *C. tenuissimus* selects APases to adapt to the surrounding environment.

Although it is unclear whether the four predicted proteins for PDEase show diesterase activity, we detected PDEase activity for *C. tenuissimus* in Pi-depleted conditions and *C. tenuissimus* grows well using nucleic acids or phospholipids as P sources (Yamaguchi et al., 2014). Two of the four predicted PDEases have a signal peptide and are highly transcribed in P deficiency (Table 1 and Fig

2). Therefore, we speculated these proteins are candidates for PDEase and may function with APase to cut off P from DOP. Measuring predicted PDEase activities should be examined in the future.

Two phytases are coded in the genome of *C. tenuissimus* NIES-3715 and both phytases were significantly transcribed under Pi-depleted conditions. We speculate one of the two phytases (CTEN210_08217) is the secretion type because we predicted a signal peptide, while the other phytase (CTEN210_08228) may localize the cytoplasm. Previous research shows *P. tricornutum* hydrolyzes phytic acid using a secreted phytase-like gene (Phatr3_J47612) and can grow using P, but less efficiently (Li et al., 2022). Whether both phytases can hydrolyze phytic acid is unclear, thus phytase localized in the cytoplasm may have been hydrolyzed by phytic acid because myo-inositol, which is a degradation product of phytase, increased in stationary growth cells (supplementary Fig. 1).

4.2. Carbohydrate metabolism

The maximal quantum yield of photosystem II (*F*v/*F*m) of *C. tenuissimus* decreased in Pi-depleted culture (Fig. 1), as well as transcription levels of Calvin cycle-related genes (Fig. 4). This low value of *F*v/*F*m and decreasing transcription levels are also seen in several diatoms (Lin et al., 2013; Qi et al., 2013; Shih et al., 2015). Therefore, Pi-depleted conditions can negatively impact carbon fixation. In metabolic analysis, however, glucose levels of *C. tenuissimus* increased under Pi-depleted conditions (Fig. 5), suggesting P deficiency drives gluconeogenesis. However, the transcription pattern does not appear to be regulated towards gluconeogenesis. The partial metabolism of carbohydrates is common among the cytoplasm, plastid, and mitochondria owing to their evolutionary history (Kroth et al., 2008; Smith et al., 2012). In the genome of *C. tenuissimus* NIES-3715, fundamental proteins related to glycolysis/gluconeogenesis metabolism functioning in the cytoplasm were found except enolase (EC:4.2.1.11, Fig. 5 and supplementary table). In gluconeogenesis, fructose 1,6-bisphosphatase (EC:3.1.3.11), which catalyzes nonreversible steps, was downregulated, while phosphofructokinase (EC:2.7.1.11, CTEN210_04188), which catalyzes

359 nonreversible steps in glycolysis, was upregulated (Fig. 5). Glucose is generated from pyruvate or
360 oxaloacetate during gluconeogenesis. A mitochondrial phosphoenolpyruvate carboxykinase
361 (EC:4.1.1.49) converts oxaloacetate to phosphoenolpyruvate (PEP) and its transcript
362 (CTEN210_14672) was significantly downregulated 1.6-fold (supplementary table). In addition,
363 pyruvate phosphate dikinase (EC:2.7.9.1), which initiates gluconeogenesis from pyruvate, was not
364 found in the genome of *C. tenuissimus* NIES-3715. However, chloroplastidal phosphoenolpyruvate
365 synthase (PEPS, EC:2.7.9.2), which converts pyruvate into PEP, was found and its transcript was
366 significantly upregulated 1,417-fold (Fig. 5 and supplementary table). Interestingly, PEPS of *C.*
367 *tenuissimus* was similar to *Myxococcales* bacterium (MCB9566101.1, 63.4% identity). PEPS was
368 identified in *T. pseudonana* and *F. cylindrus* genomes and these amino acid sequences were also
369 similar to bacterial PEPS (Smith et al., 2012). PEPS is mostly found in prokaryotes and is believed to
370 convert pyruvate to PEP in the gluconeogenic direction (Sauer and Eikmanns, 2005). Therefore,
371 diatom PEPS are may be acquired horizontally from bacteria, and the role of PEPS in *C. tenuissimus*
372 under Pi-depleted conditions may act in gluconeogenesis.

373 The low photosynthetic activity and partial transcription patterns of the gluconeogenesis makes it
374 difficult to explain why glucose increased in Pi-depleted conditions. Diatoms possess a
375 polysaccharide (chrysolaminarin) as a primary storage carbohydrate (Beattie et al., 1961; Paulsen
376 and Myklestad, 1978). The enzyme exo- and endo-1,3-ß-glucanases or ß-glucosidases degrade it and
377 produce glucose (Kroth et al., 2008). In *C. tenuissimus* NIES-3715 genome, we found three
378 exo-1,3-ß-glucanases (CTEN210_04987, CTEN210_09284, and CTEN210_12619), one
379 endo-1,3-ß-glucanase (CTEN210_07384), and one ß-glucosidase (CTEN210_08480) and
380 transcriptions of exo-1,3-ß-glucanases (CTEN210_09284) and endo-1,3-ß-glucanases
381 (CTEN210_07384) were upregulated in Pi-depleted conditions by 1.5- and 1.3-fold, respectively
382 (supplementary table), suggesting chrysolaminarin degradation may supply glucose instead of
383 photosynthetic production and gluconeogenesis for cell proliferation and energy demand.

384

4.3. Changes in lipid metabolism

In Pi-depleted conditions, transcriptions of glycerophospholipid degradation enzymes were significantly upregulated (Fig. 5). *Chaetoceros tenuissimus* highly transcribed PLA, PI-PLC, PLD, and GDPEs genes (Fig. 5). We observed these transcripts in *P. tricornutum* and *T. pseudonana* (Alipanah et al., 2018; Dyhrman et al., 2012). GDPEs of plants are also induced under P deficiency and degrade glycerophosphodiesters (L. Cheng et al., 2011; Y. Cheng et al., 2011; Mehra et al., 2019). GDPEs produce glycerol 3-phosphate and supply diacylglycerol as a substrate for nonphosphorus lipids. Glycerol 3-phosphate can also be synthesized from glyceraldehyde 3-phosphate via glycerone phosphate. Glyceraldehyde 3-phosphate is synthesized in glycolysis/gluconeogenesis, the Calvin cycle, and pentose phosphate pathways (Figs 4 and 5). In the pentose phosphate pathway, ribose-5-phosphate isomerase (EC:5.3.1.6) produces ribose 5-phosphate for nuclear acid synthesis. Although cellular localization of two ribose-5-phosphate isomerases was not clear in this study, these transcriptions were downregulated in P deficiency. This result indicates enhanced metabolic flow produces glyceraldehyde 3-phosphate rather than new nuclear acid synthesis. In fact, *Chaetoceros tenuissimus* highly transcribed a cytosolic phosphoketolase (EC:4.1.2.9) and likely produced glyceraldehyde 3-phosphate (Fig. 4). Nucleic acid degradation occurs as a P scavenging process in P-deficient responses and xylulose 5-phosphate as a substrate for phosphoketolase is produced as a by-product (Alipanah et al., 2018; Shih et al., 2015). The produced glyceraldehyde 3-phosphate was used for glycerol 3-phosphate synthesis because cytosolic triosephosphate isomerase (EC:5.3.1.1, CTEN210_02718) and glycerol-3-phosphate dehydrogenase (EC:1.1.5.3, CTEN210_03022) were highly transcribed. From these transcription changes in *C. tenuissimus*, the diacylglycerol synthesis appears to concentrate on producing nonphosphorus lipids, galactolipids (MGDG and DGDG) and sulfolipid (SQDG), of which the related genes were highly transcribed (Fig. 5). Substituting phospholipids with galactolipids in P-deficient conditions is common in plants (Nakamura et al., 2009; Verma et al., 2021). In *P. tricornutum*, gene transcription related to synthesizing galactolipids increased (Alipanah et al., 2018) and DGDG levels in P-starved

411 cells also increased (Kumar Sharma et al., 2020). Moreover, increased gene transcription, protein,
412 and lipid levels for SQDG are present in *T. pseudonana*, *T. weissflogii*, *P. tricornutum*, *C. affinis*,
413 and *S. costatum* (Alipanah et al., 2018; Dyhrman et al., 2012; Martin et al., 2011; Van Mooy et al.,
414 2009; Wang et al., 2014; Zhang et al., 2016). Although UDP-glucose is needed as a substrate for
415 UDP-sulfoquinovose synthase in SQDG synthesis, UDP-glucose in *C. tenuissimus* appeared to be
416 supplied from glucose 1-phosphate and myo-inositol 1-phosphate degradation due to these
417 transcripts (Fig. 5). However, it is possible that *C. tenuissimus* enhanced its ability to synthesize
418 SQDG within a limited number of substrates because downstream genes in SQDG synthesis were
419 highly transcribed. In contrast to synthesis of nonphosphorus lipids, transcription of genes
420 synthesizing PI, PG, and cardiolipin were significantly downregulated (Fig. 5). This result indicates
421 Pi-depleted conditions suppress *de novo* synthesis of phospholipids. Therefore, from these results, *C.*
422 *tenuissimus* degraded membrane phospholipids for complementation of P deficiency in the cell and
423 replaced them with nonphosphorus lipids.
424
425 **5. Conclusion**
426 Previous research suggests *C. tenuissimus* can survive Pi-depleted conditions using nucleic acids and
427 phospholipids as P sources (Yamaguchi et al., 2014). However, molecular mechanisms for P
428 deficiency adaptation were still unclear. In this study, we showed that Pi-depleted conditions led *C.*
429 *tenuissimus* to alter their transcriptions and metabolites. Their photosynthetic activity was weaker
430 than in the Pi-replete conditions. Consequently, transcripts related to the Calvin cycle and
431 glycolysis/gluconeogenesis pathway were downregulated. However, glucose accumulated in the cell
432 under Pi-depleted conditions. Instead of carbon fixation, chrysolaminarin degradation genes are
433 highly transcribed and may contribute to glucose supply for proliferation. To acquire phosphorus, *C.*
434 *tenuissimus* remarkably upregulated APase, PDEase, phytase, Pi-transporter, and PtPSR
435 transcriptions and was enhanced when using P from DOP. Genes for the phospholipid degradation
436 and nonphospholipid synthesis of *C. tenuissimus* were also highly transcribed. This result indicated

that substitution from phospholipids to galactolipids and sulfolipids in the cell membrane reused intracellular P sources. These molecular data reveal *C. tenuissimus* adopted strategies to survive in P-deficient environments and these genes are likely to respond to variations in environmental P availability.

6. Data availability

Sequence data generated during the current study are available in the DDBJ repository, under accession numbers DRR255165–DRR255168 and DRR255173–DRR255176.

Author contributions

Y.H. analyzed the data and wrote the manuscript. T.H. analyzed the metabolites and wrote the manuscript. H.Y. analyzed the APase activity and wrote the manuscript. Y.T. designed and performed the research and wrote the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Supplementary information

Supplementary figures 1 and 2; the pdf file.

Supplementary table; excel file.

Figure legends

Figure 1. The physiological changes between Pi-replete and Pi-depleted conditions. (a) Growth curves for each nutritional condition. Black and white circles indicate Pi-replete and Pi-depleted conditions, respectively. (b) Photo of culture flasks after seven days of culture. Left and right flasks indicate Pi-replete and Pi-depleted conditions, respectively. (c) Photosynthetic and (d) alkaline phosphatase activities in each nutritional condition (C, control; P-, Pi-depleted) after two and seven days of cultures. (e) Scattergram of principal component analysis for metabolites among growth and nutritional conditions.

Figure 2. Relative transcript levels for each gene. Vertical and horizontal axes indicate fragments per kilobase of exon per million mapped reads (FPKM) and gene locus tag ID, respectively. Red and green bar plots indicate Pi-replete and Pi-depleted conditions, respectively.

Figure 3. Unrooted maximum likelihood tree of alkaline phosphatase. Gray circles indicate branches of bootstrap values ≥80%.

Figure 4. Logarithmic fold changes of each gene in the Calvin cycle and the pentose phosphate pathway. Box colors indicate logarithmic fold changes. The slash in the box indicates no significant changes. The number to the right of the box indicates gene locus tag ID.

Figure 5. Logarithmic fold changes of each gene in glycolysis/gluconeogenesis, glycerolipid and glycerophospholipid metabolism. Box colors indicate logarithmic fold changes. The number to the right of the box indicates gene locus tag ID. The slash in the box indicates no significant changes. The bar plot of glucose shows the relative abundance under Pi-replete and Pi-depleted conditions.

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705 Table 1. Specific response genes to Pi-depleted conditions.

	local ID	Protein accession no.	LogFC	Signal Peptide
Alkaline phosphatase	CTEN210_11224	GFH54748.1		
	CTEN210_01177	GFH44703.1	11.5	+
	CTEN210_18438	GFH61962.1		
	CTEN210_14915	GFH58439.1	−0.6	
	CTEN210_12547	GFH56071.1	1.5	
	CTEN210_11546	GFH55070.1	−1.2	
Phosphodiesterase	CTEN210_06844	GFH50368.1	4.7	+
	CTEN210_06846	GFH50370.1	3.5	+
	CTEN210_06845	GFH50369.1		
	CTEN210_06843	GFH50367.1	1.6	
Phytase	CTEN210_08228	GFH51752.1	10.6	
	CTEN210_08217	GFH51741.1	12.3	+
Sodium-dependent phosphate transporter (Pi-transporter)	CTEN210_07924	GFH51448.1	6.5	
	CTEN210_07925	GFH51449.1	6.2	
	CTEN210_10191	GFH53715.1	5.6	
	CTEN210_04085	GFH47610.1		
	CTEN210_01094	GFH44620.1	−6.4	
Myb-like transcription factor, P starvation response (PtPSR)	CTEN210_12441	GFH55965.1	12.0	

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