

Transcriptional responses of the marine diatom *Chaetoceros tenuissimus* to phosphate deficiency

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Title: Transcriptional responses of the marine diatom *Chaetoceros tenuissimus* to phosphate deficiency

Yuki Hongo^{1*}, Takeshi Hano², Haruo Yamaguchi³, Yuji Tomaru²

¹Fisheries Resources Institute, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan

²Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan

³Faculty of Agriculture and Marine Science, Kochi University, 200 Otsu, Monobe, Nankoku, Kochi 783-8502, Japan

***Corresponding authors**

Yuki Hongo

Fisheries Resources Institute, Japan Fisheries Research and Education Agency

2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan

Tel: +81-45-788-7673

Fax: +81-45-788-5001

Email: hongo_yuki37@fra.go.jp

ORCID: <https://orcid.org/0000-0003-1434-1220>

25 **1. Introduction**

26 Marine diatoms play crucial roles in primary production and global carbon fluxes (Nelson et al.,
27 1995). Among marine planktonic diatoms, *Chaetoceros tenuissimus* is a picoplanktonic diatom and
28 widely distributed throughout the coastal waters of Japan (Tomaru et al., 2018), the Narragansett Bay
29 (Rines, 1988), the Mediterranean Sea (Kooistra et al., 2010; Montresor et al., 2013), North Sea
30 (Grzebyk et al., 2022), and the San Matias Gulf (Sar et al., 2002), in addition, metabarcoding data
31 record the appearance of *C. tenuissimus* on the African, Asian, and New Zealand coasts (De Luca et
32 al., 2019). In the coastal waters of Japan, *C. tenuissimus* frequently forms blooms during warm
33 seasons (Tomaru et al., 2011) due to its great growth potential. According to laboratory data, *C.*
34 *tenuissimus* clones (NIES-3714 and -3715) undergo over three divisions/day at high temperatures of
35 25°C–30°C (Tomaru et al., 2014). Furthermore, growth rates of six divisions/day have been recorded
36 under semicontinuous culture with strong photoirradiation (Tomaru et al., 2021). These
37 ecophysiological characteristics may allow *C. tenuissimus* to behave as a cosmopolitan species and
38 form blooms at euphotic layers in warm coastal waters.

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

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

51 as suggested by Suzumura et al. (1998) and Monbet et al. (2009). Although P-repleted cells of *C.*
52 *tenuissimus* prefer orthophosphate as a primary P source (Yamaguchi et al., 2022b), they can access
53 various types of phosphorus compounds, such as phospholipids and nucleic acids, as a sole
54 phosphorus source (Yamaguchi et al., 2022b, 2014). Therefore, *C. tenuissimus* can endure P-limited
55 conditions in nature. Molecular responses in P-stressed cells have been previously examined using a
56 microarray analysis approach, whereby alkaline phosphatase and phosphate transporter were
57 significantly upregulated (Yamaguchi et al., 2022a). Studies on gene responses to P deficiency were
58 performed in several diatoms, namely *Thalassiosira pseudonana* (Dyhrman et al.,
59 2012), *Thalassiosira weissflogii* (Wang et al., 2014), *Phaeodactylum tricornutum* (Cruz de Carvalho
60 et al., 2016; Feng et al., 2015; Yang et al., 2014), *Skeletonema costatum* (Zhang et al., 2016), and
61 *Chaetoceros affinis* (Shih et al., 2015). Among those studies, APases and phosphate transporters
62 were highly expressed in addition to genes related to modified cell membranes containing
63 sulfolipids. Indeed, cell membranes of phytoplankton, including *T. pseudonana*, were replaced by
64 phospholipids with nonphosphorus membrane lipids, such as sulphoquinovosyl diacylglycerol
65 (SQDG) and betain, maintained under P-deficient conditions (Martin et al., 2011; Van Mooy et al.,
66 2009). These responses are directly related to increasing uptake of inorganic phosphate (Pi) and
67 scavenging of P from dissolved organic phosphate (DOP) or intracellular P sources. Other gene
68 responses for P-deficient conditions were observed in the glycolytic pathway for *T. pseudonana*
69 (Alipanah et al., 2018; Dyhrman et al., 2012) and *P. tricornutum* regulates the transcription factor
70 that regulates P deficiency-induced genes (PtPSR) (Kumar Sharma et al., 2020). In addition,
71 P-deficient conditions influence cell proliferation and photosynthetic efficiency (Feng et al., 2015;
72 Shih et al., 2015; Wang et al., 2014; Yang et al., 2014; Zhang et al., 2016).

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

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

79

80 **2. Materials and methods**

81 **2.1. Culture conditions**

82 Algal cultures were grown in modified SWM-3 medium enriched with 2 nM Na_2SeO_3 (Imai et al.,
83 1996) under a 12/12-h light-dark cycle at 20°C. Light irradiance was $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ using white
84 LED illumination. The final concentration of additive phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) for SWM-3 is 100
85 μM (Pi-replete SWM-3 media). We also prepared a phosphate deficiency SWM-3 medium with an
86 additional final phosphate concentration of 3.3 μM (Pi-depleted SWM-3 media).

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

100

101 **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 ($\text{mol cell}^{-1} \text{time}^{-1}$).

108

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 $16,000 \times g$ 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

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

130

131 **2.4. RNA extraction and sequencing**

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

140

141 **2.5. Differential expression gene (DEG) analysis**

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

154

155 **2.6. Kegg pathway analysis**

156 The Kegg ortholog (KO) number for *C. tenuissimus* NIES-3715 was assigned using BlastKOALA
157 (Kanehisa et al., 2016) at the Kegg website (<https://www.kegg.jp/blastkoala/>), which conducts
158 BLAST searching against a nonredundant dataset generated from the KEGG GENES database and
159 assigns a KO number (Kanehisa et al., 2016). Genes related to metabolic pathways were retrieved
160 using the KO numbers from the kegg pathway: glycolysis/gluconeogenesis (00010), pentose
161 phosphate pathway (00030), carbon fixation in photosynthetic organisms (00710), glycerolipid
162 metabolism (00561), glycerophospholipid metabolism (00564), Inositol phosphate metabolism
163 (00562), Pentose and glucuronate interconversions (00040), and TCA cycles (00020).

164

165 **2.7. Prediction of protein localization**

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

171

172 **2.8. Phylogenetic relationship of alkaline phosphatase**

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

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

182

183 **3. Results**

184 **3.1. Physiological response under culture conditions**

185 Cell concentrations of both Pi-replete and Pi-depleted cultures logarithmically increased from zero to
186 three days of culture (log phases), then maintained until seven days of culture (stationary phases, Fig.
187 1a). In the stationary phase, cell concentration of Pi-depleted cultures was half that of Pi-replete
188 cultures (Fig. 1a). Consequently, the color of Pi-depleted culture at seven days was paler than
189 Pi-replete culture (Fig. 1b) and photosynthetic activity of Pi-depleted culture at seven days was also
190 significantly lower than Pi-replete culture ($t\text{-test} < 0.05$, Fig. 1c). In both growth phases, APase
191 activity in Pi-depleted culture was significantly higher than the Pi-replete culture ($t\text{-test} < 0.05$, Fig.
192 1d).

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

199

200 **3.2. Specific response genes for phosphorus deficiency**

201 We yielded an average of 11.3 million paired-end reads from 8 cDNA libraries, which corresponded
202 to four replications under Pi-replete and Pi-depleted cultures during the stationary phase. These
203 sequence reads were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive
204 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

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

234 Glucose-6-phosphate 1-dehydrogenase (EC:1.1.1.49, CTEN210_03330) and 6-phosphogluconate
235 dehydrogenase (EC:1.1.1.44, CTEN210_10904) in the pentose phosphate pathway were upregulated
236 2.1- and 1.6-fold (Fig. 4 and supplementary table), respectively. Although two transcriptions of
237 ribulose-phosphate 3-epimerase (EC:5.1.3.1) were not regulated under Pi-depleted conditions,
238 phosphoketolase (EC:4.1.2.9, CTEN210_05835) was upregulated 83-fold (Fig. 4 and supplementary
239 table). Two out of four transaldolase transcripts (EC:2.2.1.2, CTEN210_04683, and
240 CTEN210_09483) were downregulated 1.8- and 2.5-fold, while one (CTEN210_16426) was
241 upregulated 1.5-fold (Fig. 4 and supplementary table).

242

243 **3.6. DEGs for carbohydrate metabolism**

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

254

255 **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

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

335 Two phytases are coded in the genome of *C. tenuissimus* NIES-3715 and both phytases were
336 significantly transcribed under Pi-depleted conditions. We speculate one of the two phytases
337 (CTEN210_08217) is the secretion type because we predicted a signal peptide, while the other
338 phytase (CTEN210_08228) may localize the cytoplasm. Previous research shows *P. tricornutum*
339 hydrolyzes phytic acid using a secreted phytase-like gene (Phatr3_J47612) and can grow using P, but
340 less efficiently (Li et al., 2022). Whether both phytases can hydrolyze phytic acid is unclear, thus
341 phytase localized in the cytoplasm may have been hydrolyzed by phytic acid because myo-inositol,
342 which is a degradation product of phytase, increased in stationary growth cells (supplementary Fig.
343 1).

344

345 **4.2. Carbohydrate metabolism**

346 The maximal quantum yield of photosystem II (F_v/F_m) of *C. tenuissimus* decreased in Pi-depleted
347 culture (Fig. 1), as well as transcription levels of Calvin cycle-related genes (Fig. 4). This low value
348 of F_v/F_m and decreasing transcription levels are also seen in several diatoms (Lin et al., 2013; Qi et
349 al., 2013; Shih et al., 2015). Therefore, Pi-depleted conditions can negatively impact carbon fixation.
350 In metabolic analysis, however, glucose levels of *C. tenuissimus* increased under Pi-depleted
351 conditions (Fig. 5), suggesting P deficiency drives gluconeogenesis. However, the transcription
352 pattern does not appear to be regulated towards gluconeogenesis. The partial metabolism
353 of carbohydrates is common among the cytoplasm, plastid, and mitochondria owing to their
354 evolutionary history (Kroth et al., 2008; Smith et al., 2012). In the genome of *C. tenuissimus*
355 NIES-3715, fundamental proteins related to glycolysis/gluconeogenesis metabolism functioning in
356 the cytoplasm were found except enolase (EC:4.2.1.11, Fig. 5 and supplementary table). In
357 gluconeogenesis, fructose 1,6-bisphosphatase (EC:3.1.3.11), which catalyzes nonreversible steps,
358 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- β -glucanases or β -glucosidases degrade it and
377 produce glucose (Kroth et al., 2008). In *C. tenuissimus* NIES-3715 genome, we found three
378 exo-1,3- β -glucanases (CTEN210_04987, CTEN210_09284, and CTEN210_12619), one
379 endo-1,3- β -glucanase (CTEN210_07384), and one β -glucosidase (CTEN210_08480) and
380 transcriptions of exo-1,3- β -glucanases (CTEN210_09284) and endo-1,3- β -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

385 4.3. Changes in lipid metabolism

386 In Pi-depleted conditions, transcriptions of glycerophospholipid degradation enzymes were
387 significantly upregulated (Fig. 5). *Chaetoceros tenuissimus* highly transcribed PLA, PI-PLC, PLD,
388 and GDPEs genes (Fig. 5). We observed these transcripts in *P. tricornutum* and *T. pseudonana*
389 (Alipanah et al., 2018; Dyhrman et al., 2012). GDPEs of plants are also induced under P deficiency
390 and degrade glycerophosphodiester (L. Cheng et al., 2011; Y. Cheng et al., 2011; Mehra et al.,
391 2019). GDPEs produce glycerol 3-phosphate and supply diacylglycerol as a substrate for
392 nonphosphorus lipids. Glycerol 3-phosphate can also be synthesized from glyceraldehyde
393 3-phosphate via glycero phosphate. Glyceraldehyde 3-phosphate is synthesized in
394 glycolysis/gluconeogenesis, the Calvin cycle, and pentose phosphate pathways (Figs 4 and 5). In the
395 pentose phosphate pathway, ribose-5-phosphate isomerase (EC:5.3.1.6) produces ribose 5-phosphate
396 for nuclear acid synthesis. Although cellular localization of two ribose-5-phosphate isomerases was
397 not clear in this study, these transcriptions were downregulated in P deficiency. This result indicates
398 enhanced metabolic flow produces glyceraldehyde 3-phosphate rather than new nuclear acid
399 synthesis. In fact, *Chaetoceros tenuissimus* highly transcribed a cytosolic phosphoketolase
400 (EC:4.1.2.9) and likely produced glyceraldehyde 3-phosphate (Fig. 4). Nucleic acid degradation
401 occurs as a P scavenging process in P-deficient responses and xylulose 5-phosphate as a substrate for
402 phosphoketolase is produced as a by-product (Alipanah et al., 2018; Shih et al., 2015). The produced
403 glyceraldehyde 3-phosphate was used for glycerol 3-phosphate synthesis because cytosolic
404 triosephosphate isomerase (EC:5.3.1.1, CTEN210_02718) and glycerol-3-phosphate dehydrogenase
405 (EC:1.1.5.3, CTEN210_03022) were highly transcribed. From these transcription changes in *C.*
406 *tenuissimus*, the diacylglycerol synthesis appears to concentrate on producing nonphosphorus lipids,
407 galactolipids (MGDG and DGDG) and sulfolipid (SQDG), of which the related genes were highly
408 transcribed (Fig. 5). Substituting phospholipids with galactolipids in P-deficient conditions is
409 common in plants (Nakamura et al., 2009; Verma et al., 2021). In *P. tricornutum*, gene transcription
410 related to synthesizing galactolipids increased (Alipanah et al., 2018) and DGDG levels in P-starved

cells also increased (Kumar Sharma et al., 2020). Moreover, increased gene transcription, protein, and lipid levels for SQDG are present in *T. pseudonana*, *T. weissflogii*, *P. tricornutum*, *C. affinis*, and *S. costatum* (Alipanah et al., 2018; Dyhrman et al., 2012; Martin et al., 2011; Van Mooy et al., 2009; Wang et al., 2014; Zhang et al., 2016). Although UDP-glucose is needed as a substrate for UDP-sulfoquinovose synthase in SQDG synthesis, UDP-glucose in *C. tenuissimus* appeared to be supplied from glucose 1-phosphate and myo-inositol 1-phosphate degradation due to these transcripts (Fig. 5). However, it is possible that *C. tenuissimus* enhanced its ability to synthesize SQDG within a limited number of substrates because downstream genes in SQDG synthesis were highly transcribed. In contrast to synthesis of nonphosphorus lipids, transcription of genes synthesizing PI, PG, and cardiolipin were significantly downregulated (Fig. 5). This result indicates Pi-depleted conditions suppress *de novo* synthesis of phospholipids. Therefore, from these results, *C. tenuissimus* degraded membrane phospholipids for complementation of P deficiency in the cell and replaced them with nonphosphorus lipids.

5. Conclusion

Previous research suggests *C. tenuissimus* can survive Pi-depleted conditions using nucleic acids and phospholipids as P sources (Yamaguchi et al., 2014). However, molecular mechanisms for P deficiency adaptation were still unclear. In this study, we showed that Pi-depleted conditions led *C. tenuissimus* to alter their transcriptions and metabolites. Their photosynthetic activity was weaker than in the Pi-replete conditions. Consequently, transcripts related to the Calvin cycle and glycolysis/gluconeogenesis pathway were downregulated. However, glucose accumulated in the cell under Pi-depleted conditions. Instead of carbon fixation, chrysolaminarin degradation genes are highly transcribed and may contribute to glucose supply for proliferation. To acquire phosphorus, *C. tenuissimus* remarkably upregulated APase, PDEase, phytase, Pi-transporter, and PtPSR transcriptions and was enhanced when using P from DOP. Genes for the phospholipid degradation and nonphospholipid synthesis of *C. tenuissimus* were also highly transcribed. This result indicated

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

441

442 **6. Data availability**

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

445

446 **Author contributions**

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

450

451 **Declaration of competing interest**

452 The authors declare no competing interests.

453

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456

457 **Supplementary information**

458 Supplementary figures 1 and 2; the pdf file.

459 Supplementary table; excel file.

460

461 **Figure legends**

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

469

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

473

474 **Figure 3.** Unrooted maximum likelihood tree of alkaline phosphatase. Gray circles indicate branches
475 of bootstrap values $\geq 80\%$.

476

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

480

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

485

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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	