Journal of Proteomics 196 (2019) 141–149

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Unraveling the molecular mechanism of the response to changing ambient phosphorus in the dinoflagellate *Alexandrium catenella* with quantitative proteomics

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ARTICLE INFO

Keywords:
Marine dinoflagellates
Harmful algal blooms
Alexandrium catenella
Phosphorus
Quantitative proteomics

ABSTRACT

Phosphorus (P) is a key macronutrient limiting cell growth and bloom formation of marine dinoflagellates. Physiological responses to changing ambient P have been investigated in dinoflagellates; however, the molecular mechanisms behind these responses remain limited. Here, we compared the protein expression profiles of a marine dinoflagellate *Alexandrium catenella* grown in inorganic P-replete, P-deficient, and inorganic- and organic-P resupplied conditions using an iTRAQ-based quantitative proteomic approach. P deficiency inhibited cell growth and enhanced alkaline phosphatase activity (APA) but had no effect on photosynthetic efficiency. After P resupply, the P-deficient cells recovered growth rapidly and APA decreased. Proteins involved in sphingolipid metabolism, organic P utilization, starch and sucrose metabolism, and photosynthesis were up-regulated in the P-deficient cells, while proteins associated with protein synthesis, nutrient assimilation and energy metabolism were down-regulated. The responses of the P-deficient *A. catenella* to the resupply of organic and inorganic P presented significant differences: more biological processes were enhanced in the organic P-resupplied cells than those in the inorganic P-resupplied cells; *A. catenella* might directly utilize G-6-P for nucleic acid synthesis through the pentose phosphate pathway. Our results indicate that *A. catenella* has evolved diverse adaptive strategies to ambient P deficiency and specific mechanisms to utilize dissolved organic P, which might be an important reason resulting in *A. catenella* bloom in the low inorganic P environment.

Biological significance: The ability of marine dinoflagellates to utilize different phosphorus (P) species and adapt to ambient P deficiency determines their success in the ocean. In this study, we investigated the response mechanisms of a dinoflagellate *Alexandrium catenella* to ambient P deficiency, and resupply of inorganic- and organic-P at the proteome level. Our results indicated that *A. catenella* initiated multiple adaptive strategies to ambient P deficiency, e.g. utilizing nonphospholipids and glycosphingolipids instead of phospholipids, enhancing expression of acid phosphatase to utilize organic P, and reallocating intracellular energy. Proteome responses of the P-deficient *A. catenella* to resupply of inorganic- and organic-P differed significantly, indicating different utilization pathways of inorganic and organic P, *A. catenella* might directly utilize low molecular weight organic P, such as G-6-P as both P and carbon sources.

1. Introduction

Dinoflagellates are not only important primary producers and primary food web components in the ocean but also the major causative agents of harmful algal blooms (HABs) [1–3]. Some dinoflagellate species can produce potent toxins that are fatal to marine organisms and humans [1,4–7]. In the past few decades, the frequency, scale and distribution of dinoflagellate blooms have increased significantly worldwide, causing global public concern about the negative impacts of these blooms on marine ecosystems and human health [3,8].

Much effort has been devoted to the formation mechanism of dinoflagellate HABs, and nutrients are regarded as a key environmental factor regulating the occurrence of HABs in the ocean [9]. Among these nutrients, phosphorus (P) plays an essential role in the formation of

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https://doi.org/10.1016/j.jprot.2018.11.004
Received 3 July 2018; Received in revised form 30 October 2018; Accepted 5 November 2018
Available online 07 November 2018
1874-3919/

HABs, and the availability of ambient P determines the scale and length of blooms [10–13]. However, the concentration of dissolved inorganic P (DIP), a preferred form of P by dinoflagellates, is very low in the ocean, and DIP always presents a limitation to dinoflagellate growth [11,14–16]. Studies have shown that phytoplankton have evolved diverse adaptive strategies to ambient P deficiency, e.g., by utilizing nonphosphorus lipids instead of phospholipids [13,15,17], expressing high-affinity phosphate transporters [18], or synthesizing enzymes to utilize dissolved organic P (DOP) [13,15,18]. However, the molecular mechanisms involved in the responses to ambient P changes, such as P deficiency and pulse supplies of DIP or DOP, are far less known in dinoflagellates than those in other phytoplankton groups [13,15,18], which impedes our understanding of the mechanisms underlying the formation of HABs.

Alexandrium is a widely distributed toxigenic dinoflagellate genus in the ocean that can produce paralytic shellfish toxins, resulting in paralytic shellfish poisoning incidents worldwide [19,20]. Moreover, *Alexandrium* is also a major causative agent of coastal HABs around the world, which threatens marine organisms and humans [20–23]. Recently, the frequency, geographical distribution and scale of blooms caused by *Alexandrium* have significantly increased [19,20]. Studies have shown that *Alexandrium* can survive in P-deficient environments and is able to utilize both DIP and DOP for cell growth [11,14,24]; however, very little is known about the molecular mechanism responsible for the adaptive response to ambient P changes. In this study, we applied an iTRAQ-based quantitative proteomic approach, combined with a transcriptomic dataset, to compare the global protein expression profiles of *Alexandrium catenella* grown in inorganic P-replete, P-deficient, inorganic and organic P-resupplied conditions, and characterized differentially expressed proteins. The goal of this study was to unveil the adaptive strategies of *A. catenella* to ambient P deficiency and the response mechanisms to the resupply of inorganic and organic P.

2. Material and methods

2.1. Organism and culture conditions

The *A. catenella* strain was kindly provided by the Culture Collection Center of Marine Algae, Xiamen University, China. *A. catenella* cells were maintained in K medium at 20 °C under a light: dark period of 14 h: 10 h. Light with an intensity of approximately 100 μmol photons $m^{-2} s^{-1}$ was provided by a cold fluorescent lamp [25].

2.2. Experimental design

Four treatments were set up in the experiment: P-replete, P-deficient, DIP-resupplied and DOP-resupplied. Each treatment had three biological replicates, and the initial cell density of *A. catenella* was approximately 3000 cells mL^{-1}. At the beginning of the experiment, 10 μM Na_2HPO_4 was added to the P-replete treatment, and 0.5 μM Na_2HPO_4 was added to the other three treatments. After a four-day growth period, the cells with the addition of 0.5 μM Na_2HPO_4 ceased growth, but the alkaline phosphatase activity (APA) increased continuously, indicating that the cells were in a P-deficient state [15]. On day 7, 10 μM Na_2HPO_4 or glucose-6-phosphate (G-6-P) was added to the P-deficient treatments as the DIP-resupplied or DOP-resupplied group, respectively.

2.3. Physiological parameter analysis

Parameters such as cell density, DIP and DOP concentrations, particulate P (PP) content, APA activity and PSII maximum photochemical yield (*Fv/Fm*) were monitored every day. Three 1 mL aliquots of each culture were collected and fixed with 2 μL Lugol's solution, and then cells were counted under a light microscope. A total of 25 mL of algal cells of each culture was filtered using a GF/F membrane, and the cell pellets on the membrane were used to determine PP, while the filtrate was used to determine the DIP and DOP concentrations. The phosphomolybdenum blue spectrophotometric method was used to measure the DIP concentration [26], while the DOP and PP concentrations were analyzed using the method reported by Jeffries et al. [27]. APA was measured using the method reported by Ou et al. [11]. *Fv/Fm* was analyzed using a PhytoPAM [15]. A Student's test was used to compare the differences of each parameter among the four treatments. A P value $< .05$ was considered to indicate a significant difference.

2.4. Protein preparation and peptide labeling

According to the physiological parameters, samples were collected for iTRAQ-based quantitative proteomic analysis, i.e., P-replete sample collected on day 4, P-deficient sample collected on day 8, and Na_2HPO_4 or G-6-P resupplied samples collected after 28 h of P resupply.

Proteins were extracted using the TRIzol extraction method, which employs TRIzol, chloroform, ethanol and isopropanol [28]. After reductively alkylation, 100 μg proteins from each sample were digested using trypsin with a ratio of protein: trypsin of 20: 1 at 37 °C for 4 h; then, the same amount of trypsin was added for the second digestion at 37 °C for 8 h. The peptides were dried using a vacuum centrifuge and resolved in 0.5 M tetraethyl-ammonium bromide. Finally, peptides were labeled using the iTRAQ labeling regent following the kit manual: Tag_{113}, P-replete; Tag_{114}, P-deficient; Tag_{115}, DIP-resupplied; Tag_{116}, DOP-resupplied.

2.5. Peptide fractionation

Peptides were fractioned using a Shimadzu LC-20AB HPLC Pump system, reconstituted with 4 mL buffer A (25 mM NaH_2PO_4 in 25% C_2H_3N, pH = 2.7) and eluted at a flow rate of 1 mL min^{-1} with a gradient of 5% buffer B (25 mM NaH_2PO_4 and 1 M KCl in 25% C_2H_3N, pH = 2.7) for 7 min, 5–60% buffer B for 20 min, and 60–100% buffer B for 2 min. Then, the system was maintained at 100% buffer B for 1 min, decreased to 5% buffer B within 1 min and maintained at 5% buffer B for 10 min. Elution was monitored by measuring absorbance at 214 nm, and the eluted peptides were pooled into 20 fractions and vacuum-dried.

2.6. LC-MS/MS analysis

Each fraction was reconstituted with buffer C (5% C_2H_3N, 0.1% HCOOH) to a concentration of 0.5 μL and centrifuged at 20,000*g* for 10 min. Approximately 5 μL supernatant was loaded on a LC-20 AD nanoHPLC. The sample was loaded at 8 μL min^{-1} for 4 min, and the following gradient was maintained at 300 nL min^{-1} for 60 min: 5% buffer D (95% C_2H_3N, 0.1% HCOOH) for 5 min, followed by a 35 min linear gradient to 35% buffer D, 35–60% buffer D for 5 min, 60%–80% buffer D for 2 min, buffer D maintained at 80% for 2 min and returned to 5% within 1 min, and 5% buffer D maintained for 10 min. Finally, the peptides after HPLC separation were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in a Q-EXACTIVE.

2.7. Bioinformatic analysis

The raw data files were converted to the MGF format using Proteome Discoverer software, and then protein identification was conducted with the search software Mascot (Version 2.3.02). The putative amino acid sequences translated from the CDS of unigenes from corresponding transcriptome sequences were used as a protein database. At least one unique peptide and peptides at the 95% confidence interval were used for protein identification. Blast2GO was used to annotate protein functions against the KEGG and NCBI-Nr databases.

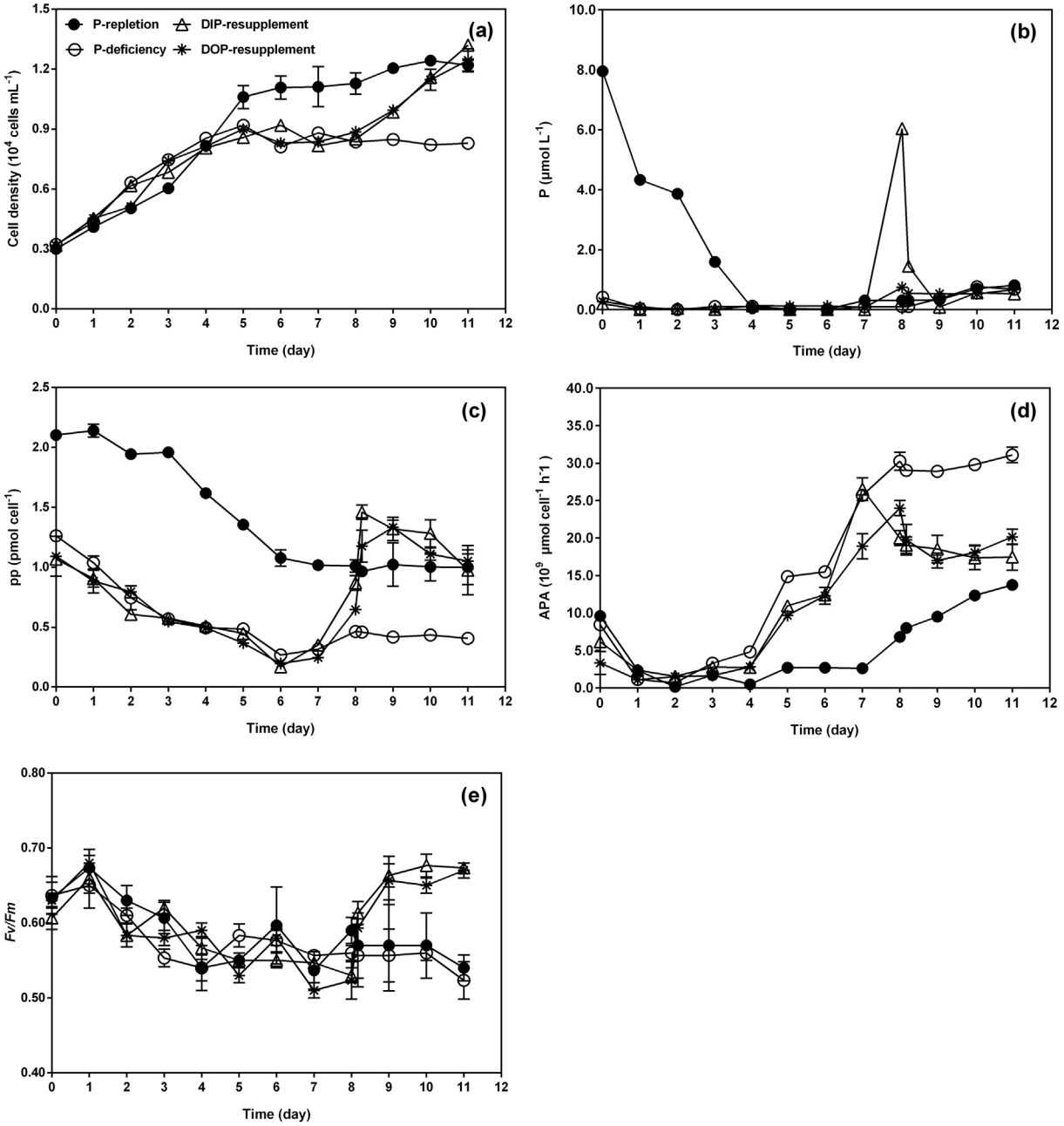

Fig. 1. Physiological responses of *A. catenella* to P deficiency and P resupply. (a) Cell density; (b) P concentration; (c) PP concentration; (d) APA; (e) *Fv/Fm*. P, concentrations of inorganic and organic phosphorus in the culture media; PP, particulate organic phosphorus; APA, alkaline phosphatase activity; *Fv/Fm*, PSII maximum photochemical yield.

The quantification of proteomic data was performed using IQuant software developed by Wen et al. [29]. In our study, six pairwise comparisons were set: P-deficient vs P-replete, DIP-resupplied vs P-deficient, DIP-resupplied vs P-replete, DOP-resupplied vs P-deficient, DOP-resupplied vs P-replete and DOP-resupplied vs DIP-resupplied. Differentially expressed proteins (DEPs) were defined with the criteria of mean fold change ≥1.2 (up-regulated) or ≤ 0.83 (down-regulated) between each pairwise comparison and p values < .05 [28].

3. Results

3.1. Physiological responses of *A. catenella* to P deficiency and resupply

The physiological responses of *A. catenella* to P deficiency and P resupply are shown in Fig. 1. For cell density, no significant difference was observed among the four treatments in the first 3 days, and cell density reached approximately 8.0×10^3 cells mL^{-1} by day 4. After that, the P-replete cells continuously grew and entered the stationary phase on day 7, while the P-deficient cells ceased growth and maintained a stable, low cell density level. After resupply of DIP or DOP on day 8, the cell growth of both groups rapidly recovered, and a cell density of 1.2×10^4 cells mL^{-1} was reached by day 11 (Fig. 1a). However, no significant difference was observed between the two cultures ($p > .05$).

The P concentration decreased rapidly in each treatment and was undetected in the P-deficient treatment on day 1, while P was exhausted in the P-replete treatment on day 4. After resupply of DIP or DOP on day 8, P was rapidly absorbed in both cultures, and DIP was consumed faster than DOP (Fig. 1b), but there was no significant difference between the two cultures. The cellular PP contents decreased

rapidly and reached the lowest level in the P-replete cells on day 6. After resupply of DIP or DOP on day 8, the PP contents increased rapidly in both cultures, but the PP content of the DIP-resupplied cells was higher than that of the DOP-resupplied cells (Fig. 1c).

APA rapidly increased from day 4 in the P-deficient cultures and reached a maximum of approximately 2.91×10^{-8} μmol L^{-1} h^{-1} on day 8. After resupply of DIP for 4 h on day 8, APA decreased immediately in the DIP-resupplied cells, while it continuously increased after DOP resupply and then decreased after DOP resupply for 4 h (Fig. 1d).

Fv/Fm presented insignificant differences among the four treatments until day 8. After P resupplied, *Fv/Fm* increased to approximately 0.68 in both the DIP- and DOP-resupplied cells. Interestingly, the *Fv/Fm* of all treatments maintained high levels (0.54–0.69) over the whole experimental period (Fig. 1e).

3.2. Differentially expressed proteins among different P treatments

A total of 47,942 unique spectra were matched to 12,339 unique peptides with a false discovery rate of 1%, and 6577 proteins were identified. Of these proteins, 3276 proteins were annotated into 112 pathways based on KEGG classification, and proteins associated with carbon metabolism, biosynthesis of amino acids, ribosome, glycolysis/gluconeogenesis, purine metabolism and ABC transporters were abundant (Fig. 2a).

With the strict criterion, a total of 480 DEPs were successfully identified between the P-replete and P-deficient cells. Among them, 210 proteins were up-regulated and 270 proteins were down-regulated in the P-deficient cells (Fig. 2b). These DEPs were significantly enriched ($p \leq .05$) in 17 metabolic pathways: proteins involved in sphingolipid metabolism, starch and sucrose metabolism, and photosynthesis were up-regulated in the P-deficient cells, while proteins associated with protein synthesis, nutrient absorption and energy metabolism were down-regulated (Supplementary Table 1).

In the comparison between the DIP-resupplied cells and the P-deficient cells, 88 proteins were up-regulated and 34 proteins down-regulated after DIP resupply (Fig. 2b). Proteins involved in nucleic acid metabolism, e.g., phosphoribosylaminoimidazolecarboxamideformyltransferase/IMP cyclohydrolase (purH), ribonucleo-sidediphosphate reductase (NrdE), and in photosynthesis, e.g., chlorophyll *a*-c binding protein and chlorophyll *a*-b binding protein, and in cytoskeleton formation, e.g., dynein light chain 2 and actin beta/gamma, were up-regulated in the DIP-resupplied cells. However, the KEGG pathway enrichment results showed that only ribosome-related proteins were enriched and significantly up-regulated ($p \leq .05$, Supplementary Table 2). Compared with the P-deficient cells, 284 DEPs were identified in the DOP-resupplied cells. Of these proteins, 154 DEPs were up-regulated and 130 DEPs were down-regulated in the DOP-resupplied cells (Fig. 2b and Supplementary Table 3). Photosynthesis, nucleic acid metabolism and protein synthesis proteins were up-regulated in the DOP-resupplied cells, while proteins in the carbon fixation and pentose phosphate pathways were down-regulated (Supplementary Table 3). However, only 18 up-regulated proteins (Fig. 3a) and 11 down-regulated proteins (Fig. 3b) were shared between DIP-resupplied and DOP-resupplied cells, respectively.

3.3. P metabolism-related proteins

Several proteins related to P metabolism, e.g., acid phosphatase (ACP), 5′-nucleotidase, ABC phosphate transporter, sugar-phosphatase and inorganic pyrophosphatase, were identified (Table 1). Among the five ACP proteins, only broad-range acid phosphatase DET1 was up-regulated in the P-deficient cells. However, no significant difference was observed between the DIP- and DOP-resupplied cells. In addition, 5′-nucleotidase, phosphate transporter, sugar-phosphatase and inorganic pyrophosphatase proteins were down-regulated in the P-deficient cells and were slightly up-regulated after P resupply. Pyrophosphate-energized vacuolar membrane proton pump was significantly up-regulated after DOP resupply. However, this protein demonstrated no significant change in the DIP-resupplied cells.

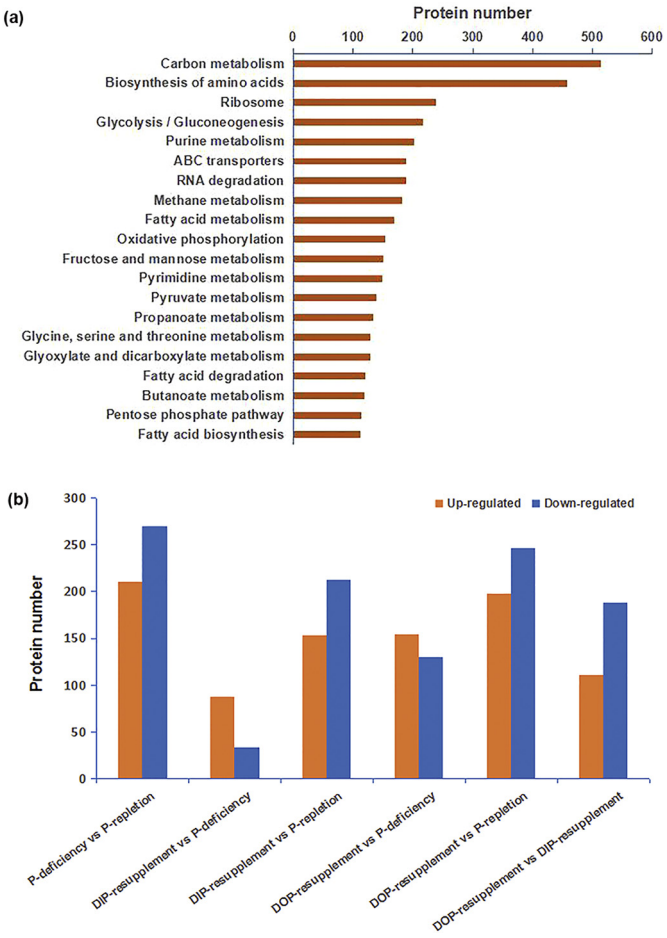

Fig. 2. Overview of proteins identified in *A. catenella*. (a) Top 20 abundant KEGG pathways of the annotated proteins; (b) Statistical analysis of differentially expressed protein number in each comparison between two samples. “A vs B", A is normalized to B. Orange represents the up-regulated proteins in the former compared with the latter, and blue represents the down-regulated proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Responses of A. catenella to ambient P deficiency

Some phytoplankton species can initiate multiple adaptive strategies, including the enhancement of phosphate transport and cellular P reallocation and the utilization of organic P-containing and non-P-containing lipids, in response to ambient P deficiency [15,18]. Our results showed that arylsulfatase, α-galactosidase and sialidase 1 were up-regulated in the P-deficient *A. catenella* cells (Fig. 4 and Supplementary Table 4). These proteins can decompose complex sphingolipid compounds such as sulfatides and ganglioside to form galactocerebroside. Sphingolipids are the major component of plant cell plasma membranes and vacuolar membranes and play an important role in membrane stability, signal transduction, cell proliferation, differentiation and apoptosis [30,31]. The up-regulation of these proteins indicated that *A. catenella* reduced the demand for P by using galactocerebroside instead of phospholipids to adapt to the P-deficient environment. ACP is a relatively nonspecific enzyme that can hydrolyze

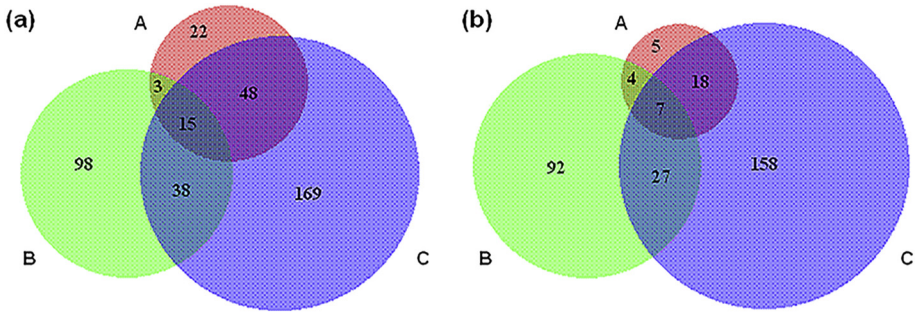

Fig. 3. Statistical analysis of differentially expressed proteins among different P conditions. The size of the circle represents the number of differential expression proteins. (a) Up-regulated proteins, (b) Down-regulated proteins. A represents P-replete vs. P-deficient, B represents DIP-resupplied vs. P-deficient, C represents DOP -resupplied vs. P-deficient. "A vs. B" is A normalized to B.

Table 1
P metabolism-related proteins in *A. catenella* cells.

	Protein ID	Protein name	Ratio	Q value[a]
P-deficient vs P-replet				
Up	CL7054_Contig1_All	Broad-range acid phosphatase DET1	1.51	0.016
Down	CL6300_Contig2_All	Phosphate transport system substrate-binding protein	0.58	0.001
	CL3140_Contig3_All	5′-nucleotidase	0.57	0.001
	Unigene34875_All	Sugar-phosphatase	0.821	0.012
	CL763_Contig2_All	Inorganic pyrophosphatase	0.738	0.003
DOP-resupplied vs P-deficient				
Up	CL6232_Contig1_All	Pyrophosphate-energized vacuolar membrane proton pump	1.89	0.045

[a] Q value is the checksum value for differentially expressed proteins. Difference is considered significant when Q value is < 0.05.

the phosphomonoester bond to release phosphate ions under acid conditions. As an induced enzyme, ACP is important in the hydrolysis and mobilization of extracellular phosphomonoesters and can also act as an extracellular agent in the utilization of organic P or other P-containing compounds [32]. Under P-deficient conditions, the expression of ACP is enhanced, which regulates organic P metabolism, phospholipid hydrolysis and inorganic phosphate assimilation, playing an important role in the production and circulation of inorganic phosphate [33]. In our study, ACP was up-regulated (1.51-fold) in the P-deficient cells, while 5′-nucleotidase and sugar-phosphatase were down-regulated (Fig. 4 and Supplementary Table 4), indicating the importance of ACP in the response to ambient P deficiency in *A. catenella*. Notably, AP was not identified in our study, although APA was detected. Many types of AP such as PhoA, PhoX and PhoD have been identified in various phytoplankton species, but only PhoA has been identified in *A. catenella* [34]. Other types of AP might exist in *A. catenella* but were not annotated due to the lack of dinoflagellate genome data. Previous studies have shown that cells first synthesize inactive AP precursors, which are then hydrolyzed by a specific protease to form mature active AP [35]. Mature AP is released outside the cell to degrade ambient organic P, which results in a low abundance of intracellular AP. Furthermore, the nondetection of AP might also be caused by the substrate because AP generally displays an absolute substrate specificity. Our study indicated that *A. catenella* might initiate ACP instead of AP to utilize organic P under ambient P-deficient conditions, which needs further study. Moreover, unlike most phytoplankton, which can enhance the expression of phosphate transporters in response to P deficiency [18], two inorganic proteins related to P utilization were significantly down-regulated in the P-deficient *A. catenella* cells, suggesting that *A. catenella* mainly uses DOP instead of DIP in response to ambient P deficiency.

In the P-deficient cells, ATP synthase and enzymes for energy production via glycolysis were significantly down-regulated indicating that intracellular energy production was decreased (Fig. 3 and Supplementary Table 4). Down-regulation of ATP synthase is also observed at the transcriptional level [36]. However, proteins involved in photosystem I and II, such as photosystem II oxygen-evolving enhancer protein (OEE) 1 and 2, photosystem I subunit IV and cytochrome *c*6, were up-regulated (Fig. 4 and Supplementary Table 4). The absence of OEE2 reduces the rate of photosynthetic oxygen evolution, while the absence of OEE1 results in the complete loss of photosynthetic oxygen evolution [37]. Photosystem I subunit IV mediates electron transports from the primary acceptor to soluble ferredoxin [38]. The up-regulation of these proteins suggests that the P-deficient cells synthesized more proteins to compensate for the decreasing efficiency of photosynthetic oxygen evolution and light energy conversion caused by P deficiency to maintain normal photosynthesis. The high *Fv/Fm* value of the P-deficient cells during the experiment supports this postulation.

In our study, enzymes participating in the Calvin cycle, such as ribulose-1,5-bisphosphate- carboxylase/oxygenase (Rubisco), phosphoglycerate kinase (PGK), fructose-bisphosphate aldolase (FBA), glyceraldehyde-3-phosphate dehydrogenase (GAPD), fructose-1,6-bisphosphatase (FBPase) and phosphoribulokinase (PRK), were all down-regulated in the P-deficient cells (Fig. 4 and Supplementary Table 4). Rubisco is a rate-limiting photosynthetic enzyme that catalyzes the condensation of CO_2 and ribulose-1,5-bisphosphate to produce 3-phosphoglycerate, PGK and GAPD, which catalyze 3-phosphoglycerate to form glyceraldehyde-3-phosphate, FBA and FBPase, which catalyze glyceraldehyde-3-phosphate to form fructose-6-phosphate, and PRK, which participates in the last step of the Calvin cycle, irreversibly catalyzing the formation of RUBP by ribulose-5-phosphate to ensure the Calvin cycle continues [39–41]. The down-regulation of these proteins indicates that P deficiency inhibited carbon fixation by *A. catenella* and decreased the intracellular ATP consumption.

Nitrogen (N) and sulfur (S) are two nutrient elements essential to cell growth and development. In our study, proteins involved in N and S metabolism, such as ATP-sulfurylase (ATPS), 3′-phosphoadenosine-5′-phosphosulfate reductase (PAPSR), sulfite reductase (SIR), cysteine synthase (Cys synthase), nitrite reductase (NIR), glutamine synthetase (GS) and glutamate synthase (GOGAT), were all down-regulated in the P-deficient cells (Fig. 3 and Supplementary Table 4). S can improve the adaptability of plants to biotic and abiotic stresses, and S assimilation is well coordinated with the assimilation of N and carbon dioxide. ATPS is a key enzyme in the assimilation of sulfate in plants and catalyzes the reaction of ATP with sulfate to produce adenosine-5′-phosphosulfate (APS) [42,43]. NIR catalyzes the reduction of NO_2^- to NH_4^+, which is transformed into organic N compounds by GS/GOGAT [44,45]. The down-regulation of these proteins decreases N and S assimilation, balancing element proportions and reducing energy requirements, which might be an adaptive strategy of *A. catenella* to ambient P deficiency that saves more intracellular energy to maintain cell survival.

Proteins related to protein translation, i.e., 36 ribosomal proteins and 7 aminoacyl-tRNA synthetase, were down-regulated (Fig. 4 and Supplementary Table 4). The lack of ribosomal proteins can reduce the ribosome number and protein synthesis [46]. Aminoacyl-tRNA synthetase provides amino acids to extend polypeptide chains and is essential for protein synthesis [47]. These results suggest that P deficiency inhibits the protein translation of *A. catenella*, which reduces the energy

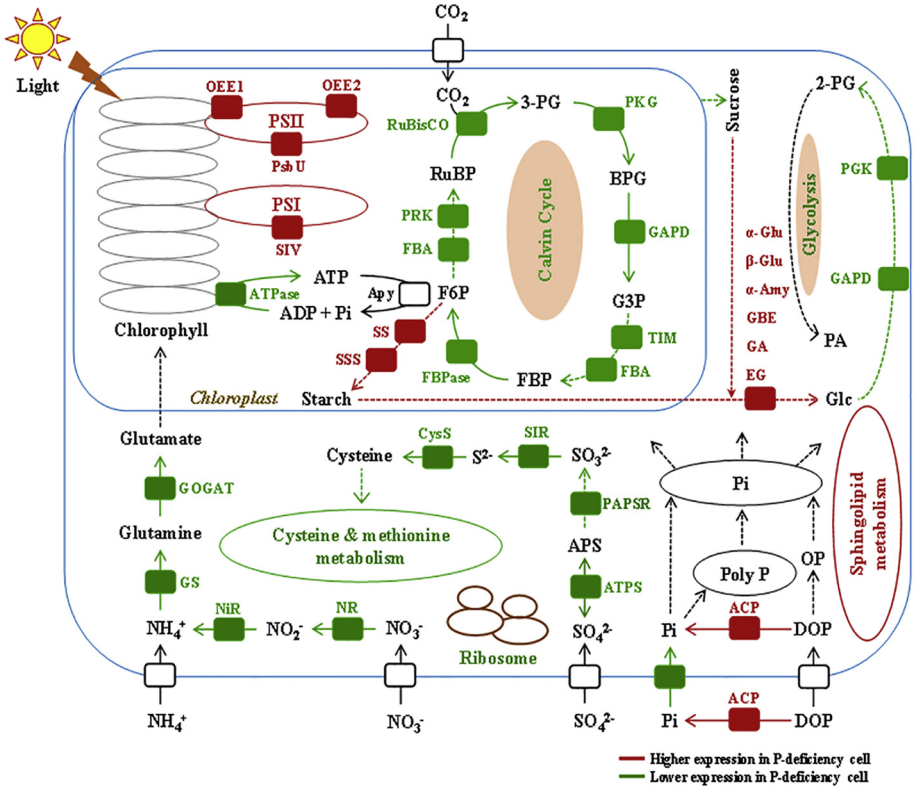

Fig. 4. The metabolic processes in the P-deficient *A. catenella*. The rectangles indicate transporters or enzymes; the black empty rectangle indicates proteins with no significant changes in the P-deficient compared to the P-replete cultures; the green solid rectangle represents down-regulated transporters or enzymes; the red solid rectangle represents up-regulated transporters or enzymes. The solid lines represent one-step reactions, and the dashed lines represent reactions with more than one step. ACP: acid phosphatase, α-Amy: α-amylase, ATPase: ATP synthase, Apy: Apyrase, ATPS: ATP-sulfurylase, CysS: cysteine synthase, EG: endoglucanase, FBA: fructose-bisphosphate aldolase, FBPase: fructose-1,6-bisphosphatase, GA: glucoamylase, GAPD: glyceraldehyde-3-phosphate dehydrogenase, GBE: 1,4-α-glucan branching enzyme, α-Glu: α-glucosidase, β-Glu: β-glucosidase, GOGAT: glutamate synthase (ferredoxin), GS: glutamine synthetase, NiR: nitrite reductase, NR: nitrate reductase, PAPSR: phosphoadenosinephosphosulfate reductase, PGK: phosphoglycerate kinase, PRK: phosphoribulokinase, RuBisCO: ribulose-1,5-bisphosphate-carboxylase/oxygenase, SIR: sulfite reductase, SS: starch synthase, SSS: soluble starch synthase, TIM: triosephosphate isomerase, APS: adenosyl phosphosulfate, BPG: 1,3-diphosphoglycerate, DOP: dissolved organic phosphorus, FBP: 1,6-fructose diphosphate, F6P: fructose 6-phosphate, G3P: glyceraldehyde 3-phosphate, Glc: Glucose, OEE: photosystem II oxygen-evolving enhancer protein, P: phosphorus, PA: pyruvic acid, PAPSR: 3′-phosphoadenosine 5′-phosphosulfate reductase, 2-PG:2-phosphoglycerate, 3-PG: 3-phosphoglycerate, Pi: phosphate, PolyP: polyphosphate, PsbU: photosystem II PsbU protein, RuBP: Ribulose 1,5-bisphosphate, SIV: photosystem I subunit IV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consumption involved in protein synthesis. This might also be a feedback response to the decrease in N assimilation in the P-deficient cells.

It has been reported that photosynthetic products are stored mainly as starch in cells to reduce P consumption under P-deficient conditions [48]. In our study, starch synthase (SS) and soluble starch synthase (SSS) were up-regulated in the P-deficient cells, indicating that more starch was synthesized. Meanwhile, proteins involved in starch and sucrose degradation were also up-regulated in the P-deficient cells, which enhanced the conversion of glucose polymers to sugars, providing fundamental materials and energy for cell survival (Fig. 4 and Supplementary Table 4). Sucrose, as a major product of photosynthesis, not only plays an important role in plant growth, development, nutrients storage, and signal transduction, but also is an important signal molecule in plant stress responses. Recent studies demonstrate that sucrose can regulate P transport and absorption of plants and improve their ability to tolerate low P environment [49–51].

Overall, the *A. catenella* cells initiated diverse strategies to adapt to ambient P deficiency, i.e., by enhancing ACP expression, utilizing nonphospholipids, reducing P consumption, and adjusting intracellular energy allocation.

4.2. Response of A. catenella to resupply of inorganic and organic P

After resupply of DIP or DOP, energy and ATP synthesis were recovered in the P-deficient cells, and proteins related to P metabolism and energy-consuming processes were also up-regulated (Fig. 5). However, significant differences were observed between the DIP- and DOP-resupplied cells.

4.3. Energy metabolism

ATP acting as a universal cellular energy cofactor fuels all life processes, including gene expression, material metabolism and transport. In our study, F0F1-type ATP synthase and ATP phosphoribosyltransferase were up-regulated in both DIP- and DOP-resupplied cells (Supplementary Tables 5 and 6). Up-regulation of the mitochondria ATP synthase gene is also observed in *Karenia mikimotoi* grown in G-6-P [52]. ATPase is an ATP synthase found in mitochondria and the chloroplast, and the alpha and beta subunits combine to form the catalytic site of F-type ATPases [53]. ATP phosphoribosyltransferase catalyzes the first step of histidine biosynthesis and produces ATP [54]. The up-regulation of these two proteins indicates that P resupply enhanced ATP synthesis, providing energy essential for cell growth and other physiological functions.

The growth and development of plants are ultimately driven by the light energy captured by photosynthesis [55]. In our study, photosystem I P700 chlorophyll *a* apoprotein A1 (PsaA), photosystem I p700 chlorophyll *a* apoprotein A2 (PsaB), photosystem II CP43 chlorophyll apoprotein (CP43), photosystem II CP47 chlorophyll apoprotein (CP47), photosystem II P680 reaction center D1 protein (D1), photosystem II P680 reaction center D2 protein (D2), photosystem II cytochrome *b*559 (Cyt b559) subunit alpha and Cyt b6f complex (PetB) were all up-regulated in the DOP-resupplied cells, while chlorophyll *a*-b binding protein and chlorophyll *a*-c binding protein F were up-regulated in the DIP-resupplied cells (Fig. 4, Supplementary Tables 5 and 6). PsaA and PsaB are involved in light capture, charge separation and electron transport [56], and CP43 and CP47 are responsible for receiving excitation energy and transferring it directly to the photosystem II reaction center [57]. The photosystem II reaction center complex is mainly composed of D1, D2 and Cyt b559 and is the core part converting light energy into chemical energy [58,59]. Pet B mainly regulates the electron transfer from plastoquinone to plastocyanin or from ferredoxin to the circulating electrons around photosystem I [60]. Chlorophyll a-c binding protein C and chlorophyll *a*-b binding protein

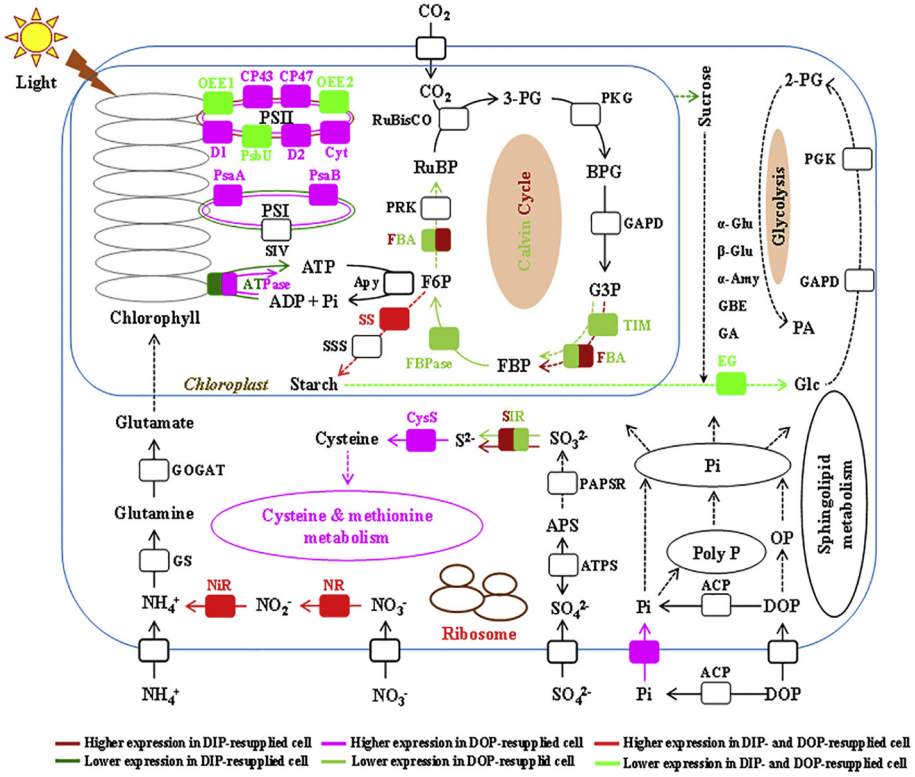

Fig. 5. The metabolic processes in the DIP- and DOP-resupplied *A. catenella* cells. The rectangles indicate transporters or enzymes; the black empty rectangle indicates proteins with no significant changes in the P-deficient compared to the P-replete cultures; the green solid rectangle represents down-regulated transporters or enzymes; the red solid rectangle represents up-regulated transporters or enzymes. The solid lines represent one-step reactions, and the dashed lines represent reactions with more than one. Cyt: cytochrome, PsaA: photosystem I P700 chlorophyll *a* apoprotein A1, PsaB: photosystem I p700 chlorophyll *a* apoprotein A2, CP43: photosystem II, CP43 chlorophyll apoprotein, CP47: photosystem II CP47 chlorophyll apoprotein, D1: photosystem II P680 reaction center D1 protein, D2: photosystem II P680 reaction center D2 protein; other abbreviations are shown in Fig. 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

play important roles in maintaining the thylakoid membrane structure, regulating the distribution of the excitation energy between PS I and PSII and photoprotection [61,62]. Furthermore, the up-regulated proteins related to photosynthesis in the P-deficient cells were all significantly down-regulated after P resupply, indicating that photosynthetic oxygen evolution and light energy conversion were recovered and that the resupply of P enhanced light reactions and subsequent photosynthesis.

4.4. Nucleic acid metabolism

P is a base constituent of nucleotides. In our study, proteins related to nucleic acid biosynthesis, including adenylosuccinate lyase (purB), adenylosuccinate synthase (purA), GMP synthase (guaA), phosphoribosylamino-imidazolecarboxamideformyltransferase/IMP cyclohydrolase (purH), carbamoyl phosphate synthase (CPS) and ribonucleoside-diphosphate reductase (nrdE), were up-regulated in the DOP-resupplied cells (Fig. 5, Supplementary Tables 5 and 6). Purine is not only the base constituent of DNA and RNA but also functions as a coenzyme in many enzymatic reactions. PurB, purH, purA and guaA are mainly involved in purine synthesis [63,64]. PurB catalyzes the 8th step of the purine de novo synthesis pathway [65]; purH is a bifunctional enzyme, catalyzing the last two steps of the purine de novo synthesis pathway and playing a key role in regulating IMP synthesis [66,67]. PurB also catalyzes the conversion of IMP to AMP with purA, and guaA catalyzes the conversion of IMP to GMP [68,69]. CPS catalyzes ATP, HCO_3^- and glutamine to form carbamoyl phosphate and is the rate-limiting step of pyrimidine nucleotide biosynthesis [68]. NrdE is a key and rate-limiting enzyme of DNA synthesis and repair and catalyzes the formation of DNA precursor deoxyribonucleotides by ribonucleotides [69]. The up-regulation of these proteins in the DOP-resupplied cells indicates that the synthesis of purine and pyrimidine was enhanced, which might promote the synthesis and repair of DNA and RNA, resulting in the enhancement of nucleic acid metabolism and the translation process. However, DNA synthesis is enhanced in *K. mikimotoi* grown in G-6-P [52]. Although purH and nrdE were also up-regulated in the DIP-resupplied cells, the nucleic acid metabolism process was not significantly enriched. Our results reflect different utilization strategies of DIP and DOP and indicate that *A. catenella* evolved special mechanisms to utilize DOP.

4.5. Protein metabolism

Ribosomal proteins (RPs), a family of RNA-binding proteins, are the essential components of the ribosome and play essential roles in ribosome biogenesis and protein translation [70]. Studies show that RPs also present other extraribosomal functions (Fig. 5, Supplementary Tables 5 and 6) independent of protein biosynthesis in the regulation of diverse cellular processes [71]. In our study, nine RPs were up-regulated in the DOP- and DIP-resupplied cells, suggesting that the resupply of P enhanced protein translation in the P-deficient cells. These results are consistent with the growth of *A. catenella*; the P-deficient cells recovered cell growth and cell density increased rapidly immediately after P resupply. The up-regulation of RPs in both the DIP- and DOP-resupplied cells suggests that protein translation might be critical for cell recovery from P deficiency. Moreover, two ATP-dependent proteases, namely, ATP-dependent Lon protease and ATP-dependent Clp protease, were down-regulated in the DOP-resupplied cells (Fig. 5, Supplementary Table 6). The former plays key roles in the degradation of misfolded and damaged proteins and metabolic reprogramming, and supports cell viability under stress conditions [72], while the later can assist to remove incorrectly processed cytoplasmic precursors and misfolded proteins and improve cell tolerance to stress [73]. The down-regulation of these two proteins in the DOP-resupplied cells resulted in the decrease in misfolded and damaged proteins and the recovery of the cells. However, there were no significant changes in the ATP-dependent proteases in the DOP-resupplied cells, reflecting different response mechanisms of *A. catenella* to DIP or DOP resupply.

4.6. Different proteomic responses of A. catenella to DIP or DOP resupply

Our study showed that *A. catenella* recovered cell growth rapidly

after the resupply of DIP (Na_2HPO_4) or DOP (G-6-P) (Fig. 1a), and cells presented no differences between the two treatments at the physiological level. However, significant differences were observed at the proteomic level (Fig. 5). In the DIP-resupplied cells, only the ribosome process was enhanced, while other processes did not vary significantly. However, proteins involved in photosynthesis and pyrimidine and purine metabolism were all up-regulated significantly in the DOP-resupplied cells, except for ribosome-associated proteins, indicating different utilization mechanisms of DIP and DOP. *A. catenella*, liked *K. mikimotoi* [52], might directly utilize G-6-P via G-6-P transport and produce 5-phosphate ribose pyrophosphate via the phosphate pentose pathway, which is used for purine and pyrimidine syntheses to promote the synthesis of DNA and RNA. Although the G-6-P transporter has not been identified in marine phytoplankton, it has been found in bacteria such as *Streptococcus lactis*, *Escherichia coli* and *Salmonella typhimurium* [35,74,75]. The rapid assimilation of G-6-P by *A. catenella* cells indicated the potential existence of a G-6-P transporter. Interestingly, after the resupply of G-6-P, proteins related to the Calvin cycle such as triosephosphate isomerase, FBA, FBPase and transketolase were down-regulated, which would block ribulose-1,5-bisphosphate regeneration and result in the subsequent decrease in photosynthetic carbon fixation. G-6-P taken up into the cells might provide not only P but also carbon for *A. catenella*. G-6-P usually originates from glucose via the catalysis of glucose-6-phosphatase [76], and the formation of glucose might inhibit photosynthetic carbon fixation. In summary, *A. catenella* could use both DIP and DOP as P sources for cell growth, which was consistent with previous studies in *K. mikimotoi* and *Skeletonema costatum* [11,52], but their utilization mechanisms are different.

Understanding the role of DIP in regulating cell growth and bloom formation of dinoflagellates has been improved significantly in the past few decades [14,18], however, little is known about the role of DOP in the formation of HABs. Studies show that considerable quantities of organic P are imported to the ocean, especially the coastal area due to the extensive use of organophosphorus fertilizers and pesticides [77–79], which might provide P source for dinoflagellates. Therefore, we postulate that massive input of organic P and the strong ability to utilize DOP might be the important reasons resulting in the increasing of dinoflagellate HABs. In future, more efforts should be devoted to studying concentration and composition of DOP in seawater, and utilization ability and mechanism of DOP among different dinoflagellate species, to unveil the role of DOP for HABs formation.

5. Conclusions

In conclusion, our study showed that *A. catenella* evolved diverse strategies to adapt to ambient P-deficient environments, e.g., by utilizing nonphospholipids and glycosphingolipids instead of phospholipids to reduce cell demand for P, by enhancing ACP expression to utilize organic P, and by reallocating intracellular energy by decreasing energy-consuming biological processes. Surprisingly, AP was not identified in our proteome data, although APA changes were detected. ACP, instead of AP, might be the main mechanism of *A. catenella* cells to utilize organic P. *A. catenella* cells may use both DIP and DOP as a source of P for cell growth, but the cellular responses to the two P forms differed; only ribosome proteins were up-regulated in the DIP-resupplied cells, while more biological processes, e.g., nucleic acid metabolism, photosynthetic energy metabolism and carbon fixation, were altered in the DOP-resupplied cells. G-6-P might be taken up into the cells and directly utilized for nucleic acid biosynthesis through the pentose phosphate pathway. Moreover, G-6-P might also provide a carbon source for *A. catenella*, which, in turn, would affect photosynthetic carbon fixation, but this needs further study by combining metabolomic and proteomic approaches to elucidate the utilization mechanism of DOP in *A. catenella*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.11.004.

Acknowledgements

This work was partially supported by research grants from the National Key R & D Program of China (Project No. 2017YFC1404300) and the National Natural Science Foundation of China (Project No. 41230961, 41706131 and 41425021). D.Z. Wang was also supported by the 'Ten Thousand Talents Program' for leading talents in science and technological innovation.

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