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1 **Multi-omics profiling reveals resource allocation and acclimation strategies to**
2 **temperature changes in a marine dinoflagellate**

3

4 Running title: Molecular response to temperature in dinoflagellate

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26 **ABSTRACT**
27 Temperature is a critical environmental factor that affects the cell growth of
28 dinoflagellates and bloom formation. To date, the molecular mechanisms underlying the
29 physiological responses to temperature variations are poorly understood. Here, we applied
30 quantitative proteomic and untargeted metabolomic approaches to investigate protein and
31 metabolite expression profiles of a bloom-forming dinoflagellate *Prorocentrum*
32 *shikokuense* at different temperatures. Of the four temperatures (19, 22, 25, 28℃)
33 investigated, *P. shikokuense* at 25℃ exhibited the maximal cell growth rate and Fv/Fm
34 value. The levels of particulate organic carbon (POC) and nitrogen (PON) decreased with
35 increasing temperature, while the POC/PON ratio increased and peaked at 25℃.
36 Proteomic analysis showed proteins related to photoreaction, light harvesting and protein
37 homeostasis were highly expressed at 28℃ when cells were under moderate heat stress.
38 Metabolomic analysis further confirmed reallocated amino acids and soluble sugars at this
39 temperature. Both omic analyses showed glutathione metabolism that scavenges the
40 excess reactive oxygen species, and transcription and lipid biosynthesis that compensate
41 for the low translation efficiency and plasma membrane fluidity were largely up-regulated
42 at sub-optimal temperature. Higher accumulations of glutathione, glutarate semialdehyde,
43 and 5-KETE at 19℃ implied their important roles in low temperature acclimation. The
44 strikingly active nitrate reduction and nitrogen flux into asparagine, glutamine and aspartic
45 acid at 19℃ indicated these three amino acids may serve as nitrogen storage pools and
46 help cells cope with low temperature. Our study provides insights into the effects of
47 temperature on dinoflagellate resource allocation, and advances our knowledge of
48 dinoflagellate bloom formation in marine environments.
49
50 **IMPORTANCE**
51 Marine phytoplankton is one of the most important nodes in global biogeochemical cycle.
52 Deciphering temperature-associated marine phytoplankton cell stoichiometric changes and
53 the underlying molecular mechanisms are therefore of great ecological concerns. However,
54 knowledge of how phytoplankton adjust the cell stoichiometry to sustain growth under
55 temperature changes is still lacking. This study investigates the variations of protein and
56 metabolite profiles in a marine dinoflagellate across temperatures at which the field
57 blooms usually occur, and highlights the temperature-dependent molecular traits and key
58 metabolites that may be associated with rapid cell growth and temperature stress
59 acclimation.
60
61 **KEYWORDS**: temperature, dinoflagellate, harmful algal blooms, quantitative proteomics,
62 metabolomics, phytoplankton stoichiometry

63 **INTRODUCTION**

64 Among the biotic and abiotic parameters, temperature is recognized as a significant factor
65 modulating the diversity and distribution of phytoplankton in global oceans (1, 2). It
66 affects phytoplankton abundance directly through its effect on the rates of cell metabolism
67 and cell division, and indirectly through its effect on ocean stratification which influences
68 the movement of bottom level nutrients to surface oligotrophic seawater (3, 4).
69 Dinoflagellates are one widespread and abundant phytoplankton group in global oceans.
70 They are the major source of harmful algal blooms (HABs) which can severely impact
71 marine ecosystems and aquaculture (5, 6). Long-term field investigation and niche model
72 analyses show that the coastal dinoflagellate blooms are increasing due to ocean warming,
73 in terms of frequency, intensity and geographic distribution (7, 8). Since the
74 phylogenetically diverse dinoflagellates exhibit distinct thermal traits (9), understanding
75 the functional response to temperature among different species is therefore a requisite for
76 the prediction and management of dinoflagellate blooms.

77

78 The phytoplankton growth-temperature relationship often exhibits a bell-shaped curve
79 with gradual increases and steep declines in growth rate at sub- and supra-optimal
80 temperatures (10). Each species is known to exhibit a narrow range of temperature for
81 optimal cell growth (11, 12). Outside the optimal temperature range, cell motility,
82 biochemical properties and stoichiometry will vary largely. Dinoflagellates and other
83 phytoplankton have adopted a series of response strategies to cope with sub- and
84 supra-optimal temperatures, such as regulating nutrient acquisition, reallocating element
85 stoichiometry, biosynthesizing protective compatible solutes and antioxidant metabolites,
86 and changing the fluxes of vital metabolic pathways (13-15). In addition, high levels of
87 photoprotective pigments at supra-optimal temperature and accumulations of
88 polyunsaturated fatty acids and toxins at sub-optimal temperature have been reported in
89 dinoflagellates (16-19). All these strategies are aimed at establishing a new balance of
90 resource allocation and energy consumption that enable cells to survive and reproduce at
91 non-optimal temperatures.

92

93 Thus, deciphering temperature acclimation related molecular events in phytoplankton is
94 important for predicting the community variations under climate change and identifying
95 traits that are subject to environmental selection. Using metatranscriptomics, Toseland et
96 al. find that phytoplankton significantly increase the rate of protein synthesis and decrease
97 the number of ribosomes and their associated rRNAs as temperature rises (20). Increasing
98 photosynthetic electron transport at low temperature and up-regulating oxidative
99 phosphorylation at high temperature are invoked to compensate for repressed
100 photosynthesis in a green alga (21). Pathways to maintain necessary protein processing
101 machinery and membrane structure are induced at non-optimal temperatures in diatoms

102 (22). To date, only several temperature stress-related genes/proteins are established in
103 dinoflagellates (23, 24), and responses of a global metabolism to temperature changes in
104 this phytoplankton group using multi-omics approaches are still less studied.
105
106 The dinoflagellate *Prorocentrum shikokuense* annually forms extensive large-scale HABs
107 in the coastal East China Sea (ECS) in spring (25, 26). Field investigations show that *P.*
108 *shikokuense* exhibits relatively high growth rates between 16℃ and 26℃ in the coastal
109 ECS (27). In the laboratory, it is able to grow at temperatures ranging from 10℃ to 31℃
110 (28). In our study, culture temperatures of 19, 22, 25 and 28℃ were used to simulate
111 temperature transitions from the early to late spring in the coastal ECS, covering the
112 temperature range of a whole bloom period. The goal of this study was to decipher the
113 underlying traits associated with temperature responses that may facilitate *P. shikokuense*
114 to form blooms in spring. The iTRAQ-based quantitative proteomics and untargeted
115 metabolomics approaches were used to investigate the protein and metabolite profiles of *P.*
116 *shikokuense*. Our results showed some proteins and macromolecules that may serve as
117 important agents of stress response and nutrient storage to sustain cell growth under
118 different temperatures.
119
120 **RESULTS**

121 **Physiological parameters at different temperatures**

122 The growth rates of *P. shikokuense* at 19, 22, 25 and 28℃ were 0.39, 0.54, 0.68 and 0.69
123 d^{-1}, respectively. Significant differences in the growth rates were observed between any
124 pair of the four temperatures except 25℃ and 28℃ (p-value < 0.05). The optimal
125 temperature of *P. shikokuense* was determined to be 26.8℃ through Boltzmann-Arrhenius
126 model analysis (Fig. 1A). Fv/Fm values and DNA contents at 19, 22, 25 and 28℃ were
127 0.55, 0.56, 0.60 and 0.58, and 7.24, 6.30, 5.71 and 6.21 pg/cell, respectively (Fig. 1B).
128
129 The POC and PON decreased with increasing temperatures, but the ratios of POC/PON
130 increased from 19℃ to 25℃ and then decreased at 28℃ (Fig. 1C). The POC and PON for
131 19, 22, 25 and 28℃ were 16.18, 13.79, 11.66 and 11.21 ng/cell and 3.80, 2.57, 1.75 and
132 1.80 ng/cell, while the ratios of POC/PON were 4.34, 5.48, 6.68 and 6.24, respectively.
133
134 **Overview of the quantitative iTRAQ proteomics**

135 In total, 70,898 of the output 336,029 mass spectra matched 22,308 peptides, which
136 resulted in the identification of 4,562 high-confidence proteins. Of the high-confidence
137 proteins, 3,933 (86.3%) and 3,044 (66.8%) proteins were annotated using the NCBInr and
138 KEGG databases, respectively. Compared with 25℃, 375, 1,274 and 1,918 differentially
139 expressed proteins (DEPs) were identified at 28, 22 and 19℃, respectively (Fig. S1A).
140 Heatmap based on protein expressions revealed that DEPs at 19℃ and 22℃, and at 25℃

141 and 28°C were clustered together with high similarities, respectively (Fig. S2A). Moreover,
142 most DEPs showed clear patterns of increase or decrease from 19°C to 28°C (Fig. S2B).
143
144 KEGG pathway enrichment analysis showed that the pathways related to protein
145 processing in the endoplasmic reticulum and photosynthesis-antenna proteins were
146 significantly enriched at 28°C relative to 25°C, whereas carbon metabolism and
147 biosynthesis of secondary metabolites and amino acids were highly but not significantly
148 enriched at 25°C relative to 28°C (Fig. 2A). A comparison between 22°C and 25°C showed
149 that the mRNA surveillance pathway and RNA transport were significantly enriched at
150 22°C, while ribosome, carbon metabolism, carbon fixation, glycolysis/gluconeogenesis,
151 fructose and mannose metabolism, pentose phosphate pathway, biosynthesis of amino
152 acids and secondary metabolites, and photosynthesis-antenna proteins were significantly
153 enriched at 25°C (Fig. 2B). A comparison between 19°C and 25°C showed that the
154 spliceosome, mRNA surveillance pathway, RNA transport and degradation,
155 2-oxocarboxylic acid metabolism, and glutathione metabolism were significantly enriched
156 at 19°C, while ribosome, carbon fixation, glycolysis/gluconeogenesis, fructose and
157 mannose metabolism, pentose phosphate pathway, porphyrin and chlorophyll metabolism,
158 photosynthesis, and photosynthesis-antenna proteins were significantly enriched at 25°C
159 (Fig. 2C).
160
161 **Overview of the untargeted metabolomics**
162 We detected a total of 331 metabolites from cells growing at 19, 25 and 28°C. Among
163 them, 142 and 116 differentially expressed metabolites (DEMs) were identified at 28°C vs
164 25°C and 19°C vs 25°C, respectively (Fig. S1B). Most of these DEMs were identified and
165 classified as amino acids, peptides and their analogues, carbohydrates and carbohydrate
166 conjugates, fatty acids and conjugates. More up-regulated DEMs belonging to purine and
167 pyridine nucleosides, fatty acids and conjugates, amino acids, peptides and analogues,
168 benzene and substituted derivatives, carbohydrates and carbohydrate conjugates were
169 observed at 28°C when compared with 25°C (Fig. 3A), while more up-regulated DEMs
170 belonging to purine and pyridine nucleosides were observed at 19°C when compared with
171 25°C (Fig. 3B).
172
173 When compared with 25°C, metabolisms of amino sugar and nucleotide sugar, arginine
174 and proline, fructose and mannose, galactose, glyoxylate and dicarboxylate, nicotinate and
175 nicotinamide, and isoquinoline alkaloid biosynthesis with more DEMs were enriched at 28°C
176 (Fig. 4A). A comparison between 19°C and 25°C showed that metabolisms of glutathione,
177 purine, arachidonic acid, beta-alanine, cysteine and methionine with more DEMs were
178 enriched at 19°C, while galactose metabolism and carbapenem biosynthesis with more
179 DEMs were enriched at 25°C (Fig. 4B).

180

181 **Cell growth and stress response-related DEPs and DEMs between 28℃ and 25℃**

182 For proteomics, DEPs involved in photosynthesis, and photosynthesis-antenna proteins,
183 such as chlorophyll a-c binding protein (CAB), light harvesting protein (LHP), PsaB, and
184 PsaC were significantly up-regulated at 28℃ relative to 25℃ (Fig. 5A). Moreover, heat
185 shock protein 90 (HSP90) was also up-regulated at 28℃. The majority of DEPs involved
186 in carbohydrate metabolism, translation, and amino acid metabolism were up-regulated at
187 25℃ relative to 28℃. For metabolomics, abundances of carbohydrates and amino acids
188 varied largely between 25℃ and 28℃ (Fig. 5B). The typical carbohydrates L-fucose,
189 mannitol, and D-mannose, and amino acids L-proline and L-lysine were largely
190 accumulated at 28℃, while amino acids L-asparagine, L-histidine, Ornithine and
191 L-glutamine were largely accumulated at 25℃.

192

193 **Cell growth and stress response-related DEPs and DEMs across 19℃, 22℃, and 25℃**

194 Cell growth-related proteins such as carbonic anhydrate (CA), ribulose 1,5-bisphosphate
195 carboxylase/oxygenase (RBC), nitrate transporter (NT), LHP and CAB were significantly
196 down-regulated at 19℃ and 22℃ relative to 25℃, while those involved in
197 stress-responses such as copper/zinc superoxide dismutase (Cu/Zn SOD) was significantly
198 up-regulated at both 19℃ and 22℃ (Fig. 6A and 6B). Specifically, more DEPs involved
199 in cold acclimation, lipid and nucleotide metabolisms, such as cold shock protein (CSP),
200 long-chain fatty acid CoA ligase (ACSBG), acetyl-CoA acyltransferase 1 (ACAA1),
201 long-chain acyl-CoA synthetase (ACSL), uridine kinase (udk), CTP synthase (pyrG),
202 GMP synthase (guaA), IMP dehydrogenase (IMPDH), and adenosine kinase (ADK) were
203 significantly up-regulated at 19℃ relative to 25℃ (Fig. 6B, Fig. S3, and Table S1).

204

205 A comparison between 19℃ and 25℃ showed that amino acids gamma-aminobutyric acid
206 (GABA), L-glutamine, L-aspartic acid and L-asparagine were accumulated at 19℃, while
207 L-lysine and L-proline were accumulated at 25℃ (Fig. 6C). Fatty acids glutarate
208 semialdehyde and 5-KETE were accumulated at 19℃, indicating their important roles at
209 low temperature acclimation. Unlike other metabolites, the majority of the differentially
210 expressed purine and pyridine nucleotides, such as IMP, AMP, thiamine, cytosine, inosine,
211 adenosine and adenine, were observed to have accumulated at 19℃ (Fig. 6C and Fig.
212 S4D).

213

214 **DISCUSSION**

215 **Changes in essential resource allocation associated with rapid cell growth when**
216 **temperature increased from 19℃ to 25℃**

217 Temperature is recognized as one of the most important drivers affecting cell growth and
218 the stoichiometry of phytoplankton in global oceans (20). In our study, cell growth rates of

219 *P. shikokuense* gradually increased from 19°C to 25°C (Fig. 1A), accompanied by the
220 increased numbers of up-regulated DEPs involved in photosynthesis-antenna proteins,
221 photosynthesis, translation, carbon fixation, glycolysis/gluconeogenesis, fructose and
222 mannose metabolism, and pentose phosphate pathway (Fig. 2 and Table S1). Among these
223 pathways, abundances of the indicative proteins, such as LHPs, photosystem genes, NT,
224 CA, RBC, and ribosomal proteins increased from 19°C to 25°C (Fig. 6A, 6B and Table
225 S1). These results suggested that *P. shikokuense* actively incorporated and allocated light,
226 CO_2, and nutrients into the necessary macromolecules of carbohydrates, chlorophylls
227 (Chls) and proteins essential for cell division to support rapid cell growth at high
228 temperature. Consistent with the proteomics screening, the majority of identified DEMs
229 belonging to carbohydrates and fatty acids were found to accumulate with increasing
230 temperatures (Fig. 3).
231
232 Cell stoichiometry is the result of cellular resource allocation across different compound
233 classes that vary in their C (carbon), N (nitrogen), and P (phosphate) contents. Generally,
234 the major constituents of polysaccharides, lipids and carbohydrates, Chls, amino acids and
235 proteins, and DNA and RNA are C, N and P, respectively (29). The proteomics and
236 metabolomics results showed that C was mainly allocated to carbohydrates, while N was
237 largely allocated to Chls and proteins with increasing temperatures. Healthy cultures of
238 phytoplankton usually exhibit a Redfield C:N ratio around 106:16 (6.63) (30). To date, the
239 global data still shows no repeatable relationship between temperature and phytoplankton
240 C:N ratios (31, 32). For *P. shikokuense*, the contents of POC and PON per cell decreased
241 from 19°C to 25°C (Fig. 1C). This can be explained by the “temperature-size rule” in
242 phytoplankton that cell body size decreases when temperature increases (31, 33).
243 Meanwhile, the POC/PON ratio of *P. shikokuense* increased and peaked at 25°C with a
244 value of 6.68 (very close to 106:16). This change in POC/PON ratio may be due to the fact
245 that the PON content decreased faster than POC when exposed to higher temperature, and
246 is consistent with the finding of a previous study that diatom cells with smaller size at high
247 temperature may increase photosynthetic efficiency per Chl *a* and boost cellular C per
248 biovolume with low N input (34). Among the detected carbohydrates, L-fucose and
249 mannitol varied largely and accumulated gradually with increasing temperatures (Fig. 6C
250 and Fig. S4B). L-fucose, a common monosaccharide produced by algae, is a major
251 component of fucose-containing sulfated polysaccharides (FCSPs). The recalcitrant
252 FCSPs are known to resist microbial enzymatic degradation and contribute largely to
253 marine C sinks after the diatom blooms (35). On the other hand, mannitol is one of the
254 major photosynthetic products that serve as important C storage and antioxidant in algae
255 (36). Field studies showed significant increases in mannitol in some algae during summer
256 compared with other seasons (37). Taken together, these results suggested that warming
257 may enhance the accumulations of L-fucose and mannitol in marine phytoplankton.

258

259 **Acclimation strategies in response to supra-optimal temperature**

260 The Fv/Fm value is a measure of the maximum quantum efficiency of photosystem II
261 (PSII) and is widely used as a stress indicator in phytoplankton studies (38). We observed
262 that *P. shikokuense* had a similar growth rate but significantly lower Fv/Fm value at 28°C
263 than 25°C (Fig. 1B), suggesting the supra-optimal temperature of 28°C had affected the
264 algal biophysiological activities. Photosystems are particularly sensitive to thermal stress
265 in photosynthetic organisms (39). DEPs in *P. shikokuense* involved in photosynthesis and
266 photosynthesis-antenna proteins were more abundant at 28°C than 25°C (Fig. 5A). Among
267 them, higher expressions of psaB and psaC, which are the essential enzymes for the
268 catalysis of light-induced water oxidation and electron transfer across thylakoid membrane,
269 reinforced photoreaction of *P. shikokuense* to compensate for the reduced quantum
270 efficiency of PSII under thermal stress. Moreover, we observed concomitant increased
271 expression levels of several CABs and LHPs at 28°C (Fig. 5A and Table S1). CABs are
272 one vital component of light harvesting complex (40). In addition to acting as light
273 harvesting agents in dinoflagellates, CABs and LHPs can prevent thermal stress-induced
274 photo-damage in cells through dissipating the excessive heat and protecting the
275 photosynthetic apparatus (41, 42).

276

277 HSPs act as molecular chaperones by preventing the aggregation of misfolded proteins at
278 high temperatures (43, 44). Accumulation of HSPs in various organisms under thermal
279 stress has been widely observed, including the temperate, tropical, and symbiotic
280 dinoflagellates (23, 24, 45). In addition, HSP70 plays an essential role in repairing
281 disassembled PSII core complex through binding to the thylakoid membrane in green
282 algae (46). Higher expression of HSP70 and HSP90 at 28°C may protect the protein
283 structure and ensure PSII stability in *P. shikokuense* (Fig. 5A, Fig. S3A, and Table S1).
284 Also, even moderate thermal stress can disrupt cellular protein homeostasis and
285 reallocation of amino acids, which was evidenced by the boosted protein processing in the
286 endoplasmic reticulum, and a large number of differentially expressed amino acids
287 between 28°C and 25°C (Fig. 2A and 3). Among these amino acids, proline was found to
288 be related to a variety of thermal resistance in numerous plants (47). The finding that
289 higher content of L-proline in *P. shikokuense* at 28°C (than 25°C) (Fig. 5B) implies that
290 L-proline is a key amino acid in thermal protection.

291

292 Carbon metabolism in *P. shikokuense* was negatively affected by the moderate thermal
293 stress at 28°C when compared with 25°C (Fig. 2A). Thermal stress normally modifies
294 carbon metabolism through inhibiting enzyme activities and down-regulating genes
295 expressions (48). In our study, carbon metabolism related-enzymes of pyruvate water
296 dikinase (PPS), transketolase (tktA), methylenetetrahydrofolate reductase (metF), and

297 glycine hydroxymethyltransferase (SHMT) were significantly down-regulated at 28℃
298 than 25℃ (Table S1). The down-regulated carbon metabolism will reallocate organic
299 carbohydrates between polysaccharides and soluble sugars. Since heat stress interrupts
300 intracellular osmotic homeostasis, accumulations of soluble sugars to reduce the negative
301 effects has been observed in plants (49). The enriched mechanisms of fructose and
302 mannose, and galactose in *P. shikokuense* at 28℃ suggested their important roles at heat
303 stress acclimation (Fig. 4A). Thus, the significantly accumulated monosaccharides of
304 L-fucose, mannitol, D-mannose, sorbitol and stachyose that involved in these two
305 processes may function at osmotic adjustment under the moderate thermal stress (Fig. S4B
306 and Table S2).
307

308 **Acclimation strategies in response to sub-optimal temperature**

309 When compared with 25°C, the sub-optimal temperatures of 19°C and 22°C seriously
310 influenced the cell growth and photosystem of *P. shikokuense* (Fig. 1). Since similar
311 expression patterns and enriched pathways were observed when temperature changed at
312 19°C and 22°C relative to 25 °C (Fig. 2 and S2), we focused on the changes in the DEPs
313 and DEMs between 19°C and 25°C to reveal low temperature acclimation strategies.
314 When photosynthetic organisms are exposed to cold stress, the photosynthetic rate
315 decreases and excessive electrons are transferred to O_2 to generate reactive oxygen species
316 (ROS) (50). The antioxidant enzymes and scavengers in plants and algae are normally
317 initiated to reduce the negative effects of excess ROS on proteins, DNA, and lipids (51).
318 The relatively low photosynthetic efficiency and highly expressed antioxidant enzymes of
319 Cu/Zn SOD and CSPs at 19 °C (Fig. 1B and 6B) may imply the excess production of ROS
320 in *P. shikokuense* at this temperature. Alternatively, organisms can over-produce
321 glutathione to relieve oxidative damage caused by ROS (52). In our study, KEGG analyses
322 of both DEPs and DEMs showed that glutathione metabolism was enriched at 19°C. Six
323 essential enzymes of glutamate-cysteine ligase catalytic subunit (GCLC), glutathione
324 S-transferase (GST), glutathione dehydrogenase/transferase (DHAR), isocitrate
325 dehydrogenase (IDH1), 6-phosphogluconate dehydrogenase (PGD), and L-ascorbate
326 peroxidase (APX) (Fig. S3A and Table S1), and three metabolites of glutathione (GSH),
327 glutathione disulfide, and ascorbate were significantly up-regulated at 19°C compared
328 with 25°C (Table S2), indicating their important roles in ROS scavenging at low
329 temperature.
330

331 Since low temperature may damage the plasma membrane, phytoplankton adjust the
332 composition of their plasma membranes to optimize the liquid/crystalline physical
333 structure necessary for proper membrane function (53, 54). Lipids, the major component
334 of algal plasma membrane, are made up of phospholipids, sterols and fatty acids. A series
335 of key proteins involved in glycerophospholipid metabolism, e.g. glycerol-3-phosphate

336 dehydrogenase (GPD1), acetylcholinesterase (ACHE), and 1-acylglycerone phosphate
337 reductase (AYR1), and steroid biosynthesis, e.g. delta14-sterol reductase (TM7SF2),
338 delta24-sterol reductase (DHCR24), sterol 24-C-methyltransferase (SMT1), and
339 cycloartenol synthase (CAS1), were significantly up-regulated at 19℃ compared with 25℃
340 (Fig. S3B and Table S1). Higher expressions of these enzymes reflected accumulations of
341 glycerophospholipid and steroid in plasma membrane to cope with low temperature stress.
342 In addition, the absolute content and relative proportion of saturated and polyunsaturated
343 fatty acids determine the plasma membrane fluidity (55). Previous studies report sharp
344 increases of total and polyunsaturated fatty acids in dinoflagellates during the transition
345 from 30℃ to 15℃ (16), and higher proportions of polyunsaturated fatty acids in
346 cold-adapted dinoflagellates than warm-adapted species (56). Enzymes such as fabD,
347 FASN, ACSL and ACSBG for fatty acid biosynthesis, and enzyme ACAA1 for
348 polyunsaturated fatty acid biosynthesis were more highly expressed at 19℃ than 25℃
349 (Fig. 6B and Fig. S4C), implying the possible greater accumulation of fatty acids in *P.*
350 *shikokuense* at low temperature. Glutarate semialdehyde, a straight chain fatty acid, and
351 5-KETE, a long-chain fatty acid, were largely accumulated at 19℃ (Fig. 6C). Since the
352 profile of fatty acids is seldom reported in phytoplankton especially under high or low
353 temperature stresses, little is known about the exact role of these fatty acids identified in
354 low temperature acclimation. However, it is reasonable to propose that the greater
355 accumulation of these two fatty acids at 19℃ was incorporated into the plasma membrane
356 of *P. shikokuense* to compensate for the reduced membrane fluidity (Fig. 7).
357

358 **Allocation of P and N for bloom occurrence**

359 Spliceosome and mRNA surveillance pathway were significantly enriched at 19℃
360 compared with 25℃ (Fig. 2C). Active alternative pre-mRNA splicing, which is controlled
361 by spliceosome, can be quickly induced to produce protein isoforms under low
362 temperature (57, 58). Hence, enriched spliceosome and mRNA surveillance pathway in *P.*
363 *shikokuense* guaranteed the correctness of gene expression and protein synthesis at low
364 temperature. More nucleotides at low temperature should be required as the spliceosome is
365 mainly composed of five small nuclear RNAs (snRNA). Consistently, the majority of
366 DEPs and DEMs involved in purine and pyrimidine nucleotide metabolism was
367 significantly up-regulated at 19℃ than 25°C (Fig. 6B and 6C). Enzymes such as ADK,
368 IMPDH and guaA, responsible for catalytic conversion of adenosine and AMP to DNA,
369 and udk and pyrG for catalytic conversion of cytosine to RNA were observed (Fig. 7).
370 Indeed, the omics results were supported by a decrease in the content of DNA per cell
371 from 19°C to 25°C (Fig. 1B). The low content of RNA at high temperature was consistent
372 with the “translation compensation hypothesis”, which states that because ribosomal
373 reaction rate increases with temperature, low ribosomal density is needed to sustain the
374 same level of protein synthesis at high temperature (20, 59). Moreover, higher contents of

375 DNA and RNA at 19°C than 25°C may be due to an arrest at G_2 phase of part of the *P. shikokuense* cell population, which needs further investigation. The central compositional element role played by P in nucleic acids (60) indicated that *P. shikokuense* largely allocated P to DNA and RNA at low temperature. The limited P availability in the coastal ECS is one major factor regulating the bloom successions from diatom to dinoflagellate *P. shikokuense* (25). The powerful P remodeling and storage capacities as reflected in the accelerated ATP cycling, the switch from phospholipids to non-phospholipids, and the formation of polyphosphate, are reported to largely contribute to the occurrence of *P. shikokuense* blooms (61, 62). Additionally, the high demand for P and the preferential allocation of P into DNA and RNA at 19°C indicated that a relatively large amount of P was retained in DNA and RNA at low temperature. Once seawater temperature increases, the elemental P in DNA and RNA can be remodeled into other essential compounds for cell growth, and therefore partially relieve the low P stress caused by the diatom blooms.
388
389 The strong metabolic capability of organic compounds, such as amino acids and peptides, is a competitive advantage that facilitates *P. shikokuense* bloom under low inorganic nutrient condition (63). In our study, nearly half of the differentially expressed amino acids such as GABA, L-glutamine, L-aspartic acid and L-asparagine was found to accumulate more at 19°C rather than 25°C (Fig. 6C). Moreover, the contents of L-asparagine, L-glutamine and GABA were included in the top 10 abundant amino acids identified at 19°C (Table S2). Accumulation of amino acids and their derivatives is a common protective mechanism for phytoplankton to relieve low temperature stress (64). The four accumulated amino acids in *P. shikokuense* at 19°C may play the same role. Indeed, greater GABA accumulation at low temperature acclimation has been reported in plants and green algae (65-67). In addition, asparagine is an ideal N storage compound that exhibits a high N:C ratio compared with other amino acids (68). It is synthesized from glutamine and aspartic acid in the ATP-dependent reaction catalyzed by the enzyme AS. AS was observed to be more (although not significantly) highly expressed at 19°C than 25°C. Moreover, NR and NiR which catalyze the reduction of nitrate to ammonia, and GS that further incorporates ammonia into glutamine were more highly expressed (although not significantly) at 19°C than 25°C (Fig. 7 and Fig. S3D). The consistent expression pattern of these enzymes and metabolites suggested that cellular N flux was largely channeled into glutamine, aspartic acid and asparagine at low temperature. Combined with the large amount of PON per cell at 19°C (Fig. 1C), these results indicated that these amino acids may serve as important N storage pools for *P. shikokuense* under low temperature. Once the seawater temperature increases, the stored N within them can be quickly reallocated into other necessary macromolecules (e.g. proteins and Chls) to sustain higher growth rates for bloom occurrence.
413

414 **CONCLUSION**

415 Our study sheds light on the response mechanisms invoked by the dinoflagellate *P.*
416 *shikokuense* to sustain growth under a temperature gradient (19-28°C), which covers the
417 whole bloom period from early to late spring in the coastal ECS. We observed that *P.*
418 *shikokuense* exhibited increasing growth rates from 19°C to 25°C. Along this gradient,
419 pathways of photosynthesis-antenna proteins, porphyrin and chlorophyll metabolism,
420 photosynthesis, carbohydrate metabolism and ribosome were consistently up-regulated.
421 Compared with 25 °C, cells at 28°C endured moderate heat stress as they showed similar
422 growth rates but a lower Fv/Fm value. Proteins involved in photoreaction, light harvesting,
423 and protein homeostasis, such as LHP, CAB and HSP, were more highly expressed to
424 compensate for the negative effects of the moderate heat stress. Soluble sugars such as
425 L-fucose, mannitol, D-mannose, sorbitol and stachyose were largely accumulated at 28°C
426 to compensate for the reduced carbon metabolism and sustain osmotic homeostasis. In
427 addition, metabolites such as glutathione, glutarate semialdehyde, 5-KETE, and GABA
428 were found, which may play important roles in low temperature acclimation. Moreover,
429 the significant accumulation of nucleotides adenosine, cytosine and AMP, and amino acids
430 L-asparagine, L-glutamine and L-aspartic acid at 19°C, may serve as important P and N
431 repositories that can be reallocated into other necessary macromolecules to form large
432 scale blooms when ocean temperature increases.

433

434 Based on the number of macromolecules detected, metabolomics showed a relatively
435 lower identification depth than proteomics. This was mainly caused by the complex
436 physicochemical property of metabolites and the limited phytoplankton reference database
437 for metabolite identification. However, it is interesting to note that the two approaches
438 were complementary and some findings could be confirmed by cross-correlations. Since
439 abiotic factors such as temperature, nutrient and light initially control the growth rate and
440 cell stoichiometry of photosynthetic organisms in global oceans (20), further combined
441 use of proteomics and metabolomics promises a better understanding of how interactions
442 among these abiotic factors affect marine dinoflagellates. Insights gained from such
443 studies will help us to comprehensively understand the community variation and
444 distribution trends of marine dinoflagellates in a warming ocean.

445

446 **MATERIALS AND METHODS**

447 **Microalga isolation and culture**

448 Strain of *P. shikokuense* (CCMA206) was originally isolated from the frequent
449 bloom-occurring coastal ECS in 2014, and was provided by the Collection Center of
450 Marine Algae (CCMA), Xiamen University, China. Batch cultures of *P. shikokuense* were
451 maintained in K medium (69) prepared with 0.22 μm filtered and autoclaved seawater (30
452 psu), and grown under cool white fluorescent light at an irradiance of 100 μmol quanta m^{-2}

453 s^{-1} with a 14:10 h light: dark cycle at 25°C. The stock cultures were maintained at the
454 exponential phase by dilution with fresh medium approximately every six days.
455 Bactericidal penicillin (100 U/mL) and streptomycin (0.1 mg/mL) were added into the
456 stock culture to minimize the growth of bacteria before the commencement of temperature
457 experiments.
458

459 **Experimental design**

460 Triplicated experiments at 19, 22, 25 and 28°C were performed in 2 L acid-washed and
461 autoclaved polycarbonate bottles containing 1.6 L medium. To obtain cells with a stable
462 metabolic activity, *P. shikokuense* at each temperature were cultured and
463 semi-continuously diluted daily for approximately 20 days before sampling. Cell cultures
464 were diluted every morning with fresh medium to an initial cell density of approximately
465 10,000 cells/mL for a diurnal cycle. Sampling was conducted every day consecutively for
466 10 days.
467

468 **Growth rate and thermal trait**

469 Culture suspension (1 mL for each sample), fixed by mixing with Lugol`s solution (5 μL),
470 was counted daily before and after the dilutions with a light microscope. The growth rate
471 was calculated using the equation: $\mu=\ln(N_2/N_1)/(t_2-t_1)$, where N_1 is the cell density after
472 dilution at day 1 (t_1) and N_2 is the cell density before dilution at day 2 (t_2), respectively. A
473 unimodal extension of the Boltzmann-Arrhenius model was performed to determine the
474 optimal growth temperature (70). We added an experiment at 30°C to increase the model
475 accuracy. The data on growth rates at different temperatures were applied to fit the
476 model: $\mu = \mu_0 \frac{e^{\frac{Ea}{k_b}\left(\frac{1}{T_0}-\frac{1}{T}\right)}}{1+\frac{Ea}{E_h-Ea}e^{\frac{Ea}{k_b}\left(\frac{1}{T_{opt}}-\frac{1}{T}\right)}}$, where μ is the specific growth rate at each

477 temperature, μ_0 is a pre-exponential constant independent of temperature, k_b is
478 Boltzmann`s constant (8.62×10^{-5} evK^{-1}), Ea is estimated as the slope of linear regression
479 of the log-transformed rate against the Boltzmann temperature-$1/k_bT$, T_{opt} is the optimal
480 temperature at which the rate reaches the maximum value, and E_h is added to describe the
481 "steepness" of the decrease of the rate when the temperature exceeds T_{opt}.
482

483 **Fv/Fm, DNA, POC and PON measurement**

484 To determine the maximum photochemical efficiency of PSII (Fv/Fm), whole cultures (5
485 mL for each sample) at 19, 22, 25 and 28°C were dark adapted for 15 min and then
486 measured daily using a PHYTO-PAM (Walz GmbH, Effeltrich, Germany). Whole cultures
487 (20 mL for each sample) at the four investigated temperatures were harvested by
488 centrifugation (6,000 g, 10 min, 4°C) at day 4. Cell pellets were suspended in 0.5 mL
489 solution I (Tris–HCl 50 mM, EDTA 50 mM, and sucrose 50 mM; pH 8.0) reagent for

490 DNA extraction. Cell pellets were frozen in liquid nitrogen for 5 min, sonicated in ice, and
491 then DNA extractions were performed following the enzyme/phenol-chloroform
492 extraction protocol (71). DNA concentration was quantified using the NanoDrop 2000
493 (Thermo Fisher Scientific, Wilmington, USA).
494
495 Whole cultures (10 mL for each sample) at the four investigated temperatures were filtered
496 through pre-combusted (500°C, 4 h) 1.6 µm GF/A membranes (diam. 25 mm) at day 4.
497 The filtration membranes were exposed to HCl fumes and oven-dried at 65°C for 24 h to
498 remove inorganic carbon and nitrogen before mass spectrometric (MS) analysis. The POC
499 and PON were determined using a PE 2400 Series II CHNS elemental analyzer (Perkin
500 Elmer, Norwalk, USA). Statistical comparisons of the treatment groups were assessed
501 with one-way ANOVA using SPSS22.0 software (SPSS Inc., Michigan Avenue, Chicago,
502 Illinois, USA).
503
504 **Protein extraction, digestion, peptide labeling and LC-MS/MS analysis**
505 Whole cultures (100 mL for each sample) at 19, 22, 25 and 28°C were harvested by
506 centrifugation (6,000 g, 10 min, 4°C) at day 4. The pelleted cells were immediately frozen
507 in liquid nitrogen and then stored at -80ºC before further processing. Cell pellets (of two
508 of the three biological repeats for each temperature) were suspended in 1 mL Trizol
509 (Invitrogen, Carlsbad, USA) reagent for protein extraction, following the previous
510 protocol (72). Protein concentration was quantified using a 2D Quant kit (GE Healthcare,
511 San Francisco, CA). After adjusting the pH to 8.5 with 1 M ammonium bicarbonate, 100
512 µg protein from each sample was first reduced with DTT (1 h) at 60°C and then alkylated
513 with iodoacetamide (45 min, in the dark) at room temperature. Each sample was digested
514 twice using Trypsin Gold (Promega, Madison, WI, USA) with a protein/trypsin ratio of
515 30:1 (w/w) for 14 h at 37°C. After desalting on a Strata X C18 solid phase extraction
516 column (Phenomenex, Torrance, CA, United States), trypsin-digested samples were
517 evaporated and reconstituted in 0.2 M triethylammonium bicarbonate (TEAB). Desalted
518 peptides of eight samples (two biological repeats for each temperature) were then labeled
519 with iTRAQ reagents 8-plex Kit (Applied Biosystems, Foster City, CA) according to the
520 manufacturer's instructions: Tag113 and Tag114, 19°C; Tag115 and Tag116, 22°C; Tag117
521 and Tag118, 25°C; Tag119 and Tag121, 28°C. After a 2 h incubation, the labeled samples
522 were combined, desalted with a Strata X C18 column (Phenomenex) and then
523 vacuum-dried.
524
525 The peptides were reconstituted with buffer A (5% ACN, pH adjusted to 9.8 with ammonia)
526 to 2 ml, and then were separated on a Shimadzu LC-20AB HPLC Pump system coupled
527 with a high pH RP column. The peptides were separated at a flow rate of 1 mL/min with
528 isocratic 5% buffer B (95% ACN, pH 9.8) for 10 min, a gradient from 5 to 35% buffer B

over 40 min, then 35 to 95% buffer B over 1 min. The system was then maintained at 95% buffer B for 3 min before being decreased to 5% within 1 min and the column was re-equilibrated with 5% buffer B for 10 min. A total of 20 fractions were collected and vacuum-dried.

Each fraction was re-suspended in buffer C (2% ACN and 0.1% FA) and centrifuged at 16,000 g for 10 min. The supernatant was loaded onto a C18 trap column 5 μL/min for 8 min using a LC-20AD nano-HPLC instrument (Shimadzu, Kyoto, Japan) auto-sampler which was interfaced to a Q EXACTIVE mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The peptides were eluted from the trap column and separated by a capillary C18 column (inner diameter 75 μm) packed in-house. The gradient was run at 300 nL/min starting from 8 to 35% of buffer D (98% ACN and 0.1% FA) in 35 min, then going up to 60% in 5 min, then maintained at 80% D for 5 min, and finally returned to 5% in 0.1 min and equilibrated for 10 min. The separated peptides were subject to nano-electrospray ionization and MS DDA (data-dependent acquisition). The parameters for MS analysis were as follows: electrospray voltage: 1.6 kV; precursor scan range: 350-1,600 m/z at a resolution of 70,000 in Orbitrap; MS/MS fragment scan range: >100 m/z at a resolution of 175,000 in HCD mode; normalized collision energy setting: 27%; dynamic exclusion time: 15 s; automatic gain control (AGC) for full MS target and MS2 target: 3e6 and 1e5, respectively; The number of MS/MS scans following one MS scan: 20 most abundant precursor ions above a threshold ion count of 20,000.

Protein identification, quantification and bioinformatics analysis

The raw MS/MS data were converted to MGF files by Proteome Discoverer 1.4 (Thermo Scientific, Waltham, MA) and the exported MGF files were searched using Mascot (v2.3.02, MatrixScience; London, UK) against a database containing translated protein sequences from transcriptomes of *P. shikokuense* pure culture (73). Mascot parameters were set as follows: trypsin was selected as the specific enzyme with a maximum of two missed cleavages permitted per peptide; fixed modifications of carbamidomethyl (C), iTRAQ8-plex (N-term) and iTRAQ8-plex (K); variable modifications consisting of oxidation (M); peptide charge, 2+, 3+, and 4+; 20 ppm of peptide mass tolerance; 0.05 Da of fragment mass tolerance. The automatic Mascot decoy database search was performed. The Mascot results were processed by IQuant utilizing MascotPercolator to re-score the peptide spectrum matches (PSMs) (74). The identified peptide sequences were assembled into a set of confident proteins using the Occam's razor approach implemented in IQuant, and the false discovery rate (FDR) at 1% was set in both PSM and protein levels. For this study, high-confidence proteins containing at least one unique peptide and two unique spectra were chosen and DEPs (differentially expressed proteins) were filtered with the cutoffs of fold ratios ≥ 1.2 or ≤ 0.83 and p-value < 0.05. Functional annotations were

568 performed against the database of NCBI non-redundant protein (NCBInr) and Kyoto
569 Encyclopedia of Genes and Genomes (KEGG), and KEGG enrichment of DEPs was
570 performed using the R package GSEA.
571

572 **Metabolite extraction and LC-ESI/MS analysis**

573 Biological activity of the cultured *P. shikokuense* cells (100 mL for each sample) was
574 firstly quenched by adding 50 μL $HgCl_2$ saturated solution. Whole cultures at 19, 25 and
575 28℃ were harvested by centrifugation (6,000 g, 10 min, 4℃) at day 4, immediately frozen
576 in liquid nitrogen and then stored at -80ºC. Cell pellets (six repeats for each temperature)
577 were suspended in 2 mL centrifuge tube with 1 mL extraction buffer (acetonitrile:
578 methanol: water; 2:2:1, v/v/v) and 100 mg glass beads. The centrifuge tubes were allowed
579 to freeze in liquid nitrogen for 5 min and then thawed at room temperature. The thawed
580 samples were then ground at 55 Hz for 2 min (the freeze-thaw-grind cycle was repeated 3
581 times). Following another round of centrifugation (12,000 g, 10 min, 4℃), the supernatant
582 was transferred to a new tube, vacuum-dried and re-dissolved in 300 μL
583 2-amino-3-(2-chloro-phenyl)-propionic acid (4 ppm). The solution was then filtered
584 through a 0.2 μm (PALL Life Sciences, USA) membrane prior to LC-MS/MS analysis.
585

586 LC-MS/MS analysis was conducted on a Vanquish UHPLC System (Thermo Fisher
587 Scientific, USA) using an ACQUITY UPLC ® HSS T3 (150×2.1 mm, 1.8 μm) (Waters,
588 Milford, MA, USA). The column was maintained at 40℃. The flow rate and injection
589 volume were set at 250 μL/min and 2 μL, respectively. For LC-ESI (+)/MS analysis, the
590 mobile phases consisted of buffer E (0.1% formic acid in CAN) and buffer F (0.1% formic
591 acid in water). Separation was conducted under the following gradient: 2% E, 0-1 min;
592 2-50% E, 1-9 min; 50-98% E, 9-12 min; 98% E, 12-13.5 min; 98-2% E, 13.5-14 min, 2%
593 E, 14-20 min. For LC-ESI (-)-MS analysis, the analysis was carried out with mobile
594 phases of ACN and ammonium formate (5mM). Separation was conducted under the
595 following gradient: 2% ACN, 0-1 min; 2-50% ACN, 1-9 min; 50-98% ACN, 9-12 min; 98%
596 ACN, 12-13.5 min; 98%-2% ACN, 13.5-14 min; 2% ACN, 14-17 min.
597

598 Mass spectrometric detection of metabolites was performed on Q Exactive (Thermo Fisher
599 Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2
600 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows:
601 sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and -2.50 kV for
602 ESI(+) and ESI(-), respectively; capillary temperature, 325℃; MS1 range, m/z 81-1000;
603 MS1 resolving power, 70000 FWHM; number of data dependent scans per cycle, 10;
604 MS/MS resolving power, 17500 FWHM; normalized collision energy, 30%; dynamic
605 exclusion time, automatic.
606

607 **Metabolite identification, quantification and bioinformatics analysis**
608 The raw data were converted to mzXML format by MSConvert in the ProteoWizard
609 software package (v3.0.8789) (75) and processed using XCMS for feature detection,
610 retention time correction and alignment. The metabolites were identified by mass accuracy
611 (<30 ppm) and MS/MS data which were matched with HMDB, Massbank, LipidMaps,
612 mzclound and KEGG. The robust LOESS signal correction (QC-RLSC) was applied for
613 data normalization to correct for any systematic bias. After normalization, only ion peaks
614 with relative standard deviations (RSDs) less than 30% in QC were kept to ensure proper
615 metabolite identification.
616
617 All the multivariate data analyses were performed using Ropls software (76). After scaling
618 data, models were built on principal component analysis, orthogonal partial least-square
619 discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA).
620 The metabolite profiles were visualized as score plot with each point representing a
621 sample. The corresponding loading plot and S-plot were generated to provide information
622 on the metabolites that influence clustering of the samples. All the models evaluated were
623 tested for over fitting with methods of permutation tests. The descriptive performance of
624 the models was determined by R2X (cumulative) (perfect model: R2X (cum) = 1) and
625 R2Y (cumulative) (perfect model: R2Y (cum) = 1) values while their prediction
626 performance was measured by Q2 (cumulative) (perfect model: Q2 (cum) = 1) and a
627 permutation test. The permuted model was applied to predict classes: R2 and Q2 values at
628 the Y-axis intercept must be lower than those of Q2 and the R2 of the non-permuted model.
629 OPLS-DA allowed the determination of discriminating metabolites using the variable
630 importance on projection (VIP). The *p*-value, VIP and fold change (FC) were applied to
631 discover the contributable-variable for classification. Finally, p-value < 0.05 and VIP
632 values > 1 were considered to be statistically differentially expressed metabolites (DEMs).
633 The identified metabolites were functionally annotated to the KEGG database, and
634 pathway enrichment and topology analyses of DEMs were performed using
635 MetaboAnalyst (77).
636 **Data availability** The mass spectrometry proteomics data were deposited to the
637 ProteomeXchange Consortium via the PRIDE partner repository with the dataset
638 identifier PXD033681.
639

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650
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652
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898 **FIGURE LEGENDS**

899

900 **FIG 1** The physiological responses of *Prorocentrum shikokuense* at different temperatures.
901 Growth rate (A), Fv/Fm value and DNA content (B), POC and PON content, and
902 POC/PON ratio (C). POC: particulate organic carbon, PON: particulate organic nitrogen.

903

904 **FIG 2** KEGG enrichment of differentially expressed proteins (DEPs) at 28℃ vs 25℃ (A),
905 22℃ vs 25℃ (B), and 19℃ vs 25℃ (C). Functional categories of DEPs are grouped at
906 KEGG level 3. GSEA-derived normalized enrichment scores (NES) for DEPs are shown.
907 A positive NES of 22℃ vs 25℃ indicates that the pathway was highly enriched at 22 ℃,
908 while a negative NES of 22℃ vs 25℃ indicates that the pathway was highly enriched at
909 25℃. p.adjust < 0.05 indicates a significant enrichment.

910

911 **FIG 3** Classification and distribution of differentially expressed metabolites (DEMs)
912 between temperature comparisons. “a vs b” means a compared with b.

913

914 **FIG 4** The top 15 enriched KEGG terms of differentially expressed metabolites (DEMs)
915 at 28℃ vs 25℃ (A) and 19℃ vs 25℃ (B). Red numbers with plus signs and blue
916 numbers with minus signs indicate up-regulated and down-regulated DEMs, respectively.

917

918 **FIG 5** Color-coded scatter plots of $\log_2$-fold change in protein (A) and metabolite (B)
919 abundances at 28℃ vs 25℃. “a vs b” means a compared with b. Functional categories of
920 differentially expressed proteins (DEPs) are grouped at the KEGG level 2.

921

922 **FIG 6** Color-coded scatter plots of $\log_2$-fold change in protein abundance at 22℃ vs 25℃
923 (A), and 19℃ vs 25℃ (B), and $\log_2$-fold change in metabolite abundance at 19℃ vs 25℃
924 (C). “a vs b” means a compared with b. Functional categories of differentially expressed
925 proteins (DEPs) are grouped at the KEGG level 2.

926

927 **FIG 7** Schematic illustration of the key differentially expressed proteins (DEPs) and
928 differentially expressed metabolites (DEMs) in *Prorocentrum shikokuense* involved in
929 nutrient storage and stress acclimation at 19℃. Red and green colors represent the
930 up-regulated and down-regulated levels at 19℃ compared with 25℃.