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Physiological and molecular responses of invasive cyanobacterium *Raphidiopsis raciborskii* to ambient phosphorus deficiency*

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Abstract *Raphidiopsis raciborskii* can cause harmful cyanobacterial blooms when concentrations of environmental phosphorus (P) are very low, thus the physiological and molecular mechanisms involved in the acclimation to P need to be characterized better. The growth, chlorophyll fluorescence, alkaline phosphatase, and expression of genes directly involved in P assimilation were compared in the *R. raciborskii* FACHB 1496 strain grown with and without inorganic P. The specific growth rate (μ), Chl *a*, and six fluorescence parameters (minimal fluorescence (F_0), maximal fluorescence (F_m), maximal variable fluorescence (F_v), electron transport flux (further than Q_A) per RC (ET_0/RC), quantum yield of the electron transport in PSII ($\emptyset_{E0}$), and the probability that an electron from a trapped exciton is moved into the electron transport chain beyond Q_A^- (ψ_0)) markedly decreased in *R. raciborskii* in response to experimental P-deficiency. In contrast, the relative variable fluorescence at the J-step (V_J), trapped energy flux (leading to Q_A reduction) per RC (TR_0/RC), and alkaline phosphatase activity significantly increased. In addition, gene expressions involved in the alkaline phosphatase (*phoA1* and *phoA2*), high-affinity inorganic P transporter (*pstS1*), phosphonate transporter and metabolism (*phnD* and *phnM*), and nucleotidase (*nucH*) were significantly upregulated under P deficiency. However, physiological and molecular responses were resumed rapidly after P re-supplementation following P-deficient conditions. Our results highlight that *R. raciborskii* can perform coordinated and complex cellular and physiological responses to cope with P deficiency, reflecting *R. raciborskii*'s multi-faceted machinery to respond to environmental P fluctuations.

Keyword: phosphorus deficiency; *Raphidiopsis raciborskii*; gene expression; chlorophyll fluorescence; alkaline phosphatase

1 INTRODUCTION

As an essential nutrient, phosphorus (P) is not only involved in cellular structure and metabolic processes (Karl, 2014; Lin et al., 2016), but influences the growth and proliferation of phytoplankton (Dyhrman, 2016). A shift in the phytoplankton community towards cyanobacteria dominance occurs when freshwater bodies are enriched with phosphorus (Burford et al., 2014; Chislock et al., 2014; Wan et al., 2019). Therefore, P availability is hypothesized to be responsible for the survival and vitality of phytoplankton, as well as the proliferation of cyanobacteria (Wu et al., 2000; Young et al., 2010; Li et al., 2016).

Phosphorus supply, however, is affected by redox-dependent P retention in sediments (Welch and Cooke, 1995), land use types in watersheds (Downing and McCauley, 1992; Carpenter et al., 1998), and the food web structure of lakes and streams (Elser et al., 1988). As a result, these lakes and streams often have very low P availability (Nausch et al., 2004). For example, during the summer months in temperate

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lakes, dissolved inorganic phosphorus (DIP), a preferred form of P to phytoplankton, often fails to meet the demand of phytoplankton growth (Smith, 1983; Wu et al., 2000; Karl, 2014). To survive under low-P conditions, phytoplankton has developed a series of encoding regulatory mechanisms to control the uptake, storage, and metabolism of phosphate (Su et al., 2007; Tetu et al., 2009; Liu et al., 2015; Luo et al., 2017; Shi et al., 2017; Zhang et al., 2019). Harke et al. (2012) found that genes involved in high-affinity phosphate-binding proteins and putative alkaline phosphatase were significantly upregulated (50- to 400-fold) when *Microcystis aeruginosa*, a bloom-forming cyanobacterium, was cultured under low inorganic phosphate conditions. Additionally, a set of genes involved in high-affinity phosphate uptake and dissolved organic phosphate (DOP) hydrolysis, e.g., the P-specific transport (*Pst*) system (Vershinina and Znamenskaya, 2002), P-inorganic transport (*Pit*) system (Vershinina and Znamenskaya, 2002; Ilikchyan et al., 2009), and DOP hydrolysis genes, *phoX* and *phoA* (Moore et al., 2005; Wu et al., 2007; Sebastian and Ammerman, 2009) have been reported to cope with P deficiency. These findings suggest phytoplankton can efficiently transport phosphate and exploit organic phosphate sources to adapt to P-deficient environments. Wan et al. (2019) has suggested that different cyanobacterial species, i.e., *Microcystis* and *Dolichospermum*, have distinct responses to P availability. Therefore, molecular patterns presented by different cyanobacteria in response to P availability will help understand the variation of P-acclimation mechanisms. Owing to genome data scarcity, however, studies regarding molecular responses to P have mostly focused on a few marine and even fewer freshwater cyanobacteria (Vershinina and Znamenskaya, 2002; Su et al., 2003; Tetu et al., 2009; Harke et al., 2012).

Raphidiopsis raciborskii is an invasive and notorious cyanobacterial species because it has recently become prevalent in temperate regions (Padisák, 1997; Saker and Griffiths, 2001; Burford et al., 2016). Wu et al. (2009) and Posselt et al. (2009) showed that P availability had an essential role in the occurrence and invasive behavior of *R. raciborskii*. For example, its high DIP uptake affinity and P storage capacity (Isvánovics et al., 2000; Willis et al., 2017), as well as superior ability to utilize DOP (Bai et al., 2014, 2020), may help *R. raciborskii* outcompete other species. Several studies found that *R. raciborskii* could grow under conditions of low and/or variable P availability (Piccini et al., 2011; Wu et al., 2012; Prentice et al., 2015; Willis et al., 2015, 2019; Guedes et al., 2019; Xiao et al., 2020), reflecting that the ability to adapt to low and variable P supply might also be a crucial factor driving the dominance of *R. raciborskii* (Amaral et al., 2014). Although previous studies indicated that this cyanobacterium could efficiently regulate its physiological metabolism to adapt to ambient inorganic phosphate availability (Wu et al., 2012; Willis et al., 2015, 2019; Guedes et al., 2019; Xiao et al., 2020), the adaptive mechanisms, especially in molecular responses, to ambient P deficiency, are not well characterized in *R. raciborskii*.

Genomic comparisons of nine co-occurring *Raphidiopsis* strains from Lake Wivenhoe, Queensland, Australia, revealed that the core genome content is 85% similar and is much conserved in the main P metabolic pathways among the nine strains (Sinha et al., 2014; Willis et al., 2018). However, Willis et al. (2019) showed that two strains of *R. raciborskii*, CS-505 and CS-506, showed a marked difference in the gene expression response to P-free conditions, which suggests that the different responses to P might result in diverse ecotypes (Burford et al., 2018; Guedes et al., 2019). Therefore, the molecular mechanisms responding to P acclimation in the diverse ecotypes of *R. raciborskii* need to be studied from different regions. Posselt et al. (2009) indicated that the cell concentrations and biovolume of *R. raciborskii* significantly increased with periodic pulsed addition of DIP. Amaral et al. (2014) also found that the growth rate increased by 2–3-fold due to multiple pulses of DIP relative to a single pulse. These findings have highlighted the ability of *R. raciborskii* to use pulsed P supplies supports its succession and dominance in lakes and reservoirs. However, little information about the molecular responses to P-Pulse conditions has been obtained for *R. raciborskii* exposed to P deficiency. Here, we hypothesized that (i) P deficiency affects the growth, photosynthesis, and gene expression in *R. raciborskii*; (ii) *R. raciborskii* can recover rapidly to grow when P was resupplied; and (iii) genes encoding phosphate uptake and transporters are upregulated significantly under P-deficiency condition, whereas rapidly downregulated transcriptions occur when P is resupplied. In the present study, therefore, the growth, chlorophyll fluorescence, alkaline phosphatase activity, and gene expression in *R. raciborskii* were characterized to reveal the adaptation strategy

Table 1 Formulae and description of Chl-*a* fluorescence parameters in this study (Strasser and Strasser, 1995)

Term	Formula	Description
Technical fluorescence parameter	$F_0 \cong F_{20\,\mu s}$	Minimal Chl-*a* fluorescence, when all reaction centers in PSII are open
	F_m	Maximal Chl-*a* fluorescence, when all reaction centers in PSII are closed
	$F_v \equiv F_m - F_0$	Maximal variable Chl-*a* fluorescence
	$V_J = (F_J - F_0)/(F_m - F_0)$	Relative Chl-*a* fluorescence at the J-step
Specific energy fluxes (per Q_A^- reducing PSII reaction center-RC)	$ABS/RC = (M_0/V_J)/(1 - F_0/F_m)$	Absorption flux per reaction centers
	$TR_0/RC = M_0/V_J$	Energy flux trapped (resulting in Q_A reduction) in each reaction center
	$ET_0/RC = M_0(1/V_J)(1 - V_J)$	Flux of electron transport (further than Q_A) in each reaction center
Flux ratios or yields	$\varnothing_{P0} = TR_0/ABS = 1 - F_0/F_m$	Maximum quantum yield for the primary photochemistry in PSII
	$\varnothing_{E0} = ET_0/ABS = (1 - F_0/F_m)(1 - V_J)$	Quantum yield for the electron transport in PSII
	$\psi_0 = ET_0/TR_0 = 1 - V_J$	Probability that an electron from a trapped exciton is moved into the electron transport chain further than Q_A^-

F_0 and F_J ($F_{2\,ms}$) are the fluorescence intensity at 50 μs and 2 ms, respectively. F_m is the maximum fluorescence intensity. ABS, RC, M_0, TR_0, and ET_0 are the photon flux absorption, reaction centers, energy flux trapping, flux of electron transport, and normalized initial slope of the fluorescence transient, respectively.

of *R. raciborskii* to ambient P deficiency and the response mechanisms to P-re-supplementation.

2 MATERIAL AND METHOD

2.1 Culture conditions and experimental design

Raphidiopsis raciborskii FACHB 1496 was selected as the experimental alga and was cultured in a liquid CT medium (Ichimura, 1979) under 30 μE/(m^2·s) white light intensity at 25±1 °C, with a 12-h:12-h light:dark cycle. Cells were harvested in the logarithmic growth phase, washed twice with sterile P-free CT medium, and inoculated in a fresh P-free CT medium for five days to exhaust intracellular P (Bai et al., 2014).

The experiment included two treatments, each in triplicate, with an initial cell density of 2×10^6 cells/L. At the beginning of the experiment, medium with 0- and 0.6-mg/L K_2HPO_4 were used for the P-deficient (-P) and P-replete (+P) treatment, respectively. The cells were harvested at 0, 24, 72, 120, and 144 h to monitor Chl *a*, alkaline phosphatase activity, orthophosphate concentrations, and RNA expression. After 144 h of culture, 0.6-mg/L K_2HPO_4 was added to both treatments to determine how phosphorus replenishment affected the parameters mentioned above after another 24 h.

2.2 Alkaline phosphatase and Chl-*a* detection

ELF®97 phosphate dye (ELFP, USA) was used for the microscopic detection of external phosphatases in the strain. The dye method for external phosphatase detection followed the protocol of Wu et al. (2009). In brief, the cells were incubated into the ELFP solution (final concentration 27 mmol/L) at darkness and 25 °C for 4 h, followed by filtration with 0.22-μm pore size filters, and 2–3 times washing. Samples were observed with a fluorescence microscope with UV-excitation (Nikon, Japan). The activities of alkaline phosphatase were determined according to the method of Shen and Song (2007) using *p*-nitrophenyl phosphate (*p*NPP, USA) as a substrate. Chl *a* was extracted with 90% acetone and measured spectrophotometrically according to Nusch (1980). Based on the cell density, the specific growth rate (μ) was analyzed according to the following equation: $\mu = (\ln C_{t_2} - \ln C_{t_1})/(t_2 - t_1)$, in which C_{t_2} and C_{t_1} are cell densities at t_2 (24 h) and t_1 (0 h) for the -P treatment, t_2 (72 h) and t_1 (24 h) for the +P treatment, and t_2 (168 h) and t_1 (144 h) for the P-added treatment.

2.3 PSII fluorescence measurement

A Plant Efficiency Analyzer (PEA, UK) was used to conduct Chl-*a* fluorescence assays with a 3 000 μmol photons (photosynthetically active radiation, PAR) /(m^2·s) actinic light after all samples were dark-adapted for 20 min. The fluorescence signals were recorded according to Yang et al. (2020). Each transient was analyzed according to the JIP-test using the following original data: F_0, $F_{300\,\mu s}$ (F_K), $F_{2\,ms}$ (F_J), and $F_{30\,ms}$ (F_I) defined as the fluorescence intensity at 20 μs, 300 μs, 2 ms, and 30 ms, respectively, as well as the maximum fluorescence intensity (F_m) (Strasser and Strasser, 1995). The parameters selected for the quantification of PSII activity are shown in Table 1.

2.4 DNA extraction, amplification, and sequencing

Total genomic DNAs of *Raphidiopsis* were

Table 2 Primer sequences and product sizes for quantitative PCR reactions in this study

	Primer 5′→3′	Product size (bp)	Efficiency (%)	Function
16sR 16sF	AGAAAAGAGGTTTACGACCCAAGAGC TGAAAGATTTATTGCCTGGAGATGAGC	267	101	16S rDNA
phoA1R phoA1F	CCGCTGCAAATACTAATCCAAACAAT TCACCAGGAGCCAAAGGACGAA	295	105	Alkaline phosphatase
phoA2R phoA2F	CACTTCGCTTTTGCTCGCTTTGT CAATCGGAGTGTTTCCCGCTTCA	199	98	Alkaline phosphatase
nucHR nucHF	CTTCACCACCGATGGCACGAC CCCCATAGGCGGAGCCAAGT	199	99	Endonuclease phosphatase
pstSR pstSF	CAAACCATTGCGGGCACAGTAGAT TACCGCTACCATCCGCACGAACT	292	97	High affinity inorganic phosphate binding protein
phnMR phnMF	GCTGCCAATTCCGCATGACGTT TGGGGCCACAGGTGAAGCTTTTA	172	98	Phosphonate metabolism protein
phnDR phnDF	GAACTTCGGTATTATTTCCACAGA AATCAGATGAGCATAGTAACCTTTT	273	97	Phosphonate ABC transporter

extracted according to Wu et al. (2010). The PCR primers designed for seven genes are listed in Table 2. PCR was carried out in a PTC-100 thermal cycler (MJ Research Inc., USA). Fifty-microliter volume (containing 5–10-ng DNA, 1-U *Taq* DNA polymerase (TaKaRa, Japan), 10 pmol of each primer, and 200 μmol/L of each deoxyribonucleoside triphosphate) was amplified with the program of 94 °C for 5 min, 35 cycles (94 °C for 40 s, 60 °C for 50 s, and 72°C for 2 min), and 72 °C for 5 min. The amplification products were purified and then cloned using a pGEM-T vector (Promega, USA) based on the protocol of the manufacturer. An ABI 3730 Automated Sequencer (Perkin-Elmer Biosystems, USA) was used to sequence all products.

2.5 RNA isolation and reverse transcription

Pelleted cells harvested by centrifuging at 7 000 r/min were re-suspended in Trizol reagent (Invitrogen, USA). Total RNAs were extracted following the Trizol reagent manual (Invitrogen, USA) after pellets were homogenized with a mini-bead beater. RQ1 RNase-free DNase (Promega, USA) was used to digest total RNAs. The random primers $p(dN)_9$ and a reverse transcriptase kit (Generay, China) were employed to reverse-transcribe DNase-treated RNA to cDNA.

2.6 Quantitative real-time PCR

Final volumes of 20 μL including 0.2 μL (10 pmol/μL) of both forward primer and reverse primer (Table 2), 1-μL cDNA, 10-μL Master Mix (SYBR Green, TOYOBO, Japan), and 8.6-μL ddH_2O, were used in the amplification reactions of quantitative real-time PCR with an iCycler Iq (Bio-Rad, Hercules, CA). The amplification conditions were 95 °C for 3 min, followed by 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. The expression levels of target genes from quantitative real-time PCR (QPCR) were evaluated using the C_t value (Livak and Schmittgen, 2001) by the normalization of the housekeeping gene 16S rRNA. The fold change of transcription was calculated using the $\Delta\Delta C_t$ method, where $\Delta\Delta C_t = C_{t,\text{ target gene}} - C_{t,\text{ 16S}}$. Samples were processed in triplicate reactions. Amplification efficiencies of 90%–110% and a ΔC_t of the two slopes less than 0.1 were considered acceptable.

2.7 Statistical analysis

The Chl-*a* concentration, fluorescence parameters, alkaline phosphatase activity, and gene transcription of *R. raciborskii* under -P and +P treatments were evaluated by paired *t*-tests. All Data were analyzed using one-way analyses of variance (ANOVA). The ANOVAs were performed by pairwise multiple comparisons using least significant differences (LSD) analysis. Significance levels of $P<0.05$ were adopted for all tests. Before running statistical analyses, gene transcriptions were normalized relative to the housekeeping gene 16S rRNA and all data were tested for homogeneity of variance and normal distribution of residuals. All analyses and graphs were performed in SPSS version 26.0 (IBM, USA) and Origin version 2021 (Origin Lab Corporation, USA).

3 RESULT

3.1 Growth experiment

The specific growth rate (μ) of *R. raciborskii* was

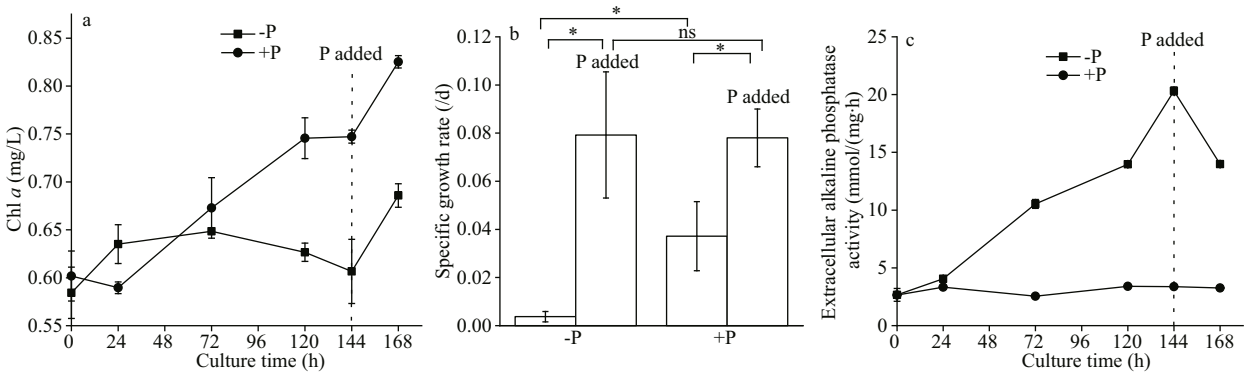

Fig.1 Chl *a*, specific growth rate (μ), and alkaline phosphatase activity (APA) responses of *R. raciborskii* to changing ambient phosphorus

a. Chl-*a* concentration; b. specific growth rate (μ); c. alkaline phosphatase activity (APA). The dotted line indicates the times K_2HPO_4 (DIP) was resupplied. *: significance at 0.05 level; ns: no significance.

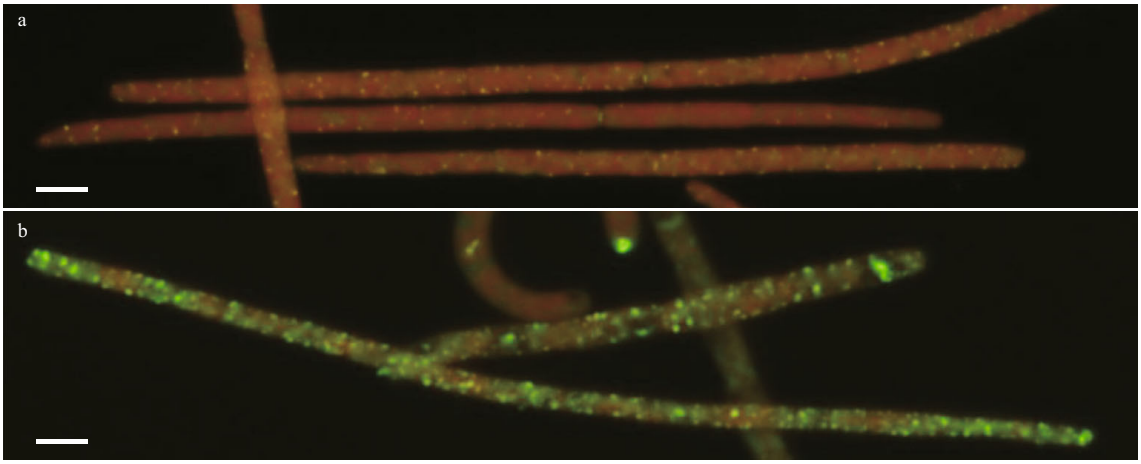

Fig.2 ELF-APA labeling of filaments of *R. raciborskii* cultured at +P and -P conditions

a. +P condition; b. -P condition. External phosphatase was dyed for green fluorescence. Scale bars: 20 μm.

significantly lower in -P than in +P treatments, and Chl *a* was significantly higher in the +P treatment (Fig.1a & b) (ANOVA, $P<0.05$). When P was added at the end of the experiments, both treatments showed comparable Chl-*a* responses, and the significant differences in absolute Chl-*a* levels between both treatments remained.

3.2 Alkaline phosphatase activities (APAs) and Chl-*a* fluorescence

After 144 h, a significant difference was found in alkaline phosphatase activity between the -P and +P treatment (Fig.1c). After 144 h, APAs in -P treatment were significantly higher (7.32- and 6.01-fold) than those at 0 h and in the +P treatment at 144 h, respectively ($P<0.05$). When phosphorus was added to the -P treatment at the end of the experiment, APAs showed a remarkable decrease. However, APAs did not significantly respond to the P addition in the +P treatment ($P>0.05$). In the +P treatment, only red chlorophyll auto-fluorescence was determined in the filaments (Fig.2a). However, predominantly green fluorescent products were observed in filaments when *R. raciborskii* was labeled by ELF in the -P treatment (Fig.2b).

After 144-h culture, Chl-*a* fluorescence parameters of *R. raciborskii* showed significant differences between treatments (Table 3). Technical fluorescence parameters, such as minimal fluorescence (F_0), maximal fluorescence (F_m), and maximal variable fluorescence (F_v), had significantly lower values in -P treatments than in +P treatments ($P<0.05$). Similar trends were observed for the flux of electron transport (further than Q_A) in each reaction center (ET_0/RC), quantum yield for the electron transport ($\varnothing_{E0}$) of PSII, and the probability that an electron from a trapped exciton is moved into the electron transport chain further than Q_A^- (ψ_0) (ANOVA, $P<0.05$). However, significantly higher values of relative variable fluorescence at the J-step (V_J) and trapped energy

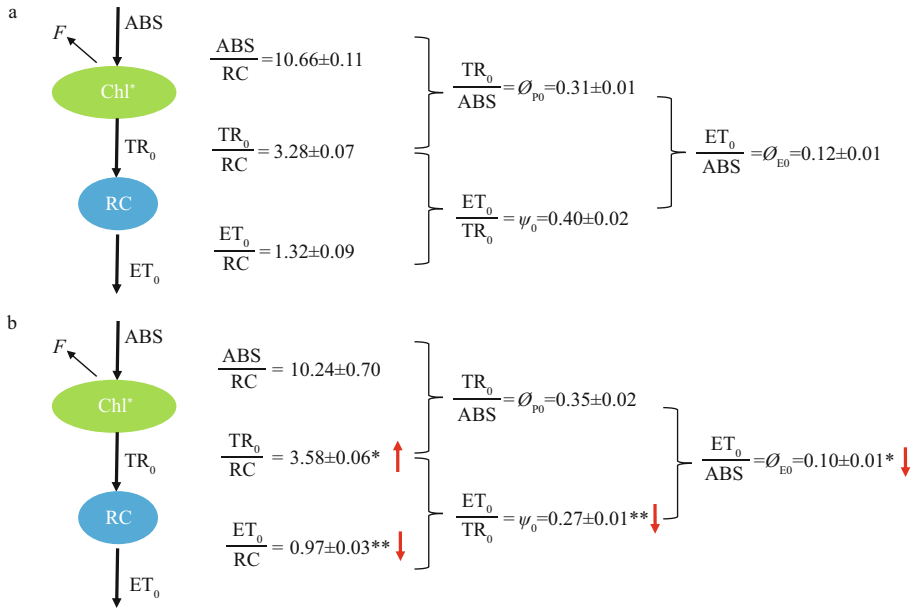

Fig.3 A simple energy flux model of PSII in *R. raciborskii* cultured at +P and -P conditions

a. +P condition; b. -P condition. Red arrows indicate a significant change in the parameter.

flux (leading to Q_A reduction) per RC (TR_0/RC) were found in -P than in +P treatments. In a simple energy flux model (Fig.3), a markedly lower energy flux was observed in -P than in +P treatments (P<0.05).

3.3 Gene responses to P deficiency

The expression of six genes involved in the phosphorus assimilation process was examined after 0, 24, 72, and 144 h in the -P and +P treatment. The expression of *phoA1* encoding alkaline phosphatase was significantly upregulated in -P compared to +P treatments at all sampling times (P<0.01; Fig.4a). The expressions at 24, 72, and 144 h in the -P treatment were 15-, 19-, and 54-fold higher than 0 h, respectively.

Moreover, the expressions of *phoA1* were 7.5-, 6.46-, and 2.70-fold higher in -P treatments at 24, 72, and 144 h, respectively, than those in +P treatments. However, the expression at 168 h decreased significantly in the -P treatment after phosphorus had been added to the medium at 144 h (P<0.01; Fig.4a), and the gene expression showed no significant difference between -P and +P treatments (P>0.05; Fig.4a). *PhoA2* encoding alkaline phosphatase-like in the -P treatment was significantly upregulated at 72 and 144 h (P<0.01; Fig.4b). The expressions of *phoA2* at 72 and 144 h were 1.72 and 1.76 times higher than those at 0 h in the -P treatments, while the expressions of *phoA2* in the -P treatment at 72 and 144 h were 1.55 and 1.74 times higher than those in the +P treatment, respectively.

Significantly higher expressions in the genes

Table 3 Different responses of Chl-*a* fluorescence parameters in *R. raciborskii* to presence and absence phosphorus in 144-h culture

Parameter	+P	-P
Technical fluorescence parameters		
F_0	1 323.33±47.89	347.67±28.47**
F_m	1 948.33±56.26	535.33±29.79**
F_v	625.00±12.99	187.67±84.64**
V_J	0.58±0.01	0.73±0.01**
Specific energy fluxes (per Q_A-reducing PSII reaction center-RC)		
ABS/RC	10.66±0.11	10.24±0.70
TR_0/RC	3.28±0.07	3.58±0.06*
ET_0/RC	1.32±0.09	0.97±0.03**
Flux ratios or yields		
$Ø_{P0}$	0.31±0.01	0.35±0.02
$Ø_{E0}$	0.12±0.01	0.10±0.01*
$ψ_0$	0.40±0.02	0.27±0.01**

*: P<0.05; **: P<0.01.

pstS1 encoding a high-affinity inorganic phosphate-binding protein, *phnD* encoding a phosphonate ATP-binding cassette (ABC) transporter, *phnM* encoding a phosphonate metabolism protein, and *nucH* encoding the endonuclease phosphatase were found in *R. raciborskii* in the -P treatment at 24 to 144 h than at 0 h (P<0.01; Fig.4c–f). Moreover, the -P treatment exhibited general increases in gene expressions of *pstS1* and *phnD* over time compared to the +P treatment (P<0.01; Fig.4c–d). However,

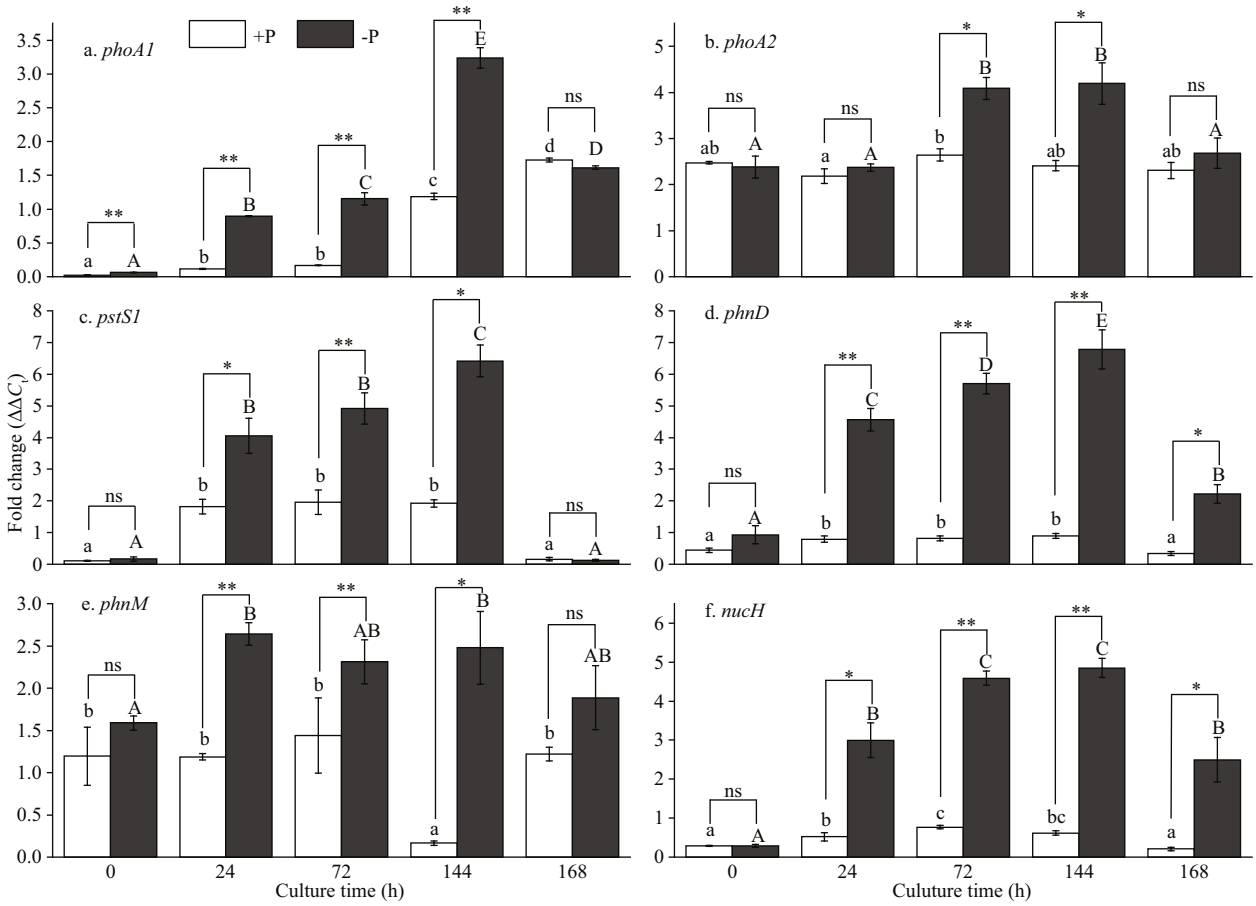

Fig.4 Relative quantitative gene expression for phosphorus metabolism genes encoding alkaline phosphatase (*phoA1* and *phoA2*), high-affinity phosphate binding proteins (*pstS1*), phosphonate transporter and metabolism (*phnD* and *phnM*), and nucleotidase (*nucH*)

*: significance at 0.05 level; **: significance at 0.01 level. The different capital and lowercase letters indicate significant difference among the controls and the treatments, respectively. ns, no significance. 168 h is the time when K_2HPO_4 (DIP) was resupplied.

the genes *phnM* and *nucH* showed a constant, high expression level at 72 and 144 h relative to 0 h (P<0.01; Fig.4e–f). Compared with the +P treatment, the gene expressions of *pstS1*, *phnD*, *phnM*, and *nucH* were markedly upregulated in the -P treatment (P<0.05, Fig.4c–f). However, the expressions at 168 h decreased significantly in the -P treatment when phosphorus had been added to the cultures at 144 h (P<0.01, Fig.4c–f).

4 DISCUSSION

4.1 Physiological responses of *R. raciborskii* to P variations

Phosphorus is a critical factor controlling the physiological ecology of phytoplankton (Lin et al., 2016; Zhang et al., 2018). However, dissolved inorganic phosphorus (DIP) in many waters often falls below the level limiting the growth of phytoplankton (Dyhrman, 2016). In the present study, P-depleted cultures grew slower and entered the stationary phase earlier than the P-enriched cultures (Fig.1a–b), supporting previous studies (Wu et al., 2012; Bai et al., 2014). Similar results were presented by Guedes et al. (2019), who found that five *R. raciborskii* strains could grow under P deficiency for 10 d by reductions in growth rate and Chl-*a* yield, suggesting that internally stored phosphorus, i.e., polyphosphate, might have been used during the 10-d endurance of P deficiency (Guedes et al., 2019). Droop (1973) proposed that the storage could be expended theoretically for 3–4 generations when grown under P-depleted conditions. In our experiment, the culture P-depleted for 5 d probably was not depleted enough to exhaust intracellular P, which resulted in a slight increase in Chl *a* in 24 h, while a significant difference was observed between -P and +P treatments.

The production of extracellular phosphatases is one possible mechanism allowing cyanobacteria to overcome P limitation (Dyhrman et al., 2012; Lin et al.,

2012; Zhang et al., 2016; Shi et al., 2017). Under the P-deficient culture conditions, alkaline phosphatase activities were markedly elevated (Fig.1c). After P supplementation to previously limited cultures, alkaline phosphatase activity was significantly downregulated after 24 h (Fig.1c). Predominantly green fluorescent products were observed when *R. raciborskii* was cultured in a P-deficient medium (Fig.2), further supporting the findings of González-Gil et al. (1998), who showed that an intensive green fluorescence ELF alcohol (ELFA) was formed and tagged at or near the sites of enzymatic activity in cells when phosphatases and enzyme-labeled fluorescence phosphate (ELFP) were present, suggesting that phytoplankton can produce alkaline phosphatase to hydrolyze organic P to compensate for P deficiency when ambient P is scarce (Zhang et al., 2019). Prentice et al. (2019) suggested that 89% of the total P demand resulted from alkaline phosphatase activity in field populations dominated by *R. raciborskii*. This suggests that high alkaline phosphatase activity could be induced as a response to the P starvation in *R. raciborskii*, supporting findings of Wu et al. (2012), Posselt et al. (2009), and Lu et al. (2021). However, Burford et al. (2018) observed that *R. raciborskii* strains isolated in Australia did not increase alkaline phosphatase activity in response to P deficiency, reflecting that *R. raciborskii* strains showed intra-specific variations in alkaline phosphatase (Guedes et al., 2019). This suggested that *R. raciborskii* has developed various strategies to adapt to P-deficient environments (Guedes et al., 2019; Xiao et al., 2020).

The reduction of the parameters F_0, F_m, F_v, and ET_0/RC showed that the electron transport was blocked in the photosynthetic system II (PSII). Additionally, φ_{E0} and ψ_0 also decreased in P deficient treatments relative to P supplemented treatments. However, an increased trapped energy flux per reaction center (TR_0/RC) was found in P deficient treatments (Table 2; Fig.3), suggesting that *R. raciborskii* in P deficient treatments could dissipate excess excitation energy by thermal and fluorescence forms to avoid the potential for photo-oxidative damage (Perron et al., 2012). Jacob and Lawlor (1993) also showed that non-photochemical dissipation of energy was raised, and PSII activity was downregulated when phytoplankton suffered long-term P deficiency. Therefore, P starvation might suppress the photophosphorylation of *R. raciborskii* cells, resulting in an increased excitation of the thylakoid membrane and a decreased probability of PSII excitation energy transfer from the antenna to the RCs, as well as relatively inefficient photosynthesis (Jacob, 1995; Wu et al., 2012). However, Pierangelini et al. (2014) proposed that the regulation of excitation energy transfer is likely driven by state transitions rather than by heat dissipation in two *R. raciborskii* strains exposed to 100 μmol photons/(m^2·s), indicating that *R. raciborskii* can develop different acclimation strategies toward different environmental factors. Additionally, the responses to P-depleted conditions were compared among five *R. raciborskii* and five *Microcystis aeruginosa* strains by Guedes et al. (2019), who observed that only one *R. raciborskii* and all *M. aeruginosa* strains showed a decrease in PSII, further supporting that intra-specific variation occurs in *R. raciborskii* populations.

4.2 Molecular mechanisms of responses to P variation

In a P-deficient environment, many phytoplankton organisms tend to increase phosphate uptake by regulating the expression of phosphate transporters (Frischkorn et al., 2014; Alexander et al., 2015; Liu et al., 2015; Shi et al., 2017; Zhang et al., 2019). All the six genes involved in the phosphorus assimilation process showed significant upregulation after 24, 72, and 144 h of P-deficient culture. However, the expression patterns differed among the six genes (Fig.4). Genes encoding alkaline phosphatase (or -like), *phoA1* and *phoA2*, were significantly upregulated, which was consistent with the result of APAs. A similar result was obtained for *R. raciborskii* CS-505 and CS-506 (Willis et al., 2019). Previous observations have indicated that *R. raciborskii* has the highest recorded active uptake rates (Willis et al., 2017). Active P uptake is below a threshold of 4.75-μg/L phosphate (Prentice et al., 2015). This suggested that the transcript expression of alkaline phosphatase allowed the acquisition of P from organic P-molecules with ester bonds in *R. raciborskii* (Liu and Wu, 2012; Bai et al., 2014). However, the different expressions of *phoA1* and *phoA2* were found at 24-, 72-, and 144-h P-free conditions (Fig.4a–b), reflecting that differential regulation of the two genes in *R. raciborskii* may be a result of their different affinities for P assimilation (Su et al., 2007; Beszteri et al., 2012).

The gene *pstS1* encoding a high-affinity phosphate-binding protein was upregulated in this study under P-deficient conditions (Fig.4c). This is consistent with the findings of Willis et al. (2019). However, the timing of upregulation was different from the

Australian strains CS-505 and CS-506, which indicates that the timing of upregulation varies greatly among different strains of *R. raciborskii* (Vershinina and Znamenskaya, 2002). Usually, the gene *pstS1* has multiple copies in the genome of cyanobacteria and pico-cyanobacteria (Su et al., 2007), but only one copy may respond to changes in P availability (Harke and Gobler, 2013). In *R. raciborskii*, however, there is only one copy of *pstS1* in a complete operon in the form of *pstS-C-A-B* (Sinha et al., 2014; Fuentes-Valdés et al., 2016), which suggests that the presence and regulation of *pstS1* are responsible for how *R. raciborskii* adapts and responds to P-limited or P-depleted conditions (Moore et al., 2005).

Like in the genomes of *Nostoc* sp. PCC7120 and *Trichodesmium* IMS101 (Su et al., 2007), in the genome of *R. raciborskii*, orthologous genes of the phosphonate transporter complex and the C-P lyase were found (Stucken et al., 2010). After 24-h culture in a P-deficient medium, the genes *phnD* encoding phosphonate ABC transporter and *phnM* encoding phosphonate metabolism protein were markedly upregulated (Fig.4d–e), suggesting that phosphonate compounds might be an important phosphorus source to *R. raciborskii* in this low DIP system. These results support a previous study showing that *R. raciborskii* can grow with phosphonate as the only available P source (Bai et al., 2014). However, Willis et al. (2019) found the gene *phnD* did not upregulate in CS-505, but a significant upregulation was found in CS-506, reflecting that intra-specific variation of the phosphonate pathway might occur in *R. raciborskii* populations, and that this pathway is active under P-deficient conditions. Dyhrman et al. (2006) showed that the ability to utilize phosphonates played an important role in the prevalence and competitive advantage of *Trichodesmium*, indicating that phosphonate metabolism might represent a crucial niche adaptation for the invasion of *R. raciborskii*.

In this study, the gene expressions of *nucH*, encoding extracellular nuclease, were markedly upregulated in P-deficient treatments in contrast to P supplemented treatments (Fig.4f). Suzuki et al. (2004) proposed that the *phoA-nucH* operon in *Synechocystis* PCC6803 is activated under P limitation. Luo et al. (2017) also found 5′-nucleotidase activities and transcript expression were upregulated when the dinoflagellate *Karenia mikimotoi* was cultured with ATP as the only available P source, further supporting that nucleotidase was able to cleave PO_4^{3-} from ATP (Dyhrman and Palenik, 2003; Dyhrman et al., 2012). This implies that *nucH* might play a crucial role in the utilization of the P moiety in nucleic acids in the environment (Su et al., 2007).

4.3 Implications for the management of *R. raciborskii* blooms

Input reduction of nutrients, e.g., nitrogen (N) and P, has been widely recognized as an effective way of controlling cyanobacterial blooms in lakes and reservoirs (Qin et al., 2007; Hamilton et al., 2016). Reducing N or P inputs or both combined to prevent blooms effectively is a controversial issue (Xu et al., 2010; Paerl et al., 2011). Wan et al. (2019) proposed that P reduction was more important for controlling blooms dominated by diazotrophic cyanobacteria than by non-diazotrophic cyanobacteria. However, our results indicated that *R. raciborskii* could endure P deficiency conditions for 144 h (6 d) by reducing its growth and photosynthesis and increasing its alkaline phosphatase (AP) activity and gene transcriptions of phosphate uptake and transporters. These results support that *R. raciborskii* can regulate physiological and molecular responses to low P availability by an increase in P uptake, the utilization of organic compounds and phosphorus storage compounds, and the induction of alkaline phosphatase (Bai et al., 2014; Guedes et al., 2019; Willis et al., 2019). Additionally, Xiao et al. (2020) indicated that P luxury storage was a strategy for the persistence of *R. raciborskii* populations under P stress conditions. Therefore, P reduction may not always control *R. raciborskii* blooms, especially in lakes and reservoirs with intermittent P pulses.

5 CONCLUSION

Here, we investigated the responses of *R. raciborskii* to ambient P deficiency and resupply at the physiological and molecular levels. The growth and photosynthesis of *R. raciborskii* were markedly inhibited, while alkaline phosphatase significantly increased under P-deficiency. The expression of genes encoding alkaline phosphatase, high-affinity phosphate-binding proteins, the phosphonate transporter, metabolism, and nucleotidase exhibited a general increase under P-deficient conditions. However, physiological and molecular responses occurred rapidly after DIP re-supplementation of P-deficient treatments, indicating that *R. raciborskii* has a strong ability to regulate its utilization of P to adapt to low P environments. However, different

strains of *R. raciborskii* may have different capacities for metabolizing different P sources. Therefore, variations between strains regarding P metabolization need to be further clarified to understand the global distribution patterns of *R. raciborskii*.

6 DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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