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Molecular insights into the circadian clock in marine diatoms

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Abstract

The circadian clock is a fundamental endogenous mechanism of adaptation that coordinates the physiology and behavior of most organisms with diel variations in the external environment to maintain temporal homeostasis. Diatoms are the major primary producers in the ocean. However, little is known about the circadian clock in marine diatoms compared with other organisms. Here, we investigated circadian clock genes, their expression patterns, and responses to environmental stimuli such as light, nitrogen and phosphorus in two marine diatoms, *Skeletonema costatum* and *Phaeodactylum tricornutum*, using a combination of qRT-PCR and bioinformatic analysis. We identified 17 and 18 circadian clock genes in *P. tricornutum* and *S. costatum*, respectively. Despite significant evolutionary differences, these genes were similar to those of the higher plant *Arabidopsis*. We also established a molecular model for the marine diatom circadian clock comprising an input pathway, core oscillator, output pathway, and valve effector. Notably, the expression patterns of core clock genes (circadian clock associated 1 (*CCA1*), late elongated hypocotyl (*LHY*) and timing of cab 1 (*TOC1*)) in both species differed from those of terrestrial plants. Furthermore, the expression of these genes was influenced by variations in ambient light, nitrogen and phosphorus availability. Although marine diatoms and higher plants share common circadian clock components, their clock genes have diverged throughout evolution, likely as a result of adapting to contrasting environments.

Key words: circadian clock, circadian genes, marine diatoms, *Phaeodactylum tricornutum*, *Skeletonema costatum*

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1 Introduction

The circadian clock is an endogenous regulatory mechanism that allows acclimation to cyclical changes in the external environment, thereby enabling organisms to predict temporal changes, adjust physiological homeostasis, improve their ability to compete, and maximize their chances of survival (Harmer, 2009). It takes about 24 h for the Earth to rotate, and the circadian clock creates a physiological and metabolic rhythm, referred to as the circadian rhythm, that mimics the cycle of the surrounding environment (Harmer, 2009). Circadian rhythms are ubiquitous and have been identified in numerous organisms ranging from prokaryotic cells to multicellular eukaryotes (Bruce, 1970; Konopka and Benzer, 1971; Feldman and Hoyle, 1973; Staiger, 2002).

The rhythm-generating oscillator is a complex network of interlocking feedback loops (Troein et al., 2009). Previous studies have shown that the circadian clock has three distinctive features: (1) as an endogenous regulatory mechanism, the clock produces a circadian rhythm in a manner that is independent signals from the external environment (Harmer, 2009); (2) the circadian clock can be reset by changes in the external environment (Dodd et al., 2005); and (3) the circadian clock can compensate for changes in temperature (Salomé et al., 2010). To synchronize time and space with the external environment, organisms use the circadian clock to regulate gene expression, as well as physiological and biochemical reactions; by synchronizing these processes, an organism can gain significant survival benefit from a changing environment (Barak et al., 2000; Harmer, 2009). The circadian clock controls protein abundance and activity in response to environmental conditions by regulating transcription, translation, and posttranslational modifications, resulting in an oscillatory period that is almost 24 h long (Greenham and McClung, 2015).

Although the mechanisms that regulate the circadian clock are complex, they are known to comprise an input path, a core oscillator, an output path, and a valve effector in higher plants (Harmer, 2009). Environmental signals, such as light and nutrient which are drivers of circadian rhythms, are transmitted to the core oscillator through the input pathway, while the output pathway amplifies the rhythmic signal generated by the core oscillator and transmits it to appropriate downstream genes (McClung, 2001, 2011). The core oscillator is the main component of the circadian clock, and is described as a transcriptional regulatory feedback loop consisting of positive and negative interactions

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among three components, namely, two MYB domain-containing transcription factors (circadian clock associated 1 (*CCA1*) and late elongated hypocotyl (*LHY*)), and a member of the pseudo-response regulator (PRR) protein family (timing of cab 1 (*TOC1*)) (Alabadí et al., 2001). The expression levels of *CCA1* and *LHY* generally peak just before or after the onset of illumination. Their products bind to the evening element (AAATATCT) on the *TOC1* promoter and inhibit its expression. The expression level of *TOC1* is known to peak in the evening owing to the reduced expression of *CCA1* and *LHY*, thereby releasing the inhibitory effect on *TOC1* (Alabadí et al., 2001; McClung, 2001, 2011). Loss of functional activity in any of the core clock genes results in a shortened clock period, whereas their overexpression confers arrhythmicity to multiple outputs (Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadí et al., 2001). Signals generated by the core oscillator result in physiological and biochemical responses via the output of downstream circadian clock genes (McClung, 2001, 2011).

Numerous studies have investigated the circadian clocks of several model species, including cyanobacteria (Kondo et al., 1994), *Chlamydomonas* (Bruce, 1970), *Drosophila melanogaster* (Konopka and Benzer, 1971) *Arabidopsis thaliana* (Harmer et al., 2000), the mouse (*Mus musculus*) (Turek et al., 2005), and humans (*Homo sapiens*) (Wright et al., 2013). These studies have helped elucidate the structural and regulatory mechanisms of the circadian clock. Several studies have also shown that marine phytoplankton exhibit 24 h period circadian rhythms in a range of processes, including physiological and biochemical pathways, gene expression patterns, and gathering behavior (Schweiger et al., 1964; Ragni and d'Alcalà, 2007; Wada et al., 2011; Filonova et al., 2013; Noordally and Millar, 2015). Transcription of the core cell cycle genes, including cyclins and cyclin-dependent kinases, is under circadian control in the unicellular green alga *Ostreococcus*, independent of the photosynthetic capacity (Moulager et al., 2007). Furthermore, the circadian clock genes *CCA1* and *TOC1* have been identified in *Ostreococcus tauri* (Noordally and Millar, 2015), and have also been characterized in *A. thaliana*. Annunziata et al. (2019) demonstrated that bHLH-PAS nuclear protein RITMO1 regulates diel rhythm in the marine diatom *P. tricornutum* and widely represents in algal genomes. Collectively, these data suggest that marine phytoplankton possess a circadian clock system similar to that of higher plants. However, at present, little is known of the circadian clock system in marine phytoplankton.

Diatoms are among the most important phytoplankton in the ocean, accounting for 20% of global carbon fixation and 40% of marine primary production (Goldman, 1993; Falkowski et al., 1998; Field et al., 1998). Consequently, diatoms play an essential role in regulating global biogeochemical cycles and climate (Goldman, 1993; Falkowski et al., 1998; Field et al., 1998). Circadian clock is important for regulating phytoplankton cell growth, gene expression, pigment synthesis, phototactic movements, and carbon fixation (Noordally and Millar, 2015). In our previous study, we identified several homologs of circadian clock genes, including *CCA1* and *PRR5*, in the transcriptome of the marine diatom *Skeletonema costatum* (Zhang et al., 2016), which suggested the likely existence of a circadian clock in marine diatoms. In the present study, we investigated the expression patterns of circadian clock genes and their responses to environmental stimuli in two marine diatoms, *S. costatum* and *Phaeodactylum tricornutum*, and constructed a molecular model for the marine diatom circadian clock. Our results demonstrated that marine diatoms possess the same genetic clock components as higher plants, but the clock genes have diverged, likely due to their contrasting living environments.

2 Materials and methods

2.1 *Organisms and culture conditions*

Strains of *Skeletonema costatum* (Greville) Cleve, 1873 and *Phaeodactylum tricornutum* Bohlin, 1897 were kindly provided by the Collection Center of Marine Algae, Xiamen University, China. Cells were routinely maintained in *f*/2 medium at 20°C under a 14 h:10 h light: dark cycle at a light intensity of 100 μmol/(m^2·s) provided by a cold fluorescent lamp, as previously described (Zhang et al., 2016).

2.2 *Cell synchronization*

Before the experiments, cultures of *S. costatum* and *P. tricornutum* were synchronized for 48 h using a dark-induction method (Wang et al., 2013). Synchronized cells were then transferred to fresh *f*/2 medium and cultured for three generations (Zhang et al., 2016). Cells were used for experiments when they were in the exponential growth phase. An initial cell density of 1×10^4 cells/mL was used for each diatom species and experiments were performed in triplicate.

2.3 *Responses of core clock genes to environment stimuli*

To elucidate the sensitivity of *S. costatum* and *P. tricornutum* core clock genes to environmental changes, the two diatom species were grown under different light regimes and nitrogen (N, $NaNO_3$) and phosphorus (P, NaH_2PO_4) conditions. Synchronized cells were transferred to fresh *f*/2 medium at 20°C at an initial cell density of 1×10^4 cells/mL. Light: dark cycles of 14 h:10 h, 24 h:0 h, and 0 h:24 h were set as the control group, continuous light group, and continuous dark group, respectively, each with three replicates. For the N experiment, synchronized cells were transferred to N-free *f*/2 medium as the N-deplete group, while N was added to the N-deplete group at a final concentration of 880 μmol/L as the N-resupplied group. For the P experiment, synchronized cells were transferred to *f*/2 medium with P concentration of 0.5 μmol/L as the P-deplete group, while P was added to the P-deplete group at a final concentration of 30 μmol/L as the P-resupplied group.

2.4 *Physiological analysis*

Three 1 mL and three 15 mL aliquots of culture media were collected daily at 11:00 for cell counting and the photochemical efficiency of photosystem II (Fv/Fm) analysis, respectively (Zhang et al., 2016). Culture medium (25 mL) from each nutrient treatment was filtered through a GF/F membrane, and dissolved N and P concentrations in the filtrate were measured using continuous flow analysis (CFA-SAN Plus/Skalar Analytik, Erkelenz, Germany).

2.5 *Identification of circadian clock genes in S. costatum and P. tricornutum*

In this study, a reference list of 26 circadian clock genes that had been previously identified in eukaryotic plants, including *A. thaliana* and rice (*Oryza sativa*) (Table S1), were used to identify circadian clock genes in *S. costatum* and *P. tricornutum*. The protein sequences of circadian clock genes from higher plants, such as *A. thaliana*, were first downloaded from the UniProt (http://www.uniprot.org/) and NCBI databases as query sequences. A local BLAST search (*e*-value<10^{-5}) was then carried out using the transcriptome of *S. costatum* and the genome of *P. tricornutum* as databases. Candidate clock genes were then screened and verified using the online BLASTP tool in the NCBI database. The core clock genes (*CCA1*, *LHY*, and *TOC1*) in *S. costatum* and *P.*

tricornutum were searched for conservative motif based on the databases SMART and Pfam, and compared with the *Arabidopsis* clock core genes to further confirm the authenticity of the core clock genes in diatoms.

2.6 *Phylogenetic analysis of core clock genes*

The deduced amino acid sequences of the core clock genes (*CCA1*, *LHY*, and *TOC1*) in *S. costatum* and *P. tricornutum* were aligned to those in the NCBI database. Phylogenetic analysis of all aligned sequences was carried out using Molecular Evolutionary Genetics Analysis (MEGA) 5.0 software. The length of *CCA1*, *LHY*, and *TOC1* are 496, 749 and 496 amino acids, respectively. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. Based on the defined haplotype sequences of the corresponding gene coding regions, molecular evolutionary trees were constructed using the neighbor-joining method.

2.7 *RNA extraction and cDNA synthesis*

Samples for gene expression analysis were collected every 3 h from 9:00 on the third day for a period of 48 h (Figs 1a–h), while samples were collected from the N- and P-resupplied media after N and P addition (Figs 1c, d, g and h). From each sample, 50 mL of culture medium was filtered through a polycarbonate membrane (pore-size 3 μm; Millipore Corporation, USA) and resuspended in 1 mL of Trizol reagent (Invitrogen, USA). The sample was then immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction (Zhang et al., 2016). Total RNA was extracted using TRI-Reagent (MRC, USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany) (Zhang et al., 2016). Then, RNA was reverse transcribed into cDNA for qPCR using the FastKing RT Kit with gDNase (Qiagen, Germany).

2.8 *Quantitative PCR analysis*

Quantitative PCR was performed using the Bio-Rad CFX96 Touch real-time PCR detection system (Applied Biosystems, USA) with the SuperReal PreMix Plus (SYBR Green) Kit (Tiangen, China). The primers for the circadian clock genes were designed using Primer Premier 5 software and are listed in Tables S2 and S3. The 18S rRNA (Kang et al., 2012) and TATA box binding protein genes (Seo et al., 2020) were used as internal reference genes for *S. costatum* and *P. tricornutum*, respectively. Amplification was performed in a 20 μL reaction volume, as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s. Gene expression profiles were subsequently calculated using the $2^{-\triangle Ct}$ method.

2.9 *Statistical analysis*

Significant differences ($p \leqslant 0.05$) among gene expression patterns were identified by one-way ANOVA followed by Duncan's multiple range test. Results are presented as mean±standard deviation of relative mRNA expression.

3 Results

3.1 *Identification of circadian clock genes in S. costatum and P. tricornutum*

In this study, 17 and 18 circadian clock genes were identified in *P. tricornutum* and *S. costatum*, respectively (Table 1). Three phytochrome genes (*PHYC*, *PHYD*, and *PHYE*), a cryptochrome circadian regulator 1 gene (*CRY1*), zeitlupe (*ZTL*), flarin binding, kelck repeat, f-box 1 (*FKF1*), and LOV kelch protein 2 (*LKP2*), which are all located in the input pathway of the circadian clock, were identified in both *S. costatum* and *P. tricornutum*. However, the phytochrome genes *PHYA* and *PHYB* were only detected in *S. costatum* (Table 1). Three key genes of the circadian clock core oscillator (*CCA1*, *LHY*, and *TOC1*) were identified in both diatom species. Based on the analysis results of databases SMART and Pfam found that *CCA1* and *LHY* of the two diatoms contained the DNA binding domains from MYB proteins, as well as the switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor (TF)IIIB (SANT) domain family, both diatom *TOC1* and *Arabidopsis TOC1* had characteristic Co, constanslike, and timing of cab expression 1 (CCT) motif (Fig. 2). The *PRR5* and *PRR7* genes, known to play a role in the morning loop of the circadian clock, were identified in *S. costatum*, whereas the *PRR9* gene was only identified in *P. tricornutum*. The *TOC1* and LUX ARRHYTHMO (*LUX*) genes, known to play a role in the evening loop, identified in both diatom species, as were *PRR3* and casein kinase 2 (*CK2*), two core oscillator genes, and one output pathway gene, chlorophyll *a*/*b* binding protein (*CAB2*) (Table 1).

3.2 *Expression patterns of core clock genes in S. costatum and P. tricornutum*

The expression profiles of three core clock genes (*CCA1*, *LHY*, and *TOC1*) in *S. costatum* and *P. tricornutum* showed clear diel oscillations (Fig. 3). The oscillatory period of *CCA1* was similar in both diatom species and lasted about 24 h. During the day, the expression level of *CCA1* increased and peaked 2 h after cells had entered the dark cycle, and then began to fall, reaching a minimum 1 h after entering the photoperiod (Figs 3a-1, b-1). The *LHY* gene had an oscillation period of about 24 h in both diatoms (Figs 3c-1, d-1). In the first cycle, *LHY* expression began to increase almost 7 h after entering the light cycle, peaking about 1 h before the dark phase. Then, 1 h after entering the dark cycle, the expression level of *LHY* began to decrease and reached a minimum level almost 1 h before the next cycle. At the beginning of the second cycle, the expression level of *LHY* began to increase and peaked 2 h after the beginning of the dark cycle (Figs 3c-1, d-1). The *TOC1* gene also exhibited a diel oscillatory pattern that was similar to those of *LHY* and *CCA1* (Figs 3e-1, f-1).

3.3 *Expression of core clock genes under different light, N and P supply conditions*

Although the expression phases of the three core clock genes *CCA1*, *LHY*, and *TOC1* in *S. costatum* and *P. tricornutum* varied under different light, N, and P supply conditions, a clear circadian pattern could still be detected (Fig. 3). Under continuous light, the peak of *CCA1* expression in *S. costatum* (Fig. 3a-2) was not significantly altered, whereas two peaks of *CCA1* expression could be detected in *P. tricornutum* (Fig. 3b-2). The oscillation period lasted about 24 h in both diatoms. The peak of *LHY* expression in the two diatom species moved forward under the continuous light regime; the period was shortened in *S. costatum*, but remained at 24 h in *P. tricornutum* (Figs 3c-2, d-2). The peak of *TOC1* expression was delayed for almost 3 h and 12 h in *S. costatum* and *P. tricornutum*, respectively, but the period remained unchanged (Figs 3e-2, f-2). Under continuous dark conditions, the peak of *CCA1* expression moved forward 12 h and 9 h in *S. costatum* and *P. tricornutum*, respectively, but the oscillation period remained unchanged (Figs 3a-3, b-3). The peak expression of *LHY* moved forward 3 h and 9 h in *S. costatum* and *P. tricornutum* within a 24 h oscillation period, respectively (Figs 3c-3, d-3). The peak of *TOC1* expression in *S. costatum* moved forward

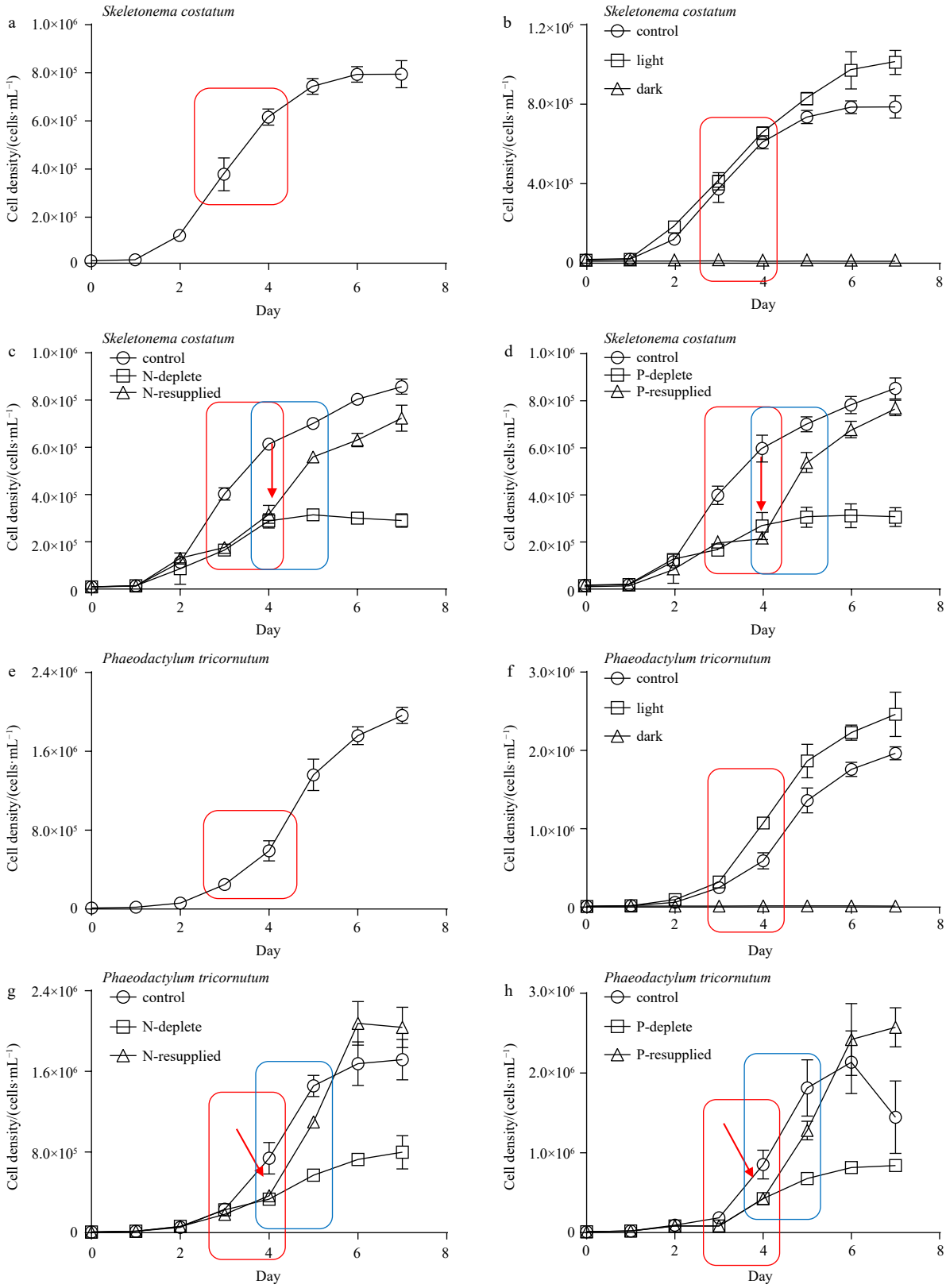

Fig. 1. Growth curves of *Skeletonema costatum* and *Phaeodactylum tricornutum* under control (a and e), continuous light and dark (b and f), N-deplete and N-resupplied (c and g), P-deplete and P-resupplied (d and h) conditions. The red and blue rectangles indicate the sampling time for molecular analysis while the blue rectangles in c, d, g and h indicate the sampling time in the N- or P-resupplied groups. The red arrow indicates the time point of N or P resupply.

3 h but remained unchanged in *P. tricornutum* (Figs 3e-3, f-3).

Compared with the control, the expression of *CCA1* was increased only in the N-depleted *S. costatum* cells (Fig. 3a-4), and the peak phase moved forward after N resupply (Fig. 3a-5);

Table 1. Circadian clock genes identified in the diatoms *Skeletonema costatum*, *Phaeodactylum tricornutum*, and other species[a]

Location	Gene name	Eukaryotic algae						Flowering plants[b]	
		Skeletonema costatum	*Phaeodactylum tricornutum*	*Ostreococcus tauri*	*Chlamydomonas reinhardtii*	*Symbiodinium* spp.	*Cyanidioschyzon merolae*	*Arabidopsis thaliana*	*Oryza sativa*
Input pathway	*PHYA*	+	nd	nd	nd	–	nd	+	+
	PYHB	+	nd	nd	nd	–	nd	+	+
	PHYC	+	+	nd	nd	+[c]	nd	+	+
	PHYD	+	+	nd	nd	–	nd	+	+
	PHYE	+	+	nd	nd	–	nd	+	+
	CRY1	+	+	nd	+	+	+	+	+
	ZTL	+	+	nd	nd	nd	nd	+	+
	LKP2	+	+	nd	nd	nd	nd	+	+
	FKF1	+	+	nd	nd	nd	nd	+	+
Core oscillator	*CCA1*	+	+	+	nd	nd	nd	+	+
	LHY	+	+	nd	nd	nd	nd	+	nd
	TOC1	+	+	+	+	nd	nd	+	nd
	PRR3	+	+	nd	nd	nd	nd	+	nd
	PRR5	+	+	nd	nd	nd	nd	+	nd
	PRR7	+	+	nd	nd	nd	nd	+	nd
	PRR9	–	+	nd	nd	nd	nd	+	nd
	CK2	+	+	+	+	nd	nd	+	+
Output pathway	*CAB2*	+	+	nd	nd	nd	nd	+	+
Valve effector	*LUX*	+	+	nd	nd	nd	nd	+	nd

Note: [a]Prokaryotes are not included in the table because they have completely different clock genes; [b]only representative flowering plants are listed; [c]unknown type. + indicates detected; nd, not detected; –, no data. The content of this table refer to Martínez-García et al. (2000), Noordally and Millar (2015) and Zhang et al. (2016).

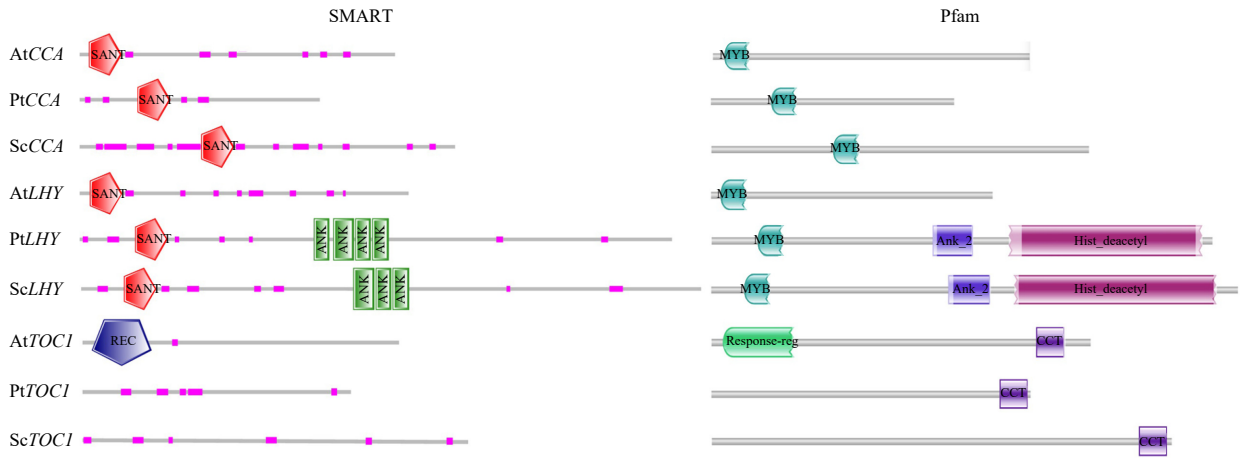

Fig. 2. Conservative motif analysis of the core clock genes (*CCA1*, *LHY*, and *TOC1*) in *Skeletonema costatum* and *Phaeodactylum tricornutum* based on the databases SMART and Pfam. At is abbreviation of *Arabidopsis thaliana*; Pt, *P. tricornutum*; Sc, *S. costatum;* REC, phosphoacceptor receiver; SANT, switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor (TF)IIIB; ANK, ankyrin; Ank2, Ankyrin-B; CCT is timing of cab expression 1.

however, the peak of *CCA1* expression moved forward in the N-deplete *P. tricornutum* cells (Fig. 3b-4), showing high expression during the daytime, but returned to the evening with N resupply (Fig. 3b-5). The peaks of expression of both *LHY* and *TOC1* moved forward in the N-deplete cells; however, the oscillation period remained at about 24 h. The peaks of *LHY* and *TOC1* expression in the N-deplete *S. costatum* cells moved forward 6 h and 9 h (Figs 3c-4, e-4), respectively, while those of the N-deplete *P. tricornutum* cells moved forward 9 h and 6 h (Figs 3d-4, f-4), respectively. After N resupply, the peaks of *LHY* and *TOC1* expression were again delayed in the two diatoms (Figs 3c-5, d-5, e-5 and f-5).

The expression patterns of *CCA1* and *LHY* in the P-deplete *S. costatum* cells remained unchanged compared with those of the control (Figs 3a-6, c-6), while the peak of *TOC1* expression was delayed for 3 h (Fig. 3e-6). However, the peaks of *CCA1*, *LHY*, and *TOC1* expression in *P. tricornutum* moved forward, and these genes were highly expressed during the day (Figs 3b-6, d-6 and f-6). After P resupply, *CCA1* expression in *S. costatum* exhibited two peaks within an oscillation period (Fig. 3a-7), *LHY* was highly expressed during the day (Fig. 3c-7), and *TOC1* expression returned to a normal pattern (Fig. 3e-7).

3.4 *Genetic evolution of core clock genes in S. costatum and P. tricornutum*

To examine the evolutionary status of core clock genes in *S.*

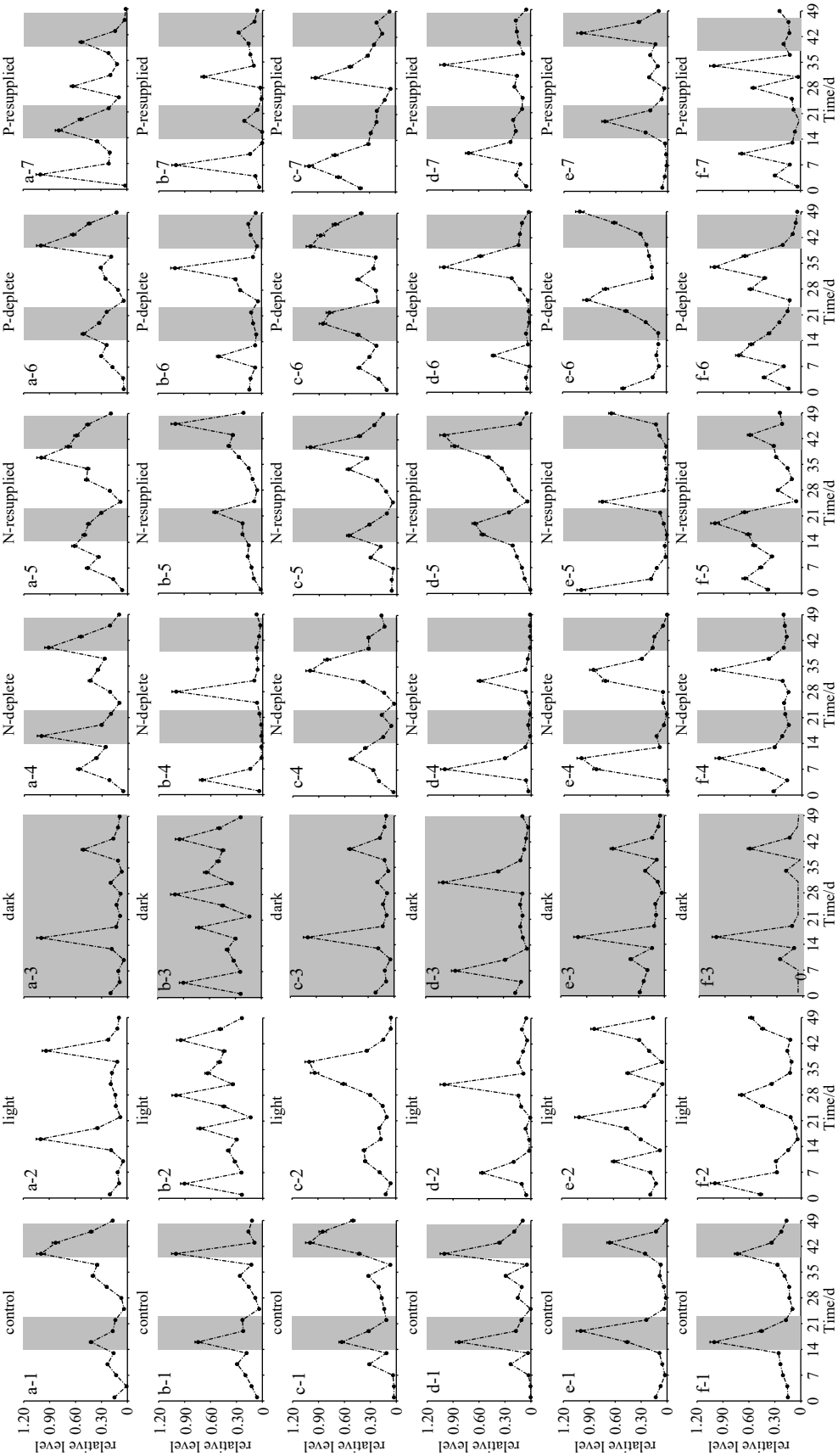

Fig. 3. Expression of *CCA1*, *LHY*, and *TOC1* in *Skeletonema costatum* and *Phaeodactylum tricornutum* within 24 h. Expression of *CCA1* in *S. costatum* and *P. tricornutum* under control (a-1 and b-1), continuous light (a-2 and b-2), continuous dark (a-3 and b-3), N-deplete (a-4 and b-4), N-resupplied (a-5 and b-5), P-deplete (a-6 and b-6) and P-resupplied (a-7 and b-7) conditions; expression of *LHY* in *S. costatum* and *P. tricornutum* under control (c-1 and d-1), continuous light (c-2 and d-2), continuous dark (c-3 and d-3), N-deplete (c-4 and d-4), N-resupplied (c-5 and d-5), P-deplete (c-6 and d-6) and P-resupplied (c-7 and d-7) conditions; and expression of *TOC1* in *S. costatum* and *P. tricornutum* under control (e-1 and f-1), continuous light (e-2 and f-2), continuous dark (e-3 and f-3), N-deplete (e-4 and f-4), N-resupplied (e-5 and f-5), P-deplete (e-6 and f-6) and P-resupplied (e-7 and f-7) conditions. Sc represents *S. costatum*; Pt, *P. tricornutum*.

costatum and *P. tricornutum*, we used the neighbor-joining method to construct phylogenetic trees. The amino acid sequences for *CCA1*, *LHY*, and *TOC1* in *Brassica campestris*, *B. napus*, *B. juncea*, *B. oleracea*, *B. carinata*, *Noccaea caerulescens*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Arabidopsis halleri*, *Ananas comosus*, *Picea abies*, *Nicotiana tabacum*, *Helianthus annuus*, *S. costatum*, *P. tricornutum*, and *Chlamydomonas reinhardtii* were selected for phylogenetic analysis (Fig. 4). The genes were well clustered into their relevant phylogenetic branches and were located at the same branchpoint as *Helianthus annuus*, *Nicotiana tabacum*, *Picea abies*, and *Arabidopsis*. However, they were clustered in different evolutionary branches from that of *Chlamydomonas reinhardtii* (Figs 4a–c). The *LHY* phylogenetic tree indicated that the two subclasses were well clustered into their relevant phylogenetic branches. The *LHY* genes of *S. costatum* and *P. tricornutum* were clustered together, while the *LHY* genes of *Vigna radiata*, *Gossypium hirsutum*, and *Cucumis melo* were clustered into one branch; these were then clustered to-

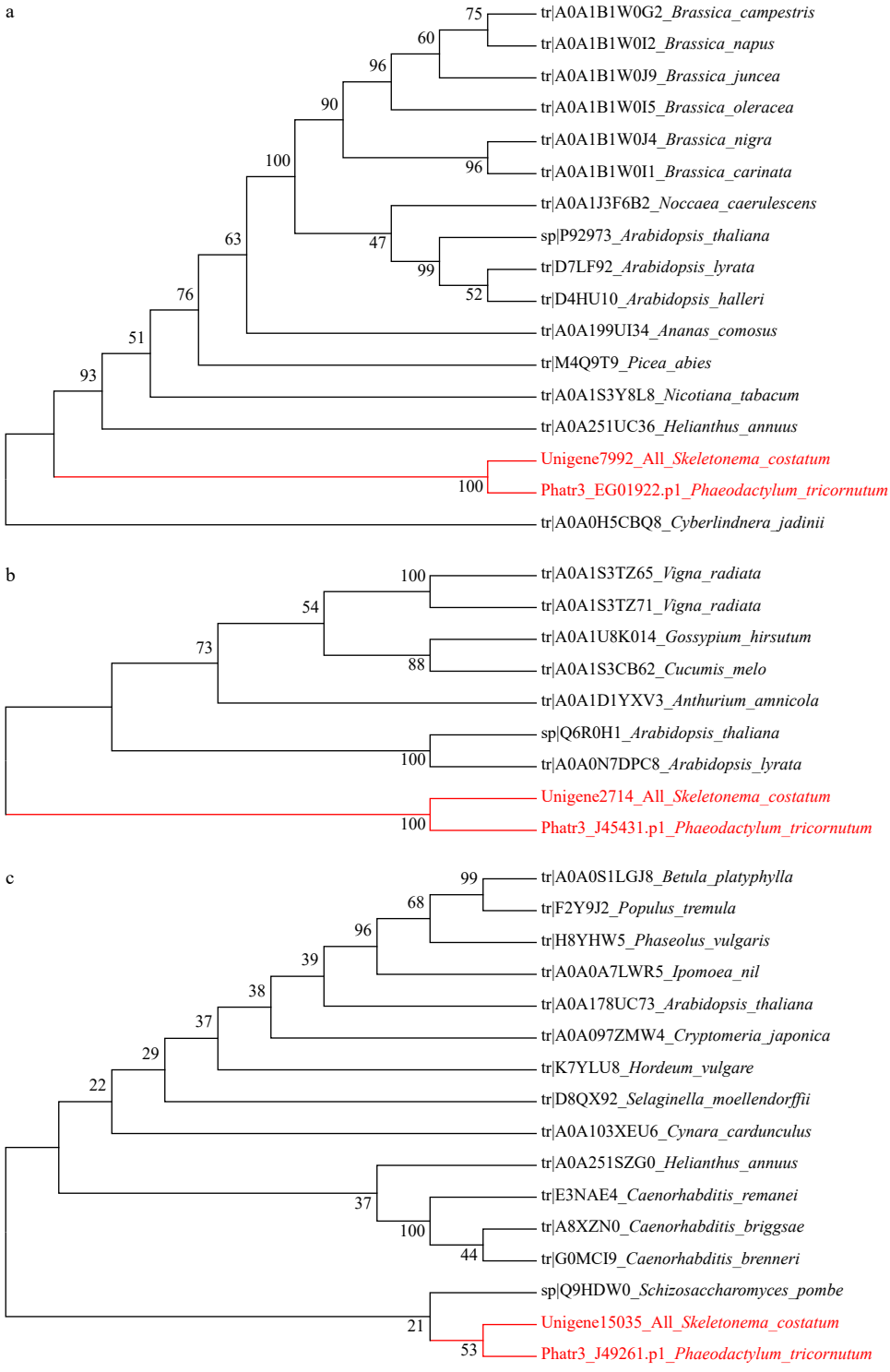

Fig. 4. The phylogenetic tree of *CCA1* (a), *LHY* (b), and *TOC1* (c).

gether with *Anthurium amnicola* and *A. thaliana* (Fig. 4b). The *TOC1* genes of *S. costatum* and *P. tricornutum* were clustered at the same branchpoint as *Schizosaccharomyces pombe*. Further analysis showed that the *TOC1* gene of *Betula platyphylla* was clustered with those of *Populus tremula*, *Phasedus vulgaris*, *Ipomoea nil*, *A. thaliana*, *Cryptomeria japonica*, and *Hordeum vulgare*, and was clustered with nematodes to form another branch (Fig. 4c).

3.5 *A speculative molecular model for the circadian clock in marine diatoms*

Based on the circadian clock genes identified in *S. costatum* and *P. tricornutum*, we established a speculative molecular model of the circadian clock in marine diatoms by referring to the model previously created for *A. thaliana* (McClung, 2001). According to our model, five red-light receptor *PHY* genes (*PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE*), a blue-light receptor gene *CRY1*, and three downstream clock regulator genes *ZTL*, *LKP2*, and *FKF1* are located in the input pathway of the circadian clock of marine diatoms, and these factors transmit environmental signals to the core oscillator (Fig. 5). Nine genes are located in the core oscillator and form a complex multiple feedback regulatory network that includes a core loop based on *CCA1*/*LHY*-*TOC1*, a morning loop featuring *CCA1*/*LHY* and *PRR7*/*PRR9*, and an evening loop under the control of the *TOC1* core gene (Fig. 5). Signals from the input pathway are passed to the complex regulatory network formed by the core oscillator to create new signals that exhibit regular diel variation. However, only the *CAB2* gene was identified in the output pathway (Fig. 5).

4 Discussion

Studies investigating the circadian clock have largely focused on the identification, expression, and regulation of clock genes in the cyanobacteria *Synechococcus* (Kondo et al., 1994) and the higher plant *A. thaliana* (Millar et al., 1995). In this study, circadian clock genes were identified in two diatom species, *S. costatum* and *P. tricornutum*, that were similar to those of *A. thaliana*, but completely different from those of the prokaryote *Synechococcus*. This suggests that marine diatoms have evolved a circadian clock system that is similar to that of higher plants.

During their growth and development, marine diatoms often undergo stress as a result of a range of abiotic factors (Irigoien et al., 2004). Light is the most important of these factors, not only affecting cell growth, but also representing the primary environ-

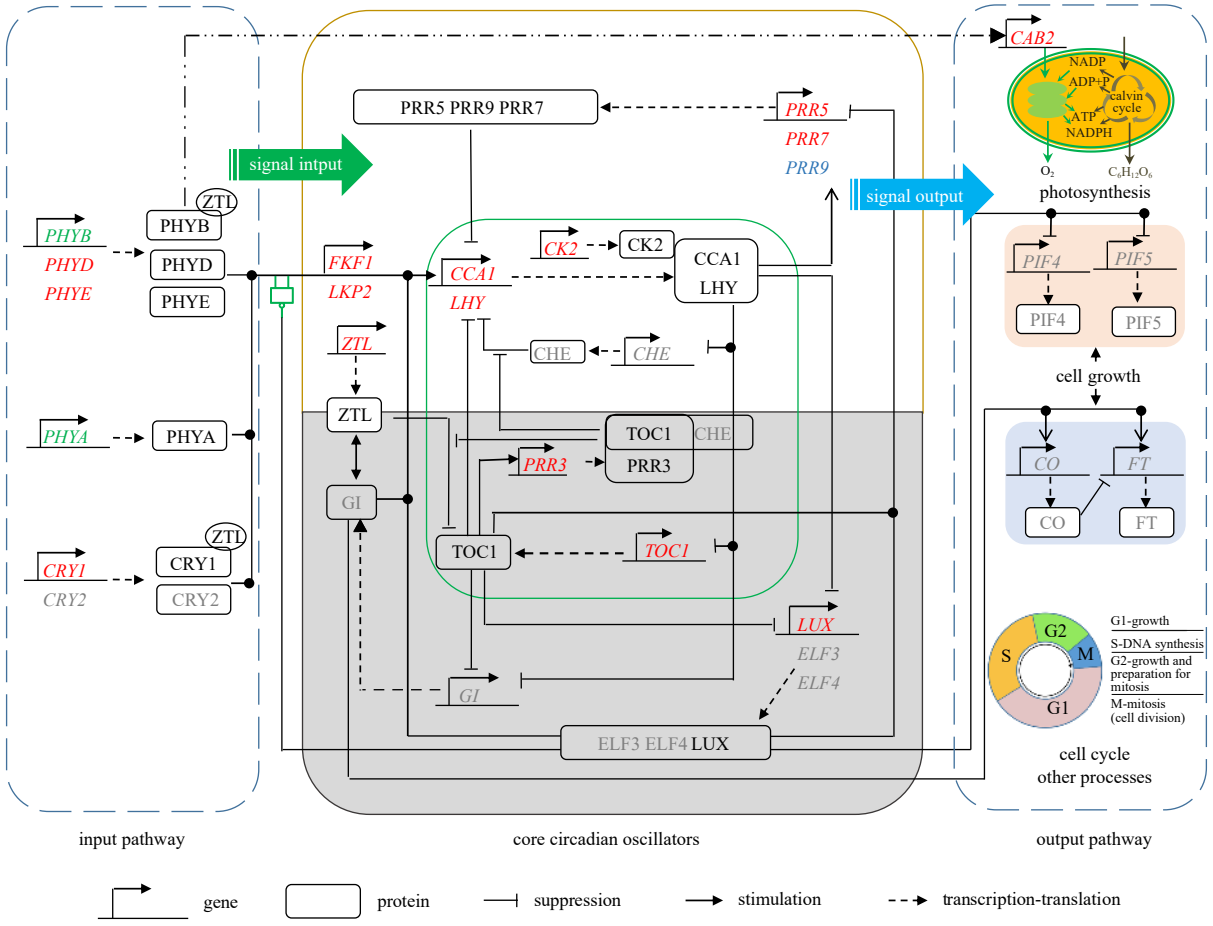

Fig. 5. The speculative molecular model of the circadian clock in marine diatoms. Genes in the figure are shown in italics, proteins are shown in normal font. Words in red represent the genes that are identified in *Skeletonema costatum* and *Phaeodactylum tricornutum*; words in gray represent the genes and proteins that are not identified in *S. costatum* and *P. tricornutum*; words in green represent the genes that are identified in *S. costatum*; words in blue represent the genes that are identified in *P. tricornutum*. The core feedback loop of the circadian oscillator is highlighted in green square frame; the white area represents daytime, while the shaded area represents nighttime. The environmental signals of the input pathway and the metabolic process of the output pathways showed in a previous study (Zhang et al., 2016).

mental input for the circadian clock (McClung, 2001). Input pathway genes such as *PHYs*, *CRY*, *ZTL*, and the valve effector gene *LUX* were identified in both diatom species (Table 1). Members of the phytochrome red-light photoreceptor *PHY* family, cryptochrome blue-light photoreceptor *CRY* family, and genes encoding the *ZTL* family of LOV domain-containing proteins, are involved in converting light signals into transcriptional regulatory signals and transmit light via *LKP2* and *FKF1* (Kevei and Nagy, 2003; Lin and Shalitin, 2003; Demarsy and Fankhauser, 2009). *LUX* acts as a valve effector and controls the strength of input signals (Hazen et al., 2005). When red or far-red light input reaches a critical value, it will be prevented from entering the circadian clock system and possibly damaging it (Hazen et al., 2005). Five genes of the phytochromes (*PHY*) family (*PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE*) were identified in *S. costatum*, whereas only three (*PHYC*, *PHYD*, and *PHYE*) were identified in *P. tricornutum* (Table 1). Moreover, only three of these genes (*PHYA*, *PHYB*, and *PHYC*) have been identified in rice (Dehesh et al., 1991; Basu et al., 2000). The *LKP2* and *FKF1* genes, known to play roles in light signaling, were also identified in both *S. costatum* and *P. tricornutum* (Table 1). In *A. thaliana*, overexpression of *LKP2* results in hypocotyl elongation and a late-flowering phenotype under long-day conditions. Furthermore, by regulating the downstream expression of *CAB2* under continuous light or darkness, such plants show disorder in their circadian rhythm (Sato et al., 2002). The same genes were identified in the photoreceptor and light-transport pathways of *S. costatum* and *P. tricornutum*, suggesting that the input pathway adopted by these species is similar to that adopted by higher plants. However, *PHYA* and *PHYB* were not identified in *P. tricornutum*, indicating that circadian clock input pathway genes in marine diatoms might differ from those of higher plants.

In the core oscillator, we identified genes involved in the core loop, morning loop, and evening loop in both diatom species, including three core genes (*CCA1*, *LHY*, and *TOC1*) (Table 1). The three genes are the main components of the core oscillator and together form a regulatory feedback loop. The *LHY* and *CCA1* gene products bind to the evening element (AAATATCT) on the *TOC1* promoter and inhibit its expression, while *TOC1* also acts as a transcriptional repressor for *CCA1* and *LHY* (Alabadí et al., 2001). The clock signal integrated by the core oscillator in marine diatoms exerts its effects on a range of physiological and biochemical processes by regulating the expression of the downstream gene *CAB2*. The morning loop, consisting of *CCA1*/*LHY* and *PRR9*/*PRR7*/*PRR5*, and the evening loop, consisting of *TOC1* and *LUX,* might also be present in marine diatoms. The *CCA1* gene is known to be involved in regulating photoperiod sensitivity, breaking seed dormancy, seed germination, and adaptation to cold stress, while also promoting hypocotyl elongation in *A. thaliana* (Hotta et al., 2007). Under continuous light, *LHY* and *CCA1* promote plant flowering by reducing the expression of the short vegetative phase (*SVP*) gene (Fujiwara et al., 2008). Homologs of the *TOC1* gene have been identified in a range of plants, including rice, alfalfa (*Medicago sativa*), soybean (*Glycine max*) and maize (*Zea mays*), demonstrating strong functional conservation in terms of protein structure and evolution (Murakami et al., 2003; Boxall et al., 2005; Liu et al., 2009). In *A. thaliana*, overexpression of *TOC1* leads to a longer circadian cycle and dysregulation of the circadian rhythm (Salter et al., 2003; Derveaux et al., 2010). In addition, *TOC1* also participates in photomorphogenesis in *A. thaliana*. However, *TOC1* mutants flower early under short-day conditions (Más et al., 2003). Among the genes involved in the morning cycle, *PRR5*, *PRR7*, and *PRR9* were identified in *P. tricornutum*; however, *PRR9* was not present in *S. costatum*. The PRR protein family has a CCT domain at the C-terminus, which is unique to plants, and includes a nuclear import signal (Matsushika et al., 2007). *Arabidopsis PRR7* and *PRR9* mutants exhibit a longer circadian clock period, while *PRR5* mutants have a shorter cycle; *PRR5* and *PRR7* can control the sensitivity of circadian clock photoresponses (Kaczorowski and Quail, 2003; Michael et al., 2003). In addition, *PRR3* was also identified in both diatom species (Table 1). The expression of the PRR3 protein in the vascular bundles of leaves affects the stability of the TOC1 protein, while a ZTL competitor preferential interacts with TOC1 (Para et al., 2007). *TOC1* and *LUX* were identified in both diatom species, while *PRR9* was identified only in *P. tricornutum*; however, GIGANTEA (*GI*), EARLY FLOWERING 3 (*ELF3*), and EARLY FLOWERING 4 (*ELF4*) were not detected in either species (Table 1). This indicated that the two marine diatom species retained the core components of the circadian clock during evolution, and that the genes missing from the evening loop might have been lost due to evolutionary selection.

The *GI*, CONSTANS (*CO*), and FLOWERING LOCUS T (*FT*) genes of the output pathway were not detected in either of the diatom species, with only *CAB2* being identified (Table 1). In etiolated monocotyledons or dicotyledons, the phytochrome promotes the transcriptional accumulation of *CAB2*, and the *CAB2* promoter is also known to be induced by light and inhibited in the dark (Meyer et al., 1989). We also identified *CK2* in both diatom species (Table 1). In *A. thaliana*, CCA1 and LHY are phosphorylated by CK2 (Lu et al., 2011). *CK2* regulates the circadian clock by influencing the DNA binding of CCA1 (Mulekar and Huq, 2014). The circadian rhythm of plants may have originated in green algae (Matsuo et al., 2008). The fact that we identified the same circadian clock genes in *S. costatum* and *P. tricornutum* as those found in higher plants indicated that marine diatoms and higher plants have evolved a similar biological clock mechanism.

The expression pattern of *CCA1* in *S. costatum* and *P. tricornutum* showed clear diel variation with a cycle of almost 24 h (Fig. 3). In *P. tricornutum*, *LHY* also exhibited a diel expression pattern that was similar to that of *CCA1*, while *LHY* oscillated for about 27 h in *S. costatum* (Fig. 3). In higher plants such as *A. thaliana*, rice, and wheat, *CCA1* and *LHY* were expressed in the morning; expression peaks before and after illumination, decreased during the daytime, and begins to increase again after darkness (Izawa et al., 2003; Murakami et al., 2007). However, in both diatom species, the expression of *CCA1* and *LHY* increased at the onset of darkness, and peaked before dawn, earlier than in higher plants such as *A. thaliana* and rice. The expression pattern of *CCA1* in the two diatoms was similar to that in the green alga *Ostreococcus tauri* (Dong et al., 2011). Moreover, the expression of *TOC1* in the two diatom species was similar to that in *A. thaliana* and other higher plants, and peaked during the night (Dong et al., 2011). The peak expression of *TOC1* in the two diatom species moved backward when compared with higher plants (Alabadí et al., 2001). Moreover, we found that variations in ambient light and levels of N and P affected the expression patterns of the three core clock genes in *S. costatum* and *P. tricornutum*, indicating that light, N and P might improve their environmental adaptability by adjusting the circadian clock. However, the expression patterns between the two diatoms under conditions of changing light and N and P availability were different, indicating species-specific responses to environmental change. Flowering plants live in a terrestrial environment while diatoms live in an aquatic environment, and these habitats differ in both light com-

position and intensity. These factors are likely to have played important roles in driving the differentiation of circadian clock genes during evolution.

Evolutionary analysis of the core clock genes revealed that, in *S. costatum*, *P. tricornutum*, and *Chlamydomonas reinhardtii*, *CCA1* was located in different evolutionary branches, and had diverged from that of *A. thaliana* and *B. campestris*, but was closely related to that of *Helianthus annuus*, *Nicotiana tabacum*, *Picea abies*, and *Ananas comosus*. In the two diatom species, *LHY* was located in evolutionary branches different from those of *Vigna radiata*, *Gossypium hirsutum*, *Cucumis melo*, *Anthurium amnicola*, and *A. thaliana*, indicating that *LHY* in the two diatoms had diverged from those of other species. The *TOC1* genes of *S. costatum*, *P. tricornutum*, and *Schizosaccharomyces pombe* were clustered at a common branchpoint and had a close evolutionary relationship. We speculate that the physiological function of the marine diatom circadian clock may have differentiated throughout evolution. That marine diatoms and higher plants possess the same circadian clock genes suggests that functional differentiation may have occurred before the emergence of terrestrial plants.

5 Conclusions

In this study, the circadian clock genes and their expression patterns in marine diatoms were investigated for the first time. We identified circadian clock genes in *S. costatum* and *P. tricornutum* that have already been described in *A. thaliana* and other higher plants. Furthermore, most of the clock genes were the same in the two diatom species, with some exceptions. Our results indicated that marine diatoms have evolved circadian clock mechanisms suited to their environment. Based on the circadian clock genes identified in marine diatoms and the established circadian clock model for higher plants, we constructed a speculative molecular model for the circadian clock in marine diatoms, including the input pathway, core oscillator, output pathway, and valve effector (Fig. 5). The core clock genes of the two diatom species, *CCA1*, *LHY*, and *TOC1*, showed circadian oscillations for periods of approximately 24 h, but changes in ambient light and N and P availability induced phase shifts of these genes which might adjust the circadian clock performance to adapt to the environmental changes. However, their expression patterns differed from those of terrestrial plants. Homology and cluster analyses further showed that, in *S. costatum* and *P. tricornutum*, the three core clock genes had evolved separately from those of other plants, even though they shared the same genetic clock components. Future efforts should be devoted to investigating circadian clock genes in other diatom species and characterizing their expression patterns under different environmental conditions, which should significantly advance our understanding of how the circadian clock allows marine diatoms to respond and adapt to environmental changes.

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Supplementary information:

Table S1. Circadian clock genes identified in eukaryotic plants.
Table S2. Primers for qRT-PCR analysis of circadian clock genes in *Skeletonema costatum*.
Table S3. Primers for qRT-PCR analysis of circadian clock genes in *Phaeodactylum tricornutum*.

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