

Cell cycle propagation is driven by light–dark stimulation in a cultured symbiotic dinoflagellate isolated from corals

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Abstract Endosymbiosis is an intriguing plant–animal interaction in the dinoflagellate–Cnidaria association. Throughout the life span of the majority of corals, the dinoflagellate *Symbiodinium* sp. is a common symbiont residing inside host gastrodermal cells. The mechanism of regulating the cell proliferation of host cells and their intracellular symbionts is critical for a stable endosymbiotic association. In the present study, the cell cycle of a cultured *Symbiodinium* sp. (clade B) isolated from the hermatypic coral *Euphyllia glabrescens* was investigated using flow cytometry. The results showed that the external light–dark (L:D) stimulation played a pivotal role in regulating the cell cycle process. The sequential light ($40\text{--}100\ \mu\text{mol m}^{-2}\ \text{s}^{-1} \sim 12\ \text{h}$) followed by dark ($0\ \mu\text{mol m}^{-2}\ \text{s}^{-1} \sim 12\ \text{h}$) treatment entrained a single cell cycle from the G_1 to the S phase, and then to the G_2/M phase, within 24 h. Blue light ($\sim 450\ \text{nm}$) alone mimicked regular white light, while lights of wavelengths in the red and infrared area of the spectrum had little or no effect in entraining the cell cycle. This diel pattern of

the cell cycle was consistent with changes in cell motility, morphology, and photosynthetic efficiency (F_v/F_m). Light treatment drove cells to enter the growing/DNA synthesis stage (i.e., G_1 to S to G_2/M), accompanied by increasing motility and photosynthetic efficiency. Inhibition of photosynthesis by 3-(3, 4-dichlorophenyl)-1, 1-dimethyl-urea (DCMU) treatment blocked the cell proliferation process. Dark treatment was required for the mitotic division stage, where cells return from G_2/M to G_1 . Two different pools of adenylyl cyclase (AC) activities were shown to be involved in the growing/DNA synthesis and mitotic division states, respectively.

Keywords Endosymbiosis · Cell division · Cnidaria · Photoperiod · Flow cytometry · *Symbiodinium*

Introduction

The mutualistic symbiosis of microalgae inside host cells of Cnidaria is an intriguing phenomenon whose cellular and molecular regulation mechanisms remain to be elucidated (Douglas 2003; Furla et al. 2005). In coral reefs, more than 10^6 dinoflagellate (*Symbiodinium* sp.) per cm^2 colony-surface area reside inside the gastrodermal cells of the host (Falkowski et al. 1993; Smith and Muscatine 1999). *Symbiodinium* density during endosymbiosis has been examined and shown to be regulated depending on the feeding status and light–dark photoperiod of the host (Fitt 2000). As a consequence, the regulation of symbiont numbers and how they may synchronize with the division and proliferation of the host cells becomes a key feature in the maintenance of a stable endosymbiotic association (Reimer 1971; Falkowski et al. 1993). Disruption of this homeostatic regulation results in a decrease in the number

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of either symbiont or host cells, which may be in accordance with cell death or coral bleaching.

So far, only one physiological explanation has been proposed for the regulation of cell proliferation between the symbionts and the host cells. Population control of both the symbionts and hosts is regulated by nutritional status during this association (Falkowski et al. 1993; Smith and Muscatine 1999). In this *Symbiodinium*–Cnidarian association, the *Symbiodinium* contribute significantly to the host nutrition through the translocation of a photosynthetic product to the host; in return, they receive high concentrations of inorganic nitrogen from the host. *Symbiodinium* in fed anemones (*Aiptasia pulchella*) exhibited more than a 2-fold higher division frequency than did starved polyps. In fact, flow cytometric and microscopic analyses of *Symbiodinium* sp. revealed that the G₁ phase of their cell cycle was lengthened dramatically in the symbiotic state. This slowing in cell cycle also reflected in the usual low percentage of dividing cells (3–5%) in sea anemones (*A. pulchella*) or scleractinian coral (*Astrangia poculata*) (Smith and Muscatine 1999; Dimond and Carrington 2008). On the other hand, under normal environmental conditions, the symbiotic *Symbiodinium* population was maintained by the low-level expulsion of *Symbiodinium* cells from the host coelenteron (Falkowski et al. 1993; Dimond and Carrington 2008). This low-level expulsion is thought to balance increases in symbiont numbers that occur through *Symbiodinium* division. A similar phenomenon has been observed in paramecium–algae endosymbiosis in which the growth phase of the host cells was found to determine the cell cycle of the symbiotic algae (Kadono et al. 2004). Based on these results, it clearly indicates that during symbiotic state, the symbiont *Symbiodinium* proliferation is under the host regulation. However, the cellular and molecular mechanisms cannot be elucidated without first examining the cell cycle regulation in free-living *Symbiodinium*.

Plant cells have developed several mechanisms to enable them to coordinate gene expression and enzymatic activities with light signals (see Lee et al. 2008 for review). The synchronous growth and division of photosynthetic cells that are exposed to alternating periods of light and darkness is a common phenomenon in nature. In dinoflagellates, the circadian phasing as control by light–dark period has been shown to be the pivotal regulation for cell proliferation (Wong and Kwok 2005). The exposure to light–dark cycles has been used as a cue for the synchronization of cell division in many phototrophic algal species. In unicellular green algae, the light-induced stimulation of cell division has been described by Spudich and Sager (1980), and the action spectrum reveals two peaks at 485 and 674 nm. This light induction of cell division was sensitive to DCMU (Munzner and Voigt

1992). In phototrophic algae such as *Euglena gracili*, cellular cAMP levels oscillate in a diel-dependent fashion, with two peaks of cAMP concentrations (Leighfield and Van Dolah 2001). Although similar results have been demonstrated in dinoflagellates (Lam et al. 2001; Leighfield and Van Dolah 2001), there has been little investigation into how light–dark photoperiods may regulate the cell proliferation of *Symbiodinium*. Therefore, in the present study, the cell cycle of cultured *Symbiodinium* was examined to investigate how light–dark treatment may regulate its cell proliferation. These results should provide crucial evidence to elucidate the mechanism by which endosymbiotic status may regulate the cell cycle of symbionts and how host cells may be inter-regulated during endosymbiotic association.

Materials and methods

Coral collection, preparation of inside-out tentacles, and gastrodermal cell dissociation

Coral colonies of *Euphyllia glabrescens* were collected by SCUBA divers from the inlet of the Third Nuclear Power Plant (21°57'22.56"N, 120°45'17.46"E) at depths of 3–8 m on fringing reefs in Southern Taiwan. Colonies were immediately transferred into fresh seawater and placed in an upright position in an outdoor aquarium with flow-through seawater (exchange rate ~2% per hour) and left to recover before use.

In order to examine the distribution of symbionts inside the gastrodermal cell of corals, an inside-out manipulation of amputated tentacles and symbiotic gastrodermal cell dissociation were performed according to published methods (Chen et al. 2005; Huang et al. 2007). Cells were then suspended in filtered seawater (FSW) at a final concentration of $1 \times 10^6 \text{ ml}^{-1}$ and used for staining.

Cryosection examination and microscopy

Portions of tentacle fragments (~0.5–1 cm in length) from *E. glabrescens* were fixed immediately with 3.6% paraformaldehyde at 4°C overnight. The samples were then sectioned at a thickness of 10 µm at –30°C using a Leica CM 1850 Cryostat (Leica Microsystems Nussloch GmbH, Heidelberg, Germany). The tissue structure of the tentacles was then investigated by microscopy as follows. Differential interference contrast (DIC) microscopy and fluorescence microscopy were performed using an upright microscope (Axioskop 2 plus, Zeiss) equipped with epifluorescence optics for various fluorophores, including Cy3 and 4', 6-diamidino-2-phenylindole (DAPI). A CCD camera (Quantix or CoolSNAP-Proc, Photometrics Ltd.,

AR) was used to obtain the microscopic images using suitable exposures. Confocal microscopy was performed using an LSM 510 confocal microscope (Zeiss).

Symbiodinium culture and clade identification

The *Symbiodinium* sp. used in this study were directly isolated from *E. glabrescens*. Tentacles were removed and homogenized in microfuge tubes containing 5 ml of FSW. The homogenate was then centrifuged for 5 min at 400g. The supernatant was discarded, and the pellet was washed with FSW three times. It was then filtered through a 20 μm nylon filter, and the filtrate centrifuged for 5 min at 400g. The pellet was washed with an artificial seawater (ASW; 420 mM NaCl, 26 mM MgSO_4 , 23 mM MgCl_2 , 9 mM KCl, 9 mM CaCl_2 , 2 mM NaHCO_3 , 10 mM Hepes, pH 8.2) at least five times to remove bacteria and other debris. Finally, the pellet was suspended in Guillard's medium (f/2, from Sigma) containing antibiotics (streptomycin 100 $\mu\text{g ml}^{-1}$ and penicillin 100 units ml^{-1}). Cells were cultured at 25°C with light (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in a cycle of 12-h light/12-h dark (12L/12D), and were replenished with fresh medium every 5 days. Only a culture that had been growing in the laboratory for at least 1 month was used for the experiment; this culture exhibited motile to static stages, indicating a free-living state and a culture free of host gastrodermal membranes.

Restriction fragment length polymorphism (RFLP) analysis of *Symbiodinium* 18S rDNA was used to identify the clade of the culture *Symbiodinium* according to the published procedure (Rowan and Powers 1991). Basically, *Symbiodinium* nuclear small subunit (n18S-rDNA) was amplified by polymerase chain reaction (PCR) from these isolates using primers ss5z and ss3z and digested with *Taq I* and *Sau3A I* (Promega) restriction enzymes. Digestion products were separated by electrophoresis in 2% 0.5 \times Tris–borate (TBE) agarose gels to generate RFLP pattern. RFLP patterns analysis was compared to the literature to assign each culture to one of these established *Symbiodinium* n18S-rDNA RFLP clade. Based on RFLP genotype pattern 885/505 bp for *Taq I*, and 765/500 bp for *Sau3A I*, it showed that the cultured *Symbiodinium* to be the clade B.

Diel light–dark photoperiods with variations in duration, intensity and wavelength

In order to study the effect of light/dark duration on the *Symbiodinium* cell cycle, cells were first cultured as usual (i.e., 12L/12D), followed by treatments with different durations of daily light (h)/dark (h) regimes, including 0L/24D, 3L/21D, 6L/18D, 9L/15D, 12L/12D, 15L/9D, 18L/6D, 21L/3D, and 24L/0D, with a light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. To study the effect of light intensity,

cells were cultured with a regular light/dark duration (i.e., 12L/12D) using various light intensities (40–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). To study the effect of light wavelength, cells were cultured in a growth chamber with an LED as the light source (700F LED, HIPOINT[®], Jih Her Tyan Scientific Co., Ltd., Taiwan). The duration of the light/dark regime was maintained at 12L/12D, while lights (intensity at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of different wavelengths were used, including blue (450 \pm 10 nm, mean \pm SD), red (660 \pm 10 nm), infrared (735 \pm 10 nm), or mixed (i.e., blue plus red plus infrared).

Inhibitory effects on photosynthesis and AC activity

Both photosynthesis and cellular cAMP level have been shown to involve in cell cycle regulation in various autotrophic dinoflagellates (Lam et al. 2001; Wong and Kwok 2005) and unicellular alga (*Euglena gracilis*; Yee and Bartholomew 1989). As a consequence, inhibitors for photosynthesis and adenyl cyclase were used to modulate photosynthesis and cellular cAMP, and to characterize how the cell cycle could be regulated. 3-(3, 4-Dichlorophenyl)-1, 1-dimethyl-urea (DCMU; Sigma, St. Louis, MO, USA) at 10^{-5} M was added to the culture of *Symbiodinium* at the beginning of the experiment. Two types of adenine nucleoside derivatives, which inhibit AC activity with isozyme-dependent sensitivity (Johnson et al. 1997), 9-(tetrahydro-2-furyl)-adenine (SQ 22536), and 9-(cyclopentyl)-adenine (9-CP-Ade) (Sigma, St. Louis, MO, USA) at 10^{-7} to 10^{-5} g ml^{-1} , were added to the culture of *Symbiodinium* at two different points in the diel cycle and the corresponding cell cycle. Cells were then harvested every 6 h throughout the diel cycle, and the cell cycle distribution was determined by flow cytometry as described below.

Cloning and purification of green fluorescent protein from *E. glabrescens*

Total RNA of *E. glabrescens* was extracted from tentacles with TRIzol (Invitrogen, Carlsbad CA, USA), and total cDNA was synthesized using the SuperScript[™]III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instruction. cDNA of the green fluorescent protein (denoted as egGFP with MW \sim 29 kDa) contains 1,050 bp and the open reading frame was 678 bp and can be translated into 225 deduced amino acids. The ORF egGFP was amplified by PCR using *Pfx50*[™] (Invitrogen). The product was sequenced to confirm that there was no deviation from the original sequence, and was subsequently cloned into the vector (pTrcHis TOPO[®] TA Expression Kits; Invitrogen). After overnight culture, TOP10 *E. coli* strain was induced to the protein expression by 0.1 M IPTG. 6XHis tag GFP protein was purified by a simple

purification process according to the manufacturer's instructions (QuickPick™ IMAC Plus, Bio-Nobile Finland, USA).

To investigate the effect of egGFP on the *Symbiodinium* cell cycle, a *Symbiodinium* culture was incubated with either bovine serum albumin (BSA) or egGFP (final concentration of 10^{-7} to 10^{-5} g ml $^{-1}$) immediately prior to 12L/12D treatment.

Flow cytometrical analyses of cell cycle progression

Cell densities of cultures from the exponential growth stage were maintained at 5×10^4 ml $^{-1}$ for the experiment. Cells ($\sim 5 \times 10^5$ – 10^6 cells ml $^{-1}$) were collected every 6 h at specific times during the photoperiod of light–dark stimulation (see Fig. 1; sampling time was designed as “T” followed by numerals representing hours after the initial light on, such as T00, T05, T11, T17, T23, etc.). After centrifugation (68g for 5 min), cells were fixed by ice-cold 70% ethanol (~ 1 h) in the presence of bright light illumination ($18 \mu\text{mol m}^{-2} \text{s}^{-1}$) to bleach the chlorophyll content. Cells were then incubated in phosphate-buffered saline (PBS) containing Triton-X 100 (0.1%), RNase ($10 \mu\text{g ml}^{-1}$), and propidium iodide ($30 \mu\text{g ml}^{-1}$) at 4°C overnight in the dark. The DNA content per cell was measured based on the DNA–PI complex fluorescence under 488/610 nm (E_x/E_m) using an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc., Fullerton CA, USA). Histograms of relative DNA content were analyzed using MultiCycle AV for Windows V5.0 (Phoenix Flow Systems, San Diego, CA) in order to quantify the percentage of cells at each stage (G_1 , S, G_2/M). Each sample testing was repeated at least three times ($N = 3$).

In some cases, cell walls (or cellulose) of *Symbiodinium* were stained with Calcofluor white M2R (CFW, from Sigma) at a final concentration of 25 μM (from a 0.1% w/v stock solution in PBS) at room temperature for 1 h.

Measurement of photosynthetic efficiency

Microscopic measurement of photosynthesis efficiency was performed using an Axiovert 200 fluorescence microscope

(Carl Zeiss) connected to a chlorophyll fluorescence microfluorometer and a Pulse Amplitude Modulation microfluorimeter (PAM; Heinz Walz GmbH, Effeltrich Germany). A single blue LED (470 nm) emitted pulsed actinic, saturation, and measuring lights for PAM measurement. For the PAM acquisition, a photomultiplier served as the fluorescence detector, which was operated in conjunction with a PC and WinControl software (Walz). *Symbiodinium* were plated on cover slides for PAM microfluorimetry. The fluorescence measured by PAM-microscopy was excited in the portion of cells that were collected. Photochemical efficiency was determined as F_v/F_m (maximum quantum yield of PSII) of individual symbiotic endoderm cells according to the reported procedures and instrument protocols (Beer et al. 1998).

Statistical analysis

The statistical significance among treatments was evaluated using the analysis of variance (one-way ANOVA followed with Duncan's multiple-range procedure, $P < 0.05$) or Student's *t*-test ($P < 0.05$).

Results

Coral–dinoflagellate endosymbiotic association occurs where *Symbiodinium* sp. resides inside the gastrodermal cells of the diploblastic host Cnidaria. Microscopic examination of cross-sections of tentacles from *E. glabrescens* showed that the average thickness of the host gastrodermal layer is ~ 70 – $80 \mu\text{m}$ (Fig. 2a), which raised the possibility that there may be multiple numbers of *Symbiodinium* ($\sim 10 \mu\text{m}$ in diameter), inside a single gastrodermal cell (Fig. 2a). This prediction was confirmed by directly examining isolated symbiotic gastrodermal cells (Chen et al. 2005; Huang et al. 2007), by which method the distribution of *Symbiodinium* in a host gastrodermal cell can be observed. As shown in Fig. 2b and c, isolated symbiotic gastrodermal cells were found to contain multiple numbers of *Symbiodinium*. This phenomenon suggests the presence

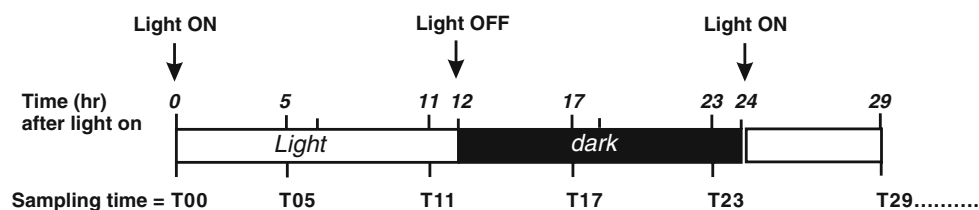


Fig. 1 Sampling time for *Symbiodinium* sp. during a light–dark photoperiod cycle. During normal 12L/12D treatment, *Symbiodinium* sp. were collected for flow cytometrical analyses at five (T05), eleven

(T11), seventeen (T17), twenty-three (T23), twenty-nine (T29), etc. hours after the initial light ON

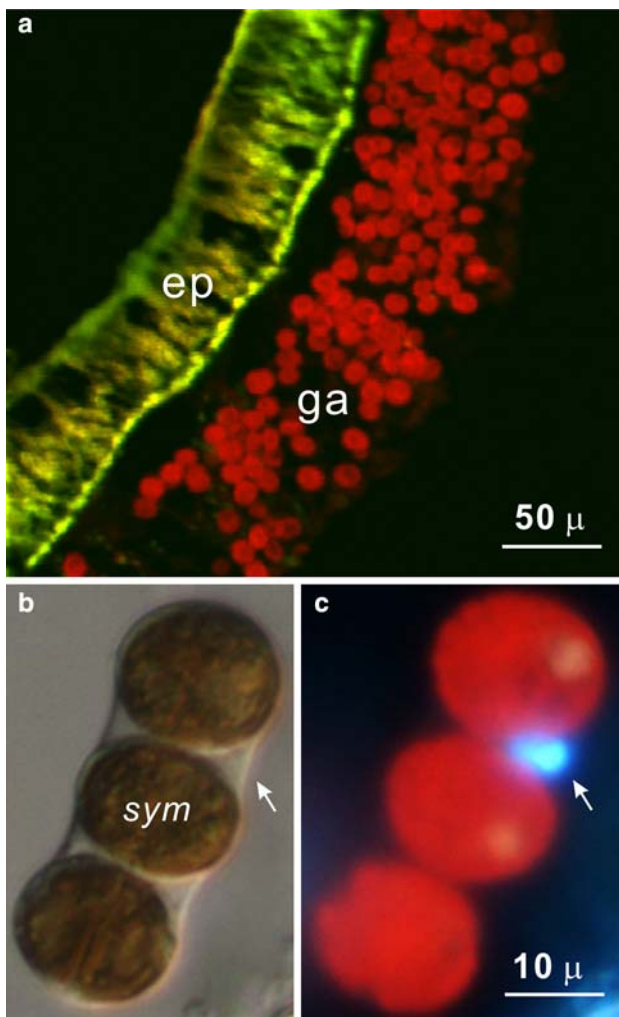


Fig. 2 In situ distribution of *Symbiodinium* sp. in *E. glabrescens*. **a** The confocal section of a tentacle shows the distribution of *Symbiodinium* sp. (red fluorescent) in the gastrodermis (ga) of the tissue. The epidermis (ep) is green fluorescent due to the presence of green fluorescent protein. **b** and **c** DIC and fluorescent microscopy images of a gastrodermal cell containing three *Symbiodinium* (sym). The nucleus of the gastrodermal cell is stained by Hoechst 33342 and is indicated by arrows

of an underlying mechanism for stable endosymbiosis that works by homeostatically regulating the cell proliferation of the host cell and its intracellular symbionts. For example, an interesting question is immediately raised: how are these multiple symbionts assorted at the time of host cell division. On the other hand, how does the host cell respond at the time of symbiont division. In order to tackle these questions, it is critical to first understand how the cell cycle of cultured *Symbiodinium* is regulated. In the present study, *Symbiodinium* sp. was isolated from the host coral *E. glabrescens* and was cultured for use. According to the RFLP analysis, this culture belongs to clade B (data not shown).

Cell cycle of *Symbiodinium* under the treatment of light–dark photoperiods

Flow cytometry analysis was used to characterize the cell cycle of cultured *Symbiodinium* for 3 days under various light–dark stimulations (Fig. 3). By varying the duration of light irradiation at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ between 0 (0L/24D, Fig. 3b), 12 (12L/12D, Fig. 3a) and 24 h (24L/0D, Fig. 3c), cell cycle progression from the growing/DNA synthesis (i.e., G_1 to S to G_2/M) and mitotic division (or “cytokinesis,” i.e., G_2/M to G_1) stages was regulated. First, under the 12L/12D treatment (Fig. 3a), cells progressed from G_1 (T05; i.e., 5 h after initial light on, see also Fig. 1) to S (T11), and then to the G_2/M phase during the first 12 h of light stimulation. Upon the photoperiod entering the dark stimulation, there were no new DNA synthesis (i.e., the S phase) initiated, while more cells entered the G_2/M phase (see T17 and T23), followed by cell division, and finally resulted in the cytokinesis to generate G_1 cells for the next cycle (T29). The cell cycle stage during the sampling time in a normal single 12L/12D period can be summarized: T05 contained G_1 cells; T11 contained G_1 , S, and G_2/M cells; T17 contained G_1 and G_2/M cells; T23 contained G_1 and G_2/M cells. As shown in T17, T23, T41, T47, T65, and T71 (Fig. 3a), approximately 40–55% of G_1 cells remained quiescent for each cycle. Furthermore, cells could be synchronized and their cell cycle could be halted at the G_1 stage in the absence of light irradiation (Fig. 3b). It is important to note that there were no DNA synthesis and cell growth under dark treatment as there was almost no cell size change (data not shown). Furthermore, prolonged light irradiation (24L/0D) also significantly altered the cell cycle, as shown in Fig. 3c. First, continuous light stimulation at day 1 entrained G_1 cells to enter the S and then G_2/M phases, and resulted in more G_2/M cells than that in the control cycle at T17 ($\sim 80\%$ vs. $\sim 50\%$). This was due to the fact that more G_1 cells at T11 were induced to enter the S and then G_2/M phases under the continual light irradiation between T11 and T17. Continual light irradiation not only stimulated cells to enter G_2/M phase until there was almost no G_1 cells, but also generated increasing number of cells containing triple and tetra copies of chromosomes by blocking the cytokinesis (see $3n-4n$ in T23, T29, T35, T41, T47, and T53). The appearance of cells with $3n-4n$ chromosomes increased until T59, when mitotic division was resumed to generate G_1 cells.

The cell cycle of the *Symbiodinium* sp. was further examined with regard to several physiological and morphological functions, including cell morphology, motility, and photosynthetic efficiency. As shown in Fig. 4, upon the initial-light irradiation (“Light ON” in Fig. 4a) during 12L/12D treatment, cells were smaller in size and motile as G_1 gymnodinoids with a visible epicone and hypocone (for

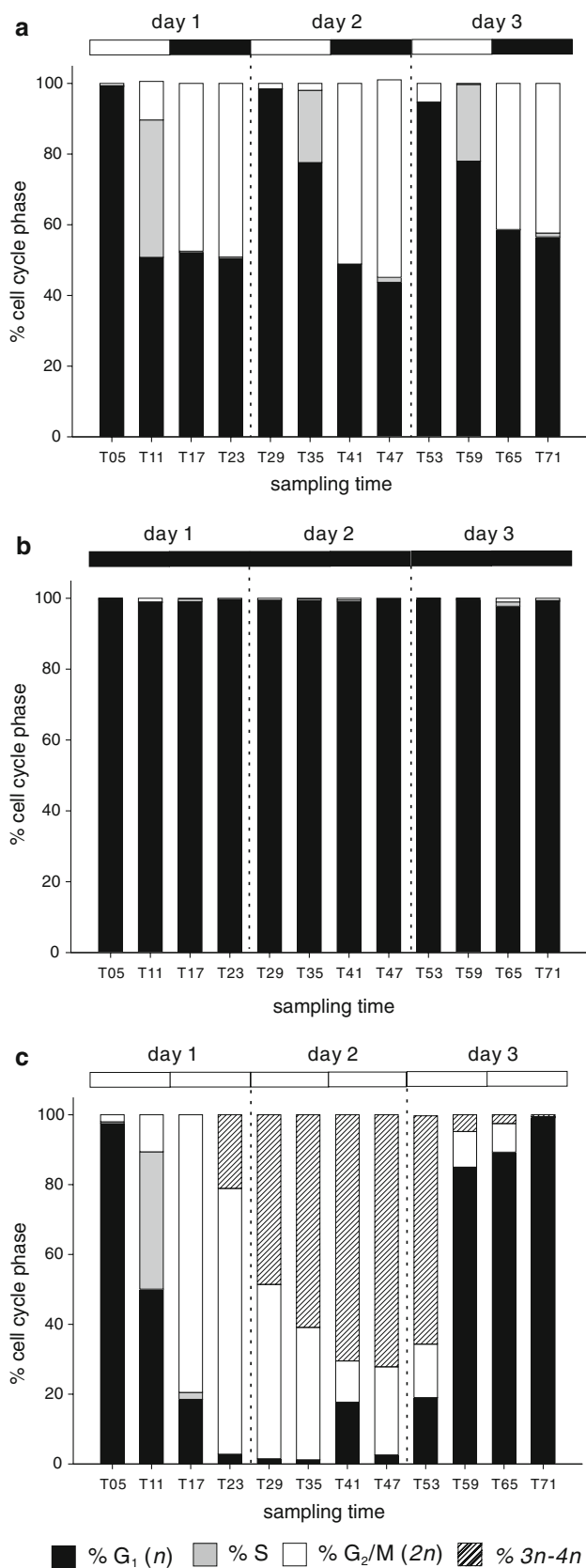


Fig. 3 Effect of the light–dark photoperiod on the cell cycle of the cultured *Symbiodinium* sp. during a 72-h incubation. Cultured *Symbiodinium* were treated for 72 h under three different photoperiods: **a** 12L/12D, **b** 0L/24D, and **c** 24L/0D. Cells were then collected at specific sampling times (see Fig. 1) and processed for cell cycle analysis using flow cytometry, as described in “Materials and methods.” Histogram values for each cell stage (G₁, S, G₂/M and 3n–4n) are averaged from triplicate experiments (N = 3)

example, see G₁ at T05, Fig. 4b). The percentage of motile G₁ gymnodinoids increased to the maximum (32.7 ± 5.3%, mean ± SD) at around 5 h after light stimulation (T05, Fig. 4a), then decreased to the basal level at T11, when cells entered the S and G₂/M phases. During the dark treatment (as indicated by the “Light OFF” arrow in Fig. 4a), cells at the G₂/M phase were larger in size and remain as immotile coccoids (see also T17 and T23 at Fig. 4b). The changes in morphology and motility were also consistent with photosynthetic efficiency (F_v/F_m). As shown in Fig. 4a, during the light irradiation, photosynthetic efficiency gradually increased to a maximal F_v/F_m (0.6 ± 0.1, mean ± SD) at T05 and then decreased, returning to background level after the dark treatment (T17 and T23).

The effect of light–dark treatment on the *Symbiodinium* cell cycle was further investigated in order to understand how duration changes in the light–dark photoperiod may affect the different stages of the cell cycle. Cells were first cultured regularly (i.e., 12L/12D), followed by a 2-day culture where the duration of light–dark photoperiod was varied as 0L/24D, 3L/21D, 6L/18D, 9L/15D, 12L/12D, 15L/9D, 18L/6D, 21L/3D, or 24L/0D. Cell stages were then examined as previously to study how (i) the growing/DNA synthesis stage (G₁ to S to G₂/M) and (ii) the mitotic division stage (i.e., cytokinesis: G₂/M to G₁) were regulated by analyzing the percentage of G₂/M cells at T23 or G₁ cells at T29, respectively (refer to Fig. 3a). As shown in Fig. 5, increasing the duration of light irradiation (i.e., decreasing the duration of dark treatment) from 0L/24D to 15L/9D at 40 μmol m⁻² s⁻¹ resulted in an increase in the transition of cells from G₁ to S to G₂/M (see filled circles “% of G₂/M phase at T23”; i.e., the growing/DNA synthesis stage). Interestingly, under this treatment, most G₂/M cells underwent mitotic division during the dark treatment and returned to G₁ at the first sampling time of next cycle, resulting in ~100% cells in G₁ stage at T29 (see “% G₁ at T29”). However, longer periods of light stimulation, such as 18L/6D, 21L/3D, and 24L/0D, not only resulted in further increased of cell numbers at G₂/M at T23, but also resulted in significantly decreased of cell numbers at G₁ at T29. Under these longer light stimulation, conversion of cells from G₁ to G₂/M increased to reach the maximum

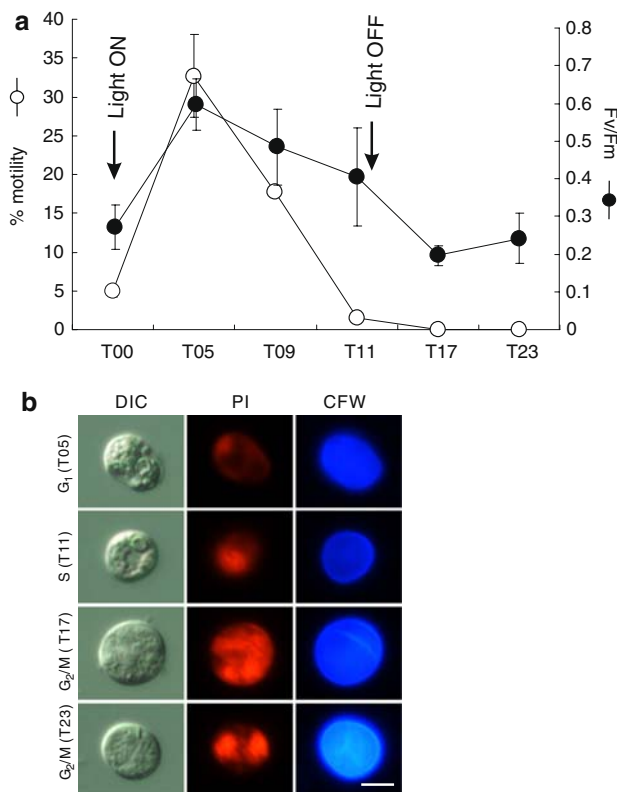


Fig. 4 Changes in morphology, motility and photosynthesis efficiency in the cultured *Symbiodinium* during the cell cycle. *Symbiodinium* sp. at different cell cycle stages (i.e., G₁, S, G₂/M) were collected at specific sampling times (i.e., T05, T11, T17 and T23) during 12L/12D treatment. **a** Cellular motility (blank circles) and photosynthesis efficiency (filled circles, F_v/F_m) were measured simultaneously using PAM-microscopy. For both measurements, five (5) repetitions were performed. Values were expressed as mean \pm SD. **b** The morphology, DNA and cellulose contents were examined by DIC and fluorescence microscopy after PI and CFW staining as described in “Materials and methods.” Scale, 5 μ m

(~76%) under the 24L/0D treatment, while the mitotic division to form G₁ cells decreased from ~100% to ~1.58%. This is due to the fact that increasing the duration of light irradiation led more cells to grow to G₂ with no further division, resulting in the formation of cells containing the 3n–4n chromosome, as also shown in Fig. 3c. This simultaneously resulted in the decrease of G₁ cell percentage at T29. Changes in light intensity (40–100 μ mol m⁻² s⁻¹) did not affect the cell cycle (data not shown).

The effect of irradiation wavelength on the cell cycle progression was then examined (Fig. 6). The *Symbiodinium* culture was irradiated with a normal 12L/12D cycle by different wavelengths (blue, red, infrared, or mixed lights) at the same intensity (40 μ mol m⁻² s⁻¹). As shown in Fig. 6a and b, irradiation with both the control mixed (i.e., blue plus red plus infrared) and blue wavelengths of light were able to complete the cell cycle progression normally.

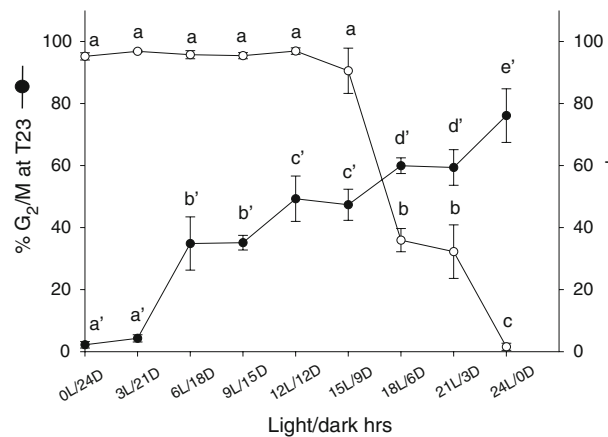
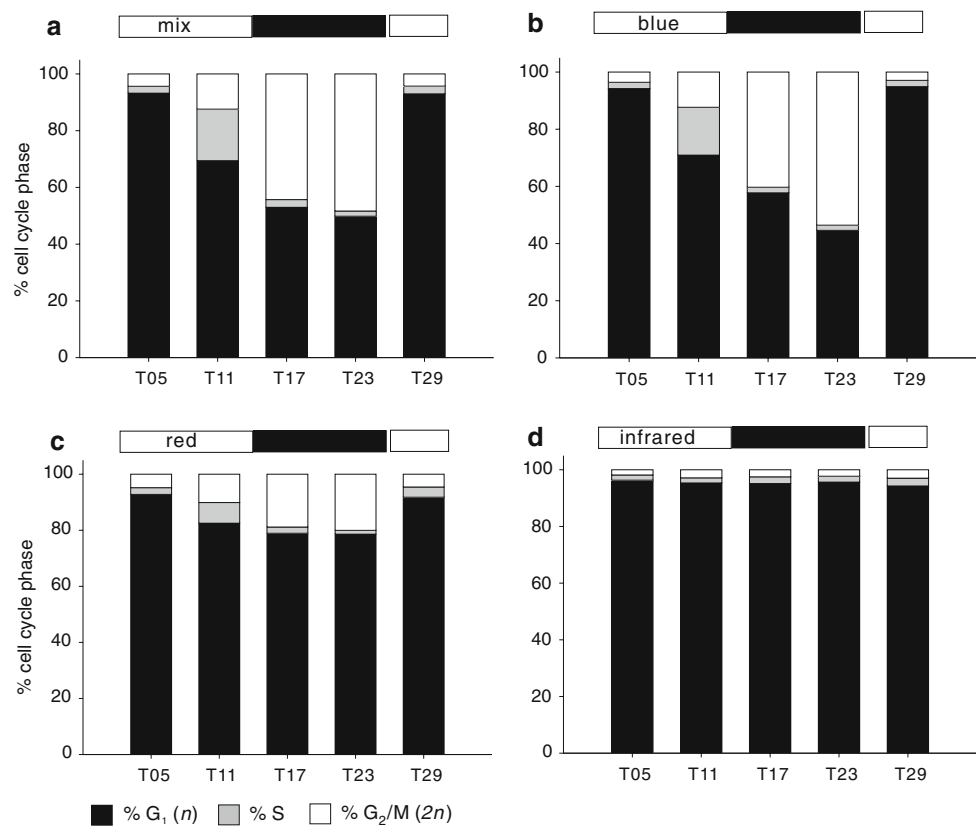


Fig. 5 Effect of the duration of the light–dark photoperiod on *Symbiodinium* growth/DNA synthesis and mitotic division. Two-day cell cycle analyses of *Symbiodinium* sp. were performed in which the light–dark duration varied from 0L/24D to 24L/0D. The cell percentage at the G₂/M phases at T23 (filled circle) and the G₁ phase at T29 (blank circle; i.e., the initial sampling time of the second day) were measured to represent the growth/DNA synthesis (i.e., G₁ to S to G₂/M) and mitotic division (i.e., G₂/M to G₁) stages, respectively. The repetition number of the experiment is 3 for each light–dark treatment. Values were expressed as mean \pm SD. Letters (a–c and a’–e’) denote statistical significance ($P < 0.05$) under different light–dark treatments as analyzed by ANOVA

However, under the red-light irradiation (Fig. 6c), the growth/DNA synthesis was significantly ($P < 0.05$) inhibited, as shown by the decreased percentage of cells at the S phase in T11 ($7.38 \pm 5.28\%$, mean \pm SD) compared to that of the control mixed wavelength treatment ($18.16 \pm 5.22\%$). The decrease of cell growth/DNA synthesis under the red-light irradiation was also reflected in the decrease in the number of cells at the G₂/M phase at T17 ($18.83 \pm 7.25\%$ vs. $44.31 \pm 8.95\%$ in control, $P < 0.05$) and T23 ($20.03 \pm 8.69\%$ vs. $44.31 \pm 8.95\%$ in control, $P < 0.05$). In contrast, cell cycle progression was almost blocked and remained at the G₁ stage under the infrared-light irradiation (Fig. 6d).

Symbiotic dinoflagellates of reef-building corals are vulnerable to strong irradiation typical of shallow tropical coral reefs (Salih et al. 1998). It has been shown that autofluorescent pigments (proteins) in the host tissues appeared to have a photoprotective effect for their symbiont by dissipating the excess irradiation (Salih et al. 2000). In *E. glabrescens*, the green fluorescence protein (egGFP) is enriched in the epidermis (Peng et al. 2008; see also Fig. 2a). Since the blue irradiation alone entrained the *Symbiodinium* cell cycle normally (see Fig. 6b), it is interesting to explore whether egGFP, which absorbs light at the blue wavelength region, may affect the *Symbiodinium* cell cycle progression or not. The cell cycle of *Symbiodinium* under a 12L/12D photoperiod was examined in the absence or presence of the expressed egGFP

Fig. 6 Effect of irradiation wavelength on the *Symbiodinium* cell cycle. Two-day cell cycle analyses of *Symbiodinium* sp. were performed in a normal 12L/12D treatment at different irradiation wavelengths: **a** mixed (i.e., blue plus red plus infrared), **b** blue, **c** red, and **d** infrared. Histogram values for each cell stage (G_1 , S and G_2/M) are averaged from triplicate experiments ($N = 3$)



(10^{-5} g ml $^{-1}$) cloned from *E. glabrescens*. As shown in Fig. 7, the number of cells at the S phase of T11 was decreased by egGFP incubation ($25.72 \pm 4.66\%$ vs. $34.04 \pm 5.51\%$ in control), and the cell cycle progression toward the G_2/M phase during the dark period (i.e., T17 and T23) was significantly retarded in the presence of egGFP (T17: $28.92 \pm 1.01\%$ vs. $56.94 \pm 7.11\%$ in control, $P < 0.05$; T23: $30.43 \pm 2.66\%$ vs. $58.29 \pm 7.14\%$, $P < 0.05$). The mitotic division that retrained cells in G_2/M at T23 to G_1 at T29 was not affected by the egGFP treatment. This indicated that egGFP reacts on the growth/DNA synthesis stage (i.e., G_1 to S to G_2/M) to retard the cell cycle. Similar results were also observed when the *Symbiodinium* sp. cell suspension was immersed inside an egGFP solution to mimic the in situ epidermal environment (data not shown).

Effect of inhibitors of photosynthesis and AC activity

Both the photosynthesis and cellular cAMP have been shown to be pivotal in regulating cell cycle in various dinoflagellates (Yee and Bartholomew 1989; Lam et al. 2001; Wong and Kwok 2005). As a consequence, inhibitors that block their generations were used to examine their effect on the *Symbiodinium* cell cycle progression. The effect of photosynthesis on the cell cycle transit of *Symbiodinium* was examined by treatment with DCMU, which

is a triazine herbicide that has been shown to interrupt photosynthetic electron transfer at the PSII reaction center by specifically binding to the D1 polypeptide of the reaction center core complex (Pfister et al. 1981; Erickson et al. 1985). Cell cycle progression under the normal 12L/12D photoperiod in the absence or presence of DCMU (10^{-7} M) was examined, and the results showed that DCMU completely blocked the cell cycle (Fig. 8).

Adenylyl cyclase degrades ATP to form cAMP, and inhibition of the enzyme activity should decrease the cAMP concentration and its subsequent signaling. Two inhibitors for AC, 9-CP-Ade and SQ 22536, were used to elucidate whether the activities of AC and/or its derivatives such as cAMP may be involved in the regulation of the *Symbiodinium* cell cycle. Treatment of *Symbiodinium* with 9-CP-Ade (10^{-5} g ml $^{-1}$) at the beginning of light irradiation (T0, indicated by the arrow in Fig. 9b) significantly retards the cell cycle progression from G_1 to S and to the G_2/M phase. In comparison with the control (Fig. 9a), the number of cells at the S phase at T11 ($19.07 \pm 0.43\%$ vs. $38.90 \pm 4.82\%$ in control, $P < 0.05$) and the G_2/M phase at T17 ($28.51 \pm 5.82\%$ vs. $51.62 \pm 3.61\%$ in control, $P < 0.05$) and T23 ($27.53 \pm 6.23\%$ vs. $51.75 \pm 5.72\%$ in control, $P < 0.05$) were significantly decreased. These decreases in cells at S and G_2/M were also accompanied by inhibition of mitotic division and resulted in a decrease of cells at G_1 in the next cycle (i.e., T29; $82.55 \pm 1.93\%$ vs.

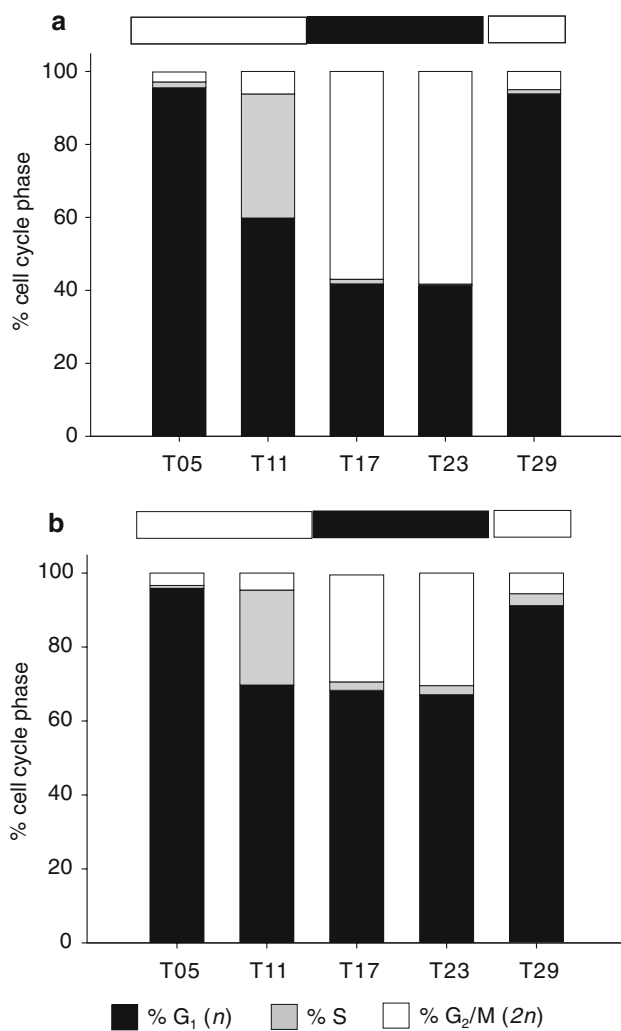


Fig. 7 Effect of egGFP on the *Symbiodinium* cell cycle. Two-day cell cycle analyses of *Symbiodinium* sp. were performed in a normal 12L/12D treatment in the absence (a) or presence (b) of egGFP (10^{-5} g ml $^{-1}$). Histogram values for each cell stage (G₁, S and G₂/M) are averaged from triplicate experiments ($N = 3$). In the presence of egGFP, the progression of cells from the S phase to the G₂/M phase at T17 and T23 was significantly retarded ($P < 0.05$)

$93.21 \pm 1.10\%$ in control, $P < 0.05$). Interestingly, application of 9-CP-Ade at the beginning of the dark period (T17, indicated by the arrow in Fig. 9c) did not affect the phasing of the cell cycle during T17 and T23, but significantly retarded mitotic division of cells and resulted in a decrease in cells at G₁ in the following cycle (T29, $83.62 \pm 2.90\%$ vs. $93.21 \pm 1.10\%$ in control, $P < 0.05$).

The growth/DNA synthesis stage was not affected by the addition of SQ 22536 (10^{-5} g ml $^{-1}$) either at the beginning of light irradiation (T0) or dark treatment (T17) (Fig. 9d, e). However, in both cases, mitotic division was inhibited and resulted in decrease in the number of G₁ cells at T29 ($70.74 \pm 9.58\%$ in Fig. 9d and $69.87 \pm 4.76\%$ in Fig. 9e vs. $93.98 \pm 0.49\%$ in control in Fig. 9a, $P < 0.05$).

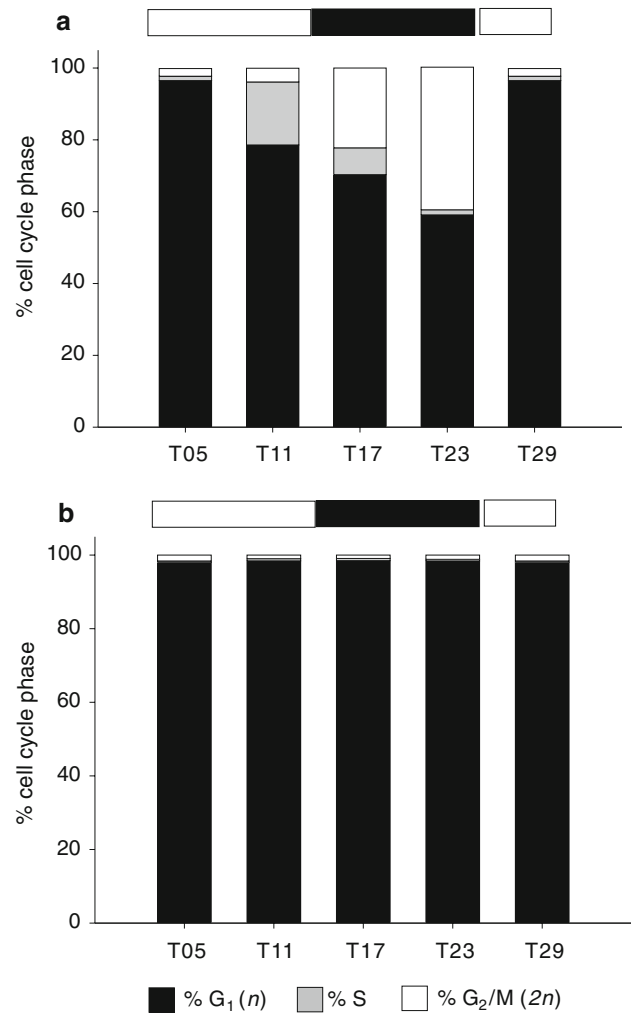
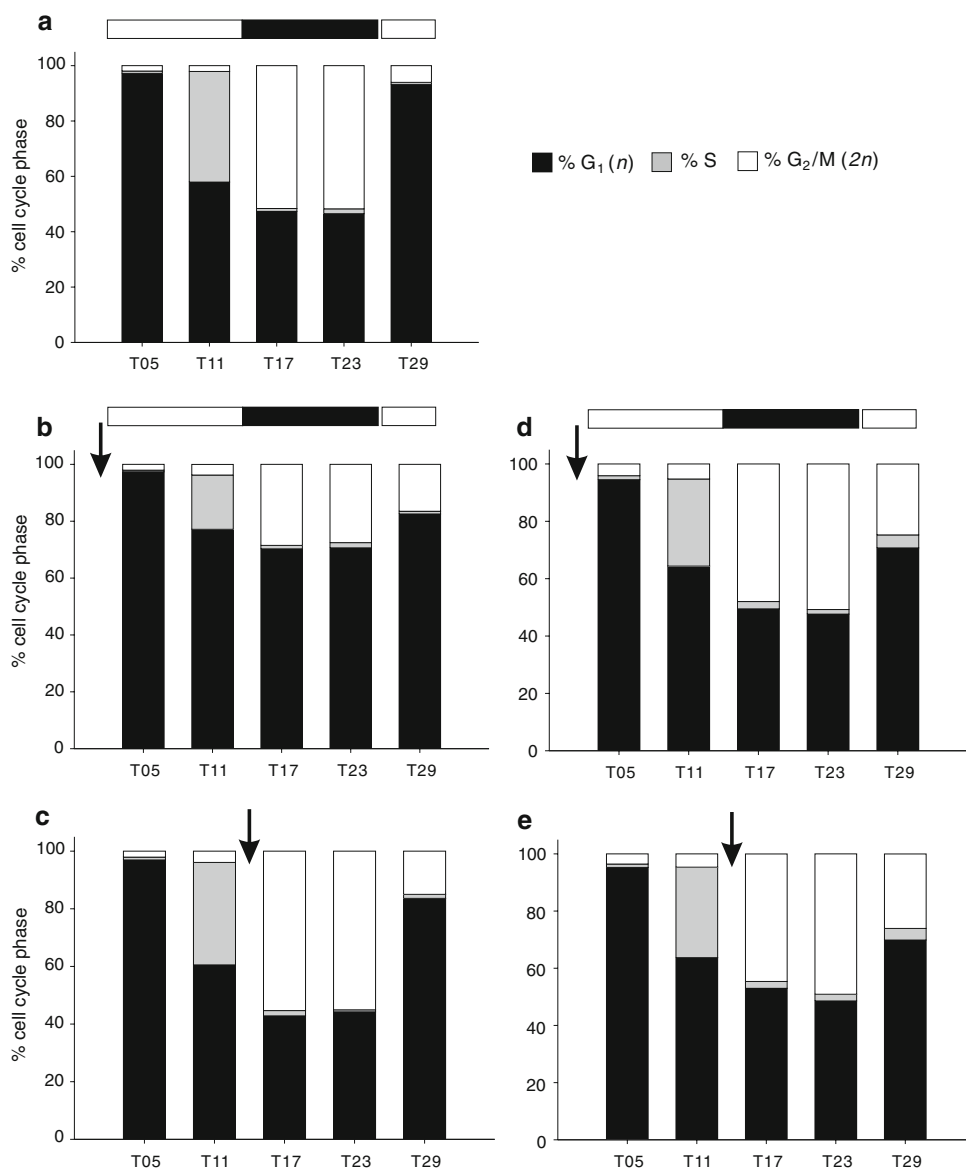


Fig. 8 Effect of DCMU on the *Symbiodinium* cell cycle. Two-day cell cycle analyses of the *Symbiodinium* sp. were performed in a normal 12L/12D treatment in the presence of DMSO (panel a, control) or DCMU (panel b, 10^{-7} M). Histogram values for each cell stage (G₁, S and G₂/M) are averaged from triplicate experiments ($N = 3$). As shown, the cell cycle was completely blocked by DCMU treatment

Discussion

Cell division regulation of symbionts and their host cells is one of the pivotal mechanisms for maintaining a stable endosymbiosis. Nevertheless, its examination at the cellular and molecular level has not been achieved. Previous studies at the physiological level concluded that the stability of symbiotic association required control of population growth of the symbionts (Falkowski et al. 1993). Results accumulated from analyses on different cnidarian species have led to the summary that there are two major factors determining this homeostatic regulation: the nutrition and the light irradiation (McAuley 1985; Smith and Muscatine 1999; Fitt 2000). The external food source immediately affected the host cell growth and division, which in turn determined the

Fig. 9 Effect of AC inhibitors on the *Symbiodinium* cell cycle. Two-day cell cycle analyses of *Symbiodinium* sp. were performed in a normal 12L/12D treatment in the absence (panel a) or presence of 9-CP-Ade (panels b and c; final concentration 10^{-5} g ml $^{-1}$) or SQ 22536 (panels d and e; final concentration 10^{-5} g ml $^{-1}$). Inhibitors were added at the start of either light (arrows in panels b and d) or dark treatment (arrows in panels c and e)



division of its intracellular symbionts, has been shown in the sea anemone *A. pulchella* (Smith and Muscatine 1999) and the hydroid *Myrionema ambionense* (Fitt 2000). Light irradiation of the host, following feeding, was required to ensure the mitosis of symbionts in the hydroid *M. ambionense*, presumably to provide carbon skeletons necessary for completing the cytokinesis. The present study has characterized the cell cycle of clade B *Symbiodinium* isolated and cultured from *E. glabrescens*, using flow cytometry. Several important regulations were identified, which could be critical for future in examining the symbiont division in situ (see below).

In corals, major environmental stresses such as water temperature, increase in irradiation and UV, trigger bleaching (Hoegh-Guldberg 1999; Fitt et al. 2001; Coles and Brown 2003). Corals exhibit interspecific differences in susceptibility to these environmental stresses, and it has

been speculated that differing susceptibility results from physiological variation in stress tolerance among their symbiont *Symbiodinium* (Perez et al. 2001). According to the RFLP analysis, the culture *Symbiodinium* sp. being examined in this study was one of the sub-generic lineages, the clade B. So far, there are at least eight clades (from A to H) of *Symbiodinium* sp. that have been identified from various Cnidaria (Baker 2003; Coffroth and Santos 2005). It is premature to conclude whether or not other clades of *Symbiodinium* exhibit similar cell cycle regulation as that in the clade B due to the lack of systematic comparison. However, among all *Symbiodinium* clades, clade B particularly dominates in corals distributed at higher latitudes in western Atlantic and Caribbean regions (Rowan et al. 1997; van Oppen et al. 2005). It has been shown to exhibit very broad geographical distribution, a large water depth range (5–40 m) and high flexibility of adaptation to large

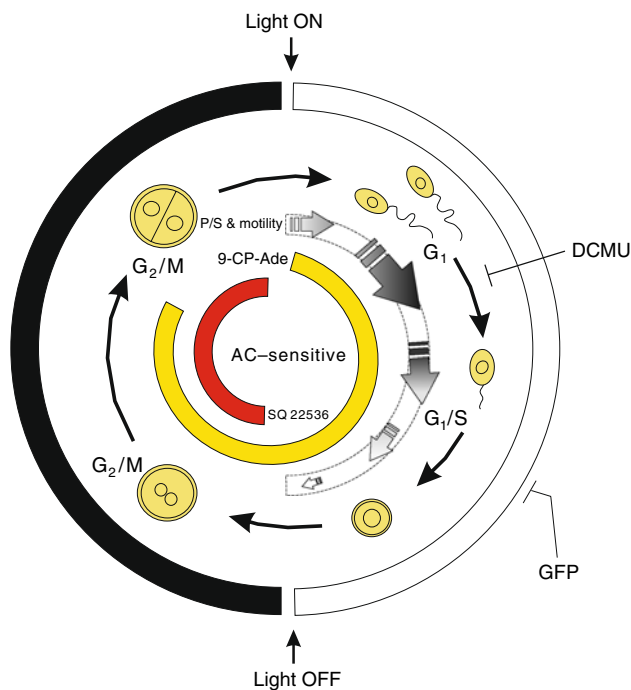


Fig. 10 Summary of the *Symbiodinium* cell cycle

range of temperature and light conditions in seawater throughout its global distribution (LaJeunesse 2004; Karako-Lampert et al. 2005; Frade et al. 2008). As a consequence, the investigation of the cell cycle of the clade B *Symbiodinium* sp. will provide new insights on how corals in different environments may respond to bleaching and other stresses. Results of the present study are summarized in Fig. 10.

The cell cycle of the *Symbiodinium* sp. is entrained by the light–dark photoperiod

As shown in Fig. 10, the light–dark photoperiod (presented as white and dark circular bars for light and dark treatment in a regular 12L/12D period), rather than an endogenous circadian rhythm, is the primary cue for *Symbiodinium* cell cycle progression. Light irradiation is required for the cell growth and DNA synthesis (i.e., G₁ to S to G₂/M), while dark treatment is required for cytokinesis of mitotic division (i.e., G₂/M to G₁). This conclusion is consistent with the finding that the cytokinesis of symbionts is usually completed at dawn or at the beginning of the light photoperiod (Fitt 2000). Furthermore, increasing the duration of light irradiation at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 0 to 24 h enhanced the percentage of cells progressing from G₁ to S and then to G₂/M (see also Fig. 5), indicating that light stimulation increased cell growth and DNA synthesis, but delayed the cytokinesis. Further, supporting evidence is presented in Fig. 3c; the appearance of undivided cells containing 3n–4n

chromosomes indicates the failure of the cytokinesis under continual light irradiation (i.e., in the absence of dark treatment), and the cell cycle was prolonged for 3 days to complete. The delay in cytokinesis and the generation of undivided cells with multiple copies of chromosomes under prolonged light irradiation have not been observed previously in other dinoflagellates. It may be an important regulation process for in situ symbiont division during endosymbiosis. Nevertheless, a similar phenomenon has been observed in green algae *Chlamydomonas reinhardtii*, where prolonged blue and white lights were shown to activate the S phase and shifted the commitment point for cell division later, resulting in the formation of undivided cells containing 8n chromosomes (Munzner and Voigt 1992; Oldenhof et al. 2006). This delayed cell division coincided with postponed kinase activity of the cell cycle regulatory cyclin–CDK complex (Oldenhof et al. 2004).

Effect of photosynthesis and the irradiation wavelength on the cell cycle

The diurnal patterns of both photosynthetic efficiency and motility of *Symbiodinium* were synchronized to the cell cycle (see the “P/S and motility” in Fig. 10). Both the photosynthetic efficiency and motility began to increase at the onset of light irradiation, which correlated with the G₁ stage of gymnodinoid cells; they then decreased when cells entered the S phase. Upon the onset of the dark period, when cells entered the G₂/M phase and became coccoid, both photosynthesis and motility decreased to the basal level. Here, photosynthesis was shown to be essential for the cell cycle progression since the blockage of PSII by DCMU at low concentration (0.1 μM) completely blocked DNA synthesis and further cell cycle progression (see also Fig. 8). The sensitivity of photosynthesis in regulating the cell cycle in *Symbiodinium* is much higher than that in the unicellular alga *Euglena. gracilis*, where DCMU at 10 μM was required to block the cell cycle (Yee and Bartholomew 1989). Furthermore, the photosynthetic efficiency of *Symbiodinium* varied significantly depending on their cell cycle stage. As shown in Figs. 4 and 10, motile gymnodinoid cells at the G₁ phase (sampled at T05, Fig. 4) showed the highest photosynthetic efficiency, while immotile coccoid cells at G₂/M (i.e., T17–T23, Fig. 4) had the lowest photosynthetic efficiency. The motility pattern of *Symbiodinium* during the cell cycle is consistent with the results given by Fitt and Trench (1983), where they concluded that motile gymnodinoid cells at the G₁ phase are newly divided after dark treatment.

Light irradiation at wavelengths in the blue range (~450 nm) has been shown to regulate the cell cycle in various dinoflagellates and green algae (Munzner and Voigt 1992; Oldenhof et al. 2004; Brunelle et al. 2007).

Illumination with blue but not red or infrared lights has been reported to delay the cell cycle by lengthening DNA synthesis phase in the unicellular green alga *Chlamydomonas reinhardtii* (Munzner and Voigt 1992). In the present study, stimulation of *Symbiodinium* with blue light alone in a normal 12L/12D photoperiod mimicked that of white light to entrain the cell cycle normally, while irradiation with either red or infrared light has little or no effect on the cell cycle progression (Fig. 6). At the moment, whether the cell cycle or the photosynthesis is the direct target of light irradiation remains to be elucidated. The action spectrum of *Symbiodinium* photosynthesis in coral tissues spans relatively broad with maxima at red (580–650 nm) and blue (430–450 nm) regions (Kuhl et al. 1995; Salih et al. 2000). It is reasonable to speculate that the irradiation may indirectly affect the cell cycle progression of *Symbiodinium* by modulating the photosynthesis through particular light absorption. If this is the case, both blue and red light should affect the cell cycle similarly since either the blue or red light could be absorbed and used for photosynthesis. In fact, preliminary results indicated that the photosynthetic efficiency (F_v/F_m) of G_1 gymnodinoids after blue or red light pre-treatment in a 12L/12D photoperiod remained similar (data not shown). However, as shown in Fig. 6, only mixed white light and blue lights but not red or infrared lights were able to entrain the cell cycle normally. This finding indicates that, besides the indirect effect, there may be direct effect of the blue light in regulating the cell cycle. The direct effect of blue light has been demonstrated in the dinoflagellate *Karenia brevis* where a cryptochrome blue-light receptor was identified as a circadian control of the cell cycle (Brunelle et al. 2007).

The role of blue light in regulating the cell cycle in the *Symbiodinium* sp. can be further confirmed by the attenuation of the S phase cell formation after the egGFP treatment as shown in Fig. 7. As shown in Fig. 2a, the epidermis of *E. glabrescens* contains significant amount of green fluorescent protein (see also Peng et al. 2008). The green fluorescent tissue layer may have an important impact on the cell cycle of symbionts in the gastrodermis. Incubation with cloned egGFP significantly suppressed the growth/DNA synthesis from G_1 to S (Figs. 7 and 10). At the moment, there is no evidence indicating that the egGFP directly affects the cell cycle or indirectly by affecting the photosynthesis. However, our results contradict the previous conclusion that fluorescent proteins (or pigments) should protect the photosynthetic efficiency (Salih et al. 2000). In the present study, egGFP was shown to retard rather than to maintain a normal cell cycle. egGFP inhibited the progression of G_1 to S to G_2/M but did not affect the cytokinesis (see Fig. 7). This indicates that the dissipation of blue light by egGFP may be the direct effect that inhibits the cell cycle progression. The role of egGFP in regulating the cell cycle

suggests an important role of tissue layer interaction in situ for symbiont proliferation. The epidermis of the *E. glabrescens* has abundant egGFP during its whole life history. The present in vitro study provides critical insight that the symbiont cell cycle in situ may be significantly retarded at the growth/DNA synthesis stage. This is consistent with the finding that G_1 phase of symbiont cell cycle was significantly lengthened, and there was unusual low percentage of dividing cells (3–5%) in both *A. pulchella* and the stony coral *Astrangia poculata* (Smith and Muscatine 1999; Dimond and Carrington 2008).

The role of cyclic AMP (cAMP)

The second messenger cAMP is a key regulator of various cellular responses in both prokaryotes and eukaryotes. Previous studies have shown that cAMP regulates the cell cycle of the dinoflagellate *Cryptothecodinium cohnii*, where the cAMP concentration was shown to peak during the G_1 phase and decrease to a minimum during the S phase (Lam et al. 2001). Similarly, cAMP-dependent protein kinase activities peaked at both the G_1 and G_2/M phases of the cell cycle, decreasing to a minimum at the S phase (Lam et al. 2001; Wong and Kwok 2005). Although cellular cAMP was not directly measured in the present study, the regulatory effect of AC that cleaves ATP to generate cAMP was examined by using two adenine nucleoside derivatives, 9-CP-Ade and SQ 22536 which exhibit isozyme-dependent sensitivity of AC on P-site-mediated inhibition (Johnson et al. 1997). As shown in Fig. 10, two pools of AC activities were shown to involve in the cell cycle regulation. First, the 9-CP-Ade sensitive AC pool presents almost throughout the light–dark period (yellow circular bar, Fig. 10). Inhibition of the AC activity in this pool retarded the cell cycle progression from G_1 to S to G_2/M (see also Fig. 9b, c). Second, the SQ 22536 sensitive AC pool exists during the dark period (red circular bar, Fig. 10). Inhibition of the AC activity in this pool retarded the cytokinesis only (i.e., from G_2/M to G_1). How the cAMP or AC activity is regulated in symbionts in situ turns out to be a critical focus for future research.

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