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Initial evidence of functional siRNA machinery in dinoflagellates

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ABSTRACT

Dinoflagellates are a major group of protists widely distributed in the aquatic environments. Many species in this lineage are able to form harmful algal blooms (HAB), some even producing toxins, making this phylum the most important contributors of HAB in the marine ecosystem. Despite the ecological importance, the molecular mechanisms underpinning the basic biology and HAB formation of dinoflagellates are poorly understood. While the high-throughput sequencing studies have documented a large and growing number of genes in dinoflagellates, their functions remained to be experimentally proven using a functional genetic tool. Unfortunately, no such tool is yet available. This study was aimed to adopt the RNA interference (RNAi) gene-silencing tool for dinoflagellate research, and to investigate the potential effects of RNAi-based silencing of proton-pump rhodopsin and CO_2-fixing enzyme Rubisco encoding genes in dinoflagellates. It was found that RNAi treatment caused a significant decrease in growth rate in both species. Compared with the non- endogenous target (GFP-siRNA) and the blank control, RNAi treatments also suppressed the expression of the target genes. These results constitute the first experimental evidence of the existence and operation of siRNA in two species of dinoflagellates, present initial evidence that dinoflagellate rhodopsins are functional as a supplemental energy acquisition mechanism, and provide technical information for future functional genetic research on dinoflagellates.

1. Introduction

Dinoflagellates are a major group of protists, including photosynthetic species (commonly known as algae), heterotrophic species, and parasitic species (Hackett et al., 2004; Lin, 2011). They are widely distributed in the aquatic environments (Morse et al., 2018; Taylor et al., 2008). In the ocean, photosynthetic dinoflagellates constitute one of the most important groups of primary producers, which through photosynthesis provide food for animals (Lin, 2011). The genus of *Symbiodinium* contains numerous species that are essential endosymbionts of reef-building corals and other invertebrates (Baker, 2003; Lin et al., 2015). Parasitic species can infect various organisms ranging from dinoflagellates, crustaceans, to fish eggs (Coats, 1999), More importantly, nearly 100 species of dinoflagellates can form blooms that have devastating consequences on the ecosystem, economy, and public health, making them the greatest contributors of harmful algal blooms (HAB) (Berdalet et al., 2016; Dia et al., 2014). Despite the ecological importance, molecular mechanisms underlying the basic biology and HAB formation are poorly understood. This is largely because no functional genetic tool is available for experimental verification of gene functions in dinoflagellates.

Dinoflagellates possess huge genomes preventing whole genome sequencing for HAB species; so far only the smallest genome representatives from the genus of *Symbiodinium* have begun to be sequenced (Aranda et al., 2016; Lin et al., 2015; Shoguchi et al., 2013). These initial genomic data have shed light on gene content, gene structure (e.g. unidirectional encoding), gene expression regulation (e.g. microRNA), and genome evolution (e.g. extensive retroposition (Song et al., 2017), but do not yet provide a clear understanding on functions and regulation of genes. Meanwhile, the rapidly growing transcriptomic data have documented numerous genes in dinoflagellates. Yet about 50% of these genes do not match any known genes, and their functions need to be experimentally established. Even for the other 50% that match characterized genes in other organisms, their functions also remain to be experimentally verified. For both, functional genetic tools such as gene knockout or silencing are required.

One of the potentially useful tools is RNA interference (RNAi). RNAi is a RNA-dependent gene silencing mechanism naturally occurring in many organisms in which the small guide strand RNA (20–30 nt) generated from a short double-stranded RNA (dsRNA) molecule form RNA-induced silencing complex (RISC) with Argonaute proteins to bind to target mRNA sequence (Lee and Ambros, 2001). The binding impedes

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Table 1
Primers used in siRNA and dsRNA synthesis.

Primer	Sequence	Source
Karve-Rhod-F1	5 '-CAACGATGCGGTGGCGATTTC-3'	This study
Karve-Rhod-R1	5 '-CTACCAATGTCTCGCCGCACT-3'	This study
Karve-Rub-F1	5 '-GGTCCTGCAACTGGGTTAATTG-3'	This study
Karve-Rub-R1	5 '-CCTCACCCGTTAAACAAGAAGC-3'	This study
Karve-Rhod-T7-F1	5 '-GGATCCTAATACGACTCACTATAGGG CAACGATGCGGTGGCGATTTC-3'	This study
Karve-Rhod-T7-R1	5 '-GGATCCTAATACGACTCACTATAGGG CTACCAATGTCTCGCCGCACT-3'	This study
Karve-Rub-T7-F1	5 '-GGATCCTAATACGACTCACTATAGGG GGTCCTGCAACTGGGTTAATTG-3'	This study
Karve-Rub-T7-R1	5 '-GGATCCTAATACGACTCACTATAGGG CCTCACCCGTTAAACAAGAAGC-3'	This study
Donghai-Rhod -F	5 '-CAACACGCTGTCGTTCGCCC-3'	This study
Donghai-Rhod -R	5 '-GCTTGCCCGCAGAGTATTCGT-3'	This study
Donghai-RUB-F	5 '-CTTCGGACAAGAACATCAGC-3'	This study
Donghai-RUB-R	5 '-GTAGGTGCCCGCCTTCCACAG-3'	This study
Donghai-Rhod-T7-F	5 '-GGATCCTAATACGACTCACTATAGGG CAACACGCTGTCGTTCGCCC-3'	This study
Donghai-Rhod-T7-R	5 '-GGATCCTAATACGACTCACTATAGGG GCTTGCCCGCAGAGTATTCGT-3'	This study
Donghai-RUB-T7-F	5 '-GGATCCTAATACGACTCACTATAGGG CTTCGGACAAGAACATCAGC-3'	This study
Donghai-RUB-T7-R	5 '-GGATCCTAATACGACTCACTATAGGG GTAGGTGCCCGCCTTCCACAG-3'	This study
GFP-T7-S	5 '-GGATCCTAATACGACTCACTATAGGG CACAAGTTCAGCGTGTCCGGCG-3'	Zhang et al. (2012)
GFP-T7-A	5 '-GGATCCTAATACGACTCACTATAGGG CGATGCGGTTCACCAGGG TGTCG-3'	Zhang et al. (2012)
GFP-A	5 '-CGATGCGGTTC ACCAGGGTGTCG-3'	Zhang et al. (2012)
GFP-S	5 '-CACAAGTTC AGCGTGTCCGGCG-3'	Zhang et al. (2012)

the expression of the cognate mRNA sequences by various mechanisms, such as RNA degradation, transcriptional repression and translation inhibition (Pilkington et al., 2011; Sakurai et al., 2009; Stroehle et al., 2010). MicorRNAs (miRNAs), Piwi-associated small RNAs (piRNA), and small interfering RNAs (siRNAs) are three major classes of small RNAs that function as RNAi effector. in vivo RNAi plays important roles in regulating development, physiological or metabolic processes, or stress responses by modulating gene expression (Ketting, 2011; Pilkington et al., 2011; Sakurai et al., 2009). This natural RNAi machinery can be exploited as a tool in research and therapeutics to silence an mRNA of interest when a siRNA complementary to the target mRNA can be designed and introduced into living organisms.

Since its initial discovery as a gene silencing mechanism in *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been found in a wide range of eukaryotes including protists, animals and plants (Cerutti and Casas-Mollano, 2006; Reinhart et al., 2002). RNA-mediated silencing pathways have also been identified in the green alga model *Chlamydomonas reinhardtii* rendering RNAi a valuable tool for algal research (Cerutti et al., 2011; Schroda, 2006). Recent transcriptomic (Shi et al., 2017) and genomic work (Lin et al., 2015) has indicated that dinoflagellates possess microRNA and potentially RNAi regulatory systems. Baumgarten and colleagues documented 8 novel miRNAs and 13 siRNAs in *S. microadriaticum* indicating that *Symbiodinium* not only produces miRNAs, but also siRNAs (Baumgarten et al., 2013). In the genome of *Symbiodinium kawagutii*, Lin and colleagues identified 102 mature microRNAs, 49 of which were similar to animal miRNAs, 11 to plant miRNAs, and 1 to viral miRNAs (Lin et al., 2015). Northern blot analysis indicated that one of these miRNAs was down-regulated under thermal stress, and *in silico* analysis predicted heat shock proteins (HSP) 70 and 90 as target genes, suggesting a role of this miRNA in regulating HSP70 and 90 gene expression in response to thermal stress. More recently, microRNAome analysis was conducted for *P. donghaiense* grown under phosphate stress, which revealed 17 miRNAs, potentially regulating 3268 protein-coding genes (Shi et al., 2017). Whether the RNAi machinery functions to silence gene expression in dinoflagellates and whether it can be a tool for dinoflagellate functional genetic studies remain to be explored experimentally, however.

In this study, siRNA was used to examine the functionality of RNAi machinery in dinoflagellates, and to investigate the potential effects of RNAi-based silencing on two target genes in two dinoflagellates. The species *Prorocentrum donghaiense* and *Karlodinium veneficum* were chosen because they represent HAB species from two different phylogenetic and taxonomic groups, the Desmophyceae (which is thecate and with typical dinoflagellate chloroplast) and the Gymnodiniales (which is athecate and with tertiary replacement chloroplast). As a proof-of-concept work, Rubisco and rhodopsin were chosen as the target genes. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is an essential enzyme for CO_2 fixation and as such is widespread among photosynthetic species. The rhodopsin gene putatively encodes a proton-pump that absorbs light energy and converts it to ATP (Lin et al., 2010), but this important function remains to be experimentally demonstrated. The cultures of these two species were treated with different siRNA molecules, one for the non-target gene green fluorescence protein or GFP as a control, one for rhodopsin (target gene), and the other for Rubisco (target gene). Growth rates of the cultures and expression of the target genes in the treated cultures were measured and compared to that in the control cultures to assess the effects.

2. Materials and methods

2.1. Algal cultures

The culture of *Prorocentrum donghaiense* (strain CCMAXU-364) was obtained from the Collection Center of Marine Bacteria and Algae, in Xiamen University, China. The culture of *K. veneficum* (strain CCMP2778) was obtained from the National Center for Marine Algae and Microbiota (NCMA) (Bigelow Laboratory of Ocean Sciences, East Boothbay, Maine, USA). The dinoflagellate cultures were grown in L1 culture medium at 20 ℃ under a 14:10 h light : dark cycle at a photon flux of 110 ± 10 $\mu Em^{-2}s^{-1}$ provided by cool fluorescent lamps.

2.2. dsRNA and siRNA synthesis

A fragment of about 400 bp from the ORF of each target gene (Rubisco and rhodopsin) was cloned into PMD18-T (TAKARA). The correct and in-frame structure of the expression vector construct was confirmed by sequencing. Specific primers (T7S, A, T7A, S) (Table 1) with a T7 promoter were designed to generate the sense and anti-sense products. Sense and anti-sense RNA were transcribed for 30 min at 37 ˚C and analyzed by agarose gel electrophoresis. The sense and anti-sense single- stranded RNA (ssRNA) were mixed at 1:1 ratio, denatured at 94 ˚C for 10 min. then cooled to room temperature to allow annealing. The resulting double-stranded RNA (dsRNA) was precipitated in equal amount of LiCl (3 mol/L) overnight, washed using 70% ethyl alcohol three times, and finally stored at −80 ˚C for later use. Non-endogenous control dsRNA (GFP gene, *gfp*) was generated (381bp long)

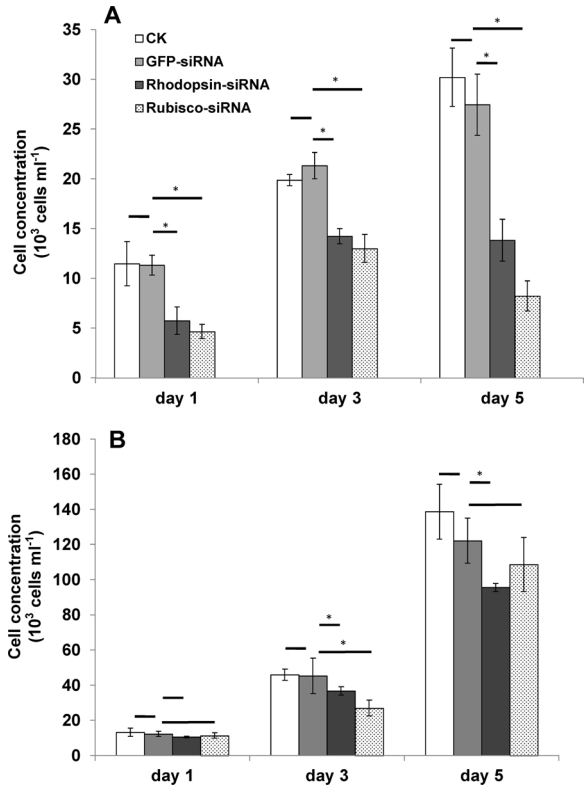

Fig. 1. Effects of siRNA treatment on growth of (a) *K. veneficum* culture and (b) *P. donghaiense*. Rubisco and Rhodopsin siRNA were added in L1 culture separately at the final concentration of 200 ng/ml; GFP-siRNA was used as a control at the same concentration. A blank control (CK) was set up in L1 growth medium without any treatment. Each of this condition was set up in triplicate. Bars indicate means and error bars standard deviations, with significant differences between treatments denoted with an asterisk on top of the bars ($P < 0.05$).

with specific primers (G-T7S, G-A, G-T7A, and G–S) (Table 1) designed from the cloning vector PYL 322-d1-GFPn (provided by Dr. R. X. Shen, College of Life-Science, South China Agricultural University, China). The dsRNA molecules of the three genes were all subjected to digestion with RNase III (TOYOBO, Japan) to generate mature siRNAs (single stranded). This was carried out by incubating 1 µg dsRNA with 1 U RNase III for 60 min at 37 °C. The resulting siRNAs were purified following the RNA extraction procedure described above.

2.3. RNAi treatment and silencing efficacy analysis

Cultures were grown in 20 ml L1 medium in triplicate for each treatment condition. For RNAi treatment, siRNA of the target gene was supplied at a final concentration of 200 ng/ml in L1 medium directly. For a non-endogenous gene siRNA control, the cultures were amended with *gfp* siRNA solution also at a final concentration of 200 ng/ml. Meanwhile, triplicated untreated L1 cultures were set up and used as a blank control. Cell concentration was measured daily under the microscope using Sedgewick-Rafter counting chamber (Phycotech, St. Joesph, MI, USA).

After 12 h and 24 h of incubation in the respective treatments (or control), 20 ml cells were harvested by centrifugation at 3000 x g for 15 min at culture incubation temperature (20 °C), and each cell pellet was suspended in Trizol Reagent, vortexed thoroughly, and stored at −80 °C. Total RNA for each treatment was extracted using Trizol procedure combined with Qiagen RNA columns, as described earlier (Zhang et al. 2014). RNA concentration and quality were checked using NanoDrop 2000 at 260, 280, and 230 nm. The first-strand cDNA was synthesized using iScript™cDNA Synthesis Kit (Bio-Rad) with 200 ng of RNA.

qPCR was performed with the first-strand cDNA samples as templates to analyze transcriptional suppression of target genes in dinoflagellate cells after RNAi treatments. Primers were the same as previously reported (*P. donghaiense* Rhodopsin: KM282617; *P. donghaiense* Rubisco: (Shi et al., 2013); *K. veneficum* Rhodopsin: ABL60988.1; *K. veneficum* Rubisco: JX292171.1) (Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes from *P. donghaiense* (KF142734) and *K. veneficum* (DQ867061.1) were used as a reference gene for these species respectively to normalize expression of target genes as reported previously (Cui et al., 2016; Shi et al., 2013). The GAPDH gene (*gapdh*) has been reported as the most stable reference gene among multiple genes examined (actin, *α-tubulin* and mitochondrial cytochrome) in *Prorocentrum donghaiense* (Shi et al., 2013), and it has also been widely used as reference gene in *Karenia mikimotoi* (Luo et al., 2017; Zhang et al., 2017). qPCR of the target and reference genes was performed on CFX-96 qPCR system (Bio-Rad Laboratories, Inc., Shanghai, China) in triplicate for each cDNA template using SsoFast EvaGreen (Bio-Rad) according to the manufacture's protocol with the following reaction conditions: 95 °C for 15 s and 60 °C for 30 s (40 cycles). Initial data analysis was carried out using the Bio-Rad CFX-96 manager software, which created Ct values and evaluated relative levels of PCR products from the standard curves. Melting curve analysis was carried out at the end, in which the detection of one single peak indicated that PCR was specific to the target; in this study this was true in all cases.

2.4. Data analysis

All data of this study were subjected to analysis of variance (ANOVA). Multiple comparisons of means were conducted using Duncan's Multiple Range Test in SAS (Release 9.2). Probability of 0.05 was used as the criterion of statistical significance.

3. Results

3.1. Influence of siRNA silencing on K. veneficum growth

All the *K. veneficum* cultures were grown in the same L1 medium except that the RNAi treatments (Rhodopsin-siRNA, Rubisco-siRNA, respectively) and non-endogenous control (GFP-siRNA) contained siRNA in the medium. All treatments were set up at a similar starting cell concentration (8,000–10,000 cells/ml). After 24 h, Rhodopsin-siRNA and Rubisco-siRNA treated groups exhibited significantly lower cell concentrations than the blank control and the GFP-siRNA control groups ($P < 0.05$) (Fig. 1a). The Rhodopsin-siRNA treatment caused the cell concentration to decrease by 49.33%, 33.23% and 49.63%, and Rubisco-siRNA treatment caused a decrease by 58.74%, 38.96% and 69.98% on day 1, day 3 and day 5, respectively.

3.2. Influence of siRNA silencing on P. donghaiense growth

All the *P. donghaiense* cells were grown in the same L1 medium, and siRNA was supplied in the treatment groups as described above except that siRNA sequences used here were based on the specific target gene sequences in this species. Starting at the same cell concentration (10,000 cells/ml), the target gene RNAi-treated groups showed significantly lower cell concentrations than the blank control and the GFP-siRNA control groups on day 3 and day 5 ($P < 0.05$) except the Rubisco-siRNA treatment on day 5 (Fig. 1b). On those two days, the Rhodopsin-siRNA treatment decreased the cell concentration by 18.48% and 21.78%, respectively relative to the GFP-siRNA control. For the Rubisco-siRNA treatment, there was a decrease of cell concentration by 40.25% ($P < 0.05$) on day 3 and 11.05% on day 5 (not significantly; $P > 0.05$), respectively. Similar growth depression was observed when the treatments were compared with the blank control

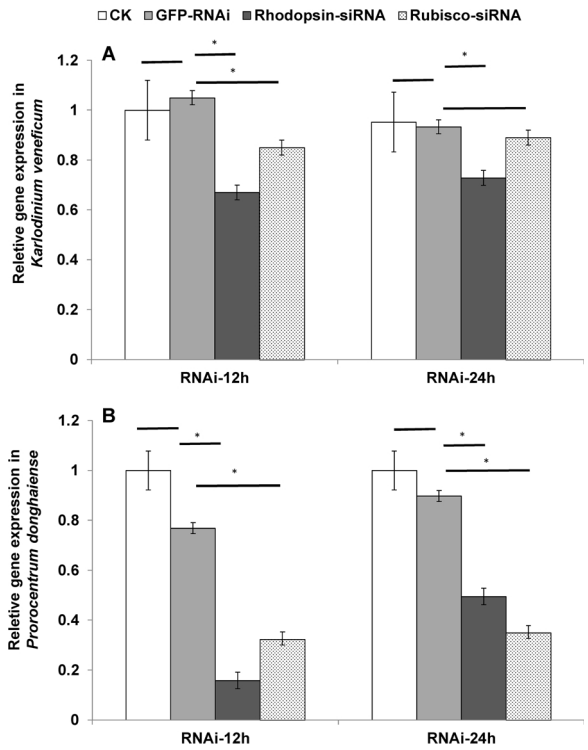

Fig. 2. Effects of siRNA treatment on expression of genes (Rhodopsin and Rubisco) in (a) *K. veneficum* and (b) *P. donghaiense*. Rubisco and Rhodopsin siRNA were added in L1 culture separately at the final concentration of 200 ng/ml; GFP-siRNA was used as control at the same concentration. A blank control (CK) was set up in L1 medium without any treatment. Each of this condition was set up in triplicate. siRNA treatment lasted for 12 h and 24 h when samples were collected for gene expression analysis. Bars indicate means and error bars standard deviations, with significant differences between treatments denoted with an asterisk on top of the bars ($P < 0.05$).

(Fig. 1).

3.3. RNAi silencing effect on Rhodopsin and Rubisco transcript abundances in K. veneficum

The transcript abundances of Rhodopsin and Rubisco in *K. veneficum* in the different treatment and control groups were determined using RT-qPCR. Relative to the GFP-siRNA control, transcript abundances of Rhodopsin and Rubisco in the Rhodopsin-siRNA group and the Rubisco-siRNA group decreased by 36.19% and 9.05% respectively after 12 h incubation, and by 23.47% and 8.16% respectively after 24 h incubation (Fig. 2a). The decreases were all statistically significant ($P < 0.05$) except for the Rubisco-siRNA group at 24 h. Similarly, when compared with the blank control, significant decreases in the transcript abundances of Rhodopsin and Rubisco were also observed (Fig. 2a). These results indicated that transcript levels of the two target genes were suppressed by their corresponding siRNAs. In contrast, the non-endogenous GFP-siRNA had no significant influence on the transcript abundances of the target genes as their transcript abundances were similar to that in the blank control.

3.4. RNAi silencing effect on transcript abundances of Rhodopsin and Rubisco in P. donghaiense

The transcript abundances of Rhodopsin and Rubisco in *P. donghaiense* in the different treatment and control groups were also determined using RT-qPCR. Compared with the GFP-siRNA control, the relative transcript abundances of Rhodopsin and Rubisco in the cells treated with Rhodopsin-siRNA and Rubisco-siRNA respectively decreased by 82.72% and 58.02% for the 12 h treatment, 45.05% and 61.54% for the 24 h treatment (Fig. 2b). The decreases were all significant statistically ($P < 0.05$). Similar decreases were observed when transcript abundances of these two genes in the treatment groups were compared with that in the blank control. These results indicated that expression of the target genes were suppressed by siRNA treatments. The non-endogenous GFP siRNA control had no significant influence on the transcript abundances of target genes because the transcript abundances in this group were similar to that in the blank control (Fig. 2b).

4. Discussion

4.1. Existence and operation of siRNA machinery in dinoflagellates

RNAi gene silencing machinery is widespread in eukaryotes. In organisms such as *C. elegans, Schizosaccharomyces pombe*, and *Arabidopsis thaliana* (Castel and Martienssen, 2013; Martienssen and Moazed, 2015; Schroda, 2006; Smialowska et al., 2014; Xu et al., 2013), RNAi functions as a mechanism to silence target genes to protect cells against viruses. Furthermore, this mechanism has proven a useful tool to silence gene expression. In the model green alga *C. reinhardtii*, a target gene can be knocked down by expressing corresponding antisense RNAs of siRNAs (Schroda, 2006). The current short report represents the first attempt, best to the authors' knowledge, to adapt the siRNA technology for dinoflagellate research, which is hoped to provide a technical foundation for future more in-depth studies.

Following the *C. reinhardtii* model, siRNA of target genes was added into the culture medium in this study. To compare the efficacy of RNAi in different species of dinoflagellates and for different genes, *K. veneficum* and *P. donghaiense*, and two target genes, Rhodopsin and Rubisco, were selected for the RNAi treatment. Growth trends were observed, and RNAi treatments for both genes were found to cause a decrease in growth rate in both species. Compared with the non- endogenous target (GFP-siRNA) and the blank control, RNAi treatments resulted in the suppression of the target genes. When normalized against the reference gene GAPDH, the transcript abundances of Rhodopsin and Rubisco were reduced by as high as 82.72% and 58.02% by siRNA treatment in *P. donghaiense*, and 36.19% and 19.05% in *K. veneficum*. The result is evidence that siRNA exists and is functional in both species for the regulation of both target genes investigated. Between the two species, there seemed to be stronger effects (i.e. higher silencing efficiencies) in *K. veneficum* on growth and in *P. donghaiense* on gene expression. The differences might be due to differential uptake efficiencies and metabolic responses to siRNA between species.

What constitutes the RNAi machinery in dinoflagellates is still unclear. Generally RNAi machinery requires the RNase III enzyme Dicer or like protein to cleave double-stranded RNAs into short fragments, which bind to the Argonaute family proteins where they are denatured into single-stranded guide RNA to form RISC complex. Besides, the production of the precursor double-stranded RNA requires a RNA-dependent RNA polymerase (RDRP). Dicer- and Argonaute-like protein genes have been identified in *Symbiodinium* spp. (Baumgarten et al., 2013; Dagenais-Bellefeuille et al., 2017; Lin et al., 2015), but no RDRP has been detected in dinoflagellates. It is worth noting, however, that different organisms may use different molecular machinery to function as RNAi; for instance gene silencing in the diatom *Phaeodactylum tricornutum* apparently uses a molecular setup independent of Dicer and RDRP (De Riso et al., 2009). RNAi mechanism in dinoflagellates still needs to be further investigated to understand what molecular machinery is involved.

4.2. Potential of siRNA as a tool to study functions of dinoflagellate genes

RNAi is a simple and effective alternative to gene knockout, because of its high sequence-specificity and effective interference activity. Meanwhile, with its simple operation, short cycle and low cost, it has

become an extremely important tool and a popular research topic in the fields of functional genetics, gene therapy and genomics. Apart from providing evidence of the existence and operation of siRNA in two species of dinoflagellates, the initial effort reported here also provides technical information expected to be valuable for future functional genetic research in dinoflagellates. Based on the similar results from three repeated experiments of all the treatments, it was found that siRNA treatment of 12 h was enough to achieve effective silencing, and extending the treatment time beyond that would not enhance RNAi efficacy. Variations exist in the duration of RNAi efficiency in different organisms, such as *C. reinhardtii, C. elegans* and *S. pombe* (Martienssen and Moazed, 2015; Schroda, 2006; Smialowska et al., 2014; Xu et al., 2013). It has been reported that RNAi efficiency decreases after rounds of cell divisions without rearrangement of the target RNAi construct in *C. reinhardtii* (Schroda, 2006). For animals, it has also been shown that siRNA exhibited strongest inhibitory effect on target gene mRNA within 12 h (Kimber et al., 2007; Wang et al., 2017; Zhang et al., 2012). A decline in the gene depressing effect was also observed in the present study when the incubation prolonged beyond two days. Furthermore, the use of the non-endogenous GFP-siRNA as a control helped exclude the possibility that the observed effects were simply a nonspecific result of the treatment procedure. Due to the small size of dinoflagellate cells, it is difficult to inject dsRNA into cells as is usually done in animal models. In the present study, however, soaking the cells in siRNA (i.e. adding siRNA into growth medium) seemed to work just fine, as in the case of *C. elegans* (Xu et al., 2013; Zhang et al., 2012), despite the apparent differential efficacies between species discussed above.

Future applications of siRNA can be made easier as siRNA can be chemically synthesized at commercial facilities. This would require prior knowledge of target motif on mRNA, however. Without the target motif information, as in the case of the present study, artificial synthesis of siRNA would require a “tiling” scheme and synthesis of many siRNAs in order to ensure coverage of the effective target motif and gene expression inhibition. Therefore, following a method that has previously been used (Zhang et al., 2012), in the present study dsRNA that was transcribed in vitro induced by T7 from the full-length cDNA was used. The dsRNA was then digested using RNase III to generate multiple siRNAs.

The initial application of siRNA has provided the first experimental evidence that Rubisco and the proton-pump rhodopsin (PPR) are functional in promoting growth, at least in *K. veneficum* and *P. donghaiense*. Between the two genes, the function of Rubisco as CO_2 fixing enzyme has been widely accepted, whereas that of PPR has remained a question. Rhodopsin is a large protein family, including sensory rhodopsin (for vision in animals), motive rhodopsin (driving flagellum motion), ion pump rhodopsin (pumping chloride or sodium), besides proton pump rhodopsin (Ernst et al., 2014). In bacteria, where PPR was initially discovered, it has been experimentally demonstrated that it absorbs light and creates a proton gradient to facilitate ATP production (Martinez et al., 2007), and its expression promotes the growth of bacteria limited by nutrient deficiency under illumination (Akram et al., 2013). It has been reported that a homolog widely occurs in dinoflagellates and its amino acid sequence is highly similar to that in bacteria, containing key residues to form the pocket to hold the retinal co-factor, residues to fine-tune absorption spectra, and those to provide and accept proton (Lin et al., 2010; Shi et al., 2015). Within dinoflagellates, however, there seems to be a diverse set of rhodopsins, with the majority being similar to a subgroup of PPR named xanthorhodopsin (Shi et al., 2015), some being similar to sensory rhodopsin (for instance in *Oxyrrhis marina*), and others being unique subgroups of PPR (found in *Karlodinium*; (Shi et al., 2015)). A xanthorhodopsin type of PPR found in *O. marina* has been shown to enhance population survival of this heterotrophic dinoflagellate under starvation under light (Guo et al., 2014). The siRNA treatment experiments reported here have just provided the first evidence in photosynthetic (*P. donghaiense*) and mixotrophic (*K. veneficum*) species that expression of their rhodopsins (xanthorhodopsin and unique PPR respectively) likely provides supplemental energy to enhance growth. The siRNA technique developed here will be useful for further studies on rhodopsin in other dinoflagellates. If verified, PPR will likely be the simplest and most efficient energy converting machinery ever known in eukaryotes. The siRNA tool will also prove helpful for verifying functions of many other genes currently poorly understood in dinoflagellates.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2018.11.014.

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