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A Genome-Wide Identification of Osmotic Stress-Responsive MicroRNAs in *Pyropia haitanensis* (Bangiales, Rhodophyta)

Min Cao[1,2†], Dongmei Wang[1,2†], Fanna Kong[1,2], Junhao Wang[1,2], Kuipeng Xu[1,2] and Yunxiang Mao[1,3,4*]*

[1] Key Laboratory of Marine Genetics and Breeding (OUC), Ministry of Education, Qingdao, China, [2] College of Marine Life Sciences, Ocean University of China, Qingdao, China, [3] Laboratory for Marine Biology and Biotechnology, Pilot Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [4] Key Laboratory of Utilization and Conservation for Tropical Marine Bioresources (Hainan Tropical Ocean University), Ministry of Education, Sanya, China

Pyropia haitanensis, one of the most economically important marine crops worldwide, can recover from up to 80% water loss during emersion in the intertidal zone. However, little is currently known regarding the molecular mechanism involved in the response to osmotic stress at the post-transcriptional level. In this study, we profiled the transcriptional patterns of microRNAs (miRNAs) in *P. haitanensis* under the conditions of dehydration and succeeding rehydration. Forty-seven miRNAs were sequenced and characterized, which included two evolutionarily conserved and 45 novel miRNAs. Among these, 12 miRNAs were differentially expressed under osmotic stress conditions. The target genes were predicted to be related to catalytic activity, binding, transporter activity, transcription factor activity, signal transducer activity, and antioxidant activity. The miRNAPC-3p-99769_194 exhibited constant upregulation during dehydrated stress, whereas its target gene, a carbonic anhydrase, was downregulated as detected via qPCR. Furthermore, we identified genes that encode Dicer and Argonaute (AGO) proteins. The Dicer protein is encoded by two genes, each partially harboring several conserved domains. In this study, we discovered that an inverse expression profiles of miRNAPC-3p-99769_194 and its target CA under osmotic stress, which revealed the essential role of miRNAs in the suppression of carbon fixation to maintain basic living conditions. Additionally, the *Pyropia*-specific organization of Dicer genes may be indicative of the distinctive structural features of miRNAs. This study provided novel insights into the miRNA-mediated regulatory mechanisms that are essential for desiccation-tolerance in *P. haitanensis*.

Keywords: *Pyropia haitanensis*, genomics, miRNA, osmotic stress, Dicer, AGO

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Jianhua Fan,
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****Correspondence:***
Dongmei Wang
wangdm@ouc.edu.cn
Yunxiang Mao
yxmao@hnt.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Pyropia, a genus of red algae, is one of the most economically important marine crops worldwide, especially in East Asian countries such as China, South Korea, and Japan (Sahoo et al., 2002). Its annual fresh weight can reach 1,806,000 tons according to statistics provided by the Food and Agriculture Organization in 2014[1]. *P. haitanensis* is a native species that is distributed and

[1] http://www.fao.org/fishery/en

intensively farmed in southern China. The production of *P. haitanensis* represents 75–80% of the total production of cultivated *Pyropia* in China (Zhang et al., 2005).

Most *Pyropia* species are naturally distributed in the intertidal zone, with a drastically changing environment (Nakamura et al., 2013). Due to their living environment, they are periodically exposed to an environment in which they experience a variety of potentially stressful environmental conditions, which include nutrient limitation, high and low temperature, intense light, and osmotic stress caused by tidal changes (Davison and Pearson, 1996). The stress caused by environmental factors and long-term evolution make *Pyropia* highly environmentally tolerant, especially toward osmotic stress (Hwang et al., 1997). For example, many species of *Pyropia* (or species related to *Porphyra*) can survive after they lose 85–95% of their cellular water content during low tide in the daytime (Blouin et al., 2011). Therefore, *Pyropia* is an ideal model organism for the study of the molecular mechanism involved in the tolerance of seaweed to osmotic stress in the intertidal zone (Cock and Coelho, 2011).

Studies show that *Pyropia* adapt and tolerate osmotic stress via many physiological and molecular mechanisms, including signal transduction systems, antioxidant systems, molecular chaperones, protective proteins, soluble permeants, and changes to cell wall components (Davison and Pearson, 1996). For example, "floridoside" protects the cell when the gametophytes of *Porphyra purpurea* are exposed to the stress of salinity (Reed et al., 1980). Additionally, putative fucosyltransferase is located in the nucleus and confers tolerance to desiccation and osmotic stress in *P. tenera* (Wi et al., 2018). In addition, the genes that change under natural hydration and desiccation conditions are involved in protein synthesis, processing, and degradation, photosynthesis, and respiration in *P. columbina* (Contreras-Porcia et al., 2013). Using dynamic stochastic general equilibrium modeling (DGE) to characterize the tolerance mechanism of *P. haitanensis* under dehydration and rehydration conditions, it is clear that these differential genes are related to the synthesis of unsaturated fatty acids, transcription factor families, and molecular chaperones (Wang et al., 2015). Unigenes related to glutathione transferase, superoxide dismutase, heat shock proteins, serine/threonine kinases, and phospholipases under osmotic stress are upregulated significantly, which implies that these genes play important roles in the prevention of damage from osmotic stress (Sun et al., 2019). However, little is currently known regarding the molecular mechanisms involved in the response to osmotic stress at the post-transcriptional level.

MicroRNAs (miRNAs) are endogenous small RNAs that are 21–25 nucleotides (nt) long. They play an important role in the regulation of animal and plant growth and development, hormone signaling, programmed cell death, and disease infection (Bartel, 2004; Lin and Gregory, 2015; Thomson and Dinger, 2016; Gao et al., 2019). Recent studies show that miRNAs play important roles in a variety of stress conditions. For example, the expression of miR169, miR394, miR159, miR395, and miR160 is induced when plants are subjected to drought stress, some of which are involved in plant root development and transcriptional regulation; thus, allow the plant to tolerate water stress (Ni et al., 2013). Additionally, the expression of several miRNAs, such as miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, and miR158 is altered in response to salinity and consequently affects the expression of their targets in *Arabidopsis* (Liu et al., 2008) and rice (Zhao et al., 2009). Drought stress in wheat enhances protein storage via the regulation of the expression of miRNAs and their target genes (Chen et al., 2017). In Yu et al. (2019), overexpressed miRNA169c in *Arabidopsis* and discovered that the transgenic strain is more sensitive to drought stress. However, little is known about the osmotic stress response and regulatory molecular mechanisms in intertidal seaweeds compared with that in higher plants.

To understand the molecular mechanisms underlying osmotic acclimation and adaptation to intertidal environments at the post-transcriptional level, a comprehensive analysis of miRNA expression in response to dehydration and rehydration in *P. haitanensis* based on the genome was performed. This study was undertaken to identify conserved and novel miRNAs expressed in *P. haitanensis* and characterize osmotic-regulated miRNAs. The results of this study will lay the foundation for the elucidation of the molecular mechanisms involved in osmotic acclimation.

MATERIALS AND METHODS

Seaweed Culture and Osmotic Treatments

A lab-cultured pure line PH-40 of *P. haitanensis* was used in the experiments to eliminate the interference caused by genotypic differences. The sporophytes (conchocelis) of this pure line were directly developed from a single somatic cell that was isolated using 2% snail enzyme from a farmed gametophyte collected from Putian, Fujian Province, China. The gametophytes of *P. haitanensis* were cultured in a light incubator under the following conditions: 20 ± 1°C with 50–60 μmol photons·m^{-2}·s^{-1} of illumination during a 12: 12 h light: dark cycle. Provasoli-Enriched Seawater (PES) medium was changed every 3 days. Material processing was performed when the blade was 10–15 cm long. For miRNA identification, gametophytes were exposed to dehydration and rehydration. The gametophytes were harvested in the following conditions: normal conditions as control (CON), 30 ± 5% water loss rate (moderate water loss, MWL), 50% ± 5% (high water loss, HML), and 80% ± 5% (severe water loss, SWL). The gametophytes from SWL were then put back into seawater to rehydrate for 30 min (REH) according to a previous study (Wang et al., 2015). The different water loss levels were determined according to a previous study (Kim et al., 2009). Three biological replicates were performed for each treatment. After treatment, all samples were stored in liquid nitrogen for subsequent RNA extraction.

RNA Extraction and miRNA Library Construction

Total RNA was separately isolated from gametophytes (control and osmotic-treated samples) using Trizol reagent (Invitrogen,

Carlsbad, CA, United States) according to the manufacturer's protocol. Concentrations and qualities of the isolated RNAs were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Approximately 2.5 μg of total RNA was used to prepare a small RNA library, according to the protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, United States). Then, the libraries were sequenced by Illumina Hiseq2500 50SE (single-end) at LC-Bio (Hangzhou, China).

Data Processing

The raw reads were processed using Illumina's Genome Analyzer Pipeline software (Solexa 0.3) and the ACGT V3.1 program developed by LC Sciences (LC Sciences, Houston, TX, United States). After removing adapter dimers, junk, and low complexity sequences from the small RNA reads, total read counts for the small RNAs were established. Then, small RNAs that mapped to common RNA families [ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA)] were discarded. Subsequently, the remaining sequences were mapped to the assembled genome of *P. haitanensis* using the Bowtie software (Langmead et al., 2009; Cao et al., 2019). For alignment, one mismatch in the first 16 nt was allowed. Mapped reads were used for further analysis. The mapped sequences that were 18–25 nt long were mapped to specific species precursors in miRBase 21.0 by Bowtie search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. Length variation at 3′ and 5′ ends and one mismatch inside of the sequence were allowed in the alignment. Meanwhile, the remaining sequences that did not match known miRNAs were used to identify novel miRNAs in *P. haitanensis*.

Differential miRNA Expression

miRNA differential expression based on normalized deep sequencing counts was analyzed by selectively using DeSeq. The miRNA expression level fold-change was calculated using the formula mentioned in a previous report (Marsit et al., 2006): Fold-change = $\log_2$ of RPKM value. The significance threshold (p-value) was set at 0.01 and 0.05 for each test. The fold-change (>1) and p-values ($p < 0.05$) were combined to identify differentially expressed genes, which were defined as osmotic stress-responsive miRNAs.

Target Prediction and Annotation of Differentially Expressed miRNAs

To further understand the function of the differentially expressed miRNAs, computational target prediction algorithms (TargetScan5.0 and miRanda 3.3a) were used to predict the miRNA binding sites (potential target genes) (Yang et al., 2012). The function of the target genes of these differentially expressed miRNAs were annotated using the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[2] databases.

[2]http://www.genome.jp/kegg

Verification of miRNA PC-3p-99769 and Its Target CA Using qPCR

To validate the osmotic stress-responsive miRNAs identified by high-throughput sequencing and expression trends, the differentially expressed miRNA and its target gene was confirmed by qPCR (PC-3p-99769_194-ph08980.t1). The gametophytes were collected as described for miRNA library preparation and sequencing. Then, small RNAs (<200 nt) were extracted from the samples following the manufacturer's instructions for the RNA isolation for Small RNA Kit (TaKaRa). Equal amounts of high-quality small RNAs were reverse transcribed to complementary DNA (cDNA). Reverse transcription (RT) was performed using the PrimeScript RT reagent Kit (TaKaRa, China) according to the manufacturer's instructions but with specific stem-loop RT primers for miRNAs (**Supplementary Table S1**). The 5.8S and 18S subunits were used as internal controls for miRNA and mRNAs, respectively. The expression levels of these genes were analyzed by qRT-PCR with a Light-Cycle® 480 Real-Time PCR System. The reactions were performed using 20 μL volumes containing 10 μL of SYBR® Premix Ex Taq (TaKaRa Biotech Co., Dalian, China), 0.6 μL of each primer (forward and reverse primer), 2 μL of diluted cDNA, and 6.8 μL of RNA-free water using the following protocol: 95°C for 30 s, 40 cycles at 95°C for 5 s, 57°C for 30 s, and 72°C for 30 s. The relative expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

RESULTS

Small RNA Sequencing

To profile small RNAs and identify the osmotic stress-responsive miRNAs in *P. haitanensis*, small RNA sequencing for *Pyropia* gametophytes subjected to varying degrees of dehydration and rehydration (CON, MWL, HWL, SWL, and REH) were performed. After filtering low-quality raw reads and mRNA, Rfam, and Repbase mappable reads, the average reads of these samples were 2,507,531, 2,705,002, 2,620,137, 2,741,703, and 2,842,799 bp, respectively (**Table 1**). These reads were used for further miRNA identification and prediction.

Identification and Analysis of miRNAs in *P. haitanensis*

Small RNA sequences were mapped to miRBase and the *P. haitanensis* genome to identify conserved and novel miRNAs. Forty-seven miRNAs were predicted and 95.74% of these were 20–24 nt long, with the 21 nt as the dominant length (**Figure 1**). In the first nucleotide bias analysis of miRNAs in *P. haitanensis*, the first residue of the 19 nt miRNAs was either predominantly uridine (U) or cytosine (C). The first residue of the 20, 21, and 22 nt miRNAs was predominantly U, whereas the 24 nt miRNAs predominantly started with guanine (G).

Among the 47 miRNAs, 2 could be mapped to specific miRNA sequences in miRBase and the extended *Pyropia* genome sequences from their genomic loci could form hairpins. Thus,

TABLE 1 | Data statistics of small RNAs produced by Illumina deep sequencing.

	CON_1	CON_2	CON_3	MWL_1	MWL_2	MWL_3	HWL_1	HWL_2	HWL_3	SWL_1	SWL_2	SWL_3	REH_1	REH_2	REH_3
Raw reads	3388376	3994155	3362531	3731044	4167760	3521290	3965306	3517722	3299367	3698517	3657563	3294530	3556995	3991948	3519499
3ADT&length filter	906932	880562	1260524	924957	1256603	926192	1029051	919171	766724	701474	615483	897112	668749	936647	725914
Junk reads	21230	30868	20239	24536	25756	24168	26746	23170	22677	26337	28990	25302	25884	30259	26760
Rfam	20750	29070	16104	27712	20126	31862	28298	31181	32802	23060	26248	27442	17108	28334	24022
mRNA	9444	20539	8163	15167	14015	15966	14250	18036	12361	20148	21344	14733	18843	17787	21645
Repeats	83	355	74	204	119	302	182	304	171	261	224	165	200	197	310
valid reads	2430566	3034067	2057960	2739316	2851851	2523839	2867928	2526901	2465582	2928286	2966283	2330540	2826896	2979696	2721806
rRNA	13800	17807	10547	17688	13792	18815	17122	18501	19185	15733	17454	16510	10240	15725	12896
tRNA	6340	10127	5049	9262	5665	11863	10011	11669	12555	6271	7771	9956	6169	11467	10016
snoRNA	8	62	10	33	18	43	42	38	16	46	35	21	29	39	43
snRNA	4	30	4	15	9	28	16	32	14	18	19	18	11	11	30
other Rfam RNA	598	1044	494	714	642	1113	1107	941	1032	992	969	937	659	1092	1037

they were defined as conserved miRNAs for this study. The other 45 are novel miRNAs (**Table 2**).

To trace the evolutionary scenario of miRNAs in the red algae lineage, we downloaded the published miRNA data for the *Pyropia* species *P. yezoensis* and the sister red algal clade Floriceadens *Chondrus crispus* for phylogenetic comparative analysis. We discovered that the pre-miRNA sequences of 15 miRNAs could be aligned to both *P. yezoensis* and *Porphyra umbilicalis*, which indicated that these sequences were well conserved in *Pyropia* species. Only two were also observed in *C. crispus* (**Supplementary Table S2**). The target genes of these conserved miRNAs are mainly involved in DNA synthesis (DNA repair proteins and DNA mismatch repair proteins), signal pathways (mitogen-activated protein kinase and serine/threonine protein kinase), transporters (ABC transport system and zinc transporter proteins), and some transcriptional regulators.

Differential Expression Analysis of miRNAs

Many genes undergo significant expressional variation in response to osmotic stress (Wang et al., 2015). Our identification of miRNAs in the *P. haitanensis* genome caused us to investigate whether miRNAs contribute to the post-transcriptional regulation of key genes that lead to physiological modulation to adapt to stressful conditions. Therefore, we further investigated the expression levels of miRNAs under different degrees of dehydration and subsequent rehydration. Twelve miRNAs exhibited significant variation in at least one dehydrated or rehydrated condition (**Figure 2**). According to their transcriptional patterns along the time course of osmotic stresses, they can be classified into three clusters (**Figure 2**). In cluster I, three miRNAs exhibited slight variations in response to most of the stress conditions, while the levels of PC-3P-45934_389 and PC-3P-75147_255 were transiently down-regulated in HWL and SWL, respectively. There were four miRNAs in cluster II. Although there was no significant change in their expression under dehydrated conditions, they were induced when *Pyropia* thalli were rehydrated after severe water loss. The final five miRNAs were grouped in cluster III. All of which showed a sharp increase in expression when *Pyropia* underwent water loss, except for PC-5P-180717_97, which was upregulated only until HWL. When rehydrated, the levels of most of the miRNAs in cluster III barely changed, and only PC-5p-210644_78 showed marked downregulation.

PC-3p-99769_194 Regulated Photosynthetic Carbon Fixation Under Dehydration via the Repression of the Expression of Carbonic Anhydrase (CA)

To understand the specific functions of these differentially expressed miRNAs under osmotic stress in *P. haitanensis*, we predicted the target genes of differentially expressed miRNAs and analyzed the enriched functional categories

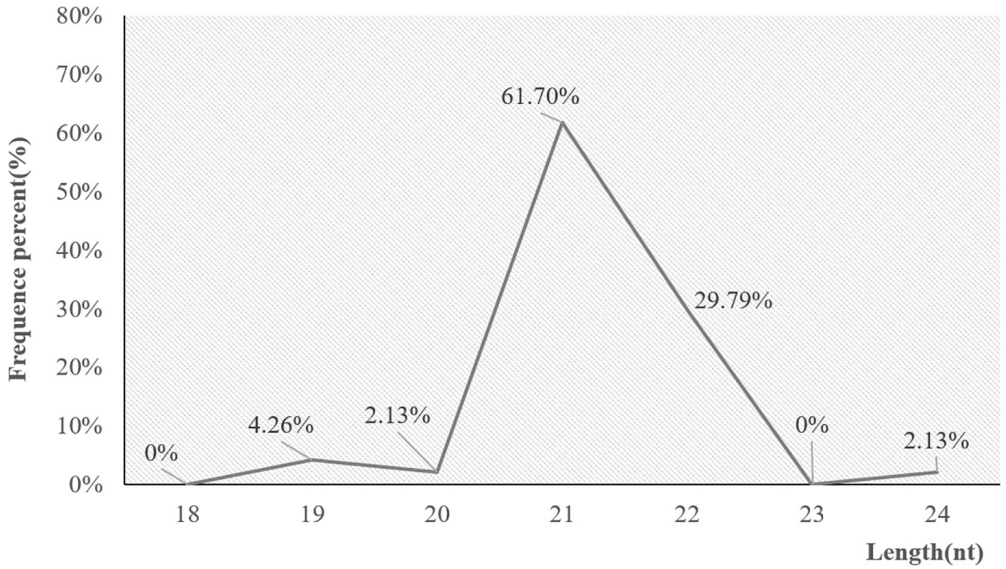

FIGURE 1 | The length distribution of identified miRNAs.

that they encoded in terms of GO and KEGG pathways. Overall, 60 target genes were predicted for 12 miRNAs. According to the GO classification system, these genes were classified into molecular function categories related to catalytic activity, binding, transporter activity, transcription factor activity, signal transducer activity, and antioxidant activity (**Supplementary Table S3**). The results of KEGG metabolic pathway annotation showed that cell cycle, meiosis, nucleotide excision repair, DNA replication, glycerolipid metabolism, and base excision repair pathways were significantly enriched ($P < 0.01$) (**Supplementary Table S3**). Nucleotide excision repair, base excision repair, cell cycle, and DNA replication were also significantly enriched in differentially transcribed genes under osmotic stress in *P. haitanensis* as previously described (Wang et al., 2015). The consistency between the two datasets supports the regulatory role of miRNAs in response to osmotic stress.

miRNA PC-3p-99769_194 from cluster III was predicted to bind to the 523–542 bp region of the CA gene and may regulate its expression by degrading mRNA. The miRNA PC-3p-99769_194 was upregulated during the whole dehydrated process. Accordingly, the target CA gene had declined in transcriptional activity as observed in the published transcriptomic data under dehydration in *P. haitanensis*. The CA gene exhibited 0.77- and 0.44-fold downregulation under 30 and 80% dehydration and 1.3-fold upregulation under rehydration conditions.

PC-5p-335121_37 and PC-5p-350045_35 were highly expressed in samples under dehydration stress. The target genes of PC-5p-335121_37 are ph07921.t1 and ph07931.t1 which encode the U5 snRNP complex subunit and superoxide dismutase, respectively. PC-5p-350045_35 targets cid-thiol ligase and glycosyltransferase (*ph09862.t1* and *ph00598.t1*), respectively. PTC-MIR482D-P3_2SS14CG18CG exhibited upregulated expression in rehydration samples. The target gene of ptc-MIR482D-P3_2SS14CG18CG is *ph04985.t1*, which encodes epoxyqueuosine reductase (**Supplementary Table S3**).

Validation of the Expression of PC-3p-99769_194 and Its Target CA Using qPCR

To validate the expression of miRNAs obtained from the high throughput sequencing approach, qPCR was performed on three miRNAs among samples of CON, MWL, HWL, SWL, and REH. PC-3p-99769_194 exhibited 2. 21-, 3. 46-, 1. 71-, and 1.83-fold upregulation in *P. haitanensis* after MWL, HWL, SWL, and REH, respectively. The expression of PC-5p-350045_35 in dehydration and rehydration samples were higher than those in the control (**Figure 3**). Its target gene CA (*ph0980.t1*) was downregulated 0. 87-, 0. 84-, and 0. 51-fold during MWL, HWL, SWL, respectively. And underwent a slight 1.39-fold increase during REH. These inverse transcriptional profiles suggest that PC-3p-99769_194A might function as a negative regulator of CA expression.

Small RNA Biogenesis and Function Apparatus in *P. haitanensis*

To unravel the RNA silencing machinery for miRNA generation and function in *Pyropia,* we identified genes encoding the two proteins that mediate the central activities required for miRNA and small RNA biogenesis and functioning, Dicer and Argonaute (AGO), respectively. Dicer proteins are characterized in many organisms, have multiple functional domains from the N- to C-terminus, and are approximately 2000 amino acids (aa) long. In *P. haitanensis*, we performed a Basic Local Alignment Search Tool (BLAST) search against the genome using Dicer proteins in model organisms as the query and discovered two candidate genes that exhibited high sequence similarity (*ph07342.t1* and

TABLE 2 | Summary of known and predicted miRNA in *P. haitanensis*.

miRNA	miRNA sequences	Known/ Novel
PHO_PC-3p-622531_14	CGCCGCGGTGCCGGCGCCC	Known
PHO_PC-5p-141452_132	TGGCGGGGTTCCTCTGCGG	Known
PHO_PC-3p-525521_19	TCGGTGGTGGGCGAGGCTTC	Novel
PHO_PC-3p-131912_144	TAACATGTTATCCAACCCCCC	Novel
PHO_PC-3p-196970_86	TCCAGCAACGTCGAGACCAAC	Novel
PHO_PC-3p-371143_32	TCCGTGGCGGCGGCGGCGACA	Novel
PHO_PC-3p-417402_27	TCCGAGATAGTCATGACCATT	Novel
PHO_PC-3p-468992_23	TCAAGCCGCACATAGTCGAAT	Novel
PHO_PC-3p-4725_2053	TCACGTCGGGGCCGCACTCGG	Novel
PHO_PC-3p-4725_2053	TCACGTCGGGGCCGCACTCGG	Novel
PHO_PC-3p-505409_21	TGTTATCGAACCCCCCTCACC	Novel
PHO_PC-3p-547179_18	TCACCGTTGCCGCCGGCGTCC	Novel
PHO_PC-3p-633762_14	CCGCGCGCGGCGCTGCGCCTA	Novel
PHO_PC-3p-693516_11	TCGCGCGCCGCCGTCGCCACC	Novel
PHO_PC-3p-729018_10	GCGGGCGTACAATGGGGAGGA	Novel
PHO_PC-3p-75147_255	TTATCAAACCCCCCTCACGAC	Novel
PHO_PC-3p-98937_196	TATCGAACCCCCCTCACGACC	Novel
PHO_PC-3p-99769_194	TCAACCGGGCGGCGCTGTCGG	Novel
PHO_PC-5p-101936_190	TGAGTTGGTCCTGACGTTGCT	Novel
PHO_PC-5p-166473_108	TATCAAACCCCCCTCACGACA	Novel
PHO_PC-5p-210644_78	TGCGGAGTGGAAATGTCTCGA	Novel
PHO_PC-5p-273263_52	TCGAAGTCAAGGGCCGGGTCC	Novel
PHO_PC-5p-335121_37	TCCGCGAACTCAGACTGCCGC	Novel
PHO_PC-5p-350045_35	TCTGCCGTGTTCGCGCCGCGG	Novel
PHO_PC-5p-393688_30	TGTTATCAAACCCCCCTCACC	Novel
PHO_PC-5p-409256_28	TGTCTCGAGTCGGAGCGGAGT	Novel
PHO_PC-5p-480556_23	TCGTGCTGTCGTTTGCCGCCT	Novel
PHO_PC-5p-505409_21	TGTTATCGAACCCCCCTCACC	Novel
PHO_PC-5p-517777_20	TCGTCGCACTCAGAAAGAGGT	Novel
PHO_PC-5p-632100_14	CGCCGCCCGCCCGGCCGCCCG	Novel
PHO_PC-3p-45934_389	TCACGTCGGGGCCGCACTCGGG	Novel
PHO_PC-3p-45934_389	TCACGTCGGGGCCGCACTCGGG	Novel
PHO_PC-3p-528659_19	TGGCCAGCAGGAGGAGGTCTGC	Novel
PHO_PC-3p-639141_14	TGGAAATGTAGGTACAGGGTGT	Novel
PHO_PC-3p-639141_14	TGGAAATGTAGGTACAGGGTGT	Novel
PHO_PC-5p-180717_97	TCCACGGCTGTGGGACGGCCCC	Novel
PHO_PC-5p-274854_52	TCAAACCCCCCTCACGACCTCC	Novel
PHO_PC-5p-399919_29	TCGGGCGCGGCGGTGGCCGGCT	Novel
PHO_PC-5p-416633_27	TCAAAGTCAAGACTGGGTCCGG	Novel
PHO_PC-5p-516530_20	CTATCAAACCCCCCTCACGACC	Novel
PHO_PC-5p-563372_17	TGACGCCCGTCTGCCATCGCTT	Novel
PHO_PC-5p-563372_17	TGACGCCCGTCTGCCATCGCTT	Novel
PHO_PC-5p-605903_15	TGGCGCCGTCGCCGAACGCCTC	Novel
PHO_PC-5p-625345_14	GTGCGCGCGCCGGCTGCAGGTG	Novel
PHO_PC-5p-388767_30	GGCAGCGGCGGCGGCGGTAACGGC	Novel
ptc-MIR482d-p3_2ss14C G18CG	TTCTTGCCGACCCGACCGATG	Novel
sbi-MIR399d-p3_2ss1 GT19AG	TCGGTCATCGTCGTCGTCGTC	Novel

ph05047.t1 at 562 aa and 1163 aa long, respectively). *Ph07342.t1* contains a DEAD-like helicase domain (DEXDc) and a helicase C (HELIc) domain and was aligned to the N-terminal part of reference Dicer. *Ph05047.t1* has two parallel RIBO (ribonuclease III) domains. The two genes are located in two separate loci and are not adjacent to each other, which is different from the gene arrangement in other species (**Figure 4**). This phenomenon was verified in *Po. umbilicalis*. However, red macroalgae *C. crispus* and *Gracilariopsis chorda*, as well as the unicellular red algae *Porphyridium purpureum*, have one full-length canonical Dicer gene with multiple functional domains. To trace the evolutionary history of the two segmented Dicer genes, we identified Dicer homologs in available species and performed phylogenetic analyses. In the evolutionary trees constructed based on model Dicer sequences for ph07342.t1 and ph05047.t1, both closely clustered with the two *Po. umbilicalis* proteins and formed a unique branch, whereas the Dicer proteins from other species united as a single group (**Supplementary Figure S1**). For AGO proteins, there were 1, 2, 1, 3, and 3 candidate genes found in *P. haitanensis*, *Po. umbilicalis*, *G. chorda*, *C. crispus*, and *P. purpureum* genomes, respectively. All had canonical structures with an AGO-specific domain, a PAZ domain, and a PIWI domain at the C-terminal. *P. haitanensis* AGO exhibited close phylogeny with homologs in other red algae (**Supplementary Figure S2**). Conserved amino acids in the PIWI domain that facilitate siRNA binding were observed in *P. haitanensis* AGO (**Figure 5**). Moreover, it harbored a classical glutamate as a key residue of the catalytic DDE motif in RNase H. The DDH motif is required for metal ion coordination in the catalytic center, albeit "H" residue was not found in the corresponding locus (**Figure 5**). The identification of the key components of miRNA biogenesis machinery in *P. haitanensis*, as well as their expressional variation under desiccation, indicated the potential involvement of an miRNA-based regulatory mechanism in response to osmotic stress and gene engineering via artificial miRNA and siRNA. Although AGO that exhibited contractive gene copies, albeit well-conserved sequences, the distinctive structure of Dicer proteins lets us envisage some unique features possibly existing in *Pyropia* and *Porphyra*. In addition, we searched the transcriptional levels of two Dicer genes and the AGO gene in the published transcriptomic data under dehydration in *P. haitanensis* and discovered that the two Dicer genes were increased under dehydration and rehydration conditions. The AGO gene was 1.14-fold upregulated under medium dehydration, whereas AGO was 0.69- and 0.52-fold downregulated under severe dehydration and rehydration conditions.

DISCUSSION

miRNAs Regulate Carbon Fixation by Downregulating CA Under Osmotic Stress

The expression of miRNAs, an important class of gene regulators, is altered by abiotic stress treatment such as light, temperature, moisture, salt, and heavy metal ions (Jia et al., 2009; Lv et al., 2010). However, as most of these studies were performed using model organisms, little is known about seaweeds. CA catalyzes the interconversion between carbon dioxide and dissolved

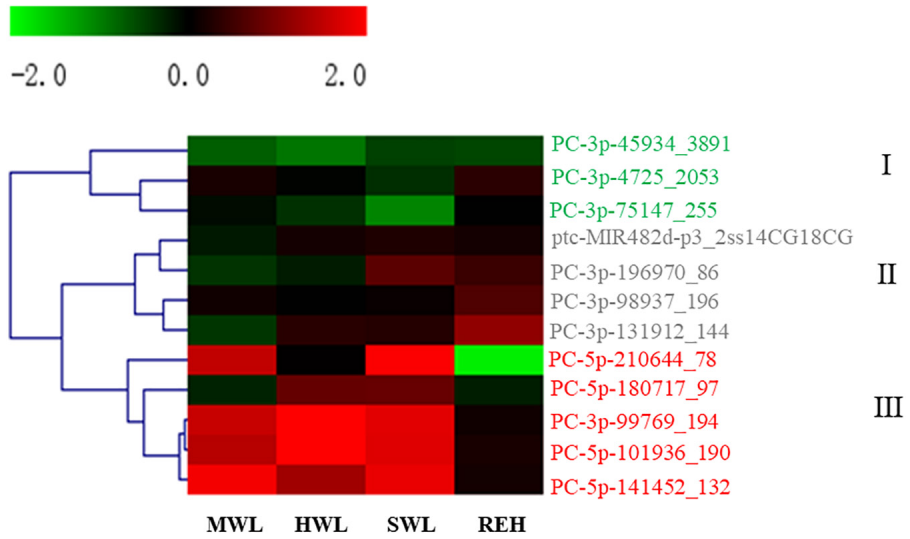

FIGURE 2 | Expressional patterns of miRNAs during desiccated stresses. CON, Normal conditions (control); MWL, 30 ± 5% water loss rate (moderate water loss); HML, 50% ± 5% (severe water loss); SWL, 80% ± 5% (Severe water loss); REH, the gametophytes from SWL were put back into seawater to rehydrate for 30 min. The expressional variation was represented by fold-change = $\log_2$(FPKM under stress/FPKM under control). The miRNAs in cluster I, II, and III have been depicted in green, gray and red, respectivley.

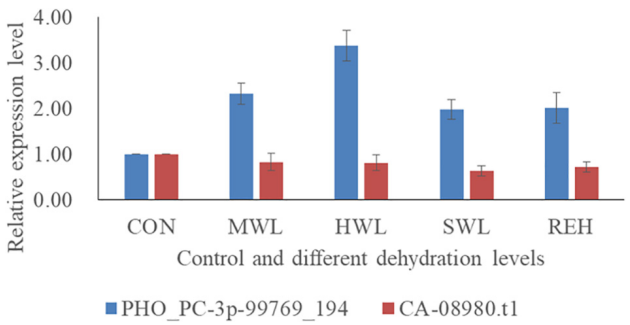

FIGURE 3 | Verification of the expression of PC-3p-99769_194 and its target CA gene.

bicarbonate. It is the key component of the carbon concentration mechanism in aquatic photosynthetic organisms by facilitating the accumulation of sufficient carbon dioxide in the vicinity of rubisco (Badger and Price, 1994). However, the exact molecular mechanism involved in the control of the expression of CA is unclear. In this study, we demonstrated a post-transcriptional regulatory mechanism of CA and identified an miRNA as the key element involved in the regulation of the expression of the CA gene. The raised level of expression of PC-3p-99769_194 under dehydration stress suggested its increased negative regulation of the target CA gene. This is further supported by the decreased transcriptional level of CA gene in our previous study (Wang et al., 2015). Three enzymes involved in photosynthesis (rubisco, fumarate and nitrate reductase, and CA are downregulated in desiccated individuals compared to naturally hydrated controls by 2DE and LC-MS/MS analyses (López-Cristoffanini et al., 2015). Under high salinity treatment, the algae *Nitzschia closterium f. minutissima* adjusts its photosynthetic pigment content to decrease photosynthesis and inhibits the activity of CA (Yu et al., 2011). Combined with our study, we speculate that *Pyropia* reduce their photosynthetic activities in response to osmotic stress via miRNA-based regulatory mechanisms.

Rehydration can induce various metabolic processes that responding to osmotic stress in seaweed. The target gene of upregulated ptc-MIR482D-P3_2SS14CG18CG is related to epoxyqueuosine reductase, which catalyzes the last step of the two-electron reduction of epoxyqueuosine to queuosine during the synthesis of tRNA (Nishimura, 1983) and plays an important role in translational efficiency, accuracy, and structural stabilization of tRNA (El Yacoubi et al., 2012). In our study, genes encoding epoxyqueuosine reductase showed high expression when *P. haitanensis* was subjected to osmotic stress and its expression was suppressed during rehydration. Salt treatment in *Anabaena fertilissima* leads to the hyperaccumulation of epoxyqueuosine reductase (Rai and Swapnil, 2019). Therefore, we speculate that suppressed miRNA leads to the increased expression of epoxyqueuosine reductase in response to osmotic stress in *Pyropia*.

The miRNA Biogenesis in *P. haitanensis*

Dicer and AGO are the key components in miRNA biogenesis and are characterized in almost all organisms that have miRNA regulatory mechanisms (Bernstein et al., 2003; Yang et al., 2013). Both have well-conserved homologs with similarly organized functional domains and motifs. However, in this study, we identified that in *Pyropia*, Dicer was divided into two genes, *ph07342.t1* that harbors the N-terminal functional domains and an open reading frame (ORF) that contains the other

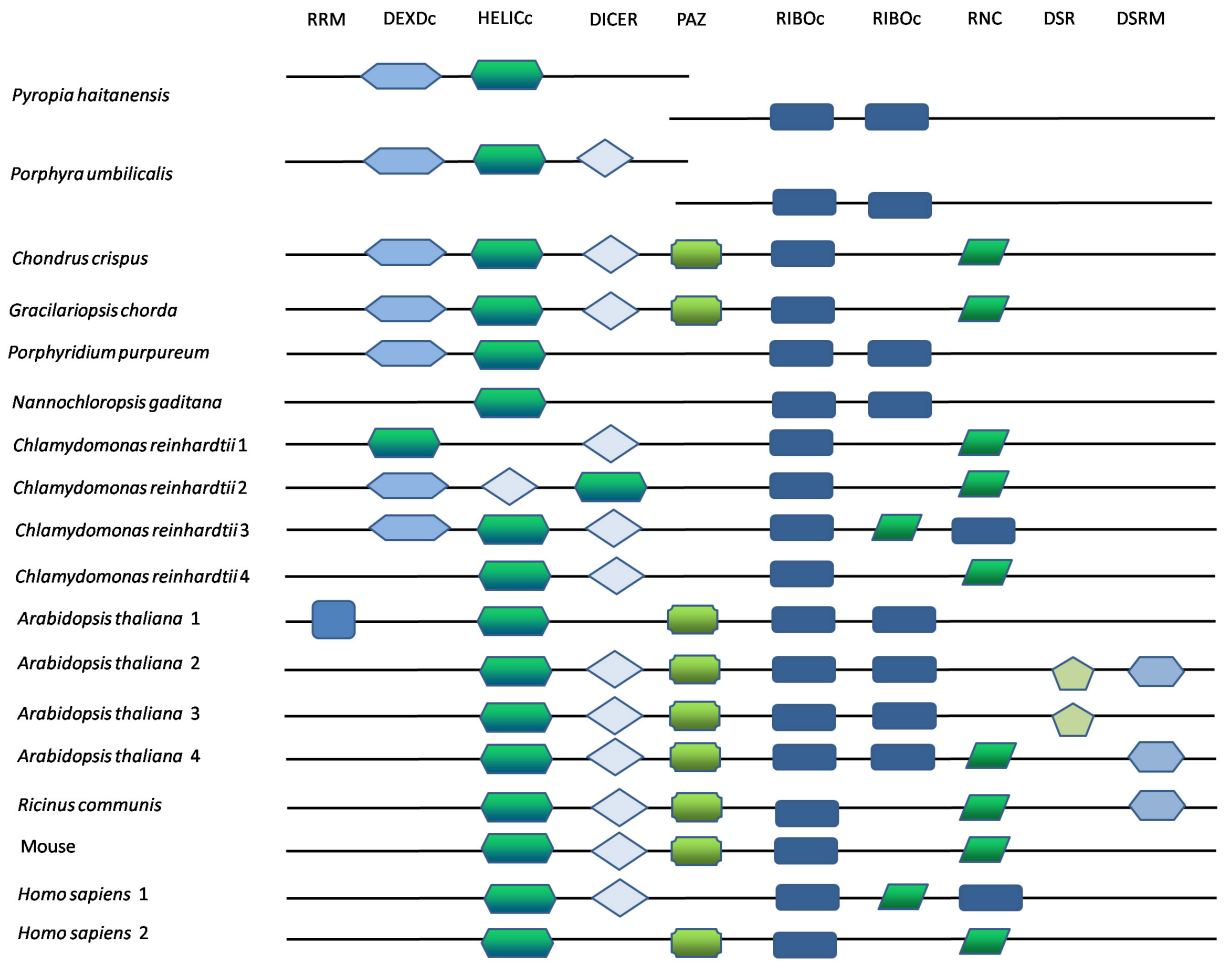

FIGURE 4 | Structural features of Dicers in *P. haitanensis* and other species. Each conserved domains were represented as different shapes. RRM, RNA recognition motif; DEXDc, DEAD-like helicase domain; HELICc, helicase C; RIBOc, Ribonuclease III C terminal domain; RNC, dsRNA-specific ribonuclease.

domains. The segmentation of Dicer was also observed in other Bangiales including *P. yezoensis* and *Po. umbilicalis*, whereas in the Florideophyceae genomes, such as *C. crispus*, *G. chorda*, and *P. purpureum*, full-length canonical Dicer was present. This particular distribution of segmented Dicer protein suggested that it might be generated after the evolutionary diversification of Bangiales. The length of a small RNA is mainly related to the type of enzymes involved in its processing. For example, a 21 nt segment is generally generated by Dicer-like 1 (DCL1), a 22 nt segment by RNase endonuclease, and a 24 nt segment by Dicer-like 3 (DCL3) (Xie et al., 2004; Vazquez, 2006; Ou et al., 2012). The characteristic length distribution of small RNAs, with 21 nt as the dominant length in *Pyropia*, suggests that *Pyropia* Dicer protein might function in a DCL1-like way in miRNA biogenesis.

Partial and truncated Dicer with a C-terminal fragment was observed in *Caenorhabditis elegans* before and characterized to be a negative regulator of miRNA biogenesis via its competitive interaction with AGO proteins (Sawh and Duchaine, 2013). However, the truncated small Dicer was generated by *in vivo* proteolytic cleavage, rather than by alternative splicing or individual "truncated" genes as in *Pyropia*. Thus, we envisage that they might function in a different way. The action mode is "two-peptides," which means that the two peptides encoded by the two ORFs serve as the "subunits" and form a heterodimer. In *Pyropia* AGO, although a close phylogenetic relationship with its red algal counterparts was observed, it was encoded by only one gene, whereas three were observed in other red algae. Therefore, the segmented structure of Dicer and the contracted copy numbers of AGO represented the distinctive feature of miRNA biogenesis in *Pyropia* and imply a diverse miRNA regulatory mechanism. The latter was further reinforced by the dominant length of miRNA and a relative lack of universally conserved miRNAs.

CONCLUSION

To investigate the miRNA-based regulatory mechanisms in response to desiccation stress in *Pyropia*, we profiled the miRNAs

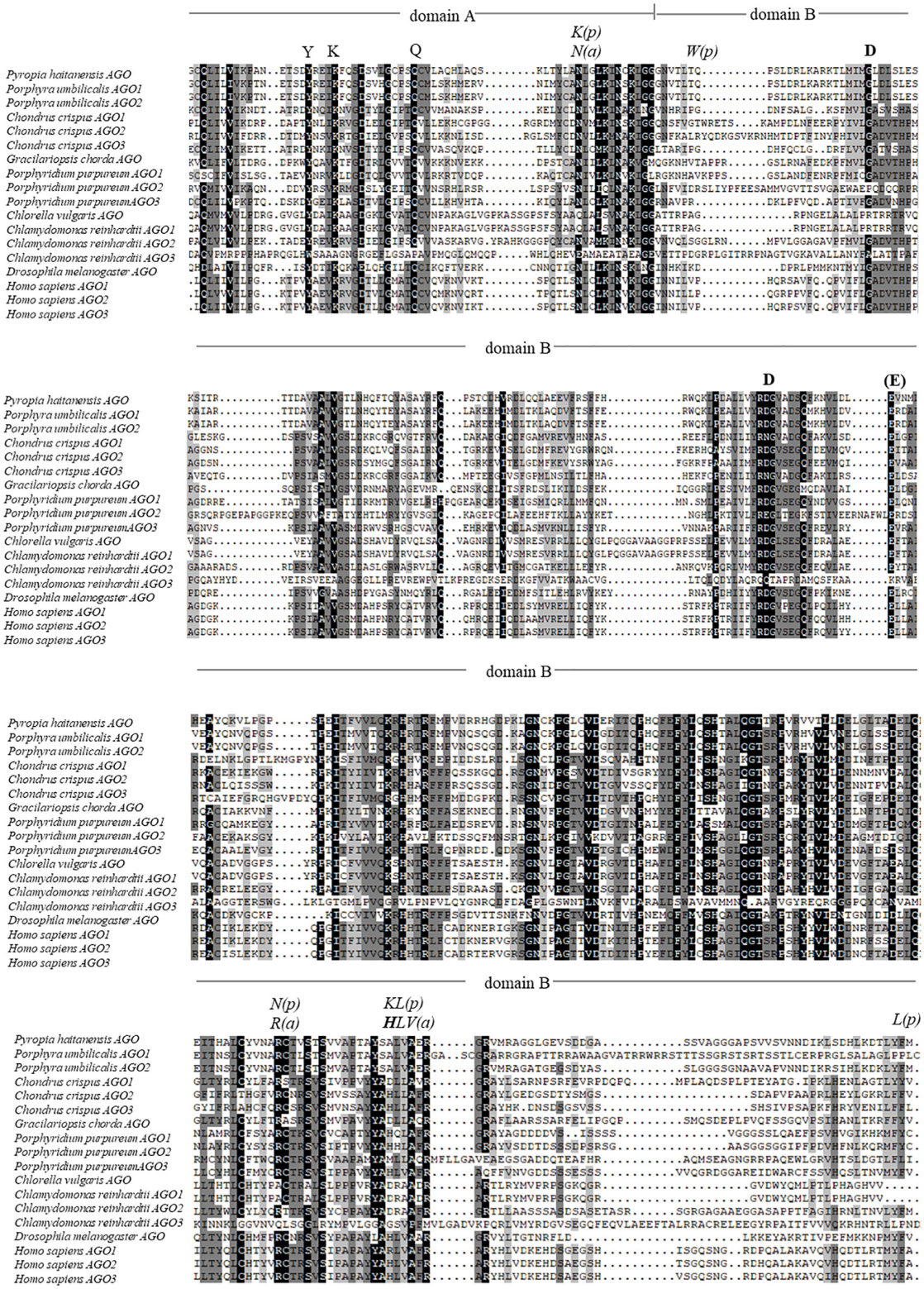

FIGURE 5 | Sequence alignment of Argonaute PIWI domains. Accession numbers of AGOs in P. haitanensis and other species were listed in **Supplementary Table S4**. Residues highlighted in dark gray are shared ≥75% similarities, media gray are those shared ≥50% similarities, light gray are those shared ≥30% similarities in all sequences. Conserved amino acids were marked as N/Q, D/E, R/K, S/T, F/Y, A/G, and V/I/L/M in this figure. D, D and H amino acid residues in bold letters represent the DDH motif. Italicized residues are class-switch residues that attribute a given protein either to the (p)iwi or to the (a)rgonaute subfamily.

from a series of dehydrated and rehydrated *Pyropia* thalli by high-throughput sequencing. Analysis of their transcriptional variations, as well as target genes, revealed that miRNAs play essential roles in suppressing carbon fixation by downregulating the CA gene to survive in the intertidal zone. The genetic structural and phylogenetic analysis of Dicer and AGO proteins disclosed the distinctive features of miRNA-mediated mechanism in *Pyropia*. Our discoveries provide valuable information for further functional characterization of miRNAs in response to abiotic stress in *Pyropia* and shed light on the evolution of miRNA-mediated mechanism in algae.

DATA AVAILABILITY STATEMENT

The sequencing data have been deposited into the NCBI Sequence Read Archive under the BioProject: PRJNA573823.

AUTHOR CONTRIBUTIONS

YM conceived the study. MC performed the experiments. KX, FK, and JW analyzed the results. MC and DW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00766/full#supplementary-material

FIGURE S1 | Phylogenetic relationships of Dicer genes.

FIGURE S2 | Phylogenetic relationships of AGO genes.

TABLE S1 | Specific primers for miRNAs and mRNAs.

TABLE S2 | Conserved miRNAs in *Pyropia* and *C. crispus*.

TABLE S3 | Significant expressed miRNAs and the function of their target genes.

TABLE S4 | The AGO and Dicer sequences used in this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.