RNA BIOLOGY
2016, VOL. 13, NO. 3, 343–352
http://dx.doi.org/10.1080/15476286.2015.1125075

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RESEARCH PAPER

Identification and characterization of microRNAs in Eucheuma denticulatum by high-throughput sequencing and bioinformatics analysis

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ABSTRACT

Eucheuma denticulatum, an economically and industrially important red alga, is a valuable marine resource. Although microRNAs (miRNAs) play an essential role in gene post-transcriptional regulation, no research has been conducted to identify and characterize miRNAs in *E. denticulatum*. In this study, we identified 134 miRNAs (133 conserved miRNAs and one novel miRNA) from 2,997,135 small-RNA reads by high-throughput sequencing combined with bioinformatics analysis. BLAST searching against miRBase uncovered 126 potential miRNA families. A conservation and diversity analysis of predicted miRNA families in different plant species was performed by comparative alignment and homology searching. A total of 4 and 13 randomly selected miRNAs were respectively validated by northern blotting and stem-loop reverse transcription PCR, thereby demonstrating the reliability of the miRNA sequencing data. Altogether, 871 potential target genes were predicted using psRobot and TargetFinder. Target genes classification and enrichment were conducted based on Gene Ontology analysis. The functions of target gene products and associated metabolic pathways were predicted by Kyoto Encyclopedia of Genes and Genomes pathway analysis. A Cytoscape network was constructed to explore the interrelationships of miRNAs, miRNA-target genes and target genes. A large number of miRNAs with diverse target genes will play important roles for further understanding some essential biological processes in *E. denticulatum*. The uncovered information can serve as an important reference for the protection and utilization of this unique red alga in the future.

Abbreviations: miRNA, microRNA; RT-PCR, reverse transcription polymerase chain reaction; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; HiSeq, high-throughput sequencing; MFE, minimum free energy of folding; BP, biological process; CC, cellular component; MF, molecular function

ARTICLE HISTORY
Received 28 October 2015
Revised 19 November 2015
Accepted 22 November 2015

KEYWORDS
Bioinformatics; characterization; eucheuma denticulatum; high-throughput sequencing; identification; microRNA

Introduction

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs approximately 21 nt long that play pivotal roles in post-transcriptional processing via mRNA cleavage or translational repression.[1,2] Their pri-miRNA precursors, which have a characteristic stem-loop structure, are recognized and cleaved by a Dicer-containing protein complex to yield pre-miRNAs.[3] In plants, a pre-miRNA is then cleaved by Dicer-like 1 protein to generate an approximately 23-nt miRNA/miRNA* duplex.[4] The mature miRNA on the 5′ end of the duplex is generally incorporated into the RNA-induced silencing complex (RISC), [5] while the miRNA* on the 3′ end is usually degraded following miRNA-RISC target recognition.[6] Finally, translational repression or mRNA degradation is induced.[7]

Since their initial discovery in *Caenorhabditis elegans*,[8] many miRNAs have been identified in model organisms and found to play crucial roles in various biological processes.[9] With the rapid development of next-generation sequencing technology[10] and new bioinformatics software,[11] an increasing number of miRNAs have been detected in crop plant species such as rice, tobacco, maize, potato and wheat.[4-7,12] Much evidence is accruing that miRNAs play an essential role in processes such as tissue development, signal transduction and environmental stress response in model organisms and higher plants.[12-17] In non-model algae, however, studies on unique small RNAs have been limited.[18]

Eucheuma denticulatum or eucheuma, frequently dubbed a "marine crop," is one of the main economic marine algae typically growing on reefs in tropical areas.[19] As the main raw material of carrageenan,[20] eucheuma has been extensively used in the food industry.[21] In addition to carrageenan, eucheuma is rich in dietary fiber, a substance that according to some reported studies can reduce blood lipids.[22,23] Notably, some research has demonstrated that seleno-polysaccharides of eucheuma can inhibit tumor cells and virus growth,[24-27] possibly by blocking cell growth and inducing cell apoptosis in the former case and by preventing viral adsorption normal cells in the latter.[28] Despite its importance, eucheuma germplasm resources are undergoing severe degradation as a result of frequent introduction and over-reproduction, environmental

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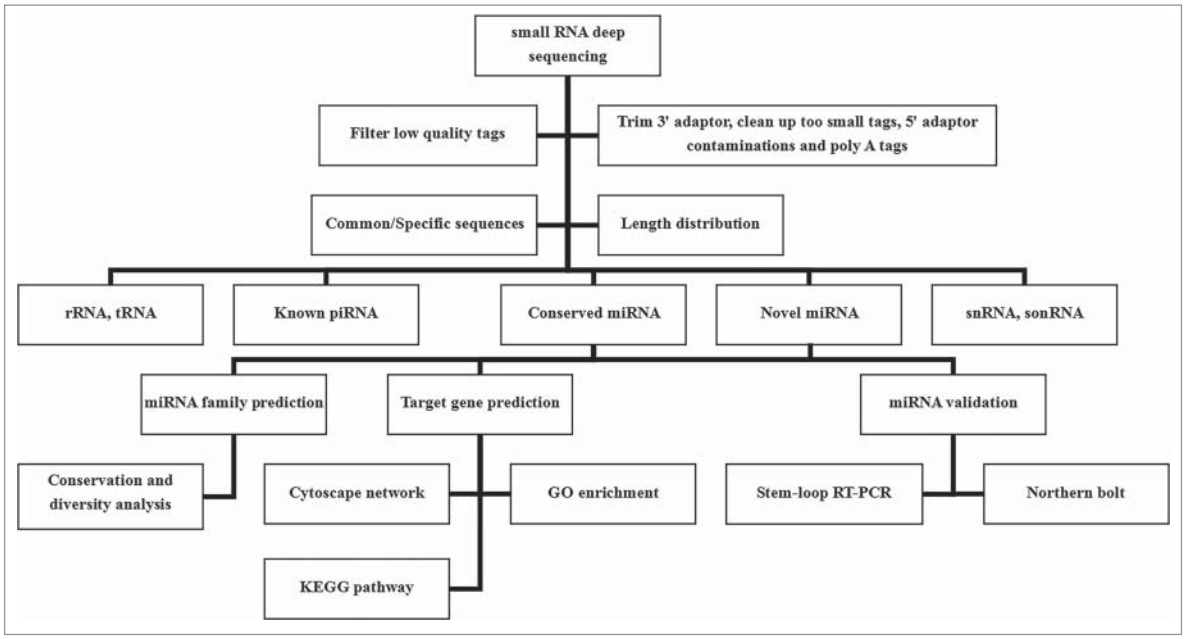

Figure 1. Overview of experiments and bioinformatics analyses applied to *Eucheuma denticulatum*.

pollution, disease interference, competition from epiphytic algae and herbivore animal invasion.[29-32] The protection and development of eucheuma resources is an urgent priority in the main countries of production, such as South Africa, Australia, Japan, the Philippines, Indonesia and China.[33,34] Identifying as many miRNAs as possible in this red alga is very important for this purpose.

Thus far, most studies on eucheuma have focused on its growth and cultivation,[35,36] photosynthesis and respiration,[37] processing technology,[38] bioactive components,[39] genetic variation,[40] and cloning and expression of key enzyme genes.[41] Although over 35,000 miRNAs from 223 species have been submitted to the miRNA database (miRBase 21.0, June 2014, http://www.mirbase.org/),[42] no miRNAs have yet been found in *E. denticulatum*. Because the *E. denticulatum* genome has not been sequenced, the lack of extensive sequence information in the database impedes miRNA identification using bioinformatics technology. However, new high-throughput sequencing (HiSeq) combined with bioinformatics[43,44] may be an effective strategy for identification of as many miRNAs as possible in this red alga. Their identification would be extremely important to comprehensively understand the roles of these non-coding RNAs in various biological regulatory processes and metabolic pathways.[45] Capture of useful information from miRNA data, prediction of target genes, analysis of related biological processes and metabolic pathways will be very meaningful to further protect and develop this valuable marine crop in the future.[46,47]

Results and discussion

HiSeq of small RNAs of E. denticulatum

As shown in the overview in Fig. 1, a high-quality small RNA population (its concentration was about 42 ng/L) isolated from total RNA of *E. denticulatum* was subjected to HiSeq, thereby generating 11,444,177 clean reads (Table 1). The most frequent small-RNA length was 19 nt (Fig. 2). The numbers and proportions of unique and total small RNAs mapped to expressed sequence tags (ESTs) can be seen in Table 2. After removing RNAs such as rRNAs, tRNAs, snoRNAs and snRNAs identified by BLASTn searching, the remaining sequences were annotated as miRNAs and classified in this study as either known or conserved. Among the various small RNA categories shown in Fig. S1A and S1B, 267,018 reads (7,919 unique reads) were annotated as conserved miRNAs. Another 11,073,638 reads (267,018 unique reads) could not be annotated; these novel miRNAs were further analyzed.

Identification of novel miRNAs in E. denticulatum

To predict novel miRNAs in *E. denticulatum*, we aligned the unannotated sequences to ESTs of this red alga. After screening the candidates based on stringent identification criteria described in Materials and Methods, only one novel miRNA (temporarily named as ede-miR1) could be identified. The secondary structure of this novel miRNA precursor can be seen in Fig. 3. In this study, we therefore identified a total of 134 miRNAs (133 conserved and 1 novel one) in *E. denticulatum* (Table S1). The length

Table 1. Summary of reads generated from sequencing of *Eucheuma denticulatum* small-RNA libraries.

Type	Reads	Percent (%)
Total reads	11556963	
High quality	11536642	100%
3′adapter null	1392	0.01%
Insert null	2306	0.02%
5′adapter contaminants	26879	0.23%
Smaller than 18nt	61785	0.54%
PolyA	103	0.00%
Clean reads	11444177	99.20%

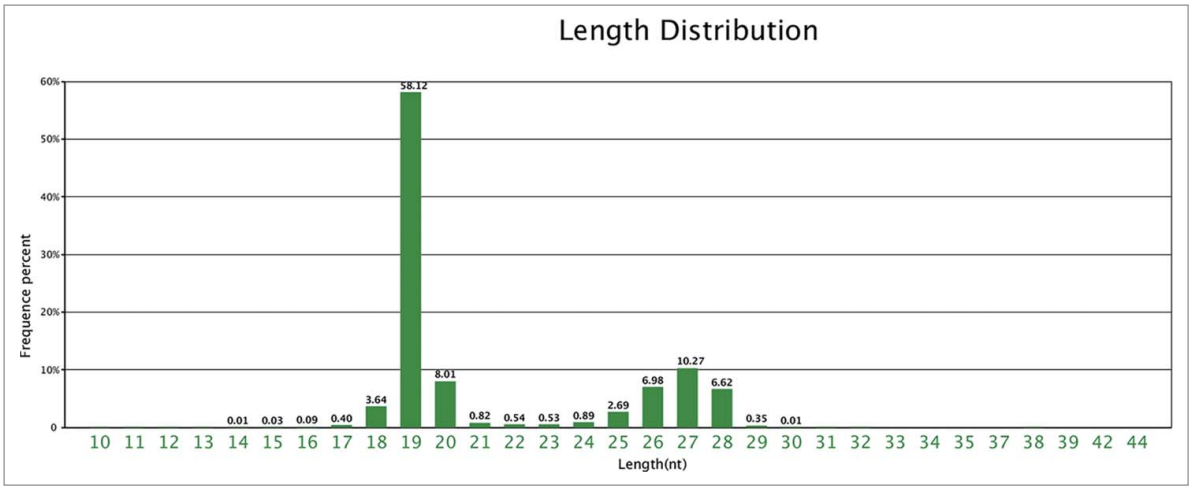

Figure 2. Length distribution of small RNAs in *Eucheuma denticulatum*. As shown by the indicated frequency percentages, 19-nt-long reads were the most abundant.

distribution of identified miRNAs ranged from 18 to 27 nt, with 19 nt the most frequent length (Fig. S2A). These sizes are typical of plant miRNAs. According to the distribution of nucleotides in different positions of the detected miRNAs, the least common nucleotide was C (Fig. S2B), and U was the most biased first-position nucleotide (Fig. S2C), which is consistent with the basic characteristics of most identified miRNA sequences.[48]

Conservation and diversity of miRNA families

Potential miRNA families can be predicted on the basis of sequence homology between predicted and known miRNAs stored in miRBase. Altogether, 126 miRNA families with 133 members were predicted in this study (Table S2). As shown in Table 3, most families had only one representative in *E. denticulatum*, with miR164, miR166, miR169, miR319, miR397, miR398 and miR5562 having 2 members. To investigate the conservation and diversity of these predicted miRNA families across plants, we additionally performed a BLAST search of 21 randomly selected new predicted miRNA families in *E. denticulatum* against miRNA families in other plant species (Table S3). As shown in Fig. 4, the 21 families were found in 11 different plant species. Among them, 8 and 6 families had homologs in *Oryza sativa* and *Arabidopsis thaliana*, respectively. Moreover, miR6463 homologs were found in *Populus trichocarpa, A. thaliana, Medicago truncatula, Glycine max* and *Brachypodium distachyon*, indicating that these RNA sequences may originate from a common sequence in an ancestral species. Finally, these identified miRNA families were found in both bryophytes and angiosperms, revealing the diversity of these miRNA families in different plant species. Whether there is a potential relationship among conservation and diversity of miRNA families, small RNAs evolution as well as species evolution remains to be explored in the future.

Table 2. Summary of small RNAs mapped to transcripts and ESTs of *Eucheuma denticulatum*.

	Unique reads	Percent (%)	Total reads	Percent (%)
Total small RNAs	2997135	100%	11444177	100%
Mapped to transcripts and ESTs	66052	2.2%	1477411	12.91%

Validation of miRNAs in E. denticulatum

To validate the credibility of identified miRNAs, 4 miRNAs were first randomly selected for RNA gel blot detection[49] using the digoxigenin-labeled oligonucleotide probes listed in Table S4. Second, 13 miRNAs (12 conserved and one novel) were randomly chosen and subjected to stem-loop reverse transcription (RT)-PCR validation[50] using the primer sequences given in Table S5. As shown in Fig. 5A, 3 of the 4 miRNAs could be detected by probe hybridization, while all 13 miRNAs were detectable by stem-loop RT-PCR (Fig. 5B). The results of these 2 validation approaches demonstrate that the majority of miRNAs identified in this study are credible. As is well known, some miRNAs are expressed at low levels.[51] In addition, some genes are expressed in different tissues or at different stages.[52] This spatial and temporal variation in miRNAs expression may be the main factor hindering miRNA validation, such as the negative result seen for miR7734-5p and the low expression of miR2667a observed in our northern blot analysis (Fig. 5A). In spite of this fact, the 2 validation rates in this study (75% and 100%) are still high based on the principle of random sampling, which indicates that our applied methods were effective. Furthermore, the spatial and temporal expression analysis on some miRNAs will be conducted.

Prediction of E. denticulatum miRNA target genes

Plant miRNAs can bind nearly perfectly with their targets by complementary matching to regulate the mRNA post-transcriptional process by mRNA cleavage or translational inhibition.[53] To predict as many target genes as possible in *E. denticulatum*, both psRobot[54] and TargetFinder [55] were used to predict putative targets. As shown in Table 4, 542 and 621 target genes were predicted based on 627 targets predicted by psRobot and 813 targets predicted by TargetFinder, respectively. After removing duplicates, 871 target genes were identified (Fig. S3). Moreover, 155 and 214 targets respectively

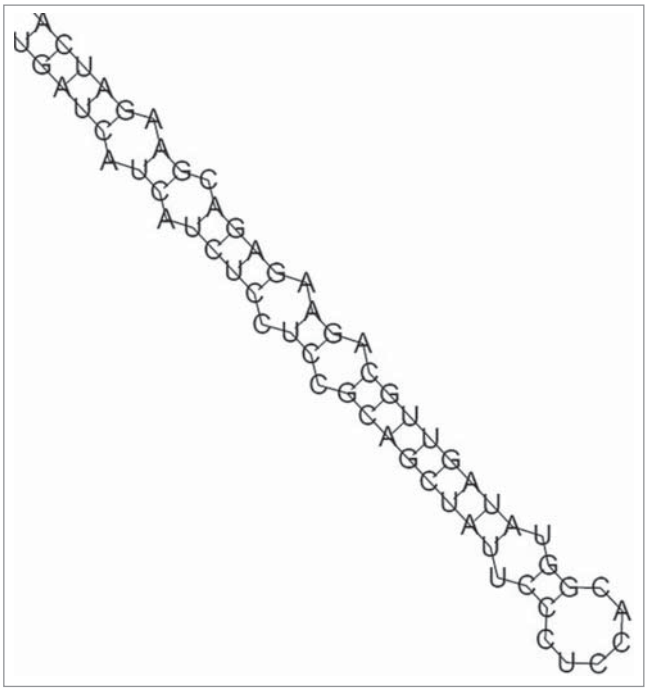

Figure 3. Secondary structure of predicted pre-miRNAs in *Eucheuma denticulatum*. The stem-loop structure of the novel miRNA precursor ede-miR1 in *E. denticulatum* is indicated.

obtained by psRobot and TargetFinder were predicted as potential translational inhibition locations based on psRNA-Target analysis [56] (Table S6). The observed percentages of translational inhibition (32.8% and 35.7%) demonstrate that cleavage may be the main miRNA inhibition pattern during the process of mRNA post-transcription in *E. denticulatum*.

Gene ontology (GO) analysis

Statistical results of GO classification demonstrate that the majority of putative target genes were related to BP and CC categories (Fig. 6).[57] Directed acyclic graphs of the top 10 enriched GO terms based on the 3 ontologies (BP, CC and MF) can be seen in Fig. S4A–S4C. As shown in Table S7, the 3 most highly enriched GO terms in BP, CC and MF categories were related to porphyrin-containing compound biosynthetic process, plastid stroma, and catalytic activity, respectively. Notably, some enriched GO terms may provide reference information for protection and utilization of this red algal resource.[58] For example, the enriched target genes (GO: 0050896) regulated by miR535d were associated with *E. denticulatum* response to stimulus (Tables S6 and S7), possibly providing some clues for future study of the adaption of eucheuma to various environmental stresses.

Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis

A KEGG reference pathway analysis was carried out based on the functional hierarchy of target genes in the KEGG orthology system.[59] As shown in Table S8, the most significantly enriched pathways were biosynthesis of secondary metabolites (pathway ID: ko01110) and metabolic pathways (pathway ID: ko01100). Because these metabolic categories are so broad,[60] however, no pathway maps could be identified in the KEGG database. Nevertheless, the porphyrin and chlorophyll metabolism category (pathway ID: ko000860) attracted our attention because of its essential role in plant growth and development (Fig. 7).[61] The potential target gene, *HemB* (including 2 types: *HemB1* and *HemB2*), is involved in the synthesis of chlorophyll precursor and plays an essential role in plant early embryonic development.[62] Research has demonstrated that transcription factors FHY3/FAR1 can regulate *HemB1* gene expression via 2 regulatory modes: [63] either through binding of FHY3/FAR1 to the promoter of *HemB1* to activate its expression, or by direct interaction of the regulation factor PIF1 with FHY3 or FAR1 to partly repress the activation of *HemB1* expression by those transcription factors. As the mechanism underlying post-transcriptional regulation of *HemB1* expression is still unclear, however, further study is needed in conjunction with future explorations of the eucheuma growth and development process.

Cytoscape network analysis

Cytoscape networks were constructed to illustrate the relationships of miRNAs, miRNA-target gene and target genes in

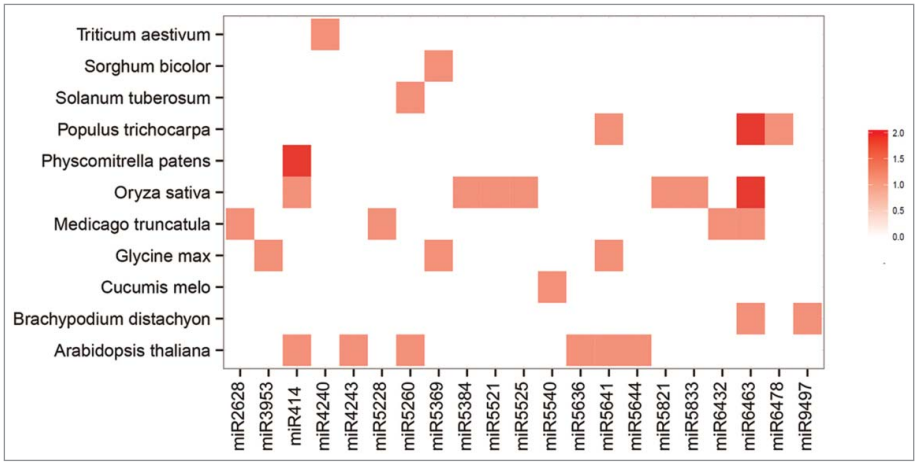

Figure 4. Distribution of predicted conserved miRNA families in different plant species. Data for mature miRNAs in different plant species are from miRBase21.0. Color coding is used to indicate the number of miRNA members in each family, with dark red corresponding to the highest number and white the lowest.

Table 3. Sizes of miRNA families identified in *Eucheuma denticulatum*.

Size of family member	Number of miRNA family	Percent of miRNA family (%)
1	126	94.73%
2	7	5.27%

E. denticulatum.[64] As shown in Fig. S5 the most enriched and cross-linked networks were revealed to be miR5260-110 target genes and miR398b-3p-miR398a-5p-miR418-miR1919a-miR159g-3p-miR5565e-target genes. The number of miRNAs and target genes and the comprehensive co-regulatory networks among them demonstrate that some important biological processes in the organism may be regulated in a complicated, indirect fashion.[65]

Our described findings, which correspond to the first reported identification of miRNAs in *E. denticulatum*, narrow the existing research gap and lay a foundation for more detailed study of eucheuma small RNAs. Future investigations that involve mining of some key miRNAs, validation of target genes and their functions, and exploration of their regulatory mechanisms and potential metabolic pathways will be necessary for future protection and utilization of this marine alga.

Materials and methods

Plant material

Three samples of *E. denticulatum* at the sporophyte stage were collected on 1 June, 2014 from the southeastern coast of Hainan near the South China Sea (about 19° N latitude and 110° E longitude) by researchers from the College of Marine Life, Ocean University of China (Qingdao, China). Six tissue portions from different locations were removed from each sample and sheared into approximately 1.5–2-cm cubes. The fresh tissues from the different samples were pooled together, immediately frozen in liquid nitrogen and stored at −75°C.

Small RNA library construction and deep sequencing

After extraction of high-quality total RNA (its concentration was about 450ng/uL, and its value of OD260/280 was between 1.8 and 2.2) from approximately 80 mg of the collected tissue pool with Trizol reagent (Invitrogen, Carlsbad, California, USA), about 10 μg of enriched small RNA was isolated using an Illumina TruSeq Small RNA Sample Prep kit (Illumina, San Diego, California, USA) and used for small-RNA library construction. Small-RNA library quality assessment was performed on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, California, USA) and a StepOnePlus Real-Time PCR system (ABI, Carlsbad, California, USA). Deep sequencing was performed on an Illumina HiSeq 2000 system (Illumina).

Standard bioinformatics analysis of small RNAs

As shown in Fig. 1, raw sequencing reads were filtered to remove unclean reads, including low-quality tags, insert null tags, 3′ adaptor null tags, and tags with 5′ adaptor contaminants, poly-A tails or lengths less than 18 nt. The remaining clean reads were annotated by alignment against *E. denticulatum* ESTs in GenBank (July 2014) using SOAP (http://soap.genomics.org.cn/)[66] and against all plant miRNAs in miRBase 21.0 (July 2014) using BLAST. After removing tRNAs, rRNAs, snRNAs, snoRNAs and known miRNAs identified in Rfam 11.0 (http://rfam.sanger.ac.uk),[67] GenBank and miRBase 21.0 databases, the remaining unannotated small RNAs were used for novel miRNA prediction.

Identification of novel miRNAs

Novel miRNAs and their precursors were identified by aligning the unannotated reads against eucheuma ESTs using Mireap (http://sourceforge.net/projects/mireap/).[68] We filtered out unreasonable reads that did not meet the following criteria:[69] (i) the precursor could form a perfect stem-loop structure; (ii) the miRNA and miRNA* formed a duplex with no more than 2 nucleotides on 5′- and 3′-end overhangs; (iii) no loops and bulges larger than 4 nt were located within the miRNA-miRNA* duplex and (iv) the value of the precursor's minimum free energy of folding (MFE) was no more than −18 kcal/mol.[70] The remaining high-confidence sequences were retained as potential novel miRNAs, and the secondary structures of their precursors were obtained using Mireap.

Prediction of miRNA families

After using ClustalX 2.0 [71] and MEGA 5 [72] to conduct an alignment analysis and BLAST search of sequences of conserved miRNAs combined with their precursors against miRNA sequences deposited in miRBase 21.0, we considered sequences

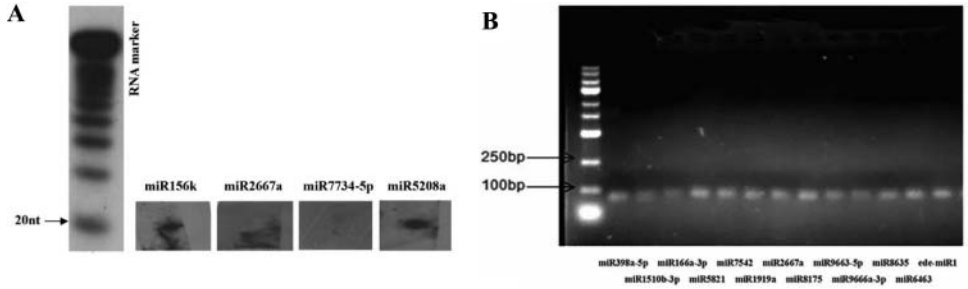

Figure 5. Gel-based detection of *Eucheuma denticulatum* miRNAs. (A) RNA gel blot hybridization of digoxigenin-labeled probes for 4 *E. denticulatum* miRNAs. (B) Agarose gel of stem-loop reverse transcription PCR products based on 13 *E. denticulatum* miRNAs.

Table 4. Summary of predicted conserved miRNA targets in *Eucheuma denticulatum*.

Software	miRNA number	Target gene number	Count of targets	Target location number
psRobot	67	542	626	627
TargetFinder	92	621	810	813
Total	100	871	1079	

with homology percentages of at least 98% and with no more than 2 mismatches among them to belong to the same family. To explore the conservation and diversity of *E. denticulatum* miRNA families in different plant species, we also conducted a BLAST comparison of 21 randomly selected miRNA families identified in *E. denticulatum* vs. plant miRNA families in miRBase.

Northern blot validation

Four miRNAs were randomly selected and subjected to RNA gel blot detection.[73] First, approximately 30 μg of total RNA was resolved on a 15% denaturing polyacrylamide gel and electrically transferred to Hybond-N+nylon membrane (Amersham Biosciences, London, UK). Blot hybridization was then carried out with miRNA-complementary oligonucleotides labeled with digoxigenin (Roche, Basel, Switzerland). The oligo sequences are listed in Table S7. In brief, membranes were subjected to the following steps:[74] (i) pre-hybridization incubation at 62°C for 2 h followed by hybridization with incubation at 42°C for 18 h; (ii) 2 washings with 2× saline sodium citrate/0.1% sodium dodecyl sulfate for 5 min at room temperature and then 2 washings with 0.5× saline sodium citrate/0.1% sodium dodecyl sulfate for 15 min at 68°C; (iii) incubation in blocking buffer for 30 min followed by addition of 20 mL antibody buffers and incubation for 30 min at room temperature; (iv) immersion in detection buffer for 5 min and then incubation at 25°C for 5 min with 1 mL added CDP-Star (a chemical luminescence material with the molecular formula $C_{18}H_{19}Cl_2Na_2O_7P$); and (v) incubation at 37°C for 10 min and exposure to X-ray film at 25°C for 15 min.

RT-PCR validation

To further validate our identification results, 13 miRNAs, including 12 conserved miRNAs and one novel miRNA, were randomly selected for stem-loop RT-PCR detection.[75] RT primer, forward primer and universal primer sequences used in this study are given in Table S8. First, the RT reaction was carried out according to the HiScript 1st Strand cDNA Synthesis kit protocol (Vazyme Biotech, New York, USA) in 20-μL reaction volumes consisting of 1 μg RNA, 0.5 μL RT primer, 5 μL of 2× RT Mix, 1 μL RT Enzyme Mix and RNase-free double-distilled H_2O. Reaction conditions were 25°C for 5 min, 42°C for 20 min, 85°C for 10 min and 4°C for 5 min. PCR amplification was then carried out according to the 2× Taq PCR Master Mix kit instructions (Vazyme Biotech) in reaction mixtures of 1 μL cDNA, 0.5 μL universal primer, 0.5 μL forward primer, 5 μL 2× Taq PCR Master Mix and 3 μL distilled H_2O. The PCR cycling program was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 15 s, with a final step of 72°C for 7 min and then 4°C for 2 min. The resulting PCR products were electrophoretically analyzed on a 1.2% agarose gel.

Target gene prediction

Prediction of miRNA target genes was performed by running psRobot (http://omicslab.genetics.ac.cn/psRobot/)[76] and

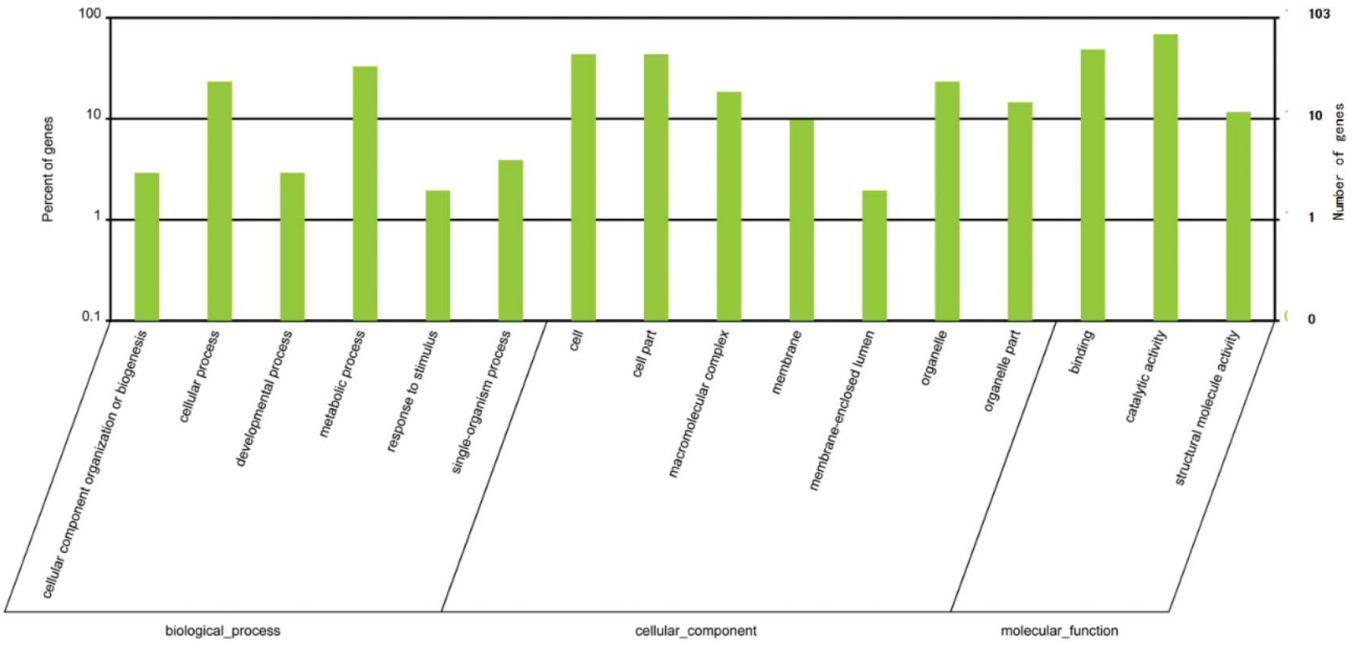

Figure 6. Gene Ontology (GO) classification of target genes. The x-axis shows the diverse biological functions of target genes based on 3 GO categories (biological process, cellular component and molecular function). The y-axis shows the percentage and number of these target genes.

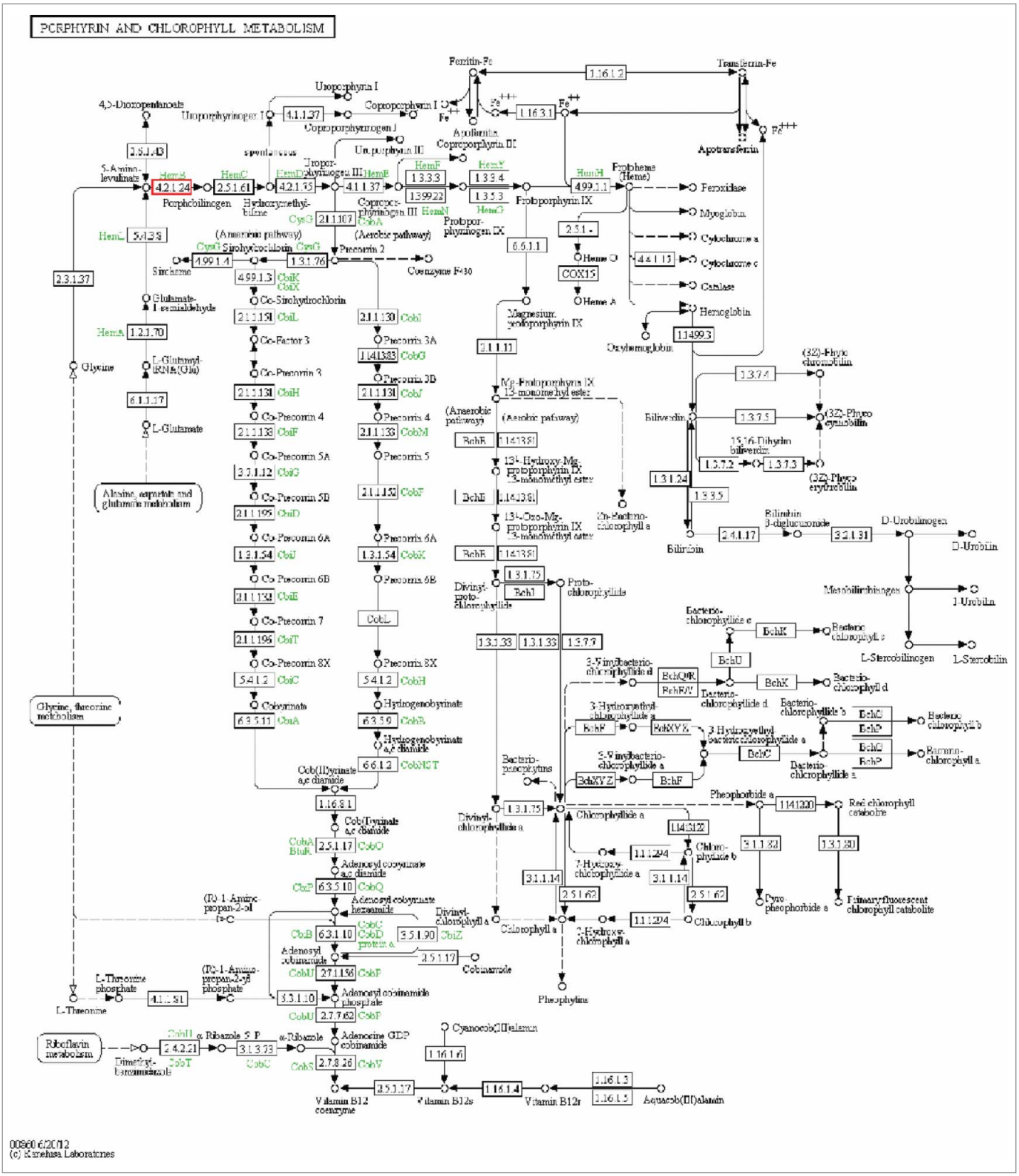

Figure 7. Porphyrin and chlorophyll metabolic pathway. The porphyrin and chlorophyll metabolic pathway was revealed by KEGG analysis. Small boxes represent proteins or enzymes (with EC nos.), with red boxes corresponding to the candidate target genes encoding them. Specific genes or enzymes are indicated in green for some species. Small circles indicate metabolites, and arrows represent the different metabolic pathways.

TargetFinder (http://carringtonlab.org/).[77] All identified miRNAs were used as queries against eucheuma transcripts and ESTs deposited in GenBank. Based on perfect complementary and close homology between miRNAs and target transcripts in plants,[78] potential targets were those with the following characteristics:[79] (i) no more than 4 mismatches in all, with no more than one mismatch in positions 1–9 and no mismatches at positions 10 and 11; (ii) no deletions or insertions; (iii) a perfect duplex at positions 8–12; (iv) no loops or bulges in either strand; (v) overhangs on 5′ and 3′ ends of no more than one nucleotide

and (vi) a MFE value of less than −18 kcal/mol between the miRNA and its complementary sequence. Moreover, potential translational inhibition was predicted based on whether one mismatch could be detected in positions 9–11 by using psRNATarget (http://plantgrn.noble.org/psRNATarget/).[56]

GO classification and enrichment

We analyzed GO functions using the following equation:[80]

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

In this equation, N is the total number of genes with GO annotations, n is number of target gene candidates in N, M is the total number of genes annotated to a certain GO term and m is the number of target gene candidates in M. GO terms with Bonferroni-corrected p-values ≤ 0.05 were defined as significantly enriched in target genes. In addition, GO classification into BP, CC and MF categories was conducted using the AmiGO tool of the Gene Ontology Consortium (http://geneontology.org/).[81] Enriched GO terms and their topological structures were obtained using the Goseq package.[82] We also constructed directed acyclic graphs of the top 10 enriched terms based on the 3 classifications. Because of the scattered, sparse nature of the gene functional distribution obtained in this study, GO classification and enrichment analysis of target genes regulated by novel miRNAs could not be conducted effectively.

KEGG pathway prediction

We inferred the main referenced metabolic pathways based on the diverse target gene functions uncovered by KEGG enrichment analysis. Target genes with KEGG orthology IDs were obtained based on similar functional products deposited in KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do).[83] The same equation used in the GO analysis was applicable to the KEGG pathway analysis, except that N was the total number of genes with KEGG annotations and M was the total number of genes annotated to a certain pathway. Only genes with a false discovery rate ≤ 0.05 were considered as significantly enriched target genes. Based on the results of the KEGG enrichment analysis, reference metabolic pathway maps were obtained using KegSketch software (http://genome.jp/kegg/).[84] For the same reason given for the GO analysis, target genes regulated by novel miRNAs could not be effectively subjected to KEGG pathway analysis.

Cytoscape network construction

To reveal correlations among miRNAs, miRNA-target genes and target genes in *E. denticulatum*, Cytoscape networks were constructed according to the Cytoscape software manual (http://www.cytoscape.org).[85,86]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Dr. Barbara Goodson (University of Texas, USA) for her critical reviews of the manuscript and editorial assistance with the English. We thank Professor Tao Liu in College of Marine Life, Ocean University of China for the support of Eucheuma dentticulatum samples, and Beijing genomics of institute (BGI) for assistance with sequencing.

Funding

This work was supported by National Science Foundation of China [Shulian Xie, 31370239, Jia Feng, 31200164].

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