



Characterization and Comparative Analysis of MicroRNAs in 3 Representative Red Algae

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Background: MicroRNA (miRNA) is a key regulator at the gene posttranscriptional regulation level. We have previously identified miRNAs and their putative targets in 3 representative red algae, *Chondrus crispus*, *Galdieria sulphuraria* and *Porphyridium purpureum*.

Objectives: In this study, unique molecular and evolutionary characterization of miRNAs were revealed in the 3 red algae based on the comparative miRNAs profiling.

Materials and Methods: Genome locations of small RNAs (sRNAs), miRNAs and *MIRNAs* (*MIRs*) in the 3 red algae were shown by collinearity analysis. Characterization of miRNAs and *MIRs* were profiled via bioinformatics analysis. Taken *MIR156s* and miR156s for examples, red algae miRNAs evolutionary features were demonstrated via phylogenetic and evolutionary information analysis. MiRNA targets main inhibition type was validated via performing data statistics and RLM-RACE PCR. Key target genes and their function were predicted by the common Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Results: Quantity, nucleotide bias and common sequences of miRNAs were analyzed in the 3 red algae. Four typical precursor structures and primary molecular features of red algae miRNAs were profiled. Genome-wide collinearity analysis of sRNAs, miRNAs and *MIRs* in the 3 red algae was performed to show their distribution and interrelation based on the deep sequencing data. Taken red algae *MIR156s* for example, their family members and sequences divergence were demonstrated. The whole evolutionary processes of miR156s and pre-miR156s in red algae were steady with negative selected pressure though diverse phylogenetic relationships and evolutionary parameters showed. Through 3 red algae miR156 targets validation, cleavage was validated as their main miRNA targets inhibition type. The common target genes (GO:0009536) enriched significantly for plastid formation will provide important insights for red algal biopigment research. The common KEGG pathways (ko01100) enriched significantly were predicted without a detailed reference metabolic map.

Conclusions: MiRNA plays an essential role in gene expression regulation involved in diverse biological processes of red algae. Comprehensive molecular and evolutionary features of miRNAs in the 3 red algae will provide insights for further utilizing the algae resources at the molecular level.

Keywords: *Chondrus crispus*, *Galdieria sulphuraria*, MicroRNA, *MIRNA*, *Porphyridium purpureum*

1. Background

The red algae (Rhodophyta), distributed in a diverse environment, are essential for understanding plant evolution and stress adaptation (1). Red algae contain different species such as multicellular and unicellular species, aquatic and semiterrestrial species, species with sexual and asexual reproduction, and species with

alternating and nonalternating generations. Currently, Rhodophyta can be divided into two subphyla, seven classes, 760 genera and approximately 4410 species (2). Increasing studies showed that some red algae had unique biological functions with their utilization in industry (3) medicine (4) and food fields (5). The multicellular marine red alga, namely, *Chondrus*

crispus, is rich in carrageenan and polysaccharides and has been found to reduce blood fat and cholesterol to some extent (6). Both *Porphyridium purpureum* and *Galdieria sulphuraria* are unicellular microalgae. The former's natural phycobiliproteins and polyunsaturated fatty acids have been applied in the food-processing and healthcare fields (7). The latter's metal ion absorption from wastewater and unique abiotic stress response mechanisms have been the research focus on extremophiles (8).

Endogenously produced microRNA (miRNA), with a length of ~21 nts, regulates gene expression posttranscriptionally (9). Studies demonstrated that miRNA derived from the non-protein-coding gene—*MIRNA (MIR)*, acts as a negative regulator of target gene expression (10). Intracellular two splicing events with Dicer like enzymes, Argonature protein family and other factors degrade the primary miRNA (pri-miRNA) and precursor miRNA (pre-miRNA) into approximately 21-nt mature miRNA (11). Then, mature miRNA with an RNA-induced silencing complex inhibits corresponding target gene expression via cleavage or translation inhibition (12). Studies have showed that miRNA has diverse functions in growth, development, metabolism and evolution (13).

We have previously identified 503 miRNAs belonging to 469 *MIR* families in the 3 representative red algae described above by high-throughput sequencing combined with bioinformatics analysis (14-16). However, the detailed molecular information including pri-miRNA sequences, pre-miRNA structures, *MIRs* and their promoters, etc. are still unknown so far. Genome-wide characterization of miRNAs and *MIRs* in the 3 red algae have not been investigated. Unique phylogenetic relationship and evolutionary pattern of miRNAs in red algae have not been uncovered. The main inhibition type of red algae miRNA targets and their experimental validation need to be further explored. Potential key target genes and their pathways in the 3 red algae need to be mined.

2. Objectives

We summarized and revealed the unique molecular and evolutionary characterization of miRNAs in the 3 red algae for the first time, which will provide an important reference for miRNAome research on other algae. Their putative targets experimental validation and key target genes functions and pathways prediction will lay

a foundation for uncovering algae miRNA regulatory mechanism. Research results will be helpful to utilizing red algae resources at molecular level in future.

3. Materials and Methods

3.1. Prediction of Pri-MiRNA, MIR and MIR Promoter

Based on the known reference genomes information and our previous sRNA high-throughput sequencing data in *C. crispus*, *G. Sulphuraria* and *P. purpureum*, we obtained their intergenic (upstream and downstream area of miRNA ≤ 2000 bp) and intronic miRNAs. Then the pri-miRNA could be screened according to the following filtering conditions: (i) a harpin secondary structure could be constructed with a deletion of no more than 4-nt via running Mfold 6.5; (ii) the minimum free energy value of the structure should be ≤ -30 kcal/mol; and (iii) length of the structure should be at least 200 bp. Based on the upstream sequence (≤ 1800 bp) of pri-miRNA, *MIR*, *MIR*-promoter, TATA box and transcription start sites (TSSs) of *MIRs* were predicted via running Promoter Scan and Eponine software.

3.2. Genome-Wide Collinearity Analysis

The previous identified sRNAs, miRNAs and *MIRs* were mapped onto the reference genomes of *C. crispus*, *P. purpureum* and *G. Sulphuraria* via running Bowtie 2.0 and Circos software 0.55, respectively. Based on sequences alignment analysis, the homologous relationships of *MIRs* in the 3 red algae were constructed by Blast n search.

3.3. Statistical Analysis

The quantity of miRNAs including their common sequences, length distribution and nucleotide bias was analyzed statistically with SPSS and SAS software. The quantity of *MIR156* family members in 11 representative plant species including the 3 red algae was calculated by querying the miRBase 22.0 database. Significance was estimated by *p* tests in SAS software. Windows and Linux systems were used for the Perl or R language environment.

3.4. Conservation and Divergence Analysis

Taken *MIR156* for example, multiple sequences alignment of pre-miR156s and mature miR156s was performed using Clustal W 2.0. The identity percentages were analyzed by a Kolmogorov-Smirnov test in

GeneDoc 2.7. The consensus secondary structure of pre-miR156s in plants, including the 3 red algae, was reanalyzed online via running Rfam 12.0.

3.5. Phylogenetic Analysis

Taken miR156 for example, the basic data for miR156s phylogenetic analysis involved all 140 precursors in plants including the 3 red algae. The codon positions included 1st+2nd+3rd+noncoding. All nucleotide positions above containing gaps and missing data were eliminated. A phylogenetic tree was constructed based on Bayes model of MEGA 5.0. The bootstrap consensus values were calculated from 1,000 replicates.

3.6. Evolutionary Rate Analysis

Taken miR156 for example, sequences of miR156s and their targets in plants and the 3 red algae were converted to an applicable data format according to the instructions of MEGA 5.0 and Clustal W 2.0 software. Due to heterogeneity in the relative evolutionary rates of different regions of pre-miRNAs, the K of the miR156s processed from the 5' and 3' ends were estimated using the baseml module in PAML. The Ka and Ks of miR156 targets were calculated by the y00 subroutine in PAML. Their neutral tests (Tajima's D and Fu's Fs) was estimated in Arlequin 3.11.

3.7. Cleavage Inhibition and RLM-RACE Validation

MiRNA target cleavage inhibition type was predicted by psRNATarget with no mismatches between 9 and 11 nt in the miRNA::target pair (17). Conversely, translation inhibition was predicted with at least one mismatch in the complementary region above. Total RNAs of the 3 red algae were isolated using TRIzol reagent. After the RNA purified and ligated to the RNA oligo adapter, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) PCR was performed using the FirstChoice®RLM-RACE kit. CDNA synthesis was performed with oligo (dT) primer and reverse transcriptase according to One Step RT-PCR kit description. The first cycle of amplification was performed with cDNA using the P1 and P2 primers to generate nonspecific 5' RACE products. The second cycle of amplification was performed with the P3 and P4 primers to generate the final 5' RACE products with three replicates. The sequencing was performed by Sangon Biotech Co., Ltd.

3.8. GO and KEGG Pathway Enrichment Analysis

GO terms assigned by Blast2GO 3.1 were enriched with GO Enrichment Analysis Software Toolkit. The enriched terms ($p \leq 0.05$) were categorized into three function groups with GO Consortium and AmiGO: biological processes (BP), molecular functions (MF), or cellular components (CC). Then the common GO terms in the 3 red algae were screened. Final top 17 significant ones ($p \leq 0.01$) were further screened. Similar to the above analysis, the top 24 significantly enriched common KEGG pathways ($p \leq 0.01$) in the 3 red algae were illustrated with KegSketch software. KO terms were obtained in KOBAS 2.0.

4. Results

4.1. Molecular and Collinear Characterization of MiRNAs in the 3 Red Algae

Five common miRNAs, including miR2916, miR4414b, miR6173, miR6194 and miR7536a, were detected among 503 miRNAs (429 conserved and 74 novel miRNAs) (**Fig. 1A**). Lengths of 21 and 24 nts were the most frequent (149) and least frequent (5) miRNA sizes, respectively (**Fig. 1B**). Except for the first base, the base proportion of these miRNAs was balanced, with an AU percentage of 52.42% (**Fig. 1C**). The first base was biased towards U. By investigating all miRNA precursor structures in the 3 red algae, we classified them into 4 types (**Table S1A**). Type I was the standard stem-loop structure with one closed effective loop at the top and one stem without any bulges or branched loops (**Fig. S1A**). Other types (II-IV) with diverse loops, stems, or bulges at different positions of the precursor are allosteric ones (**Figs. S1B-D**), which would lead to diverse mature miRNAs and targets (18). Furthermore, 19, 50, and 7 pri-miRNAs with length distribution ranged from 201 to 2886 nts were predicted in *C. crispus*, *P. purpureum*, and *G. sulphuraria*, respectively. Additionally, 47 pri-miRNAs were located in the intronic region of coding genes, and others were located in the intergenic region (**Table S1B**). Altogether, 34 MIR promoters and 27 TATA boxes were predicted in the 3 red algae (**Table S1C**). Genome-wide collinearity analysis results showed that 4.9 M, 5.4 M and 2.4 M sRNAs were detected in the reference genomes of *C. crispus* (104.8M), *P. purpureum* (19.2M), and *G. sulphuraria* (13.6 M), respectively (**Fig. 1D**). However, much more miRNAs

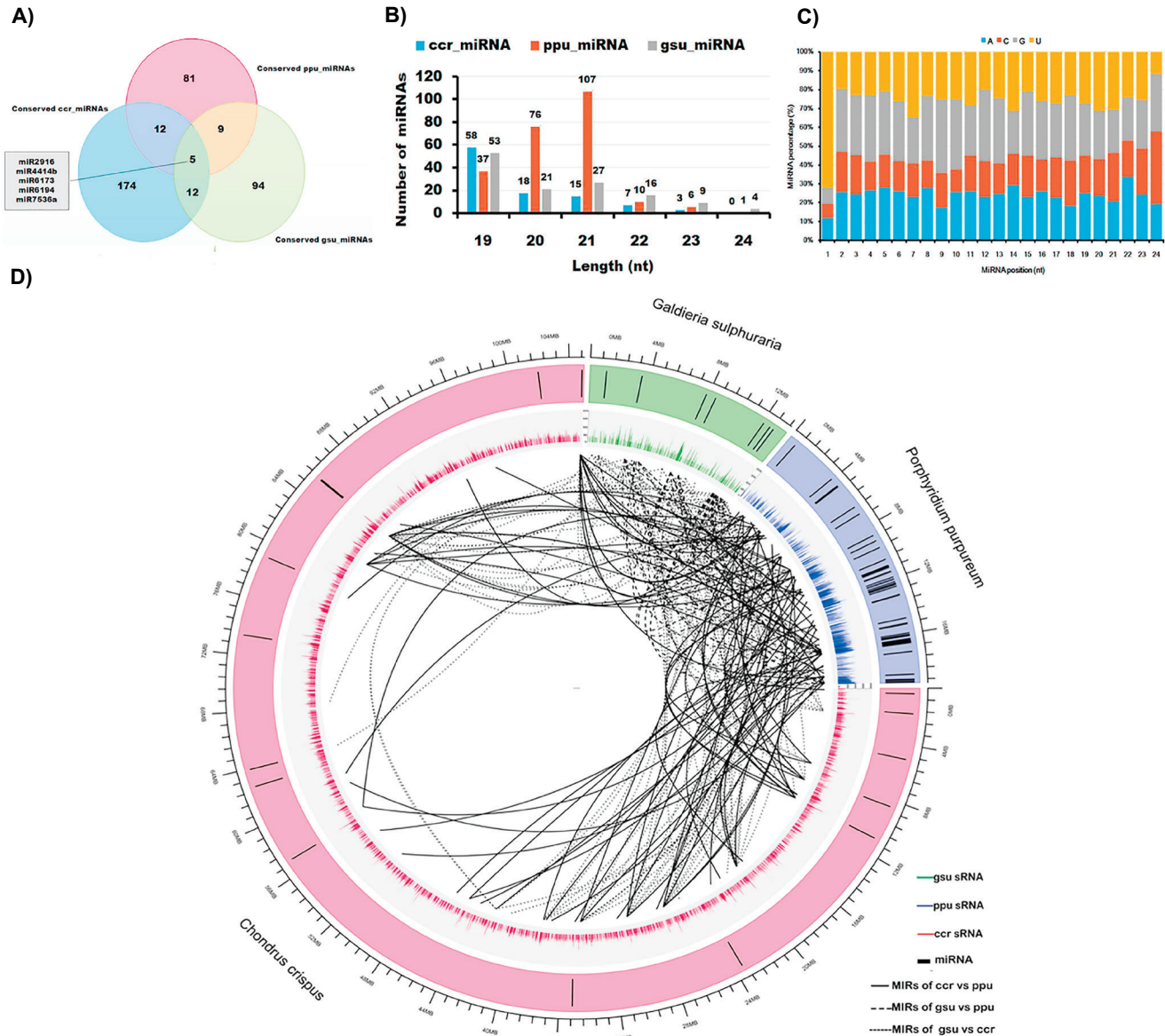


Figure 1. Molecular characterization of miRNAs in the 3 red algae. **A)** Venn diagram of the common miRNAs number; **B)** Length distribution of miRNAs (19-24nt); **C)** Nucleotide distribution of miRNAs (19-24nt); **D)** Circos plot of sRNAs, miRNAs and MIRs on the reference genomes.

(254 miRNAs) were identified in *P. purpureum*, which indicated that lots of potential unknown miRNAs have not been identified in *C. crispus* and *G. sulphuraria*. Moreover, one miRNA high-expression region (84.8~85.6 M) was detected in the *C. crispus* reference genome. Three miRNA high-expression regions (11.2~12.8 M, 15.2~16.8 M and 19.2~20.0 M) were detected in the *P. purpureum* reference genome.

Regrettably, no regions with significant miRNA high-expression were found in the *G. sulphuraria* reference genome. Moreover, the 186 homologous MIRs were detected between *C. crispus* and *G. sulphuraria* (Table S1D). Total 72 homologous MIRs were predicted between *C. crispus* and *P. purpureum* (Table S1E). Total 64 homologous MIRs were obtained between *P. purpureum* and *G. sulphuraria* (Table S1F).

4.2. Conservation and Divergence of 3 Red Algae MIR Families by Comparing Their MIR156s across Diverse Plant Species

Altogether, total 469 MIR families were predicted in the 3 red algae, and the majority of them had only one member (92.92% on average) (Table S2A). Based on the conserved chloroplast gene *rbcl*, a ML phylogenetic tree (19) of 11 representative plant species including the

3 red algae combined with 18 random MIR families was constructed (Fig. 2A). The distribution of family members was diverse in these species, which was irrelevant to the clustering of their host species. Furthermore, taken MIR156s for example, which could be detected in all typical plant species with 99 family members, both of 3 family members were founded in *Physcomitrella patens* (a transitional species between

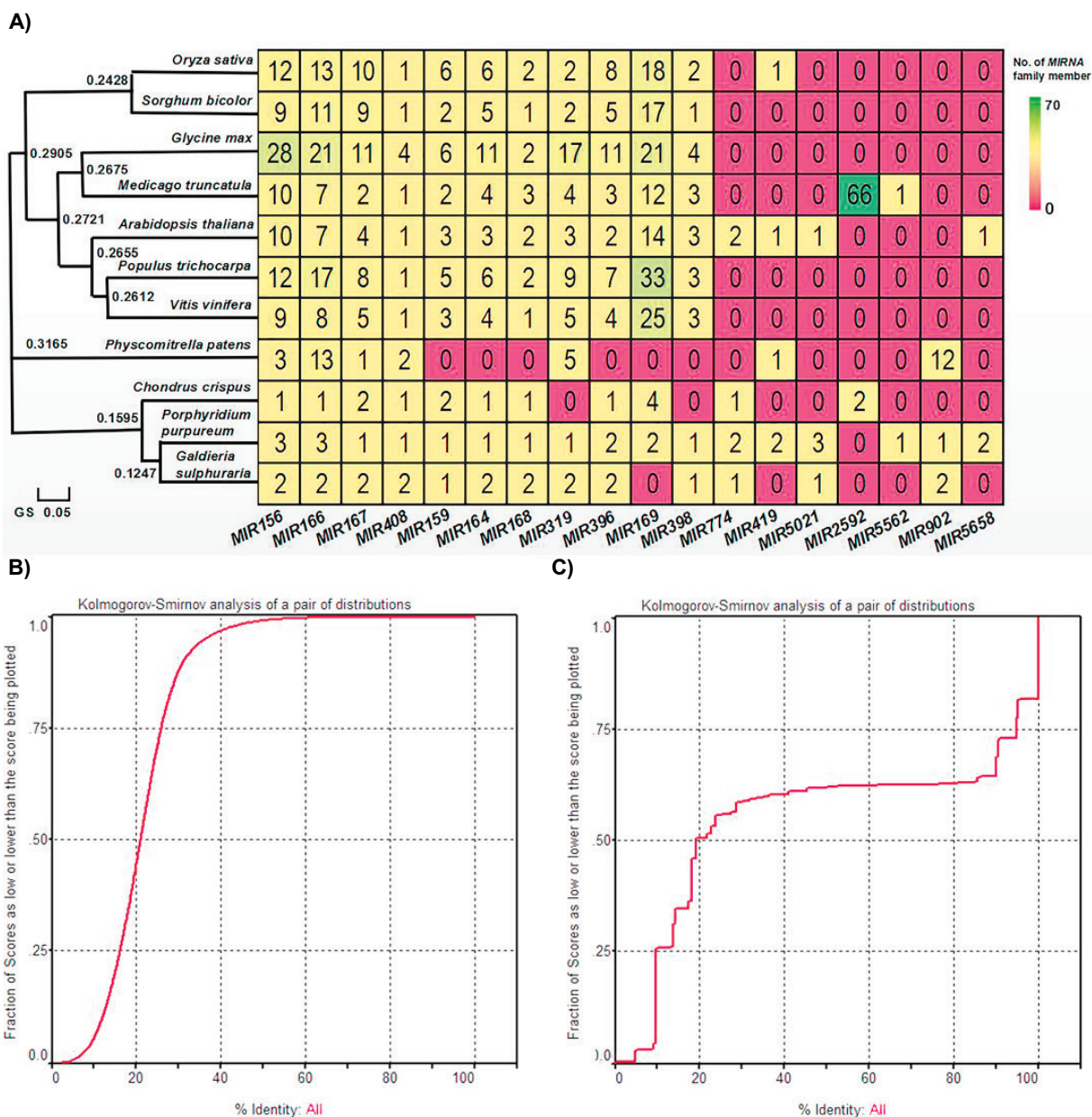


Figure 2. Conservation and divergence of MIRs between the 3 red algae and other plant species. **A)** Distribution of some MIR families among diverse plant species; **B)** The percentage identity of the aligned pre-miR156s in plants; **C)** The percentage identity of the aligned miR156s in plants.

aquatic and land plants) and *P. purpureum* though the latter having a closer phylogenetic relationship with *G. sulphuraria*. Moreover, as two important food crops, *Glycine max* has the most 28 *MIR156* members, whereas *Sorghum bicolor* has only nine.

Based on 294 known *MIR156* matures reported in 47 plant species (Table S2B), we found that most of them were processed from the 5' end of the precursors (accounting for 84.01%), and 98 sequences were validated. Based on the Kolmogorov-Smirnov statistical test (19), the proportion of *MIR156* precursors with >22% sequence identity was approximately 0.375 (Fig. 2B). The percentage identity of aligned *MIR156* matures showed that ~90% sequence identity

was ~0.375 (Fig. 2C). This result indicated that *MIR156* matures should be more conserved than their precursors in plants including red algae. Furthermore, the consensus structures of matures and precursors of *MIR156* showed their conserved or diverged nucleotide region (Fig. S2A, B). Moreover, their mature sequence alignments analysis demonstrated that miR156-5p is more conserved than miR156-3p (Fig. S2C).

4.3. Phylogeny and Evolution of 3 Red Algae MiR156s Across Diverse Plant Species

A MrBayes tree (20) of pre-miR156s in diverse plant species, including the 3 red algae (Table S3A), was divided into group 1 and group 2 (Fig. 3A). As a small

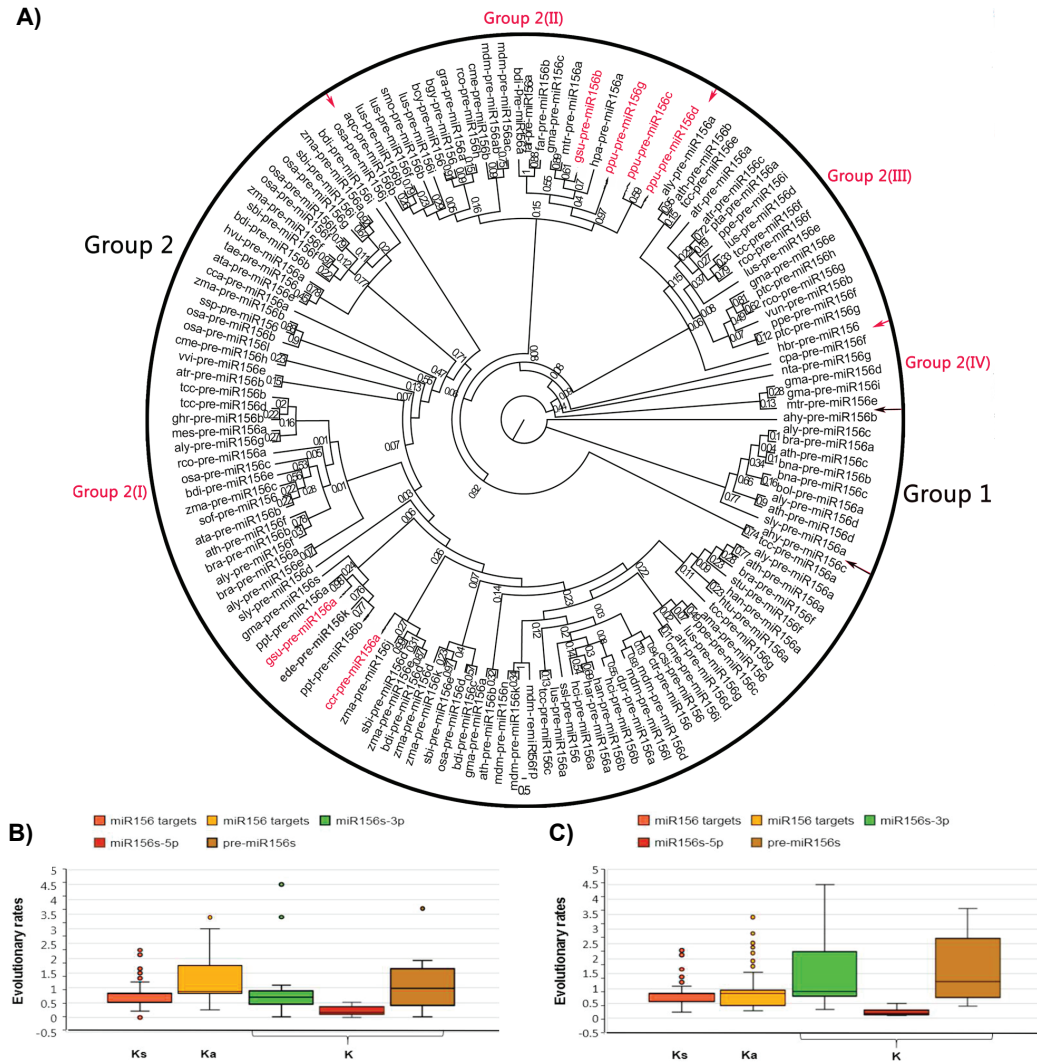


Figure 3. Phylogenetic and evolutionary analyses of red algal miR156s across different plants. **A)** Phylogenetic tree of pre-miR156s in different plants; **B)** Evolutionary rate of miR156s, pre-miR156s and their targets in plants including red algae; **C)** Evolutionary rate of miR156s, pre-miR156s and their targets in the 3 red algae.

group, group 1 comprised ten pre-miR156s. Ahy-pre-miR156b formed one cluster with a long branch, implying a faster evolutionary rate than in the other nine miR156s in group 1. As a large group, group 2 containing all remaining pre-miR156s in plants, branched into multiple subgroups or clades. Group 2 was divided into four subgroups, each comprising a variable number of pre-miR156s and branching off multiple times. As a large subgroup with 84 pre-miR156s, group 2 (I) produced many branches, which comprised three pre-miR156s in *G. sulphuraria* and *C. crispus*. In group 2 (II), four pre-miR156s in *P. purpureum* and *G. sulphuraria* and another 6 pre-miR156s in five different species constructed one branch. Group 2 (III), which consisted of 18 pre-miR156s, formed 4 branches that shared one common ancestor. Similarly, in group 2 (IV), the precursors gma-pre-miR156d/i and mtr-pre-miR156e shared a common ancestor with cpa-pre-miR156f and nta-pre-miR156g.

We selected miR156s (including miR156s-5p/3p and pre-miR156s) and their targets as examples for evolutionary rates estimating (21). The overall trends of nucleotide divergence (K) (22) of miR156 matures and precursors were similar between red algae and other plants with the negative selected pressure (values of Tajima's D and Fu's Fs <0 significantly) (**Fig. 3B**, **Table S3B**). However, the sequences processed from the 3' end vary greatly, with faster evolutionary rates (higher K values) in the 3 red algae than in other plants (lower K values). Moreover, the level of synonymous substitution (Ks) in miR156 targets was lower than that of nonsynonymous substitution (Ka) in both red algae and other plants (**Fig. 3C**), which indicated that some positive selection events happened during the evolution of miR156 targets in the plant kingdom. However, both Tajima's D and Fu's Fs (23) of miR156 targets in the 3 red algae were less than zero, indicating that some negative selection events happened.

4.4. Prediction and Validation of MiRNA Target Inhibition Type in the 3 Red Algae

Based on our previous predicted 18723 de-redundant miRNA targets in the 3 red algae, we speculated that cleavage was the main miRNA target inhibition type (accounting for 68% on average) (**Fig. 4A**).

We selected ccr-miR156, ppu-miR156g, and gsu-miR156a and their corresponding putative targets to validate the cleavage inhibition type in the 3 red algae.

Their RLM-RACE results showed that the first PCR amplification with a pair of adapter primers (P1 and P2 seen in **Table S4**), no any specific miR156 cleaved products could be detected (**Fig. 4B**, **Fig. S3**). After the second PCR amplification with a 5' nested adaptor forward primer (P3) and a gene-specific reverse primer (P4) (**Table S4**), we obtained the desired specific targeted products (721 bp for ccr-miR156, 399 bp for gsu-miR156a, 267 bp for ppu-miR156g) (**Figs. 4C-E**). Moreover, sequences alignment analysis combined with products sequencing data confirmed that the 3 targeted mRNAs (CHC_T00006037001 for ccr-miR156, XM_005703296.1 for gsu-miR156a, HS828387.1 for ppu-miR156g) were cleaved by miR156s with 10th position as the cleavage sites (**Figs. 4F-H**).

4.5. The Common GO and KEGG Pathway Enrichment

A total of 17 significant enriched common GO terms of miRNA target genes (p -value ≤ 0.01) in the 3 red algae were screened (**Fig. 5A**, **Figs. S4A-C**). Their categories quantity order was MF (8) > CC (6) > BP (3). The highly enriched target genes (genes number=10034, p -value=0.0013) function was plastid formation (GO:0009536) (**Table S5A**). Furthermore, 24 significant enriched common KEGG pathways miRNA target genes involved in (p -value ≤ 0.01) were screened in the 3 red algae (**Fig. 5B**, **Fig. S4D**). Among the pathways, metabolic pathways (ko01100) was highly enriched (totals of 30262 genes, p -value=0.0078) especially for *P. purpureum* (**Table S5B**).

5. Discussion

Although a number of miRNAs were identified in the 3 red algae, many potential miRNAs have not been founded, which could be speculated through fewer common miRNAs and miRNAs distribution on the reference genomes. Moreover, most of the pre-miRNAs (73.68%) predicted in the intergenic regions indicated that the 3' UTR should not be the main miRNA mapped region in red algae. Lots of homologous *MIRs* detected on the reference genomes indicated that the non-coding-gene duplication event might occur in the evolutionary processes of red algae.

MIR family divergence could be reflected in the following two aspects: on the one hand, the diverse member numbers were revealed in this study with the most 146 members in *MIR169* and the fewest 2 in *MIR5562*. On the other hand, the precursor was more

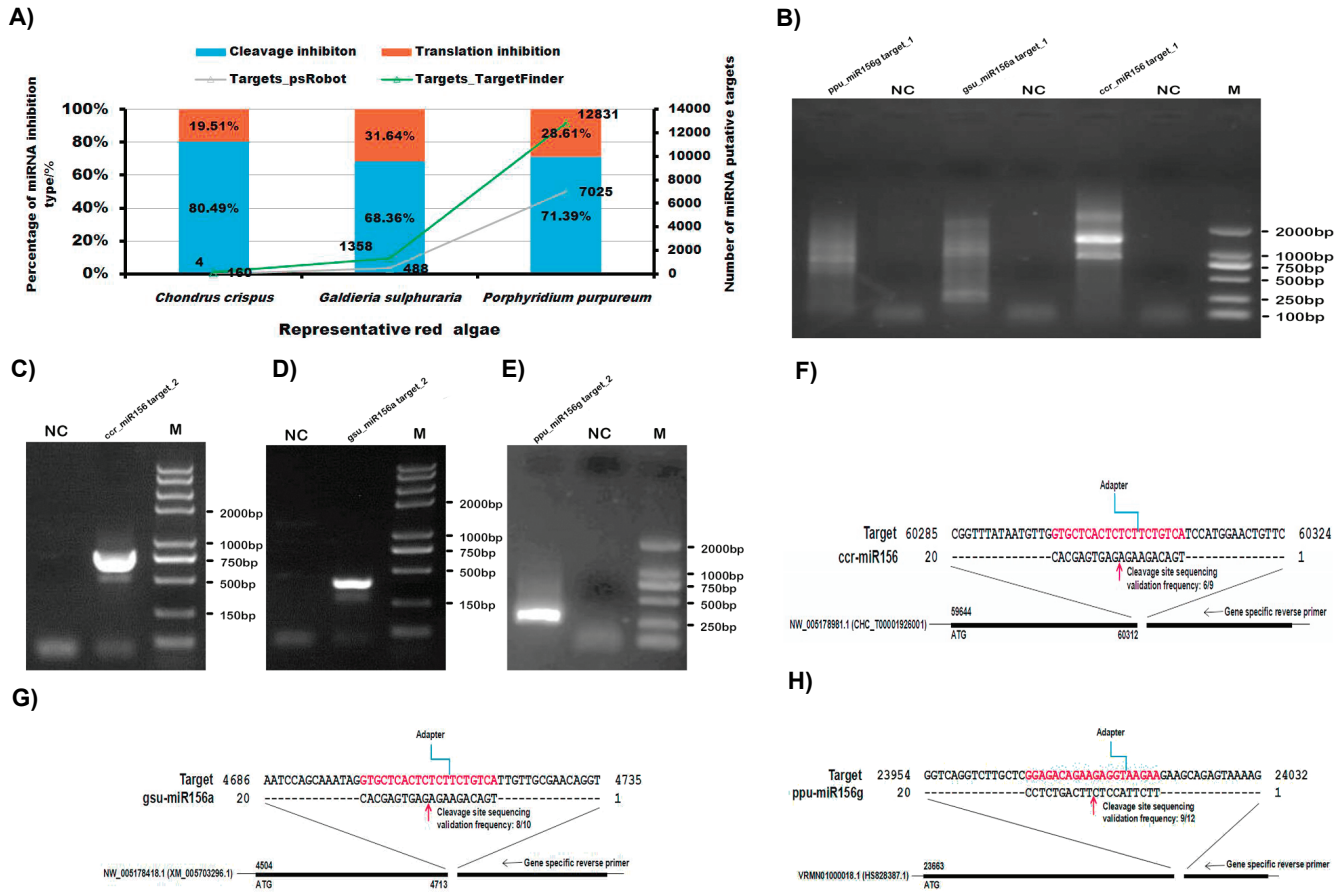


Figure 4. MiRNA targets in the 3 red algae. **A)** Number of putative miRNA targets and inhibition type; **B)** 1st RLM RACE PCR of miR156 targeted products; 2nd RLM RACE PCR of ccr-miR156 C) gsu-miR156a **D)** ppu-miR156g **E)** targeted product; Schematic presentation of ccr-miR156:target **F)** gsu-miR156a:target **G)** ppu-miR156g:target **H).**

diverse than the mature with the miR156s-5p played a leading role in posttranscriptional gene control. As an essential regulator in the processes of plant growth and development (24), miR156 matures are relatively conserved than pre-miR156s (complex phylogenetic relationships and different evolutionary rates) in different plants including red algae. But red algal Precursor's evolutionary rates were faster than other higher plants. Although inconsistent miR156 targets evolutionary parameters predicted, we could reveal the evolution of miR156s and pre-miR156s in plants including the 3 red algae was steady with negative selected pressure. But their neutral evolutionary event to be omitted could not be confirmed based on fewer species and no significant data tests.

Three putative miR156 targets and their cleavage sites in the 3 red algae were validated, which indicated that cleavage should be the main miRNA target inhibition type in red algae. Next, we will determine detailed target gene information and validate their function in the red algae. Additionally, the highly enriched target genes (GO:0046872) closely related to the synthesis of plastids will provide the important information for synthesis and application of red algal pigments especially for *P. purpureum*, whose B-phycoobiliprotein is an important food colorant and a new tool used for fluorescence labeling (25). Due to the absence of explicit red algae metabolism annotation information in KEGG, the significantly enriched metabolic pathways (ko11100) without the detailed reference map need to

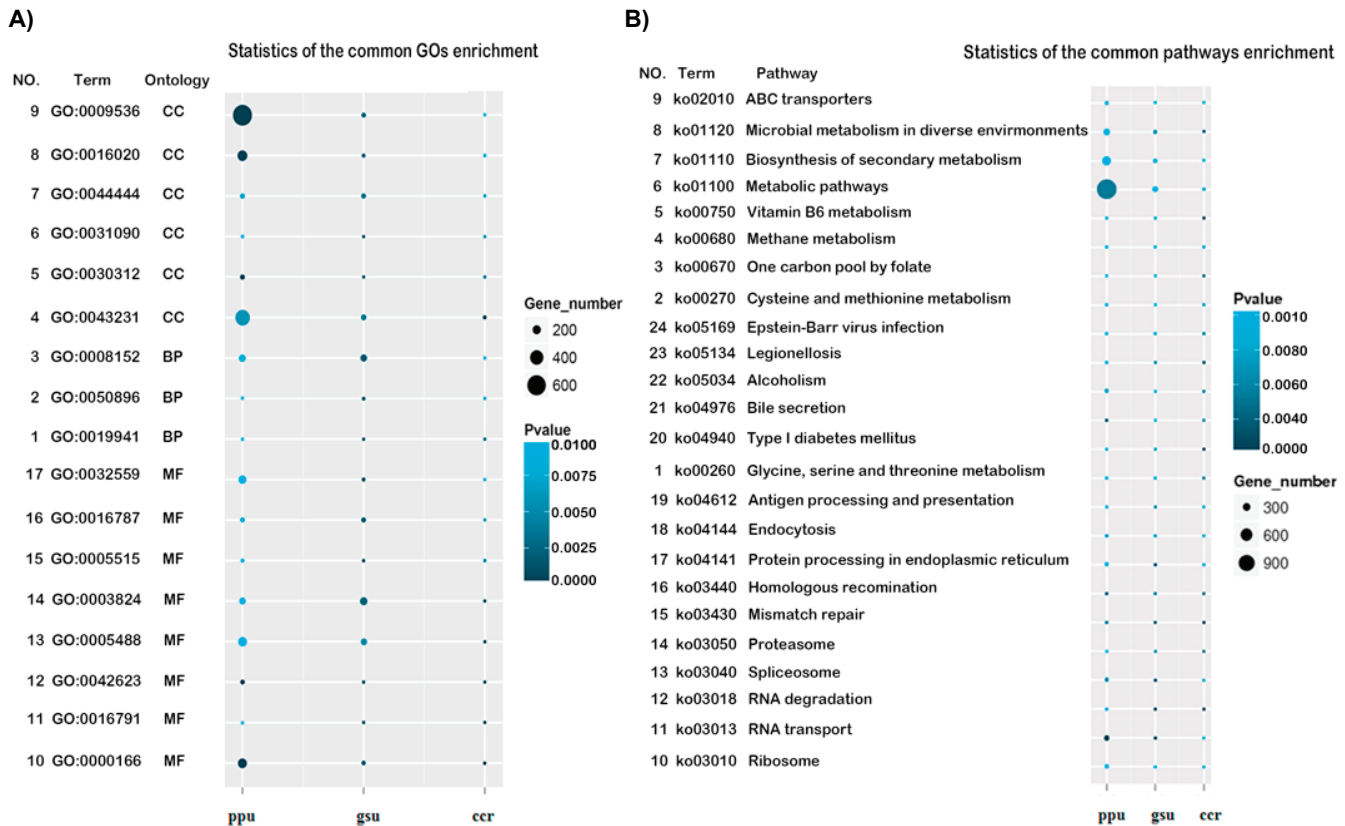


Figure 5. Significant enriched common GO and KEGG pathways in the 3 red algae. **A)** Bubble chart of top 17 GOs enrichment. **B)** Bubble chart of top 22 pathways enrichment.

be further researched at the metabolic level.

6. Conclusions

We profiled unique molecular features of miRNA and *MIR* families in *C. crispus*, *G. sulphuraria* and *P. purpureum* comprehensively via comparing their sequences information across other plants. Taken red algae *MIR156s* including family members, miR156 precursor, mature miR156, and miR156 target for example, we further revealed red algal phylogenetic and evolutionary characterization. MiR156 targets were validated in the 3 red algae, and the common GO and KEGG pathways of target genes were enriched. These results will lay a foundation for elucidating unique characteristics in algae and provide insights that can be used to further explore and utilize red algae resources.

Acknowledgments

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Data Availability Statement

The raw sRNA sequencing data have been deposited in the NCBI SRA database with the accession numbers: SRP066538/SRS1172958/SRX1445023/SRR3228749 for *C. crispus*, SRP071761/SRR3228731/SRS1338707/SRX1631643 for *P. purpureum* and SRP071954/SRR3234543/SRS1359098/SRX1640152 for *G. sulphuraria*.

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