miR8181 is Involved in the Cell Growth and Development Regulation of Saccharina Japonica

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Abstract

**Background:** Aureochrome, a blue-light receptor found in photosynthetic stramenopiles, plays an important role in brown algal growth and development. Aureochrome preserves the reversed effector-sensor domain for blue light reception and acts as the candidate optogenetic tool for light induced post-transcriptional regulation, but the inner rapid regulation of *aureochrome* remains to be studied. MicroRNA (miRNAs) of plant can cleavage the specific base-pairing site of mRNA by RNA interference mechanism, and such post-transcriptional regulation of miRNAs to photoreceptor has received attention due to the flexible regulation pathway. However, the targeting relationship between *aureochrome* and miRNA is unclear.

**Results:** In this study, the potential regulatory network between miRNAs and *aureochrome* were explored by transcriptome and sRNA sequencing in *Saccharina japonica*. Our results found that 18 miRNAs perfectly paired with *aureochrome*. Among the screened miRNAs, miR8181-x was negatively correlated with *aureochrome* with high credibility and exhibited tissue-specific expression in *S. japonica*. Degradome sequencing detected the exact cleavage site of miR8181-x on *aureochrome*, confirming their targeting regulation relationship. Among the 54 target genes of miR8181-x, nine genes of ABC transporters, E3 ubiquitin-protein protein ligase, Hsp90, Mx1, PetC, EF2, GSA, HAD-superfamily hydrolase and SET2 that exhibited similar expression with *aureochrome* competed with the same binding site, thus constructing the competing endogenous RNA network. Functional analysis of miR8181-x target genes revealed that regulation of cell differentiation and development was enriched, indicating the potential role of miR8181-x in the regulation of growth and development.

**Conclusion:** Our study found that miR8181-x negatively regulated the expression of *aureochrome*. The exact cleavage site in *aureochrome* were verified by degradome sequencing, confirming the targeting relationships. Functional enrichment of miR8181-x target genes revealed that miR8181-x involved in the cell growth and development regulation of *S. japonica*.

**Background**

Light serves as a biological stimulus to trigger signal transduction pathways via specific photoreceptors. The responses of photoreceptors to light wavelength, direction and duration of light illumination are important for marine plant growth and development [1, 2, 3]. Similar to green algae, photosynthetic stramenopiles acquire the blue light responses of phototropism, chloroplast photorelocation and photomorphogenesis from secondary symbiosis [4]. Aureochrome is classified as new type of blue light receptor in photosynthetic stramenopiles [5], and it consists of a basic region/leucine zipper (bZIP) domain at the N-terminus and a light-oxygen-voltage (LOV) domain at the C-terminus. The DNA-binding region in the bZIP domain includes a region that is rich in basic amino acids and a heptad leucine repeat zipper [6]. The LOV domain is composed of 11 well-conserved amino acids that are responsible for FMN binding and receiving the environmental signal [7, 8]. SjAUREO from the brown alga *Saccharina japonica* exhibits 40%-92% similarities with other photosynthetic stramenopiles [9]. Yeast two-hybrid screening has demonstrated a strong interaction between SjAUREO and the 40S ribosomal protein S6, which might be involved in blue light-mediated cellular division and photomorphogenesis [10]. In the single cellular diatom *Phaeodactylum tricornutum*, PtAUREO1a and PtAUREO1b mutants exhibit significantly decreased photoacclimation to blue light [11, 12]. AUREO1 in *Heterosigma akashiwois* may act as a blue light-sensitive negative gene regulator, which is driven by dark-to-light transitional cues [13]. Although the function of aureochrome has been verified in various species, further details regarding the transcription regulation of aureochrome remain to be further explored.
MicroRNAs (miRNAs) play an important role in post-transcriptional regulation [14, 15], and plant microRNAs can trigger endonucleolytic cleavage of base-pairing mRNA targets via a RNA interference mechanism [16]. Animal-like mechanisms that repress mRNA translation with the assistance of both agronaute proteins and a microtubule-severing enzyme also exist in plants [17, 18]. Additionally, competing endogenous RNAs (ceRNAs) that share the common miRNA response element reduce miRNA activity, leading to the depression of specific mRNAs [19].

MiRNAs from different lineages are nonhomologous but have strongly conserved structures, such as the presence of uracil at the first residue. The developmental plasticity mediated by miRNAs allows plants to efficiently cope with environmental stress [20]. A previous study has revealed that mature miRNAs are involved in the red light signalling pathway, which is involved in plant photomorphogenesis [21]. The majority of miRNA target genes have been identified as transcription factors and may function in plant development, boundary formation and organ polarity [22].

For low tidal zone inhabitants with blue light irradiance, light reception and subsequent signal amplification are crucial for the S. japonica development and growth. Although miRNAs from the lineage of S. japonica have been identified [23, 24], the interaction between miRNAs and target genes have received less attention. With integrative analysis of the transcriptome, sRNA and degradome sequencing data, the miRNAs that target to aureochrome were screened, and the exact cleavage sites were verified in this study. By functional enrichment of targeting genes at genome-wide level, miRNA functions were also further investigated. Our aim was to verify the candidate miRNAs that target aureochrome and to explore the regulatory role of aureochrome in brown algae.

Results

Identification of miRNAs targeting aureochromes

By searching the whole genome data of S. japonica, five homologous sequences encoding aureochrome were screened. Apart from aureochrome2 and aureochrome3, the remaining three homologs, namely aureochrome1, aureochrome4 and aureochrome5, were significantly upregulated in response to blue light (Tukey’s tests, p < 0.05; Fig. 1A), indicating their sensitivity in response to blue light. Based on the complementarity interactions between miRNAs and target genes, we performed homology predictions for the miRNA target genes with Patmatch (v1.2) software. In total, we obtained 18 miRNAs that may target aureochromes (Table S1), and constructed a miRNA-aureochrome network (Fig. 1B).

The transcription level of miR818-x was downregulated in response to blue light, which was opposite to the aureochrome5. qRT-PCR analysis verified the opposite expression tendency between aureochrome5 and miR8181-x (Fig. 2, 3), indicating the negative regulation relationship. Moreover, the transcription levels of miR8181-x in the kelp blade was significantly higher than that in the holdfast (Tukey’s tests, p < 0.05; Fig. 3), suggesting that miR8181-x exhibits tissue-specific expression patterns in S. japonica.

Cluster analysis of miR8181-x target genes

By integrating high-throughput sequencing of mRNAs and sRNAs, Patmatch software predicted 1915 mRNAs as potential targets of miR8181-x (Fig. 4A). According to their transcription patterns, we separately grouped these targets by hierarchical clustering. These target genes were classified into six clusters based on their degrees of transcription. In cluster 4, 311 genes were upregulated in blue light compared to dark and white light conditions.
(Fig. 4B), exhibiting similar expression patterns with the *aureochrome5* transcript, thus screening as the candidate ceRNAs.

**Target gene identification for miR8181-x by degradome sequencing**

To validate the target cleavage relationship between *miR8181-x* and mRNAs, we constructed three degradome libraries (BL, DR, and WL) and performed sequencing using the Illumina Hiseq 2500. After removing low-quality sequences, 6019083, 4907605, and 6578179 clean reads were obtained for the BL, DR, and WL libraries, respectively (Table 1). The obtained sequences were mapped to the reference genome of *S. japonica* registered in the NCBI database (accession: MEHQ00000000). Finally, 2614536, 2018907, and 2387139 sequences were mapped to the reference genome (Table 1). These mapped sequences were analyzed to identify target genes of miRNAs. In total, 55 mRNAs that included *aureochrome5* were identified to be cleaved by *miR8181-x* (Table 2). DAVID was used to determine the functional analysis of *miR8181-x* target genes. The results showed that *miR8181-x* might be involved in the cellular component (CC) category with the following enriched terms: “organelle”, “cell part”, “macromolecular complex” and “membrane”. The following terms in the molecular function (MF) category were significantly enriched: “binding”, “catalytic activity”, and “transporter activity” (Fig. 5). In the biological process (BP) category, the following terms were significantly enriched: “growth”, “reproduction”, “single-organism process”, “localization”, “response to stimulus” and “developmental process” (Fig. 5). Together, these results indicated that *miR8181-x* plays important roles in growth and development of brown alga. Moreover, *ABC transporters*, *E3 ubiquitin-protein ligase*, *Hsp90*, *Mx1*, *PetC*, *EF2*, *GSA*, *HAD-superfamily hydrolase* and *SET2*, which were previously classified in cluster 4 (Fig. 4B), were verified as the target mRNAs (Fig. 6, 7A). The scores ranged from 2 to 5, and their functions were various, including defense, energy production and conversion, coenzyme transport and post-translational modification (Fig. 7B). These nine mRNAs may serve as ceRNAs of *aureochrome5* as they share common miRNA response elements and inhibit normal *miR8181-x*-targeting activity.

| Library | Clean reads | Mapped reads | Percent |
|---------|-------------|--------------|---------|
| BL      | 6019083     | 2614536      | 71.02%  |
| DK      | 4907605     | 2018907      | 68.58%  |
| WL      | 6578179     | 2387139      | 70.68%  |
Table 2
The alignment and cleavage information of the target genes of *miR8181*-x.

| Target      | Alignment Score | Alignment Range | Cleavage Site | Target      | Alignment Score | Alignment Range | Cleavage Site |
|-------------|-----------------|-----------------|--------------|-------------|-----------------|-----------------|--------------|
| EVM0016004  | 4               | 503–520         | 511          | EVM0005054  | 5               | 6078–6096       | 6087         |
| EVM0016561  | 4.5             | 580–597         | 588          | EVM0000423  | 4.5             | 2405–2423       | 2414         |
| EVM0015607  | 5               | 764–781         | 772          | EVM0012110  | 5               | 1085–1102       | 1093         |
| EVM0013568  | 4               | 1075–1092       | 1083         | EVM0006977  | 3               | 1227–1244       | 1235         |
| EVM0014908  | 4.5             | 2602–2619       | 2610         | EVM0003777  | 5               | 3868–3885       | 3876         |
| EVM0003612  | 4               | 3025–3042       | 3033         | EVM0005629  | 3.5             | 691–708         | 699          |
| EVM0006721  | 2.5             | 2365–2382       | 2373         | EVM0000411  | 4.5             | 2391–2408       | 2399         |
| EVM0009953  | 3               | 1332–1349       | 1340         | EVM0000744  | 4.5             | 653–670         | 661          |
| EVM0013590  | 3.5             | 2602–2619       | 2610         | EVM0002624  | 5               | 2326–2343       | 2334         |
| EVM0016449  | 4.5             | 1055–1072       | 1063         | EVM0000710  | 5               | 78–95           | 86           |
| EVM0012744  | 5               | 1243–1260       | 1251         | EVM0010320  | 4.5             | 1815–1832       | 1823         |
| EVM0009697  | 4.5             | 1091–1108       | 1099         | EVM0010609  | 3               | 481–498         | 489          |
| EVM0000151  | 4.5             | 4079–4096       | 4087         | EVM0003731  | 4.5             | 3124–3141       | 3132         |
| EVM0004836  | 5               | 1554–1571       | 1562         | EVM0004377  | 4               | 2410–2428       | 2419         |
| EVM0004383  | 2               | 396–413         | 404          | EVM0013427  | 4.5             | 2435–2453       | 2444         |
| EVM0011126  | 4.5             | 451–468         | 459          | EVM0013645  | 4.5             | 309–326         | 317          |
| EVM0015897  | 5               | 2457–2474       | 2465         | EVM0007444  | 5               | 2757–2774       | 2765         |
| EVM0014625  | 5               | 1245–1262       | 1253         | EVM0002057  | 5               | 1149–1167       | 1158         |
| EVM0014965  | 5               | 226–243         | 234          | EVM0001832  | 3               | 5467–5484       | 5475         |
### Discussion

By integration analysis of the miRNA and mRNA high-throughput sequencing data, we found 18 candidate miRNAs targeting *aureochrome* orthologs. Among the candidate miRNAs, the level of novel miRNAs was significantly higher than that of known miRNAs. The high levels of novel miRNAs in brown algae are attributed to the rapid evolution process [24], which had significant consequences for miRNA function. Among the 18 candidate miRNAs, *miR8181-x* was identified to target the blue light receptor, *aureochrome5*, with high reliability. Moreover, the cleavage site of *miR8181-x* on *aureochrome5* was identified by degradome sequencing, verifying their targeting relationship. Plant miRNAs cleave the RNA strand at the binding site by a RNA-induced silencing complex with negative correlation between the expression of miRNA and target gene [25]. In our study, *miR8181-x* showed a negative correlation with *aureochrome5* in response to light irradiation, and this negative correlation was also found in different tissues of *S. japonica*. The tissue-specific expression pattern of *miR8181-x* suggested that its fundamental roles include maintaining tissue development.

A previous study has reported that ceRNAs play important roles in miRNA-mediated posttranscriptional regulation of gene expression [26]. Here, based on targeting relationship and transcriptional expression profiles, we constructed the ceRNA regulation network of *miR8181-mRNA*, which may regulate the *aureochrome5* transcript. The mRNAs in the ceRNA network were involved in various pathways, indicating the intricate regulatory relationship. In the ceRNA network, one nuclear gene encoding *petC* had competing roles with *aureochrome5* for light sensing in kelp. *PetC* plays a regulatory role with other cytochrome b6f subunits [27], and overexpression of *petC* enhances the light conversion efficiency and CO₂ assimilation rate [28]. The competing endogenous
relationship between aureochrome5 and petC might act as bridge to connect the light sensing and energy production in kelp.

In green plants, the miR8181 family is involved in anthocyanin biosynthesis [29], but the function of miR8181 in algae remains unclear. Integrated analyses of target verification and functional enrichment provided clues for elucidating the precise underlying mechanisms of miR8181-x. We found that the miR8181-x-mediated regulatory pathway involved glycolysis/gluconeogenesis for energy supply, and the most common processes included cell differentiation, cell cycle and cell development in the kelp. Therefore, we speculated that miR8181-x might regulate cell growth and development in S. japonica.

Conclusions

miR8181-x negatively targeted the aureochrome5 in a tissue-specific manner and was found to be involved in the cell growth and development regulation of S. japonica.

Methods

Sample collection and treatment

S. japonica juvenile sporophytes (“Zhongke No. 1”) were cultivated and collected from the Gaolv Aquacultural Ltd. Co. in Lidao, Rongcheng, Shandong, China. After eliminating the epiphytes via sterilized seawater washing, kelps were kept in cold conditions during shipping. The collected kelps were pre-cultured in the dark at 10 °C for 24 h and were exposed to the dark, white light (70 µmol photons m^{-2} s^{-1}) and blue light (25 µmol photons m^{-2} s^{-1}) for 3 h, respectively. White fluorescent lamps (Philips, Shanghai, China) and blue light-emitting diodes (460 ~ 475 nm wavelengths; Ichia, Japan) were applied as light sources. After exposure, the juvenile sporophytes were immediately frozen with liquid nitrogen and stored at -80 °C for subsequent RNA extraction. The kelp (“Zhongke No. 1”) was originally cultivated after nine generations of hybridization with long and wide S. japonica phenotypes. One voucher specimen of S. japonica was deposited in the Resource-sharing Platform of Specimens Marine Biological Museum Chinese Academy of Sciences (No. MBM436715).

RNA isolation and library preparation

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA). The extracted RNA was qualitatively examined using 1% agarose gel electrophoresis, and the concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). After extraction, RNAs ranging from 18 to 30 nt were separated by 15% polyacrylamide gel electrophoresis (PAGE). Next, 3’ adapters were added, and the 36–44 nt RNAs were enriched. Subsequently, 5’ adapters were ligated to the RNAs. The ligation products were reverse transcribed and the 140–160 bp PCR products were enriched to generate a cDNA library. Finally, sequencing was conducted on an Illumina HiSeqTM 2500 at Gene Denovo Biotechnology Co. (Guangzhou, China). The RNA integrity and concentration were further measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). The small RNAs and mRNAs were reverse transcribed to cDNA and then sequenced with a HiSeq 2500 and HiSeq 4000 (Illumina, San Diego, CA, USA), respectively.

Identification of miRNAs and target gene prediction
After removing reads containing adapters or low-quality bases, all clean tags were aligned with small RNAs in the Rfam and GenBank databases (Release 209.0) with our previous *S. japonica* genome data registered in the NCBI database (accession: MEHQ00000000). The rRNA, scRNA, snoRNA, snRNA and tRNA sequences were filtered out. All clean tags were validated using the miRBase database with known miRNAs. We selected the following prediction criteria for novel miRNAs: length, 18–25 nt; maximal free energy allowed for a miRNA precursor, 18 kcal/mol; space between miRNA and miRNA*, 14–35 nt; maximal asymmetry of the miRNA/miRNA* duplex, 5 nt; and flank sequence length of miRNA precursor, 10 nt. Finally, the identified miRNAs were predicted by Patmatch (v1.2) software. The minimum free energy of the miRNA/target duplex was set at ≥ 74% and there were no more than two adjacent mismatches in the miRNA/target duplex and no mismatches at positions 10–11 of the miRNA/target duplex.

**Target gene function enrichment analysis**

Blast2Go was employed for exploring the Gene Ontology (GO) annotation terms. Database for Annotation, Visualization and Integrated Discovery (DAVID) was adopted for pathway analysis with the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [30, 31]. *P* values less than 0.05 indicated enriched gene sets.

**Analysis of miRNAs and target genes by qRT-PCR**

Total RNAs were reverse transcribed using the PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan). qRT-PCR was conducted with the SYBR® PrimeScript™ miRNA RT-PCR Kit (RR716, Takara, China). Poly(A) tail addition and reverse transcription reactions were conducted in a total volume of 20 µL. The miRNA PrimeScript RT enzyme mix from the SYBR® PrimeScript™ miRNA RT-PCR Kit was used in the reverse transcription reaction mixtures, and the qRT-PCR primers used are listed in Table S2. Actin and U6 were adopted as internal control markers, and the relative expression of the miRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method. All qRT-PCR tests were performed with three biological replicates.

**Degradome library construction and data analysis**

Three degradome libraries (DR, BL and WL) were constructed using the juvenile sporophytes of *S. japonica*. Following the enrichment of mRNA, the obtained poly(A)-enriched RNA was ligated to oligonucleotide adaptors harbouring an MmeI recognition site. First-strand cDNA was generated from the ligated sequence via reverse transcription. After PCR amplification, the additional DNA products were yielded. After purification, digestion and ligation, the cDNA library was subjected for sequencing with Illumina Hiseq 2500.

Raw data obtained from HiSeq sequencing were processed to filter out the low-quality tags. The 5’ adapters, 3’ adapter contaminants, insert tags, and reads shorter than 18 nts were removed to obtain clean data, which were further mapped to the reference genome of *S. japonica*. By performing Blastn searches against the Rfam and National Centre for Biotechnology Information (NCBI) databases with an E-value cutoff of $10^{-2}$, the full-length sRNA tags were annotated to non-coding RNAs, and all of which were discarded. Additionally, t-plots were constructed according to the category of sites to analyse the miRNA targets and RNA degradation patterns.

**Statistical analysis**

Statistical differences were examined using one-way analysis of variance (ANOVA) by SPSS 22.0. *P*-values less than 0.05 were considered to be significant.
Abbreviations

Analysis of variance: ANOVA; Basic region/leucine zipper: bZIP; Biological process: BP; Cellular component: CC; Competing endogenous RNA: ceRNA; Database for Annotation, Visualization and Integrated Discovery: DAVID; Gene Ontology: GO; Kyoto Encyclopaedia of Genes and Genomes: KEGG; Light-oxygen-voltage: LOV; MicroRNA: miRNA; Molecular function: MF; National Centre for Biotechnology Information: NCBI; Polyacrylamide gel electrophoresis: PAGE.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Competing Interests

All the authors have approved the manuscript and agree with submission to your esteemed journal. There are no conflicts of interest to declare.

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Authors’ contributions

XY, WL and DD were responsible for data analysis, interpretation, and manuscript preparation. XW and JY gave contribution to the experimental design and editing of the final manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

Targeting regulation relationship between microRNAs and Aureochrome. (A) Relative expression profiles of five Aureochrome homologs transcripts of Saccharina japonica in response to dark (DR) and blue light (BL). Values are normalized for β-actin expression levels and represent means ± S.E. for n = 3. *P<0.05 by one-way analysis of variance. (B) The predicted regulatory network between microRNAs and Aureochrome homologs in S. japonica.
Figure 2

Negative regulation between miR8181-x and Aureochrome5 transcripts. qRT-PCR analyses of Aureochrome5 (A) and miR8181-x (B) transcripts in response to blue light (jewelry blue) and white light (light grey). Values are normalized for β-actin expression levels and represent means ± S.E. for n = 3. *P<0.05 by one-way analysis of variance.
Figure 3

Tissue-specific expression pattern of miR8181-x and Aureochrome5 in S. japonica. Relative expression profiles of Aureochrome5 (A) and miR8181-x (B) transcripts in different tissues of Saccharina japonica. Values are normalized for β-actin expression levels and represent means ± S.E. for n = 3. *P<0.05 by one-way analysis of variance.
Figure 4

(A) The regulatory network between miR8181-x and its target genes in S. japonica. (B) A set of diagrams showing six patterns of dynamic transcript expression in the target genes of miR8181-x in response to dark (DR), blue light (BL), and white light (WL) conditions. (C) KEGG functional enrichment of target genes in cluster 4.
Figure 5

Targets plots of the targets cleaved by the miR8181-x. The T-plots showed the distribution of the degradome tags along the full-length of the target mRNA sequence.
Figure 6

(A) Sequences and pairing between miR8181-x and its targeted mRNAs that exhibit similar expression profile with Aureochrome5 identified by high-throughput sequencing and degradome analysis in Saccharina japonica. The miR8181-x is in blue and the targeted mRNAs is in black. The red base represents the cleavage site of the miR8181-x. (B) Identification of the score of targeted mRNAs cleaved by the same miRNA of miR8181-x by degradome analysis in Saccharina japonica.
Figure 7

Functional enrichment analysis of miR8181-x in Saccharina japonica. Go analysis for the identified target genes of miR8181-x based on high-throughput sequencing and degradome analysis.

Supplementary Files

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