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Transcriptome Analysis and Physiological Responses of Economic Macroalga *Gracilariopsis lemaneiformis* under Sulfate Deficiency

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ABSTRACT

Sulfur is an essential macronutrient for the growth of all photosynthetic organisms and plays important roles in different metabolic pathways. However, sulfur metabolism and its related research on macroalgae with important ecological value is rather limited. In this study, marine ecological valued macroalga *Gracilariopsis lemaneiformis* was used to study the general physiological responses and transcriptome profiling under the sulfate deficiency. The relative growth rate of algae under sulfate deficiency was statistically significantly lower than that of control after 6 days. However, no significant differences were observed in the pigments content and Fv/Fm value, indicating that the photosynthesis was not obviously affected under the sulfate deficiency. Furthermore, the significantly increased soluble protein and carbohydrate contents, and indistinctively differentially expressed sulfate transporter/permease (ST) and ATP sulfurylase (ATPS) genes suggesting that *G. lemaneiformis* cells did not use sulfur from internal protein and carbohydrate pools but utilize stored sulfur from vacuole to satisfy their nutritional requirements during the sulfate deficiency. Transcriptome data showed that most annotated differentially expressed genes (DEGs) were down-regulated under the sulfate deficiency, including a large proportion of genes involved in the pathway of sulfur metabolism. Therefore, it was concluded that the pathway of sulfur metabolism was suppressed under the sulfate deficiency in *G. lemaneiformis*. The results and analysis in this study provide a comprehensive understanding for the physiological and molecular response of *G. lemaneiformis* to sulfate deficiency.

KEYWORDS

Gracilariopsis lemaneiformis; sulfate deficiency; sulfate sufficiency; transcriptome

1 Introduction

Sulfur is an essential macronutrient for all photosynthetic organisms [1]. Simultaneously, sulfur is one of the most abundant elements in the cells of photosynthetic organisms [2]. The major form of sulfur acquired by photosynthetic organisms is inorganic sulfate. Inorganic sulfate is firstly transported into the cells via sulfate transporter/permease. Intracellular sulfate will be activated by ATP sulfurylase (ATPS) and to form adenosine 5'-phosphosulfate (APS). Afterwards, APS is used for the synthesis of sulfur-containing metabolites through the reductive or phosphorylated pathways, such as amino acids (e.g., cysteine and

methionine) and its derivatives (e.g., methionine and glutathione), vitamins (e.g., biotin and thiamine), and sulfated polysaccharides (e.g., agar and carrageenan) [3–5]. In contrast to the low sulfate concentration in freshwater and terrestrial ecosystems (10-50 μM), the sulfate concentration in the marine ecosystem remains stable and can reach 25–28 mM [1]. It was proposed that high sulfate concentration is an evolutionary driver in the expansion of modern marine phytoplankton [6]. Therefore, what are the effects of sulfate deficiency on the marine algae?

Sulfur metabolism and its related research had been extensively studied in different algae [5,7–11]. Among these studies, transcriptome responses of algae under sulfate deficiency mainly focus on some model algae, such as green alga *Chlamydomonas reinhardtii*, cyanobacteria *Synechocystis* sp. and diatom *Emiliania huxleyi* [1,12–14]. Previous transcriptome data showed that genes encoding sulfate transporters and extracellular arylsulfatase were up-regulated, the reductive capacity of sulfate was enhanced, the metabolism of amino acids/metabolites containing sulfur was reinforced during sulfate deficiency [15]. Additionally, a recent study indicated that sulfur deprivation can significantly influence microalgal physiological characterizations, including the content of metabolites (e.g., starch, protein and alcohol), photosynthetic rate, the maximum effective quantum yield of photosystem II, the activities of antioxidant enzymes and metabolic pathways [16–18]. However, the study on physiological and transcriptome responses of macroalgae with important ecological value is rather limited [5]. Consequently, it is very curious to study if different algae, especially between microalgae and economical macroalgae, have diverse responses to sulfate deficiency?

Gracilariopsis lemaneiformis is widely cultured along the coasts of China, and it is a red macroalga of great economic value for the biotechnology industry because it produces raw materials, such as agar [19,20]. It was reported that *Gracilariopsis* genus account for 60–80% of the total agar production [21]. Besides, *G. lemaneiformis* can also be used as food for human and fish, and plays an important role in bioremediation [22,23]. In this study, biochemical methods were used to analyze the general physiological responses (growth rate, the contents of pigments, soluble protein and soluble carbohydrate) of *G. lemaneiformis*. The growth rate was significantly reduced, the contents of pigments were not obviously changed, while the contents of soluble protein and carbohydrate were significantly increased under the sulfate deficiency of *G. lemaneiformis*. Furthermore, next-generation sequencing was used to obtain a transcriptome profiling of *G. lemaneiformis* responses to sulfate deficiency. The transcriptome data presented that the sulfur metabolism pathway was suppressed under the sulfate deficiency. The results and analysis in this study provide a comprehensive understanding for the physiological and molecular responses of *G. lemaneiformis* to sulfate deficiency.

2 Materials and Methods

2.1 Algal Growth and Treatments

Healthy *G. lemaneiformis* with dark red and dense branches were pre-cultured for two weeks under the following conditions: temperature 20°C, photoperiod Light:Dark = 14 h:10 h, light intensity 50–60 μmol photons m^{-2} s^{-1}. After preculture, 2 g of healthy *G. lemaneiformis* were cultured in 1 L of sterile artificial seawater using 3 L conical flask. In control group (sulfate sufficiency) 25 mmol L^{-1} sulfate was used, while sulfate was not added in the sulfate deficiency group. Samples and control groups were cultured in triplet. The seawater was changed in each two days with freshly filtered sterile seawater during the period of culture.

2.2 Physiological Measurements

Algae were collected, dried, and weighed after the culturing of 0, 2, 4, 8, 10, 12, 14 and 16 days. The relative growth rate was calculated by the formula: $[(W_t/W_0)^{1/t}-1] \times 100\%$, W_t is the fresh weight of algae at time t (g), while W_0 is the fresh weight of algae at the beginning (g), t is the time between the two-detection time (day) [20,24].

After the culture of 0, 8 and 16 days, 100 mg dried algae were collected and finely powdered in liquid nitrogen using a mortar. Powdered samples were used to analyze the physiological data. Phycoerythrin (PE), chlorophyll a, soluble carbohydrate and soluble protein were measured via UV-VIS spectrophotometry at different wavelengths [23,25]. The maximum effective quantum yield of photosystem II (PSII) was determined by Fv/Fm value. The Fv/Fm ratio was measured with a pulse amplitude modulated (PAM) fluorometer (Imaging PAM, Walz, Effelrich, Germany) as introduced in our previous papers [23,24,26]. After the culture of 0, 8 and 16 days, 2 mg algae were collected and dried to constant weight at 60°C. Dried samples were well powdered in liquid nitrogen using a mortar. Finally, the samples were used to analyze the amounts of C, N and S elements by elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

2.3 Data Statistics and Analysis

All the original data were calculated and statistical analyzed with SPSS 20.0 software for windows. Significant differences among different groups were analyzed by student's t test ($\alpha = 0.05$). All data were calculated with three biological replicates and reported as the means ± SD.

2.4 Transcriptome Analysis

2.4.1 RNA Isolation, Library Preparation and Sequencing

The total RNA from each sample (16th day culture) was extracted using the DP432 kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China) following manufacturer's recommendation. RNA quality and concentration were measured by a 2100 Bioanalyzer at 260 nm and 280 nm. RNA integrity number of all samples was above 6.0. Isolated RNA was used for sequencing and qRT-PCR (Quantitative Reverse Transcriptase Polymerase Chain Reaction) analysis.

A total of 1 μg RNA per sample was used to generate paired-end RNA-seq library using NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). The library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). The library quantity was assessed using the Agilent Bioanalyzer 2100 system.

2.4.2 Quality Controls, De Novo Assembly and Clustering

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library was sequenced on an Illumina Hiseq 2000 platform.

Based on the technology of edge synthesis and edge sequencing (Sequencing By Synthesis, SBS), the cDNA library was sequenced using Illumina Hiseq high throughput sequencing platform. The raw data was filtered to remove the connector sequence and low-quality reads to obtain clean data with high quality. After obtaining high quality sequencing data, trinity platform was used for transcriptome assembly.

2.4.3 Gene Function Annotation and Classification

The Unigene sequences were compared with NR, Swiss-Prot, GO, COG, KOG, eggNOG, KEGG by using BLAST software. KEGG Orthology results of Unigene in KEGG were obtained by using KOBAS2.0. After predicting the amino acid sequences of Unigene, the comment information of Unigene was obtained by comparing HMMER software with Pfam database. The E-value for BLAST parameter was smaller than 1e–5, for HMMER parameter was smaller than 1e–10. Finally, Unigenes with annotated information were obtained.

2.4.4 Identification of Differentially Expressed Genes (DEGs)

DESeq2 was used to analyze the differentially expressed genes among the samples. During the process of differential expression analysis, the recognized effective Benjamini-Hochberg method was used to correct

the significant *P* value obtained from the original hypothesis test. The key index of gene screening was to reduce the false positive caused by independent statistical hypothesis test of many gene expression values. The screening criterion was as following: the false discovery rate (FDR) was less than 0.001, the difference multiple Fold Change (FC) was greater than or equal to 2. FC represents the ratio of expression levels between the two groups.

2.4.5 Real-Time qRT-PCR

Verification of DEGs was carried out on the same RNA samples for RNA-Seq experiment. Totally, the expression levels of nine unigenes were verified by qRT-PCR. The primers were shown in Table S2. 1 mL RNA with high quality was used to synthesize cDNA via KP118-02 kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The expression levels of genes were measured with a BIO-RAD-T100 using Talent qPCR PreMix (SYBE Green) (Tiangen Biochemical Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The experiments were done as following: 95°C for 3 min, followed by 40 cycles of amplification (95°C for 5 s and 60°C for 10 s, 72°C for 15 s). $2^{-\Delta\Delta Ct}$ method was used to obtain the raw Ct values [5,27]. Finally, the expression levels of these selected genes from qRT-PCR were compared to that of differentially expressed genes (DEGs) from RNA-Seq.

2.5 Ethics Statement

The authors confirmed that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as macroalga was used as material in this study.

3 Results and Discussion

3.1 Physiological Responses of G. lemaneiformis under Sulfate Deficiency

The relative growth rate of *G. lemaneiformis* under sulfate sufficiency and deficiency was shown in Fig. 1. During the first 6 days, the relative growth rate did not show significant difference between sulfate sufficiency and deficiency ($P > 0.05$). However, the relative growth rate of algae under sulfate deficiency was significantly lower than that of sulfate sufficiency after 6 days ($P < 0.05$). Consistent phenomenon was observed in other algae. For example, the growth of cells were severely diminished during sulfur limitation in the green microalga *Chlamydomonas reinhardtii* [28]. Compared to sulfur sufficiency, sulfur limitation/deprivation reduced the growth rate of the halotolerant chlorophyte *Dunaliella salina* [17,29]. These results indicated that sulfur is an essential macronutrient for the growth of algae.
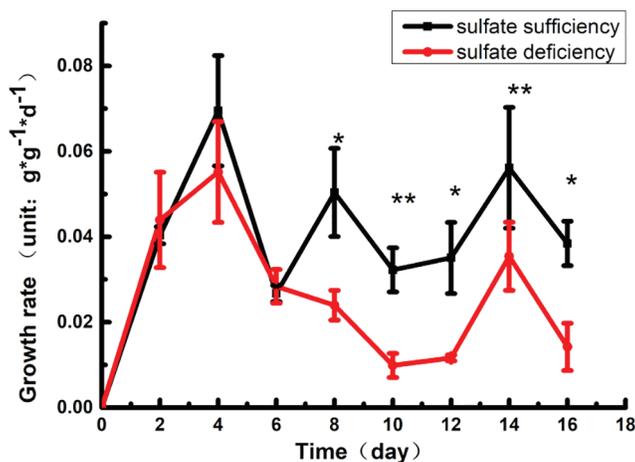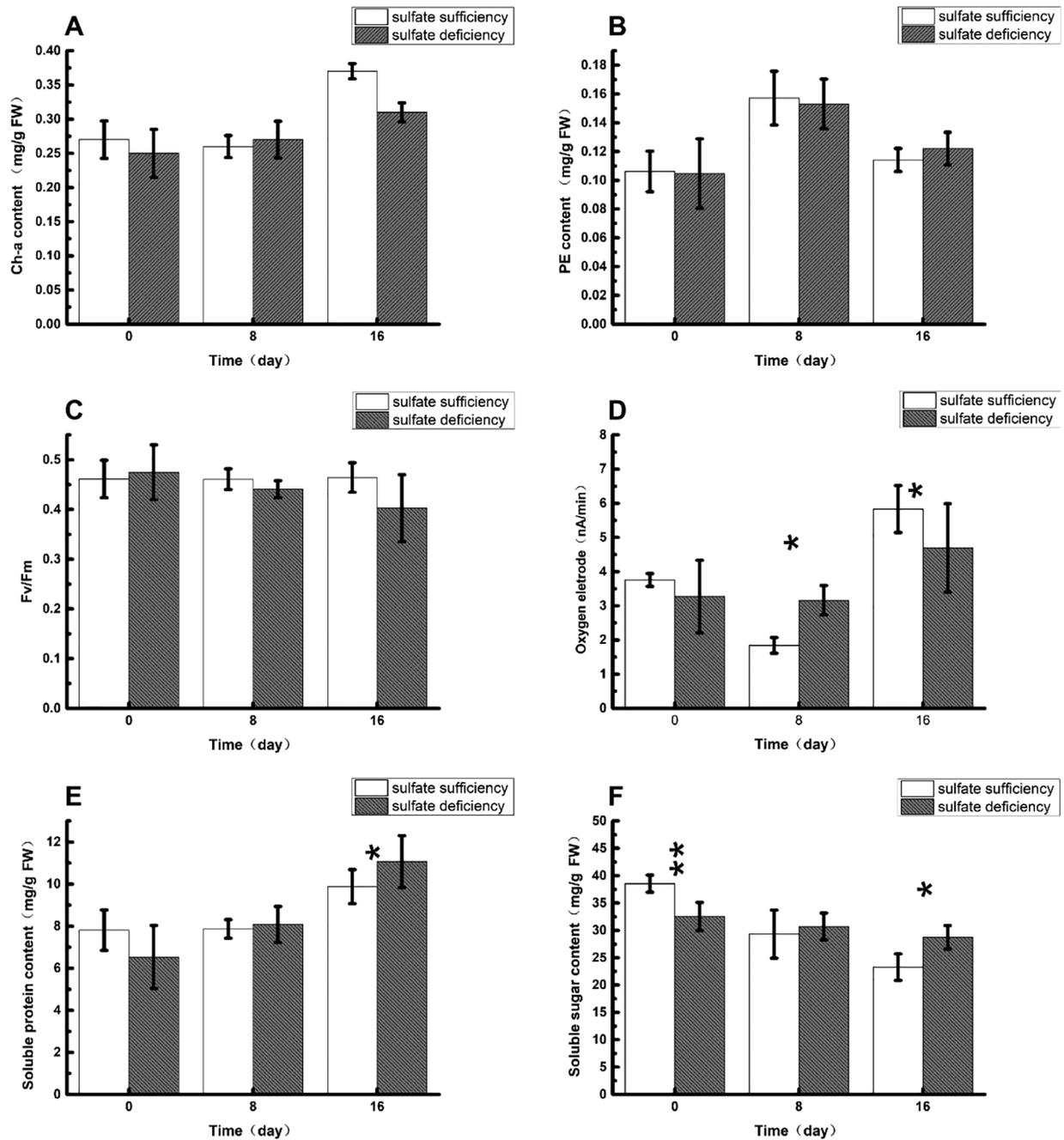

Figure 1: Relative growth rate of *G. lemaneiformis* under sulfate deficiency. *represents significant difference between sulfate sufficiency and deficiency, **represents extremely significantly difference between sulfate sufficiency and deficiency

The changes of chlorophyll a, phycoerythrin content, Fv/Fm value, photosynthetic oxygen release capacity, soluble carbohydrate content and soluble protein of *G. lemaneiformis* were shown in Fig. 2 under sulfate sufficiency and deficiency conditions. Considering the invariable color of algae during sulfate deficiency, the contents of the photosynthetic pigment chlorophyll a and phycoerythrin were analyzed. Although the content of chlorophyll a was gradually increased during the culture, compared to that of sulfate sufficiency the accumulation of chlorophyll a was not significantly difference on the 8th and 16th days of culture in sulfate deficiency ($P > 0.05$) (Fig. 2A). Chlorophyll a content was also increased under the sulfur deprivation in *D. salina* and higher than that on the 8th day of culture in sulfur sufficiency [17]. These results implicated that there was a similar defense mechanism between *D. salina* and *G. lemaneiformis* during sulfate deficiency. However, total chlorophyll content was found to be unaffected by the sulfur limitation in *D. salina* [29]. The PE content showed no significant difference between sulfate sufficiency and deficiency on days 0, 8 and 16 (Fig. 2B). However, compared to the beginning of culture chlorophyll and PE contents were increased on the 8th and 16th days. PE is a major phycobiliprotein in red macroalgae, which works on the capture of light and transfer the light to photosystem II. Owing to its antioxidant property, anti-tumor activity and solubility in water, PE was used in different industries, including cosmetic, clinical diagnostics and food industries and so on [30]. It is well known that phycoerythrin plays a decisive role in the red color of normal *G. lemaneiformis*. Therefore, the content of PE without significant difference explained the invariable color of algae under the sulfate deficiency.

The value of Fv/Fm under the sulfate deficiency was reduced during the culture, however, it was not significantly different to that on the 8th and 16th days of the sulfate sufficiency ($P > 0.05$) (Fig. 2C). It was reported that sulfur limitation or deprivation affected the photosynthetic efficiency and structures of the photosynthetic apparatus, and resulted in the reduction of photosynthetic capacity and the decrease of proteins related with photosynthesis (e.g., rubisco and chlorophyll a/b binding proteins) in algae [29,31,32]. Light-saturated photosynthesis rate was declined in *D. salina* in S-deprived medium [18]. These reports explained the reduced Fv/Fm value under the sulfate deficiency in *G. lemaneiformis*. The data of photosynthetic oxygen release showed that the photosynthetic oxygen release in the sulfate deficiency was significantly higher than that in the sulfate sufficiency group on the 8th day ($P < 0.05$). However, the photosynthetic oxygen release in the sulfate deficiency was observably lower than that in the sulfate sufficiency group on the 16th day ($P < 0.05$) (Fig. 2D). These results were thought to be a specific response necessary to deal with the S limitation. However, there are still no references to explain why the results of Fv/Fm and photosynthetic oxygen release are so different.

The soluble protein data showed that the content was increased, especially, the content of soluble protein under the sulfate deficiency was prominently higher than that of sulfate sufficiency on the day 16 ($P < 0.05$) (Fig. 2E). Sulfate deficiency resulted in the increase of soluble protein content in *G. lemaneiformis*, similarly to the case in autotrophic *Galdieria phlegrea*, but not in heterotrophic *G. phlegrea* [9], *Chlorella sorokiniana* [8] and *Chlamydomonas reinhardtii* [33]. The content of soluble carbohydrate was gradually decreased during sulfate sufficiency and sulfate deficiency (Fig. 2F). It was shown that the content of soluble carbohydrate during sulfate deficiency was significantly higher than that in sulfate sufficiency on the day 16th ($P < 0.05$). Soluble protein and carbohydrate were significantly increased, it could be proposed that the sulfate deficiency cells of *G. lemaneiformis* did not utilize S from the internal protein and carbohydrate pools to redistribute the resource in order to satisfy their nutritional requirements [34]. It was reported that excess sulfate will be stored in the vacuole [31]. Therefore, our results indicated that *G. lemaneiformis* cells used the stored sulfate from algal vacuoles during sulfate deficiency.

Figure 2: Physiological data of *G. lemaneiformis* under sulfate deficiency (A) is chlorophyll a content, (B) is phycoerythrin (PE) content, (C) is Fv/Fm, (D) is photosynthetic oxygen release capacity, (E) is the content of soluble protein, (F) is the content of soluble carbohydrate. *represents significant difference between sulfate sufficiency and deficiency, **represents extremely significantly difference between sulfate sufficiency and deficiency

As shown in Fig. 3, the percentage of sulfur under sulfate deficiency was significantly lower than that under the condition of sulfate sufficiency on the day 8th and 16th ($P < 0.05$) (Fig. 3A). However, no significant difference of total C percentage, total N percentage and C/N ratio were observed between the two groups (Figs. 3B and 3C). Similar results were also observed in a previous paper where showed that total C and N were not strongly affected, while total S was decreased under S starvation in autotrophic microalga *Galdieria phlegrea* [9]. Our results suggested that sulfur, but not carbon and nitrogen was significantly affected by sulfate deficiency in *G. lemaneiformis*.
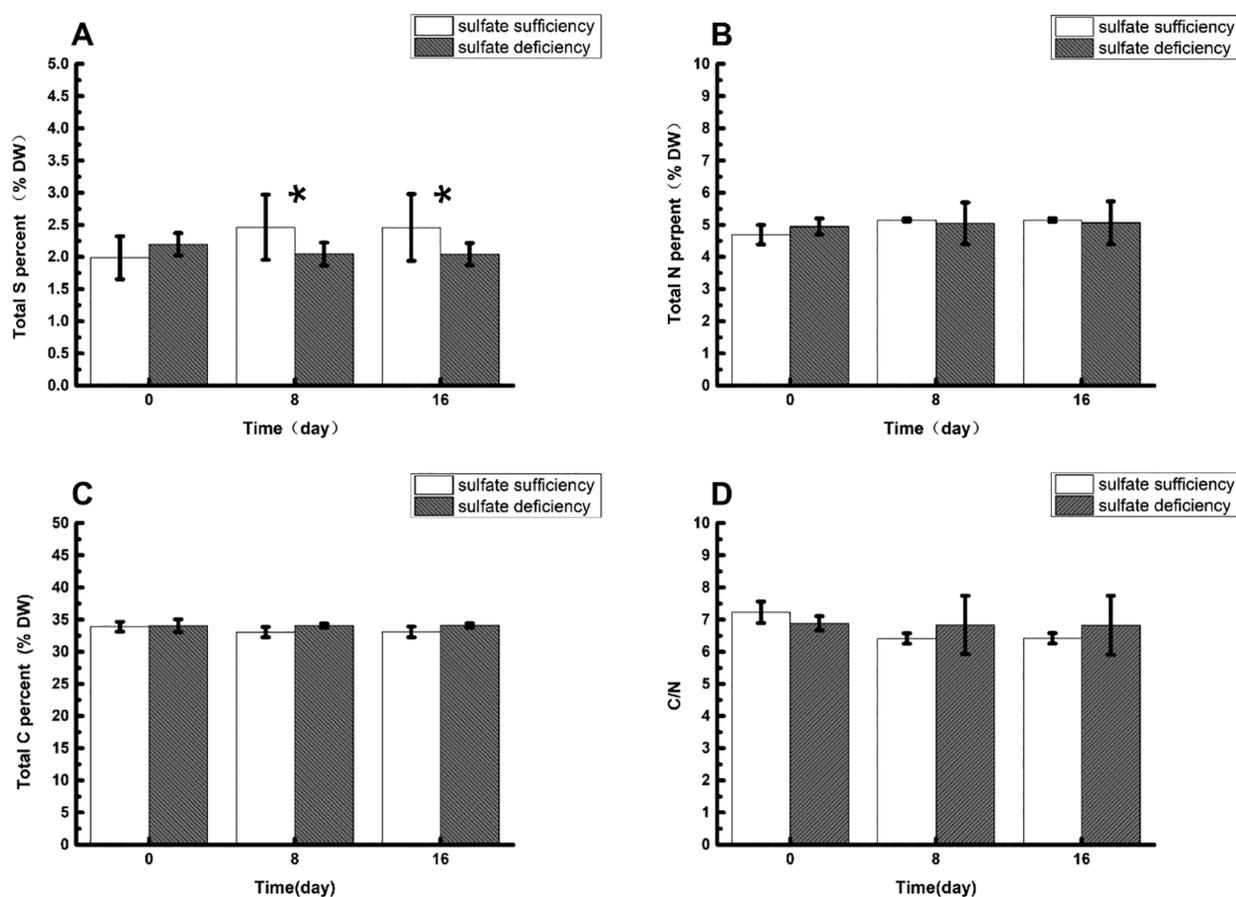
Figure 3: Elements of *G. lemaneiformis* under sulfate deficiency (A) is total sulfur percent, (B) is total nitrogen percent, (C) is total carbon percent, (D) is the ratio of total carbon/ nitrogen. *represents significant difference

3.2 General Transcriptome Analysis of G. lemaneiformis Response to Sulfate Deficiency

Because no-reference genome can be used in this transcriptome analysis, clean reads obtained from this project were assembled into different length contigs using overlap information until the contigs could not be extended anymore [35]. After the assembling, 55,443 transcripts and 39,440 unigenes (>300 bp) were generated, with an N50 of 4,484 and 2,513 bp, respectively. Besides, it was shown that the length of transcripts and unigenes was mainly focused on the range of 300–500 bp. The total lengths of transcripts and unigenes were respective 107,954,061 and 48,908,123 bp, with a mean length of 1,947 and 1,240 bp.

3.2.1 Functional Categories of Genes Affected by Sulfate Deficiency in G. lemaneiformis

Comparing unigene sequences with major databases, a total of 25,023 unigenes were commented on GO, COG, KEGG, KOG, Pfam, Swissprot, eggNOG and NR database to 13,367 (53.42%), 11,759 (46.99%), 12,938 (51.70%), 16,725 (66.84%), 24,389 (97.47%), 14,216 (56.81%), 21,202 (84.73%) and 22,905 (91.54%), respectively.

The distribution of GO, KOG and KEGG annotation was shown in Fig. 4. 13,667 unigenes were annotated in the GO database. In the cellular component, most of the unigenes were involved in the cell, cell part and organelle. In the molecular function, unigenes were mostly related to catalytic activity, binding, and structural molecular activity. In the biological process, most of the unigenes were annotated to the cellular process, metabolic process, and the single-organism process (Fig. 4A).

KOG analysis showed that a total of 11,759 unigenes were annotated (Fig. 4B). Among them most of the annotated genes (2,436, 19.46%) were involved in translation, ribosomal structure, and biogenesis. Afterwards, 1,452 (11.6%) genes were related to post-translational modification, protein turnover and chaperones.

Unigenes marked with the KEGG database were divided into 5 pathways (Fig. 4C). These were cellular processes, environmental information processing, genetic information processing and biological systems, metabolism, and organic systems. The number of abundant unigenes of each pathway was different. The most abundant unigenes were endocytosis-related genes (367) in the cellular process. The most abundant unigenes were phosphatidylinositol signaling-related genes (118) in environmental information processing. The largest number of unigenes was ribosome-related genes (2,107) in genetic information processing and biological systems. The most abundant unigenes were related to carbon metabolism (590), followed by genes related to biosynthesis of amino acids (466) in the metabolic process. The largest number of unigenes were associated with plant-pathogen interactions (94) in organic synthesis.

3.2.2 Differentially Expressed Genes (DEGs) Affected by Sulfate Deficiency in G. lemaneiformis

The great majority of unigenes in this study did not have high expression fold-change in the samples of sulfate sufficiency and deficiency being compared, therefore, two expression fold-change as a threshold value in defining DEGs was carried out. Totally, 2,238 differentially expressed unigenes were obtained, 139 up-regulated genes (red dots) and 2,099 down-regulated genes (green dots) were found in sulfate deficiency samples compared to that in sulfate sufficiency samples. Most of genes (33,853) did not have significant difference in expression (black dots) (Fig. 5A). Subsequently, all DEGs were subjected to GO term and KEGG pathway enrichment analysis. Totally, 875 DEGs were annotated in Go database. In the biological process, most of the DEGs were annotated to the cellular process, metabolic process, and the single-organism process. In the cellular component, most of the DEGs were involved in the cell, cell part and organelle. In the molecular function, DEGs were mostly related to catalytic activity, binding and structural molecular activity (Fig. 5B). Overall, 2,238 DEGs were classified into 20 KEGG pathways. Among the 20 KEGG pathways, the pathway related with ribosome (200, 8.9%) represented the largest group, with the high reliable in the enrichment significance of DEGs in the ribosome pathway (Fig. 5C).
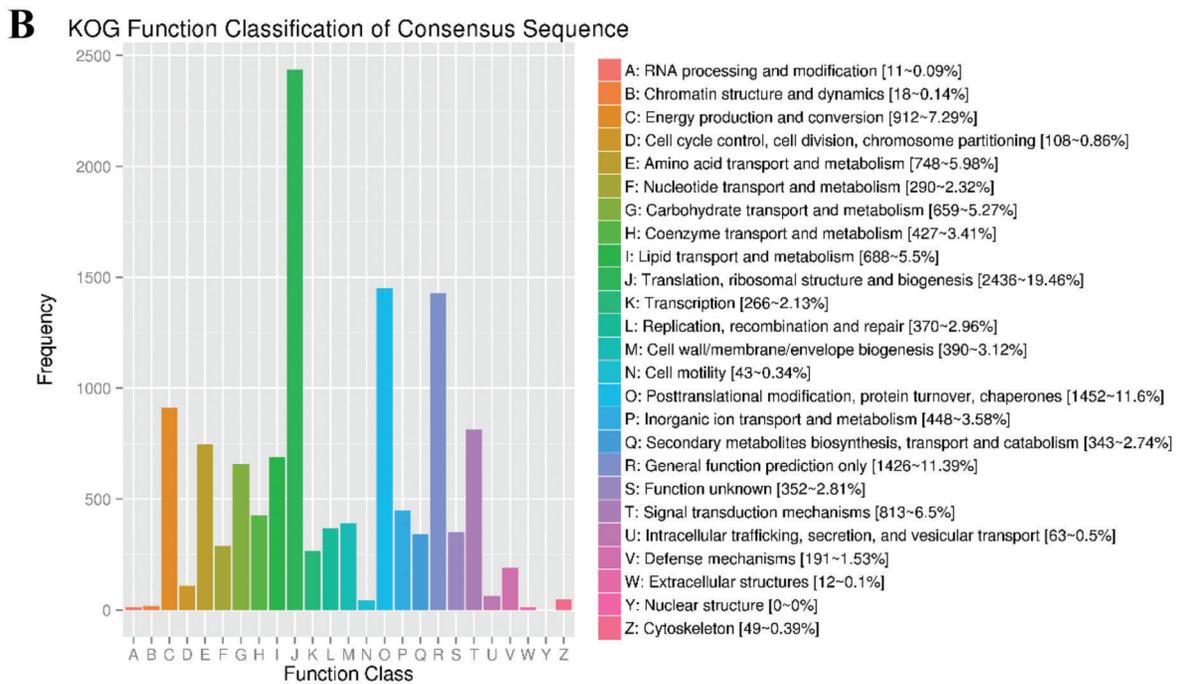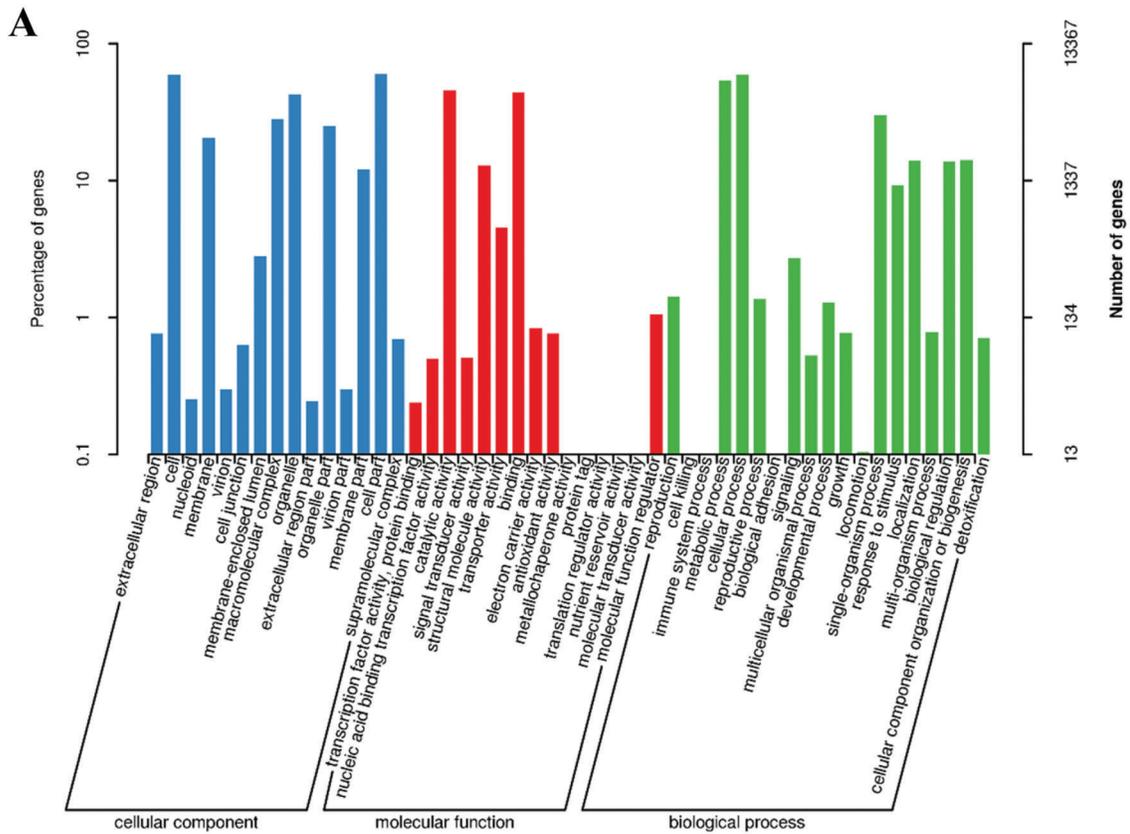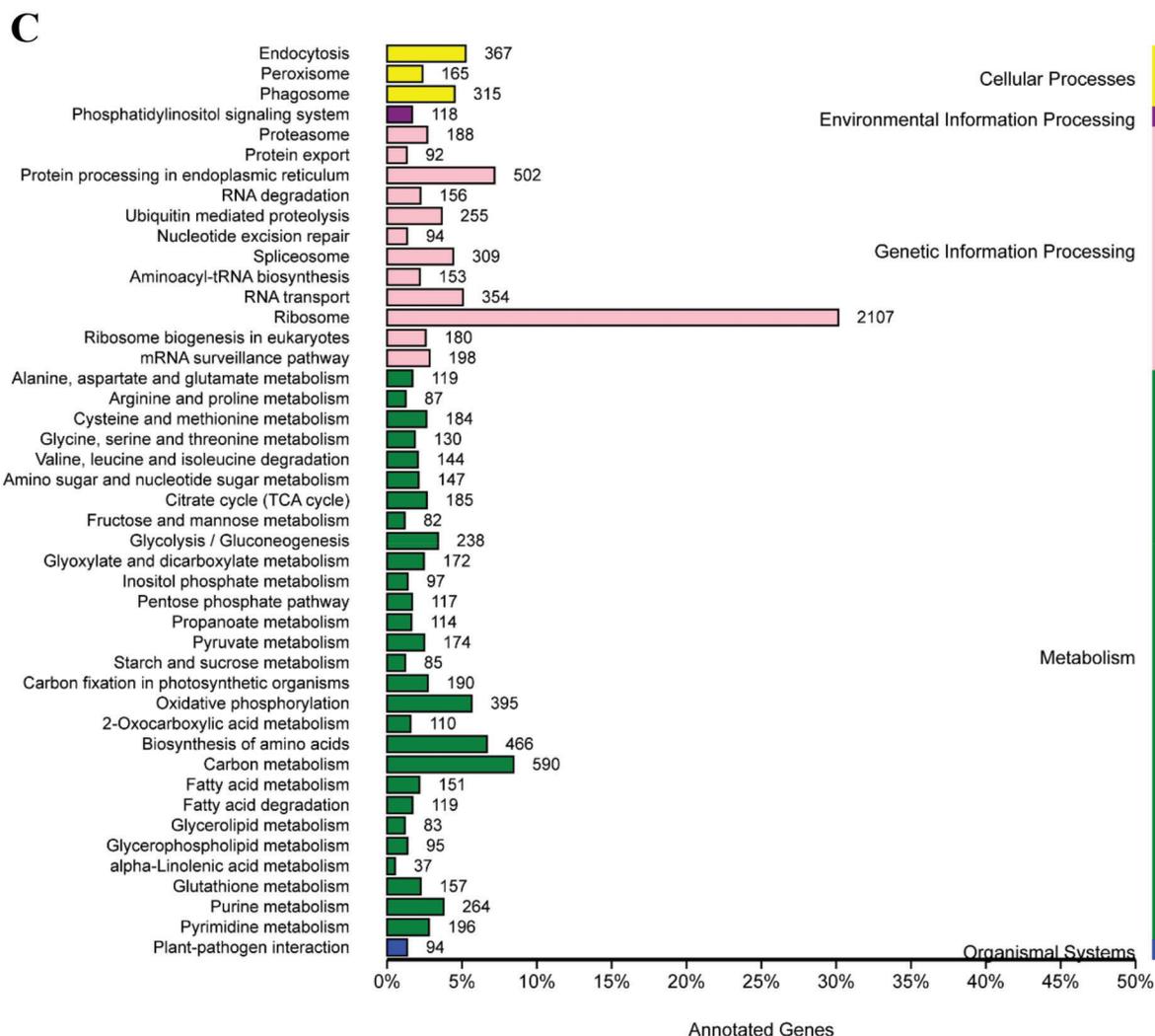
A

B KOG Function Classification of Consensus Sequence

A: RNA processing and modification [11~0.09%]
B: Chromatin structure and dynamics [18~0.14%]
C: Energy production and conversion [912~7.29%]
D: Cell cycle control, cell division, chromosome partitioning [108~0.86%]
E: Amino acid transport and metabolism [748~5.98%]
F: Nucleotide transport and metabolism [290~2.32%]
G: Carbohydrate transport and metabolism [659~5.27%]
H: Coenzyme transport and metabolism [427~3.41%]
I: Lipid transport and metabolism [688~5.5%]
J: Translation, ribosomal structure and biogenesis [2436~19.46%]
K: Transcription [266~2.13%]
L: Replication, recombination and repair [370~2.96%]
M: Cell wall/membrane/envelope biogenesis [390~3.12%]
N: Cell motility [43~0.34%]
O: Posttranslational modification, protein turnover, chaperones [1452~11.6%]
P: Inorganic ion transport and metabolism [448~3.58%]
Q: Secondary metabolites biosynthesis, transport and catabolism [343~2.74%]
R: General function prediction only [1426~11.39%]
S: Function unknown [352~2.81%]
T: Signal transduction mechanisms [813~6.5%]
U: Intracellular trafficking, secretion, and vesicular transport [63~0.5%]
V: Defense mechanisms [191~1.53%]
W: Extracellular structures [12~0.1%]
Y: Nuclear structure [0~0%]
Z: Cytoskeleton [49~0.39%]

Figure 4: (Continued)

C

Figure 4: Functional annotation of all unigenes from *G. lemaneiformis* (A): Gene Ontology (GO) annotation based on the three major categories: cellular component, molecular function, and biological process. (B): The eukaryotic Orthologous Groups database (KOG) analysis of the annotated unigenes. (C): Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways classification of the annotated unigenes

3.3 Transcriptional Regulation of Sulfur Metabolism in G. lemaneiformis under the Sulfate Deficiency

3.3.1 Sulfur Acquisition and Assimilation

The genes encoding sulfate transporter/permease (ST) and ATP sulfurylase (ATPS) were identified in the transcriptome data of *G. lemaneiformis*, but they were not differentially expressed under the sulfate deficiency compared to that in sulfate sufficiency (Fig. 6). The expression of *ST* and *ATPS* genes was not affected, indicating that *G. lemaneiformis* used some other sulfate transporter/permease and/or ATP sulfurylase to transport or assimilate sulfate from surrounding environment under the condition of sulfate deficiency. The sulfur storage of most photosynthetic organisms in cells was very limited, a main response of photosynthetic organisms to sulfate deficiency is to increase the capacity of the cell for transporting and/or assimilating sulfate from surrounding environment [31,36]. Consistent with the results from physiology research, it was speculated that unlike other photosynthetic organisms *G. lemaneiformis*

could use vacuolar sulfur to satisfy their nutritional requirements. Subsequently, adenosine 5'-phosphosulfate (APS) will be assimilated for the synthesis of sulfur-containing metabolites through the reductive or phosphorylated pathways. Genes encoding APS reductase (APR) and APS kinase (APK) are the key node in the reductive and phosphorylated pathways. Nevertheless, the two genes were not found in the transcriptome data of *G. lemaneiformis* under sulfate deficiency. Similar with *G. lemaneiformis*, *APR* gene was also not found in *Gracilaria changii* and *Gracilaria salicornia* under sulfate deprivation [5]. Therefore, it was speculated that some alternative reductase/phosphorylase or pathways exist to meet the demands of the reduction and phosphorylation of APS. In this study, a gene encoding sulfohydrolase/sulfurylase (SH) was down regulated in sulfate deficiency *G. lemaneiformis*.

The pathway of sulfur metabolism was modified from previous papers [3,5]. Unigenes in red or green rectangles indicate that the expression of the unigenes in *G. lemaneiformis* under sulfate deficiency was up-regulated or down-regulated compared to those in sulfate sufficiency, respectively. Unigenes in blue rectangles indicate that they are only present in *G. lemaneiformis* under sulfate deficiency and sufficiency, but do not have up-and down-regulation. Whereas grey rectangles indicate these unigenes were not found in *G. lemaneiformis* under sulfate deficiency and sufficiency. APS, adenosine 5'-phosphosulfate; APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; BPNT, 3'(2'), 5'-bisphosphate nucleotidase; CBL, cystathionine beta-lyase; CGS, cystathionine gamma-lyase; GGCS, γ-glutamylcysteine synthetase; GSHS, glutathione synthetase; GST, glutathione-S-transferase; MS, methionine synthase; OAST-TL, O-acetylserine (thiol)-lyase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, S-adenosylmethionine; SAMS, SAM synthetase; SAT, serine acetyltransferase; SH, sulfohydrolase/ sulfurylase; SiR, sulfite reductase; SO, sulfite oxidase; SOT, sulfotransferase; ST, sulfate transporter/permease.

Additionally, one gene encoding sulfite oxidase (SO) was down regulated in this experiment. Consistency result was also observed in sulfate-deficiency *G. salicornia*, but not in sulfate deficiency *C. reinhardtii* [5,12]. The diverse results might be due to different algal species. SO plays a role in the recycling of sulfite to form intracellular sulfate. This means that the regeneration of free sulfate was decreased, excess sulfite was used to synthesize cysteine in *G. lemaneiformis*. Together with *SO* gene, a gene encoding sulfite reductase (SiR) was also down regulated. Since most genes were down regulated during the pathway of sulfate reduction, therefore, it was suggested that the sulfate reduction pathway was inhibited in sulfate deficiency *G. lemaneiformis*.

3.3.2 Metabolism of Glutathione and Methionine

The metabolisms of glutathione and methionine are the extend of sulfate reductive pathways. Serine acetyltransferase (SAT), the only up-regulated gene in the pathway of sulfur metabolism in sulfate deficiency *G. lemaneiformis*, is responsible for the biosynthesis of O-acetylserine from serine (Fig. 6). The up regulation of *SAT* gene might be explained by that serine was continually synthesized from nitrogen metabolism. Hydrogen sulfide derived from the reduction of sulfate is inserted into the construction of O-acetylserine to form cysteine under the action of O-acetylserine(thiol)lyase (OAS-TL). Cysteine is the first stable organic sulfur compound during the pathway of sulfur metabolism. It was reported that the synthesis of cysteine is regulated by the assemblage and dis-assemblage of SAT and OAS-TL complex [37]. SAT is only active when it is associated in the complex, while OAS-TL is active when it is free [38]. However, the gene encoding OAS-TL was not found from the transcriptome data in this study. The content of cysteine showed that it was significantly increased under the sulfate deficiency compared to that in sulfate sufficiency on the 16th day (Fig. S1A). Consequently, it was speculated that an alternative gene exists in *G. lemaneiformis* function as OAS-TL, however, the expression of alternative gene was restrained.
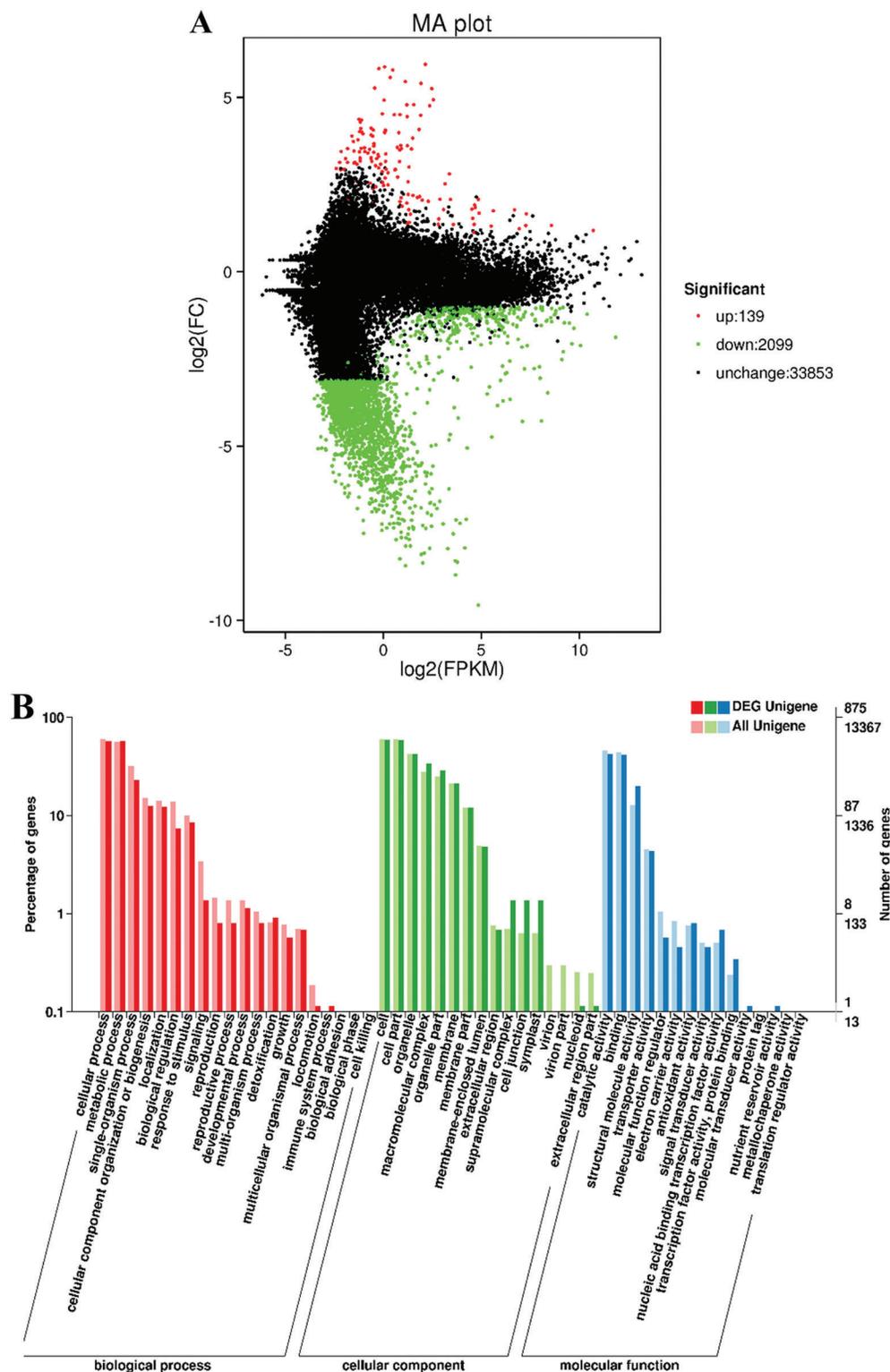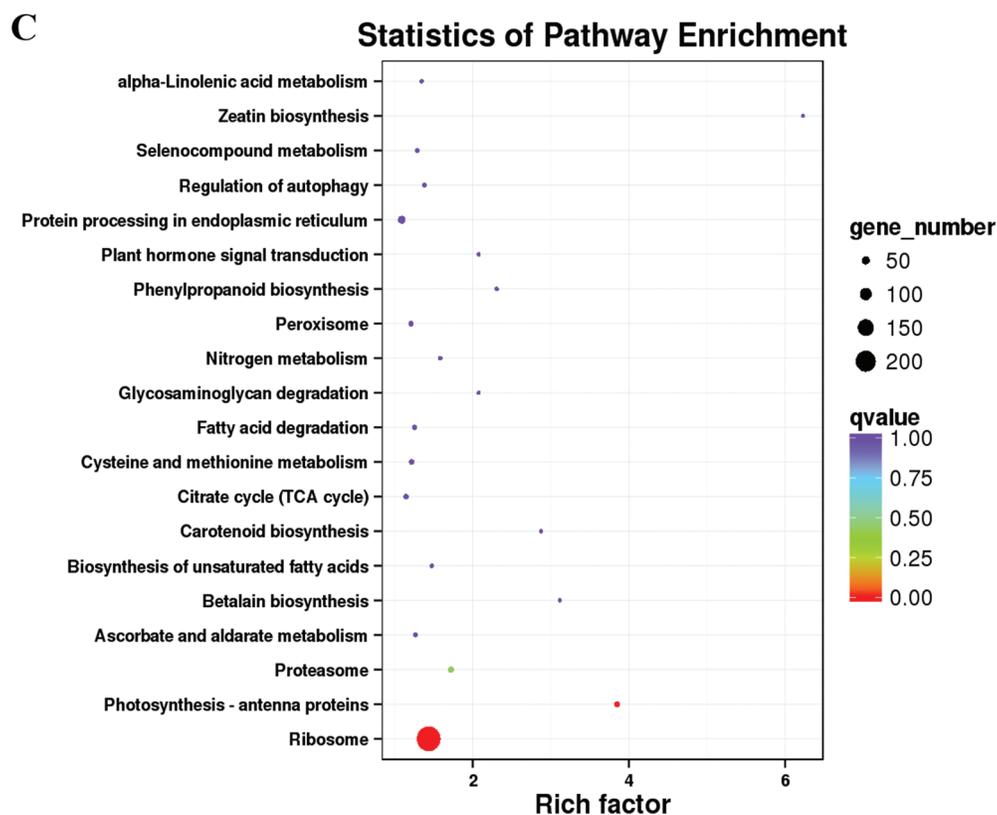
A

B

Figure 5: (Continued)

C

Statistics of Pathway Enrichment

Figure 5: Differentially expressed genes (DEGs) in *G. lemaneiformis* (A): Volcano plot of gene expression difference between control and sulfate deficiency samples. Each dot represents a gene. Log2 (FDR) is the logarithmic value of the mean expression in the two samples, which is used to show the difference in expression. (B): GO analysis of the DEGs. The abscissa is GO classification. The left side of the ordinate is the percentage of the number of genes, and the right side is the number of genes. (C): KEGG pathway functional enrichment analysis of the DEGs. Each circle in the graph represents a KEGG pathway, the longitudinal coordinate represents the name of the pathway, and the abscissa is the enrichment factor

It was known that genes encoding cystathionine gamma-lyase (CGS), cystathionine beta-lyase (CBL), methionine synthase (MS) and SAM synthetase (SAMS) are in-charge of the methionine synthesis. Additionally, genes encoding γ-glutamylcysteine synthetase (GGCS) and glutathione synthetase (GSHS) are responsible for the synthesis of glutathione [3,5]. Nevertheless, all these genes were down-regulated to different extents in *G. lemaneiformis* during sulfate deficiency. Our results indicated that the biosynthesis of methionine and glutathione was suppressed by sulfate deficiency in *G. lemaneiformis*. However, it was observed that the content of methionine during sulfate deficiency was not significantly reduced from that of sulfate sufficiency (Fig. S1B). Therefore, it was proposed that methionine biosynthesis in alga *G. lemaneiformis* was regulated by the combination of transcriptional and post-transcriptional mechanisms.

3.3.3 Other Identified Differentially Expressed Genes Involved in Sulfur Metabolism

Some unigenes involved in sulfur metabolism were also analyzed in *G. lemaneiformis*, the data of selected differentially expressed unigenes were shown in Table S2. It was found that genes encoding heparan-sulfate-6-O-sulfotransferase (HSS) and glycosyltransferase (GT) were down-regulated in sulfate-deficient *G. lemaneiformis*. Previous study showed that the down-regulation of *HSS* gene was consistent

with the significant decrease of sulfate content in agar, and proposed that the different expression of *GT* gene might increase the gel strength of gel [5]. Therefore, the down regulated *HSS* and *GT* genes might change the quality of agar in *G. lemaneiformis*. Furthermore, some unigenes related with sulfate transport were also analyzed from the transcriptome of *G. lemaneiformis* during sulfate deficiency. The results showed that genes encoding S-type anion channel, ABC transporter related protein, ABC transporter permease and mitochondrial carrier protein/S adenosylmethionine carrier were all down-regulated in their sulfate deficient algal samples (Table S2). Therefore, it was concluded that the whole sulfur metabolism was suppressed in *G. lemaneiformis* under the sulfate deficiency.
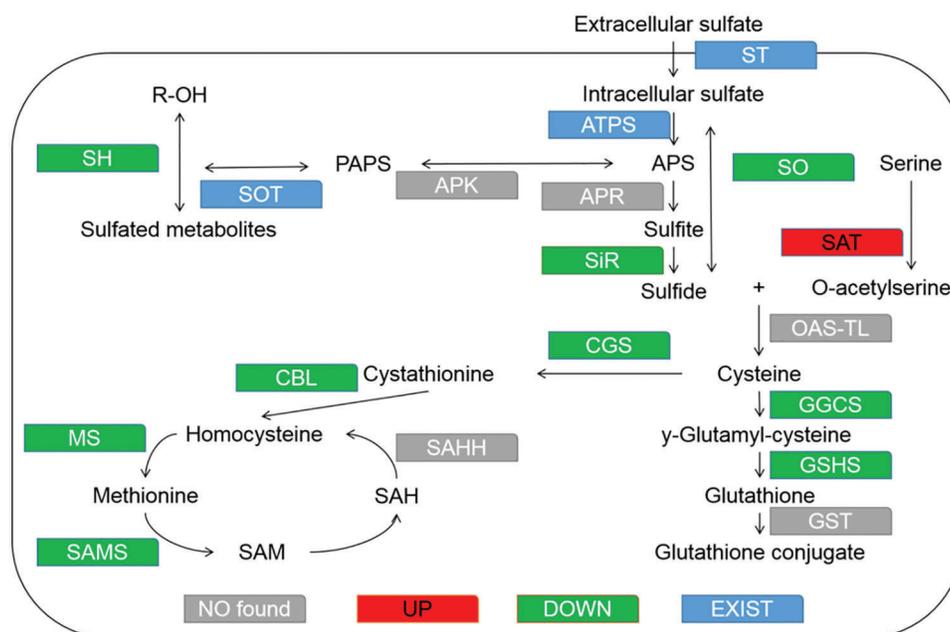
Figure 6: The pathway of sulfur metabolism in *G. lemaneiformis*

3.4 Verification of DEGs with qRT-PCR

Nine genes were selected from *G. lemaneiformis* and were verified by qRT-PCR (Fig. 7). Three genes are related with sulfur metabolism, including C87474, C137071 and C133127. Three genes are involved in nitrogen metabolism, including C116395, C116845 and C129388. While the other three genes (C135653, C135893 and C137592) participate the process of photosynthesis. As a result, the expression profiles of nine unigenes in *G. lemaneiformis*, generated by RNA-Seq, corroborated with those obtained from qRT-PCR (Fig. 7). The highly consistency between the results obtained from RNA-Seq and qRT-PCR indicated that transcriptome data of *G. lemaneiformis* under the sulfate sufficiency and deficiency were credible.
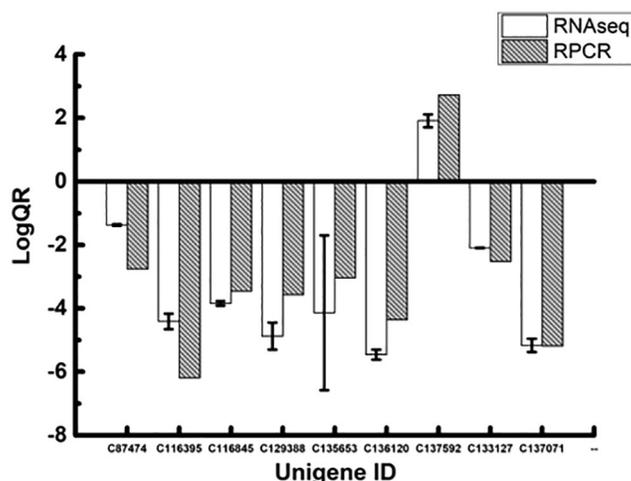
Figure 7: Validation of DEGs via qRT-PCR

4 Conclusions

In this study, the relative growth rate of algae was significantly inhibited after 6 days under sulfate deficiency. However, pigments content and Fv/Fm value were not significantly decreased, indicating that the photosynthesis was not obviously affected under the sulfate deficiency. The soluble protein and carbohydrate contents were increased, suggesting that *G. lemaneiformis* cells did not use sulfur from internal protein and carbohydrate pools but utilize stored sulfur from vacuole to satisfy their nutritional requirements during the sulfate deficiency. Furthermore, it was concluded that most annotated differentially expressed genes (DEGs) were down regulated under the sulfate deficiency via transcriptome analysis, including large number of genes involved in the sulfur metabolism pathway. Therefore, it was speculated that the pathway of sulfur metabolism was inhibited in *G. lemaneiformis* under the sulfate deficiency.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request. Sequence reads are available by the NCBI sequence read archives sulfate sufficiency (SRR12206572; SRR12213124; SRR12215316) and sulfate deficiency (SRR12223594; SRR12226568; SRR12235007).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Table S1: Primers for Real-Time PCR

Gene ID	Forward primer (5'-3')	Reverse primer (5'-3')
C87474	AGCATCTTGCCCGAAGTCT	CCTAAGTCCATTCTCAAAGCAC
C116395	AGCGTGATAAAGACTTCCATCG	CAAATGCTCAACAGGCTGAAA
C116845	GCATTAGTGGCAGAAGATAAGC	CCATTTCAGCAACACCAACT
C129388	CATACAGATGCCAGCACAA	GCTCCTCCATAAGGTAAGTGA
C135653	TGAAGTTCGTCTCTGCCTG	TCCTCTCCAACAACCTCTG
C136120	TGGATGCGAGCGTAGAAGA	GATGTATCGGACGGGAACA
C137592	TGCTGGTTCTCTACTTTACGG	GCTTAGTTCCCATTCACGA
C133127	TCAAGGAAGAGACAGACAACC	CGATGATGAAGTGTGTGGC
C137071	AGAGGGAGATGCTACTGTCGG	TGCTTGTCTGACCATTTGAATC
Actin	CTACTCGTTTACCACTTCTGCTGA	TTCCATTCCGACCAACTCTG

Table S2: Expression level of selected differentially expressed genes involved in sulfur metabolism in *G. lemaneiformis*

Functional category	Gene name	Unigene ID	Fold change expression(−s/+s)
1. Sulfur acquisition and assimilation	ST	No found	
	SO	c119006	−3.38
		c65490	−2.09
		c199940	−2.14
	ATPS	c154691	2.86
		c176997	−2.08
		c119872	−4.44
	SiR	c84656	−2.14
	APR	No found	
	APK	No found	
	BPNT	c187624	−2.22
2. Metabolism of glutathione and methionine	SAT	c179346	2.76
	OAS-TL	No found	
	GGCS	c180194	−3.11
		c129988	−8.07
		c137461	−6.69
	GSHS	c112293	−8.32
		c113137	−7.11
		c120694	−6.21

(Continued)

Table S2 (continued)

Functional category	Gene name	Unigene ID	Fold change expression(−s/+s)
		c121103	−6.1
		c124601	−4.88
		c132624	−3.9
		c135494	−2.23
	GST	No found	
	CGS	c233886	−2.08
		c160766	−2.69
		c113517	2.21
		c124193	−8.25
		c125721	−5.94
	CBL	c233886	−2.08
		c160766	−2.69
		c124193	−8.25
		c125721	−5.94
		c68155	−2.37
		c103766	−2.14
		c159948	−2.22
		c132321	−2.24
		c235781	−2.37
		c158621	−3.32
		c62066	2.05
	SAMS	c123904	−2.08
		c162436	−2.08
		c149877	−2.57
		c232173	−2.14
		c66069	−3.11
		c156784	−2.78
		c149103	−2.782
		c139605	−4.75
		c59566	−2.14
		c126053	5.63
		c229368	−2.37
		c119146	−5.18
		c124225	−7.57
	SAHH	c123122	−2.78
		c150275	−4.26
	SH	No change	

Table S2 (continued)

Functional category	Gene name	Unigene ID	Fold change expression(−s/+s)
3. Others	HS6ST	c123122	−2.78
	SuOx	c119006	−3.38
	GT	c156138	−2.88
	ABCtrp	c231480	−2.22
	SAMC	c134921	−2.46
	ABCtp	c61051	−2.38
	SLAC	c128172	−2.9

Note: Genes with a fold change ≥2-fold in transcript abundance were considered as up-regulated/down-regulated genes in sulfate deficiency sample (−S) compared to the control sample (+S). The up- or down-regulated genes were classified into different functional categories. All expression values were based on the biological averaging of pooled samples from three individual algae. APS, adenosine 5′-phosphosulfate; APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; BPNT, 3′(2′), 5′-bisphosphate nucleotidase; CBL, cystathionine beta-lyase; CGS, cystathionine gamma-lyase; GGCS, γ-glutamylcysteine synthetase; GSHS, glutathione synthetase; GST, glutathione-S-transferase; MS, methionine synthase; OAST-TL, O-acetylserine (thiol)-lyase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, S-adenosylmethionine; SAMS, SAM synthetase; SAT, serine acetyltransferase; SH, sulfohydrolase/sulfurylase; SiR, sulfite reductase; SO, sulfite oxidase; SOT, sulfotransferase; ST, sulfate transporter/permease; HS6ST2, Heparan sulfate 6-O sulfotransferase; SuOx, Sulfite oxidase; GT, Glycosyltransferase; ABCtrp, ABC transporter related protein; SAMC; Mitochondrial carrier protein/Sadenosylmethionine carrier; ABCtp, ABC transporter permease; SLAC, S-type anion channel.
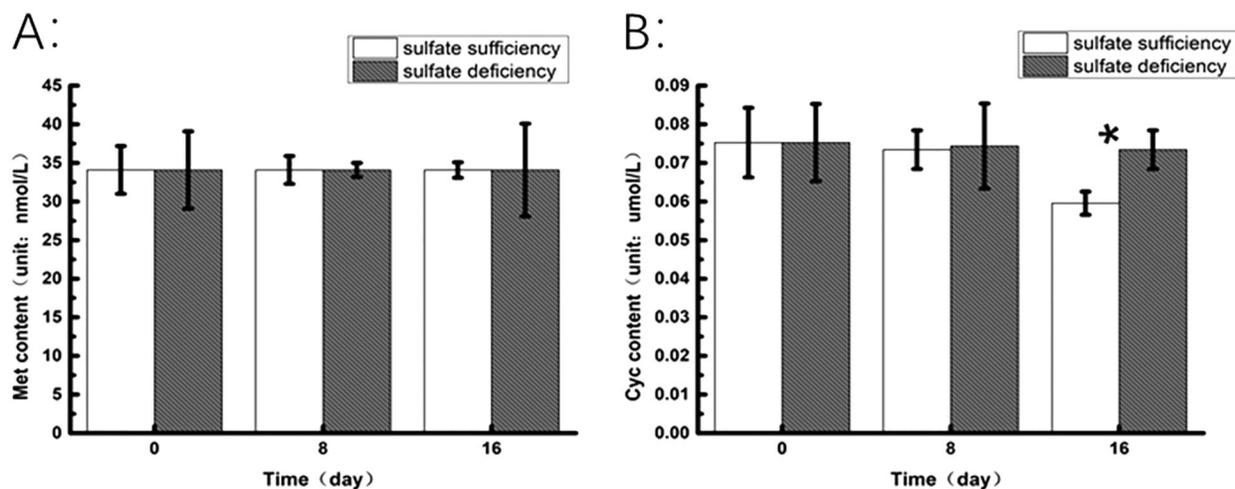
Figure S1: Contents of cysteine and methionine amino acids. (A) The content of methionine. (B) The content of cysteine