



# Physiological and Transcriptome Analysis of Exogenous L-Arginine in the Alleviation of High-Temperature Stress in *Gracilariopsis lemaneiformis*

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### Specialty section:

This article was submitted to  
Global Change and the Future Ocean,  
a section of the journal  
Frontiers in Marine Science

**Received:** 28 September 2021

**Accepted:** 01 November 2021

**Published:** 10 December 2021

### Citation:

Zhang J, Liu S, Hu C, Chen X,  
Sun X and Xu N (2021) Physiological  
and Transcriptome Analysis  
of Exogenous L-Arginine  
in the Alleviation of  
High-Temperature Stress in  
*Gracilariopsis lemaneiformis*.  
*Front. Mar. Sci.* 8:784586.  
doi: 10.3389/fmars.2021.784586

*Gracilariopsis lemaneiformis* (*G. lemaneiformis*) is an important marine red macroalgae with high economic and ecological value all over the world. To date, global warming is a key issue that has a great impact on all living organisms, such as macroalgae. L-arginine (Arg) is a precursor of nitric oxide (NO) and polyamines (PAs), which can induce stress defense responses in land plants. However, its role in inducing algae resistance at high temperature (HT) is unclear. In this study, *G. lemaneiformis* thalli were treated with different concentrations of Arg to investigate its effect and the mechanism on the tolerance of *G. lemaneiformis* against HT stress. It turned out that exogenous Arg significantly alleviated the HT-induced oxidative damage as indicated by a markedly decrease in malondialdehyde (MDA) content. Notably, Arg remarkably improved the relative growth rate (RGR) and phycobiliprotein (PBP) contents of *G. lemaneiformis* at HT. Moreover, Arg significantly elevated the activities of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), to efficiently scavenge reactive oxygen species (ROS). In addition, it also promoted the accumulation of free amino acids (AAs) as compared to those in the control treatment (CK) group under HT conditions. To investigate the mechanism of *G. lemaneiformis* to Arg, a transcriptome analysis was performed and revealed 1,414 and 3,825 differentially expressed genes (DEGs) in Arg-treated groups as compared to CK groups at 24 and 48 h of HT stress, respectively. Results showed that Arg significantly upregulated the expression of genes encoding antioxidant enzymes, heat shock proteins, and triggered transcription factors (TFs) signaling during HT stress. Moreover, Arg enhanced the DEGs involved in arginine and proline (Pro) metabolism, AAs biosynthesis, glycolysis, tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. These results may help in understanding the role of Arg in *G. lemaneiformis* resistance to HT and provide a practical viewpoint for obtaining heat-tolerant *G. lemaneiformis* to further promote the development of the cultivated seaweed industry in the future.

**Keywords:** *Gracilariopsis lemaneiformis*, L-arginine, high-temperature stress, heat resistance, transcriptome

## INTRODUCTION

*Gracilariopsis lemaneiformis* (*G. lemaneiformis*; Rhodophyta) is one of the most cultivated seaweeds around the world, especially in China and Indonesia (Kim et al., 2017), ranking second in Chinese cultivated macroalgae production with an annual yield exceeding 300,000 tons based on data in 2019 (China Fishery Statistics Yearbook, 2020). *Gracilariopsis lemaneiformis* is a valuable resource with high protein and low lipid and rich in polysaccharides, dietary fiber, minerals, essential amino acids (AAs), and trace elements (Han et al., 2012). It is not only widely used in the agar industry, but also be applied to mitigate red tide and eutrophication for marine ecological restoration (Zhou et al., 2006; Sun et al., 2018).

*Gracilariopsis lemaneiformis* 981, the thermo-tolerant cultivar, is still unable to survive in summer in the South China Sea area. When it is exposed to a temperature higher than 26°C, its normal metabolism will be disrupted and lead to algae decay and disease (Fu et al., 2014; Lv et al., 2019). Especially with the rising global and local temperatures, the *G. lemaneiformis* cultivation in the southern coast of China has been seriously affected.

High temperature (HT) exerts an adverse influence on the membrane stability of algae, ultimately reducing the algal growth rate and yield (Wang et al., 2017). The most adverse effect of HT stress is the overaccumulation of reactive oxygen species (ROS), which resulted in nucleic acids, protein, and membrane lipid peroxidation (Xu et al., 2006). However, plants can protect themselves from oxidative damage *via* regulating antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Anderson, 2002). Additionally, the enzymes, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), thioredoxin (TRX), and peroxiredoxins (PRXR), are also important in H<sub>2</sub>O<sub>2</sub> scavenging (Pérez-Pérez et al., 2009; Smirnov, 2010). Previous studies have demonstrated that exogenous salicylic acid and methyl jasmonate can improve heat tolerance by enhancing those genes encoding antioxidant enzymes in marine algae (Wang et al., 2017). Besides the phytohormones, AAs have also various prominent functions in plant stress response. Arginine, as one of the most functional diverse AAs in living cells, has been reported to modify plant stress tolerance through the manipulation of antioxidant mechanisms (Karpets et al., 2018). However, the roles of arginine in alleviating HT stress-induced ROS damage in *G. lemaneiformis* are not yet known.

Arginine is a precursor for the biosynthesis of nitric oxide (NO), polyamines (PAs), which are related to stress resistance (Liu et al., 2006). Exogenous or pre-treated with arginine has been reported its positive effect *via* modification of those arginine-derived stress-related substances under abiotic stress, such as heat, salinity, water deficit, and heavy metal, respectively, in rice, wheat, sugarcane, and *Hyoscyamus niger* (Kakkar et al., 2000; Nasibi et al., 2013; Karpets et al., 2018; Silveira et al., 2021). The application of arginine can also play a vital role in enhancing the defense resistance system against diseases in tomato fruits *via* its effects on NO biosynthesis and defensive enzyme activity (Zheng et al., 2011). In terms of temperature stress, exogenously applied arginine could alleviate the adverse effects of fluctuating

temperature stress on maize growth and development (Matysiak et al., 2020) and enhance the seedling resistance to the damaging heating in collaboration with the NO-signaling system (Karpets et al., 2018). However, it remains unclear whether the unique roles of arginine in *G. lemaneiformis* are attributable to stress-related substances under HT conditions.

The present study investigated the possible involvement of exogenous arginine in *G. lemaneiformis* resistance to HT stress by combining physiological assays with transcriptome analysis. The aim of our study was to investigate the effect of arginine on algae growth, antioxidant enzymes, and AAs changes in *G. lemaneiformis* under HT stress. Further RNA-seq indicated the underlying role of arginine in regulating various processes, such as antioxidant defense, AAs biosynthesis, and energy metabolism, which allowed the algae to adapt to alleviating HT stress in *G. lemaneiformis*.

## MATERIALS AND METHODS

### Algal Culture and Arginine Treatments

*Gracilariopsis lemaneiformis* 981 was originally collected in March 2020 with great growth, more lateral whiskers, purple-red color, no disease, and less miscellaneous algae from aquaculture base of Xiapu (26°65'N, 119°66'E), Fujian Province, China. Then algae were sampled into a bubble chamber containing ice mass and delivered to the laboratory quickly. The tender thalli (approximately 8 cm) with good growth status were washed repeatedly in filtered seawater to remove sediment and epiphytes. Materials were cultivated in an illumination incubator (GXZ-280B, Dongnan Instrument Factory, Ningbo, China) at 23°C with a photoperiod of 12 h light and 12 h darkness, and 40 μmol photons m<sup>-2</sup> s<sup>-1</sup> light intensity as pre-treatment, sterile seawater with a salinity of 25 was conducted as the medium and was renewed every 3 days during this period. After pretreatment for 2 weeks, the thalli were transferred into sterile seawater enriched with Provasoli medium (Provasoli, 1968).

The concentration of L-arginine (Arg; 0.5 mM) was chosen based on a preliminary test with a range of concentrations (0, 0.1, 0.5, and 1 mM) for the most effective and economic concentration on phenotypic changes. Then algae thalli were subjected to 30°C conditions (day/night = 12:12) with or without 0.5 mM Arg treatment for variable time periods. Each concentration was performed in biological triplicate. After treatment, *G. lemaneiformis* samples were frozen with liquid nitrogen and stored at -80°C for subsequent experiments.

### Relative Growth Rate Determination

The effects of Arg on the growth of *G. lemaneiformis* at 30°C were based on the change of fresh weight (FW) after 7 days. The calculation formula for Relative Growth Rate (RGR) was conducted as follows:

$$\text{RGR} (\% \text{ d}^{-1}) = \ln (W_t/W_0)/t \times 100$$
 where  $W_t$  represents the final weight after  $t$  days,  $W_0$  is the weight at the initial, and  $t$  refers to the treatment time (d).

## Malondialdehyde, Reactive Oxygen Species, and Antioxidant Enzyme Activity Measurements

For the determination of Malondialdehyde (MDA), tissues (0.1 g) were homogenized with 1 ml 10% tricarboxylic acid (TCA) solution for each sample, the supernatant was measured using the MDA test kit (Jianchengbio, Nanjing, China) after centrifuged at 8,000 g for 10 min at 4°C.

Measurement of H<sub>2</sub>O<sub>2</sub> content and SOD, POD, and CAT enzyme activity determination can be referred to the method described by Sun et al. (2016) and Zhang et al. (2019). For the antioxidant enzyme activity, briefly, approximately 0.1 g fresh seaweeds were powdered with liquid nitrogen, then extracted separately with 1 ml extracting solution from the detection kit. After centrifugation at 8,000 g for 10 min, the absorbance at 560, 470, and 240 nm was recorded and used to calculate, respectively, according to the instructions of the manufacturer (Cominbio, Suzhou, China).

## Phycobiliprotein Content Calculation

Phycocyanin (PC) and phycoerythrin (PE) were measured according to the method reported with slight modification (Wang et al., 2015). Approximately 0.1 g powder was weighed and extracted with 10 ml phosphate buffer (10 mM, pH 5.5) at 4°C. After centrifugation at 15,000 g for 30 min, the supernatant was used and determined by UV-Vis spectrophotometer (UV-6100, METASH, Shanghai, China).

The contents of PE and PC were calculated using the following formulas:

$$PE(\text{mg/g}) = (155.8A_{498} - 40.0A_{614} - 10.5A_{651}) \times 1000 \times V/g;$$

$$PC(\text{mg/g}) = (151.1A_{614} - 99.1A_{651}) \times 1000 \times V/g;$$

where V represents the volume of extract solution (ml), and g refers to the weight of algae powder (g).

## Proline Measurement

Proline (Pro) content was determined with the reagents in assay kits (Cominbio, Suzhou, China). Approximate 0.1 g fresh seaweeds were powdered in liquid nitrogen and extracted with 1 ml extracting solution from the detection kit. After centrifugation at 8,000 g for 10 min, the absorbance of the supernatant was measured at 520 nm and used to calculate Pro content according to the instructions of the manufacturer.

## Free Amino Acid Content Measurement

An autoanalyzer based on the ninhydrin method was used to quantify the 17 free AA contents according to the Chinese National Standard (GB 5009.124-2016).

Approximately 0.2 g of fresh *G. lemaneiformis* was weighed into the beaker with 25 ml 0.02 mol/L HCl, the solution was passed through the C18 column after ultrasonic extraction, then the mixture was filtrated through a 0.22 μm ultrafiltration

membrane (Millipore, Temecula, CA, United States), and analyzed using AA automatic analyzer (LA8080, Hitachi, Japan). The content of free AA was calculated according to the following equations:  $X = A \times D \times V_1/V_2/10,000/F_w$ . In this study, X, A, D, V<sub>1</sub>, V<sub>2</sub>, and F<sub>w</sub> refer to AA content (g/100 g), quality of AAs in the sample solution (ng), dilution ratio, the volume of extract solution (ml), sample liquid volume (μl), and FW of samples, respectively.

## RNA Isolation and cDNA Library Construction

Total RNA was extracted from *G. lemaneiformis* thalli after 30°C treatment for 24 and 48 h using the RNeasy Plant Mini Kit (Qiagen, Shanghai, China) according to the instructions of the manufacturer. Briefly, the quality and quantification of RNA were separately determined by agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). More accurate RNA quantification was performed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States) for 28S/18S and RNA integrity number (RIN).

A total of 12 samples were performed by mRNA library preparation kit (MGIEasy, MGI, Shenzhen, China) for the establishment of the cDNA library. The mRNAs were enriched with oligo magnetic adsorption, fragmented, then reversed into double-strand cDNAs, which were linked with sequencing adaptors after purification. The single-stranded circular DNA libraries were conducted by polymerase chain reaction (PCR) amplification and then sequenced according to 50 bp single-end sequencing method by using BGISEQ-500 platform performed by Wuhan Genomic Institution (BGI, Shenzhen, China).

## Transcriptome Sequencing and Differentially Expressed Gene Analysis

RNA-seq, reads filtering, and unigene annotations were performed by using SOAPnuke software (BGI, Shenzhen, China). Adaptor sequences and reads with unknown bases > 10% and low-quality reads (reads with a mass value below 15 which accounted for more than 50% of the total base number) (Cock et al., 2010) were removed to obtain clean reads. Clean reads were then mapped to a mixed reference transcriptome assembled by using HISAT (Kim et al., 2015) and Bowtie2 (Langmead and Salzberg, 2012), respectively. The expression levels of the genes and transcripts were represented by fragments per kilobase per million fragments (FPKM) mapped values and were calculated by RSEM software (Li and Dewey, 2011). The DEGs were identified using the DESeq analysis (Wang et al., 2010). The criteria of adjusted  $p$ -value  $\leq 0.001$  and  $\log_2$  (fold change)  $\geq 1$  were set as the threshold for DEGs. Q value was obtained from  $p$ -value and was adjusted using KOBAS 2.0 according to the Beyer-Hardwick (BH) method (Mao et al., 2005). The significance enrichment levels of gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were set at Q value < 0.05.

## Verification of RNA-seq Data by Quantitative Real-Time-Polymerase Chain Reaction

Ten genes were chosen randomly from the transcriptomic results for validation by qRT-PCR. The total RNA of each sample was prepared as described in Section “RNA Isolation and cDNA Library Construction.” First-strand cDNA was synthesized from 1  $\mu$ g of total RNA by reverse transcription using the HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China) according to the protocol of the manufacturer. qPCR was performed on an ABI7500 qRT-PCR System (Applied Biosystems, Foster City, CA, United States) and each reaction contained 10  $\mu$ l ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.1  $\mu$ g cDNA, and 7.5 pmol of each gene-specific primer in a final volume of 20  $\mu$ l. Three independent biological replicates were used for analyses and the relative expression level of genes was determined by the  $2^{-\Delta\Delta CT}$  method using 18S rRNA as the respective reference gene. Primers for qPCR are listed in **Supplementary Table 1**.

## Statistical Analysis

The data are expressed as the mean and SD. Statistical difference of data was evaluated with one-way ANOVA and Duncan's multiple range tests at the  $p < 0.05$  level of significance using SPSS (version 19.0, SPSS Institute, Chicago, IL, United States).

## RESULTS

### Growth and Morphological Changes of *Gracilariopsis lemaneiformis* Under High Temperature Stress

To gain insights into the possible function of Arg on *G. lemaneiformis* under HT stress, we analyzed the effects of different Arg concentrations (0, 0.1, 0.5, and 1 mM) on morphological changes of algal thalli under HT stress. Under prolonged stress, it showed that the algae thalli in the control treatment (CK; 0 mM Arg) group exhibited an obvious accelerated yellowing phenotype as compared with those of 0.5 and 1 mM Arg treatment, however, no obvious morphological change in 0.1 mM Arg treatment as compared with the CK group (**Supplementary Figure 1A**) was observed. Then, we measured the content of PBP present in the red algae, such as PE and PC. Results showed that *G. lemaneiformis* with 0.5 and 1 mM Arg treatment contained higher content of PBP as compared with the CK group at 7 d under HT stress treatment, while 0.1 mM Arg treatment was comparable and remained unchanged to the CK group. Both 0.5 and 1 mM Arg treatments were efficient in alleviating HT stress-triggered decrease of algal PBP, but there was no significant difference between the two concentrations (**Supplementary Figures 1B,C**). Thus, 0.5 mM arginine was chosen in the following experiments for economic consideration.

Exogenous application of 0.5 mM Arg was significantly decreased MDA content by 56.01% as compared with the CK group after 7 days of HT treatment (**Figure 1A**). Importantly, the RGR furtherly indicated that 0.5 mM Arg promoted the growth of

*G. lemaneiformis* as compared with those without Arg treatment under HT stress (**Figure 1B**) since the RGR of *G. lemaneiformis* under HT stress (30°C) was significantly decreased by 20.45% compared with those cultivated under optimal temperature (23°C; **Supplementary Figure 2**). Collectively, these results indicated that the application of Arg could remarkably alleviate the membrane lipid peroxidation and the growth decline of *G. lemaneiformis* under HT stress.

### Enzymatic Antioxidant Systems Under High Temperature Stress

To explore the involvement of Arg in ROS scavenging against HT stress, we firstly measured the content of H<sub>2</sub>O<sub>2</sub> in thalli under HT with or without Arg treatments after 1, 3, and 7 days of incubation. H<sub>2</sub>O<sub>2</sub> content was continued to accumulate in algal thalli without Arg treatment, showing a significant increase at day 7, whereas the H<sub>2</sub>O<sub>2</sub> content in the Arg group was declining in 7 days (**Figure 2A**).

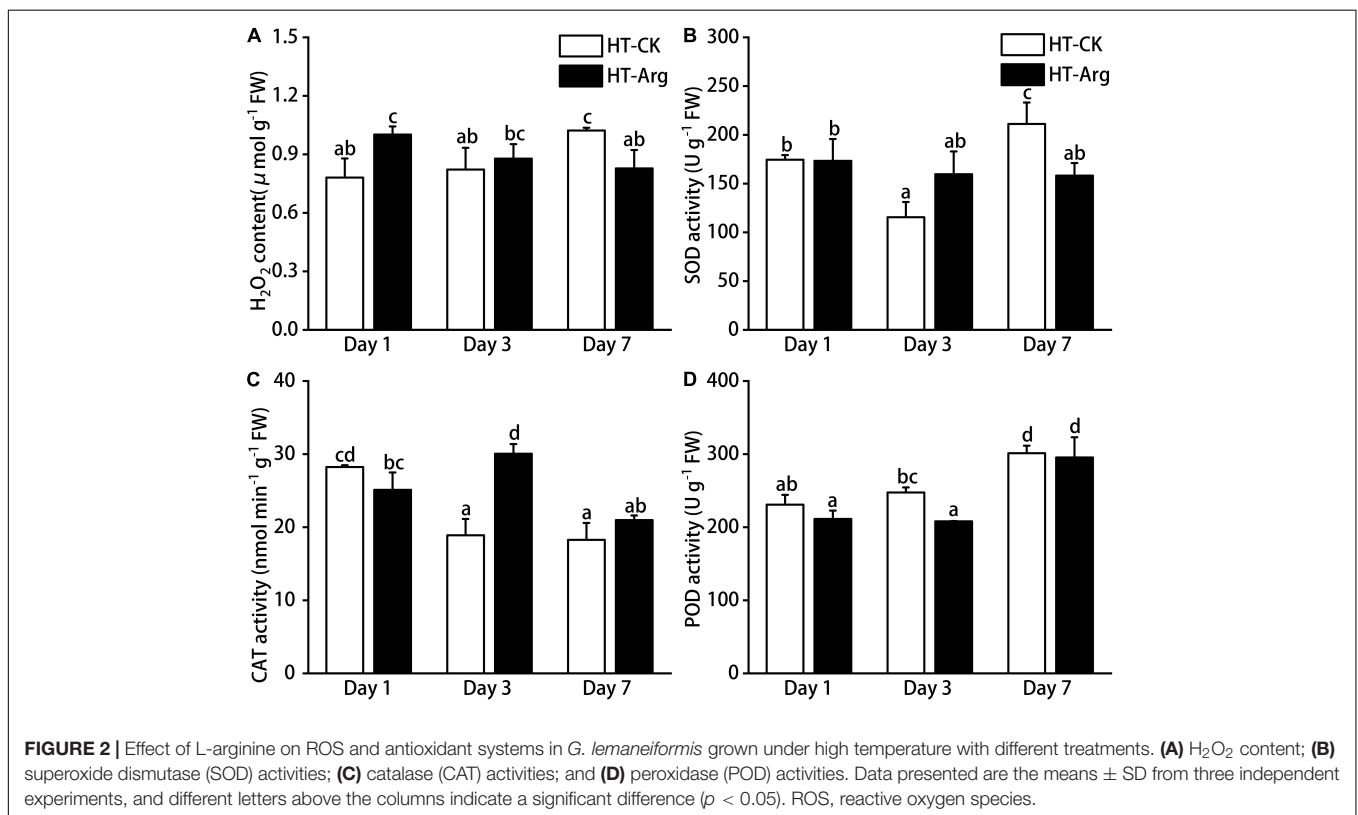
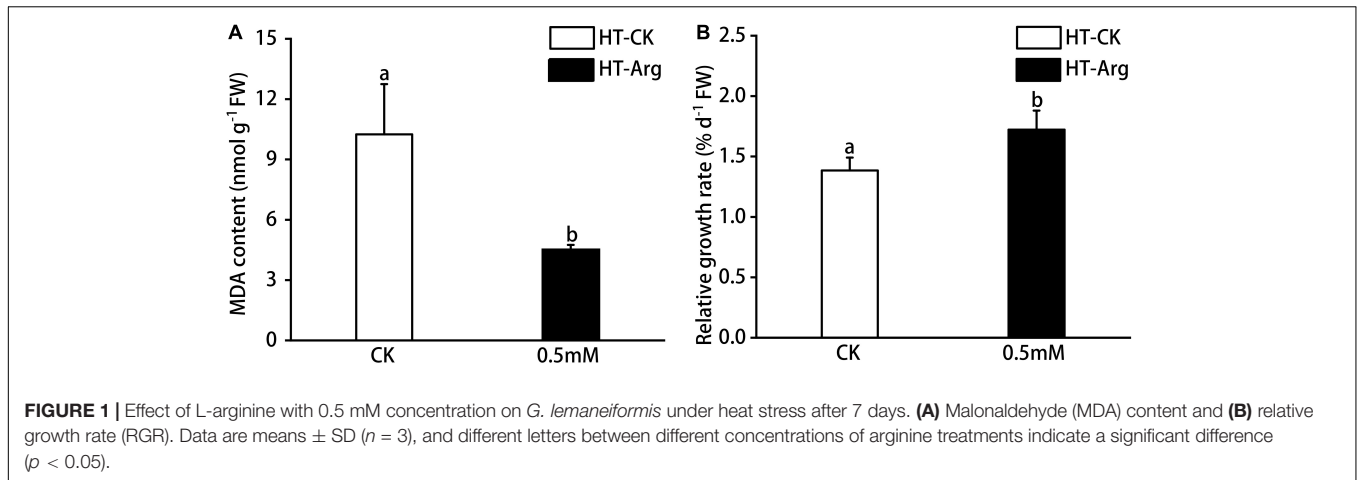
We then measured the antioxidant enzyme (CAT, POD, and SOD) activities. CAT activity was remarkably activated in the Arg-treated group, reached a maximum at 3 days, with an obviously 59.11% increase in contrast to control (**Figure 2C**). Superoxide dismutase activity was slightly activated in the Arg-treated group as compared with the CK group at 3 days under HT stress (**Figure 2B**). But Arg treatment had no significant promotion in POD activity relative to CK under HT stress (**Figure 2D**). These results indicated that Arg could upregulate the activities of antioxidant enzymes, especially activities of CAT, thus effectively protecting the algal cells from heat damage.

### Sequence Assembly and Gene Identification

To reveal the mechanism of the effect of Arg in enhancing *G. lemaneiformis* tolerance to HT stress, the algal thalli from both CK and Arg groups after HT treatment at 24 and 48 h were subjected to RNA-seq analysis. RNA samples were tested using the BGISEQ platform, generated 21.58 M clean reads average in each sample, and obtained a total of 23,479 unigenes, the detailed information of each sample was listed in **Supplementary Table 2**. The total mapping genome and gene ratio were average at 94.30 and 96.42%, respectively. Then all the unigenes were blasted against public databases (such as TFs, PRG, GO, KEGG, and NR) for identification of gene function, of which approximately 73.34% of unigenes annotated at least in one database except TFs and PRG (**Supplementary Table 3**).

### Principal Component Analysis and Gene Expression Comparison

Principal Component Analysis (PCA) was performed to describe transcriptome differences between Arg and CK groups under HT stress at 24 and 48 h. It suggested that Arg changed the transcriptome of *G. lemaneiformis* under HT stress, especially at 48 h (**Figures 3A,B**). The volcano plots of DEGs between Arg-treated and CK groups revealed a total of 1,414 unigenes (629 upregulated and 785 downregulated) at 24 h, and a total of 3,825 unigenes (2,219 upregulated and 1,606 downregulated)

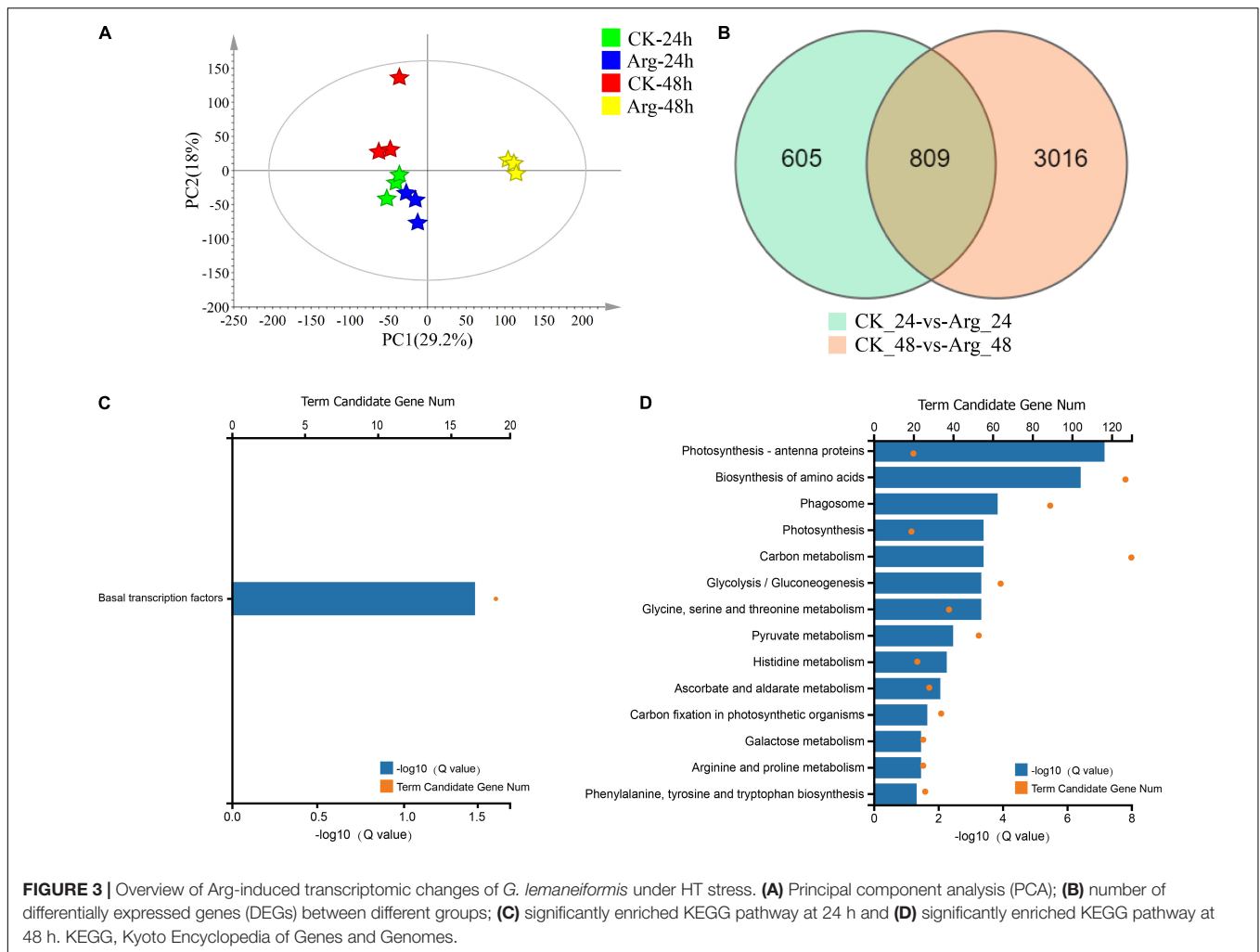


at 48 h (**Supplementary Figure 3**). To validate the reliability of the RNA-Seq results, 10 unigenes were randomly selected for RT-PCR analysis. The expression patterns of the 10 unigenes were generally in agreement with the RNA-Seq results (**Supplementary Figure 4**).

## Gene Oncology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analyses

To analyze the functions of the DEGs, GO and KEGG enrichment analyses were performed. GO analysis showed that

DEGs at 24 h were enriched in membrane part, membrane, POD activity, oxidoreductase activity, an integral component of membrane, an intrinsic component of membrane, and antioxidant activity, etc. However, DEGs at 48 h were associated with the structural constituent of the cytoskeleton, generation of precursor metabolites and energy, thylakoid, and photosynthesis (**Supplementary Figure 5**). Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed that there was only one remarkably enriched pathway at 24 h, the basal transcription factors (TFs; **Figure 3C**). While the top three significantly enriched KEGG pathways at 48 h were related to metabolisms and were mostly involved in AA metabolism, energy metabolism,



global and overview maps, and carbohydrate metabolism (glycolysis, pyruvate metabolism, ascorbate, aldarate metabolism, and alactose metabolism; **Figure 3D**).

## Functional Analysis of Differentially Expressed Genes

### Differentially Expressed Genes Encoding Antioxidant Enzymes

Transcriptome analysis revealed that the expression of unigenes encoding ROS scavenging enzymes was significantly changed by Arg treatment under heat stress. As shown in **Figure 4**, most genes had no significant difference at 24 h, but they were generally upregulated at 48 h. Genes encoding SOD (four up and one downregulated), APX (two up and one downregulated), MDAR (one upregulated), PRXR (four up and one downregulated), and TRX (seven up and one downregulated) were markedly upregulated at 48 h. However, genes (21 upregulated and 20 downregulated) encoding POD exhibited different expression patterns in *G. lemaneiformis* with Arg treatment under HT stress (**Figure 4**). These data indicated that most of the DEGs encoding ROS scavenging enzymes in *G. lemaneiformis*

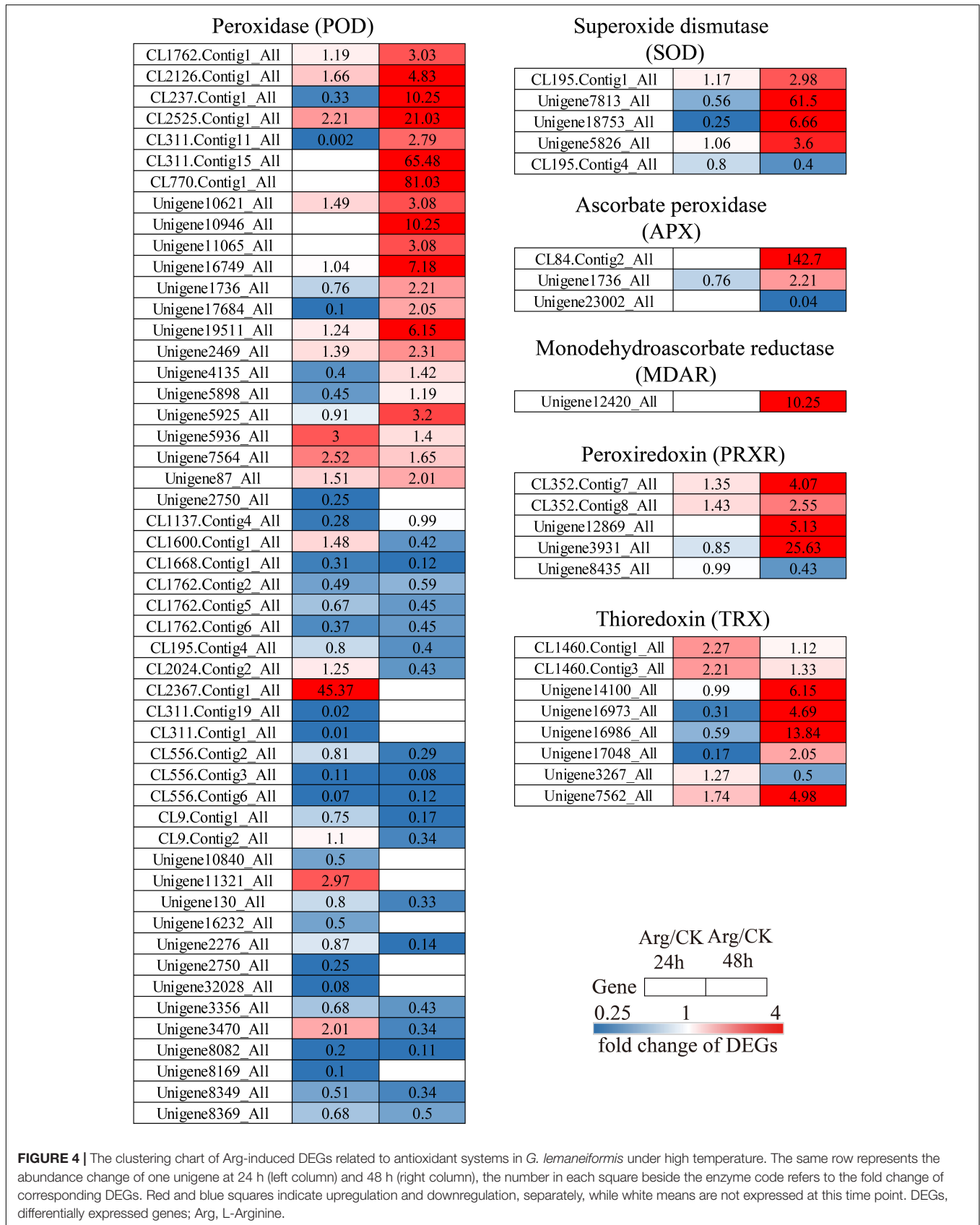
could be significantly upregulated by Arg under HT stress at 48 h treatment.

### Differentially Expressed Genes Encoding Heat Shock Proteins

In the present study, we found that genes involved in Heat Shock Proteins (HSPs) were markedly activated in *G. lemaneiformis* with Arg treatment under HT stress. Based on the results listed in **Table 1**, a total of four HSP families, containing 41 DEGs were identified. Out of these HSPs, the genes (24 genes, 14 upregulated) related to the HSP70 family were found to be the most abundant, followed by the HSP20 family (11 genes, 9 upregulated), HSP40 family (3 genes, 1 upregulated), and HSP90 family (3 genes, 2 upregulated) at 48 h. In summary, 26 up and 11 downregulated genes related to HSPs were dramatically activated in the Arg-treated groups, particularly at 48 h, while many of those genes were not significantly expressed at 24 h.

### Differentially Expressed Genes Encoding Transcription Factors

As shown in **Table 2**, many genes were annotated into TFs and sorted into 12 families, such as C2H2, TIG, HSF, and MYB.



**TABLE 1** | The DEGs encoding HSPs.

Gene ID	Family	Description	Accession	FC (Arg/CK) at 24 h	FC (Arg/CK) at 48 h
<b>HSP90 family</b>					
CL1151.Contig4_All	heat shock protein 90kDa beta	Endoplasmic-like [ <i>Gracilariopsis chorda</i> ]	PXF40343.1	2.96	2.62
CL2039.Contig1_All	heat shock protein 90kDa beta	hypothetical protein BWQ96_01140 [ <i>Gracilariopsis chorda</i> ]	PXF49002.1	0.35	0.41
Unigene7007_All	heat shock protein 90kDa beta	hypothetical protein GUITHDRAFT_160160 [ <i>Guillardia theta</i> CCMP2712]	XP_005823214.1	0.72	5.64
<b>HSP70 family</b>					
CL1722.Contig5_All	heat shock 70kDa protein 1/2/6/8	Transmembrane and coiled-coil domain-containing protein 4 [ <i>Gracilariopsis chorda</i> ]	PXF39670.1	2.19	0.58
CL833.Contig3_All	heat shock 70kDa protein 1/2/6/8	heat shock 70 kDa protein 5 [ <i>Hevea brasiliensis</i> ]	XP_021691174.1	0.09	-
CL87.Contig1_All	heat shock 70kDa protein 1/2/6/8	heat shock 70 kDa protein 2 isoform X1 [ <i>Quercus suber</i> ]	XP_023911439.1	0.2	0.03
Unigene31833_All	heat shock 70kDa protein 1/2/6/8	heat shock 70 kDa protein 2 isoform X2 [ <i>Quercus suber</i> ]	XP_023911440.1	-	98.4
Unigene322_All	heat shock 70kDa protein 1/2/6/8	heat shock 70 kDa protein 2 isoform X3 [ <i>Quercus suber</i> ]	XP_023911441.1	0.4	0.25
Unigene10379_All	heat shock 70kDa protein 1/2/6/8	DNase I-like protein [ <i>Fragilariopsis cylindrus</i> CCMP1102]	OEU10210.1	0.5	-
Unigene16737_All	heat shock 70kDa protein 1/2/6/8	heat shock cognate 70 kDa protein 2-like [ <i>Spinacia oleracea</i> ]	XP_021866290.1	0.99	3.08
Unigene32093_All	heat shock 70kDa protein 1/2/6/8	heat shock cognate 70 kDa protein 2-like isoform X2 [ <i>Cynara cardunculus</i> var. <i>scolymus</i> ]	XP_024966991.1	0.17	2.08
Unigene29252_All	heat shock 70kDa protein 1/2/6/8	DnaK chaperone protein (chloroplast) [ <i>Gracilariopsis lemaneiformis</i> ]	YP_009237795.1	0.5	0.51
Unigene3300_All	heat shock 70kDa protein 1/2/6/8	DnaK chaperone protein (chloroplast) [ <i>Gracilariopsis lemaneiformis</i> ]	YP_009237795.1	0.79	0.44
Unigene32092_All	heat shock 70kDa protein 1/2/6/8	hypothetical protein EUGRSUZ_F03980 [ <i>Eucalyptus grandis</i> ]	KCW70839.1	-	24.6
Unigene9253_All	heat shock 70kDa protein 1/2/6/8	HSP70 [ <i>Ascophyllum nodosum</i> ]	CAA62443.1	-	44.56
CL617.Contig2_All	heat shock 70kDa protein 4	heat shock 70 kDa protein 14 [ <i>Selaginella moellendorffii</i> ]	XP_002986462.2	-	14.35
CL617.Contig3_All	heat shock 70kDa protein 4	heat shock 70 kDa protein 15-like [ <i>Asparagus officinalis</i> ]	XP_020249769.1	0.3	36.9
Unigene467_All	heat shock 70kDa protein 4	Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily [ <i>Handroanthus impetiginosus</i> ]	PIN04454.1	0.57	0.06
Unigene32194_All	heat shock 70kDa protein 5	Molecular chaperones GRP78/BiP/KAR2, HSP70 superfamily [ <i>Ectocarpus siliculosus</i> ]	CBJ48460.1	-	22.55
Unigene9275_All	heat shock 70kDa protein 5	hypothetical protein CBR_g34293 [ <i>Chara braunii</i> ]	GBG62922.1	-	6.15
CL682.Contig3_All	heat shock 70kDa protein 5	Ubiquitin carboxyl-terminal hydrolase 42 [ <i>Gracilariopsis chorda</i> ]	PXF48005.1	0.002	0.06
CL682.Contig4_All	heat shock 70kDa protein 5	Ubiquitin carboxyl-terminal hydrolase 42 [ <i>Gracilariopsis chorda</i> ]	PXF48005.1	1.3	2.09
Unigene31602_All	heat shock 70kDa protein 5	luminal-binding protein 5-like isoform X1 [ <i>Daucus carota</i> subsp. <i>sativus</i> ]	XP_017238302.1	-	139.41
CL2449.Contig1_All	heat shock 70kDa protein 5	Luminal-binding protein 5 [ <i>Gracilariopsis chorda</i> ]	PXF45897.1	2	2.57
Unigene446_All	heat shock 70kDa protein 5	luminal-binding protein 5-like [ <i>Coffea arabica</i> ]	XP_027081731.1	0.57	11.71
Unigene8535_All	heat shock 70kDa protein 5	luminal-binding protein 5-like [ <i>Gossypium hirsutum</i> ]	XP_016686399.1	0.21	0.03
Unigene9249_All	heat shock 70kDa protein 5	Luminal-binding protein 5 [ <i>Gracilariopsis chorda</i> ]	PXF45897.1	1.96	3.12

(Continued)



TABLE 1 | (Continued)

Gene ID	Family	Description	Accession	FC (Arg/CK) at 24 h	FC (Arg/CK) at 48 h
<b>HSP40 family</b>					
CL208.Contig13_All	DnaJ homolog subfamily A member 2	Chaperone protein dnaJ 2 [ <i>Gracilariopsis chorda</i> ]	PXF46816.1	0.87	0.001
CL208.Contig16_All	DnaJ homolog subfamily A member 2	Chaperone protein dnaJ 2 [ <i>Gracilariopsis chorda</i> ]	PXF46816.1	-	2.41
CL208.Contig17_All	DnaJ homolog subfamily A member 2	Chaperone protein dnaJ 2 [ <i>Gracilariopsis chorda</i> ]	PXF46816.1	2.41	0.29
<b>HSP20 family</b>					
CL1223.Contig1_All	HSP20 family protein	heat shock protein [ <i>Gracilariopsis lemaneiformis</i> ]	AVT44469.1	2.6	9.12
CL2381.Contig3_All	HSP20 family protein	Heat shock protein-like [ <i>Gracilariopsis chorda</i> ]	PXF48860.1	3.83	24.51
CL816.Contig1_All	HSP20 family protein	heat shock protein [ <i>Klebsormidium nitens</i> ]	GAQ81917.1	-	971.64
CL816.Contig2_All	HSP20 family protein	heat shock protein [ <i>Klebsormidium nitens</i> ]	GAQ81917.1	-	51.25
CL2381.Contig1_All	HSP20 family protein	Small heat shock protein C4 [ <i>Gracilariopsis chorda</i> ]	PXF48858.1	32.14	24.17
CL2381.Contig2_All	HSP20 family protein	Small heat shock protein C4 [ <i>Gracilariopsis chorda</i> ]	PXF48858.1	1.03	41.83
Unigene31718_All	HSP20 family protein	small heat shock protein;heat shock protein 20 [ <i>Ectocarpus siliculosus</i> ]	CBJ31752.1	-	37
Unigene7732_All	HSP20 family protein	Small heat shock protein HSP [ <i>Parasponia andersonii</i> ]	PON44062.1	0.64	0.44
CL954.Contig1_All	HSP20 family protein	18.1 kDa class I heat shock protein-like [ <i>Coffea eugenioides</i> ]	XP_027161977.1	0.3	0.14
Unigene31601_All	HSP20 family protein	22.0 kDa heat shock protein-like [ <i>Camelina sativa</i> ]	XP_010478759.1	-	670.38
Unigene9512_All	HSP20 family protein	18 class III heat shock protein [ <i>Zea mays</i> ]	PWZ20736.1	-	148.63

DEGs, differentially expressed genes.

Of these genes, a total of 29 transcripts showed a significantly difference in transcript abundance compared with control, i.e., 13 and 21 upregulated genes in the Arg-treated groups at 24 and 48 h, respectively. Notably, the expression of DEGs involved in HSF was only upregulated at 24 h, and genes related to C2C2-GATA, Alfin-like, C3H, MADS, bZIP, and G2-like were significantly overexpressed at 48 h. Moreover, the five unigenes involved in TIG, FHA, and MYB were markedly upregulated at both 24 and 48 h.

### Differentially Expressed Genes Involved in Arginine Metabolism

The DEGs in arginine metabolism to Pro, PA, and  $\gamma$ -aminobutyric acid (GABA) synthesis and metabolism were significantly induced by Arg. For Pro synthesis, Pro can be converted by the glutamate metabolism via pyrroline-5-carboxylate (P5C) synthetase (P5CS, EC:2.7.2.11 1.2.1.41) and P5C reductase (P5CR, EC:1.5.1.2). We identified one DEG (Unigene4599\_All) encoding P5CS and one DEG (Unigene3468\_All) encoding P5CR, respectively. Arginine application did not change the expression level of P5CS, whereas upregulated the expression level of P5CR at 48 h, leading to a 1.74-fold change (Supplementary Table 4). In Pro metabolism, one unigene (Unigene12856\_All) encoding 1-pyrroline-5-carboxylate dehydrogenase (P5CDH, EC:1.2.1.88)

was upregulated in the Arg-treated groups at 48 h (Figure 5 and Supplementary Table 4).

For PA synthesis, ornithine can be converted into polyamine putrescine (PUT), then be further processed to spermidine (SPD) and spermine (SPM). In this process, it involves ornithine aminotransferase (OAT, EC:2.6.1.13), ornithine decarboxylase (ODC, EC:4.1.1.17), SPD synthase (SPDS), and SPM synthase (SPMS) enzymes. In addition, SPM can be oxidated to generate SPD by spermine oxidase (SPMO, EC:1.5.3.16). In the present study, the genes encoding OAT (one upregulated gene) and SPDS (one upregulated) exhibited different expression patterns in Arg-treated *G. lemaneiformis* at 48 h, while most genes were downregulated at 24 h (Figure 5 and Supplementary Table 4). In addition, the genes encoding SPMO (three upregulated) were markedly activated both at 24 and 48 h. Moreover, PA can also be obtained via methionine (Met) metabolism involving S-adenosylmethionine synthetase (SAMS, EC:2.5.1.6) and S-adenosylmethionine decarboxylase (SAMDC, EC:4.1.1.50). Most genes encoding SAMS (three up and one downregulated) and SAMDC (one upregulated gene) were upregulated to generate S-Adenosyl-methioninamine (dcSAM) at 48 h, which further regulated the activity of SPDS and SPMS to produce PA (Figure 5 and Supplementary Table 4).

For GABA synthesis, PUT can be converted into 4-amino-butanal, then be processed into GABA. During this

**TABLE 2** | The DEGs encoding TFs.

Gene ID	Family	Description	Accession	FC (Arg/CK) at 24 h	FC (Arg/CK) at 48 h
<b>24h up TFs</b>					
CL1348.Contig2_All	C2H2	Protein odd-skipped-related 2 [ <i>Gracilariopsis chorda</i> ]	PXF46341.1	2.26	0.85
CL1630.Contig2_All	C2H2	hypothetical protein BU14_0892s0004 [ <i>Porphyra umbilicalis</i> ]	OSX70125.1	5.41	0.86
CL2367.Contig1_All	C2H2	hypothetical protein BWQ96_02331 [ <i>Gracilariopsis chorda</i> ]	PXF47945.1	45.37	-
CL279.Contig3_All	C2H2	unnamed protein product [ <i>Chondrus crispus</i> ]	XP_005714787.1	25.78	-
CL1569.Contig1_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	55.5	13420.84
CL2476.Contig4_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	2.46	2.25
CL2476.Contig8_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	15.21	694.26
CL2112.Contig2_All	HSF	Heat stress transcription factor A-1d [ <i>Gracilariopsis chorda</i> ]	PXF45840.1	2.3	0.85
Unigene1229_All	MYB	Acyl-CoA-binding domain-containing protein 4 [ <i>Gracilariopsis chorda</i> ]	PXF47510.1	2.88	-
Unigene5065_All	MYB	hypothetical protein BWQ96_05869 [ <i>Gracilariopsis chorda</i> ]	PXF44426.1	2.13	4.79
Unigene15224_All	FHA	conserved unknown protein [ <i>Ectocarpus siliculosus</i> ]	CBJ27794.1	5.95	4.61
Unigene1614_All	bHLH	hypothetical protein BWQ96_05867 [ <i>Gracilariopsis chorda</i> ]	PXF44424.1	225.22	0.19
Unigene1671_All	bHLH	hypothetical protein BWQ96_05867 [ <i>Gracilariopsis chorda</i> ]	PXF44424.1	728.53	0.18
<b>48h up TFs</b>					
CL1212.Contig1_All	C2H2	hypothetical protein BWQ96_10349 [ <i>Gracilariopsis chorda</i> ]	PXF39939.1	0.9	2.55
CL2098.Contig2_All	C2H2	hypothetical protein BWQ96_06973 [ <i>Gracilariopsis chorda</i> ]	PXF43276.1	1.05	3.15
Unigene288_All	C2H2	MDS1 and EVI1 complex locus protein EVI1-A [ <i>Gracilariopsis chorda</i> ]	PXF49627.1	0.89	3.39
Unigene3641_All	C2H2	Protein glass [ <i>Gracilariopsis chorda</i> ]	PXF44077.1	1.13	3.42
CL57.Contig4_All	C2C2-GATA	GATA zinc finger domain-containing protein 12 [ <i>Gracilariopsis chorda</i> ]	PXF47703.1	1.002	2.07
Unigene28_All	G2-like	unnamed protein product [ <i>Chondrus crispus</i> ]	XP_005709848.1	1.18	16.55
CL1569.Contig1_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	55.5	13420.84
CL1569.Contig3_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	1.11	3.19
CL2476.Contig4_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	2.46	2.25
CL2476.Contig8_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	15.21	694.26
Unigene17153_All	TIG	Involucrin [ <i>Symbiodinium microadriaticum</i> ]	OLP81887.1	0.81	4.44
CL2459.Contig1_All	bHLH	hypothetical protein BWQ96_02427 [ <i>Gracilariopsis chorda</i> ]	PXF47745.1	1.42	2.18
CL692.Contig4_All	Alfin-like	putative histone deacetylase complex subunit cti6 [ <i>Gracilariopsis chorda</i> ]	PXF44865.1	1.02	12.37
CL692.Contig5_All	Alfin-like	putative histone deacetylase complex subunit cti6 [ <i>Gracilariopsis chorda</i> ]	PXF44865.1	0.96	2.18
Unigene15224_All	FHA	conserved unknown protein [ <i>Ectocarpus siliculosus</i> ]	CBJ27794.1	5.95	4.61
Unigene17473_All	C3H	zinc finger CCCH domain-containing 11 [ <i>Chlorella sorokiniana</i> ]	PRW34000.1	0.99	12.3
Unigene18013_All	MADS	Myocyte-specific enhancer factor 2D [ <i>Gracilariopsis chorda</i> ]	PXF42545.1	0.4	10.25
Unigene18217_All	bZIP	hypothetical protein CHLRE_13g579566v5 [ <i>Chlamydomonas reinhardtii</i> ]	PNW73955.1	0.33	6.15
Unigene2500_All	MYB	Protein REVEILLE 8 [ <i>Gracilariopsis chorda</i> ]	PXF46547.1	1.34	11.85
Unigene5065_All	MYB	hypothetical protein BWQ96_05869 [ <i>Gracilariopsis chorda</i> ]	PXF44426.1	2.13	4.79
Unigene948_All	MYB	Transcriptional adapter 2 [ <i>Gracilariopsis chorda</i> ]	PXF46737.1	1.9	2.23
<b>24h + 48h up TFs</b>					
CL1569.Contig1_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	55.5	13420.84
CL2476.Contig4_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	2.46	2.25
CL2476.Contig8_All	TIG	Bile salt-activated lipase [ <i>Gracilariopsis chorda</i> ]	PXF41700.1	15.21	694.26
Unigene15224_All	FHA	conserved unknown protein [ <i>Ectocarpus siliculosus</i> ]	CBJ27794.1	5.95	4.61
Unigene5065_All	MYB	hypothetical protein BWQ96_05869 [ <i>Gracilariopsis chorda</i> ]	PXF44426.1	2.13	4.79

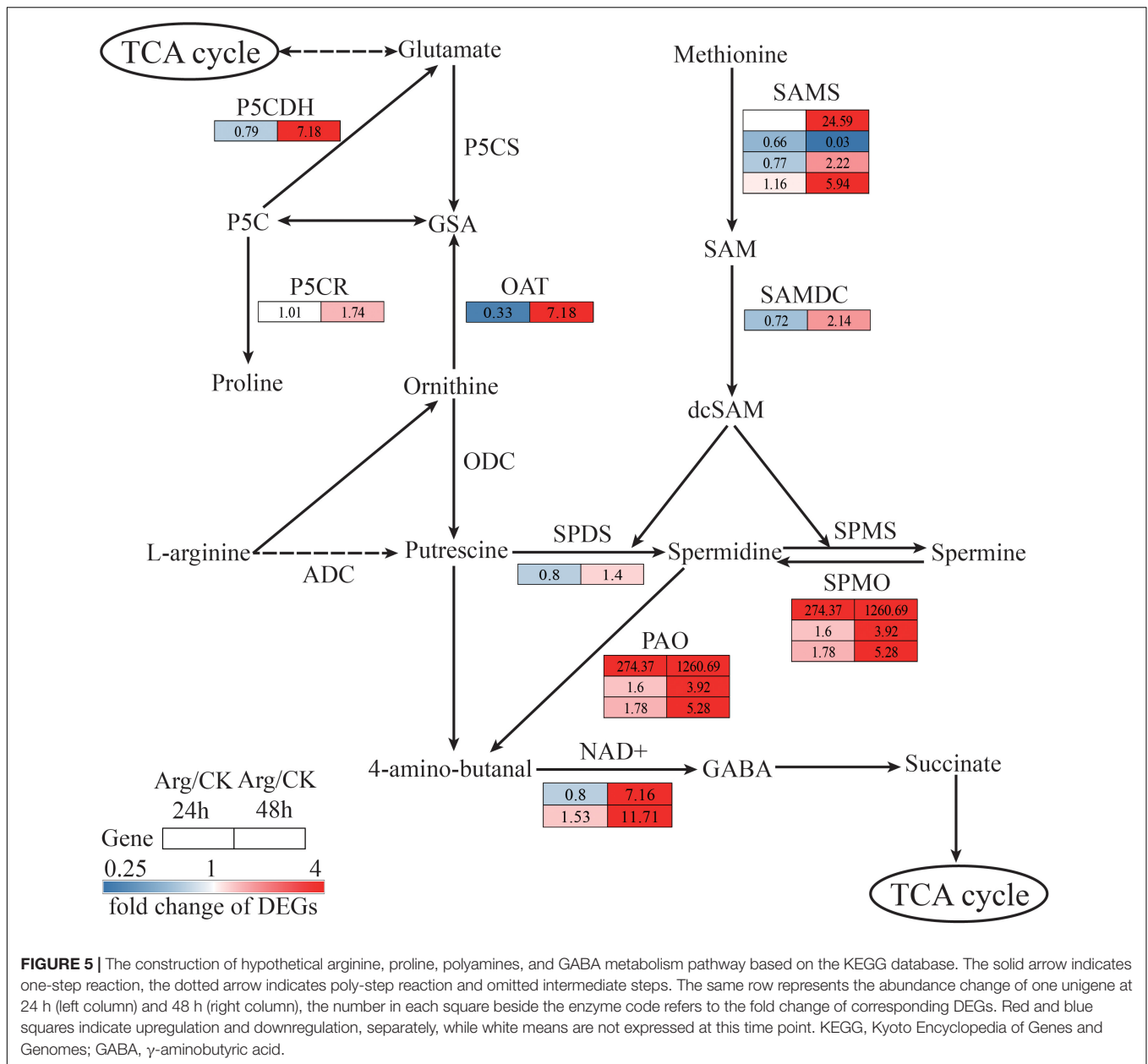
DEGs, differentially expressed genes; TFs, transcription factors.

process, it involves polyamine oxidase (PAO, EC:1.5.3.-) and aldehyde dehydrogenase (NAD<sup>+</sup>, EC:1.2.1.3). The unigenes (CL2334.Contig1\_All, CL2334.Contig4\_All and Unigene2164\_All) encoding PAO were all significantly upregulated in the Arg-treated groups both at 24 and 48 h. Besides, genes encoding NAD<sup>+</sup> were markedly upregulated in Arg-treated *G. lemaneiformis* after 48 h of HT (Figure 5 and Supplementary Table 4).

## Overview of Amino Acids Changes in Energy Metabolic Processes

Besides the change of Arg metabolic process, biosynthesis of other AA pathways was also significantly activated induced by the application of exogenous Arg.

For aspartate (Asp) biosynthesis, oxaloacetate from the TCA cycle could be converted into Asp by Asp aminotransferase (GOT2, EC: 2.6.1.1), and the unigenes (four transcripts, four

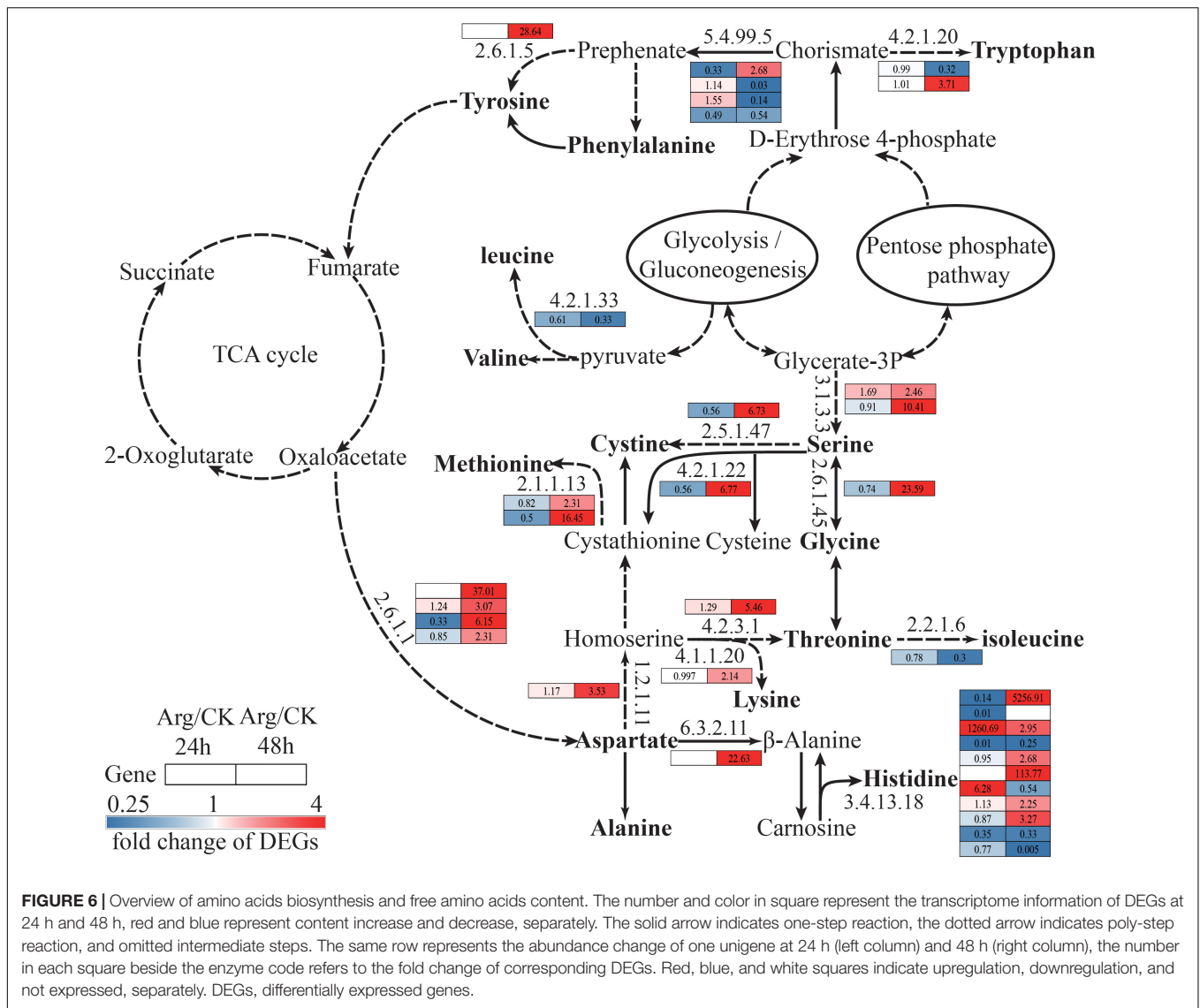


upregulated) encoding GOT2 were upregulated. Asp could be converted into Met, Thr, Lys, and histidine (His), in this process, it involves threonine synthase (THRS, EC:4.2.3.1), diaminopimelate decarboxylase (LYSA, EC:4.1.1.20), 5-methyltetrahydrofolate-homocysteine methyltransferase (METH, EC:2.1.1.13), cytosolic non-specific dipeptidase (CNDP2, EC:3.4.13.18), etc. Most genes encoding THRS, LYSA, and METH were significantly upregulated (Figure 6 and Supplementary Table 5).

Moreover, some AAs [serine (Ser), glycine (Gly), Cys, and Ile] could be synthesized from glycerate-3P and D-erythrose 4-phosphate through glycolysis and pentose phosphate pathways. During this process, it involves phosphoserine phosphatase (PSPH, EC: 3.1.3.3), cysteine synthase (CYSK,

EC:2.5.1.47), serine-glyoxylate transaminase (AGXT, EC: 2.6.1.45), cystathionine beta-synthase (CBS, EC: 4.2.1.22), etc. Many genes encoding PSPH, CYSK, and AGXT were all remarkably upregulated in *G. lemaneiformis* with Arg treatment at 48 h (Figure 6 and Supplementary Table 5).

Furthermore, we measured the content of 17 free AAs (14 detected and 3 undetected) at 72 h under HT condition by an AA automatic analyzer and found that 13 AA contents were increased (Table 3). Among those upregulated AAs, Asp was the most significantly increased AA, followed by Thr, Arg, Cys, His, Ser, and Gly, leading a 5.27-, 4.52-, 1.75-, 1.4-, 1.39-, 1.28-, and 1.25-fold changes compared to that of the CK treatment under HT stress, respectively. However, the stress-related AA Pro was not detected, thus we measured it in the laboratory



using a different method. It showed Arg treatment significantly promoted the accumulation of Pro at 3 d, leading 2.51-fold of increase as compared with the CK group (Supplementary Figure 6). In general, the content of AAs was consistent with the gene expression of their respective encoding AA synthase.

### Differentially Expressed Genes Involved in Glycolysis, Tricarboxylic Acid Cycle, and Oxidative Phosphorylation

Arginine remarkably changed the energy metabolism pathway, i.e., glycolysis, TCA cycle, and oxidative phosphorylation. In the glycolysis pathway, glucose could be converted into pyruvate to generate energy with a series of reactions, this process involves enolase (ENO, EC: 4.2.1.11) and pyruvate kinase (PK, EC: 2.7.1.40). Most genes encoding ENO (nine upregulated) and PK (two upregulated) were markedly upregulated by Arg under HT stress (Figure 7 and Supplementary Table 6). In addition, pyruvate could be converted into acetyl-CoA enter the TCA

cycle by pyruvate dehydrogenase complex (PDH). The genes (19 transcripts, 14 upregulated) encoding PDH were markedly upregulated at 48 h (Figure 7 and Supplementary Table 6). Citrate synthase (CSY, EC: 2.3.3.1), isocitrate dehydrogenase (IDH, EC: 1.1.1.42), and 2-oxoglutarate dehydrogenase complex (2-OGDH) are three key enzymes of the TCA cycle. Most genes encoding CSY (two upregulated), IDH (three upregulated), and 2-OGDH (seven upregulated) were markedly upregulated in Arg treatment under HT stress at 48 h (Figure 7 and Supplementary Table 6).

As for oxidative phosphorylation, exogenous Arg significantly activated the most genes at 48 h, but the majority of these genes had no significant difference at 24 h (Figure 8 and Supplementary Table 7). The genes (63 transcripts, 40 up and 23 downregulated) related to oxidative phosphorylation, such as genes encoding NADH dehydrogenase (10 upregulated genes), cytochrome c reductase (6 upregulated), cytochrome c oxidase (7 upregulated), F-type ATPase (10 upregulated), and V-type

**TABLE 3** | The change of free amino acids content in different treatments at 72 h under HT stress.

Free amino acids	Content (g/100g)		FC (Arg/CK) at 72 h
	HT-CK (means ± SD)	HT-Arg (means ± SD)	
Aspartate	0.00686309408941033 ± 0.000667316	0.0361609853669141 ± 0.000936299	5.27
Threonine	0.00724353944730369 ± 0.000542315	0.0327769295896092 ± 0.00220879	4.52
Serine	0.0015074225253748 ± 1.41941E-05	0.00193343776832226 ± 9.10971E-05	1.28
Glutamate	0.0358960613227125 ± 0.002270182	0.0386420335551825 ± 0.002497559	1.08
Glycine	0.000918963680774632 ± 3.57005E-05	0.00114661259632434 ± 2.24364E-05	1.25
Alanine	0.00119976923607486 ± 5.45795E-05	0.00167941748492396 ± 0.000196297	1.4
Cysteine	0.000428258910887235 ± 0.000123614	0.0005208714536165 ± 5.48702E-05	1.22
Valine	0.00342172805655155 ± 6.10373E-05	0.00415951288390824 ± 0.000202539	1.22
Methionine	0.00088166461694521 ± 0.000235557	0.000672658627090747 ± 0.000147011	0.76
Isoleucine	0.00108361643418038 ± 0.000274125	0.00130646195500967 ± 0.000360142	1.21
Leucine	0.000975307382598845 ± 6.58291E-05	0.00106542210173025 ± 0.000164473	1.09
Tyrosine	<0.0095	<0.0095	-
Phenylalanine	<0.0083	<0.0083	-
Lysine	0.00153016023842193 ± 6.13771E-05	0.00160703990690037 ± 4.48779E-05	1.05
Histidine	0.000552151573214045 ± 7.60337E-05	0.000766016866412289 ± 0.000103808	1.39
Arginine	0.0586762122944169 ± 0.002275341	0.102959823747071 ± 0.010378066	1.75
Proline	<0.0087	<0.0087	-
Total	0.12117795	0.225397224	1.86

ATPase (7 upregulated), which were remarkably upregulated in Arg-treated group at 48 h (Figure 8 and Supplementary Table 7). Generally, we speculate that exogenous Arg can increase the RGR of *G. lemaneiformis* via promoting glycolysis, TCA cycle, and oxidative phosphorylation which possibly offers more energy.

## DISCUSSION

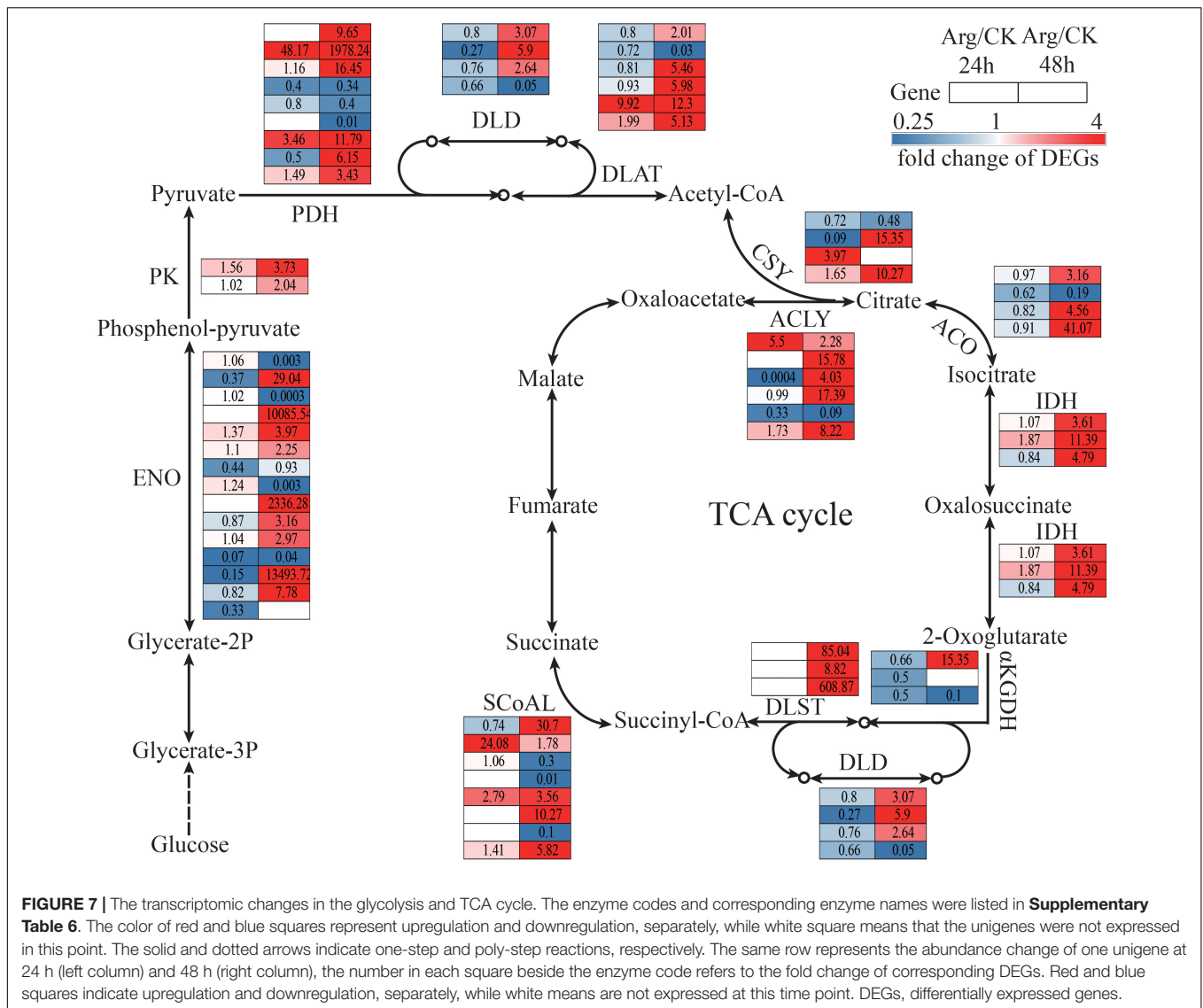
High temperature negatively affects the morphology and physiology of algae and land plants. As for *G. lemaneiformis*, one of the world's most cultivated seaweeds, an increase in temperature leads to large-scale production reduction. AAs are good candidates to be used as plant biostimulants, playing a beneficial role in growth and development under stress conditions (Haghighi et al., 2020). Especially for Arg, an AA with a high N/C ratio is involved in regulating many physiological processes under stress (Lv et al., 2020). Exogenous Arg could alleviate the adverse effects of temperature stress on maize height and the biomass of shoots and roots (Matysiak et al., 2020). However, no evidence has been found of the effects of Arg in the alleviation of HT stress in *G. lemaneiformis*. In the present study, the application of Arg significantly alleviated the HT stress-induced decrease in algae growth and the content of PBP, as algae not treated with Arg showed a much lower RGR and exhibited a more severe yellowing phenotype than Arg-treated algae (Figure 1B and Supplementary Figure 1). According to the results of RNA-seq analysis, Arg can activate a series of pathways genes in *G. lemaneiformis*, such as antioxidant system, HSPs, TFs, stress-related AAs, PAs, and energy metabolism, which may improve the heat tolerance of *G. lemaneiformis*, suggesting a potential role of Arg in increasing the yield of *G. lemaneiformis* in aquaculture as well.

## Influence of Arg on Antioxidant System of *Gracilariopsis lemaneiformis* Under High Temperature Stress

During stress, ROS, such as superoxide radicals ( $O_2^-$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $OH^\bullet$ ), are produced, which exert a detrimental impact on plant cells and result in cellular damage and death (Thorpe et al., 2013). However, plants have evolved corresponding mechanisms to eliminate excessive ROS via ROS scavenging enzymes, such as SOD, CAT, POD, APX, MDAR, TRX, and PRXR (Anderson, 2002; Pérez-Pérez et al., 2009; Smirnov, 2010; Das and Roychoudhury, 2014). In our study, DEG analysis showed that Arg treatment enhanced the transcript levels of most genes encoding those protective enzymes, such as SOD, APX, MDAR, PRXR, and TRX (Figure 4). Moreover, exogenous Arg reduced the content of  $H_2O_2$  and elevated the activities of antioxidant enzymes, such as SOD and CAT in *G. lemaneiformis* under HT stress (Figure 2). However, it was an interesting phenomenon that the  $H_2O_2$  content of the Arg-treated group was increased at 1 day. SPD could be catalyzed into 4-amino-butanol to produce  $H_2O_2$  by PAO, and the expression of genes encoding PAO could be upregulated by Arg, which may be the reason for the  $H_2O_2$  increase (Figure 5 and Supplementary Table 4). These results indicated the positive effects of Arg in regulating thermotolerance via upregulation of the antioxidant enzymes under HT stress.

## Influence of Arg on Heat Shock Proteins of *Gracilariopsis lemaneiformis* Under High Temperature Stress

Heat shock proteins, as vital cellular chaperones, play a pivotal role in conferring abiotic stress tolerance. Moreover, HSP can



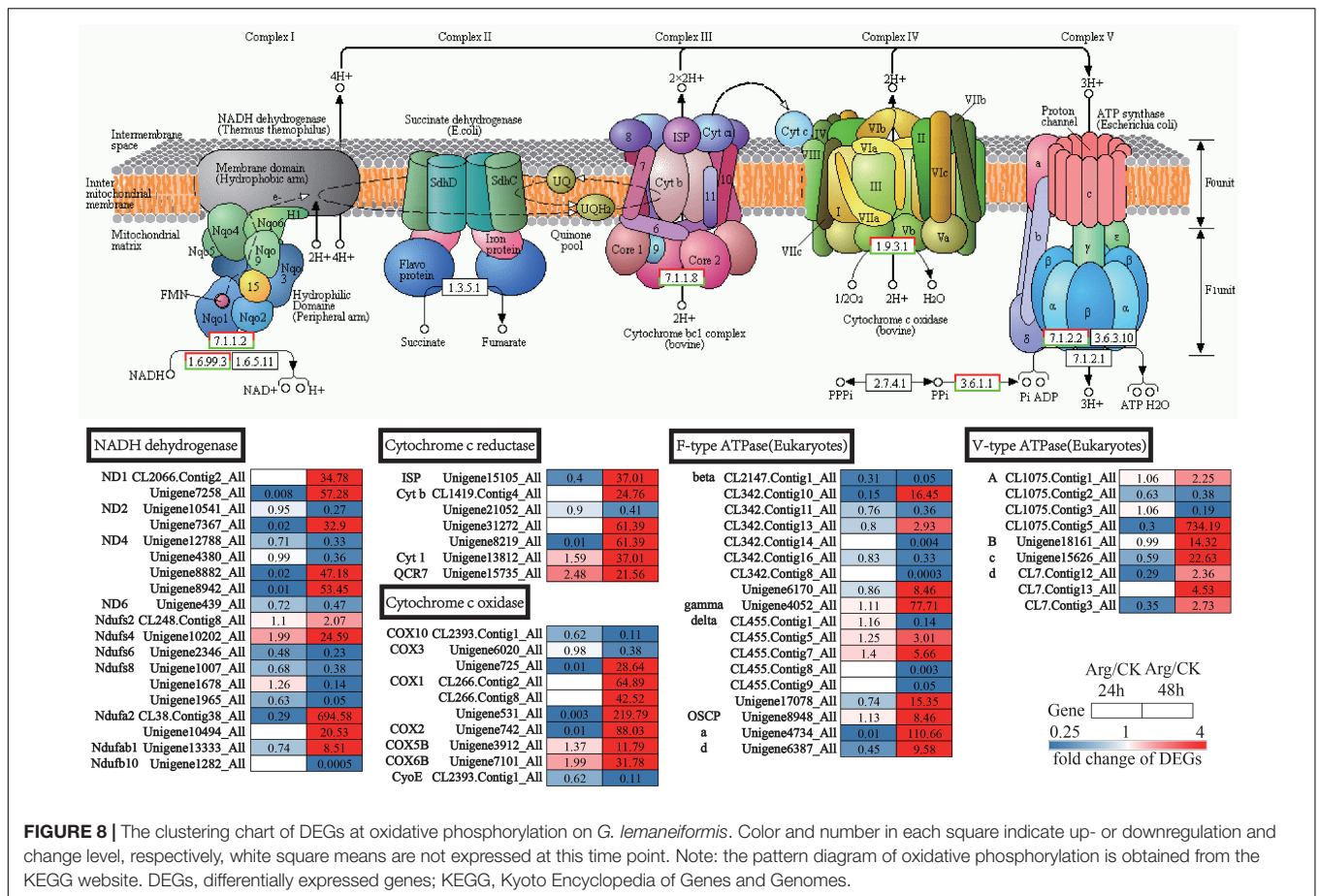
**FIGURE 7 |** The transcriptomic changes in the glycolysis and TCA cycle. The enzyme codes and corresponding enzyme names were listed in **Supplementary Table 6**. The color of red and blue squares represent upregulation and downregulation, separately, while white square means that the unigenes were not expressed in this point. The solid and dotted arrows indicate one-step and poly-step reactions, respectively. The same row represents the abundance change of one unigene at 24 h (left column) and 48 h (right column), the number in each square beside the enzyme code refers to the fold change of corresponding DEGs. Red and blue squares indicate upregulation and downregulation, separately, while white means are not expressed at this time point. DEGs, differentially expressed genes.

detoxify ROS by positively regulating the antioxidant enzymes system and improving the stability of cell membranes (ul Haq et al., 2019). At least six types of HSPs, i.e., HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (sHSPs), have been identified in higher plants. HSP90 and HSP70 can interact with some co-chaperone proteins, such as HSP20 which regulates its activities and aid in the folding of specific substrate proteins to ameliorate heat damage and maintain cell homeostasis (Wang et al., 2004; Guo et al., 2020). HSP40 is an obligatory co-chaperone partner of HSP70 families, which provides HSP70s with the activities and functional specificity to ensure their chaperone function effectively (Craig et al., 2006). Many pieces of evidence have indicated the importance of HSPs and sHSPs in regulating high-temperature tolerance in algae, such as in red algae *G. lemaneiformis*, *Cyanidioschyzon merolae*, and green algae *Chlamydomonas reinhardtii* (Gu et al., 2012; Kobayashi et al., 2014). Our previous study showed that HSPs were involved in the regulation of phytohormone-mediated heat tolerance in response

to heat stress (Wang et al., 2017). In the present study, most of the genes encoding HSP90, HSP70, HSP40, and sHSPs were induced by Arg application, particularly at 48 h, while many of those genes were not significantly expressed at 24 h. We speculate that these HSPs respond to the Arg-induced HT tolerance mainly at 48 h (Table 1), which is generally consistent with the expression pattern of other possibly HT-responsive DEGs analyzed in our study. Therefore, it indicated the crucial role of Arg in regulating thermotolerance via upregulation of HSPs when *G. lemaneiformis* responds to heat stress.

### Influence of Arg on Transcription Factors of *Gracilariopsis lemaneiformis* Under High Temperature Stress

In the signaling network from the stress signals perception to the downstream expression of stress-responsive genes, TFs play an essential role (Hussain et al., 2011). TFs have also emerged



**FIGURE 8 |** The clustering chart of DEGs at oxidative phosphorylation on *G. lemaneiformis*. Color and number in each square indicate up- or downregulation and change level, respectively, white square means are not expressed at this time point. Note: the pattern diagram of oxidative phosphorylation is obtained from the KEGG website. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

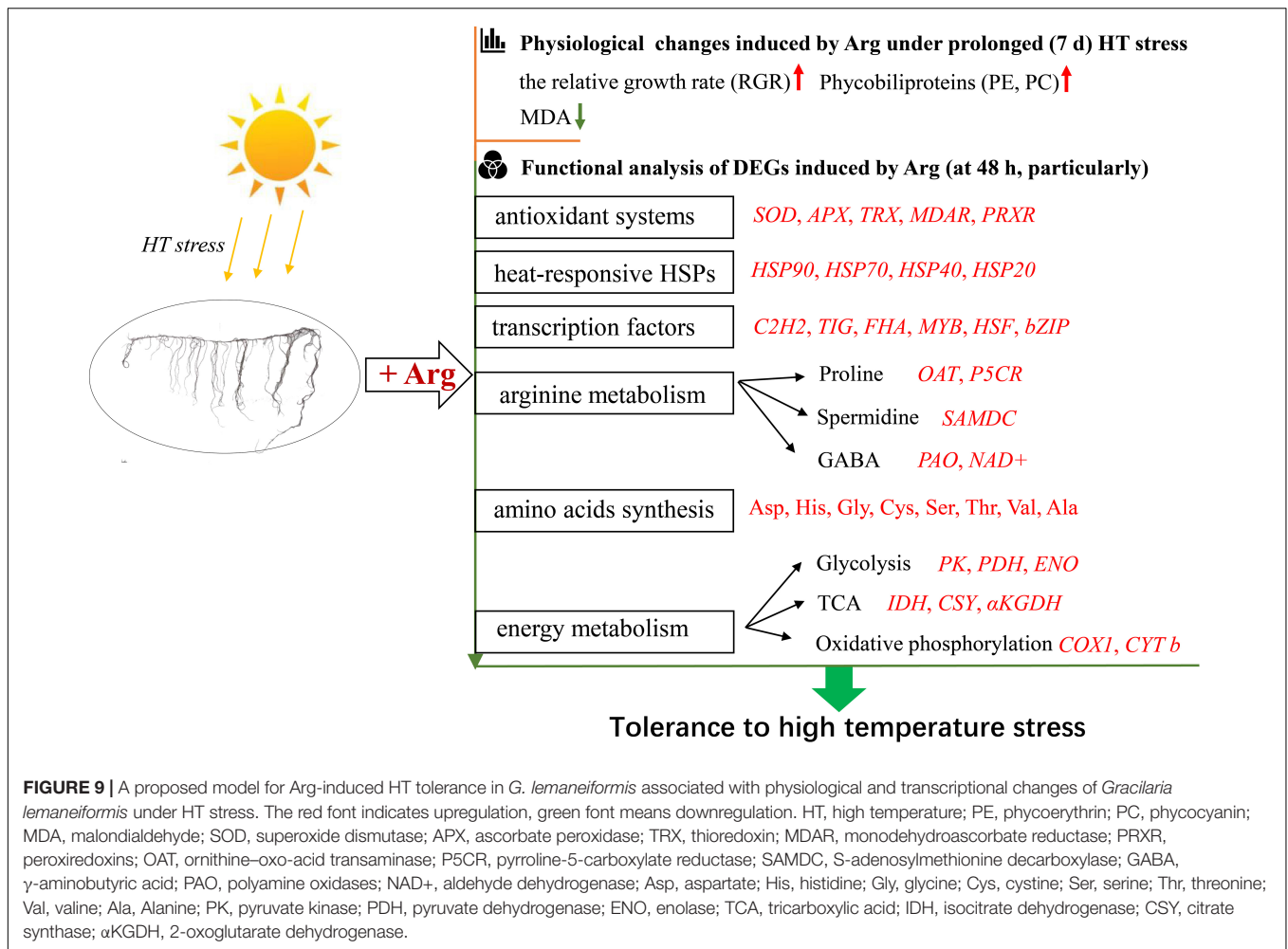
as powerful tools for the manipulation of complex metabolic pathways (Hussain et al., 2011). Members of the HSF, MYB, ERF, bZIP, and WRKY TF families have already been implicated in the regulation of stress responses in algae (Strenkert et al., 2011; Ritter et al., 2014; Liang and Jiang, 2017; Ji et al., 2018). Notably, the MYB group of TFs renders tolerance to elevated temperatures, and this high tolerance is associated with enhanced AA metabolism (El-kereamy et al., 2012; Xing et al., 2021). In the present study, Arg remarkably enhanced several TF encoding genes, covering 12 families, such as HSF, C2H2, C2C2-GATA, bHLH, MADS, MYB, and bZIP (Table 2). Particularly, the transcript level of genes encoding MYB showed considerable enhancement both at 24 and 48 h in response to HT stress. It indicated that the upregulation of TFs may contribute to Arg-induced heat tolerance in *G. lemaneiformis*.

### Influence of Arg on Polyamine, $\gamma$ -Aminobutyric Acid, and Other Stress-Related Amino Acids Under High Temperature Stress

Polyamines, such as PUT, SPM, and SPD, as second messengers, manifest a multifunctional defensive role in conferring plant abiotic stresses tolerance (Liu et al., 2007; Hasanuzzaman et al., 2019). PA metabolism may lead to increased GABA

accumulation, which also mediates plant responses to several environmental stresses (Al-Quraan, 2015). The synthesis of PA in plants requires Arg and Met metabolism (de Oliveira et al., 2018). The present study showed that Arg treatment enhanced the transcript levels of most PA synthesis-related genes (Figure 5 and Supplementary Table 4). This is similar to the results of several studies that the overexpression of PA biosynthetic genes provides increased stress tolerance in the plant (Liu et al., 2015). Moreover, exogenous application of Arg significantly upregulated the genes involved in the catabolism of PA to produce GABA (Figure 5). Then GABA from this step converts into succinate, which further enters the TCA cycle to generate more energy for stress acclimation. Collectively, these data indicated that exogenous Arg could promote PA and GABA syntheses that effectively improved the adaptive heat stress response in *G. lemaneiformis*.

Besides PA and GABA, Pro has also been proved that it can improve the resistance of plants under abiotic stress (Sairam et al., 2002; Haghghi et al., 2020; Wang X. et al., 2020). Algae and higher plants tend to accumulate Pro in response to salt and heavy metal stresses (Zhang et al., 2008; Cheng et al., 2014). In the present study, Arg treatments significantly increased Pro content, as compared to the non-treated group under high-temperature stress (Supplementary Figure 6) and upregulated the expression of the Pro synthesis genes (*P5CR* genes; Figure 5). Besides Pro, some other AAs, such as His, Ser, Met, Gly, alanine (Ala), Asp,



and valine (Val), were also reported to play vital roles in the protection of plants against various stresses (Di Martino et al., 2003; Sharma and Dietz, 2006; Liu and Lin, 2020). In the present study, we measured the contents of 17 free AAs between the Arg and CK treatments under HT stress for 3 d. The levels of most AAs were increased, particularly, Asp, Thr, Arg, Ala, and His, which were markedly increased as compared to CK treatments under HT stress. This is similar to the transcriptome data that Arg upregulated the expression of AA synthesis genes encoding Gly, Cys, Ser, Pro, Met, His, Thr, Val, Asp, and Ala in the Arg-treated group, especially at 48 h (Figure 6 and Table 3). Collectively, these results indicated that Arg might promote algae HT tolerance by upregulating the synthesis of Pro and other stress-related AAs in *G. lemaneiformis*.

### Influence of Arg on Energy Metabolism of *Gracilariopsis lemaneiformis* Under High Temperature Stress

Glycolysis, TCA cycle, and oxidative phosphorylation are cellular interconnected energy metabolic pathways, which generate carbon and nitrogen skeletons, the electron transfer chain (ETC), and provide ATP for plant and algae growth or

defense (Fernie et al., 2004; Fan et al., 2018). Genes related to glycolysis and the TCA cycle, such as HK, PFK, GAPDH, PK, SDH, and MDH, were reported to be involved in the tolerance to temperature stress (Dong et al., 2020; Wang L. et al., 2020). As reported, HT stress downregulated energy metabolisms, such as glycolysis/gluconeogenesis, TCA cycle, oxidative phosphorylation, and starch and sucrose metabolism (Wang et al., 2018). In the present study, Arg remarkably enhanced the energy metabolism at 48 h by activating the expression of genes encoding numerous supplementary enzymes in carbon and nitrogen metabolism (e.g., pyruvate kinase, PDH, CSY, IDH, and 2-OGDH) and electron transport chain (e.g., NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase, and ATPase; Figures 7, 8), which were consistent with the enhanced RGR of *G. lemaneiformis* under HT stress (Figure 1B and Supplementary Figure 1).

### CONCLUSION

Exogenous Arg can alleviate the growth decline of algae at HT. It upregulated the activities of enzymatic antioxidants and promoted the accumulation of free AAs. RNA-seq analysis



further revealed that Arg upregulated the expression levels of genes encoding antioxidant enzymes (SOD, APX, MDAR, PRXR, and TRX), HSPs (HSP70s, HSP90s, and sHSPs), and triggered TF signaling during HT stress. Moreover, Arg enhanced the DEGs involved in arginine metabolism (Pro, PA, and GABA synthesis), AA biosynthesis, and energy metabolism (glycolysis, TCA cycle, and oxidative phosphorylation). Collectively, these results supported that exogenous Arg could increase the yield of *G. lemaneiformis* at HT and revealed the mechanism of Arg to *G. lemaneiformis* under HT stress, providing a potential gene pool for obtaining heat-tolerant *G. lemaneiformis* in the future (Figure 9).

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories (SRA data, PRJNA774936).

## AUTHOR CONTRIBUTIONS

SL and NX designed the study. JZ performed the experiments and drafted the manuscript. SL, XC, and XS provided substantial contributions in this period. JZ, SL, and CH analyzed the data. SL, CH, and NX made important suggestions and provided

manuscript revision. All the authors contributed to the article and approved the final manuscript.

## FUNDING

This work was supported by the National Key R&D Program of China (2018YFD0901502), the National Natural Science Foundation of China (31672674 and 32001889), the Natural Science Foundation of Zhejiang Province (LY19C190003), and the Key Program of Science and Technology of the Ningbo Government (2019B10009).

## ACKNOWLEDGMENTS

We would like to thank SL, XC, and XS for helpful comments and technical assistance in the experiment. We would also like to thank SL, CH, and NX for writing—review.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.784586/full#supplementary-material>

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