

Transcriptome analysis of the red marine alga *Gracilaria vermiculophylla* grown under different light intensities

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Abstract

Light plays a vital role in seaweed growth by influencing photosynthesis and signaling metabolic processes. Seaweeds are subjected to various dynamic light conditions because of environmental factors such as weather and tidal changes. Despite the knowledge of the effects of light intensity on red algal growth, the gene expression patterns regulating growth under fluctuating light conditions remain unclear.

After investigating whether varying light intensity affects growth rates in the economically important red alga *Gracilaria vermiculophylla*, a comprehensive gene expression analysis using RNA-Seq was performed to determine whether these differences were due to changes in the transcriptome. Under high-light conditions, *G. vermiculophylla* showed upregulation of nitrogen and carbon metabolism genes such as nitrate reductase and carbonic anhydrase crucial for growth and biomass production.

This study provides insights into the molecular mechanisms underlying the growth responses of red marine algae to varying light intensities. The identified genes and their functions could be used to guide genetic engineering approaches to enhance red algal productivity in aquaculture.

Introduction

Light is the primary factor that affects seaweed growth directly by regulating photosynthesis and signaling metabolic processes. Seaweeds grow under diverse and dynamic light regimes in their natural environments. Factors such as clouds, water transparency, tidal ebb, and flood considerably influence the amount of light reaching seaweed growing sites (Hurd et al. 2014). Thus, seaweeds require a highly sensitive regulatory system to adapt to different light intensities to balance photosynthetic light responses and downstream metabolic constraints for their growth.

Rhodophyta (red algae) is a distinct phototrophic eukaryotic lineage containing approximately 7,000 species (Guiry and Guiry 2015), which include numerous economically important species. The bangiophytes (members of the class Bangiophyceae), one of the multicellular red algae groups, are major lucrative marine crops, such as the food “nori,” including *Pyropia* and *Porphyra* (laver). *Pyropia* and *Porphyra* are widely cultivated in East Asian countries to supply a billion-dollar (US) annual market (Blouin et al. 2011). Florideophytes form the second group of multicellular red algae and include economically important agarophytes and carrageenophytes, which have a variety of industrial uses owing to their unique cell wall composition containing polysaccharides, such as agar and carrageenan (Cardozo et al. 2007). Among florideophytes, several species in the order Gracilariales are used as a source of agar and other compounds (Armisen 1995).

A thorough understanding of the lifecycle of red algae has enabled stable seedling, leading to a considerable expansion of the aquaculture industry. After the elucidation of their life cycle, the knowledge of environmental factors affecting the growth of commercial red algae has been accumulating in the laboratory culture. For example, the optimal intensity for growth has been studied in

Gracilaria species (Dawes et al. 1999; Yokoya et al. 1999; Raikar et al. 2001). Despite the knowledge of the effect of light intensity on the growth, gene expression patterns controlling growth under fluctuating light conditions remain poorly understood.

To understand the relationship between light intensity and growth at the molecular level, we performed a comprehensive gene expression analysis using RNA-Seq in this study after examining whether different light intensities affect growth rates in the economically important red alga *Gracilaria vermiculophylla*. The gene expression patterns in response to various light intensities provide a molecular basis for their environmental adaptation. Meanwhile, understanding the potential regulators of these adaptations might help develop novel varieties with increased algal productivity.

Materials and Methods

Algal materials and treatments

Tetrasporophytes of *G. vermiculophylla* previously collected in Shinori-cho, Hakodate (Hokkaido Prefecture, Japan) were used as materials and cultivated in the laboratory. *Gracilaria vermiculophylla* was cultured at 15°C in sterile vitamin-free Provasoli's enriched seawater (PES; Provasoli 1968) under a 12-h light/12-h dark photoperiod at a light intensity of 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The culture medium for *G. vermiculophylla* was renewed weekly.

Effect of light intensity on algal growth

The growth rate of *G. vermiculophylla* was examined under low light (LL) treatment with light levels of 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and the high-light (HL) treatment with 230 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The growth rate was calculated as the percentage of length increase per day using the following equation:

$$\text{growth rate (\% day}^{-1}\text{)} = [100(\text{BL}_t - \text{BL}_0)/\text{BL}_0]/t,$$

where BL_0 = initial blade length, BL_t = blade length measured on day t of culture, and t = culture time in days.

RNA preparation and sequencing

Total RNA from algal samples was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in liquid nitrogen using a mortar and pestle, following the manufacturer's instructions. The extracted RNA was purified using a TURBO DNA-free kit (Invitrogen/Life Technologies, Carlsbad, CA) to obtain DNA-free RNA. The quantity and integrity of RNA samples were assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). A total of

six libraries of complementary DNA (2 conditions: LL, HL × 3 replicates) for *G. vermiculophylla* were constructed and subsequently sequenced using an Illumina NovaSeq 6000 instrument at Rhelixa Inc.

RNA sequencing analysis

The obtained reads were trimmed for low-quality reads and adapter sequences using fastp (Chen et al. 2018). RNA-Seq data of *G. vermiculophylla* were *de novo* assembled using Trinity (Grabherr et al. 2011). The clean reads of *G. vermiculophylla* were *de novo* assembled using Trinity and quantified using salmon (Patro et al. 2017). The TPM was calculated using RSEM (Li et al. 2011). DEGs were then identified using edgeR considering the FDR significance score < 0.05 and $|\log_2 \text{fold change}| > 1$ (Robinson et al. 2010).

Functional annotation and Gene Ontology enrichment analysis

Gene annotation was performed according to the following methods: The gene coding regions of the amino acid sequence were inferred from the contig assembled in Trinity using Transdecoder. The sequences of the obtained gene coding regions were functionally annotated by EnTAP (Hart et al. 2020).

To assess the biological significance of the DEGs, we conducted Gene Ontology enrichment analyses. GO terms were assigned to all genes using eggNOG-mapper v2 online (<http://eggno-mapper.embl.de/>) (Huerta-Cepas et al. 2019; Cantalapiedra et al. 2021) with the default parameters except that the min_hit_e-value was set to 0.05. The topGO (Alexa and Rahnenfuhrer 2023) R package was used for GO enrichment analysis, and GO terms with $P < 0.05$ were considered significantly enriched in the DEGs.

Quantitative PCR

Quantitative PCR (qPCR) analysis was performed as described by Uji et al. (2020), with minor modifications. First-strand cDNA was synthesized from 0.5 µg of total RNA using the PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). The cDNA was diluted 10-fold for qPCR analysis, and 1.0 µL of the diluted cDNA was used as a template in a 20-µL reaction volume using KOD SYBR® qPCR Mix (TOYOBO, Osaka, Japan) following the manufacturer's instructions. Realtime PCR was performed with a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland) under the following conditions: 2 min at 98°C followed by 40 cycles of 10 s at 98°C, 10 s at 55°C and 30 s at 68°C. The mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to the levels of the 18S ribosomal RNA gene. The relative expression level was calculated as the ratio of the mRNA level to the transcription level at LL. All experiments were performed in triplicate. Table S1 lists all primers used in this study.

Statistical analysis

Data are expressed as the means ± standard deviation and analyzed using Steel's test. For all analyses, $p < 0.05$ was considered statistically significant.

Results and Discussion

Effect of light intensity on the growth of *G. vermiculophylla*

After 3 days of incubation at different light levels, differences in growth rates were observed as follows: 3.8% in the LL treatment and 11.5% in the HL treatment (Fig. 1). After 10 days of incubation at different light levels, differences in growth rates were observed as follows: 6.9% for LL treatment and 25.2% for HL treatment.

Identification of DEGs under different light intensities in *G. vermiculophylla*

We used RNA-Seq to examine transcripts in the thalli of *G. vermiculophylla* cultivated in LL and HL for 3 days to identify candidate genes affecting the growth rate. Approximately 119 million reads were obtained from the deep sequencing with 19.8 million reads per library for six samples (LL, HL × 3 replicates). The mapping rates obtained from the six libraries from LL and HL were 70.6% and 73.2%, respectively (Table 1).

Table 1
Summary of transcriptome analysis in *G. vermiculophylla* under different light intensity

Sample name	Raw reads	Clean reads	GC content (%)	Mapping rate (%)
Gv_LL1	18,255,934	17,610,812	54.8	70.3
Gv_LL2	20,732,664	19,974,806	55.2	69.4
Gv_LL3	22,638,554	21,851,456	54.6	72.3
Gv_HL1	19,258,422	18,623,806	55.0	70.9
Gv_HL2	20,121,462	19,478,900	54.2	73.6
Gv_HL3	22,257,046	21,558,358	53.8	75.2

A total of 593 DEGs were obtained between LL and HL, including 56 upregulated and 537 downregulated genes under different light intensities (Supplementary Table 2, 3). Four DEGs were chosen for qPCR analysis to validate the RNA-Seq findings. As shown in Fig. 2, the tested genes had similar expression levels in RNA-Seq and qPCR analyses, indicating that the expression dataset was reliable.

To determine the biological processes of DEGs under different light intensities, GO analysis was performed (Figs. 3 and 4). In the upregulated DEGs, nucleolus organization mitotic chromosome condensation, and sister chromatid cohesion were enriched groups within the biological process category. Within the cellular component category, DEGs were prominently enriched in single-stranded DNA binding, ATP-dependent activity acting on DNA, and protein heterodimerization activity. In the molecular function category, DEGs were classified into condensed nuclear chromosome, chromosome, centromeric region, and nuclear speck.

In the downregulated DEGs, endoplasmic reticulum organization, negative regulation of protein ubiquitin, and protein exit from endoplasmic reticulum were enriched groups within the biological process category. Within the cellular component category, DEGs were prominently enriched in peptidase regulator activity, ubiquitin protein ligase binding, and structural constituent of ribosome. In the molecular function category, DEGs were classified into cytosolic large ribosomal subunit, proteasome complex, and chromatin.

The upregulated DEGs at HL conditions, included the genes associated with nitrogen and carbon metabolisms, such as nitrate reductase, ammonium transporter, and carbonic anhydrase (Table 2). Nitrogen is one of the macronutrients essential for algal growth and development, and thus is often a major limiting factor for productivity (Kumar and Bera 2020). Nitrogen is a constituent of key cell molecules, including amino acids, nucleic acids, chlorophyll, and ATP and is also the pivotal regulator of numerous biological processes, including carbon metabolism, amino acid metabolism, and protein synthesis (Foyer et al. 2003; Nunes-Nesi et al. 2010).

Table 2
The list of upregulated genes in *G. vermiculophylla* under different light intensity

Contig ID	Functional categories	Description	Fold Change
DN2981_c0_g1_i1	Nitrogen metabolism	Nitrate reductase	7.46
DN19587_c0_g1_i2	Polysaccharide catabolism	Beta-glucanase	6.50
DN1297_c0_g3_i1	Signal transduction	Plexin	5.83
DN19_c0_g1_i2	Nitrogen metabolism	Ammonium transporter	4.51
DN619_c0_g3_i1	Methyl transfer	SAM-dependent methyltransferase	3.60
DN33786_c0_g1_i1	Proteasome	Ubiquitin-like protein	3.56
DN55795_c0_g1_i1	Carbon metabolism	Carbonic anhydrase	3.31
DN68_c0_g1_i1	ATP regeneration	Phosphate translocator	3.24
DN64501_c0_g1_i1	Transfer of sugar moiety	Glycosyl transferase family	3.17
DN21276_c0_g1_i1	Transcription factor	Transcription factor MYB44	2.81
DN17557_c0_g1_i1	Oxidized base repair	8-oxoguanine deaminase	2.48
DN63904_c0_g1_i1	Membrane channels	Aquaporin	2.43
DN305_c0_g1_i7	Sugar epimerase enzyme	NAD-dependent epimerase	2.31

Nitrate reductase is a key nitrogen reduction and assimilation enzyme. It catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is then reduced to ammonia (NH_4^+) by nitrite reductase before

being assimilated into the amino acids and the nitrogen compounds of the cell (Campbell 1999). Nitrate reductase catalyzes the initial phase of nitrate assimilation in all organisms, which appears to be a rate-limiting mechanism in acquiring nitrogen in most cases. Light regulation of nitrate reductase activity has been reported in *Gracilaria tenuistipitata* (Lopes et al. 2002) and *Gracilaria chilensis* (Chow et al. 2004). High light-induced expression of nitrate reductase genes in *Gracilaria* species is responsible for the activation of nitrate assimilation, coupled with the activation of photosynthesis and the maintenance of balanced levels of carbohydrates and amino acids.

In macroalgae, NH_4^{4+} accumulating in cells, either through uptake from seawater via ammonium transporters (AMTs) or through reduction of NO_3^- , may be directed into the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Fan et al. 2020; Kakinuma et al. 2017; Shahar et al. 2020). Because NH_4^{4+} requires less energy for uptake and assimilation than NO_3^- , NH_4^{4+} is the preferred nitrogen source for uptake in certain macroalgae (Wang et al. 2014). On the other hand, excessive NH_4^{4+} concentrations are toxic and inhibit algal growth (Wang et al. 2023). AMTs in mesophyll cells of plants might be involved in retrieving $\text{NH}_3/\text{NH}_4^{4+}$ derived from photorespiration, which can reduce the efficiency of photosynthetic carbon assimilation (Gazzarrini et al. 1999). Photorespiration is a critical process, releasing substantial quantities of ammonium at rates that can exceed 10-fold the rate of primary nitrate assimilation in plants (Keys et al. 1978). Thus, the well-regulated homeostasis of internal NH_4^{4+} concentrations via AMT under HL conditions is critical for the health and productivity of *G. vermiculophylla*.

Carbon is also essential for algae to maintain their basic growth and development. Various carbohydrates, such as glucose and organic acids, provide energy and carbon skeletons for NH_4^{4+} absorption to produce amino acids and proteins (Lancien et al. 2000). The efficiency of photosynthesis and metabolism is affected by numerous environmental factors, such as CO_2 concentration, nitrogen availability, and light intensity (Morales et al. 2018). Because of aquatic environmental constraints on CO_2 supply, photoautotrophs usually need a regulated acquisition system for dissolved inorganic carbon to maintain rapid and efficient photosynthesis (Matsui et al. 2018). Macroalgae may use HCO_3^- as a source of inorganic carbon, and their photosynthetic rates in natural seawater are often limited by inorganic carbon sources (Lignell and Pedersen 1989; Gao et al. 1993). Carbonic anhydrase (CA) activity is usually linked to the ability of algae to use HCO_3^- (Mercado et al. 1998). CA is a zinc metalloenzyme that catalyzes CO_2 and HCO_3^- interconversion (Moroney et al. 2001).

External CA in algae has been proposed to serve two functions: (1) conversion of HCO_3^- to CO_2 , which would enter the cells through the plasma membrane, either by diffusion or via active transport, and (2) conversion of CO_2 from the air to HCO_3^- , which is then actively or passively transported across the plasma membrane (Mercado et al. 1999). Furthermore, internal CA is considered necessary to provide CO_2 from the internal pool of inorganic carbon to Rubisco (Mercado et al. 1998). A previous study found that the photorespiratory glycolate pathway in *C. reinhardtii* is closely involved in inorganic carbon

concentrating mechanisms through the induction of CA activity, thus playing a significant role in adapting photosynthesizing algae to low CO₂ concentration (Ramazanov and Cárdenas 1992). The upregulation of the CA gene in this study enhanced the availability of inorganic carbon for photosynthesis, resulting in an increased growth rate of *G. vermiculophylla*. Our previous study indicated the orchestration of the diurnal patterns of genes encoding enzymes involved in nitrogen and carbon assimilation in *P. yezoensis* (Kominami et al. 2022). The RNA-Seq data of *G. vermiculophylla* obtained in this study also support the coordination and optimal functioning of the metabolic pathways driving nitrogen and carbon assimilation in red algae, which are critical for growth and biomass.

In addition to the genes associated with nitrogen and carbon metabolism, our RNA-Seq data showed that aquaporin transcripts increased under HL conditions. Aquaporins are membrane-intrinsic proteins initially defined as water channels in all taxonomic kingdoms and subsequently found to have multiple substrate specificities, such as CO₂ (Groszmann et al. 2017). The roles of the CO₂-permeable aquaporins have been extensively characterized in C3 plants. Aquaporins localized in both the plasma membrane and the chloroplast envelope of these plants have been shown to facilitate CO₂ diffusion from the intercellular airspace to the site of Rubisco in chloroplasts, with an essential function of supplying CO₂ for photosynthetic reactions (Kaldenhoff 2012; Uehlein et al. 2008). Similarly, plasma membrane-type aquaporins in a marine diatom may regulate membrane permeability to CO₂ to facilitate the desired substrate fluxes (Matsui et al. 2018). Aquaporins in red algae are poorly understood. Nonetheless, the expression patterns of *P. yezoensis* aquaporins have been examined at the transcriptional and translational levels under various abiotic stress conditions, including dehydration, salinity, osmotic pressure, and temperature (Kong et al. 2017). In addition to gene expression analysis, further research is required to unravel the substrate specificity and localization of red algal aquaporins.

Conclusions

We identified the genes whose expression increases when red macroalga show a high-growth rate under high-light conditions. The genes associated with nitrogen and carbon metabolism had a large abundance of transcripts under high-light conditions, indicating that the regulation and optimal functioning of metabolic pathways that control nitrogen and carbon assimilation are critical for growth and biomass in *G. vermiculophylla*. The use of genetic engineering and genome editing technologies to elucidate the regulation and function of genes involved in carbon/nitrogen metabolisms might provide a roadmap for enhancing red algal productivity.

List of abbreviations

AMT, Ammonium transporters

CA, Carbonic anhydrase

DEG, Differentially expressed genes

FDR, False discovery rate

HL, High light

LL, Low light

PES, Provasoli's enriched seawater

TPM, Transcripts per kilobase million

Declarations

Availability of data and materials

RNA sequence data were deposited in DDBJ under the BioProject accession number PRJDB16860 (For editors and reviewers: currently we are already submitted but not open to the public.).

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Authors' contributions

TU designed the experiments and interpreted the data. TK and TU performed the experiments. TK, TU, and HM wrote the manuscript. All authors have read and approved the final version of the manuscript.

Supplementary Information

Supplementary data to this article can be found online at @@@@@.

Ethics declarations

Competing Interests

The authors declare that they have no competing interests.

Ethical Approval

Not applicable.

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Figures

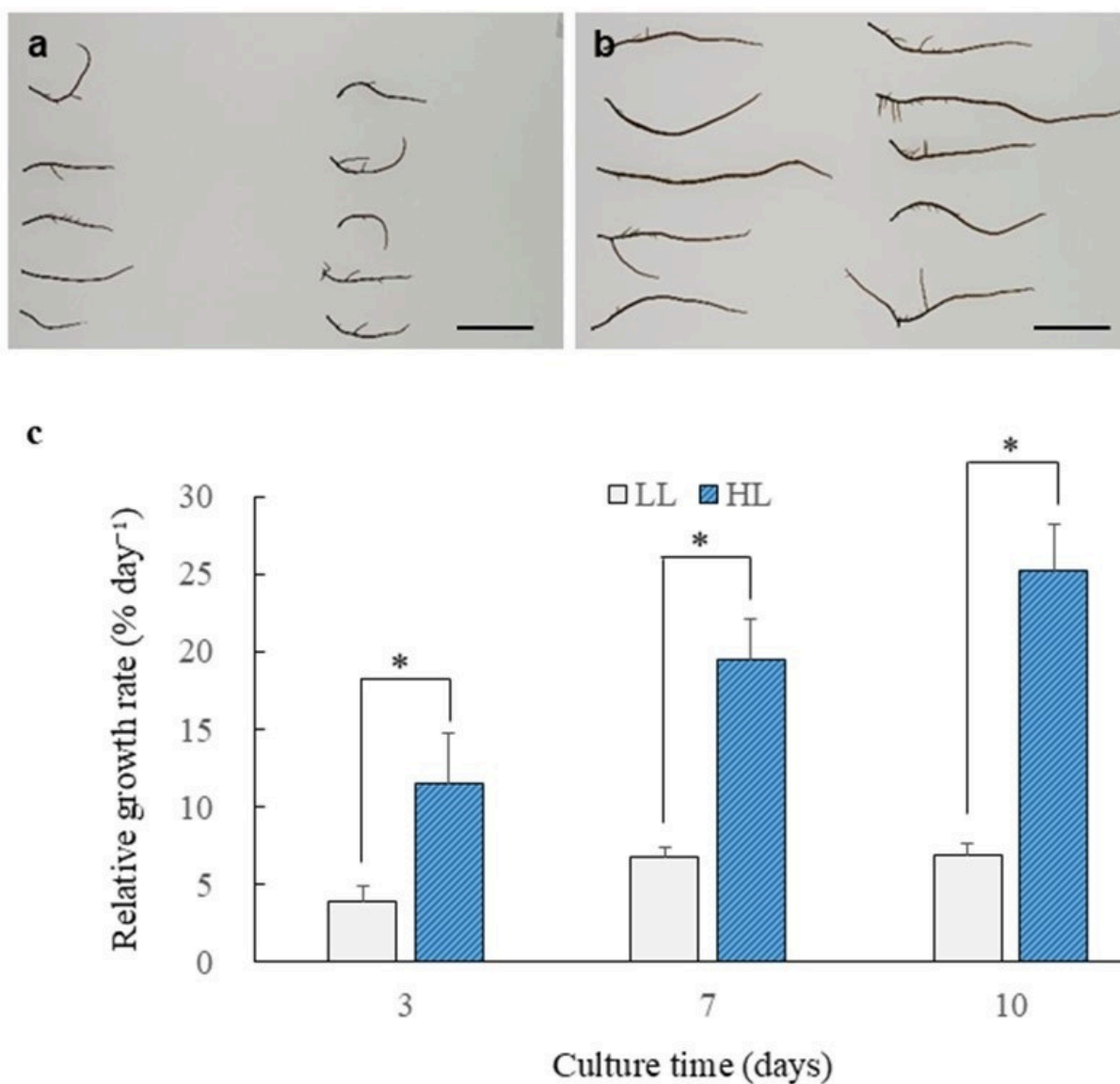


Figure 1

Effect of light intensity on the growth of *G. vermiculophylla*. (a) Algal growth on the 10th day of culture under LL: 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and (b) HL: 230 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Scale bar = 10 mm. (d) Growth rate of thalli cultured under LL and HL. The presented data are means $\pm$ standard deviations of 10 individuals for each condition. Asterisks above the bars indicate significant differences ($p < 0.05$).

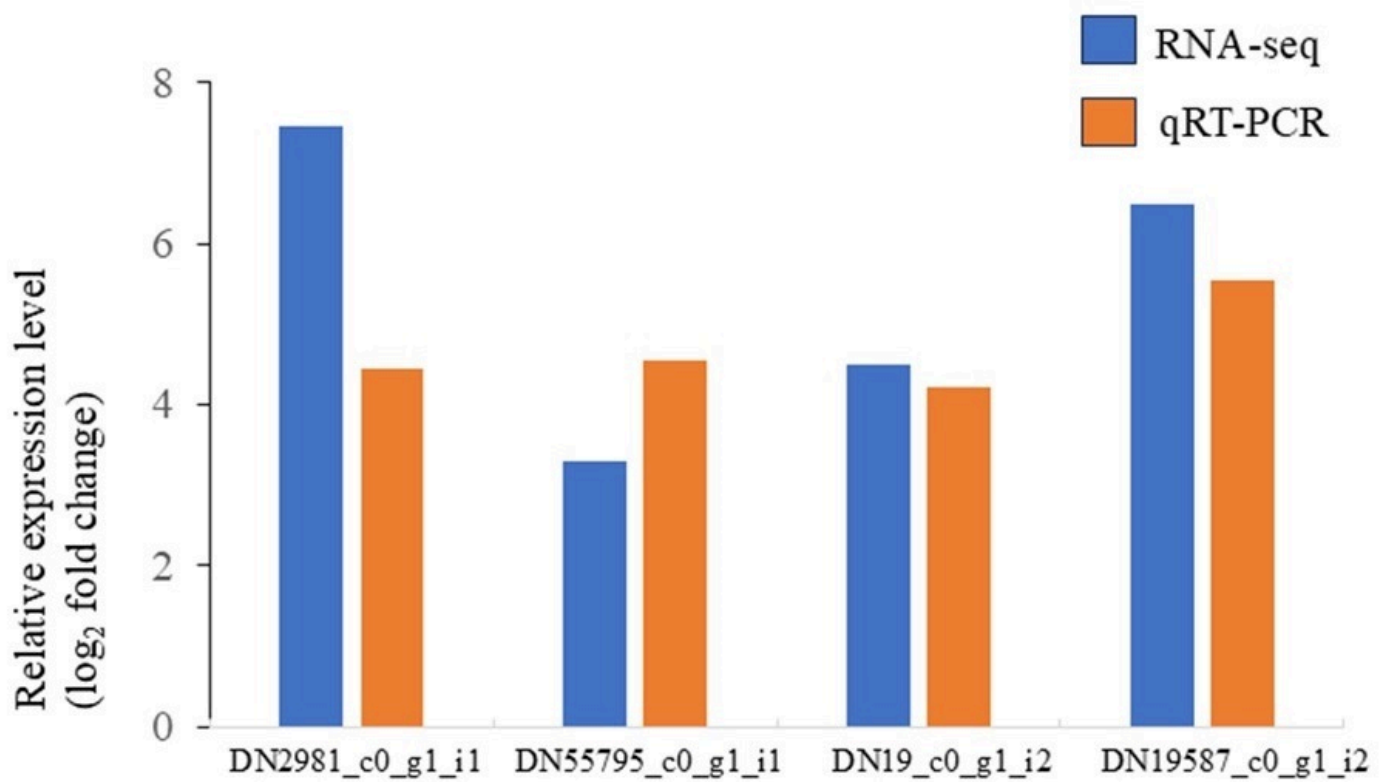


Figure 2

Comparison of gene expression data obtained by RNA-Seq and qPCR in *G. vermiculophylla*. qPCR analysis was performed on four selected genes that exhibited differences in expression levels between LL and HL in *G. vermiculophylla* (orange boxes). The gene expression levels obtained from the RNA-Seq data are indicated by blue boxes (RNA-Seq). The results are presented as relative expressions compared with those in LL.

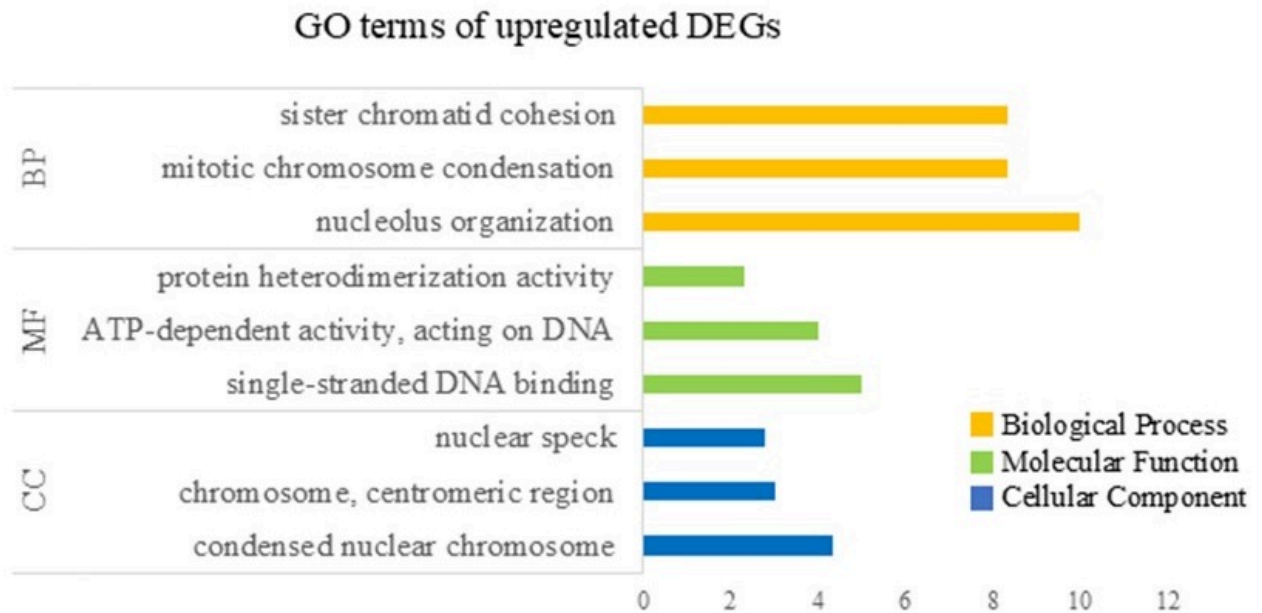


Figure 3

Numbers of enriched GO terms for upregulated DEGs. GO terms are presented for three main categories: biological processes, molecular functions, and cellular components. Each GO term is listed in ascending order by p -value.

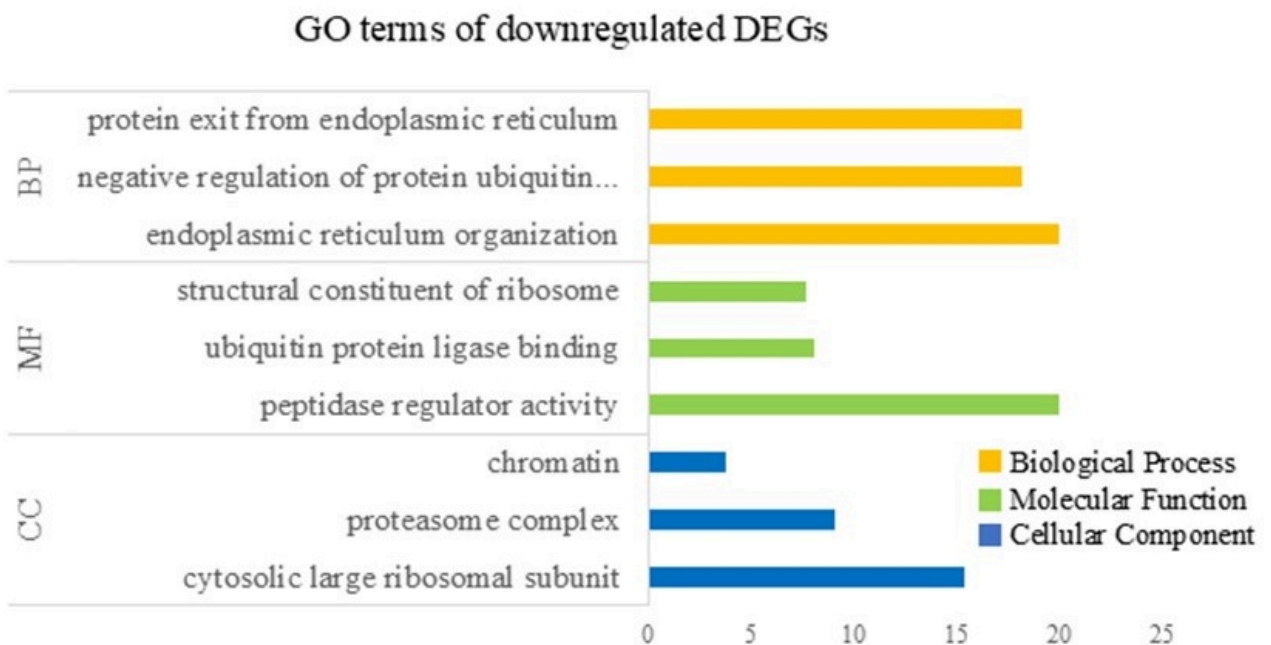


Figure 4

Numbers of enriched GO terms for downregulated DEGs. GO terms are presented for three main categories: biological processes, molecular functions, and cellular components. Each GO term is listed in ascending order by p -value.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS2.xlsx](#)
- [TableS1.xlsx](#)
- [TableS3.xlsx](#)