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Transcriptome analysis of carotenoid biosynthesis in *Dunaliella salina* under red and blue light*

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Abstract The quality of light is an important abiotic factor that affects the growth and development of photosynthetic organisms. In this study, we exposed the unicellular green alga *Dunaliella salina* to red (660 nm) and blue (450 nm) light and analyzed the cell growth, total carotenoid content, and transcriptomes. The growth of *D. salina* was enhanced by illumination with red light, whereas blue light was not able to promote the algal growth. In contrast, the total carotenoid content increased under both red and blue light. The RNA of *D. salina* was sequenced and the transcriptomic response of algal cells to red and blue light was investigated. Six transcripts encoding for the blue light receptor cryptochrome were identified, and transcripts involved in the carotenoid metabolism were up-regulated under both red and blue light. Transcripts encoding for photoprotective enzymes related to the scavenging of reactive oxygen species were up-regulated under blue light. The present transcriptomic study provides a more comprehensive understanding of carotenoid biosynthesis in *D. salina* under different wavelengths of light.

Keyword: blue light; carotenoid; *Dunaliella*; red light; transcriptome

Abbreviation: BL: blue light; Cbr: carotene biosynthesis-related protein; DEG: differentially expressed genes; GPX: glutathione peroxidase; LCY: lycopene β-cyclase; LED: light-emitting diode; PSY: phytoene synthase; RL: red light; ROS: reactive oxygen species; SOD: superoxide dismutase; WL: white light

1 INTRODUCTION

Carotenoids are light-harvesting pigments that are closely associated with chlorophylls in the photosynthetic membranes of algae and higher plants. These pigments absorb energy in the blue-green wavelength, one that is poorly absorbed by the chlorophylls (Siefermann-Harms, 1987). Carotenoids also play a protective role in the photosynthetic apparatus by quenching the chlorophyll triplet state and singlet oxygen (Young and Frank, 1996). Among the carotenoids, β-carotene has attracted great attention due to its commercial value in the food, pharmaceutical and cosmetic industries. Daily intake of natural β-carotene has also been shown to reduce the risk of lung cancer in humans (Mayne, 1996). Thus, in recent years, researchers have shown an increased interest in producing natural carotene to meet the growing demand of the market.

The unicellular green alga, *Dunaliella salina* has a potential to accumulate a large amount of natural β-carotene. Studies have shown that many abiotic stresses (high light, high temperature, nutrient deficiency, etc.) induce the accumulation of β-carotene

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in *D. salina* (Ben-Amotz and Avron, 1981; Hejazi and Wijffels, 2003). Overproduction of β-carotene was observed in response to a high light stress (Lamers et al., 2010). Following a salinity up-shock from 10% to 20% NaCl, the total carotenoid content likewise increased, which was mainly due to an increase in β-carotene (Borowitzka et al., 1990). Ultraviolet-A radiation has also been found to induce accumulation of total carotenoids (Raja et al., 2007).

Light quality has an important effect on the growth and development of photosynthetic organisms, especially for algae, of which the natural habitat may be dominated by light in blue-green wavelength because of the absorption properties of the water column. Studies involving blue light have shown this to induce high contents of total fatty acid and astaxanthin in *Haematococcus pluvialis* (Ma et al., 2018). In contrast, red light has been proven the optimal wavelength for growth in most algal species (Matthijs et al., 1996; Wang et al., 2014). Green light promoted chlorophyll production in *Chlorella vulgaris* (Mohsenpour and Willoughby, 2013). Accordingly, appropriate applications of light-emitting diodes (LED) in different wavelengths might enhance the growth and carotene accumulation in *D. salina*.

Studies on the effects of light quality on *Dunaliella* arose with the emergence of LED technology. It was found that the narrow-spectrum red light was the most efficient for promoting *D. salina* growth and accumulation of β-carotene compared with blue and white LED (Han et al., 2019). Blue light led to decreased cell numbers, whereas a combination of red and blue LED increased accumulation of β-carotene (Fu et al., 2013). However, there is so far very few investigations of the regulation mechanism whereby light quality affects the carotenoid biosynthesis genes in *D. salina*. Studies of the nuclear and organelle genomes of *D. salina* have led to significant advances in genetic engineering and the study of genome evolution (Smith et al., 2010; Polle et al., 2017). The full-scale transcriptome studies of *D. salina* have established foundations for comparative genomic studies on osmolyte and glycerol metabolisms (Fang et al., 2017; Hong et al., 2017). Therefore, transcriptional profiling of *D. salina* could be useful in determining the role played by transcription in realizing the observed changes in carotene biosynthesis that result from exposure to different wavelengths of light.

In this study, we investigated the growth and total carotenoid content of *D. salina* grown under illumination by blue (450 nm) and red (660 nm) light. The transcriptome of *D. salina* grown under each of these light conditions was investigated, and changes in the expression of genes involved in photoreceptors, carotenoid biosynthesis, and reactive oxygen species (ROS) scavenging were investigated and analyzed.

2 MATERIAL AND METHOD

2.1 Algal strains and culture conditions

Dunaliella salina HG-01 was isolated from salterns in Hangu, Tianjin, China. To confirm the identity and phylogenetic relationship of the strain, a phylogenetic tree was constructed (Supplementary Fig.S1). The cells were cultured in DM medium (Pick et al., 1986). A Multi-Cultivator MC 1000-OD (Photon Systems Instruments, Czech Republic) were used for cultivation at 22±1°C with 50±5 μmol photons/(m^2·s) in a 14-h:10-h light:dark cycle. Sterilized bubbles of air were aerated into the culture at a flow rate of 0.5 L/min. In the mid-exponential growth phase, algal cells were harvested and inoculated into fresh medium by centrifugation at 2 000×*g* for 5 min. Irradiance was shifted from cold white light-emitting diode (LED) light (50 μmol photons/(m^2·s)) to blue (450 nm, 50 μmol photons/(m^2·s)) and red light (660 nm, 50 μmol photons/(m^2·s)) for 168 h. Cell growth was monitored using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter Co., Ltd., United States).

2.2 Pigment extraction and quantification

Dunaliella salina cells grown under different wavelengths for 168 h were harvested by centrifugation for 4 min at 2 000×*g* at room temperature. Cells were covered by methanol and acetone (1:1, v:v), and the extracts were ground thoroughly with an electric tissue grinder OSEY10 (Tiangen, China). The extracts were centrifuged at 4°C for 5 min at 8 000×*g* and the supernatant was collected. Pigments were separated and identified using an Essentia LC-16 apparatus (Shimadzu Corporation, Tokyo, Japan) according to the procedure used by Xie et al. (2016).

2.3 Preparation of cDNA Library, sequencing and quality control

Total RNA was extracted from *D. salina* grown for 168 h under different wavelengths using an RNAprep Pure Plant kit (Tiangen Biotech, Beijing, China).

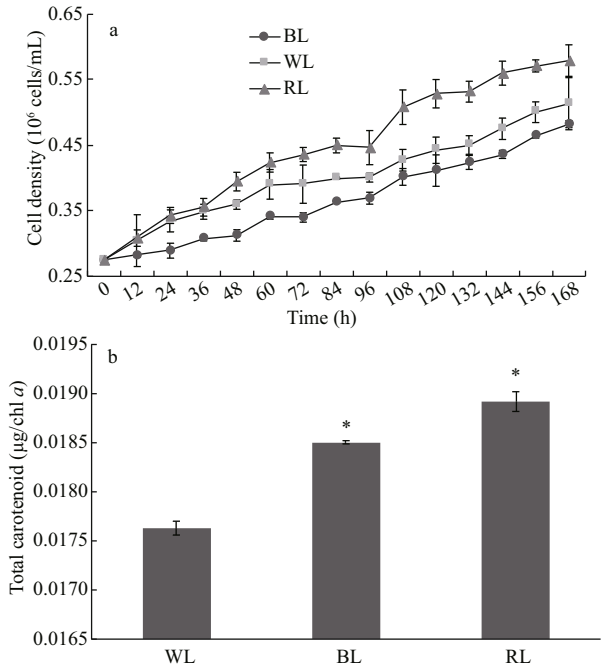

Fig.1 Growth of *D. salina* under different wavelengths of light

a. growth curve of *D. salina*; b. total carotenoid contents of *D. salina* under different wavelengths of light; *: $P<0.05$. WL: control, white light; BL: blue light; RL: red light.

RNA purity and concentration were monitored using a NanoPhotometer spectrophotometer (IMPLEN, USA). Total RNA (1.5 μg) from each sample was used to create normalized cDNA. mRNA-seq libraries were generated as described previously (Li et al., 2019) and named WL (control, white light), BL (blue light), RL (red light). Each library consisted of three biological replicates. Sequence data were deposited in the NCBI Gene Expression Omnibus (GEO) with the accession number GSE128019. By removing reads with adapters and filtering the low-quality reads, sequencing quality was controlled, generating clean reads.

2.4 Transcriptome assembly, functional gene annotation

Transcripts of the high quality clean data were assembled using Trinity (Grabherr et al., 2011). Unigenes were generated by aligning the subcomponent and selecting the longest one. The unigenes were annotated according to the GenBank non-redundant (NR) protein database, the Swiss-Prot database, and the EuKaryotic Orthologous Groups of proteins (KOG). Gene ontology (GO) classification was carried out using Blast2GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was performed using KAAS (Moriya et al., 2007).

2.5 Differential expression analysis

Differential expression analysis of two groups (BL vs WL and RL vs WL) was performed by counting and screening for differentially expressed genes (DEGs) using the DESeq R package (Anders and Huber, 2010). After calculating the *P* value, multiple hypothesis tests were performed to reduce false rates, and adjusted *P* values (padj) were generated. Genes with padj <0.05 were assigned as differentially expressed.

2.6 Quantitative Real-time PCR

Randomly selected transcriptomic sequenced genes under different light conditions were verified by quantitative real-time PCR (qPCR). Total RNA and cDNA was prepared as described previously (Li et al., 2019). Primers used for qPCR were shown in Supplementary Table S1. qPCR was performed as previously described (Li et al., 2019) using SYBR-Green as the fluorescent marker. Melting curves were collected for all PCR procedures, and PCR was performed in triplicates.

2.7 Statistical analysis

Statistically significant differences between samples were determined by two-tailed student *t* tests and one-way analysis of variance. All statistical analysis was conducted by SPSS software (IBM Co., Armonk, NY, USA). Samples with $P<0.05$ were considered to be significant different.

3 RESULT AND DISCUSSION

3.1 Characteristics of *D. salina* growth

To investigate the effects of red and blue light on growth and carotene accumulation in *D. salina*, cell density and total carotenoid contents were examined. In Fig.1a, different cell numbers of *D. salina* are shown, which correspond to the wavelengths of light used. Of all the wavelengths of light applied, red light was the most efficient in promoting algal growth. This result is consistent with previous findings in *C. vulgaris* and *Nannochloropsis oculata* (Kim et al., 2014; Schulze et al., 2016). Under blue light, cell numbers of *D. salina* at 168 h were lower than under white light, indicating that blue light was not able to promote algal growth.

The highest level of total carotenoids was observed under red light, which increased 7.31% compared with that under white light ($P<0.05$, Fig.1b). This result is consistent with the increased growth

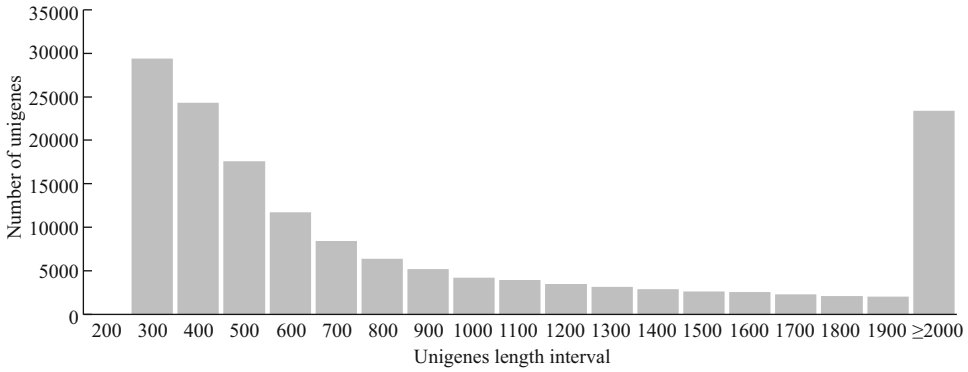

Fig.2 Length distributions of *D. salina* unigenes

Table 1 Summary of *D. salina* transcriptome sequencing and assembly

Sequencing and assembly	
Number of clean reads	132 176 860
Q20	95.80%
GC content (%)	53.04
Number of unigenes	115 975
N50	1 974
Min length (bp)	201
Max length (bp)	17 643
Average length (bp)	1 140

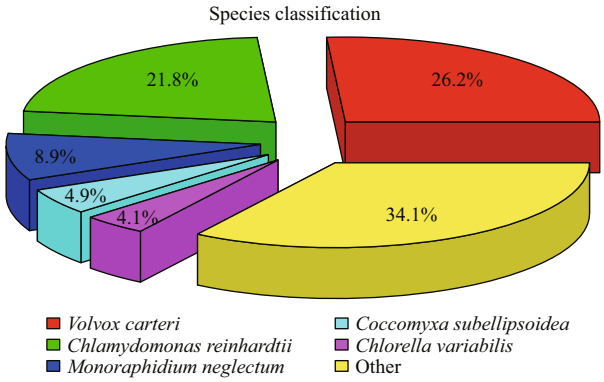

Fig.3 Top-hit species distribution of *D. salina* unigenes against NCBI NR database

efficiency under red light due to the absorption of red wavelengths by chlorophyll. Although the highest amount of β-carotene was obtained under red LED illumination, the total carotenoid content of *D. salina* grown under blue light was also 4.95% greater than that grown under white light (P<0.05). Due to its absorption in blue-green wavelength, it seems possible that the accumulation of carotenoids in *D. salina* increased to protected the photosynthetic apparatus from blue-light damage. In *D. bardawil*, high levels of β-carotene were also observed under blue light, presumably also for protection against photoinhibition (Ben-Amotz et al., 1989).

3.2 Annotation of *D. salina* transcriptome

To further investigate the response of *D. salina* to different wavelengths of light, we analyzed the transcriptome of *D. salina* exposed to WL, BL, and RL. There were 132 176 860 clean reads with average GC content of 53.04% generated from *D. salina*; 95.80% of the clean reads had a Q-value ≥20 (Table 1). There were 115 975 unigenes generated in assembly with an average length of 1140 bp and N50 of 1974 bp. The length distribution of unigenes is illustrated in Fig.2.

The acquired unigenes were annotated by the NR protein database, and the species classification was obtained and shown in Fig.3. Of all the related species, *Volvox carteri* had the most sequence similarity to unigenes of *D. salina* with 26.2%, followed by *Chlamydomonas reinhardtii* with 21.8%.

Of all the obtained sequences, 12 796 were assigned to the KOG/COG (Clusters of Orthologous Groups of proteins) database. Figure 4 illustrated that 26 categories were divided by function, and the largest number of unigenes were classified into 'general functional prediction'.

3.3 Gene ontology (GO) classification

The GO database was applied to classify functions of *D. salina* unigenes. Of all the unigenes assembled, 48 259 were assigned at least one GO term (Fig.5), among which 148 558 were assigned in the biological process category, 103 666 in the cellular component category and 58 594 in the molecular function category. In the biological process, the sequences were divided into 24 subcategories with the first two clusters, cellular (19.93%) and metabolic processes (17.85%). There were 21 subcategories divided in

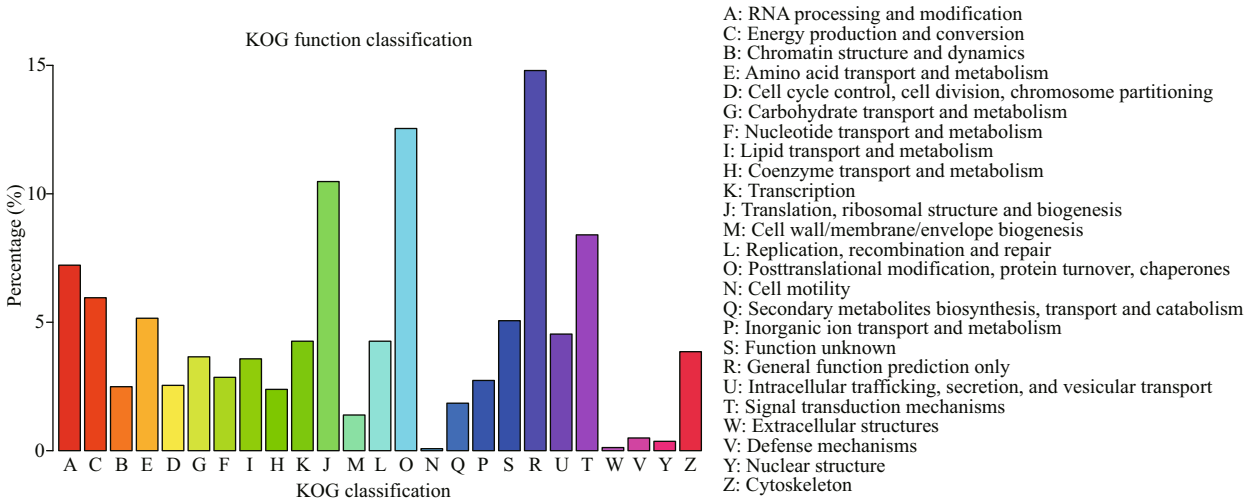

Fig.4 Eukaryotic orthologous groups (KOG) function classifications of *D. salina*

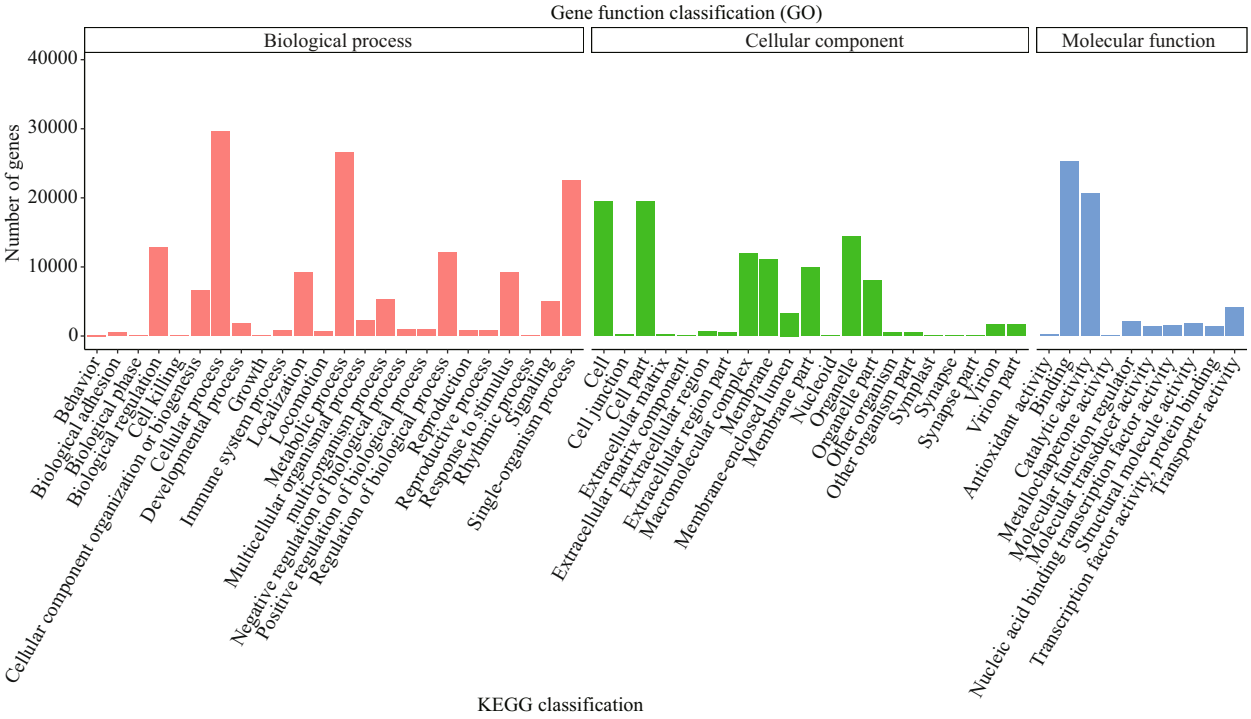

Fig.5 Gene ontology classification analysis of unigenes

The unigenes were classified into three functional categories: biological process, cellular component, and molecular function.

cellular components, in which ‘cell’ (18.83%) and ‘cell part’ (18.82%) were the most dominant. The sequences were divided into 10 subcategories, the most dominant subcategories of which were ‘binding’ (43.17%) and ‘catalytic activity’ (35.11%) in the molecular function category.

3.4 Kyoto Encyclopedia of Genes and Genomes (KEGG) classification

Assembled unigenes were then queried against the KEGG database. A total of 9 464 unigenes were assigned to KEGG pathways (Fig.6). There were five pathways in the classification: cellular processes (578), environmental information processing (235), genetic information processing (2 841), metabolism (5 616) and organic systems (190). Of all the pathways, metabolism occupied the largest number of genes (59.34%), which included the following with the most representation: energy metabolism (10.49%), carbohydrate metabolism (10.46%), overview (9.17%), amino acid metabolism (7.48%), metabolism of cofactors and vitamins (5.79%).

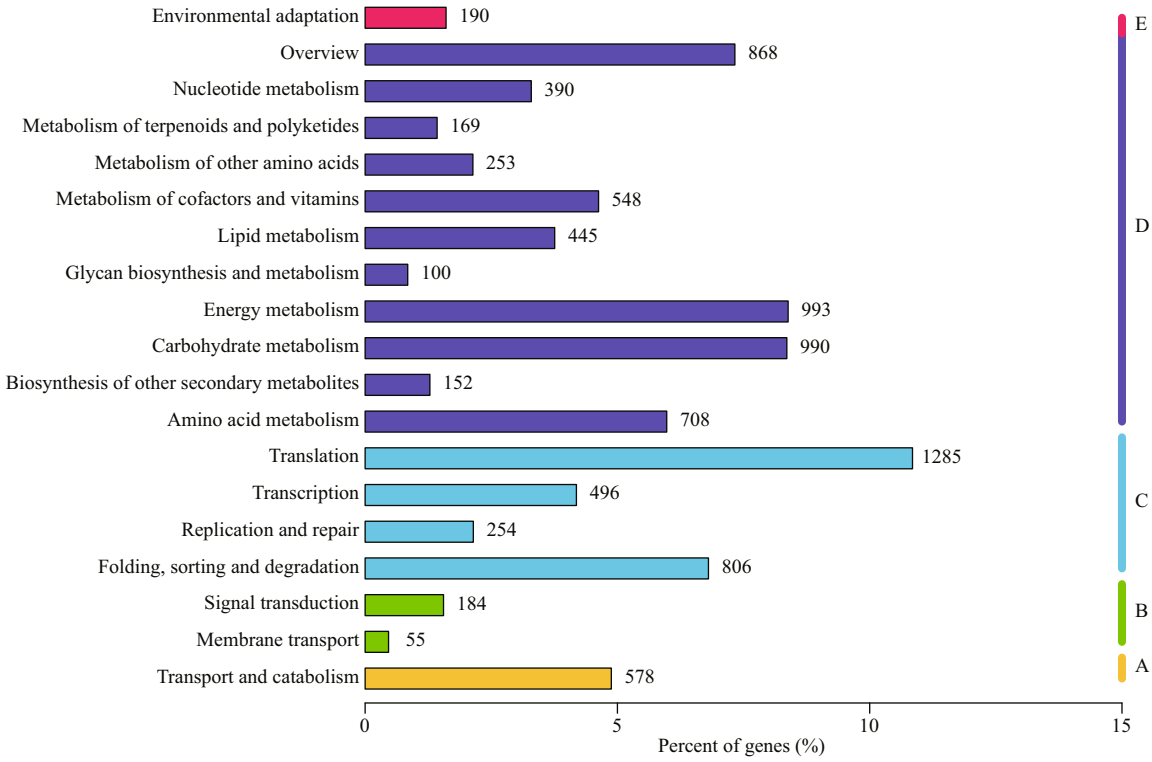

Fig.6 KEGG classification analysis of unigenes

The unigenes were classified into five main categories: A: cellular processes; B: environmental information processing; C: genetic information processing; D: metabolism; E: organic systems.

3.5 Differential expression analysis

Differential expression analysis of two groups (BL vs WL and RL vs WL) was performed using DESeq. Genes with an adjusted *P*-value <0.05 found by DESeq were assigned as differentially expressed. Ten randomly selected transcriptomic genes under different light conditions were verified by qPCR. Supplementary Fig.S2 illustrated a positive correlation between mRNA-Seq and qPCR results with eight genes matched. Differential expression analysis were based on the qualified unique sequences.

3.6 Differentially expressed genes related to photoreceptors

Plants utilize photoreceptors to regulate growth and development under different wavelengths of light conditions (Chen et al., 2004). Cryptochromes are one type of blue light receptors, which signal transduction is conserved in different plants (Lin, 2002). In the present study, six transcripts encoding for cryptochrome were identified under different wavelengths. As shown in Tables 2 & 3, they were up-regulated under both red and blue light, indicating that expression of photoreceptor genes in *D. salina* was induced by both red and blue light. Similar results were found in *Chlamydomonas reinhardtii*, where examination genes involved in chlorophyll and carotenoid biosynthesis exhibited a gradual rise during exposure under blue and red light (Beel et al., 2012).

3.7 Differentially expressed genes related to carotenoid biosynthesis

The transcript levels of genes involved in carotenoid biosynthesis in response to blue and red light were determined. Differentially expressed transcript levels of five genes involved in carotenoid biosynthesis under different wavelengths were also identified (Tables 2 & 3).

Phytoene synthase (PSY), which catalyzes the first step of the carotenoid biosynthesis pathway, is considered to be a rate-limiting enzyme in carotenoid biosynthesis (Sandmann et al., 2006). Constitutive PSY expression has enhanced total carotenoid contents and increased synthesis of β-carotene in *Dunaliella* species under various stress conditions (Pick, 1998; Coesel et al., 2008). Under blue light, two transcripts identified as PSY were up-regulated ~2.47–3.46-fold (Table 2). Increased expression of PSY has been shown to enhanced total astaxanthin contents in *H. pluvialis* grown under blue light (Ma et

Table 2 mRNA-Seq data for the genes involved in photoreceptors, carotene biosynthesis, and ROS-scavenging enzymes under blue light in *D. salina*

Gene name	Transcript ID	Fold change	padj
Light receptor			
Cryptochrome	Cluster-15 686.340 08	13.74	7.38E-04
Cryptochrome	Cluster-15 686.771 15	36.03	5.74E-03
Cryptochrome	Cluster-15 686.605 06	7.86	2.66E-02
Carotene biosynthesis and related genes			
Phytoene synthase	Cluster-15 686.594 92	3.46	1.30E-03
Phytoene synthase	Cluster-15 686.633 49	2.47	2.06E-02
Lycopene beta-cyclase	Cluster-15 686.522 53	7.12	1.46E-16
Lycopene beta-cyclase	Cluster-15 686.594 51	3.74	1.45E-08
Lycopene beta-cyclase	Cluster-15 686.321 50	3.39	2.58E-05
Lycopene beta-cyclase	Cluster-15 686.429 20	3.43	6.83E-05
Lycopene beta-cyclase	Cluster-15 686.369 46	2.01	1.53E-03
Lycopene beta-cyclase	Cluster-15 686.366 60	2.40	1.99E-03
Lycopene beta-cyclase	Cluster-15 686.614 42	12.07	4.56E-03
Lycopene beta-cyclase	Cluster-15 686.345 00	28.71	1.92E-02
Lycopene beta-cyclase	Cluster-15 686.771 44	21.04	3.12E-02
Lycopene beta-cyclase	Cluster-15 686.589 8	18.18	4.85E-02
Carotene globule protein	Cluster-15 686.529 05	5.71	2.29E-40
Carotene globule protein	Cluster-15 686.502 71	2.34	1.36E-08
ROS- scavenging enzymes			
Superoxide dismutase	Cluster-15 686.328 34	2.11	4.91E-03
Glutathione peroxidase	Cluster-15 686.281 83	4.05	1.64E-02

al., 2018). Lycopene β-cyclase (LCY) catalyzes the flux from lycopene to β-carotene. In our blue light experiment, ten transcripts encoding for LCY were up-regulated ~2.01–28.71-fold. Transcripts encoding for phytoene desaturase and ζ-carotene desaturase were not detected. Two transcripts identified as carotene globule proteins were up-regulated ~2.34–5.71-fold.

Transcripts expressed under red light were similar to those expressed under blue light (Table 3). Two transcripts encoding for PSY were up-regulated ~2.58–8.16-fold, and LCY was up-regulated ~2.23–2.34-fold. Increased expression of PSY under continuous red light has been previously observed in *Arabidopsis thaliana* (Von Lintig et al., 1997). The expression of carotene biosynthesis-related proteins (Cbr) for *D. bardawil* and *D. salina* is known to be induced during light stress (Banet et al., 2000). Single transcript for Cbr was up-regulated 8.21-fold. From the results of total carotenoids measurement results

Table 3 mRNA-Seq data for the genes involved in photoreceptors, carotene biosynthesis, and ROS-scavenging enzymes under red light in *D. salina*

Gene name	Transcript ID	Fold change	padj
Light receptor			
Cryptochrome	Cluster-15 686.841 31	6.97	2.01E-03
Cryptochrome	Cluster-15 686.862 1	6.29	3.72E-03
Cryptochrome	Cluster-15 686.605 06	8.07	2.63E-02
Carotene biosynthesis and related genes			
Phytoene synthase	Cluster-15 686.633 49	2.58	9.19E-03
Phytoene synthase	Cluster-15 686.315 49	8.16	3.68E-02
Lycopene beta-cyclase	Cluster-15 686.321 50	2.34	7.78E-03
Lycopene beta-cyclase	Cluster-15 686.300 11	2.23	1.74E-02
Carotenoid isomerase	Cluster-15 686.237 03	6.41	4.83E-03
Carotene biosynthesis-related protein	Cluster-15 686.545 05	8.21	1.84E-02

(Fig.1b), it could be observed that total carotenoid contents increased under both blue and red light compared with growth under white light. Therefore, we concluded that the increase of carotenoid contents under blue and red light occurred via the upregulation of carotenoid biosynthesis genes.

3.8 Differentially expressed genes involved in ROS generation

Several studies have reported increased level of ROS induced by blue light illumination (Consentino et al., 2015; El-Esawi et al., 2017). Here, the expression of two transcripts coding for ROS scavenging enzymes was detected: superoxide dismutase (SOD) and glutathione peroxidase (GPX). Under blue light, single transcript for SOD and GPX were identified to be up-regulated ~2.11–4.05-fold (Table 2). A greater induction of SOD activity in red alga *Gracilariopsis tenuifrons* was also observed under blue light compared with that under red and green light (Rossa et al., 2002).

Cryptochromes are blue light sensing receptors that regulate multiple processes in plants and algae (Lin, 2002). Several studies reported that cryptochrome activation induced expression of ROS-regulated transcripts (Jourdan et al., 2015). Enhancement of carotene generation induced by ROS had also been reported in *D. bardawil* (Shaish et al., 1993). Therefore, it is speculated that, blue light illumination induced higher cellular ROS, which in turn led to induction of carotenoid biosynthesis in the expressed genes.

4 CONCLUSION

The present study demostrates that red light was most efficient for promoting algal growth, and the expression of carotenoid biosynthesis genes was up-regulated compared with growth under white light. Blue light induced higher content of cellular carotenoids and the expression of ROS scavenging transcripts, which serve to protect algal cells from damage during photosynthesis. The identified cryptochrome transcripts may provide useful tools for developing a better understanding of photoreceptors in microalgae. The current findings will serve as a basis for future studies seeking to understand the mechanisms associated with the responses of *D. salina* to illumination by specific wavelengths of light.

5 DATA AVAILABILITY STATEMENT

The raw reads and processed data generated by this study have been submitted to the NCBI GEO database, the accession number is GSE128019.

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Electronic supplementary material

Supplementary material (Supplementary Table S1 and Figs.S1–S2) is available in the online version of this article at https://doi.org/10.1007/s00343-019-9064-2.