

Comparative De Novo Transcriptome Analysis and Random UV mutagenesis: Application in High Biomass and Astaxanthin Production Enhancement for *Haematococcus pluvialis*

Murat Telli (✉ tellim@ibu.edu.tr)

Bolu Abant İzzet Baysal University: Bolu Abant İzzet Baysal Üniversitesi <https://orcid.org/0000-0001-5546-0379>

Ünlü Ercan Selçuk

Bolu Abant İzzet Baysal Üniversitesi

Research Article

Keywords: De novo Transcriptomics, Astaxanthin, UV, Random Mutagenesis, *Haematococcus pluvialis*

Posted Date: July 7th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1835856/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Astaxanthin is a natural carotenoid with strong antioxidant capacity. The high demand on astaxanthin by cosmetic, food, pharmaceutical and nutraceutical industries promote its value in the biotechnological research. Several organisms have been characterized as direct or indirect source for astaxanthin. *Haematococcus pluvialis* has been characterized as one of the most promising species for astaxanthin biosynthesis. Even though *H. pluvialis* as an advantage in producing astaxanthin, its slow grow-yield limits usage of the species for large-scale production. In this study we generated mutated *H. pluvialis* strain by using one-step random UV mutagenesis approach for higher biomass production in the green flagellated period and in turn higher astaxanthin accumulation in red stage. Isolated mutant strains were tested for the astaxanthin accumulation and yield of biomass. Among tested strains only mutant strain designated as only MT-3-7-2 showed a consistent and higher growth pattern, the rest had shown a fluctuated and then decreased growth rate than wild type. To demonstrate the phenotypical changes in MT-3-7-2 is associated with transcriptome, we carried out comparative analysis of transcriptome profiles between MT-3-7-2 and the wild type strains. De novo assembly was carried out to obtain the transcripts. Differential expression levels for the transcripts were evaluated by functional annotation analysis. Data showed that increased biomass for the MT-3-7-2 strain was different from wild type with expression of transcripts upregulated in carbohydrate metabolism and downregulated in lipid metabolisms. Our data suggests a switching mechanism is enrolled between carbohydrate and lipid metabolism to regulate cell proliferation and stress responses.

Introduction

Astaxanthin ((3S-3'S)-dihydroxy- β,β -carotene- 4,4'- dione) is a xanthophyll carotenoid naturally synthesized by some plants, algae, fungi, and bacteria (Boussiba and Vonshak 1991; Johnson and An 1991). It shows a strong antioxidant activity to scavenge reactive oxygen species (ROS) due to unique configuration of thirteen conjugated double bonds in its chemical formula (Vershini 1999). Because of higher antioxidant activity compared to other antioxidant such as vitamin E, β -carotene and vitamin-C, astaxanthin have received considerable attention in application of cosmetic, food, pharmaceutical, nutraceutical and aquaculture industries (Hussein et al. 2006; Guerin et al. 2003; Lorenz and Cysewski 2000).

Haematococcus pluvialis Flotow 1844 (Chlamydomonadales, Chlorophyceae) freshwater green microalga, has been recorded as the most potent source of organism for natural astaxanthin biosynthesis (Li et al. 2011). A two-stage culture system of *H. pluvialis*, donated as green and red stages, has been commonly used to produce natural astaxanthin in commercial applications. In green stage, it is aimed to increase cell proliferation by providing optimum growth conditions of the culture. Subsequently, in red stage, hyperaccumulation of astaxanthin production in the cyst form of *H. pluvialis* is

accomplished by induction of stressors, such as salt addition, high light supply, or nutrient deficiency (Xi et al. 2016; Wang and Zarka 2003; Sarada et al. 2002).

Although, *H. pluvialis* is the richest source of natural astaxanthin, mass production for commercial production is challenging because of its slow growth rate, complex life cycle and sensitivity to fungal and bacterial contamination (Fujii et al. 2006). Consequently, developing mutagenic strains to improve growth rate and enhance astaxanthin accumulations has been widely applied in commercial applications. Random mutation induced by UV exposure, ethyl methane sulphonates (EMS) or N-methyl- N-nitro-N-nitrosoguanidine (NTG) treatments and subsequently selection of suitable mutant strains has been widely used to obtain more productive algal stains (Kamath et al. 2008; Chen et al. 2003; Fischer 1998). Astaxanthin-hyper producing mutants of *H. pluvialis* were isolated by UV exposure and EMS application in the studies of Chen et al. (2003) and Tripathi et al. (2001) at around 2.5mg/100 mg dry weight and 0.76 5mg/100 mg dry weight respectively. In the study of Chumpolkulwong et al. (1997), 2.0-fold higher astaxanthin was yielded from *Phaffa rhodozyma* mutants than wild type strains. The effectiveness of random mutation with UV and chemical treatments varied between 6% to 225% of higher astaxanthin accumulation than wild strains of *H. pluvialis* (Le-Feuvre et al. 2020).

Despite numerous studies revealing improving effects of random mutagenesis on astaxanthin accumulation, limited number of studies addressing corresponding genomic changing in those mutated strains for *H. pluvialis*. Ke et al. (2017) is the only study applied transcriptome-based analysis on a mutant strain of *H. pluvialis* to reveal genes related with photosynthesis and carbon fixation under high CO₂ exposure. Recently, several studies using transcriptomics, RNA-seq and proteomics approaches revealed transcriptional differentiation of *H. pluvialis* under different stress conditions (Gao et al. 2015; Su et al. 2018; Bangxiang et al. 2018). Moreover, Luo et al (2018) published a whole genome sequencing and transcriptomics data of wild type *H. pluvialis*. These studies helped to set up genome scale transcriptome structure and transcriptional expression profiles that improve our understanding on functional genes and associated pathways related with cell cycle and astaxanthin biosynthesis for *H. pluvialis*.

In this study we first aimed to develop mutated *H. pluvialis* strain for higher proliferation and biomass in green flagellated period and consequently higher astaxanthin accumulation in red stage per unit algae harvest by using one-step random UV mutagenesis. The second aim is to perform comparative *de novo* transcriptomics of wild and mutated strains to reveal putative transcripts and associated pathways enhancing proliferation and biomass in *H. pluvialis*.

Materials And Methods

Strains and culture conditions: Wild types of *H. pluvialis* was obtained from the culture collection of algae at Göttingen University, Germany (SAG 34-1b). The axenic stock culture was maintained in a 250 mL conical flask or on agar plates containing 200 mL of modified BG-11 medium (Stanier et al. 1971). The medium consisted of NaNO₃, 1.5 g; K₂HPO₄, 0.4 g; MgSO₄*7H₂O, 75 mg; CaCl₂*2H₂O, 36 mg; C₆H₈O₇, 6 mg; Ammonium Fe (III) citrate, 0.006 g; Na₂ EDTA*2H₂O, 1 mg; Na₂CO₃, 20 mg; H₃BO₃, 2.9 mg; MnCl₂*4H₂O, 1.8 mg; ZnSO₄*7H₂O, 0.22 mg; Na₂MoO₄*2H₂O, 0.39 mg; CuSO₄*5H₂O, 0.08 mg; Co(NO₃)*6H₂O, 0.05 mg Thiamine-HCl, 0.1 mg per liter of deionized water. The algal cultures were externally illuminated from the bottom of conical flask at a light intensity of 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light sourced by RGB LED light at a constant room temperature of 25 $\pm$ 0.5 °C using a cycle of 18 h light and 6 h dark. Aeration was supplied continuously through a sterile membrane filter of 0.2 μm in pore size at a rate of 250 mL min⁻¹, supplemented with 2 % CO₂ to maintain the pH between 7.2 and 7.5.

Screening and isolation of UV mutants: 10 ml of liquid *H. pluvialis* wild strain culture in early exponential phase ($\sim 6 \times 10^4$ cells/ml) were transferred petri dishes and exposed to UV-C (254 nm wavelength) light by portable UV-C lamp located from a distance of 20 cm for three different time periods of 3-5- and 8-min. Exposed cells were kept in dark for 24 h at 4 °C, then 50 μl of the cultures were spread on series of BG-11 agar plates and incubated at a light intensity of 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white LED light at a constant room temperature of 25 $\pm$ 0.5 °C using a cycle of 18 h light and 6 h dark. Light intensities were measured by OHSP-350 portable light meter (Hopocolor, China) from the center of the culture flask. Colony formations, cell size and morphology of UV mutated *H. pluvialis* were screened and recorded by Motic stereomicroscope mounted with a camera during 20 days of incubation period (Figure 1). Early developed large green colonies were chosen and transferred to sterile 24-well tissue culture plates containing 4 ml of BG-11 liquid medium. Growth rate of selected mutants were monitored for 3 subsequent generations by spectrophotometrically measured optical density (OD) at 669 nm (Figure 2). Each strain was replicated three times. Liquid cultures were incubated the same condition of the wild culture stock without aeration. Then, both wild type and selected mutant strains were transferred to 250 ml conical flask containing 200 ml BG-11 medium and incubated in the same condition with aeration of 250 mL min⁻¹, supplemented with 2% CO₂ for 3 more subsequent generations (Figure 3).

Astaxanthin accumulation: Liquid cultures of selected mutant and wild strains in mid of exponential phase are exposed to gradually increased high white light induction for astaxanthin accumulation. Three different light intensities were gradually applied from the bottom of the culture flask as continuous illumination by increasing the light intensity from 45 to 85 and finally 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at three-day intervals.

Growth analyses: The growth rate of isolated mutant and wild strains were monitored by cell counting in a Neubauer chamber and measuring OD measured at 669 nm using VWR (UV-6300 PC model) double beam UV visible spectrophotometer. Biomass was measured by dry mass. 10 mL culture were filtered through pre-dried and pre-weighted 0.2-µm cellulose nitrate membrane filters (Sartorius) and dried at 60°C overnight. Chlorophyll-a concentrations were measured spectrophotometrically according to Seely et al. (1972) with slight modifications: 4mL homogenized sample was centrifuged for 5 min at 5000 rpm, and the remaining pellet was re-suspended in 10 mL of 90 % (v/v) acetone and incubated overnight in dark at 4 °C after vigorous vortex. Then, the solution was centrifuged for 5 min at 5000 rpm, and the supernatant quantified spectrophotometrically at a wavelength of 665 nm.

Astaxanthin extraction: Astaxanthin extraction was conducted according to Sedmak et al. (1990) with some modifications: 4 mL culture was centrifuged at 3500 rpm for 5 minutes, and the retained samples were rinsed with distilled water. Subsequently, the samples were mixed with 5 % KOH in 30 % (v/v) methanol at 70 °C and vigorously vortexed for 5 min. Then the samples were centrifuged 3500 rpm again, the supernatant removed, and astaxanthin content extracted from the remaining pellet with 10 mL of dimethyl sulfoxide (DMSO) by using ultra sonification at 860 watt (Sonorex Super RK514, UK) at 55 °C for 10 min. DMSO and ultrasonic procedure was repeated until it resulted in a white pellet. The astaxanthin concentration was calculated spectrophotometrically at 490 nm using the equation of $c \text{ (mg/L)} = 4.5 \times A_{490} \times (V_a/V_b) \times f$, where c is astaxanthin concentration, V_a (ml) is the volume of DMSO, V_b (ml) is the volume of algal sample, f is the dilution factor (Davis, 1976).

Statistical analysis: The data obtained from growth rate, dry weight, cell size, chlorophyll-a and astaxanthin extraction of mutated and wild strains were statistical analyses utilizing Tukey HSD test to reveal the effects of mutagenesis. A p-value of < 0.05 was considered as statistically significant.

RNA extraction and cDNA library construction:

The frozen samples obtained at the end of the green stage of cultures were shipped to BGI facilities for sequencing. Following procedures were applied to each sample. MGIEasy RNA Library Prep Set (MGI, China) were used for total RNA extraction, mRNA enrichment and purification and cDNA library construction. RNA integrity was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA integrity ≥ 6.8 were chosen for subsequent analysis. Poly (A)-containing mRNA molecules were purified from total RNA using poly (T) oligo-attached magnetic beads, then mRNAs were fragmented into small pieces using fragmentation reagents. Then, first-strand cDNA was generated using random hexamer-primed reverse transcription followed by the second-strand cDNA synthesis and cDNA purification using purification reagents. cDNA fragments were then 3' end poly adenylated where adaptors were ligated subsequently. After purification of ligation products on TAE agarose gel, the purified cDNA templates were amplified by PCR amplification. Agilent Technologies 2100 Bio-analyzer (Agilent Technologies, USA) was used for the quality of final cDNA library. 100 bp pair-end readings from the cDNA sequencing library were sequenced by BGI (Shenzhen, China) using Illumina HiSeq4000 (Illumina, San Diego, CA, USA).

Sequence assembly and functional annotation:

After sequencing, adapter sequences, reads with unknown bases more than 5% and low-quality reads were filtered by using Short Oligonucleotide Analysis Package (SOAPnuke v.2x) (Chen et al 2018) developed by BGI to obtain clean reads. Then quality of the clean reads was checked by FastQC (v0.11.9) software (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). MultiQC (v1.11.0) (Ewels et al 2016) was used to merge FastQC results. 37.4 Gb pair-end reads were assembled into 288,504 clean transcripts by using Trinity tool (v2.12.0) (k-mer=25) (Has et al. 2013). The raw reads are deposited into NCBI SRA archive under PRJNA827483 Bioproject number. Clean transcripts were deposited into NCBI TSA archive under GJYM000000000. De novo transcriptome completeness analysis was performed by Benchmarking Universal Single-Copy Orthologs (BUSCO) v.5 (Eukaryota) (Simão et al. 2015). After completeness analyses CD-HIT (v4.8.1) (cd-hit-est, -c 0.90 -n 5 -d 0) was used to reduce the number of redundant sequences (Fu et al., 2012). Trinotate annotation suite (v2.0.2) (Bryant et al. 2017) was used for functional annotation of transcripts. Open reading frames with a minimum of 100 amino acids were predicted by using Transdecoder (<https://github.com/TransDecoder>) that the longest ORF were retained for further steps for overlapping ORFs. The longest ORFs were searched through the SwissProt/UniProt database (UniRef90) using BLASTX and BLASTP. Hmmscan tool (version 3.3.2) was used to search for homologies among protein domains through the Pfam database (Pfam-A.hmm). Trinotate annotations were transferred into the tab-delimited report file by 1e-5 cut-off e-value. A detailed annotation of the assembly data is provided in Supplementary Table 1.

Gene expression levels was calculated with RSEM (v1.3.3) (Li and Dewey 2011) and Kallisto (v0.46.2.0) (Bray et al. 2016). Differential expressed genes (DEGs) were identified between wild and mutated groups by using DEBrowser with DESeq2 (v1.32.0) (Love et al 2014). The distribution of DEGs of wild versus mutated types was shown in a scatter plot, MA plot, volcano plot and heatmap graphics. The Trinotate v2.0.2 pipeline (<https://trinotate.github.io/>) was used for the annotation of differentially expressed transcripts following similar pipeline described above.

Results

Growth Rates and Astaxanthin Accumulation of Selected Strains: Wild strain of *H. pluvialis* exposed to UV irradiation showed survival rate of $68,3 \pm 4,5$; $53,5 \pm 6,2$ and $38,7 \pm 3,2$ % with exposure times of 3, 5 and 8 min respectively. The first mutated green colonies were observed after 10 days of incubation in solid BG-11 medium. Around 60 algal colonies for each treatment were monitored based on color, colony size and morphology to identify potential fast-growing strains. Morphology of some of selected strain is given in Figure 1. The selected colonies were transferred to sterile 24-well tissue culture plates containing 4 ml

of BG-11 liquid medium and incubated the same conditions as stock culture without aeration and CO₂ supply. After strains turn to green in well plates, they were re-transferred to fresh medium with equal optical density. Then, among the 60 strains, only seven strains showing faster grown than wild type was selected to monitor their growth rate. Figure 2 show the growth rate of these selected seven strains after three subsequent generations cultured in 24-well tissue culture plates. Except for MT-3-7-4, all the mutated strains show higher growth rate than wild types of *H. pluvialis*. Selected strains were then transferred to 250 mL culture flaks containing 200 mL BG-11 aerated with 2% CO₂ supplementation and cultured three subsequent generation to test if there is a consistent in the growth rate performance in scale up process. However, only MT-3-7-2 showed a consistent and higher growth pattern, the rest had shown a fluctuated and then decreased growth rate than wild type. Inconsistent strains were excluded from analyses, and we used only MT-3-7-2 and two lower growth strains for further experiments. Figure 3 show growth rate of those mutated and wild strains cultured in 250 mL culture flaks containing 200 mL BG-11 aerated with 2% CO₂ after three subsequent generations. Table 1 show astaxanthin accumulation measured after 14 days of high light induction from mutated strains and wild type. Astaxanthin accumulation in MT-3-7-2 is around 45% higher ($P < 0.001$, Tukey HSD test) after being cultured six generation compared to wild type.

Overview of transcriptome assembly. After quality control, *H. pluvialis* RNA-Seq data containing 37.4 Gb paired-end raw reads assembled into 185,312 transcripts with mean length of 1083 bp and GC content of %59.41%. The N50 length was 1614 (Table 2). BUSCO completeness analyses against eukaryote database showed that 82.4% complete, 9.4% partial, and 8.20% missing core genes among a 255 total number of core genes queried. 76.5 % of detected core genes showed more than one ortholog genes (Table 3).

Functional annotation of differentially expressed transcripts:

Analysis of differentially expressed gene analysis revealed that a total of 66 transcripts were differentially expressed in mutant strain compared to wild type. Statistical summary of differentially expressed transcripts are provided in Supplementary table 2. In order to estimate the functional distribution of DEGs we carried out functional annotation analysis.

Among 38 upregulated transcripts (Supplementary table 3), 12 nonredundant “cellular component (CC)”, 29 nonredundant “molecular function (MF)” and 24 nonredundant “biological process (BP)” terms were identified for a total of 18 transcripts. We observed that ATP binding (GO:0005524) was enriched among the upregulated genes in addition to transcripts related with amino acid metabolism such as L-leucine transaminase activity (GO:0052654), L-valine transaminase activity (GO:0052655), L-isoleucine

transaminase activity (GO:0052656). We also observed enriched BP term related with carbohydrate metabolism such as maltose metabolic process (GO:0000023), starch catabolic process (GO:0005983), glucose metabolic process, (GO:0006006), pentose-phosphate shunt (GO:0006098) among upregulated transcripts. In parallel with the BP terms, carbohydrate metabolism related terms were dominated in CC terms among upregulated genes such as mitochondrion (GO:0005739), amyloplast (GO:0009501), chloroplast (GO:0009507), chloroplast stroma (GO:0009570), chloroplast envelope (GO:0009941).

Five of 27 downregulated transcripts did not match any entry in the databases analyzed (Supplementary table 4). Among 22 annotated transcripts, 21 nonredundant “molecular function (MF)”, 14 nonredundant “biological process” and 8 nonredundant “cellular component (CC)” terms were identified for a total of 16 transcripts. Under MF terms that might be related with signal transduction and protein modification translation were dominated such as 3-beta-hydroxy-delta5-steroid dehydrogenase activity (GO:0003854), calcium ion binding (GO:0005509), GTP binding (GO:0005525), serine-type carboxypeptidase activity (GO:0004185), serine-type endopeptidase activity (GO:0004252). In addition, two terms related with translation (structural constituent of ribosome) (GO:0003735), translation initiation factor activity (GO:0003743) were observed. Signaling (steroid biosynthetic process) (GO:0006694), small GTPase mediated signal transduction (GO:0007264), protein biosynthesis (rRNA processing) (GO:0006364), translation (GO:0006412) and protein modification (protein folding) (GO:0006457) terms were also identified under BP terms for down-regulated transcripts which were also dominated in CC terms such as ribosome (GO:0005840) and endoplasmic reticulum (GO:0005783). CC terms related with light harvesting and/or photoprotection were observed such as chloroplast thylakoid membrane (GO:0009535), photosystem I reaction center (GO:0009538), chloroplast envelope (GO:0009941), plastoglobuli (GO:0010287).

Cluster of ortholog analysis (COG) showed that 25 terms were assigned for the up regulated and 12 terms were assigned for down regulated transcripts (Figure 4). The highest number of differentially expressed genes were dominated under “posttranslational modification, protein turnover, chaperones”, and “amino acid transport and metabolism” function classes. In addition, six function classes “cell cycle control, cell division, chromosome partitioning”, “lipid transport and metabolism”, “signal transduction mechanisms”, “intracellular trafficking, secretion, and vesicular transport”, “replication, recombination and repair”, and “cytoskeleton” were specifically assigned for up regulated transcripts.

KEGG analysis results showed that all differentially expressed transcripts were clustered under 4 main groups (Figure 5). Data suggested that most of the upregulated transcripts were dominated under “Metabolism”, “Genetic Information Processing”, and “Environmental Information Processing”. On the

other hand, “Signaling and cellular processing category” was dominated by downregulated transcripts except for cytoskeleton proteins.

Discussion

Random mutagenesis by physical or chemical agents to acquire high-biomass and high-yield astaxanthin strains of *H. pluvialis* has been widely applied for commercial purposes. Such improved strains of microalgae have also provided unparalleled opportunity to improve our knowledge of metabolic process associated with cell proliferation and astaxanthin biosynthesis. However, there are limited study addressing corresponding molecular changing of those mutated strains at transcriptional or genomic level. Accumulation of transcriptomics data obtained from physically and chemically treated strains would enlighten the mutation mechanisms of exogenous and indigenous agents in chromosomal structure of algae cells and associated phenotypic responses. Different mutagenic agents may target or able to penetrate different parts of genome based on chromosomal structure that led to alteration of different molecular pathways.

In the case *H. pluvialis* species, seems that while UV mediated mutagenesis generated high biomass strains, chemically induced mutagenesis resulted in astaxanthin overproducing mutants. Wang et al (2008) reported a higher biomass strain of *H. pluvialis* treated by UV mediated mutagenesis, and then a high astaxanthin accumulated strain treated by subsequent EMS mutagens. Chemical mutagenesis such as EMS and NTG shortened the growth rate and enhanced astaxanthin accumulation (Bon et al. 1997; Shaish et al 1991). Sandesh et al (2008) reported up to 59% astaxanthin increased in *H. pluvialis* treated by combination of UV and 1-methyl 3-nitro 1-nitrosoguanidine (NTG) along with increased transcript level of astaxanthin pathways genes. In this study, a one-step random UV mutagenesis was applied to obtain a fast grown and high biomass strain of *H. pluvialis*. Based on fast colony forming screening strategy after 3 minutes UV exposure, we obtained MT-3-7-2 strain showing 47% increase of dry weight and 38 % increase of cell number at the end of green stage compared to wild type. Astaxanthin accumulation is also show 45% increment by culture volume in MT-3-7-2 after high light induction. Astaxanthin accumulation ratio by dry weight in wild type is 3.25% which is just slightly higher than wild type %3.19. That might indicate, acquired 45% astaxanthin increment by culture volume bases in mutated strain is possibly resulted from the improved biomass in MT-3-7-2.

The molecular mechanisms underlying algal cell proliferations and growth is poorly studied and there is only limited information in the algae literature (Oldenhof et al., 2004). Our comparative transcriptomics assembly results between high biomass mutated and wild strains revealed 64 DEGs ($\log_2 [\text{ratio}] \geq 1$ and $P_{\text{adj}} \leq 0.05$), with 37 upregulated and 27 downregulated transcripts (Supplementary table 2) that may indicate gens regulating cell proliferations and/or associated mechanisms. KEGG enrichment analyses

clustered the DEGs into 22 groups and 4 classes of metabolism, genetic information processing, environmental information processing and signaling and cellular process (Figure 5). In mutated strain, while transcripts associated with amino acid metabolisms, carbohydrate metabolisms, mitochondrion, amyloplast and chloroplast biosynthesis are upregulated, transcripts associated with lipid metabolisms, signal transduction, protein modification and translation were down regulated. Functional annotation analyses of DEG transcripts (Supplementary tables 3 and 4) revealed more carbohydrate centric and lipid-less metabolism at transcriptional level accompanying with fast growth and high biomass phenotype in MT3-7-2 than wild type of *H. pluvialis*.

Endosymbiotic association between eukaryotic host and cyanobacterial cells which has been transformed into a plastid has a synchronized cell division in cell cycle (Rodriguez-Ezpeleta and Philippe 2006). Several regulatory systems have been evolved for the coordinated cell proliferation between chloroplast and algae cell. For example, cytokinesis of algae cell is only completed following chloroplast divided once per cell cycle (Miyagishima et al 2012). Synchronization of such plastid and host genome is coordinated by retrograde signaling genes involving known seven different signal classes, yet the mechanisms and genes involved in these retrograde signaling pathways are still largely unknown (Kleine and Leister, 2013). One of those signaling transduction classes is related with sugar metabolisms and it may play a central role in regulation photosynthesis rate and cell proliferation. Carbohydrates as an end-product of photosynthetic CO₂ assimilation can be act as signaling element to regulate photosynthesis rates and cell division by modulating expression of nuclear and chloroplast encoded genes (Hausler et al 2014). Therefore, it can be concluded that chloroplast can sense concentration of carbohydrates in cytosol that regulates plastome-encoded expression for chloroplast division and cell proliferation subsequently (Hausler et al. 2014, Schmitz et al. 2014). In this study, 2 upregulated transcripts were enriched (TRINITY_DN2891_c0_g1_i2 (NCBI accession: GJYM01113419) and TRINITY_DN3921_c0_g1_i4 (NCBI accession: GJYM01144298) with fold changes of 61.6 and 3.1 respectively) in our mutated strain with GO terms related to carbohydrate metabolism of chloroplast. These upregulated transcripts might support the above-mentioned studies that high carbohydrate biosynthesis may act as signaling transducers to propagate cell proliferation by regulating nuclear and plastid encoded gene of expression.

During the photosynthesis, both carbohydrates and nonpolar lipids like triacylglycerol (TAG) are produced as energy storage in microalgae cells. Microalgae also produce polar lipids like glycerophospholipids, and some sterols involved in cellular structures and cell signaling pathways and plays key roles to regulate cellular response mediated by environmental stressors (Yonghua et al. 2019). However, under favorable conditions, microalgae strains producing high biomass tend to produce relatively low lipid content compared to carbohydrates (Alishah et al. 2019). In our transcriptomics assembly, we annotated several downregulated DEG transcripts associated with lipid synthesis and putative signaling pathways may

indicate regulating genes for such carbohydrate centric and lipid-less metabolism at transcriptional level in high biomass MT-3-7-2 mutated strain of *H. pluvialis*.

The sequence data of the downregulated transcript, TRINITY_DN1082_c0_g1_i5 (NCBI accession: GJYM01069039), was in mutated strain suggest that this gene is associated with acetyl-CoA C-acetyltransferase activity. Acetyl-CoA C-acetyltransferase involve in steroid biosynthesis through mevalonate (MVA) pathway in cytosol and plastid by condensation of two acetyl-CoA into acetoacetyl-CoA which is initiation steps of steroid and TAG synthesis (Fabris et al. 2014). Moreover, another downregulated DEGs transcript (TRINITY_DN3970_c0_g2_i2 (NCBI accession: GJYM01144659)) was enriched under GO terms related with steroid and lipid biosynthesis. Annotation data suggest that these two transcripts were involved in signaling pathways related with steroids and other lipids via 3-beta-hydroxy-delta5-steroid dehydrogenase activity. We also identified two differentially expressed transcripts associated with signaling pathways such as small GTPase mediated signal transduction (TRINITY_DN2892_c0_g1_i1 (NCBI accession: GJYM01112732)) and calcium cell signaling in endoplasmic reticulum (TRINITY_DN4735_c0_g1_i6 (NCBI accession: GJYM01141616)) where cytoplasmic TAG is synthesized. The downregulated transcript TRINITY_DN1140_c0_g1_i1 (NCBI accession: GJYM01111457) was associated with plastoglobules of PS-I reaction center. Plastoglobules in chloroplast are monolayer globular lipoprotein droplets attached to thylakoids, and it is suggested that they are involved in environmental stress responses by changing its size and number (Brehelin and Kessler 2008). Although, they are generally considered as passive lipid storage droplets, a series of recent studies indicate that plastoglobules proteins may play a role in plastid differentiation and lipid remodeling by exchanging lipid molecules between compartments of thylakoid membrane (Kessler and Vidi 2007; Brehelin and Kessler 2008; Rottet et al. 2015).

Switching energy storage form from carbohydrate to lipid, protein or carotenoids under stress conditions is well studied for commercial large scale microalgae production. However underlying molecular mechanisms remains poorly studied (Merchant et al 2012). Although our transcriptomics results do not address stress related transcriptional profile, DEGs in our high biomass mutated strain would enlighten candidate genes playing roles in lipid biosynthesis, as well as genes prominent in cell proliferation. Nevertheless, we partially identified and annotated DEGs transcripts, 16 of 38 upregulated and 21 of 27 downregulated transcripts are not annotated in or GO analyses. These DEG transcripts might also be unique genes for *H. pluvialis* or unidentified transcripts regulating cell proliferation, carbohydrate and lipid metabolisms or related of those signaling pathways genes.

Conclusion

Comparative *de novo* assembly of transcriptome results revealed a clear picture in gene ontology of differentially expressed transcripts between wild and mutated strains of *H. pluvialis*. High biomass

mutated strain diverged from wild type with expression of transcripts upregulated in carbohydrate metabolism and downregulated in lipid metabolisms. It can be concluded that a switching between carbohydrate and lipid metabolism is a mechanism regulating cell proliferation and stress responses. GO enrichment analyses of DEGs transcripts annotated as hypothetical or uncharacterized proteins might be a useful source to study if they are involved in signal transduction regulating carbohydrate and lipid metabolisms in *H. pluvialis*.

Declarations

ACKNOWLEDGEMENTS

This study was supported by Republic of Turkey Ministry of Agricultural and Forestry, General Directorate of Agricultural Research and Policies (TAGEM, Project No: 18/AR-GE/60) and Bolu Abant İzzet Baysal University Research Foundation (Project No: BAP – 2018.03.01.1317). We would like to thank the Bolu Abant İzzet Baysal University Department of Biological Sciences and Beta ALG Biotechnology Company for their support. The bioinformatics analysis steps reported the manuscript were performed at TUBITAK ULAKBIM, High Performance and Grid Computing Center (TRUBA Resources).

References

Alishah Aratboni H, Rafiei N, Garcia-Granados R. et al. (2019) Biomass and lipid induction strategies in microalgae for biofuel production and other applications. *Microb Cell Fact*,18: 178.

<https://doi.org/10.1186/s12934-019-1228-4>

Bangxiang H, Lulu H, Manman D, Jiawei S, Xiaoyun H, Yating D, Xiaomei C, Feng Z, Xuecheng Z and Xiaonan Z (2018) Transcriptome Analysis in *Haematococcus pluvialis*: Astaxanthin Induction by High Light with Acetate and Fe^{2+} . *International Journal of Molecular Science*: 19: 175.

doi:10.3390/ijms19010175

Boussiba S and Vonshak A (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol*. 32:1077-1082.

Bray NL, Pimentel H, Melsted P, Pachter L. (2016) "Near-optimal probabilistic RNA-seq quantification." *Nature biotechnology* 34 (5): 525-527.

Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Dogru DP, Tae J. Lee et al. (2017) "A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors." *Cell reports* 18 (3): 762-776.

Chen Y, Li D, Lu W, Xing J, Hui B, Han Y. (2003) Screening and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. *Biotechnol Lett*;25:527– 529.

Chumpolkulwong N, Kakizono T, Nagai S, Nishio N (1997) Increased astaxanthin production by *Phaffia rhodozyma* mutants isolated as resistant to diphenylamine. *J. Ferment. Bioeng.* 83: 429–434.

Chen Y, Chen Y, Shi C, et al. (2018) SOAPnuke: a MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. *Gigascience.* 7(1):1-6. doi:10.1093/gigascience/gix120.

Davies BH (1976). Carotenoid. In Goodwin, T.W (Ed). *Chemistry and Biochemistry of Plant Pigments*, Vol.2. Academic Press, London, pp. 38-153.

Hussein,G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006). Astaxanthin, a carotenoid with potential in human health and nutrition. *Journal of Natural Products*, 69(3): 443 – 449. <https://doi.org/10.1021/np050354+> PMID:16562856

Gao Z, et al. 2015. Transcriptome analysis in *Haematococcus pluvialis*: astaxanthin induction by salicylic acid (SA) and jasmonic acid (JA). *PLoS One* 10 (10): e0140609.

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends Biotechnol* 21: 210–216.

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB et al. (2013) "De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis." *Nature protocols* 8 (8): 1494-1512.

Häusler RE, Heinrichs L, Schmitz J, Flügge UI (2014). How sugars might coordinate chloroplast and nuclear gene expression during acclimation to high light intensities. *Mol. Plant.* 7: 1121–1137.

- Ewels P, Magnusson M, Lundin S, Käller M (2016) MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. PMID: [27312411](#). doi: [10.1093/bioinformatics/btw354](#).
- Fabris MM, Matthijs S, Carbonelle T, Pollier MJ, Dasseville R, Baart GJ, Vyverman W, Goossens A (2014) Tracking the sterol biosynthesis pathway of the diatom *Phaeodactylum tricornutum*, *The New phytologist*, 204: 521-535.
- Fischer R. (1998) Isolation of mutants, a key for the analysis of complex pathways and for strain improvement. In: Verma, A. (Ed.), *Microbes for Health, Wealth and Sustainable Environment*. Malhotra Publishing House, New Delhi, India, pp. 739–751.
- Fu L, Niu B, Zhu Z, Wu S, Li W (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*, 28(23): 3150–3152. <https://doi.org/10.1093/bioinformatics/bts565>
- Fujii K, Imazato E, Nakashima H, Ooi O, Saeki A. (2006) Isolation of the non-fastidious microalga with astaxanthin-accumulating property and its potential for application to aquaculture. *Aquaculture* 262:285 – 293.
- Johnson EA, An GH (1991) Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* 11:297-326.
- Kamath BS, Vidhyavathi R, Sarada R, Ravishankar GA. (2008) Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis*. *Biosour Technol.* 99:8667-8673.
- Kim DK, et al. (2011) Transcriptomic analysis of *Haematococcus lacustris* during astaxanthin accumulation under high irradiance and nutrient starvation. *Biotechnol. Bioprocess Eng.* 16(4): 698.
- Kleine T, and Leister D (2013). Retrograde signals galore. *Front. Plant Sci.* 4, Article 45.
- Le-Feuvre R, Moraga-Suazo P, Gonzalez J, et al. (2020) Biotechnology applied to *Haematococcus pluvialis* Fotow: challenges and prospects for the enhancement of astaxanthin accumulation. *J Appl Phycol.* 32: 3831–3852. <https://doi.org/10.1007/s10811-020-02231-z>.
- Li B, Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12: 323. <https://doi.org/10.1186/1471-2105-12-323>

Li J, Zhu D, Niu J, Shen S, Wang G (2011). An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis*. *Biotechnology Advances*, 29(6), 568 – 574. <https://doi.org/10.1016/j.biotechadv.2011.04.001> PMID:21497650 .

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Maréchal-Drouard L, et al. (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*. 318: 245–250

Miyagishima SY, Suzuki K, Okazaki K, Kabeya Y (2012) Expression of the nucleus-encoded chloroplast division genes and proteins regulated by the algal cell cycle. *Mol. Biol. Evol.* 29: 2957–2970.

Luo Q, Bian C, Tao M, Huang Y, Zheng Y, Yunyun L, Li J, Wang C, You X, Jia B, Xu J, Li J, Li Z, Shi Q, Hu Z (2018) Genome and Transcriptome Sequencing of the Astaxanthin-Producing Green Microalga, *Haematococcus pluvialis*. *Genome Biol. Evol.* 11(1):166–173. doi:10.1093/gbe/evy263

Li K, Cheng J, Lu H, Yang W, Zhou J, Cen K (2017) Transcriptome-based analysis on carbon metabolism of *Haematococcus pluvialis* mutant under 15% CO₂. *Bioresource Technology*. 233: 313–321.

Kim DK, et al. (2011) Transcriptomic analysis of *Haematococcus lacustris* during astaxanthin accumulation under high irradiance and nutrient starvation. *Biotechnol. Bioprocess Eng.* 16(4):698.

Lorenz RT, Cysewski GR (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends in Biotechnology*, 18(4), 160 –167. [https://doi.org/10.1016/S0167-7799\(00\)01433-5](https://doi.org/10.1016/S0167-7799(00)01433-5) PMID:10740262

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, p. 550.

Nishimura O, Hara Y, Kuraku S (2017) gVolante for standardizing completeness assessment of genome and transcriptome assemblies *Bioinforma. Oxf. Engl.* 33, pp. 3635-3637, <https://doi.org/10.1093/bioinformatics/btx445>.

Oldenhof H, Biová K, Van Den Ende H, Zachleder V (2004) Effect of red and blue light on the timing of cyclin-dependent kinase activity and the timing of cell division in *Chlamydomonas reinhardtii*. *Plant Physiol. Biochem.* 42: 341–348.

Häusler RE, Heinrichs L, Schmitz J, Flügge UI (2014) How Sugars Might Coordinate Chloroplast and Nuclear Gene Expression during Acclimation to High Light Intensities. *Molecular Plant* 7: 1121–1137.

Rodriguez-Ezpeleta N, Philippe H (2006) Plastid origin: replaying the tape. *Curr. Biol.* 16: R53–R56.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM (2015) BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinforma. Oxf. Engl.* 31: pp. 3210-3212, <https://doi.org/10.1093/bioinformatics/btv351>.

Sarada R, Tripathi U, Ravishankar GA (2002). Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. *Process Biochemistry*, 37(6): 623 – 627. [https://doi.org/10.1016/S0032-9592\(01\)00246-1](https://doi.org/10.1016/S0032-9592(01)00246-1).

Schmitz J, Heinrichs L, Scossa F, Fernie AR, Oelze ML, Dietz KJ, Rothbart M, Grimm B, Flügge UI, Häusler RE (2014). The essential role of sugar metabolism in the acclimation response of *Arabidopsis thaliana* to high light intensities. *J. Exp. Bot.* 65: 1619–1636.

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*, 35(2): 171–205. <https://doi.org/10.1128/MMBR.35.2.171-205.1971> PMID:4998365

Su Y, et al. 2014. Metabolomic and network analysis of astaxanthin producing *Haematococcus pluvialis* under various stress conditions. *Bioresour Technol.* 170:522–529.

Wang B, Zarka A (2003) Astaxanthin accumulation in *Haematococcus pluvialis* (chlorophyceae) as an active photoprotective process under high irradiance. *J Phycol.* 39:1116–24.

Michiel VB, Proost S, Neste CV, Deforce D, de Peer YV, Vandepoele K (2013) "TRAPID: an efficient online tool for the functional and comparative analysis of de novo RNA-Seq transcriptomes." *Genome biology* 14 (12): 1-10.

Vershinin A (1999) Biological functions of carotenoids: diversity and evolution. *Biofactors* 10:99-104.

Beissona YL, Jay J, Fedosejevsb TE, Harwoodc JL(2019) The lipid biochemistry of eukaryotic algae. Progress in Lipid Research, 74: 31-68.

Xi, T, Kim D G, Roh SW, Choi JS, Choi YE (2016). Enhancement of astaxanthin production using Haematococcus pluvialis with novel LED wavelength shift strategy. Applied Microbiology and Biotechnology, 100(14), 6231-6238. <https://doi.org/10.1007/s00253-016-7301-6>

Tables

Table 1: Dry weight and astaxanthin accumulation of wild type and mutated strains at the end of the light induction. Chlorophyll-a and -b concentrations were measured before light induction at the day of 20.

Strains	Wild Type	MT-3-7-2	MT-8-1-1	MT-3-7-3a
Astaxanthin (mg L ⁻¹)	20.48 ± 1.93	29.70 ± 1.65	13.16 ± 1.95	18.84 ± 0.56
Dry Weight (g L ⁻¹)	00.63 ± 0.07	00.93 ± 0.07	00.76 ± 0.04	00.82 ± 0.03
Astaxanthin/ Dry Weight %	03.25 ± 0.01	03.19 ± 0.02	01.73 ± 0.02	02.30 ± 0.01
Chlorophyll-a (µg mL ⁻¹)	16.39 ± 0.51	23.74 ± 1.69	17.08 ± 1.91	19.11 ± 2.16
Chlorophyll-b (µg mL ⁻¹)	05.22 ± 0.14	07.18 ± 1.25	05.65 ± 0.42	05.92 ± 0.57

Table 2: Transcriptome summary statistics.

Total Pair-End Reads	37.4 Gb
Total Number of Assembled Sequences	185,312
Total Number of Assembled Bases	200,739,303
Mean Length (nt)	1083
N50	1614
N70	966
N90	450
GC Content (%)	59.41

Table 3. BUSCO de novo assembly completeness assessment results against eukaryote database.

Total number of core genes queried	255
Number of core genes detected	Complete 210 (76.5%) Complete + Partial 234 (82,4%)
Number of missing core genes	21 (8.2%)
% of detected core genes that have more than 1 ortholog	76.5
Scores in BUSCO format	C:82.4% [S:5.9%,D:76.5%],F:9.4%,M:8.2%

Figures

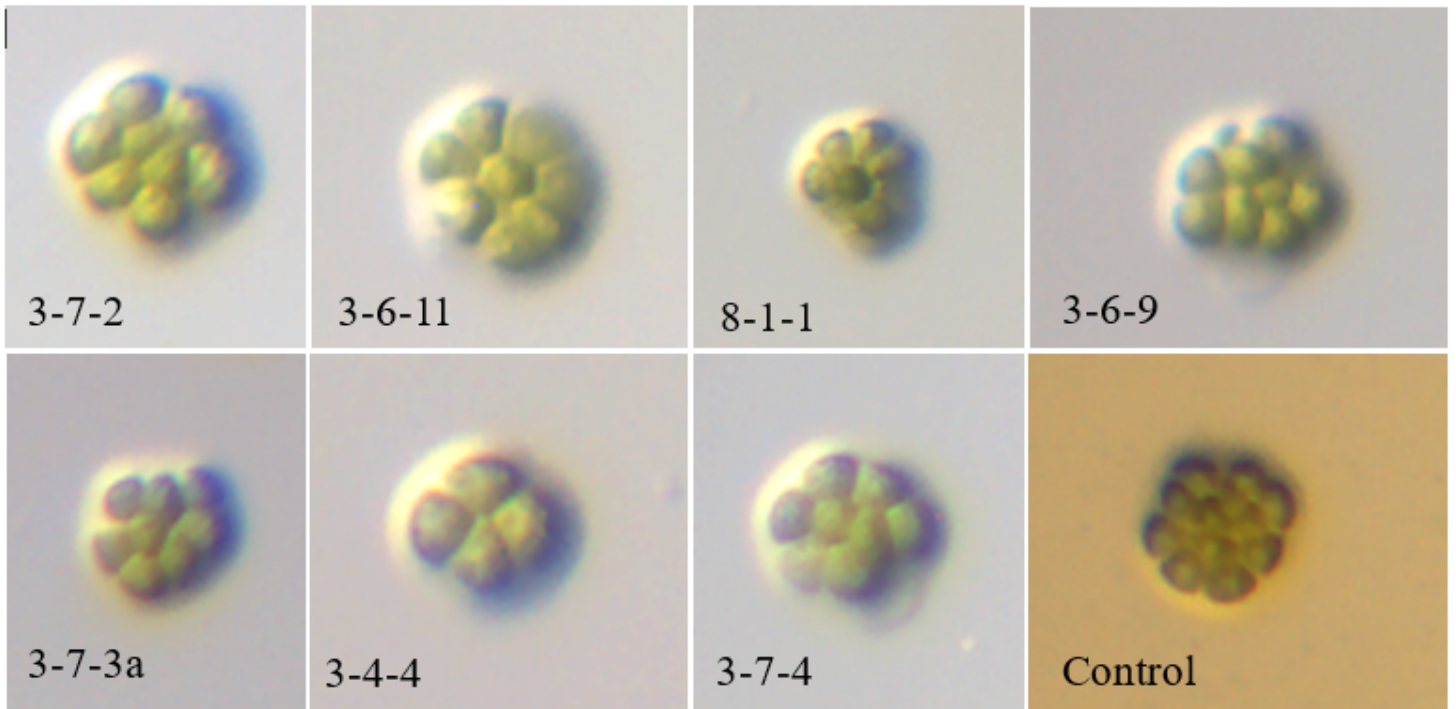


Figure 1

Colony morphology of control and mutant strains. Picture of the colony is taken by a stereomicroscope with 10x5 magnification.

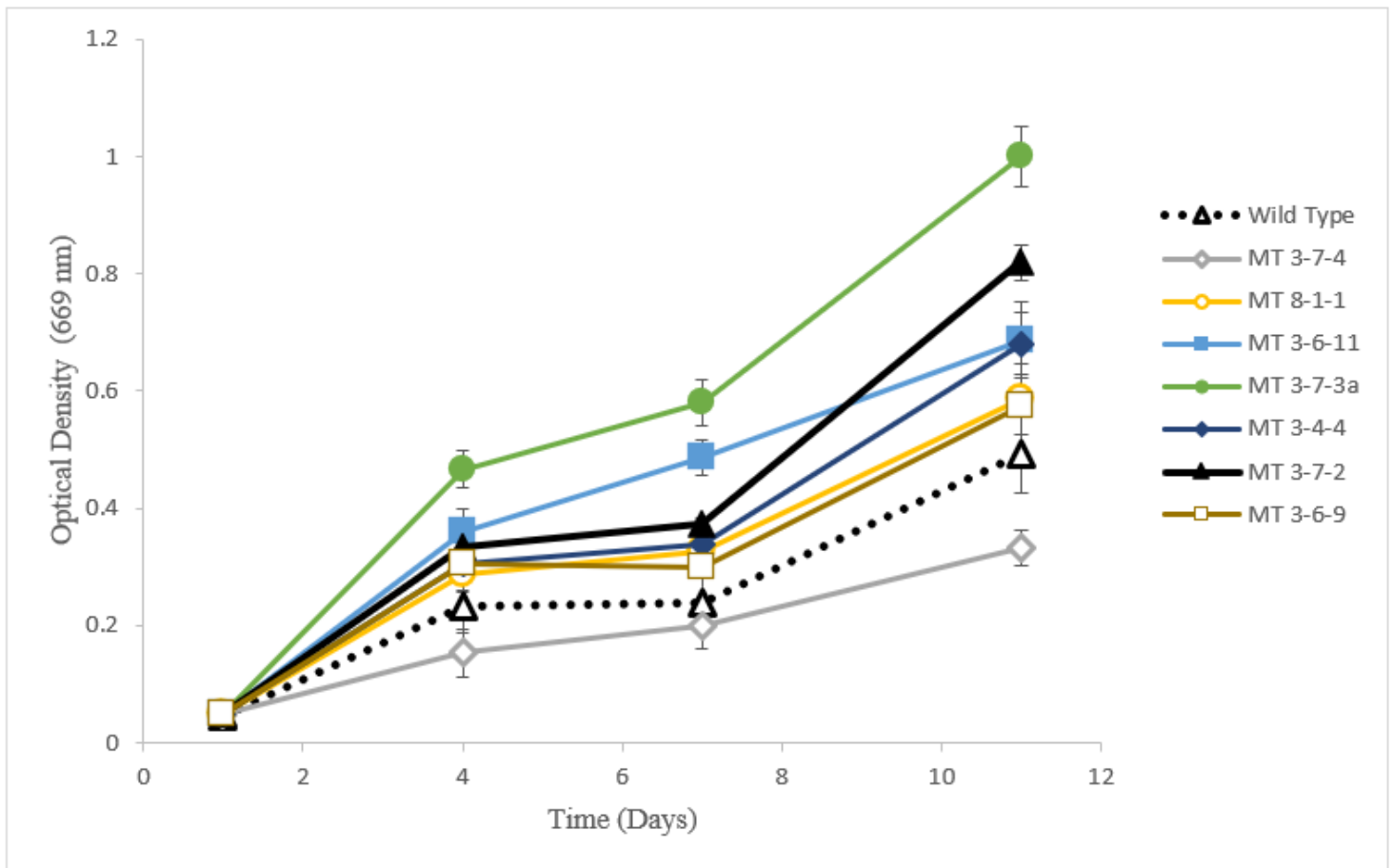


Figure 2

Growth rate of selected mutated strains and wild type of *H. pluvialis* grown in 24-well tissue culture plates containing 4 ml of BG-11 liquid medium without aeration and CO₂ supply. The data was obtained from the third generation.

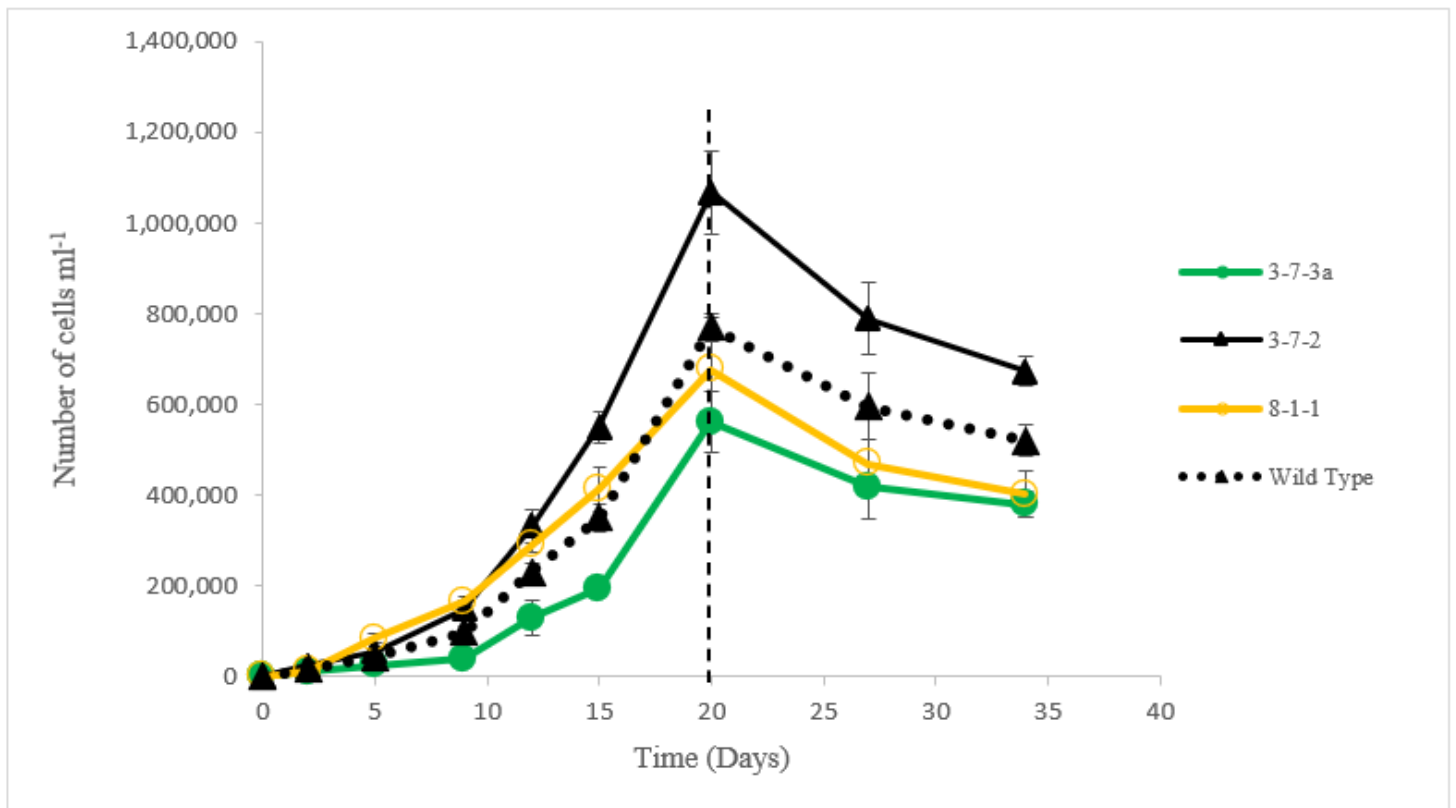


Figure 3

Growth rate of selected mutated strains and wild type of *H. pluvialis* grown in 250 mL culture flasks containing 200 mL BG-11 aerated with 2% CO₂. Cultures were induced high light intensity for astaxanthin accumulation after 20 days for 14 days. The data was obtained from the sixth generation.

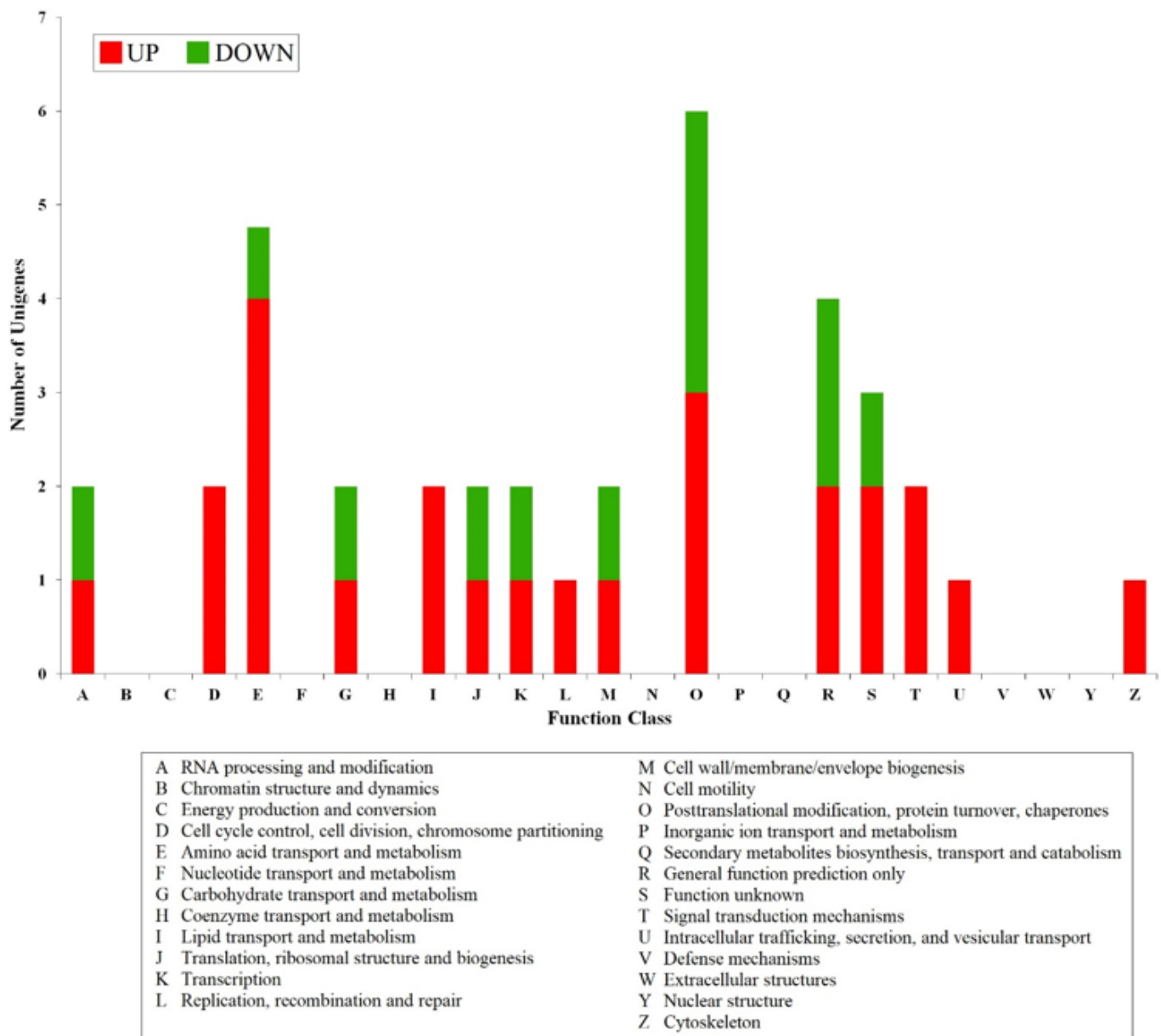


Figure 4

COG annotations for differentially expressed transcripts in wild type and MT-3-7-2.

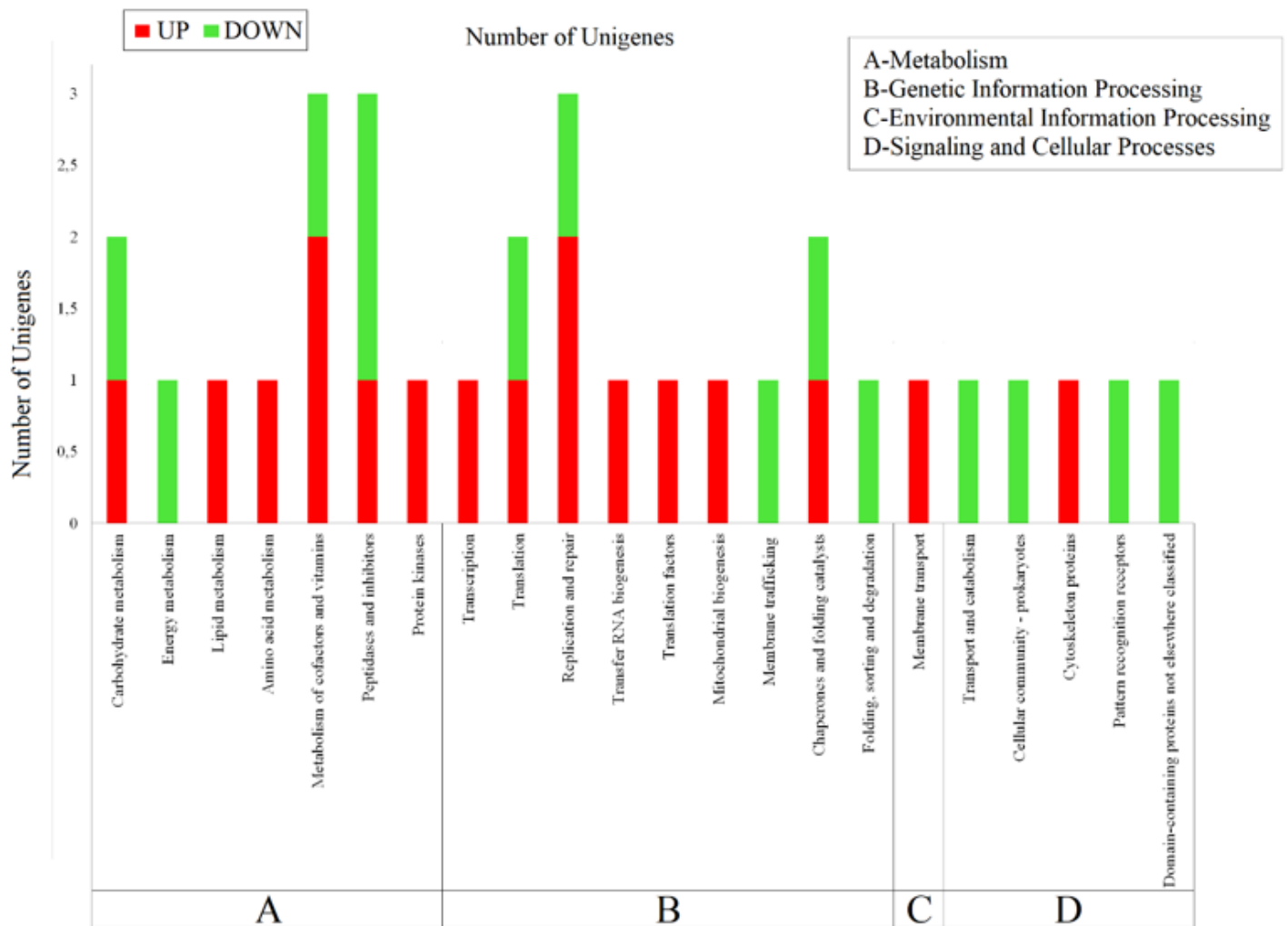


Figure 5

KEGG annotations for differentially expressed transcripts wild type and MT-3-7-2.

Supplementary Files

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

- [Supplementarytable1.xlsx](#)
- [Supplementarytable2.xlsx](#)
- [Supplementarytable3.xlsx](#)
- [Supplementarytable4.xlsx](#)