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Identification of $NaHCO_3$ Stress Responsive Proteins in *Dunaliella salina* HTBS using iTRAQ-based Analysis

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Abstract

Converting CO_2 to HCO_3^- through CO_3^{2-} as a carbon source for microalgae growth offers an alternative route for CO_2 fixation. There, however, are concerns over algae growth inhibition caused by high HCO_3^- concentration. The exact mechanism of the microalgae response to $NaHCO_3$ stress also needs further understanding. This study evaluated the effect of high HCO_3^- on enhancing growth of algae *Dunaliella salina* HTBS by identifying a series of differentially expressed proteins in responding to $NaHCO_3$ stress using iTRAQ (isobaric tags for relative and absolute quantitation) assay. Results showed that HTBS featured with adaptability to broad HCO_3^- concentrations (25 to 70 g/L $NaHCO_3$). The specific growth rate (OD_{750}) reached 1.08 day^{-1} at 25 g/L $NaHCO_3$. A total of 196 proteins were observed responsive to 25 g/L $NaHCO_3$ stress. Among these proteins, 84 proteins were up-regulated, whereas 112 down-regulated. Gene Ontology and KEGG pathway analysis showed that most of the up-regulated proteins were involved in transport and respiratory metabolism, and that the majority of the down-regulated proteins accounted for fatty acid oxidative metabolism and photosynthesis. These results provide new insights into the regulation of HCO_3^- tolerant mechanism in *Dunaliella salina* HTBS. These findings may be applicable to genetic engineering microalgae for high CO_2 fixation efficiency.

Keywords: *Dunaliella salina*; $NaHCO_3$ stress; Quantitative proteomics; Metabolite profiling

Introduction

Microalgae are a large and diverse group of photosynthetic organisms which can use of solar energy to fixed inorganic carbon (Ci) with efficiency (2-10%) that are ten times greater than higher plants (<1%) due to their high growth rate (1- to 3-fold increases in biomass per day) [1,2]. Although CO_2 is the predominant carbon source for microalgal cultivation, the low level of dissolution of CO_2 in water often leads to escape of large amounts of CO_2 from the medium during algae culture. To address this limitation, converting CO_2 to bicarbonate in alkali solution for high efficiency of absorption and storage of inorganic carbon (Ci) for microalgae culture is desirable [3]. HCO_3^- is also more advantageous to maintain the pH range of the culture medium from 6.4 to 10.3, whereas CO_2 tends to lower pH below 6.4 [4]. Furthermore, using bicarbonate would also provide a superior alternative for delivering CO_2 to an algae culture system [5].

The effects of various HCO_3^- concentrations (0.5, 1 or 2 g/L) on cell growth and biochemical composition have been well studied. Results on *Tetraselmis suecica*, *Nannochloropsis salina* and *Scenedesmus* sp. *CCNM 1077* demonstrated that the cell density, lipid and pigments contents were significantly improved with bicarbonate addition [6,7]. However, reports on responsive proteins to high HCO_3^- concentrations are very limited. Many fundamental biological questions related to biosynthesis under high HCO_3^- conditions and regulation of HCO_3^- tolerance need to be answered to understand how algae balance cation-anion and cope with the osmotic stress in the cell. Answering these questions also allows developing strategies to achieve high carbon utilization rate. However, the characteristics, diversities and evolutions of these high HCO_3^- tolerance microalgae genes and proteome related to Ci fixation are still not clear.

On the other hand, proteomics has been shown to be an effective way to study the changes of proteins as the final products of gene regulation from transcription until post-translational modifications. Proteomics has been used to study the responses to high NaCl and $NaHCO_3$ stress of tomato roots [8]. Similarly, proteomics was also employed for investigating effects of long-term nitrogen starvation or NaCl stress of *Chlamydomonas reinhardtii* [9], *Nannochloropsis oceanica* [10], *Chlorella protothecoides* [11] and *Anabaena doliolum* [12]. The metabolic reaction of microalgae is different from higher plant under stress as different proteins are responsive to different stress.

In the present study, a green microalga HTBS with high HCO_3^- tolerance was isolated. Important characteristics of HTBS were investigated with various $NaHCO_3$ concentrations (0-70 g/L). The algae reached highest specific growth rate (OD_{750}) of 1.08 day^{-1} with the initial $NaHCO_3$ concentration at 25 g/L. Then the comparative proteome differences of HTBS under 25 g/L HCO_3^- stress was analyzed using iTRAQ. A total of 2277 proteins were identified among which 196 proteins were found to be responsive to $NaHCO_3$ stress. These proteins mainly affect the ion transport and energy metabolism of HTBS. This highlights the effectiveness of proteomics approach for understanding bicarbonate response mechanism in HCO_3^- tolerance microalgae. These results provide new knowledge for understanding the mechanism of HCO_3^- tolerance of *Dunaliella salina* HTBS.

Materials and Methods

Strains and growth conditions

The HCO_3^- tolerant strain *Dunaliella salina* HTBS studied was isolated from the seawater in the Bohai Gulf (Tianjin, China). HTBS were cultured in f/2 medium under continuous artificial illumination at

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75 ± 10 μmol m^{-2} s^{-1}. Temperature and pH were controlled at 25 ± 1°C and 7.5 ± 0.2, respectively [13].

The $NaHCO_3$ treatment was carried out in two-steps. Firstly, cells were grown in sterilized f/2 medium in triangular flasks to reach the logarithmic phase, then 50 mL of the strains were transferred to columns diluted with 250 mL of f/2 medium, aerated with nitrogen to avoid the influence of CO_2 for 1 ~ 2 h; Secondly, 0 g/L, 25 g/L, 50 g/L, 70 g/L $NaHCO_3$ were added to the culture medium, respectively. All cultures were incubated under the same condition to monitor the change of biomass and total protein content caused by $NaHCO_3$ addition. Optical density was measured at 750 nm (OD_{750}) with spectrometer as described in Chi et al. [3]. Chlorophyll extraction and measurement were performed as described in Ma et al. [14].

Protein extraction, quantification and digestion

For each sample, cells were suspended in Plant Total Protein Lysis Buffer (7 M Urea, 4% CHAPS (3-[(3-cholamidop ropyl) dimethylammonio]-1-propanesulfonate), 2 M Thiourea, 40 mM Tris-HCl, pH 8.5, 2mM EDTA, 1 mM PMSF) and sonication cracking for 15 min in ice. Then the proteins were reduced with 10 mM DTT (final concentration) at 56°C for 1 h, alkylated by a final concentration of 55 mM IAM for 1 h in the darkroom. The reduced and alkylated protein mixtures were precipitated by adding 4x volume of chilled acetone at -20°C overnight. Protein pellet from previous step was dissolved in 0.5 M TEAB (Applied Biosystems, Milan, Italy) and sonicated in ice. After centrifuging at 30,000x g at 4°C, an aliquot of the supernatant was taken for determination of protein concentration by Bradford method [15] and SDS-PAGE. 100 μg proteins were taken out from each sample solution for treatment. The protein samples were digested with Trypsin Gold (Promega, Madison, WI, USA) with the ratio of trypsin: protein=1:30 for 16 h at 37°C.

iTRAQ labeling

Peptides were dried by vacuum centrifugation after trypsin digestion, then reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ Reagent kit (Applied Biosystems). Peptides were labeled with the iTRAQ tags as follows: Control (118 tag) and 25 g/L $NaHCO_3$ treatment (121 tag) were incubated for 2 h at room temperature. Then the labeled peptide mixtures were pooled and dried by vacuum centrifugation.

Fractionation by strong cationic exchange (SCX)

SCX chromatography was performed with LC-20AB HPLC system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled mixture peptides were reconstituted in Buffer A (25 mM NaH_2PO_4 in 25% acetonitrile, pH 2.7) and loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5 μm particle (Phenomenex). The mixture peptides were eluted with a gradient of buffer A at a flow rate of 1.0 mL/min for 10 min; 5-60% buffer B (1 M KCl, 25 mM NaH_2PO_4 in 25% ACN, pH 2.7) for 27 min; 60-100% buffer B for 1 min, maintained in 100% buffer B for 1 min; then equilibrated with buffer A for 10 min prior to the next injection. Elution process was monitored by measuring absorbance at 214 nm and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, each fraction was desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

LC-ESI-MS/MS analysis based on Triple TOF 5600

Each fraction was resuspended in buffer C (0.1% FA, 5% ACN) at a final peptide concentration of about 0.5 μg/μL on average, centrifuged at 20,000x g for 10 min. 10 μL supernatant was loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column. Then, the peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house. Samples were loaded at 8 μL/min for 4 min, then the 35 min gradient was run at 300 nL/min starting from 2% to 35% Buffer D (0.1% FA, 95% ACN), followed first by 5 min linear gradient to 60%, then by linear gradient to 80% for 2 min, and 4 min maintenance at 80% B, and finally returned to 5% in 1 min.

Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using 2.5 kV ion spray voltage, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150°C. The MS was operated with a RP of greater than or equal to 30,000 FWHM for TOF MS scans. For IDA, survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts/s and with a 2+ to 5+ charge-state. Total cycle time was fixed to 3.3 s. Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC (Time to Digital Convert) detector with four-anode channel detect ion. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (15 s), and then the precursor was refreshed off the exclusion list.

Database search and quantification

Discoverer 1.2 (PD 1.2, Thermo), [5600 msconverter] and the MGF file data were searched. Proteins identification and quantification were performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against NCBInrplant database (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=33090&lvl=3&lin=f&keep=1&srchmode=1&unlock) containing 1495258 sequences (Table S1).

For protein identification, 0.1 Da for fragmented ions and a mass tolerance of 0.05 Da (ppm) were permitted for intact peptide masses, with allowance for one missed cleavages in the trypsin digests. Gln->pyro-Glu (N-term Q), Oxidation (M), Deamidated (NQ) as the potential variable modifications, and Carbamidomethyl (C), iTRAQ8plex (N-term), iTRAQ8plex (K) as fixed modifications. Peptides charge states were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of database was generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides at the 95% confidence (P<0.05) interval by a Mascot probability analysis greater than "identity" were counted as identified. And each confident protein identification involved at least one unique peptide.

For protein quantitation, it was required that a protein contains at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in MASCOT. Only ratios with p-values <0.05 were used, and only fold changes >1.2 were considered as significant.

Function method description

Functional annotations of proteins identified were conducted using Gene Ontology (GO) annotation (http://www.geneontology.org/) against the non-redundant protein database (NR; NCBI). Proteins

were categorized according to their biological process, molecular function and cellular localization [16]. The differentially accumulated proteins were further assigned to the Clusters of Orthologous Groups of proteins (COG) database (http://www.ncbi.nlm.nih.gov/COG/) [17] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) [18,19]. GO and pathway enrichment analysis were performed to determine the functional subcategories and metabolic pathways in which the differentially accumulated proteins were significantly enriched.

Results and Discussion

Effect of $NaHCO_3$ concentration on HTBS cell growth

The effects of sodium bicarbonate concentrations ranged from 0 g/L to 70 g/L studied on the growth of HTBS are shown in Figure 1. The cells could grow well at high concentration of HCO_3^-. The control group without $NaHCO_3$ had a lower growth rate than 25 g/L, 50 g/L and 70 g/L $NaHCO_3$ treatment groups. Compared to 25 g/L $NaHCO_3$, cells growth rates in 50 g/L and 70 g/L $NaHCO_3$ decreased slightly, but were still higher than that of control group. The results indicated that HTBS could process high level of HCO_3^- in the medium, converting CO_2 to HCO_3^- through CO_3^{2-} as a carbon source for growth. The highest specific growth rate was measured as 1.08 day^{-1} in the culture with 25 g/L $NaHCO_3$. It implied that sodium bicarbonate supplementation enhances metabolic process and cell division in HTBS. Thus, 25 g/L $NaHCO_3$ was used in later experiments.

Effects of $NaHCO_3$ stress on expression changes of HTBS proteome

Total proteins were extracted from HTBS treated with 25 g/L $NaHCO_3$ for 24 h and from the corresponding control in two independent biological experiments [20]. Large-scale proteome analysis with quantitative information on proteins modulated under $NaHCO_3$ stress was explored using iTRAQ technique. 5% local false discovery rate (FDR) estimation was applied to ensure that erroneous quantification reports on proteome responses were minimized [21].

After data analysis, 300,848 spectra were generated of which 9,121 spectra matched known peptides, 2,908 unique peptides and 3,426 peptides. A total of 2277 proteins were identified in the experiment (Table S2-Sheet 1), 196 of these proteins were significantly regulated under $NaHCO_3$ stress comparing with control group. 84 proteins were up-regulated (Table S2-Sheet2) and 112 proteins were down-regulated (Table S2-Sheet 3) of these 196 proteins in the control *vs.* 25 g/L $NaHCO_3$ treatment (118 tags *vs.* 121 tags). These results indicate that $NaHCO_3$ stress had various effects on HTBS, including HCO_3^-, nitrogen and pH related reactions in a complex network involving multiple metabolic and physiological pathways. This study documented the variation in abundance of these differentially expressed proteins in response to HCO_3^- stress and implied that the algae detected the extent of HCO_3^--induced stress and alleviated it by modulating the expression of stress-responsive proteins.

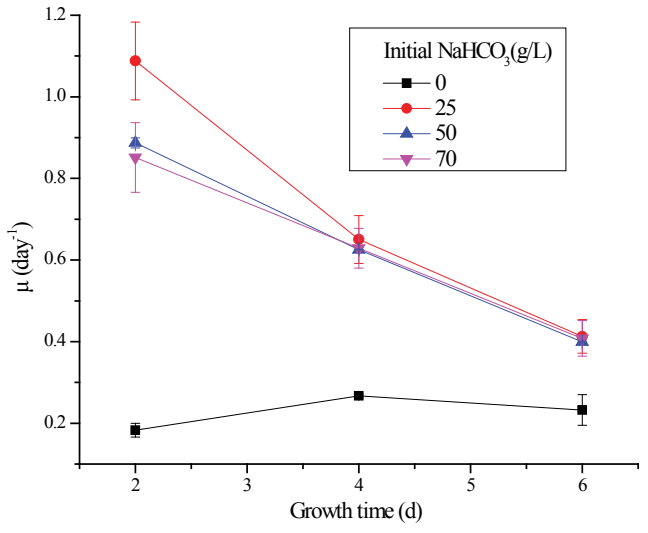

Figure 1: Specific growth rate of HTBS in different $NaHCO_3$ concentrations growth medium.

HCO_3^--responsive proteins by gene ontology analysis

To gain further biological functionality knowledge of the HCO_3^--responsive, 1917 protein out of the 2908 significantly unique peptides of HTBS in control and 25 g/L NaHCO3 treated groups were analyzed by Gene Ontology (GO) enrichment analysis ($P<0.01$, $FDR<0.05$) in the Protein Information Resource (PIR) database with three sets of ontologies: biological process (GO-BP), cellular component (GO-CC) and molecular function (GO-MF) [22] (Figure S1). The distribution of functional categories was shown in Figure 2. The main biological functional categories represented were cellular process, metabolic process, single-organism process, response to stimulus and cellular component organization or biogenesis. The most highly-enriched GO-BP category was metabolic process (20.53%), the second highly-enriched GO-BP category was cellular process (19.91%). This demonstrated that these processes were of functional importance for HCO_3^- responses in HTBS (Figure S2 A). In addition, the majority of the most abundant proteins in HTBS were related to twelve GO-MF categories involved in antioxidant activity contributed to the largest portion of the proteins (43.30%), with binding activity the second (42.97%) (Figure S2 B). For the GO-CC ontology, the top two categories were cell (21.70%) and cell part (21.70%), with organelle the third (19.38%) (Figure S2 C). A comparison with a comprehensive proteomics study of tomato roots under $NaHCO_3$ stress revealed 199 proteins that were uniquely identified in previous study [8].

Then 196 differentially accumulated proteins were assigned to 22 categories using the COG database. The main functional categories were metabolite transport and metabolism (43.37%), posttranslational modification, protein turnover, chaperones (13.27%), energy production and conversion (11.73%), translation, ribosomal structure and biogenesis (11.73%), Cytoskeleton (5.10%), cell wall/membrane/envelope biogenesis (2.04%), and transcription (1.02%) (Figure 3).

KEGG pathway analysis was used to evaluate whether the proteins with significant changes in abundance due to $NaHCO_3$ stress were clustered in specific metabolic pathways. These differentially accumulated proteins were further investigated using the KEGG database (P value ranges from 0 ~ 1) and were found to be enriched in metabolic pathways (24.21%), biosynthesis of secondary metabolites (10.90%), photosynthesis (4.84%), carbon fixation in photosynthetic organisms (3.87%), oxidative phosphorylation (3.63%), glyoxylate and dicarboxylate metabolism (3.39%), ribosome (3.39%), glycolysis/gluconeogenesis (2.42%), pyruvate metabolism (2.42%), and phagosome (2.42%) (Table 1).

Extension of the biological process model related with $NaHCO_3$ stress revealed by iTRAQ

As an overview of the regulated proteins, Table S2-Sheets 2 and 3 summarize all changed proteins in HTBS with 25 g/L $NaHCO_3$

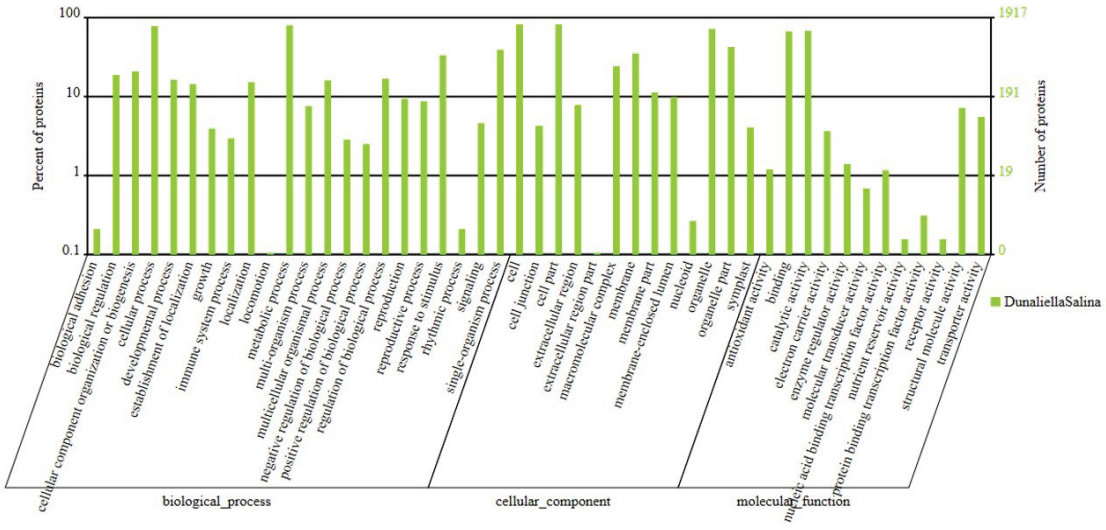

Figure 2: The biological process, cellular component and molecular function of HTBS in iTRAQ analysis.

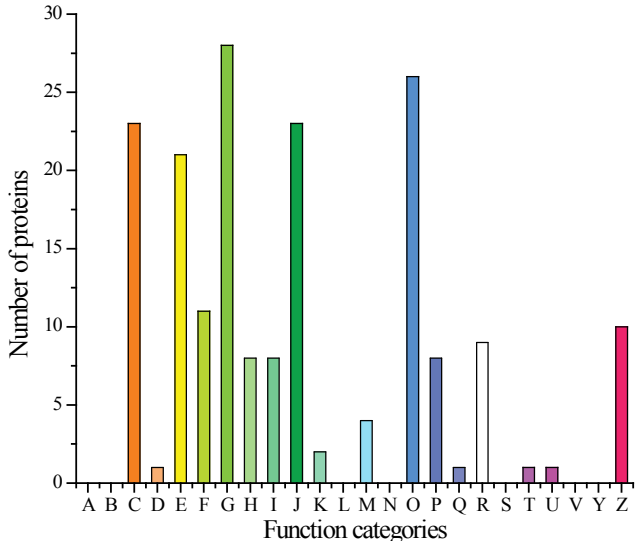

Figure 3: Clusters of orthologous groups of proteins (COG) classification of the differentially accumulated proteins in control and 25 $g \cdot L^{-1}$ $NaHCO_3$ treated. A: RNA processing and modification; B: Chromatin structure and dynamics; C: Energy production and conversion; D: Cell cycle control, cell division, chromosome partitioning; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; G: Carbohydrate transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; J: Translation, ribosomal structure and biogenesis; K: Transcription; L: Replication, recombination and repair; M: Cell wall/membrane/envelope biogenesis; O: Posttranslational modification, protein turnover, chaperones; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport and catabolism; R: General function prediction only; S: Function unknown; T: Signal transduction mechanisms; U: Intracellular trafficking, secretion, and vesicular transport; V: Defense mechanisms; Z: Cytoskeleton.

Pathway	Number of proteins	Pathway ID
Metabolic pathways	100	ko01100
Biosynthesis of secondary metabolites	45	ko01110
Photosynthesis	20	ko00195
Carbon fixation in photosynthetic organisms	16	ko00710
Oxidative phosphorylation	15	ko00190
Glyoxylate and dicarboxylate metabolism	14	ko00630
Ribosome	14	ko03010
Glycolysis/gluconeogenesis	10	ko00010
Pyruvate metabolism	10	ko00620
Phagosome	10	ko04145

Table 1: Pathway enrichment analysis of differentially accumulated proteins (P value ranges from 0 ~ 1).

treatment. A massive metabolic reprogramming was observed in response to $NaHCO_3$ stress. Functional classification showed that the differentially expressed proteins were mainly involved in mitochondrial metabolism, photosynthesis, lipid metabolism, vesicle trafficking and other functions. As the main responses to HCO_3^- stress, proteins regulating metabolism and energy conversion, carbon fixation in photosynthetic organisms and transport are summarized in Figure 4. When microalgae were allowed for adaptation under stress conditions, multiple changes occurred in proteins involved in nitrogen, carbon, and energy metabolism. As expected, there was an increase in abundance of many proteins involved in amino acid metabolism as well as in carbon assimilation and metabolism in response to HCO_3^- stress. There was also an increase in some proteins required for oxidative phosphorylation and glycolysis, indicating an increased need for ATP and energy (Figure S3).

Metabolism and energy conversion: To address $NaHCO_3$ stress, metabolic adjustments in HTBS were compensated by increasing cell growth rates and photosynthetic components, while accumulating amino acid, starch and citrate. Nitrogen metabolism enzymes including GS, GOGAT were up-regulation (Figure 4). It indicated that HTBS accumulated a range of metabolically benign solutes and many of which were N containing compounds, such as amino acids and amides, used to balance the osmotic stress as a result of high $NaHCO_3$ concentration. In a similar study, Pancha [7] also found that uptake of nitrate from the growth medium increased with the bicarbonate addition to the growth medium. Hence the nitrogen metabolism is of central importance not only in growth but also in stressful conditions. The results also show that ACCase and ACS were down-regulated under $NaHCO_3$ stress, implying significantly degradation of lipid accumulation in HTBS. It

was speculated that membrane system was injured under high $NaHCO_3$ conditions. Glutathione-S-transferases (GST), which is the enzyme involved in lipid metabolism, were overexpressed during adaptive evolution. Similarly research has been reported in Gong et al. [8]. The role of TCA cycle in HTBS had been defined well; only one protein (SDH) was up-regulated. There were four proteins (PDH, DLDH, DLAT, and PEPCK) associated with aerobic respiration being down-regulated by $NaHCO_3$ stress. As an essential enzyme catalyzed pyruvic acid to acetyl-CoA, PDH was down-regulated to decelerate TCA cycle. In addition, socitrate lyase (ICL), the major enzyme involved in glyoxylate shunt was also found to be depressed.

Carbon fixation in photosynthetic organisms: Observations of a decreased number of differentially expressed chloroplast proteins (especially lower abundance and down-regulation of Calvin cycle enzymes) revealed the potential role of carbon uptake and fixation pathways during $NaHCO_3$ stress in HTBS. It is reported that stress conditions significantly modulated anabolic and photosynthesis processes in *Chlamydomonas reinhardtii* [23]. Under some circumstances, the intracellular mechanisms are activated to sense total Ci levels in the medium in order to balance the use of carbon and nitrogen for protein or nucleotide biosynthesis [24]. On the contrary, the reduction in chlorophyll content was observed in this study (Table 2), enzymes involved in carbon fixation, carbon uptake and the major chlorophyll enzymes were depressed, especially Rubisco, Rubisco activase (RuBA), chlorophyll-ab-binding proteins (CBP) and light harvesting proteins (LCBP) (Table S2-Sheets 2 and 3 and Figure 4). Because the culture

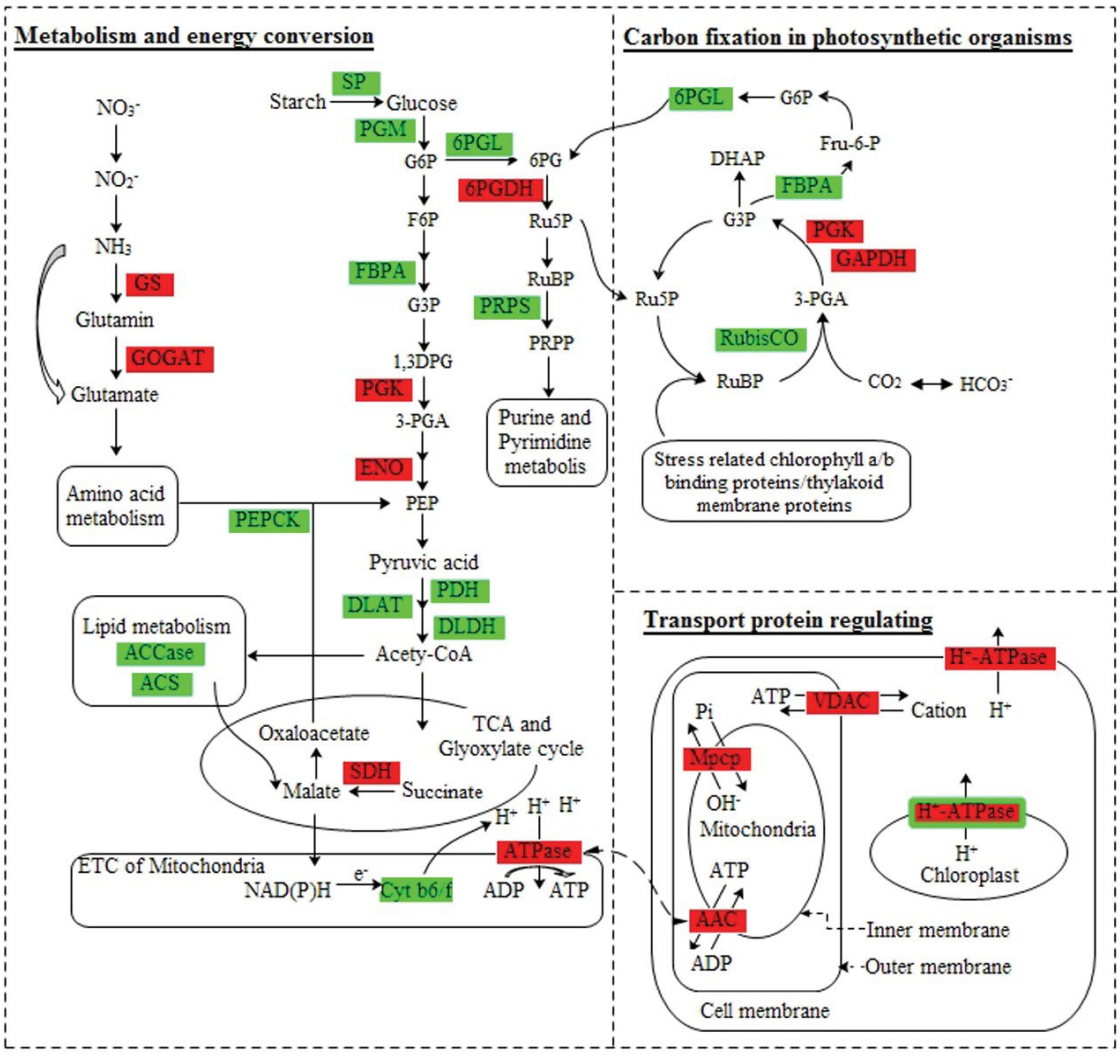

Figure 4: Summary of biological pathways affected under $NaHCO_3$ stresses in HTBS. Red boxes represent up-regulated proteins in $NaHCO_3$ stress, and green boxes indicate down-regulated proteins in $NaHCO_3$ stress, The Dual Color boxes represent proteins shown to have mixed expression patterns (both up-regulation and down-regulation). GS, Glutamine Synthetase; GOGAT, Glutamate Synthase; ACCase, Acetyl-CoA Carboxylase; ACS, Acyl-CoA Synthetase (long-chain-fatty-acid-CoA ligase); SP, Starch Phosphorylase; PGM, Phosphoglucomutase; FBPA, Fructose-Bisphosphate Aldolase; PGK, Phosphoglycerate Kinase; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; RubisCO, Ribulose-Bisphosphate Carboxylase; ENO, Enolase; PDH, Pyruvate Dehydrogenase; DLDH, Dihydrolipoamide Dehydrogenase; DLAT, Dihydrolipoyllysine-residue Acetyltransferase; SDH, Succinate Dehydrogenase; PEPCK, Phosphoenolpyruvate Carboxykinase; 6PGL, 6-Phosphogluconolactonase; 6PGDH, 6-Phosphogluconic Dehydrogenase (NADP+-dependent, decarboxylating); PRPS1, Ribose-Phosphate Pyrophosphokinase 1; H^+-ATPase, vacuolar H^+-ATPase; VDAC, Mitochondrial Porin (Voltage-Dependent Anion Channel) Outer Membrane Protein; Mpcp, Mitochondrial Phosphate Carrier Protein; AAC, ADP/ATP Carrier Protein.

$NaHCO_3$ concentrations	Different stages (h)	chlorophyll content (mg/L)
0 g/L	0	0.417
	24	0.536
25 g/L	0	0.417
	24	0.485

Table 2: Chlorophyll content of HTBS in 0 g/L $NaHCO_3$ and 25 g/L $NaHCO_3$ growth medium.

medium had sufficient nitrogen so that the concentration of CO_2 was very low. In addition, 6-phosphogluconate dehydrogenase (6PGDH), involved in the oxidative pentose-phosphate pathway, was found to be overexpressed (Table S2-Sheet 2). 6PGDH reportedly oxidizes the glucose-6-phosphate into 6-phosphoglucolactone and further yields 6-phosphogluconate followed by Calvin cycle protein ribulose-5-phosphate (Ru5P) [25]. It is one of the key enzymes involved in carbon and nitrogen cycles.

Transport protein regulating: Five proteins related to transport were identified in this study, distributed in the cell membrane, mitochondria and chloroplast (Figure 4). H^+-ATPase catalyzed the translocation of protons across the membranes and played key roles in re-establishment of ion homeostasis under $NaHCO_3$ stress [26]. The Mpcp (mitochondrial phosphate carrier protein) and AAC (ADP/ATP carrier protein) were the most abundant proteins in the mitochondrial carrier, catalyzing the exchange of Pi/OH- and ADP/ATP separately across the inner mitochondrial membrane [27]. According to their function, HTBS could amend the pH of cell and accumulate H^+ in chloroplast through up-regulate H^+-ATPase in a high $NaHCO_3$ environment.

Conclusion

This study identified a number of novel proteins whose expression and abundance were significantly altered in response to $NaHCO_3$ stress. Differentially expressed proteins showed that most of the up-regulated proteins were involved in respiratory metabolism and transport, the majority of the down-regulated proteins accounted for fatty acid oxidative metabolism and photosynthesis. The results suggested that the $NaHCO_3$ stress mainly affected energy metabolism and ion transportation of *Dunaliella salina* HTBS. The results provide useful information for further research for more comprehensive understanding of molecular mechanisms through which *Dunaliella salina* adapt to conditions of $NaHCO_3$ stress.

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