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Mutation adaptation and genotoxicity of microalgae induced by Long-Term high CO_2 stress

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ABSTRACT

This study determined that 60% is the most appropriate concentration of CO_2 for the domestication of microalgae to obtain strains with improved flue gas CO_2-adapting ability. The effect of long-term high CO_2 stress (6–99% concentrations) on microalgal gene mutations was first clarified with genomic and transcriptomic analyses. The most beneficial long fragment indel/SV gene mutations in microalgae were obtained under 60% CO_2. However, > 60% CO_2 domestication caused genotoxicity of the microalgae cells via the following mechanisms: (1) it was not conducive to forming more stable long fragment indel/SV gene mutations, thus preventing further gene mutation; (2) gene mutations did not generate successful linkage to the regulation in transcription and translation; and (3) inhibition of the mismatch repair damaged the specialized ability of genetic variation, leading to the disrepair of harmful gene mismatches and fewer beneficial mutations. These novel results revealed that higher concentrations of CO_2 for microalgal domestication did not necessarily result in microalgae that were tolerant to CO_2 owing to the genotoxicity of long-term high CO_2 stress. This conclusion informs futures efforts of domestication in the microalgae industry.

1. Introduction

The rapid development of industrial production and increasing demand for fossil fuels have resulted in widespread problems related to global warming. In the past few decades, the amount of CO_2 in the atmosphere has increased to 400 parts per million, of which 22% is caused by coal-fired power plants[1]. Accordingly, numerous countries have formulated laws and policies that require the relevant industries to reduce their emissions of greenhouse gases, such as carbon dioxide, in their production processes[2]. Therefore, treatment of CO_2 and other emissions from the industrial sector is an urgent problem in the fields of energy and environmental science.

Microalgae are among the most promising biofuels that absorb and utilize carbon dioxide[3]. They have many characteristics suitable for industrial applications[4], such as a high yield per unit volume, a strong ability to fix CO_2, and high environmental adaptability, and they are easily obtained. Therefore, microalgae are considered an important means of transferring carbon from industrial production. The efficiency of CO_2 fixation and biomass accumulation by microalgae is basically affected by the concentration of CO_2 [5], which is generally between 10% and 99% in industrial flue gas; however, microalgal growth is generally inhibited by CO_2 concentrations > 10% [6,7]. With increasing applications for microalgae in treating industrial flue gas, the influence of high CO_2 environments on microalgae has become an intensive focus of recent research.

In the field of flue gas fixation, many factors can affect the growth and carbon fixation of microalgae, including temperature, light intensity, nutrition, and toxic impurities from flue gas[8]. Therefore, there have been abundant studies examining how those parameters influence microalgae and can clearly explain their effects. For example, most microalgae usually tolerate temperatures>35 °C[9]; SOx and NOx from flue gas inhibit microalgae through peroxidation, damage to the membrane structure, the inhibition of respiration (NOx) or a decrease in pH (SOx) [10,11]. However, as the primary component of industrial flue gas, high concentrations of CO_2 may influence the microalgae through very comprehensive mechanisms.

For this reason, many studies have tried to explain the cellular effects of short-term high concentrations of CO_2 in detail. Of particular interest is how microalgae survive the initial 24-h growth retardation period after being transferred to a high CO_2 environment and then gradually adapt to the CO_2 growth process within a 7–10-day cultivation period [12]. A high concentration of CO_2 has been shown to promote carbon

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metabolism and the accumulation of lipids by microalgae through related gene translation and protein expression[13]. For example, it was demonstrated that the intracellular carbonic anhydrase of *Chlorella* PY-ZU1 cells decreased sharply until it was almost 0 under conditions of high CO_2 (15%), while the transcriptional abundances of *RuBisCO* and other key genes involved in carbon fixation were higher than those in cells cultured in ambient conditions[14]. A concentration of 15% CO_2 also caused the upregulation of genes related to photosynthesis and an increase in the rate of photosynthesis in *Haematococcus pluvialis*[15]. In addition, extra CO_2 may improve the metabolism of carbohydrates and increase the accumulation of triacylglycerol [16], while the intracellular antioxidative responses increased with elevated CO_2[12].

However, concentrations of CO_2 that are too high inhibit microalgal growth. Some researchers concluded that the inhibition caused by CO_2 was only induced by the decrease in pH because their results showed that controlling the extracellular pH could negate the inhibitory effect on growth in high CO_2 environments[17]. However, microalgal transcriptomes have revealed that the levels of transcription of microalgal genes differed greatly under growth inhibition caused solely by pH compared with that caused solely by CO_2; most genes were not synchronously regulated[18]. This past research revealed the various levels of performance when microalgae are transferred to environments with high concentrations of CO_2, clarifying the response of microalgae to high CO_2.

Although previous studies have provided considerable insight into the influence of CO_2 on microalgae, they only focused on the short-term effects of a high concentration of CO_2 on microalgae, which was often reflected in the transcription of microalgal genes because changes in gene transcription are typically a short-term physiological regulatory mechanism. In contrast, few studies have examined the long-term effects of high concentrations of CO_2 on microalgae. In addition, most previous studies focused on $< 30\%$ CO_2 concentrations. Moreover, the majority of these studies only explored the physiological responses of microalgae under a certain concentration of CO_2. Systematic studies on the long-term effects of high concentrations of CO_2 on microalgae across the entire 6–99% range of concentrations are especially lacking.

In recognition of the inhibition of microalgal growth under excessive CO_2 concentrations [19], numerous studies have focused on improving the adaptability of microalgae to high concentrations of CO_2. CO_2-tolerant microalgae from nature or generated from random mutagenesis have been screened to obtain many special microalgal strains that can tolerate concentrations exceeding 10% CO_2 with satisfactory growth [20-25]. However, obtaining such strains by screening is always an endeavor with uncertain results and a high risk of failure. Thus, it is difficult for this technique to be used widely as a reliable and practical method. Genetic engineering has been used to achieve two very successful cases to improve the tolerance of microalgae to CO_2. The first experiment limited the degree of intracellular environmental acidification to reduce inhibition by CO_2 through blocking the CO_2 concentrating mechanism (CCM) [26], which increased the microalgal growth rate in 5% CO_2 by 30% compared with the wild-type strain. The other successful experiment prevented the excessive intracellular accumulation of H^+ ions, which increased the final biomass yield from approximately 0.9 g/L to 3.2 g/L [18]. These two applications of genetic engineering were both highly effective at improving the tolerance of microalgae to CO_2. However, the operation and implementation of gene editing technology on microalgae involves some challenges. Overall, it is expensive and has a low success rate, with a long experimental cycle and stringent requirements for technical personnel. Therefore, while this approach is fairly suitable for laboratory research, it is impracticable in large-scale industrial settings involving various industrial microalgal species.

Owing to these issues with the experimental methods, domestication has gradually become one method to cultivate microalgae that are tolerant to high concentrations of CO_2, and this process is known as adaptive laboratory evolution (ALE). There have been many successful attempts to obtain microalgal strains that are highly tolerant to high concentrations of CO_2 with different species in multicycle-cultured microalgae using CO_2 gradient acclimation[27-29]. However, most research only focuses on the macroscopic results of the domestication experiment, but the mechanism of ALE under high levels of CO_2 is rarely discussed. In addition, this type of research usually directly establishes a specific CO_2 concentration as the condition for microalgal domestication, but no comparison has been made of the CO_2 conditions most suitable for domestication across the 6–99% CO_2 concentration range. Our previous study first demonstrated that CO_2 gradient domestication could be used to obtain microalgae with a high tolerance to CO_2 through the creation of directed gene mutations and natural selection [30]. However, this previous study only focused on gradient acclimation at concentrations of CO_2 that were$<$15% and did not reveal the different effects and relevant reasons for those differences of microalgal domestication under different CO_2 conditions across the very broad range of 6–99%. It is still unclear what types of genetic changes are produced in microalgal strains under different types of long-term domestication to high concentrations of CO_2. Simultaneously, a very important question remains unanswered. What CO_2 concentration is best for long-term domestication of microalgae in order to improve their ability to tolerate CO_2?

Therefore, in this study, the same microalgal strain (*Nannochloropsis oceanica CCMP1779*) was divided into nine groups and cultivated under 6–99% CO_2 for 2 months of adaptation cultivation. Genomic and transcriptome analyses were then used to determine the influence of different long-term CO_2 stress levels on the microalgal genome. This research first showed the different long-term effects of varying high CO_2 concentrations on microalgal genomes, which contrasted remarkably with the results of previous studies that primarily focused on the short-term effects of high concentrations of CO_2. Accordingly, this study focused on developing a foundation for practical applications and first delineated the different conditions of CO_2 that enabled various domestication effects and determined the most effective CO_2 conditions for microalgal domestication to achieve a higher tolerance to CO_2.

2. Experimental methods

2.1. Long-term adaptation of microalgae

In the first step, wild-type *N. oceanica* CCMP1779 was divided to nine photoreactors cultivated with 6, 15, 30, 40, 50, 60, 70, 80, and 99% CO_2, respectively. The bubbling rate of mixed CO_2 gas (x% CO_2 and [100–x]% N_2) was 2 mL/min for each photoreactor and controlled by an electric flow meter. This adaptation cultivation lasted for 2 months. During this period, a portion of microalgal suspension in every photobioreactor was discarded, and the optical density (OD) at 750 nm was readjusted to 1.0 every 7 days.

Photoreactors for microalgal cultivation were composed of a series of 300 mL column bioreactors (160 mm high, 56 mm inner diameter, and 62 mm outer diameter). The experiment was conducted in an artificial greenhouse where temperature and light were continuously maintained at 25 °C and 40 μmol m^2 s^{-1}, respectively. A dark/light cycle was not implemented, so that the growth process would be accelerated.

Seawater culture medium, consisting of artificial seawater (ESAW medium) and optimized f/2 medium[31], was the only cultivation medium used for the microalgae throughout the experiment. To control for other variables except for CO_2 gas, such as extracellular pH, 50 mM HEPES and saturated NaOH were used to maintain the extracellular pH value in the microalgal suspension at 7.5. In the domestication experiments, many safeguards were implemented to avoid contamination, such as keeping the photoreactors relatively sealed and sterilization of both utensils and solutions prior to use. The details are provided in the supplementary file.

2.2. Growth test

After 2 months of adaptation cultivation, nine different microalgal strains were collected from the nine photobioreactors and designated *N. oceanica* 6D, 15D, 30D, 40D, 50D, 60D, 70D, 80D and 99D, respectively, depending on the CO_2 cultivation concentration. These nine strains were cultivated from an initial microalgal concentration of $OD_{750} = 0.37 \pm 0.02$ with artificial seawater (ESAW medium) and optimized f/2 medium. The temperature, light, and pH were maintained as before. The CO_2 concentration for this growth test was 99% with a 3 mL/min bubbling rate.

During the 6-day growth test, the OD_{750} of microalgal suspension in each photoreactor was measured daily using a UV/visible spectrophotometer (UV2600, Unico, USA). If the measured $OD_{750} > 1.0$, the sample was diluted until an $OD_{750} < 1.0$ was achieved. During cultivation, 10 mL of microalgal culture samples were randomly harvested, and their OD_{750} was measured. They were dewatered via centrifugation and then washed three times with deionized water. The pellet was collected, and the mass was measured after drying at 80 °C for 72 h. Owing to the linear relationship between the biomass yield and OD_{750}, the dry weight of calculated microalgal biomass was obtained by fitting, and the biomass yield for each day was calculated based on OD_{750}.

2.3. Genome resequencing for domesticated strains

Following the 2-month adaption cultivation with 6–99% CO_2, two parallel samples were collected from each strain utilized for genome resequencing to identify the mutations in the genome ($n = 18$ samples for genomics). Each sample contained approximately 0.2 L microalgal suspension and 10^9–10^{10}*N. oceanica* CCMP1779 cells. DNA was extracted based on the CTAB method [32]. DNA samples were randomly broken into 350 bp fragments by a Covaris crusher (S220; Covaris, LLC, Woburn, MA, USA), which were then used to build the library with a TruSeq Library Construction Kit (Novo Nordisk, Bagsværd, Denmark). After preparation via end repair and phosphorylation, the addition of A-tails, ligating index adapters, denaturation and amplification, the DNA library for each sample was constructed and quantified with a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA) then finally diluted to 1 ng/μL. After the expected insert size was obtained, the effective library concentration was accurately quantified by qPCR to ensure the quality. The effective concentration was determined as > 2 nM. The library was sequenced by Illumina HiSeq (NovaSeq 6000; Illumina, San Diego, CA, USA), and the original image data files obtained were converted into sequenced reads (raw data) by base calling analysis. Raw data were filtered to exclude paired-end reads with a ratio of N in the single-end sequencing reads that exceeded 10% of the read length, a ratio of low-quality bases ($Q \leq 5$) in single-end sequencing read that exceeded 50% of the read length, and adapters. Basic quality information is listed in Table S1.

The effective sequencing data were compared to the reference genome using BWA software (http://bio-bwa.sourceforge.net/; parameter: mem-t 4-k 32-m), and the repeated results with the reference genome were removed by SAMTOOLS (parameter: rmdup)[33]. In this study, the genome of *N. oceanica* CCMP1779 (https://mycocosm.jgi.doe.gov/Nanoce1779/Nanoce1779.home.html) was used as the reference genome[34]. According to the reference genome, single nucleotide polymorphisms (SNP) and indel mutations in every sample were identified using SAMTOOLS when reads ≥ 4 and mapping quality (MQ) ≥ 20. SV mutations were identified using BreakDancer[35].

2.4. Transcriptome sequencing for domesticated strains

Three strains were chosen from the growth stage to represent the changes in transcriptome induced by long-term CO_2 stress: 6D (low carbon), 60D (middle carbon), and 99D (high carbon). Two parallel samples for each strain were collected after the growth test under 99% CO_2 stress. RNA was extracted from the samples using the TRIzol method. A total of 1 μg RNA per sample was used as input material for sample preparation. Sequencing libraries were generated using the NEBNext Ultra™ RNA Library Prep Kit (NEB, Ipswich, MA, USA) for Illumina following the manufacturer's recommendations, and index codes were added to assign sequences to each sample. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads, and cDNA was synthesized for library construction. The index-coded samples were clustered on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq platform (NovaSeq 6000, Illumina), and 150 bp paired-end reads were generated as raw data.

Raw data (FASTQ format) were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained from the raw data by removing reads that contained adapters and poly-N sequences, as well as low quality reads. The Q20, Q30, and GC content of the clean data were calculated. All downstream analyses were based on the high-quality clean data. The basic quality of transcriptome sequencing is listed in Table S2. Clean paired-end reads were aligned to the *N. oceanica* CCMP1779 reference genome using Hisat2 (v2.0.5)[36]. The mapped reads for each sample were assembled by StringTie (v1.3.3b) using a reference-based approach [37]. FeatureCounts (v1.5.0-p3) was used to count the read numbers mapped to each gene[38]. Finally, the fragments per kilobase of exon per million reads mapped (FPKM) of each gene were calculated based on the length of the gene and read counts mapped to the gene.

2.5. Genomics data analysis methods

First, for each mutant entry, including SNP and indels, the allele frequency was calculated for every sample. For the SV mutation, the frequency is 1 for mutants and 0 otherwise. The average value and standard error of each strain were then calculated based on the values in two parallels. To ensure the reliability of the following analysis and the significance of mutations, the following two criteria were used to pre-filter the mutation entries: 1) entries that satisfied the equation in the control group (6D was regarded as the control group in the analysis): the standard deviation of allele frequency – the mean allele frequency = 0, were discarded; 2) entries with an average allele frequency difference between the control and other domesticated groups of < 0.125 were discarded.

For filtered mutation entries, to identify genetic mutation trends following domestication with different CO_2 concentrations, a sliding average fitting trend line was made for each entry (decay = 0.64), and their shapes were automatically determined by observing the following two boundary principles: 1) small fluctuations within the scope of slope in the range of ± 0.03 were neglected; and 2) when the slope in the middle of a part of the curve was zero, if the signs of slope before and after this point were the same, this part of the curve was still considered as monotonous. For the following analysis, mutation entries with frequent fluctuations of allele frequency and regular instability under different CO_2 concentrations were discarded. Therefore, only mutations with four types of changing curves would be retained: monotone increasing, monotone decreasing, increasing and then decreasing, as well as decreasing and then increasing. For those significant mutation entries, bioinformatic databases, including GO (Gene Ontology), KEGG (the Kyoto Encyclopedia of Genes and Genomes), and Pfam (Protein Family database), were used for functional annotation.

To further analyze the functional characteristics of significant gene mutations, functional information of the gene fragments directly related to gene mutation sites was summarized and classified based on the GO and KEGG database entries. According to the total number of gene mutations under each GO entry, the first 45 entries were selected, and for each KEGG entry, the first 15 entries were selected. In addition, when performing GO functional statistics of the gene fragment where the

mutation occurred, four items that were too general were deleted to make the results more meaningful, including metabolic process, cellular process, cell, and intracellular.

To further determine the effects of long-term high CO_2 stress on genes related to photosynthesis and respiration, all the significant gene fragments associated with effective gene mutation sites located in chloroplasts and mitochondria were selected for analysis.

All the scripts used in the data analysis process described above were written in Python.

2.6. Transcriptomics data analysis methods

Out of the three transcriptome groups (6D, 99D, and 60D), 6D was selected as the control group. Two comparison groups were established: 60D versus 6D (60D/6D) and 99D versus 6D (99D/6D). In both comparison groups, the fold change (FC) of each gene was calculated based on the FPKM ratio of the experimental group to the control group, and log base 2 was calculated. Significance was determined by a Student's *t*-test, and genes with *P-value* $\geq$ 0.05 were removed for every group. The remaining genes were determined to be significantly differentially regulated.

To analyze the functional characteristics of the differentially regulated genes, they were annotated with the PF, GO, and KEGG databases. When performing GO functional statistics of the gene fragment where the mutation occurred, four items that were too general were deleted to make the results more meaningful as stated before, including metabolic process, cellular process, cell, and intracellular. The mean and standard deviation of the Log_2 FC of differentially regulated genes under each GO and KEGG category were calculated separately, and then the significance of categories was determined by a Student *t*-test. Categories with *P-value* $\geq$ 0.05 were not considered significant and were removed, and the remaining categories were ordered by the average Log_2 FC. For GO categories, only the top 10 positive and bottom 10 categories were retained and analyzed.

To further observe the effects of long-term high CO_2 stress on gene transcription related to photosynthesis and respiration, all the significantly differentially regulated genes located in chloroplasts and mitochondria were selected for analysis. All the scripts used in the data analysis process described above were written in Python and VBA.

2.7. Associated genome and transcriptome analysis

To analyze the linkage effect of gene mutations on transcriptome expression, the PF database was first used to perform functional classification on the significant gene mutations and significantly differentially regulated genes. The number of each type of gene mutation and the average Log_2 FC in the comparison groups of 99D/6D and 60D/6D in each category were calculated. When the number of mutation points > 0 and the average log_2 FC (99D/6D) or (60D/6D) had at least one non-null value, the following two screening criteria were used to select the PF categories that were successfully linked: 1) mutation points > 5, or (2) $|\text{average Log}_2\text{ FC}_{99D/6D}|>2$ or $|\text{average Log}_2\text{ FC}_{60D/6D}|>2$.

To further analyze the successful linkage types between mutation and transcription, the DBSCAN method [39] was used automatically to classify the genes according to characteristic variables based on the proportion of each gene mutation type, the total number of gene mutations, and the upregulation/downregulation of transcriptome expression of the PF entries described above. Scripts used in the data analysis process described above were written in Python and VBA.

3. Results

3.1. Growth curve for N. Oceanica strains after long-term CO_2 stress

After 2-months of cultivation with 2 mL/min 6–99% CO_2 to domesticate *N. oceanica* CCMP1779, nine different strains (6D, 15D, 30D, 40D, 50D, 60D, 70D, 80D, and 99D) were collected. They were then subjected to greater CO_2 stress (3 mL/min, 99% CO_2) to test their ability to adapt to a high CO_2 environment and showed very different growth curves (Fig. 1) after one day of cultivation. The best microalgal strain was 60D, domesticated by 60% CO_2, which achieved 1.41 g/L biomass yield after 6 days of cultivation. When the domesticated CO_2 concentration was < 60%, the biomass yield decreased, and the final biomass yield of 6D was 1.12 g/L. However, when the domesticated CO_2 concentration was > 60.

%, the biomass yield decreased with the increasing domestication of the CO_2 concentration. The final biomass yield of 99D decreased to a minimum of 1.04 g/L. Therefore, higher long-term CO_2 concentrations were not more favorable for microalgae to adapt to high CO_2. The optimal CO_2 concentration for domestication was approximately 60%.

3.2. Gene mutations in N. Oceanica strains after long-term CO_2 stress

Genome resequencing showed that long-term CO_2 domestication created some stable gene mutations in microalgal strains. These relatively stable mutations showed different trends among the CO_2 concentrations (Fig. 2). Overall, among all the significant mutations, the number of single point mutations, SNPs (1,471) were almost twice as abundant as the number of indel mutations < 50 bp (820), while only 3 SV mutations > 50 bp were identified. In indel and SNP, mutations with allele frequency increased first and then decreased the most. Even the three SV mutations all increased first and then decreased. Moderate domestication with CO_2 was more conducive to obtaining new genotypes in microalgae. The most significant difference in the type of distribution between the SNP and indel groups was monotonically increasing. The number of monotonically increasing SNP mutations was as high as 583, while the indel increases were only 139. Fig. 2a and e show many allele frequencies in the trend line bonding in the upper left corner, demonstrating that most monotone-increasing types of mutations were the mutations in the original strain that were strengthened during the process of domestication. Furthermore, the number of SNP mutations with allele frequency increased from 0 to > 0.5, much greater than that of indel mutations, indicating that new mutations created entirely by domestication primarily occurred in the SNP types.

3.3. Functional aggregation of gene mutations after long-term CO_2 stress

GO functional statistics of the genes directly associated with the

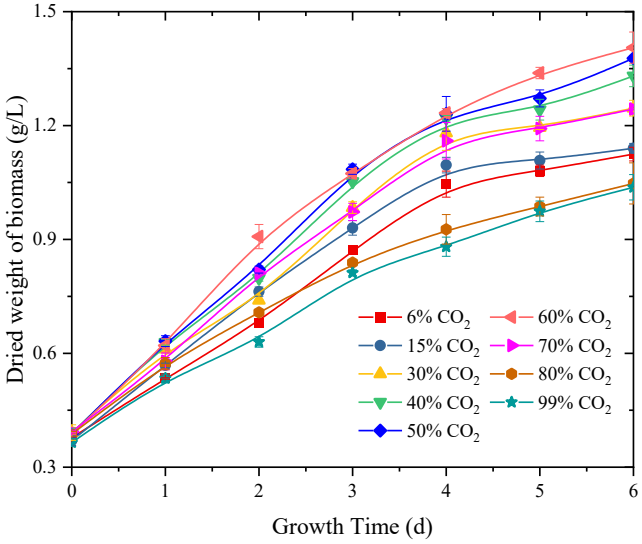

Fig. 1. Growth curves of *Nannochloropsis oceanica* under 3 mL/min 99% CO_2 stress after long-term adaptative cultivation with 6%-99% CO_2.

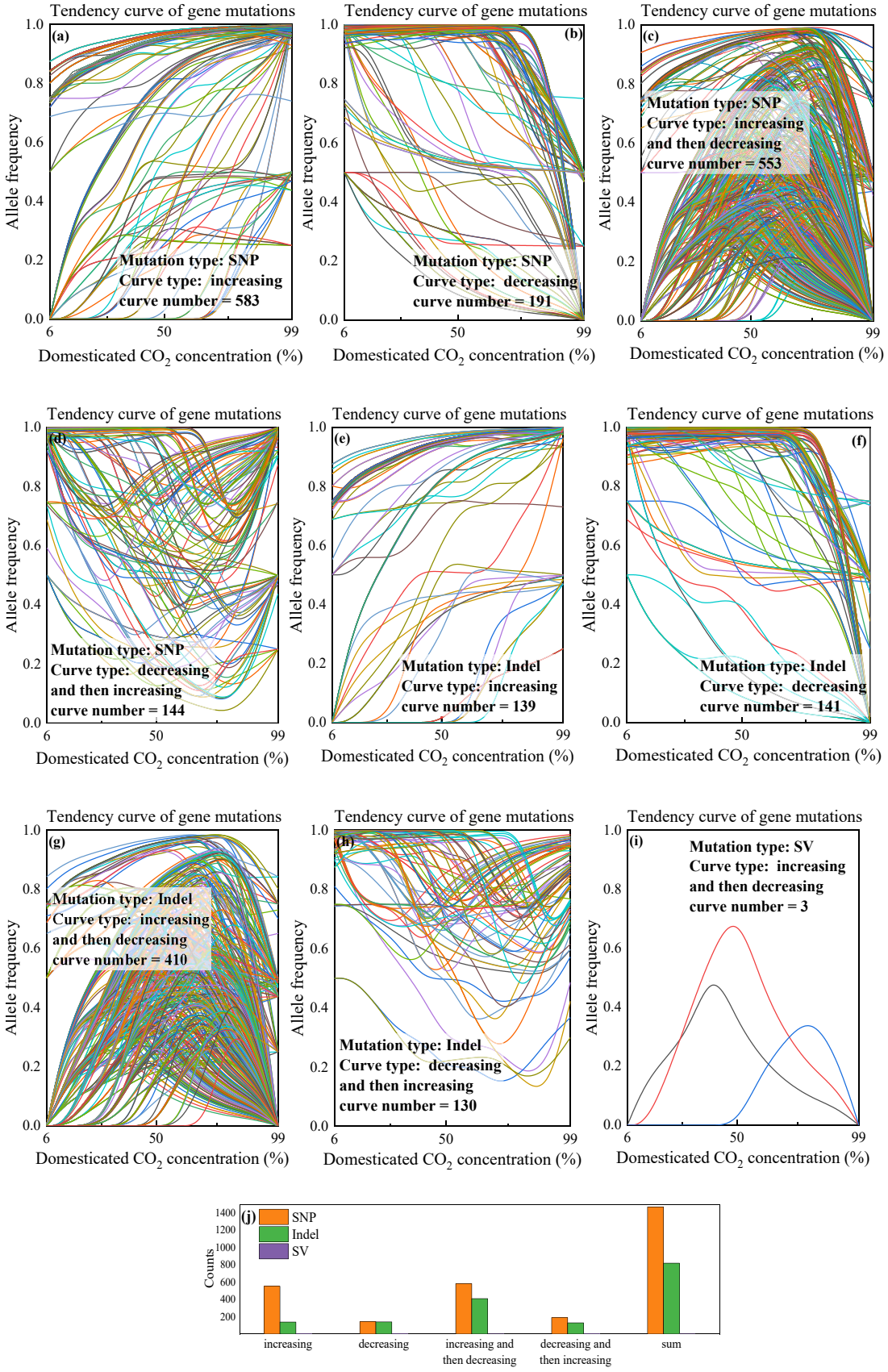

Fig. 2. Statistics for gene mutation types of *Nannochloropsis oceanica* with long-term adaptative cultivation with 6%-99% CO_2. (a)-(d) SNP, (e)-(h) Indel, (i) SV and (j) Number of gene mutations in various types.

occurrence sites of SNP and indels showed very similar distributions (Fig. 3a and b). However, in indel binding, ion binding, protein binding, and carbohydrate derivative binding obviously occupied a more important position than those in the SNP, and the ranking was higher according to the number of gene mutation sites. When the KEGG database was used (Fig. 3c and d), carbon metabolism and the biosynthesis of secondary metabolites were the most important functions of both SNPs and indels. However, the SNP mutations were more concentrated in the mRNA surveillance pathway, ABC transporters, and metabolism of fatty acid and sugar, while the indel mutations were more

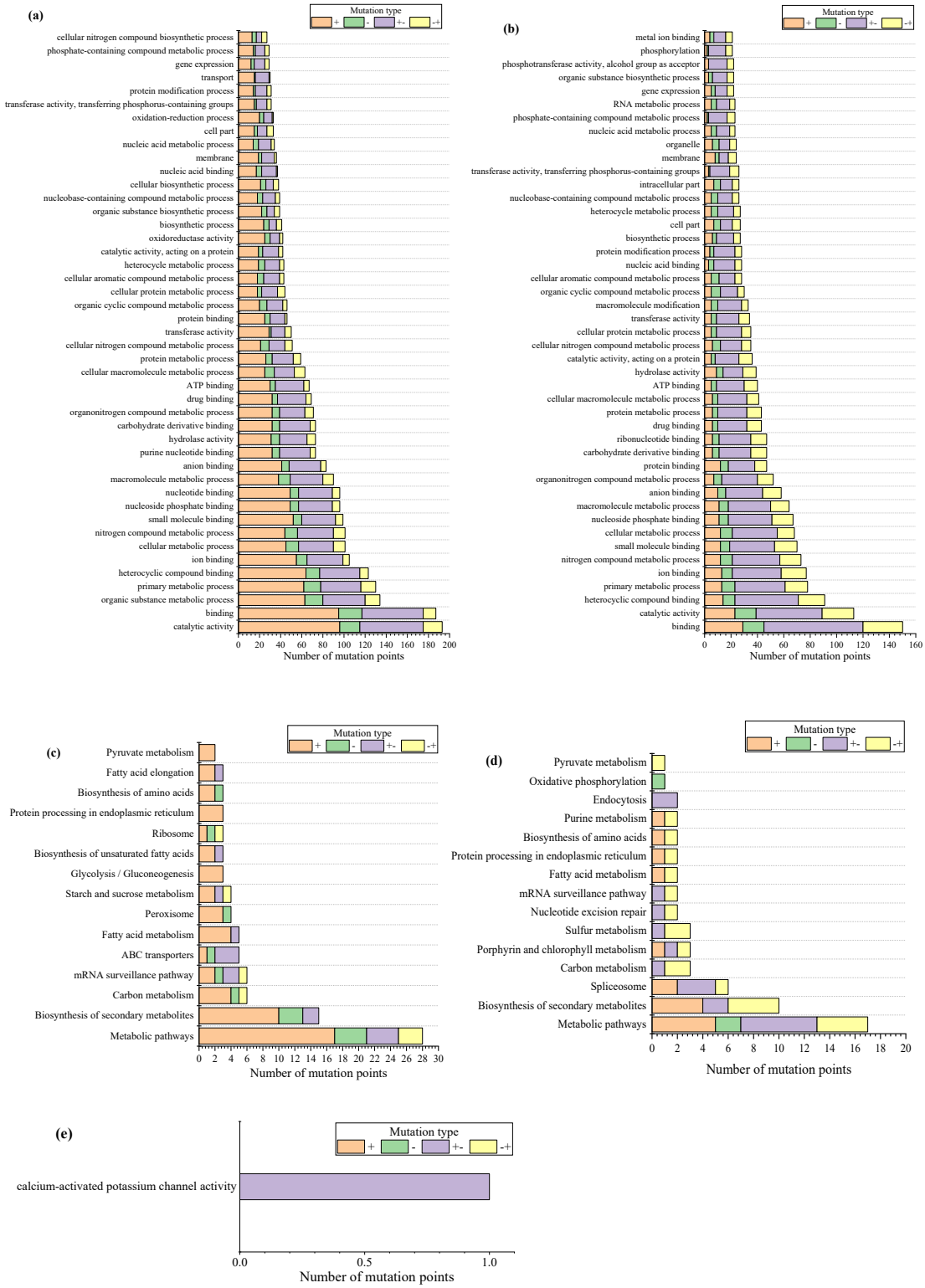

Fig. 3. Functional aggregation of gene fragments associated with gene mutations. Aggregations of (a) SNP mutations on GO entries, (b) Indel mutations on GO entries, (c) SNP mutations on KEGG entries, (d) Indel mutations on KEGG entries, and (e) SV mutations on GO entries. GO, Gene Ontology; KEGG, the Kyoto Encyclopedia of Genes and Genomes; SNP, single nucleotide polymorphism.

concentrated in “spliceosome” and “porphyrin and chlorophyll metabolism.” In addition, the only GO category for SV was a calcium-activated potassium channel (Fig. 3e).

From the SNPs to Indels, and then the SV, the proportion of those “increasing and then decreasing” in the primary functional categories continuously increased, and the type of monotone increasing became less important.

Further observations of the mutations in chloroplast and mitochondria showed that the SNPs in chloroplast and mitochondria were evenly distributed, while there were a greater number of indels in the chloroplast compared with the mitochondria (Fig. 4). This could indicate that the chloroplasts had more opportunities for longer stable mutations. In the chloroplast, many genes involved in photosynthetic light harvesting were mutated, including chlorophyll *a*-b binding protein L1818, chlorophyll synthase, zeaxanthin epoxidase, chlorophyll *a*-b binding protein of LHCII type I, magnesium-chelatase subunit *ChlH* (key enzyme for chlorophyll synthesis) [40], and 1-deoxy-D-xylulose-5-phosphate synthase (key enzyme for carotenoid synthesis) [41]. Obviously, gene mutations invoked by high CO_2 stress had a certain tendency of directional mutation to light harvesting proteins.

3.4. Association between gene mutations and transcriptional regulation

As shown in Fig. 5a, successful linkage between gene mutation and transcription regulation primarily occurred in the following functional category: > 10 SNPs in the ABC transporter linked to universally

Fig. 4. Gene fragments associated with gene mutations located in chloroplasts and mitochondria. (a) SNP mutations in chloroplasts, (b) SNP mutations in mitochondria, (c) Indel mutations in chloroplasts, and (d) Indel mutations in mitochondria.

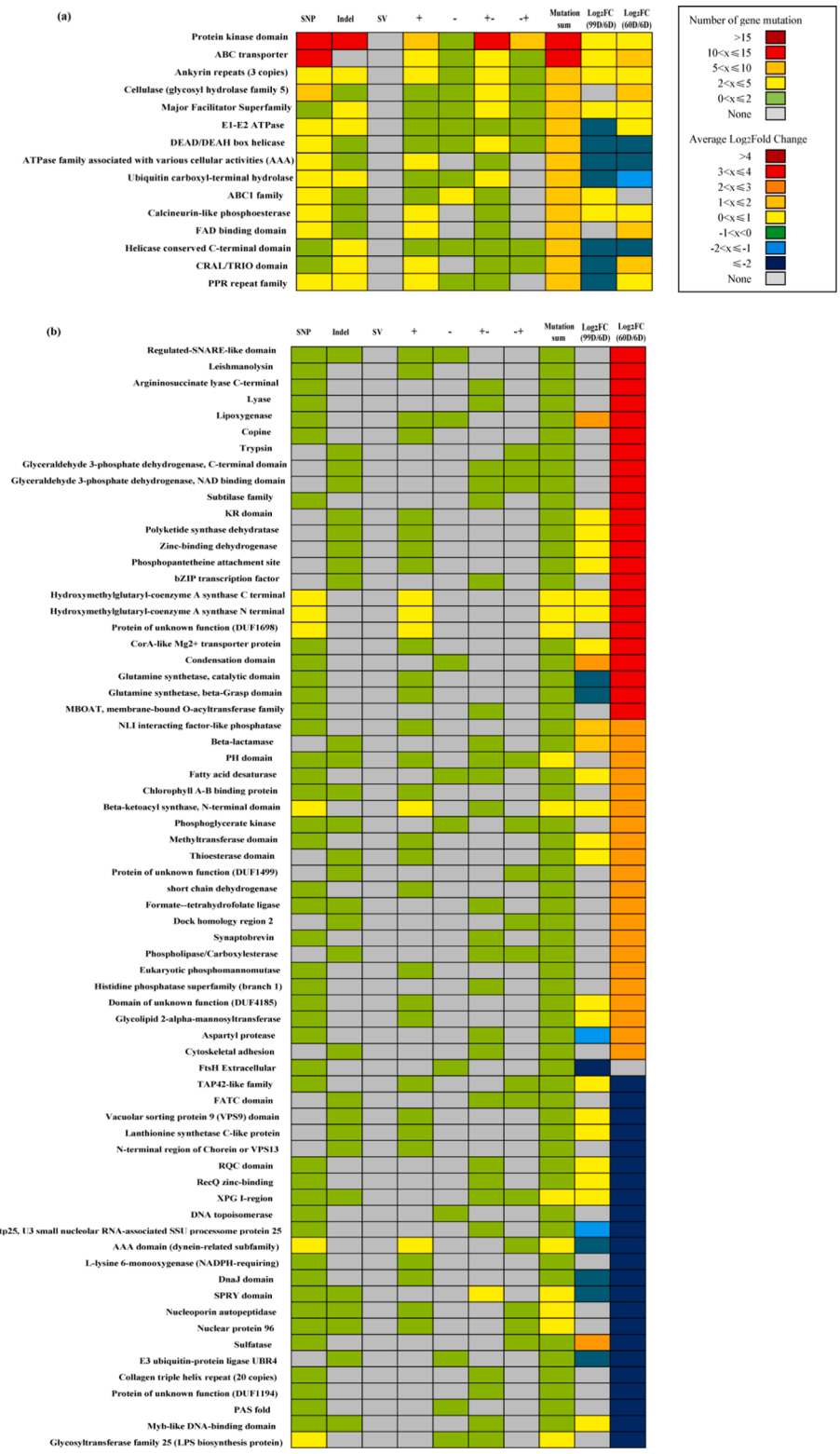

Fig. 5. Functional aggregation of gene fragments whose mutation successfully links to significant differences in transcription. (Notes: for mutation types, '+': increasing; '-': decreasing; '+-': increasing and then decreasing; '-+': decreasing and then increasing) (a) PF families with > 5 mutation points. (b) PF families with | average Log_2Fold $Change_{99D/6D}$|>2 or |average Log_2Fold $Change_{60D/6D}$|>2.

upregulation in the 60D group, and > 5 SNPs and 0 ~ 2 Indels in cellulase linked to upregulation in the 60D group. The FAD binding domain and CRAL/TRIO domain also showed similar linkage, while the > 2 '+-' type mutations caused universal downregulation of ubiquitin carboxyl-terminal hydrolase in the 60D group.

Fewer than five mutations were linked to large upregulation/downregulation in gene expression (Fig. 5b). For example, light-harvesting protein (Chlorophyll *A*-B binding protein) made a successful linkage between mutations of only increasing type and upregulation in 60D/6D. In addition, some protein families responsible for cell cycle

and division, such as copine and the FATC domain, also showed similar linkages. In these protein families, mutations with four types of changing trends of allele frequency all induced expression in 60D/6D. Obviously, it was not only curve types in which extreme points were taken in the middle of the gene frequency curve that caused significant regulation in the 60D/6D group. Although 37.3% of the significantly differentially regulated protein families in 60D/6D linked to a single mutation method of monotony increasing, only 5.9% linked to a single mutation method of monotony decreasing. Compared with 60D/6D, it was difficult to induce expression in 99D/6D. When the number of

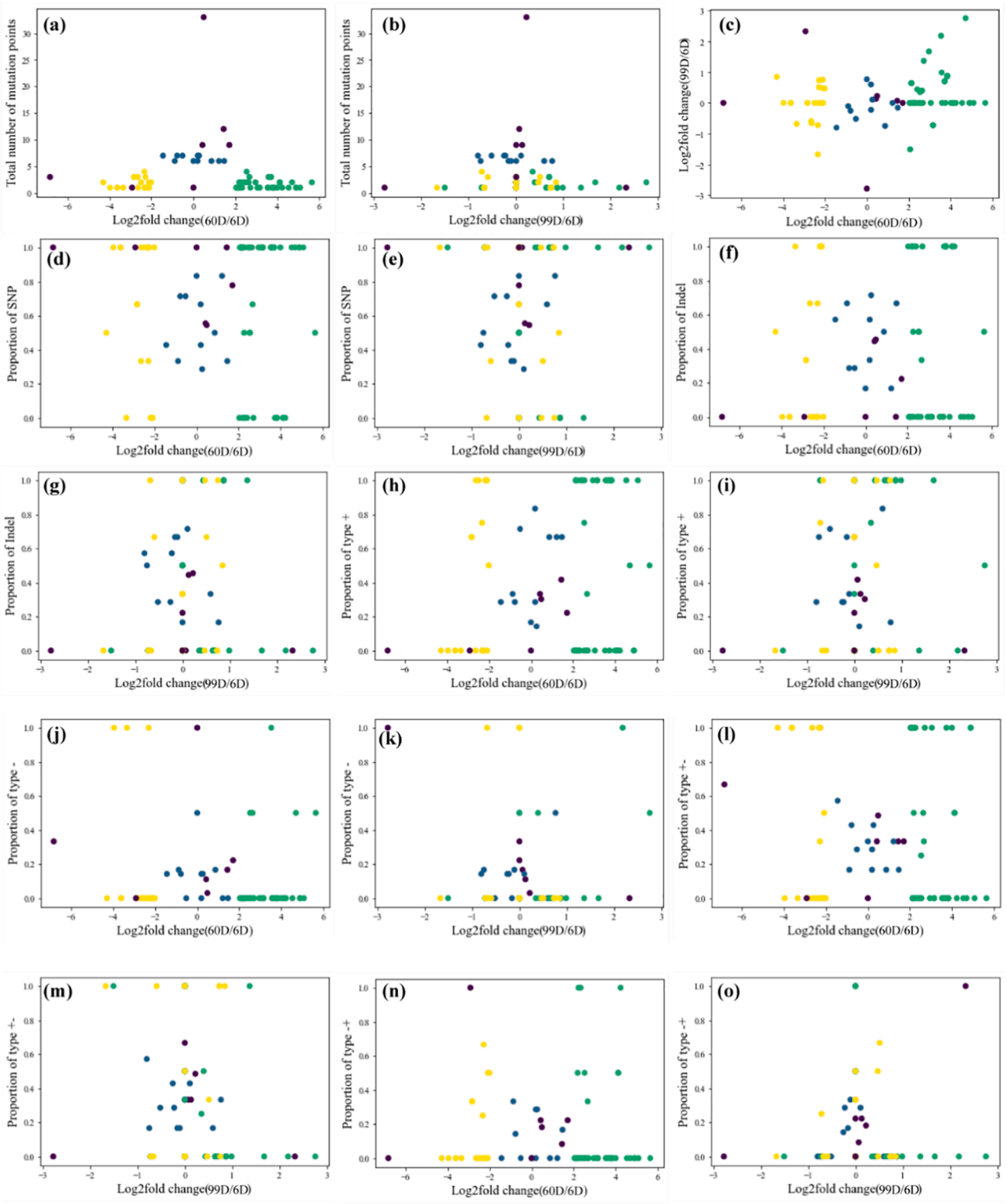

Fig. 6. Classification results of linkage types of gene mutations and the regulation of transcriptome expression with the unsupervised learning algorithm DBSCAN, which are painted in each characteristic dimension. (Notes: A different color represents a differently classified group, while purple indicates outlier points that cannot be classified.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutations > 5, 86.7% of the protein families induced regulation in 99D/6D, but when the number of mutations ≤ 5, this proportion decreased to 50.7%. Among those, in 73.5% of the protein families regulated in 99D/6D, there were mutations in which monotony increased or decreased, and 55.9% of the regulations in 99D/6D were induced by only monotony increasing or decreasing.

To determine the linkage type between mutations and the regulation of expression, unsupervised learning automatically classified the protein family data points described above. As shown in Fig. 6a, the most important differentiation of the three categories (yellow, blue, and green groups) after classification was the difference in expression changes in the 60D/6D group. Average Log_2 FC in the yellow group was < -2; the green group was > 2, and the blue group was between − 2 and 2. When those three groups expanded in the dimension of average Log_2 FC 99D/6D (Fig. 6c), the points in yellow and blue groups were evenly distributed up and down the 0 axis, while most points in the green group were scattered above the 0 axis. Simultaneously, the total numbers of mutations in the blue group were > 5, while those in the yellow and green groups were < 5 (Fig. 6b). Therefore, when the Log_2 FC of the 60D/6D group increased to > 2, the 99D/6D group was likely to follow the 60D/6D group up, but when the Log_2 FC increased by < 2 or even lower, it was not possible to estimate the trend of expression in 99D/6D group.

In terms of the relevance between the types of mutation types and regulation of expression in the different color groups; the green group primarily consisted of SNP types compared with indel types, while in the yellow and blue groups, the proportions of SNP and indel were more equal. Furthermore, the SNPs induced a larger distribution of Log_2 FC in the 99D/6D group, while the indels induced a narrower distribution of Log_2 FC in the 99D/6D group. There was no significant difference between the two gene mutations in the 60D/6D group (Fig. 6d–g). The proportion of increasing types in the yellow group was much smaller than in the other groups, while it was the primary type of mutation in the blue group (Fig. 6h and i), while the decreasing type primarily existed in the blue group (Fig. 6j and k). Increasing and then decreasing types of mutations limited the Log_2 FC in 99D/6D group of all the points to − 1 to 1 (Fig. 6m and o).

3.5. Functional analysis for transcriptional regulation

The functional analysis showed that, compared with 6D, 60D primarily upregulated the proteins involved in photosynthesis (light reaction) and carbon metabolism (primarily for the dark reaction), related sugar metabolism (galactose metabolism, glycolysis, cellular carbohydrate catabolic process, and acetyl-CoA carboxylase), and fatty acid biosynthesis and metabolism (Fig. 7a and b). Therefore, via genetic mutations and successful transcription linkage, the microalgal strain domesticated in the 60D group (60% CO_2) already had a comprehensive ability to fix carbon to accommodate high concentrations of CO_2.Fig. 8.

In contrast, similar transcription group functional chains did not appear in the 99D group strain (99% CO_2) (Fig. 7c and d). Carbon

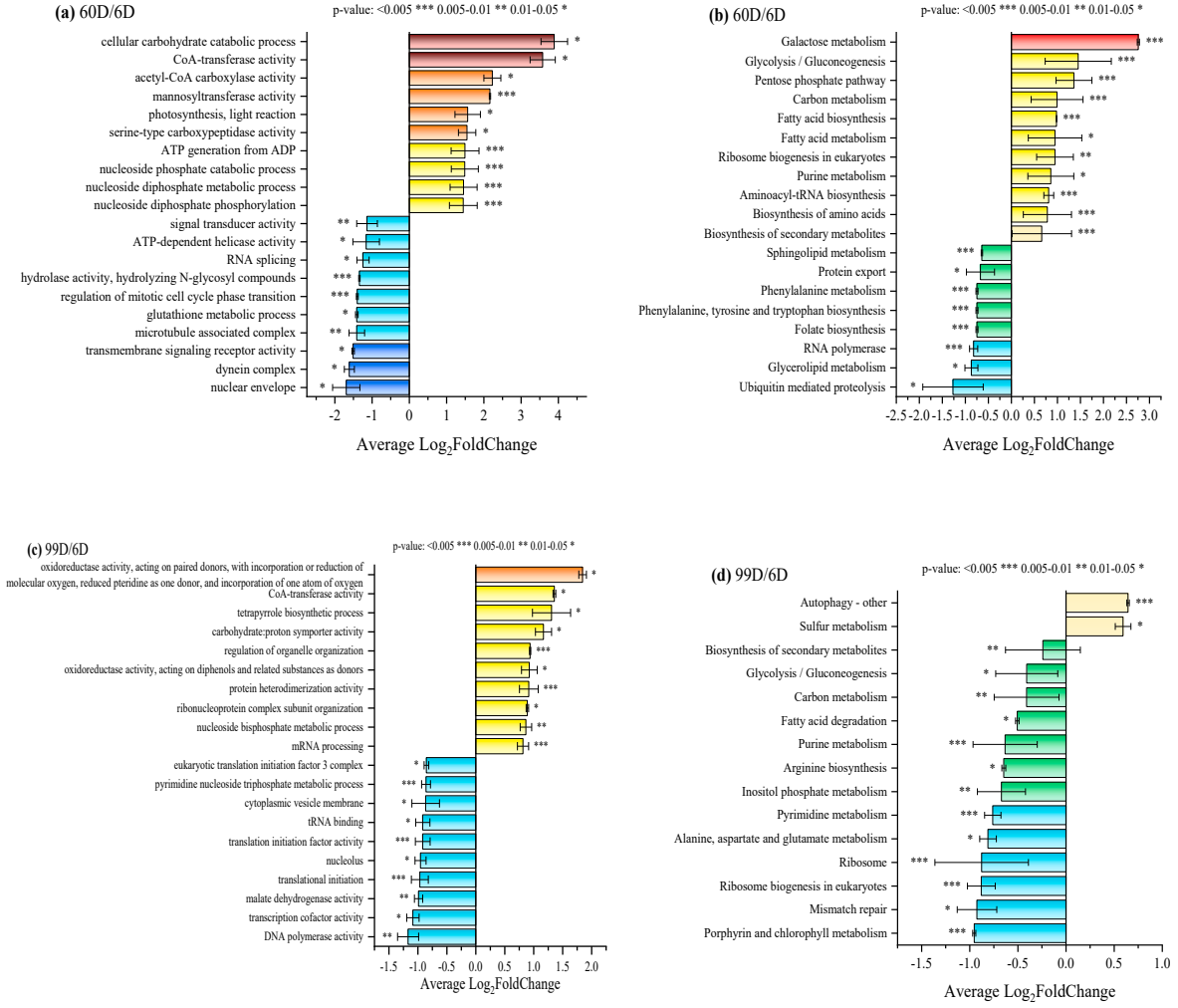

Fig. 7. Functional aggregation of significantly regulated gene fragments in transcriptome expression. (a) Significantly regulated GO entries in 60D/6D group, (b) significantly regulated KEGG entries in 60D/6D group, (c) significantly regulated GO entries in 99D/6D group, and (d) significantly regulated KEGG entries in 99D/6D group.

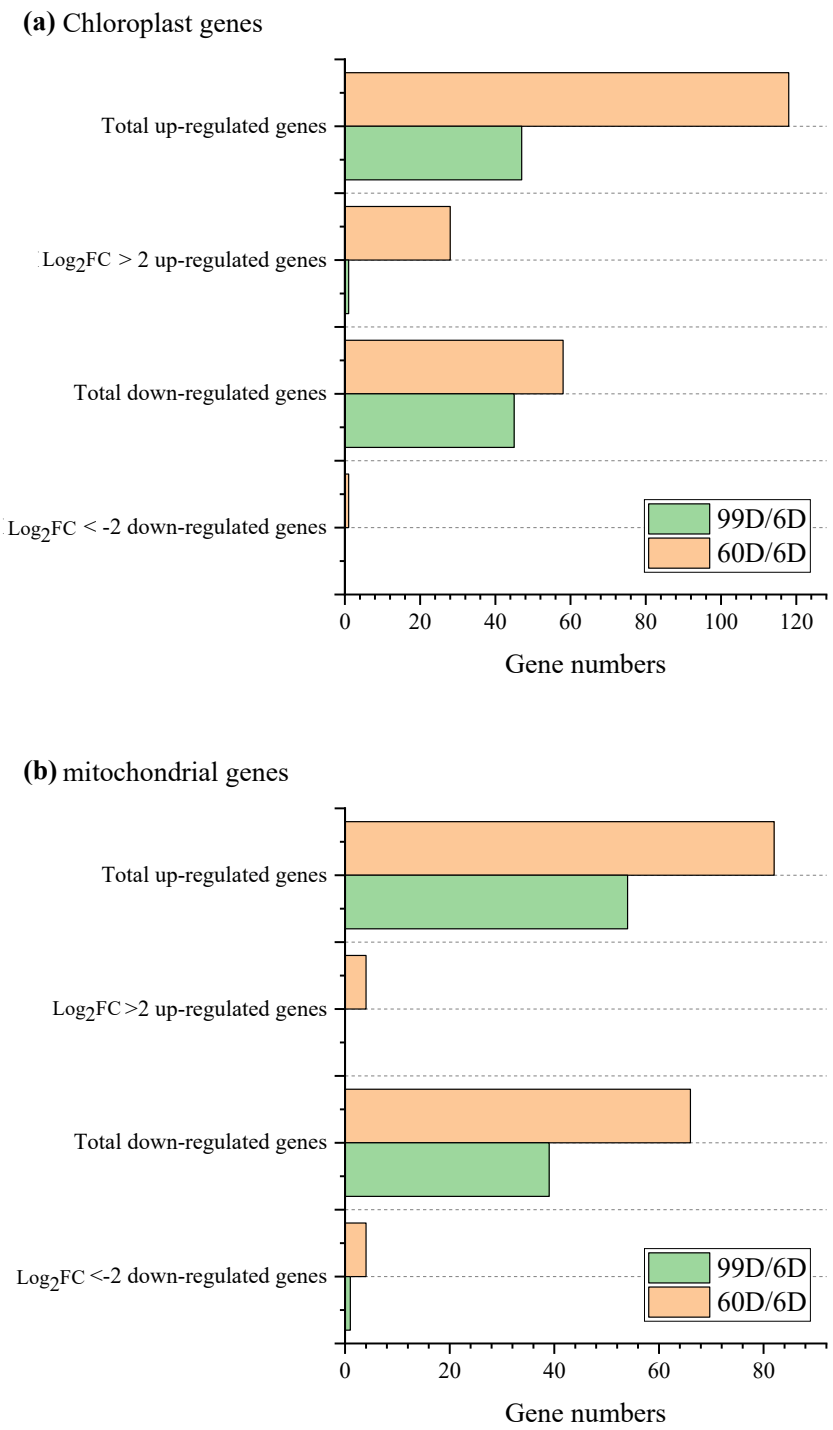

Fig. 8. Numbers of significantly regulated genes located in chloroplasts and mitochondria. (a) Numbers of significantly regulated genes in the 60D/6D and 99D/6D group in chloroplasts, and (b) in mitochondria.

metabolism and fatty acid metabolism were not only enhanced but even showed significant downregulation. Furthermore, in the 99D/6D group, the average Log_2 FC of porphyrin and chlorophyll metabolism was significantly reduced to almost − 0.95, although the types of multiple gene mutations associated with chlorophyll synthesis and chlorophyll-related light harvesting were monotonically increasing, i.e., the higher the domesticated carbon concentration, the stronger the gene mutation. As a result, the same gene mutations showed very different phenomena in the 60D and 99D groups, which may be related to the significant downregulation of a series of pathways associated with DNA synthesis, transcriptional processes, and repair of mismatch in the 99D group. The average Log_2 FC of tRNA binding, translation initiation factor activity, translational initiation, transcription cofactor activity, DNA polymerase activity, ribosome, ribosome biogenesis in eukaryotes, and mismatch repair were downregulated to − 0.92, −0.92, −0.97, −1.09, −1.17, −0.88, −0.88 and − 0.93.

Several proteins in chloroplast and mitochondria showed significant differences in expression (Fig. 8. and Fig. S1). In the 60D/6D group, 118 chloroplast and 82 mitochondrial genes were upregulated, while 58 chloroplast and 66 mitochondrial genes were downregulated. Among these, 23.7% of the upregulated and 1.7% of the downregulated chloroplast genes, and 4.8% of the upregulated and 6.1% of the

downregulated mitochondrial genes showed a $|Log_2 FC| > 2$. However, in the 99D/6D group, only 47 chloroplast and 54 mitochondrial genes were upregulated, while 45 chloroplast and 39 mitochondrial genes were downregulated. Among these, 2.1% upregulated and 0% downregulated were chloroplast genes, and 0% upregulated and 2.6% downregulated mitochondrial genes showed a $|Log_2 FC| > 2$. In other words, there was no strong up-regulation or down-regulation of gene expression in chloroplasts and mitochondria in 99D. The regulation of gene expression in 99D was relatively weak on the whole.

4. Discussion

Sustained acclimation to high CO_2 is a simple way for microalgal strains to acquire higher tolerance to CO_2[27]. This study aimed to determine whether higher concentrations of CO_2 were better for the adaption of microalgae. In previous studies, many successful experiments of ALE have been conducted in different laboratories to obtain microalgae that could more effectively adapt to CO_2 [28]. However, in many of the studies, the CO_2 conditions used for microalgal domestication were directly selected by the authors, without any exploration of a range of CO_2 concentrations[25,42], so there was a lack of discussion on the differences in the effects of domestication of microalgae under different CO_2 conditions. It is widely believed that higher concentrations of CO_2 are better for domestication to achieve higher tolerance to CO_2. Some researchers even used pure CO_2 directly to domesticate microalgae[29]. Some other ALE experiments demonstrated that not all the domestication conditions were effective with microalgae used in other fields, such as the bioremediation of heavy metals[43]. Similarly, when the primary goal was domestication to tolerate CO_2, the ideal domestication effect may be obtained at a certain CO_2 concentration. In this experiment, the effect of long-term CO_2 adaptation of microalgae reached a peak when the CO_2 concentration was approximately 60%. This method enables *N. oceanica* to grow prolifically under 3 mL/min 99% CO_2. Most industrial flue gas has high CO_2 concentrations and a large gas flux; for example, coal-fired power plants and blast furnaces emit 7–27% CO_2, while the fermentation industry and chemical plants emit ~100% CO_2 [44]. Therefore, it is necessary to continuously optimize the conditions for microalgal domestication and explore a higher ability of microalgae to tolerate CO_2.

Genome resequencing showed that long-term high CO_2 stress resulted in a large number of gene mutations. However, the number and intensity of gene mutations peaked when the CO_2 concentration was in the middle of the 6–99% range (approximately 60%). This trend became more obvious for long-fragment gene mutations. The evidence to support this hypothesis included the following: (1) to SNP (1 bp mutation), the number of mutations whose allele frequency shows a monotonous trend with increasing CO_2 concentrations was slightly higher than the number of mutations with a peak or valley allele frequency in the middle of 6–99% CO_2 concentration. (2) When the length of a gene mutation fragment $>$ 1 bp (indel), the number of monotonously changed gene mutations was sharply reduced. (3) When the length of a gene mutation fragment $>$ 50 bp (SV), the only type of mutation is 'increasing and then decreasing'. This meant that a high concentration of CO_2 was not conducive to the formation of more stable long-fragment gene mutations by microalgae. Numerous possibilities for gene mutation were prevented by the inhibition of forming indels[45].

However, preventing the formation of long-fragment mutations was not the only genotoxic component from high CO_2 concentrations. In terms of the functional distribution among SNP and indels, or the four different trend types for allele frequency of gene mutations, there were no significant distinctions among them. Ion binding, carbon metabolism, light harvesting, and sugar and fatty acid metabolism all played important roles in the functional distribution of mutation sites. However, a successful linkage to gene transcription could not be created with their mRNA concentration at 99% CO_2 domestication. In the strains with 99% CO_2 domestication, numerous concentrated gene mutation sites were able to force the regulation of transcription expression. Even genetic mutations at similar positions, which could lead to significant up- or downregulation in expression at relatively moderate concentrations of carbon (60% CO_2), did not result in similar effects under 99% CO_2. On the one hand, many gene fragments associated with transcription and protein translation were significantly downregulated after long-term exposure to high CO_2. Thus, transcription and translation were greatly inhibited, making it difficult for beneficial gene mutations to manifest in transcription. Alternatively, the inhibition mismatch repair in transcription had a significant influence on it. The function of "mismatch repair" enzymes not only eliminates genetic errors and maintains genetic stability but also plays a highly specialized role in promoting genetic variation[46]. *N. oceanica* strains lost this function owing to the genotoxicity of long-term high CO_2 stress, resulting in the inability of harmful gene mismatches to be repaired, while beneficial gene mutations were not targeted for induction.

Under the influence of the gene inhibition described above, excessive carbon concentration (>80% CO_2) caused the inhibition of growth and apoptosis of microalgae in the domestication stage, which was likely to induce inhibition from other factors. It is possible that owing to the continuous state of massive apoptosis, the disrupted cells and contents that had not been completely dissolved and digested accumulated in the bioreactors. The increase in the density of those fragments may have prevented external light from reaching the surviving cells[47]. Therefore, it is possible that the domestication effect was inhibited owing to the reduced penetration of light. In addition, as a byproduct of cell wall lysis, cell wall remnants have been reported to inhibit the growth of microalgae[48]. Thus, the growth of microalgae was possibly further hindered by those cell wall remnants. In contrast, normal cells secreted excess polysaccharides and carbohydrates to the extracellular medium [49,50], which could be used as carbon sources and nutrients for continuous cell growth. Thus, the microalgal strains that grew better during the domestication process (under approximately 60% CO_2) may obtain a nutritionally sustainable enhancement effect. Ultimately, the severe differentiation of domestication effects was exacerbated.

It is worth noting that it may be feasible to use 100% CO_2 to domesticate microalgal colonies composed of microorganisms of multiple species because during the slow domestication process, some species that were particularly unsuitable for high CO_2 were eliminated[29]. Although altering the gene expression of dominant species may also have some obstacles and degradation, owing to the elimination of some species, the overall adaptability of the colony to CO_2 could be significantly improved. However, for the domestication of pure-bred microalgae, the effect of genotoxicity is expected to be particularly prominent. Thus, an excessively high concentration of CO_2 hindered effective domestication. Currently, it is imperative to select the appropriate concentration of CO_2 for domestication.

From the other view, previous research has examined the influence of a certain concentration of high CO_2 on microalgae[51,52]. However, this research primarily focused on the short-term effect of high CO_2, particularly the influence on transcriptome during the first 7 days after the culture was transferred to high CO_2[53]. The long-term effect on genomics was always neglected, particularly the different long-term effects from varying high concentrations of CO_2 in the range of 6–99%. As a result, although some directional mutations related to chloroplasts and photosynthesis occurred, they could not enhance the ability to fix CO_2 and tolerate 99% CO_2 domestication. Therefore, long-term domestication with a high CO_2 concentration could not provide ideal adaptation ability of *N. oceanica* to high CO_2.

5. Conclusions

When microalgae (*Nannochloropsis oceanica* CCMP1779) were cultured in 6–99% CO_2 for 2 months, some changes were apparent in their genes and transcription. A concentration of 60% CO_2 for acclimation was the most effective at obtaining gene mutations and

transcription changes that were more favorable to CO_2 tolerance. This research delineated that a long-term concentration of high CO_2 would cause multiple genotoxic effects on microalgae. Therefore, increasing the CO_2 concentration without limits during domestication did not necessarily result in microalgae with better CO_2 tolerance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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