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Exogenous arginine induced metabolic modulation and biomass enhancement in *Nannochloropsis oceanica* under high CO_2 stress

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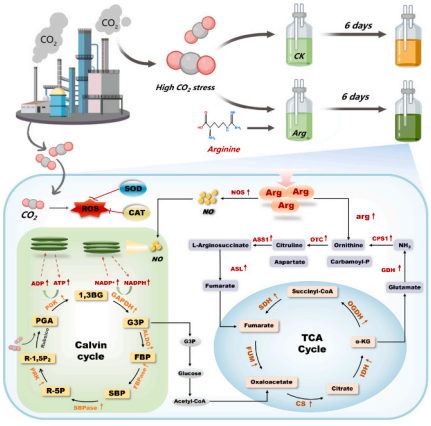
HIGHLIGHTS

- Arginine enhances biomass of *N. oceanica* by 46 % under high CO_2 stress.
- Arginine promotes pigments synthesis, prolonged G1 phase, and activated NO signaling.
- Arginine upregulates antioxidant enzymes and improved cell viability.
- Arginine upregulates key enzymes in Calvin and TCA cycles under CO_2 stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Given that excessive CO_2 induces reactive oxygen species (ROS) which inhibit photosynthesis and microalgal survival, this study explores how exogenous arginine enhances stress resilience and biomass yield of *Nannochloropsis oceanica* under high CO_2 stress. Arginine promoted NO synthesis, enhancing antioxidant enzyme activities to combat ROS damage. Transcriptomic analysis revealed that key enzymes in the Calvin cycle (PRK, GAPDH, FBPase and SBPase) and TCA cycle (CS, IDH, SCS and MDH) were significantly up-regulated, supporting carbon fixation and energy metabolism. Arginine also alleviated cell necrosis by extending the G1 phase, promoting DNA replication and protein synthesis, which increased the proportion of viable cells from 47 % to 84 %. Furthermore, the synthesis of chlorophyll *a* and carotenoid were enhanced, leading to 46 % increase in biomass yield. The results highlighted arginine as a promising metabolic regulator for enhancing stress tolerance and carbon fixation efficiency of *N. oceanica* under high CO_2 conditions.

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1. Introduction

Rising CO_2 emissions from fossil fuel combustion have accelerated climate change and environmental degradation (Yao et al., 2023). Among various mitigation strategies, the Non-Aqueous Solvent (NAS) CO_2 capture process has emerged as a promising approach for industrial carbon removal (Sepehri et al., 2020). In parallel, Microalgae offers a biologically driven solution for not only efficient CO_2 fixation and high-value bioproduct generation (Cheng et al., 2019). However, industrial flue gases and biogas often contain high CO_2 levels (15–40 vol%) (Yang et al., 2023a), which cause acidification and oxidative stress, impairing microalgal growth, thus limiting carbon capture (Kikuchi et al., 2024). Current mitigation strategies, such as pH buffers (Aditya et al., 2023) or adaptive laboratory evolution (ALE), are costly and time-consuming, and incomplete resolution of oxidative stress (Wang et al., 2022). Therefore, developing efficient approaches to enhance microalgal tolerance to high CO_2 is essential for future carbon mitigation.

High CO_2 stress in microalgae is primarily driven by rapid CO_2 influx, which increases photosynthetic electron transport chain excitation pressure and ROS production (Choi et al., 2022). Exogenous compounds like proline, spermidine, and phytohormones have been shown to enhance ROS scavenging, antioxidant enzyme activity, and stress resilience in microalgae under abiotic stress. For instance, proline supplementation reduced ROS and MDA levels while boosting biomass and astaxanthin in *Haematococcus pluvialis* under high light (Xing et al., 2023), and spermidine improved superoxide dismutase (SOD) activity in *Chlorella* sp. exposed to flue gas with SO_2 (Wang et al., 2020). Similarly, gibberellins (GAs) applied in *Chlorella vulgaris* upregulated antioxidase genes under sulfamethoxazole stress (Yang et al., 2023b), while γ-aminobutyric acid (GABA) induced osmolyte accumulation providing energy for cellular defense response against drought-salinity stresses in pomegranates (Zarbakhsh and Shahsavar, 2023). However, the effectiveness of these exogenous compounds differs depending on the species and type of stress, highlighting the need for targeted strategies to enhance the tolerance of *N. oceanica* to high CO_2 conditions.

Arginine, a proteinogenic amino acid with the highest nitrogen-to-carbon ratio, plays vital roles in stress responses and nitrogen transport in photosynthetic organisms (Babalar et al., 2018). As a precursor for polyamines and nitric oxide (NO), arginine regulates growth, metabolism, and ROS scavenging (Dai et al., 2019). Exogenous arginine has been shown to enhance antioxidant enzyme activity against ROS and facilitate biomass accumulation and astaxanthin synthesis in *Haematococcus pluvialis* under high light (Acheampong et al., 2024), reduce MDA levels in *Gracilariopsis lemaneiformis* during high-temperature stress (Zhang et al., 2021), and improve root growth and pigments synthesis in drought-stressed wheat (Hussein et al., 2022). However, the potential effects of arginine on metabolic regulation and biomass accumulation in *N. oceanica* under 40 vol% CO_2 stress remain unexplored, highlighting the need to elucidate the underlying regulatory mechanisms in this context.

Therefore, the aim of this study was to investigate the effects of exogenous arginine on cell viability and biomass yield in *N. oceanica* under 40 vol% CO_2 stress conditions. The mechanism of protection conferred by arginine on *N. oceanica* was studied in terms of photosynthesis and chlorophyll production. Transcriptomic analysis was performed to establish the effect of exogenous arginine on the activity of antioxidant enzymes involved in energy metabolism in *N. oceanica*. This work provides a theoretical basis for developing strategies to enhance the tolerance of microalgae to high CO_2 concentrations.

2. Materials and methods

2.1. Strain and culture condition

The *N. oceanica* strain (CCMP1779), obtained from the State University of New Jersey (USA), was used as the experimental strain for this study. Cultivation was carried out in 300 mL column photobioreactors (160 mm height, 56 mm inner diameter, and 62 mm outer diameter) containing a seawater culture medium, which consisted of artificial seawater (ESAW medium) supplemented with optimized f/2 medium (Wang et al., 2022). To maintain an initial pH of 7.5 ± 0.2, a saturated NaOH solution was added to the culture medium. The initial optical density at 750 nm (OD750) was adjusted to 1 at the start of cultivation. Exogenous arginine (Aladdin, >99.0 % purity by HPLC) was added to the algal suspension at concentrations of 0, 1 mM, 2 mM, and 3 mM. Two parallel replicates were performed for every condition with each experiment repeated at least twice to ensure reproducibility. The photobioreactors were incubated in an artificial greenhouse maintained at 25 °C, with an illumination intensity of 80 μmol m^{-2} s^{-1}. The microalgae was exposed to CO_2 with a gas mixture of 40 vol% CO_2 and 60 vol% N_2 continuously injected into the photobioreactors at a flow rate of 5 mL/min, controlled by flowmeter (CS200, Seven Stars).

2.2. Measurement of biomass yield and pH

During cultivation, OD_{750} value of microalgae was measured at 24-hour intervals using an UV/visible spectrophotometer (Unico UV2600, US). When the measured OD_{750} value exceeded 1.0, the sample was diluted to obtain a reading below 1.0. The biomass yield of the microalgae was calculated based on the OD_{750} value due to the linear relationship between the two. To establish this standard relationship formula, 10 mL microalgal culture samples were collected, and their OD_{750} values were recorded. The samples were then dewatered via centrifugation (Beckman Avanti J26-XP, USA) at 4000 rpm for 10 min. The resulting cell pellets were washed three times with deionized water and subsequently dried at 80 °C for 72 h. The dry weight of the biomass was determined, and a formula for calculating biomass yield was derived through data fitting (Feng et al., 2023), as shown in Eq. (1):

$$Dried\ weight\ of\ biomass\ (g/L) = 0.381 \times OD_{750},\ R^2 = 0.9904 \quad (1)$$

The pH value of microalgae suspension was measured daily by using a pH-meter (FE20; Mettler Toledo, Greifensee, Switzerland).

2.3. Measurement of cell pigments concentration

The concentrations of chlorophyll and carotenoid in *N. oceanica* cells were determined daily using spectrophotometry. Specifically, 1 mL of algal suspension was collected and centrifuged. The resulting pellet was mixed with 3 mL of anhydrous methanol (>99 %), thoroughly homogenized, and incubated in the dark for 30 min. The mixture was then centrifuged at 8000 rpm for 3 min, and the absorbance of the supernatant was measured at 480 nm, 652 nm, and 665 nm using an ultraviolet–visible spectrophotometer (*P*6 UV–vis spectrophotometer, MAPADA, China). The absorbance values were recorded as A480, A652, and A665. If any absorbance exceeded 1.0, the supernatant was diluted to ensure readings below 1.0. Since *N. oceanica* does not contain chlorophyll *b*, only the chlorophyll *a* and carotenoid were calculated (Chua and Schenk, 2017). The formula used are as follows:

$$[Chl\ a]\mu g \bullet mL^{-1} = -8.0962 \times A_{652} + 16.5169 \times A_{665} \quad (2)$$

$$[Carotenoids]\mu g \bullet mL^{-1} = 4 \times A_{480} \quad (3)$$

2.4. Measurement of photosynthetic fluorescence parameters

In order to investigate the photosynthetic characteristics of microalgae, the pulse modulation fluorometer FMS (FMS-2, Hansatech, UK) was used to measure the key fluorescence parameters (Roháček, 2002). The basic parameters include F_v/F_m, Φ_{PSII} , q_P, where F_v/F_m represents the maximum quantum yield of PSII photochemistry, Φ_{PSII} is the effective quantum yield of PSII photochemical energy conversion, and q_P indicates the photochemical quenching of variable chlorophyll

fluorescence. Prior to measurement, 1 ml of algal suspension was collected and kept in complete darkness for 30 min. This dark adaptation process ensured that all PSII reaction centers were fully relaxed and ready for accurate fluorescence measurement. The sample was then immediately transferred to the fluorometer for testing. Fluorescence measurements were performed under all arginine treatment conditions (0, 1 mM, 2 mM, and 3 mM), with each condition analyzed in duplicate every 24 h. Results were expressed as the average of the two measurements.

$$F_v/F_m = (F_m - F_o)/F_m = 1 - F_o/F_m;\ 0 < F_v/F_m < 1 \quad (4)$$

$$\Phi_{PSII} = (F_m^{'} - F_s)/F_m^{'} = \Delta F/F_m^{'} = q_P \cdot F_v^{'}/F_m^{'};\ 0 \le \Phi_{PSII} < 1 \quad (5)$$

$$q_P = \left(F_m^{'} - F_s\right)\Big/F_v^{'} = \Delta F\Big/F_v^{'} = 1 - \left(F_s - F_o^{'}\right)\Big/\left(F_m^{'} - F_o^{'}\right);\ 0 \le q_P \le 1 \quad (6)$$

2.5. Extraction and analysis of extracellular polymeric substances and organic element

For the quantitative analysis of microalgae cells, Extracellular polymeric substances (EPS) was analyzed using three-dimensional fluorescence spectroscopy (Feng et al., 2023). A 5 mL sample of algal suspension was centrifuged at 5500 rpm for 15 min to collect the supernatant containing soluble EPS (S-EPS). The cells were then resuspended in 0.06 % NaCl solution and centrifuged at 9000 rpm for 15 min to obtain the loosely bound EPS (LB-EPS) supernatant. The remaining microalgae cells were resuspended again in 0.06 % NaCl solution, heated at 60 °C for 30 min, and centrifuged at 12000 rpm for 20 min to collect the supernatant containing tightly bound EPS (TB-EPS). All extracts were filtered through a sterile 0.45 μm acetate syringe filter and analyzed quantitatively using a three-dimensional fluorescence excitation-emission matrix (EEM) spectrometer (F-7000 FL Spectrophotometer, Hitachi, Japan). The scanning parameters for the spectrometer were as follows: emission spectrum (Em) range of 240–800 nm, excitation spectrum (Ex) range of 240–600 nm, step size of 5 nm, and scanning speed of 2400 nm/min. Fluorescence Region Integration (FRI) was employed to calculate the volume of different regions in the EEM images, enabling quantitative evaluation of EPS.

In addition, after cultivation, microalgal suspensions from each sample were washed and harvested by centrifugation at 8000 rpm for 10 min to remove excess water. The resulting pellets were dried at 80 °C for 72 h (Wang et al., 2023). Finally, the carbon (C), hydrogen (H), nitrogen (N) and sulfur (S) contents of the harvested microalgae were determined using elemental analyzer (Elementar Unicube, Germany).

2.6. Detection of cell cycle and apoptotic activity in microalgae

To evaluate the cell cycle and apoptotic activity of the microalgae (Xin et al., 2024), a small amount of algal samples from each group were taken at the end of cultivation. The samples were fixed and stained using a Cell Cycle Detection Kit and an Annexin V-FITC/PI Double Staining Apoptosis Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). The stained samples were then analyzed using a flow cytometer (CytoFLEX, Beckman Coulter, USA). Data analysis was performed using FlowJo V10 software (FlowJo LLC, USA).

2.7. Transcriptome methods and analysis

To investigate gene expression changes in *N. oceanica*, transcriptomic analysis was performed using RNA-seq (Pardo-Palacios et al., 2024). Total RNA was extracted using TRIzol® Reagent and qualified by 5300 Bioanalyser (Agilent) and ND-2000 (NanoDrop Technologies). High-quality RNA samples were used to construct sequencing libraries using either Illumina® Stranded mRNA Prep (for ≥1 μg RNA), followed by sequencing on the NovaSeq X Plus platform.

Raw reads were filtered and trimmed using fastp, and clean reads were aligned to the *N. oceanica* reference genome using HISAT2. Transcript assembly was performed by StringTie, and gene expression was quantified using RSEM in terms of TPM (transcripts per million). The TPM ratio of the experimental group to the control group was calculated and expressed as the fold change (FC). Differential expression analysis was conducted with DESeq2; genes with $|\log_2 FC| \ge 1$ and FDR < 0.05 (DESeq2) were considered significantly differentially expressed genes (DEGs).

Significantly DEGs were further categorized according to KEGG, GO, and PF annotations. For GO and PF categories, functional classifications with p-values < 0.05 and $|\text{average } \text{Log}_2(FC)| > 0.5$ were retained as significantly different. For KEGG categories, genes with $|\text{average } \text{Log}_2(FC)| > 0$ were sorted by p-value ($p < 0.5$) and selected for subsequent significant difference analyses.

2.8. Data analysis

Data were analyzed and presented as mean ± SD (standard deviation, n = 4). Statistical significance was determined using one-way analysis of variance (ANOVA). For the data of gene expression, post hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Arginine promoted the growth of Nannochloropsis oceanica under high CO_2 stress

To investigate the effects of exogenous arginine on the growth and stress responses of *N. oceanica* under high CO_2 conditions, three treatment groups were established with varying arginine concentrations (1, 2, and 3 mM), along with a control check group (0 mM arginine, CK group), allowing the role of arginine in microalgal growth and its underlying regulatory mechanisms to be assessed comparatively.

As shown in Fig. 1a, the dry weight of microalgal biomass (DW) increased steadily over time in all groups. By the end of the cultivation period, the biomass of groups treated with arginine was significantly higher than that of the CK. Among all the treatment groups, the group exposed to 2 mM arginine exhibited the most substantial increase, reaching a maximum DW of 0.98 g/L, corresponding to a 46 % increase as compared to the CK's DW of 0.67 g/L (the growth difference was exhibited, see supplementary material). Conversely, the DW achieved in the 1 mM arginine- treated group was nearly identical to that of the CK, while the 3 mM arginine-treated group exhibited an intermediate level of biomass accumulation. These findings suggested that a suitable concentration of exogenous arginine can significantly enhance the tolerance of *N. oceanica* to high CO_2 stress and promote sustained biomass accumulation.

Biomass accumulation in microalgae is primarily attributed to the Calvin cycle, which utilizes ATP and NADPH generated during photosynthetic reactions to fix CO_2 and synthesize carbohydrates through a series of enzymatic processes (Yang et al., 2017). Transcriptomic analysis was performed to elucidate the role of arginine in Calvin cycle regulation, comparing gene expression in the 2 mM arginine-treated group (hereafter referred to as "Arg group") and the CK. Results revealed significant upregulation of genes encoding most Calvin cycle-related enzymes in three arginine-treated groups (see supplementary material). Notably, the expression levels of four key enzymes: phosphoribosyl kinase (PRK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase (FBPase), and sedoheptulose -1,7-bisphosphatase (SBPase), were found to be significantly elevated in Arg as compared to the CK (Fig. 1c). Among them, PRK catalyzes the phosphorylation of ribulose 5-phosphate (Ru5P) by ATP to produce

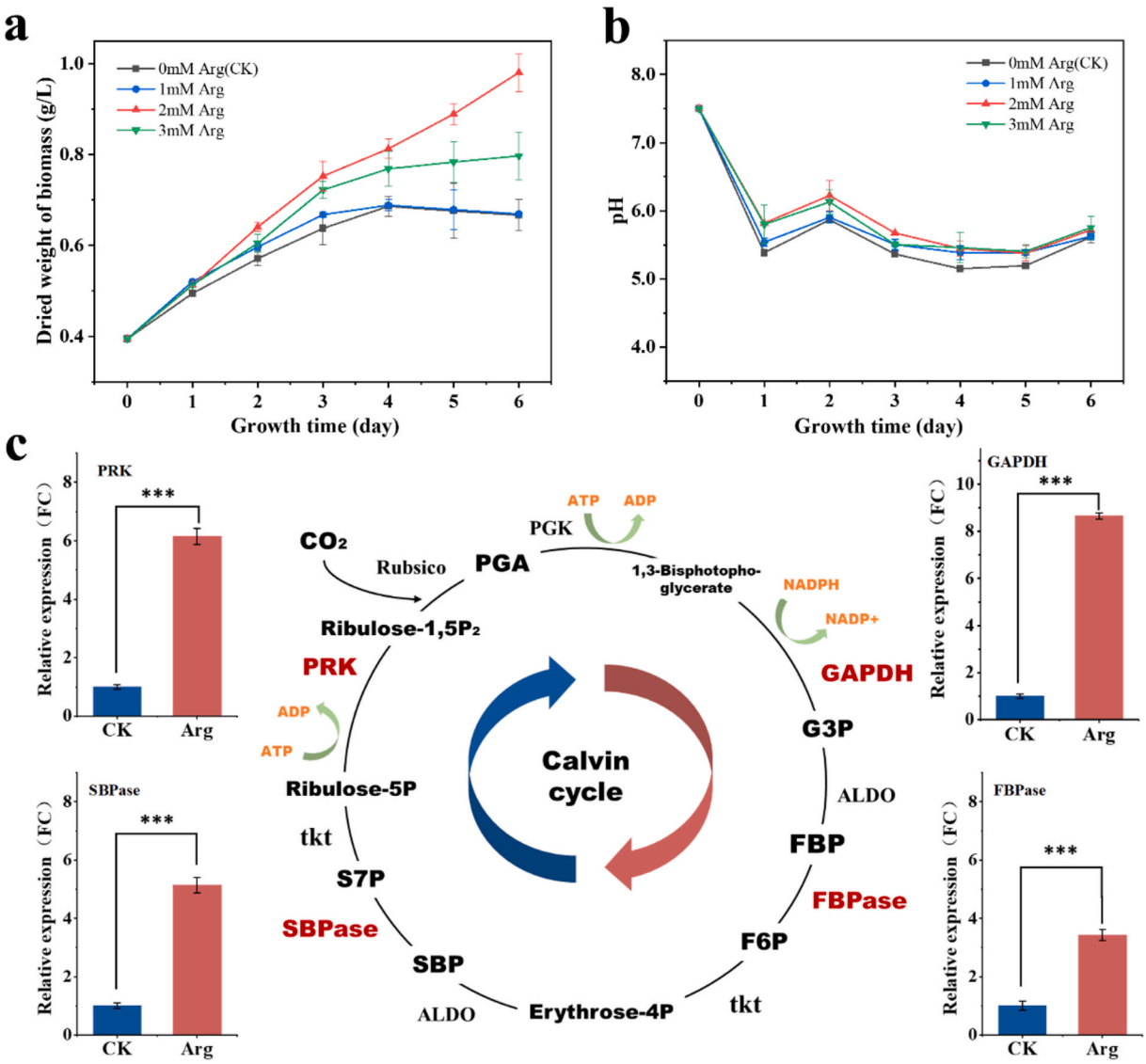

Fig. 1. Exogenous arginine promoted Calvin cycle-related enzyme activity and carbon fixation in *Nannochloropsis oceanica* under high CO_2 stress. (a) Biomass dried weight ($p < 0.01$); (b) pH value (ns); (c) Up-regulated key enzymes involved in Calvin cycle: phosphoribosyl kinase (PRK), sedoheptulose -1,7-bisphosphatase (SBPase), fructose-1,6-bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *** $p < 0.001$.

ribulose 1,5-bisphosphate (RuBP), the primary CO_2 acceptor molecule in the Calvin cycle (Yu et al., 2020). As the final step in the regeneration phase, PRK activity is essential for maintaining Calvin cycle efficiency (Meloni et al., 2023). In the Arg group, PRK expression increased by 5.7-fold compared to the CK, to help enhance the rate of carbon fixation. GAPDH facilitates the conversion of 1,3-bisphosphoglycerate (1,3BPG) into glyceraldehyde 3-phosphate (G3P), a core intermediate for CO_2 fixation and carbohydrate synthesis. A portion of G3P exits the cycle and is converted into organic molecules such as glucose (Wei et al., 2022). In the Arg group, GAPDH expression exhibited an 8.9-fold increase, strongly indicating substantial levels of carbohydrate accumulation. Similarly, FBPase and SBPase play vital roles in the generation of intermediates and the regeneration of RuBP, which is essential to sustain the Calvin cycle. In the Arg group, the expression of FBPase and SBPase were upregulated by 3.8- and 5.0-fold, respectively, accelerating the Calvin cycle and leading to enhanced biomass accumulation (Raines, 2003).

The introduction of high concentrations of CO_2 often leads to acidification, which can negatively affect microalgal growth as *N. oceanica* thrive at an optimal pH of around 7.5 (Wang et al., 2023). Although arginine-treated groups showed slightly higher pH value than the control, the difference was not significant. Thus, its role in alleviating CO_2-induced acidification appears limited based on current research.

3.2. *Arginine increased pigments synthesis and enhanced photosynthetic efficiency in Nannochloropsis oceanica*

Photosynthesis is a critical process by which algae convert light energy into chemical energy, with photosynthetic pigments playing a central role in the mechanism. The chlorophyll content of microalgal cells has been shown to directly influence their photosynthetic efficiency (Fareen et al., 2021). Additionally, carotenoids serve as accessory pigments in the light-harvesting complex (LHC), protecting the photosynthetic system against oxidative damage (Varela et al., 2015). Under high CO_2 conditions, the photosynthetic system of microalgal cells can become highly susceptible to damage, highlighting the importance of investigating the mechanism by which arginine influences the synthesis and accumulation of photosynthetic pigments under stress conditions.

Experiments were conducted to measure the chlorophyll *a* and carotenoid contents of cells in each group (Fig. 2a & b). Results showed

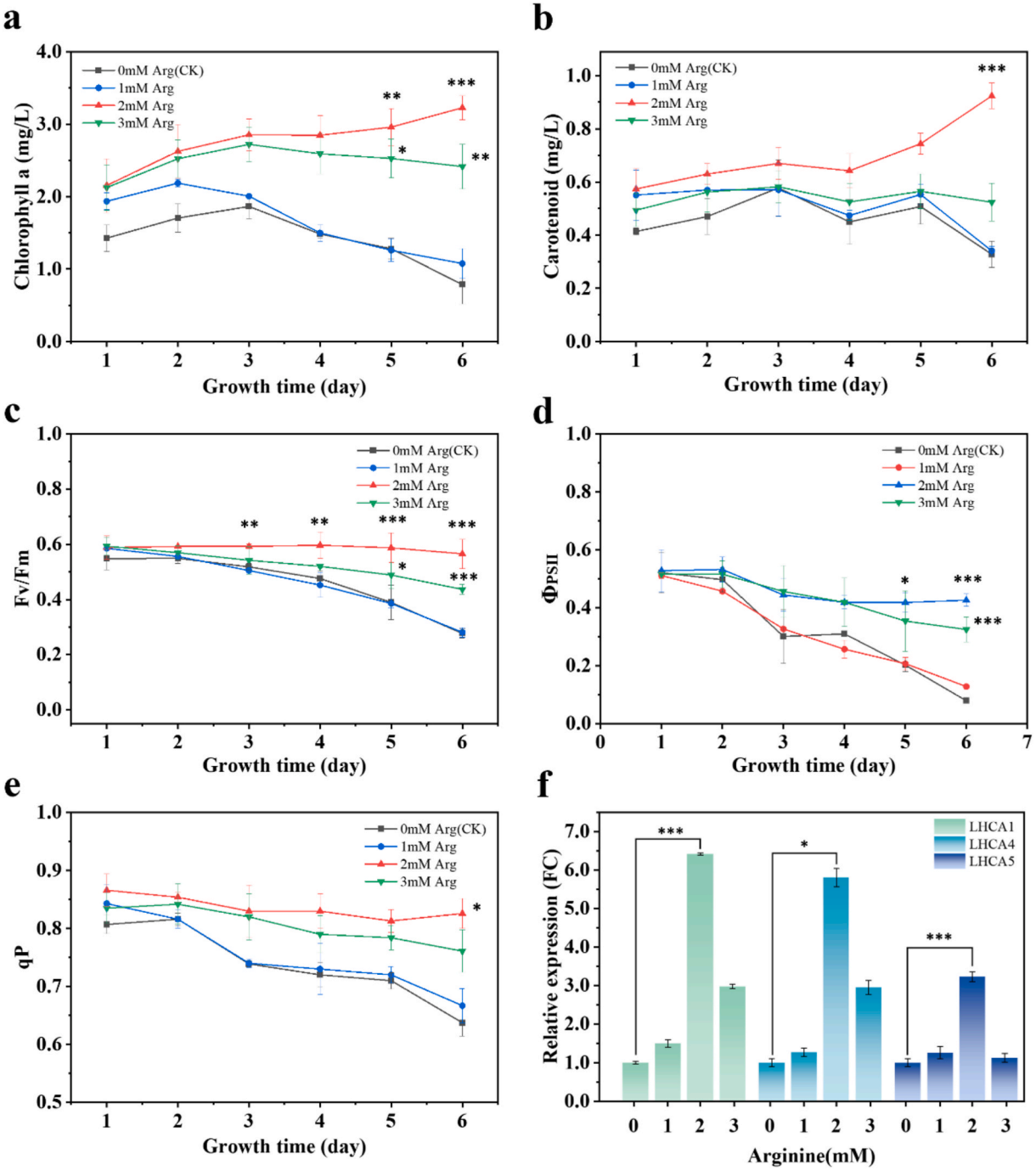

Fig. 2. Arginine enhanced photosynthetic pigments and fluorescence parameters (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$): (a) chlorophyll *a*; (b) carotenoids; (c) Fv/Fm, maximum quantum yield of Photosystem II photochemistry; (d) Φ_{PSII}, effective quantum yield of photochemical energy conversion in Photosystem II and (e) qP, the photochemical quenching of variable chlorophyll fluorescence; (f) relative gene expression of light-harvesting compelexes (LHCs).

that the addition of arginine significantly increased the content of both pigments under high carbon conditions, as compared to the CK. In all groups, chlorophyll and carotenoid levels initially exhibited an increasing trend during the first three days of treatment, followed by a gradual decline during the last three days. However, the pigment levels in three arginine-treated groups remained consistently higher than in the CK group, suggesting that prolonged exposure to high CO_2 concentrations may damage the photosynthetic system, eventually leading to photosynthetic collapse. Notably, the chlorophyll *a* content in Arg group continued to rise throughout the experiment, reaching 3.23 mg/L, corresponding to a 4.1-fold increase as compared to the CK group (0.79 mg/L). Similarly, the carotenoid content in Arg group reached 0.92 mg/L, showing a 2.8-fold increase versus the CK group (0.33 mg/L). The light-harvesting chlorophyll complex (LHC) is a pigment-protein complex located in the thylakoid membrane, which is an essential component of photosynthesis, formed through the non-covalent association of chlorophylls and carotenoids with polypeptides (Kilian et al., 2008). LHCs ensure the proper orientation of pigment molecules, enabling the rapid transfer of excited light energy towards photosynthetic reaction centers via resonance energy transfer (Bischoff et al., 2023). Transcriptomic analysis of LHC-related genes—LHCA1, LHCA4, and LHCA5, revealed that they were significantly upregulated in the Arg group (Fig. 2c), with

their expression increased by 5.6-, 4.4-, and 4.0-fold, respectively, versus the CK group. This indicated that arginine supplementation promoted the coordinated enhancement of photosynthetic pigments and LHCs, improving the light energy capture and conversion efficiency of microalgal cells.

To further assess the photosynthetic efficiency of *N. oceanica* in the presence or absence of arginine supplementation, the photosynthetic parameters of each group were determined, where Fv/Fm denotes the maximum photochemical quantum yield (Fig. 2d), Φ_{PSII} indicates the effective quantum yield of photochemical energy conversion (Fig. 2e), and qP corresponds to the photochemical quenching of chlorophyll fluorescence (Fig. 2f). In the CK group, all three parameters exhibited a decreasing trend under continuous high carbon supply, reflecting the damage and suppression of the photosynthetic system under high carbon stress. In contrast, the arginine-treated groups exhibited significantly higher values for the three photochemical parameters. Notably, in the Arg group, Fv/Fm remained stable throughout the cultivation period, increasing to 2.0-fold higher than the CK group. Similarly, the parameters Φ_{PSII} and qP in Arg group were 5.4- and 1.3-fold higher than in the CK group, respectively. These results demonstrated that arginine enhanced pigment synthesis and LHC expression in *N. oceanica*, thereby stabilizing the photosynthetic system and improving light energy conversion under high CO_2 stress (Sunku et al., 2013).

3.3. Arginine enhanced antioxidant enzymes activity and alleviated apoptosis

In general, CO_2 plays a key role in cell division, influencing various cellular processes, such as metabolism and signaling pathways that regulate the cell cycle. However, when CO_2 concentrations become excessive, they can lead to the overproduction of ROS, resulting in oxidative stress and significant DNA damage. If this cell damage is not repaired, programmed cell death (apoptosis) is triggered (Wang et al., 2023). The combined effects of accelerated cell division and apoptosis can inhibit microalgal biomass yields (Peng et al., 2023). In the present study, exogenous arginine was found to significantly increase antioxidant enzyme activities, improve cell survival, alleviate apoptosis, and moderate rapid cell division in *N. oceanica* under high CO_2 concentrations, thereby facilitating sustained microalgal biomass production.

Transcriptomic analysis revealed that arginine treatment significantly upregulated nitric oxide synthase (NOS) expression to 1.4-fold, as compared to the level observed in the CK group (Fig. 3a). Increased NOS expression promoted NO production, which stimulated the expression of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) by 2.8-, 2.7-, and 3.2-fold, respectively, versus the CK group (Fig. 3b–d). This phenomenon occurred because high CO_2 levels promoted excessive ROS generation, with ROS subsequently attacking essential cellular components, including iron-sulfur clusters ($[4Fe\text{-}4S]^{2+}$), which function as critical cofactors during DNA replication and repair. ROS, such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), induce oxidative DNA damage and impair DNA repair mechanisms, ultimately causing apoptosis or necrosis (Huang, 2022). Furthermore, SOD, CAT, and GPx are essential antioxidant enzymes that are required for ROS scavenging from various cell compartments and cell responses to stressful situations (Wang et al., 2020). Therefore, these findings indicate that arginine augments cellular antioxidant defenses, safeguarding cellular integrity and promoting sustained growth under CO_2 stress conditions.

Both biotic and abiotic stresses disrupt cell cycle processes and have the potential to precipitate programmed cell death (Qi and Zhang, 2019). As shown in Fig. 3e, comparative analysis of the cell cycle process revealed substantial differences between the Arg and CK groups. The cell cycle consists of four distinct phases: G1 (postmitotic interphase), S (DNA synthesis phase), G2 (premitotic interphase), and M (mitosis/cytokinesis). In the G1 phase, the Arg group exhibited a significantly higher proportion of cells on day 5 (68 %) and day 6 (61 %) as compared

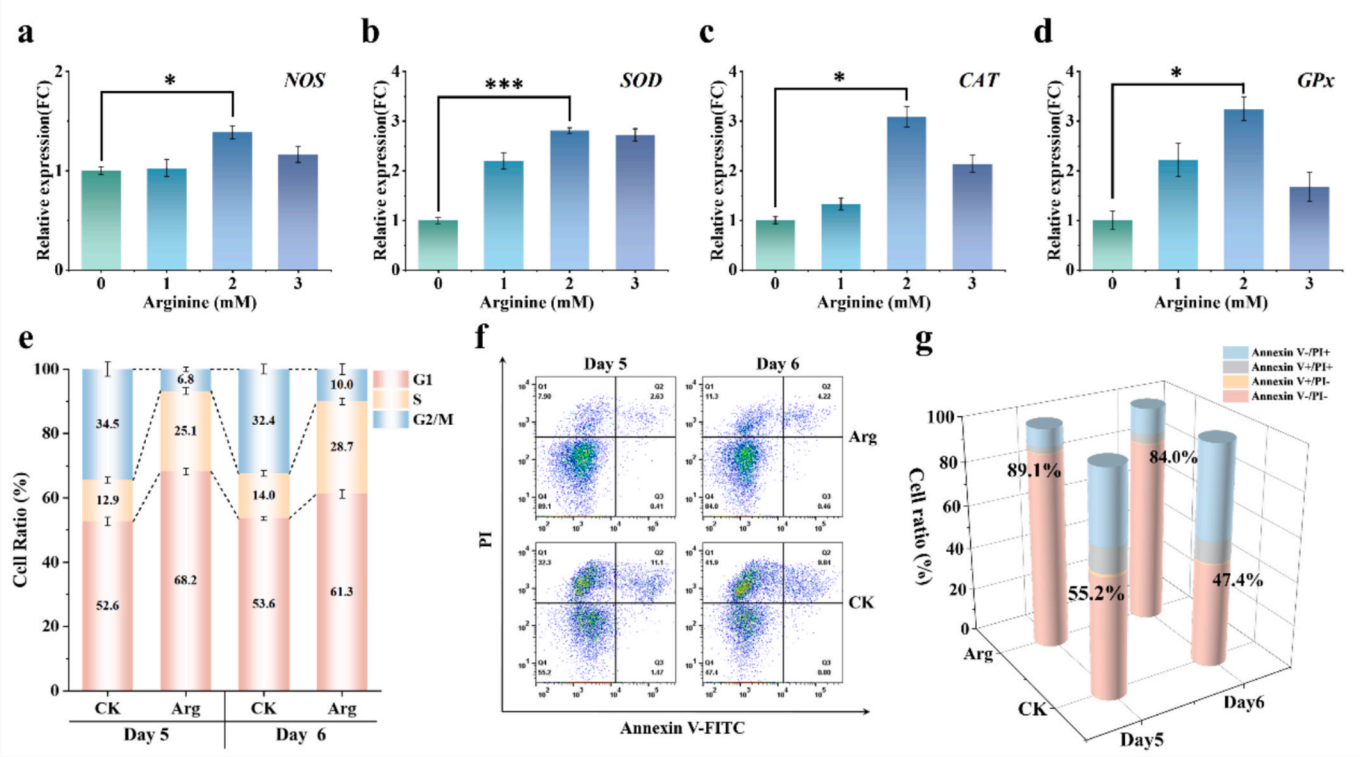

Fig. 3. Exogenous arginine enhanced antioxidant enzyme activities and cell viability in *Nannochloropsis oceanica* under high CO_2 stress. Transcriptomic levels of (a) nitric oxide synthase (NOS), (b) superoxide dismutase (SOD), (c) catalase (CAT), and (d) glutathione peroxidase (GPx), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; (e) cell cycle distribution ($p < 0.05$); (f) cell apoptosis: viable cell (Annexin V−/PI−), early apoptotic cell (Annexin V+/PI−), late apoptotic cell (Annexin V+/PI+), necrosis cell (Annexin V−/PI+); and (g) proportion of different cell states ($p < 0.001$).

to the CK group (53 % and 54 %, respectively). This extended G1 phase duration suggested that arginine may contribute to cell cycle modulation by providing additional time for cell growth, antioxidant enzyme synthesis, and metabolic pathway repair prior to DNA replication (Bertoli et al., 2013). Furthermore, a study proposed that arginine treatment helped balance carbon and nitrogen metabolism, highlighting the critical role of nitrogen metabolism in the tolerance of *Dunaliella* sp. cells to long-term salinity stress (Bamary and Einali, 2023). In the present study, an increase in nitrogen content was observed in the Arg group (42 % higher over the CK group, see supplementary material), which correlated with enhanced protein synthesis. This observation was consistent with the extended G1 phase, suggesting that the cells were prepared more effectively for cell division (Wang et al., 2023).

The S phase data further corroborated the observed protective effects of arginine. The proportion of cells in S phase was substantially higher in the Arg group (25 % on day 5 and 29 % on day 6), as compared to the CK group (13 % on day 5 and 14 % on day 6). These results suggested that arginine helped maintain DNA replication activity and reduce cell cycle arrest in the G1 or G2 phases under high CO_2 stress (Xin et al., 2024).

To elucidate the molecular mechanisms underpinning arginine-mediated cell cycle regulation, its role in NO signaling was investigated. Arginine serves as a precursor for NO, which has been shown to positively regulate the activity of protein arginine methyltransferase 5 (PRMT5) via S-nitrosylation at Cys-125 under external stress conditions (Hu et al., 2017). PRMTs are key enzymes that can catalyze protein arginine methylation, which is a crucial post-translational modification involved in gene expression, pre-mRNA splicing, DNA damage repair, mRNA translation, and cell cycle progression (Hu et al., 2017). PRMTs

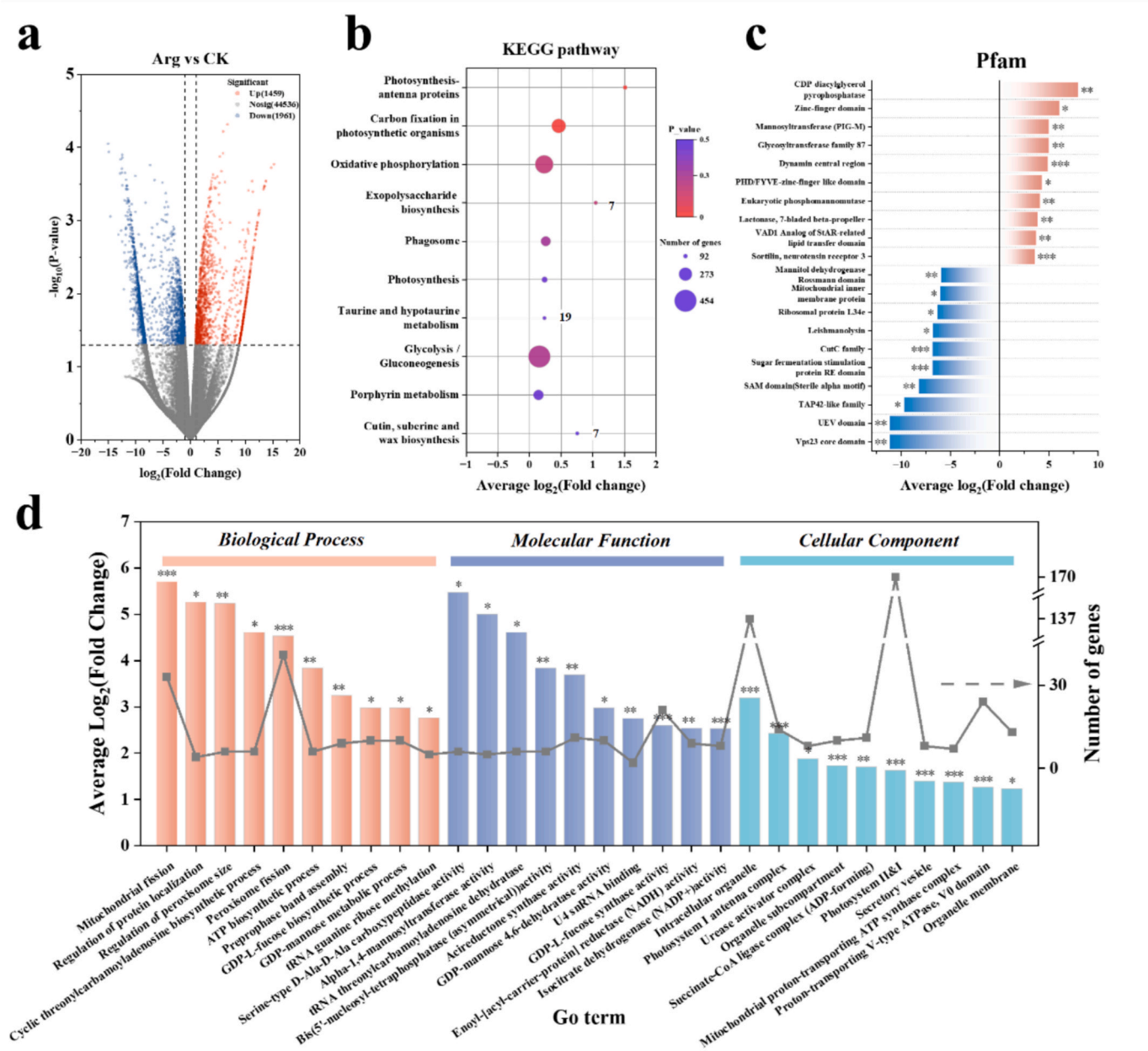

Fig. 4. Transcriptomic analysis of *N. oceanica* under high CO_2 stress with exogenous arginine. (a) Volcano plot of differentially expressed genes (DEGs): red and blue dots indicated significantly up- and down-regulated genes ($p < 0.05$), respectively; (b) KEGG pathway: bubble size denoted number of DEGs and color indicated p-values ($p < 0.5$); (c) Pfam database analysis of DEGs: top 10 up- and down-regulated genes ranked by fold change (*$p < 0.1$, **$p < 0.05$, ***$p < 0.01$,); (d) Gene Ontology (GO) enrichment analysis in terms of biological process, molecular function, and cellular component (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can influence cell cycle regulation either by modulating gene expression or by directly methylating cell cycle-related proteins (Yang et al., 2016). Transcriptomic analysis of the Arg group showed increased PRMT expression (see supplementary material), further supporting the hypothesis that arginine-mediated signaling pathways contribute to normal cell cycle progression and stress adaptation responses.

To validate the impact of arginine on cell viability, apoptosis, and necrosis, an Annexin V-FITC/PI double staining kit was employed (Fig. 3f & g). On day 5 of the treatment period, the proportion of viable cells in the Arg group was 89 %, which was 1.6-fold higher over the CK (55.2 %), while the necrosis rate was significantly lower at only 8 %, as compared to 32 % in the CK group. By day 6, both groups exhibited modest declines in cell viability (84 % in Arg vs 47 % in CK), paralleled by distinct necrosis at 11 % and 41 %, respectively. These findings unequivocally demonstrate that arginine supplementation significantly enhanced cell viability and mitigated the development of apoptosis and necrosis under high CO_2 stress (Goodwin et al., 2004).

3.4. Effect of arginine treatment on differentially expressed genes in Nannochloropsis oceanica

To investigate the impact of arginine on the gene expression profile of microalgae under high carbon conditions, differentially expressed genes (DEGs) were analyzed (Fig. 4). As shown in the volcano plot in Fig. 4a, a total of 1459 genes were significantly up-regulated and 1961 genes were significantly down-regulated, based on the criteria of a p-value < 0.05 and $\log_2(FC) > 1$. These findings indicated that arginine significantly influenced the transcriptomic response of *N. oceanica* under high carbon stress conditions. To further elucidate the functions of these differentially expressed genes, functional annotation and pathway analysis were conducted.

Based on KEGG pathway enrichment analysis (Fig. 4b), several biological pathways were significantly affected by arginine treatment. Notably, the Photosynthesis-antenna proteins and Carbon fixation in photosynthetic organisms pathways exhibited the most significant alterations ($p < 0.01$), with both pathways showing clear up-regulation. In the Photosynthesis-antenna proteins pathway, 86 % of 92 DEGs were up-regulated, with an average fold change of 2.9. Similarly, in the Carbon fixation pathway, 294 genes were significantly up-regulated, with an average up-regulation of 1.4-fold. These findings indicated that arginine supplementation significantly enhanced both the light-harvesting capacity and carbon assimilation efficiency of *N. oceanica* under high CO_2 stress. In addition, genes associated with Oxidative phosphorylation were broadly up-regulated (375 DEGs, average fold change of 1.2), suggesting improved cellular energy metabolism. The Phagosome pathway was also enriched, potentially contributing to the removal of metabolic byproducts and oxidative components, thus supporting intracellular homeostasis under CO_2 stress conditions. Of particular interest, the Exopolysaccharide biosynthesis pathway was also up-regulated, with an average up-regulation of 2.0-fold. To validate this transcriptomic observation, EPS content was measured using three-dimensional EEM fluorescence spectroscopy. The results confirmed that EPS levels in the arginine-treated group were significantly higher than in the CK group, particularly for the S-EPS, which was 2.5-fold higher (see supplementary material). EPS is a known protective response to external stress (e.g. high CO_2), providing structural support and aiding ROS scavenging, which aligns with previous reports (Zhang et al., 2020). Other up-regulated pathways included the Photosynthesis, Taurine and hypotaurine metabolism, Glycolysis/Gluconeogenesis, Porphyrin metabolism, and Cutin, suberine, and wax biosynthesis, all of which contributed synergistically to improving photosynthetic performance, antioxidant capacity, metabolic resilience and membrane integrity.

Further annotation analysis was performed using the Pfam database, revealing that several domains were significantly up-regulated (Fig. 4c), including CDP-diacylglycerol pyrophosphatase, the Zinc-finger domain, Glycosyltransferase family 87, Mannosyltransferase (PIG-M), and the Dynamin central region. These domains are mainly involved in essential cellular processes such as metabolism, membrane repair, gene expression regulation, signaling, and material transport (Cassandri et al., 2017), suggesting that these processes may play a key role in the survival and growth of *N. oceanica* under high carbon stress conditions. Notably, the UEV and Vps23 protein domains were significantly down-regulated, both of which are linked to ubiquitination, a key mechanism for regulating protein localization and stability in eukaryotic cells (Teo et al., 2004). It was hypothesized that the down-regulation of ubiquitination in the Arg group may have regulated the dynamic balance of intracellular proteins, enhancing resource utilization and the adaptability of cells to high carbon conditions.

GO term functional annotation was used to categorize the significantly expressed DEGs into three main groups: molecular functions (MF), biological processes (BP), and cellular components (CC). As shown in Fig. 4d, the top 10 up-regulated terms were selected for each category, with all p-values being <0.05. Among these, the average $\log_2(FC)$ for the MF categories of Mitochondrial fission, Peroxisome fission, and ATP biosynthetic processes were 5.7, 4.5, and 3.8, respectively. The up-regulation of these processes effectively enhanced the antioxidant capacity and metabolic adaptation of microalgal cells, while the significant increase in ATP biosynthesis provided sufficient energy for cell division, photosynthesis, and antioxidant responses. For the BP category, Isocitrate dehydrogenase ($NADP^+$) activity also increased significantly, with an average $\log_2(FC)$ of 2.5. Furthermore, the GDP-L-fucose biosynthetic process and GDP-L-fucose synthase activity were both significantly up-regulated, indicating that arginine may enhance the structural protection of the cell wall under high carbon stress conditions, thereby supporting cell growth and division (Sun et al., 2024). In the CC category, significant changes were observed in 137 genes related to Intracellular organelles and 170 genes related to Photosystems II & I, with average $\log_2(FC)$ values of 3.2 and 1.6, respectively. These findings confirmed that *N. oceanica* was able to adapt to high levels of carbon stress by enhancing organelle metabolism and optimizing photosynthesis, assisting rapid growth under these conditions.

3.5. Mechanisms of exogenous arginine on photosynthesis and metabolism in Nannochloropsis oceanica

In the present study, arginine metabolic pathway in *N. oceanica* was investigated comprehensively, and the expression of related enzymes was systematically analyzed using transcriptomics approach. This study revealed a previously uncharacterized metabolic utilization of arginine that connected NO signaling to enhance carbon fixation and energy metabolism under high CO_2 stress (Fig. 5).

Exogenous arginine in *N. oceanica* is metabolized through two primary routes: conversion to ornithine via arginase (arg, 1.6-fold increased) or to NO and citrulline via NOS, initiating downstream signaling and metabolic cascades (Yang and Gao, 2007). During photosynthesis, although the activitiy of the enzyme Rubisco tends to reach a saturated level under high carbon conditions, arginine significantly also up-regulated the activities of other Calvin cycle-related enzymes. Except for PRK, GAPDH, FBPase, SBPase metioned above, Phosphoglycerate kinase (PGK), transketolase (tkt), and fructose-bisphosphate aldolase (ALDO) are enhanced as well, 4.9-,2.3-and2.3-fold respectively (see supplementary material). The upregulation of these enzymes facilitated the efficient progression of dark reactions, accelerating carbon fixation and the conversion of CO_2 into cellular organic matter. Consequently, the biomass accumulation capacity of *N. oceanica* was enhanced, improving its survival ability under high carbon stress conditions.

As shown in supplementary figure (see supplementary material), the downregulation of amino-acid *N*-acetyltransferase (argA or NAGS), acetylornithine/*N*-succinyldiaminopimelate aminotransferase (argD), acetylornithine deacetylase (argE), and aminoacylase (ACY1)—enzymes

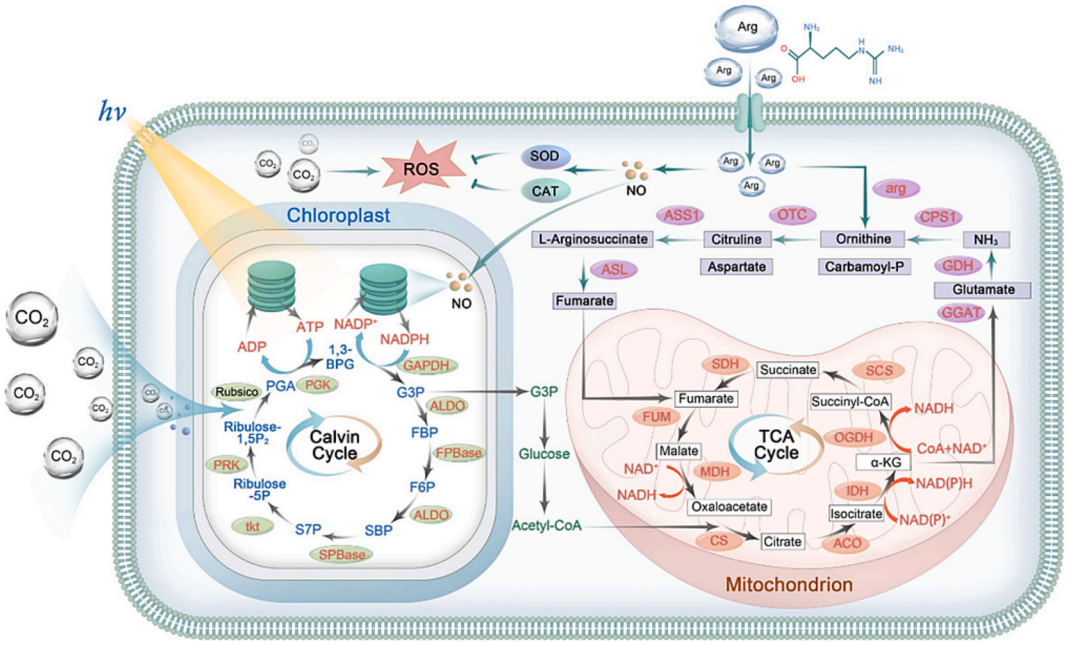

Fig. 5. Proposed mechanism of regulated metabolic pathways in *Nannochloropsis oceanica* with exogenous arginine under high CO_2 stress. Arginine enhanced antioxidant enzyme activities: superoxide dismutase (SOD); catalase (CAT) to combat reactive oxygen species (ROS), promoted Calvin cycle activity within chloroplast, and tricarboxylic acid cycle (TCA cycle) efficiency in mitochondrion to strengthen carbon fixation and energy metabolism. The expression of enzymes in red fonts are all upregulated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

catalyzing the conversion from glutamate to ornithine(precursor of endogenous arginine), demonstrating exogenous arginine triggered transcriptional feedback inhibition of endogenous biosynthesis (Xu et al., 2020). In particular, argA (NAGS) is the first rate-limiting enzyme and the main target of arginine feedback inhibition (Cheng et al., 2016). Meanwhile, ornithine transcarbamylase (OTC, 2.1-fold), argininosuccinate synthase (ASS1, 2.9-fold), and argininosuccinate lyase (ASL, 4.0-fold) were upregulated, suggesting that argininosuccinate was diverted toward fumarate production and tricarboxylic acid cycle (TCA cycle) entry, rather than recycled for arginine regeneration. In addition, glutamate-glyoxylate aminotransferase (GGAT), glutamate dehydrogenase (GDH) and carbamoyl-phosphate synthase 1 (CPS1) were also enhanced, 5.9-, 2.8- and 4.1-fold, respectively. Glyceraldehyde-3-phosphate (G3P) produced by the Calvin cycle was converted to pyruvate, generating acetyl-CoA, which also enters the TCA cycle. The transcriptional upregulation of key TCA cycle enzymes revealed a coordinated enhancement of mitochondrial energy metabolism in *N. oceanica* under high CO_2 stress. Citrate synthase (CS, 2.9-fold) and aconitase (ACO, 3.1-fold) —critical for initiating substrate flux into the cycle—showed significant induction, paralleled by the dramatic activation of isocitrate dehydrogenase (IDH, 5.3-fold), a rate-limiting enzyme governing NADPH generation, which is essential for driving antioxidant systems including, CAT, and GPx, thereby contributing to ROS mitgation under CO_2-induced oxidative stress (Li et al., 2024). Subsequent oxidative decarboxylation steps were reinforced through enhanced expression of oxoglutarate dehydrogenase (OGDH, 1.7-fold), which converts α-ketoglutarate to succinyl-CoA and generates NADH, supporting mitochondrial electron transport and ATP synthesis critical for stress adaptation (Che-Othman et al., 2020). The up-regulation of succinyl-CoA synthetase (SCS, 3.2-fold) and succinate dehydrogenase (SDH, 2.5-fold) enhanced both substrate-level phosphorylation (via SCS-derived GTP) and electron transport chain activity (via SDH-linked $FADH_2$). In addition, fumarate is converted to malate by the enzyme fumarate hydratase (FUM, 2.6-fold). Malate dehydrogenase (MDH, 4.1-fold)—the terminal enzyme of the cycle—exhibited the highest upregulation, facilitating oxaloacetate regeneration and maintaining TCA cycle continuity. Oxaloacetate also serves as a key precursor for amino acid biosynthesis, including aspartate, linking mitochondrial activity to nitrogen assimilation and anabolic growth (Zhao et al., 2020). Altogether, arginine functioned as both a metabolic substrate and regulatory signal, coordinating nitrogen metabolism, redox balance, and carbon fixation to enhance energy metabolism and cellular resilience under high CO_2 stress.

4. Conclusions

Exogenous arginine effectively alleviated high CO_2-induced oxidative stress in *Nannochloropsis oceanica* by enhancing NO production and upregulating antioxidant enzymes. Arginine extended the G1 phase to ensure accurate DNA replication, reducing cell necrosis. Differentially expressed genes (DEGs) especially related to Calvin cycle (e.g., PRK, GAPDH) and TCA cycle (e.g., CS, IDH) were significantly up-regulated, supporting celluar growth. Arginine also boosted the synthesis of chlorophyll *a* and carotenoids, ultimately increasing biomass yield. These findings elucidated arginine's role in metabolic regulation and provided a basis for further development of arginine-based strategies to improve microalgal performance under abiotic stress.

CRediT authorship contribution statement

Ruixue Ma: Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Jun Cheng:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis. **Ying Liu:** Resources. **Dongwei Jia:** Resources. **Jiahao Wang:** Writing – review & editing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.133080.

Data availability

Data will be made available on request.

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