

Article

Study on the Short-Term High-Temperature Response Mechanisms and Ethanolamine Metabolic Regulation in Desert *Chlorella*

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Abstract

Understanding the molecular basis of heat tolerance in microalgae is crucial for developing resilient strains for industrial biotechnology. This study identified two desert *Chlorella* strains, XDA024 (thermotolerant) and XDA121 (heat-sensitive), through short-term thermal screening. The thermotolerant strain XDA024 survived exposure to 50 °C for 3 h, whereas XDA121 succumbed within 1 h at 40 °C. Physiological analyses revealed that the superior heat resistance of XDA024 was associated with enhanced activities of key antioxidant enzymes, including superoxide dismutase, catalase, and peroxidase, which effectively mitigated oxidative damage, alongside an elevated proline content contributing to osmoregulation. Transcriptomic profiling under acute heat stress (45 °C, 3 h) revealed that the unique thermotolerance of XDA024 was underpinned by the upregulation of genes related to photosystem stability and lipid synthesis, processes supported by activated calcium signaling and antioxidant pathways. In contrast, XDA121 exhibited significant downregulation of photosynthesis-related genes and promoted lipid degradation, resulting in membrane instability. Exogenous application of phosphatidylethanolamine (PE) and monoethanolamine (MEA) markedly increased the survival rate of XDA121 by more than threefold, primarily by alleviating membrane damage through enhanced membrane integrity and modulated antioxidant enzyme activities. These findings indicate that thermotolerance in desert *Chlorella* (Chlorophyta) is governed by the integrated coordination of antioxidant defense mechanisms, lipid metabolism, and photosystem protection. This research provides crucial insights and practical strategies for engineering heat-resistant microalgal strains for sustainable biofuel and bioproduct production.

Keywords: *Chlorella*; short-term high temperature; response; lipid metabolism; ethanolamine



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1. Introduction

Chlorella sp., a genus of unicellular microalgae, is widely utilized in numerous sectors such as food and pharmaceutical industries, animal feed production, bioenergy generation, biofertilizer applications, soil enhancement, and environmental remediation [1–3]. To meet the diverse demands of these applications, industrial-scale cultivation of *Chlorella*

is essential for achieving sufficient biomass yield. In the context of mass microalgal cultivation, temperature stands out as a critical environmental factor influencing cellular proliferation [4]. During outdoor cultivation in summer, midday temperatures often exceed the optimal range for *Chlorella* growth, thereby adversely affecting cellular viability and productivity [5].

Elevated temperatures induce multifaceted detrimental effects on the photosynthetic apparatus. Research indicates that heat stress can cause irreversible damage to photosystem II (PSII) by disrupting its intermolecular interactions and inhibiting its activity, ultimately leading to a decline in the maximum photochemical efficiency (F_v/F_m) [6,7]. Concurrently, high temperatures accelerate the degradation of photosynthetic pigments, particularly chlorophyll a (Chla) and chlorophyll b (Chlb), thereby impairing light-harvesting capacity and energy conversion efficiency [8]. Furthermore, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is markedly inhibited under thermal stress, disrupting the Calvin cycle and reducing CO_2 fixation capacity, which ultimately constrains the synthesis of photosynthetic products [9]. Under heat stress, the structure integrity of thylakoid membrane is also compromised, resulting in diminished ATP and NADPH production that further limits carbon assimilation [10]. Thermally induced alterations in membrane lipid composition are characterized by a decreased ratio of unsaturated fatty acids and an increased ratio of saturated fatty acids [11]. This shift reduced membrane fluidity, compromise integrity, and elevate electrolyte leakage, collectively disrupting cellular homeostasis [12,13]. Additionally, high temperatures diminish the energy transfer efficiency of light-harvesting antenna proteins. It is worth noting, however, that a moderate temperature increase may enhance the activity of the photosynthetic electron transport chain and facilitate photophosphorylation, thereby temporarily improving photosynthetic efficiency [14].

Under elevated temperature stress, *Chlorella* activates a multi-level defense strategy to maintain homeostasis. The initial response involves the enhancement of antioxidant system, where key enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are upregulated to effectively scavenge reactive oxygen species (ROS) and alleviate oxidative damage [12]. Concurrently, heat shock proteins (HSPs), notably HSP70 and HSP90, are significantly induced. These molecular chaperones assist in refolding denatured proteins and maintaining proteostasis balance under thermal stress [15]. Moreover, *Chlorella* accumulates compatible solutes such as proline and trehalose, which contributes to osmotic adjustment and cellular integrity [15]. At the membrane level, the *Chlorella* modulates lipid composition by increasing the saturation of fatty acids, thereby reinforcing membrane stability and preventing hyperfluidity [16]. Furthermore, remodeling of membrane phospholipid composition, beyond mere lipid saturation, is vital for stress adaptation. Phosphatidylethanolamine (PE) is a major phospholipid essential for membrane biogenesis and repair. Thus, ethanolamine metabolism, which provides the direct precursor for PE synthesis, constitutes a key regulatory node for maintaining membrane integrity and fluidity under stress. Indeed, studies in plants link enhanced ethanolamine metabolism to improved tolerance under abiotic stresses, including salt and heat [17,18]. Different heat-tolerant *Chlorella* strains maintain cellular thermotolerance through distinct mechanisms or synergistic interactions of multiple mechanisms.

The genus *Chlorella* exhibits considerable interspecific variation in high-temperature tolerance, reflecting adaptations to adverse ecological niches. Under heterotrophic cultivation, the widely studied *Auxenochlorella pyrenoidosa* (formerly *Chlorella pyrenoidosa*) maintains robust growth at 40 °C, with enhanced harvesting efficiency attributed to increased cell size and reduced surface charge [13]. In contrast, the thermophilic species *Chlorella sorokiniana*, originally isolated from a hot spring in India, thrives at temperatures

up to 44 °C, exhibiting rapid growth rates and elevated lipid accumulation [19]. Polar strains such as *Chlorella-Ant* and *Chlorella-Arc* exhibit broad thermal tolerance, surviving at temperatures well beyond those of their native habitats. Conversely, the tropical species like *Chlorella-Trop* show greater susceptibility to temperature fluctuations, with inhibited growth and reduced photosynthetic efficiency near its upper thermal limits [9]. Temperate *Chlorella-Temp* achieves optimal growth at approximately 28 °C but experiences significant photosynthetic inhibition and growth decline above 35 °C. Strain-specific adaptations are further highlighted by the contrast between the Antarctic strain *Chlorella* UMACC 229, adapted to low temperatures (6 to 9 °C) and showing limited recovery under heat stress, and the engineered thermotolerant strain *Chlorella sorokiniana* TR42, which sustains vigorous growth and biomass production even at 42 °C [12,20]. In open cultivation systems, transient temperature rises can reversibly affect photosystem II efficiency in high-light acclimated *Chloroidium saccharophilum* (formerly *Chlorella saccharophila*) [6]. These studies collectively underscore that thermotolerance in *Chlorella* is shaped by a complex interplay of physiological traits, with different species employing distinct strategies under high-temperature stress.

Desert algae, notably green algae (Chlorophyta) and cyanobacteria (Cyanophyceae), have evolved a suite of physiological and ecological adaptations that enable them to thrive in extreme environments. These include the rapid recovery of photosynthetic efficiency, increased chlorophyll content, induction of soluble proteins and lipids, and enhanced production of extracellular polysaccharides. Furthermore, pigment migration facilitates the formation of chemical gradients, allowing these organisms to acclimate to high-light conditions and persist in desert ecosystems [21,22].

To address the challenges of high temperatures in the application of *Chlorella* and explore its inherent thermotolerance mechanisms, we screened 16 desert *Chlorella* strains (Table A1) from Xinjiang Special Environmental Algae Culture Collection (XJSAC) for thermotolerance. The heat-resistant strains were subsequently subjected to RNA-Seq analysis under high-temperature stress, systematically revealing their transcriptomic responses. Key metabolic pathways and gene expression regulatory patterns associated with thermal adaptation were identified. Exogenous intervention experiments further validated the molecular networks involved in high-temperature stress responses, shedding light on the mechanistic basis of thermotolerance in *Chlorella*. This integrated approach provides both candidate strains and a methodological framework for breeding resilient algae, while advancing the fundamental understanding of stress adaptation mechanisms in microalgae to inform future engineering efforts.

2. Materials and Methods

2.1. Algal Strain Screening and Culture Conditions

Sixteen *Chlorella* strains (Table A1) in consecutive order of their catalog numbers from the XJSAC for preliminary thermotolerance screening. The strains were initially cultured on BG11 solid medium at 28 °C with an illumination intensity of 20,000 Lux, following a light/dark (L:D) cycle of 16:8 h light/dark cycle in a ZRG-800A-L artificial climate chamber (Binglin Electronic Technology, Shanghai, China). Prior to experiments, a single colony from each algal strain, following resuscitation on BG11 solid medium, was aseptically transferred to BG11 liquid medium and sub-cultured for 15 days under identical conditions to prepare the inoculum. When the OD₆₈₀ of the sub-culture reached approximately 0.5, 100 µL of the culture was inoculated into 100 mL of fresh BG11 liquid medium and incubated with shaking at 150 rpm in a ZQZY-88BGV illuminated shaking incubator (Zhichu Instrument, Shanghai, China). The cultures were subsequently subjected to high-temperature treatment once the OD₆₈₀ reached 0.8.

2.2. High-Temperature Treatment and Thermotolerance Screening of Algae

When the OD₆₈₀ of the algal cultures reached 0.8, high-temperature stress treatment was conducted by placing the constant-temperature water bath (DK-8D, Yiheng Instrument, Shanghai, China) inside ZRG-800A-L artificial climate chamber. The water temperature was set to 35 °C, 40 °C, 45 °C, and 50 °C, with treatment durations ranging from 1 to 5 h, while cultures maintained at 28 °C served as the control. After heat treatment, 100 µL aliquots were transferred to 10 mL of fresh BG11 medium and incubated at 28 °C for recovery, with three replicates per treatment. Following a 10-day recovery period, the OD values were measured to evaluate short-term heat resistance. Based on these results, one strain with the highest tolerance and one with the lowest tolerance were selected for further physiological and biochemical analyses. Post high-temperature stress, algal cells were collected immediately via centrifugation, weighed, flash-frozen in liquid nitrogen, and ground using tissue grinder (JXFSIPRP-24L, Jingxin, Shanghai, China) at 50 Hz for 30 s per cycle, repeated 3 times with 5 s intervals between cycles. Oxidative stress indicators, including PRO and MDA contents, as well as the activities of CAT, SOD, and POD, were quantified using commercial assay kits (Grace Biotechnology Co., Ltd., Suzhou, China) according to the manufacturer's instructions. Chlorophyll content was determined following the method described by Chen et al. [23]. The photosynthetic performance was evaluated by measuring chlorophyll fluorescence parameters using a pulse-amplitude modulation chlorophyll fluorometer (Phyto-PAM, Heinz Walz GmbH, Effeltrich, Germany). Prior to measurement, samples were dark-adapted for 20 min to ensure the full oxidation of PSII reaction centers. The maximum quantum yield of PSII (Fv/Fm) was then measured using a saturating light pulse. Subsequently, the effective photochemical quantum yield of PSII (Y(II)) was determined under an actinic light intensity of 3000 µmol·m⁻²·s⁻¹ with a duration of 0.8 s.

2.3. Transcriptome Sequencing and Metabolic Pathway Analysis of DEGs

The selected heat-tolerant and non-heat-tolerant algal strains were subjected to a 3h thermal stress at 45 °C. Cells were then harvested by centrifugation, rapidly frozen in liquid nitrogen, and stored, with three biological replicates per group. Total RNA was extracted utilizing the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into complementary DNA (cDNA). RNA sequencing (RNA-Seq) analysis was performed on the Illumina NovaSeq 6000 platform by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China), generating over 6 Gb of data per sample. Raw reads were processed using fastp (<https://github.com/OpenGene/fastp>, accessed on 8 November 2025) to remove: (1) reads containing more than 50% of low-quality bases (Q-value ≤ 20), (2) adapter contaminants, and (3) reads with over 10% unknown nucleotides. The resulting high-quality clean reads were de novo with Trinity (v2.8.4); the k-mer length was set to 31, with a minimum frequency of 6. Gene expression was quantified in TPM using feature Counts, and DEGs were identified with DESeq2 ($|\log_2\text{FoldChange}| \geq 1$ and a false discovery rate (FDR) < 0.05). Functional annotation of DEGs was carried out using the Gene Ontology (GO, <https://www.geneontology.org/>, accessed on 8 November 2025), Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/>, accessed on 8 November 2025), and Clusters of Orthologous Groups (COG, <https://www.ncbi.nlm.nih.gov/research/cog>, accessed on 8 November 2025) databases. Enriched metabolic pathways under high-temperature stress were identified using hypergeometric testing (FDR corrected *p*-value < 0.05). From these pathways, as key DEGs for further analysis genes that were upregulated under high-temperature stress in the thermotolerant strain XDA024 but showed no upregulation in the corresponding metabolic pathway nodes of the heat-sensitive strain XDA121.

2.4. Effects of Exogenous PE and MEA on the Thermotolerance of *Chlorella*

To evaluate the enhancement of thermotolerance, the heat-sensitive strain XDA121 was cultured in BG11 liquid medium supplemented with 10 mg/L PE or 0.001 mg/L MEA. The concentrations of PE and MEA were determined as the minimal effective doses based on preliminary internal experiments that assessed cell viability across a range of concentrations, ensuring no detrimental effects at the selected levels. After 24 h of cultivation at 28 °C, the cultures were subjected to a heat stress of 45 °C for 3 h, with three biological replicates per treatment. Algal cells viability was evaluated utilizing 1% methylene blue staining according to Freshney [24]. In this method, viable cells remain green while non-viable cells stain blue under microscopy. Viability was calculated as: $\text{Viability (\%)} = (\text{Number of viable cells} / \text{Total cells}) \times 100$ based on random field counts. Additionally, key physiological and biochemical parameters, including PRO, MDA, CAT, SOD, POD, and chlorophyll content, were assessed in PE- and MEA-treated algal cells both before and after heat stress, following the procedures detailed in Section 4.2.

To analyze changes in gene expression following PE and MEA treatment, quantitative real-time PCR (qRT-PCR) was conducted using TB Green Premix Ex Taq II (TaKaRa) on a QuantStudio 1 Plus real-time PCR system. The primer sequences are listed in Table S1, with *18S RNA* serving as the internal reference gene for normalization. The reaction protocol began with an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melting curve analysis was performed after amplification to verify the specificity of the PCR products. Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [25].

2.5. Statistical Analysis

Statistical analysis and graph editing were performed using GraphPad Prism software (version 10, GraphPad Software, San Diego, CA, USA). Data from three independent biological replicates were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Significant differences were considered at $p < 0.05$ (different lowercase letters) and highly significant differences at $p < 0.01$ (different uppercase letters).

3. Results

3.1. Screening of Thermotolerant Algal Strains

Following various heat treatments, all 16 algal strains underwent a 10-day recovery culture at 28 °C. After 5 h of exposure to 45 °C, nine strains (XDA006, XDA021, XDA024, XDA038, XDA051, XDA072, XDA086, XDA100, and XDA102) exhibited survival capacity (Figure 1). Among them, XDA024 showed the highest thermotolerance, maintaining viability even after 3 h at 50 °C. Conversely, strains XDA061 and XDA121 displayed limited heat resistance, with XDA121 succumbing within 1 h at 40 °C. Based on these distinct thermotolerance profiles, the most heat-resistant strain (XDA024) and the most heat-sensitive strain (XDA121) were selected for further experimentation.

To evaluate the effects of high-temperature exposure on algal activity, the recovery growth curves of strains XDA024 and XDA121 were compared following heat treatments of varying duration (Figure 2A). The results revealed significant differences in thermotolerance between the two strains ($p < 0.01$). Under control conditions (no heat stress), their growth curves were nearly identical. However, after exposure to temperatures ranging from 40 °C to 50 °C for 1 to 5 h, XDA024 displayed significantly greater heat resistance. Notably, even after 3 h at 50 °C, XDA024 sustained an OD₆₈₀ of 0.5 following a 15-day recovery period, with consistent increases in absorbance observed across all treatment groups. Conversely, XDA121 exhibited a heat tolerance of ≤ 40 °C and lost the capacity

to proliferate after only 1 h at 45 °C. These findings confirm the exceptional short-term high-temperature tolerance of XDA024.

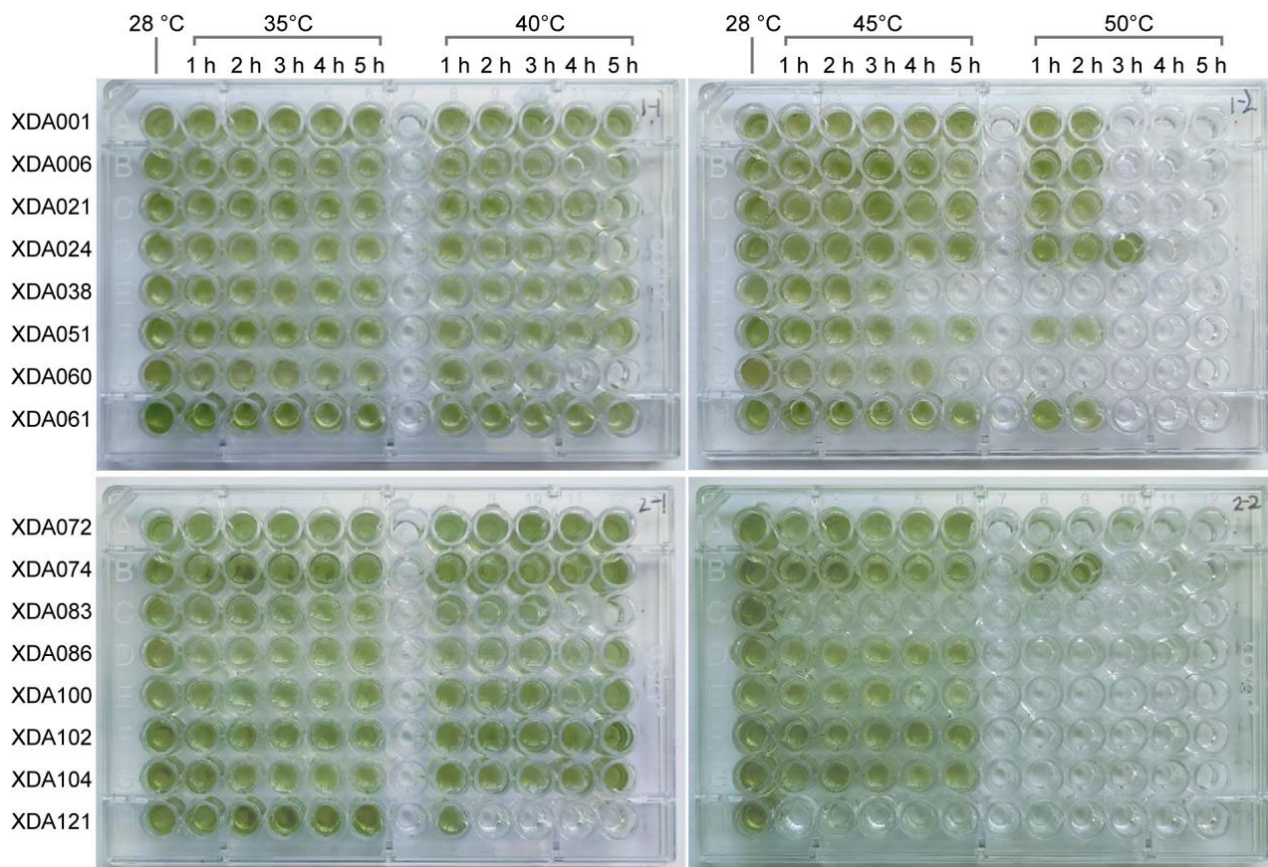


Figure 1. Growth performance of 16 algal strains post heat treatment. Sixteen algal strains were subjected to graded thermal stress (35–50 °C for 1–5 h) with 28 °C as control. Then 100 µL aliquots were transferred to fresh BG11 medium (10 mL) post-treatment and cultured at 28 °C ($n = 3$). Measurement of optical density (OD) for assessing short-term heat resistance across algal strains.

3.2. Physiological and Biochemical Responses of XDA024 and XDA121 to Heat Treatment

The investigation into the physiological and biochemical responses of different algal species to short-term high-temperature stress revealed a progressive decline in chlorophyll content (including chlorophyll a and chlorophyll b) in both XDA024 and XDA121 as temperatures increased (Figure 2B). Significant differences in chlorophyll content compared to the control group ($p < 0.05$) were observed when temperatures exceeded 35 °C. Notably, at 40 °C, XDA024 maintained significantly higher chlorophyll levels than XDA121 ($p < 0.01$), indicating its superior capacity to preserve the stability of the photosynthetic pigment system under optimal and moderately elevated temperatures. A distinct difference in chlorophyll a/b ratio was observed between the two strains. Under optimal temperatures (28–40 °C), the ratio was consistently higher in XDA024 than in XDA121 ($p < 0.05$). However, under extreme heat stress (>40 °C), this relationship reversed, with XDA024 exhibiting a lower ratio attributable to a relative accumulation of chlorophyll b ($p < 0.05$) (Figure 2B), implying variety-specific responses in chlorophyll metabolism. Consistent with this physiological distinction, PSII functionality was markedly more stable in XDA024, which maintained significantly higher F_v/F_m and $Y(II)$ values, underscoring its superior ability to preserve light-use efficiency under high temperature. Conversely, XDA121 experienced a drastic reduction in photosynthetic efficiency above 40 °C, revealing profound PSII damage and greater vulnerability to stress.

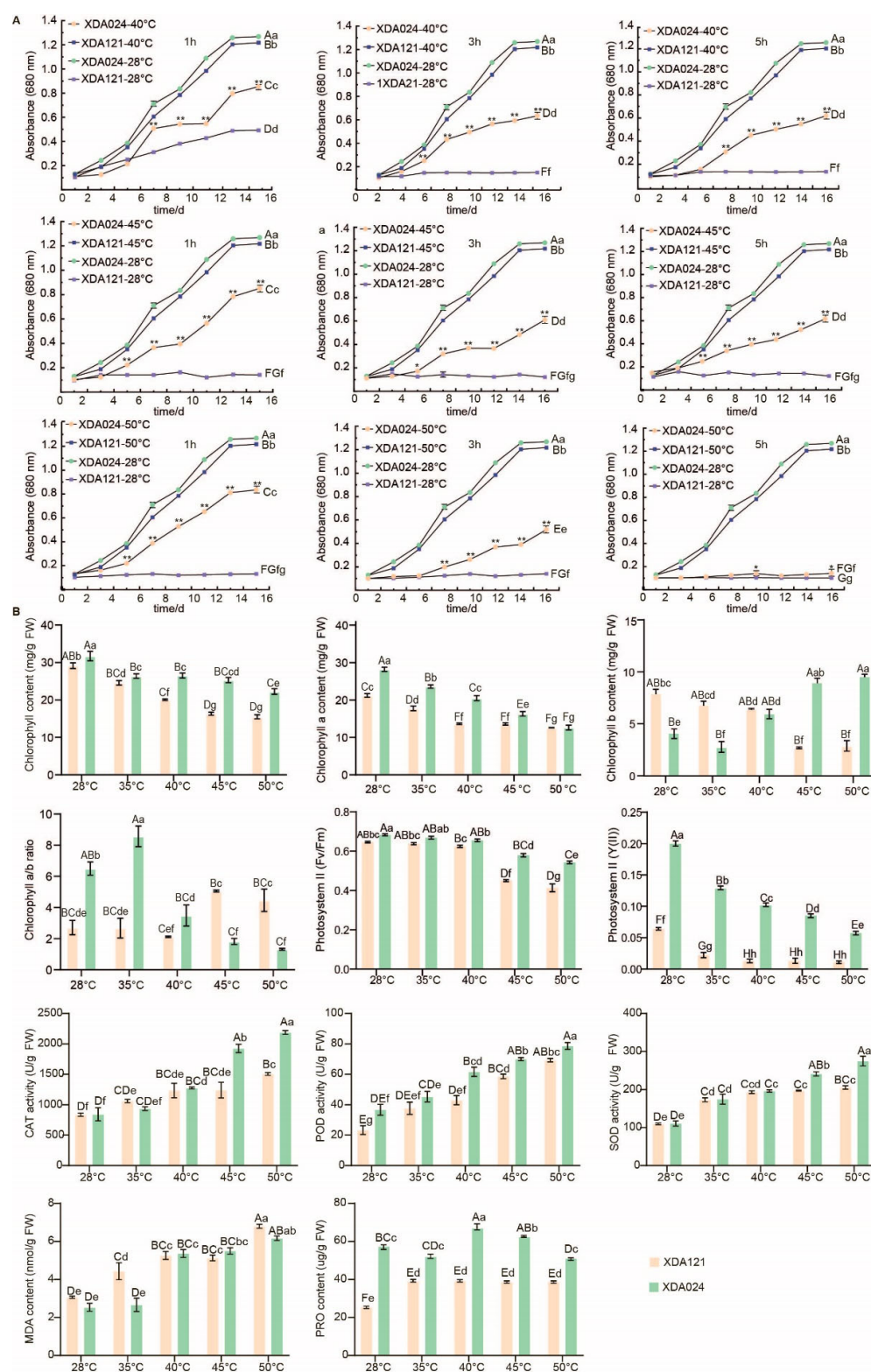


Figure 2. Impact of short-term thermal stress on the growth performance of *Chlorella* strains XDA024 and XDA121. (A) Post-treatment growth trajectories of XDA024 and XDA121 subjected to brief thermal exposure at varying temperatures. (B) Physiological stress indices of XDA024 and XDA121 following a 3h exposure to different thermal stress conditions. Different lowercase letters and “*” signify statistically significant differences among treatments at $p < 0.05$, while different uppercase letters and “***” indicate significant differences at $p < 0.01$.

In addition, XDA024 exhibited consistently higher activities of SOD, CAT, and peroxidase (POD) than XDA121 under various high-temperature conditions (Figure 2B). After a 3 h exposure to 50 °C, the activities of SOD, CAT, and POD in XDA024 were significantly higher than those in XDA121 by approximately 36.02% ($p < 0.01$), 44.96% ($p < 0.01$), and 13.48% ($p < 0.05$), respectively. This indicating a more effective ROS scavenging capability, which effectively mitigated oxidative damage in algal cells. As a key marker of membrane lipid peroxidation, MDA content increased significantly in both strains under rising temperatures. However, the accumulation was markedly greater in XDA121, suggesting that its weaker antioxidant enzyme activity resulted in ROS accumulation and more severe damage to the cell membrane system. Furthermore, following high temperature stress, XDA024 showed a substantially higher Proline (PRO) content compared to XDA121 ($p < 0.01$), indicating its stronger ability to maintain cellular homeostasis via osmoregulation. These findings collectively suggest that the thermotolerance of XDA024 may be attributed to its robust antioxidant enzyme activity and osmoregulatory capabilities. In contrast, the heat sensitivity of XDA121 likely stems from its insufficient antioxidant defense and membrane protection mechanisms.

3.3. Transcriptomic Profiling of Heat Stress Responses and KEGG Enrichment Analysis of Thermotolerance-Related Differentially Expressed Genes (DEGs)

To investigate the thermotolerance mechanisms of the heat-tolerant alga, transcriptome analysis was conducted on the heat-tolerant strain XDA024 and the heat-sensitive strain XDA121 after 3 h of treatment at 45 °C, with cultures grown at 28 °C serving as controls (Figure 3). Although most genes showed reduced expression under heat stress, a distinct subset was markedly upregulated (Figure 3A). Quantitative analysis identified 5363 DEGs in XDA024, of which 1350 were upregulated and 4013 were downregulated. In XDA121, 7091 DEGs were detected, including 2089 upregulated and 5812 downregulated genes (Figure 3B, Table S2). These findings indicate that XDA121 mounts a more extensive transcriptional response to heat stress, exhibiting 32.3% more total DEGs and a 54.7% greater number of upregulated genes than XDA024, suggesting a heightened genomic sensitivity to thermal perturbation. Given the concomitant physiological evidence of severe oxidative damage and membrane injury in XDA121 (Figure 2B), this more-extensive and less -discriminate transcriptional response likely reflects profound cellular distress and a loss of regulatory control.

KEGG enrichment analysis of heat-responsive DEGs in XDA024 and XDA121 (Figure 3C) showed that XDA024 exhibits a broader and more pronounced transcriptional response across twelve key metabolic and repair pathways following heat exposure. These enriched pathways included glycosylphosphatidylinositol (GPI)-anchor biosynthesis, lipoic acid metabolism, homologous recombination, sphingolipid metabolism, DNA replication, ether lipid metabolism, non-homologous end-joining, selenocompound metabolism, glycerophospholipid metabolism, nucleotide excision repair, SNARE interactions in vesicular transport, autophagy-other. The notable enrichment of DEGs in DNA repair pathways, especially homologous recombination and nucleotide excision repair, implies that high temperatures likely induced DNA damage, thereby activating corresponding repair mechanisms to preserve genomic integrity. This transcriptomic response aligns with physiological evidence of heat-induced oxidative stress, marked by elevated activities of antioxidant enzymes (SOD, CAT, POD) and accumulation of the lipid peroxidation marker MDA (Figure 2B). ROS overproduction, which these enzymes counteract, is a well-known cause of DNA damage. Thus, the concurrent upregulation of DNA repair and antioxidant pathways in XDA024 points to an integrated cellular defense against heat-induced genotoxic stress. Furthermore, the pronounced enrichment of genes related to Glycosphingolipid biosynthesis and Glycerophospholipid metabolism suggests a substantial remodeling

in cell membrane architecture and related signal transduction pathways under thermal stress conditions.

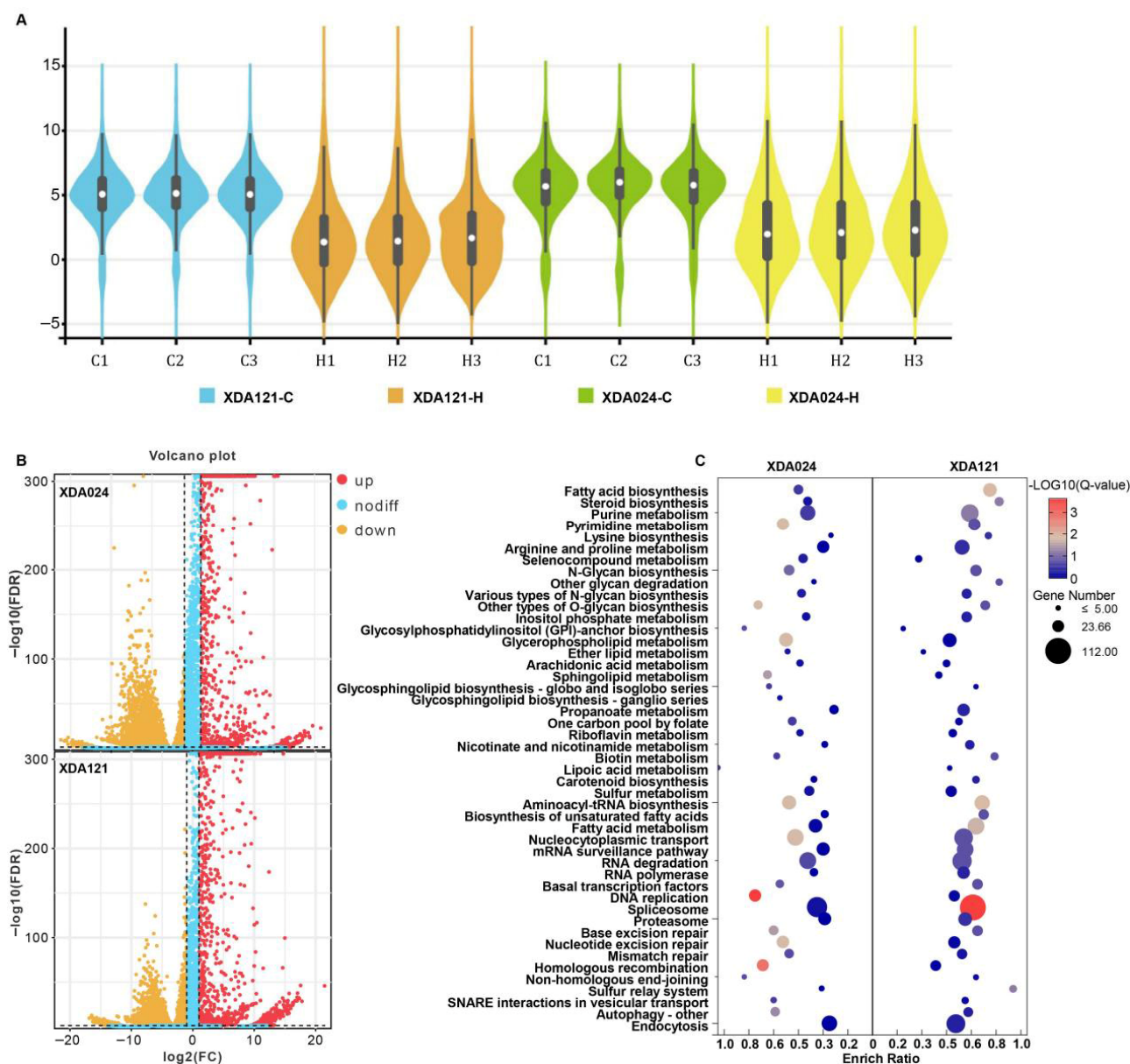


Figure 3. Transcriptomic analysis of *Chlorella* strains XDA024 and XDA121 under conditions of heat stress. (A) Violin plots illustrate DEGs, highlighting distinct subsets with significant upregulation following treatment. (B) Volcano plot analysis indicates that strain XDA121 exhibits a more pronounced transcriptional response to thermal stress relative to strain XDA024. (C) KEGG pathway enrichment analysis of DEGs reveals strain-specific metabolic reprogramming in response to acute heat exposure.

3.4. Functional Analysis of Thermotolerance-Associated DEGs

3.4.1. DEGs Related to HSP

Under heat stress, both XDA024 and XDA121 significantly upregulated molecular chaperone genes to preserve protein homeostasis. Notably, XDA121 mounted a more pronounced heat-shock response, with total chaperone transcript levels increasing 6.79-fold (relative to its 28 °C control; Log_2FC ; hereafter), compared to a 3.00-fold increase in XDA024 (relative to its 28 °C control) (Figure 4(A1)). Distinct induction patterns were observed for HSP70 and HSP90 family members: in XDA024, *HSP70* (Unigene0009468) reached 15,786.35 TPM (transcripts per million), a 5.98-fold induction, whereas XDA121 exhib-

ited a remarkable upregulation of *HSP83* (an HSP90 family member) to 172,936.86 TPM, representing a 9.40-fold increase, potentially linked to membrane stabilization and antioxidant functions. Interestingly, *dnaJ* chaperone (Unigene0011383) in XDA121 (Figure 4(A2)), which displayed the highest baseline expression (389.21 TPM) at 28 °C, was downregulated 5.60-fold under heat stress, suggesting a possible negative regulatory mechanism within its chaperone networks. The differential expression patterns highlight distinct proteotoxic strategies. Specifically, XDA121 exhibits a stronger but potentially less coordinated chaperone response induction. This overactivation of HSPs, without precise regulatory coordination, may itself reflect a state of stress-induced proteotoxicity and could contribute to its higher heat sensitivity. In contrast, XDA024 shows a more moderate yet balanced response, likely supporting its superior thermotolerance.

3.4.2. DEGs Related to Lipid Metabolism

Significant differences in lipid metabolism were observed between XDA024 and XDA121 under heat stress. As illustrated in Figure 4(B1), XDA024 exhibited enhanced lipid biosynthesis activity after heat treatment. Key genes involved in fatty acid synthesis were notably upregulated, including *acetyl-CoA carboxylase* (*accD*, Unigene0003291), which increased by 4.29-fold, and *malonyl-ACP transacylase* (*MCAT*, Unigene0010560), which rose by 1.93-fold, collectively promoting the production of long-chain fatty acids. Under high temperature stress, the expression of *sphingolipid 4-desaturase/C4-monooxygenase* (*DES*, Unigene0013863) and *serine decarboxylase* (*SDC*, Unigene0019576), which are involved in sphingosine and ethanolamine synthesis, was up-regulated by 1.20-fold and 1.65-fold, respectively. Moreover, phosphatide *cytidyltransferase* (*CDS*, Unigene0019735), a key enzyme in phospholipid homeostasis, was upregulated by 4.0-fold.

In contrast, XDA121 demonstrated a pronounced shift toward lipid degradation (Figure 4(B2)), marked by the significant downregulation of *DES* and *SDC* by 5.50-fold and 3.53-fold, respectively. These findings suggest that XDA024 accumulates phytosphingosine through active sphingolipid biosynthesis, potentially triggering antioxidant or programmed cell death signaling pathways. Conversely, the metabolic shift in XDA121 may compromise membrane integrity. Furthermore, the expression of key genes involved in membrane stability, including *accD* (Unigene0014177), *phosphatidylinositol 4-kinase GAMMA 7* (*PI4KG7*, Unigene0010654), and *adaptor protein complex AP-2 subunit mu A12* (*APA12*, Unigene0017208), was significantly upregulated in response to heat stress. This coordinated upregulation points to a potential compensatory mechanism that reinforces membrane structure to mitigate thermal damage.

Furthermore, XDA024 and XDA121 exhibited markedly distinct regulatory patterns in antioxidant capacity. In XDA024, the expression of *CAT2* (Unigene0000949) increased from 897.0 TPM to 2831.1 TPM (1.66-fold, log₂FC), whereas in XDA121 (Unigene0012173), it decreased from 315.4 TPM to 51.8 TPM (decreased by 2.61-fold). This suggests that elevated *CAT2* expression in XDA024, potentially acting in concert with lipid-mediated signaling pathways, contributes to enhanced resistance to oxidative stress. Additionally, calcium ions, serving as key secondary messengers, participate in the modulation of multiple cellular processes. The thermotolerant strain XDA024 exhibited a 5.19-fold upregulation of Calcium-dependent kinase 29 (*CDK29*, Unigene0000795), a major intracellular calcium sensor and decoder, suggesting a potential role for enhanced calcium signaling in its heat-stress adaptation.

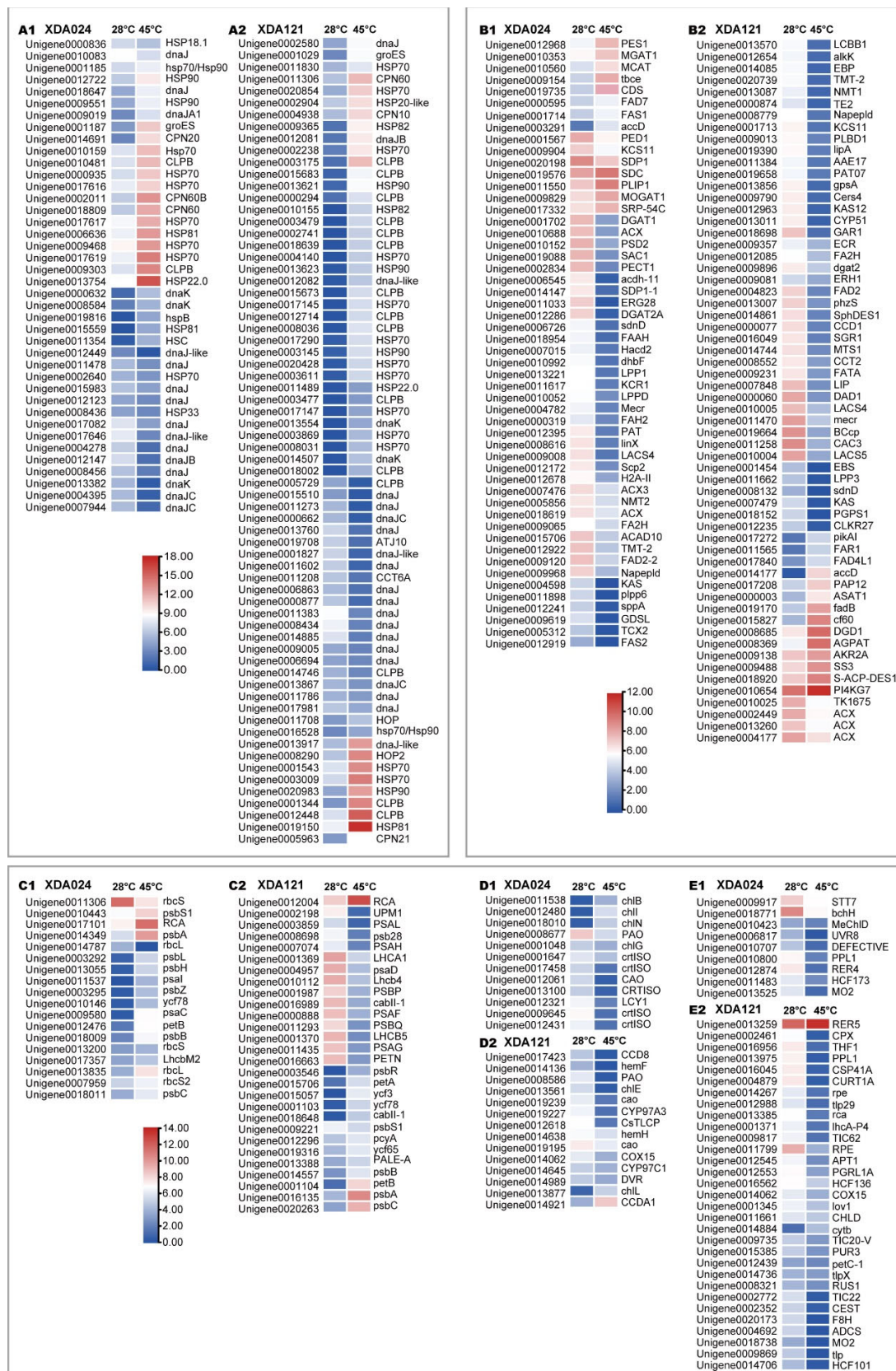


Figure 4. Differentially expressed genes (DEGs) in XDA024 and XDA121 before and after short-term high-temperature stress. (A1,A2) DEGs related to heat shock proteins (HSPs); (B1,B2) DEGs related to lipid metabolism; (C1,C2) DEGs related to the core photosynthetic system; (D1,D2) DEGs related to pigment metabolism and substance synthesis; (E1,E2) DEGs related to environmental response and systemic regulation.

3.4.3. DEGs Related to Photosynthesis

In response to short-term elevated temperatures, the expression of genes encoding photosynthetic complexes in XDA024 was significantly upregulated, indicating an active repair mechanism for the photosystem. Key upregulated genes included those encoding photosystem I/II core protein, such as *psaC* (Unigene0009580, 9.04-fold, TPM 116.68), *psbA* (Unigene0014349, 4.43-fold, TPM 964.76), and *psbB* (Unigene0018009, 4.77-fold, TPM 90.11). The expression levels (TPM) for rubisco activase (*RCA*, Unigene0017101) increased by 4.27-fold, while the chlorophyll synthesis gene *chlB* (Unigene0011538) was upregulated by 2.5-fold (Figure 4(C1,D1)). The log2FC for photosystem II reaction center genes, such as *psbA*, exceeded 3.0, indicating enhanced light energy capture efficiency under elevated temperatures. This coordinated upregulation of repair and synthesis genes provides a molecular explanation for XDA024's ability to sustain high photosynthetic performance (Fv/Fm, Y(II)) and chlorophyll content under stress. Conversely, genes associated with chlorophyll synthesis, such as Chloroplast Protein Kinase (*STT7*, Unigene0009917, decreased by 1.48-fold) and magnesium chelatase (*bchH*, Unigene0018771, decreased by 3.59-fold) (Figure 4(E1)), along with calvin cycle genes (*rbcS*, Unigene0011306, decreased by 3.90-fold), were downregulated, suggesting a partial impairment in chloroplast metabolism.

In the XDA121 sample, the expression of *RCA* (Unigene0012004) was markedly upregulated by 4.17-fold, reaching a TPM value of 6016.2 (Figure 4(C2)). The photosystem I subunit *psaA* (Unigene0016135) also showed increased expression (1.8-fold). Despite these inductions, the downregulation of the light-harvesting complex protein *cabII-1* (Unigene0016989) and the photosystem II reaction center gene *psb28* (Unigene0008698) by 5.05-fold likely limits the energy input to the photosystems. This expression pattern suggests a shift toward enhanced reliance on photosystem I-driven cyclic electron flow to maintain cellular energy equilibrium, which may be insufficient to prevent the severe drop in Fv/Fm and Y(II). Furthermore, in XDA121, the downregulation of the electron transport chain component *PETN* (Unigene0016663) and the light-harvesting antenna genes (*LHCA1*, Unigene0001369) and *LHCB5* (Unigene0001370) likely impairs the coordination of photosynthetic electron transport. The dysregulation may increase the susceptibility to photodamage, which is inconsistent with the observed severe decline in photosynthetic efficiency and elevated MDA accumulation in this strain under heat stress (Figure 2B). This risk may be partially compensated by the observed upregulation of *RCA*. Collectively, this less coordinated and more disruptive transcriptional response aligns with XDA121's pronounced physiological sensitivity.

3.5. Effects of Exogenous Phosphatidylethanolamine (PE) and Monoethanolamine (MEA) on Algal Thermotolerance

Transcriptome analysis revealed that, compared with the heat-sensitive alga XDA121, multiple genes in the lipid metabolism pathway of the heat-tolerant alga XDA024 were up-regulated under high-temperature stress. The up-regulation of *SDC* and *CDS* may lead to the accumulation of PE or MEA, thereby enhancing the thermotolerance of algal cells. To evaluate this possibility, exogenous PE and MEA were supplemented to XDA121 to examine their effects on the algal cells' thermotolerance.

Methylene blue staining revealed that both treatments significantly improved membrane integrity under heat stress, increasing the survival rate by approximately 3.3- and 3.5-fold for PE and MEA, respectively, compared to the untreated control (Figure 5A,B). This protective effect was corroborated by a pronounced reduction in MDA levels in supplemented groups, indicating attenuated membrane lipid peroxidation (Figure 5C). Physiological and biochemical analyses further demonstrated that although chlorophyll loss under heat stress was not rescued by either compound, oxidative stress markers were

distinctly modulated ($p < 0.01$). While MEA further enhanced CAT and POD activities, SOD activity was suppressed in both PE- and MEA-treated cells, likely due to diminished superoxide anion generation resulting from membrane stabilization.

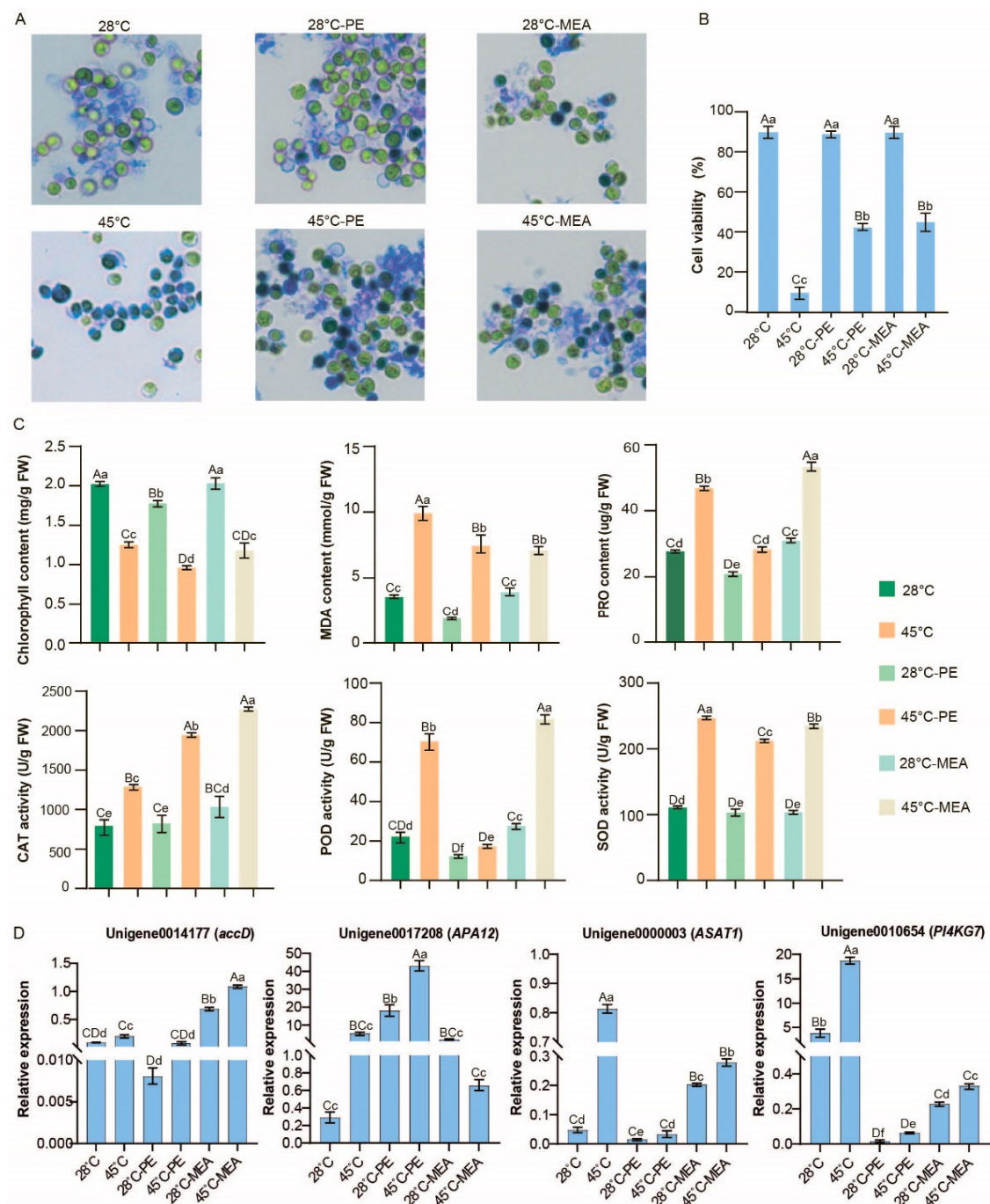


Figure 5. Effects of MEA and PE supplementation on the thermosensitive algal strain XDA121 after 3 h exposure to 28 °C or 45 °C. **(A)** Viability assessment by methylene blue staining; blue and green coloration indicate dead and live cells, respectively. **(B)** Survival rates of algal cells under different treatment conditions. **(C)** Changes in physiological indicators in XDA121 under heat stress. **(D)** Expression levels of lipid metabolism-related genes in XDA121. Error bars represent standard deviations ($n = 3$). Statistical significance was determined by one-way ANOVA; different lowercase and uppercase letters denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

These physiological responses were consistent with gene expression patterns related to membrane stability and oxidative defense. In the absence of supplements, heat stress

strongly upregulated *accD*, *APA12*, *PI4KG7*, and *ASAT1* (Figure 5D), reflecting a coordinated stress response involving lipid synthesis, membrane trafficking, and antioxidant defense. PE supplementation led to feedback inhibition of *accD* and strong upregulation of *APA12*, suggesting direct membrane incorporation and activation of endocytic trafficking. In contrast, MEA maintained high *accD* expression, supporting continued PE synthesis, but only weakly induced *APA12*. Both additives downregulated *PI4KG7* and *ASAT1*, with the suppression being more pronounced with PE, aligning with its stronger membrane-stabilizing and antioxidant effects.

Together, these results showed that PE and MEA enhance thermotolerance through shared and distinct mechanisms: both alleviate oxidative membrane damage, but PE acts more directly on membrane structure and trafficking, whereas MEA sustains lipid synthesis while offering moderate oxidative protection.

4. Discussion

4.1. Differential Thermotolerance in *Chlorella* Strains

Through short-term high-temperature stress screening of 16 algal strains, two *Chlorella* strains with markedly different thermotolerance profiles were identified: the heat-tolerant XDA024 and the heat-sensitive XDA121. Physiological assessments revealed that XDA024 exhibited significantly higher activities of SOD, CAT, and POD, along with substantial PRO accumulation under heat stress (Figure 2B). These responses collectively contributed to reduced oxidative damage, as evidenced by lower MDA accumulation [26–28]. Proline likely acts as a compatible solute and non-enzymatic antioxidant to further stabilize cellular structures under stress, as observed in other microalgae [12,28]. Transcriptomic analysis provided a molecular basis for these observed physiological advantages in XDA024. The significant enrichment of pathways related to glycerophospholipid, sphingolipid, and ether lipid metabolism in XDA024 (Figure 3C) supports active membrane remodeling, which is crucial for maintaining integrity under thermal stress [29,30]. This likely underpins the observed reduction in membrane lipid peroxidation. Furthermore, the enrichment of GPI-anchor biosynthesis suggests enhanced capacity for properly anchoring proteins to the membrane, which could contribute to the stability of membrane-associated protein complexes, including those in the photosynthetic apparatus.

Under heat stress, the efficiency and stability of the photosynthetic apparatus are critical for algal survival. Evidence shows that photosynthetic systems adapt to elevated temperature through multiple mechanisms, including regulation of photosynthetic pigment composition, enhancement of antioxidant enzyme activity, and induction of heat shock protein expression [12,31]. Both strains exhibited downregulation of genes related to photosynthetic pigment synthesis (Figure 4C,D), a conserved response that may alleviate photoinhibition by reducing light absorption [32]. However, their core strategies diverged. In response to heat stress, the tolerant strain XDA024 upregulated genes associated with photosystem I/II core proteins, electron transport, and the Calvin cycle, suggesting an enhancement of energy transfer. Notably, chlorophyll b is indispensable for protecting Photosystem I (PSI) and stabilizing the electron transport chain under high-temperature stress [33], and this protective role contributed to the superior thermotolerance observed in the XDA024 strain. Also, this alteration may broaden the spectrum of absorbed light and enhance the efficiency of energy capture. The consequent increase in energy flux to the reaction center could thereby significantly improve the photosynthetic rate under thermal stress conditions. This differential response implies that thermotolerant strains may adapt to high-temperature environments by preferentially repairing damage to the photosynthetic apparatus.

Crucially, the sophisticated multi-level defense in XDA024 did not incur a growth penalty under optimal conditions (28 °C), and it conferred superior recovery post-stress (Figures 1 and 2A). This aligns with observations in other extremophile algae, where adaptation to harsh environments like deserts involves coordinated enhancement of antioxidant capacity and photosynthetic apparatus preservation [12,34]. The fact that robust thermotolerance and growth are not mutually exclusive in XDA024 underscores the value of screening naturally resilient germplasm. The distinct responses of XDA024 and XDA121 likely stem from fundamental differences in their capacity to coordinate antioxidant defense, membrane stabilization, osmoprotection, and photosynthetic resilience—a suite of traits that can be leveraged for developing industrial algal strains suited to fluctuating thermal environments.

4.2. Lipid Metabolism Plays a Central Regulatory Role in *Chlorella* Thermotolerance

As primary constituents of the cell membrane, lipids not only provide structural integrity but also function as key signaling mediators in pathways governing stress responses and gene expression. In this study, transcriptomic analysis identified a significant activation of the lipid metabolic pathway in the thermotolerant algal strain XDA024 (Figure 3C), characterized by a pronounced upregulation of *accD*, *CDS* and *DES*. This coordinated gene expression profile suggests an enhanced biosynthetic capacity for long-chain fatty acids, glycerophospholipid and sphingolipids. Notably, the upregulation of *SDC*, the rate-limiting enzyme that catalyzes the conversion of serine to ethanolamine, points to the activation of a specific serine to ethanolamine branch pathway as an adaptive response to thermal stress. The concurrent upregulation of *CDS* further supports the hypothesis that the metabolic route linking ethanolamine and CDP-diacylglycerol contributes to thermotolerance. Notably, in XDA024, *CDS* expression was induced approximately 4-fold under 45 °C stress, surpassing the 2.26-fold upregulation reported for the thermotolerant strain *Chlorella sorokiniana* at 42 °C [12]. The resulting sphingolipids, such as phytosphingosine, potentially enhance membrane integrity by improving fluidity and promoting the formation of lipid raft structures [35]. In contrast, XDA121 showed suppressed lipid metabolism, with a 5.5-fold downregulation of sphingolipid 4-desaturase leading to impaired membrane stability and increased heat susceptibility. These findings suggest that XDA024 adapts to high-temperature environments by modulating lipid metabolism, thereby strategically adjusting membrane fluidity and stability to confer enhanced thermotolerance.

In XDA024, lipid metabolism interacts synergistically with the antioxidant system, as indicated by the 1.66-fold upregulation of *CAT2*. The synergy may involve lipid metabolites, such as sphingosine and sphingosine-1-phosphate (S1P), which are known to modulate the cellular redox status. Potential mechanisms include regulating calcium ion flux to influence antioxidant enzyme activity and activating MAPK signaling pathways (e.g., *JNK* and *ERK*) to alter antioxidant gene expression [36,37]. Furthermore, the interplay between sphingolipid metabolism and endoplasmic reticulum calcium homeostasis could influence autophagy, thereby impacting the overall redox balance. This intricate network underscores the multifaceted role of lipid metabolites in antioxidant defenses [38]. Consistent with this, pathways like “Autophagy-other” were significantly more enriched in heat-treated XDA024 than in XDA121, with *CDK29* (Unigene0000795) showing a notable 5.19-fold upregulation. These findings align with existing literature and highlight the pivotal regulatory function of lipid metabolism in short-term heat stress response of *Chlorella*.

4.3. Exogenous Metabolite Intervention Significantly Enhances *Chlorella* Thermotolerance

The strategic application of key lipid metabolites, PE and its precursor MEA, significantly enhanced the thermotolerance of the heat-sensitive algal strain XDA121. This

finding directly supports the viewpoint that lipid metabolism plays a central role in heat stress adaptation [39]. Our results demonstrate that this enhancement is achieved primarily through the preservation of membrane integrity and the modulation of oxidative stress responses, with PE and MEA operating via distinct yet complementary mechanisms.

The protective role of PE is consistent with its well-established function as a fundamental structural phospholipid essential for maintaining membrane fluidity and stability [40–42]. Its incorporation into membranes is supported by the feedback inhibition of the fatty acid synthesis gene *accD* and the strong upregulation of the endocytic trafficking gene *APA12* (Figure 5D). This suggests that PE directly reinforces membrane architecture and promotes repair processes. This direct membrane stabilization led to a reduced oxidative burden, as inferred from the decrease in SOD activity (Figure 5C). The subsequent downregulation of the antioxidant peroxidase gene *ASAT1* and the signaling kinase gene *PI4KG7* indicates a lowered oxidative stress state and a reduced urgency for stress signaling, consistent with a direct mitigation of the primary membrane injury. The significant reduction in MDA content further corroborates that PE effectively attenuates membrane lipid peroxidation. The inability of PE to restore chlorophyll content aligns with previous reports that its primary protective role is related to membrane system support rather than direct protection of photosynthetic organelles [43].

In contrast, MEA, as a biosynthetic precursor, functions indirectly by sustaining the production of PE and other membrane lipids [44]. This is evidenced by the maintained high expression of *accD*, ensuring a supply of fatty acids for ongoing membrane lipid synthesis. However, the weaker induction of *APA12* and the more modest downregulation of *ASAT1* and *PI4KG7* suggest a less direct impact on membrane trafficking and stress signaling compared to PE. Instead, MEA treatment markedly enhanced the activities of CAT and POD, indicating a shift in the oxidative defense strategy towards specific peroxide detoxification pathways. This coordinated modulation effectively reduced MDA accumulation and contributed to heat tolerance [45], highlighting that MEA's protection is partly mediated by modulating the antioxidant enzyme network. It is plausible that other mechanisms, such as the induction of HSPs or non-enzymatic antioxidant systems, also contribute to acquired thermotolerance [46–48], suggesting that the adaptive response is multifaceted.

In summary, our integrated analysis demonstrates that microalgal thermotolerance is achieved through the synergistic coordination of four core defense systems, as visually synthesized in Figure 6. The antioxidant system (SOD, POD, CAT) and molecular chaperones (e.g., HSPs, CLPB) collaboratively maintain protein homeostasis and cellular redox balance. This foundational protection is essential for preserving membrane integrity and safeguarding the photosynthetic apparatus under heat stress. While the tolerant strain XDA024 inherently possesses robust defense mechanisms, the sensitive strain XDA121 suffers from metabolic dysregulation under heat stress. Our study demonstrates that this sensitivity can be partially ameliorated by supplementing key lipid pathway metabolites. The focus on short-term heat stress may not fully capture the complex metabolic demands of long-term industrial cultivation. Therefore, future research should prioritize comparative lipidomics between XDA121 and XDA024 under continuous high-temperature stress to reveal specific lipid alterations underlying sustained thermotolerance. Furthermore, genetic engineering of key genes identified here, such as *accD* or *APA12*, presents a promising strategy for enhancing thermotolerance in economically important algal species. Also, to bridge the gap between laboratory findings and industrial application, targeted functional validation of these candidates is essential.

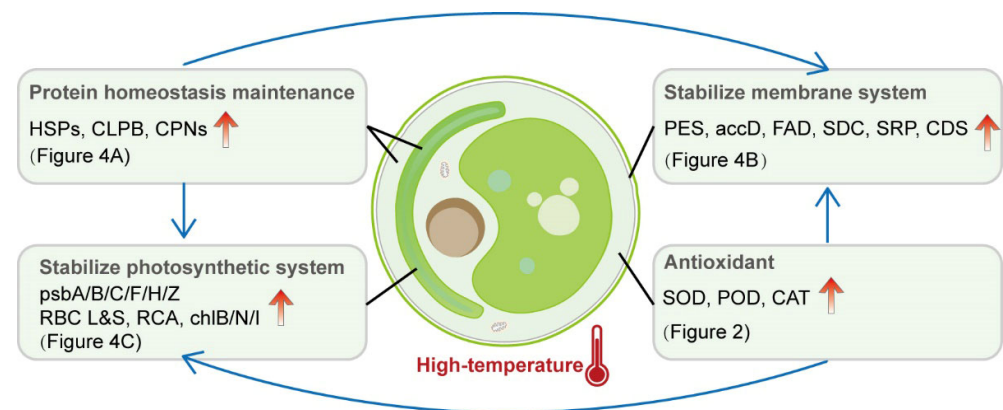


Figure 6. Schematic model illustrating the integrated thermotolerance network in *Chlorella* XDA024 under high-temperature stress.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/phycolgy6010013/s1>, Table S1: qRT-PCR primer sequences for gene quantification in XDA121 after PE and MEA treatment; Table S2: Differentially expressed genes in thermotolerant *Chlorella* (XDA024) and heat-sensitive *Chlorella* (XDA121) before and after heat treatment.

Author Contributions: Conceptualization, Y.C. and S.A.; methodology, Y.C. and N.Y.; software, N.Y. and L.Y.; validation, N.Y., L.Y., M.S. and X.T.; formal analysis, Y.C., and L.C.; resources, S.A. and A.T.; data curation, L.C.; writing—original draft preparation, N.Y. and L.C.; writing—review and editing, Y.C., S.A. and L.C.; supervision, Y.C. and S.A.; funding acquisition, Y.C. and S.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The differentially expressed gene profiles are provided in Supplementary Table S2, and all other datasets are available from the corresponding author upon reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

Chla	Chlorophyll a
Chlb	Chlorophyll b
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase

ROS	Reactive oxygen species
HSPs	heat shock proteins
POD	Peroxidase
PRO	Proline
MDA	Malondialdehyde
DEGs	Differentially expressed genes
GPI	Glycosylphosphatidylinositol
accD	Acetyl-CoA carboxylase
APA12	Adaptor Protein complex AP-2 subunit mu A12
ASAT1	L-Ascorbate Peroxidase 1
PI4KG7	Phosphatidylinositol 4-Kinase GAMMA 7
MCAT	Malonyl-ACP transacylase
DES	sphingolipid 4-desaturase/C4-monooxygenase
SDC	Serine decarboxylase
LHCA	Light-harvesting antenna
PE	Phosphatidylethanolamine
MEA	Monoethanolamine
ETA	Ethanolamine
RCA	Rubisco activase
S1P	Sphingosine-1-phosphate
CDK	Calcium-dependent kinase
PS	Phosphatidylserine
PC	Phosphatidylcholine
cDNA	Complementary DNA
RNA-Seq	RNA sequencing
TPM	Transcripts per million
FDR	False discovery rate
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
COG	Clusters of Orthologous Groups

Appendix A

Table A1. Chlorella strain information.

No.	Code	18S Sequence BLAST Hit ID	ITS Sequence BLAST Hit ID	Latin Name
1	XDA001	MZ266546.1	KR905186.1	Chlorella sorokiniana
2	XDA006	MH703759.1	KJ564285.1	Chlorella sorokiniana
3	XDA021	FR865682.1	GQ487232.1	Chlorella sp.
4	XDA024	KJ149805.1	MF483440.1	Chlorella sorokiniana
5	XDA038	OR393071.1	KR905186.1	Chlorella sorokiniana
6	XDA051	OM472011.1	MZ333647.1	Chlorella sp.
7	XDA060	MZ374011.1	MF629793.1	Chlorella sorokiniana
8	XDA061	AY197628.1	OW834972.1	Chlorella sorokiniana
9	XDA072	KF444207.1	KT778107.1	Chlorella sorokiniana
10	XDA074	LK021940.1	KT778107.1	Chlorella sorokiniana
11	XDA083	MN248530.1	LR215790.1	Chlorella sp.
12	XDA086	MZ736628.1	KF689556.1	Chlorella sp.
13	XDA100	MK541792.1	MT735072.1	Chlorella sp.
14	XDA102	FN298918.1	KP726219.1	Chlorella sp.
15	XDA104	MZ736630.1	OM472006.1	Chlorella sp.
16	XDA121	PQ479384.1	MN160396.1	Chlorella sorokiniana

NCBI BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 8 January 2025.

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