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GENOME-WIDE INSIGHTS INTO LONG NON-CODING RNA RESPONSES TO CO_2 STRESS IN NANNOCHLOROPSIS OCEANICA

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ABSTRACT

Microalgae are **photosynthetic, microscopic, mostly unicellular organisms** found in freshwater and marine environments. Long non-coding RNAs (lncRNAs) are emerging as critical regulators of gene expression in eukaryotes, particularly in response to environmental stresses. However, their functional roles in microalgae remain poorly understood. In this study, strand-specific RNA sequencing was performed to investigate genome-wide lncRNA responses in the marine microalga *Nannochloropsis oceanica* under fluctuating carbon dioxide (CO_2) conditions. A total of 134 lncRNAs were identified, among which 51 exhibited significant differential expression between high and low CO_2 treatments, including 33 upregulated and 18 downregulated transcripts. Functional enrichment analysis revealed that CO_2 -responsive lncRNAs are associated with cellulose, glucan, and polysaccharide metabolism, as well as transmembrane transport processes. Furthermore, lncRNA–mRNA co-expression and co-location network analyses suggested potential roles in transcriptional regulation, protein expression, and epigenetic modulation. A total of 2051 alternative splicing events were detected under CO_2 stress, indicating a possible regulatory interplay between lncRNAs and splicing mechanisms. Collectively, these findings provide new insights into lncRNA-mediated regulatory responses to CO_2 stress and offer potential targets for improving carbon utilization and stress tolerance in microalgal biotechnology.

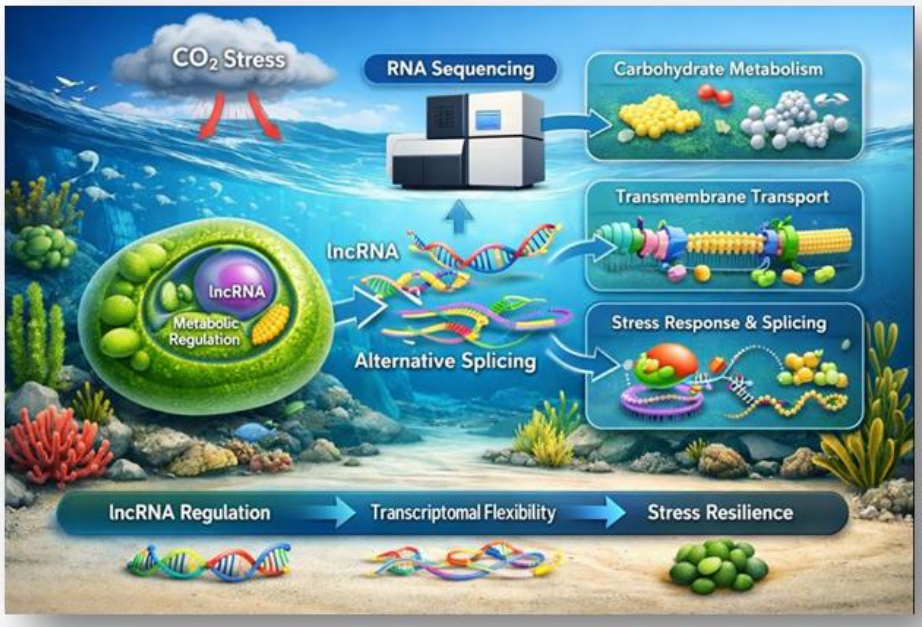
KEYWORDS: *Nannocloropsis oceanica, CO2 fluctuations lncRNAs, Microalgae.*

INTRODUCTION

Nannochloropsis oceanica is a unicellular marine microalga belonging to the class Eustigmatophyceae within the phylum Ochrophyta.[1] Initially, long non-coding RNAs (lncRNAs) were considered transcriptional noise; however, accumulating evidence now recognizes them as key regulators of chromatin remodeling, transcriptional control, alternative splicing, and RNA stability.[2] In plants, extensive studies have demonstrated that lncRNAs play crucial roles in growth and development, nutrient sensing, and responses to various abiotic stresses, including drought, salinity, and carbon dioxide (CO_2) fluctuations.[3] In contrast, the functional characterization of lncRNAs in microalgae remains limited, creating a significant knowledge gap in understanding their regulatory mechanisms.[4] *Nannochloropsis oceanica* has emerged as an important model photosynthetic microalga due to its rapid growth rate, high stress tolerance, and suitability for genetic manipulation.[5] Despite the availability of genomic and transcriptomic resources, the role of lncRNAs in CO_2 adaptation and metabolic regulation in *N. oceanica* is still poorly understood. Therefore, the present study aims to systematically identify CO_2-responsive lncRNAs at the genome-wide level and to characterize their potential regulatory roles under fluctuating CO_2 conditions.

Importance of the Study

- This study provides the first genome-wide insight into carbon dioxide (CO_2)–responsive long non-coding RNAs (lncRNAs) in the marine microalga *Nannochloropsis oceanica*,[6] addressing a major knowledge gap in microalgal gene regulation.
- It reveals lncRNAs as dynamic regulators involved in microalgal adaptation to fluctuating CO_2 levels, highlighting an additional layer of control beyond protein-coding genes.
- The functional association of lncRNAs with carbohydrate metabolism, transmembrane transport, and regulatory pathways emphasizes their role in metabolic reprogramming under CO_2 stress.
- The identification of extensive alternative splicing events suggests that lncRNAs contribute to transcriptome flexibility and stress resilience in photosynthetic microalgae.



AIM AND OBJECTIVES

The aim of this study is to **identify and characterize long non-coding RNAs (lncRNAs) involved in the response of the marine microalga *Nannochloropsis oceanica* to fluctuating carbon dioxide (CO_2) conditions**, and to elucidate their potential regulatory roles in gene expression, metabolic pathways, and stress adaptation mechanisms.

- To perform a genome-wide identification of long non-coding RNAs (lncRNAs) in the marine microalga *Nannochloropsis oceanica* under fluctuating carbon dioxide (CO_2) conditions.
- To analyze the expression dynamics of CO_2-responsive lncRNAs across low and high CO_2 treatments using strand-specific transcriptome sequencing.
- To characterize the potential regulatory roles of CO_2-responsive lncRNAs in carbon-associated metabolic pathways, including polysaccharide and carbohydrate metabolism.
- To investigate the involvement of lncRNAs in transmembrane transport, transcriptional regulation, and epigenetic control mechanisms under CO_2 stress.
- To examine the association between lncRNAs and alternative splicing events as a component of post-transcriptional regulation during CO_2 adaptation.
- To identify key lncRNA-mediated regulatory networks that contribute to microalgal stress tolerance and carbon utilization efficiency, with potential applications in microalgal biotechnology.

Classification of Microalgae

Microalgae are **photosynthetic, microscopic, mostly unicellular organisms** found in freshwater and marine environments. They are taxonomically diverse and distributed across several evolutionary lineages.[7]

1. Based on Cellular Organization

- **Unicellular microalgae** – *Nannochloropsis, Chlorella*
- **Colonial microalgae** – *Volvox*
- **Filamentous microalgae** – *Spirogyra*

2. Based on Prokaryotic vs Eukaryotic Nature

A. *Prokaryotic Microalgae*

- **Cyanobacteria (Blue-green algae)**

Example: *Anabaena, Nostoc*

B. *Eukaryotic Microalgae*

True algae with membrane-bound nucleus and organelles.

3. Major Taxonomic Groups of Eukaryotic Microalgae

A. *Chlorophyta (Green Algae)*

- Pigments: Chlorophyll a & b
- Storage: Starch
- Examples: *Chlorella, Scenedesmus*

B. *Rhodophyta (Red Algae – Micro forms)*

- Pigments: Phycoerythrin
- Storage: Floridean starch
- Examples: *Porphyridium*

C. *Heterokontophyta / Stramenopiles*

- **Bacillariophyceae (Diatoms)**
- **Eustigmatophyceae**
- **Xanthophyceae (Yellow-green algae)**

4. Class Eustigmatophyceae **(Where *Nannochloropsis* belongs)**

- Unicellular
- Lacks flagella
- High lipid content
- Marine and freshwater species
- Important for biofuel and CO_2 studies

Genera

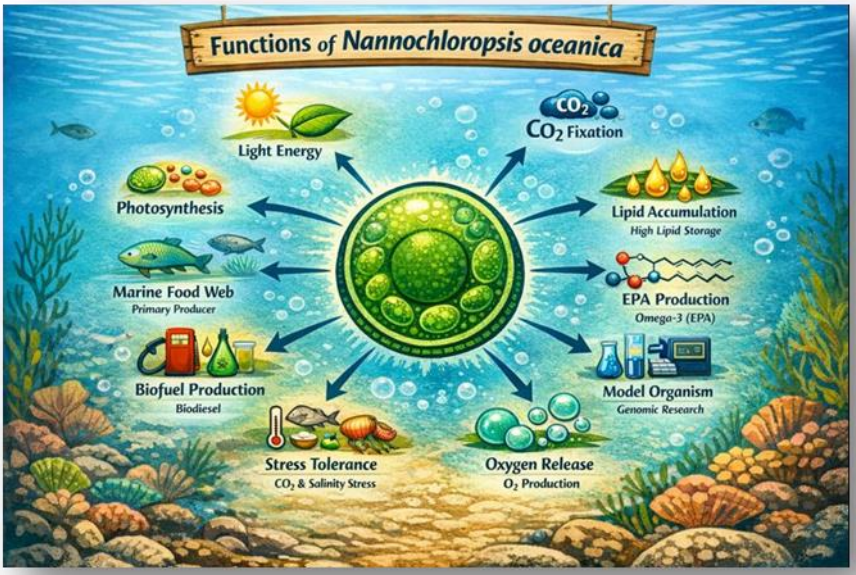
Nannochloropsis, Monodus.

Taxonomic Classification of *Nannochloropsis*

Rank	Classification
Domain	Eukaryote
Kingdom	Chromista
Phylum	Ochrophyta
Class	Eustigmatophyceae
Order	Eustigmatales
Family	Monodopsidaceae
Genus	*Nannochloropsi*
Species	Nannochloropsis oceanica

Functions Of Nannochloropsis

- Performs **photosynthesis** by utilizing light energy to synthesize organic compounds.
- Fixes **carbon dioxide (CO_2)** efficiently and supports carbon sequestration in marine systems.
- Accumulates **high levels of neutral lipids (TAGs)** as an energy storage reserve.[8]
- Produces **omega-3 fatty acids (EPA)** that are nutritionally important.
- Serves as a potential source for **biofuel and biodiesel production** due to high lipid yield.
- Functions as a **primary producer** forming the base of the marine food web.
- Used widely as **live feed in aquaculture** for fish larvae and shellfish.
- Exhibits strong **stress tolerance** to elevated CO_2 and salinity conditions.
- Releases **molecular oxygen (O_2)** as a by-product of photosynthesis.
- Acts as a **model organism** for genomic and metabolic research studies.



MATERIALS AND METHODS

Materials

Algal Strain

The marine eustigmatophyte microalga *Nannochloropsis oceanica* IMET1 was used as the experimental organism in this study.

Culture Medium and Chemicals

Modified f/2 liquid medium was prepared using artificial seawater containing 35 g L^{-1} sea salt.[9] The medium was supplemented with 1 g L^{-1} sodium nitrate ($NaNO_3$) as the nitrogen source and 67 mg L^{-1} sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) as the phosphorus source. Iron and chelating agents were supplied as 3.65 mg L^{-1} ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 4.37 mg L^{-1} disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$), respectively. Trace metal and vitamin solutions were added according to the standard f/2 formulation described by Kang et al. (2015).

Reagents and Consumables

- TRIzol reagent (Invitrogen, USA)
- rRNA depletion kit
- RNase-free water
- Agarose
- Ethanol (75%)
- Chloroform and isopropanol

Instruments and Software

- NanoDrop spectrophotometer
- High-speed refrigerated centrifuge
- Illumina sequencing platform
- HISAT2
- StringTie
- CPC2
- CNCI
- Pfam database
- DESeq2 package

Methods

Culture Conditions and CO_2 Treatments

Cultures were grown under controlled laboratory conditions and exposed to three CO_2 treatments: low CO_2 (0.01%), ambient air (control, approximately 0.04% CO_2), and high CO_2 (5%). Each treatment consisted of three independent biological replicates. CO_2 concentrations were maintained using a controlled aeration system. Algal cells were harvested during the exponential growth phase and immediately processed for RNA extraction.

RNA Extraction and Quality Assessment

Algal cells were harvested by centrifugation and rapidly frozen in liquid nitrogen. Cell disruption was performed by grinding under liquid nitrogen to ensure efficient breakage of the rigid cell wall. Total RNA was extracted using a phenol–chloroform-based method with TRIzol reagent following the manufacturer's instructions. RNA was separated using chloroform, precipitated with isopropanol, washed with 75% ethanol, and resuspended in RNase-free water.

RNA concentration and purity were assessed using a NanoDrop spectrophotometer by measuring A260/A280 and A260/A230 ratios. RNA integrity was evaluated by agarose gel electrophoresis. Only high-quality RNA samples were used for library preparation.

RNA Library Preparation and Sequencing

Ribosomal RNA was removed from total RNA samples using a commercial rRNA depletion kit. Strand-specific RNA sequencing libraries were constructed to retain transcript orientation. A total of nine libraries (three per

CO_2 treatment) were prepared and sequenced using an Illumina high-throughput sequencing platform, generating paired-end reads.

Sequencing Quality Control and Read Processing
RNA sequencing generated 47.39 million raw reads, with a minimum of 5.28 million reads per library. Raw reads were subjected to quality filtering to remove adaptor sequences, low-quality reads, and ambiguous bases. Clean reads obtained after filtering were used for downstream analyses.[10]

Identification of Long Non-Coding RNAs
Clean reads were aligned to the *N. oceanica* IMET1 reference genome using HISAT2. Transcript assembly was performed using StringTie. Candidate long non-coding RNAs were identified based on transcript length (≥200 nucleotides) and lack of protein-coding potential. Coding potential was assessed using CPC2, CNCI, and Pfam database searches. Only transcripts predicted as non-coding by all tools were retained and classified as intergenic, sense, antisense, or intronic lncRNAs based on genomic location.

Differential Expression Analysis
Transcript expression levels were normalized to account for differences in sequencing depth and library size. Differential expression analysis was conducted using the DESeq2 package. Long non-coding RNAs showing statistically significant expression changes between CO_2 treatments were identified as CO_2-responsive lncRNAs and categorized as upregulated or down regulated.[11]

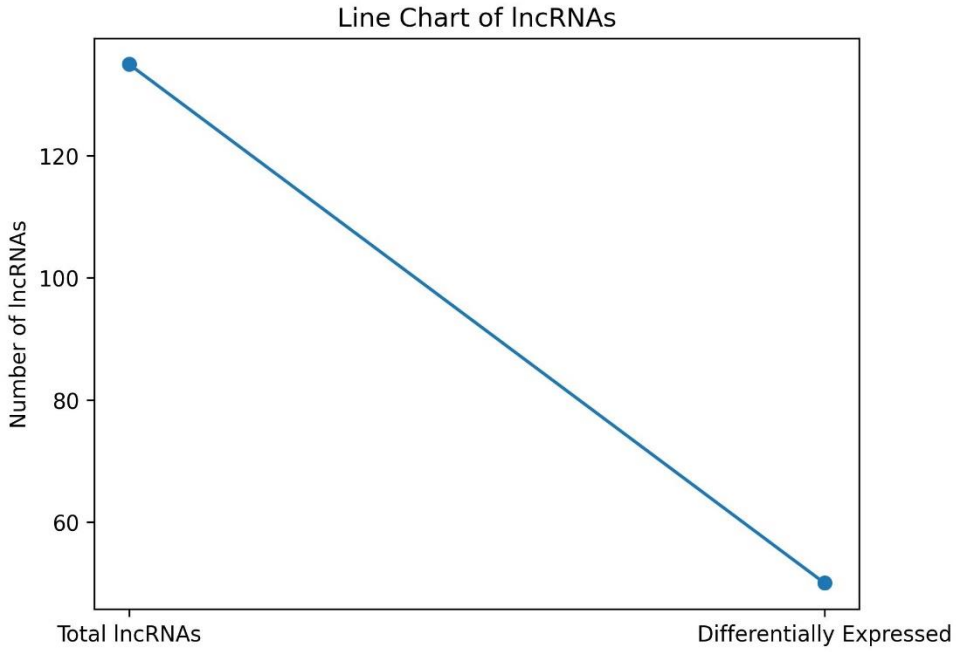
Findings
This study provides genome-wide evidence that long non-coding RNAs (lncRNAs) are actively involved in carbon dioxide (CO_2) stress responses in the marine microalga *Nannochloropsis oceanica*. Strand-specific transcriptome analysis identified **134 lncRNAs**, revealing an additional regulatory layer in this species.

Comparative expression analysis under contrasting CO_2 conditions showed that **51 lncRNAs were differentially expressed**, with **33 upregulated** and **18 downregulated** under high CO_2 stress, indicating a dynamic lncRNA-mediated response to carbon availability.

Functional enrichment analysis of predicted lncRNA target genes revealed significant associations with **polysaccharide metabolism**, including cellulose and glucan biosynthesis, as well as **transmembrane transport processes**, suggesting roles in carbon allocation and metabolic adjustment. Additionally, **2051 alternative splicing events** were detected under CO_2 stress, highlighting extensive transcriptome remodeling and a potential involvement of lncRNAs in post-transcriptional regulation.

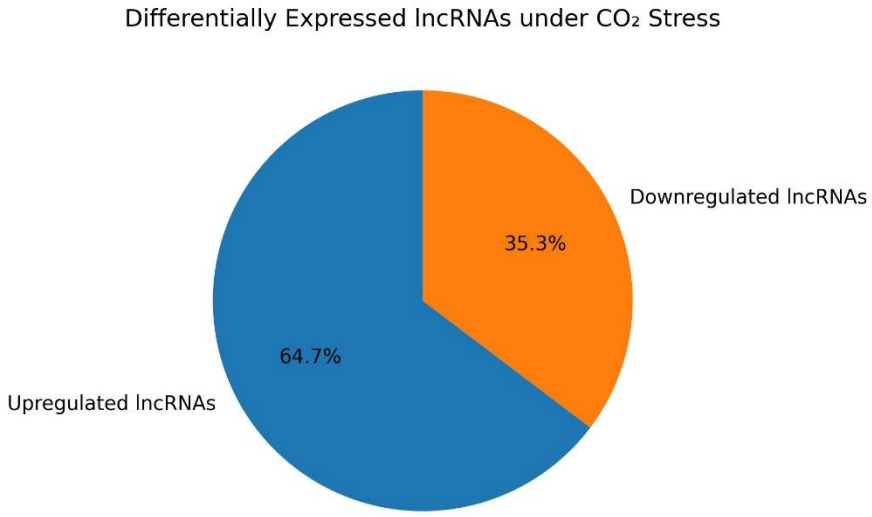
Collectively, these findings establish lncRNAs as key regulators of CO_2 adaptation, contributing to metabolic reprogramming and stress tolerance in *Nannochloropsis oceanica*.

RESULTS
Genome-wide Identification of lncRNAsA total of 134 lncRNAs were identified from the N. oceanica transcriptome and categorized based on their genomic origin.[12]



CO_2- Responsive Differentially Expressed lncRNAs

Comparative analysis between high and low CO_2 treatments revealed 51 differentially expressed lncRNAs, including 33 upregulated and 18 downregulated transcripts, indicating dynamic lncRNA regulation under CO_2 stress.[13]



Functional Enrichment Analysis

Functional enrichment of predicted lncRNA target genes showed significant associations with cellulose, glucan, and polysaccharide metabolism, as well as transmembrane transporter activity, suggesting roles in carbon allocation and cellular transport.[14]

Alternative Splicing Events

A total of 2051 alternative splicing events were detected under CO_2 stress conditions, highlighting an additional regulatory layer potentially mediated by lncRNAs.[15]

DISCUSSION

The present study demonstrates that lncRNAs play important regulatory roles in mediating CO_2 stress responses in *Nannochloropsis Oceanica*.[15] The identification of CO_2-responsive lncRNAs involved in carbohydrate metabolism and transport suggests that these molecules may fine-tune metabolic reprogramming under fluctuating CO_2 conditions. Furthermore, the association of lncRNAs with extensive alternative splicing events indicates their possible involvement in transcriptome plasticity and stress adaptation.[16] These findings are consistent with previous reports in plants and extend lncRNA regulatory paradigms to photosynthetic microalgae.

CONCLUSION

This study presents the first genome-wide characterization of carbon dioxide (CO_2)–responsive long non-coding RNAs (lncRNAs) in the marine microalga *Nannochloropsis oceanica*. Using strand-specific transcriptome analysis, we identified 134 lncRNAs, several of which showed significant and condition-dependent expression changes under fluctuating CO_2 levels. These results reveal lncRNAs as dynamic regulators of microalgal responses to carbon availability.

Functional and network analyses linked CO_2-responsive lncRNAs to key metabolic and regulatory pathways, including carbon-associated polysaccharide metabolism, transmembrane transport, transcriptional control, and epigenetic regulation. In addition, the widespread alternative splicing events detected under CO_2 stress suggest a potential role for lncRNAs in post-transcriptional regulation.

Overall, these findings expand current knowledge of lncRNA-mediated regulatory mechanisms in microalgae. The identified CO_2-responsive lncRNAs provide promising targets for future studies aimed at enhancing carbon fixation efficiency, stress tolerance, and microalgal biotechnological productivity.

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