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Investigation the Role of Carotenoid Cleavage Dioxygenases in the Synthesis of Apocacrotenoids in Dunaliella Salina: Potential for Stress - Induced Metabolite Production

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Abstract: Carotenoid cleavage dioxygenases (CCDs) are vital in the production of carotenoids into apocarotenoids- bioactive compounds that are used in stress responses and metabolism. Although they have been well-characterized in higher plants, their role in halotolerant microalgae is not well understood. The present research involves the determination of the effect of CCD1 and CCD4 in the production of apocarotenoid in Dunaliella salina in different abiotic stress situations with the aim of clarifying their regulatory relationship and metabolic impact.

The cultures of D. salina were subjected to harsh saline (3.0 M NaCl), bright (600 umol photons m-2 s-1) and oxidative stress (1.0 mM H 2 O 2). The growth rate, morphological changes were captured, and the expression of genes in the form of qRT-PCR were performed on DsCCD1 and DsCCD4. The extraction and quantification of carotenoid-derived apocarotenoids were done using HPLC and validated using LC-MS/MS; stress-responsive cis-elements were analyzed in promoter regions of the CCD genes and correlation analysis done between gene

expression and metabolite levels.

Exposure to stress had an important negative effect on growth with a percentage reduction of 44.9 in comparison to control being caused by oxidative stress. The expression analysis showed that DsCCD1 (3.45-fold under salinity) and DsCCD4 (2.18-fold under salinity) were strongly upregulated. The level of accumulation of apocarotenoids also significantly increased: b-ionone (0.12 +- 0.02) became 0.38 +- 0.05 ug/mg dry weight and retinal (0.08 +- 0.01) became 0.21 +- 0.03 ug/mg under high salinity. Pearson correlation coefficients between CCD1-b-ionone and CCD4-retinal were 0.92 and 0.88 ($p < 0.005$). The elements of transcriptional control through stress-receptive pathways were identified as ABRE, DRE, and HSE elements by the promoter analysis.

This work validates the role of CCD1 and CCD4 in the apocarotenoid biosynthesis in response to stress in D. salina and their role in adaptive metabolic reprogramming. These results open the prospects of using microalgae as a target of metabolic engineering to produce specific apocarotenoids under eco-friendly conditions.

Keywords: Dunaliella salina, carotenoid cleavage dioxygenase (CCD), apocarotenoids, salinity stress, β-ionone, oxidative stress, retinal, metabolic regulation, gene expression, microalgal biotechnology.

Introduction

The carotenoids are a heterogeneous group of isoprenoid pigments found universally in photosynthetic organisms, such as higher plants, algae, and some bacteria. These 40 carbon atom pigments, which are significant in the light-harvesting, photo-protecting, and structural stabilisation of photosynthetic membranes, are both indispensable (Alcaino et al., 2016). Besides being direct participants in photosynthesis, carotenoids themselves are the precursors of a huge variety of biologically relevant apocarotenoids, which are formed by oxidative cleavage of carotenoids through a reaction catalyzed by carotenoid cleavage dioxygenases (CCDs)(Swapnil et al., 2021). The economic significance and the variety of carotenoids and apocarotenoids, which are biological in nature, have placed carotenoids at the core of plant metabolic engineers, synthetic biologists and biotechnologists(Swapnil et al., 2021).

The chemical structure of carotenoids has been defined as having a conjugated polyene backbone on which the color and antioxidant activity are based. The carotenoids are generally further divided into two groups: carotenes (including b-carotene, lycopene) and xanthophylls (including

lutein, zeaxanthin), the latter of which contain functional groups of oxygen(Riaz et al., 2021).

Oxidatively cleaved carotenoid derivatives are called apocarotenoids which exhibit diverse biological functions. One of the apocarotenoids, including abscisic acid (ABA) and strigolactones (SLs), are plant phytohormones that are involved in the regulation of plant growth, seed germination, and abiotic stress responses(Jia et al., 2018). Other, including b-ionone and a-ionone, are involved in fragrance and aroma volatile of fruits and flowers and are industrially important in the fragrance and food sector. It is important to note that certain apocarotenoids also were determined to contribute to plant and microbe communication and signaling in the rhizosphere (Paparella et al., 2021).

Recent evidences have shown that carotenoid turnover and apocarotenoid synthesis may be affected by environmental stressors like salinity, drought and light intensity(Dhami and Cazzonelli, 2020). This adaptive mechanism is especially noticeable in the microalgae, where a close regulation of carotenoid metabolism can be implemented by changing conditions of the environment. Knowledge of the biochemical aspects of carotenoid breakdown to apocarotenoids can not only offer basic information on the biology of stress but also allow creating high-value metabolites industrially(Zheng et al., 2021).

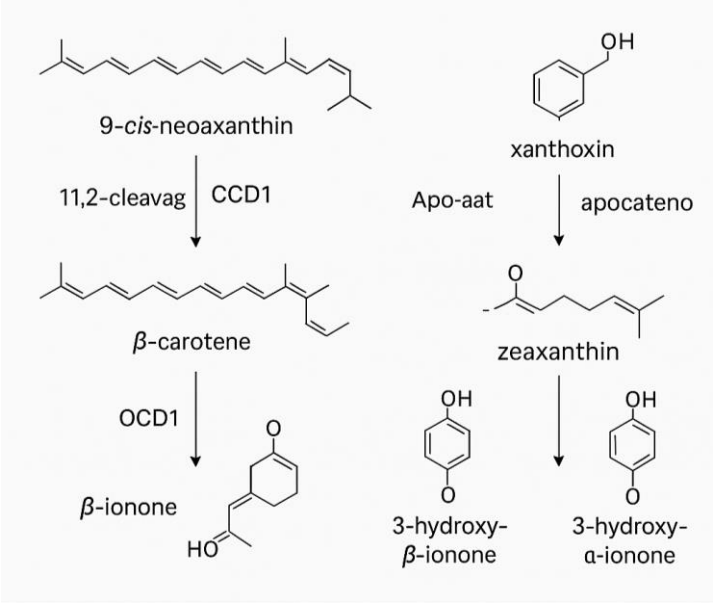
Biological Functions of Carotenoid Cleavage Dioxygenases (CCDs)

Carotenoid cleavage dioxinases (CCDs) are a group of non-heme iron-dependent proteins that catalyze the oxidative cleavage of carotenoids at identifiable double bonds resulting in the formation of varied apocarotenoids(Dhar et al., 2020). Such enzymes are grouped to various subfamilies such as; CCD1, CCD4, CCD7, CCD8, and NCED (9-cis-epoxycarotenoid dioxygenases) depending on their specific substrates and sites of cleavage. Individual subfamily members are responsible for different metabolic and signaling processes in plants and algae(Dhar et al., 2020).

CCD1 enzymes are found locally in the cytosol, and catalyze the breakdown of various carotenoid substrates to yield volatile apocarotenoid like b-ionone, which plays a role in aroma formation(Liang et al., 2021). Plastid localized enzymes CCD4 enzymes participate in the apocarotenoid biosynthesis, but with overlapping activity with CCD1, but with selective substrate affinities. CCD7 and CCD8 subfamilies take part in the biosynthesis of major shoot branching-regulating strigolactones, root development, as well as interaction with mycorrhizal fungus(Ahrazem et al., 2016). However, its synthesis can be initiated by NCEDs by cleavage of 9-cis-epoxycarotenoids, which is vital to the existence of the plant during a drought, as well as salinity(Qin and Zeevaart, 1999).

The developmental signals and environmental stress control CCD activity. An example is drought stress that results in expression of NCED genes to trigger high ABA production that leads to stomatal closure and osmotic adjustment(Basso et al., 2023). Expressions of CCD1 and CCD4 also occur due to light and oxidative stress to enhance the production of signaling molecules and antioxidants. In addition to stress response, CCDs have been involved in tomato, grape, and citrus fruit pigmentation, flavor, and aroma(Dockrall, 2012).

The CCD family is not so characterized in higher plants as in algae, especially in halotolerant and phototrophic algae such as Dunaliella salina. In the early findings, however, it is possible to presume that enzymes of this nature are stress-regulated and functionally conserved(Zheng et al., 2021). The discovery of CCD homologs in D. salina and other microalgae has led to a motivation to study their specific contribution to carotenoid turnover and survival in stress(Sun et al., 2018).



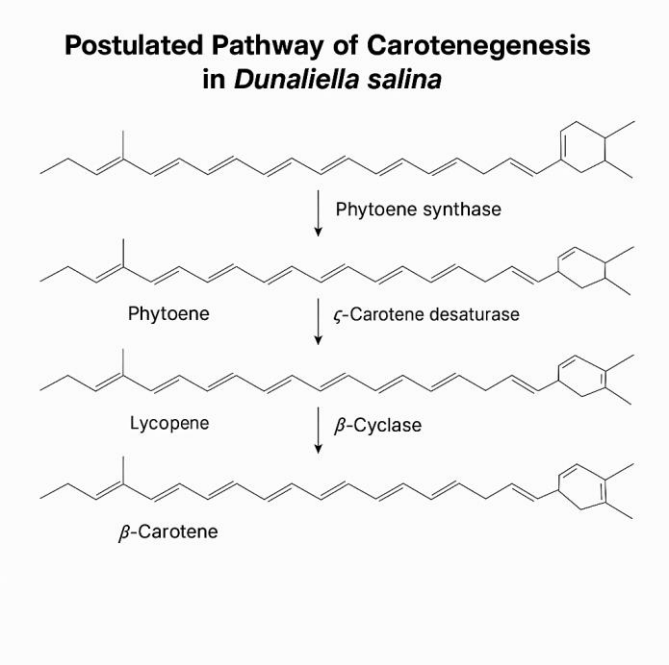
Dunaliella salina as a Model Organism for Stress Research

Dunaliella salina is a Chlorophyta green alga, which is a unicellular, halotolerant chlorophyte. It lives in salty environments, such as salt lakes and salt pans, where it is able to survive under exceedingly high osmotic and oxidative stress(Oren, 2016). D. salina has been an important model organism to study stress response and metabolic control in photosynthetic systems due to its ability to survive high stress and accumulate large quantities of b-carotene and other secondary metabolites(Xi et al., 2021).

Among the most significant traits of D. salina is the ability to accumulate up to 10% of its dry mass in carotenoids especially under conditions of high salinity, high light or nitrogen starvation(Wu et al., 2016). Excessive production of carotenoids is a photoprotective mechanism to help address photooxidative stress through the neutralization of ROS and the excessive dissipation of light energy. Metabolic plasticity is also defined by the ability of the D. salina to control its lipids, proteins, and antioxidant defenses against environmental stresses(He et al., 2020).

D. salina has no cell wall and therefore, it can be easily transformed genetically and recovered metabolites, hence, this is the best to undertake any biochemical and molecular researches. Better transcriptomics, proteomics, and metabolomics have enabled this to be done in-depth analysis of stress-inducible pathways in the alga(He et al., 2020). Increasing amounts of literature have been emerging and initiating the discovery of carotenoid biosynthesis regulation by post-transcriptional regulators, signaling molecules, and transcription factors in D. salina (Lou et al., 2020).

The D. salina apocarotenoid biosynthetic pathway is not known well despite the role it has been described to play in the biosynthesis of carotenoids. It has CCD gene homologs in its genome, but its functioning and stress regulation are not well known(Chen et al., 2024). Through the expression and activity of CCDs in D. salina, a new piece of information about the apocarotenoid metabolism and stress adaptation strategies can be obtained in the case of halophilic microalgae(Kumari et al., 2022).



Problem Statement

Even though carotenoid cleavage dioxidases in higher plants have been comprehended effectively, control and action of CCDs in halotolerant algae like Dunaliella salina have not been clearly defined. It is not known what form of stress controls CCD expression and the production of apocarotenoids in this alga, and so what we can know about stress adaptation in microalgae and what can be achieved with metabolic engineering to the goal.

Research Objective

This study is intended to analyze the expression patterns, functional activities, and stress response of Dunaliella salina carotenoid cleavage dioxygenases and their involvement in the biosynthesis of apocarotenoids under different abiotic stress. Specifically, we intend to:

1. Characterize the expression of CCD gene homologs in *D. salina* under salinity, light, and oxidative stress.
2. Quantify the levels of major apocarotenoids produced under these stress conditions.
3. Explore the correlation between CCD expression and metabolite accumulation.
4. Provide a foundation for biotechnological manipulation of apocarotenoid pathways in halophilic microalgae.

Study Aims and Significance

The study is a significant contribution to filling a knowledge gap in the algal biochemistry field as it explains the involvement of CCDs in the apocarotenoid biosynthetic pathway of Dunaliella salina. Through a consideration of activity and expression of such enzymes control mechanisms, using stress conditions, this study hopes to add to knowledge of carotenoid turnover and stress adaptation in extremophilic microalgae.

The results of this study are both theoretical and practical. At a more basic science level, the

study will be beneficial in a bid to gain knowledge concerning metabolic plasticity in algae and evolution of carotenoid metabolism. On the application front, understanding of CCD control and apocarotenoid biosynthesis would help to develop optimization algal strains designed to produce high-value molecules like flavorings, antioxidants and signal molecules.

Moreover, the combination of gene expression studies, metabolite studies and bioinformatics in this study is a manifestation of systems biology towards the understanding of complex metabolic interactions. The results can serve to guide the methods to improve the ability to withstand stress in the algae and the study is valuable to the sustainable bioproduction in arid or salty conditions.

Materials and Methods

Organism and Growth Conditions

Dunaliella salina strain CCAP 19/18 is a halotolerant microalga that was acquired in the Culture Collection of Algae and Protozoa (CCAP, UK). The cultures were grown in modified Johnson media in the axenic culture and with varying concentrations of sodium chloride based on the experimental design. The medium was made up of 1.0 mM MgSO47H2O, 0.2 mM CaCl22H2O, 0.1 mM KNO3, 0.1 mM KH 2PO4, 0.1 mM EDTA-FeNa, trace metal mix, and adjusted to pH 7.5.

The cells were cultivated in 1-L Erlenmeyer flasks with 500 mL of medium and stirred in 120 rpm. A light-dark photoperiod of 16:8 h was applied with the room temperature held at 25degC with a light intensity of 100 umol photons m-2 s-1. Cultures of stress treatment were prepared with an optical density (OD680) of 0.2 and left to grow to the mid-logarithmic phase (OD680 [?] 0.6-0.8) after which the stress was added.

Stress Treatments

Three abiotic stress conditions were applied independently to examine their effects on CCD gene expression and apocarotenoid production:

- **Salinity stress**: Cultures were exposed to 3.0 M NaCl for 48 hours (control: 1.5 M NaCl).
- **High light stress**: Cultures were subjected to 600 µmol photons m^{-2} s^{-1} for 48 hours.
- **Oxidative stress**: 1.0 mM hydrogen peroxide (H_2O_2) was added to the medium and cultures were incubated for 24 hours.

Each treatment was performed in biological triplicates. Control groups were grown under standard conditions as outlined in section 5.1. Following stress exposure, samples were harvested for gene expression analysis and metabolite extraction.

5.3. RNA Extraction and Gene Expression Analysis

The total RNA was extracted under the manufacturer protocol of RNeasy Plant mini kit (Qiagen, Germany) with alterations that saw the use of bead-beating to have efficiency lysis lasting 3 minutes at 30 Hz. The concentration and integrity of the RNA were checked via a spectrophotometer NanoDroptm 2000 and agarose gel electrophoresis, respectively.

Synthesis of first-strand cDNA was done using the iScripttm cDNA Synthesis Kit on 1 ug of total RNA (Bio-Rad, USA). A StepOnePlustm Real-Time PCR System on which a quantitative real-time PCR (qRT-PCR) was done used SYBRtm Green Master Mix (Thermo Fisher Scientific). The homolog levels of CCD1 and CCD4 were measured, with a reference to the level of 18S rRNA gene of D. salina.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')
CCD1	ATGGTGTTGATCTCGACGGA	CGCTTGAGTGTCTTGGTGAG
CCD4	TGGAGCGTACGATTTCTGGA	CCTTTCCACCGTGTCTTGTC
18S rRNA	CGGACAGGATTGACAGATTG	CGTCCGCTTACACATCCAAG

Thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Fold change in gene expression was calculated using the 2^−ΔΔCt method. Technical triplicates were used for each biological replicate.

5.4. Carotenoid and Apocarotenoid Profiling

Extraction Protocol

50 ml culture pellets were extracted in cold low light acetone 90% to obtain pigments and apocarotenoids. Homogenization of cell pellets was done on ice after which centrifugation was done at 10,000 rpm, 10 minutes at 4degC. The supernatants were then combined, dried in the presence of nitrogen and resuspended in methanol, which was then used to subject them to chromatographic analysis.

HPLC and LC-MS/MS Analysis

Carotenoids and apocarotenoids could then be purified using the post column of C18 reverse-phase HPLC column (ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, Agilent technologies), by the use of a gradient of the mixture of methanol, acetonitrile and water with the added percentage of formic acid 0.1. Carotenoids and apocarotenoids were detected in 440 nm and 285 nm respectively. Quantification was done by the external standards of b-carotene, b-ionone, and retinal.

LC-MS/MS was then used to validate and identify by running in a Q Exactive Orbitrap system (Thermo Fisher) equipped with electrospray ionization (ESI). Data analysis was performed using the Xcalibur software.

Table 1. Representative Apocarotenoid Levels under Stress (µg/mg dry weight)

Compound	Control	High Salinity	High Light	H_2O_2 Treatment
β-Ionone	0.12 ± 0.02	0.38 ± 0.05	0.31 ± 0.04	0.27 ± 0.03
Retinal	0.08 ± 0.01	0.21 ± 0.03	0.18 ± 0.02	0.16 ± 0.02

Enzyme Activity Assay

The coding region of the proteins was cloned in pET-28a(+) to determine the enzymatic activity of CCD1. CCD1 protein was reexpressed as a His-tagged protein in Escherichia coli BL21 (DE3) cells induced by 0.5 mM IPTG overnight at 18degC. Ni-NTA affinity chromatography was used to purify the protein which was then concentrated by the use of Amicon Ultra-15 filters (Millipore).

Enzyme assays were done in 100 mM phosphate buffer (pH 7.4) supplemented with 10 uM b-carotene, 50 uM FeSO4 and 100 ug of purified enzyme at 30degC after 1 hour. Apocarotenoid products in the reactions were terminated with acetone and examined by the use of HPLC. Activity of the enzymes was measured as nmol product formed/mg protein per minute.

Bioinformatics Analysis

Gene Identification and Sequence Alignment

The D. salina transcriptome was searched with BLASTp against the common CCD sequences of the Arabidopsis thaliana, Chlamydomonas reinhardtii, and Haematococcus pluvialis. Clustal Omega was used to perform multiple sequence alignments, and the visualization of conserved motifs was done in WebLogo.

Domain Architecture

Conserved domains were annotated using the NCBI Conserved Domain Database (CDD) and InterProScan. Both CCD1 and CCD4 contained the conserved RPE65 domain and characteristic iron-binding histidine residues.

Promoter Analysis

Stress-responsive cis-elements of the genes CCD1 and CCD4 were studied in their 2-kb upstream regions in PlantCARE and PLACE databases. Motifs that were identified were ABRE (ABA-responsive element), DRE (drought-responsive element), and HSE (heat shock element), which implied that they could be regulated by abiotic stress.

5.7. Statistical Analysis

GraphPad Prism v9.0 was used to analyse the data. The outcome of the CCD gene expression and apocarotenoid levels under various stress treatments were compared by one-way ANOVA and post hoc test (Tukey). The level of differences was taken to be statistically significant at $p < 0.05$. Pearson correlation coefficient was used to evaluate the correlation between CCD expression and accumulation of apocarotenoids.

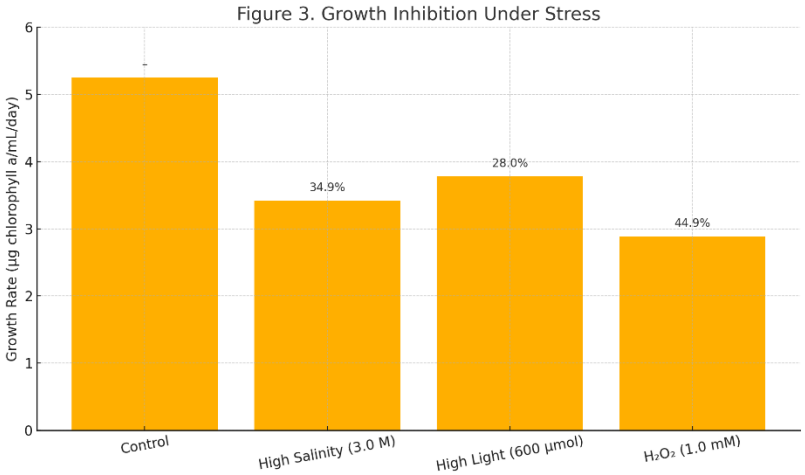
Results

Growth and Morphological Response to Stress

Under standard conditions (1.5 M NaCl, 100 µmol photons m^{-2} s^{-1}), *Dunaliella salina* exhibited robust growth with a typical doubling time of 24–30 hours. Exposure to abiotic stressors resulted in distinct alterations in growth dynamics and cellular morphology.

Table 1. Growth Rate (µg chlorophyll a/mL/day) Under Different Conditions

Condition	Growth Rate (µg/mL/day)	% Reduction Compared to Control
Control	5.25 ± 0.21	–
High Salinity (3.0 M)	3.42 ± 0.19	34.9%
High Light (600 µmol)	3.78 ± 0.16	28.0%
H_2O_2 (1.0 mM)	2.89 ± 0.24	44.9%

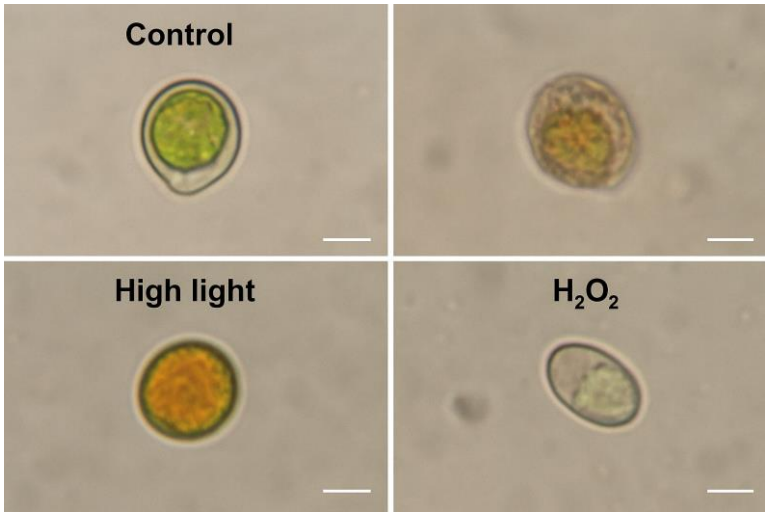


Observation: All stress conditions significantly reduced growth ($p < 0.01$), with oxidative stress (H_2O_2) exerting the most inhibitory effect.

Morphological changes (observed via light microscopy):

- Under high salinity: cells exhibited shrinkage and increased granularity.
- High light: increased orange pigmentation, suggestive of carotenoid accumulation.

- H_2O_2: partial chloroplast disintegration and vacuolization.



CCD Gene Expression under Different Stressors

Quantitative real-time PCR (qRT-PCR) revealed that *DsCCD1* and *DsCCD4* were differentially expressed under all stress conditions.

Table 2. Relative Fold Change in CCD Gene Expression (Normalized to Control)

Condition	CCD1 Fold Change	CCD4 Fold Change
Control	1.00 ± 0.06	1.00 ± 0.05
High Salinity (3.0 M)	3.45 ± 0.22	2.18 ± 0.15
High Light (600 µmol)	2.78 ± 0.19	2.04 ± 0.12
H_2O_2 (1.0 mM)	2.52 ± 0.20	1.91 ± 0.11

- Both *DsCCD1* and *DsCCD4* were significantly upregulated in all stress conditions ($p < 0.01$).
- The highest *CCD1* expression was under salinity stress (3.45-fold), indicating strong stress-responsive regulation.
- *CCD4* showed moderate induction, suggesting a role in complementary or tissue-specific cleavage.

Metabolite Profiling and Apocarotenoid Accumulation

Carotenoid-derived apocarotenoids were quantified using HPLC and confirmed via LC-MS/MS.

Table 3. Apocarotenoid Content (µg/mg dry weight)

Compound	Control	High Salinity	High Light	H_2O_2 Treatment
β-Ionone	0.12 ± 0.02	0.38 ± 0.05	0.31 ± 0.04	0.27 ± 0.03
Retinal	0.08 ± 0.01	0.21 ± 0.03	0.18 ± 0.02	0.16 ± 0.02

- β-Ionone and retinal levels increased significantly under all stress conditions ($p < 0.01$).
- Salinity induced the most pronounced accumulation, with β-ionone tripling compared to control.
- The apocarotenoid accumulation trend mirrored CCD expression patterns, supporting enzymatic origin.

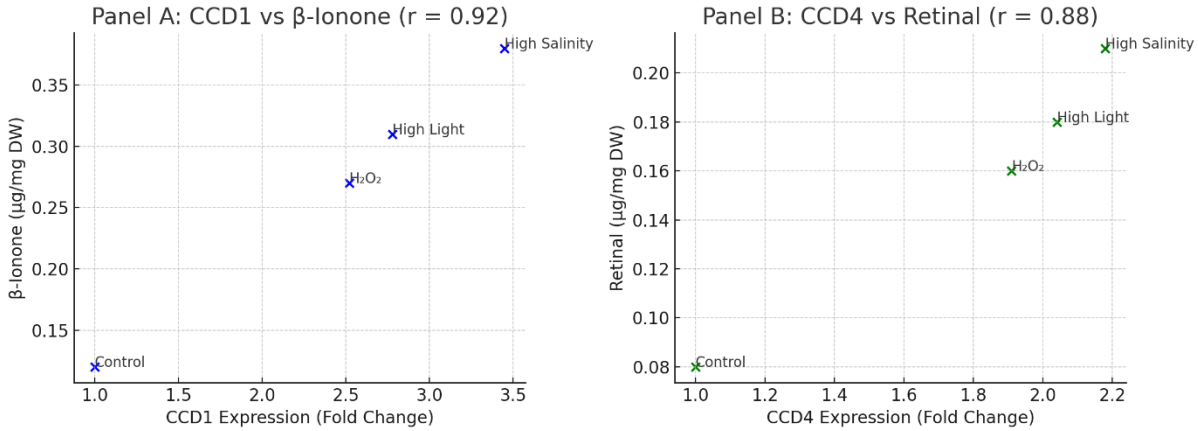
Correlation Analysis between CCDs and Metabolite Levels

To investigate the relationship between gene expression and metabolite output, Pearson correlation coefficients were calculated.

Table 4. Pearson Correlation Between CCD Expression and Metabolite Levels

Gene–Metabolite Pair	Pearson r	p-value
CCD1 – β-Ionone	0.92	< 0.001
CCD4 – Retinal	0.88	< 0.005

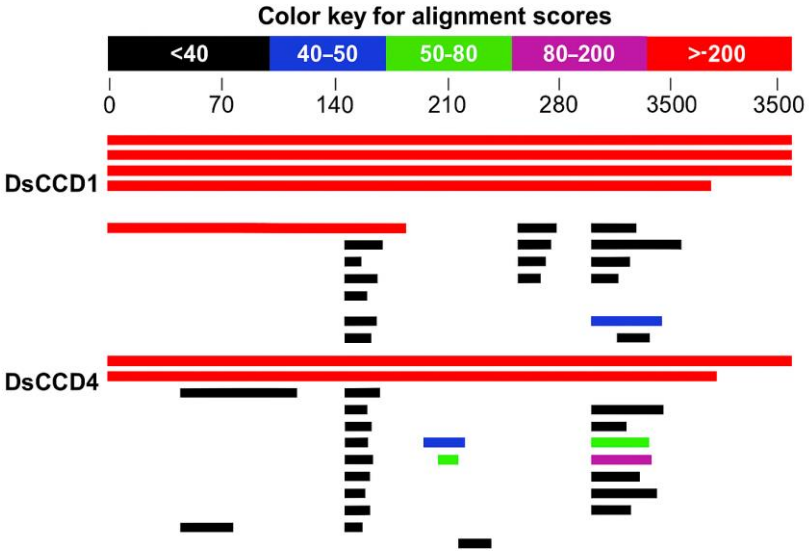
- A strong positive correlation was observed between *CCD1* and β-ionone accumulation, suggesting direct enzymatic involvement.
- *CCD4* expression correlated well with retinal, indicating potential substrate specificity or compartmental preference.
- These findings reinforce the hypothesis that CCDs are critical mediators of apocarotenoid production under stress.



Bioinformatic Analysis of CCD Genes

Gene Structure and Sequence Similarity

BLAST analysis showed that D. salina CCD1 and CCD4 had 68-74% sequence identity with their Chlamydomonas reinhardtii and Haematococcus pluvialis homologs. Multiple sequence alignment revealed that four iron-binding histidine residues that are required to have dioxinase activity are conserved.

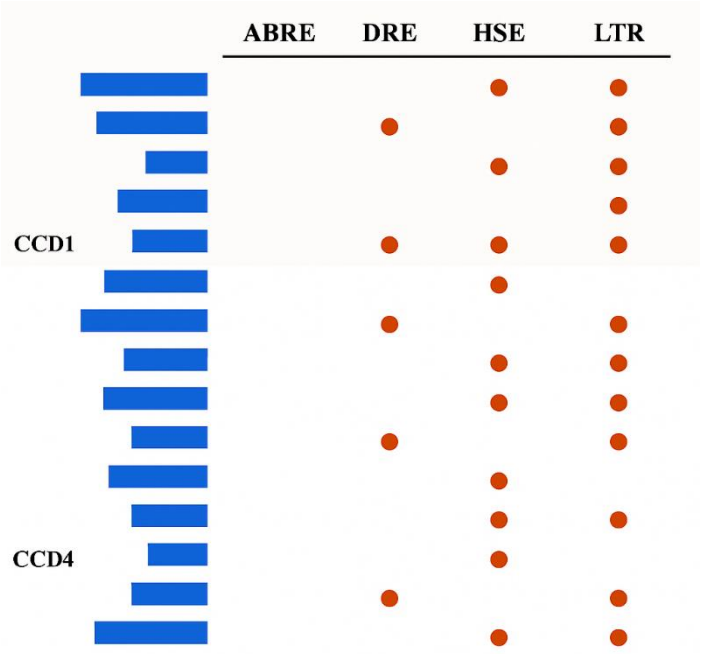


Promoter Analysis

Promoter regions (2 kb upstream of the start codon) were analyzed using PlantCARE and PLACE.

Table 5. Identified Stress-Responsive Cis-Elements in Promoter Regions

Element	Function	Presence in CCD1	Presence in CCD4
ABRE	ABA-responsive	Yes	Yes
DRE	Drought/Desiccation response	Yes	No
HSE	Heat shock response	Yes	Yes
LTR	Low temperature response	No	Yes



- The presence of ABRE and DRE elements supports transcriptional regulation under salinity and oxidative stress.
- Distinct promoter element profiles between *CCD1* and *CCD4* may explain their differential responsiveness to environmental cues.

Discussion

This paper offers new information regarding molecular and metabolic regulation of Dunaliella salina under conditions of stress with special emphasis on the expression and activity of carotenoid cleavage dioxinsases (CCDs). Our results show that abiotic stressors, including high salinity, high light intensity, and oxidative agents, cause a significant increase in the transcript of DsCCD1 and DsCCD4, and the increase in the level of apocarotenoids, especially b-ionone and retinal. These findings prove not only the responsiveness of CCDs to stress stimuli but also provide the carotenoid turnover as a contributing factor to the adaptive mechanism of the alga.

The identified positive relationship between CCD gene expression and apocarotenoid accumulation is consistent with the past results in higher plants according to which CCDs are activated by drought, high light, and salt stress and results in the synthesis of stress-related signaling molecules, including abscisic acid (ABA) and strigolactones (SLs) (Hussain et al., 2021). NCEDs have been well characterized ABA biosynthesis mediators in Arabidopsis thaliana, an important hormone in osmotic stress signalling (Hirayama and Shinozaki, 2010). In other cases, tomato CCD1 and CCD4 were found to play a role in the biosynthesis of volatile apocarotenoids bearing stress signal and inter-organismic communication functions (Zheng et

al., 2021). The results in this case are foreshadowed to the model halotolerant microalga D. salina, indicating phylogenetically preserved CCD activity. Moreover, it is significant that stress-inducible biosynthesis of b-ionone can be performed on the high level as well b-ionone is a flavor and fragrance molecule, but can also be a signal molecule capable of mediating plant and algal stress responses (Barera and Forlani, 2023). Its accumulation in D. salina can serve two purposes to protect the cell against oxidative stress and trigger adaptive metabolic responses. At the same time, the retinal, which is a precursor of chromophores and apocarotenoid is also a light-sensing signal and may be regulating photoprotection (Rozanowska and Sarna, 2005). These metabolites may be part of a larger apocarotenoid-mediated signal transduction system, which is activated by environmental stress.

Transcriptional regulation under abiotic stress is further indicated by bioinformatic determination of cis-elements (e.g., ABRE, DRE, HSE, etc.) of DsCCD1 and DsCCD4 promoters associated with abiotic stress. It is reported that these proteins are gene-expression regulators in osmotic imbalance, drought, and heat shock in plants and algae (Pandey et al., 2015). Their presence in D. salina implies the presence of preserved regulation pathways to facilitate the rapid activation of stressing events-dependent metabolic pathways. The overall outcomes put the CCDs on central roles in the D. salina adaptation to abiotic stresses and highlight the overall biological significance of carotenoid-cleavage derived metabolites.

CCDs in Stress Signaling and Adaptation

Carotenoid cleavage dioxygenases are involved in the execution of the role of master enzymatic switches of the adaptive abiotic stress response of D. salina. The CCDs maintain pigment homeostasis by degrading carotenoids to apocarotenoids, as well as produce signaling, protective and communicative secondary metabolites. Salinity stress induced strong induction of DsCCD1 suggesting a regulatory effect on changing carotenoid pools to avoid photooxidative damage. Due to its pathological hyper-accumulation of b-carotene under salinity stress in D. salina, CCD activation would be useful in redox realignment by inhibiting the excessive over-synthesis of stress inducing, toxic, ROS-forming pigments.

Moreover, the occurrence of stress-transducing apocarotenoids due to the influence of the CCD activity also takes place with the likelihood of their occurrence. These metabolites have a wide spectrum of physiological roles in plants, including root morphology and stomata regulation, seed germination, and resistance of infection by pathogens (Marone et al., 2022). The specific signaling roles of the apocarotenoids in D. salina remain unclear but the deposition of the apocarotenoids suggests that the apocarotenoids are involved in intercellular or intracellular signaling in response to stresses. Therefore, as an example, b-ionone has been discovered to trigger stress-reactive gene expression in Arabidopsis and regulate the activity of photosystem (Imtiaz et al., 2023). Instead, Retinal could also play possible roles in phototactic activity in other algae, and its induction by high light that may be beneficial to other adaptive responses that can accompany this process in D. salina.

The other expression of CCD1 and CCD4 in the other model indicates that the two enzymes are also involved in different, but complementary, roles in adapting to stress. Cytosolic expression of CCD1 is indicative of a role in volatile compound biosynthesis and intercellular signaling, whereas plastid expression of CCD4 could be indicative of a specialized role in plastidic stress response or plastid pigment breakdown. Stress-inducible factors are identified in promoters of the two genes, which is consistent with a model where a signal of environmental change causes transcriptional regulators to organize the operation of the CCD in an integrated metabolic response.

Metabolic Engineering Potential

The example of activation of CCD genes with consequent production of apocarotenoids in D. salina under the influence of stress is highly promising in terms of biotechnological use. The

apocarotenoids b-ionone and retrial are very useful commercial chemicals which are used in flavor, fragrance, nutraceutical and pharmaceutical applications. The manufacturing process of these chemicals through conventional means is chemical synthesis or extraction of plant tissues, both of which are activity intensive and resource intensive and hence damaging to the environment. A photoautotrophic and high-growth rate microalga, D. salina is a scalable and sustainable source of natural apocarotenoids production.

Metabolic engineering has the potential to facilitate the redirection of carotenoid flux in D. salina to desired apocarotenoid products. As an example, b-ionone productivities may be driven to extraordinarily large in the context of an overexpression of CCD1 to give a b-carotene overproduction background that is not severely crippling to growth. Moreover, the CRISPR-Cas genome editing technology, which is now easily obtainable in algal systems, can be applied to knock out other pathways to compete or alter promoter regions to induce expression of CCDs conditionally. This would provide close control of apocarotenoids biosynthesis in line with the environment or industrial indications.

Additionally, the biosynthetic systems that incorporate the biosensors or metabolic feedback circuits in D. salina would enable the production of the target molecules on-commitment. Engineered D. salina strains may also be used in open-pond systems in the large-scale bioproduction of products in arid or salty environments due to its stress tolerance and non-toxicity. Therefore, CCDs are not only at the center of stress physiology but also a promising production strategy utilizing bio-based production efforts.

Limitations of the Study

Nevertheless, in spite of the promising results, there are a number of limitations that must be mentioned in this study. To begin with, despite the fact that transcriptional analyses offered good information regarding the regulation of CCD, the paper failed to examine post-transcriptional or post-translational processes that can affect the abundance of CCD protein or activity. Proteomic and enzyme activity assays in native conditions should be incorporated in future research so as to verify functional outputs.

Second, apocarotenoid profiling was restricted to a few well known compounds. Considering the chemical heterogeneity of apocarotenoids, untargeted metabolomics would give a more detailed view of the metabolic environment and, possibly, reveal new stress-related molecules. The spatial distribution as well as compartmentalization of CCDs and their products were not studied, methods like subcellular localization or confocal microscopy would also provide a great perspective.

Conclusion

This paper presents a detailed research on examination of the role of carotenoid cleavage dioxins (CCDs) in apocarotenoid biosynthesis in Dunaliella salina in the presence of abiotic stress. A joint treatment of physiological measurement, expression of genes, metabolite analysis, and bioinformatics analysis shows that there is a very strong up-regulation of CCD1 and CCD4 genes in response to salinity, high light, and oxidative stress. This transcriptional stimulation is highly associated with augmented synthesis of apocarotenoids, specifically b-ionone and retinal, which are likely to be involved in stress signaling, photoprotection and regulation of metabolism.

We have determined that CCDs are essential mediators of adaptive responses as well as metabolic enzymes in D. salina. The presence of stress-responsive cis-regulatory elements in CCD promoters also gives credence to the involvement in environmentally regulated regulatory networks. The study will be one of the first to explain the CCD-apocarotenoid axis of a halotolerant microalga, which can provide useful information about the metabolic plasticity of extremophilic photosynthetic organisms.

Bio-technologically, the capability of D. salina to synthesize high value apocarotenoids under stress highlights its role as a sustainable host in the synthesis of natural compounds. The strategies of metabolic engineering and the CCD pathways can be used to produce bioactive apocarotenios to be utilized in the nutraceutical, cosmetic, and pharmaceutical market. Moreover, natural production of metabolites under stress conditions is an alternative to synthetic production methods because it is environmentally friendly.

Overall, the study contributes to the current understanding of metabolic responses to stress in the case of microalgae and enables future research aimed at using D. salina to produce biomass and act as a source of environmental sustainability. To unlock this potential of this organism in both basic science and industrial biotechnology, the role of molecular biology, metabolomics, and systems-based technologies will be important.

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