

Identification of salinity-responsive microRNAs in *Dunaliella salina* and their regulatory influence on carotenogenic genes

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Abstract

Carotenoid biosynthesis in *Dunaliella* species is regulated by a complex network of molecular mechanisms, which remain largely unexplored. MicroRNAs (miRNAs) function as post-transcriptional regulators, acting as molecular switches that modulate gene expression. However, their role in the regulation of carotenogenesis pathway genes (*Crt* genes) in *D. salina* has not been investigated. This study aimed to identify and characterize miRNAs involved in the regulation of *Crt* genes in *D. salina* using publicly available Sequence Read Archive (SRA) data and a homology-based mapping approach. A total of 30 miRNAs were mapped from the *D. salina*SRA dataset, and their potential targets among *Crt* genes were predicted using psRNATarget. The miRNA-target interactions were validated through RNA hybridization analysis using RNAHybrid. Five miRNAs—*dsa-miR1163.2*, *dsa-miR917*, *dsa-miR1145.1*, *dsa-miR166a-3p*, and *dsa-miR414*—were identified in *D. salina* as putative regulators of *Crt* genes and were further analyzed for differential expression under salinity stress using reverse transcription quantitative PCR (RT-qPCR) was performed to confirm their expression profiles. This study represents the first report of miRNAs from a *D. salina* strain isolated from Sambhar Lake, India, providing novel insights into miRNA-mediated regulation of *Crt* genes. These findings pave the way for future research on engineering *D. salina* strains with enhanced carotenoid production for commercial applications.

Introduction

Dunaliella salina is a unicellular green alga (Chlorophyceae) that lacks a true cell wall. The algal cells are enveloped in a glycoprotein layer and have two flagella allowing the cells to swim freely in aquatic habitat (Borowitzka 2013). It has an extraordinary property of growing at hypersaline concentrations ranging from 50 mM to 5 M of sodium chloride (NaCl) by maintaining the osmotic pressure inside and outside the cell through glycerol metabolism (Ben-Amotz 1980). Additionally, it is also widely known for producing several bio-active compounds, including carotenoids, glycerol, essential proteins, lipids and vitamins that have pharmaceutical and nutraceutical properties. Major carotenoids including beta (β)-carotene, lutein, phytoene, zeaxanthin and astaxanthin have been reported from *D. salina* (Liang *et al.* 2019). β -carotene is a vital anti-oxidant that is also critical in vitamin-A synthesis in both plants and animals. Moreover, *D. salina* is known to accumulate β -carotene up to 10-12% of its dry biomass (Ben-Amotz & Avron 1981). Owing to its anti-oxidative properties, β -carotene has become an essential component of food supplements, animal fodder, aquafeed, food additives, cosmetics and nutraceutical supplements. It has anti-tumour and anti-cancerous properties leading to its use in treatments of tumours, skin cancer, melanoma and gastrointestinal cancer. Furthermore, it has also proven to be effective in treating coronary diseases such as angina pectoris, myocardial infarction, and other cardiovascular problems (Elleuch *et al.*, 2021).

The carotenoid synthesis pathway (carotenogenesis) is the key pathway for β -carotene synthesis that occurs in chloroplasts and has been identified in both algae and plants (Howitt & Pogson 2006). The biochemical and molecular system of carotenogenesis is well-investigated in algal species such as *Chlamydomonas*, *Dunaliella*, *Haematococcus*, and *Chlorella* (Tan *et al.* 2007; Kathiresan *et al.* 2015; Rammuni *et al.* 2019). Several genes regulating this pathway have also been identified in *D. salina* (Figure 5). The phytoene synthase gene (*Psy*) first reported in *D. salina* showed similarity with other algal and cyanobacterial *Psy* genes (Yan *et al.* 2005). It is responsible for catalysing the dimerization of C20 geranyl-geranyl pyrophosphate (GGPP) into a C40 compound, phytoene. This is a rate-determining step for β -carotene synthesis in *D. salina*. Another gene, i.e., Phytoene desaturase (*Pds*) was first reported in *D. salina* by Yan *et al.*, (2005). It is involved in the conversion of Phytoene to Lycopene through dehydrogenation. The lycopene-beta-cyclase gene (*LycB*) further converts the Lycopene to β -carotene and was characterized in *D. salina* by Zhu *et al.*, (2008). In the last two decades, several other genes related to carotenoid biosynthesis have also been studied in *D. salina* including Zeta (ζ)-carotene desaturase (*Zds*), Geranylgeranyl pyrophosphate synthase (*GGPS*), Carotenoid isomerase (*CRTISO*) (Ye & Jiang, 2010; Zhu *et al.*, 2020; Chen *et al.*, 2023). Nevertheless, their expression regulation and the underlying molecular mechanism remain obscure.

MiRNAs are considered a primeval gene-regulatory system that existed in unicellular organisms before the emergence of multicellular organisms. These are 18-24 nucleotides long, endogenous non-coding RNA sequences responsible for post-transcriptional gene regulation through translation repression or cleavage of target mRNAs (Obernosterer *et al.* 2006). Their role in regulating gene expression at post-translational levels in algae was first reported in *Chlamydomonas reinhardtii* (Zhao *et al.* 2007), with further studies investigating miRNAs in other microalgae including *Volvox*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Coccomyxa* etc. (Li *et al.* 2014; Yang *et al.* 2019; Azaman *et al.* 2020; Lou *et al.* 2020). However, a clear miRNA biogenesis pathway in algae remains to be established. Nevertheless, genes involved in miRNA biogenesis such as *Dicer-like-3* (*DCL3*) and *Argonaute* (*Ago3*) have been identified in *Chlamydomonas reinhardtii* (Voshall *et al.* 2015; Chung *et al.* 2019).

Focus on identifying miRNAs in *Dunaliella* has gained attention in the last decade with the first report being as early as 2017 (Winfrey *et al.* 2017). The availability of the draft genome of *D. salina* along with a large volume of sequence read archive (SRA) and expressed sequence tags (ESTs) generated from cDNA libraries of *D. salina* in different studies, has opened the avenue to study the role of miRNAs in the stress-response pathway of *D. salina*. These investigations have reported miRNAs under high salinity, light, and osmotic stress (Lou *et al.* 2018, 2020; Gao *et al.* 2019). Lou *et al.*, (2020) in their work, have identified a novel miRNA, targeting the malate dehydrogenase-encoding gene, that has

been stipulated to play a role in carbon partitioning under osmotic stress. Hitherto, only a limited number of miRNAs and their specific target genes have been identified in *D. salina*. Owing to the diverse habitat distribution and complex molecular architecture, genome sequencing data is not available for all species of *Dunaliella*. Presently, the National Centre for Biotechnology Information (NCBI) hosts genome assemblies of three *Dunaliella* species *vis-a-vis*: *D. salina* strain CCAP 19/18 (PRJNA32771); *D. primolecta* (PRJEB47344) and *D. tertiolecta* strain CCAP 19/42 (PRJNA496373). Thus, due to limited genomic information, *in-silico* miRNA prediction and identification using publicly available genomic data such as ESTs, SRA and Genome Survey Sequences (GSS) becomes a fast and reliable tool for miRNA identification with several miRNAs already characterized in higher plants using this approach (Gupta *et al.* 2017; Vivek 2018; Yasin *et al.* 2020; Chaudhary *et al.* 2022). Hence homology-based sequence alignment remains a reliable approach for identifying known miRNAs in *D. salina* (Lou *et al.* 2020).

This study is the first to report miRNAs from the *D. salina* strain found in the salt pans of Sambhar Lake, Rajasthan, India using SRA-based identification method. The *Dunaliella* species flourishing in Sambhar Lake has been identified as *D. salina* in previous morphological and molecular-based studies (Sharma *et al.* 2012; Kulshreshtha & Singh 2013). Evolutionarily, *Dunaliella* is classified within the order Chlamydomonadales and exhibits morphological traits typical of *Chlamydomonas reinhardtii*, a well-studied model organism for research due to its status as a unicellular, photosynthetic eukaryote (Zhao *et al.* 2007). Several studies investigating the morphological and biochemical features of *C. reinhardtii* have yielded much molecular data, adding to its utility for algal miRNA identification (Molnár *et al.* 2007).

However, *Dunaliella* has evolved as a separate lineage with robust physiological and biochemical mechanisms of stress tolerance and has gained attention as a model organism to study abiotic stress regulation in photosynthetic eukaryotes (Oren 2005; Zhao *et al.* 2007; Takaichi 2011; Preetha *et al.* 2012). With the growing importance of bioengineering processes for the bioprospection of *Dunaliella*, miRNAs have become potential tools for augmenting underlying genetic regulatory pathways responsible for abiotic stress regulation and β -carotene accumulation. Thus it becomes imperative to identify miRNAs responsible for regulating these pathways via targeting specific genes, to develop additional molecular tools that can help in commercial β -carotene production. Apart from *C. reinhardtii*, *Arabidopsis thaliana* is a widely studied plant model with a well-defined miRNA biogenesis pathway (Voinnet 2009). Several miRNAs including miR156, miR159, miR166, miR414 etc. are reportedly found to be conserved in *A. thaliana* and different algal species (Chung *et al.* 2017; Yang *et al.* 2019; Lou *et al.* 2020; Li *et al.* 2023). Thus both *C. reinhardtii* and *A. thaliana* serve as a repository of miRNAs with numerous miRNAs already reported along with their target genes. These model organisms hence can be utilized as a source of known miRNAs for identifying miRNA families in other organisms. Therefore, to identify conserved miRNA families in *D. salina*, mature miRNAs sequences of *C. reinhardtii* and *A. thaliana* were retrieved from miRBase for homology-based mapping against the publicly available SRA dataset of *D. salina*. Further, *in-silico* tools designed for predicting miRNA-target gene interactions have proven to be quick and efficient for identifying binding sites of miRNA on their target genes, analyzing hybridization energies and formulating strategies for gene expression regulation (Srivastava *et al.* 2024). This study focuses on determining miRNAs targeting *Crt* genes in *D. salina* and their expression validation. The findings illustrate the role of miRNAs in *Crt* gene regulation in *D. salina* and hold the potential to carry out future research for studying the role of miRNAs in the abiotic stress-regulatory pathway and β -carotene accumulation through regulating the expression of target *Crt* genes.

Material and methods

Area of Study and Sample Collection

Sambhar Salt Lake is situated 80 km southwest in the Jaipur district of Rajasthan, India. It is the largest inland salt lake in the country, located in the depressions of the Aravalli hill ranges. Due to its unique hydro-ecological characteristics, the lake was included in the International Ramsar Convention as a wetland of international importance in 1990. The water in the lake is brackish and drained by seasonal rivers during monsoon, hence the water levels of the lake vary seasonally, dropping to as low as 60 centimetres in summer and rising to 3 meters in monsoon. The lake also houses one of the largest salt refineries in the country and has a distinct, extensive saline wetland (Singh *et al.*, 2013; L. Sharma *et al.*, 2019). Apart from its commercial and international significance, the lake is a critical migratory habitat for flamingos, shorebirds, ducks etc. due to its location on the Central Asian-Indian Flyway route (Sambhar Lake (India) - BirdLife IBA Factsheet). Moreover, Sambhar Lake has unique flora and fauna including species of halophilic shrimps, avifauna, fungi and green algae such as *Dunaliella*. Previous studies have successfully characterized the *Dunaliella* strain found in Sambhar Lake, India (Sharma *et al.* 2012; Singh *et al.* 2019). These studies reported the strain to be of *D. salina* based on 18S-rRNA gene characterization. The samples for this research work were collected in February 2020 from the salt pans of the lake at 30-40 cm depth (Figure 1a & b) in 500 mL sterile collection bottles. The samples were checked for pH in the laboratory (8.9 ± 0.2). The samples were transferred in open tubs and stored in the culture chamber with a photoperiod of 18:6 hours (Light: Dark) at $24^\circ\text{C} \pm 2^\circ\text{C}$, illuminated with 50 mmol photons $\text{m}^{-2} \text{s}^{-1}$ cool-white light (Syska LED lamps, India) and were processed for further studies (Figure 1c). All subsequent cultures were maintained under the same growth conditions throughout the study.

Isolation, Culturing and Maintenance of *D. salina*

10 mL of the collected sample was initially subjected to centrifugation in 15 mL falcon tubes (Tarsons) at 3000 rpm for 1-2 minutes which resulted in the accumulation of *Dunaliella* cells on the periphery of the top layer of the supernatant. The cells were picked without disturbing

the supernatant using a 1000 mL micropipette (BR Biochem Life Sciences) and were transferred to 100 mL Erlenmeyer flasks containing 50 mL Johnson's medium (Johnson *et al.* 1968; Borowitzka *et al.* 1984). Flasks were then incubated in the growth chamber for two weeks (Figure 1d). Periodic observations of the pre-cultures were carried out under Metzer microscope (India) attached with a Fixed Microscope Adapter (FMA050) and Metzer-M Capture Pro imaging system to monitor the general health status and to check the absence of any possible contamination. Subsequent culturing was done through the abovementioned process by centrifuging the pre-culture and inoculating in fresh media to maintain stock cultures. Sterile conditions were maintained during the entire experimentation by using autoclaved media, equipment, glassware and the use of laminar airflow.

Selection of optimal salinity for culturing local D. salina strain

Salinity regulation is a common and efficient method for maintaining *D. salina* cultures. Therefore, determining the optimum salinity for culturing the local strain of *D. salina* for optimum biomass yield for RNA isolation was yet another objective of this study. To achieve this, *D. salina* cells from stock cultures were inoculated in fresh Modified Johnson's media with trends of salinity ranging from 0.5M, 2M, 2.5M, 3M, 4M and 5M. Cells were cultured in triplicates of 50 mL fresh media in 100 mL Erlenmeyer flasks by inoculating an average of 2×10^5 cells from stock cultures. The growth parameters between these six salinities were observed for 5 weeks.

For comparative growth analysis of cultures at different salinities, cell density was determined by weekly cell count for each set of salinity. 1 mL of well-mixed algal suspension culture from each set was taken in a 1.5 mL Eppendorf tube and fixed with 5 μ L formaldehyde. An aliquot from this was then placed on a Neubauer haemocytometer to count the cell numbers and monitor cell growth. The volume and dilution method was used to estimate total cell numbers in the culture (Khatoon *et al.* 2020). The specific growth rate (SGR) defined as the growth rate per day ($\mu \text{ day}^{-1}$) was calculated by the method suggested by (Clesceri *et al.* 1989). SGR was determined by using the formula $\ln X_2 - \ln X_1 / T_2 - T_1$ where X_1 is the cell count on the day of inoculation, X_2 is the cell count at the end of 5 weeks, $T_2 - T_1$ is the time interval (5 weeks = 35 days) for the determination of cell density of *D. salina*. The quantitative analysis of cell growth at different salinities was done using the one-way analysis of variance (ANOVA). The salinity concentration showing maximum growth over 5 weeks was selected as the optimum concentration to grow the Sambhar Lake strain of *D. salina* throughout this study.

RNA isolation from *D. salina*

RNA was isolated directly from 3-week-old algal cultures through the modified Trizol method. RNA isolation was performed in triplicate for *D. salina* cultures grown in media with 2M NaCl concentration. 50 mL culture was centrifuged at 3000 rpm for 5 minutes. The obtained algal pellet was washed by resuspending in 2 mL DEPC water, followed by transferring the suspension to a 2 mL Eppendorf tube and pelleting again at 3000 rpm for 5 minutes. After discarding the supernatant, the pellet was resuspended in 1 mL Trizol solution and the tube was vortexed for 10 minutes followed by the addition of 200 μ L chloroform and a quick spin. The tube was then incubated for 10 minutes at room temperature followed by centrifugation at 10,000 rpm for 20 minutes in a cooling centrifuge. The upper aqueous phase was collected in a fresh Eppendorf, and 500 μ L of chilled isopropanol was added followed by the addition of 50 μ L of freshly prepared ammonium acetate solution. The tube was then incubated at -80°C for 1 hour. Further, the tube was thawed and spun to obtain the RNA pellet. The supernatant was discarded and the pellet was washed twice with 1 mL 75% ethanol, air-dried and dissolved in nuclease-free water. The concentration of the RNA samples was determined using Nanodrop and the same were stored at -80°C for further use.

Identification of conserved miRNAs in *D. salina*

Retrieval of MicroRNAs and Homology-based mapping against SRA dataset of D. salina

51 and 428 miRNA sequences of *C. reinhardtii* and *A. thaliana* respectively, were retrieved from miRBase Version 22.1 (<https://www.mirbase.org/>) (Griffiths-Jones *et al.* 2006) to map the known miRNAs in *D. salina*. Sequence Read Archive (SRA) files are a collection of both coding and non-coding sequences, generated from multi-omics studies and deposited in the SRA repository of the National Centre of Biotechnology Information (NCBI). In this study, the SRA dataset of *D. salina* was used for homology-based sequence alignment analysis using the BLASTN tool of NCBI at default settings. miRNA sequences with a minimum number of 17 nucleotides, showing maximum homology (95-100%) against SRA sequences with 100% query coverage were selected for target prediction (Supplementary Table S1 and S2).

Target gene prediction of mapped miRNAs in D. salina

In this study, we predicted the target genes of the mapped miRNAs using psRNATarget. Since *D. salina* is a rich source of carotenoids with commercial importance, recent studies have focused on identifying miRNAs involved in the carotenoid synthesis pathway. Therefore, intending to identify miRNAs involved in the regulation of β -carotene synthesis, we focused on the genes involved in the carotenogenesis pathway in *D. salina* and used them as candidate target genes for miRNA-target prediction. The gene sequences were retrieved from NCBI. Table 1 provides the list of *Crt* genes used as candidate targets along with their functions and accession numbers. Using psRNATarget (Dai *et al.* 2018), the target gene prediction was performed for the mapped miRNAs. The mapped miRNAs and fasta sequences of the four *Crt* genes were

submitted in the psRNATarget for target prediction. The parameters of psRNATarget were: E-value between 0-5; length of seed region- 2 to 8 nt.; Maximum number of top targets- 10 and a sum of gaps and mismatches in the seed region not more than two, rest other parameters were set to default.

Table 1- List of *Crt* genes involved in carotenogenesis used as candidates for miRNA-target gene prediction

S.No.	Gene Name	Accession Number	Length of mRNA	Coding Sequence Region	Function	References
1.	Lycopene-β-cyclase (<i>LycB</i>)	KX218392.1	1794	1-1794	Conversion of Lycopene to β-carotene	(Liang <i>et al.</i> 2017)
2.	Phytoene synthase (<i>Psy</i>)	AY601075.1	1260	1-1260	Conversion of Geranyl-geranyl pyrophosphate (GGPP) to Phytoene	(Yan <i>et al.</i> 2005)
3.	Phytoene Desaturase (<i>Pds</i>)	DQ243892.1	2290	22-1770	Conversion of Phytoene to Lycopene	(Sun <i>et al.</i> , 2008)
4.	Zeta-carotene desaturase (<i>Zds</i>)	HM754265.2	2144	36-1766	Conversion of Zeta-carotene to Lycopene	(Ye & Jiang, 2010)

Expression validation of mapped miRNAs in *D. salina*

Primer construction for expression validation of miRNAs and target *Crt* gene in *D. salina*

Based on the results sequence homology and psRNATarget, miRNA sequences showing maximum alignment with the SRA dataset of *D. salina* and targeting *Crt* genes were selected for expression validation and primers were designed for the same. Stem-loop, forward and reverse primers of specific miRNAs and target genes were designed according to Chen *et al.*, (2005) with some modifications to investigate their expression in the isolated RNA samples from the cultured *D. salina* cells via stem-loop reverse transcription (SL-RT) PCR. The primers were obtained from Eurofins Genomics Pvt. Ltd. (India). The list of primers used in this research work has been provided in supplementary data (Supplementary Table S3).

First-strand cDNA synthesis

Single-stranded cDNAs are used in Stem-Loop Reverse Transcription (SL-RT) PCR studies to check the expression of different miRNAs. The obtained cDNAs can then be used for semi-quantitative PCR studies to investigate the expression of specific miRNAs in various RNA samples. In our study, first-strand cDNA for each selected miRNA was constructed using 3 µg of the isolated RNA using Hi-cDNA Synthesis Kit (HiMedia) as per manufacturer’s instructions in a thermomixer. All cDNAs were stored at -20° C for further use.

PCR for miRNA expression analysis

To check the expression of selected miRNAs in cultured *D. salina* cells, semi-quantitative PCR was performed in a 10 µL reaction, using HiChrom PCR master mix as per the manufacturer’s instructions using Biorad PCR machine with the following thermal profile; initial denaturation- 95° C for 3 minutes, followed by 40 cycles of denaturation at 95° C for 30 seconds, 45 seconds for annealing at 55°C for cre-miR917and ath-miR166a3p; 60°C for cre-miR1163.2, cre-miR1145.1 and 62°C for ath-miR414; extension at 72°C for 1 minute, final extension at 72°C for 10 minutes and hold at 25°C for 5 minutes. The PCR products were then run on 4% agarose gel to check the expressions. The PCR product size of specific miRNAs was calculated by using the length of respective SL-RT and forward primers.

Identifying potential target binding sites of miRNAs on *Crt* genes

There are several computational tools to identify putative target binding sites of miRNAs. Moreover, the prediction of binding locations can vary depending on the length of the miRNA, the target sequence and the specificity of the underlying algorithm of the tool (Srivastava *et al.* 2024). In this work, the miRNA-target gene alignments obtained from psRNATarget were further analyzed using RNAHybrid to corroborate psRNATarget results and determine hybridization pattern and Minimum Free Energy (MFE) (Rehmsmeier *et al.* 2004). The MEF was a critical factor in selecting the potential miRNA-target gene configurations. The potential matches of miRNA-target genes were selected based on MEF ≤ -25 kcal mol⁻¹ in the top five hits and a sum of gaps and mismatches not more than two in the seed region (2-8nt.) (Mückstein *et al.* 2006; Kertesz *et al.* 2007). The miRNA-target gene sequences exhibiting MEF ≤ -25 kcal mol⁻¹ in the target binding regions have been selected as potential target binding sites of miRNAs on their respective target *Crt* genes (Kalarikkal & Sundaram 2021) and such miRNAs have been identified for their likely involvement in carotenoid synthesis regulation (Supplementary Table S5).

Differential expression analysis of miRNAs targeting *Crt* genes in *D. salina* under salinity stress

Quantitative PCR (qPCR) of miRNAs targeting *Crt* genes

qPCR was performed to determine the differential expression of potential miRNAs targeting *Crt* genes in *D. salina* under hypersalinity stress (HS). 18-day-old *D. salina* cultures grown in 2M ASWM were subjected to 72 hours of salinity stress at 4M by adding NaCl. RNA isolation was then performed as described above. For cDNA synthesis, stem-loop inversion was performed using 2 µg isolated RNA from control and treated samples, respectively, using Hi- cDNA Synthesis Kit (HiMedia) as per the manufacturer's instructions in a thermomixer. *18S-rRNA* was used as a reference gene. The qPCR was performed using the Qiagen QuantiNova qPCR master mix as per the manufacturer's instructions. The reaction conditions were as follows: initial activation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and combined annealing and extension at 60°C for 45 seconds. All the reactions for each miRNA sample were performed in triplicate. The qPCR data were analyzed using the second derivative method ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = Ct_{\text{sample}} - Ct_{18S-rRNA}$. Ct_{sample} and $Ct_{18S-rRNA}$ are the Ct values for the miRNA and the internal reference gene (*18S-rRNA*), respectively.

qPCR of a potential target gene of miRNAs

One of the *Crt* genes vis-à-vis *Pds* was selected as a candidate gene for qPCR analysis (based on psRNATarget and RNAHybrid results) to establish a potential regulatory relationship between miRNAs and their target *Crt* genes. qPCR was performed using the Qiagen QuantiNova qPCR master mix according to the manufacturer's instructions. The reaction conditions were as follows: initial activation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and a combined annealing and extension at 60°C for 30 seconds. The reactions were conducted in triplicate. The qPCR data were analyzed using the second derivative method ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = Ct_{Pds} - Ct_{18S-rRNA}$. Ct_{Pds} and $Ct_{18S-rRNA}$ represent the Ct values for the *Pds* gene and the internal reference gene (*18S-rRNA*), respectively.

Statistical analysis

Changes in the level of miRNAs and target *Crt* gene under HS conditions are represented as fold-change relative to control. The experiments were carried out in triplicates and the results are presented as mean ± standard error (SE). Student's t-test was conducted to determine the statistically significant expression with p-value > 0.05 marked as "NS"; 0.01 < p < 0.05 marked as "*" and p < 0.01 marked as "**" (Figure 6 & 7).

Results

Growth analysis of *D. salina* cultures

Pure cultures of *D. salina* were obtained through regular serial dilution and sub-culturing. *D. salina* cells maintained in Johnson's media appeared green and pear-shaped when observed under a microscope at 40X (Figure 1e). Wholesome cell growth of *D. salina* was observed in salinities 2 and 2.5M. However, at 2M the cell growth was sustained for up to 5 weeks with the highest cell number 4.48×10^6 cell mL⁻¹. The cell count at 2.5M after 5 weeks of growth was 4.17×10^6 cells mL⁻¹. Cultures at 0.5M salinity levels showed maximum growth up to the 4th week (2.57×10^6 cells mL⁻¹) after which the cell count declined reaching 2.18×10^6 cells mL⁻¹ after 5 weeks. While high salinity has been shown to affect carotenoid production in *D. salina*, cellular growth remained minimal at 3M, 4M and 5M concentrations in this study with a cell count of 1.06×10^6 cells mL⁻¹, 9.08×10^5 cells mL⁻¹ and 5.64×10^5 cells mL⁻¹ respectively (Figure 2). The ANOVA test was performed to determine the effect of salinity on cell growth at a P-value < 0.05 (obtained P-value: 0.015 was significantly lower than the threshold value) which was considered significant. Further, the algal suspensions at 2M showed the highest SGR followed by cultures maintained at 2.5M (Figure 3). Based on the analysis of cell density, it was found that 2M salinity concentration was optimum for growing the Sambhar Lake strain of *D. salina* under laboratory conditions to produce higher biomass with a significant growth difference (P < 0.05) when compared to growth at different salinity levels.

Identification of known miRNAs targeting *Crt* genes in *D. salina*

51 and 428 mature miRNA sequences of *C. reinhardtii* and *A. thaliana*, respectively were blasted against the SRA dataset of *D. salina*. The blast was performed using default parameters (Blast results provided in Supplementary Table S1 and S2). A total of 30 miRNAs were mapped from the SRA dataset of *D. salina*. 16 miRNAs of *A. thaliana* and 14 miRNAs of *C. reinhardtii* showed maximum homology with the SRA dataset. After mapping miRNA sequences showing the highest homology with SRA sequences of *D. salina*, the 30 miRNAs were subjected to target prediction against the four selected *Crt* genes using psRNATarget. Five miRNA sequences: three belonging to *C. reinhardtii* i.e. miR1163.2, miR917 and miR1145.1 while two miRNAs of *A. thaliana* i.e., miR166 and miR414 were selected for expression validation in *D. salina* (Table 2) based on the results of psRNATarget. The analysis showed all five miRNAs to target more than one *Crt* gene and exhibited a sum of gaps and mismatches of not more than two in the seed region (Supplementary Table S4). Table 3 describes the different miRNAs targeting specific *Crt* genes along with their binding sites and the type of inhibition exhibited by the miRNAs on their respective target genes. Although the results of psRNATarget yielded multiple binding sites for miRNA-target genes, only the top results have been included in the table.

Table 2- Known miRNAs identified in *D. salina* through homology-based mapping against the SRA dataset

S.No.	Name and Sequence of miRNA (5' – 3')	No. of nucleotides in miRNA	SRA Accession Number	Identified miRNA sequence from SRA of <i>D. salina</i>	No. of nucleotides in identified miRNA of <i>D. salina</i>
1.	cre-miR1163.2 AGGGCAUGCUGCGUGCCAUGG	21	SRA:ERR2041056.4978782.1	dsa-miR1163.2: 33- AGGGCAUGCUGCGUGCCAUGG- 13	21
2.	cre-miR917 UUCACGGUUAUGUUCGAAG	19	SRA:ERR2041055.9098914.1	dsa-miR917: 35-UUCACGGUUAUGUUGGAAG- 53	19
3.	cre-miR1145.1 UUGGGGCCCAGCAGGUCC	18	SRA:ERR2041055.11303051.1	dsa-miR1145.1: 66- UUGGGGCCCAGCAGGUCC- 83	18
4.	ath-miR166a3p UCGGACCAGGCUUCAUCCCC	21	SRA:ERR2041055.227925.2	dsa-miR166: 49- CGGACCAGGUUCAUCCCC- 68	20
5.	ath-miR414 CAUCCUCAUCAUCCUCGUCC	20	SRA:ERR2041055.11534020.1	dsa-miR414: 49- CAUCCUCAUCAUCCUCGUCC- 30	20

Table 3- Crt genes found to be potential targets of mapped miRNAs based on psRNATarget

S.No.	Mapped miRNAs	Potential Target Gene(s)	Target binding region (5'-3')	Type of binding site on target gene	Type of Inhibition
1.	dsa-miR1163.2	<i>LycB</i>	1146-1166	CDS	Cleavage
		<i>Psy</i>	202-221	CDS	Cleavage
		<i>Pds</i>	1820-1840	3' UTR	Translation
		<i>Zds</i>	862-882	CDS	Cleavage/ Translation
2.	dsa-miR917	<i>Psy</i>	131-149	CDS	Cleavage
		<i>Pds</i>	2151-2169	3' UTR	Translation
		<i>Zds</i>	1694-1711	CDS	Cleavage/ Translation
3.	dsa-miR1145.1	<i>LycB</i>	30-47	CDS	Cleavage
		<i>Psy</i>	318-335	CDS	Cleavage
		<i>Pds</i>	327-344	CDS	Cleavage/ Translation
		<i>Zds</i>	141-158	CDS	Cleavage/ Translation
4.	dsa-miR166a3p	<i>Pds</i>	642-661	CDS	Cleavage
		<i>Zds</i>	1554-1573	CDS	Translation
5.	dsa-miR414	<i>LycB</i>	1701-1720	CDS	Translation
		<i>Psy</i>	1056-1075	CDS	Cleavage
		<i>Pds</i>	912-930	CDS	Translation

Expression validation of miRNAs targeting *Crt* genes

The total RNA extracted using the modified Trizol method was quantified using a nanodrop (Thermo Scientific NanoDrop One). An optimum quantity of total RNA in a range of 35-50 µg per 50 mL algal culture with an absorbance ratio (260/280 nm and 260/230 nm) greater than 2 was obtained. This RNA was further used for cDNA synthesis. Based on the *in-silico* results obtained from psRNATarget, five miRNAs targeting *Crt* genes were selected for expression validation in the local strain of *D. salina*. All five miRNAs including miR1163.2, miR917, miR1145.1, miR166 and miR414 were found to be expressed in *D. salina* cultured at 2M salinity concentrations (Figure 4). Further, the location of PCR products between 50 and 100 bp as visualized through gel electrophoresis corroborated the PCR product length calculated for each miRNA: dsa-miR1163.2-63; dsa-miR917-65; dsa-miR1145.1-62; dsa-miR166-64; and dsa-miR414-66.

Prediction of potential miRNA-target gene configurations

Based on the results of RNAHybrid, three out of five miRNAs: dsa-miR1163.2, dsa-miR1145.1 and dsa-miR414 were found to exhibit $MFE \leq -25$ kcal mol⁻¹ in the miRNA-target gene duplexes. These miRNAs were selected as candidate miRNAs that could potentially regulate target *Crt* genes and carotenogenesis. miR1163.2 and miR1145.1 were found to target all four *Crt* genes in their coding sequence (CDS) regions. Meanwhile, miR414 was found to target all *Crt* genes except *Zds* (Supplementary Table S4). Figure 5 depicts the potential role of the five miRNAs in regulating the expression of different *Crt* genes in the carotenoid synthesis pathway. Single miRNA was found to have multiple binding sites on each target gene upon RNAHybrid analysis (Supplementary Table S5). The energy values (kcal mol⁻¹) shown in the figure refer to the miRNA-target gene binding configurations exhibiting the highest negative MFE in the range of -25 to -32 kcal mol⁻¹, thus having stability akin to plant miRNA-target gene duplexes (Chaudhary *et al.* 2022). miR917 and miR166a3p exhibited $MFE > -25$ kcal mol⁻¹ and more than 2 gaps/mismatches in the seed region, therefore these miRNA-target duplexes have not been considered as potential candidate miRNAs having a role in carotenoid regulation in *D. salina*. However, their role in other molecular pathways of *D. salina* remains to be discovered.

Possible role of differentially expressed miRNAs in regulating carotenoid synthesis in *D. salina* under salinity stress

qPCR results revealed that Ds-miR1163.2, dsa-miR917, dsa-miR1145.1, dsa-miR166a-3p and dsa-miR414 were differentially expressed in *D. salina* cultures upon salinity stress (4M NaCl). Only dsa-miR1145.1 and dsa-miR414 were found to be upregulated under salinity shock, while all miRNAs were downregulated. Of the downregulated miRNAs, dsa-miR1163.2 was downregulated by 33.8% as compared to control and this was statistically significant with $p < 0.01$. The downregulation of dsa-miR917 and dsa-miR166a-3p was not statistically significant at $p < 0.05$. Of the two upregulated miRNAs, dsa-miR1145.1 was found to be upregulated by 0.58-fold but it was not statistically significant at $p < 0.05$. dsa-miR414 was found to be upregulated by 1.65-fold and this was significant at $p < 0.05$ (Figure 6).

The *Pds* gene is a key pathway gene in carotenoid synthesis in *D. salina*. It catalyzes the desaturation of phytoene. This gene was selected for differential expression analysis under HS conditions as it exhibited the lowest MFE and, therefore, a more stable hybridization with dsa-miR414 when analyzed through RNAHybrid. qPCR analysis revealed the downregulation of the *Pds* gene under HS conditions by 31%. However, this downregulation was not significant at $p < 0.05$.

Based on the cumulative analysis of target prediction studies and qPCR analysis, dsa-miR414 presented significant differential expression between control and HS conditions. The corresponding target gene, i.e., *Pds* of this significantly expressed miRNA, was predicted through psRNATarget and RNAHybrid. Later, the expression of the *Pds* gene was verified using qPCR. dsa-miR414 was markedly upregulated by nearly > 2-fold while its target gene, *Pds* was down-regulated by < 1-fold under HS (Figure 7).

Discussion

miRNAs in *D. salina* isolated from Sambhar Lake, India

Numerous known and novel miRNAs have been recently identified in *D. salina*. However, target genes for only a limited number of these miRNAs have been determined, and their expression levels correlated (Gao *et al.* 2019; Lou *et al.* 2020). This study is the first of its kind to report miRNAs from the *D. salina* strain isolated from Sambhar Lake, India. Identification of miRNAs in *D. salina* has recently gained traction due to its commercial importance in carotenoid production. Therefore, our research work reports 5 miRNAs (dsa-miR1163.2, dsa-miR917, dsa-miR1145.1, dsa-miR116 and dsa-miR414) in *D. salina* and highlights three miRNAs: dsa-miR1163.2, dsa-miR1145.1 and dsa-miR414 having a potential role in the regulation of carotenoid synthesis. These findings are based on the *in-silico* identification of these miRNAs and their subsequent expression validation in the *D. salina* strain found in Sambhar Lake, India. Additionally, differential expression validation. Nevertheless, further research is required to determine the exact relation between these miRNAs and their potential target *Crt* genes and how the identified miRNAs regulate the expressions of *Crt* genes.

Functional analysis of identified miRNAs in *D. salina*

miRNA sequences belonging to miR166 and miR414 families have been reported in several other higher plants and algae. Their identification and expression in *D. salina* confirm the conserved nature of these miRNAs in unicellular green algae as well as in higher plants. Owing to their conserved nature and putative role in abiotic stress regulation these miRNAs can be further examined to identify precursor miRNAs and also establish a correlation with their target *Crt* gene/s. This work underscores the potential for using miRNAs and their target genes as advanced biotechnological systems that can be further manipulated to improve stress tolerance in *D. salina* and enhance β -carotene production, paving the way for more efficient and commercially viable algal bioengineering strategies.

Lou et al., (2020) in their study reported 7 known and 16 novel miRNAs in *D. salina*. One of these known miRNAs i.e., miR166 was found common in our study with identical sequences and has been reported to play a crucial role in stress tolerance by targeting the HD-ZIP transcription factors (Yadav *et al.* 2021). Further, the role of miR166 as a stress biomarker has been established in several plant models, therefore similar studies can be further carried out to investigate the function and target genes of miR166 in *D. salina*. miR166 has been identified in *C. sorokiniana*, another unicellular green alga (Azaman *et al.* 2020). A recent study has investigated its role in the abscisic acid (ABA) signaling pathway of *Glycine max* during drought stress (Zhao *et al.* 2024). Based on the results of target prediction in this study, miR166 was found to target 2 key *Crt* genes: *Pds* and *Zds* in their respective CDS regions. However, in our study, miR166 has not been considered a candidate miRNA regulating expression of *Crt* genes as it exhibited higher MFE upon RNAHybrid analysis. Despite this, a correlation can be hypothesized based on the targeting of *Crt* genes by miR166 and its functional role in the regulation of stress responses investigated in other studies. Nevertheless, due to a lack of studies based on knockdown techniques and the development of transgenics lacking miR166, there remains a gap in establishing a direct evidence-based relation between miR166 and its target *Crt* genes. Additionally, miR414 is also a conserved miRNA with reports in several higher plants. It has been suggested to play a role in plant morphogenesis, metabolism, growth and defense responses in *Stevia rebaudiana* (Guleria & Yadav 2011). In our investigation, miR414 was found to target *Psy*, *Pds* and *Zds* genes of the carotenoid synthesis pathway and exhibited high negative MFE in their miRNA-target mRNA duplexes during RNAHybrid analysis. However, the regulatory function of this miRNA remains uninvestigated in other plant systems and thus further in-depth research is required to establish its role in plant gene regulation. The present study underscores the role of miR414 in the regulation of *Crt* genes in *D. salina* and opens another line of evidence to carry out future research based on miR414 in other plant and algal models.

Species-specific miRNAs: cre-miR1163.2, cre-miR917 and cre-miR1145.1 have not yet been investigated for their role in gene regulation in *C. reinhardtii*. The hairpin structure and mature sequences of these miRNAs are available in miRBase (Molnár *et al.* 2007). miR1163.2 and miR1145.1 have not been previously reported in *D. salina*. However, the high negative MFE displayed by the duplexes of these miRNAs and mRNAs of *D. salina* anticipates their potential role in carotenoid synthesis (Figure 5). miR917 was found to target *Psy*, *Pds* and *Zds* genes, but due to low negative MFE values and more than 2 gaps/mismatches in seed-region, it has not been considered as a candidate miRNA regulating *Crt* gene expression. Interestingly, 2 known miRNA sequences belonging to the miR917 family have been reported by Lou et al., (2020) in *D. salina* strain CCAP 19/18. However, neither of the two sequences shows homology with the ones reported in *C. reinhardtii* and that identified in our study. Nevertheless, these miRNAs can be further studied to determine their expression under different physiological conditions and investigate their function in different biological processes through gene ontology (GO). The findings of this study demonstrate the conserved nature of these miRNAs as both *C. reinhardtii* and *D. salina* belong to the same order of algal species. In future, similar sequences and their functions can be investigated in other algae and higher plants to understand the molecular mechanism of stress responses and carotenoid accumulation.

dsa-miR414 may play a role in regulating carotenoid synthesis in *D. salina*

This study primarily focuses on dsa-miR414 in regulating the expression of *Crt* genes in *D. salina* and in turn, moderating the accumulation of carotenoids under salinity stress. Dsa-miR414 possibly plays an inhibitory role against *Pds* gene. There is not enough direct evidence to fully establish the correlation between dsa-miR414 and carotenoid accumulation under hypersalinity conditions in *D. salina* or other algal and plant models. However, according to the current experimental data, it is very likely that dsa-miR414 inhibits the *Pds* gene in this *D. salina* strain under HS conditions. Further work is required to illustrate how this dsa-miR414 regulates the accumulation of carotenoids particularly β -carotene in *D. salina*. Additionally, studies can be conducted to decipher other target genes of dsa-miR414.

In conclusion, our study predicts five conserved miRNAs: dsa-miR1163.2; dsa-miR917; dsa-miR1145.1; dsa-miR166a3p and dsa-miR414 that were found to be expressed in the *D. salina* strain isolated from Sambhar Lake, India using SLRT-PCR. Furthermore, the expression levels of these miRNAs were compared between control and HS conditions through qPCR analysis. The potential role of dsa-miR1163.2, dsa-miR1145.1 and dsa-miR414 in carotenoid synthesis was thus established. Aided through computational analysis, these three miRNAs have been suggested to play a potential role in carotenoid synthesis in *D. salina*. Additionally, differential expression analysis for one of the *Crt* genes, i.e., *Pds* was performed to validate the finding of *in-silico* target prediction. The findings add to the currently expanding list of miRNAs and their target genes being identified in *D. salina*. An initial assessment suggests that dsa-miR414 may play a role in regulating the *Pds* gene in *D. salina*, supporting the need for further research on miRNA-mediated carotenoid accumulation in this microalgal strain. The identified miRNAs

can be further investigated for their evolutionary conservation in algae and higher plants along with their prospective role in carotenoid synthesis and other biological processes.

Declarations

Ethical Statement

Consent to Publish

All authors read and approved the final version of the manuscript and agreed to publish the research work.

Research content

The manuscript has not been submitted to any other journal for simultaneous consideration. The submitted work is original and has not been published elsewhere in any form. The work represents a single study.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

N/A

Informed Consent

N/A

Author Contribution

Yogita Sharma: Formal analysis, methodology, data curation and writing original draft; Rekha Yadav: Drafting, reviewing and editing; Hemakshi Sharma: reviewing and editing; Chandra Pal Singh: Conceptualization, methodology, funding acquisition, supervision and research administration. All authors read and approved the final version of the manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

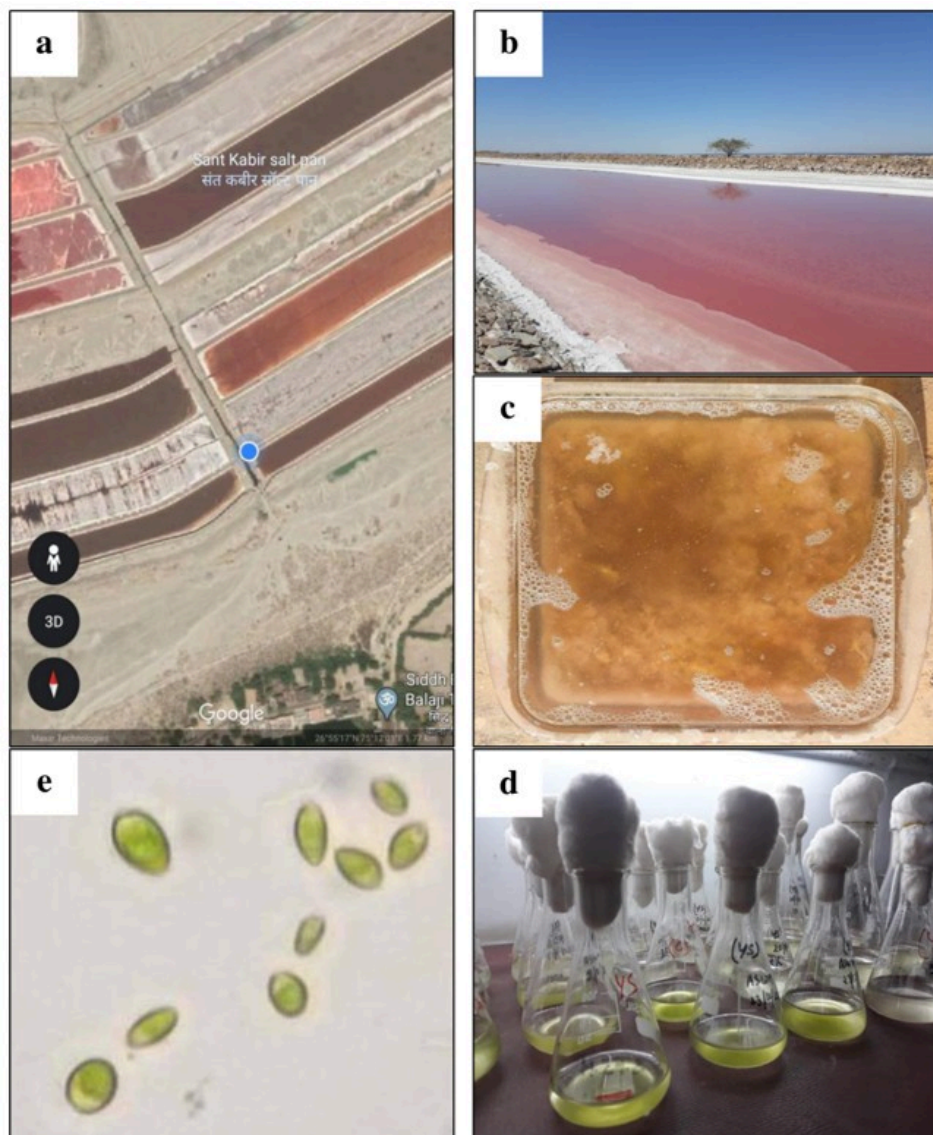


Figure 1

(a) Sample collection site- Sambhar Lake, Rajasthan, India ($26^{\circ}55'17''\text{N}$ $75^{\circ}12'01''\text{E}$); (b) Saltpans at Sambhar Lake, Rajasthan, India appear pink due to the presence of *D. salina*; (c) Collected water samples stored in open tubs; (d) Stock cultures of *D. salina* growing in Johnson's media at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and a photoperiod of 18:6 hours (Light: Dark) under white light; (e) *D. salina* cells under microscope (40X magnification)

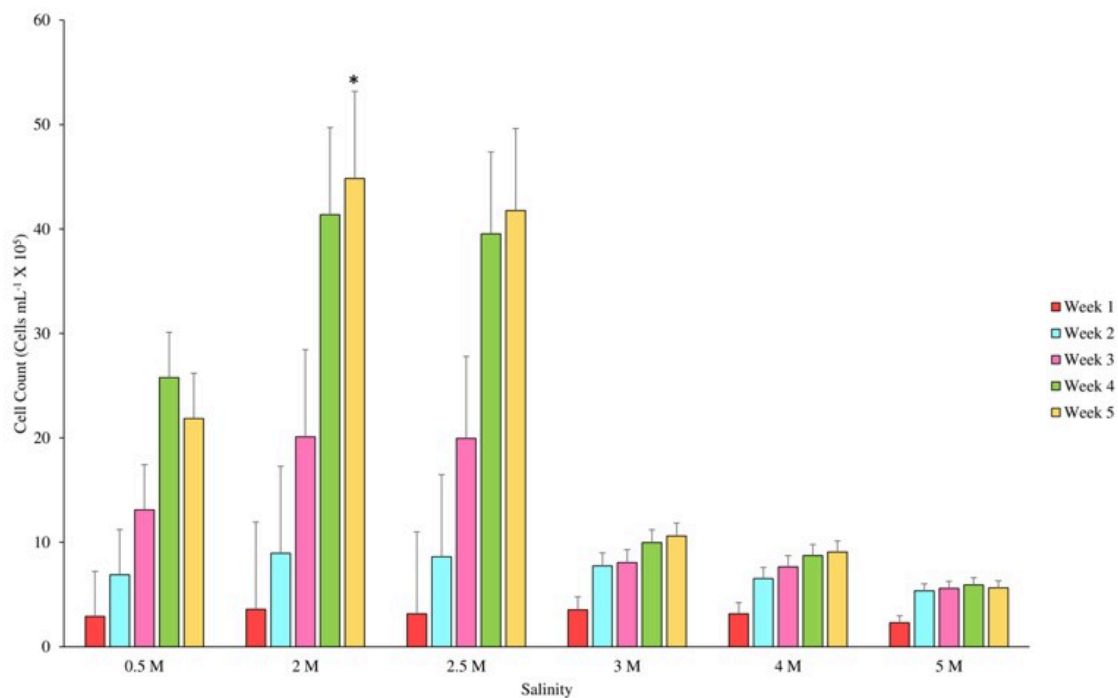


Figure 2

Cell count of *D. salina* ($\times 10^5$) cells mL^{-1} cultured at different salinity concentrations. Values represent Mean $\pm$ Standard Deviation ($*p < 0.015$)

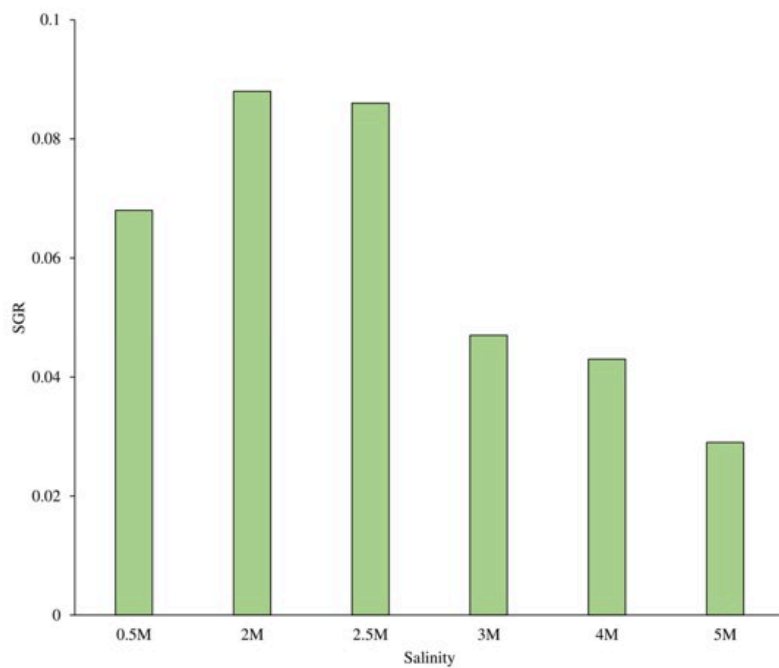


Figure 3

Specific growth rate ($\mu \text{ day}^{-1}$) of *D. salina* at different salinity concentrations

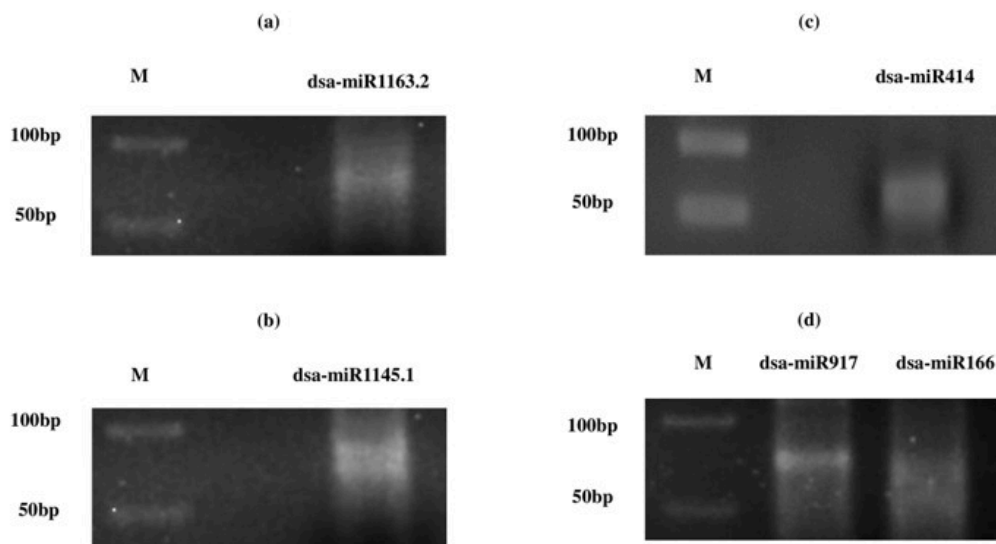


Figure 4

Expression validation of known miRNAs in *D. salina* isolated from Sambhar Lake, India. a) dsa-miR1163.2; b) dsa-miR1145.1; c) dsa-miR414 and d) dsa-miR917 & dsa-miR166. M- 50 bp ladder

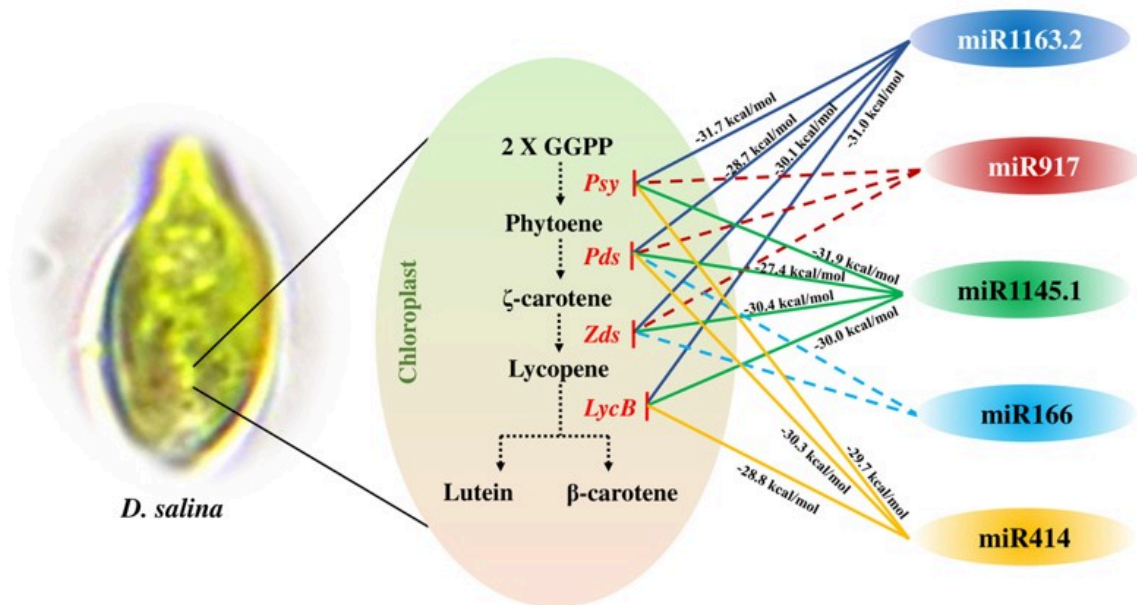


Figure 5

Identified miRNAs in *D. salina* targeting *Crt* genes involved in the carotenoid synthesis pathway. The energy values refer to the most stable miRNA-target gene configurations analyzed through RNAHybrid. The dotted lines represent miRNA-target gene configurations with MFEs > -25 kcal mol⁻¹

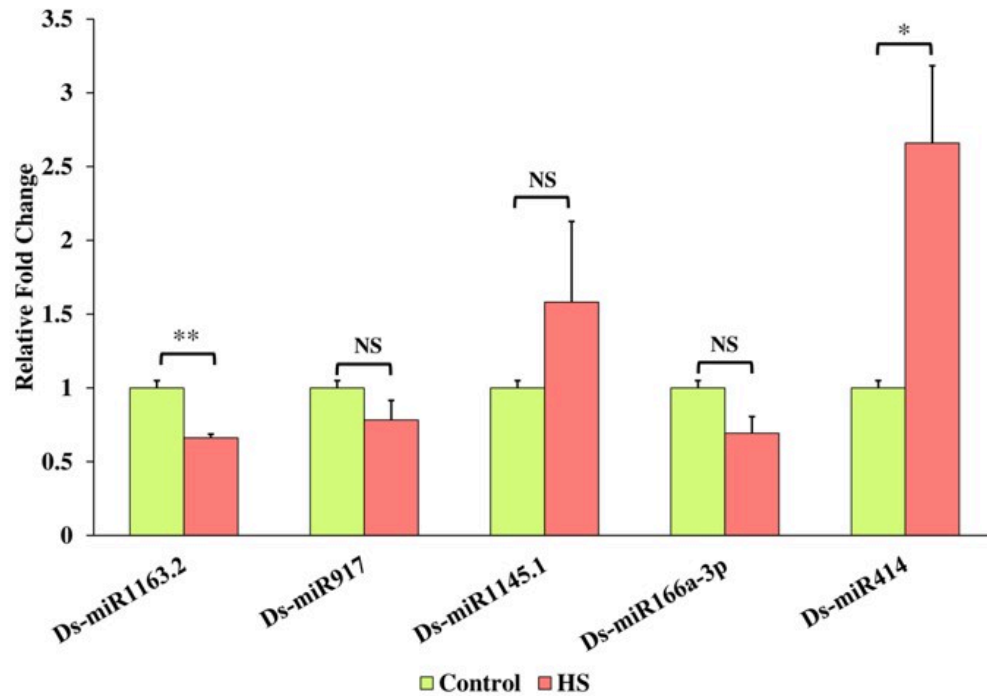


Figure 6

qPCR analysis of dsa-miR1163.2, dsa-miR917, dsa-miR1145.1, dsa-miR166a3p and dsa-miR414 under control and hypersalinity conditions. Error bars indicate standard error (SE). Students's t-test was performed with P-value > 0.05 marked as "NS", 0.05 < P < 0.01 marked as "*" and P < 0.01 marked as "**"

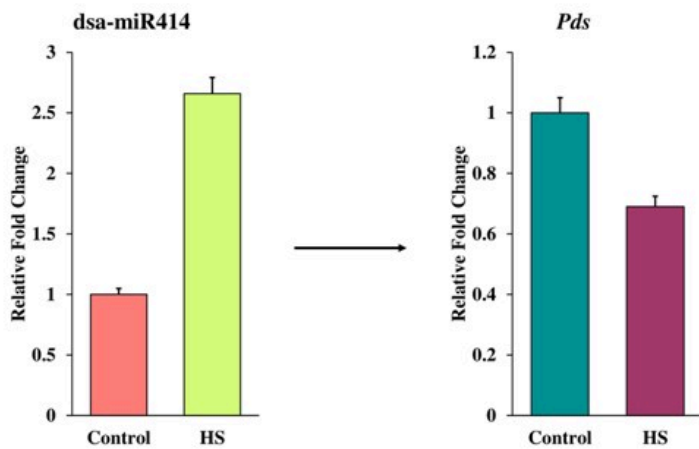


Figure 7

qPCR analysis of dsa-miR414 and its putative target gene *Pds* under control and hypersalinity conditions

Supplementary Files

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