Regulation of *Dunaliella salina* Malate Dehydrogenase Gene Expression by Interfering Ribonucleotides

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Received: 2025-02-12 Accepted: 2025-05-10

Abstract

The microalga *Dunaliella salina* is one of the most resilient organisms adapted to harsh environments. Research indicates that the organisms, especially plants, respond to various environmental stresses differently. *D. salina* has emerged as a halotolerant model organism for studying stress adaptation due to its ability to thrive under extreme salinity, light, and nutrient-deficient conditions. It produces a vital carotenoid, 9-cis beta-carotene, which is utilized in medical industry. One of the significant interferences in stress responses is mediated by 21-24 nucleotide interfering RNAs. Malate dehydrogenase is a key enzyme involved in energy metabolism in both mitochondria and chloroplasts, and its transcription and activity regulation are highly significant. This study investigated the number of miRNA binding sites to the malate dehydrogenase transcript. The involvement of some miRNAs, including novel-m0533-3p, in energy-related metabolism has been identified. The results showed that the mitochondrial transcript had 5 binding sites and the chloroplast transcript had 1 binding site for novel-m0533-3p miRNA. The low number of miRNA binding sites to the chloroplast malate dehydrogenase mRNA sequence indicates that perhaps other gene expression regulation methods control the chloroplast malate dehydrogenase gene or probably, Chloroplastic Malat Dehydrogenase is regulated by enzyme activity, and also the 5 point of binding sites of the miRNA to the mitochondrial malate dehydrogenase mRNA, indicates that this type of gene expression regulation is more dominant. Our results suggest that miRNAs act as dynamic regulators that modulate MDH expression in a stress-type-dependent manner. These findings align with previous studies emphasizing post-transcriptional regulation as a key mechanism for microalgae adaptation to harsh environments.

Keywords: *Dunaliella salina*, Malate Dehydrogenase, Interfering Ribonucleotide, Gene regulation, Transcription

Introduction

Dunaliella salina, a halotolerant microalga, has emerged as a model organism for studying stress adaptation due to its ability to thrive under extreme salinity, light, and nutrient-deficient conditions. This resilience

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is closely linked to its capacity for high-value metabolite biosynthesis, particularly 9-cis beta-carotene, a carotenoid of significant medical and industrial interest (Zarandi-Miandoab et al., 2019). Environmental stressors such as variation in light intensity, salinity fluctuations, and nitrogen deprivation trigger complex molecular responses in *D. salina*. These responses encompass differential gene expression and metabolic rewiring to prioritize protective compounds like beta-carotene over fatty acids (Zarandi-Miandoab et al., 2015; Barczak-Brzyżek et al., 2022).

Gene expression regulation (a process determining the type and quantity of proteins) plays a pivotal role in stress adaptation (Ni et al., 2009). Various methods of gene expression regulation are used to balance the concentration of enzyme proteins, and various other methods are used to regulate the activity of said enzyme proteins (Lackner and Bähler, 2008). In conditions of environmental stress, it is crucial to regulate and control the energy status of the cell (Kansal et al., 2021). Malate dehydrogenase is a key enzyme that plays a significant rolein energy metabolism and the production of nicotinamide adenine dinucleotide (NADH); thus, the regulation of its transcription and activity is vital. The enzyme malate dehydrogenase (MDH) is important in metabolic cycles occurring in two types of cellular organelles, mitochondria and chloroplasts (Xiao et al., 2018). Mitochondrial MDH (mMDH) is integral to the tricarboxylic acid (TCA) cycle, driving ATP production and maintaining the $NADH/NAD^+$ balance, while chloroplastic MDH (cMDH) facilitates carbon fixation and photoprotection (Ermakova et al., 2024; Fabian et al., 2009). Recent studies highlight the role of small non-coding RNAs, particularly microRNAs (miRNAs), in the post-transcriptional regulation of stress-responsive genes, including MDH isoforms (Fang and Rajewsky, 2011; Hurschler et al., 2010). For instance, miRNAs can silence target mRNAs via sequence complementarity, influencing metabolic pathways critical for stress survival (Infantino et al., 2021). However, the mechanisms underlying miRNA-MDH interactions in *D. salina* remain poorly characterized. This study investigates how miRNA-mediated regulation of MDH isoforms contributes to the alga's stress adaptability, with implications for biotechnological applications

Material and methods

Gene sequences were retrieved from the NCBI database, including mitochondrial (KT001001.1, KT001002.1) and chloroplastic MDH isoforms (AF522057.1, EU352600.1, EU352601.1). Putative miRNA binding sites were predicted using miRBase (v22) and MirGeneDB, followed by in silico interaction analysis with TargetScan and miRWalk under stringent criteria: minimum free energy (MFE $\leq$ -15 kcal/mol), seed region complementarity ($\geq$6 nucleotides), and evolutionary conservation across algal species. RNAhybrid, IntaRNA, and RNAfold were employed to validate miRNA-mRNA interactions, focusing on thermodynamic stability and structural accessibility. For physiological validation, VARNAv3.9 was utilized to model RNA secondary structures and assess the binding feasibility of

novel-m0533-3p under stress-mimicked conditions. This tool confirmed robust interactions between novel-m0533-3p and chloroplasts MDH transcripts, highlighting sequence-specific binding at conserved motifs within the 3'UTR, which likely modulates post-transcriptional repression under environmental stress.

Results and Discussion

The differential Targeting of miRNA on MDH Isoforms for *D. salina* malate dehydrogenase as identified in the NCBI database,) illustrated that mitochondrial MDH transcripts exhibited 44 predicted miRNA binding sites,whereas fewer sites were present in chloroplastic MDH (Table 1).

Four transcripts detailing the characteristics of *D. salina* malate dehydrogenase are presented in Table 1. As can be seen in the Table, the first row corresponds to the mitochondrial enzyme (KT001001.1), which has the capacity to bind and interact with miRNA at 5 distinct points. The second, third, fourth rows pertain to the chloroplast enzymes, whose genes reside in the nucleus, while the resulting translation product is located in the chloroplast, contributing to the stroma and the Calvin cycle. All three transcripts of the chloroplast malate dehydrogenase gene (AF522057.1, EU352600.1, EU352601.1) can interact with miRNA at only a single point. The estimate of the number of points that can potentially bind to m0533-3p miRNA acts as a confirmation that interfering nucleotide affects the transcript of the nuclear malate dehydrogenase gene, which may alter the regulation of gene expression. The novel-m0533-3p sequence binds to the RNA of the malate dehydrogenase gene and alters its expression levels during stress (Lou et al., 2020).

The limited number of miRNA binding sites in the mRNA sequence of chloroplast malate dehydrogenase suggests the presence of significant gene expression regulation pathways that control the chloroplast malate dehydrogenase gene. Conversely, the high abundance of the mRNA binding site within the mRNA sequence of mitochondrial malate dehydrogenase implies that this type of gene expression regulation is more dominant than other regulatory mechanisms of gene expression (Afonso-Grunz and Müller, 2015; Fang and Rajewsky, 2011; Wang et al., 2016). miRNA sequences are recognised as significant regulators of gene expression; however, their effects are typically varies based on the specific conditions of the plant and the nature of environmental stress. A study conducted by Brzyżek (2022) showed that miRNAs can affect the expression of certain chloroplast genes, yet their effects are often less than the effect of light (Barczak-Brzyżek et al., 2022).

The regulation of chloroplast malate dehydrogenase activity appears to be largely influenced by redox regulation via the thioredoxin system and is influenced by light (Yoshida et al., 2015; Miginiac-Maslow et al., 2000). It seems logical that the need for regulation of the function of such a key enzyme would require a high speed of action, mediated by light and at the post-translational level in the stroma. Certainly, regulation at the transcriptional level for a nuclear gene whose product is to function in the chloroplast requires more time. Generally, the

Table 1. Activity of gene characteristics, malate dehydrogenase transcripts, and number of predicted miRNA sites and m0533-3p miRNA binding sites in *D. salina*

Gene (Transcript ID)/ Organelle	mRNA	Predicted miRNA Sites	Number of adhesion points	miRNA novel-m0533-3p
Dunaltella salina putative Malate Dehydrogenase (MDH) gene, complete cds GenBank: KU001001.1 LOCUS KT001001 4445 bp DNA **353aa** linear PLN 31-OCT-2016 >ALY05463.1 putative malate dehydrogenase MLAYKRVAGSAAAQTSRGPSSRVTGLSPRVSRR KIVKCEAKKVALLGAGGGIGQPLSLLLKMNKLV TELSLYDIAGVTGVGADISHCNTPVKVSAFNGPE ELEGALKGAELIIIPAGVPRKPGMTRDDLFNVNA GIVKALVEAASKHCPEAIIQLVTNPVNSTVPIAAE VMKKAGTHNPAKIMGVSTLDVVRANTFVAEAK KLDVKDVDVPVVGGHAGATILPLLSQATPPVSFT DAEKKMMTEKIQNAGTVVVEAKAGKGSATLSM AYAAARMAESTLMGLNGEPNIYECSYVQSDVVP DCPFFASKLLLGPGGVAKVLPLGNMDAFEQACF DAMLPELKASIQKGIDFANA Mitochondria	[illegible]	44	5 point	UCCUGGACGCCCGGACUAUCAU
Dunaltella salina putative malate dehydrogenase (MDH) mRNA, complete cds. KT001002, VERSION KT001002.1 **352 aa** protein_id="ALY05464.1" >MLAYKRVAGSAAAQTSRGPSSRVTGLSPRVSR RKIVKCEAKKVALLGAGGGIGQPLSLLLKMNKL VTELSLYDIAGVTGVGADISHCNTPVKVSAFNGP EEEGALKGAELIIIPAGVPRKPGMTRDDLFNVNA GIVKALVEAASKHCPEAIIQLVTNPVNSTVPIAAE VMKKAGTHNPAKIMGVSTLDVVRANTFVAEAK KLDVKDVDVPVVGGHAGATILPLLSQATPPVSFT DAEKKMMTEKIQNAGTVVVEAKAGKGSATLSM AYAAARMAESTLMGLNGEPNIYECSYVQSDVVP DCPFFASKLLLGPGGVAKVLPLGNMDAFEQACF HAMLPELKASTQKGIDFANA Mitochondria	[illegible]	36	2 point	UCCUGGACGCCCGGACUA UCAU
Dunaltella salina cultivar CCAP 19/30 chloroplast Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: EU352601.1 LOCUS EU352601 **429aa** 1288 bp mRNA linear PLN 15-JAN-2008 >ABY61961.1 chloroplast malate dehydrogenase, partial MQLNLQKNVGLAQKQATVTPAFNVRGNVARKV QAGSNAAARRASSVVRAVDAPEKAAKQFGVFR LSYDVNNEDKEMMKNWKKTINV AVTGASGMIANHLLFMLASGEVYGKDQPIALQL LGSERSYEALEGVAMELEDSLYPLLREVSIGIDPY EIFAGADWALMVGAQPRGPGMERSDLIQKNGQI FQTQGRALNEVADRNCKVVVVGNPCCTNAFIA MENAPNLPRKNFHALTRLDENRAKSQLAMKAG KFYTSVSRVAVWGNHSTTQVPDFLNAKIGGLPA MNVIRDNQWFKDQFTPKVAMRGGALIKKWGRS SAASTAVSVADSIRSLITPTPPGDCFSSGVCSDGN LYGIQEGLNFSFPCRSKGDGDYEICNDFIIDDWLR MKIKAAEEELIQERDCVSHLIGREGGACAIGPNTP DTSVPGEK Chloroplast	[illegible]	44	1 point	UCCUGGACGCCCGGACUAUCAU
Dunaltella salina cultivar CCAP 19/25 chloroplast Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: EU352600.1 EU352600 **434aa** 1303 bp mRNA linear PLN 15-JAN-2008 >ABY61960.1 chloroplast malate dehydrogenase, partial MQLNLQKNVGLAQKQATAPSPAFSVRGNAVRS KLSAQLGSKAGFTRRAPVARAVAEAEDKASKQF GVFRLSYDVSNEDKEVMKNWKKTINVAVTGAS GTIANHLLFMLASGEVYGKDQPISLHLLGSERSY EALEGVAMELEDSLYPLLREVSIGIDPYEIFAGAD WALMVGAQPRGPGMERSDLIQKNGQIFQVQGR ALNEVADRNCKVIVVGNPCCTNALIAMENAPNL PRKNFHALTRLDENRAKSQLALKAGKFYTSVSR VAIWGNHSTTQVPDFLNAKIGNLPAMNVIRDNQ WFKEHFTPKVAMRGGALIKKWGRSSAASTAVS VADSIRSLITPTPPGDCFSSGVISDGNLYGIQEGLN FSFPCRSKGDGDYEICNDFIIDDWLRMKIKAAED ELIQERDCVSHLIGREGGACAIGPNTPDTSVPGEK Chloroplast	[illegible]	35	1 point	UCCUGGACGCCCGGACUAUCAU
Dunaliella salina Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: AF522057.1 LOCUS AF522057_1 **230 aa** >AAM83089.1 malate dehydrogenase, partial DRNCKVIVVGNPCCTNALIAMENAPNLPRKNFN ALTRLDENRAKSQLALKAGKFYTSVNRVAVWG NHSTTQVPDFLNAKIGGLPAMNVIRDNQWFKEE FTPKVALRGGALIKKWGRSSAASTAVSVADSIRS LITPTPPGDCFSSGVCSDGNLYGIQEGLNFSFPCR SKGDGDYEICNDFIIDDWLRMKIKAAEEELIQER DCVSHLIGREGGACQIGPGAPDTSVPGEN Chloroplast	[illegible]	34	1 point	UCCUGGACGCCCGG ACUAUCAU

differences in the expression of these two genes are related to the type of metabolism, environmental conditions, and the specific requirements of cells in response to both internal and external factors (Schwartzbach, 2017). This difference in expression allows cells to respond more effectively to their metabolic and energy demands. The higher miRNA targeting of mitochondrial MDH may reflect its central role in energy production and consumption during stress, requiring precise regulation to balance ATP production and redox homeostasis. In contrast, chloroplastic MDH suppression under stress (e.g., via novel-m0533-3p) could prioritize photoprotective carotenoid synthesis over carbon fixation, aligning with *D. salina's* stress-response strategy (Li et al., 2024).

The biological Implications of this variation in interaction with miRNA may be associated with the duration required to react and respond to environmental conditions. It appears that in response to environmental stimuli such as light, the chloroplast must adapt its energy state very quickly, which is why it employs the thioredoxin system to regulate MDH enzyme activity. However, to manage the energy state of the cell under various conditions, the cell nucleus has enough time to regulate at the transcriptional level through the intervention of the miRNA. This variation in performance leads to the dual regulatory role of miRNAs. A single miRNA can upregulate or downregulate gene expression, depending on the cellular context (Fabian et al., 2009). There is an emphasis on multi-miRNA targeting of miRNAs. Multiple miRNAs can target a single gene, and their combined activity determines the expression of a given gene. miRNA can act through binding to the 3'UTR of target mRNA (Fang and Rajewsky, 2011), mRNA Degradation, Translation Inhibition (Afonso-Grunz and Müller, 2015), Nascent protein degradation, mRNA storage in P-bodies (Fabian et al., 2009; Hurschler et al., 2010), and transcription inhibition (Fabian et al., 2009).

The findings of this study highlight the critical role of miRNAs in regulating the expression of mitochondrial and chloroplastic malate dehydrogenase (MDH) genes in the microalga *D. salina* under various environmental stresses. Validation using three bioinformatics tools, RNAhybrid, IntaRNA, and RNAfold, demonstrated that mitochondrial MDH exhibits significantly stronger constructive regulatory interactions compared to its chloroplastic counterpart, suggesting distinct miRNA-mediated post-transcriptional control mechanisms between the two organelles. Our results indicate that miRNAs act as dynamic regulators that modulate MDH expression in a stress-type-dependent manner. These findings align with previous studies highlighting posttranscriptional regulation as a crucial mechanism for microalgal adaptation to extreme environments (Wang et al., 2016).

A key discovery was the identification of novel-m0533-3p, which selectively binds to all three chloroplastic MDH transcripts (AF522057.1, EU352600.1, EU352601.1) and suppresses their expression (Li et al., 2023). The specificity of this miRNA for chloroplastic MDH suggests a compartmentalized regulatory strategy that may prioritize mitochondrial energy metabo-

lism during stress (Huang et al., 2018). Such compartmentalization is consistent with plant studies in which miRNAs regulate organelle functions to maintain cellular homeostasis (Nalawade and Singh, 2023). Notably, the mitochondrial MDH sequence (KT001001.1) showed minimal interaction with this miRNA, supporting the hypothesis of preferential activation of mitochondrial pathways for ATP production under stress. This mechanism enables stable β-carotene synthesis and osmotic balance.

The dual regulatory roles of miRNAs, both upregulating and downregulating gene expression, were evident in this study. For example, under hypoxic conditions, miR-206-like sequences likely enhance mMDH expression by binding to the 5'UTR region, a mechanism previously observed in animal systems (Rao et al., 2016). While speculative, this hypothesis could explain enhanced mitochondrial ATP output during stress, warranting validation via hypoxia-responsive miRNA profiling.

This contrasts with the canonical miRNA-mRNA interaction at the 3'UTR and underscores the tissue-specific nature of miRNA activity. Such functional flexibility may explain how *D. salina* dynamically regulates its metabolic network to balance energy demands, antioxidant production, and carotenoid synthesis under fluctuating environmental conditions.

Our findings reveal a compartment-specific miRNA regulatory network: mitochondrial MDH is spared from miRNA silencing, ensuring sustained energy production. At the same time, chloroplastic MDH is downregulated to redirect resources toward beta-carotene synthesis. This aligns with studies in Arabidopsis, where miRNAs fine-tune organellar functions under stress (Lou et al., 2020). For example, novel-m0533-3p-mediated cMDH suppression mirrors miR398-mediated silencing of Cu/Zn superoxide dismutase in plants under oxidative stress (Martinez-Vaz et al., 2024). The results align with *D. salina's* metabolic prioritization of beta-carotene under stress (Minarik et al., 2002), but reliance on in silico predictions introduces false positives. Tissue-specific RNA-binding proteins (RBPs) and alternative polyadenylation may further modulate miRNA accessibility factors unaddressed here. Comparative studies in *Chlamydomonas reinhardtii* could clarify the evolutionary conservation of these regulatory motifs (Musrati et al., 1998).

Conclusion

Gene expression regulation in *D. salina* is a complex process influenced by various environmental factors. The regulation of the malate dehydrogenase gene expression has a direct impact on cellular function and energy metabolism. Given the presence of two malate dehydrogenase isozymes in *D. salina*, regulation at different levels during transcription, post-transcription, translation, and post-translational modification and creating a proper balance is crucial. Once understood, these mechanisms go a long way in explaining how this small organism survives and resists.

This study elucidates a miRNA-driven regulatory framework enabling *D. salina* to balance energy metabolism and stress adaptation.

Significant findings highlight the specific targeting of MDH isoforms by miRNAs in different compartments. Notably, novel-m0533-3p acts as a suppressor of chloroplastic MDH during stress conditions, while mitochondrial MDH is emphasized for maintaining energy homeostasis.

Future studies should focus on integrating multi-omics approaches (e.g., transcriptomics and proteomics) to comprehensively map miRNA-MDH interactions. Techniques like CLIP-Seq can elucidate tissue-specific miRNA targeting, whereas CRISPR interference (CRISPRi) may reveal causal relationships between specific miRNAs and stress phenotypes. In addition, comparative analyses across microalga species identified conserved miRNA regulatory motifs, providing insights into universal stress adaptation mechanisms. These insights advance microalgal biotechnology, offering strategies to engineer high-beta-carotene strains resilient to environmental stressors. The carotenoids and other secondary metabolites produced under harsh living conditions in *D. salina* are useful and effective in metabolic diseases and cancer therapy.

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