



Article

The Impact of Mitochondrial DNA Depletion on Mitochondrial Ultrastructure, Photosynthesis, and the mTERF Gene Family in *Chlamydomonas reinhardtii*

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Abstract

Mitochondrial biogenesis requires coordinated expression from both nuclear and mitochondrial genomes. To understand the consequences of mitochondrial genome loss, we generated a mitochondrial DNA-depleted line (*crm*⁻) in *Chlamydomonas reinhardtii* via long-term ethidium bromide treatment. We then examined how mtDNA disruption affects mitochondrial ultrastructure, chloroplast function, and the mitochondrial transcription termination factor (mTERF) gene family. Our results reveal that mitochondrial dysfunction is associated with severe organelle remodeling, including mitochondrial elongation, matrix condensation, and cristae collapse. Consequently, mitochondria reduce the electron sink capacity which appears to over-reduce the chloroplast electron transport chain, correlating with causing damage to photosystem II (PSII), as indicated by higher plastoquinone PQ redox state and PSII excitation pressure and lower non-photochemical quantum yield [Y(NPQ)]. Furthermore, we identified and characterized eight nuclear-encoded *mTERF* genes in *C. reinhardtii* (CrmTERFs). Phylogenetic analysis grouped them into three clades with potential functional conservation. Collinearity analysis suggested potential evolutionary relationships between mTERF genes in *Chlamydomonas* and *Marchantia polymorpha*. Gene ontology annotation linked CrmTERFs to transcription termination and RNA biosynthesis regulation. Additionally, in silico prediction identified twelve putative miRNAs targeting seven of the eight CrmTERFs, with CrmTERF3 as the only exception, providing candidates for future experimental validation. This study provides the first comprehensive analysis of the nuclear encoded *mTERF* gene family in *Chlamydomonas* and demonstrates that mtDNA loss is correlated with mTERF genes expression, as well as mitochondrial structure and chloroplast photoprotective impairments. These findings suggest a potential role for CrmTERFs in mitochondrial retrograde signaling and organellar crosstalk, though functional validation is required to establish causality.

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1. Introduction

In eukaryotic cells, genetic information is shared between the nucleus and organellar genomes such as chloroplasts and mitochondria. However, with the passage of time, most organellar genomes have become shorter over time, mainly through gene loss during their evolution from their bacterial ancestors or mass transfer of genetic material to the nuclear genome [1–3]. Consequently, only a limited number of genes associated with electron transport chains, photosynthesis and respiration persist in organellar genomes and the expression of the remaining organellar functions, largely dependent on nucleus-encoded proteins [4,5]. Among these are the mitochondrial transcription termination factor (*mTERF*), a family characterized by a conserved *mTERF* motif, encoded by a nuclear gene [6]. This protein functions in mitochondria and is known to regulate mitochondrial gene expression by binding nucleic acids to carry out replication, transcription, and translation.

The *mTERF* gene family, characterized by a unique and conserved protein motif of approximately 30–32 amino acids that often includes three leucine zipper-like structures, is central to organellar gene regulation [7,8]. Initially identified in humans (*Homo sapiens*), where *mTERF1* was shown to bind a specific 28-nucleotide sequence to terminate transcription of the mitochondrial 16S rRNA gene, research has since revealed a family of proteins with diverse and critical functions [9,10]. A previous study suggested that *mTERF* proteins may play multiple roles in intracellular regulation. In metazoans, the knockout of *mTERF2* [11] and tissue-specific inactivation of *mTERF3* [12] in mice resulted in severe mitochondrial abnormalities arising from aberrant transcription of the organellar genome, potentially at the stage of transcription initiation. However, plant *mTERFs* have been implicated in a wide array of processes, from organellar transcription and RNA processing to plant groCKh and stress responses. For instance, in *Arabidopsis*, the loss of *mTERF5* alters Absciscic Acid (ABA) sensitivity and salt tolerance, while the mitochondrial *mTERF18* is crucial for heat tolerance [13,14]. The *SLmTERF13* gene was reported to regulate the abiotic stress response in tomato [15]. Differential expression patterns were observed in the grape *mTERF* gene in response to various biotic stressors and phytohormone treatments [16]. In addition to mammals and higher plants, a nuclear *mTERF*-like gene of *Chlamydomonas reinhardtii* (*MOC1*) has been identified, which contributes to the regulation of mitochondrial transcription in response to light variations [17].

The importance of *mTERF* genes in various species and their functions in controlling mitochondrial gene expression have drawn a lot of interest. [18]. Bioinformatic analysis have revealed that plants and metazoan share a huge and complicated family of *mTERF* genes [19]. There are at least 35 *mTERFs* identified in *Arabidopsis thaliana* [14], capsicum (*Capsicum annuum* L.) [20], 31 potential *mTERF* genes in maize (*Zea mays* L.) [21], 25 *mTERF* genes in grape [16] and 28 *mTERF* [15] genes in the tomato genome. Clearly, these studies have demonstrated the importance of the identification of genome-wide *mTERF* genes to study their role in plant groCKh and stress response.

A commonly used approach to study the nuclear response to impaired mitochondrial function is the use of ρ^0 (ρ^0) cell lines. These cells are depleted of mitochondrial DNA (mtDNA) through long-term treatment with low, non-lethal concentrations of ethidium bromide (EthBr). For instance, Affymetrix technology was employed to study profile changes in nuclear gene expression of Nawalma cells resulting from the absence of mtDNA [22]. Similarly, radioactive microarrays were employed to compare the transcriptome of a human breast cancer cell line and its ρ^0 derivative [23]. More recently, mtDNA-

depleted cells (*crm⁻* cells) in *Chlamydomonas* were used to study the comparison of retrograde signaling with Antimycin A-inhibited cells [24]. This approach can be used to investigate the nuclear mTERF genes' response to the impairment of mitochondrial function, as well as their impact on mitochondria and chloroplast.

This study directly investigates the effects of Ethidium Bromide (EB)-induced mitochondrial DNA (mtDNA) depletion on three interconnected levels in *Chlamydomonas*: organelle structure, cellular physiology, and nuclear gene regulation. We hypothesize that the loss of mtDNA will initiate a cascading failure that (1) severely disrupts mitochondrial ultrastructure (e.g., cristae integrity), which in turn (2) impairs chloroplast photoprotection and compromises photosynthetic efficiency due to a breakdown in inter-organellar energy and signaling homeostasis. Furthermore, given their primary role in mitochondrial gene expression, we hypothesize that (3) the nuclear-encoded mTERF gene family will undergo significant transcriptional and/or post-transcriptional reprogramming in response to the loss of its functional target, since they play primary roles in regulating mitochondrial transcription, replication, and translation. We predict this response will not be a simple, uniform downregulation but a selective adaptation, potentially indicative of a stress-induced re-wiring of organelle-targeted genetic programs, positioning *mTERFs* as potential regulatory nodes in the retrograde signaling network that maintains mitochondrial–chloroplast energy balance under stress.

2. Results

2.1. EB Treatment to Eliminate mtDNA from *Chlamydomonas reinhardtii*

The use of ethidium bromide (EB) to generate mtDNA-depleted (ρ^0) lines is a long-established methodology in mitochondrial research, having been successfully applied in diverse eukaryotic systems including yeast, mammalian cells, and algae to study the nuclear response to mitochondrial dysfunction [25–28]. In *Chlamydomonas reinhardtii*, the specific elimination of mtDNA by intercalating dyes like acriflavine and ethidium bromide has been rigorously characterized, confirming that the primary effect is mtDNA loss without pervasive, nonspecific nuclear toxicity under the employed conditions [25]. While we acknowledge that high concentrations or prolonged exposure to intercalating agents can have off-target effects, our protocol uses a low, non-lethal concentration (6 μM) and a defined treatment period (five days) based on these established models and our previous study [29], where we generated *crm⁻* cells depleting mtDNA using three different chemicals, including the treatment of cells with ethidium bromide (EB), dideoxydidehydrocytidine (ddC) and acriflavine (AF). In this study we created *crm⁻* cells by treating algae with EB to study the impact of EB on mitochondrial structure, photosynthesis and *mTERF* gene family. Quantitative PCR confirmed that a five-day treatment with 6 μM EB reduced the mtDNA copy number from approximately 4500 copies in wild-type cells to fewer than 10 copies, representing a >99.9% depletion (Figure 1). Thus, the EB treatment protocol significantly produced cells with near-complete mtDNA loss, enabling the subsequent functional analyses (Figure 1).

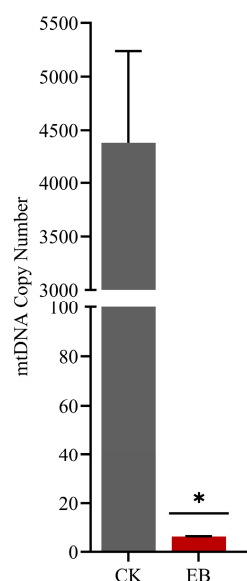


Figure 1. The mtDNA copy number levels of the EB treatment and control determined through qPCR. Data are presented as mean $\pm$ SD ($n = 3$), * $p < 0.05$ indicates significant difference with CK/EB as determined by Student's t -test. The mean and SD values were derived from three biological repetitions.

2.2. Effect of mtDNA Elimination on Mitochondrial Structure and Ultimately on Photosynthesis

Transmission electron microscopy (TEM) was employed to examine the alterations in the subcellular ultrastructure of EB-treated cells compared with CK (Figure 2). The mitochondria of EB-treated cells or *crm⁻* cells seem to have abnormal morphology with elongated size, condensed matrix and collapsed cristae.

In mitochondria, the transfer of electrons from the substrates NADH and succinate is catalyzed by the enzymatic complexes I, II, III, and IV, with molecular oxygen being the final acceptor. The mitochondrial genome comprises seven protein-coding genes of the respiratory chain (*nad1*, *nad2*, *nad4*, *nad5*, *nad6*, *cob*, and *cox1*) and the partial elimination of mtDNA affects the expression of these genes. These genes belong to Complex I, Complex III and Complex IV of the mitochondrial electron transport chain (mETC). This reduction in mETC activity is predicted to diminish the mitochondrial electron sink capacity, which is known to be critical for preventing the over-reduction of the chloroplast electron transport chain (cETC) under illumination. To confirm whether this disruption in the mitochondrial electron flow directly impacts chloroplast function, we compared photosynthetic parameters of CK and EB-treated cells across a range of measurement irradiances (0–1162 PPFD). In control, the effective photochemical quantum yield of PSII [Y(II)] declined with increasing irradiance and was similar to the trend for EB treatment but lower than CK from 33 to 365 PPFD (Figure 3A). In CK, the regulated non-photochemical quantum yield of PSII [Y(NPQ)] increased with irradiance and showed a similar trend to EB but lower all the way along the irradiance (Figure 3B). The PSII excitation pressure [1-qP; the fraction of closed (reduced) PSII reaction centers], the redox state of the Qa pool and the non-regulated non-photochemical quantum yield of PSII [Y(NO)] showed a similar trend to that seen after treatment; they increased with measurement irradiance as compared to CK, but became closer to CK as it reached 621 PPFD (Figure 3C–E). The light response curve of the photosystem II electron transport rate (ETR_{II}) in the wild type increases up to 200 PPFD with irradiance and then keeps falling until 1162 PPFD; moreover, a similar trend was observed for EB but lower as compared to CK.

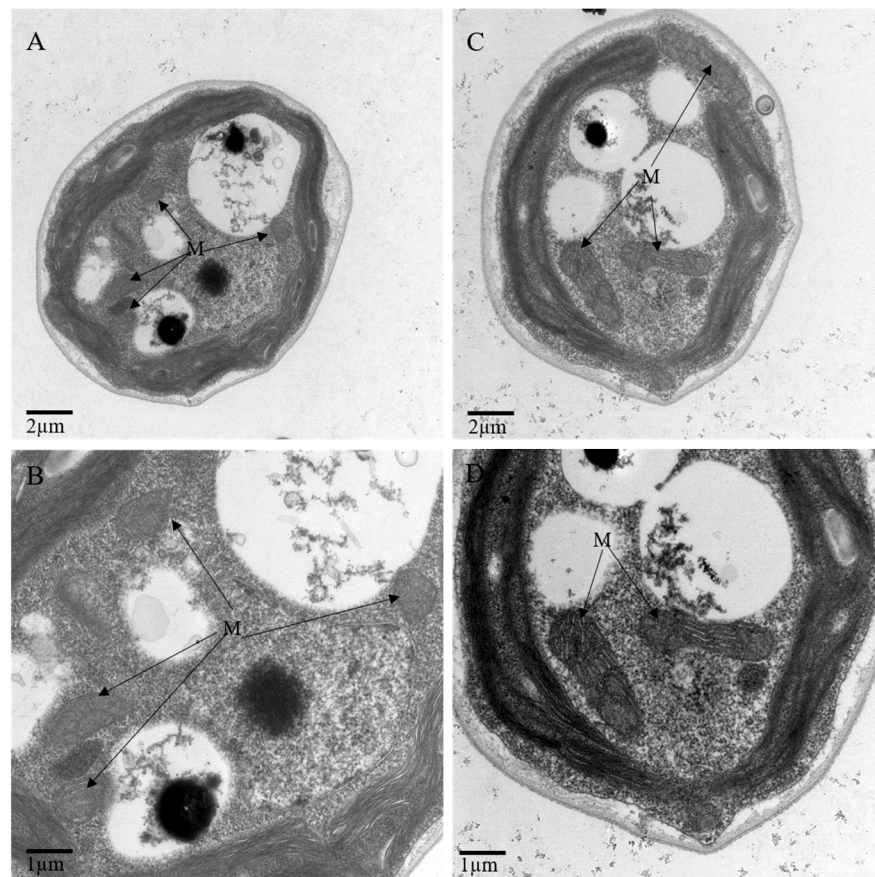


Figure 2. Transmission electron microscopic images of *chlamydomonas* between the CK and the EB-treated cells. (A,B) Solitary mitochondrion (M) in untreated control cell. (C,D) EB-treated cell mitochondria in network with an elongated, electron-dense, condensed matrix and collapsed cristae. M: Mitochondria.

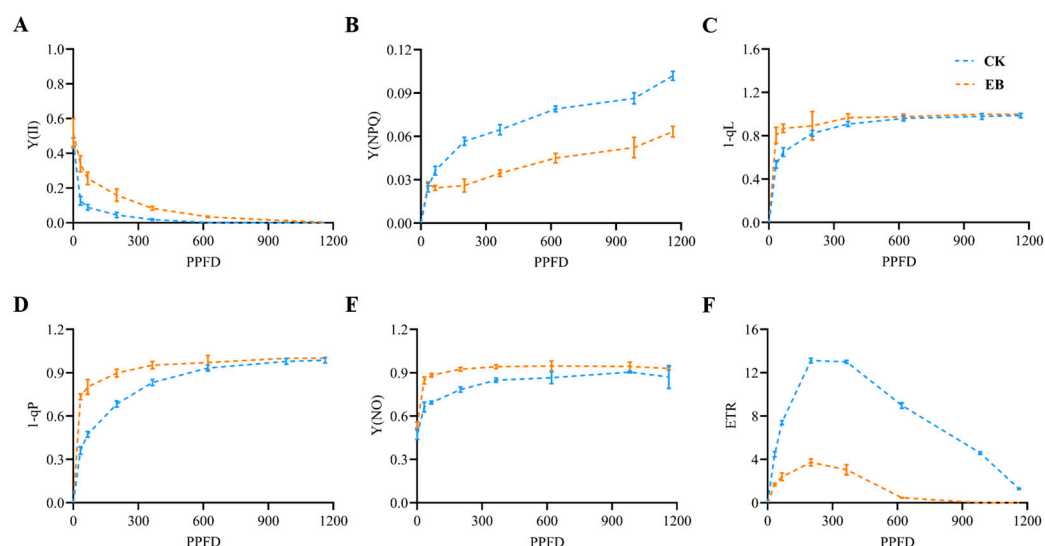


Figure 3. Light response curves of chlorophyll a fluorescence parameters in wild-type (CK, blue dotted line) and EB-treated (orange dotted line) *chlamydomonas* cells: photochemical quantum yield, $Y(II)$ (A); regulated non-photochemical quantum yield, $Y(NPQ)$ (B); redox state of the QA pool, $1-qL$ (C); excitation pressure on PSII, $1-qP$ (D); non-regulated non-photochemical quantum yield, $Y(NO)$ (E); and electron transport rate, ETR (F). Data points show means $\pm$ SD of three biological replicates ($n = 3$); error bars not visible or smaller indicate replicates ($n = 3$) were highly consistent.

Mann–Whitney test was used to predict differences between CK and EB at corresponding photo-synthetic photon flux density (PPFD) levels.

2.3. Identification and Characterization of *CrmTERF* Genes

We identified eight nuclear-encoded *CrmTERF* genes in *C. reinhardtii* (Table 1). Protein length varied considerably, from 212 amino acids (*CrmTERF7*) to 986 amino acids (*CrmTERF5*), with corresponding molecular weights ranging from 22.7 kDa to 102.5 kDa. Most *CrmTERF* proteins were acidic (pI 5.28–6.18), except for *CrmTERF3*, *CrmTERF7*, and *CrmTERF8*, which exhibited basic pI values (>9.0). Hydrophilicity analysis indicated predominantly hydrophilic proteins, though *CrmTERF3*, *CrmTERF4*, and *CrmTERF8* were predicted to be more hydrophobic. The instability index suggested that *CrmTERF7* is stable (26.98), whereas *CrmTERF3* is likely unstable (58.20). Chromosomal mapping revealed an uneven distribution across the genome, with two genes located on chromosome 12 (*CrmTERF3* and *CrmTERF4*) and the others dispersed on chromosomes 3, 6, 9, 10, 14, and 16. All *CrmTERF* genes exhibited Ka/Ks ratios below 1, indicating they have primarily undergone purifying selection during evolution.

Table 1. The characteristics of the eight *CrmTERF* genes identified in *C. reinhardtii*.

Gene Number	Phytozome Identifier	Chromosome	Localization PSL (bp)	PSL (aa)	MW (Da)	Theoretical pI	Hydrophilicity	II	Ka/Ks Value
<i>CrmTERF1</i>	Cre10.g427000	Chr.10:	1,291,940–1,296,761	558	60,009.72	6.06	0.055	38.06	0.9435
<i>CrmTERF2</i>	Cre09.g408051	Chr.9:	5,372,586–5,379,754	635	64,787.65	5.94	0.033	44.32	0.8184
<i>CrmTERF3</i>	Cre12.g542500	Chr.12:	9,285,129–9,286,681	251	27,462.49	10.00	−0.215	58.20	0.8184
<i>CrmTERF4</i>	Cre12.g560750	Chr.12:	6,993,378–6,997,261	618	65,704.57	6.14	−0.021	55.70	0.8448
<i>CrmTERF5</i>	Cre06.g278129	Chr.6:	3,588,777–3,594,398	986	10,2540.78	6.18	0.026	48.07	0.8448
<i>CrmTERF6</i>	Cre03.g155850	Chr.3:	2,004,455–2,010,120	615	63,553.61	5.28	0.333	43.57	0.9688
<i>CrmTERF7</i>	Cre16.g651550	Chr.16:	1,307,348–1,310,194	212	22,706.23	9.36	0.051	26.98	0.9435
<i>CrmTERF8</i>	Cre14.g611200	Chr.10:	499,117–502,070	234	25,718.87	9.46	−0.213	44.67	0.9688

Note: PSL—Protein sequence length, MW—Molecular Weight, II—Instability index.

2.4. Phylogenetic Relation, Gene Structure Analysis and Distributions of Conserved Motifs in *CrmTERFs*

A phylogenetic tree was constructed to explore the evolutionary relationships among the *CrmTERF* proteins, which grouped the eight members into four distinct clades (Figure 4A), reflecting sequence divergence and potential functional specialization. Analysis of exon–intron structures revealed considerable diversity among *CrmTERF* genes (Figure 4B). *CrmTERF1*, *CrmTERF2*, and *CrmTERF5* contained a higher number of exons, consistent with their longer coding sequences, whereas *CrmTERF3*, *CrmTERF7*, and *CrmTERF8* exhibited fewer exons, reflecting their shorter gene lengths. Intron numbers and lengths also varied, suggesting structural divergence within the family.

To investigate conserved protein domains, motif composition analysis identified ten conserved motifs across the *CrmTERF* proteins (Figure 4C). Motif distribution varied notably among members, indicating possible functional diversification. *CrmTERF1* and *CrmTERF4* displayed the highest motif diversity, containing Motifs 1, 2, 3, and 4 in different arrangements. *CrmTERF5* and *CrmTERF8* shared a similar motif architecture (Motifs 4, 5, and 10), suggesting functional similarity, while *CrmTERF7* contained only Motif 1, implying it may represent a more specialized member. Motifs 1 and 4 were the most widely distributed across the family, suggesting they represent core functional domains.

Overall, these structural and motif analyses indicate that *CrmTERF* proteins share conserved domains essential for their function, while variations in motif composition may underlie functional divergence and specialization.

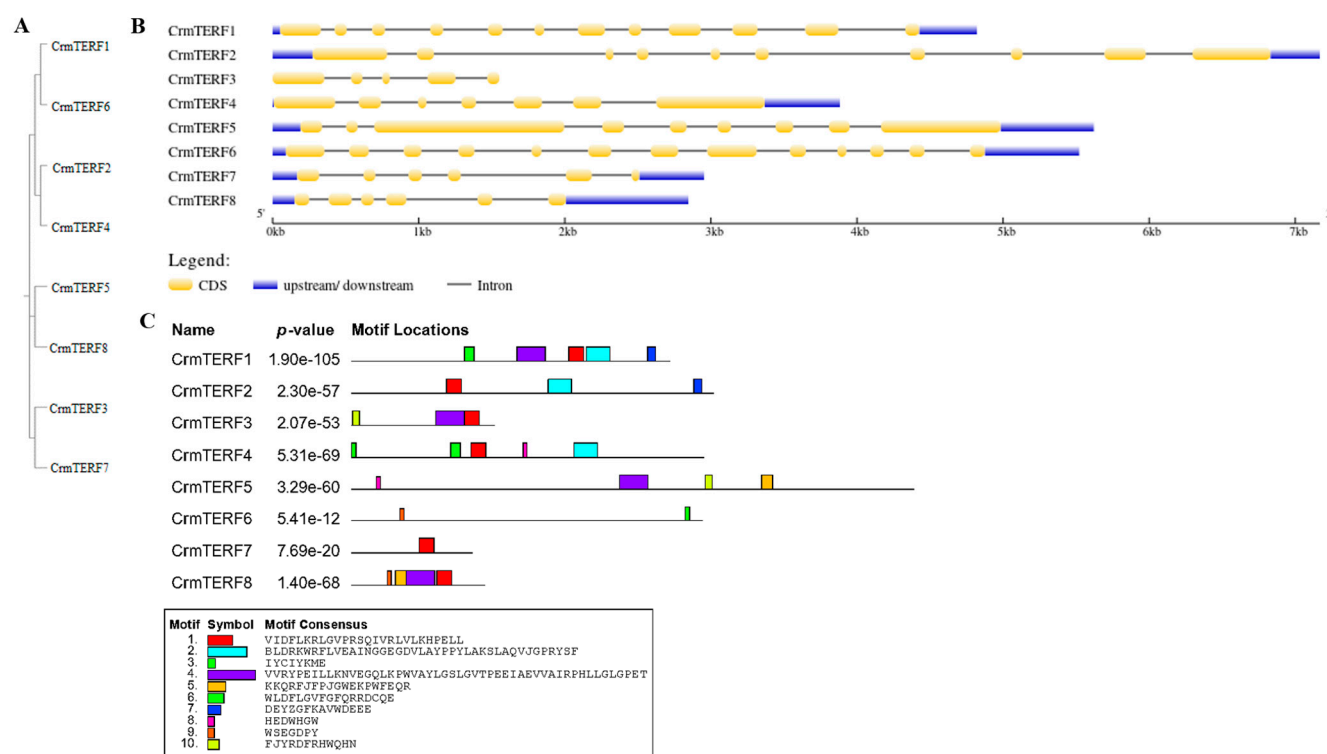


Figure 4. Phylogenetic relationship, gene structure analysis and distribution of conserved motifs of *CrmTERFs*. (A) Phylogenetic tree of 8 *CrmTERFs*. (B) Gene structure for *mTERFs*. The gray horizontal line denotes intron regions, while the yellow horizontal line denotes exon regions. (C) Distributions of conserved motifs in *CrmTERFs*. Ten putative motifs are indicated with different colored boxes.

2.5. Chromosomal Location and 3D Protein Structures

Chromosomal mapping revealed that the eight *CrmTERF* genes are distributed across seven chromosomes in *C. reinhardtii* (Figure 5A). *CrmTERF3* and *CrmTERF4* are both located on chromosome 12, suggesting a possible local duplication event, while the remaining genes are dispersed singly across chromosomes 3, 6, 9, 10, 14, and 16.

Computational 3D structural models were generated to predict the tertiary organization of *CrmTERF* proteins (Figure 5B). All models exhibited well-folded, α -helix-rich structures, consistent with transcription-associated DNA-binding proteins. Despite variations in protein length, the core folding pattern was conserved. The largest protein, *CrmTERF5*, displayed an extended conformation with additional helices and loops, potentially indicating extra functional domains. In contrast, *CrmTERF6*, *CrmTERF7*, and *CrmTERF8* adopted simpler, more compact folds, which may reflect functional specialization. These *in silico* predictions provide a preliminary structural framework suggesting that *CrmTERF* proteins are structurally conserved but exhibit variations that could underlie functional diversification.

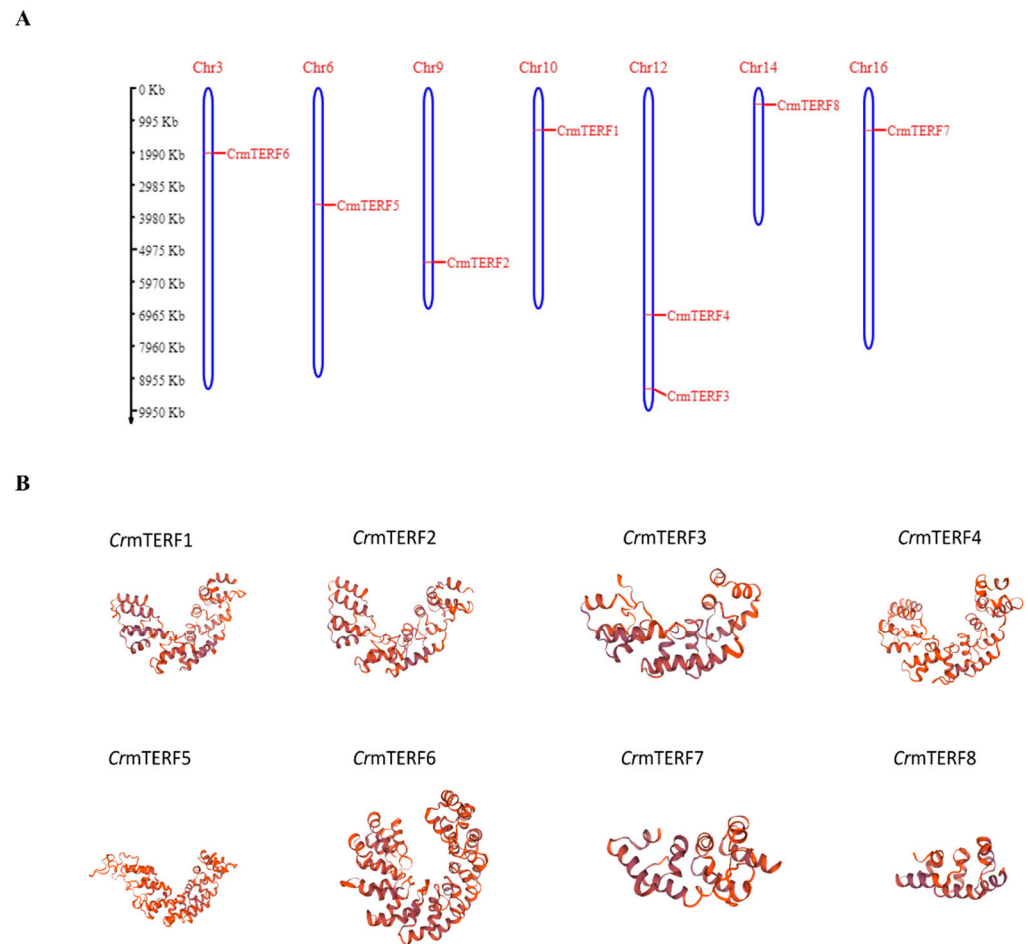


Figure 5. Chromosomal location and 3D structures. **(A)** The *mTERF* gene distribution on seven *Chlamydomonas reinhardtii* chromosomes is shown schematically, along with the gene names in red on the left side. The scale on the left side indicates the location of the *mTERF* genes on chromosomes. The top of each chromosome (Chr) is where you may find the chromosomal numbers. **(B)** *mTERF* family 3D structures displaying functional sites.

2.6. Phylogenetics and Collinearity Analysis

Phylogenetic analysis of mTERF proteins from *C. reinhardtii* and five other species (*Marchantia polymorpha*, *Coccomyxa subellipsoidea*, *Volvox carteri*, *Physcomitrella patens*, and *Arabidopsis thaliana*) placed the proteins into three major clades (Figure 6A). The eight CrmTERF genes were distributed across all three clades—five in the green clade, two in the gray clade, and one in the blue clade—indicating both conservation and diversification within the gene family across lineages.

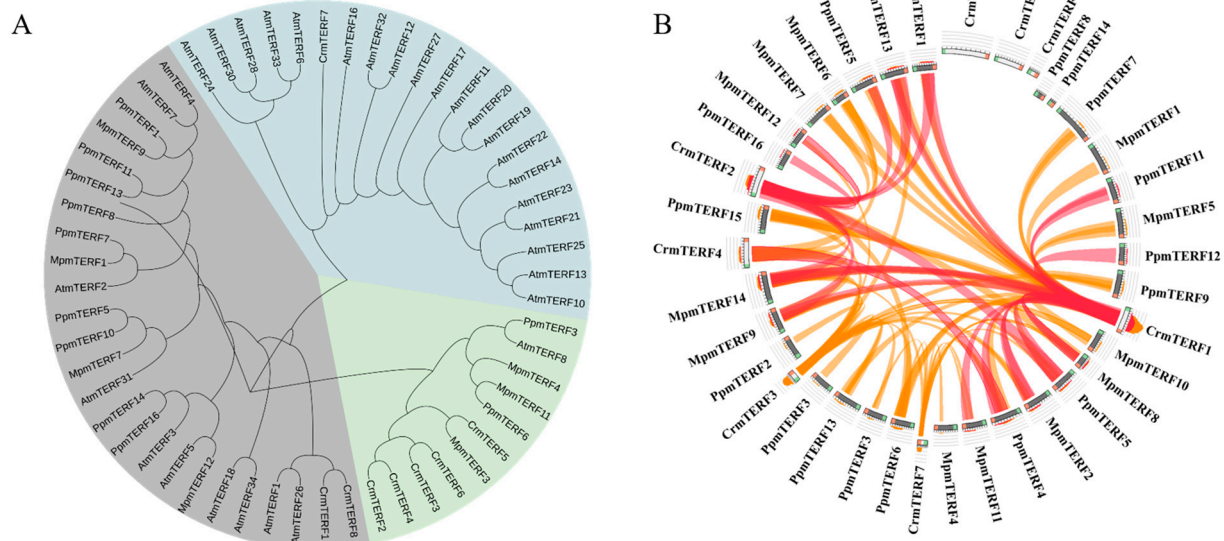


Figure 6. A phylogenetic and collinearity analysis of *mTERF* proteins. **(A)** A phylogenetic analysis of *mTERF* proteins from *Chlamydomonas reinhardtii* (Cr), *Marchantia polymorpha* (Mp), *Coccomyxa subellipsoidea* (Cs), *Volvox carteri* (Vc), *Physcomitrella patens* (Pp) and *Arabidopsis thaliana* (At) was carried out using the maximum likelihood method. There are three groups of *mTERF* proteins which include *CrmTERF*, each of which is represented by a gray, green and blue color. Genes from *Chlamydomonas reinhardtii* (Cr) are highlighted in blue. **(B)** Collinearity analysis of *mTERF* proteins between *Chlamydomonas reinhardtii* (Cr), *Marchantia polymorpha* (Mp), *Volvox carteri* (Vc), *Physcomitrella patens* (Pp) and *Arabidopsis thaliana* (At). The red and orange colors represent $\leq 80\%$ and $\leq 60\%$ identity, respectively.

Collinearity analysis revealed extensive syntenic relationships, with the strongest conservation observed between *C. reinhardtii* and bryophytes (*M. polymorpha* and *P. patens*), suggesting shared ancestral *mTERF* genes (Figure 6B). In contrast, fewer syntenic connections were detected with *A. thaliana*, highlighting the divergence of the *mTERF* family in higher plants. These results indicate that while *mTERF* genes have diversified during evolution, a core set has remained conserved from algae to early land plants.

2.7. Gene Ontology (GO) Annotation and miRNAs Analysis

The GO enrichment analysis primarily linked *CrmTERF* proteins to DNA-templated transcription termination and regulation of RNA biosynthesis (Figure 7A,B), supporting their predicted role in transcriptional control. In addition, 12 putative miRNAs were identified as targeting seven of the eight *CrmTERF* genes (Figure 8). *CrmTERF5* and *CrmTERF6* were each targeted by four miRNAs, indicating particularly tight post-transcriptional regulation. Only *CrmTERF3* lacked predicted miRNA targets. Notably, cre-miR1145.1 targeted both *CrmTERF1* and *CrmTERF5*, suggesting potential coordinated regulation of these genes. These miRNA-*CrmTERF* interactions highlight a layer of potential post-transcriptional control that may fine-tune *CrmTERF* expression under stress.

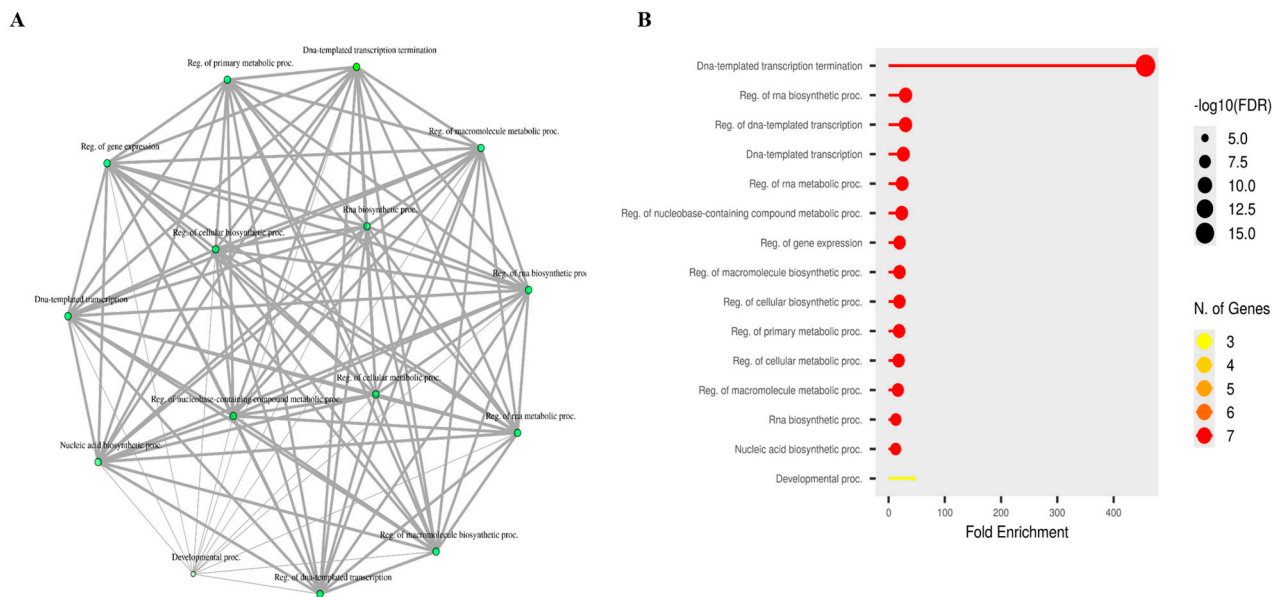


Figure 7. Gene ontology enrichment analysis. **(A)** Enriched GO biological process terms visualized as a network. **(B)** Enriched GO biological process terms visualized as a chart. GO enrichment analysis of *CrmTERF* genes was performed and visualized using the online tool ShinyGO 0.80.

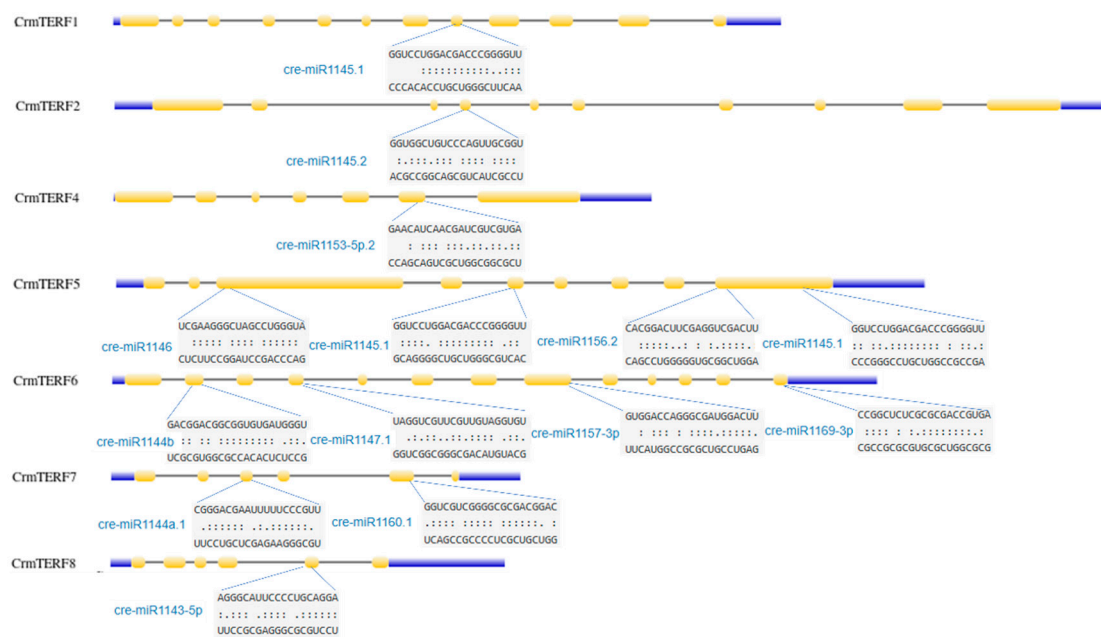


Figure 8. Predicted target cleavage sites of *CrmiRNAs* in *CrmTERF* in *C. reinhardtii*.

2.8. *mTERF* Genes Expression Patterns After mtDNA Depletion

The transcript levels of *mTERF* genes in *C. reinhardtii* after five days of EB treatment were investigated using qRT-PCR. *CrmTERF2* showed a significantly lower gene expression while *CrmTERF4*, *CrmTERF7* and *CrmTERF8* showed significantly higher gene expression while the expression of *CrmTERF1* was lower but not significantly. *CrmTERF3*, *CrmTERF5* and *CrmTERF6* were higher but not significantly (Figure 9).

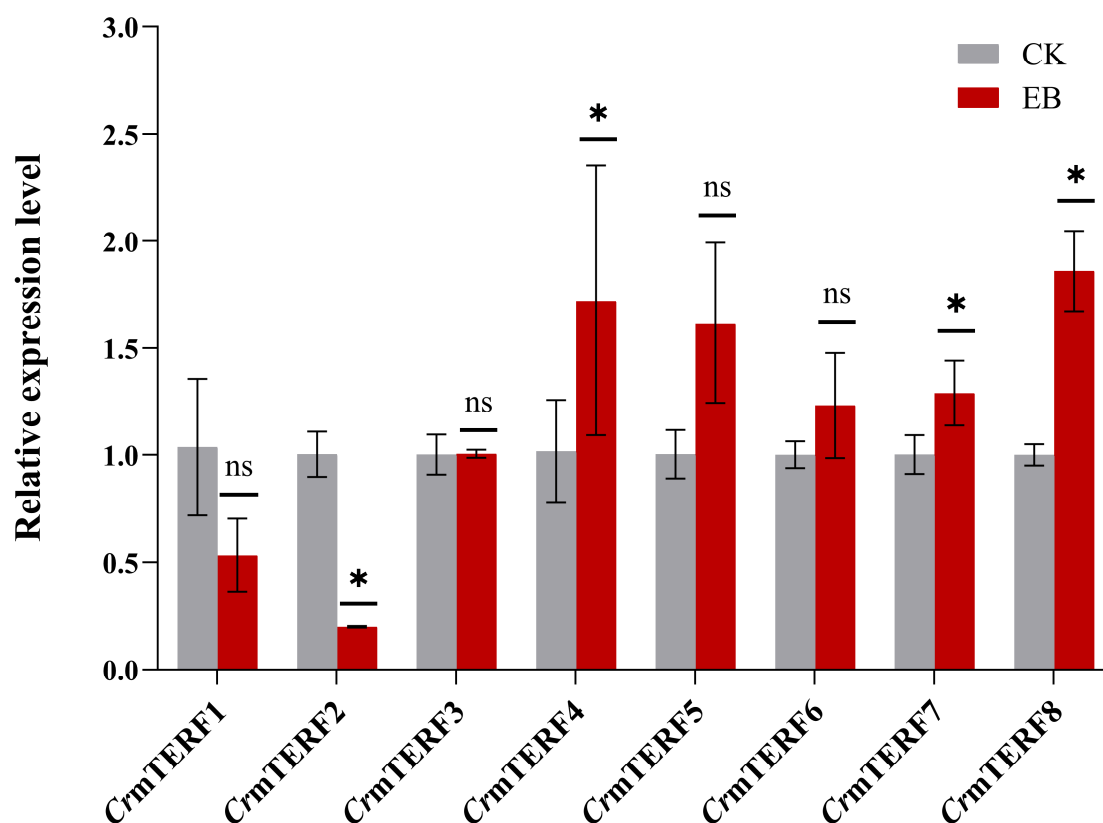


Figure 9. Relative expression levels of *CrmTERF* genes for EB-treated cells. *t*-test was employed to determine significance (* $p < 0.05$). The mean and standard deviation values were obtained from three biological replicates.

3. Discussion

Mitochondrial transcription termination factor (mTERF) is a nuclear encoded DNA-binding protein, that typically functions in mitochondria and is implicated in gene expression regulation. EB intercalates into mtDNA and inhibits its replication, leading to a reduced copy number, a well-established method for generating q^0 (mtDNA-depleted) lines in diverse eukaryotic systems, including *Chlamydomonas*. It was established that low-concentration EB treatment in *Chlamydomonas* specifically eliminates mtDNA while chloroplast and nuclear DNA remain intact. This selectivity is attributed to preferential mitochondrial dye accumulation, limited nuclear permeability, and absence of mtDNA repair factors not applicable to chloroplasts under these conditions [25]. Nevertheless, we acknowledge chemical approaches cannot fully exclude off-target effects. To address this, we validated our findings through multi-chemical replication in our previous study [29] where we generated *crm*[−] cells using EB, acriflavine, and ddC, all yielding consistent mtDNA depletion and comparable phenotypes. Three chemically distinct compounds producing identical effects strongly supports the conclusion that observed phenotypes arise from mtDNA loss itself rather than non-specific toxicity.

Fewer mtDNA copies are associated with reduced transcription of mitochondrial-encoded genes, which are essential subunits of the Electron Transport Chain (ETC) complexes I, III and IV. Depletion of mtDNA with EB, resulted in impaired mitochondrial function, consistent with the classical q^0 phenotype observed in yeast, mammalian, and algal models [26,28]. It is hypothesized that the mitochondrion senses this stress and sends signals to the nucleus to alter gene expression, potentially activating specific transcription factors that bind to the promoters of nuclear genes, including mTERF genes. The primary

role of mTERF proteins is thought to be the regulation of mitochondrial transcription, replication, and translation. Thus, mtDNA depletion may act as a signal to the nucleus, possibly triggering increased expression of factors like *mTERFs* to modulate the remaining mtDNA.

Interestingly, we observed a significant four-fold decrease in *mTERF3* gene (*MOC1*) expression in *crm⁻* cells compared to the wild-type control. Given that *MOC1* is known to terminate mitochondrial transcription in *Chlamydomonas* [17], its downregulation could represent a compensatory adjustment to the drastically reduced mtDNA template (>99.9% depleted). This pattern is consistent with the concept of mitochondrial retrograde signaling and aligns with reported nuclear transcriptional shifts following mtDNA depletion or ETC inhibition in *C. reinhardtii* [24]. However, this interpretation remains speculative in the absence of direct evidence for a signaling cascade or controls excluding potential off-target effects of EB. Alternatively, reduced *MOC1* might dysregulate the processing of remaining mitochondrial transcripts, potentially exacerbating the molecular phenotype. These observations highlight a correlation rather than a causal relationship. Future studies using genetic mtDNA depletion or complementation are needed to directly test these hypotheses and establish causality.

It is well-studied that the respiratory metabolism in the mitochondrion and the photosynthetic metabolism in the chloroplast interact and play a vital role in the energetic metabolism of photosynthetic organisms [30]. Their activity in photosynthetic eukaryotes is tightly linked, suggesting that the disruption of mitochondrial function might also perturb chloroplast function [31]. Our results support the existence of inter-organellar communication, as a reduction in the mtDNA copy number was correlated with disrupted mitochondrial structure, showing abnormalities such as elongated size, condensed matrix and collapsed cristae, as well as altered photosynthesis. A reduction in the copy number of mtDNA appeared to reduce the electron sink capacity, as indicated by increased chloroplast PQ redox state and elevated PSII excitation pressure [32]. These results suggest that the mETC may serve as an important sink for chloroplast-derived electrons during photosynthesis, aligning with studies in plants that identified the mitochondrion as an important sink for photo-generated reductants [33]. *Y(NPQ)* and *Y(II)* remained low following EB treatment despite having similar higher excitation pressure which is similar to Antimycin A inhibitor but distinct from Myxothiazol inhibition. This implies that EB might also affect the chloroplast electron transport chain as observed previously [30]. *Y(NO)* denotes the proportion of energy in PSII that is dissipated passively as heat and fluorescence. An elevation in *Y(NO)* indicates an excess of excitation energy in the PSII reaction center resulting from inadequate energy dissipation through alternative pathways [*Y(II)*, *Y(NPQ)*], which are significantly lower in EB and hence could be linked to PSII damage [34]. Collectively, these observations support the model that the function of the mitochondrion, showing the capacity to act as an electron sink, is critical in supporting cETC activity and may help avoid PSII damage.

In this study, we also explored the *Chlamydomonas reinhardtii* genome to identify *mTERF* genes, and eight potential mTERF genes were identified in the green algae *Chlamydomonas reinhardtii*. Comparative analysis revealed that land plant species such as Arabidopsis, capsicum, maize, grape, and tomato possess a larger number of mTERF genes (25–35), suggesting gene family expansion during plant evolution.

Structural analysis of the exon and intron borders of the *CrmTERF* genes provides insight into their evolution. Unlike *mTERFs* has no intron in higher plants like grape (*Vitis vinifera* L.), capsicum (*Capsicum annuum* L.), maize (*Zea mays* L.) [16,20,21], implying evolution via retrotransposition, while *Chlamydomonas* has introns in all mTERF genes. In *C. reinhardtii*, introns may contain regulatory elements influencing transcript stability, nu-

cleo–cytoplasmic export, and alternative splicing, potentially enabling functional diversification. Despite the variations in exon/intron numbers, most genes showed a conserved arrangement, suggesting evolutionary conservation within the family. Interestingly, closely related gene pairs identified in the phylogenetic tree (e.g., *CrmTERF1/CrmTERF6* and *CrmTERF2/CrmTERF4*) exhibited partially similar exon–intron organizations, supporting their evolutionary relatedness. However, distinct intron/exon patterns in other members (e.g., *CrmTERF5* vs. *CrmTERF3*) highlight structural divergence that may contribute to functional diversification.

The phylogenetic tree and collinearity analysis was performed among *Chlamydomonas reinhardtii*, *Volvox carteri*, *Marchantia polymorpha*, *Physcomitrella patens* and *Arabidopsis thaliana*. The *CrmTERFs* were distributed across all major clades, with some clustering alongside bryophyte and algal proteins, while others grouped more closely with higher plant sequences, signifying, as suggested by previous work, that genes existing in the same group could exhibit the same functions [35]. This pattern indicates that while nuclear-encoded *mTERF* gene family has diversified significantly during evolution, certain lineages have maintained strong conservation from algae through to land plants. The collinearity network demonstrates that the *mTERF* family in *C. reinhardtii* conserved syntenic relationships with bryophytes, while showing partial divergence from higher plants. This suggests that while functional diversification occurred during plant evolution, a conserved ancestral core of *mTERFs* persists across algae and early land plants, reflecting their essential role in organelle transcriptional regulation.

In our study, 12 putative miRNAs pointing to the *CrmTERF* genes except *mTERF3* were found. The roles of some of these miRNAs were reported in the past and implicated in other processes. *miR1145.1* targeting *mTERF1* and *mTERF5* has been linked to carotenoid synthesis in *Dunaliella salina* [36], *cre-miR1147.1* is involved in proteolysis and peptidase regulation, while *cre-miR1156.2* is associated with amino acid metabolism [37]. These miRNA–*mTERF* interactions suggest potential post-transcriptional regulatory layers that could be explored in future reverse genetics studies.

GO annotation analysis associated *CrmTERFs* with DNA-templated transcription termination of RNA biosynthesis regulation and transcriptional regulation. Functional associations have been reported in other systems; the knockout of *mTERF2* [11] and tissue-specific inactivation of *mTERF3* [12] in mice led to aberrant mitochondrial transcription and severe phenotypes [14]. Additionally, the *Drosophila mTERF* homolog *DmTTF* is responsible for transcription termination [38]. Together, these results support the hypothesis that *CrmTERF* genes may play a role in transcription termination and regulation. Most of the *mTERF* genes show higher gene expression, which could reflect a compensatory mechanism to enhance the transcription and replication efficiency of the few remaining mtDNA copies. However, these expression changes remain correlative, and functional studies are needed to confirm their regulatory roles.

4. Materials and Methods

4.1. Microalgae Strains, GroCKh Conditions and EB Treatment

The experiments used *Chlamydomonas reinhardtii* strain CC-124 (wild-type, mt[−], background 137c) obtained from our laboratory algal repository. Cultures were maintained in laboratory-scale photobioreactors (PBRs) under continuous shaking (120 rpm) with aeration and ambient CO₂ concentration (~0.04%). Standard growth conditions were 25 °C in Tris-acetate-phosphate (TAP) medium under constant illumination (100 μmol photons m^{−2} s^{−1}, 50% red:50% blue; Philips Amsterdam, NL, USA), cultivating the algae mixotrophically.

To initiate the EB treatment, cells were first grown to the stationary phase (~5 × 10⁶ cells mL^{−1}), then diluted to 3 × 10⁵ cells mL^{−1} in 300 mL of fresh TAP medium supplemented

with 6 μM ethidium bromide (EB). Cultures were incubated mixotrophically with continuous shaking (120 rpm) for up to 5 days. Samples were harvested in triplicate after this period, and mtDNA depletion was confirmed by quantifying the copy number using quantitative PCR (qPCR). Control cultures were treated identically but without the addition of EB.

4.2. Analysis of Mitochondrial DNA (mtDNA) Copy Number

Genomic DNA extraction was performed by using a Genomic DNA Kit (Transgen, Beijing, China, EE101). Sample quality was assessed through a device named the NanoDrop2000 Ultra spectrophotometer to set a unified concentration of 50 ng/ μL in order to perform qPCR. The detailed method for the analysis of the mtDNA copy number has been described previously [39]. Briefly, to estimate the number of mitochondrial DNA (mtDNA) copies per cell, we compared the DNA from mitochondria to the DNA from the cell's nucleus. We used a specific, stable gene from each location: the *rrnL6* gene (for mtDNA) and the *CLPD24* gene (for nuclear DNA). The difference in their detection cycles (ΔCt) allows us to calculate the relative amount. The number of mtDNA copies per cell was calculated as $2 \times 2^{(\Delta\text{Ct})}$, where $\Delta\text{Ct} = \text{Ct}(\text{CLPD24}) - \text{Ct}(\text{rrnL6})$.

4.3. Transmission Electron Microscopy

Samples for transmission electron microscopy (TEM) were prepared using a standard chemical fixation protocol. Cells were initially fixed in 2.5% (*v/v*) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight (~12 h) at room temperature. After three buffer washes, samples were post-fixed in 1% (*w/v*) osmium tetroxide in the same buffer for 4 h at 4 °C. Following dehydration through a graded ethanol series (30%, 50%, 70%, 85%, 95%, and 100%; 15 min per step), the specimens were stained en bloc with 2% uranyl acetate in 50% ethanol for 1 h. Dehydration was completed with two changes in anhydrous ethanol and two changes in propylene oxide (15 min each). Samples were then infiltrated and embedded in Epon 812 resin (Electron Microscopy Sciences, , Fort Washington, PA, USA). Ultrathin sections (70–90 nm) were collected on copper grids and post-stained with lead citrate for 5 min. Sections were examined using a Hitachi HT 7800 transmission electron microscope operated at 80 kV, and images were acquired with a CMOS camera in high-contrast (HC) mode at magnifications of 3000 $\times$, 4000 $\times$, and 8000 $\times$.

4.4. Analysis of Photosynthetic Parameters

For all fluorescence measurements, three biological replicates were taken. We used a PAM2500 device (Heinz Walz GmbH, Effeltrich, Germany) to measure photosynthetic parameters. Measurements were conducted using 2 mL, 15 min dark-adapted cells at mid-log phase (containing 20 μg Chl) at room temperature (25 °C). For the construction of rapid light curves (RLCs), eight incrementally increasing actinic light (wavelength of 630 nm) intensities (0, 2, 4, 6, 8, 10, 12, 14) with PAR range (0, 33, 66, 200, 365, 621, 983, 1162 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were set in a light-curve edit. This actinic light was provided continuously without dark interval for 10 s each and followed by a saturation pulse. Chlorophyll a fluorescence was utilized to investigate photochemical and non-photochemical processes. Photochemical energy quenching (qP), also known as excitation pressure, is calculated as 1-qP and redox state of Qa pool as (1-qL). The electron transport rate (ETR), effective PSII quantum yield [Y(II)], the quantum yield of regulated thermal energy dissipation [Y(NPQ)], and the quantum yield of non-regulated thermal energy dissipation [Y(NO)] were also evaluated.

4.5. Identification of Nuclear Encoded *mTERF* Gene Family in *C. reinhardtii*

Protein data for *C. reinhardtii* at the whole-genome level were obtained from the Phytozome database (<http://phytozome.jgi.doe.gov/pz/portal.html>, accessed on 15 August 2024) [40]. The *Arabidopsis* genome database (<https://www.arabidopsis.org/>, accessed on 15 August 2024) supplied the protein sequences of the *mTERF* family from *Arabidopsis thaliana*, which were utilized as query sequences for alignment with *C. reinhardtii* protein files. The Pfam ID of *mTERF* (PF02536) was acquired by visiting the Pfam website (<http://pfam.janelia.org/>; visited on 15 August 2024). The *mTERF*.hmm file, containing the conserved domain information of the *mTERF* family, was retrieved from the Pfam website. Protein sequences of *C. reinhardtii*, *mTERF*.hmm, and the Pfam ID of *mTERF* (PF02536) were utilized in a straightforward HMM search using TBtools (v1.068) to identify the proteins containing a *mTERF* domain, resulting in the detection of 9 proteins having a *mTERF* domain. Initially, the data was filtered using an e-value threshold of $<1 \times 10^{-5}$, after which we manually eliminated the redundant sequences. The *mTERF* structural domain was predicted within the amino acid sequence of the chosen *mTERF* protein family member in *C. reinhardtii* utilizing the Conserved Domain Database (CDD, <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>; accessed on 15 August 2024) from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>, accessed on 15 August 2024). The candidate genes lacking the *mTERF* structural domain were eliminated. Consequently, eight potential *CrmTERF* genes were found in *C. reinhardtii*.

The physicochemical properties of the *mTERF* protein from *C. reinhardtii*, including molecular weight, protein length, instability index, theoretical pI, and hydrophilicity, were obtained using the online tool ExPASy-Prot (<https://web.expasy.org/protparam/>; accessed on 15 August 2024) [41]. The Ks_Ka calculator (<https://sourceforge.net/projects/kakscalculator2/>) was used to calculate Ka/Ks ratios of each *CrmTERF* gene. The protein sequences of the eight *mTERF* proteins were analyzed using the default parameters of the web program MEME v5.4.1 (<https://meme-suite.org/meme/tools/glam2scan>; accessed on 15 August 2024) to identify conserved areas [42]. The application employed the following settings: the sequencing of alphabet DNA, RNA, or protein; site distribution limited to zero or one occurrence per sequence (zoops); the motif-finding mode set to classic mode; and 10 motifs.

4.6. *mTERF* Gene Structure and Chromosomal Distribution

The chromosomal locations and protein sequences of all *mTERF* genes in *C. reinhardtii* were obtained by using the internet database Phytozome (<http://phytozome.jgi.doe.gov/pz/portal.html>, viewed on 15 August 2024) [40]. We assessed the distribution of *mTERF* genes throughout the chromosomes via an online software called MapGene2 Chromosome V2.1 (http://mg2c.iask.in/mg2c_v2.1/index.html; accessed on 15 August 2024) [43] by submitting the gene ID, the start position of the gene, the end position of the gene, and the chromosome ID of the gene in one box, and chromosome ID and the chromosome sequence length in other box. We used the kb scale and blue color for the chromosome, while red color was used for the gene ID, gene line and connecting wire. This is how we discovered the *mTERF* genes' position on the chromosomes of *C. reinhardtii*.

The *mTERF* gene structure diagram was constructed utilizing the web program GSDS V2.0 (<https://gsds.gao-lab.org/>; accessed on 15 August 2024) [44] by submitting CDS and genomic sequences followed by quantitative analysis of introns and exons.

4.7. Phylogenetic and Collinearity Analysis

The protein sequences of *mTERF* genes from *Chlamydomonas reinhardtii* (Cr), *Marchantia polymorpha* (Mp), *Coccomyxa subellipsoidea* (Cs), *Volvox carteri* (Vc), *Physcomitrella patens*

(Pp) and *Arabidopsis thaliana* (At) were utilized for phylogenetic analysis, and the evolutionary relation of the *C. reinhardtii* *mTERF* gene family to other species was displayed. ClustalW 2.0 software was used to conduct multiple alignments of the *mTERF* protein sequences and the phylogenetic tree was built with MEGA 7.0 software [45] using the neighbor-joining (NJ) method with 1000 bootstrap repetitions. The online website iTOL tool (<https://itol.embl.de/login.cgi>, accessed on 15 August 2024) [46] was used to visualize the phylogenetic tree by using these settings: mode = circular, cover = clade, branch length = ignored, inverted tree = no, alignment = left, rotation = on and colors = gray, green and blue.

Comparative collinearity analysis to find the evolutionary relationships and conservation among *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Volvox carteri*, *Physcomitrella patens* and *Arabidopsis thaliana* *mTERF* proteins was conducted by using an online tool named the synteny viewer circoletto tool (<https://tools.bat.infospire.org/circoletto/>; accessed on 15 August 2024), using the homology thresholds defined by the tool's default BLAST (Basic Local Alignment Search Tool) settings: an E-value cutoff of 10 to the -10 (normal) and a minimum identity percentage of 60%, as indicated by the orange and red color scheme ($\leq 60\%$ and $\leq 80\%$ identity, respectively) [47].

4.8. Visualization of CrmiRNA's Predicted Cleavage Sites and GO Enrichment Analysis

CrmiRNAs targeting CrmTERFs were identified using the online website tool psRNA-Target Schema V2 (2017 release) (<https://www.zhaolab.org/psRNATarget/>; accessed on 15 August 2024) [48] using expectation threshold = 5.0; length for complementarity scoring (HSP size) = 19 bp; penalty for G:U pair = 0.5; extra weight in seed region = 1.5; mismatches allowed in seed region (2–13 nt) = 2; penalty for opening gap = 2.0; penalty for extending gap = 0.5; translation inhibition range considered. Hits with a maximum expectation value ≤ 2.0 were considered putative targets for subsequent analysis. Gene structures were designed using an offline tool named TBtools where yellow triangles depict the CrmiRNA probable targeted sites in the exons of CrmTERF genes. An online tool named ShinyGO v 0.80 (<http://bioinformatics.sdstate.edu/go/>; accessed on 15 August 2024) [49] with FDR cutoff = 0.05, minimum pathway size = 2, redundancy removal enabled, and maximum pathways to display = 20 was used to carry out the GO enrichment analysis of the CrmTERF genes and visually draw the chart to make it clear.

4.9. Extraction and Purification of RNA and RNA Reverse Transcription

Total RNA was extracted from approximately 1×10^7 *Chlamydomonas* cells (equivalent to ~50 mg fresh weight) per biological replicate using the TIANAMP Genomic RNA Extraction Kit (Tiangen Biotech, Beijing, China). The extraction was performed strictly following the manufacturer's protocol, which included on-column DNase I digestion to remove genomic DNA contamination. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). All RNA samples used for cDNA synthesis exhibited A260/A280 ratios between 1.9 and 2.1 and A260/A230 ratios greater than 2.0, confirming high purity. First-strand cDNA was synthesized [50] from 500 ng of total RNA using the Quantscript RT Kit (Tiangen Biotech, Beijing, China) in a 20 μ L reaction volume. The reverse transcription protocol was as follows: genomic DNA removal at 42 °C for 2 min, reverse transcription at 37 °C for 15 min, followed by enzyme inactivation at 85 °C for 3 min. The resulting cDNA was diluted 1:5 with nuclease-free water and stored at -20 °C until use for qPCR analysis.

4.10. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

cDNA obtained in the last step was used in real-time PCR analyses using the Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA)

on an [Applied Biosystems ViiA 7 Real-Time PCR System]. Reactions were assembled in a 10 µL volume containing 5 µL of SYBER Green, 1 µL of forward and reverse primer each, 1.5 µL of diluted cDNA template, and 2.5 µL of nuclease-free water. Thermocycling was performed as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (combined annealing/extension). The PCR primer sets used to include the housekeeping gene *RACK1* are shown in Table 2 and were designed from the published database, BLAST. Comparative gene expression analysis using qRT-PCR was performed according to previously described methods [51] and the calculated relative expression with $2^{(-\Delta\Delta Ct)}$ formula, with three biological replicates.

Table 2. List of primers utilized in this study's qRT-PCR gene expression investigation and mtDNA copy number.

Gene Name	Primer Name	Sequence (5'-3')	Length
<i>mTERF1</i>	1-F	ATGTTTCGCAACCTCTTTTCGG	20
	1-R	CTCCTCGAAGAGGCAAGTGG	20
<i>mTERF2</i>	2-F	CGATGCCACACTTCAGTTGC	20
	2-R	CCGATGCCCAGCAAGTCTAA	20
<i>mTERF3</i>	3-F	TATTGGGGTATCGCCGAACG	20
	3-R	GATGCTCCCAGCAGTAGGTC	20
<i>mTERF4</i>	4-F	TGAGATTGAGGCGGAGTGATG	21
	4-R	CTTGAATGCGACCGGTGAAC	20
<i>mTERF5</i>	5-F	TACAAACAGCAACAGCACGC	20
	5-R	GGATCCGGAAGAGAGCTGC	19
<i>mTERF6</i>	6-F	CTGACCGACCCACATGGAC	19
	6-R	CGCCGACCAGAGCTCTTTAT	20
<i>mTERF7</i>	7-F	GCAAAGCCCCGACACTAAGA	20
	7-R	CGATAGAGGAGGGCAGCTTG	20
<i>mTERF8</i>	8-F	ACTACATGACCAGCATCGGC	20
	8-R	TATTTTCCGCAGGTCGCGT	20
<i>RACK1</i>	9-F	CTTCTCGCCCATGACCAC	18
	9-R	CCCACCAGGTGTCTTCTCAG	20
<i>rrnL6</i>	10-F	ACAATTACGCTGAAAACAGTACCA	24
	10-R	TCACTGTTTGTTATGCAAAACCTT	24
<i>CLPD24</i>	11-F	TGTTTCTCCTTGTTCCACCTCTG	23
	11-R	CCGGGTTGACGTCTGTCTTG	20

4.11. Statistical Analysis

All data are presented as the mean ± standard deviation (SD) from three independent biological replicates ($n = 3$). For comparisons between two independent groups, including the qPCR results for mtDNA copy number and gene expression comparisons, the data were checked for normality using the Shapiro–Wilk test and for homogeneity of variance using the F-test. The Shapiro–Wilk test was used to assess whether the data followed a normal distribution, with p-values greater than 0.05 indicating that the data did not deviate significantly from normality. To test the assumption of equal variances, an F-test was performed for each gene comparing the variances of the control and treatment, as automatically provided by GraphPad Prism v10 when running an unpaired *t*-test with the assumption of equal standard deviations. For data that satisfied both normality and equal variance assumptions, Student's *t*-test (assuming equal variances) was used to compare treatment groups. In cases where normality was satisfied but variances were unequal, Welch's *t*-test (not assuming equal variances) was applied.

Chlorophyll fluorescence parameters [Y(II), Y(NPQ), 1-qP, 1-qL, Y(NO), and ETR] are bounded ratios (0–1 scale for quantum yields, and 0 to a theoretical maximum for ETR) that do not meet the assumptions of parametric tests. Therefore, comparisons between control and treatment at each PPFD level were performed using the two-tailed Mann–Whitney U test. Statistical significance levels are denoted as * $p < 0.05$ with exact p -values reported in the figure legends.

5. Conclusions

The experimental results of this study correlate with our central hypotheses. We observed that mtDNA depletion is associated with severe mitochondrial ultrastructural defects and is associated with significant impairment of chloroplast photoprotection, supporting a functional link between these organelles. Furthermore, we present the first genomic inventory of the *mTERF* family in green algae, identifying eight *CrmTERF* genes and documenting their altered expression under mitochondrial stress. Based on these findings, we propose a model in which the nucleus may orchestrate adaptive responses and the *CrmTERF* family acts as a potential mediator in retrograde signaling and organelle crosstalk. This model provides a testable hypothesis and a foundation for future functional validation to establish causal mechanisms.

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