Characterization of the nuclear proteome of Chlamydomonas in response to salt stress

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Abstract

Microalgae biomass is considered a promising alternative feedstock for biodiesel production due to its high productivity of neutral lipids, specially under abiotic stress conditions. Among the unicellular microalgae that show this characteristic, *Chlamydomonas reinhardtii* appears as one of the most important model species that have increased lipids production under abiotic stress conditions. In this study, we show that cells cultivated under mixotrophic condition supplemented with 0.1 M of NaCl rapidly raises their amount of neutral lipids in *C. reinhardtii* without reduction of their cellular growth rate, being therefore a promising condition for biomass towards bioenergy production. The nuclear proteome of these cells was investigated where we identified 323 proteins with an enrichment of almost 60% of

nuclear proteins in the total dataset. We found 61 proteins differentially regulated upon salt treatment, including proteins annotated in functional categories related to translation and nucleosome assembly functions, among others. Additionally, we identified Transcription factor proteins (TFs) and analyzed their likely Transcription factors-binding regulatory elements identifying target genes related to lipids metabolism and kinase functions, indicating possible regulatory pathways of lipids biosynthesis. Together these data can help understand regulatory nuclear mechanisms leading to an increase of lipids in the first 24h of salt stress 0.1M NaCl.

Keywords: lipids, regulation, microalgae, transcription factors

1. Introduction

In recent decades, research in renewable energy has received increased interest and funding. This has been driven mainly by Climate Change-related concerns, demanding a transition from fossil fuel dependency to alternative, more sustainable energy sources, as indiscriminate fossil fuel usage has led to worsening the greenhouse effect and atmospheric pollution [1,2]. In this context, biomass production from microalgae cultures poses a valuable solution for sustainable consumption of atmospheric carbon dioxide coupled to bioenergy generation [3,4]. Through photosynthesis, these fast-growing microorganisms are capable of assimilating carbon dioxide into their biomass generating, for instance, starch and lipids which serve as a potential feedstock for biofuels production. Furthermore, microalgal biomass might be also converted to a plethora of industrialized products such as bioplastics (biopolymers industry), pigments (food and cosmetic industry), and nutraceuticals (food industry) [5].

When facing salt stress and nutrient deprivation, some microalgae species can increase lipids biosynthesis and storage [6–9]. However, this stress can also limit cell growth, consequently hindering the biomass production [10]. *Chlamydomonas reinhardtii* (from now on *C. reinhardtii*) is a green unicellular flagellate microalga that has been widely employed as a model organism for chloroplast, and more recently, systems biology studies of cellular stress responses [11,12]. The genome of *C. reinhardtii* was sequenced in 2007 [13], which has since allowed for expanding systems and genetic analysis, as well as enabled genome annotation and functional genomics analysis through omics studies [14]. Aiming to increase lipid yields, such studies have been pivotal to unravel the pathways and molecular mechanisms associated with stress-related responses and consequences to biomass production in microalgae.

The cellular response to salt stress in *C. reinhardtii* has been widely studied [15–17]. The exposure of cells to 0.2 M NaCl is highly toxic, as it raises the levels of intracellular peroxides with subsequent inhibition of photosynthesis and cell growth [17]. In contrast, the exposure to lower concentrations of NaCl, specifically 0.1 M, drive increased production of lipids [16]. The regulatory proteins involved in the adaptation to salt stress and increased lipid yields are not yet fully characterized. Although there are some studies analyzing the nuclear proteome during physiological conditions and under CO_2 deprivation [18,19], the identity of the Transcriptional Regulators proteins (TRs) and Transcription Factor proteins (TFs) involved in the cell response to salt stress (0.1 M NaCl) are still unknown. In this study, we analyzed the nuclear proteomics of *C. reinhardtii* during acclimation to salt stress, identifying differentially expressed proteins and stress response-associated target genes that may be regulated by the TFs identified in the nuclear fraction analyzed.

2. Material and methods

2.1. Cell culture and growth conditions

Chlamydomonas reinhardtii (strain cc-503 cw92 mt+) was obtained from the Chlamydomonas Resource Center (University of Minnesota). Cells were cultivated under mixotrophic and temperature-controlled conditions in Tris-Acetate-Phosphate (TAP) [20] medium with continuous shaking and under light ($\sim$100 µE $\cdot$ m^{-2} $\cdot$s^{-1}).

Cells were cultivated in 2 L flasks containing 0.5 L medium with an initial cell density of 1 x 10^5 cells/mL and were cultivated up to the exponential growth phase with either TAP medium (Control) or TAP supplemented with 0.1 M NaCl (Treatment). Samples were taken every 24 h for cell growth monitoring and counting with a Countess II FL Automated Cell Counter (Thermo Fisher Scientific). Proteomic studies were performed for the Control and treatment (0.1M NaCl) conditions sampled at 24 h time point counted after initial cell inoculation. All the experimental data is expressed as the mean value of three independently performed experiments with their standard deviation (SD).

2.2 Neutral lipids quantification

Neutral lipids were quantified by fluorescence measurement adapted from a method previously described by Gorman and colleagues [21]. Briefly, cells were centrifuged at 5.000 g for 10 minutes at 4 °C and resuspended in TAP media to a final density of 1 x 10^6 cells$\cdot$mL^{-1}. Then, DMSO was added to a final concentration of 1% and left for 5 minutes. After this time, the cell suspension was incubated with Nile Red (1µg/mL) for 10 minutes in an amber flask. Fluorescence was measured in 96 well plates with wavelengths at 530 nm excitation

and 580 nm emission in a Synergy H1 Hybrid Reader (Biotek, USA). Each measurement was repeated three times.

2.3. Nuclei isolation and extraction of nuclear proteins

The nuclear protein extraction protocol was adapted from Winck [22] with a CelLytic PN extraction kit (Sigma, USA). To avoid proteolytic degradation, Plant protease inhibitor (Sigma, USA) was added, and all steps performed at 4° C. Pellet of 250 mL cell culture was re-suspended in 600 µL of NIB solution, harvested, macerated in liquid nitrogen using a mortar and pestle. The powdered material was suspended in 10 mL of NIB solution, filtered through a miracloth membrane (22–25 µm; Calbiochem, Bad Soden, Germany) and centrifuged at 1,260 g for 10 minutes. To the cell pellet, NIBA buffer containing 1% Triton X-100 was added. After homogenization, the sample was centrifuged at 1,000 g for 30 minutes. The cell pellet was washed twice by homogenization in 1 mL of NIB solution and centrifugation at 600 g for 10 minutes. The pellet enriched with nuclei was resuspended in 1 mL NIBA and extraction buffer (1.2 M potassium acetate, 25 mM Hepes-KOH, 10% glycerol, 1 mM EDTA). The resulting nuclei was disrupted by 1 minute sonication (60% amplitude, 1 min. with 10 sec. on and 20 sec. off cycles) using a sonicator Vibra Cell VCX 130 device (Sonics, CT, U.S.A.). Finally, samples were centrifuged at 13,000 g for 15 minutes and nuclear proteins were collected from the supernatant for quantification by Bradford method using the Bio-Rad protein assay kit (Bio-Rad, USA) [23].

2.4 Mass spectrometry analysis

Amount of 2 µg from protein extracts were solubilized with 8M urea in a proportion 1:1, reduced with 5 mM DTT, alkylated with 14 mM iodoacetamide (IAA), treated with 5

mM DTT for 15 min in the dark and digested with Trypsin (Promega, WI, USA) in the proportion 1:50 for 16 h at 37 °C. Peptides solution was desalted with STAGE tips with C18 disks (3M, Neuss, Germany). Peptides were eluted in 0.1% formic acid. The peptides were separated and analyzed by LC-MS/MS using the Mass Spectrometer Q-ToF Maxis 3G (Brucker Daltonics, USA) coupled to a Nano LC Acquity (Waters, USA). The CaptiveSpray ion source used in spectrometry and the electrospray voltage was adjusted to 2 kV, with the source temperature at 150 °C. Peptide separation was performed in two steps, with accumulation of peptides in nanoAcquity UPLC® 2G-V/MTrap Symmetry® C18 column (180 µm x 20 mm, 5 µm) for 3min at a flow of 7 µL/min in 0.1% acid formic, followed by an analytical separation using nanoAcquity UPLC® BEH130 (100 µm x 100 mm, 1.7 µm), with acetonitrile gradient in 0.1% formic acid as mobile phase in gradient between 2–85% in 0.1% formic acid in flow of 0.3 µL/min for 85 min.

2.5 Data analysis

Proteins were identified using MaxQuant software v.1.6.17.0 [24] available in our Galaxy server platform (http://143.107.55.132/galaxy/) against the reference proteome database of *C. reinhardtii* v5.5 annotation (17,741 proteins), retrieved from Phytozome 12 [25]. Maximum error of 6 ppm was accepted for precursor ion search (MS search), 0.5 Da for fragment search (MS/MS search) and a maximum of 2 missed cleavages, with a maximum limit of 1% False Discovery Rate (FDR) for identification of peptides and proteins, using a Reverse proteome database as decoy. Cysteine carbamidomethylation was set as fixed modification and methionine oxidation was set as variable modification.

Statistical analyses were carried out using Perseus software (version 1.6.14.0) [26]. Protein hits were accepted if they were quantified in at least two of the three experimental

replicates performed and were present in at least one experimental condition. Identified by site and reverse entries were excluded from further analysis. Significant differentially expressed proteins were calculated using Two sample T-test (T-student) with a Benjamini-Hochberg FDR threshold of 0.05 as post-hoc test [27].

Enrichment analysis of the protein annotation terms of Gene Ontology (Gene Ontology; www.geneontology.org) was applied for the data mining of the proteomics data. For detecting significant enriched terms of the selected list of proteins identified we applied BiNGO tool [28] test and p-value correction by Benjamini-Hochberg method. The visualization of the network of GO terms was performed using the AutoAnnotate application of Cytoscape [28,29]. The node color (orange-red gradient) indicates the scale of calculated corrected p-value [30].

Protein subcellular localization was predicted using SUBA prediction tool (http://suba.live/) [31] with an Arabidopsis TAIR annotation for homologs of *C. reinhardtii* proteins [32]. Furthermore, prediction of nuclear location was performed using the NucPred prediction server (https://nucpred.bioinfo.se/nucpred/) [33] and PredAlgo Subcellular localization prediction tool (https://giavap-genomes.ibpc. fr/predalgo/) [34].

2.6 Microscopy

To evaluate nuclei integrity and enrichment, an aliquot of purified nuclei was stained with DAPI (Sigma, USA) and observed with fluorescence microscopy (microscope Dmi8 (Leica, USA) and confocal microscope CTR6 LED (Leica, USA).
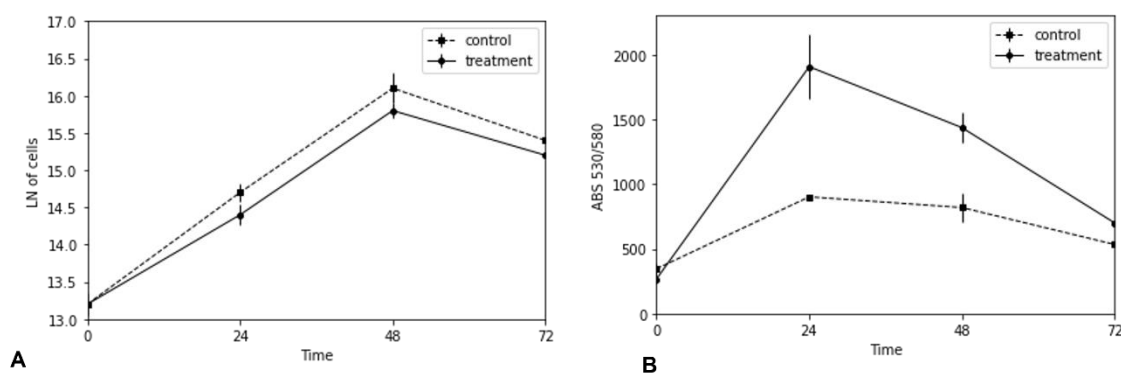
2.7 Prediction of transcription factor target genes

Transcription Factors and Transcription Regulators were identified according to the annotation available at the PlnTFDB database (http://plntfdb.bio.uni-potsdam.de/) [35] followed by the identification of their homologs in *Arabidopsis thaliana* (Araport11 annotation [36]) using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of known cis-element binding motifs recognized by these Arabidopsis TFs homologues and annotated in the Plant Transcription factor Database v4.0 (http://planttfdb.cbi.pku.edu.cn/) [37] were then searched in the promoter regions ($\leq$ 1500 bp upstream of the transcription start site) of *Chlamydomonas* genes (v5.6 genome, Phytozome13) using the MAST tool (http://memesuite.org/tools/mast) [38]. Sequence hits with an e-value $\leq$ 1 were considered for further analysis.

3. Results

3.1 The impact of 0.1 M NaCl on growth and lipid accumulation in *C. reinhardtii*

C. reinhardtii has been postulated as a prominent organism for biomass production and thus, a potential source for sustainable energy production. Importantly, during adaptation to some adverse conditions, prominently salt stress, the ability of *C. reinhardtii* to produce biomass might be reduced. To assess this, a comparison between cultures with the supplementation of 0.1 M NaCl (Treatment) and without adding NaCl (Control) was performed. Cell growth and the concentration of neutral lipids were analyzed over time (0, 24, 48 and 72 hours) **(Figure 1)**.

Figure 1. *C. reinhardtii* **growth and lipid production under saline stress with 0.1M NaCl**

A: Logarithmic transformed growth curve of non-stressed (dotted line) and salt (0.1 M NaCl) stressed (solid curve) *C. reinhardtii* cultures; **B:** Relative quantification of neutral lipids of non-stressed (dotted line) and salt (0.1 M NaCl) stressed (solid curve) *C. reinhardtii* cultures measured by fluorescence at 530/580 nm with Nile Red staining. X axis indicates time in hours.

As shown in **Figure 1**, the treatment of cells culture with 0.1 M NaCl only marginally affected the growth rate of *C. reinhardtii* while strongly stimulated the lipids synthesis, especially in the first 48 h of cultivation under salt treatment. After this time, the rate of lipids production sharply decreases, reaching similar levels to that of non-stressed cells. Thus, our study indicates that slight deviations from physiological conditions are sufficient to trigger increased lipids production without affecting cell growth. Although it is reported that *C. reinhardtii* can survive up to 0.2 M NaCl [16,39], this condition is reported to cause growth arrest and loss of biomass, and thus is not well-suited for bioenergy production purposes. Therefore, we aimed to identify possible target nuclear regulatory proteins and their target genes that may be associated to the increased lipids biosynthesis without affecting cell growth performance, revealing the identity of novel targets for microalgae transcriptional engineering.

3.3. Proteomics analysis

3.1 Nuclear protein extraction and proteomic analysis

Having determined that 0.1 M salt stress does not impact growth but boost intracellular neutral lipids synthesis, we performed the nuclear proteomics analysis to unravel stress-related factors. During the time-course experiment, cells were collected for nuclei isolation and protein extraction after 24 h of exposure (please see the Methods section). Firstly, the integrity of isolated nuclei was analyzed using 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence micrograph (**Figure S1**) shows uniform spheres with an average diameter of approximately 3 µm with no visible contamination with other cellular compartments.

3.2 Protein identification and subcellular localization

Nuclear proteins extracted from *C. reinhardtii* were then analyzed using nano-LC-MS/MS. During data analysis, we identified a total of 472 proteins, value that was narrowed to 323 proteins after data pre-processing analysis to removing contaminants and filtering missing values and considering two valid values in all three replicates in at least one experimental condition. Multi-scatter plots showing Pearson correlation maps were used to assess the data quality (**Figure S2**). In **Table S1** we summarized the functional categories and expression levels of all 323 proteins identified.
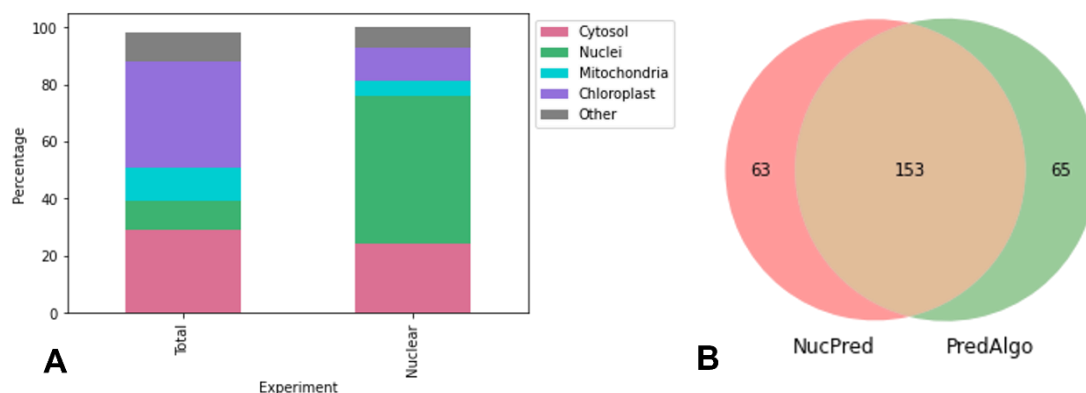
3.3 Enrichment of nuclei sample

An important aspect of the subcellular proteome analysis is the purity and enrichment of the sample since it can include contamination of other organelles and chloroplasts. The prediction of the subcellular localization of the identified proteins and assessment of the

presence of potential contaminants was performed using three prediction tools for subcellular location: SUBA [31], NucPred [33], and PredAlgo [34].

At first, to estimate the enrichment of nuclear samples with nuclear proteins, we compared the total proteome and nuclear proteome of cells culture under the same conditions of 0.1 M NaCl for 24hours (**Figure 2A**). SUBA location was used to predict the subcellular location of proteins using as input their homolog sequences from *Arabidopsis thaliana* available at *Arabidopsis Information Resource (TAIR)* database (https://www.arabidopsis.org/). In our analysis, we found an increment superior to 50% of annotated nuclear proteins when observing the 10% of annotated nuclear proteins identified in the whole-cell proteome compared to the 68% of annotated nuclear proteins identified in the nuclear proteome (**Figure 2B**).

PredAlgo and NucPred prediction servers were used to estimate the presence of nuclear proteins in the nuclear proteome analyzed (**Figure 2B**). The combination of PredAlgo and NucPred predictions revealed that 153 (47%) out of 323 proteins analyzed are predicted to be nuclear localized. PredAlgo analysis revealed that 216 (67%) proteins are indeed nuclear-related proteins, while 62 proteins (19% of total proteins) were associated with the chloroplast, 21 proteins (6.5% of total proteins) to the mitochondria, and the remaining 10 proteins (3% of total proteins) were classified as extracellular proteins.

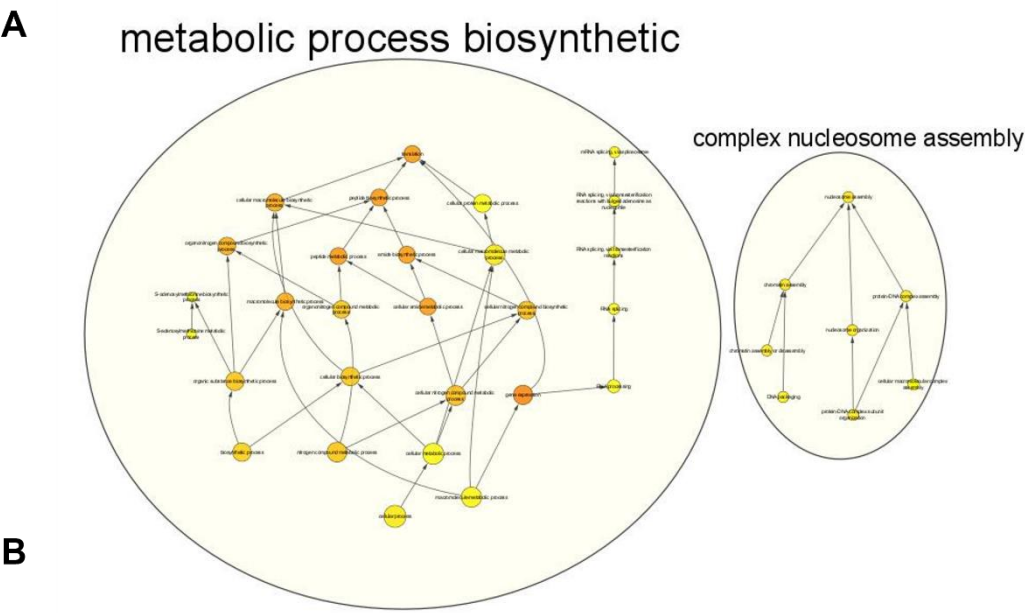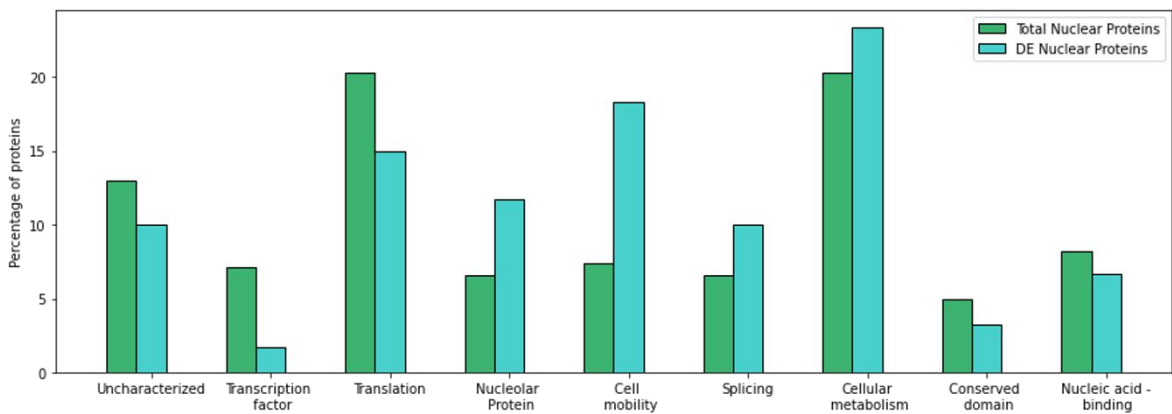**Figure 2**. **Prediction of subcellular localization of identified proteins**

(**A**) Percentage of categories of subcellular localization prediction for nuclear and whole-cell proteins performed with SUBA4 (Arabidopsis Subcellular Database); (**B**) Venn diagram of groups of nuclear-predicted proteins identified by PredAlgo (green circle) (https://giavapgenomes.ibpc.fr/predalgo/) and by NucPred (https://nucpred.bioinfo.se/cgi-bin/single.cgi) (red circle). Overlapping region represents proteins that were identified by both predictors as nuclear proteins.

3.4 Differentially expressed and exclusive nuclear proteins

For the identification of differentially expressed proteins (DEs), we performed a Student's T-test (p < 0.05) analysis, with further Benjamini-Hochberg post-hoc test correction. We found 61 proteins differentially expressed during exposure to 0.1 M NaCl out of the 323 proteins identified, as summarized in **Table 1**. Besides that, we include in this set proteins that were unique in Control and Treatment samples.

We conducted Gene Ontology (GO) terms enrichment analysis for all 323 proteins and the 61 DEs to annotate their molecular function and to describe the possible biological role of proteins using the GO categories Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) with analysis of the distribution of proteins identified into their corresponding GO annotations. The results of GO annotation of the DEs and unique proteins

indicated that their annotation are mostly related to the categories of process biosynthetic metabolic (ribosomal proteins in translation section in **Table 1**) and complex nucleosome assembly (Histone H1 - Cre13.g567450.t1.2 and Cre06.g275900.t1.2) (**Table 1** and **Figure 3A**). In the CC category, DEs were mainly enriched in nucleus annotation category, as expected, and in terms of MF, the DEs were mainly associated with nucleobase containing compound kinase activity, represented by Cre16.g654300.t1.2 e Cre01.g029750.t1.1 (**Figure S3**).

Figure 3. Functional classification of overrepresented Gene Ontology (GO) terms in the dataset of total and differentially proteins

A: Bar plot showing a comparison of the percentage distribution of the GO terms categories retrieved for the datasets of total and DEs proteins; **B**: Networks of the enriched Biological Process GO terms in the analysis of the differentially expressed proteins using BiNGO. Illustration of downregulated gene GO enrichment category of Biological Process (BP). The color gradient of distribution networks is the enrichment scale (*orange* stands for the highest significance of enrichment and *yellow* the minimum significance of enrichment above the cutoff (FDR corrected = 0.05) and node size represents the GO hierarchy. The threshold of hypergeometric distribution of the functional annotation was set as p-value < 0.05).

The most significant GO categories annotated for the dataset of nuclear proteomics and nuclear DEs are cellular metabolism and translation. The nuclear proteome list of DEs were annotated to the categories translation, nucleolar proteins, cell mobility, and cellular metabolism. Previous studies of the nuclear proteome of *C. reinhardtii* have also shown the presence of flagellar proteins in the nuclear fraction [18]. These proteins are expected to be found in nuclear proteome since they have an association with nuclear transport and even may play role in nuclear function [40]. However, these annotated flagellar proteins were absent in our results from cells cultivated in 0.1M NaCl treatment. This result may be connected to the paralysis or loss of flagella, which is one the first cellular responses to stress [41,42].

Table 1. Differentially expressed nuclear proteins in response to 0.1 M salt stress

Phytozome ID	Phytozome description	SUBA	PredAlgo	NucPred	Fold Change
Uncharacterized					
Cre08.g382150.t1.1#	uncharacterized		Nuclear	Not Nuclear	-15.579728
Cre02.g118750.t1.1#	uncharacterized		Nuclear	Nuclear	-15.490067
Cre14.g610900.t1.1#	PROGESTERONE INDUCED BLOCKING FACTOR 1		Nuclear	Not Nuclear	-14.833532
Cre14.g633950.t1.2	uncharacterized		Secretory Pathway	Nuclear	-0.9458238

Cre02.g095146.t1.1	uncharacterized		Nuclear	Not Nuclear	-0.9081868
Cre16.g687182.t1.1	uncharacterized		Mitochondrion	Nuclear	-0.8512069

Translation and ribosome biogenesis

Cre02.g091100.t1.2#	Cytosolic 80S ribosomal protein L15	Cytosol	Nuclear	Not Nuclear	-16.058077
Cre08.g360900.t1.2#	Cytosolic 80S ribosomal protein S15	Cytosol	Nuclear	Nuclear	-15.928843
Cre06.g264300.t1.2#	Chloroplast ribosomal protein S15	Mitochondrion	Chloroplast	Nuclear	-15.800218
Cre12.g512600.t1.2#	Cytosolic 80S ribosomal protein L18	Cytosol	Chloroplast	Nuclear	-15.686391
Cre10.g417700.t1.2	Cytosolic 80S ribosomal protein L3	Cytosol	Chloroplast	Nuclear	-0.9360618
Cre09.g397697.t1.1	Ribosomal protein L4	Cytosol	Nuclear	Nuclear	-0.9273469
Cre10.g420750.t1.2	Cytosolic 80S ribosomal protein L30	Cytosol	Nuclear	Nuclear	-0.9272525
Cre12.g489153.t1.1	Cytosolic 80S ribosomal protein L31	Cytosol	Nuclear	Nuclear	-0.9179496
Cre01.g011000.t1.2	Cytosolic 80S ribosomal protein L6	Cytosol	Nuclear	Nuclear	-0.8997668

Nucleolar protein - nucleosome biogenesis

Cre06.g260850.t1.2#	Nucleolar protein	Nuclear	Chloroplast	Not Nuclear	-14.923977

Gene ID	Annotation				Value
Cre10.g442000.t 1.2	U3 small nucleolar RNA associated protein 6 (UTP6)	Nuclear	Nuclear	Nuclear	-0.9217898
Cre12.g548000.t 1.2	U3 small nucleolar RNA associated protein 10 (UTP10)	Plasma Membrane	Mitochondrion	Not Nuclear	-0.9089622
Cre12.g525200.t 1.2	Nucleolar protein	Nuclear	Nuclear	Nuclear	-0.9059913
Cre16.g656250.t 1.1	U1 small nuclear ribonucleoprotein	Nuclear	Nuclear	Not Nuclear	-0.9035496
Cre17.g737250.t 1.2	U5 small nuclear ribonucleoprotein protein	Cytosol	Nuclear	Nuclear	-0.8846926
Cre01.g055400.t 1.2	NUCLEOLAR AND COILED BODY PHOSPHOPROTEIN 1	Nuclear	Nuclear	Not Nuclear	-0.8817377

Cell mobility related

Gene ID	Annotation				Value
Cre02.g091700.t 1.2#	Protofilament ribbon protein of flagellar microtubules	Mitochondrion	Nuclear	Nuclear	-16.528622
Cre16.g655750.t 1.2#	Tektin		Nuclear	Nuclear	-16.511042
Cre02.g141350.t 1.2	Nexin dynein regulatory complex 10		Nuclear	Not Nuclear	-0.8690188
Cre12.g536100.t 1.2#	Flagellar Associated Protein 126		Nuclear	Nuclear	-15.967271
Cre10.g427300.t 1.2#	Radial Spoke Protein 2		Nuclear	Nuclear	-15.789686
Cre12.g528000.t 1.2#	Flagellar Associated Protein 303	Golgi	Mitochondrion	Nuclear	-15.748193
Cre01.g025450.t 1.2#	Nexin dynein regulatory complex 9		Nuclear	Nuclear	-15.415973

Cre10.g465250.t1.2#	Radial Spoke Protein 11		Nuclear	Nuclear	-15.266677
Cre16.g654300.t1.2#	Radial Spoke Protein 23	extracellular	Nuclear	Nuclear	-15.187778
Cre12.g556250.t1.2#	Septin like protein		Nuclear	Nuclear	-15.125212
Cre17.g703600.t2.1	Basal body protein		Nuclear	Not Nuclear	-0.9299278

Splicing

Cre12.g513500.t1.2#	Transcription coupled DNA repair protein	Nuclear	Nuclear	Nuclear	-16.807381
Cre04.g226450.t1.1	Nuclear pre mRNA splicing factor	Nuclear	Nuclear	Nuclear	-0.9098076
Cre08.g372800.t1.1	Nuclear pre mRNA splicing factor involved in polyadenylation	Nuclear	Nuclear	Nuclear	-0.907742
Cre14.g621000.t1.2	Pre mRNA splicing factor	Nuclear	Nuclear	Not Nuclear	-0.8972515
Cre06.g259800.t1.2	Cwc22 pre mRNA splicing factor	Nuclear	Chloroplast	Not Nuclear	-0.8688461
Cre02.g075650.t1.2	pre mRNA splicing factor RBM22/SLT11 (RBM22)	Nuclear	Nuclear	Nuclear	-0.8473909

cellular nitrogen compound metabolic process

Cre07.g322176.t1.1	DNA directed RNA polymerase I subunit RPA2 (RPA2	Nuclear	Nuclear	Nuclear	-0.9289727
Cre03.g172950.t1.2	TruB family RNA pseudouridine synthase	Nuclear	Nuclear	Nuclear	-0.915942
Cre01.g051100.t1.2	SNW domain containing protein 1 (SNW1	Nuclear	Nuclear	Not Nuclear	-0.8950347
Cre06.g300750.t1.1	intron binding protein aquarius (AQR)	Cytosol	Nuclear	Nuclear	-0.8681914

Process metabolic cellular					
Cre06.g282800.t1.2#	Isocitrate lyase	Peroxisome	Nuclear	Nuclear	-15.573973
Cre06.g257601.t1.2#	2 cys Peroxiredoxin	Chloroplast	Chloroplast	Nuclear	-15.168163
Cre01.g010900.t1.2	Glyceraldehyde Phosphate Dehydrogenase	Chloroplast	Chloroplast	Nuclear	-0.945159
Cre06.g250200.t1.2	S adenosylmethionine synthase	Cytosol	Nuclear	Nuclear	-0.9164602
Cre06.g278254.t1.1	serine/threonine protein kinase SRPK3 [EC:2.7.11.1] (SRPK3	Nuclear	Chloroplast	Nuclear	-0.9144799
Cre03.g149100.t1.2	Citrate synthase	Peroxisome	Mitochondrion	Nuclear	-0.8881439

Cre01.g044800.t1.2	Pyruvate formate lyase		Chloroplast	Nuclear	-0.9242933
Cre06.g288750.t1.2	Nuclear RNA cap binding protein	Nuclear	Nuclear	Nuclear	-0.8752861

Conserved Domain

Cre01.g035000.t1.2#	periodic tryptophan protein 1 (PWP1)	Nuclear	Nuclear	Nuclear	-15.201243

Ribosome biogenesis

Cre10.g450879.t1.2#	ribosome biogenesis protein SSF1/2 (SSF1_2)	Nuclear	Nuclear	Not Nuclear	-16.726006
Cre07.g314100.t1.2	ribosome biogenesis protein UTP30 (UTP30	Nuclear, Cytosol	Nuclear	Nuclear	-0.8590539

RNA binding/RNA metabolism

Cre12.g528950.t1.1#	Puf protein	Nuclear, Cytosol	Nuclear	Nuclear	-16.174809
Cre04.g213905.t2.1#	RNA binding protein 39 (RBM39)	Nuclear	Nuclear	Not Nuclear	-15.477695
Cre07.g349100.t1.1#	ATP dependent RNA helicase	Nuclear	Secretory Pathway	Not Nuclear	-15.236351
Cre10.g439700.t1.2	conserved expressed putative RNA binding protein	Nuclear	Nuclear	Not Nuclear	-0.9235499

Transcription Factor

Cre09.g393728.t1.1#	SET domain-containing protein 6 (SE TD6)	Chloroplast	Nuclear	Nuclear	-14.438726
Histone					
Cre13.g567450.t1.2	Histone H1	Nuclear	Nuclear	Nuclear	-0.9387458
Cre06.g275900.t1.2	Histone H1	Nuclear	Nuclear	Nuclear	-0.895312

Unique proteins from Control samples

3.5 Prediction of nuclear Transcription factors target genes in *C. reinhardtii* under salt stress

We took all 323 proteins present in the nuclear proteome and searched for known Transcription Factors (TFs) according to the annotation available at the PlnTFDB database (http://plntfdb.bio.uni-potsdam.de/). We identified 19 *C. reinhardtii* TFs, and their amino acids sequences were identified and used on a further searching for their homolog sequences in *A. thaliana*. Their respective cis-element recognition motifs were then searched in PlantPan. Starting with 323 proteins in *C. reinhardtii*, we found 16 *A. thaliana* TFs homologs, all of which featured at least one reported cis-element recognition motifs. With the motifs retrieved, we performed a MEME/MAST analysis to find putative target motifs in the promoter regions of *Chlamydomonas* genes. Overall, we identified 733 unique targets associated to 8 of the TFs identified (**Table S2).**

Table 2. *C. reinhardtii* **Transcription Factors and Transcriptional Regulators homologs identified in** *A. thaliana*

C. reinhardtii Phytozome gene ID	TF family[1]	*A. thaliana* homolog TAIR ID[2]	Motif ID[3]	Number of targets[4]
Cre01.g062172.t1.1	CCAAT	N / A		
Cre02.g073650.t2.1	**Top**	N / A		
Cre02.g115250.t1.1	**Top**	N / A		
Cre03.g152150.t1.2	C2H2	AT5G04390.1	TFmatrixID_0216, TFmatrixID_0953	3
Cre03.g176651.t1.1	**MYB-related**	AT3G09600.2	TFmatrixID_1236, TFmatrixID_1247, TFmatrixID_1521	11
Cre03.g197350.t1.2	**MYB**	AT1G09770.1	TFmatrixID_0099, TFmatrixID_0524, TFmatrixID_0547	115
Cre06.g250950.t1.2	C3H	AT5G42820.2	TFmatrixID_0992	0
Cre06.g261450.t1.2	HMG	AT4G11080.1	TFmatrixID_1071, TFmatrixID_1072	585
Cre06.g264750.t1.2	CCAAT	AT1G54830.3	TF_motif_seq_0257	0
Cre06.g268600.t1.2	CSD	AT2G21060.1	TFmatrixID_0222	0
Cre07.g344050.t1.1	Top	AT3G54320.3	TFmatrixID_0516, TFmatrixID_0539	0

Cre09.g393728.t1.1	SET	AT1G12890.1	TFmatrixID_0053, TFmatrixID_0075, TFmatrixID_0101	0
Cre10.g446900.t1.2	Top	AT2G31370.5	TFmatrixID_0188	0
Cre10.g455600.t1.1	PHD	AT3G19510.1	TFmatrixID_0285	0
Cre10.g466250.t1.2	**Top**	N / A		
Cre11.g482700.t1.2	HMG	AT1G04880.1	TFmatrixID_0788, TFmatrixID_0793	17
Cre12.g508150.t1.2	SNF2	AT5G08520.1	TFmatrixID_0356, TFmatrixID_1252, TFmatrixID_1257	3
Cre12.g520650.t1.2	TUB	N / A		
Cre13.g570050.t1.2	CCAAT	N / A		
Cre14.g620850.t1.2	bHLH	AT1G69010.1	TFmatrixID_0162, TFmatrixID_0801, TFmatrixID_0802	0
Cre16.g672300.t1.2	HMG	AT4G11080.1	TFmatrixID_1071, TFmatrixID_1072	585
Cre17.g702650.t1.1	HMG	AT4G11080.1	TFmatrixID_1071, TFmatrixID_1072	585

Differentially expressed proteins($p \leq 0.05$) in the nuclear proteome are highlighted in **Bold font.** 1) TF/TRs and their respective families identified with PlnTFDB v3.0. 2) BLASTP hit with the highest bit-score (e-value ≤ 1) for Arabidopsis thaliana (sequences source: Phytozome v13); non-TF hits omitted for clarity; marked as "N / A" if no valid hits.

3) Motif(s) reported on the PlantPAN 3.0 database. 4) Number of target motifs found using MAST software (v5.4.1), hit e-value ≤ 1.

4. Discussion

4.1 Proteomic analysis of *C. reinhardtii* nuclear proteins in salt stress

In this report, we present the characterization of the nuclear proteome of *C. reinhardtii* under salt stress by supplementation of cell culture with 0.1 M NaCl for 24hours. The increase of lipids has been previously reported in salt stress conditions with 0.2 M NaCl [16,39]. Under salt stress 0.1 M NaCl cells have been reported to increase 50-fold their Triacylglycerol (TAG) content in a wild-type CC124 *C. reinhardtii* [43]. However, the regulatory aspects of the response are not yet completely understood. Therefore, we performed the nuclear proteome analysis of *C. reinhardtii* cells cultivated with supplement of 0.1M of NaCl, which did not affect the cell growth but stimulated the increase in lipids accumulation.

In our analysis, we identified 323 proteins, with 68% predicted to be nuclear using the SUBA database. We found that 19% of the proteins present in our sample were annotated to chloroplast, which is somehow expected that protein contaminants from the chloroplast, the largest organelle that occupies almost half of the cell volume. Therefore, even without observing visible contamination of chloroplast in our fluorescence microscopy analysis of isolated nuclei we believe that some portion of the proteins identified may be derived from organelles other than nucleus. PredAlgo and NucPred analyses revealed a great enrichment of nuclear proteins (about 58 %) in our nuclear proteome with just a few protein contaminants

from other cellular compartments. Notably, the presence of such contaminants did not impact further analysis, and it is similar to the results observed in previous publications [18,19]. Nevertheless, proteins present in both nuclear and cellular fractions (**Figure 2B**) may also be cytoplasmic proteins that may exert their function in the nucleus at some point during the cellular response to salt stress.

The nucleus is a dynamic organelle, changing its proteome in response to intracellular and environmental stimuli [44]. The characterization of proteins under salt stress conditions can provide novel information about mechanisms of the lipid's accumulation and adaptation to salt stress at the molecular level. The function of most proteins found in the nuclear proteome seems to be related to cellular metabolism (nitrogen/organic cyclic compound metabolic process) and translation. In the set of DE proteins, we found the proteins related to translation, nucleolar proteins, cell mobility, and cellular metabolism downregulated or unique in the non-stressed sample (Control), indicating targets proteins that may be involved in the regulation of ribosome assembly under abiotic stress. At 24h under salt stress, even though the cells did not show significant alteration of cell growth rate, it seems that cells already respond modulating the cellular machinery of protein synthesis.

Among the differentially expressed proteins identified, we highlight the several ribosomal and nucleolar proteins observed. Although their presence is expected in a nuclear isolate their differential expression indicates a drastic change in the ribosomal protein profile, which is known to influence translational efficiency [45,46]. This has been reported to have a regulatory role in other plant models, especially in stress response mechanisms [47–50]. In *Chlamydomonas*, specifically, drastic changes in ribosomal protein expression have been observed to accompany transcriptome changes in other phenotypes such as nutrient

deprivation responses [18,51–53] suggesting the possibility for similar regulatory mechanisms for salt stress and other stress-related responses.

This translation-level regulatory response is further supported by the identification of a range of differentially expressed pre-mRNA splicing factors. However, alternative splicing is unlikely to fully explain these findings, as only roughly 10.1% of *Chlamydomonas*'s protein-coding loci have annotated alternative transcripts as of the latest genome annotation [13,54] compared to ~30% for flowering plants [55]. Alternatively, the changes in pre-mRNA processing introduced by these differential proteins could be related to non-coding RNAs (ncRNAs), which have been found to act in the regulation of certain stress response behaviors and chloroplast metabolism in *Chlamydomonas* [56–59]. Along with the changes observed in ribosomal proteins, these nuclear proteome findings highlight the early regulatory shift into a sustained, long-term salt stress response phenotype, markedly characterized by alterations in ribosome-related mechanisms and protein translation rate, even though the results observed for treatment condition did not indicate significant changes in the cell growth performance.

4.2. Transcription factors

Among the proteins identified on the nuclear proteome, we also observed 15 TFs and 7 TRs providing new insights into the regulatory cascades that establish the middle and long-term response of *C. reinhardtii* to salt stress beyond the 24h time point analyzed here. These regulatory proteins feature limited experimental characterization in the literature, as most *Chlamydomonas* TFs and TRs [60,61], so their confirmed presence in the nuclear proteome under salt stress is deeply impactful. Particularly, we bring attention to the five differentially

expressed TFs (**Table 2**), split among the Top and MYB families. Members of the later, in particular, are well known in the literature for their roles in various biotic and abiotic stress responses in other plants [62–64]. The two downregulated MYB TFs we found, Cre03.g176651.t1.1 and Cre03.g197350.t1.2, have been previously identified as histone H2A deubiquitinase MYSM1 and Cell Division Cycle 5 (CDC5) [65,66] respectively. As a histone deubiquitinase, MYSM1 acts in transcriptional regulation through chromatin modifications, but specifically features limited characterization in plants as very few plant species have been found to possess MYSM1-like proteins [67,68] thus, this differential expression of *C. reinhardtii* MYSM1 under salt stress may be vital to understanding its role and what processes it may be involved in. On the other hand, CDC5 is well characterized in land plants and eukaryotes in general as a crucial regulator of the cell cycle [69]. More interestingly, *Chlamydomonas* CDC5 mutants have been shown to accumulate neutral lipids [66] in a manner similar to what we observed under salt stress, further corroborating this protein's crucial role we propose herein.

Furthermore, the TRs with the highest number of identified target genes – (Cre06.g261450.t1.2, Cre16.g672300.t1.2, and Cre17.g702650.t1.1) were all three mapped to a single *A. thaliana* TR homologue sequence (AT4G11080), with high conservation of sequences on and around their high mobility group-box (HMG_box, PF00505, http://pfam.xfam.org/) motifs. This motif is known to interact with DNA, both in sequence-specific and nonspecific manners, and in some cases mediate chromatin remodeling processes [70,71]. Furthermore, the HMG-box-containing proteins which are found in plants are divided into four main groups: 1) HMGB proteins; 2) structure-specific recognition protein 1 (SSRP1) homologs; 3) proteins containing both an HMG-box domain and AT-rich

interaction domain (ARID); and 4) proteins containing three HMG-box domains ($3 \times$ HMG-box). The Arabidopsis protein in question, AT4G11080, has recently been identified as one of the two $3 \times$ HMG-box proteins in this species, and found to specifically interacting with mitotic and meiotic chromosomes [72].

While *Chlamydomonas* does not feature, from our knowledge, any $3 \times$ HMG-box proteins, the three TRs identified in our proteomics analysis show relatively high conservation on and around their HMG-box domains, as well as on the N-terminus (data not shown). This leads us to believe that the reported recognition motif for AT4G11080 might be reasonably conserved in *Chlamydomonas*, providing the most promising insight into the biological role of the four algae TRs, which have very limited functional characterization. Analyzing the 585 gene targets identified for these TFs, we observed an enrichment of GO annotation terms related to kinase activity and protein modification, which may be indicative of the signal transduction processes necessary to sustain over time the metabolic changes observed that activates lipids accumulation while minoring affecting the cell growth (**Figure S4**). Further functional characterization of these target genes is necessary, and it would bring more details on stress-related signaling cascades in Chlamydomonas and novel targets for biotechnological applications.

5. Conclusion

In this study, we demonstrated that mild osmotic abiotic stress triggered enhanced lipid biosynthesis in *C. reinhardtii*, while not impairing cell growth. Triggering such a physiological status is highly desirable for biomass production towards sustainable lipids accumulation, thus, bioenergy production. In addition, we provided important insights into

the nuclear proteome of this biomass-producing phenotype of *C. reinhardtii*. Interestingly, we identified 61 downregulated DEs proteins related to functions like translation and nucleosome assembly under salt stress. We anticipate that our results correlating the alterations of abundance for some nuclear proteins during the short-term acclimation of cells to salt stress and the increase of neutral lipids production will contribute to the studies of bioenergy production from *C. reinhardtii* and it highlights the potential of salt stress as a viable industrial approach for inducing lipids accumulation. However, further studies must be performed to identify and characterize the function of some target differential regulated proteins to address their regulatory role in this type of stress response.

5. Supplementary Materials

Figure S1. Analysis of the structural integrity of the isolated nucleus of *C. reinhardtii* under 0.1 M NaCl salt stress

Figure S2. Reproducibility between replicates of proteome datasets.

Figure S3. Functional classification of overrepresented Gene Ontology (GO) annotation terms in the dataset of differentially expressed (DEs) nuclear proteins under salt stress (0.1 M NaCl) according to BiNGO (Cytoscape software) tool.

Figure S4. Functional classification of overrepresented Gene Ontology (GO) terms in the dataset of putative targets of Cre17.g702650.t1.1 according to BiNGO (Cytoscape software) tool.

Table S1. All proteins identified in nuclear proteome of *Chlamydomonas reinhardtii* in salt stress.

Table S2. *C. reinhardtii* targets for the Transcription Factors and Transcriptional Regulators homologs identified in *A. thaliana.*

Table S3. ProteinGroups file of protein identification without data pre-processing.

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8. Author Contributions

Conceptualization, L.O.M., F.N.M and F.V.W.; Methodology, L.O.M. and F.N.M.; Formal Analysis, L.O.M., F.N.M., F.V.W.; Investigation, L.O.M, F.N.M. and F.V.W.; Resources, F.V.W.; Writing – Original Draft Preparation, L.O.M., F.N.M.; Writing – Review

& Editing, L.O.M. and F.V.W.; Visualization, L.O.M. and F.N.M.; Supervision, F.V.W.;

Project Administration, F.V.W.; Funding Acquisition, F.V.W."

9. Data availability statement

All proteomic raw and processed data was submitted to the ProteomeXchange repository under Submission Reference: 1-20220329-125025; Submission Path: /nfs/pride/drop/pride-drop-005/flaviavw_20220329_123940. The complete file of proteinGroups identified by Mass Spectrometry containing the protein identification and expression data without data pre-processing or data filtering is available in **Table S3**.

10. Conflicts of interest

The authors declare no conflict of interest.

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