Expression of lipid metabolism-related genes in Chlamydomonas reinhardtii treated with lipid-inducing small molecules

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Abstract

A large scale in vivo high throughput screen was performed to identify small molecules that induce lipid accumulation in the model organism *Chlamydomonas reinhardtii*. From a screening library of 43,736 compounds, a total of 367 active compounds were identified that induce lipid accumulation to at least 2.5-fold compared with controls. Of these, 4 compounds were selected for gene expression analysis. Samples were collected every 24h for 72h. Growth and lipid accumulation (Nile Red) were recorded. Relative expression of 18 genes were analyzed via qPCR from 3 biological replicates. The final growth of compound treated cells was on average 90% over controls, 2-fold higher than nitrogen-starved cells, and lipid accumulation was up to 6-fold higher than controls. Via hierarchical clustering and principal component analysis (PCA), it was shown that compound treatment and nitrogen starvation have different effects on lipid metabolism-related gene expression. Unlike nitrogen starvation, the compound treatments do not suppress de novo fatty acid synthesis and have less repressive effects on photosynthesis. Both nitrogen starvation and compound treatment increased expression of diglyceride acyltransferase, which catalyzes the committed step in TAG synthesis. It is proposed that citrate efflux from mitochondria may play an important role in the lipid accumulation induced by the compounds.

Background

Microalgae, a very large and diverse group of photosynthetic organisms, have attracted global attention as a renewable energy feedstock. Previous studies conclude that nutrient stresses, especially nitrogen starvation, induce significant lipid accumulation that might be used for the production of biofuels. However, nitrogen starvation also causes in degradation of the photosynthetic apparatus, severely limiting the rate of growth. Additionally as a lipid induction method, nitrogen limitation is impractical in large-scale production due to technical and financial drawbacks. This led us to develop a high throughput screening (HTS) system, which we have employed to identify synthetic chemical compounds that increase lipid production without severely compromising cell growth or photosynthetic capacity.

Real-time quantitative PCR (qPCR) has become the gold standard for quantifying differential gene expression between samples over the past 10 years. Although the $2^{-\Delta\Delta Ct}$ method is most commonly used, large errors can be introduced by baseline subtraction and amplification efficiency estimation. In this study, we employed an algorithm proven to be effective in previous studies to analyze the raw data (shown in Study Design below). In addition to quantification, accurate liquid handling has always been critical in qPCR experiments. Therefore, an automated liquid handling system was used to prepare samples for qPCR to minimize human errors.

In addition to comprehensive differential analysis, we focused on the metabolism of citrate, which is transported across mitochondrial membrane and then cleaved by ATP-citrate lyase into oxaloacetate and acetyl-CoA, a substrate for *de novo* fatty acid biosynthesis.

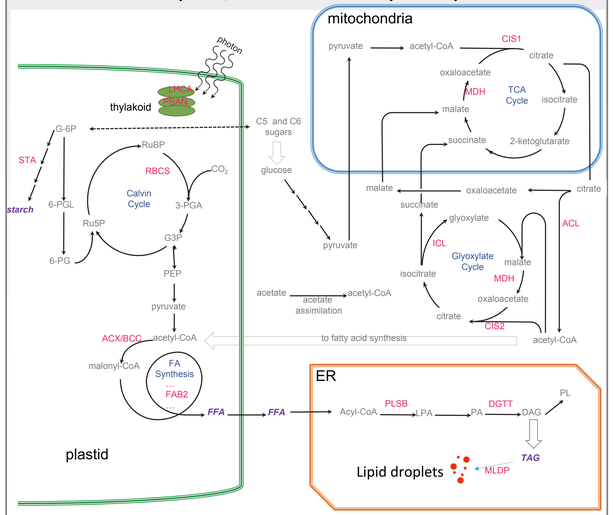
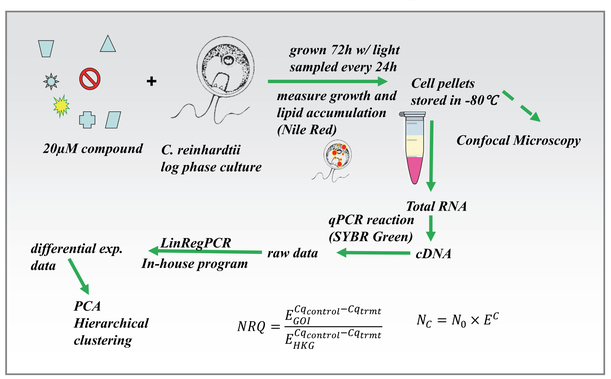


Figure 1. Lipid-metabolism related pathways in *C. reinhardtii*. The differential expression of genes (in pink) were analyzed to give a snapshot of the network.

Study Design



Results

1. Growth and lipid accumulation

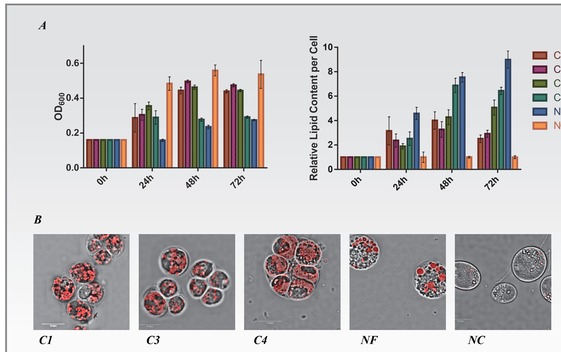


Figure 2. Growth and relative lipid content of cells with different treatments for 72h. (A) Cells in log phase were washed 2× with TAP or TAP N- media respectively. Compounds were added to a final concentration of 20 μM in the media. For each treatment and control, three biological replicates of 100 mL each in 250 mL flasks were grown with shaking under white light. 25 mL of cultures were collected at 24h, 48h and 72h of incubation. Cells were stained with Nile red and fluorescence signal measured with a BioTek Synergy plate reader. **(B)** Confocal images of algal cells stained with Nile Red to visualize lipid bodies. C1-C4: compound #1-4; NF: nitrogen-free; NC: negative control.

2. Overview of qPCR amplification data

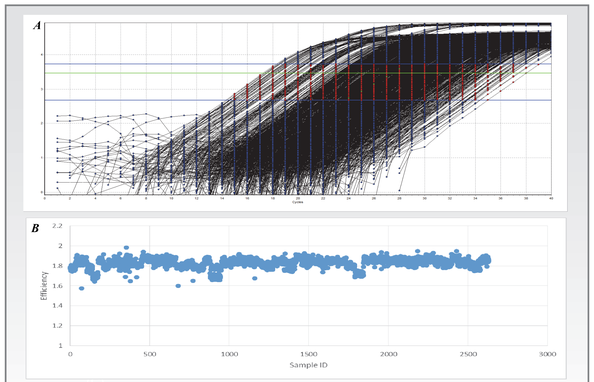


Figure 3. Assessment of gene expression after compound treatment using QPCR. (A) qPCR plots on log scale**.** The raw amplification data of all the samples was combined and analyzed with LinRegPCR. The baseline was subtracted and efficiencies calculated from common window-of-linearity (red points in the plot) per amplicon group. The mean efficiency of each amplicon group was used for quantification. **(B)** Amplification efficiencies of individual samples. Average efficiency >1.8 (90%) indicates good amplification. **(C)** Eppendorf epMotion 5075 automated liquid handling system.

3. Target information

Gene	Protein	Accession
TCA		
CIS1	citrate synthase (mitochondrial form)	XM_001702931
MDH	malate dehydrogenase	XM_001693066
ACL	ATP citrate lyase, subunit A	XM_001700848
Glyoxylate Cycle		
ICL	Isocitrate Lyase	XM_001695279
CIS2	citrate synthase (glyoxysomal form)	XM_001695519
Photosynthesis		
RBCS	RUBisCO small subunit 2	XM_001702356
LHCA1	light-harvesting protein of photosystem I	XM_001695283
PSAN	photosystem I reaction center subunit N	XM_001701648
TAG Synthesis		
DGTT	diacylglycerol acyltransferase (DGAT2)	JN815266
PLSB	glycerol-3-phosphate acyltransferase	XM_001694925
MLDP	major lipid droplet protein	XM_001697616
Starch Synthesis		
STA	ADP-glucose pyrophosphorylase small subunit	XM_001691802
Fatty acid biosynthesis		
ACX	acetyl-CoA carboxylase	XM_001696893
BCC	acetyl-coa biotin carboxyl carrier	XM_001700390
FAB2	plastid acyl-ACP desaturase	XM_001691545
Stress Response		
APG	Autophagy-related protein 8 (ATG8)	XM_001699138
BIP	binding protein 1 (HSP70-like protein)	XM_001701633
Reference Gene		
RACK1	receptor of activated protein kinase C 1 (ribosomal protein)	XM_001698013

Figure 4. Information on selected targets for qPCR analysis. 2-3 genes each from several pathways related to lipid metabolism were selected (see Fig. 1). All sequences can be found in RefSeq database of NCBI except for DGTT. The primers were designed using NCBI Primer-BLAST online tool and specificities checked in RefSeq database within the taxonomy of *C. reinhardtii*. All the amplification products have sizes between 80-150 bp. RACK1, a housekeeping gene used in this study had stable expression levels among the different treatments.

4. Hierarchical Clustering and Principle Component Analysis

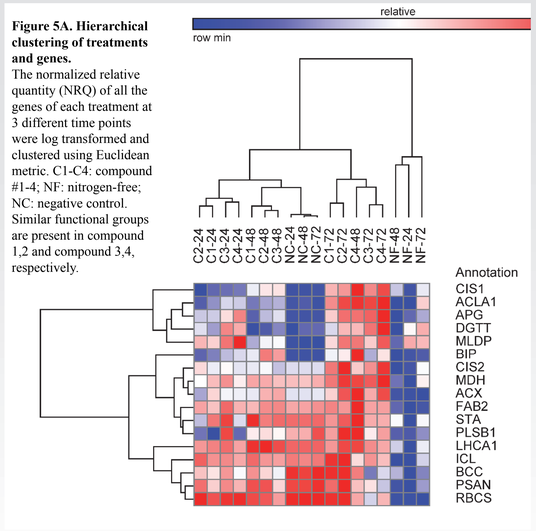


Figure 5A. Hierarchical clustering of treatments and genes. The normalized relative quantity (NRQ) of all the genes of each treatment at 3 different time points were log transformed and clustered using Euclidean metric. C1-C4: compound #1-4; NF: nitrogen-free; NC: negative control. Similar functional groups are present in compound 1,2 and compound 3,4, respectively.

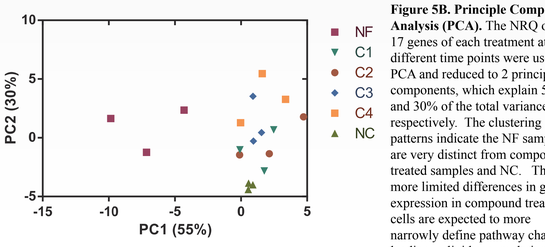


Figure 5B. Principle Component Analysis (PCA). The NRQ of all 17 genes of each treatment at different time points were used in PCA and reduced to 2 principal components, which explain 55% and 30% of the total variance, respectively. The clustering patterns indicate the NF samples are very distinct from compound treated samples and NC. Thus, more limited differences in gene expression in compound treated cells are expected to more narrowly define pathway changes leading to lipid accumulation.

5. Differential expression of selected genes

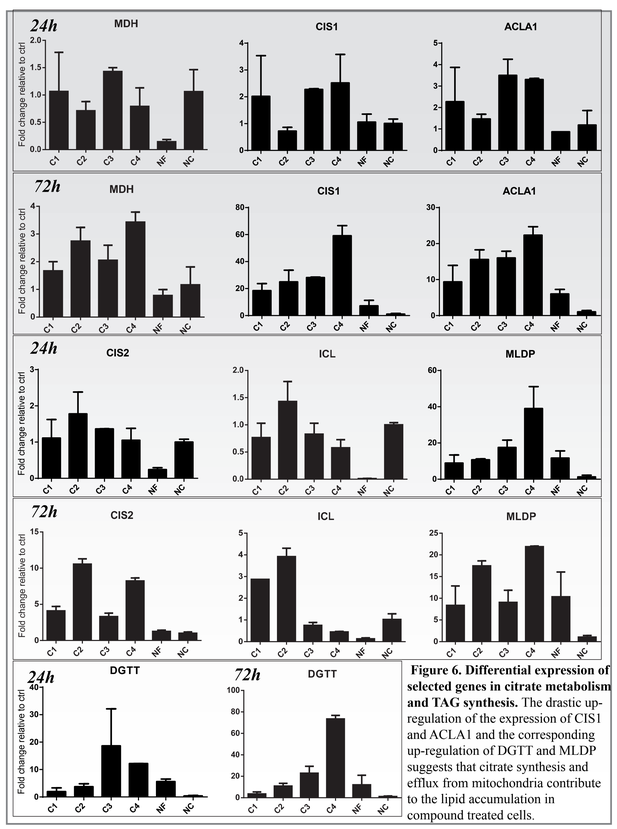


Figure 6. Differential expression of selected genes in citrate metabolism and TAG synthesis. The drastic up-regulation of the expression of CIS1 and ACLA1 and the corresponding up-regulation of DGTT and MLDP suggests that citrate synthesis and efflux from mitochondria contribute to the lipid accumulation in compound treated cells.

6. Summary of Expression Analysis All Genes

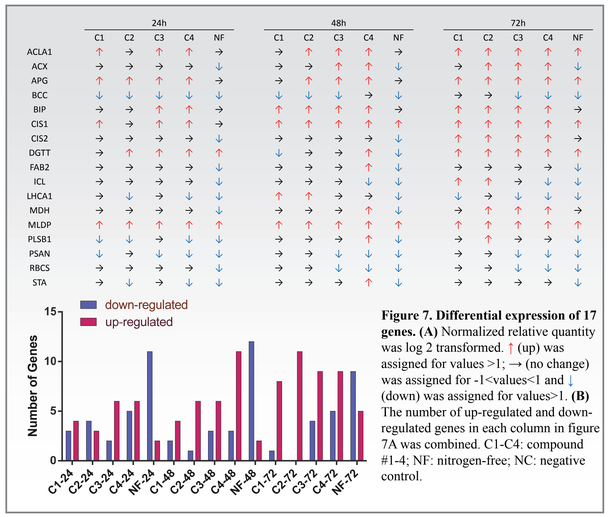


Figure 7. Differential expression of 17 genes. (A) Normalized relative quantity was log 2 transformed. ↑ (up) was assigned for values >1; → (no change) was assigned for -1<values<1 and ↓ (down) was assigned for values>1. **(B)** The number of up-regulated and down-regulated genes in each column in figure 7A was combined. C1-C4: compound #1-4; NF: nitrogen-free; NC: negative control.

Conclusions

- Each compound induced lipid accumulation up to 6-fold higher than control
- No compound severely compromised growth, whereas nitrogen deprivation did
- Compound treatment and nitrogen starvation have different effects on the regulation of lipid metabolism-related genes as indicated in PCA and clustering
- Compound treatments did not suppress de novo fatty acid synthesis and induced less down-regulation of photosynthetic genes, such as RuBisCo and light harvesting complex
- Compound 1 and 2 induced the up-regulation of acetyl-CoA carboxylase complex, whereas compound 3 and 4 mainly acted on diglyceride acyltransferase
- Citrate efflux from mitochondria might play an important role in the lipid accumulation induced by compounds as indicated by the increased expression of the citrate synthase and ATP-citrate lyase genes
- Compound treatment may be useful for identifying components and mechanisms that regulate lipid synthesis and can be utilized for biofuel production

Future Directions

- Identified changes in gene expression upon compound treatment from this targeted analyses indicate that unbiased whole transcriptome analysis is warranted
- Confirm the observed alterations in gene expression result in changes in protein levels and activities using western blots and enzyme assays
- Confirm changes in gene expression using targeted proteomics
- Employ targeted metabolomics to acquire more information on physiological changes including increased synthesis of citrate
- Perform pathway analysis and target identification employing bioinformatics tools to inform further genetic analysis for biotechnological applications

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