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Core features of triacylglyceride production in *Ettlia oleoabundans* revealed by lipidomic and gene expression profiling under distinct induction conditions

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ABSTRACT

Many algae produce triacylglycerides (TAG) when starved for assimilable nitrogen. In the model organism *Chlamydomonas reinhardtii*, and probably in other species, nitrogen starvation is also the cue to prepare for sexual reproduction. The ensuing process of gametogenesis entails a host of physiological and gene expression changes, which makes it difficult to distinguish the changes that are peculiar to TAG accumulation from other aspects of gametogenesis. As others have found, we show here that the chlorophyte *Ettlia oleoabundans* produces TAG under conditions of nitrogen deprivation and elevated NaCl concentrations. We exploit these two conditions, and the intermediate response of the organism to intermediate levels of stress, to identify physiological and gene regulatory features in common. Strikingly, TAG levels and chlorophyll concentrations are inversely correlated across both sets of conditions. Similarly, membrane lipids undergo related compositional changes under the two conditions. In contrast, RNA-seq analysis reveals substantially different expression profiles. This is useful because the gene expression changes that are in common, against this background of expression differences, are more likely to be relevant to the shared changes in physiology and lipid composition. Gene expression changes in common include transcripts related to fatty acid synthesis and degradation, TAG synthesis, and a putative TAG lipase that is substantially down-regulated. The identification of properties that are shared by distinct TAG-inducing conditions could facilitate the engineering of algal strains with improved TAG production properties.

1. Introduction

The production and storage of triacylglycerides (TAG) by algae has been the subject of intensive study [1–3]. The impetus for much of this research has been the prospect of algae becoming a feedstock for biodiesel production that is more sustainable than the likes of soybean and palm oil [4,5]. Substantial challenges remain in achieving this goal [6–10]. However, the production and accumulation of TAGs by algae is a fascinating metabolic phenomenon in its own right. Why is it that cells starved for a nutrient accumulate substantial quantities of such an energy-rich molecule? Why is it that nitrogen starvation appears to be a universal inductive signal for algal TAG accumulation, more so than the limitation of other essential compounds such as phosphate or sulfate?

The TAG-producing species about which we know the most is *Chlamydomonas reinhardtii* [3]. Unfortunately, nitrogen starvation in *Chlamydomonas* is not just an inducer of TAG synthesis, it is also the signal for gametogenesis, a complex set of physiological, morphological, biochemical and gene regulatory processes that prepare *Chlamydomonas* cells for sexual reproduction [11,12]. Whether TAG production and storage is an adaptive part of the sexual life cycle is not clear. For most algal species that produce TAGs upon nitrogen starvation we know nothing about the conditions under which sexual reproduction can occur. It is possible that all of these organisms initiate a process similar to *Chlamydomonas* gametogenesis, but it is also possible that nitrogen starvation and sexual reproduction are uncoupled (if sexual reproduction happens at all). Perhaps TAG synthesis is simply a way of dealing with excess photosynthetic reducing power that, under normal growth conditions, would be put into nitrogen-containing macromolecules such as proteins and nucleic acids. Without TAG synthesis as a sink for photosystem-generated reducing power, reactive

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oxygen species could be more of a problem for the organism.

The challenge in studying TAG synthesis is to separate TAG production and storage from the other physiological and gene regulatory events that occur during nitrogen limitation. Here, we take advantage of a chlorophyte alga, *Ettlia oleoabundans*, that produces substantial quantities of TAGs under two very different conditions: nitrate starvation and elevated concentrations of NaCl. The discovery that *Ettlia oleoabundans* produces TAGs, including the observation that TAGs are produced at moderate salt concentrations and elevated pH, was made previously [13–15]. A partial analysis of lipid composition has been reported for *Ettlia* grown under conditions of elevated pH and ionic strength, and gene expression changes have been reported for cells starved for nitrate [16,17]. However, to our knowledge there has not yet been a comprehensive comparison of lipid metabolism, morphological and physiological changes, and gene regulation performed under different TAG-producing conditions. This is true not just for *Ettlia*, in fact, but for any algal species. In part this is because few species are known to produce substantial amounts of TAG under any condition except nitrogen starvation.

The experiments described here have allowed us to identify a limited number of features that are associated with TAG synthesis in two very different physiological backgrounds. The dissimilarity of the conditions used to induce TAG accumulation, and the differences in the response of the organism to those stresses, gives added weight to those aspects of the response that are in common.

2. Materials and methods

2.1. Strains and cultivation conditions

Neochloris oleoabundans (UTEX # 1185) (now *Ettlia oleoabundans*) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). Cultures were grown photo-autotrophically in a modified Bold Basal Medium with 3-fold nitrogen (9 mM $NaNO_3$) plus vitamins B1 and B12. Cultures were inoculated with 1×10^6 cells/ml late log phase cells into 250 ml Erlenmeyer flasks containing 100 ml of medium and grown at 25 °C under white light irradiation (22–28 μmol photons $m^{-2} s^{-1}$), while being rotated at 100 rpm. $NaNO_3$ and NaCl concentrations were varied as described in the text.

2.2. Chlorophyll measurements

Chlorophyll fluorescence was measured in dimethyl sulfoxide (DMSO) using a fluorescence plate reader (SpectraMax® M5 Multi-Mode Microplate Reader; Molecular devices LLC, California, USA). The excitation wavelength was 430 nm and the detection wavelength was 690 nm. The concentration was determined from a standard curve generated from chlorophyll-*a* obtained from *Anacystis nidulans*.

2.3. Quantitation of neutral lipids by Nile Red

Nile Red fluorescence was used to estimate the quantity of triacylglycerides in cells [18]. Cells were fixed with Prefer solution (Anatech Ltd., Michigan, USA) for 10 min, washed and re-suspended in 50 mM Tris-HCl pH 7.0, and stained with Nile Red (Sigma, Saint Louis, USA) for 10 min in the dark at a final concentration of 2.5 μg/ml. Fluorescence was measured at an excitation wavelength of 488 nm and detected at 575 nm.

2.4. Microscopy

Lipid bodies stained with Nile Red were visualized using a Nikon A1Rsi Fast Laser Scanning and Spectral Confocal microscope (Nikon, Tokyo, Japan). Excitation was at 488 nm; detection employed a 560–615 nm band pass filter. Calcofluor white fluorescence was detected by excitation at 633 nm and emission at 650 nm using a long pass filter.

2.5. Lipid analyses

Triacylglycerol (TAG), phosphatidyl-glycerol (PG), phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE) sulfoquinovosyl-diacylglycerol (SQDG) and monogalactosyl-diacylglycerol (MGDG) were quantified by mass spectrometry (details provided as Supplemental Information). Lipids were extracted as described but using 2:1 chloroform:methanol [19]. Both standards and dried algal extract were dissolved in chloroform:methanol 1:1 (v:v) with a final injection volume of 20 μL. Quantifications were determined using area under curve (AUC) of the relevant ion chromatogram peaks for (a) a SIM analysis for TAG or (b) MRM analysis based on precursor ion to head group fragment transitions for PG and SQDG (81 MRM transitions) and PC and MGDG (22 MRM transitions). Normalizations were performed to internal standards except for MGDG and SQDG for which appropriate standards were unavailable. Standards were purchased from Avanti Polar Lipid: deuterated tripalmitoyl-glycerol (d5-TAG) for TAG; dimyristoylphosphatidic acid (DMPA), dimyristoylphosphatidyl glycerol (DMPG) and dimyristoylphosphatidyl ethanolamine (DMPE) for PG and dimyristoylphosphatidylcholine (DMPC) for PC. For MGDG, MRM transition intensities were normalized to the sum of the intensities for the standard-normalized species. For SQDG the signal was normalized to DMPE which elutes at a similar retention time. Essentially all TAG mass species could be explained by one C16 and two C18 fatty acids of varying saturation, or by two C16 and one C18 fatty acid, consistent with the preponderance of C16 and C18 fatty acids in *Ettlia* [15,20]. The methodology could have detected TAGs with three C16 or three C18 fatty acids had such TAGs been present at reasonable levels but none were found. Further details on the chromatography and mass spectrometry are provided as Supplemental Information.

2.6. Replication of assays

For each of the ten growth conditions, three cultures, grown months apart, were characterized by cell count, mean cell diameter, chlorophyll content, and Nile Red fluorescence. Aliquots were frozen for subsequent analysis by mass spectrometry; preparation of lipids from the frozen aliquots was done in parallel for the different biological replicates to minimize the effects of variation in solvent extraction. The values reported for lipid species, chlorophyll, and Nile Red fluorescence are the means of the three biological replicates, normalized (where described in the text) by cell count, or by both cell count and average cell volume. For each biological replicate, chlorophyll and Nile Red fluorescence values were themselves the means of at least three technical replicates. RNA-seq was performed on a single biological replicate for each of the ten conditions. However, the transcriptome analysis is effectively based on multiple samples because PCA was used to reduce a set of nine values for each transcript (fold-differences in expression relative to control) to just two (PCA dimensions 1 and 2). The values that characterize a transcript's gene expression are, in a sense, based on all nine of the fold-difference values, or an average of 4.5 values for each PCA coordinate.

2.7. Construction of sequencing libraries

Cell pellets were obtained by centrifugation at 10,000*g* for 5 min at 4 °C, snap-frozen in liquid nitrogen and transferred immediately to − 80 °C until ready for RNA extraction. RNA was extracted using a hot phenol method [21], and quality assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Purification of poly-adenylated mRNA RNA was carried out using two rounds of hybridization to Dynal oligo(dT) magnetic beads (Invitrogen, Carlsbad, CA, USA). The resulting mRNA was used to construct Illumina sequencing libraries using the mRNA-Seq Kit (Illumina, San Diego, CA,