Algal Research 33 (2018) 142–155

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Transcriptome analysis reveals the genetic foundation for the dynamics of starch and lipid production in *Ettlia oleoabundans*

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ARTICLE INFO

Keywords:
Microalgae
Nitrogen starvation
Starch
Lipids
Carbon-partitioning
Transcriptome

ABSTRACT

The oleaginous microalga *Ettlia oleoabundans* accumulates both starch and lipids to high levels under stress conditions such as nitrogen starvation (N−). To steer biosynthesis towards starch or lipids only, it is important to understand the regulatory mechanisms involved. Here physiological and transcriptional changes under nitrogen starvation were analysed in controlled flat-panel photobioreactors at both short and long time-scales. Starch accumulation was transient and occurred rapidly within 24 h upon starvation, while lipid accumulation was gradual and reached a maximum after 4 days. The major fraction of accumulated lipids was composed of de novo synthesized neutral lipids - triacylglycerides (TAG) - and was characterized by a decreased composition of the polyunsaturated fatty acids (PUFAs) C18:3 and C16:3 and an increased composition of the mono-unsaturated (MUFAs) and saturated (SFAs) fatty acids C18:1/C16:1 and C18:0/C16:0, respectively. RNA-sequencing revealed that starch biosynthesis and degradation genes show different expression dynamics from lipid biosynthesis ones. An immediate rapid increase in starch synthetic transcripts was followed by an increase in starch degrading transcripts and a decrease in the starch synthetic ones. In contrast, increased gene expression for fatty acid and TAG synthesis was initiated later and occurred more gradually. Expression of several fatty acid desaturase (FAD) genes was decreased upon starvation, which corresponds to the observed changes to higher levels of MUFAs and SFAs. Moreover, several homologs of transcription regulators that were implicated in controlling starch and lipid metabolism in other microalgae showed differential gene expression and might be key regulators of starch and lipid metabolism in *E. oleoabundans* as well. Our data provide insights into the genetic foundation of starch and lipid metabolism in *E. oleoabundans* under nitrogen starvation and should facilitate metabolic engineering towards tailored strains with desired storage compound composition.

1. Introduction

With a growing world population and declining natural oil and gas reserves, the global demand for sustainable and renewable resources becomes ever more relevant. In this respect, microalgae are a promising source for sustainable production of food additives, chemical building blocks and cosmetic ingredients. Microalgae can accumulate high levels of storage compounds such as carbohydrates, lipids and pigments under stress conditions and can serve as a renewable source of proteins. They can be cultivated on marginal lands and in (semi-)arid regions and outdoor cultivation of marine and halotolerant microalgae will require a lower freshwater input. Thereby they compete less with agricultural crop production and have a smaller effect on drinking water supply. Moreover, they are a particularly good substitute for some vegetable oils since the negative environmental impact of e.g. palm, one of the main oil sources currently used in food and cosmetics applications, can potentially be mitigated [1–3]. Nevertheless, the market for most microalgae-derived products currently is not economically competitive with those derived from existing plant-based and petrochemical resources. To overcome this gap, microalgal strain development in combination with improved biorefineries is required [4,5]. Many studies for microalgal strain development have focused on metabolic engineering by the introduction or mutagenesis of single target genes involved in lipid, starch or pigment biosynthesis [6,7] and less on engineering of

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https://doi.org/10.1016/j.algal.2018.05.004
Received 13 December 2017; Received in revised form 11 April 2018; Accepted 4 May 2018

multiple genes or complete pathways [8]. The selected gene targets for metabolic engineering have been identified by transcriptomics or were based on detailed biochemical and genetic information from other microalgal or plant models. Most of these studies focused on microalgae that accumulate only lipids or starch under stress conditions such as nitrogen or phosphorus starvation, while only a few have focused on transcriptomics and metabolic engineering of so-called "hybrid" producers: oleaginous microalgae that are capable of simultaneous accumulation of starch and neutral lipids such as triacylglycerides (TAG). The oleaginous microalgae *Ettlia oleoabundans* and *Acutodesmus obliquus* and several *Chlorella* species are known hybrid producers of industrial relevance that accumulate high levels of both starch and lipids under nitrogen starvation [9–11]. To achieve economically viable production of these hybrid producers multi-product biorefineries and cultivation optimization are important factors, but for many industrial applications it is also desired that carbon-partitioning can be altered to produce mainly TAG or starch and to genetically control the switch between starch and lipid metabolism. The genetic foundation for this dual accumulation strategy however is unknown in most of these microalgae, due to a lack of well-annotated genome sequences and a limited number of transcriptome studies. It is also plausible that the regulatory mechanisms for storage compound accumulation in hybrid producers are more complex than in microalgae mainly accumulating a single storage compound. *E. oleoabundans* is a hybrid producer with several properties that make it a very suitable candidate for industrial production. It can accumulate up to 56% of its cell dry weight as lipids under nitrogen starvation, mainly in the form of TAG, and while isolated as a freshwater strain it can also grow and accumulate storage compounds under saline conditions and high pH as well [12–15]. Furthermore, continuous TAG production in continuously growing cells has been reported [16]. However, the genomic foundation for these favourable industrial traits of *E. oleoabundans* currently is not yet established. The few studies that addressed the transcriptional changes upon nitrogen starvation in this species, have analysed either a single timepoint at an early-stage (< 24 h) [15] or a late-stage (11 days) during batch N-starvation [17] or investigated transcriptomes during nitrogen limitation under turbidostat cultivation [18]. For a full understanding of the cellular and transcriptional changes upon N-starvation it is necessary to track the temporal dynamics of metabolite concentrations, physiological parameters and transcript abundances over both a short and long timespan, to identify key regulatory genes controlling metabolic switch-points. Some recent studies in e.g. *Chlamydomonas reinhardtii*, *Chlorella* spp., *Nannochloropsis* spp. and *Monoraphidium neglectum* have performed this correlation analysis of storage compound accumulation and transcriptome patterns, which indicated possible transcription factors and metabolic nodes that are involved in or even control the "switch" between starch and lipid metabolism [19–24]. In addition, integration of transcriptomics with genome-scale metabolic models could aid in pinpointing the critical metabolic nodes that should be targeted in metabolic engineering.

In this study, we therefore set out to perform an in-depth analysis of the temporal dynamics of storage compound accumulation and transcriptome changes during nitrogen starvation in *E.oleoabundans*, with the aim to identify the metabolic genes and transcriptional factors that are involved in the switch between starch and lipid metabolism in this microalga. Our results revealed the differences in temporal dynamics of gene expression for the starch and lipid pathways upon nitrogen starvation, and identified transcriptional regulators that might be involved. These insights are valuable for rational metabolic engineering in this important industrial microalga.

2. Materials & methods

2.1. Culture conditions and nitrogen starvation experiment

Ettlia oleoabundans UTEX 1185 (culture collection of Algae, University of Texas, Austin) was grown in Bold's Basal Medium (BBM) at pH 7.5 and 25 °C, with 25.2 mM KNO_3 (N-replete conditions) or without nitrate (N-starvation). Culture purity during experiments was monitored by microscopical observation and analysis on a Multisizer™ 3 Coulter Counter® (Beckman Coulter). Experiments were performed in flat panel air-lift photobioreactors (Labfors 5 Lux, Infors HT, Switzerland) with a working volume of 1.7 L, at pH 7.5 and 25 °C, and sparged with 2% CO_2 at an air flow of 1.2 L min^{-1} and photon flux density (PFD) of 800 μmol s^{-1} m^{-2} at a 12:12 light:dark cycle.

The pH was maintained at pH 7.5 by the on-demand supply of 0.1 M HCl. A nitrogen-replete batch pre-culture of *E. oleoabundans* (250 mL) in exponential growth phase was used to inoculate all three photobioreactors, and microalgae were further batch cultivated until a biomass concentration of 2 g cell dry weight (CDW)/L was reached. Subsequently, continuous nitrogen-replete cultivation (N+) was continued for several days until a stable biomass concentration was maintained. For the nitrogen starvation experiment biomass from the nitrogen-replete continuous culture was collected, spun down, rinsed and resuspended in nitrogen-free BBM medium. The reactors were rinsed with distilled water, filled with nitrogen-free BBM medium and inoculated with the same volume of the collected microalgae. During the nitrogen-depleted phase (N−), the reactors were run in non-steady state continuous mode. Medium was fed continuously at 800 mL/d to compensate for the volume-loss due to sampling. Samples for analysis of biomass and cell parameters from the nitrogen-replete continuous phase (N+) were collected at four timepoints in the light-phase preceding nitrogen starvation (−25, −23, −19 and −15 h; Fig. 1). Samples for biomass, cell parameter and molecular analysis from the onset of nitrogen starvation (N−) were collected at time-points at the start, middle and end of each light-phase and at the end of each dark-phase over a 4-day period (0, 2, 6, 10, 23, 29, 33, 46, 53, 71, 77 and 99 h; Fig. 1). Samples were centrifuged, and cell pellets snap frozen in liquid nitrogen and stored at −80 °C. The experiments were performed as three biological replicates.

2.2. Biomass analysis

2.2.1. Cell dry weight, cell count and cell parameters

Cell dry weight (CDW) concentrations were determined as described by Kliphuis et al. [25], by filtering culture broth (around 10 mg of biomass) through pre-dried (100 °C overnight) and pre-weight Whatman glass fibre filter paper (GF/F; Whatman International Ltd., Maidstone, UK). The filter was washed twice with filtered demineralized water to remove adhering inorganic salts and trace elements and subsequently dried overnight at 100 °C before weighing. Cell numbers were determined using a Multisizer™ 3 Coulter Counter® (Beckman Coulter). Quantum yield (QY) was determined using an Aquapen-C (Photon Systems Instruments, Czech Republic).

2.2.2. Total fatty acids

Extraction and quantification of total fatty acids (TFA) were adapted from Breuer et al. [26]. Around 20 mg of pellet was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized overnight. Freeze-dried cells were disrupted by a bead beating step in a Precellys® 24 bead beater (Bertin Technologies) for 2× (3 × 60 s with 120 s pause in between at 2500 rpm), followed by 1× (2 × 60 s with 120 s pause in between at 2500 rpm) in the presence of a chloroform:methanol mixture (1:1.25) to extract the lipids from the biomass. The internal standards C15:0 (tripentadecanoin - T4257; Sigma-Aldrich, St Louis, MO, USA) and C19:0 (trinonadecanoin - T4632; Sigma-Aldrich, St Louis, MO, USA) were added to the extraction mixture to enable fatty acid quantification. Methylation of the fatty acids to fatty acid methyl esters (FAMEs) and the quantification of the FAMEs by GC/MS analysis were performed as described by Breuer et al. [26]. TFA concentration was calculated as the sum of triacylglycerides (TAG) and polar lipids (PL).