Synthetic Biology in Oleaginous Green Algae

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Project Goals: The fundamental research goal of this project is to design and engineer photoautotrophic green algae for scalable production of biofuel precursors and higher value bioproducts. Results from a series of "multi-omic"systems analyses of the model Chlorophyte, *Chlamydomonas reinhardtii*, and an oleaginous relative, *Chromochloris zofingiensis*, have improved our understanding of the genomic basis for remodeling of energy metabolism as a consequence of carbon source. Additional transcriptomic and proteomic analyses of Chromochloris under macro- and micro-nutrient stress conditions will provide information about how the organism maintains photosynthesis. Leveraging discoveries from green algal genomics, transcriptomics and proteomics, we are now building and applying new synthetic biology tools to modify metabolism in the genetically tractable, oleaginous Trebouxiophyte, *Auxenochlorella protothecoides*.

Rationale: Continued combustion of fossil fuels and production of greenhouse gases is incompatible with maintaining a stable climate regime. Various biological systems are being explored as platforms for the sustainable production of replacement fuel and chemical precursors that are commonly derived from petroleum. Crop plants provide readily scalable systems for synthetic biology, but raise concerns around diversion of arable land away from food production. Engineered prokaryotes and fungi have long been used for synthesis of alcohols, carboxylic acids, enzymes, therapeutic proteins and pharmaceuticals, but these organisms require a reduced carbon feedstock for growth. Facilities for large-scale heterotrophic fermentation are expensive and energy-intensive, and it can be difficult to reduce production costs to a sufficiently low level to justify making fuel or industrial biochemicals. Utilization of photosynthetic microalgae as host organisms for bioproduction could bridge some of the problems with crop plant and heterotrophic microorganisms. While it is not possible to achieve the high cell densities of heterotrophic fermenters, many algae have substantially higher photosynthetic productivity per unit area than plants. They can also be grown in marginal areas that are inappropriate for agriculture, and some can tolerate brackish or salt water and so do not increase pressure on potable water sources. While not as inexpensive as planting seeds, capital outlays to build algae ponds and bioreactors are substantially less than for industrial fermentors. Problems with large-scale outdoor algae production include reduced productivity due to self-shading, maintenance of monoculture, predation, and obtaining a positive energy balance when considering mixing, harvesting, de-watering and extraction of energy-rich compounds such as lipids.

Progress: Over the course of this project, systems analyses of the oleaginous freshwater Chlorophyte, *Chromochloris zofingiensis*, during trophic transitions between photoautotrophic growth and heterotrophic growth in the presence of glucose, have revealed the genomic basis for remodeling of cellular metabolism in the presence of different carbon sources[1–3]. Of particular interest to our group is how the organism responds to trace metal starvation, a situation faced by algae in nature and one that limits photosynthetic productivity. We have performed initial physiological characterization of photoautotrophic Chromochloris cells in order to define concentrations of copper, iron and zinc that are limiting, deficient and replete for growth, and we have used ICP-MS measurements to understand how starvation affects cellular quotas of macro- and micronutrients. Transcriptomic and proteomic analyses of how Chromochloris responds to trace metal starvation are in progress.

Chromochloris is a robust producer of triacylglycerides when supplied with glucose or another reduced carbon source. However, the Chromochloris cell wall has hindered efforts to

transform and engineer this species. Therefore, we have developed *Auxenchlorella protothecoides*, an oleaginous Trebouxiophyte that undergoes analogous trophic transitions, as a potential host strain for synthetic biology. We have developed methods for Auxenochlorella transformation, nuclear gene targeting and high-level transgene expression by means of homologous recombination, and we demonstrate the utility of this species as both a reference organism for basic research and as a potential biotechnology platform. To develop the system, we took advantage of public genome sequences for *A. protothecoides* Cp0710/UTEX 25 and UTEX 2341. These haploid assemblies were all highly fragmented, but we were able leverage these sequences to resolve polymorphic diploid alleles at multiple loci for *A. protothecoides* UTEX 250. Public transcriptome datasets were used to improve models for genes involved in central carbon metabolism, fatty acid and lipid biosynthesis, chlorophyll biosynthesis, light-harvesting and photosynthetic electron transfer.

We chose to demonstrate application of the system for reverse genetics and transgene expression by disrupting chlorophyll biosynthesis, since as the primary light-harvesting and photochemical pigment, chlorophyll is fundamental to bioenergy, and chlorophyll biosynthetic mutants have readily scorable, visual phenotypes. The *CHL27* gene encodes the di-iron component of Mg-protoporphyrin IX monomethylester (Mg-PIXMME) cyclase, a key enzyme that catalyzes formation of the chlorophyll isocyclic E ring. Sequential ablation of both *CHL27* alleles resulted in non-photosynthetic mutants that were completely chlorophyll-deficient and accumulated a red intermediate pigment with an absorption spectrum that was consistent with Mg-PIXMME. Chlorophyll biosynthesis and photosynthetic growth were restored to *chl27* double knockouts by knock-in of a synthetic, codon-optimized *CHL27* gene. Knock-ins could be targeted to a neutral locus, *DAO1*, encoding a non-essential D-amino acid oxidase, and expression of the *CHL27* transgene could be driven by any of three strong promoters that were expressed in photoautotrophic cells. Alternatively, knock-ins could be targeted to one of the mutated *chl27* alleles, and *in situ* expression of the transgene was driven by the native regulatory elements at *CHL27*.

We recently reported discovery of evolutionarily conserved polycistronic transcripts in several species of green algae[4]. Auxenochlorella strains transformed with synthetic bicistronic constructs co-expressed both a *SUC2* selectable marker gene, enabling growth on sucrose, and a *BKT1* reporter gene, encoding beta-carotene ketolase and catalyzing synthesis of red keto-carotenoids. *In vivo* comparison of SUC2 and BKT1 activity from polycistronic transcripts driven by two different promoters suggested an inverse relationship between the amount of translation from the upstream and downstream ORFs. This would be consistent with the hypothesis, suggested by *in vitro* results, that the abundance of proteins produced by polycistronic transcripts is determined by the relative strength of the Kozak sequences associated with each ORF. Collectively, these results demonstrate our ability to precisely and reversibly target Auxenochlorella nuclear genome sequences, and to control transgene expression, which will facilitate future metabolic engineering of strains for photosynthetic production of biofuels and value-added bioproducts.

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