

Biological Design of *Lemnaceae* Aquatic Plants for Biodiesel Production

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Project Goals:

- 1. Leveraging our established *Agrobacterium*-mediated transformation methods, we will develop a comprehensive toolset for genetic manipulation of *Lemnaceae*. We will establish CRISPR/Cas9 genome editing to complement our previously demonstrated artificial miRNA silencing methods. We will construct artificial chromosomes in *Lemna minor* to potentiate whole pathway engineering.**
- 2. Resting and over-wintering fronds have higher starch content than corn kernels, but the energy density of oil is more than twice that of starch. We will use regulatory network and metabolic flux modeling to re-engineer the carbon allocation pathways to optimize triacylglyceride (TAG).**
- 3. Enabled by multiple *Lemnaceae* genome sequences and annotations and an extensive living collection of global accessions, we will use comparative genomics and systems network analysis to catalog transcription factors and promoters in gene expression networks underpinning developmental and environmental responses to maximize bioenergy products while preserving rapid biomass accumulation. Nutrient deprivation and CO₂ irrigation will be used to enhance yield.**

Lemnaceae species (commonly called duckweeds) are the world's smallest aquatic flowering plants. They have a much reduced morphology comprising leaf-like growing fronds, starch-filled resting fronds, and simple roots. While they are sometimes regarded as invasive due to their ability to rapidly cover the surface of freshwater ponds in the presence of nitrogen-rich agricultural runoff, duckweeds are native to all continents but Antarctica, and their extreme growth rate is ideal for vast biomass production. *Lemnaceae* in optimal conditions have an exponential growth rate that can double the number of fronds in 30 hours and produce 64 grams of biomass per gram starting weight in a week, which is far beyond that of terrestrial crops such as corn (2.3 g/g /week), and unencumbered by secondary products such as lignin. *Lemnaceae* offer an attractive alternative to algae as biofuel feedstocks because of their robust growth in open ponds and the relative ease of harvesting dry material. Convenient metabolic labeling in culture makes *Lemna* a good system for pathway modeling and engineering, as nutrients are taken up from liquid growth media, and non-responsive stomata can utilize very high levels of atmospheric CO₂. Existing commercial strains of *Lemnaceae* have been optimized for protein production, environmental sensing, or wastewater remediation; we propose to redesign these strains for biofuel production. Our goal is to divert a substantial portion of accumulated carbon from starch to oil metabolism in *Lemnaceae*, using resting fronds as the storage tissue. Clonal propagation, limited seed set, and variable chromosome number are shared with sugarcane and *Miscanthus*, and many of the design principles and technologies we develop will have applications in other energy crops.

Under prior support from DOE, the Long Island Biofuels Alliance has achieved significant milestones in harnessing the potential of duckweed as a bioenergy crop. The Shanklin Lab completed a survey of fatty acid and TAG composition across 30 *Lemnaceae* species, while the Schwender lab has constructed a constraint-based model of carbon flux. A reliable and rapid protocol for stable transformation of *Lemna minor* was published by the Martienssen and Shanklin labs, along with gene-knockdown by artificial

miRNA. The Lam and Martienssen labs have contributed to the sequence and gene content of three *Lemna* genomes complete with chromosome structures, methylomes, small RNA transcriptomes, and structural variant analysis across accessions. Current genome assemblies have yielded validated orthologs in all the major lipid biosynthesis pathways. Critically, we have already developed engineered *L. minor* exhibiting a significant increase in oil content, building on the successful engineering of sugarcane to achieve 2-5% leaf TAG in the Shanklin lab under ARPA-E support. By the end of the 4-year funding period, we will have dramatically increased oil content in fast growing aquatic plants, providing a novel, robust and highly productive source of biodiesel.

Publications

1. Yan, Yiheng, Jason Candreva, Hai Shi, Evan Ernst, Robert Martienssen, Jorg Schwender, and John Shanklin. 2013. "Survey of the Total Fatty Acid and Triacylglycerol Composition and Content of 30 Duckweed Species and Cloning of a $\Delta 6$ -Desaturase Responsible for the Production of γ -Linolenic and Stearidonic Acids in *Lemna Gibba*." *BMC Plant Biology* 13 (1). BioMed Central:201.
2. Cantó-Pastor, A., A. Mollá-Morales, E. Ernst, W. Dahl, J. Zhai, Y. Yan, B. C. Meyers, J. Shanklin, and R. Martienssen. 2015. "Efficient Transformation and Artificial miRNA Gene Silencing in *Lemna Minor*." *Plant Biology* 17 Suppl 1 (January). Wiley Online Library:59–65.
3. Michael, Todd P., Douglas Bryant, Ryan Gutierrez, Nikolai Borisjuk, Philomena Chu, Hanzhong Zhang, Jing Xia, et al. 2017. "Comprehensive Definition of Genome Features in *Spirodela Polyrrhiza* by High-Depth Physical Mapping and Short-Read DNA Sequencing Strategies." *The Plant Journal: For Cell and Molecular Biology* 89 (3). Wiley Online Library:617–35.

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