

Biological Design of *Lemnaceae* Aquatic Plants for Biodiesel Production

Evan Ernst,^{1,2} James Birchler,³ Eric Lam,⁴ Jorg Schwender,⁵ John Shanklin,⁵ and **Robert A. Martienssen*** (martiens@cshl.edu)^{1,2}

¹Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; ²Howard Hughes Medical Institute, Cold Spring Harbor, NY; ³University of Missouri, Columbia, MO; ⁴Rutgers University, New Brunswick, NJ; and ⁵Brookhaven National Laboratory, Brookhaven, NY

Project Goals:

- 1. Leveraging our transformation methods, we will develop a comprehensive toolset for genetic manipulation of *Lemnaceae*. We will establish CRISPR/Cas9 genome editing to complement our 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. We will use comparative genomics of multiple *Lemnaceae* genome sequences, an extensive living collection of global accessions, and systems network analysis to characterize 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. *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. 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 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.

We have performed single molecule long-read genome sequencing of diploid *L. gibba* and allotetraploid *L. minor* clones, using Oxford Nanopore technology followed with Hi-C to link scaffolds into chromosome-scale assemblies. The 21 chromosomes of diploid *L. gibba* are highly colinear with each of the subgenomes of allotetraploid *L. minor*. A comparison with genome sequences of *S. polyrhiza* and *W. australiana* reveals similar gene content that is highly reduced compared to terrestrial monocots such as rice and Brachypodium. Whole methylome sequencing has shown a dramatic reduction in asymmetric cytosine methylation in *Lemna* spp., which is similar to *Spirodela polyrhiza* in this respect. *Spirodela* has a much-reduced retrotransposon content, which accounts for further reductions in symmetric CG methylation, while *L. minor* retains a similar retrotransposon content to other monocot genomes. Small RNA sequencing has revealed dramatic differences between the three genera consistent with known pathways of RNA directed DNA methylation. We have analyzed orthologous gene content across the *Lemnaceae*, 17 other monocots and 11 non-monocots, revealing variations that likely account for some of these differences, as well as for reduced morphology, clonal reproduction, and aquatic growth habit. With these tools at hand we will be able to more easily identify gene-regulatory bottlenecks limiting oil production.

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. Engineered lines include stable overexpressors of WRINKLED, DGAT and PDAT1, all exhibiting marked increases in TAG content. We are addressing expected growth defects in the lines by developing CRISPR/Cas9 knockouts targeting SDP1, and multigene overexpression lines including OLE1 which have proven to mitigate FA cytotoxicity in other systems. The groundwork for construction of artificial chromosomes and transgene stacking systems is being established by the introduction of a landing pad construct developed in the Birchler Lab.

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 Polyrhiza* 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.

Funding for this project is provided by the DOE Office of Biological & Environmental Research (DE-SC0018244).