Duckweed (Lemnaceae): Biological design for alkane and biodiesel production

Rob Martienssen, Evan Ernst, Almudena Molla-Morales, Alex Canto Pastor, Will Dahl, Seung Cho Lee
Duckweed (Lemnaceae): A clonal aquatic flowering plant
Existing energy crops

- Corn/sugarcane/swichgrass
  - Compete with food crops
  - High energy input
  - High lignin content
  - Production of bioethanol

- Algae
  - High biomass output
  - Difficult to harvest
  - Contamination problems
  - Can produce oil
Replacing fossil fuels

<table>
<thead>
<tr>
<th>Biofuel feedstock</th>
<th>Fossil energy balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulosic ethanol <em>(Miscanthus</em> grass)</td>
<td>2 -36</td>
</tr>
<tr>
<td>Palm oil</td>
<td>9</td>
</tr>
<tr>
<td>Ethanol (sugarcane)</td>
<td>8</td>
</tr>
<tr>
<td>Rapeseed (canola) oil</td>
<td>2-3</td>
</tr>
<tr>
<td>Corn ethanol</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The best energy crops are clones……..
Oil palm is the most productive oil crop...

But competes with rainforest and food production.
The oil palm *SHELL* gene controls oil yield and encodes a homologue of *SEEDSTICK*

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**Figure 1**

A diagram showing the comparison between Dura, Pisifera, and Tenera genotypes in terms of gene expression. The graph illustrates the expression levels of various genes during oil accumulation in kernel development.
Lemnaceae (duckweed) are the world’s smallest, but fastest growing aquatic flowering plants. Used for basic research, environmental monitoring and waste water remediation. Very high rates of biomass accumulation make them an attractive target for engineering biofuel feedstocks.
**Lemnaceae** as energy crop

- Does not compete with food production
- Can be grown on wastewater
- Rapid growth
- Low lignin content
- Long production period
- Spread all over the world
- Cheap and easy to grow
Lemnaceae (family Araceae, monocots)
Lemna gibba flower

pollen

style

pollen

Lemna gibba flower
Lemnaceae

Liopsida class
Aridae subclass
Arales order
Araceae family

Wolfia microscopica

- Reduced morphology
  - Fronds (leaf-like structures)
  - Meristem-like stem cell “pocket”
- Clonal reproduction
  - 48 hours duplication by budding
  - Limited flowering

Spirodela polyrhiza
  (Landolt, 1986)
Lemnaceae

- Wide natural variation:
  - Growing rate
  - Starch 12-48%
  - Protein 11-40%
  - Lipid 2-9%
  - Low lignin content (5%)

- Needs genetic modification for lipid production
Chemical properties of biodiesel

The transesterification process:

- Triglyceride + methanol → Catalyst + heat → Glycerine + methyl ester

Table 1. Fuel Properties as a Function of Fuel Composition in Diesel Engines

<table>
<thead>
<tr>
<th>Chemical property</th>
<th>Saturated 12:0, 14:0, 16:0, 18:0, 20:0, 22:0</th>
<th>Monounsaturated 16:1, 18:1, 20:1, 22:1</th>
<th>Polyunsaturated 18:2, 18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetane number</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Cloud point</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Stability</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>NO\textsubscript{x} emissions</td>
<td>Reduction</td>
<td>Slight increase</td>
<td>Large increase</td>
</tr>
</tbody>
</table>
Photosynthetic rate responds well to increased Carbon

Andersen, 1985
Our goal

- Develop duckweeds as biofuel feedstock
  - Create/improve molecular tools to study duckweed
  - Study duckweed development and metabolism
  - Generation of transgenic lines for biofuel production
    - Increase expression of genes related to the production of TAG
    - Silence the genes that have a role in the oxidation of lipid bodies
    - Redirect the starch metabolism silencing the key genes that lead to its accumulation
**Lemna gibba genome sequence**

http://www.lemna.org

### Lemna gibba G3 DWC131 Assembly (450 Mbp)

<table>
<thead>
<tr>
<th></th>
<th># &gt;200nt</th>
<th># &gt;100Knt</th>
<th>N50 (bp)</th>
<th>NG50 (bp)</th>
<th>Longest (bp)</th>
<th>Size (Mbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contigs</td>
<td>471,436</td>
<td>-</td>
<td>1,876</td>
<td>1,476</td>
<td>33,244</td>
<td>401</td>
</tr>
<tr>
<td>Scaffolds</td>
<td>140,499</td>
<td>54</td>
<td>16,085</td>
<td>18,907</td>
<td>270,981</td>
<td>507</td>
</tr>
</tbody>
</table>
PICKLE: a regulatory gene for Oil biosynthesis
Molecular tools

Aim:

- Develop a stable transformation protocol suitable for high throughput application
  - Study of promoters for overexpression of genes of interest
  - Design artificial microRNA using endogenous miRNA precursors to silence undesired pathways
Transformation: regeneration

- Green Fluorescent Protein (GFP) gene from Jellyfish
- Efficiency of stable transformants 90%
- 6 week regeneration
- Comparable with Arabidopsis

Alex Cantó Pastor, Almudena Molla Morales
Diverting carbon from starch to oil

Increasing the energy density of vegetative tissues by diverting carbon from starch to oil biosynthesis in transgenic Arabidopsis

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(a) TAG (nmol/mg DW)

WT  5  7  9
35S-WRI1  **  **

B33-WRI1  7  9  10

35S-AGPRNAi  2  5  6

AGPRNAi-WRI1  14  17  28
Climate change: we’ve been here before...

- In the early Eocene (~49 million years ago), atmospheric CO$_2$ concentrations were 5 times current levels.
- Arctic sea surface temperatures averaged 13°C in contrast to today’s -9°C.
- Isolation of the Arctic Ocean from deep water currents led to a surface layer of fresh water.

“Azolla event” & global climate change

- Arctic sediment core samples revealed alternating layers of freshwater *Azolla* fossils measuring 8-20 meters thick.

- *Azolla* blooms alone may have drawn 80% of the CO\(_2\) out of the atmosphere contributing to the climate change that converted the ancient Greenhouse to the current Icehouse.

Azolla on a river in New Zealand.


Effects of Prolonged Near Weightlessness on Growth and Gas Exchange of Photosynthetic Plants

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An experiment was designed to determine the effects of long-duration (30 days) exposure to near weightlessness on growth and gas exchange of the unicellular green alga Chlorella sorokiniana and the giant duckweed Spirodela polyrhiza. Instrumentation was provided for in-flight monitoring of carbon dioxide, oxygen, temperature, and pressure. Transmittance of light through the cultures was measured with photo-cells to indicate relative growth. Twelve hour light-dark cycles and data acquisition were controlled by programmer. The experiment was launched into near circular east-west orbit at Vandenberg Air Force Base on 30 March 1966 as part of the Air Force Office of Aerospace Research nonrecoverable OV-1 satellite program. Data were taken every 3 hours, stored on a satellite tape recorder, periodically transmitted to tracking stations, and accumulated at Cape Kennedy for decommutation. Computer reduction of data was performed at Brooks Air Force Base. Following data reduction, programmed control experiments were performed to simulate conditions, especially temperature, experienced in orbit. The alga experiment developed a gas leak during launch and lost pressure rapidly upon exposure to the vacuum of space. Data from the duckweed experiment were obtained for 230 hours prior to failure of the satellite power system. A nonstatistical comparison of flight and ground control data indicates that photosynthetic and respiratory gas exchange of Spirodela polyrhiza was not affected by exposure to near weightlessness for a period of 230 hours. Accuracy of comparison of flight and ground control data was compromised because of inability to quantitatively duplicate the amount of experimental plant material under conditions required for maintenance of axenic culture.
Conclusions

- *Lemnaceae* are perfect candidates for biofuel production
- The use of *Lemnaceae* as an oil source requires genetic modification
- Genetic tools for *Lemnaceae* transformation are ready
- We are testing strategies for overexpression and silencing of genes involved in TAG and starch metabolism, respectively
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