Objectives:

1. Generate a model for the organization of glycerolipid synthesis
2. Test the validity of the model of glycerolipid synthesis

References:


Glycerolipids

Membrane lipids

Monogalactosyldiacylglycerol (MGDG)
Each Membrane of the Cell has a Unique Glycerolipid Composition

Chloroplast
- Stroma
- Granum (stack of thylakoids)
- Intermembrane space
- Inner membrane
- Outer membrane

Thylakoid
- thylakoid interior
- membrane components required for the light reactions
Glycerolipid Synthesis

Radioactive labeling experiments of leaves using $^{14}$C-acetate lead to the proposal that glycerolipid synthesis proceeds in 2 stages:

1. Sequential addition of fatty acids to glycerol-3-phosphate (G3P)

\[
\text{G3P} \rightarrow 18:1-\text{ACP} \rightarrow \text{ACP} \rightarrow 18:1, 16:0-\text{ACP} \rightarrow \text{ACP} \rightarrow 18:1, 16:0-\text{PA} \rightarrow \text{PA}
\]

2. Addition of the head group (defines the class of lipid)

\[
\text{Headgroup} \rightarrow 18:1, 16:0 \rightarrow \text{Glycerolipid}
\]

The enzymes involved and location of the pathway were not known.
Glycerolipid Synthesis

Additional $^{14}$C-acetate labeling experiments of spinach leaves by Roughan and Slack in the 1970ies led to an observation that there are 2 types of PA in leaves:

- **Prokaryotic** (found in cyanobacteria)
  - PA
    - $18:1$
    - $16:0$
  - MGDG
    - $18:1$
    - $16:0$
    - Gal
  - DGDG
    - $18:1$
    - $16:0$
    - GalGal
  - SL
    - $18:1$
    - $16:0$
    - SQD
  - PG
    - $18:1$
    - $16:0$
    - PG

- **Eukaryotic** (found in eukaryotes)
  - PA
    - $18:1$
    - $18:1$
  - MGDG
    - $18:1$
    - $18:1$
    - Gal
  - DGDG
    - $18:1$
    - $18:1$
    - GalGal
  - SL
    - $18:1$
    - $18:1$
    - SQD
  - PG
    - $18:1$
    - $18:1$
    - PG

$+$ PC, PE, PI
Glycerolipid Synthesis

Hypothesis:
There are two pathways for glycerolipid synthesis in leaves: prokaryotic and eukaryotic. These pathways are likely located in two different cellular compartments.

Model:

Which compartments?
Glycerolipid Synthesis

Hypothesis:

There are two pathways for glycerolipid synthesis in leaves: prokaryotic and eukaryotic. These pathways are likely located in two different cellular compartments.

Model:
Glycerolipid Synthesis

Experimental data:
MGDG, DGDG and SL are found only in the plastid membranes

New model:

Eukaryotic MGDG, DGDG and SL are also made in the plastid from substrate made in the ER
Glycerolipid Synthesis

Plastidial compartment

Extraplastidial compartment

18:1-ACP (16:0-ACP) → ACP
18:1(16:0) → 16:0
16:0 → DAG

18:1-CoA (16:0-CoA) → CoA
18:1(16:0) → 18:1-CoA
18:1 → DAG

CDP-DG → Pi, PPI

PG, SL, MGDG, DGDG

PC, PE, PG, PI
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach

Marker for prokaryotic pathway
What are “18:3” plants?

“18:3” plants only have eukaryotic type MGDG, DGDG and SL.

Glycerolipid Synthesis

“18:3” plants – barley leaves, pea leaves

Plastid

Extraplastidial - ER?
Glycerolipid Synthesis

“18:3” plants – barley leaves, pea leaves

ER

Plastid
Testing the Glycerolipid Synthesis Model

How?

Isolate mutants with defects in glycerolipid synthesis.

Can we isolate such mutants?
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach

Marker for prokaryotic pathway
Testing the Glycerolipid Synthesis Model

How?
Isolate mutants with defects in glycerolipid synthesis.

Can we isolate such mutants?

1. Make predictions concerning the phenotype of mutants lacking activity of enzymes of the prokaryotic pathway for glycolipid synthesis

   • Potential biosynthetic steps affected
   • Fatty acid phenotype of such mutants
   • Lipid composition of such mutants
   • Flux (=carbon flow) through the eukaryotic pathway
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach
Testing the Glycerolipid Synthesis Model

1. Make predictions concerning the phenotype of mutants lacking a functional prokaryotic pathway for glycerolipid synthesis
   - Potential biosynthetic steps affected (act1, act2, pap)
   - Fatty acid phenotype of such mutants (loss of 16:3)
   - Lipid composition of such mutants (loss of prokaryotic type of MGD, DGD, SL, perhaps PG)
   - Flux through the eukaryotic pathway (a lot greater)

2. Isolate mutants with the predicted phenotype
Summary of the Screen for Mutants With Altered Leaf Fatty Acid Composition

~4,000 leaf samples taken from mutagenized ($M_2$) plants analyzed by GC-FID

9 putative mutants identified
## Isolation of Mutants

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<tr>
<th>Fatty acid</th>
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What next? How would you proceed?
Genetic analyses of Mutants Lacking 16:3 Fatty Acid in Leaves

1. Is the mutation heritable?
   Yes - in all mutants that have reduced 16:3 levels (JB67, LK3, JB1, LK9, JB25, LK8)

2. Is the mutation recessive/dominant?
   \( F_1 \) progeny from a cross between each mutant x WT has normal 16:3 levels demonstrating that...

3. Is the phenotype caused by a mutation in a single nuclear gene?
   \( F_2 \) progeny from a cross between each mutant x WT segregates 3:1 (WT:mutant) demonstrating that...

4. Do mutants with a similar phenotype have mutations in the same gene?
## Isolation of Mutants

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Genetic analyses of Mutants Lacking 16:3 Fatty Acid in Leaves

4. Do mutants with a similar phenotype have mutations in the same gene?

F<sub>1</sub> progeny from a complementation cross between:

- JB67 x LK3 -> has wildtype levels of 16:3
- JB67 x JB25 -> has wildtype levels of 16:3
- JB67 x LK8 -> has wildtype levels of 16:3
- LK3 x JB25 -> has wildtype levels of 16:3
- LK3 x LK8 -> has wildtype levels of 16:3
- JB1 x LK9 -> has 3-4% of 16:3
- JB25 x LK8 -> lacks 16:3

demonstrating that...
Genetic analyses of Mutants Lacking 16:3 Fatty Acid in Leaves

4. Do mutants with a similar phenotype have mutations in the same gene?

\( F_1 \) progeny from a complementation cross between:

- JB67 x LK3 -> has wildtype levels of 16:3
- JB67 x JB25 -> has wildtype levels of 16:3
- JB67 x LK8 -> has wildtype levels of 16:3
- LK3 x JB25 -> has wildtype levels of 16:3
- LK3 x LK8 -> has wildtype levels of 16:3
- JB1 x LK9 -> has 3-4% of 16:3
- JB25 x LK8 -> lacks 16:3

...demonstrating that JB1 and LK9, as well as JB25 and LK8 have mutations in the same gene, i.e. they are allelic
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</table>
## Fatty acid composition of total leaf lipids

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>WT (mean ± SD, n = 10)</th>
<th>(WT × JB25)F₁ (mean ± SD, n = 10)</th>
<th>JB25 (mean ± SD, n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₆:₀</td>
<td>14.1 ± 0.5</td>
<td>14.0 ± 0.5</td>
<td>12.6 ± 0.3</td>
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<td>C₁₆:₁</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.5</td>
<td>1.4 ± 0.4</td>
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<td>C₁₆:₁t*</td>
<td>1.6 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.0 ± 0.4</td>
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<td>C₁₆:₂</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>0.5 ± 0.3</td>
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<td>C₁₆:₃</td>
<td>11.4 ± 0.3</td>
<td>10.5 ± 0.6</td>
<td>1.5 ± 0.3</td>
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<tr>
<td>C₁₈:₀</td>
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<td>1.6 ± 0.1</td>
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<td>C₁₈:₁</td>
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<td>C₁₈:₂</td>
<td>13.4 ± 0.5</td>
<td>14.2 ± 0.8</td>
<td>17.8 ± 0.8</td>
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<td>C₁₈:₃</td>
<td>52.5 ± 0.4</td>
<td>51.7 ± 1.7</td>
<td>55.1 ± 1.5</td>
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*Trans isomer.
**Glycerolipid Synthesis**

“16:3” plants – Arabidopsis, spinach

![Diagram of Glycerolipid Synthesis](image)

**ER**

16:0-CoA  18:1-CoA

**Plastid**

18:2 16:1 (16:0)

Marker for prokaryotic pathway
Glycerolipid Synthesis

Radioactive labeling experiments of isolated chloroplasts using $^{14}$C-G3P to determine which enzyme is defective in JB25 mutant

1. Sequential addition of fatty acids to glycerol-3-phosphate (G3P)

2. Addition of the head group (defines the class of lipid)
Distribution of radioactivity among the polar lipids after $[^{14}\text{C}]\text{G3P}$ labeling of isolated chloroplasts for 20 minutes in the light and 20 minutes in the dark. The same amount of radioactivity was applied to each lane.
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach

**ER**

**Plastid**

Marker for prokaryotic pathway
Glycerolipid Synthesis

Radioactive labeling experiments of chloroplasts or leaf extracts using $^{14}$C-18:1-ACP or $^{14}$C-16:0-ACP to determine which enzyme is defective in JB25 mutant

1. Sequential addition of fatty acids to glycerol-3-phosphate (G3P)
Enzyme activities in chloroplasts or leaf extracts labeled with $[^{14}\text{C}]18:1\text{-ACP}$ or $[^{14}\text{C}]16:0\text{-ACP}$

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<thead>
<tr>
<th>Enzyme</th>
<th>Activity, pmol/min per mg of protein</th>
<th>Chloroplast</th>
<th>Whole cell</th>
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<td><strong>G3P acyltransferase (ACT1)</strong></td>
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<tr>
<td>Wild type</td>
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<td>JB25</td>
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<tr>
<td>Wild type + JB25†</td>
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<tr>
<td>LK8</td>
<td>ND</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td><strong>LPA acyltransferase (ACT2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>170</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>JB25</td>
<td>200</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>LK8</td>
<td>ND</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td><strong>RUBISCO</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>400</td>
<td>570</td>
<td></td>
</tr>
<tr>
<td>JB25</td>
<td>490</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td><strong>PEP carboxylase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.224</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>JB25</td>
<td>0.239</td>
<td>12.6</td>
<td></td>
</tr>
</tbody>
</table>

JB25/LK8 mutant lines have mutations in the G3P acyltransferase gene – ACT1
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach

Marker for prokaryotic athway
Distribution of radioactivity in leaf lipids after labeling with $[^{14}C]$-acetate for 142 hrs

$[^{14}C]$-acetate labels fatty acids, which means that all lipids will be labeled
Distribution of radioactivity in leaf lipids after labeling with $^{14}$C-acetate for 142 hrs.
Distribution of radioactivity in leaf lipids after labeling with $^{14}$C-acetate for 142 hrs

**Summary**

Labeling kinetics demonstrates parallel operation of 2 pathways of glycerolipid synthesis in *wild type* plants:

- High accumulation of $^{14}$C-labeled MGDG (major prokaryotic lipid) and PC (major eukaryotic lipid) at early times

- Gradual decline of $^{14}$C label in PC and accumulation in MGDG and DGDG indicates transfer of substrate from the ER into the plastid where eukaryotic type MGDG and DGDG synthesis takes place

In the *act1* mutant only PC is highly $^{14}$C-labeled at early times demonstrating that only the eukaryotic pathway of glycerolipid synthesis is operating.

Steady decline of $^{14}$C label in PC and accumulation in MGDG, DGDG and SL indicates transfer of substrate from the ER into the plastid where eukaryotic type MGDG and DGDG and SL synthesis takes place.
Distribution of radioactivity in leaf lipids after labeling with $[{}^{14}\text{C}]$-acetate for 142 hrs is similar in WT and mutant at the end of experiment.
### Fatty acid composition of leaf lipids

The fatty acid composition of leaf lipids is of particular interest. Why?

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MGDG (mol %)</th>
<th>DGDG (mol %)</th>
<th>SL (mol %)</th>
<th>PG (mol %)</th>
<th>PC (mol %)</th>
<th>PE (mol %)</th>
<th>PI (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:2&lt;/sub&gt;</td>
<td>WT: 1.9, JB25: -</td>
<td>WT: -</td>
<td>WT: -</td>
<td>WT: 22.6, JB25: 29.9</td>
<td>WT: -</td>
<td>WT: -</td>
<td>WT: -</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:3&lt;/sub&gt;</td>
<td>WT: 33.0, JB25: 1.7</td>
<td>WT: 1.7, JB25: 0.8</td>
<td>WT: -</td>
<td>WT: -</td>
<td>WT: -</td>
<td>WT: -</td>
<td>WT: -</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:0&lt;/sub&gt;</td>
<td>-</td>
<td>WT: 0.2, JB25: 0.7</td>
<td>WT: 0.8</td>
<td>WT: 1.2, JB25: 1.5</td>
<td>WT: 2.4, JB25: 2.8</td>
<td>WT: 2.0, JB25: 1.8</td>
<td>WT: 2.4, JB25: 2.3</td>
</tr>
</tbody>
</table>


- , Not detected.
Glycerolipid Synthesis

“16:3” plants – Arabidopsis, spinach
Glycerolipid Synthesis

“18:3” plants – barley leaves, pea leaves
Distribution of radioactivity among the polar lipids after $^{14}$C]G3P labeling of isolated chloroplasts for 20 minutes in the light and 20 minutes in the dark. The same amount of radioactivity was applied to each lane.
Fatty acid composition of leaf lipids

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MGDG</th>
<th>DGDG</th>
<th>SL</th>
<th>PG</th>
<th>PC</th>
<th>PE</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{16:0}</td>
<td>1.1</td>
<td>1.1</td>
<td>12.0</td>
<td>7.6</td>
<td>43.3</td>
<td>26.5</td>
<td>32.0</td>
</tr>
<tr>
<td>C_{16:1}</td>
<td>1.9</td>
<td>0.2</td>
<td>1.2</td>
<td>1.2</td>
<td>2.1</td>
<td>8.8</td>
<td>7.0</td>
</tr>
<tr>
<td>C_{16:2}</td>
<td>33.0</td>
<td>1.7</td>
<td>1.7</td>
<td>0.8</td>
<td>2.4</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td>C_{16:3}</td>
<td>61.1</td>
<td>90.5</td>
<td>80.5</td>
<td>86.9</td>
<td>48.7</td>
<td>54.5</td>
<td>28.0</td>
</tr>
<tr>
<td>(%)</td>
<td>39.0</td>
<td>34.6</td>
<td>15.2</td>
<td>18.0</td>
<td>2.8</td>
<td>2.0</td>
<td>8.1</td>
</tr>
</tbody>
</table>

---

PG fatty acid composition is relatively normal – t16:1 present

\[ \text{mol } \% \]
Conclusions

1. *act1* mutation does not result in a block in chloroplast lipid synthesis, but redirection of carbon into the eukaryotic pathway for production of chloroplast lipids.

2. Nearly normal amounts of all lipids required for chloroplast biogenesis and similar lipid composition of leaves in the mutant and WT indicate that the lack of synthesis of prokaryotic MGDG, DGDG and SL in the mutant is largely compensated for by increased synthesis of eukaryotic MGDG, DGDG and SL.

3. PG is made in the mutant, but its levels are reduced. Positional analysis of C16:0 fatty acid indicated that it is esterified to the *sn*-2 position on the glycerol backbone. Also, t16:1 made only in the chloroplast is present in PG. These results showed that plastids in the *act1* mutant can make PG via the prokaryotic pathway, suggesting that most of the PA still available in the plastid of this leaky mutant is channeled towards PG production.
Flux Diagrams for WT and act1 Mutant
Chloroplast lipid synthesis in the *act1* mutant resembles “18:3” plants

Non-green tissues of “16:3” plants (seeds, roots) also only use eukaryotic pathway to make plastid membranes