Fast and cheap metabolic phenotyping using microplate technology

Metabolic phenotyping?
Assay principles & technology
Examples of application
- Enzyme profiles in pine needles
- Major metabolites across a Maize NAM population

Yves Gibon – INRA-Bordeaux
Metabolic phenotyping

Human metabolic phenotype diversity and its association with diet and blood pressure

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Figure 1 | Hierarchical cluster analysis using group average linkage based on median ¹H NMR urine spectra, by population sample and gender (n = 4,630).
Studying metabolism in plants

Primary metabolic pathways are well described.

Our knowledge about their regulation is expanding.

The contribution to plant performance is far from being understood.
What metabolic levers to influence plant performance?

Genotypes
- Growth conditions
- Detailed experiments

Sample handling

Transcripts

Proteins

Enzymes

Metabolites

Fast and cheap

Bioinformatics

Data mining

Eco-Physiology

Xeml Lab
Hannemann et al. PCE 2009

Data management

Sink

Light

Source

Sink

Sugars

Amino acids

CO₂

SO₄²⁻

NO₃⁻

PO₄³⁻
What high throughput for metabolic phenotyping?

Horizontal high throughput
- Microplate technology
- Microfluidics
- Targeted – ‘biased’
- Low costs / sample
- n samples >> n variables

Vertical high throughput
- Transcriptomics, proteomics
- Metabolomics
- Untargeted – ‘unbiased’
- High costs / sample
- n samples << n variables
Microplate technology

• **1951** Invention by Takatsky
• **70’s** Elisa assays popular
• **80’s** First standard liquid handling robots
• **2003** “SBS standards” proposed by the Society for Biomolecular Screening
• **2009** Mature technology at decent prices
  – One standard microplate <0.25€ (125.10^6 / year worldwide)
  – Microplate readers: filter based <5k€, multifunction <50k€
  – Multichannel pipettes: <1k€
  – 96-head robot + external gripper + incubators <100 k€
**Assay principles**

- **Beer-Lambert**-assays: visible, UV
  - Major metabolites
  - High enzyme activities
  - nmol range

- Fluorescence
  - Fluorescamine derivates
  - Umbelliferone
  - Peroxidase/Resorufin
  - Amino acids
  - Low enzyme activities
  - pmol range

- Kinetic assays
  - Phosphorylated intermediates
  - Low enzyme activities
  - pmol range
  - Coenzyme A
  - NAD(H)
  - NADP(H)
  - Glycerol-3P & DHAP
Cycling assays were discovered in 1935 by Otto Warburg & colleagues.
Many possible assays

A few principles
Major metabolites

- **Protein content**
- **Carbohydrates**
  - Starch
  - Sucrose
  - Hexoses
  - Glucose-6P
- **Organic acids**
  - Citrate
  - Malate
  - Fumarate
- **Nitrogen**
  - Nitrate
  - Total amino acids
  - Proline
  - Glutamate
  - Aspartate
- **Chlorophyll** a and b

- <20 mg fresh weight required
- Ethanolic extraction
- Up to 200 samples/week (per hand)
- Costs: 0.05-0.5 €/analyte/sample
## Minor metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Principle</th>
<th>Sensitivity pmol/well</th>
<th>Sensitivity µg DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol-3P</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>PPI</td>
<td></td>
<td>1-2</td>
<td>6</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ADP-Glc</td>
<td></td>
<td>1-2</td>
<td>20</td>
</tr>
<tr>
<td>3-PGA</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Glc6P</td>
<td>Glc6P DH / PMS-MTT</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Glc1P</td>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Fru6P</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>MDH-CS / PTA</td>
<td>0.2</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

### Arabidopsis developing seeds
- 1 mg DW (10 siliques)
- TCA extraction
Enzyme profiling

- #50 enzymes from central metabolism
  - Benson-Calvin cycle
  - Starch metabolism
  - Oxidative pentose phosphate cycle
  - Sucrose metabolism
  - Glycolysis
  - TCA cycle
  - N assimilation
- <100 mg FW
- 0.1-1€ / analyte / sample
- up to 12 enzymes in 100 samples / week (per hand)
Current capacity

• **Metabolites**
  – Sugars, starch, organic acids, amino acids, protein content, chlorophylls, antioxidant capacity
  – 12-15 analytes in up to 2000 samples / week

• **Enzymes**
  – $V_{\text{max}}$, $K_m$, $K_i$, $T^\circ \text{C}$
  – 12 analytes in up to 500 samples / week
Labs doing metabolic phenotyping on microplates

Alistair Rogers
Brookhaven Institute

Stitt Lab
MPI MPP Golm

Bordeaux
• How do evergreen conifers cope with extreme temperatures?

✓ *Pinus sylvestris* needles were harvested throughout a year in 3 locations somewhere in the north of Saskatchewan (Canada)

✓ Central metabolism enzymes were profiled
Inside pine needles

15 enzyme activities expressed as nmol.gFW⁻¹.min⁻¹ were determined at 25°C in 216 samples collected throughout one year.
What about 12,000 samples?

Nengyi Zhang

Ed Buckler
The ultimate germplasm resource to date for localizing QTLs

**Figure 1.**—Diagram of genome reshuffling between 25 diverse founders and the common parent and the resulting 5000 immortal genotypes. Due to diminishing chances of recombination over short genetic distance and a given number of generations, the genomes of these recombinant inbred lines (RILs) are essentially mosaics of the founder genomes. ×, crossing; ⊙, selfing; SSD, single-seed descent.

Genetic Design and Statistical Power of Nested Association Mapping in Maize

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• **NAM Population** (NY 2007)

• **6,000 lines = 6,000 rows**

• **2 samples / row**
  one from end and one from middle plants, respectively.

• **In total, 12,000 samples**

• **12 analytes determined**
  sucrose, glucose, fructose, starch, malate, fumarate, nitrate,
  glutamate, amino acids, protein content, chlorophyll a, chlorophyll b,
## QTL mapping for metabolites

<table>
<thead>
<tr>
<th>Trait</th>
<th>No. of QTL</th>
<th>$R^2$ for Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>8</td>
<td>0.40</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>13</td>
<td>0.39</td>
</tr>
<tr>
<td>Malate</td>
<td>8</td>
<td>0.43</td>
</tr>
<tr>
<td>Glutamate</td>
<td>7</td>
<td>0.31</td>
</tr>
<tr>
<td>Fumarate</td>
<td>15</td>
<td>0.31</td>
</tr>
<tr>
<td>Amino acids</td>
<td>20</td>
<td>0.46</td>
</tr>
<tr>
<td>Protein content</td>
<td>6</td>
<td>0.34</td>
</tr>
<tr>
<td>Starch</td>
<td>14</td>
<td>0.55</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5</td>
<td>0.55</td>
</tr>
<tr>
<td>Glucose</td>
<td>12</td>
<td>0.40</td>
</tr>
<tr>
<td>Fructose</td>
<td>7</td>
<td>0.38</td>
</tr>
<tr>
<td>Nitrate</td>
<td>11</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Consensus map (1106 markers across the whole population)

GLM model
- FDR = 0.05
- DTA as covariable
- Population

The 26 parent lines are being genotyped with much higher resolution

The high resolution genotypic sequence data can be projected from the parents onto the RIL offspring

The combination of the statistical power of QTL mapping with the very high resolution of association mapping should ultimately reveal the genes controlling the levels of these metabolites. Just a matter of time. Then, we might think about even larger experiments with more factors (120,000 samples?)
Conclusion

• Metabolic phenotyping in very large-scale experiments is possible
• Robust, fast and cheap assays
• From generation of new questions to functional genomics
Acknowledgements

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