Metabolism-based herbicide resistance in *Lolium rigidum*: Using RNA-Seq transcriptome analysis to identify resistance-endowing genes

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Metabolism-Based Herbicide Resistance (MBHR)

- Increased gene expression
  - Metabolize herbicides
  - e.g., cytochrome P450, glucosyl transferase
- Possible multiple resistance to
  - Herbicides from other modes of action
  - New herbicides not yet discovered
- Generalist, broad-spectrum adaptive response
Diclofop-methyl metabolism pathway in wheat

Genetic diversity in function and regulation

305 gene models in Arabidopsis

278 gene models in Arabidopsis
Australia - Resistance to Multiple, Different Herbicides is Common

MBHR? Role of low dose?

www.ahri.uwa.edu.au
Next-Gen Sequencing

Grass Weeds and Enhanced Metabolism
Sequencing Costs

Cost per human-size genome

Next-generation sequencing

Moore's Law

NIH National Human Genome Research Institute

genome.gov/sequencingcosts
Project Goals

- How many genetic traits are involved in MBHR?
  - Gene expression regulation?
  - Cytochrome P450, GST, glucosyl transferase

- RNA-Seq: useful in non-model species?

- Goals:
  - Molecular diagnostics for MBHR
  - Identify new targets to inhibit metabolism
Enhanced metabolism genes in diclofop resistant *Lolium rigidum*

- 3 generations selection
  - 0.1, 0.5, and 2.0 × field dose
  - Neve & Powles, 2005
- \( \text{LD}_{50} \) ratio: 40
- No target site mutations in ACCase gene
- Enhanced diclofop metabolism
Experimental Approach

A - Reference Transcriptome

Normalized cDNA Library, 454 Sequencing
Annotation: UniProt, Pfam

B - RNA Seq – Alignment and DE Analysis

Biological Replicates and Treatments
100 bp Paired Illumina Reads

C – Validation – F2, 2,4-D Induction, Populations

qRT-PCR
2 normalization genes
Low dose recurrent selected *Lolium* has increased diclofop metabolism & no ACCase TSR.  

![Graph showing metabolites percentage]

- Non-Phytotoxic: R (30%) vs. S (10%)
- Diclofop acid: S (40%) vs. R (30%)

$n=4$
### De Novo Reference Transcriptome: 454 Pyrosequencing

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>L. rigidum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg. Read Length</td>
<td>448</td>
</tr>
<tr>
<td>Avg. Size</td>
<td>1,049</td>
</tr>
<tr>
<td>Largest Contig</td>
<td>8,222</td>
</tr>
<tr>
<td># All Contigs</td>
<td>19,623</td>
</tr>
</tbody>
</table>

- 1 R individual
- Leaves only
- cDNA library
- 57 CytP450 contigs
- 56 GST contigs
RNA-Seq Experiment

- 24 total RNA libraries - Illumina
  - 8 channels, 100 bp paired end
- 4 biological replications
  - 4 individuals, 3 clones each
- Susceptible population (S): VLR1
- Diclofop 1X (375 g/ha)
- Bowtie alignments
- Genedata Refiner Genome
- Quantify FPKM
- Analysis: DESeq in R

2 cm of stem and 4 cm of expanding leaf sampled for RNA
1 Month After Treatment – RNA-Seq Samples

S

R

0 surfactant 375 g/ha

0 surfactant 375 g/ha
Number of Contigs with Differential Expression (DE), $P < 0.05$ (DESeq)

- **Rn vs Sn**: 307 contigs
- **Ra vs Sa**: 87 contigs
- **Rt vs St**: 275 contigs
- **57** common contigs between Rn vs Sn and Ra vs Sa
- **278** common contigs between Rt vs St
- **134** common contigs between Rn vs Sn and Rt vs St
- **55** common contigs between Ra vs Sa and Rt vs St

**Constitutive Differences**
- **57** contigs
- **278** contigs

**Diclofop-induced Differences**
- **87** contigs

**Notes**
- **n** = not treated
- **a** = adjuvant
- **t** = diclofop treated
Are Diclofop-induced Changes in S Similar to Constitutive Differences between R & S?

Differences Between Rn and Sn

- R-n vs S-n
  - 776

Diclofop-Induced Changes In Expression in S

- S-s vs S-t
  - 92
- n = not treated
- s = surfactant
- t = diclofop treated

- 764
- 12
- 80
278 Contigs: Constitutive Expression Differences

- Selected 28 with relevant annotations

CYP72A2
28 Contigs Chosen
Expression Differences: R and S

Not Treated Samples

Reads Mapped Per Kilobase of Contig Model

- R-n
- S-n
Validation 1: Forward Genetics

- Individual plants cloned
  - Susceptible F₂: dead at 1/2X diclofop
  - Resistant F₂: survived 1X diclofop
- qRT-PCR
- 9 contigs out of 28 tested significant between F₂-R and F₂-S

<table>
<thead>
<tr>
<th>Contig</th>
<th>Annotation</th>
<th>R/S</th>
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</thead>
<tbody>
<tr>
<td>03513</td>
<td>Glucosyl transferase (GT)</td>
<td>7</td>
</tr>
<tr>
<td>01604</td>
<td>Cytochrome P450 (CYP72A1)</td>
<td>4</td>
</tr>
<tr>
<td>02218</td>
<td>Cytochrome P450 (CYP72A2)</td>
<td>9</td>
</tr>
<tr>
<td>05345</td>
<td>Nitronate monooxygenase (NMO)</td>
<td>13</td>
</tr>
<tr>
<td>06783</td>
<td>Cytochrome P450 (CYP83A)</td>
<td>4</td>
</tr>
<tr>
<td>05390</td>
<td>Glutathione-S-transferase (GST)</td>
<td>7</td>
</tr>
</tbody>
</table>
Principal Components Analysis – F2

Untreated Samples

FPKM from RNA-Seq

qPCR on F2
2,4-D antagonizes diclofop and chlorsulfuron in *Lolium*

2,4-D induces herbicide metabolism

Han et al., 2013, Pest Manage Sci 69: 996-1000.
Validation 2: Does 2,4-D induce similar gene expression?

No Diclofop Treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative Expression Value</th>
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<tbody>
<tr>
<td>GT</td>
<td>29X</td>
</tr>
<tr>
<td>CYP72A1</td>
<td>42X</td>
</tr>
<tr>
<td>CYP72A2</td>
<td>19X</td>
</tr>
<tr>
<td>NMO</td>
<td>49X</td>
</tr>
<tr>
<td>CYP83A</td>
<td>n.s.</td>
</tr>
<tr>
<td>GST</td>
<td>5X</td>
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24 hr after 2,4-D

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative Expression Value</th>
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</thead>
<tbody>
<tr>
<td>GT</td>
<td>+dic, -2,4-D</td>
</tr>
<tr>
<td>CYP72A1</td>
<td>+dic, +2,4-D</td>
</tr>
</tbody>
</table>

Cloned Plants

No 2,4-D | +2,4-D

<table>
<thead>
<tr>
<th>Gene</th>
<th>No 2,4-D</th>
<th>+2,4-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT</td>
<td>n=4</td>
<td>P&lt;0.001</td>
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</table>

P < 0.001

24X

19X

49X

n.s.

5X
Validation 3: Gene Expression in MBHR Field Populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Metabolic Resistance to:</th>
<th>CYP72A1</th>
<th>CYP72A2</th>
<th>NMO</th>
<th>GT</th>
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</thead>
<tbody>
<tr>
<td>France 2005</td>
<td>Diclofop</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>1</td>
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<tr>
<td>France 2006</td>
<td>Diclofop</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>France 2007A</td>
<td>Diclofop + ALS</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>174</td>
</tr>
<tr>
<td>France 2007B</td>
<td>Diclofop + ALS</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>France 2007C</td>
<td>Diclofop</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>16</td>
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<tr>
<td>France 2008</td>
<td>Diclofop + ALS</td>
<td>23</td>
<td>14</td>
<td>30</td>
<td>292</td>
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<tr>
<td>France 2009</td>
<td>Diclofop + ALS</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>107</td>
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<tr>
<td>France 2010</td>
<td>Diclofop + ALS</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Australia SLR31</td>
<td>Diclofop + ALS</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

No target-site mutations in ACCase or ALS genes, highly resistant
Transcriptional Marker Validation

French Populations Ward

Need multiple individuals to diagnose population as MBHR

Susceptible
Validation experiments essential
  - Test hypothesis generated by RNA-Seq

Increased expression of at least 4 genes involved in MBHR
  - Fits with low dose recurrent selection model
  - More pathways & genes remain to be found

Next: Biochemical and functional validation
Limitations:
- Study transcriptome – can’t see post-translational regulation of proteins
- Diversity of metabolic mechanisms
  - Each low dose experiment could have a different outcome
  - Populations show high diversity
  - We’ve found some genes for MBHR, just the start
- Recommend full dose
- Avoid low dose that allows partial survival – this selects for MBHR
  - ACCase herbicide use patterns in Australia
  - Low dose selection experiments
Cluster analysis of all SNP data

Binary distance, Ward linkage, in R

Shows very low technical variation relative to biological variation

**Divergence in 3 generations**
Thank You

Questions?