Plant Research & Biosafety
Plants constitute the majority of biomass on Earth.

Bar-On et al., *Proc. Natl. Acad. Sci.* 2018
Plants are remarkable chemists
New approach to develop plant-inspired medicines

Li and Weng, *Nature Plants* 2017
Outline

1. Use of metabolomics for assessing genetically engineered plants, particularly crops
2. The joy and pain of conducting research on non-model organisms
What is metabolomics

**Targeted metabolomics**

**Question:** What are the levels of specific metabolites in a sample?

- Standard metabolites
- LC/MS of standard metabolites
- Selected reaction monitoring
- Optimization and standard curve for quantification
- Samples: Tissue lysates, Cells, Blood and other biofluids
- LC/MS of metabolite extracts
- Data analysis by comparison of sample groups and/or standards
- Quantification of specific metabolites in biological samples

**Untargeted metabolomics**

**Question:** What is the global metabolic profile of a sample?

- Samples: Tissue lysates, Cells, Blood and other biofluids
- LC/MS of metabolite extracts
- Overlayed extracted ion chromatograms
- Data alignment and analysis
- Validation with MS/MS from standards
- Global metabolic profile of biological samples

Adapted from Patti, G et al. Nat. Rev. (2012)
Surprising observations

**No direct link** between absence of CHLOROPHYLLASE 2 and the accumulation of acetyl-aminodipate and acetyl-tryptophan.
Accumulation of acetylated amino acids in T-DNA mutants

![Acetyl-aminoadipate](image)

![Acetyl-tryptophan](image)

**Phosphinothricin resistance**

**Kanamycin resistance**

**Sulfadiazine resistance**
Mechanism for phosphinothricin resistance

**phosphinothricin**
= BASTA® = Ignite®
= glufosinate ammonium
**non-selective herbicide / antibiotic**

*BIALAPHOS RESISTANCE (BAR)*
*PHOSPHINOOTHRICIN-N-ACETYLTRANSFERASE (PAT)*

**N-acetyl-phosphinothricin**
inactive

Phosphinothricin resistance is a major trait of GM crops

Percent of planted acres in the United States

![Graph showing percent of planted acres in the United States for different crops over time, with data for each crop category including varieties with both HT and Bt (stacked) traits.]

Phosphinothricin resistance trait is present in 5-20% of herbicide-resistant crops (estimation)

USDA, Economic Research Service
Safety evaluation of BAR and PAT

Safety of the BAR and PAT DNA coding sequences and proteins

Characterization of BAR and PAT specificity in vitro ($K_M$ 25 µm)

Competition assays with amino acids (40 µM phosphinothricin, AAs in 50-fold excess)
Structure-guided engineering of BAR

Close-up view of the BAR active site

Activity of WT BAR, Y92F and N35T toward aminoadipate and tryptophan
Non-specific activities of the major herbicide-resistance gene BAR

Bastien Christ¹, Ramon Hochstrasser²,⁴, Luzia Guyer², Rita Francisco², Sylvain Aubry³,⁴, Stefan Hörtensteiner³,²* and Jing-Ke Weng¹,³,*

Opinion

Contribution of Untargeted Metabolomics for Future Assessment of Biotech Crops

Bastien Christ,¹ Tomáš Pluskal,¹ Sylvain Aubry,²,³,* and Jing-Ke Weng¹,³,*
Highlights

The metabolome of food crops significantly contributes to their quality and safety.

An updated framework for biotech crop risk assessment is needed in light of the rapid emergence of new genetic engineering tools for altering crop traits.

Recent advances in the field of metabolomics enable comprehensive characterization of plant metabolomes in a high-throughput manner.

Implementation of state-of-the-art untargeted metabolomics technologies could improve the safety assessment process for future biotech crops.
Case-by-case study design

New event
- Genomic engineering technology
  - Transgenics
  - (Agrobacterium-mediated or gene gun-mediated transformation)
  - Gene editing (ZFNs, TALENs and CRISPR/Cas systems)

Key questions:
- What are the genetic and biochemical properties of the new trait?
- Is the new trait intended to modify the metabolic capabilities of the host?
- Can indirect effects on the host metabolome be predicted?

Reference set
- Conventional counterparts
- Existing genetic diversity
- Historical data

Metabolomics
- Metabolite fingerprinting
  - Detection of large difference across the metabolome
- Metabolite profiling of selected classes of metabolites
  - Detection of metabolite abundance difference at the single-metabolite level

Statistics
- Estimation of metabolic variation within the existing genetic diversity
- Estimation of significant metabolic variation due to the new trait
- Identification of selected metabolites

Risk characterization
- Identification of potentially hazardous metabolic alterations due to the new trait?

Targeted metabolomics
- Metabolite absolute quantification

Hazard characterization
- Exposure assessment

Standardized protocols and public databases
1. Use of metabolomics for assessing genetically engineered plants, particularly crops
2. The joy and pain of conducting research on non-model organisms
Whitehead Institute greenhouse
Transgenic *Arabidopsis thaliana*

**Target gene**

ATGTCGAAGACGGTGAGGAT
CGGCAACCGCATCAGGGCAAG
GGCGCGCAACAGGTGGCC
ATCGGAACGGCTACGCCGGCCAATGTGGTGTACCA...

**Binary vector with T-DNA borders**

**Agrobacterium tumefaciens**

**Arabidopsis thaliana**

[Images of plants labeled a, b, c, d]

**Transformant selection**

+ glufosinate

~4 months!

Transient gene expression in *Nicotiana benthamiana*

ATGTCAAGACGTTGAGGAT
CGGGCAGCGCAGCGGGCAAAGGGGCCGGCAACAGTGCTGGCCATCGGCACGGCTACGCCGGCCAATGTGGTGTACCA...

Target gene

Binary vector with T-DNA borders

*Agrobacterium tumefaciens*

Nicotiana benthamiana

~1 week
Main biosafety concerns when working with non-model species

- Adherence to regulations
- Safety of employees
- Import of plant (and other) species
- Soil safety
- Pest management
Whitehead Institute Biosafety Office risk assessment process

The movement, use, possession, or release of exotic or potentially harmful plant-associated arthropods, biological control agents, plant pests, plant pathogens, noxious weeds, and invasive plants are regulated by

- **State of Massachusetts**
- **USDA Animal and Plant Health Inspection Service** (APHIS)
- Research with genetically engineered plants, genetically engineered plant-associated microbes, and genetically engineered plant-associated microorganisms (arthropods and nematodes) is covered by the


Whitehead Institute Biosafety Office risk assessment process

For plants, care must be taken to
• avoid the unintentional transfer of plant genes, recombinant or otherwise, to other plants
• minimize unanticipated, harmful effects to organisms or the environment outside the experimental site/facility
• avoid the inadvertent spread of pathogens or noxious weeds to crops or native vegetation
• prevent the introduction of unwanted exotic organisms into a new habitat
• Since plant research usually (but not always) does not pose a human health hazard, biosafety principles are designed instead to protect the natural and agricultural environment.

A risk assessment that factors in the
• specific organism(s) under study
• geographic, ecological, and agricultural environment surrounding the study site
• physical/mechanical barriers available, and
• scientifically accepted culture techniques
Stinging nettles (*Urtica dioica*)

- Causes irritating stings and rash
- Not regulated by USDA
- Use of PPE (safety goggles, lab coat, gloves) and protective covers for the plants

![Moroidin molecule](image_url)
Poison ivy (*Toxicodendron radicans*)

- Causes strong allergic reaction known as urushiol-induced contact dermatitis
- Not regulated by USDA in MA (listed as *Prohibited noxious weed* in MN)
Euphorbia spurge 
(*E. lactea, E. tirucalli, E. resinifera*)

- Contain highly poisonous sap
- Not regulated by USDA
- Use of PPE (safety goggles, lab coat, gloves)

![Resiniferatoxin](image)
Goat’s rue (*Galega officinalis*)

- Rich in galegine, a substance with blood glucose-lowering activity
- Classified as noxious weed by USDA
- Obtaining a USDA permit was fairly straightforward, existing greenhouse space certified for transgenic plants was sufficient

![Galegine](attachment:Galegine.png)
Fireflies (*Photinus pyralis*)

*Photinus pyralis* (Big Dipper Firefly)

**Luciferase**

Dr. W.D. McElroy (1917-1999)

Tim Fallon

**Reaction Scheme**

- **Firefly D-luciferin** (luciferase) + ATP → **D-luciferyl adenylate**
- **D-luciferyl adenylate** + Mg$^{2+}$ → **Oxyluciferin**

Biosafety and regulatory control in practice

- **Is your species regulated?** If it is not a major model organism, and is a terrestrial invertebrate, likely yes. The investigator will need to submit a Plant Protection and Quarantine (PPQ) Form 526 permit and a containment plan.

<table>
<thead>
<tr>
<th>“No jurisdiction species”</th>
<th>Regulated</th>
<th>USDA PPQ Form 526 permit maybe required</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No USDA permit required for international import and/or environmental release</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humans¹</td>
<td>Mosquitoes² (CDC regulated)</td>
<td>Organisms collected or purchased within the same state¹</td>
</tr>
<tr>
<td>C. elegans²</td>
<td>Spiders³ (USFWS regulated)</td>
<td>Dead organisms¹</td>
</tr>
<tr>
<td>E. coli²</td>
<td>Scorpions³ (USFWS regulated)</td>
<td>Saccharomyces cerevisiae²</td>
</tr>
<tr>
<td>tail-less whip scorpions</td>
<td>Centipedes³ (USFWS regulated)</td>
<td></td>
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<tr>
<td>Vinegaroons³ (USFWS regulated)</td>
<td>Sun spiders³ (USFWS regulated)</td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Most fungi²</td>
<td>Native fireflies²</td>
</tr>
<tr>
<td></td>
<td>Essentially all arthropods / insects²</td>
<td>Bacillus subtilis² (Pathogen of invertebrate – biocontrol)</td>
</tr>
<tr>
<td></td>
<td>Bladder Snails²</td>
<td>D. melanogaster*</td>
</tr>
</tbody>
</table>

*informally exempted from 7 CFR 330. Will be formally exempted with 7 CFR 330 update.

Working with USDA to enable your research

Containment guidelines are not codified in regulation. USDA does have many internal policies, and rules-of-thumb with respect to containment, but some flexibility does exist. Biocontainment has the following broad goals:

1) Escape proof caging
2) Escape safeguards during handling when outside caging
3) Prevention from theft by unauthorized personnel.
4) Organisms cannot escape from and are killed before going into solid or liquid waste streams.
5) The escape of unintended associated microbial or virus/viroid plant pests must be considered in addition to the macroscopic organism, especially from international sources.

Strains that have been in laboratory culture for several years (‘pure culture’) are viewed more favorably by USDA.

For those species where the lack of associated microbial or virus/viroid plant pests cannot be inferred, a biosafety cabinet and more stringent waste treatment procedures are required.
Japanese aquatic fireflies (*Aquatica lateralis*)

Considered biocontrol organisms
- Needed USDA biocontainment facility + inspection
- ~9 month permitting process.
- USDA requires that investigators complete all steps of process. E.g., permit submission and communication is handled by PI
- Required submission of in-depth (22 page) standard operating procedures biocontainment protocols
Firefly biocontainment facility is a single dedicated room with:
1) Card access door.
2) Door sweeps under door
3) Mesh over HVAC
4) Sub-bacterial/viral wastewater filtering or bleaching
5) Autoclaved solid waste
6) Escape proof mesh box for handling
7) Lockable cabinet w/ firefly aquaria
*Note, (7) is perhaps more stringent than USDA requires. We are practicing rearing procedures that could allow for dropping of the dedicated room requirement.
(8) Unregulated plant pest snail rearing (as food for firefly larvae)
Firefly genomes illuminate parallel origins of bioluminescence in beetles

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