Genetic Manipulation of Susceptibility to Fusarium Head Blight

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Fusarium Head Blight

- Few sources of effective wheat or barley germplasm conferring resistant to FHB in wheat or barley. This makes crop improvement difficult.

- Transgenics are an alternative but gene assays in transgenic wheat or barley is slow and expensive and has only a moderate capacity. OK for testing single genes, but not efficient enough for de novo gene discovery.

- Approach: Use higher capacity model plants to assay genes for their ability to confer resistance to Fusarium.
A pipeline for gene discovery and deployment

1. **Select potential antifungal genes**
   - Based on genetic, molecular data from wheat and other species

2. **Isolate selected genes**
   - Clone and sequence genes

3. **Physcomitrella: Create gene knockouts and overexpressors**
   - Mutate or express genes in Physcomitrella

4. **Physcomitrella: Assay plants for FHB resistance**
   - Expose mutant plants to FHB, DON

5. **Wheat: assay selected genes for FHB resistance**
   - Transient assay in wheat using VIGS (Scofield Lab)

6. **Wheat: Stable Transgenics**
   - Transformed FHB-Resistant Wheat, Barley (Dahleen Lab)
**Physcomitrella patens**

Grows like yeast but is a multicellular plant

Haploid gametophyte dominates life cycle

Genome size: 511Mbp; 27 chromosomes

Genome sequence completed in 2007

Functional conservation with higher plants and yeast

Undergoes high efficiency homologous recombination

Allows targeted gene replacement for gene knockout or site-specific mutation etc.

**Model system for functional genomics**

Our Targets:

Disease, Cell Death, Toxin Action, Induced Immunity, Cell Wall
Gene knockout by homologous recombination

Genomic DNA

Double homologous recombination

Result: gene disruption by allele replacement

3-10% reversion to Neo\(^s\) following transient expression of CRE recombinase

Possible reversion of targeted gene following site-specific recombination
Recombination rates in Physcomitrella

Efficiency (GT/GT+IR):  4% (.5kb) up to 100% (2-4 kb) in Physco

0.005-0.1% in angiosperms
95% in S. cerevisae
1-30% in N. crassa
0.1-1% in mouse ES cells

- Essentially any gene or genomic sequence in the *Physcomitrella* genome can be deleted quickly and precisely.
- Can complement knockout mutants with genes from other plants.
- Foreign genes can be introduced into a specific locus and expressed in a predictable manner (no position effects).
Can *Fusarium* infect *Physcomitrella patens*?

Can it be infected?

Is it a relevant model for diseases of crop plants?

[www.wheatlifemagazine.com](http://www.wheatlifemagazine.com)
Infection of Physcomitrella by *Fusarium graminearum*

<table>
<thead>
<tr>
<th>Time</th>
<th>Fusarium::GFP</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>48h</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
<td>72h</td>
<td><img src="image5.png" alt="Image" /></td>
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<tr>
<td>96h</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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</table>
Physcomitrella is sensitive to multiple FHB mycotoxins

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>0h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
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<tbody>
<tr>
<td>DON</td>
<td><img src="https://example.com/don-0h.jpg" alt="Image" /></td>
<td><img src="https://example.com/don-24h.jpg" alt="Image" /></td>
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<tr>
<td>DAS</td>
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<td>T-2</td>
<td><img src="https://example.com/t2-0h.jpg" alt="Image" /></td>
<td><img src="https://example.com/t2-24h.jpg" alt="Image" /></td>
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<tr>
<td>ZON</td>
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<td><img src="https://example.com/zon-24h.jpg" alt="Image" /></td>
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Tricothecenes contribute to *F. graminearum* virulence

(A)  

<table>
<thead>
<tr>
<th>Fusarium-GFP</th>
<th>Fusarium-WT</th>
<th>Symptoms</th>
<th>Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δtri5</td>
<td></td>
<td></td>
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</tbody>
</table>

48h 72h 48h 48h

(B)

The Δtri5 strain of *F. graminearum* does not produce DON or DAS

The Δtri5 strain of *F. graminearum* does not produce DON or DAS

25 50 75 100

% CELL DEATH

WT

Δtri5

0 24 48 72 96

hours post inoculation

0 25 50 75 100
Programmed Cell Death (PCD)
Is Programmed Cell Death a target for Fusarium toxins?

- *Fusarium* mycotoxins contribute to virulence on Physcomitrella and wheat
- Both *Fusarium* and mycotoxins cause cell death on host plants
- **Programmed Cell Death (PCD)** is a genetically controlled process
- Mutating genes that control host cell death may suppress symptoms and attenuate virulence

**Mycotoxins**

0h | 24h | 48h | 72h
---|---|---|---
DON | ![image](image1.png) | ![image](image2.png) | ![image](image3.png)
DAS | ![image](image4.png) | ![image](image5.png) | ![image](image6.png)
T-2 | ![image](image7.png) | ![image](image8.png) | ![image](image9.png)
ZON | ![image](image10.png) | ![image](image11.png) | ![image](image12.png)

**Fusarium graminearum**

- *Fusarium graminearum* Mycotoxins
  - DON, DAS, T-2, ZON
- GFP-FHB SYMPTOMS: CELL DEATH
  - Uninoculated, WT
PCD is required for host cell death and sensitivity to DON.

Plant Cell Death

% CELL DEATH

WT L3Δ ERG1KO AtBI-1OE PPBI-1KO VPErKO

Deoxynivalenol (DON)
PCD mutant plants are resistant to multiple mycotoxins

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L3Δ</th>
<th>ERG1-KO</th>
<th>AtBl1-OE</th>
<th>PpBl1-KO</th>
<th>VPEγ-KO</th>
</tr>
</thead>
</table>

Plant Cell Symptoms
Some PCD mutant plants are resistant to FHB

Fusarium-GFP

KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant
Medium Throughput Assay for Gene Function
Assay Gene Knockout Plants for Reduction of Symptoms

Lots of ‘failures’ – you need a high capacity system in order to select those rare genes that do confer resistance Fusarium.
Fusarium graminearum infection in TP-KO plants

TP-KO

24h
48h
72h
96h

Cell death %

WT
TP-KO

0
24
48
72
96

**Fusarium graminearum** infection in PC-KO plants

**PC-KO**

24h 48h 72h 96h

**Cell death %**

<table>
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<th>Time</th>
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<th>PC-KO</th>
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<tbody>
<tr>
<td>24h</td>
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</tbody>
</table>

**Graph Legend**

- 0
- 24
- 48
- 72
- 96
Fusarium graminearum infection in HP-OE plants

HP-OE

24h 48h 72h 96h

Cell death %

WT HP-OE
Mutation of tb1 affects the ability of Fusarium to enter into cells.

Effect are manifested at or near the plant cell surface and vacuole.

Mechanism is not understood, but may be revealing something important about how Fusarium colonizes its host. Interactions at the cell surface may be important.
Pipeline for discovery and deployment of genes effective against FHB

Select potential antifungal genes
Isolate selected genes
Physcomitrella: Create gene knockouts and overexpressors
Physcomitrella: Assay plants for FHB resistance
Wheat: assay selected genes for FHB resistance
Wheat: Stable Transgenics

Based on genetic, molecular data from wheat and other species
Clone and sequence genes
Mutate or express genes in Physcomitrella
Expose mutant plants to FHB, DON
Transient assay in wheat using VIGS (Scofield Lab)
Transformed FHB-Resistant Wheat, Barley (Dahleen Lab)
Induced Immunity
Chitosan induces immunity in *Physcomitrella* and wheat. Chitosan pre-treatment induces immunity against *Fusarium graminearum* in both *Physcomitrella* and wheat. However, the response is more pronounced in *Physcomitrella*.
## Induced Immunity in Physcomitrella

### RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>- CHITOSAN + FHB</th>
<th>+ CHITOSAN (12h pre) + FHB</th>
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</thead>
<tbody>
<tr>
<td><strong>PCD</strong></td>
<td><img src="image1" alt="PCD" /></td>
<td><img src="image2" alt="PCD" /></td>
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<td><strong>ER stress</strong></td>
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<td><strong>Defense</strong></td>
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<td><strong>Immunity</strong></td>
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**WT plants inoculated with**
*F. graminearum*

**WT plants pre-treated with**
chitosan for 12h and then inoculated with
*F. graminearum*
Role of nucleases in infection of *F. graminearum*

**RNase activity gel assay**
- 56 kDa
- 35 kDa
- 32 kDa

**DNase activity gel assay**
- 32KDa

**Gene Expression - Northern blot**
- RNS1
- BEN1
- rRNA

**Gene Expression - RT-PCR**
- RNS1
- BEN1
- Actin1
Role of nucleases in infection of *F. graminearum*

**Sensitivity to FHB**

WT

RNS1-OE

BEN1-OE

**Sensitivity to DON**

Plant Cell Death

Note that plants overexpressing RNS1 are significantly more resistant to FHB than WT but are still sensitive to DON (as is the WT).

This is consistent with RNS1 having a direct antifungal effect on *F. graminearum*.

N.B. Exogenous RNase is lethal to *F. graminearum*.
Chitosan pre-treatment induces many of the components involved in chitosan and other PAMP (elicitor) signaling.

PAMP = Pathogen Associated Molecular Pattern

* "Non-quantitative" RT-PCR
Chitosan-induced immunity in WT, CEBiP OE and CEBiP KO *Physcomitrella* plants

![Images of WT, CEBiP-OE, and CEBiP-KO plants infected with F. graminearum with and without chitosan treatment]

**Survival %**

- **F. graminearum (F.g.)**
- **F. graminearum + Chitosan (F.g. + Chit)**

WT | CEBiP-OE | CEBiP-KO
Yeast Gene Knockout Library Screen

Nilgun Tumer’s Lab
Rutgers University
Screens for new cellular targets of trichothecene toxins

Screen for new toxin targets in yeast

Select potential antifungal genes

Isolate selected genes

Physcomitrella: Create gene knockouts and overexpressors

Physcomitrella: Assay plants for FHB resistance

Wheat: assay selected genes for FHB resistance

Wheat: Stable Transgenics

T2 Toxin

Yeast Gene Knockout Library

Based on genetic, molecular data from wheat and other species

Clone and sequence genes

Mutate or express genes in Physcomitrella

Expose mutant plants to FHB, DON

Transient assay in wheat using VIGS (Scofield Lab)

Transformed FHB-Resistant Wheat (or other crop)

• Arabidopsis T-DNA Knockouts
• RNAi in wheat, barley
Selection of resistant strains on plates using different concentrations of T-cin

Double printed omniplates identified mutants that were resistant only at 4 μM (circled white) or at 8 μM (circled black) T-cin

McLaughlin, Tumer et al., 2009 PNAS in press.
A genome-wide high throughput screen of yeast gene deletion library to identify novel tricothecene targets

Controls (No toxin)

Working Glycerol Plate

Original Glycerol Plate

Backup Glycerol Plate

Toxin Treatments

PCR Verification “Barcode”

Phenotype Comparisons (OD 600)

- Inhibited Growth (Susceptibility)
- Increased Growth (Resistance)
- False Positive

4720 strains on 72 plates
350 slow growing strains on 4 plates
Screened twice at 4, 8, 12 and 24 µM

McLaughlin, Tumer et al., 2009 PNAS in press.
Mitochondria play a critical role in Tricothecin toxicity

These NUCLEAR-encoded genes, targeted to the mitochondrion, represent novel tricothecene targets. Their contribution to the susceptibility of plants to Fusarium can now be tested in Physcomitrella, Arabidopsis and wheat.
Gene Knockouts Help Define Cellular Mechanisms

PCD

Reactive Oxygen Species

Induced Immunity

Cell Wall

Can we simulate the effects of these mutants by chemical treatment?
Chemical suppression of PCD controls *Fusarium*

(A) Prevention of plant cell death in *Physcomitrella*

(B) Suppression of *Fusarium* growth *in planta*

(C) Reduced *Fusarium* growth *in planta*

*Physcomitrella* plants infected with GFP-labeled *Fusarium graminearum*

CC1 and CC2 PCD suppressors are NOT toxic to *Fusarium*
Chemical suppression of PCD and *Fusarium*

PCD suppressors protect plants from *Fusarium* infection
### Chemically Induced Defense Against FHB (non-PCD)

#### Physcomitrella patens

<table>
<thead>
<tr>
<th>Condition</th>
<th>GFP</th>
<th>Autofluorescence</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHB</td>
<td>![GFP Image]</td>
<td>![Autofluorescence Image]</td>
<td>![Merge Image]</td>
</tr>
<tr>
<td>FHB + G17-1</td>
<td>![GFP Image]</td>
<td>![Autofluorescence Image]</td>
<td>![Merge Image]</td>
</tr>
<tr>
<td>FHB + B49</td>
<td>![GFP Image]</td>
<td>![Autofluorescence Image]</td>
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#### Wheat

<table>
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#### Cell Death Analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Cell Death</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Fg</td>
<td>30</td>
</tr>
<tr>
<td>Fg+G17-1</td>
<td>40</td>
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<tr>
<td>Fg+B49</td>
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<td>Fg+B49</td>
<td>50</td>
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</table>
An end-run around the gene discovery and deployment pipeline?

Select potential antifungal genes

Isolate selected genes

Physcomitrella: Create gene knockouts and overexpressors

Physcomitrella: Assay plants for FHB resistance

Wheat: VIGS assay of selected genes

Wheat, Barley Transgenics

Design chemical interventions based on mechanisms revealed by mutant studies

Test efficacy in Physcomitrella, wheat, etc.

Chemical studies can help us understand mechanisms of FHB susceptibility and resistance

May provide novel approaches for chemical control of FHB
Conclusions

• Physcomitrella is a rapid and sensitive assay for genes that control sensitivity to DON and susceptibility to Fusarium.

• We have identified a number of genes whose mutation alters sensitivity to Fusarium.

• There are multiple ways to enhance resistance to Fusarium.
  – Suppress PCD pathway
  – Enhance innate immunity/defense responses
  – Alter the plant cell wall/cell surface

• It is important to test the efficacy of these genes in crop plants
  – Test by VIGS assay in wheat (Scofield, Purdue University)
  – Test by in transgenic crop plants (Dahleen, BioEn-USP)

• Chemical approaches to controlling FHB
  – Physcomitrella gene mutants can suggest novel approaches for the chemical control of FHB (non-toxic, non-fungicidal chemicals)
  – This may have some practical applications
Support

Acknowledgements

Hemalatha Saidasan
Mark Diamond

Eric Lam
Nilgun Tumer

Steve Scofield
Lynn Dahleen
Overexpression of the Ribosomal Protein L3 confers resistance to FHB

Fusarium-GFP

Uninoculated  Inoculated
ScL3Δ-OE  TaL3Δ

TaL3Δ: Wheat L3Δ gene
ScL3Δ: Yeast L3Δ gene

Plant Cell Death

% cell death

0 25 50 75 100

0 24 48 72 96

WT  TaL3Δ  ScL3Δ

96h