Physcomitrella patens: A model system for studying disease susceptibility and the plant cell wall

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Biotech Center
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Talking Points

• Joe Varner was right –everything leads back to the cell wall

• We need to understanding the basics (Goldemberg, Buckeridge)

• 10% of Genome devoted to the cell wall –many, many of these genes are of unknown function (Carpita)

• The regulation of genes is not always predictable (Carpita, VIGS)

• There are many, many candidate genes ‘coming down the pike’ (Souza) whose effects on the cell wall (and on other traits) need to be tested *in vivo* (Carrer).

• There is a pressing need for functional methods that allow the effects of mutating and altering large numbers of genes to be tested *in vivo*.
  – Must be efficient and rapid
  – Allow genes to be added, removed and altered
  – Biologically relevant

• Physcomitrella –a rapid assay system for functional genomics
  – Applications to study disease
  – Applications to study the cell wall
Why is disease important? Yield Loss

Dry death of sugarcane
Associated with
Fusarium subglutinans
Fusarium spp.
Pythium spp.
Colletotrichum spp.
Meloidogyne spp.

[Botanic Gardens Trust
NSW Australia]

Stem Borers

*Chilo terrenellus*  *Diatraea saccharalis*

insects.tamu.edu/extension/bulletins/mp-1777.html

Red Rot Disease
caused by *Colletotrichum falcatum*

[Sugarcane Breeding Station, Coimbatore, India]
sugarcane-breeding.tn.nic.in/pathology.htm
Buchanan, Gruissem, Jones  
The role of plant cell wall polysaccharide composition in disease resistance. Trends Plant Sci. 9(4):203-9
Disease and the Plant Cell Wall

- Pathogens are an important source of cell wall-degrading enzymes.

- Cell Wall is an important barrier to infection.

- Cell Wall can also actively contribute to disease resistance (cross-linking, peroxidases, secreted proteins).

- Exposure to pathogens can reveal the underlying biological functions of cell wall genes – assay system.

- Molecular genetics provides a direct approach to modify the plant cell wall to prevent disease in the field and enhance post-harvest processing for biofuels.
The Cell Wall is a Battleground

Pathogen

Bacterium

Fungus

Plant Cell Wall

Virulence Effectors Toxins etc.

TTSS

Lytic Enzymes

Plant

Physcomitrella patens

Good Molecular Genetics?

Nucleus
What is *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

Targeted gene replacement for knockout or site-specific mutation etc.

**Model system for functional genomics**

- Disease, Cell Death, Cell Wall
Physcomitrella Patens Lifecycle

Spore germination
Protonemal Growth
Chloronema
Caulonema
Physcomitrella Patens Lifecycle

Bud cell development  Colony Growth  Gametophore
Physcomitrella Patens Lifecycle

Gametophore with rhizoids

U: Antheridia
L: Archegonia

Spore Capsule
The Moss *Physcomitrella patens*, Now and Then. Didier G. Schaefer and Jean-Pierre Zryd
Gene knockout by homologous recombination

Possible reversion of targeted gene following site-specific recombination

3-10% reversion to Neo<sup>s</sup> following transient expression of CRE recombinase

Result: gene disruption by allele replacement

Double homologous recombination

Genomic DNA

ATG  TAA

\[ \text{A} \quad \text{B} \quad \text{C} \quad \text{D} \]

\[ \text{B} \quad \text{Neo} \quad \text{C} \]

\[ \text{lox} \quad \text{lox} \]

\[ \text{B} \quad \text{wild type sequence} \]

\[ \text{B} \quad \text{mutant sequence} \]

\[ \text{Neo} \quad \text{Selectable marker} \]

\[ \text{Lox site} \]
<table>
<thead>
<tr>
<th>Metric</th>
<th>Values</th>
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<tr>
<td>Efficiency (GT/GT+IR)</td>
<td>4% (.5kb) up to 100% (2-4 kb) in <em>Physco</em></td>
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<tr>
<td></td>
<td>0.005-0.1% in other plants</td>
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<tr>
<td></td>
<td>95% in <em>S. cerevisae</em></td>
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<tr>
<td></td>
<td>1-30% in <em>N. crassa</em></td>
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<tr>
<td></td>
<td>0.1-1% in mouse ES cells</td>
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<tr>
<td>Efficiency/ug DNA</td>
<td>0.5 to 30 (size dependent) in <em>Physco</em></td>
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<tr>
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<td>0.001-0.02 in other plants</td>
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<tr>
<td></td>
<td>1-10 in yeast, <em>A. nidulans</em></td>
</tr>
<tr>
<td>Efficiency/living cell</td>
<td>10^-3 to 10^-4 (size dependent) in <em>Physco</em></td>
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<tr>
<td></td>
<td>10^-6 in other plants</td>
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<td></td>
<td>10^-4 in mouse ES cells</td>
</tr>
<tr>
<td></td>
<td>10^-5 in <em>Dictyostelium</em></td>
</tr>
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</table>
Efficient Gene Targeting increases capacity to address gene function through the creation of mutant plants.

Examine relationship between genotype and phenotype.

High Efficiency means you can afford to be wrong, much of the time…

…as long as you are right, occasionally.

i.e. Hit Rate is acceptable.
Infection Assay of Gene Knockout Plants

Lots of ‘failures’ – you need a high capacity system in order to select those rare genes that do confer resistance.
**Working hypothesis:**
That some, and perhaps many of the cellular mechanisms involved in the expression of disease resistance and susceptibility are shared between mosses and angiosperms.
The Cell Wall is a Battleground

*Fusarium graminearum*

*Physcomitrella patens*

Nucleus

Plant Cell Wall

Fungus

Lytic Enzymes

Virulence Effectors
Toxins etc.
Fusarium spp. cause disease in many other important crops.

Fusarium infection of wheat and barley and contamination with tricothecene mycotoxins (DON, DAS etc) is a health concern for humans and animals and an economic problem for farmers and processors.

Excellent genetic and molecular resources are available for Fusarium (sequenced genome, gene knockouts)
Infection of Physcomitrella by *Fusarium graminearum*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Fusarium-GFP</th>
<th>Symptoms</th>
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<td><img src="image2.png" alt="Image" /></td>
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<tr>
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<tr>
<td>72h</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>
</tr>
</tbody>
</table>
Infection of Physcomitrella by *Fusarium graminearum*

Thanks to Stephan Rensing and the Biozentrum, Basel for cryo em images
Physcomitrella is sensitive to *Fusarium* mycotoxins

<table>
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<th></th>
<th>0h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
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<td><img src="image16.png" alt="Image" /></td>
</tr>
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</table>
**Trichothecenes contribute to *F. graminearum* virulence**

**A**

<table>
<thead>
<tr>
<th></th>
<th><em>Fusarium</em>-GFP</th>
<th><em>Fusarium</em>-WT</th>
<th>Symptoms</th>
<th>Cell Death</th>
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<td><strong>Δtri5</strong></td>
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<td><img src="image7.jpg" alt="Image" /></td>
<td><img src="image8.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>

48h 72h 48h 48h

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

**B**

![Graph](image9.jpg)

Cell death is clearly important:

So we focus on genes that control Programmed Cell Death (PCD).
Plant Functional Genomic Targets

Plant genes that affect disease susceptibility:

- Programmed Cell Death (PCD)
- Induced Immunity (antimicrobials)
- Cell Wall
Bax Inhibitor-1 (BI-1) is a conserved inhibitor of PCD.

BI-1 is an antagonist of the pro-apoptotic protein Bax.

It is the only component of the animal Bax/Bcl2 regulatory mechanism that is conserved in plants.

Its targets in plants are unknown.

The sole Physcomitrella BI-1 gene was cloned and verified.
Plants that have undergone homologous recombination are verified by PCR using primers that flank the predicted sites of integration.

E.g. primers 1+8 can only amplify a fragment if the HPT gene of the replacement vector integrates next to the appropriate flanking genomic sequences.
The PpBI-1 gene is inducible by chitosan (and other treatments).
PpBI1 KO plants express no BI-1 mRNA or protein.
AtBI1 OE plants constitutively express BI-1 mRNA and protein.
PpBI-1 KO and AtBI-1 OE lines: toxin sensitivity

KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant
AtBI-1 OE and PpBI-1 KO plants: susceptibility to *F. graminearum*

**A**

- **GFP-FHB**
  - Uninoculated
  - WT
  - AtBI1-OE
  - PpBI1-KO

- **CELL DEATH**
  - Uninoculated
  - WT
  - AtBI1-OE
  - PpBI1-KO

- **72h**

- **KO** = Gene Knockout Plant
- **OE** = Overexpressing Transgenic Plant

**Graphs**

- % CELL DEATH vs. Hours post inoculation
Other gene knockout plants show enhanced resistance to *Fusarium graminearum*.

KO = Gene Knockout Plant

OE = Overexpressing Transgenic Plant
• Cell death is under genetic control. Manipulation of PpBI-1, VPEγ and other genes alters sensitivity to PCD inducers.

• Manipulation of these genes also alters sensitivity to PCD-inducing pathogens (necrotrophs)
Pipeline for discovery and deployment of genes effective against Fusarium

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 Fusarium 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 Fusarium-Resistant Wheat (Or Sugarcane)
Anoikis and Disease Susceptibility

Anoikis ("without a home") is a form of apoptosis which occurs when anchorage-dependent animal cells detach from the extracellular matrix (ECM).
Anoikis: detachment from ECM leads to apoptosis in mammalian cells

“Bit1 may be a guardian of cell attachment”

A Mitochondrial Protein **Bit1**, Mediates Apoptosis Regulated by Integrins and Groucho/TLE Corepressors


In mammalian cells

- Anoikis: loss of attachment to the ECM → PCD
- Bit1 promotes attachment-dependent PCD
- Integrins regulate Bit1 mediated PCD
- There is a homolog of Bit1 in Physcomitrella (and Arabidopsis) –function?
- No integrins in plants –other cell surface proteins? AGPs?
Induction of PpBit1 by Yariv Reagent

Yariv does not induce other pro-PCD genes
Yariv does induce the anti-PCD BI-1 gene
Yariv Induces PCD in Arabidopsis and Physcomitrella

Bit1 Knockout (KO): Effect on Response to Yariv

KO = Gene Knockout Plant

Yariv reagent
Bit1 Overexpression (OE): Effect on Response to Yariv

OE = Overexpressing Transgenic Plant

Yariv reagent
WT, Bit1-KO and Bit1-OE: Effect on Response to Yariv

KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant
Model of Yariv Action

Yariv

AGPs

Bit1

PCD
Fusarium infection of WT, Bit1-KO and Bit1-OE plants

KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant
**Fusarium infection of WT, Bit1-KO and Bit1-OE plants**

*Physcomitrella patens* infected with *Fusarium graminearum*:GFP

- **WT**
- **Bit1-KO**
- **Bit1-OE**

72h

Mutation of Bit1 affects the ability of *Fusarium* to enter into cells.

Mechanism is not understood

Effect is manifested *at or near the plant cell surface*
Plant Functional Genomic Targets

Plant genes that affect disease susceptibility:

- Programmed Cell Death (PCD)
- Induced Immunity (antimicrobials)
- Cell Wall
  - Glycosyl Transferases
  - Other Cell Wall Modifiers
*Fusarium graminearum* infection in *Physcomitrella XT-KO* (β 1,2 Xylosyl Transferase) plants

ΔXT

24h 48h 72h 96h

*Fusarium-GFP*

Cell death %

KO = Gene Knockout Plant
Fusarium graminearum infection in Physcomitrella FT-KO (α-1,3 Fucosyl Transferase) plants

ΔFT

24h
48h
72h
96h

Fusarium-GFP

Cell death %

KO = Gene Knockout Plant
Fusarium graminearum infection in Physcomitrella FT-KO (α-1,4 Fucosyl Transferase) plants

ΔSKO

Fusarium-GFP

Cell death %

0 25 50 75 100

WT HPGP-OE

24h 48h 72h 96h

KO = Gene Knockout Plant
Fusarium graminearum infection in GNT-KO (N-acetyl glucosaminyl transferase) plants.
The Cell Wall is a Battleground

**Lysobacter enzymogenes**

**Physcomitrella patens**

**Bacterium**

Lytic Enzymes

Plant Cell Wall

TTSS

Virulence Effectors, Toxins etc.

Nucleus
Infection of Physcomitrella with *Lysobacter enzymogenes*

*L. enzymogenes* enters **living** Physcomitrella cells

**GFP labeled *Lysobacter enzymogenes***
Physcomitrella Fasciclin-Like Protein KO infected with *L. enzymogenes*:GFP

Fasciclin-LP KO

% cell death

Fasciclin-LP KO

WT

WT

FLP-KO

Fasciclin-LP KO

Fasciclin comprise a large family of predicted cell surface proteins in plants

KO = Gene Knockout Plant
*L. enzymogenes* on *Physcomitrella* Pectin Methylesterase KO, OE Plants

KO = Gene Knockout Plant  
OE = Overexpressing Transgenic Plant
Effect of \textit{L. enzymogenes} on Xyloglucan endotransglycosylase/hydrolase mutants

KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant
L. enzymogenes: GFP on Physcomitrella Xyloglucan endotransglycosylase/hydrolase Knockout (KO) and Overexpressing (OE) plants
*L. enzymogenes*:GFP on *Physcomitrella* Xyloglucan endotransglycosylase/Overexpressing (OE) plants

**PpXTH-OE**
What else is needed to exploit this system?

Structural Information on the Physcomitrella Cell Wall
Physcomitrella Cellulose Synthase Genes

The cellulose synthase (CESA) gene superfamily of the moss Physcomitrella patens

Alison W. Roberts - John T. Bushoven

Fig. 1 Gene structure of all P. patens CESA and CSL genes for which cDNA sequences are available. Introns were identified by comparing cDNA sequences with contigs assembled from genomic shotgun sequences. Three genomic sequences were incomplete, resulting in uncertainty of intron or position. Introns shared by members of different families are indicated.
Parsimony phylograms corresponding to the majority consensus of 1,000 bootstrap replicates (% values above 74 shown, * indicate polytomies).

a Unrooted phylogram of all full-length CESA-, CSLB-, CSLD-, CSLE-, CSLF-, CSLG-, and CSLH-deduced amino acid sequences from *P. patens*, rice and Arabidopsis.

b Phylogram of full-length CESA-deduced amino acid sequences from *P. patens*, Arabidopsis, rice, maize and *Pinus taeda* rooted with CESA1 from *M. caldariorum* (Mc; GenBank accession number AF525360). Shaded and open boxes distinguish Arabidopsis CESA*s thought to function as heterotrimers in primary and secondary cell wall syntheses, respectively.

c Unrooted phylogram of all full-length CSLA- and CSLC-deduced amino acid sequences from *P. patens*, rice, and Arabidopsis.

Roberts, 2007 Plant Mol Biol 63:207
### Physcomitrella Cell Wall Carbohydrate Linkage Analysis

#### Table 2: Linkage analysis of 6-8-day-old *P. patens* protonemal tissue showing the relative abundance of linkage positions for major monosaccharides

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<th>Monosaccharide</th>
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<td>Galp</td>
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<td>2-</td>
<td>0.8</td>
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<td>4-</td>
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<tr>
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<td>4,6-</td>
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<tr>
<td>Galactitol</td>
<td>Terminal</td>
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<td>Glucitol</td>
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Peña et al, 2008 Glycobiology, in press
Comprehensive Microarray Polymer Profiling (CoMPP)

**Arabidopsis**

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<th>Polymer Type</th>
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<tr>
<td>Glucans</td>
<td>789</td>
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<td>Glycoproteins</td>
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**Physcomitrella**

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<td>Proteoglycans</td>
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Helps define limitations & utility of model system
Physcomitrella is a rapid and sensitive system ‘companion system’ for plant reverse genetics and functional genomics.

We have established its utility to identify a number of mechanisms involved in susceptibility to fungal pathogens (PCD, immune responses, cell wall).

Genes identified in Physcomitrella can be exploited through transgenesis in crops, including wheat and sugarcane.

Modifications to genes that affect cell surface and cell wall function can influence the degree and course of infection.

This system should be useful for examining the effects of modifying cell wall genes on other properties of the plant cell wall, e.g. biogenesis, maturation, cross-linking, accessibility to digestion.

Conclusions
Special Thanks To:

FAPESP

Marcos Buckeridge
Helaine Carrer
Marcio de Castro Silva-Filho
Glaucia Mendes Souza

And all our colleagues and partners at USP

Obrigado pela sua atenção!
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- Luciana Ambrozevecius (ESALQ)
- Carmen Collazo
- Devino Rajah
- Bozehna Lisko,
- Jared Cohen

**Pathology and PCD**
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- Brad Hillman, Rutgers
- Nilgun Tumer, Rutgers
- Eric Lam, Rutgers
- Joyce Loper, Oregon State
- Susan von Bodman, U. Conn
- Jin-Rong Xu, Purdue
- Louise Brisson, Laval

**C. elegans**
- Monica Driscoll, Rutgers
- Barth Grant, Rutgers

**Genomo-Physcology**
- Ralf Reski, Freiburg
- Stephan Rensing, Freiburg
- Eva Decker, Freiburg
- Wolfgang Frank, Freiburg
- Gertrud Wiedemann, Freiburg

**Sulfur metabolism**
- Stan Kopriva JIC

**Cryo em**
- Biozentrum Basel e.m. Facility

**ABI-mutants**
- Yoichi Sakata, Tokyo Univ. of Agriculture

**GFP Physcomitrella lines**
- Tomomichi Fujita, Hokkaido University

**VIGS wheat assays**
- Steve Scofield, Purdue

---

**Funding**
- US Wheat and Barley Scab Initiative
- Rutgers Biotech Center
- Charles and Joanna Busch Foundation
The anti-(1→5)-α-L-arabinan monoclonal antibody LM6 binds to some AGPs in *Physcomitrella patens*. Preferentially found on growing cell tips. JIM5 is a control that labels all cells.

**Effect of gene knockout of AGP1:** Retarded cell extension growth