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

Michael Lawton

Biotech Center

Rutgers University

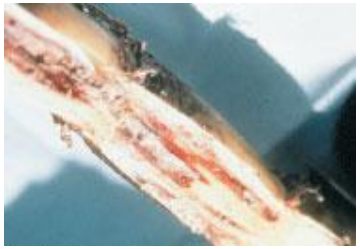
Talking Points

- 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.

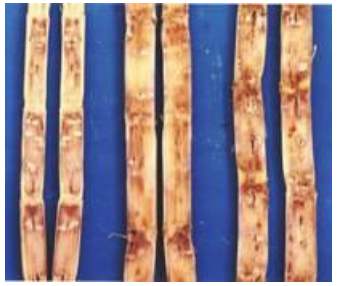
[Botanic Gardens Trust
NSW Australia



Stem Borers

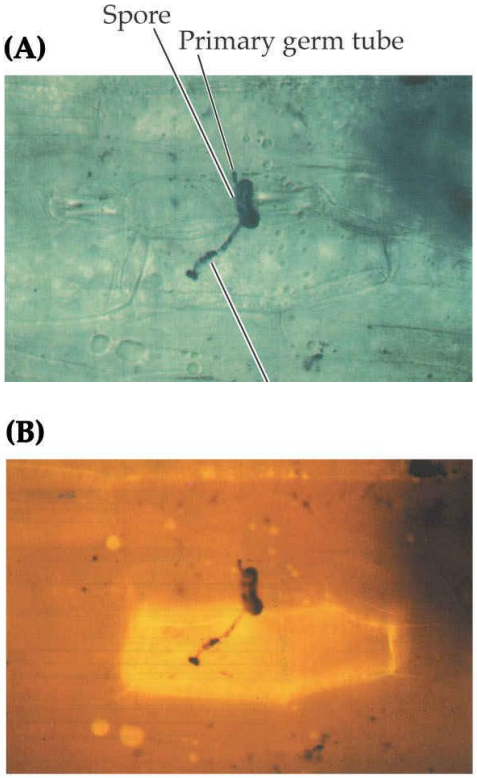
Chilo terrenellus *Diatraea saccharalis*

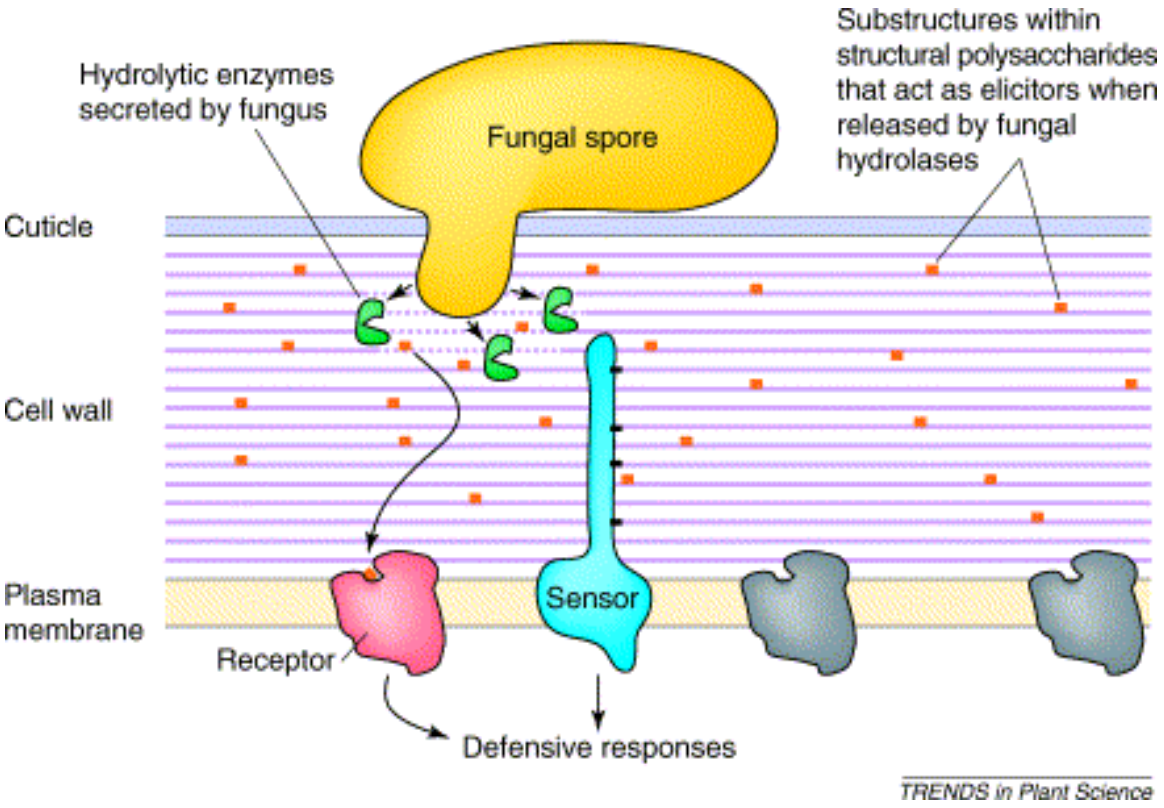
insects.tamu.edu/extension/bulletins/mp-1777.html



Red Rot Disease
caused by *Colletotrichum falcatum*
[Sugarcane Breeding Station, Coimbatore, India]
sugarcane-breeding.tn.nic.in/pathology.htm

Role of the Cell Wall in Resistance and Susceptibility





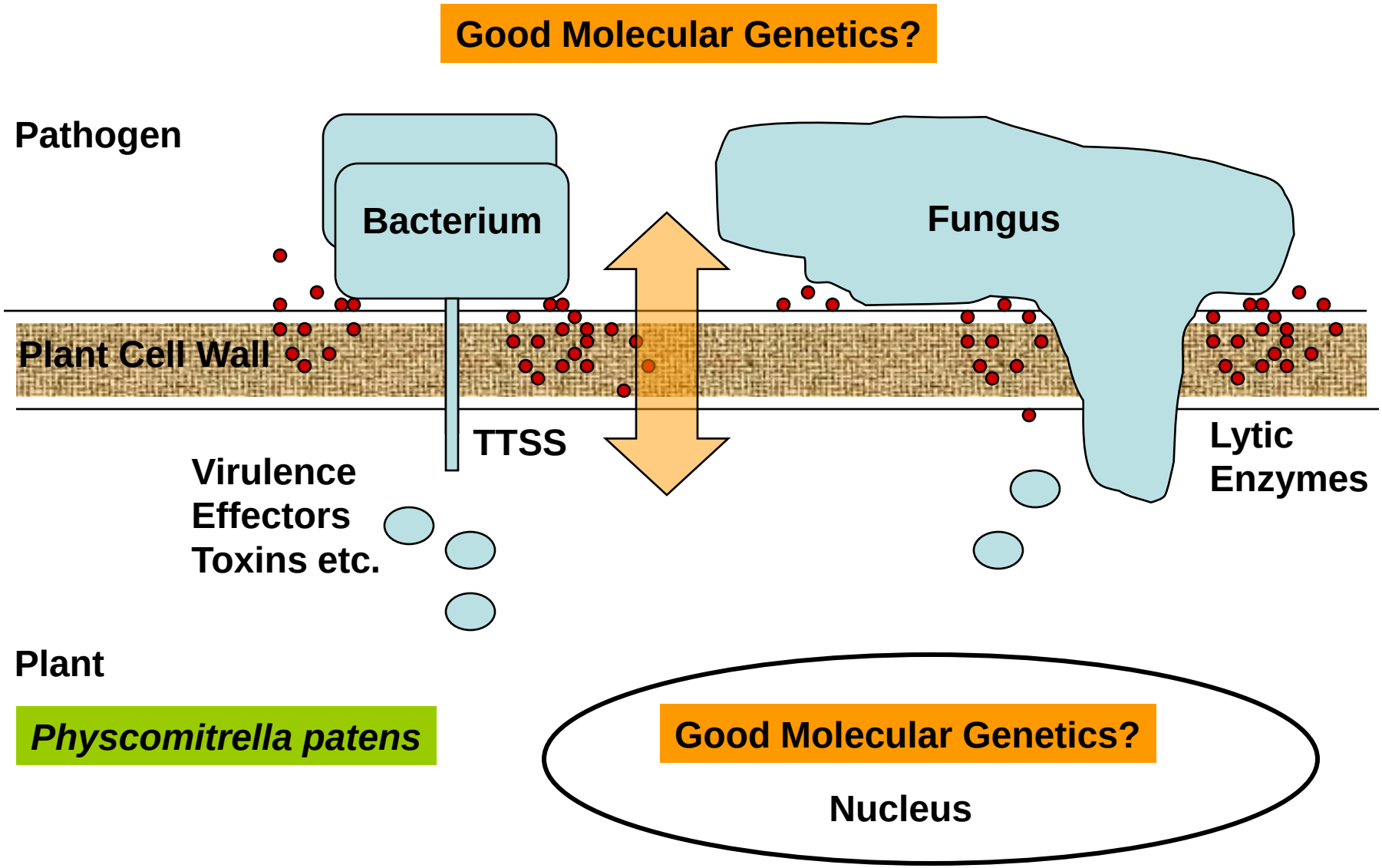
Buchanan, Gruissem, Jones

Vorwerk S, Somerville S, Somerville C. (2004)
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



What is *Physcomitrella patens*?

Grows like yeast but is a multicellular plant

Haploid gametophyte dominates life cycle

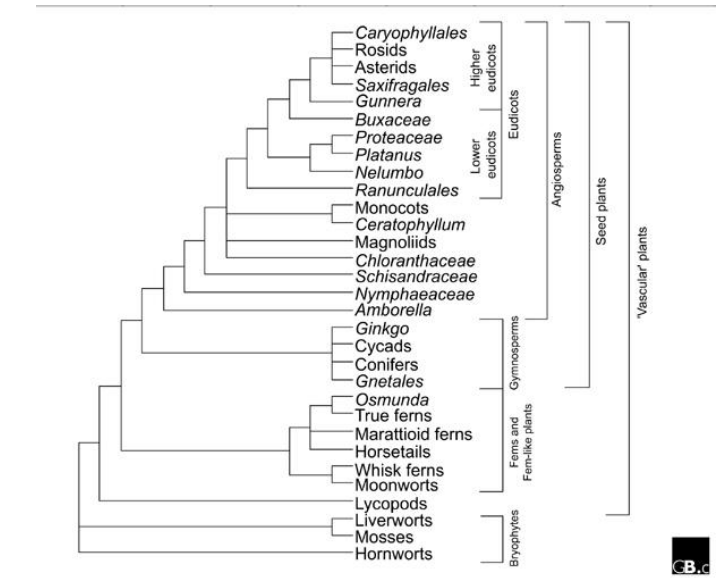
Genome size: 511Mbp; 27 chromosomes

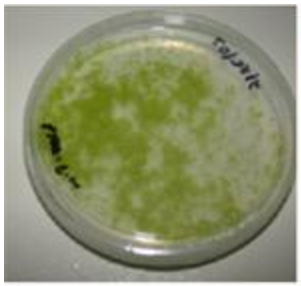
Genome sequence completed in 2007

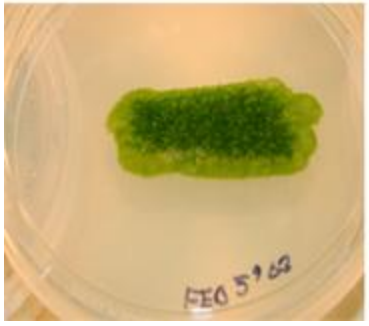
Functional conservation with higher plants and yeast

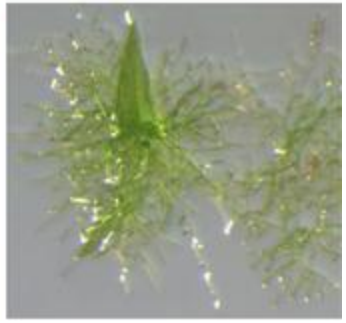
Undergoes high efficiency homologous recombination

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









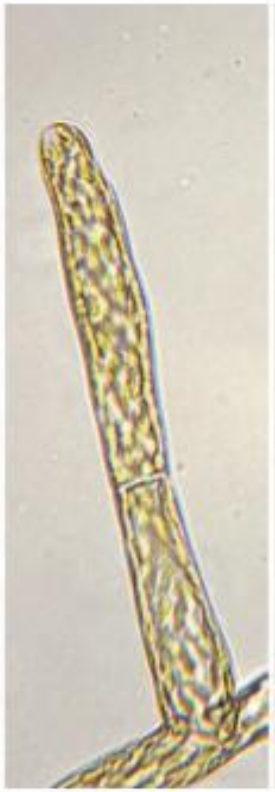
Model system for functional genomics

- Disease, Cell Death, Cell Wall

Physcomitrella Patens Lifecycle

Spore germination

Protonemal Growth

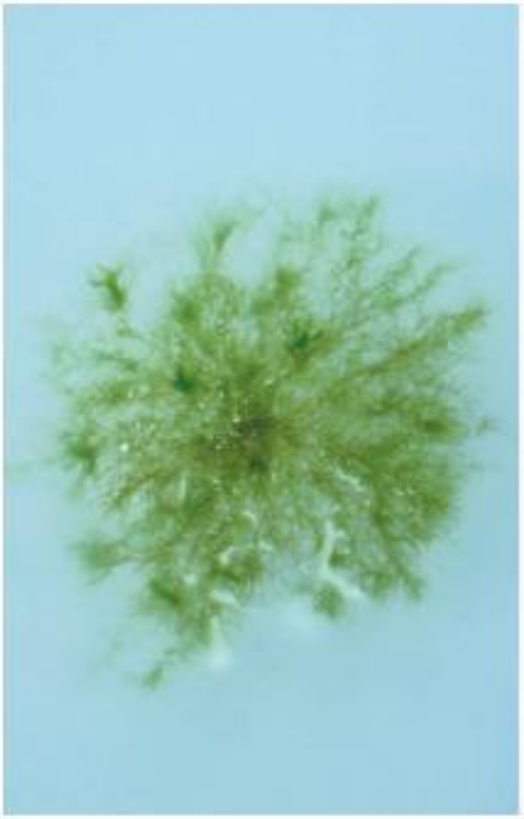


Chloronema

Caulonema

Physcomitrella Patens Lifecycle

Bud cell development



Colony Growth

Gametophore

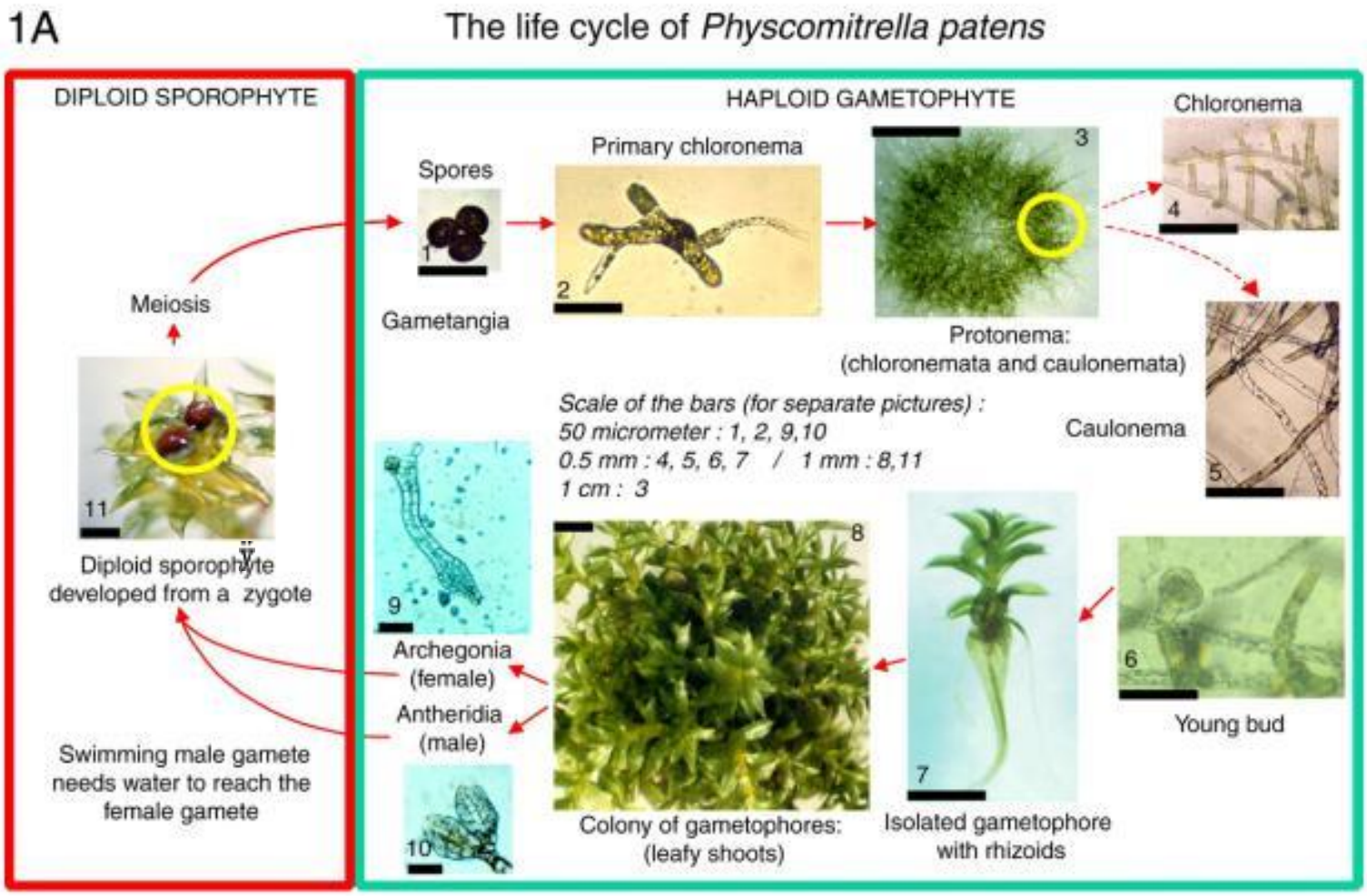
Physcomitrella Patens Lifecycle

Gametophore
with rhizoids

U: Antheridia
L: Archegonia

Spore Capsule

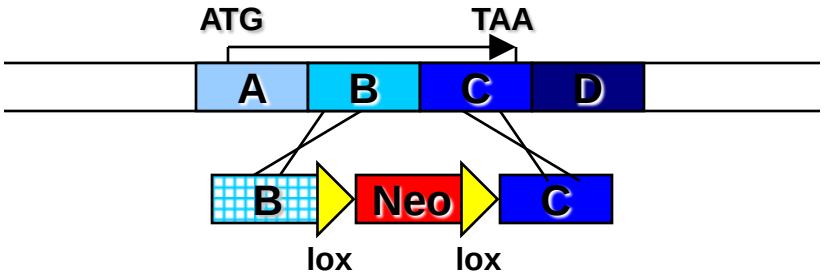
Haploid Phase Dominates: Reveals Mutant Phenotypes



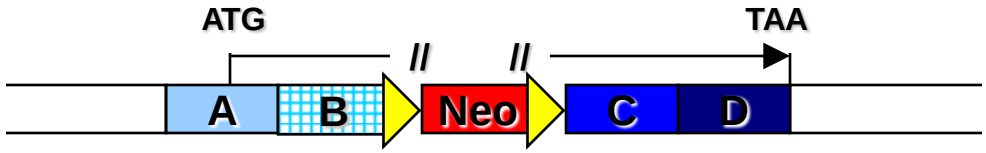
Plant Physiol, December 2001, Vol. 127, pp. 1430-1438
The Moss *Physcomitrella patens*, Now and Then. Didier G. Schaefer and Jean-Pierre Zrÿd

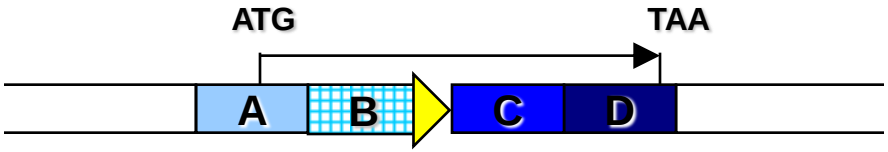
Gene knockout by homologous recombination

Genomic DNA

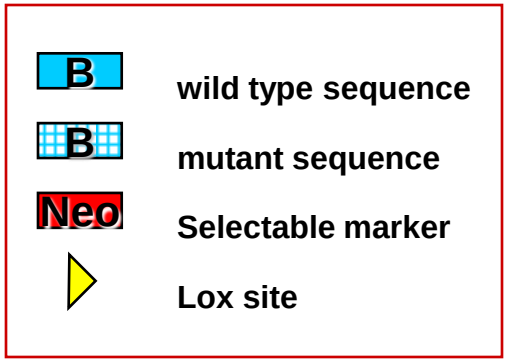


Double homologous recombination





Possible reversion of targeted gene following site-specific recombination



Result: gene disruption by allele replacement

3-10% reversion to Neo^{S} following transient expression of CRE recombinase

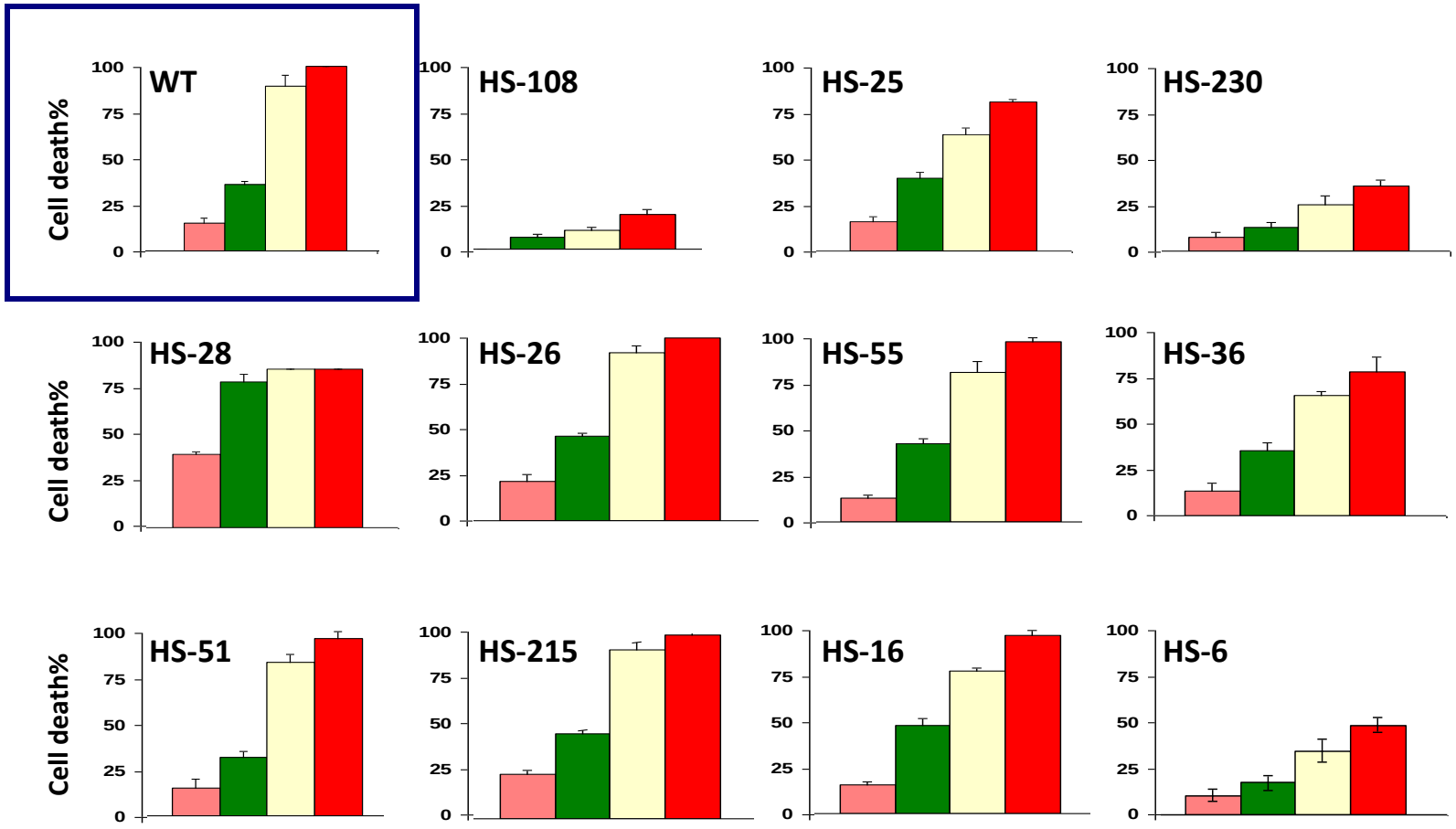
Recombination rates in Physcomitrella

Efficiency (GT/GT+IR):	**4% (.5kb) up to 100% (2-4 kb) in *Physco***
	0.005-0.1% in other plants
	95% in *S. cerevisae*
	1-30% in *N. crassa*
	0.1-1% in mouse ES cells
Efficiency/ug DNA:	**0.5 to 30 (size dependent) in *Physco***
	0.001-0.02 in other plants
	1-10 in yeast, *A. nidulans*
Efficiency/living cell	**10^{-3} to 10^{-4} (size dependent) in *Physco***
	10^{-6} in other plants
	10^{-4} in mouse ES cells
	10^{-5} in *Dictyostelium*

Physcomitrella Functional Genomics

- 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

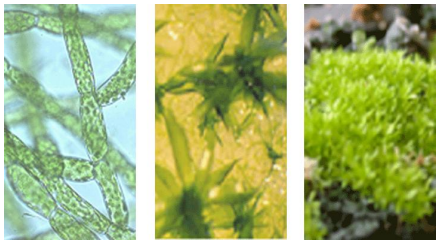
Infection Assay of Gene Knockout Plants

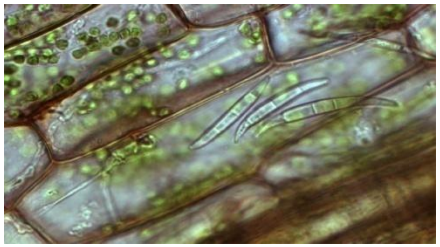


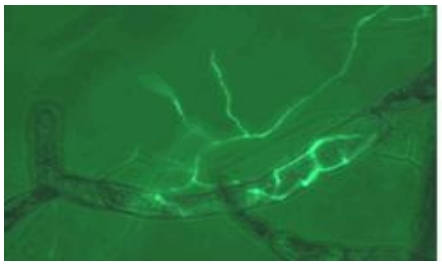
Lots of 'failures' –you need a high capacity system in order to select those rare genes that do confer resistance.

“Physcopathology”

Physcomitrella



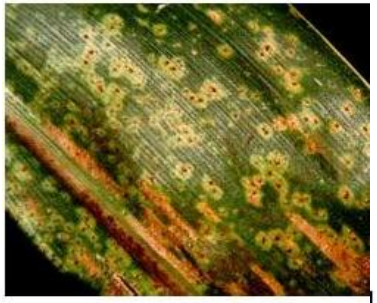




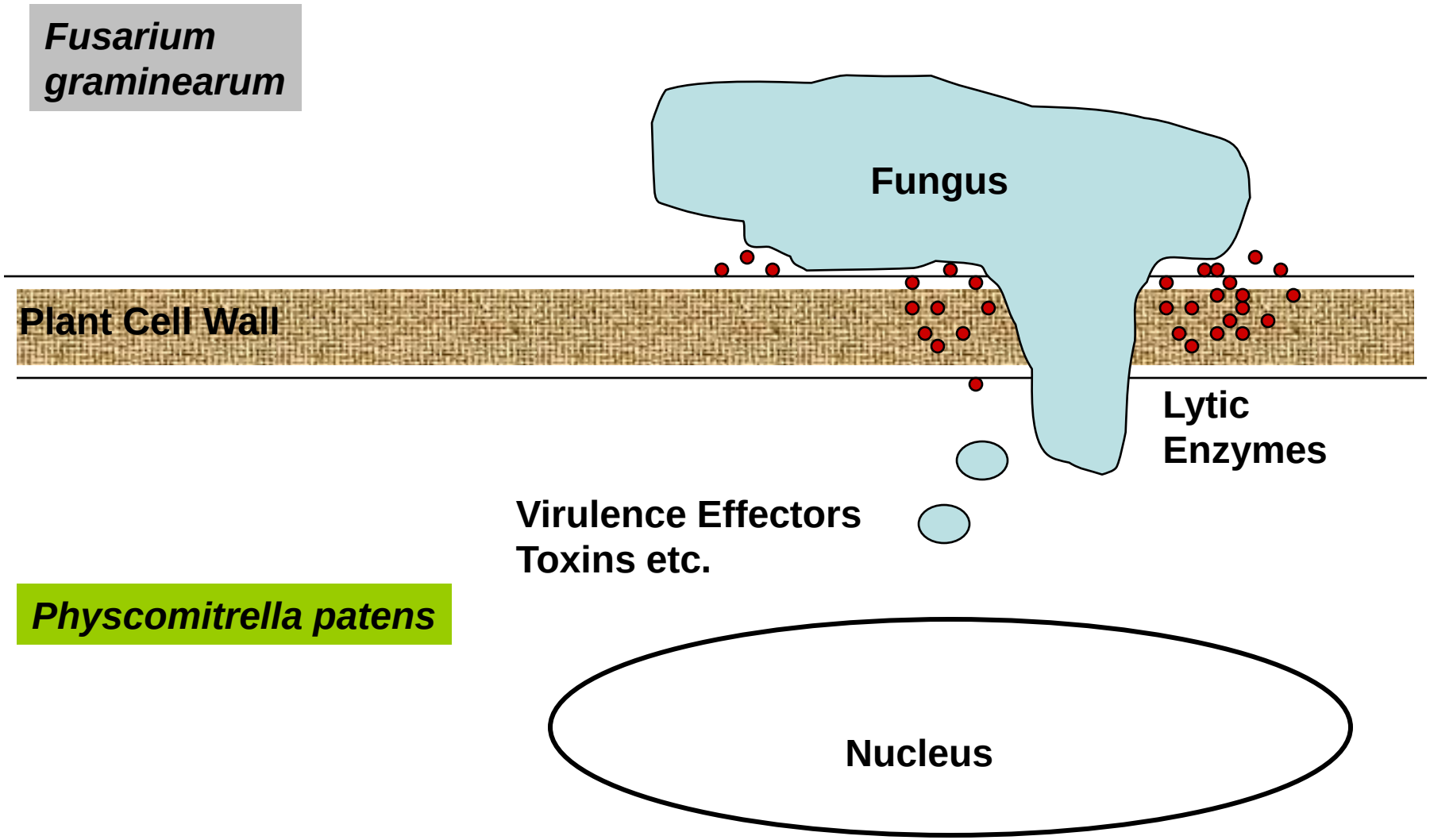
Working hypothesis:
That some, and perhaps many of the cellular mechanisms involved in the expression of disease resistance and susceptibility are shared between mosses and angiosperms.

functional comparison

Crop Plants



The Cell Wall is a Battleground



Fusarium graminearum

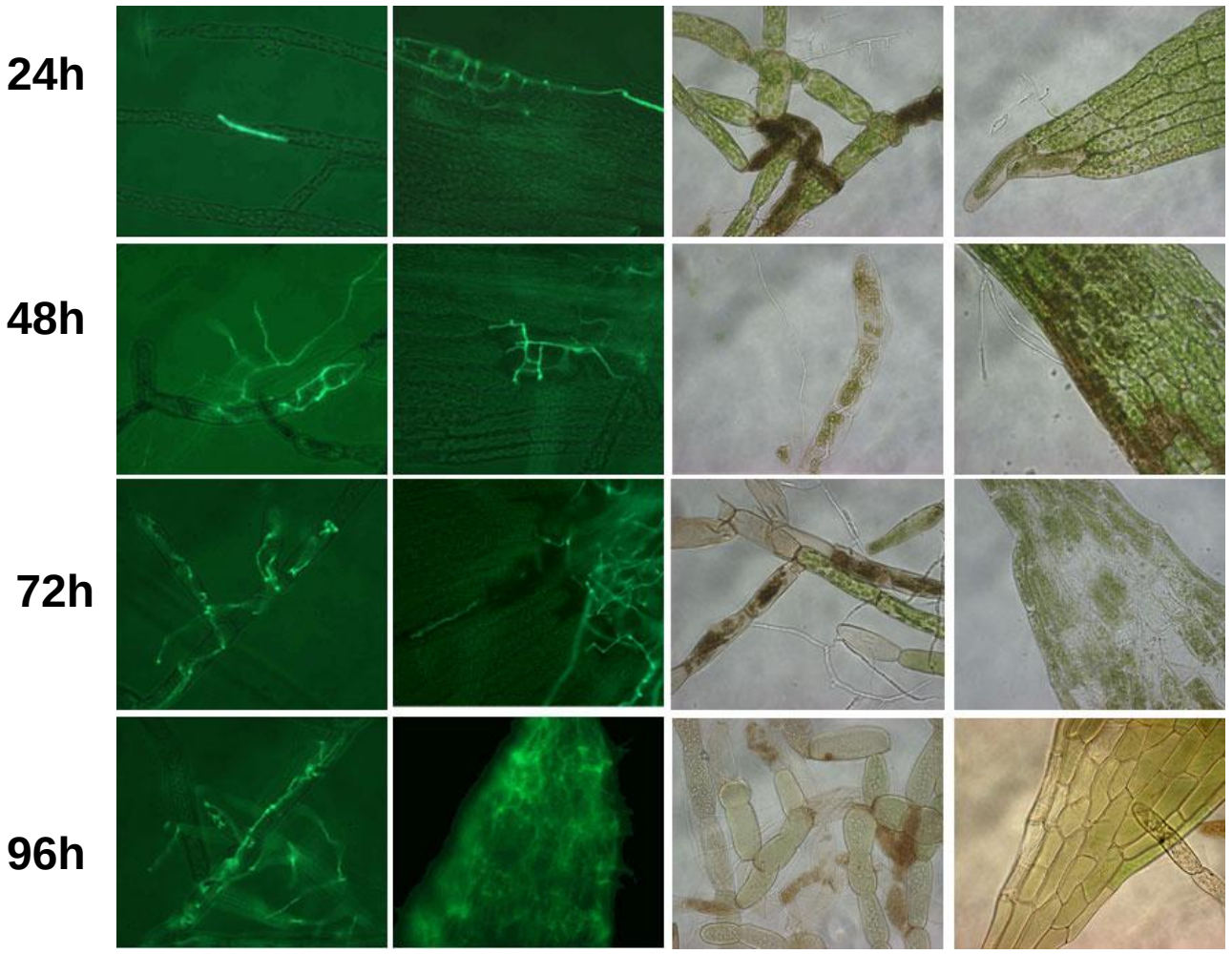
Wheat heads with symptoms of Fusarium head blight.

Penn State *Agricultural Research and Cooperative Extension*

Fusarium on barley seed

- **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*



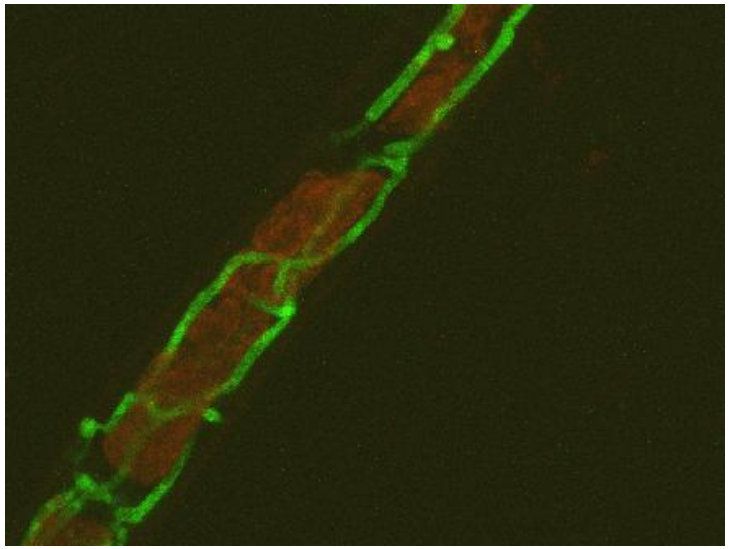
Infection of Physcomitrella by *Fusarium graminearum*

Confocal

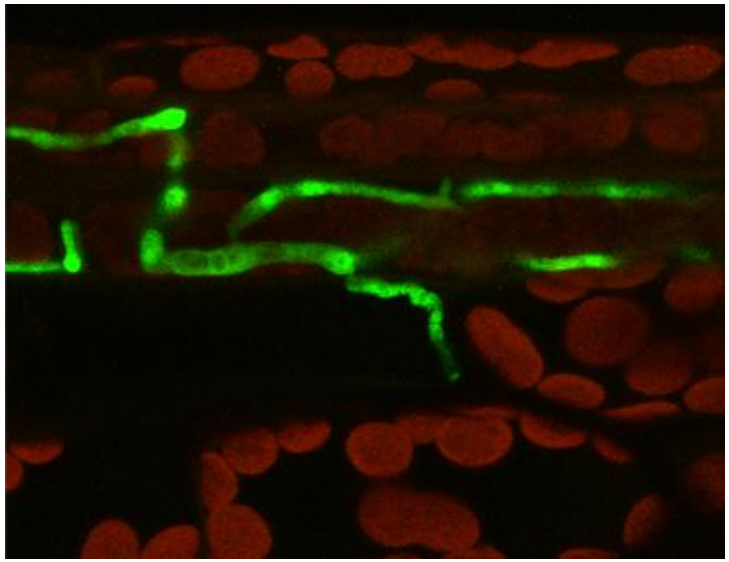
Cryo-e.m.

GFP-labeled *Fusarium graminearum*

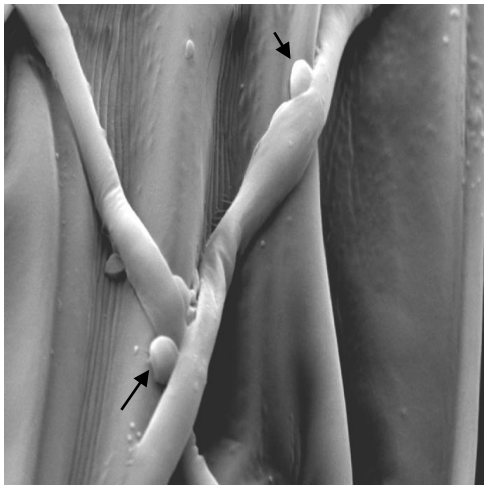
Protonema



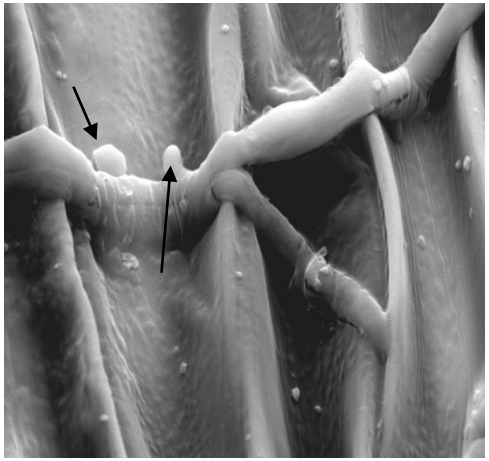
Gametophore



24h



Gametophore

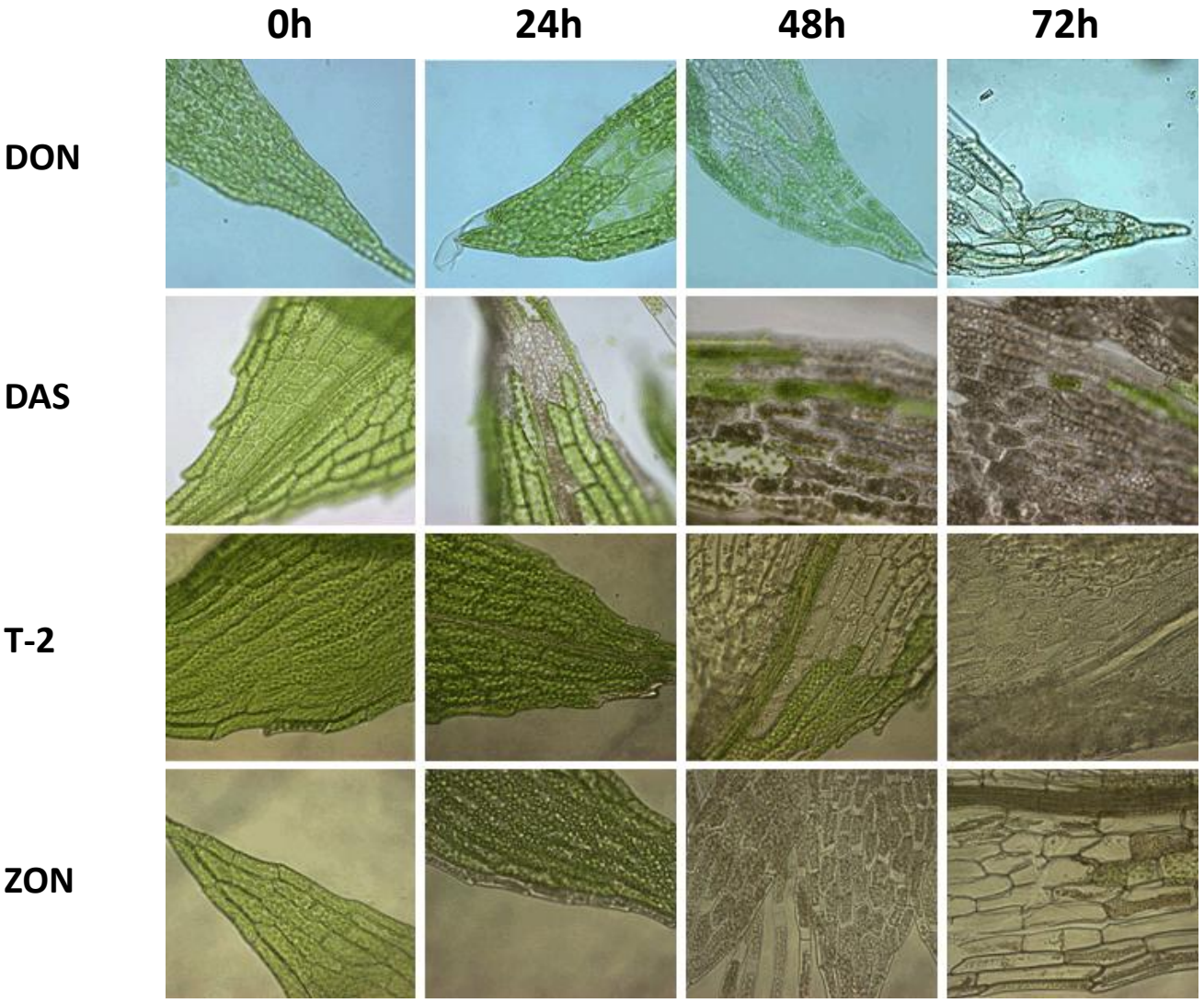


Gametophore

24h

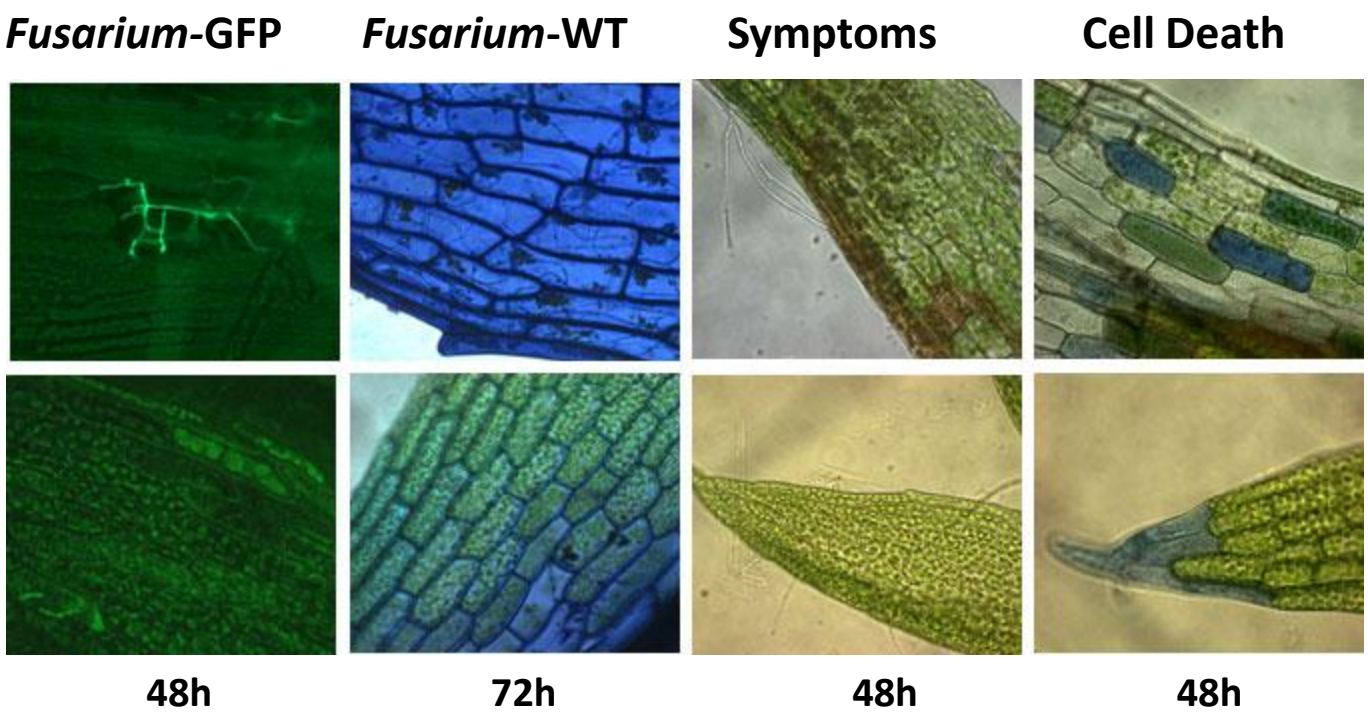
Thanks to Stephan Rensing and the Biozentrum, Basel for cryo em images

Physcomitrella is sensitive to *Fusarium* mycotoxins



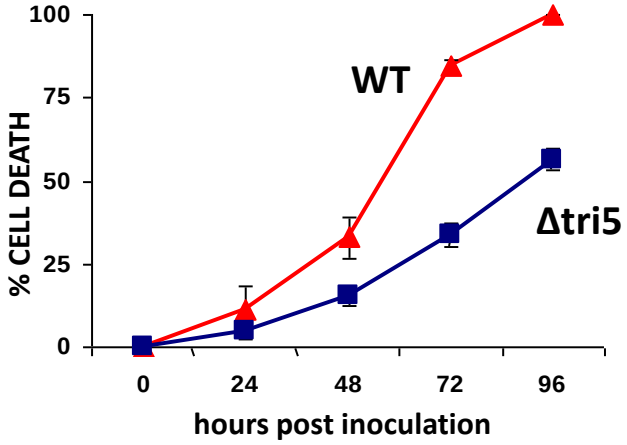
Tricothecenes contribute to *F. graminearum* virulence

A



The Δ*tri5 strain of F. graminearum* does not produce DON or DAS

B



Cell death is clearly important

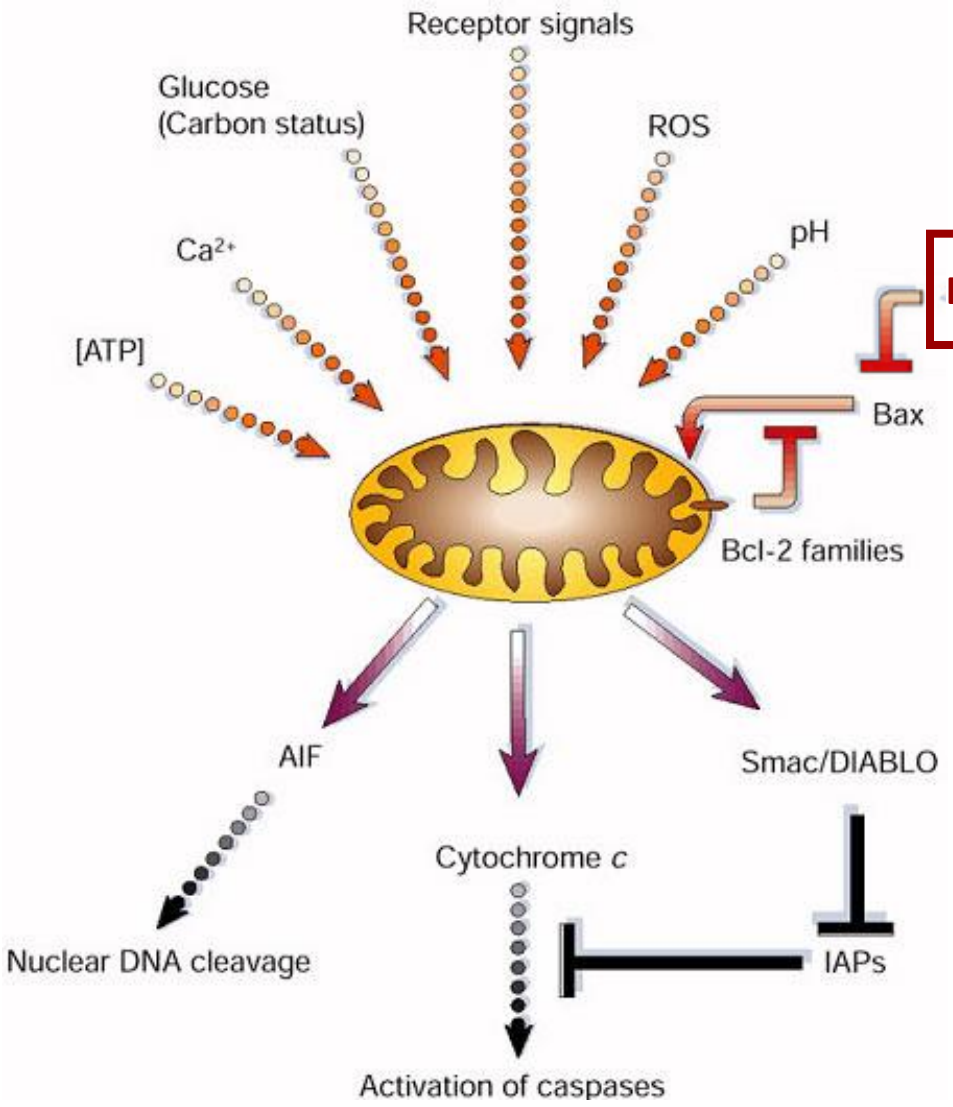
So we focus on genes that control Programmed Cell Death (PCD)

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



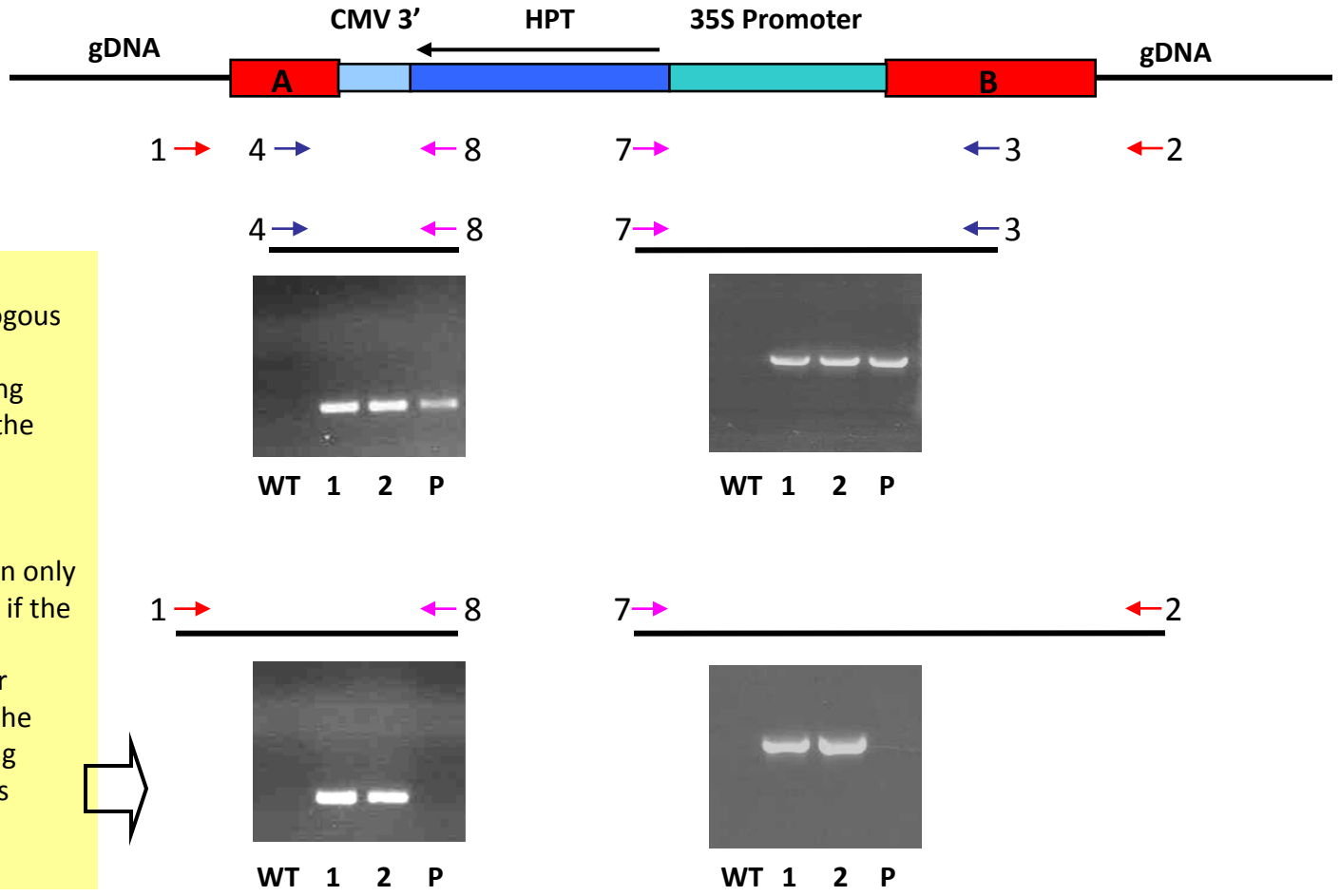
BI-1 is an antagonist of the pro-apoptotic protein Bax.

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

PpBI-1 knockout (PpBI1-KO): structural verification

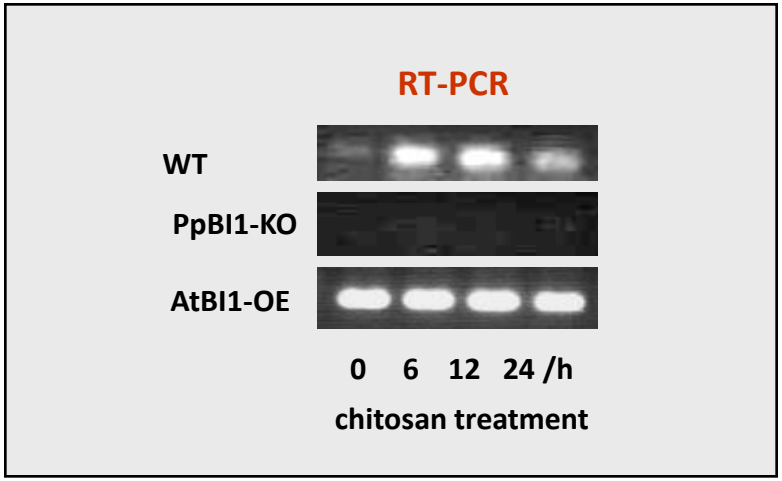


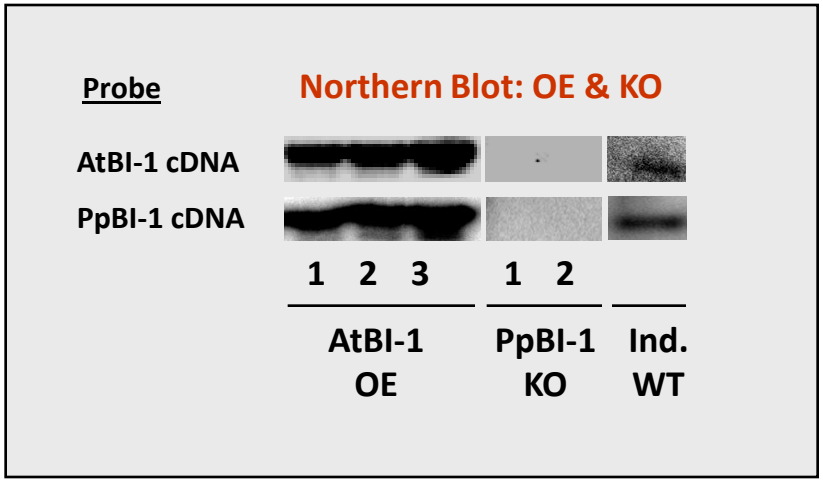
Plants that have undergone homologous recombination are verified by PCR using primers that flank the predicted sites of integration.

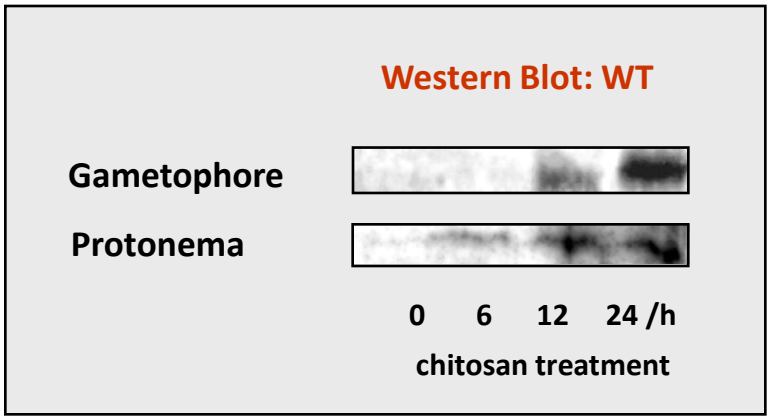
E.g. primers 1+8 can only amplify a fragment if the HPT gene of the replacement vector integrates next to the appropriate flanking genomic sequences

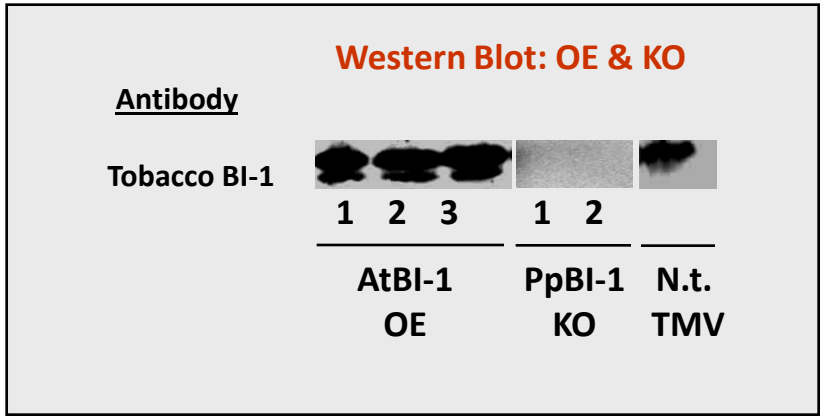
WT: Wild Type **1: PpBI-1 KO line-1** **2: PpBI-1 KO line-2** **P: KO-plasmid**

PpBI-1 KO and AtBI-1 OE lines: expression studies



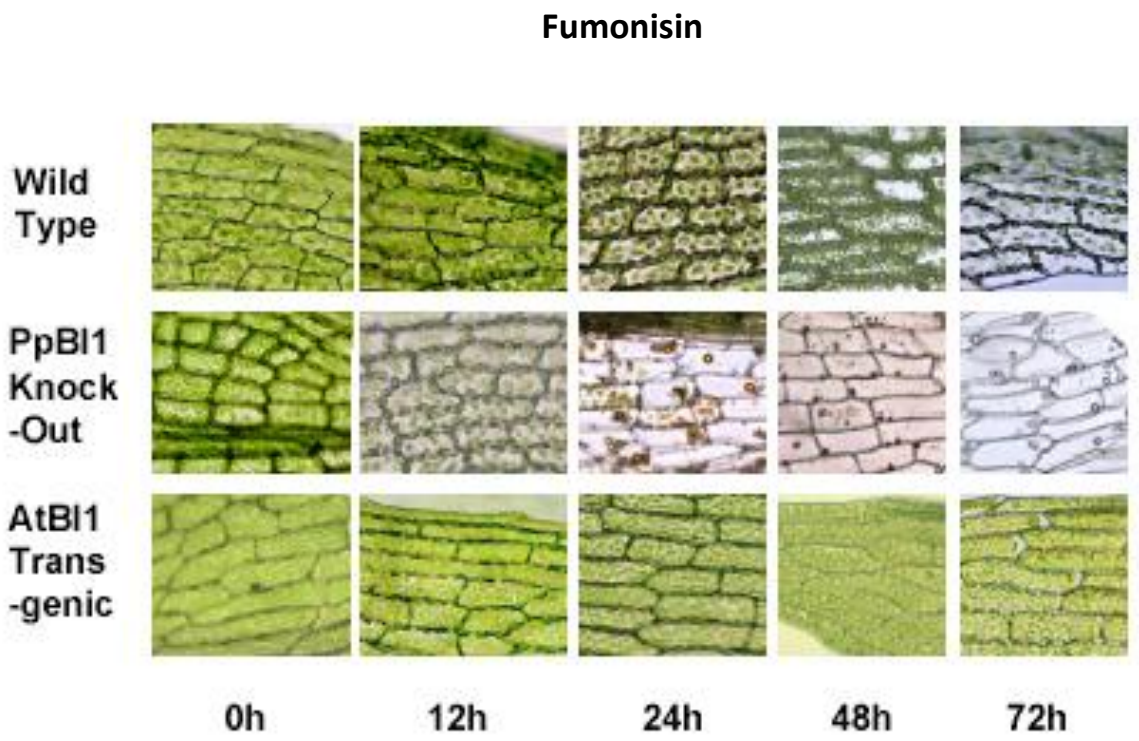


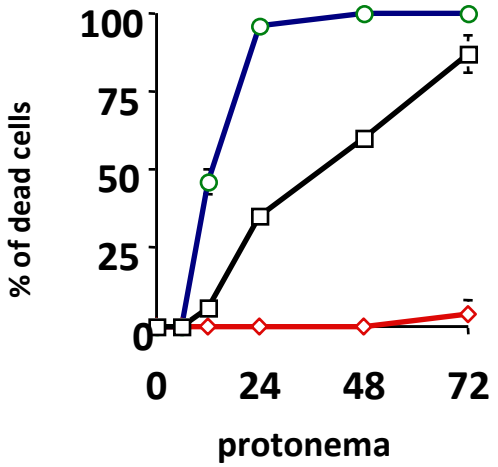


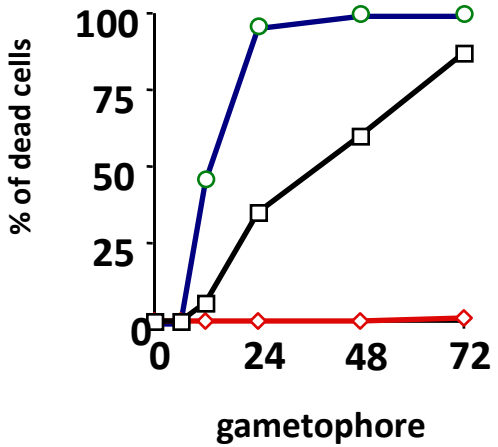


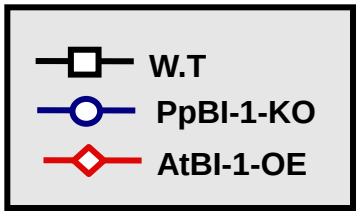
The PpBI-1 gene is inducible by chitosan (and other treatments)
PpBI1 KO plants express no BI-1 mRNA or protein
AtBI1 OE plants constitutively express BI-1 mRNA and protein

PpBI-1 KO and AtBI-1 OE lines: toxin sensitivity







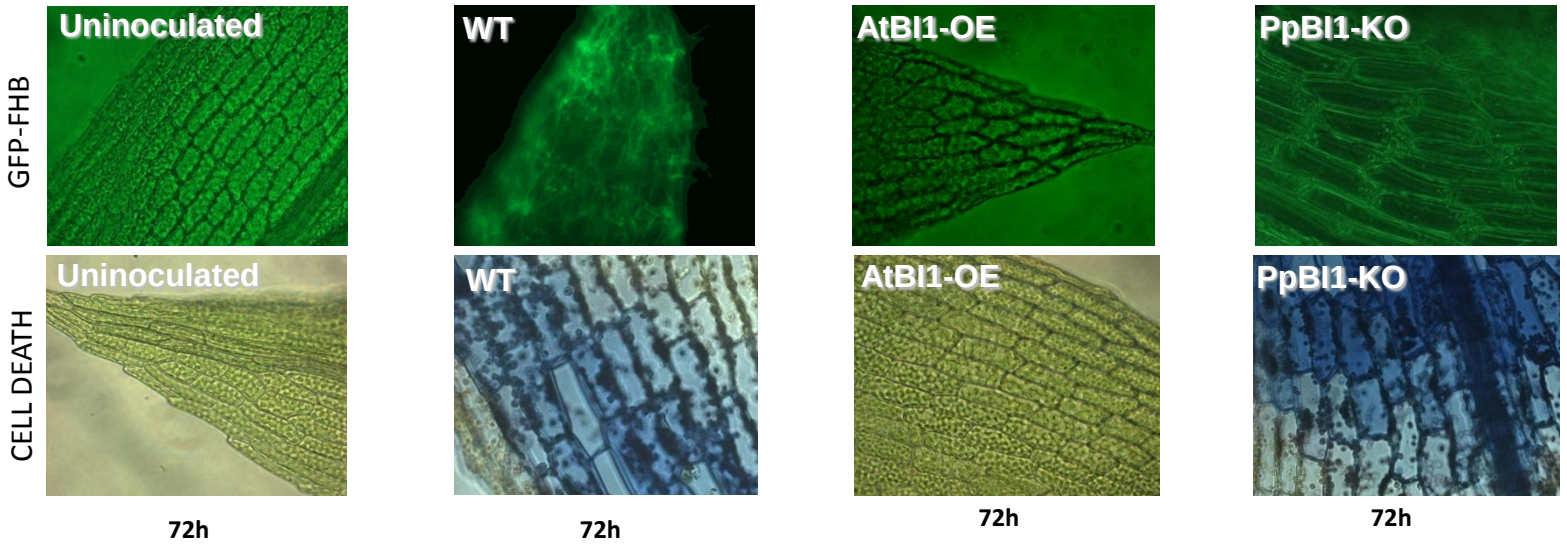


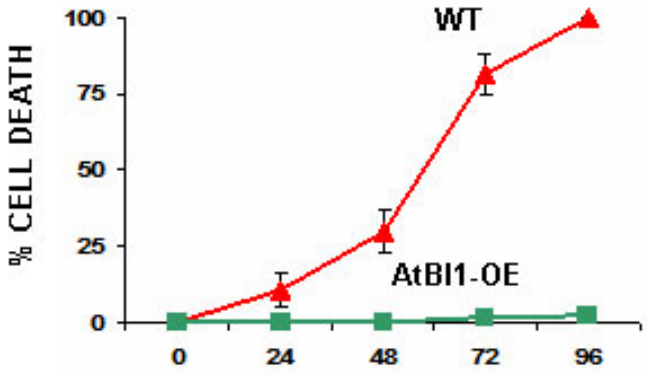
KO = Gene Knockout Plant

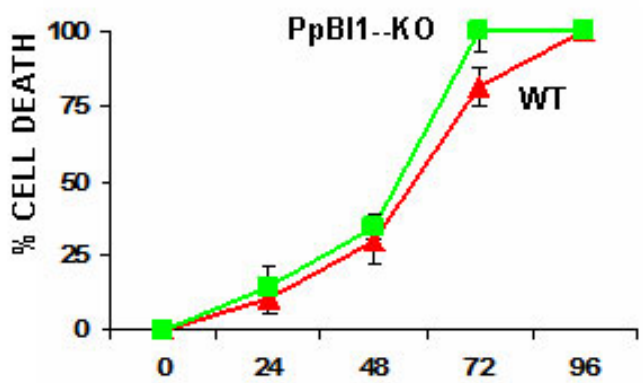
OE = Overexpressing Transgenic Plant

AtBI-1 OE and PpBI-1 KO plants: susceptibility to *F. graminearum*

A



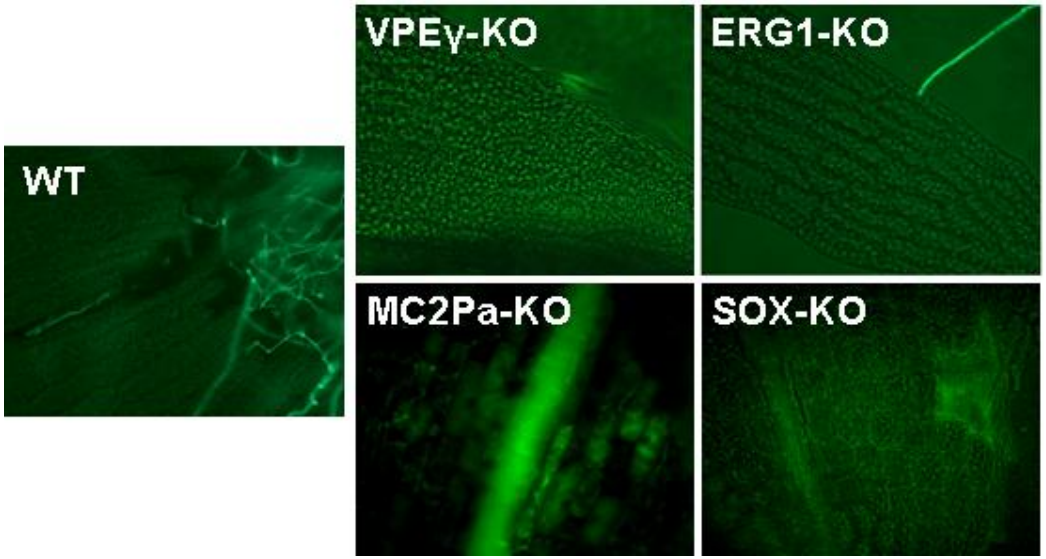


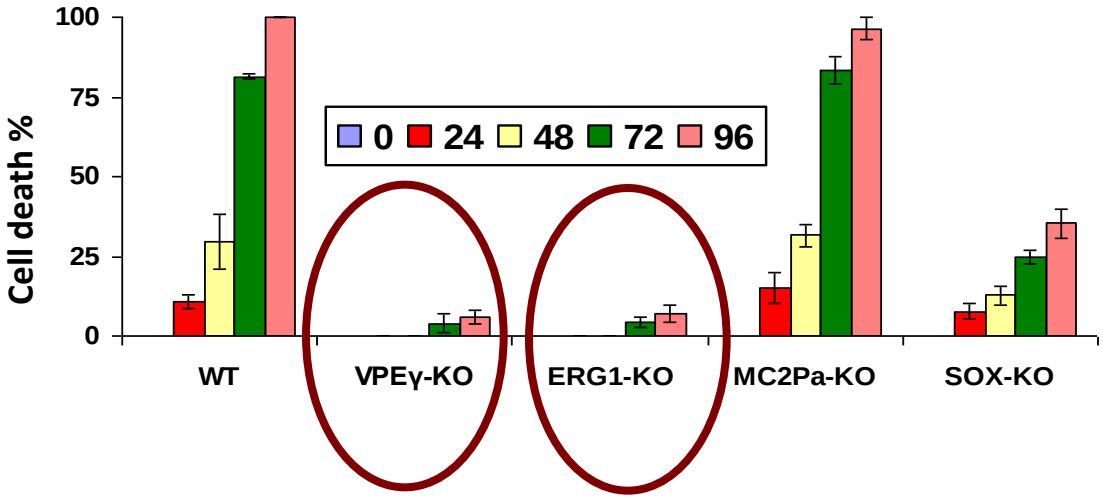


KO = Gene Knockout Plant

OE = Overexpressing Transgenic Plant

Other gene knockout plants show enhanced resistance to *Fusarium graminearum*



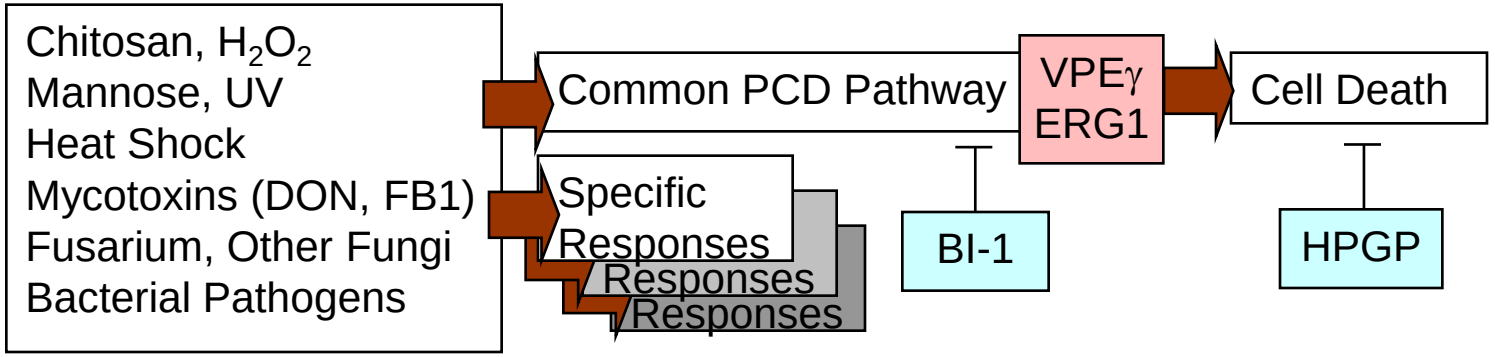


KO = Gene Knockout Plant

OE = Overexpressing Transgenic Plant

Programmed Cell Death and Disease Suseptibility

- **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

Select potential antifungal genes

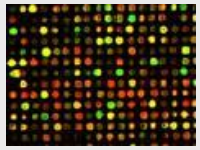
Isolate selected genes

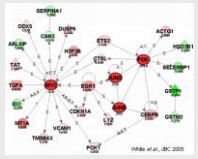
Physcomitrella: Create gene knockouts and overexpressors

Physcomitrella: Assay plants for Fusarium resistance

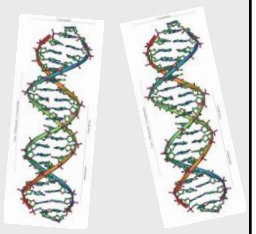
Wheat: assay selected genes for FHB resistance

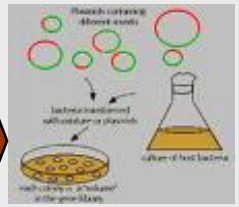
Wheat: Stable Transgenics

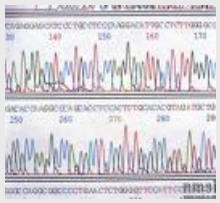




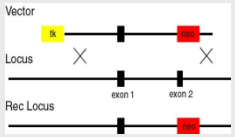
Based on genetic, molecular data from wheat and other species

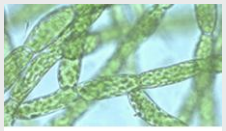




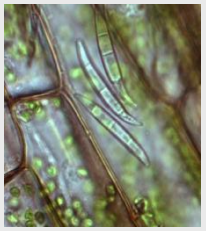


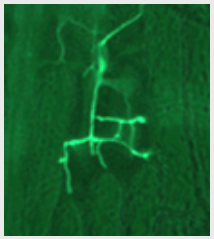
Clone and sequence genes

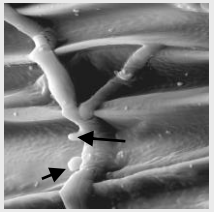




Mutate or express genes in Physcomitrella







Expose mutant plants to FHB, DON

Transient assay in wheat using VIGS (Scofield Lab)

Transformed Fusarium-Resistant Wheat

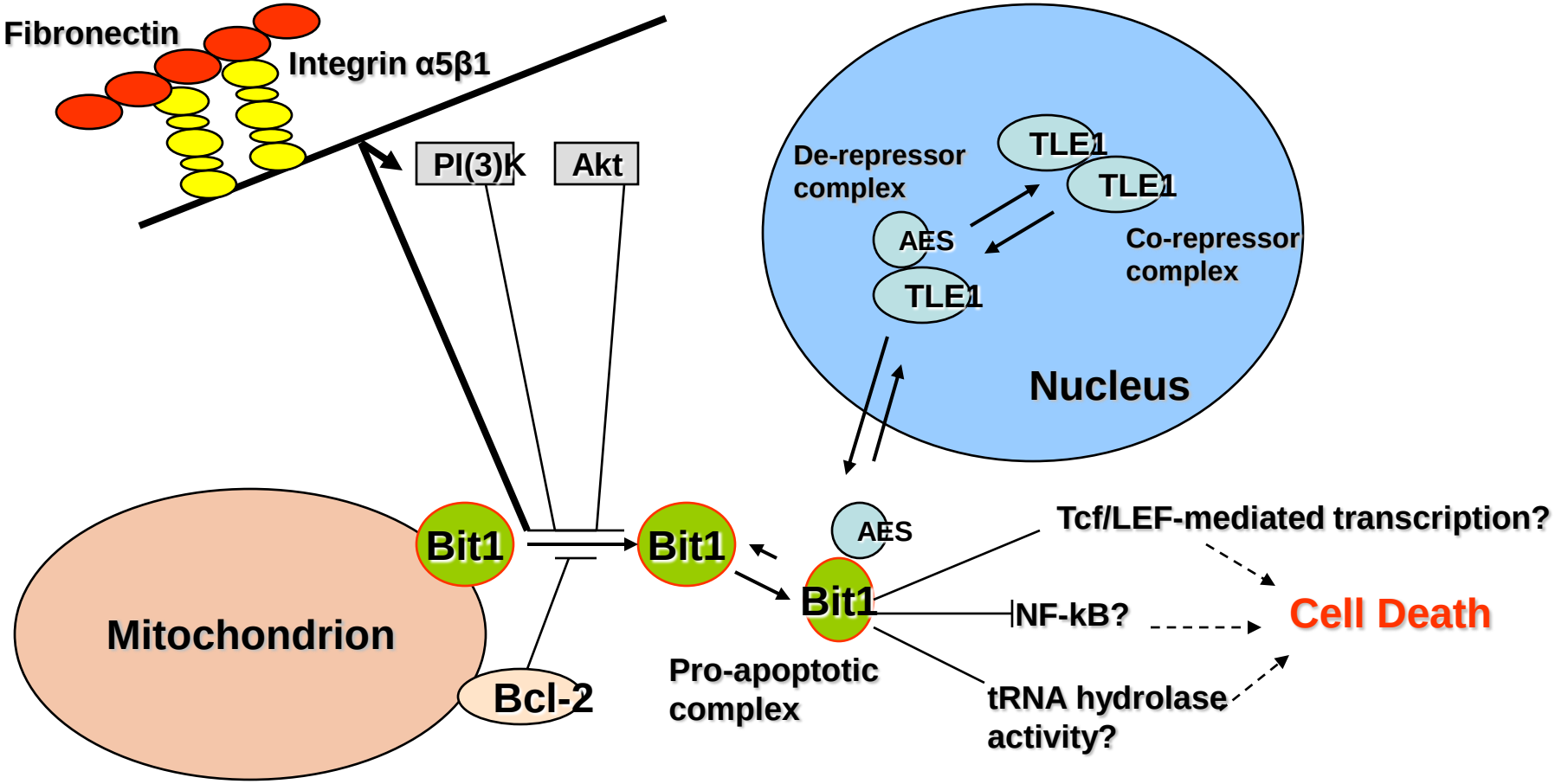
(Or Sugarcane)

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"



Stupack & Cheresh, Nature Cell Biology, 6: 388-389, May 2004.

Bit1 and Anoikis

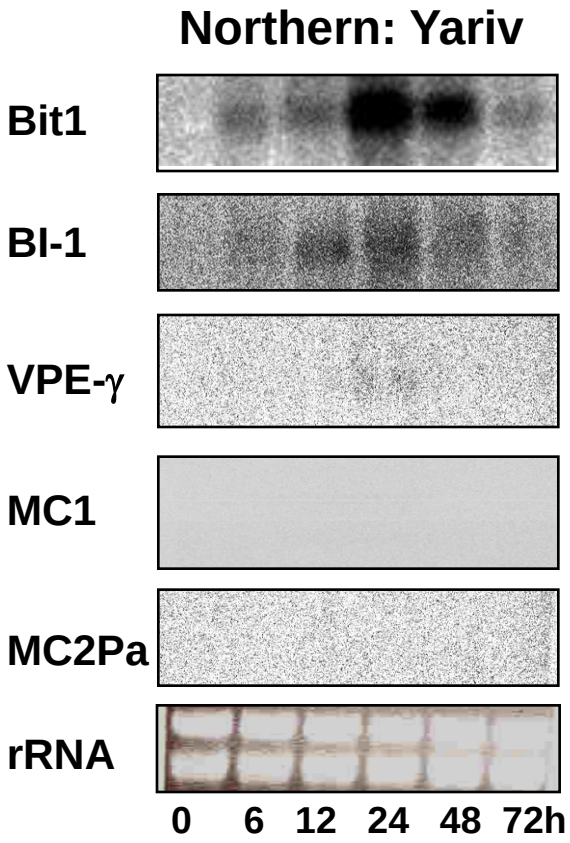
A Mitochondrial Protein **Bit1**, Mediates Apoptosis Regulated by Integrins and Groucho/TLE Corepressors

Ruoslahti et al, Cell 116:751-762, March 2004.

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



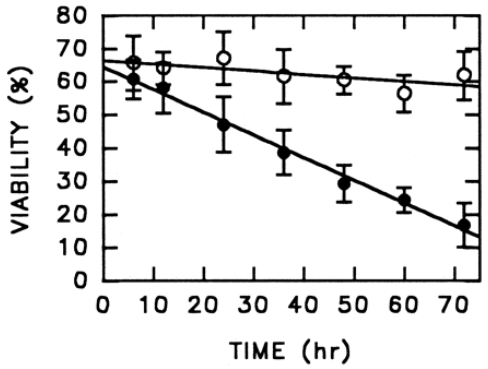
Yariv does not induce other pro-PCD genes

Yariv does induce the anti-PCD BI-1 gene

Yariv Induces PCD in Arabidopsis and Physcomitrella

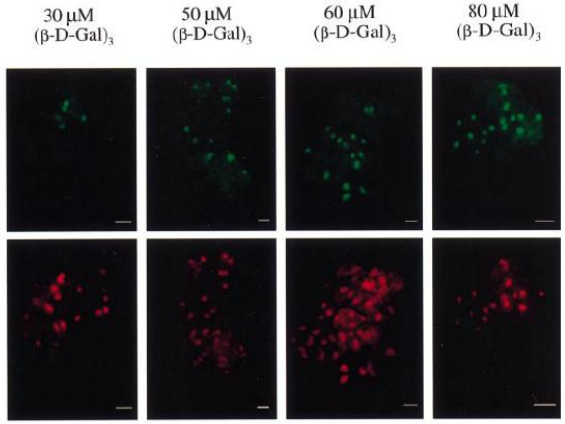
Arabidopsis

Viability

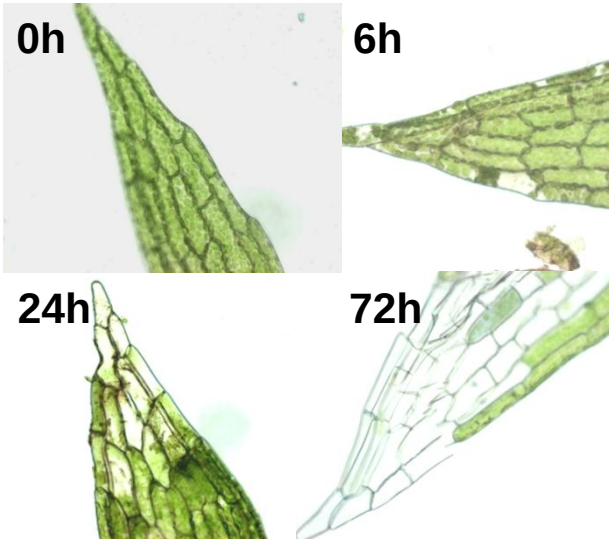


TUNEL

Propidium Iodide



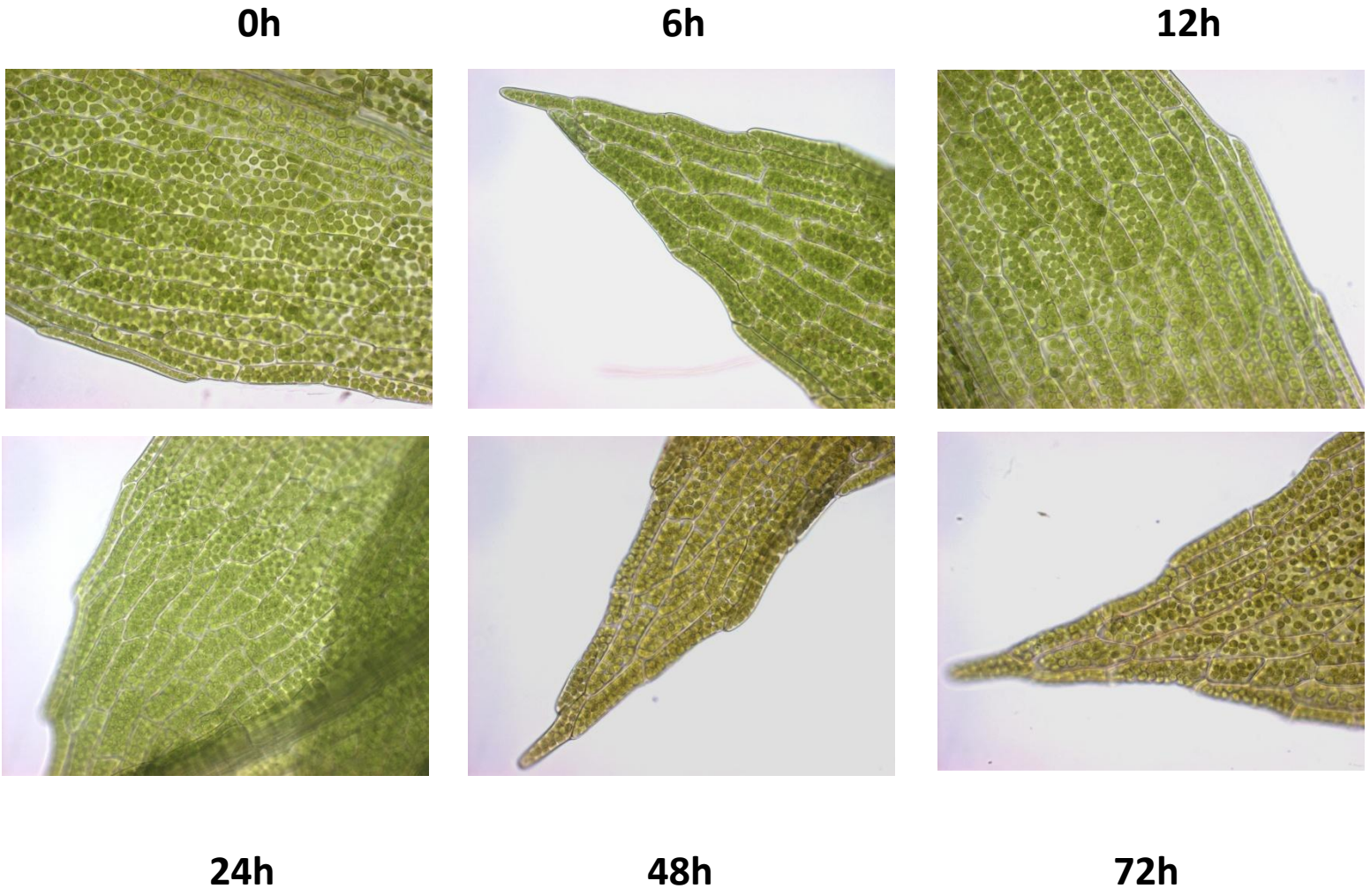
Physcomitrella



Gao and Showalter, Plant J. Volume 19, Issue 3, Pages 321-331 (1999)

Guan and Nothnagel, *Plant Physiology* 135:1346-1366 (2004)

Bit1 Knockout (KO): Effect on Response to Yariv

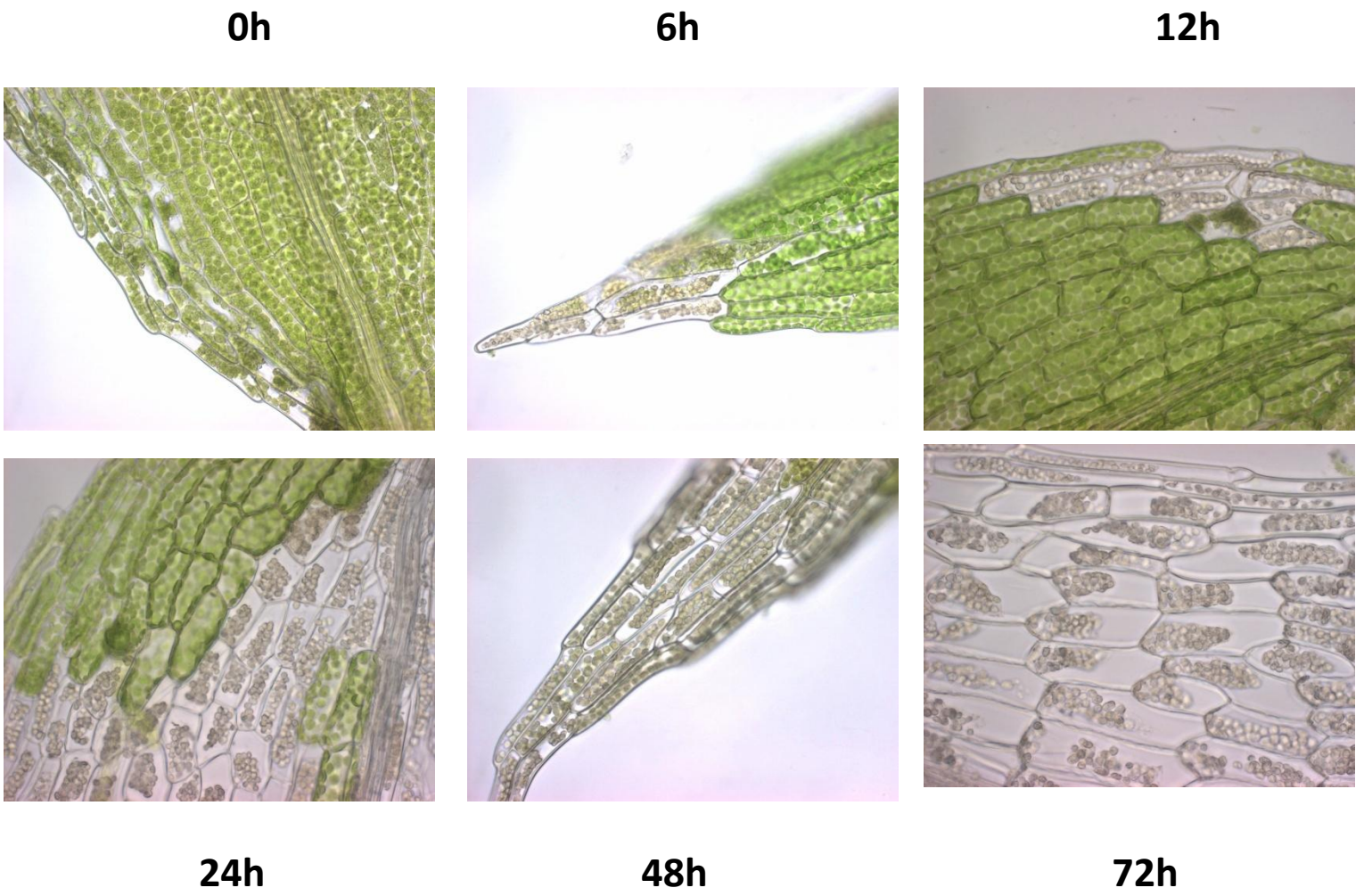


Bit1-KO

KO = Gene Knockout Plant

Yariv reagent

Bit1 Overexpression (OE): Effect on Response to Yariv

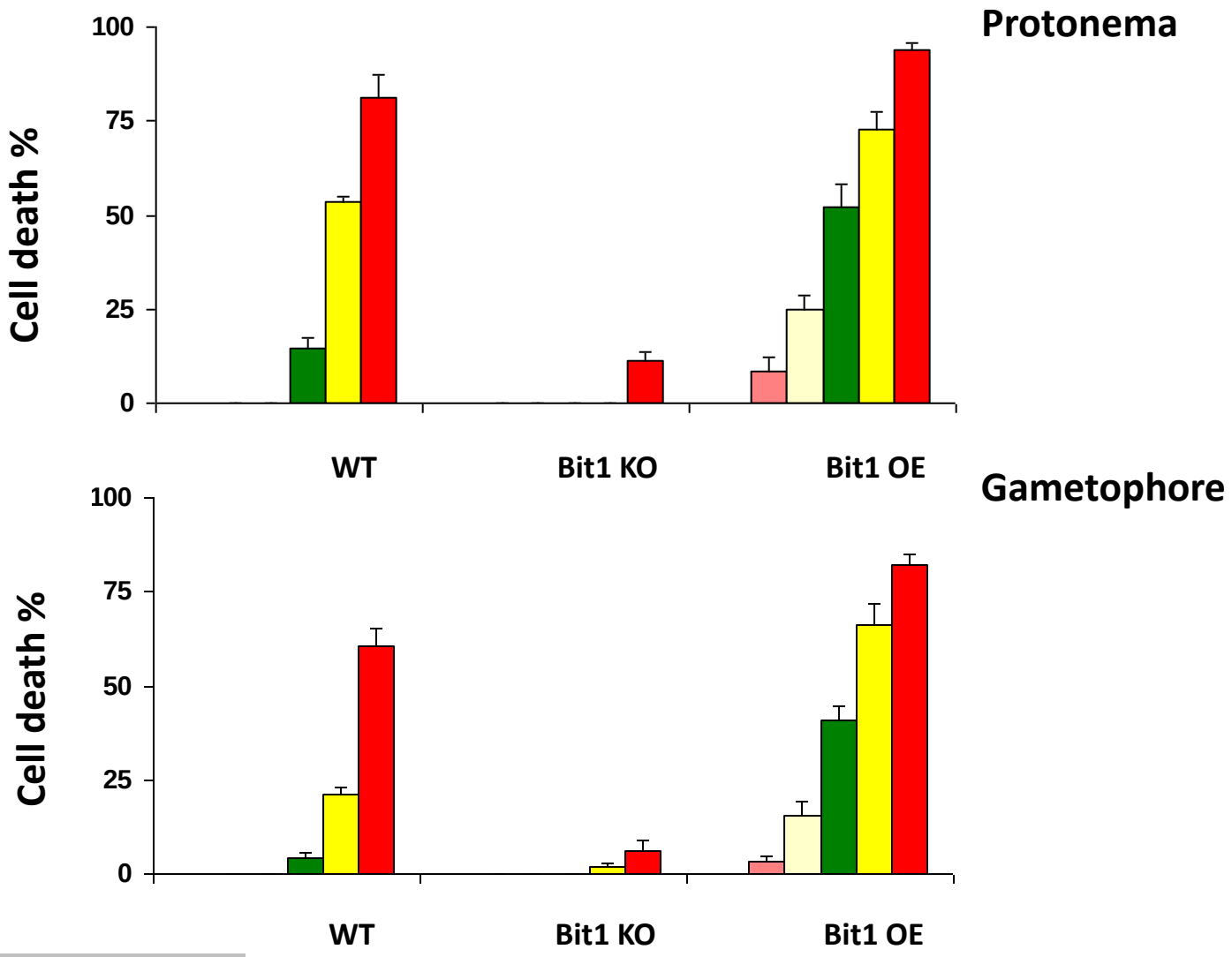


Bit1-OE

OE = Overexpressing Transgenic Plant

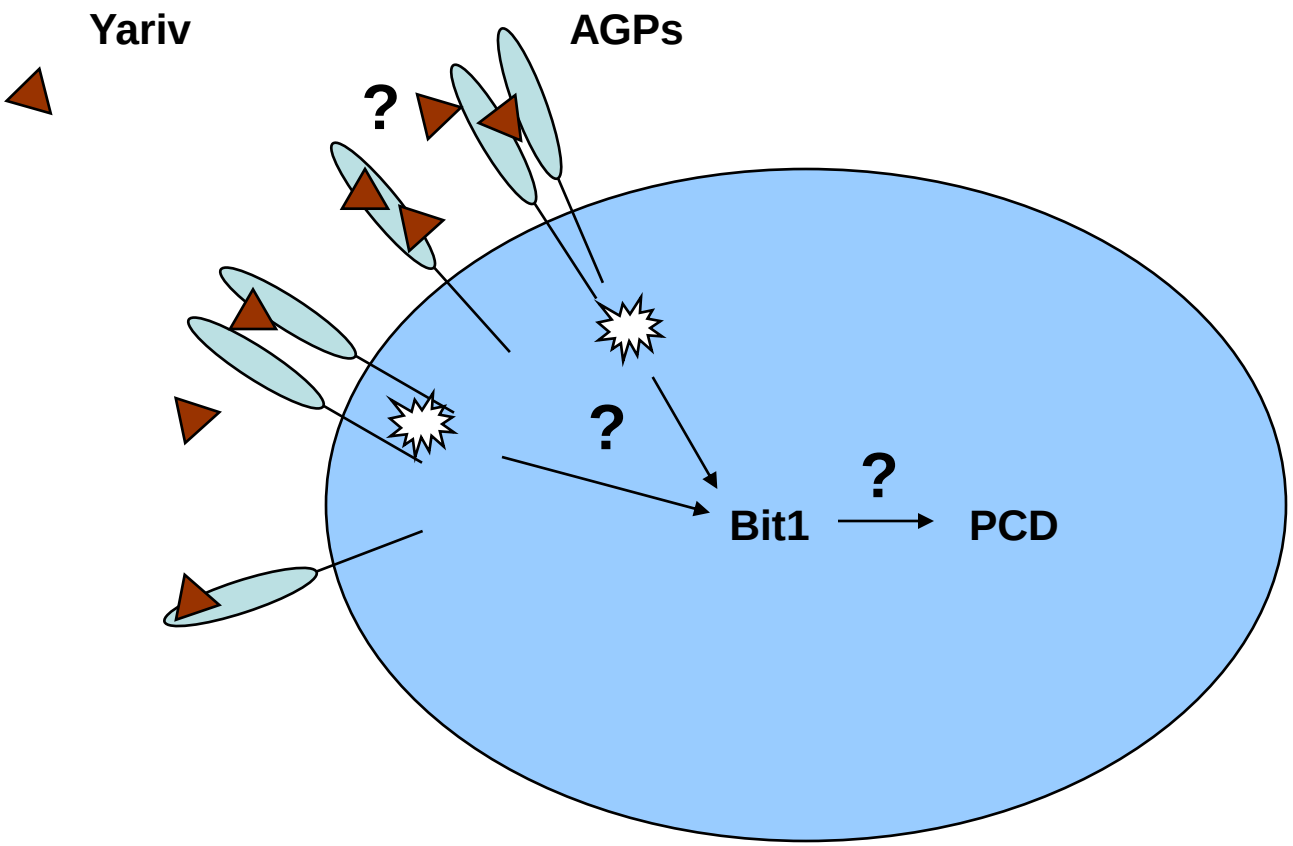
Yariv reagent

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

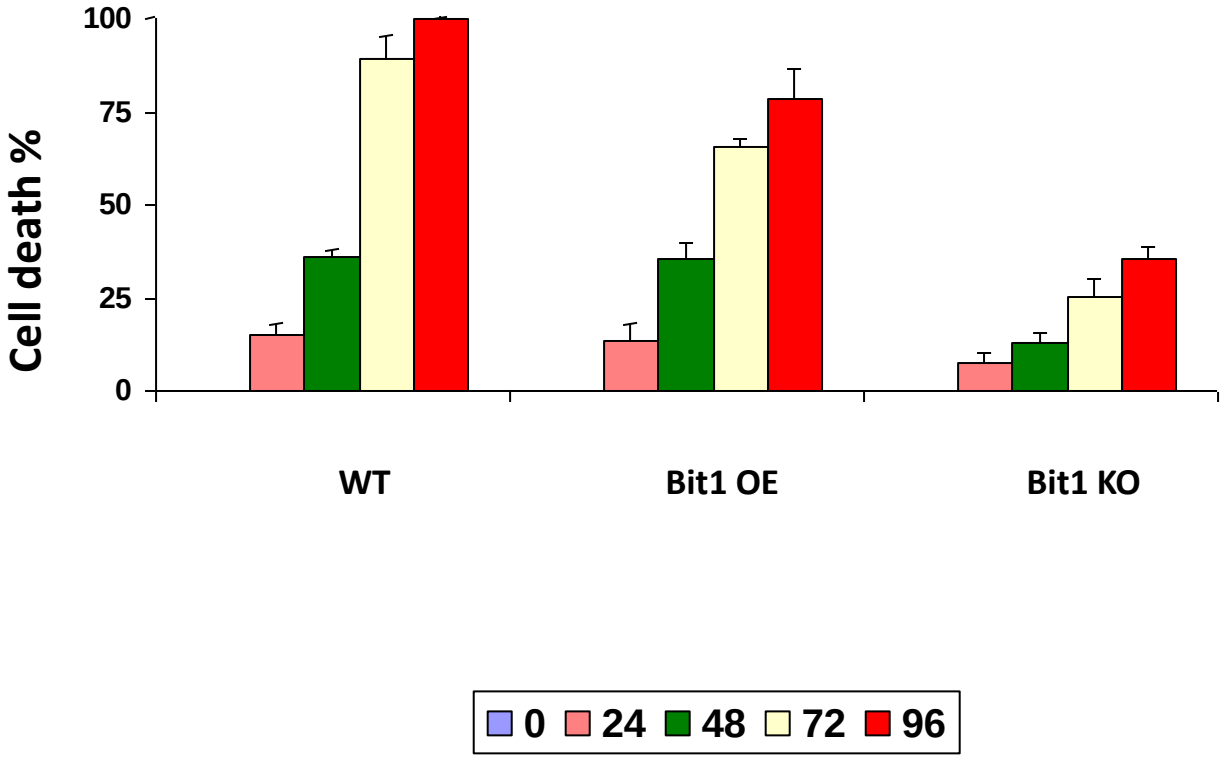


KO = Gene Knockout Plant
OE = Overexpressing Transgenic Plant

Model of Yariv Action



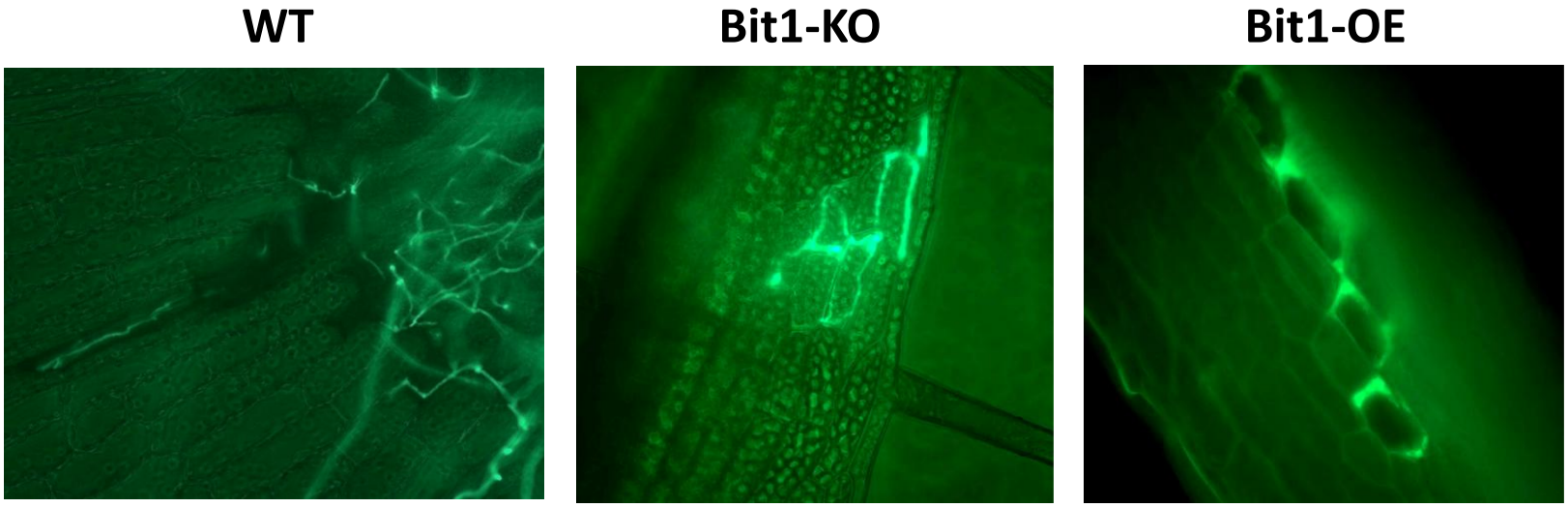
Fusarium infection of WT, Bit1-KO and Bit1-OE plants



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**

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

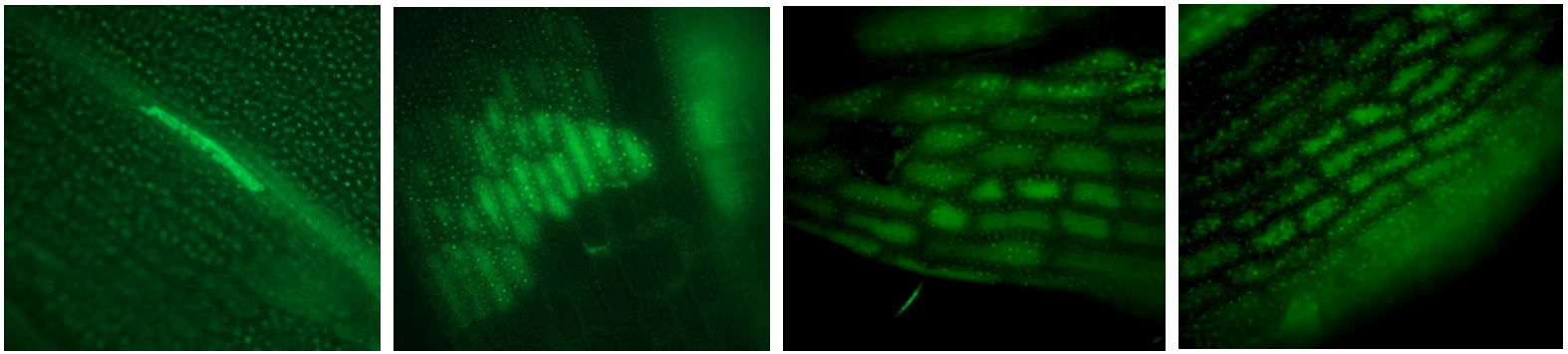
Plant Functional Genomic Targets

Plant genes that affect disease susceptibility:

- Programmed Cell Death (PCD)
- Induced Immunity (antimicrobials)
- Cell Wall
  - Glycosyl Transferases
  - Other Cell Wall Modifiers

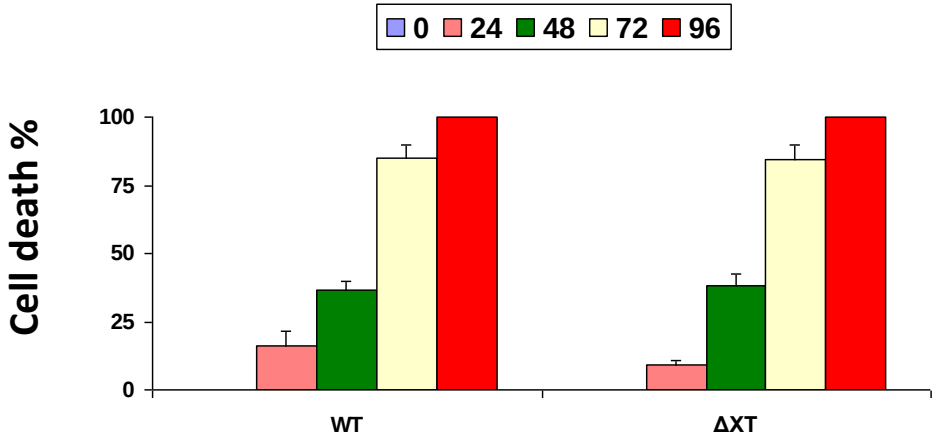
Fusarium graminearum infection in Physcomitrella XT-KO (β 1,2 Xylosyl Transferase) plants

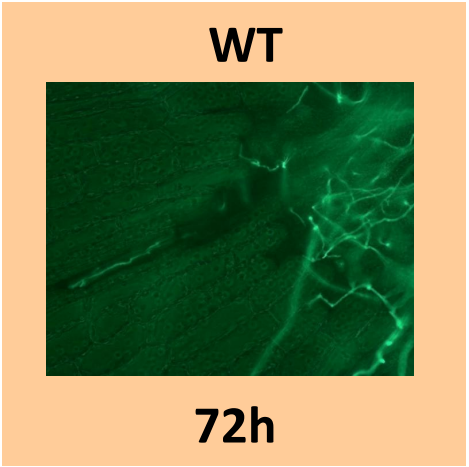
ΔXT



24h 48h 72h 96h

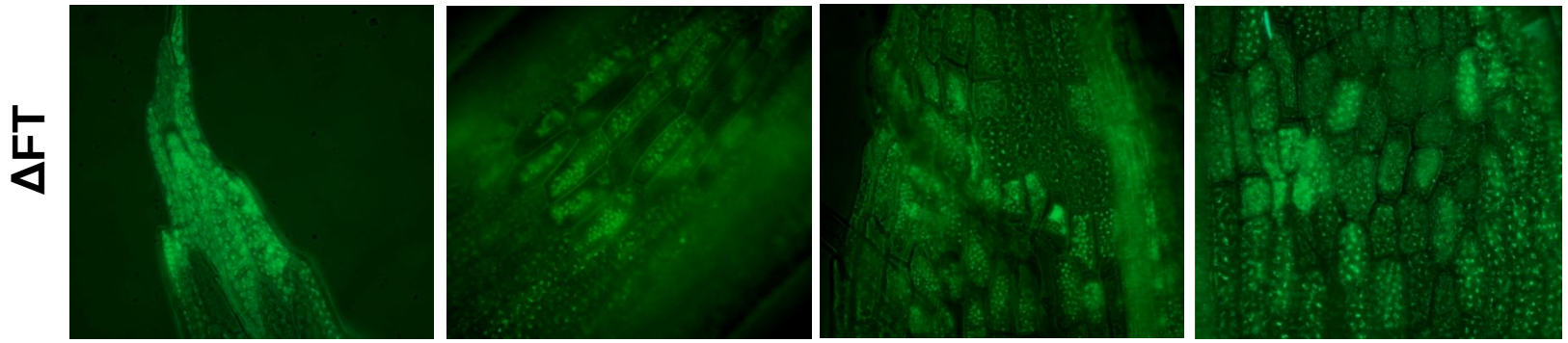
***Fusarium*-GFP**



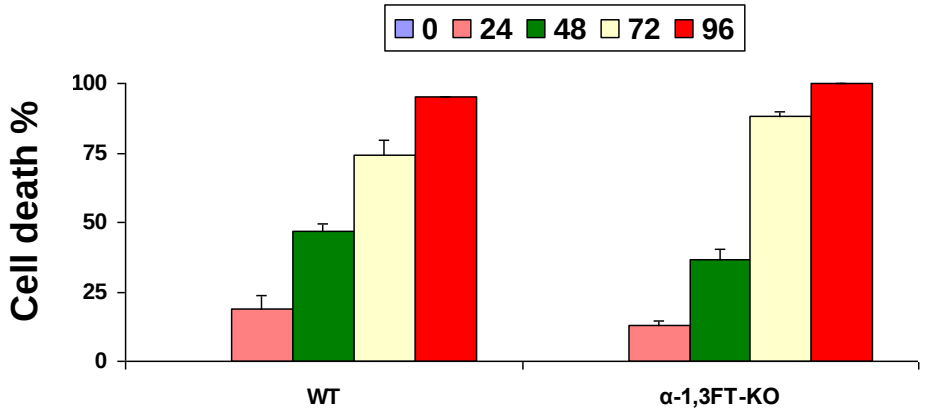


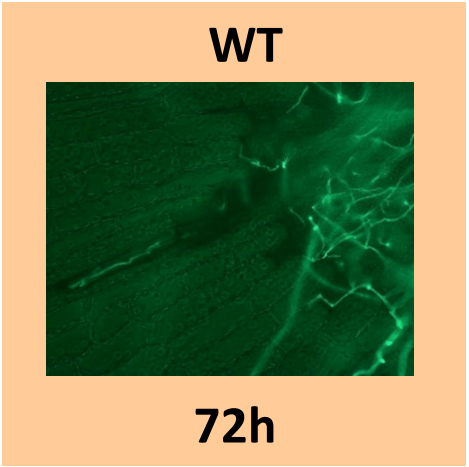
KO = Gene Knockout Plant

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



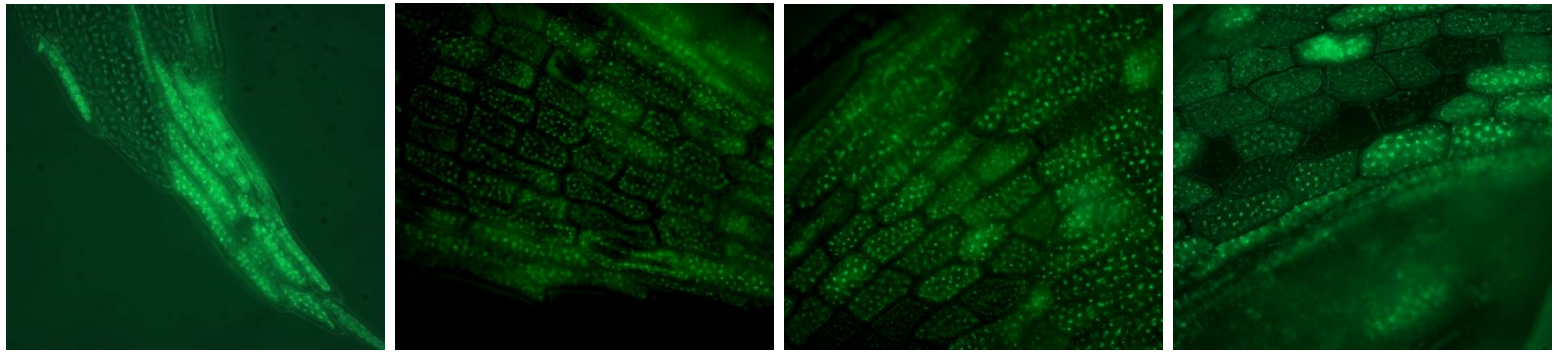
Fusarium-GFP



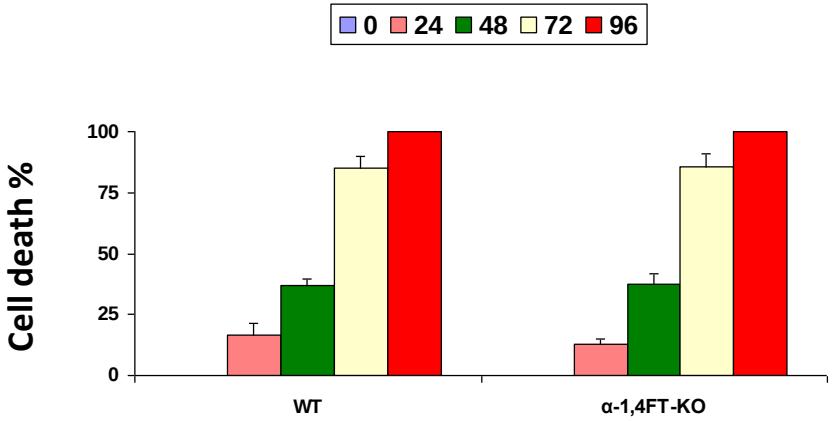


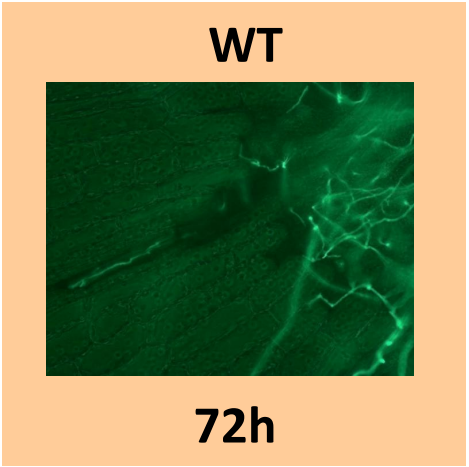
KO = Gene Knockout Plant

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



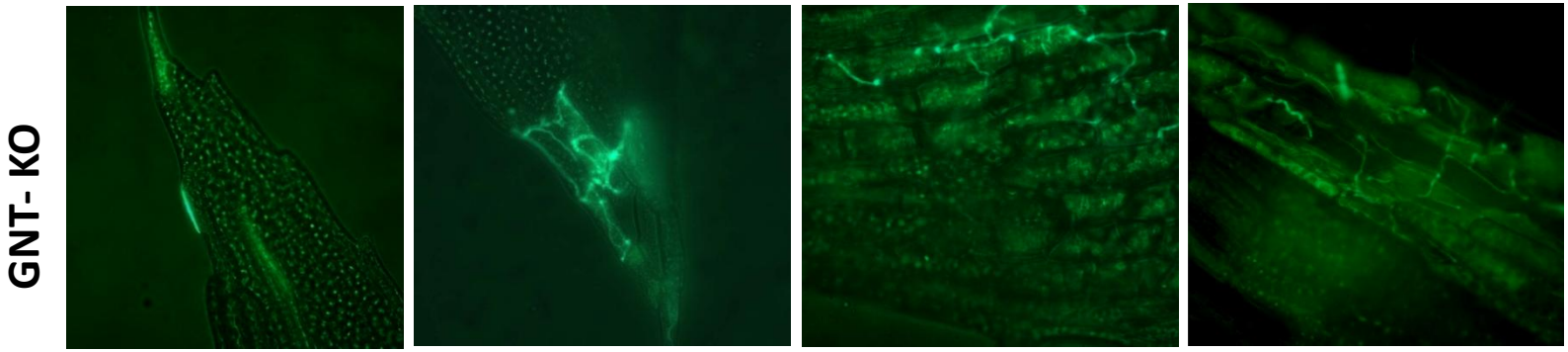
Fusarium-GFP



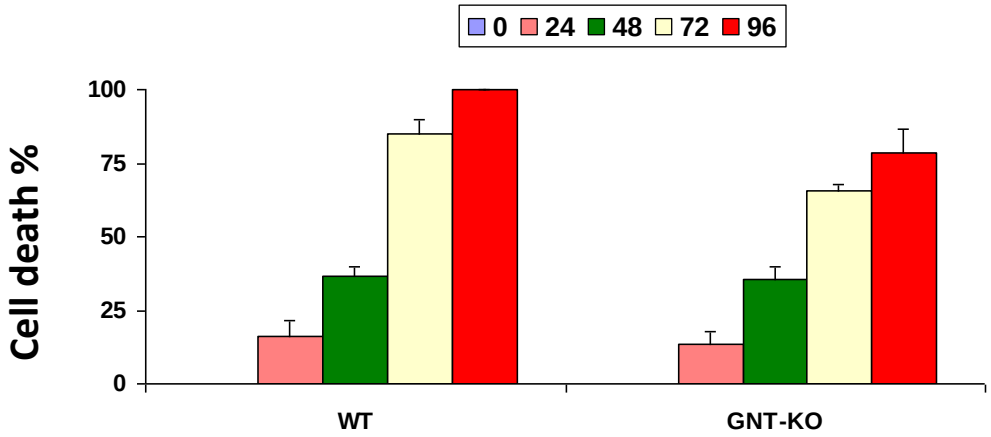


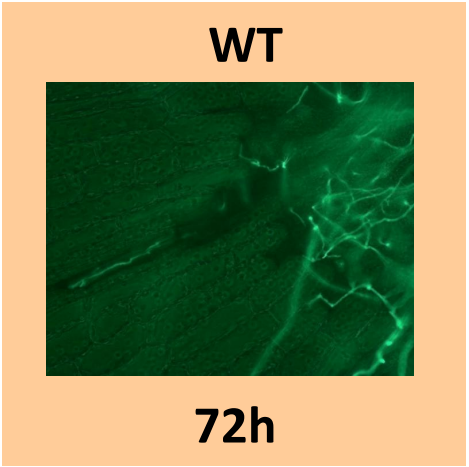
KO = Gene Knockout Plant

Fusarium graminearum infection in GNT-KO (N-acetyl glucosaminyl transferase) plants



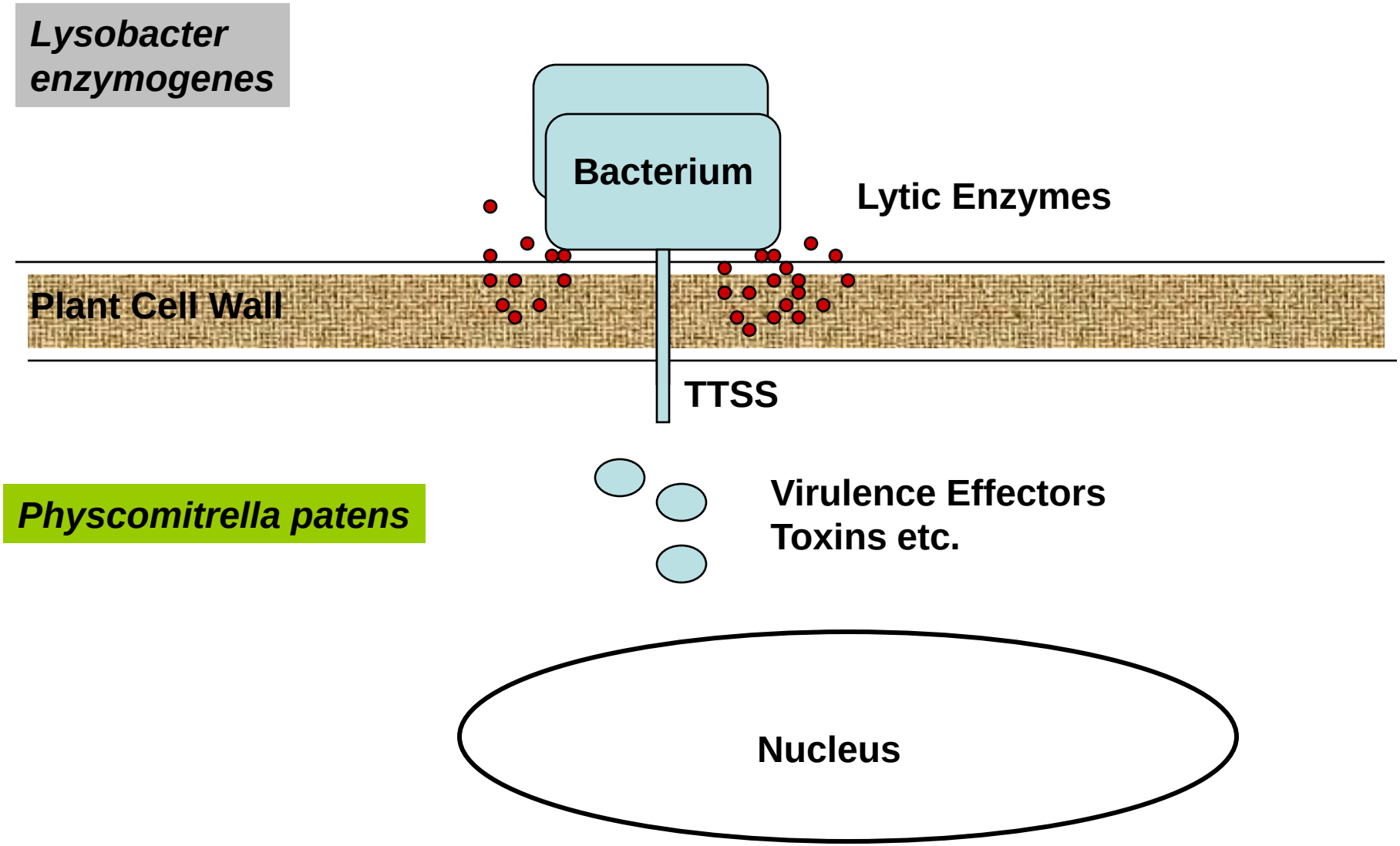
Fusarium-GFP



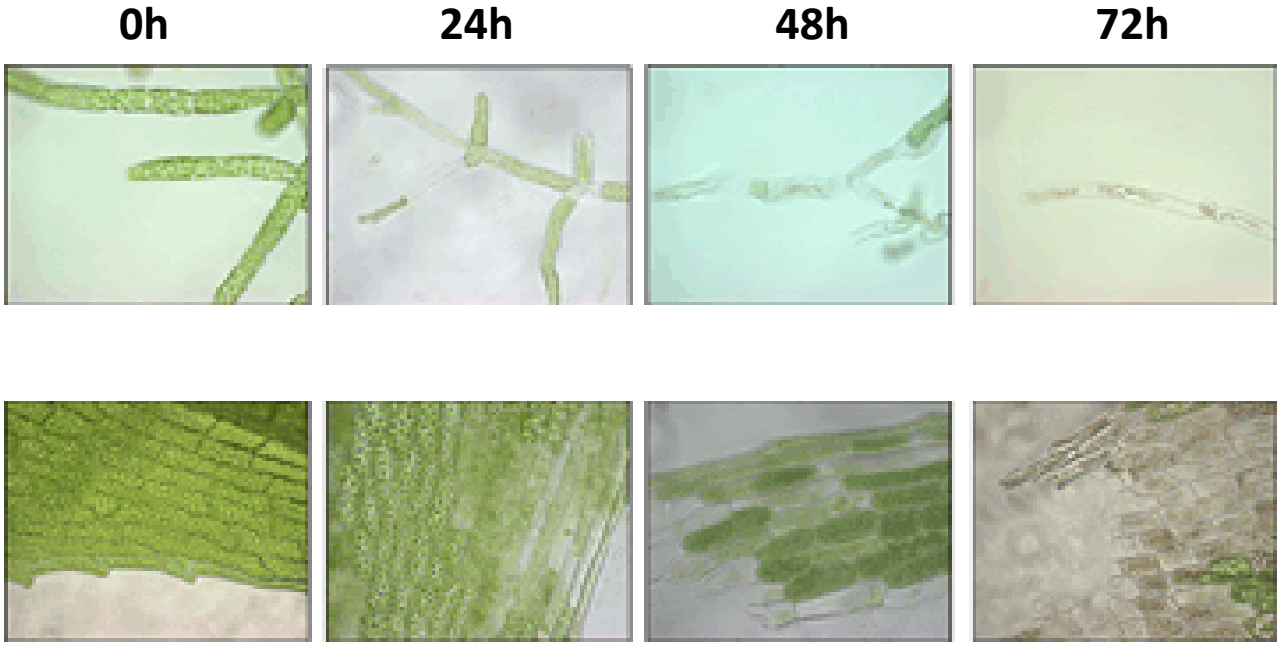


KO = Gene Knockout Plant

The Cell Wall is a Battleground

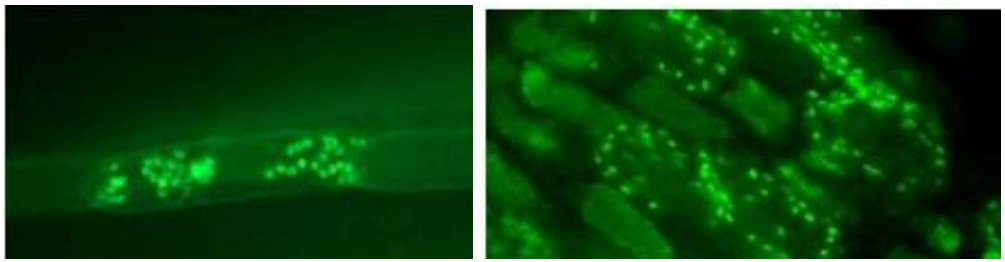


Infection of Physcomitrella with *Lysobacter enzymogenes*



L. enzymogenes **enters <u>living</u> Physcomitrella cells**

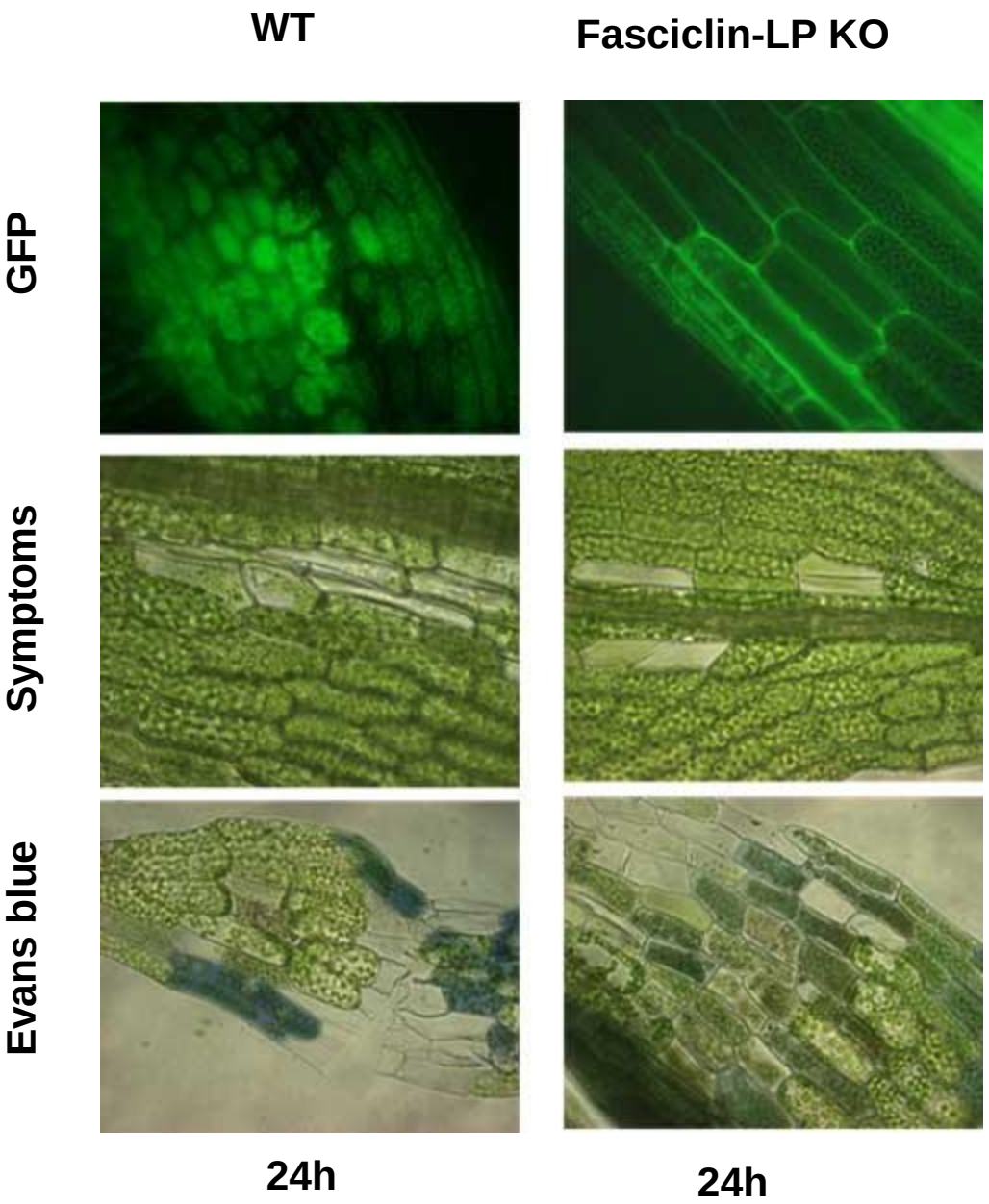
GFP labeled *Lysobacter enzymogenes*

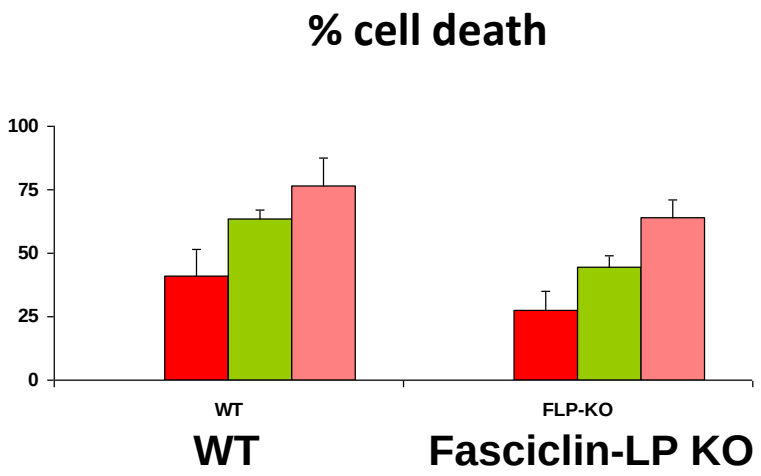


Protonema **Gametophore**

48h

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

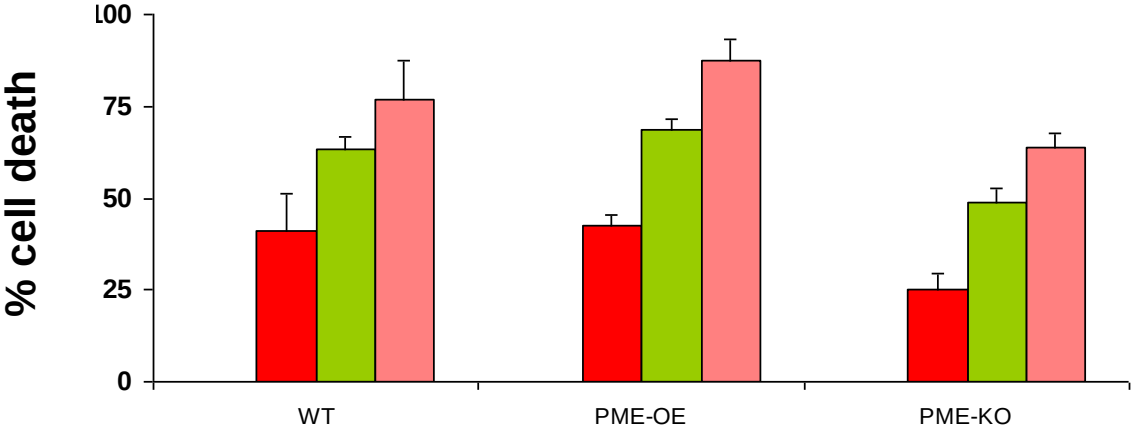




Fasciclins comprise a large family of predicted cell surface proteins in plants

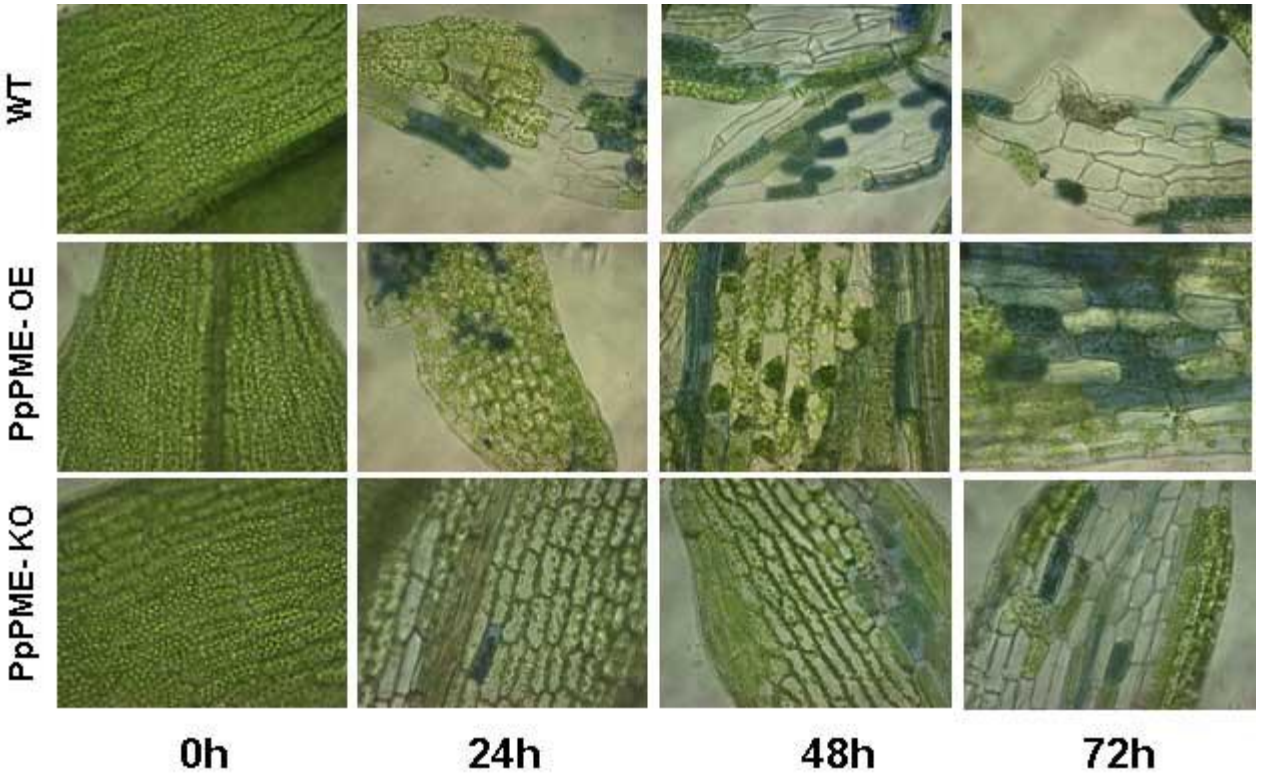
KO = Gene Knockout Plant

L. enzymogenes on Physcomitrella Pectin Methylesterase KO, OE Plants

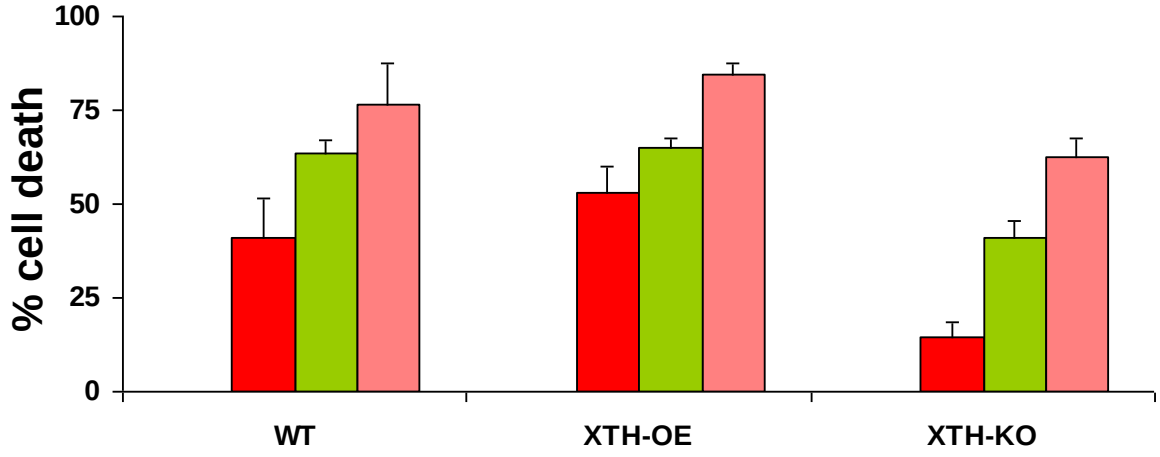


KO = Gene Knockout Plant

OE = Overexpressing Transgenic Plant



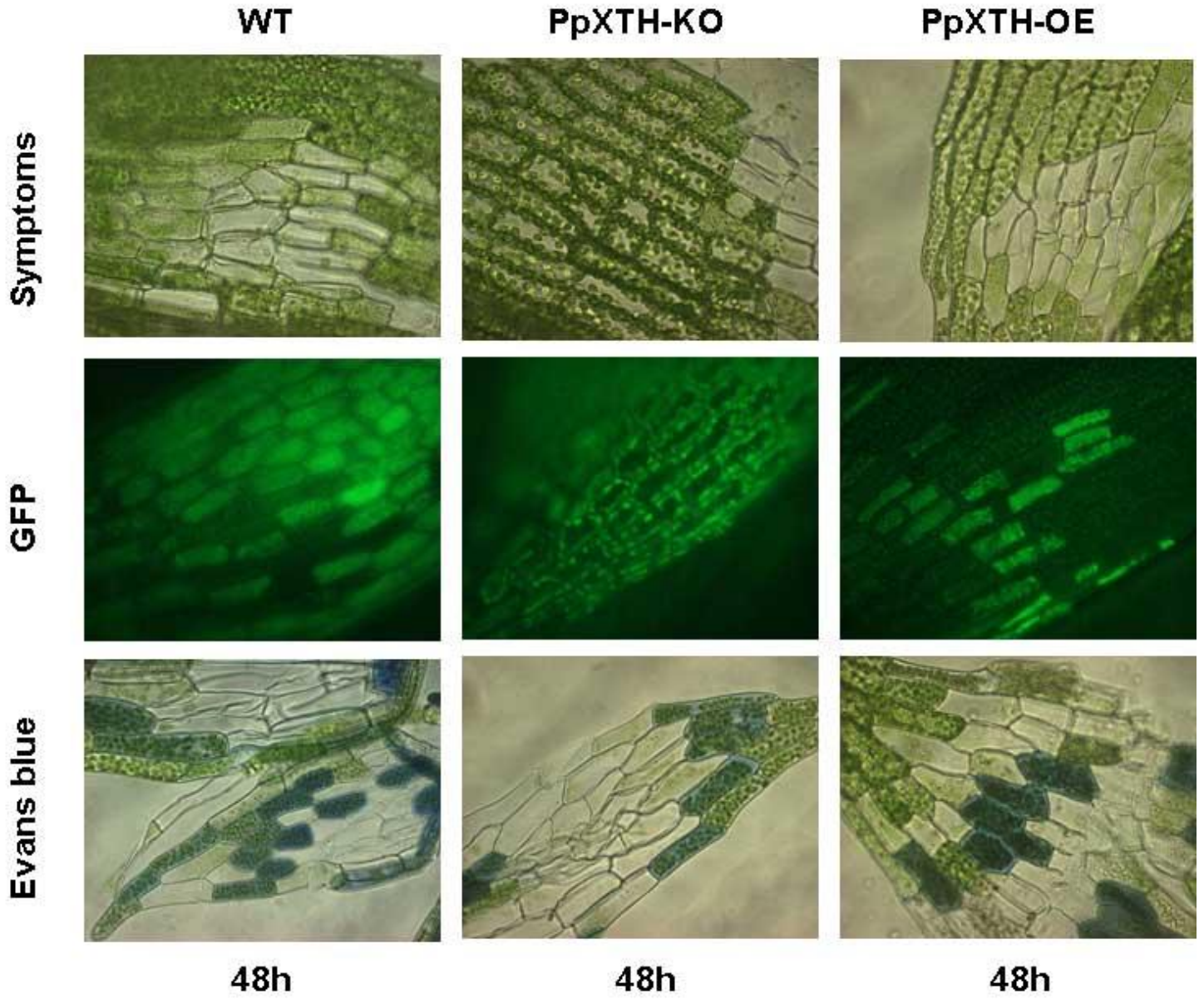
Effect of *L. enzymogenes* on Xyloglucan endotransglycosylase/hydrolase mutants



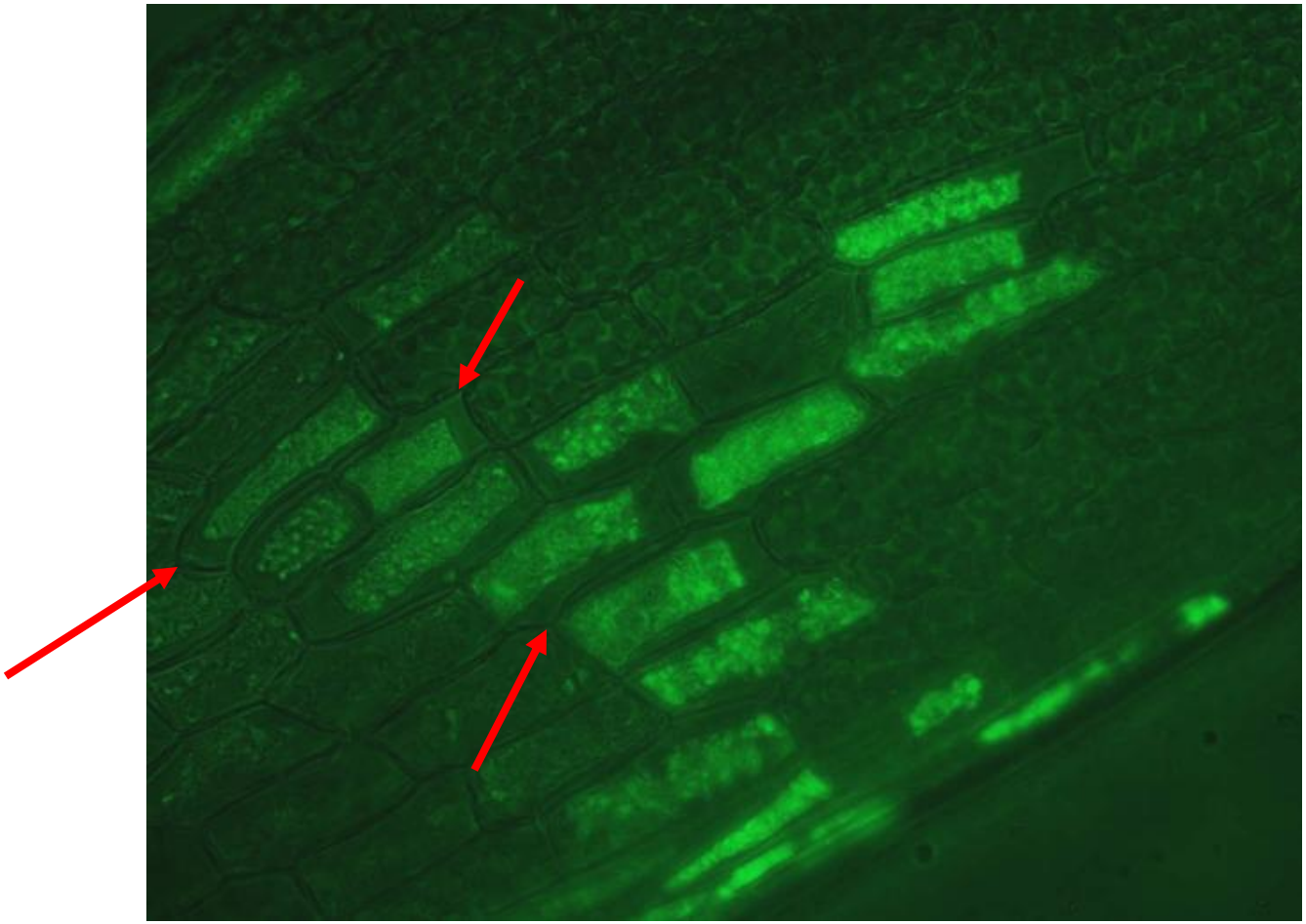
KO = Gene Knockout Plant

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

Plant Mol Biol (2007) 63:207–219
DOI 10.1007/s11103-006-9083-1

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

Alison W. Roberts · John T. Bushoven

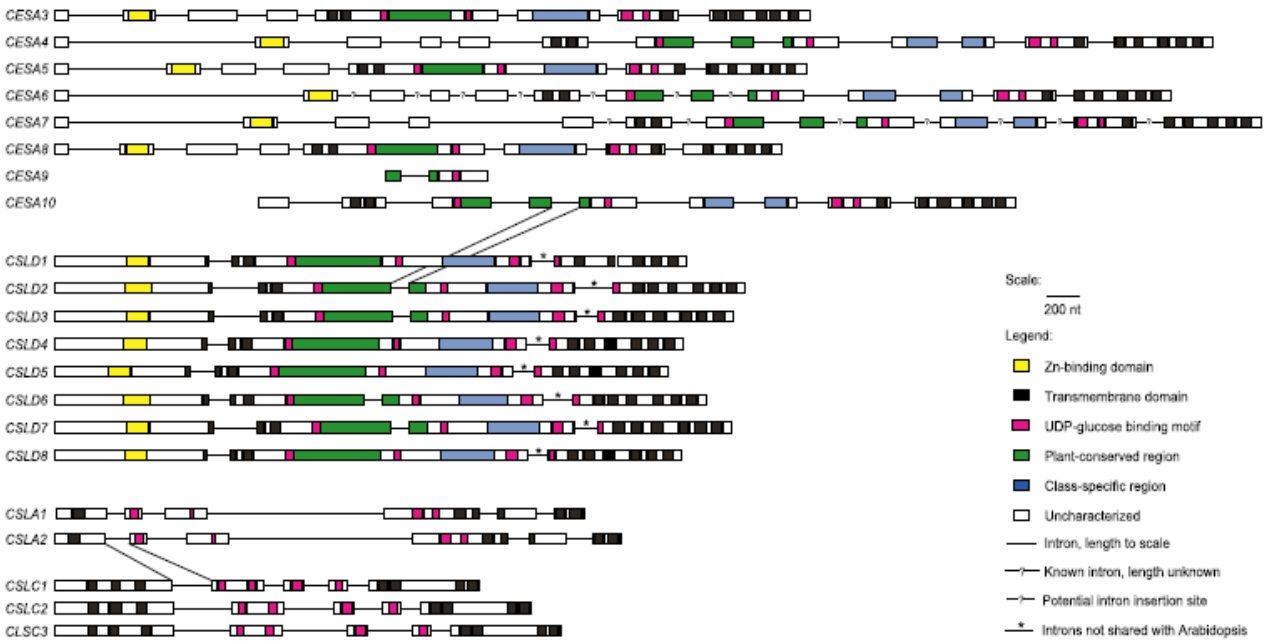
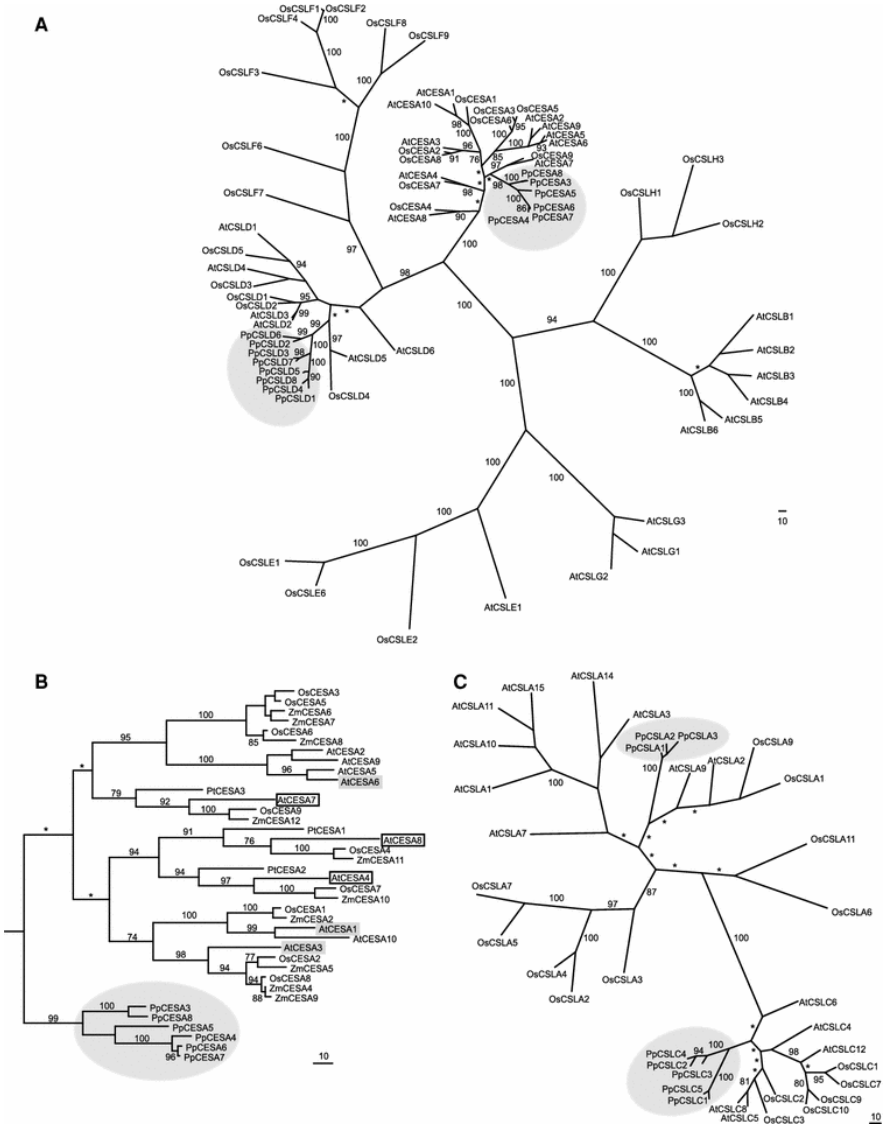


Fig. 1 Gene structure of all *P. patens CESA* and *CSL* genes for which cDNA sequences are available. Introns were identified by comparing cDNA sequences with contigs assembled from genomic shotgun sequences. Three genomic sequences were incomplete, resulting in uncertainty of intron or position. Introns shared by members of different families are indicated

Roberts, 2007 Plant Mol Biol 63:207

Physcomitrella Cellulose Synthase Genes



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

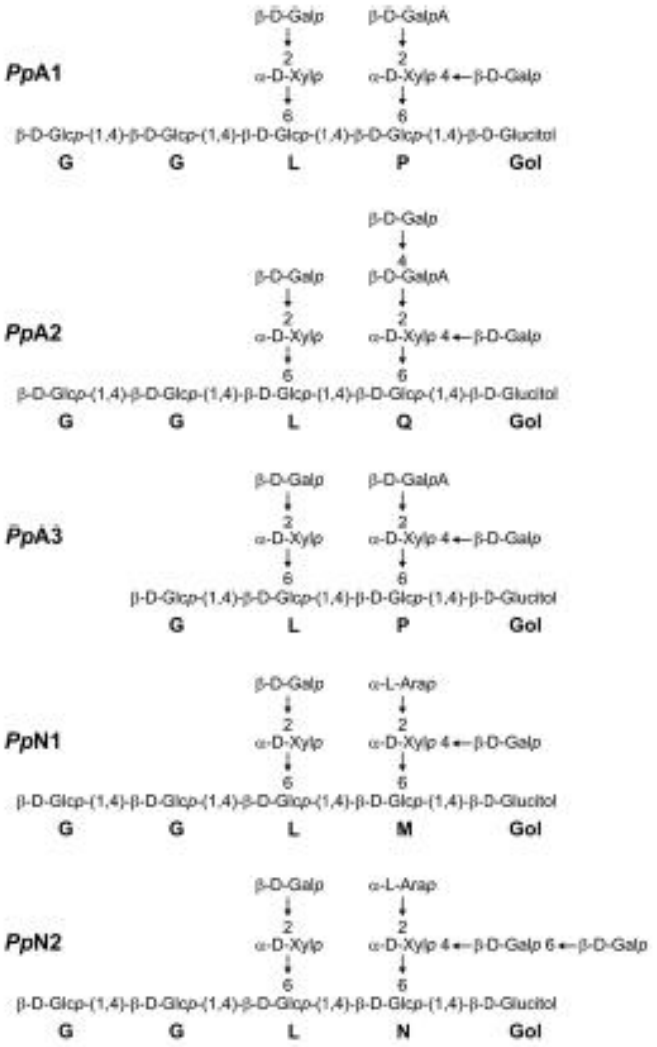
Roberts, 2007 Plant Mol Biol 63:207

Physcomitrella Cell Wall Carbohydrate Linkage Analysis

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

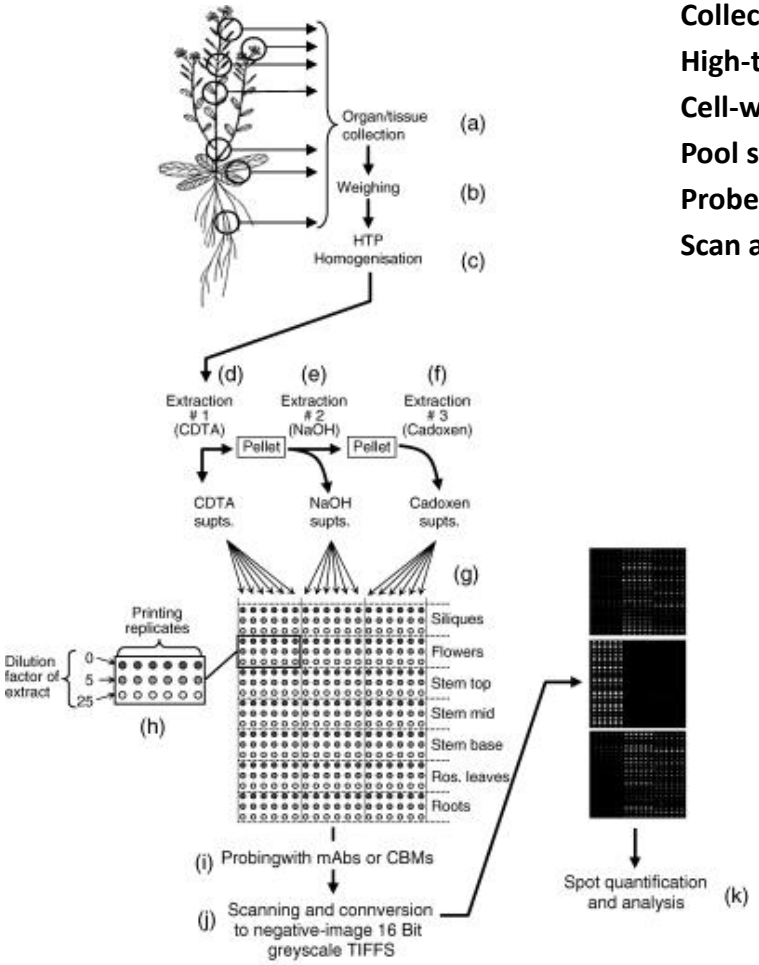
Monosaccharide	Deduced linkage	*Mol* %
Gal*p*	Terminal	7.3
	2-	0.8
	3-	2.0
	4-	0.1
	6-	0.6
	3,6-	0.1
	4,6-	0.1
	Galactitol	0.2
Fuc*p*	Terminal	0.1
	4-	0.5
GalA*p*	Terminal	0.8
	4-	13
Rha*p*	Terminal	0.2
	2-	1.3
	2,4-	2.2
Ara*p*	Terminal	0.8
Ara*f*	Terminal	1.1
	3-	0.4
	5-	0.2
Xyl*p*	Terminal	0.5
	2-	1.2
	4-	2.4
	2,4-	1.0
Man*p*	Terminal	0.3
	4-	6.2
	4,6	0.7
Glc*p*	Terminal	1.7
	3-	0.3
	4-	48.6
	2,4-	0.3
	3,4	0.2
	4,6-	4.5
	Glucitol	0.3

Isabel Moller, et al. (2007) The Plant Journal, Volume 50, Issue 6, Pages 1118-1128



Peña et al, 2008 Glycobiology, in press

Comprehensive Microarray Polymer Profiling (CoMPP)



Collect Samples (50 mg fresh weight) from each tissue/organ

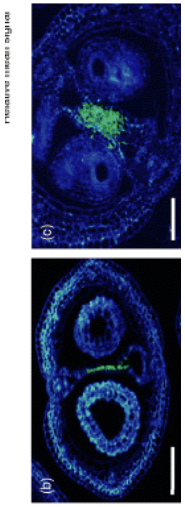
High-throughput (HTP) homogenization

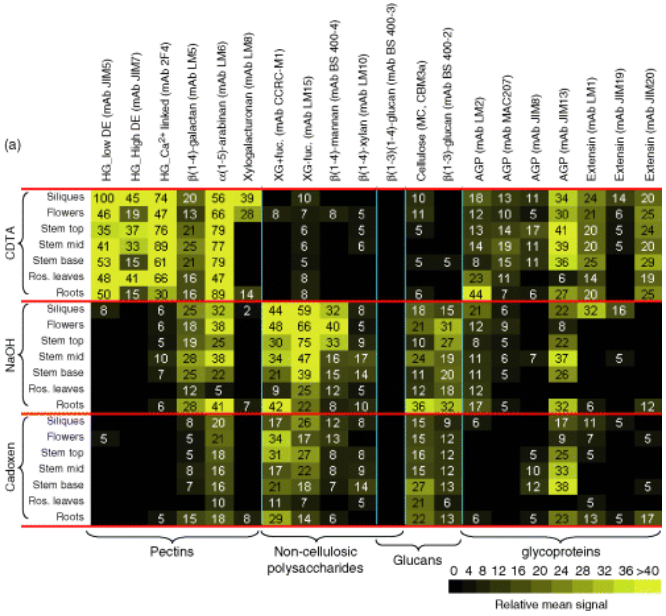
Cell-wall polymers are sequentially extracted using CDTA, NaOH and cadoxen

Pool supernatants and print dilutions printed in sextuplet onto nitrocellulose.

Probe arrays are probing with mAbs or CBMs

Scan and quantifyusing ImaGene 6.0 microarray analysis software.

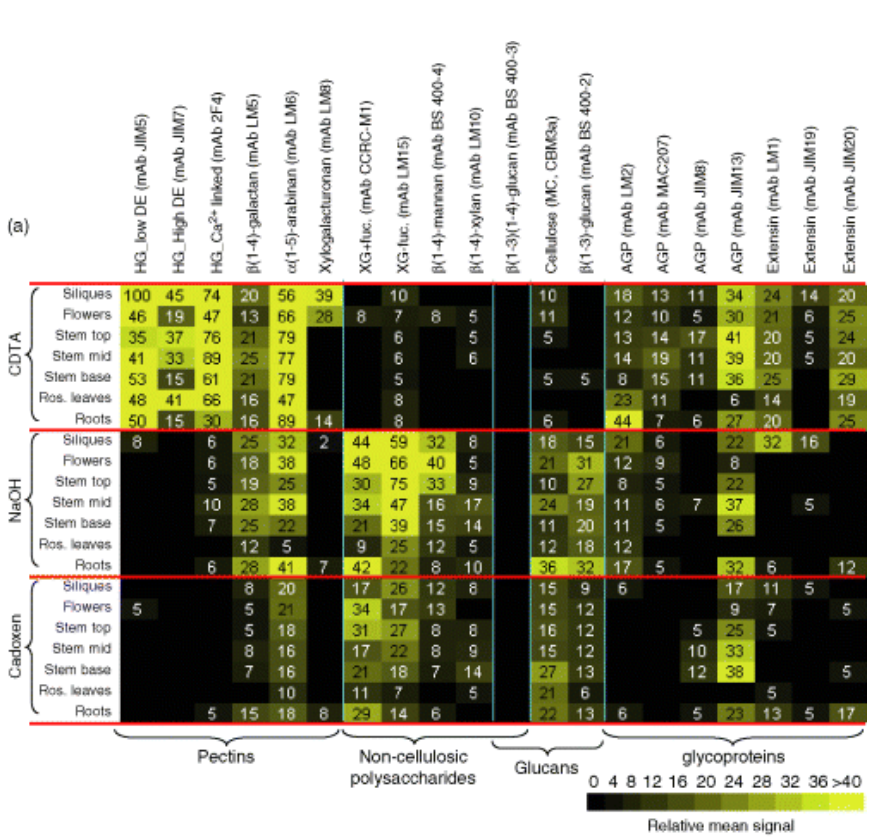




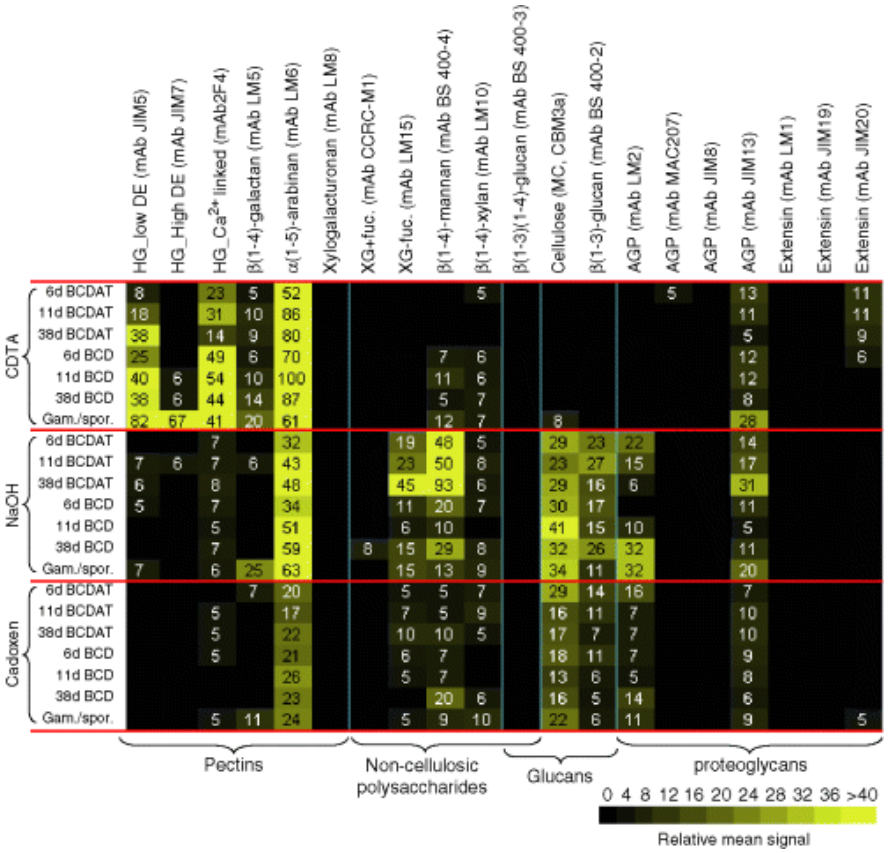
Isabel Moller, Iben Sørensen, Adriana J. Bernal, Claudia Blaukopf, Kieran Lee, Jens Øbro, Filomena Pettolino, Alison Roberts, Jørn Dalgaard Mikkelsen, J. Paul Knox, Antony Bacic and William G.T. Willats. (2007) High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. The Plant Journal, Volume 50, Issue 6, Pages 1118-1128

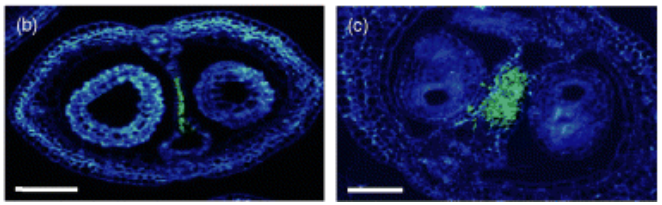
Comprehensive Microarray Polymer Profiling (CoMPP)

Arabidopsis



Physcomitrella





Helps define limitations & utility of model system

Isabel Moller, Iben Sørensen, Adriana J. Bernal, Claudia Blaukopf, Kieran Lee, Jens Øbro, Filomena Pettolino, Alison Roberts, Jørn Dalgaard Mikkelsen, J. Paul Knox, Antony Bacic and William G.T. Willats. (2007) High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. The Plant Journal, Volume 50, Issue 6, Pages 1118-1128

Conclusions

- 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

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!

Acknowledgments

Lawton Lab
Hemalatha Saidasan
Mark Diamond
Chi-Tai Tang (U Texas S.Western)
Luciana Ambrozevecius (ESALQ)
Carmen Collazo
Devino Rajah
Bozehna Lisko,
Jared Cohen

Pathology and PCD
Don Kobayashi, Rutgers
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

Physcology
Didier Schaefer, INRA
Andrija Finka, UNIL
Jean-Pierre Zrÿd, UNIL
Younousse Saidi, UNIL

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

Arabinogalactan Proteins in Physcomitrella

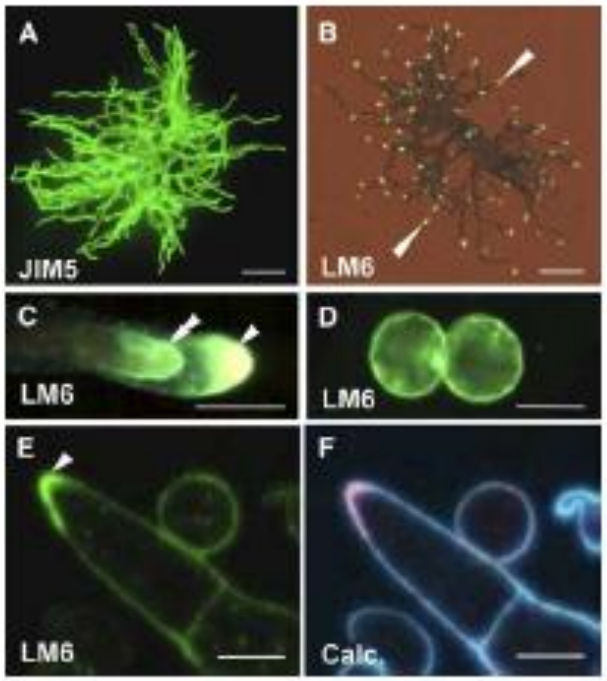


Figure 5. The Distribution of Pectic Polysaccharides and AGPs in *P. patens*.

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

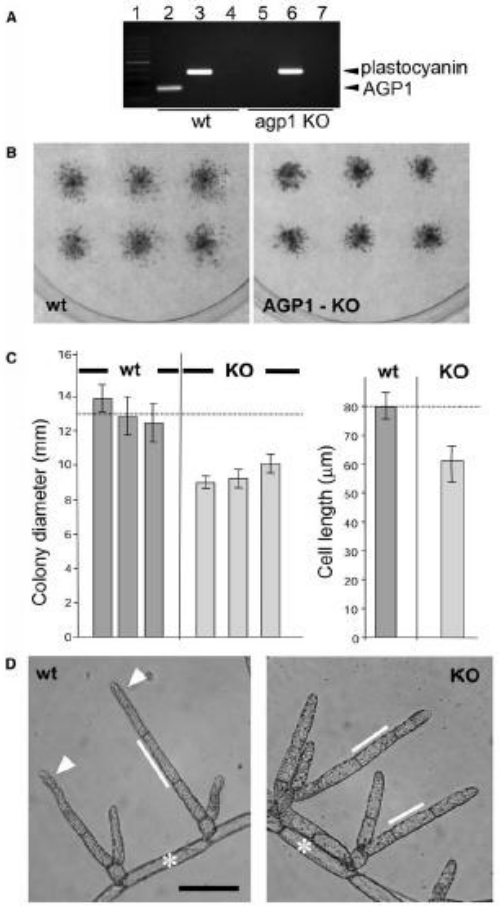


Figure 9. Expression of *AGP1* and Phenotype of the Wild Type and a Transgenic KO Line of *P. patens*.

Effect of gene knockout of AGP1: Retarded cell extension growth

Lee et al, **Arabinogalactan Proteins Are Required for Apical Cell Extension in the Moss *Physcomitrella patens*** Plant Cell 2005 17: 3051-3065.