Looking for LOV: Location of LOV1 function in *Nicotiana benthamiana* cells

By:
Patrick Rutledge[1]
Dr. Jennifer Lorang[2,3], Dr. Marc Curtis[2,3], Dr. Thomas Wolpert[2,3]
BioResource Research[1], Botany and Plant Pathology[2], Center for Genome Research and Biocomputing[3],
Oregon State University, Corvallis, Oregon 97331

Introduction

- Plants able to recognize and confer resistance to pathogens in two ways

  1) Recognition of pathogen associated molecular patterns (PAMPs)

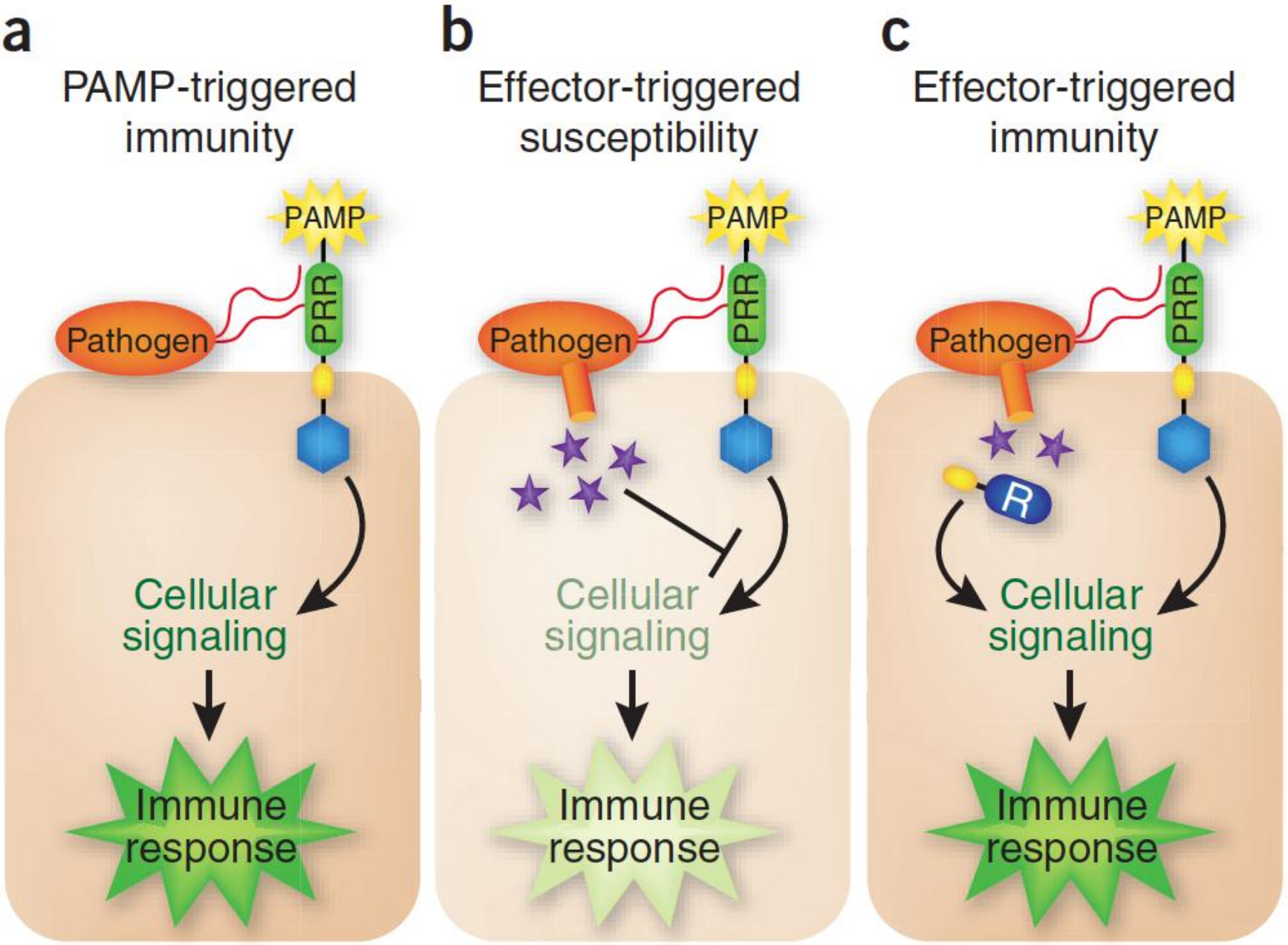
- Successful pathogens must suppress immunity in host
  - Inhibit pathways or molecules involved in defense response by using effectors

- 2) Recognition of effectors by resistance proteins

- Plants have evolved to produce resistance proteins that “guard” molecules targeted by pathogen effectors

Virulence Effectors

- Secreted by both biotrophic and necrotrophic pathogens
  - **Biotrophs**- Can only live and replicate on living hosts
  - **Necrotrophs**- Kills living cells of host and feeds on dead tissue
- Virulence effector, **victorin**, secreted by *Cochliobolus victoriae*

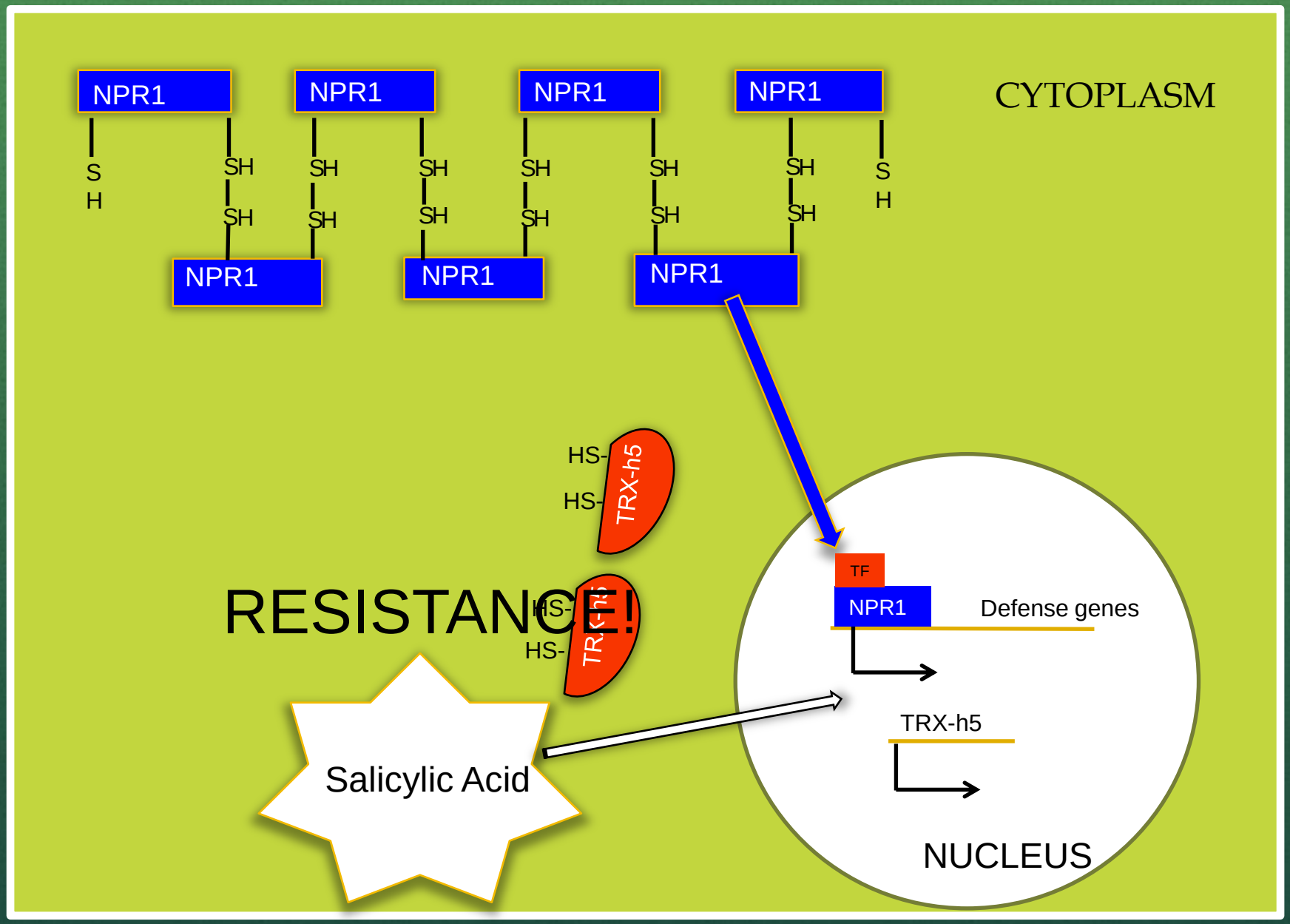


Pieterse et al 2009

- Isolated **LOV1**
  - **L**ocus **O**rchestrating **V**ictorin Effects 1
  - Encodes for resistance (R) protein that “guards” thioredoxin 5 (TRX-h5)

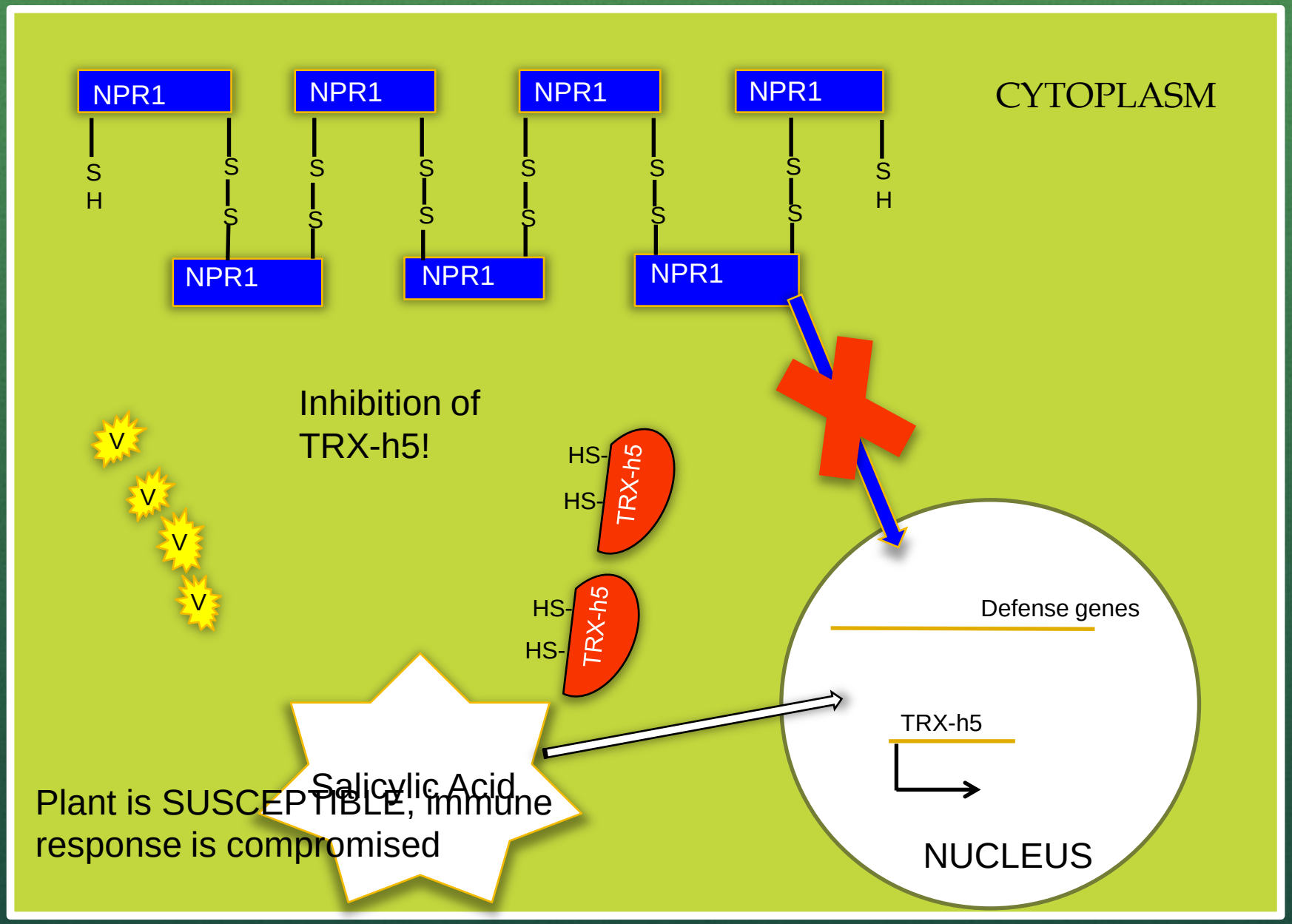
Arabidopsis thaliana

Leaf Cell



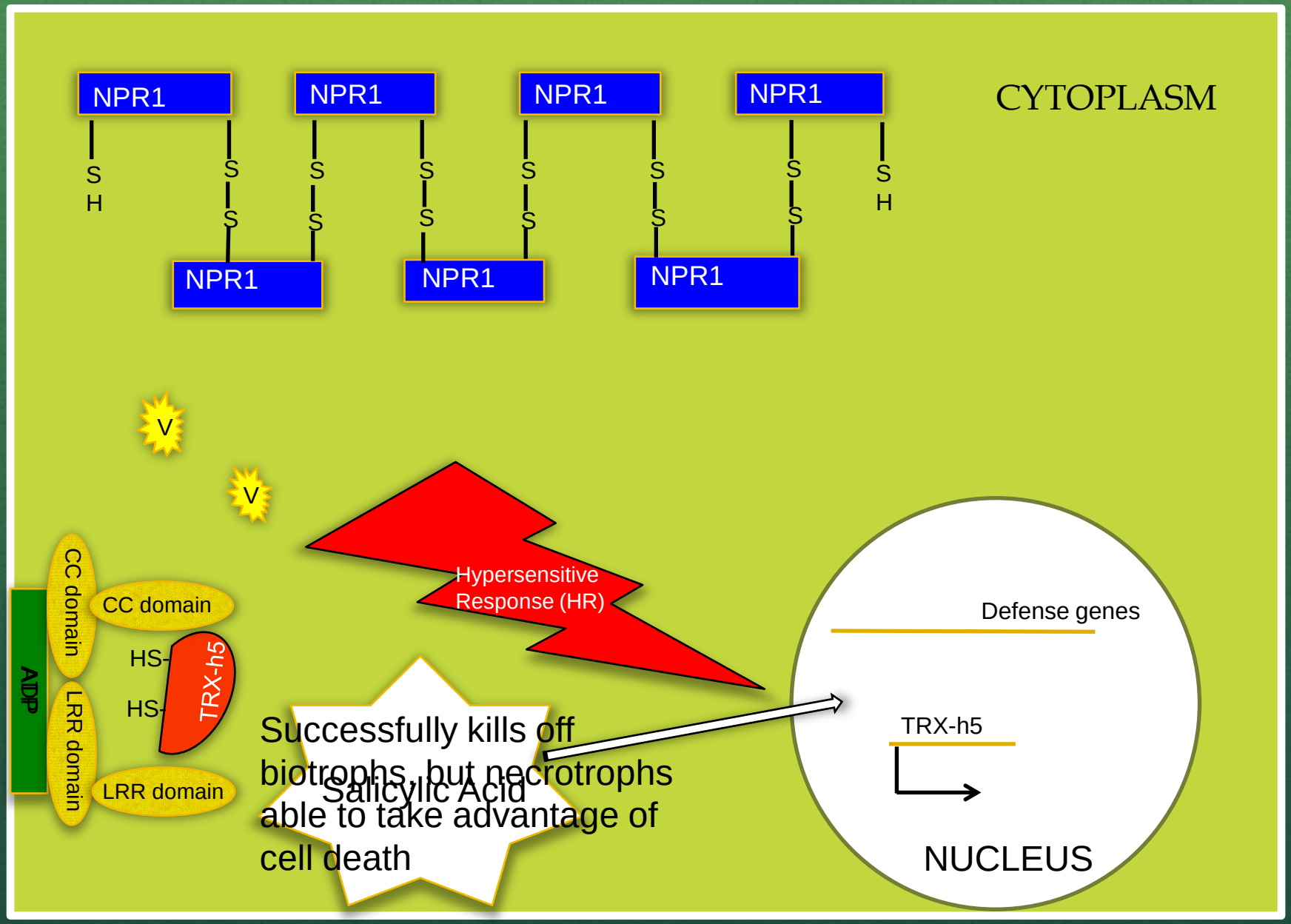
Design credit: Thomas Wolpert

Leaf Cell + Victorin



Design credit: Thomas Wolpert

Leaf Cell + LOV1 + Victorin

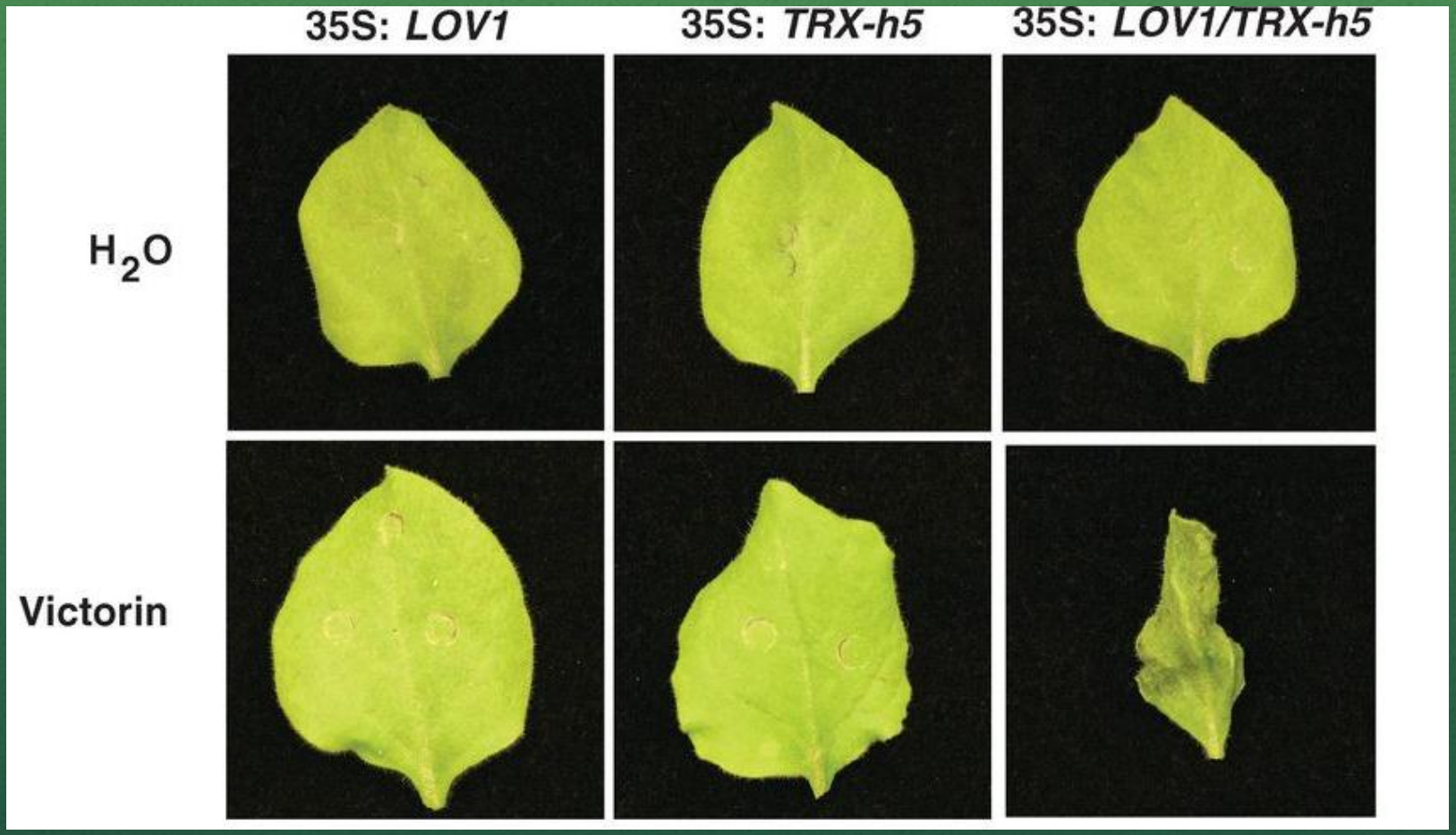


Design credit: Thomas Wolpert

Nicotiana benthamiana

- Close relative to tobacco
  - Not for smoking!
- Model organism used to study plan
- Able to transiently express proteins
  - Very susceptible to *Agrobacterium*
  - Allows for study of protein localizati
  - Study of plant immunity and defens

Nicotiana benthamiana leaves



Phenotypic cell death response exhibited only when LOV1 + TRX-h5 expressed together

Research Objectives

Overall Objective: Understanding where LOV1 protein exerts its effects to regulate cell death

- R proteins present in all plants, and even humans!
- Some R proteins translocate to the nucleus, study attempts to show LOV1 can function solely in the plasma membrane.

Specific Objectives:

1) Quantifying a specific victorin concentration to run effective time courses of cell death

2) Clarifying where LOV1 protein is activated in *N. benthamiana* cells

3) Understanding where LOV1 localizes and functions after activation of cell death

Methods

- Various genetic tags engineered onto LOV1
  - Nuclear Export Signal (NES) and mutant NES (nes)
  - CBL & mCBL
- Electrolyte leakage assays (ELAs) of tobacco using various concentrations of victorin solution
- Direct phenotype response
- Fluorescent microscopy on light and confocal microscopes

Genetic Constructs of LOV1

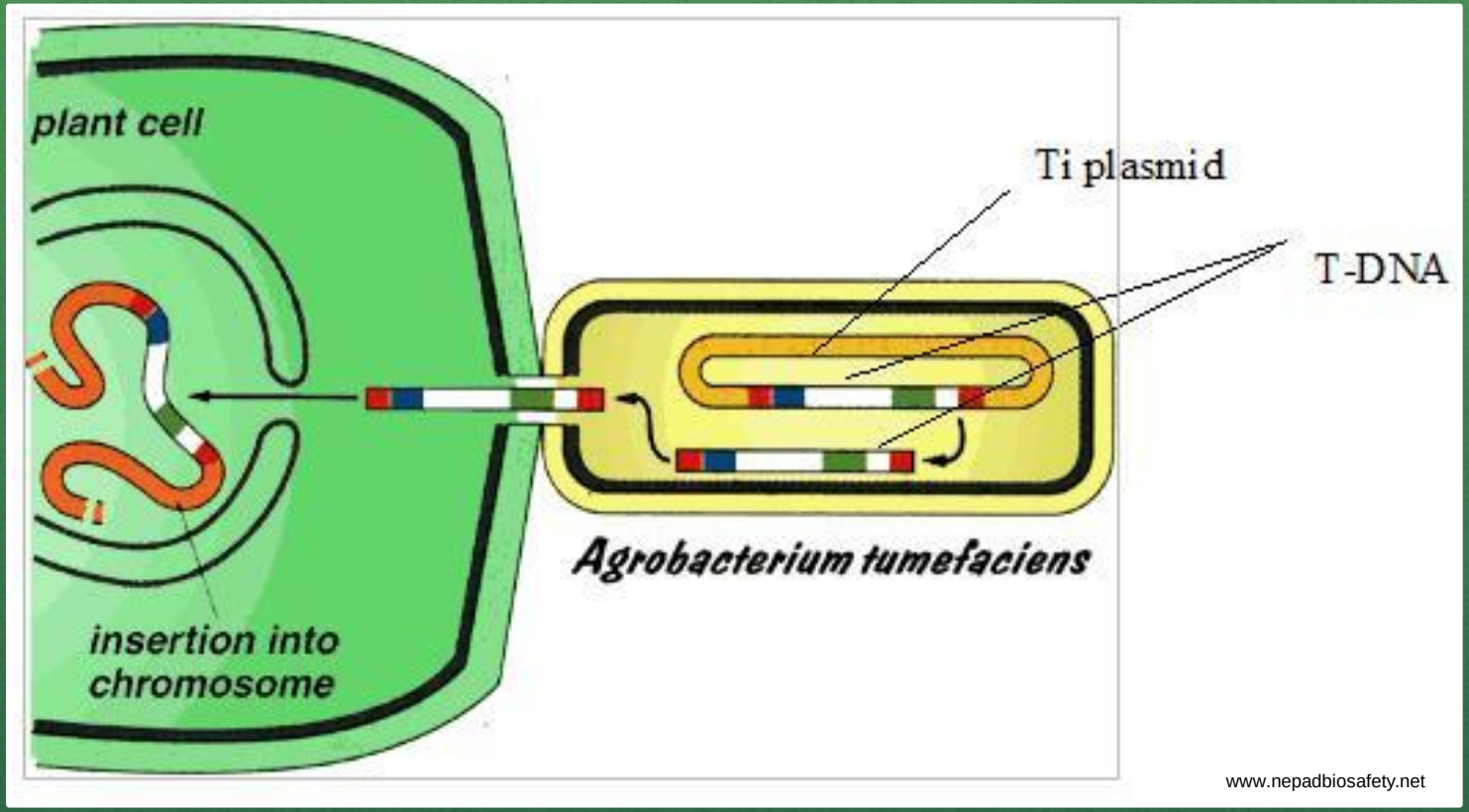
- NES & nes tags fused to C-terminal
  - NES-LOV1 prevents localization to nucleus
  - nes-LOV1 as control
- CBL & mCBL tags fused to N-terminal of LOV1
  - CBL-LOV1 irreversibly tethers LOV1 to plasma membrane
  - mCBL-LOV1 as control

NES 5'-atggacgagctgtacaagaacgagcttgctcttaagttggctggacttgatattaacaag-3'

nes 5'-atggacgagctgtacaagaacgagcttgctcttaa**ggca**gctgga**gca**gatgctaacaag-3’

CBL 5’-atgggctgcttccactcaaaggcagcaaaagaattt-3’

mCBL 5’-atg**gcc**agcttccactcaaaggcagcaaaagaattt-3’



www.nepadbiosafety.net

Mechanism of *A. tumefaciens* infection

Nature's "genetic engineer"

www.lifesciencesfoundation.org

microbewiki.kenyon.edu

Tumors produced by *A. tumefaciens* infection

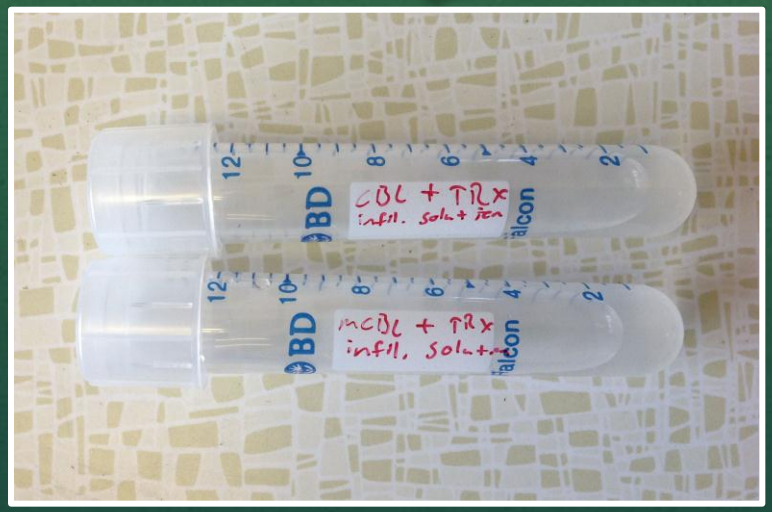
Genetic variations
of LOV1

+

TRX-h5

Incubated 3 days
(30 °C)

Grown overnight



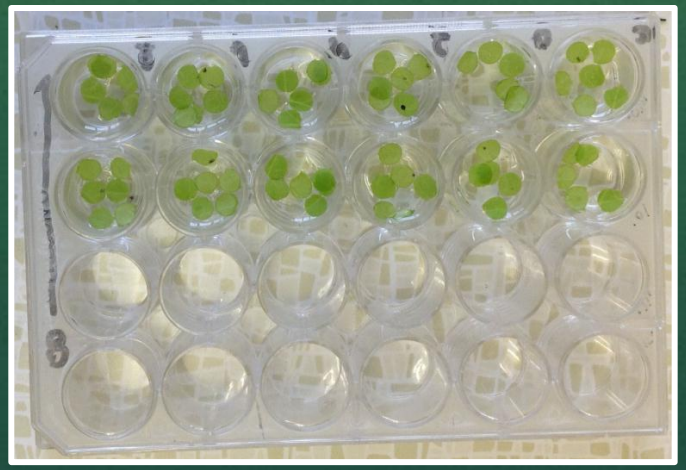
Infiltration Solution

Injection

3 week old tobacco plants

Infiltrated tobacco leaves

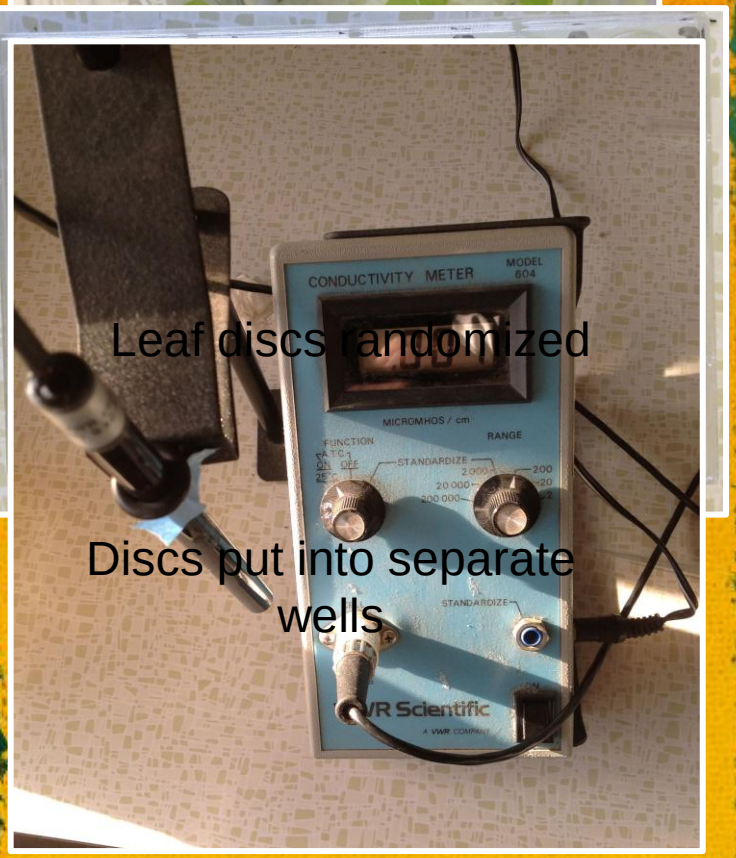
Confocal Microscopy



Electrolyte Leakage Assay

Phenotypic Response

http://www.vmrf.org/researchcenters/confocal/instrumentation.html



Leaf discs randomized

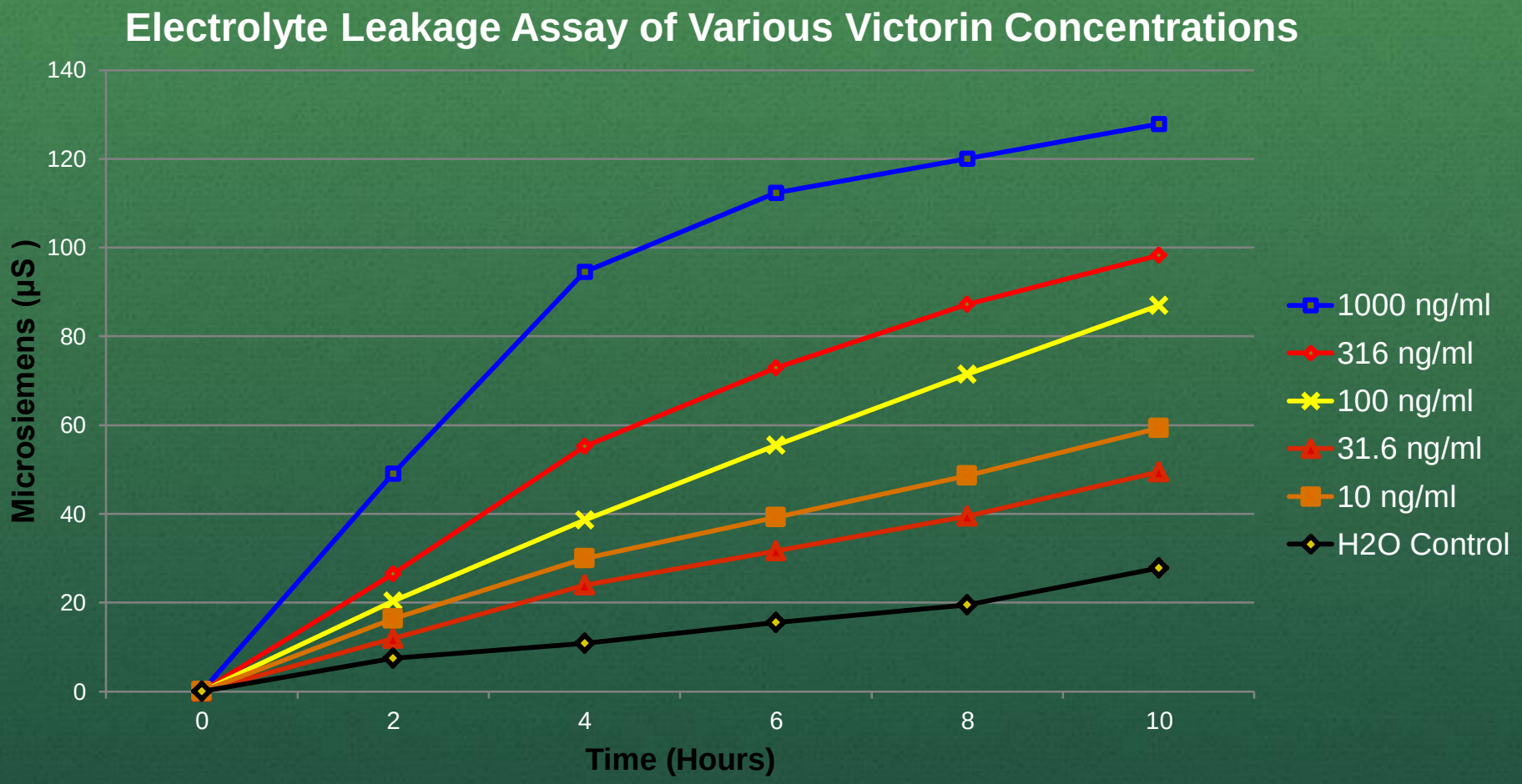
Discs put into separate wells

Conductivity meter used to measure ion leakage

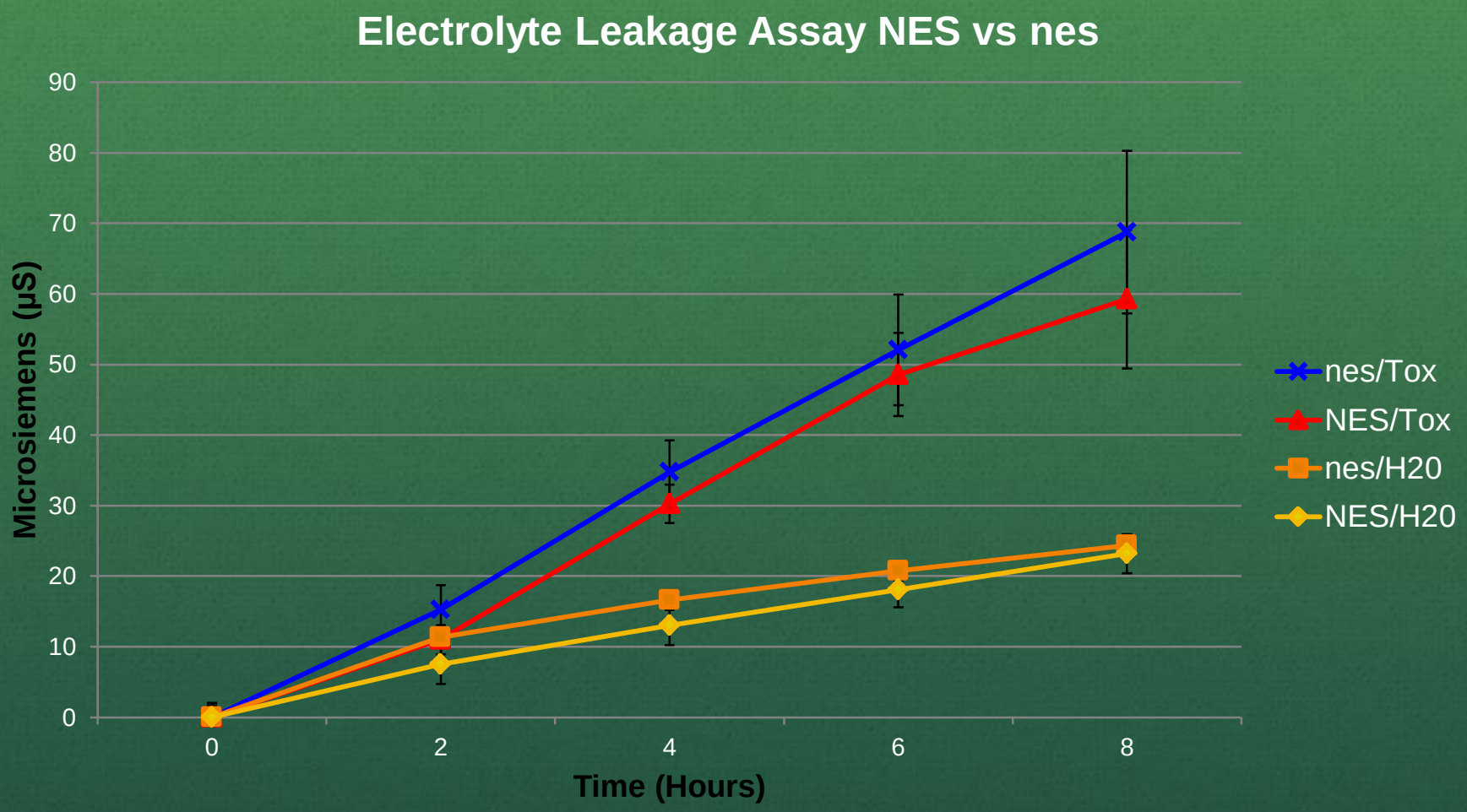
Electrolyte Leakage Assay

- Method of quantifying cell death
- Wells contain water and victorin
  - 6 leaf discs per well
  - Replicates of 3-5 wells
- Repeatable using different genetic constructs
  - Useful comparison of protein's functionality

Results



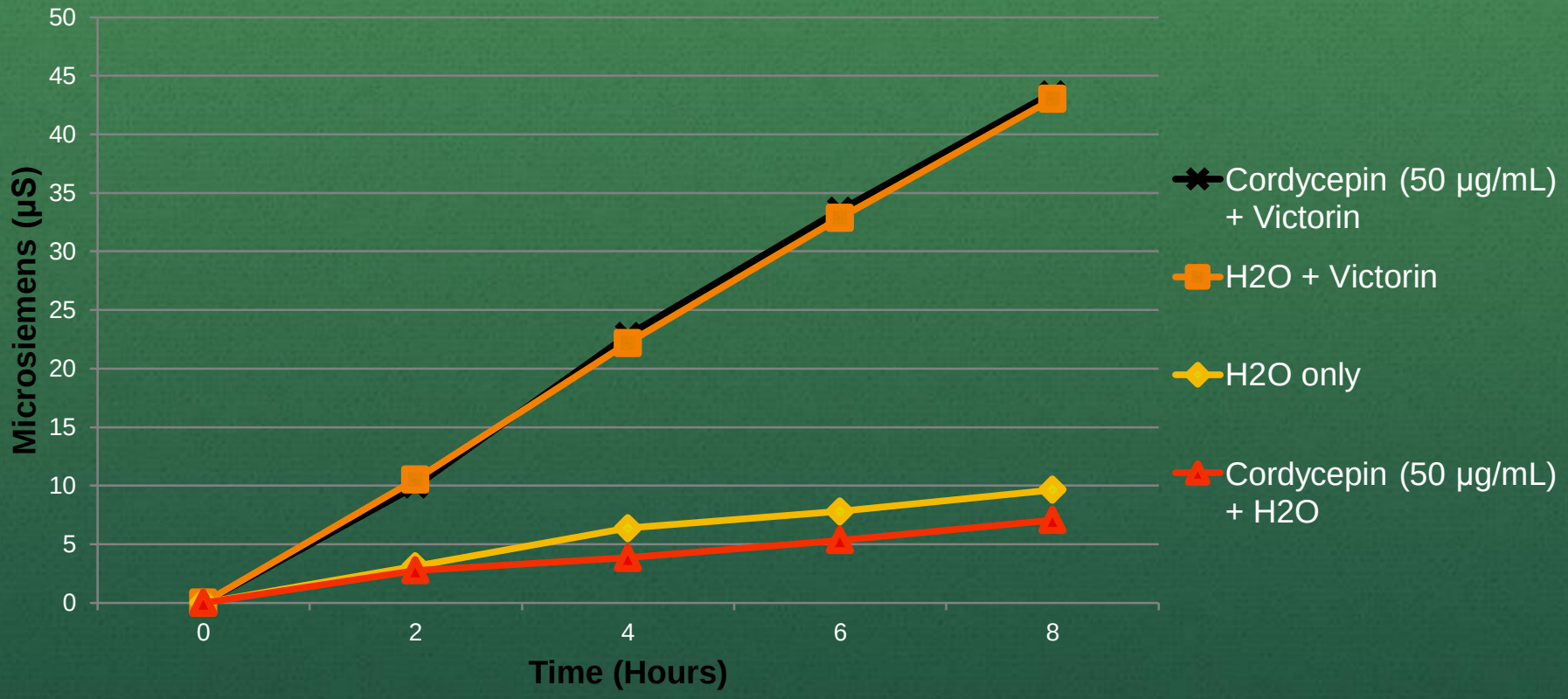
Graph 1: Victorin mediated cell death in *N. benthamiana* transiently expressing LOV1. The amount of cell death induced by victorin is directly correlated with the amount of electrolyte leakage.



Graph 2: A nuclear exclusion signal (NES) was fused to LOV1. As a control, a mutant NES (nes) was also fused to LOV1. Treatment with 384 ng/ml victorin concentration shows no significant difference in cell death.

Phenotype response to victorin. An example of victorin induced cell death in *N. benthamiana* transiently expressing NES LOV1 + TRX-h5. 2 hours post-infiltration

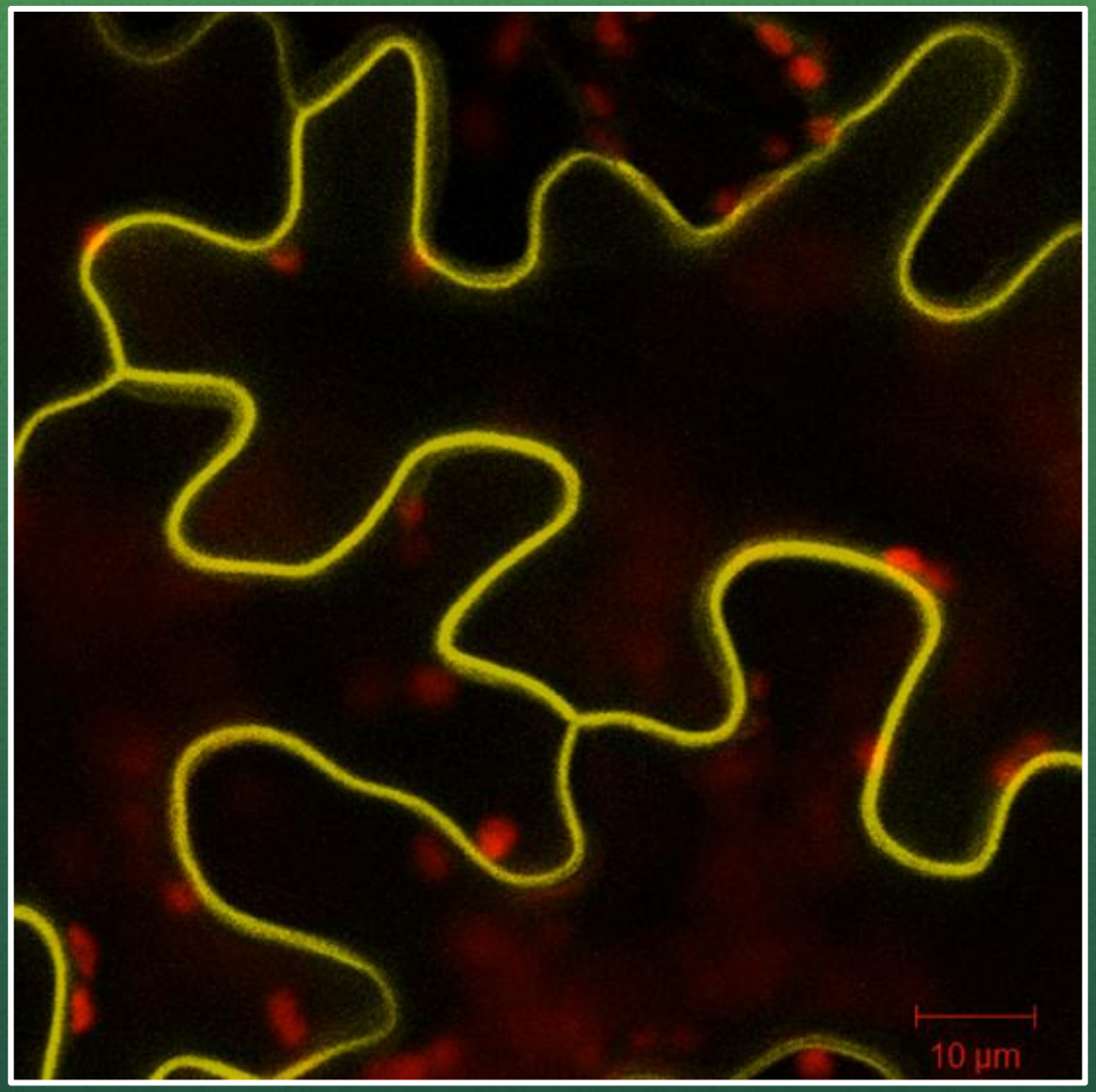
Electrolyte Leakage Assay using Cordycepin (50 µg/mL)



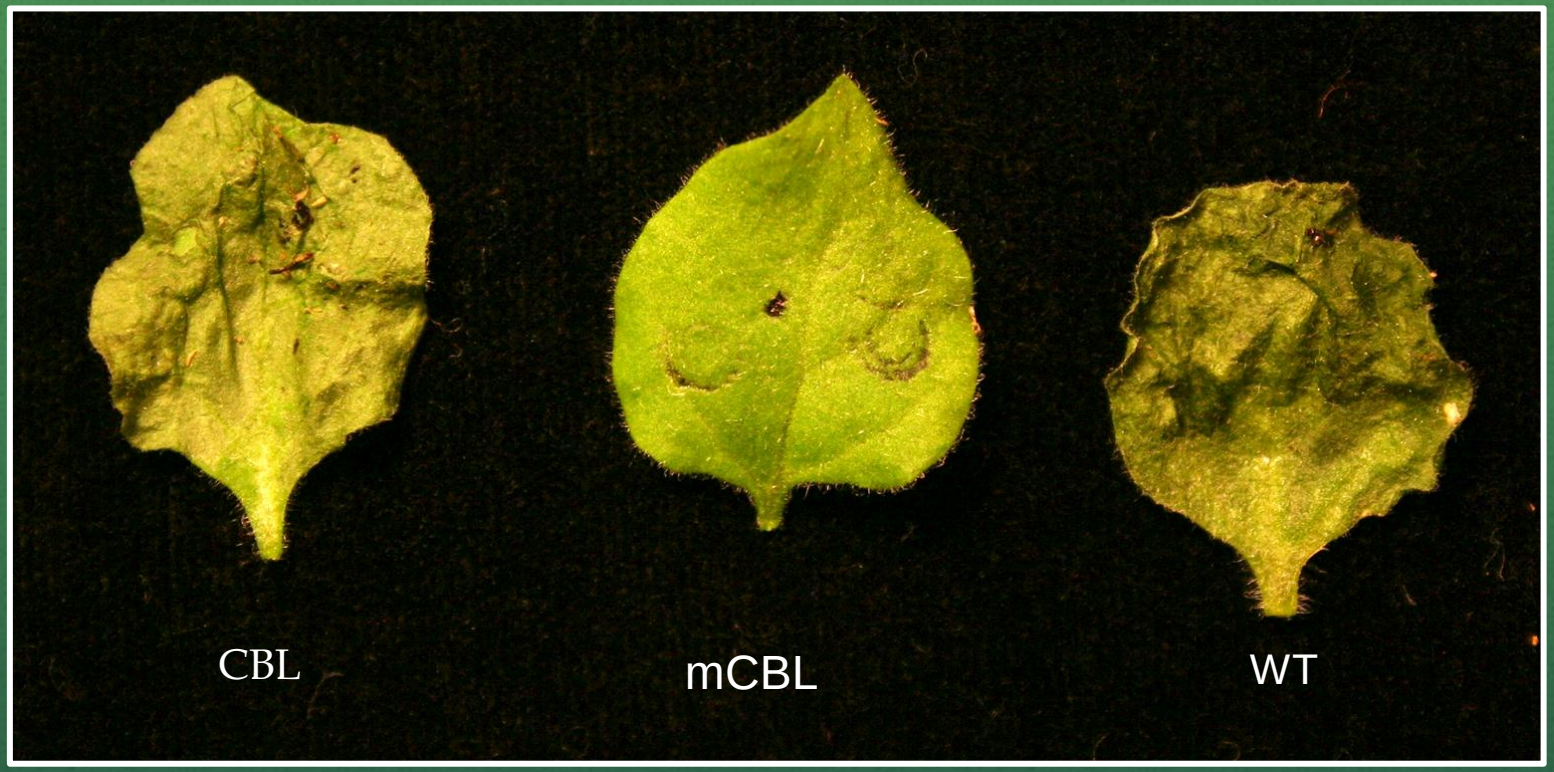
Graph 3: Transcriptional inhibitor Cordycepin used to determine further transcription of downstream defense genes needed for hypersensitive-response function. Treatment with 384 ng/ml victorin concentration shows no significant difference in cell death.

Confocal Microscopy

- To operate, need to take classes
  - Microscopes cost over $300,000!
- Yellow Fluorescence Protein (YFP) fused onto C-terminal of LOV1
  - Allows for detection and tracking by use of UV-irradiation
- Can be used to observe localization of genetic constructs of LOV1



LOV1 localizes to the plasma membrane. Confocal microscopy of yellow fluorescence protein (YFP) tagged LOV1 shows protein localizing to the plasma membrane of *N. benthamiana* cells



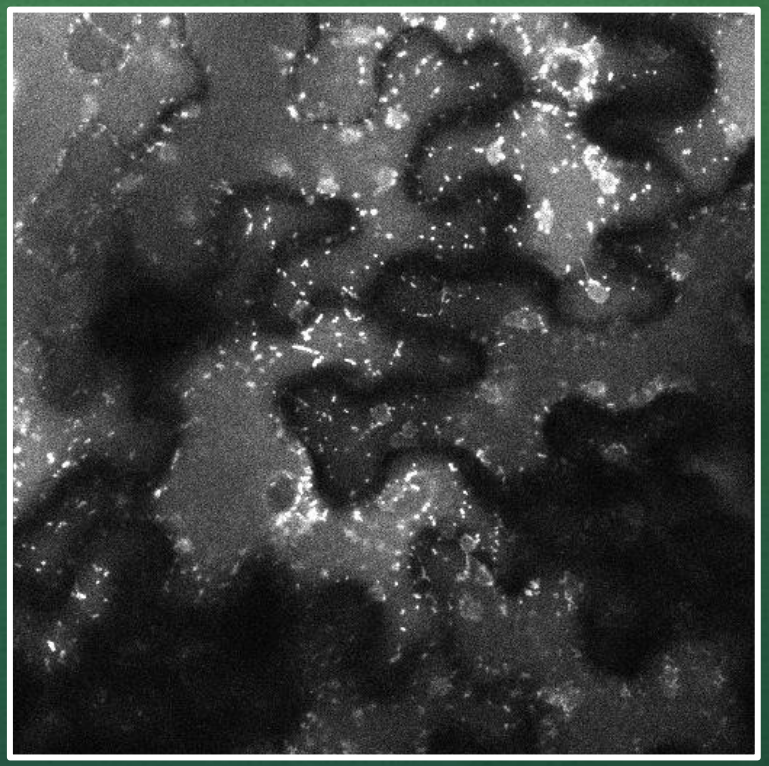
Phenotype response to victorin. CBL construct designed to irreversibly tether LOV1 to plasma membrane. mCBL acts as control for testing protein functionality. 30 hours post-infilitration.

Discussion/Conclusion

- LOV1 tagged with a functional NES is **as sensitive** to victorin as LOV1 tagged with a nonfunctional NES (nes).
  - Implies LOV1 does not need to translocate to the nucleus to confer sensitivity to victorin.
- Transcription of other defense genes after programmed cell death begins is not required.
- YFP tagged LOV1 localizes to the plasma membrane.
- LOV1 tethered to plasma membrane still functional throughout a victorin-induced cell-death time course.

Future Experiments

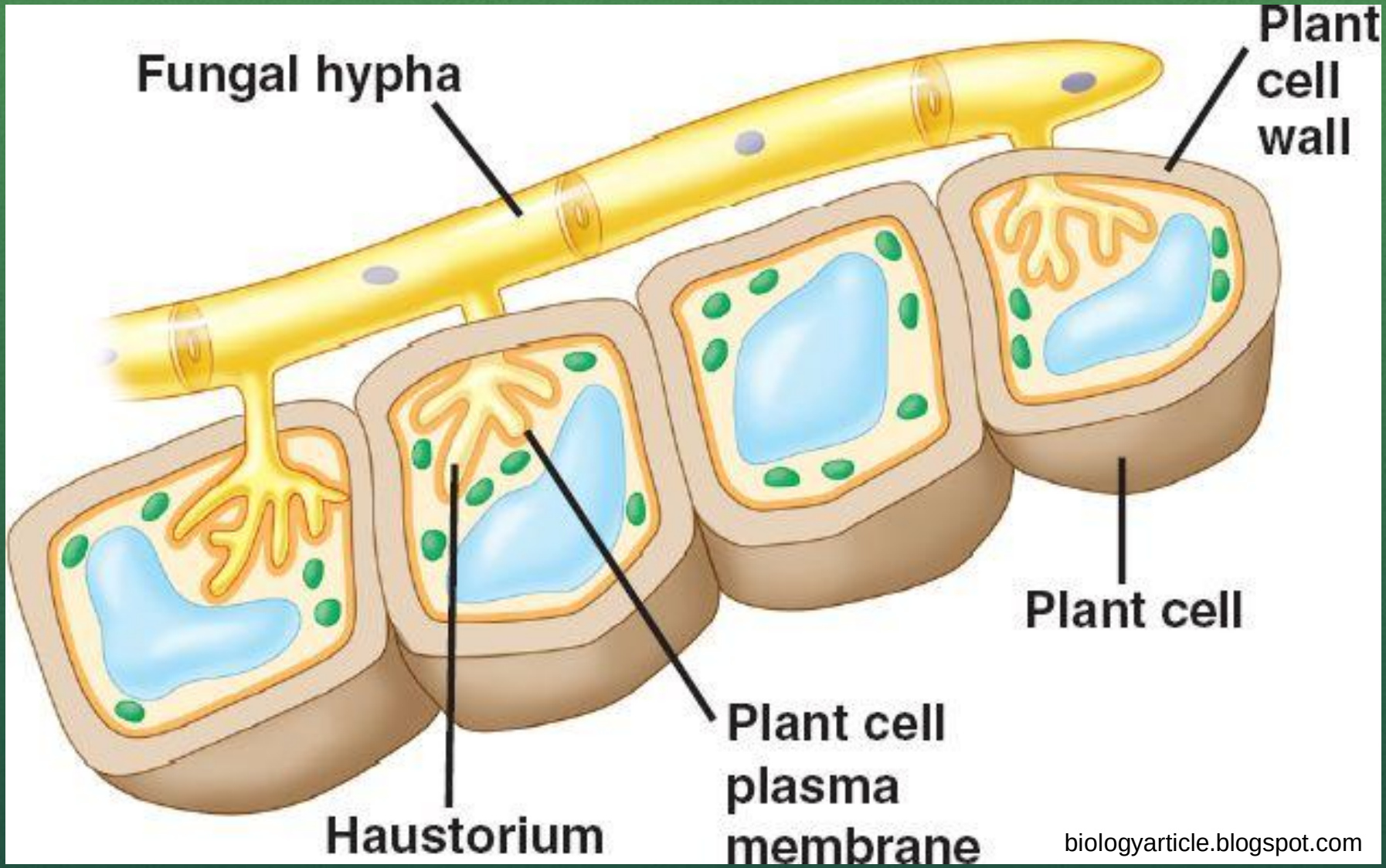
- Mitochondrial dye experiments
  - More accurate cell death indication
- Electrolyte Leakage Assays using CBL & mCBL



Fluorescent tagging of mitochondria using Tetramethyl Rhodamine (TMRM) dye

Acknowledgements

- Many thanks to:
  - Dr. Thomas Wolpert, Dr. Jennifer Lorang, Dr. Marc Curtis
  - BRR advisor Wanda Crannell
  - BRR director Kate Field
  - E.R. Jackman Internship Support Group
  - Audience!


Fungal hypha
Plant cell wall
Plant cell
Plant cell plasma membrane
Haustorium
biologyarticle.blogspot.com