

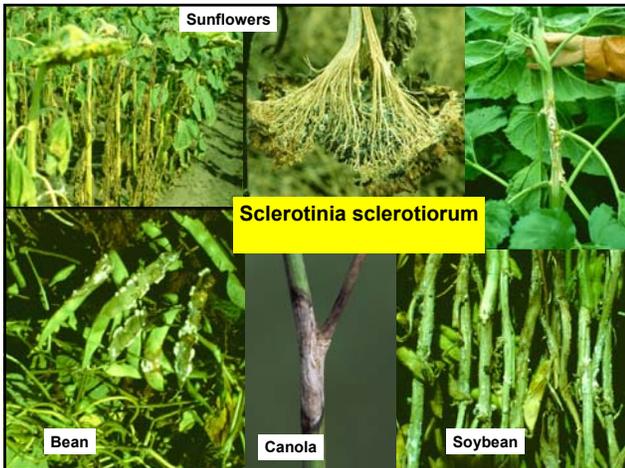
Innovative Methods to Identify Resistance to *Sclerotinia sclerotiorum*

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Disease control is the ultimate objective of the initiative !!

We need improved host resistance!!





What other methods could we use to examine host resistance???

Could we examine the amount of the pathogen in host tissue as a measurement of resistance?

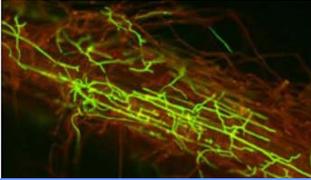
There are two ways to approach this idea.

Quantify the pathogen hyphal biomass in host tissue using DNA technology –

Use real time PCR and specific primers for *S. sclerotiorum* to quantify the DNA of the pathogen in host tissue.

Quantify the pathogen hyphal biomass in host tissue using reporter genes.

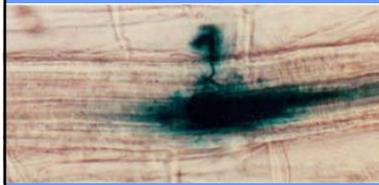
Transform *Sclerotinia* with a reporter gene such as β -glucuronidase (GUS) gene or the green fluorescent protein (gfp) gene and measure the reporter gene activity as an indication of hyphal biomass.



Oliver, R. et al. 1993. Use of fungal transformants expressing β -glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Mol. Plant-Microbe Interact.* 6:521-525

de la Pena, R and Murray, T. 1994. Identifying wheat genotypes resistant to eyespot disease with a β -glucuronidase-transformed strain of *Pseudocercospora herpotrichoides*. *Phytopathology* 84:972-977.

β -glucuronidase-transformed strain of *Pseudocercospora herpotrichoides*



Decided to use GFP-

We could have some fun doing the transformations and maybe have a tool to study infection, resistance and other aspects of the biology of *S. sclerotiorum*

Investigators who have transformed Pathogen with gfp:

Jeff A. Rollins, U of FL, Plant Pathology

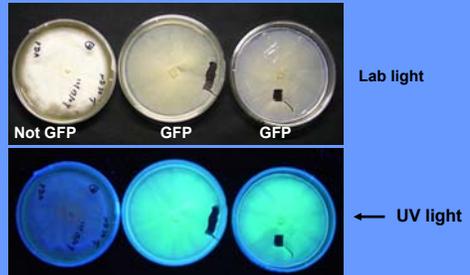
Adrienne Sexton & Barbara Howlett, Black Leg group, U of Melbourne, Australia

? Geraldine Vautard-Mey, U Claude Bernard, Villeurbanne, France



J. M. Lorang, R. P. Tuori, J. P. Martinez, T. L. Sawyer, R. Redman, J. A. Rollins, T. J. Wolpert, K. B. Johnson, R. Rodriguez, M. B. Dickman, and L. M. Ciuffetti. 2001. Green Fluorescent Protein: Lighting up Fungal Biology. *Applied and Environmental Microbiology* 67:1987-1994

Green fluorescent protein is a small protein produced by a gene from the jelly fish *Aequorea victoria*. Formation of this fluorescent chromophore is not species dependent. Blue or UV light and oxygen are the only requirements to induce green fluorescence.



There are two methods of using GFP to determine hyphal biomass.

1. Measure the fluorescence of the mycelium in the host tissue using a photometer - similar to the method of determining ploidy of nuclei in fungi using fluorescent stains.
2. Perform a protein extraction from the tissue and measure the fluorescence level using something like a MicroArray Fluorescence Reader for High-Throughput Protein Evaluation.

OBJECTIVES.

- 1) Genetically transform isolates of *S. sclerotiorum* with the green fluorescent protein (GFP) gene using a variety of *gfp* expression vectors, then select those with the strongest constitutive expression.
- 2) The selected transformed isolates will be grown on four crops of known susceptible and partially resistant genotypes and GFP expression will be used to detect and quantify the growth of the pathogen in host tissue. Our hypothesis is that quantifying the fungus biomass in host tissue can be used to measure resistance to *Sclerotinia* in plant tissue.

Need high expression of *gfp* in fungus and no loss of pathogenicity

The GFP plasmid vectors:

gGFP and tGFP from Amir Sharon, Israel,
Aspergillus nidulans promoters

pGV3 and pGV2 from G. Vautard-Mey, France,
cre1 Promoter from *Sclerotinia*

pCT74 from Lynda Ciuffeti, Oregon.
ToxA promoter of *Pyrenophora*

pTEFEGFP from Dan Cullen, Madison.
TEF promoter from *A. pullulans*

Transformation protocols:

BioRad gene gun at USDA facilities.
Using DNA coated tungsten particles. Mycelium bombarded.
Selection on PDA amended with 100 µg Hygromycin/ ml medium

Standard protoplast-polyethylene glycol (PEG) (Liljeroth et al., 1993)
Protoplasts produced with β-D-glucanase and driselase.
14-20 µg vector DNA per 1 x10⁶ protoplasts
Selection on PDA amended with 100 µg Hygromycin/ ml medium

Agrobacterium mediated (Bundock et al., 1995)
In progress

We concentrated efforts on two vectors, gGFP and pCT74
and two isolates, ND 30 and 21

RESULTS

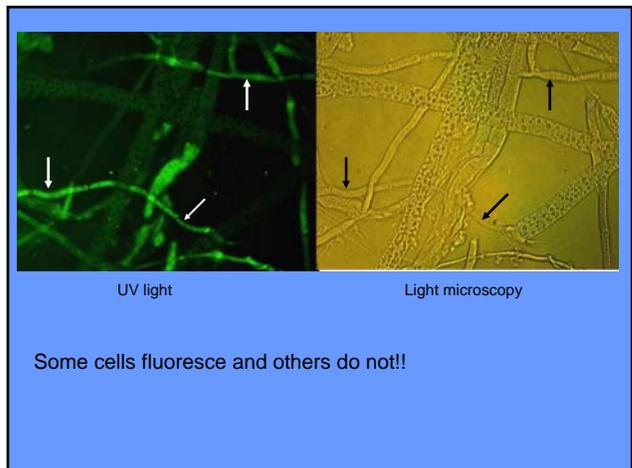
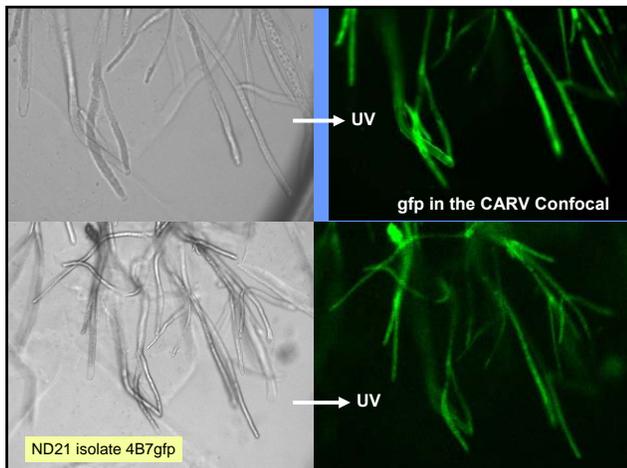
No transformants using gene gun.

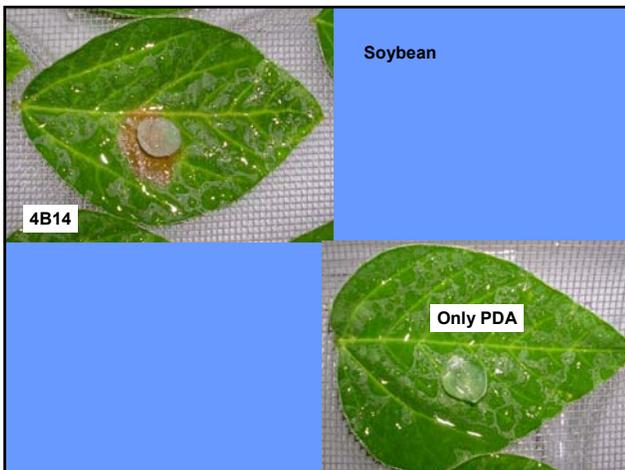
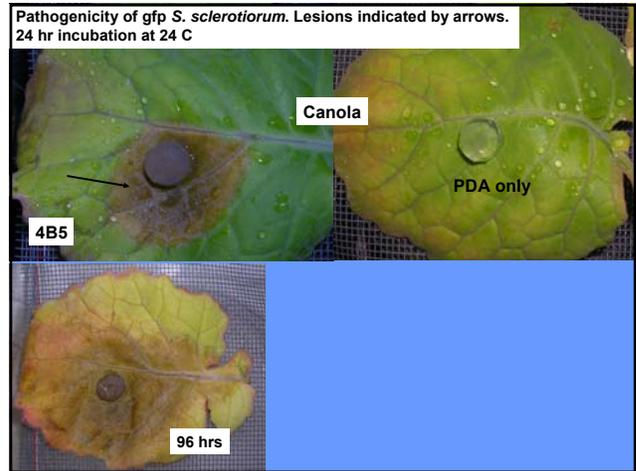
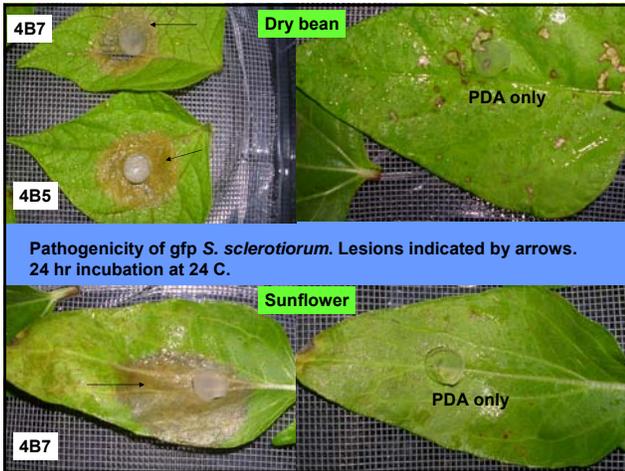
With protoplast-PEG method, putative transformants of ND21 appeared on the surface of the selection medium in 7 to 12 days and were subcultured twice on PDA amended with 100 µg/ml Hygromycin B. Cultures growing on the second subculture were evaluated for gfp expression and then pathogenicity.

RESULTS

20 hygromycin resistant transformants were obtained and 8 expressed gfp and grew normally. In two transformants, 4B7 and 4A12 the gfp expression was reasonably strong, but in the others it was poor. We believe it should be stronger for our host experiments. Some hyphal cells will fluoresce and others will not. Transformation experiments are continuing with other isolates and methods are being tried to improve the gfp expression of our putative transformants.

The pathogenicity of 8 putative transformants was confirmed on soybean, bean, canola and sunflower leaves.





Interactions of gfp *S. sclerotiorum* and hosts

Initiating studies with soybean, bean, sunflower and canola to view gfp pathogen in host tissue and measure biomass.

Examined host tissue to determine which tissues autofluoresce.

