Innovative Methods to Identify Resistance to *Sclerotinia sclerotiorum*

Berlin D. Nelson, Melvin D. Bolton and Amal de Silva, Dept. Plant Pathology, North Dakota State University, Fargo, ND

ABSTRACT:

The objective of this research is to utilize the green fluorescent protein gene (gfp) as a tool to measure the amount of the pathogen in host tissue. Our hypothesis is that quantifying the amount of fungus biomass in host tissue could be used to detect resistance. Research continued on the transformation of isolates of *Sclerotinia sclerotiorum* with gfp. We obtained a variety of plasmid vectors, each with different promotors: pTEFEGFP; pCT74; gGFP and tGFP; and pGV2 and pGV3. Plasmid DNA was produced via the transformation of competent *Escherichia coli* and extracted and purified. We initially used the Bio-Rad gene gun and bombarded 3 day old cultures with DNA coated tungsten particles. However, due to difficulty in access to the gene gun we switched to protoplast mediated transformation. Protoplasts were generated and a standard protoplast-PEG (polyethylene glycol) transformation method was employed. We concentrated our efforts on two pDNA's, pCT74 and gGPF, and two isolates, ND 30 and ND 21. Selection for transformants was on media amended with 100 µg/ml Hygromycin B. An *Agrobacterium* mediated protocol is also currently being attempted. Putative transformants via the PEG method appeared on the surface of the selection medium in 7 to 12 days and were transferred to potato dextrose agar amended with 100 µg/ml Hygromycin B. Twenty hygromycin resistant transformants were obtained, and 8 expressed gfp. In two transformants the gfp expression was reasonably strong, but in the others it was poor. All 8 putative transformants were pathogenic on leaves of host plants. Transformation experiments are continuing. In preparation for the eventual examinations of interactions between gfp *S. sclerotiorum* and host tissue we examined host tissue under UV light to determine the autofluorescence of stem and petiole tissue of soybean, bean, and canola and the root tissue of sunflower. Autofluorescence could interfere with the measurement of gfp in host tissue, thus it is necessary to know where the tissues autofluoresce. The bases of trichomes strongly autofluoresced and some vascular tissue fluoresced in soybean, bean and canola while the vascular tissue and the endodermis in sunflower roots strongly autofluoresced.

INTRODUCTION

Sclerotinia sclerotiorum is a plant pathogenic fungus that causes important diseases known as white mold, Sclerotinia wilt, stalk and stem rot, or head rot on a wide variety of broadleaf crops grown in the USA. Epidemics and serve outbreaks of Sclerotinia diseases have been reported in many areas of the Midwest. One of the greatest needs for disease control is resistant cultivars. Although there has been considerable work on identifying resistance in susceptible crops, the progress of this research has been slow. Incorporation of resistance into commercial cultivars remains a difficult objective.

One of the major difficulties in advancing the use of resistance has been the lack of rapid and easy methods for greenhouse or laboratory screening. Field screening has been the primary method to identify resistance, but is not always reliable and only allows one generation per year. Advances in breeding for resistance require reliable techniques for identifying physiological resistance. New methods to identify and study resistance in susceptible crops are needed. Not only would these be useful in detection and screening, but they would also allow more in-depth study of the host-pathogen relationship. A recent development in studying host-pathogen interactions is the use of reporter genes such as β-glucuronidase (GUS) and the green fluorescent protein (GFP) to observe and quantify the presence of a pathogen in the host (Candresse et al., 2001; De la Pena and Murray,1994) . Reporter genes have wide use in studying many aspects of pathogen biology (Chalfie et al., 1994; Lorang et al., 2001; Oliver et al., 1993)

This project proposes to genetically transform *S. sclerotiorum* with the green fluorescent protein (GFP) gene and use the fluorescent protein as a tool to examine resistance of four crops (canola, dry beans, soybeans and sunflower) to *Sclerotinia*. **The emphasis will be to determine the methodology and feasibility of using GFP to identify resistance in selected field crops. Our hypothesis is that quantifying the fungus biomass in host tissue can be used to measure resistance to *Sclerotinia* in young plant tissue.** We know of no published reports where GFP has been used as a tool to examine host resistance to *Sclerotinia*, although the fungus has been transformed with this reporter gene (Lorang et al., 2001; Sexton and Howlett, 2004) and gfp has been used to study some aspects of the biology of the pathogen (Guimaraes and Stotz, 2004; Sexton and Howlett, 2004).

METHODS

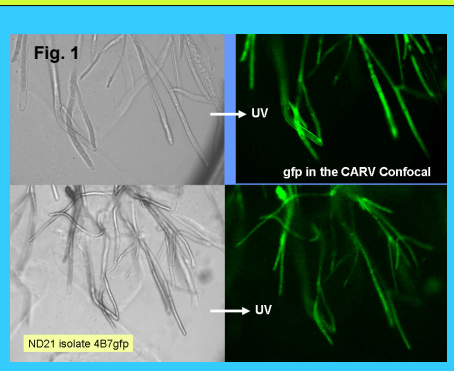
A variety of plasmid vectors with various promoters were obtained: gGFP and tGFP with *Aspergillus nidulans* promoters from Amir Sharon, Israel; pGV3 and pGV2 with the *cre1* promoter from *Sclerotinia* from G. Vautard-Mey, France; pCT74 with the ToxA promoter of *Pyrenophora* from Lynda Ciuffeti, Oregon; and pTEFEGFP with the TEF promoter from *A. pullulans* from Dan Cullen, Madison. Plasmid DNA (pDNA) was produced by the transformation of competent *Escherichia coli* following the procedure outlined by Sambrook et al. (1989). Transformed cells were selected by growth on LB (Luria-Bertani) medium containing 50 µg ampicillin/ml and then increased on the same medium. The pDNA was extracted and purified according to the procedure by Sambrook et al. (1989). The *E. coli* cells were collected by centrifugation and pDNA extracted and purified using the Wizard DNA plasmid purification kit. The pDNA was dissolved in TE buffer, pH 8.0, and stored at -20 C.

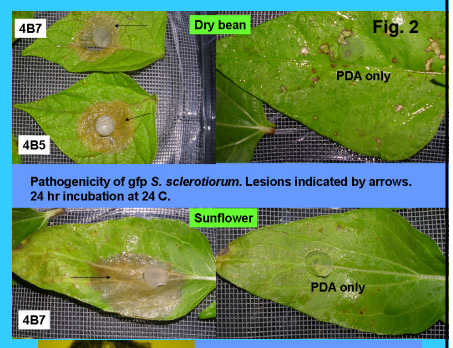
Transformation experiments were attempted with a BioRad gene gun (PDS-1000/Helium System) in the USDA facility in Fargo using pDNA coated tungsten particles and bombardment of mycelium on PDA. A slightly modified procedure of Bommineni et al. (1993) was followed for coating of the tungsten particles with pDNA. Colonies were bombarded at 63.27 or 77.33 kg/cm2 at a vacuum of 0.17 MPa or 0.18-0.19 MPa. The Petri dish (100 x 20 mm) containing the 2-3 day old colony was placed 9 cm below the flying disc and was bombarded twice. Following bombardment, colonies were allowed to incubate for 48 h at 25EC. Then colonies were overlaid with hygromycin-PDA medium to select transformants. All initial attempts at transformation used the pDNA from pCT74 and gGFP and *S. sclerotiorum* isolates ND 30 and 21.

Transformation was also attempted with a standard protoplast-PEG (polyethylene glycol) method (Liljeroth et al., 1993). Protoplasts were produced in a β-D-Glucanase plus Driselase cocktail with a 3.5 hr incubation at 24° C. The PEG method used 15 to 20 µg of pDNA per 1 x 106 protoplasts. Protoplasts were suspended in the plating media and poured into Petri dishes. The plating medium was overlaid with PDA amended with 100 µg hygromycin B/ml PDA and incubated at 23° C. Putative transformants were transferred to fresh hygromycin/PDA for 4 to 7 days, then transferred again to fresh hygromycin/PDA for storage. Hyphae from all colonies developing on hygromycin/PDA were examined at 400 X on a Leitz Wetzlar or Olympus fluorescence microscope, or a Nikon E600 CARV Confocal cell imaging system with filters for gfp excitation and emission.

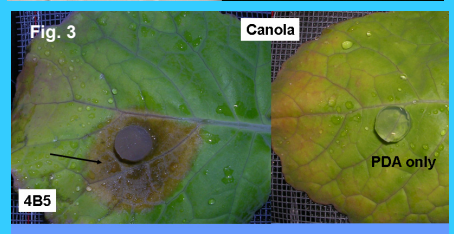
Putative gfp transformants were tested for pathogenicity on soybean, sunflower, canola and bean leaves. Fresh leaves from month old plants were removed, washed, sprayed with a mist of distilled water and placed in humid chambers. One cm diameter plugs of mycelium on PDA were place mycelium side down on the leaves and incubated at 23° C. Lesion development was recorded over 5 days. Controls were plugs of only PDA.

In preparation for the eventual examinations of interactions between gfp *S. sclerotiorum* and host tissue, we examined host tissue under UV light to determine the autofluorescence of stem and petiole tissue of soybean (cv. MN0301), bean (cv. UI114), and canola (cv. Hyola 401) and the root tissue of sunflower (cv. HA 89). Autofluorescence could interfere with the measurement of gfp in host tissue, thus it is necessary to know where the tissues autofluoresce.





Pathogenicity of gfp *S. sclerotiorum*. Lesions indicated by arrows. 24 hr incubation at 24 C.



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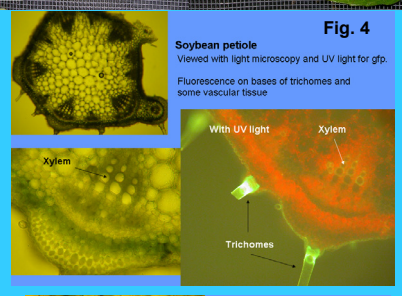

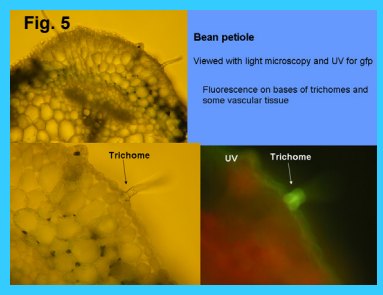

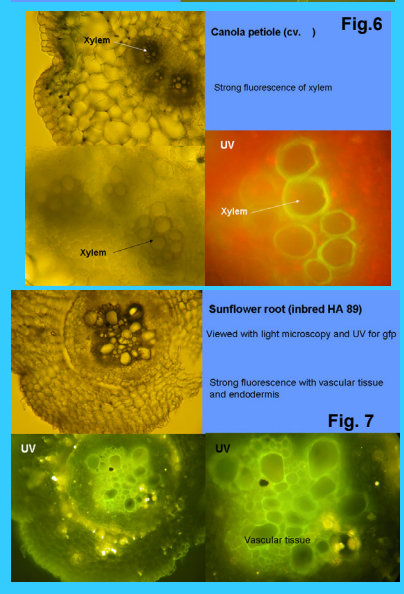

RESULTS & DISCUSSION

No transformants were obtained using the gene gun, but putative transformants were obtained with pCT74 and isolate ND 21 with the protoplast-PEG method. Colonies appeared on the hygromycin/PDA selection medium in 7 to 12 days. A total of 20 putative transformants grew on the first and second transfer to hygromycin/PDA.

Some colonies grew similar to the wild type while others grew very slowly. Of those, 8 expressed gfp when examined with UV light (Fig 1). In two transformants, 4B7 and 4A12 the gfp expression was reasonably strong, but in the others it was poor. Several characteristics of these transformants are that not all the hyphal cells fluoresce and the percentage of cells that fluoresce differ between transformants. The stability of these transformants is currently being evaluated. All 8 putative gfp transformants were pathogenic on leaves of host plants, but the aggressiveness of these isolates compared to the wild type has not been examined.

Under UV light using filters for gfp, the bases of trichomes strongly autofluoresced and some vascular tissue fluoresced in soybean, bean and canola while the vascular tissue and the endodermis in sunflower roots strongly autofluoresced. Autofluorescence of host tissue will need to be considered when measuring gfp in host tissue.

Transformation experiments are continuing with other isolates and pDNA, and methods are being tried to improve the gfp expression of our putative transformants. We believe gfp expression should be greater to study the interaction of the pathogen with hosts. We are currently examining the gfp isolates in host tissue to determine the feasibility of using them to detect resistance in host crops.

Literature cited:

Bommineni, V. R., Chibbar, R. N., Datla, R. S. S., and Tsang, E. W. T. 1993. Transformation of white spruce (*Picea glauca*) somatic embryos by microprojectile bombardment. Plant Cell Rep. 13:17-23.

Candresse, T., Le Gall, O., Maisonneuve, B., German-Retana, S. and Redondo, E. 2001. The use of green fluorescent protein-tagged recombinant viruses to test *Lettuce mosaic virus* resistance in Lettuce. Phytopathology 92: 169-176.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. 1993. Green fluorescent protein as marker for gene expression. Science 263:802-805.

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

Guimaraes, R. L., and Stotz, H. U. 2004. Oxalate production by Sclerotinia sclerotiorum deregulates guard cells during infection. Plant Physiology 136:3703-3711.

Liljeroth, E., Jansson, H-B., and Schafer, W. 1993. Transformation of *Bipolaris sorokiniana* with the GUS gene and use for studying fungal colonization of barley roots. Phytopathology 83:1484-1489.

Lorang. J., Tuori, R., Martinez, J., Sawyer, T., Redman, R., Rollins, J., Wolpert, T., Johnson, K., Rodriguez, R., Dickman, M., and Ciuffetti, L. 2001. Green fluorescent protein is lighting up fungal biology. Appl. Environ. Microbiol. 67:1987-1994.

Oliver, R. P., Farman, M. L., Jones, J. G., and Hammond-Kosack, K. E. 1993. Use of fungal transformants expressing β-glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. MPMI 6:52-525.

Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sexton, A., and Howlett B. J.. 2004. Sclerotinia sclerotiorum stem rot of Canola in Australia. Abstract, 7th European Conference on Fungal Genetics, Copenhagen, April 17-21, 2004.