

Transformation of *Sclerotinia sclerotiorum* with the green fluorescent protein gene and fluorescence of hyphae in four inoculated hosts

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To obtain a genetic marker to observe and study the interaction of *Sclerotinia sclerotiorum* with its hosts, isolates ND30 and ND21 were transformed using pCT74 and gGFP constructs, both containing genes for the green fluorescent protein (GFP) and hygromycin B phosphotransferase. Putative transformants were obtained using polyethylene glycol-mediated transformation of protoplasts. Seven stable *gfp* transformants were identified and evaluated for fluorescence *in vitro* and *in planta*, pathogenicity and colonization of host tissues. Real-time quantitative polymerase chain reaction detected a single copy of the *gfp* gene in transformants. Fluorescence was quantified directly from mycelium and protein extracted from hyphae. The seven transformants (four from ND30 and three from ND21) were pathogenic on dry bean, canola, soybean and sunflower. However, depending on the host, three transformants differed significantly ($P = 0.05$) in the length of lesions formed compared to the wild-type. Hyphae fluoresced in plant tissue and could clearly be distinguished from plant cells. Infection and colonization of tissues were clearly visible with a fluorescent microscope. Transformants differed in the intensity of GFP expression both *in vitro* and *in planta*.

Keywords: bean, canola, sclerotinia wilt, soybean, sunflower, white mould

Introduction

Sclerotinia sclerotiorum is a necrotrophic pathogen that causes important diseases known as white mould, sclerotinia wilt and stalk, stem or head rot on a wide variety of broadleaf crops such as bean, canola, pea, soybean, sunflower and tomato (Bolton *et al.*, 2006). The wide range of susceptible crops (Bolland & Hall, 1994) contributes to crop losses in many countries (Clarkson *et al.*, 2004; Kora *et al.*, 2005). Since diseases caused by *S. sclerotiorum* have traditionally been difficult to control and may rely on the use of fungicides (Abawi & Grogan, 1979), the search for host resistance has been an important objective of research programmes on this pathogen (Zhao *et al.*, 2004). However, levels of resistance that can be relied upon as a primary control measure have been difficult to achieve. New methods of studying the interactions of *S. sclerotiorum* with its many hosts may lead to a better understanding of host-pathogen interactions.

A recent development in studying host-pathogen interactions is the use of transgenes that encode the green fluorescent protein (GFP) or β -glucuronidase (GUS) to

observe the presence of a pathogen in the host (De la Pena & Murray, 1994; Candresse *et al.*, 2002). However, the application of traditional transgenes such as *uidA*, the ‘GUS gene’, to study fungal-plant interactions is limited because of destruction of tissues caused by the application of substrates required for activity, leakiness of the product, and cellular permeabilization of substrates (Lorang *et al.*, 2001). On the other hand, GFP can be used without these limitations to study fungal-plant interactions, allowing researchers to monitor GFP production *in planta* in real time. Expression of *gfp* in filamentous fungi requires a *gfp* variant as the wild-type (WT) *Aequorea victoria gfp* gene is not efficiently translated in many fungi (Spellig *et al.*, 1996; Cormack *et al.*, 1997). An improved *gfp* gene, *sgfp*, which contains a serine-to-threonine substitution at amino acid 65 (S65T) was developed for higher eukaryotes (Chiu *et al.*, 1996).

A variety of fungi such as *Ustilago maydis*, *Cochliobolus heterostrophus*, *Aspergillus nidulans* and the oomycete *Phytophthora parasitica* have been transformed with the *gfp* reporter gene (Spellig *et al.*, 1996; Fernández-Ábalos *et al.*, 1998; Maor *et al.*, 1998; Bottin *et al.*, 1999). Maor *et al.* (1998) quantified the fluorescence intensity of a *gfp*-transformed *C. heterostrophus* and found a correlation between fluorescence and the amount of mycelium observed in pure culture and in inoculated maize leaves.

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Recently, fungal biomass was quantified by measuring the green fluorescence intensity from *gfp*-transformed *Colletotrichum* in crude plant extracts (Chen *et al.*, 2003).

Lorang *et al.* (2001), in a team effort to transform eight ascomycete plant pathogens, were the first to report transformation and expression of *gfp* in *S. sclerotiorum*. Guimarães & Stotz (2004) used the same transgenic isolate to examine the effect of the pathogen and oxalic acid on stomatal guard cells of *Vicia faba*. There have been reports of transformations of *S. sclerotiorum* other than with *gfp*; mycelial fragments and ascospores were transformed using *Agrobacterium*-mediated transformation, primarily for targeted mutagenesis (Liberti & Dobinson, 2006; Weld *et al.*, 2006). There have been no studies examining the pathogenicity of *gfp* transformants of *S. sclerotiorum* on important crops or attempting to quantify the expression of *gfp* *in vitro* and *in planta*. The objective of this research was to produce stable *gfp* transformants of *S. sclerotiorum* and evaluate their pathogenicity and fluorescence *in vitro* and *in planta* on four economically important crop hosts. A preliminary report of this work has been published (de Silva *et al.*, 2005).

Materials and methods

Transformation vector

Two transformation vectors were chosen that utilized different promoters. Plasmid vectors pCT74, containing *sgfp* driven by the *ToxA* promoter of *Pyrenophora tritici-repentis* (Lorang *et al.*, 2001), and gGFP, containing *sgfp* driven by the *A. nidulans gpd* promoter (Maor *et al.*, 1998), were obtained from Lynda Ciuffetti, Oregon State University and from Amir Sharon, Tel Aviv University, Israel, respectively. Both vectors contain the *hph* gene for resistance to hygromycin B. *Escherichia coli* cells were transformed and cultured with pCT74 or gGFP constructs according to Sambrook & Russell (2001). Plasmid DNA was purified using the Wizard plus SV miniprep (Promega Corporation) purification system. Plasmid pCT74 was linearized with *EcoRI*, gel-extracted using the QIAquick gel extraction kit (Qiagen Inc.) and dissolved in TE buffer, pH 8.0. The gGFP plasmid was not linearized.

Fungal transformation

Transformation was performed on *S. sclerotiorum* WT isolates ND30 and ND21. ND21 was originally grown from a sclerotium collected from a North Dakota sunflower field and ND30 from a dry bean field and both isolates were virulent on local crops. Transformation was carried out with either pCT74 or gGFP constructs using polyethylene glycol (PEG)-mediated transformation of protoplasts (Liljeroth *et al.*, 1993). For transformation of ND21, only pCT74 was used. Isolates were grown in potato dextrose broth for 4–5 days and 1 g fresh weight of mycelium was used to produce protoplasts in an enzyme cocktail containing 400 mg β -D-glucanase and 200 mg driselase (Interspex Products) with a 3.5-h incubation at

24°C. After incubation, the enzyme/protoplast solution was filtered through four layers of sterile cheesecloth. The protoplasts were collected by centrifugation at 400 g and resuspended in STC (1.2 M sorbitol, 10 mM Tris, pH 7.5, and 10 mM CaCl₂). Plasmid DNA (10–15 μ g) was added to 1–2.5 $\times 10^6$ protoplasts in 500 μ L STC solution and incubated on ice for 15 min, then 500 μ L PEG was added to the protoplast solution and incubated on ice again for 5 min. PEG contained 500 μ L of 3 \times PEG amendments (1.8 M KCl and 150 mM Tris, pH 7.4) and 1 mL of 30% (w/v) polyethylene glycol 3350. Protoplasts were collected by centrifugation, resuspended in 100 μ L STC and added to 20 mL cooled plating medium (198.18 g dextrose, 1 g tryptone, 1 g yeast extract and 8 g technical agar L⁻¹; autoclaved and kept at 65°C until use) which was poured into Petri plates to solidify. After 3 h, plating medium was overlaid with 10 mL potato dextrose agar (PDA) amended with hygromycin B for a final selection concentration of 100 μ g mL⁻¹ and incubated at 23°C.

Putative transformants were transferred to fresh hygromycin-amended PDA and recultured from hyphal tips several times on selective medium to enhance purity of the cultures. All putative transformants were examined for fluorescence with either a Leitz Wetzlar epifluorescence microscope or a Nikon E600 CARV Confocal cell imaging system using filters at 475 nm excitation and 510 nm emission. Putative *gfp* transformants were stored at –80°C as mycelium on PDA in 50% glycerol or grown on autoclaved barley seeds for 14 days then stored at –80°C until further analysis. All experiments were initiated from stored cultures.

Evaluation of transformants

Transgene stability was tested in putative *gfp* transformants by placing a mycelial plug from the leading edge of a 2-week-old hygromycin-amended PDA culture onto the edge of PDA without hygromycin in 100 \times 15-mm Petri plates. Once hyphal growth had reached the opposite side of the plate (6–7 days), a transfer was made from that side to another plate of PDA where growth was again initiated on the edge of the PDA. This process was repeated until the fungus had grown across four plates of PDA (24–28 days on PDA). A transfer was then made to PDA amended with hygromycin B at 100 μ g mL⁻¹. Fluorescence of hyphae was examined with either the epifluorescence microscope or the cell imaging system. There were three replications per transformant.

Determining *gfp* presence and copy number

Stable *gfp* transformants and the progenitor WT isolates were grown on autoclaved cellulose nitrate membrane filters (0.45 μ m; Whatman International) on PDA at 23°C for DNA and protein extractions. Mycelial mats were harvested from the filters after 10–14 days and frozen at –80°C. Genomic DNA was isolated from mycelium as described by Möller *et al.* (1992) with minor modifications. To confirm the presence of the *gfp* gene, PCR was

performed on transformed and WT isolates using forward 5'-GCGACGTAACGGCCACAAG-3' and reverse 5'-CCAGCAGGACCATGTGTGATCG-3' primers which amplified a 606-bp fragment of the *gfp* sequence. Amplification of the *gfp* gene sequence consisted of 35 cycles of 1 min at 94°C, 2 min at 53°C and 1.5 min at 72°C, and was followed by 5 min at 72°C. PCR reactions contained 2.5 µL of 25 mM Mg buffer, 2.5 µL of 10 × buffer, 1 mM dNTPs, 0.25 µM of each primer and 1 unit Taq DNA polymerase (Invitrogen) for a total volume of 25 µL.

Real-time quantitative polymerase chain reaction (qPCR) (Heid *et al.*, 1996; Bubner & Baldwin, 2004) was used to determine the copy number of the *gfp* gene integrated in the fungal genome. This technique can distinguish copy number with high reliability (Bubner & Baldwin, 2004). Oligonucleotide primers and TaqMan probes were designed using PRIMERQUEST (Integrated DNA Technologies) for the *sgfp* sequence (NCBI accession EF090408) and the endogenous control actin gene (NCBI accession AJ000335) (Table 1). qPCR was performed with IQ Supermix (Bio-Rad) according to the manufacturer's instructions using an iCycler (Bio-Rad). Each reaction mixture of 25 µL contained 1 × PCR Supermix, 1 µM primer and 100 µM probe. The conditions for the PCR programme were as follows: 3 min at 95°C and 50 cycles of 30 s at 95°C, 1 min at 53°C and 1 min at 72°C. The DNA concentration of the samples was 100 ng µL⁻¹. Each sample was assayed in triplicate and the experiment was repeated. The *sgfp* amplicon was amplified as a singleplex assay. Standard curves were obtained with transformant ND30-43 for the *sgfp* and actin genes using a 10-fold serial dilution.

Fluorescence quantification

Fluorescence of GFP was quantified directly from mycelium growing on PDA. Stable *gfp* transformants and the WT of both ND30 and ND21 were grown on PDA for 4 days. Mycelial plugs (7 mm diameter) were taken from the leading edge of actively growing colonies and were placed directly into individual wells of 96-well, U-bottom polystyrene assay plates (Becton Dickinson) and plug fluorescence was measured. The experimental design was a randomized complete block with four replications and the experiment was repeated. Each replication consisted

of one plug from a single culture. Treatments were WT, *gfp* transformants and the control PDA plugs.

Fluorescence of GFP was also quantified from total protein extracted from mycelium. Total protein was extracted from mycelium as described by Remans *et al.* (1999). Mycelial mats were produced as previously described and ground while frozen in liquid nitrogen. Screw-cap tubes (2 mL) (Bio Plas) each containing five 3-mm glass beads (VWR) were filled with 500 µL ground mycelia. Extraction buffer (1 mL) containing 10 mM TrisEDTA (pH 8.0) and 0.02% (w/v) sodium azide was added to each sample. Samples were shaken for 45 s at speed 6.5 in the Bio-Savant FastPrep shaker (Qbiogene), transferred to ice for 10 min, then centrifuged twice at 11 000 g at 4°C, and the supernatant transferred to a fresh 1.5-mL tube after each centrifugation. Total protein concentration from the WTs and the transformants was determined using a protein assay kit (BioRad) (Bradford, 1976). All samples were standardized for total protein by diluting with extraction buffer to 70 µg mL⁻¹. Protein samples were stored at -80°C until further analysis. The experiment was completely randomized with three replications of protein extracts of WT cultures, *gfp* transformants and the buffer controls, and the experiment was repeated (a replication was the protein extracted from one culture grown on PDA). The fluorescence of both mycelial plugs and extracted protein was measured with a Synergy HT multi-detection microplate reader (BioTek) with 10 reads per well with an excitation wavelength of 485/20 nm, an emission wavelength of 528/20 nm and a sensitivity factor between 97 and 105 for the plugs and 105 and 106 for mycelial protein. The sensitivity factor ensures a signal to be within a linear range of the reader. The units of measurement provided by the microplate reader are termed relative fluorescence units.

Pathogenicity of transformants

Stable fluorescing transformants ND30-31, ND30-41, ND30-Y41, ND30-43, ND21-7, ND21-14 and ND21-17 were chosen for pathogenicity assays on stems of the following crops: soybean (*Glycine max*) cv. MN 0301; sunflower (*Helianthus annuus*) cv. HA 89; canola (*Brassica napus*) cv. Hyola 401; and bean (*Phaseolus vulgaris*) cv. U1114. All plants were grown in potting mix in containers (164-mL volume; Stuewe and Sons, Inc.) for 3 weeks in a greenhouse under natural light and high-pressure sodium lamps (1000 µE m⁻² s⁻¹) for 16 h day⁻¹. A cut-stem method of inoculation (Kull *et al.*, 2003) was employed. The wide end of pipette tips (1000 µL; 200 µL for canola) were pressed twice into the leading edge of mycelium of 4- to 5-day-old cultures grown on PDA so that a 12-mm length of agar was inside the tip. The pipette tip was then placed over the ends of freshly cut stems so that the cut end was inserted into the agar. The inoculated plants were maintained under humid conditions (≥ 90% RH) for 36 h, then kept in a growth room at 23 ± 2°C under fluorescent lights for a 12 h day⁻¹. Lesion length was recorded 4 days after inoculation. Controls received pipette tips with PDA.

Table 1 Primer pairs and fluorogenic probes for real-time quantitative PCR (TaqMan) assay used to quantify *sgfp* and control actin genes in *Sclerotinia sclerotiorum* isolates

Name	Orientation	Sequence (5'-3')
Actin	Forward	AGATCTTGGCTGAGCGTGGTTACA
Actin	Reverse	TGACTGGCGGTTTGGATTTCTTGC
Actin	Probe ^a	TTTCTCCACCACTGCCGAGCGTGAAAT
SGFP	Forward	TGACCCTGAAGTTCATCTGCACCA
SGFP	Reverse	TCTTGTAGTTGCCGTCGTCCTTGA
SGFP	Probe	AGCACGACTTCTCAAGTCCGCCAT

^aProbes were labelled with the fluorescent reporter dye 6-FAM at the 5' end and the fluorescent quencher dye TAMRA at the 3' end.

The experiment was a randomized complete block with four replications and was repeated once. Each crop was considered a separate experiment.

Transformants ND30-31, ND30-41, ND21-7 and ND21-14 and the progenitor WT isolates were inoculated onto petioles of the four crops to examine colonization of tissues. Pipette tips (1000 μ L) were pressed into the leading edge of mycelium of 4- to 5-day-old cultures grown on PDA and then placed over the ends of freshly cut petioles (del Rio *et al.*, 2001) on 40- to 50-day-old plants. Three petioles on each of three plants per crop were inoculated with transformants or WT. Plants were incubated in humid chambers at $23 \pm 2^\circ\text{C}$ under fluorescent lights for 12 h day⁻¹. At various times over 1 week, infected plant tissue was excised from the plants and thin sections (cross and longitudinal) were made with a razor blade. Sections were mounted in water or 50% glycerol and examined with the previously mentioned microscopes. All inoculations were repeated to verify results.

Data analysis

All data from the pathogenicity and GFP quantification experiments were analysed with analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute Inc.). Data was pooled for analysis when variances between experiments were homogeneous. Least significant differences (Fisher's protected LSD) were calculated to compare means following significant *F*-tests.

Results

Generation and confirmation of transformants

Twenty putative transformants (with pCT74) were obtained from ND21 and 10 from ND30 (five with pCT74 and five with gGFP). Colonies appeared on the hygromycin-amended PDA selection medium in 7–12 days. Of these 30 putative transformants, eight displaying the greatest GFP fluorescence with the UV microscope were selected to test expression stability. Seven of those putative transformants retained stable fluorescence of GFP after 24 or more days on PDA: ND30-31, ND30-41, ND30-Y41, ND30-43, ND21-7, ND21-14 and ND21-17. These seven transformants were selected for further study. Of the seven transformants, only ND30-31 was created with the gGFP construct. There was no apparent visual difference in the fluorescence between ND30-31 and the other transformants. Although the colony colour of ND21 transformants was similar to that of the WT, these transformants had a greater density of mycelium in a zone just behind the leading edge of the colony on PDA than did the WT. Transformants of ND30 had colony morphology similar to the WT. Transformants of ND30, but not ND21, readily produced sclerotia on PDA; ND21 transformants produced sclerotia on autoclaved barley seeds. The medulla tissue in sclerotia also expressed GFP. Sclerotia of transformants were morphologically similar to those of the WT.

All seven selected transformants from ND30 and ND21 were tested for the presence of the *gfp* gene. PCR detected a 606-bp gene sequence from the *gfp* gene for all transformed isolates (data not shown). To determine copy number, relative quantification by the standard curve method was used in qPCR assays (Shou *et al.*, 2004). The correlation coefficients of the standard curves were more than 0.99. To account for variation in the starting quantities between samples, normalization was performed by dividing starting quantities of *gfp* in transformants by the starting quantities of the actin gene in samples. The copy number was calculated with relative amounts of DNA for each transformant divided by that of the calibrator ($r_{\text{transgene}}/r_{\text{calibrator}}$) (r = relative amount) (Shou *et al.*, 2004). The copy number of the calibrator ND21-14 was determined to be one according to a standard Southern blot analysis. Since the calibrated copy number of the other six transformants was equal to or less than the copy number of the calibrator, it was estimated that the transformants had a single integration of the *gfp* gene (Table 2).

Quantification of fluorescence

Fluorescence measurements of ND30-41, ND30-Y41, ND21-14 and ND21-17 mycelial plugs showed significantly ($P < 0.05$) higher fluorescence values than the WT in all three runs of the experiment (Table 3). Transformants ND30-31 and ND30-43 showed significantly ($P < 0.05$) higher fluorescence values than the WT in two experiments. Transformant ND21-7 showed a significantly ($P < 0.05$) higher fluorescence value in one experiment. Fluorescence measurements of PDA plugs were not different from those for the WT.

Transformant ND30-43 had significantly ($P < 0.05$) higher fluorescence of the protein extract than the WT and other transformants in both experiments (Table 4).

Table 2 Calculated starting quantities of transgene DNA (relative to that of endogenous gene) and estimated copy number of *gfp* gene in transformants of *Sclerotinia sclerotiorum*

Transformant	Relative amount of DNA ^a	Calculated copy number ^b	Estimated copy number
ND30-31	0.288	0.453	1
ND30-41	0.592	0.932	1
ND30-Y41	0.60	0.944	1
ND30-43	0.505	0.795	1
ND21-7	0.715	1.12	1
ND21-14	0.635	1	–
ND21-17 ^c	0.612	0.963	1

^aCalculated by dividing starting quantity of transgene by starting quantity of endogenous gene.

^bCalculated using relative amount of DNA for each transformant divided by that of calibrator.

^cThe calibrator ND21-14 had a single copy of the *gfp* gene according to a standard southern blot analysis. Accordingly, the calculated copy numbers of the other six transformants were based on ($r_{\text{transgene}}/r_{\text{calibrator}}$) (r = relative amount) (Shou *et al.*, 2004).

Table 3 Fluorescence of mycelium-colonized potato dextrose agar plugs of wild-type and *gfp*-transformed *Sclerotinia sclerotiorum*^a

Isolate/Transformant	Fluorescence values ^b		
	Experiment 1	Experiment 2	Experiment 3
ND 30 WT	43 442	42 176	48 913
ND30-31	46 527	55 106	64 324
ND30-41	67 914	55 343	88 879
ND30-Y41	77 213	61 647	60 157
ND30-43	55 102	61 636	74 416
LSD ^c ($P < 0.05$)	14 289	12 828	11 156
ND 21 WT	49 102	44 732	57 329
ND21-7	51 047	55 311	86 061
ND21-14	84 783	60 059	79 745
ND21-17	60 395	71 580	72 229
LSD ($P < 0.05$)	7 879	18 839	8 399

^a7.0-mm-diameter potato dextrose agar (PDA) plugs taken from the leading edge of actively growing wild-type (WT) and green-fluorescent-protein-transformed colonies (4 days old) were placed directly into individual wells of assay plates. Fluorescence was measured with a Synergy HT multi-detection microplate reader with 10 reads per well, at an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. Sensitivity levels were set at 97, 100 and 105 for experiments 1, 2 and 3, respectively. Fluorescence readings of the PDA control plugs for the three experiments were 46974, 34467 and 53969, respectively.

^bRelative fluorescence units. Values are the mean of four replications.

^cLeast significant difference for comparing within isolate ND30 or ND21.

However, the protein extract of the other three ND30 transformants did not show higher fluorescence than the WT. In contrast, all ND21 transformants had significantly ($P < 0.05$) higher fluorescence of the protein extract than the WT (Table 4). Fluorescence of the extract of transformant ND21-7 was two-fold higher than that of the WT.

Pathogenicity

All transformants were pathogenic (Fig. 1; Table 5) and initial lesion formation was visible 24 h after inoculation. All ND30 transformants on dry bean and canola caused lesions equal to those caused by the WT. In sunflower, two transformants, ND30-41 and ND30-43, consistently caused significantly ($P < 0.05$) shorter lesions than the WT over runs of the experiment. On soybean, transformant ND30-43 caused significantly ($P < 0.05$) shorter lesions than the WT and transformant ND30-31 caused significantly ($P < 0.05$) larger lesions. Lesion lengths caused by ND21 transformants on the four crops were not significantly ($P < 0.05$) different from those caused by the WT (Table 5).

Microscopy of the pathogen in host tissue

Tissues of petioles were examined at various times between 2 and 7 days following inoculation with ND30-31, ND30-41, ND21-7 and ND21-14. The pathogen

Table 4 Fluorescence of protein extracts from mycelium of wild-type and *gfp*-transformed *Sclerotinia sclerotiorum*^a

Isolate/Transformant	Fluorescence values ^b	
	Experiment 1	Experiment 2
ND30 WT	26 408	30 917
ND30-31	26 088	28 592
ND30-41	24 310	29 119
ND30-Y41	24 813	22 291
ND30-43	43 062	36 613
LSD ^c ($P < 0.05$)	4 149	4 758
ND21 WT	25 166	29 018
ND21-7	65 095	75 354
ND21-14	49 956	54 267
ND21-17	45 940	52 345
LSD ($P < 0.05$)	4 376	4 737

^aWild-type (WT) and transformants were grown for 10–14 days at 23°C on membrane filters placed on potato dextrose agar. Protein was extracted and fluorescence was measured on a Synergy HT multi-detection microplate reader with 10 reads per well, an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. Fluorescence readings of the extraction buffer for the two experiments were 16077 and 19598, respectively. Sensitivity levels for experiment 1 and 2 were 105 and 106, respectively.

^bRelative fluorescence units. Values are the mean of three replications.

^cLeast significant difference for comparing within isolate ND30 or ND21.

Table 5 Pathogenicity of *Sclerotinia sclerotiorum* wild-types and *gfp* transformants on stems of four crops^a

Isolate/Transformant	Lesion length ^b (cm)			
	Dry bean	Sunflower	Soybean	Canola
ND 30 WT	2.08	4.95	2.65	3.71
ND30-31	2.37	5.35	3.75	4.10
ND30-41	1.43	1.52	1.77	1.75
ND30-Y41	2.41	5.46	3.63	3.57
ND30-43	1.21	2.13	1.21	2.18
LSD ^c ($P < 0.05$)	NS	1.04	1.05	NS
ND 21 WT	1.67	3.16	2.11	2.38
ND21-7	1.66	3.91	2.38	2.33
ND21-14	1.81	3.75	2.51	2.57
ND21-17	1.86	2.71	2.18	2.76
LSD ($P < 0.05$)	NS	NS	NS	NS

^aPlants were grown in the greenhouse then 3 weeks after planting placed in a growth room at 23°C and inoculated. Pipette tips (1000 μ L; 200 μ L for canola) were pressed into the leading edge of mycelium of 4- to 5-day-old wild-type (WT) and transformant cultures grown on potato dextrose agar and then placed over the ends of freshly cut stems so the cut end was inserted into the agar.

^bLesion length was measured 4 days after inoculation. Values are the means of four replications averaged over two experiments.

^cLeast significant difference for comparing the means of individual isolates at ($P < 0.05$).

NS = not significant at $P \leq 0.05$ for the *F*-test of the ANOVA.

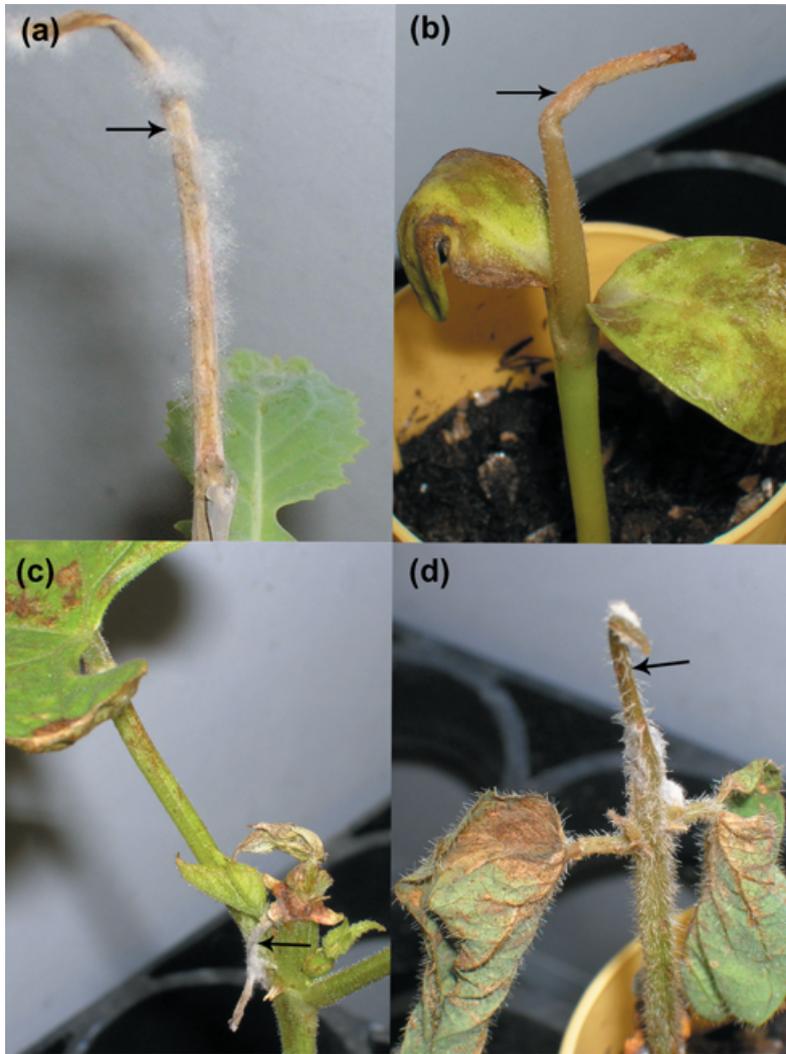


Figure 1 Lesion development (arrows) on stems of four field crops inoculated with *gfp* transformants of *Sclerotinia sclerotiorum* 4–6 days after inoculation. (a) ND30-31 on canola; (b) ND21-17 on sunflower; (c) ND21-14 on dry bean; (d) ND30-31 on soybean. White mycelium is visible on stems of canola, dry bean and soybean. Plants were grown in the greenhouse then 3 weeks after planting placed in a growth room at 23°C and inoculated.

directly penetrated tissue through the cut end of petioles, but also grew down the outside of the petiole. This allowed examination of infection through the epidermis. On petioles, lesions were visible within 1–2 days after inoculation. Within 4 days, broad lesions appeared along petioles of all the inoculated crops. Mycelia and individual hyphae were observed fluorescing on and in petiole tissue of sunflower, dry bean, soybean and canola (Fig. 2). The fluorescing hyphae were clearly detected in plant tissue and could be differentiated from plant cells. Microscopic observation of cross and longitudinal sections showed hyphae beginning to ramify through the cortex and parenchyma tissues 4–5 days following inoculation. Hyphal branches were observed penetrating the parenchyma cells and were seen running parallel to and around vessels (Fig. 2). No definitive penetration of xylem tissue could be confirmed. The extent of colonization varied greatly between hosts and samples, even when there were obvious lesions. When tissue samples were observed with standard light microscopy, it was difficult to observe the hyphae in host tissues and cells. Under UV light, however,

fluorescing hyphae were obvious. An important observation of these transformants was that not all the hyphal cells fluoresced in host tissue and the percentage of cells that fluoresced differed between transformants (Fig. 3). The two transformants of ND21, ND21-7 and ND21-14 appeared to have more fluorescing hyphae in host tissue than the two of ND30. The most intense fluorescence was usually associated with hyphal tip cells and adjacent cells (Fig. 3). Older hyphal cells were often highly vacuolated and GFP expression was negligible in these cells (Fig. 2).

Epidermal peels were also made to examine the transformants on the surface of tissues of all four crops. Penetration by the fungus through stomates and direct penetration through epidermal cells were observed (Fig. 3). Infection was usually initiated from swollen ends of hyphae by direct penetration of the epidermis wall. The penetration pegs were observed to branch with subsequent development of intracellular hyphae.

Autofluorescence of plant tissue occurred in these observations. The bases of trichomes strongly fluoresced and some vascular tissue also fluoresced green, but most

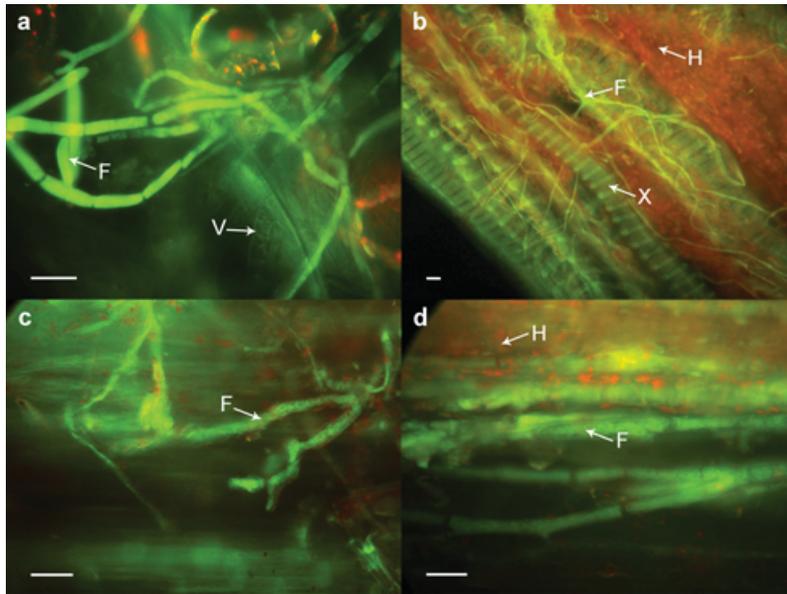


Figure 2 Longitudinal sections of *Sclerotinia sclerotiorum gfp* transformants colonizing petiole tissue of (a) sunflower, (b) dry bean, (c) soybean and (d) canola photographed with an epifluorescence microscope at 475 nm excitation and 510 nm emission 7 days after inoculation. In soybean the hyphae were visible in the host, but hyphae with low fluorescence were sometimes difficult to discern because of the autofluorescence of the host tissue. Older hyphae [V in section (a)] often had numerous vacuoles and did not fluoresce. F = fluorescing hyphae; V = older hypha with numerous vacuoles and no fluorescence; H = host tissue; X = xylem. Scale bar = 10 μ m.

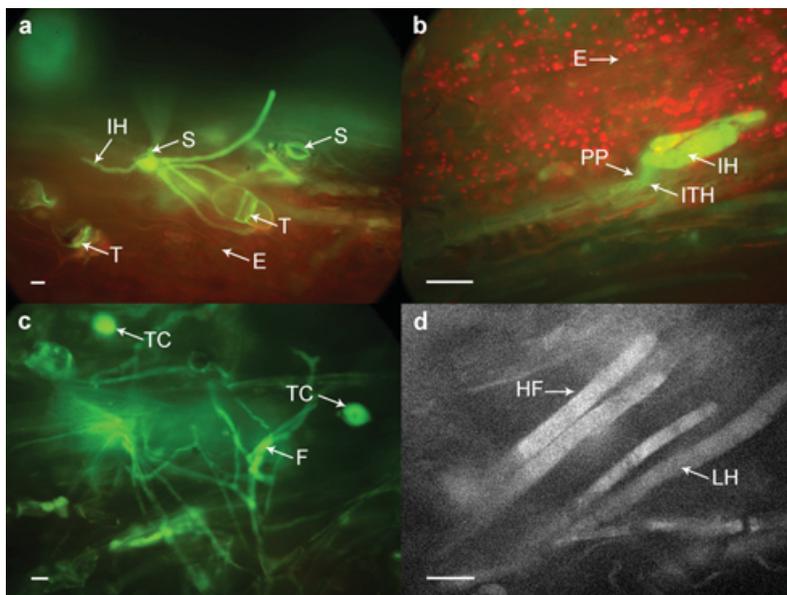


Figure 3 *Sclerotinia sclerotiorum gfp* transformants in petiole tissue of canola and sunflower photographed with an epifluorescence microscope at 475 nm excitation and 510 nm emission 7 days after inoculation. (a) Infection of canola by penetration of a stomate. Note fluorescing penetrating hypha. Red-brown area denotes host tissue. (b) Infection of canola by direct penetration of epidermis. Note fluorescing swollen hyphal tip cell of an infection hypha on surface of petiole giving rise to the penetration peg and the beginning of an intracellular hypha. (c) Longitudinal section of a colonized petiole tissue of sunflower. Bright round spots are tip cells of fluorescing hyphae perpendicular to the section, which always showed brighter fluorescence than other hyphal cells. (d) Monochrome image of a longitudinal section of infected sunflower petiole tissue photographed by Nikon E600 CARV Confocal cell imaging system using filters at 475 nm excitation and 510 nm emission. Note that fluorescence differs in brightness between hyphae. S = stomata; IH = infection hypha; T = trichome; E = epidermis; PP = penetration peg; ITH = intracellular hypha; TC = tip cells of fluorescing hyphae; F = fluorescing hyphae; HF = high-fluorescing hyphal tip; LH = low-fluorescing hypha. Scale bar = 10 μ m.

of the plant cells and tissues were brown to red under the UV conditions employed in this study (Fig. 3). When autofluorescence was strong it partially masked adjacent fluorescing hyphae. In infected soybean, however, petiole tissue fluoresced a light green, thus fluorescing hyphae, especially when not strongly fluorescent, were not as readily differentiated from host tissue.

Discussion

The necrotrophic pathogen *S. sclerotiorum* was successfully transformed using PEG-mediated transformation of protoplasts from hyphae with two different *gfp* vectors. All the transformants were pathogenic on four important crops. No other studies have shown the pathogenicity of *gfp* transformants of *S. sclerotiorum* on a variety of crops or measured GFP fluorescence in this pathogen. Somatic hyphae of transformants of *S. sclerotiorum* produced GFP *in vitro* and *in planta* in tissues of dry bean, canola, soybean and sunflower. Furthermore, GFP was also expressed in medulla tissue of sclerotia, as observed by Lorang *et al.* (2001).

Infection and establishment of fluorescing somatic hyphae in plant tissues were readily observed in all four crops. This pathogen is reported to produce infection cushions (Jamaux *et al.*, 1995), but for transformants and WT isolates in this study, the infecting structures on the surface of these hosts were swollen terminal cells of hyphae, as observed by Nelson (2005) in soybean and similar to those observed by Tariq & Jeffries (1984) in rapeseed and bean. GFP allowed a clear view of infection and establishment within epidermal cells without any special preparation of the tissue other than a wet mount. In the only other study to examine a *gfp*-transformed *S. sclerotiorum* on host tissue, Guimarães & Stotz (2004) inoculated leaves of *V. faba* (broad bean) to study the effect of the pathogen on stomatal guard cells. They observed fungal growth on the host surface and ramifying hyphae emerging through stomata from the inside of the leaf, but they did not observe the transformed pathogen within leaf tissue. In contrast to the findings in this study, they reported that initial penetration occurred via infection cushions (Guimarães & Stotz, 2004).

Of the four crops where infected petioles were examined, only soybean tissue autofluoresced and interfered with viewing transformants *in planta*. The autofluorescence sometimes made it difficult to clearly distinguish hyphal cells from host cells, especially when hyphal cells did not fluoresce brightly. This autofluorescence was not observed in non-inoculated petiole tissue. Why this occurred only in soybean and not in the other crops is unknown. Diseased tissue or phenolic compounds secreted as a defence response could be responsible for this tissue autofluorescence. Numerous studies suggest that phenols are a common component in the defence response and such molecules and their derivatives are thought to contribute to the discoloration and autofluorescence of host tissues at the site of infection (Nicholson & Hammerschmidt, 1992). Oxalic acid may also have contributed to autofluorescence

since it is considered to be important in pathogenicity of *S. sclerotiorum* (Cessana *et al.*, 2000; Bolton *et al.*, 2006) and is produced in advance of fungal hyphae (Guimarães & Stotz, 2004). This toxin may affect host cell membranes, possibly liberating compounds that fluoresce.

Transformants differed in the intensity of GFP production both *in vitro* and *in planta*. Although fluorescence was quantified *in vitro* and in the protein extract, it could not be quantified *in planta* since those observations were conducted visually and instruments to quantify UV intensity with the microscope were not available. To determine whether quantifying fluorescence is a useful technique to identify and select those transformants with the most intense fluorescence, measurements from mycelial plugs and total protein extracts were made on the Synergy HT microplate reader. Measurements from the mycelial plugs, a fast and easy technique, would be useful for preliminary screening, especially if there are many transformants. It could also be used to evaluate sectoring of GFP production in a colony. However, final selection of transformants used to examine the host-pathogen relationship should be based on visual observations with a UV microscope. Even though the Synergy HT microplate reader is a highly sensitive instrument, it cannot distinguish differences between transformants as well as visual observations of infected plant tissue through a UV microscope. For example, transformant ND21-7 always appeared to fluoresce more brightly than other transformants in plant tissue when examined by UV microscope, but the Synergy HT microplate reader did not indicate that the mycelium *in vitro* fluoresced more than several other transformants (Table 3). This may have been caused by the ability of ND21-7 to produce more GFP while in a pathogenic mode, resulting in greater fluorescence of hyphae in plant tissue compared to the other transformants. Transformant ND21-7 had the highest fluorescence values of the protein extracts from mycelium grown *in vitro*.

Measurements of the fluorescence of total protein extracts from *in vitro* hyphal growth appear to be less reliable for detecting and evaluating *gfp* transformants. Three out of four of the ND30 transformants could not be distinguished from the WT with this method, whereas the three ND21 transformants were clearly distinguished from the WT. Since all four ND30 transformants fluoresced and the WT did not, the reasons why the protein extracts of those transformants could not be differentiated from the WT are unclear. A likely explanation which is supported by the data shown in Table 4 is that ND30 transformants produce less fluorescent protein in hyphal cells than ND21 transformants. It is important to note that ND21 transformants always fluoresced brighter than ND30 transformants when viewed in UV light under the microscope.

An important observation in this study was that sectoring of GFP fluorescence occurred in stable transformants. These sectors varied from hyphae with weak fluorescence to strong fluorescence. This was only detectable by examining fluorescence of mycelium in several regions of the colonies using the UV microscope, since there were no

colony characteristics associated with these sectors. Lorang *et al.* (2001) also reported that sectoring in the intensity of GFP expression was observed in transformants of *S. sclerotiorum*. The reason for these sectors is probably associated with the multinucleate nature of hyphal cells. The multinucleate cells of *S. sclerotiorum* can vary in number of nuclei. During transformation, most likely only one or more nuclei in the original protoplast had the *gfp* gene inserted into the fungal genome. However, during the formation of new hyphal tip cells or branches, which includes mitosis and the migration of nuclei into the newly formed cells, these new hyphal cells could receive fewer or no transformed nuclei and the quantity of GFP and thus the intensity of fluorescence would be affected.

GFP transformants of *S. sclerotiorum* can be useful tools for studying the basic biology of diseases caused by this pathogen, since this study showed they were pathogenic on stems of four crops and GFP was expressed in the tissues of those crops. They may also be valuable for studying the relationships between this pathogen and other microorganisms in the environment.

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