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Use of green fluorescent protein to quantify the growth of *Colletotrichum* during infection of tobacco

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Abstract

To develop a quantitative assay of fungal growth inside plant tissues, strains of *Colletotrichum destructivum* and *Colletotrichum orbiculare* were transformed with a modified green fluorescent protein (GFP) gene fused with a glyceraldehyde-3-phosphate dehydrogenase promoter from *Aspergillus nidulans*. Transformants expressed GFP in culture and had the same growth rate and general appearance as the wild type. GFP was observed in all fungal structures during infection of leaves of *Nicotiana benthamiana*, except for the melanized appressoria and setae. The timing and appearance of the fungal structures in the host appeared to be identical to that of the wild type. GFP accumulation in inoculated leaves of *N. benthamiana* was quantified in leaf extracts using a fluorescence microplate reader, and the quantity of fluorescence was strongly correlated with the growth of the fungus as measured by the amount of fungal actin gene expression using Northern blot hybridizations. These results demonstrated that assaying green fluorescence levels from a GFP-transformed fungus is an accurate, fast and easy means of quantifying fungal growth inside host plant cells.

Keywords: Anthracnose; *Colletotrichum* disease; Green fluorescent protein (GFP); Detection; Hyphae; Infection

1. Introduction

Examination of disease progression by quantifying the amount of fungal biomass in infected host tissues is important in the study of fungal–plant interactions. An easily measured and distinctive visual marker of fungal growth would be useful for this purpose. A promising candidate is the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, that is characterized by its unique ability to emit green fluorescence upon excitation by UV or blue light (Cody et al., 1993). Other than its requirement for light and oxygen, GFP requires no other co-factors for visualization. Its fluorescence is species-independent and can be studied in living tissues without cell lysis or tissue distortion (Chiu et al., 1996). GFP fluorescence is also stable under diverse conditions, including temperatures up to 65 °C, a pH range of 3–12, and in inorganic solvents, such as 1% SDS, 8 M urea, glutaraldehyde or formaldehyde (Yang et al., 1996).

GFP has been expressed in a wide variety of organisms including bacteria, fungi, plants and animals (e.g., Chalfie et al., 1994; Casper and Holt,

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1996; Ludin et al., 1996; Spellig et al., 1996). Although the original GFP gene from *A. victoria* has been expressed in many organisms, it has been difficult to obtain detectable expression of this gene in filamentous fungi (Maor et al., 1998). However, a modified version of the *gfp* gene, *sgfp*, where Ser65 was replaced with a Thr (Chiu et al., 1996), has been successfully expressed at high levels in a number of filamentous fungi (Spellig et al., 1996; Maor et al., 1998; Dumas et al., 1999; Robinson and Sharon, 1999; Frietag et al., 2001; Sexton and Howlett, 2001; Horowitz et al., 2002). For example, *sgfp* expression in *Ustilago maydis*, a basidiomycete, resulted in fluorescence that was used to visualize the pathogen in vivo, and *sgfp* did not significantly interfere with fungal development during infection of maize (Spellig et al., 1996). For *Cochliobolus heterostrophus*, an ascomycete, the intense fluorescence of GFP provided a highly efficient marker to detect cytoplasmic and developmental changes in the fungus, and permitted the monitoring of fungal structures both on and inside inoculated maize leaves (Maor et al., 1998).

One advantage of using GFP as a marker is that its fluorescence intensity is directly proportional to the amount of GFP. Therefore, if the GFP gene is controlled by a constitutive promoter, then measuring the amount of GFP will provide a means of quantifying fungal growth. Although there was no attempt to extract and quantify GFP, Maor et al. (1998) noted that green fluorescence intensity was highly correlated with the amount of mycelium of a transformed strain of *C. heterostrophus*.

This work describes the transformation and expression of the *sgfp* gene in two fungal pathogens of tobacco (*Nicotiana* spp.), *Colletotrichum destructivum* and *Colletotrichum orbiculare*, that have differences in their intracellular hemibiotrophic infection processes, but both exhibit an initial biotrophic phase followed by a necrotrophic phase (Shen et al., 2001a,b). GFP was used to quantify the biomass of these fungi in infected *Nicotiana benthamiana* plants and to monitor their infection processes. For comparison, a more established method of fungal quantification was also used, which was the quantification of transcript levels of a constitutively expressed fungal actin gene by Northern blot hybridizations.

2. Materials and methods

2.1. Fungal and plant materials

Isolates *C. destructivum* N150 (Shen et al., 2001a) and *C. orbiculare* ATCC20767 (Shen et al., 2001b) were cultured on sodium chloride–yeast extract–sucrose agar medium (SYAS) (Mandanhar et al., 1986) and potato dextrose agar medium (PDA) (Difco, Detroit, MI, USA), respectively. Plates were incubated under continuous fluorescent lighting at 25 °C. Conidia were obtained from 7- to 10-day-old cultures for plant inoculations and fungal transformations. Cultures were stored at −70 °C in 18% (v/v) sterile glycerol. *N. benthamiana* plants were grown from seed in a growth room with 16 h light at 200 μmol s^{-1} m^{-2} and 8 h dark with 25:17 °C day/night temperatures.

2.2. Inoculation

Each plant at the five- to seven-leaf stage was inoculated by spraying a 300-μl suspension of 2×10^6 conidia ml^{-1} in sterile distilled water with an atomizer. Control plants were sprayed with sterile distilled water. The sprayed plants were then enclosed immediately in a loosely sealed, plastic-lined container with >95% relative humidity, and incubated at 25 °C under low light (15 μmol s^{-1} m^{-2}) for up to 7 days.

2.3. Fungal transformation

Fungal transformation was performed by electroporation of germinated conidia following Robinson and Sharon (1999) with the following modifications. Conidial germination was induced by incubating 15 ml of 5×10^5 conidia ml^{-1} in pea juice (Robinson and Sharon, 1999) in a 250-ml flask at 30 °C with shaking for 5 h for N150 and 10 h for ATCC20767, at which time 50% to 80% of the conidia had germinated. The final concentration of spores prior to electroporation was adjusted to 5×10^7 spores ml^{-1} in cold electroporation buffer. Prior to electroporation, gGFP (Maor et al., 1998) was linearized with the restriction enzyme, *Hin*dIII (New England Biolabs, Mississauga, ON, Canada) at 37 °C for 16 h in 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT, pH 7.9. A high-voltage electric pulse (Gene

Pulser, Bio-Rad Laboratories, Hercules, CA, USA) was selected at 1.5 kV, 600 W resistance and 25 mF capacitance resulting in time constants of 9–13 ms. *C. destructivum* N150 was incubated on regeneration medium at 27 °C for 24 h and then overlaid with 10 ml of regeneration medium with 1% agar containing 50 μg ml^{-1} hygromycin B (Life Technologies, Burlington, ON, Canada). However, for *C. orbiculare* ATCC20767, the regeneration plates were incubated at 27 °C for 15 h, and then overlaid with 10 ml of regeneration medium with 1% agar containing 70 μg ml^{-1} hygromycin B. After 6 to 10 days, colonies were examined by fluorescence microscopy, and green fluorescent colonies were transferred to PDA or SYAS plates not amended with hygromycin B.

2.4. Fluorescence microscopy and protein extraction

An Eclipse E600 fluorescence microscope (Nikon, Mississauga, ON, Canada) was used to view fungal colonies on media and in inoculated plants. The microscope has a mercury lamp with an excitation filter of 460–500 nm and a barrier filter of 510–560 nm. For quantification of GFP, conidia were washed from 7- to 10-day-old SYAS cultures or 2.8-cm-diameter leaf discs were excised and then placed in 1.5 ml microcentrifuge tubes. Soluble proteins were extracted by thoroughly grinding the leaf discs with a drill-powered pestle in 200 μl of pre-chilled extraction buffer (30 mM Tris–HCl, 10 mM EDTA pH 8, 10 mM NaCl, 5 mM DTT). The homogenate was centrifuged at 15000 × *g* for 15 min at 4 °C. An aliquot of supernatant (200 μl) was measured for green fluorescence using a Wallac Victor, 1420 Multilabel Counter with the lamp filter F485 nm and the emission filter F510 nm (Perkin-Elmer Life Sciences, St. Laurent, PQ). Each experiment was repeated at least twice. Data are presented as the mean of three replicates with standard errors, or linear regressions with regression coefficients and *p*-values.

2.5. DNA extraction

Isolation of the plasmid gGFP was done according to Sambrook et al. (1989). To obtain fungal genomic DNA, the fungus was grown on PDA overlaid with a cellophane sheet (Flexel, Atlanta, GA, USA) for 7 days. The mycelium was separated from the culture medium by removing the cellophane from the agar and grinding it to a fine powder in liquid nitrogen. Genomic DNA was then extracted using the method of Raeder and Broda (1985).

2.6. Actin gene amplification by PCR

A pair of degenerate oligonucleotide primers, ACTAP1 (sense) (5′-AGA(AG)GA(AG) GT(CT)GC (CT)GC(ACT)CT-3′) and ACTAP2 (antisense) (5′ TTAGAAGCACTTG CGGTG 3′), was designed based on conserved amino acid sequences (EEVAAL and XFCKRH) of *actA* from *C. gloeosporioides* f. sp. *malvae* (Jin et al., 1999) and several actin protein sequences from other fungal species. PCR was then performed using *C. destructivum* N150 genomic DNA as the template. The PCR reaction contained 10 ng of template DNA, 0.2 mM dNTP, 1.2 mM $MgCl_2$, 0.5 mM primers, 2.5 U *Tsg* DNA Polymerase (Biobasic, Toronto, ON) and 1 × *Tsg* buffer (50 mM KCl, 10 mM Tris–HCl pH 9.5 at 25 °C). The PCR amplification conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 67 °C for 1 min and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. A PCR product of 1327 bp was cloned into plasmid pUC57 (BioBasic, Markham, ON, Canada), and the resulting plasmid, designated pCDACT, was sequenced at the Guelph Molecular Supercentre (University of Guelph). Initial DNA sequence comparisons were performed with BLASTN of the GenBank nonredundant nucleotide sequence database to confirm whether the cloned DNA sequence was from the actin gene.

2.7. Northern blot hybridization

Total RNA was isolated following the method of Chen et al. (1999) with the following modifications. Leaf tissue (3 g) was homogenized in liquid nitrogen in a chilled mortar, and then 2.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1) and 2.5 ml of extraction buffer were added to the mortar and mixed thoroughly. The homogenate was transferred to a sterile 10 ml polypropylene tube and centrifuged at 12 000 × *g* for 10 min at 4 °C . The supernatant was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The RNA was then precipitated by adding (1/4) volume of 10 M LiCl to the supernatant and leaving the sample at 4 °C overnight. RNA was

harvested by centrifugation at 12 000 × *g* for 30 min at 4 °C, and the pellet was washed in 1 ml 70% ethanol and dried.

For Northern blot analysis, 10 μg of total RNA was separated by gel electrophoresis in a 1% formaldehyde agarose gel (0.4 g agarose, 34.8 ml DEPC-H_2O, 4 ml 10 × MOPS, 1.2 ml 37% formaldehyde) in 1 × MOPS buffer (Sambrook et al., 1989), and transferred onto positively charged, 0.45-μm pore size, microporous nylon 66 membranes (Roche Diagnostics, Laval, PQ, Canada). Northern blots were performed using the DIG System (Roche Diagnostics) following the manufacturer's instructions. The probe used for hybridization was the cloned actin gene from *C. destructivum* amplified using primers ACTAP1 and ACTAP2 as described above. The transcript amount was quantified by scanning the band images and quantifying the band intensities with the National Institute of Health Scion Image program (Scion, Frederick, MD, USA). Relative transcript levels were calculated by dividing the actin transcript band intensity by the corresponding amount of total RNA in each lane. Total RNA was quantified by image analysis of the ethidium bromide-stained RNA gel prior to blotting. Northern blot analysis was repeated twice.

3. Results

3.1. Generation of gfp transgenic C. destructivum and C. orbiculare

Nine hygromycin-resistant colonies of *C. destructivum* N150 and eight hygromycin-resistant colonies of *C. orbiculare* ATCC20767 were obtained after several rounds of electroporation with the plasmid, gGFP. Transformants with the strongest fluorescence, designated N150P3 and A20767P1, respectively, for *C. destructivum* and *C. orbiculare*, were selected. Only weak fluorescence was observed in the wild type controls. Southern blots with genomic DNA derived from N150P3, A20767P1, and their wild type strains showed the presence of *sgfp* only in the transformants (data not shown). The level of GFP fluorescence in the transformants was stable even when the transformants were cultured without hygromycin selection for many generations.

No visible changes in hyphae and colony morphology of N150P3 and A20767P1 were observed compared to the wild type strains, and the growth rates of N150P3 and A20767P1 on PDA and SYAS plates were similar to the corresponding wild type strains (data not shown). On *N. benthamiana* plants inoculated with N150P3 or A20767P1, light green or water-soaked symptoms were first visible by 72 or 96 h after inoculation, respectively. Over the next 48 h, the lesions expanded resulting in the death of the plants. The lesion type and timing of symptoms produced by the transformants appeared to be identical to that produced by the wild type strains. These results suggest that the *gfp* transformation did not visibly affect the cultural characteristics or pathogenicity of *C. destructivum* or *C. orbiculare*.

3.2. Quantification of fungal biomass in infected plants by green fluorescence intensity level versus Northern blot hybridization of a fungal actin gene

GFP quantification of conidia of A20767P1 showed that there was a strong linear relationship ($R^2 = 0.988$, $P = 0.0001$) between the $\log_{10}$ of relative fluorescence units (RFUs) and the $\log_{10}$ of number of conidia (Fig. 1). For plants inoculated with A20767P1, the RFUs extracted from leaves containing lesions increased slightly up to 96 h after inoculation. However, by 120 h after inoculation, the amount of RFUs had increased greatly (Fig. 2). From 0 to 96 h after inoculation, RFUs ranged from 3 to 5, but from 120 to 144 h after inoculation, plants showed the most severe symptoms,

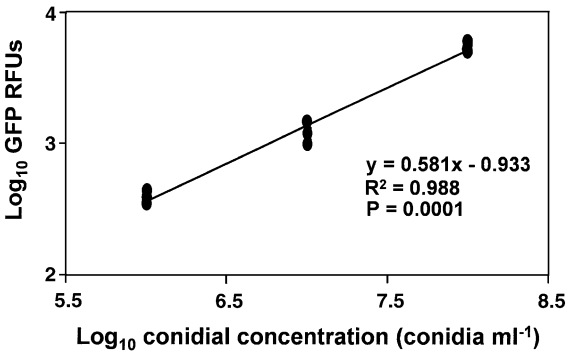

Fig. 1. Relative fluorescence units (RFUs) of GFP in conidial suspensions of *C. orbiculare* transgenic strain A20767P1. Three observations were made at each conidial concentration.

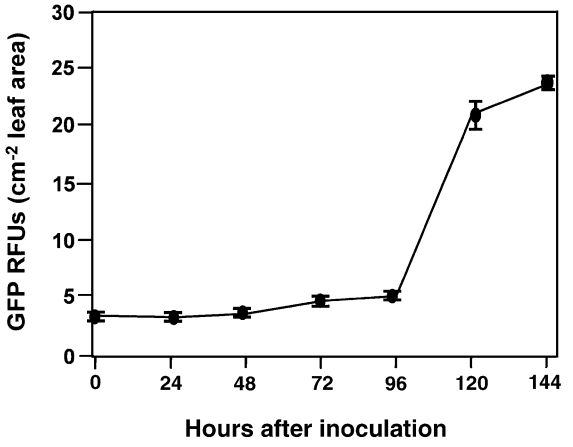

Fig. 2. Relative fluorescence units (RFUs) of GFP in leaves of *N. benthamiana* inoculated with transgenic *C. orbiculare* A20767P1. Fluorescence intensity was measured from non-inoculated leaf extracts at 0 h after inoculation or from inoculated leaf extracts at 24–144 h. Each point represents a mean of three observations from three plants and bars indicate standard errors.

and the RFUs had also increased to their highest values, between 20 to 25.

For plants infected with N150P3, the RFUs did not changed from 0 to 48 h after inoculation (2 RFUs), but then increased quickly by 72 h (10 RFUs) and 96 h (19 RFUs) after inoculation (Fig. 3). From 96 to 120 h after inoculation, the pathogen appeared to have heavily colonized the plant tissues, and the RFUs reached their maximum observed values (20 RFUs). This corresponded with the observation by microscopy of the greatest amounts of fungal biomass in the plants at that stage of the infection process.

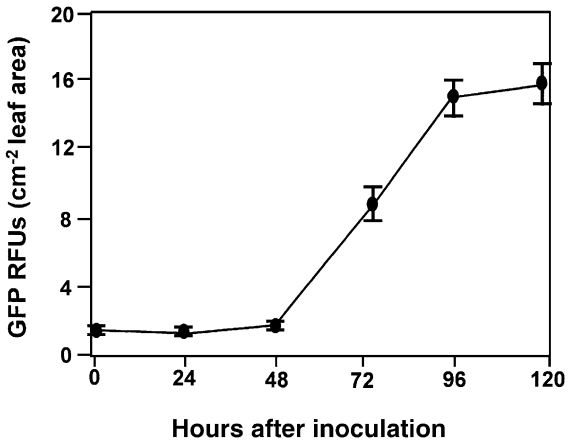

Fig. 3. Relative fluorescence units (RFUs) of GFP in leaves of *N. benthamiana* inoculated with transgenic *C. destructivum* N150P3. Fluorescence intensity was measured from non-inoculated leaf extracts at 0 h after inoculation or from inoculated leaf extracts at 24–120 h. Each point represents a mean of three observations from three plants and bars indicate standard errors.

To confirm the accuracy of the GFP measurements, a portion of the actin gene of *C. destructivum* was amplified and cloned. Sequence analysis of the cloned 1258-bp PCR product, designated *CDact*, (GenBank accession no. AY157843) showed that it shared 95% nucleotide identity and 99% amino acid identity with an actin gene, *actA* of *C. gloeosporioides* f. sp. *malvae* (Jin et al., 1999). Northern blots of RNA of plant tissues infected with N150P3 were probed with *CDact* and showed a pattern of expression (Fig. 4) similar to that of GFP (Fig. 3). Expression of *CDact* was first detected at a low level at 24 h after inoculation, and increased slowly until 48 h after inoculation, after

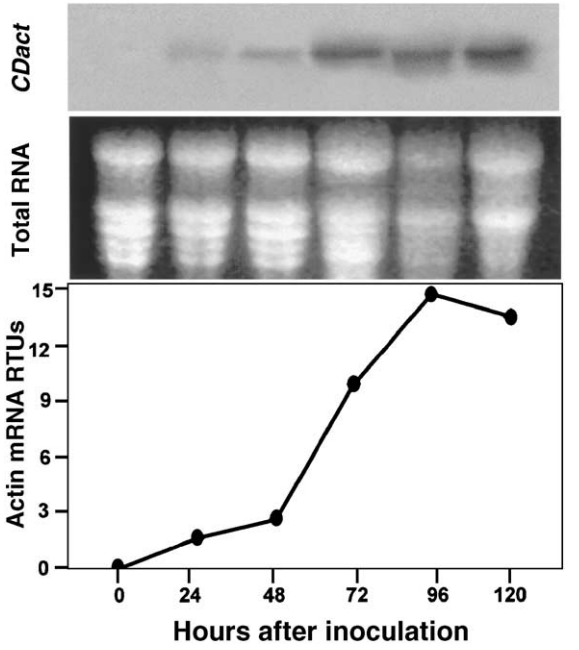

Fig. 4. Fungal biomass in infected tobacco indicated by relative transcript units (RTUs) of expression of the actin gene, *CDact*, from *C. destructivum* N150P3. Top panel, northern blot of total RNA extracted from non-inoculated leaves of *N. benthamiana* at 0 h after inoculation or from *C. destructivum* N150P3-inoculated leaves of *N. benthamiana* at 24–120 h. Middle panel, the ethidium bromide-stained gel before blotting. Bottom panel, quantification of the actin expression level normalized to the total amount of RNA. Two replicate northern blots showed the same pattern of expression.

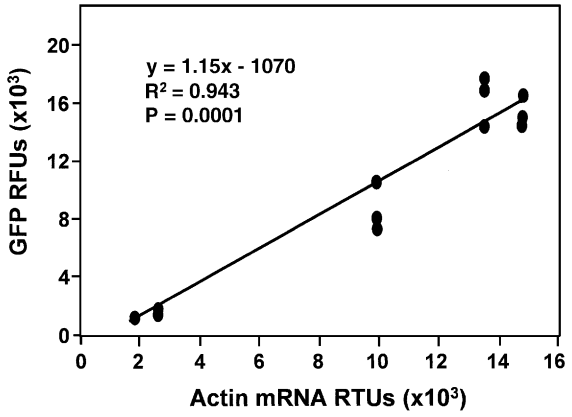

Fig. 5. Relationship between mRNA expression levels of the *C. destructivum* actin gene, *CDact*, and GFP fluorescence intensity in *N. benthamiana* plants infected with *C. destructivum* N150P3. Relative transcript levels of mRNA were obtained by dividing the band intensity in Northern blots of the actin gene by the combined band intensities of total RNA from the ethidium bromide-stained gel prior to blotting.

which there was a faster increase until 96 h after inoculation. The highest expression of *CDact* was observed at 96 h after inoculation. There was a strong linear relationship ($R^2 = 0.943$, $P = 0.0001$) between the GFP RFU levels and fungal actin gene expression during fungal growth in the plant (Fig. 5).

3.3. Microscopy of pathogen development in N. benthamiana infected with the transgenic fungi

No GFP fluorescence was detected in leaves infected with the wild type strain of *C. orbiculare*, A20767 (Fig. 6A). For plants infected with A20767P1, only large primary hyphae were observed in the penetrated epidermal cells up to 72 h after inoculation (Fig. 6B). This is the period of biotrophic infection (Shen et al., 2001b), and no leaf lesions were observed. The large primary hyphae continued to grow penetrating adjacent cells (Fig. 6C). Secondary hyphae was

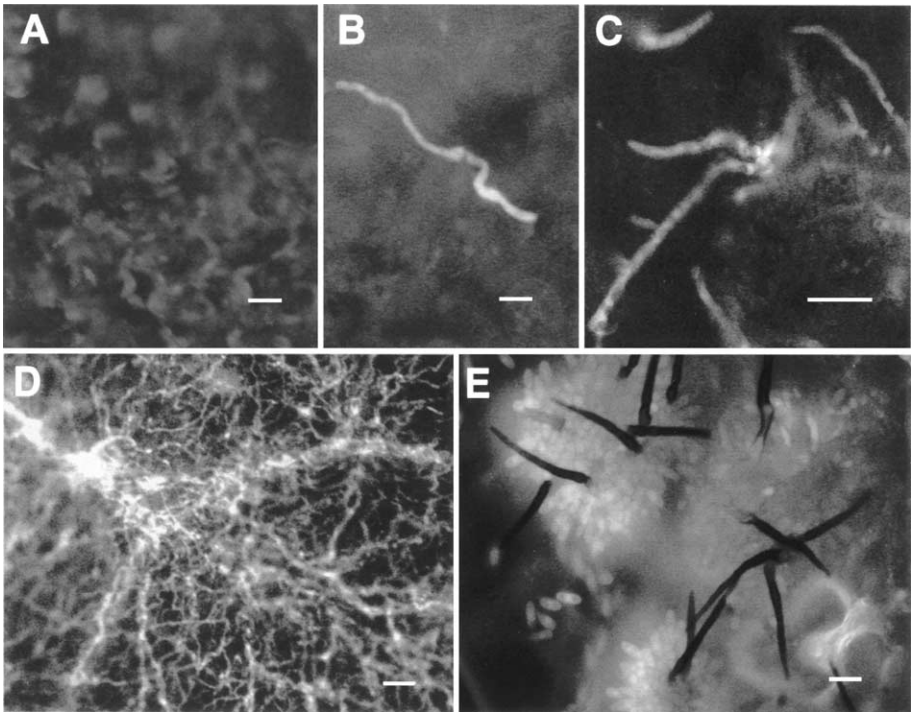

Fig. 6. Development of transgenic isolate *C. orbiculare* A20767P1 in infected *N. benthamiana* indicated by GFP. (A) Plant infected with wild type A20767 at 96 h after inoculation; (B to E) plants infected with transgenic *C. orbiculare* A20767P1. Primary hyphae at 72 h (B) and 96 h (C), respectively; (D) secondary hyphae at 120 h; (E) Acervulus at 144 h. Bar = 12.5 μm.

observed by 96 h after inoculation (Fig. 6D) corresponding with the appearance of lesions. Strongly fluorescent masses of conidia with melanized setae formed on the leaf surface at 120 h after inoculation (Fig. 6E).

No GFP fluorescence was detected in the leaves infected with the wild type strain, *C. destructivum* N150 (Fig. 7A). By 36 h after inoculation, fluorescent germinating conidia and melanized appressoria were detected on leaves infected with N150P3 (Fig. 7B). Fluorescent multi-lobed vesicles were visible in leaf epidermal cells by 48 h after inoculation (Fig. 7C), but no lesions were visible during this period, which is the biotrophic phase of infection (Shen et al., 2001a). The multi-lobed vesicles continued to grow and later began to produce thin secondary hyphae from the tips of the vesicles (Fig. 7D). After 72 h, widespread secondary hyphae of the transformant were visible (Fig. 7E), and the growth of the secondary hyphae corresponded with the development of necrotrophy and the appearance of lesions.

4. Discussion

Although quantification of plant pathogenic fungi in infected plants is important for the study of fungal–plant interactions, the methods available to achieve this have limitations. Chitin and ergosterol levels have often been used for fungal quantification. The former is a cell wall constituent (Manibhushanrao and Mani, 1993; Roche et al., 1993), and the latter is a predominant sterol component of many fungi (Gardner et al., 1993; Gretenkort and Ingram, 1993). However, both compounds exist in many fungal species, and therefore their measurement is not species specific. Also, the chitin assay is a colorimetric assay dependent on the detection of the chitin substrates, glucosamine and/or *N*-acetyglucosamine, both of which have been detected in uninfected plants, and thus the quantity of chitin may not be well correlated with fungal biomass (Schnüer, 1993), particularly at lower fungal biomass levels.

Fungal biomass has also been calculated based on determining the quantity of specific proteins or frag-

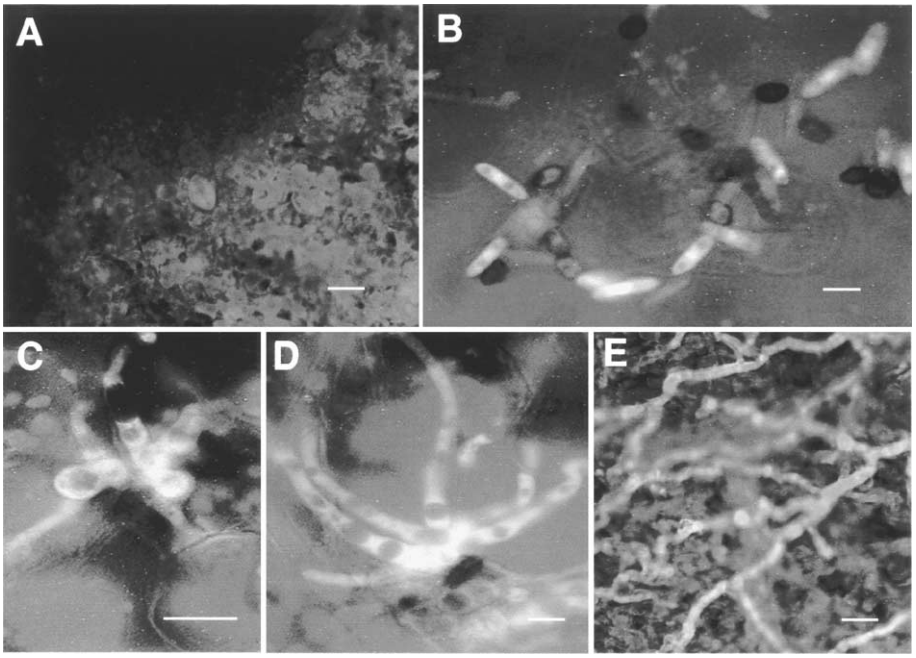

Fig. 7. Development of transgenic *C. destructivum* N150P3 in infected *N. benthamiana* revealed by GFP. (A) Leaf infected with wild type N150 at 72 h after inoculation; (B to E) plants infected with transgenic *C. destructivum* N150P3. (B) Conidia and appressoria at 36 h; (C) a multi-lobed vesicle at 48 h; (D) a multi-lobed vesicle with secondary hyphae growing from the tips of the vesicles at 60 h; (E) secondary hyphae at 72 h. Bar = 12.5 μm.

ments of DNA or RNA. For example, the quantity of a fungal actin protein can be measured by an enzyme-linked immunosorbent assay, but such an assay usually requires the production of monoclonal antibodies to ensure high specificity, which can be difficult and costly to obtain (Harrison et al., 1990; Newton and Reglinski, 1993). The amount of fungal DNA can be quantified by methods such as competitive PCR, in which the amount of PCR product from the target gene is determined relative to the amount of PCR product that was co-amplified from a defined quantity of competitor template (e.g., Mahuku et al., 1995; Turner et al., 2002). Although sensitive and accurate, competitive PCR can be time consuming to develop, and the number of amplification cycles has to be carefully calibrated to ensure that the final PCR products are not saturated. Another alternative for quantifying fungal growth is Northern blot analysis to measure the amount of RNA from a constitutively expressed fungal gene, such as the actin gene (e.g., Mahe et al., 1992; Jin et al., 1999). However, the disadvantages of using northern blot analysis are that the method usually takes several days to complete and requires the extraction of RNA, which can be vulnerable to degradation.

In this study, isolates *C. destructivum* N150 and *C. orbiculare* ATCC20767 were successfully transformed with *sgfp* and showed strong fluorescence under blue light. These two transgenic isolates exhibited stable expression of GFP after numerous successive transfers onto nutrient media without antibiotic selection and during growth in infected plants. Because GFP fluorescence intensity is known to be proportional to its protein content, and a promoter of a constitutively expressing gene was used to drive the *sgfp* gene, the amount of GFP could be used to estimate the relative amounts of fungal biomass at different stages after inoculation of the host. In this study, the *gpd* promoter from *Aspergillus nidulans* was used for the *sgfp* gene, and this promoter is known to provide high level, constitutive expression for genes expressed in a large number of ascomycete fungi (Maor et al., 1998). The validity of using GFP with the *gpd* promoter to quantify fungal growth was demonstrated in this study by the close agreement between the RFUs of green fluorescence and the level of expression of the constitutively expressed *C. destructivum* actin gene, *CDact*, as determined by Northern blot analysis.

Thus far, there are no reports of using GFP fluorescence to quantify fungal biomass directly in infected plants. Maor et al. (1998) found that the copy number of *sgfp* in *C. heterostrophus* was directly related to both the amount of GFP protein detected by Western blot analysis and the level of GFP fluorescence. Moreover, when the average fluorescence was measured by digital analysis of microscopic images of the *gfp*-transformed strain, there was a good correlation between fluorescence and the amount of mycelium observed under the microscope in both pure culture and inoculated leaves. This suggested that fungal biomass and disease levels could be determined by monitoring GFP levels in inoculated plant tissues. In our study, the biomass of the transgenic fungi in infected plants was quantified by directly measuring the amount of green fluorescence intensity in crude plant extracts using a fluorescence microplate reader, which is easier than fluorescence microscopy imaging analysis. The GFP extraction method described in this work is simple to perform, and hundreds of samples can be quickly assessed with a fluorescence microplate reader. GFP was also stable for several hours in extracted protein solutions, and the results of fluorescence quantification from infected tissue were highly reproducible.

The accumulation of GFP and expression of the actin gene of *C. destructivum* N150P3 in infected tobacco leaves showed similar patterns as that of actin gene expression for *C. orbiculare* ATCC20767 (syn. *C. gloeosporioides* f. sp. *malvae*) during infection of round-leaved mallow (Jin et al., 1999). In infected mallow tissue, the levels of actin mRNA of *C. gloeosporioides* f. sp. *malvae* also revealed two periods of fungal growth, a slower period of increase corresponding with the biotrophic phase of the infection, followed by a greater increase in actin expression as the necrotrophic phase developed. The changes in actin gene expression for hemibiotrophic *Colletotrichum* spp. during infection of different hosts appear to correspond with the conversion from relatively slower growing infection vesicles or intracellular primary hyphae in the biotrophic phase to comparatively faster growing narrow secondary hyphae in the necrotrophic phase (Mahe et al., 1992; Jin et al., 1999).

A comparison of the changes in green fluorescence levels in tobacco plants infected with the GFP-transformed strains of *C. destructivum* and *C. orbiculare* revealed differences in the time of the shift in the fungal growth rate during infection. For *C. orbiculare*, the timing of the shift to a faster rate of GFP accumulation occurred later than that of *C. destructivum* in infected leaves, which was related to difference in the timing of the shift from biotrophic to necrotrophic growth and the timing of symptom appearance between these two fungi, which has previously been observed by microscopy (Shen et al., 2001a,b).

The GFP-transformed strains of both *C. destructivum* and *C. orbiculare* exhibited a normal phenotype in culture and during infection, indicating that the accumulation of GFP did not alter the morphology or pathogenicity of these fungi. Thus, it appears that GFP is a suitable vital marker for *C. destructivum* and *C. orbiculare*. As previously reported for other fungi (Maor et al., 1998; Dumas et al., 1999; Robinson and Sharon, 1999), GFP accumulated in the fungal cytoplasm serving as a vital stain, and the amount of green fluorescence of the transformants was sufficient to be visualized under fluorescent microscopy for the different cell types of *C. destructivum* and *C. orbiculare*, except for melanized appressoria and setae, which appeared to have blocked the emission of fluorescence. GFP detection under a fluorescent microscope was a relatively easy approach to observe these fungi, requiring no reagents or treatments, such as fixation and sectioning, thus reducing the chances of producing artifacts.

A limitation of using GFP is the need to produce a transgenic strain for each fungus to be studied. However, this may prove to be less of a limitation as alternative transformation methods, such as electroporation, should make fungal transformations simpler and quicker than traditional approaches (Robinson and Sharon, 1999). Thus far, for *Colletotrichum* spp., *sgfp* has also been expressed in *C. lindemuthianum, C. gloeosporioides* f. sp. *aeschynomene*, and *C. acutatum* (Dumas et al., 1999; Robinson and Sharon, 1999; Horowitz et al., 2002). Expression of *sgfp* had no observable effect on the morphology or pathogenicity of these fungi and provided an efficient marker for monitoring fungal colonization of susceptible and symptomless host plants.

This research has shown that GFP fluorescence can be used as a reporter of fungal biomass as well as serving as a vital stain for monitoring fungi by fluorescence microscopy. These result help demonstrate some of the benefits of using *gfp*-transformed fungi in studying pathogen–plant interactions.

Acknowledgements

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