CSIRO PUBLISHING

www.publish.csiro.au/journals/app *Australasian Plant Pathology*, 2004, **33**, 69–75

Transformation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of Fusarium wilt of banana, with the green fluorescent protein (GFP) gene

Marinda Visser[A,D], *Thomas R. Gordon*[C], *Brenda D. Wingfield*[B], *Michael J. Wingfield*[A] *and Altus Viljoen*[A]

[A]Department of Microbiology, Plant Pathology and [B]Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.
[C]Department of Plant Pathology, University of California, Davis, California 95616, USA.
[D]Corresponding author; email: marinda.visser@fabi.up.ac.za

Abstract. *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is the causal agent of Fusarium wilt (Panama disease) of bananas in most tropical and subtropical banana-producing regions of the world. The fungus infects through roots, colonises the rhizomes and eventually blocks the vascular system of the pseudostems, resulting in plant death. The green fluorescent protein (GFP) emits green fluorescence when excited by blue light, making it a useful tool to study early stages of fungal infection. The objective of this study was to transform *Foc* isolates with the GFP gene. Isolates representing 'subtropical' race 4 of the fungus were transformed with the *sGFP* derivative using hygromycin as a selectable marker. Efficiency and transformation of spheroplasts depended on mycelium age, the choice of enzymes and the temperature and duration of incubation. The transformed isolates did not differ markedly from the wild type isolates in growth and morphological characteristics *in vitro*. Fluorescence microscopy showed expression of the green fluorescent protein in fungal structures. The presence of the GFP DNA in the fungal cells was confirmed by PCR using a GFP-specific primer pair and Southern blot analysis. Pathogenicity tests showed that the transformation process did not alter pathogenicity of *Foc* isolates. Fungal hyphae within tissues of infected plants could be seen to fluoresce and the transformed fungus was re-isolated from artificially inoculated plants. Transformants of *Foc* will facilitate future infection studies with this pathogen on banana.

Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been developed as a reporter for gene expression (Prasher 1992; Chalfie *et al*. 1994; Cubitt *et al*. 1995; Niedenthal *et al*. 1996; Spellig *et al*. 1996; Tsien 1998), a tracer for studying cellular dynamics of filamentous fungi (Suelmann and Fischer 1997; Suelmann and Fischer 2000) and a label to follow development of pathogens within their plant hosts (Sheen *et al*. 1995; Chalfie and Kain 1998; Bottin *et al*. 1999). GFP was first expressed in *Escherichia coli* and *Caenorhabditis elegans* (Chalfie *et al*. 1994).

Expression of the GFP has subsequently been successful in plants (Haseloff and Amos 1995; Sheen *et al*. 1995; Chiu *et al*. 1996) mammals (Pines 1995) and yeasts (Cormack *et al*. 1997). The GFP has also been expressed in numerous filamentous fungi such as *Ustilago maydis* (Spellig *et al*. 1996), *Podospora anserina* (Berteaux-Lecellier *et al*. 1998), *Magnaporthe grisea* (Kershaw *et al*. 1998), *Cochliobolus heterostrophus* (Maor *et al*. 1998), *Mycosphaerella graminicola* (Skinner *et al*. 1998), *Colletotrichum lindemuthianum* (Dumas *et al*. 1999), *Phytophthora parasitica* (Bottin *et al*. 1999), *Aspergillus niger* (Du *et al*. 1999) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Lagopodi *et al*. 2002).

The formation of the fluorescent chromophore is apparently not species dependent and this has resulted in success with expression in several heterologous systems. It requires only ultraviolet (UV) or blue light and oxygen to fluoresce. The detection of GFP is non-invasive and non-destructive. There is no need for staining with special dyes or fixing of the sample using heat. However, successful expression of GFP in filamentous fungi requires a GFP derivative that is efficiently translated in fungi, the development of a transformation system in the target species, and a strong fungal promoter that drives strong constitutive expression of the GFP (Lorang *et al*. 2001). The *sGFP*

 10.1071/AP03084 0815-3191/04/010069

derivative has been shown to be efficiently translated and expressed in most filamentous fungi tested (Lorang *et al*. 2001).

The fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*) causes a highly destructive vascular wilt disease of banana plants (Stover 1972). *Foc* is a soilborne fungus that infects banana plants through their roots, then colonises the rhizomes and eventually blocks the vascular vessels of the pseudostems (Stover 1972).

The objectives of this study were to transform isolates of *Foc* with a plasmid harbouring the s*GFP* derivative, then determine whether the transformed isolates retained their integrity and pathogenicity, and finally confirm the expression of the GFP in infected plant material.

Methods

Fungal isolates and culture conditions

Five virulent 'subtropical' race 4 isolates of *Foc* were selected for transformation with the GFP. These included isolates from South Africa (*Foc* 1, 6, 42), Taiwan (*Foc* 28), and Australia (*Foc* 46). All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Preparation of fungal spheroplasts

Spheroplasts of *Foc* were prepared using the method described by Vollmer and Yanofsky (1986) with minor modifications. Isolates were grown on potato-dextrose agar (PDA) and the aerial mycelium was harvested after 9–10 days. Conidial suspensions (3.5×10^9 conidia/mL) were prepared and inoculated into 500 mL potato-dextrose broth (PDB) in 2 L Erlenmeyer flasks and placed on a shaker at 15.5 rcf for 18 h. Cultures were grown overnight, vacuum filtered and then washed with 100 mL of 0.6 M $MgSO_4.7\ H_2O$ solution. Fungal mycelium (1.5 g) was transferred to pre-weighed sterile 50 mL disposable centrifuge tubes and placed on ice. The cells were resuspended in osmotic medium (OM) (0.98 M $MgSO_4$, 8.4 mM Na_2HPO_4, 1.6 mM NaH_2PO_4; 10 mL/g mycelium) and the suspension was transferred to a sterile 250 mL flask on ice.

To ensure complete digestion of fungal cell walls, a modification of the enzyme mixture described by Churchill *et al*. (1990) was used. The enzyme mixture (2 mL/g mycelium) consisted of lysing enzyme (0.15 mg/mL) (Novo Industries 7367155), chitinase (10.4 mg/mL) (Fluka) and β-D-gluconase (21.6 mg/mL) (Sigma). After adding 9 mL of enzyme mixture to each flask, helicase (Sigma) was added (0.3 mL/g mycelium), mixed gently, and incubated on ice for 5 min. Bovine serum albumin (BSA, Sigma) (0.75 mL/g mycelium) was added and the mixture shaken at 6.7 rcf at 30°C for 3–4 h. The yield of spheroplasts was checked microscopically to ensure complete digestion of the cell walls.

Aliquots (5 mL) of the spheroplast solution were mixed with 5 mL of OM. This suspension was carefully overlaid with 10 mL of trapping buffer (0.4 M sorbitol in 100 mM Tris-HCl, pH 7) and centrifuged for 15 min at 3824 rcf, 4°C to produce two separate phases. The spheroplasts were removed and pooled, placed on ice, and diluted with 2 volumes of 1 M sorbitol. The spheroplast solution was centrifuged in a bench top centrifuge at 2655 rcf at 4°C for 5 min. The pellet was washed with 500 µL of ice-cold sorbitol and centrifuged at 2655 rcf for 5 min at 4°C. The pellet was resuspended in 500 µL STC [1 M sorbitol, 50 mM Tris HCl (pH 8), 50 mM $CaCl_2$] and centrifuged at 2151 rcf for 5 min at 4°C. The spheroplasts (at 3×10^8 cells/mL) were finally resuspended in a spheroplast storage buffer containing four parts STC and one part 60% polyethylene glycol (PEG) 6000 and a 1% (v/v) dimethylsulfoxide (DMSO). Cells were directly used or frozen in vials at –80°C for later use.

Transformation of fungal spheroplasts

A plasmid construct harbouring the s*GFP* derivative was used to express GFP in *Foc*. The construct also contained a hygromycin-B resistance gene, which was used as a selective marker by culturing transformants on a medium containing the antibiotic. The transformation vector (pCT74), which expresses s*GFP* from the ToxA promoter of *Pyrenophora tritici-repentis*, was obtained from LM Ciuffetti (Freitag *et al*. 2001; Lorang *et al*. 2001).

For the transformation, spheroplasts ($2–3 \times 10^8$/mL in 100 µL) were mixed with 20 µL (0.1–1 µg) plasmid, suspended in 5 µL distilled water, and 25 µL of 2 × STC in a 15 mL screw cap tube. This was followed by the addition of 25 µL 60% PEG 4000 with gentle mixing and incubation at room temperature for 20 min. An additional 1.2 mL of 60% PEG 4000 was then added, mixed gently and the mixture incubated for exactly 5 min at room temperature. Cells clumped together at this stage. Four millilitres of 1 × STC was added and mixed.

Transformed spheroplasts were plated using 500 µL of protoplast suspension per 20 mL of molten regeneration agar pre-cooled to 50°C. The regeneration medium was prepared by dissolving 24 g PDB and 9 g agar in 400 mL distilled water. In a separate bottle, 273.84 g of sucrose was dissolved in 600 mL distilled water. The contents of both bottles were then autoclaved and mixed while the temperature was still above 50°C. The plates were incubated right side up, overnight. After 16–18 h, each plate was overlaid with 10–12 mL of 1% water agar containing hygromycin-B at a concentration of 150 µg/mL. A control plate was included which was not overlaid with hygromycin-B. This was done in order to check the viability of the spheroplasts. After the overlaid agar solidified, plates were incubated right side up at room temperature. Transformed isolates grew through the overlay in the presence of hygromycin-B within 2–7 days.

Morphological and cultural characteristics

Transformants were transferred to water agar, and PDA (supplemented with hygromycin-B) to examine the morphology and cultural characteristics of *Foc* transformants. Non-transformed cultures of the same isolates were used as controls. Slide preparations using a drop of sterile water and a strand of hyphae were studied under the microscope using white and UV light.

Detection of the GFP gene using GFP specific PCR primers

Transformed isolates were grown on PDA plates containing hygromycin-B for 7–10 days. Conidia from actively growing cultures on PDA were used to inoculate PDB medium in a total volume of 100 mL in 250 mL flasks. Fungal isolates were grown in PDB in still culture for 10–14 days, harvested and freeze dried. Mycelial masses were ground to a fine powder in liquid nitrogen. Total DNA from each isolate was extracted using the phenol/chloroform-based extraction method described by Raeder and Broda (1985).

Specific primers designed by Lorang *et al*. (2001) were used to confirm the presence of the GFP in *Foc*. The 25 µL PCR reaction cocktail contained 0.4 mM of each deoxynucleoside triphosphates (dNTPs), 10 × PCR buffer, 10 pMol of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), and 2 ng of DNA. Both diluted (1:10) and undiluted DNA templates were used in the optimisation of the PCR. The GFP-specific primers sequences were GFP1 5′ TAG TGG ACT GAT TGG AAT GCA TGG AGG AGT; GFP2 5′ GAT AGA ACC CAT GGC CTA TAT TCA TTC. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited). Reaction conditions were initial denaturation at 96°C for 2 min, denaturation at 94°C for 30 s, primer annealing at 58°C for 45 s, elongation at 72°C for 45 s, for 30 cycles, and a final extension

72°C for 7 min. Amplified product was resolved on a 1.5% agarose gel in Tris Acetic acid EDTA (TAE) (242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA at pH 8.0) and stained in ethidium bromide and visualised under UV illumination. Sizes of the PCR fragments were estimated using a molecular weight standard (100 bp ladder, Promega).

Confirmation of integration of GFP using Southern blots

The method described by Sambrook *et al*. (1989) was used for Southern blot analysis. Total isolated DNA was digested with *Eco*RI and fragments were separated on a 1% agarose gel in 1 × TAE buffer and blotted onto a positively charged nylon membrane (Roche Molecular Biochemicals). For probe labelling, the Gene Image random prime labelling kit was used (Amersham Life Science). Labelled probes (GFP PCR amplified product) were allowed to hybridise to blotted DNA. This was followed by different stringency washes and incubation in a liquid blocking solution as recommended by the supplier of the probe. Membranes were then incubated with a 500-fold diluted anti-fluorescein-AP conjugate to obtain a fluorescence signal. After washing, fluorescence signals on the membrane were detected using a Gene Images CDP-Star detection kit (Amersham Life Science), which was followed by exposure to an X-ray film.

Pathogenicity tests

Five transformed isolates of *Foc* were used to test whether pathogenicity to bananas had been retained in these cultures. Transformants were grown on half-strength PDA supplemented with hygromycin-B. Conidia were harvested after 5 days and inoculated into Armstrong's *Fusarium* medium (Booth 1971) to enhance sporulation. After 5 days, the conidial suspensions were passed through sterile cheesecloth to separate the mycelium from the conidia. The conidial suspension was adjusted to a final concentration of 5×10^6 conidia/mL.

Tissue culture-derived Cavendish banana plants were inoculated with the transformed isolates. A hydroponics system where plants were grown in 250 mL plastic cups in sterile distilled water for 7 days was used. The plants were fertilised with a hydroponic mixture of 0.6 g/L $Ca(NO_3)_2H_2O$, 0.9 g/L Agrasol'O and 3 g/L Micromax. For inoculation, the plants were removed from plastic cups, the roots slightly wounded by crushing gently and the conidial suspension added to the medium to obtain a final concentration of 5×10^5 conidia/mL. Plants were returned to the cups and maintained in a greenhouse at 25°C. Five plants per inoculum were used for the pathogenicity trial. Control plants were treated in the same manner and maintained in sterile water. Disease severity was evaluated 6 weeks after addition of the conidial suspension. Plants were cut just above the roots and internal symptoms rated, using a standardised disease rating scale (Orjeda 1998) for Fusarium wilt of banana.

To determine whether banana tissue had been colonised with transformed *Foc*, thin sections (8–12 μm) of the rhizome and pseudostem were made using a microtome. Sections were prepared, mounted on slides in sterile distilled water and observed under white and UV light using fluorescence microscopy (Zeiss, Mannheim, Germany) equipped with filter blocks (Nikon standard fluorescence filter cubes 78648) with spectral properties matching those of GFP 480 nm excitation, 515 nm emissions. Images were captured with an AxioCam HR camera (Carl Zeiss Ltd, Mannheim, Germany) and processed with Corel DRAW-Version 10.0 software (Corel Corporation Ltd Ontario, Canada).

Results

Preparation of spheroplasts and the transformation process

Approximately 4×10^7 uninucleate spheroplasts were generated for each of the *Foc* isolates used in this study. Mycelial age had a significant effect on the frequency of spheroplast regeneration. Younger mycelial networks resulted in positive and higher frequency of spheroplast regeneration. The type of osmotic stabiliser (sucrose, NaCl and KCl) used in the regeneration medium also influenced the regeneration of spheroplasts. Sucrose was found to result in the optimal regeneration of spheroplasts.

One hundred hygromycin-B-resistant transformants were obtained after the transformation procedure. Sixty percent of transformants were stable when transferred to fresh hygromycin-containing medium. Thus, the efficiency of the transformation process with the plasmid containing the transformation vector (pCT74) that expresses *sGFP* from the *ToxA* promoter of *P. tritici-repentis* was sufficient for the purposes of this study.

Morphological and cultural characteristics

Strong constitutive expression of *sGFP* occurred and could be visualised in fungal hyphae, microconidia and macroconidia as well as on the dying vascular tissue, pseudostem and rhizome (Fig. 1). All transformants generated from spheroplasts appeared bright green and fluoresced uniformly. No morphological changes in size and shape of vegetative structures were observed. Transformed and wild-type isolates varied slightly in mycelial growth but not in sporulation and colony appearance. Transformed strains retained the colony morphology typical of the wild type, including cottony growth of aerial mycelium and pink pigmentation (Fig. 2).

Detection of the GFP gene using GFP-specific PCR primers

A 417 base pair PCR product was consistently amplified using GFP-specific primers and the genomic DNA (diluted or undiluted) of transformed isolates as template. A primer annealing temperature of 58°C was optimal for the PCR conditions. All untransformed isolates were consistently negative after the amplification reaction (Fig. 3).

Confirmation of the detection of GFP using Southern blots

The presence of the GFP gene in the fungal genome was confirmed with Southern blot analysis. Successful hybridisation occurred using the 417 bp PCR product as a probe against the DNA of the transformed isolates. The gene was not detected in the negative control (Fig. 4).

Inoculation experiments

In the pathogenicity tests, all *Foc* GFP transformants were as pathogenic as the isolates from which they were derived. After 7–14 days, wilt symptoms became visible on banana plants inoculated with both transformed and wild-type isolates of *Foc*. Severe yellowing and subsequent necrosis of the roots appeared 6 weeks after inoculation (Fig. 5). Characteristic internal symptoms were reddish to dark brown discoloration of infected roots and the vascular tissue. All

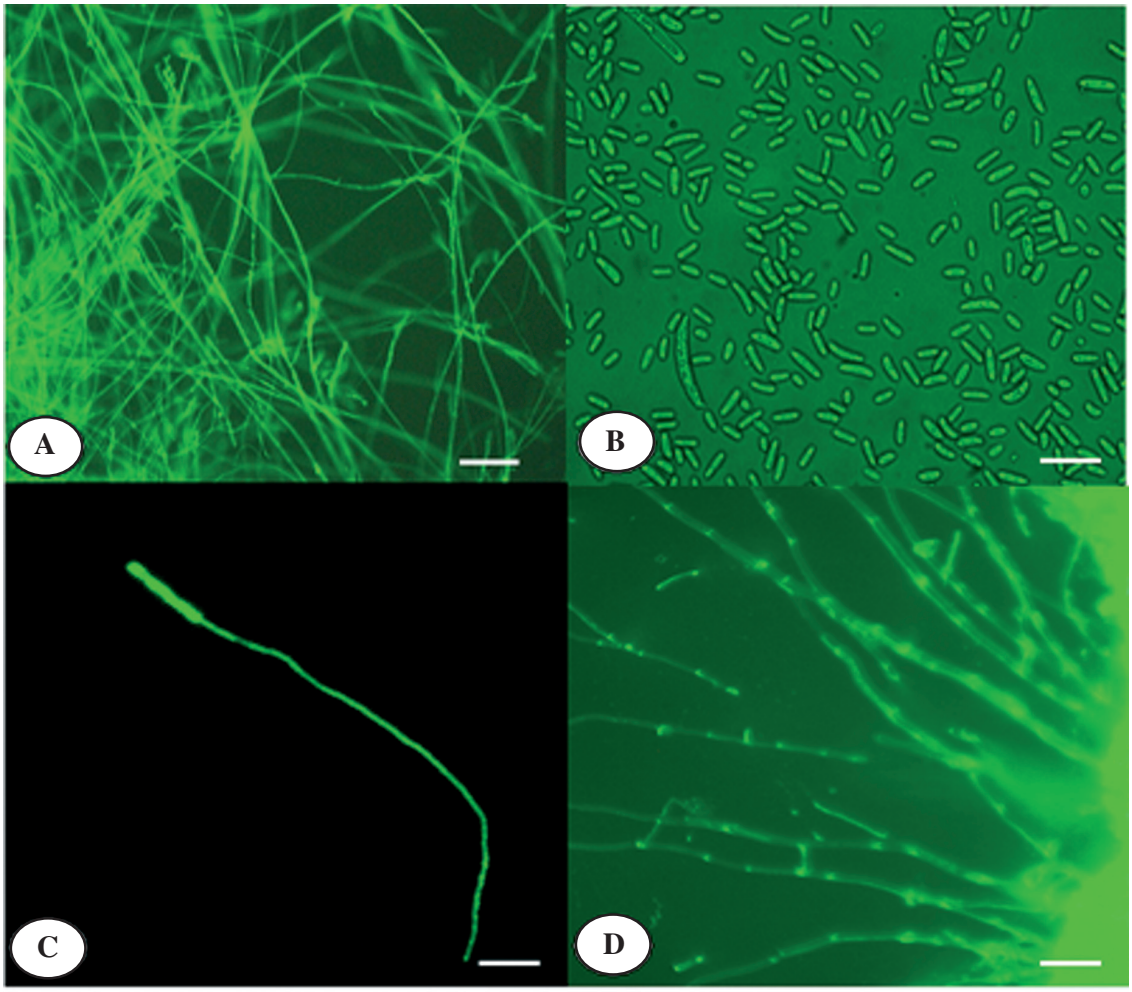

Fig. 1. Structures of transformed isolates of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) fluorescing bright green. (*A*) Fluorescing hyphal mass. (*B*) Typical size and shape of microconidia and macroconidia of *Foc*. (*C*) A germinating macroconidium on a glass slide confirming viability of the fungus. (*D*) Cross section of banana pseudostem showing network of hyphae of *Foc* through the plant tissue. (Scale bar: 10 mm = 24 μm.)

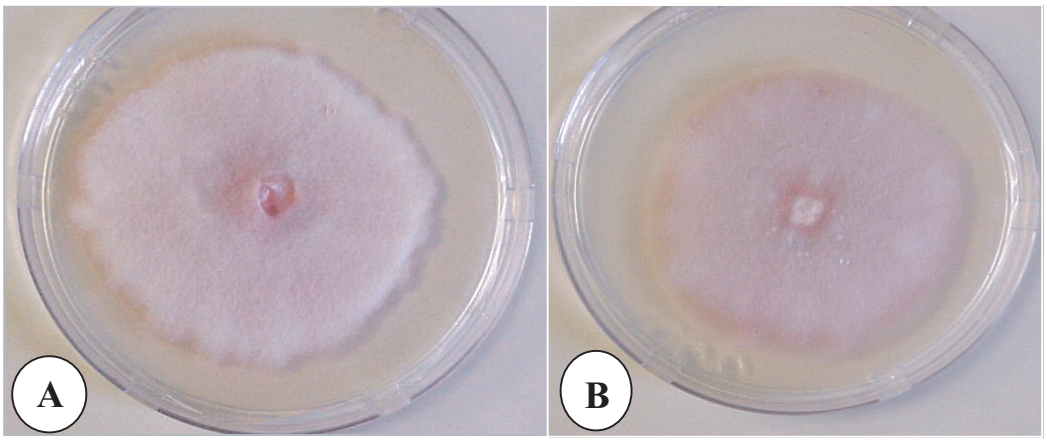

Fig. 2. Cultural characteristics of transformed and wild-type isolates of *Fusarium oxysporum* f. sp. *cubense*. (*A*) Typical cottony growth of wild type. (*B*) Typical cottony growth of transformed isolate.

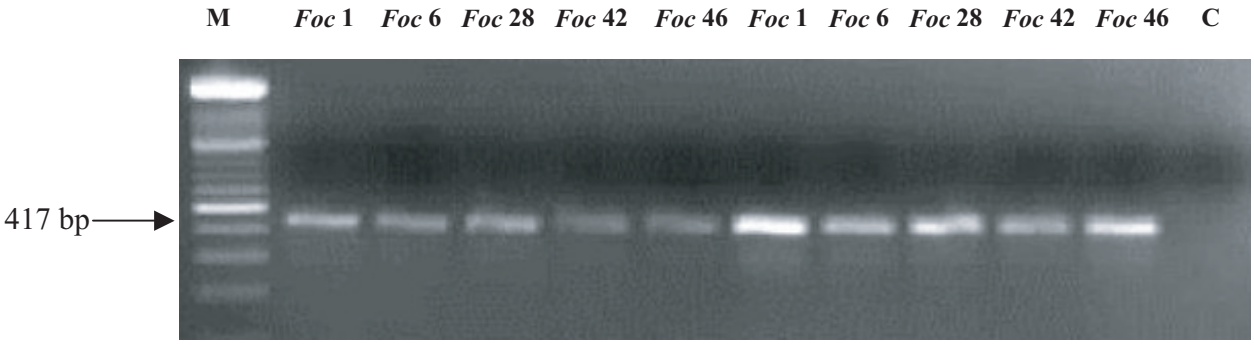

Fig. 3. Confirmation of transformation of GFP gene in the genome of *Fusarium oxysporum* f. sp. *cubense* by PCR using GFP-specific PCR primers. A 1.5% agarose gel stained in ethidium bromide and run in 1 × TAE to resolve amplified product. Size estimates of the amplified products were estimated using a 100 bp molecular weight standard. Lanes 1, molecular weight standard; 2–6 amplified product using undiluted template DNA; 7–11 amplified product using 1:10 dilution of template DNA; lane 12 negative control using genomic DNA of an untransformed *Foc* 145 strain.

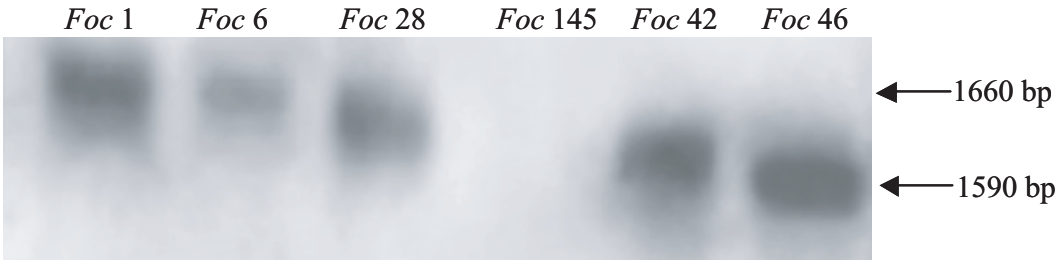

Fig. 4. Southern blot analysis of digested genomic DNA showing the presence of the GFP in the genome of *Fusarium oxysporum* f. sp. c*ubense*. Lanes 1–3, *Foc* 1, *Foc* 6, *Foc* 28, lane 4 not loaded, lane 5 untransformed *Foc* 145, lane 6–7, *Foc* 42, *Foc* 46.

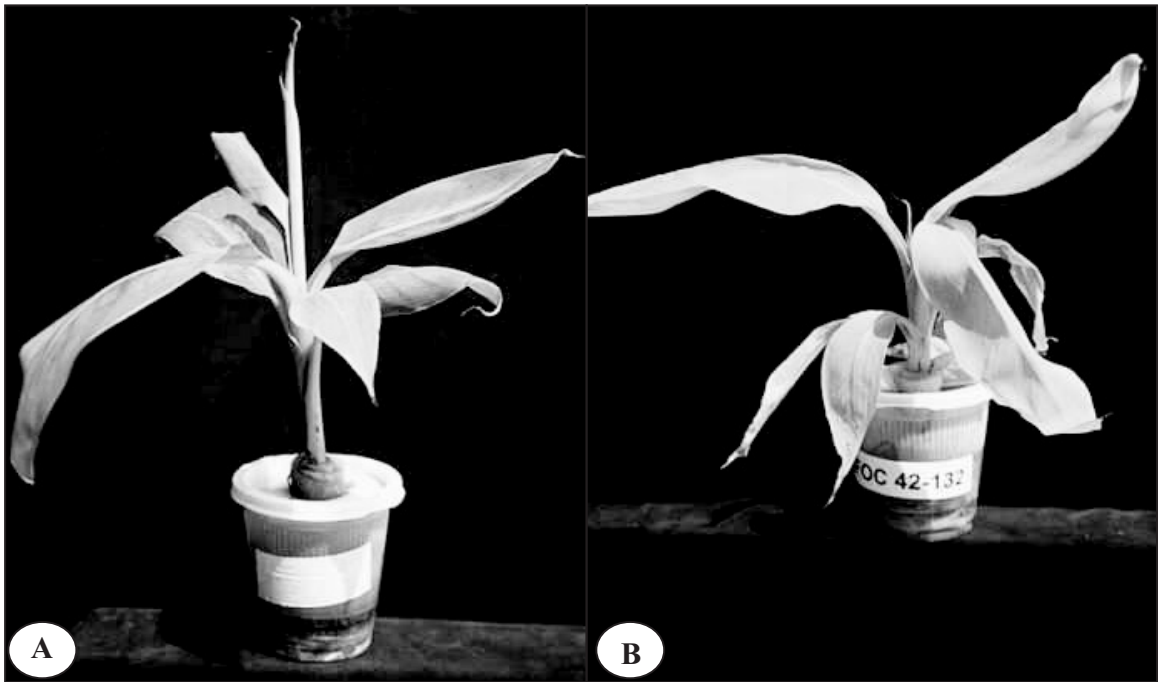

Fig. 5. (*A*) Control plant showing no disease symptoms. (*B*) Plant inoculated with transformed isolate of *Fusarium oxysporum* f. sp. c*ubense* showing symptoms of Fusarium wilt.

control plants remained asymptomatic and the pathogen could not be isolated from them.

Foc transformed with the GFP was observed in the vascular tissue of inoculated banana plants. The movement and colonisation of the fungus could be traced into the stem. Auto fluorescence of the plant did not prevent visualisation of GFP expression. Transformants were successfully re-isolated from diseased plant tissue.

Discussion

This study reports on the first transformation of *Foc* with the gene coding the GFP. Similar to results obtained for other fungi transformed with the GFP (Freitag *et al*. 2001; Lorang *et al*. 2001), transformed isolates of *Foc* were able to fluoresce uniformly and the integration of GFP proved to be stable. This emphasises the effectiveness of this gene as a marker. The slight variation in GFP intensity experienced in the current study can probably be explained by the integration of the plasmid into different chromosomal sites as has been suggested previously (Lorang *et al*. 2001; Lagopodi *et al*. 2002).

Fusarium species are known to change form and colour in response to environmental constraints due to small mutations (Follin and Laville 1966; Booth 1971; Nelson *et al*. 1983; Hawksworth *et al*. 1995). These changes are expressed in *Foc* by means of their growth, colony colour and presence of sclerotia on artificial media (Waite and Stover 1960). Transformation with the GFP in this study, however, did not affect colony morphology, growth, pigmentation, or sporulation.

Similar to studies involving the GFP transformation of *C. heterostrophus* (Maor *et al*. 1998), *U. maydis* (Spellig *et al*. 1996) and *Phytophthora palmivora* (Van West *et al*. 1999), the transformed *Foc* retained its virulence. Spellig *et al*. (1996) found that GFP expression did not significantly interfere with *U. maydis* development *in planta*. Furthermore, Maor *et al*. (1998) and Van West *et al*. (1999) found that transformed isolates were as virulent as the wild type isolates, and that high expression levels of GFP did not affect pathogenicity.

The ability to visualise *Foc* in banana tissue infested with GFP-transformed isolates will make it possible to study the infection processes of this fungus in greater detail than has previously been possible. Transformation of other fungal pathogens has facilitated infection studies in the past (Spellig *et al*. 1996; Van West *et al*. 1999; Freitag *et al*. 2001). Thus, we expect to study details of the disease process, such as the exact mode of penetration and the growth of *Foc* within the host in future.

Acknowledgements

We thank the National Research Foundation (NRF), the THRIP initiative of the Department of Trade and Industry, South Africa, the United States Department of Agriculture, the Banana Growers Association of South Africa (BGASA) and the University of Pretoria, South Africa, for financial support. We also thank Jeness Petersen, University of California, Davis for technical assistance with the GFP transformation process.

References

Berteaux-Lecellier V, Zickler D, Debuchy R, Panvier-Adoutte A, Thompson-Coffe C, Picard M (1998) A homologue of the yeast *SHE4* gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus *Podospora anserina*. *EMBO Journal* **17**, 1248–1258.

Booth C (1971) 'The genus *Fusarium*.' (Commonwealth Mycological Institute: Kew, England)

Bottin A, Larche L, Villalba F, Gaulin E, Esquerre-Tugaye MT, Rickauer M (1999) Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe–plant interaction in the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. *FEMS Microbiology Letters* **176**, 51–56. doi:10.1016/S0378-1097(99)00218-9

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* **263**, 802–805.

Chalfie M, Kain S (1998) 'Green fluorescent protein: properties, applications and protocols.' (John Wiley & Sons Inc.: New York, USA)

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Current Biology* **6**, 325–330.

Churchill ACL, Ciuffetti LM, Hansen DR, Van Etten HD, Van Alfen NK (1990) Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. *Current Genetics* **17**, 25–31.

Cormack BP, Bertram G, Egerton M, Gow NA, Falkow S, Brown AJ (1997) Yeast-enhanced green fluorescent protein (yEGFP), a reporter of gene expression in *Candida albicans*. *Microbiology* **143**, 303–311.

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. *Trends in Biochemical Sciences* **20**, 448–455. doi:10.1016/S0968-0004(00)89099-4

Du W, Huang Z, Flaherty JE, Wells K, Payne GA (1999) Green fluorescent protein as a reporter to monitor gene expression and food colonization by *Aspergillus flavus*. *Applied and Environmental Microbiology* **65**, 834–836.

Dumas B, Centis S, Sarrazin N, Esquerre-Tugaye MT (1999) Use of green fluorescent protein to detect expression of an endopolygalcturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Applied and Environmental Microbiology* **65**, 1769–1771.

Follin JC, Laville E (1966) Variations chez le *Fusarium oxysporum*. Comportement des lignees issuees des differents organs de multiplication. *Fruits* **21**, 261–268.

Freitag M, Ciuffetti LM, Selker EU (2001) Expression and visualization of green fluorescent protein (GFP) in *Neurospora crassa*. *Fungal Genetics Newsletter* **48**, 15–19.

Haseloff J, Amos B (1995) GFP in plants. *Trends in Genetics* **11**, 328–329. doi:10.1016/0168-9525(95)90186-8

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) 'Dictionary of the fungi.' (CAB International: Wallingford, UK)

Kershaw MJ, Walkey G, Talbot NJ (1998) Complementation of the Mpg1 mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. *The EMBO Journal* **17**, 3838–3849. doi:10.1093/EMBOJ/17.14.3838

Lagopodi AL, Ram AFJ, Lamers GEM, Punt PJ, Van den Hondel CAMJJ, Lugtenberg BJJ, Bloemberg GV (2002) Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Molecular Plant–Microbe Interactions* **15**, 172–179.

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, *et al*. (2001) Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology* **67**, 1987–1994. doi:10.1128/AEM.67.5.1987-1994.2001

Maor R, Puyesky M, Horwitz BA, Sharon A (1998) Use of green fluorescent protein (GFP) for studying development and fungal–plant interaction in *Cochliobolus heterostrophus*. *Mycological Research* **102**, 491–496. doi:10.1017/S095375629 7005789

Nelson PE, Toussoun TA, Marasas WFO (1983) '*Fusarium* Species: an illustrated manual for identification.' (Pennsylvania State University Press: University Park, USA)

Niedenthal RK, Riles L, Johnston M, Hegemann JH (1996) Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. *Yeast (Chichester, England)* **12**, 773–786. doi:10.1002/(SICI)1097-0061(19960630)12:83.3.CO; 2-C

Orjeda G (1998) 'Evaluation of *Musa* germplasm for resistance to Sigatoka diseases and Fusarium wilt.' (International Plant Genetic Resources Institute, Rome, Italy (IPGRI), International Network for the Improvement of Banana and Plantain, (INIBAP), Montpellier, France, ACP-EU Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, The Netherlands)

Pines J (1995) GFP in mammalian cells. *Trends in Genetics* **11**, 326–327. doi:10.1016/S0168-9525(00)89092-7

Prasher DC (1992) Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* **111**, 229–233. doi:10.1016/0378-1119(92)90691-H

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**, 17–20.

Sambrook J, Fritsch EF, Maniatis T (1989) 'Molecular cloning: a laboratory manual.' (Cold Spring Harbor Press: New York, USA)

Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DM (1995) Green fluorescent protein as a new vital marker in plant cells. *The Plant Journal* **8**, 777–784. doi:10.1046/J.1365-313X.1995.08050777.X

Skinner W, Bailey A, Renwick A, Keon J, Gurr S, Hargreaves J (1998) A single amino-acid substitution in the iron–sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. *Current Genetics* **34**, 393–398. doi:10.1007/S002940050412

Spellig T, Bottin A, Kahmann R (1996) Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Molecular & General Genetics* **252**, 503–509. doi:10.1007/S004380050257

Stover RH (1972) Fusarial wilt (Panama Disease) of bananas and other *Musa* species. In 'Banana, plaintain and abaca diseases'. (Commonwealth Mycological Institute: Kew, England)

Suelmann R, Fischer R (1997) Nuclear traffic in fungal hyphae: *in vivo* study of nuclear migration and positioning in *Aspergillus nidulans*. *Molecular Microbiology* **25**, 757–769. doi:10.1046/J.1365-2958. 1997.5131873.X

Suelmann R, Fischer R (2000) Mitochondrial movement and morphology depend on an intact actin cytoskeleton in *Aspergillus nidulans*. *Cell Motility and the Cytoskeleton* **45**, 42–50. doi:10.1002/(SICI)1097-0169(200001)45:13.3.CO;2-3

Tsien RY (1998) The green fluorescent protein. *Annual Review of Biochemistry* **67**, 509–544. doi:10.1146/ANNUREV.BIOCHEM. 67.1.509

Van West P, Reid B, Campbell TA, Sandrock RW, Fry WE, Kamoun S, Gow NAR (1999) Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiology Letters* **178**, 71–80. doi:10.1016/S0378-1097(99)00320-1

Vollmer SJ, Yanofsky C (1986) Efficient cloning of genes of *Neurospora crassa*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 4869–4873.

Waite BH, Stover RH (1960) Studies on Fusarium wilt of bananas, VI. Variability and the cultivar concept in *Fusarium oxysporum* f. *cubense*. *Canadian Journal of Botany* **38**, 985–994.

Received 28 May 2003, accepted 1 August 2003