Agrobacterium tumefaciens-mediated transformation of *Fusarium oxysporum* f. sp. *cubense* for pathogenicity gene analysis

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DECLARATION

I, the undersigned, hereby declare that the work in this thesis is the result of my own investigation and that it has not previously in its entirety or partially been submitted for a degree at any other university.

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PREFACE

Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most destructive plant diseases in recorded history. The disease was first discovered in Australia in 1874 but became renowned for the severe losses it caused to export banana plantations during the 1960s in Central America. The banana export industry was saved only by replacing Gros Michel bananas, the dessert banana grown for the export market, with highly resistant Cavendish banana cultivars. Despite this apparent solution, the fungus was found to attack Cavendish bananas in the sub-tropics, where plants were believed to be predisposed to the disease by the cool winter climate. Good management practices and conventional disease management strategies have not been sufficient to reduce losses and stop the disease from spreading, and today Fusarium wilt can be found in almost all banana-producing countries of the world. Since 1988, *Foc* has been responsible for significant losses of Cavendish bananas in tropical Asia. The only sustainable control measure, the use of resistant varieties, is not always popular as people prefer to eat locally adopted varieties that, unfortunately, are susceptible to *Foc*.

Sustainable Fusarium wilt management in banana depends on the improvement of existing banana cultivars or the development of novel disease management strategies. Molecular biology and biotechnology provide opportunities to introduce foreign resistance genes into existing cultivars and to develop new, environmentally friendly products that can protect susceptible bananas from *Foc*. Better knowledge of the Fusarium wilt pathogen, its diversity, and its mechanisms of pathogenesis will contribute significantly to developing these novel approaches for control of the disease. Molecular information on the pathogenicity of *Foc,* however, is limited, whereas other *formae speciales* of *F. oxysporum* have been better studied. In this thesis, *Agrobacterium tumefaciens*-mediated transformation of (ATMT) was employed to investigate genes responsible for pathogenicity of *Foc* to banana.

Chapter 1 provides an overview of pathogenicity in *F. oxysporum*. Pathogenic and non-pathogenic forms of the fungus are first introduced to the reader, and then the biology, epidemiology and etiology of pathogenic forms of *F. oxysporum* are discussed. The genetic make-up and ability of the Fusarium wilt fungus to cause disease in plants concludes the first part of the review. In recent years, there has been a noted increase in the number of techniques available to study host-pathogen interactions. The second part of the review concentrates on these techniques and their applications in studying pathogenicity of the Fusarium wilt pathogen.

In **Chapter 2**, an ATMT and screening system for *Foc* was developed. Five *A. tumefaciens* strains were evaluated for their efficiency to transform *Foc* with a randomly integrating vector that confers hygromycin B resistance and expression of green fluorescent protein (GFP). A small insertion mutant library of *Foc* was created, and a subset of transformants was characterized by determining

the number of T-DNA inserts present, the location and identity of predicted genes disrupted by T-DNA insertion, and whether transformants of *Foc* were altered in their virulence against susceptible banana plants.

In **Chapter 3**, the role of a known pathogenicity gene, *Frp1*, of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*) was investigated in *Foc*. The first objective was to isolate and characterize the *Frp1* gene in *Foc*, and to compare it to the homologous gene in *Fol*. A vector containing a modified *Fol Frp1* gene was then obtained and used for targeted disruption of the gene in *Foc* via ATMT. Mutants in which the *Frp1* gene was disrupted were then analyzed for GFP expression, culture morphology, and alterations in pathogenicity to banana.

CHAPTER 1

PATHOGENICITY IN *FUSARIUM OXYSPORUM*: A REVIEW

INTRODUCTION

Fusarium oxysporum Schlecht. is one of the most important and cosmopolitan fungi in the world. Plant pathogenic strains of *F. oxysporum* are responsible for vascular wilting in most vegetables, field crops and flowers (Booth, 1984). Vascular wilt diseases have been responsible for some of the most important epidemics in modern agriculture, such as Panama disease (Fusarium wilt of banana), Bayoud disease (Fusarium wilt of date palm), and Fusarium wilt of cotton. Once introduced into a field, the Fusarium wilt fungus survives for decades as saprophytes in soil and plant debris until a suitable host is found. After infection, mostly through wounds, the pathogen damages its host by blocking off the xylem vessels, thereby restricting the movement of water and nutrients from the soil. This results in reductions in respiration and, consequently, fruit production. With severe infections of a susceptible host, the entire plant wilts and dies. Not all *F. oxysporum* strains necessarily cause disease in plants. Some strains are non-pathogenic to agricultural crops and may protect them against diseases and pests (Athman, 2006; McLeod *et al*., 2006; Nel *et al*., 2006; Paparu *et al*., 2007), while others cause mycotoxicosis in mammals (including immunocompromised humans) (Roncero *et al*., 2003), chickens (Desjardins, 2006), and shrimp (Souheil *et al*., 1999).

Fusarium wilt is responsible for severe economic losses worldwide. Panama disease almost destroyed the international banana export industry in Central America before 1960, with losses estimated at approximately US$ 400 million (US$ 2.3 billion in 2000-value) (Ploetz, 2005). This estimate excluded costs for unemployment, abandoned villages, unrealized income, and expenditures to maintain social stability. Similarly, Fusarium wilt resulted in losses of AUS$ 100 million per annum to the Australian cotton industry (http://www.tpp.uq.edu.au/Research/SubProgram1/Fusariumwiltincotton/tabid/716/Default.aspx). Fusarium wilt diseases are most effectively controlled by using disease resistant plants (Ploetz, 2005). Chemical, cultural and biological control strategies can be used to manage Fusarium wilt diseases of some annual crops. Despite implementation of these practices, severe economic losses still occur, which suggests that novel disease management strategies need to be developed.

A good understanding of pathogenesis in *F. oxysporum* will provide essential information for the development of novel disease management strategies. Such an approach focuses on targeted and highly specific disease control that aims to reduce dependency on chemical control and encourages the use of environmentally safe and consumer acceptable technologies. The basis of all cellular functions lies within the genetic material of an organism. The identification and characterization of pathogenicity genes and gene products, therefore, is an important first step in understanding the capacity of a pathogen to inflict damage in plants. Such knowledge is one channel, amongst others, for developing the means to counter the disease-causing abilities of

pathogens, which are often specific to particular host-pathogen interactions. In this review, current knowledge of Fusarium wilt pathogens, pathogenicity of *F. oxysporum,* and the genes underlying pathogenicity are summarized. Methodologies to study pathogenicity in plant pathogens, from genome to metabolome, will also be discussed.

FUSARIUM OXYSPORUM - THE MODEL ORGANISM

Pathogenic and Non-Pathogenic Forms

Fusarium oxysporum can be divided into pathogenic and non-pathogenic strains. According to Alabouvette (1990) and Trouvelot *et al*. (2002), non-pathogenic *F. oxysporum* strains play an important role in soil suppressiveness to diseases induced by pathogenic strains. This is done by competing for nutrients and infection sites, root colonization, and induced systemic resistance. They also play an important role in soil microbial ecology. Non-pathogenic strains of *F. oxysporum* are often parasitic, but not pathogenic, to a non-host (Trouvelot *et al*., 2002). According to Olivain and Alabouvette (1999), the difference between pathogenic and non-pathogenic strains is quantitative and not qualitative. Beckman (1990), however, believes that non-specific host responses are elicited in response to invasion by any organism due to recognition as non-self. A virulent pathogen will, however, be able to tolerate or overcome the host resistance responses and cause disease (Recorbet *et al*., 2003).

Pathogenic forms of *F. oxysporum* may have evolved from previously non-pathogenic strains (Gordon and Martyn, 1997). It has been noted that, despite the presence of pathogenic strains in soil, plants in native areas do not succumb to disease. The relationship between *F. oxysporum* and plant hosts is, therefore, believed to be mostly of a non-pathogenic nature (Gordon and Martyn, 1997). Isolates of *F. oxysporum* that cause disease in different hosts most commonly have evolved from a single ancestor (Hua-Van *et al*., 2001), and new outbreaks of the disease are most likely due to movement of a pre-existing strain rather than the evolution of new, independent pathotypes (Appel and Gordon, 1996).

Differences between pathogenic and non-pathogenic strains of *F. oxysporum* are difficult to capture. The colonization of flax roots and their early physiological cell responses have been used to distinguish between pathogenic and non-pathogenic strains (Olivain *et al*., 2003). Trouvelot *et al*. (2002) determined that the factor that makes Fo47 a potential biocontrol agent may be expressed during initial contact with the host plant. The exact trigger(s) that causes a non-pathogen to develop into a pathogen, or what differentiates the two from each other, still needs to be determined.

Host Specialization

Based on host specificity, more than 150 different *formae speciales* (f. sp.) of *F. oxysporum* have been recognized (Table 1) (Baayen *et al*., 2000; Di Pietro *et al*., 2003). This specialization is based on the presence of one or more pathogenicity and/or virulence genes against the host. The host has inherent defense systems that are pre-emptive (constitutive defense) but also induces defense responses that are activated with infection (induced defense). The genes and gene combinations that determine resistance to a *forma specialis* are only found in the specific host and probably a small number of related plants (Alabouvette *et al*., 2003; Rep *et al*., 2004). *Formae speciales* of *F. oxysporum* can be further separated into races, based on cultivars of a crop that are susceptible to specific individuals of a *forma specialis*. Currently, the only valid method to identify *formae speciales* and races is by means of bioassays in which the recognized host plants are challenged with the fungus (Jeger *et al*., 1995; Alabouvette *et al*., 2003).

The specificity of fungal virulence genes determines host specialization and will distinguish between host and non-host (Nimchuk *et al*., 2003). Based on dominant monogenic resistance, the traditional gene-for-gene link is believed to exist in a host for a specific race of *F. oxysporum* (Simons *et al*., 1998). The avirulence gene *six1*, for instance, has been demonstrated to induce *I-3*-mediated resistance in tomato following inoculation with *F. oxysporum* f. sp. *lycopersici* (*Fol*) (Rep *et al*., 2004). The tomato – *Fol* interaction, however, is the only Fusarium wilt pathosystem where the gene-for-gene concept has been demonstrated. In the *Fusarium oxysporum* Schlecht f. sp. *cubense* (E.F. Smith) Snyder and Hansen (*Foc*) - banana interaction, plant resistance appears to be dependent on multiple defence-related genes (Ploetz, 2005; Van den Berg *et al*., 2007). Several other genes have been associated with resistance in banana, including a dominant gene for resistance against *Foc* race 1 and a recessive gene against race 4 (Ortiz, 1995; Ploetz, 2005).

Host specificity to a particular *forma specialis* of *F. oxysporum* can be changed. This happens when a new or modified resistance gene is introduced into the host, thereby conferring resistance to the pathogen. Both classical breeding for disease resistance and genetic engineering of the host can, therefore, bring about resistance to Fusarium wilt. This resistance will be sustained only until changes in the pathogens' genome occur. A *forma specialis* can acquire virulence genes by horizontal gene transfer or become more virulent/pathogenic through mutations, although the exact mechanisms are not well understood (Kistler, 2001; Van der Does and Rep, 2007). In the asexual *F. oxysporum*, however, a non-virulent form will predominantly become virulent by mutation, which is a slow and uncommon event (Ploetz, 1990).

The Etiology of Fusarium wilt

F. oxysporum enters plants through their roots before colonizing the xylem vessels (Beckman, 1987). The pathogen progresses up the vascular bundles until the fungus is blocked by sieve cells, which it grows into to release microconidia that will be carried with the vascular flow to the next sieve cells. Progressive blocking of vascular bundles eventually results in loss of nutrient and/or

water uptake and the consequential wilting of the plant. Leaves of wilted plants will first turn yellow, with necrosis following chlorosis from the older to the youngest leaf until all leaves of the wilted plant are affected. After the death of the plant, the fungus will move from the vascular vessels to adjacent tissues and sporulate abundantly. It has been observed that the pathogen extensively colonizes the vascular system of susceptible plants. Growth of non-pathogenic forms, alternatively, is limited to the basal parts of the plant, such as the root cortex, and sometimes can advance to the xylem vessels (Beckman, 1987; Beckman and Roberts, 1995; Gordon and Martyn, 1997). Strategies that the pathogen employs to promote successful infections include avoiding or overcoming plant defence responses, such as pathogen recognition, callus deposition, vascular gelation, vascular blockage by tylose occlusions or vessel crushing, or the suppression of host cellular responses that lead to a reduction in respiration (Beckman, 1987). At every stage of the plant-pathogen interaction, a different set of virulence factors presumably are activated to respond to or attack the host (Figure 1).

Genetics and Genomics

In 2003, the genome size of *F. oxysporum* was estimated broadly as ranging from 18 - 51 Mb in size (Roncero *et al*., 2003). In 2007, the genome sequence of *Fol* was completed at the Broad Institute, where the genome size was estimated to be 59.9 Mb (Broad Institute, Cambridge, MA, USA: http://www.broad.mit.edu/annotation/genome/fusarium_group/GenomesIndex.html). Annotation of the genome, composed of 15 chromosomes with an estimated 17 735 genes, is currently underway.

F. oxysporum is a phylogenetically diverse, monophyletic complex of filamentous ascomycetous fungi (O'Donnell *et al*., 2004) with a great amount of genetic variation among its different *formae speciales.* Two possible reasons for this variation are the presence of active transposable elements, for which many families are found in *F. oxysporum* (Roncero *et al*., 2003), and the presence of a parasexual cycle, which is the result of heterokaryon formation (Teunissen *et al*., 2002). No sexual state (teleomorph) has ever been observed in *F. oxysporum* (Jeger *et al*., 1995).

Virulence and Pathogenicity Factors

Fungi produce a wide array of toxins, enzymes and hormones during pathogenesis. Pathogenicity factors are necessary for successful infection and disease development in the host and include plant cell wall-degrading enzymes, toxins, and polysaccharides. Factors predicted to play a role in pathogenesis in *F. oxysporum* include various polysaccharidases or cell wall-degrading enzymes (CWDEs) like arabinase, galactanase, pectin methylesterase, polygalacturonases (PGs), pectin transeliminase, xylanase, cellulase, hemicellulases, ß-galactosidase, (McHardy and Beckman, 1981; Vidhyasekaran, 1997), cutinase, esterase (Ahn *et al*., 2006), and a glucan containing β-1,3-bonds (Konnova *et al*., 1995). Previous studies linked these factors to colonization, overcoming host resistance and symptom induction (McHardy and Beckman, 1981). Despite these findings, gene disruption studies showed that CWDEs do not clearly play a major role in *F. oxysporum*

virulence (Suga and Hyakumachi, 2001; Di Pietro *et al.*, 2003). Genes shown by molecular methods not to be involved in virulence include, amongst others, *pg1* (endopolygalacturonase) (Garcia-Maceira *et al.*, 2001), *pl1* (pectate lyase) (Huertas-Gonzalez *et al.*, 1999), *prt1* (serine protease) (Di Pietro *et al.*, 2001b), *xyl3* (family 10 endoxylanase) (Gómez-Gómez *et al.*, 2002), and *fcd1* (quinone oxidoreductase) (Kawabe *et al.*, 2006). This may be due to the functional redundancy of multiple genes having similar activities that are able to compensate for the loss-of-function of the disrupted gene (Walton, 1994). Two forms of endo-pectic lyase (endo-PL) were suggested to be required for virulence in *F. oxysporum* f. sp. *ciceris*, where an isolate with low virulence produced only one form of the enzyme (Vidhyasekaran, 1997). This enzyme has been proposed to be a possible marker of pathogenicity and virulence in this system (Perez-Artes *et al.*, 2004).

Two roles have been hypothesized for toxins in *F. oxysporum*: overcoming the host's resistance mechanisms during infection, and causing symptom formation during growth and reproduction (McHardy and Beckman, 1981; Yoder and Turgeon, 1985). Toxins are further recognized as substances that can protect the pathogen from the host in other necrotrophic plant pathogenic fungi (Mayer *et al.*, 2000). A range of toxins has been found in *F. oxysporum* (Table 2), with fusaric acid and lycomarasmin being the most important (Desjardins, 2006). Fusaric acid causes wilt symptoms in plants and may also influence the interactions between fungi and bacteria in the plant. Sequence data are not yet available for genes involved in the biosynthesis of this toxin (Desjardins, 2006; Wu *et al.*, 2007). Lycomarasmin has been shown to cause wilting and curling of removed tomato leaves (Woolley, 1948). More recently, *F. oxysporum* has been shown to synthesize the toxins beauvericin and enniatin (Song *et al.*, 2008), the latter of which was shown to be a virulence factor for *F. avenaceum* (Fr.) Sacc. (Herrmann *et al.*, 1996). Long-term storage methods of *F. oxysporum* can influence its ability to produce toxins (Rodríguez *et al.*, 2006). Desjardin (2006) has proposed that *F. oxysporum* seems to be of relatively minor importance in terms of mycotoxin production.

MOLECULAR METHODS TO STUDY PATHOGENICITY IN *F. OXYSPORUM*

Pathogenicity studies in *F. oxysporum* initially involved methods such as *in planta* screening, microscopy of host-pathogen interactions (Gold *et al.*, 2001; Aboul-Soud *et al.*, 2004), and enzyme pattern analysis (Ho *et al.*, 1985). Current methods of investigation in Fusarium wilt development include molecular genetic studies and targeted gene disruption. In *F. oxysporum*, several virulence and pathogenicity genes have been characterized (Table 3). *F. oxysporum* acts like a typical soil-borne pathogen, has a wide host-range, and disease mechanisms that can be applied to other soil-borne pathogens, making it a good model for virulence studies in soil-borne plant pathogens. Researchers at the University of Córdoba have further shown that the fungus can be used as a multi-host model for virulence gene characterization in both plants and mammals (Ortoneda *et al.*,

2003).

The number of genes responsible for pathogenesis in filamentous fungi is unknown and will depend on the fungus and its infection processes. Idnurm and Howlett (2001) estimated that a phytopathogenic fungus might have between 60 and 360 pathogenicity genes. They based the estimate on the fact that between 0.5% and 2% of non-pathogenic transformants are produced during random mutagenesis. They also predicted that half of the fungal genome codes for functional genes (10,000), and half of these encode 'essential' survival genes. Pathogenicity genes have been best studied in the rice blast fungus *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, where only 16 unique genes linked to pathogenicity had been recognized by 2001 (Idnurm and Howlett, 2001). However, the rate at which pathogenicity genes are being identified in this fungus has increased significantly with ever-improving methodologies, such as transformation efficiencies of up to 3600 transformants per µg DNA (Di Pietro *et al.*, 2003), straightforward culture conditions, multiple successful infection studies, and the increasing amounts of genetic and biological information that follow the availability of a sequenced genome (Ebolle, 2007).

Identification of Unknown Genes

Random or non-specific mutations that disrupt virulence genes in *F. oxysporum* may involve both deletions or insertions. Disruptions generally occur in the open reading frame of the virulence gene and can lead to non-functional or shortened protein structures. Early methods of inducing mutations involved treating pathogens with mutagenizing chemicals and ultra-violet (UV) radiation (Sanchez *et al.*, 1975). The mutants that survived generally had reduced genetic stability and multiple site-mutations. This resulted in extensive screening experiments in which it was difficult to locate and identify the genes of interest (Recorbet *et al.*, 2003). Despite these drawbacks, Freeman *et al.* (2002) utilized UV-induced mutagenesis to isolate a non-pathogenic mutant of *F. oxysporum* f. sp. *melonis* for biological control in muskmelon. Since the objective was to generate a loss-of-pathogenicity mutant for biocontrol and not for pathogenicity gene identification, no tag was required and the use of this method was appropriate.

Methods whereby genes are cloned randomly from a pathogen's genome, without prior knowledge of the end product, are called "black-box" approaches (Timberlake, 1991; Sweigard and Ebbole, 2001). The most recent and promising random insertional method of gene disruption is *Agrobacterium tumefaciens*-mediated transformation (ATMT). With ATMT, a plasmid vector containing a selectable marker gene is introduced into the fungal cell by means of *A. tumefaciens* transformation. The most commonly used selectable marker for fungi is the *Escherichia coli* hygromycin resistance gene (*hph*) (Michielse *et al.*, 2005). The vector is processed by *Agrobacterium* by cleavage of the vector outside the left- and right border repeats, resulting in a linear molecule. This modified version of the vector (T-DNA) is then incorporated into the host genome through nonhomologous recombination, resulting in random gene disruption events (Michielse *et al.*, 2005). ATMT often improves the low transformation efficiencies obtained using

protoplast-mediated transformation or restriction enzyme-mediated integration (REMI) (Migheli *et al*., 2000; Gold *et al*., 2001). Other advantages of ATMT include its relative ease of implementation, the ability to transform spores and mycelia directly without protoplasting, low insert copy numbers, fewer genomic lesions in unlinked insertion sites, and the ability to recover recipient DNA flanking the insertion site due to the presence of a vector tag (Mullins and Kang, 2001; Weld *et al*., 2006). ATMT has been used with great success for random gene disruption in a variety of plants and fungi (De Groot *et al*., 1998; Mullins *et al*., 2001; Gelvin, 2003; Michielse *et al*., 2005). Other random insertional transformation methods include $CaCl_2$/polyethylene glycol (PEG) transformation of protoplasts (Kistler and Benny, 1988; Jain *et al*., 2002), electroporation of protoplasts or germinating conidia (Ward *et al*., 1989), and particle bombardment of intact fungal tissue (Lorito *et al*., 1993).

A variation of insertional mutagenesis is the use of naturally occurring transposons for random gene disruption in *F. oxysporum*. A transposon is a mobile segment of DNA that moves from one location of the genome to another as a DNA entity (Fairbanks and Andersen, 1999; Mullins and Kang, 2001). A range of transposons has been described in *F. oxysporum* (Table 4), of which *impala* has been characterized most extensively. Transposons in *F. oxysporum* can be categorized into 17 families based on their function and composition and are estimated to make up approximately 5% of the genome (Hua-Van *et al*., 2000; Roncero *et al*., 2003). By tagging these transposons, the insertion point into the host genome can be located, and subsequent selection of mutants can be done. Tagging and recovery of the disrupted gene, therefore, allows for higher efficiencies through selection and reversible mutagenesis (Migheli *et al*., 2000; Hamer *et al*., 2001). In addition, modified mini-Tn5 transposons or plasposons, which are plasmids that allow for high-throughput recovery of flanking genomic DNA after mutagenesis with a transposon, may be of use with fungi in the future. A plasposon is a suicide plasmid consisting of a selectable gene flanked by two inverted repeats and a transposase gene. The transposase gene allows for random integration into the host genome, and the selectable gene or the plasposon serves as a tag for gene recovery. Although this technique has only recently been developed for bacteria, some potential exists for its use in fungi (Leveau *et al*., 2006).

Another method that creates gene mutations at random is REMI (Sweigard, 1996; Kahmann and Basse, 1999; Maier and Schäfer, 1999; Gold *et al*., 2001). REMI uses a restriction enzyme (RE) to partially digest protoplast-bound genomic DNA, providing sites for integration of either circular or linearized plasmid DNA (digested with the same RE as used for the genome). When plasmid integration occurs at the corresponding RE site in the pathogen genome, the RE recognition site is often restored (Sweigard, 1996; Kahmann and Basse, 1999). In addition to generating tagged mutations, this technique tends to create non-tagged mutations and mutational hotspots, hampering the identification of unknown genes (Sweigard, 1996; Idnurm and Howlett, 2001). Despite the drawbacks, REMI has been the most successful random insertion method used in fungi until relatively recently when advances in ATMT were realized.

To verify a successful mutational event or confirm a predicted gene function, complementation studies may be done. In this instance, mutants with a disrupted gene are transformed with plasmid DNA that contains the full-length, undisrupted gene of interest in addition to a second selective marker. Infection assays or other bioassays are then performed to determine whether the function lost by mutagenesis is restored. Complementation studies can also be used to study the function of a gene in a related organism that has an inherent loss of that function (Di Pietro *et al*., 2001a; Goins *et al*., 2006).

Characterization of Known Genes

Known pathogenicity and virulence genes in fungi can be characterized by disrupting/knocking-out (KO) target genes with vectors via ATMT or other transformation methods. In order for a recombination event to occur that targets a specific gene, the DNA used for transformation typically contains a selective marker gene that is flanked on each end by a fragment of the target gene. Gene disruption is achieved by homologous recombination between the vector and host genome, resulting in targeted mutagenesis (Timberlake, 1991; Sweigard and Ebbole, 2001; Michielse *et al*., 2005). Methods to generate KO vectors include traditional restriction enzyme digestion, re-ligation and cloning; PCR fusion; restriction-free (RF) cloning; and split-marker recombination. The PCR fusion method requires two rounds of PCR to generate a DNA fragment that contains a selectable marker within the target gene region (Catlett *et al*., 2002). RF cloning is a high-throughput and restriction-free method for generating plasmids containing the gene of interest (Van den Ent and Lowe, 2006). For the split-marker recombination technique, either the PCR fusion method (plasmid-free) or the plasmid-based method can be applied, but only with the use of protoplasts (Catlett *et al*., 2002). Another plasmid-free method to generate targeted mutations is by using linear minimal element (LME) constructs. These LMEs contain a partial target gene sequence at the one end and a selectable marker gene at the other end. Re-circularisation of the LME in the cell then allows for gene disruption by a single recombination event. Classic linear constructs, however, have been shown to circularise and produce unwanted and less specific results (Cho *et al*., 2006).

The classical and most widely used method for vector preparation remains RE digestion and re-ligation (Covert *et al*., 2001; Takken *et al*., 2004). A technique that shows great potential, but has not yet been used in *Fusarium*, involves gene replacement and conditional expression (GRACE™) and has been developed by Elitra Pharmaceuticals (http://www.bioportfolio.com/biocorporate/3751-Elitra_Pharmaceuticals.html). In this method, *Candida albicans* (C.P. Robin) Berkhout was used as the model organism for a two-step strategy in which the target gene is replaced with a PCR-generated cassette upon which, through promoter replacement, conditional expression occurs (Firon and D'Enfert, 2002). Once the DNA insert has been developed, it is transported to the cell by electroporation of protoplasts, ATMT or other transformation methods (Riach and Kinghorn, 1996).

Various other methods can be employed for the characterization of a gene. Using PCR,

amplification of a known gene with conserved primers can be achieved to determine its diversity across strains (Alabouvette *et al.*, 2003), or real time PCR (RT-PCR) can be used for the quantitative amplification of expressed genes under various growth conditions (Hatsch *et al.*, 2006; Ramos *et al.*, 2007). Microarray slides can be spotted with a subset of known genes or whole genomes and probed with RNA obtained from the organism of interest, which has been subjected to a range of environmental stimuli. Expression levels of the known genes are then analyzed. *Fusarium graminearum* Schwabe gene subsets and whole-genome chips are commercially available (Gold *et al.*, 2001; Güldener *et al.*, 2006; Seong *et al.*, 2008).

RNA Inhibition Approaches

Gene disruption methods may not always be effective due to gene duplication (Kistler *et al.*, 1995) or because of the diploid status of some organisms (Firon and D'Enfert, 2002). Disruption of one gene could result in the activation of a separate pathway with the same functionality. In order to overcome this limitation, post-transcriptional gene silencing may be applied, resulting in the simultaneous disruption of expression of potentially redundant genes of the same family (Firon and D'Enfert, 2002). During RNA interference (RNAi), the phenotype is knocked out, as opposed to the genotype in gene disruption studies (Michielse *et al.*, 2005). One constraint is that genes that normally are expressed at low levels are difficult to detect and, therefore, detecting loss of expression of such genes by RNAi is even more challenging (Firon and D'Enfert, 2002). Researchers at the Third Military Medical University (Jian *et al.*, 2006) have devised a strategy that uses two opposing polymerase III promoters, decreasing the cost and risk of sequence errors in the synthesis of RNAi knockdown constructs. RNAi has not been applied widely to *F. oxysporum* (Ito *et al.*, 2002) but has been adapted for studies in *F. graminearum* (McDonald *et al.*, 2005) and *F. solani* (Ha *et al.*, 2006).

Genomics

To fully understand pathogenesis, an accurate genomic blueprint of the pathogen is essential. Genome sequences are the basis of functional genomics, proteomics and metabolomics (Firon and D'Enfert, 2002; Talbot, 2003). More than 40 fungal genome sequencing projects have been completed (Yoder and Turgeon, 2001; Collemare *et al.*, 2008), including *F. oxysporum* (Broad Institute) and three close relatives, namely *F. sporotrichioides* Sherb. (Oklahoma University: http://www.genome.ou.edu/fsporo.html), *F. graminearum* Schwabe (Broad Institute), and *F. verticillioides* (Sacc.) Nirenberg (Broad Institute). Although sequence information for *F. oxysporum* might be deduced from *F. graminearum*, there are some major differences between the two fungi, including genome size, reproduction strategies, and stability (Table 5) (Di Pietro *et al.*, 2003). Once an annotated genome is available, genes with predicted or unknown functions can be identified, disrupted, and systematically characterized. Similarity searches can be performed using the online basic local alignment search tool (BLAST), a search tool that enables one to compare uncharacterized gene sequences with those of other fungi in which sequence is available but function may or may not be known (Talbot, 2003; Jewett *et al.*, 2006).

Methods for *de novo* whole genome sequencing include the high-throughput method of pyrosequencing (http://www.454.com) (Margulies *et al.*, 2005), amongst others (Lizardi, 2008; Stratton, 2008). Various methods are available to complement full genome sequencing efforts, including the generation of BAC clones, as is used by the Whitehead Institute for Biomedical Research (Cambridge, MA, USA) (Talbot, 2003). RE maps offer valuable information for the confirmation and alignment of sequence data. An optical map for the *F. oxysporum* genome was assembled by Dr. David Schwartz at the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/FOMapping.html). This map was constructed by microscopy and RE digestion, consists of 15 linkage groups, and corresponds to approximately 55X physical coverage of the genome. Large (250-3,000 kb) DNA fragments were used, which allowed for the detection of fragments that were larger than 5 kb.

Gene Expression Profiling

Differential gene expression profiles can lead to the identification of known and unknown genes that respond to a variety of specific stimuli (Dickinson, 2003; Emmersen *et al.*, 2007). Until recently, a lack of fully sequenced and annotated fungal genomes and few high-throughput tools for the characterization of fungal genes and genomes made the broad characterization of differentially expressed genes in fungi quite complicated. For instance, when investigating the interaction between pathogens and plants, the mRNA collected will yield a mixture of cDNA from both plant and pathogen, making it difficult to trace the origin of a sequenced fragment of interest. Recently, a web-based screening tool (http://mips.gsf.de/proj/est3) was devised to distinguish between the host plant and pathogen sources of expressed genes during infection studies by looking at triplet nucleotide frequencies rather than codon frequencies (Emmersen *et al.*, 2007).

Libraries of expressed sequence tags (ESTs) have been developed for many fungi, but to date these have proven to be of limited use in characterizing pathogenicity genes of *F. oxysporum* due to the incomplete availability of genetic information for this complex group of species (Idnurm and Howlett, 2001; Lorenz, 2002; Iida *et al.*, 2006). ESTs are nucleotide sequences of cDNA clones (Fairbanks and Andersen, 1999) that are derived from an organism in response to certain environmental stimuli. In two recent studies, conidial germination of *F. oxysporum* was studied by constructing EST libraries derived from sporulating and non-sporulating fungal cultures. Gene profiles were compared and several unique conidiation-related transcripts were predicted (Deng *et al.*, 2006; Iida *et al.*, 2006). An EST library has been prepared for *F. sporotrichioides* by the Genome Center at the University of Oklahoma (http://www.genome.ou.edu/fsporo.html). Together with microarray technologies, ESTs are a valuable complement to genome sequencing efforts, serving to confirm predicted ORFs as potentially functional genes.

Microarrays are a high-throughput method for the expression analysis of thousands of genes on a small slide. The technique can be used to detect hundreds of different cellular functions simultaneously (Hughes *et al.*, 2000; Idnurm and Howlett, 2001; Lorenz, 2002) with no previous

information about the function of the responsive transcripts required (Hughes *et al.*, 2000). Microarrays are based on reverse northern blot analyses and involve the spotting of unique sets of genes (cDNA or genomic) on a solid slide. The spotted nucleotides are then probed with labeled RNA from the organism of interest. A vast amount of data is usually generated, with hybridization signals being both quantitative and qualitative, and requiring careful analyses (Dickinson, 2003). With microarrays, the use of whole genome sequence data or very large and complex EST libraries are most informative. A good example is that of researchers at North Carolina State University (USA), who assessed nitrogen metabolism in *M. grisea* by comparing the complement of genes expressed in a nitrogen poor vs nitrogen rich growth environment. Thirty nine genes were predicted to be involved in nitrogen metabolism (Donofrio *et al.*, 2006). A similar study focused on gene expression during macroconidial germination (Seong *et al.*, 2008).

Two more gene expression methods are suppression subtractive hybridization (SSH) and complimentary DNA-amplified fragment length polymorphism (cDNA-AFLP). SSH involves differential expression of two RNA populations. Genes expressed to the same degree are eliminated by hybridization methodologies, whereas differentially expressed genes are enriched and remain for identification. The presence of low-abundance genes is also enriched in these steps (Diatchenko *et al.*, 1996; Birch *et al.*, 2000; Gold *et al.*, 2001; Dickinson, 2003). cDNA-AFLP (Bachem *et al.*, 1998; Oomen *et al.*, 2003) is a modification of AFLP, where double-stranded DNA is made from mRNA and then used for restriction digestion. By ligation of linkers to the ends of the digested fragments, a PCR can be performed and the products compared on a polyacrylamide gel. Differentially expressed bands are excised, sequenced and identified (Dickinson, 2003). These methods have great potential for gene identification, and minimal sequence data are required.

Less commonly used methods for gene expression profiling are also available. One-dimensional microfluidic bead array, for example, is a variation on a DNA array technique and can be used for multiple mRNA detection. This technique was used to detect a set of known genes expressed by different cell lines exposed to external stimuli (Wen *et al.*, 2007). Gene analysis or genotyping can also be done by using padlock probes. This methodology is based on analytical DNA circularization reactions, where closing-replicating circles of DNA are used as probes for multiplex DNA analysis (Nilsson *et al.*, 2006). Padlock probes are strands of DNA containing the gene or single nucleotide polymorphisms (SNPs) of interest and a marker gene. These probes are circularized during hybridization to target DNA, and the ends are closed by ligation or by a molecular inversion probe (MIP) assay. The circularized probes are amplified exponentially by PCR and identified and quantified by microarray or size separation by electrophoretic analyses. Despite not having been used for *Fusarium* yet, this methodology has some advantages, such as high specificity, distinction between allelic variants, and multiplexing of reactions.

Proteomics and Metabolomics

A relatively new analysis paradigm for studying plant-pathogen interactions is by means of protein characterization (Kahmann and Basse, 2001; Dickinson, 2003; Rep *et al.*, 2004; Rep, 2005; Xu *et al.*, 2007). The proteome is defined as a "global set of proteins expressed in a cell at a given time and biological state" (Kim *et al.*, 2007). Protein samples are typically run on 1- or 2-dimensional gels, allowing the identification of different protein fingerprints. The protein profiles then can be analysed by a number of methods such as mass spectrometry (Villas-Bôas *et al.*, 2005), peptide sequencing, or protein array technology. Similar to gene studies, differential protein production can be analysed and unknown proteins detected and characterized. The sequence of a gene encoding a particular protein can be obtained by PCR with degenerate primers based on known amino acid sequences derived from the protein. An additional method for protein detection is based on sequence conservation, where fungal libraries are screened with heterologous nucleic acid probes that identify particular protein-encoding genes of interest (Gold *et al.*, 2001).

Metabolomics or metabolite profiling involves the identification and quantification of intracellular and extracellular primary or secondary metabolites, providing more detailed insights into the functioning of an organism. These metabolites determine the phenotype of the organism. Metabolomics has been widely used in the food and pharmaceutical industry (Dickinson, 2003; Jewett *et al.*, 2006). Different metabolite profiles have been demonstrated in *F. oxysporum* subjected to varying carbon sources and oxygen supply, and to a less significant degree during the cultivation process (Panagiotou *et al.*, 2005). Comprehensive analysis of an entire metabolome is technically a great challenge, but techniques are constantly being improved. Mathematics and complicated analytical tools have played a significant role in functional genomics and metabolomics (Jewett *et al.*, 2006).

Chemical Genomics

Chemical genomics is based on the genome-wide response of biological systems to chemical compounds. The chemicals used can be any low molecular weight compound that affects entire gene families in either the pathogen or in the host, thereby influencing disease development. This approach essentially captures chemical control of a pathogen on a different level. Chemical genomics has been applied for many years and was recently revived when it was used to detect functional systems influencing the non-host resistance of pea to *Fusarium solani* f. sp. *phaseoli*. Analyses of the genomic responses varied from microscopic monitoring of microbial growth to RT-PCR gene expression (Hartney *et al.*, 2007). An alternate view of chemical genomics is described by the *laeA* gene, which has been characterized in *Aspergillus* spp. *LaeA* is an example of a global transcriptional regulator that modulates the production of multiple secondary metabolite gene clusters, as well as fungal development, in *Aspergillus* (Perrin *et al.*, 2007; Bayram *et al.*, 2008). Knowledge of such regulators of primary and secondary metabolism in fungi can provide new opportunities to suppress the expression of metabolic pathways required for fungal pathogenicity.

CONCLUSION

Fusarium wilt diseases, caused by the soil-borne fungal pathogen *F. oxysporum*, are of great economic importance worldwide and affect many valuable agricultural crops. The diseases caused by *F. oxysporum* are difficult to control, with resistant plant varieties offering the best opportunity to reduce crop losses. More often than not, resistant varieties are not available or acceptable to local producers and consumer markets. Traditional disease management practices, therefore, have to be replaced by innovative disease management strategies. The field of molecular plant-pathogen interactions has developed rapidly in recent years and offers new strategies for increasing plant resistance against specific Fusarium wilt pathogens by exploiting the molecular and cellular basis of pathogenicity.

The genome of *F. graminearum* (*Gibberella zeae*), an airborne pathogen closely related to *F. oxysporum*, was fully sequenced and annotated between 2002 - 2007 (Di Pietro *et al.*, 2003). The genome sequences of *F. verticillioides* and *Fol* followed, and data were released on-line to the public from 2005 - 2007 (http://www.broad.mit.edu/annotation/genome/fusarium_group/Info.html). Although the genomes of the latter species have not been completely annotated, their sequence information is vital to studies on *F. oxysporum*. Despite differences in genome size and sexual reproduction, important comparisons and deductions can be made for the purposes of gene identification. To date, this has been achieved primarily by generating mutant libraries that are screened for loss of pathogenicity or reductions in virulence, followed by targeted disruption of promising gene candidates (Duyvesteijn *et al.*, 2005).

Molecular technologies for studying host-pathogen interactions are constantly evolving. Forward and reverse genetics today offer an opportunity for the high-throughput study of in-depth host-pathogen interactions. While studies on pathogens were initially limited to greenhouse and field inoculations, high-throughput technologies, such as ATMT, have now increased the efficiency and accuracy of pathogenicity and virulence investigations. Research on fungal pathogenicity is further enhanced by suitable and rapid screening techniques, enhanced vectors for increased transformation efficiencies, whole-genome sequences and appropriate bioassays for mutant infection studies. The fields of proteomics and metabolomics further provide opportunities to better understand the genetic and biochemical pathways involved in pathogenesis and virulence to develop targets for fungicides, biocontrol agents and novel antifungal drugs.

To date, most research on plant pathogenesis and virulence in the F. *oxysporum* wilt pathogen complex has been focused primarily on *Fol*. The genome of *Fol* has been sequenced, and at least 10 of its genes have been associated with pathogenicity. In *Fol*, a classic gene-for-gene resistance response to races of the fungus has been demonstrated, making it the first example of this type for *F. oxysporum*. Little, however, is known about the genetic factors conferring pathogenicity to the

Fusarium wilt pathogen of bananas, as only a limited amount of research has been done on aspects related to plant-pathogen interactions, fungal genomics, pathogenicity and resilience to chemical control. Fusarium wilt of banana is arguably the most destructive *formae specialis* of *F. oxysporum* and almost destroyed the banana export industry based on Gros Michel bananas in Central America in the mid-20th century. Apart from plant resistance, no sustainable solution has been found to control the disease since its discovery in 1876 (Ploetz and Pegg, 2000).

The size and structure of the *Foc* genome varies from 9-12 chromosomes, with a predicted genome size of 32-59 Mbp (Boehm *et al.*, 1994). Transposons such as *impala* has been found in *Foc* and could influence the size of its genome (Hua-Van *et al.*, 2001). To date, 13 *Foc* genes or gene regions have been sequenced and submitted to Genbank (Table 6). In terms of potential virulence or pathogenicity genes, only the mitogen-activated protein kinase (*fmk1*), mitochondrial carrier protein (*fow1*), polygalacturonase 1 (*pg1*) and xylanase (*xyl3*) genes have been identified to date in *Foc* (Gevers, 2004; Groenewald, 2005). The banana Fusarium wilt pathogen is different from other *formae speciales* of *F. oxysporum* in that the Cavendish banana is sterile, making classical resistance breeding to *Foc* impossible (Ploetz, 2005). *Foc* has no definite race structure, which complicates pathogenicity studies that are laborious and often inaccurate. Also, there is only a limited amount of genetic information available on *Foc*. Due to these limitations in *Foc* research, it is crucial to look at related *formae speciales* for clues, where more information is available, though it may be somewhat nonconforming. An in-depth understanding of the molecular basis of pathogenicity of the *Foc* banana pathogen is required if any progress is to be made towards disease control.

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Table 1. Some common *formae speciales* of *Fusarium oxysporum* and their associated hosts (Armstrong and Armstrong, 1981)

Formae speciales	**Host**
Cubense	Banana
Dianthi	Carnation
Lycopersici	Tomato
Melonis	Muskmelon
Phaseoli	Common bean (*Phaseolus vulgaris* L.)
Vasinfectum	Cotton, Alfalfa, Soybean, Tobacco

Table 2. Mycotoxins in *Fusarium oxysporum* and their functions (Woolley, 1948; Marasas *et al.*, 1984; Moretti *et al.*, 2002; Bell *et al.*, 2003; Desjardins, 2006; Rodríguez *et al.*, 2006)

Mycotoxin	Chemical Structure	Host symptoms / Biology
Beauvericin	http://commons.wikimedia.org/ wiki/Image:Beauvericin.svg	• Natural infection of maize • Common metabolite • Not related to pathogenicity
Bikaverin / Norbikaverin	Bikaverin: R = $-CH_3$ Norbikaverin: R = -H	• Natural infection of sorghum
Cyclosporine A	http://www.qwbio.com/cn/ BS_productshow.asp?Articleid=993	• From non-pathogenic *F. oxysporum* • Suppression of *Sclerotinia sclerotiorum*
Diacetoxyscirpenol		• Type A Trichothecene • Natural infection of safflower
Diacetylnivalenol		• From culture isolates
7,8-Dihydroxydiacetoxyscirpenol		• From culture isolates
Eniatin		• Wilting of tomato shoots • Growth inhibition of wheat seedlings • Dry rot of potato tubers • Cation chelating agent • Ionophore • General antibiotic • Low - moderate toxicity
Fumonisin B1	www.fermentek.co.il/fumonisin_B1.htm	• Human esophageal cancer • Colonization of corn • Toxic and carcinogenic in rats and pigs • Pulmonary edema in pigs

Mycotoxin	Chemical Structure	Host symptoms / Biology
Fusarenon-X		• Type B Trichothecene • From culture isolates • Natural infection of cereals
Fusaric acid		• Wilt in rice seedlings • Wide biological activity in animals • Unknown function • Low - moderate toxicity
7-Hydroxydiacetoxyscirpenol		• Trichothecene • From grain-infecting species
Lycomarasmin		• Wilting and curling of excised tomato leaves
Moniliformin		• Toxic to 1-day-old chickens • Less toxic to larger mammals • Unknown symptoms in plants • Inhibition of the oxidation of pyruvate and α-ketoglutarate • Inactivation of pyruvate dehydrogenase • Inhibition of mitochondrial oxidation • Pathological effect on heart tissue • Low - moderate toxicity
Naphtoquinone		• Toxicity to microbes, plants and some animals • Forms free radical intermediates • Broad toxicity range
Neosolaniol		• Type A Trichothecene
Sambutoxin		• Toxic to rats - hemorrhage of the digestive system • Cytotoxic to chicken embryos • Unknown function

Mycotoxin	Chemical Structure	Host symptoms / Biology
T-2 toxin	http://www.fermentek.co.il/t2_toxin.htm	• Type A Trichothecene • Highly toxic • From infected grain
Zearalenone	www.micotoxinas.com.br	• Natural infection of cereals • Effect on reproductive system of mammals

Table 3. Genes linked to virulence/pathogenicity in *Fusarium oxysporum**

Gene	Product / Protein	Function	Fungus	Reference
*abf*B	α-$_L$-Arabinofuranosisdase B	CWDE – hydrolyses terminal non-reducing linkages from α-$_L$-Arabinofuranoside, arabinans, arabinoxylans & arabinogalactans. *Possible* role in virulence.	*Fod*	(Chacón-Martínez *et al*., 2004)
arg1	Argininosuccinate lyase	Arginine biosynthesis	*Fom*	(Namiki *et al*., 2001)
chsV	Class V chitin synthase	Resistance to α-tomatine & H_2O_2	*Fol*	(Madrid *et al*., 2003)
chs1, chs2, chs3 & chs7	Structural chitin synthase (1-3) – Class II, & chaperone-like protein (7)- Class IV	Chitin anabolism, virulence	*Fol*	(Martín-Udíroz *et al*., 2004)
cyp55A1	Cytochrome $P450_{norA}$ & $P450_{norB}$	Nitric oxide reduction	*Fo*	(Van den Brink *et al*., 1998)
fga1	G protein α subunit (a)	Conidiation, heat resistance & reduced pathogenicity	*Foc*	(Jain *et al*., 2002)
fga2	G protein α subunit (b)	Heat resistance & loss of pathogenicity	*Foc*	(Jain *et al*., 2005)
fgb1	G protein β subunit	Conidiation, developmental regulation & pathogenicity	*Foc, Fol*	(Jain *et al*., 2003; Prados-Rosales *et al*., 2006)
fmk1	Mitogen Activated Protein Kinase (MAPK)	Pathogenicity, root attachment & invasive growth	*Fol*	(Di Pietro *et al*., 2001a; Prados-Rosales *et al*., 2006)
fnr1	*Fusarium* nitrogen regulator	Fungal fitness during infection, regulation of secondary nitrogen acquisition	*Fol*	(Divon *et al*., 2006)
FoTom1	Tomatinase	Degrades plant α-tomatine to non-toxic compounds tetrasaccharide lycotetraose and tomatidine.	*For*	(Ito *et al*., 2004a; Ito *et al*., 2004b)

Gene	Product / Protein	Function	Fungus	Reference
fow1	Mitochondrial carrier protein	Plant colonization & pathogenicity	*Fol*	(Inoue *et al*., 2002)
fow2	Zn(II)2Cys6-type transcription regulator	Plant infection	*Fom*	(Imazaki *et al*., 2007)
frp1	F-box motif protein	Pathogenicity, interaction with *skp1*, colonization	*Fol*	(Duyvesteijn *et al*., 2005)
ftf1	*Fusarium* transcription factor 1	Fungal establishment / progress in the host, increased virulence with multiple copies	*Foph*	(Ramos *et al*., 2007)
gas1	β-1,3-glucanosyltransferase	Virulence on tomato & growth on solid substrates	*Fol*	(Caracuel *et al*., 2005)
pacC	pH signaling transcription factor	Negative regulator of virulence by preventing transcription of acid-expressed genes important for infection.	*Fol*	(Caracuel *et al*., 2003)
PEP cluster	Pathogenicity gene cluster	Normal growth in culture & pathogenicity	*Fopi*	(Temporini and VanEtter, 2004)
pir2	Cell wall glycoprotein	Protection against plant osmotin (PR-5)	*Fon*	(Narasimhan *et al*., 2003)
ren1	Homolog of *MedA* of *Aspergillus nidulans* and *Acr1* of *Magnaporthe grisea* – presumed conidiogenesis transcription regulator	Development of micro- and macroconidia, but not chlamydospores	*Fo*	(Ohara *et al*., 2004)
snf1	Sucrose non-fermenting protein	Virulence, cell-wall degradation and infection	*Fo*	(Ospina-Giraldo *et al*., 2003)
sti35	Thiamine biosynthesis protein	Thiamine biosynthesis and oxidative stress. Early & late infection.	*Fol*	(Ruiz-Roldan *et al*., 2008)

* *Fo*: *Fusarium oxysporum*; *Foc*: *F. oxysporum* f. sp. *cucumerinum*; *Fod*: *F. oxysporum* f. sp. *dianthi*; *Fol*: *F. oxysporum* f. sp. *lycopersici*; *Fom*: *F. oxysporum* f. sp *melonis*; *Fon*: *F. oxysporum* f. sp. *nicotianae*; *Foph*: *F. oxysporum* f. sp. *phaseoli*; *Fopi*: *F. oxysporum* f. sp. *pisi*; and *For*: *F. oxysporum* f. sp. *radicis-lycopersici*.

Table 4. Transposons present in *Fusarium oxysporum*

Transposon	**Reference**
drifter	(Rep *et al.*, 2005)
hop	(Davière *et al.*, 2001)
impala	(Langin *et al.*, 1995; Brown and Holden, 1998; Hua-Van *et al.*, 2001)
folyt	(Gómez-Gómez *et al.*, 1999)
fot1	(Migheli *et al.*, 2000; Trouvelot *et al.*, 2002)
palm	(Davière *et al.*, 2001)
skippy	(Anaya and Roncero, 1995)
tfo1	(Okuda *et al.*, 1998)

Table 5. Differences between *Fusarium oxysporum* and *Fusarium graminearum* (Di Pietro *et al.* 2003, (Di Pietro *et al.*, 2003; Broad Institute, Cambridge, MA, USA)

Fusarium oxysporum	***Fusarium graminearum***
Soilborne, vascular pathogen	Aerial pathogen
Asexual	Complete sexual cycle
Genome size: 61.26 Mb	Genome size: 36.45 Mb
Genome more dynamic: 7-14 chromosomes	Genome less dynamic: 5 chromosomes
Whole genome sequenced, but not completely annotated	Whole genome sequenced and annotated

Table 6. Known *Fusarium oxysporum* f. sp. *cubense* (*Foc*) nucleotide sequences (Genbank)

Entry	Accession	Definition	Purpose of study	Year
1[a]	EF155534	ITS 1; ITS 2; 5.8S ribosomal RNA gene; 18S ribosomal RNA gene; and 28S ribosomal RNA gene	Molecular detection of *Foc*	2007
2[a]	EF155535	*Foc*-FT marker	Detection and quantification of *Foc* from infected plant tissue and soil	2007
3[b]	AY527732	28S ribosomal RNA gene and IGS region	Genetic Diversity Studies	2004
4[c]	AY217201	Mitochondrial direct repeat region	Phylogenetics	2004
5[c]	AY217169	Translation elongation factor-like gene	Phylogenetics	2004
6[c]	AY217168	Translation elongation factor gene	Phylogenetics	2004
7[c]	AY209176	MAT-2 protein gene	Mating potential of *Foc* race 4	2004
8[d]	AY040734	MAT-1 protein gene	Mating type genes	2002
9[e]	AF393303	Mitochondrial tRNA-Tyr / Cytochrome C oxidase subunit 3; ITS region and tRNA-Ser gene, complete sequence	Uniparental mitochondrial inheritance	2001
10[f]	AF363414	Transposon *impala* Cu-12	Evolutionary history of the impala	2001
11[f]	AF363415	Transposon *impala* Cu-15	Evolutionary history of impala	2001
12[g]	AF008460	SSU rRNA gene	Evolutionary origins	1998
13[h]	AF008529	ß-tubulin gene	ß-tubulin gene	1998

[a] Chen, Q. *et al*., (2007).. Unpublished Data
[b] O'Donnell, K. *et al*., (2004). *Journal of Clinical Microbiology* 42: 5109-5120
[c] Visser, M. *et al.,* (2004). Unpublished Data
[d] Kuhn *et al*., (2002). Unpublished Data
[e] D'alessio, N. *et al*., (2001). Unpublished Data
[f] Hua-Van, A. *et al*., (2001). *Molecular Biology and Evolution* 18: 1959-1969
[g] O-Donnell, K. *et al*., (1998). *PNAS* 95: 2044-2049
[h] Cigelnik, E. and O'Donnell, K. (1997). Unpublished Data

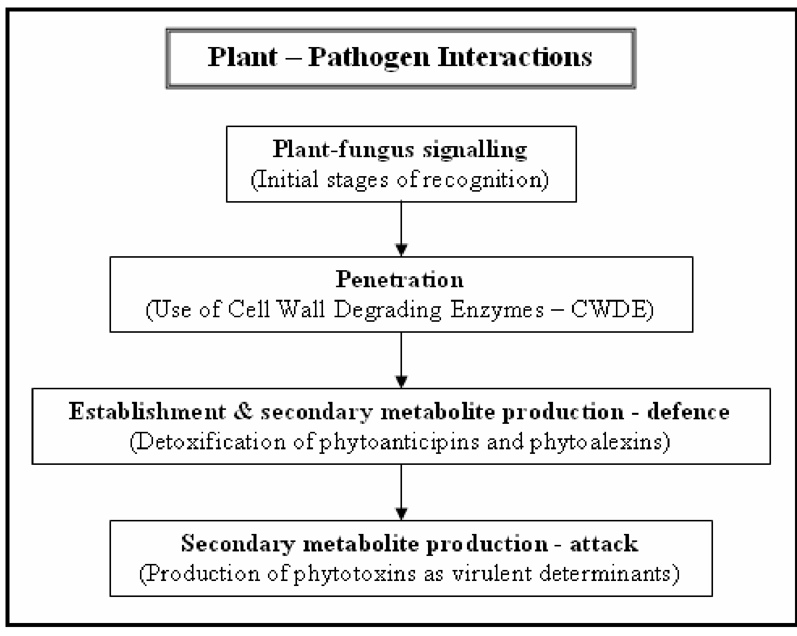

Figure 1. Plant-pathogen interactions in *Fusarium oxysporum* (Roncero *et al*., 2003).

CHAPTER 2

DEVELOPMENT OF AN *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION SYSTEM FOR *FUSARIUM OXYSPORUM* F. SP. *CUBENSE*

ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*), causal agent of Fusarium wilt of bananas, is one of the most destructive pathogens in the world. Apart from the development of plant resistance, no sustainable solution has been found to control Fusarium wilt. Understanding the genetic basis of pathogenesis in *Foc* might, however, contribute to developing novel strategies to control the disease. A number of molecular methods have been developed in recent years to study pathogenicity in fungal phytopathogens. *Agrobacterium tumefaciens*-mediated transformation (ATMT), for instance, has been used for random and targeted gene disruption to determine the function of genes involved in fungal pathogenicity. The objectives of this study were to develop an efficient ATMT system conferring random vector integration into the genome of *Foc*, to rescue genome sequences flanking inserted T-DNAs for gene function analyses, and to possibly identify new *Foc* pathogenicity genes disrupted by ATMT vector insertion. Transformation was achieved with four *A. tumefaciens* strains using a vector conferring Hygromycin B resistance and expression of green fluorescent protein. Vector insertion was confirmed with molecular methods and fluorescence microscopy. We initiated the creation of a random insertion mutant library of *Foc*, and transformants were screened for a) number of T-DNA inserts, b) location/identity of T-DNA insertions, and c) *in planta* for alterations in pathogenicity. Results showed the best transformation efficiency was obtained with *A. tumefaciens* strain EHA105/S. One or two inserts were detected for the majority of transformants. Genomic DNA flanking the individual T-DNA inserts revealed 20 unique right border fragments, but limited gene predictions could be made. Pathogenicity studies revealed three isolates with significantly reduced virulence but analyses of DNA flanking these T-DNA inserts did not reveal definitive gene identities. Information arising from this research will contribute to an enhanced understanding of fungal pathogenesis in *Foc* and assist in the development of alternative control approaches for the management of Fusarium wilt of banana.

INTRODUCTION

Fusarium oxysporum Schlecht. consists of more than 150 different *formae speciales* that are determined by host specificity (Baayen *et al.*, 2000; Di Pietro *et al.*, 2003). One of the most destructive is *F. oxysporum* f. sp. *cubense* (E.F. Sm.) W.C. Snyder & H.N. Hansen (*Foc*), causal agent of Fusarium wilt of banana. *Foc* race 1 became notorious when it almost destroyed Gros Michel banana production in Central America during the mid-20th century, necessitating replacement of Gros Michel with resistant Cavendish cultivars. Cavendish bananas have now proven to be highly susceptible to a different race of *Foc*, called race 4 (Ploetz, 1990; Ploetz and Pegg, 2000). Apart from the development of plant resistance, no sustainable solution has been found to control Fusarium wilt. Little is known about the genetic factors conferring pathogenicity of *Foc* to banana. Understanding the genetic basis of pathogenesis in *Foc* might, however, contribute to developing novel strategies to control the disease.

A number of molecular techniques have been developed to study pathogenicity in fungal phytopathogens, including *Fusarium* spp. For example, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been used as an efficient tool for random and targeted gene disruption in a variety of fungi and plants for the ultimate purpose of determining gene function (De Groot *et al.*, 1998; Covert *et al.*, 2001; Mullins and Kang, 2001; Mullins *et al.*, 2001; Gelvin, 2003). Other less efficient transformation methods include protoplast-mediated transformation (Kistler and Benny, 1988; Fincham, 1989; Ballance, 1991; Betts *et al.*, 2007), restriction enzyme-mediated integration (REMI) (Sweigard *et al.*, 1998; Kahmann and Basse, 1999; Maier and Schäfer, 1999) and electroporation (Ward *et al.*, 1989). Protoplast-mediated transformation has already been achieved in *Foc* through expression of green fluorescent protein (GFP) (Visser *et al.*, 2004) and DsRed (red fluorescent protein) (McLeod *et al.*, 2006) genes.

ATMT is a method that has been applied in various studies since 1996, but has only been fully utilized in the new millennium. The first ATMT of fungi was done by Bundock and Hooykaas (1996) with *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, and that of the filamentous fungi followed with great success in 1998 (De Groot *et al.*, 1998). ATMT is carried out by integrating transfer (T)-DNA from *A. tumefaciens* into the genome of a recipient cell. For such integration, the T-DNA first has to be cleaved at the right and left border sequences (Meng *et al.*, 2007), and the T-DNA carrying a selectable marker is randomly inserted resulting in random mutations of genomic DNA (Michielse *et al.*, 2005a; Weld *et al.*, 2006). More than 50 fungi have been transformed using this method (Michielse *et al.*, 2005a), which has led to the identification of many functional genes, including those involved in fungal pathogenicity (Di Pietro *et al.*, 2003; Betts *et al.*, 2007; Chapter 1). Advantages of ATMT include relatively high transformation efficiencies, the ability to transform spores and mycelia directly without protoplasting, low insert copy numbers, the ability to recover DNA sequences flanking insertion sites due to the presence of a tag, and limited rearrangements of

genomic insertion sites (Mullins and Kang, 2001; Mullins *et al.*, 2001; Khang *et al.*, 2005; Malz *et al.*, 2005; Michielse *et al.*, 2005a; Michielse *et al.*, 2005b; Sugui *et al.*, 2005; Weld *et al.*, 2006; Betts *et al.*, 2007). Transformation of different types of fungal tissue, such as protoplasts, hyphae, spores, and blocks of mushroom mycelia (De Groot *et al.*, 1998; Mullins *et al.*, 2001), is also possible. To date, ATMT is regarded as the most efficient method for transformation and gene disruption studies (Mullins *et al.*, 2001; Betts *et al.*, 2007).

A successful random mutagenesis transformation project relies on the following essential points: 1) creation of a random mutant library containing transformants with phenotypic differences, single T-DNA insertions and minimal genomic disturbances, 2) a high-throughput, reproducible screening method to detect altered phenotypes, 3) the recovery of sequences of tagged genes from the genome, and 4) verification that a disrupted gene is responsible for a specific phenotypic change (Mullins and Kang, 2001). The objectives of this study were to develop an efficient transformation system for *Foc*, to demonstrate that genome sequences flanking the inserted T-DNAs could be rescued for gene function analyses, and to possibly identify new *Foc* pathogenicity genes that were disrupted by ATMT vector insertion.

MATERIALS AND METHODS

Fungal and Bacterial Isolates

An isolate of *Foc*, CAV 105, with proven ability to cause disease in Cavendish bananas in the subtropics was used for ATMT. The pathogen was grown on half strength potato dextrose agar (½X PDA) (Merck, Whitehouse Station, USA) at 25°C under cool white and near-ultraviolet fluorescent light with a 12-hour photoperiod for 7–10 days. Hygromycin B sensitivity of CAV 105 was tested by plating the isolate onto PDA with hygromycin concentrations of 0, 50, 100 or 150 mg/l in triplicate. After ATMT, fungal isolates were grown routinely on ½X PDA with hygromycin B (100 mg/l) to maintain selection of the vector.

Agrobacterium tumefaciens strains assayed for ability to function in ATMT of *Foc* included AGL1, EHA105, LBA4404 and GV3101 (provided by B.G.G. Donzelli and A.C.L. Churchill, Cornell University, USA) (Table 1). Each strain was first selected and single-colony purified on antibiotic-containing yeast extract peptone (YEP) medium grown at 28°C for 24-48 hours (Table 1), then transformed by electroporation with the random gene disruption plasmid pBDg-GFP-A (B.G.G. Donzelli and A.C.L. Churchill, unpublished data). Recombinant *A. tumefaciens* cells containing pBDg-GFP-A were provided by Dr. Donzelli (Cornell University, USA) and created essentially as described previously (Moon *et al.*, 2008). pBDg-GFP-A consists of the binary vector pPK_2 (Covert *et al.*, 2001), which contains the hygromycin B gene (Punt *et al.*, 1987) as a selectable marker and *Aspergillus nidulans* (Eidam) G. Winter promoter (*Pgpd*) and terminator (*trpC*) fragments. An sGFP-fragment from pAN7.1 was inserted on the inside of the right border of vector pPK_2 to

produce the 13.92 kb pBDg-GFP-A plasmid (Figure 1). It is notable that *A. tumefaciens* strain EHA105 consistently grew as both small and large colonies on selection plates; derivative single-colony isolates used for ATMT were subsequently annotated as EHA105/S and EHA105/L, respectively.

Agrobacterium tumefaciens*-Mediated Transformation of *Foc

ATMT of *Foc* was carried out at Cornell University, Ithaca, NY, (USA) as described by Covert *et al.* (2001) with modifications essentially as described by Moon et al. (2008) unless otherwise noted here. *A. tumefaciens* cells containing pBDg-GFP-A were plated from frozen glycerol stocks onto YEP plates containing antibiotic (Table 1). After growing overnight (16 - 20 hours), a single colony was transferred to 7 ml YEP broth with antibiotic and incubated overnight with shaking at 29°C. The cells were then resuspended in induction medium (IM) (Bundock and Hooykaas, 1996) containing 200 µM acetosyringone (AS) (Acros Organics, Geel, Belgium) (IMAS) to a volume of 10 ml with an OD_{660} of 0.05. The culture was then grown overnight to a final OD_{600} of 0.6 - 0.8.

Foc spores were harvested from ½X PDA plates using IMAS broth, and the spore suspension was diluted with IMAS to 10^5 spores/ml. An aliquot (100 µl) of the diluted fungal spore suspension was then mixed with 100 µl of IMAS-induced *A. tumefaciens* cells and spread evenly on sterile black filter papers (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) placed on IMAS medium (1.5% agar). These plates were incubated at 21°C for 2 days in the dark. After co-cultivation, the black filter papers supporting the *A. tumefaciens* and *Foc* spore mixture were transferred to a selection medium (M-100 containing 300 mg/l mefoxin or carbenicillin, and 75 mg/l hygromycin), and covered with an overlay prepared from M-100 medium (0.9% agar) containing the same antibiotics (Moon *et al.*, 2008). Since AGL1 is resistant to carbenicillin, mefoxin was used for selection of this strain. Plates were incubated in the dark at 21°C. Putative transformants were visible as rapidly growing circular colonies and were transferred to fresh M-100 plates containing 300 mg/l mefoxin or carbenicillin and 75 mg/l hygromycin and incubated at 25°C under light.

Colony counts to assess transformation efficiency were performed up to 10 days after selection. For each of the five *A. tumefaciens* strains assayed for ATMT, 30 *Foc* transformants were selected for long-term storage at -70°C, to give a final collection of 150 ATMT *Foc* isolates. These ATMT *Foc* isolates were sent to South Africa, where they were sub-cultured, and monoconidial isolations prepared. The 150 isolates are maintained in 15% glycerol at -70°C, and as freeze-dried cultures at 5°C, at the facilities of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

A total of 25 *Foc* transformants, five from each *A. tumefaciens* strain, were then randomly selected for further screening. GFP expression was confirmed by epifluorescence microscopy. Mitotic stability of the transformants was tested by plating each onto ½X PDA without antibiotic selection,

followed by sequential transfers onto fresh plates each week for 5 consecutive weeks. During the 6th week, each transformant was transferred back to ½X PDA containing 50 mg/l hygromycin to confirm stable resistance to the drug (Covert *et al.*, 2001).

Genetic Analysis of Putative Transformants

DNA extraction: DNA was extracted from ATMT *Foc* isolates according to a modified phenol-chloroform method (Groenewald *et al.*, 2006). DNA pellets were suspended in 200 µl double distilled SABAX water (Adcock-Ingram, Bryanston, South Africa) and treated with 5 µl of RNase (1mg/ml) (Fermentas, Burlington, Ontario, Canada). The integrity of each DNA sample was determined on a 1% agarose gel (Roche Molecular Biochemicals, Mannheim, Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and aliquots of each sample were prepared for PCR amplification at a final concentration of 100 ng/µl.

PCR amplification of the hygromycin-gene-and-terminator (HT) fragment: Genomic DNA of the ATMT *Foc* isolates and plasmid pBDg-GFP-A DNA were used independently for amplification of the 1.58 kb *hph-TrpC* fragment, which encodes the hygromycin gene and TrpC terminator. Primers for detection of the *hph-TrpC* fragment (Table 2) were designed from the DNA sequence of pAN7.1 (Genbank accession number: Z32698.1) using DNAMan Demo Version 4.13 (Lynnon Biosoft, Vaudreuil, Canada). PCR reactions included 2.5 µl 10X PCR buffer, 1.4 µl 50 mM $MgCl_2$, 2.5 µl 2.5 mM dNTP mix (Fermentas), 0.5 µl each of 10 mM primers HT-F (Table 2) and HT-R (Inqaba, Pretoria, South Africa), 0.2 µl 5 U/µl Taq polymerase (Bioline, London, UK), 1 µl template DNA, and dH_2O to a total volume of 25 µl. The PCR thermocycle profile consisted of an initial denaturation temperature of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 65°C for 35 s and elongation at 72°C for 90 s, and was completed with a final elongation at 72°C for 7 min. PCR products (5 µl) were analyzed on a 2% agarose gel run at 80V in 1X TAE buffer. For size determination, the O'GeneRuler™ 100 bp DNA Ladder Plus marker (Fermentas) was used.

DNA sequencing and analysis of the HT fragment: Sequencing of the plasmid HT-fragment was performed as a control to confirm that the correct product had been amplified with the designed primers. HT-fragment PCR amplicon was purified using the High Pure PCR Purification kit (Roche Molecular Biochemicals) according to manufacturer's instructions, and eluted with 30 µl elution buffer. These fragments were then sequenced using the BigDye kit, version 3.1 (Roche Applied Sciences, Penzberg, Germany). Sequencing was performed on an ABI3100 instrument (AME Bioscience, Toroed, Norway). DNA sequences were visualized, verified and analyzed using Chromas 1.45 (www.technelysium.com.au/chromas.html) and BioEdit Sequence Alignment Editor 6.0.7 (Tom Hall Isis Pharmaceuticals, Inc.) software. PCR amplicons were aligned to the sequence on Genbank (www.ncbi.nlm.nih.gov), and BLASTN searches (www.ncbi.nlm.nih.gov/BLAST) were done to confirm the identity of the fragment. This fragment was also used as template for probe

synthesis for Southern analysis.

Southern Analysis

Probe preparation: The random gene disruption vector pBDg-GFP-A was cloned and amplified in competent *E. coli* (JM109) cells (Promega Corporation, Madison, WI, USA) by heat-shock according to manufacturer's instructions, using half reactions. Plasmid-containing colonies were selected by growing the cells on LB plates containing 100 mg/l kanamycin (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany). Plasmids were extracted from positive transformants using the Genelute plasmid mini-prep kit (Sigma-Aldrich Chemie), following the manufacturer's instructions. The HT probe was synthesized from purified plasmid template by PCR DIG-labeling and amplification of the hygromycin-terminator fragment according to the PCR DIG Probe Synthesis Kit (Roche). Labeling of the probe occurred in a labeling mixture with a 1:6 ratio of DIG-dUTP:dTTP using primers HT-F and HT-R (Table 2). The labeled probe was run on a 1% agarose gel with 1X TAE buffer to assess its integrity and concentration.

Southern Blot: DNA of each of 25 ATMT *Foc* isolates was restriction digested in a 100 µl reaction volume containing 10 µg fungal genomic DNA, 5 µl each of FastDigest *Eco*RV and *Kpn*I (Fermentas), 10 µl 10X FastDigest buffer and sterile SABAX water, at 37°C for 16 hours. An aliquot of 5 µl of each digest was then run on a 1% agarose gel with 1X TAE buffer to confirm complete digestion. The digested DNA was precipitated with 100% ethanol and 0.1% NaOAc, and resuspended in 10 µl 1X TE buffer. Samples were then denatured at 65°C for 10 min, cooled on ice, and run on a 1% agarose gel with 1X TAE buffer and 0.0001% EtBr at 2 V/cm for approximately 6 hours. Controls included digestions of plasmid and wild-type DNA (CAV 105) with the same enzymes, as positive and negative control, respectively, and a DIG-labeled DNA molecular weight marker VI (Roche). Southern analysis was performed according to the DIG labeling and detection manual (Roche). Hybridization was done at 42°C in a hybridizing oven (Techne Hybridizer HB-1D, Techne, Cambridge, England). Colorimetric visualization was achieved with the NBT/BCIP solution (Roche), after which the membrane was dried, scanned and analyzed to determine T-DNA copy number.

TAIL-PCR Analysis of Genomic DNA Flanking the T-DNA Insert

Thermal Asymmetric Interlaced (TAIL)-PCR amplification, purification of tertiary products, and the sequencing thereof, were carried out according to methods described by Liu and Whittier (1995) and Mullins *et al*. (2001). TAIL-PCR amplicons of the wild-type *Foc* isolate were loaded onto gels in lanes adjacent to transformants to indicate which PCR products in the transformant lanes were non-specific. Left-border amplification could not be achieved, but right-border products were obtained by making use of specific (RB1, RB2 and RB3) and AD2 degenerate primers (5'tcmaggaigcygcyac3') (C.B. Michielse, University of Amsterdam, Netherlands, personal communication). Following sequencing with the RB3 primer, TAIL fragments were submitted to the *Fusarium* group (*F. graminearum*, *F. verticillioides* and *F. oxysporum* f.sp. *lycopersici*) BLAST

database (http://www.broad.mit.edu/annotation/genome/fusarium_group/Blast.html?sp=Sblastn) and the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) for identification and possible gene function prediction.

Pathogenicity Studies

To determine whether pathogenicity had been impaired in the ATMT *Foc* isolates, a spore suspension for each of 25 transformants (5 per *Agrobacterium* strain assayed) was prepared for pathogenicity testing. The wild-type pathogenic *Foc* isolate (CAV 105) and a mixture of two other pathogenic isolates of *Foc* (CAV 045 and CAV 092) were used as positive controls. Conidial suspensions were prepared by adding sterile, distilled water to each of the 10-day-old cultures; dislodged conidia were filtered through cheese cloth and adjusted to a final concentration of 1 x 10^5 spores/ml. Water was used as negative control. Pathogenicity tests were conducted using 10 cm tissue culture-derived Cavendish banana plantlets (cultivar Williams) according to the methodology described by Nel *et al.* (2006), with some modifications. The roots of the banana plantlets were first slightly compressed to produce entry sites for the fungus before they were introduced into 250 ml polystyrene cups containing 200 ml spore suspension. The plants were kept in a greenhouse at 28°C day/21°C night with a 12-hour photoperiod, and each plant was fertilized every 2 weeks with 2 ml liquid banana fertilizer consisting of 0.6 g/l calcium nitrate monohydrate, 0.9 g/l Agrasol® 'O =3:2:8 (Fleuron, Braamfontein, South Africa), and 3 g/l Micromax® (Fleuron). Four weeks after inoculation, Fusarium wilt symptoms in the inner rhizome were rated according to a modified method of Carlier *et al.* (2002), where disease severity was rated from 0 to 5 (Figure 2), rather than from 1 to 6. Ten replicates were used for each isolate, and the experiment was repeated three times. For statistical comparisons, a factorial ANOVA was used by means of Statistica Version 7.1 (Statsoft, 2006). The Tukey HSD test was used for *post-hoc* analysis.

RESULTS

Agrobacterium tumefaciens-Mediated Transformation of *Foc*

Successful ATMT of *Foc* isolate CAV 105 was achieved with all five *A. tumefaciens* strains (including EHA105/S and EHA105L). Transformants developed as early as 7 days post-transformation (dpt) and were collected daily for 4 days. Transformation efficiencies ranged from 100 to 300 transformants per 1 × 10^6 conidia (Table 3), but an accurate calculation could not be made as the plates became completely overgrown after 12 days. The best transformation efficiency was obtained with EHA105/S, which produced 122 transformants at an average rate of 31 transformants per plate (Table 3). GV3101 was the least efficient in ATMT, with only 34 transformants being produced at a rate of nine transformants per plate. The three other *A. tumefaciens* strains produced more or less equivalent numbers of transformants. Early developing transformants had a faster growth rate in comparison to the ones emerging later.

The 25 ATMT *Foc* isolates that were analyzed further were similar to the wild type isolate in cultural morphology, except for four isolates that were not mitotically stable and grew substantially slower on the ½X PDA amended with antibiotics. Three of these isolates had been generated via ATMT with EHA105/L and one with AGL1, and were excluded from further screening. The stable transformants produced white aerial mycelia, with a light to dark purple pigmentation visible on the bottom side of the culture dishes. Micro- and macroconidia and chlamydospores produced by transformants were similar to those described for *F. oxysporum* (Leslie and Summerell, 2006). T-DNA insertion was established by visual observation of GFP expression in the spores and mycelium of transformed isolates (Figure 3). Results obtained by epifluorescence microscopy were confirmed by PCR analyses, as *Foc* isolates that were transformed via ATMT produced a 1580 bp hygromycin gene amplicon after PCR, compared to the wild type CAV 105 isolate, which lacks the hygromycin B gene (Figure 4).

Southern Analysis

Incorporation of the DIG-labeled probe into the hygromycin gene fragment resulted in a PCR amplicon of approximately 2000 bp compared to the unlabelled amplicon of 1580 bp (Figure 5). When separated on a 1% agarose gel, the concentration of probe was estimated at 200 ng/ul. Restriction enzyme digestion of genomic DNA of the ATMT *Foc* isolates produced a smear that ranged approximately from 0.3-10 kb (Figure 6). The uncut genomic DNA, in contrast, produced a single broad band of ~25 Mb. When subjected to Southern hybridization, the probe hybridized to homologous sequences at different positions on the blot, suggesting that random integration of the T-DNA took place (Figure 7). No hybridization was observed in the untransformed wild-type *Foc* isolate (data not shown). A single T-DNA insert was found in 32% of the transformants, while 64% of the ATMT *Foc* isolates contained two inserts, and 4% contained four inserts (Figure 8 and data not shown). All the *A. tumefaciens* strains generated *Foc* transformants that hosted two inserts, while all but EHA105/L also produced transformants hosting one insert. EHA105/L, however, was the only strain that produced transformants hosting four inserts.

TAIL-PCR Analysis

TAIL-PCR analysis of the genomic DNA flanking the individual T-DNA inserts revealed unique right border fragments in 20 of the 25 isolates. These fragments ranged from 300-700 bp in size. Products of similar size present in all isolates, including the wild type strain, were presumed to be the result of non-specific amplification (Figure 9). From the 20 isolates, 23 fragments were excised, purified and sequenced. Comparison to known databases showed that the majority of these fragments had low similarities to hypothetical protein sequences and conserved regions in other *Fusarium* spp. (Table 4). Furthermore, they showed no similarity to each other, except for TAIL sequences obtained from transformants CAV 1214, CAV 1579 and CAV 1580, which showed homology to the same *F. oxysporum* hypothetical protein, FOXG_02653. Alignment to each other showed that they were identical. This predicted protein-coding gene consists of two coding regions and has the strongest homology (E-values of ca. 4e-55) to hypothetical proteins in *Gibberella zeae*

(Schwein.) Petch (GI:46117058), *Chaetomium globosum* Kunze (GI:88181029), and *Aspergillus oryzae* (Ahlb.) E. Cohn (GI:83766607). Lower homology was observed to NmrA-like family protein (GI:150377395). In contrast, the E-values for most of the predicted proteins were too high to suggest biological function. No significant similarities were observed between the TAIL fragments of *Foc* and any of the sequences in the GenBank database.

Pathogenicity Studies

Pathogenicity testing indicated that, of the 25 transformants, only three (CAV 1214, 1246 and 1216) showed a significant reduction in virulence when compared to the wild-type *Foc* isolate (Figure 10). None of these transformants, however, reduced disease severity by more than 25%. The wild-type *Foc* isolate and 15 of the transformants developed a maximum disease rating of 5, while the other seven T-DNA transformants had lower disease ratings that were not statistically different from the wild type isolate (Figure 10). No disease symptoms developed in plants treated with water only. External plant symptoms reflected the ratings obtained with internal disease symptoms (Figure 11). Comparable results were obtained after three independent replications of the pathogenicity assay.

DISCUSSION

An ATMT system was developed for the first time in *Foc* as an initial step towards identifying pathogenicity genes from this fungus. Despite the small scale of the current study, 97% of the 25 ATMT isolates screened were mitotically stable and were preserved for later analyses. In a similar study of *Magnaporthe oryzae*, 86% of ATMT-generated isolates could be processed for storage (Betts *et al*., 2007). Our results with *Foc* suggest that a random insertion mutant library of several thousand transformants can be generated efficiently using ATMT. Furthermore, methodologies are now in place to screen transformants for changes in virulence or other phenotypes relevant to host-pathogen interactions.

Three ATMT *Foc* isolates in the current study showed a slight but statistically significant reduction in virulence compared to the wild type *Foc* isolate. These three isolates, interestingly, all had two insertions. A possible explanation for their reductions in virulence is that the three isolates could have been slightly debilitated by multiple insertions of the vector. Other isolates with two insertions, however, had growth characteristics and pathogenic properties similar to that of the wild-type *Foc*. Alternatively, vector insertions may have disrupted the function of one or more genes required for full virulence in banana. Targeted disruption of each of the specific ATMT-tagged genes is necessary to determine whether either or both of the mutated genes of each transformant played a role in the observed virulence reductions. Reproducible virulence reduction rates have been shown to vary between 3.8% in *Leptosphaeria maculans* and 12% in *Magnaporthe oryzae* (Betts *et al*., 2007; Blaise *et al*., 2007), where library numbers were in the thousands. Similar results were

obtained for a REMI library of *F. oxysporum* (1.5%) (Inoue *et al.*, 2001). In this small study with *Foc*, approximately 1.2% of transformants exhibited reduced virulence against bananas.

Cultures of *A. tumefaciens* strain EHA105, originally derived from single colonies, contained both large and small colonies of the bacterium, suggesting a degree of genetic instability in this strain. These observations were made with multiple strains of EHA105 obtained from different sources (B.G.G. Donzelli and A.C.L. Churchill, personal communication). EHA105/S proved to be the strain of *A. tumefaciens* that yielded the most efficient ATMT of *Foc*. This strain was also the most efficient when the rice blast pathogen *M. oryzae* was transformed with either AGL1 or EHA105 (Betts *et al.*, 2007). Within the small number of ATMT *Foc* isolates screened, EHA105/L caused a higher number of insertions on average than the other *A. tumefaciens* strains, suggesting it may be the least optimal for generation of a random insertion library. The results reported here suggest that strain EHA105/S can be used for the generation of random insertion libraries in *Foc* because 1) it generates the highest number of transformants, 2) the transformants grow up relatively fast on transformation plates, and 3) like the other strains (except EHA105/L), most of the transformants have only one or two insertions.

DNA-mediated transformation can lead to various integration events, the most common of which include homologous recombination (disruption or replacement of a specific gene), non-homologous integration (integration at a random site), and ectopic integration (integration of the vector fragment into a specific gene at a non-homologous site elsewhere in the genome) (Fincham, 1989). The number of vector inserts and the randomness thereof is determined by analyzing hybridization banding patterns by Southern analysis. Non-homologous or 'illegitimate' integration at random sites in the genomic DNA of the Fusarium wilt pathogen was expected since the disruption vector contains no genomic *Foc* sequence that would otherwise target the vector to a specific site. Further analyses with larger numbers of transformants are needed to characterize the overall nature of the insertions in *Foc*. Mullins *et al.* (2001) demonstrated that the number of single insertions in *F. oxysporum,* transformed with a random disruption vector, was increased from 53 to 80% in the absence of AS, but also resulted in reduced transformation efficiencies (Mullins *et al.*, 2001). Further analyses are necessary to determine whether the number of single-site insertions in *Foc* might also increase by conducting ATMT in the absence of AS.

TAIL-PCR had a good success rate in *Foc*, as 80% of flanking regions on one side of the insertion point could be amplified and sequenced. Mullins *et. al.* (2001) achieved a similar result of 90% in *F. oxysporum*. Failure to recover all the flanking regions in this study could be due to deletions in the T-DNA borders, complex insertion sites, or the failure of the primer set to amplify flanks. Analyses of flanking regions of T-DNA inserts can also be achieved by inverse PCR (Meng *et al.*, 2007), single oligonucleotide nested (SON)-PCR (Antal *et al.*, 2004; Blaise *et al.*, 2007), and genome walking (Riach and Kinghorn, 1996). Sequence analyses of the *Foc* flanking regions by TAIL-PCR showed that T-DNA insertion sites consist primarily of hypothetical proteins, some with conserved

functional domains. Isolates CAV 1214, CAV 1579 and CAV 1580 have 2, 1 and 1 vector insertions, respectively, and showed the highest homology to the same hypothetical protein, and lower homology was observed to NmrA-like family protein, which is a negative transcriptional regulator, and linked to controlling nitrogen metabolite repression in fungi. Further investigation revealed that DNA sequences were identical, suggesting they had been amplified from the same DNA sequence. These results suggest that the gene linked to the second, uncharacterized vector insertion point in CAV 1214 is a candidate for contributing to the reduced virulence phenotype of this isolate. Further analysis is required to determine if its mutation is directly responsible for the reduction in virulence of CAV 1214.

In *Foc*, random insertion seems to be taking place within coding sequences since the same gene sequence was not isolated from multiple transformants, except for the isolates mentioned above, although relatively few numbers were screened. It has been shown that different fungi have different preferred sites of integration, but in *Fusarium* integration seems to favor coding or conserved sequences (Mullins *et al.*, 2001), which increases the efficiency of this method. In this study, screening of more isolates would have allowed for more rigorous interpretations of the data. The flanking sequences representing putative genes obtained here also mainly consist of unknown *Foc* genomic DNA, which only aligns with very short fragments of the database sequences. Limited gene identification information can, therefore, be deduced from these results, as complete gene annotation of genomes of related species and sequencing of the *Foc* genome have not yet been accomplished. Progress in the annotation of the genome of *F. oxysporum* f. sp. *lycopersici* is expected to aid greatly in gene discovery and functional analyses of the genes isolated from *Foc*.

For gene function determination, the recovered gene sequence may not necessarily be responsible for phenotypic alterations. The possibility of small genome deletions or rearrangements at sites not linked to T-DNA integration, even with single insertions, exists (Mullins *et al.*, 2001; Weld *et al.*, 2006; Betts *et al.*, 2007). In sexual fungi, genetic segregation can be used for functional analysis, and sexual crosses can be performed to confirm invariable linkage of the DNA tag with the mutant phenotype. In asexual fungi, like *Foc*, complementation studies are necessary for verification (Mullins *et al.*, 2001; Weld *et al.*, 2006; Betts *et al.*, 2007). Alternatively, confirmation of gene function can be done by independent targeted disruption of the gene using a construct created with DNA rescued from the original ATMT insertion site.

ATMT and gene analyses in *Fusarium* have a wide range of applications. Null mutants generated through gene disruption may assist in the removal of undesirable contaminating proteins from industrial fermentations (Ballance, 1991) or aid in the commercial production of enzymes and other proteins (Fincham, 1989). The ATMT method may also be adapted for gene-specific disruptions by alteration of the T-DNA to include targeted genome sequences. For *Foc*, this approach will contribute to an enhanced understanding of fungal pathogenesis and assist in the development of alternative control approaches for the management of Fusarium wilt of banana. Utilizing ATMT, a

large random insertion mutant library of *Foc* race 4 can be created, and this study represents the first step in this process.

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Table 1. Bacteria used for *Agrobacterium tumefaciens*-mediated transformation of *Fusarium oxysporum* f. sp *cubense* and the antibiotics used for their selection.

CAV Number	Strains	Strain	Selection antibiotic (mg/l)
CAV 1745	*A. tumefaciens*	AGL1	Carbenicillin 100
CAV 1747	*A. tumefaciens*	EHA105	Rifampicin 70
CAV 1749	*A. tumefaciens*	LBA 4404	Rifampicin 70
CAV 1750	*A. tumefaciens*	GV 3101	Gentamycin 35
CAV 1748	*E. coli* + pBDg-GFP-A	JM109	Kanamycin 50

A. tumefaciens: *Agrobacterium tumefaciens*; *E. coli*: *Escherichia coli*

Table 2. Primers used during PCR detection of the hygromycin (HygR) and green fluorescent protein (GFP) genes.

Primer target	Primer ID	Primer sequence (5' -> 3')	Annealing temperature
Hygromycin gene-terminator from pBDg-GFP-A	HT-F HT-R	TGAACTCACCGCGACGTCT ATGTGGAGTGGGCGCTTAC	65 ℃
GFP fragment of pBDg-GFP-A	SgfpF SgfpR	TCTTCAAGGACGACGGCAACT TCGTCCATGCCGTGAGTGA	55 ℃

Table 3. Transformation efficacies of strains of *Agrobacterium tumefaciens* used to transfer the binary integration vector pBDg-GFP-A to *Fusarium oxysporum* f. sp. *cubense.*

Strain	Day of last count*	Total number of transformants	Average number of transformants/plate	Average number of T-DNA inserts#
AGL1	10	76	19	1.6
EHA105/L	8	70	18	2.4
EHA105/S	7	122	31	1.6
GV3101	12	34	9	1.6
LBA4404	8	61	15	1.6

* After the given day (day post transformation), no accurate counts could be made due to plate overgrowth.

Five transformants analyzed for each bacterial strain assayed for ATMT.

Table 4. Summary of *Agrobacterium tumefaciens*-mediated transformation-derived isolates of *Fusarium oxysporum* f. sp. *cubense* with their stability status, T-DNA copy number, and predicted sequence identity of fragments flanking the T-DNA recovered by TAIL-PCR.

Strain*	CAV	Stable	T-DNA Insert Copy Number	TAIL Sequence Identity	Aligning organism	E-value
AGL1	1231	✓	1	No sequence available	-	-
	1209	✓	2	Predicted protein (FOXG_15393)	*F. oxysporum*	0.053
	1214	✓	2	Conserved hypothetical protein (FOXG_02653)	*F. oxysporum*	4e-55
	1216	✓	2	No TAIL product available	-	-
	1632	x	1	Hypothetical protein similar to CRAL/TRIO domain protein (FOXG_07621)	*F. oxysporum*	0.4
EHA105/L	1393	x	2	Antiviral helicase SKI2 (FGSG_02781)	*F. graminearum*	1.5
	1396	x	2	No TAIL product available	-	-
	1398	✓	2	A) Predicted protein (FOXG_13990)	*F. oxysporum*	0.24
				B) Predicted protein (FOXG_14195)	*F. oxysporum*	0.16
	1401	x	4	No TAIL product available	-	-
	1434	✓	2	Hypothetical protein similar to trehalase precursor (FOXG_09264)	*F. oxysporum*	1.5
EHA105/S	1579	✓	1	Conserved hypothetical protein (FOXG_02653)	*F. oxysporum*	4e-56
	1504	✓	2	Conserved hypothetical protein (FOXG_13024)	*F. oxysporum*	1
	1580	✓	1	Conserved hypothetical protein (FOXG_02653)	*F. oxysporum*	4e-55
	1619	✓	2	hypothetical protein similar to heterokaryon incompatibility protein (FGSG_02393)	*F. graminearum*	0.23
	1622	✓	2	Glycogen phosphorylase (FGSG_09613)	*F. graminearum*	0.82

Strain*	CAV	Stable	T-DNA Insert Copy Number	TAIL Sequence Identity	Aligning organism	E-value
GV3101	1210	✓	2	Hypothetical protein similar to CRAL/TRIO domain protein (FOXG_07621)	*F. oxysporum*	0.41
	1246	✓	2	Hypothetical protein similar to U1 snRNP splicing complex (FOXG_04112)	*F. oxysporum*	0.25
	1249	✓	2	Hypothetical protein similar to small nucleolar ribonucleotide (FGSG_02648)	*F. graminearum*	4.2
	1301	✓	1	Predicted protein (FOXG_14447)	*F. oxysporum*	0.88
	1319	✓	1	Conserved hypothetical protein (FVEG_10327)	*F. verticillioides*	0.086
LBA4404	1352	✓	2	ATP synthase subunit alpha, mitochondrial precursor (FOXG_00308)	*F. oxysporum*	0.06
	1355	✓	2	A) Hypothetical protein similar to CRAL/TRIO domain protein (FOXG_07621)	*F. oxysporum*	0.36
				B) Predicted protein (FOXG_02549)	*F. oxysporum*	5e-98
	1358	✓	2	Hypothetical protein similar to CRAL/TRIO domain protein (FOXG_07621)	*F. oxysporum*	0.44
	1377	✓	1	No sequence available	-	-
	1380	✓	1	Predicted protein (FOXG_07918)	*F. oxysporum*	0.047

* Strain of *A. tumefaciens* used during the transformation.

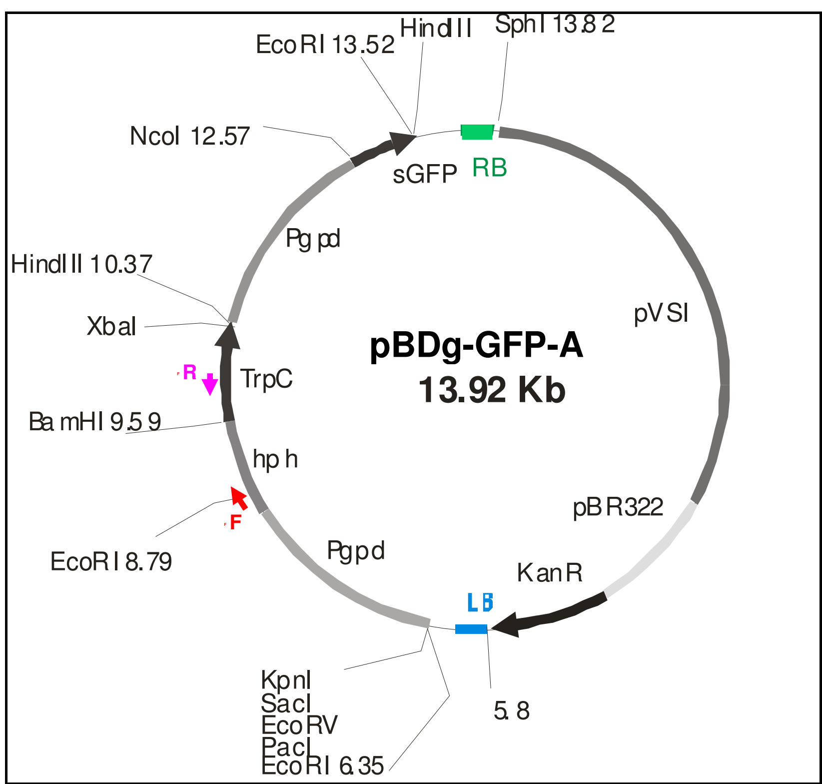

Figure 1. Restriction enzyme map of the binary vector pBDg-GFP-A used for ATMT of *Fusarium oxysporum* f. sp. *cubense* (LB: left border; RB: right border; F: PCR Primer HT-F position; R: PCR Primer HT-R position; *KanR*: Kanamycin resistance gene for bacterial selection; *Pgpd*: *Aspergillus nidulans gpd* promoter; *hph*: hygromycin B resistance gene for fungal selection; *TrpC*, *A. nidulans trpC* terminator; map is approximate) (B.G.G. Donzelli and A.C.L. Churchill, personal communication).

Rating	*Internal Disease Symptoms*
0	*Corm completely clean, no vascular discoloration*
1	*Isolated points of discoloration in vascular tissue*
2	*Discoloration of up to one-third of vascular tissue*
3	*Discoloration of between one-third and two thirds of vascular tissue*
4	*Discoloration of greater than two-thirds of vascular tissue*
5	*Total discoloration of vascular tissue*

Figure 2. Internal Fusarium wilt symptoms of banana according to rating scale by Nel *et al.* (2006) (Photos: Carlier *et al*., 2000).

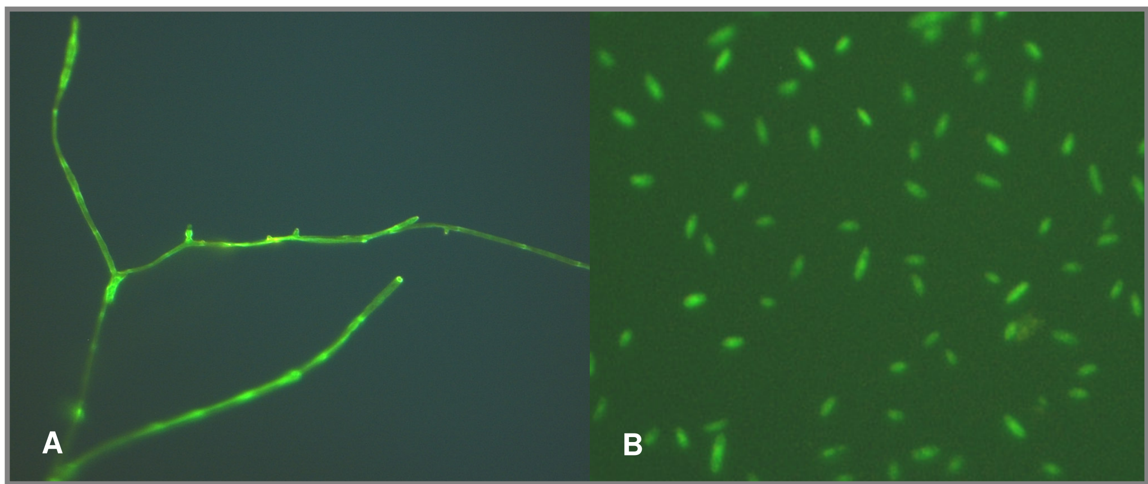

Figure 3. Mycelia (A) and microconidia (B) of a pathogenic isolate of *Fusarium oxysporum* f. sp. *cubense* (CAV 105) transformed with random insertion vector pBDg-GFP-A containing the GFP gene, as viewed using epifluorescence microscopy.

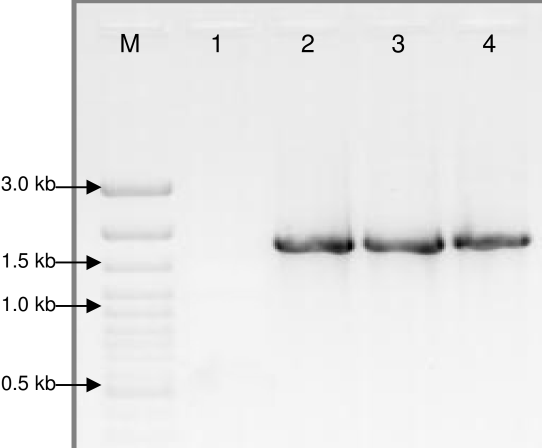

Figure 4. PCR amplification of the Hygromycin B (*hph*)-terminator (*trpC*) region, with an expected fragment size of 1580 bp (M: DNA Molecular Weight Marker, 1: wild-type CAV 105, and 2-4: transformants CAV 1619, CAV 1622, and CAV 1632).

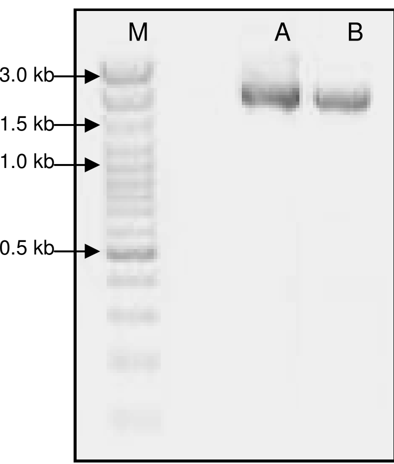

Figure 5. Agarose gel electrophoresis separation of a DIG-labeled (A) and unlabeled (B) hygromycin gene fragment, as used during Southern analyses of transformants. The DIG-labeled PCR product is slightly larger due to the incorporation of the DIG molecule (M: DNA Molecular Weight Marker).

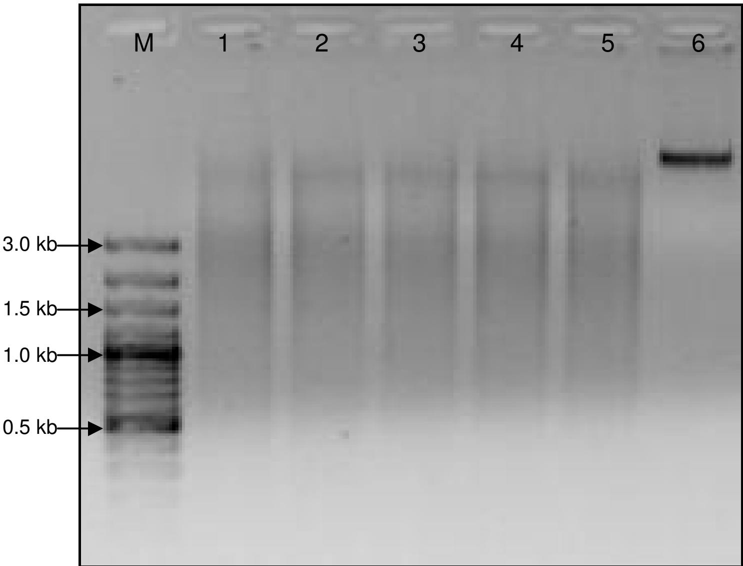

Figure 6. Digestion of genomic DNA with *Eco*RV and *Kpn*I in preparation for Southern analyses (M: DNA Molecular Weight Marker; 1-5: Digested genomic DNA, 6: Uncut genomic DNA control).

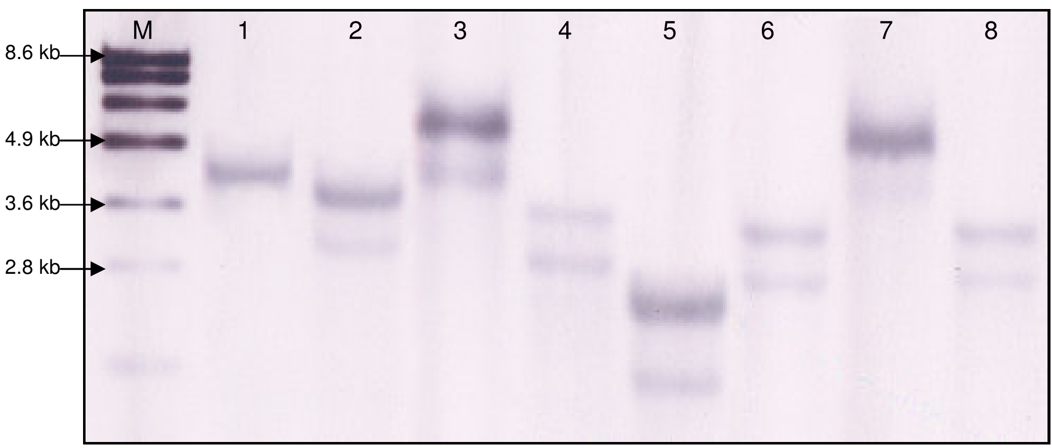

Figure 7. Southern analysis of *Eco*RV/*Kpn*I-digested DNA of *Fusarium oxysporum* f. sp. *cubense* transformants. DNA was hybridized with the Hygromycin B (*hph*)-terminator (*TrpC*) fragment. (M: DNA Molecular Weight Marker VII; Lanes 1-8: Hybridization bands of transformants isolated after *Agrobacterium tumefaciens*-mediated transformation - 1: CAV 1434, 2: CAV 1352, 3: CAV 1507, 4: CAV 1504, 5: 1619, 6: CAV 1210, 7: CAV 1301, and 8: CAV 1246).

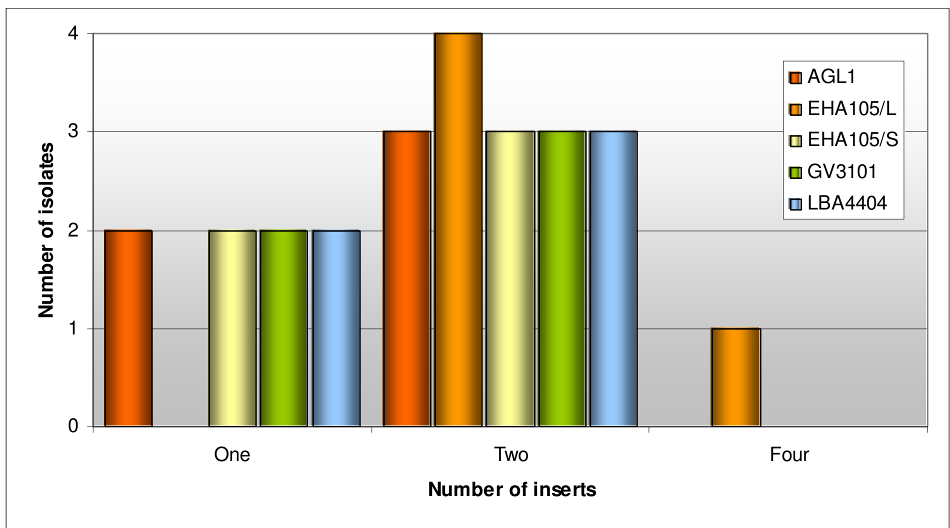

Figure 8. The number of transformants containing one, two or four T-DNA inserts for the various *Agrobacterium tumefaciens* strains used during transformation of *Fusarium oxysporum* f. sp. *cubense*.

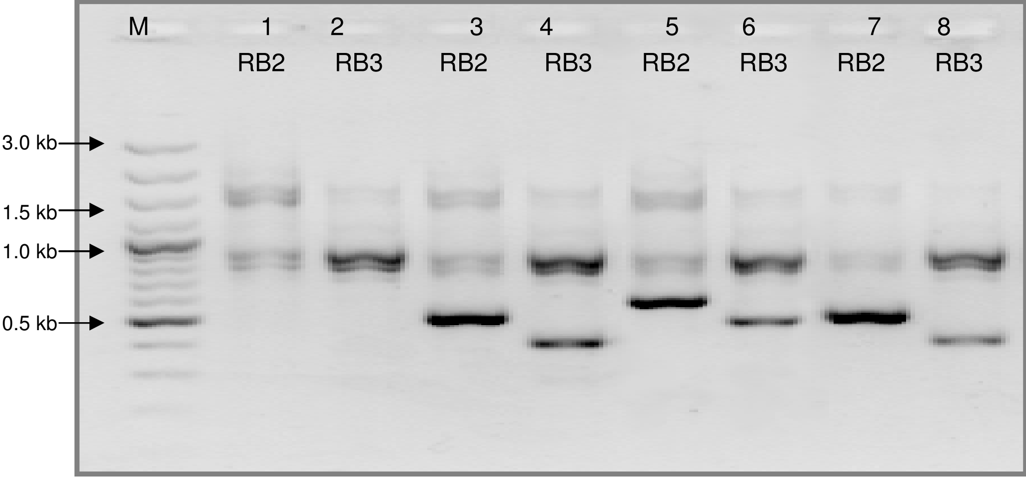

Figure 9. Thermal asymmetric interlaced (TAIL) PCR products of wild-type CAV 105 *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Lane 1-2) and three transformants (Lanes 3-8) separated on a 2% agarose gel. A fragment shift of approximately 100 bp can be seen when comparing right border TAIL2 (RB2) and right border TAIL3 (RB3) products of the same isolate: a reduction in product size is due to the presence of the RB3 primer, which is a nested primer that amplifies a smaller fragment than does primer RB2 (M: DNA Molecular Weight Marker).

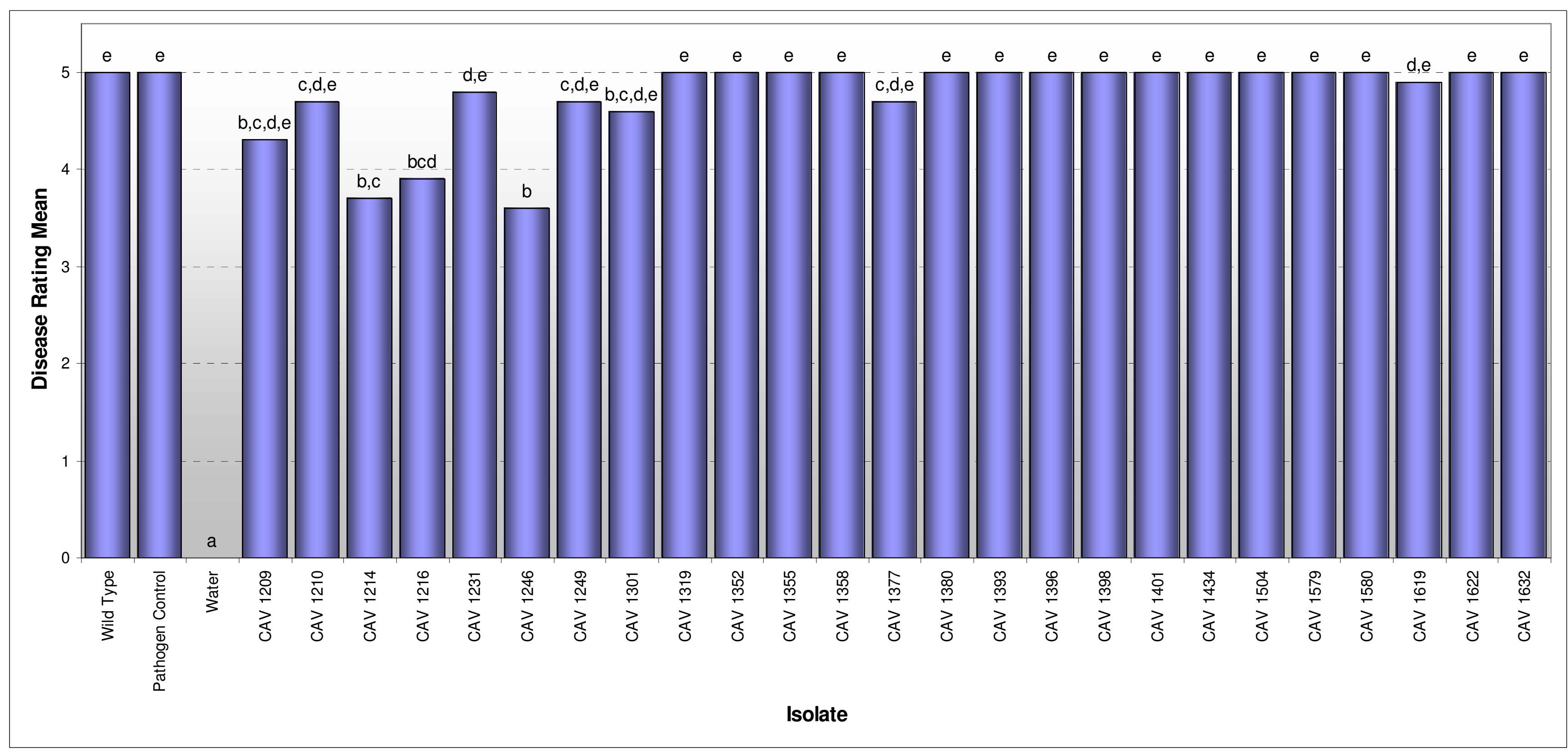

Figure 10. Disease severity of 25 random transformants of *Fusarium oxysporum* f. sp. *cubense* generated by *Agrobacterium tumefaciens*-mediated transformation with the pBDg-GFP-A vector. Pathogenicity ratings were done on a scale of 0 - 5, where the presence of complete internal rhizome discoloration is rated as 5 and the absence thereof is rated as 0. Bars having the same letters are not significantly different in disease severity between transformants. Ten replicates were used for each isolate, and the experiment was repeated three times. The pathogen control contains a mixture of highly virulent CAV 045 and CAV 092. For statistical comparisons, a factorial ANOVA was used (Statistica). The Tukey HSD test was used for *post-hoc* analysis.

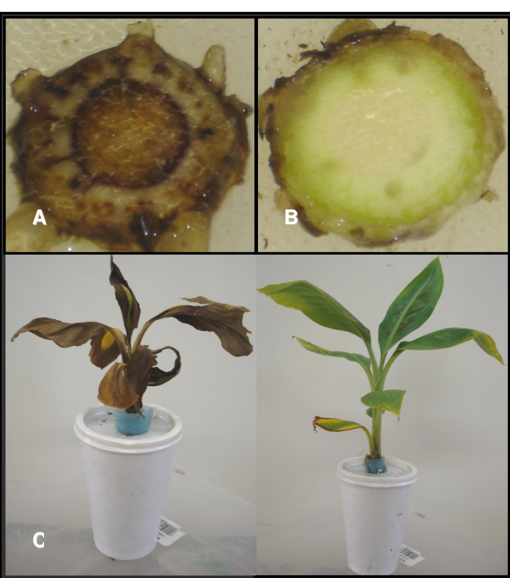

Figure 11. Internal disease symptoms of banana plantlets infected with A) pathogenic *Fusarium oxysporum* f. sp. *cubense* CAV 105 (*Foc*) and B) water control, where disease is rated as 5 and 0, respectively. C) External disease symptoms of a banana plant infected with *Foc* CAV 105 (left) compared with a healthy banana (right).

CHAPTER 3

TARGETED DISRUPTION OF THE *FRP1* GENE IN *FUSARIUM OXYSPORUM* F. SP. *CUBENSE*

ABSTRACT

Fusarium wilt of banana is one of the most destructive plant diseases in recorded history. Despite this repute, little is known about the ability of the causal organism, *Fusarium oxysporum* f. sp. *cubense* (*Foc*), to cause disease. Twenty-two virulence or pathogenicity genes have been characterized in *F. oxysporum*, but none of these have been examined in *Foc*. One of these pathogenicity genes is *Frp1*, a gene responsible for attachment of *F. oxysporum* f. sp. *lycopersici* (*Fol*) to tomato roots during infection. The objectives of this study were to isolate *Frp1* from *Foc* (*FocFrp1*), compare it to its homologue in *Fol* (*FolFrp1*), assess *FocFrp1* expression *in vitro*, and determine whether *FocFrp1* plays a role in pathogenesis of *Foc* on banana by targeted gene disruption. Targeted *Agrobacterium tumefaciens*-mediated transformation (ATMT) was used to disrupt *FocFrp1* by transfer of a binary disruption vector containing a partial *FolFrp1* gene, a hygromycin B resistance gene, and a green fluorescent protein (GFP) gene to *Foc*. Transformants were examined for GFP expression by epifluorescence microscopy and characterized by PCR for recombination events, followed by screening of *FocFrp1* mutants for changes in phenotype and pathogenicity against bananas. DNA sequences of the *Foc* and *Fol Frp1* genes were 99.4% similar, and transcripts of the two genes were identical. Levels of *Frp1* gene expression in a minimal medium were comparable for both fungi. ATMT transformation generated three presumptive *FocFrp1* disruption mutants, two of which exhibited possible reductions in virulence, compared to the 3rd mutant, when inoculated onto susceptible banana plantlets. Unfortunately, these results could not be validated due to the consistent failure of a mixture of wild-type *Foc* isolates to produce disease symptoms in these experiments. Because of the high similarity of the *FolFrp1* and *FocFrp1* genes and predicted proteins, a reduction in, or loss of, pathogenicity of the *FocFrp1* mutants on banana was hypothesized, but further studies are required to confirm preliminary results reported here. This study, however, represents the first report of targeted gene disruption in *Foc.*

INTRODUCTION

Fusarium oxysporum Schlecht. is a ubiquitous soil-borne fungus responsible for wilt diseases of a large variety of flowers, vegetables and fruit trees (Booth, 1984). The species is divided into more than 150 *formae speciales* based on host specificity, where a single *forma specialis* primarily causes disease on only a single host plant (Baayen *et al.*, 2000; Di Pietro *et al.*, 2003). The specificity of *F. oxysporum* may be attributed to only one or a few pathogenicity genes with the ability to overcome defence responses in a particular plant host (Di Pietro *et al.*, 2003). Pathogenicity genes in the Fusarium wilt fungus have not been fully elucidated, but they are anticipated to encode enzymes, toxins, growth regulators and polysaccharides that are involved in disease development (Beckman, 1987; Tjamos and Beckman, 1989). Efforts to identify pathogenicity genes in *F. oxysporum* by random and targeted gene knock-out (KO) have so far yielded 22 virulence or pathogenicity gene candidates (Chapter 1).

Targeted gene KO of pathogenicity genes in filamentous fungi can be achieved by transforming protoplasts with various DNA vectors (Kistler and Benny, 1988; Fincham, 1989; Hynes, 1996; Riach and Kinghorn, 1996; Michielse *et al.*, 2005b). A recently developed method of choice for targeted gene disruption in fungi is *Agrobacterium tumefaciens*-mediated transformation (ATMT). In the past decade, ATMT has been applied extensively for gene discovery in many fungal pathogens, and insertion mutant libraries have been generated for the purpose of ultimately identifying potential virulence or pathogenicity genes. More than 55,000 transformants were generated in a *Magnaporthe oryzae* B.C. Couch study (Betts *et al.*, 2007), and 3,000 transformants were produced to study pathogenicity in *Leptosphaeria maculans* (Sowerby) P. Karst. (Blaise *et al.*, 2007). ATMT has many advantages compared to other transformation methodologies, such as relatively high transformation efficiencies, integration of single copies of T-DNA, the facilitation of targeted homologous recombination, and generally reduced numbers of rearrangement events at the genomic insertion site (Mullins and Kang, 2001; Mullins *et al.*, 2001; Khang *et al.*, 2005; Malz *et al.*, 2005; Michielse *et al.*, 2005a; Michielse *et al.*, 2005b; Sugui *et al.*, 2005; Weld *et al.*, 2006). It also allows for the integration of multiple copies of a gene from a single vector for increased expression, depending on the design of the vector (Gouka *et al.*, 1999). Targeted gene replacement/disruption or KO vectors typically consist of a selectable marker gene flanked by fragments of the target gene intended for disruption in the wild type strain. Integration occurs either by homologous recombination, leading to a targeted gene disruption event, or by an ectopic, illegitimate integration event (Timberlake, 1991; Weld *et al.*, 2006).

A pathogenicity gene recently discovered by random mutagenesis in the Fusarium wilt pathogen of tomato, *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen (*Fol*), is *Frp1*, an F-box protein required for pathogenicity (Duyvesteijn *et al.*, 2005). *FolFrp1* is involved in attachment to tomato roots, and KO mutants lacking a functional *Frp1* gene are unable to colonize the roots of

tomato plants and cause disease. *FolFrp1* KO mutants had normal growth, spore formation and spore germination on artificial media. After extensive analysis of the gene and protein structure, it was found that *FolFrp1* is constitutively expressed in artificial media and *in planta*, and that an F-box motif exists near the amino terminal of the predicted protein. F-box proteins are thought to mediate protein-protein interactions in many cellular processes (Kipreos and Pagano, 2000), specifically, as a component of the SCF (Skp1, a cullin [Rbx1/Roc1/Hrt1], and an F-box) ubiquitin-ligase complex. The exact function of the SCF ubiquitin-ligase complex is not known, but it is thought to be related to pathogenicity.

The objective of this study was to study *Frp1* in the pathogen responsible for Fusarium wilt of banana, *F. oxysporum* f. sp. c*ubense* (E.F. Sm.) W.C. Snyder & H.N. Hansen (*Foc*). *Foc* is one of the most notorious plant pathogens in agricultural history and almost destroyed banana production in Central America during the mid-1900's (Ploetz, 2005). The replacement of Gros Michel bananas with Cavendish cultivars rescued the industry in Central America, but Cavendish bananas are now under attack by a new race of the pathogen in the subtropics and in tropical Asia (Ploetz, 2005). In this study, the *Frp1* gene of *Foc* (*FocFrp1*) is compared to that present in *Fol (FolFrp1).* The potential role of *FocFrp1* in pathogenesis of the Fusarium wilt pathogen of banana was analysed by targeted gene KO.

MATERIALS AND METHODS

Characterization of *Frp1* in *Foc*

Isolates used: An isolate of *Foc* 'subtropical' race 4 (CAV 105) was selected for *Frp1* characterization and targeted gene disruption. CAV 105 was isolated from a Cavendish banana plant with Fusarium wilt symptoms in Kiepersol, South Africa. The pathogenicity of this isolate was confirmed by means of small plant inoculation studies (Groenewald *et al*., 2006b). In addition, an isolate of *Fol* used in this study (CAV 315/ PPRI 5456) was obtained from the Plant Protection Research Institute in Pretoria, South Africa. For DNA extraction, both cultures were grown on half-strength potato dextrose agar (½X PDA) for 7-10 days at 25°C under cool-white light and near-ultraviolet fluorescent light using a 12-hour photoperiod.

PCR amplification and analysis of the Frp1 *gene*: Genomic DNA of *Foc* CAV 105 or *Fol* CAV 315 was used as template for the amplification of a 1.88-kb *Frp1* fragment from each strain. Genomic DNA was prepared as follows. Fungal propagules (hyphae and spores) of *Foc* CAV 105 or *Fol* CAV 315 were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. Fungal tissue was then placed in a 2-ml Eppendorf tube to approximately 600 µl volume, and 1 ml of DNA extraction buffer was added (Raeder and Broda, 1985). Extraction was performed according to a modified phenol-chloroform method (Groenewald *et al*., 2006a). DNA pellets were suspended in 200 µl double distilled SABAX water (Adcock-Ingram, Bryanston, South Africa) and treated with 5

µl of RNase (1mg/ml) (Fermentas, Burlington, Ontario, Canada). The integrity of each DNA sample was determined on a 1% agarose gel (Roche Molecular Biochemicals, Mannheim, Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and aliquots of each sample were prepared for PCR amplification at a final concentration of 100 ng/µl.

Primers for amplification of the *FocFrp1* gene were designed from the DNA sequence available for *FolFrp1* at Genbank (Genbank accession number: AY673970) using DNAMan Demo Version 4.13 (Lynnon Biosoft, Vaudreuil, Canada). Genomic DNA of *Foc* and *Fol* was used as template in 25-µl PCR reactions that included 2 µl 10X PCR buffer, 1.4 µl $MgCl_2$, 2.5 µl 2.5 mM dNTP mix (Fermentas), 0.5 µl each of 10 mM primers Frp1F and Frp1R (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) (Table 1), 0.2 µl 5 U/µl Taq polymerase (Bioline, London, United Kingdom), 1 µl DNA (100 ng/µl), and dH_2O. The PCR program was set at an initial denaturation temperature of 94℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 35 s, annealing at 65℃ for 35 s, elongation at 72℃ for 90 s, and was completed with a final elongation step at 72℃ for 7 min. PCR products (5 µl) were analyzed on a 2% agarose gel run at 80 V in 1X TAE buffer. For size determination, the O'GeneRuler^{TM} 100 bp DNA Ladder Plus marker (Fermentas) was loaded onto the gel.

PCR amplicons were purified using the High Pure PCR Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions and eluted with 30 µl elution buffer. The fragments were then sequenced on an ABI3100 instrument (AME Bioscience, Toroed, Norway) using the BigDye kit, version 3.1 (Roche Applied Sciences, Penzberg, Germany). DNA sequences were visualized, verified and analyzed using Chromas 1.45 (www.technelysium.com.au/chromas.html) and BioEdit Sequence Alignment Editor 6.0.7 (Tom Hall Isis Pharmaceuticals Inc., Carlsbad, CA, USA) software. PCR amplicon sequences were aligned to the *FolFrp1* sequence (AY673970) at Genbank (www.ncbi.nlm.nih.gov) using BioEdit.

RT-PCR amplification and analysis of Frp1 *transcripts*: Mycelial plugs (5/flask; 5 mm in diameter) of *Foc* isolate CAV 105 and *Fol* isolate CAV 315 were inoculated into individual 250 ml flasks containing 100 ml minimal medium broth (Leslie and Summerell, 2006) and rotated at 90 rpm at 25℃ on a rotary shaker (Shake-O-Mat, Labotec, Midrand South Africa). After 10 days, the mycelia were harvested, frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. For each fungus, RNA was extracted from 1 ml of ground tissue using the Qiazol extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNase (Fermentas) treatment was done using 10 µg nucleic acid in a 100 µl reaction volume according to the manufacturer's instructions, and the RNA was purified using the RNeasy MiniElute Cleanup kit (Qiagen). RNA concentration was measured on the NanoDrop spectrophotometer, and a 0.5 µl sample was run on a 1% denaturing / RNA gel to determine if any degradation had taken place.

Single-stranded cDNA was prepared from 1 µg total RNA with the cDNA synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) using half-reactions and the oligo dT_{15} primer according to the manufacturer's instructions. The cDNA was then tested for DNA contamination and integrity by PCR amplification of the translation elongation factor-1α (EF-1α) using the EF1 and EF2 primer pair previously used for species identification (O'Donnell *et al.*, 1998) (Table 1). PCR reaction conditions were as described previously for *Frp1* gene amplification. For quantitative real-time (qRT)-PCR optimization, the endogenous control primers EF1α-F and EF1α-R (Table 1), used in the original *FolFrp1* study (Duyvesteijn *et al.*, 2005), were tested for amplifying single products from *Foc* and *Fol* cDNAs at annealing temperatures of 53, 55 and 58°C. Genomic DNA from *Foc* served as a negative control.

For qRT-PCR, the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Indianapolis, IN, USA) was used. A dilution series (1:10, 1:100 and 1:1000) was set up with *Foc* cDNA to prepare standard curves for the endogenous EF1α control and the *Frp1* gene. PCR reaction mixes (10 µl) included 2 µl PCR grade water (Roche Diagnostics), 1 µl each of forward and reverse primers (10 µM), 5 µl LightCycler 480 SYBR Green I Master Mix, and 1 µl of diluted cDNA template. Thermocycle conditions were as follows: pre-incubation for 10 min at 95°C, and 55 cycles of denaturing for 10 s at 95°C, annealing for 10 s at 55 °C, primer extension for 10 s at 72°C and data acquisition / Tm calling at 95°C. For PCR amplification of *Frp1*, a 1:10 *Foc* or *Fol* cDNA template dilution was used with primers FRP1-F3 and FRP1-R2. All reactions were done in triplicate, and molecular grade water was used as a negative control. Expression data were normalized using the standard curve for the specific target gene and the endogenous control gene EF-1α.

The 625-bp *Frp1* cDNA fragment was amplified from either *Fol* or *Foc* cDNA according to the procedures described above. PCR amplicons were purified using the High Pure PCR Purification kit (Roche) according to the manufacturer's instructions and eluted with 30 µl elution buffer. The fragments were then purified and sequenced using the BigDye kit, version 3.1. DNA sequences were visualized, verified and analyzed using Chromas 1.45 and BioEdit Sequence Alignment Editor 6.0.7 software. PCR amplicons were aligned to the *FolFrp1* mRNA sequence from Genbank (AY673970) and the *FolFrp1* DNA sequence isolated in this study.

Targeted Gene Knock-out of *Frp1* in *Foc*

Agrobacterium tumefaciens-*mediated transformation of* Foc: A binary vector, ∆*FRP1*::*hph* (Duyvesteijn *et al.*, 2005), was modified by changing the backbone to pPK2 (Covert *et al.*, 2001) and by addition of a green fluorescent protein (GFP) gene, resulting in pPK2::HPHgfpFRP1KO (W. Jonkers and M. Rep, personal communication). This KO vector was generated by using pPK2 as the backbone with two insertions as flanks; one in the *Pac*I-*Kpn*I site at the left border and the second one in the *Xba*I-*Hind*III site at the right border (Figure 1). In the *Apa*I site of the *FolFrp1* gene, a hygromycin B resistance gene fused to a GFP gene, was inserted. The resultant plasmid

(13.133 kb) was then transformed into *A. tumefaciens* strain EHA105. This recombinant *A. tumefaciens* strain containing the vector was made available by Dr. M. Rep (Swammerdam Institute for Life Sciences, University of Amsterdam, Netherlands). This KO vector, constructed for *FolFrp1* gene disruption, was used for targeted disruption of *Frp1* in *Foc*.

A. tumefaciens strain EHA105 containing the *FolFrp1* disruption vector was grown on yeast extract peptone (YEP) agar medium containing kanamycin (50 mg/l) and rifampicin (20 mg/l) for vector and strain selection, respectively. The cultures were incubated at 28℃ for 24-48 hours, and colonies that developed were transferred to YEP broth amended with kanamycin (50 mg/l) and rifampicin (20 mg/l). The cells were incubated in the broth for another 24-48 hours at 28℃ while shaking at 250 rpm. Transformation was carried out following the method described by Covert *et al*. (2001) with a single modification that involved the addition of an antibiotic-containing overlay medium for stringent selection of fungal transformants and growth suppression of EH105, i.e., the co-cultivation plates with the *Foc* - *Agrobacterium* mixture were overlaid with M-100 medium containing 300 µg/ml carbenicillin and 100 µg/ml hygromycin (Moon *et al*., 2008). Putative transformants became visible as rapidly growing fungal colonies after 2-10 days and were transferred to fresh M-100 plates containing 300 µg/ml carbenicillin and 100 µg/ml hygromycin, followed by incubation at 25℃ under light. The transformation experiment was performed four times, and a total of approximately 500 putative transformants were counted and transferred individually to new plates until the original transformation plates were completely overgrown with fungal mycelia. Putative transformants were screened for normal growth characteristics, and GFP fluorescence was confirmed for 100 homokaryotic culture isolates, which were stored in 15% glycerol at -70℃ at the facilities of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Screening for putative FocFrp1 *KO mutants*: Crude genomic DNA was extracted from each of 100 *Foc* transformants by gently mixing fungal mycelia with 100 µl PrepMan Ultra (Applied Biosystems, Foster City, CA). The mixture was then incubated in a thermal cycler (Eppendorf, Hamburg, Germany) at 95℃ for 10 min, pelleted by centrifugation for 10 min at maximum speed in a 5810 R Eppendorf centrifuge (Eppendorf), and stored at 4℃. An aliquot (2 µl) of DNA of each transformant was subjected to PCR amplification of the 625-bp *Frp1* fragment using primers FRP1-F3 and FRP1-R2 (Table 1) with the same PCR conditions described above. PCR products were analyzed at 80 V on a 2% agarose gel in 1X TAE buffer. The O'GeneRuler™ 100-bp DNA Ladder Plus marker (Fermentas) was loaded to determine amplicon size. Absence of an amplicon indicated that the *Frp1* gene was disrupted by plasmid insertion. Isolates from which no PCR product was amplified were transferred to a minimal medium (MM) and an amended MM (MM+EtOH), where sucrose was replaced with 2% ethanol as the carbon source, to compare their growth characteristics to those of wild-type isolates (W. Jonkers and M. Rep, personal communication). Wild-type *Foc* CAV 105 was included as control. Three isolates were identified as putative KO mutants and the following numbers were assigned: CAV 2562, 2563 and 2564.

Pathogenicity Studies

Tissue culture-derived Cavendish banana plantlets were inoculated with a conidial suspension (1X 10^5 spores/ml), prepared from 10 day-old cultures flooded with sterile, distilled water (Nel *et al.*, 2006), of each fungus strain assayed. A mixture of the wild-type *Foc* isolate CAV 105 and two other virulent *Foc* isolates (CAV 045 and CAV 092) was used as a positive control, while sterile water was used as a negative control. Roots of plantlets were first slightly compressed to mildly damage them before they were transferred to the fungal spore suspension in 250-ml polystyrene cups. Plants were grown at 28°C with a 12-hour photoperiod for 4 weeks. Each plant was fertilized biweekly with 2 ml of liquid banana fertilizer consisting of 0.6 g/l calcium nitrate monohydrate, 0.9 g/l Agrasol® 'O 3:2:8 (Fleuron, Braamfontein, South Africa), and 3 g/l Micromax® (Fleuron). Disease symptoms that developed in the rhizomes were rated according to a modified scale of Carlier *et al.* (2002), where disease severity was rated from 0 to 5 (Chapter 2). Ten plants were inoculated per isolate, and the experiment was repeated twice. For statistical comparisons, a factorial ANOVA was used by means of Statistica Version 7.1 (Statsoft, 2006). The Tukey HSD test was used for *post-hoc* analysis.

RESULTS

Characterization of *Frp1* in *Foc*

The *FocFrp1* gene was 99.4% similar to that of *FolFrp1* (Figure 2). Only 11 individual nucleic acid substitutions from a total length of 1.88 kb could be detected between the two sequences. The cDNA synthesized from *Foc* RNA was free of DNA contamination, as shown by PCR amplification of the EF1-α fragment (Figure 3). cDNA amplicons had an expected size of ~350 bp, compared to the full-length, intron-containing DNA amplicon of ~800 bp. Good quality, single amplification products without primer dimers were produced with the endogenous control primers as well (Figure 4). RT-PCR analyses confirmed the expression of *Frp1* in *Foc* grown in MM at levels comparable to that of *Fol* grown under the same conditions (Figure 5).

Comparison of the *Frp1* cDNA transcripts of *Foc* and *Fol* showed that these products were identical. The *Frp1* coding region consisted of two open reading frames (ORFs) separated by a 55-bp intron at DNA position 511 bp (Figure 6). A conserved untranslated ORF (uORF) consisting of 10 amino acids was found 130 bp upstream of the start codon and had the nucleic acid sequence of 5' ATGTCGAGTATATTCTCATTCAGTGCGCAATAG 3'.

Targeted Gene Knock-out of *Frp1* in *Foc*

Successful transformation of *Foc* was achieved with the *FolFrp1* KO vector but with varying levels of efficiency. Transformants usually appeared 4 days post-transformation (dpt). Colonies were picked over a period of 6 additional days. High transformation efficiencies were obtained, but an accurate calculation could not be made due to complete overgrowth of plates by 10 dpt.

Approximately 500 transformants were picked, of which 100 had normal growth on M-100 medium supplemented with 300 μg/ml carbenicillin and 100 μg/ml hygromycin. The rest of the transformants grew extremely slowly and were discarded. For transformants that grew at rates comparable to the untransformed wild type strain, T-DNA insertion was established by observing green fluorescence in ~100 mutants when examined using epifluorescence microscopy. Both fungal mycelium and spores exhibited green fluorescence.

PCR amplification of genomic DNA template from each of the 100 putative *FocFrp1* KO mutants with the *Frp1* internal gene primers resulted in the generation of amplicons for all but three of the mutants. PCR amplification of the EF-1α gene confirmed that absence of a PCR product in these three transformants was not due to a faulty DNA extraction method or poor DNA integrity or quality but was most likely due to disruption of the *FocFrp1* gene. The putative *FocFrp1* KO mutants and the wild type *Foc* isolate had similar growth characteristics on MM with the production of abundant aerial mycelia. In contrast, on the MM+EtOH medium, the growth of aerial mycelia by the *FocFrp1* KO mutants, as well as the rate of growth, was substantially reduced compared with that of the wild type isolate (Figure 7; data not shown).

Pathogenicity Studies

Despite repeated pathogenicity testing, plants inoculated with the mix of wild-type *Foc* isolates (CAV 105, CAV 045, and CAV 092) failed to succumb to disease as expected and produced an average disease rating of 1 (out of 5, which represents fully diseased), whereas those inoculated with the three putative *FocFrp1* KO mutants produced inconsistent results (Table 2). Transformant 4 (CAV 2562) showed the highest disease severity of 2.5, while plantlets inoculated with transformants 10 or 64 (CAV 2563 or CAV 2564, respectively) either produced no internal disease symptoms (rating of 0), or exhibited much reduced symptom development (ratings of 1 or 2). No symptoms developed on the plantlets treated with water. External symptoms reflected those assigned for internal disease symptoms.

DISCUSSION

Foc was successfully transformed with the *FolFrp1* gene disruption vector in this study. Twenty percent of the transformants contained the vector, as evidenced by their ability to express GFP, while *FocFrp1* was disrupted in 3% of the GFP-expressing transformants. Targeted gene disruption efficiencies in *Aspergillus fumigatus* were shown to range from 0.07% (6/9000) to 66% (Sugui *et al.*, 2005) and from 0 to 55% in *A. awamori* (Michielse *et al.*, 2005b). In *F. pseudograminearum*, targeted gene disruption was as low as 0.3% (6/1910) (Malz *et al.*, 2005), while it ranged from 14 to 75% in other fungi (Michielse *et al.*, 2005a). A great deal of variation in individual transformation and gene disruption experiments was also seen with *Foc*. This variation is consistent with previous studies of *F. oxysporum* (Mullins *et al.*, 2001; Weld *et al.*, 2006).

A range of factors could affect the efficiency of transformation and gene KO in *Foc*. These include choice of the specific *A. tumefaciens* and host strains used (Michielse *et al*., 2005a), as well as the combination thereof. The acetosyringone concentration is thought to have an effect on transformation efficiency, as well as the number of T-DNAs inserted into the genome (Mullins *et al*., 2001). Other factors include co-cultivation conditions and the choice of selective antibiotics; the extent to which the host organism's DNA repair mechanisms are activated (Michielse *et al*., 2005b); and the length of homologous regions, since homologous flanks longer than 1000 bp generally increase recombination efficiency in filamentous fungi (Timberlake, 1991; Michielse *et al*., 2005b). Also, G/C content, the transcriptional status of the gene of interest, chromatin structure, gene locus on the chromosome, and the source of the promoter (Turgeon *et al*., 1987) may affect transformation efficiencies. Transformation efficiency may be increased by performing double marker enrichment. This is achieved by including a second dominant selection marker, which flanks the gene replacement cassette and thereby discriminates between ectopic and homologous recombination (Khang *et al*., 2005; Michielse *et al*., 2005a; Michielse *et al*., 2005b). With further replication of the transformation experiment and consideration of the abovementioned factors, the success rate for targeted gene disruption in *Foc* may be increased.

Despite the successful disruption of the *Frp1* gene in *Foc*, it was not possible to demonstrate whether the virulence phenotype had been affected. No disease developed in susceptible banana plants inoculated with a mixture of three highly virulent *Foc* isolates (CAV 105, CAV 045, and CAV 092), despite several attempts, which made the results of a reduction in pathogenesis obtained with *FocFrp1* KO transformants 10 and 64 unreliable. Pathogenicity testing in *Foc* is known to be inconsistent, and inoculated plants do not always produce disease symptoms. Several factors can influence Fusarium wilt development in greenhouse pathogenicity trials, such as the presence or absence of wounds/infection points on roots, plant age, light intensity, transpiration rate (Beckman, 1987), and the use of a hydroponic system as opposed to soil drenching. Recently, it was suggested that *Foc* spore composition and the methods used for spore preparation and application could play a role in disease development (Smith *et al*., 2008). The possibility that the *FocFrp1* gene plays a role in the pathogenicity of *Foc* against banana, thus, has not been demonstrated, and further pathogenicity testing is required to establish whether the gene has a function similar to *Frp1* in *Fol*.

The function of a protein is determined by the chemical properties of the R groups of amino acids, their position and their relative amounts (Fairbanks and Andersen, 1999). When comparing the cDNA generated from the *Frp1* gene in *Fol* to that in *Foc*, no nucleic acid differences were observed. Translation of this sequence allows one to predict that identical proteins should be produced and would be expected to have similar functions in closely related fungi. Gene disruption of *Frp1* in *Fol* resulted in transformants being unable to utilize ethanol as a sole carbon source, thereby affecting the phenotype of these isolates (M. Rep, and W. Jonkers, personal

communication). Similar observations were made with the *FocFrp1* KO mutants. Because of the similarity of the *Frp1* gene sequence and predicted protein, as well as the similar response of KO mutants on ethanol-containing minimal medium, *Frp1* gene disruption in *Foc* was expected also to result in a loss of pathogenicity and, therefore, an inability to cause disease in susceptible banana plants.

Disrupting the *Frp1* gene in *Foc* produced genetic and phenotypic characters similar to those of *Frp1* KO mutants of *Fol*. This being the case, it can be presumed that this is not the gene that is responsible for host recognition and, therefore, also the host specificity that exists in *formae speciales* of *F. oxysporum*. Genetic information such as this on pathogens assists breeders in identifying and setting up new resources of durable resistance (Hammond-Kosack *et al.*, 2004; Gurr and Rushton, 2005). The approach for sensible molecular pathogenicity clarification in *Foc* should be: 1) Analysis of novel metabolites involved in pathogenesis, 2) Investigation of the distribution/presence of homologous pathogenicity genes in *Foc* and related organisms, 3) Gene function analysis and the regulation thereof, and 4) Application of information for pathogen control (Yoder and Turgeon, 1985; Gurr and Rushton, 2005).

Comparison of genomic and cDNA sequences of the *FocFrp1* and *FolFrp1* genes showed very high similarity between the two genes and the production of identical transcripts in cultures grown in minimal medium. High DNA sequence similarity is essential for homologous recombination leading to a knock-out event to occur, given that the vector used for the *FocFrp1* KO experiments was constructed with the partial *Frp1* gene of *Fol*. The value hereof has been emphasized by Weld *et al.* (2006), who suggest that the target gene needs to be almost 100% identical to the KO vector fragments for efficient gene disruption to occur, as significant sequence differences may lead to failure of recombination between the KO vector and target gene. It was therefore expected that recombination would take place between the *FolFrp1* gene fragments in the vector and the homologous sequence in *Foc*. This assumption was confirmed when three presumptive *FocFrp1* KO strains (3% of total) were identified by a negative PCR amplification assay (i.e., failure to amplify the wild-type gene) and by their reduced ability to grow on ethanol-containing MM in comparison with wild type CAV 105.

Minor variation in the conserved uORF of some genes has been found between species of *Fusarium* in terms of composition and location relative to the start codon. Inspection of this region in the *Foc* and *Fol Frp1* genes revealed no differences in the nucleic acid and predicted amino acid sequences or in the position of the predicted start codon. This uORF may be responsible for translational regulation by restriction of ribosome progression or by obstruction of the synthesized peptide (Duyvesteijn *et al.*, 2005).

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Table 1. PCR Primers used for the amplification of *Frp1* and elongation factor 1-α genes and gene transcripts in *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *lycopersici.*

Primer Name	Primer Sequence (5'→3')*	Annealing Temperature	Expected Product Size	Purpose
Frp1F Frp1R	CTG GAT CTC CGG TCC GTA GTT AAC TGG TGC CGT TTA ATC CG	54 ℃	1880 bp (DNA)	To clone *FocFrp1* gene
FRP1-F3 FRP1-R2	ATC CGC CAC ATA TCA CCG AA GTA ATC GAT GCC ATA TCC CG	55 ℃	650 bp (DNA)	To screen transformants for *Frp1* disruption
EF1 EF2	ATG GGT AAG GAR GAC AAG AC GGA RGT ACC AGT SAT CAT GTT	60 ℃	~350 bp (cDNA)	cDNA screening
EF-1α-F EF-1α-R	TCG TCG TCA TCG GCC ACG TC CGA TGA CGG TGA CAT AGT AG	55 ℃	~300 bp (cDNA)	Endogenous control

* R = (A/G); S = (G/C)

Table 2. Disease severity of three transformants of *Fusarium oxysporum* f. sp. *cubense* generated by *Agrobacterium tumefaciens*-mediated transformation with the *FolFrp1* disruption vector. Pathogenicity ratings were done on a scale of 0-5, where the presence of full internal disease is rated as 5 and the absence thereof is rated as 0. The experiment was repeated twice with similar results obtained each time.

Isolate	CAV Number	Plant Replicate										Average
		1	2	3	4	5	6	7	8	9	10	
Pathogen	105/045/092	0	0	1	0	1	2	1	2	2	3	1.2
H_20	-	0	0	0	0	0	0	1	1	0	0	0.2
Transformant 4	2562	0	2	5	4	1	1	5	4	1	2	2.5
Transformant 10	2563	1	0	1	0	0	0	2	0	1	1	0.6
Transformant 64	2564	2	0	0	1	1	2	0	1	1	0	0.8

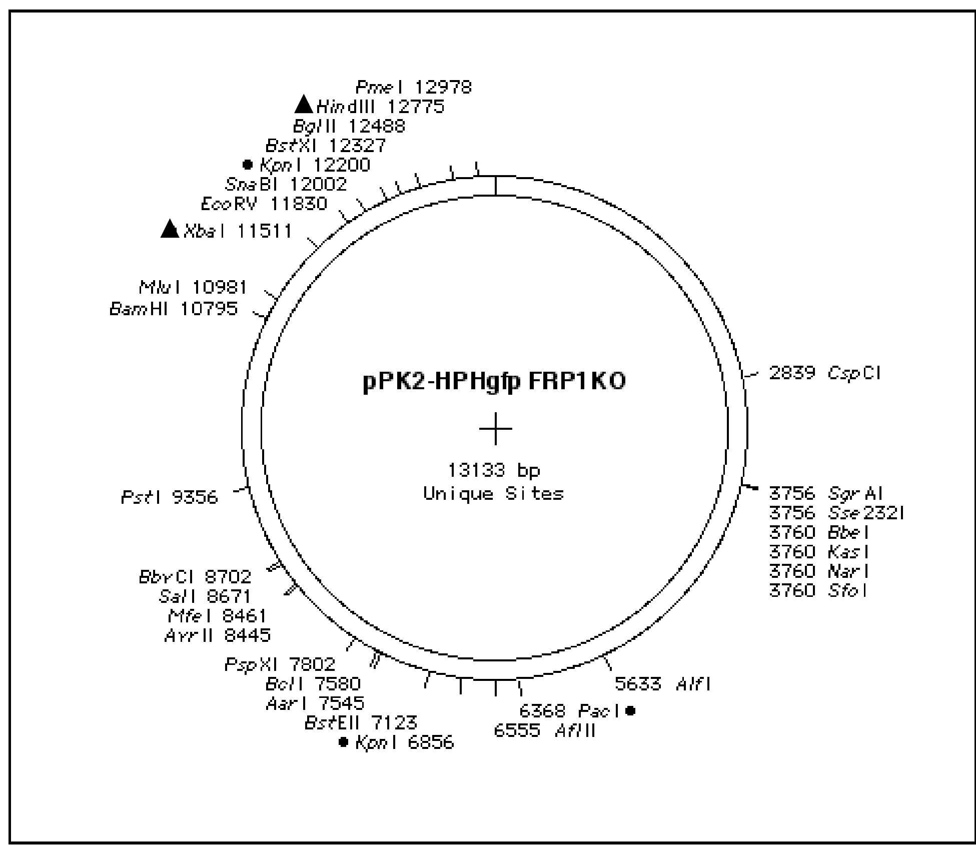

Figure 1. The *FolFrp1* disruption vector was generated by insertion of two flanks in the *Pac*I-*Kpn*I site (●) at the left border, and the second one in the *Xba*I-*Hind*III site (▲) at the right border. In the *Apa*I site of the *Frp1* gene, a hygromycin B resistance gene was fused with the GFP gene (W. Jonkers and M. Rep, personal communication).

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                         10        20        30        40        50        60        70
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  ATGTCGAGTATATTCTCATTCAGTGCGCAATAGAGACGGACGTCTGTAGCCATTTAGGCCTGCTCAGCAA
Foc (CAV105)    ...................................................................A..

                         80        90       100       110       120       130       140
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  AGGCGACACCGTCCTTTCTAACACGCCGCGGCCCTTGCGCCCTCTCGTCGATAGCGTCGAACGTCGCTCG
Foc (CAV105)    .............T...........T............................................

                        150       160       170       180       190       200       210
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  TTTTCTCTTCGACGCCCGTCATCATGGCTCATCATCAAGCGCGGACGATGGCTATGGATCCGCCACATAT
Foc (CAV105)    ......................................................................

                        220       230       240       250       260       270       280
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  CACCGAATTTGCTTCTGAGCGTTACTTCGAAAAGTTAAGCCAGCTCAATGCACACCAACATCAGCGTCAA
Foc (CAV105)    ......................................................................

                        290       300       310       320       330       340       350
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  CCTCCTCCGACTCGAGATAATCTTGATGCTTCCACCTCGTCGCCTTCGAGATTTATTCTTCCTCTACGAG
Foc (CAV105)    ......................................................................

                        360       370       380       390       400       410       420
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  AAACGAAAACTGCCGATGTTGAACCTCTAAGGCCCCTTGACCAGAAGCGTGATAAATCAAGGTTTTTCGG
Foc (CAV105)    ..........C............................................G..............

                        430       440       450       460       470       480       490
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  CCTTCGCTCAAAGGTCTCGATCCTCCACTCTAAATCGAGTGTCGCGTCTGGTCATGCGCCTCGAGTTTCT
Foc (CAV105)    ......................................................................

                        500       510       520       530       540       550       560
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  GTTGAGACGCCGCAAAAAAGGTAATGCTCTTTTGGCTGAATATTAATCCTTTCACTTTACTGACCTGTGA
Foc (CAV105)    ......................................................................

                        570       580       590       600       610       620       630
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  GATAGTGCTAGCTTCGATCAGCTTTTCCTAGGACTTCCCAACGAATTACAGATTCAAATCATTTCTGCCC
Foc (CAV105)    ....................................................C.................

                        640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  TCCCACTAACCGACGTCCTGAACCTCCGACTTGCCTCCAAATCGTGGCATACTCTCGTCACTTTCAACGA
Foc (CAV105)    ......................................................................

                        710       720       730       740       750       760       770
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  AAATACTATCGCTCGATATCATCTCGAACATCATATTCCCGTTTACGCCTCCCGCCTCTACCCCATCACA
Foc (CAV105)    ......................................................................

                        780       790       800       810       820       830       840
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  GACCCGAGCGAGATCAACTTCCAACATCTATGCGGGATATGGCATCGATTACACGTCGCTGCCAAGCTCG
Foc (CAV105)    ......................................................................

                        850       860       870       880       890       900       910
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  CCTTTCTAATGTGTGAATGGATTACAAAAGATATCTTTCTTCGCCAAACTGAAGCTCAACGACTTGCATT
Foc (CAV105)    ......................................................................

                        920       930       940       950       960       970       980
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Fol (AY673970)  TGCACCCCAGAACGAGCGCATGCGGCGACGCCTAATACCTCTTCTATTTACCATATTCCACTTCTTCGAG
Foc (CAV105)    ........................A.............................................
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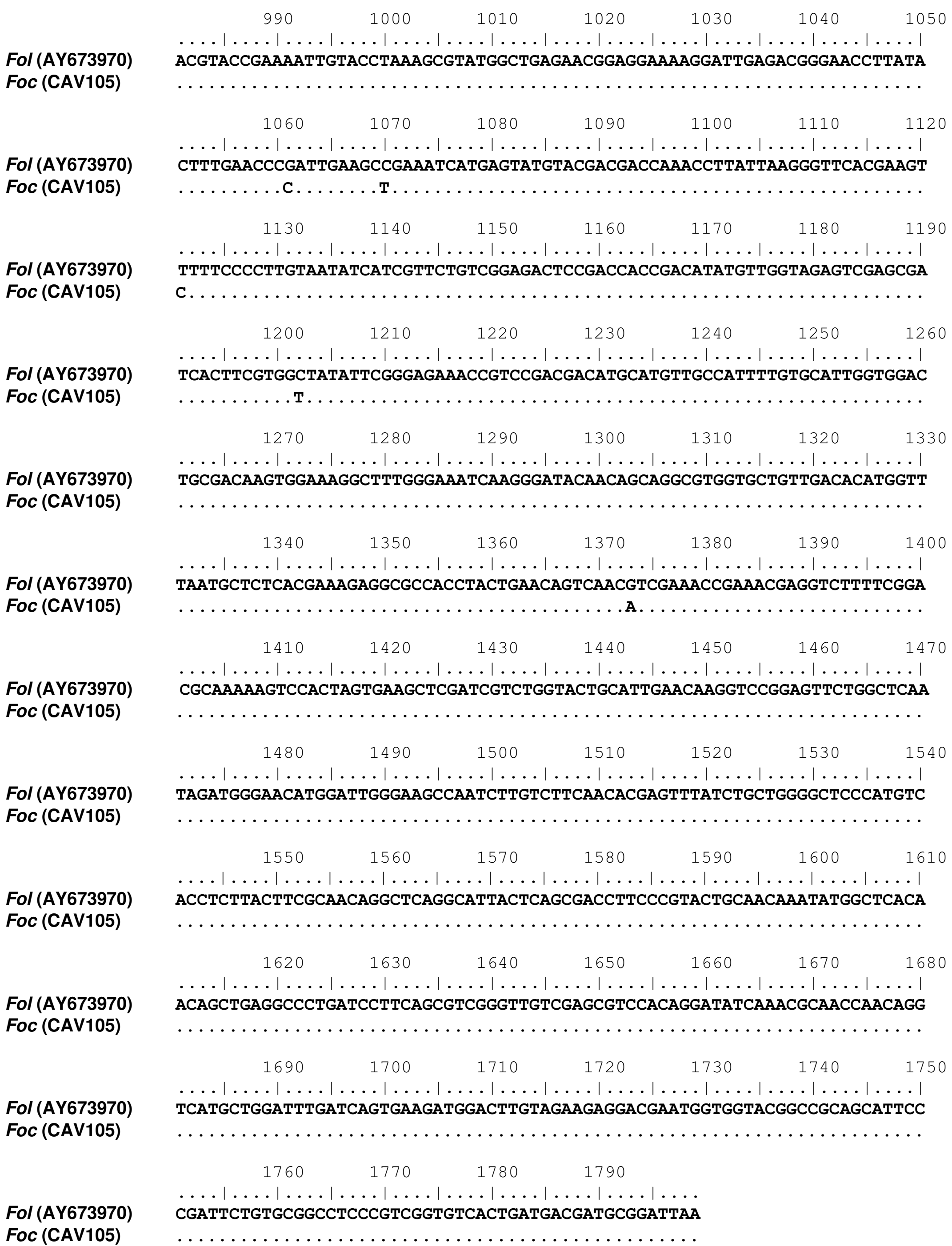

Figure 2. Alignment of the 1799-bp *Frp1* gene of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) from Genbank and that of *F. oxysporum* f. sp. *cubense* (*Foc*), which shows a 99.4% homology. Individual nucleic acid differences are indicated.

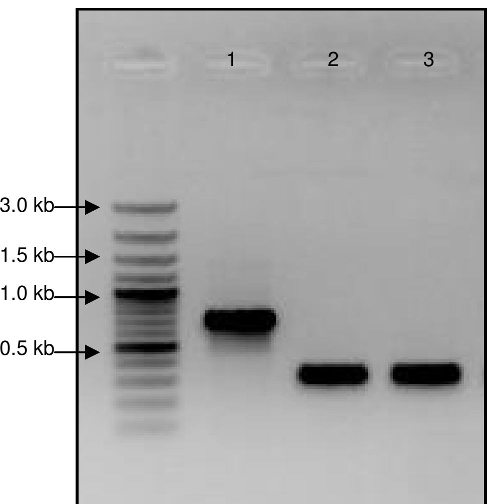

Figure 3. Electrophoretic separation of translation elongation factor 1-α amplicons (using primers EF1 and EF2) from *Fusarium oxysporum* f. sp. *cubense* control DNA (Lane 1) and cDNA to be used for RT-PCR from *Foc* CAV 105 and *Fol* CAV 315 (Lanes 2 and 3, respectively) on a 2% agarose gel. No contaminating DNA was detected in the cDNA samples as indicated by the absence of amplicons in lanes 2 and 3 of the same size as the 0.8 kb intron-containing amplicon from the DNA template in lane 1.

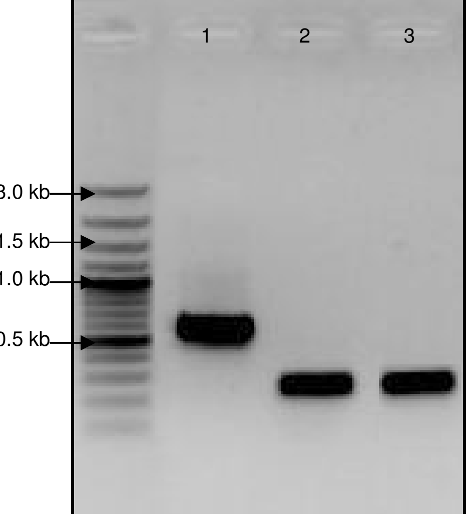

Figure 4. Electrophoretic separation of the endogenous control gene translation elongation factor 1-α amplicons (using primers EF-1α-F and EF-1α-R) from *Fusarium oxysporum* f. sp. *cubense* control DNA (Lane 1) and cDNA from *Foc* CAV 105 and *Fol* CAV 315 (Lanes 2 and 3, respectively) on a 2% agarose gel to confirm the quality of amplification, presence of single bands, and the absence of primer dimers.

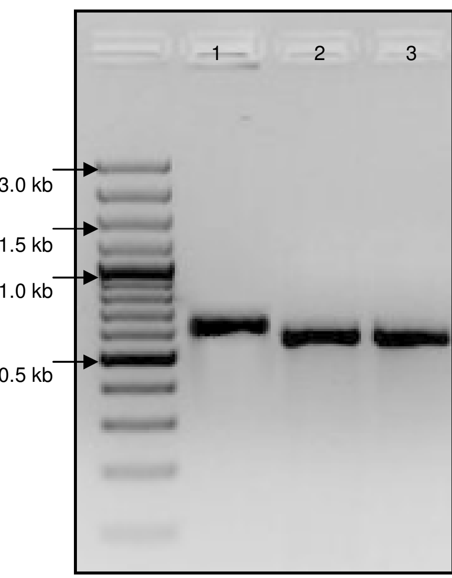

Figure 5. Electrophoretic separation on a 2% agarose gel of the 625-bp *Frp1* cDNA transcript of *Foc* CAV 105 and *Fol* CAV 315 (Lanes 2 and 3, respectively), as analyzed by *in vitro* expression in a minimal medium. *Fusarium oxysporum* f. sp. *cubense* control *Frp1* genomic DNA amplicon (650 bp) was loaded into Lane 1. *Frp1* expression levels in *Foc* CAV 105 and Fol CAV 315 appear to be comparable.

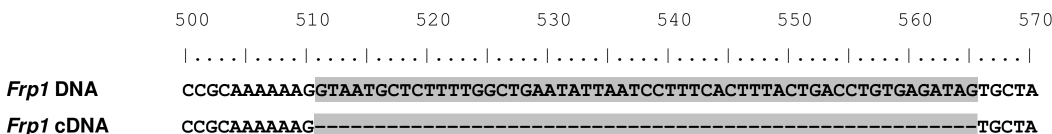

Figure 6. The 55-bp intron found in the *Frp1* transcript of *Fusarium oxysporum* f. sp. *cubense* beginning at DNA position 511 bp.

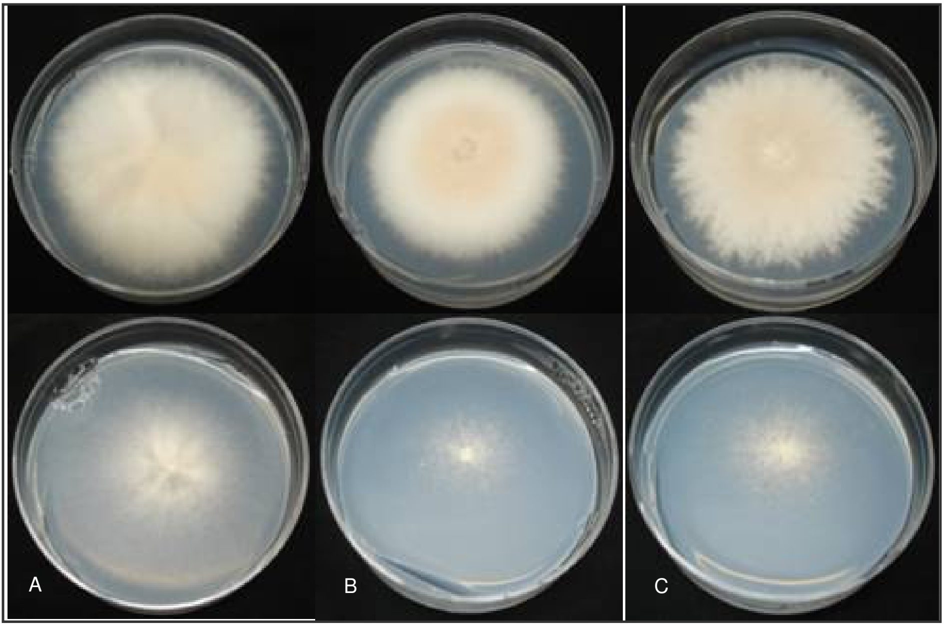

Figure 7. Growth of three *Fusarium oxysporum* f. sp. *cubense Frp1* knock-out mutants (A-C, CAV 2562, CAV 2563, and CAV 2564, respectively) can be differentiated by normal growth on minimal medium (top row) and reduced growth on a minimal medium in which ethanol replaced sucrose as the carbon source (bottom row). Wild type Foc CAV 105 grows with more fluffy, aerial mycelium than the Frp1 KO mutants on both media (data not shown).

SUMMARY

Fusarium oxysporum is a soil-borne fungus responsible for Fusarium wilt of many agricultural crops. Depending on the crop it attacks, *F. oxysporum* can be divided into several special forms, of which *F. oxysporum* f. sp. *cubense* (*Foc*), the pathogen that attacks bananas, is considered one of the most important. *Foc* caused the near collapse of the export banana industry in Central America during the mid-1900s. Apart from resistance breeding, conventional disease management strategies have failed to find methods to adequately control the disease. Understanding pathogenicity in *Foc* could provide a means to develop plants with increased resistance, or develop novel products to protect plants against the banana pathogen.

Fusarium oxysporum has been extensively studied, and its full genome sequence made available in 2007. Even though complete annotation has not been achieved, the available information will assist in gene identification and characterization in important members of the species, such as *Foc*. *Fusarium oxysporum* can be divided into pathogenic and non-pathogenic individuals, which are similar in biology, epidemiology and etiology. The pathogenic forms, however, have the ability to overcome resistance in specific crops. This ability is currently being investigated by forward and reverse genetics. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is one of the most successful techniques to investigate pathogenicity in *F. oxysporum*. The objective of this study was, therefore, to develop an efficient ATMT system to study known and unknown pathogenicity genes of *Foc*.

ATMT can be used for the random disruption of unknown pathogenesis-related genes in *F. oxysporum*. To obtain a random disruption library, *Foc* was subjected to ATMT in this study. Five *A. tumefaciens* strains were evaluated for their efficiency to transform *Foc* with a random disruption vector. *A. tumefaciens* strain EHA105/S gave the best transformation efficiency. Twenty-five transformants were screened by determining the number of T-DNA inserts, establishing the location/identity of T-DNA insertion, and examining alterations in, or loss of, pathogenicity *in planta*. T-DNA flanking genomic DNA was recovered for 20 isolates, but no known pathogenicity genes were disrupted. Pathogenicity trials resulted in three isolates with a reduced virulence, but no loss of pathogenicity. Generation of more transformants is required for a comprehensive random gene disruption study.

The *Fusarium Frp1* gene (F-box protein required for pathogenicity) is responsible for pathogenicity of *Fol* upon infection of tomato. Knock-out mutants of this gene were unable to colonize the roots of tomato plants and cause disease. The *Frp1* gene and gene product in *Foc* were compared and found to be highly similar *Fol.* Targeted disruption of *Frp1 in Foc* was carried out with a construct containing a non-functional *Fol Frp1* gene. Transformants had hygromycin resistance and expressed the green fluorescent protein (GFP). Cultural screening identified three isolates as

putative knock-out mutants and this was confirmed by unsuccessful PCR amplification of the wild-type *Frp1* gene. *In planta* screening of these isolates showed a potential loss of pathogenicity in two isolates. This result could not be substantiated, as the control inoculation of Cavendish banana plantlets with a wild type *Foc* isolate failed repeatedly. *Frp1* in *Foc* is, however, believed to play the same role in banana infection as *Fol* in tomato, as the genes and gene product of the two fungi appear to be identical.

An in-depth understanding of pathogenicity in the banana pathogen is required if any progress is to be made with the control of Fusarium wilt of banana. Thousands of ATMT *Foc* isolates need to be generated and screened for disease development in order to accurately identify genes responsible for pathogenicity. Information obtained from *Fol* could further provide valuable insights regarding the pathogenicity in *Foc.* The progress made in this study on establishing ATMT as a valuable technology for random and targeted (*Frp1*) gene disruption in *Foc* could be considered a first step in developing a larger scale project on pathogenicity gene analysis in *Foc*.