Pathogenicity associated genes in *Fusarium oxysporum* f. sp. *cubense* race 4

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DATES:
Received: 30 July 2012
Revised: 21 Sep. 2012
Accepted: 05 Nov. 2012

KEYWORDS:
banana; cDNA-AFLP; *Fusarium oxysporum*; pathogenicity; virulence

HOW TO CITE:
Sutherland R, Viljoen A, Myburg AA, Van den Berg N. Pathogenicity associated genes in *Fusarium oxysporum* f. sp. *cubense* race 4. S Afr J Sci. 2013;109(5/6), Art. #0023, 10 pages. http://dx.doi.org/10.1590/sajs.2013/20120023

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Fusarium oxysporum f. sp. *cubense* (*Foc*) is a fungus that infects banana roots and causes a destructive plant disease called Fusarium wilt. *Foc* consists of three pathogenic races (*Foc* races 1, 2 and 4), classified according to their selective impairment of banana cultivars. *Foc* race 4 is economically important as it comprises strains that infect Cavendish bananas, which are the most widely planted variety of bananas in the world, in both the tropics (*Foc* TR4) and subtropics (*Foc* STR4). The aim of this study was to investigate which genes are potentially involved in fungal pathogenicity by comparing transcript-derived cDNA fragments (TDFs) from *Foc* STR4 and TR4 to those from non-pathogenic *F. oxysporum* using cDNA-AFLP analysis. This comparison resulted in the identification of 229 unique gene fragments which include the putative pathogenicity-related TDFs encoding chitinase class V (*chsV*), GTPase activating protein, Major Facilitator Superfamily (MFS) multidrug transporter and serine/threonine protein kinase (*ste12*) genes. Quantitative analysis of transcript abundance showed a significant increase in expression of *chsV*, MFS multidrug transporter and *ste12* genes in *Foc* STR4 and TR4 compared with the non-pathogenic *F. oxysporum*. These genes play a role in escaping host defence responses and in cell wall degradation. In addition, pathogenicity-related genes from other *formae speciales* of *F. oxysporum*, such as the sucrose non-fermenting, cytochrome P450 and F-box protein required for pathogenicity genes, were significantly up-regulated in *Foc* STR4 and TR4 but not in *F. oxysporum* isolates non-pathogenic to banana. This study provides the first *in vitro* comparative analysis of TDFs expressed in pathogenic *Foc* race 4 isolates and non-pathogenic *F. oxysporum* isolates from banana.

Introduction

The vascular wilt fungus *Fusarium oxysporum* is a soil-borne facultative parasite that causes disease in more than 100 plant species, including important agricultural crops.[1] The fungus is a morphospecies that is divided into specialised groups (i.e. *formae speciales*) according to the hosts they attack, and subdivided into races according to the susceptibility of specific host cultivars.[2] Host specificity is believed to have evolved independently in *F. oxysporum*, and does not necessarily reflect phylogenetic relatedness among pathogenic members of the individual hosts.[2] In *F. oxysporum*, host specificity has been attributed to mutations in avirulence genes and lateral chromosome transfer that overcome defence responses in the host plant.[3,4]

Fungal pathogenicity genes are responsible for events such as spore attachment and germination, infection and colonisation of the host, and are divided into categories such as formation of infection structures, cell wall degradation, toxin biosynthesis and signalling.[5-7] Certain pathogenicity genes also encode proteins that are involved in the suppression or disruption of host defence mechanisms.[8,9] In *F. oxysporum*, genes that encode cell wall degrading enzymes (CWDEs), such as endo-polygalacturonase (*pg1*), exo-polygalacturonase (*pgx4*), pectate lyase (*pl1),* xylanase and a plant defence detoxifying enzyme like tomatinase, have been identified in *F. oxysporum* f. sp. *lycopersici* (*Fol*).[10-14] Pathogenicity is also influenced by the expression of CWDEs which are regulated by sucrose non-fermenting (*snf*) gene in *F. oxysporum* strain O-685 and the F-box protein required for pathogenicity (*frp1*) gene in *Fol.*[15-17] Signalling genes expressed during pathogenesis have also been identified in *Fol* (e.g. *Fusarium* mitogen-activated protein kinase (*fmk1*))[15] and *F. oxysporum* f. sp. *cucumerinum* (e.g. G protein α subunit (*fga1)* and G protein β subunit (*fgb1)*).[18,19] Several transcription factors that regulate pathogenicity genes during infection have been discovered in *F. oxysporum*, such as serine/threonine protein kinases (*ste12*),[20] a Zn(II)2Cys6-type transcription regulator (*fow2*)[21] and *F. oxysporum ste12* homolog (*fost12*).[22]

Strains of *F. oxysporum* pathogenic to bananas are known as *F. oxysporum* f. sp. *cubense* (*Foc*). Three races of *Foc* are recognised based on their ability to cause disease in a set of different banana cultivars, with *Foc* race 1 affecting Gros Michel, Silk and Pome bananas and *Foc* race 2 affecting Bluggoe and other cooking bananas.[23] *Foc* race 4 affects Cavendish bananas, which make up 80% of the world's banana export, as well as *Foc* race 1 and 2 susceptible bananas.[24] *Foc* race 4 is further subdivided into 'tropical' and 'subtropical' strains. Those belonging to *Foc* 'tropical' race 4 (TR4) are limited to tropical Asia and northern Australia, while *Foc* 'subtropical' race 4 (STR4) strains are mostly associated with Cavendish bananas in subtropical countries like South Africa, Australia, Taiwan and the Canary Islands. *Foc* TR4 is more virulent than *Foc* STR4, and can infect Cavendish bananas under stressed and non-stressed conditions, whereas *Foc* STR4 typically only infects bananas after the host has been exposed to stressful environments.[23]

Despite the economic importance of *Foc,*[24] the mechanisms of pathogenesis to banana are still poorly understood. Additionally, non-pathogenic strains of *F. oxysporum* are known to infect and colonise the cambium tissue of banana roots, but do not enter the xylem to cause Fusarium wilt. Occasionally, the non-pathogens even protect the banana plant from damage caused by *Foc*[25,26] and nematodes.[27] It is not known why non-pathogenic strains of *F. oxysporum* are unable to cause disease to banana. Therefore, the objective of this study was to identify gene transcripts that are present in *Foc* TR4 and *Foc* STR 4 but absent in non-pathogenic *F. oxysporum* using cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis. In addition, a reverse transcriptase–quantitative polymerase chain

reaction (RT-qPCR) was employed to study the transcript abundance of eight previously described pathogenicity genes from other *formae speciales* of *F. oxysporum.*[15-17,21,28-31]

Materials and methods

Fungal isolates and culture conditions

A total of 27 *F. oxysporum* isolates were selected for this study. These isolates included *Foc* STR4 from South Africa, Australia and the Canary Islands, *Foc* TR4 from Malaysia, Indonesia and Northern Australia, and non-pathogenic *F. oxysporum* obtained from Cavendish banana roots in South Africa (Table 1). The non-pathogenic *F. oxysporum* isolates were shown to be non-pathogenic as no internal disease symptoms developed after inoculating banana roots with a spore suspension (1x10^5 spores/mL) in a hydroponic system.[25,27,32,33] All isolates were maintained in 15% glycerol at -80 °C at the Department of Plant Pathology, Stellenbosch University.

RNA extraction

RNA was extracted from fungal mycelia grown *in vitro* rather than *in planta*, as insufficient genes of fungal origin were previously detected in the roots of tissue-cultured banana plants 14 d after inoculation with *Foc* race 4 (1x10^5 spore/mL). The *F. oxysporum* isolates were first grown on half strength potato dextrose agar (PDA) (19.5 g/L PDA and 10 g/L agar) for 5 days at ±25 °C, and then transferred to liquid minimal medium (MM) without a carbon source to enhance the transcript abundance of pathogenicity genes.[34Ohzd] After culturing the isolates in MM on a rotary shaker set at 90 rpm for 5 days at 25 °C, the medium was filtered through sterile cheesecloth. The mycelial mass was scraped and frozen in liquid nitrogen, ground to a fine powder with a basic analytical mill (IKA A111, United Scientific (Pty) Ltd., San Diego, CA, USA), and stored at -80 °C until RNA was extracted.

RNA of each isolate was extracted from mycelia using Qiazol (Qiagen, Valencia, CA, USA), quantified with a NanoDrop ND-1000

Table 1: *Fusarium oxysporum* isolates used for cDNA-amplified fragment length polymorphism and reverse transcription real-time quantitative PCR analysis

CAV number[a]	Strain	Vegetative Compatibility Group	Host	Region	Origin	Group
CAV 045	*Foc* STR4[b]	120	Williams	Port Edward, South Africa	South Africa	S1[e]
CAV 092	*Foc* STR4	120	Grande Naine	Kiepersol, South Africa	South Africa	S1
CAV 105	*Foc* STR4	120	Cavendish	Kiepersol, South Africa	South Africa	S1
CAV 179	*Foc* STR4	120	Not available	Wamuran, Queensland, Australia	Australia	S2
CAV 1116	*Foc* STR4	120	Cavendish	Wamuran, Queensland, Australia	Australia	S2
CAV 1180	*Foc* STR4	120	Cavendish	Byron Bay, New South Wales, Australia	Australia	S2
CAV 291	*Foc* STR4	120	Cavendish	Canary Islands	Canary Islands	S3
CAV 292	*Foc* STR4	120	Dwarf Cavendish	Las Galletas, Canary Islands	Canary Islands	S3
CAV 981	*Foc* STR4	0120/15	Grande Naine	Canary Islands	Canary Island	S3
CAV 858	*Foc* TR4[c]	1216	Cavendish	Malaysia	Malaysia	T1
CAV 865	*Foc* TR4	1216	Cavendish	Malaysia	Malaysia	T1
CAV 870	*Foc* TR4	1216	Cavendish	Malaysia	Malaysia	T1
CAV 302	*Foc* TR4	1213	Williams	Southeast Sumatra, Indonesia	Indonesia	T2
CAV 604	*Foc* TR4	1216	Grande Naine	Indonesia	Indonesia	T2
CAV 811	*Foc* TR4	1213	Cavendish	Indonesia	Indonesia	T2
CAV 789	*Foc* TR4	01213/16	Cavendish	Middle point, Northern Territory, Australia	Australia	T3
CAV 1065	*Foc* TR4	01213/16	Grande Naine	Lambell's Lagoon, Northern Territory, Australia	Australia	T3
CAV 1072	*Foc* TR4	01213/16	Cavendish	Darwin, Northern Territory, Australia	Australia	T3
CAV 255	*F. oxysporum*[d]		Soil, *Musa* sp.	Kiepersol, South Africa	South Africa	N1
CAV 241	*F. oxysporum*		Soil, *Musa* sp.	Kiepersol, South Africa	South Africa	N1
CAV 282	*F. oxysporum*		Soil, *Musa* sp.	Kiepersol, South Africa	South Africa	N1
CAV 552	*F. oxysporum*		Roots, *Musa* sp.	Kiepersol, South Africa	South Africa	N2
CAV 553	*F. oxysporum*		Roots, *Musa* sp.	Kiepersol, South Africa	South Africa	N2
CAV 560	*F. oxysporum*		Roots, *Musa* sp.	Kiepersol, South Africa	South Africa	N2
CAV 744	*F. oxysporum*		Roots, *Musa* sp.	Tzaneen, South Africa	South Africa	N3
CAV 745	*F. oxysporum*		Roots, *Musa* sp.	Tzaneen, South Africa	South Africa	N3
CAV 750	*F. oxysporum*		Roots, *Musa* sp.	Tzaneen, South Africa	South Africa	N3

[a]*Number of the isolate in the culture collection of Altus Viljoen*

[b]Fusarium oxysporum *f. sp.* cubense *subtropical race 4*

[c]Fusarium oxysporum *f. sp.* cubense *tropical race 4*

[d]*Non-pathogenic* Fusarium oxysporum

[e]*Isolates with the same designation were grouped for DNA and RNA extraction.*

spectrophotometer (Nanodrop Technologies Inc., Montchanin, DE, USA) and assessed by formaldehyde agarose gel electrophoresis (1.2%). RNA from three isolates collected from the same country or location were pooled (Table 1), *DNaseI*-treated (Fermentas, Life Sciences, Hanover, MD, USA) and column purified with an RNeasy mini kit (Qiagen). Messenger RNA (mRNA) was isolated using the Oligotex mRNA mini kit (Qiagen). Double-stranded cDNA was synthesised with the cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany) using oligo dT_{15} primers and contamination was assessed by performing a PCR with the intron flanking primers EF1 and EF2.[35]

cDNA-AFLP analysis

Transcript expression levels of putative pathogenicity genes in *F. oxysporum* were assessed by cDNA-AFLP analysis. The AFLP® Expression Analysis Kit (LICOR, Lincoln, NE, USA) was employed according to the manufacturer's instructions to determine differential gene expression patterns. Briefly, cDNA was digested with the restriction enzymes *Taq*I and *Mse*I, followed by ligating adapters using T4 DNA ligase. Pre-selective amplification was performed with *Taq*I+0/*Mse*I+0 primers, and 31 different *Taq*I+2/*Mse*I+2 primer combinations were used during selective amplification (Table 2). Band intensities of differentially expressed fragments on cDNA-AFLP gels were visually assessed and divided into four groups: (1) no transcripts detected (–), (2) low level of transcript abundance (+), (3) moderate level of transcript abundance (++) and (4) high level of transcript abundance (+++). Band intensities correspond to the original expression level.

Table 2: Primers used for the selective amplification of cDNA-amplified fragment length polymorphism fragments in *Fusarium oxysporum*

Labelled *Taq*I primer +2	*Mse*I primer +2
T[a]-GA	M[b]-TG
T-TG	M-AC
T-GT	M-TC
T-TC	M-GT
T-AC	M-AC
T-AG	M-AG
T-TC	M-TC
T-AG	M-AC
T-GT	M-GA
T-TG	M-TG
T-CA	M-TG
T-GA	M-CT
T-AG	M-GT
T-TC	M-CA
T-CT	M-TC
T-CT	M-AG
T-GT	M-AG
T-AG	M-TC
T-CA	M-GA
T-TC	M-CT
T-AC	M-GT
T-GA	M-GT
T-TC	M-AC
T-GT	M-TG
T-AC	M-AG
T-AC	M-CA
T-TC	M-AC
T-TC	M-CT
T-TG	M-GA
T-CA	M-CA
T-GA	M-AC

[a]*T*=Taq*I primer: 5' CTCGTAGACTGCGTAC 3'*

[b]*M*=Mse*I primer: 5' GATGAGTCCTGAGTAA 3'*

Isolation of polymorphic fragments and sequence data analysis

After polyacrylamide gels were resolved on the LICOR analyser and scanned with the Odyssey® infrared imaging system (LICOR), unique bands were identified using Quantity One 1-D analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Bands were excised and cloned into a vector with the InsTAclone™ PCR cloning kit (Fermentas) and sequenced in both directions. Vector sequences were manually removed from the raw sequences by Chromas 1.45 (www.technelysium.com.au/chromas.html), while BioEdit Sequence Alignment Editor 7.0.5.3 software[36] was used to create a consensus sequence for each individual fragment. The consensus sequences were compared with *Fusarium* genome sequences of the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/Blast.html) and on the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. The transcript-derived fragments (TDFs) obtained with cDNA-AFLPs were further characterised using Desktop cDNA Annotation System (dCAS, NIAID, Bethesda, MD, USA).[37] Functional groups were defined according to the Munich Information Center for Protein Sequences (MIPS)[38] and Gene Ontology (GO)[39] databases.

Quantitative analysis of transcript abundance

The transcript abundance of six putative pathogenicity genes identified by cDNA-AFLP in the current study, and eight known pathogenicity genes of *F. oxysporum* (Table 3), was assessed using a LightCycler 480 instrument (Roche Diagnostics). Five reference genes – elongation factor 1α (*TEF*), ß-tubulin (*TUB*), isocitrate dehydrogenase (*IDH*), glucose-6-phosphate 1-dehydrogenase (*G6DH*) and glyceraldehyde 3-phosphate (*GAPDH*) – were also evaluated (Table 3). Primers for the putative and known pathogenicity and reference genes were designed using Primer3 (Whitehead Institute, MIT, Cambridge, MA, USA) and Netprimer (Premier Biosoft, Palo Alto, CA, USA) (Table 3) and synthesised by Operon Biotechnologies GmbH (Cologne, Germany).

RT-qPCR reactions were performed in 10-μL volumes containing cDNA template (1:10 dilution), 1 μM of each of the forward and reverse primers and 5 μL DNA Master^{PLUS} SYBR Green mix (Roche Diagnostic). The protocol included 10 min at 95 °C followed by 55 cycles of 10 s at 95 °C, 10 s at 57 °C and 10 s at 72 °C. The amplification process was completed by a melting cycle from 55 °C to 95 °C to assess specificity. The fluorescence reading was recorded at 72 °C at the end of the elongation cycles. The PCR products were analysed by electrophoresis on a 2% agarose gel to verify that a single product of the expected size was produced. All reactions were performed in triplicate with three independent biological replicates and a negative control (no template) for all genes. A standard curve was generated by preparing a dilution series (1:10, 1:100 and 1:1000) for each pathogenicity and reference gene. Gene expression stability (M-value) and pairwise variation (V-values) were determined using Genorm.[40] Ct values were imported into qbase^{PLUS} (Biogazelle, Ghent, Belgium) for further analysis. The difference in Ct values was determined statistically by one-way analysis of variance, followed by Tukey's post-hoc analysis; $p<0.05$ was considered statistically significant.

Results

cDNA-AFLP analysis

cDNA expression patterns of approximately 3150 transcripts were examined with 31 different *Taq*I+2/*Mse*I+2 primer combinations. For each primer combination, 63–138 TDFs were visualised and varied from 100 bp to 700 bp with approximately 8% of the TDF showing differential expression. cDNA-AFLP analysis allowed the identification and isolation of 229 differentially expressed TDFs of between 103 bp and 546 bp in size (Table 4). The TDFs were classified into these functional categories: hypothetical proteins from *Fusarium* (90) and other fungal species (6), energy metabolism (13), transport (13), cell division and growth (11), protein turnover (8), cell signalling (9),

Table 3: Primer sequences of genes from *Fusarium* spp. used in reverse transcription real-time quantitative PCR analysis

Primer ID	NCBI accession number	Gene identity	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size	Reference
TDF9	-	Major facilitator superfamily multidrug transporter	CATGGGCCTCGTGAATATGT	CCTGGATGCCTTGTCAAGTT	97	
TDF52	-	Aspartyl-tRNA synthetase	CGAAGACGATGAAGGGTGAT	GCTTACCCCTCAACTGCAAC	96	
TDF64	-	L-aminoadipate-semialdehyde dehydrogenase large subunit	CGAACACCAAGAGTGGATCA	ACCATGACAGCTCCGATCTC	87	
TDF107	-	Putative chitinase class V	TGCAATTCCTTGAGGCTCTT	TCACCAGCAAAGTGCTTGAC	145	
TDF214	-	Serine/threonine protein kinase	ACCTTGGCTCACTCGAAGAA	TACTTGAGGGTGGGGTTGAG	99	
TDF223	-	GTPase-binding protein gene	CTGCCAAGGTCTCCCTATCA	GGCTTCTGACTGGTCTTTCG	96	
snf1	AF420488.1	*Fusarium oxysporum* protein kinase SNF1 gene	GGTCGGTATCTTGCCTTCAA	GGGAGGTTCGTCGTTGATAA	115	17
frp1	AY673970.1	*Fusarium oxysporum* f. sp. *lycopersici* Frp1 gene	CCTCCAAATCGTGGCATACT	CCCGCATAGATGTTGGAAGT	143	16
cyp55	D14517.1	*Fusarium oxysporum* cyp55A1 gene for cytochrome P450nor	TTATCGCATCCAACCAGTCA	GCAAGATGCTCAGCGATACA	142	31
fmk1	AF286533.1	*Fusarium oxysporum* f. sp. *lycopersici* mitogen-activated protein kinase gene	GGAGCTGATGGAGACGGATA	CGGAGGGTCTGGTAGATGAA	90	15
clc	EU030436.1	*Fusarium oxysporum* f. sp. *lycopersici* CLC voltage-gated chloride channel gene	ACCATATCCGTGGTGGTCAT	AATTCGCTGACAGCTTTGGT	101	28
fow2	AB266616.1	*Fusarium oxysporum* FOW2 gene for Zn(II)2Cys6 transcription factor	ATGCCACCCTGTTTGAGAAG	GAGGAGCCATCGTCGAGTAG	148	21
arg1	AB045736.1	*Fusarium oxysporum* ARG1 gene for argininosuccinate lyase	GCATGGTCTGCTTGAAGTGA	GACGCTCGTTTGCAGTATGA	145	30
fow1	AB078975.1	*Fusarium oxysporum* plasmid pWB60SI FOW1 gene for putative mitochondrial carrier protein	CGAGATCACCAAGCACAAGA	CGTTGACACCCTTGTTGATG	116	29
TEF	AF008486.1	Elongation factor	TCGTCGTCATCGGCCACGTC	CGATGACGGTGACATAGTAG	243	34
TUB	AF008529.1	ß-tubulin gene	CCCCGAGGACTTACGATGTC	CGCTTGAAGAGCTCCTGGAT	68	
IDH	XM_385909.1	*Gibberella zeae* PH-1 isocitrate dehydrogenase	AGTCCGTCGCTTCTCTCAAG	AAGCTGATGCTGGCGTAAAT	133	
G6DH	XM_381455.1	*Gibberella zeae* PH-1 glucose-6-phosphate 1-dehydrogenase	ATATTCCCCGAAACGAGCTT	ATGCTGAGACCAGGCAACTT	88	
GAPDH	XM_386433.1	*Gibberella zeae* PH-1 glyceraldehyde 3-phosphate	CCAGATCAAGCAGGTCATCA	GTTGGTGTTGCCGTTAAGGT	106	

lipid or fatty acid metabolism (5), transcription and translation factors (6), and those with no significant homology (68) (Figure 1). BLAST analysis with an rRNA operon showed that only one TDF (0.4%) had homology to rRNA. Several TDFs represent genes with numerous functions, including pathogenicity. These TDFs included the putative chitinase class V (*chsV*) (TDF107), GTPase activating protein (*rhoI*) (TDF223), Major Facilitator Superfamily (MFS) multidrug transporter (TDF9), laccase (*lcc*) (TDF168), Ca^{2+} ATPase (TDF24) and serine/threonine protein kinase (*ste12*) (TDF214) genes (Table 4). The TDFs corresponding to *chsV*, *rhoI*, *lcc*, Ca^{2+} ATPase, and *ste12* showed low intensity levels in *Foc* STR4 and *Foc* TR4 compared with non-pathogenic *F. oxysporum* where transcripts were not visually detected. The TDF representing the MFS multidrug transporter gene displayed moderate intensity in *Foc* STR4 and *Foc* TR4 compared with undetectable levels in non-pathogenic *F. oxysporum* (Figure 2a).

Several different transcript abundance patterns were detected during cDNA-AFLP gel analysis (Table 4). In the first pattern, high transcript abundance was detected in *Foc* STR4 with no transcripts detected in *Foc* TR4 or non-pathogenic *F. oxysporum*. Examples showing high abundance include TDFs corresponding to the aspartyl-tRNA synthetase (TDF52), galactokinase (TDF57) and O-acetylhomoserine (TDF215). The second transcript abundance pattern showed an increase in transcripts in *Foc* TR4 with no transcripts detected in *Foc* STR4 or non-pathogenic *F. oxysporum.* TDFs that exhibited this pattern were 60S ribosomal protein L2 (TDF12), meiosis induction protein (TDF13), L-aminoadipate semialdehyde dehydrogenase large subunit (TDF64), fatty acid synthase subunit alpha reductase (TDF105), small G-protein Gsp1p (TDF174) and glutamine-dependent NAD+ synthetase (TDF190). In the third pattern, transcripts were detected in *Foc* STR4 and *Foc* TR4 with no detection in non-pathogenic *F. oxysporum*. Examples of these transcripts include

Table 4: Putative identities of selected genes identified in *Fusarium oxysporum* using cDNA-amplified fragment length polymorphism analysis based on BLAST results obtained from the Broad Institute database

Transcript-derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF8	Hypothetical protein similar to glycosyl transferase	*F. verticillioides*	FVEG_12066	4.0E-05	GO: 0016757	+++	+++	++
TDF9	MFS multidrug transporter	*Aspergillus fumigatus*	EDP53405.1	2.0E-10	GO: 0015238	++	++	-
TDF12	60S ribosomal protein L2	*F. oxysporum*	FOXG_01889	3.0E-47	GO: 0003735	-	+	-
TDF13	Hypothetical protein similar to meiosis induction protein kinase Ime2	*F. verticillioides*	FVEG_11244	7.0E-41	GO: 0004672	-	+	-
TDF15	Heat shock protein 60, mitochondrial precursor	*F. oxysporum*	FOXG_07996	7.0E-18	GO: 0005524	+++	++	+++
TDF24	Hypothetical protein similar to Ca2+ ATPase	*F. oxysporum*	FOXG_10713	2.0E-16	GO: 0005388	+	+	-
TDF25	Hypothetical protein similar to coatomer subunit delta	*F. verticillioides*	FVEG_07091	2.0E-13	GO: 0042802	+++	-	-
TDF32	Vacuolar protease A precursor	*F. oxysporum*	FOXG_12714	3.0E-12	GO: 0004175	+++	+++	+
TDF39	Hypothetical protein similar to RING-8 protein	*F. oxysporum*	FOXG_00847	1.0E-04	GO: 0005515	++	+++	+++
TDF42	Hypothetical protein similar to FAD-dependent oxidoreductase	*F. graminearum*	FGSG_09373	1.0E-13	GO: 0016491	+	+	-
TDF52	Aspartyl-tRNA synthetase	*F. graminearum*	FGSG_04934	0.0E+00	GO: 0005524	+++	-	-
TDF53	Ubiquitin carboxyl-terminal hydrolase 6	*F. oxysporum*	FOXG_03651	2.0E-97	GO: 0004843	++	++	+
TDF55	Hypothetical protein similar to protein kinase	*F. oxysporum*	FOXG_03168	3.0E-17	GO: 0004672	+++	+	-
TDF56	Origin recognition complex subunit 1	*F. oxysporum*	FOXG_00048	4.0E-17	GO: 0003677	++	+++	++
TDF57	Hypothetical protein similar to galactokinase	*F. oxysporum*	FOXG_11551	3.0E-31	GO: 0005353	+++	-	-
TDF59	Hypothetical protein similar to hexose transporter	*F. oxysporum*	FOXG_12267	1.0E-07	GO: 0005353	++	+	-
TDF64	L-aminoadipate-semialdehyde dehydrogenase large subunit	*F. oxysporum*	FOXG_11115	5.0E-39	GO: 0004043	-	+	-
TDF70	Hypothetical protein similar to transporter protein smf2	*F. verticillioides*	FVEG_03655	2.0E-15	GO: 0005384	++	++	-
TDF80	ATP-dependent helicase NAM7	*F. oxysporum*	FOXG_05494	2.0E-35	GO: 0003682	++	++	-
TDF89	ER lumen protein retaining receptor 1	*F. oxysporum*	FOXG_11078	8.0E-30	GO: 0005046	++	+	+
TDF105	Fatty acid synthase subunit alpha reductase	*F. verticillioides*	FVEG_04241	3.0E-15	GO: 0004315	-	+	-
TDF107	Hypothetical protein similar to class V chitinase	*F. graminearum*	FGSG_02354	4.0E-17	GO: 0004568	+	+	-
TDF108	Urease	*F. oxysporum*	FOXG_01071	3.0E-68	GO: 0004497	++	++	-
TDF115	Eukaryotic translation initiation factor 2 subunit gamma	*F. oxysporum*	FOXG_01983	1.0E-21	GO: 0003743	++	-	++
TDF138	Protein SEY1	*F. verticillioides*	FVEG_00725	2.0E-20	GO: 0005525	+	+	-
TDF140	Serine/threonine-protein kinase hal4	*F. graminearum*	FGSG_06939	1.0E-08	GO: 0004674	+	+	-
TDF141	Protein SEY1	*F. verticillioides*	FVEG_00725	6.0E-25	GO: 0005525	++	-	++
TDF144	Glutamine synthetase	*F. graminearum*	FGSG_10264	3.0E-04	GO: 0006541	+++	+++	-
TDF147	Hypothetical protein similar to BET3 family protein	*F. verticillioides*	FVEG_04550	5.0E-36	GO: 0006888	+	+	-
TDF151	Hypothetical protein similar to HAD-superfamily hydrolase subfamily IIB	*F. oxysporum*	FOXG_16804	3.0E-37	GO: 0016787	+	-	-
TDF154	Frequency clock protein	*F. verticillioides*	FVEG_04686	3.0E-23	GO: 0097167	+	+	-
TDF156	Hypothetical protein similar to coenzyme A transferase	*F. graminearum*	FGSG_02146	1.0E-30	GO: 0008260	++	++	-
TDF158	Histone deacetylase phd1	*F. oxysporum*	FOXG_00027	4.0E-23	GO: 0017136	+	++	-
TDF161	Hypothetical protein similar to cohesin complex subunit Psm1	*F. oxysporum*	FOXG_04230	4.0E-12	GO: 0008280	++	++	-
TDF168	Hypothetical protein similar to laccase	*F. oxysporum*	FOXG_06344	7.0E-23	GO: 0052716	+	+	-
TDF174	Small G-protein Gsp1p	*Magnaporthe grisea*	MGG_09952	5.0E-13	GO: 0003924	-	+	-
TDF175	C-4 methylsterol oxidase	*F. oxysporum*	FOXG_08223	3.0E-53	GO: 0000254	+	+	-
TDF176	Hypothetical protein similar to chitin biosynthesis protein	*F. oxysporum*	FOXG_03146	4.0E-19	GO: 0006031	++	-	-
TDF179	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase precursor	*F. oxysporum*	FOXG_04115	3.0E-30	GO: 0004067	+	+	-
TDF186	Hypothetical protein similar to MutT/nudix family protein	*F. oxysporum*	FOXG_01294	9.0E-15	GO: 0005515	+	-	-
TDF190	Hypothetical protein similar to glutamine-dependent NAD(+) synthetase	*F. verticillioides*	FVEG_07876	1.0E-16	GO: 0003952	-	+	-
TDF193	Hypothetical protein similar to SAC3/GANP domain protein	*Neurospora crassa*	NCU06594	1.0E-53	GO: 0005515	++	++	+
TDF204	Hypothetical protein similar to ATP-cone	*F. oxysporum*	FOXG_11977	2.0E-53	GO: 0031250	+	-	+
TDF206	Hypothetical protein similar to vacuole-associated enzyme activator complex component Vac14	*F. graminearum*	FGSG_09846	5.0E-24	GO: 0008047	-	++	-
TDF214	Hypothetical protein similar to serine/threonine protein kinase	*F. graminearum*	FGSG_05764	3.0E-12	GO: 0004674	+	+	-
TDF215	O-acetylhomoserine	*F. oxysporum*	FOXG_11296	4.0E-16	GO: 0003961	+++	-	-
TDF217	Hypothetical protein similar to DUF895 domain membrane protein	*F. verticillioides*	FVEG_08610	9.0E-18	GO: 0015572	+	+	-
TDF223	GTPase activating protein	*Verticillium albo-atrum*	XM_003005785.1	1.0E-20	GO: 0003779	+	+	-

[a]*Indicates the visual intensity of a specific cDNA-AFLP band in* Fusarium oxysporum *f. sp.* cubense (Foc) *subtropical race 4 (STR4),* Foc *tropical race 4 (TR4) and non-pathogenic* F. oxysporum *(NP).*

–, no transcripts detected; +, low level of transcript abundance; ++, moderate level of transcript abundance; +++, high level of transcript abundance

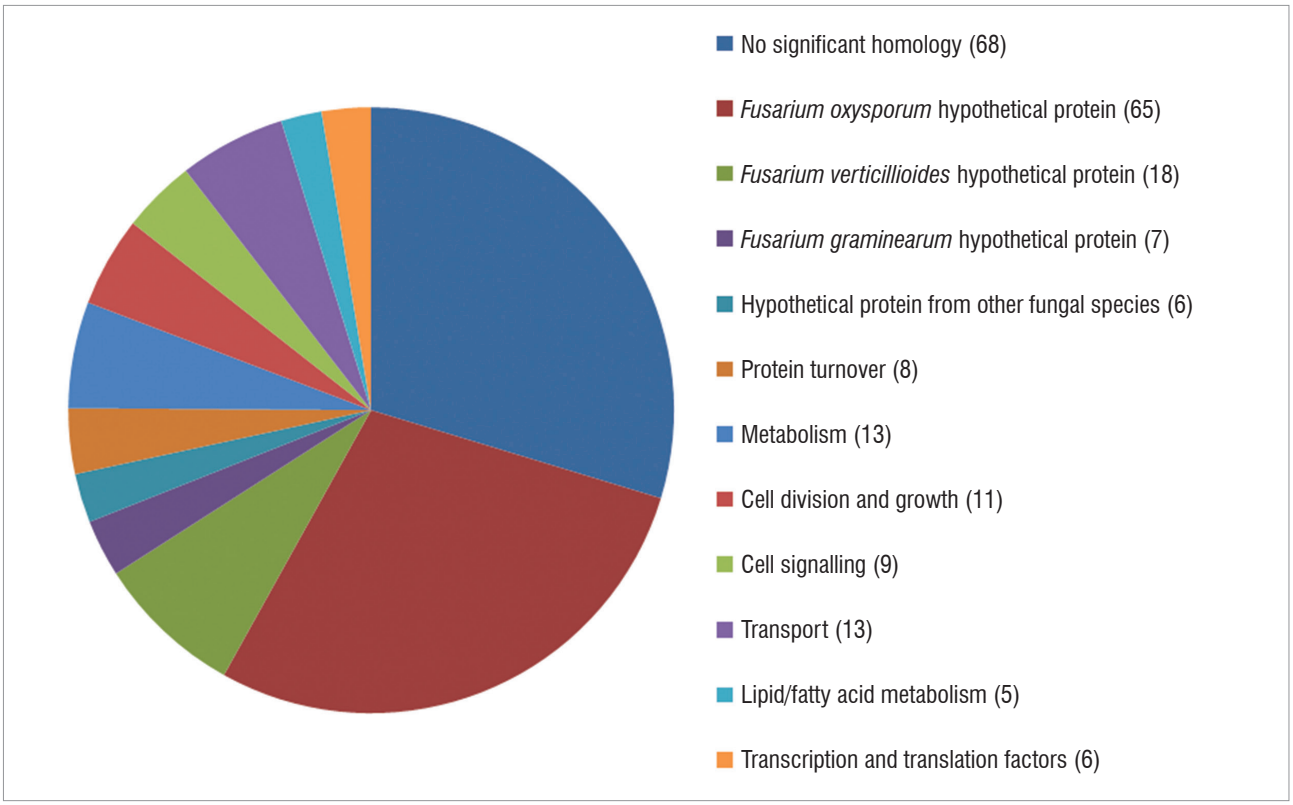

Figure 1: Classification of differentially accumulated transcript derived fragments (TDFs) after growth of *Fusarium oxysporum* f. sp. *cubense* and non-pathogenic *F. oxysporum* in minimal medium without a carbon source. A total of 229 TDFs were classified based on the BLASTX homology search on the Broad Institute database. Values in parentheses indicate the number of TDFs found in each category.

chsV (TDF107), Ca^{2+} ATPase (TDF24), FAD-dependent oxidoreductase (TDF42), *ste12* (TDF214), GTPase activating protein (TDF223) and *lcc* (TDF168). Other transcript abundance patterns included transcript presence in *Foc* STR4 and the non-pathogenic strains, with no transcripts detected in *Foc* TR4. These transcripts included eukaryotic translation initiation factor 2 subunit gamma (TDF115) and ATP-cone (TDF204). Another pattern displayed low levels of transcript abundance in *Foc* STR4 with high transcript abundance in *Foc* TR4 and non-pathogenic *F. oxysporum*.

Quantitative verification of cDNA-AFLP

Five reference genes (Table 3) were evaluated for stable expression. The average pairwise variation (V-value) calculated for *IDH, G6DH* and *GAPDH* was 0.113, with *TEF* and *TUB* showing less stable expression levels (V=0.225). As a result, the reference genes *IDH, G6DH* and *GAPDH* were used to normalise the data as suggested by Vandesompele et al.[40]

The relative transcript abundance of six genes measured by cDNA-AFLP analysis – encoding a MFS multidrug transporter (TDF9), a L-aminoadipate-semialdehyde dehydrogenase large subunit (TDF64), an aspartyl-tRNA synthetase (TDF52), a *chsV* (TDF107), a *ste12* (TDF214) and *rhoI* (TDF223) – was compared with results obtained by qRT-PCR (Figure 2). Both cDNA-AFLP and qRT-PCR analyses showed an increased abundance of the MFS multidrug transporter gene in *Foc* STR4 and *Foc* TR4 when compared with non-pathogenic *F. oxysporum* (Figure 2a). When the abundance levels of L-aminoadipate-semialdehyde dehydrogenase large subunit were compared, the cDNA-AFLP analysis demonstrated that the transcript was present in *Foc* TR4 but absent in the transcript in *Foc* STR4 and the non-pathogenic *F. oxysporum*. The qRT-PCR data showed similar levels of transcript abundance in *Foc* TR4, *Foc* STR4 and the non-pathogenic *F. oxysporum* (Figure 2b). cDNA-AFLP analyses showed an increased abundance of transcripts of aspartyl-tRNA synthetase in *Foc* STR4 compared with *Foc* TR4 and the non-pathogenic *F. oxysporum*, while qRT-PCR revealed similar transcript levels among the different isolates (Figure 2c). An increase in transcript abundance of *chsV* was found in *Foc* STR4 and *Foc* TR4 when compared with the non-pathogenic *F. oxysporum* using both cDNA-AFLP analysis and qRT-PCR (Figure 2d). Transcript abundance profiles determined for *ste12* by cDNA-AFLP and qRT-PCR were similar, and showed an increase in *Foc* STR4 and *Foc* TR4 compared with the non-pathogenic *F. oxysporum* (Figure 2e). In the case of *rhoI*, cDNA-AFLP analysis showed an increase in the number of transcripts in *Foc* race 4 compared with the non-pathogenic *F. oxysporum* (Figure 2f). However, qRT-PCR showed a higher number of transcripts in *Foc* STR4 than in *Foc* TR4 and the non-pathogenic *F. oxysporum*. Thus, the transcript abundance patterns measured by qRT-PCR were similar to those measured for the corresponding TDFs analysed using cDNA-AFLP.

Transcript abundance of known pathogenicity genes using qRT-PCR

Foc STR4 and *Foc* TR4 expressed the pathogenicity genes *snf* (Figure 3a), *frp1* (Figure 3b) and *cyp55* (Figure 3c) at significantly higher levels than non-pathogenic *F. oxysporum*. The transcript abundance of *snf* was 2.6-fold higher in *Foc* STR4 than in the non-pathogenic *F. oxysporum*. The transcript abundance levels of *frp1* were lower in non-pathogenic *F. oxysporum* isolates than in pathogenic *Foc* STR4 and *Foc* TR4 isolates, by 3.6-fold and 2.5-fold, respectively. *Snf* and *frp1* are involved in the degradation of plant cell walls.[17,41] *Cyp55* had a 1.6-fold higher expression in *Foc* TR4 compared with *Foc* STR4, but this difference was not statistically significant. *Cyp55* is a nitric oxide reductase involved in the nitrogen response pathway, which is fundamental for pathogenicity.

Fmk1 is responsible for maintaining fungal cell wall architecture and signalling. *Fmk1* was expressed significantly more in *Foc* STR4 than in either *Foc* TR4 or non-pathogenic *F. oxysporum* (Figure 3d). *Fmk1* expression was 2.9-fold higher in *Foc* STR4 than in the non-pathogenic

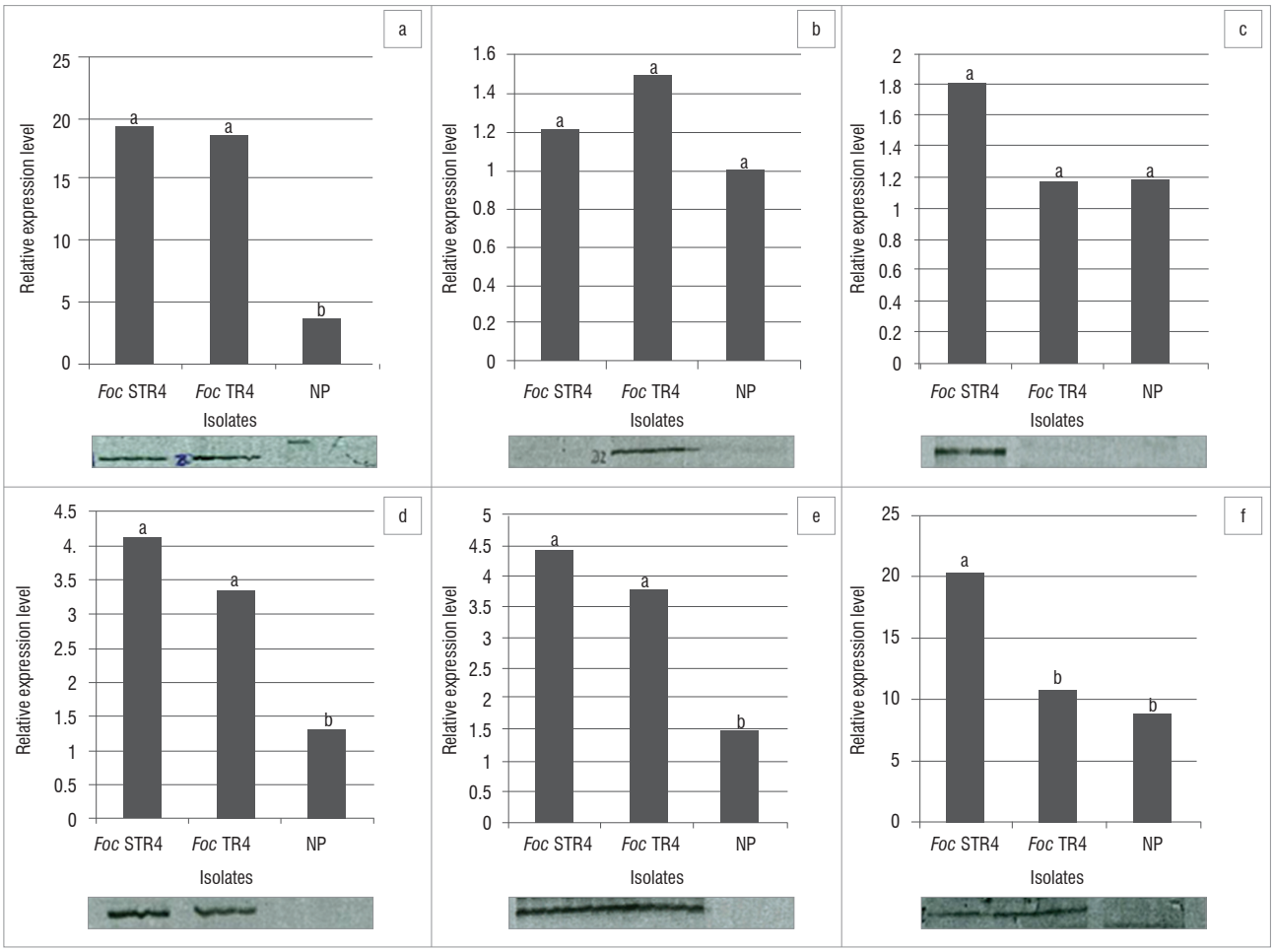

Figure 2: Verification of the relative expression of selected genes by quantitative real-time PCR in *Fusarium oxysporum* f. sp. *cubense* (*Foc*) sub-tropical race 4 (STR4), *Foc* tropical race 4 (TR4) and non-pathogenic *F. oxysporum* (NP): (a) MFS multidrug transporter (TDF9), (b) L-aminoadipate-semialdehyde dehydrogenase (TDF64), (c) aspartyl-tRNA synthetase (TDF52), (d) chitinase class V (*chsV*) (TDF107), (e) serine/threonine protein kinases (*ste12*) (TDF214) and (f) GTPase activating protein (*rhoI*) (TDF223). Segments of the original polyacrylamide cDNA-AFLP gels are shown below the horizontal axis. The letters above the bars indicate significant differences between the genotypes. The difference in Ct values was determined statistically by one-way ANOVA, followed by Tukey's post-hoc analysis; $p<0.05$ was considered statistically significant.

F. oxysporum (Figure 3d). In addition, transcript abundance of *fmk1* was a significant 2.1-fold higher in *Foc* STR4 than in *Foc* TR4. Expression of the chloride channel (*clc*) gene, which controls laccase activity, was also significantly higher in *Foc* STR4 than in the non-pathogenic *F. oxysporum* (Figure 3e). In contrast, *fow2,* the fungal gene involved in regulating pathogenicity-related transcription, was expressed significantly more in *Foc* TR4 than in the non-pathogenic *F. oxysporum*, but not significantly more than in *Foc* STR4 (Figure 3f). There were no significant differences observed in the transcript abundance profiles of the arginine biosynthesis gene (*arg1*) (Figure 3g) or mitochondrial protein gene (*fow1*) (Figure 3h).

Discussion and conclusion

The transcriptomes of *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* isolates on MM (without carbon source) were visually detected with cDNA-AFLP. More than 3000 TDFs were detected, of which 8% showed differential expression patterns. A total of 3% of these TDFs were putatively involved in pathogenicity. Several fungal gene transcripts that have previously been associated with pathogenicity in other fungal organisms have been identified for the first time in the banana pathogen *Foc*. These genes include *chsV*, *rhoI*, MFS multidrug transporter and *stel2*. In addition, the genes *snf*, *frp1* and *cyp55*, which result in diseases of crops other than banana, were more abundantly expressed in *Foc* STR4 and *Foc* TR4 than in non-pathogenic *F. oxysporum.*

The genes *chsV* and *rhoI* have previously been associated with pathogenicity in *Fol* on tomato.[42,43] *ChsV* restricts toxic substances produced by the plant for defence against pathogens,[42] whereas *rho1* plays a role in preventing the host plant from recognising the pathogen.[43] Both genes, therefore, protect the pathogen against the host's defence response. Because *chsV* and *rho1* showed higher transcript abundances in *Foc* STR4 and *Foc* TR4 than in the non-pathogen, we hypothesise that *Foc* expresses these genes when infecting the xylem vessels of Cavendish bananas to avoid the plant's defence responses.

The transcript abundance of the MFS multidrug transporter was five-fold higher in pathogenic *Foc* than in the non-pathogen. This family of transporters regulate the movement of sugars, Krebs-cycle metabolites, phosphorylated glycolytic intermediates, amino acids, peptides, osmolites, iron-siderophores, nucleosides and organic and inorganic anions and cations.[44] In addition, MFS transporters have been linked to fungal pathogenicity by avoiding toxic compounds produced by the pathogen, or by protection against plant defence compounds.[45] MFS transporter gene in the ascomycete *Verticillium dahlia* – a vascular pathogen – is essential for pathogenicity on lettuce plants.[46] With a significantly higher transcript abundance of the MFS multidrug transporter, *Foc* STR4 and *Foc* TR4 may possibly protect themselves from toxic substances produced by the plant during defence.

The transcription factor *ste12* is important during fungal infection of plant roots where it regulates genes involved in the MAPK cascade.[20,47] In a study by Garcia-Sanchez et al.[22], a *ste12*-like gene, *fost12*, showed an increased expression after 12–24 h of infection of bean plants by *F.*

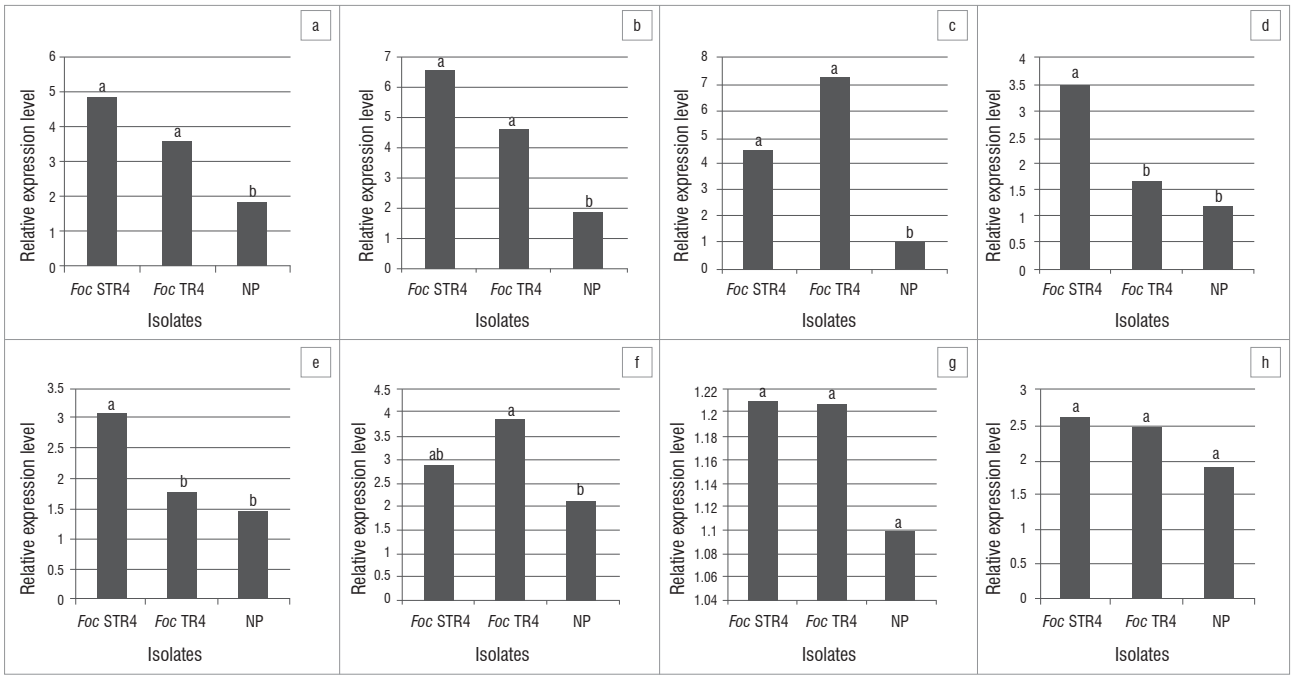

Figure 3: Relative transcript abundance of known virulence genes by reverse transcription real-time quantitative PCR in *Fusarium oxysporum* f. sp. *cubense (Foc)* sub-tropical race 4 (STR4), *Foc* tropical race 4 (TR4) and non-pathogenic *F. oxysporum* (NP): (a) sucrose non-fermenting gene (*snf*), (b) F-box protein required for pathogenicity (*frp1*), (c) cytochrome P450 (*cyp55*), (d) Fusarium MAP kinase (*fmk1*), (e) chloride channel gene (*clc*), (f) a Zn(II)2Cys6-type transcription regulator (*fow2*), (g) arginine biosynthesis gene (*arg1*) and (h) mitochondrial protein (*fow1*). The letters above the bars indicate the significant differences between the samples. The difference in Ct values was determined statistically by one-way ANOVA, followed by Tukey's post-hoc analysis; $p < 0.05$ was considered statistically significant.

oxysporum f. sp. *phaseoli*. A significant increase in transcript abundance of *ste12* in *Foc* STR4 and *Foc* TR4 can activate the MAPK signalling pathways, thereby increasing CWDE during the infection process. A second transcription factor, *fow2,*[21] a Zn(II)2Cys6-type transcription regulator involved in pathogenicity in *F. oxysporum* f. sp. *melonis*, was significantly higher in *Foc* TR4 than in the non-pathogen, but there was no significant difference between *Foc* STR4 and the non-pathogen. Because *Foc* TR4 is a more virulent pathogen than *Foc* STR4, *fow2* may assist in the more rapid invasion of root tissue or may be differentially regulated in *Foc* STR4 and *Foc* TR4.

Two well-studied pathogenicity genes previously isolated from *F. oxysporum* that regulate the abundance of CWDEs are *snf* and *frp1.*[16,17,41] Both *snf* and *frp1* were significantly higher in *Foc* STR4 and *Foc* TR4 than in the non-pathogen, which suggests that these genes are important for the Fusarium wilt pathogen to enter the host xylem tissue. As an endophyte, the non-pathogenic *F. oxysporum* isolates are usually restricted to the root cortex, and do not enter the xylem vessels.[48] In contrast, *Foc* STR4 and *Foc* TR4 both degrade the xylem cell walls to enter the vascular tissue.

Pathogenicity and cell wall degradation are affected by the enhanced expression of MAP kinases in several fungi, for example *Fol,*[15] *Fusarium graminearum,*[49] *Magnaporthe grisea*[50] and *Ustilago maydis*[51]. In *Fol, fmk1* also aids in root attachment, penetration, invasive growth and increased CWDE activity.[15] The significant increase in *fmk1* in *Foc* STR4 and non-significant increase in *Foc* TR4 compared with non-pathogenic *F. oxysporum* may explain pathogenesis in the banana Fusarium wilt pathogen, that is by accelerating invasive growth as in other *Fusarium* species.[15,52] Pathogenic *Foc* isolates are able to colonise both the cortex and the xylem tissue, resulting in severe discoloration of the corm and blocking of the vascular bundles. In contrast, the non-pathogenic strains are restricted to the root cortex, which results in no symptoms developing. The reason that *fmk1* did not show a significant increase in transcript abundance in *Foc* TR4 is not certain, but one possible explanation could be that *fmk1* transcripts amplified during pathogenicity at earlier time points were not sampled in this study. Genes expressed during the early time points are either translated into proteins or the RNA is degraded as the half-life of RNA is short and therefore the RNA cannot be detected at later time points.

Cyp55 was more abundant in *Foc* race 4 than in non-pathogenic *F. oxysporum.* This gene plays a role in the ability to regulate the nitrogen response pathway, which is essential for pathogenicity.[53] *Cyp55*, a cytochrome P450 gene involved in the reduction of nitric oxide in *F. oxysporum*, was first characterised by Kizawa et al.[54] The c*yp55* gene of *F. oxysporum* f. sp. *vasinfectum* has been previously reported to be highly expressed in cotton plants following root inoculation.[55]

Laccases serve as virulence factors in fungal pathogens by playing a role in pigmentation, appressorium formation and protection against toxic phytoalexins.[56] qRT-PCR analysis in this study revealed a significant increase in *clc* transcripts in *Foc* STR4 compared with the non-pathogen. In *Fol*, mutations of *lcc1*, *lcc3* and *lcc5* had no effect on pathogenicity in tomato plants.[57] As six *lcc* genes have been identified in *F. oxysporum*, Cañero and Roncero[28] suggested that a mutation in one of them may not necessarily prevent laccase activity, as the other isozymes fulfil their role.[57] However, c*lc* mutants showed a decrease in laccase activity with a reduction in virulence to tomato seedlings.[28] Increased *clc* expression and the role of laccases and chloride transport in the banana Fusarium wilt pathogen may be important pathogenicity determinants.

The cDNA-AFLP technique was useful in differentiating the transcript abundance of genes present in *Foc* race 4 and non-pathogenic *F. oxysporum*. However, DNA sequence differences could result in the absence or presence of a TDF not necessarily implicating differential expression. To minimise these single nucleotide polymorphisms, nine isolates from different geographical regions were combined for each of the *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* fungal samples. Most of the gene expression patterns measured by cDNA-AFLPs were confirmed by qRT-PCR analyses. However, next-generation

DNA and RNA sequencing could provide significantly better results for identifying pathogenicity genes in *Foc*, both in STR4 and TR4, especially once the full genome sequence of the Fusarium wilt fungus becomes available. Comparison of the *Foc* genome with that of other *forma speciales* of *F. oxysporum* will elucidate the ability of *Foc* to infect banana roots. Virulence factors can be studied when the genomes of *Foc* TR4, a more virulent pathogen, are compared with *Foc* STR4. Furthermore, the function of putative pathogenicity genes during infection should be investigated by gene knockout studies and RNAi silencing. Knockout mutants would help to identify additional genes required for pathogenicity in *Foc* race 4.

An in-depth understanding of pathogenicity in *Foc* is required if novel approaches to disease management are to be developed. We have identified several transcripts in *Foc* race 4 that are more abundant in the pathogenic strains compared with the non-pathogens. Many of these TDFs have been shown to play a role in host infection and colonisation by other *Fusarium* spp. These TDFs encode for CWDEs and proteins involved in avoiding toxic substances produced during plant defence. To establish function, knockout mutants of genes underlying these transcripts need to be generated, and the role of genes such as *chsV, rhoI*, MFS multidrug transporter, *ste12, snf, frp1, cyp55* and *fmk1* needs further investigation. With the rapid advancement in molecular techniques in recent years, new strategies for increasing plant resistance against specific Fusarium wilt pathogens can be generated by exploiting the molecular and cellular bases of pathogenicity.

Acknowledgements

This research was funded by The Human Resources for Industry Programme (THRIP), an initiative of the Department of Trade and Industry (DTI); the National Research Foundation Thuthuka programme; and the Banana Growers Association of South Africa.

Authors' contributions

R.S. performed the experiments; A.V., A.A.M., N.v.d.B. and R.S. were involved in the experimental design and data analysis; and all authors contributed in writing the manuscript.

References

1. Michielse CB, Rep M. Pathogen profile update: *Fusarium oxysporum*. Mol Plant Pathol. 2009;10(3):311–324. http://dx.doi.org/10.1111/j.1364-3703.2009.00538.x
2. Gordon TR, Martyn RD. The evolutionary biology of *Fusarium oxysporum*. Annu Rev Phytopathol. 1997;35(1):111–128. http://dx.doi.org/10.1146/annurev.phyto.35.1.111
3. Ma L-J, Van der Does HC, Borkovich KA, Coleman JJ, Daboussi M-J, Di Pietro A, et al. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature. 2010;464(7287):367–373. http://dx.doi.org/10.1038/nature08850
4. Rep M, Kistler HC. The genomic organization of plant pathogenicity in *Fusarium* species. Curr Opin Plant Biol. 2010;13(4):420–426. http://dx.doi.org/10.1016/j.pbi.2010.04.004
5. Idnurm A, Howlett BJ. Pathogenicity genes of phytopathogenic fungi. Mol Plant Pathol. 2001;2(4):241–255. http://dx.doi.org/10.1046/j.1464-6722.2001.00070.x
6. Möbius N, Hertweck C. Fungal phytotoxins as mediators of virulence. Curr Opin Plant Biol. 2009;12(4):390–398. http://dx.doi.org/10.1016/j.pbi.2009.06.004
7. Werner S, Sugui JA, Steinberg G, Deising HB. A chitin synthase with a myosin-like motor domain is essential for hyphal growth, appressorium differentiation, and pathogenicity of the maize anthracnose fungus *Colletotrichum graminicola*. Mol Plant Microbe In. 2007;20(12):1555–1567. http://dx.doi.org/10.1094/MPMI-20-12-1555
8. Chi M-H, Park S-Y, Kim S, Lee Y-H. A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host. PLoSPathog. 2009;5(4):e1000401. http://dx.doi.org/10.1371/journal.ppat.1000401
9. De Wit PJGM, Mehrabi R, Van Den Burg HA, Stergiopoulos I. Fungal effector proteins: Past, present and future. Mol Plant Pathol. 2009;10(6):735–747. http://dx.doi.org/10.1111/j.1364-3703.2009.00591.x
10. Di Pietro A, Roncero MIG. Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol Plant Microbe In. 1998;11(2):91–98. http://dx.doi.org/10.1094/MPMI.1998.11.2.91
11. García-Maceira FI, Di Pietro A, Roncero MIG. Cloning and disruption of *pgx4* encoding an *in planta* expressed exopolygalacturonase from *Fusarium oxysporum*. Mol Plant Microbe In. 2000;13(4):359–365. http://dx.doi.org/10.1094/MPMI.2000.13.4.359
12. Huertas-González MD, Ruiz-Roldán MC, García Maceira FI, Roncero MIG, Di Pietro A. Cloning and characterization of *pl1* encoding an *in planta*-secreted pectate lyase of *Fusarium oxysporum*. Curr Genet. 1999;35(1):36–40. http://dx.doi.org/10.1007/s002940050430
13. Roldán-Arjona T, Pérez-Espinosa A, Ruiz-Rubio M. Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* defines a new class of saponinases. Mol Plant Microbe In. 1999;12(10):852–861. http://dx.doi.org/10.1094/MPMI.1999.12.10.852
14. Ruiz-Roldán MC, Di Pietro A, Huertas-González MD, Roncero MIG. Two xylanase genes of the vascular wilt pathogen *Fusarium oxysporum* are differentially expressed during infection of tomato plants. Mol Gen Genet. 1999;261(3):530–536. http://dx.doi.org/10.1007/s004380050997
15. Di Pietro A, García-Maceira FI, Méglecz E, Roncero MIG. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Mol Microbiol. 2001;39(5):1140–1152. http://dx.doi.org/10.1111/j.1365-2958.2001.02307.x
16. Duyvesteijn RGE, Van Wijk R, Boer Y, Rep M, Cornelissen BJC, Haring MA. Frp1 is a *Fusarium oxysporum* F-box protein required for pathogenicity on tomato. Mol Microbiol. 2005;57(4):1051–1063. http://dx.doi.org/10.1111/j.1365-2958.2005.04751.x
17. Ospina-Giraldo MD, Mullins E, Kang S. Loss of function of the *Fusarium oxysporum SNF1* gene reduces virulence on cabbage and *Arabidopsis*. Curr Genet.. 2003;44:49–57. http://dx.doi.org/10.1007/s00294-003-0419-y
18. Jain S, Akiyama K, Kan T, Ohguchi T, Takata R. The G protein ß subunit FGB1 regulates development and pathogenicity in *Fusarium oxysporum*. Curr Genet.. 2003;43(2):79–86.
19. Jain S, Akiyama K, Mae K, Ohguchi T, Takata R. Targeted disruption of a G protein a subunit gene results in reduced pathogenicity in *Fusarium oxysporum*. Curr Genet.. 2002;41(6):407–413. http://dx.doi.org/10.1007/s00294-002-0322-y
20. Rispail N, Di Pietro A. *Fusarium oxysporum ste12* controls invasive growth and virulence downstream of the *Fmk1* MAPK cascade. Mol Plant Microbe In. 2009;22(7):830–839. http://dx.doi.org/10.1094/MPMI-22-7-0830
21. Imazaki I, Kurahashi M, Iida Y, Tsuge T. Fow2, a Zn(II)2Cys6-type transcription regulator, controls plant infection of the vascular wilt fungus *Fusarium oxysporum*. Mol Microbiol. 2007;63(3):737–753. http://dx.doi.org/10.1111/j.1365-2958.2006.05554.x
22. Garcia-Sanchez AM, Martin-Rodrigues N, Ramos B, De Vega-Bartol JJ, Perlin MH, Diaz-Minguez JM. *Fost12*, the *Fusarium oxysporum* homolog of the transcription factor Ste12, is upregulated during plant infection and required for virulence. Fungal Genet Biol. 2010;47:216–225. http://dx.doi.org/10.1016/j.fgb.2009.11.006
23. Ploetz RC, Pegg KG. Fungal diseases of the root, corm and pseudostem: Fusarium wilt. In: Jones DR, editor. Diseases of banana, abacá and enset. London: CAB International; 2000. p. 143–159. http://dx.doi.org/10.1094/PHP-2005-1221-01-RV
24. Ploetz RC. Panama disease: An old nemesis rears its ugly head. Part 1. The beginnings of the banana export trades. Plant Health Progress. 2005; doi:10.1094/PHP-2005-1221-01-RV.
25. Belgrove A, Steinberg C, Viljoen A. Evaluation of non-pathogenic *Fusarium oxysporum* and *Pseudomonas fluorescens* for Panama disease control. Plant Dis. 2011;95(8):951–959. http://dx.doi.org/10.1094/PDIS-06-10-0409
26. Thangavelu R, Jayanthi A. RFLP analysis of rDNA-ITS regions of native non-pathogenic *Fusarium oxysporum* isolates and their field evaluation for the suppression of Fusarium wilt disease of banana. Australas Plant Path. 2009;38(1):13–21. http://dx.doi.org/10.1071/AP08071

27. Athman SY, Dubois T, Viljoen A, Labuschagne N, Coyne D, Ragama P, et al. *In vitro* antagonism of endophytic *Fusarium oxysporum* isolates against the burrowing nematode *Radopholus similis*. Nematology. 2006;8(4):627–636. http://dx.doi.org/10.1163/156854106778613976

28. Cañero DC, Roncero MIG. Influence of the chloride channel of *Fusarium oxysporum* on extracellular laccase activity and virulence on tomato plants. Microbiology. 2008;154:1474–1481. http://dx.doi.org/10.1099/mic.0.2007/015388-0

29. Inoue I, Namiki F, Tsuge T. Plant colonization by the vascular wilt fungus *Fusarium oxysporum* requires *FOW1*, a gene encoding a mitochondrial protein. Plant Cell. 2002;14:1869–1883. http://dx.doi.org/10.1105/tpc.002576

30. Namiki F, Matsunaga M, Okuda M, Inoue I, Nishi K, Fujita Y, et al. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *melonis*. Mol Plant Microbe In. 2001;14(4):580–584. http://dx.doi.org/10.1094/MPMI.2001.14.4.580

31. Tomura D, Obika K, Fukamizu A, Shoun H. Nitric oxide reductase cytochrome P-450 gene, *CYP55*, of the fungus *Fusarium oxysporum* containing a potential binding-site for FNR, the transcription factor involved in the regulation of anaerobic growth of *Escherichia coli*. J Biochem. 1994;116(1):88–94.

32. Nel B, Steinberg C, Labuschagne N, Viljoen A. Isolation and characterization of nonpathogenic *Fusarium oxysporum* isolates from the rhizosphere of healthy banana plants. Plant Pathol. 2006;55:207–216. http://dx.doi.org/10.1111/j.1365-3059.2006.01343.x

33. Van den Berg N, Berger DK, Hein I, Birch PRJ, Wingfield MJ, Viljoen A. Tolerance in banana to Fusarium wilt is associated with early up-regulation of cell wall-strengthening genes in the roots. Mol Plant Pathol. 2007;8(3):333–341. http://dx.doi.org/10.1111/j.1364-3703.2007.00389.x

34. Trail F, Xu J-R, Miguel PS, Halgren RG, Corby Kistler H. Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). Fungal Genet Biol. 2003;38(2):187–197. http://dx.doi.org/10.1016/S1087-1845(02)00529-7

35. O'Donnell K, Corby Kistler H, Cigelnik E, Ploetz R. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci USA. 1998;95:2044–2049. http://dx.doi.org/10.1073/pnas.95.5.2044

36. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acid S. 1999;41:95–98.

37. Guo Y, Ribeiro JM, Anderson JM, Bour S. dCAS: A desktop application for cDNA sequence annotation. Bioinformatics. 2009;25(9):1195–1196. http://dx.doi.org/10.1093/bioinformatics/btp129

38. Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, et al. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res. 2004 October 14, 2004;32(18):5539–5545.

39. The Gene Ontology Consortium. Gene ontology: Tool for the unification of biology. Nature Genetics. 2000;25(1):25–29. http://dx.doi.org/10.1038/75556

40. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:research0034.

41. Jonkers W, Rodrigues CDA, Rep M. Impaired colonization and infection of tomato roots by the Δfrp1 mutant of *Fusarium oxysporum* correlates with reduced CWDE gene expression. Mol Plant Microbe In. 2009;22(5):507–518. http://dx.doi.org/10.1094/MPMI-22-5-0507

42. Madrid MP, Di Pietro A, Roncero MIG. Class V chitin synthase determines pathogenesis in the vascular wilt fungus *Fusarium oxysporum* and mediates resistance to plant defence compounds. Mol Microbiol. 2003;47(1):257–266. http://dx.doi.org/10.1046/j.1365-2958.2003.03299.x

43. Martínez-Rocha AL, Roncero MIG, López-Ramirez A, Mariné M, Guarro J, Martínez-Cadena G, et al. Rho1 has distinct functions in morphogenesis, cell wall biosynthesis and virulence of *Fusarium oxysporum*. Cell Microbiol. 2008;10(6):1339–1351. http://dx.doi.org/10.1111/j.1462-5822.2008.01130.x

44. Stergiopoulos I, Zwiers L-H, De Waard MA. Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. Eur J Plant Pathol. 2002;108(7):719–734. http://dx.doi.org/10.1023/A:1020604716500

45. Del Sorbo G, Schoonbeek H-J, De Waard MA. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol. 2000;30(1):1–15. http://dx.doi.org/10.1006/fgbi.2000.1206

46. Maruthachalam K, Klosterman S, Kang S, Hayes R, Subbarao K. Identification of pathogenicity-related genes in the vascular wilt fungus *Verticillium dahliae* by *Agrobacterium tumefaciens*-mediated T-DNA insertional mutagenesis. Mol Biotechnol. 2011;49(3):209–221. http://dx.doi.org/10.1007/s12033-011-9392-8

47. Rispail N, Di Pietro A. The homeodomain transcription factor Ste12: Connecting fungal MAPK signaling to plant pathogenicity. Commun Integr Biol. 2010;3(4):327–332. http://dx.doi.org/10.4161/cib.3.4.11908

48. MacHardy WE, Beckman CH. Vascular wilt Fusaria: Infection and pathogenesis. In: Nelson PE, Toussoun TA, Cook RJ, editors. *Fusarium*: Diseases, biology, and taxonomy. London: Pennsylvania State University Press; 1981. p. 365–390.

49. Jenczmionka NJ, Schäfer W. The *Gpmk1* MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. Curr Genet. 2005;47(1):29–36. http://dx.doi.org/10.1007/s00294-004-0547-z

50. Xu J-R, Hamer JE. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. Gene Dev. 1996;10:2696–2706. http://dx.doi.org/10.1101/gad.10.21.2696

51. Mayorga ME, Gold SE. A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. Mol Microbiol. 1999;34(3):485–497. http://dx.doi.org/10.1046/j.1365-2958.1999.01610.x

52. Zhang Y, Choi Y-E, Zou X, Xu J-R. The *FvMK1* mitogen-activated protein kinase gene regulates conidiation, pathogenesis, and fumonisin production in *Fusarium verticillioides*. Fungal Genet Biol. 2011;48(2):71–79. http://dx.doi.org/10.1016/j.fgb.2010.09.004

53. Lopez-Berges MS, Rispail N, Prados-Rosales RC, Di Pietro A. A nitrogen response pathway regulates virulence functions in *Fusarium oxysporum* via the protein kinase TOR and the bZIP protein MeaB. Plant Cell. 2010;22(7):2459–2475. http://dx.doi.org/10.1105/tpc.110.075937

54. Kizawa H, Tomura D, Oda M, Fukamizu A, Hoshino T, Gotoh O, et al. Nucleotide sequence of the unique nitrate/nitrite-inducible cytochrome P-450 cDNA from *Fusarium oxysporum*. J Biol Chem. 1991;266(16):10632–10637.

55. McFadden HG, Wilson IW, Chapple RM, Dowd C. Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*) genes expressed during infection of cotton (*Gossypium hirsutum*). Mol Plant Pathol. 2006;7(2):87–101. http://dx.doi.org/10.1111/j.1364-3703.2006.00327.x

56. Mayer AM, Staples RC. Laccase: New functions for an old enzyme. Phytochemistry. 2002;60:551–565. http://dx.doi.org/10.1016/S0031-9422(02)00171-1

57. Cañero DC, Roncero MIG. Functional analyses of laccase genes from *Fusarium oxysporum*. Phytopathology. 2008;98:509–518. http://dx.doi.org/10.1094/PHYTO-98-5-0509