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RT-qPCR PROFILING OF PATHOGENESIS RELATED GENES IN *MUSA ACUMINATA* CV. 'BERANGAN' SEEDLINGS CHALLENGED WITH *Fusarium oxysporum* F. SP. *Cubense* TROPICAL RACE 4

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The expression profile of pathogenesis related genes are signatures of an infection response in plant cells. Pathogenic infections can increase or reduce gene expression in a plant system in a relatively specific pattern. These expression patterns can be used as standards in pathogenicity studies and, where phenotypic expression is normally used to gauge a plants response to infection, it could additionally present a more rapid and early screening reference tool. Three genes: catalase (CAT), pathogen related protein (PR10), and phenylalanine ammonia (PAL) all implicitly implicated in the plant disease response pathway were targeted for analysis during the infection of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (FOCR4) in banana *Musa acuminata* cv. Berangan seedlings after a standard challenge under growth room conditions. Distinct patterns of gene expression were observed at three infection time points by real time expression analysis. There was a sequential 10-fold reduction in expression for the PR gene while, the PAL and CAT genes were both upregulated. These results present a set of reference genes that could be used for screening of a plant's response to *Fusarium* before the onset of symptoms.
Keywords: Fusarium wilt, *Musa acuminata*, tropical race 4, pathogen attack, PAL gene.

INTRODUCTION

Bananas are monocotyledonous plants within the genus Musa (Musaceae, Zingiberales) (Heslop-Harrison and Schwarzacher, 2007). Banana cultivars are mainly from *M. acuminata* (A genome) and *M. balbisiana* (B genome) which are diploid or sometimes exists as triploids and also tetraploids (D'Hont *et al.,* 2012). They are amongst the world's most popular fruit and consumed as a dessert or in cooking or for making by-products such as chips or beer (Peraza-Echeverria *et al.,* 2008). It is a significant contributor to food security, nutrition and poverty alleviation in many regions (Passos *et al.,* 2013). Banana has been in the top five among fruit crops in most countries across the tropical and subtropical regions where they are cultivated (Chen *et al.,* 2011; Nik Hassan *et al.,* 2003; Swarupa *et al.,* 2014; Shekhawat and Ganaspathi, 2013; Muhammad *et al*., 2017).

A major constraint for banana production is Fusarium wilt (Yim *et al.,* 2014; Dean *et al.,* 2012) described as a vascular wilt disease (Sutherland *et al.,* 2013) that invades vascular tissue (Magliano and Kikot, 2010) through roots causing the discolouration and wilting phenomena and finally collapse (Wu *et al.,* 2010; Nel *et al.,* 2007; Mak *et al.,* 2001). The causative agent, fungal pathogen *Fusarium* also attacks other crops such as guava (Amadi *et al.,* 2014), lettuce (Cabral and Reis, 2013), tomato (Singh *et al.,* 2010), and water melon (Tziros *et al.,* 2007). This host range phenomena is likely due to the complex pathogenic structure of this fungus. In banana, three races 1, 2 and 4, (a classification based on host range) have been identified as the most common infecting cultivated banana. Race 4 is thought to be able to attack all cultivars susceptible to both race 1 and 2. However, race 4 is further grouped into subtropical and tropical races and are generally reported not to attack cultivars across the two environments (Wu *et al.,* 2010). Tropical race 4 has been particularly devastating and recently has attracted much attention due to its apparent spread into new previously unreported regions including Africa and Australia and most recently in Pakistan and Lebanon (Ordonez *et al*., 2015). As yet no lasting solution is available to overcome this pathogen other than through continuous breeding of genetically resistant varieties. Development of tools to rapidly assess plants during challenge assays would certainly assist in the arduous selection process. Hence the present study aimed to demonstrate specific gene expression patterns in banana cv. berangan during infection with *F. oxysporum* Tropical Race 4 (FOCR4) using 3 candidate genes catalase (CAT), pathogen related protein (PR10), and phenylalanine ammonia (PAL) that could be used in early assessment of cultivars. The genes

were selected as candidate reference genes due to their intrinsic involvement in the plant pathogen defence pathway and have been shown in studies on the transcriptome of banana during infection with FOC to be either upregulated or down regulated. The PR gene is a good candidate to indicate induction of an immune response in plants upon fungal attack (Hoffmann-Sommeergruber, 2002). While, CAT are a group of enzymes involved in regulating the cellular level of active oxygen species from the damaging effect of pathogen attack (Gayatridevi *et al.,* 2012) and the PAL gene plays an important role in modulating the resistance of plant tissues in the defence reaction against infection by pathogens in many higher plants (Alvarez *et al.*, 2013). The genes were assayed over 4 time points post infection in a simple and efficient qPCR assay which is a fast and sensitive tool to quantitatively detect specific changes in mRNA expression in the plant during pathogen attack (Lin *et al.,* 2014; Jimenez-Fernandez *et al.,* 2010) and will be able to generate insights into *Musa acuminata* cv Berangan's response to FOCR4 infection.

MATERIALS AND METHODS

***Source of fungal isolate and culture conditions*:** *Fusarium oxysporum* f. sp. *cubense* tropical race 4 FOCR4 (Isolate C1HIR_9899) was originally provided by Prof. Baharuddin Salleh from the Plant Pathology and Mycology Laboratory, School of Biological Sciences, Universiti Sains Malaysia and maintained in the University of Malaya. The isolate was grown on PDA agar/broth at 27°C.

***Preparation of plant material*:** Tissue culture plantlets of *Musa acuminata* cv. Berangan were purchased from the Plant Biotechnology Incubator Unit, CEBAR, University Malaya Kuala Lumpur, Malaysia. The plantlet was hardened for 9 weeks prior to infection (Fig. 1). Inoculation was carried out by a double tray method (Mak *et al.,* 2001) using the FOCR4 (C1 HIR_9899) as inoculum.

***Total RNA isolation and cDNA library construction*:** Total RNA was extracted with an Rneasy® Plant Mini Kit, (Qiagen, Germany) according to the manufacturer`s instructions. RNA quality was evaluated using an Agilent 2100 Bioanalyzer with minimum integrity number value 8. cDNA was synthesized by using a TransScript One-Step gDNA removal and cDNA synthesis SuperMix kit, (Beijing TransGen Biotech Co., Ltd). 20 µl of the reaction mixture contained 10 µl of 2X TS II Reaction Mix, 1 µl of 0.1µg/µl random primer TranScript® II RT/RI Enzyme Mix, gDNA remover and 7 µl of total RNA (50ng-5 µg/5-500ng). The reaction mixture containing RNA and random primers were incubated at 65°C for 5 min followed with incubation on ice for 2 min. Once the rest of the components were added, the mixture was incubated at 25°C for 10 min, 42°C for 30 min and finally 85°C for 5 min to inactivate the reaction. The synthesized cDNAs were assayed for genomic DNA contamination on 1% agarose gel electrophoresis.

Figure 1. ***Musa acuminata*** **cv. 'Berangan' seedlings acclimatized for 9 weeks prior to inoculation.**

***Quantitation and integrity of total RNA*:** The quantity and quality of the RNA obtained were assessed spectrophotometrically at 230, 260 and 280 nm. The $A_{260/280}$ ratio was used to detect protein contamination and the $A_{260/230}$ ratio to determine carbohydrate contamination. The quality and integrity of the isolated RNA was verified by electrophoresing the RNA sample on 1.5% agarose gels in 1x TBE buffer and stained with ethidium bromide (Sambrook *et al.,* 1989). The bands were visualized and photographed using an Alpha Innotech AlphaImager™ 2200 UV Gel Video Image Analysis System.

***Primer design and validation of the Real-Time PCR primers*:** Three genes (CAT, PAL, PR10) were used for the expression studies in *Musa acuminata* cv. Berangan banana plantlets. Primers were designed by using Primer 3 software. The list of primer sequences are listed in Table 1.

***qPCR analysis*:** Real-time analysis was performed in an Applied Biosystem 7500 Fast Real Time System using KAFA SYBR FAST qPCR Kit Master Mix (2X) Universal, United States. The reaction mixture consisted of 1 µl of cDNA sample, 10 µl of KAPA SYBR FAST qPCR Master Mix (2x) Universal, 0.4 µl of forward and reverse real time primers and 0.4 µl ROX low. Non-template reaction (NTC) contained nuclease free water instead of cDNA as template. 20 µl aliquots of the mixture were distributed evenly into the

MicroAMP™ Optical 8-Tube Strip (Applied Biosystem, USA). Amplification cycles were conducted as follows: Initial denaturation at 95°C for 10 min, thermal cycling was performed for 40 cycles with 92°C for 15 seconds, and 60°C for 120 seconds with the fluorescence being read at the end of each cycle. The dissociation curve was analyzed at 95°C followed with 60°C after each completed run. To confirm that there were no nonspecific amplifications, or formation of heterodimer or self-primer dimers PCR amplicons for each gene were visualized on mass fraction of 1% agarose gel and the bands were visualized and photographed using Alpha Innotech AlphaImager™ 2200 UV Gel Video Image Analysis System. Executions of the PCR program and data collection were processed using 7500 software version 2.0. Each real time PCR reaction was performed in triplicate with a final volume of 20 µl. The fluorescence signals were captured, quantification cycle (Ct) values were tabulated and each Ct value was used to calculate the relative quantities using the formula $2^{\Delta\Delta ct}$.

Table 1. PCR and qPCR primers used in this study.

Primer name	T_m°C	Nucleotide sequence 5` to 3`	Application
Pr10F	49.2	CTCCGAGAAGCAGTACTACGA	qPCR
Pr10R	43.3	GATGGCCGTGGACGAA	qPCR
PALF	48.7	ACAGGAGGACCAAGCAAGGA	qPCR
PALR	47.5	CGTCCCGGAGCCGAATAT	qPCR
CatF	49.2	AAGGTCTCACCGCTTGTCTCA	qPCR
CatR	48.1	CGTCGCGGATGAAGAACAC	qPCR
RPS2F	46.0	TAGGGATTCCGACGATTTGTTT	qPCR
RPS2R	49.2.	TAGCGTCATCATTGGCTGGGA	qPCR
UBQ2F	48.7	GGCACCACAAACAACACAGG	qPCR
UBQ2R	46.7	AGACGAGCAAGGCTTCCATT	qPCR
GAPDHF	52.3	ACCACAAATTGCCTTGCTCCCTTG	qPCR
GAPDHR	52.3	ATCAACGGTCTTCTGAGTGGCTGT	qPCR

Reference gene selection: Determination of gene expression stability of the reference genes in non-treated and treated root samples were analyzed by NormFinder which is a Microsoft-Excel based software. The obtained $2^{\Delta\Delta ct}$ values were subjected into Norm Finder to identify the optimal gene with lowest M value (stable gene) among a set of chosen reference genes. These includes the RPS2 (ribosomal protein S2) gene, UBIq and GAPDH.

***Data analysis*:** The mRNA transcript levels of the three banana defense-related genes (CAT, PR10 and PAL) were analysed and normalized with reference gene in samples obtained from infected and non-infected banana roots at different stages using the same formula as above. All data were subjected to statistical analysis. The significant difference between the treated and non-treated sample set was analysed by one-way ANOVA and the means were compared using least significant difference (LSD) < 0.05.

RESULTS AND DISCUSSION

The study assayed mRNA levels by qPCR of the i) CAT gene that encodes for catalases which are highly expressed enzymes and are integrated with the plant antioxidative system (Mhamdi *et al.,* 2010) ii) PR10 which functions in parallel with the SAR system (Zhang and D Shapiro, 2002), and iii) the PAL gene from the phenylpropanoid pathway (Ali and McNear, 2014). The results obtained were consistent with reported studies in other plant systems where the genes were either highly expressed or suppressed during fungal infection. Each of the selected genes showed a specific pattern of expression. (Table 2)

Table 2. Expression levels of the selected pathogen responsive gene by qPCR. All data were subject to one way ANOVA.

Incubation Day	Gene		
	PR/RSP2	**CAT/RSP2**	**PAL/RSP2**
0	10.91 ± 1.40^{a}	0.13 ± 0.02^{b}	1.01 ± 0.17^{b}
2	7.32 ± 0.88^{c}	0.39 ± 0.05^{d}	16.82 ± 3.15^{e}
4	0.00 ± 0.00^{f}	0.42 ± 0.13^{f}	96.66 ± 35.6^{g}

The data shown are expressed as mean (%) of three replicates ± SE from three representative experiments. SE indicates standard error bar, same letter denotes not significant ($p \geq 0.05$) by Duncan test.

The expression levels of the PR10 gene in Banana cv. 'Berangan' plantlets were highly upregulated from day zero of the infection with p equal to 0. This is consistent with published findings in other plants which showed that PR10 was induced early upon microbial attack. This induction is thought to be an attempt to maximize the early defense response by the plant (Hoffmann-Sommergruber, 2002; Dolezal *et al.,* 2014). For the PAL gene, it was up-regulated significantly with *p* equivalent to 0.03. Concurrently the CAT genes were only minimally up-regulated following the FOC challenge with *p* equivalent to 0.010. This is consistent with reports by Van Den Berg *et al*. (2007) showing that CAT gene was similarly affected in tolerant and susceptible Cavendish banana plantlet upon infection. Increase in the production of PAL is associated with the increase in lignification in cell walls of banana roots upon infection to inhibit the penetration of fungal growth and development (Mohd Fishal *et al.,* 2010). In this study, we have demonstrated the differential gene expression pattern in Banana cv. 'Berangan' roots infected with *Fusarium* within 4 days post infection well before the onset of observable symptoms. Chen *et al.* (2011) in studies on gene expression in banana fruit under different experimental conditions verified that real-time PCR is a quantifiable and sensitive method for gene expression analysis. Other considerations for the successful applications of this assay include standardised upstream processes including the quality and quantity of RNA and standardisation of reference house-keeping genes. RIN value measures the RNA integrities of 25s, 18s and 16s bands as indicators of

Table 3. RNA integrity values (RIN).

9889	RIN (value)
Day 0 replicate 1	6.80
Day 0 replicate 2	7.20

Day 0 replicate 3	7.20
Day 2 replicate 1	7.60
Day 2 replicate 2	7.40
Day 2 replicate 3	7.60
Day 3 replicate 1	6.90
Day 3 replicate 2	8.00
Day 3 replicate 3	6.30

*RIN values ranges from 10 (intact) to 1 (totally degraded).

RNA purity (Table 3). Although it is generally thought that high-quality, undegraded RNA is required for all types of analysis, it is not necessarily true for qRT-PCR. Many of the primer/probe sequences used for qRT-PCR is quite short, ranging from ~50 to 200 bases, allowing even significantly degraded RNA to be used for analysis, without detectable effects. The quantity of RNA considered as optimal for this assay was set at 1 µg of total RNA (Fig. 2). The selection of the reference housekeeping gene was carried out using NormFinder. This package implements popular optimal reference gene finding algorithms in the widely used statistical software for genomic analysis (Vandesompele *et al.*, 2002). The stability of the reference gene for this study, measured based on the M values, showed that the RPS2 (ribosomal protein S2) gene had the lowest M_{value}=0.015 (most stably expressed gene) compared to UBIq and GAPDH (Fig. 3). Therefore, it was selected as the best reference gene to normalize the expression level of the 3 target genes. PCR efficiencies of each pair of primers, whether housekeeping primers or experimental primers, were first validated through insilico techniques.

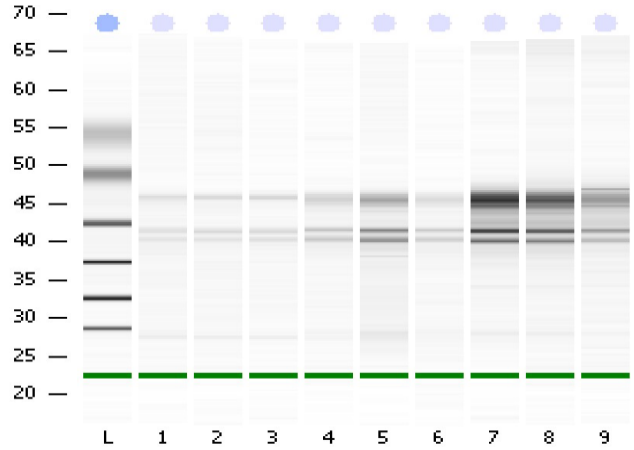

Figure 2. Electrophoresis of total RNA from root tissues infected with *Fusarium oxysporum* Tropical Race 4 (FOC4) on day 0, 2 and 4. Three biological replicates were pooled and the experiments were repeated three times.

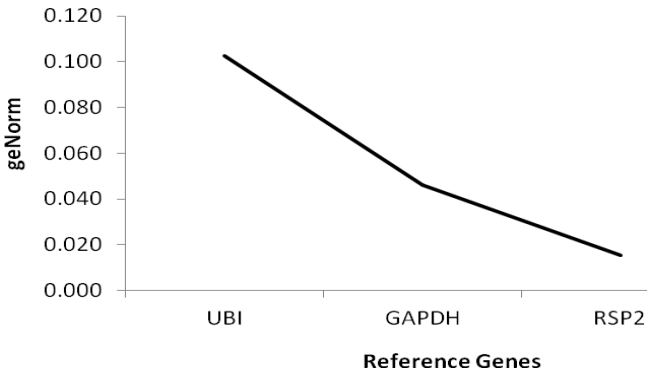

Figure 3. Average expression stability values of reference genes by NormFinder in *Musa acuminata* cv. 'Berangan' seedlings infected with *Fusarium oxysporum* Race 4 (FOC4) pathogen.

Primers that produced single peaks in the melting curve analysis at 85°C were further selected for the assay as it offers high specificity and reproducibility (Lin *et al.*, 2014). A single peak is representative of a single species of DNA molecule in the reaction while the double peaks were indicative of the presence of primer dimer or non-specific amplification. Figure 4 shows that all primers produced only single peaks. This melting curve analysis ensured that the correct-sized products had been amplified for all relevant primers pairs (Hao *et al.*, 2013).

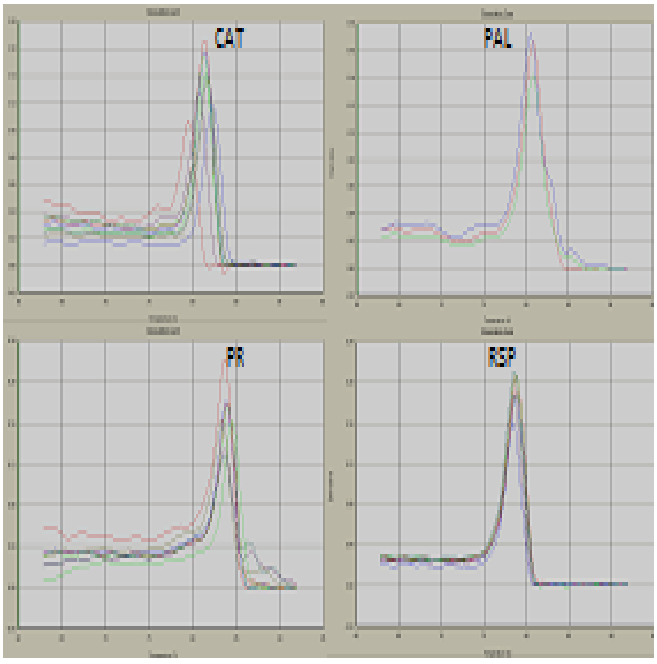

Figure 4. The dissociation curve of the amplified amplicon using Applied Biosystem 7500 Fast Real Time System. The T_m values are indicated throughout the dissociation curve.

The results of the assay are highly reproducible and present a relatively simple approach to visualising a plant's pattern of response to fungal attack. Generally identification of new *Fusarium* resistant or tolerant line is carried out using visual scoring of disease symptoms, and presents an indirect measure of the level of resistance against the fungus. A combination of both phenotypic as well as molecular signatures that could indicate the status of tolerance of a plant to pathogen attack can accelerate the screening of breeding lines for the desired traits. Here we present 3 candidate genes that could be applied to create profiles of early disease response in different banana in a simple protocol that could easily be expanded to include other useful indicator genes.

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