CHAPTER 5

IDENTIFICATION OF GENES ASSOCIATED WITH TOLERANCE TO *FUSARIUM OXYSPORUM* F.SP *CUBENSE* IN CAVENDISH BANANAS

ABSTRACT

A banana SSH library was constructed after a tolerant Cavendish selection, GCTCV-218, had been challenged with *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4. Seventy-nine of the derived clones were then selected using the cDNA microarray screening, sequenced and subjected to BLAST searches. Fifty-five clones had homology to plant genes, while 24 showed no homology to genes of interest. Twenty non-redundant gene fragments were present and several of these showed homology to defence-associated genes. Multiple alignments showed that the genes had significant homology to sequences in Genbank and provided confidence in the library. Expression profiles of four gene fragments encoding catalase 2, pectin acetylesterase, pathogenesis-related protein (*PR*)-*1* and *PR-3* were assessed using TaqMan® and Light Cycler technology. All four gene fragments were shown to be up-regulated and differentially expressed 6 hrs after infection in the tolerant GCTCV-218 in response to *Foc* when compared to susceptible cv. Williams.

INTRODUCTION

The Cavendish selection, GCTCV-218, was shown to have tolerance to Fusarium wilt, caused by *Fusarium oxysporum* Schlect. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*), in the greenhouse and under field conditions (Chapter 2). The basis for this tolerance is however poorly understood and the search for genes conferring resistance to diseases and pests has become an important step towards developing genetically improved banana plants.

At least three different classes of genes play a role in the defence strategy of plants (Glazebrook *et al*., 1997). One of these comprises genes for constitutive (passive) defence and is not directly involved in defence responses. These genes may play a role in plant resistance by inhibiting pathogen entry by, for example, forming a thick waxy cuticular layer that protects against penetration. Another class of genes are those that serve in non-specific plant defence through the production of phytoalexins, glucanases, chitinases, lignin, callose and enzymes for oxidative stress protection. In addition, antimicrobial secondary metabolites and genes coding for thionins, glutathionine S-transferases, lipoxygenases and phenylalanine ammonia-lyase (PAL) are also induced (Glazebrook *et al*., 1997). Genes in these two classes are known as minor genes for resistance and are present in all plants. A third class of genes is required for race-specific resistance and comprises genes such as major resistance (*R*) genes that result in the arrest of pathogen growth (Jørgensen, 1994).

Contemporary molecular plant pathology has focussed on plant-pathogen systems where the interaction is controlled by a gene-for-gene relationship. Comparatively little is known regarding interactions that are controlled by a complex of defence associated genes. To study multiple gene expression profiles in plants, a reliable and sensitive technique is required (Gachon *et al*., 2004). Real-time polymerase chain reaction (PCR) is able to meet this requirement and can be used to test the expression of numerous genes in the same RNA preparation. The technique is particularly useful when studying multigenic families.

Analysing all the members of a gene family is necessary in order to obtain an accurate view of its overall function (Gachon *et al.*, 2004). A widely used strategy is to select genes of interest with microarray experiments first, and then confirm their value by real-time reverse transcriptase (RT)-PCR analysis (Klok *et al.*, 2002). RT-PCR has replaced Northern blot analysis in studying gene expression profiles, as Northern blot analysis is tedious (Dong *et al.*, 2003) and does not detect genes that are expressed at a very low level readily (Brown *et al.*, 2003; Jakab *et al.*, 2003).

Little is known regarding the molecular processes involved in resistance mechanisms, metabolic pathways and downstream signalling of the banana-*Foc* interaction. An analysis of pathogen-induced genes may lead to a better understanding of the molecular processes involved in resistance, and may contribute to the development of biotechnological strategies to combat the disease. In this study, genes associated with tolerance in Cavendish bananas to *Foc* 'subtropical' race 4 (Chapter 3) are sequenced and subjected to BLAST searches to determine their putative identities. Suppression Subtractive Hybridisation (SSH) and microarray screening for selected putative defence-related gene fragments are confirmed and their expression profiles studied over time using quantitative real-time RT-PCR.

MATERIALS AND METHODS

Sequencing and Analysis of cDNA Clones

Seventy-nine cDNA clones associated with tolerance in Cavendish bananas to *Foc* (Chapter 3) were prepared for sequencing using the Qiaprep® plasmid purification kit (Qiagen, Valencia, CA, USA). Sequencing reactions were conducted at the Sequencing facility of the Scottish Crop Research Institute, Scotland. Reactions were carried out in 10 µl volume with 150 ηg template DNA, 2 µl Big Dye termination reaction mix, 2 µl primer T7 (10 µM) (5'-ATTATGCTGAGTGATATCCC-3') and 0.8 µl 5x dilution buffer (400 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$). Samples were cycled 35 times at 94ºC for 10 s, 54ºC for 5 s and 60ºC for 4 min. Sequenced products were then purified by adding 1 µl 3 M NaOAc (pH 4.6) and 25 µl absolute ethanol to the

reactions, followed by 15 min incubation at 4ºC and centrifugation at 3000 x g for 30 min. The supernatant was removed and the product washed twice with 40 µl ethanol. Samples were dried in a heating block for 2-5 min at 65ºC. Sequencing products were stored at –20ºC and analysed on an ABI PRISM 377 DNA analyser (Perkin Elmer, Ontario, Canada).

DNA sequences were edited and compared using Phred (Ewing *et al*., 1998; Richterich, 1998) and BLAST software (Altschul *et al*., 1990), respectively. Protein homologies were identified by BLASTX (Altschul *et al*., 1990), and similarities at the nucleotide level were identified using BLASTN (Altschul *et al*., 1990). The DBEST search engine was used to identify similarities with ESTs. Genes were assigned to functional categories according to the putative role the gene product plays.

Nine clones with high E-values and homology to defence-associated genes were selected for additional sequence analysis to predict their functions using Vector NTI Advance™ 9 software (Invitrogen Life Sciences, USA). Nucleotide sequences of at least two of the best BLASTX hits for each SSH clone were used for multiple alignments, using AlignX (Vector NTI Advance™ 9 software). Amino acid sequences of each of the best BLASTX hits were used to search for motifs and domains using Motif Scan (http://us.expasy.org) and Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de).

Real Time Reverse Transcriptase-PCR

Template preparation

RNA was extracted from *Foc*-inoculated Cavendish banana varieties tolerant (GCTCV-218) and susceptible (Williams) to *Foc* for real time RT-PCR analysis (Chapter 3). cDNA was synthesized using a random hexamer primer (Fermentas Life Sciences, Hanover, USA) and Power Script TM Reverse Transcriptase (BD, Biosciences, Belgium).

Primer design

Four genes were studied for expression analysis in GCTCV-218 and Williams bananas using real-time RT-PCR. These genes include those coding for PR1, pectin acetylesterase (PAE) and catalase 2, previously selected from the SSH library constructed for resistance to *Foc* in Cavendish bananas (Chapter 3), and endochitinase. Three genes, namely a banana 25S rDNA (AF 399949), ubiquitin (AY651067) and actin gene (cloned gene) were selected as possible endogenous controls.

Primers for the four defence-related genes were designed from sequences of cDNA fragments in the banana SSH library using Primer3 (Whitehead Institute, MIT, Cambridge, MA, USA) and Netprimer (Premier Biosoft, Palo Alto, CA, USA), and synthesized by either Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, Pretoria, South Africa) or Operon Biotechnologies GmbH (Cologne, Germany). Primers for the endogenous control genes were designed from sequences obtained form the NCBI database. All the primers (Table 1) were designed to have a Tm between 58 and 61°C, no more than two G's or C's within the last five nucleotides of the 3' end of the oligonucleotide, and an amplicon length of between 75 and 154 bp to ensure efficient replication in the short PCR cycles applied. Primer pairs were evaluated for efficiency by conducting a conventional PCR experiment, using GCTCV-218 cDNA as template.

RT-PCR optimisation

Primer concentrations were optimised by setting up PCR reactions using 50, 300 and 900 nM each of forward and reverse primers (i.e. forward:reverse primer concentration ratios of 50:50, 50:300, 50:900, 300:50, 300:300, 300:900, 900:50, 900:300, 900:900) of the different genes (Table 1). The PCR reactions for the nine conditions were carried out using TaqMan® technology. The ABI Prism® 7700 thermocycler (Perkin Elmer, Norwalk, CT, USA) was used for thermal cycling and to record changes in fluorescence intensity. The thermal cycling conditions were as follows: one hold at 95°C for 15 min for denaturation of DNA and activation of polymerase, 40 cycles at 95°C for 15 s, 59°C for 30 s and 72°C for 30 s.

Endogenous control genes were evaluated for their efficiency to serve as controls by conducting a real-time PCR experiment, using known amounts (30 ηg) of Williams and GCTCV-218 cDNA as template at 0 and 48 hrs after infection. Each reaction was done in duplicate. The threshold cycle (C_T) values of each of the genes were compared to identify the gene with the least variation over time.

A real-time quantitative PCR control experiment was performed to examine the linearity of amplification over the dynamic range. A serial dilution (1:10, 1:20 1:30, 1:40, 1:60 and 1:80) on 2 µl of cDNA (GCTCV-218) and each of the primer sets (300 nM of each primer) for the different genes (Table 1) was used to calculate the standard regression curves. Each dilution point on the standard curve was done in triplicate. The standard curve was calculated with the following formula: y = mx + b, where b = y-intercept of standard curve line (Crossing point) and m = slope of the standard curve line (Function of PCR efficiency). A slope of -3.32 would indicate that the PCR reaction is 100% efficient. Deviations from 100% efficiency can be calculated by putting the value of the slope (s) into the following equation: PCR efficiency = $(10^{(1/-s)}) - 1$ (Ginzinger, 2002).

Quantitative Expression Assays

The expression profiles of the four putative defence related genes, *pr1*, *pr3*, PAE and catalase 2, in GCTCV-218 and Williams bananas were assessed in triplicate (*n*=3) using TaqMan® and Light cycler technology. Expression profiles were presented as a ratio for each gene fragment at 0, 3, 6, 24 and 48 hrs after inoculation in comparison with the expression of the gene fragments in the calibrator, Williams 0 hrs after infection. Independent biological replicates were used for the Light cycler and TaqMan®

TaqMan® amplification was carried out with the ABI 7700 Sequence Detection System (Perkin Elmer) in 96-well PCR plates with optical lids (Applied Biosystems). The components per 25 µl were: 12.5 µl 2x QuantiTect SYBR Green mix, HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTPs (including dUTP for optional uracil-N-glycosylase treatment), SYBR Green I, and ROX (passive reference

dye) (Qiagen), 2 µl forward primer (300 nM), 2 µl reverse primer (300 nM), 6.5 µl H_2O and 2 µl of template cDNA (1:10 dilution). The PCR program was as follows: one hold at 95°C for 15 min for denaturation of DNA and activation of polymerase, 40 cycles of 95°C for 15 s, 59°C for 30 s and 72°C for 30 s. No template control (NTC) reactions were set up using water as template.

For Light cycler amplification, 20-µl PCR amplification reactions contained a master mixture of Taq DNA polymerase (Roche Diagnostics), dNTP mixture, $MgCl_2$ and buffer (LightCycler FastStart DNA MasterPLUS SYBR Green I) (Roche Diagnostics), 5 µM of each primer and 2 µl of a 1:10 dilution cDNA in a glass capillary tube (Roche Diagnostics). NTC reactions contained water as template. The cycling conditions were as follows: pre-incubation for 10 min at 95°C (hot start) followed by 40 cycles, each consisting of 10 s denaturing at 95°C, 15 s annealing at primer specific temperatures (Table 1), 10 s primer extension at 72°C and data acquisition at 80°C.

Data analysis

Expression data (c) for TaqMan® and Light Cycler experiments was normalized making use of the standard curve for the specific target gene and the endogenous control gene, *Musa* 25S rRNA. C_T indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The log input amount was calculated as follows: Log input = ([C_T-value] –b)/m, where b = y-intercept of the standard curve line (Crossing point) and m = slope of the standard curve line. The input amount (c_N-value) in ηg was calculated by: Input amount (c_N-value) = 10^[Log input].

The normalised expression value for each specific gene was determined by dividing the average c_N-value of the target gene by the average endogenous control c_N-value. The normalised amount of target (c_N-value) (expression level) is a unitless number that can be used to compare the relative amount of target in different samples. One sample (Williams 0 hrs after infection) was designated the calibrator and the other samples are presented in relation to the calibrator by dividing them by the calibrator value (Applied Biosystems, User Bulletin No.2, 2001).

The Standard Deviation (SD) and average input of each treatment of the target gene ($SD_{Target\ gene}$) and the endogenous control ($SD_{Endogenous\ control}$) was calculated prior to calculating the normalised values. The normalised SD for each different treatment was calculated using the following formula:
SQRT [($SD_{Endogenous\ control}$ / Average input $_{Endogenous\ control}$)^2 + ($SD_{Target\ gene}$ / Average input $_{Target\ gene}$)]* Normalised amount of target (c_N-value) for each different treatment.
The relative SD was then calculated by dividing the normalised SD with the calibrator input for each individual treatment.

RESULTS

Sequencing and Analysis of cDNA Clones

Of the 79 sequences analysed, 55 showed significant homology to plant gene sequences and 24 had no significant homology to genes of plants or any other organism (Fig. 1A; Table 2). Of the 55 sequences analogous to plant genes, ten had homology to two putative peroxidases, eight were unknown proteins, seven showed homology to unspecific monooxygenase cytochrome P450 and four to a trypsin inhibitor and a hypothetical protein. Three clones showed similarities to senescence-associated proteins and Bowman Birk protease inhibitors, two each showed homologies to *PR-1*, pectin acetylesterase precursor, xylanase inhibitor and metallothionein, respectively. Finally, there was one clone each for root control, ribosomal S3a, response regulator 6, salt stress, inhibitor CII, catalase 2, ferredoxin and an unknown protein related to lectin (Fig. 1A; Table 2). Fourteen percent of the gene fragments were associated with defence while another 4% were stress-related (Fig. 1B).

There were 20 non-redundant differentially expressed gene fragments isolated from the tolerant GCTCV-218 banana cultivar after *Foc* infection (Table 3). Of these, 11 clones either had non-significant E-values or their putative identities were not associated with defence. Nine gene fragments, however, had significant E-values and

showed multiple alignments with putative defence-associated genes, and also contained some domains and motifs that provided confidence in the sequence identities.

Gene fragment 1-59 (155 aa) shared 69% identity with the amino acid sequence of a response regulator 6 from maize (BAB 20580) and contained a response regulator receiver domain (Fig. 2). The catalase 2 fragment (76 aa) from this study was contained in the Pfam catalase region (Fig. 3) and shared 92.1% identity with the amino acid sequence of a catalase from *Zantedeschia aethiopica* (L.) (AA611AA40). This gene fragment also had the amino acid proline (P) involved in proximal heme binding (Fig. 3). Gene fragment 1-136 (143 aa) contained 52 amino acids from the Pfam metallothionein region and six cysteine residues (Fig. 4) and shared 100% identity with the amino acid sequence of a metallothionein from banana (AAG 44757).

Gene fragment 1-158 (224 aa) shared 70.5% identity with the amino acid sequence of a rice PAE (NP_918013) and was contained in the Pfam PAE and IMP dehydrogenase GMP reductase domains (Fig. 5). Gene fragment 2-45 (205 aa) only contained a part of the IMP dehydrogenase GMP reductase domain (Fig. 6) and shared 47.6% identity with the amino acid sequence of a cytochrome P450 from tobacco (T02995). The gene fragment 2-70 (116 aa) shared 79.3% identity with the amino acid sequence of a peroxidase from rice (CAH 69319) and was situated just down-stream from the signal peptide. The fragment contained two class III peroxidase conserved domains as well as the plant heme peroxidase family profile (Fig. 7). This fragment was also situated within the Pfam peroxidase region, the IMP dehydrogenase GMP reductase site and the peroxidase active site (Fig. 7).

The gene fragment 3-7 (85 aa) contained the last 73 amino acids of the super family acid proteases site (Fig. 8) and shared 100% identity with the amino acid sequence of a xylanase inhibitor from barley (CAE 46330). Gene fragment 3-167 (82 aa) contained a conserved plant *PR-1* motif and (cysteine-rich secretory protein-1 precursor) crisp1family signature as well as the segregation and condensation protein (SCP) -like extra-cellular protein region (Fig. 9). The fragment shared 52.5% identity

with the amino acid sequence of a *PR-1* from maize (AAC 25629). The gene fragment 3-169 (94 aa) was contained in the Pfam ribosomal protein S3a region (Fig. 10) and shared 87.2% identity with the amino acid sequence of the same gene from *Cicer arietinum* L. (CAD 56219).

Real-Time Reverse Transcriptase PCR

Primer design and RT-PCR optimisation

PCR products amplified with defence-related gene primers produced single bands of between 75–150 bp, depending on the primer sets used (Fig. 11). The annealing temperatures for all the different primers were optimal at 59°C. Primer concentrations for the TaqMan® were optimal at a ratio of 300:300 nM, while primer concentrations for the Light Cycler were selected as 5:5 µM, based on suggestions made in the Light Cycler manual.

The three primer pairs for the endogenous controls successfully amplified PCR products of the desired size. Actin and ubiquitin showed 1-3 C_T differences between the different treatments, while the *Musa* 25S rRNA clone showed only a 0.9 Ct difference between treatments (Table 4). To obtain accurate measurement of gene expression with real-time RT-PCR, the expression of the endogenous control should not differ between treatments. *Musa* 25S rRNA consistently exhibited the best uniform expression across several treatments and was chosen as the endogenous control for normalising the data.

PCR efficiency over a dynamic range was evaluated and a regression curve was obtained after amplification of a serial dilution for each primer set (Fig. 12). The standard curve provided a validation or insight into the PCR efficiency for a particular primer set. The PCR efficiency of gene fragments for PAE and endochitinase was 135 and 145%, respectively, while catalase 2, PR-1 and the endogenous control had efficiencies of 107, 95 and 92%, respectively (Table 5). If the PCR efficiency is greater than 100%, then the pipetting of "knowns" (endogenous control) could be inaccurate or there is a PCR inhibitor in the standard (Ginzinger, 2002). If this occurs and is not noticed it could lead to an overestimate of the amount of template in the

"unknowns" if it does not have the same PCR inhibitor or pipetting error, when using the standard-curve quantitation method.

Quantitative Expression Assays

Light cycler and TaqMan® technology revealed that four defence-associated genes, catalase 2, PAE, *PR-1* and endochitinase were up-regulated, relative to Williams, in the tolerant Cavendish banana, GCTCV-218, in response to *Foc.*

Catalase 2 (clone 1-77) was up-regulated in GCTCV-218 6 and 48 hrs after inoculation with *Foc*. This was evident in both biological replicates, whether using Light cycler or TaqMan®. In Williams, catalase 2 was up-regulated after 6 and 48 hrs when using TaqMan®, but not the Light cycler. However, catalase 2 expression was significantly higher in the tolerant GCTCV-218 than in susceptible Williams 6 hrs after infection (Fig. 13), one of the time-points selected for SSH extraction of genes (Chapter 3).

PAE (clone 1-158) was significantly up-regulated in GCTCV-218 3 hrs after infection with *Foc*, while no up-regulation was observed in Williams. *PAE* up-regulation dropped significantly after 6 and 24 as determined on TaqMan® and the Light cycler, respectively, but then increased significantly again after 48 hrs (Fig. 14).

Up-regulation of *PR-1* (clone 3-167) occurred in both GCTCV-218 and Williams (Fig. 15). However, the expression ratio of *PR-1* was significantly more substantial after 3 and 6 hours in GCTCV-218, depending on quantification of data using the TaqMan® and Light cycler systems. *PR-1* expression is most significant after 6 hrs in GCTCV, after which it is reduced.

Endochitinase (*PR-3*) was significantly up-regulated in both GCTCV-218 and Williams following inoculation with *Foc*. Optimal expression in Williams occurred after 3 hrs, upon which production is reduced. However, in GCTCV-218, chitinase

induction was most significant after 6 hrs, after which expression levels dropped significantly (Fig. 16).

DISCUSSION

In this study, twenty non-redundant gene fragments associated with tolerance to *Foc* 'subtropical' race 4 were identified in the tolerant Cavendish banana selection GCTCV-218. Nine of the 20 clones showed significant similarities to defence-associated genes, indicating that the tolerant GCTCV-218 banana recognises *Foc* and is able to respond early at the transcriptional level, with the induction of defence genes. Multiple alignments of selected clones and the presence of gene specific domains and motifs provided confidence in the identities of the clones that we isolated and sequenced.

Four defence-related genes investigated in this study were significantly up-regulated in GCTCV-218. These include genes encoding for catalase 2, PAE, *PR-1* and *PR-3* (chitinase).

In this study, catalase production was significantly increased twice in GCTCV-218, once after 3 hrs, and the second time after 48 hrs. Class II catalases have been expressed at high levels in vascular tissue (Willekens *et al*., 1994a; Bagnoli *et al.,* 2004), and CAT2 of tobacco was shown to be regulated during environmental stress responses (Willekens *et al*. 1994b). In support of our findings, Garcia-Limones *et al*. (2002) showed that catalase activities are enhanced in the incompatible interaction between chickpeas and *F. oxysporum* f.sp. *ciceri* (Padwick), and suggested that the expression of catalases in the roots is an early response to *Fusarium* infection. Early induction of catalase is most probably related to the oxidative burst, a process associated with the antioxidant defence system of plants (Foyer *et al.,* 1994). The later increase might have been associated with signal transduction (Bagnoli *et al*., 2004). Together with ascorbate peroxidase, catalases are known to modulate the levels of H_2O_2, which acts downstream of salicylic acid (SA) as a second messenger for the activation of plant defence responses (Clark *et a*l., 2000). Results from this study may suggest that the oxidative burst is initiated in the tolerant GCTCV-218 banana and that it is accompanied by antioxidant enzymes, such as catalases. They play an important role in scavenging the radicals and providing a balance between the production of toxic oxygen derivatives and protecting the plant as part of the

antioxidant defence system of plants (Foyer *et al.,* 1994). The up-regulation of catalase 2 in GCTCV-218 during the defence response might suggest that the levels of H_2O_2 are damaging to the plant and, therefore, need to be removed by scavenging enzymes.

The significance of PAE's up-regulation in GCTCV-218 found in this study is most likely related to root modification and cell wall strengthening. PAEs are known to catalyze the deacetylation of pectin, a major compound of primary cell walls of plants (Vercauteren *et al*., 2002). More specifically, PAE hydrolyzes acetyl esters in the homogalacturonan regions of pectin, thereby modifying cell walls during root development and pathogen interactions (Savary *et al*., 2003). PAE has previously also been demonstrated to be up-regulated in *Arabidopsis thaliana* (L.) roots shortly after nematode infection (Vercauteren *et al.,* 2002).

The rapid induction of *PR-1* and *PR-3* (chitinase) in GCTCV-218 following *Foc* infection, and the marked increase of *PR-1* over time, suggests that these proteins play a significant role in early plant defence in banana. PR proteins have been associated with active defence of plants against many different fungal pathogens (Pritsch *et al*., 2000; Agrios, 2004). In incompatible interactions, PR-protein production is very effective and invasion of the pathogen is blocked at a very early stage without noticeable damage to the plant (Bol *et al*., 1990; Van't Klooster *et al*., 1999). Time-course experiments to determine the role of *PR-1* and *CHI* in pear resistance to Japanese pear scab (*Venturia nashicola* S. Tanaka & S. Yamamoto) revealed a much faster and higher induction of mRNA's encoding *PR-1* and *CHI* gene in resistant than susceptible pear cultivars (Faize *et al*., 2004).

The early induction and increase of *PR-1* in GCTCV-218 after *Foc* infection could play a role in the successful containment of the pathogen. PR-1 proteins are the only family members for which no biochemical function is known, however, several studies indicate that they may have antifungal properties (Alexander *et al*., 1993; Niderman *et al*., 1995). The association of PR-1 proteins with cell wall deposits implies a more structural role, possibly in the formation of physical barriers to prevent pathogen spread (Cutt & Klessig, 1992). Immunological studies by Carr *et al*. (1987)

and Benhamou *et al.* (1988) indicated that PR-1 protein was located within tissue deposited adjacent to the outer cell wall and within xylem elements of the vascular tissue of tobacco infected by the tobacco mosaic virus (TMV). Beckman *et al.* (1982) reported callose deposition 6-12 hrs after infection in paravascular parenchyma cells of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans.. Callose deposition was possibly a result of *PR-1* expression. The rate of deposition was higher in resistant tomato lines compared to susceptible lines. In this study, the significant expression of *PR-1* in GCTCV-218 could have had two major roles in the defence reaction; firstly inactivating the pathogen due to its antifungal properties and secondly a structural role containing the pathogen by the deposition of callose.

The induction of endochitinase in GCTCV-218 could inhibit *Foc* growth due to a biochemical degradation of fungal cell walls. It could also be linked to the systemic activation of defence responses away from the initial site of infection due to their role in releasing elicitors. Chitinases are known to hydrolyze the chitin present in cell walls of fungi and can, therefore, serve as inhibitors of fungal growth (Collinge *et al*., 1993). Chitinases also release elicitor compounds from the pathogen or host cell walls, which in turn stimulates the defence system (Keen & Yoshikawa, 1983; Boller, 1987). Pegg and Young (1982) reported that the release of β-1-3 glucanase and chitinase might serve to destroy *Foc* in banana. *PR 3* also been induced by the vascular wilt pathogen *Verticillium dahliae* Kleb. in cotton stems (Hill *et al*., 1999; McFadden *et al*., 2001; Dowd *et al*., 2004). Chitinases have been proposed to form part of the biochemical defence strategy against pathogens (Boller, 1985). *PR3* expression in GCTCV-218 could inhibit *Foc* growth due to a biochemical degradation of fungal cell walls, it however could also possibly be linked to the systemic activation of defence responses away from the initial site of infection due to the their role in releasing elicitors.

An effective resistance response against Fusarium wilt diseases depends on the rate and extent of recognition and activation of the defence mechanisms (Beckman, 1987; Beckman, 1990). GCTCV-218 showed that it is able to respond rapidly to *Foc* infection by inducing genes involved in biochemical and structural defence

mechanisms. Two genes in this study, *PR-1* and PAE were induced very early (3 hrs after infection) in the tolerant defence response, while *PR3* and catalase 2 followed with a significant induction at 6 hrs after infection. In Williams, *PR-1* was induced after 6 hrs, indicating that *PR-1* is induced in this variety, but slower and at lower concentrations than in GCTCV-218. The induction of *PR-1* in Williams is not unexpected, as this Cavendish banana variety is highly resistant to *Foc* race 1 (Ploetz, 2005). Genes encoding for catalase 2, PAE and chitanase were either not up-regulated in Williams, or were up-regulated at a much lesser extent than in GCTCV-218. This might explain why Williams is not able to resist infection by *Foc* race 4.

The metallothionein (MT) fragment found in this study shared 100% identity with a type-2 MT from banana associated with fruit-ripening and leaf-senescence (Liu *et al*., 2002). MT's are cysteine-rich polypeptides that are involved in the stress response against metals, by playing a role in metal detoxification (Liu *et al*., 2002). MT's are generally more abundant in banana fruit and flowers (Liu *et al*., 2002), but a study by Clendennen & May (1997) showed that they were also strongly expressed in corm and leaf tissue. This is the first time that a MT has been isolated from banana roots that have been infected with a pathogen. A MT-like protein was isolated from rice seedlings after infection with the rice blast fungus (Xiong *et al*., 2001). This supports our data indicating that MT might indeed be up-regulated in response to infection.

Cytochrome P450 was expressed in GCTCV-218 after *Foc* attack and plays a role in secondary metabolism by being involved in the phenylpropanoid biosynthesis pathway that leads to lignin production (Dowd *et al*., 2004). Lignin deposition is a known defence response to *Foc* in resistant banana varieties (Vander Molen *et al*., 1987). Lignin biosynthesis and the induction of a cytochrome P450 were previously reported as a defence strategy in cotton against *F. oxysporum* f. sp. *vasinfectum* (Atk.) (Dowd *et al*., 2004). In this study, the induction of cytochrome P450, a gene associated with lignin biosynthesis, might therefore, translate in lignin deposition in banana roots to prevent pathogen invasion of the xylem vessels.

Ten of the 79 banana cDNA clones isolated from GCTCV-218 6 hrs after *Foc* infection showed significant homology to two class III peroxidases. These are basic

peroxidases that show oxidase activity in the absence of H_2O_2 (Pomar *et al*., 2002). Peroxidases are stored and preformed in various localised sites in banana (Mace & Wilson, 1964; Mueller & Beckman, 1974; Mueller & Beckman, 1978). The number of peroxidase isozymes and the levels produced are greatest in the roots, where they might play a role in protecting the plant against infection by root pathogens (Ploetz, 1993). Peroxidases are important in the formation of phenolic compounds that lignify host cell walls and vascular gels (MacHardy and Beckman, 1981; Beckman, 1987; Pegg, 1985). Constitutive levels of peroxidase have previously been reported in the *Foc*-resistant banana hybrid SH-3362. A resistant synthetic AA hybrid produced at the breeding programme of the Fundación Hondurereña de Investigacón Agrícola (FHIA) in Honduras had peroxidase levels 10-fold higher than in Pisang Mas, a susceptible AA cultivar (Novak, 1992). The presence of 10 peroxidase transcripts among the 79 clones identified in the tolerant banana GCTCV-218 as early as 6 hours after infection could indicate that the banana disease response involves lignin production and cell wall strengthening through the incorporation of phenolic compounds into host cell walls.

Plant cell wall degrading enzymes like xylanase have been isolated from many *Fusarium* spp. (Ruiz-Roldán *et al*., 1999; Gómez-Gómez *et al*., 2001; 2002), including *Foc* (Groenewald *et al*., unpublished data, FABI, South Africa). The presence of a xylanase inhibitor in the tolerant GCTCV-218 cultivar, therefore, has an important implication. Endoxylanases play an important role in plant infection by pathogens (Giesbert, *et al*., 1998). Substances like xylanase inhibitors, which inhibit the hydrolytic activity of xylanase, affect the functionality and performance of many of these enzymes and may play an important role in the plants' ability to protect itself against pathogen invasion.

Twenty four of the 79 sequenced clones induced in GCTCV-218 had no homology to plant sequences, and the 'no match' clones were probably a result of their short query sequences as a result of *Rsa*I digestion of the cDNA's during the SSH procedure (Chapter 3). Clones with homology to unknown proteins were not further investigated in this study, but they could potentially be unique. These genes might play a role in disease resistance and should, therefore, be considered for future studies. None of the

identified clones showed homology to fungal genes and this provided confidence in the specific inoculation and root harvest techniques we applied. Roots were harvested at least 1 cm away from the point of inoculation to minimize the amount of fungal tissue within the root sample.

Results of this study have provided information on a tolerant plant-pathogen interaction and a soil-borne root pathogen. Both areas of study are relatively poorly explored. The results shed light on the genes involved in defence and provide a step towards understanding Fusarium wilt of banana and thereby developing an effective disease management strategy.

REFERENCES

Agrios, G.N. 2004. Plant Pathology. 5th Edition. Academic Press, London.

Alexander, D., Goodman, R.M., Gut Rella, M., Glascock, C., Weymann, K., Friederich, L., Maddox, D., Ahl Goy, P., Luntz, T., Ward, E. and Ryals, J. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceedings of the National Academy of Science of the USA* 90: 7327-7331.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

Applied Biosystems 2001. ABI PRISM 7700 Sequence Detection System. User Bulletin No. 2.

Bagnoli, F., Danti, S., Magherini, V., Cozza, R., Innocenti, A.M. and Racchi, M.L. 2004. Molecular cloning and characterisation and expression of two catalases from peach. *Functional Plant Biology* 31: 349-357.

Beckman, C.H. 1987. The nature of wilt disease of plants. APS Press, St Paul, Minnesota, USA.

Beckman, C.H. 1990. Host response to the pathogen. In: Fusarium wilt of banana. (Ed. R.C. Ploetz). APS Press, St Paul, Minnesota, USA.

Beckman, C.H., Mueller, W.C., Tessier, B.J. and Harrison, N.A. 1982. Recognition and callose deposition in response to vascular infection in fusarium wilt-resistant or susceptible tomato plants. *Physiological Plant Pathology* 20: 1-10.

Benhamou, N., Côte, F., Greiner, J. and Asselin, A. 1988. Immunocytochemical localization of pathogenesis-related *PR-1* proteins in TMV-infected *Nicotiana tabacum* cv. Xanthi-nc. *Canadian Journal of Plant Pathology* 11: 185.

Bol, J.F., Linthorst, H.J.M. and Cornelissen, B.J.C. 1990. Plant pathogenesis-related proteins induced by virus infection. *Annual Review of Phytopathology* 28: 113-138.

Boller, T. 1985. Induction of hydrolases as a defence reaction against pathogens. *UCLA Symposium on Molecular and Cellular Biology N.S.* 22: 247-262.

Boller, T. 1987. Hydrolytic enzymes in plant disease resistance. In: Plant-microbe interactions, molecular and general aspects. Vol. 2. (Eds. T. Kosuge and F.W. Nester). MacMillan, New York.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J. and Manners, J.M. 2003. A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of *Arabidopsis*. *Plant Physiology* 132: 1020-1032.

Carr, J.P., Dixon, D.C., Nikolau, B.J., Voelkerding, K.V. and Klessig, D.F. 1987. Synthesis and localization of pathogenesis-related proteins in tobacco. *Molecular Cell Biology* 7: 1580-1583.

Clark, D., Durner, J. Navarre, D.A. and Klessig, D.F. 2000. Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Molecular Plant Microbe Interactions* 13: 1380-1384.

Clendennen, S. and May, G.D. 1997. Differential gene expression in ripening banana fruit. *Plant Physiology* 115: 463-469.

Collinge, B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U. and Vad, K. 1993. Plant chitinases. *Plant Journal* 3: 31-40.

Cutt, J.R. and Klessig, D.F. 1992. Pathogenesis-related proteins. In: Plant Gene Research: Genes involved in plant defence. (Eds. T. Boller and F. Meins). Springer-Verlag, Wien, Austria.

Dong, J.X., Chen, C.H. and Chen, Z.X. 2003. Expression profiles of the *Arabidopsis* WRKY gene super family during plant defence response. *Plant Molecular Biology* 51: 21-37.

Dowd, C., Wilson, I.W. and McFadden, H. 2004. Gene expression profile changes in cotton root and hypocotyls tissues in response to infection with *Fusarium oxysporum* f. sp. *vasinfectum. Molecular Plant Microbe Interactions* 17: 654-667.

Ewing, B., Hillier, L., Wendl, M.C. and Green, P. 1998. Base-Calling of automated sequenced traces using *Phred*. I. Accuracy assessment. *Genome Research* 8: 175-185.

Faize, M., Faize, L. Ishizaka, M. and Ishii, H. 2004. Expression of potential defence responses of Asian and European pears to infection with *Venturia nashicola*. *Physiological and Molecular Plant Pathology* 64: 319-330.

Foyer, C.H., Descourvières, P. and Kunert, K.J. 1994. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant Cell Environment* 17: 199-218.

Gachon, C., Mingam, A. and Charrier, B. 2004. Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany* 55: 1445-1454.

García-Limones, C., Hervás, A., Navas-Cortés, J.A., Jiménez-Díaz, R.M. and Tena, M. 2002. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp.*ciceris***.** *Physiological and Molecular Plant Pathology* 61: 325-337.

Giesbert, S., Lepping, H.B., Tenberge, P. and Tudzynski, P. 1998. The xylanolytic system of *Claviceps purpurea*: cytological evidence for secretion of xylanases in infected rye tissue and molecular characterization of two xylanase genes. *Phytopathology* 88: 1020-1030.

Ginzinger, D.G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology* 30: 503-512.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. 1997. Use of *Arabidopsis* for genetic dissection of plant defence responses. *Annual Review of Genetics* 31: 547-569.

Gómez-Gómez, E., Roncero, M.I.G., Di Pietro, A. and Hera, C. 2001. Molecular characterization of a novel endo-β-1,4-xylanase gene from the vascular wilt fungus *Fusarium oxysporum. Current Genetics* 40: 268-275.

Gómez-Gómez, E., Ruiz-Roldán, M.C., Di Pietro, A., Roncero, M.I.G., and Hera, C. 2002. Role in pathogenesis of two endo- β-1,4-xylanase genes from the vascular wilt fungus *Fusarium oxysporum*. *Genetics and Biology* 35: 213-222.

Hill, M.K., Lyon, K.J. and Lyon, B.R. 1999. Identification of disease response genes expressed in *Gossypium hirsutum* upon infection with the wilt pathogen *Verticillium dahliae*. *Plant Molecular Biology* 40: 289-296.

Jakab, G., Manrique, A., Zimmerli, L. Métraux, J.P. and Mauch-Mani, B. 2003. Molecular characterization of a novel lipase-like pathogen-inducible gene family of *Arabidopsis*. *Plant Physiology* 132: 2230-2239.

Jørgensen, J.H. 1994. Genetics of powdery mildew resistance in barley. *Critical Review in Plant Sciences* 13: 97-119.

Keen, N.T. and Yoshikawa, M. 1983. β-1,3-endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. *Plant Physiology* 71: 460-465.

Klok, E.J., Wilson, I.W., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R. and Dennis, E.S. 2002. Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. *The Plant Cell* 14: 2481-2494.

Liu, P., Goh, C.-J., Loh, C.-S. and Pua, E.-C. 2002. Differential expression and characterization of three metallothionein-like genes in Cavendish banana (*Musa acuminata*). *Physiologia Plantarum* 114: 241-250.

Mace, M.E. and Wilson E.M. 1964. Phenol oxidases and their relation to vascular browning in *Fusarium* infected banana roots. *Phytopathology* 54: 840-842.

MacHardy, W.E. and Beckman C.H. 1981. Vascular wilt fusaria: infection and pathogenesis. In: *Fusarium*: Disease, Biology and Taxonomy. (Eds. T.A. Tousson and R.J. Cook). Blackwell Publishing, United Kingdom.

McFadden, H.G., Chapple, R., de Feyter, R. and Dennis, E. 2001. Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*. *Physiological Molecular Plant Pathology* 58: 119-131.

Mueller, W.C. and Beckman C.H. 1974. Ultrastructure of the phenol-storing cells in roots of banana. *Physiological Plant Pathology* 4: 187-190.

Mueller, W.C. and Beckman C.H. 1978. Ultrastructure localization of polyphenol oxidase and peroxidase in roots and hypocotyls of cotton seedlings. *Canadian Journal of Botany* 56: 1579-1587.

Niderman, T., Genete, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mosinger, E. 1995. Pathogenesis-related PR-1 proteins are antifungal: Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiology* 108: 17-27.

Novak, F.J. 1992. *Musa* (bananas and plantains). In: Biotechnology of Perennial Fruit Crops. (Eds. F.A. Hammerschlag and R.E. Litz) CAB International, Oxfordshire, United Kingdom.

Passardi, F., Longet, D., Penel, C. and Dunand, C. 2004. The class III peroxidase multigenic family in rice and its evolution in land plants. *Phytochemistry* 65: 1879-1893.

Pegg, G.F. 1985. Life in a black hole: the micro-environment of the vascular pathogen. *Transactions of the British Mycological Society* 85: 1-20.

Pegg, G.F. and Young, D.H. 1982. Purification and characterization of chitinase enzymes from healthy and *Verticillium albo-atrum*- infected tomato plants and from *V. albo-atrum*. *Physiological Plant Pathology* 21: 389-409.

Ploetz, R.C. 1993. Molecular approaches to identifying Fusarium wilt resistance. In: Biotechnology applications for banana and plantain improvement. INIBAP, France.

Ploetz, R.C. 2005. Panama Disease: An old nemesis rears its ugly head. Part 2: The Cavendish era and beyond. APSnet Feature, October 2005.

Pomar, F., Caballero, N., Pedreño, A. and Barceló, R. 2002. H_2O_2 generation during the auto-oxidation of coniferyl alcohol derives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. *FEBS letters* 529: 198-202.

Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A. and Vance, C.P. 2000. Fungal development and induction of defence response genes during early infection of wheat spikes by *Fusarium graminearum*. *Molecular Plant-Microbe Interactions* 13: 159-169.

Richterich, P. 1998. Estimation of errors in "raw" DNA sequences: a validation study. *Genome Research* 8: 251-259.

Ruiz-Roldán, M.C., Di Pietro, A., Huartas-González, M.D. and Roncero, M.I.G. 1999. Two xylanase genes of the vascular wilt pathogen *Fusarium oxysporum* are

differentially expressed during infection of tomato plants. *Molecular and General Genetics* 261: 530-536.

Savary, B.J., Nunez, A., Liu, L.S. and Yoo, S. 2003. Pectin Acetylesterase - Analysis and Application for Sugar Beet Pectin utilization. *Proceedings of The 1st Joint International Beet Research* - American Society Of Sugar Beet Technologists Congress. Beet Sugar Development Foundation, Denver, Co.

Vander Molen, G.E., Beckman, C.H. and Rodehorst, E. 1987. The ultrastructure of tylose formation in resistant banana following inoculation with *F. oxysporum* f. sp. *cubense*. *Physiol. Mol. Plant. Pathol*. 11: 185-200.

Van't Klooster, J.W., Vleeshouwers, V.G.A.A., Kamoun, S. and Govers, F. 1999. Characterisation of a cDNA encoding pathogenesis-related protein PR-1 from potato (*Solanum tuberosum*) (Accession No. AJ250136). *Plant Physiology* 121: 1384.

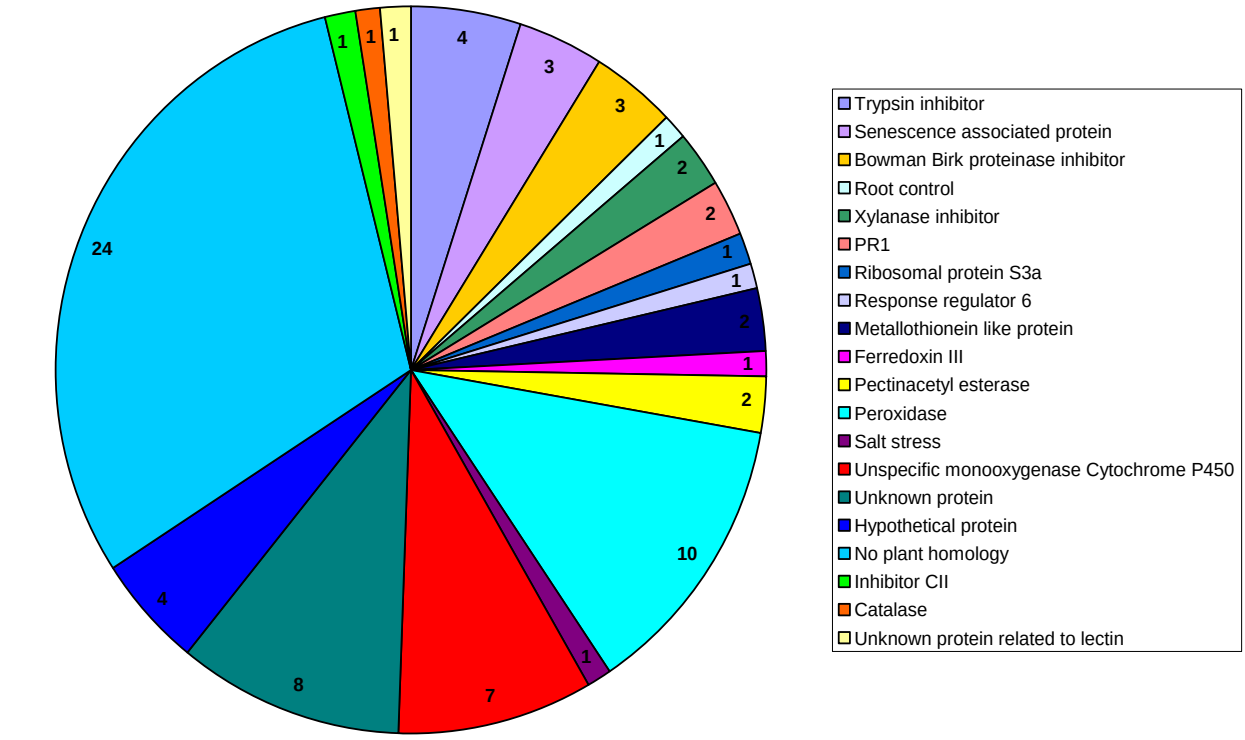
Vercauteren, I., de Almeida, E. J., De Groodt, R., and Gheysen, G. 2002. An *Arabidopsis thaliana* pectin acetylesterase gene is up-regulated in nematode feeding sites induced by root-knot and cyst nematodes. *Molecular Plant-Microbe Interactions* 15: 404-407.

Willekens, H., Langebartels, C., Tiré, C., Van Montagu, M., Inzé, D. and Van Camp, W. 1994a. Differential expression of catalase genes in *Nicotiana plumbaginifolia* (L.). *Proceedings of the National Academy of Science of the USA* 91: 10450-10454.

Willekens, H., Van Camp, W., Van Montagu, M., Inzé, D., Sandermann, H. Jr and Langebartels, C. 1994b. Ozone, sulphur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* (L.). *Plant Physiology* 106: 1007-1014.

Xiong, L., Lee, M.-W., Qi, M. and Yang, Y. 2001. Identification of defence-related rice genes by suppression subtractive hybridization and differential screening. *Molecular Plant-Microbe Interactions* 14: 685-692.

A.


4
3
3
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24
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Trypsin inhibitor
Senescence associated protein
Bowman Birk proteinase inhibitor
Root control
Xylanase inhibitor
PR1
Ribosomal protein S3a
Response regulator 6
Metallothionein like protein
Ferredoxin III
Pectinacetyl esterase
Peroxidase
Salt stress
Unspecific monooxygenase Cytochrome P450
Unknown protein
Hypothetical protein
No plant homology
Inhibitor CII
Catalase
Unknown protein related to lectin

B.

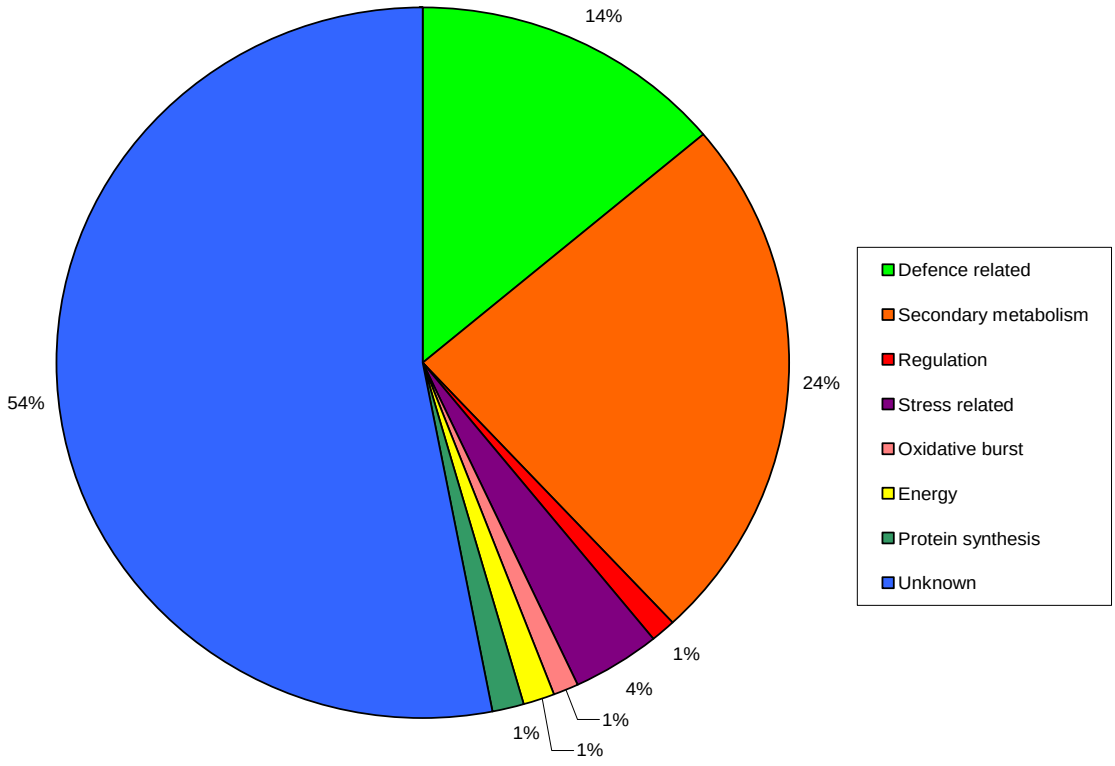

14%
24%
1%
4%
1%
1%
1%
54%
Defence related
Secondary metabolism
Regulation
Stress related
Oxidative burst
Energy
Protein synthesis
Unknown

Figure 1. Pie charts summarising the number of clones based on putative identities (A) and the percentage (%) of clones in a functional categories (B).

This was based on amino acid sequences for 79 clones from the banana enriched cDNA Suppression Subtraction Hibridisation library after infection with *Fusarium oxysporum* f.sp. *cubense*.

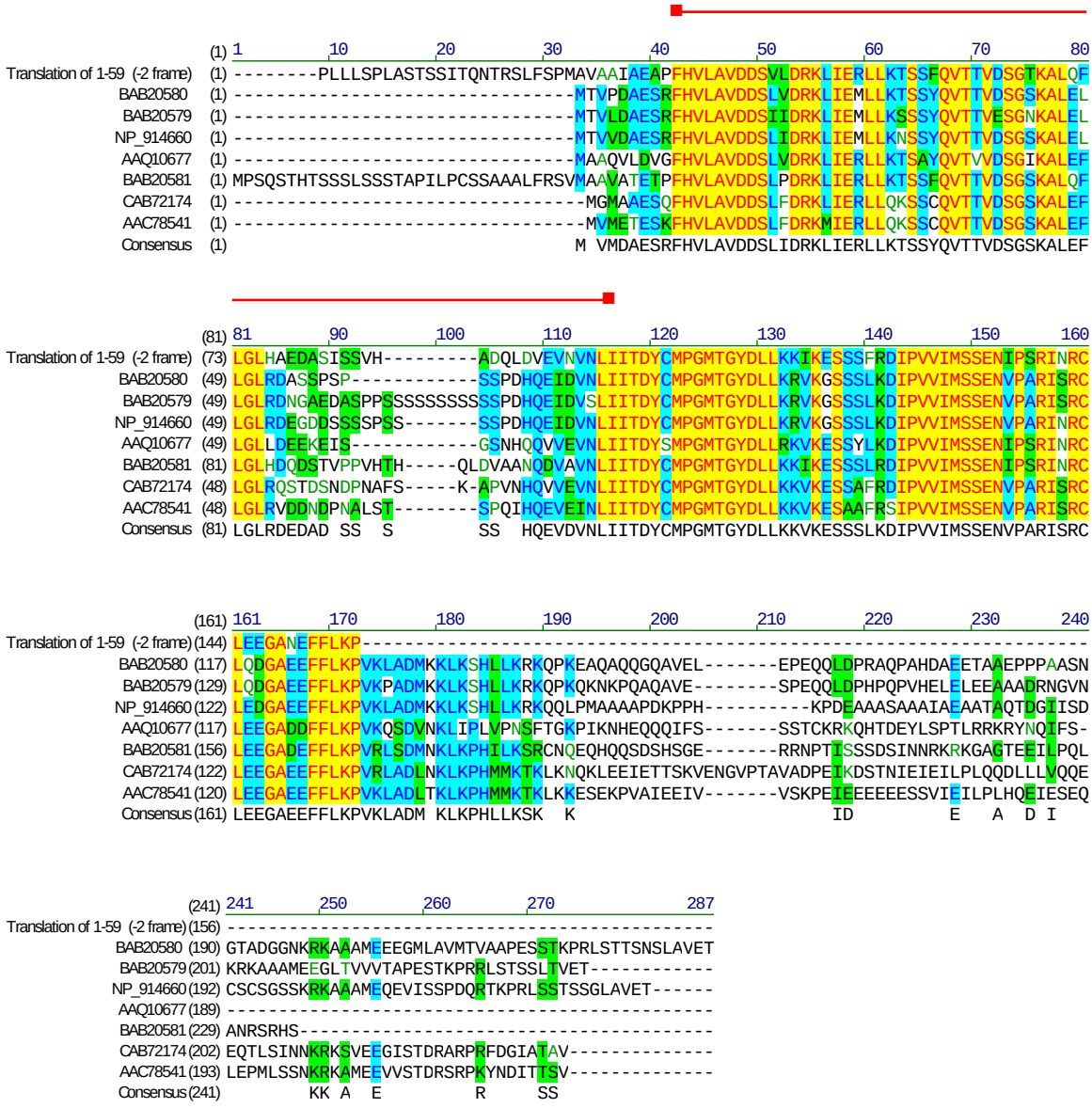
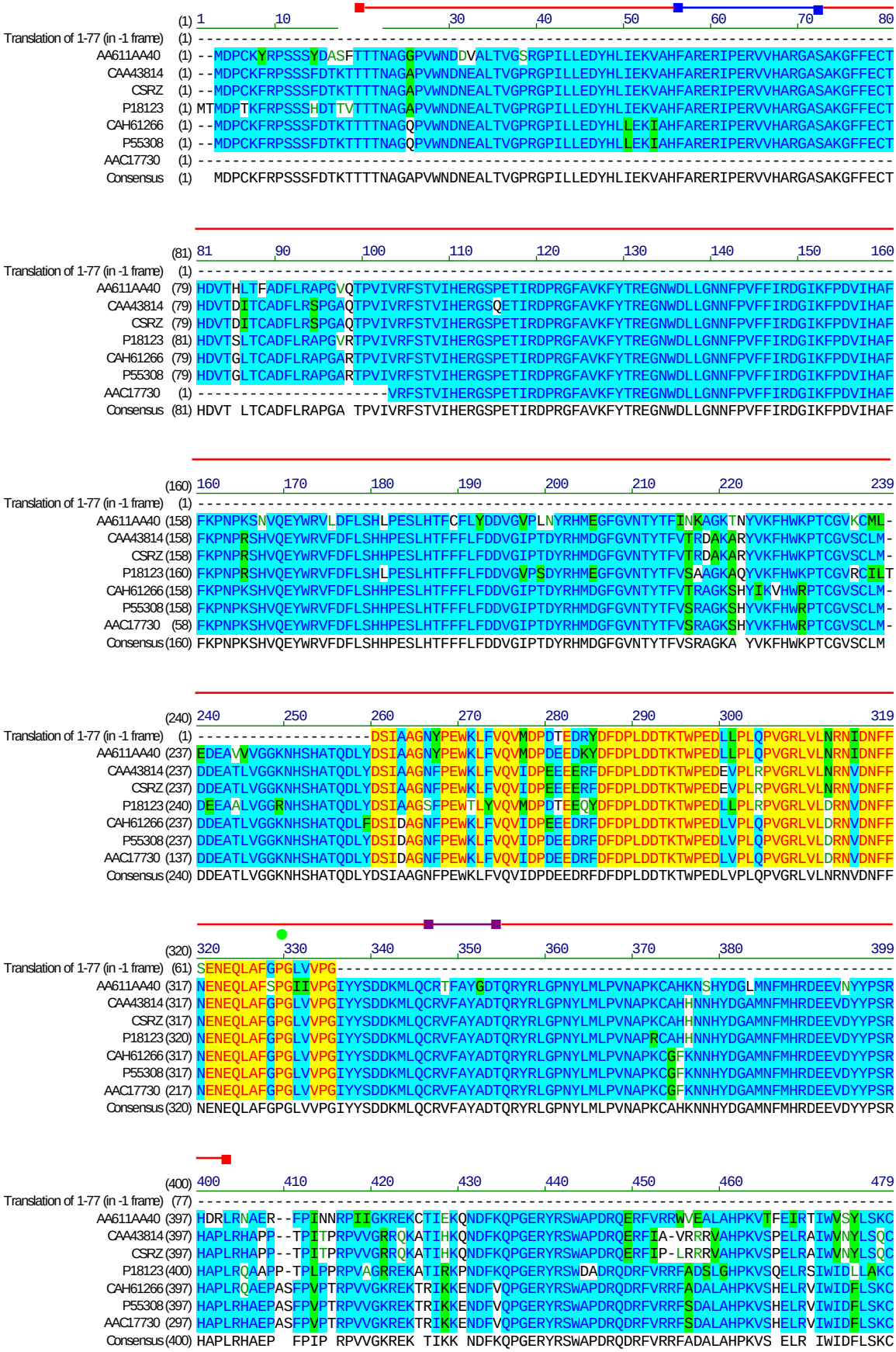


Figure 2. Alignment of deduced amino acid sequences of plant response regulators.

Clone 1-59 (from this study) is compared to BAB 20580 (*Zea mays*), BAB 20579 (*Z. mays*), NP_914660 (*O. sativa*), AAQ 10677 (*Catharanthus roseus*), BAB 20581 (*Z. mays*), CAB 72174 (*Arabidopsis thaliana*) and AAC 78541 (*A. thaliana*). The response regulator receiver domain is indicated with (■). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue

at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.


Translation of 1-77 (in -1 frame)
AA611AA40
CAA43814
CSRZ
P18123
CAH61266
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Consensus
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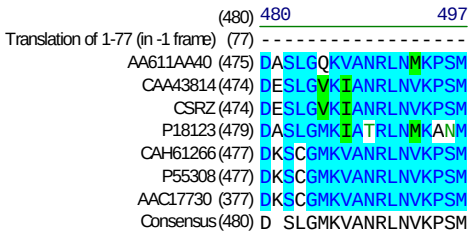


Figure 3. Alignment of deduced amino acid sequences of plant catalases.

Clone 1-77 (from this study) is compared to AA611AA40 (*Zantedeschia aetopica*), CAA 43814 (*O. sativa*), CSRZ (*O. sativa*), P 18123 (*Zea mays*), CAH 61266 (*Secale cereale*), P 55308 (*Hordeum vulgare*) and AAC 17730 (*H. vulgare*). The following domains and motifs are indicated with symbols and colours: Pfam catalase (■), catalase proximal heme-ligand signature (■), catalase proximal active site signature (■), P involved in proximal heme binding (●). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

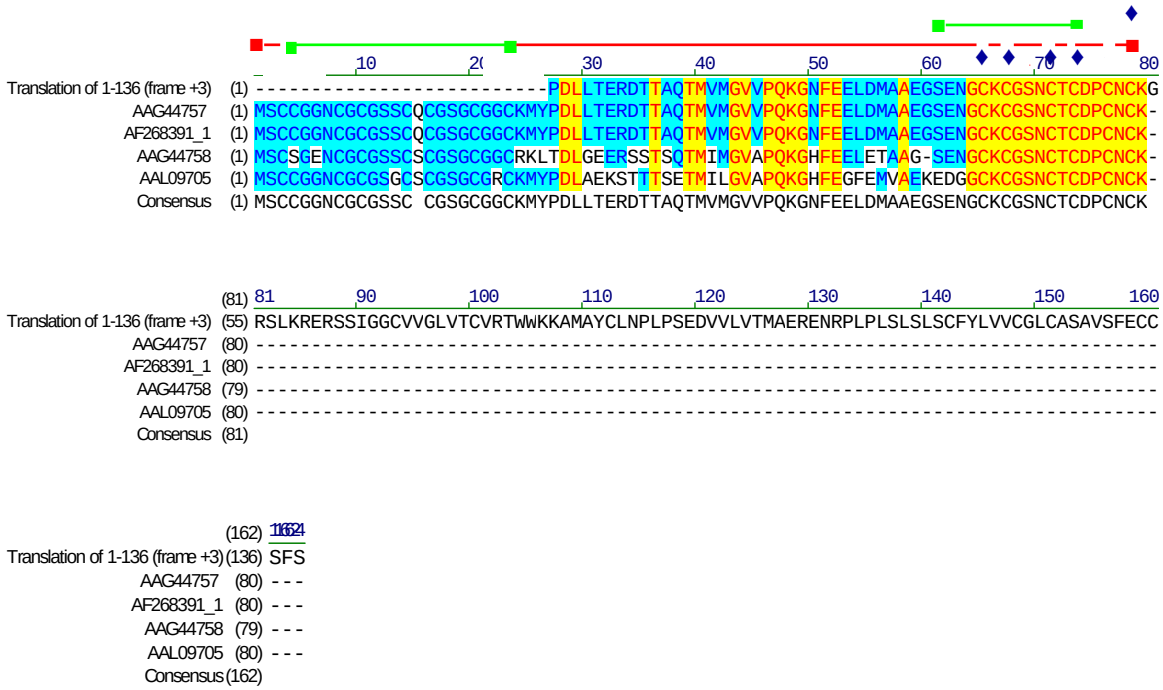


Figure 4. Alignment of deduced amino acid sequences of plant metallothionein.

Clone 1-136 (from this study) is compared to AAG 44757 (*Musa acuminata*), AF 268391_1 (*M. acuminata*), AAG 44758 (*M. acuminata*) and AAL 09705 (*Typha latifolia*). The following domains and motifs are indicated with symbols and colours: Pfam metallothionein 2 (■), cysteine rich region (■) and cysteine residues (♦). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

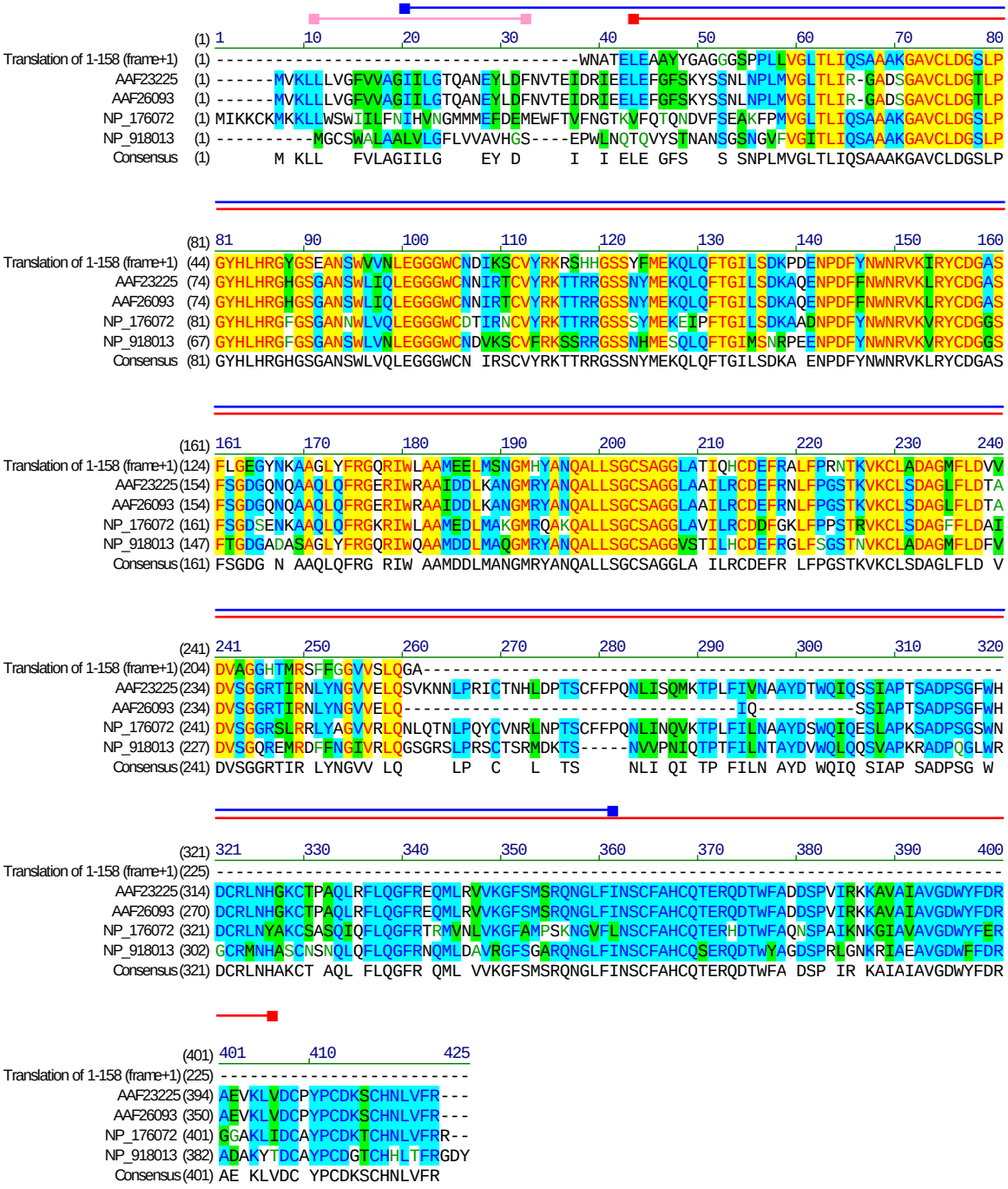


Figure 5. Alignment of deduced amino acid sequences of plant pectin acetylesterase.

Clone 1-158 (from this study) is compared to AAF 23225 (*Arabidopsis thaliana*), AAF 26093 (*A. thaliana*), NP_176072 (*A. thaliana*) and NP_918013 (*Oryza sativa*). The following domains and motifs are indicated with symbols and colours: Signal peptide (■) Pfam pectin acetylesterase (■), IMP dehydrogenase GMP reductase

domain (■). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

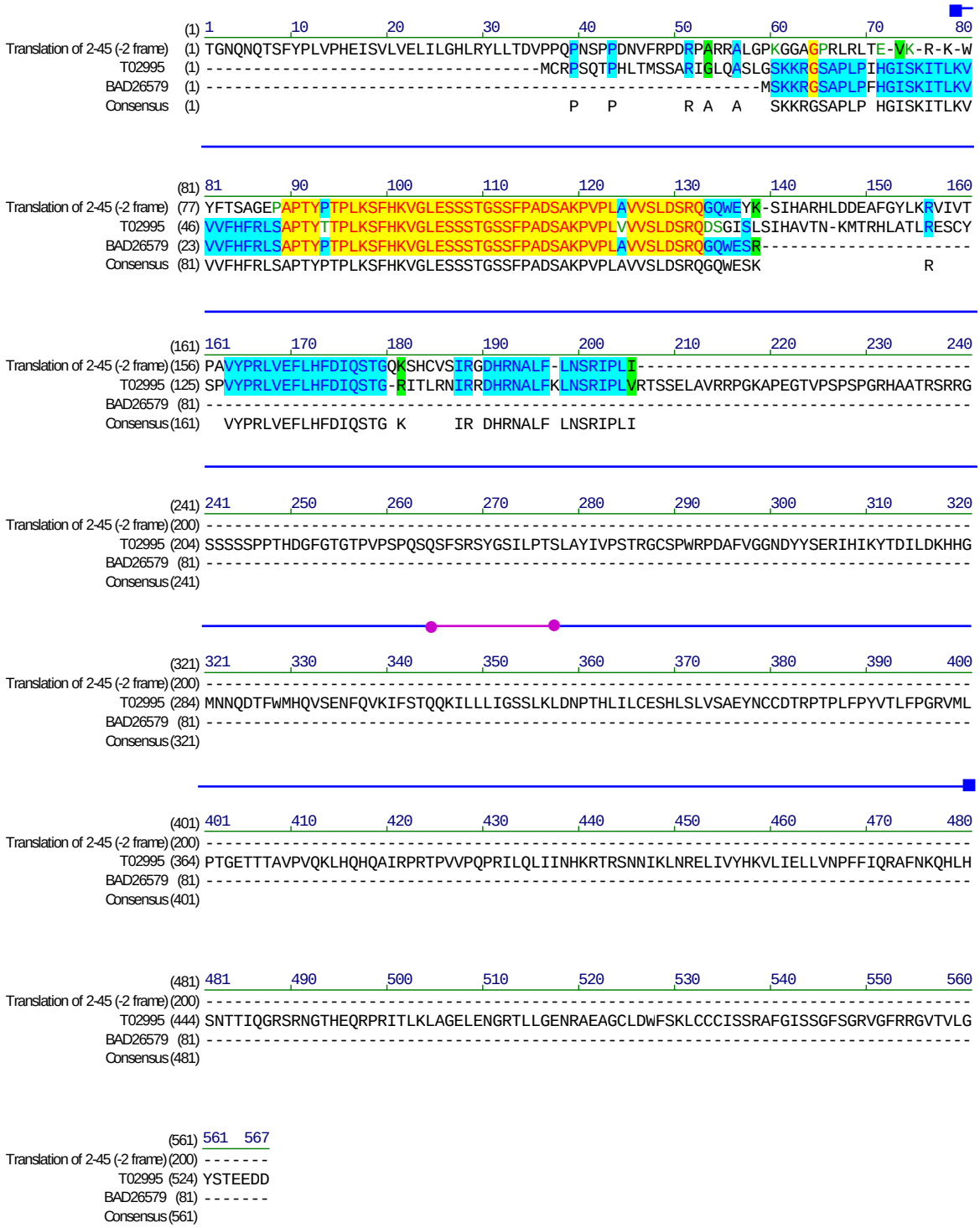


Figure 6. Alignment of deduced amino acid sequences of plant unspecific monooxygenase cytochrome P450.

Clone 2-45 (from this study) is compared to T02995 (*Nicotiana tabacum*), BAD 26579 (*Citrullus lanatus*). The following domains and motifs are indicated with

symbols and colours: IMP dehydrogenase GMP reductase domain (■) and low complexity region (●). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

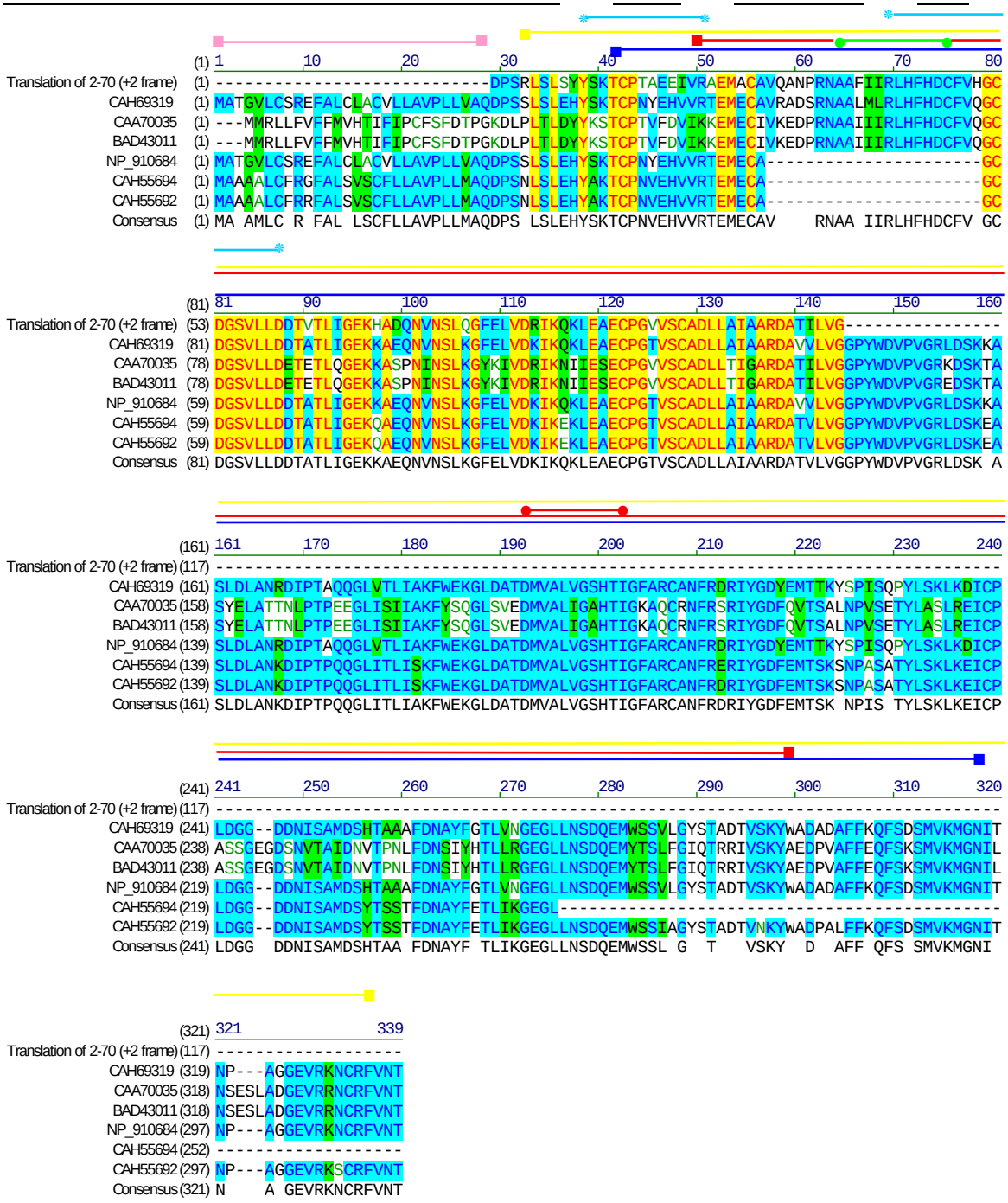


Figure 7. Alignment of deduced amino acid sequences of plant peroxidases.

Clone 2-70 (from this study) is compared to CAH 69319 (*Oryza sativa*), CAA 70035 (*Arabidopsis thaliana*), BAD 43011 (*A. thaliana*), NP_910684 (*O. sativa*), CAH 55694 (*Lolium perenne*), CAH 55692 (*Schedonorus pratensis*). The following domains and motifs are indicated with symbols and colours: Signal peptide (■), Pfam peroxidase (■), class III peroxidase conserved domain (*), IMP Dehydrogenase GMP reductase (■), peroxidase proximal heme-ligand signature (●), peroxidase active site signature (■) and plant heme peroxidase family profile (●). Colour key: Black on

window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

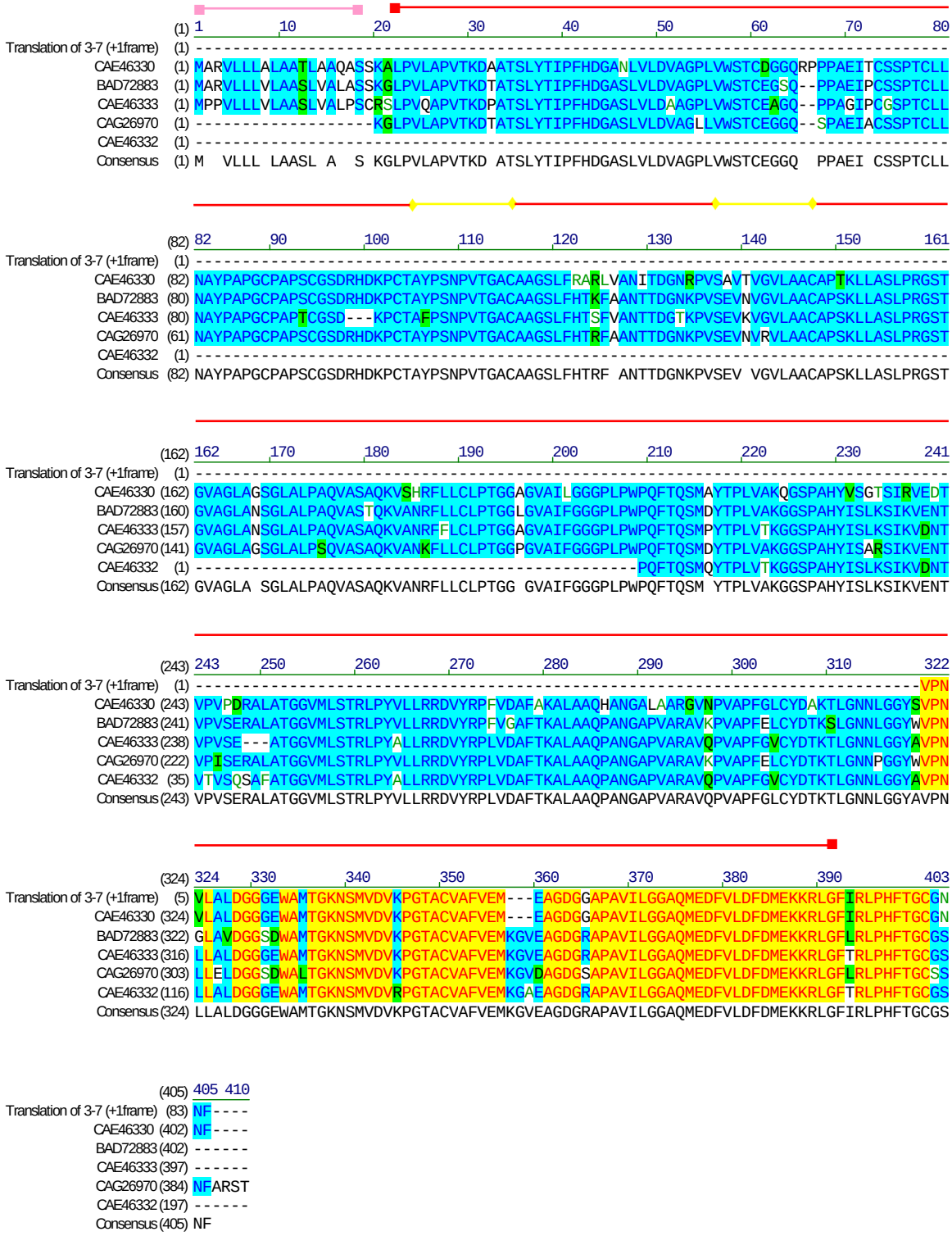

Translation of 3-7 (+1frame)
CAE46330
BAD72883
CAE46333
CAG26970
CAE46332
Consensus

Figure 8. Alignment of deduced amino acid sequences of plant xylanase inhibitors.

Clone 3-7 (from this study) is compared to CAE 46330 (*Hordeum vulgare*), BAD 72883 (*Triticum aestivum*), CAE 46333 (*Secale cereale*), CAG 26970 (*T. aestivum*) and CAE 46322 (*S. cereale*). The following domains and motifs are indicated with symbols and colours: Signal peptide (■) super family acid proteases (■) and prokaryotic membrane lipoprotein lipid attachment site (♦). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

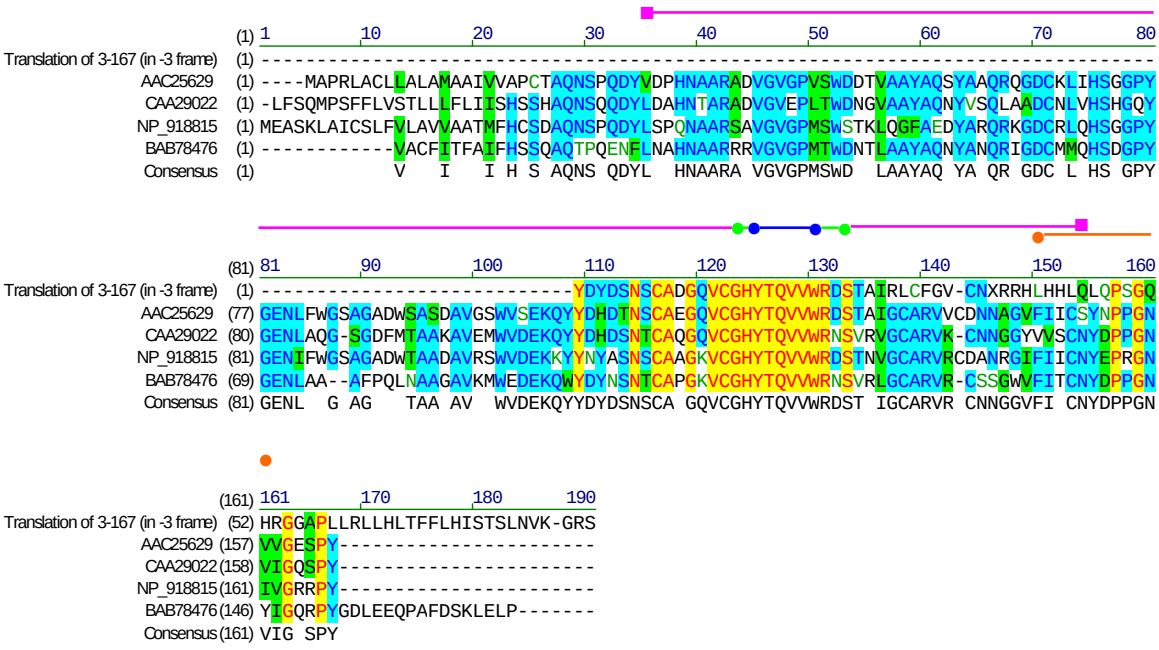


Figure 9. Alignment of deduced amino acid sequences of plant pathogenesis-related protein 1.

Clone 3-167 (from this study) is compared to AAC 25629 (*Zea mays*), CAA 29022 (*Nicotiana tabacum*), NP_918815 (*Oryza sativa*) and BAB 78476 (*Solanum torvum*). The following domains and motifs are indicated with symbols and colours: Conserved motif in all plant PR1's (●), crisp 1 family signature (●), crisp2 family signature (●), SCP-like extra-cellular protein (■). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

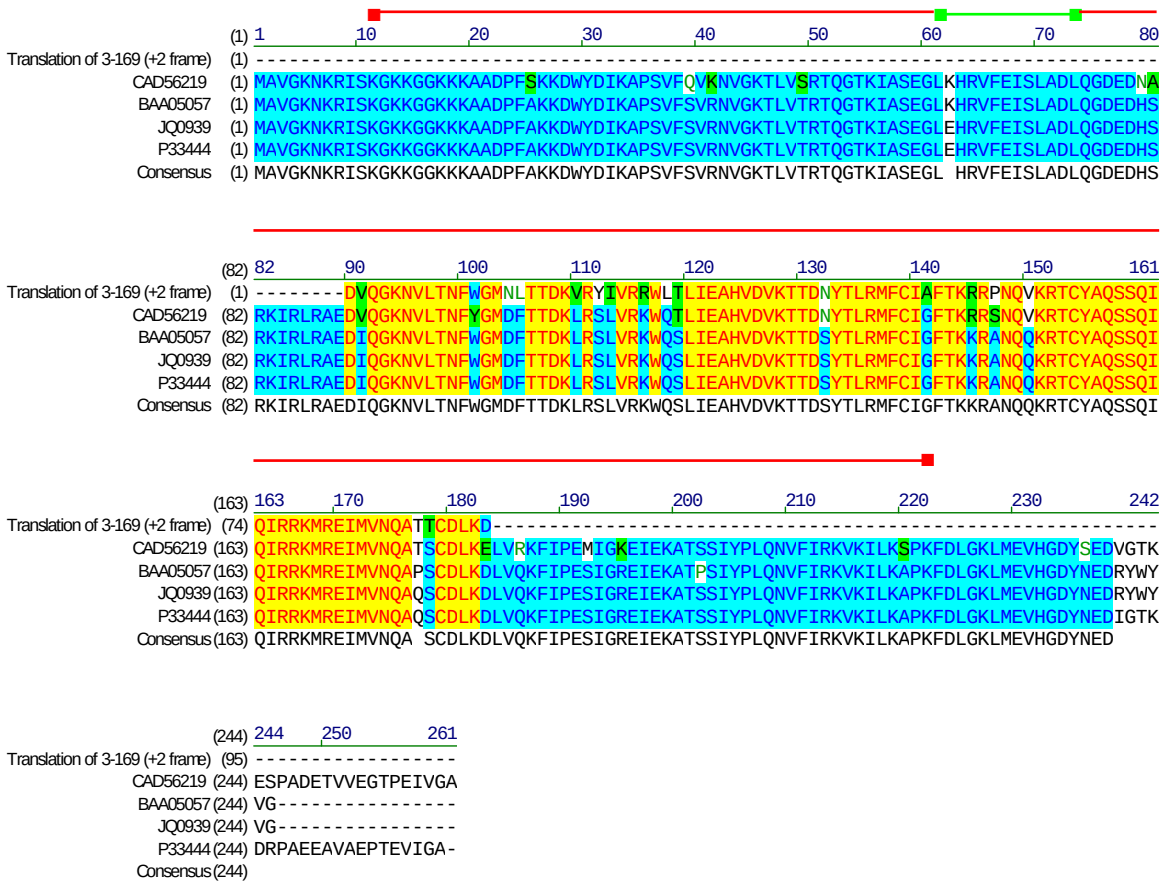


Figure 10. Alignment of deduced amino acid sequences of plant ribosomal 5S3a.

Clone 3-169 (from this study) is compared to CAD 56219 (*Cicer arietinum*), BAA 05057 (*C. roseus*), JQ 0939 (Madagascar periwinkle) and P 33444 (*C. roseus*). The following domains and motifs are indicated with symbols and colours: Pfam ribosomal 5S3a (■) and ribosomal 5S3a signature (■). Colour key: Black on window default colour = non-similar residues, blue on cyan = consensus residue derived from a block of similar residues at a given position, black on green = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position and red on yellow = consensus residue derived from a completely conserved residue at a given position.

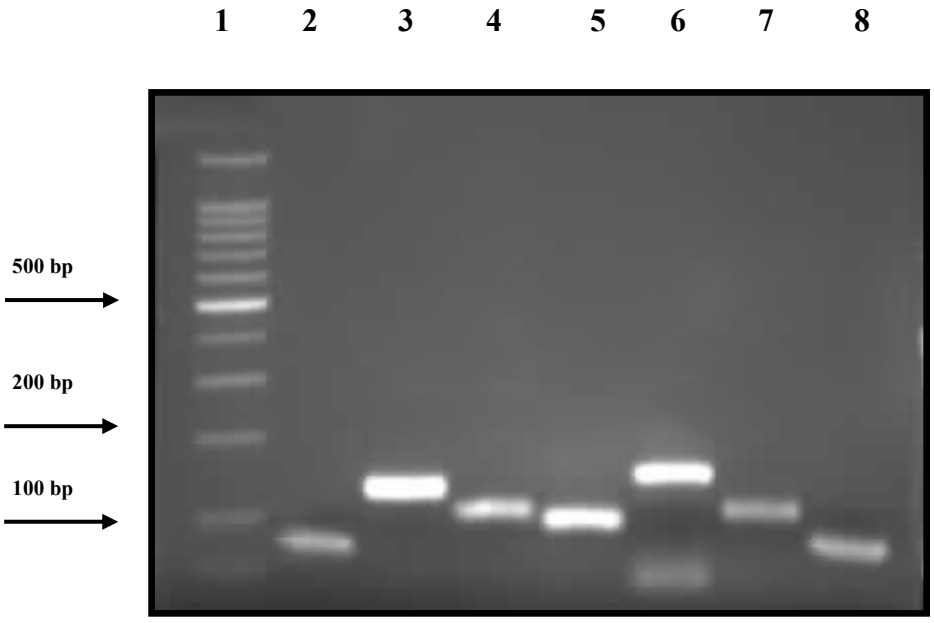


Figure 11. PCR products amplified from GCTCV-218 cDNA using clone specific primers designed from banana cDNA clones. Fragments were separated by electrophoresis through a 2% (w/v) agarose gel. Lane 1 contains the 100 bp molecular marker (Roche Diagnostics), lane 2 actin (78 bp), lane 3 clone 3-167 (*PR1*) (126 bp), Lane 4 clone 1-158 (pectin acetylesterase) (105 bp), lane 5 clone 1-77 (catalase 2) (96 bp), lane 6 endochitinase (*PR3*) (149 bp), lane 7 ubiquitin (106 bp) and *Musa* 25S rRNA (77 bp).

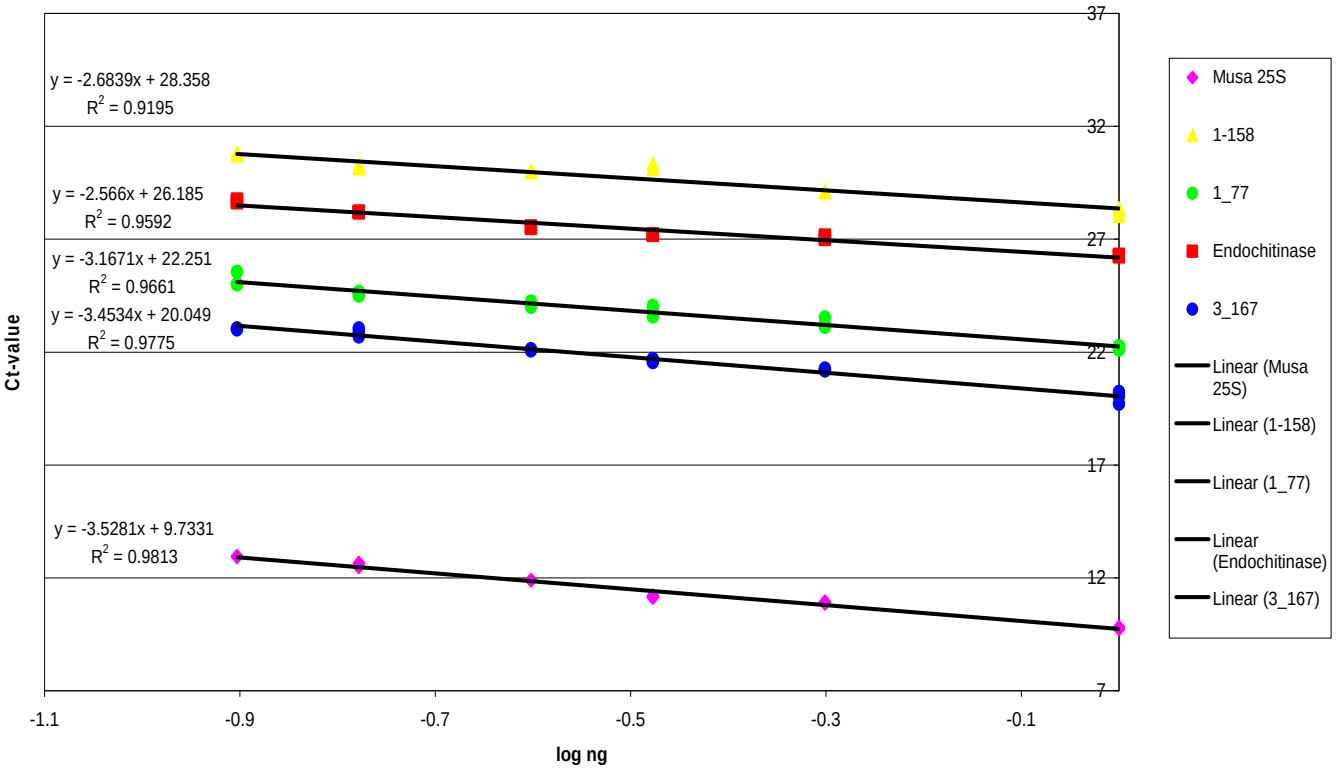
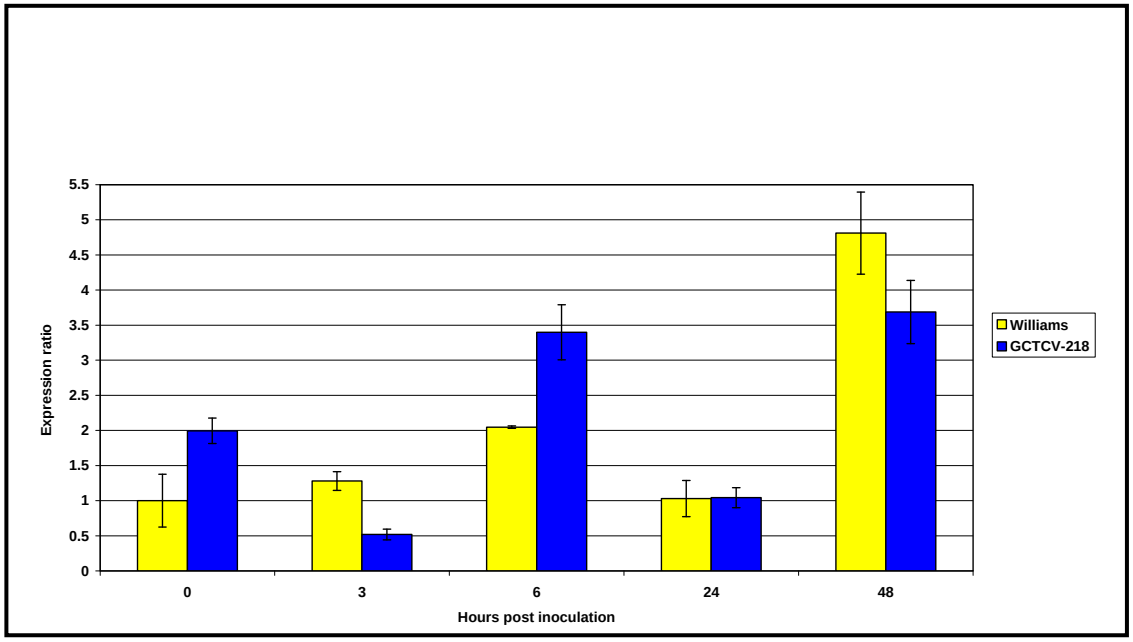


Figure 12. Standard-curve plots for calculation of PCR efficiency and quantification for different primer pairs. Ten-fold dilution series of GCTCV-218 cDNA (1:10, 1:20, 1:30, 1:40, 1:60 and 1:80) amplified with Musa 25S, 1-158, 1-77, endochitinase and 3-167 are used to generate the standard curve for each separate primer pair. The resulting Ct-values for each input amount of template are plotted as a function of the log[10] concentration of input amounts and a linear trendline is fit to the data.

A.



B.

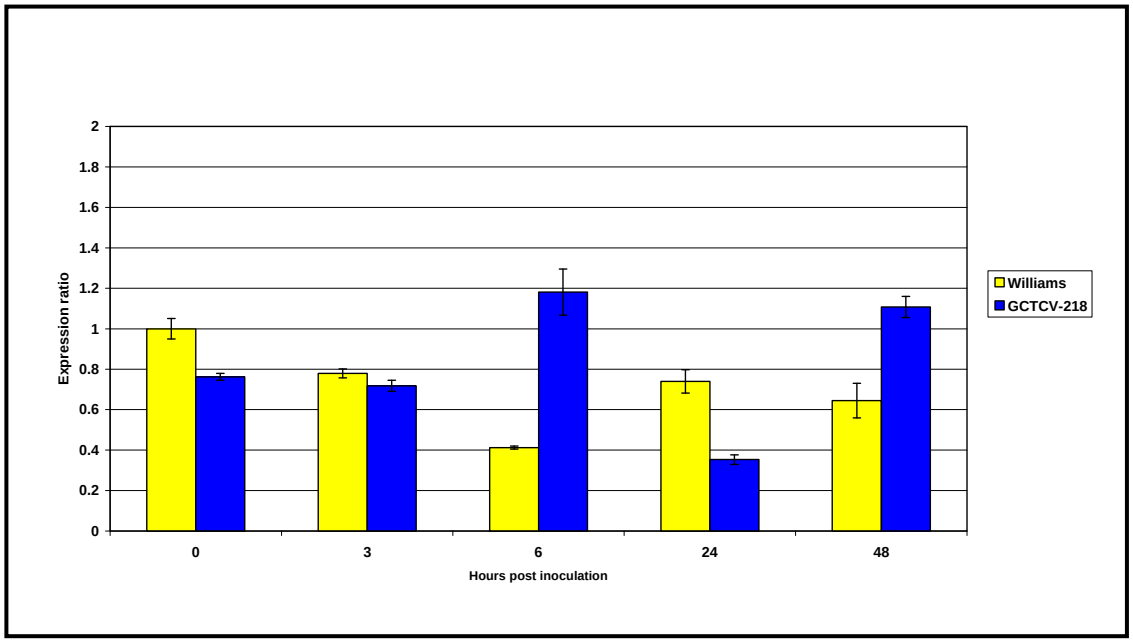
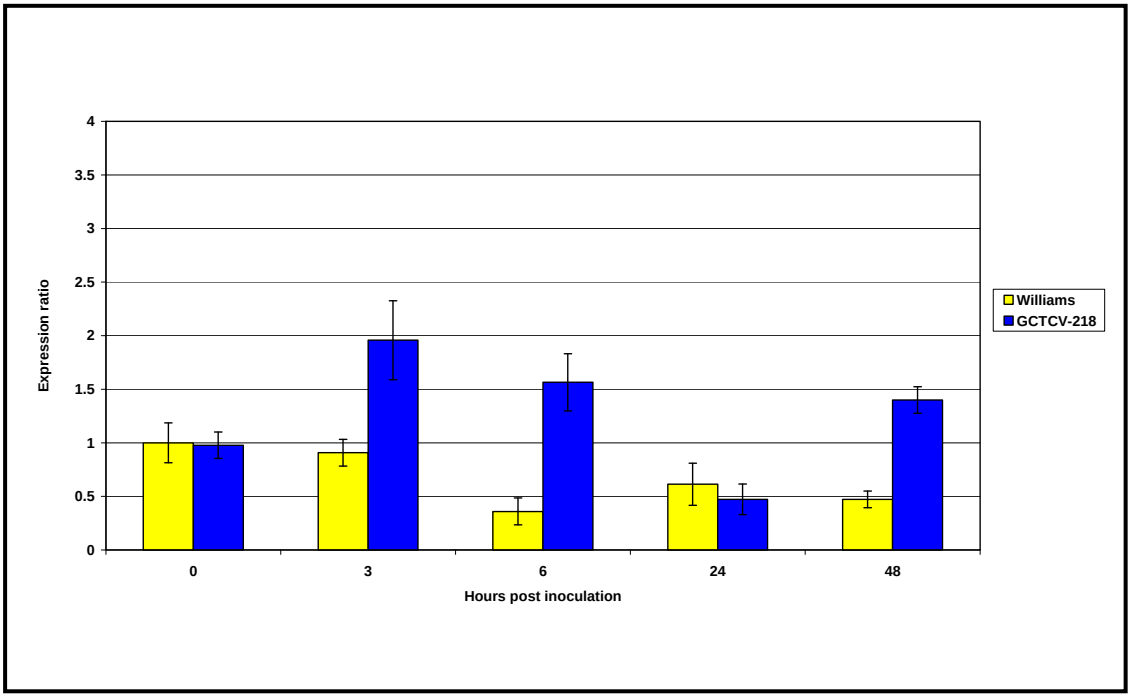


Figure 13. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of catalase 2 in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams 0 hrs after infection. Error bar is Standard Deviation, with *n*=3 for each data point.

A.



B.

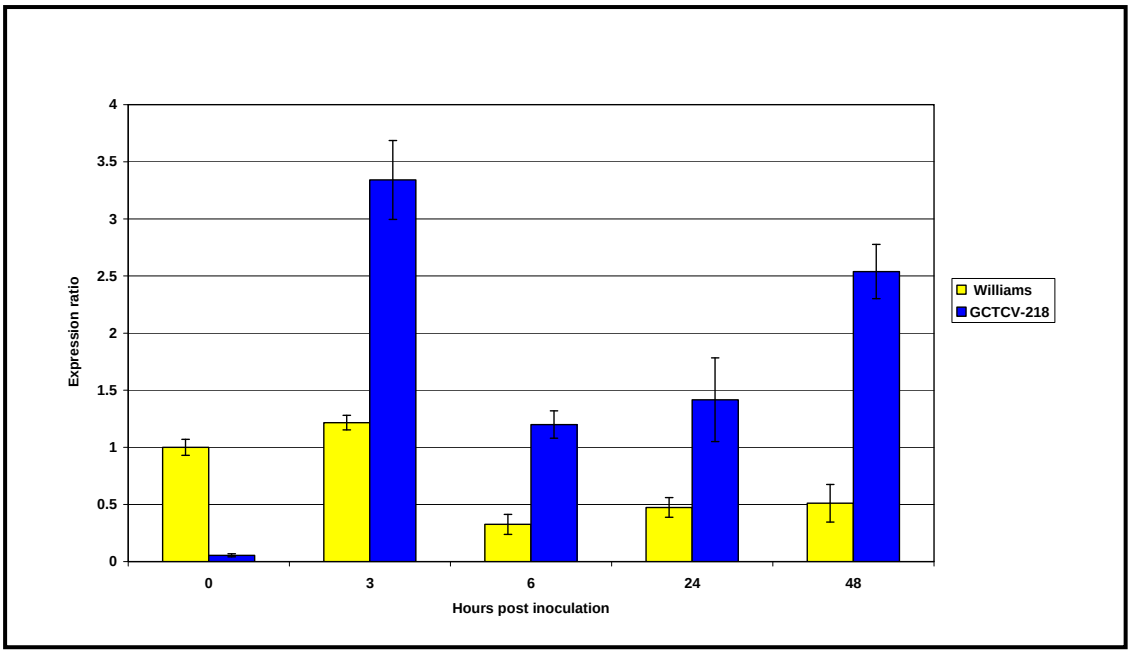
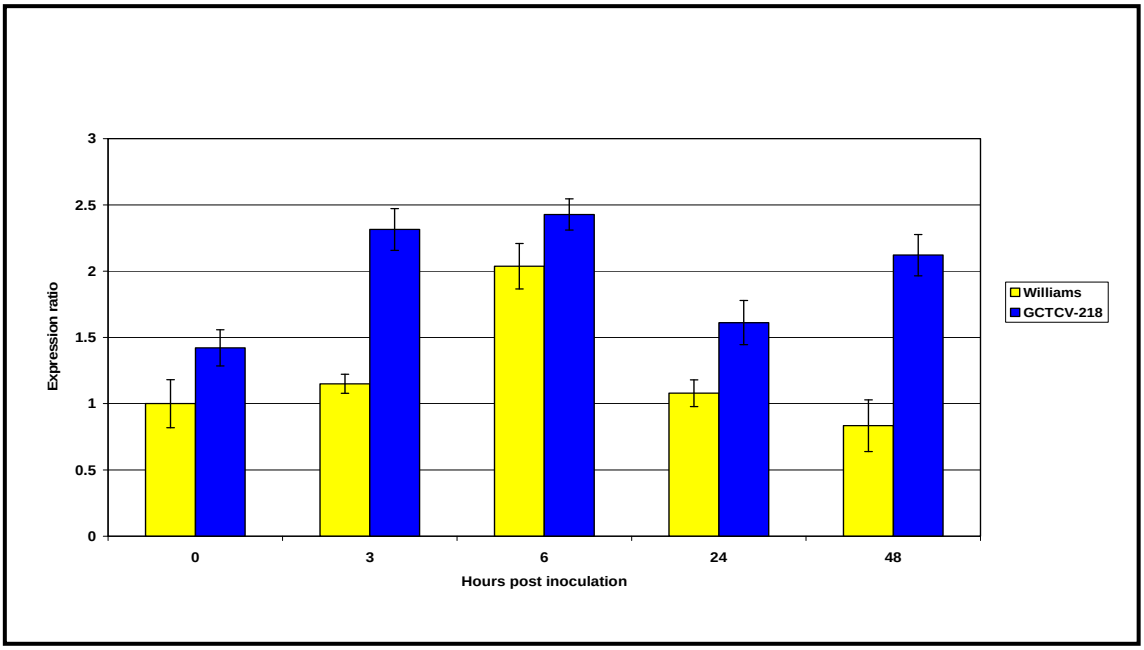


Figure 14. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of pectin acetylesterase in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams 0 hrs after infection. Error bar is Standard Deviation, with *n*=3 for each data point.

A.



B.

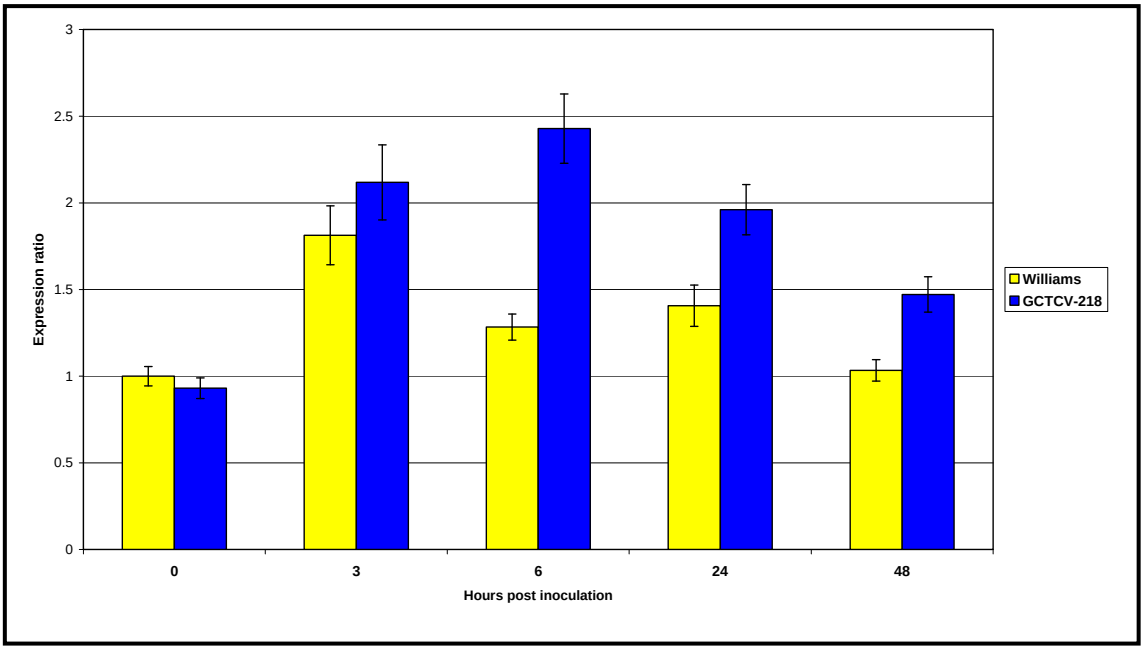


Figure 15. Relative gene expression level at 0, 3, 6, 24 and 48 hrs after of *PR*1 in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams 0 hrs after infection. Error bar is Standard Deviation, with *n*=3 for each data point.

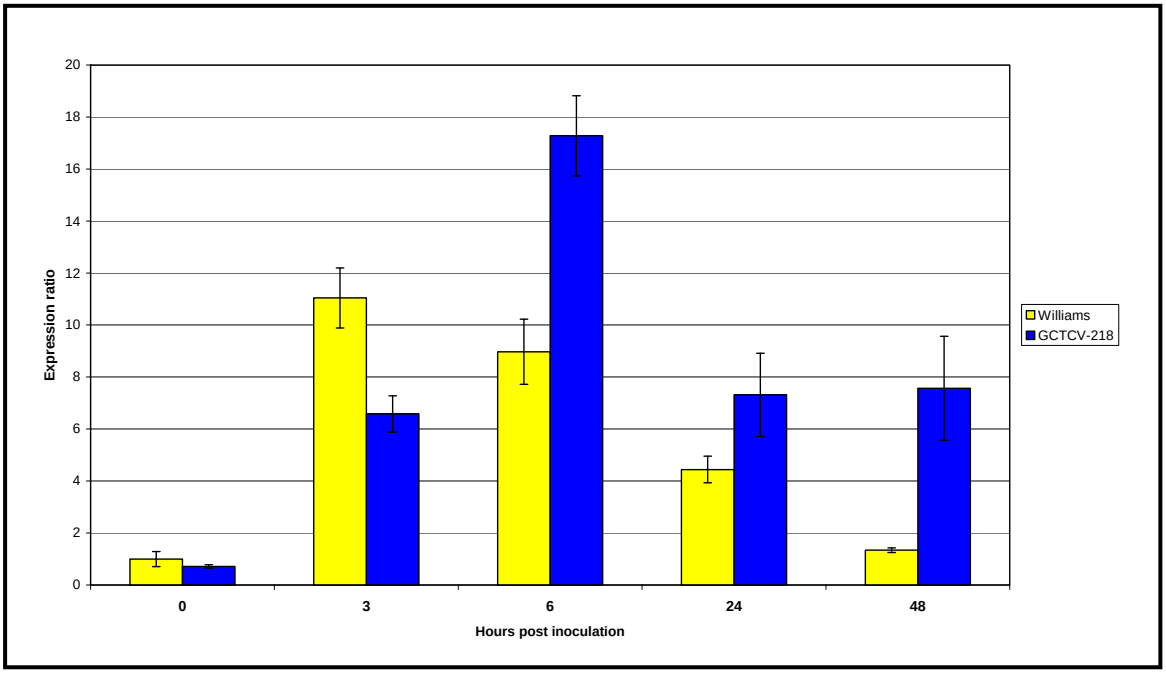


Figure 16. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of endochitinase in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the Light Cycler. Expression is given in terms of a calibrator, Williams 0 hrs after infection. Error bar is Standard Deviation, with *n*=3 for each data point.

Table 1. Base composition of oligonucleotides designed for each different gene for detection of the selected banana cDNA clones in response to *Fusarium oxysporum* f. sp. *cubense*.

Oligonucleotide	Putative identity	Sequence (5'-3')	Product size	Tm °C	Annealing temp °C
1-158 F	Pectin acetylesterase	GGCTCTCCTTTCTGGATGTTC	105	62.57	59
1-158 R		TCAGCAAGGCACTTGACTTTT		58.66	
1-77 F	Catalase 2	AAGCATCTTGTCGTCGGAGTA	96	60.61	59
1-77 R		CGCAACATCGACAACTTCTTC		60.61	
3-167 F	PR1	TCCGGCCTTATTTCACATTC	126	58.35	59
3-167 R		GCCATCTTCATCATCTGCAA		58.35	
Endochit F	Endochitinase	GGCTCTGTGGTTCTGGATGA	149	62.45	59
Endochit R		CCAACCCTCCATTGATGATG		60.4	
Actin F	Cloned actin gene	GCTATTCAGGGCGTCCTTTC	78	62.45	59
Actin R	(*Musa sp.*)	GCTGACACCATCACCAGAATC		62.45	
Ubiquitin F	Ubiquitin (*Musa* sp.)	AGGCCTGCTGCTAGAGTTCA	77	62.45	59
Ubiquitin R	(AF 399949)	TAGCAACCACCAACCAGATG		60.4	
Musa 25S F	25S rRNA (*Musa* sp.)	ACATTGTCAGGTGGGGAGTT	106	60.4	59
Musa 25S R	(AY651067)	CCTTTTGTTCCACACGAGATT		58.66	

Table 2. Sequence similarities of 79 banana (*Musa acuminata*) cDNA clones selected following Suppression Subtractive Hybridisation and microarray screening.

Clone no.	Protein similarity	Origin of similar sequence	BLASTX		BLASTN		D BEST	
			Acc. No. of similar sequence	Similarity	Acc. No. of similar sequence	Similarity	Acc. No. of similar sequence	Similarity
1-5	Putative peroxidase	*Arabidopsis thaliana*	AB013389	2e-05(89%, 25 aa)	-	-	-	-
1-6	No plant homology	-	-	-	-	-	-	-
1-8	No plant homology	-	-	-	-	-	-	-
1-13	No plant homology	-	-	-	-	-	-	-
1-17	No plant homology							
1-18	No plant homology	-	-	-	-	-	-	-
1-22	Unknown	*Hordeum vulgare*	AAL 77110.1	4e-45 (60%, 89 aa)	-	-	CF 483611	8e-13 (84%, 89 bp)
1-41	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	9e-32 (74%, 88 aa)	AY 095471.1	0 (97%, 534 bp)	-	-
1-48	No plant homology	-	-	-	-	-	-	-
1-57	Unnamed protein product	-	CAA 29122.1	2e-04 (44%, 23 aa)	-	-	-	-
1-59	Response regulator 6	*Zea mays*	BAB 20581.1	7E-56 (78%, 112 aa)	AK058585.1	3E-44 (87%, 160 bp)	CK 125116.1	0 (99%, 467 bp)
1-60	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	6e-31 (73%, 87 aa)	AY 095471.1	0 (98%, 606 bp)	-	-
1-77	Catalase2	*Zantedeschia aethiopica*	AAG61140.2	5e-50 (92%, 89 aa)	AF021939	4e-51 (84%, 243 bp)	CA273470.1	6e-61 (85%, 247 bp)

1-113	No plant homology	-	-	-	-	-	-	-
1-121	No plant homology	*Rattus norvegicus*	AAP92584.1	1e-22 (52%, 50 aa)	AF069222	0 (98%, 409 bp)	AJ602708.1	0 (96%, 421 bp)
1-136	Metallothionein-like protein	*Musa acuminata*	AAG44757.1	4E-27 (100%, 54 aa)	AF268391.1	0 (1005, 338 bp)	CK763677.1|	5e-16 (95%, 57aa)
1-141	No plant homology	-	-	-	-	-	-	-
1-149	Ferredoxin III	*Zea mays*	P27788	3E-45 (75%, 83 aa)	BX820968	0.00002 (84%, 65 bp)	CD725680	2e-12 (79&, 192 bp)
1-150	No plant homology	-	-	-	-	-	-	-
1-158	Putative pectinacetylesterase precursor	*Oryza sativa*	BAC 07121.1	6e-92 (75%, 155 aa)	AK 065122.1	4e-19 (80%, 178 bp)	CD 879787	4e-24 (80%, 240 bp)
1-159	No plant homology	-	-	-	-	-	-	-
1-162	No plant homology	-	-	-	-	-	-	-
1-163	Metallothionein-like protein	*Musa acuminata*	AAG44757.1|	2E-26 (98%, 53 aa)	AF268391.1	e-173 (97%, 329 bp)	AY095471.1	0 (98%, 406 bp)
1-165	No plant homology	-	-	-	AF069226.1	0 (98% 493 bp)	AJ774535.1	0 (96%, 492 bp)
1-171	Cytochrome P450 like_TBP	*Citrullus lanatus*	BAD26579.1	5e-30 (100% 50 aa)	AY106495.1	0 (96%, 498 bp)	CN127734.1	0 (95%, 511 bp)
1-174	Unknown	*Oryza sativa*	CAE02910.3	0.0000000000009 (51%, 33 aa)	-	-	-	-
1-177	Putative peroxidase	*Oryza sativa*	BAB19339.1	6E-68 (80%, 124 aa)	AY106495.1	6E-55 (82%, 313 bp)	CN127734.1	2E-44 (81%, 298 bp)
1-192	No plant homology	-	-	-	-	-	-	-
1-193	Hypothetical protein	*Oryza sativa*	NP 910619.1	0.0000000000003 (100&, 37 aa)	AF069222.1	0 (96%, 350 bp)	CA 736051.1	0 (95%, 352 bp)
1-199	No plant homology	*Rattus norvegicus*	AAP 92584.1	5E-26 (58%, 73 aa)	AF069222	0 (99%, 390 bp)	AJ 799053.1	0 (97%, 354 bp)
1-200	Unknown protein	*Hordeum vulgare*	AAL 77110.1	2e-45 (77%, 117 aa)	AF 474373.1	6e-12 (85%, 82 bp)	-	-
2-5	Trypsin inhibitor	*Vigna unguiculata subsp. unguiculata*	CAA 29122.1	3e-04 (51%, 27 aa)	-	-	-	-

2-18	Unknown	*Hordeum vulgare*	AAL 77110.1	2E-38 (58%, 80 aa)	AF 474373.1	0.000000000006 (85%, 82 bp)	CF483611	0.0000000000007 (84%, 89 bp)
2-23	Unknown	*Hordeum vulgare*	AAL 77110.1	8e-18 (51%, 44 aa)	-	-	BG 158940	0.008 (90%, 36 bp)
2-28	Salt stress	*Helianthus paradoxus*	CF 083631	0.003 (100%, 23 aa)	-	-	AK 065122.1	4e-19 (80%, 178 bp)
2-35	Trypsin inhibitor	*Vigna unguiculata subsp. unguiculata*	CAA 29122.1	3e-04 (51%, 27 aa)	-	-	-	-
2-37	Unknown	*Hordeum vulgare*	AAL 77110.1	3e-38 (59%, 78aa)	AF 474373.1	2e-14 (85%, 92 bp)	CB 878582.1	1e-14(85%, bp)
2-38	Putative pectinacetylesterase precursor	*Oryza sativa*	BAC 07121.1	1e-81 (75%, 140 aa)	AK 065122.1	2e-14 (84%, 98 bp)	BU 043125	5e-23 (86%, 112 bp)
2-45	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	2E-41(68%, 81 aa)	-	-	CF830219	0 (96%, 591 bp)
2-47	Putative peroxidase	*Oryza sativa*	BAB 19339,1	8e-69 (76%, 126 aa)	AY 106495.1	7e-54 (84%, 236 bp)	-	-
2-61	Unknown	*Hordeum vulgare*	AAL 77110.1	8e-10 (45%, 29 aa)	-	-	-	-
2-70	Putative peroxidase	*Oryza sativa*	BAB 19339.1	6e-49 (79%, 92 aa)	AY 106495.1	5e-54 (84%, 236 bp)	-	-
2-76	Trypsin inhibitor	*Vigna unguiculata subsp. unguiculata*	AAO43979.1|	0.0001 (41%, 23 aa)			AY095460.1	0 (97%, 610 bp)
2-86	Bowman-Birk type proteinase inhibitor II	*kidney bean*	P01060	0.00004 (37%, 23 aa)	-	-	CK 168162.1	0.0007 (100%, 26 bp)
2-110	Unknown	*Hordeum vulgare*	AAL 77110.1	1e-28 (53%, 51 aa)	AF 474373.1	3e-04 (85%, 53 bp)	CF 483611	6e-07 (87%, 54 bp)
2-116	Trypsin inhibitor	*Vigna unguiculata subsp. unguiculata*	CAA 29122.1	3e-04 (51%, 27 aa)	-	-	-	-

2-120	Bowman-Birk type proteinase inhibitor II	*Phaseolus vulgaris*	P01060	0.00004 (37%, 23 aa)	-	-	CK 168162	0.0007 (100%, 26 bp)
2-122	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	1e-31(74%, 88 aa)	AY 095471.1	0 (98%, 606 bp)	-	-
2-124	Bowman-Birk type proteinase inhibitor II	*kidney bean*	P01060	0.00004 (37%, 23 aa)			CK168162.1	0.0007 (100%, 26 aa)
2-130	Putative peroxidase	*Oryza sativa*	BAB 19339.1	1e-83 (77%, 149 aa)	-	-	CA 180787	2e-43 (81%, 308 bp)
2-131	Putative peroxidase	*Oryza sativa*	BAB 19339.1	3e-86 (77%, 154 aa)	AY 106495.1	9e-54 (84%, 236 bp)	CA 180787	1e-42 (81%, 298 bp)
2-136	Root control	*Pinus taeda*	-	-	-	-	CF663368	0.008 (100%, 24 bp)
2-137	Putative peroxidase	*Oryza sativa*	NP_910684.1	5E-38 (59%, 88 aa)	NM_185795.1	0.0000000000003 (87%, 75 bp)	CA180787.1	7e-15 (89%, 73 bp)
2-138	Putative peroxidase	*Oryza sativa*	BAB 19339.1	8E-60 (77%, 113 aa)	AY106495,1	9E-23 (81%, 184 bp)	CN127734	5E-20 (81%, 173 bp)
2-160	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	7e-32 (74%, 88 aa)	AY 095471.1	0 (97%, 479 bp)	-	-
2-168	Xylanase inhibitor	*Triticum aestivum*	CAD 27730.1	1e-34 (83%, 72 aa)	TAE 438880	3e-83 (90%, 227 bp)	CA 004002	e-167 (99%, 308 bp)
2-169	No plant homology	-	-	-	-	-	-	-
2-184	Putative peroxidase	*Oryza sativa*	BAB 19339.1	2e-86 (76%, 154 aa)	AY 106495.1	6e-55 (82%, 313 bp)	CA 180787	6e-41 (80%, 307 bp)
2-190	Unspecific monooxygenase	*Nicotiana tabacum*	T02995	1e-31(74%, 88 aa)	AY 095471.1	0 (98%, 606 bp)	-	-
3-7	Xylanase inhibitor	*Triticum aestivum*	CAD 27730.1	1e-34 (83%, 72 aa)	TAE 438880	3e-83 (90%, 227 bp)	CA 004002	e-167 (99%, 308 bp)
3-16	Putative senescence associated protein	*Pyrus communis*	AAR25995.1	0.0000000002 (96%, 30 aa)	AY428812.1	6E-65 (96%, 147 bp)	CD725233.1	4E-63 (99%, 128 bp)
3-25	Hypothetical protein	*Oryza sativa*	BAC 10355.1	5e-23 (64%, 48 aa)	-	-	-	-
3-37	No plant homology	-	-	-	-	-	-	-
3-41	Hypothetical protein	*Oryza sativa*	BAC 20633.1	1e-13 (100%, 37 aa)	MITTARRNG	0 (97%, 368 bp)	CB 645279	0 (97%, 368 bp)
3-56	No plant homology	*Rattus norvegicus*	AAP92584.1	2E-25 (58%, 72 aa)	AF069222.1	0 (98%, 355 bp)	AJ799053.1	e-171 (96%, 349 bp)

3-61	Unknown protein relatede to lectin	*Polygonatum multiflorum*	AAC49412.1	4e-17 (62%, 59 aa)	-	-	AJ603393.1	0.000002 (1005, 30 bp)
3-81	No plant homology	*Rattus norvegicus*	AAP92584.1|	2e-20 (53%, 59 aa)	AF069222.1	0 (98%, 358 bp)	AJ799053.1	e-174 (96%, 352 bp)
3-105	Inhibitor CII	*Glycine max*	763679A	0.7 (57%, 22 aa)	BT009458.1	0.026 (100%, 24 bp)	-	-
3-109	No plant homology	-	-	-	-	-		-
3-113	Pathogenesis-related protein 1	*Zea mays*	A33155	2E-21 (68%, 42 aa)	AY106735.1	0.00000004 (85%, 66 bp)	CF441577.1	0.000000000007 (90%, 57 bp)
3-114	No plant homology	-	-	-	-	-	-	-
3-125	No plant homology	-	-	-	BQ537443.1	0 (96%, 384 bp)	AF069226.1	0 (98%, 392 bp)
3-138	Hypothetical protein	-	BAC20633.1|	0.000000003 (100%, 29 aa)	AK109349.2	0 (97%, 377 bp)	CF923653.1|	0 (97%, 394 bp)
3-143	No plant homology	*Rattus norvegicus*	AAP92584.1	7E-22(54%, 67 aa)	AF293755.1	1E-89 (100%, 170 bp)	CN446975.1|	7E-90 (100%, 170 bp)
3-146	Putative senescence-associated protein	*Pisum sativum*	BAB33421.1|	4E-43 (78%, 86 aa)	AY292882	e-163 (99%, 296 bp)	CF923987	e-177 (98%, 332 bp)
3-160	Putative senescence associated protein	*Pisum sativum*	BAB33421.1|	0.000000000000005 (78%, 40 aa)	AF399947.1|	e-101 (95%, 251 bp)	CD725233.1	4E-63 (99%, 128 bp)
3-167	Pathogenesis related protein-1	*Zea mays*	AAC25629.1	4e-17 (74%, 29 aa)	AY106735.1	0.0000000000007(85%, 84 bp)	BE367183.1	0.00000000000003(87%, 76 bp)
3-169	Ribosomal protein S3a	*Cicer arietinum*	CAD 56219	2e-42 (87%, 82 aa)	AF 542188.1	2e-37 (82%, 226 bp)	CD 938508	4e-40 (82%, 233 bp)
3-174	No plant homology	-	-	-	-	-	-	-

Table 3. BLASTX identities of non-redundant clones derived from the banana Suppression Subtractive Hybridization library, microarray Enrichment Ratio 1, Enrichment Ratio 2 and unsubtracted "tester" (UT)/unsubtracted "driver" (UD) data and their putative functional categories.

Clone no.	Accession no.	Putative Identity	Species	E-value	Functional category	Microarray Data (From Chapter 3)			Inverse Northern Blot data [b] (From Chapter 3)
						ER1	ER2	UT/UD[a]	
1-5	AB 013389	Peroxidase	*Arabidopsis thaliana*	$2e^{-05}$	Secondary metabolism - Lignin biosynthesis	2.4	-0.2	1.7	ND
1-22	AAL 77110	Unknown protein	*Hordeum vulgare*	$4e^{-45}$	Unknown	1.4	1.4	1.0	ND
1-59*	BAB 20581.1	Response Regulator 6	*Zea mays*	$7e^{-56}$	Regulation	0.1	0.1	1.0	ND
1-77*	AAG61140.2	Catalase 2	*Zantedeschia aethiopica*	$5e^{-50}$	Oxidative burst	0.8	0.6	1.2	2.1
1-136*	AAG44757.1	Metallothionein	*Musa acuminata*	$4e^{-27}$	Cell rescue/defence	-0.1	-0.1	1	1.2
1-149	P27788	Ferredoxin III	*Zea mays*	$3e^{-45}$	Energy	0	-0.1	1.1	1.4
1-158*	BAC 07121.1	Pectin acetylesterase precursor	*Oryza sativa*	$6e^{-92}$	Degradation of pectin	0.7	0.6	1.1	2.0
1-174	CAE 02910	Unknown protein	*O. sativa*	$9e^{-12}$	Unknown	-0.1	-0.2	1.1	ND
2-28	CF 083631	Salt stress	*Helianthus paradoxus*	$3e^{-03}$	Stress response	0.6	0.3	1.2	2.1
2-35	CAA 29122.1	Trypsin inhibitor	*Vigna unguiculata subsp.*	$3e^{-04}$	Proteases/inhibitors	0.3	-0.1	1.3	6.9

			unguiculata						
2-45*	T02995	Unspecific monooxygenase, cytochrome P450	*Nicotiana tabacum*	$2e^{-41}$	Secondary metabolism – phenylpropanoid biosynthesis	1.4	1.0	1.3	1.0
2-70*	BAB 19339.1	Peroxidase	*O. sativa*	$6e^{-49}$	Secondary metabolism - Lignin biosynthesis	1.9	1.2	1.6	1.2
2-86	P01060	Bowman Birk proteinase inhibitor	*kidney bean*	$4e^{-05}$	Proteases/inhibitors	-0.3	-0.6	1.2	ND
2-136	CF663368	Root control	*Pinus taeda*	$8e^{-03}$		1.1	1.0	1.1	ND
3-7*	CAD 27730.1	Xylanase inhibitor	*Triticum aestivum*	$1e^{-34}$	Unknown function	0.6	0.3	1.2	1.7
3-105	763679A	Inhibitor CII	*Glycine max*	0.7	Cell rescue/defence	-0.3	-0.6	1.2	8.6
3-138	BAC 20633	Hypothetical protein	*O. sativa*	$5e^{-23}$	Unknown	0.2	0.2	1.0	ND
3-146	BAB33421.1|	Putative senescence-associated protein	*Pisum sativum*	$4e^{-43}$	Unknown function	-1.8	-1.8	1.0	ND
3-167*	AAC25629.1	PR1	*Zea mays*	$4e^{-17}$	Unknown function In vitro antifungal activity (defence)	-1.5	-1.5	1.0	ND
3-169*	CAD 56219	Ribosomal protein S3a	*Cicer arietinum*	$2e^{-42}$	Protein synthesis - translation	1.0	0.7	1.2	3.1

* These clones were selected for multiple alignments and domain and motif searches. ND=not determined

a. UT/UD = antilog of (ER1-ER2) in the base 2.

b. The inverse Northern expression ratio was calculated as follows: density of "tester"/"driver" samples after normalization of the data using an rDNA clone

Table 4. Evaluation of uniform expression of candidate endogenous control clones (actin, ubiquitin and *Musa* 25S rRNA) over different treatments containing identical cDNA concentrations using TaqMan® technology. Ct-values are defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level.

Clone	cDNA template	Treatment (after infection)	Ct value (Replicate 1)	Ct value (Replicate 2)
Actin	GCTCV-218	0 hrs	27.96	28.12
		48 hrs	23.19	23.09
	Williams	0 hrs	22.4	22.6
		48 hrs	24.17	24.0
Ubiquitin	GCTCV-218	0 hrs	25.75	25.72
		48 hrs	24.2	24.45
	Williams	0 hrs	24.1	24.63
		48 hrs	21.81	21.72
***Musa* 25S rRNA**	GCTCV-218	0 hrs	8.05	8.07
		48 hrs	8.90	8.93
	Williams	0 hrs	8.18	8.15
		48 hrs	8.98	8.99

Table 5. Calibration function, correlation coefficient and PCR efficiency of the 5 primer pairs (1-158, 1-77, 3-167, Endochitinase and *Musa* 25S rRNA) during TaqMan® -PCR assays.

Gene	Calibration function (y = mx + b)	Correlation coefficient (R^2)	PCR efficiency (%)[a]
1-158	y = -2.6839x + 28.358	0.920	135
1-77	y = -3.1671x + 22.251	0.966	107
3-167	y = -3.4534x + 20.049	0.978	95
Endochitinase	y = -2.566x + 26.185	0.959	145
Musa 25S rRNA	y = -3.5281x + 9.733	0.981	92

[a] PCR efficiency = $[(10^{(1/-s)})-1]* 100$, where S is the slope