IDENTIFICATION OF GENES ASSOCIATED WITH TOLERANCE IN THE CAVENDISH BANANA SELECTION, GCTCV-218, AGAINST *FUSARIUM OXYSPORUM* F. SP. *CUBENSE*, 'SUBTROPICAL' RACE 4

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DECLARATION

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation and that no part of this thesis has been submitted to any other university.

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TABLE OF CONTENTS

Acknowledgements i

Preface iii

Abbreviations and symbols v

CHAPTER 1 Resistance to Fusarium wilt in banana: A Review 1

INTRODUCTION 2

FUSARIUM WILT OF BANANA (PANAMA DISEASE) 3

THE HOST: BANANA 5

RESISTANCE IN PLANTS TO PATHOGENS 7

Terminology 7

The Constitutive Defence Response 8

The Induced Defence Response 9

Recognition 9

Hypersensitive response 9

Oxidative burst 11

Ion fluxes 12

Cell wall strengthening and vascular occlusion 12

Lignification 12

Callose deposition 13

Phenolic compounds 14

Indole acetic acid 15

Other Defence Responses 16

Pathogenesis-related proteins (PR proteins) 16

Peroxidases 16

Phytoalexins 18

Signal Transduction 18

Salicylic acid 19

Jasmonic acid 20

Ethylene 20

Interaction between the SA, JA and ET pathways 21

Systemic resistance 21

COMPONENTS OF PLANT DEFENCE MECHANISMS AGAINST FUSARIUM WILT 23

Structural Defence 23

Biochemical Defence 25
IMPROVEMENT OF BANANA FOR FUSARIUM WILT RESISTANCE 27
Genes Associated with Resistance to Fusarium Wilt 27
Conventional Breeding 29
Unconventional Improvement 30
Somaclonal variation 30
Induced mutations 30
Protoplast fusion 31
Genetic modification 31
CONCLUSION 32
REFERENCES 35
CHAPTER 2 Evaluation of the Cavendish banana GCTCV-218 for tolerance to *Fusarium Oxysporum* f. sp. *cubense* 'subtropical' race 4 (VCG 0120) 65
INTRODUCTION 67
MATERIALS AND METHODS 69
Greenhouse Trials 69
Plant material 69
Inoculation 69
Disease rating 70
Field Trial 70
Plant material 70
Disease rating 70
Phenolic Assays 71
Plant material 71
Extraction of phenolics 71
Total soluble phenolic acids 72
Non-conjugated phenolic acids (Free acids) 72
Glycoside-bound phenolics 72
Ester-bound phenolics 72
Cell wall-bound phenolics 73
RESULTS 73
Greenhouse Trials 73
Field Trials 74
Phenolic Assays 74

Total soluble phenolics 74
Non-conjugated phenolic acids (Free acids) 74
Glycoside-bound phenolics 74
Ester-bound phenolics 74
Cell wall-bound phenolics 75
DISCUSSION 75
REFERENCES 78
CHAPTER 3 Construction of a cDNA library with genes associated with tolerance to *Fusarium oxysporum* f. sp. *cubense* in Cavendish bananas 91
ABSTRACT 92
INTRODUCTION 93
INTRODUCTION 93
MATERIALS AND METHODS 96
Inoculation of Banana Plants with *Foc* 96
Plant material and growth conditions 96
Preparation of inoculum 96
Inoculation and sample collection 97
Total RNA isolation 97
cDNA synthesis 97
Suppression Subtractive Hybridisation (SSH) 98
Monitoring SSH efficiency 99
Southern analysis 100
Subtracted library construction 101
Colony PCR and sequencing of selected clones 101
RESULTS 103
Inoculation of Banana Plants with Foc 103
RNA isolation 103
cDNA synthesis 103
Suppression Subtractive Hybridisation (SSH) 103
Southern Blot analysis 104
Subtracted library construction 104
Colony PCR and sequencing 105
DISCUSSION 105
REFERENCES 109

CHAPTER 4 High-throughput screening of a Banana cDNA library using DNA microarray analysis 125

ABSTRACT 126

INTRODUCTION 127

MATERIALS AND METHODS 128

cDNA Microarray Analysis of the SSH Library 128

Preparation of SSH clones and slide spotting 128

Probe preparation 128

Hybridization and washing 129

cDNA Microarray screening of the SSH library 130

Inverse Northern Dot Blots 131

Membrane preparation 131

Probe preparation, hybridisation and detection 131

RESULTS AND DISCUSSION 132

cDNA Microarray Screening of the SSH Library 132

Inverse Northern Blot Analysis of Selected Transcripts 133

REFERENCES 136

CHAPTER 5 Identification of genes associated with tolerance to *Fusarium oxysporum* f.sp *cubense* in Cavendish bananas 141

ABSTRACT 142

INTRODUCTION 143

MATERIALS AND METHODS 144

Sequencing and Analysis of cDNA Clones 144

Real Time Reverse Treanscriptase-PCR 145

Template preparation 145

Primer design 145

RT-PCR optimisation 146

Quantitative Expression Assays 147

Data analysis 148

RESULTS 149

Sequencing and Analysis of cDNA Clones 149

Real-Time Reverse Transcriptase PCR 151

Primer design and RT-PCR optimisation 151

Quantitative Expression Assays 152

DISCUSSION 154
REFERENCES 160
SUMMARY 200

ANNEXURE: High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis. Noëlani van den Berg, Bridget G. Crampton, Ingo Hein, Paul R.J. Birch, and Dave K. Berger.

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**People who make their own rules when they know they're right…
People who get a special pleasure out of doing something well (even if only for themselves)…
People who know there's more to this whole living thing than meets the eye: they'll be with Jonathan all the way.**

**Jonathan Livingstone Seagull
(Richard Bach)**

PREFACE

Musa acuminata Colla (banana) is one of the most important food crops in the world and provides a staple food and source of income in many households, especially in Africa. However, bananas worldwide are under serious threat by *Fusarium oxysporum* Schlect. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*). There exists no control strategy against the pathogen and control involves the use of resistant cultivars and cultural practices that prevent the introduction and spread of the disease into disease-free areas. Natural disease resistance exists in wild-type bananas and a few hybrids, but these bananas are not acceptable to the Cavendish market and the search for a new tolerant or resistant Cavendish banana is underway. Conventional breeding strategies are however hindered by the fact that Cavendish bananas are sterile and do not produce seed. Therefore, non-conventional strategies such as transformation are more realistic and could be more successful. Unfortunately, very few banana genes have been isolated and characterised up to date and the banana-*Foc* interaction has not yet been studied extensively, if at all, on the molecular level. This leads to a lack of knowledge in understanding disease resistance mechanisms in banana and complicates the matter of transforming susceptible bananas with resistance genes.

This thesis firstly aims to evaluate the disease tolerance of a Cavendish banana, GCTCV-218, infected with *Foc* and secondly to isolate the disease resistance genes expressed early in the banana-*Foc* interaction. Fusarium wilt is a root pathogen and few molecular studies have been done on the plant response in roots to pathogens. This is, so far, known the first molecular study on the Cavendish banana-*Foc* interaction.

Chapter 1 provides the reader with a short review of banana, and the pathogen, *Foc*. The chapter then gives a broad overview of disease resistance in plants and further provides information on Fusarium resistance in banana and other crops with reference to the type of resistance (i.e. constitutive or actively induced chemical or structural resistance).

The Cavendish banana, GCTCV-218, is a somaclonal variety selected by researchers at the Taiwan Banana Research Institute (TBRI) in Taiwan and showed promising results in disease resistance trials against 'tropical' race 4 (VCG 0121). **Chapter 2** evaluates the disease tolerance of GCTCV-218, under South African conditions against *Foc* 'subtropical' race 4 (VCG 0120). Literature has reported that phenolic compounds may be involved in resistance against *Foc* and this chapter will also study the different phenolic compounds in GCTCV-218 compared to the susceptible Williams at different time intervals after *Foc* infection.

Chapter 3 describes the construction of a banana cDNA library containing gene fragments that are differentially expressed in GCTCV-218 in response to *Foc* compared to the susceptible Williams cultivar. A highly effective PCR-based technique, termed Suppression Subtractive Hybridisation (SSH), was applied in this chapter.

Chapter 4 reports on the development of a high-throughput screening method of the banana SSH cDNA library using DNA microarray analysis. This is a novel approach in screening SSH libraries for false positives that have escaped the subtraction process and has been published in **Bio Techniques (2004) 37: 818-824**.

Seventy-nine gene fragments were selected for sequencing after screening the library. In **Chapter 5**, the selected gene fragments were sequenced and subjected to BLASTX, BLASTN and DBEST searches. A table containing non-redundant gene fragments was compiled and some of these gene fragments were subjected to alignments with known corresponding genes from the NCBI database. The expression profile of four defence related genes was further investigated by quantitative Reverse Transcriptase-PCR.

ABBREVIATIONS AND SYMBOLS

α	Alpha
AC	Acetate
ADP	Adenosine diphosphate
AFLP	Amplified Fragment Length Polymorphism
AIR	Alcohol insoluble residue
AMV	Avian Myeloblastosis Virus
AOS	Active oxygen species
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Avr	Avirulence
β	Beta
BLAST	Basic Local Alignment Search Tool
BLASTX	BLAST algorithm to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
BLASTN	BLAST algorithm to compare a nucleotide query sequence against a nucleotide sequence database.
bp	base pairs
BTH	benzo-(1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester
Ca^{2+}	Calcium (II) ions
cADPR	Cyclic ADP ribose
CAV	Culture collection, Altus Viljoen
$Ca(NO_3)_2.H_20$	Calcium nitrate
°C	Degrees Celsius
CDPK	Calcium-dependant protein kinase
cDNA	complementary Deoxyribonucleic Acid
CIRAD-FLHOR	Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Département des productions fruitières et horticoles
cm	centimetres
Ct	Cycle number at which the fluorescence signal crosses a fixed

	threshold
cv	cultivar
DIG-dUTP	Digoxygenin deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
dITP	Deoxyinosinetriphosphate
DIG	Digoxygenin
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine tetraacetic acid
EMBRAPA-CNPMF	Empresa Brasileira de Pesquisa Agropecuária – Mandioca e Fruticultura Tropical
EF1	Elongation factor 1
ER	Enrichment ratio
EST	Expressed sequence tags
ET	Ethylene
FABI	Forestry and Agricultural Biotechnology Institute
FHIA	Fundación Hondurereña de Investigacón Agrícola
f.sp.	Formae speciales
Foc	*Fusarium oxysporum* f. sp. *cubense*
Fod	*Fusarium oxysporum* f. sp. *dianthi*
Fol	*Fusarium oxysporum* f.sp. *lycopersici*
g	gram
g/l	gram per litre
GMP	Guanosine monophosphate
GSII	1,3-β-glucan synthase
h	Hour
HCl	Hydrochloric acid

hpi	Hours post inoculation
H_2O	Water
H_2O_2	Hydrogen peroxide
HR	Hypersensitive Response
H	Hour
hrs	Hours
H^+	Hydrogen
IAA	Indole acetic acid
IAEA	International Agricultural Exchange Association
IITA	International Institute for Tropical Agriculture
IMP	Inosine 5' monophosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Induced resistance
ISR	Induced systemic resistance
ITS	Internal transcribed spaces
JA	Jasmonic acid
JME	Jasmonic methyl ester
K^+	Potassium
KCl	Potassium chloride
LAR	Local acquired resistance
LB	Luria-Bertani
LMP	Low melting point
LRR	Leucine-rich repeats
M	Molarity
MAPK	Mitogen-activated protein kinases
MeJA	Methyl jasmonate
MeOH	Methyl hydroxide
min	Minutes
mg	Milligrams
$MgCl_2$	Magnesium Chloride
ml	Millilitres
ml^{-1}	Per millilitre
ml/l	millilitre per litre

mm	Millimetres
mM	millimolar
mRNA	messenger Ribonucleic Acid
N_2	Nitrogen
$NaCO_3$	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NBS	Nucleotide-Binding Site
NCBI	National Centre for Biotechnology Information
ηg	Nanogram
nm	nanometre
No.	Number
NO	Nitric oxide
NOS	NO synthase
O_2^-	Super oxide
OH	Hydroxyl radical
P	Proline
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
pH	Log hydrogen ion concentration
pI	Isoelectric point
POD	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related
R	Resistance
R^2	Correlation coefficient
RDA	Representational difference analysis
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid

RT	Reverse transcriptase
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SD	Standard Deviation
SAR	Systemic acquired resistance
SDS	Sodium Dodecyl Sulfate
SDW	Sterile distilled water
SNP	Sodium nitroprusside
spp	species
SSC	Sodium Saline Citrate
SSH	Suppression Subtractive Hybridisation
ST	Subtracted tester
TAE	Tris-acetate tetraacetic acid
TBRI	Taiwan Banana Research Institute
TFA	Triflouroacetic acid
Tm	Melting temperature
TMV	Tobacco mosaic virus
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol chloride
tRNA	Total ribonucleic acid
UD	Unsubtracted driver
UDP	Uridine diphosphate
µg	Micrograms
µl	Micro litre
µM	Micro molar
UT	Unsubtracted tester
UV	Ultra violet
VCG	Vegetative Compatibility Group
v/v	Volume per volume
v/v/v	Volume per volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
λ	Lambda (wavelength)
%	Percentage

Σ	Sum of

CHAPTER 1

RESISTANCE TO FUSARIUM WILT IN BANANA: A REVIEW

INTRODUCTION

In nature, plants are constantly challenged by aspiring pathogens, but disease rarely occurs because most plants are able to defend themselves against microbial attack. Plants have an extensive repertoire of responses, by which they are able to localize, inhibit and isolate infections (Beckman *et al.*, 1982; Schenk *et al.*, 2000). These defence responses are dependent upon early recognition of the pathogen and rapid mobilization of the response system (Schenk *et al.*, 2000). Although defence mechanisms have been studied extensively in several plants, little is known about either defence responses of monocotyledonous plants or, more generally, plant responses against root invading pathogens.

Fusarium wilts are economically important soil borne diseases that affect a wide variety of crops throughout the world (Beckman, 1987; Fuchs *et al*., 1999). One of the most important Fusarium wilt diseases is Fusarium wilt of banana (*Musa* spp.). Bananas are regarded as the fourth most important staple fruit crop, and provide a food source and income to millions of people in the world. Fusarium wilt devastated banana plantations in Central America during the 1960's, and the international export industry was saved from destruction by replacing Gros Michel bananas with resistant Cavendish bananas (Ploetz, 2005a). Cavendish bananas, however, now succumb to a new race (race 4) of the Fusarium wilt pathogen that is, once again, threatening sustainable cultivation of bananas globally (Ploetz, 2005b).

A clear understanding of disease resistance is essential to facilitate the breeding or development of Fusarium wilt resistance in banana. Since the introduction of Cavendish as a replacement dessert banana to the world, very few studies addressed the fundamentals of resistance to Fusarium wilt. In fact, the topic was arguably best summarised by Wardlaw (1961) and Stover (1962) more than 40 years ago. Much of what is known about Fusarium wilt resistance today is still based on the work of Beckman (1987). The objective of this review, therefore, is to summarise the literature related to resistance in banana to Fusarium. The review first recapitulates

information relevant to the Fusarium wilt pathogen, the host plant, and resistance responses in plants in general. It then discusses defence mechanisms in banana and other crops to Fusarium wilt pathogens, and considers how such resistance can be utilised to obtain disease resistance in susceptible banana varieties. Finally it proposes ways to study resistance mechanisms and resistance genes in banana to Fusarium wilt.

FUSARIUM WILT OF BANANA (PANAMA DISEASE)

Fusarium wilt or Panama disease of banana is regarded as one of the most destructive diseases of agricultural crops in the world (Wardlaw, 1961; Stover, 1962; Simmonds, 1966, Ploetz, 2005 a,b). The disease was first reported from Australia in 1874, but became renowned for the destruction it caused to export banana plantations in Central America during the first half of the 20th century (Jones, 2000). The Fusarium wilt pathogen was disseminated throughout the world in infected planting material, and today the disease occurs in all countries where banana is grown except the South Pacific Islands and some of the countries bordering the Mediterranean (Jeger *et al*., 1996).

Fusarium wilt of banana is caused by the soil-borne fungus *Fusarium oxysporum* Schlect. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*) (Stover and Waite, 1954). The fungus survives as immobile and dormant chlamydospores in decayed banana tissue and soil until it is stimulated to germinate by the host roots, root excretions from non-host roots, or contact with pieces of fresh non-colonized plant remains (Stover, 1962). The pathogen enters the plant through the root tips, moves through the xylem vessels and colonises the rhizome (Beckman, 1990). It then produces microconidia that invade the water-conducting tissue (xylem) of the pseudostem, plugging the vascular vessels and reducing the movement of water. Further spread of the conidia is hindered by sieve cells and, consequently, the spores germinate and grow through the sieve cells to continue their spread until the entire xylem system is blocked (Stover *et al*., 1961; Jeger *et al*., 1995). After the plant dies, the fungus grows into the surrounding tissue where they form chlamydospores that are released back into the soil when the plant decays (Jones, 2000).

Classical external symptoms of Fusarium wilt of banana appear as a yellowing of the leaf margins of older leaves (Moore *et al.*, 1995). As the disease progresses, the yellowing advances from the oldest to the youngest leaves. Leaves gradually collapse at the petiole or towards the base of the midrib and hang down to form a "skirt" of dead leaves around the pseudostem. A longitudinal splitting of the outer leaf-bases of the pseudostem is often observed just above soil level (Brandes, 1919; Wardlaw, 1961; Stover, 1962). Internal symptoms are characterised by the discoloration of vascular tissue. Discoloration begins with yellowing of the vascular tissue in the roots and corm, which then progresses to form continuous yellow, red or brown discoloured vascular strands in the pseudostem (Wardlaw, 1961). No disease symptoms have been observed in or on fruit. Susceptible banana plants infected with *Foc* rarely recover.

Three races of *Foc* cause Fusarium wilt to a set of differential banana cultivars (Stover and Buddenhagen 1986; Ploetz, 2005). The race that devastated almost 100 000 acres of Gros Michel bananas in Central America during the first half of the 1900's, is race 1. Race 1 also attacks cultivars of the AAB genotype, 'Silk' and 'Pome'. The outbreaks of Fusarium wilt in Gros Michel bananas were stopped by replacing them with Cavendish varieties, which are resistant to *Foc* race 1 (Ploetz, 2005). However, Cavendish bananas are attacked by race 4 of the pathogen in countries such as Taiwan, Canary Islands, Australia, South Africa, Philippines and Malaysia (Gowen, 1995; Bentley *et al.*, 1998). Strains of *Foc* that attack Cavendish bananas in the subtropics are referred to as 'subtropical' race 4, and those attacking Cavendish bananas in the tropics are referred to as 'tropical' race 4. Race 4 also attacks bananas susceptible to all other races of *Foc*. Another race of *Foc*, race 2, attacks Bluggoe and other closely related cooking bananas, while a fourth race, *Foc* race 3, was recently discarded as a race of *Foc* (Ploetz, 2005b). This race attacks *Heliconia* spp., a plant distantly related to *Musa*, and was found to be genetically distinct from *Foc* (Waite, 1963).

Numerous disease control strategies have been investigated for the control of Fusarium wilt. Of these, quarantine and exclusion practices have been successful in preventing pathogen dissemination by restricting the movement of infected corms, suckers and soil (Moore *et al.,* 1995). Flood fallowing, crop rotation and the use of organic amendments were unsuccessful in controlling the disease effectively (Moore

et al., 1995). Chemical control measures do not exist, and partial control of the pathogen has largely relied on the use of methyl bromide. However, fumigated areas were thoroughly reinvaded by the pathogen (Herbert and Marx, 1990), and the use of methyl bromide has now also been restricted in many countries. Biological control and the use of chemical or biological products to induce systemic resistance in plants have not been properly investigated. It is now generally accepted that an effective way of combating the disease is by the use of resistant genotypes (Jones, 2000).

THE HOST: BANANA

The banana plant is a large perennial monocotyledonous herb consisting of an underground stem (rhizome), a pseudostem and a terminal crown of leaves (Jones, 2000). Both the root system and the aerial parts arise from a sympodial rhizome (Gowen, 1995). The terminal growing point or meristem of the rhizome extends within the pseudostem and is eventually transformed into an inflorescence that forms the bunch (Robinson, 1996). The bunch consists of female, male and hermaphrodite flowers, of which only the female flowers develop into fruit. Most edible bananas are seedless due to female sterility and a lack of pollen due to triploidy (Robinson, 1996). Reproduction in banana, therefore, occurs in the form of vegetative propagation by the production of suckers, which are outgrowths of vegetative buds formed on the rhizome (Simmonds, 1959).

More than 1000 varieties of bananas have been identified in the world. The greatest diversity of bananas is found in Southeast Asia, while secondary centres of evolution include India and the east African Highlands (Simmonds, 1959). All bananas and plantains (cooking bananas) belong to the genus *Musa* (Family *Musaceae*, Order *Zingiberales*), which includes five sections, divided into 40 species. *Eumusa* is the largest and best known section and includes *M. acuminata* Colla and *M. balbisiana* Colla, which are the principal progenitors of most edible banana cultivars (Simmonds, 1959; Stover, 1962; Waite, 1963). Their origin is considered to be Southeast Asia for *M. accuminata* (genome AA) and the Indian subcontinent for *M. balbisiana* (genome BB) (Simmonds, 1966). *Musa acuminata* is generally considered to be sweet, and *M. balbisiana* starchy with resistance to biotic and abiotic stresses.

Inedible, seed-bearing diploids are still found in Southeast Asia and the western Pacific regions and are regarded as the ancestors of modern-day bananas and plantains (Stover, 1986; Ploetz and Pegg, 1997). Natural evolution over time has resulted in many inedible diploids crossing naturally and resulting in intraspecific hybrids (Buddenhagen, 1990; Robinson, 1996). To obtain seedless, edible bananas and plantains, early breeders crossed *M. acuminata* with *M. balbisiana* to produce diploid, triploid and tetraploid hybrids (Simmonds, 1966). A triploid banana consisting of one set of chromosomes from *M. acuminata* and two sets from *M. balbisiana* is referred to as ABB, and a tetraploid banana consisting of three sets of chromosomes from *M. acuminata* and one set from *M. balbisiana* is referred to as AAAB. Bananas in the Gros Michel and Cavendish groups are classified as AAA, signifying that they are triploid with all chromosomes coming from *M. acuminata*.

Edible bananas are divided into two major groups: cooking bananas and dessert bananas (Jones, 2000). Cooking bananas form 57% of the world banana production, and comprise a staple food that needs to be baked, boiled, fried or roasted before consumption (Jones, 2000). Plantains are one of the best-known cooking bananas that are produced by many small-scale farmers throughout the tropics as an important food, fibre and income source (Swennen, 1990). Dessert bananas are eaten raw when ripe, and account for the remaining 43% of the world's production. Bananas from the Cavendish subgroup are the most popular dessert bananas. The banana export market, consisting mainly of Cavendish bananas, constitutes only 13% of all bananas grown in the world. The rest are grown for local consumption in the tropics and subtropics (Jones, 2000).

Humans have been responsible for moving vegetative banana planting material (suckers) outside Asia and around the world. The mode and time of introduction of bananas to Africa is believed to be from India around the 1400s. They were then spread across the African continent from east to west (Simmonds, 1959; Robinson, 1996). The Portuguese carried the plant to the Canary Islands some time after 1402 and from there to the New World (Simmonds, 1959). Dessert and cooking varieties were introduced into the Americas from Southeast Asia before 1750 (Wardlaw, 1961). Gros Michel was first introduced into Panama before 1866, and with the

expanding export industry at the time, was distributed throughout the entire Central America (Stover, 1962). The Silk (ABB) variety was introduced into Australia before 1876 and the Gros Michel cultivar was introduced only around 1910 (Stover, 1962). This set the stage for the cultivation of bananas as a dessert and as a staple crop, around the world. Edible bananas are now cultivated in many tropical and subtropical regions of the world, including, Asia, Africa, South and Central America, Oceania and the Caribbean.

RESISTANCE IN PLANTS TO PATHOGENS

Terminology

A diverse range of organisms constantly challenge plants, but not all of them are able to cause disease. When an organism is able to invade and multiply within plants, they are referred to as **pathogens**. Sometimes pathogens can live on a susceptible host without causing any disease. In such case they are called **saprophytes**. If, however, conditions become favourable for infection and disease, they are called **parasites**.

The ability of a pathogen to cause disease to a host plant is often dependent on how a plant responds. If infection takes place with subsequent disease development, a plant is considered **susceptible** to the pathogen. Susceptibility may be caused by an inability of the plant to recognise the pathogen and/or produce an effective and rapid defence response. Such an interaction between plant and pathogen is termed a **compatible** interaction. If, however, plants are able to restrict pathogen multiplication or movement from the initial site of infection they are **resistant** (Dempsey and Klessig, 1995), and the interaction is **incompatible**. The speed and extent of the defence response often establishes whether a plant is resistant or susceptible (Lamb *et al*., 1989). **Tolerance/partial resistance** is the ability of a plant to sustain the effects of a disease without suffering serious yield losses and dying as a result of infection. Partial resistance is also known as field resistance. Some crops that are resistant comprise varieties that do not develop any disease, despite challenge by a known pathogen under favourable environmental conditions. Such varieties are then considered as **immune**. When an entire plant species is resistant to a pathogen, it is

called **non-host resistance** (Heath, 2000a; Nürnberger and Lipka, 2005). The prerequisite for successful invasion of a plant by a pathogen, therefore, is basic compatibility, where a potential pathogen has attained pathogenicity factors in co-evolution with the plant in order to overcome non-host resistance (Heath, 1981). Plants are able to produce or accumulate **stress metabolites** in response to stresses such as pathogen invasion, drought or heat shock. **Minor resistance gene** are present in all plants and play a role in non-specific plant defence through the production of phytoalexins, glucanases, chitinases, lignin, callose and enzymes for oxidative stress protection.

The Constitutive Defence Response

Constitutive (passive) resistance is due to the presence of preformed physical and chemical factors (Dangl and Jones, 2001). The cell wall in plants is the physical barrier that keeps most organisms from developing close contact with the plant (Johal *et al.*, 1995; Ride, 1992; Nürnberger and Lipka, 2005). Preformed physical factors also include the thickness or hardness of the cuticle, the amount and quality of wax that cover the epidermal cells (Dangl and Jones, 2001; Nürnberger and Lipka, 2005), the size and shape of stoma and the root pericycle (Keen, 1992). Early barriers of defence in plants include preformed secondary metabolites, peptides, protein inhibitors and proteins that play a role in the normal programme of growth and development, but may bring about resistance to various pathogens (Heath, 2000b; Dixon, 2001). Preformed chemical defence factors in plants are often also due to the presence of high concentrations of alkaloids and phenolics in cells, as well as the presence of prohibitins or phytoanticipins (Grayer & Kokubun, 2001). Phytoanticipins are low molecular weight compounds that are present in plants before challenge by micro-organisms or are produced after infection solely from pre-existing constituents (VanEtten *et al.*, 1994). Plants often also release fungitoxic exudates that inhibit the germination of spores (Agrios, 2004). If all these preformed plant weapons are not sufficient to stop pathogen invasion the plant activates inducible defences.

The Induced Defence Response

Induced (active) defence responses involve both physical and chemical factors (Agrios, 2004) that are activated upon pathogen attack. Inducible defence responses in non-host plants comprise the synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, and translation products from pathogenesis-related genes as well as the localized reinforcement of cell walls and hypersensitive, programmed cell death (Nürnberger and Lipka, 2005). Disease resistance in plants relies on complex mechanisms of molecular recognition and cellular signal transduction (Fig. 1).

Recognition

The ability of plants to invoke defence reactions is mediated by the initial recognition of pathogens by plants (Dixon *et al.*, 1994; Schenk *et al.*, 2000). The activation of inducible plant defence responses is brought about by the recognitions of pathogen associated molecular patterns (PAMP) (Nürnberger and Lipka, 2005). Once the plant has recognized an attacking pathogen, the race is on. The plant attempts to prevent infection and to minimize potential damage, while the pathogen attempts to gain access to nutrients for reproduction and growth (Schmelzer, 2002). Resistance in plants is innate owing to the presence of genes coding receptor-related or receptor-like proteins, which can either directly bind pathogen-derived molecules or represent constituents of larger signal perception protein complexes (Schmelzer, 2002). This results in a cascade of biochemical events in the plant that lead to the induction of defence responses (Keen, 1992; Dixon *et al.*, 1994; Baron and Zambryski, 1995). Plant cells are capable of defending themselves through a wide variety of mechanisms that can either be a local or systemic, constitutive or inducible response (Dixon, 1986; Keen, 1990; Ryals *et al.*, 1994).

Hypersensitive response

The hypersensitive response (HR) is regarded as one of the most frequently occurring defence responses in crop plants against pathogens (De Wit, 1992) and is an active process of the host and may be a form of programmed cell death (Greenberg and Yao,

2004). The HR is associated with a phenomenon termed the resistance response (RR) and is considered to be the cell death component of the RR (Greenberg and Yao, 2004). The RR involves the coordinate activation of several defence responses that limit pathogen growth in the host (Greenberg, 1997)

The HR occurs only in specific host-pathogen systems in which the plant and the pathogen are incompatible and the pathogen fails to infect the host (Agrios, 2004). This might be due to the presence of a resistance (*R*) gene in the plant, which recognizes and is triggered by the elicitor molecule produced by the pathogen avirulence (*Avr*) gene (Greenberg and Yao, 2004). The HR involves only single cells or very few cells, and results in rapid cell death around the penetration site that could be responsible for restricting pathogen growth (Tomiyama, 1982; Keen, 1992; Schenk *et al.*, 2000). According to Agrios (2004), an effective hypersensitive response is not always visible with the eye and may remain unnoticed when a plant remains resistant to pathogen attack. Ultrastructural analysis has revealed morphological events that occur during the HR including changes in mitochondrial morphology, membrane dysfunction and progressive vacuolization of the cytoplasm (Greenberg and Yao, 2004).

The HR is sufficient to restrict the growth of biotrophic and hemibiotrophic pathogens, which require nutrition from living plant cells for at least part of their infection cycle. However, to contain necrotrophs, the HR has to be supported by other defence mechanisms. In this case the early response is followed by a cascade of other defence responses (De Wit, 1992).

Mechanisms which accompany the HR include rapid burst of oxidative reactions, ion fluxes, especially K^+ and H^+, cellular decompartmentation and strengthening of cell walls through increased synthesis and deposition of callose, lignin, hydroxyproline-rich glycoproteins and phenolic compounds (Bowles, 1990; Agrios, 2004). Enzymes of the phenolic pathway such as peroxidases as well as glucanases and chitanases are also expressed (Dixon and Lamb, 1990). Resistance modulated by the HR is a cellular and tissue response and, therefore, the spatial and temporal expression of defence response genes are strategically important (Keen, 1992).

In addition to playing a role in limiting pathogen growth directly, the HR may have additional contributions, such as activating specific defences in neighbouring tissue. Such systemic signalling is important for protecting plants from future infections (Greenberg and Yao, 2004).

Oxidative burst

The HR has several rapid processes that involve the activation of pre-existing components rather than changes in gene expression. One of these processes is the release of reactive oxygen species (ROS) (Apostel *et al.*, 1989; Sutherland, 1991; Legendre *et al.*, 1993; Mehdy, 1994; Ebel and Scheel, 1997). The predominant species detected in plant-pathogen interactions are super oxide (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). These highly reactive oxygen radicals are thought to be released by the multi-subunit NADPH oxidase enzyme complex of the plant cell plasma membrane (Parker, 2000). They appear to be released in affected cells within seconds or minutes from contact of the plant cell with the pathogen or its elicitors (Bradley *et al.*, 1992; Agrios, 2004), and reach maximum activity within minutes to a few hours (Agrios 2004).

The oxidative burst leads to the cross-linking of cell wall proteins, rendering plant cell walls more resistant to attack by fungal enzymes (Bradley *et al.*, 1992; Keen, 1999). Researchers have reported that soybean and bean suspension cultures treated with fungal elicitors have shown an increase in lignifications and cross-linking of cell wall hydroxyproline-rich glycoproteins, responses linked to increased H_2O_2 levels (Bradley *et al.*, 1992; Brisson *et al.*, 1994). ROS have also been considered as signalling agents for the induction of hypersensitive cell death and additional active defence responses (Levine *et al.*, 1994; Alvarez *et al.*, 1998). The ROS may serve as second messengers for the activation of genes expressing protective proteins, such as glutathione peroxidase, glutathione S-transferase and polyubiquitin, as well as for enzymes involved in scavenging ROS (Lamb and Dixon, 1997). The highly reactive oxidants, O_2^- and OH may exert potent antimicrobial activity and contribute to lipid peroxidation of both plant and pathogen membranes (Vera-Estrella *et al.*, 1994; Parker, 2000). ROS are also said to be toxic to pathogens (Peng and Kúc, 1992; Mehdy, 1994). Peng and Kúc (1992) showed that spore germination for a number of

different fungal pathogens was inhibited by micromolar concentrations of H_2O_2. ROS has further been connected with the production of phytoalexins (Mehdy, 1994; Lamb and Dixon, 1997), but there may be a ROS-independent phytoalexin synthesis pathway (Mithöfer *et al*., 1997).

Ion fluxes

Changes in ion fluxes and membrane depolarisation in plant cells occur within minutes in response to pathogen attack (Zimmermann *et al.*, 1997). Ion fluxes are caused by changes in the permeability of the plasma membrane and *R* gene- and receptor-mediated regulation of the ion channels within the plasma membrane, resulting in calcium (Ca^{2+}) and proton (H^+) influx and potassium (K^+) and chloride (Cl^-) efflux (Scheel, 1998; Nürnberger and Scheel, 2001). It appears that ion fluxes are necessary in most plant-pathogen interactions for the induction of the oxidative burst and defence gene activation (Scheel, 1998).

Cell wall strengthening and vascular occlusion

In several plant diseases the cell walls of the host are able to produce, modify or accumulate defence-related substances that reinforce the walls, resulting in enhanced resistance. Among the substances produced or deposited by plant cell walls in reaction to infection are callose, glycoproteins, amino acid hydroxyproline, phenolic compounds such as lignins and suberin, and mineral elements such as silicon and calcium (Agrios, 2004). Many of these substances react and cross-link with one another to form more insoluble cell wall structures that confine the pathogen. Plant cells also respond to invading pathogens by producing vascular occlusions such as tyloses (Agrios, 2004) and gels (Mace, 1963). They serve to cut off the transpiration stream in the xylem, thereby immobilising the pathogen. Tyloses and gels are infused with stress metabolites and become lignified and highly resistant to physical and chemical degradation (Mace, 1963).

Lignification

Lignification is one of many biochemical events resulting in an ultra-structurally modified reinforced cell wall (Walter, 1992). Lignin provides rigidity and mechanical support to plant tissue, waterproofs xylem elements and allows for defence strategies against pathogen attack. It is commonly formed in response to microbial penetration and mechanical damage (Wardrop, 1971), and is resistant to degradation by most micro-organisms (Kirk, 1971; Kirk *et al*., 1979). Lignin is a matrix polymer enclosing cellulosic fibrilic material that restricts the diffusion of enzymes and toxins from the fungus to the plant, and of nutrients and water from the plant to the fungus, thereby starving the fungus (Ride, 1978).

Lignification follows the elicitor-receptor theory. Vance *et al.* (1980) proposed that more than one elicitor might interact with a single receptor and that two or more receptors might independently activate a resistance mechanism. Low molecular weight lignin precursors are produced by the plant and could act as elicitors. These lignin precursors are possibly able to react with fungal walls and membrane polymers to activate a recognition system, or to block the activation by other compounds (Vance *et al*., 1980). Lignin and lignin-like polymers of host plants have indefinite structure and configuration and are unlikely candidates for receptors. There is no evidence that minor changes in lignin molecules lead to major property changes. Unlike other cell wall proteins and saccharides, lignin is not a preformed constituent of epidermal cells prior to initial pathogen penetration (Vance *et al*., 1980).

Callose deposition

Callose deposition is known to be a very rapid and localized event in response to mechanical injury or pathogen invasion (Bowles, 1990). In many host-pathogen interactions callose appears to be locally deposited directly from the plasma membrane onto the adjacent cell wall (Aist, 1976). Localised deposition of callose may be important in the first line of defence against pathogens. Callose is also a major component of papillae or cell wall appositions which are formed at the sites of attempted penetration by invading fungal hyphae (Aist, 1976; Bell, 1981).

Callose is a polysaccharide containing a high proportion of 1, 3 - β-linked glucose. Callose synthetase or 1, 3 - β-glucan synthase (GSII) catalyses the formation of

callose, and this protein is a functional component of the plasma membrane (Bowles, 1990). GSII is abundant in plant homogenates but is latent in healthy living cells. Callose synthesis can be initiated in a Ca^{2+}-dependent or in a Ca^{2+}- independent manner. Localised deposition of callose may start when Ca^{2+} influx increases and activates the GSII (Bowles, 1990). Callose deposition can also be caused by polycations such as chitosan and poly-L-lysine (Köhle *et al.*, 1985). These elicitors were shown to interfere with the negatively charged phospholipids of the plasma membrane, generating a local Ca^{2+} influx into cells, K^+ efflux and an external alkalisation of the medium, followed by callose formation (Köhle *et al.*, 1985; Waldmann *et al.*, 1988). In the absence of Ca^{2+}, callose synthase can be activated by polyamines, ruthenium red, trypsinization (Kauss *et al.*, 1990) and by phospholipids.

Phenolic compounds

Antibiotic phenols have been found in all plants that have been investigated (Nicholson and Hammerschmidt, 1992). Specialized plant cells synthesize phenolics and store them in their vacuoles during normal processes of differentiation. These phenolic-storing cells are distributed within most plant tissues, either uniformly in all cells or randomly scattered or strategically located at potential points of entry (Beckman, 2000). During injury or infection, phenolic-storing cells burst with a chemical reaction that oxidizes the plant phenolics. This then serves to lignify and/or suberize the site of infection.

One of the main roles of phenolics in plants is to protect them against diseases caused by bacteria, fungi and viruses (Swain *et al.*, 1979). Some phenols occur constitutively and function as preformed inhibitors associated with non-host resistance (Mansfield, 1983; Nicholson and Hammerschmidt, 1992). Others are formed in response to pathogen invasion and are considered to form part of the active induced defence response (Nicholson and Hammerschmidt, 1992). The rapid accumulation of phenols may result in the effective isolation of the pathogen at the original penetration site (Friend, 1981; Ride 1983). Phenolics also serve as signalling molecules during vascular defence (Wink, 1997).

Many phenolic compounds have anti-microbial activity *in vitro* (Friend, 1981). Certain common phenolic compounds, such as chlorogenic acid, caffeic acid and ferulic acid, are toxic to pathogens, and are produced and accumulate at a faster tempo after infection, especially in resistant plants compared to susceptible plants (Agrios, 2004). Walker and Link (1935) showed that catechol and protocatechuic acid had a toxic effect on onion parasites. Although individual phenolics may reach toxic concentrations, several of them appear concurrently in the same infected tissue, and it is possible that their combined fungitoxic effect is responsible for the inhibition of pathogen infection in resistant plants (Agrios, 2004).

Indole acetic acid

Indole acetic acid (IAA) builds up rapidly in plants when decompartmentation occurs (Beckman, 2000). Gordon and Paleg (1961) made the discovery that phenolics mediated the conversion of tryptophan to IAA (3-indoleacetic acid). Two processes cause the level of IAA to raise sharply in the affected xylem tissues. Firstly, the release of flavenols inhibits the ATPase pumps that are responsible for downward transport of IAA, thus causing accumulation of IAA above the infection point (Lomax *et al*., 1995; Normanly *et al*., 1995). Secondly, oxidized phenolics have been shown to inhibit the destructive oxidation of IAA, therefore permitting a severe build-up of IAA (Sequeira, 1964). This type of build-up in the host plant has been demonstrated to occur in infected vascular tissues of tomatoes infected with *Fusarium* (Matta, 1970).

The action of IAA in affected tissue is indicated by an oxidative burst (Lamb and Dixon, 1997), the activation of H^+ pumps, and a drop in apoplastic pH (Cleland, 1987; Grignon and Sentenac, 1991). Beckman (1969) demonstrated such a drop in pH in the walls of banana parenchyma cells that were in contact with vessels infected with *Fusarium*. Studies on cotton infected with *Verticillium* indicated that there is a dramatic shift in IAA/cytokinin balance in infected vascular tissues at and around the site of perturbation (Misaghi *et al*., 1972). According to Fosket (1994) and Kaufman and Song (1987) this kind of shift in hormone balance in plants was shown to promote lateral growth of cells in the affected area. The treatment of banana roots with IAA promotes lateral growth in adjacent paravascular parenchyma cells to form tyloses in

the lumina of vessels (Mace and Solit, 1966), and Beckman (1987) showed that an oxidative burst occurs in plant cells during this period.

Other Defence Responses

The expression of defence-related genes such as those involved in lignin and suberin pathways, those for signal transduction proteins, pathogenesis related (PR) proteins as well as proteins that are necessary for the accumulation of phytoalexins and phenyl propanoids, is induced concurrently or downstream of the HR (Reymond and Farmer, 1998; Greenberg and Yao, 2004). Defence responses, such as the production of PR proteins (1, 3 β-glucanases and chitanases), can be induced many hours or even days after infection (Lamb *et al.*, 1989; Bowles, 1990).

Pathogenesis-related proteins (PR proteins)

PR proteins have been identified in many monocots and dicots and appear to be ubiquitous in higher plants (Cutt and Klessig, 1992). They can be induced in response to a diverse range of pathogens and biotic and abiotic elicitors (Bowles, 1990). PR proteins are induced both locally and around the infection site and systemically away from the initial infection site. Some PR proteins inhibit spore release and germination, while others are associated with strengthening of the plant cell wall and its outgrowths and papillae (Agrios, 2004). PR proteins are either extremely acidic or extremely basic and are, therefore, highly soluble and reactive (Agrios, 2004). They are grouped into families based on acid solubility and their resistance to proteinases (Bowles, 1990). Currently there are 11 recognised groups of PR proteins (Van Loon *et al.*, 1994; Koiwa *et al.*, 1997; Van Loon, 1997). The better-known PR proteins are PR-1 proteins, β-1,3-glucanases (PR-2), osmotin-like proteins, thaumatin-like proteins (PR-5), glycine and cysteine-rich proteins, proteinase inhibitors (PR-6), proteinases (PR-7), chitosanases (PR-8) and peroxidases (PR-9) (Agrios, 2004).

Peroxidases

The induction of plant peroxidases appears to be an early event in plant-microbe interactions (Cook *et al.*, 1995; Harrison *et al.*, 1995). Peroxidases are haem-containing proteins that catalyse the reduction of hydroperoxides, especially hydrogen peroxide, to water. Most higher plants possess a number of different isoenzymes and at least 12 distinguishable isoenzymes that fall into three sub-groups have been characterised from tobacco: the anionic (pI 3.5-4.0), moderately anionic (pI 4.5-6.5) and the cationic (pI 8.1-11) isoenzymes. Peroxidase isoenzyme expression is tissue-specific, developmentally regulated and influenced by environmental factors (Lagrimini *et al.*, 1987). Each group is thought to serve a different function in the cell.

Peroxidases are involved in several plant defence responses including lignification (Walter, 1992), suberization and wound-healing (Sherf *et al.*, 1993) as well as in the production of antimicrobial radicals (Peng and Kúc, 1992; Kobayashi *et al.*, 1994). The function of highly anionic peroxidase isoenzymes is understood best. This group is cell wall-associated and has high activity for the polymerization of cinnamyl alcohols in *vitro* (Mäder, 1986). They function in lignification and the cross-linking of cellulose, pectin, hydroxy-proline-rich glycoproteins and lignin during secondary cell wall formation (Lagrimini *et al.*, 1987). Anionic peroxidases show a high affinity towards cinnamylalcohols *in vitro*, but can probably also cross-link extensin monomers and feruloylated polysaccharides (Walter, 1992). They may also function in suberization or wound healing by forming water-tight barriers over the wound (Espelie *et al.*, 1986). The moderately anionic peroxidases are localized in the cell walls, only have moderate activity towards lignin precursors, and may be involved in suberization and wound healing (Lagrimini and Rothstein, 1987; Walter, 1992). The cationic isoenzymes efficiently catalyse the synthesis of H_2O_2 from NADH and H_2O and have been localized to the central vacuole (Mäder, 1986). It is speculated that this group of isoenzymes regulates auxin levels, form ethylene from 1-aminocyclopropane-1-carboxylic acid and provides H_2O_2 for other peroxidase isoenzymes (Lagrimini *et al.*, 1987). Peroxidases are also involved in other processes, such as the inactivation of host and pathogen enzymes by oxidized phenolics (Matern and Kneusel, 1988).

Phytoalexins

Phytoalexins are toxic antimicrobial substances produced in appreciable amounts in plants after stimulation by phytopathogenic micro-organisms, or by chemical or mechanical injury (Agrios, 2004). They are thought to be important components of the active plant defence response, despite the absence of genetic proof using plant mutants (Keen, 1999). Phytoalexins accumulate in healthy plant cells surrounding wounded or infected cells and are stimulated by substances diffusing from damaged cells into the adjacent healthy tissue (Agrios, 2004). Resistance occurs when one or a few phytoalexins reach a concentration sufficient to restrict pathogen development. Most phytoalexins are produced in response to fungal infection and inhibit the growth of the fungus, but some are also toxic to nematodes and bacteria.

Signal Transduction

In most eukaryotic organisms pathogen recognition and defence responses are linked by means of signal transduction cascades (Nürnberger and Scheel, 2001). Plant tissues acquire resistance by relying on transmissible signal molecules that, at low concentration, can activate resistance mechanisms in cells not directly invaded by the pathogen (Ross, 1961; Schenk *et al.* 2000). Molecules are classified as signal molecules if they are synthesised in the plant, increase systemically following pathogen attack, move throughout the plant, induce defence-related proteins and phytochemicals and if they enhance resistance in the plant against pathogens.

The events following pathogen recognition are still poorly understood. Downstream pathways seem to vary for different host plants, resistance genes, elicitors and pathogens (Bent, 1996; Ebel and Mïthofer, 1998; Glazebrook, 2001). Consequently, general models are unable to explain signal transduction and resistance for every plant-pathogen interaction. Despite the complexity and diversity of plant-pathogen interactions, signalling events and resistance mechanisms for many similar plant responses have been identified.

During early signal transduction events, cells in and around the recognition site experience large ion fluxes and undergo changes in lipid oxidation, protein phosphorylation, and concentration and accumulation of ROS (Dixon *et al*., 1994; Hammond-Kosack and Jones, 1996; Ebel and Mïthofer, 1998; Holt *et al*., 2000). Different kinases are involved downstream of pathogen recognition in different plant-pathogen systems. These include receptor-like kinases, protein kinases, calcium-dependant protein kinase (CDPK's) and mitogen-activated protein kinases (MAPKs) (Fig. 1) (Torii, 2000; Guillaume *et al*., 2001; Romeis, 2001; Asai *et al*., 2002).

Following the early defence responses and subsequent biochemical and signalling alteration within the infected plant cells, a second signalling wave induces specific defence responses. These defence responses are induced either by positive feedback loops or signal cross-talk and influence the metabolic flow or activity of housekeeping genes to ensure high levels of required precursor compounds (Hammond-Kosack and Jones, 1996). The induction of defence-related genes seems to be regulated by a small number of signalling pathways that are dependent on low molecular mass regulators. Salicylic acid (SA), jasmonic acid (JA), ethylene (ET), possibly hydrogen peroxide (H_2O_2) and nitric oxide (NO) play a role in cross-talk and convergence points between pathways (Reymond and Farmer, 1998; Kumar and Klessig, 2000).

Salicylic acid

SA, a phenolic signalling molecule, has been shown to play a central role as a signalling molecule involved in efficient generation of the local HR (Gaffney *et al*., 1993), local acquired resistance (LAR) and systemic acquired resistance (SAR) (Chamnongpol *et al*., 1998; Reymond and Farmer, 1998). An intricate relationship between SA and cell death has been discovered, indicating that SA is both required for and induced by cell death (Nawrath and Métraux, 2002). Whether or not SA acts as the primary systemic signal for SAR, however, is still under debate. Still, SA accumulation and activity almost invariably precede the expression of *PR-1* transcripts (Cao *et al*., 1994; Delaney *et al*., 1994). Little progress has been made in characterising the SA biosynthesis pathway itself, although a number of experiments have demonstrated that SA is synthesized from phenylalanine via cinnamic and benzoic acid (Lee *et al*., 1995; Coquoz *et al*., 1998). Additionally, oxidative stress

caused by ultraviolet light or ozone also triggers SA biosynthesis (Yalpani *et al.*, 1994).

Jasmonic acid

The signalling molecule JA and its counterpart methyl jasmonate (MeJA) are involved in several plant biological aspects such as pollen and seed development, fruit ripening, tuber formation and defence reactions against wounding, insects and microbial pathogens (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Li *et al.*, 2001; Lorenzo and Solano, 2005). JAs are fatty acid derivatives with a 12-carbon backbone and are involved in basal resistance against specific pathogens (Pieterse *et al.*, 2002). JA and MeJA accumulation is associated with lipid oxidation mediated by either ROS or by lipoxygenases and are, therefore, part of the lipid-based intracellular signalling pathway (Farmer and Ryan, 1992; Ebel and Mithofer, 1998). JA signalling is important in limiting the growth of certain pathogens, such as *Alternaria* spp. and *Pythium* spp. and has systemic effects resulting in gene expression throughout the plant (Glazebrook, 1999). JA and ET-mediated defence responses are associated with induced expression of an antifungal plant defensin (*PDF1.2*), *PR3* and *PR4* (Penninckx *et al.*, 1996; 1998) and a thionin (*Thi2.1*) (Epple *et al.*, 1997). JA is also involved in induced systemic resistance (ISR) against pathogen infection in plants first challenged with a non pathogenic, root colonizing bacterium, *Pseudomonas fluorescens* Migula (Pieterse *et al.*, 1998).

Ethylene

ET is involved in many plant development processes such as root hair development, root nodulation, seed germination, flower senescence and fruit ripening, but has also been implicated in the defence response to pathogen attack and abiotic stresses, including wounding, ozone, chilling and freezing (Johnson and Ecker, 1998). Five ethylene receptors (ETR 1, ETR 2, ERS 1, ERS 2 and EIN 4) have been identified and were shown to play a role in ET signalling (Solano and Ecker, 1998). Additionally, the *Ctr1* gene encoding for a serine/threonine protein kinase is required for ET signalling. ET may play a very different role in disease resistance, depending on the

type of pathogen and the plant species (Thomma *et al*., 1999). It is involved in symptom inhibition and resistance after infection of plants by necrotrophic pathogens, but enhances cell death caused by other pathogens (Wang *et al*., 2002).

Interaction between the SA, JA and ET pathways

Many stress responses in plants require the coordinated interaction of signalling pathways such as JA, ET, SA and abscisic acid (Lorenzo and Solano, 2005). Little is known about how plants integrate signals that are generated by different inducers of resistance, into specific defence responses (Pieterse *et al*., 2002). A well-accepted hypothesis is that this might be accomplished by the modulation of different signalling pathways (Fig. 2). Despite the fact that the SA-dependent and JA-ET-dependent pathways induce the expression of different *PR* genes and also confer resistance to different pathogens, there is ample evidence that they share common pathways and interact with each other, either positively or negatively (Feys and Parker, 2000; Schenk *et al.*, 2000 Ton *et al*., 2002; Wang *et al*., 2002; Lorenzo and Solano, 2005). Pieterse *et al*. (1998) identified a convergence point between different pathways in NPR1, which is required for both SA-dependent SAR and JA-ET-dependent ISR. The gene product SSI1 (suppressor of SA insensitivity) possibly acts as a switch in the cross-talk between the SA- and JA-ET-resistance signalling pathways (Shah *et al*., 1999). JA and ET can either cooperate or act as antagonists in the regulation of defferent stress responses such as pathogen attack and wounding (Lorenzo and Solano, 2005). ET and JA also act synergistically in the wound-response and stimulate the biosynthesis of each other (O'Donnell *et al*., 1996). This so-called cross-talk between pathways provides a regulatory potential for activating multiple resistance mechanisms in varying combinations. This enables the plant to prioritise the activation of a specific defence pathway over another, thereby providing optimal defence strategies against the invading pathogen (Pieterse *et al*., 2002).

Systemic resistance

Systemic resistance (SR) is the activation of defence responses in tissues far removed from the initial site of pathogen attack or wounding (Ryals *et al*., 1994; 1996; Maleck

and Dietrich, 1999). This is due to the expression of a characteristic set of defence-related proteins that are induced systemically throughout the entire plant (Schmelzer, 2002). A number of genes associated with SR have been characterised as ones coding for β-1,3 glucanases, chitinases and thaumatin-like proteins (Bowles, 1990; Ward *et al*., 1991; Ryals *et al*., 1994). Linthorst (1991) also confirmed that at least five families of PR proteins have been associated with SAR.

It is hypothesised that the HR and other pathogen-induced local necrosis trigger SR (Kessmann *et al*., 1994; Greenberg and Yao, 2004), and that SR targets the majority of fungal, bacterial and viral pathogens. Underlying the systemic response must be a long distance signal transduction network which is capable of initiating a complex set of coordinated events resulting in a broad spectrum defence barrier (Cutt and Klessig, 1992). It has been reported that the accumulation of SA in local and systemic plant tissues contributes to SR (Malamy *et al*., 1990; Métraux *et al*., 1990; Rasmussen *et al*., 1991).

Certain micro-organisms can protect plants by inducing SR to diseases (Kúc, 1982; Matta, 1989). Dependent on the organism and signalling pathway involved, either SAR or ISR is obtained. SAR to pathogenic forms of *F. oxysporum* has been induced by using non-pathogenic strains (Mandeel and Baker, 1991; Hervas *et al*., 1995) and formae speciales of *F. oxysporum* (Gessler and Kúc, 1982; Kroon *et al*., 1991). In 1997, a non-pathogenic *F. oxysporum* strain (Fo47) was shown to induce resistance to Fusarium wilt in tomato (Fuchs *et al*., 1999). Isolates of the rhizobacterium *Pseudomonas fluorescens* systemically induced resistance against Fusarium wilt of chickpea caused by *F. oxysporum* f.sp. *ciceri* and significantly reduced the wilt disease by 20-50% compared to the control (Saikia *et al*., 2003). ISR requires JA and ET and is independent of SA accumulation (Pieterse *et al*., 1996; van Wees *et al*., 1997). *PR-3*, *PR-4*, *Thi2.1* and *PDF1.2* are the local and systemic marker genes of ISR activation (Thomma *et al*., 1998). ISR also appears to provide protection against pathogens such as *B. cinerea* and *Alternaria brassicicola*, for which SAR is ineffective (Thomma *et al*., 1998). Evidence exists for overlap between the mechanisms regulating resistance in ISR and SAR (Barker, 2000).

COMPONENTS OF PLANT DEFENCE MECHANISMS AGAINST FUSARIUM WILT

Each plant cell has acquired the capability to respond to infection and must possess a preformed and/or inducible defence strategy (Hammond-Kosack and Jones, 1997; Ebel and Mithöfer, 1998). These defence strategies consist of structural or biochemical defence systems.

Structural Defence

The epidermis, of young roots, forms the first constitutively formed structural barrier to fungal invasion. The endodermis and later the phellem surrounding the vascular tissues further form a particularly strong barrier to colonization (Baayen, 1987). A study by Brammall and Higgins (1988) shed light on constitutive defence responses in tomato against *F. oxysporum lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. (*Fol*). Results suggested that the hypodermis was an important constitutive barrier hindering colonization of the inner cortex of the tomato. It is generally accepted that Fusarium wilt infection of bananas takes place though the roots. Evidence form one study has shown that root injury and cutting, to exposes the xylem, is a major factor in the infection of banana roots (Sequeira *et al.*, 1958).

Plants are able to respond to pathogens by forming physical barriers that enclose and exclude them from nutrient access (Schmelzer, 2002). These responses are induced when the plant recognises a structural and/or chemical feature of the pathogen, or the damage associated with pathogen invasion. The depositioning of substances like suberin, hydroxyproline-rich glycoproteins, callose and many mono- or polymeric phenolic materials, on or into the cell wall all contribute to barrier formation (Walter, 1992). A typical example is the local apposition of material to the cell wall, resulting in a massive plug, the papilla.

Banana tissue responds to *Foc* conidial uptake in the xylem by forming vascular occluding gels 24-48 hrs after inoculation (Beckman, 1987). Light microscopy studies indicated that gels might arise from perforation plates, end-walls and side-

walls of vessels (Beckman and Zaroogian, 1967). These gels are produced just above the spore-trapping sites to cut off the transpiration stream and immobilise the secondary spores of the pathogen. After 2-3 days, the vascular parenchyma cells form tyloses that block the lumena of infected vessels. The walls of tyloses also become infused with stress metabolites so that the gel and tylose occlusion is lignified and highly resistant to physical and chemical degradation. These gels and coatings may contribute to the failure of the water transport system, thus contributing to the typical wilt symptoms.

Vascular occlusion represents a general, non-specific defence response that is the norm in both susceptible and resistant hosts. They persist for several days in resistant reactions, during which time they become infused with phenolics at the infection-response interface (Mace, 1963; Beckman and Talboys, 1981). In susceptible plant-pathogen interactions, gels seem to weaken and shear, thereby failing to stop the advance of the pathogen (Vander Molen *et al*., 1977; 1987). Only, when a virulent pathogen was introduced into a susceptible host at the optimum temperature for disease development did the defence response become disrupted and permit extensive systemic colonisation (Beckman, 1987).

In carnation infected with *F. oxysporum* f.sp. *dianthi* (Prill. & Delacr.) (*Fod*), microscopy studies of stems and roots have revealed constitutive and induced structural defence responses. The epidermis, endodermis and later the phellem surrounding the vascular tissues formed constitutive structural barriers to fungal invasion (Baayen, 1987). Higuera and Ebrahim-Nesbat (1999) showed that *Fod* is able to colonize both the susceptible and resistant carnation cultivars. Carnation stems of resistant varieties responded with vascular gelation and hyperplasia of xylem parenchyma bordering infection sites, while susceptible varieties were intensively colonized by the pathogen (Baayen, 1988). Baayen *et al.* (1989) also described occlusion of infected root xylem vessels with gums and lignification responses in the xylem parenchyma. Vascular plugs, vessel coatings, callose deposits and phenolic compounds were observed in resistant pea plants when infected with *F. oxysporum* f.sp. *pisi* (Lindf.) (Tessier *et al*., 1990).

The hypodermis of tomato serves as an important constitutive barrier to colonization of the inner cortex by *Fol* (Brammall and Higgins, 1988). Histochemical studies of tomato parenchyma infected by *Fol* provided evidence that deposits of callose-containing substances were laid down in response to infection and that they later became lignified (Mueller and Beckman 1988; Beckman *et al*., 1989). The formation of papillae is also induced as soon as the pathogen is able to penetrate the preformed defence barrier, the hypodermis. Papillae may protect the hypodermal protoplast of tomato from both physical and chemical contact with *Fol* (Brammal and Higgins, 1988). Other features of great significance involved deposition of phenolics, lignins and nonlamellar suberin in the cortical cells. The authors concluded that phenolic structural defence barriers (papillae and modified cortical cell walls) appear to be important in limiting *Fol* colonization in tomato cultivars possessing single dominant gene resistance to this disease.

Biochemical Defence

The success of plant resistance is dependent on the rate and extent of the host response. Resistance responses in banana are based on the ability of tolerant or resistant plants to produce phenolics, deposit lignin, and increase enzymes involved in cell wall strengthening (Beckman, 1990; De Ascensao and Dubery, 2000). Phenolic compounds play an important role in the resistance process against vascular diseases and are present in the xylem tissue of banana, cotton and potato (Beckman, 2000). The role of phenolics is the inactivation of hydrolytic enzymes of the pathogen and/or their incorporation into fungal and host cell walls and vascular gels to lignify them (MacHardy and Beckman, 1981; Beckman, 1987). By inhibiting hydrolytic enzymes and lignifying these structures, phenolics lock up the infection site physically and isolate the pathogen.

Enzymes that are important in the formation of phenolics are phenol-oxidizing enzymes such as peroxidases and polyphenol oxidases, which are associated with many different vascular diseases (Pegg, 1985). Peroxidases and polyphenol oxidases are stored and preformed in various localised sites in banana and cotton (Mace and Wilson, 1964; Mueller and Beckman, 1974; Mueller and Beckman, 1978). Different

isoforms of these enzymes are known to differ among different banana genotypes (Jarret and Litz, 1986). The levels and number of peroxidase isozymes produced are greatest in the roots of banana and it has been postulated that they may play a role in protecting the plant against infection by root pathogens (Bonner *et al.*, 1974; Lagrimini and Rothstein, 1987). Novak (1992) cited unpublished work by Morpurgo that indicated that constitutive levels of peroxidase were present in a resistant hybrid banana. SH-3362, a *Foc* race 4 resistant, synthetic AAA hybrid produced at FHIA, had peroxidase levels 10-fold higher than in Pisang Mas, a susceptible AA cultivar.

The biochemical basis of tolerance in banana to Fusarium wilt was investigated by De Ascensao and Dubery (2000) using fungal elicitors from the mycelial cell walls of *Foc*. Root tissue of the tolerant hybrid FHIA-01 (AAAB) responded to the fungal elicitor through strong deposition of lignin, preceded by the induction or activation of the enzyme activities involved in the synthesis and polymerisation thereof, whereas only slight increases were observed in the susceptible cv. Williams. Phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO) activity were all induced at higher levels in the resistant cultivar. The production of stress metabolites by host plants also helps to inhibit *Foc*, and the release of PR proteins (β-1-3 glucanase and chitinase) may serve to destroy the pathogen (Pegg and Young, 1982).

Responses in cotton to *F. oxysporum* f. sp. *vasinfectum* (Atk.) include the possible biosynthesis of secondary metabolites such as lignan, syringyl lignin polymers, and terpenoid indole alkaloids in the hypocotyls (Dowd *et al.*, 2004). High syringyl content has been associated with pathogen defence response in wheat (Ride, 1975) and lignans are known to possess antifungal activity (Davin and Lewis, 1992). In roots, however, genes associated with tannin, anthocyanin and lignin biosynthesis were repressed by the pathogen (Dowd *et al.*, 2004). The importance of the preformed steroidal glycoalkaloid, tomatine, as an important resistance factor in tomato against *Fusarium* infection has been demonstrated by De Fago *et al.* (1983).

IMPROVEMENT OF BANANA FOR FUSARIUM WILT RESISTANCE

Natural sources of resistance are present in wild banana species, some cultivars and in synthetic diploids developed by breeding programs (Jeger *et al*., 1995). *Foc* infects both resistant and tolerant cultivars, and host resistance is only expressed after infection (Beckman, 1987; Beckman, 1990).

Genes Associated with Resistance to Fusarium Wilt

At least three different classes of genes play a role in the defence strategy of a plant to disease. The first class comprises genes for constitutive (passive) defence and is not directly involved in defence responses, but may play a role in plant resistance by inhibiting pathogen entry by, for example, forming a thick waxy cuticular layer that protects against penetration. The second class contains genes that serve in the non-specific plant defence, for example the production of phytoalexins, glucanases, chitinases, lignin, callose and enzymes for oxidative stress protection. In addition to antimicrobial secondary metabolites, genes also coding for thionins, glutathionine S-transferases, lipoxygenases and phenylalanine ammonia-lyase (PAL) are induced (Glazebrook *et al*., 1997). The third class is required for race-specific resistance and comprises genes such as *R* genes and results in the arrest of pathogen growth (Jørgensen, 1994).

Genetic analysis of host-pathogen interactions has shown that there are gene-for-gene interactions between the products of resistance (*R*) alleles and corresponding avirulence (*Avr*) alleles in the pathogen that lead to recognition and resistance (Flor, 1971). Plant *R* genes confer the capacity to recognize invasion by specific races of pathogens and to induce defence responses such as the HR (De Wit, 1992). However, plants are also able to activate several general defence responses that are under the control of a set of genes known as minor resistance genes (Dong, 1998). Major *R* genes confer immunity to the plant against specific pathogens, while the minor genes are linked to tolerance, which is a more general response that remains even though the pathogen might mutate.

R genes are grouped into six major classes, based on DNA sequence information (Jones, 2001). These classes include the following: Leucine-rich-repeat (LRR) kinase, extracellular (e) LRRs, *Pseudomonas* tomato resistance (*Pto*), Toll and Interleukin-1 receptor (TIR): nucleotide-binding (NB): LRR, coiled coil (CC): NB: LLR and signal anchor (SA):CC. The majority of *R* genes encode proteins containing a NB site and LRRs. R proteins are structurally similar and determine the recognition of a diversity of AVR proteins (Nimchuk *et al*., 2001). There are *Avr* genes in races of a pathogen for every *R* gene, defining resistance in the host plant (Baron and Zambryski, 1995; Staskawicz *et al*., 1995; Hammond-Kosack and Jones, 1997; Heath, 2000a; Agrios, 2004). When *avr* genes are lost or modified, the pathogen becomes virulent and causes disease again (Baron and Zambryski, 1995).

A gene-for-gene relationship has been proposed for the interaction between *F. oxysporum* races and plant host cultivars (Ori *et al*., 1997). This gene-for-gene relationship was confirmed for the *Fol* / tomato interaction, where the tomato resistance gene *I-2* confers resistance to race 2 of the pathogen (Ori *et al*., 1997; Mes *et al*., 2000). However, gene-for-gene interactions could not be demonstrated for the *Fol* race 1 / tomato interaction. It is apparent from both the studies of Beckman *et al.* (1982) and Beckman *et al.* (1989) that cells of the cultivar having either the homozygous recessive (ii) or the homozygous dominant (II) gene condition are capable of responding more strongly to non-pathogenic root flora than to *Fol* race 1. It therefore seems likely that the “I” gene does not directly provide for the synthesis and deposition of callose, but is rather involved in the regulation of the process through recognition, transduction or modulation.

A gene expression study on the interaction between susceptible cotton and *F. oxysporum* f. sp. *vasinfectum* demonstrated that defence-related genes were induced in the hypocotyls in contrast to being constitutively expressed in the root tissue (Dowd *et al*., 2004). Known defence genes identified in this study include two classes of *PR10* (cotton *PR10* and ribonuclease-like *PR10*), *PR5* (thaumatin), *PR2* (glucanase), *PR3* (chitinase, class I and IV) and cotton phytoalexin (gossypol) biosynthesis genes, such as those encoding for ATP citrase lyase b-subunit, Acetyl CoA C-acyltransferase, HMG CoA reductase mevalonate disphosphate decarboxylate and (+)-Δ-cadinene synthase.

In the banana/*Foc* interaction races have not been well defined (Ploetz, 1994) and should, therefore, not be confused with races in other pathosystems for which host genes for resistance and susceptibility are known (Stover and Buddenhagen, 1986). The current race structure is based on field evaluations of a limited number of banana cultivars and disease development is significantly influenced by climatic conditions. Different climatic conditions determine the disease development in Cavendish bananas caused by *Foc* in the tropics and sub-tropics (Ploetz *et al.*, 1990). 'Tropical' strains of *Foc* belong to VCG 01213/16, while 'subtropical' strains belong to VCG 0120. It is, therefore, rather difficult to propose a gene-for gene hypothesis for the banana/*Foc* interaction. The presence of tolerant banana cultivars, such as GCTCV (Hwang and Ko, 2004) further suggests that defence mechanisms are under the control of minor resistance genes, rather than one single dominant *R* gene.

Conventional Breeding

There are five major conventional banana breeding programmes in the world. These are Empresa Brasileira de Pesquisa Agropecuária – Mandioca e Fruticultura Tropical (EMBRAPA-CNPMF) in Brazil, the Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Département des Productions f Fruitières et Horticoles (CIRAD-FLHOR) in Guadeloupe, the Fundación Hondurereña de Investigacón Agrícola (FHIA) in Honduras, CARBAP in Cameroon, and the International Institute for Tropical Agriculture (IITA) in Nigeria (Jones, 2000).

The first banana breeding programme at FHIA was established in 1959. The aim of this programme was to develop banana cultivars with resistance to *Foc* race 1 (Jones, 2000). To achieve this, fertile diploid pollen was taken from resistant male plants and applied to female flowers of Gros Michel and other triploid varieties with female fertility, in order to obtain resistant tetraploid hybrids (Cheeseman, 1932; Jones, 2000). The banana breeding process, however, is extremely slow due to the low number of fertile seeds that are obtained. Still, the FHIA breeding programme has produced several hybrids with resistance to both *Foc* race 1 and 4 over the past few

years, such as FHIA-01 (AAAB) (Moore *et al*., 1995; Jones, 2000) and SH-3640/10 ('High Noon') (Eckstein *et al*., 1996; De Beer, 1997). At EMBRAPA, conventional breeding was used in combination with unconventional banana improvement programmes. In the process, Prata, Maçã and Prata Anã tetraploid hybrids were developed that also showed resistance to *Foc* (De Matos *et al*., 1999). Although these tetraploid bananas have disease resistance, their taste is unacceptable to consumers of Cavendish bananas. Attempts are, therefore, being made to breed for triploid hybrid bananas in Honduras (Rowe and Richardson, 1975) and Guadeloupe (Vakili, 1967; Stover and Buddenhagen, 1986).

Unconventional Improvement

Cavendish cultivars do not produce seed and are, therefore, not suitable for use in conventional breeding programmes. Consequently, researchers have been investigating unconventional methods to improve disease resistance in these bananas. Programmes for the unconventional improvement of banana have been established at several institutions, such as the Taiwan Banana Research Institute (TBRI) and the International Atomic Energy Agency (IAEA) in Austria. These programmes improve banana by means of somaclonal variation, induced mutations, protoplast fusion and genetic modification.

Somaclonal variation

When meristem cultures of banana are micro-propagated for more than six cycles, natural somatic mutations occur that are genetically stable (Hwang *et al*., 1984). Such somaclonal variants can be grouped according to changes in plant stature, pseudostem colour, leaf morphology and bunch characteristics (Hwang and Tang, 2000). Researchers at the TBRI were able to select Cavendish banana clones with resistance to *Foc* race 4 from somaclonal variants since 1983. These include the moderately resistant clone (GCTCV-215-1) and the highly resistant clone (GCTCV-218), both derived from Giant Cavendish (Hwang, 1999).

Induced mutations

Mutation breeding is the use of mutagens to develop variants that increase agricultural traits. Mutations are alterations in the nucleotide sequence of a DNA molecule and can be induced by irradiation (Novak *et al.,* 1990) or chemicals (Omar *et al.,* 1989). Mutations by gamma irradiation in banana plants can be induced either by *in vitro* irradiation of the sucker or irradiation of the shoot tip culture (Novak *et al.,* 1990). Chemicals such as ethyl methanesulphonate, sodium azide and diethyl sulphate induce mutations in banana (Omar *et al.,* 1989).

Protoplast fusion

Protoplast fusion was used to form triploid banana plants by fusing a diploid banana cell with a monoploid cell (Novak *et al*., 1989; Assani *et al*., 2001). Protoplasts (single cell systems) are cells from which the cell walls have been removed enzymatically and/or mechanically. Under suitable conditions the protoplasts are able to reform cell walls and multiply. These protoplast cells form clusters that develop into callus and plants are then regenerated from the callus.

Genetic modification

At least four classes of resistance genes have been identified for cloning into susceptible plants (Walsh, 2000). The first set of resistance genes encodes components of receptor systems that directly or indirectly detect the presence of potential pathogens. Genes involved in the gene-for-gene interactions are included in this class. The activation of such receptors probably initiates a signal transduction pathway that in turn results in the induction of generic response genes (Godiard *et al*., 1994). A second class of resistance genes encode products that detoxify and inactivate compounds produced by the pathogen in order to cause disease (Walsh, 2000). The third class of resistance genes codes for altered targets for pathogen-derived molecules that are essential for pathogenesis. In this case, a resistance allele could possibly code for a product that did not interact with the pathogen (Walsh, 2000). The fourth class of resistance genes include those that encode for constitutive biochemical or structural barriers to pathogens (Walsh, 2000).

Researchers have explored the possibility of introducing foreign genes into banana by means of particle bombardment (Sági *et al.*, 1995; Becker *et al.*, 2000) and *Agrobacterium tumefaciens*-mediated transformation (Ganapathi *et al.*, 2001; Chakrabarti *et al.*, 2003). Biolistic-mediated transformation has been used to transform cv. 'Mas' banana plantlets with the pSOC1 construct which contains sequences encoding for a MADS-box transcription factor associated with early flowering (Wong *et al.,* 2004). *Agrobacterium tumefaciens*-mediated transformation has successfully been used to transform 'Rastali' bananas with a β-1, 3-endoglucanase gene. Transgenic plants exhibited three times higher enzyme activity that untransformed plants (Sreeramanan *et al.,* 2004). Khanna *et al.* (2004) also reported successful transformation of Cavendish and 'Lady Finger' bananas using *A. tumefaciens*-mediated transformation. Marker-gene expression and molecular analysis showed that the four transgenes, hygromycin phosphotransferase, β-glucuronidase, neomycin phosphotransferase and the green fluorescent protein had integrated stably in the banana genome.

CONCLUSION

Fusarium wilt of banana became known as one of the most devastating agricultural diseases of the past century, destroying thousands of virgin forests in Central America. The disease was eventually brought under control following the introduction of resistant varieties to replace the susceptible Gros Michel variety. No other means of control seemed to be effective. Today, the international banana industry faces, once again, destruction by one of the most important soil-pathogens, *Foc*. Only this time there is no replacement variety for the sweet Cavendish banana, a seedless variety that cannot be improved by conventional breeding.

The banana-*Foc* interaction, although very important for understanding the principles underlying disease resistance, has received little attention in the years that followed the solving of the epidemics in Central America. In studies by Stover (1962) and Beckman (1987; 1990) on the resistance response in banana roots, it was shown that vascular occluding gels and tyloses were produced in response to *Foc* infection. These

gels and tyloses then become infused with phenolics and stress metabolites to prevent progress of the pathogen. In resistant varieties, enzymes such as PAL, POD and PPO, and the PR-proteins are produced. The genetics underlying these responses, however, are still unknown, despite the fact that Fusarium wilt resistant banana hybrids have been developed in conventional banana breeding programmes. Our current understanding of the genetics of resistance against Fusarium wilt diseases, therefore, is limited to the interaction between *F. oxysporum* and crops such as tomato (Beckman *et al.*, 1982; Brammal and Higgins, 1988; Beckman *et al.*, 1989) and carnation (Baayen 1987; 1989). In this respect, the role of transcription factors such ERF1 and antifungal compounds such as *PRs* and peroxidases have been described.

The pathosystem and plant response to variants and special forms of *F. oxysporum* are not always consistent. In tomato, for instance, a true gene-for-gene interaction between race 2 in *Fol* has been described. However, a race-for-race interaction seems to be unlikely in the case of the *Foc*-banana interaction. One of the most important questions related to resistance in banana, therefore, is whether resistance is determined by *R* genes or minor genes, such as those involved in the production of glucanases, chitinases, phytoalexins, lignin, callose, enzymes for oxidative stress protection and antimicrobial secondary metabolites. The latter scenario seems to be more likely, as various levels of tolerance and resistance in banana to certain races of *Foc* has previously been described. Race definition in *Foc* is further complicated by the role of the environment that appears to influence disease development in certain *Foc*-banana interactions.

The search for genes involved in plant resistance to pathogens and pests has become a fast growing area of interest. Techniques that are currently employed to identify resistance genes include studies on segregating plant populations and differential gene expression. To study genes that are differentially expressed, methods such as differential display, differential hybridization (Liang and Pardee, 1992), representational difference analysis (RDA) (Lisitsyn and Wigler, 1993), subtractive library construction (Tedder *et al.*, 1988), Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.*, 1996; 1999), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), and cDNA microarray analysis (Schummer *et al.*, 1997) have been employed. For the seedless Cavendish banana, a technique such as SSH

seems to be most appropriate, since the common genes that are present in the isogenic lines would be removed and only the differentially expressed genes would be isolated.

The isolation and characterization of resistance and defence genes in banana to *Foc* can be of great significance. Banana resistance genes could be used as markers for the rapid identification of tolerant or resistant traits in banana selections. The identification of banana genes associated with defence would contribute to the understanding of the molecular basis of the banana/*Foc* interaction. These banana genes can further be used for genetic improvement of susceptible bananas via transformation. These genetically modified bananas would be more acceptable to the consumer market than those improved with genes from other crops.

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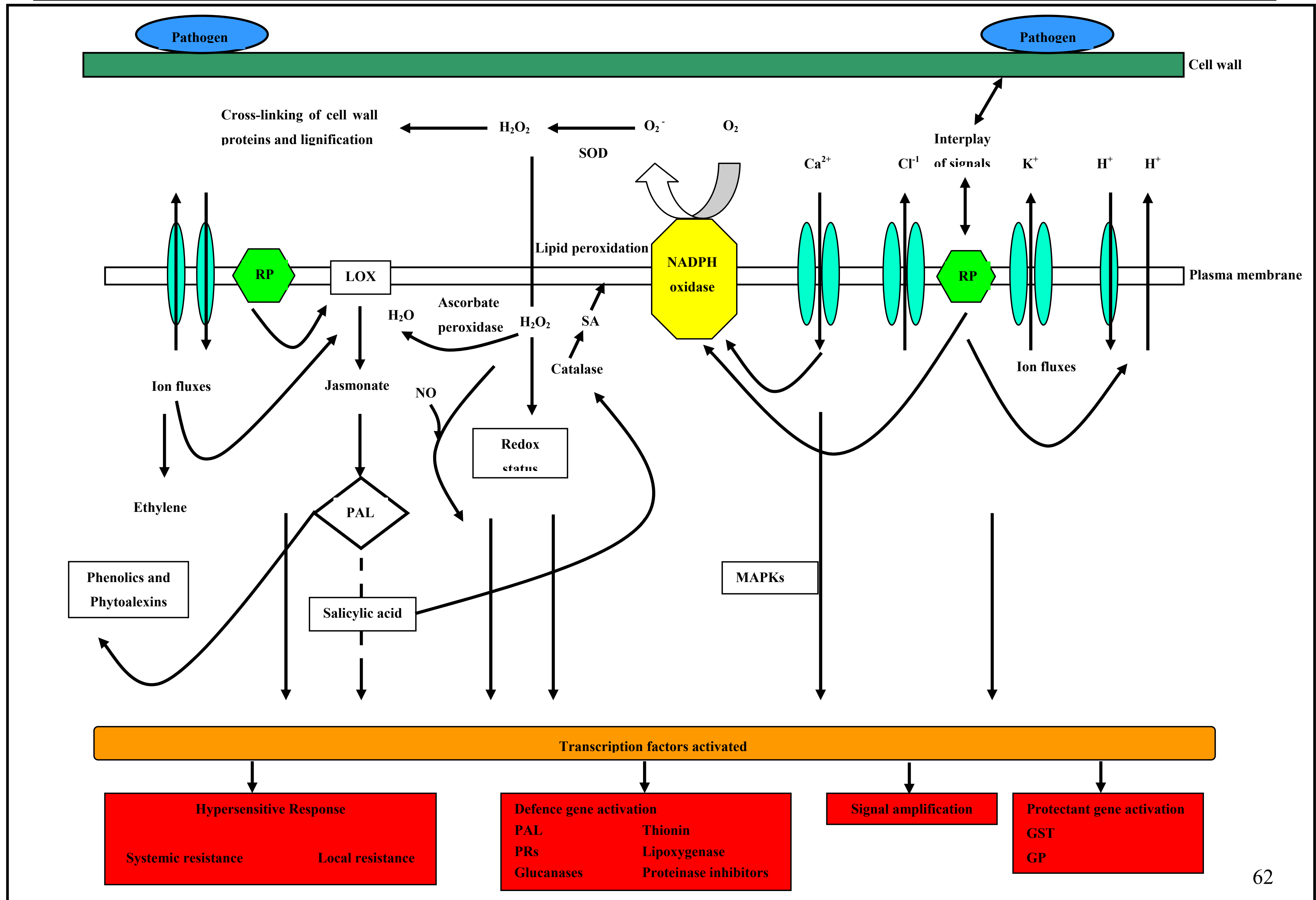
Pathogen
Pathogen
Cell wall
Cross-linking of cell wall proteins and lignification
H_2O_2
O_2^-
O_2
SOD
Interplay of signals
Ca^{2+}
Cl^{-1}
K^+
H^+
H^+
Lipid peroxidation
RP
LOX
NADPH oxidase
RP
Plasma membrane
H_2O
Ascorbate peroxidase
H_2O_2
SA
Catalase
Ion fluxes
Jasmonate
NO
Ion fluxes
Redox status
Ethylene
PAL
Phenolics and Phytoalexins
Salicylic acid
MAPKs
Transcription factors activated
Hypersensitive Response
Systemic resistance
Local resistance
Defence gene activation
PAL
Thionin
PRs
Lipoxygenase
Glucanases
Proteinase inhibitors
Signal amplification
Protectant gene activation
GST
GP

Figure 1. Overview of signalling events controlling the activation of plant defence responses.

The plasma membrane is considered to be the primary site for elicitation of different signalling cascades involved in plant-pathogen recognition and defence response. These defence responses include the production of reactive oxygen intermediates by a plasma membrane-associated complex, the induction of various ion fluxes (calcium (Ca^{2+}), proton (H^+) influx, and potassium (K^+), chloride (Cl^{-1}) efflux) across the plasma membrane. Activated signalling cascades at the plasma membrane in turn induce other plant defence mechanisms, including the accumulation of secondary signalling molecules salicylic acid (SA), ethylene (ET), jasmonate (JA), synthesis of phytoalexins and phenolics and pathogenesis-related (PR) proteins. Mitogen-activated protein kinase (MAPK) activation is placed downstream of ion fluxes and leads to defence gene induction by a process that is independent of the oxidative burst. Resistance responses are also often accompanied by the induction of the hypersensitive response (HR). (Abbreviations: superoxide dismutase (SOD), receptor (RP), lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), mitogen-activated protein kinase (MAPK), glutathione S-transferase (GST), glutathione peroxidase (GP), pathogenesis-related proteins (PRs).

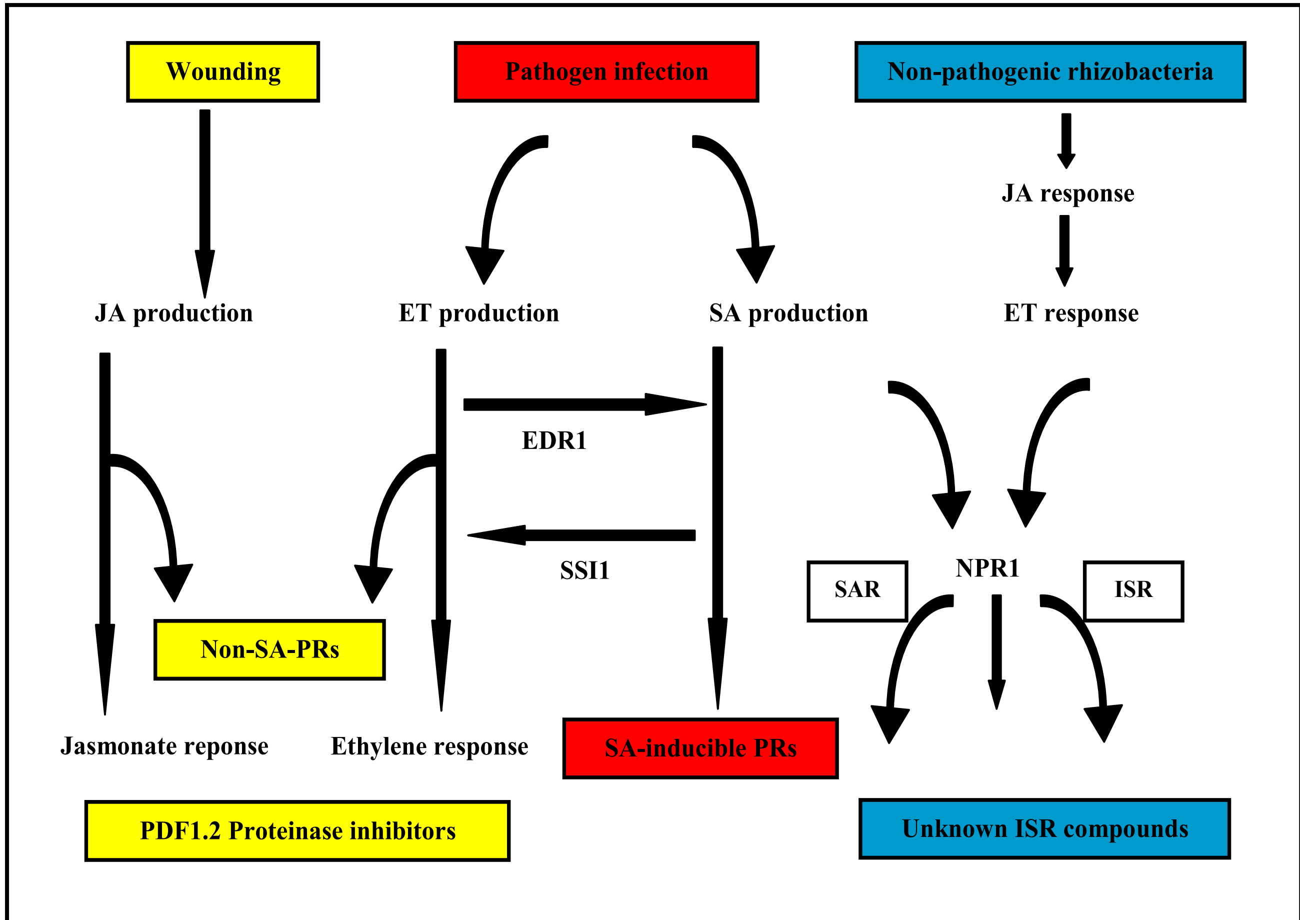

Figure 2. **Simplified model of SA-,ET-, and JA-dependant signalling, convergence points and possible cross-talk between different stimuli.**

The figure has been adapted from Pieterse and Van Loon (1999) and Wang *et al.* (2002). (Abbreviations: SA: salicylic acid, ET: ethylene, JA: jasmonate, SAR: systemic acquired resistance, ISR: induced systemic resistance, PRs: pathogenesis related proteins).

CHAPTER 2

EVALUATION OF THE CAVENDISH BANANA GCTCV-218 FOR TOLERANCE TO *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* 'SUBTROPICAL' RACE 4 (VCG 0120)

ABSTRACT

Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most destructive diseases of bananas in the world. In South Africa, all Cavendish banana varieties are highly susceptible to *Foc* 'subtropical' race 4 (VCG 0120). Since no replacement variety is acceptable to the local market, the only way to control Fusarium wilt is by reducing the spread of the pathogen by means of cultural practices such as the use of micro-propagated planting material. A Fusarium wilt-tolerant Cavendish plant known as GCTCV-218 was recently selected in a field severely affected by the disease in Taiwan. In this study, GCTCV-218 is evaluated for tolerance to Fusarium wilt of bananas in South Africa, both under greenhouse and field conditions. The production of phenolics in GCTCV-218 was also compared to that in the susceptible Cavendish variety, Williams, after challenging it with the pathogen. Greenhouse and field results showed that GCTCV-218 is significantly more tolerant to *Foc* than Williams. Significantly more ester-bound and cell wall-bound phenolics were also produced in GCTCV-218, 24 hrs after pathogen attack. GCTCV-218 was tolerant to *Foc* 'subtropical' race 4 (VCG 0120) and the tolerance appeared to be linked to the ability to produce greater amounts of phenolic compounds at an early stage after infection.

INTRODUCTION

Bananas (*Musa* spp.) are regarded as one of the most important agricultural crops produced in tropical and subtropical countries of the world. However, the continued production of bananas is threatened by highly virulent fungal pathogens, such as *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*). *Foc* is responsible for an important vascular disease that was first reported from Australia in 1876, known as Fusarium wilt or Panama disease (Stover, 1962). Today, Fusarium wilt has been reported from all banana-growing countries except those bordering the Mediterranean (Ploetz, 1990).

Three pathogenic races of *Foc* have been identified. Strains that infect the varieties Gros Michel (AAA), Apple (AAB), Silk (AAB), Taiwan Latundan (AAB) and IC2 (AAAA) belong to race 1. Those that cause disease to Bluggoe bananas (ABB) and close relatives and some Jamaican tetraploids (AAAA) are referred to as race 2, while race 4 causes disease to Cavendish bananas (AAA) as well as race 1 and 2 susceptible varieties (Stover and Buddenhagen, 1986; Hwang, 1999).

Fusarium wilt became known as one of the most important diseases in agricultural history following the demise of more than 40 000 ha of Gros Michel bananas in Central and South America over a period of 50 years (Stover, 1962). In the early 1960's, the international banana export trade in Central America was rescued by the timely replacement of *Foc* race 1-susceptible Gros Michel bananas with resistant Cavendish varieties. Losses of Cavendish bananas to *Foc* race 4, first in the subtropics (Ploetz, 1990), and more recently in the tropics (Pegg *et al*., 1993; Ploetz, 1994), have raised fears that the world trade in banana might once again be threatened. In South Africa, where bananas are planted in the sub-tropics, Fusarium wilt has already been reported from four of the six banana production areas (Viljoen, 2002). Likewise, the disease has destroyed many thousands of hectares of Cavendish bananas in tropical countries such as Indonesia and Malaysia (Hwang and Ko, 2004) and no replacement variety with resistance to *Foc* race 4 exists for the seedless Cavendish banana.

No sustainable control strategy exists for Fusarium wilt of banana, other than replacing susceptible varieties with those resistant to the disease. Conventional breeding efforts to find a resistant replacement for Cavendish bananas have had limited success, often because of the reluctance by local markets to accept the new hybrids (Stover and Buddenhagen, 1986; Rowe and Rosales, 1993; Daniells *et al*., 1995). Hwang and Tang (1996), therefore, initiated a program using unconventional improvement methods for Cavendish bananas in Taiwan for Fusarium wilt resistance by generating somaclonal variants. Two clones, GCTCV-215-1 and 217, with good resistance to *Foc* 'tropical' race 4 (VCG 0121) were found (Hwang, 1999). However, a field selection from Giant Cavendish, known as GCTCV-218, eventually rescued the banana industry in Taiwan from destruction (Hwang and Ko, 2004).

Resistance to Fusarium wilt of banana is dependant on cell wall strengthening and occlusion to prevent fungal colonization of the vascular tissue (Beckman, 1990). This involves the synthesis and deposition of lignin in the cell walls, and the accumulation of cell-wall appositions in the xylem vessels, respectively (Shiraishi *et al*., 1989). Cell wall strengthening and xylem occlusion depend on phenolic-based defence responses by the plant (Matern and Kneusel, 1988). Phenolics are substances that are constitutively expressed and widely distributed in plants. Mace (1963) established that healthy banana roots have specialised cells that contain high concentrations of phenols. Upon challenge by pathogens and pests, a substantial increase in phenolic synthesis takes place as part of the active defence response (Mace, 1963; Mace and Solit, 1966; Beckman and Mueller, 1970; Nicholson and Hammerschmidt, 1992; Matern *et al*., 1995). A successful resistance response depends on the rate and extent of recognition and activation of the defence mechanisms (Beckman, 1987; Beckman, 1990).

In a recent investigation, Groenewald *et al*. (unpublished data, FABI, South Africa) showed that *Foc* strains in Taiwan (VCG 0121) are genetically only distantly related to those causing disease to Cavendish bananas in South Africa (*Foc* 'subtropical' race 4, VCG 0120). The aim of this study, therefore, was to determine whether the Cavendish selection from Taiwan (GCTCV-218) also has tolerance to *Foc* 'subtropical' race 4 (VCG 0120). The role of phenolics production in the resistance response was also considered.

MATERIALS AND METHODS

Greenhouse Trials

Plant material

Cavendish banana varieties GCTCV-218 (tolerant to *Foc*) and Williams (susceptible to *Foc*) were micro propagated at Du Roi Laboratories in Letsitele, South Africa. Once the plantlets were grown to 10-cm plants, they were removed from seedling trays and transplanted into 250-ml polystyrene cups containing water. Plants were maintained in a greenhouse at 18/25ºC with a 16 hrs natural sunlight/8 hrs dark photoperiod, and fertilized once a week with a nutrient solution (0.6 g/l $Ca(NO_3)_2.H_20$, 0.9 g/l Agrasol-0® (Scotts, Scotts-Europe B.V., The Netherlands), 1 ml/l Micromax® Stock solution (3 g/l) (Fleuron, P.O. Box 31245, Braamfontein 2017). Inoculation with *Foc* was carried out after 21 days to allow the young banana plants to recover from stress related to the planting procedure, and to develop a strong root system.

Inoculation

Single-spore cultures of the highly virulent *Foc* isolates (CAV 045, 092, 105), belonging to 'subtropical' race 4, vegetative compatibility group (VCG) 0120, were used as starter inoculum. The isolates were first grown on 90-cm-diameter Petri dishes with half-strength Potato Dextrose Agar (PDA) (Biolab, Merck Laboratories, Wadeville, Gauteng, South Africa) for 7-10 days. The inoculum was prepared by pouring sterile distilled water onto the surface of Petri dishes containing the isolates for inoculation, and loosening the fungal spores with a sterile glass rod. The spore suspensions were then filtered through sterile cheese cloth, combined, and the spore concentration was adjusted to 2.5×10^5 conidia.ml^{-1} using a haemocytometer.

The roots of all the banana plantlets were slightly wounded by crushing the entire root system, prior to inoculation. Each plantlet was inoculated by adding 2.5 ml of the spore suspension to each cup containing 250 ml water in order to achieve a final inoculum concentration of 2.5×10^3 conidia.ml^{-1}. Sterile distilled water was added to cups containing the control plants. Plants were kept in the greenhouse for a further 6 weeks. They were watered only when approximately 50 ml water was left in the

cups. Five replicates with six plants in each replicate were inoculated for both GCTCV-218 and Williams, and the entire experiment was repeated.

Disease rating

Disease development was evaluated 5-6 weeks after inoculation using a disease modified version of the severity rating scale for Fusarium wilt of banana (Carlier *et al*., 2002). The rating scale ranged from 0-5, with plants showing no internal symptoms scoring a 0 and plants showing 100% vascular discoloration scoring 5 (Fig. 1). Disease severity was calculated using the formula of Sherwood and Hagedorn (1958):

> Disease severity (%) = [∑ (No. plants in a disease scale category)*(Specific disease scale category)/ (Total no. of plants in the trial)*(Maximum disease scale category)] *100

Statistical analysis for the data was conducted using the General Linear Models (GLM) procedure of *STATISTICA*, version 7 (STATSOFT Inc. 2004). Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Significance was evaluated at $P<0.05$.

Field Trial

Plant material

Three separate field trials were conducted over a period of 2 years in the Kiepersol area, Mpumalanga, South Africa. Tissue-cultured banana plantlets approximately 40 cm high were planted in three different fields infested with *Foc* ‘subtropical’ race 4 (VCG 0120) in January 2002. Experimental plots consisted of a completely randomised block design, with 15 or 20 plants of either GCTCV-218 or Williams per block, dependent on the trial site, and five replicate blocks randomised in the plantation. Standard banana cultivation methods were applied to the trial.

Disease rating

Disease development was rated according to the presence or absence of external disease symptoms. Healthy plants were awarded a value of 0, while diseased plants were scored as 1. Disease severity was calculated using the formula of Sherwood and Hagedorn (1958). Statistical analysis for the data was conducted as described above.

Phenolic Assays

Plant material

GCTCV-218 and Williams tissue cultured banana plantlets were grown in the polystyrene cup-system described above. Plants were inoculated with a *Foc* spore suspension with a final concentration of 2.5 x 10^3 conidia.ml^{-1} and maintained in the greenhouse until sampling. Control plants were treated with sterile distilled water. Roots were harvested at 0, 6, 24 and 48 hrs after inoculation, and immediately placed in liquid N_2. The roots were then ground with an electric homogeniser (IKA A11 Basic analytical Mill, United Scientific (Pty) Ltd., San Diego, USA), freeze dried and stored at -80°C until the phenol assays were performed. Each assay was performed in triplicate.

Extraction of phenolics

Phenolics were extracted using a modification to the method described by De Ascensao and Dubery (2003). Phenolics from the root material (0.05 g) of the control and treated plants (GCTCV-218 and Williams) were extracted with 1 ml of a solution containing MeOH/AC/H_2O (7:7:1 (v/v/v)). The suspension was homogenised for 1 min before being shaken for 1 h at 200 rpm and centrifuged for 5 min at 12 000 x g. After centrifugation, the supernatant was saved. The remaining precipitate was re-homogenised and centrifuged as above. The second supernatant was combined with the first and the procedure was repeated two more times. The four combined supernatants were concentrated to 1 ml. Aliquots were made in order to determine total soluble phenolic acids, free phenolic acids, MeOH soluble ester-bound phenolic acids and MeOH soluble glycoside-bound phenolic acids. The remaining precipitate was dried at 70°C for 24 hrs. The resulting alcohol insoluble residue (AIR) yielded the cell wall material that was used to extract the ester-bound cell wall phenolic acids.

Total soluble phenolic acids

The method used to determine the total soluble phenolic content is based on the reduction of the phospho-molybdene/phosphor-tungstate present in the Folin-Ciocalteau reagent (Swain and Hillis, 1959). The aliquoted supernatant was concentrated in a speedy vac (SPD111V vacuum centrifuge) (Savant, Holbrook, New York, USA) to 5 µl and diluted to 175 µl with water and added to 25 µl of 20% (v/v) Folin-Ciocalteau reagent and mixed. After 3 min, 50 µl of saturated aqueous sodium carbonate ($NaCO_3$) was added, mixed and incubated at 30°C for 30 min. A blank of water was used as control. Gallic acid was used as a phenolic standard to construct a standard curve ranging from 0 - 400 µg.ml^2 ($y = 1.3527x – 0.0109$, $R^2 = 0.9986$). The concentration of phenols in the various extracts was calculated from the standard curve and expressed as µg gallic acid g^{-1} dry weight.

Non-conjugated phenolic acids (Free acids)

Twenty-five µl of triflouroacetic acid (TFA) was added to 500 µl of aliquoted supernatant to acidify the solution prior to extraction with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories) (Cvikrová *et al*., 1993). The ether extract was dried and the resulting precipitate was resuspended in 250 µl 50% aqueous MeOH. This solution was used to determine the free phenolic content with Folin-Ciocalteau reagent.

Glycoside-bound phenolics

The aliquoted supernatant (500 µl) for MeOH soluble glycoside-bound phenolic content determination was hydrolysed in 50 µl concentrated pure HCl for 1 h at 96°C, directly placed on ice for 15 min and then extracted three times with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories). The ether extract was dried in a speedy vac and the remaining precipitate was resuspended in 250 µl 50% aqueous MeOH. This solution was used to determine the glycoside phenolic content with Folin-Ciocalteau reagent in the same way as described for total phenolic acids.

Ester-bound phenolics

Soluble ester-bound phenolic acids were extracted after alkaline hydrolysis under mild conditions (Cvikrová *et al.,* 1993). One hundred and twenty five µl of 2 M NaOH was added to the aliquot of supernatant and the tubes were left to stand at room

temperature for 3 hrs and then placed on ice for 15 min. After hydrolysis, 150 µl 1 M HCl was added and the phenolics extracted with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories) as described above. The final solution was used to determine the phenolic ester content using the Folin-Ciocalteau reagent.

Cell wall-bound phenolics

The ester-bound phenols incorporated into the cell wall were extracted following alkaline hydrolysis (Campbell and Ellis, 1992). Dry cell wall material (AIR) was weighed (0.01 g) and resuspended in 0.5 M NaOH (1 ml for 10 mg) for 1 h at 96°C. Cell wall-esterified hydroxycinnamic acid derivatives were selectively released under these mild saponification conditions. The supernatant was acidified to pH 2 with HCl, centrifuged at 12 000 x g for 10 min and then extracted with 1 ml diethyl ether (Saarchem, Merck Laboratories). The extract was dried in a speedy vac and the precipitate was resuspended in 250 µl 50% aqueous MeOH. This solution was used to determine the cell wall-esterified phenolic acids content with Folin-Ciocalteau reagent.

Statistical analysis for the phenolic assay data was conducted using the General Linear Models (GLM) procedure of *STATISTICA*, version 7 (STATSOFT Inc. 2004). Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Significance was evaluated at $P<0.05$.

RESULTS

Greenhouse Trials

Yellowing of banana leaves and wilting appeared 4-5 weeks after inoculation with *Foc*. After 6 weeks, the Williams plants developed more severe internal symptoms than GCTCV-218. Many of the Williams plants scored 5 compared to 1 (few) or 0 (no) internal symptoms in GCTCV-218. Disease severity values for Williams were 65 and 57% for the two trials, compared to disease severity values of 34 and 38% in

GCTCV-218 (Fig. 2). No symptoms developed in the control plants of either GCTCV-218 or Williams.

Field Trials

GCTCV-218 consistently showed better tolerance to Fusarium wilt in the field than Williams. In the three sites, GCTCV-218 plants had a disease severity of 10, 34 and 14%, compared to the disease severity rating for Williams of 52, 76 and 72% (Fig. 3).

Phenolic Assays

Total soluble phenolics

GCTCV-218 produced a significantly higher total phenolic content when compared to Williams at 0 and 6 hrs after inoculation with *Foc* (Fig. 4). Although there were no significant differences between the treatments, an induction in total phenolics was observed in GCTCV-218 after 6 hrs, while induction in Williams occurred only after 24 hrs. No significant differences in total phenolic production in GCTCV-218 and Williams were obvious at 24 and 48 hrs after inoculation.

Non-conjugated phenolic acids (Free acids)

Free acids were significantly higher in GCTCV-218 when compared to Williams before inoculation with *Foc*, but decreased after inoculation in both varieties. GCTCV-218 also had a significantly higher level of free acids at 24 hrs after inoculation (Fig. 5).

Glycoside-bound phenolics

A significant decrease in glycoside phenolics occurred in GCTCV-218 6 hrs after inoculation with *Foc*, with an increased induction taking place after 24 and 48 hrs (Fig. 6). GCTCV-218 showed a significant induction of phenolics after 24 hrs, and Williams only at 48 hrs. Glycoside-bound phenolics were significantly higher in GCTCV-218, 24 and 48 hrs after inoculation when compared to Williams.

Ester-bound phenolics

Ester-bound phenolics were expressed at similar levels in GCTCV-218 at 0 and 6 hrs after inoculation, but a significant increase in content was observed at 24 hrs and reached a plateau at 48 hrs after inoculation with *Foc* (Fig. 7). The ester-bound phenolics in Williams, however, only increased at 48 hrs. At 24 hrs, GCTCV-218 further had a slightly higher ester-bound content when compared with the susceptible cultivar Williams.

Cell wall-bound phenolics

Williams showed a significantly higher basal content of cell-wall bound phenolics at 0 and 6 hrs after inoculation with *Foc* when compared to GCTCV-218. After *Foc* infection however, there was a significant decrease in the content (Fig. 8). GCTCV-218 responded to pathogen infection at 24 and 48 hrs after inoculation with a significant increase in cell-wall bound phenolics as well as a significantly higher phenolic content when compared to Williams.

DISCUSSION

Greenhouse and field evaluation in this study showed that GCTCV-218 developed significantly less Fusarium wilt than Williams bananas in South Africa, and could be considered tolerant to *Foc* ‘subtropical’ race 4 (VCG 0120). This result has major implications for the continued cultivation of Cavendish bananas in countries affected by *Foc* race 4. In Taiwan, GCTCV-218 was highly resistant against race 4 and also showed high yields of high quality fruit (Hwang and Ko, 2004). Hybrids with good resistance to the pathogen, such as FHIA-01 (‘Goldfinger’) (Moore *et al*., 1995; Jones, 2000) and SH-3640/10 (‘High Noon’) (Eckstein *et al*., 1996; De Beer, 1997), are not always acceptable to the Cavendish-dominated markets. In these situations GCTCV-218 could be considered a good replacement for susceptible Cavendish varieties in countries affected by *Foc* race 4.

Planting of a tolerant Cavendish selection such as GCTCV-218 would need to be supported by an integrated disease management strategy. This might also include treatment of plants with chemicals that induce systemically acquired resistance,

biological control organisms, and fertilisers that reduce pathogen growth and enhance plant vigour (Nel, 2004).

Evaluation and confirmation of disease tolerance in GCTCV-218 provides an opportunity to study resistance mechanisms in Cavendish bananas against *Foc*. The plant could be considered an isogenic line of Giant Cavendish, the susceptible Cavendish variety from which it was selected. Techniques such as Suppression Subtractive Hybridisation (SSH) (Diatchenko *et al*., 1996 and 1999) provide an opportunity for isolating genes differentially expressed in response to *Foc* race 4. If these genes are linked to resistance, they could be used as markers for the early identification of tolerant somaclonal variants, or they could be re-introduced into high-yielding susceptible plants to improve disease tolerance.

Higher levels of total soluble phenolics in GCTCV-218 compared to Williams in this study suggests that constitutive defence compounds are present in higher concentrations in tolerant bananas, even before contact with the pathogen. This increased presence of phenolics could contribute to an enhanced plant response after infection. The role of phenolics in defence responses in banana has been well illustrated previously. De Ascensao and Dubery (2000) reported a prominent increase in total soluble phenolics in FHIA banana roots, 8 hrs after treatment with elicitors from *Foc* race 4. Williams, however, only responded after 12 hrs and did not show the same prominent increase as the tolerant hybrid. Histochemical observations of root sections of banana varieties susceptible (Poyo) and partially resistant (Yangambi Km5) to nematodes also revealed striking differences in phenolic content (Valette *et al*., 1998). Resistant roots had high levels of lignin, flavanoids, dopamine, cafeic esters and ferulic esters that were associated with a very low rate of nematode root penetration in the resistant cultivar.

Early induction of phenolic compounds could contribute to tolerance in GCTCV-218 against *Foc* race 4 compared to Williams, where no phenolics were induced after 48 hrs. This early response may be a key factor in preventing pathogen spread into and throughout the vascular system of the tolerant plant. Phenolics are precursors of several secondary metabolites and proteins involved in disease resistance, such as phytoalexins and lignin (Matern *et al*., 1995). In this study, ester-bound phenolics

were expressed at increased levels in GCTCV-218, 24 hrs after pathogen attack. Evidence strongly suggests that esterification of phenols to cell wall materials, is a common theme in the expression of disease resistance (Fry, 1987). The early increase of glycoside-bound phenolics in GCTCV-218 may further contribute to disease tolerance by being toxic to the pathogen and thereby preventing pathogen spread. Cell wall-bound phenolics were significantly induced at 24 and 48 hrs after pathogen attack. De Ascensao and Dubery (2003) reported a 3-fold increase of glycoside-bound phenolic, a 4.2-fold increase of ester-bound phenolics and a 6.3-fold increase in cell wall-bound phenolics in the resistant Goldfinger 36 hrs after *Foc* – elicitor treatment.

In this study, GCTCV-218 had a greater capacity for phenolic metabolism in response to *Foc* and it also displayed a high basal level of total soluble phenolics, free acids and glycoside-bound phenolics before pathogen infection. Strengthening of cell walls is generally accomplished by infusion of phenolics into, or the apposition of phenolic-containing material against the cell wall (Beckman *et al*., 1974; Ride, 1975). The constitutive presence of phenolics suggests that GCTCV-218 has pre-existing defence mechanisms that protect the plant. GCTCV-218 responded strongly to pathogen attack by producing high levels of ester-bound and cell wall-bound esterified phenolics. This suggests that cell wall strengthening and subsequent lignin deposition is taking place. The high level of phenolics found in GCTCV-218 may also possibly contribute to the effective and timeous production of papillae and gels in response to *Foc*. GCTCV-218, therefore, is able to actively induce a structural and biochemical defence response against *Foc*. Apart from simply inducing strong defence responses, GCTCV-218 appears to be able to induce them early enough to contain *Foc* and prevent further spread.

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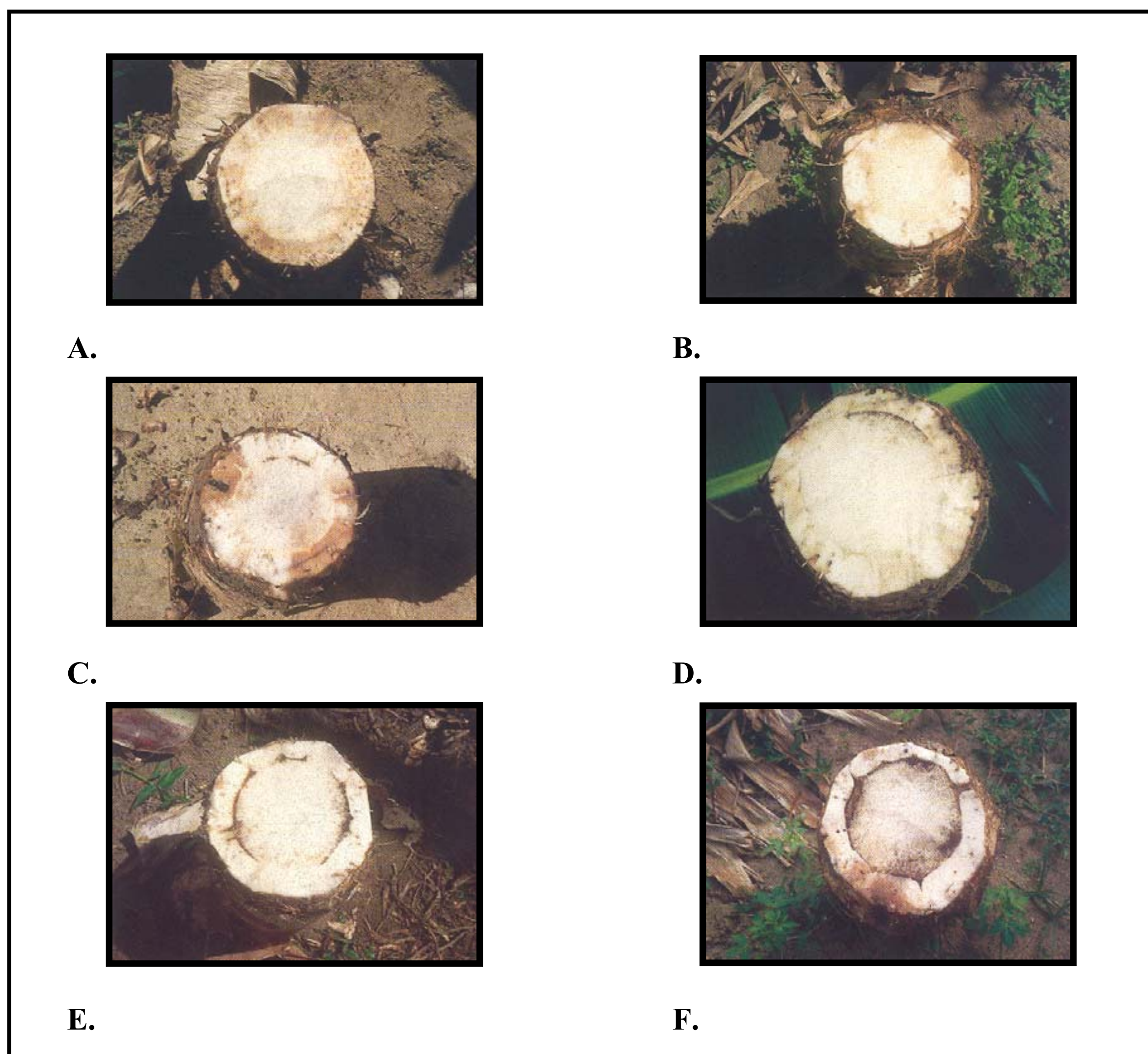

Photo's Zilton Cordeiro EMBRAPA-CNPMF (Inibap Technical Guidelines)

Figure 1. Visual internal disease rating scale used to evaluate disease severity of *Fusarium oxysporum* f. sp. *cubense* in bananas.

Disease severity	Internal disease symptoms in banana corm
0 (Fig. 1A)	Corm completely clean, no vascular discoloration
1 (Fig. 1B)	Isolated points of discoloration in vascular tissue
2 (Fig. 1C)	Discoloration of up to 1/3 of vascular tissue
3 (Fig. 1D)	Discoloration of between 1/3 and 2/3 of vascular tissue
4 (Fig. 1E)	Discoloration greater than 2/3 of vascular tissue
5 (Fig. 1F)	Total discoloration of vascular tissue (dark purple)

(Carlier *et al.*, 2002)

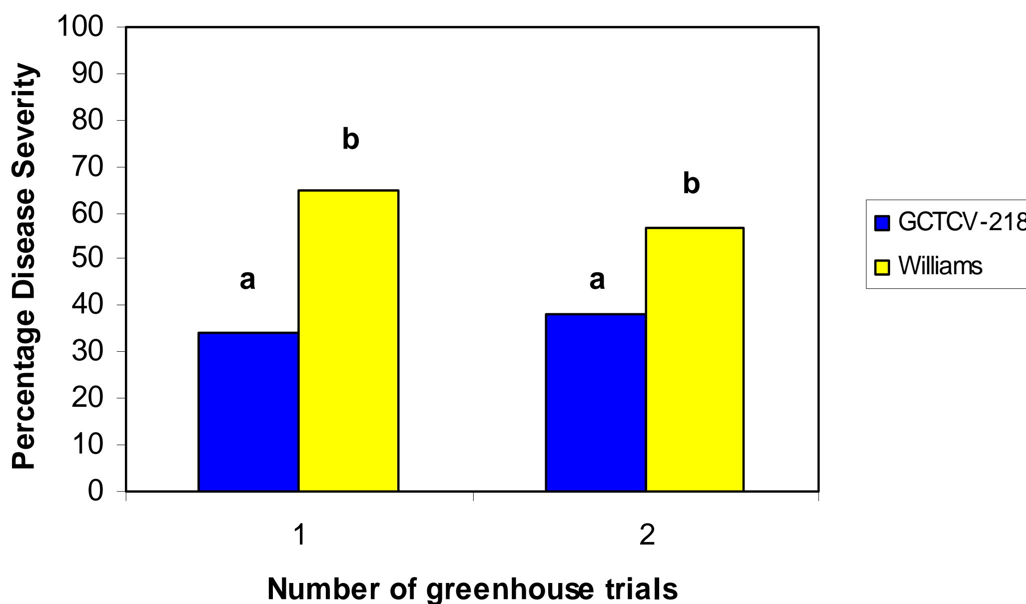

Figure 2. Disease severity index of GCTCV-218 and Williams bananas infected with *Fusarium oxysporum* f. sp. *cubense* during two independent greenhouse trials. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b] Percentage disease severity values in the same trial with the same letter are not significantly different at $P<0.05$.

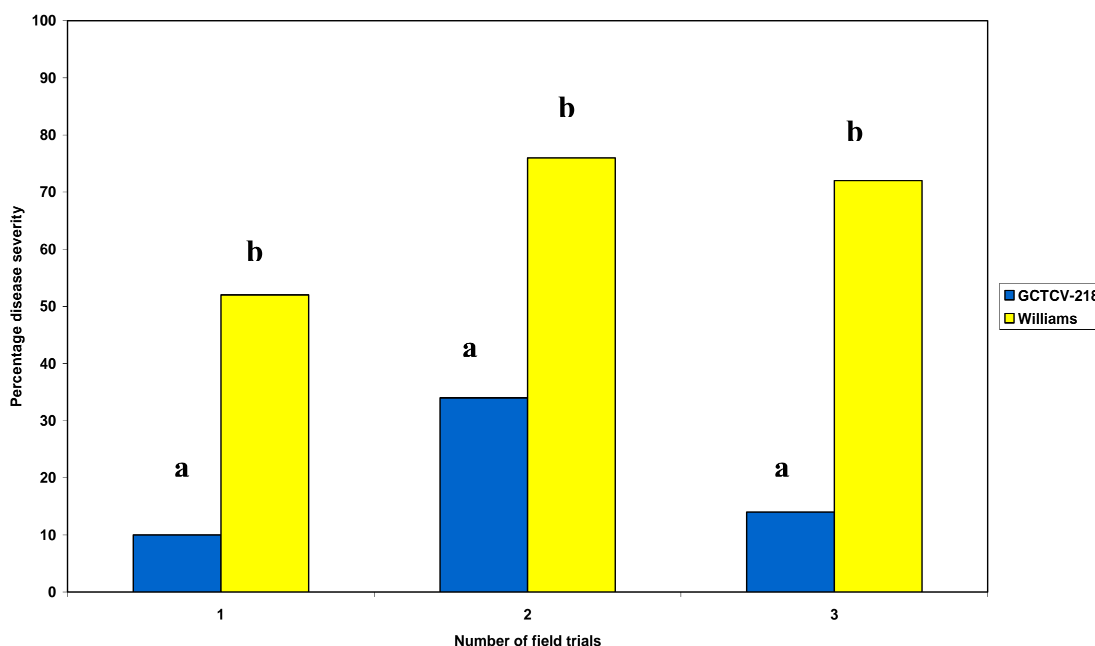

Figure 3. Disease severity index of GCTCV-218 and Williams bananas during three independent field trials in *Fusarium oxysporum* f. sp. *cubense* infected areas in South Africa. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b] Percentage disease severity values in the same trial with the same letter are not significantly different at $P<0.05$.

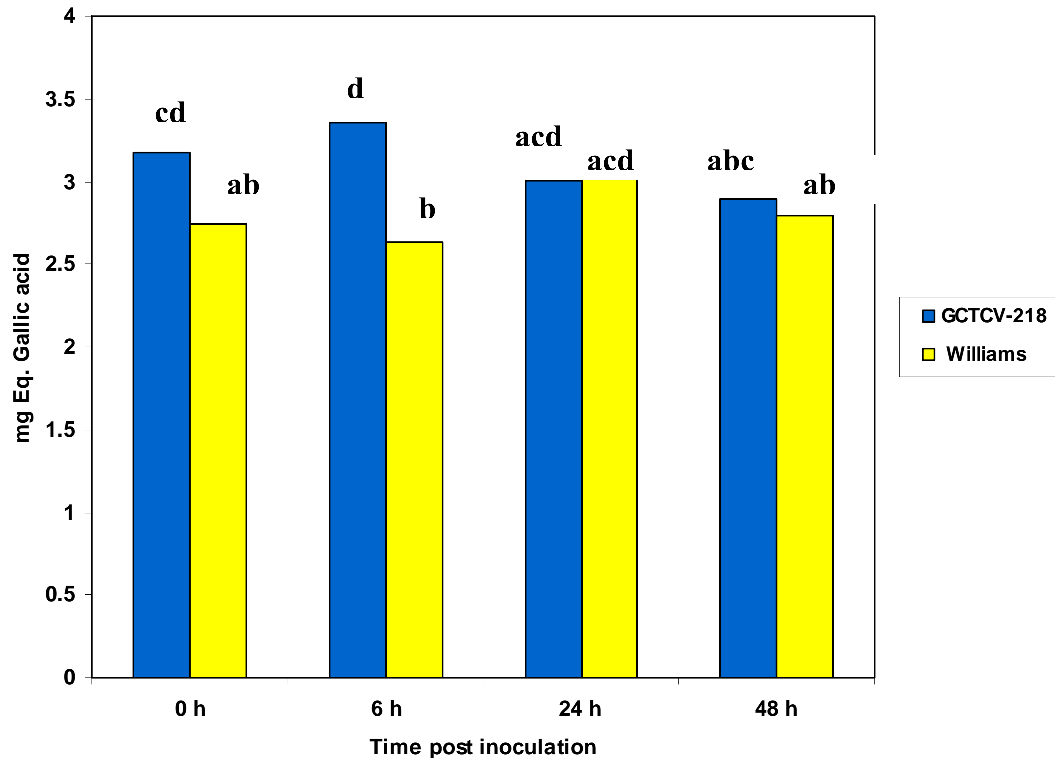

Figure 4. Total phenolic content in GCTCV-218 and Williams banana cultivars at different time points after inoculation with *Fusarium oxysporum* f. sp. *cubense.* Phenolics were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b,c,d] Bars presented with the same letter are not significantly different at $P<0.05$.

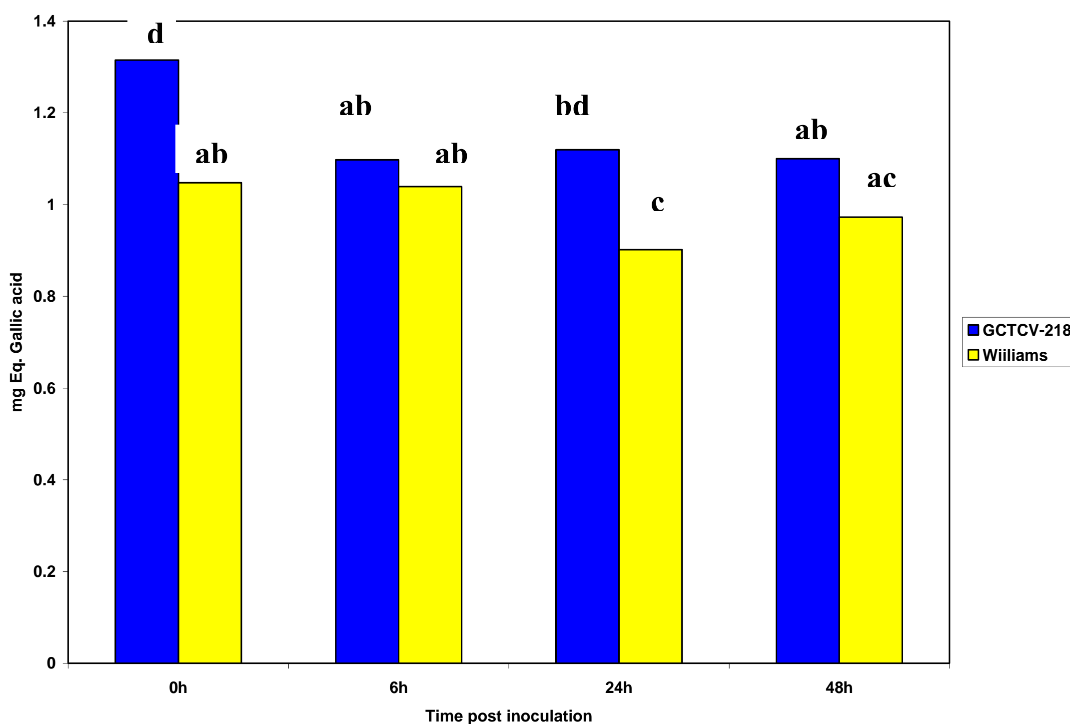

Figure 5. Non-conjugated phenolic acid content in GCTCV-218 and Williams banana cultivars at different time points after inoculation with *Fusarium oxysporum* f. sp. *cubense.* Phenolics were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b,c,d] Bars presented with the same letter are not significantly different at $P<0.05$.

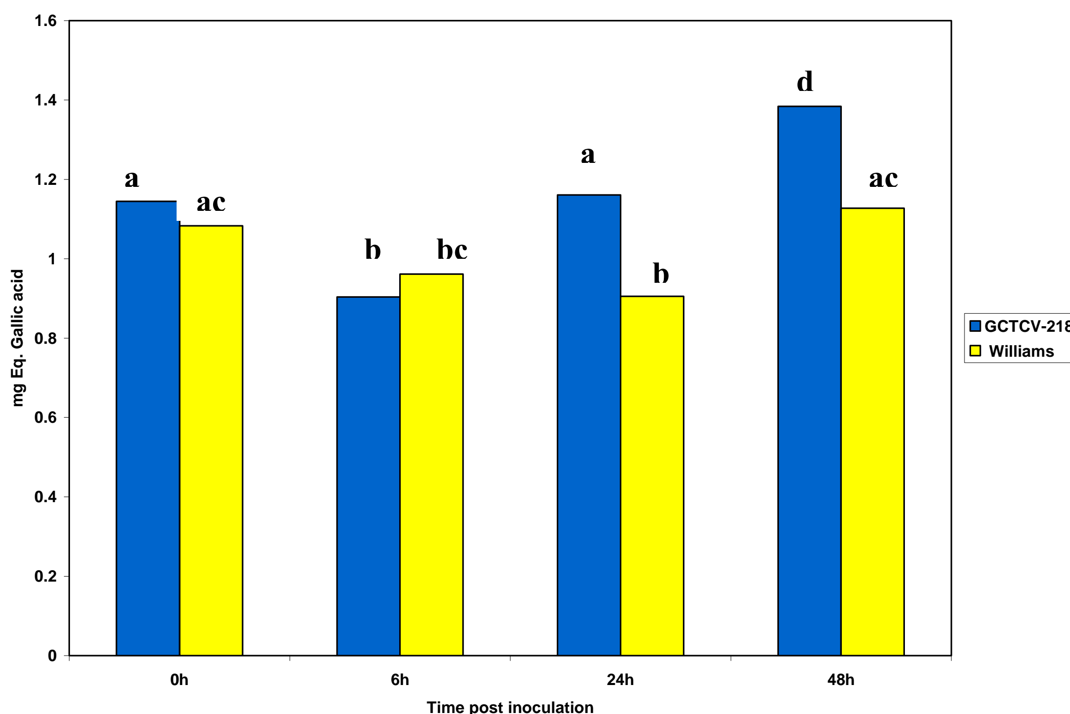

Figure 6. Glycoside-bound phenolic content in GCTCV-218 and Williams banana cultivars at different time points after inoculation with *Fusarium oxysporum* f. sp. *cubense.* Phenolics were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b,c,d] Bars presented with the same letter are not significantly different at $P<0.05$.

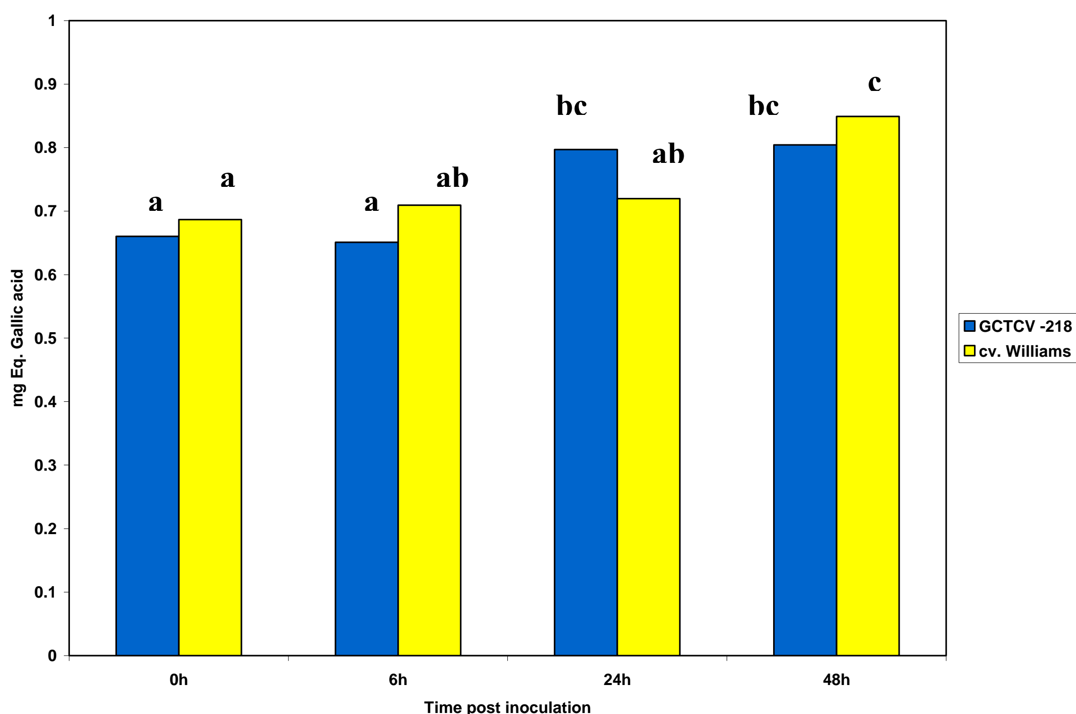

Figure 7. Ester-bound phenolic content in GCTCV-218 and Williams banana cultivars at different time points after inoculation with *Fusarium oxysporum* f. sp. *cubense*. Phenolics were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight**.** Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b,c,d] Bars presented with the same letter are not significantly different at $P<0.05$.

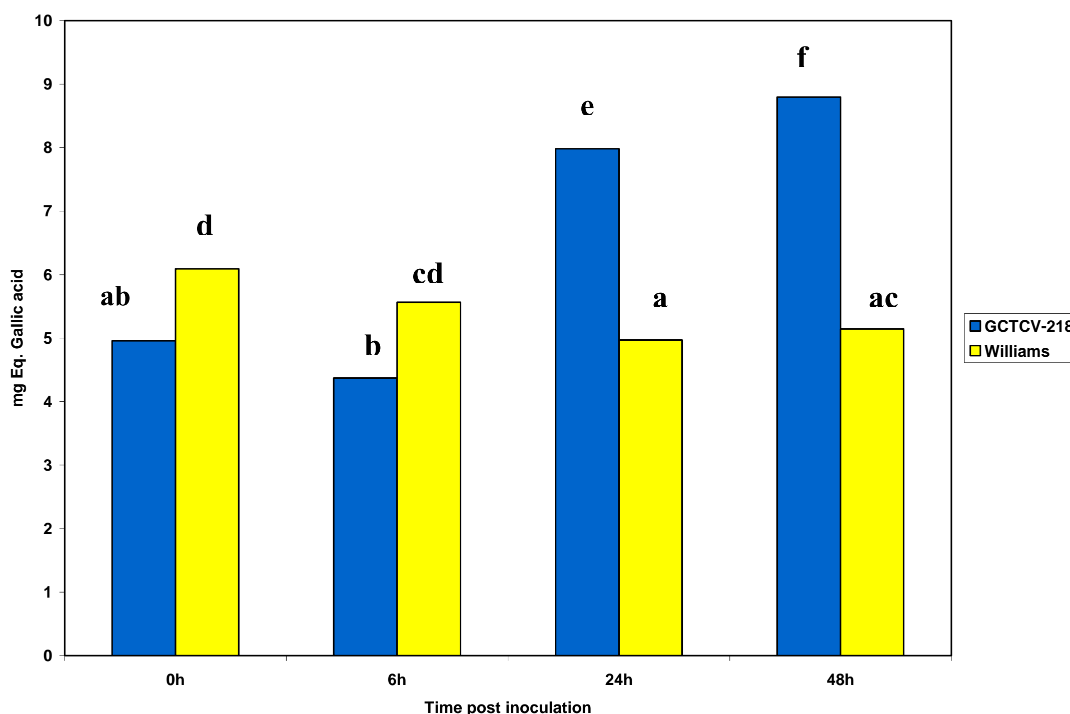

Figure 8. Cell wall-bound phenolic content in GCTCV-218 and Williams banana cultivars at different time points after inoculation with *Fusarium oxysporum* f. sp. *cubense*. Phenolics were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight. Experiments were analysed using One-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. [a,b,c,d,e,f] Bars presented with the same letter are not significantly different at $P<0.05$.

CHAPTER 3

CONSTRUCTION OF A cDNA LIBRARY WITH GENES ASSOCIATED WITH TOLERANCE TO *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* IN CAVENDISH BANANAS

ABSTRACT

Identification of banana genes involved in the defence response against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) represents an important step towards understanding disease resistance mechanisms in bananas. Suppression Subtractive Hybridisation (SSH) was used to isolate differentially expressed genes in a tolerant banana cultivar (GCTCV-218), compared to the susceptible cultivar Williams, in response to infection by *Foc*, 6 hrs post inoculation. Southern Blot analysis and a PCR showing the reduction of the housekeeping gene, actin, after the SSH, indicated that the subtraction was efficient. A cDNA library containing 736 cDNA clones was constructed. Sequencing results and BLASTX searches indicated that the cDNA library contained cDNA fragments that are associated with defence responses in other plants. The construction of a cDNA library enriched for differentially expressed transcripts is a valuble first step supporting future studies concerning resistance to *Foc* in bananas.

INTRODUCTION

Bananas (*Musa* spp.) are one of the most important sources of human nutrition, providing food and income to millions of people in the world (Jones, 2000). The crop includes dessert and cooking bananas and is planted widely throughout the tropics and subtropics. The most important dessert bananas are Cavendish (AAA) varieties, which comprise almost 90% of the export market and 40% of all bananas cultivated (Jones, 2000). Because of the clonal nature of the plant, sustainable production of bananas is threatened by diseases such as Fusarium wilt, caused by *Fusarium oxysporum* Schechtend.:Fr. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*).

No effective control strategy exists for Fusarium wilt other than replacing susceptible bananas with resistant banana varieties. For example, to continue banana production in Central America during the 1960s, Cavendish varieties had to be introduced to replace Gros Michel bananas that were highly susceptible to *Foc* race 1 (Stover, 1962). In the past three decades, however, a new race of the pathogen, *Foc* race 4, has caused devastating losses to Cavendish bananas both in the tropics and subtropics (Ploetz, 1994). Despite conventional and unconventional breeding efforts, no dessert banana variety has been developed to replace Cavendish bananas in areas that are severely affected by Fusarium wilt.

Resistance to *Foc* race 4 is found in wild banana varieties such as Calcutta 4 (AB) (Jeger *et al.*, 1995). This resistance has been introduced into banana hybrids in breeding programmes such as Fundación Hondurereña de Investigacón Agrícolan (FHIA) in Honduras (Moore *et al.*, 1995; Jones, 2000). The introduction of resistance into tetraploid hybrids involves the transfer of a set of chromosomes from *Musa balbisiana* Colla, a starchy banana species known for its ability to tolerate biotic and abiotic stresses (Jones, 2000). Tetraploid hybrids developed in banana breeding programmes have seldom been accepted as a replacement for sweet triploid Cavendish banana varieties, consisting exclusively of sets of chromosomes from *M. acuminata* Colla (Jones, 2000). Other efforts to introduce resistance to Fusarium wilt involve unconventional plant improvement efforts, such as induced mutations, the

production of somaclonal variants, and field selection of disease resistant plants (Hwang and Tang, 1996).

Substantial progress in the development of *Foc* race 4-resistant Cavendish bananas has been made at the Taiwan Banana Research Institute (TBRI) in Taiwan. The most promising plant from this institute is GCTCV-218, a Cavendish selection with good tolerance to *Foc* race 4 isolates belonging to vegetative compatibility groups (VCGs) 0120 (Chapter 2) and 0121 (Hwang and Ko, 2004). VCG0120 is widely distributed throughout the world, but became particularly notorious for causing disease to Cavendish bananas in the subtropics (Ploetz, 1994).

The search for genes conferring resistance to diseases and pests has become an important objective towards developing genetically improved banana plants. At least three different classes of genes play a role in the defence strategy of a plant (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 hinders 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. These include major resistance (*R*) genes and result in the inhibition of pathogen growth (Jørgensen, 1994). A plant that possesses an *R*-gene has resistance to a specific pathogen containing the corresponding *avr*-gene. A *R*-gene to *Foc* in banana has recently been identified in *M. acuminata* Colla *malaccensis* Simmonds (Pereza-Escheverria *et al*., 2004), and transgenic plants containing the *RGC*-2 gene are currently being evaluated to ascertain whether this gene confers resistance to *Foc*.

Studying genes that are expressed in plants when they are infected by pathogens is an important step towards understanding gene function and molecular mechanisms underlying plant defence responses. These genes can be isolated and identified using a highly effective and very powerful Polymerase Chain Reaction (PCR)-based technique for generating enriched complimentary (c)DNA libraries, known as Suppression Subtractive Hybridisation (SSH) (Diatchenko *et al.* 1996; Diatchenko *et al.* 1999). SSH has numerous applications, mostly in clinical medical studies (Carmeci *et al.,* 1998; Kuang *et al.,* 1998). Over the past few years, SSH has also had an impact on agriculture, where it has been successfully applied for gene discovery in plant-pathogen interactions (Birch *et al.,* 1999; Beyer *et al.,* 2001; Hein *et al.,* 2004; Lu *et al.,* 2004).

SSH involves the hybridisation of cDNA, generated from mRNA that is transcribed upon pathogen attack, from plants that contain the target genes ("tester") and plants that serve as reference material ("driver"). Gene sequences that are similar in the "tester" and "driver" material will hybridise and be removed during the SSH reaction, while those genes that are uniquely expressed in the "tester" but not in the "driver" will not be hybridised. In the process, differentially expressed genes will be selectively amplified by means of suppression PCR (Diatchenko *et al.,* 1996; Gurskaya *et al.,* 1996).

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 further contribute to the development of biotechnological strategies to fight the disease. The aim of this study, therefore, was to use SSH to isolate genes that are differentially up-regulated in GCTCV-218, but not in the susceptible Cavendish banana variety, Williams, upon challenge with *Foc* 'subtropical' race 4 VCG 0120.

MATERIALS AND METHODS

Inoculation of Banana Plants with *Foc*

Plant material and growth conditions

Micropropagated Cavendish banana plantlets tolerant (GCTCV-218) and susceptible (Williams) to *Foc* race 4 were obtained from Du Roi Laboratories in Letsitele, South Africa. All plantlets were transplanted into plastic cups containing water, fertilised regularly with a nutrient solution and maintained in a greenhouse at 18/25ºC with a 16-hrs natural sunlight/8-hrs dark photoperiod (Chapter 2). After 4 weeks the plantlets developed a strong root structure, and were transferred to an aeroponic system. The aeroponic system comprised five separated compartments that could accommodate six plants per compartment. Strips of sponge were wrapped around the pseudostems of each plant before they were stabilized in holes drilled through the Perspex lids of each compartment, with their roots hanging above a fine mist sprayer. The automated mist sprayer was set to irrigate the roots for 30 seconds every 2 min. To allow the banana roots to recover from stress related to the planting procedure, plants were kept in the aeroponic system for at least 10-14 days before inoculations were carried out. All the plants were kept under the same light and temperature conditions and were fertilised every 2 days.

Preparation of inoculum

Three highly virulent *Foc* isolates representing VCG 0120, CAV 045, 092 and 105, were used to inoculate the roots of banana plantlets. Each isolate was first grown on half strength potato dextrose agar (PDA) medium in 90-cm-diameter Petri dishes at 25°C for 7-10 days. Sterile distilled water (SDW) was then poured onto the sporulating cultures, and the hyphae abraded with a sterile glass rod to loosen the spores. The spore suspensions were then removed from the Petri dishes, mixed, filtered through sterile cheesecloth, and diluted to a final concentration of 10^5 spores.ml^{-1}.

Inoculation and sample collection

Once the banana plantlets developed into 15-cm plants, their roots were inoculated with *Foc*. For inoculation, a small wound was made by first puncturing the healthy root with a sterile needle. A single droplet of the *Foc* spore suspension was then placed onto the wound, and the inoculation points sealed with Parafilm (Pechiney Plastic Packaging, Menasha WI, USA) to ensure that the spores were not washed off during irrigation. Six plants and six roots per plant were inoculated.

Root tissue for RNA extraction was collected 0, 3 and 6 hrs after inoculation from three plants. The conidia of *Foc* germinate within in 3 hrs and by 6 hrs the plant would most likely have responded in some way. To ensure that the root tissue was fungus-free, samples were taken 1 cm away from the point of inoculation. Root tissue from three different plants was pooled for each individual time point, placed in liquid nitrogen, ground with a homogenizer (IKA A11 Basic analytical Mill, United Scientific (Pty) Ltd., San Diego, USA) and stored at -80ºC. The remaining three inoculated plants of both GCTCV-218 and Williams were maintained in the greenhouse for six weeks, after which they were evaluated for disease development. Disease rating was done using the rating scale for Fusarium wilt of banana (Carlier *et al*., 2002).

Total RNA isolation

Total RNA was extracted from 100 mg banana roots per treatment, using the RNeasy Plant Mini kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. Isolated RNA samples from the same time points were combined and stored at –80ºC. The quantity of RNA was determined with a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The RNA was analyzed for the presence of distinct ribosomal bands and the absence of degraded RNA by gel electrophoresis under non-denaturing conditions on 1% (w/v) agarose gels. Total RNA samples were treated with RNase-free DNase I (Fermentas, Life Sciences, Hanover, USA) to degrade single-stranded (ss) and double-stranded (ds) DNA (Ausubel *et al.,* 1999).

cDNA synthesis

Ds cDNA was synthesized from total RNA using a cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. First strand synthesis was carried out in a reaction volume of 21 µl, containing 20 µg RNA, 2 µl oligo dT_{15} primer (200 µM) and RNase free water. The sample was incubated at 70ºC for 10 min and immediately placed on ice. The following components were added: 8 µl 5x Reverse Transcriptase-buffer, Avian Myeloblastosis Virus (AMV), 4 µl 0.1 M dithiothreitol (DTT), 2 µl AMV RT (25 U/µl), 1 µl RNase Inhibitor (25U/µl) and 4 µl 10 mM dNTP-mix. After mixing, the sample was incubated at 42ºC for 60 min and immediately placed on ice to terminate the reaction. Second strand synthesis followed immediately by taking 40 µl of the cDNA from the first strand reaction, 30 µl 5x second strand buffer, 1.5 µl 10 mM dNTP-mix, 6.5 µl second strand enzyme blend (mixture of DNA polymerase I, *Escherichia coli* ligase and RNase H) and 72 µl redistilled water. The reaction was mixed gently and incubated at 16ºC for 2 hrs, followed by the addition of 20 µl (20 U) T4 DNA polymerase and incubation for a further 5 min. The reaction was terminated by adding 17 µl 0.2 M EDTA (pH 8.0). Ds cDNA was purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

To determine whether cDNA was contaminated with genomic DNA, a cDNA sample was screened for a small fragment of the banana actin gene by PCR-amplification, using the primers ActinF (5'ACCGAAGCCCCTCTTAACCC-3') and ActinR (5'-GTATGGCTGACACCATCACC- 3') (Fig. 1). PCR amplifications were carried out using first strand cDNA and genomic DNA as templates. The PCR was conducted in 20 µl and contained 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM dNTP's (Roche Diagnostics), 0.25 µM of each primer, 1 U Taq polymerase (Roche Diagnostics) and 10 ηg DNA. The samples were heated at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min, followed by a final elongation step at 72ºC for 7 min. The PCR was conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, Perkin Elmer, Ontario, Canada), and the PCR products were separated by electrophoresis on a 2% agarose gel.

Suppression Subtractive Hybridisation (SSH)

cDNA derived from RNA extracted 3 and 6 hrs after inoculation of GCTCV-218 with *Foc* was pooled and served as "tester", and cDNA derived from RNA extracted from Williams plants at the same time points after inoculation by *Foc* served as "driver". "Tester" and "driver" material was then subjected to SSH analysis (Diatchenko *et al.* 1996; 1999) using a PCR-Select cDNA Subtraction kit ™ (Clontech, BD Biosciences, Palo Alto, California) according to the manufacturer's instructions, but with a ratio of 300:1 "tester" to "driver" material in the primary hybridisation.

Monitoring SSH efficiency

Reduction in actin levels. cDNA enriched for differentially expressed transcripts was referred to as "subtracted tester" (ST), cDNA from infected tolerant and susceptible banana plants was referred to as "unsubtracted tester" (UT) and unsubtracted "driver" (UD), respectively. Reduction in actin levels during the SSH procedure was determined by comparing cDNA levels in the ST with that of UT and UD. Ten ηg of the ST, UT or UD was amplified with 0.33 µl of the primer pair ActinF and ActinR (15 µM stock) in a 20 µl PCR reaction. The reaction further contained 2.0 µl PCR buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl), 2.0 µl dNTP mix (2.5 mM stock) (Roche Diagnostics) and 0.2 µl Taq polymerase (5 U/µl) (Roche Diagnostics). Primers were designed to amplify a cDNA fragment of the actin gene that flanked an intron and did not contain an *Rsa*I restriction site (Fig. 1). Each sample was denatured at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min, before a final elongation step at 72ºC for 7 min. Amplification products (5 µl) were collected after 20, 25 and 30 cycles and separated on a 2% agarose gel. The amplified fragment was also sequenced to confirm that it was from the actin gene.

Presence of a banana defence-associated gene. Primers were designed from the genebank nucleotide sequence of endochitinase (AF 416677) to amplify a cDNA fragment of 149 bp that did not contain a *Rsa*I restriction site. The presence of endochitinase in the ST material was then determined by PCR amplification using 10 ηg of the ST, 2.0 µl PCR buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl), 2.0 µl dNTP mix (2.5 mM) (Roche Diagnostics), 0.33 µl of EndochitF (5'-GCCACCGTCAGAGGTTATACAG- 3') and EndochitR (5'-GACTATTAAGGGCTCCGTGGTT- 3') (15 µM stock) and 0.2 µl Taq polymerase

(5U/µl) (Roche Diagnostics). Samples were denatured at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 1 min, followed by a final elongation step at 72ºC for 7 min. PCR products were then visualised on a 2% agarose gel and sequenced to confirm their identity.

Southern analysis

Adaptor sequences were removed by digesting 10 µl each of ST, UT and UD cDNA with RsaI (Roche Diagnostics). PCR products were then separated through a 1.5% low melting point (LMP) agarose gel, excised and purified using a QIAquick® Gel Extraction Kit (Qiagen). The ST, UT and UD cDNA (25-50 ηg) was diluted to 47.5 µl with SDW, denatured at 95°C for 3 min, and placed on ice for 2 min. Denatured cDNA was labelled with Ready-To-Go ™ labelling beads (-dCTP) (Amersham Biosciences UK Limited, England) in a reaction mixture containing buffer, dATP, dGTP, dTTP, FPLCpure™ Klenow fragment and random nonamers. Twenty five µCi radioactive labelled [α-^{32}P] dCTP was added and the reaction mixture incubated at 37°C for 20 min. Unincorporated radiolabeled nucleotides were removed using MicroSpin™G-50 Columns (Amersham Biosciences) using the manufacturer's instructions. Prior to use, the labelled ds DNA was denatured at 95°C for 10 min followed by incubation on ice for 3 min. The probe was centrifuged briefly and immediately used for the hybridization

Southern-based screening was performed as described by Southern (1975). PCR products from ST, UT and UD material were separated on three replicate 2% gels, visualized under UV light and photographed, and 5 µl of each sample was transferred to a Hybond-N$^+$ nylon transfer membrane (Amersham-Pharmacia). DNA transfer was carried out via alkaline capillary blotting with transfer buffer (1.5 M NaCl, 0.5 M NaOH) for 16 hrs. The membrane was rinsed in 2x Sodium Saline Citrate (SSC) (300 mM NaCl, 30 mM Na_3citrate-2$H_2$0 (pH 7.0), air-dried and cross-linked on top of an Ultraviolet Cross linker CL-508 (UVI-tec, St John's Innovation Centre, Cambridge, England) at 0.167 J (312 nm) for 2 min. Blots were wrapped in cling film and stored at –20°C. Hybridization of membranes was carried out as described by Ausubel (1999) with ^{32}P-radiolabelled PCR-amplified ST, UT and UD probes.

Subtracted library construction

To construct a cDNA library, SSH products were size fractioned on a 1.5% LMP agarose gel and separated into fragments ranging from 150-400 bp and others ranging from 400-700 bp. The fragments were then extracted from the agarose gel using a Qiaquick® Gel Extraction kit (Qiagen). Non-fractionated DNA was also purified with the Qiaquick® PCR purification kit (Qiagen) and included in the library. The cDNA clones were all ligated with pGEM-T Easy vector (Promega, Madison, USA). Ligation reactions were carried out in 10 µl reaction volumes containing 10-50 ηg insert DNA, 50 ηg pGEM-T Easy vector, 1x rapid ligation buffer and 3 Weiss units T4-DNA-Ligase (Promega). Samples were incubated for 16 hrs at 4ºC and stored at -20ºC.

Escherichia coli JM 109 competent cells (Promega) were transformed with cDNA fragments by heat-shock as recommended by the manufacturer. Following the transformation, cells were plated on Luria-Bertani (LB) agar plates (10 g l^{-1} peptone, 5 g l^{-1} yeast, 5 g l^{-1} NaCl, 15 g l^{-1} agar, 100 µg ml^{-1} ampicillin, 60 µg ml^{-1} X-gal and 60 µg ml^{-1} Isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37ºC for 16-18 hrs. Identification of the recombinant clones was done by colour screening on Xgal/IPTG indicator plates. A blue/white selection was used and 300 recombinant (white) colonies were picked for each ligation reaction (non-fractionated, and size fractionated). The library was replicated by transferring recombinant transformants to 1.5 ml tubes containing 700 µl LB amended with 50µg/ml ampicillin and grown for 20-24 hrs at 37ºC on a rotary shaker (250 rpm), and 300 µl 50% glycerol was added to each tube prior to storing at -80ºC.

Colony PCR and sequencing of selected clones

Colony PCR was conducted on 15 randomly selected clones in 25 µl reaction volumes containing 2.5 µl PCR buffer (10 mM Tris-HCl; 1.5 mM MgCl2; 50 mM KCl), 2.0 µl dNTP mix (2.5mM) (Roche Diagnostics), 0.5 µl of each of the primers T7 (5'-ATTATGCTGAGTGATATCCC-3') and SP6 (10 µM) (5'-ATTTAGGTGACACTATAGAAT-3'), 0.5 µl Taq DNA polymerase (5 U/µl) (Roche

Diagnostics), 13.5 µl SDW and 0.5 µl of the bacterial colony grown in LB. PCR conditions were as follows: Initial denaturation at 94ºC to avoid the preferential cloning of highly abundant or small DNA molecules for 2 min, 25 cycles of 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 1 min, and a final elongation step at 72ºC for 5 min. PCR products were then separated by electrophoresis on a 1.5% agarose gel to confirm successful insertion of cDNA fragments.

Cycle sequencing (Griffin and Griffin, 1993) was carried out in 10 µl reaction volumes containing 150 ηg template DNA (derived from colony PCR products), 2 µl Big Dye termination reaction mix (V3) (Applied Biosystems, Foster City, CA, USA), 2 µl primer T7 (10 µM) and 4 µl 5x dilution buffer (400 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$). Samples were cycled 25 times at 96ºC for 10 sec, 50ºC for 5 sec and 60ºC for 4 min. Sequenced products were diluted 1:20 and purified by adding 2 µl 3 M NaOAc (pH 4.6) and 50 µl cold absolute ethanol to the reactions, followed by 10 min incubation on ice and centrifugation at 10 000 rpm for 30 min at 4ºC. The supernatant was removed and the product washed to remove salts and excess dye terminators, using 250 µl 70% ethanol. This was followed by centrifugation for 10 min at 10 000 rpm at 4ºC. Samples were dried in an incubator at 37ºC for 5 min and stored at –20ºC. DNA sequences were analyzed on an ABI PRISM 377 DNA analyzer (Perkin Elmer) at the DNA Sequencing Facility of the University of Pretoria, South Africa.

Vector and SSH adaptor sequences were removed manually using Vector NTI® Suite V.6 (InforMax®, North Bethesda, USA) and database searching utilized BLAST software (Altschul *et al.,* 1990), available through the National Centre for Biotechnology Information (NCBI) (website http://www.ncbi.nih.gov/BLAST). Homologies were identified by BLASTX. The degree of sequence similarity between banana cDNA clones and known sequences was represented by E-values.

RESULTS

Inoculation of Banana Plants with *Foc*

GCTCV-218 and Williams plants point-inoculated with *Foc* developed typical internal Fusarium wilt symptoms after six weeks. The entire corm of susceptible Williams plants showed brown-purple discoloration, while the corms of tolerant GCTCV-218 plants only showed one or two brown specs. All three Williams plants had a disease severity score of 5, while the three GCTCV-218 plants had disease ratings of either 1 or 2. These results showed that the inoculation technique was effective in causing disease.

RNA isolation

Yields of between 15-30 μg total RNA for individual samples of GCTCV-218 and Williams were extracted from banana roots. However, this concentration was reduced substantially when RNA was passed through the Qiagen cleanup column, and numerous extractions had to be done to obtain sufficient material for cDNA synthesis. No degradation of RNA was visible for GCTCV-218 and Williams RNA samples at 0, 3 and 6 hrs after inoculation on 1% agarose gels, and two distinct bands were present (Fig. 2).

cDNA synthesis

Successful synthesis of cDNA for both GCTCV-218 and Williams was confirmed by PCR amplification from complementary and genomic DNA of the two banana varieties. Amplification of genomic DNA with the Actin gene primer pair resulted in a 260 bp fragment. This fragment is larger than the 170 bp fragment produced for first strand cDNA (Fig. 3). The difference in size is a result of the absence of a 90-bp intron sequence in cDNA, indicating that there was no genomic DNA present in the cDNA samples. Sequencing confirmed that the amplified product was a fragment from the actin gene.

Suppression Subtractive Hybridisation (SSH)

Reduction in actin levels. Amplification of the actin gene from ST material yielded no visible PCR product, even after 30 PCR-cycles (Fig. 4). However, specific PCR-products of 170 bp became visible after 20 cycles for the UT and UD, with intensities increasing after further PCR-cycles. Assuming that PCR amplification is 100% efficient during all amplification cycles and is, therefore, exponential, each cycle (x) difference represents a 2x times less actin template in subtracted material compared to unsubtracted material. In this subtraction procedure, ST would be expected to contain at least 210 times less actin template than UT and UD. Although these figures are theoretical, they emphasise the success of removing common sequences during the SSH.

Presence of a banana defence-associated gene. A 149 bp fragment of endochitinase was amplified in the ST material to show successful enrichment of a defence gene in the subtracted material in response to pathogen infection (Fig. 5). Sequencing results confirmed that the amplified fragment was from endochitinase.

Southern Blot analysis

Distinct bands were produced by the ST during PCR amplification compared to the product "smear" produced for the UT and UD (Fig. 6), indicating that the ST had been enriched for a number of differentially expressed products. During Southern analysis, ST probes bound primarily to the ST PCR product, with substantially less hybridization to UT and UD PCR products (Fig. 7). UT hybridised mainly to both UT and UD, representing common genes in UT. Furthermore, very little UD probe hybridized to the ST sample (Fig. 7), demonstrating that gene transcripts common to both "driver" and "tester" had been removed by the subtraction, and thus implying an enrichment for "tester" specific transcripts.

Subtracted library construction

A cDNA library of 736 banana clones expressed early after *Foc* infection was constructed, with cDNA clones ranging in size from 150 to 700 bp. The non-fractionated ST material yielded 250 clones. The fraction containing fragments between 150 and 400 bp long yielded 240 clones, and the fraction containing fragments between 400 and 700 bp long yielded 246 clones.

Colony PCR and sequencing

Colony PCR indicated that transformation of cDNA clones was successful and that the majority of vectors had only single inserts that varied in size from 150 to 600 bp (Fig. 8). Clones that had more than one insert were discarded from the library. A small subset of 15 cDNA clones was sequenced to check the quality of the cDNA library. Most cDNA clones that were sequenced (except clone 2-8) showed significant similarities to plant genes and thus were assumed to be derived from the host and not from the pathogen (Table 2).

Two of the 15 clones putatively identified by BLASTX searches showed homology to peroxidases from rice, and one each to an unspecific monooxygenase cytochrome P450, isoflavone reductase, Bowman Birk proteinase inhibitor, putative senescence associated protein, auxin protein, cytosolic monohydroascorbate reductase, UTP-glucose glucosyltransferase, reversibly glycosylated polypeptide and a putative transcription factor. Two clones had no significant homology and two showed homology to unknown proteins from barley (Table 2). Six of the 15 clones sequenced, were grouped in the functional category associated with defence/stress responses, two clones had sequences involved in metabolism and one clone had a sequence that could play a role in transcription. The level of redundancy in the 15 sequenced clones was low; only two clones for a peroxidase and an unknown protein occurred twice.

DISCUSSION

In this study, SSH was successfully utilised to isolate more than 700 cDNA clones in the tolerant Cavendish banana cultivar, GCTCV-218, in response to infection by *Foc*. A number of these clones showed significant sequence similarities to defence-associated genes. The variety of putative gene functions that were assigned to the banana clones selected for sequencing, provided confidence in the SSH library. The isolation of theses genes at an early time point reveals that the tolerant banana, GCTCV-218, recognises *Foc* and responds at the transcriptional level.

Two of the clones showed homology to a peroxidase gene from *Oryza sativa* L. This is relevant because peroxidases have been implicated in several physiological processes of importance in plant-pathogen interactions including lignification (Lagrimini et *al.,* 1987), cross-linking of cell-wall proteins (Bradley *et al.,* 1992), wound healing (Sherf *et al.,* 1993) and papillae formation (Cadena-Gomez and Nicholson, 1987). Enhanced peroxidase activity after pathogen infection has also been correlated with resistance in many different host pathogen systems.

The BLASTX search showed a clone sharing similarity with an isoflavone reductase from *Pyrus communis* L. This enzyme catalyzes the penultimate step in the synthesis of isoflavonoid phytoalexins that play a role in plant defence (Ibrahim and Varin, 1993). One clone had homology to a Bowman-Birk type protease inhibitor from kidney bean. The synthesis of theses inhibitors may be induced by pathogen infection or wounding (Qi *et al.,* 2005) and they have been linked with enhanced protection of plants against insects and microorganisms (Deshimaru *et al.,* 2004). The BLASTX search also showed a clone with homology to cytochrome P450. This compound plays a role in secondary metabolism by being involved in the phenylpropanoid biosynthesis pathway (Dowd *et al*., 2004) that leads to lignin production.

Results of this study represent the first effort to isolate genes in banana with resistance to *Foc*. Even though conventional breeding has been used by several banana improvement programmes to introduce resistance to pathogens and pests (Hwang and Tang, 1996), almost nothing is known about the genes underlying such resistance. Although several technologies are available to discover these genes (Cochran *et al.,* 1983; Duguid and Dinauer, 1990; Liang *et al.,* 1993; Lisitsyn and Wigler, 1993; Velculescu *et al.,* 1995; Schena *et al.,* 1995), SSH used in this study has numerous advantages above other gene isolation methods, and it was effective in isolating differentially expressed genes from banana. An advantage of SSH is that it allows the detection of low-abundance, differentially expressed cDNAs, such as those involved in signalling and signal transduction (Birch *et al*., 2000).

The SSH procedure appeared to be efficiently executed. Small quantities (20 μg) of RNA from banana roots were sufficient to set up the SSH experiment. The isolation of mRNA from total RNA yielded very little material (data not shown) and total RNA was thus used for the procedure. This is important in an experiment where only a limited amount of starting material can be obtained. Constitutively expressed genes, such as the actin gene were removed during subtraction hybridisation, while induced resistance genes, such as endochitinase, were found in the SSH final product. Southern blot analysis further confirmed the subtraction efficiency of the SSH technique. Southern blot data suggested that the subtracted "tester" had been enriched for differentially expressed transcripts, and that "housekeeping" gene transcripts that are common to both "driver" and "tester" had been removed by the subtraction.

From the limited number of clones sequenced in this study, it was clear that SSH was effective in enriching for pathogen-induced transcripts from as early as 3 and 6 hrs after inoculation with *Foc*. Rare transcripts, such as the putative transcription factor isolated in this study, are often not detected by other methods such as differential display (DD) (Liang and Pardee, 1992) reverse transcriptase (RT) PCR and cDNA-amplified fragment length polymorphisms (AFLP) due to competition within the PCR (Birch *et al*., 2000).

In most cases, the Clontech PCR-Select cDNA subtraction method greatly enriches for differentially expressed genes. Nevertheless, the subtracted "tester" will still contain some cDNAs that have escaped the subtraction and correspond to mRNAs that are common to both "tester" and "driver" samples. This type of background (false positives) may somewhat depend on RNA quality and purification and the performance of the specific subtraction, but it mainly arises when very few mRNA species are differentially expressed in "tester" and "driver" (Clontech PCR-Select™ cDNA Subtraction Kit User Manual, 2000). It is important and essential to subject the library to a differential screening method. Therefore, the cDNA library was screened (Chapter 4) enabling us to select clones for a more comprehensive study.

The cDNA library constructed from genes differentially expressed in Cavendish bananas after infection by *Foc* could be applied usefully in a number of ways. Firstly,

the identification of host genes involved in defence responses is one of the most critical steps leading to the understanding of disease resistance mechanisms in plants (Xiong *et al.,* 2001). Secondly, the genes can be used as markers for resistance traits in plant improvement programmes, and thirdly, the genes can be used for genetic improvement of plants via transformation. The manipulation of defence-associated genes and their signalling pathways by transgenic expression is a promising strategy to improve disease resistance in plants (Martin, 1999).

This study is the first of its kind, utilising SSH for isolating defence associated genes in banana against *Foc*. It could contribute significantly to the understanding of gene expression profiles associated with defence mechanisms in monocotyledonous plants.

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GCAACTGTTTATGATANGGTTGAAGATCTGGCACCATACCTTCAACAATG     50
                                                  5'
AGCTCCGTGTTGCTCCTGAGGAGCATCCTATTCTGCTCACCGAAGCCCCT    100
                                       specific primer
              3'
CTTAACCCAAAGACAAACAGGGAAAAGATGACCCAGATAATGTCTGAAAC    150
ACTINF

TTTCATTGTACCAGCTATGTATGTTGCTATTCAGGGCGTCCTTTCGCTCT    200

ATGYWAGTGSCSGTACTAGTGGTTAGTATCTTGTGATCGCTTACCTGATG    250

TGRAGTTGGTCATTCTTTTTATTTTCAACTRAGATGCCCAAGYTAAATGC    300
                            3'                 5'
TATTTTGACAGGCATTGTACTTGATTCTGGTGATGGTGTCAGCCATACCG    350
                            specific primer ACTINR

TTCCTATCTATGAAGGATATGCTCTACCTCATGCCATTCTTCGTTTGGAT    400

CTTGCTGGTCGTGATCTCACGGATTGCCTGATGAANATCCTGACAGAGAG    450

GGGTTATTCATTCACTACTACTGCAGAACGGGAAATCGTAAGGGACATCA    500

AGGAGAARCTTGCCTACGTTGCTGKTGACTATGAACAGGAGCTGGATACT    550

GSCGAGACTAGCTCTGCTGTGGAGAAAASYWWKRAMCTTCCCGTTGGGCA    600

RGKTATCACGAWTGGGGCTNRARRWTYANGAGCCCCGGANGTGCTNTYCC    650

AGCCATCATTGATTGGCATGGAARCCNNTGGAGTTCATGAGACAACATAC    700

AACTCTATTATGAAGTGTGATGTGGATATCAGGAAAGATCTGTATGGCAA    750

TATTGTGCTTAGCGGTGGATCAGCAATGTTCCCTGGTATTGCCGATCGCA    800

TGAGCAAGGAGATCACAGCGCTTCCACCAAACAGCATGAAGATAAAGGTG    850

GTTGCCCCACCCGAAAGGAAATACAGCGTCTGGAT                   885
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Figure 1. Sequence of the cloned actin gene used for Actin gene-based PCRs.

Yellow: Actin Forward primer, Blue: Actin Reverse primer and Red: Intron (90 bp).

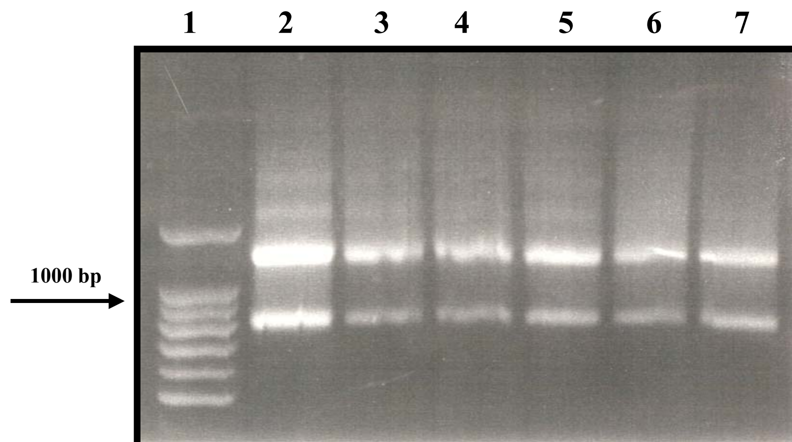

Figure 2. RNA samples from banana root material under non-denaturing conditions. Lane 1 contains the 100 bp marker (Roche Diagnostics), lanes 2-4 GCTCV-218 RNA and lanes 5-7 Williams RNA.

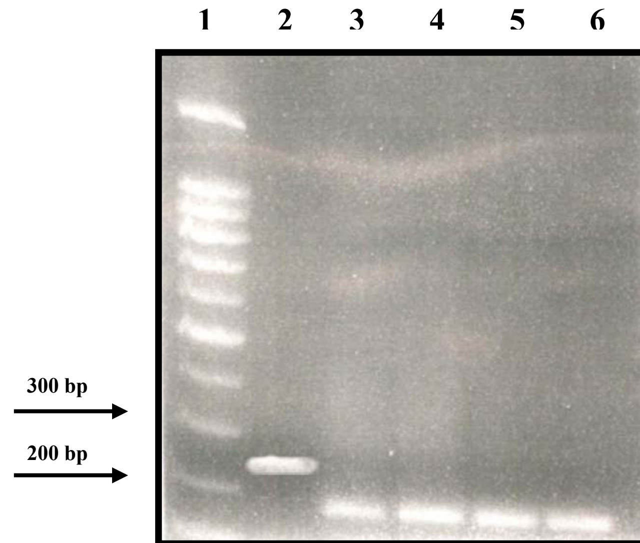

Figure 3. Actin-based control for monitoring contamination of cDNA with genomic DNA. PCR products from amplification (30 cycles) of genomic DNA (260 bp) (Lane 2), PCR products from second strand GCTCV-218 cDNA (Lanes 3 and 4) and Williams cDNA (Lanes 5 and 6) (170 bp) using primer ActinF and ActinR, separated by electrophoresis through a 2% (w/v) agarose gel. 100 bp molecular marker (Roche Diagnostics) (Lane 1) was used to estimate the size of DNA fragments.

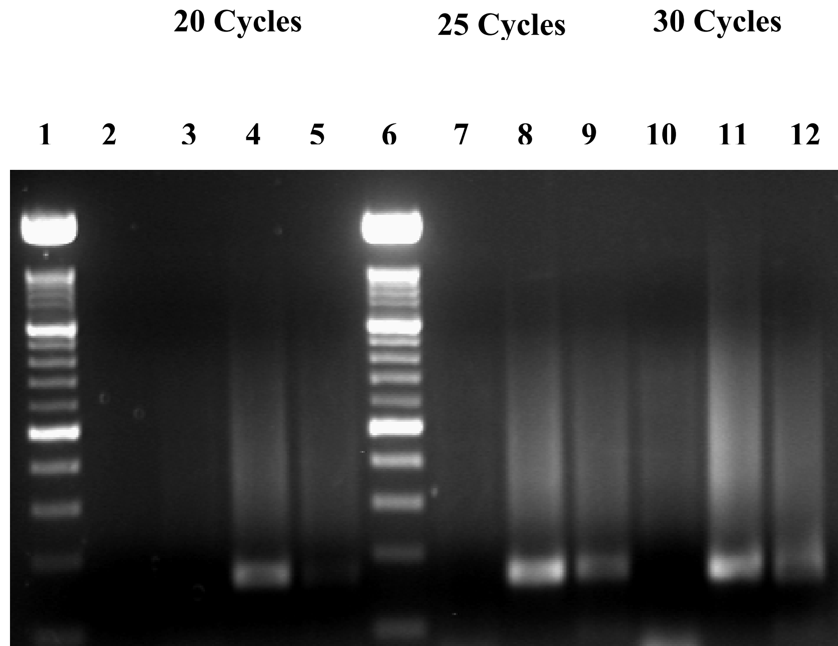

Figure 4. PCR-based assessment of the decrease in actin cDNA levels following Suppression Subtractive Hybridisation. Amplification of the banana actin gene from subtracted "tester" (ST), unsubtracted "tester" (UT) and unsubtracted "driver" (UD) cDNA samples. Lanes 1 and 6, 100 bp marker (Roche Diagnostics); lane 2, water control; lanes 3-5, ST, UT and UD after 20 amplification cycles; lanes 7-9, ST, UT and UD after 25 amplification cycles; lanes 10-12, ST, UT and UD after 30 amplification cycles.

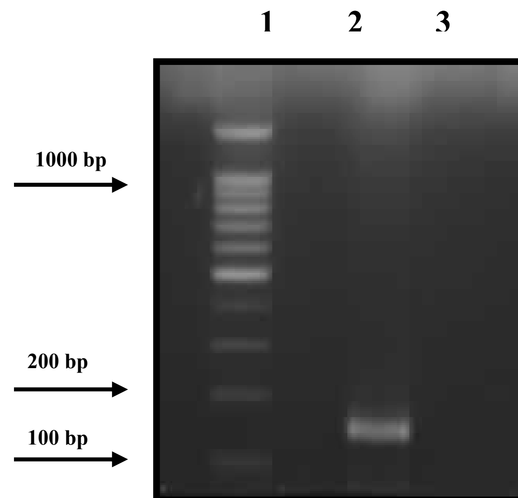

Figure 5. PCR-based assessment of the successful amplification of endochitinase in subtracted "tester" cDNA following Suppression Subtractive Hybridisation.

Lane 1, 100 bp marker (Roche Diagnostics); lane 2, water control; lane 3 endochitinase fragment (149 bp).

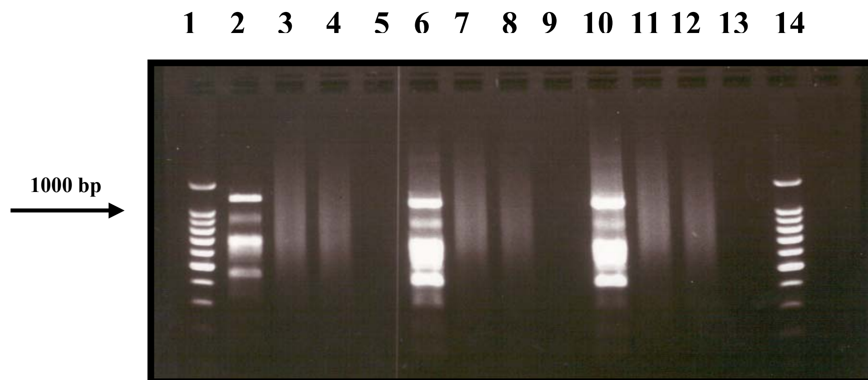

Figure 6. Agarose gel electrophoresis of Suppression Subtractive Hybridisation amplification products. Lane 1 and 14, 100 bp marker (Roche Diagnostics); lane 2, 6 and 10, subtracted "tester" PCR products; lane 3, 7 and 11, unsubtracted "tester" PCR products; lane 4, 8 and 12, unsubtracted "driver" PCR products. Lanes 5, 9 and 13, water controls.

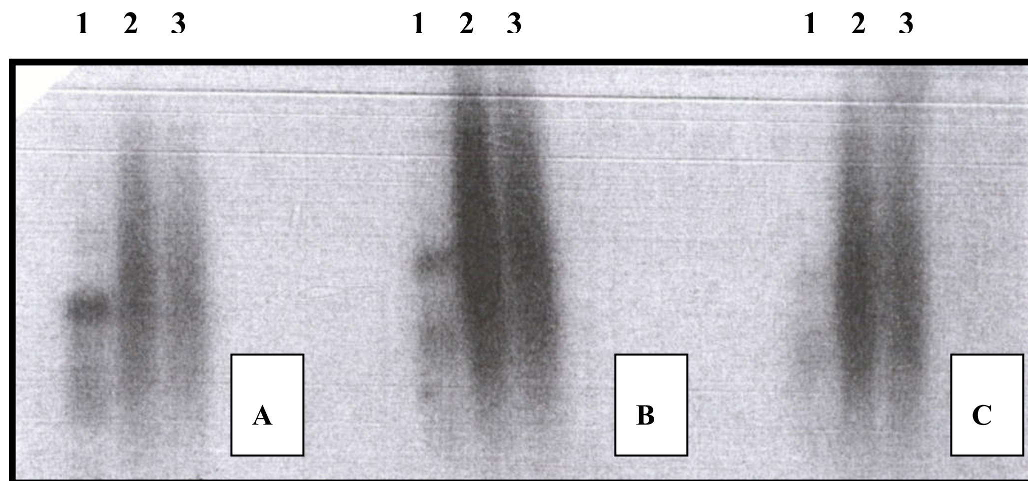

Figure 7. Southern analysis of subtracted "tester" (ST), unsubtracted "tester" (UT) and unsubtracted "driver" (UD) cDNA amplification products.

Amplification products in Fig. 6 were transferred to a positively charged nylon membrane and identical filters were independently hybridized with complex probes derived from forward ST cDNA (Filter A), UT cDNA (Filter B) and UD cDNA (Filter C). Lane 1, ST PCR products; lane 2, UT PCR products; lane 3, UD PCR products.

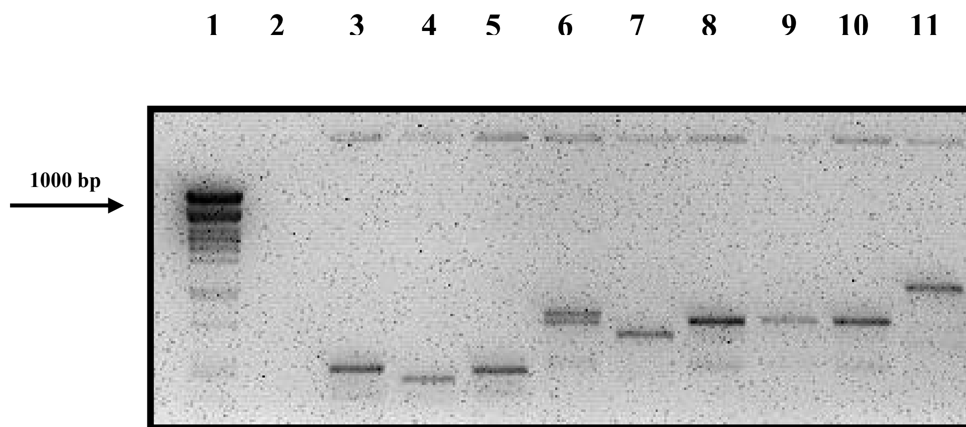

Figure 8. Colony PCR of nine selected Suppression Subtractive Hybridisation cDNA clones indicating single inserts (except lane 6) of variable sizes between 150 and 600 bp. Lane 1 contains the 100 bp molecular marker (Roche Diagnostics) used for size estimations.

Table 1. Primer and adaptor sequences used in the Suppression Subtractive Hybridisation procedure.

Primer or Adaptor name	Nucleotide sequence
PCR primer 1	5'-CTAATACGACTCACTATAGGGC-3'
Nested PCR primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested PCR2	5'-AGCGTGGTCGCGGCCGAGGT-3'
Adaptor 1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GATTATGCTGAGTGATATCCCGAGCTCGCCGGCGGGCCCGTCCA-5'
Adaptor 2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' 3'-GATTATGCTGAGTGATATCCCGTCGCACCAGCGCCGGCTCCA-5'

Table 2. Selected banana cDNA clones expressed in a tolerant banana, GCTCV-218, after *Fusarium oxysporum* f. sp. *cubense* infection, following Suppression Subtractive Hybridisation.

Clone number	Protein similarity	Origin of similar sequence	BLASTX Acc. No. of similar sequence	E-value	Putative Function
2-25	Unspecific monooxygenase, cytochrome P450	*Nicotiana tabacum* L. (tobacco)	pir|T02995	$7e^{-42}$	Defence
2-18	Isoflavone reductase related protein	*Pyrus communis* L. (pear)	gb|AA24001.1	$2e^{-70}$	Defence
1-1	Putative peroxidase	*Oryza sativa* L. (rice)	dbj|BAB19339.1	$6e^{-86}$	Defence
1-5	Putative peroxidase	*O. sativa*	emb| CAE05415.1	$2e^{-05}$	Defence/Stress
2-39	Bowman Birk type proteinase inhibitor	*Phaseolus vulgaris* L. (kidney bean)	sp| P01060| IBB2_PHAVU	$2e^{-05}$	Defence/stress
3-146	Putative senescence associated protein	*Pisum sativa* L. (pea)	dbj| BAB33421.1	$4e^{-43}$	Defence/Stress
3-130	Aux/IAA protein	*Solanum tuberosum* L. (potato)	gb| AAM29182.1	$5e^{-21}$	Auxin-related
3-91	Cytosolic monohydroascorbate reductase	*O. sativa*	dbj|BAA77214.1	$3e^{-04}$	Metabolism
3-26	UTP-Glucose Glucosyltransferase	*Arabidopsis thaliana* (L.) (thale cress)	pir|T01732	$3e^{-04}$	Metabolism (primary)
2-2	Unknown protein	*Hordeum vulgare* (L.) (barley)	gb| AAL77110.1	$5e^{-55}$	Other
2-147	Reversibly glycosylated polypeptide	*Gossypium hirsutum* L. (cotton)	emb|CAC83750.1	$1e^{-14}$	Other
1-97	No significant homology	-	-	-	Other
3-31	No significant homology	-	-		Other
1-95	Unknown protein	*H. vulgare*	AF 474373_7	$1e^{-49}$	Other
2-8	Putative transcription factor	*Periplaneta americana* L. (cockroach)	emb| CAB51041.1 (AJ243883)	$2e^{-14}$	Transcription

CHAPTER 4

HIGH-THROUGHPUT SCREENING OF A BANANA cDNA LIBRARY USING DNA MICROARRAY ANALYSIS

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High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis. Noëlani van den Berg, Bridget G. Crampton, Ingo Hein, Paul R.J. Birch, and Dave K. Berger.

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ABSTRACT

The efficient construction of a cDNA library enriched for differentially expressed transcripts is an important first step in many biological studies. This study presents a quantitative procedure for screening a banana cDNA library constructed by Suppression Subtractive Hybridisation (SSH). Following two-colour cyanin dye labelling and hybridisation of subtracted "tester" with either unsubtracted "driver" or unsubtracted "tester" cDNAs to the SSH library arrayed on glass slides, two values were calculated for each clone; an enrichment ratio 1 (ER1) and an enrichment ratio 2 (ER2). Graphical representation of ER1 and ER2 enabled the identification of clones that were likely to represent up-regulated transcripts. Normalization of each clone by the SSH process was determined from the ER2 values, thereby indicating whether clones represented rare or abundant transcripts. Differential expression of banana clones identified by this quantitative approach was verified by inverse Northern blot analysis.

INTRODUCTION

Genes that are differentially expressed in plants, and their pattern of expression, provide important information about processes such as cell differentiation, morphological and metabolic changes, and disease development. Various molecular techniques exist for studying differential gene expression. These include representational difference analysis (RDA) (Lisitsyn and Wigler, 1993), Suppression Subtractive Hybridization (SSH) (Diatchenko *et al*., 1996; 1999), differential display, differential hybridization (Liang and Pardee, 1992), subtractive library construction (Tedder *et al*., 1988), serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995), and cDNA microarray analysis (Schummer *et al*., 1997). The technology of cDNA microarray hybridization has become a standard tool for genome-wide monitoring of gene expression in animal studies, and is now also starting to contribute to plant biology (Reymond, 2001). cDNA microarray analysis offers the possibility of providing a rapid, high throughput method for screening SSH cDNA libraries for differentially expressed genes (Yang *et al*., 1999).

Genes that were up-regulated in Cavendish bananas tolerant to *Fusarium oxysporum* Schechtend.:Fr. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*) were isolated by means of SSH and compiled in a cDNA library (Chapter 3). In this study, microarray analysis was used to screen the banana cDNA library to identify genes expressed in response to *Foc* infection and to obtain information about the relative abundance of these gene transcripts upon the induction of the plant defence response. The aim was to identify and discard genes that escaped the differential subtraction process and to select defence response-associated genes for sequencing and expression studies. Such a quantitative approach would allow me to determine the extent to which a transcript was induced upon pathogen infection, to determine whether a transcript was rare or abundant, and to assess the redundancy of clone sequences comprising the SSH library.

MATERIALS AND METHODS

cDNA Microarray Analysis of the SSH Library

Preparation of SSH clones and slide spotting

A banana SSH library that contains 736 clones, expressed in tolerant GCTCV-218 but not in susceptible Williams bananas upon infection with *Foc* (Chapter 3), was used for cDNA microarray analysis. Clones from the library were PCR-amplified using T7 (5'-ATTATGCTGAGTGATATCCC-3') and SP6 (5'-ATTTAGGTGACACTATAGAAT-3') primers. The amplification procedure was conducted in a 100 µl reaction volume containing the following reagents: 10 µl 10x NH_4 buffer, 5 µl $MgCl_2$, 8.0 µl dNTP mix (2.5 mM) (Roche Diagnostics, Mannheim, Germany), 3.0 µl of T7 and SP6 (10 µM) and 0.2 µl BIOTAQ™ DNA polymerase (5 U/µl) (Bioline Ltd., London), 1.0 µl of the bacterial colony grown in Luria-Bertani (LB) broth and 70.2 µl sterile distilled water (SDW). PCR conditions were as follows: Initial denaturation at 94ºC for 2 min, 30 cycles of 94ºC for 30 s, 50ºC for 30 s, 72ºC for 1 min and a final elongation step at 72ºC for 5 min. PCR products were separated by electrophoresis on a 1% agarose Electro-Fast® Stretch gel (Abgene, Epson, UK), and purified using Multiscreen® PCR Purification Plates (Millipore, Molsheim, France). They were then eluted in 45 µl SDW prior to being robotically printed onto silanized microarray slides (Amersham Biosciences, Little Chalfont, UK) using an Array Spotter Generation III (Molecular Dynamics, Sunnyvale, CA, USA). On average, 300 ηg/µl of each banana SSH fragment was spotted onto each slide, in duplicate. For global normilisation, four fragments, *uidA* (300 ρg), *luc* (30 ρg) and *bar* (3000 ρg) genes, and a fungal rDNA internal transcribed spaces (ITS) fragment (3000 ρg), were spotted to serve as controls.

Probe preparation

Probes were prepared from cDNA transcripts called the subtracted "tester" (ST), unsubtracted "tester" (UT) and unsubtracted "driver" (UD). The ST represents cDNA transcripts expressed in tolerant (GCTCV-218) but not in susceptible (Williams) bananas, while the UT and UD represent those transcripts expressed in GCTCV-218 and Williams, respectively (Chapter 3). Prior to labelling PCR products of ST, UT

and UD were purified with a Mini Elute™ PCR purification kit (Qiagen, Valencia, California, USA) and digested with *Rsa*I (Roche Diagnostics) to remove the adaptors. Samples were separated on a 1.5% low melting point (LMP) agarose gel and the desired products without the adaptors were cut out and cleaned using the QIAquick® Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Two hundred nanograms of each purified ST, UT and UD probe were transferred to two tubes. Probes in one set of tubes were then labelled with Cy™5, while the other set was labelled with Cy3 dUTP (Amersham Biosciences) using Klenow enzyme (USB, Cleveland, OH, USA) (Ramonell *et al.*, 2002). Each probe was thereafter spiked with Cy-labelled *uidA* (0.3 ηg), *luc* (0.03 ηg) and *bar* (3 ηg) genes, and a Cy-labelled fungal ITS fragment (3 ηg). After incubation at 37ºC for 20 hrs, the probes were purified using a Multiscreen® PCR Purification Plate (Millipore) and eluted in 45 µl SDW. The probes were then pooled and dried in a SPD111V vacuum centrifuge (Savant, Holbrook, New York, USA), before being resuspended in Hybridization buffer (Amersham Biosciences).

Hybridization and washing

The glass slide spotted with the SSH library was incubated in pre-treatment solution (3.5x Sodium Saline Citrate (SSC); 0.2% Sodium Dodecyl Sulfate (SDS); 1% bovine serum albumin) at 60ºC for 20 min. Microarray slides were then placed face-up in a HybUP hybridization chamber (NB Engineering, Pretoria, South Africa) and covered with 20 µl of MilliQ water (Millipore, Simplicity 185) (Millipore). Thirty- five µl of the probe was placed on each slide, allowed to spread evenly, and covered with a cover slip. The slides were then hybridised at 42ºC for 16 hrs. After hybridization, the slides were washed with 1x SSC/0.2% SDS, 0.1x SSC/0.2% SDS for 4 min at 42ºC (twice), followed by three washes in 0.1x SSC/0.2% SDS, 0.1x SSC/0.2% SDS (twice) and three washes in 0.1x SSC for 1 min at room temperature. After the final wash, the slides were briefly rinsed with distilled water and dried with high-pressure nitrogen, and then scanned with a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA).

cDNA Microarray screening of the SSH library

The Array-Vision™ programme (Imaging Research Inc., Ontario, Canada) was used to localize and integrate the spots on the microarray slides. Green spots visible on the computer image represented transcripts that were more abundant in the probe labelled with Cy3, while red spots represented transcripts that were more abundant in the probe labelled with Cy™5. Yellow spots represented transcripts that were present in equal amounts in probes labelled with Cy3 and Cy™5.

Prior to mathematical quantification of the computer image of each clone, background signal intensities were subtracted, the signal intensities of duplicate spots averaged, and spots with a signal/noise ratio of less than 2 were rejected. Global normalization of data for the cyanin dye effect was performed using a control gene set to calculate normalization functions c and c' for each pair of dye swap slides (Yang *et al.*, 2002). Enrichment ratios of ST:UD (ER1) and ST:UT (ER2) were calculated from experiments performed in duplicate. To determine ER1, slides hybridized with ST and UD were used, and for ER2, slides hybridised with ST and UT were used. ER1 and ER2 were calculated using the formula of Yang *et al.* (2002):

$$\frac{1}{2} [\log_2 \text{Cy3 ST/Cy5 UD} - c - (\log_2 \text{Cy3 UD/Cy5 ST} - c')]$$

UT/UD values were calculated from the ER1 and ER2 values as follows:

Because $\text{ER1} - \text{ER2} \sim \log_2 \text{ST/UD} - \log_2 \text{ST/UT} = \log_2 \text{UT/UD}$, therefore:

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

Enrichment ratios ER1 and ER2 provided data on the abundance of transcripts. A positive ER1 and ER2 value indicated transcripts that were enriched during subtraction relative to their levels in UD or UT, respectively. Conversely, negative ER1 and ER2 values indicated transcripts that were reduced in abundance during SSH relative to their levels in UD or UT, respectively, due to normalization.

rRNA redundancy for the library was determined by hybridising the microarray slide with a banana rRNA probe. A clone was considered to have hybridised to the rRNA probe if fluorescence was more than two standard deviations above local background

fluorescence (Leung and Cavalieri, 2003). When artefacts were visible on spots, they were flagged and the data rejected.

Inverse Northern Dot Blots

Membrane preparation

A Hybond-N^+ nylon transfer membrane (Amersham Biosciences) was pre-wetted for 10 min in distilled water, fitted into a 96-well BioRad Biodot ™ manifold (Bio-Rad Laboratories Inc., Hercules, CA, USA) and flushed with 500 µl SDW. Three hundred nanograms of each SSH clone was made up to a 100 µl with SDW, denatured in a total volume of 200 µl containing 2 M NaCl and 0.8 M NaOH and incubated at 37°C for 15 min. One hundred µl DNA of each clone was then loaded into separate wells on the membrane. The clones were fixed to the membrane by applying a vacuum of 20 inches of Hg, followed by flushing the wells with a 100 µl solution consisting of 1 M NaCl and 0.4 M NaOH. The SSH clones were arrayed in duplicate on each membrane. After loading of the clones, the membranes were washed in 2x SSC and cross-linked on an Ultraviolet Cross linker CL-508 (UVI-tec, St John's Innovation Centre, Cambridge, England) at 0.167 J for 3 min and stored at –20°C.

Probe preparation, hybridisation and detection

Banana "tester" (GCTCV-218) and "driver" (Williams) probes were prepared from 30 µg total RNA collected from roots 3 and 6 hrs after infection with *Foc*. cDNA was synthesised from total RNA and labelled with DIG-dUTP using a DIG DNA Labelling and detection Kit (Roche Diagnostics). Hybridizations (Southern, 1975) were then performed using 40 ηg/µl of either the DIG-labelled "tester" or "driver" cDNA. Membranes containing the SSH clones were first pre-hybridized in 15 ml DIG Easy Hyb (Roche Diagnostics) at 42°C for 3 to 5 hrs. For hybridization, the probes were denatured at 96°C for 10 min, placed on ice and then added to 15 ml new Dig Easy Hyb, pre-warmed to 42°C. Membranes were incubated overnight at 42°C in a Hyb Oven. They were then washed twice under low stringency conditions (2x SSC/0.1% SDS) for 15 min at room temperature, followed by two washes under high stringency (0.5x SSC/0.1% SDS) for 15 min at 68°C. Hybridization signals were detected using CDP Star as outlined by the manufacturer (Roche Diagnostics) and

exposed to X-ray film. X-ray images were captured on the VersaDoc BIO-RAD imaging system Model 3000 (Bio-Rad Laboratories, Inc.) using Quantity One 4.4.1 software (Bio-Rad Laboratories, Inc.), and exported to Array vision™ (Imaging Research Inc.). Array vision™ (Imaging Research Inc.) was then used to calculate signal density for each spot, following normalization, by comparing values of rDNA dots. Inverse Northern expression ratios were calculated by dividing normalised density measurements for each clone hybridised with "tester" probes, with measurements for the same clones hybridised with the "driver" probe.

RESULTS AND DISCUSSION

cDNA Microarray Screening of the SSH Library

Hybridized glass slides were of high quality, with very low background, high signal/noise ratios and very few artefacts. Microarray slides where the ST was labelled with Cy3 and the UD labelled with Cy^{TM}5 produced a computer image with more green spots compared to red spots (Fig.1). This indicated that the SSH library mostly contained transcripts that were up-regulated in response to *Foc*. It is, however, important to bear in mind that the computer image does not provide quantitative data and it, therefore, was necessary to present the computer images as numerical data.

Enrichment ratios ER1 and ER2 provided data on the abundance of transcripts. A positive ER1 and ER2 value indicated transcripts that were enriched during subtraction relative to their levels in UD or UT, respectively. Conversely, negative ER1 and ER2 values indicated transcripts that were reduced in abundance during SSH relative to their levels in UD or UT, respectively, due to normalization. Normalization equalizes the concentration of individual transcripts, which may be present at very different concentrations prior to normalization (Diatchenko *et al.*, 1996). This therefore has the advantage of enriching for rare transcripts in the ST samples and reducing levels of abundant transcripts.

A relative abundance of cDNAs in UT and UD was found when plotting ER1 against ER2 for individual banana clones (Fig. 2). Clones that were plotted on the diagonal

line were derived from transcripts of equal abundance in UT and UD. The majority of banana clones (63%) were plotted above the line (ER1>ER2), indicating a greater abundance of these transcripts in UT than in UD. This confirmed that SSH enriched for transcripts that are up-regulated in the "tester" in response to *Foc* infection. Clones above the diagonal line with positive ER2 values (86%) were likely to be derived from low abundance transcripts. Expression of some of these clones might be difficult to detect in Northern blots, and accurate comparisons of gene expression between "tester" and "driver" might require real-time reverse transcription PCR (RT-PCR). Several clones above the diagonal line showed negative ER2 values (14%), representing transcripts enriched relative to levels in UD and reduced in abundance during normalization relative to levels in UT. These clones represent abundant transcripts specifically up-regulated in the "tester" that should be more easily detected in Northern blot analyses.

The SSH library used in the current study was prepared from total RNA, since isolation of mRNA proved to be difficult and resulted in extremely low concentrations. When the SSH library was screened with rDNA probes, they hybridised to 28% of the banana clones, showing that a substantial number of rDNA clones escaped the subtraction process. In future, it is suggested that a technique be used to isolate sufficient amounts of mRNA from banana roots for cDNA synthesis in order to increase the selection for poly $(A)^+$-tail fragments.

Inverse Northern Blot Analysis of Selected Transcripts

Results obtained by microarray analysis were confirmed by inverse Northern blot analyses. Clones with ER1>ER2 had inverse Northern expression ratios greater or equal to one (Table 1), confirming that these clones represented transcripts that were up-regulated in the "tester" compared to the "driver" (Fig. 2). Clones where ER1<ER2 showed inverse Northern expression ratios of less than one (Table 1), confirming that they represented transcripts that were more abundant in the "driver" than the "tester", and that these clones had escaped the subtraction process. In order to relate the inverse Northern and microarray data, a ratio of UT/UD was calculated from ER1 and ER2 ratios (Table 1). Inverse Northern ratios correlated with UT/UD

ratios (i.e., clones with inverse ratios >1 had UT/UD values ≥1, whereas clones with inverse Northern ratios <1 gave UT/UD values <1) (Table 1). This means that these transcripts were more abundant in the tolerant GCTCV-218 after *Foc* infection compared to the susceptible Williams.

The banana SSH library was screened by a high-throughput DNA microarray technique, to ensure that false positives were removed before the library was sequenced. The majority of clones showed ER1>ER2. The library yielded more clones that were reduced in abundance in ST relative to UT. Many of the clones that were enriched during the SSH were of equal or greater abundance in UD than UT and therefore many of the clones would be eliminated from further analysis. The experimental set-up of the SSH (challenging both "tester" and "driver" with *Foc*) led to many possible defence genes being eliminated from the subtracted material, as they were present in both the "tester" and "driver" samples. However, the scientific question was to investigate the unique difference in gene expression in the tolerant GCTCV-218 cultivar compared to the susceptible Williams upon infection with the Fusarium wilt pathogen.

In the past, SSH libraries were screened by reverse northern blot analysis or cDNA-AFLP to identify cDNA clones that were differentially expressed in plants (Birch *et al*., 1999; Mahalingam *et al*., 2003). However, these methods were time consuming, and did not allow for the quantitative analysis of the level of induction or abundance of a transcript upon treatment. cDNA microarrays proved to be highly effective for screening SSH libraries in a rapid, high-throughput manner. The advantages of this screening method are that it provides an objective and quantitative way to identify differentially expressed genes as well as to determine the relative abundance of transcripts in the original UT samples. In contrast to membrane-based methods where different membranes are compared with each other, hybridisation to glass slide cDNA microarrays can be performed with different fluorescent tags, which allows direct comparison of the relative abundance of transcripts in ST, UT and UD. Furthermore, hybridisations are performed on a small surface area, which reduces the amount of labelled probe needed. Finally, the computerised scanning of the array provides a

high-throughput quantitative method to choose which genes to sequence and study further using Northern blot analysis, real-time RT-PCR, or custom microarrays.

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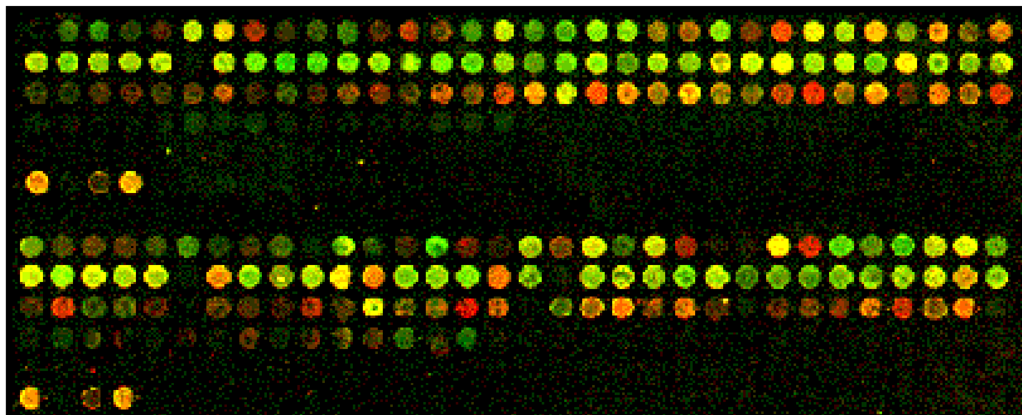

Figure 1. Microarray glass slide image of banana Suppression Subtractive Hybridisation clones spotted onto a glass slide and hybridised with subtracted "tester" (ST) (Cy3) and unsubtracted "driver" (UD) (Cy™5) probes. The Array-Vision™ programme (Imaging Research Inc., Ontario, Canada) was used to localize and integrate the spots on the microarray slides. Green spots represent transcripts that are more abundant in the ST, while red spots represent transcripts that are present in the UD. Yellow spots are present in equal amounts in both the ST and UD.

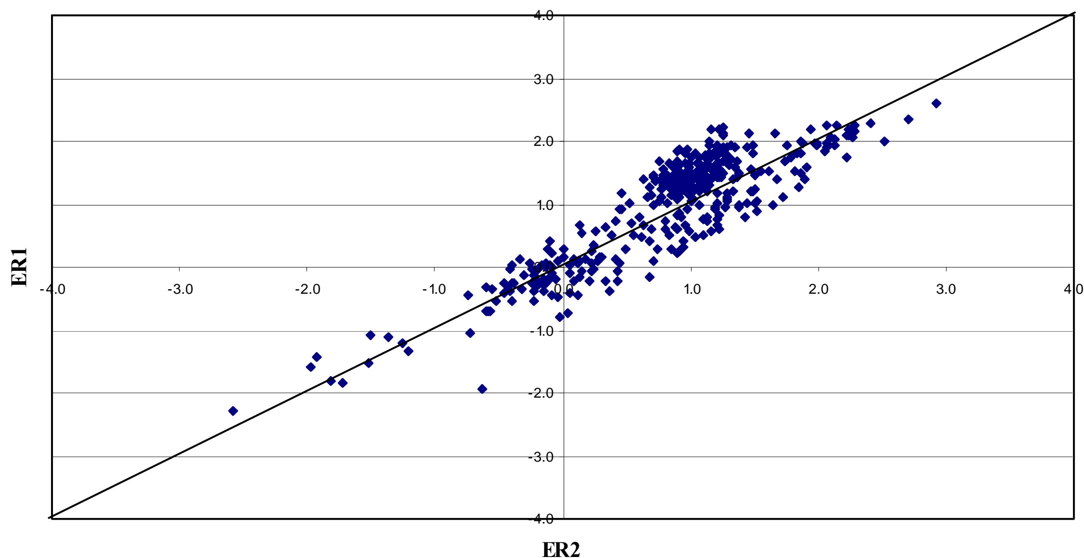

Figure 2. Suppression Subtractive Hybridisation (SSH) enrichment ratio 1 (ER1) is plotted against SSH enrichment ratio 2 (ER2). ER1 was calculated for each clone by $\log_2$ transforming the value of the subtractive "tester" (ST) fluorescence divided by the unsubtracted "driver" fluorescence (UD). ER2 for each clone was calculated by $\log_2$ transforming the value of the subtractive "tester" (ST) fluorescence divided by the unsubtracted "tester" (UT) fluorescence. The diagonal line indicates clones derived from transcripts of equal abundance in UD and UT (i.e. ER1=ER2). Clones that lie above the line indicate transcripts that are induced upon treatment (ER1>ER2), while those below the line indicate transcripts that have escaped the subtraction (ER1<ER2). Clones above the line with positive ER2 values represent rare transcripts, whereas clones above the line with a negative ER2 value are regarded as abundant and have been reduced in relative concentration during normalization.

Table 1. Validation of Microarray screening of selected banana Suppression Subtractive Hybridisation clones by Inverse Northern data.

Clone no.	ER1[a]	ER2[b]	UT/UD Ratio[c]	Inverse Northern Expression Ratio[d]
3-105	-0.3	-0.6	1.2	8.6
2-35	0.3	-0.1	1.3	6.9
3-169	1.0	0.7	1.2	3.1
2-28	0.6	0.3	1.2	2.1
1-77	0.8	0.6	1.2	2.1
1-158	0.7	0.6	1.1	2.0
3-7	0.6	0.3	1.2	1.7
2-21	1.8	0.9	1.9	1.5
1-149	0	-0.1	1.1	1.4
2-18	2.1	1.5	1.6	1.4
2-70	1.9	1.2	1.6	1.2
1-136	-0.1	-0.1	1.0	1.2
2-45	1.4	1.0	1.3	1.0
1-1	0.8	0.9	0.9	0.98
2-14	0.7	0.9	0.9	0.97
3-94	-0.1	0.2	0.8	0.94
2-134	1.5	1.6	0.9	0.1
3-156	0.6	0.8	0.9	0.1

SSH, suppression subtractive hybridisation; ST, subtracted "tester"; UD, unsubtracted "driver"; UT, unsubtracted "tester".

[a,b] Enrichment ratio 1 and 2 (ER1 and ER2) were calculated from microarray screening as $\log_2$(ST/UD) and $\log_2$(ST/UT), respectively.

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

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

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.

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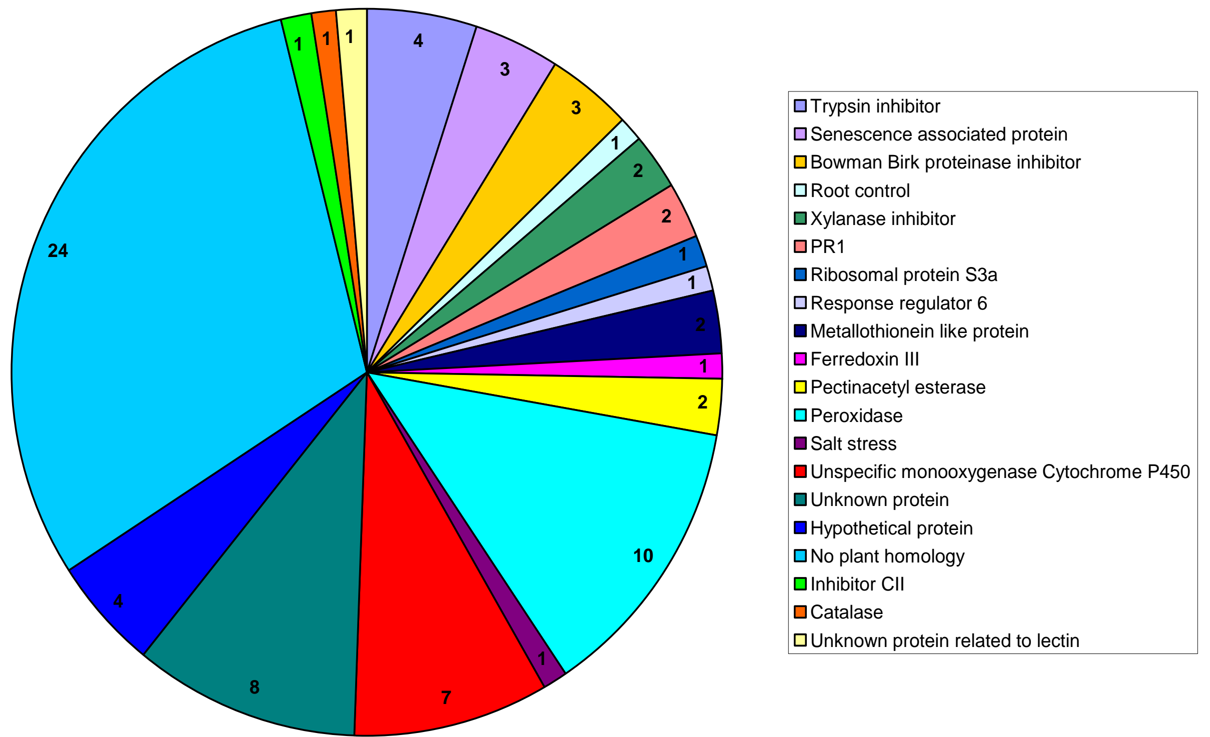
A.



B.

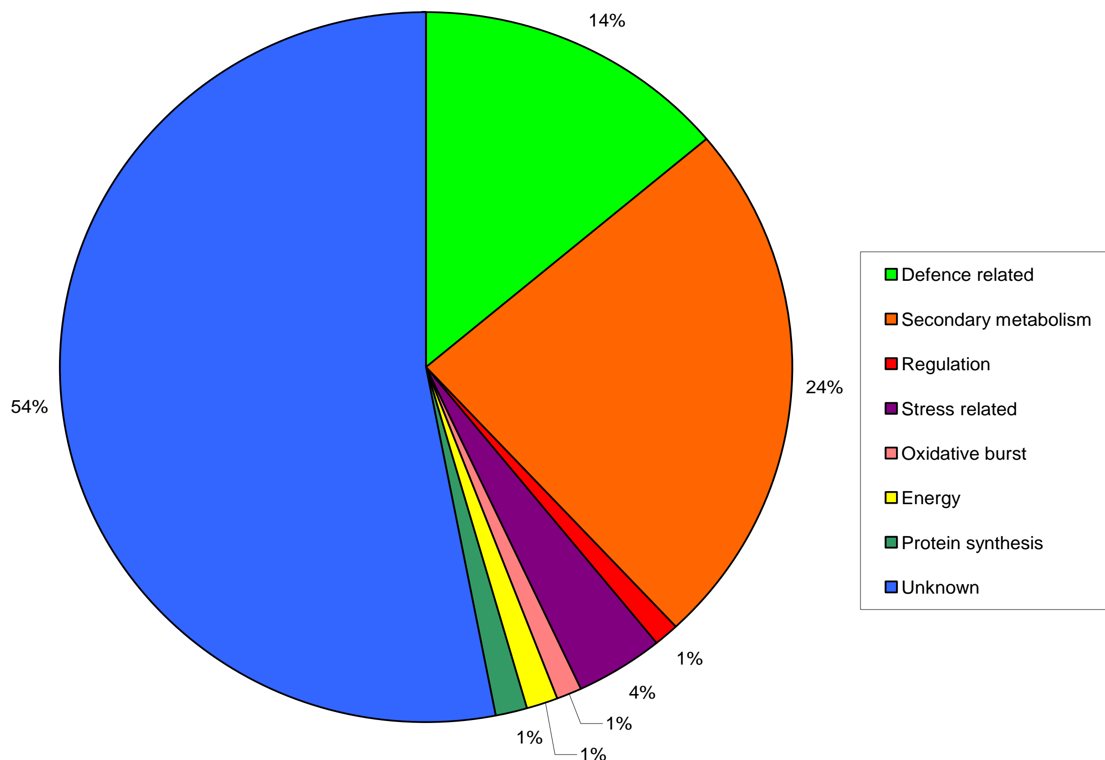


Figure 1. Pie charts summarising the number of clones based on putative identities (A) and the percentage (%) of clones in a functional categories (B).

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*.

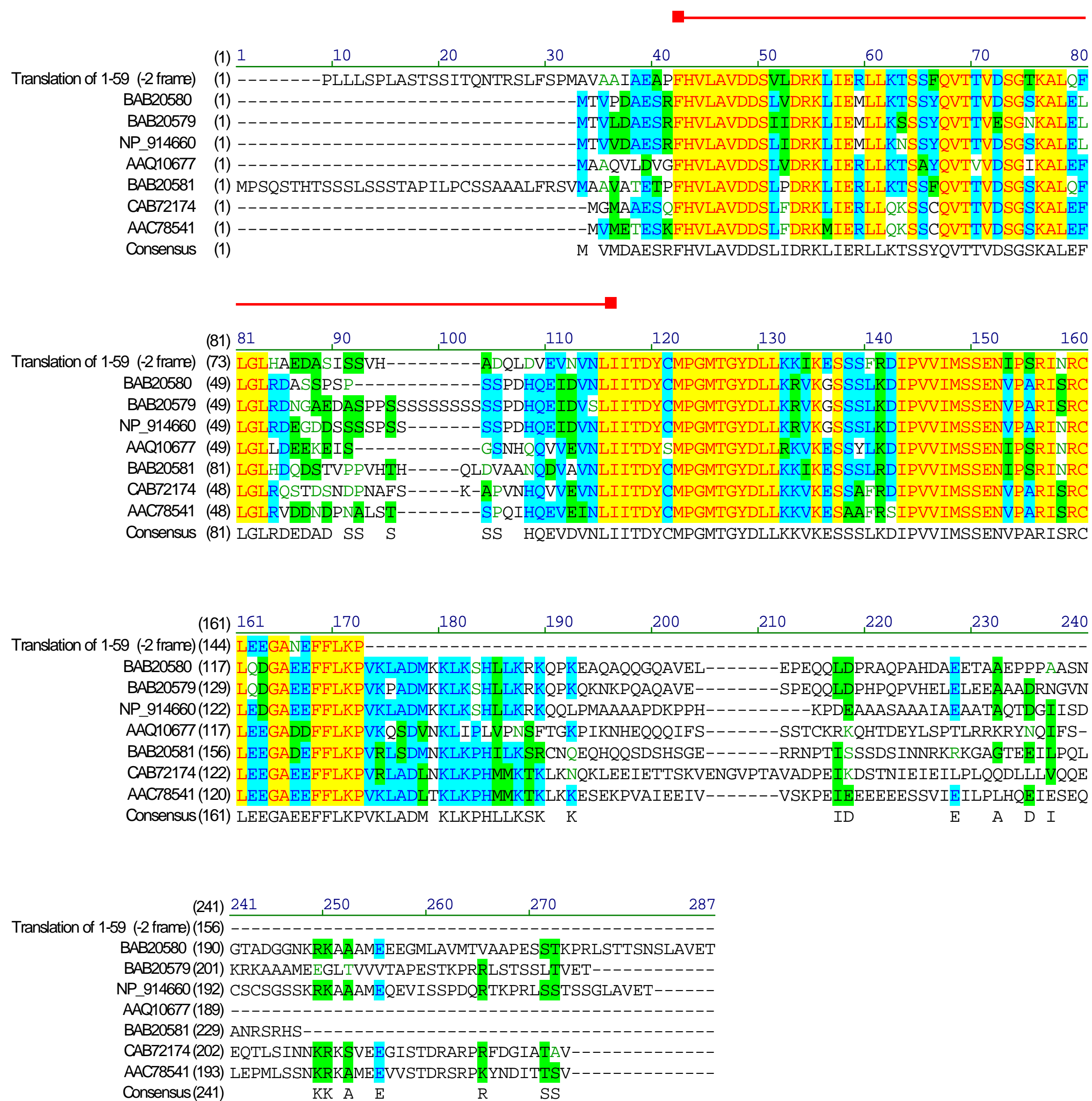


Figure 2. Alignment of deduced amino acid sequences of plant response regulators.

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.

```
                              (1) 1        10                  30          40          50          60          70        80
Translation of 1-77 (in -1 frame) (1) --------------------------------------------------------------------------------
                   AA611AA40  (1) --MDPCKYRPSSSYDASFTTTNAGGPVWNDDVALTVGSRGPILLEDYHLIEKVAHFARERIPERVVHARGASAKGFFECT
                    CAA43814  (1) --MDPCKFRPSSSFDTKTTTTNAGAPVWNDNEALTVGPRGPILLEDYHLIEKVAHFARERIPERVVHARGASAKGFFECT
                        CSRZ  (1) --MDPCKFRPSSSFDTKTTTTNAGAPVWNDNEALTVGPRGPILLEDYHLIEKVAHFARERIPERVVHARGASAKGFFECT
                      P18123  (1) MTMDPTKFRPSSSHDTTVTTTNAGAPVWNDNEALTVGPRGPILLEDYHLIEKVAHFARERIPERVVHARGASAKGFFECT
                    CAH61266  (1) --MDPCKFRPSSSFDTKTTTTNAGQPVWNDNEALTVGPRGPILLEDYHLLEKIAHFARERIPERVVHARGASAKGFFECT
                      P55308  (1) --MDPCKFRPSSSFDTKTTTTNAGQPVWNDNEALTVGPRGPILLEDYHLLEKIAHFARERIPERVVHARGASAKGFFECT
                    AAC17730  (1) --------------------------------------------------------------------------------
                   Consensus  (1)   MDPCKFRPSSSFDTKTTTTNAGAPVWNDNEALTVGPRGPILLEDYHLIEKVAHFARERIPERVVHARGASAKGFFECT

                             (81) 81       90        100       110       120       130       140       150       160
Translation of 1-77 (in -1 frame) (1) --------------------------------------------------------------------------------
                   AA611AA40 (79) HDVTHLTFADFLRAPGVQTPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                    CAA43814 (79) HDVTDITCADFLRSPGAQTPVIVRFSTVIHERGSQETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                        CSRZ (79) HDVTDITCADFLRSPGAQTPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                      P18123 (81) HDVTSLTCADFLRAPGVRTPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                    CAH61266 (79) HDVTGLTCADFLRAPGARTPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                      P55308 (79) HDVTGLTCADFLRAPGARTPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                    AAC17730  (1) ----------------------VRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF
                   Consensus (81) HDVT LTCADFLRAPGA TPVIVRFSTVIHERGSPETIRDPRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAF

                            (160) 160       170       180       190       200       210       220                239
Translation of 1-77 (in -1 frame) (1) --------------------------------------------------------------------------------
                  AA611AA40 (158) FKPNPKSNVQEYWRVLDFLSHLPESLHTFCFLYDDVGVPLNYRHMEGFGVNTYTFINKAGKTNYVKFHWKPTCGVKCML-
                   CAA43814 (158) FKPNPRSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVTRDAKARYVKFHWKPTCGVSCLM-
                       CSRZ (158) FKPNPRSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVTRDAKARYVKFHWKPTCGVSCLM-
                     P18123 (160) FKPNPRSHVQEYWRVFDFLSHLPESLHTFFFLFDDVGVPSDYRHMEGFGVNTYTFVSAAGKAQYVKFHWKPTCGVRCILT
                   CAH61266 (158) FKPNPKSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVTRAGKSHYIKVHWRPTCGVSCLM-
                     P55308 (158) FKPNPKSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVSRAGKSHYVKFHWRPTCGVSCLM-
                    AAC17730 (58) FKPNPKSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVSRAGKSHYVKFHWRPTCGVSCLM-
                  Consensus (160) FKPNPKSHVQEYWRVFDFLSHHPESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVSRAGKA YVKFHWKPTCGVSCLM

                            (240) 240       250       260       270       280       290       300                319
Translation of 1-77 (in -1 frame) (1) --------------------DSIAAGNYPEWKLFVQVMDPDTEDRYDFDPLDDTKTWPEDLLPLQPVGRLVLNRNIDNFF
                  AA611AA40 (237) EDEAVVVGGKNHSHATQDLYDSIAAGNYPEWKLFVQVMDPDEEDKYDFDPLDDTKTWPEDLLPLQPVGRLVLNRNIDNFF
                   CAA43814 (237) DDEATLVGGKNHSHATQDLYDSIAAGNFPEWKLFVQVIDPEEEERFDFDPLDDTKTWPEDEVPLRPVGRLVLNRNVDNFF
                       CSRZ (237) DDEATLVGGKNHSHATQDLYDSIAAGNFPEWKLFVQVIDPEEEERFDFDPLDDTKTWPEDEVPLRPVGRLVLNRNVDNFF
                     P18123 (240) DEEAALVGGRNHSHATQDLYDSIAAGSFPEWTLYVQVMDPDTEEQYDFDPLDDTKTWPEDLLPLRPVGRLVLDRNVDNFF
                   CAH61266 (237) DDEATLVGGKNHSHATQDLFDSIDAGNFPEWKLFVQVIDPEEEDRFDFDPLDDTKTWPEDLVPLQPVGRLVLDRNVDNFF
                     P55308 (237) DDEATLVGGKNHSHATQDLYDSIDAGNFPEWKLFVQVIDPDEEDRFDFDPLDDTKTWPEDLVPLQPVGRLVLDRNVDNFF
                   AAC17730 (137) DDEATLVGGKNHSHATQDLYDSIDAGNFPEWKLFVQVIDPDEEDRFDFDPLDDTKTWPEDLVPLQPVGRLVLDRNVDNFF
                  Consensus (240) DDEATLVGGKNHSHATQDLYDSIAAGNFPEWKLFVQVIDPDEEDRFDFDPLDDTKTWPEDLVPLQPVGRLVLNRNVDNFF

                            (320) 320       330       340       350       360       370       380                399
Translation of 1-77 (in -1 frame) (61) SENEQLAFGPGLVVPG----------------------------------------------------------------
                  AA611AA40 (317) NENEQLAFSPGIIVPGIYYSDDKMLQCRTFAYGDTQRYRLGPNYLMLPVNAPKCAHKNSHYDGLMNFMHRDEEVNYYPSR
                   CAA43814 (317) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCAHHNNHYDGAMNFMHRDEEVDYYPSR
                       CSRZ (317) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCAHHNNHYDGAMNFMHRDEEVDYYPSR
                     P18123 (320) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPRCAHHNNHYDGAMNFMHRDEEVDYYPSR
                   CAH61266 (317) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCGFKNNHYDGAMNFMHRDEEVDYYPSR
                     P55308 (317) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCGFKNNHYDGAMNFMHRDEEVDYYPSR
                   AAC17730 (217) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCGFKNNHYDGAMNFMHRDEEVDYYPSR
                  Consensus (320) NENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNYLMLPVNAPKCAHKNNHYDGAMNFMHRDEEVDYYPSR

                            (400) 400       410       420       430       440       450       460                479
Translation of 1-77 (in -1 frame) (77) --------------------------------------------------------------------------------
                  AA611AA40 (397) HDRLRNAER--FPINNRPIIGKREKCTIEKQNDFKQPGERYRSWAPDRQERFVRRWVEALAHPKVTFEIRTIWVSYLSKC
                   CAA43814 (397) HAPLRHAPP--TPITPRPVVGRRQKATIHKQNDFKQPGERYRSWAPDRQERFIA-VRRRVAHPKVSPELRAIWVNYLSQC
                       CSRZ (397) HAPLRHAPP--TPITPRPVVGRRQKATIHKQNDFKQPGERYRSWAPDRQERFIP-LRRRVAHPKVSPELRAIWVNYLSQC
                     P18123 (400) HAPLRQAAPP-TPLPPRPVAGRREKATIRKPNDFKQPGERYRSWDADRQDRFVRRFADSLGHPKVSQELRSIWIDLLAKC
                   CAH61266 (397) HAPLRQAEPASFPVPTRPVVGKREKTRIKKENDFVQPGERYRSWAPDRQDRFVRRFADALAHPKVSHELRVIWIDFLSKC
                     P55308 (397) HAPLRHAEPASFPVPTRPVVGKREKTRIKKENDFVQPGERYRSWAPDRQDRFVRRFSDALAHPKVSHELRVIWIDFLSKC
                   AAC17730 (297) HAPLRHAEPASFPVPTRPVVGKREKTRIKKENDFVQPGERYRSWAPDRQDRFVRRFSDALAHPKVSHELRVIWIDFLSKC
                  Consensus (400) HAPLRHAEP  FPIP RPVVGKREK TIKK NDFKQPGERYRSWAPDRQDRFVRRFADALAHPKVS ELR IWIDFLSKC
```

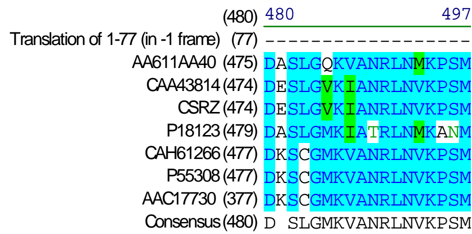


Figure 3. Alignment of deduced amino acid sequences of plant catalases.

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.

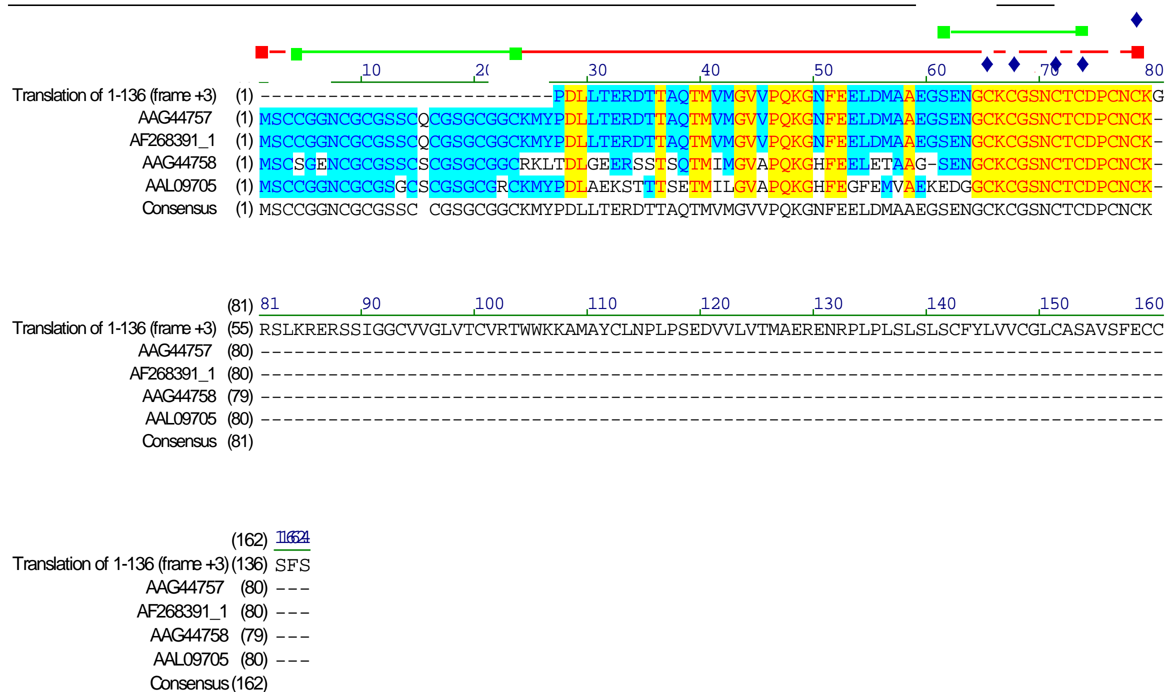


Figure 4. Alignment of deduced amino acid sequences of plant metallothionein.

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.

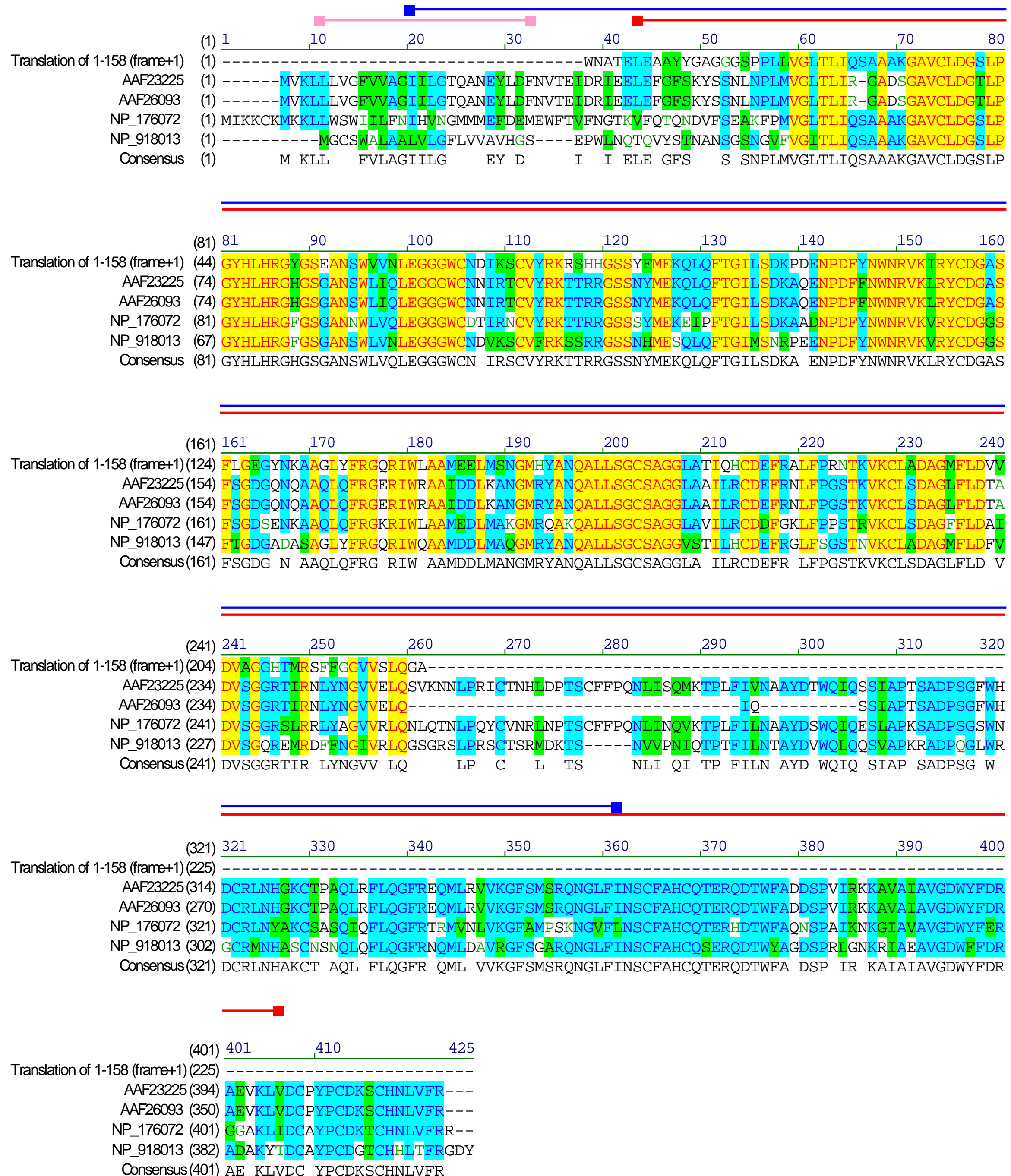


Figure 5. Alignment of deduced amino acid sequences of plant pectin acetylesterase.

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.

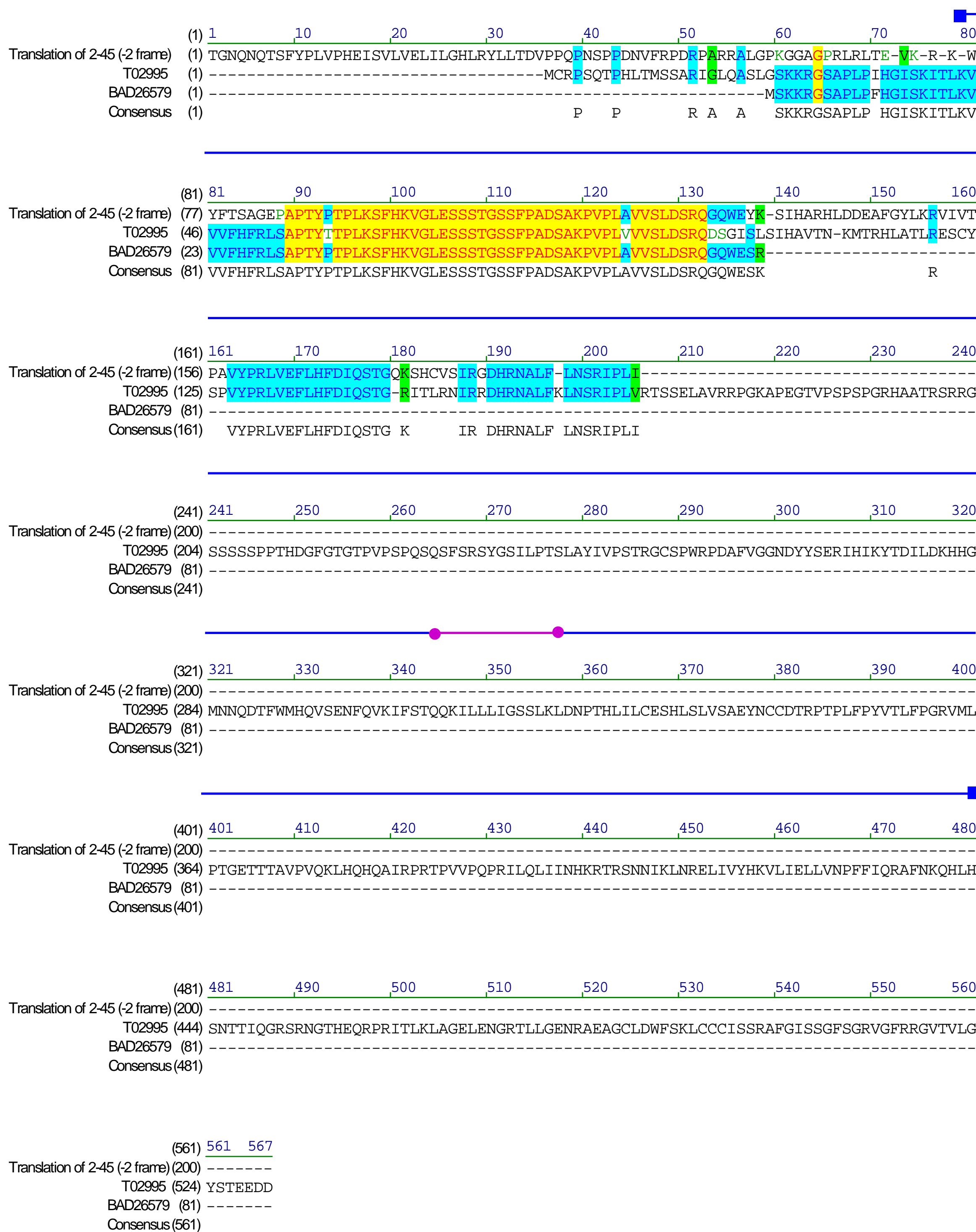


Figure 6. Alignment of deduced amino acid sequences of plant unspecific monooxygenase cytochrome P450.

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.

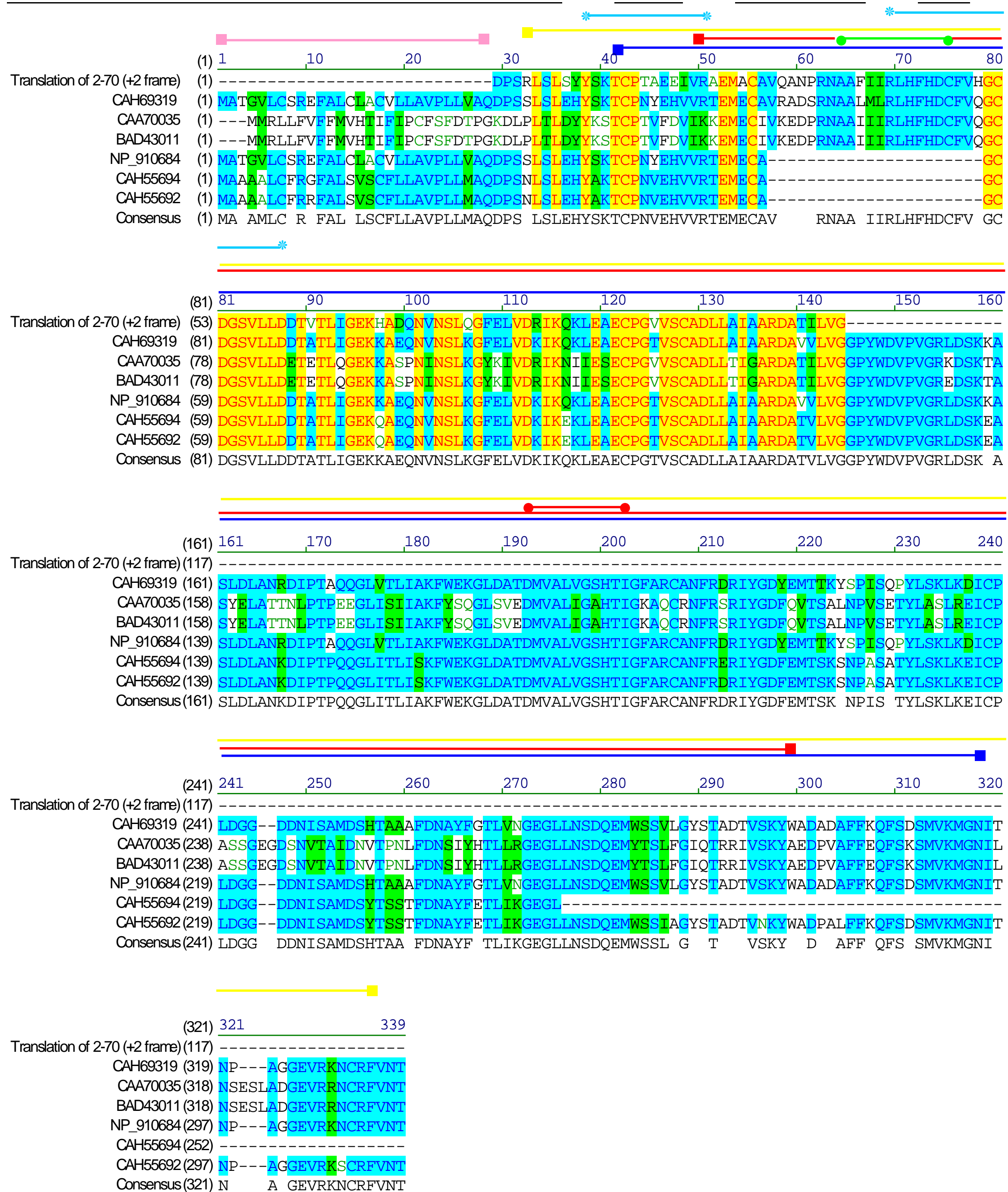


Figure 7. Alignment of deduced amino acid sequences of plant peroxidases.

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.

```
                              (1) 1        10         20         30         40         50         60         70        80
Translation of 3-7 (+1frame)  (1) --------------------------------------------------------------------------------
CAE46330                      (1) MARVLLLALAATLAAQASSKALPVLAPVTKDAATSLYTIPFHDGANLVLDVAGPLVWSTCDGGQRPPPAEITCSSPTCLL
BAD72883                      (1) MARVLLLVLAASLVALASSKGLPVLAPVTKDTATSLYTIPFHDGASLVLDVAGPLVWSTCEGSQ--PPAEIPCSSPTCLL
CAE46333                      (1) MPPVLLLVLAASLVALPSCRSLPVQAPVTKDPATSLYTIPFHDGASLVLDAAGPLVWSTCEAGQ--PPAGIPCGSPTCLL
CAG26970                      (1) ------------------KGLPVLAPVTKDTATSLYTIPFHDGASLVLDVAGLLVWSTCEGGQ--SPAEIACSSPTCLL
CAE46332                      (1) --------------------------------------------------------------------------------
Consensus                     (1) M  VLLL LAASL A   S KGLPVLAPVTKD ATSLYTIPFHDGASLVLDVAGPLVWSTCEGGQ  PPAEI CSSPTCLL

                             (82) 82      90         100        110        120        130        140        150       161
Translation of 3-7 (+1frame)  (1) --------------------------------------------------------------------------------
CAE46330                     (82) NAYPAPGCPAPSCGSDRHDKPCTAYPSNPVTGACAAGSLFRARLVANITDGNRPVSAVTVGVLAACAPTKLLASLPRGST
BAD72883                     (80) NAYPAPGCPAPSCGSDRHDKPCTAYPSNPVTGACAAGSLFHTKFAANTTDGNKPVSEVNVGVLAACAPSKLLASLPRGST
CAE46333                     (80) NAYPAPGCPAPTCGSD---KPCTAFPSNPVTGACAAGSLFHTSFVANTTDGTKPVSEVKVGVLAACAPSKLLASLPRGST
CAG26970                     (61) NAYPAPGCPAPSCGSDRHDKPCTAYPSNPVTGACAAGSLFHTRFAANTTDGNKPVSEVNVRVLAACAPSKLLASLPRGST
CAE46332                      (1) --------------------------------------------------------------------------------
Consensus                    (82) NAYPAPGCPAPSCGSDRHDKPCTAYPSNPVTGACAAGSLFHTRF ANTTDGNKPVSEV VGVLAACAPSKLLASLPRGST

                            (162) 162     170        180        190        200        210        220        230       241
Translation of 3-7 (+1frame)  (1) --------------------------------------------------------------------------------
CAE46330                    (162) GVAGLAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVAKQGSPAHYVSGTSIRVEDT
BAD72883                    (160) GVAGLANSGLALPAQVASTQKVANRFLLCLPTGGLGVAIFGGGPLPWPQFTQSMDYTPLVAKGGSPAHYISLKSIKVENT
CAE46333                    (157) GVAGLANSGLALPAQVASAQKVANRFFLCLPTGGAGVAIFGGGPLPWPQFTQSMPYTPLVTKGGSPAHYISLKSIKVDNT
CAG26970                    (141) GVAGLAGSGLALPSQVASAQKVANKFLLCLPTGGPGVAIFGGGPLPWPQFTQSMDYTPLVAKGGSPAHYISARSIKVENT
CAE46332                      (1) ----------------------------------------------PQFTQSMQYTPLVTKGGSPAHYISLKSIKVDNT
Consensus                   (162) GVAGLA SGLALPAQVASAQKVANRFLLCLPTGG GVAIFGGGPLPWPQFTQSM YTPLVAKGGSPAHYISLKSIKVENT

                            (243) 243     250        260        270        280        290        300        310       322
Translation of 3-7 (+1frame)  (1) -----------------------------------------------------------------------------VPN
CAE46330                    (243) VPVPDRALATGGVMLSTRLPYVLLRRDVYRPFVDAFAKALAAQHANGALAARGVNPVAPFGLCYDAKTLGNNLGGYSVPN
BAD72883                    (241) VPVSERALATGGVMLSTRLPYVLLRRDVYRPFVGAFTKALAAQPANGAPVARAVKPVAPFELCYDTKSLGNNLGGYWVPN
CAE46333                    (238) VPVSE---ATGGVMLSTRLPYALLRRDVYRPLVDAFTKALAAQPANGAPVARAVQPVAPFGVCYDTKTLGNNLGGYAVPN
CAG26970                    (222) VPISERALATGGVMLSTRLPYVLLRRDVYRPLVDAFTKALAAQPANGAPVARAVKPVAPFELCYDTKTLGNNPGGYWVPN
CAE46332                     (35) VTVSQSAFATGGVMLSTRLPYALLRRDVYRPLVDAFTKALAAQPANGAPVARAVQPVAPFGVCYDTKTLGNNLGGYAVPN
Consensus                   (243) VPVSERALATGGVMLSTRLPYVLLRRDVYRPLVDAFTKALAAQPANGAPVARAVQPVAPFGLCYDTKTLGNNLGGYAVPN

                            (324) 324    330        340        350        360        370        380        390       403
Translation of 3-7 (+1frame)  (5) VLALDGGGEWAMTGKNSMVDVKPGTACVAFVEM---EAGDGGAPAVILGGAQMEDFVLDFDMEKKRLGFIRLPHFTGCGN
CAE46330                    (324) VLALDGGGEWAMTGKNSMVDVKPGTACVAFVEM---EAGDGGAPAVILGGAQMEDFVLDFDMEKKRLGFIRLPHFTGCGN
BAD72883                    (322) GLAVDGGSDWAMTGKNSMVDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFLRLPHFTGCGS
CAE46333                    (316) LLALDGGGEWAMTGKNSMVDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFTRLPHFTGCGS
CAG26970                    (303) LLELDGGSDWALTGKNSMVDVKPGTACVAFVEMKGVDAGDGSAPAVILGGAQMEDFVLDFDMEKKRLGFLRLPHFTGCSS
CAE46332                    (116) LLALDGGGEWAMTGKNSMVDVRPGTACVAFVEMKGAEAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFTRLPHFTGCGS
Consensus                   (324) LLALDGGGEWAMTGKNSMVDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFIRLPHFTGCGS

                            (405) 405 410
Translation of 3-7 (+1frame) (83) NF----
CAE46330                    (402) NF----
BAD72883                    (402) ------
CAE46333                    (397) ------
CAG26970                    (384) NFARST
CAE46332                    (197) ------
Consensus                   (405) NF
```

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.

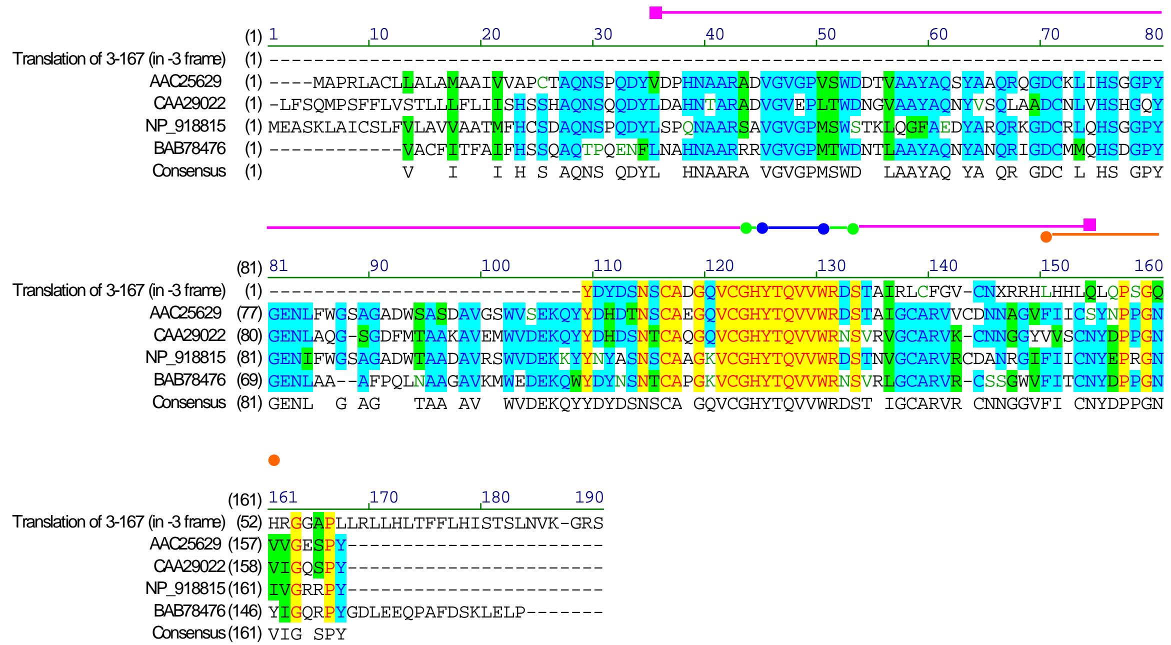


Figure 9. Alignment of deduced amino acid sequences of plant pathogenesis-related protein 1.

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.

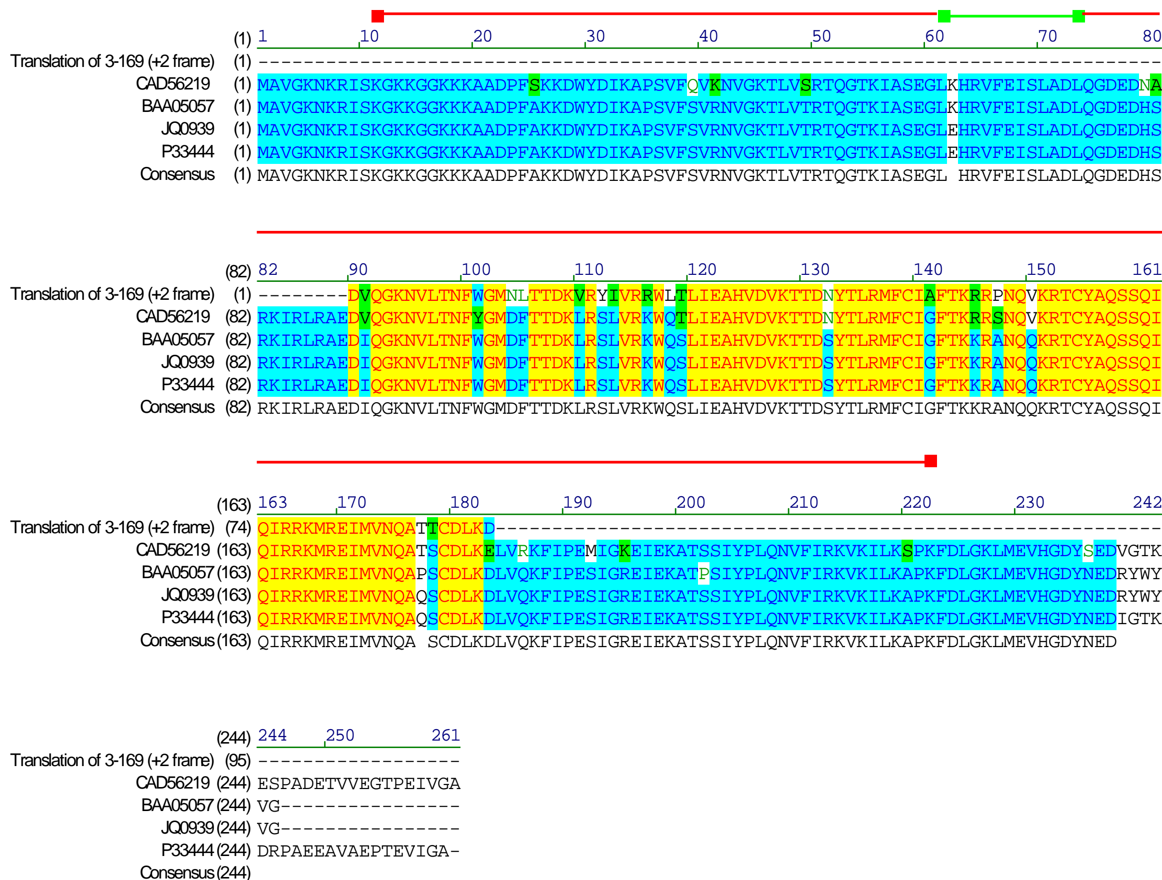


Figure 10. Alignment of deduced amino acid sequences of plant ribosomal 5S3a.

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.

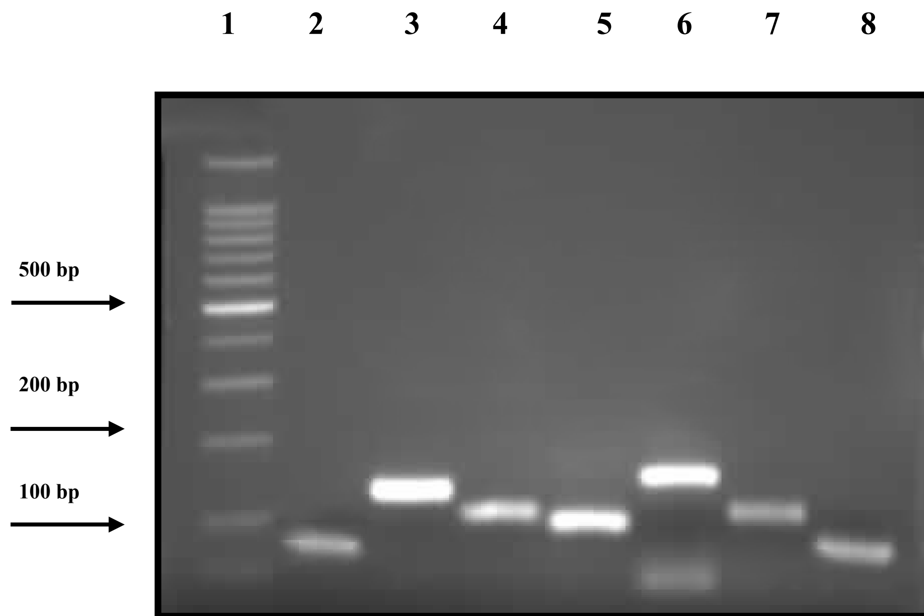


Figure 11. PCR products amplified from GCTCV-218 cDNA using clone specific primers designed from banana cDNA clones. Fragments were separated by electrophoresis through a 2% (w/v) agarose gel. Lane 1 contains the 100 bp molecular marker (Roche Diagnostics), lane 2 actin (78 bp), lane 3 clone 3-167 (*PR1*) (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).

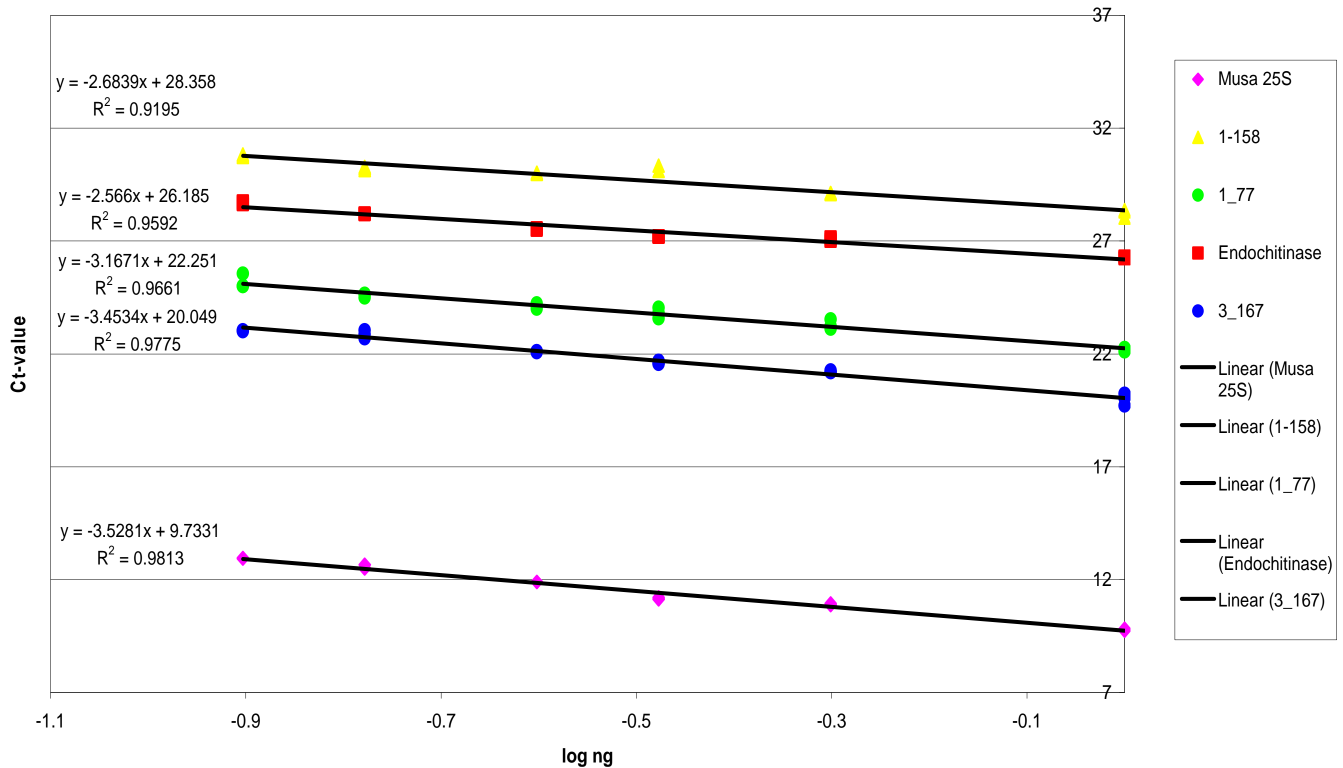
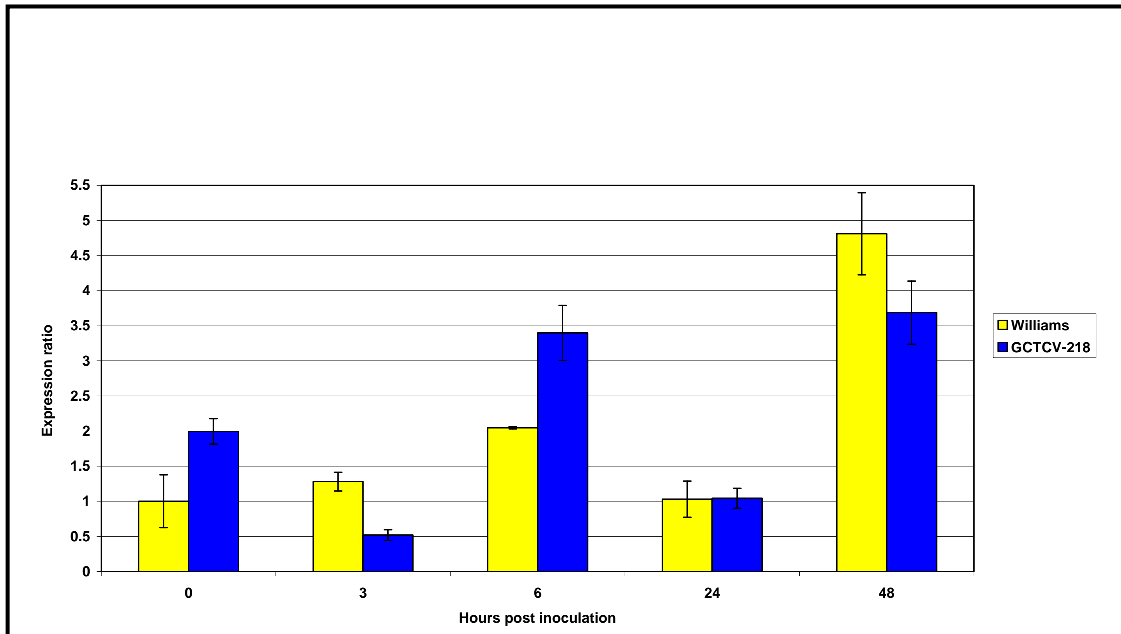


Figure 12. Standard-curve plots for calculation of PCR efficiency and quantification for different primer pairs. Ten-fold dilution series of GCTCV-218 cDNA (1:10, 1:20, 1:30, 1:40, 1:60 and 1:80) amplified with Musa 25S, 1-158, 1-77, endochitinase and 3-167 are used to generate the standard curve for 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.

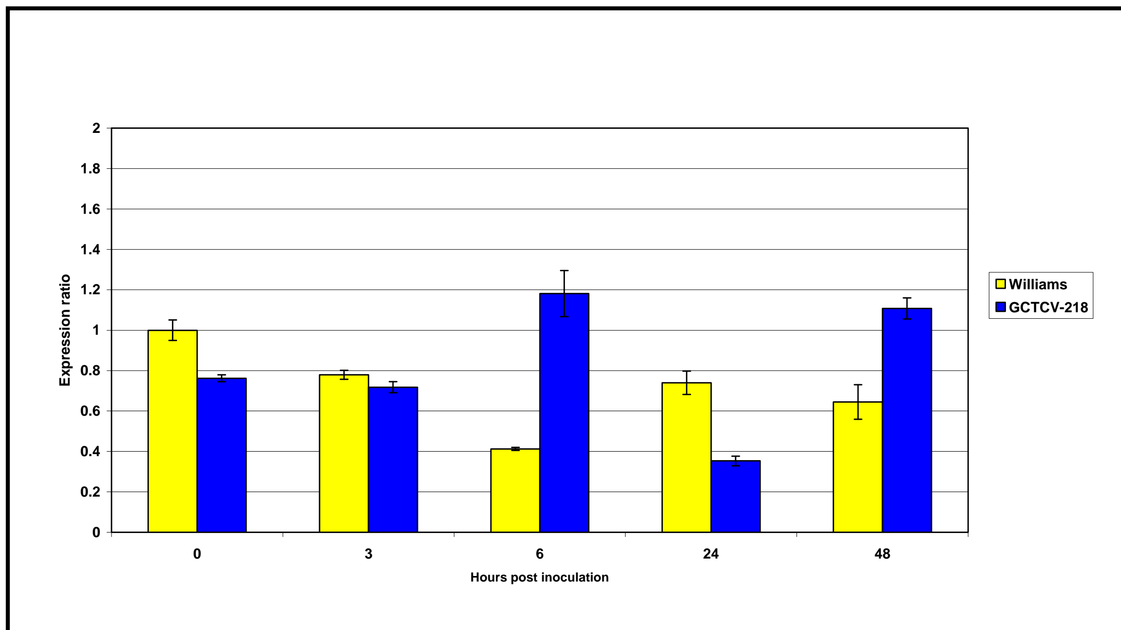
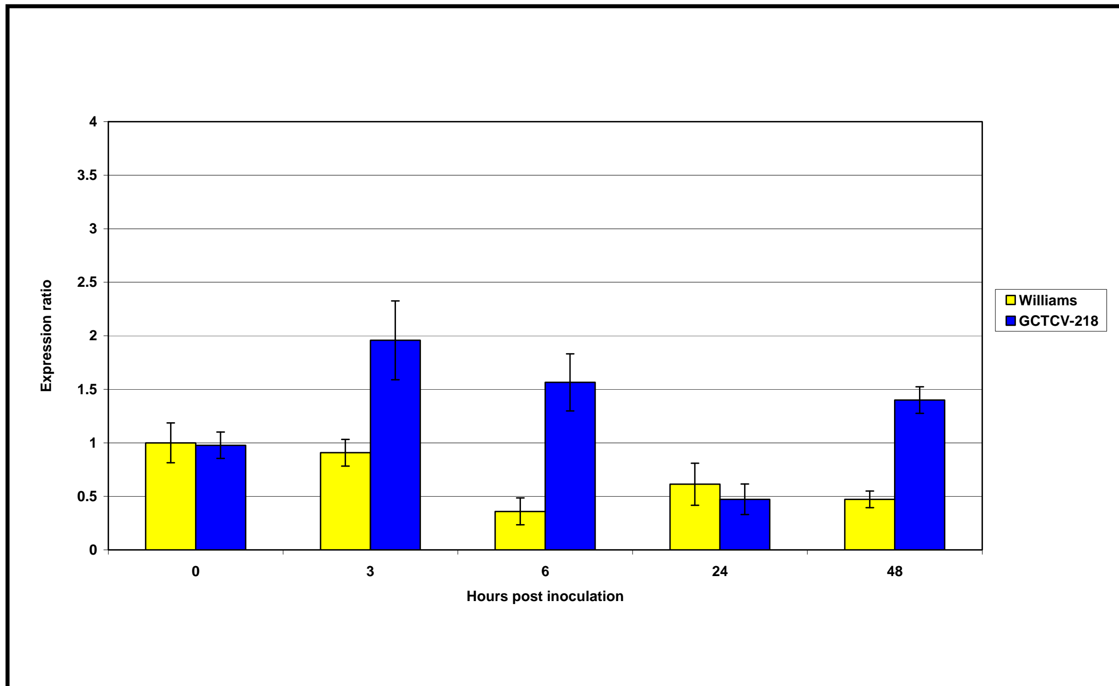


Figure 13. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of catalase 2 in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams 0 hrs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

A.



B.

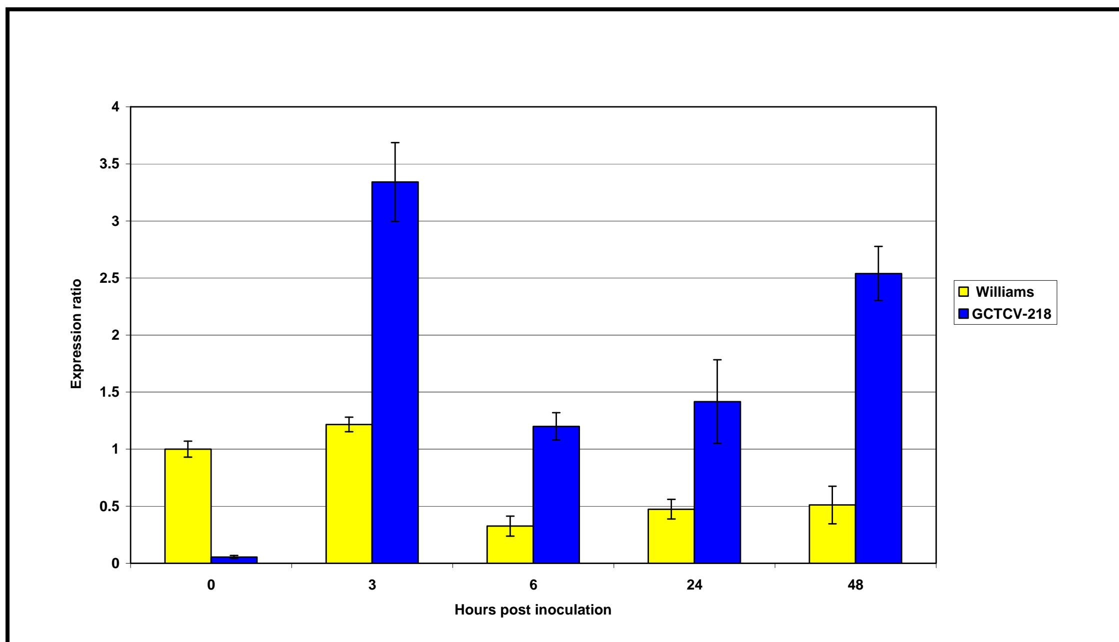
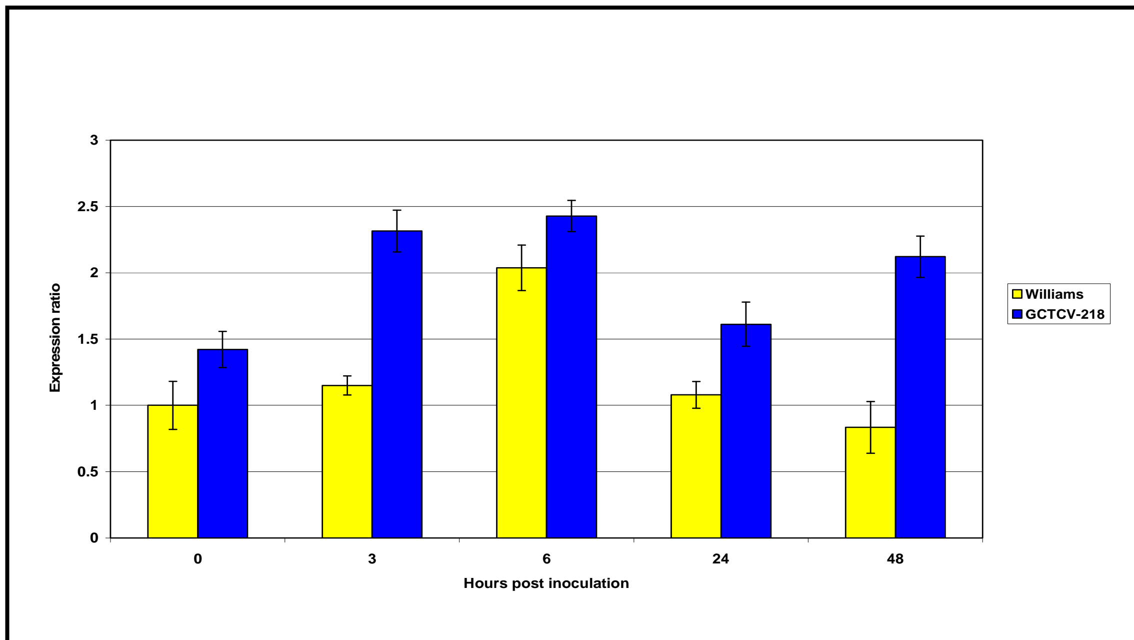


Figure 14. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of pectin acetylesterase in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams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

A.



B.

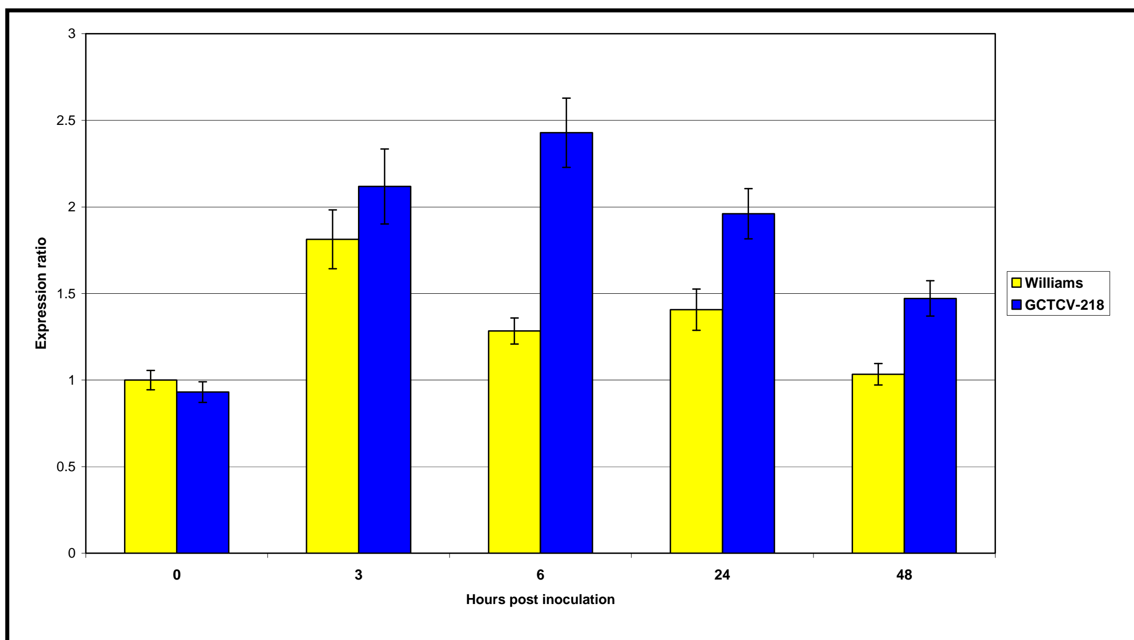


Figure 15. Relative gene expression level at 0, 3, 6, 24 and 48 hrs after of *PR*1 in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the TaqMan® system (A) and the Light Cycler (B). Expression is given in terms of a calibrator, Williams 0 hrs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

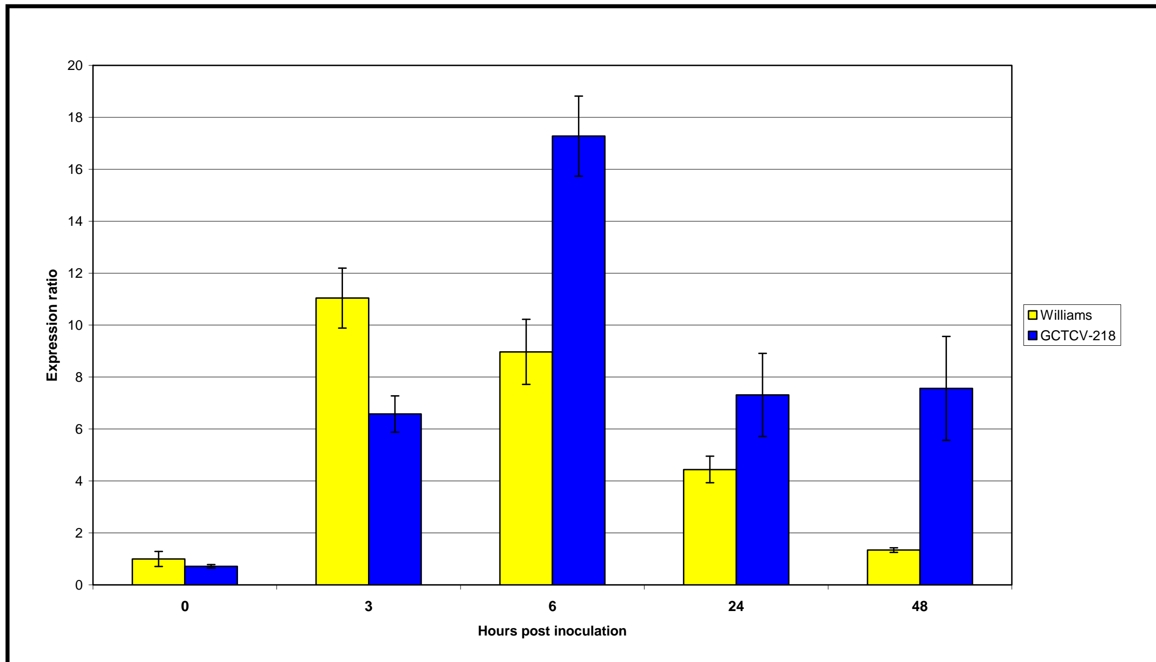


Figure 16. Relative gene expression level at 0, 3, 6, 24 and 48 hrs of endochitinase in GCTCV-218 and Williams bananas after infection with *Fusarium oxysporum* f.sp. *cubense*, quantified using the Light Cycler. Expression is given in terms of a calibrator, Williams 0 hrs after infection. Error bar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

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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)	-	-	-	-

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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)	-	-	-	-

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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 Unknown function	-1.8	-1.8	1.0	ND
3-167*	AAC25629.1	PR1	*Zea mays*	$4e^{-17}$	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

SUMMARY

Fusarium wilt of banana has a long and devastating history in many of the world's banana producing countries. The most pronounced damage caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), the Fusarium wilt pathogen, occurred during the 20th century in Central America, where tens of thousands of virgin forests were lost to further banana production. No control strategy is effective against Fusarium wilt other than replacement of susceptible by resistant varieties. It is, therefore, important to develop or identify resistant replacements that would not only be able to resist the pathogen, but also be acceptable to consumers.

Resistance in wild banana varieties has been identified, and hybrids have been developed by breeding programmes with good resistance to Fusarium wilt. These varieties, unfortunately, appear not to be acceptable replacements for Cavendish bananas, the sweet desert banana variety that serves as the primary export banana and constitutes almost 40% of all bananas planted in the world today. A field selection, GCTCV-218, now proved to be the Cavendish plant with the most resistance to *Foc* 'tropical' race 4 (VCG 0121) has saved the Cavendish-based banana industry in Taiwan from devastation. In this thesis, GCTCV-218 has been evaluated against *Foc* 'subtropical' race 4 (VCG 0120), the primary variant of the pathogen in subtropical banana-producing countries such as South Africa, Australia and the Canary Islands. Defence-associated genes that are differentially expressed and that were up-regulated early in the defence response against the pathogen were isolated and identified.

Greenhouse and field trials conducted at the research facilities of the Forestry and Agricultural Biotechnology Institute, University of Pretoria and in Kiepersol, South Africa, respectively, showed that GCTCV-218 had a significantly higher level of disease tolerance against *Foc* 'subtropical' race 4 (VCG 0120) when compared to the commercially grown Williams cultivar. Phenolic assays revealed that total phenolics and cell-wall bound phenolics were expressed at higher levels in GCTCV-218 after pathogen attack and seemed to play an important role in the tolerance of GCTCV-218. It was, therefore, proposed that GCTCV-218 could be considered a replacement for other Cavendish banana varieties planted in South Africa.

The genetic basis of defence mechanisms in banana to *Foc* is unknown. In this investigation, Suppression Subtractive Hybridisation (SSH) was used to construct a cDNA library, containing banana genes that were up-regulated early (3 & 6 hours after infection), in the GCTCV-218/*Foc* interaction. The efficiency of the procedure was confirmed by PCR amplification of a known defence gene (endochitinase) present in the subtracted tester material, as well as analysing the reduction of a known housekeeping gene, actin, in the subtracted material compared to unsubtracted material. Southern blot data further provided confidence in the subtraction process. A cDNA library containing 736 gene fragments was constructed and then subjected to a screening procedure to remove false positives that escaped the subtraction process.

The screening of a banana cDNA library for defence-related genes involved the development of a high-throughput cDNA microarray technique. This novel technique removed all false positives, such as housekeeping genes that escaped the subtraction as well as clones representing rDNAs. Seventy-nine genes differentially expressed in GCTCV-218 and not in Williams were selected, sequenced and subjected to BLASTX, BLASTN and DBest searches. Of these, several gene fragments showed homology to defence-associated genes, and 20 unique genes fragments were identified. These include two different peroxidases, response regulator 6, catalase 2, metallothionein, pectin acetylesterase (PAE), two different unknown proteins, salt stress, trypsin inhibitor, unspecific monooxygenase cytochrome P450, Bowman Birk proteinase inhibitor, root control, xylanase inhibitor, inhibitor CII, hypothetical protein, putative senescence-associated protein, pathogenesis-related protein 1 (*PR1*) and ribosomal protein S3a.

The significance of the defence reaction to Fusarium wilt diseases in agricultural crops depends on the tempo of plant response. When a host plant is able to respond early to pathogen invasion the pathogen is successfully contained, preventing further spread throughout the plant. The expression of genes with antimicrobial activity, such as endochitinase, suggests an induced biochemical defence response against *Foc*. The expression of PAE and *PR1* results in the deposition of lignin and callose production for cell wall strengthening. Four defence associated genes (catalase 2, pectin acetyl esterase (PAE), *PR-1* and endochitinase) were selected for expression profile analysis using Real-time reverse transcriptase PCR, with TaqMan® and Light Cycler

technology. All four genes were shown to be differentially expressed in GCTCV-218 at 3 and 6 hrs after infection, confirming SSH results. *PR-1* and PAE were induced very early (3 hrs after infection) in the GCTCV-218, while *PR3* and catalase 2 followed with a significant induction at 6 hrs after infection. This study concludes that GCTCV-218 is able to respond rapidly in response to *Foc* infection by activating both a biochemical and structural defence mechanism.

High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis

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Efficient construction of cDNA libraries enriched for differentially expressed transcripts is an important first step in many biological investigations. We present a quantitative procedure for screening cDNA libraries constructed by suppression subtractive hybridization (SSH). The methodology was applied to two independent SSHs from pearl millet and banana. Following two-color cyanin dye labeling and hybridization of subtracted tester with either unsubtracted driver or unsubtracted tester cDNAs to the SSH libraries arrayed on glass slides, two values were calculated for each clone, an enrichment ratio 1 (ER1) and an enrichment ratio 2 (ER2). Graphical representation of ER1 and ER2 enabled the identification of clones that were likely to represent up-regulated transcripts. Normalization of each clone by the SSH process was determined from the ER2 values, thereby indicating whether clones represented rare or abundant transcripts. Differential expression of pearl millet and banana clones identified from both libraries by this quantitative approach was verified by inverse Northern blot analysis.

INTRODUCTION

The identification of differentially expressed genes and examination of their patterns of expression are important to gain information about the functions relevant to processes such as cell differentiation, morphological or metabolic changes, and disease development. Various molecular techniques exist for studying differential gene expression, including representational difference analysis, suppression subtractive hybridization (SSH), differential display, differential hybridization, subtractive library construction, serial analysis of gene expression, and cDNA microarrays (1–6). In previous studies, SSH libraries were screened to identify cloned differentially expressed genes by colony blot hybridization or cDNA amplified fragment length polymorphism (AFLP) (7,8). However, these methods are time-consuming and do not allow the level of enrichment of a transcript to be quantified. SSH has also been used as a method to generate a cDNA library to use in subsequent cDNA microarray expression profiling (9). We used cDNA microarrays to screen PCR-amplified clones from SSH libraries to identify genes from pearl millet [*Pennisetum glaucum* (L.) R.Br] and banana (*Musa acuminata*) that are up-regulated during defense responses. This quantitative approach of determining the extent to which transcripts were enriched by the SSH process allowed us to identify and exclude clones that were not derived from up-regulated transcripts and to determine whether transcripts were rare or abundant.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pearl millet breeding lines ICML12=P7 and 842B were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) India and ICRISAT Zimbabwe, respectively. The seed was sterilized and germinated on half-strength Murashige and Skoog (MS) medium (10) at 25°C with a 16 h light/8 h dark photoperiod. Banana plants of a *Fusarium oxysporum* f.sp. *cubense* (*Foc*)-tolerant Cavendish selection and a *Foc*-susceptible Williams cultivar were transferred into plastic cups containing water and maintained in a greenhouse at 18°–25°C with a 16 h light/8 h dark photoperiod. Prior to inoculation, the plants were transferred to an aeroponic system for root inoculation.

Plant Treatments

Leaves of 10-day-old ICML12=P7 and 842B pearl millet seedlings were wounded by pricking leaves at 1 cm intervals with a sterile needle. The undersurfaces of the leaves were inoculated with a total of 100 µL of either 100 mg/mL chitin or a crude boiled extract of flagellin from *Bacillus* sp. Control pearl millet 842B seedlings

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were treated with deionized water. Plates containing pearl millet seedlings were sealed with Micropore™ tape (3M, Isando, South Africa) and incubated at 25°C with a 16 h light/8 h dark photoperiod.

Roots of 15 cm banana plants were inoculated with a 10^5 spores/mL *Foc* spore suspension by injecting the roots with a sterile needle. The inoculation point was sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA) to ensure that spores were not washed off by the aeroponic irrigation system. The plants were maintained under the same conditions as described above until sampling.

RNA Isolation and cDNA Synthesis

RNA was isolated from leaf and root tissue at different time intervals after the treatment and pooled prior to cDNA synthesis and SSH. Pearl millet leaves were harvested 5, 14, and 24 h post-elicitor treatment (hpe). Banana root material was sampled at 3 and 6.5 h post-inoculation (hpi) and immediately placed in liquid nitrogen.

Total RNA was prepared as previously described (11). Poly(A)$^+$ RNA was purified from total RNA using an Oligotex® mRNA Mini Kit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized from mRNA (pearl millet) and total RNA (banana) using a cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany).

Suppression Subtractive Hybridization

SSH was performed as previously described (11). Pearl millet cDNA prepared from the elicitor-treated samples was used as the "tester" and from the control sample as the "driver." Similarly, banana cDNA from *Foc*-infected Cavendish variety plants (tolerant) was used as the tester and from infected Williams (susceptible) plants as the driver. The cDNA enriched for differentially expressed transcripts was termed subtracted tester (ST), whereas unsubtracted tester (UT) cDNA was prepared from treated pearl millet plants and infected tolerant banana plants, and unsubtracted driver (UD) cDNA was prepared from control pearl millet plants and infected susceptible banana plants. The ratio of tester to driver in both experiments was 300:1. ST PCR amplification products ranging from 0.2–1.2 kb were purified and cloned as previously described (11). The banana and pearl millet ST libraries were constructed independently by two researchers.

PCR-Based Control with Actin Primers

ST, UT, and UD were used as templates in PCR with actin primers, actinF (5′-ACCGAAGCCCCTCTTAACCC-3′) and actinR (5′-GTATGGCTGACACCATCACC-3′). Primers were designed to amplify a cDNA fragment of the actin gene that flanked an intron and did not contain an *Rsa*I restriction site. PCR amplification was performed using *Taq* DNA polymerase (Roche Diagnostics) at 55°C. Aliquots (5 µL) were removed after 20, 25, and 30 PCR cycles and electrophoresed on a 2% agarose gel.

Southern Blot Analysis

Ten microliters of each ST, UT, or UD cDNAs were digested with *Rsa*I (Roche Diagnostics) to remove adaptors. PCR products minus adaptors were excised from a 1.5% low melting point agarose gel and purified using a QIAquick® Gel Extraction Kit (Qiagen). Products were labeled with digoxigenin (DIG)-dUTP using a DIG DNA Labeling and Detection Kit (Roche Diagnostics), according to the manufacturer's instructions. Hybridizations were performed as described previously (12), using either 5 ng/µL DIG-labeled ST, UT, or UD cDNAs. Hybridization signals were detected using CDP-Star® as outlined by the manufacturer (Roche Diagnostics).

cDNA Microarray Analysis

Libraries containing 960 pearl millet and 736 banana SSH clones were arrayed onto silanized microarray slides (Amersham Biosciences, Little Chalfont, UK) using an Array Spotter Generation III (Molecular Dynamics, Sunnyvale, CA, USA). Cloned inserts were PCR-amplified using SP6 and T7 primers, purified using Multiscreen® PCR Purification Plates (Millipore, Molsheim, France), and visualized on a 1% agarose Electro-Fast® Stretch gel (ABgene, Epsom, UK) prior to being robotically printed onto glass slides. On average, 200 and 300 pg each of pearl millet and banana SSH fragments, respectively, were spotted. The *uidA*, *luc*, and *bar* genes and a fungal rDNA internal transcribed spacer (ITS) fragment were also printed to serve as controls for global normalization. Two hundred nanograms of ST, UT, or UD cDNA probes, following the removal of adaptor sequences, were labeled by the incorporation of Cy™5 or Cy3 dUTP (Amersham Biosciences) using Klenow enzyme (USB, Cleveland, OH, USA) as previously described (13). Each hybridization was performed in duplicate with the reverse cyanin dye labeling of the probes. To ensure that high background hybridization does not occur due to incomplete removal of adaptor sequences, we recommend prehybridization with unlabeled adaptor sequences. Reactions were spiked with cyanin-labeled *uidA* (0.3 ng), *luc* (0.03 ng), and *bar* (3 ng) genes and a fungal ITS fragment (3 ng). After incubation at 37°C for 20 h, the probes were purified using a Multiscreen® PCR Purification Plate (Millipore, Molsheim, France) and eluted in 45 µL sterile distilled water. The probe was dried in a SPD111V vacuum centrifuge (Savant, Holbrook, NY, USA) and resuspended in DIG Easy Hyb Buffer (Amersham Biosciences). The glass slide was initially incubated in pretreatment solution [3.5× standard saline citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA)] without probe at 60°C for 20 min. The glass slide was placed in a HybUP hybridization chamber (NB Engineering, Pretoria, South Africa) with the probe at 42°C for 16 h. After hybridization, the slides were washed for 4 min at 42°C with 1× SSC/0.2%

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Table 1. Primary Microarray Data

http://fabinet.up.ac.za/microarray/SSH/Banana_raw_data.xls
http://fabinet.up.ac.za/microarray/SSH/Banana_modified_data.xls
http://fabinet.up.ac.za/microarray/SSH/Banana_microarray_image.jpg
http://fabinet.up.ac.za/microarray/SSH/PearlMillet_raw_and_modified_data.xls
http://fabinet.up.ac.za/microarray/SSH/PearlMillet_microarray_image.jpg

SDS, 0.1× SSC/0.2% SDS (twice), followed by three washes in 0.1× SSC for 1 min at room temperature. The slides were rinsed with distilled water, dried with high-pressure nitrogen, and scanned with a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA). The computer program ArrayVision™ (Molecular Dynamics) was used to localize and integrate every spot on the array (Table 1).

Enrichment ratios of ST:UD (ER1) and ST:UT (ER2) were calculated from experiments performed in duplicate. For each clone, background signal intensities were subtracted, signal intensities of duplicate spots on glass slides were averaged, and spots with a signal-to-noise ratio of less than 2 (banana) or 3 (pearl millet) were rejected. Global normalization of data for the cyanin dye effect was performed using a control gene set to calculate normalization functions c and c′ for each pair of dye swap slides (14). To determine ER1, slides were hybridized with ST and UD. ER1 was calculated using the following formula (14):

$$½[\log_2 \text{Cy3 ST/Cy5 UD} - c - (\log_2 \text{Cy3 UD/Cy5 ST} - c')]$$

ER2 was calculated in the same way, following hybridizations with ST and UT. UT/UD values (Table 2) were calculated from the ER1 and ER2 values as follows. Because ER1 - ER2 ~ $\log_2$ ST/UD - $\log_2$ ST/UT = $\log_2$ UT/UD, therefore UT/UD = antilog of (ER1 - ER2) in the base 2. The primary data sets, along with a figure showing hybridization to the microarrays, are shown in Table 1.

rRNA redundancy for pearl millet and banana libraries was determined by hybridizing glass microarray slides with pearl millet and banana rDNA probes, respectively. A clone was considered to have hybridized to an rDNA probe if its fluorescence was more than two standard deviations above local background fluorescence (15).

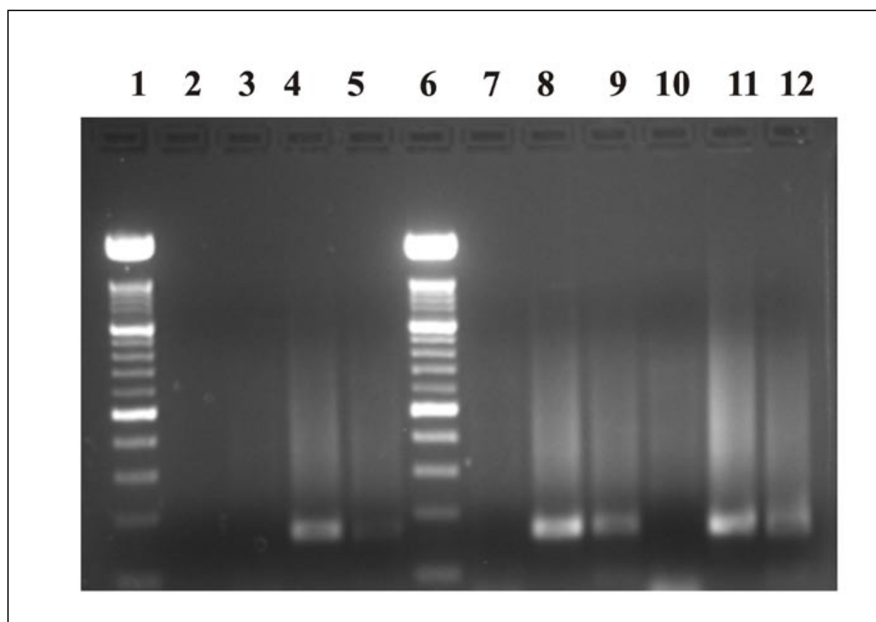

Figure 1. Amplification of the banana actin gene from subtracted tester, unsubtracted tester, and unsubtracted driver cDNA samples. Lanes 1 and 6, DNA Molecular Weight Marker XIV, 100 bp ladder (Roche Diagnostics); lane 2, water control; lanes 3–5, subtracted tester (ST), unsubtracted tester (UT), and unsubtracted driver (UD) after 20 amplification cycles; lanes 7–9, ST, UT, and UD after 25 amplification cycles; lanes 10–12, ST, UT, and UD after 30 amplification cycles.

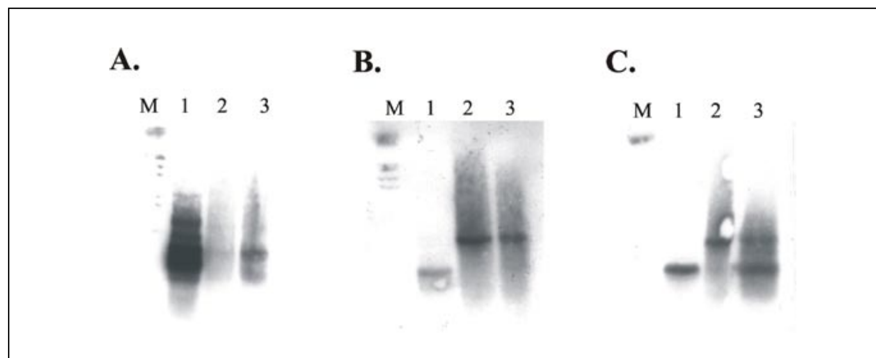

Figure 2. Southern hybridizations to pearl millet subtracted tester (lane 1), unsubtracted tester (lane 2), and unsubtracted driver (lane 3) to analyze library-specific enrichment following SSH. Identical filters were independently hybridized with complex probes derived from (A) subtracted tester (ST), (B) unsubtracted tester (UT), and (C) unsubtracted driver (UD). M represents a digoxigenin (DIG)-labeled Marker III (Roche Diagnostics). SSH, suppression subtractive hybridization.

Inverse Northern Dot Blots

Amplified, denatured inserts of selected clones from banana and pearl millet SSH libraries were applied to a positively charged Hybond™ nylon membrane (Amersham Biosciences) as previously described (11). Poly(A)$^+$ mRNA, used for pearl millet probe generation, was isolated from 50 µg freshly prepared total RNA (tester and driver). Banana tester and driver probes were prepared from 30

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μg total RNA. cDNA was labeled with DIG-dUTP using the DIG DNA Labeling and Detection Kit. Hybridizations were performed as described previously (12), using 20 ng/μL cDNA. Hybridization signals were detected using CDP-Star.

ArrayVision was used to calculate signal density, following normalization by comparing values of rDNA dots. Inverse Northern expression ratios were calculated by dividing normalized density measurements for each clone hybridized with the tester

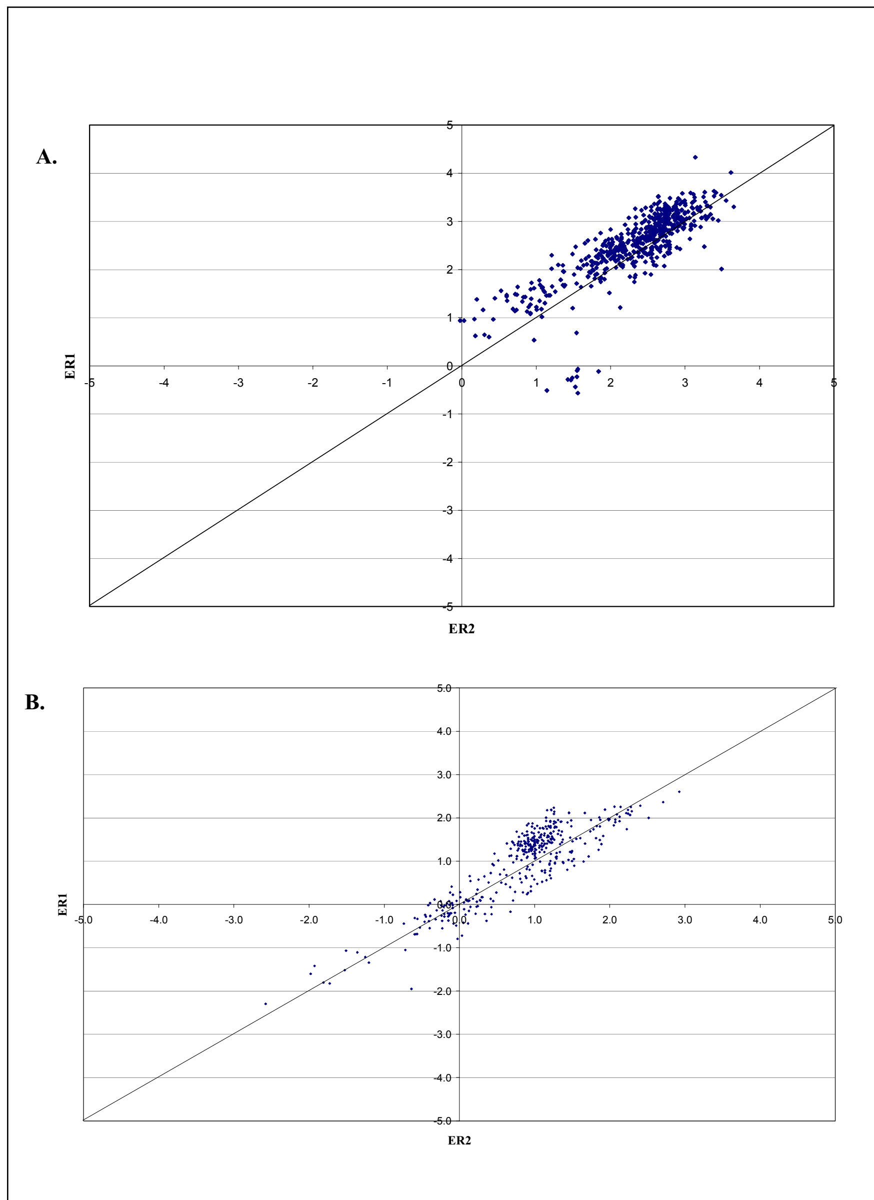

Figure 3. Screening pearl millet (A) and banana (B) SSH libraries on glass microarray slides. Suppression subtractive hybridization (SSH) enrichment ratio 1 (ER1) is plotted against SSH enrichment ratio 2 (ER2). ER1 was calculated for each clone by $\log_2$ transforming the value of the subtractive tester (ST) fluorescence divided by the unsubtracted driver (UD) fluorescence. ER2 for each clone was calculated by $\log_2$ transforming the value of the ST fluorescence divided by the unsubtracted tester (UT) fluorescence. The diagonal line indicates clones derived from transcripts of equal abundance in UD and UT (i.e., ER1 = ER2). Clones that lie above the diagonal line represent transcripts that are induced upon treatment (ER1 > ER2), while those below the line indicate transcripts that have escaped the subtraction (ER1 < ER2). Clones above the line with a positive ER2 value represent rare transcripts, whereas clones above the line with a negative ER2 value are regarded as abundant and have been reduced in relative concentration during normalization.

probes, with values for the same clones hybridized with the driver probe.

RESULTS AND DISCUSSION

SSH cDNA Library Construction

To identify differentially expressed pearl millet defense response genes, SSH was performed between cDNAs from the downy mildew-resistant line ICML12=P7 that had been wounded and treated with elicitors and cDNAs from an untreated downy mildew susceptible line, 842B. SSH experiments for banana were designed to isolate unique defense response genes from a tolerant variety in response to *Foc*. SSH was therefore performed using two near-isogenic banana cultivars, a Cavendish selection (tolerant) and Williams (susceptible) from the Cavendish group of banana varieties.

Two tests were performed to investigate the efficiency of the SSH. First, PCR was used to amplify a representative housekeeping gene (actin) to show the successful removal (reduction in the levels) of this gene in the ST material (result for the banana SSH shown in Figure 1). Specific PCR products were visible after 20 cycles from the UT and UD material, whereas no PCR product was detected in the ST material even after 30 PCR cycles. In addition, the efficiency of subtraction was evaluated by Southern blot analysis. Complex ST probes hybridized mainly to the ST PCR products, with far less hybridization to UT and UD PCR products (Figure 2A). Furthermore, very little UT and UD probe hybridized to the ST material (Figure 2, B and C), demonstrating gene transcripts common to both UD and UT had been removed by the subtraction and thus implying enrichment for tester-specific transcripts. Similar results were obtained with the banana SSH (data not shown). The SSH procedure yielded cDNA libraries of 960 pearl millet clones and 736 banana clones.

Table 2. Validation of Microarray Screening of Selected Pearl Millet and Banana SSH Clones by Inverse Northern Data

Clone No.	ER1[a]	ER2[b]	UT/UD Ratio[c]	Inverse Northern Expression Ratio[d]
Pearl Millet				
3-D5	1.4	0.2	2.3	34.3
4-H11	1.2	0.3	1.9	21.1
10-B7	1.0	0.2	1.7	11.1
4-H9	0.6	0.3	1.2	9.3
5-C3	1.5	0.7	1.7	7.9
1-B7	1.6	0.5	2.1	7.2
6-A2	1.2	0.7	1.4	6.3
8-D7	1.0	0.4	1.5	6.2
4-A2	0.9	0.03	1.8	5.8
4-E12	0.9	-0.02	1.9	5.4
7-D7	1.6	0.8	1.7	5.3
2-A12	3.6	3.4	1.1	3.6
6-F1	2.6	2.0	1.5	3.3
2-A8	3.6	3.3	1.2	2.6
6-H1	2.2	1.9	1.2	2.5
6-C2	3.0	2.7	1.2	2.3
6-D1	2.6	2.5	1.1	1.5
3-H3	4.3	3.1	2.3	1.3
6-G2	2.0	1.7	1.2	1.1
10-C6	-0.3	1.5	0.3	0.9
Banana				
2-35	0.3	-0.1	1.3	6.9
2-21	1.8	0.9	1.9	1.5
2-18	2.1	1.5	1.6	1.4
2-45	1.4	1.0	1.3	1.0
1-1	0.8	0.9	0.9	0.98
2-14	0.7	0.9	0.9	0.97
3-94	-0.1	0.2	0.8	0.94
2-134	1.5	1.6	0.9	0.1
3-156	0.6	0.8	0.9	0.1

SSH, suppression subtractive hybridization; ST, subtracted tester; UD, unsubtracted driver; UT, unsubtracted tester.
[a,b]Enrichment ratio 1 and 2 (ER1 and ER2) were calculated from the microarray screening as $\log_2$(ST/UD) and $\log_2$(ST/UT), respectively.
[c]UT/UD = antilog of (ER1 - ER2) in the base 2.
[d]The inverse Northern expression ratio was calculated as follows: density of tester/density of driver samples after normalization of the data using an rDNA clone.

Screening of the SSH Libraries Using Glass Slide Microarrays

cDNA microarray technology was used to perform a high-throughput screen of the pearl millet and banana SSH cDNA libraries to identify genes expressed in response to pathogen elicitor treatment and *Foc* infection, respectively, and to obtain information about the relative abundance of these gene transcripts upon induction of plant defense responses. The aim was to identify and discard housekeeping and rRNA genes that had escaped subtraction and to select defense response-associated genes for sequencing.

The SSH libraries arrayed on the glass slides were screened with rDNA clones from pearl millet or banana. The rDNA probes hybridized to 5% of the pearl millet clones and 28% of the banana clones (data not shown). The banana library had been made from total RNA, whereas the pearl millet library had been made from mRNA, which may explain why more banana rDNA clones escaped subtraction.

After hybridization with combinations of the SSH cDNAs, enrichment ratios were calculated for ST:UD (ER1) and ST:UT (ER2). A positive ER1 or ER2 value indicates transcripts that have been enriched during subtraction relative to their levels in UD or UT, respectively. Conversely, negative ER1 or ER2 values indicate transcripts that have been reduced in abundance during SSH relative to their levels in UD or UT, respectively, due to normalization. Normalization equalizes the concentration of individual transcripts, which may be present at very different concentrations prior to normalization (2). This has the advantage of enriching for rare transcripts in the subtracted tester samples and reducing the levels of abundant transcripts.

Relative abundance of cDNAs in UD and UT was visualized by plotting ER1 versus ER2 for individual pearl millet and banana clones (Figure 3). The diagonal line on each graph (ER1 = ER2) in Figure 3 represents similar levels of enrichment/normalization during SSH relative to UD and UT. Clones lying on this line are derived from transcripts of equal abundance in UD and UT. The majority of clones (77% for the pearl millet SSH and 63% for the banana SSH) lie above this line (ER1 > ER2), indicating a greater abundance of these transcripts in UT than in UD and confirming that each SSH has enriched for transcripts that are up-regulated in the tester. Clones above the diagonal line with positive ER2 values are likely to be derived from low abundance transcripts; expression of some of these may be difficult to detect in Northern blots, and accurate comparisons of gene expression between tester and driver may require real-time reverse transcription PCR (RT-PCR). Several clones above the diagonal line showed negative ER2 values, representing transcripts enriched relative to levels in UD and reduced in abundance during normalization relative to levels in UT. This was more evident in the banana SSH (Figure 3B). Such clones represent abundant transcripts specifically up-regulated in the tester that should be readily detected in Northern blot analyses.

Inverse Northern Blot Analysis of Selected Transcripts

To validate conclusions drawn from comparisons of ER1 and ER2 and to confirm that clones are derived from differentially expressed transcripts, clones from each library were selected for inverse Northern blot analyses. These clones were arrayed in duplicate on dot blots and hybridized to freshly prepared nonamplified tester and driver cDNAs.

ER1 and ER2 ratios from the microarray screening (Figure 3), together with the inverse Northern expression ratios of the selected pearl millet and banana clones, are shown in Table 2. Clones with ER1 > ER2 had inverse Northern expression ratios greater than one, confirming that these clones represented transcripts that were up-regulated in the tester compared to the driver (data for 19 pearl millet and 4 banana clones are shown in Table 2). Clones where ER1 < ER2 showed inverse Northern expression ratios of less than one (10-C6, 1-1, 2-14, 3-94, 2-134, 3-156) confirmed that they represented transcripts that were more abundant in the driver than the tester and that these clones had escaped the subtraction process. In order to relate the inverse Northern and microarray data, a ratio of UT/UD was calculated from the ER1 and ER2 ratios (Table 2). As expected, inverse Northern ratios correlated with UT/UD ratios (i.e., clones with inverse Northern ratios >1 had UT/UD values >1), whereas clones with inverse Northern ratios <1 gave UT/UD <1 (Table 2).

Although the majority of clones in the two SSHs showed ER1 > ER2, there were differences between the two SSHs (Figure 3). The pearl millet SSH, which used mRNA for the subtraction and was based on treated tester and untreated driver material, yielded a majority of clones that were enriched in ST relative to UT. In contrast, the banana SSH, based on total RNA and involving tester and driver samples treated in the same way (i.e., both challenged with *Foc*), but in which different defense responses were anticipated, yielded more clones that were reduced in abundance in ST relative to UT. Many of the clones

enriched in this latter SSH were of equal or greater abundance in UD than UT. Therefore, whereas the vast majority of clones in the pearl millet SSH could be confidently included in downstream analyses as likely derived from up-regulated genes, many of the clones in the banana SSH would be eliminated from further analyses. This emphasizes the value of the approach for quantitative characterization of the SSH and in selecting clones for further study.

We have effectively demonstrated the use of cDNA microarrays to screen two independently constructed pearl millet and banana SSH libraries in a rapid, high-throughput manner. The major advantages of our screening method are that it provides an objective and quantitative way to identify differentially expressed genes as well as to determine the relative abundance of transcripts in the original UT samples.

Previous studies have used inverse Northern blot analysis to screen SSH libraries in which PCR products or colonies are dotted onto nylon membranes, and the driver and tester cDNAs are labeled with radioactivity (2,7). This method has disadvantages; namely, that comparisons are made between two separate membrane hybridizations, which introduces error, and the interpretation is qualitative (7) unless a laboratory has access to a phosphorimager. In contrast to membrane-based methods, hybridization to glass slide cDNA microarrays can be performed with different fluorescent tags, which allows a direct comparison of the relative abundance of transcripts in ST, UT, and UD. Furthermore, hybridizations are performed on a small surface area, which reduces the amount of labeled probe needed. Finally, the computerized scanning of the array provides a high-throughput quantitative method to choose which genes to sequence and study further using Northern blot analysis, real-time RT-PCR, or a custom microarray.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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