Identification of defence-related genes in banana against Fusarium Wilt

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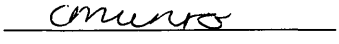
DECLARATION

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree M.Sc. to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.



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I avoid looking forward or backward, and try to keep looking upward

~~~ Charlotte Brontë ~~~

PREFACE

Banana is one of the most important but undervalued staple food crops in the world. It provides food for millions of people in the tropics and subtropics and is a major source of income for millions of households, especially in Africa. Bananas are, however, under threat by many pathogens and pests, one of the most serious threats being Fusarium wilt caused by *Fusarium oxysporum* Schlect. f.sp. *cubense* (E.F. Smith) Snyder & Hanson (*Foc*). To date, there is no effective disease control strategy in the form of integrated disease management, chemical control or otherwise. Natural disease resistance exists in wild-type bananas and in a few hybrids but in both instances these bananas are not acceptable to the commercial market. Conventional breeding strategies to generate disease resistant plants are slow, and to date, not very successful due to the long generation time of the plant, the large amount of space and manpower required and the fact that bananas acceptable to the consumer's palate are sterile and do not produce seeds. Therefore, biotechnological strategies such as defence-gene identification and plant transformation could successfully speed up the generation of a banana resistant to *Foc*. Unfortunately, little is known about the interaction between banana and *Foc*. The molecular mechanisms underlying resistance to *Foc* in bananas is, consequently, also not well understood. The aim of this thesis, therefore, was to investigate the differential expression of defence-related genes in four banana varieties differing in susceptibility to *Foc*, namely Williams, FHIA-17, Rose and Calcutta IV.

In **Chapter 1**, the genetic and molecular basis of plant defence responses against Fusarium wilt diseases is reviewed. The first part of this chapter introduces Fusarium wilt diseases, their responsible pathogen and their control. The second half of the review provides an overview of plant defence strategies, both preformed and induced as well as the genetic basis of the defence response. Where applicable, examples of how these defence mechanism apply to plant defence against Fusarium wilts are included. Furthermore the different technologies available to study disease resistance in plants are also discussed.

Chapter 2 evaluates the disease susceptibility of four banana varieties under South African conditions in a field infested with *Foc* 'subtropical' race 4. Plants were simultaneously challenged with *Foc* in the greenhouse at the University of Pretorial and a gene expression analysis of six defence-related genes

isolated in previous studies on banana defence was conducted using quantitative real-time reverse transcriptase PCR. These genes include *peroxidase, endochitinase, phenylalanine ammonia lyase, PR-1, catalase* and *pectin acetylesterase,* of which c*atalase*, *peroxidase* and *pectin acetylesterase* were strongly up-regulated in the tolerant and resistant banana varieties, and can possibly be linked to their defence response.

Chapter 3 describes the generation of gene expression profiles in four banana cultivars (Williams, FHIA-17, Rose and Calcutta IV) challenged with *Foc*, and identifies genes differentially expressed. This has been done using a cDNA fingerprinting technique termed cDNA-Amplified Fragment Length Polymorphism. Transcript derived fragments have been selected for sequencing and subjected to BLASTX and BLASTN searches. Expression profiles of genes further investigated by qRT-PCR are presented.

CHAPTER 1

THE GENETIC AND MOLECULAR BASIS OF PLANT DEFENCE RESPONSES AGAINST FUSARIUM WILT DISEASES

INTRODUCTION

Fusarium wilt diseases are widespread and highly destructive whether they occur in cultivated crops or in indigenous plant species. Losses are often so substantial that it becomes no longer profitable, or even possible to continue growing the crop without control of the disease (Mace *et al.*, 1981). Most Fusarium wilt diseases cannot be managed efficiently by using chemical, biological and cultural control strategies, and rely entirely on the use of resistant planting material (Mace *et al.*, 1981). The narrowing of the genetic base of agronomic and horticultural crops through selection and plant breeding, and the intensive monoculture of these crops, however, has contributed to the damage caused by at least some Fusarium wilt pathogens (Mace *et al.*, 1981). A good example is Fusarium wilt of banana (Panama Disease) which devastated export banana (*Musa*) fields in Central America homogeneously planted with the 'Gros Michel' cultivar in the first half of the 19th century (Stover, 1962).

Most plants are resistant to most microbes and only specialist organisms have evolved the capacity to overcome plant defences. Broadly, there are three reasons for pathogen failure: the plant is unable to support the niche requirements of a potential pathogen and is a non-host, it possesses preformed structural barriers and biochemical substances that prevent colonization (Shibuya and Minami, 2001), or plant defence mechanisms are activated upon recognition of the pathogen to contain infection (Hammond-Kosack and Jones, 1996). Successful pathogen invasion ensues if the preformed plant defences are not applicable to the specific pathogen launching the attack, the plant does not detect the pathogen, or the activated defence responses are ineffective (Hammond-Kosack and Jones, 1996). A detailed understanding of the individual components that define the molecular and genetic basis of the defence response of host plants to Fusarium wilt pathogens, and an intimate understanding of the technology available to study these mechanisms, is a necessary first step towards the development of complete and durable disease resistance.

FUSARIUM WILT

THE PATHOGEN

The Fusarium wilt pathogen *Fusarium oxysporum* Schlecht. is a cosmopolitan fungus consisting of a number of pathogenic and non-pathogenic individuals. The pathogenic members of *F. oxysporum* are divided into special forms (*formae speciale*) (Booth, 1971), defined by the particular host crop that they attack. Most *formae speciales* are pathogenic to a single host crop, for example *F. oxysporum* f.sp *cubense (Foc)* (E.F. Smith) Snyder & Hansen to banana, *F. oxysporum* f.sp. *vasinfectum* (*Fov*) (Atk.) to cotton (*Gossypium hirsutum* L.) and *F. oxysporum* f.sp. *dianthi* (*Fod*) (Prill. & Delacr) Snyder & Hansen to carnation (*Dianthus caryophyllus* L.). Some *formae speciales*, however, cause disease to more than one host, for instance *F. oxysporum* f.sp. *radicis-lycopersici*, which at least in greenhouse studies can cause disease on hosts from several other plant families as well as tomato (Kistler, 1997). There are at least 150 *formae speciales* within *F. oxysporum* (Baayen *et al.*, 2000) that can be further divided into races (Armstrong and Armstrong, 1981). A race is based upon the virulence of individuals in a *formae speciales* to a set of differential host cultivars (Correll, 1991). Race designation in *F. oxysporum* can be a simple subdivision with a single cultivar defining a single race, or a more complex subdivision where several cultivars are host to a single pathogenic race. Single-cultivar races are found in the tomato (*Solanum lycopersicum* L.)/*F. oxysporum* f.sp. *lycopersici* (Fol) (Sacc.) W.C. Snyder & H.N. Hans interaction where three races of *Fol* and three cognate *R* genes in tomato have been identified (Ori *et al.*, 1997), while multiple-cultivar races are present in *Foc* where race 4 attacks Cavendish bananas as well as all cultivars that are attacked by races 1 and 2.

ETIOLOGY AND SYMPTOMS

Fusarium oxysporum survives in the soil and plant debris as dormant chlamydospores or conidia (Stover, 1962). The chlamydospores and conidia are stimulated to germinate by host roots, root excretions from non-host roots, or when coming in contact with pieces of fresh non-colonised plant remains (Stover, 1962). Infection takes place mostly through wounds and rapidly elongating root tips where the fungus moves into

the xylem and xylem parenchyma of susceptible plants (Roberts and Boothroyd, 1984). Both pathogenic and non-pathogenic forms of *F. oxysporum* can colonise roots, but the spread of the non-pathogens are limited to the cortex (MacHardy and Beckman, 1981) and do not enter the xylem vessels. The colonised vessels may be clogged with mycelia, spores, or polysaccharides produced by the fungus (Roberts and Boothroyd, 1984). The xylem may be further plugged by gels and gums derived from enzymatically hydrolysed constituents of affected cell walls or with by-products from the pathogen. Once colonised with the fungus vascular tissues turn brown, providing a diagnostic symptom for the vascular wilt disease (Roberts and Boothroyd, 1984). As a result of the blockage and breakdown of xylem, symptoms such as leaf wilting and yellowing appear before the plant eventually dies. As long as the infected plant is alive, the wilt-causing fungi remain in the vascular tissues and a few surrounding cells. Only when the infected plant is killed by the disease do these fungi move into other surrounding tissues and sporulate at or near the surface of the dead plant (Ploetz and Pegg, 2000). The resulting spores can then be used as new inoculum for further spread of the fungus. *Fusarium oxysporum* is spread over short distances by irrigation water and contaminated farm equipment (Ploetz, 1994), over long distance in infected plant material.

CONTROL

Fusarium oxysporum and its many special forms affect a wide variety of hosts, begetting its management to differ among pathosystems. In general, chemical treatments, biological control, cultural control and resistant cultivars can be included in an integrated disease management strategy.

Chemicals for the control of plant diseases can be subdivided into four categories, namely fungicides, surface sterilants, fumigants and plant activators. Fungicides that have been used effectively for the control of Fusarium wilt of tomato grown in hydroponic systems (Song *et al.*, 2004) and partial control of ornamental plants such as carnation and chrysanthemum (*Chrysanthemum* spp. L) (Gullino *et al.*, 2002) include Prochloraz, carbendazim and benzimidazoles respectively. However, Fusarium wilt of several important crops such as banana (Nel *et al.*, 2007) and conventionally grown tomatoes (Borrero *et al.*, 2006), could not be controlled by fungicides. Surface sterilants are of great importance for preventing the introduction and spread of Fusarium wilt diseases in fields (Moore *et al.*, 1999). Today, sterilants such as

copperoxycloride, chlorine bleach and methylated spirits have been replaced by environmentally more friendly products such as 'Farmcleanse ®' and Sporekill® (Moore *et al.*, 1999; Nel *et al.*, 2007). Soil fumigation has been a widely used strategy for the eradication of soil pathogens. This technology involves introducing gas-forming chemicals such as carbon disulfide, methyl bromide, or chloropicrin into soil to kill target pathogens (Maloy, 2005). However, undesirable side effects such as killing beneficial organisms, contamination of groundwater, and toxicity of these chemicals have resulted in less reliance on this approach for disease management (Maloy, 2005). Plant activators such as BION®, Messenger® and Vacciplant® have been tested on various crops. BION® has successfully controlled Fusarium wilt of cotton, however, Messenger® and Vacciplant® were less successful in the prevention of Fusarium root rot in pea plants (Grunwald *et al.*, 2003).

Biological control is an environmentally sound and effective means of reducing the ability of soil pathogens to infect their hosts through the use of natural enemies and/or competing microorganisms (Fravel *et al.*, 2003). Many groups of micro-organisms have been proposed as having a role protecting host plants from Fusarium wilt pathogens. Of these, non-pathogenic *F. oxysporum* and the fluorescent pseudomonads proved to be the most effective (Alabouvette *et al.*, 1993). Biological control of Fusarium wilt diseases can be achieved by means of antibiosis, competition and induced resistance (Fravel *et al.*, 2003). While biological control of many Fusarium wilt diseases have been demonstrated in the greenhouse (Paulitz and Belanger, 2001), only a few have been controlled in the field. This lack of field control can be contributed to factors such as inconsistent biotic and abiotic parameters (Knudsen *et al.*, 1997), for example fluctuations in temperature and rainfall or the varying composition of soil nutrients and microorganisms between fields. Biological control, therefore, would probably be most effective as a component in an integrated disease management system.

Cultural control measures for the control of Fusarium wilt include quarantine, sanitation practices and soil management aimed at reducing or eliminating the amount of inoculum in a field or plant. Quarantine as a measure to limit the spread of the pathogen involves limiting the movement of pathogens in infected planting material between countries or between fields. Sanitation involves the use of disease-free planting material (most importantly plants grown in tissue culture labs), planting in fields known to be pathogen free, using clean water for irrigation and clean field equipment when ploughing, harvesting or maintaining the

plantations. Soil management includes crop rotation, soil amendments and fertilizers and flood fallowing (Ferro, 2006). Crop rotation as a measure to control Fusarium wilt diseases by withholding the pathogen's host has been met with mixed success but was generally found to be ineffective as the fungus is able to survive in the soil for long periods of time without a host plant. Soil amendments and fertilizers have the potential to control Fusarium wilts by improving plant vigor, thus improving plant resistance and affecting the microbial populations within the soil (Alabouvette *et al.*, 2004).

As many integrated disease management programs are not always sufficient to control Fusarium wilt diseases, disease resistant plants are a necessary alternative. An understanding of how plants defend themselves on a molecular level is a crucial step towards generating resistant plants where resistance is not readily available in closely related species and wild progenitors.

PLANT DEFENCE MECHANISMS

Plant resistance is correlated with the activation of a diverse set of defence mechanisms. The response involves the transcription of numerous defence-related genes, opening of ion channels, modifications of protein phosphorylation status, and activation of preformed enzymes to undertake specific modifications to primary and secondary metabolism. In addition, a range of secondary signalling molecules are generated to ensure coordination of the defence response both temporally and spatially, resulting in rapid containment of the pathogen (Hammond-Kosack and Jones, 2000). Some of the responses are constitutive and pathogen non-specific, but the majority of them are induced after recognition of some feature of the pathogen (Jalali *et al.*, 2006). The ability of potential hosts to discriminate between self and non-self is a key to the activation of innate defence mechanisms in response to attempted microbial infection (Nurnberger and Lipka, 2005).

STRUCTURAL DEFENCE

The first line of plant defence is its surface, which the pathogen must penetrate if it is to cause infection. The cell wall in plants is the physical barrier that keeps most organisms from developing close enough

contact with the plant for disease to ensue (Nurnberger and Lipka, 2005). Preformed structural factors not specifically involved in the *F. oxysporum* defence response include the thickness or hardness of the cuticle and the amount or quality of wax and cuticle that cover the epidermal cells (Dangl and Jones, 2001; Nurnberger and Lipka, 2005). These form a barrier against all pathogens apart from those that have the necessary pathogenicity factors for direct penetration or cell wall degradation, or vectors that are carrying pathogens, such as insects. Other pre-formed defence mechanisms include the size, location and shape of the stoma, at what time of day the stomata open, the presence of lenticels and the root pericycle (Keen, 1992).

Structural plant defence mechanisms against *Fol* have been studied in resistant and susceptible tomato plants (Brammall and Higgins, 1988). Their results suggested that the hypodermis is an important constitutive barrier hindering colonisation of the inner root cortex by the pathogen. The structural integrity of banana roots is also a key barrier to infection by *Foc* as several studies have shown that root injury and cutting, to expose the xylem, is a major factor influencing infection (Sequeira *et al.*, 1958). In carnation, microscopy studies of stems and roots have revealed constitutive and induced structural defence responses to *Fod*. Baayen (1987) found that the epidermis, endodermis and later the periderm surrounding the vascular tissues formed constitutive, structural barriers upon *Fod* invasion.

When attacked, plants physically reinforce cell walls to stall or prevent pathogen penetration (Maor and Shirasu, 2005). One of the proposed strategies by which plants do this is the deposition of callose, a sugar polymer that consists of (1-3)-ß-D-glucan subunits and is present between the plasma membrane and the cell wall (Maor and Shirasu, 2005). Plants deposit callose in proximity to the attacking pathogen whereas immuno-deficient plants do not, indicating that it may play a role in induced structural defence. Histochemical studies of tomato parenchyma infected by *Fol* provided evidence that callose-containing substances were deposited in response to infection which then become lignified or suberized by the infusion of phenolics or lipids respectively (Mueller and Beckman, 1988; Beckman *et al.*, 1989; Beckman, 2000). However, some researchers have shown that plants deficient in callose production show enhanced resistance to powdery mildew pathogens; they postulate that the callose deposits which form a structural barrier around the invading pathogens serve to prevent the perception of pathogen-derived elicitors and effectively seal off the pathogen from the action of plant antimicrobials (Jacobs *et al.*, 2003; Nishimura *et*

al., 2003). Histochemical studies of tomato parenchyma infected with *Fol* provided evidence that callose-containing substances were deposited in response to infection and that later lignification was detected (Mueller and Beckman, 1988; Beckman *et al.*, 1989). In pea plants (*Pisum sativum* L.) Tessier *et al.* (1990) noted vascular plugs, vessel coatings, callose deposits and phenolic compounds in resistant plants when infected with *F. oxysporum* f.sp. *pisi* (*Fop*) (Lindf.).

Banana responds to *Foc* conidial uptake in the xylem by forming vascular occluding gels (composed of neutral sugars such as arabinose, glucose and xylose and uronic acids that are also found in the cell walls of the host) 24-48 hrs after inoculation (Beckman, 1987). The gels are produced just above the spore-trapping sieve vessels to cut off the transpiration stream and immobilise the secondary spores of the pathogen. Two to 3 days after inoculation the vascular parenchyma cells form tyloses that block the lumena of infected vessels. In general, these vascular occlusions 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). In carnation, *Fod* is able to colonise resistant and susceptible cultivars (Higuera and Ebrahim-Neabat, 1999). The stems of the resistant carnation cultivar, however, respond with vascular gelation and hyperplasia of xylem parenchyma bordering infection sites, while susceptible varieties were intensively colonised 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.

BIOCHEMICAL DEFENCE

Plants constitutively synthesize a variety of chemical substances in their leaves and roots, called phytoanticipins, which prevent pathogens from causing disease. Some examples of pre-formed antimicrobial compounds include alkaloid and phenolic compounds, fungitoxic exudates which can inhibit fungal spore germination, tannins, dienes, saponins, plant lectins as well as hydrolytic enzymes such as glucanases and chitinases which may cause breakdown of the fungal pathogen cell wall (Heath, 2000). Additionally, protease inhibitors, once believed to be an exclusive defence mechanism against insects,

have been shown to have anti-fungal properties (Joshi *et al.*, 1999; Heath, 2000). Phenolic compounds play an important role in the resistance process against vascular diseases and are present in the xylem tissue of plants (Beckman, 2000). Enzymes that are important for 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 preformed and stored in various localised sites (Mace and Wilson, 1964; Mueller and Beckman, 1974, 1978) in order to be more readily available during the defence response. This fact is consistent with findings by Novak (1992) that SH-3362, a *Foc* race 4-resistant, synthetic AAA hybrid banana produced at FHIA, had constitutive levels of peroxidase ten-fold higher than Pisang Mas, a susceptible diploid.

Inducible defence responses in non-host plants comprise the synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, pathogenesis-related (PR) proteins, localised reinforcement of the plant cell wall and hypersensitive programmed cell death (Nurnberger and Brunner, 2002; Thordal-Christensen, 2003; Mysore and Ryu, 2004; Nurnberger and Lipka, 2005). All of these mechanisms are preceded by some form of recognition and signal transduction within the plant cells. Several alterations in cellular metabolism occur as an outcome of recognition, such as calcium influx, generation of active oxygen species and activation of protein kinases (Jalali *et al.*, 2006). These alterations result in the activation of an array of resistance responses, including a form of programmed cell death called the hypersensitive response (HR), and the production of antimicrobial proteins in a process referred to as effector-triggered immunity (Martin *et al.*, 2003; Jones and Takemoto, 2004; Jones and Dangl, 2006). The events following pathogen recognition are not well understood and downstream pathways seem to vary for different host plants, resistance genes, elicitors and pathogens (Bent, 1996; Ebel and Mithofer, 1998; Glazebrook, 2001). Despite the complexity and diversity of plant-pathogen interactions, signalling events and resistance mechanisms for many plant responses have been identified. When elicitors are recognised, three kinds of signals are produced: intracellular signals, short distance intercellular signals, and systemic signals (Vidhyasekaran, 1997).

The hypersensitive response

The HR is regarded as one of the most frequently occurring defence responses in host-pathogen systems in which the plant and the pathogen are incompatible and the pathogen fails to infect the host (De Wit,

1992). The HR varies from other mechanisms of resistance to plant disease; it is in fact the antithesis. Whereas other resistance mechanisms all involve the synthesis of a protective cell-sparing entity, the HR involves the extremely rapid death of host cells, which limits the progression of the infection (Goodman and Novacky, 1996). Upon exposure to certain pathogens, plant cells in the immediately affected area undergo a rapid cell suicide response that is theoretically intended to kill cells near the site of infection by cutting the pathogen off from healthy host cells, thereby limiting the spread of pathogens.

The HR is usually preceded by rapid and transient responses, including ion fluxes, especially K^+ and H^+, alterations in protein phosphorylation patterns, pH changes, changes in membrane potential, release of reactive oxygen species (ROS) and oxidative cross-linking of plant cell wall proteins (Richberg *et al.*, 1998; Dickinson, 2003; Dickman, 2004). Enzymes of the phenolic pathway such as peroxidases (POX), are expressed as well as glucanases and chitinases (Dixon and Lamb, 1990).

Some of the first responses activated in many incompatible interactions are the production of ROS, also known as active oxygen species or reaction oxygen intermediates, the production of nitric oxide, and phosphorylation cascades (Dickinson, 2003). The predominant species detected in plant-pathogen interactions are super oxides (O_2^-), hydroxyl radicals (OH) and hydrogen peroxides (H_2O_2) that can be detected within or less than 5 minutes after pathogen attack (Hammond-Kosack and Jones, 2000). During defence responses, ROS are produced by plant cells because of the enhanced enzymatic activities of plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases (Kawano, 2003; Laloi *et al.*, 2004) and amine oxidases in the apoplast (Allan and Fluhr, 1997; Laloi *et al.*, 2004). It was found in a study of elicitor-induced plant defence responses of parsley, that ion fluxes are required for the induction of the oxidative burst (Jabs *et al.*, 1997; McDowell and Dangl, 2000). In addition, calmodulin family members and calmodulin-like domain protein kinases have been implicated in regulation of the oxidative burst and other defence responses (Romeis *et al.*, 2000; 2001; Kim *et al.*, 2002; Nimchuk *et al.*, 2003).

Several roles in plant defence have been proposed for ROS. H_2O_2 may be directly toxic to pathogens (Peng and Kuc, 1992), give rise to the extremely reactive OH in the presence of iron (Fe^{2+}), and contribute to the structural reinforcements of the plant cell walls either by cross-linking or by increasing the rate of lignin polymer formation (Hammond-Kosack and Jones, 2000). ROS is also likely to induce some

signalling pathways (Levine *et al.*, 1994; Alvarez *et al.*, 1998). H_2O_2 induces benzoic acid 2-hydroxylyase enzyme activity, which is required for biosynthesis of salicylic acid (SA), and genes for proteins involved in certain cell protection mechanisms, for example glutathione-S-transferase and phenylalanine ammonia-lyase (PAL) (Hammond-Kosack and Jones, 2000). Reversible protein phosphorylation is the key biochemical event in most cell signalling pathways, and signal transduction involving ROS is no exception. Several reports have shown that mitogen-activated protein kinases (MAPK's) are activated by H_2O_2 (Hancock *et al.*, 2001; Asai *et al.*, 2002), which could lead to the modulation of gene expression. Whether H_2O_2 has a direct effect on MAPK's or activating upstream effectors needs to be established (Hancock *et al.*, 2001).

Production of ROS may further substantially alter the redox balance in the responding cells to regulate specific plant transcription factors for genes related to plant defence (Hammond-Kosack and Jones, 2000). It is now becoming apparent that the redox status inside a cell is crucial to the correct functioning of many enzymes, and can be used to alter enzyme activity (Gamaley and Klyubin, 1999). Thus, alteration of the redox status could act as a signalling mechanism (Gamaley and Klyubin, 1999). It is suggested that enzymes such as ribonucleotide reductase and thioredoxin reductase, as well as transcription factors, might be among the targets for altered redox status (Kirlin *et al.*, 1999; Hancock *et al.*, 2001). An important family of transcription factors are the WRKY transcription factors. The general binding preference of WRKY proteins for W boxes results in genes containing these promoter elements becoming likely targets of WRKY factors. This includes the WRKY genes themselves, as well as a large variety of defence-related genes of the *PR* type (Eulgem *et al.*, 2000).

Phytoalexins are low molecular mass, lipophilic antimicrobial compounds that accumulate rapidly at sites of incompatible pathogen infection (Hammond-Kosack and Jones, 2000). They are formed by the phenylpropanoid pathway. Phenylpropanoids are natural products derived from the amino acid L-phenylalanine via deamination by L-PAL (Dixon *et al.*, 2002). Phytoalexins are produced in healthy cells adjacent to localised damaged and necrotic cells in response to materials diffusing from the damaged cells. Resistance occurs when one or a few phytoalexins reach a concentration sufficient to restrict pathogen development. PR proteins also accumulate in plant cells in response to pathogen infection or other signals related to plant defence responses. A number of PR protein classes have been found to

have direct antimicrobial activity, which may explain the relevance of induction upon pathogen inoculation (Van Loon, 1997).

Responses in cotton to *Fov* include the possible biosynthesis of secondary metabolites such as lignin, syringyl lignin polymers, and terpanoid indole alkaloids in the hypocotyls (Dowd *et al.*, 2004). High syringyl content has been associated with pathogen defence response in wheat (*Triticum aestivum* L.) (Ride, 1975) and lignins are known to possess antifungal activity (Pegg and Young, 1982). In roots, however, genes associated with tannin, anthocyanin and lignin biosynthesis were repressed by the pathogen (Dowd *et al.*, 2004). In a study by De Ascensao and Dubery (2000), root tissue of the tolerant banana variety 'Goldfinger' responded to the fungal elicitor (from the mycelial cell walls of *Foc*) through the strong deposition of lignin, preceded by the induction or activation of the enzyme activities involved in the synthesis and polymerization thereof, whereas only slight increases were observed for the susceptible banana variety Williams.

Local and systemic resistance responses

Defence responses are expressed through the accumulation of downstream signalling molecules like SA, jasmonate (JA) and ethylene (ET), which regulate expression of localized cell death. In addition, these molecules lead to the expression of local and systemic induced resistance (Jalali *et al.*, 2006). Another plant hormone, abscisic acid (ABA) is also known to regulate responses to pathogens. Defence responses are seen to involve complex reiterative signalling networks with extensive signal amplification and crosstalk between pathways, as reviewed by Jalali *et al.* (2006).

It has long been known that plants develop a generalized resistance in response to infection by a pathogen or to treatment with certain natural or synthetic compounds (Chester, 1933; Da Rocha and Hammerschmidt, 2005). The classic form of systemic resistance is referred to as systemic acquired resistance (SAR) and occurs in distal plant parts upon localised infection by a necrosis-inducing pathogen (Ryals *et al.*, 1996; Van Hulten *et al.*, 2006). SAR is a broad spectrum resistance controlled by a signalling pathway that depends on endogenous accumulation of SA and is predominantly effective against biotrophic pathogens (Dong, 2004; Van Hulten *et al.*, 2006). It is also associated with the accumulation of PR proteins and the HR (Da Rocha and Hammerschmidt, 2005). Selected strains of non-pathogenic

rhizobacteria, also known as plant growth promoting rhizobacteria (PGRB), can also induce systemic resistance, which is referred to as induced systemic resistance (ISR) (Van Loon *et al.*, 1998; Van Hulten *et al.*, 2006). This type of resistance shows host specificity in regard to eliciting resistance (Van Wees *et al.*, 1997; Ton *et al.*, 2001; Da Rocha and Hammerschmidt, 2005). This suggests that specific recognition between protective bacteria and the plant is a pre-requisite for the activation of the signalling cascade leading to ISR. ISR is not mediated by the SA pathway, but instead by the JA/ET pathway (Pieterse *et al.*, 1998; Da Rocha and Hammerschmidt, 2005). A third type of induced resistance is activated upon application of the chemical ß-aminobutyric acid (BABA) (Van Hulten *et al.*, 2006). The signalling pathway controlling BABA-induced resistance differs from that of SAR and ISR; against pathogenic fungi and oomycetes it is controlled by a pathway that involves abscisic acid- and phosphoinositide- dependent signalling (Van Hulten *et al.*, 2006).

There are several examples of induced resistance being used to control Fusarium wilt diseases in various crops. For example, non-pathogenic *F. oxysporum* strain *Fo*47 induced resistance to Fusarium wilt in tomato (Fuchs *et al.*, 1997) by increasing the levels of chitinase, ß-1,3-glucanase and ß-1,4-glucosidase activity within the plant. Similar results were observed in pea root tissue inoculated with *Fo*47 (Benhamou and Garand, 2001). Benhamou and Garand's (2001) data indicated that pea root cells undergo marked ultra-structural modifications upon inoculation with strain *Fo*47, which correlates with the creation of a fungitoxic environment that protects the roots by restricting fungal growth and development to the outermost tissues. In banana, *Pseudomonas fluorescens* reduced the vascular discolouration associated with Fusarium wilt and induced the accumulation of resistance associated enzymes in roots such as POX, polyphenol oxidase (PPO) and PAL (Saravanan *et al.*, 2004). Low doses and frequent exogenous applications of indoleacetic acid was also shown to induce resistance in banana to *Foc* (Fernandez-Falcon *et al.*, 2003). In carnation, application of *Pseudomonas* sp. strain WCS 417r protected plants systemically against *Fod* (Van Peer *et al.*, 1991; Ramamoorthy *et al.*, 2001).

GENETIC BASIS OF THE PLANT DEFENCE RESPONSE

RECOGNITION OF *F. OXYSPORUM* BY PLANTS

The simplest mode of elicitor perception by the plant cell would be a direct interaction of pathogen-derived elicitors with specific receptors on host cells (Ebel and Cosio, 1994; Gomez-Gomez, 2004). Plant disease resistance genes (*R*) encode proteins (R) that act as receptors that recognize corresponding avirulence (*Avr*) gene products/pathogen effectors to initiate defence responses (Meyers *et al.*, 2005) (Fig. 1).

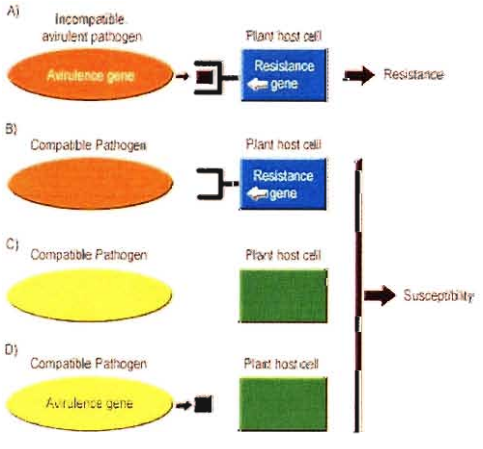

Figure 1. The gene-for-gene interaction. Basic interaction of pathogen avirulence with host resistance genes (International Rice Research Institute, http://www.knowledgebank.irri.org).

R genes have been cloned from a variety of plant species. Most *R* genes encode proteins that have a putative amino-terminal signalling domain, a nucleotide binding site (NBS) and a series of carboxy-terminal leucine-rich repeats (LRRs) (Meyers *et al.*, 2005) (Fig. 2). These 'NBS-LRR' proteins have been divided into two major classes: those with an amino-terminal TIR (Toll/interleukin receptor) domain (which are known as TIR-NBS-LRR or TNL proteins) and those that encode an amino-terminal coiled-coiled motif (CC-NBS-LRR or CNL proteins). The details of the molecular functions of these protein domains and their interacting partners are still being established. However, the consistent identification of this class of proteins across diverse plant species demonstrates that NBS-LRR genes are a pillar of plant defences

(Meyers *et al.*, 2005). Several classes of plant *R* genes have been identified in addition to the seemingly pervasive NBS-LRR class of *R* genes. These include cytoplasmic signal-transducing serine-threonine kinases, extracellular LRRs with transmembrane anchor and extracellular LRRs with transmembrane receptor and cytoplasmic serine-threonine kinase. The presence of cytoplasmic and transmembrane classes of R protein indicates that some are specialized in the detection of secreted ligands or surface components from the pathogen, and some are dedicated to recognize ligands that appear inside the cell (Fig. 2) (Gomez-Gomez, 2004).

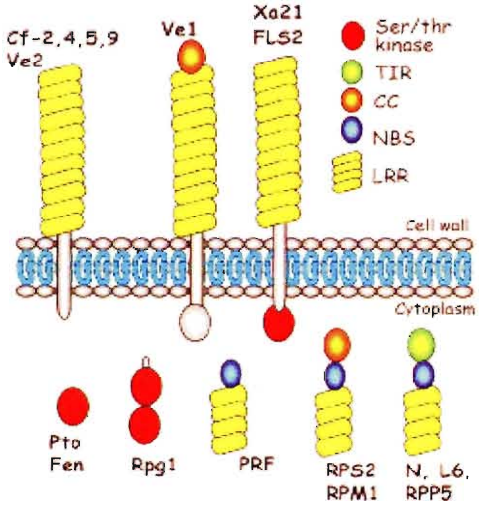

Figure 2. Representation of the location and structure of the main classes of R proteins (Gomez-Gomez, 2004).

Little is known regarding the mechanisms of host recognition during Fusarium wilt/host interactions. The only example where a gene-for-gene mechanism has been proposed is in the tomato/*Fol* interaction where the tomato resistance gene *I-2* confers resistance to race 2 of the pathogen (Ori *et al.*, 1997). Taylor (2004) isolated four resistance gene candidates from *Musa* spp. that showed similarity to previously isolated monocotyledonous *R* genes, and two which showed homology to the *Fol*-resistance locus in tomato. It does, however, remain to be determined whether the isolated resistance gene candidates confer resistance to any pathogens of significance, particularly *Foc*. Additionally, Peraza-Echeverria *et al.* (2007) amplified and identified a serine/threonine kinase (STK) from banana similar to the tomato Pto

protein. They found that multiple sequence alignments of the banana *Pto* resistance gene candidate products revealed that the sequences contain several conserved sub-domains present in most STKs, and also several conserved residues that are crucial for Pto function. The implications of this finding for disease resistance development in banana are potentially great. Priya and Subramanian (2007) reported the presence of an *R* gene of CC-NBS-LRR class in resistant *Zingiber officinale* Roscoe (ginger) varieties against *F. oxysporum* f.sp. *zingiberi (Foz)*. An interesting observation from their study is the presence of the putative *R*-gene in only the resistant ginger varieties. Neither the partially resistant or susceptible varieties showed the presence of this gene sequence.

Most plant defence responses to Fusarium wilt diseases do not involve *R*-genes. In the case of Fusarium wilt host plants, a secondary response takes place that involves a multi-'minor' gene qualitative resistance. Pathogen recognition in non-host plants can be brought about by pathogen effectors which seem to fall into two broad categories. The "virulence/Avr determinants" act as elicitors which are specific and unique for a particular pathogen. Each group of pathogens have developed a unique strategy for survival, resulting in multiple virulence factors that can vary between different strains and species of pathogens (Gomez-Gomez, 2004). By contrast, the second category of pathogen effectors, the non-specific elicitors, are constitutively present in the pathogen, some of them being physiologically equivalent to the 'microbial- or pathogen-associated molecular patterns, otherwise known as MAMPs or PAMPs (exogenous elicitors), described for mammals and *Drosophila* (Parker, 2003; Gomez-Gomez, 2004; Jones and Dangl, 2006) (Fig. 3). These may include surface-derived structures such as fungal cell wall constituents (chitin, glucan, protein and glycoprotein), which elicit defence responses in a wide range of plant species (Montesano *et al.*, 2003; Jones and Takemoto, 2004). Cell wall-degrading enzymes, i luding endopolygalacturonase and xylanase, are ubiquitous as virulence effectors (Shibuya and Minami, 2001; Jones and Takemoto, 2004), but can also function as elicitors.

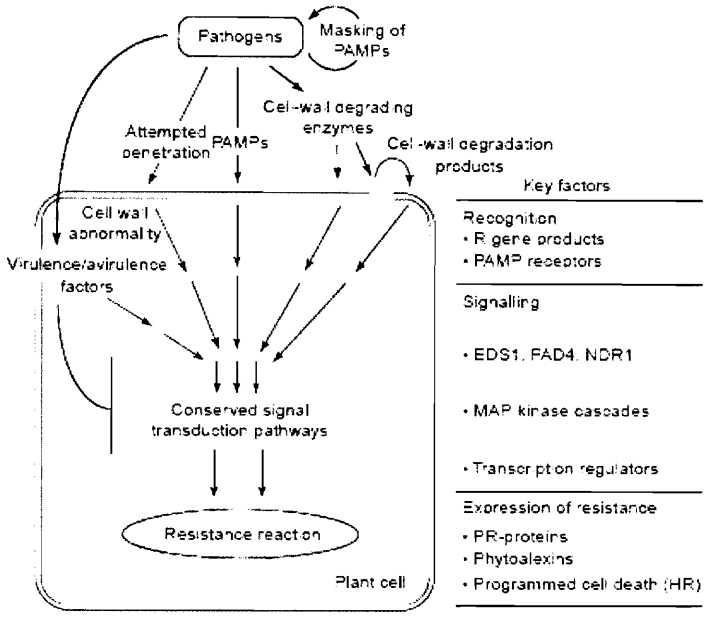

Figure 3. A simplified model for plant recognition and signalling responses induced by various pathogen elicitors (Shibuya and Minami, 2001; Jones and Takemoto, 2004). The model illustrates the multifaceted nature of pathogen attack and the broad spectrum of elicitors produced as a consequence, ranging from non-specific elicitors (PAMPs), through to highly specific elicitors (virulence effector/avirulence factors) with narrow specificity. One role of the latter may be to suppress plant mechanisms capable of responding to the former. Red arrows indicate pathogen strategies for infection and black arrows indicate plant signalling for resistance. Although detection of PAMPs is shown at the cell surface, and the action of virulence effector proteins and their detection as avirulence factors is shown in the cytosol, these locations are not mutually exclusive. (EDS1, PAD4 and NDR1 are downstream regulators of *R* gene signalling).

SIGNAL TRANSDUCTION PATHWAYS

Recent studies identified a number of components that may be involved in the ROS signal transduction of plants. These include the MAPKKK, *At*ANP1 (also NPK1), the MAPKs, AtMPK3/6, Ntp46MAPK, and calmodulin (Fig. 4). A sensor that might be a two-component histidine kinase, or a receptor-like protein kinase, is thought to sense H_2O_2. Calmodulin and a MAPK cascade are then activated resulting in the

induction/activation/suppression of a number of transcription factors. These regulate the response of plants to oxidative stress. Cross-talk with the pathogen-response signal transduction pathway (gene-for-gene) also occurs and may involve interactions between different MAPK pathways, feedback loops, and the action of SA and NO (Fig. 4).

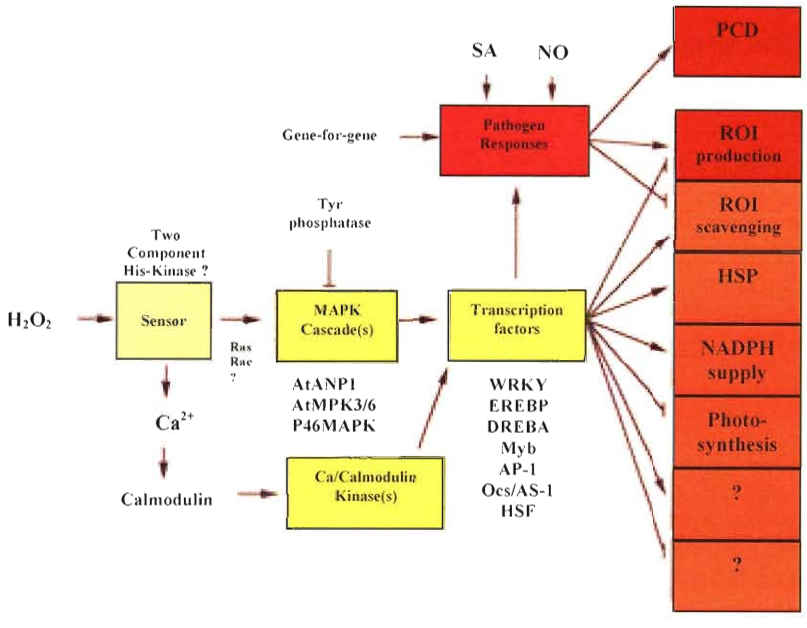

Figure 4. Theoretical components of reactive oxygen species signal transduction in plants (University of Nevada Reno, http://www.ag.unr.edu/ROS/).

After recognition, transcripts accumulate which code for signalling molecules (Eulgem *et al.*, 2004; Jalali *et al.*, 2006). The plant hormones SA, JA and ET are major players in the network of defence signalling pathways. Cross-talk between SA-, JA- and ET-dependent signalling pathways is thought to be involved in fine-tuning the defence reaction, eventually leading to the activation of an optimal mix of defence responses to resist the pathogen (Pieterse *et al.*, 2001). Generally, SA-dependent defences are activated more strongly in response to necrosis-inducing microbial pathogens, and JA- and ET-dependent defences are activated to a higher extent in response to insect herbivory (Reymond and Farmer, 1998; Bostock, 1999; Maleck and Dietrich, 1999; Pieterse and van Loon, 1999; Pieterse *et al.*, 2001).

SA is required for SAR which is associated with the expression of PR proteins (Malamy *et al.*, 1990; Ryals *et al.*, 1996; Baker *et al.*, 1997). Several *Arabidopsis* mutants provided insights into the genetics involved in the signalling mechanisms leading to SAR. Mutant *Arabidopsis* loci showing increased levels of SA and constitutive expression of *PR* genes as well as enhanced resistance to virulent bacterial and fungal pathogens include *cpr1* (Bowling et al., 1994) and *lsd2* (Dietrich *et al.*, 1994). *lsd2* plants show a constitutive lesion phenotype, which suggests that *LSD2* encodes a negative regulator acting upstream of SA synthesis or perception but downstream of the HR (Baker *et al.*, 1997). Another class of mutant loci, including *npr1* and *nim1* (Delaney *et al.*, 1995), induced a normal HR and SA accumulation in response to pathogen infection but fails to express *PR* genes upon treatment with chemical inducers such as SA. *NPR1*, therefore, appears to function downstream of SA accumulation, and may act as a transcriptional regulator of *PR* gene expression. Mutants affected in the *NPR1* gene accumulate normal levels of SA in response to pathogen infection, but fail to mount SAR (Pieterse *et al.*, 2001).

The *Arabidopsis* mutants *ndr1* (Century *et al.*, 1995) and *eds1* (Parker *et al.*, 1996) provided evidence for convergence of signals downstream of different R-Avr interacting partners into a single signalling pathway (Baker *et al.*, 1997). These mutants differ from *nim1* and *npr1* because they retain the ability to induce SAR. The *EDS1* and *NDR1* products may act upstream of SA accumulation but downstream of the initial recognition step. Additionally, *EDS1* may act upstream of *NDR1* (Baker *et al.*, 1997). Mutational screens in *Arabidopsis* identified several other plant defence signalling genes that are components of SA signalling in the plant response against pathogens, such as *PAD4* (Glazebrook *et al.*, 1997; Zhou *et al.*, 1998) and *SID1*/*EDS5* and *SID2*/EDS16 (Rogers and Ausubel, 1997; Nawrath and Metraux, 1999). Several of these genes have been mapped onto a schematic representation of the plant defence signalling pathway (Fig. 5).

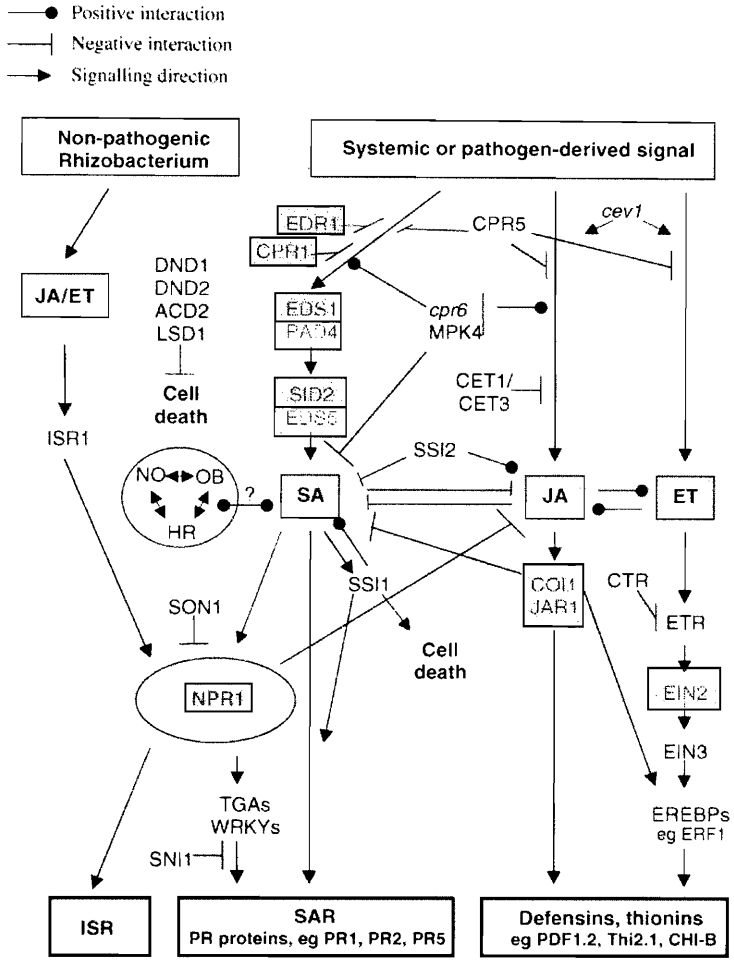

Figure 5. A schematic representation of the genes involved in the plant defence signalling pathway (CSIRO Publishing, www.publish.csiro.au/temp/FP04135_F3.gif).

ET and JA regulate expression of genes encoding antimicrobial peptides such as thionin and defensin (Epple *et al.*, 1998). Thionin gene expression is up-regulated by methyl jasmonate and is down-regulated by the ET-insensitive *ein2* and JA-insensitive *jar1* mutations. Similarly, *pdf1-2* (plant defensin1-2) expression is induced by JA and necrotrophic pathogen infection (Penninckx *et al.*, 1996; 1998). This induction is eliminated in the JA-insensitive *coi1* mutant and *ein2*. Neither thionin nor *pdf1-2* gene activation is affected by *NahG* expression, suggesting SA independence. However, epistasis analysis has revealed evidence for antagonism and crosstalk between the SA-dependent and the JA/ET-dependent defence pathways (Clarke *et al.*, 1998). ISR requires NPR1, which also operates downstream from SA (Pieterse *et al.*, 1998).

INDUCED PLANT DEFENCE RESPONSES

Many individual genes or families of genes are induced by pathogens or elicitors in a quantitative resistance response to Fusarium wilt pathogens.

Regulatory proteins

Several large-scale expression profiling studies revealed that a multitude of transcription factor genes are expressed in response to a wide variety of different defence–related stimuli (Durrant *et al.*, 2000; Maleck *et al.*, 2000; Chen *et al.*, 2002; Mysore *et al.*, 2002; Nimchuk *et al.*, 2003). Members of the large ERF/AP2-domain, bZIP, homeodomain, Myb, WRKY families as well as other zinc-finger factors were found to be up-regulated during multiple incompatible and compatible interactions (Nimchuk *et al.*, 2003). Elevated expression of such potential regulator genes in certain defence situations by no means proves a role of the respective factors in these processes, and their up-regulation may be an indirect consequence of the activation of the defence program rather than its cause. However, several independent studies indicated that products of transcription factor genes showing defence-associated up-regulation can specifically bind to promoters of *PR*- or other defence-related genes and may participate in their regulation (Korfhage *et al.*, 1994; Rushton *et al.*, 1996; Zhou *et al.*, 1997; Eulgem *et al.*, 1999; Nimchuk *et al.*, 2003). Transcription factor activity can be linked to upstream signalling events by phosphorylation (Karin and Hunter, 1995). Both ERF and WRKY transcription factors may be targeted by defence-activated protein-kinases (Nimchuk *et al.*, 2003).

Antifungal proteins

Plants have evolved a variety of potent defence mechanisms to protect themselves against pathogenic organisms, including the synthesis of low molecular weight compounds, proteins, and peptides that have antifungal activity (Selitrennikoff, 2001). Antifungal proteins include the PR proteins, defensins, cyclophilin-like protein, lipid transfer proteins and protease inhibitors; low molecular weight antimicrobial compounds include phytoalexins, antimicrobial peptides, and small proteins (e.g. thionins), hevein-like proteins, and knottin-like peptides. PR proteins are some of the best studied antifungal proteins, and have been classified into no less than 17 groups (van Loon *et al.*, 2006).

Table 1. The Recognised families of pathegenesis-related proteins (van Loon *et al.*, 2006).

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3*, *Chia*
PR-4	Tobacco 'R'	Chitinase type I, II	*Ypr4*, *Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6*, *Pis* ('*Pin*')
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8*, *Chib*
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase	*Ypr9*, *Prx*
PR-10	Parsley "PR1"	Ribonuclease-like	*Ypr10*
PR-11	Tobacco "class V" chitinase	Chitinase, type 1	*Ypr11*, *Chk*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13*, *Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14*, *Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

A gene expression study on the interaction between susceptible cotton and *Fov*, demonstrated that defence-related genes, in particular *PR*-2 (glucanases) and *PR*-3 (chitinase, class I and IV), *PR*-5 (thaumatin) and *PR*-10 were induced in the hypocotyls in contrast to being constitutively expressed in the root tissue (Dowd *et al.*, 2004), thus confirming a role in the defence response against the cotton wilt pathogen. The *Arabidopsis esal* mutant, which is susceptible to a variety of *Fusarium* species, notably the banana pathogen *Foc*, had its resistant phenotype restored upon expression of tobacco PR protein genes, *PR*-1 and *PR*-5 (Hemelrijck *et al.*, 2006).

Active-oxygen species/detoxification

H_2O_2 is continuously generated in plant cells as a by product of photosynthesis, photorespiration, fatty acid β-oxidation, and oxidative phosphorylation (Dempsey *et al.*, 1999). In addition, rapid and transient increases in ROS, including O_2^- and H_2O_2, are generated during the oxidative burst in plants resisting pathogen attack (Baker and Orlandi, 1995; Low and Merida, 1996). In many plant species, a membrane-bound NADPH oxidase similar to that identified in neutrophils appears to generate O_2^- after infection or elicitor treatment; the O_2^- is subsequently dismutated to H_2O_2 by superoxide dismutase (SOD) (Hammond-Kosack and Jones, 1996; Allan and Fluhr, 1997). Increased levels of H_2O_2 can also be generated by extracellular peroxidases in elicitor-treated tobacco and bean cells (Dempsey *et al.*, 1999). Catalases are tetrameric heme-containing enzymes that convert 2 $H_2O_2 \rightarrow O_2 + 2\ H_2O$, thus protecting the cell from the

damaging effects of H_2O_2 accumulation (Sanchez-Casas and Klessig, 1994; Dat *et al.*, 2000). The other major H_2O_2-scavenging enzyme in plant cells is ascorbate peroxidase (Dempsey *et al.*, 1999). These two enzymes act as cell protectants in *Arabidopsis*, SA has been shown to inhibit the *in vivo* and *in vitro* activity of ascorbate peroxidase, as well as catalase (Rao *et al.*, 1997). The discovery that SA inhibits the two major H_2O_2 -degrading enzymes in plant cells prompted speculation that SA might transduce the resistance signal by elevating the level of H_2O_2 or other ROS derived from H_2O_2 (Chen *et al.*, 1993). Increased levels of ROS might then act as second messengers to induce *PR* gene expression and disease resistance. This theory has, however, been refuted by several additional studies (Neuenschwander *et al.*, 1995; Allan and Fluhr, 1997).

Phenylpropanoid pathway

Phenylpropanoid pathway enzymes (Fig. 6) are important defence-related compounds which include PAL, chalcone synthase, 4-coumarate CoA ligase, chalcone isomerase, chalcone reductase, cinnamic acid 4-hydroxylase, cinnamyl alcohol dehydrogenase, chalcone flavone isomerase, NADPH:isoflavone oxidoreductase, caffeoyl-CoA 3-O-methyltransferase and tyrosine decarboxylase (Dixon *et al.*, 2002). The up-regulation of these genes may result in the increased production of lignin for cell wall strengthening, flavanoids, isoflavonoids and stilbenes (Dixon *et al.*, 2002). It is becoming increasingly clear that phenylpropanoid natural products may also play important roles as signal molecules, both in plant development and plant defence. The best-known examples of regulatory roles for phenylpropanoids include the activities of SA as a regulator of both local and systemic pathogen-induced defence gene activation, the oxidative burst, and pathogen-induced cell death (Dempsey *et al.*, 1999; Nimchuk *et al.*, 2003).

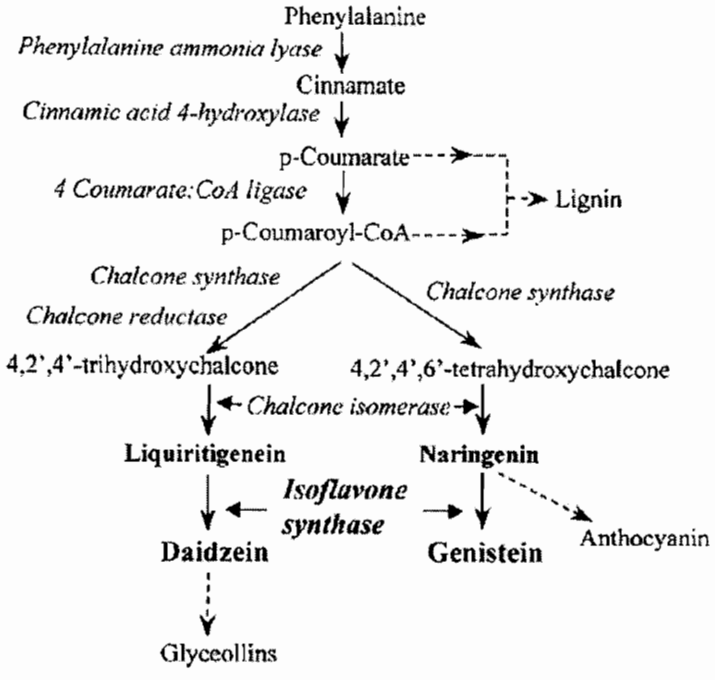

Figure 6. A simplified diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis. Branches for lignin and anthocyanin synthesis are marked. Dotted arrows represent multiple enzymatic steps (Jung *et al.*, 2000).

Isoprenoid phytoalexins

In plants, isoprenoid compounds such as sterols and ubiquinones, growth regulators (gibberellins, abscisic acid, brassinosteroid, cytokinin), phytoalexins and other specialized terpenes are essential for normal growth, development and defence against pathogens (Bach, 1995). In isoprenoid biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase converts acetyl CoA and acetoacetyl CoA to HMG CoA, and HMGR reduces HMG CoA to mevalonate, which is subsequently converted to isopentenyl pyrophosphate, the universal precursor for isoprenoids (Alex *et al.*, 2000).

Carbohydrate binding/hydrolyzing enzymes

Many plant species contain carbohydrate-binding proteins, which are commonly referred to as either lectins or agglutinins. Generally speaking, lectins are proteins that bind reversibly to specific mono- or oligosaccharides (Peumans and Van Damme, 1995). All plant lectins are artificially classified together

solely on the basis of their ability to recognize and bind carbohydrates. Several chitin-binding lectins have a role in the plant's defence, specifically against fungi. The first group is the chitin-binding merolectins, which are small proteins composed of a single chitin-binding domain. Hevein, a 43-amino acid polypeptide from the latex of the rubber tree *(Hevea brasiliensis* Müll. Arg.) has an antifungal activity comparable to that of nettle lectin (Parijs *et al.*, 1992). Chimerolectins are fungicidal plant lectins that belong to the class I chitinases (Parijs *et al.*, 1992; Peumans and Van Damme, 1995).

DISCOVERY AND ANALYSIS OF GENES RELATED TO DEFENCE AGAINST FUSARIUM WILT

Forward and reverse genetics are terms used to broadly describe the techniques researchers use to discover and characterize genes. Forward genetics begins with a mutant phenotype and asks the question "what is the genotype?" In other words, forward genetics largely refers to the search for a gene that underlies a specific plant response or trait of interest. Reverse genetics begins with a mutant gene sequence and asks "what is the resulting change in phenotype?" Thus, approaches taken to elucidate the function and role of a gene, usually by the manipulation of its expression, have been termed "reverse genetics." Molecular techniques which fall under the umbrella of reverse genetics include, for example, transcriptional profiling by cDNA-amplified fragment length polymorphisms (AFLP) (Bachem *et al.*, 1996), expressed sequence tag analysis, expression microarrays (Schena *et al.*, 1996), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), massively parallel signature sequencing (Brenner *et al.*, 2000) and proteomics. Gene knockout and mutant generation are commonly used reverse genetics techniques.

FORWARD GENETICS

Mutant analysis

Most of what researchers know today about plant defence and signalling pathways have been a direct result of random gene knockout and phenotype identification (Hammond-Kosack and Parker, 2003). The

common approach to obtain a mutant entails the identification (phenotyping) of a plant after seed germination, which has been mutated by chemicals, radiation or insertional mutagenesis either via transposable elements or *Agrobacterium tumefaciens* -mediated transfer (T)-DNA (Hammond-Kosack and Parker, 2003). The mutant phenotype generally exhibits characteristics that may be of particular interest. The next stage involves the identification and characterization of the mutated gene which resulted in the phenotype. Novel mutants either compromised or enhanced in defence are being identified through increasingly sophisticated screening and selection strategies. For example, mutagenised transgenic *Arabidopsis* populations can be monitored for atypical activity of a defence component by using a promoter fused to a luciferase reporter (Murray *et al.*, 2002; Hammond-Kosack and Parker, 2003). Another means by which mutant genes can be identified is by the use of the PCR Targeting Induced Local Lesions in Genomes (TILLING) technique (Till *et al.*, 2003). TILLING is a relatively high-throughput and simple way of identifying variations in a specific DNA sequence, typically 1000 bp in length, without the use of DNA sequencing. Its utility has already been demonstrated for diploid and polyploid organisms with significantly larger genomes.

REVERSE GENETICS

Transcriptomics

The goal of reverse genetics is the identification of a phenotype that is caused by a mutation in a particular gene. For example the *Arabidopsis* defence response mutant (*esa1*) which shows enhanced susceptibility to *Alternaria brassicicola* (Schwein.) has proven to be an ideal model for research on the plant's defence response against fungal pathogens in general and *Fusarium* species in particular. However, the *ESA1* gene which confers this phenotype is yet to be identified (Hemelrijck *et al.*, 2006). Bouche and Bouchez (2001) and Hammond-Kosack and Parker (2003) have listed *Arabidopsis* mutations whose disease resistant or susceptible phenotypes were characterised by reverse genetics. To prove definitively that the insertional mutation causes the phenotype, one must either isolate additional mutant alleles for the locus (by using for example TILLING) or complement the mutation by introducing a wild-type copy of the gene into the mutant plants by using transgenic technology (Krysan *et al.*, 1999).

To fully understand the function of a particular gene it is essential not only to study its temporal and spatial expression, but also that of other genes that may be similarly co-regulated (Wilson *et al.*, 2002). An analysis of the frequency with which gene sequences occur in individual libraries has been one possibility, but in most instances, this approach, which requires the sequencing of large numbers of different cDNAs from multiple libraries, has been time consuming and extremely expensive. Technologies that allow high-throughput analysis of differential gene expression constitute powerful tools for both novel gene discovery and for acquiring additional data about biological events on a genomic scale. All these tools share the common goal of identifying genes/transcripts that are expressed in ways which indicates them as possible candidate genes for a specific process or function. The goal is to analyze a pool of transcripts and hopefully gain a number of candidates for further analysis, as even a genome-wide expression analysis cannot prove a gene function relationship, and often other supplementary experimental data is required (Pereira, 2000). Another crucial aspect of the differential expression experiments is their design. A well-contrasted pair of samples could result in the identification of genes which are, at least in part, responsible for the differences between the samples (Ranik, 2005).

Widely used methods for the large-scale study of gene expression include SAGE, MPSS, gene macro- and microarrays, oligonucleotide chips (Lockhart and Winzeler, 2000), complementary (c)DNA-amplified fragment length polymorphism (Bachem *et al.*, 1996) (AFLP)-based techniques for RNA fingerprinting, and GeneCalling by CuraGen (Shimkets *et al.*, 1999; Pereira, 2000). Each of these techniques is powerful in identifying genes that are differentially expressed in normal and diseased cells. Other techniques to study differential gene expression include subtractive techniques such as suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996; 1999) and representational difference analysis (Lisitsyn and Wigler, 1993).

SAGE represents an efficient open-system method with which to systemically analyze the patterns of gene expression in a qualitative and quantitative way. This is also true of cDNA-AFLP's. However, in SAGE, rarely transcribed genes cannot be distinguished in a statistically significant manner (Nakagawa *et al.*, 2004). On the other hand, macroarray or microarray systems can be used to analyze gene expression with a high resolution. These approaches, however, do not exclude the possibility that unidentified genes or unknown genes are overlooked. To the advantage of the SAGE technique is the fact that for wheat, barley (*Hordeum vugare* L.), rice (*Oryza sativa* L.) and maize (*Zea Mays* L.) there are between 1221K

(rice) and 1052K (wheat) sequences in dbEST (http://www.ncbi.nlm.nih.gov/dbEST//index.html. accessed 2008). This means that there are potentially many more EST's that can be matched to SAGE tags than there are unigenes on array available (Wilson *et al.*, 2002). At the single gene level, quantitative real-time PCR has proved to be a very precise system that complements the above approach for quantifying the levels of transcription (Wilson *et al.*, 2002).

Reverse genetics has been successfully applied to better understand host-pathogen interactions and plant defence responses to pathogens. In cotton, for example, microarray analysis of large-scale temporal and tissue-specific plant gene expression changes occurring during a susceptible plant–pathogen interaction in the roots and hypocotyl tissues revealed different gene expression profiles (Dowd *et al.*, 2004). cDNA-AFLP was employed for gene expression analysis in barley after powdery mildew inoculation (Eckey *et al.*, 2004), and was also used to reveal genes differentially expressed during the hypersensitive response of cassava leaves in response to an incompatible *Pseudomonas syringae* pathovar (Kemp *et al.*, 2005). Seventy-eight transcript-derived fragments showing differential expression were identified using this technique, of which many encoded putative homologues of known defence-related genes. These included genes involved in signalling (e.g. calcium transport and binding, ACC oxidases and a WRKY transcription factor), cell wall strengthening (e.g. cinnamoyl coenzyme A reductase and peroxidase), programmed cell death (e.g. proteases, 26S proteosome), antimicrobial activity (e.g. proteases and beta-1,3-glucanases) and the production of antimicrobial compounds (e.g. DAHP synthase and cytochrome P450s) (Kemp *et al.*, 2005).

In a microarray study of the interaction between hypocotyl tissues of cotton and *Fov*, increased expression of defence-related genes was observed, whereas few changes in the expression levels of defence-related genes were found in infected root tissues (Dowd *et al.*, 2004). In infected roots, more plant genes were repressed than were induced, especially at the earlier stages of infection. Although many known cotton defence responses were identified using the microarray, including *PR* genes and gossypol biosynthesis genes, potential new defence responses were also identified, such as the biosynthesis of lignins which are a class of phytoestrogens (Dowd *et al.*, 2004). In banana, four defence-related genes were up-regulated in a tolerant banana selection GCTCV-218 after inoculation with *Foc*. These genes included *catalase*, pectin acetylesterase (*PAE)*, *PR-1* and *PR-3*, which implied that the tolerant phenotype observed in this banana

selection to *Foc* was possibly due to the up-regulation of cell wall strengthening enzymes (Van den Berg *et al.*, 2007).

SSH was used to identify differentially expressed defence-related genes in pearl millet and banana (Van den Berg *et al.*, 2004). SSH was performed between cDNAs from the downy mildew-resistant pearl mllet line ICML 12=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 defence-related genes from a tolerant (GCTCV-218) and a susceptible (Williams) variety in response to *Foc*. A cDNA microarray was then 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.

The most complete picture of gene expression in plants can, however, be offered by proteomics (Dove, 1999; Pereira, 2000). This addresses the protein expression of a cell type, the protein modifications, and can reveal actual interactions in a cell. The protein content of tomato xylem sap was found to change dramatically upon infection with *F. oxysporum (*Rep *et al., 2002)*. Peptide mass fingerprinting and mass spectrometric sequencing were used to identify the most abundant proteins appearing during compatible and incompatible interactions, and a new member of the PR-5 family was identified that accumulated early in both types of interaction. Other PR proteins appeared in compatible interactions only, simultaneously with disease development. This study demonstrated the feasibility of using proteomics for the identification of known and novel proteins in xylem sap, which will provide insight into plant-pathogen interactions in vascular wilt diseases (Rep *et al.*, 2002).

CONCLUSION

Fusarium oxysporum is an important vascular wilt pathogen that has caused worldwide devastation of many plant species. The most sustainable and environmentally friendly means to control this important pathogen involves the use of disease resistant plants. Despite the significant progress that has been made in recent years in understanding the biochemistry and molecular biology of plant-microbe

interactions and plant defence responses, similar research on the interaction between plants and *F. oxysporum* has been slow. Much of our current knowledge on the interaction between *F. oxysporum* and its host plants is still limited to histopathological and histochemical studies performed by Beckman and Roberts (1995) and others (Vander Molen *et al.*, 1977; Shi *et al.*, 1991; Gao *et al.*, 1995a; 1995b). The molecular and genetic basis of resistance to *F. oxysporum* has been studied in tomato (Sela-Buurlage *et al.*, 2001), while the biochemical interactions of carnation (Baayen, 1987; Baayen *et al.*, 1989; Van Peer *et al.*, 1991; Higuera and Ebrahim-Neabat, 1999; Curir *et al.*, 2005) and banana (De Ascensao and Dubery, 2000) with the wilt pathogen has been investigated. More recently, Taylor (2004), Forsyth (2006) and Van den Berg (2007) studied defence-related genes in banana.

Knowledge of the genetic and molecular basis of plant defence against *F. oxysporum* isolates attacking agricultural crops, vegetables and ornamental plants is greatly lacking when compared to the vast amount of knowledge available on the molecular interactions between rice and *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (Kim *et al.*, 2001), wheat and *Fusarium graminearum* (Schwein.) Petch (Pritsch *et al.*, 2001), and grapevines (*Vitis vinifera* L.) and *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni. A thorough investigation of the role that known defence-related genes play in plants is a necessary first step to elucidate means of generating resistant crop plants. Known defence-related genes against Fusarium wilt pathogens can be identified by searching for homologues in plants affected by *F. oxysporum* and by studying the expression profiles of known defence-related genes. If these genes do not offer conclusive solutions or shed enough light as to how the plants in question defend themselves, then the search for novel defence mechanisms involved in host specificity, pathogen recognition and plant response can continue by using techniques such a SSH, cDNA-AFLP and microarray analysis.

The search for genes conferring resistance to Fusarium wilt has become an important objective towards developing genetically improved banana plants. Little is known about the molecular processes underlying resistance responses, metabolic pathways and downstream signalling of the banana-*Foc* interaction. We do, however, know that resistance is due to quantitative resistance and as genetic mapping (an ideal technique for identifying disease resistance genes) of banana will be a difficult task due to the seedless nature of the fruit and the long generation time, an analysis of pathogen-induced genes may lead to a better understanding of the molecular processes involved in resistance and the identification of defence

pathways. This will make an important contribution to the development of biotechnological strategies to fight this disease. Transcriptional profiling using cDNA-AFLP can be used to analyze pathogen induced genes as well as expression analysis of known fungal defence-related genes using real-time PCR. Once identified, defence-related genes could also be used as markers for the rapid detection of resistant traits in agricultural crops, or for the genetic improvement of susceptible plants via transformation.

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CHAPTER 2

EXPRESSION OF DEFENCE-RELATED GENES IN RESPONSE TO *FUSARIUM OXYSPORUM* F.SP *CUBENSE* IN FOUR BANANA (*MUSA* SPP.) VARIETIES

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ABSTRACT

Bananas (*Musa* spp), an important staple food crop worldwide, are threatened by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), a soil-borne fungal pathogen responsible for Fusarium wilt in many parts of the world. Resistance appears to be the only sustainable means to control Fusarium wilt. Little, however, is known about the molecular and genetic basis of resistance in wild banana varieties and in certain purpose-bred cultivars. Four banana varieties were assessed in the field for resistance to *Foc* 'subtropical' race 4. Of these, Rose and Calcutta IV were found to be resistant, and Williams and FHIA-17 highly susceptible and tolerant to the pathogen, respectively. Williams, FHIA-17, Rose and Calcutta IV were then analysed for the expression of six well-characterised plant defence-related genes upon challenge by *Foc*. *Catalase*, *Peroxidase* and *Pectin acetylesterase* were strongly up-regulated in the tolerant and resistant banana varieties, and can possibly be linked to their defence response. This implies that the mode of action of the plant to defend itself from *Foc* is possibly due to a strong oxidative burst, or due to the induction of secondary metabolism that includes the phenylpropanoid pathway and cell wall strengthening.

INTRODUCTION

Bananas (*Musa* spp.) are one of the most important, but undervalued, food crops in the world. In terms of food security, bananas are considered the world's fourth most important staple food crop after rice, wheat and maize (Arias *et al.*, 2003). Over 100 million metric tons of bananas and plantains are produced annually and that which enters international commerce is worth US$ 5 billion per year (Ploetz, 2005). The export trades in dessert bananas, which account for 13% of global production, depend almost entirely on Cavendish cultivars. The total global production of Cavendish fruit, however, amounts to 41% of all bananas grown in the world. The rest of the global production is made up of plantains (21%), cooking bananas (24%) and varieties of dessert bananas (14%). Apart from the cultivated, seedless bananas and plantains, several wild species and interspecific hybrids of *Musa* can be found growing in the wild in Southeast Asia and India, which are known to be the centre of origin for the cultivated banana (Ploetz, 2005).

Bananas are susceptible to a wide range of pathogens and pests (Jones, 2000). One of their most important diseases is Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cubense* Snyder & Hansen (*Foc*), also known as Panama disease (Stover, 1962). *Foc* enters the banana plant through the roots, and then colonizes the xylem vessels of the rhizome and pseudostem where they block nutrient and water transport to cause a lethal wilting (Jones, 2000). The damage caused by Fusarium wilt during the first half of the 20th century established the disease as one of the greatest epidemics in agricultural history (Jones, 2000). Like the banana plant, the origin of *Foc* is believed to be Southeast Asia (Stover, 1962). Three pathogenic races of *Foc* are recognized, with race 1 causing disease to Gros Michel (AAA) and the AAB genotypes 'Silk' and 'Pome'; race 2 affecting Bluggoe (ABB) and other cooking bananas, and race 4 causing disease to Cavendish bananas (AAA) and all race 1 and race 2 susceptible cultivars (Waite and Stover, 1960). *Foc* race 4 is further subdivided into 'tropical' and 'subtropical' strains, depending on their ability to cause disease to Cavendish bananas under tropical and subtropical conditions, respectively.

The control of Fusarium wilt is difficult (Jones, 2000) as no conventional control strategies have been developed to reduce the damage caused by the disease. In Central America, almost 40,000 ha of Gros Michel plantations, planted in virgin forest soils, were lost because of Fusarium wilt (*Foc* race 1) up to the 1960's (Jones, 2000). As a means of control, Gros Michel plants were replaced with *Foc* race 1-resistant Cavendish cultivars. Cavendish bananas, however, now succumb to *Foc* race 4 in the subtropics and in the tropics in Southeast Asia and, this time, there are no replacement cultivars for the dessert bananas (Ploetz, 2005). Cavendish bananas cannot be used in conventional breeding programmes as they do not produce seeds. The disease is also difficult to manage in the field, as *Foc* is able to survive in infested soil for up to 30 years (De Vries and Toenniessen, 2001).

Genes conferring resistance to diseases and pests of banana can be found in wild progenitors of *Musa* spp. One such a variety, Calcutta IV, a wild diploid banana that produces an extremely low quality, virtually inedible fruit, has resistance to most pathogens, including *Foc*, *Mycosphaerella fijiensis* Mortlet (black Sigatoka) and *M. musicola* Mulder (yellow Sigatoka) (Jeger *et al.*, 1995). Several genes with putative homology to *R*-genes have previously been isolated form Calcutta IV by Taylor (2004) as well as by Peraza-Echeverria *et al.* (2007). Improved Cavendish varieties with tolerance to Fusarium wilt have also been selected for in the field and developed in tissue culture laboratories by means of somaclonal variation (Hwang and Ko, 2004). One such selection with good tolerance to Fusarium wilt, GCTCV-218, expressed a range of genes when challenged with *Foc* that were not induced when susceptible Cavendish plants were challenged with the pathogen (Van den Berg *et al.*, 2007). The aim of the current study is to elucidated whether selected defence related genes play a role in the defence response of four banana varieties. The four banana varieties with varied susceptibility to Fusarium wilt were evaluated for resistance to *Foc* 'subtropical' race 4 under subtropical climatic field conditions in order to ascertain whether the susceptibility status of these plants was accurately represented in South Africa. Expression of selected defence-related genes were then studied over time following colonization of these varieties by *Foc* 'sub-tropical' race 4 in order to study the role these genes play in the banana defence response.

MATERIALS AND METHODS

PLANT MATERIAL

Four banana varieties were selected based on published information regarding the status of their resistance to *Foc* (Tang and Hwang, 1999; Orjeda, 2000). These varieties included the Cavendish cultivar Williams (genomic group AAA) (highly susceptible to *Foc* 'tropical' and 'subtropical' race 4), Calcutta IV (genomic group AA), Rose (genomic group AA) and FHIA-17 (genomic group AAAA) (all resistant to *Foc* race 1 and 4). All four varieties were obtained from the Bioversity International Banana Transit Center based at the University of Leuven in Belgium. The micropropagated banana plantlets were multiplied at the tissue culture facility of the Forestry and Biotechnology Institute (FABI) at the University of Pretoria in South Africa. After rooting, the plantlets were transplanted into composted soil in 1 L plastic bags for hardening-off in mist beds at the University of Pretoria's experimental farm.

FIELD EVALUATION OF BANANA VARIETIES

After hardening-off, plants of approximately 40 cm high were transported to the Kiepersol area in Mpumalanga, South Africa, where they were evaluated for resistance to Fusarium wilt. The four varieties were planted in blocks of five plants that were completely randomized in a field heavily infested with *Foc* 'subtropical' race 4 (VCG 0120). Twenty plants of each variety were included in the study, and the experiment was conducted over a period of 2 years. The development of Fusarium wilt was rated using a modified version of the disease severity rating scale developed by (Carlier *et al.*, 2002). According to this rating scale, banana plants were cut down at ground level and scored according to the internal discoloration of their inner rhizome using a rating scale ranging from 0-5. Plants that developed no internal symptoms were given a value of 0, and those showing vascular discolouration of 100% of the inner rhizome were awarded a value of 5. Disease incidence was calculated as the number of plants showing disease symptoms divided by the total number of plants and percentage disease severity was calculated for each variety using the formula of Sherwood and Hagedorn (1958), whereby:

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

EXPRESSION OF DEFENCE-RELATED GENES

Challenge of banana plants with Foc

Approximately six weeks after the tissue cultured plants were transplanted, they were removed from the bags and their roots washed to rid them of excess soil. They were then transplanted to 500-ml polystyrene cups filled with water to enhance their root development (Groenewald *et al.*, 2006). Each cup was fertilized weekly with 2.5 ml of plant nutrient solution consisting of 0.45 g Agrasol® 'O 3:2:8 (Fleuron, South Africa), 0.3 g calcium nitrate monohydrate and 0.15 g Micromix® (Fleuron) L^{-1} distilled water with a pH of 7. The plants were kept in the greenhouse at 25°C to acclimatize for approximately 3 weeks prior to inoculation.

Three highly virulent isolates of *Foc* 'subtropical' race 4 representing VCG 0120 (CAV 105, CAV 045 and CAV 092) were used as inoculum. Each isolate was first grown on half strength potato dextrose agar (PDA) medium in 90-cm-diameter Petri dishes at 25ºC for 7 days. Thereafter, sterile Armstrong *Fusarium* medium (Booth, 1977) was inoculated with mycelia and shaken at 25ºC at 177 rpm for 1 week to enhance sporulation. The spores of each suspension were filtered through sterile cheesecloth and mixed to form a *Foc* cocktail for inoculation. The spores were then counted using a haemocytometer and the suspension diluted with sterile distilled water to achieve a final concentration of 10^5 spores ml^{-1}. One hundred ml of the spore suspension was used to replace the water in each polystyrene cup, so that only the banana root tips were submerged. Plant tissue was collected from the root material above the water level 0, 6 and 72 hours after inoculation. Root material from three plants per banana variety was harvested per time point. Root tissue for RNA extraction was frozen in liquid nitrogen, ground with an IKA A11 Basic analytical mill homogenizer (United Scientific, San Diego, USA) and stored at -80ºC.

RNA extraction and cDNA synthesis

RNA was extracted from banana roots using the RNeasy Plant Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions, and stored at -80ºC. The quality and quantity of RNA was determined with a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). RNA quality was analyzed under UV illumination for the presence of distinct ribosomal bands and for the absence of degraded RNA by gel electrophoresis under non-denaturing conditions on a 1% (w/v) agarose gel. DNase treatment of RNA was performed by the addition of 1 µl RNase-free DNase (1 U/µl) (Fermentas Life Sciences, Hanover, USA), 1 µl 10X reaction buffer with $MgCl_2$, 1 µg RNA and up to 9 µl DEPC-treated water. The mixture was incubated at 37ºC for 30 min followed by the addition of 1 µl 25 mM EDTA and an incubation step of 65ºC for 10 min. DNase-treated RNA was column purified using the RNeasy® MiniElute™ Cleanup kit (Qiagen) according to the manufacturer's instructions.

First strand cDNA synthesis was carried out in an 11.6-µl reaction volume by adding 1 µl random hexamer primer (Fermentas), 0.5 µl RNase inhibitor (40 U/µl) (Fermentas) and RNase free water to 1 µg RNA. The mixture was incubated at 70ºC for 1 min and chilled on ice for 5 min. This was followed by the addition of 1 µl 10 mM dNTPs (Fermentas), 2.4 µl 15 mM $MgCl_2$, 4 µl 5X ImProm-II™ reaction buffer and 1 µl ImProm-II™ Reverse Transcriptase (Promega Corporation, Madison, Wisconsin, USA). This mixture was incubated at 25ºC for 10 min, 42ºC for 60 min, and 70ºC for 15 min. The cDNAs were then assayed for genomic DNA contamination by PCR, using an intron-exon boundary spanning primer pair specific for the banana actin gene: ActinF (5'-ACCGAAGCCCCTCTTAACCC-3') and ActinR (5'-GTATGGCTGACACCATCACC-3'). PCR amplifications were carried out using first strand cDNA as the template. The PCR was conducted in a 20-µl reaction volume that contained 1.5 µl 15 mM $MgCl_2$, 2.5 µl 10 X NH_4^+ buffer, 2 µl 2.5 mM dNTPs, 0.4 µl of each primer (10 µM), 0.5 U *Taq* polymerase (Bioline Ltd, London, UK), 0.4 µl DNA and SABAX water. The PCR was conducted in an Eppendorf Mastercycler gradient PCR Machine (Eppendorf Scientific, Hamburg, Germany). PCR conditions consisted of an initial denaturation step of 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min. Final elongation was carried out at 72ºC for 7 min. PCR products were separated by electrophoresis on a 2% (w/v) agarose gel and visualized under UV illumination.

Gene expression analysis using qRT-PCR

The expression of six putative defence-related genes (Table 1) in banana roots was analyzed using quantitative real-time reverse-transcription PCR (qRT-PCR). Four of these genes, *PR-1*, *catalase*, *pectin acetylesterase* (*PAE*) and *endochitinase* (*PR-3*), were reported to be up-regulated in a tolerant Cavendish cultivar (GCTCV-218) following inoculation with *Foc* (Van den Berg *et al.*, 2007). The other genes p*henylalanine ammonia lyase* (*PAL*) and *peroxidase (POX)* were found to be up-regulated in Lady Finger and Cavendish banana roots following *Foc* challenge of plants previously inoculated with non-pathogenic *F. oxysporum* (Forsyth, 2006). An endogenous control gene, *Musa* 25S rRNA (AF 399949) was used, as its expression remains relatively constant across all treatments. Primers for these genes were synthesized by either Operon Biotechnologies GmbH (Cologne, Germany) or Inqaba Biotec (Hatfield, South Africa) (Table 1). The primers were tested in conventional PCR reactions in a total volume of 12.0 µl containing 0.7 µl 15 mM $MgCl_2$, 1.25 µl 10 X NH_4^+ buffer, 1.25 µl 2.5 mM dNTP mix, 1.0 µl of each specific primer (5 µM stock) and 0.1 U *Taq* DNA polymerase (1 U/µl) (Bioline Ltd) and 1.0 µl of template cDNA. The samples were heated at 95ºC for 3 min, then cycled 44 times at 94ºC for 30 s, 65ºC for 30 s, and 72ºC for 30 s, with a final elongation step at 72ºC for 10 min. The PCR was conducted in an Eppendorf Mastercycler gradient PCR Machine (Eppendorf Scientific). PCR products were separated on a 2% (w/v) agarose gel and visualized under UV illumination.

qRT-PCR was performed using the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) using the first strand cDNAs as template, as specified by the instruction manual. Dilution series and standard curves were generated to examine the linearity of amplification over the dynamic range (three orders of magnitude). A serial dilution (1:10, 1:100 and 1:1000) was performed on 1 µl of cDNA from one of the resistant cultivars (Rose) inoculated with the pathogen, and qRT-PCR run for each primer set. A 10-µl reaction for PCR amplification contained 5 µl LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics), 1 µl of each of the forward and reverse primers (10 µM), 1 µl of diluted cDNA template and 2 µl PCR grade water (Roche Diagnostics). Control treatments contained water instead of cDNA template. All PCR reactions were performed in triplicate on each of three biological replications. The cycling conditions were as follows: pre-incubation for 10 min at 95°C followed by 55 cycles, each consisting of 10 s denaturing at 95°C, 10 s annealing at 65°C, 10 s primer extension at 72°C, and data acquisition at 95°C. For PCR amplification of all experimental samples, 1:10 cDNA template

dilutions were used. Melting curve analysis of the qRT-PCR products was performed to confirm that the individual qRT-PCR products corresponded to single homogenous DNA species.

DATA ANALYSES

Data for field resistance in four banana varieties to Fusarium wilt was analyzed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test using STATISTICA v. 7.1. For defence gene expression studies, standard regression curves were calculated from amplification data from each of the target genes' serial dilutions as follows: $y = mx + b$, where $b = y$ - intercept of standard curve line (crossing point) and m = slope of the standard curve line (function of PCR efficiency) (Ginzinger, 2002). Expression data was normalized using the standard curve for the specific target gene and the endogenous control gene, *Musa* 25S rRNA. The treatment Williams 0 h was designated as the calibrator and the expression levels in the other samples were determined relative to the calibrator, by dividing them by the calibrator expression value. Data from the qRT-PCR was analyzed using ANOVA and the Duncan's Multiple Range Test. In all cases significance was evaluated at $P < 0.05$.

RESULTS

FIELD EVALUATION OF BANANA VARIETIES

External symptoms, typical to that of Fusarium wilt of banana, developed on Cavendish bananas in a banana field known to be severely infested by *Foc* 'subtropical' race 4. These symptoms included the yellowing of older leaves, which progressed upward to the younger leaves until the entire plant was affected and died off. The suckers of Cavendish plants were often disease free, but became affected after winter or during bunching. FHIA-17 bananas were less affected by Fusarium wilt, and external symptoms became visible only during bunching and after winter. Since it was not clear whether leaf yellowing in Calcutta IV and Rose was a result of the cold winter temperatures or Fusarium wilt, we decided to base the evaluation of Fusarium wilt incidence and severity on the rating of internal symptoms following destructive sampling. When cut down at soil level, Calcutta and Rose showed no internal disease

symptoms 2 years after planting. By contrast, the Cavendish banana cultivar Williams had a disease incidence of 92% and a disease severity index of 77%. FHIA-17 proved to be moderately tolerant and developed a disease incidence of 36% and a disease severity index of 25% (Fig. 1).

EXPRESSION OF DEFENCE-RELATED GENES

RNA extraction and cDNA synthesis

RNA purified from the banana roots was found to be of high quality (Fig. 2A) and the absence of contaminating genomic DNA was confirmed (Fig. 2B). The amplification of a region of the actin gene from cDNA yielded the expected 170-bp mRNA-derived amplicon (Fig. 2B), which was clearly distinguishable from the faint 260-bp genomic DNA-derived, intron-containing fragment that can be seen in lane 11.

Gene expression analysis using RT-PCR

PCR efficiency was evaluated over a dynamic range and a regression curve was obtained after amplification of a serial dilution for each primer set (Fig. 3). The expression profiles for the six defence-related genes in four banana varieties differed significantly over time (Fig. 4).

Constitutive (0 h) expression of defence-related genes

The constitutive expression of genes encoding the antifungal proteins PR-1 (Fig. 4A) and PR-3 (Fig. 4B) was significantly higher in the susceptible and tolerant banana varieties (Williams and FHIA-17) than in the resistant varieties (Rose and Calcutta IV) before being challenged with *Foc*. *PAL* (Fig. 4C) was also expressed significantly more in Williams and FHIA-17 than in the resistant varieties, but not as considerable as the *PR*- genes. The expression of *catalase* (Fig. 4D) and *POX* (Fig. 4E) was significantly lower in the resistant Calcutta IV variety when compared to the expression in the other varieties. Constitutive expression of *PAE* was significantly lower in Williams than in any of the other cultivars. *PAE* was expressed most prolifically in cultivar Rose (Fig. 4F).

Early (6 hpi) up- and down-regulation of defence-related genes

Expression of *PR-3* was significantly increased in all four banana varieties 6 h after inoculation with *Foc* (Fig. 4B). In contrast, *PR-1* was significantly down-regulated in these varieties (Fig 4A). *POX* was significantly up-regulated in the resistant varieties Rose (98.8 %, Table 2) and Calcutta IV (57.6 %), but much less so in the tolerant FHIA-17 variety (10.3 %). This gene, however, was significantly down-regulated in Williams (46.7 %). In a similar manner, although not as pronounced, *catalase* was up-regulated in the resistant and tolerant banana varieties, but not in the susceptible Cavendish variety. Despite its down-regulation in Williams, overall levels of *catalase* expression were still higher than the amount expressed in Calcutta IV. *PAL* and *PAE* were significantly up-regulated in the susceptible Williams plants, but not so in any of the other varieties, with the exception of *PAE* in Rose. *PAL* was significantly down-regulated in the highly resistant Calcutta IV, but not *PAE*.

Six hours after infection of banana roots with *Foc*, *PR-3* expression was significantly higher in the Williams (susceptible) and FHIA (tolerant) varieties than in Rose and Calcutta IV (both resistant) varieties. Expression levels for PR-1 proteins, however, were similar between Williams, FHIA-17 and Rose, but significantly less in Calcutta IV. Of the other genes that were studied, *POX* was expressed significantly more in the tolerant and resistant banana varieties than in the susceptible Cavendish variety, while *PAL* was expressed almost three times more in Williams than in FHIA-17 (tolerant), Rose and Calcutta IV (resistant). *Catalase* was expressed in higher quantities in FHIA-17 and Rose than in Williams (susceptible) and Calcutta IV (resistant), while *PAE* was expressed most in cultivar Rose.

Late (72 hpi) up- and down-regulation of defence-related genes

After 72 hpi, *catalase* was the only gene significantly up-regulated in the tolerant FHIA-17 and resistant Calcutta IV varieties, but significantly down-regulated in the susceptible Williams variety. There was no significant up- or down-regulation of this gene in cultivar Rose. At 72 hpi *PR-1* was significantly up-regulated in the resistant variety Calcutta IV, but also in the susceptible Williams cultivar. There were no significant changes in expression levels of *PR-1* in FHIA-17 and cultivar Rose. *PR-3*, which was significantly up-regulated in all the banana varieties after 6 hpi, was significantly down-regulated after 72 hpi, except in Calcutta IV, which showed no significant difference in expression levels. *PAL* was significantly up-regulated in Rose (resistant) and significantly down-regulated in Williams (susceptible), but

its regulation did not change in the tolerant FHIA-17 and resistant Calcutta IV varieties. Genes which encode POX and PAE were significantly down-regulated in the *Foc*-resistant banana varieties, cultivar Rose and Calcutta IV, and in Williams, but not in the tolerant FHIA-17 variety.

When expression levels of genes were compared in the four banana varieties 72 h after infection with *Foc*, *catalase* was significantly more in the tolerant and resistant banana varieties than in the susceptible Williams plants. In contrast, expression levels of *PAL* were significantly higher in the susceptible Williams plants than in the tolerant and resistant varieties. *PR-1* was strongly up-regulated in the highly resistant Calcutta IV, but was still expressed at lower levels than in the susceptible Cavendish variety. *POX*, similar to *PAE*, was expressed at significantly higher levels in FHIA-17 and cultivar Rose than in either Williams or in Calcutta IV. *PR-3* was expressed most prolifically in FHIA-17.

DISCUSSION

A proper understanding of the Fusarium wilt pathosystem is required to interpret responses of different banana varieties to *Foc* attack. Cavendish bananas in subtropical countries, such as South Africa, have proven to be highly susceptible to *Foc* 'subtropical' race 4, VCG 0120 (Viljoen, 2002), as the current study confirmed. This specific variant of the pathogen, however, does not cause disease to Cavendish bananas in the tropics. The reason for this discrepancy is believed to be a function of predisposition by abiotic stress; in the subtropics this stress is caused by the cold winter temperatures (Viljoen, 2002; Ploetz, 2005). In this study, Calcutta IV and Rose proved to be fully resistant to *Foc* in the subtropics, which is consistent with their performance in tropical banana-production areas (Orjeda, 2000). FHIA-17 only proved to be tolerant to Fusarium wilt infection in our study, which differs from its response to *Foc* race 1 isolates in the tropics (Sharrock *et al.*, 1998). This reduction in field resistance can be the result of predisposition by the cooler winter temperatures, the different race of *Foc*, or a combination of the two.

The defence response of a plant to a pathogen is a highly regulated chain of events and can be broadly classed into the early and late response. Upon pathogen recognition, signaling events trigger early cellular responses that include changes in ion fluxes, synthesis of reactive oxygen species and changes in gene

transcription. In this case, a localized hypersensitive cell death event is often observed. Delayed defence responses include the production of antimicrobial compounds, cell wall fortification, and the activation of systemic acquired resistance (SAR), which results in a long-lasting resistance that is established in non-infected areas of the plant (Romeis, 2001). The genes investigated in this study fall into these two categories and can be further divided into basic functional groups. *Catalase* and *POX* are genes involved in the oxidative burst; *POX* is also involved in secondary metabolism along with *PAL* and *PAE*, specifically in the phenylpropanoid pathway and cell wall strengthening. PR-1 and PR-3 are antifungal proteins. Additionally, PR-3 is possibly also involved in the degradation of fungal cell wall components.

Several genes investigated in this study have shown that they are potentially key genes in the defence response of the tolerant and/or resistant banana cultivars to *Foc*. Genes which were expressed constitutively at higher levels in the tolerant and resistant banana varieties include *POX*, *PR-3*, *PAE* and *catalase*. In the case of *PAL* and *PR-1*, constitutive expression was higher in the susceptible and tolerant varieties. The antifungal *PR* genes are especially interesting in Williams and FHIA-17. In both cases these genes were expressed at a significantly higher level in the susceptible and tolerant varieties than in the resistant ones, suggesting increased levels of anti-fungal proteins being produced constitutively in these varieties. It was further interesting to find that *catalase* and *POX* had a high constitutive expression in the susceptible and tolerant cultivars, which perhaps allows a more rapid response to pathogen attack in these varieties. The absence of a high constitutive expression in Calcutta IV may imply that this variety can resist *Foc* effectively without allocating cellular resources to constitutive gene expression of *catalase* and *POX*.

Plant cells are protected against damage from active oxygen species generated during the hypersensitive response by a complex antioxidant system (Zhang and Kirkham, 1994). Enzymatic antioxidants are produced by genes analysed in this study, namely *catalase* and *POX*. Early expression of catalase in a cell is related to the oxidative burst, where they play an important role in scavenging for free 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). *Catalase* was up-regulated 6 h after pathogen challenge in the tolerant and resistant banana cultivars, implying that the oxidative burst was initiated in these plants. Catalase activities were shown to enhance the incompatible interaction between

chick pea (*Cicer arietinum* L.) and *F. oxysporum* f.sp. *ciceris* (Padwick) (Garcia-Limones *et al.*, 2002), and Van den Berg *et al.* (2007) showed that the expression of *catalase* increased in a tolerant banana variety (GCTCV 218) at 6 and 48 hpi in response to *Foc* 'sub-tropical' race 4. In this study, the expression of *catalase* in the tolerant and resistant plants continued to increase to 72 hpi, but conversely decreased further in the susceptible Williams. This suggests that *catalase* may be associated with its additional function in the signal transduction pathway in the tolerant and resistant plants, but not in the susceptible Williams plant. Signalling, as a result of *catalase* up-regulation, would result in the development of SAR (Bagnoli *et al.*, 2004).

POXs have multiple functions within the plant cell. During the course of the plant-pathogen interaction, POXs are key enzymes in the plant's detoxification systems and are involved in scavenging reactive oxygen whose increased generation is closely associated with the induction of plant defence reactions (Gay and Tuzun, 2000; Morkunas and Gmerek, 2007). Other functions of POXs include the oxidation of isophenols to forms that are more toxic towards the pathogens (Takahama and Hirota, 2000; Morkunas and Gmerek, 2007), the cross-linking of cell wall proteins and lignification of cell walls (Lagrimini and Rothstein, 1987; Morkunas and Gmerek, 2007). The early and substantial up-regulation of *POX*, particularly in the resistant banana varieties, indicates that a strong oxidative burst occurred and that the defence response of these cultivars might involve lignin production and cell wall fortification. Alternatively the drop-off in expression levels after the early response may also imply that the defence response of these plants is more heavily reliant on oxidative processes than secondary metabolic pathways and cell wall fortification.

Lignin is a polymer formed via the phenylpropanoid pathway. The first step in this pathway is the deamination of phenylalanine to cinamic acid which is catalyzed by the enzyme PAL. Other than lignin, PAL is a precursor for phenylpropanoid-derived secondary plant products such as salicylic acid and isoflavonoid phytoalexins that are involved in defence (Ward *et al.*, 1991). The expression of *PAL* was highest in the susceptible Williams plant 6 and 72 hpi. This may imply that, whilst the resistant plants had a large initial oxidative burst, the susceptible plant did not and instead channeled resources into the phenylpropanoid pathway. The expression of this gene in the resistant cultivars increased at 72 hpi, but at levels lower than that in Williams. This late response in the resistant varieties may indicate that systemic

responses have been activated, but that early responses sufficiently protect resistant plants against further progress of the pathogen. For Fusarium wilt diseases, resistance has often been related to early response time of plant defence mechanisms (Beckman and Roberts, 1995).

PAEs are known to catalyze the deacetylation of pectin, a major compound of primary cell walls of plants (Vercauteren *et al.*, 2002). When activated, PAE contributes to cell wall modifications and strengthening. *PAE* was expressed constitutively at higher levels in the tolerant and resistant banana varieties than in the susceptible Williams cultivar, with expression levels of *PAE* in Rose almost five times more than that in Williams. At 6 hpi, the up-regulation of *PAE* in Rose increased further. While this increase in gene expression was not sustained at 72 hpi, Rose expressed a significantly higher amount of *PAE* than any of the other banana varieties. It is, therefore, evident that *PAE* plays a possibly important role in the defence response of cultivar Rose and to a lesser extent in FHIA-17 where expression of this gene is down regulated at 6 hpi but increases again by 72 hpi. Previously *PAE* was found to be up-regulated in a tolerant banana variety GCTCV-218 when challenged with *Foc* (Van den Berg *et al.*, 2007) and was also shown to be up-regulated in *Arabidopsis thaliana* (L.) Heynh. roots in response to attack by root-knot and cyst nematodes (Vercauteren *et al.*, 2002).

PR-proteins are defined as proteins in the host plant that are induced in response to pathogen attack or a related event (Van Loon *et al.*, 1994). The precise role that PR proteins play in defence and in SAR has yet to be determined but their expression is often coincident with the development of resistance. The induction/activation of *PR* genes, therefore, is used routinely as a convenient marker of SAR (Ward *et al.*, 1991). Since 1991, the function of most PR-proteins has been elucidated, barring one – PR-1. The expression of two PR proteins was analyzed in this study, namely PR-1 and PR-3. PR-1 is an abundant PR protein that has been associated with antifungal properties, such as the hydrolysis of fungal cell walls (Niderman *et al.*, 1995; Van Loon, 1997) and the initiation of structural defences (Cutt and Klessig, 1992). The expression of the *PR-1* gene is also one of the most significant indicators of SAR in crop plants (Brederode *et al.*, 1991; Ward *et al.*, 1991; Cordelier *et al.*, 2003; Menard *et al.*, 2005). In all four of the banana cultivars tested, *PR-1* was down-regulated at 6 hpi. Expression of *PR-1* in Williams increased by 72 hpi, but was further reduced in the tolerant and resistant varieties. It, therefore, appears that *PR-1* is not associated with the defence response to *Foc* attack; an alternative hypothesis is that *Foc* is able to

suppress PR-1 gene expression. Van Loon (1997) mentioned that the accumulation of *PR* genes is not a prerequisite for the induction of resistance. *PR-3*, on the other hand, was strongly up-regulated at 6 hpi. Endochitinase (chitinases) has a dual role in plant defence. It serves to hydrolyze the chitin present in the cell walls of fungi and could also be linked to the systemic activation of defence responses due to their role in releasing elicitors (Collinge *et al.*, 1993). Williams and FHIA-17 showed a markedly higher expression of this gene than the resistant banana varieties Rose and Calcutta IV. At 72 hpi, *PR-3* expression was significantly reduced in all the varieties, but remained high in FHIA-17. This up-regulation of *PR-3* suggests that this gene plays an important role in the defence response of all the banana varieties.

The current study has made a contribution to the understanding of how resistant and tolerant plants are able to defend themselves against *Foc* 'subtropical' race 4. Defence-related genes, such as *catalase*, *POX* and *PAE* were clearly implicated to have a possible role in the active defence response, while *PR-1* is expressed constitutively (0 hpi) at much higher levels in the susceptible Williams cultivar and the tolerant FHIA-17 hybrid. A better understanding of the function of defence-related genes should in future include histopathological and histochemical studies to determine what structural and biochemical defence responses prevent Fusarium wilt development in tolerant and resistant banana varieties. Additional defence-related genes in banana should also be identified in an effort to better understand defence pathways and how banana plants defend themselves against *Foc*. Microarray analysis of gene expression or mutant analysis will be an excellent means to study this problem further to shed some more light on this incomplete picture.

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Table 1. Primer sequences of defence-related genes studied in banana roots by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) following infection by *Fusarium oxysporum* f.sp. *cubense*.

Target gene[a]	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size (bp)
Peroxidase[1]	CGGTAGGATCCAAAGAAAGC	TTCAGAGCATCGGATCAAGG	150
Endochitinase[2]	GTCACCACCAACATCATCAA	CCAGCAAGTCGCAGTACCTC	150
PAL[1]	CCATCGGCAAACTCATGTTC	GTCCAAGCTCGGGTTTCTTC	150
PR-1[2]	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA	126
Catalase[2]	AAGCATCTTGTCGTCGGAGTA	CGCAACATCGACAACTTCTTC	96
PAE[2]	GGCTCTCCTTTCTGGATGTTC	TCAGCAAGGCACTTGACTTTT	105
Musa 25S rRNA [2]	GTAAACGGCGGGAGTCACTA	TCCCTTTGGTCTGTGGTTTC	106

[1] Primer sequences previously identified by Forsyth (2006) and [2] primer sequences previously identified by Van den Berg *et al.* (2007).

PAL = Phenylalanine ammonia lyase, PR = pathogenesis-related and PAE = pectin acetylesterase

Table 2. Percentage increase (black text) or decrease (red text) of gene expression in each banana variety at each time point relative to the calibrator Williams 0 hpi.

PAL = Phenylalanine ammonia lyase, PAL = pectin acetylesterase and PR-1 = Pathogenesis related-1

	Catalase	Endochitinase	PAE	PAL	Peroxidase	PR-1
Williams 0 hpi	-	-	-	-	-	-
Williams 6 hpi	**13.6**	**129.3**	**89.1**	**216.2**	**46.7**	**72.0**
Williams 72 hpi	**77.2**	**70.5**	**30.8**	**101.9**	**50.9**	**55.3**
FHIA-17 0 hpi	**7.5**	**10.9**	**149.8**	**30.9**	**15.6**	**8.9**
FHIA-17 6 hpi	**7.2**	**103.8**	**32.1**	**24.0**	**10.3**	**68.4**
FHIA-17 72 hpi	**22.3**	**31.2**	**191.8**	**32.0**	**15.7**	**70.2**
Rose 0 hpi	**8.3**	**75.1**	**404.7**	**51.1**	**14.0**	**47.2**
Rose 6 hpi	**33.6**	**37.8**	**617.2**	**36.8**	**98.8**	**73.6**
Rose 72 hpi	**27.5**	**80.2**	**251.4**	**27.3**	**26.1**	**77.7**
Cal IV 0 hpi	**92.4**	**72.8**	**105.1**	**18.2**	**78.4**	**49.1**
Cal IV 6 hpi	**55.0**	**43.2**	**146.6**	**68.9**	**57.6**	**97.7**
Cal IV 72 hpi	**23.0**	**47.5**	**9.3**	**54.4**	**56.2**	**79.8**

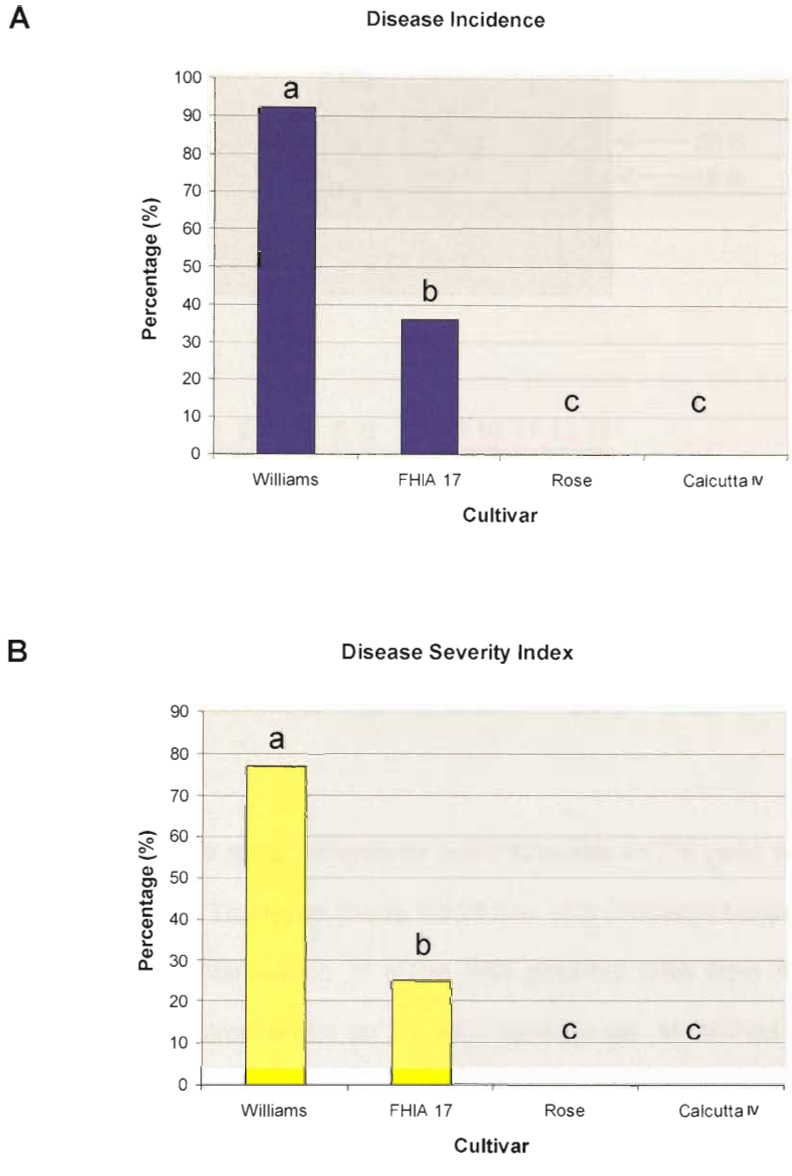


Figure 1: Disease incidence (A) and severity (B) of Fusarium wilt development in four banana varieties in the field. Data were analyzed using a one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P<0.05$.

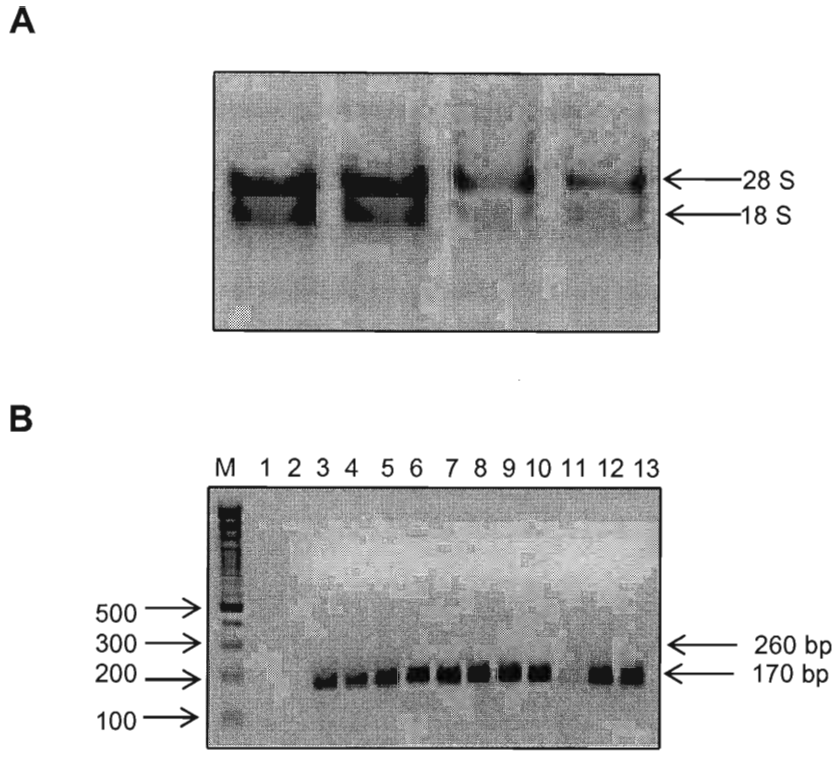
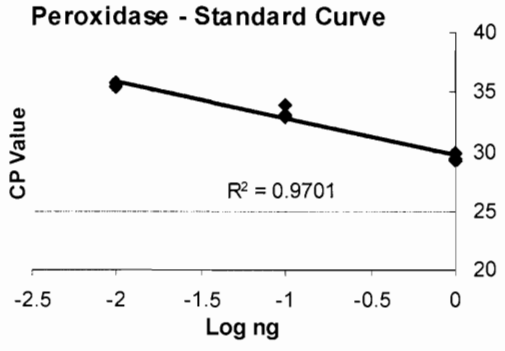


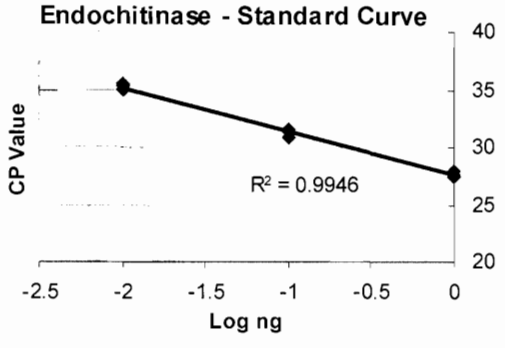
Figure 2. (A) Total RNA from banana roots assayed by electrophoresis on 2% (w/v) agarose gel under non-denaturing conditions. The figure shows the 28 and 18 S ribosomal bands. (B) Actin-based control for monitoring contamination of cDNA with genomic DNA from first strand cDNA synthesis assayed by electrophoresis on 2% (w/v) agarose gel. M: 100-bp molecular weight standard, Lanes 1 and 2: Water control, Lanes 3-10, 12-13: 170-bp PCR product from banana root cDNA. Lane 11: cDNA contaminated with genomic DNA as a faint band is visible at 260 bp.

Figure 3: Standard regression curve plots. A dilution series of the banana cultivar Rose cDNA spanning three orders of magnitude (1:10, 1:100, 1:1000) amplified with *Musa* 25S (G), peroxidase (POX) (A), endochitinase (PR-3) (B), phenylalanine ammonia lyase (PAL) (C), Pathogenesis Related (PR-1) (D), catalase (E) and pectin acetylesterase (PAE) (F) primers was used to generate the standard curve for each separate primer pair. The resulting crossing point (CP) values for each input amount of template are plotted as a function of the log[10] concentration of input amounts and a linear trend-line is fit to the data. R^2 is the proportion of variability the data set that is accounted for by a statistical model, thus, R^2 = 1 indicates that the fitted model explains all variability in *y*, while R^2 = 0 indicates no 'linear' relationship between the response variable and regressors.

A


Peroxidase - Standard Curve
CP Value
R² = 0.9701
Log ng
40
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

B


Endochitinase - Standard Curve
CP Value
R² = 0.9946
Log ng
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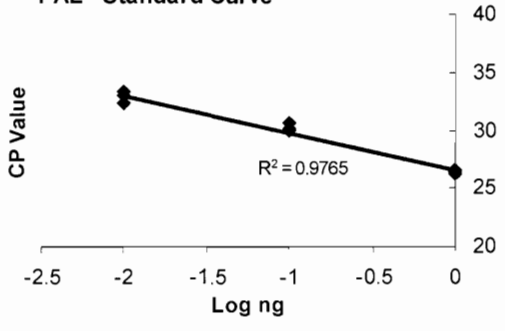
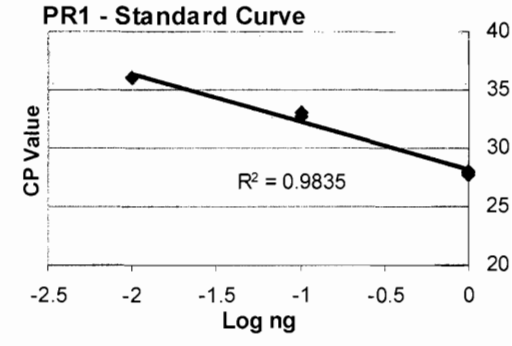
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

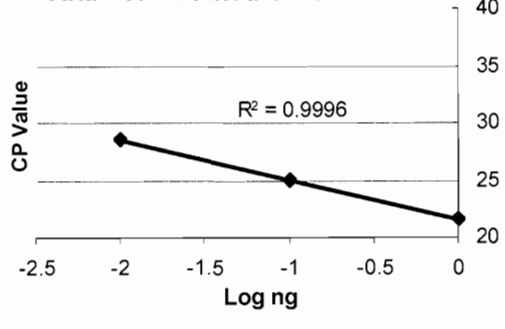
C


PAL - Standard Curve
CP Value
R² = 0.9765
Log ng
40
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

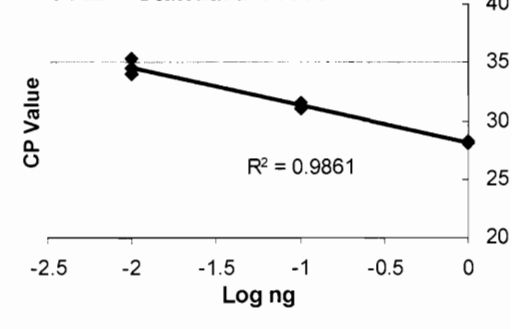
D


PR1 - Standard Curve
CP Value
R² = 0.9835
Log ng
40
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

E


Catalase - Standard Curve
CP Value
R² = 0.9996
Log ng
40
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

F


PAE – Standard Curve
CP Value
R² = 0.9861
Log ng
40
35
30
25
20
-2.5
-2
-1.5
-1
-0.5
0

G

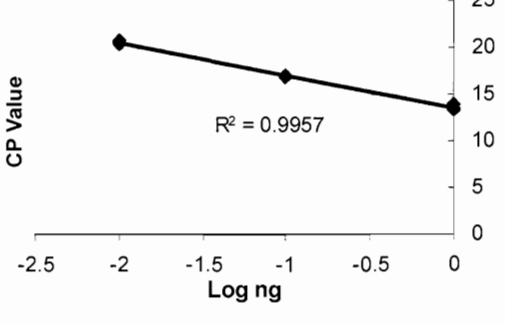
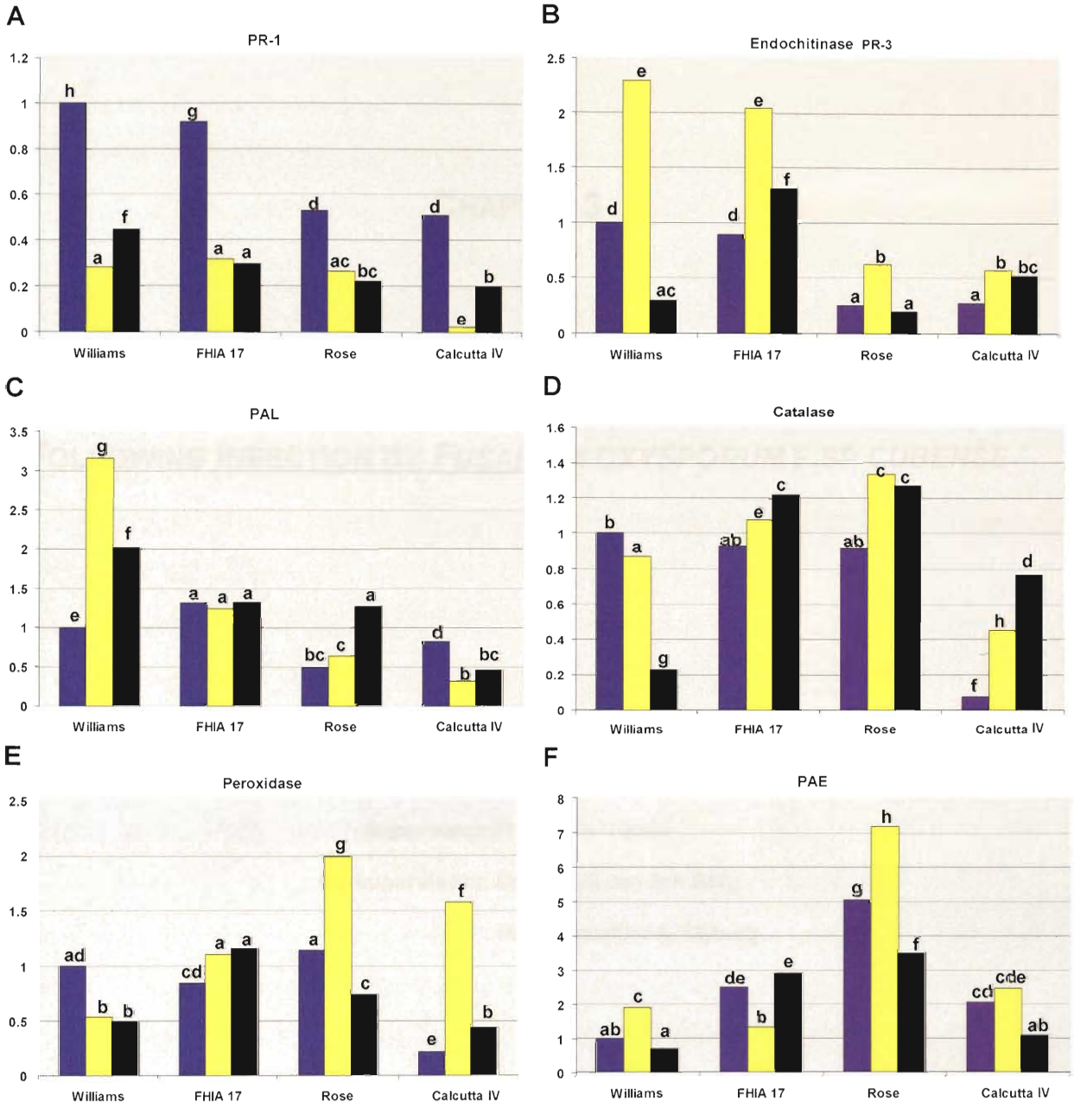

Musa Control - Standard Curve
CP Value
R² = 0.9957
Log ng
25
20
15
10
5
0
-2.5
-2
-1.5
-1
-0.5
0

Figure 4: Relative gene expression levels at 0 (blue), 6 (yellow) and 72 hrs (black) post inoculation (hpi) with *Fusarium oxysporum* f.sp *cubense* of *PR-1* (A), Endochitinase (*PR-3)* (B), *Phenylalanine ammonia lyase (PAL)* (C), *Catalase* (D), Peroxidase (*POX)* (E), and *Pectin Acetylesterase (PAE)* (F). Expression ratios were determined by quantitative RT-PCR and are expressed relative to a 'calibrator', the expression level for the particular transcript in Williams roots at 0 h. Data sets were analyzed using ANOVA and the Duncan's Multiple Range Test. Bars presented with the same letter are not significantly different at $P < 0.05$. The 'X' axis represents the banana variety and the 'Y' axis represent the gene expression level.


A
PR-1
B
Endochitinase PR-3
C
PAL
D
Catalase
E
Peroxidase
F
PAE
Williams
FHIA 17
Rose
Calcutta IV

CHAPTER 3

IDENTIFICATION OF DEFENCE-RELATED GENES INDUCED IN BANANA FOLLOWING INFECTION BY *FUSARIUM OXYSPORUM* F.SP *CUBENSE*

Claire Munro

Supervisor: Prof. Altus Viljoen

Co-supervisors: Dr Noëlani van den Berg

Prof. Alexander A. Myburg

ABSTRACT

Bananas are an important source of nutrition for millions of people worldwide. Fungal diseases, such as Fusarium wilt, pose a major threat to the continued production of this important crop. Genetic improvement of susceptible banana cultivars offers the most sustainable means of Fusarium wilt control. In recent years, biotechnological approaches for developing disease resistant banana cultivars have become popular, as conventional breeding with this crop has many obstacles. Elucidating factors related to banana disease defence mechanisms has, therefore, become important for improving existing high-yielding but susceptible cultivars. In this study, gene expression profiles were investigated in the Cavendish cultivar "Williams" that is highly susceptible to the Fusarium wilt pathogen, *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4, and compared to those expressed in tolerant (FHIA-17) and resistant (Rose and Calcutta IV) banana varieties. A cocktail of three *Foc* isolates was inoculated onto the roots of the four banana varieties, and RNA collected 0, 6 and 72 hours post inoculation for cDNA-amplified fragment length polymorphism (AFLP) analysis. cDNA-AFLP bands associated with differential gene expression patterns were selected on the basis of their presence or up-regulation in the resistant and tolerant plants and not in the susceptible plants. The transcript derived fragments (TDFs) were then excised from the polyacrylamide gels and identified. Many genes of unknown function and identity were isolated; others had general metabolic and photosynthetic functions in the plant, whilst some had functions which could be involved in the Fusarium wilt defence response. TDFs representing genes such as *S-adenosylmethionine synthase* and *isoflavone reductase*, which are potentially involved in the production of cell wall strengthening compounds such as lignin, were identified. Quantitative reverse-transcriptase real-time PCR (qRT-PCR) was used to confirm the expression patterns of selected TDFs as seen on the cDNA-AFLP gels. Two TDFs which displayed constitutive expression on the cDNA-AFLP gels were isolated and sequenced and were optimised for use as additional endogenous control genes for the normalisation of the qRT-PCR data. qRT-PCR results confirmed in most cases what was seen on the cDNA-AFLP gels. cDNA-AFLP was a good technique for the identification of defence-related genes in banana, however, additional experiments such as microarray analysis of gene expression in the four banana varieties would be beneficial for the interpretation of some of the results obtained in this study.

INTRODUCTION

Considerable efforts have been directed towards understanding the molecular mechanisms underlying plant-microbe interactions (Hammond-Kosack and Jones, 1996). During the early stages of the plant-microbe interaction when the plant recognizes the pathogen as potentially infectious, local defence responses aid in isolating the pathogen from non-infected tissues. Defence responses are also induced in plant tissue away from the infection point by a series of signalling events that ensures systemic resistance to the pathogen (Ryals *et al.*, 1996). Events of recognition, signalling and defence by a host plant to its fungal pathogen, and the ability of the pathogen to overcome the plant's defence system, implies a complex, dynamic and interactive molecular network. Induction of molecular responses necessitates the up- and down-regulation of numerous, but specific genes (Nimbalkar *et al.*, 2006). In the past, large-scale differential gene expression analyses have been restricted to the model plant *Arabidopsis thaliana* (L.) Heynh. and a few agricultural crops such as sugarcane (*Saccharum* spp. L.) (Carmona *et al.*, 2004), tomato (*Solanum lycopersicum* L.) (Durrant *et al.*, 2000), coffee (*Coffea* spp. L.) (Fernandez *et al.*, 2004), cassava (*Manihot esculenta* Crantz) (Kemp *et al.*, 2005) and rice (*Oryza sativa* L.) (Zhang *et al.*, 2004; Nimbalkar *et al.*, 2006).

Several factors, abiotic and biotic, limit banana (*Musa* spp. L.) production. Among the latter, the most important are two fungal diseases, black Sigatoka caused by *Mycosphaerella fijiensis* Morelet. and Fusarium wilt (Panama disease) caused by *Fusarium oxysporum* Schlechtend:Fr. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*). After disastrous outbreaks of Fusarium wilt in Central America during the first half of the 19th century, the *Foc* race 1-susceptible Gros Michel export banana was replaced by resistant Cavendish cultivars. A new race of *Foc* (race 4) emerged, however, that has overcome the resistance in Cavendish bananas. The new *Foc* race 4 appeared, presumably as independent events, in the Canary Islands, Australia, Taiwan and South Africa (Stover, 1990; Morpurgo *et al.*, 1994). More recently, outbreaks of *Foc* race 4 have been reported from Malaysia, Indonesia and northern Australia, and is now spreading rapidly in other banana producing countries in Asia (Ploetz, 2006).

Selecting plant genotypes resistant to pathogens has become a major tool for combating agricultural losses and increasing productivity. For a disease such as Fusarium wilt of banana, resistant plants probably provide the only means of sustainable production, as all conventional disease management strategies investigated so far failed to provide lasting control (Ploetz, 2005). Breeding for banana resistance is particularly difficult due to the sterile and polyploid nature of the plant and the saprophytic - pathogenic nature of the fungus (Novak, 1992; Morpurgo *et al.*, 1994). The relationship of *Foc* with the banana host and the complex interplay occurring between them under different environmental conditions result in an extremely complex situation (Morpurgo *et al.*, 1994). Field screening is time consuming and requires a large amount of space and intensive manpower inputs. Biotechnology has, thus, become a feasible alternative for elucidating factors related to banana disease defence mechanisms in resistant, inedible banana varieties. Once understood, such plant defence mechanisms can be exploited to generate resistance in popular high-yielding, edible banana cultivars to *Foc*.

Genes underlying defence-related responses in plants can be discovered and characterized by means of forward and reverse genetics. Forward genetics involves mutant analysis and the search for the gene relating to the altered phenotype, whilst reverse genetics aims to elucidate the function and role of a gene by manipulating its expression. One of the most recognized techniques that forms part of both forward and reverse genetics includes transcriptional profiling by cDNA-amplified fragment length polymorphism (AFLP) analysis (Bachem *et al.*, 1996). The AFLP technique was first used by Bachem *et al.* (1998) for generating mRNA fingerprints to study differential gene expression during potato tuber formation (Nimbalkar *et al.*, 2006). An advantage of cDNA-AFLP over a microarray analysis of expressed genes lies in the fact that microarray transcript-profiling is a closed system, which means that transcripts not represented on the array are not quantified. cDNA-AFLP analysis, on the contrary, represents an open gene discovery system with an essentially unlimited number of primer-enzyme combinations to be assayed (Breyne *et al.*, 2003). cDNA-AFLP has proven to be an excellent tool to identify novel genes related with plant resistance to pathogens (Durrant *et al.*, 2000). Carmona *et al.* (2004) used this technique to isolate and identify genes related to resistance in the sugarcane-*Puccinia melanocephala* Syd. & P. Syd., interaction, and Kemp *et al.* (2005) isolated cassava genes differentially expressed during the hypersensitive response of leaves in response to an incompatible *Pseudomonas syringae* pathovar.

Identification and characterization of defence-related genes in the banana-*Foc* interaction is required for understanding plant resistance responses against Fusarium wilt for future improvement of this important commercial and staple food crop. As the *de novo* description of genes involved in the banana defence response requires an overview of gene expression in both resistant and susceptible cultivars, the objectives of this investigation were to generate reliable expression profiles of the banana transcriptome in a high-throughput manner using cDNA-AFLP analysis. Expression patterns of partially isolated candidate defence-related genes were then further characterized using quantitative real-time reverse transcriptase PCR (qRT-PCR).

MATERIALS AND METHODS

INOCULATION OF BANANA PLANTS WITH *FOC* IN THE GREENHOUSE

Plant material and growth conditions

Four tissue-cultured banana varieties that differ in their level of resistance to Fusarium wilt were obtained from the Bioversity International *Musa* Transit Center at the University of Leuven in Belgium. These varieties include the resistant varieties Rose (AA), Calcutta IV (AA), the tolerant variety FHIA-17 (AAAA) and the susceptible Cavendish cultivar 'Williams' (AAA). The plantlets were then multiplied by micropropagation at the tissue culture facilities of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria in South Africa. After rooting, the plantlets were transplanted into a composted bark medium in 1 L plastic bags for hardening-off. After approximately 6 weeks in the mist beds at the University of Pretoria's experimental farm, the plants were removed from their bags and their roots washed to rid them of excess soil. Each banana plantlet was then replanted to a 500-ml polystyrene cup filled with water and 2.5 ml of plant nutrient solution consisting of 0.45 g Agrasol® 'O 3:2:8 (Fleuron, South Africa), 0.3 g calcium nitrate monohydrate and 0.15 g Micromix® (Fleuron) L^{-1} distilled water with a pH of 7 (Groenewald *et al.*, 2006). The plants were allowed to acclimatize in the greenhouse for approximately 3 weeks prior to inoculation with *Foc*.

Preparation of inoculum

Three highly virulent isolates of *Foc* representing VCG 0120 (CAV 105, CAV 045 and CAV 092) were chosen to inoculate the roots of banana plantlets. The isolates were initially cultured on half strength PDA for 1 week. Thereafter, Armstrong *Fusarium* medium (Booth, 1977) was inoculated with mycelia from the Petri dishes and allowed to shake at 25°C at 177 rpm for 1 week to enhance sporulation of the *Foc* isolates. Spore suspensions from each of the isolates were mixed to form a "spore cocktail", and filtered through sterile cheesecloth to remove the mycelia. The spores were then counted using a haemocytometer.

Inoculation and sample collection

Once the banana plantlets had developed into 10-cm plants their roots were inoculated with the *Foc* suspension. Root tips were first submerged in 100 ml water, and the appropriate volume of the spore cocktail added to achieve a final concentration of 10^5 spores ml^{-1}. Three plants per time point from each banana variety were inoculated to harvest roots at 0, 6 and 72 hrs post inoculation (hpi). Samples were taken from the root material growing above the water level, frozen in liquid nitrogen, ground with a homogenizer (IKA A11 Basic analytical mill, United Scientific, San Diego, USA) and stored at -80°C.

cDNA-AFLP Analysis

Template preparation for cDNA-AFLP analysis

The ground root material from two plants per treatment was bulked and total RNA extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. 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. Poly A^+ RNA was isolated from the total RNA using the Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Double-stranded (ds)cDNA was synthesized from 300 ng of purified poly A^+ RNA using the cDNA Synthesis System (Roche Applied Science, Penzberg, Germany) according to the manufacturer's

instructions. First strand cDNA synthesis was carried out in a reaction, containing 300 ng poly A^+ RNA, 2 µl oligo dT_{15} primer (200 µM) and RNase-free water up to 21 µl. The sample was incubated at 70ºC for 10 min and immediately placed on ice. After incubation the following components were added: 8 µl 5X Reverse Transcriptase (RT) buffer, 4 µl 0.1 M dithiothreitol (DTT), 50 U µl AMV RT, 1 µl RNAse inhibitor (25 U/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 cDNA synthesis followed immediately by using 40 µl cDNA from the first strand reaction and adding to it 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. This was followed by the addition of 20 U T4 DNA polymerase and incubation for a further 5 min. The reaction was terminated by the addition of 17 µl 0.2 M EDTA (pH 8.0). The double stranded cDNA was column-purified using the High Pure PCR product purification kit (Roche Applied Science) and the cDNAs were assayed for genomic DNA contamination by PCR using an intron-exon boundary spanning primer pair specific for the banana actin gene as previously described (Van den Berg *et al.*, 2007).

Generation of transcript profiles

cDNA-AFLP analysis was performed as described by Bachem *et al.* (1998) using the AFLP Expression Analysis Kit (LI-COR Biosciences, Lincoln, Nebraska). Generation of TaqI+0/MseI+0 pre-amplification PCR products was performed in three steps: Restriction digestion of the cDNA template, adapter ligation and the pre-amplification PCR.

TaqI restriction digestion was carried out in a 20-µl reaction volume consisting of 100 ng of double-stranded cDNA template, 4 µl 5X RL buffer, 0.5 µl TaqI enzyme and sterile distilled water. This mixture was incubated at 65ºC for 2 hrs and then immediately placed on ice. *MseI* restriction digestion followed immediately by the addition of 1 µl 5X RL buffer, 0.5 µl *MseI* restriction enzyme and 3.5 µl sterile distilled water to the TaqI digestion mix. The mixture was incubated at 37ºC for 2 hrs and then at 80ºC for 20 min to inactivate the two restriction enzymes. The reaction was then placed on ice. Adapter ligation followed with the addition of 4.5 µl adapter ligation mix and 0.5 µl T4 DNA ligase to the 25 µl TaqI/MseI restriction digestion mix. The reaction was mixed gently and incubated at 20ºC for 2 hrs. Pre-amplification was conducted in a 12.5-µl reaction volume by adding 1 µl cDNA from the adapter ligation step, 10 µl pre-amp

primer mix, 1.25 µl 10X amplification buffer (Roche Diagnostics, Mannheim, Germany) and 1.25 U *Taq* DNA polymerase (Roche Diagnostics) to a 0.2 ml PCR tube. The reaction mixture was mixed gently, centrifuged briefly and cycled 20 times at 94ºC for 30 s, 56ºC for 1 min, and 72ºC for 1 min. The pre-amplification products were diluted 1:300 in sterile distilled water and used as template for final selective amplification. The unused portion of the pre-amp template mixture was stored at -20ºC for long-term use.

Selective PCRs were performed by combining eight TaqI+2 primers and eight *MseI*I+2 primers (+GA, +GT, +TC, +TG, +CT, +CA, +AG and +AC on both adapter primers) provided in the AFLP Expression Analysis Kit to afford a total of 64 primer combinations. The TaqI+2 primers in this kit are fluorescently labeled with infrared dye (IRDye700, LI-COR) for the purpose of fragment visualization. The selective amplification reaction mix consisted of 3 µl *Taq* DNA polymerase working mix, 1 µl diluted pre-amp DNA, 1 µl *MseI*I primer containing dNTP's and 0.25 µl IRDye^{TM} 700-labelled TaqI primer. PCR amplification was performed using a touchdown program consisting of one cycle of 94ºC for 30 s, 65ºC for 30 s and 72ºC for 1 min, followed by 12 cycles of subsequently lowering the annealing temperature by 0.7ºC per cycle while keeping the other steps 94ºC for 30 s (denaturation) and 72ºC (amplification), and 23 cycles of 94ºC for 30 s, 56ºC for 30 s and 72ºC for 1 min. Following the 23 cycles the reaction mixtures were soaked at 4ºC. Ten µl of loading buffer was added to each reaction, mixed well, centrifuged briefly and denatured at 94ºC for 3 min. Selective PCR products were resolved on 8% denaturing polyacrylamide gels in model 4200S LI-COR DNA Analyzers (LI-COR Biosciences) as previously described (Myburg *et al.*, 2001). cDNA-AFLP images were saved in 16-bit TIFF format for image analysis.

Image analysis

cDNA-AFLP band sizes and intensities were determined using the AFLP-QuantarPro software (KeyGene products B.V., Wageningen, The Netherlands). Lane finding, band finding and sizing were performed as described in the AFLP-QuantarPro user manual, with band finding and scoring parameters previously described for LI-COR gels (Myburg *et al.*, 2001). Only differentially expressed Transcript Derived Fragments (TDFs) (based on visual inspection) were quantified in AFLP-QuantarPro. Band intensities were automatically lane-to-lane normalized by the AFLP-QuantarPro software based on the total lane intensity to correct for loading inconsistencies and other technical artifacts (Ranik *et al.*, 2005). Band intensities were exported to Microsoft Excel for further analysis.

Clustering and identification of gene expression patterns

Cluster analysis was performed on the normalized band intensities using CLUSTER [version 3.0 (Eisen *et al.*, 1998)] and the open-source software Java TreeView (Saldanha, 2004), in order to identify groups of TDFs with similar expression patterns across the four plants and three time points in the tree. Following mean centering and standardization, distances were calculated using the standard Pearson's correlation and the expression profiles clustered using the hierarchical centroid linkage algorithm in CLUSTER. The output of the clustering algorithm was visualized using Java TreeView.

TDF isolation and identification

Polyacrylamide gels containing fragments of interest were scanned using the Odyssey Infrared Imager (LI-COR Biosciences). Fragments of interest were selected on the basis of their presence or up-regulation in the resistant and tolerant plants and not in the susceptible banana. These fragments were excised from the gel using a sterile scalpel blade and suspended in 20 µl sterile distilled water. Additionally, TDFs identified as being down-regulated in the susceptible plants were excised. Elution of the PCR product from the polyacrylamide gel was achieved by 10-20 cycles of freezing (-70°C) and thawing to room temperature. Eluted PCR products were re-amplified using the specific *MseI*+2 primer used in the final amplification reaction, in combination with a non-fluorescently labelled TaqI+0 adapter-specific primer. The PCR was performed using the same touchdown program used in the final amplification step, modified with a final elongation step of 20 min. Re-amplified fragments were visualized on a 2% (w/v) agarose gel.

Re-amplified TDFs cloned into plasmids which were transformed in in competent *Escherichia coli* cells using the InsTAclone Cloning Kit (MBI Fermentas, Hanover, Maryland, USA) according to the manufacturer's instructions. Following transformation, the cells were plated out on Luria-Bertani (LB) agar plates containing 250 µg ml^{-1} ampicillin, 60 µg.ml^{-1} X-gal and 60 µg.ml^{-1} Isopropanol-ß-D-thiogalactopyranoside (IPTG), and incubated overnight at 37ºC. A blue/white selection was used and recombinant (white) colonies were picked for each ligation reaction. Transformants were transferred to 700 µl LB broth amended with 100 µg.ml^{-1} ampicillin and grown overnight at 37ºC on a rotary shaker at 200 rpm. Thereafter, 300 µl 50% glycerol was added to each tube prior to storing at -80ºC.

A colony PCR was performed on all the selected clones using the M13F-pUC(-40) (5'-GTTTTCCCAGTCACGAC–3') and M13R-pUC(-26) (5'-CAGGAAACAGCTATGAC-3') universal primers, and 2 µl of cells grown overnight in broth as template DNA. The colony PCR was conducted in a 20-µl reaction volume and contained 1.5 µl $MgCl_2$, 2.5 µl NH_4^+ buffer, 2 µl 2.5 mM dNTP's, 0.4 µl of each primer (10 µM), 0.5 U *Taq* polymerase (Bioline, London, UK), 2 µl of bacterial culture in broth which was boiled for 10 min (prior to its addition to the PCR reaction mixture) as template DNA, and sterile distilled water. The PCR was conducted in an Eppendorf Mastercycler gradient PCR Machine (Eppendorf Scientific). PCR conditions consisted of an initial denaturation of 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min. Final elongation was carried out at 72ºC for 7 min. PCR products were separated by electrophoresis on a 2% (w/v) agarose gel to confirm successful insertion of the re-amplified TDFs.

The TDF inserts were sequenced using ABI BigDye terminator chemistry on ABI3100 instruments with the universal M13-pUC vector primers (Macrogen Corp., Rockville, USA). Once sequenced, the vector and cDNA-AFLP adapter sequences were removed manually using BioEdit Sequence Alignment Editor (Hall, 1999). Sequences were assigned putative identities by translating BLAST (BLASTX) against the non-redundant protein database in Genbank (Altschul *et al.*, 1990). Additionally, all sequences were subjected to similarity searches using nucleotide blast (BLASTN), mainly to identify hits to possible contaminants such as rRNA and genomic DNA products. An E-value greater than 10^{-5} is commonly used as the threshold for a significant hit, however, owing to the lack of available sequence data for banana and the small size of many of the TDFs the most similar homologue for each TDF was noted in the results regardless of the E-value.

QUANTITATIVE RT-PCR CONFIRMATION OF DIFFERENTIAL GENE EXPRESSION

Template Preparation

One µg of total RNA was extracted from banana roots of each of three plants of Williams, FHIA-17, Rose and Calcutta IV newly challenged with *Foc*, which had been subjected to conditions identical to the plant material initially prepared for cDNA-AFLP analysis. The RNA was DNAse-treated and purified using the

RNeasy® MiniElute™ Cleanup kit (Qiagen) according to the manufacturers' instructions. First strand cDNA was then synthesized using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, USA).

Generation, optimization and geNORM analysis of endogenous control genes for qRT-PCR

Four bands, whose expression across the 12 treatments appeared to be constitutive, were excised from the cDNA-AFLP gel, re-amplified, cloned, sequenced and identified as described above. Primers were designed for the four TDFs and dilution series and standard curves were generated to examine the linearity of amplification over the dynamic range (three orders of magnitude). A serial dilution (1:10, 1:100 and 1:1000) was performed from 1 µl of cDNA from one of the resistant cultivars (Rose) inoculated with the pathogen, and qRT-PCR run for each primer set. A 10-µl reaction for PCR amplification contained 5 µl LightCycler® 480 SYBR Green I Master mix, 1 µl of each of the forward and reverse primers (10 µM), 1 µl of diluted cDNA template and 2 µl PCR grade water (Roche Diagnostics). Control treatments contained water instead of cDNA template. All PCR reactions were performed in triplicate on three biological replications. The cycling conditions were as follows: Pre-incubation for 10 min at 95°C (hot start) followed by 55 cycles, each consisting of 10 s denaturing at 95°C, 10 s annealing at 65°C, 10 s primer extension at 72°C, and data acquisition at 95°C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used. Melting curve analysis of the qRT-PCR products was performed to confirm that the individual qRT-PCR products corresponded to single homogenous DNA species. The expression of the four genes were analyzed across the three time points (0, 6 and 72 hpi) and their expression data normalized against the *Musa* 25S gene in order to assess whether they were expressed constitutively for use as additional endogenous control genes for the normalization of the qRT-PCR data. Raw expression data from the newly-selected endogenous controls as well as the *Musa* 25S endogenous control gene were analyzed in geNORM version 3.4 (Vandesompele *et al.*, 2002) to determine which two genes were the most stably expressed (lowest M values) and the most suitable for use in gene expression normalization. geNORM version 3.4 calculates the gene expression stability measure for a control gene as the average pair-wise variation, *V*, for that gene with all other tested control genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability.

Real-time RT-primer design and data analysis

Gene-specific primers were designed for seven selected TDF sequences using DNAMAN version 4.13 (Lynnon BioSoft). The primers were tested using a dilution series, and standard regression curves were generated as described before. qRT-PCR was performed using a LightCycler version 480 instrument (Roche Diagnostics) to confirm the TDF expression patterns identified by cDNA-AFLP. All PCR reactions were performed in triplicate (technical replications) and three biological replications for each treatment were included and analyzed. The expression data from the biological replications for each of the target genes were averaged and the gene expression data were normalized using the geometric means of the two most stable endogenous control genes [the geometric mean for the accurate averaging of control gene expression is required in place of the arithmetic mean, as the former makes allowances for possible outlying values and abundance differences between different genes (Vandesompele *et al.*, 2002)]. This was done in order to create a consensus graph of the expression data from the three plants and multiple endogenous controls. Data sets were analysed using ANOVA and the Duncan's Multiple Range Test (Van den Berg *et al.*, 2007). Bars presented with the same letter are not significantly different at $P < 0.05$.

RESULTS

cDNA-AFLP ANALYSIS

RNA and cDNA quality

RNA purified from the banana roots was found to be of high quality and the absence of contaminating genomic DNA was confirmed for all cDNA samples. The amplification of a region of the actin gene from cDNA yielded the expected 170-bp mRNA-derived amplicon, which was clearly distinguishable from the 260-bp genomic DNA-derived, intron-containing fragment (Chapter 2).

cDNA-AFLP expression patterns and clustering

cDNA-AFLP analysis on LI-COR DNA analysers allowed high-throughput, high-resolution identification of differentially expressed TDFs. Many different expression profiles were generated using the eight selected primer combinations across the 12 treatments, including absolute presence or absence of TDFs and

changes in relative abundance of TDFs (Fig. 1). A total of 76 TDFs were selected for isolation and characterization. These include TDFs expressed in the tolerant and resistant varieties and not the susceptible Williams, and TDFs differentially expressed over time for the same banana variety challenged by *Foc*.

Seven major expression clusters were identified (based on a visual analysis) in the JAVATree View output tree generated from the cluster analysis (Fig 2). Cluster "i" represents transcripts which are up-regulated (red bands) in FHIA-17 at 72 hpi and in the Rose cultivar at all time points. Calcutta IV and Williams are seen to have increased gene expression at 6 hpi. Cluster "ii" depicts genes that are up-regulated/expressed in FHIA-17 and Rose. Expression decreases in Calcutta IV and is down-regulated in Williams. Expression cluster "iii" represents transcripts expressed in all varieties except Calcutta IV. Cluster "iv" represents transient expression in all varieties. Cluster "v" indicates an increase in expression in all but the Rose variety. Cluster "vi" indicates increased expression in the resistant varieties only. Lastly, cluster "vii" depicts up-regulation in the resistant varieties and transient expression in Williams. The cluster analysis potentially indicates gene expression changes in the plants following challenge with *Foc*. The tree does indicate that gene expression changes in response to the pathogen in the susceptible plant are not the same as that seen in the resistant and tolerant plants.

TDF isolation and characterization

TDFs were successfully excised from the polyacrylamide gel and re-amplified (Fig. 3), cloned (Fig. 4), sequenced and assigned putative identities using BLASTX (Table 1). Putative identities were assigned to 36 of the isolated TDFs (excluding TDFs with no hits) along with the measure of similarity (E-value) and representation of the relative expression pattern of each TDF in relation to its uninoculated control (time 0 hrs). Based on the putative functions of the proteins inferred by similarity, the TDFs were broadly classified into eight functional categories (Fig. 5). These eight categories include TDFs involved in gene expression regulation (8%), protein biosynthesis and modification (12%), similarity to defence-related genes (5%), photosynthesis (3%), primary cell metabolism (5%) and transport (9%). Eight percent of the TDFs had similarity to proteins with unknown function and 50% of the sequenced TDFs had no homology to any sequences in public databases.

geNORM analysis of endogenous control genes

*Musa*Cont1 and *Musa*Cont2 were selected as additional controls for normalization of the qRT-PCR data. These two new genes had the lowest M value, which means that they were more stable than the *Musa* 25S endogenous control gene for this particular data set (Table 2).

QUANTITATIVE RT-PCR CONFIRMATION OF DIFFERENTIAL GENE EXPRESSION

TDF expression as quantified by qRT-PCR

Six TDFs were chosen to represent varying expression profiles across the four cultivars (Table 3). These include fragments with similarity to an *S-adenosylmethionine synthase* (*SAMS*) (Clone: ACAC 290), two unknown proteins (ACAC 238 and CTCT 183), a putative *heparanase* (CAAG 301), *elongation factor* (*EF*)-*1 alpha* (CTCT 178) and an *AAA ATPase 26S proteosome subunit P45* (GTTC 198). SAMS (ACAC 290), is an enzyme which catalyses reactions upstream of secondary metabolic processes such as lignin biosynthesis, and was selected as it appeared to be up-regulated in the resistant banana varieties Rose and Calcutta IV 6 hpi with *Foc* (Fig. 6A). An Unknown Protein 1 (ACAC 238) was selected as it was down-regulated in the susceptible Williams at 72 hpi and displayed up-regulation or high constitutive expression in Calcutta IV (Fig. 6B). A TDF with similarity to a putative *heparanase* (*ß-glucuronidase*) (CAAG 301) was selected because of its strong expression in the resistant Rose banana variety (Fig. 6C). Heparanase is thought to be indirectly involved in H_2O_2 degradation (Morimoto *et al.*, 1998), thus acting as a cell protectant, and is also involved in the generation of phenolic compounds that may be used for cell wall fortification (Eckey *et al.*, 2004). Expression data for the TDF with similarity to *a-EF1* (CTCT 178) indicated that it was highly expressed in Calcutta IV 6 hpi (Fig. 6D). The TDF representing an *AAA ATPase 26S proteosome subunit P45* (GTTC 198) was up-regulated in FHIA-17 and in Rose (Fig. 6E). Unknown Protein 2 (CTCT 183), although expressed at all time points, was selected as Williams expressed this gene at a relatively low level when compared to the tolerant and resistant cultivars (Fig. 6F). The qRT-PCR data for each of these TDFs (and the three biological replications) was normalized using the geometric mean of the *Musa*Cont1 and *Musa*Cont2 expression data, and a "consensus" graph was successfully generated representing the expression pattern for each of the selected genes.

The up-regulation of *SAMS* in the resistant Rose and Calcutta IV varieties, as visualized by cDNA-AFLP gel band intensities, was confirmed by the qRT-PCR data. *SAMS* shows a similar pattern of expression in the susceptible and resistant banana cultivars, being up-regulated at 6 hpi, with expression decreasing thereafter (Fig. 6A). The susceptible Williams plants showed expression levels of *SAMS* comparable to that of the resistant Rose cultivar at 6 hpi, but Rose maintained a higher level of *SAMS* expression at 72 hpi. Calcutta IV had the highest *SAMS* expression at 6 hpi, but expression levels of this gene returned to a level comparable to the uninoculated control. A correlation between the qRT-PCR data and the band intensities on the cDNA-AFLP was observed.

The qRT-PCR data for the TDF corresponding to a *heparanase* confirmed that the overall expression of this gene was highest in the resistant Rose cultivar and that marginal increases of this enzyme were observed at 6 hpi in Williams and FHIA-17 (Fig. 6C). The expression trend in Calcutta IV was confirmed but the intensity of the response at 6 hpi was surprising, having an expression level higher than that of Rose. qRT-PCR data for the Unknown protein 1 displayed a vague correlation between the gel band intensities and expression data within a cultivar but, relative expression between the cultivars was not accurately represented. *a-EF* qRT-PCR data was an accurate representation of what was seen on the cDNA-AFLP gel. qRT-PCR data for Unknown Protein 2 accurately reflected what was seen on the gel. *ATPase 26S proteosome subunit P45* qRT-PCR profiles did not correspond well to what was seen on the cDNA-AFLP gel.

Differential expression of plant specific TDFs:

Due to the finding that the cDNA-AFLP and qRT-PCR results displayed correlations albeit vague, one can tentatively assume that the cDNA-AFLP gel patterns of the isolated TDFs are semi-quantitative. Several defence-related genes were identified in the tolerant and resistant banana varieties that were not present in the susceptible Williams cultivar (Table 1). *Cytochrome P450-like TBP* (ACGA 209) was down-regulated in the susceptible Williams cultivar after infection with the pathogen, but displayed transient expression in the two resistant cultivars Rose and Calcutta IV, as it was expressed at 0 hrs and 72 hpi in Rose and at 6 hpi in Calcutta IV (Table 1). A TDF representing a putative *transketolase* (*TK*) was constitutively expressed in the tolerant FHIA-17 and resistant Rose (Table 1). The *TK* gene was up-regulated after pathogen attack in the susceptible Williams cultivar, but expression decreased after 6 h. *Isoflavone*

reductase (*IFR*) was not expressed in any of the uninoculated control plants but was up-regulated in Williams, Rose and Calcutta IV 6 hpi, after which the gene appeared to be down regulated in these plants. Two TDFs with homology to an *ascorbate POX* and a *haem POX* were observed in the Williams cultivar to be up-regulated after 6 hrs, but expression was seen to decrease thereafter. *Ascorbate POX* was up-regulated in Rose at 6 hpi and down-regulated at 72 hpi. The TDF representing *haem POX* in FHIA-17 was constitutively expressed (Table 1).

A putative WRKY6 (CTCT 178) and two bZIP transcription factors (ACGA 192 and GTTC 184) were identified from TDFs with very short sequences (138 bp, 155 bp and 178 bp respectively). As a result of this, combined with the fact that there is very little sequence data available for banana these three TDFs had very high E-values (0,11, 0.15 and 0.96 respectively) which indicates a high probability that these TDFs could in fact represent entirely different genes. They were, however, discussed further as their putative identities are representative of genes implicated in the plant defence response of several well characterized plant-pathogen interactions.

The putative WRKY6 TDF was down-regulated in Williams and FHIA-17 at 6 hpi and expression increased in the resistant Rose (Table 1). A TDF representing a *bZIP* transcription factor (GTTC 219) from *Glycine max* L. was identified from the cDNA-AFLP profiles as being down-regulated in the susceptible Williams and up-regulated at 72 hpi in FHIA-17 and Rose. This TDF was constitutively expressed. The second bZIP transcription factor (ACGA 192) was down-regulated in Williams at 6 hpi but its expression increased by 72 hpi. FHIA-17, Rose and Calcutta IV all expressed this TDF constitutively; Rose had the highest constitutive expression (Table 1). The TDF with homology to a putative ring-box Roc1/Rbx1/Hrt1 protein (GTTC 277) was induced at 6 hpi in both the resistant varieties and expressed constitutively in the susceptible and tolerant varieties, at low levels. A kinase-like TDF (CTCT 256) was identified. This TDF was expressed constitutively in Williams and FHIA-17, but was up-regulated slightly in Rose at 6 and 72 hpi. This TDF was present in Calcutta IV at 0 hrs and was down-regulated after pathogen challenge. All of the mentioned expression patterns are represented in Table 1.

DISCUSSION

Important TDFs associated with primary cellular metabolism, secondary metabolites and gene expression were identified in four banana varieties of variable susceptibility to *Foc* in this study. The genes encoding enzymes for primary cellular metabolism include *SAMS* (ACAC 290) and a putative *heparanase* (CAAG 301), and those for secondary cellular metabolism include *Cytochrome P450 TBP* (ACGA 209), a putative *TK* (AGAG 292), *IFR* (AGAG 268) and an *ascorbate* and *haem POX* (CACA 289 and CAAG 301). Two TDFs, whose E-values were not significant enough for conclusive identification of the fragment, are associated with gene expression and regulation. These include a putative WRKY6 and bZIP transcription factors. The most numerous group of sequenced TDFs represented proteins involved in protein processing, synthesis and degradation. Peptides in this group included a putative ring-box Roc1/Rbx1/Hrt1 protein (GTTC 277), EF -1 alpha and 2 (ACGA 173 and CTCT 229), two protein kinases (PK) (CACA 166 and CTCT 256) and a cysteine proteases (ACGA 183).

A gene of special interest involved in primary cellular metabolism is one encoding for SAMS, a precursor for secondary metabolism which leads to lignin synthesis. This gene was up-regulated to the greatest extent 6 hpi in Calcutta IV, a possible rapid response to pathogen attack, and was lowest in the susceptible Williams cultivar compared to the other varieties at 72 hpi. The deposition of lignin is a previously confirmed defence response to *Foc* in resistant banana varieties (Vander Molen *et al.*, 1987). This finding was confirmed by Van den Berg *et al.* (2007) who showed that cell wall strengthening enzymes accumulated to a greater extent in a tolerant banana selection (GCTCV-218) than in the susceptible Williams cultivar. The putative *heparanase*, shown to be highly up-regulated in Rose and Calcutta IV, has a possible role in the detoxification of H_2O_2 (Morimoto *et al.*, 1998), thus acting as a cell protectant. This may be indicative of a strong oxidative burst in the resistant plants. *Heparanase* has also been linked to the production of lignin (Eckey *et al.*, 2004).

In this study, several TDFs had homologies to enzymes which are directly or indirectly related to the phenylpropanoid pathway and lignin biosynthesis, such as a cytochrome P450-like TBP, a putative TK and an IFR. The most common basic structural unit of lignin is a phenylpropanoid. Phenylpropanoid

metabolism, a secondary metabolic pathway, comprises a series of branching biochemical reactions which provide the plant with a host of important phenolic compounds (Weisshaar and Jenkins, 1998). One such branch leads to the production of flavonols, anthrocyanins and tannins. Phenylpropanoids have a range of important functions in plants, including structural components such as lignin, protectants against biotic and abiotic stresses (antipathogenic phytoalexins, antioxidants and UV absorbing products), pigments and signalling molecules.

Cytochrome P450-like TBP is an enzyme involved in secondary metabolism leading to lignin production (Dowd *et al.*, 2004). The induction of *cytochrome P450* in the resistant banana cultivars may result in lignin deposition in the root, thereby halting the ingress of the pathogen into the banana vascular system. Cytochrome P450 has an additional possible function in the generation of reactive oxygen species (Puntarulo and Cederbaum, 1998). The oxidative burst, a well-studied defence response involving reactive oxygen species, leads to the cross-linking of cell wall proteins which renders cells more able to resist fungal enzymatic attack (Bradley *et al.*, 1992; Keen, 1999). In their article, Dowd *et al.* (2004) reported that lignin biosynthesis and the induction of *cytochrome P450* were defence strategies employed in the *F. oxysporum* f.sp. *vasinfectum* (Atk.) – cotton interaction.

TK is an enzyme which acts as a catalyst in the Calvin cycle and in the oxidative pentose phosphate pathway and produces erythrose-4-phosphate, a precursor for the Shikimate pathway leading to phenylpropanoid metabolism (Henkes *et al.*, 2001). Henkes *et al.* (2001) silenced the *TK* gene in tobacco which resulted in an inhibition of ribulose-1,5-bisphosphate regeneration and photosynthesis when expression of *TK* was reduced by 20-40%. When expression of *TK* was decreased by up to 50%, decreased levels of aromatic amino acids and the precursors and products of the phenylpropanoid pathway were observed. The down-regulation of this gene in the susceptible Williams could possibly translate into decreased or insufficient lignin production as a defence strategy and thus allowing pathogen entry into the roots. Furthermore, the constitutive expression of this gene in Rose and FHIA-17 may imply that these varieties have inherently stronger cell walls than the susceptible plant, thus limiting pathogen entry. Phenylpropanoid metabolism does not, however, lead to the exclusive production of lignin. Phytoalexins such as flavanoids and isoflavonoids, which are natural pathogen-induced antimicrobial compounds, are also produced as a result of this metabolic pathway (Dixon *et al.*, 2002). A decreased

level of *TK* expression could, therefore, translate into a decrease in phytoalexin production allowing the pathogen to survive and advance into the banana roots.

A key enzyme in the branch of phenylpropanoid metabolism involved in the production of phytoalexins such as flavanoids and isoflavonoids, is IFR. This enzyme catalyses the conversion of isoflavones to isoflavanones which results in the synthesis of prenylated isoflav(an)ones and pterocarpan phytoalexins (Dixon, 2001). The up-regulation of this gene 6 hpi in Williams, Rose and Calcutta IV is an indication that this secondary metabolic pathway is being activated by the pathogen in both the resistant and susceptible plants. The observation that the susceptible plant displayed a similar response to the resistant plant begs the question as to whether the response is perhaps not as highly up-regulated in the susceptible plant as in the resistant one. This could be answered by investigating an earlier time-point post-inoculation, as it is possible that the resistant plant expresses this gene within minutes of pathogen perception, whereas the susceptible plant may mount a response a few hours thereafter. An alternative hypothesis is that this is not the primary mode of defence that the resistant plants employ.

POXs are enzymes which are also involved in lignin biosynthetic metabolism (Vidhyasekaran, 1997). The constitutive expression of *POX* (CAAG 301) in FHIA-17 is consistent with previous research by Novak (1992), indicating that a FHIA hybrid SH-3362, a resistant synthetic AA hybrid had POX levels 10-fold higher than Pisang Mas, a susceptible AA cultivar. In Chapter 2, the *POX* gene in FHIA-17 was up-regulated at 6 hpi and was expressed constitutively over the rest of the time course. Furthermore, FHIA-17 had significantly higher levels of expression than in Williams, Rose and Calcutta IV at 72 hpi (Chapter 2). The expression of this TDF decreased 72 hpi in the susceptible Williams variety which was also observed in the expression pattern of the TDF representing an ascorbate POX (CACA 289). The expression of this TDF increased in the resistant Rose variety at 6 hpi and decreased again at 72 hpi, which implicates a role in the early plant defence response.

The observed pattern of expression whereby genes encoding lignin-biosynthetic enzymes, such as SAMS, cytochrome P450 TBP and TK were down-regulated in Williams could be a result of the pathogen suppressing such enzymes. This was seen in the *Puccinia graminis* f.sp. *tritici* – wheat interaction, where

PAL, 4-courmarate/CoA ligase, cinnamyl-alcohol dehydrogenase and POX-increases were suppressed by the pathogen in a susceptible wheat line. The same study further indicated that these lignin biosynthetic enzymes continued to increase for as long as 7 days after inoculation in the resistant wheat lines (Moerschbacher *et al.*, 1988; Vidhyasekaran, 1997). Although no notable increases were seen in the tolerant FHIA-17 and resistant Calcutta IV plants there was a clear decrease of POX expression in the susceptible plant, as was demonstrated earlier (Chapter 2).

Several TDFs whose most similar homologues were to proteins involved in gene-regulation were identified. These included a WRKY transcription factor, a reverse transcriptase and a bZIP transcription factor. Both the putative WRKY transcription factor and bZIP transcription factors had non-significant E-values. The E-value is the expected number of false hits per sequence query. If the E-value is between 1 x 10^{-50} and 1 x 10^{-2}, the hit has some similarity to the query sequence and may be related. These values can indicate that the sample sequence is in the same family as the hit or it may have closely related functional domains. If the E-value is between 1 X 10^{-2} and 1, the hit has a slight possibility of being related to the query which may indicate a distant evolutionary relationship. If the E-value is above 1, the hit is not very closely related to any sequence in the database (BLAST tutorials, accessed 2007, http://www.swbic.org/origin/proc_man/Blast/BLAST_tutorial.html). The putative identities of these TDFs are, however, discussed as their possible identities have a definite role in the plant defence response.

Dong *et al.* (2003) found the early induction of transcription factors after pathogen attack for 49 different WRKY proteins in *Arabidopsis*. In barley the transient knockdown of the barley homologue resulted in enhanced resistance of epidermal cells towards powdery mildew (Eckey *et al.*, 2004). The WRKY transcription factor, therefore, seems to be able to play a negative regulatory role in plant defence. bZIPs are a large family of transcription factors in plants; *Arabidopsis* has 75 members (Singh *et al.*, 2002). The bZIP transcription factor-like TDF isolated in this study may be involved in the defence response of banana, as a class of these transcription factors comprising of TGA/*octopine synthase* (ocs)-element binding factor (OBF) proteins has previously been linked to stress responses (Singh *et al.*, 2002). The OBF proteins bind to the activation sequence-1 (ac-10/ocs) element, which regulates the expression of some stress responsive genes such as the *PR-1* and *glutathione-S-Transferase6* genes (Lebel *et al.*, 1998; Chen and Singh, 1999; Singh *et al.*, 2002).

There is no evidence supporting a gene-for-gene resistance mechanism in the banana-*Foc* interaction. A TDF corresponding to a putative ring-box Roc1/Rbx1/Hrt1 protein, which is a component of SCF ubiquitin ligase and anaphase-promoting complex was, however, identified. The anaphase-promoting complex is involved in post-translational modification, protein turnover, chaperones, cell division and chromosome partitioning (Devoto *et al.*, 2003). SCF ubiquitin ligase is involved in regulatory mechanisms which control a range of cellular processes in plants and may play a role in plant disease resistance regulation (Devoto *et al.*, 2003). It has been proposed that regulators of SCF ubiquitin ligases are essential components of *R*-gene-mediated resistance. Devoto *et al.* (2003) that the anaphase-promoting complex may regulate plant defences against pathogens, although no ubiquitin ligase targets that are associated with disease resistance have yet been identified in plants.

For SCF ubiquitin ligase to carry out its function its target(s) must be phosphorylated before serving as a substrate (Deshaies, 1999). This may account for the identification of two kinase-like TDFs in banana in this investigation. The expression of the kinase-like TDFs may be interpreted in several ways. The absence of the second kinases-like TDF in Williams, FHIA-17 and Calcutta IV could be due to genotypic differences of the plants which are reflected in the expression profiles. Alternatively, and very theoretically, Rose could be the only cultivar activating the additional phosphorylation of proteins for secondary metabolic pathways. Calcutta IV may also down-regulate this gene after pathogen attack as a result of steady-state phosphorylation prior to pathogen attack, at which point the necessary phosphorylated proteins are rapidly channeled into secondary metabolism/defence pathways, leaving this enzyme redundant.

An AAA ATPase from the 26S proteasome subunit P45 was identified in addition to the ring-box protein/SCF ubiquitin ligase. The SCF ubiquitin ligase complex plays a key role in making a variety of regulatory proteins for destruction by the 26S proteosome (Deshaies, 1999). Protein degradation is necessary for modulation of the steady-state abundance of proteins, and to switch cellular regulatory circuits from one state to another. The coincidence of the increased expression of both these TDFs in the resistant and tolerant banana varieties suggests a highly regulated chain of events leading to the resistance response. The qRT-PCR data confirmed that this TDF was highly expressed in the resistant

cultivars but indicated that the AAA ATPase was highly expressed at both 0 hrs and 6 hpi in the susceptible Williams at which point expression was almost undetectable at 72 hpi.

A major draw-back of studying a crop which is a non-model monocot is the lack of sequence data. This is evident by the fact that the largest group of characterized TDFs contained open reading frames with no significant similarity to any known plant protein sequences. It is highly likely that some of these TDFs are derived from the 3' regions of transcripts due to the fact that cDNA synthesis is primed at the mRNA poly A+ tail, and full-length cDNA copies are less abundant than partial cDNAs. This is in direct conflict with the manner in which the majority of ESTs in databases are sequenced. As a general rule sequencing begins at the 5' end of a gene, thus lowering the likelihood of overlap with 3' biased TDFs (Ranik *et al.*, 2005). Lastly, 8% of the sequenced TDFs exhibited significant homology to proteins of unknown function, possibly representing transcripts which are uniquely expressed in banana and not yet characterized in this monocotyledonous plant species or its associated model plants, rice and maize.

A major criticism regarding PCR-based expression profiling techniques (such as cDNA-AFLP) has stemmed from the fact that conventional PCR is not accurately quantitative and thus not a good technique for the analysis of gene expression. This is despite clear evidence demonstrating the ability of cDNA-AFLP to accurately quantify relative gene expression levels at the whole-genome level (Breyne *et al.*, 2003; Reijans *et al.*, 2003; Ranik *et al.*, 2005). This problem can be circumvented using qRT-PCR which is based on monitoring the accumulation of products during the exponential phase of the PCR (Higuchi *et al.*, 1993). This difference can possibly be attributed to the limited number of PCR cycles performed during pre-amplification and selective amplification, ensuring that the relative TDF quantities are directly proportional to the initial transcript abundance in the mRNA samples.

The qRT-PCR validation of cDNA-AFLP data in this study demonstrated that cDNA-AFLP was largely, an accurate representation of gene expression. Discrepancies in the patterns of expression of selected genes as they appear on the cDNA-AFLP gel and the qRT-PCR data could be attributed to the experimental design. These discrepancies could be due to independent RNA preparations being used for the cDNA-AFLP analysis and qRT-PCR studies. This finding is consistent with results obtained by Robatzek and Somssich (2002) in their study of WRKY6 transcription factors during plant senescence and

pathogen defence using cDNA-AFLP. They were only able to validate 70% of their TDFs from roots and 50% of the TDFs from leaves using qRT-PCR when independent RNA samples were used. Alternatively, these discrepancies could be attributed to a genotypic bias. The cDNA-AFLP process with its many stages of digestion, ligation, pre- and selective amplification, could be influenced by the different banana varieties genotypes used in this study. It is possible that these same differences which bias the cDNA-AFLP do not bias the results of the qRT-PCR, resulting in the observed differences.

In this study, the cDNA-AFLP technique was successfully applied in identifying genes related to the defence response of banana. Although the sequence of events during the defence response of banana is not yet clear, several genes relating to secondary metabolism, specifically the synthesis of cell wall strengthening compounds, were identified. Our results also correlate well with previous research conducted on banana defence-related genes (Van den Berg *et al.*, 2007). Future work on this subject should include expression microarray analysis using model monocots such as rice and maize, as well as histochemical studies on the roots of the resistant banana varieties inoculated with the pathogen, in order to gain a holistic understanding of the resistance response. It would be of additional value to transform susceptible banana varieties with genes such as *SAMS* or *isoflavone reductase* to see if this results in a resistant phenotype or, alternatively, to silence the expression of these genes in the resistant plants to note whether a susceptible phenotype is observed.

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Table 1. Putative identities and plant-specific expression patterns of 36 selected TDFs in 4 banana varieties challenged with *Fusarium oxysporum* f.sp. *cubense*. Darker blocks indicate highly expressed TDFs or up-regulation and lighter blocks indicated low expression or down-regulation. White blocks indicate the absence of the TDF at a particular time point or banana variety.

Clone	TDF size (bp)	BLASTX Accession of similar sequence	Most Similar Homologue	E-value	Expression pattern											
					Williams			FHIA-17			Rose			Calcutta IV		
					0	6	72	0	6	72	0	6	72	0	6	72
ACAC 290	249	CAC82203	S-adenosylmethionine synthetase, *Oryza sativa*	1e-15												
ACAC 238	199	NP_850546	Unknown protein, *Arabidopsis thaliana*	1e-04												
ACAC 187	147	AAG21898	Putative peptide transport protein, *O. sativa*	8e-16												
ACAC 166	128	CAD91335	Monosaccharide transporter, *Glycine max*	2e-13												
ACGA 441	400	AAM08545	Putative pyridoxamine 5-phosphate oxidase, *O. sativa*	1e-36												
ACGA 281	243	NP_191281	UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosamine-phosphotransferase, *A. thaliana*	2e-18												
ACGA 209	169	BAA10929	Cytochrome P450 like TBP, *Nicotiana tabacum*	5e-11												
ACGA 192	155	ABI34662	bZIP transcription factor bZIP111, *G. max*	0.15												
ACGA 183	144	CAA84378	Cysteine Proteinase, *Vicia sativa*	1e-07												
ACGA 173	134	CAC12818	Elongation factor 2, *N. tabacum*	5e-15												
ACGA 170	130	AAD32766	Putative Na+-dependent inorganic phosphate cotransporter, *A. thaliana*	4e-9												
ACGA 163	124	AAW66799	Cysteine protease, *Pinus taeda*	2.8												
AGAG 292	250	AAO33154	Putative transketolase, *O. sativa*	2e-16												
AGAG 268	227	AAT67247	Isoflavone reductase, *Musa acuminata*	1e-36												
AGAG 247	206	CAH59632	Mitochondrial phosphate translocator, *Medicago truncatula*	1e-23												
AGAG 242	201	EAZ21685	Hypothetical protein OsJ_005168, *O. sativa*	1e-20												
AGAG 217	175	NP_198638	Catalytic, *A. thaliana*	3e-04												
AGAG 200	157	ABE83303	Reverse transcriptase, *M. truncatula*	4e-17												

Clone	TDF size (bp)	BLASTX Accession of similar sequence	Most Similar Homologue	E-value	Expression pattern											
					Williams			FHIA-17			Rose			Calcutta IV		
					0	6	72	0	6	72	0	6	72	0	6	72
AGCA 287	246	BAF46352	Alpha chain of nascent polypeptide associated complex, *Nicotiana benthamiana*.	2e-32												
AGCA 148	103	NP_199218	Antiporter/ drug transporter/ transporter, *A. thaliana*	0.011												
CACA 289	248	ABH10015	Ascorbate peroxidase, *Eucalyptus camaldulensis*	6e-14												
CACA 245	204	O24045	RuBisCO small subunit, *M. acuminata*	0.34												
CACA 166	125	AAG31752	Protein kinase AKINbetagamma-2, *Zea mays*	0.001												
CAAG 301	262	ABE85163	Haem peroxidase, plant/fungal/bacterial, *M. truncatula*	1e-13												
CAAG 301	262	CAD42650	Putative heparanase, *Hordeum vulgare* subsp. *vulgare*	2e-29												
CAAG 152	111	ABK28712	Unknown protein, *A. thaliana*	0.99												
CAAG 177	136	ABE93756	Synaptobrevin, *M. truncatula*	1e-17												
CTCT 393	349	BAE71196	Putative hydroxymethylglutaryl-CoA lyase, *Tritifolium pretense*	7e-39												
CTCT 256	211	NP_565453	Kinase, *A. thaliana*	5e-28												
CTCT 229	188	AAF42980	EF-1 alpha, *Z. mays*	0.018												
CTCT 178	138	CAH68822	Putative WRKY6 protein, *H. vulgare* subsp. *vulgare*	0.11												
CTCT 183	143	AAU10659	Unknown protein, *O. sativa*	2e-12												
GTTC 184	139	ABA94172	Expressed protein, *O. sativa*	3e-06												
GTTC 219	179	ABI34675	bZIP transcription factor bZIP28, *G. max*	0.96												
GTTC 198	157	ABE80601	AAA ATPase; 26S proteasome subunit P45, *M. trunculata*	2e-19												
GTTC 277	236	ABA18098	putative ring-box Roc1/Rbx1/Hrt1 protein, *Olimarabidopsis pumila*	1e-26												

Table 2. Primer sequences of potential endogenous control genes for quantitative real-time PCR analysis of defence related genes in banana against *Fusarium oxysporum* f.sp. *cubense*.

Potential Endogenous Control	BLASTX Accession of similar sequence	Gene Identity	Forward Primer (5'-3')	Reverse Primer (5'-3')	geNORM M value
*Musa*Cont1	ABE78299	Ribosomal protein S23 *Medicago truncatula*	TGACGAAGTCTTGATCGCTGG	AATAGCGCCAACAGCGACA	**0.108**
*Musa*Cont2	NP_001058571	Os06g0714100, *Oryza sativa*	TTGAAGCCAACAAACATGCG	ATTTGGAGCCACCAGGAGC	**0.092**
Musa 25S	AF 399949	Ribosomal protein	GTAAACGGCGGGAGTCACTA	TCCCTTTGGTCTGTGGTTTC	**0.153**

Table 3: Primer sequences from genes identified by cDNA library of banana varieties challenged with *Fusarium oxysporum* f.sp. *cubense*. These primers were used for confirmation of gene expression by quantitative real-time PCR.

Clone	Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACAC290	S-adenosylmethionine synthetase	CAAGTGGGAGAAGCCTGCTG	GGAGTTCTGGTGCAGCAAATG
ACAC238	Unknown protein	GCACAGAGCAGCAAGCAACA	CAACTGAAGGTGTGGCAAAGC
CAAG301	Putative heparanase	CTCGGCCAGAATGACGTTG	TTCCGTGGATATCAGTGGCTC
CTCT229	EF-1 alpha	GCATAACTGCTCCATTTCCTTCC	GACCCACCATTCTTGAAAGCA
CTCT183	Unknown protein	TGCTGATTGGGCTCTAGGGA	CATAAACTGAGCGCCGGGA
GTTC277	AAA-ATPase; 26S Proteosome subunit P45	TCTAGCCCGTGCTTCTTCAGA	CTTGATCCTGCTCTTGTGCG

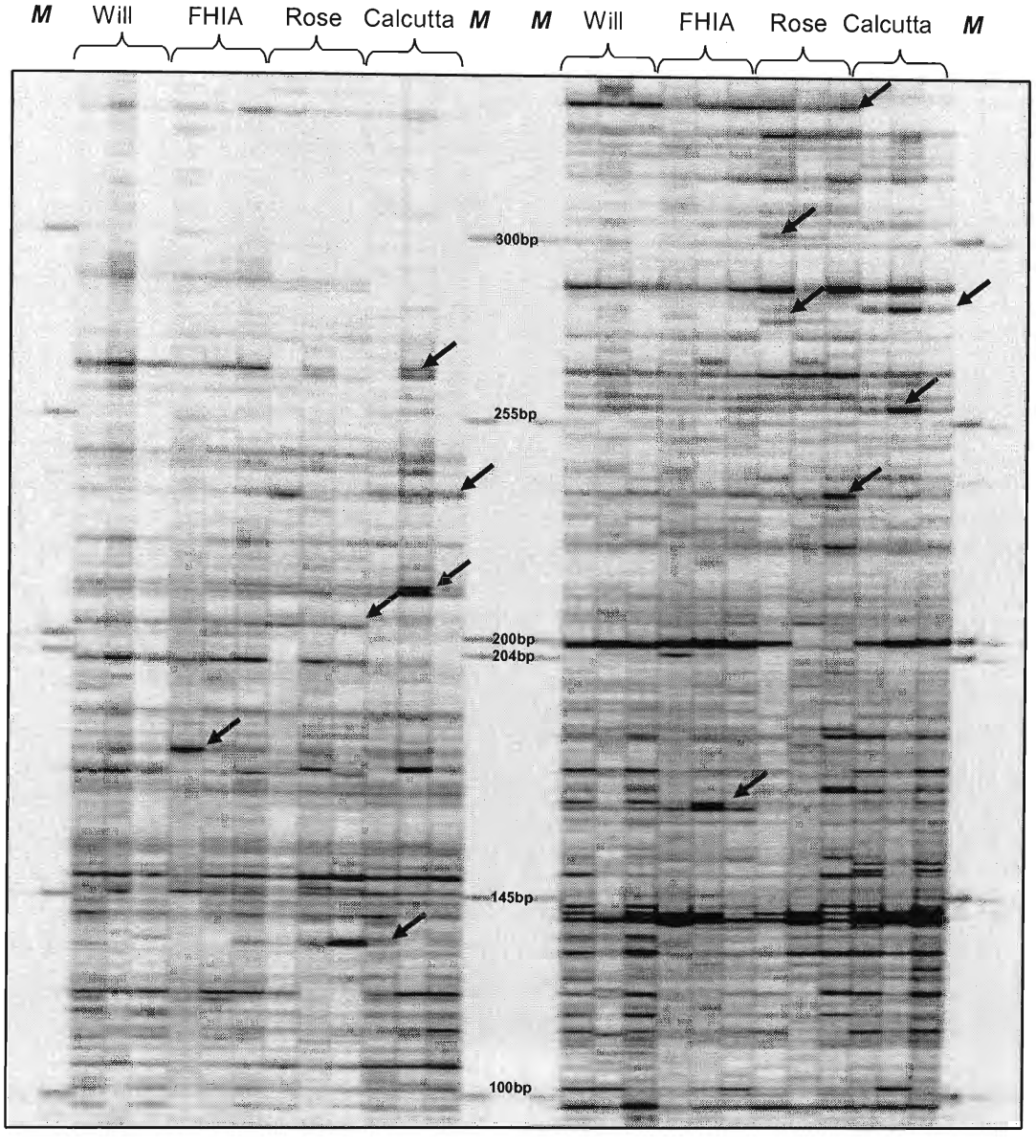


Figure 1. cDNA-AFLP expression patterns in susceptible and resistant banana varieties following infection by *Fusarium oxysporum* f.sp *cubense*. The transcript derived fragments indicated with arrows are examples of gene fragments differentially expressed in either the susceptible or resistant plants. The three lanes within a banana variety represent time points of 0, 6 and 72 hrs. M is the molecular weight standard (IRD 700, LI-COR Biosciences), and fragment lengths are indicated in base pairs (bp).

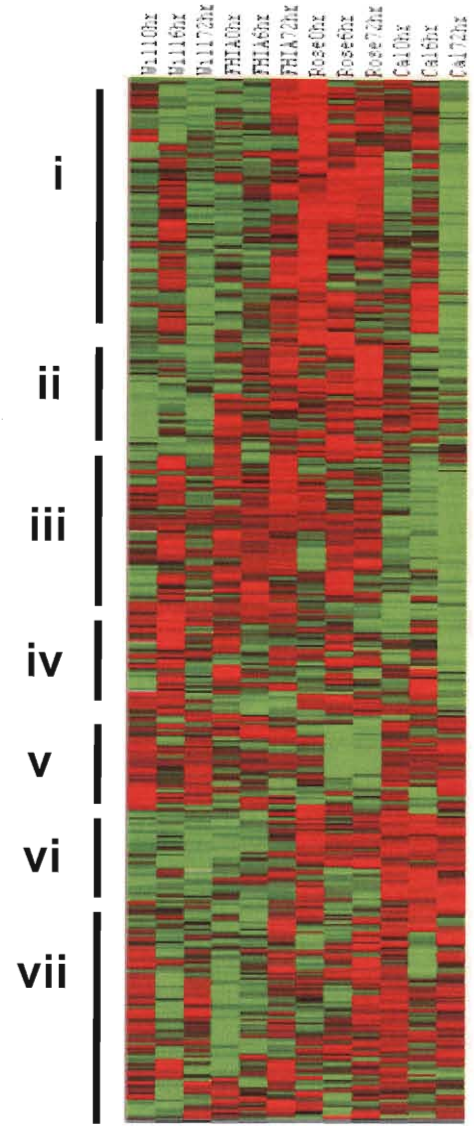


Figure 2: Hierarchical clustering of differentially expressed transcript derived fragments (TDFs) in four banana varieties challenged with *Fusarium oxysporum* f.sp. *cubense* at three different time intervals. Relative expression levels (mean centered and normalized) are represented by a red/green colour continuum with green signifying down-regulation, red indicating relative up–regulation, and black representing TDFs that are absent. The rows correspond to the quantified, differentially expressed TDFs, and columns to the banana varieties Williams, FHIA-17, Rose and Calcutta IV collected at 0, 6 and 72 hrs post inoculation. i- vii represent the seven major expression clusters based on visual inspection.

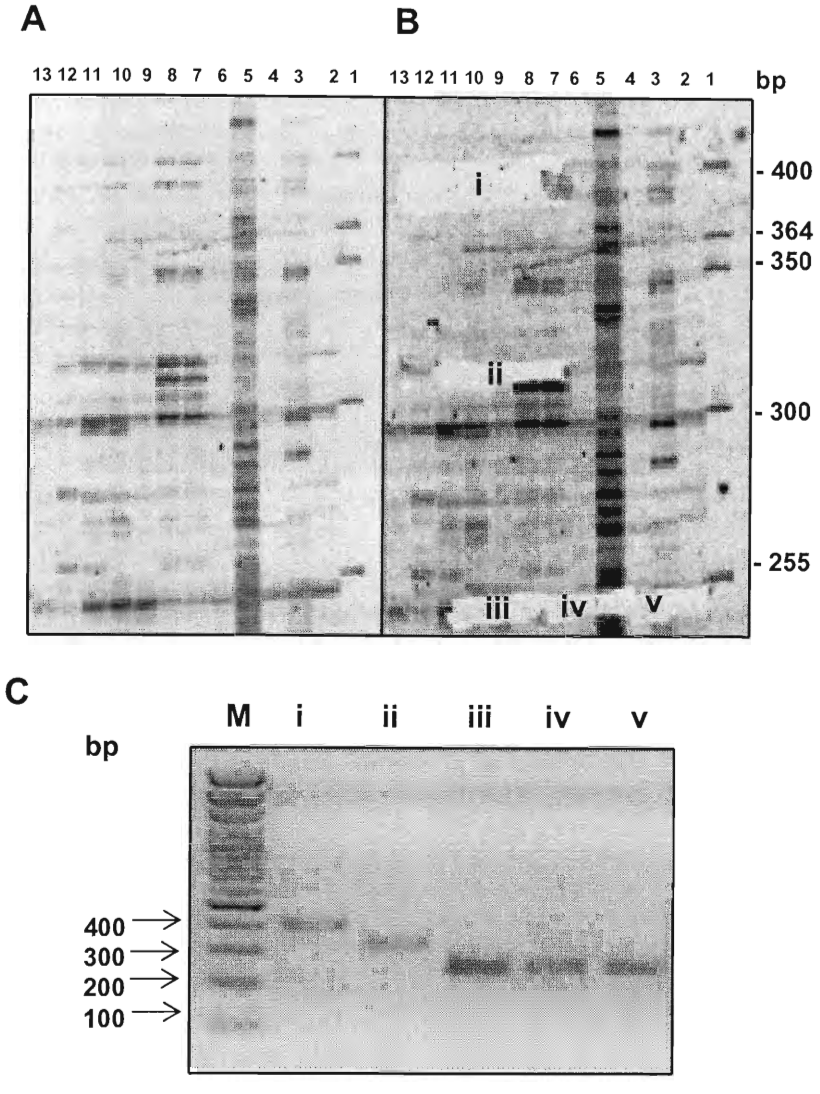


Figure 3. Section of a LI-COR gel image scanned on the Odyssey infrared scanner showing transcript derived fragment profiles in banana infected with *Fusarium oxysporum* f.sp. *cubense* using a single primer combination across 12 treatments. Lane 1: IRD700-labeled molecular size standard (IRD 700, LI-COR Biosciences); Lanes 2-4: Williams 0, 6 and 72 hours post inoculation; Lanes 5-7: FHIA 17 0, 6 and 72 hpi; Lanes 7-10: Rose 0, 6 and 72 hpi; and Lanes 11-13: Calcutta IV 0, 6 and 72 hpi. B. The identical section of gel image as in A after band excision and re-scanning. (i) – (v) indicates the excised fragments with approximate sizes of 400, 300 and 250 bp respectively. C. An agarose gel of re-amplified fragments in lanes (i) – (v). Lane M: 100-bp molecular weight standard (Fermentas, Hanover, USA).

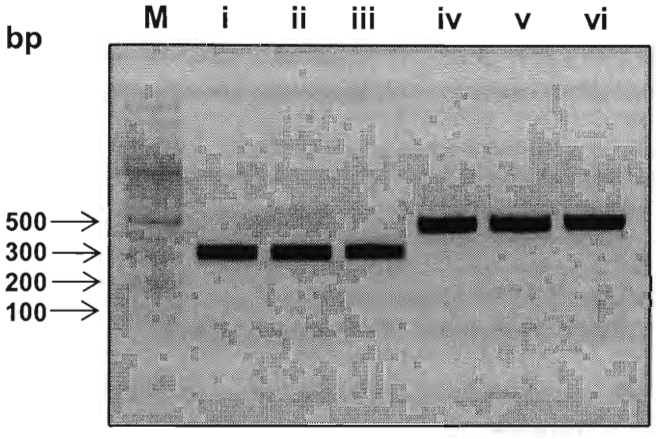


Figure 4. Colony PCR of transcript derived fragments (TDFs) successfully ligated into the pTZ57R/T cloning vector from the InsTAclone™ Cloning Kit (MBI Fermentas, Hanover, Maryland, USA). Lanes i-iii: colony PCR of a TDF performed in triplicate; Lanes iv-vi: colony PCR of a TDF performed in triplicate. Lane M: 100-bp molecular weight standard (Fermentas).

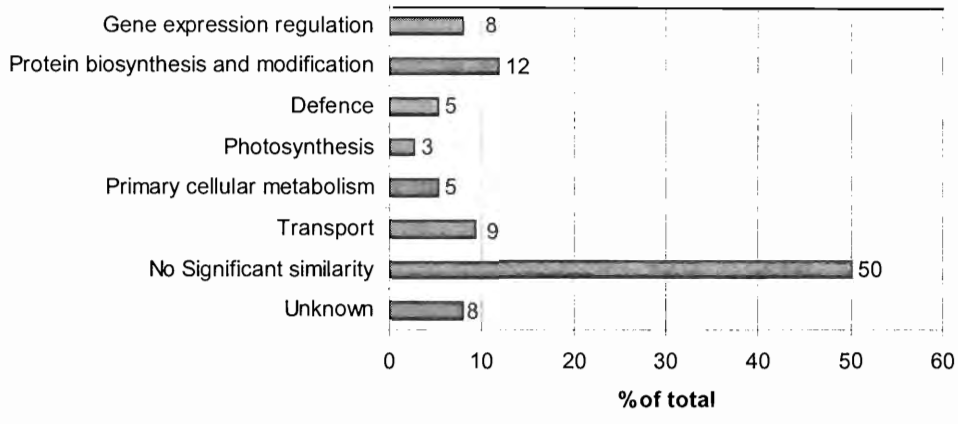
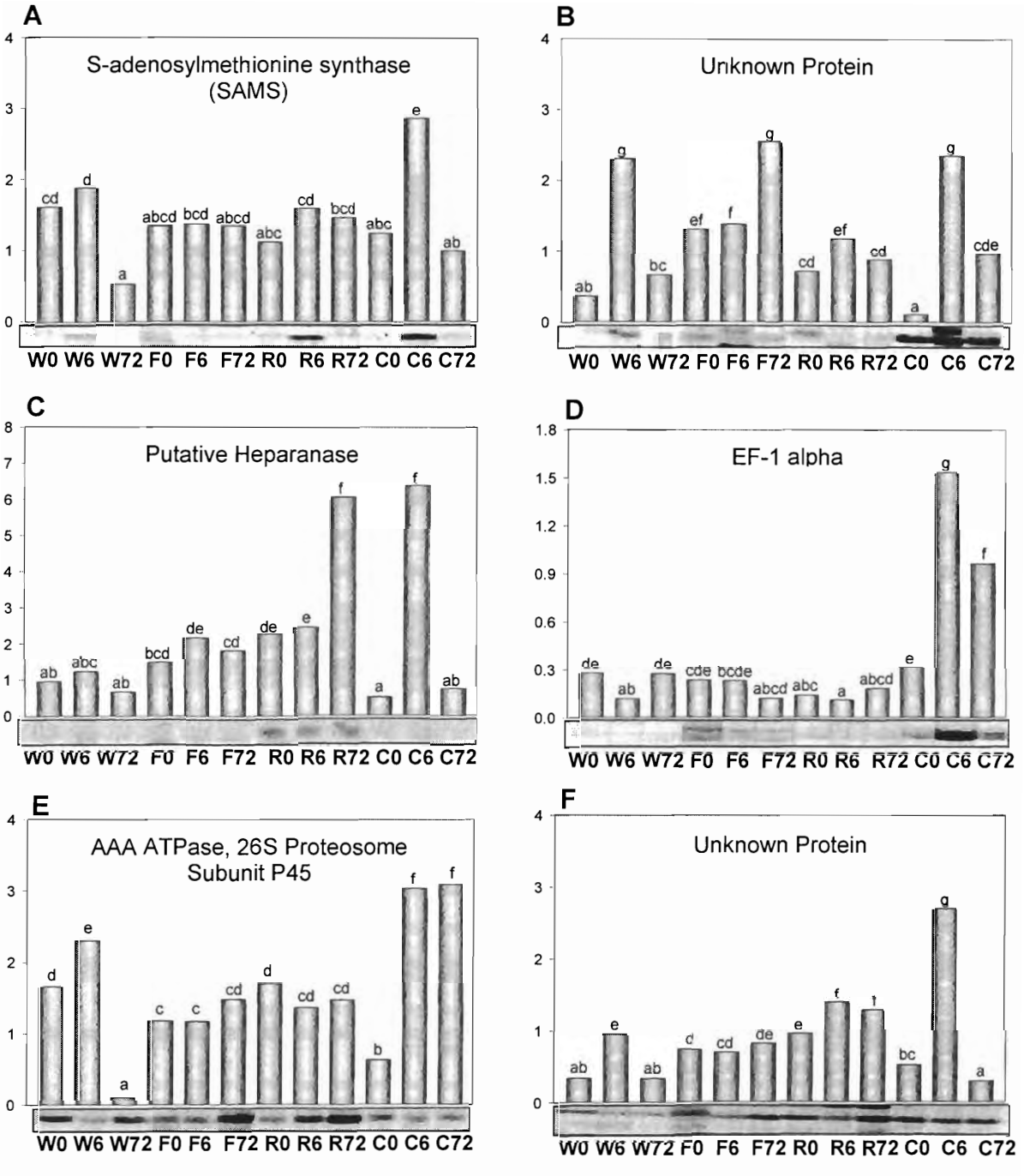


Figure 5. Classification of similarity-inferred identities of 36 transcript derived fragments (TDF) isolated from banana infected by *Fusarium oxysporum* f.sp. *cubense* based on BLASTX results. "No significant similarity" are TDFs that have no homology to any previously characterized protein or had no homology to plant proteins. "Unknown" denotes significant similarity to proteins of unknown function.

Figure 6: Expression patterns of six putative defence-related genes, *S-adenosylmethionine synthetase* (A), *Unknown Protein* (B), *Putative Heparanase* (C), *EF-1 alpha* (D), *an AAA ATPase, 26S Proteosome Subunit P45* (E), and an *Unknown Protein* (F) in four banana varieties at different time intervals following infection with *Fusarium oxyporum* f.sp. *cubense*, using qRT-PCR. Vertical axes represent the measure of transcript derived fragment abundance within the roots relative to the expression level of the geometric mean of expression data from *Musa*Cont1 and *Musa*Cont2. Segments of the original cDNA-AFLP gel images containing the TDFs are shown below the horizontal axes for comparison. W = "Williams", F = "FHIA-17", R = "Rose" and C = "Calcutta IV". 0, 6 and 72 represent hours post inoculation. Data sets were analysed using ANOVA and the Duncan's Multiple Range Test. Bars presented with the same letter are not significantly different at $P < 0.05$.


A
S-adenosylmethionine synthase (SAMS)
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72
B
Unknown Protein
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72
C
Putative Heparanase
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72
D
EF-1 alpha
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72
E
AAA ATPase, 26S Proteosome Subunit P45
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72
F
Unknown Protein
W0 W6 W72 F0 F6 F72 R0 R6 R72 C0 C6 C72

SUMMARY

Bananas (*Musa* sp.) serve as a staple diet and source of income for millions of people worldwide. The crop, however, is vulnerable to several important diseases such as Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*). During the mid-20th century, thousands of acres of export banana plantations were lost in Central America due to Fusarium wilt. The epidemic was brought under control only by replacing the highly susceptible Gros Michel banana with Cavendish cultivars. Cavendish bananas, however, are susceptible to *Foc* race 4, a highly virulent variant of the pathogen that also causes disease to banana varieties susceptible to *Foc* races 1 and 2. Only this time, no resistant replacement varieties acceptable to the commercial market exist, while cultural and/or chemical control strategies proved to be ineffective for disease control. The only sustainable solution, thus, would be the improvement of existing banana varieties that are acceptable to consumers for resistance to Fusarium wilt.

An understanding of how plants defend themselves against pathogens is an imperative first step towards the development of disease-resistant plants. Two broad defence mechanisms against pathogen attack exist in plants: Constitutive defence mechanisms that are pathogen non-specific and induced plant defence responses following recognition of specific pathogen features. A primary objective of this study was to elucidate induced banana defence responses. Induced defence responses may include the induction of regulator and antifungal proteins, the production of active-oxygen species or products from the phenylpropanoid pathway. A few studies have attempted to elucidate the genetic factors involved in the banana defence response but to date no clear answer has arisen. Forward and reverse genetics encompass approached by which plant defence responses can be studied; a particular technique of interest which can be classified under both forward and reverse genetics is cDNA-Amplified Fragment Length Polymorphism (AFLP)

In a field trial conducted in an *Foc*-infested field in Kiepersol, South Africa, cultivars Rose and Calcutta IV proved resistant, and FHIA-17 tolerant to *Foc* 'subtropical' race 4 (VCG 0120) when compared to the highly susceptible Cavendish cultivar 'Williams'. The genetic basis of defence to *Foc* in FHIA-17, Rose and Calcutta IV was verified using quantitative real-time reverse transcriptase PCR (qRT-RT-PCR).

Catalase, *POX* and *PAE* were strongly up-regulated in the tolerant and resistant banana varieties. These genes are involved in the oxidative burst and secondary metabolism leading to the phenylpropanoid pathway and cell wall strengthening. Resistance to *Foc*, 'subtropical' race 4, thus seems to depend on the early recognition of the pathogen and subsequent blocking of its progress into the plant's vascular system.

The response of resistant and tolerant banana varieties to *Foc* was elucidated by analysing the banana transcriptome 6 and 72 hours post inoculation (hpi) using cDNA-AFLP. Seventy-six differentially expressed transcript derived fragments (TDFs) were isolated, sequenced and subjected to BLASTX and BLASTN searches. Many of the sequences were not significantly similar to any other sequences in the databases, but several genes fragments showed homology to defence-related genes. TDFs representing genes such as *S-adenosylmethionine synthase* (SAMS) and *isoflavone reductase*, which are potentially involved in the production of cell wall strengthening compounds such as lignin, were identified. Expression patterns of selected TDFs as seen on the cDNA-AFLP gels were confirmed using qRT-PCR. As additional endogenous controls, two TDFs which displayed constitutive expression on the cDNA-AFLP gels, were isolated, sequenced and were optimised for use as endogenous control genes for the normalisation of the qRT-PCR data. In most cases, the expression patterns seen on the cDNA-AFLP gels were replicated by qRT-PCR. This study concludes that the tolerant (FHIA-17) and resistant (Rose and Calcutta IV) banana varieties induced defence-related genes upon attack by *Foc* and that the cDNA-AFLP technique was further effective in identifying additional defence-related genes.

Looking to the future, the greatest understanding of the defence responses induced during the banana/*Foc* interaction would be obtained by using additional molecular approaches or techniques. It is in some cases sufficient to look exclusively at transcriptomic i.e. cDNA-AFLP and microarray data to study the plant's response, however, a look at differential protein and metabolite expression would complement transcriptomic data and add insight into the fate of certain expressed genes. This would possibly speed up the identification of the defence pathways used by resistant bananas to resist infection by *Foc*, once identified these pathways can be manipulated in the susceptible plants and thus the generation of a *Foc* resistant banana could finally become a reality.