

**THE IDENTIFICATION AND CHARACTERIZATION OF RESISTANCE IN *MUSA*  
TO *FUSARIUM OXYSPOURUM* F.SP *CUBENSE* RACE 1**

by

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## SUMMARY

*Fusarium oxysporum* f. sp. *cubense* (Foc), a soil-borne fungus affecting bananas (*Musa spp.*), is considered one of the most devastating pathogens in agricultural history. The fungus infects banana roots, colonises the rhizome and pseudo stem, and causes a lethal wilting disease called Fusarium wilt. Fusarium wilt can cause losses of up to 100% in banana fields planted with susceptible genotypes, without any known cure. Host plant resistance to Foc, which has been identified in the *Musa* gene pool, is widely considered the only feasible method to control the disease. However, conventional breeding to improve susceptible banana varieties is hampered by male and female sterility and the long generation period of the crop.

The inheritance of resistance in *Musa* to Foc race 1 in the 'SN8075F<sub>2</sub>' population, derived from the cross of cultivar 'Sukali Ndiizi' and the diploid banana 'TMB2X8075-7', was investigated in this study. One hundred and sixty three F<sub>2</sub> progenies were evaluated for their response to Fusarium wilt in a screen house experiment. The test plants were inoculated by mixing loam soil with millet grains, colonized by Foc race 1, in polythene pots. One hundred and fifteen genotypes were categorized as susceptible and 48 as resistant based on rhizome discolouration. Mendelian segregation analysis for susceptible vs. resistant fitted the segregation ratio of 3:1 ( $X^2 = 1.72$ ,  $P = 0.81$ ), suggesting that resistance to Fusarium wilt in the diploid line 'TMB2X8075-7' is provided by a single recessive gene. The name *pd1* (Panama disease 1) has been proposed for the recessive gene responsible for resistance to Fusarium wilt in the diploid line 'TMB2X8075-7'.

DArT markers were identified in a segregating population following a cross between the susceptible banana cultivar 'Sukali Ndiizi' and a resistant diploid banana 'TMB2X8075-7'. The markers were in qualitative linkage disequilibrium, with 13 markers linked to resistance and 88 markers associated with susceptibility to Foc race 1. Putative functions have been assigned to candidate genes through *in-silico* database analysis including Laccase-25 (LAC25), Homeobox-leucine zipper protein (HOX32), SWIM zinc finger family protein, Transcription factor MYB3, GDSL esterase/lipase EXL3 among others. The candidate markers and genes closely associated with resistance/susceptibility could also be used in genetic engineering or for marker-assisted selection (MAS) in breeding for Fusarium wilt resistance.

The Foc race 1-banana binomial interaction of three genotypes ('Sukali Ndiizi' AAB, 'Mbwazirume' AAA and 'TMB2X8075-7' AA) was investigated by deep sequencing of the root transcriptome to study Fusarium wilt resistance in bananas. A total of 299 million raw reads, each about 100-nucleotides long, were derived from cDNA libraries constructed at four time

points: 0, 48, 96 and 192 hrs after inoculation with Foc race1. From the 10136 differentially expressed genes (DEGs), 5640 (55.7%) were uniquely up-regulated and 4496 (44.4%) uniquely down-regulated in the libraries of 'Mbwazirume', 'TMB2X28075-7' and 'Sukali Ndiizi at 48, 96 and 192 hrs post inoculation. The DEGs were annotated with Gene Ontology (GO) terms and pathway enrichment analysis, and significant pathway categories identified included the 'Metabolic', 'Ribosome', 'Plant-pathogen interaction' and 'Plant hormone signal transduction' pathways. Salicylic acid and ethylene were stimulated in the 'Plant hormone signal transduction' pathways in all the three genotypes. Fifteen defence-related genes were identified as candidate genes contributing to Fusarium wilt resistance in banana. These candidate genes could be used to improve susceptible banana genotypes to enhance levels of fungal disease resistance to Foc race 1.

## OPSOMMING

*Fusarium oxysporum* f. sp. *cubense*, 'n grondgedraagde swam wat piesangs (*Musa spp.*) affekteer, word beskou as een van die mees vernietigende siektes in die geskiedenis van landbou. Die swam infekteer piesangwortels, koloniseer die rhizoom en pseudostam, en veroorsaak 'n dodelike verwelksiekte, genoemd Fusarium verwelk. Fusarium verwelksiekte kan verliese van tot 100% veroorsaak in plantasies wat met vatbare genotipes geplant is, sonder enige kuur. Gasheerplantweerstand teen Foc, wat in die *Musa* genepoel beskikbaar, word lank reeds beskou as die enigste haalbare manier om die siekte te beheer. Maar konvensionele teling word belemmer deur manlike en vroulike onvrugbaarheid en die lang generasie tydperk van die gewas.

Die erfenis van weerstand in *Musa* teenoor Foc ras 1 in 'SN8075F2, 'n afstammeling van die kruis tussen kultivar 'Sukali Ndiizi' en die diploïede piesang 'TMB2X8075-7' word in hierdie studie ondersoek. Een honderd en sestig F<sub>2</sub> nasate is vir hul reaksie op Fusarium verwelking in 'n glashuis eksperiment geëvalueer. Die plante is geïnkuleer deur leemgrond te meng met millet saad wat deur Foc ras 1 gekoloniseer is, en in plastiek potte geplant is. Een honderd en vyftien (115) genotipes was vatbaar, en 48 bestand ten opsigte van die verkleuring van hul rhizoom. Mendeliese segregasie analise vir vatbaar teen bestand pas die segregasie verhouding van 3: 1 ( $X^2 = 1,72$ ,  $P = 0,81$ ), wat daarop dui dat die weerstand teen Fusarium verwelking in diploïede lyn 'TMB2X8075-7' deur 'n enkele resessiewe geen bepaal word. Die naam *pd1* (Panama siekte 1) is voorgestel vir die resessiewe geen wat weerstand teen Fusarium verwelking in die diploïede lyn 'TMB2X8075-7' verskaf.

DARt merkers is geïdentifiseer in 'n segregerende populasie na 'n kruis tussen 'Sukali Ndiizi' en 'n weerstandige diploid piesang 'TMB2X8075-7'. Die merkers was onewewigtig in kwalitatiewe koppeling, met 13 merkers wat gekoppel was aan weerstand en 88 merkers aan vatbaarheid vir Foc ras 1. Funksies aan kandidaatgene toegeken deur in-silico databasis analise sluit in Laccase-25 (LAC25), Homeobox-leucine zipper proteïen (HOX32), SWIM zinc 'finger family protein', 'Transcription factor MYB3', GDGL esterase/lipase EXL3. Hierdie kandidaat merkers en gene wat nou verband hou met weerstand/vatbaarheid kan ook in die genetiese modifikasie van piesangs, of vir merker-geassosieerde seleksie (MAS) vir die teling vir Fusarium verwelking weerstand gebruik word.

Die Foc ras 1-piesang binomiaal interaksie van drie genotipes ('Sukali Ndiizi' AAB, 'Mbwazirume' AAA en 'TMB2X8075-7' AA) was ondersoek deur analise van hul wortel transkriptoom. 'n Totaal van 299 miljoen basispare, wat elkeen bestaan uit sowat 100 basispare, is bepaal tydens vier tydspunte: 0, 48, 96 en 192 ure na inokulasie. Van die

10136 gene differensieel uitgedrukte gene (DEGs) was 5640 (55,7%) uniek uitgedruk en 4496 (44,4%) uniek onderdruk in 'Mbwazirume', 'TMB2X28075-7' en 'Sukali Ndiizi' teen 48, 96 en 192 uur na inokulasie. Die DEGs is met Gene Ontologie (GO) terme en pad verryking analise geannoteer. Die beduidende geenkategorieë wat geïdentifiseer is het die volgende ingesluit: 'Metaboliese', 'Ribosoom', 'Plant-patogeen interaksie' en 'Plant hormoon seintransduksie'. Salisiensuur en etileen is gestimuleer in die 'Plant hormoon seintransduksie' bane in al die drie genotipes. Vyftien verdediging-verwante gene is geïdentifiseer as kandidate wat bydra tot weerstand teen Fusarium verwelking in piesangs. Hierdie kandidaatgene kan gebruik word om vatbaar genotipes te verbeter vir verhoogde weerstand teen Foc ras 1.

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## CHAPTER 1

### **The defence response of bananas to *Fusarium oxysporum* f. sp. *cubense*: Implications for banana improvement**

#### **INTRODUCTION**

Banana (*Musa* sp.) is the eighth most important global food commodity after maize, wheat, rice, potato, cassava, soybean and barley (FAOSTAT, 2013). It is grown in more than 100 countries, with an annual production of around 150 million metric tonnes. The fruit is nutritious and contains high levels of potassium, vitamin C and vitamin B6 (Samson, 1986; Robinson, 1996). Bananas are cheap to produce, can grow in a range of environments and produce fruit all year-round. They are consumed either as a staple food (cooking banana), beverage or dietary supplement (dessert banana) (Jones, 2000). Cooking bananas are peeled and cooked into a dish, while dessert banana are ripened and eaten raw (Robinson, 1996). Some banana types can be used to brew alcoholic drinks (Jones, 2000). In some countries, especially in Latin America (Ecuador, Costa Rica, Colombia and Panama), the Caribbean and Asia, bananas are a major export commodity. Currently, all banana export cultivars are selections from somatic mutants of the group Cavendish. The banana export industry constitutes about 15% of global production and is valued at about US\$10 billion (FAOSTAT, 2013). The remaining 85% of bananas are grown in the developing world, and are used as food and beverages for domestic consumption in both rural and urban areas. The banana crop is a very important staple food crop and source of income for over 400 million people in the tropics (FAOSTAT, 2013).

A number of pests and diseases threaten the international banana industry (Robinson, 1996). The burrowing nematode (*Radopholus similis* Cobb) and the banana weevil (*Cosmopolitus sordidus* Germar) are the most destructive banana pests (Gowen, 1995; Sarah, 2000). Common diseases of bananas include Fusarium wilt (caused by *Fusarium oxysporum* f. sp. *cubense* (Smith) Snyder and Hans.), black Sigatoka (caused by *Mycosphaerella fijiensis* Morelet), yellow Sigatoka (caused by *Mycosphaerella musae* (Speg.) Syd. and Syd), banana bunchy top disease (caused by the *banana bunchy top virus* (BBTV)), banana mosaic disease (caused by the *cucumber mosaic virus* (CMV)), anthracnose (caused by *Colletotrichum musae* (Berk. and Curtis) Arx.), banana bacterial wilt (caused by *Xanthomonas vasicola* pv. *musacearum* (Yirg. and Brad.) Aritua), Moko disease (caused by *Ralstoniasolanacearum* (Smith) Yabuuchi) and rhizome rot (caused by *Erwinia*

*carotovora* Winslow) (Carlier *et al.*, 2000; Ploetz and Pegg, 2000; Thawites *et al.*, 2000; Thomas *et al.*, 2000; Aritua *et al.*, 2009).

Fusarium wilt, which is also known as Panama disease, is one of the most devastating diseases of banana globally (Moore *et al.*, 2001). The disease became notorious when it destroyed thousands of hectares of 'Gros Michel' bananas in Central America during the 1900s (Stover, 1962). Numerous control strategies have been devised to prevent damage caused by Fusarium wilt of bananas. Crop rotation, flood following, chemical fumigation and the use of organic amendments were unsuccessful in controlling the disease effectively (Herbert and Marx, 1990; Moore *et al.*, 1995). *Fusarium oxysporum* f.sp. *cubense* (Foc) survives in organic matter and in the soil as dormant chlamydospores in the absence of a suitable host. This survival has made attempts to apply cultural or chemical control options futile. Therefore, host plant resistance remains the most effective, economical and environmentally friendly approach to control Fusarium wilt of bananas (Moore *et al.*, 1999).

Plants respond to pathogen attack to either hinder it completely (resistant plants) or to minimize its effect (tolerant plants), but sometimes the pathogen succeeds, leading to disease (susceptible plants) (Swarupa *et al.*, 2014). Developing cultivated bananas with resistance to the major diseases and pests is one of the greatest challenges in sustainable banana production (Becker *et al.*, 2000). The development of such bananas can be achieved by means of conventional cross breeding and biotechnology-facilitated improvement (Ortiz and Swennen, 2014). Some improvement methods, such as a long-term breeding program, have many limitations due to sterility of cultivated bananas, long growth cycles, low seed set and hybrids that are often not accepted by local consumers (Crouch *et al.*, 1998; Sági, 2000). Improving disease resistance is vital for the future survival of bananas (Pearce, 2003). This review, therefore, deals with host factors responsible for resistance to Foc in bananas and the genetic tools available for improving banana cultivars for Foc resistance.

## **THE ORIGIN, DOMESTICATION AND GLOBAL SPREAD OF BANANAS**

Bananas belong to the genus *Musa* L., family *Musaceae* Juss. and order *Zingiberales* Griseb. *Musa* comprises of five sections, divided into 40 species. Eumusa is the largest and best known section and includes two wild seed-forming species, *Musa acuminata* Colla and *Musa balbisiana* Colla, which are the principal progenitors of most edible banana cultivars (Simmonds, 1959; Stover, 1962; Waite, 1963). They are believed to have originated from Southeast Asia and Indochina, where the earliest domestication of bananas is believed to have happened around 8000 Before Common Era (BCE) (Simmonds, 1962). From there

bananas were introduced to the tropical and subtropical regions of the world where the crop has gained great importance.

The number of banana varieties grown globally is estimated to be more than 1000. Humans have been responsible for moving vegetative banana planting material (suckers) outside Asia and around the world. East African Highland bananas (EAHB) and plantains were introduced into Africa from Asia around 2500-3000 years ago. These have then further evolved on the African continent through somatic mutations to increase in number and diversity. They are said to be endemic to the regions where they are found (Shepherd, 1957; Simmonds, 1959). Infact, the eastern African highlands are considered a secondary centre of banana diversity (Karamura, 1996, Stover, 1962).

The origin of dessert bananas introduced into Africa is believed to be India around the 1400s. They were then spread across the African continent from east to west (Simmonds, 1959; Robinson, 1996). The Portuguese carried the plant to the Canary Islands sometime after 1402 and from there to the New World (Simmonds, 1959). Dessert and cooking varieties were introduced into the Americas from Southeast Asia before 1750 (Wardlaw, 1961). Gros Michel was first introduced into Panama before 1866, and with the expanding export industry at the time, was distributed throughout the entire Central America (Stover, 1962). The Silk (ABB) variety was introduced into Australia before 1876 and the Gros Michel cultivar was introduced only around 1910 (Stover, 1962). This set the stage for the cultivation of bananas as a dessert and as a staple crop around the world. Edible bananas are now cultivated in many tropical and subtropical regions of the world, including, Asia, Africa, South and Central America, Oceania and the Caribbean.

## **THE BANANA CROPPING SYSTEM IN EAST AFRICA**

Banana is the main staple food in the Great Lakes region of eastern Africa. They are cultivated primarily for their fruit that is used for food, juice, brew and household incomes. As an important cash crop in the regional economy, banana trade is worth US\$ 4.3 billion, which is about 5% of the East African Community's Gross Domestic Product (GDP) (EAC, 2012; FAOSTAT, 2013). It forms an indispensable part of life in the region, with the annual per capita consumption over 200 kg, the highest in the world. When grown under perennial production systems bananas produce fruit all year-round, thus bridging the 'hunger-gap' between crop harvests. Bananas also maintain soil cover throughout the year and their biomass is used for mulch and soil fertility conservation. In mixed farming systems bananas are used as ground shade and a nurse-crop for a range of shade-loving crops, including cocoa, coffee and black pepper (Sharrock and Frison, 1999).

The East African region produces half of Africa's banana crop, thereby providing a staple food and a source of income to more than 50 million people (Kalyebara *et al.*, 2006). It is the largest banana producing and consuming region in Africa, where more than 80 cultivated varieties of locally evolved bananas are grown by smallholder farmers. Uganda is the leading producer and consumer of banana in the region, and also enjoys the highest diversity of a group of bananas uniquely adapted to this region, called East African Highland bananas (EAHB). In Uganda, 75% of families grow bananas on 1.5 million hectares, which accounts for over 38% of utilised arable land.

A typical banana plantation in Uganda will constitute about 12 varieties (Karamura *et al.*, 1996). Such high diversity is attributed to a variety of end uses, better food security and the perception that each cultivar has a unique range of strengths and weaknesses. The most important cultivar group is the EAHB, which comprise both cooking and brewing types. The former is a staple for more than 17 million people, and thus important for food security. In Uganda, EAHB (AAA-EA) represent 76% of total production, while Kayinja ('Pisang Awak' subgroup ABB) contributes 8%; Sukali Ndiizi (AAB) 7%; Kisubi ('Ney Poovan' subgroup AB) 5%; Gros Michel ('Bogoya' AAA) 2%; and plantain (AAB) 2% of bananas cultivated (Gold *et al.*, 2002).

A combination of abiotic and biotic stresses constrains banana production in the Great Lakes region. Their impact, however, varies in relative importance across regions (countries). The major abiotic stresses include nutrient deficiencies and moisture limitations (drought stress). Banana production systems are prone to nutrient deficiencies because potassium (K) and nitrogen (N) are lost off the farm in bunches that are harvested and sold to distant markets over time (Taulya, 2015). Bananas require 25 mm of rainfall per week for satisfactory growth, which corresponds to 1 300 mm per annum (Purseglove, 1988). However, most of the banana growing regions in eastern Africa receive between 1 000 and 1 300 mm per annum. Irrigation is not practiced, implying that moisture stress affects the yields. Banana production in East Africa suffers from many biotic stresses, the most important being Fusarium wilt, bacterial wilt, nematodes, weevil, black Sigatoka and banana bunchy top disease (BBTD) (Tushemereirwe *et al.*, 2003; Swennen *et al.*, 2013). The devastating effects of these pests and diseases pose a great threat to the sustainability of banana production in the region (Edmeades *et al.*, 2007). Fusarium wilt is a major threat to many bananas types commonly grown by smallholders in eastern Africa, such as the dessert bananas 'Gros Michel' and 'Sukali Ndiizi' and the beer banana "Pisang Awak" (AAB) (Okech *et al.*, 2005; Bouwmeester *et al.*, 2009).

## BANANA FUSARIUM WILT

### The pathogen

The genus *Fusarium* comprises several fungal species, including species pathogenic and non-pathogenic to agricultural crops. One of the most notorious is *F. oxysporum*, known to cause vascular wilts and root rots in more than 100 species of plants (Agrios, 2005). Pathogenic isolates of *F. oxysporum* have been classified in more than 100 *forma speciales*. Members of a *forma specialis* normally cause disease in a particular range of host species, with a few *formae speciales* able to colonise a broader range of plants (Okubara and Paulitz, 2005). A *forma specialis* can be further subdivided into races based on characteristic virulence patterns on differential host cultivars (Gordon and Martyn, 1997). One of the most devastating *formae speciales* of *F. oxysporum* is the soil-borne fungus responsible for Fusarium wilt of bananas, called Foc (Stover, 1962). Foc infects bananas through the roots, colonizes the vascular system of the plant and blocks the flow of water to the leaves (Beckman, 1989). Fusarium wilt can be devastating, with losses as high as 100% in susceptible cultivars (Thangavelu *et al.*, 2001). The disease became notorious when it destroyed thousands of hectares of 'Gros Michel' bananas in Central America during the 1900s (Stover, 1962).

Three physiological Foc races (races 1, 2 and 4) have been recognised. Foc race 1 causes disease in 'Gros Michel' (AAA), 'Sukali Ndizi' (AAB), 'Kisubi' (AB) and 'Pisang Awak' (ABB) cultivars, and Foc race 2 affects Bluggoe (ABB) bananas. Foc race 4 attacks Cavendish bananas and all the cultivars susceptible to Foc races 1 and 2 (Moore *et al.*, 1995). Foc is further subdivided into 24 vegetative compatibility groups (VCGs). Of these, only Foc race 1 and VCGs 0124, 0124/125, 01212 and 0122 have been reported in Uganda (Kangire *et al.*, 2001). Foc race 1 does not affect EAHB (Kangire *et al.*, 2001).

### Life cycle

*Fusarium oxysporum* has both a saprophytic and a parasitic phase in its life cycle. The life cycle starts saprophytically in the soil as chlamydospores, which are dormant and immobile until they are stimulated to germinate by exudates from extending banana roots (Stover, 1962; Beckman and Roberts, 1995). These germinating chlamydospores develop a thallus that produces conidia after 6-8 hrs. The conidia germinate and attach to the roots of the host plant where they penetrate the epidermal cells and later enter the vascular system (Stover, 1970; Beckman and Roberts, 1995). As the fungus progresses, it obstructs the vascular system. The obstruction is caused by a combination of accumulated fungal mycelium and conidia in the vascular tissue, host defence responses like the production of gels, gums and tyloses, and vessels crushing by proliferation of adjacent parenchyma cells (Beckman,

1987). Foc then grows out of the xylem tissue and into the neighbouring parenchyma cells, where they produce vast quantities of conidia and chlamyospores. Chlamyospores are formed in either the hyphae found in infected and decaying host tissue, or in macroconidia in the soil (Nash *et al.*, 1961; Christou and Snyder, 1962). Chlamyospore formation and germination depends on nutrients available in the soil (Schippers and van Eck, 1981). Hsu and Lockwood (1973) concluded that an environment deficient in energy, but with an appropriate weak salt solution, is required for chlamyospore formation. This implies that low nutrient levels in soils favour the formation of chlamyospores while the release of nutrients during the decay of plant debris and roots stimulate germination.

The process of infection by Foc starts with the adhesion of the microconidia to the root hairs and epidermal cell surfaces of the host plant's root (Bishop and Cooper, 1983; Li *et al.*, 2011; Yin *et al.*, 2011). The microconidium attached to the root surface germinates into an infection hypha which invades the younger roots where cell division is very active, and forms germ tubes which penetrate the roots either directly through the cell wall or indirectly through wounds (Lucas, 1998). Foc has been found to penetrate the root cap and zone of elongation intercellularly in the root of banana (Li *et al.*, 2011). Mechanical wounding increases infection of Foc, but it is not essential for root infection to occur (Stover, 1962). Once inside the cells, fungal growth proceeds rapidly to produce a network of branching hyphae which expand by growing in the intercellular spaces along the junctions of root epidermal cells. The swollen hyphae enter epidermal cells by constricting in size when passing from one cell to another, resuming their original diameter upon gaining entry to the new cell (Li *et al.*, 2011). From inside cells Foc colonizes neighbouring cells through pores in cell end plates (Beckman *et al.*, 1961; Beckman *et al.*, 1962; Bishop and Cooper 1983; Li *et al.*, 2011).

### **The symptoms**

Banana plants infected by Foc develop characteristic symptoms both externally and internally (Wardlaw, 1961; Stover, 1962). The most prominent internal symptom is vascular browning of the rhizome and pseudo stem (Fig. 1) (MacHardy and Beckman, 1981). The external symptoms include premature yellowing of the older leaves, starting along the leaf margins and continue to the midrib until the leaves are completely brown and die. The yellowing progresses from the older leaves to the younger leaves and appears to be a result of severe water stress. Sometimes disease symptoms become visible only after the bunch has started to form and the plant is under stress (Brandes, 1919). Splitting of the pseudo stem just above the soil level may also occur. Eventually all the leaves die and the pseudo stem remains standing until it is removed or collapses (Brandes, 1919; Wardlaw, 1961;



Stover, 1962). Bananas also get stunted, less productive and even die when the infection is severe (MacHardy and Beckman, 1981).

### **Control**

Since the discovery of Fusarium wilt of banana, various control strategies have been devised to prevent the damage caused by the disease (Table 1). These strategies concentrate on restricting the introduction of the disease, early detection of the disease, effective quarantine and sanitation methods, lowering the amount of inoculum in a field through cultural, biological and chemical control, while enhancing plant vigour and disease tolerance. The use of cultural control measures like crop rotation provides control of many diseases, but not Fusarium wilt, as chlamydospores stay viable in the soil for extensive periods (Hwang, 1985; Su *et al.*, 1986). Chemical treatments, such as soil fumigation, have economic and environmental implications and can lead to the killing of beneficial microorganisms. Fumigation has reduced the levels of Foc in infested soils, but has not been able to eradicate it (Herbert and Marx, 1990). Biological control and Fusarium wilt suppressive soils have been receiving attention for many years, and can potentially form part of an integrated disease management program for Fusarium wilt diseases (Ploetz *et al.*, 2003). The rationale for biological control is premised on antagonistic microbes like mycoparasitic species of *Trichoderma* and *Gliocladium* spp., and the use of non-pathogenic isolates of *F. oxysporum* and arbuscular mycorrhizal fungi to induce host resistance against Foc (Nel *et al.*, 2006; Thangavelu and Mustafa, 2010; Akila *et al.*, 2011).

### **RESISTANCE IN BANANA TO FUSARIUM WILT**

Plants respond to pathogen attack either to hinder it completely (resistant plants) or to minimize its effect (tolerant plants). Sometimes the pathogen succeeds in infecting plants, leading to disease (susceptible plants) (Swarupa *et al.*, 2014). Resistance and susceptibility in plant-pathogen systems depends on the constitutive and induced defence functions of the host. Host plants have developed an innate defence system against pathogens and, in turn, pathogens have evolved strategies to suppress the plant defence system.

#### **Pathogen detection**

Recognition of a potential invader (pathogens or non-pathogens) is a requirement for an efficient defence response. Generally, the plant cell surface has pattern-recognition receptors (PRR) that detect the pathogen, called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs). This detection of the pathogen then initiates basal resistance or PAMP-triggered immunity (PTI) in both non-host and host plants (Gomez-Gomez *et al.*,

2001; Zipfel *et al.*, 2004; Zipfel *et al.*, 2006). PTI initiates several intracellular responses associated with plant defence, including changes in Ca<sup>2+</sup> flux, reactive oxygen species (ROS) and phytoalexin production, mitogen-activated protein kinase cascades, plant cell wall reinforcement at infection sites, and stomatal closure. However, if this first barrier is broken by the invading pathogen, the plant's resistance (R) genes can recognise invasion from the effectors (*Avr*) of the pathogen inside the cell to activate effector-triggered immunity (ETI) (Hammond-Kosack and Parker, 2003; Dangl and McDowell, 2006).

ETI is generally similar to PTI, but it is more specific and faster than PTI (Jones and Dangl, 2006). ETI involves defence signalling events, the expression of pathogenesis-related (*PR*) genes, systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants (Flor, 1971; Dong, 1998; Durrant and Dong, 2004). Regardless of how pathogens are detected (through effectors or PAMPs), the plant's defence system is regulated by several hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxins, gibberellins, abscisic acid (ABA), cytokinins, brassinosteroids, and peptide hormones (Bari and Jones, 2009).

SA, JA and ET are the main known molecules signalling the activation of defence genes (Thomma *et al.*, 2001). The role of SA is well-known in mediating local defence against biotrophic and hemi-biotrophic pathogens and in the establishment of systemic acquired resistance (SAR), whereas JA/ET is mainly associated with necrotrophic pathogens (Pieterse *et al.*, 2012; Fu and Dong, 2013). SA accumulation in response to pathogen detection triggers the release of non-expressor of pathogenesis related proteins 1 (*npr1*) monomers, which then translocate to the nucleus and activate the expression of *PR* genes (Zhang *et al.*, 2003; Fu and Dong, 2013).

Jasmonic acid accumulation in response to pathogen detection is perceived by a co-receptor complex consisting of the F-box protein Coronatine insensitive 1 (COI1) and the Jasmonate ZIM domain (JAZ) family of transcription repressors (Sheard *et al.*, 2010). An increasing concentration of JA promotes physical interaction between COI1 and JAZ proteins, which leads to ubiquitination and subsequent degradation of JAZs through the 26S proteasome, thereby relieving the repression on MYC transcription factors and initiating the expression of JA-responsive genes that encode PR proteins, including Plant Defensin1.2 (PDF1.2), Thionin2.1 (THI2.1), Hevein-like protein (HEL) and chitinaseB (CHIB) (Reymond and Farmer, 1998; Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008). Stimulation of the biosynthesis of ET during pathogen infection signals ET-responsive transcription factors that regulate ET-responsive genes encoding class I basic chitinases, class I  $\beta$ -1,3-glucanase and other basic-type PR proteins (Ohme-Takagi *et al.*, 2000). However, many stress responses in plants require the coordinated interaction of the JA, ET and SA signalling pathways (Lorenzo and Solano, 2005).

The understanding of the defence mechanism of bananas against Foc is very important for the control of Fusarium wilt of banana. Through transcriptome profile analysis Li *et al.* (2012) revealed that genes involved in the recognition of PAMPs and ETI, like the chitin elicitor-binding protein (CEBiP), chitin elicitor receptor, elicitor-responsive proteins (ERG), proline-rich extensin-like receptor kinases (PERKs), BRI1-associated receptor kinase 1 (BAK1), flagellin-sensing2-like genes (FLS2), somatic embryogenesis receptor-like kinases (SERKs), plant receptor-like kinases (RLKs) and mitogen-activated protein kinase (MAPK) CC-NB-LRR protein (RPM1), disease resistance protein (RPS2) and RPM1 interacting protein 4 (RIN4) are differentially expressed in the interaction of Foc TR4 with the resistant banana genotype 'Nongke No 1' and susceptible wild type 'Brazilian'.

The role of SA, JA and ET in activating defence genes against Foc in bananas is still contentious. Transcriptome profile analysis by Wang *et al.* (2012) reported up-regulation of JA biosynthetic-related genes by Foc TR4, while Li *et al.* (2013) instead reported suppression of allene oxide synthase gene, a JA biosynthetic-related gene. Similarly, Wang *et al.* (2012) did not find induction of any ET biosynthetic or signalling pathway genes, whereas Li *et al.* (2013) showed induction of *EIN3* by Foc TR4. The inconsistency could be attributed to the genetic background of the genotypes studied, and may require further analysis of the functions of genes related to the JA, ET and SA in *Musa* sp.

### **Structural defence**

The plant surface constitutes the first line of defence that pathogens must penetrate before they can cause infection (Swain, 1977; Agrios, 2005). Therefore, resistance to penetration of epidermal cells by pathogens is an important component of defence reactions (McDowell and Dangl, 2000). Structural defences are often present in the plant even before the pathogen comes in contact with the plant, and include barriers such as cell walls strengthened by lignins (Wuyts *et al.*, 2013). Structural defences of host plants can also be triggered by both pathogens and non-pathogens (Dangl and McDowell, 2006). During induced structural plant defence, plant cell walls are fortified at the sites of penetration, a phenomenon known as the cell wall apposition (CWA) (Hardham *et al.*, 2007).

Foc can also be localised in banana roots by gels and gums which trap conidia in the vessel elements (Beckman *et al.*, 1962). In some bananas the gels persist long enough to form tyloses (occlusions in xylem vessels) which contain the pathogen (Beckman *et al.*, 1962). Tyloses are considered to be a resistance factor against the attack of Foc in resistant banana cultivars due to an inhibition of the upward spread of the fungus (Beckman, 1987; 1990; 2000). Tylose formation has been successfully found 2 days after inoculation of a resistant banana cultivar with Foc (Vander-Molen *et al.*, 1987). If the gels are short-lived, tylose formation is delayed or is not formed at all, thereby allowing conidia to spread ahead

of the vascular occlusion (Beckman, 1964). Bananas have also been reported to respond to invasion by *F. oxysporum* by enlarging their cells (hypertrophy) or rapidly multiplying their cells (hyperplasy) (Wardlaw, 1930; Chambers and Corden 1963; Pennypacker and Nelson, 1972).

The early up-regulation of cell wall strengthening genes like pectin acetyl esterase (PAE) and peroxidase-related genes were observed in the roots of the Fusarium wilt-tolerant banana genotype (GCTCV-218) when compared to the susceptible genotype Williams in the *Musa-Foc* race 4 interaction (Van den Berg *et al.*, 2007). PAE hydrolyses acetyl esters in the homogalacturonan regions of pectin, thereby modifying cell walls during pathogen interactions (Savary *et al.*, 2003). Peroxidases are involved in many physiological processes in plants, such as plant response to biotic and abiotic stresses and the biosynthesis of lignin. They are involved in the polymerization of the precursors of lignin (Pegg, 1985; Beckman, 1987). High constitutive levels (10X) of peroxidase have been reported in the Foc-resistant banana hybrid SH-3362 (AA) in comparison to the susceptible diploid cultivar Pisang Mas (AA) (Novak, 1992).

### **Biochemical defence**

Plants have the ability to synthesize a large number of biochemical substances, most of which are phenols or their oxygen-substituted derivatives (Cowan, 1999). In many instances these substances serve as plant defence mechanisms against predation by insects, herbivores and microorganisms (Beckman, 2000). Phenolics can occur constitutively and function as preformed inhibitors of a pathogen (phytoanticipins) or can be produced in response to infection by the pathogen (phytoalexins). For instance, various studies have reported the phenolic content of Fusarium- and nematode-resistant bananas to be significantly higher *vis-a-vis* susceptible ones (Fogain and Gowen, 1996; Holcher *et al.*, 2014).

In the interaction of bananas with Foc TR4, transcripts of 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS), 4-coumarate:CoA ligase (4CL), polyphenol oxidase (PPO), glutathione S-transferase (GST), UDP-glucuronic acid decarboxylase and cellulose synthase genes; which act at different stages of the Shikimate-phenylpropanoid-lignin and cellulose biosynthesis pathways; were reported to be up-regulated in the compatible but not in the incompatible interaction (Li *et al.*, 2012). This suggests that the pathogen fails to overcome the basal defence mechanism of the resistant genotype to induce responses in the Shikimate-phenylpropanoid-lignin and cellulose biosynthesis pathways. In response to Foc TR4, four proteins involved in the phenylpropanoid pathway; *viz* caffeoyl-CoA O-methyltransferase (CCOMT), isoflavone reductase (IFR) and leucoanthocyanidin dioxygenase (LDOX) and S-adenosylmethionine synthase (SAM); were

up-regulated in the roots of the resistant and moderately resistant banana varieties 'Yueyoukang 1' and 'Nongke no 1', respectively, but not in the Foc TR4-susceptible 'Brazil', which implies that phytoalexins and lignification of the cell wall are important in protecting bananas against Fusarium wilt (Li *et al.*, 2013b).

A common response in plants to pathogen attack is the production of *PR* proteins, many of which have antimicrobial activity (Kitajima and Sato, 1999; Van Loon and Van Strien, 1999). For example, two *PR* proteins (*PR*-1 and *PR*3) were induced in both the resistant 'Yueyoukang 1' and susceptible 'Brazilian' bananas after pathogen infection, whereas only *PR*-1 was induced in the moderately tolerant variety 'Nongke no 1' (Li *et al.*, 2013b). Contrary to these findings, Li *et al.* (2013a) reported that no significant change occurred in the transcript level of *PR*1-like gene in Cavendish bananas following Foc race 1 and Foc TR4 infection. *PR*1 is a well-known SA-responsive gene in plants. Two *PR*5-like (thaumatin-like) genes and a *PR*4-like (endochitinase) gene were found to be up-regulated by both Foc race 1 and Foc TR4 (Li *et al.*, 2013a) in Cavendish bananas. Thaumatin-like proteins have been shown to have antifungal activity against a broad spectrum of fungal pathogens (Hajgaard *et al.*, 1991; Vigers *et al.*, 1991; Huynh *et al.*, 1992; Malehorn *et al.*, 1994; Abad *et al.*, 1996) Furthermore, the expression of a *PR*5-like (thaumatin-like) rice genes (TLP) have been reported to enhance resistance to Foc TR4 in banana (Mahdavi *et al.*, 2012).

The generation of ROS is a nearly ubiquitous response to abiotic and biotic stresses in plants. ROS, which include hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion radical (O<sub>2</sub><sup>-</sup>) and the hydroxyl radical (-OH), defend plants from pathogens by acting as antimicrobial agents, mediating the oxidative cross-linking of cell walls and acting as signalling molecules to induce defence genes and the hypersensitive response (HR) (Custers *et al.*, 2004). In the banana-Foc pathosystem, one ROS-producing Germin-like protein 12-1 (GLP) has been documented (Li *et al.*, 2013b). However, the high levels of ROS can lead to severe oxidative destruction of cell structures such as nucleic acids, proteins and lipids. Subsequently, ROS should be detoxified efficiently by ROS-scavenging systems. In the banana-Foc interaction, four antioxidant proteins; viz IN2-1, L-ascorbate peroxidase (APX), glutathione S-transferase (GSTF) and superoxide dismutase [Mn] (SOD); were activated to scavenge ROS. These enzymes maintain ROS homeostasis in different compartments of the plant cell and can be used as a biochemical marker for Foc resistance in bananas (Mittler *et al.*, 2004; Kavino *et al.*, 2007; Li *et al.*, 2011).

Fungal pathogens secrete a mixture of hydrolytic enzymes, toxins and plant hormone-like compounds to penetrate and manipulate the plant's complex defence system (Knogge, 1996). Plants, in turn, produce inhibitors to suppress these enzymes (Collmer and

Keen, 1986). In the banana-Foc pathosystem, a polygalacturonase inhibitor protein (PGIP) inhibits plant cell wall-degrading enzymes secreted by the fungus (Ravishankar *et al.*, 2011).

### **Genetics of resistance**

Resistance to *F. oxysporum* in crop plants appears to be a genetically complex trait. Depending on the host-*F.oxysporum* combination, resistance can be controlled by one gene (monogenic), by a few genes (oligogenic) or by multiple genes (multigenic) (Berrocal-Lobo and Molina, 2007). In many instances the interactions between *Fusarium* sp. and its plant host are controlled by the action of race-specific R-gene-mediated gene-for-gene type interactions (Hammond-Kosack and Jones, 1997). In eggplant (*Solanum melongena* L.), monogenic inheritance of resistance to Fusarium wilt, caused by *F. oxysporum* f. sp. *melongenae*, has been confirmed (Mutlu *et al.*, 2008). However, the gene-for-gene interaction in Fusarium wilt resistance can be complicated when pathogenic isolates secrete more than one Avr protein. In such cases Fusarium wilt resistance will be specified by multiple loci of resistance genes, like the *I*-genes in tomato (*Lycopersicon esculentum* Mill.) (Sela-Buurlage *et al.*, 2001). The gene-for-gene interaction in Fusarium wilt resistance can be complicated further by the ability for certain Avr-proteins to suppress the action of some resistance (*R*) genes in the pathogen-host interaction, like it has been described in tomato. In tomato, pathogenic isolates of *F. oxysporum* f. sp. *lycopersicum* secrete the effector protein *avr1* which induces the resistance gene *I-1*, but suppresses the action of the resistance genes *I-2* and *I-3* (Houterman *et al.*, 2008).

In melon, the Fusarium wilt resistance locus *FOM-2* has been reported to contain a single *R*-gene with complex features, indicating that the gene belongs to a tightly linked family of highly homologous genes (Joobeur *et al.*, 2004). In *Arabidopsis thaliana* (L.) Heynh, six dominant resistance loci (*RFO*) to *F. oxysporum* f. sp. *matthiolea* were identified. Interestingly *RFO1*, which was the largest contributor to resistance, also confers resistance to other *formae speciales* of *F. oxysporum*, suggesting that *RFO1* is not race-specific (Diener and Ausubel, 2005). *RFO1* encodes the cell wall-associated kinase-like 22, which belongs to the RLK protein family.

In bananas, Fusarium wilt resistance has been reported to be controlled by a single dominant gene in the resistant diploid banana Pisang Lilin (Vakili, 1965). R genes in bananas have been extensively studied using gene homologues (Miller *et al.*, 2008; Peraza-Echeverria *et al.*, 2008). However, the full genome sequencing of *Musa* has revealed that defence-related genes, encoding nucleotide-binding site leucine-rich repeat (LRR) proteins, are less represented in banana (89 genes) compared to rice (*Oryza sativa* L.) (464 genes) and grapevine (*Vitis vinifera* L.) (459 genes) (D'Hont *et al.*, 2012). The variation in response to Fusarium wilt diseases in bananas has been attributed to the rate and extent of

recognition and activation of the defence mechanisms (Beckman, 1987; Van den Berg *et al.*, 2007). Identifying the parts of the genome that contribute to the variation in such a complex trait and ultimately the genes and alleles responsible for trait variation remains a challenge for banana breeders.

## **RESISTANCE BREEDING OF BANANA**

Resistance breeding describes methods for the creation, selection and fixation of resistance against biotic constraints into superior plant phenotypes, which are suited to needs of farmers and consumers. The wide diversity of existing *Musa* germplasm can be a source for pests and disease resistance, abiotic stress resistance, and altered agronomic performance; thus a valuable resource for the improvement of the crop. The challenge is to identify and characterize the relevant genes and genetic diversity, and then to utilize this variation to improve the largely sterile and vegetatively propagated crop. Several breeding techniques have been deployed in banana improvement, including selecting somaclonal variants, induced mutation breeding, protoplast fusion, genetic engineering and conventional cross breeding (Chen *et al.*, 2011).

### **Somaclonal variation**

*In vitro* mutagenesis can lead to variability in banana clones that are generated from a single mother plant. This process, called somaclonal variation, can be the result of nuclear chromosomal re-arrangement, gene amplification, non-reciprocal mitotic recombination, transposable element activation, point mutations and reactivation of silent genes (Jain, 2001). Somaclones in banana are induced as the number of *in vitro* multiplication cycles are increased (Sahijram *et al.*, 2003). Once the number of multiplication frequency in Cavendish bananas by means of shoot tip culture exceeds 12 cycles, the number of somaclonal variants increase substantially (Ko *et al.*, 1991). Researchers at the Taiwan Banana Research Institute (TBRI) were able to select Cavendish banana clones with resistance to Foc race 4 from somaclonal variants. These include Foc tolerant clone (GCTCV-215-1) and resistant clone (GCTCV-218), both derived from Giant Cavendish (Hwang and Ko, 2004). In a similar study, three somaclones tolerant to Foc race 1 (IBP 5-61, IBP 5-B and IBP 12) were obtained from Gros Michel in Cuba (Bermúdez *et al.*, 2002).

### **Induced mutations**

Mutation breeding is the use of mutagens to develop variants that increase agricultural traits. Mutations are alterations in the nucleotide sequence of a DNA molecule and can be induced by irradiation or chemicals such as ethyl methanesulphonate, sodium azide and diethyl

sulphate (Omar *et al.*, 1989; Novak *et al.*, 1990; Bhagwat and Duncan, 1998; Smith *et al.*, 2006). Either banana suckers, shoot tip cultures or embryogenic cell suspensions can be treated with mutagens to induce variation (Novak *et al.*, 1990; Bhagwat and Duncan, 1998; Xu *et al.*, 2005; Smith *et al.*, 2006). Mutation breeding by gamma irradiation has led to the production of several valuable plants, including a Dwarf Parfitt mutant with tolerance against Foc race 4, called Novaria, which is an early flowering Grande Naine mutant (Mak *et al.*, 1996; Smith *et al.*, 2006). Most induced variants are of no commercial use, as a large number of plants need to be screened for improved properties; a process that is both time consuming and expensive (Crouch *et al.*, 1998). Mutations in banana plants cannot be controlled and can be lost in the second or third generation (Hwang and Tang, 2000; Sahijram *et al.*, 2003). Variations acquired by induced mutations can differ between different genotypes (Roux, 2004).

### **Protoplast fusion**

Protoplasts are cells from which the cell wall has been removed mechanically and/or enzymatically (with pectinase, hemicellulase and cellulase) (Haïcour *et al.*, 2004). Under suitable conditions, the protoplasts will resynthesize the removed cell wall and continue to divide. Such cells then form clusters of cells and develop into callus that can be used to generate complete *in vitro* plants (Davey *et al.*, 2005). Protoplast fusion allows for the genetic gene pool to be widened and, therefore, can overcome the hurdle of sterile cultivars in conventional breeding (Davey *et al.*, 2005). Fusion between two different protoplasts permits the transfer of useful characteristics, even if molecular knowledge of the genes is absent (Haïcour *et al.*, 2004). There is, however, a major disadvantage to protoplast culture. Protoplast cell culture is limited by the low frequency of plant regeneration from the protoplasts (Smith and Drew, 1990). However, protoplast fusion has been used to develop Foc race 4-tolerant banana plants from 'Maçã' (AAB), 'Lidi' (AA) and 'Bluggoe' (ABB) (Novak *et al.*, 1989; Sági *et al.*, 1994; Assani *et al.*, 2001; Matsumoto *et al.*, 2002; Chen *et al.*, 2011).

### **Genetic engineering**

Genetic modification involves using recombinant nucleic acid (DNA or RNA) techniques to form new combinations of heritable genetic material followed by the incorporation of that material either indirectly through a vector system or directly through micro-injection, macro-injection and micro-encapsulation techniques. The technology can be used to increase nutritional value of foods, minimize abiotic and biotic stresses of plants, produce secondary metabolites, and to gain more knowledge on plant-pathogen interactions (Chakraborty *et al.*, 2000; Melchers and Stuver, 2000; Balint-Kurti *et al.*, 2001; Wang *et al.*, 2004; Kumar *et al.*, 2005). Genetic engineering in banana has been done by means of particle bombardment



(Sági *et al.*, 1995; Becker *et al.*, 2000) and *Agrobacterium tumefaciens*-mediated transformation (Ganapathi *et al.*, 2001; Chakrabarti *et al.*, 2003; Triparthi *et al.*, 2013). Transgenic bananas with enhanced provitamin A and banana Xanthomonas wilt resistance are at the product development stage in Uganda (Tushemereirwe, personal communication; Triparthi *et al.*, 2013).

Before genetically modified plants can be released, several biosafety issues have to be addressed (Sharma *et al.*, 2002). One of the most important concerns is the containment of the transgene in the transgenic plant. Environmental groups are worried that “super” weeds can be generated when the transgene escape, in rare cases, to non-transformed plants. The effect of the transgene on non-target organisms also needs to be evaluated (Sharma *et al.*, 2002). It is further argued that the introduction of foreign genes into agricultural crops might lead to allergies (Sharma *et al.*, 2002). However, to date there is no evidence that genetically modified plants will have a negative effect on humans and on the environment (Bartsch and Schuphan, 2002).

### **Conventional cross breeding**

The conventional breeding of banana involves the transfer of pollen grains from resistant fertile diploid male plants to the female flowers of triploid clones with female fertility, to obtain resistant tetraploid hybrids (Cheeseman, 1932; Ortiz *et al.*, 1995; Jones, 2000). The genetic improvement of bananas to produce cultivars with host plant resistance and other desirable agronomic traits is complicated by the long duration of 18 months for the crop to establish from seed to seed (Pillay *et al.*, 2002). Also the high cost and space (9 m<sup>2</sup> per mat) requirements of bananas are limitations to the banana breeders (Rowe, 1984). The complex banana genetics, low genetic variability, polyploidy and the low levels of female and/or male fertility in most widely-grown triploid clones once made banana breeding an almost impossible venture (Rowe, 1984; Tezenas du Montcel *et al.*, 1996; Pillay *et al.*, 2002).

Despite several constraints, breeding programmes have managed to successfully produce several banana hybrids with resistant to various biological constraints. For instance, Foc race 1- and race 4-resistant hybrids have been developed at Fundacion Hondurena de Investigacion Agricola (FHIA) and Empresa Brasileira de Pesquisa Agropecuária Centro (EMBRAPA, Brazil), and black Sigatoka-resistant hybrids have been developed by the National Agricultural Research Organisation (NARO) (Uganda), International Institute of Tropical Agriculture (IITA) (Uganda and Nigeria) and The African Centre for Research on Banana and Plantain (CRBP, Cameroon) (Ortiz *et al.*, 1995; Eckstein *et al.*, 1996; Jones, 2000; Amodaran *et al.*, 2009; Ssali *et al.*, 2010). In Uganda two 'Matooke' hybrids, Kabana 6H and Kabana 7H, have been released and are currently being promoted by various development agencies (Nowankunda *et al.*, 2015). In Tanzania, initial adoption studies

showed that FHIA banana hybrids are grown by 29% of farmers in the districts where they were introduced (Edmeades *et al.*, 2007). The efficiency of conventional crossbreeding can be greatly enhanced to generate hybrids combining host plant resistance to pathogens and pests, short growth cycles and height, high fruit yield, parthenocarpy and desired fruit quality when complemented by biotechnology tools like molecular markers (Ortiz and Swennen, 2014).

### **Marker-assisted breeding**

Molecular markers in *Musa* have mostly been used for germplasm characterization. For instance, the genetic diversity within *Musa* has been assessed with intergenic spacers (Lanaud *et al.*, 1992), restriction fragment length polymorphisms (RFLPs) (Gawel *et al.*, 1992; Jarret *et al.*, 1992; Raboin *et al.*, 2005), random amplified polymorphic DNA markers (RAPDs) (Pillay *et al.*, 2001), intersimple sequence repeats (ISSRs) (Godwin *et al.*, 1997), microsatellites (SSR) (Creste *et al.*, 2003; 2004), amplified fragment length polymorphism (AFLP) (Ude *et al.*, 2003), inter-retroelement amplified polymorphism (IRAP) (Teo *et al.*, 2005) and diversity array technology (DARTs) (Amorim *et al.*, 2009; Risterucci *et al.*, 2009). However, beyond germplasm characterization, molecular genetics techniques have the potential to markedly enhance the efficiency of genetic improvement in *Musa* (Crouch *et al.*, 2000; Josh and Nayak, 2010).

Molecular markers provide tools for studying the genetic relationship among breeding lines (Staub and Serquen, 1996; Saghai *et al.*, 1997). When molecular markers are co-inherited with physical traits, they are most likely associated with the genes underlying the trait. RAPD, SSR, AFLP, RFLP, DArT and single nucleotide polymorphism (SNP) markers all provide framework maps to locate genes/quantitative trait loci (QTLs) for traits of interest (Wenzl *et al.*, 2004). Nucleic acid sequence data obtained from expressed sequence tags (ESTs), resistance gene analogs (RGAs) and genome sequences can be used to develop genetic markers and maps, or to identify functional genes (Pillay *et al.*, 2012). Markers and maps based on informative sequences are useful for identifying and potentially cloning genes and QTLs of agricultural and biological significance. ESTs can be used in finding genes, mapping the genome, and identification of coding regions in genomic sequences (Fulton *et al.*, 2002). The growing EST databases in different plant species, including *Musa*, provide valuable resources for development of EST-based markers.

*R*-genes isolated from plants have often been shown to occur in gene clusters (Miller *et al.*, 2008; Mohamad and Heslop-Harrison, 2008). The majority of known *R*-genes contain nucleotide-binding sites (NBS) and LRR domains. The conserved nature of motifs within these domains has been exploited to search for RGAs using a homology-dependent PCR-based approach. RGAs are genomic regions with conserved domains indicating the

likelihood to code for resistance genes, which have also been reported from the *Musa* gene pool (Miller *et al.*, 2008; Mohamad and Heslop-Harrison, 2008). Although not all RGAs may correspond to functional disease resistance genes, RGA primers have been shown to amplify the conserved sequences of LRR, NBS and serine/threonine protein kinases (PtoKin), thereby targeting genes and gene families for disease resistance, defence response, or other important signal transduction processes (Jupe *et al.*, 2012). RGAs can be considered useful not only as genetic markers but can lead to the identification of important genes such as the *Lr1* gene in wheat (*Triticum aestivum* L.). RGAs have also been used for mapping QTLs for many important characters, including disease resistance, in plants (Faino *et al.*, 2012). Despite the benefits likely to accrue from MAS breeding in bananas, no breeding programme has reported its utilisation so far. This could partly be attributed to limitations in generating appropriate segregating populations due to either male or female sterility, and the high ploidy levels of bananas that make the task of tagging molecular markers to traits of economic importance difficult (Ortiz and Swennen, 2014).

### **Diversity Arrays Technology (DArT)**

DArT is a DNA hybridization-based genotyping technology which enables low-cost whole-genome profiling of crops without prior sequence information (Jaccoud *et al.*, 2001). DArT reduces the complexity of a representative sample (such as pooled DNA representing the diversity of *Musa*) using the principle that the genomic “representation” contains two types of fragments: constant fragments found in any “representation” prepared from a DNA sample from an individual belonging to a given cultivar or species, and variable (polymorphic) fragments called molecular markers, found in some but not all of the “representations”. DArT markers are biallelic and may be dominant (present or absent) or co-dominant (two doses vs. one dose or absent) (Jaccoud *et al.*, 2001).

DArTs are attractive approaches to detect large numbers of genome-specific SNP markers (Wenzel *et al.*, 2004). Whole genome DArTs profiles can be used in characterising germplasm, QTLs and associated mapping, bulk segregant analysis (BSA) and marker-assisted selection (MAS) for multiple traits simultaneously (Jaccoud *et al.*, 2001). In comparison to other molecular markers, DArTs require availability of the array, a microarray printer and scanner, and computer infrastructure to analyse, store and manage the data produced, which limits wider application. However, DArT markers are sequence-ready and, therefore, if sequenced they can be developed for a PCR analysis using standard electrophoresis.

Sequenced DArT markers have been used successfully with *Musa* in several studies. For instance, DArTs was used to characterise the *Musa* germplasm accessions for genetic variability (Sales *et al.*, 2001; Amorim *et al.*, 2009; Risterucci *et al.*, 2009). DArTs have also

been used to successfully construct linkage maps in two diploid banana populations independently (Hippolyte *et al.*, 2010; Mbanjo *et al.*, 2012). DArT has been successfully applied in quantitative BSA, thus underscoring the ability of quantifying allele frequencies in DNA pools (Wenzel *et al.*, 2007). DArT-BSA identified genetic loci that influence phenotypic characters like pubescent leaves and aluminium tolerance in barley with at least 5 cM accuracy, and should prove useful as a generic tool for high-throughput, quantitative BSA in plants irrespective of their ploidy level (Wenzel *et al.*, 2007). This application makes DArTs particularly attractive for identifying loci linked to Fusarium wilt resistance in the vegetatively propagated banana polyploid.

## **CONCLUSION**

The fungus Foc, which infects the roots of susceptible bananas and causes a lethal wilt disease, is one of the most devastating pathogens in the history of banana production. In the Great Lakes region of eastern Africa, where bananas are a major staple food and an important cash crop, Fusarium wilt is the most serious threat to the livelihoods of many smallholder farmers. This threat has been aggravated by the recent report of Foc TR4, which attacks a wider range of hosts, in Africa (Viljoen, personal communication). Host plant resistance has long been acknowledged as the most feasible intervention to control banana Fusarium wilt (Moore *et al.*, 1999).

Banana improvement comprises of two essential steps: 1) generating diversity by: either conventional cross breeding, genetic engineering, protoplast fusion, somaclonal variation or induced mutagenesis, and 2) selecting genotypes with favourable combination of traits. Researchers have successfully developed Fusarium wilt tolerant and resistant bananas by either conventional cross breeding, somaclonal variation or induced mutagenesis (Rowe, 1990; Mak *et al.*, 1996; Hwang and Ko, 2004; Smith *et al.*, 2006). Unfortunately, the underlying host factors for Fusarium wilt resistance is poorly understood and remains unpredictable (Rowe, 1984; Tezenas du Montcel *et al.*, 1996; Pillay *et al.*, 2002). Thus, researchers cannot optimize the performance of banana varieties when their constituents for success are unknown. The hit-or-miss nature of current breeding efforts requires many years of field-testing for several rounds of selection, including evaluation for agronomic performance in early evaluation trials (EET) (based on individuals). In addition, selected hybrids/mutants have to be further evaluated for pest/disease response, yield and consumer acceptability in the preliminary yield trials (PYT). Promising hybrids/mutants from the PYTs are advanced for participatory on-farm evaluation and multi-location evaluation (Nowakunda *et al.*, 2015).

Fortunately, resistance to Foc exists within the *Musa* gene pool (Jones, 2000). To effectively utilise this resistance to improve Foc-susceptible cultivars, a better understanding is required of a) the part(s) of the banana genome that contribute to Fusarium wilt resistance, b) inheritance of Fusarium wilt resistance, c) molecular markers that are co-inherited with Fusarium wilt resistance, and d) the interaction between pathogen and host. However, the genetic basis of Fusarium wilt resistance in *Musa* is not well understood, but its elucidation could facilitate the development of new control strategies based on host factors required for resistance. For this reason a Foc-segregating banana population was generated from the susceptible genotype 'Sukali Ndiizi' and the resistant genotype 'TMB2x8075-7', and the inheritance of resistance to Foc will be investigated in Chapter 2.

Wild and cultivated diploid bananas are a valuable source of resistance in banana breeding. The diploid line 'TMB2X8075-7' (AA), derived from the cross ('SH3362' X 'Calcutta 4'), is a source of resistance to Foc race 1. However, the genetic improvement of bananas to produce cultivars with host plant resistance and other desirable agronomic traits is complicated by the long duration time of 18 months for the crop to establish from seed to seed, and the high cost and space requirements (Rowe, 1984; Pillay *et al.*, 2002). When molecular markers are co-inherited with physical traits like Foc resistance, they are most likely associated with the genes underlying the trait (Semagn *et al.*, 2006). Therefore, the host resistance genes for Foc can be identified indirectly in banana progenies using MAS. Molecular markers will make breeding efforts to improve banana for resistance to Fusarium wilt much more efficient and successful. In Chapter 3, candidate markers associated with resistance to Fusarium wilt will be identified using a DArT-Bulk segregant analysis platform.

Changes in host RNA levels during a fungal infection provide valuable information on the molecular processes underlying resistance and susceptibility. Therefore, investigating the transcriptome during the Foc-*Musa* interaction is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues. Gene expression profiles of banana roots in response to infection of Foc have been studied extensively on the commercial dessert banana Cavendish variety (Li *et al.*, 2012; Li *et al.*, 2013a). Most of these studies are lacking cultivars of local importance in Africa. Chapter 4, therefore, will unravel the Foc-banana interaction of three genotypes of local importance to the banana cropping system in eastern and central Africa using RNA-seq analysis.

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**Table 1.** Management options of Fusarium wilt of bananas.

Management option	Description	Source
Cultural control	Use of disease-free tissue culture plantlets to prevent the introduction of the disease in disease-free areas(farms)	Moore <i>et al.</i> , 1999
Quarantine and sanitation	Prevent the introduction of the disease to disease-free farms by treatment of vehicles, machinery, tools and farm wear with effective surface disinfectant like 'Farmcleanse', chlorine bleach, methylated spirits and copper oxychloride solutions.	Deacon, 1984; Jeger <i>et al.</i> , 1995; Moore <i>et al.</i> , 1999; Viljoen <i>et al.</i> , 2002.
Crop rotation	Withholds the pathogen's host to reduce the pathogen populations in the soil. Sugarcane growing combined with fallow rotations reduces the Fusarium wilt incidence significantly.	Sequeira <i>et al.</i> , 1958; Hwang, 1985; Su <i>et al.</i> , 1986; Huanga <i>et al.</i> , 2013
Flood–fallowing	Fields are watered excessively for about 6 months to create nearly anaerobic conditions that will reduce the survival of the pathogen. Only effective in the short run (crop plant) but subsequent cycles are affected by Foc.	Wardlaw, 1961; Stover, 1962
Soil amendments	Application of organic amendments has been successfully used to increase soil suppressiveness to Foc. Organic matter is associated with increases of microbial biomass and microbial activities, resulting in enhancement of general suppression. However, application of the ammonium form of nitrogen reduces the suppressiveness of the soil.	Rishbeth and Naylor, 1957; Stover 1962; Ploetz <i>et al.</i> , 1990
Biological control	<i>Antagonistic microbes:</i> mycoparasitic species of <i>Trichoderma</i> and <i>Gliocladium spp.</i> , non-pathogenic isolates of <i>Fusarium oxysporum</i> with arbuscular mychorizal fungi produce cell wall degrading enzymes, compete with the Foc for infection sites and induce host resistance against Foc.	Nel <i>et al.</i> , 2006; Thangavelu and Mustafa, 2010; Akila <i>et al.</i> , 2011

	<p><i>Botanicals</i>: Plant extracts with active principles which either act on the pathogen directly or induce systemic resistance in the host plants resulting in reduction of disease development. Combined application of botanical formulation (<i>Datura sp</i>) and biocontrol agents have been proved effective to a limited extent.</p>	Akila <i>et al.</i> , 2011; Huang <i>et al.</i> , 2012
Chemical control	<p><i>Surface sterilants</i>: Disinfecting Foc-infested soil on a small scale by drenching with copper sulphate, carbolineum, formaldehyde, formalin and carbon disulphide. Though unsuccessful and expensive, it reduces the population of Foc in the soil.</p>	Brandes, 1919; Rishbeth and Naylor, 1957; Moore <i>et al.</i> , 1999
	<p><i>Plant activators</i>: Chemicals that activate host plant defense response. These include 2,6-dichloroisonicotinic acid (INA), benzo-(1,2,3)thiadiazole-7- carbothioic acid S-methyl ester (BTH), probenazole and D,L--aminobutyric acid (BABA).</p>	Tally <i>et al.</i> , 1999
	<p><i>Soil fumigation</i>: Methyl bromide has been effective in containing outbreaks of Fusarium wilt in the short term</p>	Herbert and Marx, 1990
	<p><i>Fungicides</i>: like mercury compounds, R&amp;H-3888 (nitrile), EP-161(methyl isothiocyanate), Vapam (sodium n-methyl dithiocarbamate), allyl alcohol, and Mylone (3,5-dimethyltetrahydro- 1,3,5,2H-thiadiazine-2-thione) have been found to be effective though expensive.</p>	Meredith 1943; Lakshmanan <i>et al.</i> , 1987; Davis <i>et al.</i> , 1994



**Figure 1.** Symptoms of banana Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense*: (A) Yellowing of the older leaves, (B) splitting of the pseudo stem, (C) and (D) internal vascular browning.

## CHAPTER 2

### Inheritance of resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 in banana<sup>1</sup>

#### ABSTRACT

*Fusarium* wilt of bananas (also known as Panama disease), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is a serious problem in banana production worldwide. Genetic resistance offers the most promising means to control *Fusarium* wilt of bananas. In this study, the inheritance of resistance in *Musa* to Foc race 1 was investigated in three 'SN8075' F<sub>2</sub> populations derived from a cross between 'Sukali Ndiizi' and 'TMB2X8075-7'. A total of 163 F<sub>2</sub> genotypes were evaluated for their response to *Fusarium* wilt in a screen house experiment. The test plants were inoculated by mixing loam soil with millet grains colonized by Foc in polythene pots. One hundred and fifteen genotypes were categorized to be susceptible and 48 were resistant. Mendelian segregation analysis for susceptible vs. resistant fit the segregation ratio of 3:1 ( $\chi^2=1.72$ , P=0.81), suggesting that resistance to *Fusarium* wilt in *Musa* is conditioned by a single recessive gene. We propose *pd1* (*Panama disease 1*) to be the name of the recessive gene conditioning resistance to *Fusarium* wilt in the diploid line 'TMB2X8075-7'.

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<sup>1</sup>Ssali, R.T., Kiggundu, A., Lorenzen, J., Karamura, E., Tushemereirwe, W. and Viljoen, A. 2013. Inheritance of resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 in bananas. *Euphytica* DOI 10.1007/s10681-013-0971-6.

## INTRODUCTION

Bananas (*Musa* spp.) provide a staple food for millions of people living in the humid tropics (Arias *et al.*, 2003). Most of the cultivated banana types are diploid or triploid, and it is believed that they originated from intra- and inter-specific hybridizations between seed-bearing wild subspecies of *Musa acuminata* Colla (A genome donor) and *M. balbisiana* Colla (B genome donor) (Simmonds and Shepherd, 1955). As a result, the various bananas types can be classified on the basis of their genome constitution as AA, BB and AB (diploids), and as AAA, AAB and ABB (triploids). A great variety of banana cultivars are grown in east and central Africa. The most important of these is the East African Highland bananas (EAHB-AAA), which comprise approximately 85% of bananas produced in Uganda (Karamura *et al.*, 1996). Several dessert banana varieties are also grown, mainly for fresh eating and beer brewing, of which the cultivar 'Sukali Ndiizi' (AAB) is the most popular because of its compact bunch, short fingers and very sweet apple flavours when ripe (Gold *et al.*, 2002,). However, the economic value of this cultivar is usually not realized because it is severely affected by Fusarium wilt (Kangire *et al.*, 2001).

Fusarium wilt of bananas (Panama disease) is caused by the devastating soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen (Foc). Panama disease became famous when it destroyed thousands of hectares of Gros Michel bananas in Central America. After root infection, Foc colonizes the vascular system of the host plant and blocks the flow of water from the roots to the leaves (Moore *et al.*, 1995). This results in yellowing and wilting of leaves, which progresses from the oldest to the youngest leaves, and the eventual death of plants. In severe cases, losses in production of susceptible banana genotypes can be as much as 100%.

Foc affecting bananas is broadly divided into three physiological races (Foc races 1, 2 and 4) further subdivided into 24 vegetative compatibility groups (VCGs). Only Foc race 1 (VCGs 0124, 0124/125, 01212 and 0122) have been reported in Uganda. Foc race 1 does not affect EAHB, but severely affects dessert bananas such as 'Gros Michel' (AAA), 'Sukali Ndiizi' (AAB), 'Kisubi' (AB) and 'Psang Awak' (ABB) (Kangire *et al.*, 2001). Fusarium wilt is difficult to manage because the pathogen persists in infested soils for decades (Stover and Buddenhagen, 1986). The most effective management strategy for Fusarium wilt control, therefore, is the growing of Foc-resistant cultivars.

However, the genetic improvement of bananas to produce cultivars with host plant resistance and other desirable agronomic traits is complicated by low genetic variability, polyploidy and the low levels of female and /or male fertility in most widely grown triploid clones once made banana breeding to be considered an impossible venture (Rowe, 1984; Pillay *et al.*, 2002). Fortunately the ability of some banana clones to produce seeds when



pollinated has made banana improvement possible. For instance, Fusarium wilt-resistant lines have been developed at Fundacion Hondurena de investigacion Agricola (FHIA) in Honduras, including the diploid hybrid SH-3362 (AA), derived from intercrossing wild accessions of *Musa acuminata* subsp. *Malacensis*. 'SH3362' is resistant to Foc races 1 and 4 (Rowe, 2000). The banana diploid line 'TMB2X8075-7' was developed from a cross between 'SH3362' (AA) hybrid, kindly provided by Fundacion Hondurena de investigacion Agricola (FHIA), and 'Calcutta 4' (AA), a known Foc-resistant wild type banana (Moore *et al.*, 1995). The banana diploid line 'TMB2X8075-7' could therefore be a source of resistance for both Foc race 1 and race 4.

Resistance to *F. oxysporum* in crop plants appears to be a genetically complex trait (Berrocal-lobo and Molina, 2007). Identifying the parts of the genome that contribute to the variation in such a complex trait, and ultimately the genes and alleles responsible for trait variation, is a challenge in banana breeding. Classical genetic analysis is not sufficiently developed in autopolyploid crops and bananas, like other vegetatively propagated plants, are highly heterozygous (Pillay *et al.*, 2002). Therefore, pure inbred lines do not exist due to the difficulty of self-pollination and the random pairing of multiple homologous chromosomes. Furthermore, polyploidy genomes comprise many different homeologous chromosomes that can pair and recombine in a variety of possible combinations, which complicate genetic studies in inter-specific interploidy banana crosses.

Various types of segregating populations can be used in genetic studies to investigate the genetic basis of specific traits, including F<sub>2</sub> population, backcrosses, doubled haploids (DHs), recombinant inbred lines (RILs) and near-isogenic lines (NILs) (Collard *et al.*, 2005). F<sub>2</sub> populations are easy to develop since individuals are products of single meiotic cycle with a wide genetic assortment (Loisel *et al.*, 1994). In this study, allele(s) involved in Fusarium wilt resistance in *Musa spp.* were characterized following the development of a F<sub>2</sub> population from a cross between 'Sukali Ndiizi' and 'TMB2X 8075-7', a resistant diploid banana hybrid.

## **MATERIALS AND METHODS**

### **Development of F<sub>1</sub> and F<sub>2</sub> populations**

Crosses were made between the Foc race 1-susceptible triploid dessert banana 'Sukali Ndiizi' (AAB) and the resistant diploid banana 'TMB2X8075' (AA). During the crossing, pollen grains were harvested and rubbed onto the receptive female flowers in the field. Emerging female flowers and male pollen sources were bagged to avoid any possible contamination with alien pollen. Seeds harvested were germinated *in vitro* (Vuylesteke *et al.*, 1990) to develop an F<sub>1</sub> population of 76 progeny genotypes. The ploidy of the individuals was determined using a Partec II ploidy analyzer (Dolezel, 1997). Three diploids from this

population ('ND131k-15', 'ND131k-57' and 'ND131k-18',) were selfed to obtain 163 F<sub>2</sub> plants (Fig. 1). Both F<sub>1</sub> and F<sub>2</sub> plants were tested for their resistance response to Foc race 1.

*Preparation of innoculum:* Foc was collected from infected rhizome tissue of 'Sukali Ndiizi' bananas grown at the National Agricultural Research Laboratories, (NARL), Kawanda in Uganda. Discoloured vascular tissue (about 1cm<sup>2</sup>) was cut out and then surface disinfected and placed on potato dextrose agar (PDA) (5 g/L dextrose, 200 g potato, bacto agar 15 g/L) fortified with 100 µg/ml streptomycin sulphate. After incubation at 25°C for 5 days, developing colonies were sub-cultured under the same conditions, and single spore colonies prepared for further identification.

Inoculum for Fusarium wilt evaluation was prepared by growing Foc race 1 on finger millet. The millet was first rinsed in tap water, soaked in distilled water overnight and rinsed a second time in distilled water before it was transferred into polythene bags (500 g/bag) and autoclaved. When the grain was cool, five squares (1 cm<sup>2</sup>) of Foc were transferred from the culture plates to each of the millet bags, and the bags incubated for 14 days at 25°C. During the incubation period, bags were turned regularly to ensure thorough mixing of the inoculum.

### **Screening F<sub>1</sub> and F<sub>2</sub> hybrids for Fusarium wilt response**

The screening of F<sub>1</sub> and F<sub>2</sub> hybrids for Fusarium wilt response was conducted in a sequence of 3 completely randomised pot experiments. Tissue cultured banana plantlets (about 3 months old) of 76 F<sub>1</sub> progenies and 163 F<sub>2</sub> progenies were artificially inoculated with Foc race 1. Five plants of each line along with the resistant ('TMB2X8075-7') and susceptible parent ('Sukali Ndiizi') were planted into plastic polythene pots containing 10 kg of sterile loam soil mixed with 500 g of Foc-colonized millet grain. Millet grain that was not infected with Foc was used for control treatments. After planting, the plantlets were maintained in a screen house with day temperatures of about 28°C and approximately 12 hours of light for 8 weeks. Symptom development was scored using a method proposed by Smith *et al.*, (2008), with some modifications. Briefly, discolouration of the rhizome was scored on a six-point rating scale, where 1 = no discolouration, 2 = 1-15% discolouration, 3 = 16-33% discolouration, 4 = 34-50% discolouration, 5 = more than 50% discolouration and 6= dead plant. The pathogen was re-isolated from representative diseased plants to complete Koch's postulates. The development of Fusarium wilt symptoms on banana lines was analyzed using Assistat software (Silva and Azevedo, 2006). Differences between progenies were determined by the Scott and Knott (1974) method. This method groups the means by maximizing the sum of squares between groups. The genetic basis of Fusarium wilt resistance was hypothesized from the observed frequencies of the F<sub>2</sub> progenies.

## RESULTS

The 'Sukali Ndiizi'-'TMB2X8075-7' cross generated 76 F<sub>1</sub> progenies comprising 18 diploids, 21 triploids and 37 tetraploids (Table 1). In the F<sub>1</sub> population, two triploid and five tetraploid progenies were resistant. Thirty seven F<sub>1</sub> progeny genotypes showed tolerance to Foc. These included seven diploids, 11 triploids and 19 tetraploids. Thirty two progenies were susceptible, comprising of 11 diploids, eight triploids and 13 tetraploids. None of the diploids in the F<sub>1</sub> population showed resistance to Fusarium wilt. The Scott and Knott test of the rhizome discolouration in all the F<sub>2</sub> populations were categorized into only susceptible (clustered with the susceptible parent 'Sukali Ndiizi') and resistant (grouped with the resistant parent 'TMB2X8075-7' lines) (Fig 2). The F<sub>2</sub> population derived from the diploid hybrid 'ND131k-15' comprised of 58 susceptible lines and 28 resistant lines ( $\chi^2$  (3:1), P=2.62). The second F<sub>2</sub> population derived from 'ND131k-18' showed 35 susceptible lines and seven resistant lines ( $\chi^2$  (3:1), P=1.56). The third population derived from 'ND131k-57' showed 22 susceptible lines and 13 resistant lines ( $\chi^2$  (3:1), P=3.57). Overall, combining the three F<sub>2</sub> populations led to a segregation of 48 resistant and 115 susceptible lines, giving a good  $\chi^2$  fit for 1(resistant):3(susceptible) (Table 2).

## DISCUSSION

The identification and use of disease resistance genes is a pivotal activity for improving banana for resistance to Foc. This study provides the first evidence that resistance to Foc race 1 in the diploid line 'TMB2X8075-7' is mediated by a single recessive gene. Several traits in banana have been reported to be under the genetic control of recessive genes. Ortiz and Vuylsteke (1992; 1994) elucidated the inheritance of resistance to black Sigatoka (*Mycosphaerella fijiensis* Morelet) disease, to be under the genetic control of recessive-additive alleles *bs*, *bsr2* and *bsr3* at three loci. Other traits reported to be controlled by recessive genes include apical dominance (*ad*), pseudostem waxiness (*wx*), dwarfism (*dw*) and albinism (*a1* and *a2*) (Ortiz and Vuylsteke, 1994; 1995; Ortiz *et al.*, 1995). We propose the name, Panama disease 1 (*pd1*), for the gene mediating resistance to Foc race 1 in the diploid banana 'TMB2X8075-7'. It is important to reveal the mode of action of recessively inherited resistance to effectively utilize it in a crop improvement strategy. This study did not determine the molecular mechanism of resistance of *pd1* in *Musa*. However, molecular techniques like genetic mapping or transcription analysis could be used to elucidate mechanisms involved in this type of resistance.

An earlier study by Vakili (1965) reported resistance to Foc race 1 in the diploid banana 'Pisang Lilin' to be conditioned by a single dominant gene. One possible reason for

the discrepancy could be that these deductions were based on the 1:1 (resistant: susceptible) segregation of an  $F_1$  population in contrast to the present investigation based on segregation in the  $F_2$  population. Individuals in the  $F_1$  populations exhibit limited genetic variation in comparison to individuals of  $F_2$  populations (Ortiz, 2012). The other possibility for discrepancy is that the diploid banana 'Pisang Lilin', which Vakili (1965) used as the resistant parent, contained a different resistance gene from the one in 'TMB2X8075-7'. It is, therefore, plausible to presume resistance to Foc race 1 as a multiple allelic trait in *Musa*. It will be reasonable to further investigate the allelic relationship of the resistance gene in the diploid bananas 'Pisang Lilin' and 'TMB2X8075-7'.

Although recessive resistance is not as exploited in breeding as dominant resistance, it appears to be generally very durable. For instance in pepper, the *pvr1* allele which is associated with resistance to potyviruses, remained effective more than 50 years after its introduction (Kang *et al.*, 2005). Therefore, the diploid banana 'TMB2X 8075-7' may provide a durable source of recessive resistance to Foc race 1 in bananas. Developing molecular markers that co-segregate with *pd1* will greatly enhance improving susceptible commercial varieties like 'Gros Michel' (AAA), 'Sukali Ndiizi' (AAB) and 'Pisang Awak' (ABB) by recurrent selection using marker-assisted selection, while also paving the way for fine mapping and positional cloning of the *pd1* gene.

This study represents the first genetic investigation of the inheritance of resistance to Foc race 1 in the diploid banana hybrid 'TMB2X8075-7'. Results from this work provide impetus for further investigations to: 1) identify and develop molecular markers associated with *pd1*, 2) fine map and clone the *pd1* gene in *Musa*, and 3) determine molecular mechanism(s) involved in the resistance of *pd1* to Fusarium wilt.

In conclusion, the production of  $F_2$  diploid hybrids via triploid x diploid cross has enabled the genetic analysis of the inheritance of Fusarium wilt in the banana diploid line 'TMB2X8075-7'. The diploid banana line 'TMB2X8075-7' carrying the recessive resistance gene *pd1* can be used in a recurrent selection breeding strategy.

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**Table 1.** Response of segregating banana F<sub>1</sub> genotypes ('Sukali Ndiizi'-'TMB2X 8075-7') following inoculation with *Fusarium oxysporum* f. sp. *cubense* race 1

<b>Ploidy level</b>	<b>Resistant (RDI = 1.6-2.4)</b>	<b>Tolerant (RDI = 2.6-3.0)</b>	<b>Susceptible (RDI = 3.2-6.0)</b>
Diploids	<b>0</b>	<b>7</b> (ND131K25, ND131K33, ND131K57, ND131K64, ND131K_7, ND26K_2, , ND66K_1)	<b>11</b> ( ND131K15, ND131k-18, ND131K22, ND131k-23, ND131K63, ND131K_3, ND131K_4, ND28K, ND28K_2, ND28K_6, ND70K-8)
Triploids	<b>2</b> (ND183k, ND200k)	<b>11</b> (ND131K_1,ND18k_14, ND215K-8, ND23k, ND231K ,ND263K, ND273K, ND303K, ND40K_1, ND70K_5, ND89K_1)	<b>8</b> (ND18K_4, ND113K54, ND131K_6, ND131K26, ND131K35, ND131K44, ND131K54, ND13K_5,)
Tetraploids	<b>5</b> (ND14K,ND206K-19, ND231k-29,ND24k-6 ,ND95k-1)	<b>19</b> (ND100K_1, ND100K-3, ND119K_2, ND153K19, ND207K_7, ND25K_4, ND26K_1, ND26K_3, ND28K16, ND28k-3, ND28K-8, ND43K_1, ND66k-2, ND70K_3, ,ND70K_6, ND91k-3, ND95K_3) ND95K_4, ND95K_6)	<b>13</b> (ND131K32, ND131k-37, ND131K40, ND189K, ND28K_1, ND43K_2, ND45K_9, ND47K, ND6K_2, ND70K_1, ND70K_4, ND70K_7, ND88K_4)

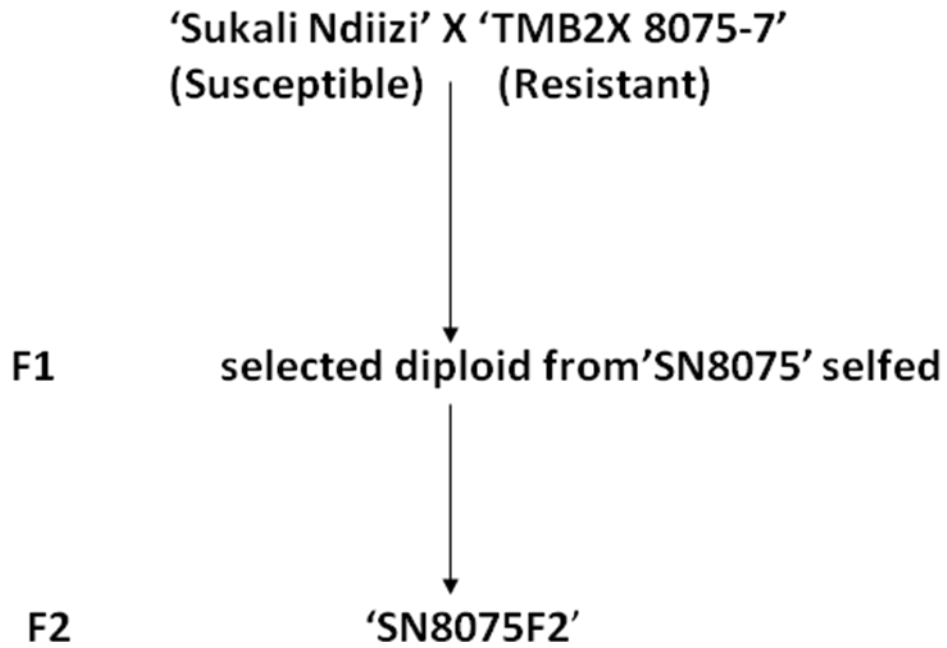
Mean internal rating of banana lines to *Fusarium oxysporum* f. sp. *cubense* race 1 was scored based on the discolouration of the rhizome (Smith *et al*, 2008). According to the rhizome discoloration index (RDI), 1 = no discolouration, 2 = 1-15% discolouration, 3 = 16-33% discolouration, 4 = 34-50% discolouration, 5 = more than 50% discolouration, and 6 = dead plant. Differences between progenies were determined by the Scott and Knott test.

**Table 2.** Single locus goodness of fit Chi square test for the segregation of Fusarium wilt resistance in the 'SN8075F2' population

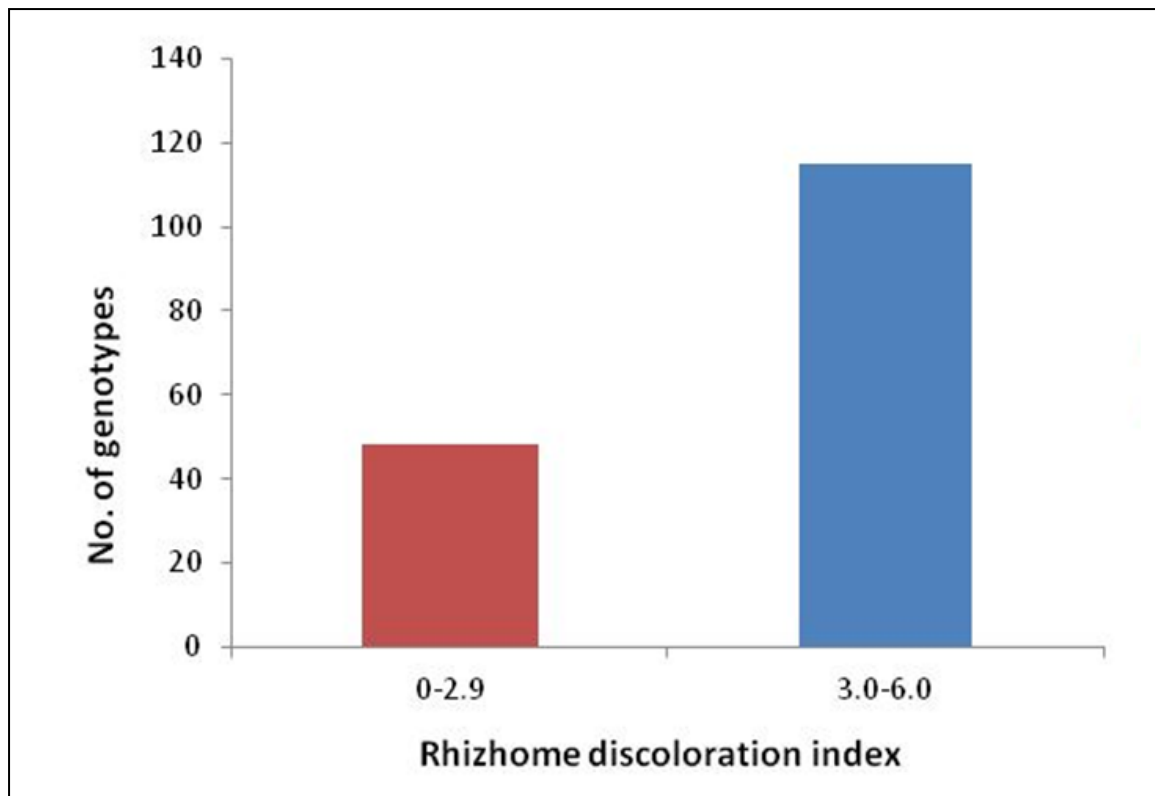
<b>Cross</b>	<b>Susceptible</b>	<b>Resistant</b>	<b><math>\chi^2(3:1)</math></b>	<b><i>P</i>(3:1)</b>
ND131k-15 X ND131k-15	58	28	2.62	0.89
ND131k-18 X ND131k-18	35	7	1.56	0.79
ND131k-57 X ND131k-57	22	13	3.57	0.94
<b>Pooled</b>	<b>115</b>	<b>48</b>	<b>1.72</b>	<b>0.81</b>

Progenies were categorized as either resistant or susceptible by the Scott and Knott test for mean internal rating of disease symptoms of *Fusarium oxysporum* f. sp. *cubense* race 1 infection (discoloration of rhizome). Chi-square tests for goodness of fit were calculated as  $\chi^2 = \frac{[Obs. - Exp.]^2}{Exp}$ , where Obs= observed; Exp=expected.





**Figure 1.** Pedigree of the *Fusarium oxysporum* f. sp. *cabense* race 1 *Musa* segregating population.



**Figure 2.** Segregation of banana genotypes for *Fusarium* wilt resistance in the SN8075F<sub>2</sub> population (Red) resistant genotypes, (Blue) susceptible genotypes based on the Scott and Knott test.

## CHAPTER 3

### Identification of DArT markers for the *Fusarium* wilt resistance gene, *pd1*, in *Musa* by bulk segregant analysis

#### ABSTRACT

*Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is a serious constraint to the production of bananas globally. A major recessive gene, *pd1*, was identified to confer resistance to Foc race 1 in a segregating F<sub>2</sub> population from a cross between a susceptible cultivar 'Sukali Ndiizi' (AAB) and a resistant genotype 'TMB2X8075-7' (AA). In the current study, quantitative bulk segregant analysis (BSA) on the Diversity Arrays Technology (DArT) platform was used to identify markers associated with resistance in banana to Foc race 1. DNA of 40 resistant and 40 susceptible F<sub>2</sub> progenies were pooled for BSA-DArT analysis, along with DNA from the resistant and the susceptible parents. A total of 14 354 DArT markers were polymorphic between the parents and the resistant and susceptible bulks, and were used to estimate the genetic similarity between the parents and bulks. The genetic similarity was highest between the resistant and susceptible bulks (0.61) and lowest between the Foc race 1-susceptible parent 'Sukali Ndiizi' and the two phenotypic bulks (0.03). One hundred and one DArT markers were in qualitative linkage disequilibrium, with 13 markers linked to resistance and 88 markers to susceptibility. Putative functions have been assigned to the DArTs that were mapped to coding sequences of the banana reference genome through *in-silico* database analysis of DArT clone sequences. DArTs closely associated with resistance/susceptibility can be used in the improving bananas with resistance to Foc race 1.

## INTRODUCTION

Banana (*Musa spp.*) is a vegetatively-propagated crop grown in the tropical and sub-tropical regions of the world. It is the eighth most important global food commodity after maize, wheat, rice, potato, cassava, soybean and barley, and the fourth most important staple food in the developing world (FAOSTAT, 2013). In some countries, especially in Latin America and the Caribbean, bananas are a major export commodity. However, 85% of the total world banana production is used as food for domestic consumption in both rural and urban areas. Most of the cultivated edible banana varieties are triploids ( $2n=3X=33$ ), believed to have originated from two wild seed-forming diploid species, *Musa acuminata* Colla and *Musa balbisiana* Colla, which provided the “A” and “B” genomes of bananas, respectively. Unfortunately banana production is often limited by pests and diseases in most banana-growing areas.

Fusarium wilt, caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (E.F. Smith) Snyder & Hansen (Foc), is one of the most important diseases of bananas worldwide. Three physiological Foc races (races 1, 2 and 4) have been recognised. Foc race 1 causes disease in ‘Gros Michel’ (AAA), ‘Sukali Ndizi’ (AAB), ‘Kisubi’ (AB) and ‘Pisang Awak’ (ABB) cultivars, and Foc race 2 affects Bluggoe (ABB) bananas. Foc race 4 attacks Cavendish bananas and all the cultivars susceptible to Foc races 1 and 2 (Moore *et al.*, 1995). Foc is further subdivided into 24 vegetative compatibility groups (VCGs). Of these, only Foc race 1 and VCGs 0124, 0124/125, 01212 and 0122 have been reported in Uganda (Kangire *et al.*, 2001). Foc race 1 does not affect East African Highland Bananas (EAHB) (Kangire *et al.*, 2001).

Numerous control strategies have been devised to prevent damage caused by Fusarium wilt of bananas. Crop rotation, flood following, chemical fumigation and the use of organic amendments were unsuccessful in controlling the disease effectively (Herbert and Marx, 1990; Moore *et al.*, 1995). Foc survives in organic matter and in the soil as dormant chlamydospore in the absence of a suitable host. This survival has made attempts to devise cultural or chemical control options futile. Growing resistant cultivars is the most effective strategy to control the disease. For example, banana plantations and the international export industry were saved from Fusarium wilt destruction by replacing Gros Michel bananas with resistant Cavendish bananas in Central America during the 1960’s (Ploetz, 2005).

Improving bananas for host plant resistance against Fusarium wilt remains the most effective, economical and environmentally friendly intervention (Moore *et al.*, 1999). Sources of resistance to Foc exist within the *Musa* gene pool (Jones, 2000) and can be used for conventional breeding. In Honduras, the FHIA breeding programme successfully developed five Fusarium wilt-resistant tetraploid hybrids, viz ‘FHIA-01’ (AAAB), ‘FHIA-17’ (AAAA),

'FHIA-18', (AAAB), 'FHIA-23' (AAAA) and 'SH-3640.10' (AAAB) (Rowe, 1984). Also in Brazil the banana breeding at Embrapa Cassava and Fruits has developed a number of Fusarium wilt resistant silk hybrid including 'Caipira' (AAA), 'Thap Maeo' (AAB), 'Prata Graúda' (AAB), 'Prata Baby' (AAB - Nam), 'Pacovan Ken' (AAAB), 'Japira' (AAAB), 'Vitória' (AAAB), 'Preciosa' (AAAB), 'Tropical' (AAAB), 'Maravilha' (AAAB), 'Caprichosa' (AAAB), 'Garantida' (AAAB), 'Princesa' (AAAB) and 'Platina' (AAAB) (Amorim *et al.* 2013). However, breeding efforts are complicated by parthenocarpy, low seed set and the slowness of field pathogenicity testing, which makes efforts to improve bananas for Fusarium wilt resistance a time-consuming and expensive process. Efforts to breed resistant hybrids could be much more efficient and successful if molecular markers can be used to rapidly identify resistant plants (Pillay *et al.*, 2012).

PCR-based molecular markers are the most appropriate assays for molecular breeding applications because they are relatively simple to handle and can be easily automated (Rafalski and Tingey, 1993). Marker-assisted selection (MAS) in plant breeding requires that markers are linked to genes of interest, are cost-effectively, and applicable to large number of samples and crosses in a breeding program (Yang and Buirchell, 2008). Molecular markers for MAS have been developed from methods such as restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR), sequence tagged sites (STS) and microsatellite or simple sequence repeat (SSR) (Ruane and Sonnino, 2007). Molecular markers that are co-inherited with a trait can be used to introgress that trait more efficiently. However, limitations in generating appropriate segregating populations due to either male or female sterility, and the high ploidy levels of bananas, make the task of tagging molecular markers to traits of economic importance difficult.

Developing molecular markers for a MAS breeding programme requires genetic linkage maps to identify molecular markers associated with disease resistance. The first genetic linkage map of *Musa* was developed by Fauré *et al.* (1993). This map was based on 58 RFLPs, four isozyme and 28 RAPD markers segregating for parthenocarpy in 92 F<sub>2</sub> progenies of an F<sub>1</sub> hybrid plant derived from a cross between 'SF265' AA × 'Banksii' AA. Several other genetic linkage maps have been developed in *Musa*, including an AFLP genetic linkage map for genes associated with resistance and susceptibility to Foc from an F<sub>1</sub> hybrid population of *M. acuminata* ssp. *malaccensis*, SSR/DArT marker parental genetic maps of *M. acuminata* 'Borneo' and 'Pisang Lilin' (Kayat *et al.*, 2007; Hippolyte *et al.*, 2010; Mbanjo *et al.*, 2012). However, as linkage maps become more saturated with random markers the process of mapping becomes less efficient. Bulk segregant analysis (BSA) provides a method to focus on regions of interest, areas sparsely populated by markers and

for locating genes that do not segregate in populations that were initially used to generate the genetic maps (James *et al.*, 2012).

A marker-based method with good application to diversity studies and molecular breeding is Diversity Array technology (DArTs) (Amorim *et al.*, 2009; Risterucci *et al.*, 2009). DArT is a DNA hybridization-based genotyping technology which enables low-cost whole-genome profiling of crops without prior sequence information (Jaccoud *et al.*, 2001). DArT markers are biallelic and may be dominant (present or absent) or co-dominant (two doses vs. one dose or absent) (Jaccoud *et al.*, 2001). An advantage of DArT is that it can detect and type DNA variation at several loci in parallel without relying on sequence information, making it the marker of choice for a non-model crop with limited genetic resources and complex genomes, like bananas. Past studies have demonstrated the feasibility of converting DArT markers into PCR markers, for instance the conversion of DArT markers closely linked to QTLs of Fusarium wilt resistance in Asiatic Lily hybrids (*Lilium* sp. L.) (Sahin *et al.*, 2009). The successful conversion of DArT markers into PCR markers has been attributed to the fact that the hybridization step in developing DArT markers selects against highly repetitive sequences, which enhances the chance to locate regions where large physical distance corresponds to small genetic distance, such as in the centromeric regions or areas of high gene density (Sahin *et al.*, 2009).

Wild and cultivated diploid bananas are a valuable source of resistance in banana breeding. The diploid line 'TMB2X8075-7' (AA), derived from the cross 'SH3362' x 'Calcutta4', is a source of resistance to Foc race 1. According to Mendelian segregation analysis, resistance to Foc race 1 in the line 'TMB2X8075-7' is conditioned by a single recessive gene, called *pd1* (Ssali *et al.*, 2013). In this study the DArT platform for bulk segregant analysis was used to identify and locate candidate markers associated with resistance to Foc race 1 on the reference *Musa* genome of a double-haploid *Musa acuminata* genotype (Dhont *et al.*, 2012).

## **MATERIALS AND METHODS**

### **Planting material used and DNA extraction**

A population segregating for resistance to Foc race 1, named 'SN8075f2', was obtained by crossing a susceptible banana cultivar 'Sukali Ndiizi' and a resistant diploid banana 'TMB2X8075-7'. Three diploids of the resultant progenies ('ND131k-15', 'ND131k-57' and 'ND131k-18') were then selfed to obtain F<sub>2</sub> plants.

DNA was extracted from the leaves of the resistant diploid banana line 'TMB2X8075-7', carrying the recessive resistance gene *pd1*, the susceptible cultivar 'Sukali Ndiizi', and 40 resistant and 40 susceptible F<sub>2</sub> plants derived from the 'SN8075f2' population'. A

modification of the CTAB method was used for DNA extraction (Crouch *et al.*, 1998). Briefly, 20 mg of frozen leaf tissue was ground to powder form in liquid nitrogen and suspended in pre-heated extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl [pH 8.0]) and 2%  $\beta$ -mercaptoethanol. The suspension was extracted with equal volumes of chloroform-isoamylalcohol (24:1). DNA was pelleted using ice-cold isopropanol, washed with 70% ethanol and dissolved in sterile double-distilled water.

### **Diversity Array Technology**

Six DNA samples were prepared for quantitative bulk segregant analysis (BSA) comprising of 'Sukali Ndiizi' (the susceptible parent), 'TMB2X8075-7' (the resistant parent), a resistant bulk (40 F<sub>2</sub> plants, 10  $\mu$ g of DNA per plant), a susceptible bulk (40 F<sub>2</sub> plants, 10  $\mu$ g of DNA per plant) and two random bulks (mix of 20 susceptible and 20 resistant F<sub>2</sub> plants, with 10  $\mu$ g of DNA per plant). The DArT-BSA assay was performed on the DNA samples by Diversity Arrays Technology, 1 Wilf Crane Crescent, Yarralumla ACT2600 in Australia.

### **Restriction enzyme digestion and adapter ligation**

A combined digestion/ligation reaction was prepared by adding 1  $\mu$ L of DNA for each sample to 9  $\mu$ L of digestion/ligation mix. The digestion/ligation reaction mixture contained a buffer (100 mM Tris-acetate (OAc), 500 mM KOAc, 100 mM Mg(OAc)<sub>2</sub>, 50 mM DTT, pH7.8), 0.1  $\mu$ L of BSA (New England/ Massachusetts, USA) 0.2  $\mu$ L of 50 mM ATP, 0.1 $\mu$ L of 5  $\mu$ M PstI adapter, 0.1  $\mu$ L of PstI (20 U/ $\mu$ L NEB), 0.1  $\mu$ L *TaqI* (20 U/ $\mu$ L NEB), 0.2  $\mu$ L of T4 DNA ligase (30 Weiss units/ $\mu$ L NEB) and 7.2  $\mu$ L of ultrapure water. The samples were incubated at 37°C for 90 minutes and at 60°C for 90 min, heat inactivated at 80°C for 20 min and stored at -20°C until used.

### **PCR amplification of the genomic representation**

PCR was used to create a genomic representation containing *PstI* fragments. Forty-nine  $\mu$ L of PCR mix were added to 1 $\mu$ L of *PstI/TaqI* digestion/ligation reaction as the PCR template. The PCR mix contained 50 $\mu$ M of each dNTP, 0.004U of RED Taq (Sigma-Aldrich Missouri, USA) 0.4 $\mu$ M of *PstI* (Sigma-Aldrich Missouri, USA), 1X PCR buffer (100mMTris-HCl pH8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>, 0.1% gelatin (Sigma-Aldrich Missouri, USA) in 39 $\mu$ L of ultrapure water. The PCR amplification program consisted of an initial step at 94°Cfor1min, 30 cycles at 94°C for 20s, 58°C for 40s, and 72°C for 1 min, and a final step at 72°C for 7 min. Five microliters of PCR product were analyzed on 1.2% agarose gels to confirm that a homogeneous smear of fragments was obtained and to visualise the size distribution. All representations and 'representation bulks' were purified, labelled with cy3 or cy5, and hybridized to DArT arrays together with the FAM-label polylinker of the Topo vector (Life

Technologies, Texas, USA). The DArT arrays contained 48 sub-arrays with 2304 polymorphism-enriched clones printed in duplicate and 384 control clones, each printed six times (6912 array features in total). The arrays were printed with a MicroGrid II arrayer (Biorobotics, Cambridge, UK) on SuperChip poly-L-lysine slides (Erie Microarray, Portsmouth NH, USA) using DArT-spotter (buffer for heavy-duty microarray printing). After an overnight hybridization at 62°C, the arrays were washed and scanned on a LS300 confocal laser scanner (Tecan, Grödig, Austria) with 10-µm resolution at 543 nm (cy3), 633 nm (cy5) and 488 nM (FAM). Array images were analyzed with DArTsoft 7.4 (Diversity Arrays Technology P/L, Canberra, Australia). The logarithm of the ratio between the two background-subtracted averages of feature pixels in the cy3 and the cy5 channel ( $\log_2 [\text{cy3}/\text{cy5}]$ ) was used as a measure of the difference in abundance of the corresponding DNA fragment in the two representations hybridized to an array.

### Sequencing DArT clones

*Escherichia coli* clones were co-cultured with polymorphic DArT markers at 30°C for 22 hrs in Terrific broth (Amresco, Ohio USA) in 384-deep-well microtiter plates. Plasmid DNA, isolated using the Eppendorf Perfect Prep Plasmid 384 procedure, and sequenced in both directions using the M13R (5'-GGAAACAGCTATGACCATG-3') and T7-ZL (5'-TAATACGACTCACTATAGGG-3') primers with the Applied Biosystems Big Dye Cycle sequencing chemistry. Following an ethanol precipitation cleanup step, the reactions were run on an Applied Biosystems 3730xl (Life Technologies, California, USA) capillary electrophoresis instrument. Vector sequences and *Pst*I sites were trimmed so as not to introduce biased similarity among DArT clones in the analyses.

### Estimating genetic distance

The scored DArT markers were used to estimate genetic similarity between the parents and the phenotypic bulks using Jaccard's similarity co-efficient (GS) (Murguia and Villasenor, 2003):

$$GS_{ij} = a / (a + b + c),$$

where  $GS_{ij}$  corresponds to the genetic similarities between genotype (or phenotypic bulk) **I** and genotype (or phenotypic bulk) **J**. 'a' is the number of polymorphic DArT clones present in both genotypes (or phenotypic bulks) **I** and **J**, 'b' is the DArT clones present in genotype (or phenotypic bulk) **I** but absent in **J**, and 'c' is the number of DArT clones present in genotype or phenotypic bulk) **J** but absent in **I**.

## **Bulk Segregant Analysis**

Theoretically, Foc race 1-resistant diploids in the 'SN8075' F<sub>2</sub> population in the resistant bulk should have inherited 100% of the alleles for resistance from the resistant parent 'TMB2X8075'. While the susceptible diploids in the susceptible bulk should have inherited 33% of the triploid susceptible parent's alleles and 50% from the male parent 'TMB2X8075-7'. A Chi square test for 'goodness of fit' was applied for each DArT marker separately to assess the extent to which the hybridisation intensity (variant frequency) at the marker site deviated from the expected inheritance probability of the resistant and the susceptible bulk. Linkage was then revealed by markers that are present in the resistant parent and the resistant bulk and absent in the susceptible parent and susceptible bulk (coupling), or markers present in the susceptible bulk and parent and absent in the resistant parent and bulk (repulsion).

## **RESULTS**

### **Genetic distances of 'TMB2X8075-7' and 'Sukali Ndiizi'**

In the DArT-BSA assay, 14354 high quality DArT markers were scored and sequenced, each with an average of 69 nucleotide bases. The presence or absence of data of DArT markers was used to estimate the genetic similarity between the parents and the phenotypic bulks by Jaccard's similarity co-efficient (GS). The genetic similarity was highest between the resistant and susceptible bulks (0.61) and lowest between the Foc race 1-susceptible parent 'Sukali Ndiizi' and the two phenotypic (resistant and susceptible) bulks (0.03). The genetic similarity between the two parents was estimated to be 0.39 (Table 1). This implies that there were more allelic differences between the parents in comparison to allelic differences between the phenotypic bulks of their F<sub>2</sub> progenies.

### **DArT markers associated with resistance to Foc race 1**

One hundred and one DArT markers showed polymorphism between the parents and bulks, thereby suggesting their association with Fusarium wilt resistance or susceptibility (Table 2). The nucleotide sequences of these 101 distinct DArT markers were mapped *in silico* to either the annotated genes of the *Musa* genome (DH Pahang genes V1 database) or the full *Musa* reference genome sequence (Pahang pseudochromosome v1 database)(<http://banana-genome.cirad.fr/blast>, E-value ≤ 1e<sup>-10</sup>). Thirteen of these DArT markers were associated in a coupling phase linkage with the gene *pd1*, and were present only in the resistant parent and the resistant bulk. Eleven of these DArT markers linked with resistance to Foc race 1 in coupling showed significant similarities with the *Musa* reference



genome DH Pahang on seven chromosomes (Fig. 1). Eighty-eight DArT markers were present in only the susceptible parent and the susceptible segregants, and were thus associated in repulsion phase linkage with the *pd1* gene.

One of the DArT makers linked with resistance to Foc race 1 in the coupling phase (100025374|F|0) had a plant resistance gene in its proximity when viewed on Gbrowse on the Banana Genome Hub ([http://banana-genome.cirad.fr/cgi-bin/gbrowse/musa\\_acuminata](http://banana-genome.cirad.fr/cgi-bin/gbrowse/musa_acuminata)). This nucleotide-binding sites (NBS) and Leucine rich repeat (LRR) putative disease resistant gene CNL B19 (Fig. 2) was identified within 20 kbs from the location of this DArT marker on chromosome 1, and could be a likely candidate for the Foc race 1-resistance gene, *pd1*. Five markers that are linked with resistance to Foc race 1 in the coupling phase had significant similarities ( $1e^{-10}$ ) to genes annotated to the *Musa* reference genome. These include genes for Laccase-25 (LAC25), SWIM zinc finger family protein, Homeobox-leucine zipper protein, Uncharacterized isochorismatase family protein and expressed proteinPomt1 (Table 3).

Eighty three DArT clones linked with resistance to Foc race1 in repulsion phase linkage showed significant similarities ( $1e^{-10}$ ) to the *Musa* reference genome DH Pahang pseudochromosome v1 database. After a blast search, 31 of the DArT markers linked with resistance to Foc race1 in repulsion phase showed significant similarity ( $1e^{-10}$ ) with genes annotated on the *Musa* reference genome (Table 4). These genes represented genomic region(s) that could be required for susceptibility to Fusarium wilt in *Musa* sp. Most of these genes were categorised to be involved in biological and molecular functions in the Panther database (<http://www.pantherdb.org>) (Fig. 3).

## DISCUSSION

A high level of molecular diversity was obtained between the two parent genotypes, 'Sukali Ndiizi' and 'TMB2X8075-7'. This could be attributed to either difference in ancestry, ploidy and/or genomic composition. 'Sukali Ndiizi' is a triploid dessert banana with the genomic composition of AAB, while 'TMB2X 8075-7' is a diploid hybrid banana with a genomic composition of AA. The high genetic dissimilarity of parents is an important determinant in attempts to successfully identify reliable molecular markers to use for MAS (Michelmore *et al.*, 1991). The phenotypic bulks are less similar from each of the parents than the two parents are from each other (Table 1). This could be attributed to allele dilution, which reduces the likelihood of detecting some of the low frequency alleles in bulk DNA (Reyez-valdez *et al.*, 2013).

Resistance to Foc race 1 has previously been demonstrated as a simple inherited trait, controlled by a single recessive gene *pd1* (Ssali *et al.*, 2013). This implies that the 13 DArT clones linked in coupling to Foc race 1 resistance could be useful in introgressing the *pd1* gene into breeding materials. The available sequence information of these candidate DArT markers, of about 69-bp each, can be used to convert the DArT markers into PCR-based molecular markers. Unfortunately, limited success has been reported in the development and use of PCR-based molecular markers to select for disease resistance in banana breeding for disease resistance. For instance, a SCAR marker linked to Sigatoka leaf spot disease (*Mycosphaerella musicola* Leach) failed to distinguish between resistant and susceptible genotypes due to either the amplification conditions, limited number of primers or, most importantly, the absence of tight linkage with the gene of interest (Nwauzoma *et al.*, 2011).

The presence of a plant *R* gene in the proximity of the DArT marker (100025374|F|0), which is linked with resistance to Foc race 1 in the coupling phase, indicates that the *R* gene could be involved in Fusarium wilt resistance. *R* genes have been isolated from various plants containing NBS and LRR domains. The conserved nature of motifs within these domains has been exploited to search for resistance gene analogues (RGAs) in the *Musa* gene pool by using a homology-dependent PCR-based approach (Miller *et al.*, 2008; Mohamed, 2008). Banana *R* genes *RGC2* and *RGC5* were also isolated from *Musa acuminata* ssp *malacensis* as candidates genes for protecting bananas against Foc race 4 (Peraza-Echeverria *et al.*, 2008). Despite these efforts, the success of *R* genes in protecting bananas against Foc race 4 has been limited. Though most *R* genes in bacterial and fungal plant-pathogen interactions show dominant inheritance, some recessively inherited *R* genes like barley *mlo* (Buschges *et al.*, 1997), *Arabidopsis* *RRS1-R* (Deslandes *et al.*, 2002), rice *xa13* (Chu *et al.*, 2006), and *xa5* (Iyer and McCouch, 2004; Jiang *et al.*, 2006) have been reported. The *R* proteins encoded by dominant *R* genes recognize specific pathogen effectors and trigger signal transduction cascades that lead to rapid disease resistance in the host plant (Dangl and Jones, 2001). However, the function of *R* proteins inherited recessively is not generally understood. Therefore, DArT maker (100025374|F|0) could be useful in validating the co-segregation of the putative *R* gene, with Foc race 1 resistance in subsequent studies and could renew interest in deploying *R* genes in protecting bananas against Foc.

Five DArT clones linked with Foc race 1 resistance in 'coupling' had significant similarities ( $1e^{-10}$ ) with genes annotated to the *Musa* reference genome. These include genes coding for Lacasse 25, Putative uncharacterized isochorismatase family protein, WIM zinc finger family protein, Homeobox-leucine zipper protein (HOX32) and expressed protein (Pomt1). None of these genes, though, are known to be involved directly as defence genes.

However, *Lacasse 25* might play a role in plant defense, as it codes for an enzyme involved in the lignin degradation and detoxification of lignin-derived products, and may be important in regulating products of the phenylpropanoid pathway. These products have been reported to be induced over 40-fold during the infection process of Foc in resistant bananas (Li *et al.*, 2013). Some genes though not known for disease resistance have been reported to play key roles in defence. For instance, the recessively inherited *R* gene *xa13* is involved in both disease resistance and pollen development (Chu *et al.*, 2006). Further investigations, by cloning and functional analysis of these genes, have potential to unravel defence roles for these genes.

The 31 genes that are significantly similar ( $1e^{-10}$ ) to DArT clones linked in 'repulsion' to Foc race 1 resistance could be genomic regions required for susceptibility to Foc race 1 in *Musa*. Genes associated with susceptibility to pests and diseases in crops have been reported before, including susceptibility to yellow stem borer in rice (Selvi *et al.*, 2002), black root rot in tobacco (Bai *et al.*, 1995) and club root rot in Brassicaceae (Pink *et al.*, 1994). Such 'susceptibility factors' could be similar to genes such as *PMR6*, which is required for susceptibility to powdery mildew in *Arabidopsis* Col-0 (Vogel *et al.*, 2002). The role of genes conferring susceptibility to Fusarium wilt in bananas can be validated by inducing gene-specific mutations using self-complementary chimeric oligonucleotides (COs) to create stable, site-specific base substitutions in these genes to generate mutants (Beetham *et al.*, 1999).

The current study demonstrated that DArT-Bulk segregant analysis is a good strategy to identify markers associated with traits of interest in banana. BSA allowed us to identify DArT markers associated with the resistance gene *pd1*, either in coupling or repulsion phase linkage. Bulk segregant analysis is the first step towards finding markers that co-segregate with a given phenotype. These candidate markers could be used in combination to predict the presence or absence of the *pd1* gene in breeding populations. MAS can be a useful strategy to improve disease resistance in crop plants (Josh and Nayak, 2010), especially in bananas, where a long generation time (18 months) and large space is required (6-9 m<sup>2</sup> per plant) to breed bananas with disease resistance. Furthermore, these markers can also be used as indicators of genetic constitution in developing reliable breeding schemes that can maximize heterosis and reliably predict the specific combining ability of parental genotypes.

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**Table 1.** Jaccard's similarity co-efficient (GS) between parents and bulks of bananas resistant and susceptible to *Fusarium oxysporum* f. sp. *cubense* race 1 estimated from markers of the DArT assay

	Sukali Ndiizi	TMB2X8075	Susceptible bulk	Resistant bulk
Sukali Ndiizi	-			
TMB2X8075	0.39	-		
Susceptible bulk	0.03	0.22	-	
Resistant bulk	0.03	0.29	0.61	-

**Table 2.** *In-silico* mapping of Diversity Array Technology-Bulk Segregant Analysis assay markers unto *Musa* reference genome (<http://banana-genome.cirad.fr>)

Description	Number
1. Total number of high quality markers scored and sequenced	14354
2. Number of DArT markers showing polymorphism between the susceptible and resistant parents and bulks	101
3. DArT markers associated with resistance to Foc race1 in coupling	13
4. DArT makers associated with resistance to Foc race 1 in coupling with significant similarities ( $1e^{-10}$ ) with genes on the <i>Musa</i> reference genome DH Pahang genes V1 database after blast search	5
5. DArT makers associated with resistance to Foc race 1 in coupling with significant similarities ( $1e^{-10}$ ) with on the <i>Musa</i> reference genome DH Pahang pseudochromosomes V1 database after blast search	11
6. DArT markers associated with resistance to Foc race1 in repulsion	88
7. DArT markers associated with resistance to Foc race1 in repulsion phase linkage with significant similarities ( $1e^{-10}$ ) with the <i>Musa</i> reference genome DH Pahang pseudochromosomes V1 database after blast search	83
8. DArT markers associated with resistance to Foc race1 in repulsion with significant similarities( $1e^{-10}$ ) with the <i>Musa</i> reference genome DH Pahang genes database V1 after a blast search	31
9. DArT markers associated with resistance to Foc race1 in repulsion with significant similarities( $1e^{-10}$ ) with the <i>Musa</i> reference genome DH Pahang database at more than one loci after a blast search	4

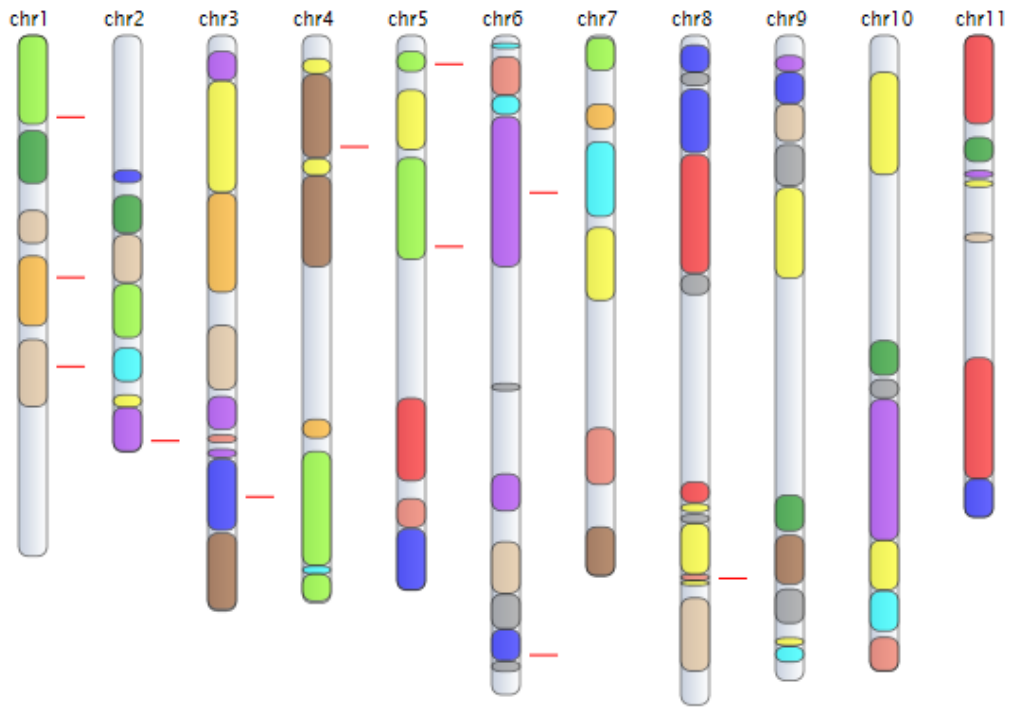
**Table 3.** BlastX identities of DArT markers associated with resistance to *Fusarium oxysporum* f. sp. *ubense* race1 in coupling phase linkage with significant similarities with the *Musa* reference genome DH Pahang genes V1 database

<b>DArT clone</b>	<b>Locus</b>	<b>Annotated Function</b>	<b>E-value</b>
100011297 F 0	GSMUA_Achr6P12470_001	Putative Uncharacterized isochorismatase family protein (hydrolases)	3E-32
100023812 F 0	GSMUA_Achr1P05220_001	SWIM zinc finger family protein, putative, expressed. Involved in early stages of the homologous recombination repair (HRR) pathway of double-stranded DNA breaks arising during DNA replication or induced by DNA-damaging agents	4E-16
100025294 F 0	GSMUA_Achr5P02420_001	Homeobox-leucine zipper protein (HOX32) complete transcriptional regulation/factor	1E-28
100011250 F 0	GSMUA_Achr6P33880_001	Laccase-25 (LAC25) complete	3E-29
100019822 F 0	GSMUA_Achr4P07960_001	Expressed proteinPomt1 complete Transfers mannosyl residues to the hydroxyl group of serine or threonine residues. Co-expression of both POMT1 and POMT2 is necessary for enzyme activity, expression of either POMT1 or POMT2 alone is insufficient	2E-27

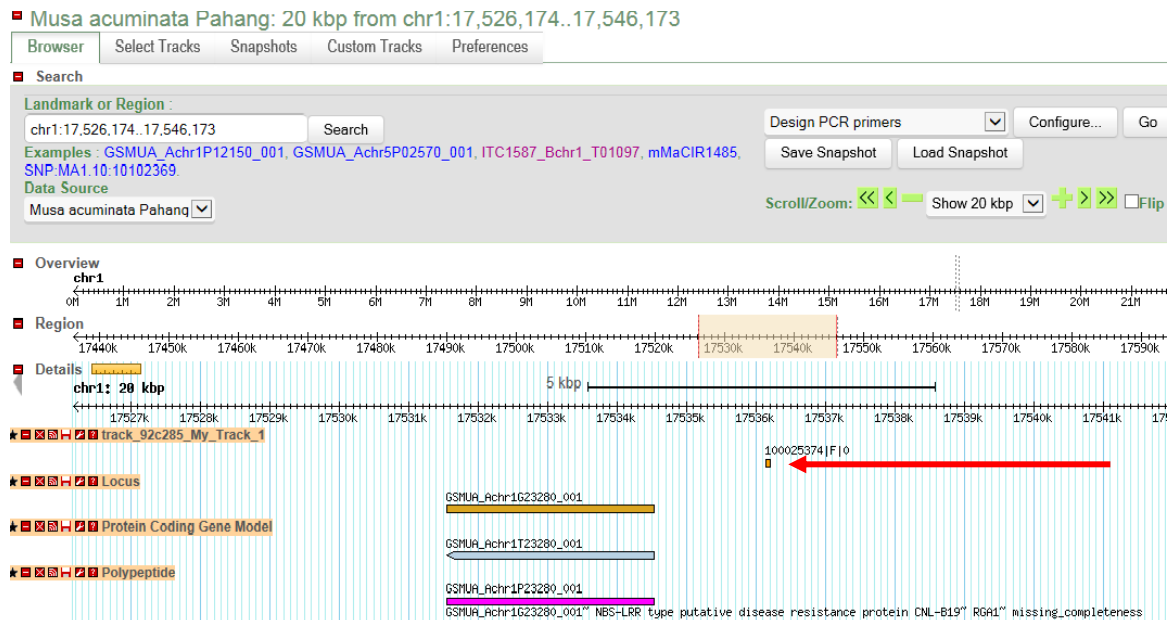
**Table 4.** BlastX identities of DArT markers associated with resistance to *Fusarium oxysporum* f. sp. *cubense* race1 in repulsion with significant similarities with genes annotated on the *Musa* reference genome

<b>DART marker</b>	<b>Locus ID</b>	<b>Annotated Function</b>	<b>Expect Value</b>
100003486 F 0	<a href="#">GSMUA_Achr1P02570_001</a>	Kinesin motor protein-related, putative, expressed~ ATK4~ fragment	8E-30
100004396 F 0	<a href="#">GSMUA_Achr10P29590_001</a>	Expressed protein~ SYDE2~ complete	3E-20
100005759 F 0	<a href="#">GSMUA_Achr8P12360_001</a>	Peroxidase 5~ GSVIVT00037159001~ complete	2E-27
100007616 F 0	<a href="#">GSMUA_Achr1P00620_001</a>	Putative Phosphorylated carbohydrates phosphatase TM_1254~ TM_1254~ modules	8E-30
100007676 F 0	<a href="#">GSMUA_Achr11P01800_001</a>	Hypothetical protein~ SDE3~ missing_functional_completeness	3E-32
100008351 F 0	<a href="#">GSMUA_Achr6P12520_001</a> <a href="#">GSMUA_Achr8P16310_001</a>	Expressed protein~ TNeu053m17.1~ complete	4E-13 3E-32
100009080 F 0	<a href="#">GSMUA_Achr2P23110_001</a>	Hypothetical protein~ bdp1~ missing_functional_completeness	3E-32
100011082 F 0	<a href="#">GSMUA_Achr5P04270_001</a>	Putative Zinc finger protein CONSTANS-LIKE 9~ COL9~ complete	5E-25
100013873 F 0	<a href="#">GSMUA_Achr6P10790_001</a>	Ribulose-phosphate 3-epimerase, chloroplastic (Fragment)~ RPE~ complete	3E-32
100016347 F 0	<a href="#">GSMUA_Achr10P12160_001</a> <a href="#">GSMUA_Achr8P13850_001</a> <a href="#">GSMUA_Achr6P03130_001</a> <a href="#">GSMUA_Achr5P23590_001</a>	Pollen-specific protein C13~ MGS1~ complete	2E-15 1E-25 3E-23 3E-23
100017821 F 0	<a href="#">GSMUA_Achr11P05050_001</a>	Tetratricopeptide repeat domain containing protein, expressed~ MBB1~ complete	3E-32
100019109 F 0	<a href="#">GSMUA_AchrUn_randomP25990_001</a>	Hypothetical protein~ PEX1~ missing_functional_completeness	8E-24
100019295 F 0	<a href="#">GSMUA_Achr4P24130_001</a>	F-box/kelch-repeat protein SKIP11~ SKIP11~ modules	7E-12
100019852 F 0	<a href="#">GSMUA_Achr11P24440_001</a>	SLT1 protein, putative, expressed~	2E-30

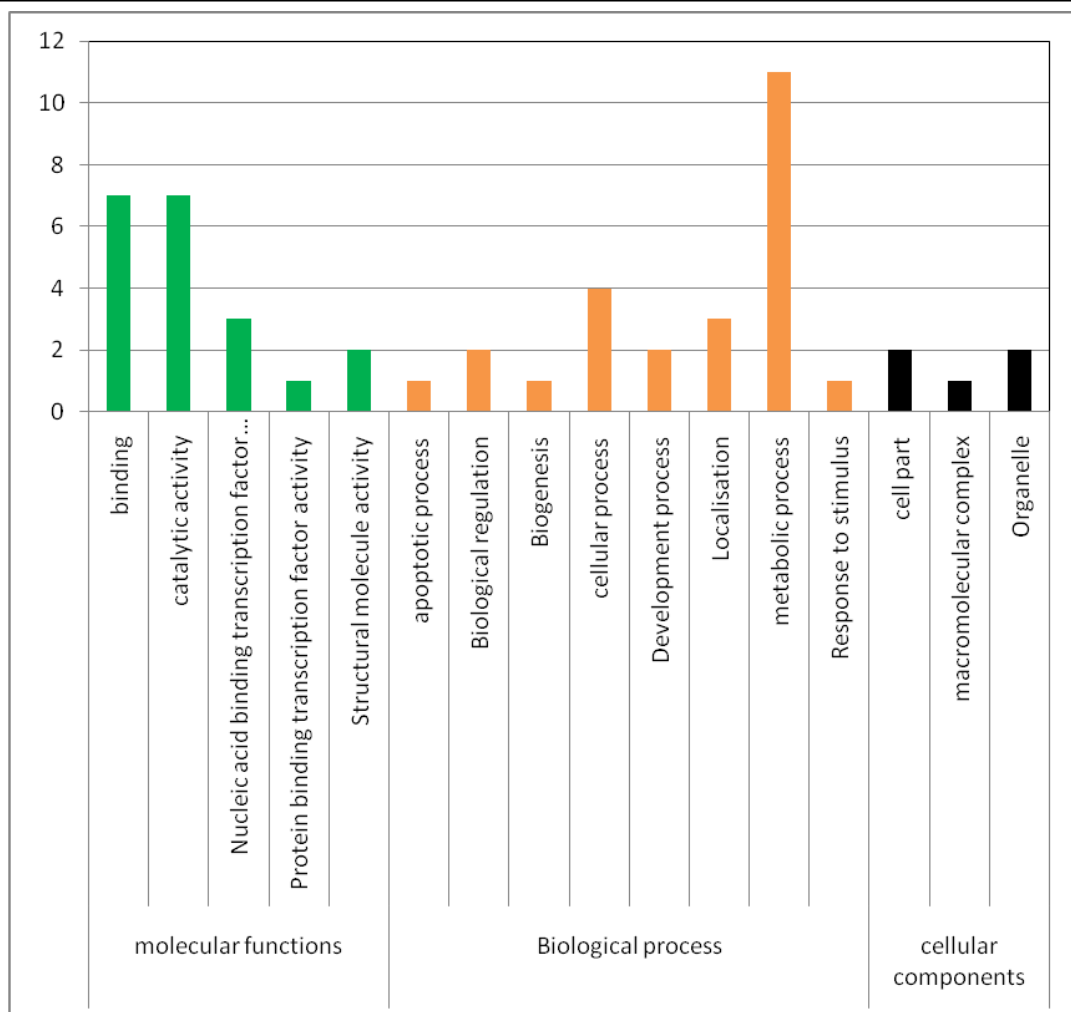
		WDR60~ complete	
100021118 F 0	<a href="#">GSMUA_Achr11P04290_001</a>	Adenine phosphoribosyltransferase 1~ APT1~ complete	3E-17
100021608 F 0	<a href="#">GSMUA_Achr9P08600_001</a>	Transcription factor MYB3~ MYB3~ complete	3E-32
100022032 F 0	<a href="#">GSMUA_Achr2P17340_001</a>	F-box protein At4g18380~ At4g18380~ complete	2E-27
100022181 F 0	<a href="#">GSMUA_Achr6P01450_001</a>	Putative DNA topoisomerase 3-alpha~ Top3a~ complete	3E-26
100022379 F 0	<a href="#">GSMUA_Achr9P22210_001</a>	Putative E3 ubiquitin-protein ligase At4g11680~ At4g11680~ fragment	4E-13
100022539 F 0	<a href="#">GSMUA_Achr1P17040_001</a>	Peptidylprolyl isomerase domain and WD repeat-containing protein 1~ PPWD1~ complete	3E-32
100022881 F 0	<a href="#">GSMUA_Achr9P00560_001</a> <a href="#">GSMUA_Achr1P21010_001</a>	Putative Predicted protein~ BEE1~ complete	3E-32 2E-21
100023686 F 0	<a href="#">GSMUA_Achr4P25550_001</a>	GDSL esterase/lipase EXL3~ complete	3E-32
100024494 F 0	<a href="#">GSMUA_Achr7P16480_001</a>	Pre-mRNA branch site p14-like protein~ At5g12190~ complete	2E-27
100024995 F 0	<a href="#">GSMUA_Achr8P03630_001</a>	22.3 kDa class VI heat shock protein~ HSP22.3~ complete	3E-32
100025437 F 0	<a href="#">GSMUA_Achr10P16700_001</a>	Hypothetical protein~ RFS2~ missing_functional_completeness	3E-32
100026327 F 0	<a href="#">GSMUA_Achr2P03770_001</a>	Endoglucanase 13~ GLU6~ complete	3E-32
100026357 F 0	<a href="#">GSMUA_Achr4P09180_001</a>	Putative Cytochrome P450 724B1~ CYP724B1~ complete	8E-30
100027383 F 0	<a href="#">GSMUA_AchrUn_randomP15600_001</a>	expressed protein~ ycf2-A~ complete	3E-23
100028187 F 0	<a href="#">GSMUA_Achr3P10360_001</a>	DNA polymerase lambda, putative, expressed~ Poll~ complete	3E-32
100029068 F 0	<a href="#">GSMUA_Achr6P05400_001</a>	Coatomer subunit beta'-1~ Os06g0143900~ complete	3E-32
100029511 F 0	<a href="#">GSMUA_Achr5P18580_001</a> <a href="#">GSMUA_Achr11P22830_001</a>	Glycosyl transferase 8 domain containing protein, putative, expressed~ Gyg1~ complete	2E-15 3E-32



**Figure 1.** Location of the DArT markers (red bars) linked to resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 in coupling phase with significant similarity on the *Musa acuminata* Pahang reference genome after Blast search (E value  $1e^{-10}$ ). Distinct colours on the chromosomes indicate the 12 *Musa*  $\alpha/\beta$  ancestral blocks (Dhont *et al.*, 2012).



**Figure 2.** Evidence of the presence of a resistance gene (NBS–LRR type putative resistance protein CNL-B19-Orange block) within 20 kbps of a DArT marker (shown by the red arrow) associated with resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 in coupling on the *Musa acuminata* Pahang reference genome on Gbrowse.



**Figure 3.** Functional categorization of genes of the *Musa* reference genome DH Pahang (genes database V1) mapped by DArT markers in linkage disequilibrium to *Fusarium oxysporum* f. sp. *ubense* race 1 in repulsion phase (annotations for molecular functions-green bar graphs, biological processes-orange bar graphs and cellular components-black bar graphs).

## CHAPTER 4

### Transcriptome analysis of African bananas following infection with *Fusarium oxysporum* f. sp. *cubense* race 1

#### ABSTRACT

*Fusarium* wilt (Panama disease), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is responsible for significant losses of bananas worldwide. Host resistance, as part of an integrated disease management strategy, offers the most effective means to control banana *Fusarium* wilt. Resistance to Foc is induced in the roots, which is the primary point of infection, to impede the progress of the fungus by means of cell wall strengthening and occlusion of the vascular vessels. An understanding of the genetic basis of the Foc-banana interaction will assist breeders and biotechnologists in developing resistant cultivars. In this study, next-generation RNA sequencing (RNA-Seq) was employed to analyse the transcriptional changes taking place in banana roots following infection by Foc. RNA was extracted from the roots of the Foc race 1-susceptible banana 'Sukali Ndiizi' (AAB), the resistant banana 'TMB2X8075-7'(AA) and the immune genotype 'Mbwazirume' (AAA), at four time points (0, 48, 96 and 192hrs) post inoculation. Complementary DNA (cDNA) libraries derived from Foc race 1-infected roots were sequenced to obtain 299.9 million (M) raw reads of about 100-nucleotides each. The sequences were mapped onto the *Musa* reference genome, and 10136 genes were found to be differentially expressed (DEGs). Of these, 5640 genes (55.7%) were uniquely up-regulated, while 4496 genes (44.4%) were uniquely down-regulated in the three genotypes. DEGs were annotated with Gene Ontology terms and by pathway enrichment analysis. The significant pathway categories identified included the following: 'Metabolic', 'Ribosome', 'Plant-pathogen interaction' and 'Plant hormone signal transduction' pathways. SA and ET were stimulated in the 'Plant hormone signal transduction' pathway in all three genotypes. Several candidate genes and pathways that may contribute to *Fusarium* wilt resistance in banana were identified. These genes could potentially be used in the improvement of bananas for *Fusarium* wilt resistance.

## INTRODUCTION

Fusarium wilt of bananas (also known as Panama disease), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is a serious problem to banana production worldwide. Three races of this pathogen (Foc races 1, 2 and 4) have been identified based on the pathogenicity of isolates to a set of banana differential cultivars (Stover and Buddenhagen, 1986; Ploetz, 2005). Prominent symptoms of banana Fusarium wilt include yellowing of the foliage, splitting of the pseudo stem base and vascular discoloration (MacHardy and Beckman, 1981). Fusarium wilt can be devastating, with losses as high as 100% in susceptible banana cultivars. The fungal pathogen infects the roots following adhesion of chlamydospores and conidia to the root hairs and root epidermal cell surfaces (Bishop and Cooper, 1983; Li *et al.*, 2011; Yin *et al.*, 2011). Once attached to the root surface, the infective propagule germinates and infects young roots in the region of active cell division. Foc can penetrate banana roots directly or enter the vascular tissue through wounds (Lucas, 1998). While mechanical wounding increases infection of banana roots with Foc, it is not essential for infection to occur (Stover, 1962). Once inside root cells, fungal growth proceeds rapidly to produce a network of branching hyphae which expands by growing in the intercellular spaces along the junctions of root epidermal cells. From inside cells, Foc colonizes neighbouring cells through pores in cell end plates (Beckman *et al.*, 1961; Beckman *et al.*, 1962; Bishop and Cooper, 1983; Li *et al.*, 2011).

Numerous control strategies have been devised to prevent damage caused by Fusarium wilt of bananas. Crop rotation, flood following, chemical fumigation and the use of organic amendments were unsuccessful in controlling the disease effectively (Herbert and Marx, 1990; Moore *et al.*, 1995). Foc can persist in infested banana fields for an extended period of time. It survives in organic matter and in the soil as dormant chlamydospore in the absence of a suitable host. This survival has made attempts to devise cultural or chemical control options futile. Therefore, host plant resistance remains the most effective, economical and environmentally friendly approach to control the disease. Resistant bananas can be developed by conventional plant breeding or genetic modification (Ortiz and Swennen, 2014). Conventional banana breeding has generated Fusarium wilt-resistant varieties in the past (Rowe, 1990; Damodaran *et al.*, 2009), but the process is slow due to the long generation period of the crop, complex banana genetics and the limited amount of genetic tools (Ortiz and Swennen, 2014).

Resistance in plant-pathogen systems is controlled by constitutive and induced defence functions of the host. The success of induced resistance depends on the rate and extent of the host response. In banana roots the resistance response is based on the ability of tolerant or resistant plants to produce phenolics, deposit lignin, and increase enzymes



involved in cell wall strengthening (Beckman, 1989; De Ascensao and Dubery, 2000). Phenolic compounds play a particularly important role as they inactivate the hydrolytic enzymes of the pathogen and are incorporated into host cell walls and vascular gels to lignify them (MacHardy and Beckman, 1981; Beckman, 1987). Vascular occluding gels are formed 24-48 hrs after infection and tyloses, which block the lumina of infected xylem vessels, within 2-3 days (Beckman, 1987). The gels and tyloses are lignified to physically block the infection site and isolate the pathogen (Beckman and Halmos, 1962). This may contribute to the failure of the water transport system, thus contributing to the typical wilt symptoms. Vascular occlusion represents a general, non-specific defence response in both susceptible and resistant hosts. In resistant hosts the gels and tyloses persist for several days (Mace, 1963; Beckman and Talboys, 1981). In susceptible plants, however, gels seem to weaken and shear, thereby failing to stop the advance of the pathogen (Vander Molen *et al.*, 1977; 1987).

The molecular basis of resistance in banana to *Fusarium* wilt has been investigated by suppression subtractive hybridisation, transcriptomic profile analysis and proteomic profile analysis (Van den Berg *et al.*, 2007; Li *et al.*, 2011; Ravishankar *et al.*, 2011; Li *et al.*, 2012; Li *et al.*, 2013a; Li *et al.*, 2013b). These investigations mostly involved the response of the commercial Cavendish banana variety to Foc race 4. Transcripts and proteins identified in these studies included those associated with the Shikimate-phenylpropanoid lignin and cellulose biosynthesis pathways (Li *et al.*, 2012; Li *et al.*, 2013a), reactive oxygen species (Li *et al.*, 2011), pathogenesis-related (PR) proteins (Li *et al.*, 2013b) and inhibitors of plant cell wall-degrading enzymes secreted by the fungus (Ravishankar *et al.*, 2011). The rate and extent of expression of defence genes differed between resistant and susceptible varieties (Van den Berg *et al.*, 2007).

The availability of the 'A' and 'B' banana reference genome sequences derived from *Musa acuminata* Colla and *M. balbisiana* Colla, respectively, provides an opportunity to analyse the defence response in bananas to Foc infection and to identify genes of interest for potential use in plant improvement programmes (Dhont *et al.*, 2012; Davey *et al.*, 2013). *Musa acuminata* and *M. balbisiana* are believed to be the progenitors of all cultivated banana varieties. The aim of this study was to identify transcripts associated with resistance of three banana varieties grown in east and central Africa to Foc race 1.

## **MATERIALS AND METHODS**

### **Inoculation of bananas with Foc**

*Foc isolate used and inoculum production:* Foc race 1 was isolated from the infected vascular tissues of a diseased 'Sukali Ndiizi' banana plant at National Agricultural Research

Laboratories (NARL), Kawanda in Uganda. The discoloured tissue was first surface disinfected by submerging it in 75% ethanol for 2 min, and then plated out onto 1/4 strength potato dextrose agar (PDA) amended with streptomycin (100 µg/ml streptomycin sulphate per Petri dish). Developing Foc colonies were purified by sub-culturing, then single-spored and maintained on carnation leaf agar (CLA) at NARL. The culture was identified at Stellenbosch University as VCG 0124 according to the methodology described by Puhalla (1985).

Fungal inoculum for plant infection was produced by cultivating the Foc race 1 isolate on 1/4 strength PDA for 7 days at 25°C. To stimulate conidial production, a 1-cm<sup>3</sup> agar plug containing fungal mycelia was then transferred from the PDA plates into Armstrong liquid medium (Booth, 1971). The Armstrong medium consisted of 20g sucrose, 400mg MgSO<sub>4</sub>.7H<sub>2</sub>O, 1.6g KCl, 1.1g KH<sub>2</sub>PO<sub>4</sub>, 5.9g Ca(NO<sub>3</sub>)<sub>2</sub>, 0.2 g/ml FeCl<sub>3</sub>, 0.2 g/ml MnSO<sub>4</sub> and 0.2 g/ml ZnSO<sub>4</sub>. The spore suspension was incubated at 25°C and shaken twice a day. After 7 days it was washed twice with sterile distilled water, and its concentration adjusted to 10<sup>6</sup> spores/ml, using a haemocytometer (Singleton *et al.*, 1992).

*Planting material and inoculation:* Tissue-cultured plantlets of three banana varieties grown in east and central Africa were used in the study. These varieties were 'Sukali Ndiizi' (AAB), a triploid dessert banana susceptible (disease development occurs) to Fusarium wilt; 'TMB2X8075' (AA), a diploid banana hybrid resistant (restricts disease development) to Fusarium wilt; and 'Mbwazirume' (AAA), a triploid East African highland banana (EAHB) land race immune (wards off pathogen attack) to Foc race 1. The plantlets were all planted in polythene pots containing 10 kg of sterile loam soil and allowed to grow for 3 months in a screen house under tropical conditions, with daily temperatures of about 28°C and with approximately 12 hours of light, before inoculation.

For inoculation, the banana plantlets were removed from their pots, their roots washed clean and soaked in 500 ml of the Foc race 1 spore suspension for 30 minutes. Control plants were soaked in sterile distilled water. Twelve plants of each treatment were then replanted into plastic polythene pots containing 10 kg of sterile loam soil. The inoculated plantlets were maintained in a screen house with day temperatures about 28°C and approximately 12 hours of light until sample collection 0, 48, 96 and 192 hrs post inoculation. Each sample was then washed to remove all potting soil, before about 1g of root tissue was excised and immediately wrapped in aluminium foil, flashed in liquid nitrogen and stored at -80 °C until further use.

### **RNA isolation, library construction and sequencing**

*RNA isolation:* Frozen banana root tissue was ground to a fine powder by using a 0.1% DEPC-treated mortar and pestle that was pre-chilled in liquid nitrogen. Total RNA was extracted from the finely-ground banana roots using the PureLink® RNA mini kit (Life Technologies, Austin, USA), following the manufacturer's instructions. Genomic DNA (gDNA) was removed from each sample by using RNase-free DNase I according to the manufacturer's instructions (Life Technologies, Austin, USA). RNA purity was confirmed with a Nanodrop 2000 spectrophotometer (Thermo scientific, Pittsburgh, PA) to ascertain a sample purity of OD<sub>260/280</sub>=1.8~2.2. Three samples (10µg each) of banana root RNA samples per treatment were packed in Gentergra tubes for transcriptome sequencing at the DNA core facility of the Bond Life Sciences Center at the University of Missouri, Columbia, USA.

*Library preparation and sequencing:* Total RNA samples sent to the University of Missouri were checked for RNA degradation and contamination on 1% agarose gels. RNA integrity was also established by using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). After quality control, RNA samples for transcriptome analysis were prepared for each treatment using Illumina's kit (San Diego, USA) by following the manufacturer's instructions. Briefly, mRNA was purified from 30 µg of total RNA using Sera-mag Magnetic Oligo (dT) Beads (Illumina) and fragmented into small pieces using divalent cations under elevated temperature. Double-stranded cDNA was then synthesized using the SuperScript double-stranded cDNA Synthesis kit (Invitrogen, Camarillo, CA) with random hexamer (n=6) primers (Illumina, San Diego, USA). These cDNA fragments went through an end-repair process, phosphorylation and ligation of adapters. Products were subsequently purified and amplified by PCR to create the final cDNA libraries. The cDNA libraries were sequenced on the Illumina HiSeq™ 2000 (San Diego, USA), with both ends of the inserts being sequenced. The 100-bp raw Paired End reads were generated by the Illumina Genome Analyzer II system (San Diego, USA).

### **Transcriptome annotation**

The raw sequence reads were assembled to the reference banana genome of the *Musa acuminata* subsp. *malaccensis* doubled-haploid Pahang (DH-Pahang) using the Geneious 8.02 software. The reads that mapped onto each coding sequences (CDS) annotation on the reference banana genome were counted to calculate the expression level of each transcript as Reads per Kilobase per Million (RPKM) values:

$$\text{RPKM} = (\text{CDS read count} * 10^9) / (\text{CDS length} * \text{total mapped read count})$$

The Median gene expression ratio was calculated for each gene between the mock inoculation libraries (at 0 hours) and libraries at 48, 96 and 192hrs post inoculation for each genotype, independently. Differential expression absolute confidence was calculated as the negative base-10 log of the p-value, to show genes that have differential expression when greater than 6. The RPKM method eliminates the influence of different gene lengths and sequencing levels on the calculation of gene expression. Therefore, the calculated gene expression could be directly used for comparing differences in gene expression among samples.

The differentially expressed genes were annotated using the online bioinformatics tool Blast2GO (Conesa and Götzt, 2008) (Fig. 1). The sequences were subjected to BLASTX searches against the National Center for Bioinformatics (NCBI) sequence database with E-value cut off of  $10^{-3}$ . Sequences were then mapped to the Gene Ontology (GO) Consortium database. KEGG Automatic Annotation Server (KAAS) was also used for functional annotation of genes by BLAST comparisons against the manually curated KEGG GENES database (Yuki *et al.*, 2007). Defence genes that were differentially expressed exclusively for the resistant, susceptible and immune genotypes were identified.

## RESULTS

### Sequence assembly and annotation

A total of 299.9 million raw reads (100-bases each) were sequenced from libraries constructed from root tissues of 'Mbwazirume', 'TMB2X8075-7' and 'Sukali Ndiizi' bananas inoculated with Foc race 1 at 0, 48, 96 and 192 hours post inoculation (Table 1). The raw reads covered more than 85.4% of the reads when mapped onto the reference banana genome in all the libraries. This match indicated that a large portion of the *Musa* reference genome was covered by the transcripts.

### Gene expression profiling

A total of 5372 genes were differentially expressed (DEGs) when banana varieties 'Mbwazirume', 'TMB2X8075-7' and 'Sukali Ndiizi' were inoculated with Foc race 1, of which 3154 (58.7%) genes were uniquely up-regulated and 2218 (41.3%) genes were uniquely down-regulated (Fig. 2). In the immune banana 'Mbwazirume', 916 (9%) genes were induced and 854 (8.4%) genes repressed during infection (Fig. 2A). In contrast, a much higher number of genes were induced and suppressed in the resistant and susceptible genotypes. In the resistant 'TMB2X8075-7' 1 920 (18.9%) genes were induced and 1 654 (16.3%) repressed, and in the susceptible genotype 'Sukali Ndiizi' 2 800 (27.6%) genes were induced and 1988 (19.6%) repressed (Figs. 2B and 2C).

### **Kyoto encyclopaedia of genes and genomes (KEGG) pathway mapping**

KAAS analysis of differentially expressed genes indicated that the most enriched pathways during Foc race 1 infection of three African banana genotypes were the 'Metabolic', 'Ribosome', 'Plant-pathogen interaction' and 'Plant hormone signal transduction' pathways (Fig. 4). KEGG Ontology (KO) terms were identified in the 'Plant-pathogen interaction' pathway of the banana genotypes during Foc race 1 infection. These include calmodulin (CALM), molecular chaperone (HtpG), heat shock protein (HSP90B), mitogen-activated protein kinase kinase 4/5, plant (MKK4\_5P), mitogen-activated protein kinase kinase 1 plant (MEKK1P), WRKY transcription factor 33 (WRKY33), respiratory burst oxidase (RBOH), calcium-binding protein (CML), pathogenesis-related protein 1 (PR1), and disease resistance protein (RPM1). Strikingly, the WRKY33 was uniquely up-regulated in the immune genotype 'Mbwazirume' but down-regulated in the resistant genotype 'TMB2X8075' and susceptible genotype 'Sukali Ndiizi', which suggests that it is a key transcriptional regulator of the immune response to Foc race 1 in bananas.

### **Genotype-specific differentially expressed genes (DEGs)**

A total of 1677 genes were uniquely expressed in the three banana genotypes. Of these, 564 genes (31.9%) were from 'Mbwazirume' and 1 113 (23.1%) from 'Sukali Ndiizi' (Fig. 3). There were no genes uniquely differentially expressed in the resistant genotype 'TMB2X8075-7'. The 'Mbwazirume'-specific Foc race 1 responsive genes were primarily assigned to 'Cellular process' (2 076 genes; 19.9%), 'Biological regulation' (3 594 genes; 34.5%) and 'Molecular functions' (4 739 genes 35.6%). Three hundred and thirty one genes determined to be exclusively induced by Foc race 1 in 'Mbwazirume' were assigned to GO categories like 'Metabolic', 'Cellular', 'Stimulus response', 'Cellular component organization processes', 'Oxidation-reduction process', 'Metabolic process', 'Oxido-reductase activity', 'Metal ion binding' and 'Hydrolase activity'. 'Mbwazirume'-specific genes repressed by Foc race 1 were largely involved in 'Translation', 'Ribosome', 'Intracellular' and 'Structural constituent of ribosome'. In 'Sukali Ndiizi', 816 and 297 genes were identified as specifically induced and repressed during Foc race 1 infection, respectively. These genes were assigned to 'Cellular process' (5 400 genes, 32%), 'Biological regulation' (5 535 genes, 32.8%) and 'Molecular functions' (5 932. 35.8%). Induced genes specific to 'Sukali Ndiizi' were assigned to 'Metabolic process', 'Membrane', 'Oxidation-reduction', 'Ribosome' and 'Structural constituent of ribosome', and those repressed to 'Involved in metal ion binding', 'Oxidation-reduction process', 'Oxido-reductase activity', 'Membrane', 'Metabolic process' and 'Integral component of membrane'.

### **Genotype-specific activation of signalling networks**

Five receptor-coding genes were uniquely induced in the immune genotype 'Mbwazirume' 48 and 96 hrs after inoculation (Table 2). These included the syntaxin-132-like (LOC103969225), syntaxin-121-like (LOC103989550), leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1 (LOC103973170), germin-like protein 3-8 (LOC103981884) and a probable carboxylesterase 15 (LOC103993738). Susceptible genotype 'Sukali Ndiizi' also induced unique receptor coding genes like leucine-rich repeat receptor-like protein kinase PXL2 (LOC103971692, LOC103980269, LOC103980977), probable inactive receptor kinase At2g26730 (LOC103991379, LOC103980977), probable receptor protein kinase TMK1 (LOC103995817) and syntaxin-related protein KNOLLE (LOC103997250) 192 hrs after inoculation (Table 4). No unique receptor coding genes were repressed by either 'Mbwazirume' or 'Sukali Ndiizi'.

DEGs specific to the immune genotype 'Mbwazirume' induced 48 and 96 hours after Foc race 1 inoculation were involved in 'Protein Kinase' and 'Calcium signalling'. These included genes coding for somatic embryogenesis receptor kinase 2-like protein kinase 2A (LOC103979424), chloroplastic-like (LOC103975808), somatic embryogenesis receptor kinase 1-like (LOC103988013), respiratory burst oxidase homolog protein B (LOC103987581), probable calcium-binding protein CML45 (LOC103996707), probable calcium-binding protein CML30 (LOC103992433) and a calcium-binding protein KIC-like (LOC103993069) (Table 2), while genes specific to the susceptible genotype 'Sukali Ndiizi' induced were involved in 'Calcium signalling' and 'GTP signalling' (Table 4). The calcium signalling genes specific to 'Sukali Ndiizi' were coding for calreticulin-3-like (LOC103971503), probable calcium-binding protein CML7 (LOC103978713), oxygen-evolving enhancer protein 2, chloroplastic-like (LOC103990328), calcineurin B-like protein 3 (LOC103997760), calmodulin-like LOC103999616 and V-type proton ATPase 16 kDa proteolipid subunit c1 (LOC103992604). Some of the GTP signalling genes induced specifically by the susceptible genotype included tubulin alpha chain-like (LOC103992122, LOC103986585), ADP-ribosylation factor-like (LOC103992981, LOC103980364), GTP-binding protein SAR1A-like (LOC103969355) among others. Genes specific to the immune genotype 'Mbwazirume' involved in kinase signalling (LOC103971224, LOC103974557), calcium signalling (LOC103969232, LOC103972398) and GTP signalling (LOC103979648 LOC103996834) were repressed during Foc race 1 infection, while only genes involved in calcium signalling (LOC103993907, LOC103983709) were uniquely repressed by 'Sukali Ndiizi'.

### Genotype-specific defence-related genes

Several defence-related genes were specific to 'Mbwazirume' (Table 2 and 3). Foc strongly induced one putative disease resistance protein RGA3 (LOC103998212) 339.8-fold at 48 and 96 hours after inoculation. Eight DEGs were induced for WRKY transcription factors, including WRKY transcription factor 18-like (LOC103978171), probable WRKY transcription factor 50 (LOC103979280), probable WRKY transcription factor 70 (LOC103992513, LOC103987931), probable WRKY transcription factor 60 (LOC103990571), probable WRKY transcription factor 33 (LOC103971078), probable WRKY transcription factor 43 (LOC103996660) and probable WRKY transcription factor 31 (LOC104001039). DEGs coding for *PR* genes included *PR4*-like (LOC103989972, LOC103986657), *PR1*-like (LOC103977651, LOC103977652, LOC103977653), non-specific lipid-transfer protein-like (LOC103981321), glucan endo-1,3-beta-glucosidase 14-like (LOC103991553), chitinase 6 (LOC103988682), cysteine proteinase 1-like (LOC103989280), zingipain-2-like (LOC103982135) and zingipain-1-like (LOC103987740). A jacalin-related lectin 35-like gene (LOC103999398) was also induced 1 million-fold 48 and 96 hours after inoculation. Genes associated with cell wall strengthening uniquely up-regulated in 'Mbwazirume' included glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a (LOC103978957), subtilisin-like protease (LOC103993250), beta-hexosaminidase 3-like (LOC103971127), mitochondrial phosphate carrier protein 3, mitochondrial-like (LOC103977542), peroxidase 4 like (LOC103987320), UDP-glucose 6-dehydrogenase 5-like (LOC103987519), V-type proton ATPase catalytic subunit A-like (LOC103982160), UDP-glucose 6-dehydrogenase 4-like (LOC103979437) and chalcone synthase (LOC104000649).

Defense response genes specific to the immune banana genotype 'Mbwazirume' down-regulated during infection by Foc race 1 included the proteasome subunit alpha type-2-A (LOC103985763) and the peroxidase A2-like (LOC103987549) at 1.9 and 13.5-fold change 96 hrs post inoculation, respectively. Down-regulation of Mbwazirume-unique genes involved in the cell wall biosynthesis was observed for V-type proton ATPase catalytic subunit A-like (LOC103982160), Low Quality Protein: citrate synthase, mitochondrial-like (LOC103983279), ketol-acid reductoisomerase, chloroplastic-like (LOC103992731), protein notum homolog (LOC103994567) and probable xyloglucan endotransglucosylase/hydrolase protein 26 (LOC103995047) 96 hrs post inoculation.

Some of the defense-related genes specific to susceptible banana genotype 'Sukali Ndiizi' (Table 4 and 5) that were up-regulated during infection by Foc race 1 included a defensin-like (LOC103982399) and a glucose-6-phosphate isomerase, cytosolic 2B-like (LOC103969976) with an 8.1- and 4.3-fold change at 48, 96 and 192 hrs post inoculation, respectively. Other defence response genes specific to Sukali Ndiizi were induced 192 hours post inoculation, and they included guanine nucleotide-binding protein subunit beta-like

protein A (LOC103968855), probable 6-phosphogluconolactonase 4, chloroplastic (LOC103971013), serine/threonine-protein kinase SAPK7-like (LOC103971820), probable mannose-1-phosphate guanylyltransferase1 (LOC103975370), uncharacterized protein LOC103987558 (LOC103987558), probable flavin-containing monooxygenase 1 (LOC103987576) and actin-depolymerizing factor 7-like (LOC103999912)

## DISCUSSION

Recognition of a potential invader (pathogens or non-pathogens) is a requirement for efficient defence response (Dangl and McDowell, 2006). Generally, the plant cell surface has pattern-recognition receptors (PRR) which detect patterns of the pathogen called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) (Zipfel, 2008). The up-regulation of unique receptor-coding transcripts like syntaxin-132-like (LOC103969225), syntaxin-121-like (LOC103989550) and leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1 (LOC103973170) in the immune genotype 'Mbwazirume' during Foc invasion indicates an innate ability to detect the pathogen and initiate defence responses. For Instance, syntaxins are target-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) on the acceptor membrane required for specific docking and fusion of the donor's vesicles via interactions with corresponding vesicular SNAREs and other proteins (Kalde *et al.*, 2007). Syntaxins have been implicated in mediating defence-related secretion in plants. For instance, SYP121 contributes significantly to resistance against the powdery mildew fungus (*Blumeria graminis* f. sp. *tritici* (DC.) Speer) in wheat (Bhat *et al.*, 2005). Most transcripts coding for receptors specific to the susceptible genotype 'Sukali Ndiizi' were induced 192 hrs post inoculation. This suggests that recognition of the pathogen is a continuous process during fungal colonisation and plants can induce defence responses after infection of the pathogen.

The plant surface constitutes the first line of defence that pathogens must penetrate before they can cause infection (Swain, 1977; Agrios, 2005). Therefore, resistance to penetration of epidermal cells by pathogens is an important component of the defence reactions (McDowell and Dangl, 2000). Several genes coding for cell wall strengthening; like subtilisin-like protease (LOC103993250), peroxidase 4 like (LOC103987320), UDP-glucose 6-dehydrogenase 5-like (LOC103987519), V-type proton ATPase catalytic subunit A-like (LOC103982160), UDP-glucose 6-dehydrogenase 4-like (LOC103979437) and chalcone synthase (LOC104000649); were induced uniquely in the immune genotype 'Mbwazirume'. For instance, a subtilisin-like protease (LOC103993250) was up-regulated a million-fold at 48 and 96 hours after inoculation with Foc race 1. Expressing high levels of subtilisin-like protease in 'Mbwazirume' enabled the plant to recognize and induce timely defence



responses. Subtilisin-like proteins are involved in pathogen recognition and immune priming (Figueredo *et al.*, 2014). The deployment of these subtilisin-like proteases in banana improvement will require further investigation into their target substrate and in the elucidation of their participation in the immune priming activation which are out of the scope of this study.

Genes involved in cell wall biosynthesis also have phytoalexin properties. For instance chalcone synthase 2-like (LOC104000649), which was up-regulated 9.7-fold in 'Mbwazirume', is a key entry enzyme committed to the production of the polyketide phenylpropanoids in plants (Dao *et al.*, 2011). Chalcone synthase helps the plant to produce more flavonoids, isoflavonoid-type phytoalexins and is involved in the SA defence pathway. These results confirmed the findings of Li *et al.* (2013) that phytoalexins and lignification of the cell wall are important to protect bananas against *Fusarium* wilt.

PR proteins inhibit the growth, multiplication and/or spread of an invading pathogen, and activate the systemic acquired resistance (SAR) response in a plant (Ward *et al.*, 1991; Kombrink and Somssich, 1997; Kitajima and Sato, 1999; van Loon and van Strien, 1999). The immune genotype 'Mbwazirume' induced specific DEGs that are coding for PR proteins 48 and 96hrs post inoculation. For instance three DEGs (LOC103977651, LOC103977652, LOC103977653) coding for PR-1 proteins were up-regulated 64.7-, 58.8- and 569.5-fold, respectively. PR-1 proteins are known to have antifungal activity against a number of plant pathogenic fungi (Kitajima and Sato, 1999). Also, DEGs unique to 'Mbwazirume' coding for PR-4 (LOC103989972, LOC103986657), chitinase 6 (LOC103988682) and glucan endo-1,3-beta-glucosidase 14-like (LOC103991553) were up-regulated 95.9-, 193.7-, 171.4- and 10.4-fold, respectively. An important structural component of fungal cell walls is chitin, a well-known elicitor of immune responses in plants. PR4 is a chitin-binding protein, which binds to insoluble chitin and enhances hydrolysis of chitin by other enzyme like chitinases (Saboki *et al.*, 2011). After hydrolysis with chitinase, fungal cell wall fragments further act as elicitors of plant defence responses such as stomatal closure and lignifications (Moerschbacher *et al.*, 1988; Vander *et al.*, 1998; Lee *et al.*, 1999). The introduction of chitinase genes into plants has augmented plant resistance to fungal pathogens (Dana *et al.*, 2006; Nirala *et al.*, 2010). For instance, transgenic rice plants with the chitinase gene *chi11* had improved resistance to sheath blight compared to non-transformed plants (Ceasar and Ignacimuthu, 2012).

PR genes have synergistic effects in protecting plants against pathogens (Saboki *et al.*, 2011). For instance plant chitinases alone usually affect only the hyphal tip of fungi and are unable to effectively degrade the harder chitin structures of mycelia. When it is combined with other PR proteins, like  $\beta$ -1,3-glucanase, a synergistic effect is usually observed. For example, tomato plants expressing tobacco class I  $\beta$ -1,3-glucanase and chitinase transgenes showed an increased tolerance to infection by *Fusarium oxysporum* f.sp.

*lycopersici* (Jongedijk *et al.*, 1995). This suggests that bananas can be defended against Foc race 1 by simultaneously expressing related number of PR proteins such as PR4, Chitinase 6 and glucan endo-1,3-beta-glucosidase.

Foc race 1 also induced a gene uniquely in 'Mbwazirume' coding Zingipain (LOC103987740, LOC103982135) by a million-fold. Zingipain is a cysteine protease reported to have anti-proliferative activity against fungi (Karnchanatat *et al.*, 2011). *Zingipain* can be another candidate defence gene to engineer into Foc race 1-susceptible genotypes. The jacalin-related lectin 35-like (LOC103999398) uniquely induced a million-fold by Foc race1 in 'Mbwazirume' represents a subgroup of proteins that have one or more domains with sequences similar to the jacalin protein isolated from *Artocarpus integrifolia* (Bunn-Moreno and Campos-Neto, 1981). JRLs are commonly regulated by the SA and JA pathways and are apparently associated with plant defence (Nakagawa *et al.*, 2000; Jiang *et al.*, 2006). For instance, transgenic tobacco plants, over-expressing the wheat Jacalin-related lectin *Ta-JA1* increased resistance to bacterial, fungal, and viral pathogens (Ma *et al.*, 2010). Another wheat Jacalin-related lectin *TaJRL1* increased susceptibility to the facultative fungal pathogen *Fusarium graminearum* Schwabe and the biotrophic fungal pathogen *Blumeria graminis* through virus-induced gene silencing, while the same gene displayed increased resistance to *F. graminearum* and *Botrytis cinerea* Pers. in *Arabidopsis thaliana* (L.) Heynh. transgenic plants (Xiang *et al.*, 2011). This *Musa* Jacalin-related lectin could also be a candidate gene for genetic modification of susceptible banana genotypes against Foc race 1. Another candidate for genetic modification of bananas against Foc race 1 is the gene coding for non-specific lipid transfer proteins (LOC103981321), uniquely induced a million-fold in the immune genotype 'Mbwazirume'. Non-specific lipid transfer proteins are located extracellularly, usually associated with plant cell walls, and are involved in plant defence mechanisms against phytopathogenic bacteria and fungi, and possibly in the assembly of hydrophobic protective layers of surface polymers such as cutin (Salcedo *et al.*, 2007). However, root specific promoters must be used in developing transgenic plants with non-specific lipid transfer proteins genes since they have also been reported to be allergens (Salcedo *et al.*, 2007).

The significant enrichment of pathways for 'Metabolic' and 'Ribosome' in response to Foc race 1 infection in the African banana genotypes at all the time points sampled suggests that these genotypes require increased energy and a more rapid syntheses of defence proteins as they respond to pathogen attack. Inducing such a wide array of defence mechanisms involves a massive redistribution of energy toward defence response. The banana genotypes studied seem to initiate all available defence mechanisms so that at least some may be effective against the pathogen (Katagiri, 2004).

In this study RNA-seq was used to investigate the response of three genotypes 'Mbwazirume', 'TMB2X8075-7' and 'Sukali Ndiizi' to Foc race1. Strict filtering and conservative matching of the sequencing data to the reference banana genome (*Musa acuminata* subsp. *malaccensis*) was used in the expression profile analysis. RNA-seq analysis results showed that bananas responded to Foc race 1 infection by activating mainly the 'primary Metabolic' and 'Ribosome' pathways. Several defence-related genes were highly induced during Foc race 1 infection in the immune genotype 'Mbwazirume', and these could be acting synergistically to enhance levels of disease resistance. These include the putative disease resistance gene *RGA3*, the syntaxin-121-like gene, gene coding for a non-specific lipid transfer protein (*nsLTPs*), a Zingipain, a subtilisin-like protease, a jacalin-related lectin 35-like gene and a WRKY transcription factor 33. Future research efforts have to be made to characterize these defence-related genes to prove their involvement in resistance to Foc race 1 so that they may be considered as promising candidate genes to engineer durable, Fusarium wilt resistant cultivars for Africa.

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**Table 1.** Mapping RNA-sequence reads from the libraries of immune banana 'Mbwazirume', resistant banana 'TMB2X8075-7' and susceptible banana 'Sukali Ndiizi' at 0, 48, 96 and 192 hours post inoculation onto the diploid banana reference genome *Musa acuminata* ssp. *malacensis*

Time	Mbwazirume			TMB2X8075-7			Sukali Ndiizi		
	Total Raw reads	Reads mapped to genome	% reads mapped	Total Raw reads	Reads mapped to genome	% reads mapped	Total Raw reads	Reads mapped to genome	% reads mapped
<b>0 Hrs</b>	25372252	23,067,689	90.9	30,707,328	27,731,971	90.3	25,563,694	22,611,917	88.5
<b>48 Hrs</b>	24,771,982	21,151,221	85.4	24,667,143	22,157,083	89.8	27,800,890	23,973,588	86.2
<b>96 Hrs</b>	20,696,506	18,564,801	89.7	23,342,048	20,966,269	89.8	31,658,384	27,627,421	87.3
<b>192 Hrs</b>	16,889,032	15,138,624	89.6	27,052,071	24,333,093	89.9	21355976	18,548,507	86.9

**Table 2.** Expression profiles of selected defence-related genes uniquely induced by *Fusarium oxysporum* f. sp. *cubense* race 1 in the immune banana genotype 'Mbwazirume' at 48, 96 and 192 hours post inoculation

Gene number	Annotation	Differential expression ratio		
	Receptors	48 Hrs	96 Hrs	192 Hrs
LOC103969225	syntaxin-132-like	6.9	6.9	-
LOC103989550	syntaxin-121-like	4	4	-
LOC103973170	leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1	9.6	9.6	-
LOC103981884	germin-like protein 3-8	13	13	-
LOC103993738	probable carboxylesterase 15	5.6	5.6	-
	<b>Kinase signaling</b>			
LOC103979424	somatic embryogenesis receptor kinase 2-like	3.5	3.5	-
LOC103975808	protein kinase 2A, chloroplastic-like	6.9	6.9	-
LOC103988013	somatic embryogenesis receptor kinase 1-like		3.4	-
	<b>Calcium signalling</b>			
LOC103992069	uncharacterized protein LOC103992069		8.8	-
LOC103987581	respiratory burst oxidase homolog protein B	5.8	5.8	-
LOC103996707	probable calcium-binding protein CML45	18.1	18.1	-
LOC103992433	probable calcium-binding protein CML30	4.8	4.8	-
LOC103993069	calcium-binding protein KIC-like	32.1	32.1	-
	<b>WRKY transcription factors</b>			
LOC103978171	WRKY transcription factor 18-like	11.9	11.9	-
LOC103979280	probable WRKY transcription factor 50	1000000	1000000	-
LOC103987931	probable WRKY transcription factor 70	552.5	552.5	-
LOC103992513	probable WRKY transcription factor 70	6.5	6.5	-
LOC103990571	probable WRKY transcription factor	5.8	5.8	-
LOC103971078	probable WRKY transcription factor 33	7	7	-
LOC103996660	probable WRKY transcription factor 43	16.8	16.8	-
LOC104001039	probable WRKY transcription factor 31	5.9	5.9	-
	<b>Defence response</b>			
LOC103989972	pathogenesis-related protein PR-4-like	95.9	95	-
LOC103977652	pathogenesis-related protein 1-like	58.8	58.8	-
LOC103977651	pathogenesis-related protein 1-like	64.7	64.7	-
LOC103977653	pathogenesis-related protein 1-like	569.5	569.5	-
LOC103986657	pathogenesis-related protein PR-4-like	193.7	193.7	-
LOC103981321	non-specific lipid-transfer protein-like	1000000	1000000	-
LOC103991553	glucan endo-1,3-beta-glucosidase 14-like	10.4	10.4	-
LOC103988682	chitinase 6	171.4	171.4	-

LOC103982135	zingipain-2-like	1000000	1000000	-
LOC103989280	cysteine proteinase 1-like	2.1	2.1	-
LOC103999398	jacalin-related lectin 35-like	1000000	1000000	-
LOC103998212	putative disease resistance protein RGA3	339.8	339.8	-
LOC103987740	zingipain-1-like	1000000	1000000	-
	<b>Cellwall biosynthesis</b>			
LOC103978957	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a	4.9	4.9	-
LOC103993250	subtilisin-like protease	1000000	1000000	-
LOC103971127	beta-hexosaminidase 3-like		1.9	-
LOC103977542	mitochondrial phosphate carrier protein 3, mitochondrial-like	3.8	3.8	-
LOC103987320	peroxidase 4 {ECO:0000250 UniProtKB:Q42578}-like		4.3	-
				-
LOC103987519	UDP-glucose 6-dehydrogenase 5-like	3.4	3.4	-
LOC103979437	UDP-glucose 6-dehydrogenase 4-like	14.4	14.4	-
LOC104000649	Chalcone synthase 2-like	9.7	9.7	-

<sup>1</sup>Median gene expression ratio between the mock inoculation library (at 0 hours) and libraries at 48, 96 and 192 hours post inoculation for each gene.

- No differential gene expression was observed

**Table 3.** Expression profiles of selected defence-related genes uniquely repressed by *Fusarium oxysporum* f. sp. *cubense* race 1 in the immune banana genotype 'Mbwazirume' at 48, 96 and 192 hours post inoculation

Gene number	Annotation	Differential expression ratio		
		48 Hrs	96 Hrs	192 Hrs
	<b>Kinase signalling</b>			
LOC103971224	pyruvate kinase, cytosolic isozyme-like	-	-1.8	-
LOC103974557	CBL-interacting protein kinase 18-like	-2.7	-2.7	-2.7
	<b>GTP signalling</b>			
LOC103979648	ADP-ribosylation factor 1-like	-	-1.6	-
LOC103996834	ras-related protein RABE1c-like	-	-1.8	-
	<b>Calcium signalling</b>			
LOC103969232	probable calcium-binding protein CML30	-5.2	-5.2	-4.5
LOC103972398	calreticulin-like	-	-1.9	-
	<b>Defence response</b>	<b>48 Hrs</b>	<b>96 Hrs</b>	<b>192 Hrs</b>
LOC103985763	proteasome subunit alpha type-2-A	-	-1.9	-
LOC103987549	peroxidase A2-like	-	-13.4	-
	<b>Cell wall biosynthesis</b>			
LOC103982160	V-type proton ATPase catalytic subunit A-like	-	-2.2	-
LOC103983279	LOW QUALITY PROTEIN: citrate synthase, mitochondrial-like	-	-2.4	-
LOC103992731	ketol-acid reductoisomerase, chloroplastic-like	-	-1.7	-
LOC103994567	protein notum homolog	-	-2.2	-
LOC103995047	probable xyloglucan endotransglucosylase/hydrolase protein 26	-	-1000000	-

<sup>1</sup>Median gene expression ratio between the mock inoculation library (at 0 hours) and libraries at 48, 96 and 192 hours post inoculation for each gene.

- No differential gene expression was observed

**Table 4.** Expression profiles of selected defence-related genes uniquely induced by *Fusarium oxysporum* f. sp. *cubense* race 1 in the susceptible banana genotype ‘Sukali Ndiizi’ at 48, 96 and 192 hours post inoculation.

Gene number	Annotation	Differential expression ratio		
	Receptors	48 Hrs	96 HRs	192 Hrs
LOC103971692	leucine-rich repeat receptor-like protein kinasePXL2	-	-	9.3
LOC103971790	leucine-rich repeat receptor-like protein kinasePXL2	-	-	3.9
LOC103975939	mitochondrial import receptor subunit TOM7-2-like	2.6	2.6	2.6
LOC103980269	leucine-rich repeat receptor-like protein kinasePXL2	-	-	6.4
LOC103980977	probable inactive receptor kinase At2g26730	-	-	2.8
LOC103982766	mitochondrial import receptor subunit TOM9-2-like	-	-	3.8
LOC103991379	probable inactive receptor kinase At1g48480	-	-	4.5
LOC103991990	probable leucine-rich repeat receptor-like protein kinase At1g68400	-	-	4.4
LOC103995817	probable receptor protein kinase TMK1	-	-	4.8
LOC103997250	syntaxin-related protein KNOLLE	-	-	4.1
	<b>Calcium signalling</b>			
LOC103990328	oxygen-evolving enhancer protein 2, chloroplastic-like	47	47	47
LOC103992604	V-type proton ATPase 16 kDa proteolipid subunit c1	-	-	2.7
LOC103997760	calcineurin B-like protein 3	2.4	2.4	2.4
LOC103999616	calmodulin-like	2.1	2.1	2.1
LOC103971503	calreticulin-3-like	1.9	1.9	1.9
LOC103978713	probable calcium-binding protein CML7	-	-	3.7
	<b>GTP signalling</b>			
LOC103992122	tubulin alpha chain-like			3.1
LOC103992981	ADP-ribosylation factor-like	196.6	196.6	196.6
LOC103994024	probable mannose-1-phosphate guanylyltransferase3	-	-	5.1
LOC103996118	rac-like GTP-binding protein 2	-	-	8.1
LOC103969133	tubulin beta chain-like	-	-	2.3
LOC103969355	GTP-binding protein SAR1A-like	-	-	2
LOC103970000	GTP-binding nuclear protein Ran1B-like	1.9	1.9	1.9
LOC103973213	ras-related protein RABA5c-like	-	-	3.2
LOC103974379	LOW QUALITY PROTEIN: elongation factor 1-alpha	2	2	2
LOC103977185	ras-related protein Rab11D-like	-	-	2.8
LOC103980242	ras-related protein RIC2	-	-	2.9
LOC103980364	ADP-ribosylation factor-like protein 5	-	-	2.9
LOC103983307	tubulin beta-2 chain-like	-	-	3.1
LOC103984452	ras-related protein RABH1b	2.3	2.3	2.3
LOC103986585	tubulin alpha chain	-	-	2.9
	<b>Defence response</b>			
LOC103968855	guanine nucleotide-binding protein subunit beta-like protein A	-	-	2.8
LOC103969976	glucose-6-phosphate isomerase, cytosolic 2B-like	4.3	4.3	4.3

LOC103971013	probable 6-phosphogluconolactonase 4, chloroplastic	-	-	2.7
LOC103971820	serine/threonine-protein kinase SAPK7-like	-	-	2.7
LOC103975370	probable mannose-1-phosphate guanylyltransferase1	-	-	3.5
LOC103982399	defensin Ec-AMP-D1 {ECO:0000303 PubMed:18625284}-like	8.1	8.1	8.1
LOC103987558	uncharacterized protein LOC103987558	-	-	4.3
LOC103987576	probable flavin-containing monooxygenase 1	-	-	91.5
LOC103999912	actin-depolymerizing factor 7-like	-	-	2.8
	<b>Cell wall modification</b>			
LOC103997147	ATP synthase subunit gamma, mitochondrial isoform X2	-	-	2.1
LOC103997615	probable xyloglucan endotransglucosylase/hydrolase protein 23	-	12.6	12.6
LOC103997729	DNA-damage-repair/toleration protein DRT100-like	-	-	7.9
LOC104000855	mitochondrial dicarboxylate/tricarboxylate transporter DTC-like	2.8	2.6	2.6

<sup>1</sup>Median gene expression ratio between the mock inoculation library (at 0 hours) and libraries at 48, 96 and 192 hours post inoculation for each gene.

- No differential gene expression was observed

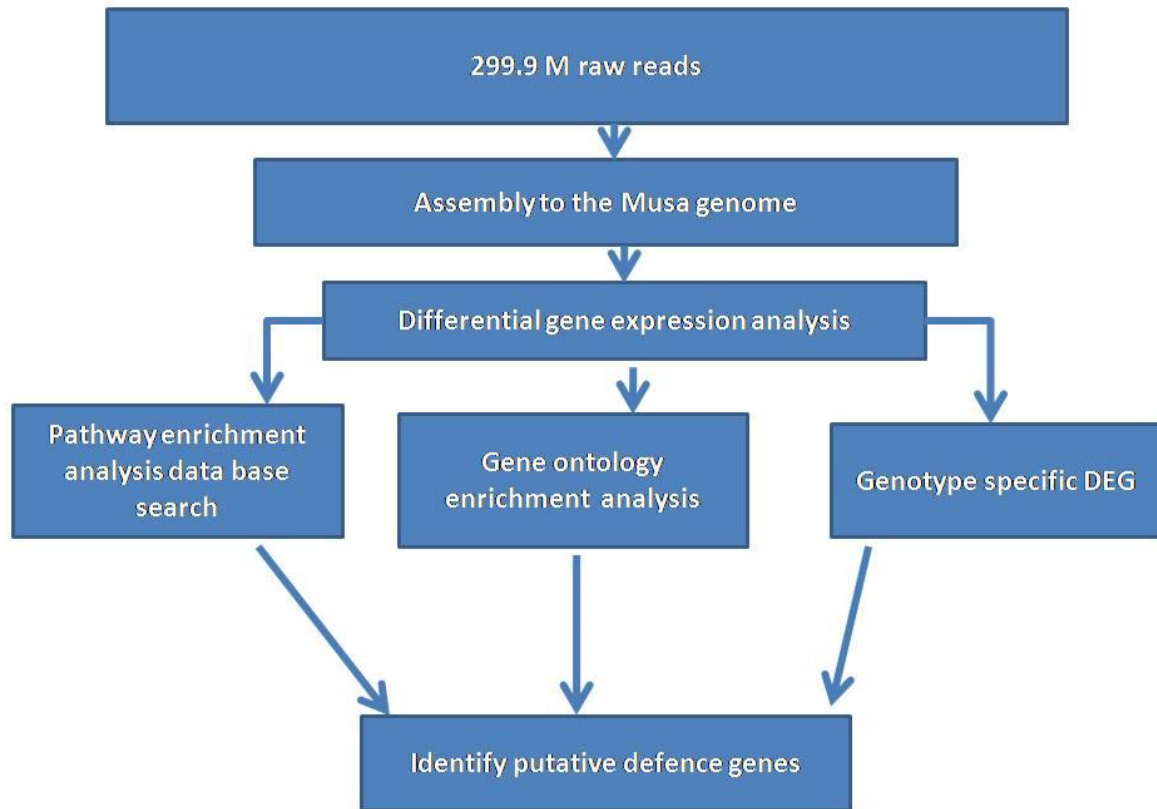


**Table 5.** Expression profiles of selected defence-related genes uniquely repressed by *Fusarium oxysporum* f. sp. *cubense* race 1 in the susceptible banana genotype ‘Sukali Ndiizi’ at 48, 96 and 192 hours post inoculation.

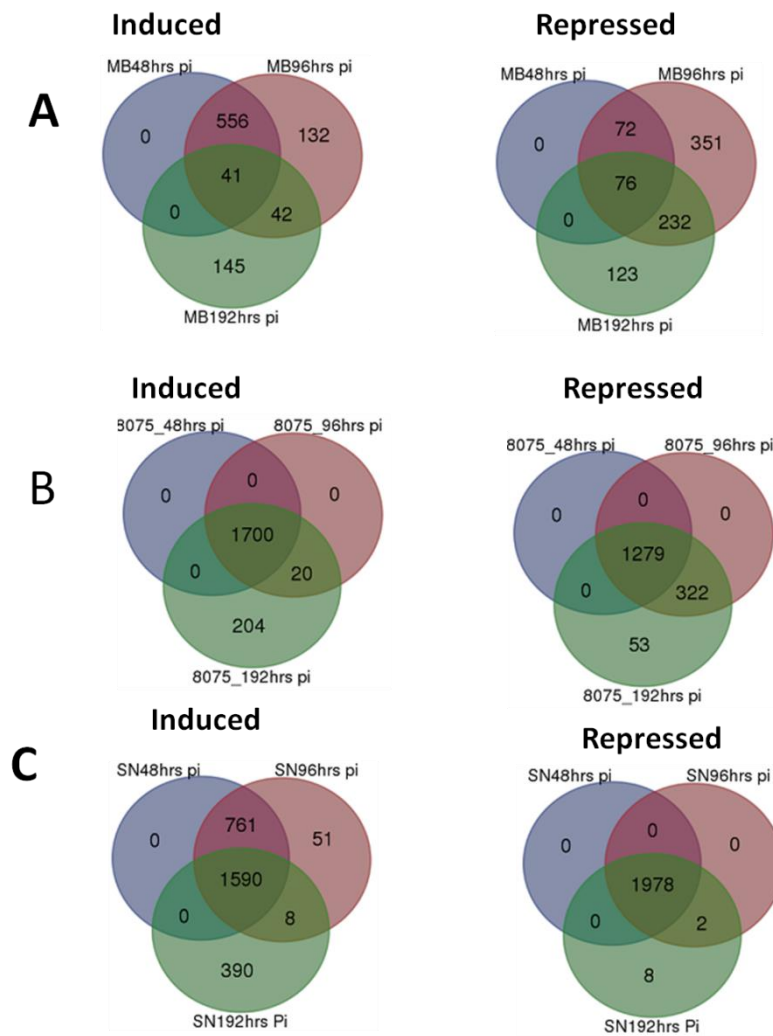
Gene number	Annotation	Differential expression ratio		
	Calcium signalling	48 Hrs	96 Hrs	192 Hrs
LOC103993907	LOW QUALITY PROTEIN: oxygen-evolving enhancer protein 1, chloroplastic-like	-19.8	-19.8	-19.8
LOC103983709	tubulin beta chain-like	-1.5	-1.5	-1.5
Gene number	Annotation	Differential expression ratio		
	Cell wall biosynthesis	48 Hrs	96 Hrs	192 Hrs
LOC103976769	probable xyloglucan endotransglucosylase/hydrolase protein 31	-19.8	-19.8	-19.8
LOC103971718	cytochrome P450 84A1-like	-22.4	-22.4	-22.4
LOC103979717	protein ASPARTIC PROTEASE IN GUARD CELL 1-like	-3.1	-3.1	-3.1

<sup>1</sup>Median gene expression ratio between the mock inoculation library (at 0 hours) and libraries at 48, 96 and 192 hours post inoculation for each gene.

- No differential gene expression was observed

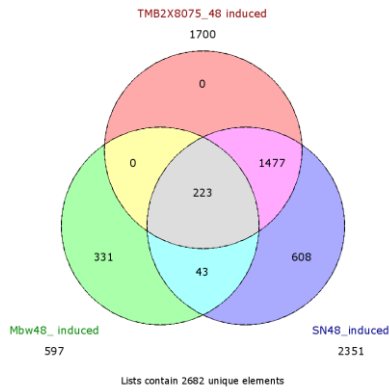


**Figure 1:** Schematic representation of the data mining strategy to identifying defence genes associated with *Fusarium oxysporum* f. sp. *cabense* race 1 resistance in *Musa* sp.

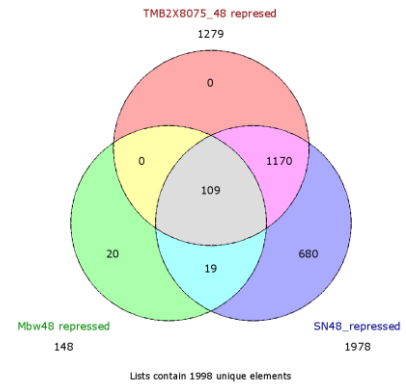


**Figure 2.** Distribution of differentially expressed genes showing significant up- and down-regulation following mock inoculation with *Fusarium oxysporum* f. sp. *cabense* race 1. A) The immune banana genotype 'Mbwazirume', B) The resistant banana genotype 'TMB2X8075', and C) The susceptible banana genotype 'Sukali Ndiizi'.

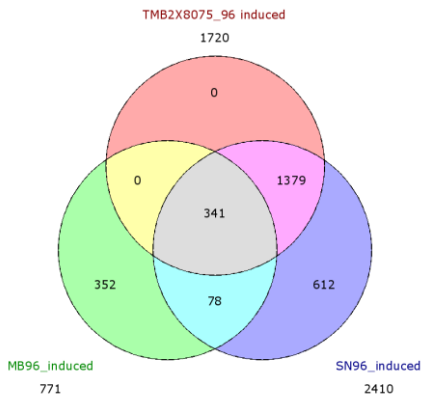
**A) Induced genes 48 hours**



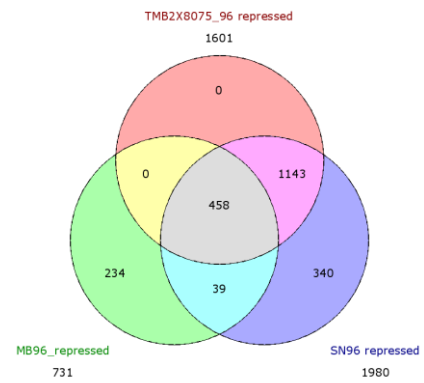
**B) Repressed genes 48 hours**



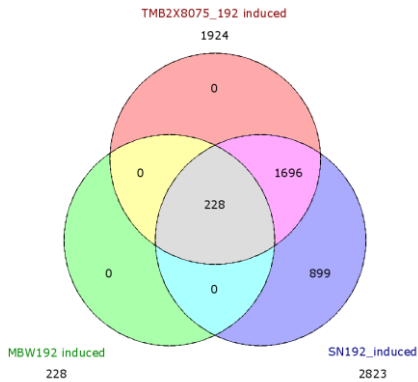
**C) Induced genes 96 hours**



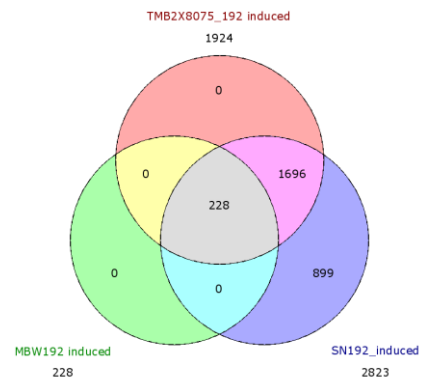
**D) Repressed genes 96 hours**



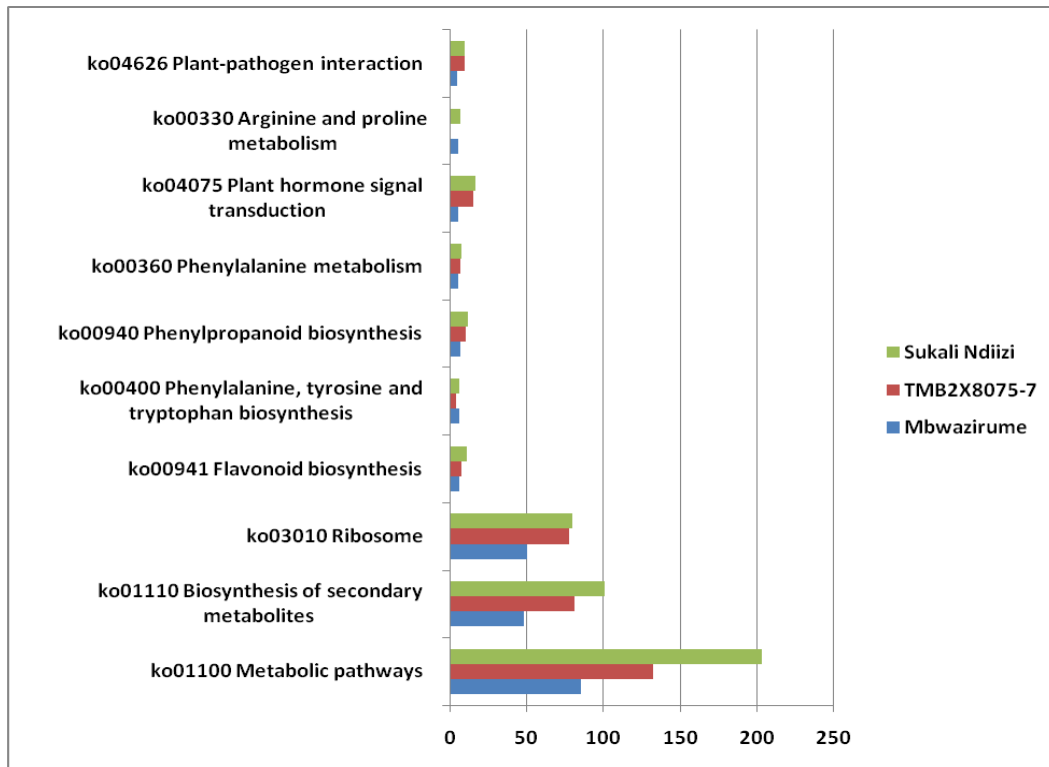
**E) Induced genes 192 hours**



**F) Repressed genes 48 hours**



**Figure 3.** Venn diagrams comparing genes induced at 48 (A), 96 (C) and 192 (E) hours post inoculation between immune banana 'Mbwarzirume', resistant banana 'TMB2X8-75-7' and susceptible banana 'Sukali Ndiizi' and genes repressed at 48 (B), 96 (D) and 192 (F) hours post inoculation between immune banana 'Mbwarzirume', resistant banana 'TMB2X8-75-7' and susceptible banana 'Sukali Ndiizi'.



No. of Kegg Ontology terms

**Figure 4.** Pathways differentially enriched in three banana genotypes 'Mbwazirume', 'TMB2X8075-7' and 'Sukali Ndiizi' in response to *Fusarium oxysporum* f. sp *cubense* race 1 inoculation. The bar graphs show the number of ontology terms mapped by differentially expressed genes of Fusarium wilt immune banana 'Mbwazirume' (blue), resistant banana 'TMB2X 8075-7'(red) and the susceptible banana 'Sukali Ndiizi' in the Kyoto encyclopedia of genes and genomes (KEGG) database.

## CHAPTER 5

### CONCLUSIONS

Banana (*Musa* spp.), including the dessert and cooking banana, is the eighth most important food crop in the world, and the fourth most important in the least developed countries (FAOSTAT, 2013). In Africa, banana is a key economic resource for rural farmers. With a total annual production estimated at 33.2 MT, the crop supports livelihoods of many poor rural farming communities in Africa. In east and central African countries, banana is a major staple food and an important cash crop in the regional economy, worth about US\$ 4.3 billion (EAC, 2012). Domestic per capita consumption of bananas in Uganda is estimated to be between 220-460 kg, the largest in the world. The potential of the banana crop has not been fully harnessed due to the challenge of pests and diseases, which include Fusarium wilt, bacterial wilt, nematodes, weevil, black Sigatoka and Banana Bunchy Top Disease (BBTD) (Tushemereirwe *et al.*, 2003; Mwangi and Nakato, 2009). The yield loss due to pests and diseases pose a great threat to the sustainability of banana production in the Great Lakes region of eastern Africa (Edmeades *et al.*, 2007; Swennen *et al.*, 2013).

Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (Foc), is one of the world's most destructive banana diseases. The disease first gained prominence when it caused significant losses to 'Gros Michel' bananas grown for export to the USA and Europe during the first half of the 20<sup>th</sup> century. The international banana industry was saved by replacing the Fusarium wilt-susceptible variety 'Gros Michel' with resistant 'Cavendish' bananas. Once the disease is present in a field it cannot be eradicated by currently available cultural practices and fungicides. The soil-borne nature and ability of the fungus to survive for extended periods makes it difficult to target with fungicides. Suckers taken from diseased areas spread the Fusarium wilt fungus over large distances, while it can be disseminated within and between fields with soil attached to shoes, tools, vehicles and in irrigation water. The only means to protect bananas is to prevent the fungus from being introduced into disease-free fields through preventive measures, or by planting resistant varieties. Unfortunately, even the 'Cavendish' varieties that once provided a solution to Fusarium wilt have now succumbed to the disease due to the emergence of a variant of the Fusarium wilt fungus, called Foc TR4.

Genetic improvement of bananas to produce Fusarium wilt-resistant cultivars is complicated by limited genetic information, low genetic variability, polyploidy and low levels of female and/or male fertility in most of the widely-grown triploid clones. Resistance breeding in bananas is also limited by the long generation time of the crop and large space requirements, which make selecting genotypes across crop cycles costly. Most cultivated bananas are

seedless and reproduce vegetatively. Their gene pool is extremely narrow, which makes them highly prone to pests and diseases. Consumer preference and international markets have further narrowed the diversity in cultivated bananas. The Cavendish banana, now commonly found in food stores around the world, makes up approximately 40% of all bananas grown on the planet. The world export market, comprising 13% of all bananas produced globally, consists almost entirely of Cavendish bananas. In Uganda, where cooking bananas are grown by smallholder farmers the East African Highland bananas (EAHB-AAA), locally called 'matooke', is the important cultivar group which comprises about 85% of bananas produced in Uganda (Karamura *et al.*, 1996).

In this study, an F<sub>2</sub> population segregating for Foc race 1-resistance was developed. The diploid banana 'TMB2X 8075-7' is an improved diploid male parent, which was derived from a cross between 'SH3362' (AA) and 'Calcutta 4' (AA); both resistant to Foc race 1 (Moore *et al.*, 1995; Rowe, 2000). By means of inheritance studies it was determined that resistance in the improved diploid banana 'TMB2X8075-7' is controlled by a single recessive gene *pd1*. Resistance mediated by recessive genes is known for being very durable, therefore 'TMB2X 8075-7' may provide a source of resistance to Foc race 1 that can be introgressed into susceptible commercial varieties like 'Gros Michel' (AAA), 'Sukali Ndiizi' (AAB) and 'Pisang Awak' (ABB) by recurrent selection. A molecular marker for *pd1*, identified by means of Diversity array technology (DArT) analysis, might also be of significant value when breeding Foc race 1-resistant hybrids, as field evaluation of bananas for Fusarium wilt resistance might not be required anymore. This will reduce the duration and costs for breeding for Fusarium wilt resistance.

Functional annotations of the DArT markers that mapped significantly ( $1e^{-10}$ ) on the *Musa* reference genome V1 database showed that these were not directly linked to plant defence. It is worth investigating the role of these genes in Fusarium wilt resistance further, either by transcript expression studies or by developing gene-specific mutant plants. These genes could also be good candidates for identifying single nucleotide polymorphisms (SNPs) associated with Fusarium wilt resistance on haplotype maps of *Musa* sp. Further studies are also required to validate the co-segregation of *pd1* with candidate DArT markers that were associated the Foc race 1-resistance in coupling phase, to confirm the role of *pd1* in Fusarium wilt resistance. The 31 genes in linkage disequilibrium with the *pd1*, which mapped to genes on the *Musa* reference genome (DH Pahang genes V1 database), could represent a genomic region required for susceptibility to Foc race 1 in *Musa*. Their role could be validated by inducing gene-specific mutations using self-complementary chimeric oligonucleotides (COs) to create stable, site-specific base substitutions, and by testing such mutants for susceptibility to Foc race 1.

RNA-seq analysis of three banana genotypes ('Mbwazirume', 'TMB2X8075-7' and 'Sukali Ndiizi') to Foc race 1 showed that bananas respond to Foc race 1 infection by activating the 'primary Metabolic' and 'Ribosome' pathways. The 'primary Metabolic' pathway enables the plant to meet the increased energy demands of the plant as it responds to pathogen attack, while the 'Ribosome' pathway provides for new ribosome's or changes in ribosome components to facilitate more rapid syntheses of defense proteins. This implies that nutrients are important factors in plant-disease interactions for the formation of mechanical barriers and synthesis of natural defence compounds (phytoalexins, antioxidants, and flavanoids). Enhanced plant nutrition could be explored to compensate the high energy demand when susceptible bananas are grown in Foc race 1-infested fields. However, we need to understand the abundance of various nutrients in bananas, and their roles in plant defence, to provide a balanced nutrient supply optimal for disease resistance. The RNA-seq results further showed that several pathogenesis-related proteins could be acting synergistically to enhance levels of resistance in banana following infection with Foc race 1. These PR protein genes could potentially be used to develop transgenic plants with Foc race 1 resistance.



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