EVALUATION OF EXPRESSION OF *Hrap* AND *Pflp* GENES IN TRANSGENIC BANANA PLANTS FOR RESISTANCE AGAINST *XANTHOMONAS CAMPESTRIS* PV. *MUSACEARUM*

By

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DECLARATION

I, Muwonge Abubaker declare that the thesis which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my original work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Date……30/04/2015……..

Signed 

This thesis has been submitted for examination with my approval as the University supervisor.

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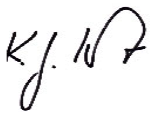
Signed 

PROF KARL KUNERT

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ABSTRACT

Banana *Xanthomonas* wilt (BXW) remains a major problem to banana production in the East and Central African region. This PhD study evaluated the expression of stacked *Hrap* (hypersensitive response assisting protein) and *Pflp* (plant ferredoxin like protein) genes to possibly provide higher resistance to *Xanthomonas campestris* pv. *musacearum* (*Xcm*) compared to single gene expression. An expression plasmid vector having stacked *Pflp* and *Hrap* genes under control of a constitutive CaMV35s promoter was constructed using PCR cloning and restriction enzymes analysis. This plasmid vector was used to transform embryogenic cells using *Agrobacterium tumefaciens* transformation system and transgenic plants of banana cultivars "Gonja manjaya" and "Sukali ndiizi" were generated. Twenty one soil potted transgenic lines each of stacked genes as well as of the single *Hrap* or *Pflp* were artificially inoculated with *Xcm* and evaluated for resistance to BXW under screen house conditions. From inoculated plants, 28.6% of single *Hrap* lines, 19.1% of single *Pflp* lines and 33.3% of stacked lines had no disease symptoms and were completely resistant to BXW. Partial resistance was observed in 28.6% of single *Hrap* lines, 33.3% of single *Pflp* lines and 28.6% of stacked lines with only inoculated leaf showing symptoms. All non-transgenic control lines were susceptible and wilted completely. Also lesion length and chlorotic area were significantly smaller in all transgenic plants in comparison to non-transgenic control plants. However, no significant difference in lesion length and chlorotic area was observed transgenic lines with single *Hrap* and *Pflp* transgenes in comparison to lines stacked genes. Histochemical staining and calorimetric assays further confirmed higher hydrogen peroxide accumulation after *Xcm* infection in all transgenic plants which was related with increased transcription of *NPR1* genes. *NPR1* gene induction was also associated with

transcription of *PR1*, *PR3* and GST genes in transgenic banana lines. However, *PR1* gene transcription was significantly lower in all transgenic lines than in the non-transgenic control plants. In contrast, transcription of *PR3* gene, and also of a gene coding for GST, was higher in all transgenic lines than the non-transgenic control after *Xcm* infection. *Hrap* and *Pflp* transgene expression thereby shifted the pathogen response to a response normally expected for a necrotrophic pathogen. Overall, this study has provided evidence that stacked expression of *Hrap* and *Pflp* genes in transgenic banana lines did not have higher or additive resistance levels to *Xcm* compared to single genes. Future field studies should be carried out to demonstrate if developed transgenic lines expressing stacked transgenes have durable resistance to *Xcm* compared to lines expressing a single transgene.

THESIS COMPOSITION

Chapter 1 of this PhD thesis provides a summary of the importance of banana and impact of the banana *Xanthomonas* wilt in the East African region. Further, current knowledge on plant defense responses and the transgenic strategies used to manipulate these responses for enhanced resistance are reviewed. Existing information on the role of hypersensitive response assisting protein (Hrap) and plant ferredoxin like protein (Pflp) proteins in plant defense are then provided as well as perception and attitude of Ugandans towards genetically modified bananas. The rationale and the objectives for carrying out this study are finally outlined at the end of the chapter. **Chapter 2** describes the construction of a plasmid vector with stacked *Hrap* and *Pflp* transgenes using the polymerase chain reaction (PCR) cloning and restriction enzyme analysis. The procedures that were used to confirm the correctness of the plasmid vector are also provided. **Chapter 3** describes the transformation of stacked or single *Hrap* and *Pflp* genes into banana cells using *Agrobacterium*-based system. Regeneration of transformed plants and detection of transferred transgenes using molecular based methods are further outlined. **Chapter 4** evaluates and compares resistance levels in transgenic plants with stacked and individual *Hrap* and *Pflp* genes against banana *Xanthomonas* wilt. Further results are outlined about the accumulation of hydrogen peroxide following infection as well as any phenotypic changes in plants over expressing the transgenes either individually or stacked. **Chapter 5** outlines the results obtained after investigating the expression of defense related genes (*PR1*, *PR3*, *NPR1*, and *GST)* in *Hrap* and *Pflp* transgenic bananas to demonstrate any possible relation of defense genes expression with resistance to *Xanthomonas campestris* pv. *musacearum*. Finally, **Chapter 6** summarizes all

new key findings generated from the work and evaluates the set working hypothesis. In additional, future actions based on the results obtained in this study are outlined.

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DEDICATION

This work is dedicated to the memory of my beloved dad EDIRISA NADIBANGA MUWONGE. You did everything to ensure I got great education but did not live to see the fruits of your sweat. May Almighty ALLAH rest you in peace and grant you jana, Ameen.

ABBREVIATIONS AND SYMBOLS

%	Percentage
µM	Micromolarity
AATF	African Agricultural Technology Foundation
ALS	Acetolactase synthase
AsA	Ascorbate
Avr	Avirulence
bp	Base pair
BSA	Bovine serine albumin
BXW	Banana *Xanthomonas* wilt
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming unit
CIAP	Calf intestine alkaline phosphatase
CRD	Completely randomized design
cv	Cultivar
DAB	3,3-diaminobenzidine
dATP	deoxyadenosine triphosphate
ddH_20	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	Days post inoculation
DSI	Disease severity index
ECS	Embryonic cell suspension

ET	Ethylene
ETI	Effector-triggered immunity
FAOSTAT	Food and Agriculture Organization Statistics
g/L	Gram per liter
GFP	Green fluorescence protein
GM	Genetically modified
GSH	Glutathione
GST	Glutathione S transferase
GusA	β-glucuronidase
HCD	Hypersensitive cell death
hr	Hour
HR	Hypersensitive response
HRAP	Hypersensitive response assisting protein
IITA	International Institute of Tropical Agriculture
ISR	Induced systemic resistance
JA	Jasmonic acid
kb	kilobase
KV	Kilovoltage
LB	Luria Bertani
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
min	Minute

mM	Millimolar
ms	Millisecond
MYB	Myeloblastosis
NADP	Nicotinamide adenine dinucleotide phosphate
NARL	National Agricultural Research Laboratories
NCBI	National Center of Biotechnology Information
ND	Ndizzi
ng	Nanogram
NLB	Northern leaf blight
Nos	Nopaline synthase
*npt*II	Neomycin phosphotransferase
NPR1	Nonexpressor of pathogenesis related 1
^{o}C	Degree celsius
OD	Optical density
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PFLP	Plant ferredoxin-like protein
PR	Pathogenesis related
PR1	Pathogenesis related 1
PR3	Pathogenesis related 3
PRR	Pattern recognition receptors

PTI	PAMP-triggered immunity
qRT-PCR	Quantitative real time polymerase chain reaction
R	Resistance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second
SA	Salicylic acid
SAR	Systemic acquired resistance
SCV	settled cell volume
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride-sodium citrate
TCA	Trichloroacetic acid
TTSS	Type three secretion system
Xcm	*Xanthomonas campestris* pv. *musacearum*
YMA	Yeast-mannitol agar
YPGA	Yeast extract peptone glucose agar
µL	Microliter

CHAPTER ONE

INTRODUCTION

1.1 Importance of banana

Banana, a term used in the text of this study, to refer to bananas and plantains, are monocotyledonous plants belonging to the *Musaceae* family, and are believed to have originated from South East Asia and the Pacific Islands including Papua New Guinea, Malaysia and Indonesia (Simmonds, 1962; Langhe, 1996). Bananas are further categorized based on ploidy levels, genome composition, place of cultivation, and use of their end products (Karamura *et al*., 1998). Banana is the eighth most important staple food crop in the tropics and subtropics, with an annual world production of around 133 million tons (Tripathi *et al* 2014a). They are grown in more than 120 countries throughout the tropics and sub-tropics (FAOSTAT, 2011). The vast majority of producers are small scale-farmers growing the crop for home consumption and local markets (IITA, 2008). In Africa, bananas supply up to 25% of the carbohydrates for more than 100 million people in humid forest and mid-altitude regions (IITA, 1998). In the Great Lakes region of Africa, bananas are important for both food security and as a cash crop (Karamura, 1998). They also find other uses such as in brewing and for medicinal purposes (Rieger, 2005). Their byproducts including peels, leaves and pseudo-stems are also used as animal feeds (Price, 1994). Despite their economic importance, production of bananas is threatened by various biotic constraints, the banana *Xanthomonas* wilt being one of them particularly in the Great Lakes region (Tripathi *et al*., 2009).

1.2 Banana *Xanthomonas* Wilt

Banana *Xanthomonas* wilt (BXW) is caused by a gram-negative bacterium, *Xanthomonas campestris* pv. *musacearum* (Xcm). Bacteria *Xanthomonas campestris* in general are a serious problem in crop productivity as they cause diseases of significant importance. This group was ranked in fifth position among the top 10 bacterial plant pathogens according to a survey by Mansfield *et al*. (2012). No wonder BXW is the most devastating disease of bananas in Africa's Great Lakes region (Kalyebara *et al*., 2006). BXW was first reported in Ethiopia in Ensete species more than 45 years ago and then in banana (Yirgou and Bradbury, 1968; 1974). Outside Ethiopia it was reported in 2001 in the Mukono district of central Uganda, since then BXW has spread to all banana growing regions of the country as well as to other countries in the Great Lakes region namely Rwanda, (Reeder *et al*., 2007), Democratic Republic of Congo (Ndungo *et al*., 2006), Burundi Tanzania, and Kenya (Mwangi *et al*., 2006). Production loss of about 53% resulting into an estimated economic loss at about $2 - $8 billion over a 10 year period was envisaged (Abele and Pillay, 2007). The disease is mainly transmitted from one plant to another within the field by flying insects and also repeated use of infected plant cuttings in new plantations (Biruma *et al*., 2007). Disease symptoms include premature and uneven ripening of the inflorescence, rotting of fruits, wilting, yellowing and eventual death of leaves, Figure 1.1. This is followed by gradual collapsing of the entire plant during the terminal stages of infection (Mwangi *et al*., 2007). A number of cultural practices have been applied to manage BXW disease. These include removal of the male bud to prevent flower infection by insects (Ssekiwoko *et al*., 2006; Biruma *et al*., 2007; Tripathi et al., 2009), complete removal of the

entire mat once there is infection of a single plant (Adikin, 2010), and sterilization of garden tools using sodium hypo-chloride (Smith *et al*., 2008).

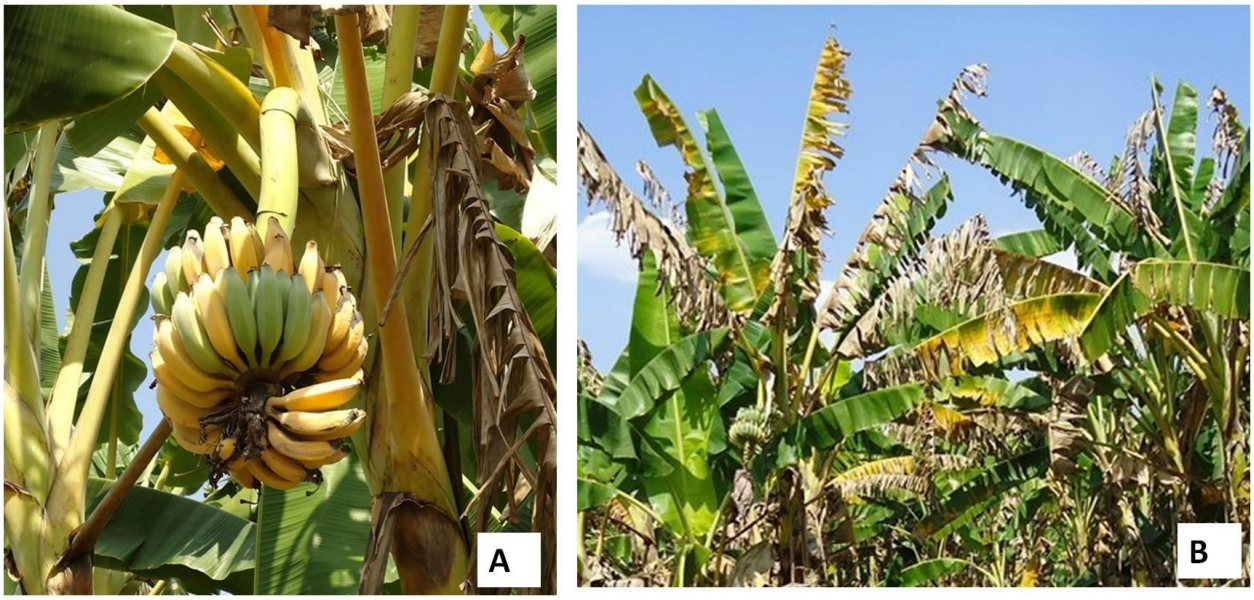

Figure 1.1: Symptoms of banana *Xanthomonas* wilt on field plants. A: premature ripening of the banana fingers; B: chlorotic and necrotic leaves.

Control of BXW is mainly based on use the above mentioned cultural practices. So far no effective bactericides have been reported for controlling BXW (Okurut *et al*., 2006). Furthermore, developing resistant varieties using conventional breeding was halted because all banana germplasm grown in the region are susceptible to BXW and so far, no resistant cultivars have yet been identified (Tripathi et al., 2014a). Although some level of tolerance was reported in wild *Musa balbisiana* (Tripathi *et al*., 2008), most of the local genotypes, such as "Gonja manjaya", "Sukali ndiizi" and *M. balbisiana* are sterile making conventional breeding impossible.

1.3 Pathogen-plant interactions

Activator pathogen proteins and plant receptor proteins are involved in pathogen-plant interactions that upon interaction; defense responses are activated (Chisholm *et al*., 2006, Jones and Dangl, 2006). A general term for pathogen activator proteins is the microbe-associated molecular pattern (MAMP) (Bent and Mackay, 2007). MAMPs are recognized by pattern recognition receptors (PRRs) on the plant cell surface (Truman *et al*., 2006; Jones and Dangl, 2006; Abouelmaatti *et al*., 2013) triggering defense responses (Segonzac and Zipfel, 2011). Recognition of MAMPs, or pathogen associated molecular patterns (PAMP), by PRR triggers a response referred to as basal resistance or innate immunity (Bittel and Robatzek, 2007), which is relatively static in evolving its recognition specificity relative to mammalian adaptive immune systems (Bent and Mackay, 2007). The relevance of MAMP receptors for improving plant disease resistance has been previously demonstrated (Zipfel *et al*., 2004; Li *et al*., 2005; Torres *et al*., 2006). Despite the ability of PRRs to recognize MAMPs, or other effectors, pathogens can suppress the plant immune system allowing them to grow inside the host (Pel *et al*., 2012). This is achieved by shedding the flagella upon entering the host (Milton *et al*., 1996; Tans-Kersten *et al*., 2001) and masking the bacterial epitopes that would trigger host defense reactions (Gonzalez *et al*., 1996; Andersen-Nissen *et al*., 2005).

Avirulence (Avr) proteins as well as hypersensitive response and pathogenicity (hrp) proteins, referred to as effectors, are pathogen-defense activating proteins. They are delivered inside the plant cytoplasm via the Type three secretion system (TTSS) in a pilus encoded by *hrp* genes (Brueggeman *et al*., 2002; Casper-Lindley *et al*., 2002; Alfano and Collmer, 2004). Between 15-

30 Avr proteins can be delivered from individual plant pathogenic bacteria (Cunnac *et al*., 2004; Buttner and Bonas, 2006; Lindeberg *et al*., 2006). The *Avr* gene products are recognized by the plant Resistance (R) genes in a gene for gene interaction. Inside the plant cell, Avr proteins can be directed to specific localization for R gene mediated recognition (Kjemtrup *et al*., 2000). Both indirect and direct interaction of Avr effectors with R proteins have been demostrated (Bent and Mackay, 2007). Directly interacting Avr effectors prevent recognition via mutation through IS elements and transposase terminal repeats (Kjemtrup *et al*., 2000). These changes disrupt the physical interaction with R protein but maintaining virulence activity (Liu *et al*., 2005, Bent and Mackay, 2007). Virulence activity of indirectly recognized effectors is however compromised for any mutation to escape recognition by the host.

Innate immune response upon detection of PAMP activates PAMP-triggered immunity (PTI), whereas recognition of the presence and activity of effectors by R proteins activates Effector-triggered immunity (ETI) (Thomma *et al*., 2001). ETI occurs much faster, is more prolonged and more robust than PTI (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). Data has shown that both the hypersensitive response (HR) and systemic acquired resistance (SAR) also occur in PTI responses (Thomma *et al*., 2001; Khatib *et al*., 2004; Mishina and Zeier, 2007).

1.4 Plant defence systems

1.4.1 Reactive oxygen species

Recognition of pathogens activates plant defense responses (Zipfel and Felix, 2005; Bittel and Robatzek, 2007) in a highly coordinated manner occurring immediately after invasion or within hours and some are delayed for days (Zhang and Zhou, 2010). The oxidative burst is one of the most rapid plant defense responses to pathogen attack and includes production of superoxide and hydrogen peroxide at the invasion site by various oxidases and peroxidases sources, such as NADPH oxidase (Low and Merida, 1996; Allan and Fluhr, 1997; Bolwell *et al*., 1998; Vanacker *et al*., 2000; Schopfer *et al*., 2001; Torres *et al*., 2006; Nuhse *et al*., 2007; Nicaise *et al*., 2009). Two possible reactive oxygen species (ROS) production patterns following pathogen attack in plants exist where the first low amplitude, transient production phase is followed by a sustainable and also much higher second phase. Such a biphasic pattern of ROS accumulation occurs in interactions where the avirulent pathogen effectors are recognized by an R gene which results in a hypersensitive response (HR) and disease resistance (Lamb and Dixon, 1997; Apel and Hirt, 2004; Mendoza, 2011). In contrast, a single transient low amplitude first phase accumulation of ROS has been found for virulent pathogens avoiding host recognition and without disease resistance development (Torres *et al*., 2006). ROS production was initially considered as a system to kill invading pathogens (Levine *et al*., 1994), but there is strong evidence that ROS are also important signals, mediating defense gene activation (Mendoza, 2011) and also directly strengthen host cell walls via cross-linking of glycoproteins (Bradley *et al*., 1992, Lamb and Dixon, 1997).

Research has shown that suppressed ROS production results in more susceptibility to the avirulent oomycete *Phytophthora infestans*, and silencing of NADPH oxidase, responsible for apoplastic ROS generation, suppresses HR in *Nicotiana benthamiana* (Yoshioka *et al*., 2003). In signal transduction pathways, ROS further trigger defense responses with ROS sensors that induce signaling cascades (Zaninotto *et al.*, 2006). ROS either directly oxidize components of the signaling pathways or modifies transcription factor activities, such as activities of zinc finger proteins and WRKY transcription factors (Mou *et al*., 2003, Apel and Hirt, 2004; Miller *et al*., 2008). Genes induced in response to ROS include mainly genes involved in carbohydrate metabolism, detoxification function, metabolite transport, defense responses and signaling function (Gasch *et al*., 2000; Causton *et al*., 2001; Desikan *et al*., 2001; Chen *et al*., 2003).

The balance between ROS production and removal by the antioxidant systems will determine the strength, lifetime and size of the ROS signaling (Sharma *et al*., 2012). Production and removal of ROS has to be strictly controlled in plants by different anti-oxidative defense components and enzymes systems (Alscher *et al*., 1997). The non-enzymatic anti-oxidants systems include ascorbate (AsA), glutathione (GSH), tocopherol, flavonoids, alkaloids and carotenoids (Apel and Hirt, 2004), whereas enzymatic systems are superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase, Fig. 1.2.

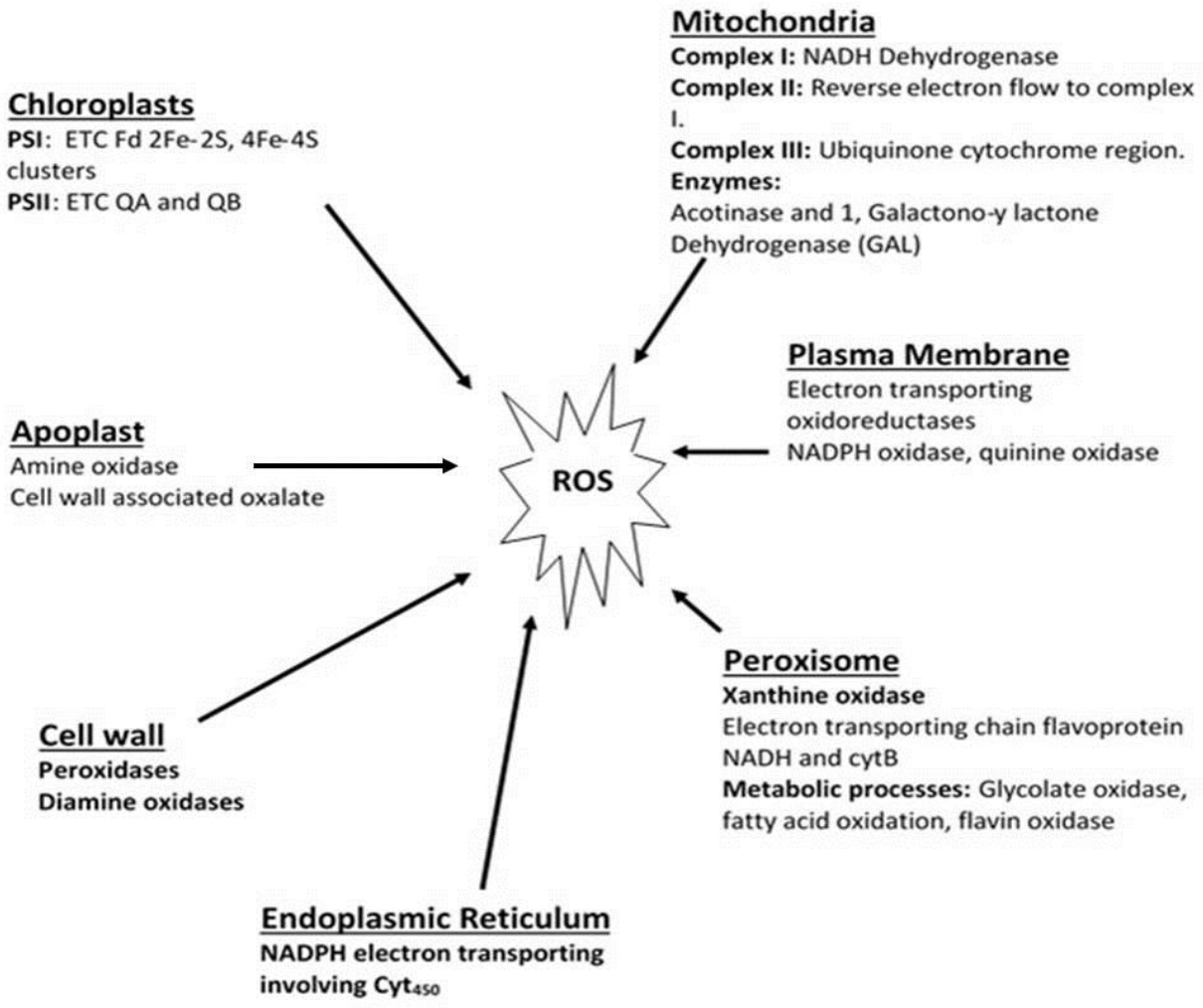

Figure 1.2: Sites of reactive oxygen species (ROS) production in plants. ROS are produced in the chloroplast, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum, and cell wall (modified from Sharma *et al*., 2012)

1.4.2 Hypersensitive response and programmed cell death

The hypersensitive response (HR) prevents the multiplication of the invading pathogen (Morel and Dangll, 1997) and is a result of recognition of an Avr protein from the pathogen by a cognate

plant resistance (*R*) gene product (Jones and Dangl, 2006). During this response, some plants cells form a layer of dead cells and circle the invading pathogen thus inhibiting the pathogen growth (Iakimova *et al*., 2005). HR is further genetically controlled and activated by increased production of ROS (Dangl and Jones, 2001). Upon HR elicitation, host defense-related genes are activated with production of anti-microbial secondary metabolites such as phytoalexins (Dangl and Jones, 2001; Dixon, 2001; Truman *et al*., 2006). HR is also not confined to a few cells but is rather macroscopic (Mur *et al*., 2007). HR formation during different plant–pathogen interactions also varies greatly in phenotype and timing at both macro and microscopic scale (Christopher-Kozjan and Heath, 2003; Krzymowska *et al*., 2007). During HR, dying plant cells further produce a physical barrier by strengthening their cell walls through depositing different phenolic compounds and synthesizing diverse toxic products (Iakimova *et al*., 2005)

Similarities between the HR and animal apoptosis have been reported. Common features are cytoskeletal rearrangements, DNA laddering, cellular shrinking, chromatin condensation, and margination and ruffling of the plasma membrane (Sasabe *et al*., 2000, Yao *et al*., 2002; Ning *et al*., 2002; Kiba *et al*., 2006; Mur *et al*., 2007). Absence of apoptotic bodies formation at the end of the HR process, unlike in apoptosis, suggests that HR has a distinctive mechanism (Jones and Dangl, 2006).

1.4.3 Systemic acquired resistance

Systemic acquired resistance (SAR) is a defense mechanism that develops in distal plant parts after local infection (Durrant and Dong, 2004; Jing *et al*., 2011), with the phloem as the major conduit of the SAR signals throughout the entire plant (Thakur and Sohal, 2013). Initially it was

thought that SAR is induced by pathogens triggering HR (Conrath, 2006), recent findings suggest that HR, or tissue necrosis, at the site of infection is not a requirement for SAR activation (Mishina and Zeier, 2007). Once induced, SAR is active against a broad spectrum of pathogens including viruses, bacteria, and fungi and can last for a plant´s lifetime (Ryals *et al*., 1994; Sticher *et al.,* 1997). In SAR, salicylic acid (SA) is a key signal molecule (Vlot *et al.*, 2009). Induction of SAR correlates with increased levels of SA in both local and systemic leaves (Malamy *et al*., 1990). Transgenic tobacco and *Arabidopsis* plants constitutively expressing a bacterial SA hydroxylase, which removes SA, are unable to accumulate high levels of SA and therefore do not acquire systemic resistance upon infection with pathogens (Gaffney *et al.,* 1993; Friedrich *et al*., 1995).

Besides SA, lipid-derived signals have also a role in SAR (Nandi *et al*., 2004; Jung *et al*., 2009; Chanda *et al*., 2011). *Arabidopsis* mutants defective in induced resistance 1 (*dir1*) strongly indicated that lipid transfer proteins play a role in transmission of mobile signals for SAR (Maldonado *et al*., 2002). In addition, ethylene has also been implicated as a signal for SAR. Using ethylene-insensitive transgenic tobacco plants, signaling of SAR was dependent on ethylene perception (Verberne *et al*., 2003). There is further a relationship between hydrogen peroxide production and SAR. Systemic hydrogen peroxide has also been found in remote un-inoculated leaves following the oxidative initial burst but in lower amounts and in small groups of cells and both the initial and secondary hydrogen peroxide bursts are required for SAR (Conrath *et al*., 2006).

1.4.4 Defense proteins

1.4.4.1 NPR1

Onset of SAR is characterized by increased levels of SA, which is sensed by redox sensitive protein referred to as the nonexpressor of pathogenesis related genes1 (NPR1) (Durrant and Dong, 2004). Available evidence shows that NPR1 acts downstream of ROS and phytohormones in the defense signaling cascade. The NPR1 protein exists in the cytosol in an oligomeric form (Spoel *et al*., 2009), but during pathogen attack, it is reduced to monomeric state due to changes in redox state of the cell and then translocates to the nucleus (Mou *et al*., 2003; Shi *et al*., 2010; Zhang and Zhou, 2010). NPR1 is a co-transcription factor required for the activation of other transcription factors (Cao *et al*., 1994; Rochon *et al*., 2006). The activated transcription factors bind to the promoter sequences of pathogenesis-related (*PR*) genes leading to the activation of *PR* genes (Johnson *et al*., 2003; Rochon *et al*., 2006). The expression of PR proteins accompany establishment of SAR (Thakur and Sohal, 2013). There is evidence however, that accumulation of ROS in the cytosol prevents NPR1 translocation to the nucleus, hence reducing NPR1 dependent PR gene expression (Peleg-Grossman *et al*., 2010).

In addition, NPR1 plays a key role in the SA-independent induced systemic resistance (ISR) (Durrant and Dong, 2004). ISR triggered by non-pathogenic root-colonizing bacteria confers resistance to bacteria and fungi in the aerial parts of the plant (Pieterse *et al*., 1998). NPR1 also mediates cross-talk between the SA signaling pathway and the jasmonic acid (JA) and ethylene (ET) signaling pathways providing resistance to insects and necrotrophic pathogens (Spoel *et al*.,

2009; Koornneef and Pieterse, 2008; Luna *et al*.2012). *NPR1* gene homogues have been characterized in *Arabidopsis*, rice, soybean, grapevine, cocoa and from banana, some of which are specifically involved in defense (Endah *et al*., 2008; 2010; Zhao *et al*., 2009; Sandhu *et al*., 2009). Two *NPR1* homologues (*MNPR1A* and *MNPR1B*) of banana are reported to be induced in response to a *Xanthomonas campestris* pv. *musacearum*, a hemibiotroph whereas the necrotroph pathogen *Fusarium oxysporum* induced only one *NPR1* gene (Endah *et al*., 2010).

Over-expression of At*NPR1* in *Arabidopsis thaliana*, rice, tomato, apple and wheat resulted in enhanced fungal and bacterial resistance (Cao *et al*., 1998; Friedrich *et al*., 2001; Lin *et al*., 2004; Fitzgerald *et al*., 2004; Chern *et al*., 2005; Makandar *et al*., 2006; Malnoy *et al*., 2007). It also resulted in activation of *PR* genes (*PR2, PR5*, and *PR8*) in apple, providing enhanced resistance to *Erwinia amylovora* (Malnoy *et al*., 2007).

1.4.4.2 PR proteins

Biosynthesis of pathogenesis related (PR) proteins in plants are among the induced defense responses in response to pathogen attack (Upadhyay *et al*., 2014). PR proteins belong to 17 families based on amino acid sequence similarity and their biological properties or functions (Table 1.1). They comprise chitinases, 1,3-glucanases, thaumatin-like proteins, proteinase inhibitors, a subtilisin-like endoproteinase, plant peroxidases, the birch allergen Betv1-related, plant defensins, thionins, nonspecific lipid-transfer proteins, germin-like proteins, and secretory protein (Van Loon *et al*., 2006).

Accumulation of PR proteins occurs locally in the infected and surrounding tissues, as well as in remote uninfected tissues. They may be acidic or basic, where the former are localized in the intercellular spaces, whereas the latter are predominantly localized in the vacuole (Van Loon and Van Strien, 1999). Expression of acidic *PR* genes during pathogen attack is mediated by salicylic acid and ROS signaling molecules while basic *PR* genes by ethylene and methyl jasmonate (Sinha *et al*., 2014). Thus *PR* genes may be used as indicators for which signaling pathway is activated during infection. Increase in the expression levels of *PR1*, *PR2* and *PR5* genes represents induction of the SA pathway (Naidoo *et al*., 2013), whereas increased expression of *PR3*, *PR4* and lipoxygenase genes are used as indicators for the onset of JA signaling pathway (Mishina and Zeier, 2007; Kusajima *et al*., 2010). Over expression of SA and JA signature defense genes have been shown to result in increased resistance to pathogens (Mishina and Zeier, 2007; Kusajima *et al*., 2010). Besides defense, PR are also be involved in other important functions in plants such as growth and development (Seo *et al*., 2008).

Table 1.1: Classification of pathogenesis related proteins (Van Loon and Van Strien, 1999)

Families	Proteins	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Endoproteinase
PR-7	Tomato P69	Chitinase type III
PR-8	Cucumber chitinase	Peroxidase
PR-9	Tobacco "lignin forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	*Arabidopsis* THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

1.4.4.3 Glutathione S-transferase (GST)

GSTs are dimeric enzymes that catalyze the conjugation of electrophilic molecules to glutathione (GSH), which are sequestered in the vacuole where they are further processed and detoxified (Dixon *et al*., 2002; Dean *et al*., 2005). The organic hydroperoxides are detoxified by conversion to the less toxic monohydroxy alcohols (Lieberherr, 2001). Organic peroxides are produced during pathogen attack as well as from detoxification of microbial toxins and from phytoalexins

produced during the hypersensitive response (Li *et al*., 1997). GSTs can also function as carriers of auxin and phenylpropanoids, as well as delivering anthocyanin into the vacuole for sequestering (Mueller *et al*., 2000; Smith *et al*., 2003; Dean *et al*., 2005). Furthermore, GSTs can activate phenylpropanoid metabolism thus serving as signaling molecules (Loyall *et al*., 2000).

Increased expression of *GST* genes is among the defense responses to pathogen attack in plants (Dean *et al*., 2005; Sappl *et al*., 2009; Liao *et al*., 2014). Together with the *PAL* gene, *GST* are induced by H_2O_2 in a time and dosage dependent manner during pathogen attack (Bhattacharjee, 2012). Induction of *GST* has been implicated in disease resistance to *Phytophthora infestans* in potato, to various diseases in rice (Wisser *et al*., 2005) as well as to southern leaf blight, gray leaf spot and northern leaf blight (NLB) in maize inbred lines (Wisser *et al*., 2011). Expression of *GST* can also be induced in response to abiotic stresses as well (Wisser *et al*., 2011). Crosstalk between GST and other players in plant defense to pathogen attack has also been demonstrated. Using *Arabidopsis*, GST homolog genes were induced early than PR1 accumulation, and required SA and ET signaling pathways (Lieberherr, 2001).

1.4.4.4 Plant ferredoxin-like proteins

Particular interest in this PhD study was the capacity of the plant ferredoxin-like protein (PFLP) as defense protein to enhance resistance to pathogens in transgenic plants. The *Pflp* gene cloned from sweet pepper (*Capsicum annum*) encodes a 12.6 kDa protein (Dayakar *et al*., 2003). Ferredoxins are generally small soluble acidic proteins that function as electron donors at reductive steps in various metabolic pathways (Mazouni *et al*., 2003). They mediate electron transfer from photosystem I to ferredoxin NADP reductase which reduces $NADP^+$ for CO_2

fixation (Hurley *et al*., 2002). Ferredoxin is also involved in other redox processes including nitrogen assimilation, sulphite reduction or fatty acid metabolism as well as in the modulation of the activity of various enzymes via the thioredoxin system (Mazouni *et al*., 2003)

Comparison of the sweet pepper PFLP amino acid sequence with sequences of other PFLPs showed that it has a 72% homology with *Lycopersicon esculentum,* 54% with *Arabidopsis thaliana,* 56% with *Oryza sativa*, 52% to *Pisum sativum*, 52% with *Spinacia oleracea,* and 48% with *Zea maize* (Dayakar *et al*., 2003). Structurally, PFLP has a signal peptide of 47 amino acids targeting the chloroplast and a putative 2Fe-2S domain responsible for redox activity (Dayakar *et al*., 2003). The cluster irons and inorganic sulfur atoms are ligated to the polypeptide backbone by the thiolate side chains of cysteine residues (Kurihara *et al*., 2003). In the peptide sequence of PFLP, three cysteine residues exist at C86, C91 and C94 in the [2Fe–2S] domain respectively (Huang *et al*., 2006). The other domain is a casein kinase II (Ck2) phosphorylation site with the general consensus sequence S/TXXD/E (Huang *et al*., 2006) which is located in the carboxyl-terminal region of PFLP.

Expression of *Pflp* gene triggered the harpin-mediated HR in transgenic plants (Dayakar *et al*., 2003), though the exact role in HR is still unclear. PFLP might interfere in the interaction between harpin and a putative receptor or with the *Avr* gene/*R*-gene mediated HR induction (Keen, 1990). Moreover, PFLP was shown to delay harpin-mediated HR (Lin *et al*., 1997), hence postulating that PFLP may interact with harpin as a putative receptor and prevents binding between the receptor and the active fragment of harpin. By this, the harpin may retain its ability to activate HR via the signal transduction system when PFLP is degraded (Lin *et al*., 1997).

In an *in*-vitro assay, recombinant PFLP was also found to have anti-microbial activity against *Escherichia coli, Erwinia carotovora, Xanthomonas campestris* var. *vesicatoria, Xanthomonas campestris* var *campestris* and *Pseudomonas syringae*. (Huang *et al*., 2006). The cysteine residue in the 2Fe-2S domain was found responsible for iron binding and its mutation resulted in loss of anti-microbial activity implying that iron binding capability of PFLP is a requirement for *in vitro* anti-microbial activity (Lin *et al*., 1997).

1.4.4.5 Hypersensitive response assisting protein

Harpins are microbial elicitors that interact with plant cell surface proteins whereby activating the plant defense response (Lee *et al*., 2001). An amphipathic protein isolated from sweet pepper was found to intensify the harpin-mediated HR in sweet pepper and the protein was named Hypersensitive Response Assisting Protein (HRAP) (Chen *et al*., 1998). HRAP dissociates multi-meric harpin into monomeric and dimeric forms that intensify harpin-mediated HR (Chen *et al*., 1998). Structurally, HRAP has three positively charged regions, a 21 amino acid NH_2-terminal hydrophobic region and a cAMP-dependent phosphorylation site (Chen *et al*., 2000). To further elucidate the interaction of HRAP protein and harpin of the pathogen, the three regions of HRAP were truncated. Findings showed that the cAMP phosphorylation region is the one that interacts with harpin and that mitogen-activated protein kinase (MAPK) pathway transduction is involved (Chou, 2005). When the *Hrap* gene from sweet pepper was cloned, the sequence had no similarity to any known sequences (Chen *et al*., 2000). In anti-microbial assays, pathogen

proliferation in the intercellular spaces of tobacco was further inhibited by a recombinant HRAP protein (Ger *et al*., 2002).

1.5 Over-expression of defence-related proteins in transgenic plants

Xanthomonas species are a serious problem as they cause diseases in a number of plants. In a survey, pathogens of the *Xanthomonas* species ranked 4th, 5th and 6th among the top 10 plant pathogenic bacteria based on economic importance (Mansfield *et al*., 2012). Transgenic approaches have been employed to develop plants resistant to *Xanthomonas* species (Table 1.2), in addition to other disease-causing microorganisms.

Pflp and *Hrap* transgenes have been overexpressed in a number of plant crops including bananas to enhance their resistance against pathogens. Constitutive expression of the *Pflp* gene isolated from sweet pepper (*Capsicum annuum*) in transgenic tobacco, rice, orchid, banana, Arabidopsis and tomato plants has been reported to confer disease resistance against various plant pathogens (Tang *et al.,* 2001; Dayakar *et al.,* 2003; Liau *et al.,* 2003; Huang *et al.*, 2007; Namukwaya *et al.,* 2012; Ger *et al*., 2014; Tripathi *et al.,* 2014b). In these plants, *Pflp* mediated resistance was attributed to increased production of ROS and activation of the harpin dependent hypersensitive response (HR) (Dayakar *et al*. 2003; Huang *et al*. 2004).

Expression of a *Hrap* transgene in transgenic potatoes, tobacco, and *Arabidopsis* also enhanced resistance to *Phytophthora infestans*, *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* respectively through induction of the HR (Ger *et al*., 2002; Pandey *et al*., 2005). Using a mutant

Erwinia carotovora subsp. *carotovora* defective in producing HrpN, it was demonstrated that disease resistance mechanism mediated by the *Hrap* gene was harpin dependent (Pandey *et al*., 2005).

Recently individual expression of *Pflp* and *Hrap* genes in banana plants showed enhanced resistance to *Xanthomonas campestris* pv. *musacearum* (Tripathi *et al*., 2010; Namukwaya *et al*., 2012). While induction and execution of defense responses studied in different plant-pathogen combinations have been found to significantly differ (Lamb and Dixon, 1997; Heath. 2000), defense response mechanisms in *Hrap* and *Pflp* expressing transgenic banana plants following infection with BXW remain less well understood.

The *AtMYB30* gene encoding an R2R3 MYB protein is among other defense genes that have been expressed in transgenic plants for enhanced resistance against pathogens. Over-expression of *AtMYB30* in *Arabidopsis* and tobacco accelerated and intensified hypersensitive cell death (HCD) in response to an avirulent pathogen attack and induced an HR-like response upon virulent pathogen attack (Vailleau *et al*., 2002). The transgenic lines exhibited up-regulation of HR marker genes; *hsr203* and *hsr515* and *Arabidopsis*, *Athsr3*, as well as *PR* genes (*PR1* and *PR5)*.

Overexpression of the *BAA1* gene encoding fruit bromelain (protease) in Chinese cabbage (*Brassica rapa*) also enhanced resistance to the soft rot pathogen *Pectobacterium carotovorum* ssp. *Carotovorum* (Jung *et al*., 2008). The proteases are involved in developmental programmed

cell death (PCD) and increase in the resistance to pathogenic infection might be the result of PCD (Beers *et al*., 2000).

Table 1.2: Transgenic approaches applied for resistance against *Xanthomonas* species

Transgenic Plant	**Gene expressed**	**Gene type**	**Pathogen**	**Reference**
Rice	*Xa21*	Resistance	*Xoo*	Rafique *et al*., 2010.
Rice	*avrXa27*	Avirulence	*Xoo*	Tian and Yin, 2009
Rice	*Xa26*	Resistance	*Xoo*	Li *et al*., 2012
Rice	*Xa23/Rxo1*	Resistance	*Xoo, Xoc*	Zhou *et al*., 2009
Rice	*Pflp*	Ferredoxin	*Xoo*	Tang *et al*., 2001
Banana	*Pflp*	Ferredoxin	*Xcm*	Namukwaya *et al*., 2012; Tripathi *et al*., 2014b
Banana	*Hrap*	Hypersensitive	*Xcm*	Tripathi *et al*., 2010; Tripathi *et al*., 2014b
Banana	*Xa21-PRR*	Recognition receptor	*Xcm*	Tripathi *et al*., 2014
Arabidopsis	*Avr-XccC*	Effector protein	*Xcc*	Wei *et al*., 2010
Orange	*Attacin A*	Pathogenesis related	*X. citri*	Cardoso and Mendes, 2010

Xoo: Xanthomonas oryzae pv. *oryzae; Xoc: Xcc: Xanthomonas campestris* pv. *campestris; Xcm: Xanthomonas campestris* pv. *musacearum*.

1.6 Genetically modified banana in Uganda

This PhD study focused on evaluation of transgenic banana transformed with stacked *Pflp* and *Hrap* transgenes for BXW resistance, a serious constraint to banana production in Uganda. Since 2000, Uganda embarked on the use of genetic engineering approaches to address various banana production constraints. Various genes for pest and disease resistance have been therefore recently introduced into local bananas using the *Agrobacterium*-mediated transformation and confined field trials (CFT) for some developed transgenic bananas have been already conducted. Despite the tremendous efforts in developing transgenic bananas which have the desired agronomic traits as well as of improved quality attributes, studies on the consumers' perception, attitudes and knowledge towards of GM crops in developing countries such as Uganda are still few (Chen *et al*., 2002; Curtis *et al*., 2004; Einsele, 2007). Kikulwe (2010) for example explored the knowledge, attitudes, and perceptions among banana-consuming households in relation to the introduction of genetically modified (GM) bananas in Uganda. The results of the survey showed that taste (89%), price (76%) and nutrition (62%) were the most important factors influencing GM banana purchase in Uganda whereas health safety (55%) and environmental safety (35%) were the least. Furthermore, 78% of the consumers were willing to purchase GM banana at a discount, whereas only 38% would buy if a higher price was charged. This was a strong motivation to undertake this PhD study to develop and evaluate GM banana for BXW resistance.

However, the study also revealed that higher educated consumers in rural and urban areas as well as high income earners, particularly in urban areas, were more unlikely to accept GM food as a result of their perceived future health concerns. When offered with quality benefits, such as

better taste and more nutritious but at the same price as traditional (non-GM) banana, over three-quarters of the households would be willing to purchase GM banana, indicating high acceptance of the GM technology under certain circumstances (Kikulwe, 2010).

1.7 Working hypothesis and objectives

Since previous studies already revealed that expression of individual *Hrap* and *Pflp* genes in transgenic bananas enhance resistance to BXW (Tripathi *et al*, 2010; Namukwaya *et al*., 2012), this PhD study particularly focused on the use of these stacked transgenes to possibly provide higher resistance to *Xcm*. If successfully executed this PhD study would therefore increase our understanding of *Hrap* and *Pflp* defense mechanisms to BXW, as well as provide new insights in the use of stacked *Hrap* and *Pflp* transgenes for developing transgenic banana plants that are resistant to BXW. In general, qualitative resistance conferred by a single transgene is often unstable, unlike quantitative resistance derived from many different transgenes with additive effects. Studies focusing on the use of stacked transgenes already showed higher resistance levels to bacterial pathogens and pests in various crops (Stewart *et al*. 2001; Maqbool *et al*. 2001; Datta *et al*., 2002; Cao *et al*., 2002; Zhao *et al*., 2003; Senthilkumar *et al*., 2010). This provided a strong motivation to evaluate also such a stacked transgene strategy in this PhD study. However, a direct comparison of level of resistance and particularly any effect on plant growth that could be triggered in banana by transgene expression (*Hrap* and *Pflp*), specifically when stacked, has not been carried out. Reduced plant growth and viability are often an undesired plant phenotype when pathogen defense proteins are constitutively expressed (Kim *et al*., 2009).

Initially it was hypothesized that expression of stacked *Hrap* and *Pflp* transgenes triggers higher resistance against *Xcm* in comparison to single transgene expression. It was also hypothesized that resistance to *Xcm* in transgenic banana plants expressing *Hrap* and *Pflp* is associated with ROS production and that over-expression of stacked *Pflp* and *Hrap* transgenes affects plant growth. The objectives of this study were therefore to first construct a plasmid vector carrying *Hrap* and *Pflp* transgenes stacked together, secondly to transform the local banana cultivar "Gonja manjaya" with single *Hrap* and *Pflp* transgenes as well as both transgenes stacked to generate transgenic plants, thirdly evaluate and compare resistance against *Xcm* and any transgene effect on growth of transgenic banana plants, fourthly characterize hydrogen peroxide production as a consequence to *Xcm* infection, and finally determine relative expression of selected defense-related genes (*PR1*, *PR3*, *Npr1* and *GST*) in transgenic bananas plants following *Xcm* infection.

CHAPTER TWO

CONSTRUCTION OF A STACKED *Hrap* and *Pflp* PLASMID VECTOR

2.1 Introduction

Plant genetic transformation is among the approaches used for crop improvement. Both *Agrobacterium*-mediated and particle bombardment have previously been used for plant transformation (Travella *et al*., 2005). *Agrobacterium* mediated transformation; the most widely used method and also applied in this PhD study, is, however, preferred because single copy numbers in addition to intact T-DNA can be delivered inside the plant cell (Zhang, 2013). Designing a correct plasmid construct containing a transgene and regulatory elements is a further important task for developing any genetically modified plant. Regulatory elements consist of a promoter and terminator sequence, or polyadenylation site, and a selectable marker gene. The promoter should be highly active, if required in various tissues. The 35S promoter of the cauliflower mosaic virus (CaMV) is therefore the most widely used promoter for constitutive expression in transgenic plants (Zhijian *et al*., 2001; Govindarajulu *et al*., 2008). This promoter causes higher level gene expression in various tissues and is not greatly influenced by environmental conditions or tissue types (Cummins, 1994). Since developing plants for disease resistance requires a relatively strong promoter, the 35S CaMV promoter is a suitable candidate which can confer constitutive transgene expression (Zhijian *et al*., 2001). Versions of 35S CaMV promoters have also been developed to further enhance their activity, this includes an enhanced double 35S promoter. The selectable marker gene, encodes a protein which provides resistance to antibiotics or herbicides, permitting growth of only transformed plants cells on a growth medium. Despite certain health concerns on the use of antibiotics in transgenic crops, no adverse effects of the neomycin phosphotransferase (*npt* II) selectable marker gene, used in this study,

has been so far reported (Miki and McHugh, 2004). This renders *npt* II the most widely used selectable marker in transgenic crops (Miki and McHugh, 2004).

The objective of this part of the study was to construct a plasmid vector for transforming banana with stacked *Hrap* and *Pflp* transgenes both derived from sweet pepper. The polymerase chain reaction (PCR) together with conventional restriction enzyme analysis procedures was used for the cloning of the genes.

2.2 Materials and Methods

2.2.1 *Hrap* and *Pflp* plasmids

The plasmid vectors pBI-*Hrap* and pBI-*Pflp* having the coding sequences of the hypersensitive response assisting protein (*Hrap*) and plant ferredoxin-like protein (*Pflp*) genes, respectively, for this study were obtained from IITA. IITA acquired these plasmids from Academia Sinica Taiwan through AATF, who negotiated the license from the owner. The pBI-*Hrap* and pBI-*Pflp* were constructed using a pBI121 binary vector (Clontech, Palo Alto, CA, USA) by replacing the beta-glucuronidase (*gus* A*)* gene coding sequences with either a full-length *Hrap* gene or *Pflp* gene coding sequences. Both *Hrap* and *Pflp* coding sequences were derived from sweet pepper (*Capsicum annum*) and were used as cDNA. The pBI-*Hrap* having the *Hrap* gene under the control of constitutive double CaMV35S promoter and a nopaline synthase (nos) terminator, together with the neomycin phosphotransferase (*npt* II*)* selectable marker gene, with its nopaline synthase (nos) promoter of *Agrobacterium tumefaciens* and the 3′-nopaline synthase terminator

is shown in Figure 2.1. The pBI-*Pflp* vector was identical to pBI-*Hrap* where the *Hrap* gene was replaced with *Pflp* gene (Fig. 2.2). Both plasmids were maintained in the *E. coli* strain JM 109 on Luria Bertani (LB) plates (10g/L trypton, 5g/L yeast extract, 10g/L NaCl, 15g/L agar) at 4^{o}C and as well as glycerol stocks at -80^{o}C.

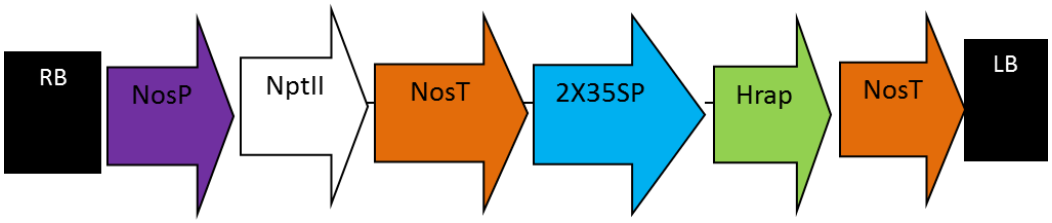

Figure 2.1: T-DNA region of pBI-*Hrap* plasmid. RB: Right border; nosP: nopaline synthase promoter; *nptII*: neomycin phosphotransferase II; nosT: nopaline synthase terminator; 2X35S P: Double cauliflower mosaic virus promoter; *Hrap*: Hypersensitive response assisting protein coding sequence; LB: left border.

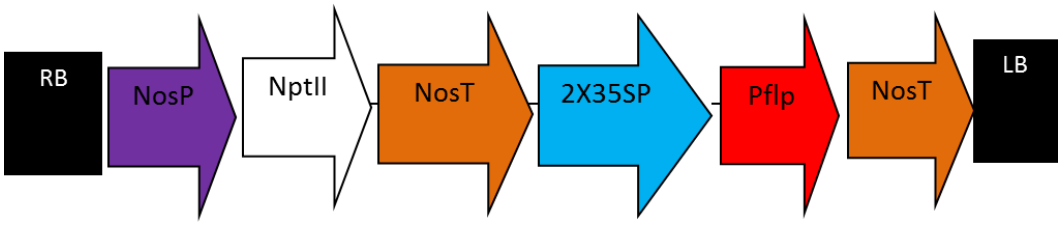

Figure 2.2: T-DNA region of the pBI-*Pflp* plasmid. RB: right border; *nos*P: nopaline synthase Promoter; *nptII*: neomycin phosphotransferase II gene; nosT: nopaline synthase terminator; *Pflp* plant ferredoxin-like protein coding sequence; 2X35S P: double cauliflower mosaic virus promoter; LB: left border

2.2.2 Amplification of *Pflp* gene cassette

The entire gene cassette (*Pflp* coding sequence, double CaMV35S promoter and nos-terminator sequence) was amplified from plasmid pBI-*Pflp* using the 35SH forward and the NosH reverse primers that were designed using 35S and nos sequences deposited in National Center of Biotechnology Information (NCBI) Genbank (Accession AF485783). The 35SH (5′-ACAAAGCTTGCATGCCTGCAGGTC-3′) flanked the 5′-end and the NosH (5′-TGTTTCGAACCGATCTAGTAACATAG-3′) primer the 3′-end with a *Hin*dIII restriction site at 5′ and 3′ end respectively. PCR mixture (50 µl) consisted of 1X *Pfu* buffer, 100 ng DNA template, dNTPs to the final concentration of 0.2 mM, $MgS0_4$ to the final concentration of 3.0 mM, 0.5 µM of each primer and 0.5 U of *Pfu* DNA polymerase (Fermentas, UK). The amplification program was an initial DNA denaturation of 5 min at 95^{o}C; followed by 35 cycles consisting of 40 s at 95^{o}C, primer annealing for 1 min at 65^{o}C, 2 min synthesis at 72^{o}C and final DNA extension of 10 min at 72^{o}C. The PCR amplification products were separated by electrophoresis on a 1% agarose gel at 100 V for 1 hr followed by gel staining in an ethidium bromide solution (5.0 µg/ml). The stained gel was visualized under UV light and the expected product according to the size excised using a sterile scalpel. The PCR product was purified from the gel using a Nucleospin extraction kit II (BD Biosciences Clontech) according to the manufacturer's instructions and the purified PCR product was eluted in 30 µl of NE buffer.

2.2.3 Adenosine tailing of PCR product and cloning into pCR 2.1-TOPO vector

The adenosine (A) overhangs was added onto the amplified PCR product using *Taq* polymerase. Briefly, 3 µl of purified blunt-ended PCR product, 1 µl of 10X *Taq* PCR buffer, 0.4 µl of $MgCl_2$

(1.0 mM final concentration), 2 µl of 1 mM dATP (0.2 mM final concentration) and 1 µl of *Taq* DNA polymerase (5 U/µl) were added and nuclease-free water to the final volume of 10 µl. Reaction was incubated for 30 min at 70^{o}C. Of this reaction (A-tailed PCR fragment), 2 µl was ligated into the pCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), together with blue/white selection of vectors having the inserts was done according to the manufacturer's instructions. Inserted *Pflp* fragment in plasmid pCR 2.1-TOPO was transferred into competent *Escherichia coli* (*E. coli*) cells.

2.2.4 Preparation and transformation of competent *E. coli* JM 109

Escherichia coli (*E. coli)* JM 109 cells on an LB agar plate were removed from 4^{o}C storage and then cultures initiated from single colonies in 10 ml LB broth medium. The cultures were incubated at 37^{o}C for 16 – 18 hr. From this overnight culture, 1 ml was inoculated into 100 ml of fresh LB broth and incubated at 37^{o}C with shaking at 200 rpm until an optical density (OD_{600}) of 0.6 was attained. The culture was chilled on ice for 5 min and then transferred to a sterile 50 ml centrifuge tube. Cells were collected by centrifugation for 5 min at 4000 x *g* at 4^{o}C. The supernatant was carefully discarded before re-suspending the cell pellet gently in 30 ml of ice-cold TFB1 buffer (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM CaCl, 15% glycerol, pH 5.8). The suspension was kept on ice for 90 min before centrifuging for 5 min at 4000 x *g* at 4^{o}C. The supernatant was discarded and the cells carefully re-suspended in 4 ml of ice-cold TFB2 buffer (10 mM MOPs, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8). Aliquots of 100 µl of competent cells in sterile 1.5 ml micro-centrifuge tubes were prepared followed by freezing in liquid nitrogen before storing at –80°C until required for transformations.

A heat shock method was used to transfer plasmid DNA into *E. coli* JM 109 cells, described in protocol 2 of Qiagen bench guide (2001) as follows. Competent cells were removed from the -80^{o}C storage and put on ice to thaw. Upon thawing, cells (100 µl) were re-suspended by flicking the tube to which 2 µl of TOPO cloning reaction (with plasmid vector having *Pflp* cassette) was added. The contents were gently mixed and left on ice for 20 min. The micro-centrifuge tube containing bacteria-plasmid mixture was then immersed in a water bath or heat block at 45^{o}C for 60 s then placed back on ice for 10 min. To the tubes, 500 µl of LB broth was added and incubated at 37^{o}C for 90 min with shaking at 200 rpm. Aliquots of 100 and 200 µl of transformation mixture were plated onto a LB agar plate supplemented with 100 mg/L kanamycin (Duchefa Biochemie, Holland). Plates were incubated at 37°C overnight for bacterial colonies to develop.

The transformed cells were confirmed by isolation of plasmid. Cultures of putative bacterial transformants were initiated from developed colonies in 10 ml LB broth supplemented with 100 mg/L kanamycin. The cultures were incubated at 37^{o}C for 16 – 18 hr with shaking at 200 rpm. The plasmid pCR 2.1-TOPO with the *Pflp* insert was isolated from JM 109 cells using a QIAprep spin mini-prep kit (Qiagen) according to the manufacturer's instructions.

2.2.5 Plasmid restriction and de-phosphorylation

Plasmids pCR 2.1-TOPO-*Pflp* and pBI-*Hrap* were separately digested with *Hin*dIII (Biolabs, England) as follows: a 20 µl reaction consisted of 1-2 µl of the plasmid DNA (1.5 µg), 2 µl of

10X NEB 2 buffer, 1 µl of *Hin*dIII (20 U), and nuclease-free water was added to obtain the final volume. The reaction mixture was incubated at 37^{o}C for 1 hr. The digested and linearized pBI-*Hrap* vector was de-phosphorylated using calf intestine alkaline phosphatase (CIAP). Dephosphorylation was done by adding 2 µl 10X phosphatase buffer and 1 µl of phosphatase to 10 µl of restricted pBI-*Hrap* plasmid. The reaction mixture was incubated for 30 min at 37^{o}C and DNA was then purified using a Qiaquick purification kit (Qiagen, UK) to remove the phosphatase according to manufacturer's instructions.

The *Hin*dIII restricted *Pflp* fragment (*Pflp* gene together with its promoter and terminator) from pCR 2.1-TOPO-*Pflp* was separated by electrophoresis on a 1% agarose gel and the expected DNA fragment according to size was then excised from the gel. The fragment was purified using Nucleospin extraction kit II according to the manufacturer's instructions.

2.2.6 Ligation of *Pflp* in pBI-*Hrap* and transformation of *E. coli* cells

The *Pflp* cassette was ligated into digested pBI-*Hrap* vector by adding 4 µl of purified *Pflp* insert, 2 µl of the pBI-*Hrap* vector, 1 µl of 10X T4-ligase buffer, 1 µl ligase (Fermentas, UK), and nuclease-free water to a final volume of 10 µl. The ligation mixture was incubated at 16^{o}C over night after which the reaction was terminated by heating for 10 min at 65^{o}C. From the ligation mixture, 2-5 µl of ligate was transformed into competent *E. coli* JM 109 cells as outlined in Section 2.2.4 above. Transformants were selected on LB agar plates supplemented with 100 mg/l of kanamycin. Bacterial cultures were initiated from transformed colonies after which

plasmid DNA was isolated using the QIAprep spin mini-prep kit (Qiagen) to confirm the presence of both *Hrap* and *Pflp* containing plasmids.

2.2.7 Confirmation of both *Hrap* and *Pflp* genes in a single plasmid

Plasmids having both *Hrap* and *Pflp* genes were verified using PCR, restriction enzyme digestion and sequencing. For PCR, *Hrap* gene specific primers (forward 5′-GAGCTCACAGCATTTTGGCCATCCC-3′ and reverse 5′-TGGAGTTGGAGGACGAGG AAC-3′) and *Pflp* gene specific primers (forward 5′-GAGCTCCCAAACGTTGGGGAAGC-3′ and reverse 5′ ACGAGTTCTGCCTCTTTGTGAGT 3′) were used. The reaction mixture of 20 µl consisted of 2 µl of 10X PCR buffer, 1.6 µl of $MgCl_2$ (2.0 mM final concentration), 0.4 µl of dNTPs (0.2 mM final concentration), 1 µl of each forward and reverse primers (0.5 mM final concentration), 0.1 µl of *Taq* polymerase (0.5 U), 1µl of template DNA, and nuclease-free water added to obtain the final volume. PCR conditions were 95^{o}C for 5 min followed by 35 cycles of 94^{o}C for 40 s, primer annealing at 55^{o}C for 40 s and 72^{o}C for 1 min, and final DNA extension at 72^{o}C for 10 min. PCR products were analyzed on an agarose gel and screened for the expected *Hrap* and *Pflp* fragments according to size.

Plasmids were further verified by restriction enzyme analysis using the following Biolabs (England) restriction enzyme combinations; *Bam*HI and *Sac*I, *Eco*RI and *Bam*HI, *Hin*dIII and *Sac*I. The reaction mixture of 20 µl consisted of 2 µl of 10X enzyme buffer, 1 µl of each enzyme (20 U), 0.25 µl of bovine serine albumin (BSA) (0.2 mg/ml), 1-2 µl of plasmid DNA, and nuclease free-water to the final volume. Restricted plasmids were separated on a 1% agarose gel

and products visualized using UV light after staining with ethidium bromide (5 µg/ml). Plasmid pBI-*Hrap-Pflp* constructs were also sent for sequencing. Plasmid construct (pBI-*Hrap-Pflp*) after validation was transformed into competent *E. coli* cells as outlined in Section 2.2.4 above.

2.2.8 Transformation of *Agrobacterium tumefaciens*

2.2.8.1 Preparation of electro-competent *Agrobacterium tumefaciens*

A fresh *Agrobacterium tumefaciens* AGL1 strain on LB agar plate supplemented with 250 mg/l carbenicillin (Duchefa Biochemie, Holland) and 50 mg/l rifampicin ((Duchefa Biochemie, Holland) was used. A culture was initiated by inoculating a single *Agrobacterium* colony in 50 ml of LB broth supplemented with antibiotics and incubated at 28^{o}C for 48 hr with shaking at 200 rpm. From this culture, 2 ml was re-inoculated into 100 ml of fresh LB broth supplemented with antibiotics and incubated at 28^{o}C until the OD_{600} of cells was 0.5-0.8. The cells were then transferred aseptically to ice-cold 50 ml centrifuge tube and kept on ice for 30 min. Cells were harvested by centrifuging at 4000 rpm for 10 min in a bench top refrigerated centrifuge machine at 4^{o}C. The supernatant was decanted and pellet re-suspended (washed) in 40 ml of sterile cold ddH_2O. Cells were washed three times and finally re-suspended in 1 ml of sterile ice-cold 10% glycerol. Aliquots of 50 µl of cells were prepared in 1.5 ml centrifugation tubes, flashed in liquid nitrogen and stored at -80^{o}C.

2.2.8.2 Transformation of electro-competent *Agrobacterium tumefaciens*

Transformation of *Agrobacterium tumefaciens* cells was done by electroporation according to the protocol of Weigel and Glazebrook (2006). Electroporation cuvettes (1 mm) and 1.5 ml micro-centrifuge tubes were placed on ice. Electro-competent AGL1 cells were placed on ice to thaw before mixing by flicking gently. To 50 µl of the cells, 1 µl (~1000 ng) plasmid was added and mixed. The AGL1 cells - plasmid mixture was carefully transferred into a chilled cuvette and then electroporated using the BTX ECM 630 Bio-Rad GenePulser electroporator at 2.1 kV, 100 Ω, and 25 µF, time constant ~2.6 ms. After electroporation, 1 ml of LB broth was added immediately to the cuvette, mixed gently by pipetting up and down before transferring to a 2 ml micro-centrifuge tube. The electroporated cells were incubated at 28^{o}C for 2 hr with shaking at 250 rpm. After the 2 hr, cells were centrifuged at 6000 rpm for 2 min and about 900 µl of the supernatant discarded and the bacterial pellet was re-suspended in about 100 µl of remaining medium. The transformation mixture was plated onto Yeast-mannitol agar (YMA: 1 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 1 g $CaCO_3$, 15 g agar, in 1 liter of water) media plates supplemented with 250 mg/l carbenicillin, 50 mg/l rifampicin and 100 mg/l kanamycin. Plate was incubated at 28^{o}C for 3 days. To confirm the presence of plasmids in transformed AGL1 cells, single colonies were inoculated in 10 ml of LB broth supplemented with appropriate antibiotics and incubated at 28^{o}C for 3 days. Presence of plasmid with stacked genes in *Agrobacterium tumefaciens* was confirmed using PCR with *Hrap* and *Pflp* gene specific primers as outlined in section 2.2.7.

2.3 Results

2.3.1 Amplification of *Pflp* cassette

The plasmid pBI-*Hrap-Pflp* with stacked genes was constructed as illustrated in Figure 2.3. An amplicon of expected 1632 bp size of the *Pflp* coding sequences together with its dual CaMV35S promoter and nos terminator was amplified by PCR from pBI-*Pflp* using 35SH and NosH primers (Fig. 2.4). The purified fragment with dATP overhangs was then cloned into the multiple cloning site (MCS) of a linearized plasmid pCR 2.1-TOPO and designated as pCR 2.1-TOPO-*Pflp*. JM 109 transformats carrying the pCR 2.1-TOPO-*Pflp* were screened by blue/white colony selection and insertion was also verified by *Hin*dIII restriction enzyme analysis to confirm ligation and 16632 bp inserts were restricted out.

Verified pCR 2.1-TOPO-*Pflp* (Fig. 2.5) and pBI-*Hrap* (Fig. 2.1) were each digested with *Hin*dIII to remove the *Pflp* cassette and linearize plasmid respectively. To prevent re-ligation of pBI-*Hrap*, plasmid DNA was de-phosphorylated using CIAP. The *Pflp* cassette was then inserted at the *Hin*dIII site of pBI-*Hrap* and the resulting vector with both *Hrap* and *Pflp* coding sequences was designated pBI-*Hrap-Pflp*.

2.3.2 Verification of plasmid vector with stacked *Hrap-Pflp* genes

Restriction analysis with double enzymes pairs of endonucleases; *Bam*HI and *Sac*I, *Eco*RI and *Bam*HI, *Hin*dIII and *Sac*I were used to verify the presence of both *Hrap* and *Pflp* stacked genes

in recombinant plasmid pTOPO vectors. The *Bam*HI/ *Sac*I restriction gave fragments of about 894 bp in stacked plasmids (R1 and R2) and pBI-*Hrap*, and fragments of about 500 bp and 300 bp as well in stacked plasmids and pBI-*Pflp* plasmid (Fig. 2.5). Furthermore, *Eco*RI/*Bam*HI, restriction gave fragments of about 900 bp and 700 bp in stacked plasmids and in pBI-*Hrap*, pBI-*Pflp* plasmid respectively (Fig. 2.5). The patterns of restriction products in stacked pTOPO plasmids were also in agreement with products in restricted pBI-*Hrap* and pBI-*Pflp* plasmids with *Hin*dIII/ *Sac*I enzymes (Fig. 2.5). PCR was also used to verify recombinant stacked plasmids. Amplicons of sizes 1000 bp and 500 bp were obtained in stacked plasmids and in pBI-*Hrap* and pBI-*Pflp* plasmids respectively (Fig. 2.6). This confirmed that both *Hrap* and *Pflp* genes were present in stacked plasmids. Furthermore, recombinant plasmids were sent for sequencing (Inqaba Biotech, South Africa). The sequencing results were searched for encoded proteins using BLAST (Altschul *et al*., 1997) and also aligned against nucleotide sequences deposited in the NCBI Genbank, (http://www.ncbi.nlm.nih.gov/). Blasting results confirmed presence of a *Pflp* transgene with 95% identity (60/63) with both full ferredoxin chloroplastic (accession q9zts2) and full ferredoxin (accession P83527) all from *Capsicum annuum*. Also, the trans-activator protein of the cauliflower mosaic virus (accession NP_056729) with 99% identity was confirmed as well as hypothetical protein from *Agrobacterium fabrum* str. c58 (accession YP_001542606) with 100% identity. Sequencing results, restriction analysis and PCR analysis therefore clearly confirmed that the constructed pBI-*Hrap-Pflp* vector had both the *Hrap* and *Pflp* genes together with CaMV35S promoter and nos-terminator (Fig. 2.7).

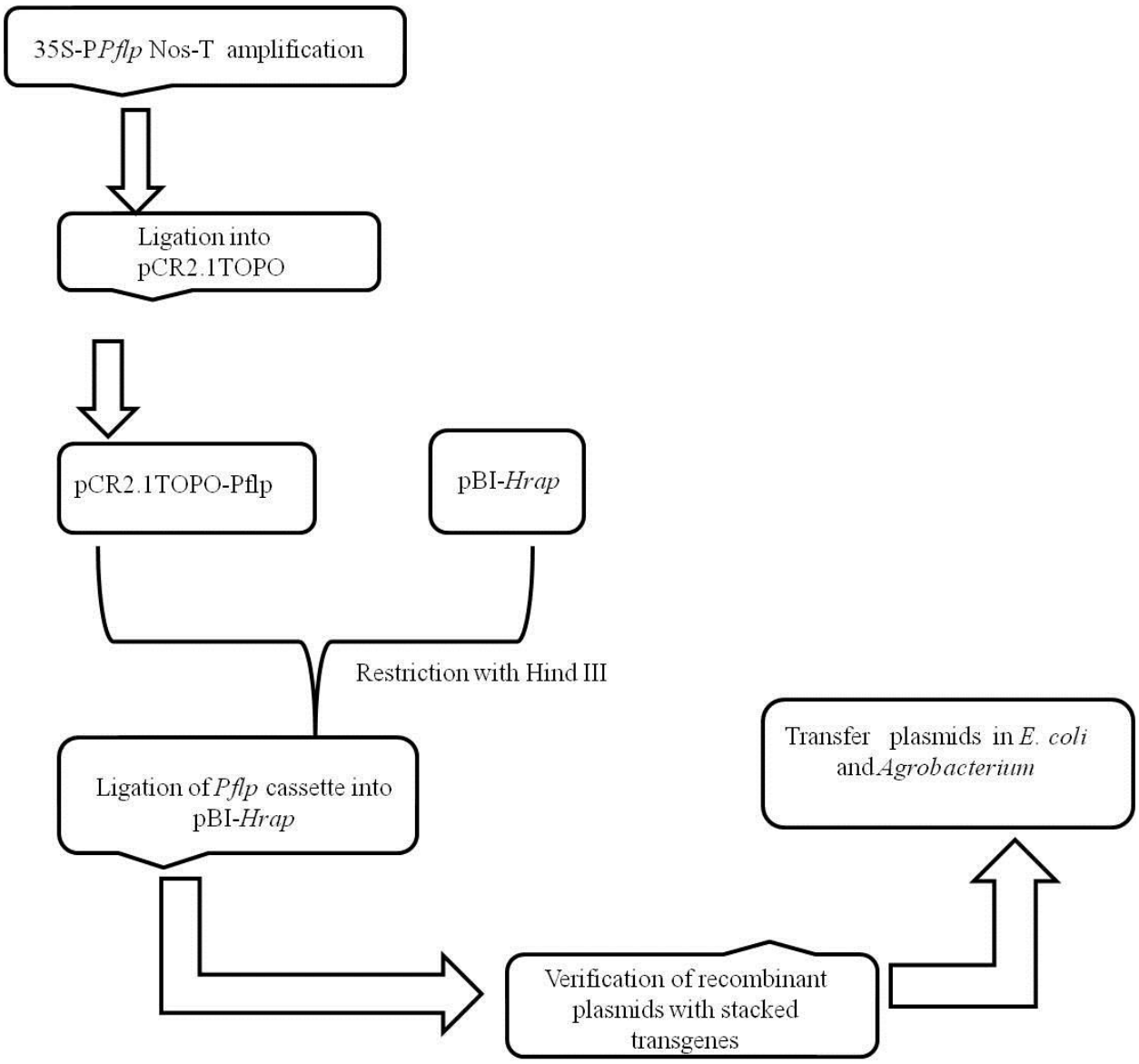

Figure 2.3: Diagrammatic illustrations of cloning of the *Pflp* cassette.

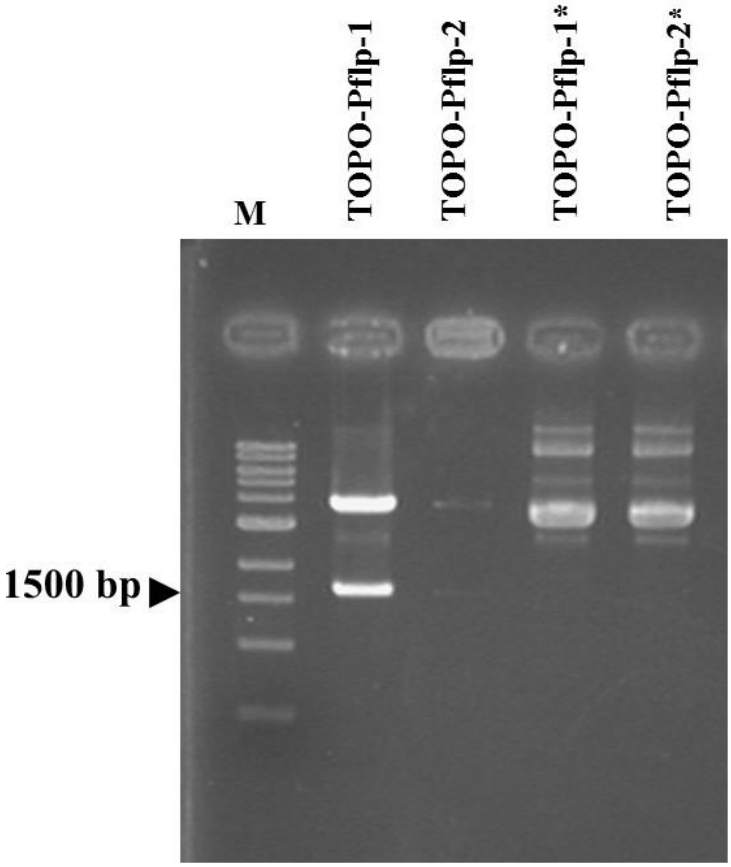

Figure 2.4: Restriction analysis of pCR 2.1-TOPO-*Pflp* to confirm ligation of *Pflp* cassette. M: 1 kb ladder (Biolabs-UK); *unrestricted pCR 2.1-TOPO-*Pflp*

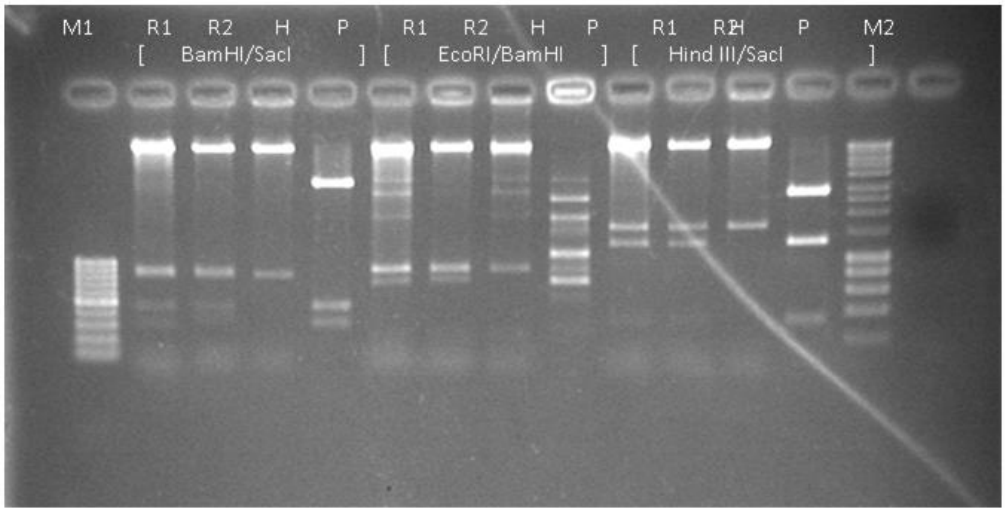

Figure 2.5: Verification of stacked pBI-*Hrap-Pflp* plasmids by restriction analysis. **.** M1: 100 bp DNA ladder plus (Fermentas), R1: stacked genes plasmid 1; R2: stacked genes plasmid 2; H: single pBI-*Hrap*; P: single pBI-*Pflp;* M2: 1 kb DNA ladder (Fermentas).

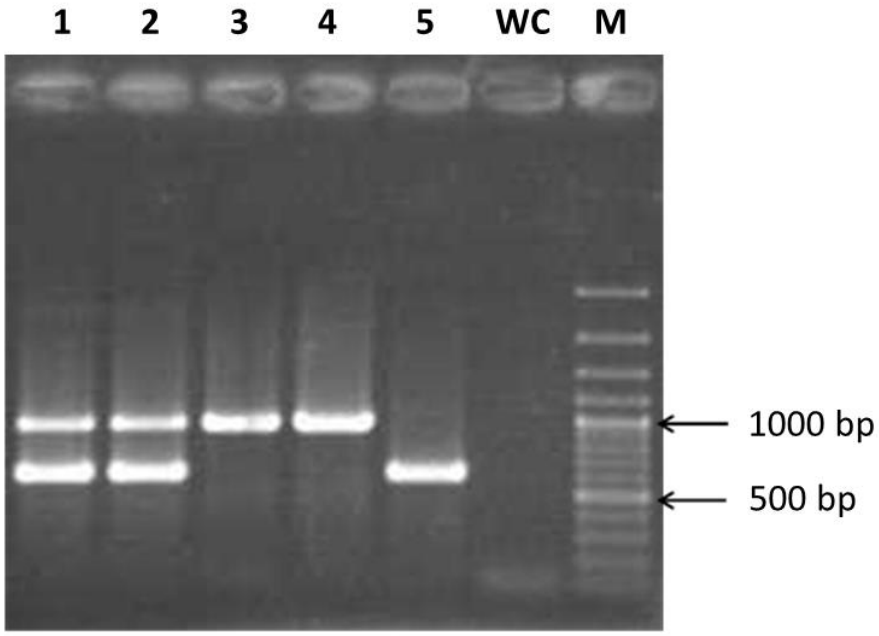

Figure 2.6: PCR verification of stacked pBI-*Hrap-Pflp* plasmids (in pTOPO)**.** 1-3: recombinant plasmids pBI-*Hrap-Pflp;* 4: pBI-*Hrap*; 5: pBI-*Pflp*; WC: reagent control (no template DNA); M: Gene-ruler 100 bp plus DNA ladder, Fermentas (UK).

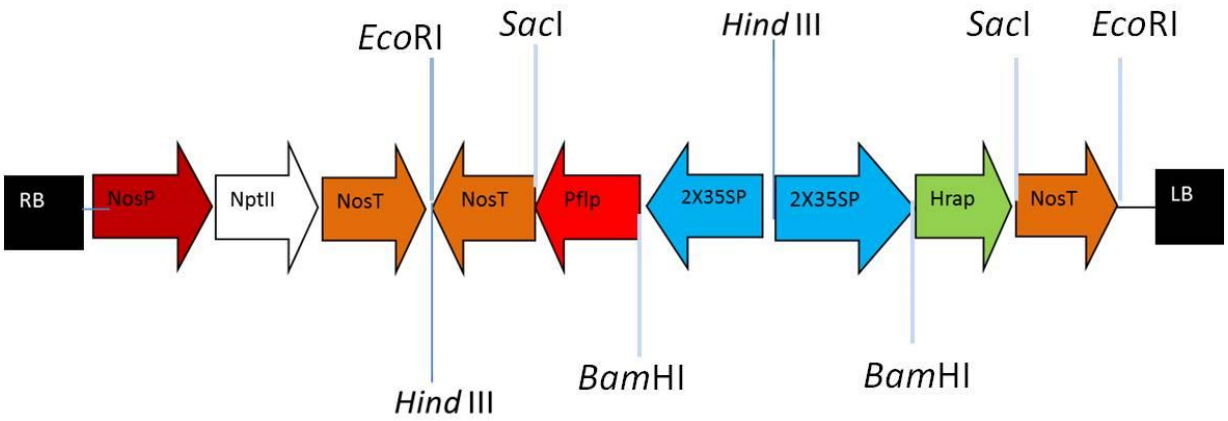

Figure 2.7: Schematic representation of T-DNA region of stacked pBI-*Hrap-Pflp* plasmid. RB: right border; nos-P: promoter region of *Agrobacterium tumefaciens* nopaline synthase gene; *nptII*: coding sequence of the neomycin phosphotransferase II gene; nos-T: terminator region of the nopaline synthase gene; *Pflp*: coding sequences of plant ferredoxin-like protein; 2X35SP: Double 35S cauliflower mosaic virus promoter; *Hrap:* coding sequence of the hypersensitive response assisting protein from sweet pepper; LB: left border.

5′-CC<u>AAGCTT</u>GCATGCCTGCAGGTCCCCAGATTAGCCTTTTCAATTTCAGAAAG
AATGCTAACCCACAGATGGTTAGAGAGGCTTACGCAGCAGGTCTCATCAAGAC
GATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTCCCAAGAAGGTTAA
AGATGCAGTCAAAAGATTCAGGACTAACTGCATCAAGAACACAGAGAAAGATA
TATTTCTCAAGATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTC
ACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCCACT
GAATCAAAGGCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGC
CGTAAAGACTGGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGA
AGAAAATCTTCGTCAACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATA
TCAAAGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGG
GTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATT
GTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAA
AGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGAC
CCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCA
AAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATC
CCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGA
GAGAACACGGGGGACTCTAGAGGATCCC**GATGGCTAGTGTCTCAGCTACCATG**
ATTAGTACCTCTTCATGCCAGAAAACCAGCTGTGACAGCTTAAACCCATCCCAA
CGTGGGAGCACTGTTGGCTAAATCAGCAATGTGCAAGTCACTTGCATGCTCAT
ACAAGTGACTATCACACTGACGACCAATGATGGAATGGCCKAAGTATGTAATC
TGGATCAGCTGAGGAAGCTGAAACATTGTACCTTTCTTTCCTTATTCGTGCAGG
GCAGGTTCTTGCTCATCTTGTGCTGGTAAAATTGCYSKTKGAGCTGTTGATCAA
ACTGATGGCAACTTTCTTGATGATGACCAATTAGAGGAGGGATGGGTGCTAAC
TTGTGTTGCTTATCCACAGTCTGATGTTACTATTGAGACTCACAAAGAAGCAGA
ACTCGTGGGCTAACGAGCTGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAG
ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTA
CGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGT
TTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAAT
ATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGA
TCGGTTCGAAACAAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGA
TCCGAGCTCGGTAC**C<u>AAGCTT-3′</u>**

Figure 2.8: Nucleotide sequences of the cloned complete Pflp cassette in pBI-*Hrap-Pflp*. *Pflp* coding sequence (red), 2xCaMV 35S promoter sequences (blue) and nos-terminator sequence (green). *Hin*dIII recognition sequences flanking the cloned cassette are bolded and underlined.

2.4 Discussion

In this study, a complete cassette comprising of *Pflp* coding sequence, double 35S promoter sequence of Cauliflower mosaic virus and the nos terminator region of the nopaline synthase gene was successfully amplified from the donor plasmid pBI-*Pflp* with conventional PCR allowing insertion into plasmid pBI-*Hrap* plasmid already carrying the *Hrap* coding sequence. This resulting plasmid pBI-*Hrap-Pflp* with stacked *Pflp* and *Hrap* genes has been used in this PhD study to transform banana for possible enhancement in resistance against the BXW disease, compared to transgenic banana plants transformed with either *Pflp* or *Hrap* individually.

Gene stacking approaches have been previously applied to either target different pathogens or to avoid early resistance breakdown (Chan *et al*., 2005; Zhu *et al*., 2012; Storer *et al*., 2012). Recently, trans-plastomic *Nicotiana benthamiana* plants expressing defense proteins in three-stacking combinations to obtain multiple resistance (insects, phyto-pathogens and abiotic stress) had synergistic and enhanced effects in comparison to plants expressing such genes individually (Chen *et al*., 2014). Single genes expression is also particularly prone to rapid breakdown necessitating strategies, such as transgene stacking, to obtain not only higher but also more durable resistance (Zhao *et al*., 2003; Datta *et al*., 2002; Collinge *et al*., 2008).

CHAPTER THREE

GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC BANANA

3.1 Introduction

It is now a common practice to introduce desired traits into bananas using a genetic transformation approach. This was necessitated by the high sterility, triploidy and seedlessness of banana rendering conventional breeding in bananas difficult. Transgenic bananas with genes for nematode resistance, Fusarium wilt resistance, banana bunchy top virus resistance, BXW resistance, enhanced vitamin A and Iron content, cyclin-D genes have been previously developed (Tripathi *et al*., 2010; Talengera, 2011; Namukwaya *et al*., 2012; Shekhawat *et al*., 2012; Magambo, 2012; Roderick *et al*., 2012; Tripathi *et al*., 2014a,b; Tripathi *et al*., 2015). In production of transgenic plants, plant regeneration is an important factor (Hansen and Martha, 1999). Different explants, including suckers (Subramanyam *et al*., 2011), meristems, and corm slices (May *et al*., 1995); apical shoot tips (Tripathi *et al*., 2005) as well as embryonic cell suspensions (Khanna *et al*., 2004; Tripathi *et al*., 2012) have been applied in banana transformation. Banana cell suspension is the most used material in banana transformation despite the difficulty involved in cell suspension generation as well as the long time required to generate transgenic banana plants (Lorenzen *et al*., 2010). Particle bombardment and *Agrobacterium*-mediated transformation method have both been used in genetic transformation of banana (Sreeramanan *et al*., 2009; Becker *et al*., 2000; Huang *et al*., 2007; Tripathi *et al*., 2012). *Agrobacterium*-mediated transformation, the most commonly used method of plant transformation, is very precise and single copy numbers and intact DNA fragments can be inserted (Gelvin, 2003). Techniques, such as sonication and centrifugation, have been further used to enhance efficiency of *Agrobacterium* transformations (Liu *et al*., 2006; Khanna *et al*., 2004).

The objective of this chapter was to transform banana with the pBI-*Hrap-Pflp* plasmid carrying the stacked genes, or the plasmids with the single *Hrap* and *Pflp* genes for comparison. Stacked and single transgenes were delivered into banana cells using the *Agrobacterium* mediated *transformation* system and transformed banana cells were regenerated into plants on different selective media. Molecular based methods were used to confirm presence and integration of the transgenes in developed transgenic banana plants.

3.2 Materials and Methods

3.2.1 Banana embryonic cell suspensions

"Gonja manjaya" (AAB) and "Sukali ndiizi" (AB) cultivars used in the study were triploids and diploids bananas respectively based on their genome composition. Embryonic cells suspensions (ECS) of the local cultivar "Gonja manjaya" used in this study was generated from scalps according to Tripathi *et al*. (2012) and provided by IITA. For the cultivar "Sukali ndiizi", ECS were generated from immature male buds and were provided by the National banana research program, National Agricultural Research laboratories (NARL). The ECS from male buds were maintained in cell suspension media (MA2) (Appendix IA) according to Namanya *et al*. (2004).

3.2.2 Plasmids and preparation of *Agrobacterium*

Plasmid pBI-*Hrap-Pflp* with stacked transgenes genes and plasmids pBI-*Hrap* and pBI-*Pflp* with single genes, were used for transformation. Four days prior to use for transformation,

Agrobacterium tumefaciens AGL1 cells carrying pBI-*Hrap-Pflp*, pBI-*Hrap* and pBI-*Pflp* were separately inoculated into 10 ml Yeast mannitol broth (YMB: 1 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 1 g $CaCO_3$, in 1 liter of water) medium supplemented with 100 mgl^{-1} kanamycin (Duchefa Biochemie, Holland), 50 mgl^{-1} rifampcin (Duchefa Biochemie, Holland) and 250 mg/l carbenicilin (Duchefa Biochemie, Holland) and incubated at 28^{o}C for 3 days with shaking at 200 rpm. Then 5 ml of the *Agrobacterium* culture was transferred to 20 ml of YMB medium supplemented with appropriate antibiotics and then incubated overnight at 28^{o}C with shaking at 200 rpm. On the day of transformation, the bacterial cells were collected by centrifugation at 6000 rpm for 10 min 25^{o}C in 50 ml falcon tubes and pellet re-suspended in 25 ml of bacterial re-suspension media (BRM) (Appendix II), supplemented with 200 µM acetosyringone (3'-5'-dimethoxy-4-hydroxyacetophenone; Sigma-Aldrich, USA). The *Agrobacterium* cells were incubated at 25^{0}C for 2 hr at 70 rpm to induce virulence of *Agrobacterium*. After the 2 hr, the OD_{600} of bacterial suspension was adjusted to 0.6 using fresh BRM, and the *Agrobacterium* suspension was then used for transformation.

3.2.3 Transformation of banana embryonic cells

Agrobacterium mediated method was used to transform banana cells according to Khanna *et al*. (2004), with minor modification described below. To increase ECS transformation efficiency, cells suspensions of "Sukali ndiizi" were sub-cultured into fresh MA2 media and "Gonja manjaya" into fresh cell suspension maintenance media for scalps (ZZ) (Appendix IB). The banana cells were transferred to sterile 50 ml falcon tubes and left to settle. The MA2 or ZZ media was removed before adding 10 ml of fresh media at 45^{o}C onto the ECS. The cell

suspension was incubated at 45°C for 5 min to heat shock the cells, then the hot/warm media was removed. The cells were subdivided into sterile 50 ml falcon tubes, with each tube containing about 500 µl settled cell volume. To each Falcon tube, 10 ml of pre-induced *Agrobacterium* suspension was added followed by adding pluronic F68 (Sigma-Aldrich, USA) surfactant to the final concentration of 0.02%. The surfactant reduces the surface tension of the medium and facilitates the contact of bacterium and banana cells. For the non-transgenic control cells, MA2 or ZZ media was added instead of *Agrobacterium* suspension. Cells were centrifuged twice at 900 rpm for 3 min at 25°C. To improve transformation efficiency, banana cells - *Agrobacterium* suspension were placed on a shaker at 50 rpm for 1 hr at room temperature. The banana cells - bacterial suspension was pipetted onto sterile Whatman glass filter papers nylon meshes. The filter papers were blot-dried on sterile adsorbent papers before transferring the filter paper on 60 mm plates of solidified bacterial co-culture medium (BCCM) (Appendix III) supplemented with 300 µM acetosyringone. Plates having cells on filters were sealed using parafilm, wrapped in aluminum foil and then co-cultured for 4 days at 22°C in the dark.

3.2.4 Selection and regeneration of transformed plants

After 4 days of co-culturing, cells on each filter paper were transferred into sterile 50 ml falcon tubes before adding 30 ml of sterile MA2 or ZZ medium supplemented with 200 mg/l timentin (Duchefa Biochemie, Holland) to kill *Agrobacterium*. The tube contents were vigorously shaken to wash off the *Agrobacterium* and *Agro*-infected cells were allowed to settle before dispensing off the bacterial supernatant. This washing was repeated four times to ensure that all *Agrobacterium* was washed off from the banana cells. Finally, washed *Agro*-infected cells were

transferred onto sterile nylon meshes, thinly spread and then blot-dried on sterile adsorbent paper. Nylon meshes with the cells were transferred on embryo development media (MA3) (Appendix IV) supplemented with antibiotics (200 mg/l timentin and 50 mg/l geneticin). Un-transformed control cells were transferred onto MA3 without antibiotics selection. MA3 plates with cells were sealed and then transferred to the dark in the growth room at 26 – 28^{o}C. Cells were transferred onto fresh MA3 selection media every 14 days, until when embryos started developing. Surviving embryos were directly transferred on regeneration media (RDI) (Appendix V) supplemented with 200 mg/l timentin and 50 mg/l geneticin for one month. After one month, developed embryos were directly transferred on embryo germination medium (MA4) (Appendix VI) supplemented with 200 mg/l timentin and 50 mg/l geneticin for one to two months. Germinated embryos and regenerating plantlets were transferred on MS proliferation media (Appendix VII) supplemented with 200 mg/l timentin and 50 mg/l geneticin in glass tubes for 30 days. Each of the regenerated plantlets was given an event or entry number. For multiplication, plantlets were routinely sub-cultured (cutting and injuring meristem) on MS proliferation to enable production of multiple clones. Fully developed plantlets were weaned, which involved washing off the nutrient medium and potting in 200 ml plastic cups containing moist pasteurized top soil and farm yard manure mixed at a ratio of 12:1. Potted plants were transferred to a humid chamber in the green house with relative humidity of 45-60% for 2-3 weeks, and then removed from the humid chamber for one week to allow acclimatization. Plants were subsequently transferred with their intact soil into 3 liter pots containing about 2 kg of the same potting substrate. Weaning, potting and plant growth to the required age (3 months) were done in the containment glasshouse (level 3), and plants were watered daily.

3.2.5 Molecular characterization of transgenic plants

3.2.5.1 Extraction of DNA

Genomic DNA was extracted from young leaves of putative transgenic banana plants using the CTAB method as described by Untergasser, (2008) with slight modifications as described below. Leaf tissues were ground to fine powder using liquid nitrogen. To about 100 mg tissue powder, 800 µl preheated CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8.0, 2% PVP40, 0.1% $NaSO_2$) was added and mixed by agitation. The mixture was incubated at 65^{o}C for 30 min. An equal volume of chloroform: isoamylalcohol (24:1) was added to the mixture, mixed by inverting tubes several times before centrifuging at 12 000 rpm for 3 min. About 700 µl of the upper phase was transferred to a new centrifuge tube and 1 µl RNAse (10 mg/ml) was added and incubated for 30 min at 37^{o}C. To the tubes 700 µl chloroform: isoamylalcohol (24:1) was added and extracted as mentioned above. About 600 µl of the upper phase was again transferred to a new centrifuge tube and 600 µl isopropanol was added and mixed by inversion ten times. The mixture was stored at -30^{o}C or 30 min and then centrifuged at 12 000 rpm for 30 min. The supernatant was discarded and pellet washed with ice cold 70% ethanol. The tubes were centrifuged at 12 000 rpm for 5 min and then supernatant discarded. The pellet was re-suspended into 100 µl nuclease free water and incubated at 65^{o}C for 15 min to dissolve the DNA. The dissolved DNA was centrifuged at 12 000 rpm for 5 min before transferring the dissolved DNA into new 1.5 ml centrifuge tubes. The concentration of extracted DNA was determined

using a Nano-drop spectrophotometer. The extracted DNA was used for PCR analysis and southern blot analysis.

3.2.5.2 Polymerase chain reaction (PCR)

Genomic DNA from putative transgenic lines was used as a template DNA for PCR analysis to confirm for the presence of *Hrap* and *Pflp* transgenes. The *Hrap* forward 5′-GAGCTCACAGCATTTTGGCCATCCC-3′ and reverse 5′-TGGAGTTGGAGGACGAGG AAC-3′ and the *Pflp* forward 5′-GAGCTCCCAAACGTTGGGGAAGC-3′ and reverse 5′-ACGAGTTCTGCCTCTTTGTGAGT-3′ gene specific primers were used to amplify genomic DNA. The PCR reaction mixture of 20 µl consisted of 2 µl of 10X PCR buffer with 15 mM $MgCl_2$, 0.4 µl of dNTPs (final concentration of 0.2 mM), 0.5 µl of each primer (0.25 µM final concentration), 0.2 µl of of *Taq* DNA polymerase (0.5 U final concentration) and 1 µl of template DNA (about 100 ng). Nuclease-free water was added to the final volume. The PCR cycling condition were initial denaturation for 4 min at 94ºC, followed by 35 cycles of 40 s at 94ºC, 40 s at 55ºC, 40 s at 72ºC, and a final DNA extension at 72ºC for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1mM EDTA) and the gel stained in ethidium bromide (5 µg/ml) solution and finally visualized using a gel documentation system.

3.2.5.3 Southern blot analysis

To further determine the copy number of *Hrap* and or *Pflp* genes that were inserted in the genome of representative stacked transgenic lines, southern blot analysis was done. The DIG

labeled probe synthesis kit, the positively charged nylon membranes, DIG Easy hybridization buffer, blocking reagent, AntiDIG-AP antibody, the DIG labeled molecular weight marker and chemiluminescent films used in the study were procured from Roche (UK). The DIG labeled probe specific for *Pflp* was prepared using the PCR DIG probe synthesis kit following the manufacturer's instructions and PCR conditions for analysis of *Pflp* genes in transgenic plants were used (Section 3.2.5.2).

About 10 µg of plant DNA from randomly selected transgenic lines with stacked *Hrap* and *Pflp* genes was used. Plasmid vector (30 µg) pBI-*Hrap*-*Pflp* DNA served as positive control. The 50 µl restriction digestion reaction contained 10 µg of genomic DNA, 1 µl *Hin*dIII enzyme, 5 µl restriction buffer and water to the final volume. The digestion mixture was incubated overnight at 37ºC. The digested DNA was electrophoresed on a 0.8% agarose gel at constant voltage of 50 V for 5 hr. The quality of restriction was checked by staining the gel in ethidium bromide for 10 min and visualized under UV.

The gel was rinsed in water and soaked in depurination solution (0.2 M HCl) with shaking at 35 strokes/min at room temperature for 15 min. The gel was rinsed in water and soaked in denaturation solution (0.5 M NaOH, 1 M NaCl) for 30 min with shaking at 35 strokes/min. The gel was rinsed in water and then washed twice in neutralization buffer (0.5 M Tris.HCl, 3 M NaCl) for 15 min each with shaking at 35 strokes/min. after final rinsing in water, the gel was equilibrated in 2X sodium chloride and sodium acetate (SSC) buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 5 min before blotting.

The wick of 3 MM Whatman paper was cut such that it dropped over the support into the buffer chamber. The wick was made wet with the 20X SSC buffer with two ends immersed in the buffer chamber. Three pieces of Whatman paper were soaked with 20X SSC buffer and placed on top of the platform. After ensuring that there were no air bubbles, a gel was placed upside down onto the Whatman paper. The nylon membrane was carefully laid on top of the gel and 2 pieces of Whatman paper soaked with 20X SSC buffer placed on top of the nylon membrane. Air bubbles were removed by rolling a clean test tube over the top. The sides around the gel were covered with plastic film so as to ensure that the capillary movement of buffer went through the gel. Paper blotting stacks were placed on top and a 500 g load/weight placed on top of paper stack before transfer was allowed to proceed overnight.

Transferred DNA was fixed on to the membrane by cross linking with UV and the membrane was rinsed in 2 X SSC buffer before hybridization. The membrane was pre-hybridized in 30 ml of pre-warmed DIG Easy hybridization buffer (5X SSC, 2% Block solution, 0.1% N-lauroylsarcosine, 0.2% SDS, Formamide) at 42^{o}C, 7 strokes/ min for 1 hr. After 1 hr of incubation, the pre-hybridization buffer was replaced with 20 ml of hybridization buffer (5X SSC, 2% Block solution, 0.1% N-lauroylsarcosine, 0.2% SDS, Formamide) containing the probe that was denatured at 100^{o}C for 10 min. The hybridization was done overnight at 42^{o}C in hybridization oven at 75 strokes/ min.

The hybridization buffer was removed and the membrane was immediately washed twice in the in 50 mls of 2X washing buffer I (2X SSC, 0.1% SDS) for 15 min per wash at room temp. The membrane was then washed twice in heated washing buffer II (0.5X SSC, 0.1% SDS) at 65^{o}C for

15 min each. The washed membrane was incubated with antibody (Anti-DIG-AP) diluted in buffer 2 for 30 min at 45 strokes/ min and then equilibrated with buffer 3 for 2 min. Onto the membrane on top of cling film, 5 drops of CPD star were added ensuring that the entire blot was covered with CPD solution. The wrapped membrane was incubated at room temperature for 5 min and exposed to chemiluminescent film for 10-30 min in the dark before it was developed.

3.3 RESULTS

3.3.1 Plant transformation and regeneration of transgenic bananas

The banana cells that were co-cultured with *Agrobacterium* started turning brown from 2 weeks after plating on MA3 media supplemented with geneticin (Fig. 3.1). Direct contact of the surviving embryos with geneticin supplemented media ensured that only transgenic embryos survived (Fig. 3.1). Non-transgenic control cells responded faster than cells co-cultured with *Agrobacterium* on a medium without selection. In terms of time, regeneration of untransformed ce1lls into plants was 20-25 days earlier than transformed cells. Regeneration frequency in cell cultures that were co-cultured with stacked and single *Hrap* and *Pflp* transgenes were lower, compared to the untransformed control, and a lower number of shoots arose from the plated embryos. Regeneration was not significantly different ($P>0.05$) among cells co-cultured with *Hrap* and *Pflp* transgenes either stacked or single. Transformed “Gonja manjaya” cells had a higher regeneration frequency of about 80 plants per 0.5 ml SCV in comparison to 50 plants per 1 ml SCV for “Sukali ndiizi”.

About 150 independent transgenic lines with stacked *Hrap-Pflp* genes were generated from cultivar "Gonja manjaya" (GM), (Fig. 3.2) and 100 independent lines of "Sukali ndiizi" (ND) were also generated. In addition, 100 independent transgenic lines each of GM with single *Hrap* and *Pflp* transgenes and 20 independent lines each of ND transformed with single *Hrap* or *Pflp* were developed. From each individual line, clones were reproduced in tissue culture through repeated sub-culturing on MS proliferation medium. The transgenic and non-transgenic plants from tissue culture were weaned and kept in a green-house (Biosafety level II) to allow them to grow and develop further to the required size (3 months) before screening in a screen-house for disease reaction to Xcm. The conditions inside the green-house were natural illumination with temperatures ranging from 28^{o}C to 30^{o}C with a relative humidity between 45-60%, and plants were watered daily In contrast, the used screen-house was an enclosed room where the conditions were relatively similar to outside field conditions with no control of temperatures and relative humidity.

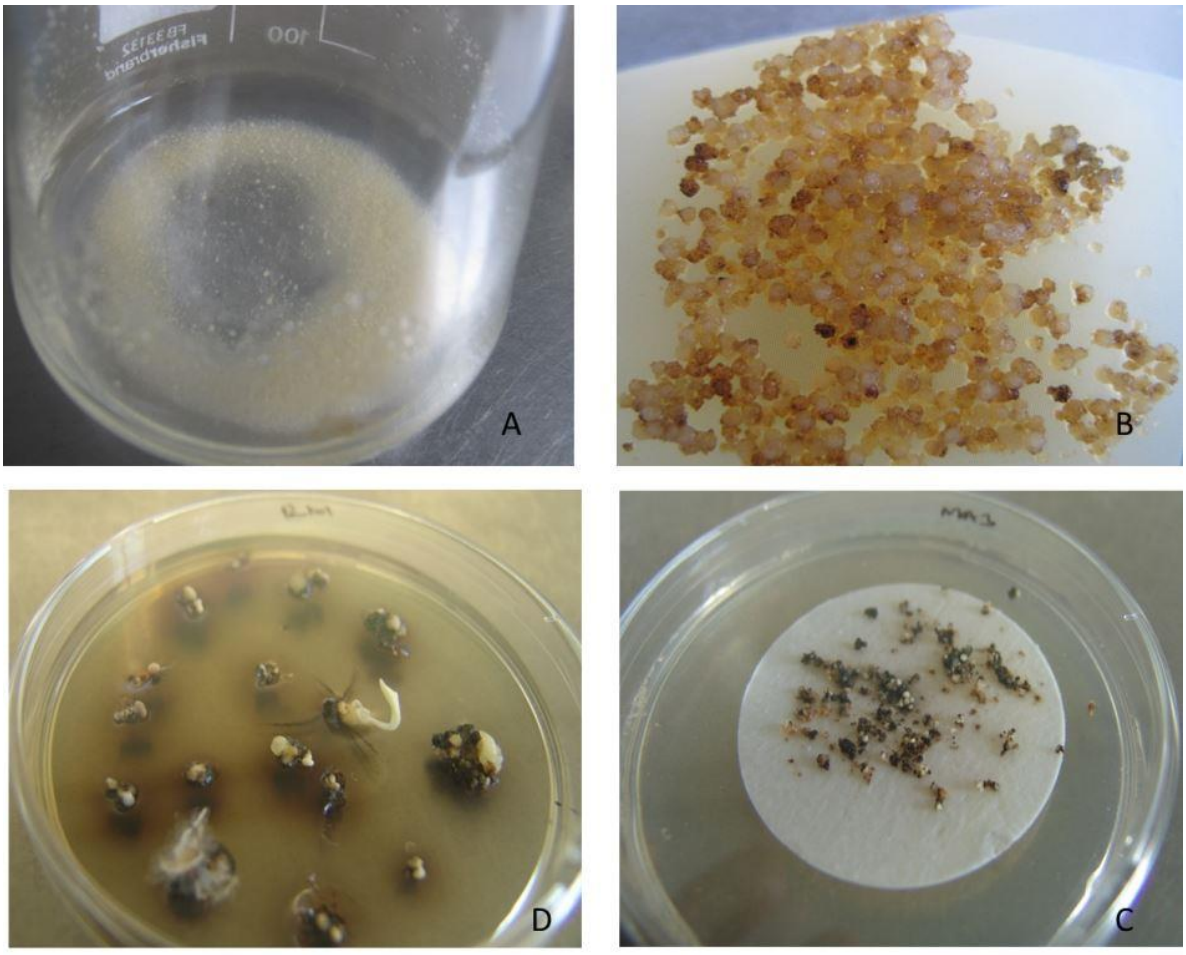

Figure 3.1: Transformation of a local cvr "Gonja manjaya" using embryonic cell suspension (ECS). A: ECS sub-cultured in fresh ZZ medium; B: Transformed ECS on MA3 media supplemented with 50 mg/l geneticin and 200 mg/l timentin, black and brown-colored cells are untransformed dying cells; C: Embryos (white) developing from transformed cells on a glass filter on MA3 medium supplemented with appropriate antibiotics; D: Shoots emerging from embryos on RDI medium supplemented with 50 mg/l geneticin and 200 mg/l timentin.

Figure 3.2: Regeneration and multiplication of transgenic plants of local cv "Gonja manjaya". A: Transgenic plantlets on MS proliferation medium supplemented with 50 mg/l geneticin; B: Weaned plants from the humid chamber; C: Potted transgenic plants in the green-house for growth and development; D: Transgenic plants ready for inoculation in the screen-house**.**

3.3.2 Molecular characterization of transgenic plants

3.3.2.1 PCR analysis

The presence of *Hrap* and *Pflp* transgenes in putative developed transgenic plants was confirmed by PCR analysis using transgene specific primers. A product of 372 bp was amplified from genomic DNA of randomly selected transgenic lines with stacked *Hrap-Pflp* genes using *Pflp* specific primers (Fig. 3.3). In addition, an amplicon of 490 bp was generated from genomic DNA of the same transgenic lines with stacked genes using *Hrap* specific primers (Fig. 3.3). Presence of *Hrap* and *Pflp* genes in transgenic lines that were transformed with single transgenes was also confirmed by amplification of 490 and 370 bp using respective gene specific primers, (Fig. 3.4 and 3.5). PCR analysis confirmed the presence of *Hrap* and *Pflp* transgenes in stacked and individual transgenic lines, and PCR positive transgenic lines were then randomly selected for screening for BXW resistance. No amplicon was detected in the non-transgenic control plants (Fig. 3.4 and 3.5).

3.3.2.2 Southern Blot analysis

A total of 8 transgenic lines with stacked Hrap and Pflp genes were selected for Southern blot analysis with Pflp probe to determine the copy number of transgenes. In two previous studies (Namukwaya *et al.*, 2012 and Tripathi *et al.*, 2010) transgenic bananas were transformed with individual Hrap and Pflp coding sequences and analyzed using Southern blot with Hrap and Pflp probes, respectively. There were no bands detected in the non-transgenic (NT) control plants.

This indicates that genes used in the study were not present in non-transgenic plants. However, H and P lines were not used in Southern blot analysis because the focus of the study was on stacked lines and not on single transgene lines (H, P) although they were also developed for comparison. Detected bands were however very faint, and almost non-detectable with Southern blotting indicating a very low copy number of transgene present in genomes (Fig. 3.6). However, possible band for S19 could be confirmed by PCR (Fig. 3.3)

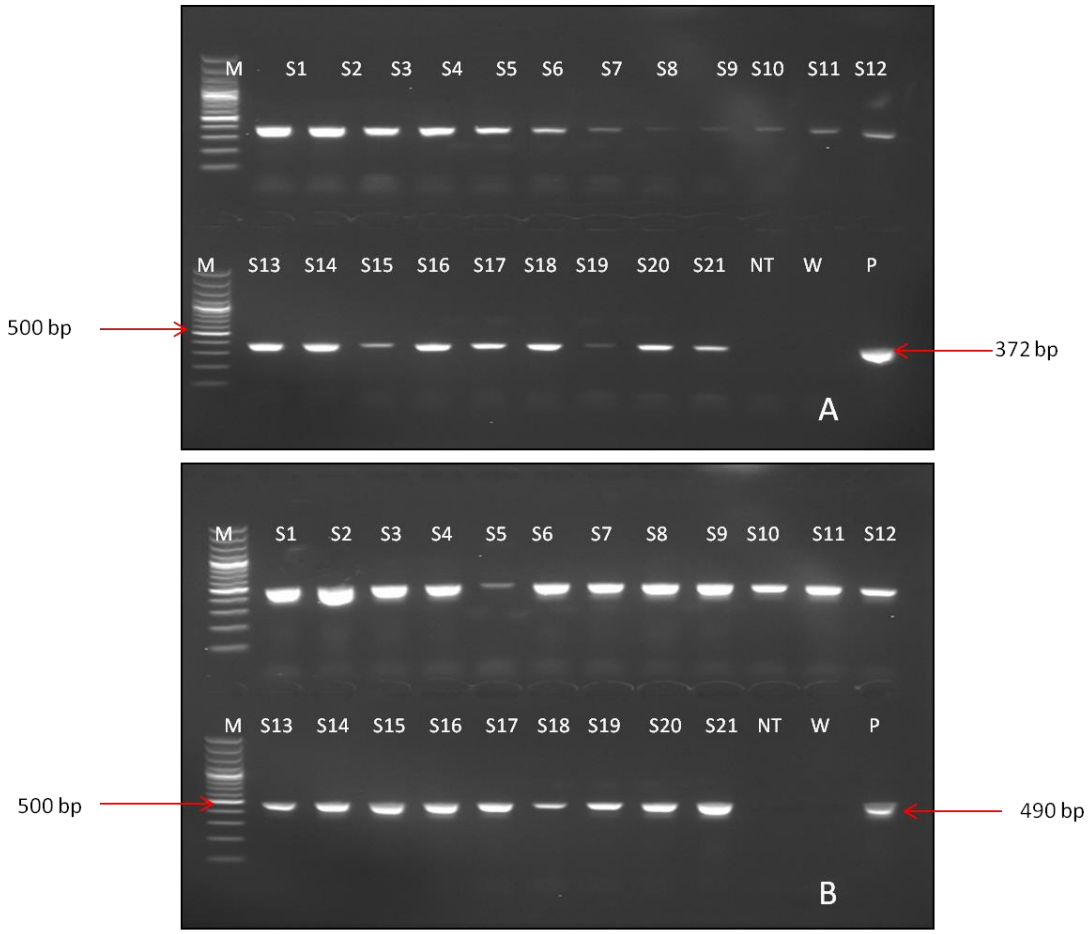

Figure 3.3: PCR analysis of representative transgenic banana plants transformed with stacked pBI-*Hrap-Pflp* genes. A: PCR with *Pflp* gene specific primers; B: PCR with *Hrap* gene specific primers. M: 100 bp plus DNA ladder GeneRuler ^{TM} (ThermoScientific, UK); S1-S21: transgenic lines with stacked genes; NT: non-transgenic control plant; W: negative control (No template DNA); P: pBI-*Hrap-Pflp* control plasmid**.**

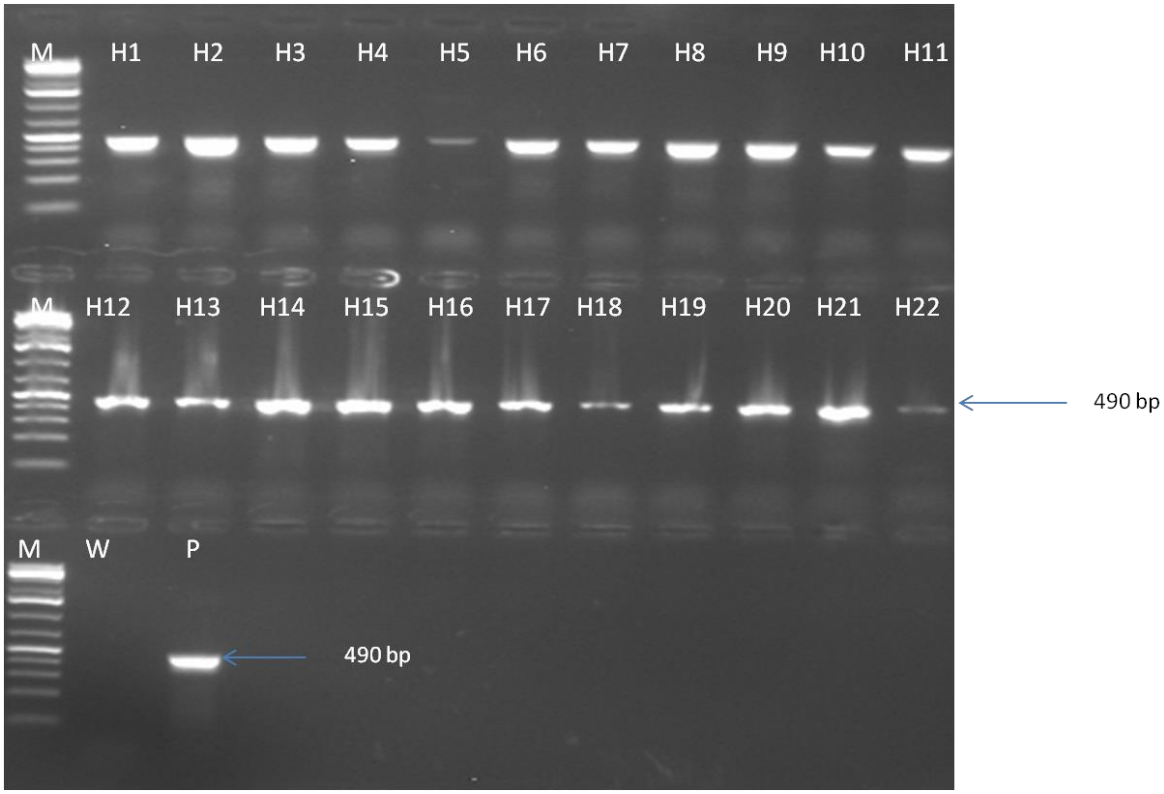

Figure 3.4: PCR analysis of representative transgenic plants transformed with *Hrap* gene. M: 100 bp plus DNA ladder GeneRuler ™ (ThermoScientific, UK); H1-H21: putative transgenic lines; W: negative control (no template DNA); P: pBI-*Hrap* plasmid DNA.

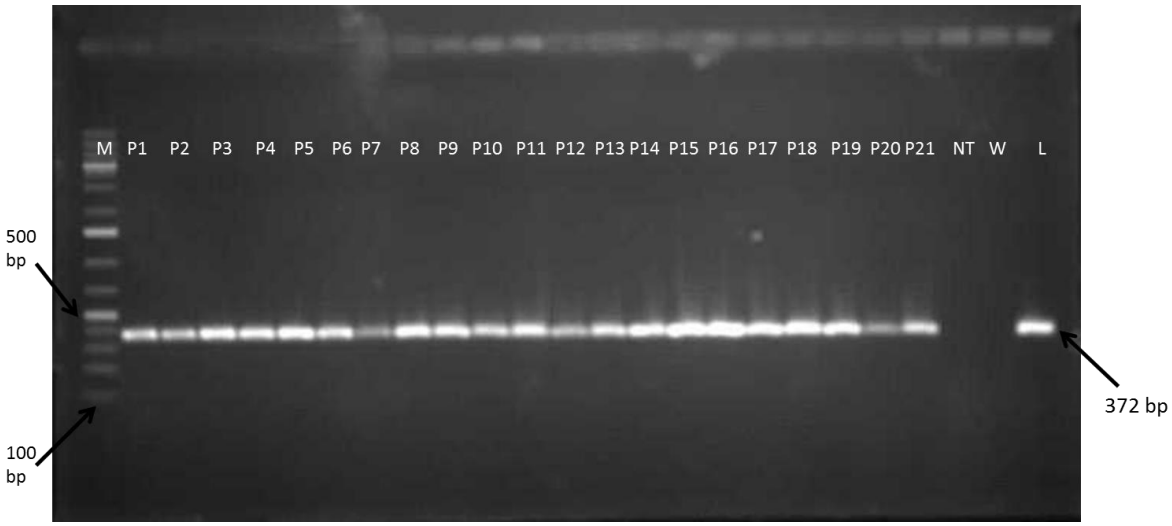

Figure 3.5: PCR analysis of representative transgenic plants transformed with *Pflp* gene. M: 100 bp plus DNA ladder GeneRuler ™ (ThermoScientific, UK); P1-P21: putative transgenic lines; NT: non-transgenic control plant; W: negative control (no template DNA); L: pBI-*Pflp* plasmid DNA used in the transformation.

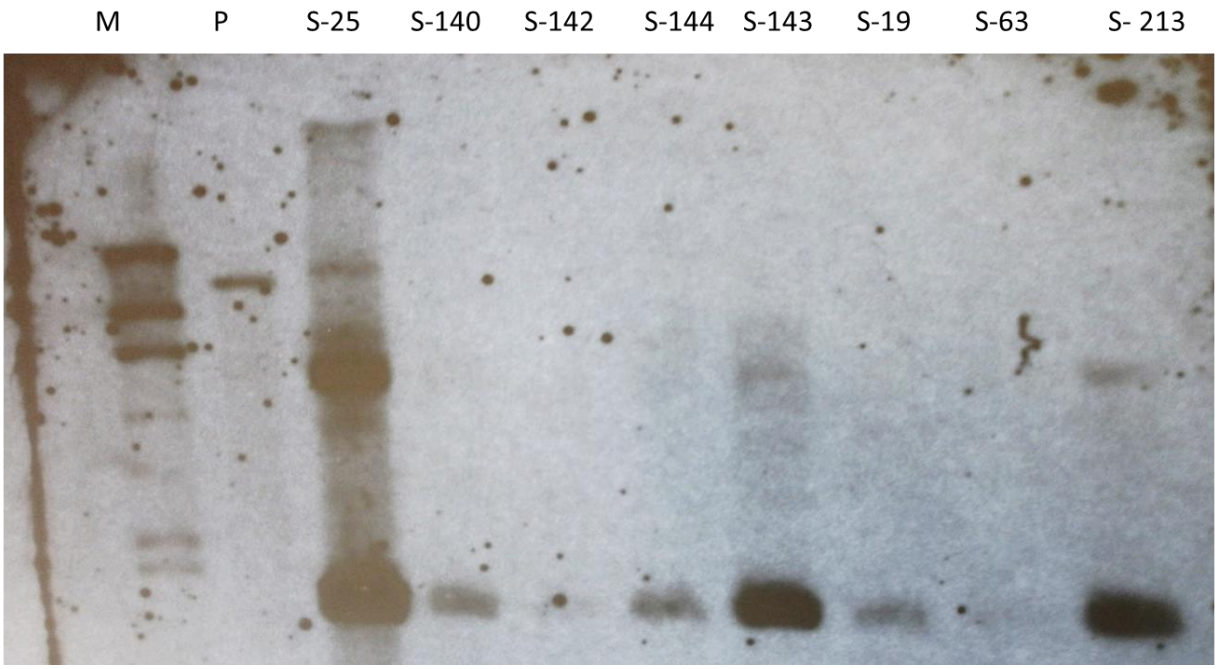

Figure 3.6: Southern analysis of representative transgenic lines with stacked genes: M: DIG labelled marker; P: Plasmid pBI-Hrap-Pflp; S-25, 140, 142, 144, 143, 19, 63, 213: transgenic lines with stacked genes.

3.4 Discussion

Transgenic lines of two bananas cultivars Gonja manjaya (AAB) and Sukali ndiizi (AB) having individual Hrap, Pflp and stacked Hrap-Pflp genes from Capsicum annum were obtained by transforming embryonic cell suspensions. *Agrobacterium*-mediated transformations of ECS of different banana cultivars have been previously reported. Ganapathi *et al*. (2001) achieved *Agrobacterium*-mediated transformation of ECS of banana cv. Rasthali (AAB) with acetolactase synthase (*ALS*) gene and *gusA* reporter gene. Pineda *et al.* (2002) also generated transgenic plants from ECS of Dominico hartón (AAB) when infected with the *Agrobacterium* harboring reporter genes *gusA* and *hph*. Furthermore, Khanna *et al*. (2004) generated transgenic banana plants from ECS of cultivar Cavendish (AAA) and Lady Finger (AAB) transformed with *hpt*, *gusA* and modified *GFP* using *Agrobacterium*-mediated transformation.

In this study, selection using geneticin was preferred over kanamycin to limit untransformed regenerates under antibiotic selection, termed as “escapes”. Kanamycin, often used in transformation instead of geneticin, is detoxified leading to a large number of escapes (Deepika *et al*., 2014). In this PhD study, non-transgenic control cells had further higher regeneration frequency than transgenic cells. The low regeneration frequency of transformed cells might have been due to geneticin that was applied for selection. A similar finding was reported by Tadesse *et al*. (2003). They found that embryonic capacity of sorghum on geneticin-containing selection medium was associated with a slower growth rate when compared to a culture on a non-selective medium. Such a considerable reduction in regeneration of transgenic sugarcane on a selective medium supplemented with 45 mg/1 geneticin was also previously reported by Bower *et al*. (1996). Since a high concentration of geneticin (50 mg/l) was used for selection, there were also

no ‘escapes’ among putative transgenic lines that were tested for transgene presence using PCR. High geneticin concentration causes rapid cell death of non-transgenic control cells, which may result in death to adjacent transformed cells, particularly early in selection when cells are used (Sant, 2011).

Cultivar “Gonja manjaya” further regenerated faster than cultivar “Sukali ndiizi”. This might be attributed to prolonged selection that causes stress in cells, and make then fail to regenerate (Talengera, 2011). Bardini *et al*. (2003) also reported that low regeneration might be caused by a stress condition due to long *in vitro* growth. Cultivar Sukali ndiizi could be therefore more sensitive to stress imposed by growth regulators than Gonja manjaya. Besides growth regulator stress, plant cultivars also vary in the response to shoot regeneration *in vitro*. Such differences were for example found in *in vitro* regeneration of two tobacco cultivars (Ali *et al*., 2007). The majority of transgenic plants of cultivar Sukali ndiizi were further non-responsive to proliferation after 3-5 sub-culturing. Some transgenic cell clusters further survived antibiotic selection but failed to regenerate into shoots. This low conversion frequency of embryos to plants has also been reported as a common problem in other plant cultivars (Gaj *et al*., 2004).

In this study, transformation efficiency of 90 independent transgenic plants per 0.5 ml SCV was obtained for cultivar “Gonja manjaya”. This was comparable to what was previously reported by Tripathi *et al*., (2012) of 50-60 transformed lines of the same cultivar. For “Sukali ndiizi”, 50 transgenic plants per 1 ml SCV obtained were less than what was previously reported (Tripathi *et al*., 2010). The differences in transformation efficiency of “Gonja manjaya” and “Sukali ndiizi” observed in different studies could be attributed to possible differences in the embryonic

suspension cell lines used and differences in in-vitro conditions. However, in banana a much higher transformation efficiency of 490 transgenic lines per 0.5 ml SCV has been reported for *Musa acuminata* cv. *Mas* (AA) (Huang *et al.*, 2007).

The presence of the *Hrap* and *Pflp* transgenes was confirmed in representative putative transgenic lines using PCR with gene specific primers. The amplified product of about 490 bp and 372 bp for *Hrap* and *Pflp* genes respectively were observed from the genomic DNA of all putative transgenic plants tested using gene specific primers, confirming the presence of both transgenes in all transgenic lines with stacked and one transgene in transgenic lines with single transgene. As a prerequisite, all transgenic lines used for the subsequent study activities were PCR positive for the transgene(s) that was inserted.

Genomic DNA of eight transgenic lines with stacked transgenes were also analyzed by Southern blot to confirm integration of *Pflp* gene in banana genome. Since *Hin*dIII restriction enzyme used was cutting at more than one site in the T-DNA, copy number determination was tried by band intensity. Observed bands would be a result of integration of the *Pflp* transgene into the banana genome. Previous studies by Namukwaya *et al*. (2012) and Tripathi *et al*. (2010) already showed that there are no *Pflp* and *Hrap* bands detected in non-transgenic banana plants when analysed by Southern blotting. Although various bands were detected and some lines seem to have more than one copy of the transgene (S-25, S-144 and S-213), bands were too faint to carry out a proper analysis.

CHAPTER FOUR

PHENOPTYPING OF TRANSGENIC BANANA PLANTS

4.1 Introduction

Plants have an array of defense mechanisms against pathogen attack by inducing various defense responses. The hypersensitive response (HR), increased production of ROS (oxidative burst), pH changes, membrane depolarization and Ca^{2+} influxes are some of the defense responses triggered upon recognition of pathogen effectors by the host ultimately culminating into resistance (Heath, 2000). However, some plants still die due to pathogen infection, despite the induction of these defense responses. This may be attributed to induction of defense responses below threshold levels that warrant resistance or manipulation of host defense responses by the pathogen so as escape recognition (Abramovitch and Martin, 2004). Studies have demonstrated that a plant's response and resistance against bacterial, viral and fungal pathogens can be enhanced by over-expression of transgenes (Rommens and Kishmore, 2000). Overexpression of *Hrap* and *Pflp* transgenes in transgenic rice and tobacco against virulent bacteria triggered an oxidative burst and induced HR rendering the plants resistant (Ger *et al*., 2002; Huang *et al*., 2004). However, biochemical responses of transgenic banana plants expressing these *Hrap* and *Pflp* transgenes in response to BXW have yet to be demonstrated in more detail. Also not well understood is whether expression of these stacked transgenes causes any phenotypic changes in a transgenic banana plant.

The objectives of this chapter were therefore to first evaluate and compare resistance levels against BXW in transgenic plants with stacked and single *Hrap* and *Pflp* transgenes. Secondly to investigate if *Xcm* enhances accumulation of hydrogen peroxide in transgenic banana tissues

following infection, and lastly to determine if expression of *Hrap* and *Pflp genes* in transgenic banana plants with stacked and single transgene changes any plant phenotypic characteristics.

4.2 Materials and Methods

4.2.1 Plant growth

Growth of transgenic lines was evaluated using potted plants in a level 2 containment facility glasshouse at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda. Rooted transgenic and non-transgenic control plants regenerated from tissue culture were weaned in a sterile loam and sandy soil mixture in 150 ml plastic cups, hardened and transferred into 3 liter plastic containers. Plants establishment was in approximately two kilograms of loam-sandy soil mixture and pots were randomly placed on 1 m high metallic tables. Plants were watered daily and illumination was by natural light at a 12/12 light/dark photoperiod. The glasshouse temperature ranged from 25-33^{o}C with relative humidity of 40-65%.

4.2.2 Plant growth parameters

4.2.2.1 Plant height and leaf growth

Growth measurement was started at one month after potting and subsequently done for three months. Plant height was used as aerial vertical plant growth and plant girth width as aerial horizontal plant growth. These growth parameters were measured once in a week for three

months. Plant height was measured from the pot rim to the junction of the petioles of the top most leaves. Girth width was measured at the base of the plant just above the soil. The total number of functional leaves on each plant from weaning was also recorded and leaf growth data were taken from the 9^{th} 10^{th} and 11^{th} leaf of the plant. Leaf growth was measured basing on the leaf length and blade width. Leaf length was measured from the point where the top most leaves meet to the tip of the leaf, whereas the blade width was measured at the broadest part of the leaf using a tape measure.

4.2.2.2 Root growth measurement

Four months after weaning, each plant was removed from the soil and remaining soil washed off from the roots with running water. Roots were excised from the plant and counted together with the length of the longest root on each plant recorded.

4.2.2.3 Biomass production

Four months after weaning each plant was removed from the pot and soil washed off from the roots with running water. The roots were blot dried before taking the fresh weight of both plant shoot and root system (biomass). Shoot and roots were dried in an oven for 48 hr at 70^{o}C, then weighed to determine the biomass production.

4.2.3 Plant resistance assays

4.2.3.1 Bacterial inoculum preparation

A pure bacteria culture of *Xcm* isolate was obtained from IITA, Uganda on a YPGA plate (yeast extract 5 g/l, peptone 10 g/l and glucose 20 g/l, agar 40 mg/l, pH 7.0). The isolates were confirmed to be virulent and were cultured routinely on YPGA medium supplemented with 50 mg/l of the antibiotic cephalexin (Duchefa Biochemie, Holland). *Xcm isolates were initially tested to be virulent to bananas.* Whether Xcm isolates used in the study harbored, or had different levels, of virulence was not tested. However, genome sequencing studies have found that Xcm isolates from the region have a high degree of similarity.

Bacterial cultures were initiated from a single colony in sterile 20 ml YPG (yeast extract 5 g/l, peptone 10 g/l and glucose 20 g/l) broth supplemented with cephalexin and then incubated at 28^{o}C with continuous shaking at 200 rpm for 48 hr. The optical density (OD) of culture at 600 nm was measured using a UV spectrophotometer. The bacterial culture was transferred in a sterile 50 ml falcon tube, and centrifuged at 6000 rpm for 10 min at 28^{o}C using a top bench centrifuge to collect the bacterial pellet. The supernatant was discarded before re-suspending the bacterial pellet in sterile water OD_{600} of 1.0 (10^{8} colony forming units (cfu/ml). The pathogen suspension was then used to artificially inoculate potted plants in the screen-house.

4.2.3.2 Inoculation and assessment of plants

Transgenic banana plants with stacked and single Hrap and Pflp transgenes were randomly selected for screening for resistance against Xcm. Plants regenerated from non-transformed ECS in tissue culture and not exposed to Agrobacterium were used as a non-transgenic control plants. Six control plants were used for growth evaluation studies and for evaluation of any somaclonal variation. Only two variant lines were identified in weaned plants which were not further used for the analysis. Three months old soil potted plants, 21 lines of each transgene category (single *Hrap* and single *Pflp* and stacked transgenes) were artificially inoculated. For inoculation, plants were placed on 1 m high metallic tables in a completely randomized design (CRD) with inoculations done using the leaf petiole inoculation assay as described by Tripathi *et al*. (2010). The inoculated leaf was tagged with a tape along the leaf stalk. Following inoculation, plants were left in the screen house at 25 – 28^{o}C under natural illumination and were watered daily. Plants were monitored for 60 days for symptoms development. Symptom development data was taken which included (i) number of days to first symptom appearance, (ii) number of days to complete wilting for plants that wilted completely, (iii) number of leaves showing symptoms out of the total number of leaves on a plant at 60 days post inoculation (dpi), and disease severity index. Photographic data of plants was also recorded for plants with symptoms, completely wilted or dead plants, as well as plants that did not show any symptoms. The disease severity index (DSI) was used to score plant's resistance to *Xcm*. The index is based on the number of leaves on a plant showing symptoms, with a scale of 0 to 5 where the scale 0 = no symptoms; scale 1 = one inoculated leaf showing symptoms; scale 2 = 2 – 3 leaves showing symptoms;

scale 3 = 4 - 5 leaves showing symptoms; scale 4 = all leaves showing symptoms but plant still alive; and scale 5 = plant dead.

In the leaf blade assay, 100 µl (10^8 cfu/ml) bacterial suspension was infiltrated into the leaf blades using a 1 ml syringe at the base where the petiole joins the leaf blade (Fig. 4.1). The area infiltrated was marked for further follow up of the disease development. Data of the number of days to symptom manifestation, lesion length and chlorotic/necrotic area of each leaf were recorded at 14 and 28 dpi. Symptom progression amongst plants with different transgenes was also studied. Lesion length was measured using a ruler whereas the chlorotic area was measured by placing a transparent plastic sheet with grids of known area. All collected data was statistically analyzed using Genstat software (Version 12.1).

4.2.3.3 *Xanthomonas* detection in inoculated plants

Explants from three biological replicates for each treatment were collected from pathogen inoculated leaf areas at 7 dpi. Samples were weighed; surface sterilized using 70% ethanol and then rinsed at least 4-times in sterile water. With a sterile mortar and pestle, pooled samples of each treatment were ground in 5 ml sterile autoclaved water to obtain a fine suspension. From the suspension, 1 ml was taken and diluted in 9 ml of sterile water to obtain 10-times dilution. Further dilutions of up to 10^{-9} were prepared. Then 20 µl from selected dilutions were spotted on YPG-CC agar (1 g/l yeast extract, 1 g/l peptone, 1 g/l glucose, 14 g/l agar, 40 mg/l cephalexin, 75 mg/l cycloheximide, and 10mg/l 5-fluorouracil). Plates were incubated at 28^{o}C for 3 - 4 days to develop colonies. The colonies were counted on each plate for the selected dilutions. *Xcm* was

also isolated and enumerated from dead tissue samples of selected plants as stated above. Results were expressed as colony forming units (cfu) per gram of tissue.

Figure 4.1: Inoculation of banana plants with *Xanthomonas campestris* pv. *musacearum*. A: Infiltration of the leaf petioles with pathogen inoculum; B: Infiltration of the leaf blades; C: plants with inoculated leaf (tagged with white tape) in the screen-house.

4.2.4 Hydrogen peroxide determination

The left and right side of the third leaf of each plant were inoculated with water and *Xcm* suspension respectively. Three leaf disc explants from water and pathogen inoculated parts of each plant were collected at each time point 0, 12 24, 48 h post inoculation (hpi). Hydrogen peroxide production was determined according to Velikova *et al*. (2000) with slight modifications as described below. Leaf tissues (80-100 mg) were homogenized in an ice bath with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 *g* for 15 min at 4^{o}C and then 0.5 ml of the supernatant was transferred into a new tube. To the supernatant, 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI were added before keeping the supernatant at room temperature in the dark for 1 h. The absorbance of the supernatant was then measured at 390 nm. The amount of H_2O_2 produced was calculated using a standard calibration curve previously made using different known concentrations of hydrogen peroxide.

Detection of hydrogen peroxide accumulation in infected leaves was also done using 3,3-diaminobenzidine (DAB) staining according to Belenghi *et al*. (2003). Leaf explants were immersed in 1mg/ml DAB solution (Sigma, USA), and then vacuum-infiltrated for 5 min before incubation for 6-8 hr at room temperature. After staining, the DAB solutions was pipetted off before de-staining the leaf explants in a boiling solution of ethanol : acetic acid (3:1). Photos of explants were then taken.

4.2.5 Transgene expression analysis of transgenic plants

4.2.5.1 RNA extraction and cDNA synthesis

Leaf samples were obtained from selected transgenic plants with known disease reaction status (resistant or susceptible). The youngest leaf was sampled from three biological replicates of each line. Total RNA was extracted from pooled samples of each line using the CTAB protocol according to Yang *et al*. (2008) with some modifications as described below. Each collected and frozen sample was ground in liquid nitrogen to a fine powder in a sterile mortar with a pestle and ground sample (200 mg) was quickly transferred to 2 ml Eppendorf tubes to which 800 µl of the extraction buffer (2% w/v CTAB, 2% w/v PVP-40, 0.5 mM EDTA pH 8.0, 1 M Tris base pH 8.0, 1.4M NaCl, 1% *β*-mercaptoethanol) was added. Samples were vortexed for 15 s before incubating at 65^{o}C for 10 min in a water bath with occasional shaking. The samples were centrifuged at 13,000 rpm at 4^{o}C for 5 min. From the top layer, 800 µl was removed to a 2 ml new tube. Samples were extracted with an equal volume of chloroform: isoamylalcohol (24:1 v/v), vortexed for 15 s and centrifuged at 13,000 rpm at 4^{o}C for 5 min. From the aqueous top layer, 700 µl was transferred to a new 2 ml tube and then 700 µl of chloroform: isoamylalcohol (24:1 v/v) added and vortexed as stated above. After centrifugation, 600 µl of the top layer was transferred to a new Eppendorf tubes before adding 600 µl of chloroform: isoamylalcohol (24:1 v/v). Samples were vortexed for 15 s and centrifuged at 13,000 rpm at 4^{o}C for 5 min. From the top layer, 600 µl was transferred to a new tube being very careful not to carry any of the bottom layer. To the sample 267 µl of 12 M LiCl (to give a final concentration of 3M) was added and

gently mixed by inversion before precipitating RNA at -20^{o}C overnight. Samples were centrifuged at 13,000 rpm 4^{o}C for 20 min. The supernatant was removed and to the pellet 1 ml of chilled 70% ethanol was added and centrifuged at 13,000 rpm at 4^{o}C for 5 min. The washing with ethanol was repeated and ethanol removed by pipetting before drying the pellets in a vacuum concentrator for 10 min at 30^{o}C. Dried pellets were re-suspended in 50 µl of RNase free water and the concentration determined by spectrophotometry using a Nano-drop 2000 spectrophotometer.

Extracted RNA was treated with DNase I to remove any contaminating genomic DNA. The reaction volume of 50 µl consisted of 3 µg re-suspended RNA, 1X final concentration of DNase I buffer, 5 U of DNase I enzyme (Fermentas #EN0521). The tube contents were mixed and spinned briefly and incubated at 37^{o}C for 30 min. The reaction was inactivated by adding 5 µl of 50 mM EDTA and then incubated at 65°C for 10 min. DNase I treated samples were verified for absence of genomic DNA with PCR with *Hrap* and *Pflp* gene specific primers (Table 4.1). Genomic DNA free of RNA was quantified using the Nano drop and 2 µg RNA was used to synthesize first-strand cDNA with random hexamer primers applying a Fermentas kit (Revertaid First Strand cDNA Synthesis #K1622, Fermentas, UK) following the manufacturer's instructions.

4.2.5.2 Quantitative real time PCR analysis

Synthesized cDNA was diluted 10 fold and primers sequences used (Table 4.1) for qRT-PCR applied were obtained from coding sequences of *Hrap* (Accession no. AF168415) and *Pflp*

(Accession no. AF039662) obtained from the NCBI genebank. The *Musa* 25S rRNA was applied as endogenous control. Amplification was done in an Applied Biosystem (USA) cycler, system 9700.

The qRT-PCR procedure was carried out on leaf samples using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) following the manufacturer's instructions. The reaction mixture constituted 2 µL of the diluted template (1/10), 0.6 µL of 10 µM of forward and reverse primers, 5 µl SYBR-Green I master mix, and 5 µl nuclease-free water were added into respective wells in a 96 well RT-PCR micro-titer plate. Non-template control reactions containing water instead of cDNA were also included in the analysis. The qRT-PCR procedure was set up as follows: DNA denaturation for 10 min at 95°C, followed by 40 cycles consisting of a denaturation step at 94°C for 10 s, annealing of primers at 60°C for 20 s and DNA extension at 72°C for 20 s. The reaction was finally subjected to a temperature of 65°C for 10 s, and the entire plate was incubated at 95°C for the fluorescence signal of samples to be assessed.

A standard curve for *Hrap*, *Pflp* and 25s genes was done to a final arbitrary concentration of 1 (stock) and sequential dilutions (factor 2) to 0.5 0.25, 0.125, and 0.0625. For each primer set, non-template control reactions containing water as template were included and each reaction was set up in triplicates.

Table 4.1: *Hrap* and *Pflp* gene specific primers used in qRT-PCR

Primer name	Primer sequences
Hrap Q-Forward	5′-CCAACACAATACTTCAATAGGG-3′
Hrap Q-Reverse	5′-AGCAGAATCACAAGGGACAAT-3′
Pflp Q-Forward	5′-CCTGACGGACCAATAGAAT-3′
Pflp Q-Reverse	5′- CACAAGATGAGCAAGAACCT-3′
Musa 25s-Forward	5′-ACATTGTCAGGTGGGGAGTT-3′
Musa 25s-Reverse	5′-CCTTTTGTTCCACACGAGATT-3′

4.3 Results

4.3.1 Growth analysis

Growth evaluation of soil-potted transgenic and non-transgenic (wild type) banana plants was done in the greenhouse over a 3 months period. Seven transgenic lines with single *Hrap* (H) or *Pflp* (P) transgene as well as stacked (S) transgenes (21 transgenic lines in total) and non-transgenic control plants, all of cv. Gonja manjaya were evaluated for growth. No significant ($p<0.05$) difference in plant height and girth width between transgenic and non-transgenic control lines was observed except for transgenic line S-156 with stacked transgenes. S-156 had significantly lower height and girth width when compared to a non-transgenic line (Table 4.2). Similarly the total number of functional leaves, leaf length and total leaf area of each line was not significantly ($p<0.05$) different among transgenic and non-transgenic control lines (Table 4.3). The only exception was *Pflp* transgenic line P107 which had a significantly higher leaf length compared to the non-transgenic control plants. Also no significant ($P<0.05$) difference

was observed in the total number of roots between transgenic plants and non-transgenic control plants (Table 4.4). Similarly there was no significant ($P<0.05$) difference in root length between transgenic lines and non-transgenic control plants with the exception of line H-55 and S-156 that had significantly lower number of root and shorter roots compared to the non-transgenic controls.

Table 4.2: Phenotypic analysis of transgenic banana plants and non-transgenic control plants

Line	Average plant height (cm)	Average girth width (cm)	Total number of functional leaves
Control	**43.75±2.9a**	**8.77±0.35a**	**11.3±0.5a**
H-100	46.33±1.1a	9.47±0.2a	12.0±0.3a
H-22	45.00±4.2a	8.97±0.5a	11.3±0.3a
H-31	44.00±1.4a	9.17±0.2a	11.3±0.6a
H-35	46.00±0.8a	9.20±0.1a	11.0±0.4a
H-55	41.67±3.8a	8.33±0.4a	10.7±0.6a
H-75	42.75±0.7a	9.18±0.9a	10.7±0.3a
H-76	44.67±0.8a	9.80±0.2a	12.0±0.6a
S-156	35.33±1.5b	7.10±0.2b	10.3±0.6a
S-214	43.67±2.9a	9.80±0.1a	12.0±0.3a
S-24	40.00±3.7a	8.17±0.7a	10.5±0.3a
S-29	46.33±0.9a	9.37±0.1a	11.0±0.9a
S-80	41.67±2.2a	8.10±0.2a	10.3±0.3a
S-82	41.56±6.4a	7.80±0.0a	10.3±0.6a
S-98	43.67±2.3a	9.27±0.2a	11.7±0.3a
P-107	39.76±4.3a	7.83±0.8a	12.0±0.3a
P-21	48.67±2.7a	9.70±0.4a	11.7±0.3a
P-37	42.50±2.0a	8.15±0.6a	11.5±0.0a
P-42	47.33±3.8a	9.53±0.5a	11.7±0.3a
P-69	48.87±2.4a	10.10±0.7a	10.3±0.3a
P-92	46.50±0.9a	9.53±0.0a	11.5±0.3a
P-93	40.33±0.7a	8.47±0.3a	11.3±0.3a

Values are the means ± SE of 6 individual plants per line measured after 3 months post potting. Letters denote significant difference determined using ANOVA at P = 0.05 within the column. Means having the same letters within the column are not significantly different.

Table 4.3: Leaf length and total leaf area of transgenic banana plants and non-transgenic control plants.

Line	Average Leaf length (cm)	Total Leaf area (cm^2)
Control	**55.40±4.3a**	**801±1.4a**
H-100	55.53±2.6a	933.0±23a
H-22	51.67±1.6a	814.0±7.2a
H-31	48.73±1.1a	827.0±9.0a
H-35	50.55±1.6a	872.0±1.1a
H-55	45.54±1.3a	790.0±18a
H-75	59.40±1.1a	1017±17a
H-76	55.33±3.5a	851.0±5.2a
S-156	50.13±1.9a	713.0±17a
S-214	49.50±1.9a	882.0±7.6a
S-24	57.70±2.2a	547.0±4.1a
S-29	53.10±3.2a	799.0±21a
S-80	47.33±1.9a	800.0±21a
S-82	60.00±2.8a	1144±5b
S-98	50.10±1.6a	909.0±20a
P-107	67.00±1.1b	1158±10b
P-21	62.00±2.3a	1026±4a
P-37	62.80±0.6a	1061±5a
P-42	60.23±1.9a	948.0±18a
P-69	62.43±2.7a	1145±14b
P-92	57.2±0.5a	917.0±6.6a
P-93	49.00±1.0a	991.0±10a

Values are the means ± SE of 6 individual plants per line at 3 months post potting. Parameters were taken on the 10^{th} leaf of each plant. Letters denote significant difference determined using ANOVA at P = 0.05 within the column. Means having the same letters within the column are not significantly different.

Table 4.4: Root growth of transgenic banana plants and non-transgenic control plants

Line	Total number of roots	Average root length (cm)
Control	**29.0±1.0a**	**68.3±8.0a**
H-100	26.3±0.7a	49.0±7.3a
H-22	24.3±0.9a	54.0±8.2a
H-31	24.7±1.2a	43.1±3.8a
H-35	23.0±1.8a	53.2±5.1a
H-55	21.7±2.4b	28.1±4.0b
H-75	25.0±2.9a	45.6±5.2a
H-76	27.7±0.7a	36.4±7.5b
S-156	16.3±2.2b	35.1±4.8b
S-214	27.7±1.8a	66.8±7.6a
S-24	21.5±2.0a	46.0±1.6a
S-29	28.3±1.7a	67.3±8.4a
S-80	23.0±2.5a	61.5±9.6a
S-82	24.0±2.1a	54.7±3.8a
S-98	31.7±2.7a	44.1±3.3a
P-107	25.7±4.6a	63.0±7.2a
P-21	28.4±3.8a	56.2±5.0a
P-37	23.0±1.6a	41.0±3.3a
P-42	31.0±1.5a	44.5±6.8a
P-69	29.7±0.9a	43.8±5.7a
P-92	36.0±1.6a	54.8±4.3a
P-93	21.3±3.3b	64.2±7.3a

Values are the means ± SE of 6 individual plants per line at 3 months post potting. Letters denote significant difference determined using ANOVA at P = 0.05 within the column. Means having the same letters within the column are not significantly different.

4.3.2 Evaluation of transgenic plants for resistance to *Xcm*

4.3.2.1 Symptom development

Twenty one transgenic lines each with stacked (S), single *Hrap* (H) and single *Pflp* (P) transgenes (PCR positive for respective transgenes) were randomly selected and screened for resistance with the leaf petiole inoculation assay (Fig. 4.1). Six individual plants (biological replicates) clonally derived from each line were screened, and the data were obtained from two independent experiments. Wilting was first observed in non-transgenic control plants with symptoms appearing in inoculated leaves on average 17 dpi. Appearance of disease symptoms varied greatly among lines. Transgenic lines with single *Hrap* (H-22) and single *Pflp* (P-25, P-4, P-48, P-49, and P-45) showed symptoms shortly after the non-transgenic control plants at 20 dpi. In other transgenic lines, appearance of disease symptoms was delayed up to 45 dpi. However, some transgenic lines did not show any disease symptoms development till the end of the experiment period (60 dpi) (Fig. 4.2).

Spread of disease symptoms from inoculated leaf to other leaves was faster in non-transgenic control plants than in transgenic plants. On average at 35 dpi, all non-transgenic control plants (30 plants) had completely wilted as a result of pathogen infection, whereas only a few transgenic lines (H-301, S-225, S-177, P-25, P-45, P-4) succumbed to pathogen infection, and the days for complete wilting was delayed to 43 dpi on average. This implies that these lines had partial resistance, unlike in non-transgenic control plants, despite eventually succumbing to *Xcm* infection.

A standard disease severity index (DSI) for *Xcm* was used to score the plant's resistance to the pathogen. The results of screened lines showed that six lines with single *Hrap* (28.6%) out of 21 lines evaluated, four lines with single *Pflp* (19.1%) and seven lines with stacked *Hrap-Pflp* (33.1%) did not show any symptoms with DSI = 0 (Table 4.5). DSI of 1 was observed in six lines with single *Hrap* (28.6%), seven lines with single *Pflp* (33.3%) and six lines with stacked *Hrap-Pflp.* Six lines with single *Hrap* (28.6%), five lines with single *Pflp* (23.8%) and four lines with stacked *Hrap-Pflp* had a DSI of 2 (Fig. 4.5). DSI of 3 was observed in one line with single *Hrap* (4.8%), two lines with single *Pflp* (9.5%), two lines with stacked *Hrap-Pflp* (9.5%). A DSI score of 5 was observed in all 18 non-transgenic control plants (100%), one line with single *Hrap* (4.8%), three lines with single *Pflp* (14.3%) and one line with stacked genes (4.8%). (Fig. 4.2; Table 4.5; 4.6).

Table 4.5: Disease severity index score of transgenic and non-transgenic control plants after artificial inoculation

Plants	**DSI**					
	0	**1**	**2**	**3**	**4**	**5**
Control	0	0	0	0	0	18
Hrap	6	6	6	1	1	1
Stacked *Hrap-Pflp*	7	6	4	2	1	1
Pflp	4	7	5	2	0	3

In total 18 non-transgenic control and 21 transgenic lines each with single *Hrap*, single *Pflp* or stacked *Hrap-Pflp* with 6 replicates for each line were score for DSI.

Table 4.6: Symptom development and DSI of transgenic lines and non-transgenic control plants

Line	Symptoms (days)	DSI	Line	Symptoms (days)	DSI
Control (NT)	**17.4±0.2**	**4.99±0.0**	**S-29**	**42.3±0.7**	**0.83±0.2**
H-154	50.0±2.0	0.16±0.2	S-32	51.0±1.0	0.50±0.1
H-156	21.9±0.7	1.83±0.2	S-40	24.2±1.2	3.67±0.3
H-158	0.00±0.0	0.00±0.0	S-45	21.8±0.8	2.83±0.2
H-22	19.2±0.5	2.00±0.1	S-3	34.5±3.5	1.71±0.1
H-240	51.5±0.5	0.50±0.2	S-5	0.00±0.0	0.00±0.0
H-243	24.0±0.7	1.83±0.1	S-76	49.3±1.3	1.00±0.0
H-255	46.3±0.3	1.00±0.0	S-8	26.7±2.0	1.68±0.0
H-273	21.7±0.3	3.17±0.5	S-89	22.3±2.3	2.00±0.0
H-301	22.8±1.8	5.00±0.1	S-9	52.6±3.3	1.00±0.3
H-309	32.3±2.3	2.00±0.1	S-98	0.00±0.0	0.00±0.0
H-310	48.0±3	0.23±0.2	P-11	35.0±0.1	1.50±0.1
H-3	30.2±0.9	2.00±0.1	P-113	26.3±3.0	1.50±0.2
H-323	26.2±0.8	1.67±0.2	P-18	22.0±0.1	2.00±0.0
H-324	33.0±0.7	1.17±0.1	P-2	29.2±1.8	2.00±0.1
H-5	50.0±2.0	0.33±0.1	P-200	34.3±4.3	1.17±0.2
H-72	47.8±1.2	0.83±0.2	P-205	27.2±2.5	1.50±0.1
H-73	0.00±0.0	0.00±0.0	P-213	40.0±2.0	1.00±0.0
H-7	22.2±0.2	3.17±0.2	P-224	34.8±1.8	1.17±0.1
H-80	23.7±3.7	2.00±0.3	P-36	0.00±0.0	0.00±0.0
H-82	34.5±0.8	1.50±0.2	P-239	26.0±1.3	2.00±0.1
H-8	0.00±0.0	0.00±0.0	P-25	18.5±0.2	5.00±0.2
S-1	0.00±0.0	0.00±0.0	P-1	31.0±0.3	1.67±0.1
S-15	22.3±2.0	2.71±0.1	P-6	0.00±0.0	0.00±0.0
S-156	47.0±2.0	0.50±0.2	P-263	26.2±1.8	2.00±0.1
S-177	24.0±4.0	4.67±0.3	P-37	37.0±2.3	2.00±0.2
S-211	0.00±0.0	0.00±0.0	P-4	17.3±1.0	4.71±0.2
S-216	41.2±1.2	1.00±0.1	P-45	20.2±0.2	4.67±0.3
S-219	43.2±1.8	1.00±0.0	P-48	20.0±1.0	2.67±0.1
S-225	21.5±0.5	4.33±0.3	P-49	21.5±1.2	2.33±0.2
S-2	0.00±0.0	0.00±0.0	P-5	53.5±0.5	0.50±0.1
S-232	0.00±0.0	0.00±0.0	P-95	0.00±0.0	0.00±0.0

H, S and P - Plants with single *Hrap* gene, stacked *Hrap-Pflp* and single *Pflp* genes respectively. Values are means of data from six individual plants ± SE of the mean.

Figure 4.2: Representative banana plants cv "Gonja manjaya" inoculated with *Xcm* at 60 dpi. A: Transgenic plants with stacked transgenes not showing any disease symptoms; B: Non-transgenic plants showing complete wilting; C: Transgenic plant with stacked genes with 3 wilted leaves showing partial resistance; D: Transgenic plants with stacked genes showing complete wilting**.**

4.3.2.2 Lesion and necrosis development

From the preliminary screening using the leaf petiole assay, five transgenic lines each with single *Hrap*, single *Pflp* and stacked genes with DSI = 1 (partial resistance) were selected to further evaluate their resistance levels using a leaf blade assay. Leaf blades inoculated plants exhibited lesions and chlorosis symptoms, but there were no wilting symptoms. Lesion development manifested first followed by chlorosis and necrosis (Fig. 4.3). Both lesions and chlorosis symptoms started from the point of inoculation and continued to spread to the leaf margins. Blackening of tissues was also observed at the point of pathogen inoculation inside the leaf petioles after 5 to 7 dpi. The blackened tissues were confirmed by Evans blue staining to be dead tissues inside the leaf petiole. To ascertain whether localized cell death at inoculation points was able to contain the pathogenic *Xcm*, bacteria were re-isolated from localized dead tissues at inoculation sites of some transgenic lines. Bacteria proliferated from dead tissues of some transgenic plants and morphological and colony characteristics showed that they were *Xcm* isolates. However, the number of colony forming units (cfu) in the non-transgenic plants was much higher than in transgenic lines (Table 4.7).

There was significant difference ($P<0.05$) in lesion size between non-transgenic and transgenic lines as well as among transgenic lines. Lesions started to develop at 14 dpi and extended up to the leaf margins by 28 dpi. Symptom progression in particular chlorosis from inoculated leaf to other leaves was slow in plants inoculated by the leaf blade assay. At 14 dpi, non-transgenic control plants, all *Pflp* transgenic lines (P-213, P-11, P-260, P-113, P-227) and stacked *Hrap-Pflp* transgenic lines (S-216, S-219, S-76, S-48) had developed lesions in comparison to only H-

243 transgenic line with *Hrap* transgene (Fig. 4.4). Lesion length at 14 dpi in non-transgenic control plants was significantly longer than in all transgenic lines (Fig. 4.4). Chlorosis manifestation in non-transgenic control plants was not significantly different from P-113 transgenic line with single *Pflp* transgene at 14 dpi (Fig. 4.5). In other transgenic lines, chlorosis development was smaller than in non-transgenic control plants (Fig. 4.5).

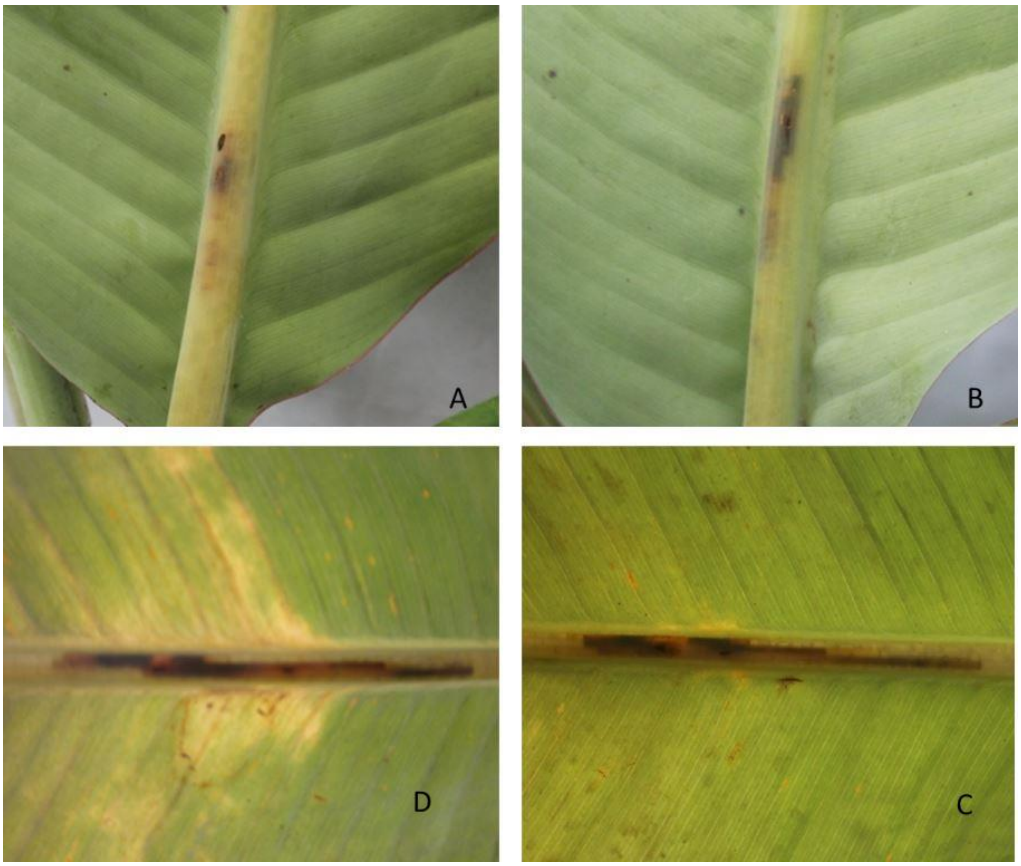

Figure 4.3: Cell death and symptom development at point of inoculation. A: Non-transgenic control plant; B: Transgenic plant; C: Dead tissues and minimal symptom development; D: Dead tissues and extensive symptoms development.

Table 4.7: Isolation of *Xanthomonas campestris* pv. *musacearum* from inoculated plants

Line	Colony forming unit (cfu)/g (log)
Control	38.25 ± 4.25a
H-5	1.00 ± 0.00c
H-78	13.5 ± 2.00b
S-39	11.0 ± 1.50b
S-82	4.00 ± 1.00bc
P-2	9.25 ± 0.75b
P-260	13.00 ± 3.00b

Samples taken at inoculation sites of transgenic plants at 7 days post inoculation. Re-isolation of bacteria was done on plants that showed localized cell death at the point of inoculation to access whether cell death was able to contain bacteria. Not all plants used for disease symptom analysis showed localized death and plants that only showed this localized cell death were used for re-isolation. Values are means of the data from three independent plants ± SE of the mean.

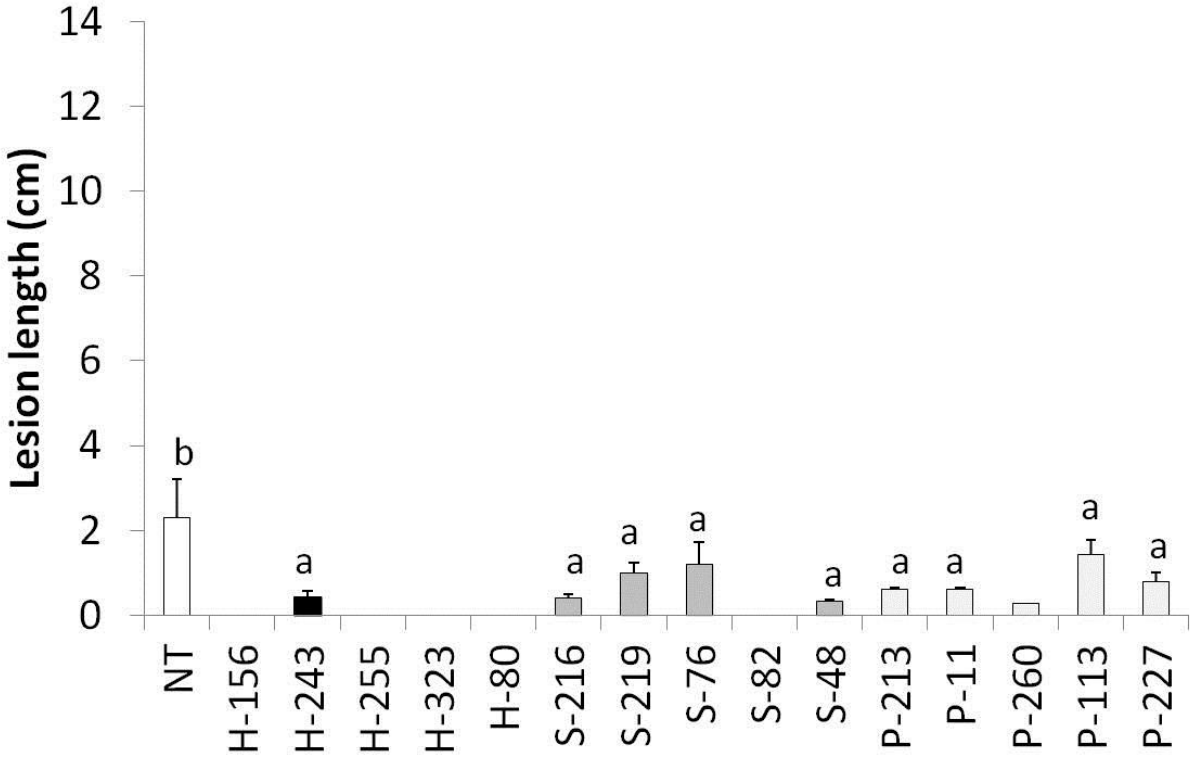

Figure 4.4: Lesion length of pathogen inoculated transgenic lines at 14 dpi. NT: non-transgenic control plants; H, S, and P: transgenic lines with *Hrap,* stacked *Hrap-Pflp* and *Pflp* transgenes respectively. Values of the graph bars with similar letters are not significantly different at P<0.05.

At 28 dpi, lesion size in non-transgenic control plants of 12 cm was significantly (P<0.05) larger than in all transgenic plants (Fig. 4.6). All transgenic lines had smaller lesions than the non-transgenic control plants but generally there was no significant difference in lesion size among individual transgenic lines with stacked and single genes, though *Hrap* lines had smaller average lesion length of 3.89 cm in comparison to 5.186 cm and 6.184 cm in stacked and *Pflp* transgenic lines respectively (Fig. 4.6).

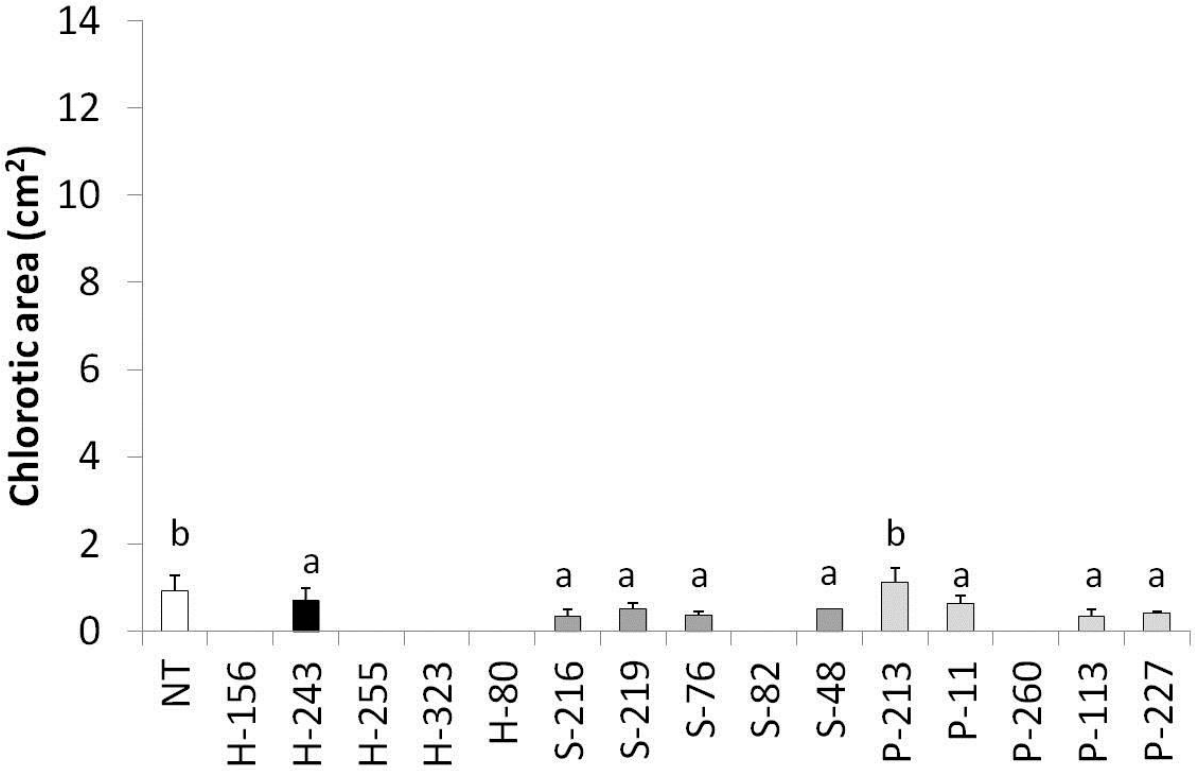

Figure 4.5: Chlorotic area of pathogen inoculated transgenic lines at 14 dpi. NT: non-transgenic control plants; H, S, P: transgenic lines with *Hrap*, stacked *Hrap-Pflp* and *Pflp* transgenes respectively. Values of the graph bars with similar letters are not significantly different at $P<0.05$.

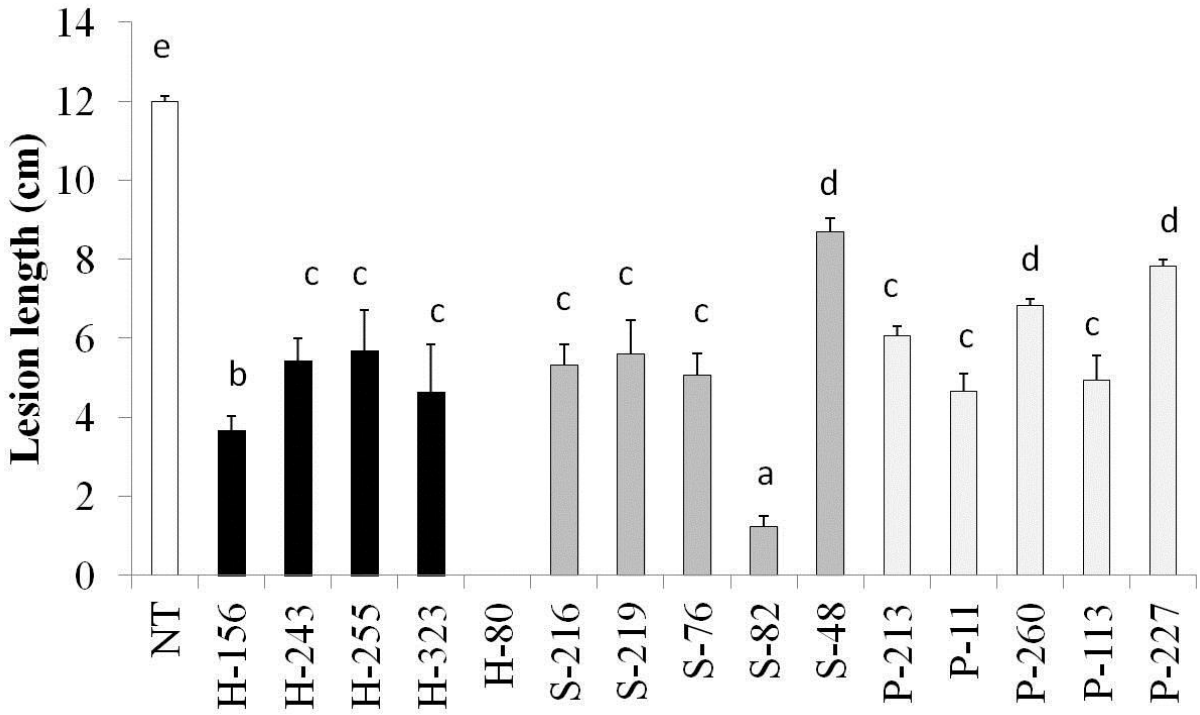

Figure 4.6: Lesion length of pathogen inoculated transgenic lines 28 dpi. NT: non-transgenic control plants. NT: non-transgenic control plants; H, S, P: transgenic lines with *Hrap*, stacked *Hrap-Pflp* and *Pflp* transgenes respectively. Values of the graph bars with similar letters are not significantly different at P<0.05.

Blackening and dead tissues were observed after chlorosis manifestation in leaves of some transgenic plants inoculated using the leaf blade assay (Fig. 4.7). Symptom manifestation continued to progress from the dead tissues up to the leaf margins. The chlorotic areas in non-transgenic control plants was significantly ($P<0.05$) larger in comparison to all transgenic lines at 28 dpi (Fig. 4.8). Among transgenic lines, all transgenic *Hrap* lines had significantly smaller chlorotic areas in comparison to transgenic lines with *Pflp* and stacked *Hrap-Pflp* transgenes (Fig. 4.8), indicating that single *Hrap* transgenic lines were more resistant to *Xcm* than lines with single *Pflp* and stacked transgenes.

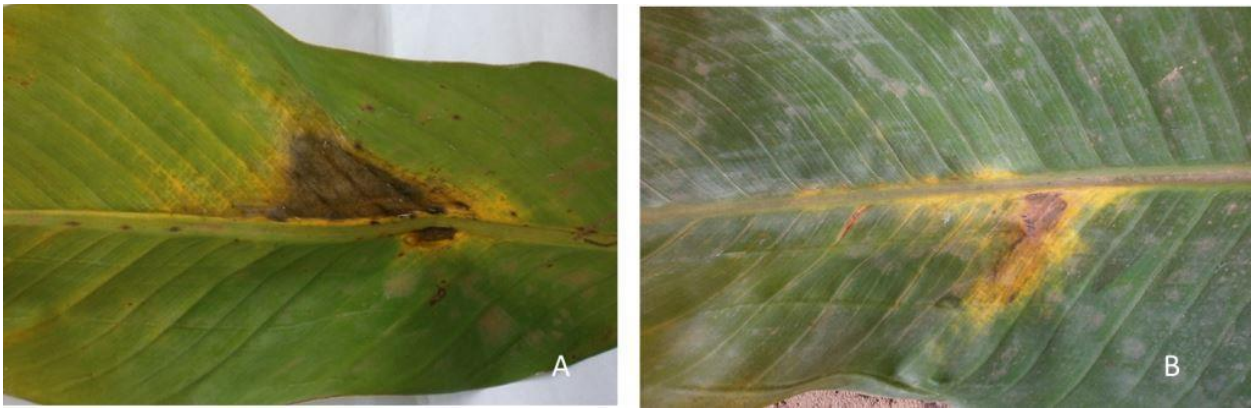

Figure 4.7: Symptom development in transgenic plants inoculated using a leaf blade assay. A: Necrotic-like symptom; B: Lesions and chlorotic symptoms.

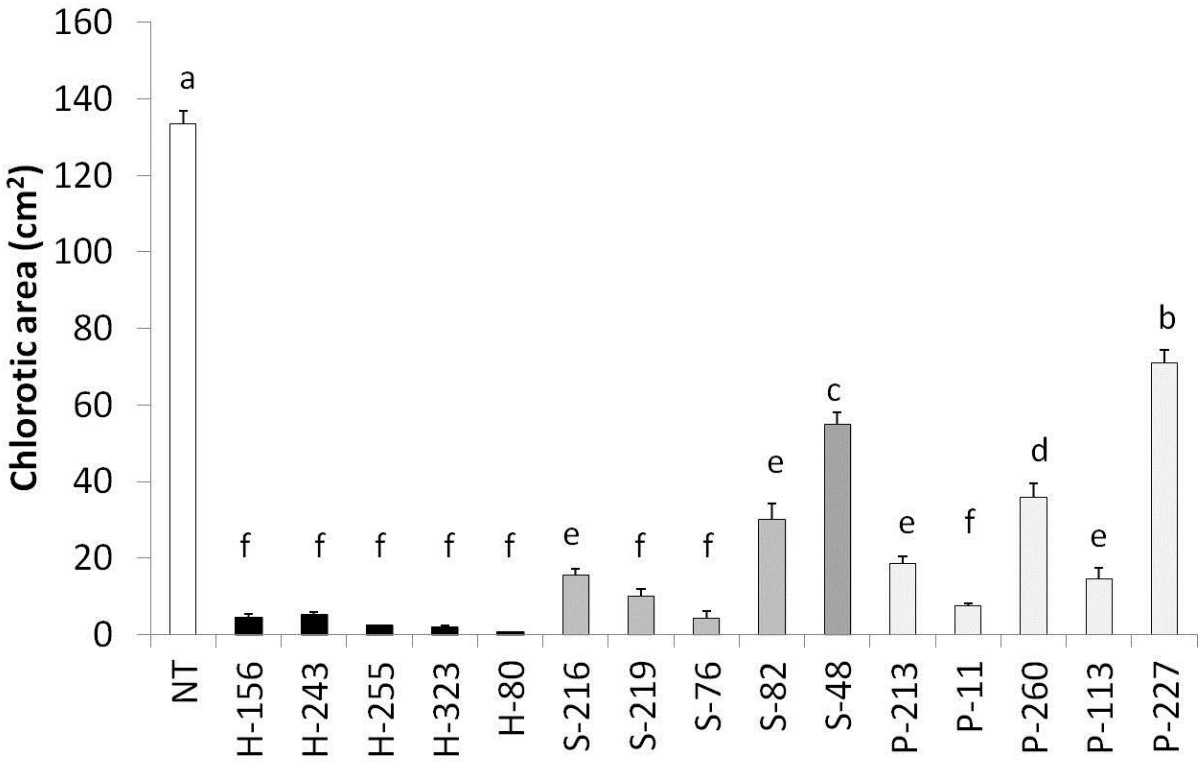

Figure 4.8: Chlorotic areas of pathogen inoculated transgenic lines at 28 dpi. NT: non-transgenic control plants; H, S, P: transgenic lines with *Hrap*, stacked *Hrap-Pflp* and *Pflp* transgenes respectively. Values of the graph bars with similar letters are not significantly different at $P<0.05$.

4.3.3 Characterization of induced resistance

4.3.3.1 Hydrogen peroxide

Hydrogen peroxide was measured in transgenic and non-transgenic control plants after inoculation with *Xcm* at 0, 12, 24, and 48 hpi. At 0 hr, there was no significant difference ($P<0.05$) in hydrogen peroxide production in non-transgenic control plants (1.70±0.68 µM/g FW) and transgenic lines except for two transgenic lines, H-7 (4.79±0.82 µM/ g FW) and P-7 (6.51±0.64 µM/g FW), which had significantly higher amounts than in the non-transgenic control plants (Table 4.8). There was significant increase and peak production of hydrogen peroxide at 12 hpi than at 0 hpi in all transgenic as well as non-transgenic control plants. Production of hydrogen peroxide in all transgenic lines was significantly ($P<0.05$) higher than in non-transgenic control plants (6.35 µM/g FW) at 12 hpi, except in transgenic lines H-5 (6.27 µM/g FW) and H-8 (4.9 µM/g FW) (Table 4.8).

Fold increase in H_2O_2 production from 0 – 12 hr was on average higher in transgenic lines with stacked genes of 11.9-folds in S-8, 13.8-folds in S-2, 7-folds in S-3 in comparison to transgenic lines with single genes as well as non-transgenic control plants (3.7-fold) except for stacked line S-5 with a 3.1-fold increase. Fold increase in H_2O_2 production relative to the non-transgenic control plants at 12 hpi were higher in most transgenic lines S-8 (1.8 fold), S-2 (1.9-fold), S-3 (1.6-fold), S-5 (1.2-fold), H-3 (1.4 fold), H-7 (2.5-fold), P-1 (1.5-fold), P-2 (1.2-fold), P-5 (1.4-fold) and P-6 (2.4-fold) unlike in transgenic lines H-5 (0.9-fold) and H-8 (0.8-fold).

At 24 hpi, hydrogen peroxide accumulation started declining in all transgenic and relative to the non-transgenic control, there was no significant difference in production except in transgenic lines H-5, H-8 and P-6 that had lower production. Hydrogen peroxide production in transgenic lines continued to decline at 48 hpi and in comparison to the non-transgenic control plants, there was no significant difference ($P<0.05$) in production except for lines S-5 and P-5 that had higher production. Furthermore, there was no significant difference in production of hydrogen peroxide in transgenic lines expressing stacked and single genes at 12 hpi, except for H5 and H8 transgenic lines that had significantly low production at 12 hpi.

Some transgenic lines had higher amounts of hydrogen peroxide at the beginning of the experiment (0 hr), which could possibly be due to transgene expression effect; however further studies involving a number of transgenic lines should be conducted to verify this hypothesis. When actual amounts of hydrogen peroxide produced over 48 hr were measured, all transgenic lines produced more hydrogen peroxide in comparison to non-transgenic control, except for transgenic lines H-5 and H-8 (Table 4.8).

Histochemical staining using DAB also confirmed production of hydrogen peroxide in artificially inoculated transgenic lines with *Xcm.* Dark-brown stained hydrogen peroxide was detected in pathogen-inoculated tissues in transgenic and non-transgenic control plants, and the dark brown stained H_2O_2 were more intense in transgenic than in non-transgenic control plants (Fig. 4.9). In both transgenic and non-transgenic control plants, brown stains were not detected in water inoculated (mock) plants (Fig. 4.9) indicating that brown stained hydrogen peroxide was due to pathogen infection and not injury during inoculation.

Table 4.8: Hydrogen peroxide production in transgenic lines and non-transgenic control plants

Line	0 hr	12 hr	24 hr	48 hr	Fold Increase (0- 12 hr)	Total Production in 48 hr
NT	**1.70±0.68^{a}**	**6.35±0.28^{a‡}**	**4.94±0.54^{a}**	**1.58±0.16^{a}**	**3.7**	**194.2**
S-8	0.99±0.03^{a}	11.8±0.62^{b‡}	3.85±1.29^{a}	2.68±0.62^{a}	11.9	248.9
S-2	0.89±0.20^{a}	12.3±0.98^{b‡}	2.44±0.35^{a}	2.77±0.44^{a}	13.8	230.0
S-3	1.44±0.77^{a}	10.11±0.75^{b‡}	2.60±0.16^{a}	2.02±0.54^{a}	7.0	200.9
S-5	2.36±0.68^{a}	7.37±0.98^{b‡}	6.57±1.97^{a}	5.81±0.89^{b}	3.1	290.5
H-3	3.69±1.86^{a}	8.54±0.49^{b‡}	4.32±0.68^{a}	3.07±0.78^{a}	2.3	261.2
H-5	3.47±0.79^{a}	6.27±0.47^{a‡}	1.11±0.51^{b}	1.44±0.09^{a}	1.8	133.3
H-7	4.79±0.82^{b}	15.9±1.95^{b‡}	6.98±1.33^{a}	3.85±0.85^{a}	3.3	388.3
H-8	1.55±0.46^{a}	4.94±1.48^{a‡}	0.80±0.41^{b}	0.44±0.32^{a}	3.2	88.1
P-2	2.83±1.41^{a}	7.29±1.44^{b‡}	4.79±0.68^{a}	2.75±0.56^{a}	2.6	273.0
P-1	3.46±0.55^{a}	9.25±0.36^{b‡}	6.59±1.31^{a}	1.89±0.67^{a}	2.7	223.7
P-5	3.85±0.75^{a}	8.93±0.55^{b‡}	4.08±0.71^{a}	4.40±0.61^{b}	2.3	256.5
P-6	6.51±0.64^{b}	14.9±2.39^{b‡}	1.03±0.27^{b}	1.03±0.23^{a}	2.3	248.8

Values represents means of 6 individual plants ± SE of the mean. Values in a column with similar letters are not significantly different at P = 0.05. Values with ‡ are significantly different at P = 0.05 across a row. NT, S, H and P represent non-transgenic control, stacked, single *Hrap* and single *Pflp* lines respectively. Total production over time calculated from individual productions at regular time intervals (12, 24, 48 hr).

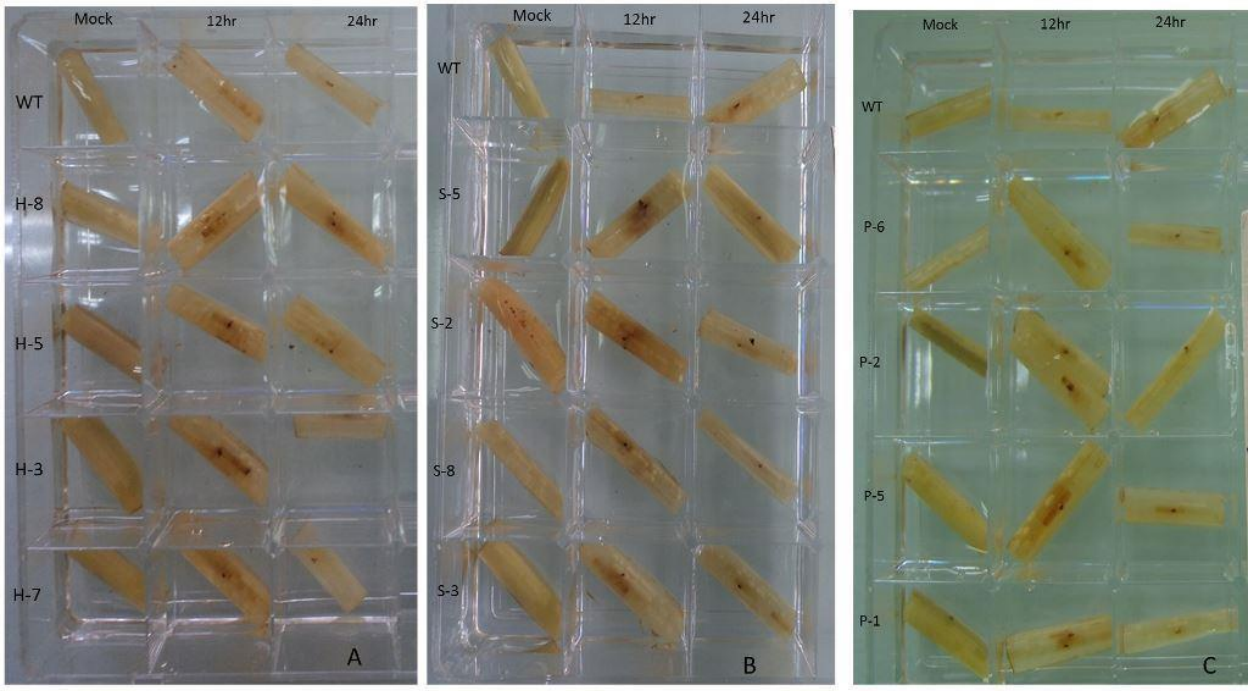

Figure 4.9: Histochemical staining of hydrogen peroxide using DAB in transgenic lines following infection with *Xcm*. A: *Hrap* lines (H); B: stacked *Hrap-Pflp* lines (S), C: *Pflp* lines (P), WT: non-transgenic control at 12 and 24 hr post inoculation in comparison to mock (water) inoculated.

4.3.4 RT-PCR analysis

Furthermore, relative expression of *Hrap* and *Pflp* transgenes was determined in representative transgenic lines with single *Hrap*, single *Pflp* or stacked genes to ascertain whether resistance to *Xcm* was additive. Transcripts of transgenes were not detected in the non-transgenic control plants unlike in transgenic lines (Fig. 4.10). Resistant *Hrap* transgenic lines, H5 and H8 had more transcripts than susceptible lines H3and H7. In *Pflp* transgenic lines, there was no direct correlation between amount of transcripts and disease phenotype. Resistant stacked lines S2 and S5 had more transcripts of *Hrap* than *Pflp* whereas susceptible lines S3 and S8 had more

transcripts of *Pflp* than *Hrap*. Transgenic lines with stacked genes had significantly lower amounts of transcripts of both transgenes compared to the transgenic lines with single genes (Fig. 4.10). The results suggest that there was no correlation between the total amount of transcripts and resistance phenotype in transgenic lines with single *Pflp* transgene unlike in transgenic lines with single *Hrap* where higher amount of transcripts correlated with resistance phenotype.

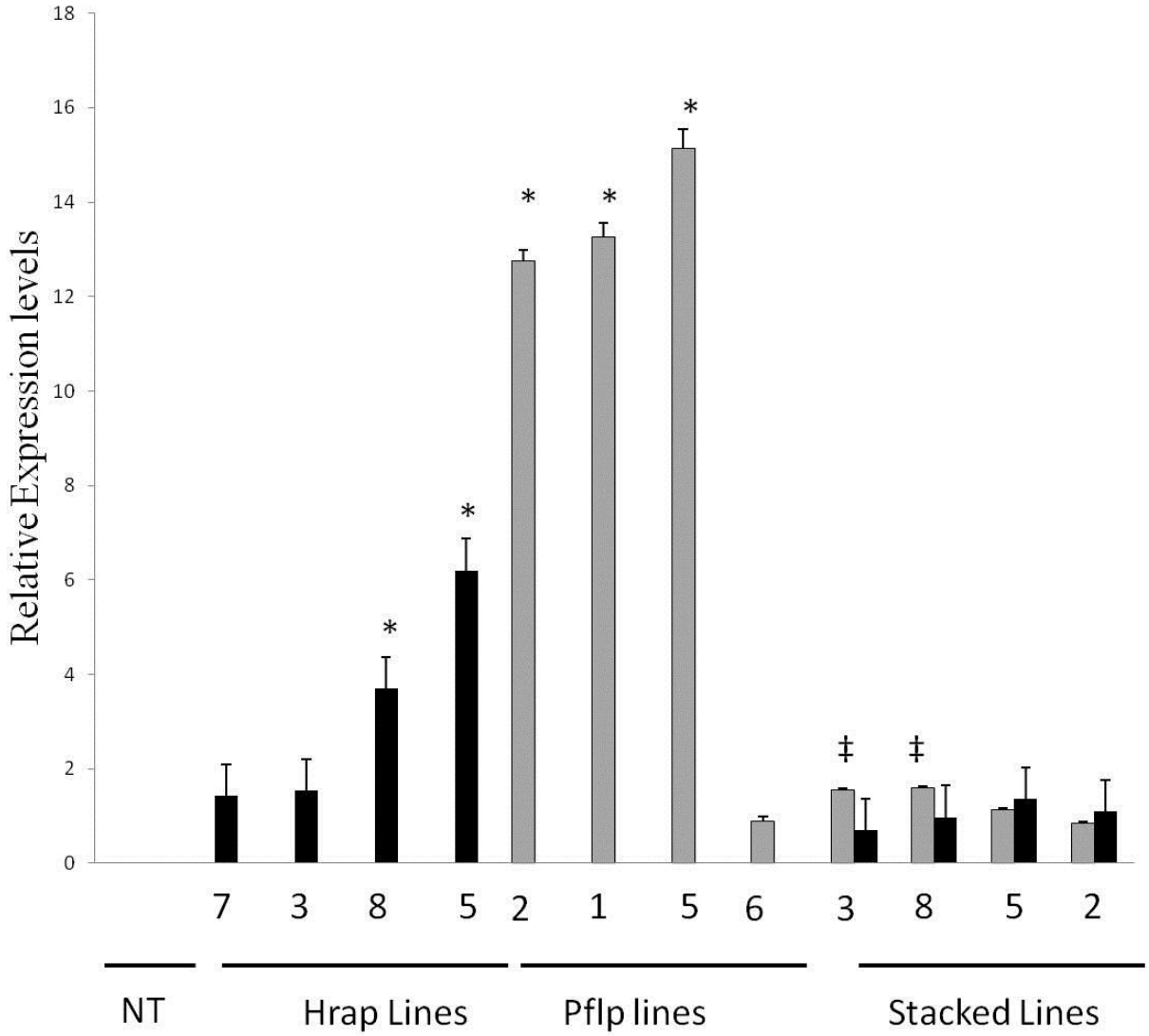

Figure 4.10: Relative expression levels of *Hrap* and *Pflp* genes in transgenic lines. NT: non-transgenic control plants. Values are the means of 3 individual plants ± SE of the mean. Values of graph bars with an asterisk are significantly different compared to stacked lines at P = 0.05. Values of graph bars with ‡ are significantly different compared to the other transgene in the same stacked line at P = 0.05.

4.4 Discussion

Banana is a vegetative propagated plant. Growth evaluation and disease analysis both required a total of 16 clones, or replicates, from a transgenic line. Such high number of clones is difficult to achieve from one transgenic line because of slow plantlet regeneration and bud production *in vitro*. This would also take a considerable time not achievable in the time frame of this PhD study. Growth analyses were therefore done separately on lines carrying the the transgenes but were not further used for pathogen treatment or gene expression analysis. Transformation using an empty vector pBI121 carrying the *gus* gene was also done and about 30 transgenic lines with the *gus* gene inserted into the banana genome were generated. However, since some clones were lost in tissue culture due to contamination thus the required number for growth evaluation was not obtained as was the case for stacked as well as single Hrap and Pflp lines.These few surviving lines had no phenotypic changes in comparison to transgenic lines exprewssing *Hrap* and *Pflp*.

The growth of *Hrap* and *Pflp* expressing transgenic banana plants was first evaluated for any phenotypic growth changes due to the integration and expression of the transgenes. Although reduced growth has been reported in some transgenic plants constitutively expressing genes for disease resistance (Kim *et al*., 2009; Goto *et al*., 2014), transgenic banana plants transformed with stacked genes generally exhibited normal plant growth in this study. Neither plant height and girth width nor leaf number changed due to the integration of *Hrap* and *Pflp* transgenes in the banana genome. In general no morphological variations and plant growth based on the growth parameters measured were observed in transgenic plants in comparison to non-transgenic

control. However one transgenic line notably S-156 had reduced growth characterized by reduced plant height, girth, total number of leaves and root length. This phenotype may have resulted perhaps from insertion mutation caused by the transgene. Since transgenic plants evaluated for phenotypic changes due to the transgenes transformation or expression were only 4 months old they had no fruits and any effect on fruit-bearing could not be carried out.

This part of the study further aimed at evaluating the resistance levels against BXW in transgenic banana plants engineered with stacked *Hrap-Pflp* genes, compared to transgenic plants with single *Hrap* and single *Pflp* genes. Throughout the study, all non-transgenic control plants were highly susceptible to *Xcm* with symptoms manifesting earlier than in transgenic lines, and eventually succumbing to BXW infection, that was signified by complete death. Six lines with single *Hrap*, four lines with single *Pflp* and seven lines with stacked *Hrap-Pflp* genes were completely resistant to *Xcm*.

Overall, the results confirm previous results by Tripathi *et al*. (2010) and Namukwaya *et al*. (2012) that banana lines engineered with the *Hrap* and *Pflp* transgene individually provide resistance against *Xcm* infection with delayed, or no, symptom development. Seven lines with stacked *Hrap-Pflp* genes were completely resistant to *Xcm* in comparison to six lines with individual *Hrap*, and four lines with individual *Pflp*. Furthermore, stacked lines did not have significantly smaller lesions and chlorotic area in comparison to transgenic lines with individual transgene. Averaged lesions and chlorotic area were smaller in transgenic lines with individual *Hrap* than in lines with stacked genes. In this study therefore, lines with stacked transgenes (*Hrap* and *Pflp*), both amplifying the hypersensitive response (HR) but with different individual

action, had no higher resistance levels against *Xcm* compared to lines engineered only with individual *Hrap* transgene.

In general, a transgene stacking approach, as applied in this study, has the potential to either delay rapid breakdown of single gene action providing higher and more durable resistance (Storer *et al*., 2012) or to simultaneously overcome different stress factors limiting plant performance (Douglas and Halpin, 2009; Naqvi *et al*., 2009). Reported ectopic overexpression of a protease inhibitor together with a chitinase in transgenic tomato enhancing nematode resistance is a recently reported successful example (Chen *et al*., 2014). Stacked transgenic lines with low transcripts of each transgene did further not result in higher resistance. Furthermore, some stacked transgenic lines having high transcript amounts of both transgenes even remained susceptible. This result suggests that resistance to *Xcm* in stacked *Hrap-Pflp* lines had no additive effect of each transgene. Therefore, expression of a single transgene was sufficient to provide pathogen protection.

In this study, it was clearly demonstrated by tissue staining that as a defense response, more hydrogen peroxide was produced in all transgenic lines following *Xcm* infection than in non-transgenic control plants. Similarly, enhanced production of hydrogen peroxide after *Xcm* infection was also confirmed using a calorimetric assay. Results obtained showed that indeed transgenic plants produced more hydrogen peroxide over a period of 48 hr than a non-transgenic control plants and had higher hydrogen peroxide production already after 12 hr as a short-term response.

However, transgenic lines H5 and H8 that were found to be resistant to *Xcm* produced less H_2O_2 in comparison to the non-transgenic control plants at 12 hpi. Also results obtained do not support the hypothesis that resistance to Xcm in transgenic banana plants expressing Hrap and Pflp is associated with ROS production. Some susceptible lines had even more H_2O_2 production than resistant lines. Since the *Hrap* and *Pflp* genes are not directly involved in the production of H_2O_2, their expression may therefore not directly correlate with amount of H_2O_2 produced. Also hydrogen peroxide has various roles, depending on its concentration. H_2O_2 productions at low concentration has been reported to act as signaling molecules for a number of physiological processes including resistance to pathogens (Gechev and Hille 2005; Petrov and Van Breusegem, 2012). Hydrogen peroxide collaborates with other signaling molecules in the plant such as nitric oxide, calcium ions and phyto-hormones to provide plant resistance to pathogens (Delledonne *et al*., 2001). Hence observed H_2O_2 concentration per se may not directly result in enhanced resistance to *Xcm* in transgenic *Hrap* and *Pflp* banana plants.

Besides concentration, the downstream biological effect of H_2O_2 was also reported to depend on the site of H_2O_2 production. (Petrov and Van Breusegem, 2012). The HRAP protein localizes to the extracellular matrices whereas PFLP to the chloroplast. Based on localization of these proteins, sites of production of hydrogen peroxide in transgenic banana plants could be different from that in non-transgenic control plants. *In situ* staining of hydrogen peroxide with 2,2′-diaminobenzidine (DAB), as done in this study however, provides only an indication of hydrogen peroxide produced and DAB oxidation further relies on *in vivo* peroxidases activity, this staining technique also provides no indication about the exact cellular location of hydrogen peroxide production (Thordal-Christensen *et al*., 1997). Further studies are recommended to

elucidate the site of H_2O_2 production in *Hrap* and *Pflp* transgenic banana plants if it is possibly responsible for observed resistance against *Xcm*. Further studies on nitric oxide, Ca^{2+} and phytohormones quantification assays should be done to underpin their possible collaborative role with H_2O_2 in resistance to *Xcm* in *Hrap* and *Pflp* transgenic bananas.

Enhanced production of hydrogen peroxide due to *Hrap* and *Pflp* transgene expression in transgenic lines has previously also been reported for rice and tobacco after challenge with bacterial pathogens (Ger *et al*., 2014). Hydrogen peroxide production as an immediate defense response to *Xcm* infection can be involved in rapid lignification of cell walls (Ros Barceló, 2005). Callose deposition strengthening the cell wall at sites of pathogen infection (Chisholm *et al*., 2006) whereby provides better protection against a pathogen.

There was, however, no strong evidence to establish any direct relation between amount of hydrogen peroxide produced and resistance to *Xanthomonas campestris* pv. *musacearum* since different transgenic lines produced significantly different amounts of hydrogen peroxide. One must, however, also be cautious with this type of quantitative data, because hydrogen peroxide assays have the potential problem of accurate hydrogen peroxide extraction and artifactual assay effects (Queval *et al*., 2008).

CHAPTER FIVE

DEFENSE RELATED GENES EXPRESSION IN TRANSGENIC BANANA PLANTS

5.1 Introduction

Plants have many defense mechanisms to protect themselves against invading pathogens and these defense responses are activated upon recognition of the invading pathogen (Naidoo *et al*., 2013). The pathogen induced plant hypersensitive response (HR) is a rapid and highly localized cell death required for neutralizing any invading pathogen. The HR starts a few hours after the pathogen enters the cell with production of hydrogen peroxide as the most stable reactive oxygen species (ROS), and considered as one of the earliest events during HR (Sutherland, 1991). However hydrogen peroxide has a dual role, being toxic in high concentrations and acting as a signaling molecule at low concentrations for adjusting cells to changed environmental conditions (Petrov and Van Breusegem, 2012; Noctor *et al*., 2014).

By using the microarray technology, hundreds of genes have been identified to be up- and down-regulated in both compatible and incompatible plant pathogen interactions. Some are involved in transcriptional regulation, signal transduction and various metabolic activities. Pathogenesis related (PR) proteins, which confer significant protection against pathogens (Senda and Ogawa, 2004; De Vos *et al*., 2005) are further activated by signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). A number of PR proteins are associated with resistance reactions of plants to various pathogens, some PR proteins are hydrolytic enzymes with either β-1,3-glucanase or chitinase activity while others are defensins with antimicrobial activities (Spoel and Dong, 2012). Distinct sets of PR proteins are further induced in response to different pathogens; *PR1*, *PR2* (a β-1,3-glucanase) and *PR5* (thaumatin) are induced by salicylic acid in response to infection with a biotrophic pathogen, whereas *PR3* (chitinase), *PR4*

(chitinase) and *PR12* (defensin) are induced by jasmonic acid in response to infection with a necrotrophic pathogen (Glazebrook, 2005). Expression of *PR1* is often also used as a marker of the systemic acquired resistance (SAR) dependent on the SA pathway (Kunkel and Brooks, 2002; Delaure *et al*., 2008).

The *Non-expressor of pathogenesis related 1* (NPR1) gene from *Arabidopsis* is also involved in the plant defense against pathogens (Endah *et al*., 2010). *NPR1* very likely acts downstream of ROS produced as well as SA, JA, ET and other phyto-hormones involved in the defense signaling cascade ultimately leading to PR induction (Endah *et al*., 2010). Two *NPR1* homologs, *NPR1A* and *NPR1B* have been found to be expressed with *NPR1A* responding to necrotrophs and *NPR1B* to biotrophs. In addition, induction of glutathione S-transferase (*GST*) in response to pathogen infection has been demonstrated (Lieberherr *et al*., 2001; Shahrtash, 2013). GSTs are dimeric enzymes that catalyze the conjugation of electrophilic molecules to glutathione (GSH) (Dean *et al*., 2005). Glutathione-deficient mutants in *Arabidopsis* were found to be highly susceptible to pathogens (Dubreuil-Maurizi and Poinssot, 2012). GSTs can also function as carriers of auxin and phenylpropanoids (Mueller *et al*., 2000; Kitamura and Tanaka, 2004) as well as acting as signaling molecules activating phenylpropanoid metabolism following exposure to UV light (Loyall *et al*., 2000). GSTs further have glutathione peroxidase activity protecting the cell from oxidative injury by detoxifying organic hydro-peroxides of fatty and nucleic acids (Dixon *et al*., 2002).

This study was undertaken to investigate if *PR1, PR3, NPR1,* and *GST* genes are induced in *Hrap* and *Pflp* transgenic banana plants of cultivar “Gonja manjaya” as possible protectants

against *Xanthomonas campestris* pv. *musacearum*. To my knowledge, this is the first study on defense-related genes expression in *Hrap* and *Pflp* transgenic bananas, aimed at possible correlation of defense genes expression with enhanced resistance against *Xcm*.

5.2 Materials and Methods

5.2.1 Plant materials and inoculation

Plants of four transgenic banana lines of cultivar "Gonja manjaya" each with stacked *Hrap-Pflp* genes (S2, S3, S5 and S8), single *Hrap* gene (H3, H5, H7 and H8) and single *Pflp* gene (P1, P2, P5, P6) and non-transgenic control plants were weaned as described in section 3.2.3. Two-month old soil potted transgenic and non-transgenic control plants with 4 - 5 open leaves were artificially inoculated with *Xanthomonas campestris* pv. *musacearum* suspension as previously described in section 4.2.1-4.2.2 (Fig. 5.1B). Transgenic and non-transgenic control plants for the control (mock) experiment were inoculated with distilled water. Three biological replicates for each line were inoculated with either pathogen or water. Leaf samples were collected at 0, 12, 24, 48 hr post inoculation (Fig. 5.1C). Samples were quickly frozen in liquid nitrogen and stored at -80^{0}C until analysis. Leaf samples were pooled from three individual plants at each time point.

Figure 5.1: Glasshouse grown transgenic plants used for qRT-PCR analysis. A: Two-month old soil-potted plants; B: Artificial inoculation of leaf petioles with *Xanthomonas campestris* pv. *musacearum* suspension or sterile water (mocks); C: Sampling of leaves at 0, 12, 24, and 48 hr post-inoculation.

5.2.2 RNA extraction and cDNA synthesis

Frozen leaf samples were ground to fine powder in liquid nitrogen and then total RNA extracted using the CTAB method according to protocol by Yang *et al*., (2008) as previously described in Section 4.2.5.1. Extracted RNA was treated with DNase I to remove any remaining genomic DNA. RNA was verified for absence of any traces of DNA by running a PCR with *Hrap* and *Pflp* gene specific primers. Complimentary DNA (cDNA) was synthesized using 2 µg of DNA-free RNA using the Revertaid First Strand cDNA Synthesis kit (Fermentas #K1622) according to manufacturer's instruction.

5.2.3 Quantitative real time polymerase chain reaction (qRT-PCR)

Primers for qRT-PCR were obtained from genes encoding the MNPR1A, MNPR1B, PR-1, PR-3, and GST proteins. Primer sequences for *MNPR1*, *PR1* and *PR3* (Table 5.1) were identical to sequences previously reported by Endah *et al.* (2008). Primer sequences for GST were designed using gene sequences from the NCBI gene bank (Table 5.1). The *Musa* 25s rRNA was used as an endogenous control gene for qRT-PCR.

Table 5.1: Defense related genes primer sequences used for qRT-PCR

Primer Name	Primer sequence
MNPR1A-forward[a]	5′-GTCGGCATTGTACCAACACA-3′
MNPR1A-reverse[a]	5′-CAGTGCAGGAGTCAGCAAAA-3′
MNPR1B-forward[a]	5′-AGGTTTGCCCGAACAAGAAG-3'
MNPR1B-reverse[a]	5′-TGAGAGGCAACAACTCAGAGAG-3′
PR-1-forward[a]	5′-TCCGGCCTTATTTCACATTC-3′
PR-1-reverse[a]	5′-GCCATCTTCATCATCTGCAA-3′
PR-3-forward[a]	5′-GGCTCTGTGGTTCTGGATGA-3′
PR-3-reverse[a]	5′-CCAACCCTCCATTGATGATG-3′
Musa 25sRNA-forward[a]	5′-ACATTGTCAGGTGGGGAGTT-3′
Musa 25sRNA-reverse[a]	5′-CCTTTTGTTCCACACGAGATT-3′
GST- forward[b]	5′-TAAGAAGCGCTTTGGGATTG-3′
GST- reverse[b]	5′-AATCTAGGCCAACGGTTCCT-3′

[a]primer sequences previously reported by Endah *et al*. (2010); [b] primer sequences designed based on NCBI deposited coding sequences.

qRT-PCR was carried out on leaf samples using a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, UK) following the manufacturer's instructions. A 10 µl reaction for PCR amplification contained 5 µl Maxima SYBR Green/Rox qPCR master mix, 2 µl of the diluted DNA template (1/10), 0.75 µl of 10 µM of forward and reverse primers, and 2.25 µl nuclease-free water. The non-template control (NTC) reaction contained water instead of cDNA. Prepared PCR reactions were added into respective wells of a 96 well RT-PCR micro-titer plate and measurements were performed in triplicate. The qRT-PCR cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 40 cycles consisting of a denaturation step at 94°C for 10 s, primer annealing at 60°C for 20 s and primer extension at 72°C for 10 s. The reaction was finally subjected to a temperature of 65°C for 10 s, and incubated at 95°C for the fluorescence signal of samples to be assessed. Melt curves were done at the start of the experiment to test for specificity of gene amplification.

5.2.4 Data analysis

With the manufacturer's software program (The Applied Biosystems 7900HT Fast Real-Time PCR System) the cycle threshold value (CT) for each sample was determined. Validation experiments for each gene were performed according to the Applied Biosystems user Bulletin No.2 (2001). The activation factor of gene expression was determined with a comparative CT method (Livak and Schmittgen, 2001): $2^{-\Delta\Delta CT}$; with $\Delta\Delta CT = \Delta CT\ (t = T_1\ h) - \Delta CT\ (t = T_0)$ and ΔCT = CT (target) – CT (reference gene) with T_0 was at 0 hr and T_1 at 12, 24 and 48 hr. The 25s rRNA gene was applied as a reference gene. The standard deviation (SD) for each target gene was calculated before calculating the normalized values. Light-cycler data were subjected to

analysis of variance (ANOVA) for significant differences for each treatment and between phenotypes. The Tukey's range test with GenStat software (Version 12.1) was applied for data analysis to find significant differences. The significant threshold value (p) was set at 0.05 and values lower than 0.05 ($p < 0.05$) were considered to be significantly different.

5.3 Results

5.3.1 *NPR1* transcription

Transcription of the two banana *NPR1* homologues, (*MNPR1A* and *MNPR1B*) was investigated in transgenic lines expressing stacked and single *Hrap* and *Pflp* genes. Transcription of the banana *MNPR1A* gene was generally higher in all tested transgenic lines at 12 and 24 hr than in the non-transgenic control (Fig. 5.2A, C, E). The control line only had a significantly higher *MNPR1A* transcription 48 hr after pathogen infection (Fig. 5.2A, C, E). Two *Hrap* lines, two *Pflp* lines and all stacked *Hrap-Pflp* lines had further higher *MNPR1A* transcription than the control at 12 hr after infection (Fig. 5.2A, C, E). However, transcription of *MNPR1A* in mock inoculated transgenic and non-transgenic plants were not significant and in the same range across time points (Fig. 5.2B, D, E).

Higher transcription of *MNPR1B* was also detected in transgenic lines infected with *Xcm*. All *Hrap* lines, *Pflp* lines and one line with stacked transgenes had higher *MNPR1B* transcription at 12 hr after infection (Fig. 5.3A, C, E). Thereafter the trend in transcription declined over time (24 and 48 hr) in transgenic lines (Fig. 5.3A, C, E). The non-transgenic control line also had a

significantly higher *MNPR1B* transcription already 12 hr after pathogen infection which persisted up to 48 hr (Fig. 5.3A, C, E). However, changes in transcription of *MNPR1B* were lower in lines with stacked transgenes when compared to lines expressing individual transgenes. Similarly there was no significant difference in expression of *MNPR1B* in mock inoculated transgenic and non-transgenic lines across time points (Fig. 5.3B, D, F). Transcripts remained relatively the same across time points (Fig. 5.3B, D, F).

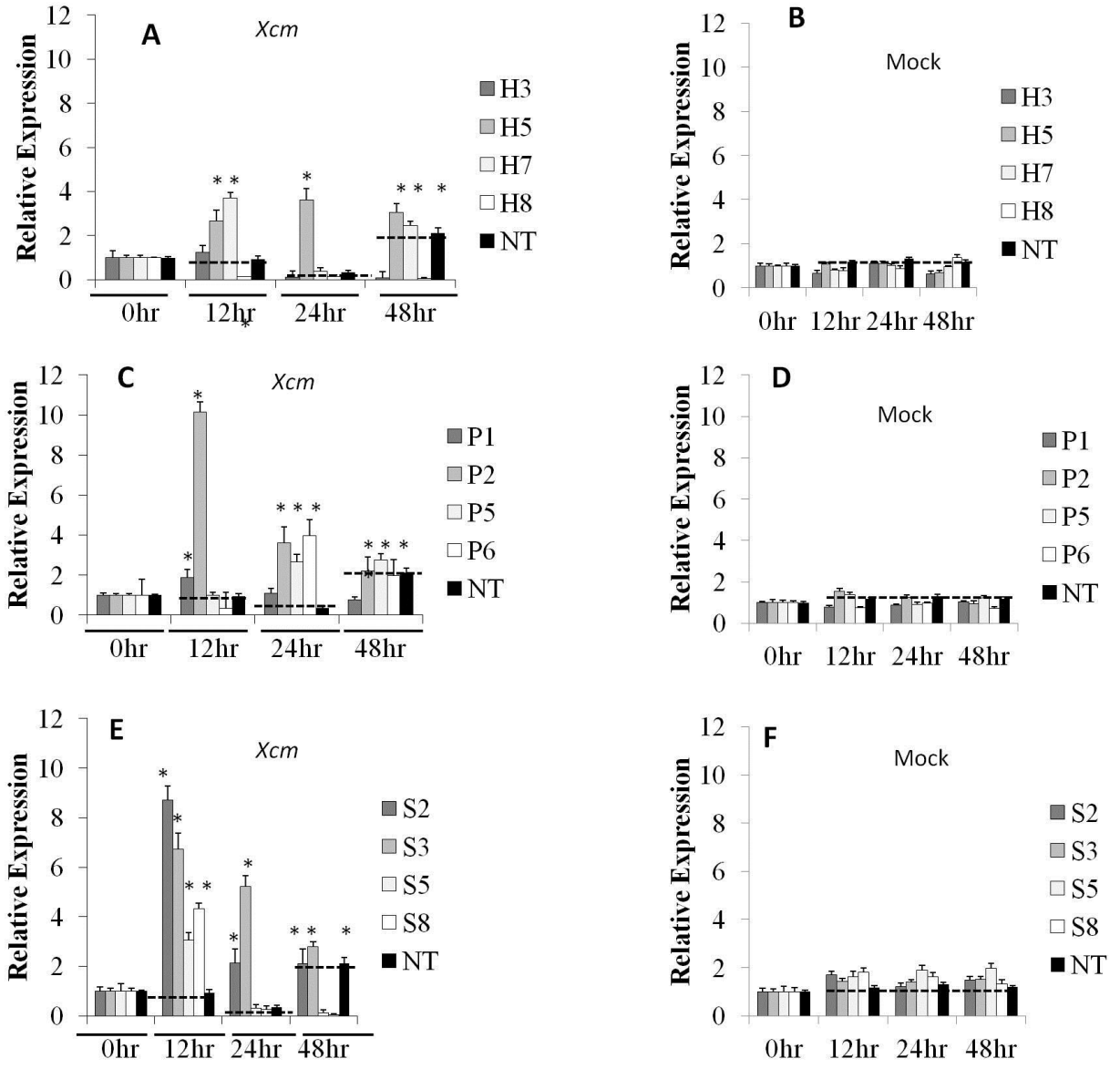

Figure 5.2: Relative gene expression of *MNPR1A* in transgenic and non-transgenic control plants. Relative gene expression was determined and compared to expression at 0 hr (set at 1) in pathogen inoculated (*Xcm*) and sterile water inoculated (Mock) cv. "Gonja manjaya" plants. *Hrap, Pflp*, stacked genes and non-transgenic control lines are designated by H, P, S and NT respectively. Results are means ±SEM of three individual plants per line. *Significant difference at $P<0.05$.

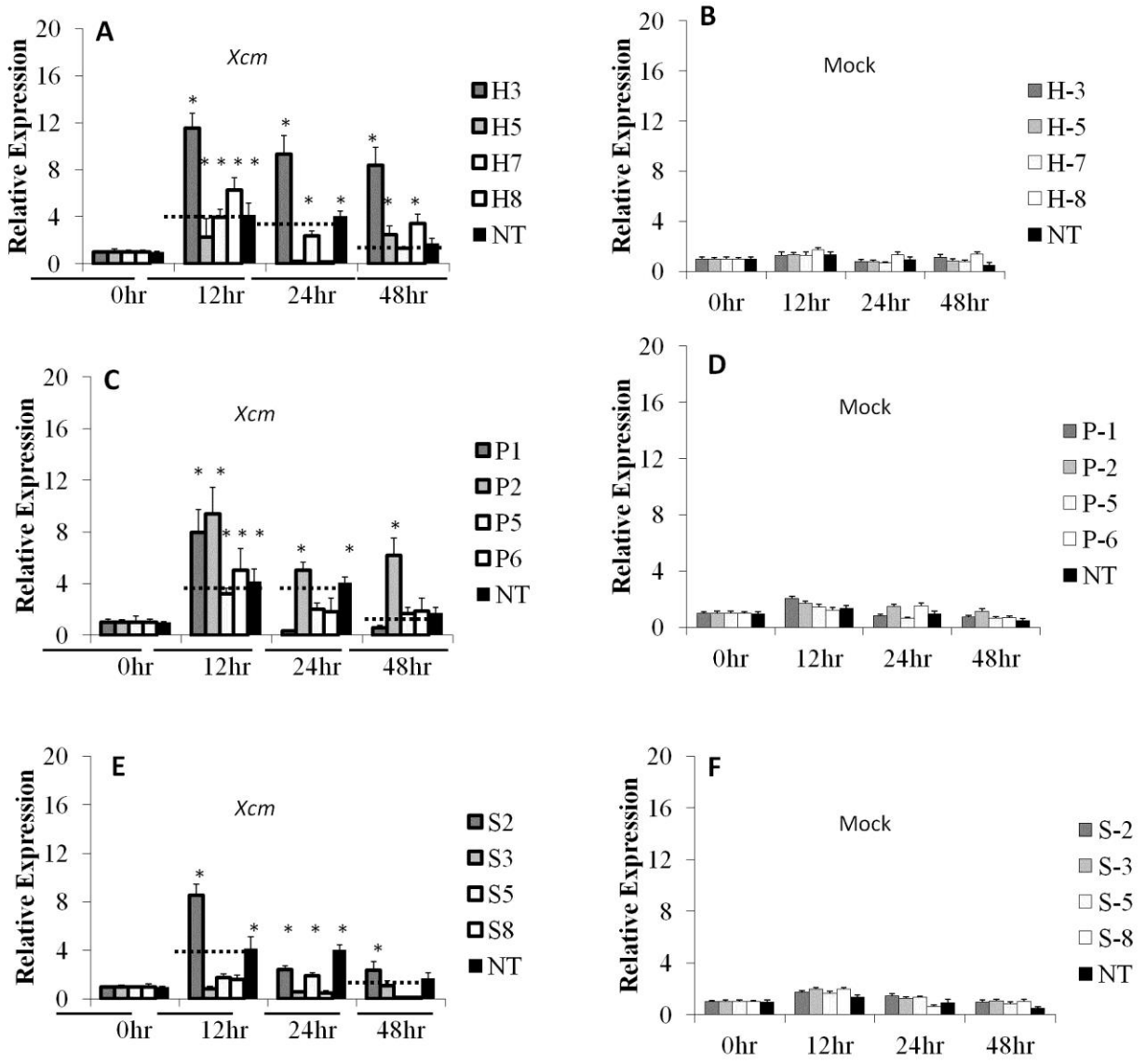

Figure 5.3: Relative gene expression of *MNPR1B* in transgenic and non-transgenic control plants. Relative gene expression was determined and compared to expression at 0 hr (set at 1) in pathogen inoculated (*Xcm*) and sterile water inoculated (Mock) cv. "Gonja manjaya" plants. *Hrap*, *Pflp*, stacked genes and non-transgenic control lines are designated by H, P, S and NT respectively. Results are means ±SEM of three individual plants per line. *Significant difference at $P<0.05$.

5.3.2 Pathogenesis-related (PR) and glutathione S-transferase transcription

Since *NPR1* activates expression of antimicrobial pathogenesis-related (PR) proteins to confer protection against a pathogen, *PR* (*PR-3* and *PR-1*) gene transcription was also measured after *Xcm* infection. *PR-3* transcription was higher in transgenic lines at 12 hr after *Xcm* infection in comparison to the non-transgenic control plants (Fig. 5.4A, C, E). The expression of *PR3* in most of transgenic lines at 12 hr was higher when compared to 24 and 48 hr post inoculation (Fig. 5.4A, C, E). Two *Hrap* line, two *Pflp* lines and two stacked lines transgenes had higher *PR3* transcription in comparison to the non-transgenic control plant at 48 hr (Fig. 5.4A, C, E). Transcription of *PR3* in non-transgenic control plants was not significantly different at 12, 24 and 48 hr after infection. Induction of *PR3* in mock inoculated transgenic and non-transgenic plants was very small and in the same range across time points (Fig. 5.4B, D, F).

In contrast, *PR-1* transcription increased in both transgenic and non-transgenic control plants at 12 hr post inoculation (Fig. 5.5A, C, E). *PR-1* transcription in non-transgenic control plants remained significantly higher at 24 hr than in transgenic lines (Fig. 5.5A, C, E). Although *PR-1* transcription increased due to pathogen infection, except for *Pflp* line (P-6), the increase in transcription in all transgenic lines was not significantly higher in comparison to the non-transgenic control plant. Similarly induction of *PR1* in mock inoculated transgenic and non-transgenic plants was in the same range and no significant difference across time points (Fig. 5.5B, D, F).

Transcription of a gene encoding GST was also measured in response to *Xanthomonas campestris* pv. *musacearum* infection. All *Hrap* and *Pflp* lines and two stacked lines had higher transcription of the *GST* gene than the non-transgenic control after *Xanthomonas* inoculation at 12 hr (Fig. 5.6A, C, E). Transcription thereafter decreased in transgenic and non-transgenic control plant at 24 hr post inoculation. Three *Hrap* lines, one *Pflp* line and two stacked lines had higher transcription of the *GST* gene in comparison to non-transgenic control plant at 48 hr after inoculation (Fig. 5.6A, C, E). Similarly transcription of *GST* in non-transgenic control plants was not significantly different at 12, 24 and 48 hr after infection Expression of *GST* in mock inoculated transgenic and non-transgenic plants was not significantly different across time points (Fig 5.6B, D, F).

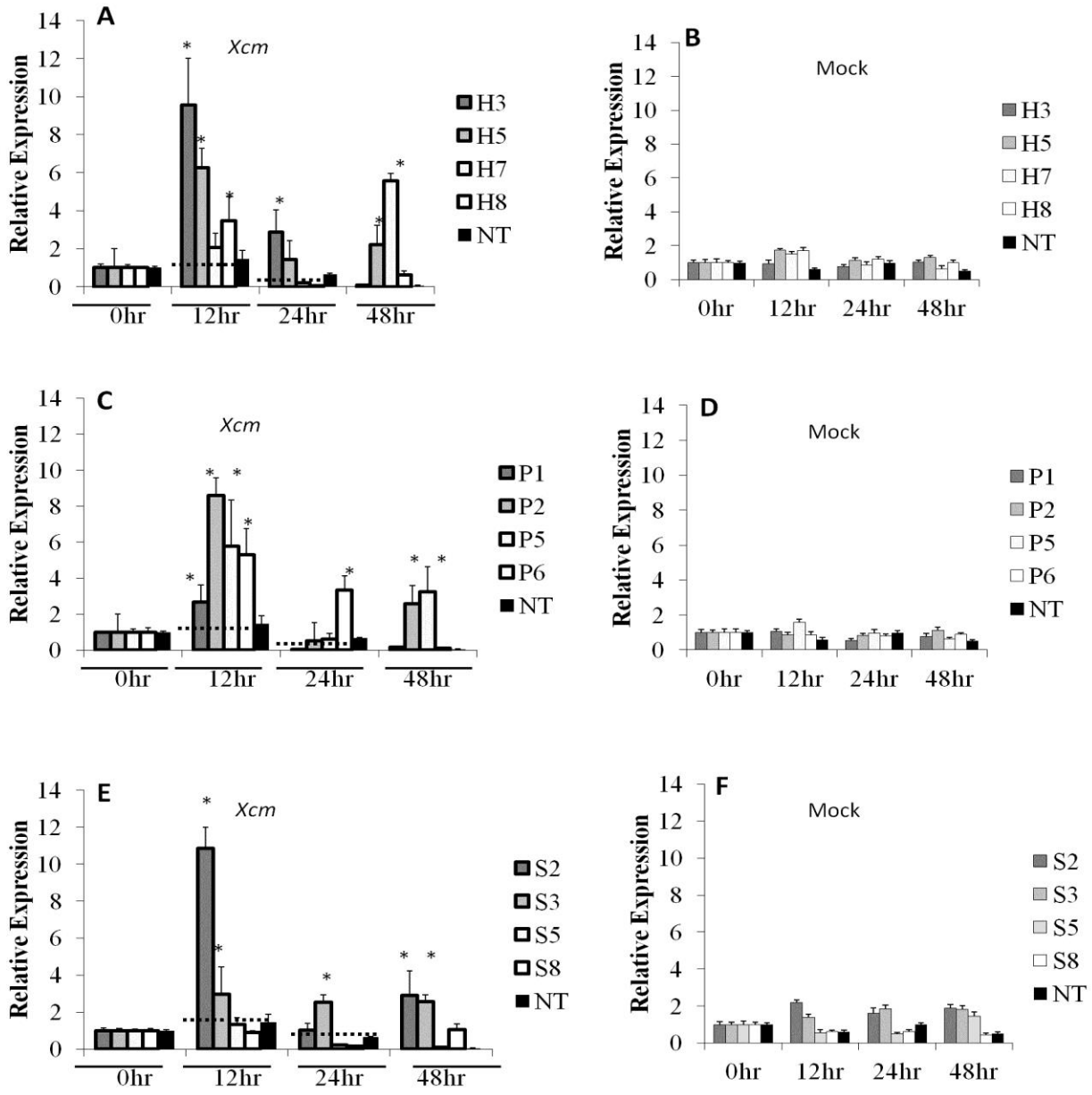

Figure 5.4: Relative gene expression of *PR3* in transgenic and non-transgenic control plants. Relative gene expression was determined and compared to expression at 0 hr (set at 1) in pathogen inoculated (*Xcm*) and sterile water inoculated (Mock) cv. "Gonja manjaya" plants. *Hrap, Pflp,* stacked genes and non-transgenic control lines are designated by H, P, S and NT respectively. Results are means ±SEM of three individual plants per line. *Significant difference at $P<0.05$.

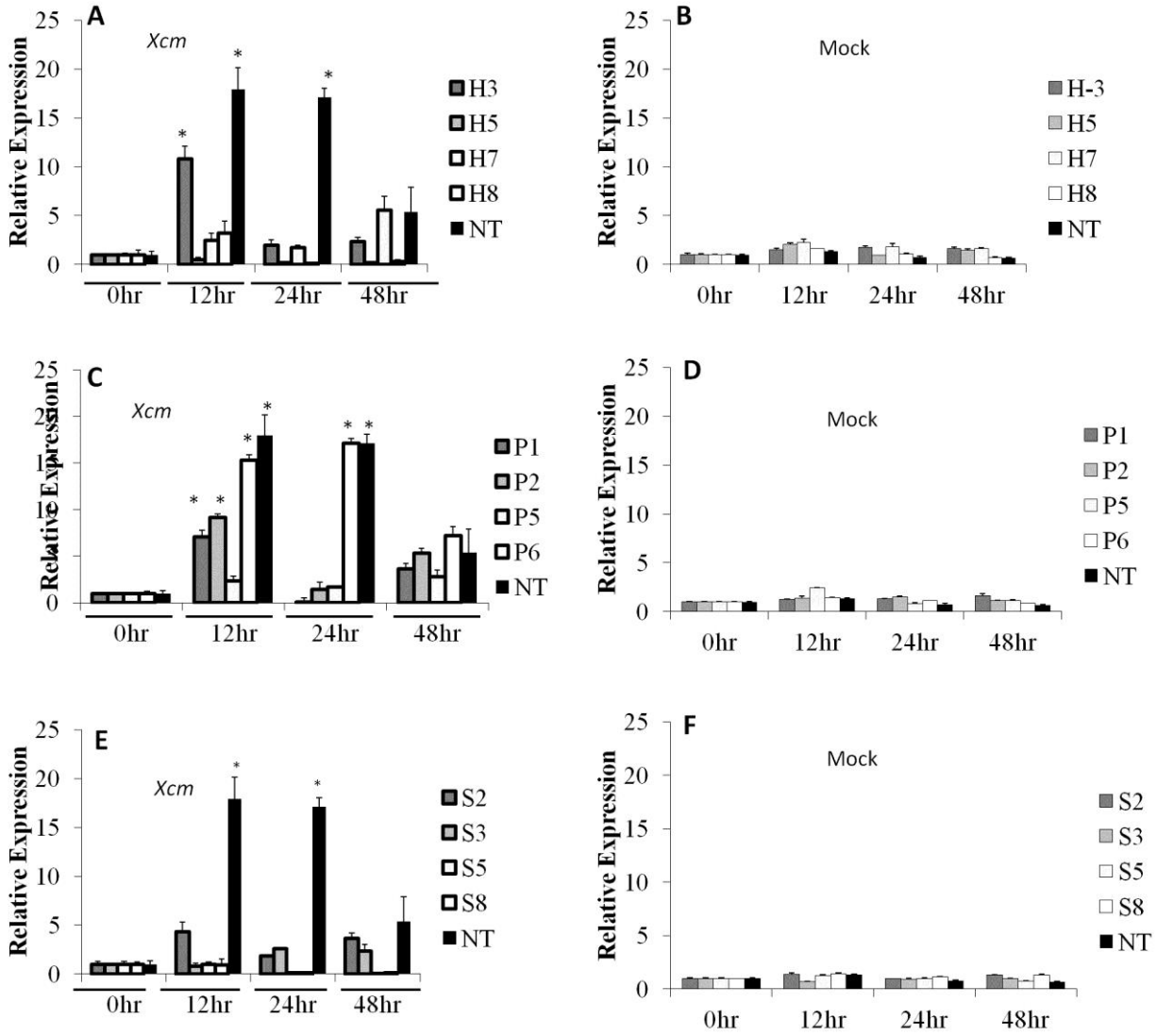

Figure 5.5: Relative gene expression of *PR1* in transgenic and non-transgenic control plants. Relative gene expression was determined and compared to expression at 0 hr (set at 1) in pathogen inoculated (*Xcm*) and sterile water inoculated (Mock) cv. "Gonja manjaya" plants. *Hrap*, *Pflp*, stacked genes and non-transgenic control lines are designated by H, P, S and NT respectively. Results are means ±SEM of three individual plants per line. *Significant difference at $P<0.05$.

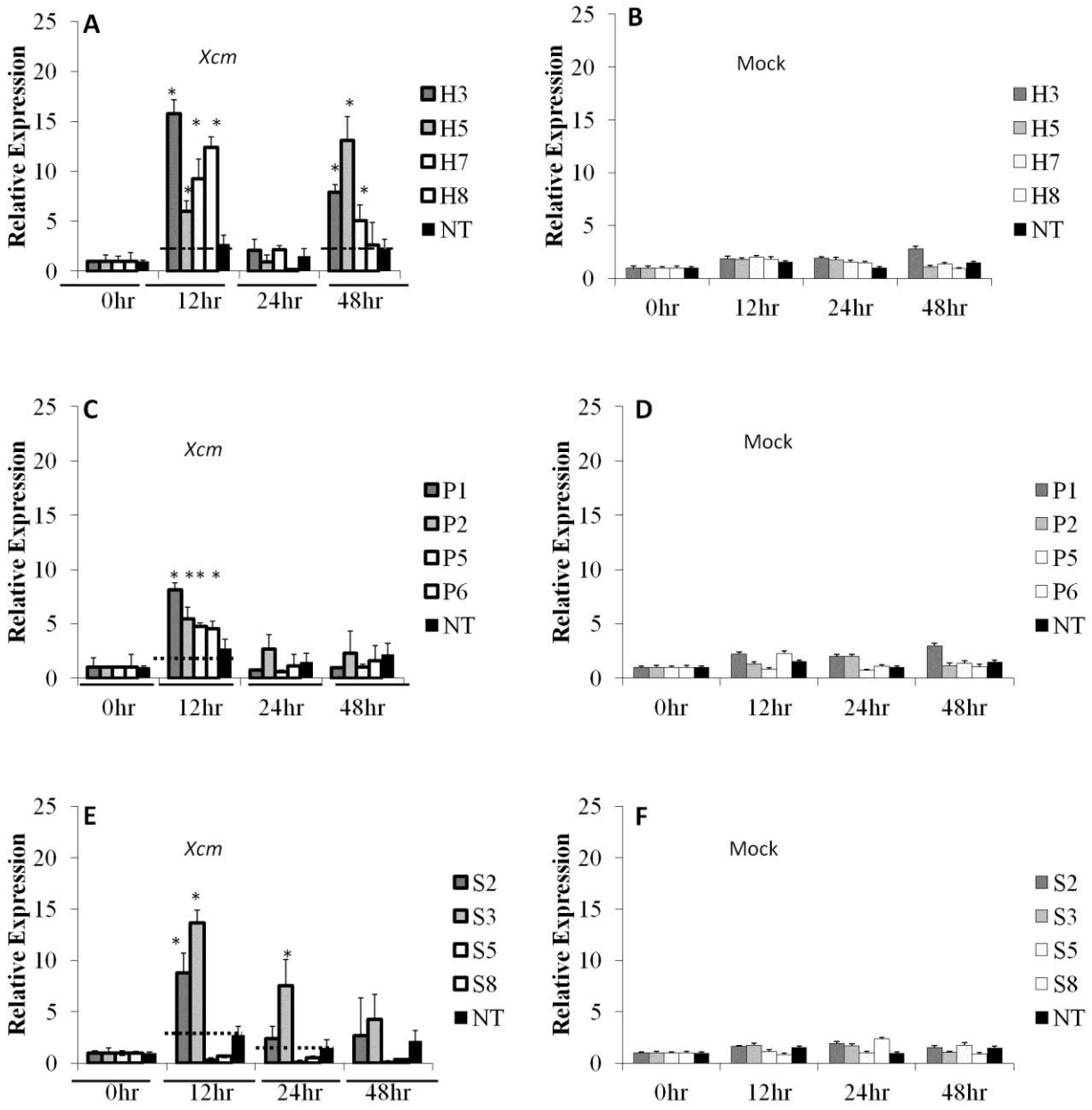

Figure 5.6: Relative gene expression of *GST* in transgenic and non-transgenic control plants. Relative gene expression was determined and compared to expression at 0 hr (set at 1) in pathogen inoculated (*Xcm*) and sterile water inoculated (Mock) cv. "Gonja manjaya" plants. *Hrap*, *Pflp*, stacked genes and non-transgenic control lines are designated by H, P, S and NT respectively. Results are means ±SEM of three individual plants per line. *Significant difference at $P<0.05$.

5.4 Discussion

In this part of the study it was demonstrated that *Xanthomonas campestris* pv. *musacearum* infection was associated with expression of the *NPR1* gene(s). This confirms previous results by Endah *et al*. (2010) that *Xanthomonas campestris* pv. *musacearum* induced transcription of both *MNPR1A* and *MNPR1B* genes in banana cultivar GCTCV-218. The NPR1 is a defense response co-transcriptor conferring resistance to pathogens, which very likely also contributes to *Xanthomonas campestris* pv. *musacearum* resistance in transgenic banana. Both banana *NPR1* genes (*MNPR1A* and *MNPR1B*) were transcribed following infection, but there was lower transcription of *MNPR1B* in lines with stacked genes. The NPR1 protein is redox-sensitive during pathogen attack and the NPR1 protein is reduced to its monomeric form before translocation to the nucleus for establishment of systemic acquired resistance (SAR). The required dissipation of disulfide bonds in the NPR1 protein involves ROS (Chern *et al*., 2001; Cumming *et al*., 2004). The potential of the NPR1 protein protecting against a pathogen has been previously demonstrated with *Arabidopsis NPR1* overexpressing in carrots where transgenic plants provided a broad-spectrum disease resistance to both necrotrophic and also biotrophic pathogens (Wally *et al*., 2009). *NPR1* gene transcription, due to *Xanthomonas* infection, further varied in this study. All transgenic lines had either increased transcription of both *NPR1* genes or higher transcription of at least one of the two *NPR1* genes in 12 h after infection. Stacked (S) lines had more induction of *MNPR1A* than *MNPR1B, Hrap* (H) and *Pflp* (P) lines had more induction of *MNPR1B* than *MNPR1A* whereas non-transgenic (NT) plants had early and more induction of *MNPR1B* than *MNPR1A*. Since NPR1 is a redox sensitive protein whose disulfide bonds formation and dissipation is regulated possibly involves ROS (Mou *et al*., 2003),

transgenic lines with higher transcription of *NPR1* genes could be having a more durable hydrogen production in contrast to transgenic lines with low transcripts. Stronger evidence however has to be provided to support this idea.

A direct relation between *NPR1* gene transcription and induction of *PR-3* gene expression was also found. The *PR-3* gene was more expressed in transgenic lines than in the non-transgenic control line. Transgene stacking provided, however, no advantage regarding amounts of *PR* gene transcripts (*PR-3* and *PR-1*) produced. Both banana *NPR1* genes have been reported to be equally active in *Arabidopsis* mutants to induce *PR-1* gene expression and restoring a pathogen-resistant phenotype (Endah *et al*., 2010). In the non-transgenic line, transcription of the *PR-1* gene was, as expected, more induced than transcription of the *PR-3* gene. The *PR-1* gene encodes a glucanase and is indicative for the salicylic pathway and establishment of SAR when a plant is infected with a biotrophic pathogen such as *Xanthomonas campestris* pv. *musacearum* (Robert-Seilaniantz *et al*., 2007). In contrast, *PR-3* gene expression, with the *PR-3* gene encoding a chitinase, is indicative for the jasmonic acid signaling pathway. The *PR-3* gene is particularly induced in response to necrotizing pathogens (Glazebrook, 2005). *PR-1* gene transcription was even suppressed in most of the transgenic lines despite that higher *PR1*-gene expression should be expected after infection with a biotrophic pathogen like *Xanthomonas campestris* pv. *musacearum*. In contrast, *PR-3* gene expression, indicative for infection with a necrotrophic pathogen, was higher in all transgenic plants than in the non-transgenic control, except for the two stacked lines (S5 and S8). The higher *PR-3* gene expression, suggests that amplifying the oxidative burst in transgenic lines has possibly shifted the response normally found against a biotrophic pathogen (*Xanthomonas* Spp) to a response more related to a necrotizing pathogen.

Future studies have, however, to demonstrate if such a shift generally occurs in transgenic lines with stacked genes by testing more lines and also more evidence has to provide if such shift would offer the advantage of improved protection against a biotrophic pathogen.

Transcription of a gene encoding GST as a response to *Xanthomonas campestris* pv. *musacearum* infection was further measured. The majority of transgenic lines had higher transcription for *GST* than the non-transgenic control. Genes for GSTs are among the most responsive genes to stress and chemical signaling treatments (Glombitza *et al*., 2004). This also includes response to oxidative stress due to ROS production (Noctor *et al*., 2014) and increased ROS production was indeed related to higher transcription of a gene encoding GST except in transgenic lines H5 and H8.

Other signaling molecules namely SA, ethylene, JA and auxin have also been implicated in the induction of *GST* genes besides H_2O_2 following infection with pathogens (Lieberherr *et al*., 2003). Ethylene production during *Colletotrichum destructivum* infection in *N. tabacum* and treatment of *Arabidopsis thaliana* with ethylene rapidly induced rapid expression of *GST*s (Shahrtash, 2013). This could perhaps explain why transgenic banana lines H5 and H8 exhibited higher induction of *GST* yet they produced lower H_2O_2 after infection with *Xcm*. Other signaling molecules may be involved in the induction of *GST* in *Hrap* and *Pflp* transgenic banana plants following infection with *Xcm* besides ROS. Quantification of SA, JA, ethylene or analysis of their respective markers genes are recommended to verify this hypothesis. The exact functions of GSTs remain, however, still elusive and it is for example not clear whether the primary GST function is acting as a conjugase or peroxidase, or both (Dixon *et al*., 2010).

Finally, simply amplifying the oxidative burst by stacking transgenes, as done in this study, might also be problematic because pathogen-triggered nuclear translocation of the NPR1 protein can be prevented by strong accumulation of hydrogen peroxide in the cytosol. This can result in inhibition of NPR1-dependent gene expression ultimately causing less PR protein production required for protection against a pathogen (Peleg-Grossman *et al.*, 2010). Furthermore, amplifying the oxidative burst due to transgene expression possibly shifts the general response of a biotrophic pathogen to a response expected more to a necrotrophic pathogen with more *PR-3* gene expression.

CHAPTER SIX

GENERAL CONCLUSIONS AND FUTURE PROSPECTS

This PhD study has overall confirmed previous results published by Tripathi *et al*. (2010) and Namukwaya *et al*. (2012) that banana lines engineered with individual *Hrap* or *Pflp* genes provide partial or complete resistance against *Xanthomonas campestris* pv. *musacearum* infection. At the beginning of the PhD thesis, it was also hypothesized that expression of stacked *Hrap* and *Pflp* transgenes triggers higher resistance against *Xanthomonas campestris* pv. *musacearum* than single transgene expression. This study provided, as a first new finding, that transgenic banana plants with stacked *Hrap* and *Pflp* transgenes had no higher resistance against *Xanthomonas campestris* pv. *musacearum* than transgenic banana plants with single *Hrap* transgene. Future longer-term studies however, should be carried out to demonstrate any other possible benefits of the stacked transgene strategy. For example, it should be investigated in more detail if a transgene stacking approach provides more durable resistance due to expression of two transgenes. This might avoid rapid loss of single transgene action due to gene silencing and early breakdown of resistance provided by a single transgene.

A further achievement of this PhD study was the successful construction of a transformation plasmid vector allowing the combined expression of the *Hrap* and *Pflp* transgenes in transformed banana. This achievement successfully addressed the first and second set objectives of the study to design a transformation vector with stacked *Hrap* and *Pflp* transgenes under the control of a CaMV 35S promoter and to genetically transform ECS of the banana with the designed plasmid vector construct. Transgenic banana lines were generated after transforming the local banana cultivar “Gonja manjaya” and Sukali ndiizi with either a single *Hrap* or *Pflp* transgene as well as with both transgenes stacked. These lines were used to carrying out both a *Xanthomonas campestris* pv. *musacearum* challenge studies and investigation of expression of endogenous

defense-related genes. A new finding of this study was that banana plants expressing the stacked transgenes were not compromised in their growth characteristics. Initially it was hypothesized that over-expression of stacked *Pflp* and *Hrap* transgenes affects plant growth, but no support for this hypothesis was found.

A further new finding was that hydrogen peroxide was produced in all transgenic banana lines following *Xanthomonas campestris* pv. *musacearum* infection. Hydrogen peroxide, as the most stable ROS, was produced as a consequence to *Xanthomonas campestris* pv. *musacearum* infection. Such enhanced production of hydrogen peroxide, due to *Hrap* and *Pflp* expression, was previously also reported for other plants (Ger *et al*., 2002; Huang *et al*., 2004; Pandey *et al*., 2005). Hydrogen peroxide causes rapid lignification of cell walls whereby providing better protection against a pathogen (Ros Barceló, 2005). In the beginning of the PhD study, it was hypothesized that resistance to *Xanthomonas campestris* pv. *musacearum* in *Hrap* and *Pflp* transgenic banana plants is associated with ROS production. Results obtained do not support this hypothesis. The study provided no strong evidence to establish any direct correlation between amount of hydrogen peroxide produced and resistance phenotype observed, since various types of transgenic lines produced significantly different amounts of hydrogen peroxide. This might be partly due to inefficient method of quantification of hydrogen peroxide. Exact hydrogen peroxide quantification is problematic (Queval *et al*., 2008), as such other hydrogen peroxide measurement techniques might be evaluated in the future to more accurately quantify hydrogen peroxide production. For future studies, other signaling molecules (NO, Ca^{2+} and phytohormones) should be measured or assayed in transgenic banana plants since they work collaboratively with H_2O_2 in eliciting resistance to pathogens. It should be also investigated if

amplifying the oxidative burst with more hydrogen peroxide produced by transgene stacking, as done in this study, could be problematic. Pathogen-triggered nuclear translocation of the NPR1 protein, also measured in this study as a defense response to *Xanthomonas campestris* pv. *musacearum* infection, can, for example, be prevented by cytoplasmic H_2O_2, consequently affecting PR protein production required for pathogen protection (Peleg-Grossman *et al*., 2010).

A further new finding was that *Hrap* and *Pflp* transgene expression mediated expression of *NPR1* genes, which are defense-response co-transcriptors conferring resistance to pathogens, and also other defense-related genes *(PR1, PR3,* and glutathione S-transferase). The transcription of *GST* in response to *Xcm* was generally higher in transgenic banana plants unlike in the non-transgenic plants. This successfully addressed the set objective of determination of relative expression of selected defense-related genes in transgenic bananas plants following *Xanthomonas campestris* pv. *musacearum* infection. However, stacked transgenes did not provide any higher expression of tested *PR1, PR3, GST* and *MNPR1A* genes in comparison to individual transgenes. Results of this study also suggest that amplifying the oxidative burst in transgenic lines possibly shifted the plant response from a biotrophic pathogen, such as *Xanthomonas campestris* pv. *musacearum*, more to a plant response found for necrotising pathogens associated with higher *PR3* gene expression. A future study has, however, to demonstrate by testing more lines if such a shift generally occurs due to *Hrap* and *Pflp* transgene expression. It might also be investigated if such shift would also offer the advantage of improved protection against necrotrophic pathogens in banana such as *Fusarium*. In future, a more detailed study might also investigate if transgene stacking provides a more durable hydrogen production and expression of defense-related genes than provided by lines only transcribing individual

transgenes. There was no correlation between disease phenotype and expression of tested defense related genes; hence expression analysis of additional defense-related genes, besides *PR1, PR3,* and *GST*, should be carried out with existing transgenic plants. In this regard, expression analysis of defense-related protease inhibitors might be carried out. These inhibitors target the gut of the banana weevil, a further major threat to banana production in Uganda (Kiggundu *et al*., 2006).

As an extension of banana transformation, future research might also focus on application of different promoter, replacing the 35S promoter sequence, for expression of either single transgenes or for both transgenes stacked. This would allow investigating transgene expression at specific time points of pathogen infection or expression in particular banana tissues specifically affected by *Xanthomonas*. Already designed constructs might also be useful to transform various other transformable banana varieties as well as to target a wider range of pathogens or even pests. A single *Pflp* transgene has already been reported to impart resistance to *Fusarium* wilt in bananas (Yip *et al*., 2011). The constructed vector in this PhD with stacked *Hrap* and *Pflp* transgenes might therefore also been tested against *Fusarium* to obtain improved resistance against this devastating fungal pathogen. Since the expression of the transgenes also generally increases defense-related gene expression, as shown in this study, transgenic plants might also be tested not only for pathogens but also for pests like nematodes and banana weevil. Finally, future studies should also consider field testing of transgenic plants with stacked transgenes produced in this study to evaluate under field conditions any benefits offered to plants when carrying stacked transgenes. Such field trial of transgenic banana expressing *Hrap* or *Pflp* individually is currently a major activity at IITA and NARO in Uganda.

References

Abele S, Pillay M. 2007. Bacterial wilt and drought stresses in banana production and their impact on economic welfare in Uganda: Implications for banana research in East African highlands. *J. Crop Improv*. **19**: 173-191.

Abouelmaatti R, Elfeil W, Reham Liu S, Wang Y. 2013. Pattern recognition receptors.*Global Animal Sci. J.* **1**: 1118-1127.

Abramovitch RB, Martin GB. 2004. Strategies used by bacterial pathogens to suppress plant defenses. *Curr. Opin. Plant Biol.* **7**: 356–364.

Adikin S. 2010. Detection and spread of *Xanthomonas vasicola* pv. *musacearum* within banana plants. MSc. thesis, Makerere University, Uganda.

Alfano JR, Collmer A. 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* **42**: 385–414.

Ali G, Hadi F, Ali Z, Tariq M, Ali Khan M. 2007. Callus induction and complete plant regeneration of different cultivars of tobacco (*Nicotiana tabacum* L) on media of different hormonal concentration. *Biotech.* **6**: 561-566.

Allan AC, Fluhr R. 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9**: 1559–1572.

Alscher RG, Donahue JH, Cramer CL. 1997. Reactive oxygen species and antioxidants: relationships in green cells. *Physiol. Plant.* **100**: 224–233.

Altschul SF, Madden T, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Res*. **25**: 3389-3402.

Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, Aderem A. 2005. Evasion of toll-like receptor 5 by flagellated bacteria. *Proc. Nat. Acad. Sci. USA* **102**: 9247–9252.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**: 373–399.

Bardini M, Labra M, Winfield M, Sala F. 2003. Antibiotic-induced DNA methylation changes in calluses of *Arabidopsis thaliana*. *Plant Cell Tiss. Org. Cult*. **72**: 157-162

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL. 2000. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain via microprojectile bombardment. *Plant Cell Rep.* **19**: 229–234.

Beers EP, Woffenden BJ, Zhao C. 2000. Plant proteolytic enzymes: possible roles during programmed cell death. *Plant Mol. Biol.* **44**: 399-415.

Belenghi B, Acconcia F, Trovato M, Perazzolli M, Bocedi A, Polticelli F, Ascenzi P, Delledonne M. 2003. AtCYS1, a cystatin from *Arabidopsis thaliana*, suppresses hypersensitive cell death. *Eur. J. Biochem* **270:** 2593–2604.

Bent AF, Mackey D. 2007. Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* **45**: 399–436.

Bhattacharjee S. 2012. The language of reactive oxygen species signaling in plants. *J. Botany*. Article ID 985298, 22 pages. http://dx.doi.org/10.1155/2012/985298

Biruma M, Pillay M, Tripathi L, Blomme G, Abele S, Mwangi M, Bandyopadhyay R, Mucugunzi P, Kassim S, Nyine M, Turyagenda L, Eden-Green S. 2007. Banana *Xanthomonas* wilt: A review of the disease, management strategies and future research directions. *Afr. J. Biotech.* **6**: 953-962.

Bittel P, Robatzek S. 2007. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Curr.Opin. Plant Biol.* **10**: 335-341.

Bolwell GP, Davies DR, Gerrish C, Auh CK, Murphy TM. 1998. Comparative biochemistry of the oxidative burst produced by rose and french bean cells reveals two distinct mechanisms. *Plant Physiol.* **116**: 1379–1385.

Bower R, Elliot AR, Potier AM, Birch RG. 1996. High efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. *Mol. Breed.* **2:** 239–249.

Bradley DJ, Kjellbom P, Lamb CJ. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cellwall protein: A novel, rapid defense response. *Cell* **70**:21-30.

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinase. *Proc. Natl. Acad. Sci. USA.*, **99**: 9328–9333.

Buttner D, Bonas U. 2006. Who comes first? How plant pathogenic bacteria orchestrate type III secretion. *Curr. Opin. Microbiol.* **9**: 193–200.

Cao H, Bowling SA, Gordon AS, Dong X. 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**: 1583–1592.

Cao H, Li X, Dong X. 1998. Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA* **95**: 6531–6536.

Cao J, Zhao JZ, Tang JD, Shelton AM, Earle ED. 2002. Broccoli plants with pyramided *cry1Ac* and *cry1C* bt genes control diamondback moths resistant to Cry1A and Cry1C proteins. *Theor. Appl. Genet*, **105**: 258–264.

Cardoso SC, Mendes JM. 2010. Transgenic sweet orange (Citrus sinensis L. Osbeck) expressing the *attacin A* gene for resistance to *Xanthomonas citri* subsp. *Citri*. *Plant Mol Biol Rep*. **28**:185–192.

Casper-Lindley C, Dahlbeck D, Clark ET, Staskawicz BJ. 2002. Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells. *Proc. Natl. Acad. Sci. USA.*, **99**: 8336–8341.

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA. 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell*, **12**: 323–337.

Chan YL, Lin KH, Sanjaya, Liao LJ, Chen WH, Chan MT. 2005. Gene stacking in phalaenopsis orchid enhances dual tolerance to pathogen attack. *Transgenic Res.* **14**: 279–288.

Chanda B, Xia Y, Mandal MK, Yu K, Sekine KT, Gao QM, Selote D, Hu Y, Stromberg A, Navarre D, Kachroo A, Kachroo P. 2011. Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat Genet*. **43**: 421–427.

Chen CH, Lin HJ, Feng TY. 1998. An amphipathic protein from sweet pepper can dissociate harpin$_{Pss}$ multimeric forms and intensify the harpin$_{Pss}$-mediated hypersensitive response. *Physiol. Mol. Plant Pathol*. **52**: 139–149.

Chen CH, Lin HJ, Ger MJ, Chow D, Feng TY. 2000. cDNA cloning and characterization of a plant protein that may be associated with the harpin$_{pss}$-mediated hypersensitive response. *Plant Mol. Biol*. **43**: 429–438.

Chen WS, Rickertsen K, Tsuboi N, Fu TT. 2002. Consumer acceptance and willingness to pay for genetically modified vegetable oil and salmon: a multiple-country assessment. *AgBioForum* **5**(3): 105-112.

Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bähler J. 2003. Global transcriptional responses of fission yeast to environmental stress. *Mol. Biol. Cell* **14**: 214–229.

Chen PJ, Senthilkumar R, Jane WN, He Y, Tian Z. Yeh KW. 2014. Transplastomic *Nicotiana benthamiana* plants expressing multiple defense genes encoding protease inhibitors and chitinase display broad-spectrum resistance against insects, pathogens and abiotic stresses. *Plant Biotech. J.* **12:** 503–515.

Chern M, Fitzgerald HA, Canlas PE, Navarre DA**,** Ronald PC. 2005. Overexpression of a rice *NPR1* homolog leads to constitutive activation of defense response and hypersensitivity to light. *Mol. Plant Microbe Inter.* **18**: 511–20.

Chern MS, Fitzgerald HA, Yadav RC, Canlas PE, Dong X, Ronald PC. 2001. Evidence for a disease-resistance pathway in rice similar to the *NPR1*-mediated signaling pathway in *Arabidopsis*. *Plant J.* **27**: 101-113.

Chisholm ST, Coaker G, Day B, Staskawicz B. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**: 803–814.

Chou HW. 2005. The study of protein interaction between harpinPss and HRAP by means of truncated *HRAP*. Thesis, National sun yat-sen University, Taiwan. http://etd.lib.nsysu.edu.tw/ETD-db/ETD-search/view_etd?URN=etd-0710106-16452 Assessed 12 January 2010.

Christopher-Kozjan CR, Heath MC. 2003. Cytological and pharmacological evidence that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. *Physiol. Mol. Plant Pathol.***62**:265-275.

Collinge DB, Lund OS, Thordal-Christensen H. 2008. What are the prospects for genetically engineered, disease resistant plants? *Eur. J. Plant Pathol.* **121**: 217 - 231.

Conrath U. 2006. Systemic acquired resistance. *Plant Signal. Behav.* **1**: 179-184.

Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J. Biol. Chem.* **279**: 21749-58.

Cummins JE. 1994. http://www.psrast.org/jccamv.htm. Retrieved on 29th August, 2013.

Cunnac S, Occhialini A, Barberis P, Boucher C, Genin S. 2004. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: Identification of novel effector proteins translocated to plant host cells through the type III secretion system. *Mol. Microbiol.* **53**: 115–128.

Curtis KR, McCluskey JJ, Wahl TI. 2004. Consumer acceptance of genetically modified food products in the developing world. *AgBioForum* **7**: 70-75.

Dangl JL, Jones JDG. 2001. Plant pathogens and integrated defence responses to infection. *Nature*, **411**: 826–833.

Datta K, Baisakh N, Thet KM, Tu J, Datta SK, 2002. Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight. *Theor. Applied Gen.* **106**: 1–8.

Dayakar BV, Lin HJ, Chen CH, Ger MJ, Lee BH, Pai CH, Chow D, Huang HE, Hwang SY, Chung MC, Feng TY. 2003. Ferredoxin from sweetpepper (*Capsicum annuum L.*) intensifying harpin(Pss)-mediated hypersensitive response shows an enhanced production of active oxygen species (AOS). *Plant Mol. Biol.* **51**: 913–924.

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC, Dicke M, Pieterse CM 2005. Signal signature and

transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Inter.***18**: 923-37

Dean JD, Goodwin PH, Hsiang T. 2005. Induction of glutathione S-transferase genes of *Nicotiana benthamiana* following infection by *Colletotrichum destructivum* and *C. orbiculare* and involvement of one in resistance. *J. Exp. Bot*. **56**: 1525-33.

Deepika R, Veale A, Cathleen M, Strauss SH, Myburg A. 2014. Genetic transformation of eucalyptus-challenges and future prospects. In KG Ramawat, J Mérillon, MR. Ahuja (ed). Tree biotechnology. ISBN: 1466597143.

Delaure SL, Hemelrijck WV, Bolle MFCD, Cammue BPA, De-Coninck BMA. 2008. Building up plant defenses by breaking down proteins. *Plant Sci.* **174**: 375–385.

Delledonne M, Zeier J, Marocco A, Lamb C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. USA* **98**: 13454–13459.

Desikan R, Mackerness SAH, Hancock JT, Neil SJ. 2001. Regulation of the *Arabidopsis* transcriptome by oxidative stress. *Plant Physiol.* **127**: 159–172.

Dixon DP, Hawkins T, Hussey PJ, Edwards R., 2010. Enzyme activities and subcellular localization of members of the *Arabidopsis* glutathione transferase superfamily. *J. Exp. Bot.* **60**: 1207–1218.

Dixon DP, Lapthorn A, Edwards R. 2002. Plant glutathione transferases. *Genome Biol.* **3.** http://genomebiology.com/2002/3/3/reviews/3004. Accessed on 10th March, 2014.

Dixon RA. 2001. Natural products and plant disease resistance. *Nature* **411**: 843–847.

Douglas R, Halpin C. 2009. Gene stacking. In: Jain SM, Brar DS. (Eds.), Molecular techniques in crop improvement. Springer, The Netherlands, pp. 613–629.

Dubreuil-Maurizi C, Poinssot B. 2012. Role of glutathione in plant signaling under biotic stress. *Plant Signal. Behav*. **7**: 210–212.

Durrant WE, Dong X. 2004. Systemic acquired resistance. *Ann. Rev. Phytopathol.* **42**: 185–209.

Einsele A. 2007. The gap between science and perception: The case of plant biotechnology in Europe. *Adv. Biochem. Engin./Biotech.* **107**: 1-11.

Endah R, Beyene G, Kiggundu A, Van den Berg N, Schlüter U, Kunert K, Chikwamba R. 2008. Elicitor and *Fusarium*-induced expression of *NPR1*-like genes in banana. *Plant Physiol. Biochem*. **46**: 1007-1014.

Endah R, Coutinho T, Chikwamba R. 2010. .*Xanthomonas campestris* pv. *musacearum* induces sequential expression of two *NPR-1* like genes in banana. Agriculture: Africa's "engine for growth" plant science and biotechnology holds the key, *Aspects Appl. Biol*. **96**: 325-330.

FAOSTAT. 2011. Top production of Bananas for 2011. http://faostat.fao.org. Accessed on 15th September, 2013.

Fitzgerald HA, Chern M, Navarre R, Ronald PC. 2004. Over expression of (*At*) *NPR1* in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Mol. Plant-Microbe Interact*. **17**: 140-151.

Friedrich L, Lawton K, Dietrich R, Willits M, Cade R, Ryals J. 2001. *NIM1* overexpression in *Arabidopsis* potentiates plant disease resistance and results in enhanced effectiveness of fungicides. *Mol. Plant-Microbe Interact*. **14**: 1114–1124.

Friedrich L, Vernooli B, Gaffney T, Morse A, Ryals J. 1995. Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Mol. Biol.* **29**: 959-68.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Sci.* **261**: 754–756.

Gaj M.D. 2004. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) *Plant Growth Regul.* **43:** 27–47**.**

Ganapathi TR, Higgs NS, Balint-Kurti PJ, Arntzen CJ, May GD, Van Eck JM. 2001. Agrobacterium -mediated transformation of embryogenic cell suspensions of the banana cultivar Rasthali (AAB). *Plant Cell Reports.* **20**:157–162

Gasch A, Spellman PT, Kao CM, Carmel-Harel O,Eisen MB, Storz G, Botstein D, Brown PO 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11**: 4241–4257.

Gechev TS, Hille J. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. *J. Cell Bio*. **168**: 17–20.

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol. Mol. Biol. Rev.* **67**: 16–37.

Ger MJ, Louh GY, Lin YH, Feng TY, Huang HE. 2014. Ectopically expressed sweet pepper ferredoxin PFLP enhances disease resistance to *Pectobacterium carotovorum* subsp. *carotovorum* affected by harpin and protease-mediated hypersensitive response in *Arabidopsis*. *Mol. Plant Pathol*. **15**(9): 892–906.

Ger MJ, Chen CH, Hwang SY, Huang HE, Podile AR, Dayakar BV, Feng TY. 2002. Constitutive expression of *Hrap* gene in transgenic tobacco plant enhances resistance

against virulent bacterial pathogens by induction of a hypersensitive response. *Mol. Plant-Microbe Interact.* **15**: 764–773.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Ann. Rev. Phytopathol.* **43**: 205–227

Glombitza S, Dubuis PH, Thulke O, Welzl G, Bovet L, Götz M, et al. 2004. Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. *Plant Mol. Biol.* **54**: 817-835.

Gonzalez JE, York GM, Walker GC. 1996. *Rhizobium* meliloti exopolysaccharides: synthesis and symbiotic function. *Genes* **179**: 141–146.

Goto S, Sasakura-Shimoda F, Suetsugu M, Selvaraj MG, Hayashi N, Yamazaki M, Ishitani M, Shimono M, Sugano S, Matsushita A, Tanabata T, Takatsuji H. 2014. Development of disease-resistant rice by optimized expression of *WRKY45*. *Plant Biotechnol. J*, 10.1111/pbi.12303

Govindarajulu M, Elmore JM, Fester T, Taylor CG. 2008. Evaluation of constitutive viral promoters in transgenic soybean roots and nodules. *Mol. Plant-Microbe Interact.* **21**: 1027–1035.

Hansen G, Martha SW. 1999. Recent advances in the transformation of plants. *Trends Plant Sci.* **4**: 226–231

Heath MC. 2000. Hypersensitive response-related death, in: Lam E, Fukuda H, Greenberg J (Eds), Programmed cell death in higher plants, Springer Netherlands, Pp. 77-90. DOI: 10.1007/978-94-010-0934-8_6.

Huang HE, Ger MJ, Chen CY, Yip MK, Chung MC, Feng TY. 2006. Plant ferredoxin-like protein (*Pflp*) exhibits an anti-microbial ability against soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* in vitro and in vivo. *Plant Sci.* 17–23.

Huang HE, Ger MJ, Chen CY, Yip MK, Pandey AK, Feng TY. 2004. A hypersensitive response was induced by virulent bacteria in transgenic tobacco plants overexpressing a plant ferredoxin-like protein (*Pflp*). *Physiol. Mol. Plant Pathol*. **64**: 103–110.

Huang X, Huang XL, Xiao W, Zhao JT, Dai XM, Chen YF, Li XJ. 2007. Highly efficient *Agrobacterium*-mediated transformation of embryogenic cell suspensions of *Musa acuminate* cv. *Mas* (AA) via a liquid co-cultivation system. *Plant Cell Rep.* **26**: 1755–1762.

Hurley JK, Morales R, Martinez-Julves M, Brodie TB, Medina M, Gomez-Moreno C, *et al*. 2002. Structure-function relationships in anabaena ferredoxin/ferredoxin:NADP(+) reductase electron transfer: insights from site-directed mutagenesis, transient absorption spectroscopy and X-ray crystallography. *Biochim. Biophys. Acta* **1554**: 5-21**.**

Iakimova ET, Michalczuk L, Woltering EJ. 2005. Hypersensitive cell death in plants – its mechanisms and role in plant defence against pathogens. *J. Fruit Ornamental Plant Res.* **13**: 135-158

IITA. (1998). Plantain and banana improvement program-annual report for 1997. *Ann. Report for 1997*: International Institute of Tropical Agriculture, Onne, Nigeria.

IITA. 2008. Banana facts. *R4D Review.* http://r4dreview.org/tag/banana/page/2/

Jing B, Xu S, Xu M, Li Y, Li S, Ding J, Zhang Y. 2011. Brush and spray: a high-throughput systemic acquired resistance assay suitable for large-scale genetic screening. *Plant Physiol.* **157**: 973–980.

Johnson C, Boden E, Arias J. 2003. Salicylic acid and *NPR1* induce the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. *Plant Cell,* **15:** 1846–58.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature* **444**: 323–329.

Jung HW, Tschaplinski TJ, Wang L, Glazebrook J, Greenberg JT. 2009. Priming in systemic plant immunity. *Sci.* **324**: 89–91.

Jung YJ, Choi CS, Park JH, Kang HW, Choi JE, Nou IS, Lee SY, Kang KK. 2008. Overexpression of the pineapple fruit bromelain gene (*BAA*) in transgenic chinese cabbage (*Brassica rapa*) results in enhanced resistance to bacterial soft rot. *Electronic J. Biotech.* **11**: 1-8.

Kalyebara MR, Ragama PE, Kagezi GH, Kubiriba J, Bagamba F, Nankinga KC, Tushemereirwe W. 2006. Economic importance of the banana bacterial wilt in Uganda. *Afr. Crop Sci. J.* **14**: 93-103.

Karamura E, Frison E, Karamura DA, Sharrock S. 1998. Banana production systems in Eastern and Southern Africa. Paper presented at the international symposium: Bananas and food security, 10-14 November Douala, Cameroon. INIBAP, 798.

Keen NT. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Ann. Rev. Genetics* **24**: 447–463.

Khanna H, Becker D, Kleidon J, Dale J. 2004. Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). *Mol. Breed.* **14**: 239–252

Khatib M, Lafitte C, Esquerre´ -Tugaye´ MT, Bottin A, Rickauer M. 2004. The CBEL elicitor of *Phytophthora parasitica* var. *nicotianae* activates defence in *Arabidopsis thaliana* via three different signaling pathways. *New Phytol*. **162**: 501–510.

Kiba A, Takata O, Ohnishi K, Hikichi Y. 2006. Comparative analysis of induction pattern of programmed cell death and defense-related responses during hypersensitive cell death and development of bacterial necrotic leaf spots in eggplant. *Planta.* **224**: 981–994.

Kiggundu A, Goulet M-C, Goulet C, Dubuc J-F, Rivard D, Benchabane M, Pépin G, van der Vyver C, Kunert K, Michaud D. 2006. Modulating the proteinase inhibitory profile of a plant cystatin by single mutations at positively selected amino acid sites. *The Plant J.* **48**: 403-413.

Kikulwe EM. 2010. On the introduction of genetically modified bananas in Uganda: Social benefits, costs, and consumer preferences. PhD thesis, Wageningen University, Wageningen, NL. (ISBN 978-90-8585-610-8).

Kim SH, Kwon S, Bhattacharjee S, Gassmann W, 2009. Regulation of defense gene expression by *Arabidopsis SRFR1*. *Plant Signaling & Behavior*, **4**(2): 149-150

Kitamura SN, Tanaka A. 2004, Transparent testa *19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *Plant J.* **37**: 104-114.

Kjemtrup S, Nimchuk Z, Dangl JL. 2000. Effect proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Curr. Opin. Microbiol.* **3:** 73-78.

Koornneef A, Pieterse CM. 2008. Cross talk in defense signaling. *Plant Physiol.* **146**: 839–844.

Krzymowska M, Konopka-Postupolska D, Sobczak M, Macioszek V, Ellis BE, Hennig J. 2007. Infection of tobacco with different *Pseudomonas syringae* pathovars leads to distinct morphotypes of programmed cell death. *Plant J.* **50**: 253–264.

Kunkel BN, Brooks DM. 2002. Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* **5:** 325–31.

Kurihara T, Mihara H, Kato S, Yoshimura T, Esaki N. 2003. Assembly of iron–sulfur clusters mediated by cysteine desulfurases, IscS, CsdB and CSD, from *Escherichia coli. Biochim: Biophys: Acta.* **1647**: 303–309.

Kusajima M, Yasuda M, Kawashima A, Nojiri H, Yamane H, Nakajima M, Akutsu K.; Nakashita H. 2010. Suppressive effect of abscisic acid on systemic acquired resistance in tobacco plants. *J. Gen. Plant Pathol.* **76**. 161–167.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**: 251–275.

Langhe ED. 1996. Banana and plantain: the earliest fruit crops? *INIBAP*. Focus paper 1: Banana and Plantain: the earliest fruit crops? Pp 6-8 in INIBAP annual report 1995 *INIBAP*, Montpellier, France.

Lee J, Klessig DF, Nürnberger T. 2001. A harpin binding site in tobacco plasma membranes mediates activation of the PR gene *HIN1* independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell.* **13**: 1079-1093.

Levine A, Tenhaken R, Dixon R, Lamb C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell.* **79**: 583–593.

Li HJ, Li XH, Xiao JH, Wing RA, Wang SP. 2012. Ortholog alleles at Xa3/Xa26 locus confer conserved race-specific resistance against *Xanthomonas oryzae* in rice. *Mol. Plant.* **5**(1): 281-90

Li X, Lin H, Zhang W, Zou Y, Zhang J, Tang X, Zhou JM. 2005. Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc. Natl. Acad: Sci. USA,* 12990–12995.

Li Z, Alfenito M, Rea PA, Walbot V, Dixon RA. 1997. Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. *Phytochem.* **45**: 689-693.

Liao W, Ji L, Wang J, Chen Z, Ye M, Ma H, An H. 2014. Identification of glutathione S-transferase genes responding to pathogen infestation in *Populus tomentosa*. *Funct. Genom.* **14**: 517-29**.**

Liau CH, Lu JC, Prasad V, Lee JT, Hsiao HH, et al. 2003. The sweet pepper ferredoxin-like protein (*Pflp*) conferred resistance against soft rot disease in *Oncidium* orchid. *Transgenic Res*. **12**: 329–336.

Lieberherr D. 2001. Analysis of pathogen-induced glutathione S-transferases in *Arabidopsis thaliana* and a gene related to systemic acquired resistance in cucumber *(Cucumis sativus L.)*. Thesis, Université de Fribourg (Suisse).

Lieberherr D, Wagner, Dubuis P, Metraux J, Mauch F. 2003. The rapid induction of glutathione S-transferases *AtGSTF2* and *AtGSTF6* by avirulent *Pseudomonas syringae* is the result of combined salicylic acid and ethylene signaling. *Plant Cell Physiol*. **44**(7):750-757

Lin HJ, Cheng HY, Chen CH, Huang HC, Feng TY. 1997. Amphipathic proteins delay the hypersensitive response caused by harpinpss and *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol.* **51**: 367–376.

Lin WC, Lu CF, Wu JW, Cheng ML, Lin YM, Yang NS, Black L, Green SK, Wang JF, Cheng CP. 2004. Transgenic tomato plants expressing the *Arabidopsis NPR1* gene display

enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* **13**: 567-581.

Lindeberg M, Cartinhour S, Myers CR, Schechter LM, Schneider DJ, Collmer A. 2006. Closing the circle on the discovery of genes encoding Hrp regulon members and type III secretion system effectors in the genomes of three model *Pseudomonas syringae* strains. *Mol. Plant Microbe Interact.* **19**: 1151–1158.

Liu Y, Yang H, Sakanishi A. 2006. Ultrasound: mechanical gene transfer into plant cells by sonoporation. *Biotech. Adv*. **24**:1–16.

Liu Z, Bos JI, Armstrong M, Whisson SC, da Cunha L, Torto-Alalibo T, Win J, Avrova AO, Wright F, Birch PR, Kamoun S. 2005. Patterns of diversifying selection in the phytotoxin-like *scr74* gene family of *Phytophthora infestans*. *Mol. Biol. Evol.* **22**: 659–672.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods*, **25**: 402–408.

Lorenzen J, Tenkouano A, Bandyopadhyay R, Vroh B, Coyne D, Tripathi L. 2010. Overview of banana and plantain (Musa spp.) improvement in Africa: past and future. *Acta Horticulturae, **879**: 595–603.*

Low PS, Merida JR. 1996. The oxidative burst in plant defense: function and signal transduction. *Plant Physiol.* **96**: 533–542.

Loyall L, Uchida K, Braun S, Furuya M, Frohnmeyer H. 2000. Glutathione and a uv light-induced glutathione S-transferase are involved in signalling to chalcone synthase in cell cultures. *Plant J.* **25**: 237–245.

Luna E, Bruce TJA, Roberts MR, Flor V, Ton J. 2012. Next-generation systemic acquired resistance. *Plant Physiol.* **158**: 844-853.

Magambo B. 2012. Generating transgenic banana (cv. *Sukali ndizi*) resistant to *Fusarium* wilt. MSc. thesis, Queensland University of Technology, Australia.

Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J. 2006. Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis NPR1*. *Mol. Plant-Microbe Interact.* **19**: 123-29.

Malamy J, Carr JP, Klessig DF, Raskin I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Sci.* **250**: 1002–1004.

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK. 2002. A putative lipid transfer protein involved in systemic resistance signaling in *Arabidopsis*. *Nature*, **419**: 399–403.

Malnoy M, Jin Q, Borejesza-Wysocka EE, He SY, Aldwinckle HS. 2007. Overexpression of the apple *MpNPR1* gene confers increased disease resistance in *Malus X. domestica. Mol. Plant-Microbe Interact*. **20**: 1568-1580.

Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV et al. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology**.** *Mol. Plant Pathol.* Rev. doi: 10.1111/j.1364-3703.2012.00804.x

Maqbool SB, Riazuddin S, Loc NT, Gatehouse AMR, Gatehouse JA, Christou P. 2001. Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Mol. Breed*. **7**: 85–93.

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ. 1995. Generation of transgenic banana (*Musa accuminata*) plants via *Agrobacterium* mediated transformation. *Biotech.* **13**: 486–492.

Mazouni K, Domain F, Chauvat F, Cassier-Chauvat C. 2003. Expression and regulation of the crucial plant-like ferredoxin of cyanobacteria. *Mol. Microbiol.* **49**: 1019–29.

Mendoza M. 2011. Oxidative burst in plant-pathogen interaction. *Biotecnol. Vegetal.* **11**: 67-75.

Miki B, McHugh S. 2004. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J. Biotech.* **107**: 193–232

Miller G, Shulaev V, Mittler R. 2008. Reactive oxygen signaling and abiotic stress. *Physiol. Plant.* **133**: 481–489.

Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.* **178**: 1310–1319.

Mishina TE, Zeier J. 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis*. *Plant J.* **50**: 500–513.

Morel JB, Dangl JL. 1997. The hypersensitive response and the induction of cell death in plants. *Cell Death Differ*. **4:** 671–683.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell,* **113**: 935-944.

Mueller LA, Goodman CD, Silady RA, Walbot V. 2000. AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. *Plant Physiol.* **123**: 1561–1570.

Mur Luis AJ, Kenton P, Lloyd AJ, Ougham H, Prats E. 2007. The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* **59**: 501-20.

Mwangi M, Bandyopadhyay R. 2006. Managing banana *Xanthomonas* wilt: information packaged for the crop crisis control project (C3P). IITA, Ibadan. 33p.

http://eastafrica.usaidgov/proxy/document.1017.aspx. Accessed on December 8th, 2013

Mwangi M, Bandyopadhyay R, Ragama P, Tushemereirwe WK. 2007. Assessment of banana planting practices and cultivar tolerance in relation to management of soil borne *Xanthomonas campestris* pv. *musacearum*. *Crop Protect*. **26**: 1203-1208.

Naidoo R, Ferreira L, Berger DK, Myburg AA, Naidoo S. 2013. The identification and differential expression of *Eucalyptus grandis* pathogenesis-related genes in response to salicylic acid and methyl jasmonate. *Frontiers Plant Sci.* **4**: 43. doi: 10.3389/fpls.2013.00043

Namanya P, Magambo SM, Mutumba G, Tushemereirwe W. 2004. Somatic embryogenesis from immature male inflorescences of East African highland banana cv 'Nakyetengu'. *Afr. Crop Sci. J.* **12**: 47-53.

Namukwaya B, Tripathi L, Tripathi JN, Arinaitwe G, Mukasa SB, Tushemereirwe WK. 2012. Transgenic banana expressing *Pflp* gene confers enhanced resistance to *Xanthomonas* wilt disease. *Transgenic Res.* **21**, 855–865.

Nandi A, Welti R, Shah J. 2004. The *Arabidopsis thaliana* di-hydroxyacetone phosphate reductase gene suppresssor of fatty acid desaturase deficiency1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell*, **16**: 465–477.

Naqvi S, Farre G, Sanahuja G, Capell T, Zhu, C, Christou P. 2009. When more is better: multigene engineering in plants. *Trends Plant Sci.* **15**: 48-56.

Ndungo V, Eden-Green S, Blomme G, Crozier J, Smith J. 2006. Presence of banana *Xanthomonas* wilt (*Xanthomonas campestris* pv. *musacearum*) in the DRC. *Plant Pathol.* **55**(2): 294.

Nicaise V, Roux M, Zipfel C. 2009. Recent advances in PAMP-triggered immunity against bacteria: Pattern recognition receptors watch over and raise the alarm. *Plant Physiol.* **150**: 1638–1647.

Ning SB, Guo HL, Wang L, Song YC. 2002. Salt stress induces programmed cell death in prokaryotic organism Anabaena. *J. Applied Microbiol.* **93**: 15–28.

Noctor G, Mhamdi A, Foyer CH. 2014. The roles of reactive oxygen metabolism in drought: Not so cut and dried. *Plant Physiol.* **164**: 1636–1648.

Nuhse TS, Bottrill AR, Jones AM, Peck SC. 2007. Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *Plant J.* **51**: 931–940

Okurut AW, Tushemereirwe WK, Aritua V, Ragama PE. 2006. Use of herbicides for the control of banana bacterial wilt in Uganda. *Afr. Crop Sci. J.* **14**: 143-149.

Pandey AK, Ger MJ, Huang HE, Yip MK, Zeng JP, Feng TY, 2005. Expression of the hypersensitive response-assisting protein in *Arabidopsis* results in harpin-dependent hypersensitive cell death in response to *Erwinia carotovora*. *Plant Mol. Biol.* **59:** 771-780

Pel MJC, Pieterse CMJ. 2012. Microbial recognition and evasion of host immunity. *J. Exp. Bot.* **64**: 1237–1248

Peleg-Grossman S, Melamed-Book N, Cohen G, Levine A. 2010. Cytoplasmic H_2O_2 prevents translocation of NPR1 to the nucleus and inhibits the induction of *PR* genes in *Arabidopsis*. *Plant Signal. Behav.* **5**(11): 1401-1406.

Petrov VD, Van Breusegem F. 2012. Hydrogen peroxide - a central hub for information flow in plant cells. *AoB Plants*. Vol.2012. *doi: 10.1093/aobpla/pls014*.

Pieterse CMJ, van Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, van Loon LC. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell,* **10**: 1571-1580.

Pineda CR, Toro Perea N, Narvaez J, Orozco ML, Laignelet A, Cárdenas H. 2002. Genetic transformation by *Agrobacterium tumefaciens* of embryogenic cell suspensions of plantain 'Dominico hartón' (Musa AAB Simmonds). *Infomusa*. **11**(2): 9-13

Price NS. 1994. The origin and development of banana and plantain cultivation. In: Gowen S. (Ed.) *Bananas and plantains* London: Chapman and Hall.

Queval G, Hager J, Gakiere B and Noctor G. 2008. Why are literature data for H_2O_2 contents so variable? A discussion of potential difficulties in the quantitative assay of leaf extracts. *J. Exp. Bot.* **59**: 135–146.

Rafique MZ, Zia M, Rashid H, Chaudhary MF, Chaudhry Z, 2010. Comparison of transgenic plant production for bacterial blight resistance in Pakistani local rice (*Oryza sativa L*.) cultivars. *Afr. J. Biotech*. **9**(13): 1892-1904

Reeder R, Muhinyuza JB, Opolot O, Aritua A, Crozier J, Smith J. 2007. Presence of banana bacterial wilt (*Xanthomonas campestris* pv. *musacearum*) in Rwanda. *Plant pathol*. **56**(6):1036

Rieger M. 2005. Banana and plantain – *Musa* spp. University of Georgia, Atlanta. http://www.uga.edu/fruit/banana.htm. Accessed on 3rd January, 2011.

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD. 2007. Pathological hormone imbalances. *Curr. Opin. Plant Biol.* **10**: 372-379.

Rochon A, Boyle P, Wignes T, Fobert PR, Despre C. 2006. The coactivator function of *Arabidopsis* npr1 requires the core of its BTB/POZ domain and the oxidation of c-terminal cysteines. *Plant Cell*, **18**: 3670-3685.

Roderick H., Tripathi L, Babirye A, Wang D, Tripathi J, Urwin PE, Atkinson H. 2012. Generation of transgenic plantain (*Musa* spp.) with resistance to plant pathogenic nematodes. *Mol. Plant Pathol.* **18**: 842-851.

Rommens CM, Kishmore GM. 2000. Exploiting the full potential of disease-resistance genes for agricutural use. *Curr. Opin. Biotech*. **11**: 120-125.

Ros Barceló A. 2005. Planta xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels. *Planta*. **220**: 747-756.

Ryals J, Uknes S, Ward E. 1994. Systemic acquired resistance. *Plant Physiol.* **704**: 1109-l 112.

Sandhu D, Tasma MI, Frasch R, Bhattacharyya MK. 2009. Systemic acquired resistance in soybean is regulated by two proteins orthologous to *Arabidopsis NPR1*. *Plant Biol*. **9**: 105-119.

Sant RRP. 2011. Development of a transformation system for sorghum. PhD thesis, Queensland University of Technology, Australia. Pg 210.

Sappl PG, Carroll AJ, Clifton R, Lister R, Whelan J, Millar AH, Singh KB. 2009. The *Arabidopsis* glutathione transferase gene family displays complex stress regulation and cosilencing multiple genes results in altered metabolic sensitivity to oxidative stress. *Plant J.* **58**: 53-68.

Sasabe M, Takeuchi K, Kamoun S, Ichinose Y, Govers F, Toyoda K, Shiraishi T, Yamada T. 2000. Independent pathways leading to apoptotic cell death, oxidative burst and defense

gene expression in response to elicitin in tobacco cell suspension culture. *J. Biochem.* **267**: 5005–5013.

Schopfer P, Plachy C, Frahry G. 2001. Release of reactive oxygen intermediates and peroxidase in germinating radish seeds controlled by light, gibberellin and abscisic acid. *Plant Physiol.* **125**: 1591–1602.

Segonzac C, Zipfel C. 2011. Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* **14**: 54–61.

Senda K, Ogawa K. 2004. Induction of *PR-1* accumulation accompanied by runaway cell death in the *lsd1 m*utant of *Arabidopsis* is dependent on glutathione levels but independent of the redox state of glutathione. *Plant Cell Physiol.* **45**: 1578–1585.

Senthilkumar R, Cheng CP, Yeh KW. 2010. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. *Plant Biotech. J.* **8**: 65-75.

Seo PJ, Lee AK, Xiang F, Park CM. 2008. Molecular and functional profiling of *Arabidopsis* pathogenesis-related genes: insights into their roles in salt response of seed germination. *Plant Cell Physiol.* **49**: 334-44.

Shahrtash M. 2013. Plant glutathione S-transferases function during environmental stresses. *Romanian J. Biol. – Plant Biol*. **58**: 19–25.

Sharma P, Jha BA, Dubey RS, Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot. http://dx.doi.org/10.1155/2012/217037*

Shekhawat UKS, Ganapathi TR, Hadapad AB. 2012. Transgenic banana plants expressing small interfering RNAs targeted against viral replication initiation gene display high-level resistance to banana bunchy top virus infection. *J. Gen. Virol.* **93**:1804-1813

Shi Z, Maximova SN, Liu Yi, Verica J, Guiltinan MJ. 2010. Functional analysis of the *Theobroma cacao NPR1* gene in *Arabidopsis. BMC Plant Biol.* **10**: 248.

Simmonds. 1962. The Evolution of the bananas. Longmans, London In: UNCST (Ed.). The biology of bananas and plantains, July 2007. http://biovisioneastafrica.com /publications/biology of bananas and plantains

Sinha M, Singh RP, Kushwaha GS, Iqbal N, Singh A, Kaushik S, Kaur P, Sharma S, Singh TP. 2014. Current overview of allergens of plant pathogenesis related protein families. *The Scientific World Journal*, **9:**543195. http://dx.doi.org/10.1155/2014/543195

Smith AP, Nourizadeh SD, Peer WA, Xu J, Bandyopadhyay A, Murphy AS, Goldsbrough PB. 2003. *Arabidopsis AtGSTF2* is regulated by ethylene and auxin, and encodes a glutathione S-transferase that interacts with flavonoids. *Plant J.* **36**: 433–442.

Smith JJ, Jones DR, Karamura E, Blomme G, Turyagyenda FL. 2008. An analysis of the risk from *Xanthomonas campestris* pv. *musacearum* to banana cultivation in Eastern, Central and Southern Africa. http://www.promusa.org/images/stories/infomusa/ features/pdf/pra.pdf

Spoel SH, Dong X. 2012. How do plants achieve immunity? Defence without specialized immune cells. *Nature Rev. Immunol.* **12**: 89-100.

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X. 2009. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell,* **137**: 860-72.

Sreeramanan S, Maziah M, Xavier R. 2009. A protocol for *Agrobacterium* -mediated transformation of banana with a rice chitinase gene. *Emirates J. Food Agricul.* **21**: 18-33.

Ssekiwoko F, Taligoola HK, Tushemereirwe WK. 2006. *Xanthomonas campestris* pv. *musacearum* host range in Uganda. *Afr. Crop Sci. J.* **14**: 111-120.

Stewart SD, Adamczyk JJ, Knighten KS, Advis FM. 2001. Impact of bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of noctuid (lepidoptera) larvae. *J. Econ. Entomol*. **94**: 752–760.

Sticher L, Mauch-Mani B, Métraux JP. 1997. Systemic acquired resistance. *Ann. Rev. Phytopathol.* **35**: 235-70.

Storer NP, Thompson GD, Head GP. 2012. Application of pyramided traits against lepidoptera in insect resistance management for bt crops. *GM Crops Food,* **3**: 154-162.

Subramanyam K, Subramanyam K, Sailaja KV, Srinivasulu M, Lakshmidevi K. 2011. Highly efficient *Agrobacterium* -mediated transformation of banana cv. *Rasthali* (AAB) via sonication and vacuum infiltration. *Plant Cell Rep.* **30**: 425-436.

Sutherland MW. 1991. The generation of oxygen radicals during host plant responses to infection. *Physiol. Mol. Plant Pathol.* **39**: 79–93.

Tadesse Y. 2003. Optimization of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) *via* microparticle bombardment. *Plant Cell Tissue Organ Cult.* **75**: 1-18.

Talengera D. 2011. Functional analysis of cyclin D2;1 type genes expressed in transgenic banana plants. PhD thesis, University of Pretoria, South Africa.

Tang X, Sun XF, Hu QN, Wu AZ, Lin CH, Lin HJ, Twyman RM, Christou P, Feng TY. 2001. Transgenic rice plants expressing the ferredoxin-like protein (*AP1*) from sweet pepper show enhanced resistance to *Xanthomonas oryzae* pv. *oryzae. Plant Sci*. **160**: 1035–1042.

Tans-Kersten J, Huang H, Allen C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. *J. Bacteriol.* **183**: 3597–3605.

Thakur M, Sohal, BS. 2013. Role of elicitors in inducing resistance in plants against pathogen infection. *ISRN Biochemistry*. 1-10

Thomma BPH, Penninckx IA, Broekaert WF, Cammue BP. 2001. The complexity of disease signaling in *Arabidopsis*. *Curr. Opin. Immunol.* **13:** 63–68.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **11**: 1187-1194.

Tian D, Yin Z. 2009. Constitutive heterologous expression of *avrXa27* in rice containing the R gene *Xa27* confers enhanced resistance to compatible *Xanthomonas oryzae* strains. *Mol Plant Pathol*. **10**: 29–39

Torres MA, Jones JDG, Dangl JL. 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* **141**: 373–378.

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA. 2005. A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. *Plant Cell Rep.* **23**: 780-789.

Tripathi L, Babirye A, Roderick H, Tripathi JN, Changa C, Urwin PE, Tushemereirwe WK, Coyne D, Atkinson HJ. 2015. Field resistance of transgenic plantain to nematodes has potential for future African food security. *Scientific reports*, **5:**8127

Tripathi JN, Muwonge A, Tripathi L. 2012. Efficient regeneration and transformation of plantain cv. "*Gonja manjaya*" (*Musa* spp. AAB) using embryogenic cell suspensions. *In Vitro Cell. Dev. Biol. – Plant*. **48:** 216–224. DOI 10.1007/s11627-011-9422-z.

Tripathi L, Mwaka H, Tripathi JN, Tushemereirwe WK. 2010. Expression of sweet pepper *Hrap* gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*. *Mol. Plant Pathol.* **11**: 721–731.

Tripathi L, Mwangi M, Abele S, Aritua V, Tushemereirwe WK, Bandyopadhyay R. 2009. *Xanthomonas* wilt: A threat to banana production in East and Central Africa. *Plant Disease*, **93**: 440–451.

Tripathi L, Tripathi JN, Hughes Jd'A. 2005. *Agrobacterium*-mediated transformation of plantain (*Musa* spp.) cultivar *Agbagba*. *Afr. J. Biotech.* **4**: 1378-1383.

Tripathi L, Tripathi JN, Kiggundu A, Korie S, Shotkoski F, Tushemereirwe WK. 2014a. Field trial of *Xanthomonas* wilt disease-resistant bananas in East Africa. *Nature Biotech.* **32**: 868–870.

Tripathi JN, Lorenzen J, Bahar O, Ronald P, Tripathi L, 2014b. Transgenic expression of the rice *Xa21* pattern-recognition receptor in banana (*Musa* sp.) confers resistance to *Xanthomonas campestris* pv. *musacearum*. *Plant Biotech. J.* 12: 663–673.

Tripathi L, Tripathi JN, Tushemereirwe WK. 2008. Rapid and efficient production of transgenic East African Highland Banana (*Musa* spp.) using intercalary meristematic tissues. *Afr. J. Biotech.* **7**: 1438-1445.

Truman W, de Zabala MT, Grant M. 2006. Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *Plant J.* **46**: 14–33.

Tsuda K, Katagiri F. 2010. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* **13**: 459-65.

Untergasser A. 2008. DNA Miniprep using CTAB. <http://www.untergasser.de/lab/protocols/miniprep_dna_ctab_v1_0.htm>. Accessed on 2nd June, 2010

Upadhyay P, Rai A, Kumar R, Singh M, Sinha B. 2014. Differential expression of pathogenesis related protein genes in tomato during inoculation with *A. Solani*. *J Plant Pathol Microbiol.* **5**: (1). http://dx.doi.org/10.4172/2157-7471.1000217.

Vailleau F, Daniel X, Tronchet M, Montillet JL, Triantaphylides C, Roby D. 2002. A *R2R3-MYB* gene, *AtMYB30*, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. *Proc. Natl. Acad. Sci.* USA **99**: 10179-84.

Van Loon LC, Rep M, Pieterse CMJ. 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol*. **44**: 135-162.

Van Loon LC, Van Strien EA. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* **55**: 85–97.

Vanacker H, Carver TLW, Foyer CH. 2000. Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. *Plant Physiol.* **123**: 1289–1300.

Velikova V, Yordanov I, Edereva A. 2000. Oxidative stress and some antioxidant systems in acid rain treated bean plants. Protective role of exogenous polyamines. *Plant Sci*. **151**: 59–66.

Verberne MC, Hoekstra J, Bol JF, Linthorst HJ. 2003. Signaling of systemic acquired resistance in tobacco depends on ethylene perception. *Plant J.* **35**: 27-32.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Ann. Rev. Phytopathol.* **47**: 177–206.

Wally O, Jayaraj J, Punja ZK. 2009. Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota L*.) expressing an *Arabidopsis NPR1* gene. *Planta*, **231**: 131-41.

Wei R, Feng F, Zhou J, He C. 2010. Effector-triggered innate immunity contributes *Arabidopsis* resistance to *Xanthomonas campestris*. *Mol Plant Pathol*. 11(6): 783-93.

Weigel D, Glazebrook J. 2006. Transformation of *Agrobacterium* using electroporation. *Cold Spring Harbor Protocols*, doi: 10.1101/pdb.prot4665

Wisser RJ, Kolkman J, Patzoldt ME, Holland JB, Yu J, Krakowsky M, Nelson RJ, Balint-Kurti PJ. 2011. Multivariate analysis of maize disease resistances suggests a pleiotropic genetic basis and implicates a *GST* gene. *Proc. Natl. Acad. Sci. USA*, **108**: 7339–7344.

Wisser RJ, Sun Q, Hulbert SH, Kresovich S, Nelson RJ. 2005. Identification and characterization of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. *Genetics*, **169**: 2277–2293.

Yang G, Zhou R, Tang T, Shi S. 2008. Simple and efficient isolation of high-quality total RNA from *hibiscus tiliaceus*, a mangrove associate and its relatives. *Prep. Biochem. Biotech.* **38**: 257-264. http://dx.doi.org/10.1080/10826060802164991

Yao N, Imai S, Tada Y, Nakayashiki H, Tosa Y, Park P. 2002. Apoptotic cell death is a common response to pathogen attack in oats. *Mol. Plant–Microbe Interact.* **15**: 1000–1007.

Yip MK, Lee SW, Su KC, Lin YH, Chen TY, Feng TY. 2011. An easy and efficient protocol in the production of *Pflp* transgenic banana against *Fusarium* wilt. *Plant Biotech. Rep.* **5**: 245-254.

Yirgou D, Bradbury JF (1968). Bacterial wilt of enset (*Ensete ventricosum*) incited by *Xanthomonas musacearum. Phytopathology*, **58**: 111-112

Yirgou D, Bradbury JF (1974). A note on wilt of banana caused by the enset wilt organism *Xanthomonas musacearum*. *East Afr. Agric. Forest. J*. **40**:111-114.

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG, Doke N. 2003. *Nicotiana benthamiana* gp91phox homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell*, **15**: 706–718.

Zaninotto F, La Camera S, Polverari A, Delledonne M. 2006. Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. *Plant Physiol*. **141**: 379-383

Zhang B. 2013. *Agrobacterium*-mediated transformation of cotton. *Methods Mol. Biol.* **958**: 31-45.

Zhang J, Zhou JM. 2010. Plant immunity triggered by microbial molecular signatures. *Mol. Plant*, **3**: 783-793.

Zhao JT, Huang X, Chen YP. 2009. Molecular cloning and characterization of an ortholog of *NPR1* gene from Dongguan Dajiao (*Musa* spp. ABB). *Plant Mol. Biol. Rep*. **27**:243-249

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nature Biotech.* **21**: 1493–1497.

Zhijian L, Subramanian J, Dennis GJ. 2001. Expression of a bifunctional green fluorescent protein *(GFP*) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). *Plant Science,* **160**: 877-887.

Zhou YL, Xu JL, Zhou SC, Yu J, Xie XW, Xu MR, Sun Y, Zhu LH, Fu BY, Gao YM, Li ZK. 2009. Pyramiding *Xa23* and *Rxo1* for resistance to two bacterial diseases into an elite indica rice variety using molecular approaches. *Mol. Breed*. **23**(2): 279-287

Zhu S, Li Y, Vossen JH, Visser RGF, Jacobsen E. 2012. Functional stacking of three resistance genes against *Phytophthora infestans* in potato. *Transgenic Res.* **21**: 89-99.

Zipfel C, Felix G. 2005. Plants and animals: a different taste for microbes? . *Curr. Opin. Plant Biol.* **8:** 353–360.

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature,* **428**: 764–767.

APPENDICES

Appendix IA. MA2 media for cell suspension from male buds

Compound	Component	Amount (mg/l)
MS macro nutrients	NH_2NO_2	1,650
	KNO_3	1,900
	$CaCl_2$	332.2
	$MgSO_4$	180.7
	KH_2PO_4	170
MS micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Ascorbic acid	40
	Myo-inostol	100
	Glycine	2.0
	Thiamine –HCl	0.5
	L-Glutamine	99.4
	Malt extract	100
	Nicotinic acid	0.5
	Pyridoxine-HCl	0.5
	Biotin	1.0
Phytohormones	2,4 D	1.0
Carbon source	Sucrose	45,000
pH		5.3

AppendixIB

ZZ maintenance media for cell suspension from scalps

Compound	**Component**	**Amount (mg/l)**
MS macro nutrients	NH_2NO_2	825
	KNO_3	950
	$CaCl_2$	166.1
	$MgSO_4$	90.35
	KH_2PO_4	85
MS micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	13.9
	$Na_2EDTA.2H_2O$	18.6
Vitamins	Ascorbic acid	40
	Myo-inostol	100
	Glycine	2.0
	Thiamine –HCl	0.5
	L-Glutamine	99.4
	Malt extract	100
	Nicotinic acid	0.5
	Pyridoxine-HCl	0.5
	Biotin	1.0
Phytohormones	2,4 D	1.1
	Zeatin	2.2
Carbon source	Sucrose	30,000
Phytagel		2.5
pH		6.12

Appendix II. BRM medium for re-suspending *Agrobacterium*

Group	**Component**	**Amount (mg/l)**
Macro nutrients	NH_2NO_2	165
	$CaCl_2$	33.2
	$MgSO_4$	18.1
	KNO_3	190
	KH_2PO_4	17
Micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$CuSO_4.5H_2O$	0.025
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
	KI	0.83
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Thiamine-HCl	10
	Glycine	2
	Pyridoxine-HCl	0.5
	Myo-inostol	50
	Nicotinic acid	0.5
	L-cystein	400
Carbon source	Sucrose	68,500
	Glucose	36,000
Gelling agent	Gelrite	2,400
Acetosyringone		49
	pH	5.3

Appendix III. Bacteria co-culturing media (BCCM)

Group	Compound	Amount (mg/l)
Macro nutrients	NH_2NO_2	82.5
	$CaCl_2$	16.6
	$MgSO_4$	9.1
	KNO_3	95
	KH_2PO_4	8.5
Micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$CuSO_4.5H_2O$	0.025
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
	KI	0.83
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Thiamine-HCl	10
	Glycine	2
	Pyridoxine-HCl	0.5
	Myo-inostol	50
	Nicotinic acid	0.5
	L-cystein	400
	L-glutamine	100
	Malt extract	100
	Proline	300
	PVP	10,000
	Ascorbic acid	10
	Biotin	1
Carbon source	Sucrose	30,000
	Glucose	10,000
	Maltose	30,000
Gelling agent	Gelrite	2400
Acetosyringone		31.4
	pH	5.3

Appendix IV. MA3 embryo development media

Group	Component	Amount (mg/l)
SH macro nutrients	$NH_2H_2PO_4$	300
	KNO_3	12,500
	$CaCl_2.2H_2O$	200
	$MgSO_4.7H_2O$	400
SH micro nutrients	$MnSO_4$. 4H2O	10
	H_3BO_3	5.0
	$CuSO_4.5H_2O$	0.2
	$ZnSO_4.7H_2O$	1.0
	$Na_2MoO_4.2H_2O$	0.1
	$CoCl_2.6H_2O$	0.1
	KI	1.0
Iron complex	$FeSO_4.7H_2O$	15
	$Na_2EDTA.2H_2O$	20
Vitamins	L-Glutamine	100
	L-proline	230
	Malt extract	100
	Pyridoxine	0.5
	Nicotinic acid	0.5
	Biotin	1.0
	Ascorbic acid	60
	Myo-inostol	100
	Glycine	2.0
	Thiamine –HCl	0.5
	L-cysteine	400
	Citric acid	60
Carbon source	Lactose monohydrate	10,000
	Sucrose	45,000
Phytohormones	NAA	0.2
	Zeatin	0.1
	Kinetin	0.2
	2iP	0.2
Gelling agent	Gelrite	2,400
	pH	5.3

Appendix V. RDI regeneration media

Group	**Component**	**Amount (mg/l)**
Macro nutrients	$MgSO_4$	9.05
	KNO_3	95
	NH_2NO_2	82.5
	$CaCl_2$	16.6
	KH_2PO_4	8.6
Micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	KI	0.83
	$CuSO_4.5H_2O$	0.025
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Thiamine-HCl	10
	Glycine	2
	Pyridoxine-HCl	0.5
	Myo-inostol	100
	Nicotinic acid	0.5
	Ascorbic acid	0.1
Carbon source	Sucrose	30,000
	Gelrite	2,400
	pH	5.8

Appendix VI. MA4 embryo germination media

Group	**Component**	**Amount (mg/l)**
Macro nutrients	$MgSO_4$	18.1
	KNO_3	190.0
	NH_2NO_2	165.0
	$CaCl_2$	33.2
	KH_2PO_4	17.2
Micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	KI	0.83
	$CuSO_4.5H_2O$	0.025
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Thiamine-HCl	10
	Glycine	2
	Pyridoxine-HCl	0.5
	Myo-inostol	50
	Nicotinic acid	0.5
Phytohormones	BAP	0.05
	IAA	0.2
Carbon source	Sucrose	30000
	Gelrite	2400
	pH	5.8

Appendix VII

MS proliferation media

Compound	**Component**	**Amount (mg/l)**
MS macro nutrients	NH_2NO_2	1,650
	KNO_3	1,900
	$CaCl_2$	332.2
	$MgSO_4$	180.7
	KH_2PO_4	170
MS micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
	Ascorbic acid	20
Vitamins	Myo-inostol	100
	Glycine	2.0
	Thiamine –HCl	0.1
	Nicotinic acid	0.5
	Pyridoxine-HCl	0.5
	Biotin	1.0
Phytohormones	BAP	1.0
Carbon source	Sucrose	30,000
phytagel		2400
pH		5.8