Xanthomonas vasicola pv. *musacearum* down-regulates selected defense genes during its interaction with both resistant and susceptible banana.

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Abstract

Expression of specific R, NPR1 and PR genes was investigated to ascertain their role in the resistance of *Musa balbisiana* to *Xanthomonas vasicola* pv. *musacearum* (Xvm). Following inoculation, wilt symptoms developed in test plants though their incidence and severity climaxed merely at 30% and 20% respectively before recovery. Xvm was limited to the inoculated and the immediate follower leaves and its population in a 50mg tissue dropped from 3.06×10^7 cfu at 18 days to 0 cfu by 140 days after inoculation (DAI). In susceptible banana, it instead increased from 3.41×10^8 to 1.08×10^{11} cfu by 21 DAI culminating in plant death. Expression of *MbNBS*, *MdNPR1* and *PR3* sharply declined during the first 6 hours, recovering in the later stages though this did not stop later wilt symptoms developed in resistant and susceptible plants. We concluded that neither Hypersensitive response, nor Systemic Acquired Resistance nor Induced systemic resistance is the resistance mechanism for *M. balbisiana* against Xvm as it suppresses key genes in these pathways.

Key words: Resistance, *Xanthomonas vasicola* pv. *musacearum* and *Musa balbisiana*

Introduction

Banana Xanthomonas wilt disease caused by *Xanthomonas vasicola* pv. *musacearum* (Xvm) formerly *Xanthomonas campestris* pv. *musacearum* (Xcm) [1] is the most devastating disease of banana in East and Central Africa threatening livelihoods of over 80millions of people in sub-Saharan Africa who depend on it. The disease causes wilting of plants, premature ripening of banana fruits and all affected plants always die leading to total yield loss. All cultivated banana varieties in the region are susceptible except *Musa balbisiana,* a wild banana which is resistant to the disease [2]. In order to effectively exploit this genotype, it the mechanism of resistance which has not been known upto this date needs to be clearly understood.

In a typical basal resistance response, host plants use specific pattern recognition receptors (PRRs) on their cell-surfaces to detect the conserved microbe associated molecular patterns (MAMPs) such as bacterial flagellins, lipopolysaccharides, elongation factors, fungal chitin and heptaglucosides of the invading microbes [3]. This recognition triggers activation of signal-transduction cascades that turn on basal defense mechanisms such as callose and silicone deposition to reinforce the cell wall, closure of stomata, production of reactive oxygen species, transcriptional induction of pathogenesis-related genes (PR) and post-transcriptional suppression of the auxin-signalling pathway [4] among others. Some pathogens have evolved ways to directly introduce effectors into the cells using the type three secretion system (TTSS), thereby evading basal defense mechanisms. Some resistant plants however use R proteins to recognize the presence of these effectors inside the plant cells and set in a chain of reactions that eventually arrest the pathogen [5].

Normally, presence of the pathogen and the subsiquent recognition of the same or its effectors leads to an increased production of superoxide (O^-_2) on the apoplastic face of the plasmalemma. At the same time a signal is translocated all over the plant to prepare such tissues for any eventual attack. Meanwhile, superoxide dismutase (SOD) at the apoplast rapidly converts O^-_2 to H_2O_2 which may not only affect the pathogen but also damage the plant cells. The higly abandunt peroxidases detoxfy H_2O_2 using ascorbate, as a reducing substrate through the ascorbate-glutathione cycle [6]. The detoxification reactions change the redox state of cells and this leads to monomerization of cytosolic Nonexpresor of pathogenesis related 1 protein (NPR1) which subsiquently leads to its translocation into the nucleus, interacts with transcription factors resulting in activation of various forms of antimicrobial pathogenesis related (PR) genes [7] [8] [9] [10]. This eventually confirs resistance to a broad spectrum of pathogens during establishment of systemic acquired resistance and induced systemic resistance [11] [12].

In this study therefore, changes in disease development and expression of specific R, NPR1 and PR genes were investigated to ascertain their role in the resistance of *Musa balbisiana* to *Xanthomonas vasicola pv. musacearum*.

Materials and methods

Experimental setup

A screen-house pot experiment was set up with tissue cultured banana plantlets using *M. balbisiana* and a cooking banana cultivar Nakinyika as resistant and susceptible genotypes respectively. Fourty two (42) tissue culture plants of each banana genotype were weaned in plastic pots and allowed to establish for 8 weeks. A compleatly randomised design was adopted for the trial in which the two treatments (injection with either water or Xvm) were randomly applied to 21 plants of each genotype. The Xvm inoculum of aproximately $X10^8$ cell per ml was prepared using one of the vilulent preserved cultures at the National Agricultural Biotechnology center Kawanda, Kampala Uganda. One tenth of a ml (100µL) of either water or Xvm as inoculum was drawn into a hypodermic syringe and then injected into the second youngest open leaf petiole of the test plants. A random sample of 3 plants for each treatment was drawn at intervals of 0hrs, 6hrs, 72hrs, and 14days post inoculation for RNA extraction. Later, another random sample of 3 plants for each treatment was drawn at 18, 25, 56, 90 and 150 days after inoculation for Xvm isolation.

Assessment of bacterial population changes within plant tissues

Part of the leaf petiole of the inoculated leaf (at 5 cm below point of inoculation) and its younger immediate follower leaf was cut, surface sterilized by washing in dilute NaOCl (1:5) with water, then rinsed 3 times in sterile water and blotted dry. Its tissues were aseptically cut, weighed and suspended in sterile water (0.5g/ml of water). After suspending for 15 minutes, this suspension was serially diluted 6 times, 10 µL of each dilution plated on semi-selective CCA medium (Mwangi et al., 2007) and incubated at 25ºC for 5 days.

RNA extraction

To collect a plant sample for RNA extraction, 10g of the apex of the follower leaf to the inoculated leaf was cut at once with a sterile surgical blade, wrapped in a labeled aluminum foil sheet and immediately frozen in liquid nitrogen. RNA from each sample was extracted following the CTAB nucleic acid extraction protocol. Generally, 0.1g of each frozen plant tissue was crushed in a separate sterile and frozen motor into a powder and 1.5ml of pre-warmed (65ºC)

extraction buffer containing: CTAB (20g/L), NaCl (81.76g/L), PVP-40 (20g/L), Tris-HCl (15.76g/L), EDTA (18.61g/L) and 2% *ß*-mercapto-ethernol was added. The mixture was incubated at 65°C for 15 mins while mixing every 2 mins by inversion after which it was centrifuged at 13000rpm for 5 mins. In a new sterile tube, 700μL of supernatant were transferred and cooled on ice for 2 mins after which 700μL of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. It was then centrifuged at 13000rpm for 5 mins at 4°C. The aqueous phase (600μL) was transferred to a new tube and an equal volume of phenol: chloroform: isoamyl alcohol (25: 24:1) added, mixed by inversion, then centrifuged at 13000rpm for 5 mins at 4°C. Finally 500μL of the aqueous phase was transferred to a new tube, and RNA precipitated by adding 10M lithium chloride to a final morality of 2.25M then incubated at -20°C for 8 hrs. The precipitate was then collected by spinning at 13000rpm for 45 mins after which the supernatant was discarded. The pellet was reconstituted in water to 450μl, then 1/9 volume of 3M sodium acetate (Ph 5.2) added followed by an equal volume of cold iso-propanol (-20°C) and mixed by inversion. The mixture was kept at -20°C overnight and then spun at 13000rpm for 2 mins at 4°C. Iso-propanol was decanted and the pellet washed twice in 500μL of 70% ethanol by tapping and spinning at 13000rpm for 2 mins at 4°C. The pellet was air dried at room temperature for 40 mins after which it was re-suspended in 30μL of sterile nuclease free water and stored at -20°C.

RNA purification

Genomic DNA traces were removed using a DNase treatment kit by Thermo scientific (EN0521) following the manufacturer's protocol. Generally, to 6μL of nuclease free water were added 1μL of 10X buffer, then 2μL of RNA (approximately 0.5μg) and 1μL of DNase I (1u/μL). The resultant 10μL reaction mixture was incubated at 37°C for 30 mins after which 1μL of 50mM EDTA was added and incubated at 65°C for 15 mins to inhibit any further DNase I reactions. The contents were immediately chilled on ice for 1 min, collected by brief spinning and then stored at -20°C awaiting cDNA synthesis.

cDNA synthesis

cDNA was synthesed using the RevertAid™ first strand cDNA synthesis Kit (Fermentas Life Sciences) according to the manufacturer's protocol. Generally, to 8μL of nuclease free water, 3μL of pure RNA sample and 1μL of random hexamer primers (0.2μg/μL) were added. This mixture was incubated at 65°C for 5 mins, then immediately chilled on ice for 5 mins and the contents were collected by brief spinning. During reverse transcription, to the primer annealed reaction mixture was added 4μL of 5x reaction buffer, 1μL Ribolock RNase inhibitor (20u/μL), 2μL of 10mM dNTP mix, and 1μL of Reverse transcriptase (200u/μL). This mixture was incubated at 25°C for 5 mins after which it was further incubated at 42°C for 1 hr followed by heating at 70°C for 5 minutes to inactivate reverse transcriptase. The contents (now cDNA) were then chilled on ice, collected by spinning and the cDNA was quantified using a nanodrop 2000 for all samples taken and was standardised to 0.08μg/μL by dilution in sterile nuclease free water before storing at -20°C.

Primer design

MbNBS Primers (Forward: 5'TGCTGGTGTTGGATGATGTT3' and Reverse: 5'GGCAGGTCTTGGTAGCTCAG3') for amplifcation of genes with NBS-LRR binding sites that are typical of detecting pathogen effectors were designed. In addition, MdNPR1 primers (Forward: 5'GATAAGGCTATGGTGGAAGA3' and Reverse:
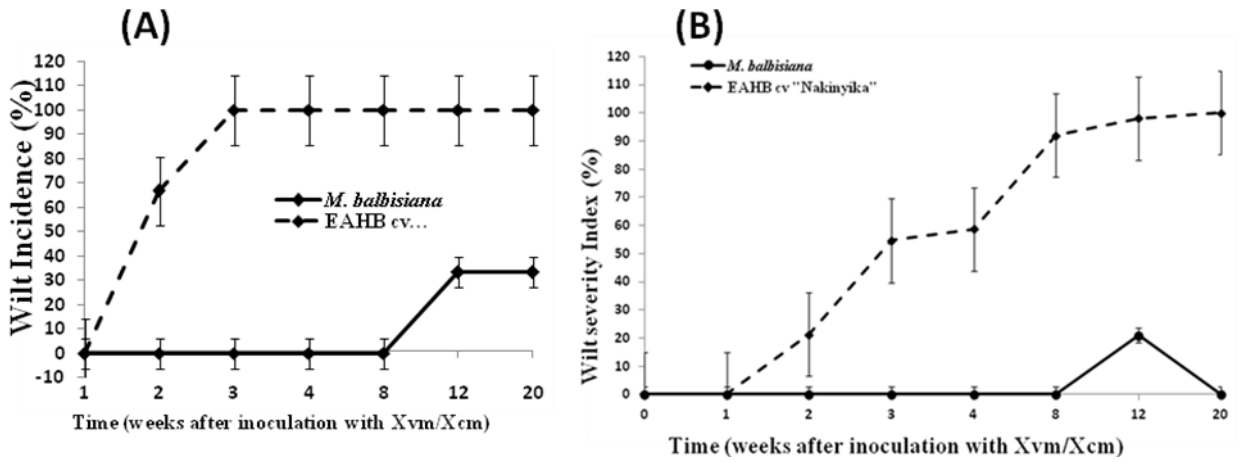
Figure 1: Change in wilt disease Incidence (A) and severity (B) over time in EAHB cv "Nakinyika" and *M. balbisiana* following inoculation with *Xvm*.

5'CGCCCTAGTTAGTCTCCTACAT3') as designed and used by Zhao *et al.,* (2009) for amplifcation of a banana NPR1, PR3 (Forward: 5'GGCTCTGTGGTTCTGGATGA3' and Reverse: 5'CCAACCCTCCATTGATGATG3') and a house keeping gene 25s primers (Forward: 5'ACATTGTCAGGTGGGGAGTT3' and Reverse: 5'CCTTTTGTTCCACACGAGATT3') as designed and used by Endah *et al.,* (2008), were synthesised.

Semiquantitative PCR

For the same set of plants at a specific sampling point, cDNA was pooled prior to running PCR. A 10μL water diluted PCR mixture containing 0.05μL taq polymerase (Qiagen), 1X Taq buffer (Quagen), dNTP mix (Qiagen), $MgCl_2$ (Qiagen), 1μL cDNA (30ng/μL), and each of the forward and reverse primers,) as detailed in table 1 was set up and run in a thermocycler at an initial activation temperature of 95°C for 5 mins, follwed by cycle denaturation at 94°C for 5s, then primer annealng for 5s and cycle extenstion at 72°C for 10s for 30cycles, then a final extension at 72°C for 20s. The contents were electrophoresed in a 2% gel for 40 mins, stained in ethidium bromide vissualised under UV and photographed.

Data collection and analysis

Wilt incidence and severity

The number of wilted plants was expressed as a percentage of the total number of inoculated plants (Incidence). In addition, the sums of the individual proportion of wilted number of leaves to the total number leaves per plant were expressed as the percentage of the total number of plants (Severity).

Bacterial population changes in planta

The number of colonies on each plate was counted, then used to estimate the bacterial load in the original plant tissue for each plant before dilution. The mean population for the plants at a particular sampling interval was estimated and the significance of their differences estimated using SAS v9 package

Changes in expression levels of selected candidate genes

Data on expression of specific genes was captured as band intensity differences on the agarose gels. This band intensity difference was converted into numerical data using GelQuantNET software. Intensities of the housekeeping 25S were standardized and factorial differences were used to estimate intensity values for targets genes. The corrected band intensity values for the target genes were log transformed and analyzed using SAS version 9. Mean band intensity due to Xvm relative to basal intensity was expressed as a ratio of band intensity due to water relative to basal intensity. The resultant ratios at a particular sampling point were plotted as Log10 ratio of relative expression against time after inoculation.

Results

Wilt incidence and severity

Wilt incidence (fig 1A) and severity (fig 1B) was noted to change over time after inoculation. In the susceptible EAHB cv "Nakinyika" wilt incidence quickly rose from 0% to 68% within 14

Table 1. Primer sequences and their optimum working conditions

Primer name	Primer sequence	Working concentration			Annealing temparature
		Primer	dNTPs	MgCl$_2$	
25s	F: ACATTGTCAGGTGGGGAGTT R: CCTTTTGTTCCACACGAGATT	0.15μM	0.2mM	1.5mM	57ºC
MbNBS	F: TGCTGGTGTTGGATGATGTT R: GGCAGGTCTTGGTAGCTCAG	0.3μM	0.2mM	1.5mM	62ºC
MdNPR1	F: GATAAGGCTATGGTGGAAGA R: CGCCCTAGTTAGTCTCCTACAT	0.1μM	0.4mM	4mM	55 ºC
PR3	F: GGCTCTGTGGTTCTGGATGA R: CCAACCCTCCATTGATGATG	0.1μM	0.4mM	4mM	60 ºC

Table 2: Relative average No. of *Xvm* cells recovered from the tissue (50mg) of *M. balbisiana* and EAHB cv "Nakinyika" at various days after inoculation (DAI) with *Xvm*.

DAI	*M. balbisiana*	EAHB cv "Nakinyika"
18	3.06×10^7	3.55×10^8
25	2.23×10^6	1.08×10^{11}
56	8.7×10^5	a
90	1.5×10^4	a
150	0	a

a= Dead plant due to *Xvm* infection

days after inoculation and by the 21 days, it had reached 100%. Similarly, severity of leaf wilting rose from 0% to 21% within 14 days and by 25 days after inoculation it had risen to 92%. By the 56 days, all plants had died. In the resistant *M. balbisiana* on the other hand, wilt symptoms delayed and were only observed 70 days after inoculation and by the 84 days, leaf wilt incidence had only risen to 33 % at which it remained even up to 140 days after inoculation. Similarly, leaf wilt severity remained at 0% for 56 days after which it rose to about 20% by the 84 days before dropping to 0% by 140 days.

Trends in Xvm proliferation and migration in planta

Xvm population in plant tissues was observed to change over the days after inoculation (Table 2). In the susceptible EAHB cv "Nakinyika", *Xvm* multiplied and migrated from the inoculated leaf to other leaves such that it was detectable by 18 days after inoculation. Xvm multiplication continued such that by 25 days after inoculation its population had reached 1.08×10^{11} cells per 50mg of plant tissue. Subsequent death and rotting of tissues could not allow recovery of any Xvm by 56 days after inoculation. In resistant *M. balbisiana*, *Xvm* was also detectable by 18 days at 3.06×10^{7} cells per 50mg of plant tissue. After then, it found to be continually reducing such that by 56 days it was 8.7×10^{5} cells per 50mg of plant tissue. By 90 days after inoculation, *Xvm* had migrated to the follower leaves though its population was only 1.5×10^{4} cells per 50mg of plant tissue. By 140 days after inoculation, it was not detectable anymore in *M. balbisiana* tissues.

Changes in the expression levels of selected defence related gene

An analysis of genes deep down in the defence pathways showed that expression levels of the *PR3* gene sharply declined below basal levels in the first 6hrs after inoculation of both resistant and susceptible banana with Xvm (fig2A). In the next period up to 72hrs after inoculation, its expression sharply increased significantly above the basal levels in the resistant before declining back to basal levels at 14 days after inoculation. Its expression however remained significantly lower than basal levels in the susceptible plant till 14 days after inoculation. Initial decline in the expression level of PR3 suggested down regulation of the gene during the initial stages of Xvm infection of banana. Further inquiry into the possibility of down regulation of this gene showed that there was a sharp decline in the expression of *MdNPR1* (one of the genes high in the NPR1-PR defence pathway) below basal levels in the first 6 hours after inoculation in both the resistant and susceptible banana. In the next period up to 72hrs after inoculation, its expression also sharply increased significantly above the basal levels in the resistant before declining back towards basal levels by 14 days after inoculation. Similarly, in the susceptible plant expression of *MdNPR1* also increased after 72hrs and went back to basal levels by 14 days except that this increase was gradual (fig2B).

Further investigations revealed that the expression levels of a putative disease resistance gene with typical NBS-LRR domains, *(MbNBS)* significantly declined sharply below basal levels in the first 6hrs after inoculation of both resistant and susceptible banana with Xvm. In the next period up to 72hrs after inoculation, its expression also sharply increased significantly above the basal levels in the resistant before declining back towards basal levels by 14 days after inoculation. In the susceptible plant however, its expression gradually increased but remained
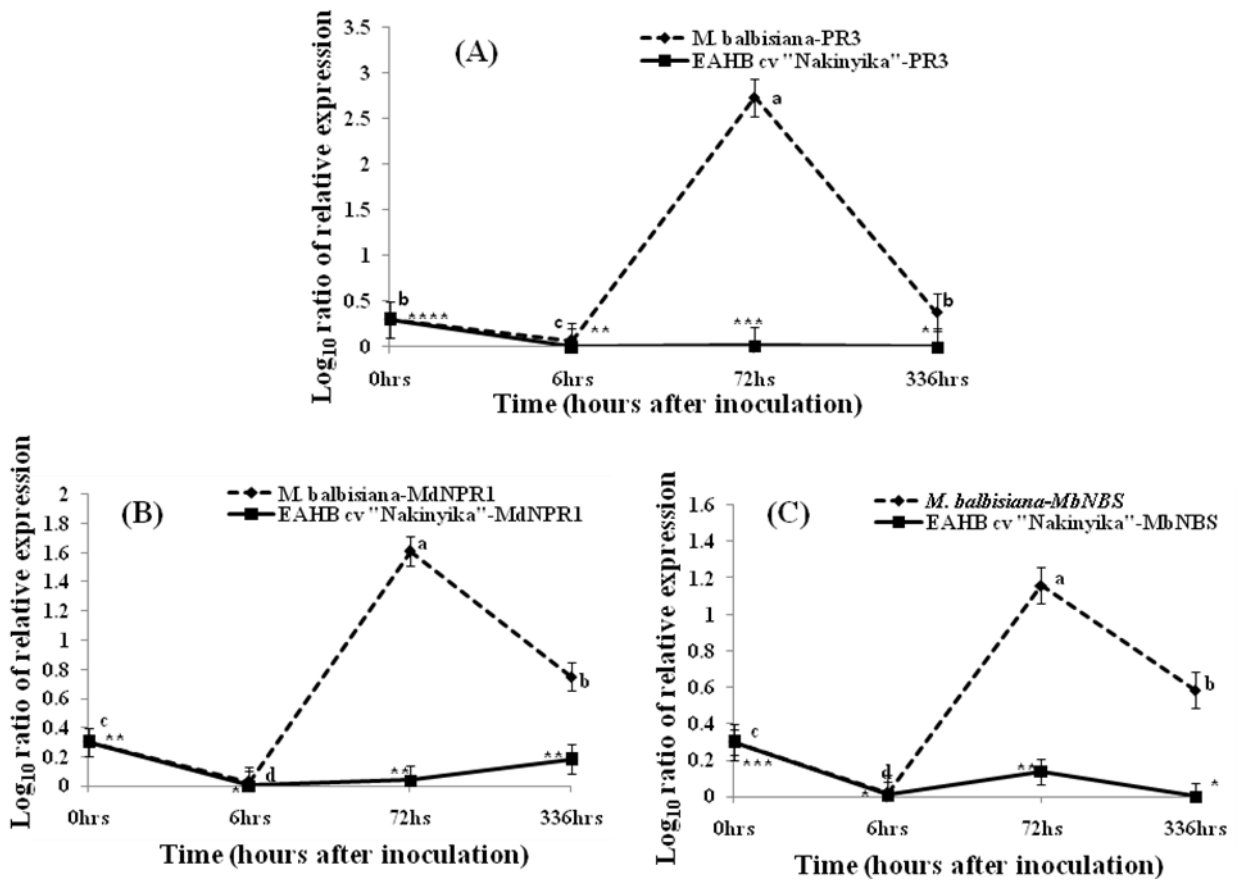
Figure 2: Relative expression levels of selected genes (A) *PR3*, (B) *MdNPR1* and (C) *MbNBS* in the banana defense pathway following inoculation of *M. balbisiana* and EAHB cv "Nakinyika" with the banana Xanthomonas wilt pathogen (Xvm).

significantly lower than basal levels and had not recovered to basal levels by 14 days after inoculation (fig3C).

Discussion

The delayed symptom expression (10 weeks after inoculation), low wilt incidence and severity in *M. balbsiana* (which only climaxed at 20% before dropping back to 0%) coupled with reduced multiplication and limited migration of Xvm within the plant tissues and the subsequent clearance from tissues suggests that *M. balbisiana* is resistant. Low bacterial population density to initiate infection (quorum sensing), presence of various plant defensive compounds such as phenolics, phytolexins and plant resistance proteins (including pathogenesis related proteins) are reported to delay disease expression and cause long incubation and latent periods [13] [14] through interference of the expression system of virulence factors [15] and direct killing of pathogens [16]. However, quorum sensing cannot explain the delayed symptom expression in *M. balbisiana* as at the time when symptoms were observed, the bacterial density in plant tissues was much lower than before symptom development. Other mechanisms were responsible for the resistance reaction. In a typical active defense response, tissues around the point of attack normally undergo a rapid programmed cell death termed hypersensitive response (HR) killing the pathogen within and conferring resistance to hosts. However, whole wilting of several leaves as observed in this resistant type were not typical of HR and this is not the resistance mechanism at play. A molecular dissection of the NPR1-PR defense pathway revealed some interesting findings where expression levels of some candidate genes varied over time after inoculation with Xvm. Firstly, while there was a general increase in the expression levels of *PR3* in both water and Xvm treated plants, the increase was greater in the water treated than the Xvm treated plants in the first 6hrs following inoculation resulting an overall initial reduction in the expression of *PR3* in both the resistant and the susceptible genotypes. While this observation is in part supported by a fact that like other pathogenesis related genes, *PR3* is generally stress induced, with its expression levels expected to be elevated under wounding and Xvm inoculation, it in part contradicted finding of Endah [17] where there was an overall increase in the expression of *PR3* in Xvm susceptible plants. The overall reduced expression of *PR3* could probably explain development of wilt disease in both resistant and susceptible banana genotypes. Further, since *PR3* gene product is an antimicrobial chitinase, this finding could mean that later recovery of expression levels of this gene to and above basal levels in the susceptible and resistant respectively could be responsible for the resistance response in the resistant. However we have often noted that elevated levels of *PR3* do not necessarily bring about resistance as was previously shown in Cavendish banana cultivar GCTCV-218 following Xvm infection [17]. More over our findings show that *M. balbisiana* developed disease symptoms 10 weeks after inoculation (a period way after 14 days when *PR3* levels were back to basal levels) suggesting that *PR3* could not have been responsible for the observed resistance response in *M. balbisiana*. It could however be responsible for the delayed symptoms. Our findings further clearly show that regardless of the host resistance status, there is a general decline in the expression of *PR3* below basal levels in the early stages of infection suggesting that Xvm has an overall effect of down regulating this gene in *M. balbisiana* and the EAHB cv "Nakinyika" alike.

In addition, the expression of an upstream NPR1 gene (*MdNPR1*) was consistent with the expression of *PR3* where it was also initially suppressed following Xvm infection. NPR1 is a known molecular switch that activates various forms of antimicrobial pathogenesis related (*PR*)

genes [7] [8] [9] [10] leading to establishment of systemic acquired resistance and induced systemic resistance [11] [12]. Its initial repression would lead to repression of *PR* genes and later induction would also lead to *PR* induction. Indeed this is what was exactly observed in this study where *MdNPR1* expression pattern was consistent with *PR3* expression pattern. *MdNPR1* expression has already been shown to be induced by an array of elicitors including SA and FOC treatment in some banana cultivars [18]. Since *NPR1* is normally induced in response to different abiotic and biotic stress factors, it would also be expected to be induced by Xvm. Our finding that its expression is repressed in the early stages of infection before subsequent induction towards and above basal expression levels in the susceptible and resistant banana respectively suggests that Xvm inoculation also causes down regulation of this gene. If this is true, it would also mean that the plant is crippled in its ability to set up SAR and ISR during attack from Xvm. Indeed both the resistant and susceptible banana develop Xanthomonas wilt disease symptoms following Xvm inoculation. Since these defence responses depend on successful recognition of pathogens and or their effectors followed by signal transduction, initial repression of the downstream genes (*NPR1* and *PR*) could be a result of either a failed signal transduction system or a failed pathogen recognition system by the host plant.

The NBS-LRR domains of *MbNBS* are characteristic of R genes that recognise and bind specific pathogen effectors setting in HR and SAR [19] [20] [21]. Genome wide sequence data for Xvm predicted this pathogen to use type three secretion effectors during host infection [22]. Work in our laboratory has already shown that Xvm expressed *HrpF* (an indispensible gene for establishment of the type three secretion system) during infection of banana. Pathogen effectors are known to variously suppress host defence mechanisms [23] [24]. Repression of *MbNBS* below basal levels in the early hours following inoculation with Xvm therefore suggests that this pathogen causes its down regulation to evade detection (of its effectors). Where as its expression is induced in the subsequent period up to 72 hrs post inoculation especially in the resistant banana leading to induction of *MdNPR1* and subsequently *PR3*, it seems rather late and consequently plants develop wilt symptoms later .

This result has shown that both the Xanthomonas wilt resistant *M. balbisiana* and the Xanthomonas wilt susceptible EAHB cv. "Nakinyika" are disabled in their ability to actively defend themselves against Xvm infection. It appears that this success on the pathogen side starts when the plants detection system is disabled. Subsequently downstream reactions are also affected and the result of this would be disease development. Indeed our finding show that Xvm attempts to multiply and migrate to distant tissues in both the resistant and susceptible banana resulting in disease as observed in this study. Despite the success in disenabling of the host banana detection system and related downstream reactions, it is not clearly known why Xvm did not sustainably establish in *M. balbisiana* in which its multiplication and migration was slowed and subsequently cleared from host tissues. Other than the HR mediated disease resistance response and the pathogenesis related proteins, various phenolic compounds have also been reported to be antimicrobial affecting the multiplication and migration of bacteria in planta [16]. The role of various phenolic compounds from *M. balbisiana* on the multiplication of Xvm are subject for future studies.

Acknowldegements

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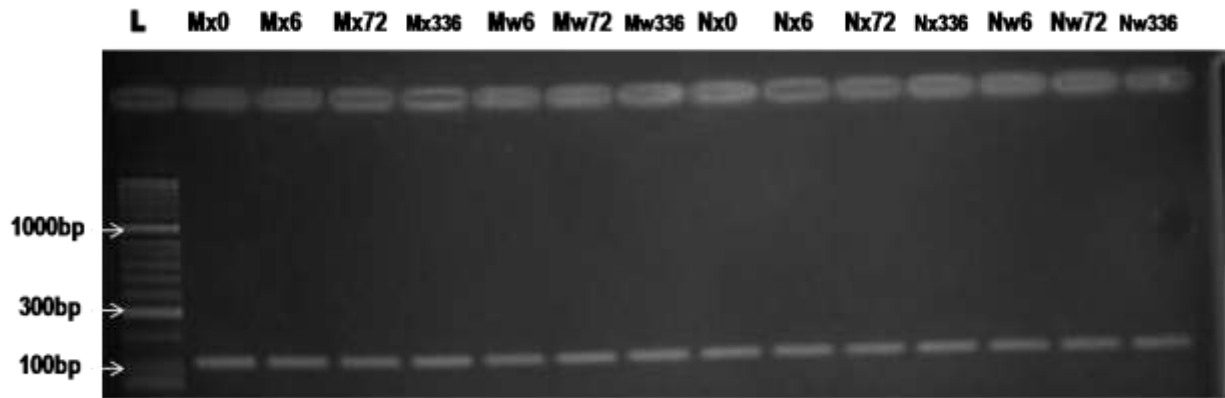## Appendix A. Supplementary data

Figure 3: Expression profile of gene *25S rRNA* in *M. balbisiana* and EAHB cv "Nakinyika" inoculated with Xvm and water. L indicates molecular ladder; Mx indicates *M. balbisiana* inoculated with Xvm, Mw indicates *M. balbisiana* inoculated with water; Nx indicates EAHB cv "Nakinyika" inoculated with Xvm, Nw indicates EAHB cv "Nakinyika" inoculated with water. 0, 6, 72, and 336 indicates the time course of sampling (hours post inoculation).
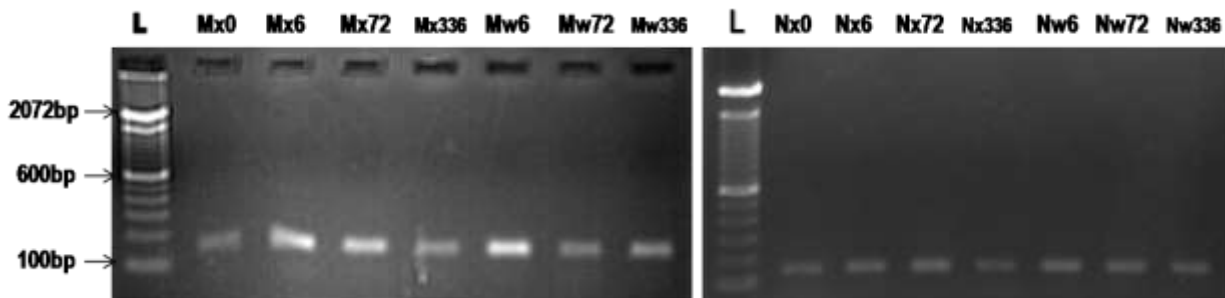

Figure 4: Expression profile of gene *PR3* in *M. balbisiana* and EAHB cv "Nakinyika" inoculated with Xvm and water. L indicates molecular ladder; Mx indicates *M. balbisiana* inoculated with Xvm, Mw indicates *M. balbisiana* inoculated with water; Nx indicates EAHB cv "Nakinyika" inoculated with Xvm, Nw indicates EAHB cv "Nakinyika" inoculated with water. 0, 6, 72, and 336 indicates the time course of sampling (hours post inoculation).
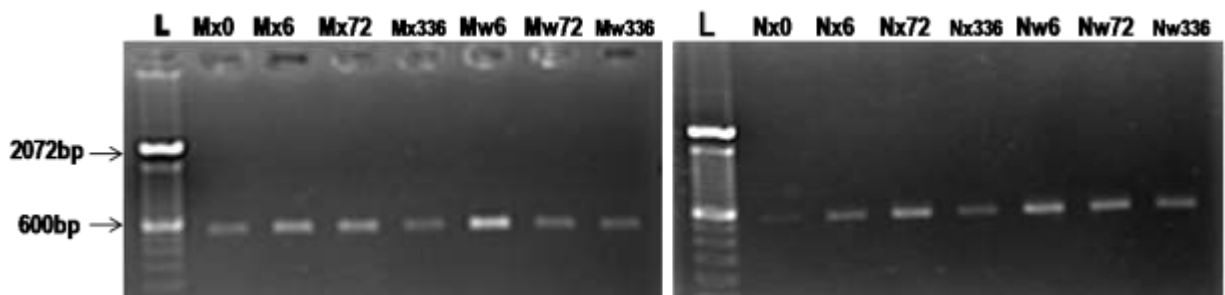

Figure 5: Expression profile of gene *MdNPR1* in *M. balbisiana* and EAHB cv "Nakinyika" inoculated with Xvm and water. L indicates molecular ladder; Mx indicates *M. balbisiana* inoculated with Xvm, Mw indicates *M. balbisiana* inoculated with water; Nx indicates EAHB cv "Nakinyika" inoculated with Xvm, Nw indicates EAHB cv "Nakinyika" inoculated with water. 0, 6, 72, and 336 indicates the time course of sampling (hours post inoculation).
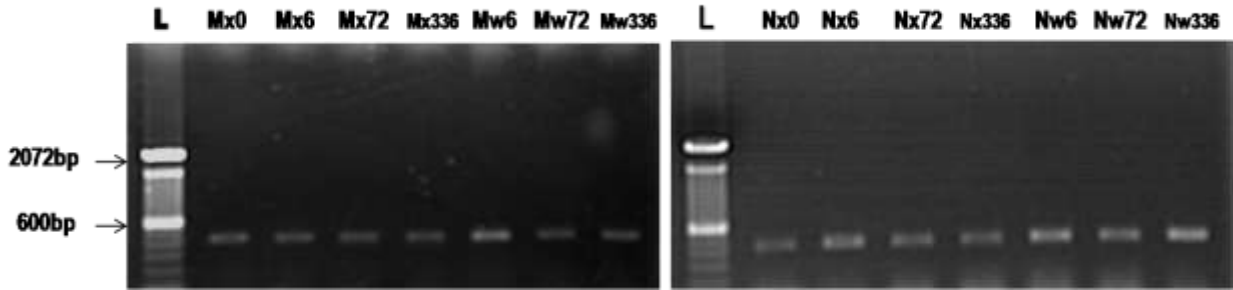
Figure 6: Expression profile of gene *MbNBS* in *M. balbisiana* and EAHB cv "Nakinyika" inoculated with Xvm and water. L indicates molecular ladder; Mx indicates *M. balbisiana* inoculated with Xvm, Mw indicates *M. balbisiana* inoculated with water; Nx indicates EAHB cv "Nakinyika" inoculated with Xvm, Nw indicates EAHB cv "Nakinyika" inoculated with water. 0, 6, 72, and 336 indicates the time course of sampling (hours post inoculation).