CHAPTER TWO

SEQUENTIAL INDUCTION OF *NPR1*-LIKE EXPRESSION IN *XANTHOMONAS* INFESTED BANANA

Endah R, Coutinho T, Chikwamba R. 2010. .*Xanthomonas campestris* pv *musacearum* induces sequential expression of two *NPR1* like genes in banana. *Aspects Appl. Biol*. **96**, Agriculture: Africa's "engine for growth" – Plant Science and Biotechnology holds the key, 325-330.

2.1 Abstract

Two *NPR1* genes have recently been isolated from banana; a jasmonic acid-inducible *MNPR1A* gene and a jasmonic acid/salicylic acid-inducible *MNPR1B* gene. These two banana *NPR1* homologues have been shown to be differentially transcribed in response to the necrotroph, *Fusarium oxysporum* f. sp *cubense* subtropical race 4 (*Foc*), salicylic acid and methyl jasmonate. Treatment with *Foc* only up-regulated *MNPR1A* in the pathogen insensitive banana cultivar GCTCV-218, while the hemibiotroph, *Xanthomonas campestris* pv. *musacearum*, induced transcription of both *MNPR1* genes in this cultivar. Up-regulation of these two genes in response to *X. campestris* pv. *musacearum* further correlated with up-regulation of the banana *PR-1* and *PR-3* genes. The up-regulation of both genes shows that *X. campestris* pv. *musacearum* sequentially induces a jasmonic acid and salicylic acid-mediated response during its infection cycle. This observation indicates the possible involvement of both these pathways in banana- *X. campestris* pv. *musacearum* interactions.

2.2 Introduction

Induction of *PR* genes requires the activation of the *non-expressor of PR1* (*NPR1*) genes (Mou *et al*., 2003; Srinivasan *et al.*, 2009; Le Henanff *et al*., 2009). These NPR1-like genes are co-regulatory proteins whose activities are affected by pathogens such as *Psuedomonas syringae* and various elicitors including salicylic acid (SA), Jasmonic acid (JA) and Ethylene (Cao *et al*., 1994, Yuan *et al*., 2007). Since its discovery, several *NPR1* homologues have been identified and isolated from many crops. In plants in which these genes have been characterised, such as *Oryza sativa* and *Vitis vinifera*, over-expression or transient expression of homologous *NPR1* genes leads to increased accumulation of *PR* genes and enhanced disease resistance to pathogens such as *Xanthomonas oryzae* pv. o*ryzae* and *Plasmopara viticola* (Quilis *et al*., 2008; Le Henanff *et al*., 2009).

PR gene produts are antimicrobial in nature, conferring resistance in plants to viruses, yeast, bacteria and fungal pathogens like Xan*t*homonas, *Magnaporthe grisea*, *Botrytis*, *Fusarium* and *Candidas albicans* (Kim *et al*., 2001; Bonasera *et al*., 2006; Van Loon *et al*., 2006; Park *et al*., 2010). Most PR proteins such as PR-1 and PR-3 are induced downstream of the *NPR1* pathway and their induction requires an interacton between fully functional NPR1 monomers and TGA2 transcription factors (Zhang *et al*., 2003) to mediate defense.

To further enhance the understanding on *NPR1* gene expression in banana against the hemi-biotrophic banana pathogen *X. campestris* pv. *musacearum*, which is the causal agent of the devastating banana bacteria wilt disease the Cavendish banana cultivar GCTCV-218 and Grand

Naine were treated with an inoculum of the pathogen. This pathogen is currently being reported to account for huge losses in the banana industry (Tushemereirwe *et al*., 2004; Ndungo *et al*., 2006). GCTCV-218 and Grand Naine were selected for this study because they have been used in previous studies to characterize the expression of the *MNPR1A* and *MNPR1B* genes to the necrotrophic pathogen *F. oxysporum* f. sp cubense (*Foc*; Endah *et al*., 2008). GCTCV-218 is an engineered resistant banana cultivar shown to have less sensitivity to *Foc* as well as better horticultural characteristics (Hwang and Ko, 2004). Grand Naine is one of the resistant Cavendish banana cultivars that replaced Gros Michel in the 80s. However, it is currently reported to be very senstivie to a range of banana pathogens including *Mycosphaerella fijiensis*, burrowing nematodes and *Foc* (Harelimana *et al*, 1997; Noupadji and Tomekpe, 1999). The specific objective was therefore to investigate if these two previously characterized *NPRI* genes together with the banana *PR-1* and *PR-3* genes are expressed in the banana cultivars in response to infection with *Xanthomonas*.

2.3 Materials and methods

2.3.1 Plant growth, inoculation and sampling

Two-week-old tissue-cultured banana plants (cv Grand Naine and GCTCV-218) were grown as previously described by Endah *et al*. (2008) and as shown in Fig. 2.1A and B. For plant infection, *X. campestris* pv. *musacearum* (Bacteria culture collection; BCC 007; FABI-South Africa) was cultured on a yeast glucose chalk agar medium. The bacteria culture was then resuspended in sterile distilled water to a final concentration of 10^7 colony-forming units per mL as outlined by Gandhi and Chand (1988). Each banana plant was then infiltrated with 100 µL of the bacteria innoculum just above the corm (Fig. 2.1C) using a 2.5 mL syringe fitted with a 21 gauge needle (New Promex Corporation, South Africa). Control plants were infiltrated with distilled water. The root system was harvested at time points 0, 12, 24 and 48 h post treatment (Fig. 2.1D) and quickly flash frozen in liquid nitrogen to stop metabolism. Samples for each time point consisted of root material pooled from three individual plants.

A

B

C

D

Figure 2.1 Growth of banana plants, plant inoculation and sampling of root materials. Two-week-old tissue cultured banana plants were grown hydroponically in 250 mL disposable cups containing 150 mL of distilled water (A) for a further 3-weeks (B). Plant pseudostems were infiltrated just above the corm and using a needle-fitted syringe (C) with either 100 µL of a 10^7 colony-forming units per mL *Xanthomonas campestris* inoculums, or with water (control plants). The entire root system was then harvest (D) 0, 12, 24 and 48 h-post treatment for qRT-PCR.

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

Total RNA from roots was extracted using the Qiagen RNeasy extraction kit following the manufacturer's recommendations (Qiagen, Germany). Complimentary DNA (cDNA) synthesis was carried out with 5 µg of DNA-free RNA using the first strand reverse transcriptase cDNA synthesis kit (Promega, USA). Primers for qRT-PCR were obtained from genes encoding the MNPR1A, MNPR1B, PR-1 and PR-3 proteins. Primer sequences were identical to sequences previously reported by Endah *et al*. (2008) (Table 2.1). The *Musa* 25s rRNA served as the endogenous control gene for the qRT-PCR procedure.

Table 2.1: Banana primers used for quantitative real-time polymerase chain reaction to measure *MNPR1* and *PR* transcription.

Primer Name	Primer sequence
MNPR1A-Forward	GTCGGCATTGTACCAACACA
MNPR1A-Reverse	CAGTGCAGGAGTCAGCAAAA
MNPR1B-Forward	AGGTTTGCCCGAACAAGAAG
MNPR1B-Reverse	TGAGAGGCAACAACTCAGAGAG
PR-1-Forward	TCCGGCCTTATTTCACATTC
PR-1-Reverse	GCCATCTTCATCATCTGCAA
PR-3-Forward	GGCTCTGTGGTTCTGGATGA
PR-3-Reverse	CCAACCCTCCATTGATGATG
Musa 25s-Forward	ACATTGTCAGGTGGGGAGTT
Musa 25sRNA-Reverse	CCTTTTGTTCCACACGAGATT

The qRT-PCR procedure was carried out on cDNA from root samples using the Sybr 480(R) kit (Sigma Aldrich, UK) following the manufacturer's instructions. The Sybrgreen master mix (Roche Diagnostics, UK) was used for qRT-PCR as follows: 5 µL of the diluted template (1/10), 1 µL of 10 µM of primers, 10 µL SYBR-Green I master mix and 3 µL nuclease-free water were added into respective wells in a 386 well RT-PCR micro-titer plate. Non-template control reactions containing water instead of cDNA as template were included in the analysis. The qRT-PCR procedure was set up as follows: DNA denaturation for 10 min at 95°C, followed by an amplification phase of 40 cycles consisting of a denaturation step at 94°C for 5 s, annealing at 60°C for 5 s and DNA extension at 72°C for 10 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 each gene was done to a final arbitrary concentration of 1 (stock) and sequential dilutions (factor 2) to 0.5 0.25, 0.125, 0.0625, 0.03125 and 0.015625. For each primer set, non template control (NTC) reactions containing water as template were included and each reaction was set in triplicates. The experiment was repeated twice with a different set of independent banana plants which had been grown, treated and the roots sampled identically as described above.

2.3.3 Data analysis

By use of the manufacturer's software program (Roche Diagnostic, Light Cycler 480) the crossing point value (CP) for each sample was obtained. All qRT-PCR data were analyzed

following steps described in the Applied Biosystems, User Bulletin No. 2 (2000) and steps described in Livak and Schmittgen (2001). The basal relative transcript amount for each sample was expressed relative to the 25s rRNA gene (Applied Biosystems, User bulletin No.2, 2001).

The crossing point value (CP) represents the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The log input amount was calculated using the formula:

Log input = (CP-value) –b)/m

where b = y intercept or crossing point and m = slope of the standard curve line. The input amount (CN-value) in nanogram (ng) was calculated using the formula:

Input amount (CN-value) = Log_{10} input

The normalised expression value of each gene was determined by dividing the average of the CN value of the target gene by the average CN-value of the control gene (*Musa* 25s rRNA). These values are unit less and represent the relative expression. Samples at time point zero were used as the calibrator and all other samples divided by their calibrator value (Applied Biosystems, User bulletin No.2, 2001).

The standard deviation (SD) and the average input per treatment for each target gene ($SD_{Targetgene}$) and the control gene ($SD_{control}$) were calculated before calculating the normalised values. The normalised SD for each treatment was calculated using the formula: SQRT [($SD_{control\ gene}$ / Average input $_{control\ gene}$)^2 + ($SD_{target\ gene}$ / Average input $_{Target\ gene}$)]* Normalised amount of

target (CN-value) for each different treatment. The relative SD was finally calculated by dividing the normalised SD with the calibrator input for each individual treatment.

Significant differences for each treatment and between the two cultivars was analysed by One-way ANOVA and the Tukey highest square difference (HSD) test using the Statistica software (StatSoft, Inc. 2006). The significant threshold value (p) was set at 0.05. Values lower than 0.05 ($P < 0.05$) were considered to be significantly different.

2.4 Results

2.4.1 *MNPR1* induction in infected banana plants

After *Xanthomonas* treatment, a six-fold significant (P<0.05) increase of *MNPR1A* transcript was observed in the less sensitive GCTCV-218 cultivar at 12 h post treatment (Fig. 2.2A). Expression subsequently decreased at 24 h post treatment but remained significantly 4.5 fold higher (P<0.05) when compared to transcription at 0 h. No significant change in expression of the *MNPR1A* gene was found for Grand Naine. In GCTCV-218, *MNPR1B* transcripts increased significantly (P<0.05) by 8.9-fold at 24 h post treatment before decreasing to the basal value at 48 h post treatment (Fig. 2.2B). Such increases were not observed in Grand Naine.

2.4.2 *PR-1* induction in infected banana plants

The banana *PR-1* transcripts in GCTCV plants increased significantly (P<0.05) by 10-fold 12 h post *Xanthomonas* treatment and were significantly 8.2-fold higher (P<0.05) at 48 h post treatment when compared to the basal expression value (one-fold) (Fig. 2.2C). Amounts of transcripts of *PR-3* were significantly higher (P<0.05) at 12 h (3.9-fold) and 24 h (2.7-fold) post treatment in GCTCV-218 when compared to basal expression at time 0 h (Fig. 2.2D). In comparison, no significant (P>0.05) change in *PR-1* and *PR-3* expression was observed for Grand Naine throughout the 48 h post infection period.

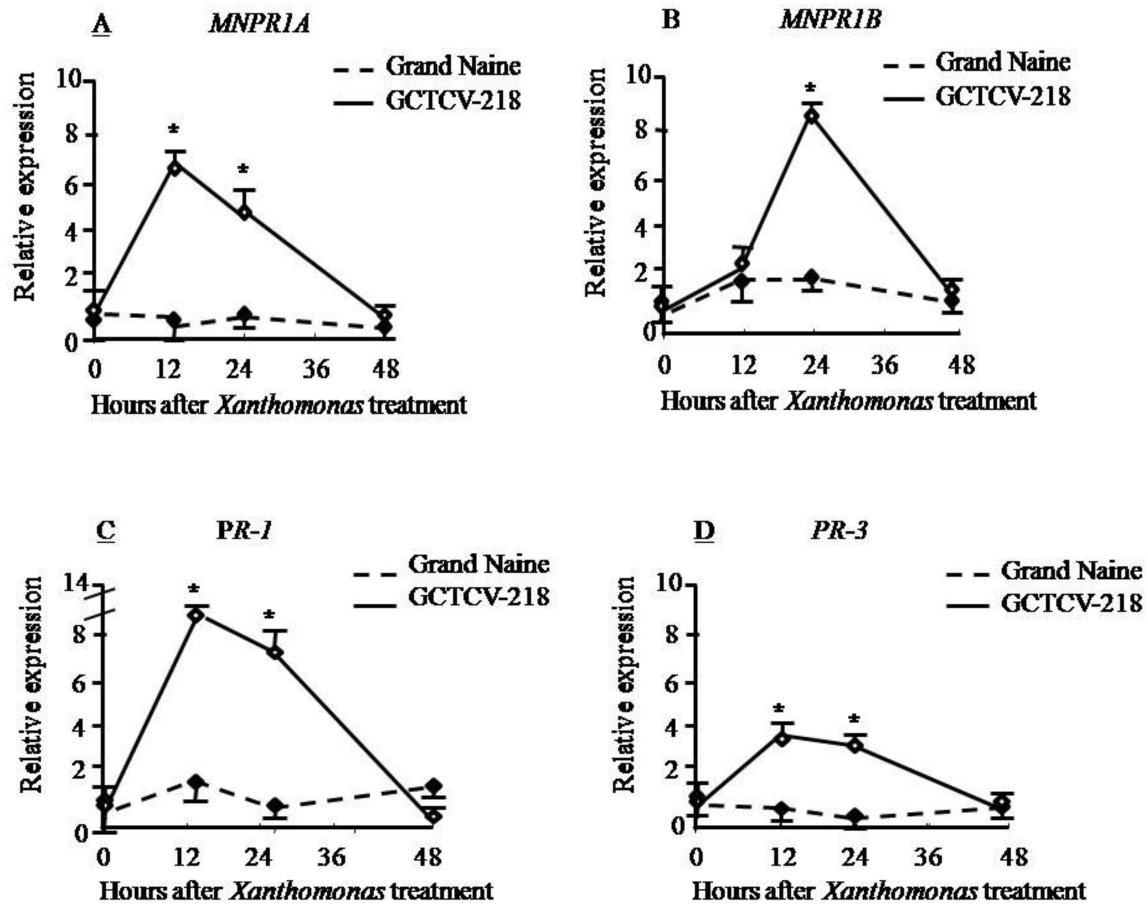

Figure 2.2 Relative gene expression of *MNPR1A*, *MNPR1B*, *PR-1* and *PR-3* in *Xanthomonas*-infected Cavendish banana cultivar Grand Naine and GCTCV-218. Relative gene expression of *MNPR1-A* and *MNPR1-B* (A and B) and *PR-1* and *PR-3* (C and D) was determined and compared in each of the cultivar relative to a 'calibrator', the expression at 0 h. The relative expression ratios obtained from control plants infiltrated with water at each time point was subtracted from those of plants infiltrated with the *Xanthomonas* inoculum to obtain the effect due to infection only. Results are means ±SEM of six individual plants.*Significant difference at P<0.05.

2.5 Discussion

In this part of the study *NPR1* gene expression has been characterized in GCTCV-218 and Grand Naine in response to *X. campestris* pv. *masacearum*. As a new result it was found that, unlike *Foc,* which has been shown to induce only the *MNPR1A* gene (Endah *et al*., 2008); *Xanthomonas* induced transcription of both *MNPR1A* and *MNPR1B* genes in GCTCV-218. *NPR1* gene transcription after *Xanthomonas* infection found in this study is further similar to the increase in transcription found for the rice *NPR1* gene after treatment of transgenic *Xa21* over-expressing rice plants with the rice pathogen *X. oryzae* pv. *oryzae* (Yuan *et al*., 2007). According to Van Loon *et al*. (2006) the ability of *Xanthomonas* to provoke the induction of these two banana genes is attributed to its mixed life style as a hemi-biotroph.

Transcription of both *NPR1* genes was further directly related to an increased transcription of the banana *PR-1* and *PR-3* genes. This is in contrast to a previous study (Endah *et al*., 2008) where *PR-3* and *MNPR1B* transcription was not induced in GCTCV-218 in response to a fungal pathogen (*Foc*). There is strong evidence, that, unlike necrotrophic pathogens, such as *Foc*, biotrophes and hemi-biotrophes, such as *Xanthomonas*, induce predominantly a SA-mediated pathogen response (Spoel *et al*., 2007; Tao *et al*., 2009) and SA elicitation has been shown to induce *MNPR1B* transcription (Endah *et al*., 2008).

Endah *et al*. (2008) further hypothesised that *MNPR1A* is responsive to the JA-defence mediated pathway. In this study with *Xanthomonas*, *MNPR1A* being JA-responsive was transcribed much earlier than SA-responsive *MNPR1B*. This suggests an early elicitation of the JA-pathway and

MNPR1A transcription which was followed by SA elicitation and *MNPR1B* transcription at a later stage of *Xanthomonas* infestation. However, *Xanthomonas* is a hemi-biotroph with a predominant biotrophic lifestyle, one would expect the biotrophic stage and SA elicitation at the beginning of infestation and a necrotrophic stage and JA elicitation later in infestation and not *vice versa* (Alfano and Collmer, 1996; Xu *et al*., 2006). However, research in cotton has already shown that during the hypersensitive response to *X. campestris* pv. *malvacearum* (*Xcm*) the JA concentration sharply increases very early (two hours after pathogen inoculation) followed by a much later increase in the SA concentration; six hours at the point of infection and 24 hours in non-infected tissues (Martinez *et al*., 2000; Delannoy *et al*., 2005). This indicates that *Xanthomonas* might not always follow a strict hemi-biotroph lifestyle. In this study with banana, we also found, comparable to cotton, that *X. campestris* pv. *musacearum* induced the sequential transcription of genes that have been previous shown to be inducible by JA and SA (Endah *et al*., 2008). This pattern might suggest that an early increase of JA concentrations could have contributed to the early activation of JA-responsive *MNPR1A* and a late activation of *MNPR1B* (Martinez *et al*., 2000; Delannoy *et al*., 2005). However, since transcripts were measured in systemic tissues and not at the point of infection, we have still to determine if the timing of JA and SA-*MNPR1* transcription might be different at the point of infection.

Overall, data presented here further supports the suggestion of a selective mode of induction of banana *MNPR1* genes to distinct classes of pathogens and the less sensitive nature of GCTCV-218 to pathogens when compared to Grand Naine. Further, elevated transcription of both *MNPR1A* and *MNPR1B* indicates that banana responds to *Xanthomonas* infestation through the sequential elicitation of both a JA and SA-mediated *NPR1*-dependent defense response

mechanism. As a next step towards addressing the function of the two genes in defense response, the next chapter of the thesis uses comparative sequence analysis tools to investigate if the two MNPR1 coding regions harbour defense-related *cis*-elements and how they relate to other identified and characterized NPR1-like sequences.