CHAPTER FIVE

CHARACTERIZATION OF TWO BANANA *NPR1*-LIKE CODING SEQUENCES FOLLOWING PATHOGEN INFECTION

5.1 Abstract

The expression of two banana *NPR1*-like coding sequences (*MNPR1A* and *MNPR1B)* and levels of resistance in transgenic *Arabidopsis* plants were studied after treatment with pathogens from three distinct classes. Treatment of various *Arabidopsis* lines with the biotroph *Hyaloperonospora arabidopsidis*, or the necrotroph *Botrytis cinerea* or the hemi-biotroph *Pseudomonas syringae* pathogens resulted in reduced pathogen proliferation in the transgenic *Arabidopsis* lines compared to the non-transformed *Arabidopsis npr1* plants. Apart from plants treated with *B*. *cinerea*, the reduced pathogen levels found in the transgenic plants were similar to those of the Wt *Arabidopsis* plants and the percentage reduction in pathogen proliferation was higher compared to the mutant plants. Reduced pathogen growth in the *MNPR1*-expressing plants further correlated with increased transcription of the two *MNPR1* transcripts and the *Arabidopsis PR-1* transcript. Put together, the banana *NPR1*-like coding regions were able to complement the *Arabidopsis npr1-2* mutant and restore its tolerance by activating *PR-1* transcription following the application of stress.

5.2 Introduction

NPR1 is a defence response co-transcription gene with a pivotal role in conferring resistance to a wide range of pathogens during the establishment of the SAR and ISR (Pieterse and van Loon 2004; Wally *et al*., 2009). While it has been clearly shown that *npr1* mutants are non-responsive to SA, very sensitive to pathogens and are hampered in their ability to activate various forms of *PR* genes (Cao *et al*., 1994), it is also known that transformation of these mutants with homologues of the *NPR1* gene under the control of defined promoters eliminates these negative effects (Kinkema *et al*., 2000). Transformation of either *npr1-1*, or *npr1-2* mutants with the native *NPR1* cDNA under the control of the 35S promoter led to reduced disease symptoms and increased amounts of *PR-1* transcripts in the transgenic plants compared to the non transformed plants (Kinkema *et al*., 2000; Yuan *et al*., 2007; Shi *et al*., 2010). These studies further highlighted than the *NPR1* gene can only activate downstream *PR-1* transcription upon activation by stressors like pathogens (Kinkema *et al*., 2000). Aside from transgenic research, non-transgenic approaches have also been used to delineate the expression pattern of the *NPR1* gene in many plant species (Endah *et al*., 2008; Zhao *et al*., 2009; Endah *et al*., 2010). This has involved the determination of the endogenous gene's transcript using mRNA gel blots, semi-quantitative RT-PCR or qRT-PCR (Cao *et al*., 1994; Endah *et al*., 2008; Zhao *et al*., 2009; Endah *et al*., 2010). Data from previous studies carried out by our research group indicates that two *NPR1*-like genes from Cavendish banana could have a differential response to various classes of pathogens (biotrophic vs necrotrophic vs hemi-biotrophs) hence, mediating different types of defence response pathways (SA vs JA) in Cavendish banana (Endah *et al*., 2008; Endah *et al*., 2010).

In this work, a transgenic approach was employed to further address the function of the banana *NPR1*-like coding regions after treatment of *MNPR1*-expressing lines with either the biotrophic *Hyaloperonospora arabidospsidis*, or the necrotroph *Botrytis cinerea*, or the hemi-biotroph *Pseudomonas syringae*. Of particular interest was to investigate if coding sequence differences in the *MNPR1* genes translates into a differential activity towards the three classes of pathogen.

5.3 Materials and methods

5.3.1 Growth of *Arabidopsis* plants

Arabidopsis Wt, *npr*, *npr1*:*35S CaMV*, *npr1*:*MNPR1A44*, *npr1*:*MNPR1A56*, *npr1*:*MNPR1A95*, *npr1*:*MNPR1B1*, *npr1*:*MNPR1B12* and *npr1*:*MNPR1B15* seeds were re-suspended in 0.1% bacterio agar solution (w/v; sigma Aldrich) and stratified at 4°C for 3-days before sowing in 4 x 6 cell trays containing peat-based M2 compost (Levington UK). The pots (one of each) where placed in trays without drainage and covered with transparent plastic lids to ensure maximum humidity. These were then transferred to a controlled environment chambers.

The growth chamber was maintained at 23°C, 10 h day/14 h dark regime and a relative humidity of 65 ± 5%. The plastic lids were removed from the trays after 2-weeks when the plants had developed at least two true leaves. Plants were then maintained under the above mentioned growth conditions for a further 3-weeks. Through out the experimental period, pots were kept moist by sub-irrigating them twice a week. Except stated otherwise, all experiments were performed on 5-weeks-old soil grown plants.

5.3.2 Pathogen growth, plant treatment and sampling

Arabidopsis plants were infected with either a virulent strain of the hemi-biotrophic bacterium *P. syringae* pv. *tomato* -luxCDABE (*Pst*DC3000-Lux; Fan *et al.*, 2008), or with the avirulent *P. syringae* pv. *tomato* avrRPM1 (*Pst*avrRPM1; JIC, England), or the necrotrophic fungus *B.*

cinerea B05.10 (kindly provided by Dr Henk-jan Schoonbeek, University of Fribourg), or the biotrophic fungus *H. arabidopsidis* (Sainsbury laboratory, JIC-England).

5.3.2.1 *Growth of P. syringae bacterial strains and plant infection*

All *P. syringae* strains were initially cultured from glycerol stocks in 90-mm Petri dishes (Plastpro Scientific, South Africa) containing King's B medium [20 gL^{-1} proteose peptone (w/v), 1.5 gL^{-1} di-potassium hydrogen phospohate (w/v), 1.5 gL^{-1} magnesium sulphate (w/v), 1.5% glycerol (v/v) and 1.2% bacterio agar (w/v)] supplemented with 50 mgL^{-1} kanamycin. Petri dishes were incubated at 28°C for 48 h. Twenty-four hours post inoculation, a loop-full of each bacteria strain was re-suspended in 1 mL of a 10 mM $MgCL_2$ solution. A portion of this inoculum (100 µL), was spread on Petri dishes containing King B's medium supplemented with the appropriate antibiotic and incubated at 28°C.

Plant infection was done in a two step process using a 1-mL syringe without a needle as follows: one leaf from each plant was infiltrated with an inoculum of *PstavrRPM1* ($5x10^5$ $cfu.mL^{-1}$) prepared in 10 mM $MgCL_2$. This was to induce a systemic acquired response (SAR). To assess the level of SAR, three additional non-inoculated leaves were infiltrated with the virulent lux-tagged *Pst*DC3000-Lux inoculum ($5x10^6$ $cfu.mL^{-1}$) 48 hours after the primary inoculation (AV treatment). Plants for the control (mock) experiment were pre-infiltrated in the same manner with a 10 mM $MgCL_2$ solution only. A secondary infiltration of of three additional non-inoculated leaves was then performed 48 hours after the primary inoculation using the virulent lux-tagged *Pst*DC3000-Lux inoculum (MV treatment). Samples for qRT-PCR were harvested 48 h after the

primary and secondary infection and quickly frozen in liquid nitrogen. For adequate statistical analysis, all the above experiments were repeated twice.

5.3.2.2 *Growth of Botrytis cinerea and plant infection*

B. cinerea B05.10 was cultured at 20°C under continuous light for 2-weeks on malt extract agar (30 gL^{-1}) and yeast extract (2 gL^{-1}) medium (MEYA; Oxoid, UK) as described by Schoonbeck *et al*. (2003). Four days prior to infection, plates were exposed overnight to UV-light to induce sporulation. An inoculum was prepared from these sporulating cultures at a concentration of 2.5 x 10^5 spores/mL in ¼ strength MEYA liquid medium. For each plant to be infected six leaves were used and 5 µL droplets of the inoculum was dropped onto the upper side of each leaf using a pipette. Plants for the control experiment were treated in the same way with 5 µL droplets of the re-suspension solution (1/4 strength MEYA liquid medium). Trays containing plants were covered with a plastic dome to achieve close to 100% humidity. Plants for qRT-PCR were however sprayed generously with either the inoculums or the control solution and trays were covered and incubated as described above. Samples for qRT-PCR were harvested at 48 h post treatment and quickly frozen in liquid nitrogen. The above experiment was repeated twice.

5.3.2.3 *Propagation of Hyaloperonospora arabidopsidis* and *plant infection*

H. arabidopsidis was propagated weekly on 2-weeks-old soil-grown *Arabidopsis* plants using a modified method described in Rairdan *et al*. (2001). Plants in trays were covered with a plastic dome and maintained at 15°C. Leaves were then detached from these plants and placed in five

50-mL Falcon tubes containing dH_2O (10 plants per tube). The tubes were shaken vigorously to release the attached spores and, the solution in all tubes was filtered using a miracloth. The spore suspension was collected into a clean 50-mL Falcon tube. The initial number of spores was counted using 3 µL droplets of the spore suspension on a haemocytometer under a light microscope. The spore count was repeated four times with separate 3-µL droplets of the spore suspension and the average spore count noted. The final spore concentration was adjusted to 2.5 x 10^4 spores/mL. Plants to be infected (3-wk-old soil grown plants) were each sprayed with 5 µL of the inoculums and trays were covered and incubated as described above. Samples for qRT-PCR were harvested at 48 h post treatment and quickly frozen in liquid nitrogen. The above experiment was repeated twice.

5.3.3 Assessment of disease severity after infection

5.3.3.1 *Bioluminescent assay for Pseudomonas syringae measurement*

Growth of *Pst*DC3000-Lux was determined *in planta* 48 h after the secondary challenge using 8-mm leaf discs that were exercised from infected leaves with a cork borer. The bacteria number within the leaf disc was measured using a FB12 luminometer (Berthold Detection Systems, http://www.berthold-ds.com/) carrying a single photon counter as described by Fan *et al*. (2008). A total of 12 plants of which a leaf disc was obtained from each of the three *Pst*DC3000-Lux-infected leaves were used to represent a single time point described by Fan *et al*. (2008). For adequate statistical analysis, all the above experiment was repeated three times with independent samples.

5.3.3.2 *Measurement of lesion diameter in Botrytis cinerea infected plants*

Lesions that developed 3 dpi on leaves treated with 5 µL droplets of *B. cinerea* were measured using a digital caliper. For each data set, lesions from six plants and a total of six leaves per plant were measured. Measurements were repeated twice using independent sets of plants treated as described above.

5.3.3.3 *Conidiophore count after Hyaloperonospora arabidopsidis infection*

Whole *Arabidopsis* rosette leaves were harvested from *H. arabidopsidis*-infected plants 7 dpi for trypan blue staining following steps described in Koch and Slusarenko, (1990). For this, the *Arabidopsis* rosettes were immediately placed in 50 mL Falcon tubes (six rosettes per tube and a total of two tubes per genotype) and trypan blue solution (20 mL of a two-part absolute ethanol to one part of a trypan blue stock v/v solution containing: 10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water and 0.02 g of trypan blue) was added to each tube. These were incubated in boiling water for 2 min to accelerate staining of the leaves and thereafter the trypan blue solution was gently decanted. Leaves were destained overnight using 20 mL of a 70% w/v chloral hydrate and after decanting the distaining solution samples were re-suspended in 80% glycerol. Individual leaves (four each) were mounted on microscope slides for conidiophore counting under a light microscope. This experiment was repeated twice using independent sets of plant material treated in the same manner as described above.

5.3.4 Quantitative real time-polymerase chain reaction

QRT-PCR was performed with leaf material treated and harvested at time points indicated above. All qRT-PCR steps were carried out as described in Chapter 5, using primers outlined in Table 5.2. All samples were normalized using values for the endogenous actin control gene and expressed relative to their control samples harvested at the same time points as the pathogen-treated samples. The Applied Biosystems, User Bulletin No. 2, (2001) and steps described in Livak and Schmittgen (2001) were used for data analysis. (See detailed description in chapter 2 of this dissertation).

5.3.5 Statistical analysis

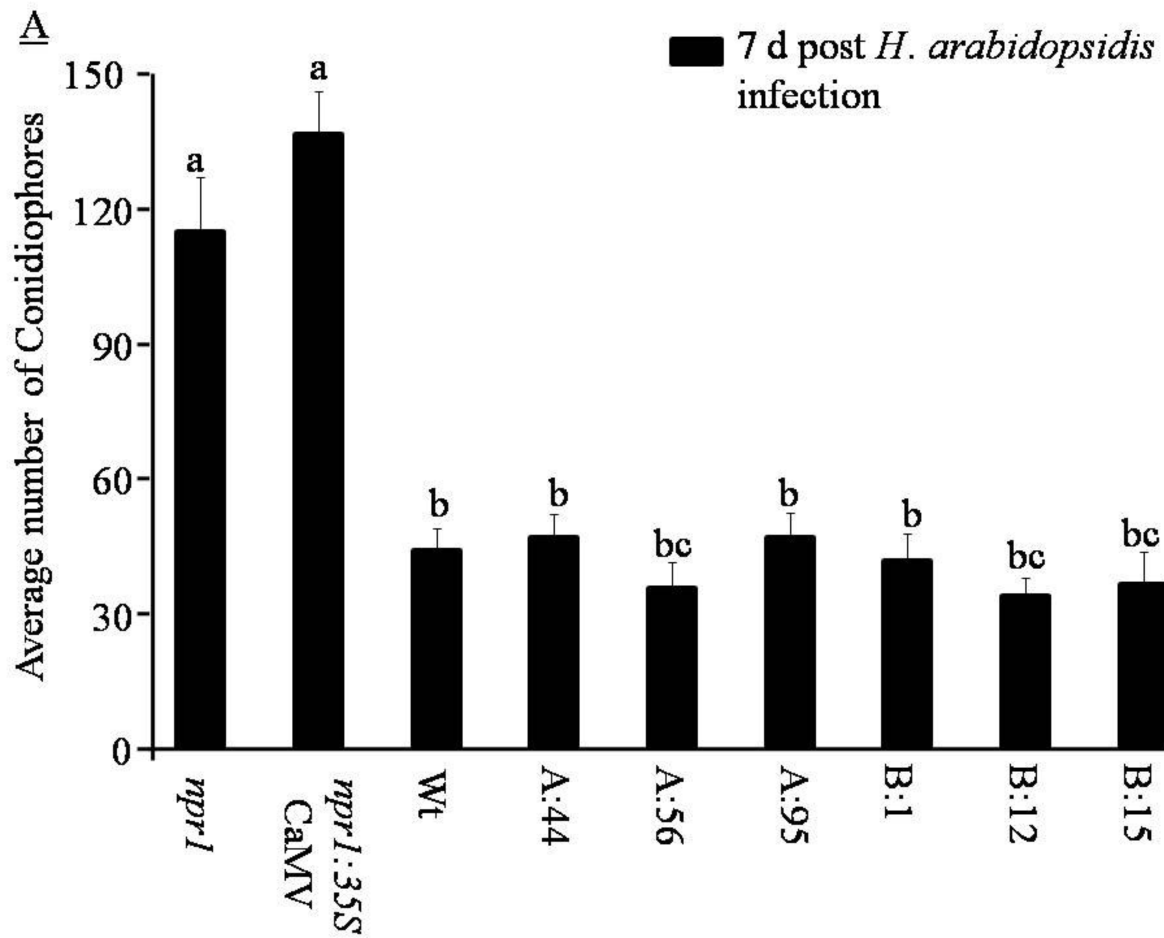
The statistical difference in each experiment over time was done using the Statistical analysis software-SAS® (SAS, USA) and Student T-Test. The cut-of value was set at $P<0.05$ for samples that were significantly different and these differences determined using ANNOVA and Tukey studentized range (HSD) test.

5.4 Results

5.4.1 Limitation of *H. arabidopsidis* conidiophores

Growth of conidiophores resulting from infection with the biotrophic fungus *H. arabidopsidis* was significantly (P<0.05) reduced by between 50% and 68% in the *MNPR1A* expressing lines and by at most 73% in the *MNPR1B* expressing lines compared to the *npr1* mutant *Arabidopsis* plants 7 dpi. In the non transformed Wt plants, *H. arabidopsidis* conidiophores were also reduced by a significant (P<0.05) 53% compared to the *npr1* mutant. However, the percentage of reduced conidiophores for the Wt plant and the complimented mutant lines were not significantly different (P>0.05) from each other (Fig. 5.1A).

Trypan blue stained leaves revealed the presence of at least six conidiophores per 4-mm leaf section in the non transgenic *npr1* mutant plants while in the Wt plants and in the *MNPR1* expressing lines, at most two conidiophores could be spotted within the same 4-mm leaf section under the microscope (Fig. 5.1B). This shows that both the Wt plants and the transgenic lines significantly (P<0.05) limited the development of conidiophores and the establishment of *H. arabidopsidis* over the 7 dpi period in comparison to the non-transformed *npr1* mutant plants whose leaves had more conidiophores.


A
7 d post *H. arabidopsidis* infection
Average number of Conidiophores
150
120
90
60
30
0
a
a
b
b
bc
b
b
bc
bc
npr1
npr1:35S CaMV
Wt
A:44
A:56
A:95
B:1
B:12
B:15

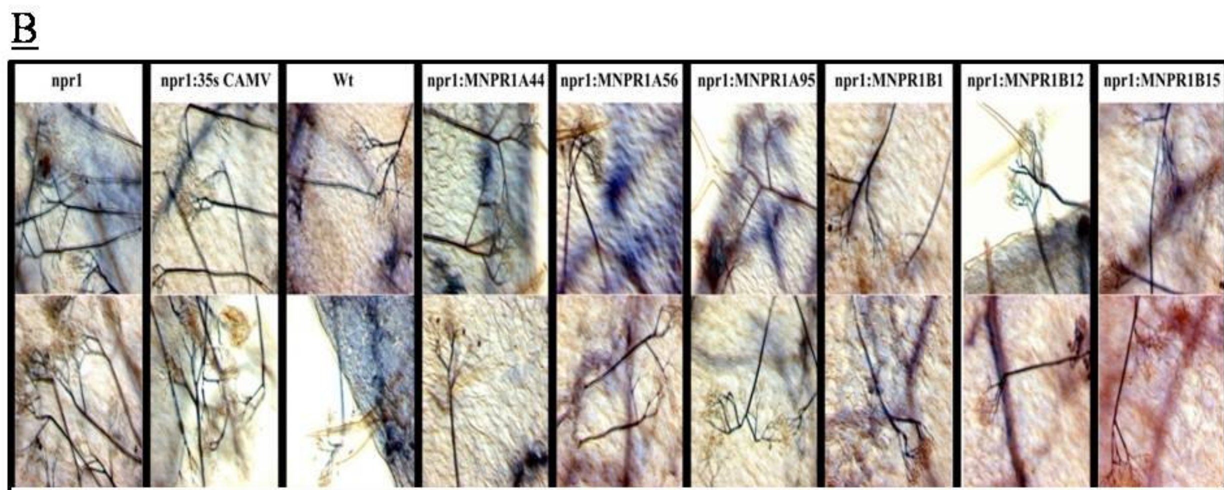

B
npr1
npr1:35s CAMV
Wt
npr1:MNPR1A44
npr1:MNPR1A56
npr1:MNPR1A95
npr1:MNPR1B1
npr1:MNPR1B12
npr1:MNPR1B15

Figure 5.1 *H*. *arabidopsidis* conidiophores present in various *Arabidopsis* genotypes 7 after infection. *Arabidopsis* genotypes (nine) were sprayed with a 2.5 x 10^4 spores/mL of a *H*. *arabidopsidis* inoculum. The mean conidiophores number on 12 plants per genotype was counted 7 dpi and plotted together with the SEM (A). The significant difference among samples was determined using SAS$^{(R)}$ software and samples which were significantly different from each other represented by unidentical letters (a, b, c, d) on the graph. Pictures obtained from a 4-mm leaf section of two different leaves after a 40x magnification are represented in B. The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

5.4.2 Limitation of *B. cinerea* infection in *MNPR1* expressing lines

Measurement of lesion diameter on leaves infected with *B. cinerea* showed that all the complemented lines had significantly (P<0.05) reduced lesion diameter ranging from 38% to 47% and 32% to 35% in the *MNPR1A* and *MNPR1B* expressing lines respectively in comparison to the non transgenic *npr1* mutant *Arabidopsis* plants (Fig. 5.2A). The average lesion diameter in the mutant plants was also not significantly (P>0.05) different from that found in the wt plant 3dpi.

In the leaves of mutant plants, symptoms resulting from infection with *B. cinerea* consisted of brownish-soaked lesions which spread from the site of infection (beyond 6 mm), further degenerating into yellowish secondary necrotic lesions as the pathogen continued to invade the plant (Fig. 5.2B). The lesion diameter on the Wt-*Botrytis* infected plants was also similar to those found on the mutant leaves i.e, spreading and necrotizing. However, in the *MNPR1* expressing lines, these lesions were limited to the site of infection and did not spread beyond 4 mm on the leaf surface during the 72 h infection period.

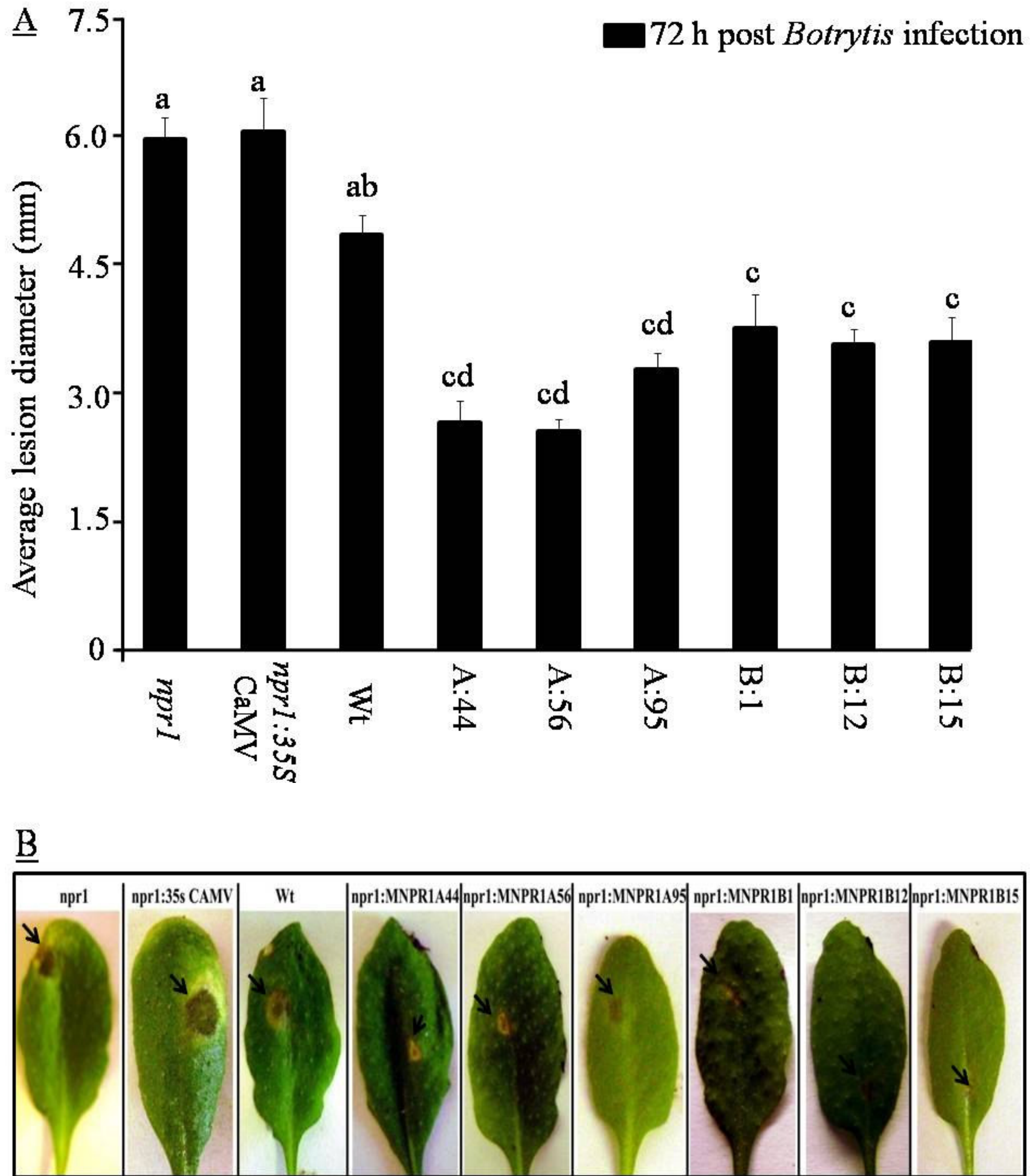


Figure 5.2 Lesions formed on leaves of various *Arabidopsis* genotypes 72 hpi with the necrotrophic fungus *B. cinerea* B05.10. Leaves (six per plant) of nine different *Arabidopsis* genotypes were infected with 5 µL droplets of a 2.5 x 10^5 spores/mL of an inoculum of *B.*

cinerea B05.10. The mean lesion diameter from 36 leaves per genotype was measured 72 hpi and plotted together with their SEM (A). The significant difference among samples was determined using SAS[(R)] software and samples which were significantly different from each other represented by unidentical letters (a, b, c, d) on the graph. Pictures of typical examples of infected leaves were taken to illustrate the lesions formed (B). Arrows on the leaves indicated the point of lesion initiation. The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

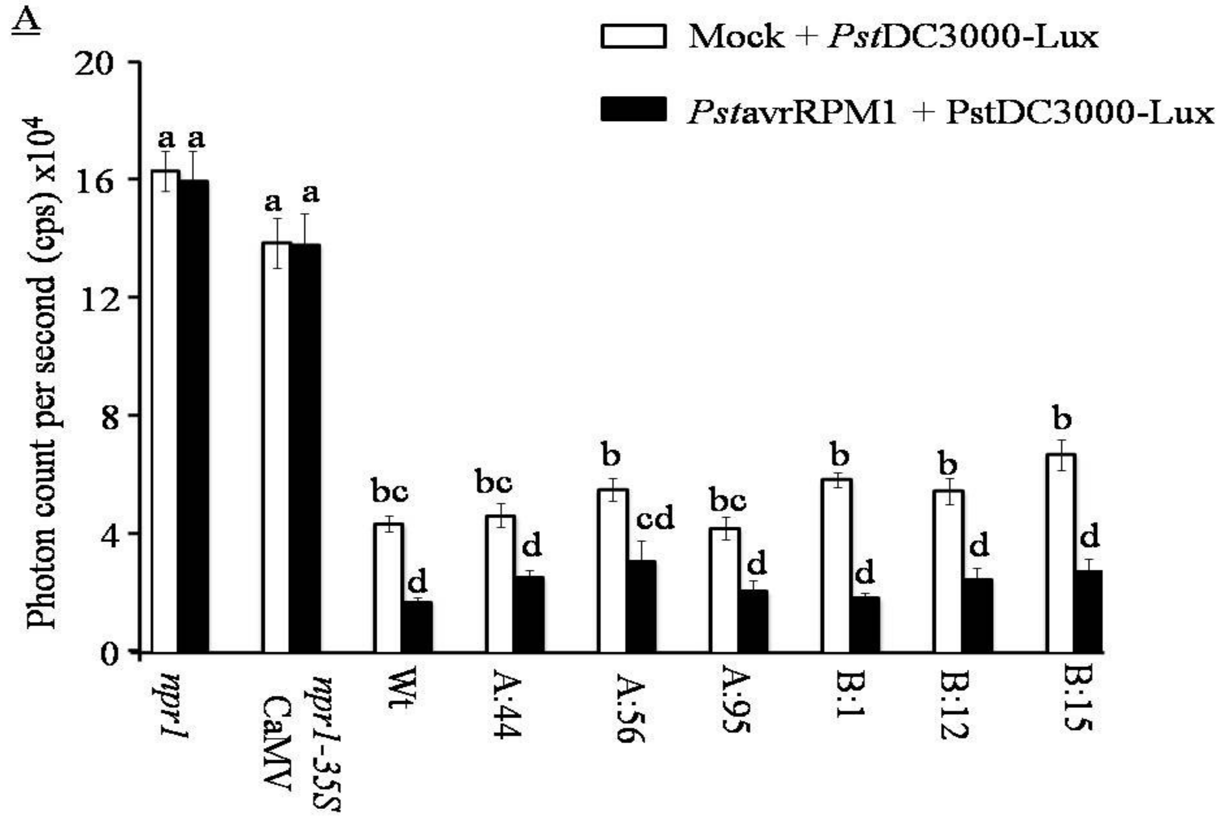
5.4.3 Limitation of *P. syringae* pv *tomato* infection in *MNPR1* expressing lines

Pst-DC3000-Lux bacteria growth was measured 48 hpi in non SAR-induced (MV-treated plants) and SAR-induced plants (AV-treated plants). In the MV-treated leaves, growth of *Pst*DC3000-Lux was significantly ($P<0.05$) reduced by up to 75% 48 h post inoculation in all transgenic lines compared to the non transgenic mutant plants. This reduced pathogen growth found in the *MNPR1* expressing plants was not significantly ($P>0.05$) different from the 72% reduction in *Pst*DC3000-Lux growth found in the Wt *Arabidopsis* plants (Fig. 5.3A; white bars).

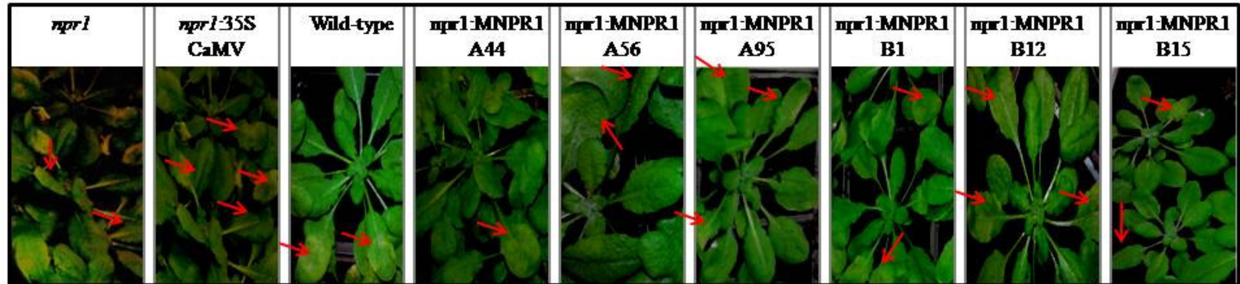
In the AV-treated leaves, 48 h following the *Pst*-lux infection and similar to the MV treatment, all transgenic lines showed a significantly ($P<0.05$) reduced *Pst*DC3000-Lux growth ranging from 83% to 86% compared to the *Arabidopsis* mutant plants. The Wt *Arabidopsis* plants also slowed down the progression of *Pst*DC3000-Lux by a significant 89% compared to the mutant plants although this was not significantly ($P>0.05$) different from the reduced pathogen growth measured in the *MNPR1* expressing plants (Fig. 5.3A; dark bars).

In comparison to the first treatment in which SAR was not induced, treatment two (AV treatment) which led to the induction of SAR limited the growth of *Pst*-lux by a significant ($P<0.05$) 61% in the Wt *Arabidopsis* plant, 43%, 68%, 55% and 59% in line *npr1*:*MNPR1A44*, *npr1*:*MNPR1A96*, *npr1*:*MNPR1B1*, *npr1*:*MNPR1B12* and *npr1*:*MNPR1B15* respectively (Fig 5.3A; white bar/dark bars).

In parallel with these bacteria counts, the *npr1* mutant leaves were more chlorotic with more spreading lesions compared to the Wt *Arabidopsis* plants and the *MNPR1* expressing plants in the non SAR induction (Fig. 5.3B) and SAR induction treatment (Fig. 5.3C).



A: Mock+*Pst*DC3000-*Lux*



B: *PstavrRPM1*+*Pst*DC3000-*Lux*

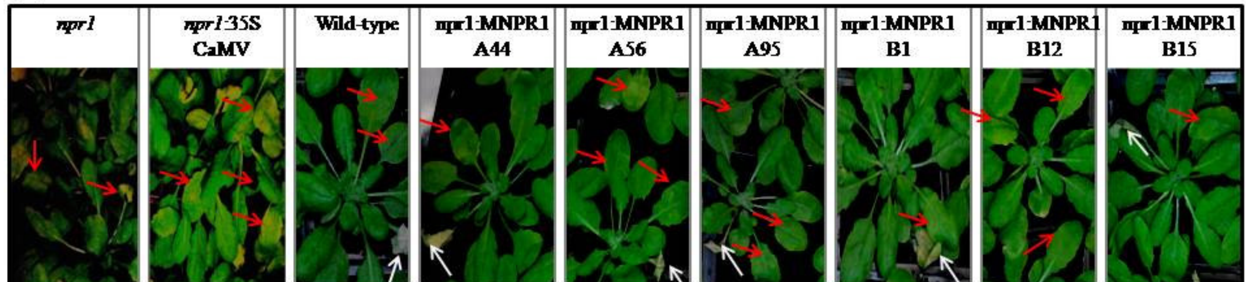


Figure 5.3 Growth of the hemi-biotrophic *Pst*DC3000-luxCDABE (*Pst*DC3000-Lux) in various *Arabidopsis* genotypes before and after SAR induction. *Pst*-DC3000-Lux bacteria growth was measured 48 hpi in non SAR-induced (Mock-*Pst*-DC3000-Lux treated plants) and SAR-induced plants (*Pst* avrRPM1-*Pst*-DC3000-Lux treated plants) using a FB12 luminometer. The mean bacteria growth from 36 leaves per genotype was plotted together with their SEM (A). The significant differences between samples was determined using SAS[(R)] software and bars with unidentical letters (a, b, c, d) were considered to be significantly different ($P<0.05$). Pictures of infected plants were taken digitally. Some of the *Pst*DC3000-Lux infected leaves (red arrows) and *Pst* avrRPM1-infected leaves (white arrows) are represented (B and C). The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

5.4.4 *MNPR1* and *PR-1* transcription

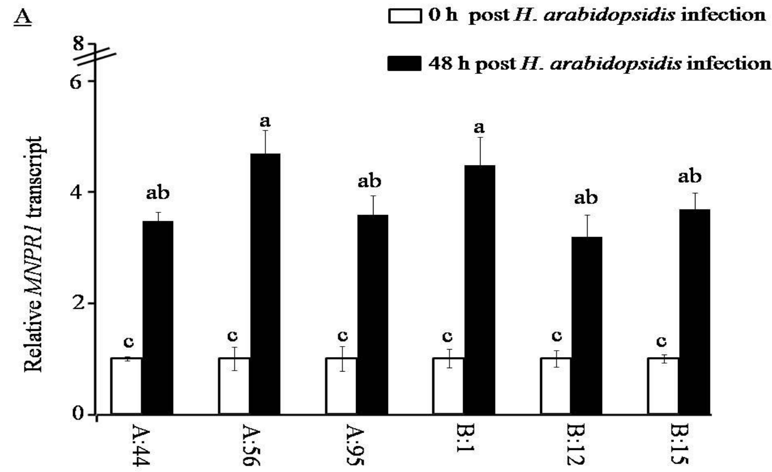
The amount of transcript of *MNPR1* and *PR*-1 genes in all plants used for the study were determined via qRT-PCR and normalized with their endogenous control actin gene before expressing them relative to their various control samples which had not been treated with the pathogen.

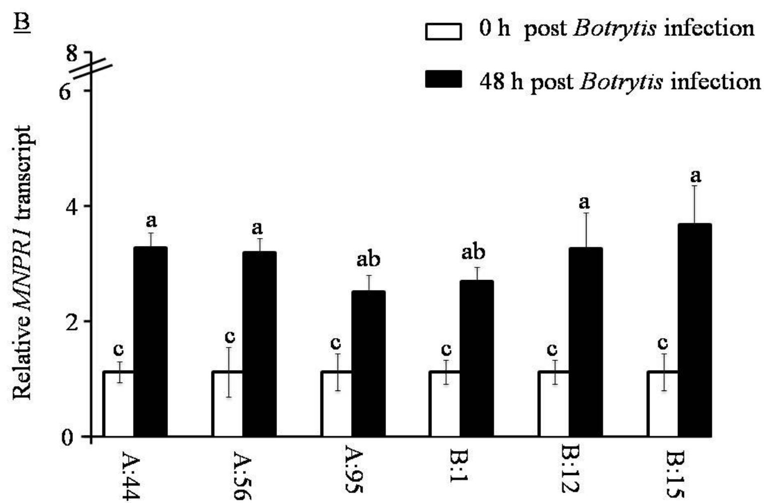
5.4.4.1 *MNPR1 transcription*

After treatment of plants with *H. arabidopsidis*, *MNPR1A* transcripts were induced by 3.5-fold, 5.7-fold and 3.6-fold in *npr1:MNPR1A44*, *npr1:MNPR1A56* and *npr1:MNPR1A95*, respectively, although these were not significantly ($P>0.05$) different from the 5.5-fold, 3.2-fold and 3.7-fold transcript amounts measured in *npr1:MNPR1B1*, *npr1:MNPR1B12* and *npr1:MNPR1B15* lines, respectively (Fig. 5.4A).

In *B. cinerea* infected plants, a 2.9-fold, 2.8-fold and 2.2-fold change in *npr1:MNPR1A44*, *npr1:MNPR1A56*, *npr1:MNPR1A95* transcription was obtained, respectively (Fig. 5.4B). These transcription amounts were also not significantly ($p>0.05$) different from the 2.4-fold, 2.9-fold and 3.3-fold transcript amounts measured in *npr1:MNPR1B1*, *npr1:MNPR1B12* and *npr1:MNPR1B15*, respectively.

The non-SAR induction treatment and SAR induction treatment activated *MNPR1* transcription in all of the transgenic lines used (Fig. 5.4). Similar to treatment with the other pathogens, no significant difference ($P<0.05$) in *MNPR1* transcription was found between the *npr1:MNPR1A* and *MNPR1B* lines.


A
0 h post H. arabidopsidis infection
48 h post H. arabidopsidis infection
Relative MNPR1 transcript
8
6
4
2
0
ab
a
ab
a
ab
ab
c
c
c
c
c
c
A:44
A:56
A:95
B:1
B:12
B:15


B
0 h post Botrytis infection
48 h post Botrytis infection
Relative MNPR1 transcript
8
6
4
2
0
a
a
ab
ab
a
a
c
c
c
c
c
c
A:44
A:56
A:95
B:1
B:12
B:15

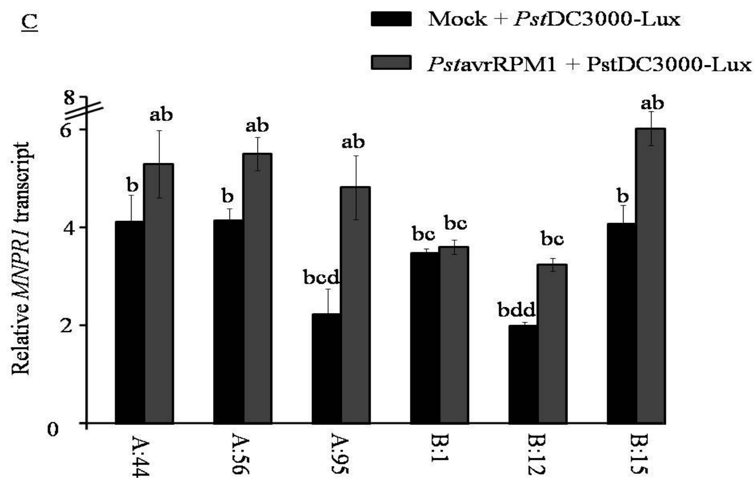

C
Mock + PstDC3000-Lux
PstavrRPM1 + PstDC3000-Lux
Relative MNPR1 transcript
8
6
4
2
0
ab
b
ab
b
ab
bcd
bc
bc
bc
bdd
ab
b
A:44
A:56
A:95
B:1
B:12
B:15

Figure 5.4 Relative *MNPR1* transcript amounts in *MNPR1*-expressing lines after pathogen infection. Various *Arabidopsis* genotypes were treated with either the biotrophic pathogen *H. arabidopsidis* isolate NOCO2 (A); or the necrotrophic fungus *B. cinerea* (B); or the hemi-biotrophic bacteria *Pst* avrRPM1 and *Pst*DC3000-Lux (C). Control plants were treated in the same manner using the re-suspension buffer for each pathogen. *MNPR1* transcript amounts were measured using cDNA from leaf samples harvested from the infected and control plants 48 hpi. Values obtained were normalized with values from their endogenous control gene (ACTIN2) and *PR-1* transcripts expressed relative to the control. The experiment was repeated once and the mean relative transcription values ± SEM of both experiments was plotted. The significant difference among samples was determined using SAS$^{(R)}$ software. Bars on the same graph with unidentical letters (a, b, c, d) are significantly different (P<0.05).

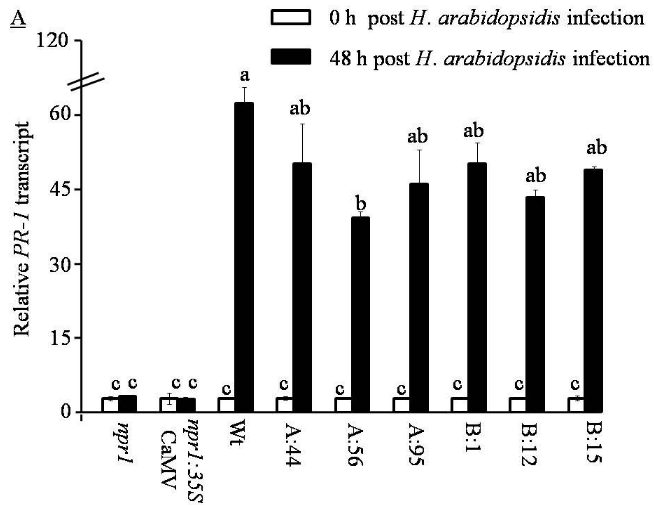
5.4.4.2 *PR-1 transcription*

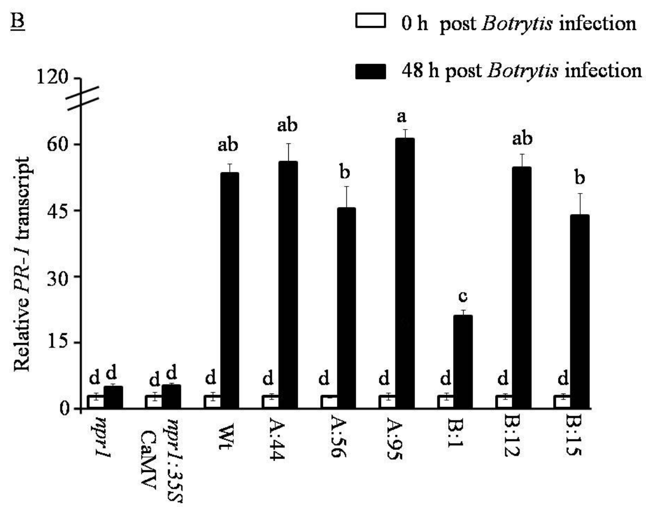
Transcription of the *Arabidopsis PR-1* gene, which is a downstream end product in the *NPR1* pathway, was measured in all the *Arabidopsis* genotypes used in this study. In all the genotypes studied, *PR-1* transcripts were differentially expressed (Fig. 5.5). In plants sprayed with *H. arabidopsidis*, *PR-1* transcripts were induced by 47.4-fold, 43.7-fold and 45.0-fold in *npr1:MNPR1A44*, *npr1:MNPR1A56* and *npr1:MNPR1A95*, respectively. These were significantly (P<0.05) different from *PR-1* expression in the control *npr1* plants but not significantly (P>0.05) different from the 47.7-fold, 41.1-fold, 46.9-fold transcript amounts measured in lines *npr1:MNPR1B1*, *npr1:MNPR1B12* and *npr1:MNPR1B15*, respectively (Fig. 5.5A). The transcript amounts in the *MNPR1* expressing lines were however significantly (P<0.05) less than the 60.8-fold *PR-1* transcript measured for the Wt *Arabidopsis* plants.

A slight but not significant (P>0.05) *PR-1* transcription of 2.7-fold and 2.0-fold was measured in the *npri* and *npr1:35S* CAMV plants after *B. cinerea* infection compared to their controls (Fig. 5.5B). However, in the transgenic lines, *PR-1* was induced by 50.1 fold, 56.9-fold and 42.8-fold in the three *MNPR1A* expressing lines respectively. This was also significantly (P<0.05) higher in comparison to transcript amounts obtained for the infected *npr1* mutant plants. Similarly, *PR-1* transcription of 22.8-fold, 50.4 fold and 40.3 fold in the three *MNPR1B* expressing lines were also significantly (P<0.05) higher than those of the *npr1* plants. Apart from npr1:MNPR1B1 whose *PR-1* transcript was significantly (P<0.05) reduced compared to all the other *MNPR1* expressing lines, the rest of the transgenic plants had no significant (P>0.05) difference in their

various *PR-1* transcript amounts and these were also not significantly (P>0.05) different from the 50-fold *PR-1* transcript measured in the Wt plants.

Following SAR induction (*Pst* avrRPM1 and *Pst*DC3000-Lux treatment), *PR-1* transcripts amount were significantly (P<0.05) higher in all the transgenic lines and the Wt plants in comparison to plants treated with 10 mM $MgCL_2$ and *Pst*DC3000-Lux. These increased *PR-1* transcription was also significantly higher than the *PR-1* transcripts in the *npr1* plants (Fig. 5.5C).


A
0 h post *H. arabidopsidis* infection
48 h post *H. arabidopsidis* infection
Relative *PR-1* transcript
120
60
45
30
15
0
npr1
npr1:35S CaMV
Wt
A:44
A:56
A:95
B:1
B:12
B:15


B
0 h post *Botrytis* infection
48 h post *Botrytis* infection
Relative *PR-1* transcript
120
60
45
30
15
0
npr1
npr1:35S CaMV
Wt
A:44
A:56
A:95
B:1
B:12
B:15

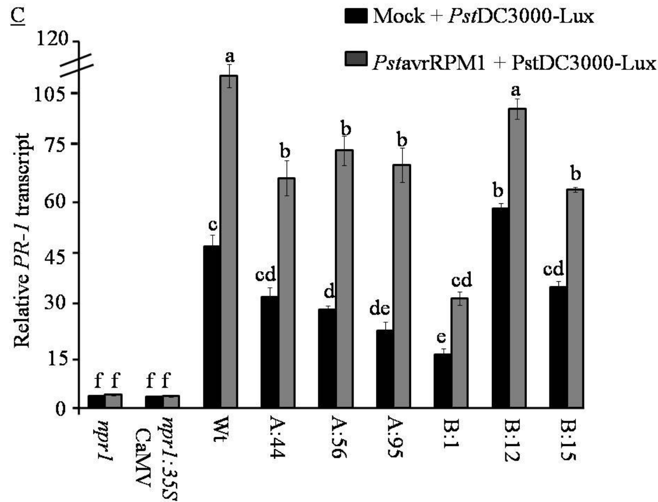


Figure 5.5 Relative *PR-1* transcript amounts in various *Arabidopsis* genotypes after pathogen infection. Various *Arabidopsis* genotypes were treated with either the biotrophic pathogen *H. arabidopsidis* isolate NOCO2 (A); or the necrotrophic fungus *B. cinerea* (B); or the hemi-biotrophic bacteria *Pst* avrRPM1 and *Pst*DC3000-Lux (C). Control plants were treated in the same manner using the re-suspension buffer for each pathogen. *PR-1* transcript amounts were measured using cDNA from leaf samples harvested from the infected and control plants 48 hpi. Values obtained were normalized with values from their endogenous control gene (ACTIN2) and *PR-1* transcripts expressed relative to the control. The experiment was repeated once and the mean relative transcription values ± SEM of both experiments was plotted. The significant difference among samples was determined using SAS$^{(R)}$ software. Bars on the same graph with unidentical letters (a, b, c, d) are significantly different ($P<0.05$).

5.5 Discussion

In the present study, *Arabidopsis* plants expressing either of the two banana *NPR1*-like coding sequences under the control of the 35S promoter and in the *npr1* mutant background were characterized in order to assess whether the *MNPR1A* or *MNPR1B* coding sequence control distinct defense response pathways (SA or JA/ET). This study clearly shows that the two *NPR1*-like genes from Cavendish banana are able to complement the *Arabidopsis npr1-2* mutant. These banana *NPR1*-like genes were transcribed in the *Arabidopsis* mutant, restoring the *NPR1* mediated component of the SAR pathway following the application of stress. Complementation of the *npr1* mutant by other *NPR1* genes is well-documented in literature and is known to restore wild-type responses to a wide array of elicitors including pathogens (Cao *et al*., 1997; Kinkema *et al*., 2000; Rochon *et al*., 2006; Sandhu *et al*., 2009). Such studies on the positive role of the *NPR1* genes in defense response have further been supported by results showing that over-expression of either a native, or homologous *NPR1* gene in *Arabidopsis*, tobacco, wheat, rice, tomato, or carrots leads to increased tolerance to either necrotrophic or biotrophic pathogens in comparison to the non-transformed plants (Cao *et al*., 1998; Fitzgerald *et al*., 2004; Lin *et al*., 2004; Makandar *et al*., 2006, Yuan *et al*., 2007; Meur *et al*., 2008; Quilis *et al*., 2008; Wally *et al*., 2009). Put together and in agreement with our previous expression studies (Endah *et al*., 2008; Endah *et al*., 2010).

Transcription of both *MNPR1* coding sequences in the transgenic lines correlated with those of their respective *PR-1* genes. The relation between *NPR1*-like gene activation and *PR-1* induction under stress conditions has been extensively studied and in most cases, increased *NPR1*

transcription correlated with *PR-1* gene activation (Cao *et al*., 1998; Endah *et al*., 2008; Le Henanff *et al*., 2009; Sandhu *et al*., 2009; Endah *et al*., 2010). For instance, transgenic *npr1-1 Arabidospsis* plants expressing the rice *NPR1* homologue (*NH1*) under the control of the 35S promoter sequence, are reported to have increased *NH1* transcription over time following treatment of the transgenic plants with SA, MeJA or *Xanthomonas* (Yuan *et al*., 2007). Hence it is possible that genes under the control of the 35S CaMV promoter can be responsive to various stresses. *NPR1*-like gene products exist as cytosolic oligomers when in an inactive state (Mou *et al*., 2003; Tada *et al*., 2008; Spoel *et al*., 2009; Lindermayr *et al*., 2010). During pathogen attack or perturbations that are sufficient to cause changes in the cell's redox environment, these oligomers dissociate into monomers and translocate to the nucleus (Mou *et al*., 2003; Tada *et al*., 2008; Le Henanff *et al*., 2009; Spoel *et al*., 2009; Lindermayr *et al*., 2010). As co-transcriptors, momoneric NPR1-like proteins interact with TGA, or WRKY family of transcription factors, facilitating their ability to recognise and bind to activation sequences found in the promoter regions of *PR-1* genes leading to the activation of these genes (Johnson *et al*., 2003; Rochon *et al*., 2006; Wang *et al*., 2006; Kesarwani *et al*., 2007; Pandey and Somssich, 2009). Hence, in accordance with the behaviour of other *NPR1*-like genes, infection of the various plant lines with a pathogen could have provoked such redox changes, activating the banana *MNPR1* genes and favouring downstream induction of PR-1 proteins in the transformed lines.

Increased *PR-1* transcription in these *MNPR1*-expressing lines led to reduced pathogen proliferation compared to the *npr1* plants not expressing the *MNPR1* coding sequences. The most pronounced reduction of up to 89% in pathogen growth was found for the hemibiotrophic *P. syringae* following the activation of the SAR. Such enhanced resistance is attributed to the

readily available defense platform initiated by the avirulent *Pst* strain 48 h before secondary infiltration of the plant with the virulent *Pst*-Lux bacteria during the AV treatment. PR proteins are the final downstream defence molecules during plant-pathogen interactions and most of them have been well studied *in vivo* and *in vitro*. They belong to a multigene family with antimicrobial properties (Niderman *et al*., 1995; Selitrennikoff, 2001; Ferreira *et al*., 2007) and have high enzymatic activities which in turn have deleterious effects on the pathogens' structures (Fritig *et al*., 1998; Ferreira *et al*., 2007; Malnoy *et al*., 2007; Shetty *et al*., 2009; Xu *et al*., 2010). Their expression in plants therefore limits the spread of pathogens, conferring resistance or tolerance to the plants during such an event (Lin *et al*., 2004; Shetty *et al*., 2009; Wally *et al*., 2009; Katilé *et al*., 2010). It is therefore not surprising that in the *MNPR1* expressing lines with increased *PR-1* transcripts, pathogen proliferation was reduced.

Although *MNPR1A, MNPR1B* and downstream *PR-1* genes were transcribed following treatment with either the biotrophic *H. arabidopsidis*, or the necrotrophic *B. cinerea* pathogens resulting to reduced pathogen proliferation, the expression levels of *MNPR1A* and *MNPR1B* were not significantly different from each other for it to be concluded that these banana *NPR1*-like coding sequences are responding differentially to the different classes of pathogens. The discrepancies between this present results and our previous hypothesis which showed a high expression of *MNPR1A* following infection with the necrotrophic *F. oxysporum* pathogen coupled with the inability for SA to induce significantly higher *MNPR1A* transcript in comparison to *MNPR1B*, could have arisen due to the use of only the coding sequence of the *MNPR1* genes under the constitutive 35S promoter. While it is well documented that as a biotroph, *H. arabidopsidis* elicits a SA-defence response pathway which can also be dependent on *NPR1* activity, literature

also highlights that the 35S CaMV promoter sequence is responsive to SA treatment and this response is sufficient for the activation of *PR-1* proteins (Qin *et al*., 1994). It is therefore possible that sensitivity of this constitutive promoter to SA could have favoured activation of the necrotrophic-responsive *MNPR1A* gene after infection with *H. arabidopsidis*. Therefore differential regulation of the two coding regions might also require the genes' specific promoters. Coupled to this, expression of the genes in a dicot rather than in banana (monocot) might have had an effect on the genes' overall responses due to the possible cross-talk among key signal molecules between the two plant systems. Looking at the expression pattern in banana, there may exist certain banana *NPR1*-like transcriptional regulators acting either upstream or downstream of the *MNPR1* gene to fine-tune its response or repress its expression to different elicitors. Whether such regulatory elements do exist in banana and which domains they control within the *MNPR1* sequence is a question which is still to be addressed. However, comparative sequence analysis has already shown that both the *MNPR1* genes are enriched in *cis*-egulatoy elements known to be responsive to SA, JA, ET and pathogens (Chapter 3). Patterns obtained in this study however highlight the complexity of plant-pathogen interaction and the dynamics involving the cross-talk between the SA and JA/ET *NPR1*-dependent defence response.

Such a complex interaction is further evident in this study from the reduced *Botrytis* growth in the *MNPR1* expressing lines compared to the wild-type *Arabidopsis* plants whose lesion diameter was comparable to those of the mutant plants. In *Arabidopsis* plants, *NPR1* is reported to play a secondary role in the local resistance to *Botrytis* as *npr1 Arabidopsis* mutants had lesion sizes comparable to wild-type plants 72 h post *Botrytis* infection (Ferarri *et al*., 2003) although this does not induce SAR (Govrin and Levine, 2002).This resistance is further believed to be

dependent on ET, JA and SA correlating with increased *PR-1* transcription (Ferarri *et al*., 2003). In this present study therefore, because samples analyzed following *Botrytis* infection were also obtained from the locally infected leaves, it might therefore be possible that defense-regulated gene products from *MNPR1*-expressing lines had a stronger responsiveness to this pathogen compared to the wild-type. However, it should also be noted that *PR-1* transcription measured for the Wt and *MNPR1*-expressing plants were not significantly different. Moreover, in the mutant *npr1* plants, although *PR-1* transcription was much reduced, other defense genes such as *PDF1.2* might have been induced in an *NPR1*-independent manner to limit the spread of *Botrytis* (Ferarri *et al*., 2003). It will therefore be interesting to investigate how these plants and their defense-related gene products respond to *Botrytis* when exposed to the pathogen for more that 72 h. A comprehensive analysis of the regulatory elements in the promoter regions of both genes to elucidate if they are different in structure and function is also a subject for future investigation as these promoters are currently being isolated. However, preliminary analysis of a 800bp region of the *MNPR1B* promoter which has already been generated reveals that together with the coding regions of the two banana *NPR1*-like genes, there exist multiple defense-related *cis*-acting elements. These *cis*-elements have been shown in previous studies to be responsive to JA, SA, ET, ABA and various classes of pathogens. Detailed molecular mutational analysis is required for a complete understanding of the mode of action and interacting elements involved in *MNPR1* gene function in response to various stresses.

Nevertheless, in this present work, the sequential expression pattern of *MNPR1A* and *MNPR1B* obtained in the transgenic lines following treatment with the hemi-biotroph *P. syringae*, is similar to that found after treatment of banana plants with the hemi-necrotrophic pathogen *X.*

campestris (Endah *et al*., 2010). This is not surprising because infection with *P. syringae* and *X. campestris* leads to the production of both SA and JA to control its defense (Block *et al*., 2005; De Vos *et al*., 2005).

It is shown here that like other *NPR1*-like genes, the two expressed banana *NPR1*-like coding sequences were transcribed after pathogen attack and further led to the induction of *PR-1* genes which limited the spread of three classes of pathogen: a biotroph (*H. arabidopsidis*), a necrotroph (*B. cinerea*) and a hemibiotroph (*P. syringae*). The next chapter in this dissertation elucidates the role of glutathione and ascorbate in *NPR1* mediated *PR-1* transcription using *Arabidopsis* mutants deficient in cytosolic glutathione.