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Functional characterization of two banana *NPR1* genes for pathogen defense response in *Arabidopsis*

By

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DECLARATION

I, Rosita Endah Yocgo 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.

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CONTENTS **PAGES**

ABSTRACT xi

THESIS COMPOSITION xiii

ACKNOWLEDGEMENT xv

ABBREVIATIONS AND SYMBOLS xix

LIST OF FIGURES xxi

LIST OF TABLES xxv

CHAPTER ONE: INTRODUCTION

1.1 Plant defense responses 2

1.2 Types of defense responses 2

1.2.1 Basal resistance or innate immunity 2

1.2.2 Hypersensitive response 3

1.2.3 Systemic resistance 6

1.3 Deciphering metabolic components of the defense network 7

1.3.1 Role of the *Non-expressor of pathogenesis-related1* genes 7

1.3.2 Structural analysis of the *Nonexpressor of pathogenesis-related1 genes* 9

1.3.3 *Non-expressor of pathogenesis-related1 genes* defense pathway 16

1.3.4 *Non-expressor of pathogenesis-related1* genes interacting elements 19

1.3.4.1 *Reactive oxygen species-antioxidant system and NPR1 interaction* 19

1.3.4.2 *Phytohormones and NPR1 interaction* 25

1.3.4.3 *Transcription factors and NPR1* 27

1.3.4.4 *Pathogenesis-related1 gene* and *NPR1* 28

1.4 Working hypothesis and aim of study 30

CHAPTER TWO: SEQUENTIAL INDUCTION OF *NPR*-1 LIKE GENE EXPRESSION IN *XANTHOMONAS* INFESTED BANANA

2.1 Abstract 34

2.2 Introduction 35

2.3 Materials and methods 37

2.3.1 Plant growth, inoculation and sampling 37

2.3.2 Quantitative real-time-polymerase chain reaction (qRT-PCR) 39

2.3.3 Data analysis 40

2.4 Results 43

2.4.1 *MNPR1* induction in infected banana plants 43

2.4.2 *PR-1* induction in infected banana plants 43

2.5 Discussion 45

CHAPTER THREE: COMPARATIVE ANALYSIS OF TWO BANANA NPR1-LIKE CODING SEQUENCES

3.1 Abstract 49

3.2 Introduction 50

3.3 Materials and methods 52

3.3.1 Alignment of NPR1-like sequencea and identification of conserved regions 52

3.3.2 Phylogenetic analysis 52

3.3.3 Identification of defense-response *cis*-elements with the *MNPR1* coding region 54

3.4 Results 55

3.4.1 NPR1 sequence analysis and identification of conserved regions 55

3.4.2 Phylogenetic grouping of MNPR1 coding sequences 65

3.4.3 Comparison of defense-related *cis*-elements within the *MNPR1* coding sequences 68

3.5 Discussion 74

CHAPTER FOUR: TRANSFORMATION OF AN *ARABIDOPSIS npr1-2* MUTANT WITH BANANA *NPR1*-LIKE CODING SEQUENCES

4.1 Abstract 79

4.2 Introduction 80

4.3 Materials and methods 82

4.3.1 Gene cassette design 82

4.3.1.1 *Plasmids* 82

4.3.1.2 *MNPR1 cloning* 84

4.3.2 *Agrobacterium* transformation 90

4.3.3 Transformation of *npr1-2 Arabidopsis* mutants 90

4.3.3.1 *Growth of npr1-2 Arabidopsis mutant plants* 90

4.3.3.2 *Plant transformation* 91

4.3.3.3 *Screening of transformed lines* 92

4.3.4 Transcription measurement 92

4.3.5 Statistical analysis 95

4.4 Results 96

4.4.1 *MNPR1* gene cassette and *Agrobacterium* transformation 96

4.4.2 Transformation and selection of homozygous transgenic lines 101

4.4.3 *MNPR1 and PR-1* gene transcription 107

4.5 Discussion 110

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

5.1 Abstract 113

5.2 Introduction 114

5.3 Materials and methods 116

5.3.1 Growth of *Arabidopsis* plants 116

5.3.2 Pathogen growth, plant treatment and sampling 116

5.3.2.1 *Growth of Pseudomonas syringae bacterial strains and plant infection* 117

5.3.2.2 *Growth of Botrytis cinerea and plant infection* 118

5.3.2.3 *Propagation of Hyaloperonospora arabidopsidis and plant infection* 118

5.3.3 Assessment of disease severity after infection 119

5.3.3.1 *Bioluminescent assay for Pseudomonas syringae measurement* 119

5.3.3.2 *Measurement of lesion length in Botrytis cinerea infected plants* 120

5.3.3.3 Conidiophore *count after Hyaloperonospora arabidopsidis infection* 120

5.3.3.4 Quantitative real time-polymerase chain reaction 121

5.3.5 Statistical analysis 121

5.4 Results 122

5.4.1 Limitation of *H. arabidopsidis* conidiophores 122

5.4.2 Limitation of *B. cinerea* infection in *MNPR1* expressing lines 125

5.4.3 Limitation of *P. syringae* pv *tomato* infection in *MNPR1* expressing lines 128

5.4.4 MNPR1 and *PR-1* transcription 132

5.4.4.1 *MNPR1 transcription* 132

5.4.4.2 *PR-1 transcription* 136

5.5 Discussion 140

CHAPTER SIX: *NPR1-DEPENDENT PR-1* TRANSCRIPTION REQUIRES A FUNCTIONAL GLUTATHIONE BIOSYNTHETIC PATHWAY

6.1 Abstract 147

6.2 Introduction 148

6.3 Materials and methods 150

6.3.1 Growth of *Arabidopsis* plants 150

6.3.2 Bacteria growth, plant treatment and sampling 151

6.3.2.1 *Growth of pathogens used for infection* 151

6.3.2.2 *Plant infection* 152

6.3.3 Chlorophyll, glutathione and ascorbate measurements 154

6.3.3.1 *Chlorophyll measurement* 154

6.3.3.2 *Measurement of total, reduced and oxidized glutathione* 155

6.3.3.3 *Measurement of reduced, total ascorbate and dehydroascorbate content* 157

6.3.4 Quantitative real time-polymerase chain reaction 159

6.3.5 Statistical analysis 160

6.4 Results 161

6.4.1 Chlorophyll content 161

6.4.2 Glutathione content 162

6.4.3 Ascorbate content 166

6.4.4 *NPR1 and PR-1* transcription 169

6.4.5 Disease progression 171

5.5 Discussion 177

CHAPTER SEVEN: GENERAL DISCUSSION AND FUTURE

PERSPECTIVE 181

REFERENCE 186

ABSTRACT

Functional characterization of two banana *NPR1* genes for pathogen defense response in *Arabidopsis*

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The *Non-expressor of pathogenesis-related1* gene *(NPR1*) mediates the induction of *pathogenesis-related* (*PR*) gene products, vital for resistance in plants. In this study, the role of two previously isolated Cavendish banana *NPR1*-like genes (*MNPR1A and MNPR1B*) has been characterized in protection against *Xanthomonas campestris*, *Hylaperonospora arabidopsidis*, *Botrytis cinerea* and *Pseudomonas syringae* pathogens. The specific aim was to investigate if sequence differences in both genes are responsible for differential activity against pathogens because in a previous expression study, *MNPR1A* and not *MNPR1B* had been more responsive to the banana necrotrophic pathogen *Fusarium oxysporum*. By challenging *Fusarium*-tolerant GCTCV-218 and susceptible Grand Naine Cavendish banana plants (which had been used in a previous characterization study) with the hemi-biotrophic *Xanthomonas* pathogen (a very important economical pathogen of banana), the two *MNPR1, PR-1 and PR-3* genes were found

to be sequentially expressed. Expression of these genes was more pronounced in the tolerant GCTCV-218 banana cultivar than in the sensitive Grand Naine cultivar. Comparative sequence analysis further showed that these two banana NPR1-like coding sequences had dissimilarities even within conserved functional domains; they grouped closely with other defense-related NPR1-like sequences and harboured defense *cis*-regulatory elements. Transformation of the coding sequences of both genes under the control of the 35S CaMV promoter/terminator sequences into *npr1-2 Arabidopsis* mutant complimented the phenotype of this mutant following infection with distinct classes of pathogens (biotrophic *Hyaloperonospora*, necrotrophic *Botrytis* and hemi-biotrophic *Pseudomonas* pathogens). These Infected-*MNPR1*-expressing plants had higher *PR-1* transcript amounts with more reduced pathogen growth compared to non-transgenic *npr1-2 Arabidopsis* mutant plants. However, the difference in the two banana coding sequences did not translate into a differential pattern of response against the three different classes of pathogens used in this study. Further detailed studies are suggested to investigate the role of the *MNPR1* promoter-coding sequences in the differential response to pathogens using a banana-pathogen system. This study also addressed the question of whether cystosolic glutathione (GSH) is necessary for *NPR1* transcription during systemic acquired resistance. Using *Arabidopsis* mutants (*clt1clt2clt3*) defective in cytosolic GSH biosynthesis and following infection with either *Pseudomonas* or *Botrytis*, *NPR1* and *PR-1* transcription was much reduced rendering the mutants more sensitive to pathogens compared to infected-wild-type *Arabidopsis* plants. Results from this study therefore implicate cytosolic glutathione as an essential antioxidant for the establishment of an effective defense response cascade.

Thesis composition

Chapter 1 of this thesis provides a summary of plant defense responses and an up-to-date review of the NPR1 defense co-transcription factor. Various elicitors required for *NPR1* activation, establishment of systemic acquired resistance and induction of *pathogenesis-related* gene products is reviewed. The rational, aim and objectives for carrying out this study is further outlined at the end of the introduction. In **Chapter 2**, the first objective to determine the expression pattern of two banana *NPR1*-like (*MNPR1*) genes and the subsequent response of downstream *PR-1 and PR-3* gene expression in response to a hemi-biotroph is addressed. Using quantitative realtime-polymerase chain reaction, the expression profiles of these genes are measured at specific time points in *Xanthomonas campestris* pv. *musacearum*-infected banana plants. In **Chapter 3** comparative sequence analysis tools such as multiple sequence aligment and phylogenetics are used to campare the two banana NPR1-like coding sequences with 39 already identified and/or characterized plant NPR1-like sequences from genbank. *Cis*-regulatory elements within these two banana *NPR1*-like sequences are also identified and described in relation to their role in defense. **Chapter 4** describes the procees of stably transforming *Arabipdosis npr1-2* mutant plants with the two *MNPR1* coding sequences under the control of the 35S cauliflower mosaic virus promoter and terminator sequences. The basal transcript amounts of the *MNPR1* coding sequences and of the *Arabidopsis PR-1* gene are further determined in homozyous transgenic lines expressing the *MNPR1* coding sequences. In **Chapter 5**, the response of the plants expressing the two banana *NPR1* coding sequences to pathogen is evaluated in greater detail with specific emphasis on whether the difference in coding sequence within these two genes leads to differential response to various classes of pathogens (necrotroph,

biotroph and hemi-biotroph). The role of cystosolic glutathione in *NPR1* transcription which mediates *PR-1* gene induction and the establishment of systemic acquired resistance is addressed in **chapter 6** using *Arabidopsis* mutants that are deficient in cytosolic gluthatione. **Chapter 7** summarises novel results generated from this work with special focus on how this study has contributed to an advanced understanding of the banana *NPR1*-like genes in defense response to pathogens. It further highlights the important role of the two banana genes in conferring resistance against a broad spectrum of pathogens. The chapter also outlines new research activities that can be applied to further our knowledge of the two *NPR1*-like genes in banana. This is followed by a **reference** list of citations used in this dissertation.

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ABBREVIATIONS AND SYMBOLS

^{o}C	Degree Celcius
%	Percentage
µg	Microgram
µL	Microlitre
bp	Base pair
cDNA	Complimentary DNA
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
g	Grams
h	hours
H_2O	Water
L	Litre
LB	Luria broth
M	Molar
mM	Millimolar
mL	Millilitres
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NaAC	Sodium acetate
ng	Nanogram
PCR	Polymerase chain reaction
DNase	Deoxyribonuclease
rpm	Revolutions per minutes
min	minute
s	Second
wk	week (s)
sd H_2O	Sterile distilled water
UV	Ultraviolet

LIST OF FIGURES

Figure 1.1

*At*NPR1 protein structure representing its three functional domains. **11**

Figure 1.2

Arabidopsis thialana NPR1 pathway (adapted from Mukhtar *et al*., 2009). **18**

Figure 1.3

The ascorbate-glutathione cycle (adapted from Smirnoff, 1996; Noctor and Foyer, 1998; Asada, 2000). **21**

Figure 1.4

GSH biosynthesis and transportation across the chloroplast. **24**

Figure 2.1

Growth of banana plants, plant inoculation and sampling of root materials **38**

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. **44**

Figure 3.1

Multiplle alignment of MNPR1A and MNPR1B with other plant NPR1-like amino acid coding sequences. **64**

Figure 3.2

Multiple alignment and evolutionary relationship of the various NPR1-like coding sequences. **66**

Figure 3.3

Defense-related *cis*-regulatory elements identified within the *MNPR1* coding

sequences. **69**

Figure 4.1

p*Bin19-LBR* vector map. **83**

Figure 4.2

Selected restriction sites within the *MNPR1A and MNPR1B* coding region. **85**

Figure 4.3

Plasmid maps of p*LBR:MNPR1A and* p*LBR:MNPR1B.* **89**

Figure 4.4

Restriction digest of p*Gem:MNPR1A and* p*Gem:MNPR1B* clones showing the 1.8 kb *MNPR1* fragment and the 3 kb p*Gem-T-Easy* plasmid backbone. **97**

Figure 4.5

Full length *MNPR1A and MNPR1B* coding region with added restriction sites. **98**

Figure 4.6

Partial sequence of the 35S CaMV promoter: *MNPR1*:35s CaMV terminator gene cassette. **100**

Figure 4.7

Kanamycin selection of plants derived from an *npr1* transgenic event. **102**

Figure 4.8

800 bp PCR products from putative transformed *Arabidopsis* plants (T-1 generation) carrying *npr1:MNPR1A and npr1:MNPR1B* DNAs after kanamycin selection **104**

Figure 4.9

PCR products from homozygous *npr1*:MNPR1A and *npr1*:MNPR1B plants. **106**

Figure 4.10

MNPR1 and *PR-1* transcript amounts in tested *Arabidopsis* plants. **109**

Figure 5.1

H. arabidopsidis conidiophores present in various *Arabidopsis* genotypes after infection. **123**

Figure 5.2

Lesions formed on leaves of various *Arabidopsis* genotypes 72 hpi with the necrotrophic fungus *B. cinerea* B05.10. **126**

Figure 5.3

Growth of the hemi-biotrophic *Pst*DC3000-LuxCDABE (*Pst*DC3000-Lux) in various *Arabidopsis* genotypes before and after SAR induction. **130**

Figure 5.4

Relative *MNPR1* transcript amounts in *MNPR1*-expressing lines after pathogen infection. **135**

Figure 5.5

Relative *PR-1* transcript amounts in various *Arabidopsis* genotypes after pathogen infection. **139**

Figure 6.1

Reduced glutathione content in *Pseudomonas*-infected wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. **164**

Figure 6.2

Total and reduced ascorbate content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. **167**

Figure 6.3

Relative *NPR1 and PR-1* transcript amounts in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. **170**

Figure 6.4

Bacteria growth measurement in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. **172**

Figure 6.5

Disease symptoms on wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. **174**

Figure 6.6

Lesion diameter and disease symptoms in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *B. cinerea*. **176**

LIST OF TABLES

Table 1.1

Selected *R* genes in plants (adapted from Liu *et al*., 2007). 5

Table 1.2

Newly identified *npr1* alleles (adapted from Canet *et al*., 2010). 14

Table 1.3

PR-1 protein family from various plants (adapted from Van Loon *et al*., 2006). 29

Table 2.1

Primers used for quantitative real-time polymerase chain reaction. 39

Table 3.1

NPR1-like amino acid sequences used for alignment. 53

Table 3.2

Percentage identity of 41 NPR1-like amino acid coding sequences. 56

Table 3.3

Elicitor/light-defense cis-elements in the coding region of *MNPR1A* and *MNPR1B* sequences. 70

Table 4.1

Primer sequences for the amplification of the *MNPR1* coding region. 84

Table 4.2

Primer sequences for determining transcripts of *PR-1 and* banana *NPR1* genes in *Arabidopsis*. 94

Table 6.1

Chlorophyll content in *Pseudomonas*-treated wild-type and *clt1clt2clt3*

mutant *Arabidopsis* plants. 161

Table 6.2

GSSG content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. 162

Table 6.3

GSH/GSSG ratio in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. 165

Table 6.4

DHA content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. 168

CHAPTER ONE

INTRODUCTION

1.1 Plant defense responses

Plants are consistently faced with multifaceted environmental challenges which impacts negatively on their development and vigor. Such challenges arise from their inevitable interactions with biotic components (pathogens, insect and animals), abiotic components (unfavorable temperature, water shortage, floods) and a combination of both stress components. These components very often act in synergy resulting in stressful conditions for the plant (Agrios, 2005). Abiotic stress factors enhance the vulnerability of plants to attack from biotic factors adding to the complexity of the stress. However, plants have evolved complex but coordinated responses to protect themselves from such stress factors. Such responses, which are of great economic importance, involve a well-organized interplay of metabolic networks aimed at limiting possible damages (Agrios, 2005) as a result of exposure to stress inducing factors.

1.2 Types of defense responses

1.2.1 Basal resistance or innate immunity

Plants constitutively synthesize cell wall components, waxy epidermis, cuticles and barks to support its architecture as well as to prevent invaders to access interior cells (Agrios, 2005). However, because these are usually not sufficient to ensure for complete protection, they have instituted a basal defense mechanism (Nürnberger *et al*., 2004; Zhang and Zhou, 2010). Such basal resistance is induced early during the invasion process and mediated by pathogen/microbe-associated molecular patterns (PAMP/MAMP). These PAMPs which can arise from either non-

pathogenic, or pathogenic organisms include elicitors such as glycoproteins from oomycetes, lipopolysaccharides, peptidoglucans, bacterial flagellin, bacterial elongation factor-Tu (EF-Tu), mannans of yeast and chitins from fungal cell wall; which are essential for the survival of the microbe (Nürnberger *et al*., 2004; Zhang and Zhou, 2010). Plants have in turn evolved pattern recognition receptors (PRR; Lacombe *et al*., 2010; Zhang and Zhou, 2010) to sense and prevent the establishment of these pathogens. These PRRs, which are mostly membrane-localized proteins, are currently being extensively studied and includes the flagellin receptor (FLS2), EF-Tu receptor (EFR) and the chitin receptor (CERK1) proteins from *Arabidopsis*. They are known to counteract the effect of the PAMPS (Lacombe *et al*., 2010; Zhang and Zhou, 2010).

1.2.2 Hypersensitive response

It is well-known that due to continuous evolution processes, pathogens have developed ways of bypassing the plant's innate immunity provoking the plant to mount an additional response known as the hypersensitive response (Liu *et al*., 2007). Comparable to programmed cell death (PCD) in animals, the hypersensitive response (HR) in plants is a form of voluntary suicide of plant cells at the site of invasion limiting access to nutrients and minerals for the pathogen (Liu *et al*., 2007) and progression to other cells. This response entails an interaction between an avirulent (*avr*) gene produced by the pathogen and a resistance (*R*) gene from the plant (Bonas and Ackerveken, 1999; Liu *et al*., 2007). Widely known as a gene-for-gene model, this form of disease resistance is highly specific and only possible if the *avr* product of the pathogen is recognized by the *R* gene in the plant. If not, the plant is susceptible and disease ensues (Bonas and Ackerveken, 1999; Liu *et al*., 2007). These *avr* genes encode chitinases and cell wall

hydrolysing enzymes which play important roles in rendering the plant's cell wall more permeable, facilitating penetration of the microbe (Agrios, 2005).

A number of the already cloned and/or characterized *R* genes encode conserved motifs consisting of nucleotide-binding sites (NBS), leucine repeat rich (LRR) region, Toll-interleukin-1 receptor (TIR), protein kinase domain (PK), Coiled-coil (CC), or leucine zipper (LZ) structure (Table 1.1; Liu *et al*., 2007) which interact with specific *avr* genes to mediate resistance.

Table 1.1 Selected *R* genes in plants (adapted from Liu *et al*., 2007).

Gene	Host	Pathogen	Protein type
L, LI, LII, LH, M, P,P2	Flux	*Melampsora lin*	TIR-NBS-LRR
***Bs*4**	Tomato	*Xanthomonas campestris*	TIR-NBS-LRR
N	Tobacco	*Tobacco mosaic virus*	TIR-NBS-LRR
RRSI-R	*Arabidopsis*	*Ralstonia solanscearum*	WRKY-TIR-NBS-LRR
***Pi*9, *Pi*2, *Piz*-t**	Rice	*Magnaporthe grisea*	NBS-LRR
***Cre*3, *Cre*1**	Wheat	*Heterodera avenuae*	NBS-LRR
***Rpg*1**	Barley	*Puccinia gramminis*	Protein kinase
***RPP*27**	*Arabidopsis*	*Peronospora parasitica*	Receptor-like protein
Pto	Tomato	*Pseudomonas syringae*	Ser/Thr protein kinase
***XA*21**	Rice	*Xanthomonas oryzae*	LRR-Ser/Thr protein kinase
***Xa*21D, *Xa*26**	Rice	*Xanthomonas oryzae*	LRR receptor-like kinase
***Cf*-9, *Cf*-2, *Cf*-4, Hcr9-4E, *Cf*-2/5**	Tomato	*Cladosporium fulvum*	LRR-TM
***RPS*2, *RPM*1**	*Arabidopsis*	*Pseudomonas syringae*	CC-NBS-LRR
RPM*1, *Prf	Tomato	*Pseudomonas syringae*	CC-NBS-LRR

1.2.3 Systemic resistance

There is evidence that the HR is not always effective in providing ample resistance and blocking pathogens from accessing the interior of cells. However, this hypersensitivity contributes in most cases to trigger an intensified state of readiness in which plant resources are assembled in preparation for future attack (Nürnberger *et al.*, 2004; Grant and Lamb, 2006). Referred to as the systemic acquired response (SAR), this leads to the activation of a chain of defense components in distal un-infected parts of the plants (Nürnberger *et al.*, 2004; Grant and Lamb, 2006). In addition, this has a long lasting effect providing protection to the plant against vast numbers of other invading pathogens (Nürnberger *et al.*, 2004; Potlakayala *et al*., 2007).

A further form of systemic resistance, which can arise independent of a HR trigger, is induced systemic resistance (ISR). ISR is well-known to be provoked by plant growth promoting rhizobacteria (PGPR) via a pathway which could be different from the SAR pathway (Pieterse and Van Loon, 1999; Hossain *et al*., 2007). The rhizobacterium *Pseudomonas putina* LSW17S and *Bacillus cerus* strain AR156 confers induce resistance to *Fusarium oxysporum* forma specialis (f. sp.) l*ycopersici* and/or *Psuedomonas syringae* pv. *tomato* (Ahn *et al*., 2007; Niu *et al*., 2011).

Although the importance of ISR is continuously being reported, there is evidence that ISR is not as effective as SAR in limiting the spread of secondary pathogens following a primary attack (Herman *et al*., 2008). Nonetheless, both ISR and SAR can act synergistically to provide an enhanced resistance (Van Wees *et al*., 2000; Herman *et al*., 2008). It is also well-established that

an organized interplay of molecules, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), products of the *Non-expressor of pathogenesis related1* (*NPR1*) genes mediate the establishment of an effective hypersensitive, or systemic defense response pathway. For instance, treatment of tomato plants with benzothiadiazole (BTH; a SA analogue) lead to elevation in both SA- and ET-mediated responses which is sufficient to reduce disease severity following *P. syringae* pv. *tomato* attack (Herman *et al*., 2008). All these molecules ultimately cause the production of antimicrobial *pathogenesis-related* (*PR*) genes (Cao *et al*., 1994; Pieterse and Van Loon, 2004; Verhagen *et al*., 2004; Wang *et al*., 2006) which act as final products in limiting the proliferation and colonization of the invading microbe in plant cells.

1.3 Deciphering metabolic components of the defense network

1.3.1 Role of the *Non-expressor of pathogenesis-related1* genes

Since its discovery in 1994, the gene encoding the Non-expressor of pathogenesis-related1 (NPR1) protein is currently widely accepted to play a central role in the defense response cascades conferring tolerance to various biotic stressors (Cao *et al*., 1994, Fitzgerald *et al*., 2004; Lin *et al*., 2004; Makandar *et al*., 2006, Meur *et al*., 2008; Wally *et al*., 2009; Shi *et al*., 2010). This gene was identified and isolated from *Arabidopsis* mutant plants that were unable to respond to SA treatment and incapable of mounting a SAR response (Cao *et al*., 1994). It has further been shown to share some similarity with the I-$_K$B protein in mammals (Ryals *et al.*, 1997). In mammals, the I-$_K$B protein interacts with NF-$_K$B transcription factors inhibiting the translocation of the protein (Ryals *et al.*, 1997).

Arabidospsis is known to harbor six *NPR1*-like sequences within its genome (*Arabidopsis* genome initiative, 2000; Liu *et al*., 2005) with the *blade-on-petiole 1* (*BOP1;* At3g57130) and *blade-on-petiole 2* (*BOP2;* At2g41370) known to be involved in plant morphogenesis (Liu *et al*., 2005). Although *NPR1* is well known as a positive mediator of defense to pathogens like *P. syringae* the role of *NPR2* has not yet been reported. However, a possible overlapping function for *NPR2* with *NPR1* has been suggested (Chen *et al*., 2008). *NPR3* and NPR4 are closely related sharing a 34% and 38% identity respectively to NPR1 at the amino acid level (Lui *et al*., 2005). These two paralogs are reported to negative mediate defense responses in *Arabidopsis* as mutant *npr3* and *npr4* plants, had increased *PR-1* transcripts following INA treatment as well as enhanced resistance to *P. sygringae* and *Hyaloperonospora parasitica* (Zhang *et al*., 2006). Rice harbors five *NPR1*-like homologues designated *OsNPR1 homolog1 - NH1*: DQ450948; *OsNPR2*: DQ450950; OsNPR3: DQ450952, *OsNPR4*: DQ450954 and *OsNPR5*: DQ450956 of which three (*OsNPR1*, *OsNPR2* and *OsNPR3)* have been isolated and implicated in defense response (Chern *et al*., 2001; 2005; Yuan *et al*., 2007). In *Vitis vinifera* (grapevine), two *NPR1*-like genes (*VvNPR1.1* and *VvNPR1.2*) have been identified (Le Hananff *et al*., 2009) and in *Theobroma cocoa* (Cocoa), one *NPR1*-like gene (*TcNPR1)* has been isolated and shown to compliment *npr1-2 Arabidopsis* mutant plants (Shi *et al*., 2010). *Glycine max* (soybean) harbours two already identified and isolated *NPR1*-like (*GmNPR1.1* and *GmNPR1*.2) sequences in its genome (Sandhu *et al*., 2009) while banana contains three already cloned *NPR1*-like gene, two of them have been isolated from Cavendish banana AAA (*MNPR1A* and *MNPR1B;* Endah *et al*., 2008) and one has been isolated from ABB Dongguan dajiao *Musa* spp. (*MdNPR1*; Zhao *et al*., 2008).

Amino acid sequence comparison shows that these three banana sequences share varying identities to each other with MNPR1A and MNPR1B being 78% identical to each other (Endah *et al*., 2008). These two banana NPR1 genes shared 47%, 48% identities with the *Arabidopsis* NPR1 sequence. However, it remains unknown if these banana *NPR1*-like genes are allelic as have been reported for the *Arabidopsis NPR1*-like genes (Kinkema *et al*., 2000). Moreover, cultivated banana plants in the genus *Musa*, are derived from an interaction of the wild diploid banana species *M. acuminata* and *M. balbisiana* (Pillay *et al*., 2006) contributing either the A or B genome, respectively. Resultant cultivars resulting from this hybridisation could either be diploid (AA, AB, BB), triploid (AAB, AAA, ABB), or tetraploid (AAAB, AABB, ABBB) (Ortiz *et al.,* 1995 and Pillay *et al*., 2006) thus additing to the complexity of the origin and possible existence of additional *NPR1*-like genes in various banana cultivars. A detailed multiple alignment study still needs to be done to understand how the ABB banana *NPR1*-like gene related with the *MNPR1* genes. This will form part of this study. In addition to this, a southern analysis also has to be done to at least determine how many copies of the genes are present in different banana cultivars with similar or dissimilar genome compositions.

1.3.2 Structural analysis of the *Non-expressor of pathogenesis-related1* genes

A considerable amount of information currently exist indicating key *cis* elements and amino acids (AA) within the NPR1 coding sequence which are important to mediate the NPR1 function. Moreover, through structural alignments and/or mutational studies, these elements have further been identified and characterized in homologous NPR1-like sequences from various other

plants (Meur *et al*., 2006; Le Henanff *et al*., 2009; Speol *et al*., 2009, Endah *et al*., 2010; Shi *et al*., 2010).

At the N-terminal (AA 10-15) of the *A. thaliana* NPR1 (*At*NPR1*)* is a phospodengron motif (Spoel *et al*., 2009). These motifs have been identified in proteasome-regulated substrates like the I-$_K$B. They are further known to play a role in protein degradation (Hayen and Ghosh, 2004). Using antibodies that specifically recognize the serine AA at position 11 and 15 in this motif, Spoel and colleagues (2009) demonstrated that very little serine 11 and serine 15 was phosphorylated in untreated wild-type and transformed NPR-GFP *Arabidopsis* plants. However, in SA-treated plants, these residues were subjected to a nuclei-specific phosphorylation. This phosphorylation in *At*NPR1 was further shown to be important for NPR1 protein turnover promoting SAR (Speol *et al*., 2009).

Downstream of the phospodengron motif of the *At*NPR1 sequence (AA 35-194) is the Bric-a-Brack Poxvirus and zinc finger (BTB/POZ) domain also located at the N-terminal. Residues at position 80-91 are described as the core of this domain (Rochon *et al*., 2006). Specifically, residues situated at position 80-84 and 87-91 within this core have been shown to directly interact with the transcriptional factor (TF) TGA2 (Rochon *et al*., 2006). This TF, which can act downstream of NPR1, is required for the activation of PR proteins (Zhang *et al*., 2003; Rochon *et al*., 2006). The BTB/POZ domain (Fig 1.1) is linked to the ankyrin repeats (position 238-371) and both domains are involve in protein-protein interactions (Coa *et al*., 1997; Becerra *et al*., 2004; Rochon *et al*., 2006) therefore accounting for the inability of the NPR1 to bind directly to DNA (Bardwell and Treisman 1994).

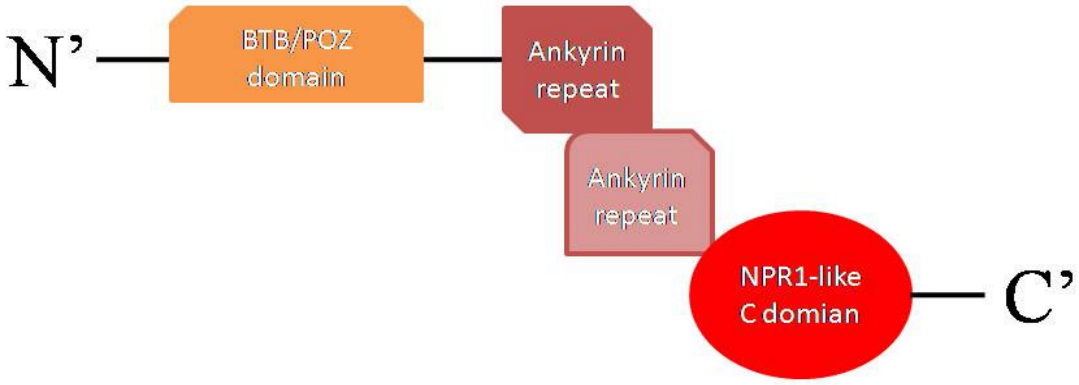


Figure 1.1 *At*NPR1 protein structure representing three of its functional domains. The *Arabidopsis thialana* NPR1 protein sequence was obtained from genbank (At1g64280) and the conserved regions deduced from the sequence. From the N-terminal (N') to the C-terminal (C') is found the Bric-a-Brack Poxvirus and zinc finger (BTB/POZ) domain, ankyrin repeats and a putative NPR1-like C domain. (http://0-www.ncbi.nlm.nih.gov.innopac.up.ac.za/protein/NP_176610.1).

Three nuclear localization signals (NLS) have been identified and characterized in *At*NPR1. These are situated in position 252-265, 541-554 and 582-593 representing NLS1, 2 and 3, respectively (Kinkema *et al*., 2000). Of these three, NLS, NLS1 is found in the first ankyrin repeat of the protein and plays no role in controlling the translocation activity of the protein (Kinkema *et al*., 2000). NLS2 and NLS3 situated in the C-terminal have been shown to be important for the translocation of the protein to the nucleus as mutation of residues in this region retained the protein in the cytosol blocking *PR*-1 induction (Kinkima *et al*., 2000).

The NPR1 protein is also enriched with conserved cysteine residues. In *At*NPR1, 17 cysteine residues exist, eight of which have been characterised. Characterized cysteine 82, 150, 155, 156, 160, 216, 521 and 529 are sensitive to SA treatment, control monomerization of the protein and S-nitrosylation (Cao *et al*., 1997; Mou *et al*., 2003; Rochon *et al*., 2006; Tada *et al*., 2008; Spoel *et al*., 2009). A typical role played by these cysteine residues in defense response has been demonstrated using a *npr1-2* mutant. In this mutant, the cysteine at position 150 is replaced by a tyrosine residue resulting in more reduced *PR-1* transcription following SA treatment or infection with *P. syringae* pv. *maculicola* (*Psm*) ES4326 pathogen (Glazebrook *et al*., 1996; Cao *et al*., 1997). However, mutant *npr1-2* plants were not completely blocked in their ability to transcribe the anti-microbial genes *BGL2* and *PR-5* following treatment with the above mentioned pathogen (Glazebrook *et al*., 1996). The residual *BGL2* and *PR-5* transcription in this mutant is believed to be independent of NPR1 (Glazebrook *et al*., 1996). However, this transcription was not effective in preventing pathogen proliferation compared to wild-type *Arabidopsis* plants (Glazebrook *et al*., 1996).

In addition to the *npr1*-2 mutant, other *npr1* mutants with altered AA residues within the NPR1 sequence have been generated. The first of these was the *npr1-1* mutant in which the histidine at position 334 has been replaced by a tyrosine. The *npr1-3* and *npr1-4* mutant resulted from entire truncation (*npr1-3*) and splicing (*npr1-4*) of the C-terminal position 400 and 432, respectively (Cao *et al*., 1997; Volko *et al*., 1998). These mutant plants have further been demonstrated to play either positive or negative roles during SAR (Glazebrook *et al*., 1996; Cao *et al*., 1997; Sigrid *et al*., 1998; Volko *et al*., 1998). Recently, Canet *et al*. (2010) further identified other *npr1* alleles in *Arabidopsis* (Table 1.2). These alleles are not responsive to both SA treatment and to infection with *P*. *syringae* pv. *tomato* DC3000 (Canet *et al*., 2010).

Table 1.2 Newly identified *npr1* alleles (adapted from Canet *et al*., 2010).

Allele	**Nucleotide mutation**	**AA mutation**	**Level of Sensitivity to *P. syringae*, SA, BTH**	***PR-1* induction 3 days post *P. syringae* infection**
***npr1*-20**	G to A	V501M and splicing 4th exon	High	Slight induction
***npr1*-21**	C to T	Q384*	High	No induction
***npr1*-22**	G to A	R544K	High	No induction
***npr1*-23**	G to A	Splicing 2nd exon	High	No induction
***npr1*-24**	C to T	L497F	High	No induction
***npr1*-25**	G to A	E443K	High	No induction
***npr1*-26**	G to A	Splicing 4th exon	High	No induction
***npr1*-27**	G to A	D428N	High	No induction
***npr1*-28**	C to T	R538*	High	No induction
***npr1*-29**	G to A	Splicing 4th exon	High	No induction
***npr1*-30**	C to T	A451V	High	No induction
***npr1*-31**	G to A	E449K	High	No induction
***npr1*-32**	G to A	R432K	High	No induction
***npr1*-33**	G to A	R432K	High	No induction
***npr1*-34**	C to T	Q526*	High	No induction
***npr1*-35**	G to A	C155Y	High	No induction
***npr1*-36**	G to A	E449K	High	No induction
***npr1*-37**	C to T	Q491*	High	No induction
***npr1*-38**	G to A	R493K	High	No induction
***npr1*-39**	C to T	S512L	High	No induction

Allele	**Nucleotide mutation**	**AA mutation**	**Level of Sensitivity to *P. syringae*, SA, BTH**	***PR-1* induction 3 days post *P. syringae* infection**
***npr1*-40**	G to A	C306Y	High	No induction
***npr1*-41**	G to A	E288K	High	Slight induction
***npr1*-42**	G to A	G504 E	High	No induction
***npr1*-43**	G to A	Splicing 4th exon	High	No induction
***npr1*-44**	C to T	H80Y	High	No induction
***npr1*-45**	C to T	Q 371*	High	No induction
***npr1*-46**	C to T	L497F	High	No induction
***npr1*-47**	C to T	L497F	High	No induction
***npr1*-48**	C to T	L515F	High	No induction
***npr1*-49**	C to T	Q491*	High	No induction
***npr1*-50**	C to T	L274F	High	No induction
***npr1*-51**	G to A	R432K	High	No induction
***npr1*-52**	Deletion	N210FS	High	No induction
***npr1*-53**	C to T	Q343*	High	No induction
***npr1*-54**	G to A	Splicing 2nd exon	High	No induction
***npr1*-55**	C to T	Q491*	High	No induction
***npr1*-56**	G to C	A496P	High	No induction
***npr1*-57**	Deletion	? DVDFM L 164-168	High	No induction
***npr1*-58**	T to A	Y64N	High	No induction
***npr1*-59**	Deletion	V194*	High	No induction
***npr1*-60**	C to T	P342S	High	No induction
***npr1*-61**	Deletion	V194*	High	No induction

1.3.3 *Non-expressor of pathogenesis-related1* genes defense pathway

Recent and accumulating evidence indicates that NPR1 acts downstream of reactive oxygen species (ROS), SA, JA, ET and also other phytohormones in the defense signaling cascade leading to PR induction. Studies have also demonstrated the possibility of a feedback mechanism for the control of an excessive production of *PR* gene products by the NPR1 protein (Spoel *et al*., 2003; Blanco *et al*., 2009; Kallenbach *et al*., 2010; Zhang *et al*., 2010).

During stable state conditions, NPR1 resides predominantly in the cytoplasm as an oligomer with only a small amount being monomeric (Fig. 1.2; Spoel *et al*., 2009). Cytoplasmic NPR1 is predominantly maintained in an oligomeric form through S-nitrosylation of the cysteine at position 216 and using electrons from S-nitrosoglutathione (GSNO; Tada *et al*., 2008). In the nucleus, these momoners are rapidly targeted for degradation through "CUL-3-based E3 ligase-mediated ubiquitinylation" (Spoel *et al*., 2009). Spoel *et al*. (2009) demonstrated the existence of the predominant oligomeric NPR1 protein in the cytosol using *Arabidopsis* NPR1-GFP plants. In their study GFP signals were very weak in the un-treated transformed plants due to the oligomeric state of the NPR1 protein. However, in SA-treated transformed plants, strong GFP signals were detected especially in the nucleus (Spoel *et al*., 2009). Stress conditions cause disturbances in the cell's redox state and the disulphide bridges associated with the oligomer are broken which releases momoneric NPR1 with a nuclear target (Kinkema *et al*., 2000; Mou *et al*., 2003; Shi *et al*., 2010). This NPR1 oligomer to monomer reduction is catalyzed by thioredoxins (Tada *et al*., 2008) and coupled with the redox-sensitive nature of some cysteine elements within the NPR1 protein, monomeric NPR1 is directed via nuclear localization signals to the nucleus (Spoel *et al*., 2009; Zhang *et al*., 2010).

The activity of NPR1 has also been shown to be influenced by glutathione when pathogen-infected or glutathione-treated plants were studied, or when plants were over-expressing the gamma-glutamylcysteine sytnthetase (γ-*ECS*) gene, or when plants with a mutation in this gene were investigated. Recently, Ghanta *et al*. (2011) demonstrated that *Nicotiana tabacum* plants over-expressing the γ-*ECS* (GSH1) gene resulted in a biotic stress response which was likely dependent on *NPR1* transcription and SA. Studies carried out by Mou *et al*. (2003) have also demonstrated that treatment of plants with GSH or a pathogen resulted in an increased GSH/GSSG ratio which promoted monomerization of the NPR1 protein. The presence of abundant monomeric NPR1 in the nucleus together with the redox status of the cells further facilitates its binding to specific redox-sensitive transcription factors (TF) belonging to the WRKY family of TFs like WRKY18 (Wang *et al*., 2006) and/or TGA TFs such as TGA2 and TGA5 (Zhang *et al*., 2003). NPR1 has therefore been described as a co-transcription factor necessary for the activation of other transcription factors (Cao *et al*., 1994; Rochon *et al*., 2006). These transcription factors recognize and bind to distinct activation sequences present in the promoter of *PR* genes leading to the activation of these *PR* gene products (Després *et al*., 2003; Johnson *et al*., 2003; Rochon *et al*., 2006). Finally, full expression of *NPR1*-target genes following pathogen attack is mediated by an efficient turnover of phosphorylated NPR1 proteins through an interaction with CUL3 unbiquitin ligase in the nucleus (Spoel *et al*., 2009).

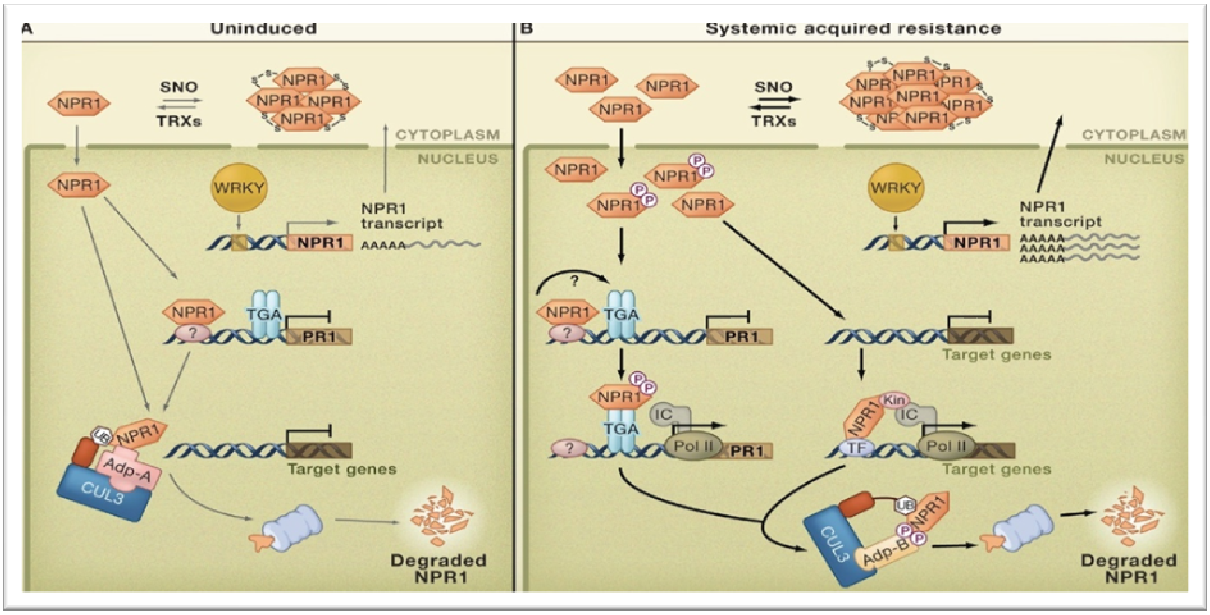


Figure 1.2 *Arabidopsis thialana* NPR1 pathway (adapted from Mukhtar *et al*., 2009). In a non-induced state (A), *A. thaliana* NPR1 proteins exist predominantly as oligomers held by disulphide bonds in the cytosol. Cytosolic oligomerization is maintained by S-nitrosylation of monomeric NPR1. Few of the monomers, which translocate to the nucleus, are quickly degraded through a "CUL-3-based E3 ligase-mediated ubiquitinylation" system; some of the monomers interact with WRKY transcription factors and are moved back to the cytoplasm while during this time, non-induced nucleic TGA and NPR1 are also unable to interact for the induction of *PR* genes. Following stress perception (B), the redox state of cells changes and thioredoxins facilitates the monomerization of more NPR1 proteins in the cytoplasm. These momoners translocate to the nucleus and are subjected to protein phosphyorylation but are also able to bind to TGAs forming a complex at the promoters of *PR* genes leading to activation of these *PR* genes. Used NPR1 is again targeted for degradation while phosphorylated NPR1 is turned-over

and recycled for the production of "new" NPR1 proteins via a process requiring WRKY transcription factors.

1.3.4 *Non-expressor of pathogenesis-related1* genes interacting elements

1.3.4.1 Reactive oxygen species-antioxidant system and NPR1 interaction

Membrane depolarization, changes in the activity of nicotinamide adenine di-nucleotide phosphate reduced tetra-sodium (NADPH)-oxidase, extracellular alkalinization and ionic fluxes are pivotal early indicators of the defense response cascade (Pike *et al*., 2005; Liu *et al*., 2007; Jeworutzki *et al*., 2010). Further reactive oxygen species (ROS), which are normally synthesized at low concentrations in unstressed plants, are required to drive some physiological and biochemical processes in cells (Kotochoni and Gachomo, 2006; Liu *et al*., 2007; Ma *et al*., 2009). ROS, such as hydrogen peroxide (H_2O_2) and superoxide, act as signaling molecules for the defense response cascade conferring tolerance to biotic stressors (Apel and Hirt, 2004; Desikan *et al*., 2005; Kotochoni and Gachomo, 2006). However, the inability of the plant to effectively scavenge ROS and maintain a favourable threshold results in a rapid oxidative damage of lipids and proteins, damages DNA and causes cell death and the appearance of necrotic lesions (Foyer *et al*., 2005; Kotchoni and Gachomo, 2006; Gills and Tuteja, 2010).

Due to the possible detrimental role of ROS during the biotic stress response, their threshold is maintained at a beneficial level by antioxidants (Foyer *et al*., 2005). The scavenging of typical H_2O_2 is well-known to be facilitated by enzymes such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and

glutathione reductase (GR), which are all involved in antioxidant recycling (Noctor and Foyer, 1998; Asada, 2000). Non-enzymatic ROS scavengers include low molecular weight antioxidants, such as glutathione (GSH) and ascorbate (AsA) that are part of the ascorbate-glutathione (Halliwell-Foyer-Asada) cycle (Fig. 1.3; Smirnoff, 1996; Noctor and Foyer, 1998; Asada, 2000).

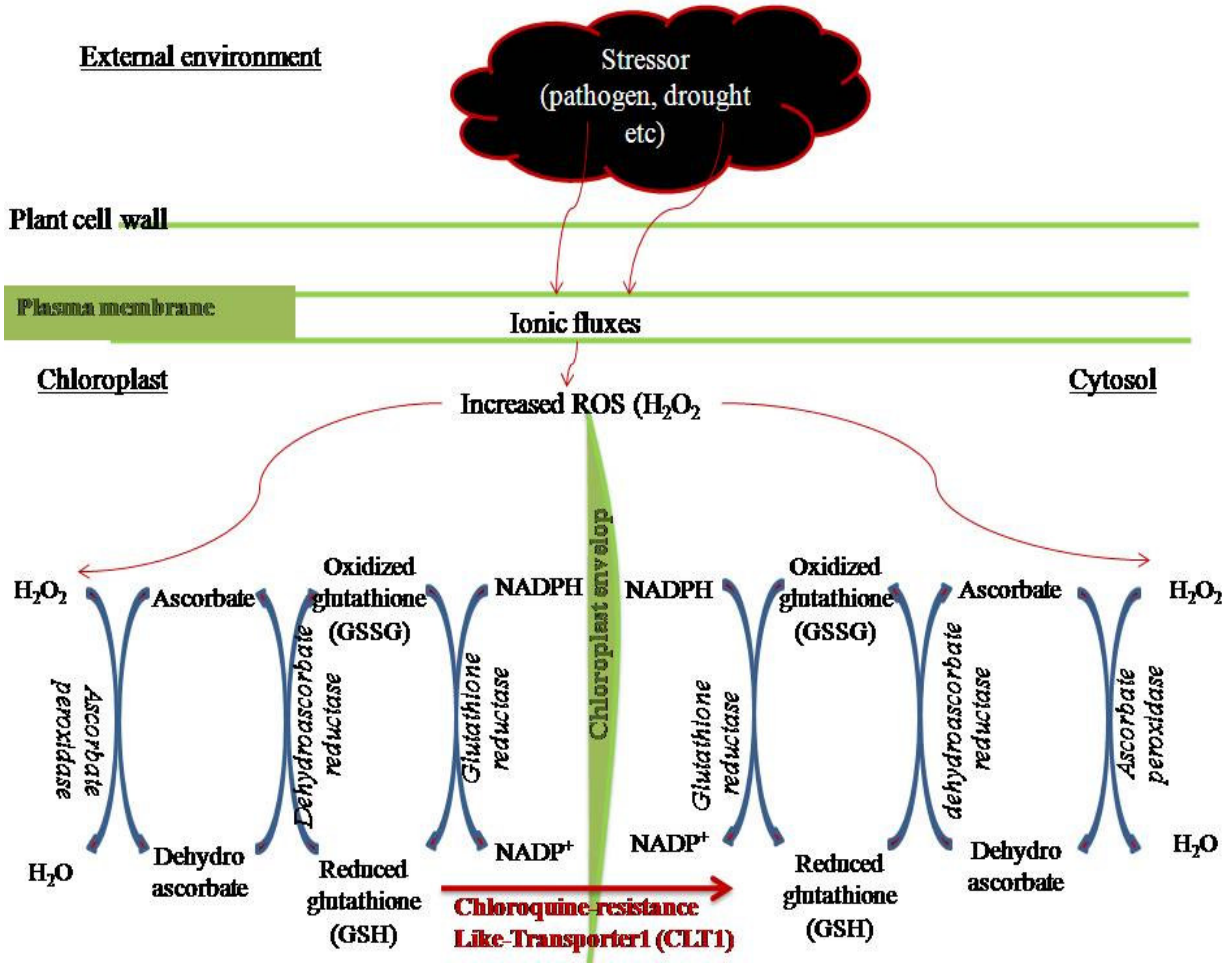


Figure 1.3 The ascorbate-glutathione cycle (adapted from Smirnoff, 1996; Noctor and Foyer 1998; Asada, 2000). Upon stress perception, changes in ionic fluxes at the plasma membrane triggers an increase in hydrogen peroxide (H_2O_2). Detoxification of H_2O_2 into water is facilitated by ascorbate (AsA) in the presence of ascorbate peroxidise (APX) which also results in the generation of dehydroascorbate (DHA). In a reaction involving DHA reductase (DHAR), AsA is regenerated using electrons transferred from reduced glutathione (GSH). This causes the formation of oxidized GSH (GSSG). The regeneration of GSH from GSSG finally requires NADPH in the presence of glutathione reductase (GR).

Gain- or loss-of-function GSH and AsA mutants have been previously generated and their functions characterized to highlight the importance of AsA or GSH in mediating downstream defense responses. In *Arabidopsis*, mutants with low ascorbate (*vtc*) content have been isolated and some of these characterized (Cronklin *et al*., 2000; Pavet *et al*., 2005; Colville and Smirnoff, 2008). The *vtc1.1* (30% less AsA), *vtc2.1* (25% less AsA), *vtc3-1* (40% less AsA) and *vtc4-1* (50% less AsA) had increased H_2O_2 concentrations, expressed *NPR1* and *PR-1* under non-stressed conditions (Pavet *et al*., 2005; Mukherjee *et al*., 2010). These mutants were also more resistant to *P. syringae* infection. They reduced the proliferation of the pathogen by 15-fold (*vtc1.1*) and 13-fold (*vtc2.1*) when compared to wild type *Arabidopsis* plants (Pavet *et al*., 2005). These mutants further compensated for AsA deficiency by increasing the GSH content (Pavet *et al*., 2005; Colville and Smirnoff, 2008). Using *vtc2-1* mutants plants, Colville and Smirnoff (2008) further found that following inhibition of GSH biosynthesis in *vtc2-1* mutant plants by treatment with the inhibitor D,L-buthionine-[S,R]-sulphoximine (BSO), *PR-1* transcription in these mutants was not completely blocked. This led to the conclusion that AsA deficiency primes plants by establishing a readily available defense response system for pathogen invaders probably through an *NPR1*-dependent mechanism.

Unlike AsA deficient mutants, the dependency for *NPR1*-dependent *PR*-1 activation has not yet been investigated in greater details using GSH deficient mutants. For instance, the *cadmium hypersensitive 2* (*cad2*) and *regulator of ascorbate peroxidase2 1.1* (*rax1.1*) are two GSH deficient mutants with high sensitive to *P. syringae* pathogen (Cobbett *et al*., 1998; Ball *et al*., 2004). Micro array data further indicates that genes involved in SA-, JA- and/or ABA-dependent defense responses are down-regulated in these GSH mutants compared to the wild-type plants

(Ball *et al*., 2004). Such findings indicate that the native GSH1enzyme contributes positively towards mediating defense but whether this is dependent on *NPR1* transcription is unknown. In fact the requirement for *GSH1* during defense has been recently demonstrated using transgenic tobacco plants over-expressing the GSH1 enzyme. These *GSH*-over expressing plants have an increased GSH content and are more tolerant to pathogen infection when compared to the wild-type plants. This tolerance is further believed to be mediated by the *NPR1* gene (Ghanta *et al*. 2011).

Transportation of GSH, or its biosynthetic metabolites, to the cytosol might also be key for NPR1 activation since NPR1 resides in the cytoplasm (Fig 1.4; Cao et al., 1994). A complete GSH biosynthesis can only occur in the chloroplast (Fig 1.4; Noctor *et al*., 2002; Maughan *et al*., 2010). Using a genetic screen based on the ability of *Arabidopsis* plants to grow in the presence of L-buthionine-SR-sulfoximine (BSO), an inhibitor of γ–ECS, a thiol transporter has been isolated and partially characterized (Maughan *et al*., 2010). This transporter has a high homology to the *Plasmodium falciparum (Pf) chloroquine-resistance transporter (PfCRT*) and has been designated *CRT-Like Transporter1* (*CLT1*). The resistance is largely due to a mutation in a single gene, *PfCRT*. Three members of this transporter family are localized in the chloroplast envelope. An *Arabidopsis* triple mutant produced and designated *clt1clt2clt3* lacks the transporter. Most, if not all, of the GSH in the leaves of the *Arabidopsis clt1clt2clt3* triple mutant is localized in the chloroplast and therefore, the cytosol is deficient in GSH and has a reduced content of *PR-1* transcript amount. The mutant, which has also been used in this study, can serve as a powerful tool to address the role of cytosolic GSH in *NPR1*-dependent *PR-1* transcription.

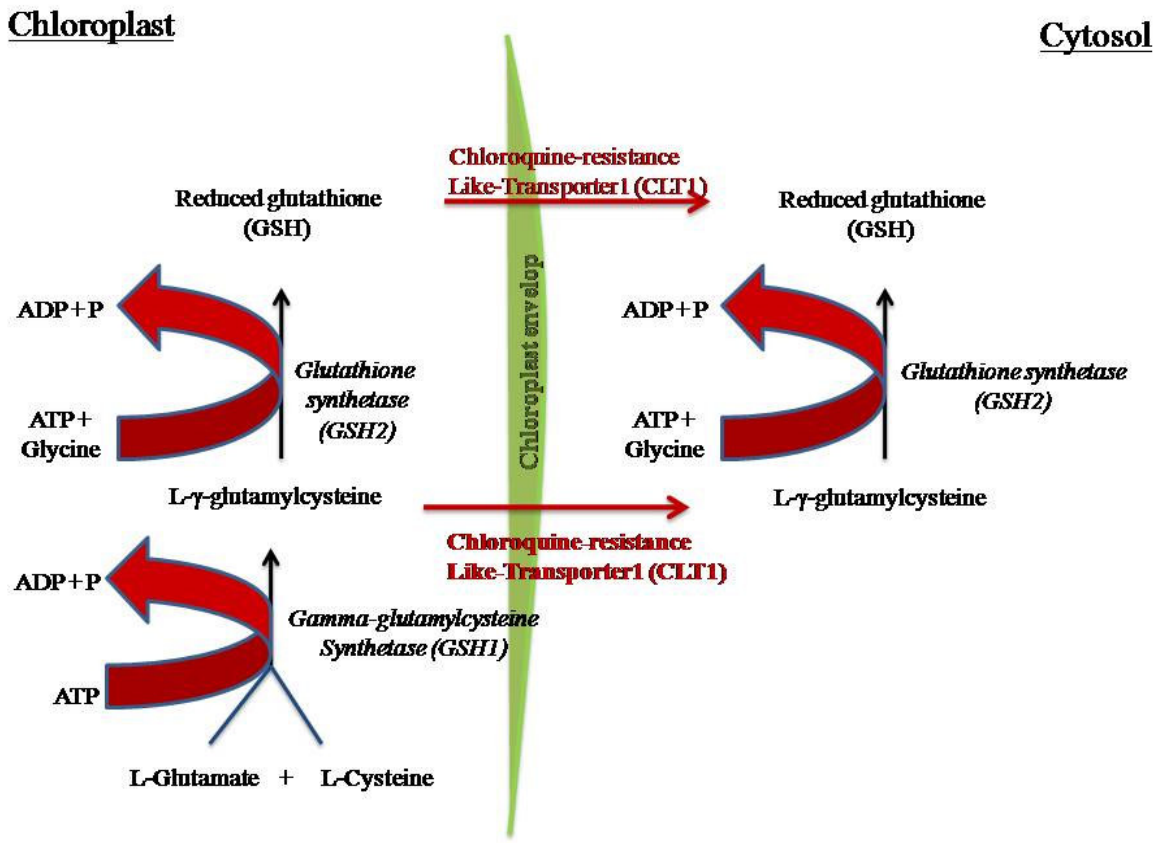


Figure 1.4 GSH biosynthesis and transportation across the chloroplast. During the synthesis of GSH which begins in the chloroplast, L-glutamine together with L-cysteine react in the presence of gamma-glutamylcysteine synthetase (γ–ECS or GSH1) in a reaction requiring ATP, leading to the formation of L-γ-glutamylcysteine (γ–EC). The γ–EC produced in this reaction is used together with glycine for the synthesis of GSH in a reaction driven by ATP and catalyzed by GSH synthetase (GSH2). γ-EC can also be transported to the cytosol by the chloroquine-resistance like-transporter1 (CLT1) for the synthesis of GSH. Similarly, GSH from the chloroplast is also transported by the CLT1 transporter into the cytosol (adapted from Noctor *et al*., 2002; Maughan *et al*., 2010).

1.3.4.2 Phytohormones and NPR1 interaction

The *NPR1* gene has been isolated from many plants and further characterized by either transient expression or over-expression studies, highlighting its role in contributing to pathogen tolerance in agronomically important crops like banana, citrus, grapevine, tomato, carrots, apple, wheat and rice (Lin *et al*., 2004; Makandar *et al*., 2006; Malnoy *et al*., 2007; Endah *et al*., 2008; Quilis *et al*., 2008; Zhao *et al*., 2008; Wally *et al*., 2009; Parkhi *et al*., 2010; Shi *et al*., 2010; Zhang *et al*., 2010). *NPR1* has also been implicated, together with JA and ET, to be important for ISR following priming by the non-pathogenic rhizobacterium *P. putina* LSW17S, which confers systemic resistance against the necrotrophic pathogen *F. oxysporum* f. sp. l*ycopersici* and *P. syringae* pv. *tomato* (Ahn *et al*., 2007). Together with SA, JA and ET, *NPR1* is also involved in the *Bacillus cereus* strain AR156-induced resistance to *P. syringae* (Niu *et al*., 2011). In *Arabidopsis*, tolerance to *F. oxysporum* (Berrocal-Lobo and Molina, 2004) and *Verticillium longisporum* (Johansson *et al*., 2006) are dependent on SA, JA, ET and cytosolic NPR1 corresponding to the induction of downstream *PR-1*, *PR-2* and *PR-4* transcripts (Johansson *et al*., 2006). Similarly, *Piriformospora indica* induced resistance in *Arabidopsis* is also dependent on the cytosolic function of NPR1 and JA (Stein *et al*., 2008).

Following treatment of *Arabidopsis npr1-1* mutant plants expressing *OsNPR1* with *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe grisea,* or the elicitors BTH, MeJA and ET, *OsNPR1* transcription was induced from as early as 4 to 8 h post treatment (Yuan *et al.*, 2007). This highlights the importance of the *OsNPR1* gene in conferring resistance to both necrotrophic and

biotrophic pathogens. However, transgenic rice plants over-expressing the *AtNPR1* and *OsNPR1* (*NH1*) gene displayed lesion mimic cell death symptoms. They were also more sensitive to light and dwarfed when compared to the untransformed plants (Fitzgerald *et al*., 2004; Chern *et al*., 2005). Transformation of rice with *NPR1*-like genes might therefore introduce undesirable traits. Also, transient over-expression of *VvNPR1.1* and *VvNPR1.2*, in *Nicotiana benthamiana* leaves triggered *PR-1* and *PR-2* expression but not *PR-3* expression in the absence of pathogen infection. Moreover, transient expression of the *VvNPR1.1* or *AtNPR1* induced expression of PR-1 proteins in response to *Plasmopa*ra *viticola* infection (Le Hananff *et al*., 2009).

Expression of the Tc*NPR1* from cocoa in *Arabidopsis npr1-2* mutant plants compliments the phenotype in these plants resulting in the induction of the SA-inducible *PR-1* transcript and the MeJA-inducible *PDF1.2* transcript following SA and MeJA treatment respectively. Expression of this *NPR1*-like gene in these transgenic plants also resulted to a 100-fold reduced *P. syringae* pv. *tomato* DC3000 bacteria growth in comparison to the non-transgenic *npr1-2* mutant plants following pathogen infection (Shi *et al*., 2010). *GmNPR1.1* and *GmNPR1*.2, from soybean when expressed in *npr1-1* mutant *Arabidopsis* plants also complimented the mutant phenotype. This resulted in the induction of increased amounts of *PR-1* transcripts following INA treatment and *BGL2* transcripts following infection with *P. syringae* pv. *tomato* (Sandhu *et al*., 2009).

Transcription of all three banana *NPR1*-like homologues has been characterized in banana and found to be differentially transcribed following treatment with either *F. oxysporum* f. sp. *cubense* race 4 (*FOC*), SA or MeJA (Endah *et al*., 2008; Zhao *et al*., 2008). Using semi-quantitative PCR, Zhao *et al.* (2008) demonstrated that SA treatment, or infection with *Fusarium*, resulted in higher

transcription of *MdNPR1* in the *Fusarium*-resistant cultivar (Dongguan Dajiao) when compared to a *Fusarium*-sensitive cultivar (Fenjiao). Endah *et al*. (2008) characterized the banana *MNPR1A* and *MNPR1B* transcription in a *F. oxysporum*-sensitive (Grand Naine; Stover and Buddenhagen, 1986) and a *Fusarium*-tolerant (GCTCV-218; Hwang *et al*., 2004) banana cultivar. Higher and earlier transcription of the two banana genes were found, as well as higher amounts of *PR-1* and *PR-3* transcripts were found in *Fusarium*-treated GCTCV-218 plants when compared to treated Grand Naine plants. Transcription of the two banana *NPR1*-like genes was also found to be differentially regulated especially *MNPR1A* which was not transcribed following SA treatment.

All these studies highlight the importance of various *NPR1*-like genes in conferring resistance to both biotrophic and/or necrotrophic pathogens and eliciting various defense response pathways leading to *PR* gene induction.

1.3.4.3 Transcription factors and NPR1

Gene transcription plays a significant role in determining proteins that will ultimately be expressed in plants (Gross and Oelgeschläger, 2006) and NPR1 has been shown to act downstream or upstream of various transcription factors. An example of such transcription factors are genes encoding the WRKY family of transcription proteins (Eulgem *et al*., 2000; 2006; 2007). They are characterised by one or more WRKYGQK domains for the recognition of W-boxes ((T)GACC/T) found in promoter regions of defense genes including the *NPR1* and *PR* genes (Eulgem *et al*., 2000; 2006; Yu *et al*., 2001). The TGA/OBF basic leucine zipper (bZIP)

family of transcription factors are a second well-characterised family of transcription factors which interact with NPR1 for the downstream activation of *PR-1* genes (Zhang *et al*., 2003). Their binding to *cis*-acting elements located at the promoters of *PR-1* genes is redox regulated requiring monomeric nucleic *NPR1* (Després *et a*l., 2000; Pieterse *et al*., 2004; Rochon *et al*., 2006). However, depending on their interaction within cells, TGA as well as WRKY transcription factors could act as suppressors rather than activators of defense responses (Kesarwani *et al*., 2007; Boyle *et al*., 2009; Agarwal *et al*., 2010). Using TGA2, Kesarwani *et al*. (2007) demonstrated that interaction with the negative regulator of N*PR1* gene (*SN1*) abolishes *PR* expression. Unlike *WRKY* transcription factors, *TGA* factors only act downstream of the *NPR1* during stress response and actually require the co-transcriptional *NPR1* activator to induce *PR* genes (Yu *et al*., 2001; Agarwal *et al*., 2007; Spoel *et al*., 2009).

1.3.4.4 *Pathogenesis-related1 gene* and *NPR1*

The genes encoding PR proteins (Table 1.3) are antimicrobial, acting as the final defense response signal for the ultimate limitation of pathogen spread (Fritig *et al*., 1998; Selitrennikoff, 2001; Ferreira *et al*., 2007). They have been isolated and characterised from a great number of plants species including rice, pepper and pumpkin (Kim *et al*., 2001; Van Loon *et al*., 2006; Park *et al*., 2010). These antimicrobial proteins confer resistance against viruses, yeasts, bacteria and fungal pathogens such as *M. grisea*, *X. oryzae* pv. *oryzae*, *Erwinia amylovora*, *B. cinera*, *F. oxysporum*, *F. solani*, *Candidas albicans* (Kim *et al*., 2001; Bonasera *et al*., 2006; Van Loon *et al*., 2006; Park *et al*., 2010). Their expression requires a network of interplay among various

molecules in the cell including *TGA* and *NPR1* genes (Mou *et al*., 2003; Zhang *et al*., 2003; Spoel *et al*., 2009).

Table 1.3 PR-1 protein family from various plants (adapted from Van Loon *et al*., 2006).

Protein family	Type member	Function
PR-1	Tobacco PR-1a	Unknown (antifungal properties)
PR-2	Tobacco PR-2	Endo-β-1-3 glucanases
PR-3	Tobacco P, Q	Chitinase type I , II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chtinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Endoproteinase
PR-7	Tomato P_{69}	Chitinase type III
PR-8	Cucumber chitinase	Peroxidase
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Pasley "PR1"	Ribonuclease-like
PR-11	Tobacco "class V" chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

1.4 Working hypothesis and aim of study

The rationale for undertaking this present study was based on previous findings that two *NPR1*-like genes with coding sequence differences were differentially transcribed in banana in response to different elicitors (Endah *et al*., 2008). In this previous study, we showed that transcription of the two genes could be cultivar or elicitor-dependent; with *MNPR1A* highly transcribed following treatment with the necrotrophic pathogen *F. oxysporum* or MeJA. Necrotrophs are known to elicit predominantly the JA/ET dependent pathway (Oliver and Ipcho, 2004; Glazebrook, 2005; Spoel *et al*., 2007). *MNPR1B* was not transcribed following treatment with *F. oxysporum*, but was transcribed following treatment with either SA, or JA (Endah *et al*., 2008). It was therefore hypothesized that the difference in coding sequence could be responsible for the observed pathogen response of the two genes, whereby *MNPR1A* could be more sensitive than *MNPR1B* to necrotrophic pathogens and JA/ET signaling rather than to biotrophic pathogens and SA signaling.

Three previously identified rice *NPR1* homologs (*NH1*, *NH2* and *NH3*) have been shown to respond differential in different plant-pathogen systems and following treatment with *P. syringae*, *M. grisea*, *X. oryzae*, MeJA, benzothiadiazole (BTH), or ET (Yuan *et al*., 2007). *Arabidopsis npr1* mutant plants expressing the *NH1*, *NH2* or *NH3* genes complimented the mutant phenotype following treatment of the transgenic plants with *P. syringae*. These three genes were also rapidly induced in transgenic rice lines over-expressing the R gene *Xa21* (which confers resistance to *X. oryzae*) and *Pir1* (which confers resistance to *M. grisae*) and also after MeJA, BTH and ET treatment. Thus indicating their involvement in both SA- and JA- mediated

responses. However, when over-expressed in rice, only the *NH1* over-expressing rice plants conferred resistant to *M. grisea* and *X. oryzae* pathogens. These *NH1* over-expressing rice plants could further transcribe *PR-1* gene products following treatment with BTH or MeJA. However, *NH2* and *NH3* over-expressing rice plants did not provide enhanced resistance to *X. oryzae*. This indicates that in rice, similar to banana, different *NPR1*-like sequences could respond differentially to various elicitors and in different plant systems.

This PhD study therefore aimed to characterize the role of *MNPR1A* and *MNPR1B* in protection against various pathogens and to investigate if sequence differences in the two banana genes are responsible for differential activity of the genes against pathogens. The first objective of the study was to determine the expression pattern of the two banana genes and the subsequent response of the downstream *PR-1* and *PR-3* genes in response to a hemi-biotroph *X. campestris* pv. *musacearum* in two banana cultivars. This was to investigate if both genes are expressed in response to this hemi-biotrophic pathogen. A second objective was to identify possible defense *cis*-regulatory elements within the two MNPR1 coding sequences and also compare the banana NPR1-like sequences with others in order to identify functional motifs already characterized in homologous NPR1 sequences. Further investigated in this study was whether the coding sequences of both genes under the control of the 35S CaMV promoter and terminator sequences could compliment the resistant phenotype of *npr1-2 Arabidopsis* mutant plants so as to address the question of their involvement in conferring tolerance to a broad range of pathogens. A fourth objective was to study whether the coding sequences of the two banana *NPR1*-like genes (*MNPR1A* and *MNPR1B)* respond differentially to three distinct classes of pathogens (biotrophic *Peronospora parasitic* currently referred to as *Hylaoperonospora arabidopsidis*, the necrotrophic

Botrytis cinerea and hemi-biotrophic *Pseudomonas syringae*) to evaluate if a particular sequence is playing a more important role in pathogen protection. Finally, the study had the last objective to investigate the effect of cytosolic GSH on *NPR1*-dependent *PR-1* transcription using *Arabidopsis* mutants (*clt1clt2clt3*) defective in cytosolic GSH biosynthesis to understand if the absence of GSH in the cytosol has any direct effect on *NPR1* transcription.

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.

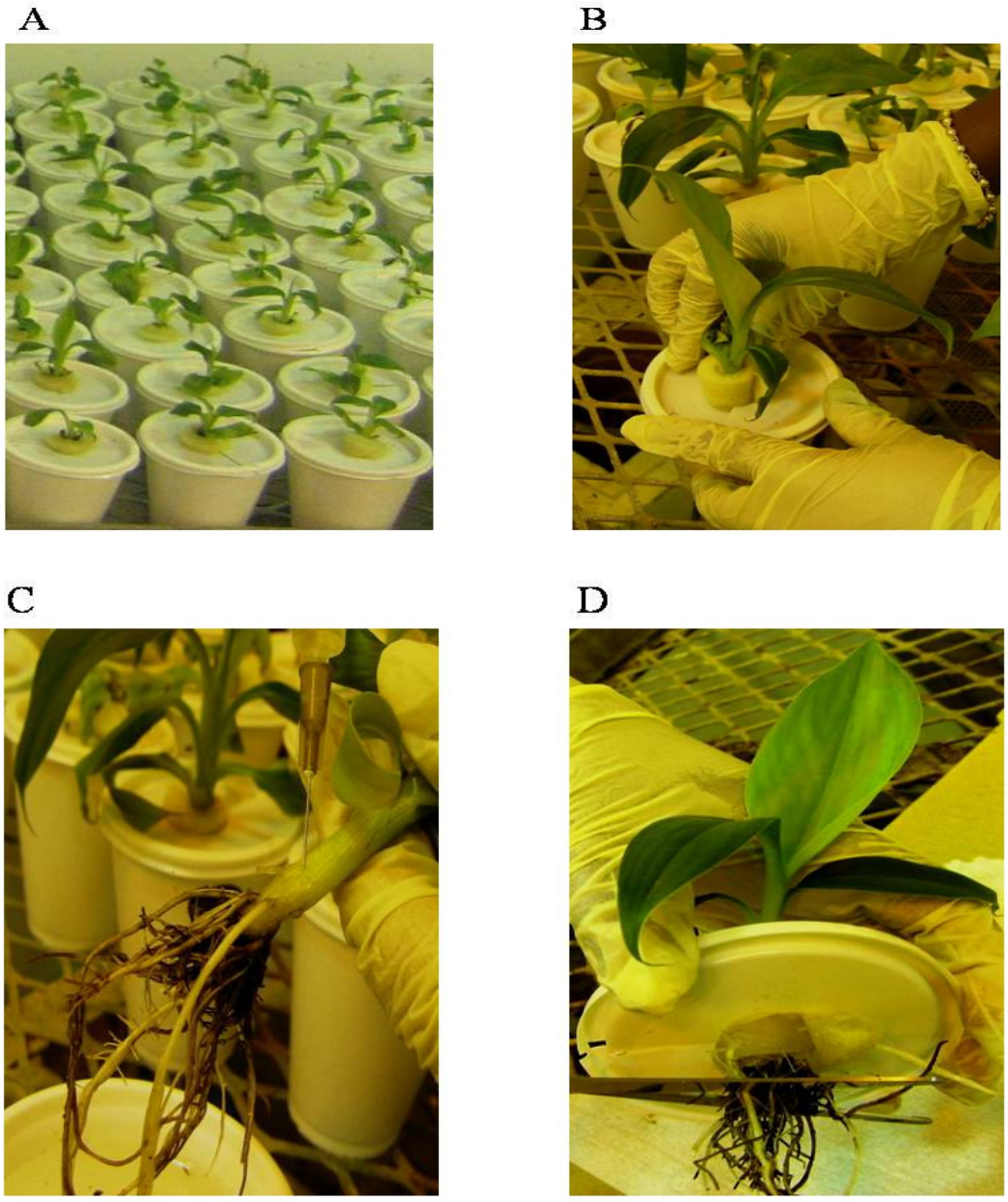


Figure 2.1 Growth of banana plants, plant inoculation and sampling of root materials. Two-week-old tissue cultured banana plants were grown hydroponically in 250 mL disposable cups containing 150 mL of distilled water (A) for a further 3-weeks (B). Plant pseudostems were infiltrated just above the corm 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.

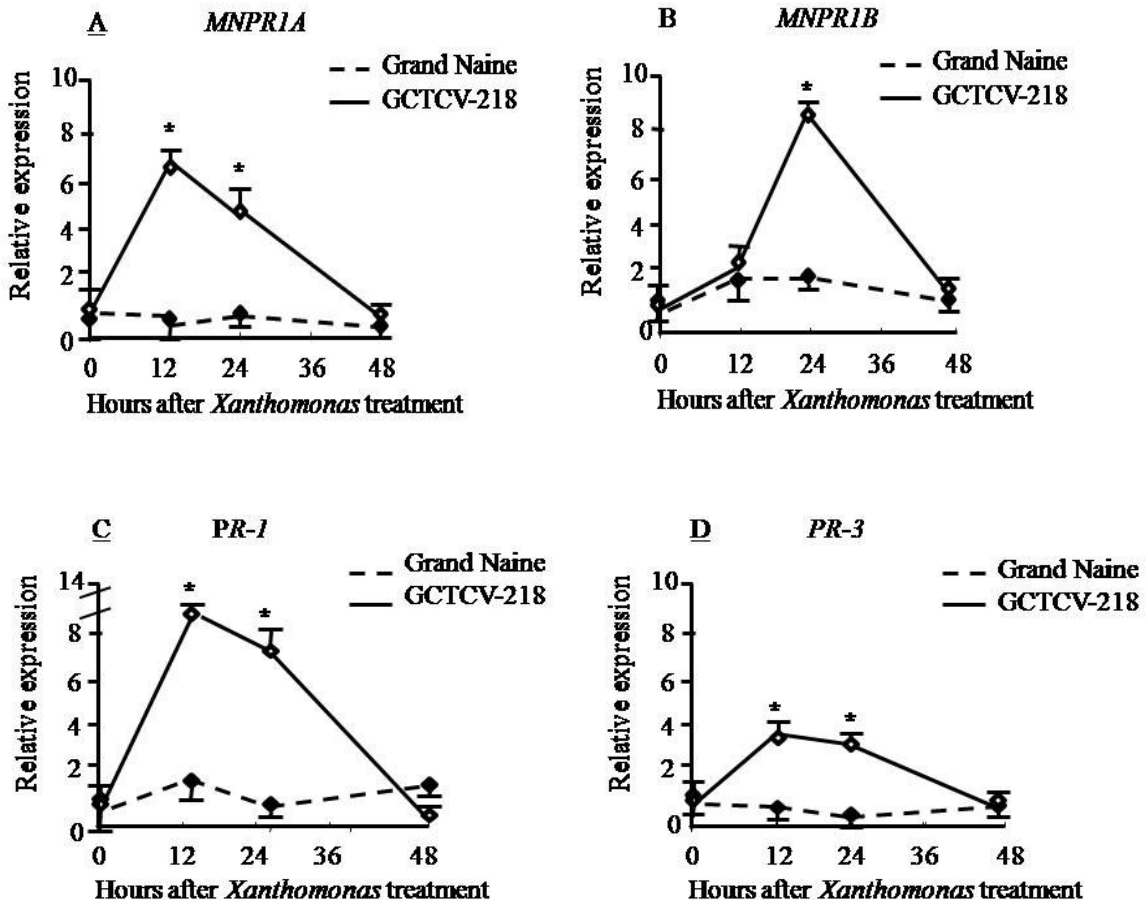


Figure 2.2 Relative gene expression of *MNPR1A*, *MNPR1B*, *PR-1* and *PR-3* in *Xanthomonas*-infected Cavendish banana cultivar Grand Naine and GCTCV-218. Relative gene expression of *MNPR1-A* and *MNPR1-B* (A and B) and *PR-1* and *PR-3* (C and D) was determined and compared in each of the cultivar 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.

CHAPTER THREE

COMPARATIVE ANALYSIS OF TWO BANANA NPR1-LIKE CODING SEQUENCES

3.1 Abstract

The *Non-expressor of pathogenesis-related*1 (*NPR1*) gene is known to exist in more that one copy in most plants like *Arabidopsis*, rice, soybean, and banana. In Banana, three *NPR1*-like genes have already been isolated. The *Musa* (*M*)*NPR1A* and *MNPR1B* genes were isolated from a Cavendish banana with an AAA genome (Grand Naine) while *MdNPR1* was isolated from a banana cultivar with a ABB genome (Dongguan dijiao). Characterization of the expression pattern of the two *MNPR1* genes in various banana cultivars following treatment with *Fusarium* and elicitors like salicylic acid depicts a differential mode of activation of these genes. However, what controls this differential response is largely unknown and the role expression of *MNPR1* genes contributie to pathogen defense, if at all, has also not been reported. To complicate this picture further, characterization of expression of the genes following treatment of banana with the hemibiotroph *Xanthomonas campestris* further unveiled a sequential expression pattern for these genes which correlated with *pathogenesis-related* (*PR*)*-1* and *PR-3*.transcription. Hence, using various sequence analysis tools, it is reported here that these banana cultivars share different levels of identities with 39 previously described NPR1-like sequences, some of which are well-known to positively mediate pathogen defense response. These MNPR1 coding sequences further harbor functional conserved motifs, in some cases with some amino acid dissimilarities within the motifs relative to known sequences. Phylogenetic analysis revealed that the banana NPR1 sequences are closely related to those of other monocotyledons like rice and *Hordeum vulgare*. *Cis*-analysis of the two AAA banana NPR1 sequences showed that they both contain various regulatory elements reported to be sensitive to pathogens and elicitors.

3.2 Introduction

Since its isolation in 1994, the gene encoding the Non expressor of pathogenesis related1 (NPR1) protein has been isolated in many monocotyledons and dicotyledons to date. In most of these plants like *Arabidopsis*, rice, soybean, grapevine, *Brassica* and banana, more that one copy of the gene has been reported (Hepworth *et al*., 2005; Yuan *et al*., 2007; Endah *et al*., 2008; Zhao *et al*., 2009; Sandhu *et al*., 2009). In banana, two copies of the *NPR1*-like genes were isolated from a Cavendish banana cultivar with an AAA genome (*MNPR1A* and *MNPR1B*; Endah *et al*., 2008) and one from a banana cultivar with an ABB genome (*MdNPR1*; Zhao *et al*., 2009).

Studies on the characterization of expression of the two *MNPR1* genes in a *Fusarium*-tolerant Cavendish banana cultivar (GCTCV-218) and a susceptible cultivar (Grand Naine) indicates that these genes respond differentially to salicylic acid (SA) treatment and to a necrotrophic pathogen *Fusarium oxysporum forma specialis* (f. Sp.) *cubense* Race 4 (*Foc*; Endah *et al*., 2008). *Foc* elicits predominantly a jasmonic acid- (JA) and or ethylene- (ET) dependent defense response pathway in plants (Oliver and Ipcho, 2004; Glazebrook, 2005; Spoel *et al*., 2007). Further, these *MNPR1* genes were sequentially expressed in response to the banana hemi-biotrophic pathogen *Xanthomonas campestris* (Endah *et al*., 2010), which, leads to increased concentrations of both SA and JA in infested plant (Martinez *et al*., 2000; Delannoy *et al*., 2005). However, the control of this differential response and that of expression of *MNPR1* genes in pathogen defense is still largely unknown. It was observed, however, that expression of these genes correlated with expression of two *pathogenesis-related* (*PR-1* and *PR-3*) genes used during these studies (Endah *et al*., 2008; Endah *et al*., 2010) hence implicating them as possible defense genes.

Preliminary sequence analysis of previously reported NPR1-like sequences has been carried out in many studies using selected sequences from the National Center for Biotechnology information (NCBI) database. In 2008 analysis done by Endah and colleagues revealed that the MNPR1 amino acid sequences share a 78% sequence identity to each other when compared with eight other NPR1 sequences. They further grouped closed with the monocotyledon NPR1 homologue (NH1 or *Os*NPR1) from rice known to mediate plant defense responses (Yuan *et al*., 2007). In 2010, a more comprehensive sequence alignment study was done by Begeault *et al*. (2010) using 31 NPR1-like sequences pooled from the database From their study, the two banana MNPR1 sequences still grouped in the same clade as the rice *Os*NPR1 sequence. What is missing from both studies, however, is how the two MNPR1 sequences compare with the third banana ABB NPR1 sequence. Moreover, more than 31 NPR1-like sequences currently exist in the NCBI database and a more detailed comparison is essential for an in-depth understanding of the *MNPR1* genes and how their structure relates to their function. Additionally, information is also lacking on the type and representation of *cis*-regulatory elements present within the *MNPR1* coding region.

The objective of this part of the study was to therefore understand at the sequence level whether these two *MNPR1* genes belong to the same group as other known NPR1-like sequences when more 39 NPR1-like sequences are compared. A further objective was to identify recently published conserved functional motifs and defense *cis*-regulatory elements present in the MNPR1 coding sequences.

3.3 Materials and methods

3.3.1 Alignment of NPR1-like sequences and identification of conserved regions

Using the *Arabidopsis* NPR1 coding sequence as a reference (At1g64280), 41 NPR1-like coding sequences from different plant species were obtained from the NCBI database (Table 3.1). These sequences were edited in BioEdit v7.0.0 to obtain the correct reading frame and aligned using the ClustalW software program (Thompson *et al*., 1994). The percentage identity among sequences was provided directly by ClustalW while conserved regions were identified using published data from well-characterized NPR1 like sequences.

3.3.2 Phylogenetic analysis

The full length amino acid coding region of the *Musa* NPR1-like sequences (Table 3.1) were used to establish a phylogenetic relationship with the other 39 *NPR1* sequences. All sequences were aligned using the multiple sequence alignment software (MAFFT version 5) program (Katoh *et al*., 2005). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 for Neighbour Joining (NJ) following the software instructions (Tamura *et al*., 2007) and the bootstrap was set to 1000 replicates.

Table 3.1 NPR1-like amino acid sequences used for alignment.

Organism	Sequence name	Genbank reference
Musa acuminate (AAA)	*M*NPR1A	ABI93182.1
M. acuminate (AAA)	*M*NPR1B	ABL63913.1
M. spp. ABB group	*Md*NPR1	ACE86413.1
Arabidopsis. Thaliana	*At*NPR1	ABR46023.1
A. thaliana	*At*NPR2	NP_194342.1
A. thaliana	*At*NPR3	NP_199324.2
A. thaliana	AtNPR4	NP_193701.2
A. thaliana	AtNPR5	ABH04470.1
A. thaliana	AtNPR6	AAU90063.1
A. lyrata	*Al*NPR1	ABR46032.1
Oryza sativa	*Os*NPR1	ABE11614.1
O. sativa	*Os*NPR2	ABE11616.1
O. sativa	*Os*NPR3	ABE11618.1
O. sativa	*Os*NPR4	ABE11620.1
O. sativa	*Os*NPR5	ABE11622.1
Brassica napus	*Bn*NPR1	AAM88865.2
B. juncea	*Bj*NPR1	ABC94642.2
Beta vulgaris	*Bv*NIM1	AAT57640.1
Capsicum annuum	*Ca*NPR1	ABG38308.1
C. chinense	*Cc*NPR1	CAP12787.1
Carica papaya	*Cp*NPR1	AAS55117.1
Glycine max	*Gm*NPR1-1	ACJ45013.1
G. max	*Gm*NPR1-2	ACJ45015.1
Gossypium hirsutum	*Gh*NPR1	ABC54558.1
Helianthus annuus	*Ha*NIM1	AAT57642.1
Hordeum vulgare	*Hv*NPR1	CAJ19095.1
Ipomoea batatas	*Ip*NPR1	ABM64782.1
Malus x *domestica*	*M*xd NPR1	ACC77697.1
Nicotiana tabacum	*Nt*NIM1	AAT57641.1
N. tabacum	*Nt*NPR1	ABH04326.1
N. glutinosa	*Ng*NPR3	ABN45747.1
Populus trichocarpa	*Pt*NIM1	XP_002300863.1
P. trichocarpa	*Pt*NPR1	XP_002322351.1
P. trichocarpa	*Pt*NIM1b	ABF48718.1
Ricinus communis	*Rc*NPR1	XP_002514127.1
R. communis	*Rc*NPR1B	XP_002520549.1
Solanum lycopersicum	*Sl*NIM1	AAT57638.1
S. lycopersicum	*Sl*NIM2	AAT57639.1
Theobroma cacao	*Tc*NPR1	ADI24348.1
Vitis vinifera.	*Vv*NPR1.1	CAO65332.1
V. vinifera	*Vv*NPR1.2	CAN67078.1

3.3.3 Identification of defense-response *cis*-elements within the *MNPR1* coding region

To identify defense response *cis*-elements within the *MNPR1* coding sequences, the coding region of *MNPR1A* and *MNPR1B* were individually analyzed using the PLACE/Signal scan web-based program as instructed by the software manufacturer (http://www.dna.affrc.go.jp/PLACE/signalup.html; Higo *et al*., 1999). Each identified *cis*-element was then individually analyzed to identify elements known to mediate elicitor and/or pathogen responses in plants.

3.4 Results

3.4.1 NPR1 sequence analysis and identification of conserved regions

Of the 41 sequences aligned, the two banana MNPR1 (MNPR1A and MNPR1B) coding sequences were 78% identical to each other (Table 3.2) but only 42% and 44%, respectively identical to the ABB banana NPR1-like sequence (MdNPR1). The rice (*Os*)NPR1 sequence was 63% and 65% identical to MNPR1A and MNPR1B, respectively while *Arabidopsis* NPR1 was only 47% and 48% identical to the two MNPR1 sequences, respectively. *Capsicum annuum* had the highest identity to both MNPR1A (64%) and MNPR1B (68%) and the least identity was with the rice NH4 sequence which was only 4% identical to MNPR1A and 10% identical to MNPR1B.

Table 3.2 Percentage identity of 41 NPR1-like amino acid coding sequences

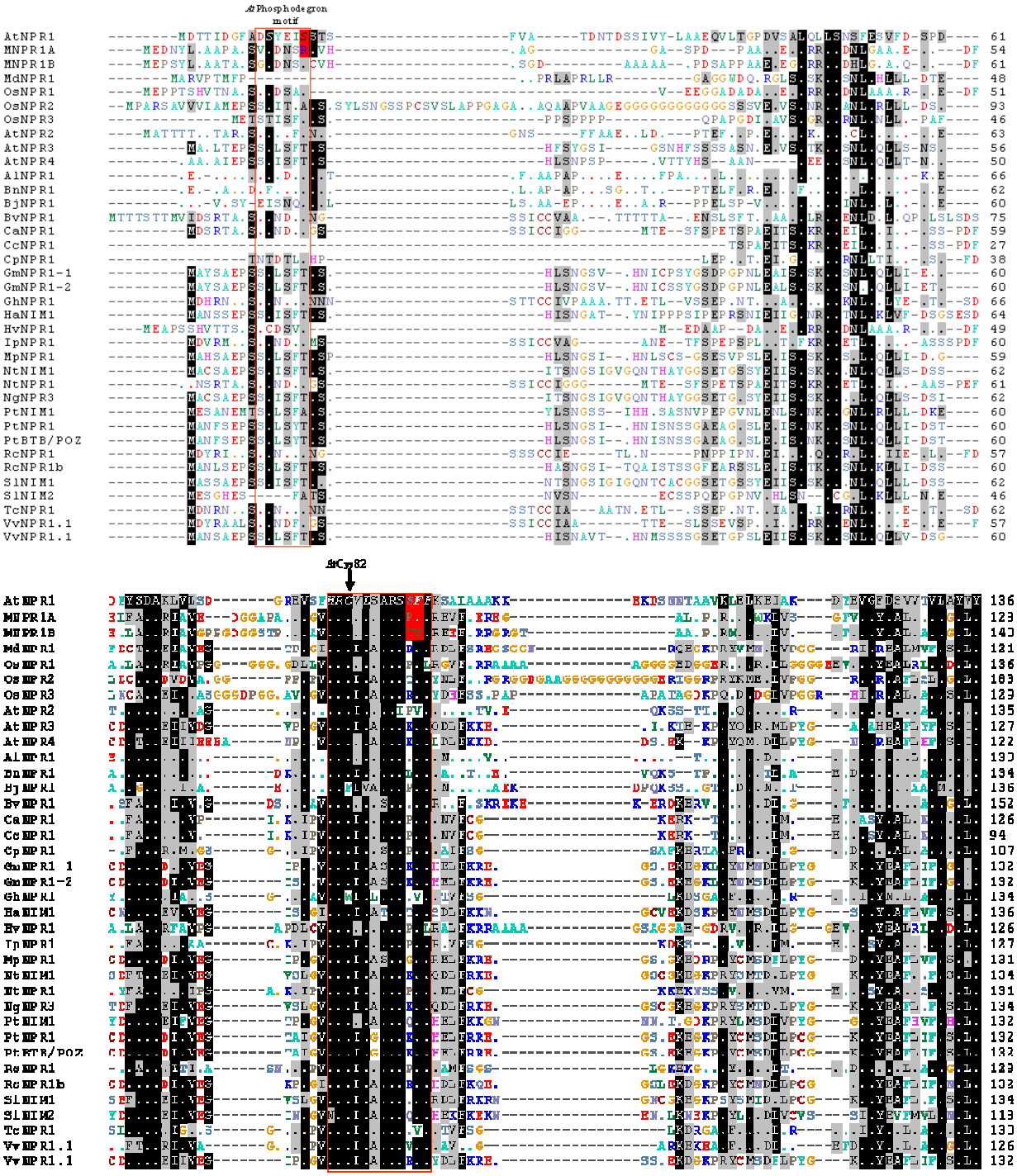
Name	MNPR1A	MNPR1B	MdNPR1	AtNPR1	AtNPR2	ATNPR3	ATNPR4	ATNPR5	ATNPR6	AlNPR1	NH1	NH2	NH3	NH4	NH5	BNNPR1	BjNPR1	BvNPR1	CaNPR1	CcNPR1	CpNPR1
MNPR1A																					
MNPR1B	78.0																				
MdNPR1	42.0	44.0																			
AtNPR1	47.0	48.0	34.0																		
AtNPR2	46.0	45.0	36.0	61.0																	
AtNPR3	39.0	40.0	52.0	35.0	36.0																
AtNPR4	40.0	40.0	52.0	36.0	37.0	71.0															
AtNPR5	22.0	24.0	25.0	21.0	20.0	25.0	25.0														
AtNPR6	20.0	21.0	26.0	20.0	18.0	24.0	24.0	83.0													
ALNPR1	49.0	49.0	36.0	89.0	60.0	36.0	37.0	22.0	20.0												
NH1	63.0	65.0	41.0	45.0	42.0	38.0	37.0	25.0	23.0	45.0											
NH2	43.0	43.0	65.0	36.0	34.0	49.0	49.0	26.0	23.0	37.0	42.0										
NH3	40.0	41.0	52.0	37.0	37.0	45.0	46.0	23.0	23.0	37.0	41.0	52.0									
NH4	4.0	10.0	8.0	9.0	7.0	7.0	8.0	9.0	9.0	9.0	13.0	10.0	11.0								
NH5	22.0	22.0	25.0	21.0	18.0	22.0	21.0	68.0	70.0	21.0	24.0	25.0	21.0	9.0							
BNNPR1	46.0	46.0	37.0	69.0	56.0	33.0	33.0	20.0	18.0	69.0	43.0	35.0	37.0	8.0	19.0						

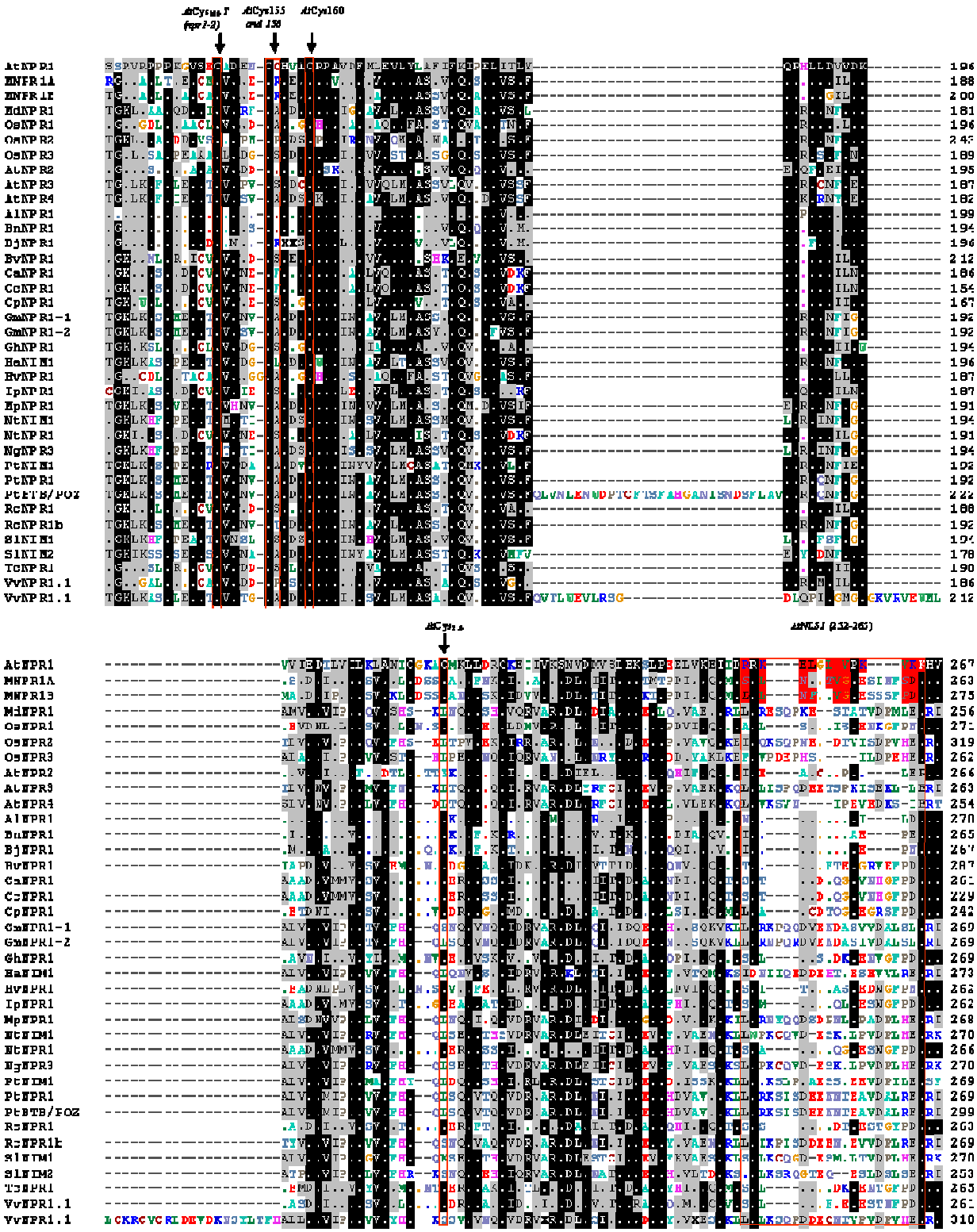
Name	MNPR1 A	MNPR1 B	MdNPR 1	AtNPR1	AtNPR 2	ATNPR 3	ATNPR 4	ATNPR 5	ATNPR 6	AlNPR1	NH1	NH2	NH3	NH4	NH5	BNNPR1	BjNPR1	BvNPR 1	CaNPR 1	CcNPR 1	CpNPR 1
BjNPR1	45.0	43.0	34.0	62.0	54.0	34.0	34.0	21.0	19.0	64.0	42.0	33.0	33.0	6.0	19.0	67.0					
BvNPR1	59.0	60.0	43.0	50.0	49.0	38.0	40.0	23.0	20.0	52.0	56.0	41.0	38.0	13.0	23.0	51.0	46.0				
CaNPR1	60.0	63.0	43.0	49.0	47.0	39.0	37.0	23.0	21.0	52.0	58.0	42.0	40.0	10.0	21.0	51.0	47.0	67.0			
CcNPR1	64.0	68.0	45.0	54.0	52.0	42.0	40.0	23.0	21.0	55.0	62.0	45.0	43.0	8.0	22.0	54.0	49.0	69.0	99.0		
CpNPR1	60.0	63.0	43.0	52.0	51.0	39.0	39.0	23.0	21.0	54.0	56.0	41.0	39.0	9.0	20.0	52.0	50.0	67.0	71.0	73.0	
GmNPR1-1	43.0	44.0	63.0	37.0	37.0	60.0	62.0	27.0	25.0	38.0	41.0	57.0	48.0	7.0	23.0	36.0	35.0	44.0	43.0	47.0	45.0
GmNPR1-2	43.0	43.0	63.0	36.0	36.0	59.0	62.0	26.0	26.0	38.0	41.0	58.0	47.0	6.0	24.0	35.0	34.0	44.0	42.0	46.0	45.0
GhNPR1	58.0	59.0	42.0	53.0	50.0	40.0	40.0	22.0	20.0	53.0	57.0	41.0	38.0	9.0	21.0	51.0	50.0	64.0	67.0	70.0	74.0
HaNIM1	40.0	38.0	58.0	36.0	36.0	54.0	53.0	23.0	22.0	37.0	39.0	53.0	47.0	7.0	22.0	34.0	34.0	42.0	41.0	45.0	40.0
HvNPR1	60.0	62.0	39.0	42.0	42.0	38.0	38.0	22.0	21.0	43.0	79.0	40.0	39.0	11.0	21.0	41.0	39.0	53.0	56.0	60.0	54.0
IpNPR1	57.0	59.0	43.0	48.0	48.0	38.0	39.0	24.0	21.0	51.0	56.0	41.0	39.0	9.0	22.0	50.0	46.0	66.0	80.0	81.0	66.0
MxdNPR1	41.0	42.0	62.0	36.0	36.0	59.0	57.0	25.0	26.0	38.0	39.0	55.0	46.0	9.0	25.0	36.0	34.0	40.0	41.0	45.0	43.0
NtNIM1	41.0	42.0	62.0	36.0	36.0	55.0	55.0	25.0	23.0	37.0	39.0	55.0	45.0	8.0	24.0	36.0	34.0	43.0	41.0	45.0	41.0
NtNPR1	58.0	62.0	43.0	50.0	49.0	39.0	39.0	23.0	21.0	52.0	55.0	42.0	40.0	8.0	22.0	48.0	45.0	65.0	92.0	91.0	69.0
NgNPR3	43.0	43.0	62.0	36.0	36.0	56.0	56.0	25.0	24.0	38.0	40.0	55.0	46.0	11.0	25.0	37.0	35.0	42.0	41.0	44.0	40.0
PtNIM1	44.0	43.0	66.0	36.0	37.0	60.0	59.0	27.0	25.0	38.0	42.0	60.0	48.0	9.0	25.0	34.0	33.0	43.0	43.0	47.0	44.0
PtNPR1	62.0	61.0	43.0	51.0	50.0	40.0	41.0	26.0	23.0	51.0	57.0	43.0	38.0	9.0	23.0	50.0	48.0	65.0	70.0	73.0	73.0
PtNPR1	62.0	61.0	43.0	51.0	50.0	40.0	41.0	26.0	23.0	51.0	57.0	43.0	38.0	9.0	23.0	50.0	48.0	65.0	70.0	73.0	73.0
PtNIM1b	40.0	39.0	59.0	32.0	32.0	50.0	50.0	27.0	27.0	34.0	35.0	53.0	45.0	8.0	24.0	32.0	34.0	40.0	38.0	41.0	39.0
RcNPR1	62.0	62.0	44.0	53.0	52.0	39.0	42.0	25.0	24.0	55.0	56.0	43.0	38.0	8.0	23.0	53.0	50.0	67.0	72.0	75.0	76.0
RcNPR1b	44.0	43.0	65.0	37.0	38.0	61.0	59.0	26.0	24.0	38.0	41.0	58.0	49.0	8.0	26.0	37.0	35.0	44.0	45.0	48.0	45.0
SlNIM1	41.0	41.0	61.0	37.0	35.0	55.0	53.0	25.0	25.0	36.0	39.0	55.0	47.0	8.0	23.0	35.0	34.0	44.0	42.0	45.0	41.0
SlNIM2	39.0	39.0	55.0	37.0	36.0	49.0	47.0	26.0	23.0	37.0	36.0	49.0	47.0	9.0	25.0	35.0	34.0	41.0	40.0	41.0	38.0
TcNPR1	60.0	61.0	42.0	54.0	53.0	39.0	40.0	24.0	24.0	55.0	58.0	41.0	39.0	10.0	21.0	53.0	50.0	67.0	71.0	74.0	76.0
VvNPR1.1	62.0	64.0	44.0	52.0	52.0	41.0	41.0	21.0	20.0	55.0	58.0	43.0	40.0	7.0	21.0	52.0	49.0	69.0	72.0	74.0	71.0
VvNPR1.2	43.0	44.0	67.0	37.0	37.0	60.0	59.0	27.0	25.0	39.0	43.0	59.0	50.0	7.0	24.0	36.0	35.0	43.0	43.0	47.0	43.0

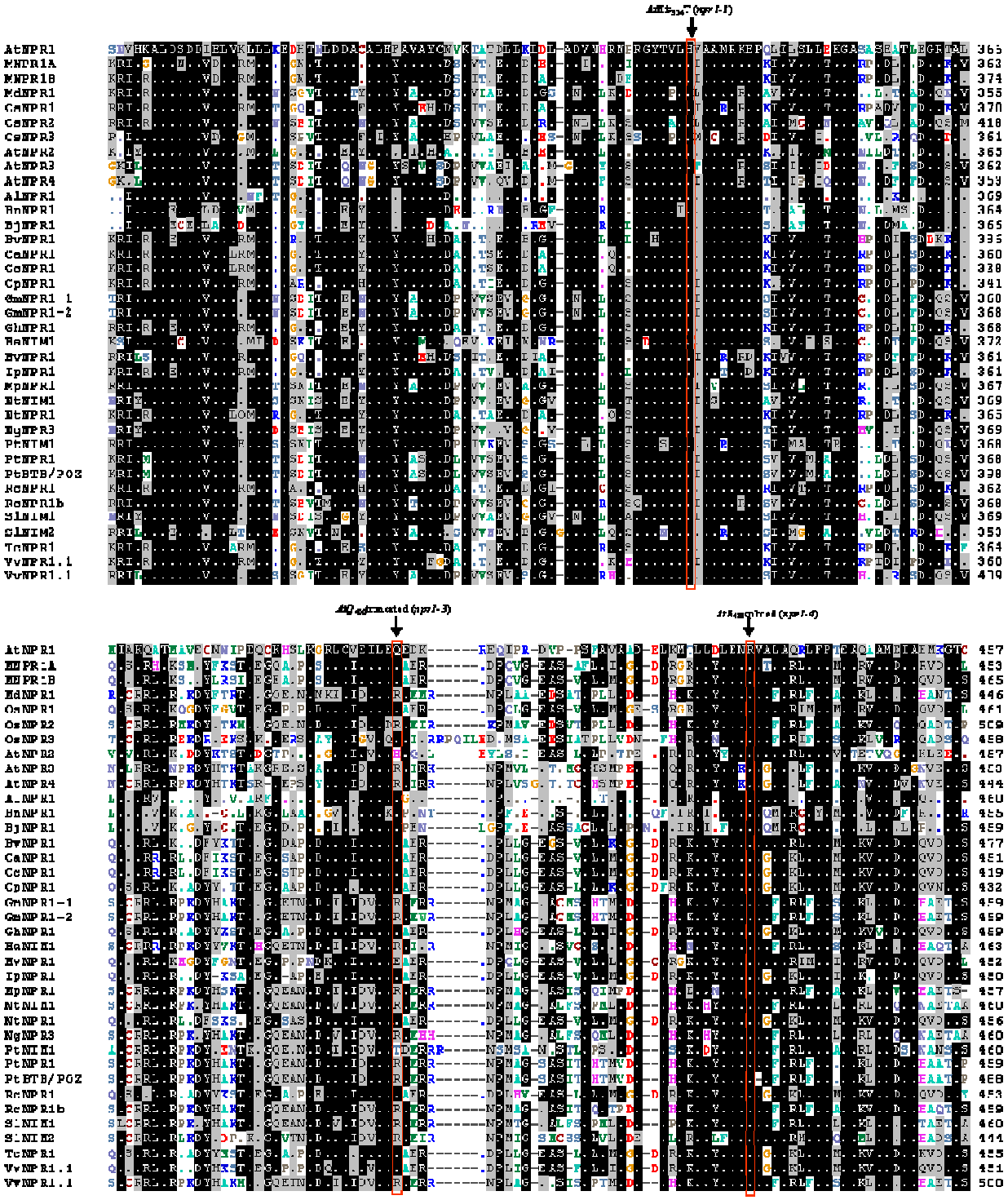
Name	GmNPR1-1	GmNPR1-2	GhNPR1	HaNIM1	HvNPR1	IpNPR1	MxdNPR1	NtNIM1	NtNPR1	PtNIM1	PtNPR1	PtNIM1b	RcNPR1	RcNPR1b	SlNIM1	SlNIM2	TcNPR1	VvNPR1.1	VvNPR1.2
GmNPR1-1																			
GmNPR1-2	96.0																		
GhNPR1	44.0	44.0																	
HaNIM1	68.0	67.0	41.0																
HvNPR1	41.0	38.0	54.0	36.0															
IpNPR1	41.0	40.0	64.0	41.0	56.0														
MxdNPR1	75.0	74.0	42.0	62.0	40.0	39.0													
NtNIM1	70.0	69.0	41.0	64.0	37.0	39.0	67.0												
NtNPR1	44.0	43.0	65.0	40.0	56.0	78.0	42.0	41.0											
NgNPR3	70.0	70.0	40.0	65.0	38.0	40.0	67.0	93.0	41.0										
PtNIM1	80.0	79.0	42.0	68.0	39.0	42.0	74.0	70.0	43.0	71.0									
PtNPR1	45.0	44.0	71.0	42.0	53.0	67.0	43.0	42.0	69.0	42.0	44.0								
PtNIM1b	62.0	61.0	38.0	57.0	35.0	37.0	58.0	58.0	38.0	58.0	61.0	41.0							
RcNPR1	45.0	44.0	73.0	40.0	54.0	70.0	43.0	40.0	71.0	40.0	44.0	80.0	40.0						
RcNPR1b	78.0	76.0	43.0	67.0	39.0	42.0	73.0	69.0	44.0	70.0	83.0	45.0	60.0	45.0					
SlNIM1	69.0	69.0	41.0	64.0	38.0	41.0	66.0	85.0	42.0	85.0	69.0	43.0	57.0	41.0	69.0				
SlNIM2	58.0	56.0	40.0	52.0	36.0	40.0	56.0	51.0	42.0	52.0	56.0	39.0	54.0	40.0	56.0	51.0			
TcNPR1	43.0	44.0	85.0	41.0	56.0	68.0	41.0	41.0	69.0	41.0	42.0	76.0	39.0	77.0	44.0	42.0	40.0		
VvNPR1.1	46.0	45.0	68.0	40.0	55.0	69.0	42.0	42.0	70.0	42.0	43.0	71.0	41.0	72.0	45.0	43.0	42.0	73.0	
VvNPR1.2	79.0	78.0	44.0	69.0	41.0	42.0	74.0	73.0	43.0	74.0	79.0	43.0	61.0	43.0	78.0	72.0	57.0	44.0	43.0

Gaps on table represent areas were sequence comparison results have already been presented

The two MNPR1 sequences also shared conserved regions that have been described in other NPR1-like genes (Fig. 3.1). These regions consist of a phosphodendron motif, the BTB/POZ domain, ankyrin repeats, and nuclear localization regions. However, within these domains, there exist some amino acid dissimilarities within the two MNPR1 sequences. Moreover, these sequence dissimilarities also exist among homologous NPR1 sequences from the same plants like rice, *Arabidopsis*, grape vine, soybean and *Brassica*. For instance, in the phosphodendron motif, the characterized serine amino acid at position 13 was present in the two MNPR1 sequences, *Os*NPR1, *Os*NPR2, *At*NPR1, *At*NPR2, *At*NPR3, but completely absent from MdNPR1 and replaced by a tyrosine in *Os*NPR3. Serine at position 15 on the other hand was present in MNPR1B, *At*NPR1, *At*NPR2, *Os*NPR1 and *Os*NPR3 but replaced by an arginine in MNPR1A. This serine was also completely absent from the MdNPR1 banana sequence. Another pronounced dissimilarity was found for the already characterized cysteine at position 529 in the *At*NPR1 sequence. This was replaced by a tyrosine in MNPR1A, MdNPR1, and *At*NPR4; by a glycine in MNPR1B; by a serine in *Os*NPR1, *At*NPR2, by aspartic acid in *Os*NPR2, by a leucine in *Os*NPR3, and by a histidine in *At*NPR3.


AtPhosphodegron motif
AtCys82


AtCys₁₄₆ F (ugr1-2)
AtCys155 and 158
AtCys160
AtCys₁₆₅
AtNLS1 (432-265)
AtNPR1
MNPR1A
MNPR1B
MdNPR1
OsNPR1
OsNPR2
OsNPR3
AtNPR2
AlNPR3
AtNPR4
AlNPR1
BnNPR1
BjNPR1
BvNPR1
CaNPR1
CcNPR1
CpNPR1
GmNPR1-1
GmNPR1-2
GhNPR1
HaNIM1
HvNPR1
IpNPR1
MpNPR1
NtNIM1
NtNPR1
NgNPR3
PtNIM1
PtNPR1
PtBTB/POZ
RcNPR1
RcNPR1b
SlNIM1
SlNIM2
TcNPR1
VvNPR1.1
VvNPR1.1



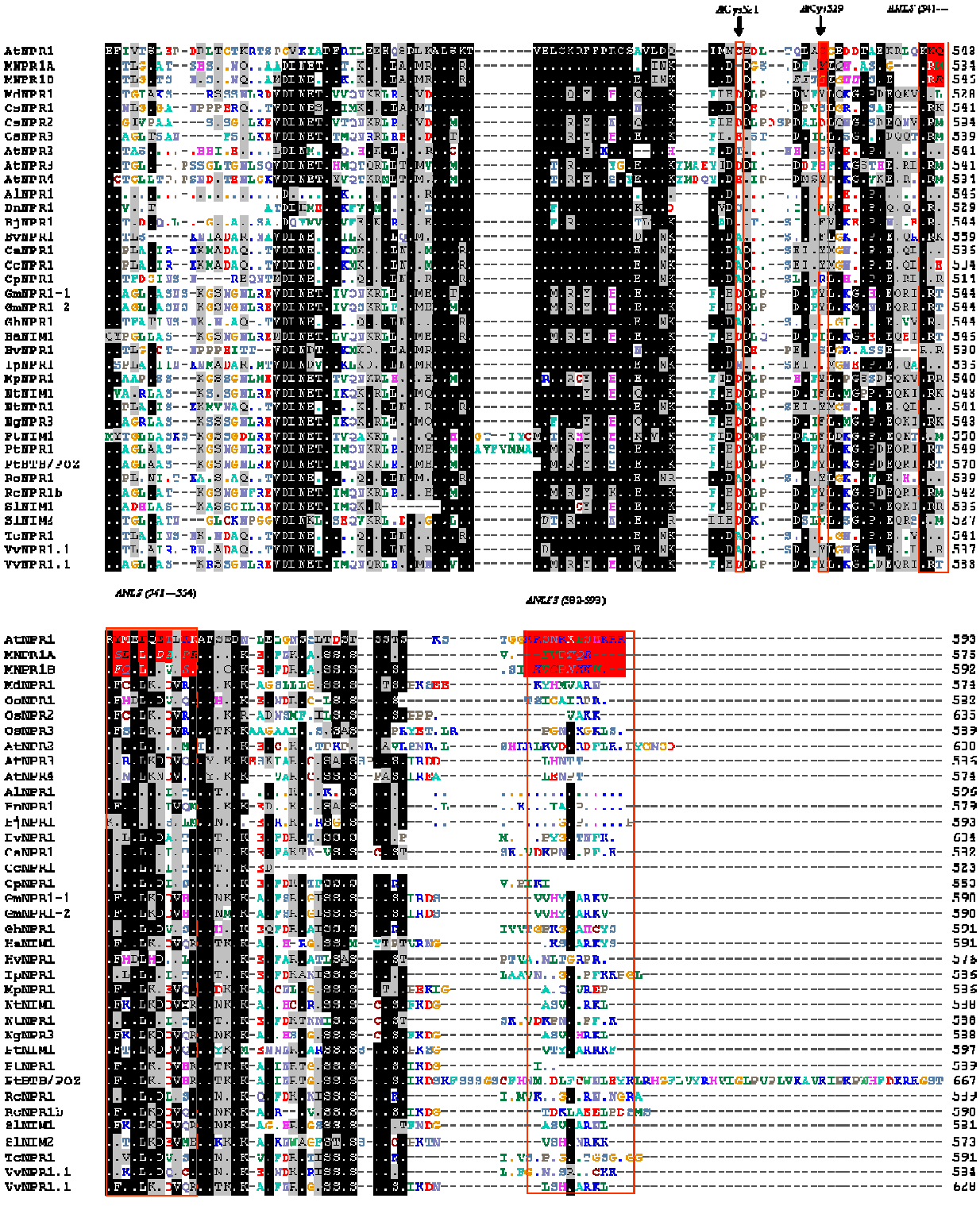

AtNPR1
MNPR1A
MNPR1B
WdNPR1
CsNPR1
CsNPR2
CsNPR3
AtNPR3
AtNPR5
AtNPR4
AlNPR1
DnNPR1
RjNPR1
BvNPR1
CaNPR1
CcNPR1
CpNPR1
GmNPR1-1
GmNPR1-2
GhNPR1
BaNIM1
BvNPR2
IpNPR1
MpNPR1
NtNIM1
NtNPR1
RcNPR1
SlNIM1
SlNIM2
TcNPR1
VvNPR1.1
VvTPR1.1
ANK5 (241—334)
ANK5 (391-393)

Figure 3.1 Multiple alignment of MNPR1A and MNPR1B with other plant NPR1-like amino acid coding sequences. Amino acid sequences were aligned using the ClustalW multiple alignment program (Thompson *et al*., 1994). Identical amino acids are represented with dots, vertical red rectangles represent conserved domains and specific arrows show other single conserved amino acids with reference to the *Arabidopsis* NPR1 sequence. Differences in amino acids in the conserved domains between the *Arabidopsis* NPR1 and banana MNPR1 sequences are shaded in red while differences between the two banana MNPR1 sequences are shaded in red and italicized.

3.4.2 Phylogenetic grouping of MNPR1 coding sequences

Bootstrap consensus for neighbour joining (NJ) produced a total of three distinct groups in the phylogenetic tree. The two MNPR1 coding sequences (Fig 3.2; red) belonged to the first group and were closely related with each other (Fig. 3.2; dark red). They further grouped closely with NPR1 sequences of two other monocotyledons (*Hordeum vulgare* and the NHI sequence from rice). Although the banana ABB NPR1-like sequence belonged to a different group (Fig. 3.2 green) from the two MNPR1 banana sequences, it however grouped closely with the rice NH2 and NH3 sequences as well as many sequences known to mediate defense responses. NH5 *At*NPR5 and *At*NPR6 were the most distant NPR1-like sequences, while NH4 was used to root the tree.

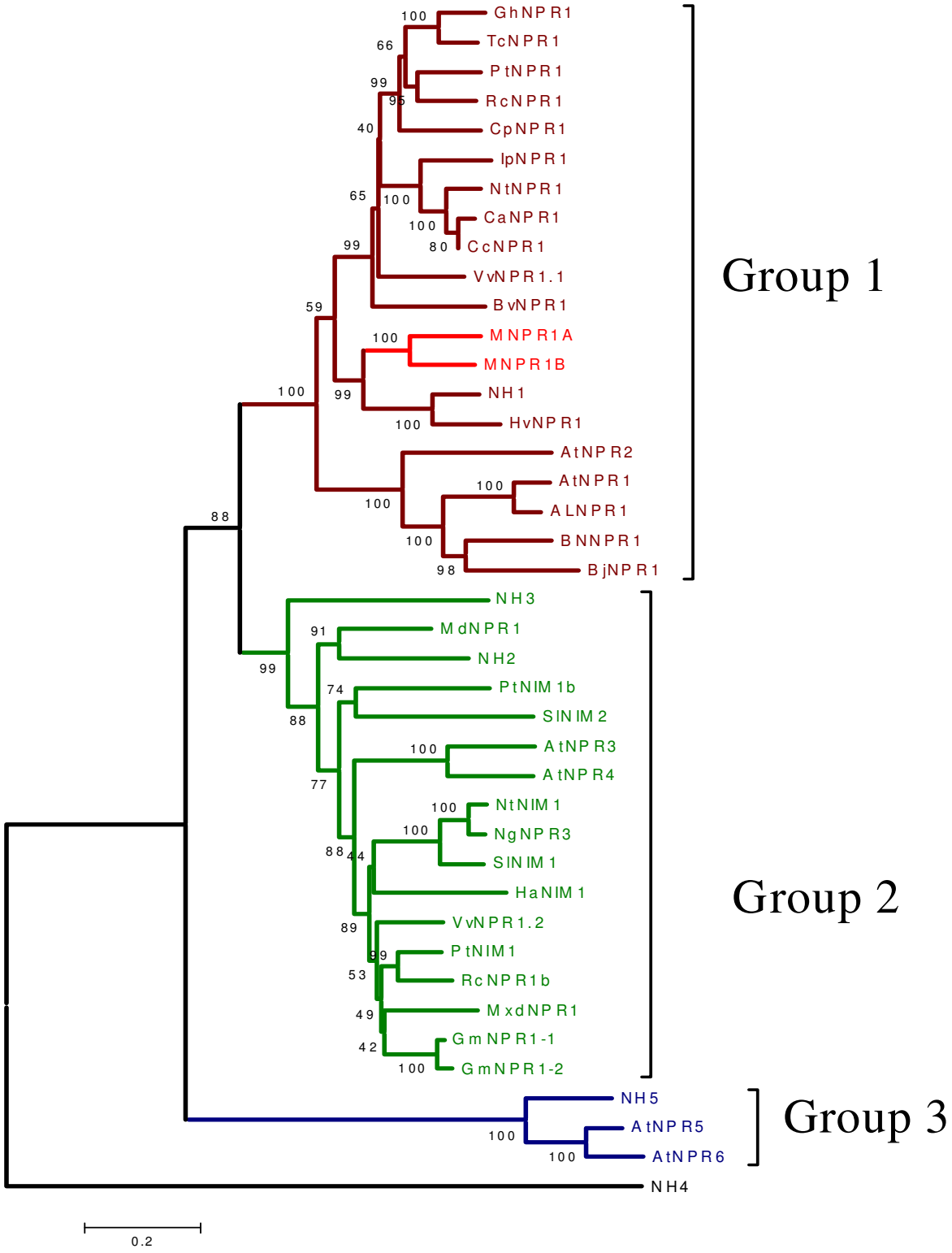


Figure 3.2 Multiple alignment and evolutionary relationship of the various plant NPR1-like coding sequences. Amino acid coding sequences from 41 plants were retrieved from Genbank

and aligned using the Mafft software program. The alignment was then edited in Bioedit to obtain the correct reading frame. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

3.4.3 Comparison of defense-related *cis*-elements within the *MNPR1* coding sequences

From PLACE/Signal scan results it was apparent that the two banana *NPR1*-like coding regions harbored known *cis*-elements involved in defense responses mediated by pathogens, JA, SA, ET and ABA (Table 3.3). The *MNPR1A* gene was more enriched in the number of occurrence of each defense *cis*-elements (Fig. 3.3). For instance the transcription factor WRKY71*Os* appeared eight times in the positive *MNPR1A* strand but only six times in the positive *MNPR1B* strand. The ET-responsive factor3 (ERF3) was also over-represented in *MNPR1A* in comparison to *MNPR1B*. However, *MNPR1B* had three additional *cis*-elements (TCA, AGCBOXPGLB, ELRECOREPCRP1 and ethylene responsive element) known to be responsive to SA which were absent in *MNPR1B* (Table 3.3).

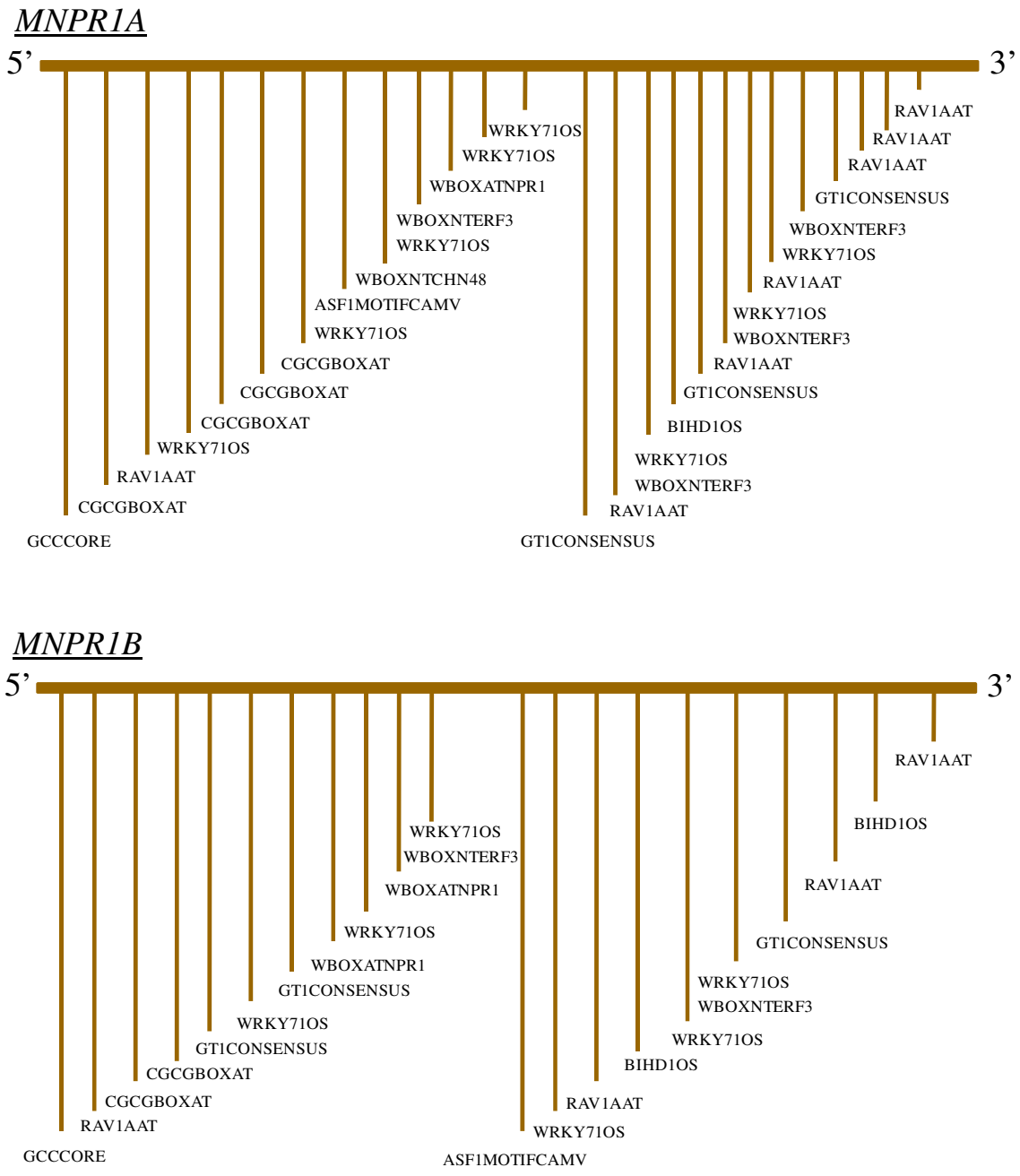


Figure 3.3 Defense-related *cis*-regulatory elements identified within the *MNPR1* coding sequences. *MNPR1* coding sequences were retrieved from the NCBI database. The defense *cis*-regulatory elements in the positive strand of *MNPR1A* and *MNPR1B* were identified on

PLACE/Signal scan database (http://www.dna.affrc.go.jp/PLACE/signalup.html; Higo *et al*., 1999) and represented linearly.

Table 3.3 Elicitor/light-responsive defense *cis*-elements in the coding region of the *MNPR1A*, and *MNPR1B* sequences.

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
GCCGCC – GCCCORE	22[a] 22[b]	73[a] 81[b]	Ethylene responsive element present in PDF1.2 Thi2.1, *PR-4*. Plays a role in JA and ET responsive gene expression	*A. thaliana*, *L. esculentum*	Brown *et al.*, (2003); Chakravarthy *et al.*, 2003); Pascual-Lobo and Molina, (2008), Diaz-Sánchez *et al.*, (2007)
GRWAAW – GT1 CONSENSUS	382[a], 778[a], 811[a], 1396[a] 363[b], 814[b], 1432[b]	771[a], 1363[a], 1505[a], 1506[a] 1540, 1541[b]	Light-regulated genes, stabilizes TATA box complex; GT1 interact with the promoter of *PR-1a* to regulate SA and SAR gene products	*L. esculentum*	Le Gourrierec *et al.*, (1999); Buchel *et al.*, (1999); Buchel *et al.*, (1996); Terzaghi *et al.*, 1995; Villain *et al.*, 1996; Zhou, (1999).
***GAAAAA* – GT1 GMSCAM4**		771[a], 1505[a] 1540[b]	Mediates pathogen- and NaCl-induced gene expression. Found in GT-1 promoters like the soybean calmodium (CaM) isoform	*L. esculentum*	Park *et al.*, (2004), Hung *et al.*, 2004;.
CCGAC – LTRECOREATCOR15	44[a], 89[a], 173[a] 44[b], 194[b]	220[a], 343[a], 1702[a] 122[b], 211[b], 379[b]	Core of low temperature responsive element (LTRE) of cor15a gene, responsive to ABA and induces BN115 gene during cold stress.	*A. thaliana*; *Brassica napus*	Baker *et al.*, (1994); Jiang *et al.*, (1996); Dunn *et al.*, (1998);

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
MACGYGB – MACGYGB		29[a]	An ABRE-related sequence found upstream of the Ca^{2+}-responsive unregulated genes	*Arabidopsis thaliana*	Kaplan *et al.*, (2006)
AGCAGC - ANAERO2CONSENSUS; TCATCAC - ANAERO3CONSENSUS, GTTTHGCAA- ANAERO4CONSENSUS	52[b], 799[b], 802[b]	85[c] 1244[a] 851[b], 1280[b] 635[a]	Anaerobic stress/ respiration	Maize (*Zea mays*); *A. thaliana*; Pea (*Pisum sativum*); Sunflower (*Helianthus vulgare*); Rice (*Oryza sativa*), Petunia (*Petunia hybrida*); Tomato (*Lycopersicon esculentum*)	Mohanty *et al.*, (2005).
RCCGAC DRECRTCOREAT	172[c] 193[b]	343[a], 1702[a]	Core motif of dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element. DRE/CRT are involve in drought, high-light; cold stress	*O. sativa*; *Z. mays*, (*H. annuus*	Dubouzet *et al.*, (2003); Qin *et al.*, (2004); Diaz-Martin *et al.*, (2005); Suzuki *et al.*, (2005) Skinner *et al.*, (2005).
TGTCA BIHD1OS	711[c], 786[c] 1061[b], 1664[b]	126[a], 558[a], 576[a] 612[b], 846[b], 1070[b]	Rice BELL homeodomain TF in disease response. Activated by DTI and *Magnaporthe grisea*. Leads to *PR-1* gene activation	*O. sativa*	Luo *et al.*, (2005)
***VCGCGB* – CGCGBCXAT**	29[a], 211[a], 245[a], 247[a] 113[b], 329[b]	29[a], 211[a], 245[a], 247[a] 113[b], 329[b]	Calmodulin-involved in multiple signaling. Induced by MeJA, SA, H_2O_2, wounding, ABA, salt, UVB stress	*A. thaliana*	Lin *et al.*, (2009), Wang *et al.*, 2009;, Yang and Poovaiah (2002), Park *et al.*, (2004)

Sequence-*Cis* element	**Strand/ position**		**Function**	**Organism**	**Reference**
	"+"	"-"			
TAACTG – MYB2AT		656[a]	Regulates water stress-related genes. Binds to *At*MYB2.	***A. thaliana***	**Urao *et al.*, (1993).**
YAACKG – MYB2CONSENSUSAT	1130[a]	656[a]	MYB recognition site present in the promoter of the dehydration-responsive gene *rd22*	*A. thaliana*	Abe *et al.*, (2003).
CNGTTR – MYBCORE	656[a], 841[a], 1523[a] 532[b], 954[b]	48[a], 920[a], 1130[a] 48[b], 956, 1419[b]	Binding site for all animal MYB and at least two *Arabidopsis* MYB proteins AtMYB2 regulates water stress responsive genes. MYB protein (MYB.Ph3) contributes in regulating the flavonoid biosynthetic pathway.	***A. thaliana*;** animal; ***P. hybrida*;**	**Luscher and Eisenman, (1990), Urao *et al.*, (1993), Solano *et al.*, (1995)**
CATGTG – MYCATERD1	420[a], 875[a] 91[b]		MYC recognition sequence necessary for expression of early responsive to dehydration (erd1) gene during dehydration	*A. thaliana*;	Chinnusamy *et al.*, (2003), Chinnusamy *et al.*, (2004); Abe *et al.*, (2003). Oh *et al.*, 2005). Lee *et al.*, (2005). Hartmann *et al.*, (2005), Agarwal *et al.*, (2006).
CANNTG – MYCCONSENSUSAT	420[a], 539[a], 875[a], 1130[a] 54[b], 675[b], 91[b], 983[b], 1088[b], 1265[b], 1489[b]	420[a], 539[a], 875[a], 1130[a] 54[b], 675[b], 91[b], 983[b], 1088[b], 1265[b], 1489[b]	MYC recognition site found in the promoters of the *dehydration-responsive* gene *rd22*. Regulates transcription of the cold stress related gene CBF/DREB1).	***A. thaliana*;**	
CACATG – MYCATRD22		420[a], 875[a] 91[b]	Drought-, ABA- regulated gene expression by MYB and MYC	*A. thaliana*	Anderson *et al.*, (2004); Abe *et al.*, (1997); Busk *et al.*, (1998).

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
TGACY – **WBOXNTERF3**	**404[a], 698[a], 906[a] 861[b], 1284[b]**	**522[a], 662[a], 1127[a] 233[b], 558[b], 693[b], 698[b], 1560[b]**	**Activators of ERF3 gene by wounding in tobacco leaves.**	***N. tabacum***	**Nishiuchi *et al.*, (2004).**
TGAC – WRKY71OS	126[a], 362[a], 404[a], 558[a], 576[a], 698[a], 906[a], 1248[a] 612[b], 846[b], 861[b], 906[b], 1070[b], 1284[b]	523, 663, 712, 787, 1128[a], 1285[a] 234[b], 559[b], 694[b], 699[b], 823[b], 1062[b], 1561[b], 1665[b], 1731[b]	Repressor of GA signaling binds to PR-10 promoters; induced by ABA. Upregulated by SA, MeJA, ACC, wounding and pathogen infection	*O. sativa*; Parsley (*Petroselinum crispum*)	Liu *et al.*, (2007). Zhang *et al.*, (2004); Xie *et al.*, (2005); Eulgem *et al.*, (1999); Eulgem *et al.*, (2000).
TTGAC – **WBOXATNPR1**	**337[a] 845[b], 860[b]**	**712[a], 787[a] 234[b], 694[b], 823[b], 1665[b]**	**WRKY- (W) –box are SA inducible. Present in *NPR1* promoters.**	***A. thaliana***	**Yu *et al.*, (2001); Chen *et al.*, (2002); Eulgem *et al.*, (2000); Chen *et al.*, (2002); Maleck *et al.*, (2000); Xu *et al.*, (2006).**
CTGACY – WBOXNTCHIN48	403[a]	522[a], 662[a] 558[b], 698[b]	WRKY1, 2, 4 in tobacco related *cis*-elements of basic chitinase gene. Possibly involve in elicitor-responsive transcription of defense genes in tobacco.	*Nicotiana tabacum*	Yamamoto *et al.*, (2004).

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
TCATCTTCTT – **TCA1MOTIF**		**1765[b]**	**SA inducible expression of genes. Found in barley beta-1,3-glucanase and over 30 different plant genes known to be induced by one or more forms of stress**	*H. vulgare*; *N. tabacum*	Goldsbrough *et al.*, (1993); Mhiri *et al.*, 1997).
CAACA – RAV1AAT	48[a], 636[a], 860[a], 920[a], 1662[a], 1694[a], 1715[a] 48[b], 956[b], 1566[b], 1736[b]	822[a], 842[a] 633[b], 858[b], 1301[b]	RAV1 box, positively regulates early responses to pathogens, SA, wounding and ET.	*A. thaliana*	Sohn *et al.*, (2006); Kagaya *et al.*, (1999).
TGACG – **ASF1MOTIFCAMV,**	**362[a] 908[b]**	**822[b], 1730[b]**	**Activation sequence factor1 (ASF-1) binding site in CaMV 35S promoter. Found in genes induced by auxins, SA genes involved in biotic and abiotic stress. TGA binding site in SA responsive *PR-1* promoters. Differentially stimulate the activity of the "as-1 element" during xenobiotic stress, SAR, Disease resistance.**	Cauliflower mosaic virus (CaMV), *N. tabacum*, *A. thaliana*	Després *et al.*, (2003); Terzaghi *et al.*, (1995); Benfey *et al.*, (1990); Katagiri *et al.*, (1989); Xiang *et al.*, (1997); Klinedinst *et al.*, (2000); Rushton *et al.*, (2002).

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
AGCCGCC – **AGCBOXNPGLB**		**81[b]**	**Ethylene responsive factors (ERFs); ERF1,2,5; activates GCC BOX dependent transcription of stress signal responsive factor; binding sites are conserved in *PR* genes and MAPK**	*N. plumbaginifolia*; *N. sylvestris*; *A.thaliana*, *O. sativa*	Hart *et al.*, (1993); Fujimoto *et al.*, (2000); Sato *et al.*, (1996); Ohme-Takagi *et al.*, (2000); Rushton *et al.*, (2002); Zhang *et al.*, (2004).
TTGACC – ELRECORFPCRPI		233[b]	Elicitor responsive elements (WRKY1, 2), PR-1, SA inducible.	*P. crispum*; *N. tabacum*	Rushton *et al.*, (1996); Chen and Chen (2000); Eulgem *et al.* (1999); eulgem *et al.*, (2000); Rushton *et al.*, (2002); Lalbi *et al.*, (2004)
CCGTCC – **PALBOXAPC**		**195[a] 225[b]**	**PAL gene family activation. Involved in the induction of transcriptional activity though not sufficient to confer UV-light or elicitor responsiveness.**	*P. crispum*	Lois *et al.*, (1989); Logemann *et al.*, (1995);

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
TGTCTC – ARFAT		**1058[a]**	**Auxin response factor (ARF) binding site, found in the promoters of primary/early auxin response genes**	***A. thaliana*; Soybean (*Glycine max*); *O. sativa***	**Ulmasov *et al.*, (1999); Nag *et al.*, (2005); Ikuta *et al.*, (2005); Harper *et al.*, (2000); Nemhauser *et al.*, (2004); Goda *et al.*, (2004); Hagen *et al.*, (2002).**
TATTAG – CPBCSPOR	929[a], 1178[a] 1214[b]		Critical for cytokinin-enhanced protein binding *in vitro*; found in the promoter of the cucumber NADPH-protochlorophyllide reductase gene;	Cucumber (*Cucumis sativus* L.)	Fusada *et al.*, (2005).
TAACAAR GAREAT	**1034[a]**		**Gibberellic acid-responsive element (GARE); Occurrence of GARE in GA-inducible, GA-responsive, and GA-nonresponsive genes. Also involve in seed germination**	***A. thaliana*;**	Ogawa *et al.*, (2003)
TAACAAA – MYBGAHV	1034[a]		Central element of gibberellin (GA) response complex (GARC) in high-pI alpha-amylase gene in barley; Similar to c-myb and v-myb consensus binding site; are partially involved in sugar synthesis.	*H. vulgare*; *O. sativa*	Gubler *et al.*, (1995); Morita *et al.*, (1998); Gubler *et al.*, (1999).
CATGCA – RYREPEATBNNAPA		**418[a], 873[a]**	**Required for seed specific expression, and response to ABA.**	***O. sativa***	**Ezcurra *et al.*, (1999). Ezcurra *et al.*, (2000)**
CAACTC – CAREOSREP1		163[a] 1319[b], 1358[b], 1648[b]	CAACTC regulatory elements (CAREs) found in the promoter region of a cysteine proteinase (REP-1) gene in rice aleurone, GAREs, and seed.	*O. sativa*	Sueh and Yamauchi, (2005).

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
RYCGAC - CBFHV	172[a] 193[b], 463[b], 1744[b]	343[a], 466[a], 1702[a] 463[b], 502[b], 1164[b]	C-repeat (CRT) binding factors; CBFs are also known as dehydration-responsive element (DRE) binding proteins (DREBs)	Barley (*Hordeum vulgare*)	Xue, (2002); Svensson *et al.*, (2006).
WAACCA – MYB1AT	954[a] 990[b]		MYB recognition site found in the promoters of the dehydration-responsive gene *rd22*. Involve in ABA signaling.	*A. thaliana*.	Abe *et al.*, (2003).
CAANNNNATC- CIACADIANLELHC	714, 1530[a] 1566[b]	550, 817[a] 853[b]	Required for circadian expression of tomato light harvesting complex (Lhc) gene;	*L. esculentum*	Piechulla *et al.*, (1998).
GATAAG - IBOX		120[a], 1565[a]	Conserved sequence upstream of light-regulated genes; Prsence in the promoter region of rbcS of plants.	L. *esculentum*; *A. thaliana*	Giuliano *et al.*, (1988); Donald and Cashmore, (1990); Rose *et al.*, (1999).
GATAA – IBOXCORE	778[a], 811[a] 814[b]	121[a], 1526[a], 1566[a]	Conserved sequence upstream of light-regulated genes of both monocots and dicots;	Monocots; dicots	Terzaghi *et al.*, (1995).
AAMAATCT – CCA1ATLHCB1		1297[b]	CCA1 binding site; CCA1 protein (myb-related transcription factor) interact with the light related gene Lhcb1*3. Related to regulation by phytochrome in shoots leaves	*A. thaliana*	Wang *et al.*, (1997).
TATTCT – -10PEHVPSBD	552[a] 588[b]	793[a] 829[b]	Involve in chloroplast gene expression, circadian rhythms and light regulation	*H. vulgare*	Thum *et al.*, (2001)

Sequence-*Cis* element	Strand/ position		Function	Organism	Reference
	"+"	"-"			
GATA – GATABOX	550[a], 613[a], 706[a], 778[a], 811[a], 949[a], 1176[a] 649[b], 709[b], 742[b], 814[b], 1212[b]	13[a], 122[a], 593[a], 1527[a], 1567[a] 272[b], 1262[b], 1306[b]	GATA motif in CaMV 35S promoter; Binding with ASF-2; found in the promoter of Petunia chlorophyll a/b binding protein, *Cab22* gene; Required for light regulated, and tissue specific expression; Conserved in the promoter of all LHCII type I Cab genes;	CaMV; *P. hybrid*, *A. thaliana*; *O. sativa*	Lam and Chua (1989); Gilmartin *et al*., (1990), Benfey and Chua, (1990) Giuliani *et al*., (1989), Teakle *et al*.,(2002). Reyes *et al*., (2004). Rubio-Somoza *et al*, (2006)
SCGAYNRNNN NNNNNNNNN NNNHD –PRECONS CRHSP70A	**44[a] 44[b], 1447[b]**	**579[b], 1983[a] 192[b]**	**Consensus sequence of (plastid response element PRE) in the promoters of HSP70A in *Chlamydomonas*; Involved in induction of HSP70A gene by light.**	***C. reinhardtii***	**von Gromoff *et al*., (2006).**
GCCAC – SORLIP1AT	25[b], 214[b]	394[a], 895[a] 215[b], 333[b], 397[b], 430[b]	One of "Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in *Arabidopsis*; over-represented in light-induced cotyledon and root common genes and root-specific genes.	*A. thaliana*;	Hudson and Quail. (2003); Jiao *et al*., (2005).
GGGCC – SORLIP2AT		**108[b]**	**one of "Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in *Arabidopsis*.**	***A. thaliana*;**	**Hudson and Quail. (2003)**

The *cis*-element in the coding regions was analyzed in place scan database. The position of the element in each gene is represented by "a" for M*NPR1A*, and "B" for *MNPR1B* sequences. Full citations in table are found in the reference section.

3.5 Discussion

New information contributed by this study is the type and relative frequency of *cis*-regulatory elements indentified within the *MNPR1A* and *MNPR1B* coding regions. The *cis*-elements were more abundant in the *MNPR1A* sequence relative to *MNPR1B*. Over-representation of such *cis*-elements such as the ERF might mediate the *MNPR1A's* activity in necrotrophic pathogens that mediate JA responses in plants (Oliver and Ipcho, 2004; Glazebrook, 2005; Spoel *et al*., 2007; Endah *et al*., 2008) Of further interest from this study was also the fact that *MNPR1B* had additional motifs like the TCA and ELRECOREPCRP1 which have been identified in tobacco and sorghum SA-inducible *WRKY1*,2 and *PR-1* genes (Goldsbrough *et al*., 1993; Mhiri *et al*., 1997; Eulgem *et al*., 1999). This might account to some extend for the increased transcription of the *MNPR1B* gene following SA treatment in our previous study (Endah *et al*., 2008). The additional ethylene responsive element found in *MNPR1B* sequence buy not in *MNPR1A* might also have a functional role in mediating JA-defense response has also observed in the gene's response to MeJA (Endah *et al*., 2008). All the *cis* elements identified during this study have also been reported to play many diverse functions in pathogen (*Pseudomonas*) and elicitor (SA, JA, ET) mediated responses as outlined in Table 3.2. How these *cis*-elements actually interact to mediate overall defense remains unknown. However, results following characterization of the expression of both genes in banana show that they are sequentially transcribed by the hemibiotrophic pathogen *X. campestris* which can elicit both a SA and JA response pathway in infested plants, differentially regulated by SA or the necrotrophic *Foc* pathogen which elicits a JA/ET dependent response, and also expressed following JA treatment (Glazebrook, 2005; Spoel *et al*., 2007; Endah *et al*., 2008; Endah *et al*., 2010).

In correlation with previous studies, the two banana MNPR1 coding sequences group closely together but, had some sequence dissimilarities even within highly conserved regions. It is well-known that most plants harbour more that a single copy of the NPR1-like gene with *Arabidopsis* having 6 copies (Hepworth et al., 2005). Food crops like rice, Soybean, and grape vine are known to harbour 5, and 2 copies each of the gene, respectively (Yuan *et al*., 2007; Endah *et al*., 2008; Zhao *et al*., 2009; Sandhu *et al*., 2009). Sequence analysis from previous studies have already revealed that these homologs share some sequence dissimilarity to each other even within such highly conserved domains as the BTB/POZ and ankyrin domains (Sandhu *et al*., 2009) and this has again been observed with the banana MNPR1 sequences in this study. However, these differences might not be very important for adifferential function as seen with soybean *NPR1* and *NPR2* genes (Sandhu *et al*., 2009) or, it might influence the genes' behaviour to pathogens and elicitors and in different plant systems as seen for the rice *NPR1* homologues (Yuan *et al*., 2007).

Phylogenetic results from this study also correlated with results using 31 NPR1-like sequences or just the ankyrin domains of 10 NPR1-like sequences. The banana MNPR1 sequences grouped closely to each other and with the rice NH1 and the NPR1-like sequence from *H. vulgare*. In previous phylogenetic studies, such close groupings of the banana MNPR1 sequences with monocotyledons have been reported (Endah *et al*., 2008; Bergeault *et al*., 2010). Both Bergeault *et al*., (2010) working with 31 NPR1-like sequences and Hepworth *et al*. (2005) working with the 6 *At*NPR1-like sequences, also found 3 main groups during their phylogenetic study also found in this current study using 41 sequences. Both the MNPR1A and MNPR1B genes grouped with the *At*NPR1 sequence which is well-know to positively mediate defense response (Speol *et*

al., 2009). MdNPR1 isolated from another banana cultivar with an ABB genome (Zhao *et al*., 2009) however grouped in another clade from the two banana AAA NPR1 sequences. Coupled to its low level of identity of less than 50% to the two MNPR1 sequences, it can be possible that this new copy of the banana NPR1 gene might have been contributed by the B genome as reported for the two *NPR1* gene copies in *Brassica juncea*, believed to have originated from two individual parental genomes (*B. rapa* and *B. nigra*; Meur *et al*., 2006). However, how the individual banana A and B genomes have evolved over-time is still unknown hence the origin of the three banana *NPR1*-like gene from the ABB genome is still highly speculative. This might however play important roles in mediating their responses during stress.

Data from this study clearly shows that two banana NPR1 like coding sequences (MNPR1A and MNPR1B) group closely with other defense response NPR1-like sequences but share sequence dissimilarities even in the highly conserved NPR1 functional regions. Furthermore, *MNPR1A* is over-represented in the occurrence of *cis* elements identified although *MNPR1B* has additional SA-responsive elements and an eythlene-responsive factor which might be important for their expression. The next part of the study focuses on a complementation study carried with *Arabidopsis npr1-2* mutants to demonstrate banana *NPR1* activity in protection against pathogens and to investigate if sequence dissimilarity in their coding sequence is responsible for differential activity of the genes.

CHAPTER FOUR

TRANSFORMATION OF *ARABIDOPSIS npr1-2* MUTANT WITH BANANA *NPR1*-LIKE CODING SEQUENCES

4.1 Abstract

The full length coding regions of the banana *NPR1*-like genes (*MNPR1A*, or *MNPR1B*) were cloned into plasmid p*Bin19-LBR* where their expression was regulated by the control of the Cauliflower mosaic virus 35S (35S CaMV) promoter and terminator sequences. These plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 cells and used to transform *npr1-2 Arabidopsis* mutant plants. Stable gene integration of the two coding sequences in the mutant plants was confirmed by PCR analysis and sequencing and, expression of the introduced genes was validated by qRT-PCR. During the first selection stage, of more than 1000 T-1 seeds from putative transformed plants screened on Murashig and skoog (MS) medium supplemented with kanamycin, a transgenic plant recovery rate of about 16-20% was obtained. However, following PCR analysis with genomic DNA from leaves of these plants, only between 16-18% of these regenerants had the gene integrated within their genome. These were considered as "true" transformants. During the second selection step (T-2 selection) of seeds, about 50% of seeds regenerated on kanamycin. While during the T-3 selection, more than 65% of seeds survived on the kanamycin-containing medium and at the T-4 selection stage more than 95% of positive transformants were obtained which were considered as homozygous and used for further analysis. Gene expression analysis of the two banana *NPR1* genes and the *Arabidopsis PR-1* gene in selected homozygous transgenic lines showed that transcription of the banana *NPR1* genes did not alter transcript amounts of the *Arabidopsis PR-1* gene.

4.2 Introduction

Rapid advances in plant molecular biology have been facilitated by the discovery of organisms, such as *Agrobacterium tumefaciens*, that can be engineered and used for gene transfer into plant genomes, facilitating expression of transgenes and evaluation of their function (Bevan, 1984; Gelvin, 2003). Binary vectors are essential tools in *Agrobacterium*-mediated transformation systems (Bevan, 1984) comprising a transfer- (T)-DNA region and the vector's backbone. The T-DNA region in most engineered binary vectors like p*Bin19* is delimited by left and right border (LB, RB) sequences and contains a complete plant selection marker expression cassette and a multiple cloning site (MCS) to facilitate introduction of the desired expression cassettes; reporter genes such as the lacz gene which encode beta- (β)-galactosidase enzyme and whose expression can easily be monitored for screening of transformed cells. The vector backbone on the other hand has selectable marker genes for the bacteria and replication functions enabling it to replicate in both *Agrobacterium* and *Escherichia coli* (*E. coli*) cells (Bevan, 1984; Komori *et al*., 2007). As early as 1987, researchers started using the *Agrobacterium* system for the transformation of *Arabidopsis*. The initial procedure involved applying *Agrobacterium* inoculum directly onto seeds, growing plants using these seeds, harvesting of progeny before selection on an antibiotic medium (Feldman and Marks, 1987). Chang *et al*. (1994) have employed the "clip 'n squirt" method which entails clipping off the reproductive inflorescences from *Arabidopsis* plants before application of the inoculum onto the rosettes. For higher transformation efficiency, the procedure was repeated after the emergence of secondary inflorescences on the plant. Seeds that developed from these inoculated plants were selected on medium containing the appropriate antibiotic. Other *Arabidopsis* transformation approaches include a tissue culture (Valvekens *et*

al., 1988), vacuum infiltration (Bechtold *et al*., 1993) and the most commonly used floral dip (Clough and Bent, 1998) methods.

The objective of this part of the study was to stably intergrate two previously isolated banana *NPR1*-like coding sequences (*MNPR1A* and *MNPR1B*; Endah *et al*, 2008) into *Arabidopsis npr1-2* mutant plants. There are four commonly used *Arabidopsis npr1* allelic mutants with different positional mutations within the *NPR1* gene sequence (Cao *et al*., 1997). Unlike the *npr1-1 Arabidopsis* mutant which has a mutated histidine at position 334, the *npr1-2* results from a mutation at position 150 from a cysteine to a tyrosine. *Arabidopsis npr1-2* mutant plants were used due to availability at the time of the study.

4.3 Materials and methods

4.3.1 Gene cassette design

4.3.1.1 *Plasmids*

p*MNPR1A* and p*MNPR1B* (-70°C glycerol stocks; FABI, South Africa) harbouring the banana *MNPR1A and MNPR1B* coding region respectively in the PCR®4-TOPO® vector backbone were streaked in 90-mm Petri dishes containing 20 mL of Luria broth (LB) agar medium [1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), 1% sodium chloride (w/v) and 1.2% bacterio- agar (w/v) pH 7.5]. The medium was supplemented with 50 mgL^{-1} carbenicillin for plasmid selection. Petri dishes containing plasmids were incubated at 37°C overnight. Resulting single white colonies from overnight plates were individually cultured in 15 mL Falcon tubes containing sterilized 5 mL of liquid LB medium [1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v) and 1% sodium chloride (w/v); pH 7.5]. Liquid cultures were supplemented with the same antibiotic as above before overnight incubation at 37°C with continuous shaking at 200 revolutions per minute (rpm).

The binary vector p*Bin19-LBR* (Fig. 4.1) containing a double 35S CaMV promoter and 35S CaMV terminator sequences was used as a host for the banana *NPR1* genes to facilitate transformation into *A. tumefaciens*. Plasmid p*Bin19-LBR* was also obtained from a -70°C glycerol stock (FABI, South Africa) and cultured as described above for the p*MNPR1* plasmids with 50 mgL^{-1} of kanamycin added to the medium.

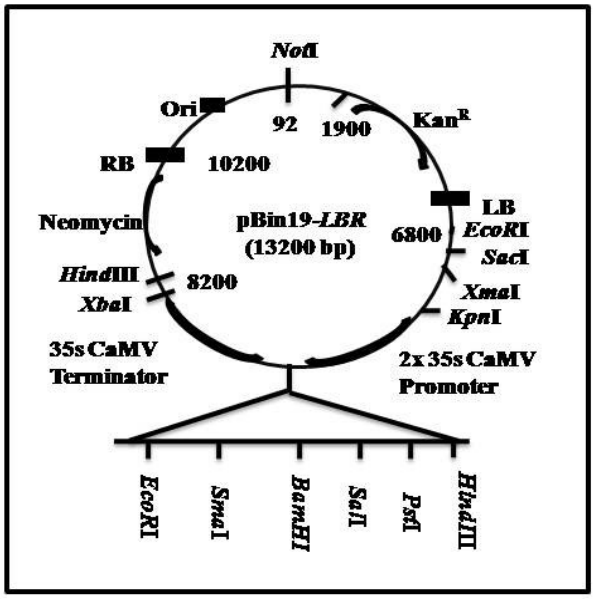


Figure 4.1 p*Bin19-LBR* vector map.

4.3.1.2 *MNPR1 cloning*

Plasmid DNA was isolated from p*MNPR1* cells using the GeneJet™ plasmid miniprep kit (Fermentas, Canada) according to the manufacturer's instructions. Primers (Table 4.1) with additional *Bam H*I, *Sma* I and *Xba* I restriction sites were designed to flank the 5' and 3' ends of the *MNPR1* coding sequences (Fig. 4.2). Designed primers were used to amplify the entire *MNPR1* coding regions which were then cloned into the plasmid p*Bin19-LBR*.

Table 4.1 Primer sequences for the amplification of the *MNPR1* coding region.

Primer name	**Primer sequence**
MNPR1A_FF	TAGGGATCCATATGGAAGACAACT
MNPR1A_RR	TAGTCTAGACCCGGGATACAGCACAAT
MNPR1B_FF	TAGGGATCCACACGGCGGGACATG
MNPR1B_RR	TAGTCTAGACCCGGGGGTATAGAATCG

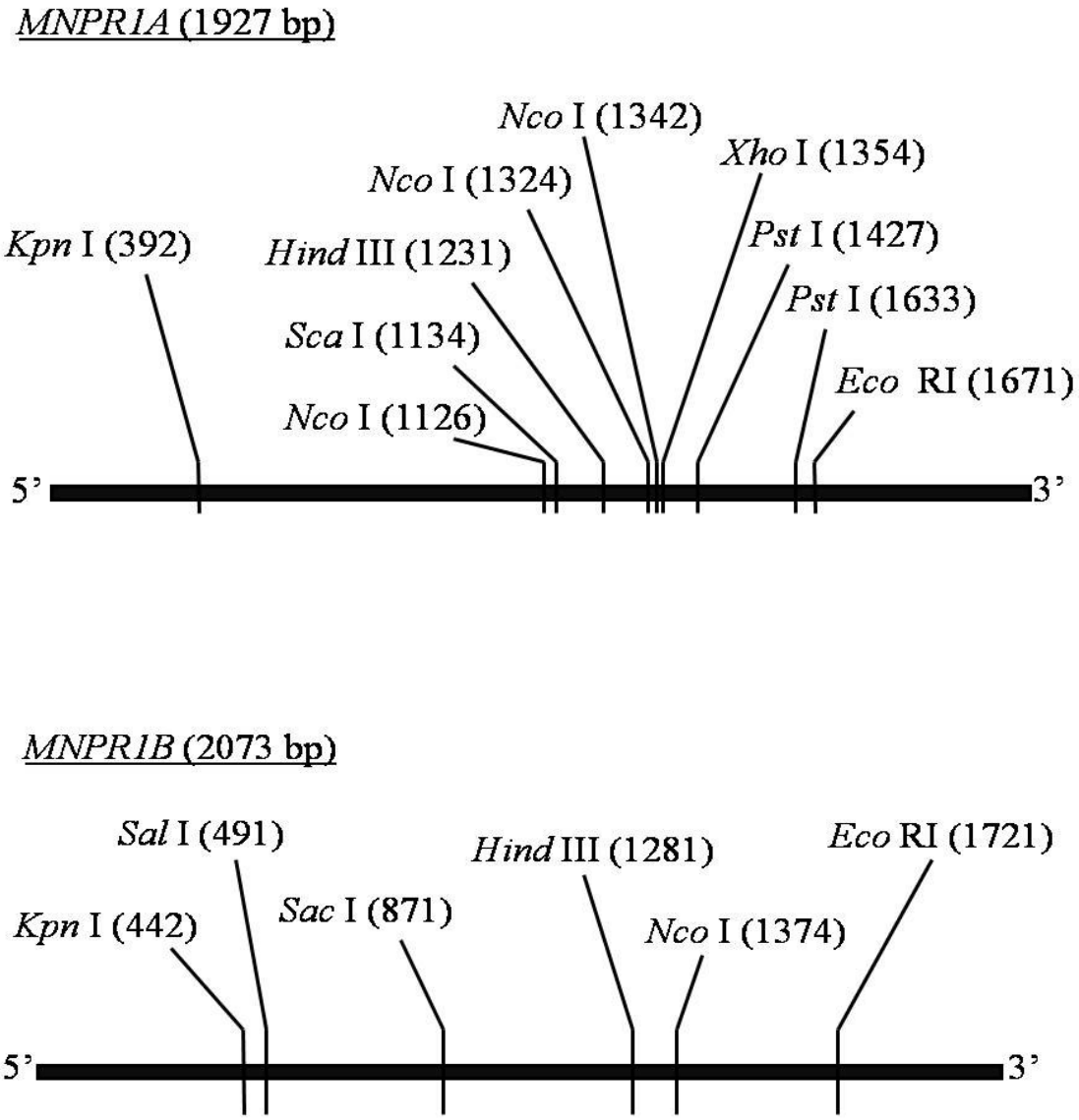


Figure 4.2 Selected restriction sites within the *MNPR1A* and *MNPR1B* coding region.

The PCR reaction was set up using the Expand long-template PCR reaction kit (Roche Diagnostics, UK) with 50 ng of plasmid DNA and following instructions recommended by the supplier. The reaction was carried out by initially denaturing the DNA at 94^{o}C for 2 min followed by a cycling step consisting of 10 cycles with DNA denaturation at 94^{o}C for 10 s, primer annealing at 64^{o}C for 30 s and DNA elongation at 68^{o}C for 1 min. This was followed by a second cycling step of 15 cycles under the same conditions as the first although during this stage, the elongation phase was increased by 10s/cycle. Final DNA elongation occurred at 68^{o}C for 10 min.

PCR products were separated by electrophoresis on a 1% (w/v) denaturing low LE agarose gel (Promega, South Africa) and a 1.8 kb *MNPR1* fragment excised from the gel. The DNA from the gel was recovered using the DNA clean and concentrator^{TM} kit (Zymo Research, USA) as recommended by the supplier. Both *MNPR1* DNA fragments (400 ng) were then individually ligated in a 10 µL reaction containing 55 ng of the p*Gem-T-easy* plasmid (Promega, UK) to which a 2X ligation buffer and 10 U of T_4 DNA ligase (Roche Diagnostic, UK) were added. Reactions were incubated overnight at 4^{o}C and used for transformation of DH5ά^{TM}-T1^{R} competent cells (Invitrogen, UK).

For transformation of *E. coli* competent cells, ligation mixture (6 µL) and competent cells (25 µL) were incubated for 30 min on ice followed by heat shock treatment of cells at 42^{o}C for 45 s. The samples were immediately placed on ice for 2 min prior to the addition of 0.2 mL of SOC medium (20% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM 10 mM $MgSO_4$, 20 mM glucose, 10 mM $MgCl_2$). For rapid replication of cells, samples were incubated with

continuous shaking at 200 rpm for 1 h at 37°C after which 0.1 mL of the transformation reaction was spread on LB agar-containing Petri dishes supplemented with 50 mgL^{-1} carbenicillin. The Petri dishes were then incubated at 37°C overnight and 10 white *E. coli* colonies from each plate were individually cultured in 5 mL of liquid broth supplemented with 50 mgL^{-1} carbenicillin. Samples were cultured overnight on a shaker at 200 rpm and 37°C. To confirm presence of the ligated *MNPR1* gene fragments and orientation of the fragments within the p*Gem-T_easy* plasmid, plasmid DNAs isolated from overnight cultures were sent for sequencing and, the plasmids with the correct *MNPR1* sequences used for cloning of the genes into the binary vector. Plasmids selected for subsequent downstream reactions were named p*Gem-MNPR1A-3* and p*Gem-MNPR1B-4*.

For cloning into p*Bin19-LBR*, 300 ng of plasmid DNA from p*Gem-MNPR1A-3* and p*Gem-MNPR1B-3* was digested with 10 U of each *Bam H*I and *Sma* I (Fermentas, Canada) in a 20 µL reaction mixture containing the required buffer as recommended by the supplier. Digestion of plasmid DNA was carried out at 37°C for 1 hr in a 1.5 mL Eppendorf tube. The resulting DNA fragments were separated by electrophoresis on a 1% agarose gel. The 1.8 kb fragment of interest was excised from the gel and the DNA recovered as described above.

A parallel digestion was also carried out using DNA from plasmid p*Bin19-LBR* under the same conditions as described above. Five µL of the digest was separated by electrophoresis on a 1% agarose gel to verify that the plasmid had been completely linearized. The remaining p*Bin19-LBR* digest was purified using the phenol: chloroform purification method (Sambrook *et al.*, 1989; Sambrook and Russell, 2001). The entire linearized and purified p*Bin19-LBR* plasmid

DNA was dephosphorylated using alkaline phosphatase enzyme and alkaline phosphatase buffer (Fermentas, Canada) as instructed by the manufacturer. Dephosphorylation of the plasmid DNA was carried out at 37^{o}C for 1 hr. After dephosphorylation, the DNA was again purified using the phenol: chloroform purification method as described above. The pellet obtained at this stage was re-suspended in 20 µL of sdH_2O.

The gel purified *MNPR1* fragments were ligated into dephosphorylated p*Bin19-LBR* and this ligation mixture was used to transform competent DH5άTM-T1^{R} cells as described above. Antibiotic selection of transformed cells was done on LB using 50 mgL^{-1} kanamycin in the medium. Isolated plasmid DNA from cells of 10 white *E. coli* colonies was analysed by restriction enzyme digestion and plasmid DNA sequencing. A glycerol stock was prepared from positively identified samples and stored at -70^{o}C. Plasmids were designated: p*LBR-MNPR1A* and p*LBR-MNPR1B (*Fig. 4.3).

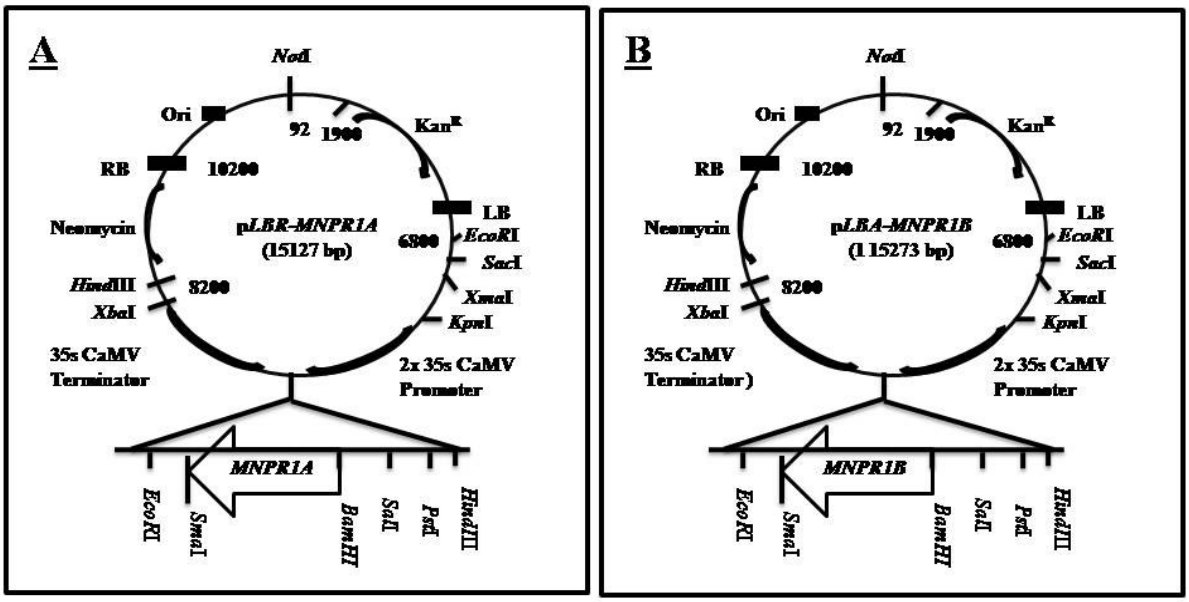


Figure 4.3 Plasmid maps of p*LBR:MNPR1 and* p*LBR:MNPR1B*.

4.3.2 *Agrobacterium* transformation

For *Agrobacterium* transformation, three gene cassettes were used. These consisted of plasmid: p*LBR-MNPR1A* and p*LBR-MNPR1B*, with plasmid p*Bin19-LBR* serving as the control. Each of these plasmids was individually transformed into *Agrobacterium* LBA4404 competent cells using the freeze thaw method as described by Chen *et al*. (1994). Positive transformants were selected after plasmid restriction enzyme digestion. Resultant *Agrobacterium* strains harbouring the plasmids were designated: p*LBA-MNPR1A*, p*LBA-MNPR1B* and p*LBA-LBR*.

4.3.3 Transformation of *npr1-2 Arabidopsis* mutants

4.3.3.1 *Growth of npr1-2 Arabidopsis mutant plants*

Arabidopsis npr1-2 mutant seeds (donated by Dr. Xinnian Dong, USA) were rinsed in 70% ethanol for 1 min followed by surface sterilization in 1.5% sodium hypochlorite (v/v) for 20 min at room temperature. Seeds were thoroughly rinsed (four times) using distilled water (dH_2O) and re-suspended in 0.1% agar (w/v). Seeds were plated onto 20 mL Murashige and Skoog (MS) containing agar plates (4.5 gL^{-1} MS salt, 20 gL^{-1} sucrose and 12 gL^{-1} Bacterio-agar). Seeds in plates were stratified for 4 days at 4^{o}C in the dark and thereafter, exposed to a 16 h light / 8 h dark regime cycle for one week. Healthy growing seedlings were potted in moist 44-mm Jiffy-7 pots (Jiffy International AS, Norway). The Jiffy pots (45 each) where placed in 40 x 30 x 9 cm trays without holes to facilitate sub-irrigation. Trays were covered with cling wraps to ensure for maximum humidity. These were then transferred to controlled environment growth chambers.

Each chamber was provided a photosynthetic photon flux of 200 µmol. m^{-2} s^{-1}, a photoperiod of 16 h day/8 h dark regime and a relative humidity of 75 ± 5%. The cling wraps were removed from the trays after 2 weeks when the plants had developed at least 2 true leaves. Plants were then maintained under the above mentioned growth conditions until the formation of inflorescences. Primary inflorescences were cut off from the plants to facilitate the formation of secondary inflorescences. Plants in which most of the inflorescences were still closed were used for transformation via floral dipping into an *Agrobacterium* inoculum.

4.3.3.2 *Plant transformation*

Agrobacterium solutions used for plant transformation were prepared from p*LBA-MNPR1A*, p*LBA-MNPR1B* and p*LBA-LBR* cultures which had been grown in liquid LB broth up to an optical density (OD_{600} nm) of between 0.8-1.0. These inocula (100 mL) were centrifuged at room temperature for 10 min at 10 000 rpm. The pellets were re-suspended in 100 mL of a 5% sucrose solution (w/v) supplemented with 0.05% Silwett L-77 (v/v; Lehle seeds, USA). Plants were transformed following a modified method of the floral dip technique as described in Clough and Bent (1998). For this, the inflorescences of each plant were dipped in the respective inoculums for 10 s. After dipping plants were placed in a horizontal position in the trays and the trays were covered with foil paper to ensure for optimum humidity. The following day, foil papers were removed from the trays and the plants were placed in an upright position. Floral dipping was repeated twice over a 2 week period to optimize the transformation efficiency and using freshly prepared inoculums during each process. The plants transformed were designated as *npr1*:*MNPR1A*, *npr1*:*MNPR1B* and *npr1*:*35S* CAMV.

4.3.3.3 *Screening of transformed lines*

Selection of *npr1* transgenic lines harbouring either the p*LBA-MNPR1A*, p*LBA-MNPR1B* and p*LBA-LBR* gene cassette were performed under sterile conditions on MS plates supplemented with 50 mgL^{-1} of kanamycin. Seedlings that germinated and formed roots in the kanamycin containing medium were potted into jiffy pots and grown until seed formation as described previously. The selection process was carried out over four generations (involving successive kanamycin selection, potting, seed formation and harvesting steps) until homozygous lines were obtained. Homozygous lines consisted of plants where by more than 80% of their seeds could vigorously regenerate on kanamycin-containing plates. After the first selection step, plantlets were potted, appropriately labelled and treated as independent transformation events. Seeds that were subsequently harvested from these independent lines were also appropriately labelled and treated separately. During each selection process, leaf material was harvested from the potted plants for DNA isolation and/or cDNA synthesis for verification of gene integration via PCR. Seeds were always harvested from a total of 10 individual plants in which the gene of interest had been amplified.

4.3.4 Transcription measurement

MNPR1A, *MNPR1B* and *PR-1* transcript level of five to six independent transformation events were determined by quantitative real time PCR (qRT-PCR) using RNA extracted from 5-wk-old leaves from homozygous transgenic lines. Leaf material for RNA extraction was also collected from non-transgenic wild-type (Wt) and *npr1* mutant *Arabidopsis* plants grown in parallel to the

homozygous transgenic lines to serve as controls. Total RNA extraction in all samples to be analyzed was performed using the Trizol reagent (Invitrogen Life Technologies, San Diego, California, USA) and following the manufacturer's instructions. Contaminants in the extracted RNA, particularly DNA, were eliminated by treating RNA samples with TurboDNA free reagent (Ambion, UK) according to steps recommended by the manufacturer. First-strand complementary DNA (cDNA) was synthesized using 2 µg of the treated RNA using the superscript II first strand cDNA synthesis kit (Invitrogen Life Technologies, San Diego, California, USA) and random hexamer primers (Invitrogen, UK) as instructed by the manufacturer.

The Netprimer3 program (Premier Biosoft, Palo Alto, CA, USA) was used to design primers for the three target genes *MNPR1A* (DQ925843), *MNPR1B* (EF137717) and also *AtPR-1* (NM_127025-2). The endogenous control primer was designed from the gene encoding the *Arabidopsis* actin protein (At3g18780). All primers were designed to have a GC content of between 50-55% consisting of 20-22 nucleotides and to amplify a product of between 80-150 bp.

Prior to the selection of primers to be used, the quality of cDNA and the efficiency of each primer pair were verified by conducting a conventional PCR reaction and detecting amplified DNA products on a 2% agarose gel (w/v) to confirm the correct size of the DNA product. Primers (Table 4.2) which produced the desired single band product were further assessed for their efficiency by conducting a qRT-PCR experiment with a serial dilution (0.5, 0.25, 0.125 and 0.1) of cDNA.

Table 4.2 Primer sequences for determining transcripts of the *Arabidopsis PR-1* and banana *NPR1* genes in *Arabidopsis*.

Primer name	Primer sequence
qMNPR1A_FF	GCTCGACAGATTTTGCTTATC
qMNPR1A_RR	GGAAGACAAAGCAGACTTGTTG
qMNPR1B_FF	CCTCCTTGGTATTCTGGACA
qMNPR1B_RR	CTTCTTCTCTAGGGTGATGG
qPR1_FF	CGGAGCTACGCAGAACAACT
qPR1_RR	CTCGCTAACCCACATGTTCA
qActin_FF	AGTGGTCGTACAACCGGTATTGT
qActin_RR	GATGGCATGGAGGAAGAGAGAAAC

The Sybrgreen master mix (Roche Diagnostics, UK) was used for the qRT-PCR as follows: 5 µL of cDNA template, 1 µL of 10µM primers, 10 µL SYBR-Green I master mix and 3 µL nuclease-free water were added into respective wells in a 96 welled-RT-PCR micro titer plate. Non-template control reactions containing water instead of cDNA as template were included in the reaction. The qRT-PCR reaction was set up as follows: denaturation for 10 min at 95°C, followed by an amplification phase of 40 cycles consisting of a DNA denaturation step at 94°C for 5 s, primer 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 fluorescent signal for each sample was assessed at 95°C.

A standard curve for each gene was also established using serial dilutions of the cDNA to a final concentration of 1, 0.5 0.25, 0.125, 0.0625, 0.03125 and 0.015625 of the original concentration. For each primer set, a non-template control (NTC) reaction containing water as a template was included and, each reaction was carried out in triplicates. The experiment was repeated twice with a different set of independent plants and leaf material was sampled identically as described above. By use of the manufacturer's software program, the relative transcript amount in each sample was determined. The Applied Biosystems, User Bulletin No. 2, (2000) and steps described in Livak and Schmittgen (2001) were used for data analysis. The basal relative transcript amount for each sample was expressed relative to the actin gene (Applied Biosystems, User bulletin No.2, 2001; and steps outlined chapter 2 of this thesis).

4.3.5 Statistical analysis

To analyze differences in transcripts amounts the statistical analysis software-SAS® (SAS, USA) was used together with a Student's T-Test. The cut-of value was set at $P<0.05$ and significant differences among samples was determined using ANNOVA and Tukey studentized range (HSD) test.

4.4 Results

4.4.1 *MNPR1* gene cassette and *Agrobacterium* transformation

Due to the absence of suitable restriction sites within the original *MNPR1* sequences, cloning was facilitated via the insertion of additional sites at the 5' and 3' flanking regions. These restriction sites were chosen due to their unique presence in the MCS of plasmid p*Bin19-LBR* (Fig. 4.1), as well as their absence in plasmid *pMNPR1A* and p*MNPR1B* (Fig. 4.2). Insertion of these restriction sites into the *Bam HI*/*Sma* I sites of p*Gem-T-easy* was first confirmed after restriction enzyme digestion using a *Bam HI*/*Sma* I digestion (Fig. 4.4). Sequence analysis of the 1.8 kb *MNPR1* fragments revealed the successful addition of *Sma* 1, *Xba* 1 and *Bam* H1 sites in their correct orientation and position (Fig. 4.5).

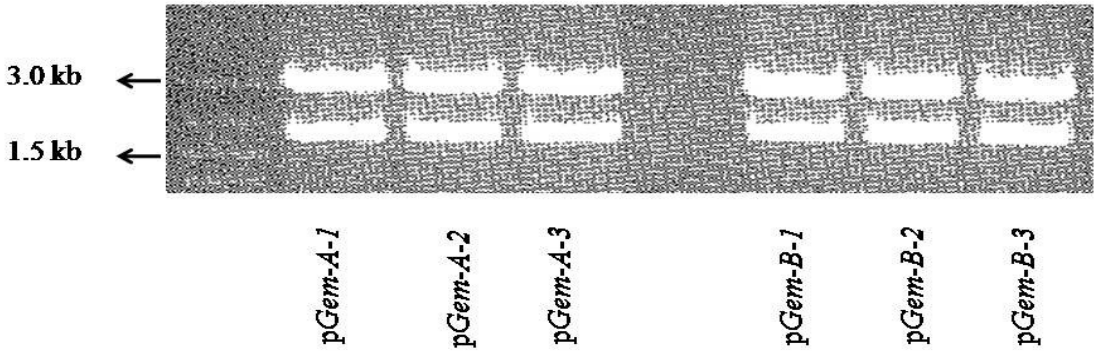


Figure 4.4 Restriction digest of p*Gem:MNPR1A* and p*Gem:MNPR1B* clones showing the 1.8 kb *MNPR1* fragment and the 3 kb p*Gem-T-Easy* plasmid backbone. The presence of an upper 3 kb fragment (backbone of p*Gem-T-easy vector*) and a lower 1.8 kb fragment (*MNPR1* inserts) were used as an indication for successful cloning.

>*MNPR1A* (1769 bp)

TAG***GGATCC***AT***ATG***GAAGACAACT ATCTCACTGCCGCCCCCGCGTTCTCCGTCTCCGACAACAGCCGCAGCGTCCACTTCGCCGGC
GGCGCATCTCCCGACCCCGCTGCCGATGTGGAGGCCCTCCGCCGCTTATCTGACAACCTTGGTGCCGCATTCGAGTCGCCGGACTT
CGAGTTGTTCGCCGACGCCCGCATCGCTGTCGAGGACGGCGGGGCGCCGGCGCGCGAGGTCGGAGTCCACCGGTGCGTGCTGTCCG
CGCGGAGCCCCTTCTTTCGGGAGGTGTTCGCGGAGCGGGAGGGGGCGCTGGCGCCGGTGAGGCTGGAGCTGTGGAAGCTGGTGAGC
GGGTTCGTGGTCGGCTACGACGCCTTGGTGACGGTGCTCGGGTACCTCTACAGAGGGAGGGTGGCGCCGCTGACCAAGGAGGTGTG
CATGTGCGTGGACGAAGAGTGCCGGCACGAGGCGTGCCGGCCGGTGGTCGATTTCATGGTCGAAGTGCTCTACGCCTCCTTCGTCT
TCCAAATCTCCGAGCTGGTCAGCCTCTTCCAGCGACACCTTCTTGATATTCTTGACAAGGTGTCTATAGATGACATTCTGGTAATC
CTATCTGTCGCTAACCTGTGTGATAGCTCTTGTGCCAAATTATTCAACAAGTGCATAGAGATTGCAGTTAAGTCAGACCTGGACAT
CATTACGCTTGAGAAGACAATGACTCCTGATATTGTCAAGCAAATCATGGATTCACGCTTGAATTTGGGAACAGTAGGACCTGAAA
GCATCAATTTTTCTGATAAACATGTCAAGAGAATACATGGGGCTCTCGATAATGATGATGTTGATTTAGTAAGAATGCTGTTGAAG
GAGGGGAATACAACACTAGATGATGCATGTGCTTTGCATTATGCAGTGGCATATTGTGACTCAAAGGTTACAACAGAGCTATTAGA
TCTTGAACTTGCAGATATTAACCATAGAAACATCAGAGGCTATACAGTGCTTCACATTGCTGCAATGCGTAAGGAACCCAAGATCA
TTGTGTCGCTACTAACAAAAGGAGCTAGACCTTCTGATCTAACACTAGATGGAAGGAAAGCAGTTCAGATCTCAAAGAGACACACC
AAGTCCATGGAGTACTTCAAGTCAACTGAAGAAGGACAAGCATCTCCCAAGAGTCGTTTGTGCATCGAGATATTAGAGCAAGCTGA
AAGAAGAGATCCGCAAGTAGGAGAAGCTTCTGCTTTTCTCGCAATAGCTGGTGATGACTTGCGGGGAAGATTACTATACCTTGAGA
ATCGAGTCACTCTTGCAAGACTATTATTCCCCATGGAGGCAAGGGTTGCCATGGACATTGCTCGAGTTGATGGCACATTAGAATTT
ACCTTAGGGTCTGCTACTAGCCATTCTACTGGAAATCAAAGAACTGCAGCAGATCTAAATGAAACACCATTCACGATCAAAGAAGA
GCATCTAGCACGTATGAGAGCACTTTCCAGAACAGTGGAACTCGGGAAGCGCTTTTTTCCCCGGTGCTCAGCGGTTATCAATAAGA
TCATGGATGATGACCCGACAGATTTTGCTTATCTTCAGCATGATGCATCAGAAGAGAAGAGGATGAGATTTTTGGAACTGCAGGAT
GCCCTGTCGAGAGCATTCAGCGAGGACAAGGAGGAATTCAACAAGTCTGCTTTGTCTTCCTCATCATCATCAACATCGGTCGGCAT
TGTACCAACACAAAGA***TGA***TACATTGTGCTGTAT***CCCGGGTCTAGA***CTA

>*MNPR1B* (1839 bp)

TAG***GGATCC***ACACGGCGGGAC***ATG***GAACCCAGCTACCTCACGGCCGCCACCGCCTTCTCGGGCTCCGACAACAGCAGCTGCGTGCA
CTTCTCCGGCGATGCGGCGGCTGCTGCAGCTCCGGACTCCGCCCCGCCCGCGGCGGAGGTCGAGGGACTCCGTCGCCTGTCGGACC
ACCTCGGCTCCGCCTTCCAGTCGCCGGACTTCGAGTTCCTCGCCGACGCCCGCATCGCGGTCGGGCCCCCAGGGGACGGCGGGTCA
ACGCCCCGCGAGGTCGCGGTACACCGCTGCGTGCTATCCGCCCGGAGCATCGTATTCCGGGAGGAGTTCGCGAGGCGGGGGAGGGG
AACGGCCGCGGCCCCGGTGAGGATGGAGCTGAAGGAGCTGGTAAAGGACTTCGAGGTCGGGTACGACGCCTTGGTGGCGGTGCTCG
GGTACCTCTACACCGGGAGGGTGGCACCGCTGCCCAAGGCGGTGTGCGCCTGCGTCGACGAGGAGTGCCGGCACGAGGCGTGCCGG
CCGGCGGTCGATTTCATGGCCGAAGTGCTCTACGCCTCCTCCGTCTTCCAAATCGCCGAGCTGGTCAGCCTCTTCCAGCGGCACCT
CCTTGGTATTCTGGACAAGATGGCAATAGATGACATACCAGTAATTCTCTCTGTTGCTAAACTATGCGATAGCTCATGCGCCAATC
TGCTCAGCAAATGCATAGACGTTGTAGTCAAGTCAGACCTAGATACCATCACCCTAGAGAAGAAGACGCCTCCTGATATTGTTAAG
CAAATTATGGATTTACGCTTGAATTTTGGGCTAGTGGGACCTGAAAGCAGCAGCTTTCCTGATAAACACGTCAAGAGAATACATAG
AGCTCTTGACAGTGATGATGTTGACTTAGTAAGAATGCTATTAAAGGAGGGGAATACAACGCTAGATGACGCATGTGCATTGCATT
ATGCGGTAGCATATTGTGATTCAAAAATCACAACAGAGCTGTTAGATCTTGCACTGGCAGATGTTAACCATAGAGACTTCAGAGGT
TATACTGTGCTTCACATAGCTGCAATGCGTAAAGAACCTAAGATCATCGTGTCACTTCTGACAAAGGGAGCCAGACCATCTGATCT
TACATTGGATGGAAGGAAAGCACTTCAGATTGCAAAGAGACTTACCAAGTCTGTGGAGTACCTCAGGTCGATTGAAGAAGGAGAAG
CATCTCCTAAGAGTCGTTTGTGCATTGAGATATTAGAGCAGGCTGAAAGAAGAGATCCACAAGTAGGTGAAGCTTCTGTATCACTT
GCAATGGCTGGTGATGACTTGCGGGGAAGATTGTTGTATCTTGAGAATCGAGTTGCTCTGGCAAGACTATTGTTCCCCATGGAGGC
AAGAGTTGCTATGGACATTGCACAAGTTGATGGCACATCGGAGTTCACCTTAGGGTCTACCAGCAACCGTTCTACTGGAAATCAAA
GGACTGCGATGGATCTAAACGAAGCACCATTCAAGATCAAGGAAGAGCATCTGGCACGAATGAGAGCACTTTCCAGAACAGTGGAA
CTTGGGAAGCGCTTTTTCCCTCGGTGCTCAGAGGTCATCAACAAGATCATGGACGACGATCTCACAGAAATCACTGGCCTCGGACA
CCACACTTCGGAGGAGAAGAGGAGGAGATTTCAGGAGTTGCAGGAAGTCCTGTCAAAAGCATTTAGCCAAGACAAGGAGGAATTCG
ACAGGTCTGCCTTGTCTTCCTCATCCTCATCGTCATCAACATCCATCGACAAGGTTTGCCCGAACAAGAAGATGAGA***TGA***TTCCCA
CCTAGTCGATTCTATACC***CCCGGGTCTAGA***CTA

Figure 4.5 Full length *MNPR1A* and *MNPR1B* coding region with added restriction sites. Primers (rectangular boxes) flanking the 5' and 3' regions of the *MNPR1* coding sequence were used for amplification of p*MNPR1A* and p*MNPR1B* plasmids. The start and stop codon (grey), *Bam H*I, *Sma* I and the *Xba* I (bold and italicized) were all identified after sequence analysis of selected clones.

Restriction enzyme digest of full length *MNPR1A* and *MNPR1B* coding sequence with *Bam HI*/*Sma* I further demonstrated successful insertion of coding sequences into the plasmid p*Bin19-LBR* to obtain plasmids p*LBR:MNPR1A* and p*LBR:MNPR1B*. Sequencing of the gene cassette in the binary vector showed that the respective banana *NPR1* sequences had been inserted in the correct orientation with the banana *NPR1* genes under the control of the 35S CaMV promoter and terminator sequence (Fig. 4.6). Plasmids were then used to transform competent LBA4404 *Agrobacterium* cells to obtain cells carrying p*LBA:LBR* (vector alone), p*LBA:MNPR1A* and p*LBA:MNPR1B* allowing *Agrobacterium*-mediated transformation of *npr1-2* mutant plants.

>p*LBR-MNPR1A* partial sequence information

```
----------------------------------------------
GTTCATTTCATTTGGAGAGGACAGCCCAAGCTTGGCTGCAGGTCGAC
GGATCCATATGGAAGACAACTATCTCACTG----------------
----------------------------------------------
TTGTACCAACACAAAGATGATACATTGTGCTGTATCCCGGGGAATTC
GGTACGCTGAAATCACCAGTCTCTCTCT------------------
```

>p*LBR-MNPR1B* partial sequence information

```
----------------------------------------------
TTCATTTGGAGAGGACAGCCCAAGCTTGGCTGCAGGTCGACGGATCC
ACACGGCGGGACATGGAACCCAGCTACCTCACGGCCGCCACC-----
----------------------------------------------
AGATGAGATGATTCCCACCTAGTCGATTCTATACCCCCGGGGAATTC
GGTACGCTGAAATCACCA-------------------------
```

Figure 4.6 Partial sequence of the 35S CaMV promoter: *MNPR1*:35S CaMV terminator gene cassette. Dashes (-----) represent sequence information not shown. Sequences in grey delineate the junction sites between the 35S promoter and 5'end of the *MNPR1* sequence; and the 3' end of the *MNPR1* sequence and the terminator sequence. The start and stop codon of the *MNPR1* sequences are underlined. Bold and italicized sequences represent sites at the 5' and 3'end of the *MNPR1* coding region.

4.4.2 Transformation and selection of homozygous transgenic lines

Using the floral dip method, *Arabidopsis npr1-2* mutant plants were successfully transformed with either plasmid p*Bin19:LBR,* p*LBR:MNPR1A*, and p*LBR:MNPR1B*. Selection of more than 1000 T-1 seeds from putative transformed plants with each of the constructs initially yielded a transgenic plant recovery rate of about 16-20% (Fig. 4.7). However, following further screening via PCR, only between 16-18% of these regenerants had the gene integrated within their genome (Fig. 4.8) and these were considered as "true" transformants. During the second selection step (T-2 selection) of seeds, about 50% of seeds regenerated on kanamycin. During the T-3 selection, more than 65% of seeds survived on the selection medium and at the T-4 selection stage more than 95% of positive transformants were obtained which were used for further analysis (Fig 4.8).

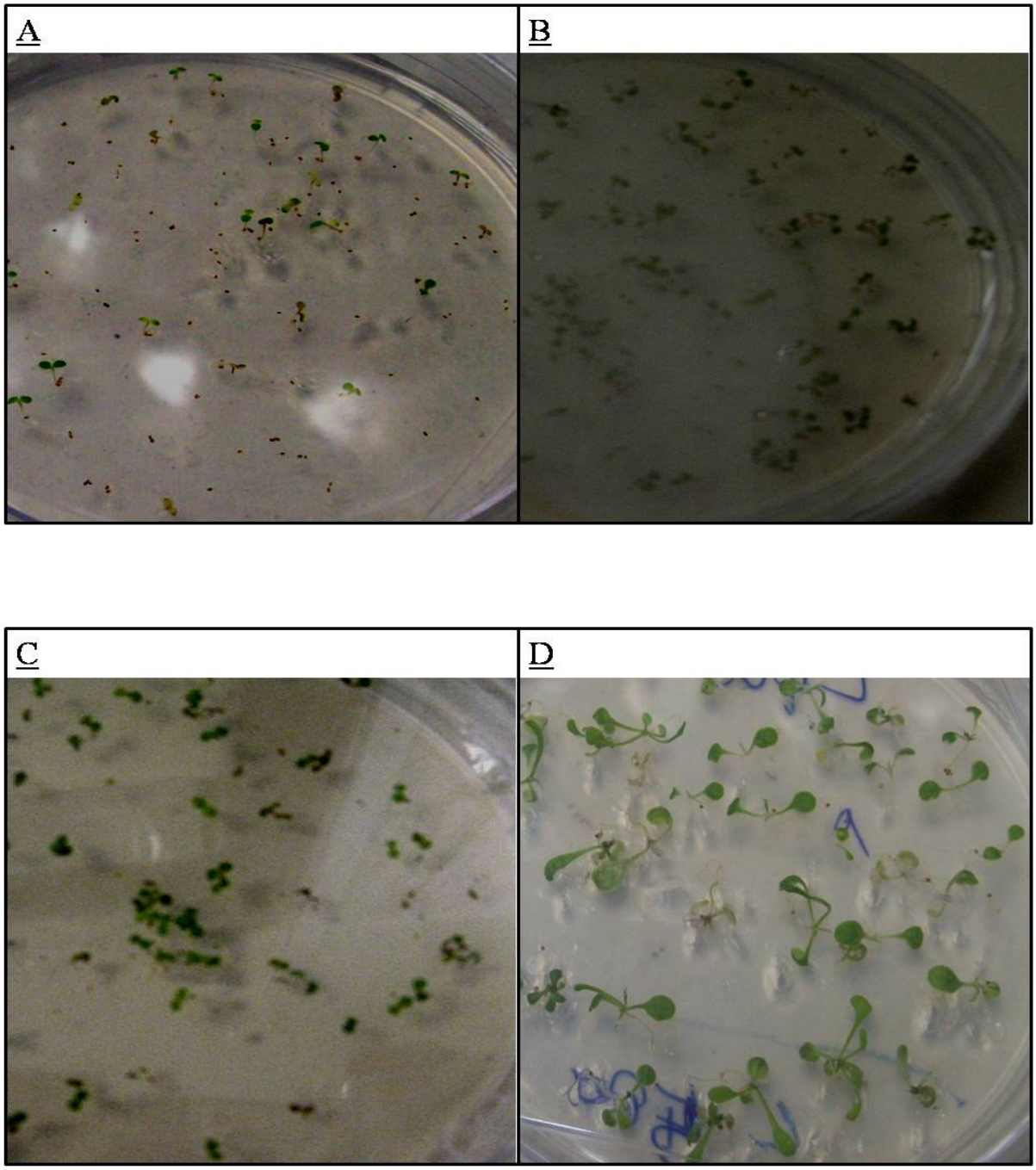


Figure 4.7 Kanamycin selection of plants derived from an *npr1* transgenic event. Following floral dipping of the *Arabidopsis npr1* mutant with p*LBA:LBR*, p*LBA:MNPR1A* and p*LBA:MNPR1B* inoculum, T-1 seeds produced were selected on kanamycin-containing MS agar

plates. Selection process was successively repeated over 4 generations (T-1 to T-4 selection) of self fertilization.

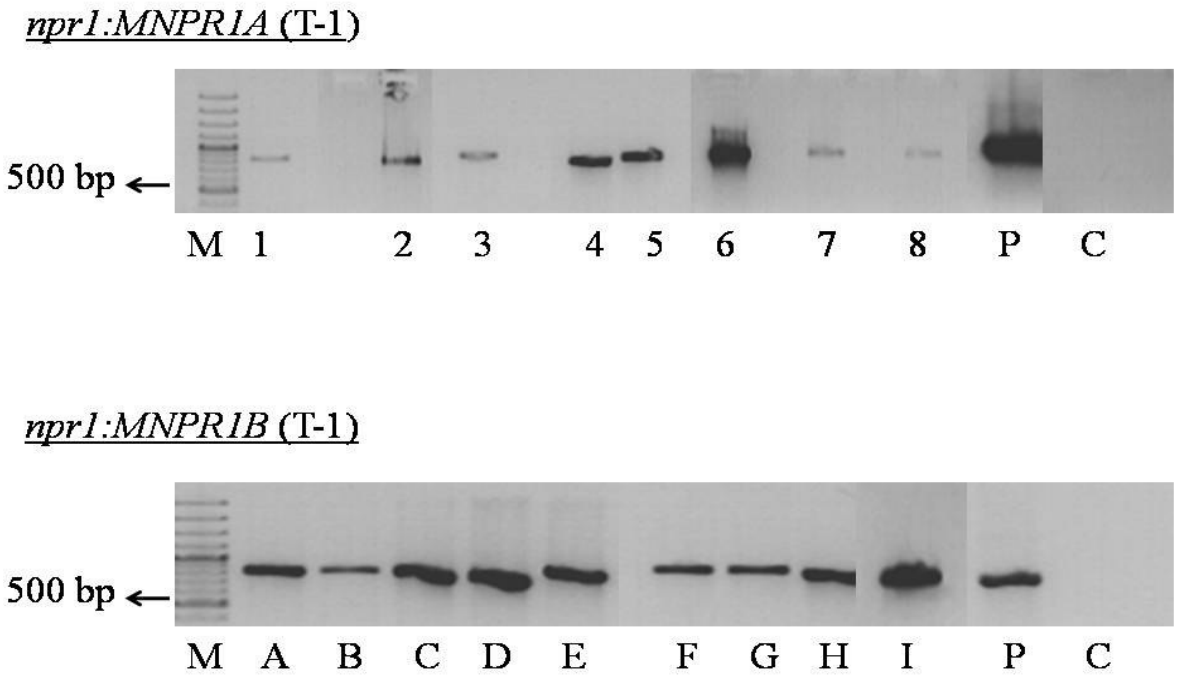


Figure 4.8 800-bp PCR products from putative transformed *Arabidopsis* plants (T-1 generation) carrying *npr1:MNPR1A* and *npr1:MNPR1B* DNAs after kanamycin selection. Numbers (1-8) represent the various independent transformation with *MNPR1A* while letters (A-I) are with the *MNPR1B* gene. The controls used during the PCR were as follows: P = plasmid (positive) control; C = *npr1* DNA (negative) control. M represents a DNA marker to determine fragment size.

Further confirmation of stable gene integration using homozygous lines obtained after four successive kanamycin selection stages showed that the *MNPR1* coding sequence could be successfully amplified from cDNA (Fig. 4.9).

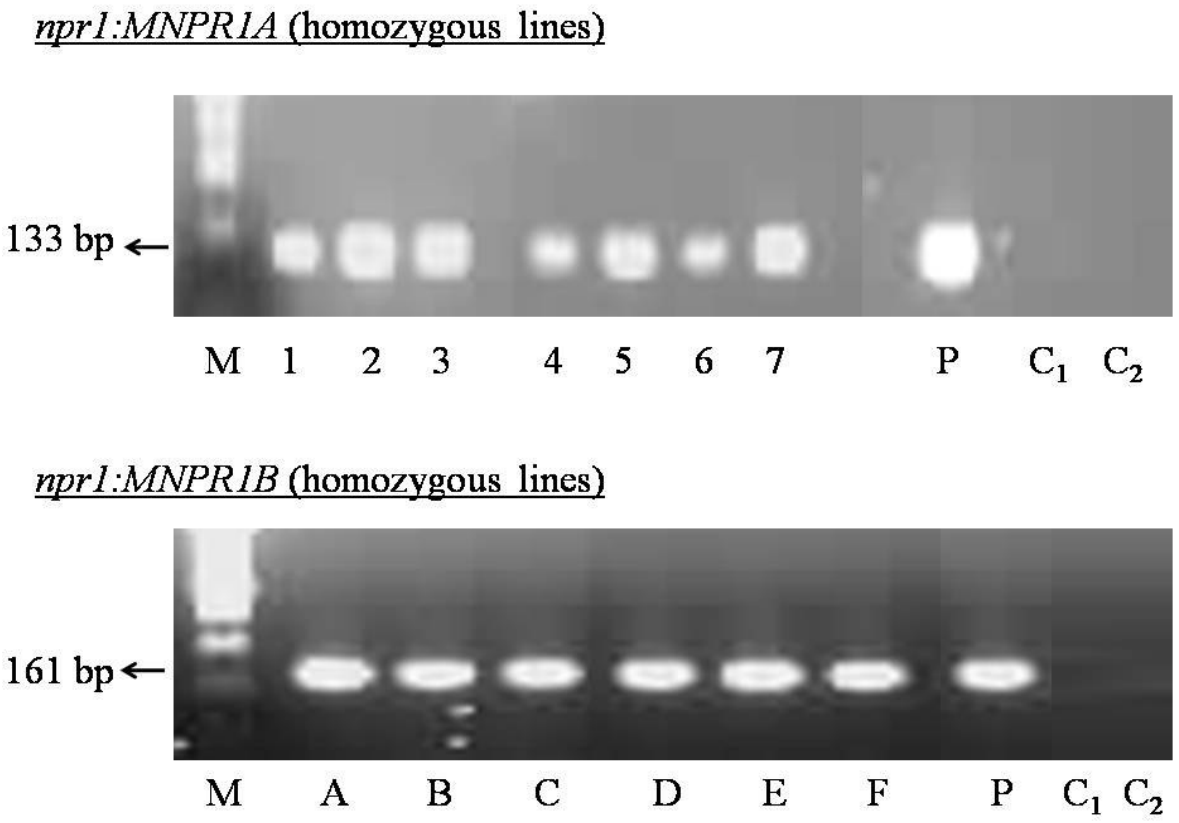


Figure 4.9 PCR products from homozygous *npr1:MNPR1A* and *npr1:MNPR1B* plants. PCR was conducted using cDNA of homozygous *npr1*:*MNPR1A* and *npr1*:MN*P*R1B transgenic plants. Numbers on gel represent *npr1:MNPR1A10*, *npr1:MNPR1A42*, *npr1:MNPR1A44*, *npr1:MNPR1A56*, *npr1:MNPR1A95*. *npr1:MNPR1A204* and *npr1:MNPR1A312*. Letters on gel represent lines: *npr1:MNPR1B1*, *npr1:MNPR1B8*, *npr1:MNPR1B9*, *npr1:MNPR1B12*, *npr1:MNPR1B15* and *npr1:MNPR1B18*. M represents the DNA marker for determination of fragment size. Controls were as follows: P = plasmid (positive), C_1 = *npr1* cDNA (negative) control, C_2 = wild-type cDNA (negative).

4.4.3 *MNPR1* and *PR-1* gene transcription

Quantification of both the *MNPR1* and *PR-1* relative to the endogenous *Arabidopsis* actin gene transcript revealed a 1- to 1.2-fold *MNPR1A* and *MNPR1B* higher transcript amount for four of the selected npr1:*MNPR1A* and npr1:*MNPR1B* transgenic plants tested (Fig. 4.10A). There was no significant differences in both *MNPR1* and *PR-1* transcript levels between the different transgenic lines ($p>0.05$). No *MNPR1A* or *MNPR1B* transcripts were detected in the various control plants (Wt, *npr1* and npr1:35S CAMV *Arabidopsis* plants). However, a 1- to 1.4-fold higher *PR-1* gene transcript level was detected in all the controls as well as the various *npr1:MNPR1A* and *npr1:MNPR1B* transgenic plants tested (Fig. 4.10B).

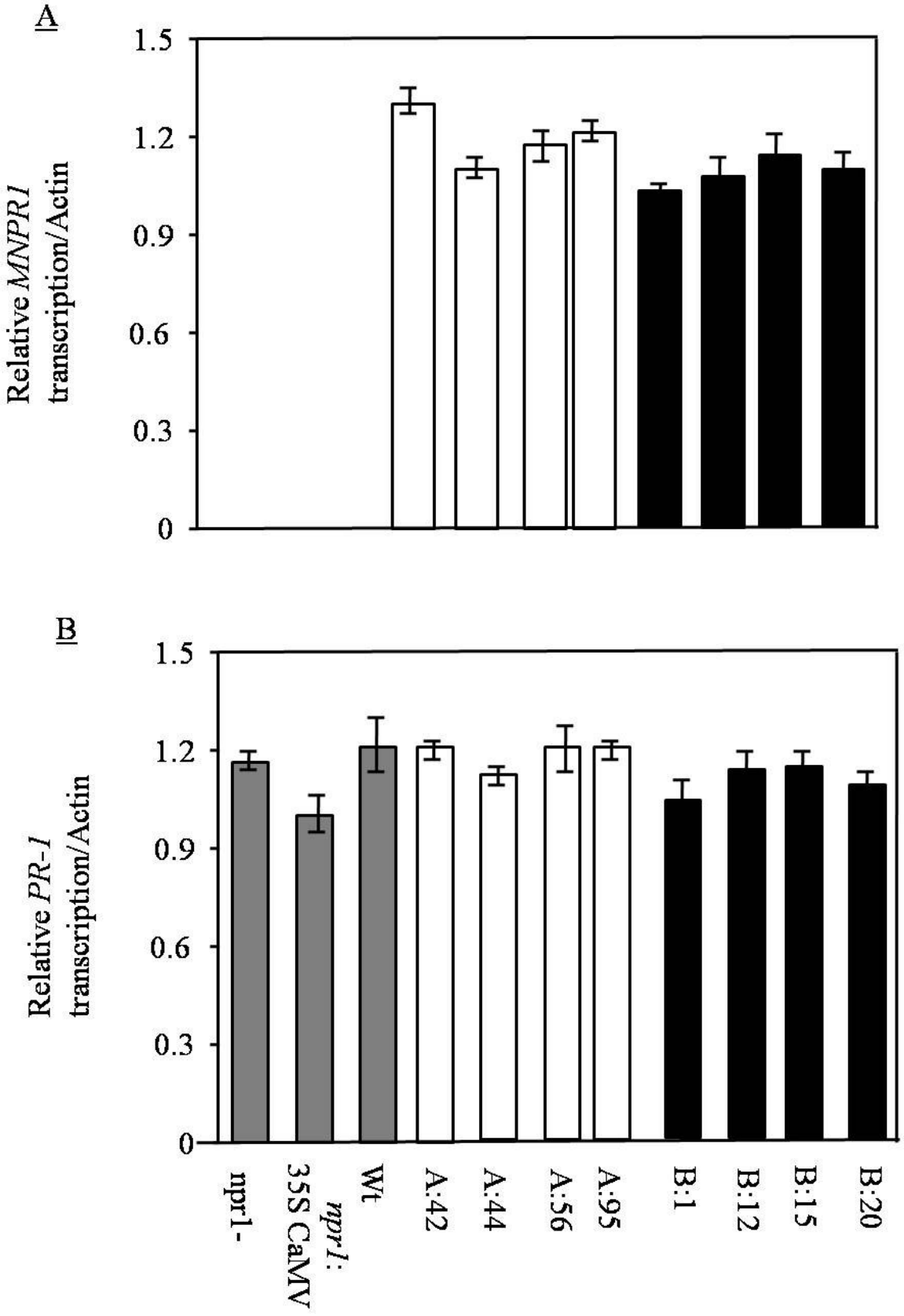

A
Relative MNPR1 transcription/Actin
1.5
1.2
0.9
0.6
0.3
0
B
Relative PR-1 transcription/Actin
1.5
1.2
0.9
0.6
0.3
0
npr1-
35S CaMV mpr1:
Wt
A:42
A:44
A:56
A:95
B:1
B:12
B:15
B:20

Figure 4.10 *MNPR1* and *PR-1* transcript amounts in tested *Arabidopsis* plants. *MNPR1A*, *MNPR1B* (A) and *PR-1* (B) transcription was measured in various *Arabidopsis* genotypes using cDNA from 5 week-old plants. The quantity of transcript was expressed relative to the endogenous actin transcript. The mean transcription values ± the SEM from two independent samples are shown. Samples represented consist of: controls (grey bars; non-transgenic *npr1*, *npr1* transformed with the 35S CaMV promoter/terminator sequence and non-transformed wild type), *npr1*:*MNPR1A* lines (white bars; consisting of line A-42, A-44, A56, A95) and n*pr*1-*MNPR1B* lines (dark bars; B-1, B12, B15, B20).

4.5 Discussion

This is the first report on the expression of banana *NPR1* genes in *Arabidopsis npr1-2* mutant plants. The model plant *Arabidopsis* was used due to the lack of an established and routine transformation system for banana at the beginning of this study. *Arabidopsis* was a suitable surrogate to study gene function as the *npr1* genes have been studied extensively in this species and, unlike banana, mutants in the *npr1* gene that are suitable for validation of gene function are available. Previous research has shown that a native *NPR1* gene can be stably integrated into *Arabidopsis npr1* mutant plants by using a homologous *NPR1* gene, or through back crossing of mutant *npr1* plants with wild-type *Arabidopsis* plants (Cao *et al*., 1994; Yuan *et al*., 2007; Sandhu *et al*., 2009).

In this study, the dipping method for *Arabidopsis* transformation produced a large number of non-transgenic "escape" plants. Although the floral dip method is well established and routinely used in *Arabidopsis* transformation, an adequate selection of F1 progenies and homozygous lines is continuously being optimized so as to obtain higher regeneration efficiencies (Clough and Bent, 1998; Xiang *et al*., 1999; Harrison *et al*., 2006; Dehestani *et al*., 2009). Harrison *et al*. (2006) reported by screening of about 3200 seeds derived from transgenic plants obtained by the floral dipping method indicates that out of 118 regenerated plants, 110 had detectable expression of the candidate gene. In this study, more than 50% of plants derived from the transformation process were not transformed. Such low frequency might be due to (i) the large number of seeds plated per 90-mm Petri dish in this study where too many seeds on the plate might have promoted detoxification of the antibiotic and hence favor the regeneration of non-transformed

seeds (Wilmink and Dons, 1993), (ii) the number of days the seeds were selected on kanamycin which might have been too long and might have allowed degradation of the antibiotic (Harrison *et al*., 2006; Dehestani *et al*., 2009) and (iii) seeds might have only transiently expressed the transgene with no expression of the transgene after potting the plants thus accounting for non-detection of the transgene in the plant genome by PCR (De Buck *et al*., 1998). However, the transgenic plants recovered were deemed adequate for the work planned in this study.

In this study, it was important to elucidate if the transformation process or the presence of the *MNPR1* genes did not result in an alteration of *PR* gene expression in the absence of pathogenic or other perturbation. In homozygous lines expressing banana *NPR1*, *Arabidopsis PR-1* transcripts were not affected by *NPR1* expression. This was found by application of qRT-PCR technique which is currently the most sensitive technique to determine amounts of transcripts (Sandhu *et al*., 2009, Divi *et al*., 2010). Further, results found in this study also confirm previous findings by Kinkema *et al*. (2000) that expression of *NPR1* genes does not activate *PR-1* transcription under non-stress conditions.

In the next chapter expression of *MNPR1A* and *MNPR1B* was investigated in addition to expression of the *Arabidopsis PR-1* gene after infection with distinct classes of pathogens.

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^{o}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^{o}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^{o}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^{o}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

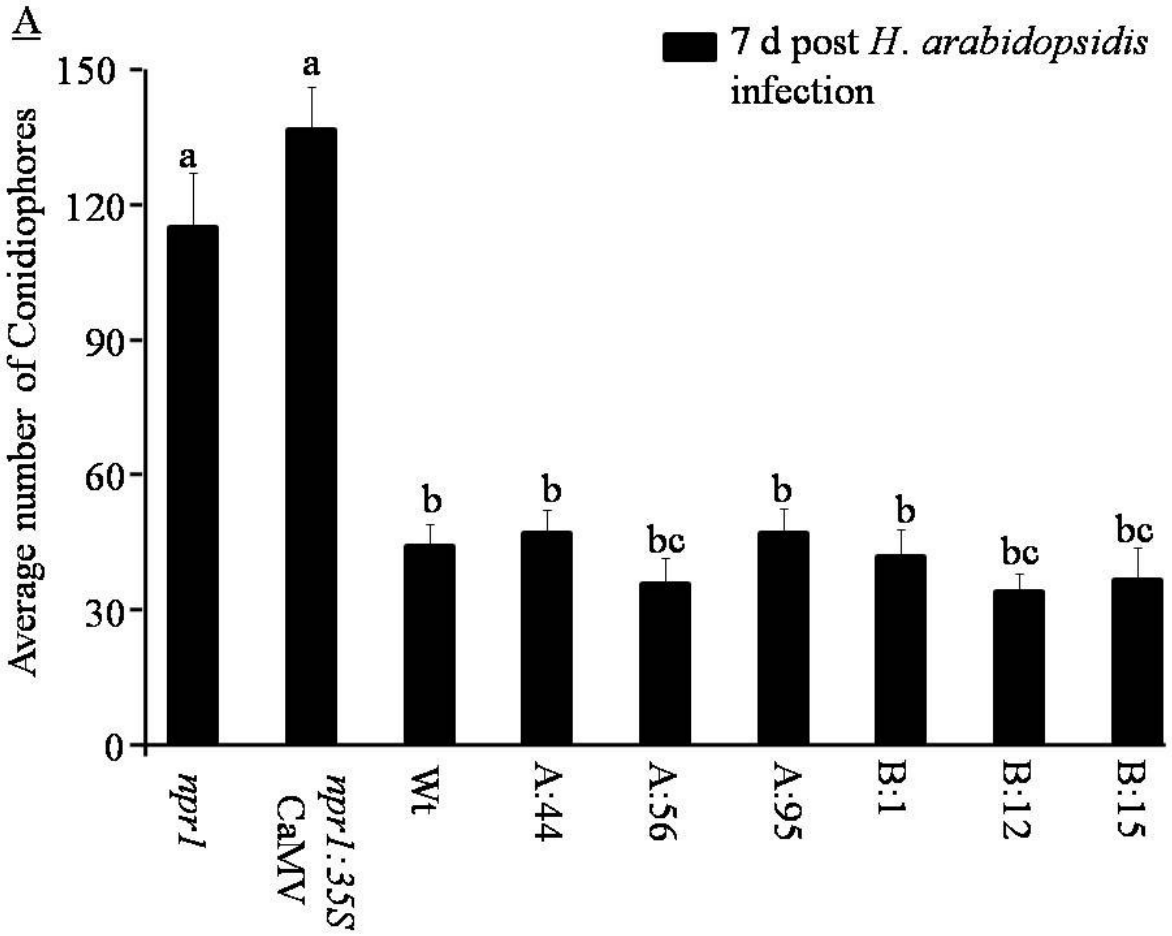
The statistical difference in each experiment over time was done using the Statistical analysis software-SAS® (SAS, USA) and Student T-Test. The cut-of value was set at $P<0.05$ for samples that were significantly different and these differences determined using ANNOVA and Tukey studentized range (HSD)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

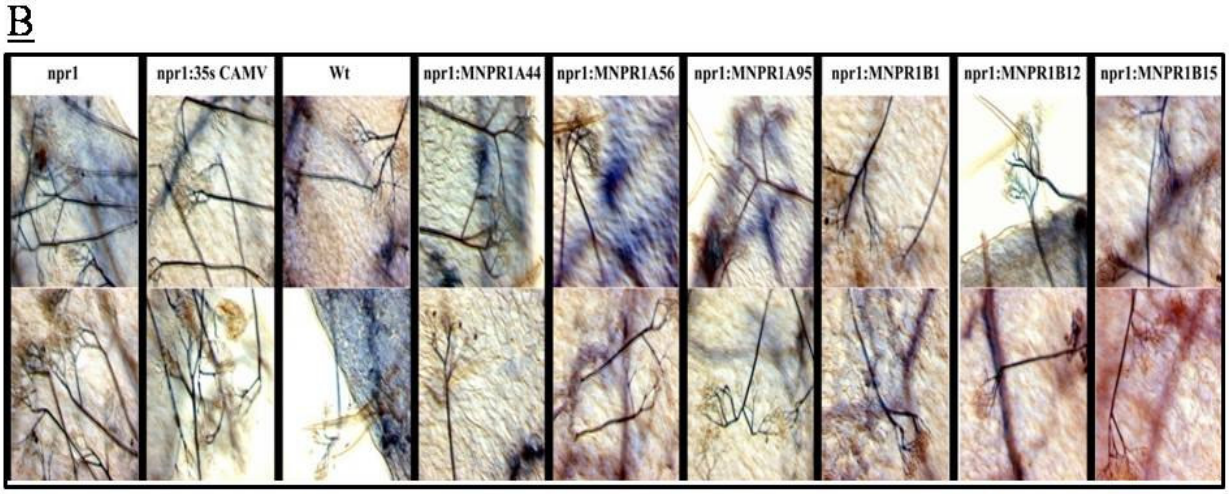

B
npr1
npr1:35s CAMV
Wt
npr1:MNPR1A44
npr1:MNPR1A56
npr1:MNPR1A95
npr1:MNPR1B1
npr1:MNPR1B12
npr1:MNPR1B15

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.

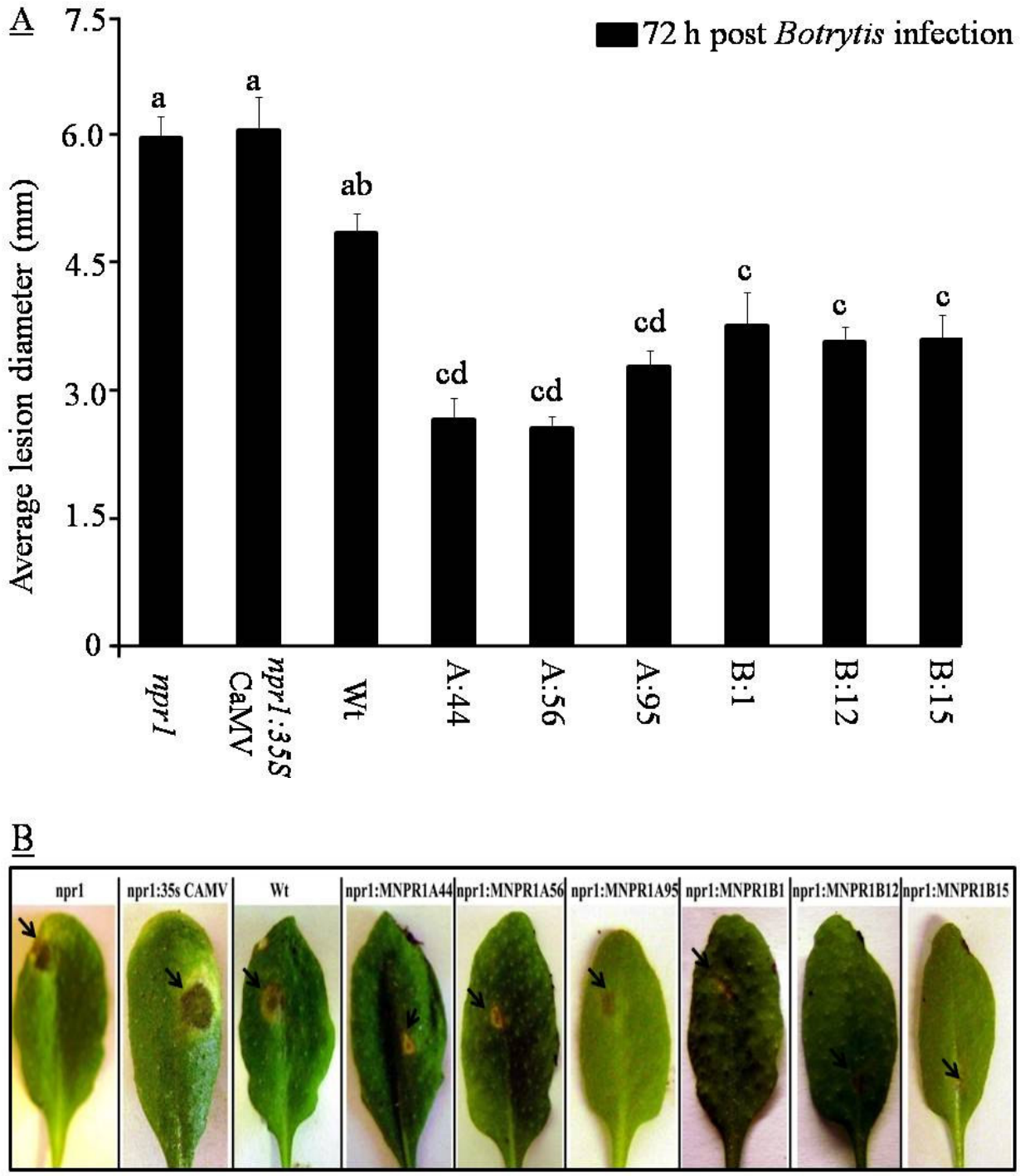


Figure 5.2 Lesions formed on leaves of various *Arabidopsis* genotypes 72 hpi with the necrotrophic fungus *B. cinerea* B05.10. Leaves (six per plant) of nine different *Arabidopsis* genotypes were infected with 5 µL droplets of a 2.5 x 10^5 spores/mL of an inoculum of *B.*

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.

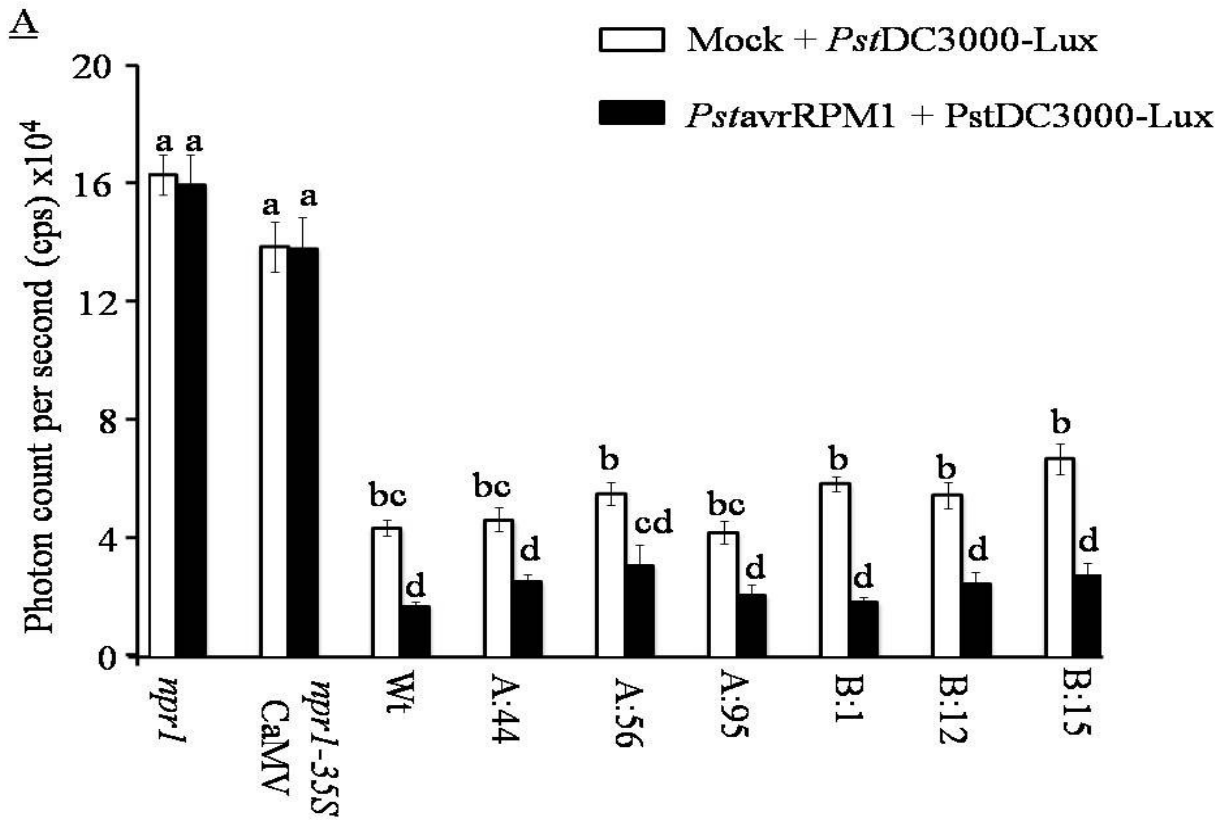
5.4.3 Limitation of *P. syringae* pv *tomato* infection in *MNPR1* expressing lines

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).

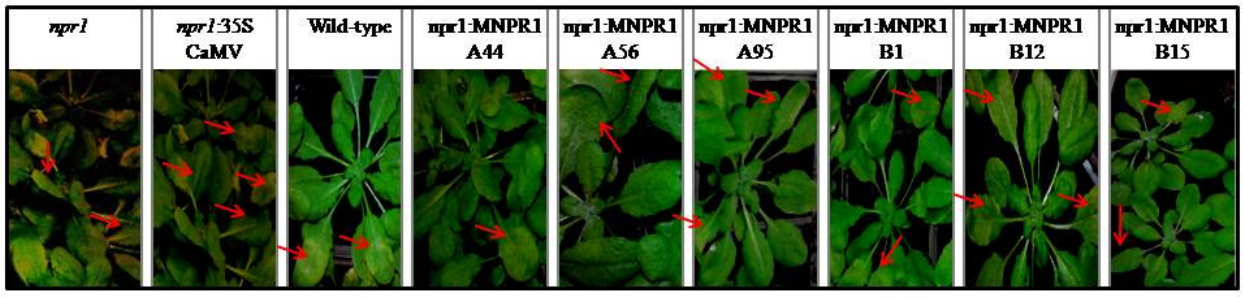
In the AV-treated leaves, 48 h following the *Pst*-lux infection and similar to the MV treatment, all transgenic lines showed a significantly ($P<0.05$) reduced *Pst*DC3000-Lux growth ranging from 83% to 86% compared to the *Arabidopsis* mutant plants. The Wt *Arabidopsis* plants also slowed down the 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*

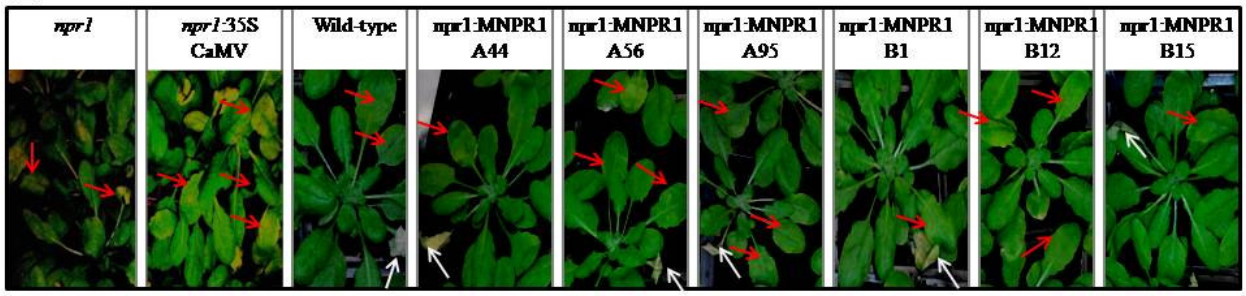


Figure 5.3 Growth of the hemi-biotrophic *Pst*DC3000-luxCDABE (*Pst*DC3000-Lux) in various *Arabidopsis* genotypes before and after SAR induction. *Pst*-DC3000-Lux bacteria growth was measured 48 hpi in non SAR-induced (Mock-*Pst*-DC3000-Lux treated plants) and SAR-induced plants (*Pst* avrRPM1-*Pst*-DC3000-Lux 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

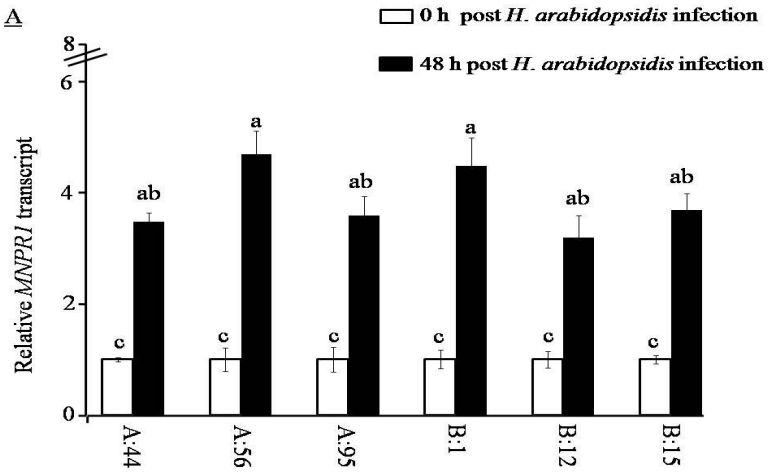
The amount of transcript of *MNPR1* and *PR*-1 genes in all plants used for the study were determined via qRT-PCR and normalized with their endogenous control actin gene before expressing them relative to their various control samples which had not been treated with the pathogen.

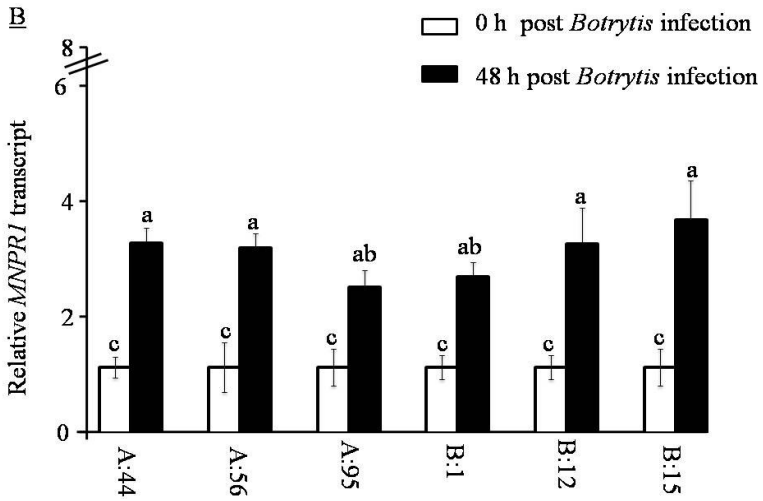
5.4.4.1 *MNPR1 transcription*

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
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
A:44
A:56
A:95
B:1
B:12
B:15

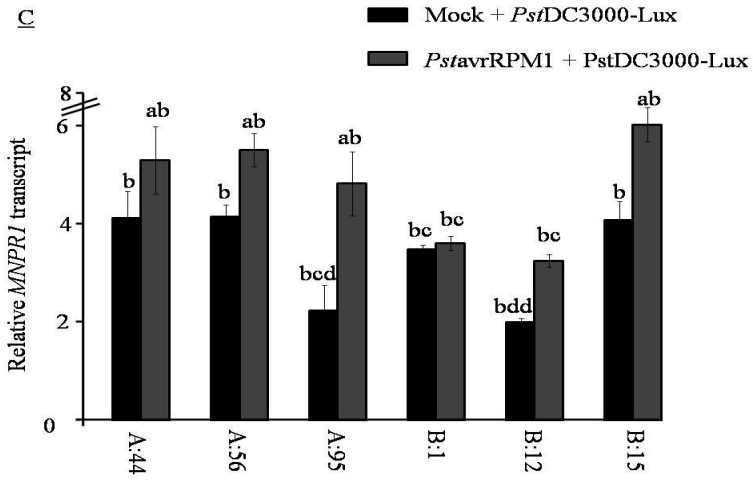

C
Mock + PstDC3000-Lux
PstavrRPM1 + PstDC3000-Lux
Relative MNPR1 transcript
A:44
A:56
A:95
B:1
B:12
B:15

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 ($P<0.05$).

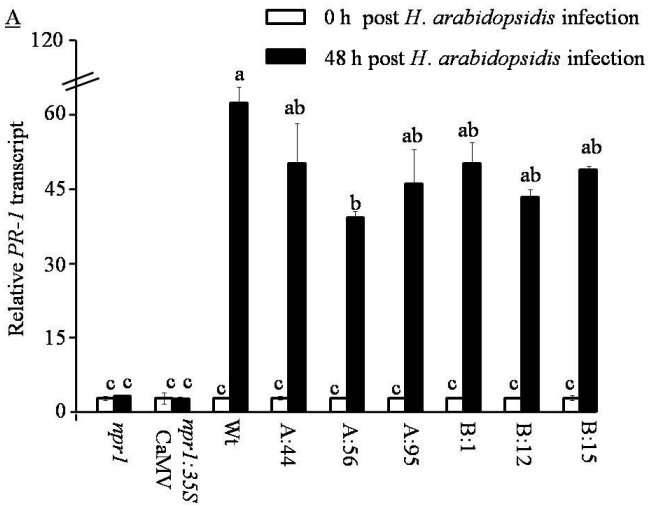
5.4.4.2 *PR-1 transcription*

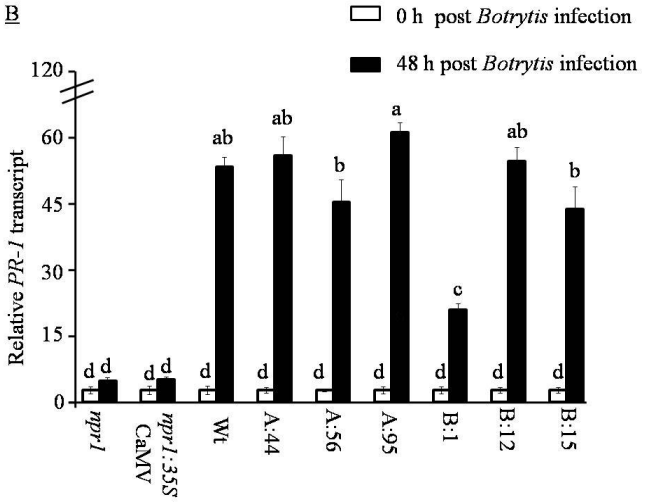
Transcription of the *Arabidopsis PR-1* gene, which is a downstream end product in the *NPR1* pathway, was measured in all the *Arabidopsis* genotypes used in this study. In all the genotypes studied, *PR-1* transcripts were differentially expressed (Fig. 5.5). In plants sprayed with *H. arabidopsidis*, *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
a
ab
b
ab
ab
ab
ab
c
npr1
CaMV
npr1:35S
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
ab
ab
b
a
c
ab
b
d
npr1
CaMV
npr1:35S
Wt
A:44
A:56
A:95
B:1
B:12
B:15

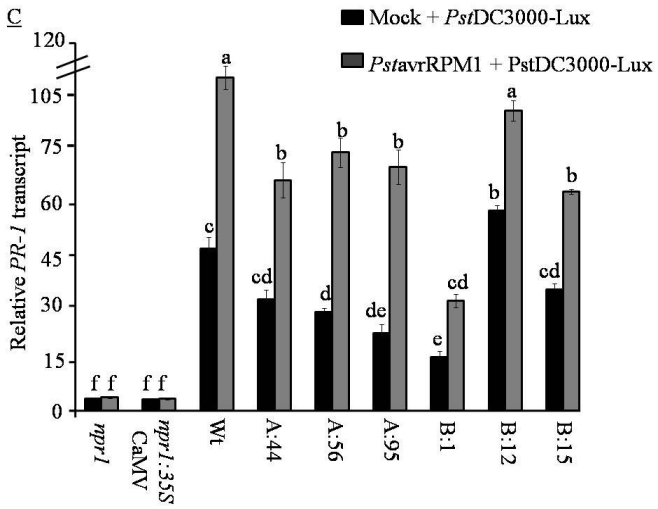


Figure 5.5 Relative *PR-1* transcript amounts in various *Arabidopsis* genotypes after pathogen infection. Various *Arabidopsis* genotypes were treated with either the biotrophic pathogen *H. arabidopsidis* isolate NOCO2 (A); or the necrotrophic fungus *B. cinerea* (B); or the hemi-biotrophic bacteria *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.

CHAPTER SIX

NPR1-DEPENDENT *PR-1* TRANSCRIPTION REQUIRES A FUNCTIONAL GLUTATHIONE BIOSYNTHETIC PATHWAY

6.1 Abstract

The glutathione-ascorbate cycle is a major pathway for reactive oxygen species (ROS) scavenging especially during abiotic and/or biotic stress conditions when syntheses of ROS escalate. This interaction further influences the activation of downstream defense signaling processes for *PR* gene activation. Most *PR* gene induction processes are dependent on the activation of cytosolic NPR1 which in itself is redox dependent. Using *Arabidopsis* mutants deficient in cytosolic reduced glutathione (GSH), the interplay between GSH and ascorbic acid (As)A in *NPR1*-dependent *PR-1* gene induction was studied. Following pathogen infection, a decreased GSH content in mutants led to an increased oxidized glutathione (GSSG) content lowering the GSH/GSSG redox pool. The GSH deficit in these plants was compensated by an increased dehydroascorbate (DHA). However, this compensatory effect had no significant effect on *NPR1* gene transcription. Interestingly, the presence of cytosolic glutathione in wild-type plants coupled with a higher GSH/GSSG ratio significantly affected the amounts of *NPR1* transcripts measured after infection. *PR-1* transcription was further substantially higher in wild-type plants than in the mutants enabling the wild-type plants to efficiently limit the spread of pathogens after infection. This study shows that *NPR1* transcription does require a functional cytosolic GSH biosynthetic pathway for an effective *PR-1* transcription and biotic stress response

6.2 Introduction

Glutathione, ascorbate and tocopherols are low molecular weight antioxidants involved in plant growth and developmental processes, as well as during plant-pathogen interactions (Foyer and Noctor, 2005; Choi *et al*., 2007). These antioxidants interact with reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), whose concentrations are known to increase when plants are exposed to various forms of stressors (Foyer and Noctor, 2005; Gills and Tuteja, 2010; Liu *et al*., 2010). H_2O_2 generated in this way during pathogen-induced oxidative burst has been associated with the hypersensitive response (HR) and systemic acquired resistance (SAR) playing a pivotal role in conferring tolerance (Mellersh *et al*., 2002; Hahlbrock *et al*, 2003; Foyer and Noctor, 2005; Davies *et al*., 2006; Liu *et al*., 2010). The major point of detoxification of H_2O_2 is the ascorbate-glutathione (AsA-GSH) cycle which occurs predominately in the mitochondria, chloroplasts, peroxisomes and the cytoplasm (Noctor and Foyer, 1998; Asada, 2000; Foyer and Noctor 2005; Kotchoni and Gachomo, 2006). During this processes, the enzyme ascorbate peroxidase (APX) uses electrons from AsA to degrade the H_2O_2 produced either from superoxide dismutase (SOD), or ROS into water and monodehydroascorbate (MDHA). The MDHA, however is relatively unstable and quickly dissociates to dehydroascorbate (DHA) in a reaction catalyzed by monodehydroascorbate reductase (MDHAR). The DHA is in a next step reduced by DHAR into AsA using electrons donated by GSH. GSH is reduced during this process to oxidized glutathione (GSSG) and in a reaction catalyzed by glutathione reductase (GR), together with electrons from NADPH; GSH is again regenerated (Noctor and Foyer, 1998; Asada, 2000). Thus, the ability of the AsA-GSH cycle to directly interact with ROS and to control the AsA and

GSH redox state of the plant has rendered the cycle a key determinant of other downstream defense response (Kiddle *et al*., 2003).

Recently, a new family of *Arabidopsis* thiol transporter has been isolated by Maughan *et al*. (2010). This transporter, which has a high homology to the *Plasmodium falciparum (Pf*) chloroquine-resistance transporter *(Pf*CRT) and designated CRT-Like Transporter1 (CLT1), has been implicated in the translocation of GSH and *gamma*-glutamylcysteine (γ EC) from the chloroplast to the cytosol. Analysis of these genotypes revealed that, while the glutathione content of the roots of the triple mutant (*clt1clt2clt3*) are significantly reduced compared to the wild-type, shoot glutathione contents are similar in both genotypes. However, it appears that most, if not all, of the GSH in the leaves of the triple *clt1clt2clt3* mutant is localized in the chloroplast and hence the cytosol is deficient in GSH. This triple mutant is therefore an ideal tool, with which to study the role of cytosolic glutathione and other redox determinants like AsA in the *NPR1*-dependent defense response pathway.

Studies aimed at delineating the role of GSH and more specifically of cytosolic GSH on *NPR1*-dependent *PR-1* activation are limited. This part of the study was therefore aimed at elucidating the effect of a deficit in cytosolic GSH on the *NPR1*-dependent *PR-1* transcription using *clt1clt2clt3 Arabidopsis* mutants and to investigate the role of cytosolic GSH in conferring resistance to plants against *Pseudomonas syringae* and *Botrytis cinerea* pathogens.

6.3 Materials and methods

6.3.1 Growth of *Arabidopsis* plants

Except indicated otherwise, *Arabidopsis* wild-type and *clt1clt2clt3* triple mutant Columbia seeds, were surface sterilized for 30 min in a 1.5% (v/v) sodium hypochlorite solution. Seeds were thoroughly rinsed (four times) in distilled water and re-suspended in 0.1% agar (w/v). The surface sterilized seeds were plated in 90-mm Petri dishes (Plastpro Scientific, South Africa) containing Murashige and Skoog (MS) agar medium and vernalized at 4^{o}C for 3-days in the dark. Petri dishes containing vernalized seeds were later transferred to a 10 h day/14 h dark regime for 10 d before sowing individual seedlings in moist 44-mm Jiffy-7 pots (Jiffy International AS, Norway). The Jiffy pots (30 each) where placed in 35 x 25 x 9 cm trays without holes to facilitate sub-irrigation. Trays were covered with cling wrap to ensure for maximum humidity. These were then transferred to controlled environment chambers.

Each chamber was provided a photosynthetic photon flux of 200 µmol. m^{-2} s^{-1}, a photoperiod of 10 h day/14 h dark regime and a relative humidity of 65 ± 5%. The cling wrap was 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 2-weeks. Throughout the experimental period, the Jiffy-7 pots were kept moist by sub-irrigating them twice a wk. All experiments were performed on 4-week-old soil grown plants.

6.3.2 Bacteria growth, plant treatment and sampling

Except stated otherwise, all reagents used for these experiments were purchased from Sigma Aldrich, USA.

6.3.2.1 *Growth of pathogens used for infection*

Arabidopsis plants were infected with either a virulent strain of *Pseudomonas syringae* pv. *maculicola* DG3 (*Pma* DG3; Guttman and Greenberg, 2001), *P. syringae* pv. *tomato* - luxCDABE (*Pst*-luxCDABE; JIC, England), the avirulent *P. syringae* pv. *tomato* avrRPM1 (*Pst*avrRPM1; JIC, England), or *Botrytis cinerea* B06.10 (kindly provided by Dr Henk-Jan Schoonbeek, University of Fribourg).

All *P. syringae* strains were initially cultured from glycerol stocks in 90-mm Petri dishes (Plastpro Scientific, South Africa) containing King B's 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)]. Petri dishes were incubated at 28 ^{o}C for 48 h.

Pma DG3 was selected on medium supplemented with 50 mgL^{-1} streptomycin and kanamycin, while the virulent *Pst*-luxCDABE strain and the avirulent *Pst*avrRPM1 strain was selected on King B's medium supplemented with 50 mgL^{-1} kanamycin only.

Twenty-four hours post infection, bacteria strains were re-suspended in a 10 mM $MgCl_2$ solution. This inoculum (100 µL), was spread on Petri dishes containing King B's medium supplemented with the appropriate antibiotic and incubated at 28°C.

B. cinerea B05.10 (Büttner *et al*. 1994) was cultured at 20°C on Malt extract agar (30 gL^{-1}) and yeast extract (2 gL^{-1}) medium (MEYA; Oxoid, UK) as described by Schoonbeck *et al*. (2003) and in section 5.3 of this thesis.

6.3.2.2 *Plant infection*

Plants for antioxidant measurements and qRT-PCR were sprayed generously with an inoculum of *Pma*DG3 (OD600nm =0.05) re-suspended in a buffer consisting of 10 mM $MgCl_2$ and supplemented with 0.02% Silwett L-77 [(v/v); Lehle seeds, USA]. Another set of plants representing the controls were sprayed in the same way with the re-suspension buffer only. Entire wild-type and *clt1clt2clt3* mutant rosettes (three per time point) were harvested at 0, 24 and 48 h post treatment. Upon harvesting, samples were immediately weight, frozen in liquid nitrogen and stored at -70°C until required. The antioxidant experiments were repeated seven times, while the qRT-PCR experiment was repeated twice with independent plants grown and infected as described above.

For the bioluminescence assay to determine bacteria growth *in planta* and qRT-PCR, a single leaf from each plant was initially syringe infiltrated with either 10 mM $MgCL_2$ followed 48 h by a secondary infiltration of three additional leaves per plant with the virulent *Pst*-DC3000-Lux

($5x10^5$ cfu.ml^{-1} in 10mM $MgCl_2$) bacteria. This represented the non-SAR inducing treatment (MV treatment). In the SAR-inducing treatment (AV), *Pst avrRPM1* ($5x10^6$ cfu.ml^{-1} in 10mM$MgCl_2$) was used for primarily infiltration of a single leaf per plant and 48 h following primary infiltration, three additional leaves were infiltrated with the virulent *Pst*-DC3000-Lux strain. Growth of *Pst*-DC3000-Lux was determined *in planta* 48 h after the secondary challenge using 8-mm leaf discs excised from *Pst*-DC3000-Lux-infiltrated leaves as described by Fan *et al*. (2008). A total of 12 plants were used to for each time point an bacteria growth was represented as the number of photon counts per second (CPS). The severity of damage caused by the pathogen was also observed visually and digital pictures of selected representative infected leaves were taken. Samples for RNA extraction were harvested 48 h after the primary and secondary infection, frozen in liquid nitrogen and stored at -70C until required. The experiment was repeated twice with independent samples.

For infection with *B. cinerea* B06.10, an innoculum (resuspended in ¼ strength MEYA medium) with a final concentration of 2.5 x 10^5 spores/mL was prepared. For each plant to be infected six leaves were used and 5 µL droplets of the inoculums were pipetted onto one side of each leaf. Trays containing plants were covered with a plastic dome to achieve close to 100% humidity. The severity of damage caused by the fungus to the plant was assessed 3 dpi. Plants for the control experiment were treated in the same way with 5 µL droplets of the re-suspension solution. Data was collected from a total of eight plants to represent a single time point and disease severity caused by the pathogen was also observed visually and pictures of selected infected leaves were digitally taken. The experiment was repeated twice with independent plants grown and infected as described above.

6.3.3 Chlorophyll, glutathione and ascorbate measurements

The methods for these assays were adapted from Queval and Noctor, (2007) with slight modifications. Except where stated otherwise, each 1 mg of plant material was homogenized in 1 mL of extraction buffer. Centrifugation steps were carried out at 4^{o}C and 12.000 revolutions per minute (rpm). Sample was assayed in triplicates in 96-welled UV-transparent micro titer plates (Corning, USA) and absorbencies were read at 25^{o}C using a SPECTRAmax plus 384 plate reader (Molecular device ltd, Ireland). Each experiment was repeated seven times with different samples that were treated and sampled in the same manner as described above in section 6.3.2.2.

Each *Arabidopsis* rosette was ground to a fine powder with a pestle in a mortar containing liquid nitrogen. The metabolite in the sample was extracted in a total volume of 2 mL using a 1-M hypochloric acid ($HClO_4$) solution and using two aliquots of the acid as follows: to each sample, 1 mL of 1 M $HClO_4$ was added, mixed properly and transferred to a 2-mL Eppendorf tube. The mortar and pestle was rinsed with the remaining 1 mL of the 1 M $HClO_4$ and this was added to the first extract in the same tube. Samples were thoroughly mixed before centrifugation at 12. 000 rpm for 10 min. The clear supernatant was retained for ascorbate and glutathione assays while the pellet was used to assay chlorophyll measurements.

6.3.3.1 *Chlorophyll measurement*

Each pellet obtained from the above centrifugation step was re-suspended in a total volume of 10 mL of an 80% acetone solution in two steps: in the first step, the pellet was re-suspended in 1 mL

of 80% acetone and mixed by vortexing. This was transferred to a 14-mL Falcon tube containing 8 mL of 80% acetone. The Eppendorf tube was rinsed with an additional 1 mL of 80% acetone and this was added to the components in the Falcon tube. The chlorophyll extract was thoroughly mixed and all samples were wrapped in foil paper and incubated in the dark at 4^{o}C overnight.

The following day, the chlorophyll extract were again mixed and a 1-mL aliquot of plant extract was transferred to a 1.5 mL Eppendorf tube. This was centrifuged at 12.000 rpm for 10 min. Samples in which the pellet still appeared green were re-suspended in an additional 1 mL of 80% acetone, mixed and centrifuged under the same conditions as the first. Both supernatants were combined and the volume noted. Once a clear pellet was obtained, 2-mL aliquots of the supernatant were used to assay chlorophyll (phaeophytin) using a quartz cuvette. Absorbance was read at 655 nm and 666 nm and these were corrected against blank readings obtained from 2 mL of an 80% acetone solution.The concentration of phaeophytin in each sample was calculated using the formula:

Phaeophytin (mg.L^{-1}) = (26.03 x A_{655}) + (6.75 x A_{666}).

6.3.3.2 *Measurement of total, reduced* and *oxidized glutathione*

Reagents for GSH assays consisted of 50 mM nicotinamide adenine di-nucleotide phosphate reduced tetra-sodium salt (NADPH; EC: 220-163-3) and 6 mM 5'5'-dithiobis-2-nitobenzoic acid (DTNB; EC: 200-714-4). These reagents were prepared fresh in a 0.12 M NaH_2PO_4 (pH 7.5) buffer which had been supplemented with 6 mM EDTA. Thereafter, aliquots were made from the NADPH solution and stored at -20^{o}C, while the DTNB solution was stored in the dark at 4^{o}C.

The enzyme for this reaction glutathione reductase (GR) was prepared fresh daily by spinning 25 µL of the GR stock and re-suspending the pellet in 567 µL of reaction buffer to obtain a final concentration of 20 UmL^{-1}.

Glutathione (reduced and oxidized glutathione) standards were prepared in a buffer consisting of 0.5 mL of a 1 M $HClO_4$ solution containing 0.1 mL of 0.12 M NaH_2PO_4 (pH 7.6). The pH of this buffer was adjusted to 6.0 with sufficient amounts of 2.5 M K_2CO_3. The pH was verified using pH strips. Excess $KClO_4$ resulting from the reaction was removed from the solution by centrifugation for 10 min and the supernatant was decanted into a clean 50-mL Falcon tube.

Standards for total glutathione were prepared from a 1 M GSH (EC:200-725-4) solution which was diluted to 200 µM to obtain a stock amount of 2 nmol in a volume of 10 µL. Serial dilutions of 2.0, 1.0, 0.5, 0.25 and 0.125 nmol were prepared from the stock using the standard buffer.

Each reaction mixture consisted of 0.16 mL of 0.12 M NaH_2PO_4 (pH 7.5), 6 mM EDTA, 0.01 mL of 6 mM DTNB, 0.01 mL of 50 mM NADPH and 0.01 mL of either standards or neutralized plant sample. Reference samples (blanks) contained the above-mentioned solutions but lacked the standard and plant supernatant. The samples were mixed in the plate reader and the absorbance at 412 nm read for zero. The reaction was initiated by adding 10 µL of GR into each well and the increase in absorbance at 412 nm was monitored for 5 min after automatic mixing.

For GSSG measurements, an aliquot of the neutralized plant supernatant was first treated with 2-vinylpyridine. This was achieved by adding aliquots of 0.2 mL neutralized plant extract to a tube

containing 5 µL of 2-vinylpyridine. The reaction mixture was incubated at 25°C for 30 min. After incubation, samples were centrifuged for 15 min and 0.15 mL of the supernatant was decanted into a fresh tube. The centrifugation step was repeated using this second supernatant and 0.13 mL of the final supernatant decanted into a fresh tube.

For GSSG standards, 0.1 M GSSG (EC: 248-170-7) was diluted to a final concentration of 4 µM to obtain a stock solution of 80 pmol in every 20 µL. The stock solution (0.2 mL) was treated with 2-vinylpyridine in the same way as the plant extract. Standards for the reaction were prepared to final amounts of 80, 40, 20, 10, 5 and 2.5 pmol from the treated stock solution. All samples were assayed as for total glutathione.

The amount of GSH for both the wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants was calculated by subtracting the GSSG content obtained for each time point from those of the total GSH content.

6.3.3.3 *Measurement of reduced, total ascorbate* and *dehydroascorbate content*

AsA contents were measured using the same acid extracts as GSH. Each supernatant (0.5 mL) obtained after centrifugation of the 1 M $HClO_4$ crude extract was decanted into a fresh tube containing 0.1 mL of a 0.12 M NaH_2PO_4 (pH 7.6) solution. The pH of the supernatant was adjusted to 6.0 by adding in a drop-wise manner an aliquot (60 µL) of 2.5 M K_2CO_3. The pH was determined using pH strips. The supernatants were thoroughly mixed after each addition to ensure a uniform and a rapid change in the pH of the extracts. The mixtures were centrifuged

again and the insoluble pellets were discarded. The final supernatants, which were used for the ascorbate and glutathione determinations, were at a pH of 6.0.

A stock solution of ascorbate oxidase (AO; EC: 232-852-6) was prepared by dissolving eight mg of the enzyme powder in a 0.2 M NaH_2PO_4 (pH 6.6) solution so as to obtain a final concentration of 40 UmL^{-1} AO. This stock solution was stored in aliquots of 0.2 mL at -20^{o}C until required. Each reaction mixture for the AsA measurement consisted of 40 µL of the neutralized plant extract (pH 6.0) and 0.16 mL of 0.12 M NaH_2PO_4 (pH 6.6) which, had been supplemented with 1 mM EDTA. The reaction was properly mixed and their absorbencies measured at $A_{266.}$ The oxidation of the AsA was initiated by adding 0.2 U AO. The reactions were again mixed and the decrease in absorbance at A_{265} was monitored until the reaction was complete.

For measurement of total ascorbate (DHA plus AsA), DHA was first reduced to AsA. This was accomplished using 1-4-dithiothreitol (DTT; EC: 222.4687) as follows: aliquots of 0.1 mL of the neutralized plant extract were added to tubes containing 0.14 mL of 0.12 M NaH_2PO_4 (pH 7.5) and 0.01 mL of 0.5 M DTT solution. The mixtures were incubated for 30 min at 25^{o}C and the samples were then assayed as described above for reduced ascorbate.

The amount of ascorbate in both wt and *clt1clt2clt3* mutant *Arabidopsis* rosettes was calculated using the extinction coefficient: A_{265} = 14000 mol/L where, mol/L = mol per litre. The amount of DHA for both the wt and *clt1clt2clt3* mutant *Arabidopsis* plants were calculated by subtracting the reduced ascorbate content obtained for each time point from those of the total ascorbate content.

6.3.4 Quantitative real time-polymerase chain reaction

Quantitative real time polymerase chain reaction (qRT-PCR) was conducted to measure the relative transcript amounts of the *Arabidopsis NPR1* (*AtNPR1*; AT1g64280) and *AtPR-1* gene (NM_127026.2) in the leaves of both wt and *clt1clt2clt3* mutant *Arabidopsis* plants treated with *P. syringae* as specified above. Primers for these reactions were designed using the Netprimer3 program (Premier Biosoft, Palo Alto, CA, USA) and following specifications described in chapter 4.3. *Arabidopsis* actin (AT3g18780) was used as the housekeeping gene. Following verification of resultant primers as described in section 4.3, the following primer combinations were used for the reaction: qNPR1_forward -TCTATCAGAGGCACTTATTGG and qNPR1_reverse – TGCCTTATGTACATTCGAGAC; qPR-1_forward – CGGAGCTACGCAGAACAACT and qPR-1_reverse – CTCGCTAACCCACATGTTCA; qActin_forward – AGTGGTCGTACAACCGGTATTGT and qActin_reverse – GATGGCATGGAGGAAGAGAGAAAC.

QRT-PCR was carried out using cDNA synthesized from total RNA of leaf material from *Pst* avrRPPM1, *Pst* avrRPPM1/*Pst*-luxCDABE and treated plants harvested at 0 and 48 h post-treatment. By use of the manufacturer's software program, the relative concentrations of the samples were determined. All samples were normalized using their endogenous actin values and the relative transcript amounts for each time point was expressed relative to the controls which served as the calibrator. The Applied Biosystems User Bulletin No. 2, (2000) and steps described in Livak and Schmittgen (2001) were used for data analysis.

6.3.5 Statistical analysis

The statistical differences from data obtained between the two genotypes and between the different time points in each experiment were calculated using the Statistical Analysis Software (SAS$^{(R)}$, USA). This was done using ANNOVA and Tukey studentized range (HSD) test and means with similar letters were considered as not being statistically different from one another. Percentage differences between two independent samples were calculated using the Student's T-Test. In both procedures, the cut-of value was set at $P>0.05$ for samples which were not significantly different from each other.

6.4 Results

6.4.1 Chlorophyll content

The chlorophyll content in the two *Arabidopsis* genotypes was measured as phaeophytin after acid extraction of plant extract. SAS$^{(R)}$ analysis showed no significant (P>0.05) differences in chlorophyll turnover in both genotypes during the time points used for this study (Table 6.1). All antioxidants measured in the following study were therefore expressed in terms of their chlorophyll content.

Table 6.1 Chlorophyll content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

Chlorophyll content (mgg^{-1}FW)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**1.2 ± 0.11^{a}**	**1.19 ± 0.07^{a}**	**1.10 ± 0.15^{a}**
***clt1clt2clt3* mutant**	**1.2 ± 0.09^{a}**	**1.21 ± 0.17^{a}**	**1.21 ± 0.13^{a}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.2 Glutathione content

GSSG content was measured after treatment of plant extracts with 2-vinyl pyridine to complex the GSH present. At the start of the experiment (0 hpi), the GSSG content was not significantly ($P>0.05$) different between the wild-type and the *clt1clt2clt3* mutant *Arabidopsis* genotypes (Table 6.2). In the mutant, a 84% significant ($P<0.05$) increase in GSSG content was found 24 hpi when compared to the GSSG amount at the 0 h time point. This increment was also 74% significantly ($P<0.05$) higher than the GSSG content in the wild-type plants 24 h post *Pseudomonas* treatment. At the 48 h time point, the GSSG content in the mutant had again increased in comparison to the 24 h GSSG content, however this was not significantly ($P>0.05$) different from that of the wild-type *Pseudomonas* treated plants analyzed 48 h after infection.

Table 6.2 GSSG content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

GSSG content ($nmolmg^{-1}$Chlorophyll)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**21.63 ± 2.14^{c}**	**24.20 ± 1.20^{c}**	**46.43 ± 7.16^{ab}**
***clt1clt2clt3* mutant**	**23.27 ± 1.12^{c}**	**42.32 ± 6.06^{ab}**	**57.29 ± 6.79^{a}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

Similar to the GSSG content, the GSH content between the two *Arabidopsis* genotypes was not significantly ($P>0.05$) different at the start of the experiment (Fig. 6.1). After 24 hpi, the GSH content in the mutant was significantly ($P<0.05$) reduced by 28% in comparison to the GSH content at the beginning of the experiment (Fig 6.1). This decrease was also significantly lower (56%) than the GSH content measured in the infected wild-type plants 24 hpi. At 48 hpi, the GSH content in both genotypes decreased without being significantly ($P>0.05$) different from each other.

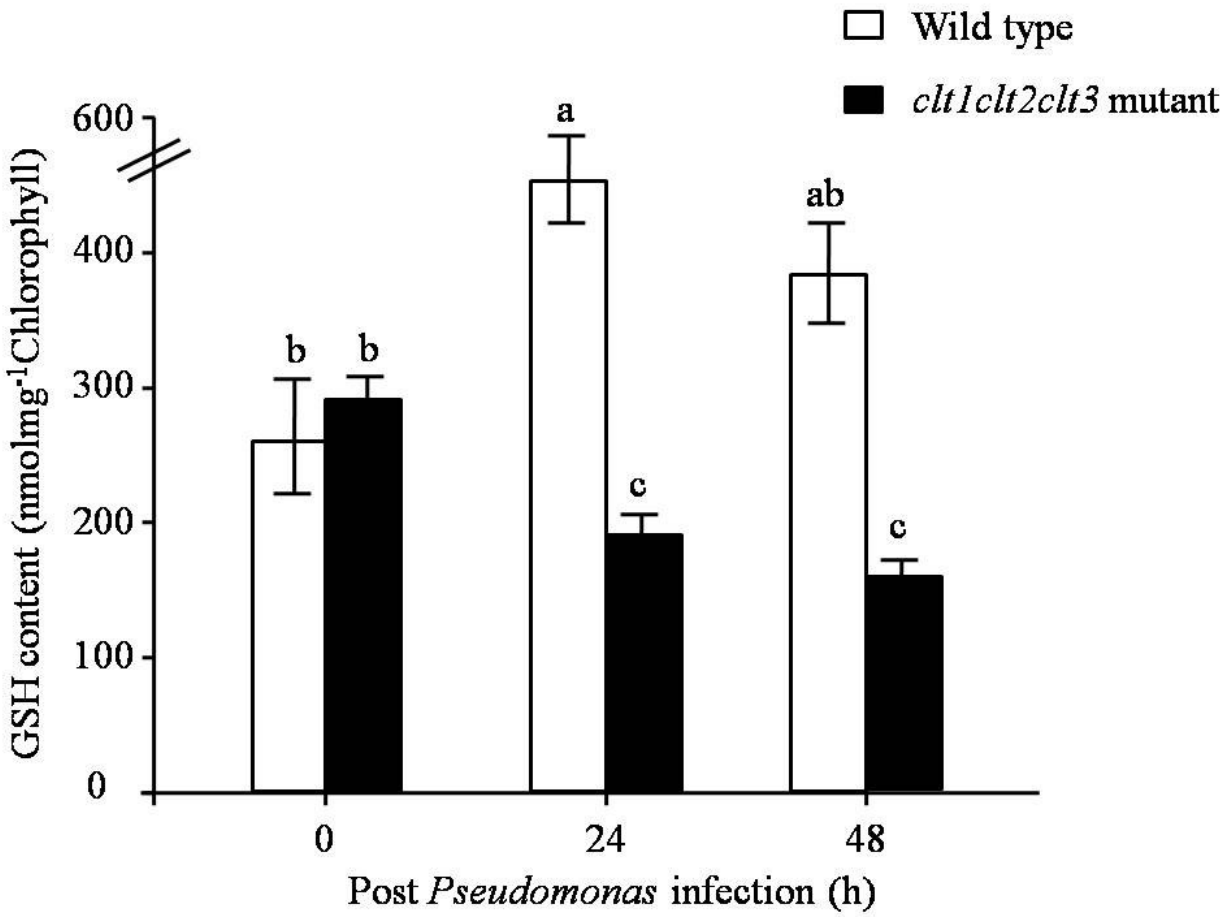


Figure 6.1 Reduced glutathione content in *Pseudomonas*-infected wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Metabolites were extracted in 1 N of a $HClO_4$ solution using *Arabidopsis* rosettes harvested at 0, 24 and 48 h post *Pma*DG3 infection. Total glutathione was measured directly from the neutralized supernatant while reduced glutathione was calculated by subtracting the GSSG content (measured after treatment of the neutralized plant supernatant with 2-vinyl pyridine) from that of the total glutathione content. All samples were assayed in triplicates by reading the absorbance at 412 nm before and after initiating the reaction with GR. The mean values ± standard error of the mean (SEM) for a total of seven repeats were plotted on the graph. Means with the same letters are not significantly different (P>0.05).

During the time points investigated, the GSH/GSSG ratio in *Pseudomonas*-treated mutant plants significantly (P<0.05) decreased by 77% at 24 hpi compared to the ratio at the start of the experiment. This was followed by a continuous significant (P<0.05) decrease in the GSH/GSSG ratio of the mutant of a further 75% at 48 hpi when compared to the GSH/GSSG ratio at 24 hpi. At both time points (24 and 48 hpi), the GSH/GSSG ratio in the mutant was always significantly (P<0.05) lower than that of the *Pseudomonas*-treated wild-type plants (Table 6.3). In the wild-type plants, the GSH/GSSG ratio initially increased significantly (P<0.05) by 46% 24 hpi in comparison to the GSH/GSSG ratio at the start of the experiment. This was followed by a 57% significant (P<0.05) decrease in this ratio 48 hpi.

Table 6.3 GSH/GSSG ratio in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

	GSH/GSSG		
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**14.70 ± 2.35^{b}**	**21.59 ± 2.07^{a}**	**08.66 ± 1.17^{c}**
***clt1clt2clt3* mutant**	**14.65 ± 1.79^{b}**	**4.89 ± 1.55^{d}**	**2.52 ± 0.09^{de}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.3 Ascorbate content

Similar to the GSH content, the absence of a functional GSH transporter in the mutant plants did not affect the total ascorbate and reduced ascorbate content at the start of the experiment (Fig. 6.2). Twenty-four h after treatment with *Pseudomonas*, both genotypes had significantly ($P<0.05$) increased amounts of total ascorbate (61%) in comparison to the content at the 0 h time point (Fig 6.2A). Thereafter, the total ascorbate content decrease in both genotypes at 48 hpi with a more dramatic and significant ($P<0.05$) decrease in the mutant plants which, was also 37% significantly ($P<0.05$) less than that of the wild-type plants.

Following pathogen infection, the content of AsA also increased significantly ($P<0.05$) by 33% in the wild-type plants when compared to the mutant plants (Fig. 6.2B) whose AsA content remained constant between the 0 and 24 hpi. At 48 hpi, the AsA content in the mutant decreased significantly ($P<0.05$) by 76% which was significantly ($P<0.05$) lower when compared to the content in wild-type plants.

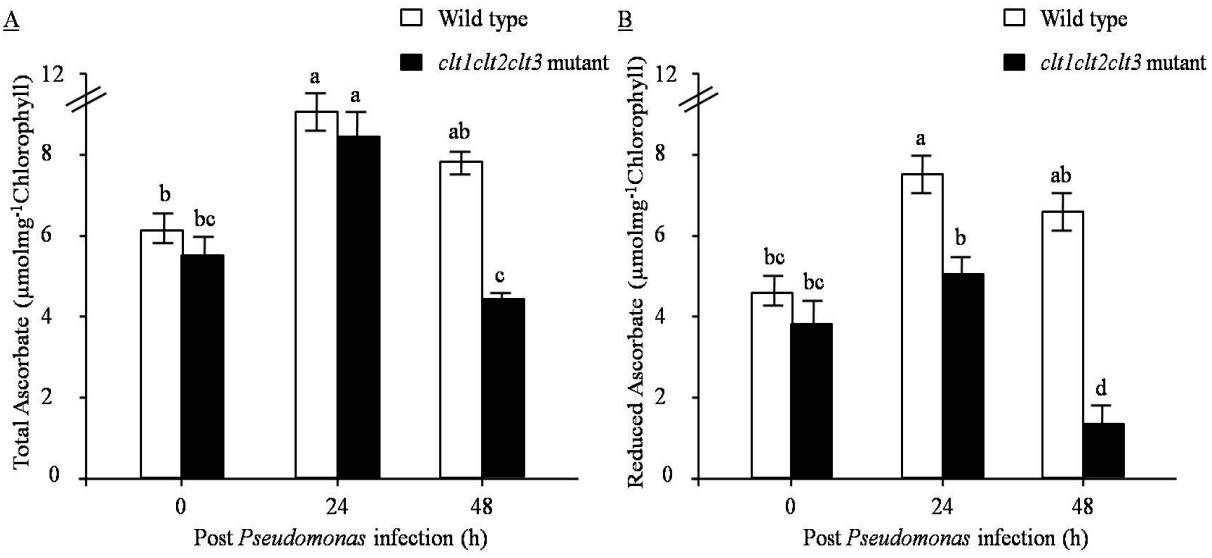


Figure 6.2 Total and reduced ascorbate content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Leaf material from three plants were pooled to represent a single time point and harvested from the two genotypes at 0, 24 and 48 h after infection with *Pma*DG3. Metabolite extraction was done in a 1 M $HClO_4$ solution and, the acid supernatant was neutralized to a pH of 6.0 with 2.5 M K_2CO_3. Total ascorbate (A) measurement was performed using samples that had been treated with DTT. Reduced ascorbate (B) content was measured directly using untreated neutralized acid supernatants. All samples were assayed by reading the absorbance at 265 nm before and after initiating the reaction with ascorbate oxidase. The mean values ± SEM for a total of seven repeats were plotted on the graphs. Means with the same letters on individual graphs are not significantly different (P>0.05).

The DHA content was measured by subtracting the AsA content at each time point from that of the total ascorbate measured. While no significant (P>0.05) difference was obtained in the DHA content between both genotypes at 0 hpi, a significant (P<0.05) 65% increase in DHA was obtained at 24 hpi for the mutant when compared to the DHA content in the wild-type (Table 6.4). The DHA content in the mutant again increased significantly (P<0.05) by 179% at 48 hpi when compared to the DHA content in the wild-type at this time point.

Table 6.4 DHA content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

DHA content ($\mu molmg^{-1}$Chlorophyll)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**1.67 ±0.15^{c}**	**2.53 ± 0.11^{b}**	**1.18 ± 0.07^{c}**
***clt1clt2clt3* mutant**	**1.79 ± 0.79^{c}**	**4.19 ± 0.42^{a}**	**3.52 ± 0.87^{ab}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.4 *NPR1* and *PR-1* transcription

Treatment of *Arabidopsis* wild-type plants and *clt1clt2clt3* mutant plants with only $MgCl_2$ (control) resulted in no significant ($P>0.05$) changes in *NPR1* transcript amounts 48 h after treatment. In the MV- and AV-treated plants, there was a slight but significant ($P<0.05$) 1.5-fold and 1.3-fold increase in *NPR1* transcript amounts for the wild-type plants compared to the $MgCl_2$ (control) plants. In the mutant plants no significant ($P<0.05$) differences in *NPR1* transcription was measured in comparison to the control plants (Fig. 6.3A). *PR-1* transcript amounts were significantly ($P<0.05$) higher in both the wild-type and mutant *Arabidopsis* genotypes after pathogen infection in comparison to the $MgCl_2$ treated samples (Fig. 6.3B). Primary infection of the plants with *Pst*-luxCDABE led to a 41-fold significant ($P<0.05$) increase of *PR-1* transcripts in the wild-type plants. This was significantly ($P<0.05$) higher than the only 5-fold increase in *PR-1* transcript amounts obtained for the mutant when compared to their $MgCl_2$ treated samples. The SA-inducing treatment (AV-treatment) resulted 48 h after the secondary infection to a significant ($P<0.05$) 77-fold increase in wild-type *PR-1* transcripts compared to a 3-fold increase in transcripts measured in the mutant.

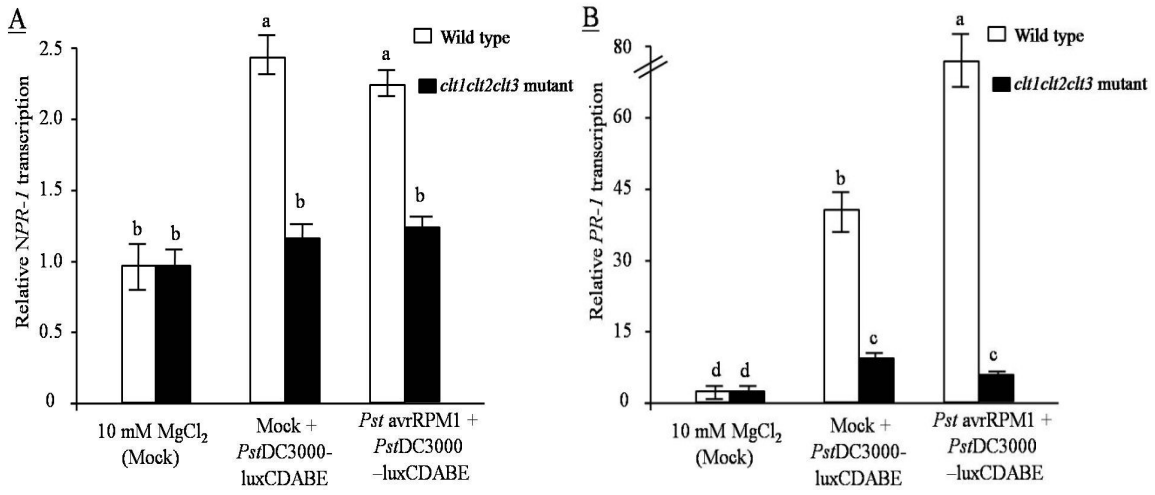


Figure 6.3 Relative *NPR1* and *PR-1* transcript amounts in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Plants of the two genotypes were treated with either $MgCl_2$ (control), *Pst*DC3000-luxCDABE or *Pstavr*RPM1/*Pst*DC3000-luxCDABE. cDNA from leaf material harvested 48 h post treatment with 10 mM $MgCl_2$ or 48 h following either a primary or the secondary challenge with *Pst*DC3000-luxCDABE were used for qRT-PCR. The transcript amounts for the *Arabidopsis* (At)-*NPR1* (A) and *At-PR-1* (B) were calculated relative to the endogenous levels of actin (reference gene) and expressed relative to the transcript amounts obtained from their respective control plant samples. The experiment was repeated twice using different sets of plant material treated and harvested as described above. The mean values ± SEM were plotted on the graph. Means with the same letters (a, b, c, d) on the same graph are not significantly different (P>0.05).

6.4.5 Disease progression

Pst-luxCDABE bacteria growth was measured 48 h post secondary infection in the MV- and AV-treated (Fig 6.4). In MV-treated (non-SAR induced) plants, a significant ($P<0.05$) 1.5-fold reduction in *Pst*-luxCDABE bacteria number was found for the wild-type plants when compared to the mutant plants 48 h post secondary infection. Similarly, in AV-treated (SAR induced) plants, growth of *Pst*-luxCDABE was significantly ($P<0.05$) suppressed (3.2-fold) in the wild-type plants in comparison to the mutant plants.

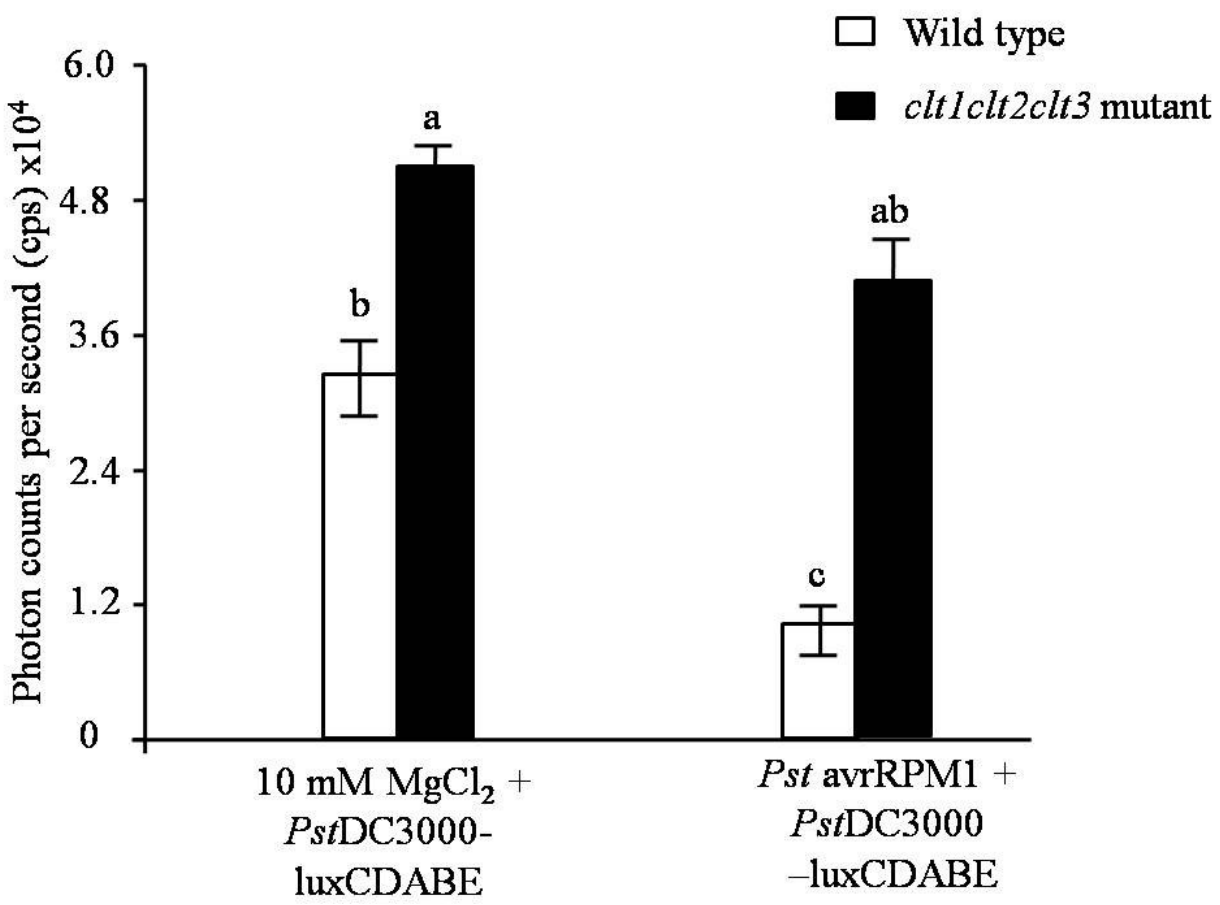


Figure 6.4 Bacteria growth measurement in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. Plants of the two genotypes were pre-treated (one leaf per plant) with either $MgCl_2$ (Control) or *Pstavr*RPM1. This was followed 48 h later by a secondary challenge (three non-inoculated leaves per plant) using the virulent *Pst*DC3000-luxCDABE strain. Leaf disks were punched from the *Pst*DC3000-luxCDABE infected leaves using a 4-mm cork borer 48 h after the secondary challenge and the luminescence detected using a luminomenter. For each experiment, 36 leaf disks per genotype were used and the experiment was repeated three-times. Values plotted represent the mean ± SEM. Means with the same letters are not significantly different (P>0.05).

The difference in bacterial growth between both genotypes was also visually apparent from the degree of damage caused on the infected leaves. The mutant leaves were more severely damaged compared to the wild-type leaves after pathogen infection (Fig. 6.5). Furthermore, wild-type plants infected with only *Pst*avrRPM1 had a significantly ($P<0.05$) reduced lesion length compared to the mutant plants even on the fourth day after infection. At this time, almost 70% of the mutant leaf had been damaged by the avirulent pathogen while the lesion on the wild-type leaf was still very much localized around the point of infection.

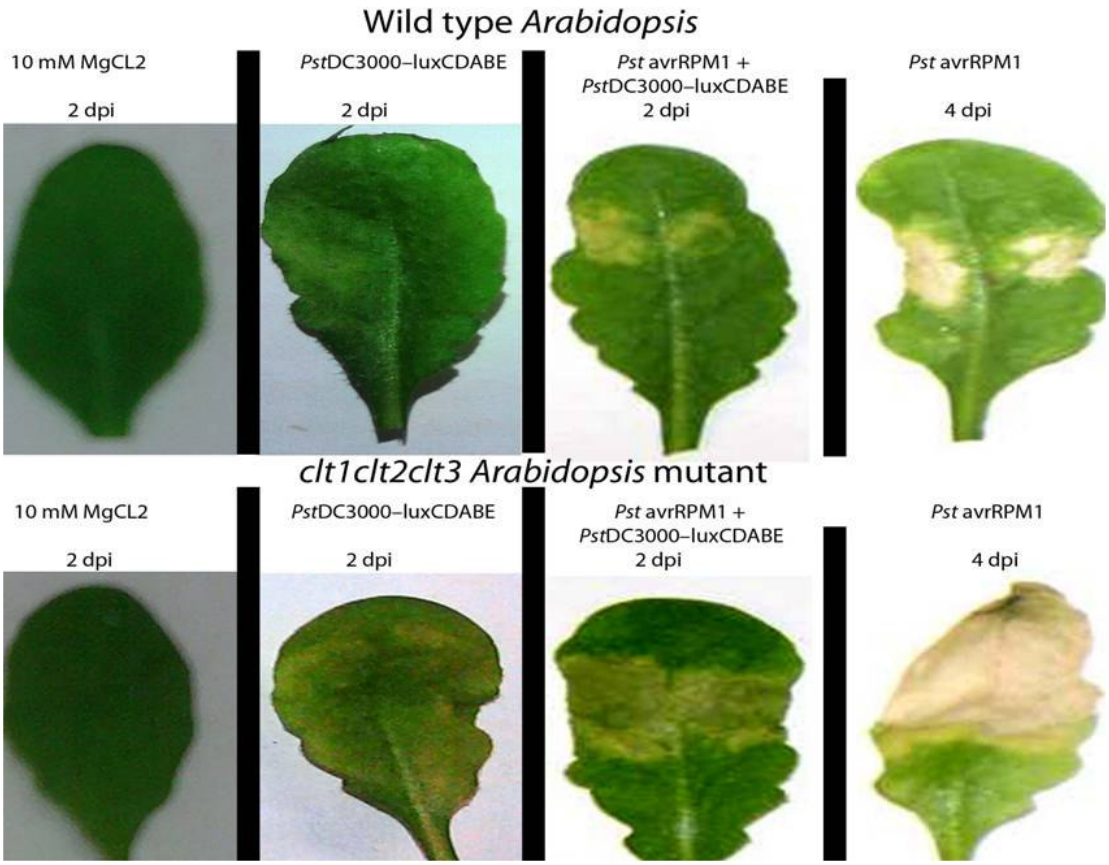


Figure 6.5 Disease symptoms in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. Plants of the two genotypes were pre-treated (one leaf per plant) with either 10 mM of $MgCl_2$ (control) or *Pstavr*RPM1. This was followed 48 h later by a secondary challenge (three non-inoculated leaves per plant) using the virulent *Pst*DC3000-luxCDABE strain. Disease progression was visually observed in infected plants at 2 d and 4 d post challenge. Pictures depict symptoms from selected leaves treated with 10 mM of $MgCl_2$ only, *Pst*DC3000-luxCDABE, *Pstavr*RPM1 + *Pst*DC3000-luxCDABE, or *Pstavr*RPM1. The experiment was repeated twice with similar results obtained for each repeat.

Disease development in the experimental plants following infection with the necrotrophic fungi *B. cinerea* was determined by measuring the lesion length formed on infected leaves 3 dpi. In wild-type infected plants, the lesion lengths formed were significantly 2.3-fold lower than those that developed on infected leaves of the mutant plants (Fig. 6.6A). These disease symptoms were also visually apparent as a greater portion of the mutant leaves became colonized by the fungi with some of their leaves becoming completely bleached compared to the spotted lesions present on the leaves of the wild-type plants (6.6B).

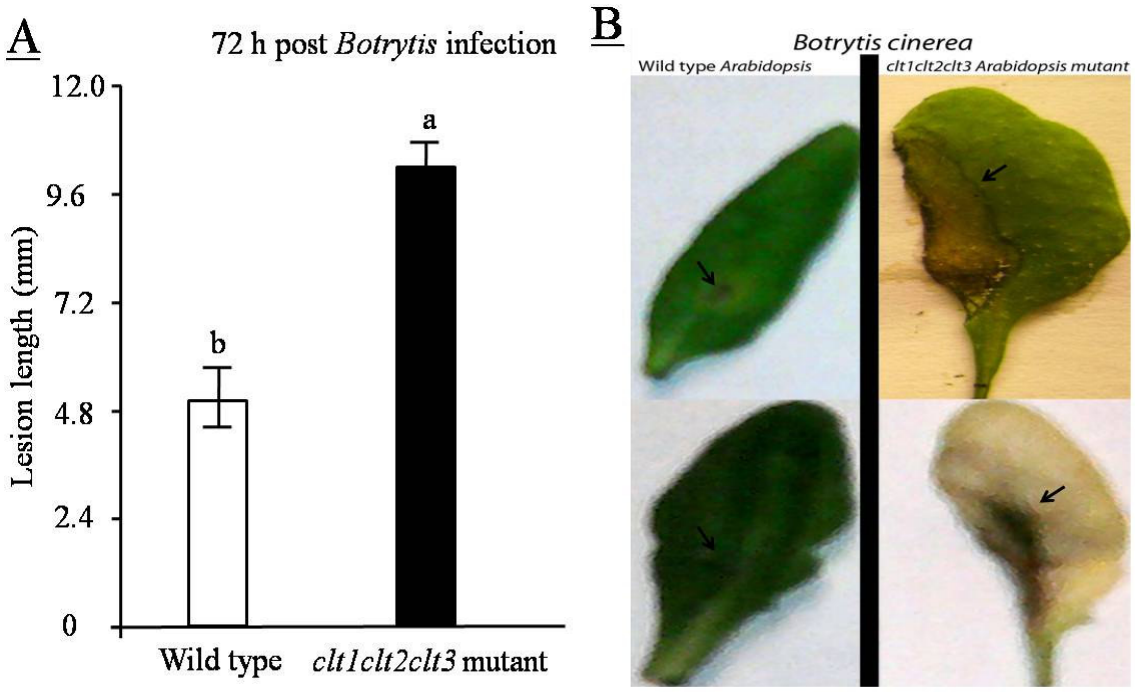


Figure 6.6 Lesion length and disease symptoms in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *B. cinerea*. Plants of the two genotypes (six leaves per plant) were treated with 5 µL of a 2.5 x 10^5 spores/mL inoculums of *B. cinerea* B06.10. The severity of damage caused by the fungus to the plant was assessed 3 dpi by measuring the lesion length (A) and visually with pictures of selected leaves taken using a digital camera (B). Data was collected from a total of eight plants to represent a single time point and the experiment was repeated twice. Values plotted represent the mean ± SEM. Means with the same letters are not significantly different (P>0.05).

6.5 Discussion

In this study, *NPR1* transcription was not significantly affected after pathogen infection in *Arabidopsis* mutant plants that were deficient in cytosolic GSH (*clt1clt2clt3*) and unable to synthesize GSH in the cytosol which affects the cellular redox state. Furthermore, the GSH amount of wild-type plants increased but decreased in *Arabidopsis* mutant plants. Such GSH increase in wild-type *Arabidopsis* plants following exposure to a pathogen, but also ozone, is well-documented in literature (Mou *et al*., 2003; Yoshida *et al*., 2009). However as a new result, this study provides evidence that the absence of the γ EC/GSH transporter in *clt1clt2clt3* mutant *Arabidopsis* plants prevents the plant's natural ability to synthesize foliar GSH to amounts comparable to the amount found in wild-type plants following pathogen infection. This reduced GSH amount in mutant plants was further directly related to an increase in the amount of oxidized glutathione (GSSG). This resulted in a greatly reduced GSH/GSSG ratio in this mutant genotype. The GSH/GSSG ratio also plays a key role in determining the redox state of cells (Pavet *et al*., 2005; Solomon *et al*., 2010) and this directly affects NPR1 oligomerization (Mou *et al*., 2003) and, as found in this current study, *NPR1* transcription was also affected.

Further, the DHA amount, but not the AsA amount, increased when the GSH amount was reduced in these mutant plants after pathogen infection. Although evidence exists for compensatory antioxidant effects with *Arabidopsis* plants deficient in either GSH (*cad2* mutant) or AsA (*vtc* mutant) having a higher AsA (GSH-deficient) or a higher GSH (AsA-deficient) amount when exposed to stresses like high light intensity (Kanwischer *et al*., 2005; Colville and Smirnoff, 2008), GSH deficiency was not compensated in the *clt1clt2clt3* mutant *Arabidopsis*

plants by an increased AsA amount. In general, in the AsA-GSH cycle for H_20_2 scavenging, AsA is directly oxidized to DHA and, regenerated in a reaction necessitating GSH (Asada, 2000). The lower AsA content measured in the *clt1clt2clt3* mutants could have been due to the usage of AsA for scavenging of H_20_2 generated by pathogen infection. In addition, a lower GSH content found in mutant plants following infection might also have limited the supply of electrons required for an adequate recycling of AsA. Unlike AsA, the DHA content in mutant plants increased during pathogen treatment causing a continuous oxidation of AsA with only a slow reduction of DHA in the AsA-GSH cycle (Asada, 2000).

In contrast to mutant plants, in wild-type *Arabidopsis* plants, which are able to synthesize GSH in both the chloroplast and the cytosol, a small but significant increase in *NPR1* transcription was measured 48 hpi not found in mutant plants. This provides evidence for a relationship between presence of cytosolic GSH and transcription of cytosol-located NPR1 and that both a functional GSH biosynthetic pathway and NPR1 transcription very likely are required for efficient protection against a pathogen such as *P. syringae*. Recent findings have already indicated such a relationship and, tobacco plants over-expressing a tomato *gamma*-glutamylcysteine synthetase gene (γ ECS) had a 2-fold higher *NPR1* transcription and synthesized more GSH, which resulted in an increased SA amount when compared to wild-type non-transformed plants (Ghanta *et al.*, 2011). Also, tobacco plants over-expressing a tomato or maize *gamma*-glutamylcysteine synthetase gene (γ ECS), or treated with a GSH solution, transcribed more *PR-1* in comparison to non-transformed or untreated plants (Gomez *et al.*, 2004). Furthermore, when Spoel *et al.* (2009) used complimented *Arabidopsis npr1-1* plants expressing the *NPR1* gene and further treated these plants with SA, an effective recruitment of NPR1 to the nucleus and a continuous

degradation and turnover occurred which promoted *PR-1* transcription. In the present study such processes have not been investigated and future research might therefore include investigating if mutant plants are also affected in proteasome-mediated degradation of already used-up nuclei localized NPR1. This might affect NPR1 turnover in the nucleus which promotes the supply of newly transcribed NPR1 transcription factors to their target promoter regions.

Downstream *PR-1* transcription was further significantly reduced in cytosolic GSH-deficient *Arabidopsis* mutant plants. These plants exhibited also a higher pathogen sensitivity following infection when compared to wild-type *Arabidopsis* plants. This confirms previous results obtained by Maughan *et al*. (2010) that *clt1clt2clt3* mutant plants have a reduced amount of *PR-1* transcripts and are more susceptible to pathogens in comparison to wild-type plants. Furthermore, findings in this study also confirm results found with *cad2 Arabidopsis* mutants that a reduced GSH content increases susceptibility to a pathogen when compared to wild-type plants (Ball *et al*., 2004). Upon pathogen infection, the GSH amount of wild-type plants increased in the present study but decreased when mutant plants were infected. When the equilibrium between GSH and GSSG production and recycling is shifted towards accumulation of oxidized glutathione (GSSG) as found for the mutant plants used in this study, the cell's environment will be more oxidized. This favors NPR1 proteins to exist predominately as an oligomer which is, in comparison to a monomer NPR1, unable to move to the nucleus to activate transcription factors such as TGA2 and TGA3 (Kinkema *et al*., 2000; Zhou *et al*., 2000; Mou *et al*., 2003). The ultimate redox dependency of transcription factors to which NPR1 monomers would bind are affected under oxidized conditions with the likely consequence of decreased

NPR1-dependent *PR-1* transcription and higher pathogen susceptibility also found in this study (Mou *et al*., 2003; Després *et al*., 2003; Pavet *et al*., 2005).

In mutant plants a reduced, but not completely blocked, *PR-1* transcription was observed 48 hpi whereas *PR-1* transcription was greatly increased at this time point in wild-type plants. This indicates that, in spite of the low GSH and also low AsA content, as well as the lack of *NPR1* transcription, *PR-1* transcription still occurred in mutant plants following infection with a pathogen. Such reduced *PR-1* transcription has also been previously reported by Colville and Smirnoff (2008) using AsA-deficient *vtc2-1* mutants with a reduced AsA content in comparison to wild-type plants. Following inhibition of GSH biosynthesis in these *vtc2-1* mutant plants by treatment with the inhibitor D,L-buthionine-[S,R]-sulphoximine (BSO), *PR-1* transcription in these mutants was still not completely blocked. Also Ball *et al*. (2004) found by using *rax1-1* and *cad2 Arabidopsis* mutants with reduced GSH amounts, that *PR-1* transcription was not completely blocked. Since *clt1clt2clt3* mutant plants also produced some *PR-1* transcript, this indicates that *PR-1* transcription might also occur independently of *NPR1* transcription. However, reduced *PR-1* transcription in mutant plants was overall not sufficient to render mutant plants more resistant to *B. cinerea* and *P. syringae* pathogens in comparison to wild-type plants in which pathogen progression were successfully prevented.

Overall, data from this study have confirmed that a functional GSH biosynthetic pathway is required to effectively mediate an *NPR1*-dependent *PR-1* biotic defense responses network in *Arabidopsis* plants and that lack of GSH causes increased susceptibility of plants against a pathogen.

CHAPTER SEVEN

GENERAL DISCUSSION AND FURTHER PERSPECTIVE

The study aimed to functionally characterize *NPR1*-like genes isolated from banana through comparative sequence analysis and complementation studies, evaluation of their expression in response to pathogen infection and their role in the pathogen response cascade through assessment of expression of downstream genes in the SA/JA responsive pathways. Also the role of known antioxidants in *NPR1* expression was explored. Overall, the study has provided novel information on the functional role of two banana *NPR1*-like homologues which highlights their involvement in mediating defense response. Further, first evidence has been provided on a possible direct relationship between cytosolic glutathione and *NPR1* transcription important for biotic stress tolerance.

New knowledge contributed was that the two *MNPR1* genes were transcribed in response to a hemi-biotrophic pathogen *Xanthomonas campestris* pv. *musacearum.* This addressed the set objective to study transcription of the two banana genes in *Xanthomonas*-infected banana. Data from this aspect of the study showed that the MeJA-inducible *MNPR1A* gene and the SA/MeJA-inducible *MNPR1B* gene were sequentially transcribed following treatment of banana plants with the hemi-biotroph *X. campestris* (Endah *et al*., 2010). This new finding is also supported by previous reports where investigations with heterologous plant systems revealed that hemi-biotrophs elicit both a SA- and JA/ET-dependent pathway (Oliver and Ipcho, 2004; Glazebrook, 2005; Spoel *et al*., 2007) and where associated increases in JA and SA in response to pathogen attack have been measured (Delannoy *et al*., 2005).

A further contribution was that *Arabidopsis npr1-2* mutants were successfully complemented via genetic transformation with both banana *NPR1* coding sequences under the control of the 35S

CaMV promoter. These transformed plants were used for the functional characterization of the two coding sequences with particular emphasis on their involvement in controlling distinct defense response pathways (SA or JA/ET-mediated defenses). Data obtained from the study revealed that both banana *NPR1*-like homologues complemented the *NPR1* function in the mutants, restoring the pathogen resistance phenotype of the *npr1 Arabidopsis* mutants to a degree comparable to the wild-type *Arabidopsis* phenotype as reported for other *NPR1*-like homologues (Kinkema *et al*., 2000; Shi *et al*., 2010).

However, both *MNPR1* coding sequences under the control of the CaMV 35S promoter did not show a differential response to the biotrophic pathogen *Hyaloperonospera arabidopsidis* and the necrotrophic pathogen *Botrytis cinerea* although both coding sequences were transcribed in the presence of these pathogens. This observation suggested that differential expression of the genes, if it exists in banana, is possibly regulated by transcriptional elements that interact with the promoter region, or with the promoter and coding sequence. Analysis of regulatory *cis* elements within the two *MNPR1* coding (MNPR1A and B) regions unveiled the existence of multiple JA, SA, ET and ABA regulatory *cis*-elements within their structure. A comparison of the MNPR1 amino acid coding sequences with those of other NPR1-like amino acid coding sequences provided additional information on the level of amino acid sequence similarity shared by these MNPR1 proteins especially in relation to conserved regions which have been well characterized in *Arabidopsis*. In the future, a follow-up mutational study using dissimilar amino acid sequence(s) from these conserved regions might provide additional information on their contribution towards defense. Further, since plant-pathogen responses are influenced by the cellular environment as well as other genes that are specific to a plant species, it is also possible

that the plant-pathogen system (*Arabidopsis* instead of banana) might have influenced the expression of these two coding sequences in *Arabidopsis*. Further studies to be carried out with banana might provide more elaborate insight into a possible differential function of the two *MNPR1* coding sequences. Such studies could entail transient expression using the agro-infiltration technique for the delivery of these *MNPR1* cDNAs or the mutated versions of these cDNA into banana plants. Transient expression systems are indeed becoming a method of choice for a more rapid and effective characterization of genes and this has been successfully applied in the characterization of grape vine *NPR1*-like genes (Le Hananff *et al*., 2009; Leicke and Stewart, 2011). Future investigations might also involve creating *MNPR1*-GUS or *MNPR1*-GFP translational fusions under the control of a 35S promoter sequence for easy visualization and sub-cellular localization of *MNPR1*. Furthermore, since the 35S promoter is also inducible by SA (Qin *et al*., 1994), this might also have contributed in this present study to trigger the transcription of the MeJA-inducible *MNPR1A* gene following infection with the biotrophic pathogen *H. arabidopsidis* which induces a SA mediated response. A transient expression study in banana using the banana *NPR1*-like genes, or the mutated versions of these genes under the control of their native promoter, might therefore provide a more robust system to study *MNPR1* expression. In such a transient expression system, all the interacting banana *cis* elements that are required to influence, or be influenced by these *MNPR1* genes would also be available allowing a complete interaction of the native elements with native transcription factors.

Finally, this study has also shown that functional GSH biosynthesis in the cytosol is required for *NPR1*-dependent *PR-1* transcription in *Arabidopsis*. This addressed the objective of shedding light on the role of GSH in *NPR1* transcription and *PR1* expression. GSH and especially the

GSH/GSSG ratio, is known to be a key indicator of the cellular redox state. This redox state controls NPR1 oligomer/monomer activity (Mou *et al*., 2003; Pavet *et al*., 2005; Solomon *et al*., 2010), which is critical for the expression of *PR* genes further downstream. *Arabidopsis* mutants deficient in cytosolic glutathione served as an ideal tool for characterization of this system. NPR1 oligomers are located in the cytosol during ambient conditions and are activated and translocated to the nucleus for the induction of downstream defense responses only during stress conditions (Kinkema *et al*., Mou *et al*., 2003; 2000; Speol *et al*., 2009). New evidence from this study shows that a dysfunctional GSH biosynthetic pathway in the cytosol, as demonstrated by the cytosolic deficient GSH mutant (*clt1clt2clt3*) *Arabidopsis* plants, compromises the plant's ability to adequately transcribe both *NPR1* and downstream *PR-1* genes. Both are important components of SAR for efficient limitation of pathogen colonization. Results obtained with wild-type *Arabidopsis* plants further confirmed previous reports of GSH-dependent *NPR1* transcription for limitation of pathogen growth. This highlights the importance of a functional GSH biosynthetic pathway for the efficient activation of *PR-1* (Gomez *et al.*, 2004; Maughan *et al*., 2010) and possibly its direct role in the process.

Overall, this study has shown that the two *NPR1*-like coding sequences from banana (*MNPR1A* and *MNPR1B*), could compliment the mutant phenotype of the *npr1-2 Arabidopsis* mutants restoring resistance to levels similar to those of wild-type *Arabidopsis* plants. However, there was no differential response patterns to the classes of pathogens investigated thereby implying that the coding regions of the *MNPR1A* and *MNPR1B* genes might not be sufficient or necessary in controlling the differential response of these genes to distinct classes of pathogens. Also, the

plant-pathogen system might also play an important role in the further evaluation of the genes to various pathogens.

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