Isolation and characterization of two

NPR1 genes in banana

By

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DECLARATION

I the undersigned hereby declare that the thesis submitted herewith, for the degree Magister Scientia, to the University of Pretoria, contains my own independent work. This work has hitherto not been submitted for any degree anywhere else.

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March 2008

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ABSTRACT

The protein encoded by the *Nonexpressor of pathogenesis-related* gene 1 (*NPR1*) also referred to as *Non inducible immunitiy 1* (*NIM1*) or *Salicylic acid insensitive 1* (*SAI1*) is a co-transcriptional regulator. The *NPR1* gene plays a pivotal role in conferring broad spectrum resistant to plants. Elicitors like salicylic acid and pathogens coupled with the accumulation of reactive oxygen species influence its activation in the cytoplasm. The downstream activity of the *NPR1* gene necessitates its translocation to the nucleus and an interaction with other transcription factors with a 'TGAC' core sequence for the induction of *Pathogenesis-related* genes. In this study I identified and isolated homologues of genes encoding the NPR1 protein in banana (*Musa* sp.). Southern blot analysis revealed the possible existence of more than one *Musa NPR1* gene in four different banana cultivars. Furthermore, two banana *NPR1* gene fragments designated (*MNPR1A* and *MNPR1B*) were isolated and their full lengths recovered and deposited in the gene bank. A preliminary phylogenetic analysis of *Musa NPR1* revealed that the two newly isolated banana genes grouped closely with other monocot *NPR1* genes. The two identified *MNPR1*-sequences differed greatly in their expression profile using quantitative real time polymerase chain reaction following either salicylic acid or methyl jasmonate treatment or treatment with *Fusarium oxysporum* Schlecht f. sp. *cubense* (Smith) Snyd. *MNPR1A* was expressed after *Fusarium* treatment with higher and earlier expression in the *Fusarium*-tolerant banana cultivar GCTCV-218 than in the *Fusarium*-susceptible banana cultivar Grand Naine. In comparison, *MNPR1B* was highly responsive to salicylic acid, but not to methyl jasmonate treatment, in both the tolerant banana

cultivar GCTCV-218 and the susceptible banana cultivar Grand Naine. Expression of the *MNPR1* genes correlated to *Pathogenesis-related* gene expression known to be involved in fungal resistance. It was found as a new result in this study that reduced sensitivity to *Fusarium* in GCTCV-218 might be partially attributed to the higher and an earlier expression of both *MNPR1A* and *Pathogenesis-related-1* genes in this cultivar after *Fusarium* treatment.

Chapter 1 of this thesis explains briefly the various types of stresses in plants and more elaborately biotic stress responses as well as the early events that occur in the plant during pathogen recognition. Further the characteristics and role of transcription factors as vital components in defense gene activation are outlined. The last part of this chapter provides information about the *nonexpressor of pathogenesis-related* genes 1 (*NPR1*). The aim and objectives of the study is then out lined at the end of the introduction. **Chapter 2** embodies the materials and methods used in this study. The use of various molecular biology techniques such as DNA isolation, gel electrophoresis, Southern hybridisation, PCR, cloning, genome walking, 3', 5' RACE, RNA extraction, cDNA synthesis, and quantitative real-time PCR are described. In **Chapter 3** the results obtained for the identification, isolation and further characterisation of expression of two novel *NPR1* genes in banana are presented. In **Chapter 4** the results obtained are discussed and the new results are highlighted contributing to an advanced understanding of *NPR1* expression due to elicitor and plant pathogen treatment. In the **Annex** plasmid maps and recipes of solutions used during the study are given and finally, a list of literature used and cited in this thesis is provided under **References**.

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ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µL	Microlitre
bp	Base pair
cDNA	Complimentary DNA
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
g	Grams
h	hours
kb	Kilo base pair
L	Litre
LB	Luria broth
M	Molar
min	minute
mL	Millilitres
mM	Millimolar
NaAc	Sodium acetate

NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
^{o}C	Degree Celcius
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotations per minutes
RT	Reverse transcriptase
s	Second
sd H_2O	Sterile distilled water
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate EDTA
Tris	2-amino-2-(hydromethyl)propane-1.3 diol
UV	Ultraviolet
ATP	Adenosine triphosphate
H^+	Hydrogen ion
ATPase	Adenosine triphosphatase
NADPH	Nicotinamide dinucleotide phosphate hydrogen
ET	Ethylene
HCl	Hydrochloric acid

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Amplification products obtained after agarose gel electrophoresis from cDNA obtained from leaves of wounded Grand Naine plants and pUBINH13100 plasmid DNA (positive control) using primers designed from the conserved ankyrin repeat region of the rice *NPR1* gene. Internal arrows point to bands that were cloned, sequenced and analysed for the existence of putative *NPR1* information.

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Full-length coding sequence of *MNPR1A* and *MNPR1B*. The full-length of *MNPR1A* and *MNPR1B* coding region was amplified from both genomic and cDNA obtained from leaves of Grand Naine plants and by applying a combination of genome walking or 5' 3' RACE techniques. The nucleotide sequences obtained from *MNPR1A* and *MNPR1B* after sequencing was translated into protein sequences in BioEdit.

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Multiple alignment of *MNPR1A* and *MNPR1B* with selected plant NPR1-like amino acid sequences. Amino acid sequences were aligned by Clustal W multiple alignment software (Thompson *et al*., 1994). Identical amino acids are represented with dots. Vertical rectangles represent conserved cysteine residues and horizontal rectangles represent BTB/POZ domain (filled) conserved ankyrin repeat domain (not-filled) in both MNPR1A and MNPR1B. Accession numbers used in the alignments are *Capsicum annum* (ABG38308.1), *Lycopersicum esculentum* (AAT57637.1), *Nicotiana tabacum* (ABH04326.1), *Oryza sativa* (NP_001042286.1), *Hordeum vulgare* (CAJ19095.1), *Arabidopsis thaliana* (AAM65726.1).

Figure 3.9 78

Multiple alignment and evolutionary relationship of the ankyrin repeats within the *NPR1* gene of various plants. Ankyrin repeat regions of the *NPR1* gene from *Musa acuminata* (119352645, 115291364), *Arabidopsis thaliana* (30696995), *Brassica juncea* (55441973), *Lycopersicum esculentum* (49182273), *Ipomea batatas* (121722583), *Zea mays* (DN218769), *Oryza sativa* (60308937) 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, maximum parsimony (MEGA4) and maximum likelihood (Phyml2.4.4) 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. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

respective elicitors. The experiment was repeated once; the relative expression was determined by quantitative RT-PCR and expressed relative to a 'calibrator', the expression level at 0 h. The relative expression ratios were plotted on the graph. Results are means ± standard error of the mean (SEM) of six plants. *Significant difference at $P<0.05$ between the two banana cultivars.

Figure 3.12   **88**

 Relative gene expression of *MNPR1A* and *PR-1* (dark bars) and *MNPR1B* and *PR-3* (white bars) in *Foc*-infected roots of Cavendish banana cultivar Grand Naine (susceptible) and GCTCV-218 (tolerant). Samples were collected for analyses at 0, 12, 24 and 48 h after treatment. Relative gene expression of *MNPR1A* and *MNPR1B* (A and B) and *PR-1* and *PR-3* (C and D) was determined and compared in each of the cultivars. The experiment was repeated once; the relative expression was determined by quantitative RT-PCR and expressed relative to a 'calibrator', the expression level at 0 h. The relative expression ratios obtained from the only wounded control plants at each time point was subtracted from those of the infected and wounded samples to obtain the effect due to infection only. The expression ratios due to infection were plotted on the graph. Results are means ± standard error of the mean (SEM) of six plants. *Significant difference at $P<0.05$ between *MNPR1A*/*MNPR1B* and *PR-1*/*PR-3*.

CHAPTER ONE

Introduction

1.1   <u>Plant stress</u>

Plants are sessile organisms and, unlike animals, they lack the ability to escape from harm. Plants are exposed to numerous environmental cues that have important effects on plant performance. Any unfavourable condition, which affects a plant's metabolism, growth and development, is considered to be a stress factor (Lichtenthaler, 1998). Stress factors are broadly categorised as either biotic or abiotic (Fig. 1.1). Biotic factors include bacterial and fungal pathogens, viruses, weeds and insect pests (Horan *et al*., 1988; Hahlbrock *et al*., 2003; Apel and Hirt, 2004; Agrios, 2005; Stange, 2006). Abiotic stress entails all stress conditions superimposed on the plant by non-living parameters (Agrios, 2005). Divergence from an optimal condition results in stress symptoms and, depending on severity, growth impairment. Moreover, abiotic stress conditions predispose the plant to biotic stress factors by weakening the plant's defense and facilitating the penetration and establishment of diseases (Agrios, 2005). Further, environmental stress factors do not act in isolation and in most cases they interact to hamper the plant's development (Agrios, 2005). The ability of an environmental factor to negatively affect plant growth and development further leads to the development of stress symptoms. The severity of stress symptoms depends on the dosage of the stress, length of exposure as well as the plant in question (Lichtenthaler, 1998; Agrios, 2005). However, plants have evolved intricate metabolic networks to combat environmental stress factors and to maintain their growth and development. Therefore, understanding processes involved in plant stress response, especially biotic stress as well as how plants counteract these stress factors to resist or tolerate these stresses will greatly contribute to plant productivity.
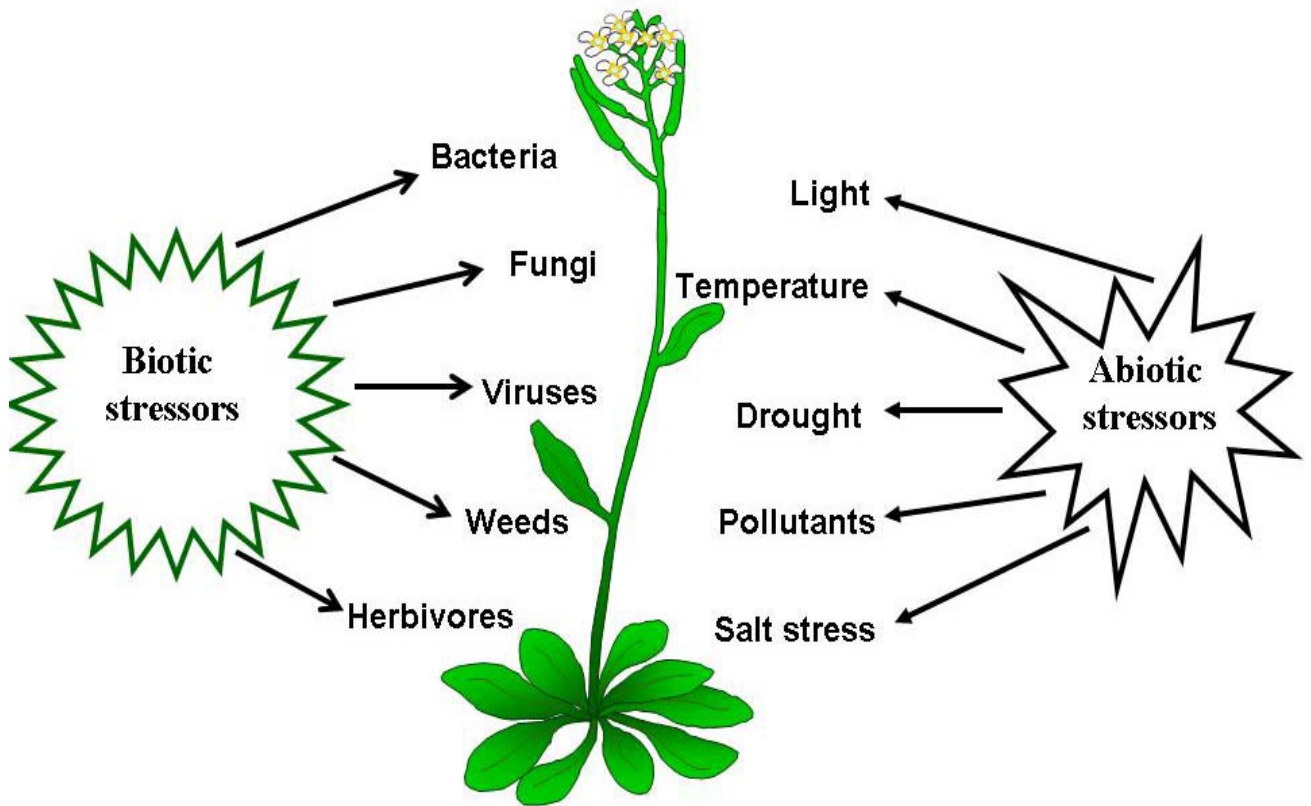
Figure 1.1 Schematic representation of plant biotic and abiotic stressors (adapted from Tippmann *et al*., 2006).

1.2 **Plant responses to biotic stress**

1.2.1 Types of plant responses

1.2.1.1 *Hypersensitive response*

One of the earliest types of responses to biotic stress in plants is the hypersensitive response (HR). The plant's HR is a localised defense response around the site of infection most often leading to localised programmed cell death (PCD) (Goodman and Novacky, 1994; Heath, 2000). Furthermore, the HR limits the spread of the pathogen to the site of infection through a gene-for-gene resistance mechanism (Heath, 2000; Belkhadir *et al*., 2004). During this process, *avirulence* (*avr*) gene products from the invading pathogens are recognised by the *resistant* (*R*) gene products produced by the plant (Belkhadir *et al*., 2004). If the plant fails to recognise the elicitor produced by the pathogen, the plant is said to be susceptible and the pathogen is able to spread and infect other parts of the plant (Heath, 2000). The pathogen is then considered to be virulent (Agrios, 2005).

It is widely accepted that, irrespective of the type of pathogen invading the plant, the hypersensitive response is usually the first form of defense response initiated by the plant (Ross, 1961; Hahlbrock *et al*., 1995). There is also evidence that, the hypersensitive response and systemic acquired response could be tightly linked, sharing certain components that, lead to the activation of other downstream events and ultimately disease resistance (Grüner *et al*., 2003). Many studies aimed at understanding early events that

occur during HR suggest that membrane depolarisation is a key and early indicator of the HR (Pike *et al.*, 2005; Liu *et al.*, 2007). Depolarisation of the plant's plasma membrane facilitates ionic fluxes within the cell. This leads to a general increase in the influx of extracellular calcium (Ca^{2+}) and hydrogen (H^+) ions (Hammond-Kosack and Jones, 1996; Heath, 2000; Hahlbrock *et al.*, 2003) and an efflux of potassium and chlorine ions (Pike *et al.*, 1998). Further, during the HR there is a general disturbance in the H^+-ATPase activity resulting in a decrease in ATP steady-state levels. This could either be due to a decrease in ATP generation or an increase in ATP consumption (Liu *et al.*, 2007). The resultant change in the cell's electrochemical potential regulates downstream signalling events such as the production of active oxygen species (AOS), protein phosphorylation and gene expression (Schaller and Oecking, 1999; Liu *et al.*, 2007; Maffei *et al.*, 2007; Stange *et al.*, 2008; Fig. 1.2).

1.2.1.2   *Systemic response*

A second form of defense response initiated by plants during pathogenic invasion is the systemic response comprising of: Induced Systemic Resistance (ISR); and Systemic Acquired Resistance (SAR). Studies on ISR are currently being intensively investigated in an attempt to understand how microorganisms signal defense responses in plants. A growing body of evidence points towards the fact that, ISR is activated by plant growth promoting *Rhizobacteria* (PGPR) via a pathway whose downstream signalling events differ in some respects from the SAR pathway (Pieterse *et al.*, 1998; Pieterse and Van Loon, 1999; Hossain *et al.*, 2007). In *Arabidopsis*, the PGPR strain *Pseudomonas*

fluorescens WCS417r was able to elicit an ISR response which, further protected the plants against *P. syringae* pv. *tomato* (Pieterse *et al.*, 1996). ISR and *pathogenesis-related* protein (*PR*) gene expression in *Arabidopsis* is also induced by the biocontrol bacterium *Paenibacillus alvei* conferring resistance to *Verticillium dahliae* (Tjamos *et al.*, 2005). It has been hypothesized that, unlike SAR, the mobile signal molecule during ISR is not SA (Ryu *et al.*, 2003; Hossain *et al.*, 2007). This was demonstrated by Ryu *et al.* (2003) using NahG [transgenic line degrading salicylic acid (SA)] *Arabidopsis* plants. After priming the plants with strains of either *Serratia marcescens* 90-166, *P. fluorescens* 89B61 and *Bacillus pimilus* T4 followed by infection with the pathogens *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato*, the plants showed resistance to the pathogens. This therefore suggested that, SA synthesis was not required for the defense process. Using various *Arabidopsis* mutants, it has further been shown that, ISR and SAR could however share certain components such as the *NPR1* gene which is also a key regulator of SAR (Van Wees *et al.*, 2000; Tjamos *et al.*, 2005, Hossain *et al.*, 2007).

SAR in plants has received increasing attention since it was first demonstrated by Ross (1961) with tobacco mosaic virus. It is a broad spectrum long lasting immune response against pathogens. Initiation of SAR in plants by one pathogen can last for several days and, unlike the hypersensitive response, which is commonly localised around the site of infection (Heath, 2000), SAR spreads to cells that are removed from the site of infection (Nürnberger *et al.*, 2004). This is comparable to the immunization process in animals and even if triggered by a single pathogen, it tends to protect the plant against a wide variety of pathogens (Nürnberger *et al.*, 2004; Lee and Hwang, 2005).

1.2.2   Downstream signalling events during plant biotic responses

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are metabolic products synthesized in cells at low concentrations during normal cellular metabolism (Apel and Hirt, 2004; Kotchoni and Gachomo, 2006; Desikan *et al*., 2005). Upon pathogen infection, their concentration increases, accumulating beyond the rate of its removal by antioxidants like ascorbate and glutathione (Noctor and Foyer, 1998; Foyer and Noctor 2005; Kotchoni and Gachomo, 2006). Failure to effectively scavenge ROS and maintain a favourable threshold under stress conditions results in a rapid oxidative damage of lipids and proteins, cell death and the appearance of necrotic lesions (Foyer *et al*., 2005; Kotchoni and Gachomo, 2006).

During plant pathogen defense responses, ROS production is controlled via an increased enzymatic activity of the plasma membrane-bound nicotinamide adenine dinucleotide phosphate hydrogen-oxidase (NADPH-oxidase) system, cell wall-bound peroxidases and amide oxidases (Hahlbrock *et al*, 1995; Apel and Hirt, 2004; Leshem *et al*., 2007; Fig. 1.2). NADPH-oxidase is able to produce ROS by reacting with oxygen to generate superoxide or cell wall-localized peroxidase which can ultimately generate hydrogen peroxide (Bolwell, 1999). Hence, NADPH oxidases are believed to play important roles during biotic and abiotic stress responses as well as during plant development (Torres *et al*., 2005). However, despite the possible detrimental effect of ROS to cells, ROS generation is one of the earliest detectable responses during pathogen invasion (Hahlbrock *et al*, 2003; Davies *et al*., 2006; Liu *et al*., 2007). This has been supported by

experiments, which further show that the removal of ROS during pathogen infection does favour the penetration of the pathogens into host cells (Mellersh *et al*., 2002).

In cells undergoing a HR, the generation of an increased concentration of ROS depends on the plant-pathogen system (Hammond-Kosack and Jones, 1996). For instance, when pepper leaves were inoculated with the avirulent pathogen *Xanthomonas campestris* pv *vesicatoria*, this resulted in the induction of a local and a systemic oxidative burst followed by cell death (Choi *et al*., 2007). In contrast, in wheat seedlings no oxidative burst or AOS scavenging was induced after infection by the Hessian fly larvae (Giovanini *et al*., 2006) indicating that HR defense responses exist that are independent of ROS accumulation (Hahlbrock *et al*., 2003; Glazebrook, 2005).

There is substantial evidence that plant pathogenic responses correlate with increased levels of secondary signalling molecules, such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA), leading to the up-regulation of defense response genes (Cao *et al*., 1994; Van Loon *et al*., 2006; Yuan *et al*., 2007). It is widely accepted that SA is essential and sufficient to induce SAR (Loake and Grant, 2007). For example, it has been demonstrated that in the absence of pathogen infection exogenously applied SA or synthetic compounds, such as 2,6, dichloroisonicotinic acid (INA) and benzol-1,2,3-thiadiazole-7-carbothionic acid s-methyl ester, can induce SAR (Cao *et al*., 1994; Lawton *et al*., 1995; Ryals *et al*., 1996; Anand *et al.,* 2007). Moreover, JA, SA and ET could interact either antagonistically, synergistically or independently in a concentration- and cultivar-dependent manner to mediate the induction of various *PR* genes like *PR-1B*, *PR-*

3, *PR-4* and *PR-12* (Pennicnckx *et al*., 1998; Thomma *et al*., 1998; Van Wees *et al*., 2000; Fan and Dong, 2002; Traw *et al*., 2003; Mur *et al*., 2006; Mei *et al*., 2006).

Intensive studies have been carried out to clearly delineate which of these signalling molecules are major players when plants are infected by different types of pathogens. Results from these studies have shown that a SA defense response pathway is elicited when plants are attacked by biotrophic pathogens, such as *P. syringae*, while necrotrophic pathogens such as *Alternaria brassicicola*, insects and herbivores elicit a JA-signalling pathway (McDowell and Dangl, 2000; Thomma *et al*., 2001; Glazebrook, 2005; Spoel *et al*., 2007). These results have been well supported by experiments using mutants that are either suppressed in their ability to accumulate one of these elicitors or mutants that have an enhanced ability to accumulate the elicitors. Typical examples of such mutants include the non immunity 1 (*nim1*-lacks response to SA) suppressor of SA insensitivity (*ssi*), SA-insensitive 1 (*sai1*), constitutive expressor of PR proteins (*cep1*-overexpressing SA), SA-induction deficient 2 (*sid2*), fatty acid desaturase (*fad3-2 fad7-2 fad8* - JA deficient), JA-insensitive 1 (*jar1*), coronatine insensitive 1 (*coi1*), enhanced disease susceptibility 16 (*eds 16*), ET-insensitive 2 (*ein 2*), non-race specific disease resistance (*ndr*) and phytolexin deficient (*pad*) mutants (Dong, 1998; Devadas *et al*., 2002; Shah, 2003; Ryu *et al*., 2003; Cipollini *et al*., 2004).
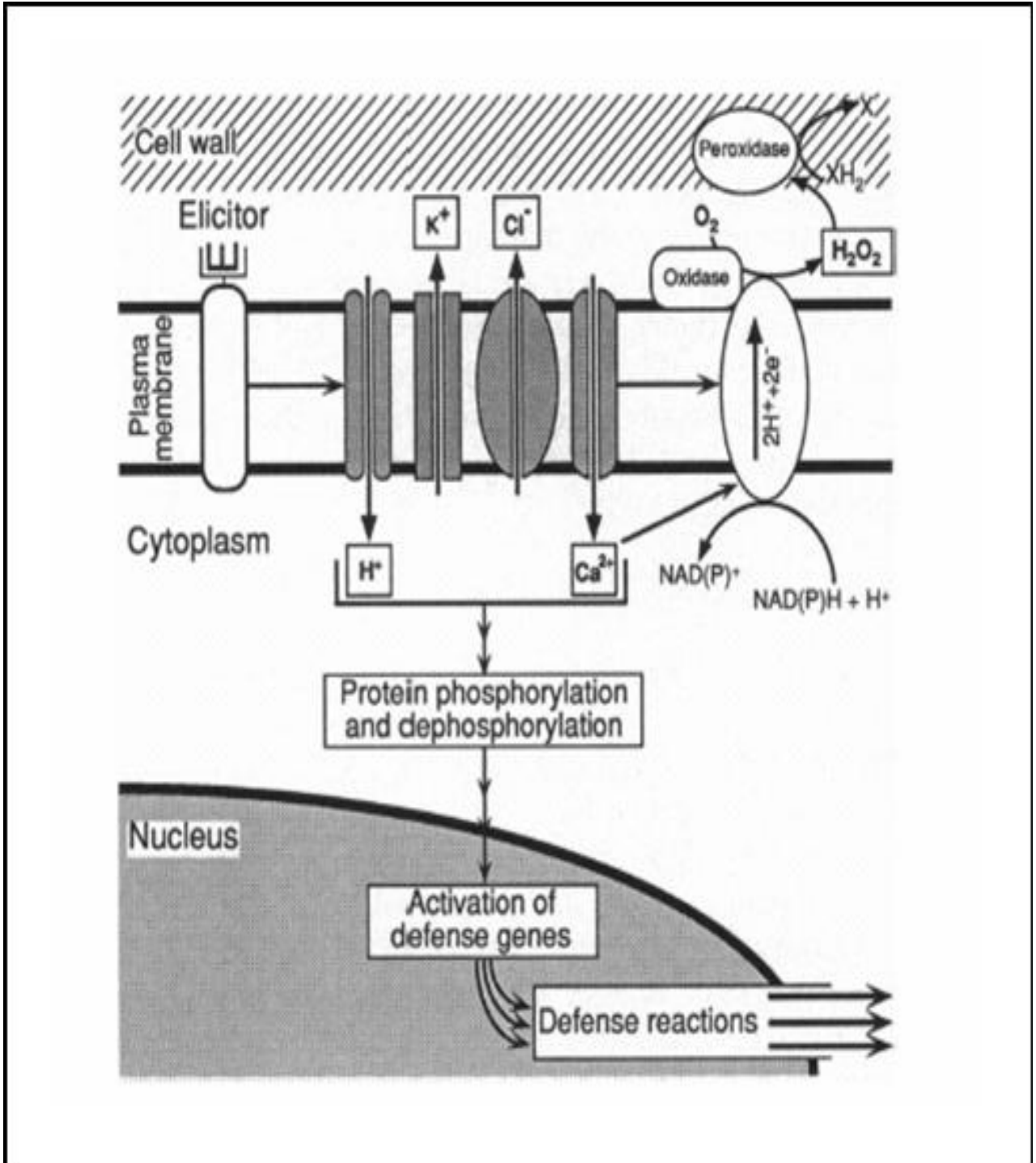
Figure 1.2 Simplified schematic illustration of the signalling cascade from elicitor recognition to defense gene activation (adapted from Hahlbrock *et al*., 1995).

1.3 **<u>Transcription and defense response</u>**

1.3.1 <u>Definition</u>

Transcription is the first step towards the selection of genes for expression, with these expressed genes controlling growth and developmental processes (Anderson and Beardall, 1991). From the time of the establishment of the central dogma describing the process from DNA transcription to RNA translation and protein synthesis, there has been a profound interest in all the steps involved in gene transcription and its regulation since these processes play important roles in overall gene expression hence, defense responses.

1.3.2 <u>Transcriptional regulation</u>

Selective pressure on plants over the course of evolution enabled them to develop special units known as transcription factors for the regulation of gene expression. Hence, the transcriptional regulatory process involves a remarkable intricate biochemical process that is in itself tightly regulated at many levels (Gross and Oelgeschläger, 2006). The transcriptional process is initiated by RNA polymerase II together with components situated around the core promoter region (Juven-Gershon *et al*., 2006). Elements found within the core promoter are refered to as general transcription factors (Lago *et al*., 2004; Juven-Gershon *et al*., 2006; Tamada *et al*., 2007). These elements include among others: the TATA box; initiator; transcription factor of RNA polymerase II; TATA box binding protein-associated factors; downstream core element and the downstream core promoter

element (Lago *et al*., 2004; Juven-Gershon *et al*., 2006; Tamada *et al*., 2007). These general transcription factors act as major players in determining the specific function of the core promoter activity. In that, they control transcription initiation by ensuring correct binding of RNA polymerase (Branden and Tooze, 1999; Watson and Murphy, 1999; Liu *et al*, 1999; Zhu *et al*., 1995; Juven-Gershon *et al*., 2006). Specific transcription factors (trans-acting elements) act upstream of the initiation site through their interaction with *cis*-acting elements (Branden and Tooze, 1999; Liu *et al*., 1999; Watson and Murphy, 1999). Some inducible transcription factors act as co-activators/co-repressors by first binding to proteins of the basal transcription apparatus to activate (enhance) or suppress the transcription process while others are environmentally sensitive requiring a signal before participating in the transcription process (Branden and Tooze, 1999, Watson and Murphy, 1999). Inducible transcription factors are directly involved in controlling the expression of genes in response to various biotic and abiotic stimuli. However, just like general transcription factors, they have specific DNA and protein domains, which help in the recognition and binding to elements along the promoter regions of genes (Branden and Tooze, 1999; Watson and Murphy, 1999).

Many transcriptional factors have been identified, isolated and characterized in plants. For instance, an analysis of the genome sequence of *Arabidopsis* using similarity searches and domain matches led to the identification of about 1709 proteins with significant similarity to know transcription factors (The *Arabidopsis* Genome Initiative, 2000). Furthermore, out of 29 classes of *Arabidopsis* transcription factors that were identified, 16 appeared to be unique to plants and most of them have been implicated in

plant defense responses (The *Arabidopsis* Genome Initiative, 2000). The majority of genes encoding transcriptional proteins are grouped into families according to specific sequences found within their protein or nucleotide chain. These sequences are highly conserved among members in the family and recognize specific elements or boxes within the promoters of genes (Table 1) (Braden and Tooze, 1999; Eulgem, 2005). The binding ability of transcription factors enables them to form homo- or hetero-dimmers with other elements further enhancing their regulatory activity. A well-studied example is the gene encoding transcriptional regulators belonging to the WRKY protein family (Eulgem *et al*., 2000, 2006, Eulgem and Somssich, 2007). These proteins harbour conserved WRKYGQK domains at their N-terminal which helps them to recognize and bind to W-boxes ((T)GACC/T) found in promoter regions of many plant defense response genes like the *Non-expressor of pathogenesis-related gene 1* (*NPR1*) (Eulgem *et al*., 2000 and 2006; Yu *et al*., 2001). WRKY transcription factors are also enriched in W-boxes hence, their ability to form homo-dimers (Eulgem *et al*., 2000).

Table 1.1 Representation of certain transcription factors involved in regulating defense-associated genes in plants (Eulgem, 2005).

Transcription factor type	Size of *Arabidopsis* family	Key feature	Consensus core motif of binding site	Comments
ERF	56	One ERF-DNA binding domain	GCCGCC (GCC-box)	Subfamily of APS transcription factors and Repressors
R2R3 Myb	125	Two repeats of Myb domain R2 and R3)	Type I: (T/C)AAC(T/G)G Type II: (G/T)T(A/T)G(G/T)T	Predominating subfamily of Myb factors in plants
TGA bZIP	10	One basic DNA binding domain; leucine zipper protein dimerization motif	TGACGTCA (TGA box): this motif usually occurs as direct repeats (e.g in as-1-like Elements	Subfamily of bZIPs: activators and repressors: from homo- or hetero-dimers
NPR1	6	Ankyrin repeat domain; BTB-POZ domain	No DNA binding site	Interact with TGA-bZIPs
Whirly	3	Whirly domain	GTCAAAAA/T	Forms homo-tetramers; binds to single stranded DNA
WRKY	74	One or two DNA WRKY domains	(T)GACCT/T (W-box)	Activators and repressors

1.3.3   Features of genes encoding transcription factors

Mature protein units have the tendency to fold and assume different shapes. The primary structure is the most basic and reflects the amino acid (AA) sequence of the polypeptide chain (Branden and Tooze, 1999). Different regions of this chain can fold to form alpha helices or beta sheets known as the secondary structure (Branden and Tooze, 1999). A

simple arrangement of these secondary structures defines a motif and when these motifs combine into compact globular structures they are referred to as domains (Branden and Tooze, 1999). Domains form the fundamental units of the tertiary structure (Branden and Tooze, 1999). Experiments in bacteria by means of x-ray crystallography have clearly demonstrated that, within DNA-binding domains, there exist highly conserved structural motifs that play important roles in determining the function and structure of protein units (Branden and Tooze, 1999).

Motifs/domains are being intensively studied in plants and most transcription factors studied harbour one or many of the following motifs/domains: a basic helix-loop-helix; a basic leucine zipper; a zinc finger, an ankyrin repeats; a Bric-a-brac POZ/virus domain (BTB); a nucleotide binding signal, a leucine rich repeat; a basic leucine zipper (bZIP) and a nuclear localization domain (Liu *et al*., 1999; Jakoby *et al*., 2002; Heim *et al*., 2003). Sequence analyses of plant proteins have demonstrated that the number of motifs/domains could vary significantly within members in the same family as well as among members of different families. For example, WRKY family of transcription regulators have a single zinc finger motif (Eulgem, 2000), while others like the gene encoding the lesion simulating disease resistance 1 (LSD 1) protein harbours three zinc finger motifs (Dangl *et al*., 1996). Similarly, most MYB-related proteins have two Myb domains (Liu *et al*., 1999). However, within a given family of transcription factors the number of binding domains could differ and this is most often used to delineate members of such families. For example, in the WRKY-family of plant transcription factors those belonging to group 1 (WRKY1 and WRKY2) have two WRKY domains located at their

C and N terminals, while those of Group II (WRKY3 and WRKY4) and group III (WRKY5) generally have a single WRKY domain located at the C-terminal (Eulgem *et al.*, 2000). The type of domain within a particular protein specifies the transcriptional regulatory activity of the individual transcription factors.

1.4   Nonexpressor of pathogenesis-related gene as transcriptional co-regulator

1.4.1   Characteristics of NPR1 proteins

The gene encoding the Nonexpressor of pathogenesis-related protein 1 *(*NPR1), also referred to as non-inducible immunity 1 (NIM1) and salicylic acid-insensitive (SAI1), (Dong, 2004) is a cytoplasmic localized transcription co-regulatory protein which was first isolated in *Arabidopsis* mutants. These *Arabidopsis* mutants were not responsive to SA treatment and were consequently highly compromised in their ability to express PR proteins and further confer resistance to *P*. *syringae* infection (Cao *et al.*, 1994). Structurally the NPR1 protein is approximately 66 KDa (Cao *et al.*, 1997) and has a high similarity to the mammalian I-$_K$B protein (Ryals *et al.*, 1997). In mammals, the I-$_K$B protein interacts with the transcription factor NF-$_K$B inhibiting its translocation to the nucleus (Baldwin, 1996). Further, structural analysis and genome searches in *Arabidopsis* depict that the NPR1 protein is characterized by a BTB/POZ (Bric-a-Brack Poxvirus and zinc finger) domain at its N-terminal, which is attached to ankyrin repeats in the middle of the protein (Cao *et al.*, 1997; Becerra *et al.*, 2004; Rochon *et al.*, 2006). Gain-of-function studies demonstrated that ankyrin repeats and BTB/POZ domains are involved

in protein-protein interactions (Bardwell and Treisman 1994; Cao *et al*., 1997), but NPR1 is unable to bind DNA and thereby controlling gene expression. Furthermore, reporter gene fusion studies, immunoprecipitation studies and studies using NPR1-GFP labelled proteins have contributed towards determining the exact sub-cellular localization of the NPR1 protein (Kinkema *et al*., 2000; Mou *et al*., 2003). Data from these studies clearly show that inactive NPR1 cluster exist as oligomers in the cytosol. Upon activation by the perception of ROS and signal molecules like SA, which causes redox-changes in the cytosol, the protein dissociates into monomeric forms and translocates to the nucleus (Kinkema *et al*., 2000; Mou *et al*., 2003).

In a study conducted by Mou *et al*. (2003), 10 conserved cysteine residues within the *NPR1* sequence have been found. These residues were present at position C82 and C216 of the sequence. These residues play a critical role in *NPR1* oligomerization. Mutations of these residues led to constitutive monomerization of the protein followed by its translocation to the nucleus. Mapping of the *NPR1* gene by crossing *npr1-1/npr1-1* mutant to wild type *NPR1/NPR1* plants has aided in obtaining information on the genome localization of the gene (Cao *et al*., 1997). These analyses revealed that the *NPR1* gene is located on chromosome 1 (Cao *et al*., 1997). To date; six paralogs of the gene have been identified in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000; Liu *et al*., 2005). These have been designated *NPR1*, *NPR2*, *NPR3*, *NPR4*, *blade-on-petiole 1* (*BOP1*) and *blade-on-petiole 2* (*BOP2*) with accession numbers At1g64280 At4g26120, At5g45110, At4g19660, At3g57130 and At2g41370, respectively (Hepworth *et al*., 2005; Liu *et al*., 2005). These five *NPR1*-like genes in *Arabidopsis* control a wide array of metabolic

events in the plant. *NPR1*, *NPR2*, *NPR3* and *NPR4* all play a role during defense responses (Cao *et al*., 1997; Liu *et al*., 2005; Zhang *et al*., 2006). *BOP1* and *BOP2* are involved in plant morphogenesis or the modulation of meristermatic activity (Ha *et al*., 2004; Hepworth *et al*., 2005). The *NPR1* gene has also been isolated and well characterised from *Nicotiana tabacum* (Liu *et al*., 2002; Weigel *et al*., 2005) and *Malus x domestica* (apple) (Malnoy *et al*., 2007).

However, apart from rice, there is limited information about *NPR1* isolation, expression and function in monocots. An *NPR1-like* gene has been isolated in *Hordeum vulgare* (barley; Acession number: CAJ19095.1) and deposited in Genbank. In rice, five *NPR1* homologues (*OsNPR1 homolog 1 - NH1* - DQ450948; *OsNPR2 - NH2* - DQ450950; *OsNPR3* - DQ450952; *OsNPR4 - DQ450954 and; OsNPR5 - DQ450956*) have been identified through computer searches (Yuan *et al*., 2007). Of these five rice *NPR1* genes, *OsNPR1*, *OsNPR2* and *OsNPR3* have been isolated but only *OsNPR1* has been well characterised (Chern *et al*., 2001 and 2005; Yuan *et al*., 2007). Similar to the *Arabidopsis NPR1*, the rice *NH1* harbours conserved cysteine residues at position C76 and C216, which are essential for monomerization. Further, *OsNPR1* is induced not only after treatment with the rice pathogens *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe grisea*, but also by benzothiadiazole, methyl jasmonate (MeJA) and ET (Yuan *et al.*, 2007). However, transgenic *Oryza sativa* plants, expressing an *Arabidopsis NPR1* gene, displayed a lesion mimicking cell death (Fitzgerald *et al*., 2004). They were more sensitive to light resulting in a dwarf phenotype (Chern *et al*., 2005). This suggests that

the native rice *NPR1* gene is more functional in rice compared to the *Arabidopsis NPR1* gene.

1.4.2 Interaction of the NPR1 protein with other cell components

1.4.2.1 Role of SA, JA/ET in *NPR1* gene activation

Its is currently widely accepted that, the NPR1 protein plays a pivotal role in many systemic defense responses conferring increased levels of resistance to a range of economically useful pathogens and herbivores (Cao *et al.*, 1994; Johansson *et al.*, 2006; Rayapuram and Baldwin, 2007; Yuan *et al.*, 2007). There is also evidence that SA is the key signal molecule required for *NPR1* gene activation (Rochon *et al.*, 2006; Loake *et al.*, 2007). However, recent findings have unveiled the existence of a JA/ET-*NPR1* dependent pathway (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000; Beroccal-Lobo and Molina, 2004) where ET acts downstream of JA and JA and ET also induce certain subsets of *PR* genes, such as *PR-3*, *PR-4*, *PR-12* (*PDF1.2*) (Penninckx *et al.*, 1998), which all have antifungal activities (Van Loon *et al.*, 2006). In *Arabidopsis,* resistance against *Fusarium oxysporum* (Berrocal-Lobo and Molina, 2004) and against *Verticillium longisporum* (Johansson *et al.*, 2006) are dependent on JA, ET in addition to SA and cytosolic *NPR1* which is further connected to expression of various defense genes such as *PR-1*, *PR-2* and *PR-4* (Johansson *et al.*, 2006).

1.4.2.2   TGA transcription factors and *NPR1*

The TGA/OBF basic leucine zipper (bZIP) family of transcription factors interact with *NPR1* to mediate defense responses (Zhou *et al*., 2000). The TGA family of transcription factors belong to a small multi-gene family with about ten members in *Arabidopsis* (Ryals *et al*., 1997; Dong, 2001). TGA factors are able to recognize and bind to activation sequence-1 (*as-1*) elements located at the promoter regions of many stress-responsive genes (Zhang *et al*., 1999; Johnson *et al*., 2003). The ability of TGA factors to recognise and bind to *cis*-acting elements that are located at the promoter site of defense response genes is solely dependent on the binding of fully functional monomeric *NPR1* to TGA in the nucleus (Després *et al*., 2000; Rochon *et al*., 2006). This mode of action for TGA has been clearly demonstrated with TGA2 whose interaction with a negative regulator of *PR* gene expression (*SN1*) suppresses the expression of *PR* genes (Kesarwani *et al*., 2007). Further, in *O. sativa* the native TGA2.1 has a negative role in the basal defense response to bacterial pathogens (Fitzgerald *et al*., 2004). The activity of TGA protein is also affected by changes in the redox-state of the cell. Electro mobility shift assays (EMSA) conducted with reduced and oxidized NPR1 and TGA proteins have shown that TGA proteins treated with dithiothreitol (DTT) resulted in stronger binding of these proteins to activation sequence 1 (AS-1) probes and that this binding is enhanced by reduced NPR1 proteins (Després *et al*., 2003; Pieterse and Van Loon, 2004). Further, the interaction of TGA2 with NPR1 has been found to require the core of the NPR1 BTB/POZ domain (residues 80 – 91) as well as the oxidation of cys-521 and cys-529 residues (Rochon *et al*., 2006).

1.4.2.3   WRKY transcription factors and *NPR1*

Despite supporting data for the interaction between *NPR1* and *TGA* transcription factors, TGA binding domains are under-represented in promoters of defense response genes (Chen *et al*., 2002). TGA core binding domains represent only about 63% of the genome while WRKY transcription factors represent 80%. When the plants were exposed to cold stress, 58% of the genes expressed were represented TGA transcription factors while 73% of the genes were WRKY transcription factors (Chen *et al*., 2002; Maleck *et al*., 2000). TGA factors are therefore not the only transcription factors acting downstream of *NPR1* for *PR* gene activation. W-boxes contain the core sequence motif (T)(T)TGAC(C)/T) and are recognized by a family of plant transcription factors referred to as WRKY (Eulgem *et al*., 2000). There are approximately 74-100 members in *Arabidopsis* harbouring one or two conserved WRKY domains made of 60 amino acids (Eulgem *et al*., 2000). This novel family of transcription regulators mediate both up-stream and down-stream activities of *NPR1* (Wang *et al*., 2006; Yu *et al*., 2001). For instance, WRKY 18 plays a positive regulatory role in SAR as a target transcription factor for *NPR1*-dependent gene expression. Other members in this family, such as WRKY70 and WRKY58, are believed to act as possible regulators to prevent excessive accumulation of SA (Wang *et al*., 2006). Computer analysis of the promoter site of the *Arabidopsis NPR1* gene has also revealed the existence of three W-boxes located within the *NPR1* promoter (Yu *et al*., 2001). All three W-boxes have been shown to be responsive to SA-induced WRKY DNA binding proteins and being essential for basal expression of the *NPR1* gene as well as for *PR-1* gene expression (Yu *et al*., 2001).

1.4.2.4   *NPR1* and *PR* gene induction

PR genes also referred to as SAR genes belong to a multigene family that are actively involved in defense responses (Van Loon *et al*., 2006). Most PR proteins studied are constitutively expressed in plants under normal developmental conditions (Vidhyasekaran, 1997). However, under various kinds of biotic and abiotic stress conditions, their concentrations increase. Since they exhibit antimicrobial activity, they can further provide protection against attack from fungi, bacteria and viruses (Vidhyasekaran, 1997; Van Loon *et al*., 2006). A huge number of PR proteins have been isolated and characterised in plants and about 17 families of PR proteins exist (Table 2; Van Loon *et al*., 2006).

Table 1.2 Families of pathogenesis-related proteins (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'	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	Ribonuclease-like
PR-11	Tobacco "class V" chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
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

Various signal molecules like SA, JA and ET can further induce the expression of a wide variety of PR proteins. The expression of some PR proteins is regulated by the *NPR1* gene through its interaction with TGA or WRKY transcription factors (Vidhyasekaran, 1997; Zhou *et al*., 2000; Loake and Grant, 2001; Kesarwani, 2007). In *Arabidopsis*,

resistance to *F. oxysporum* (Berrocal-Lobo and Molina, 2004) and to *V. longisporum* are dependent on JA, ET, SA and cytosolic NPR1 (Berrocal-Lobo and Molina, 2004, Johansson *et al.*, 2006) and these molecules induce the expression of *PR-1*, *PR-2* and *PR-4* genes. In *Malus x domestica* (Apple) a *NPR1* gene (*MpNPR1*) was isolated and characterised (Malnoy *et al.*, 2007). Over-expression of this *NPR1* gene induced the expression of *PR-2*, *PR-5* and *PR-8*. In *O. sativa*, the *NPR1* homologue *NHI* up-regulates the *PR-1* gene after benzothiadiazole (SA analogue), MeJA, *X. oryzae* pv. *oryzae* or *M. grisea* treatment or treatment with MeJA or ET (Yuan *et al.*, 2007).

1.4.3   <u>Mode of action of *NPR1* during defense responses</u>

For many years after the isolation of the first *NPR1* gene in *Arabidopsis*, the mode of action of the gene in mediating defense responses was unknown due to the inability of the protein to directly bind to DNA (Dong, 2004). It was speculated that the *NPR1* gene, due to the presence of its protein-protein interaction domains, could possible act as a transcription regulator (Zhou *et al.*, 2000). Due to the discovery of downstream transcription factors with a TGA core sequence (Zhou *et al.*, 2000), a signalling pathway mediated by the *NPR1* gene was proposed (Fig. 1.3).
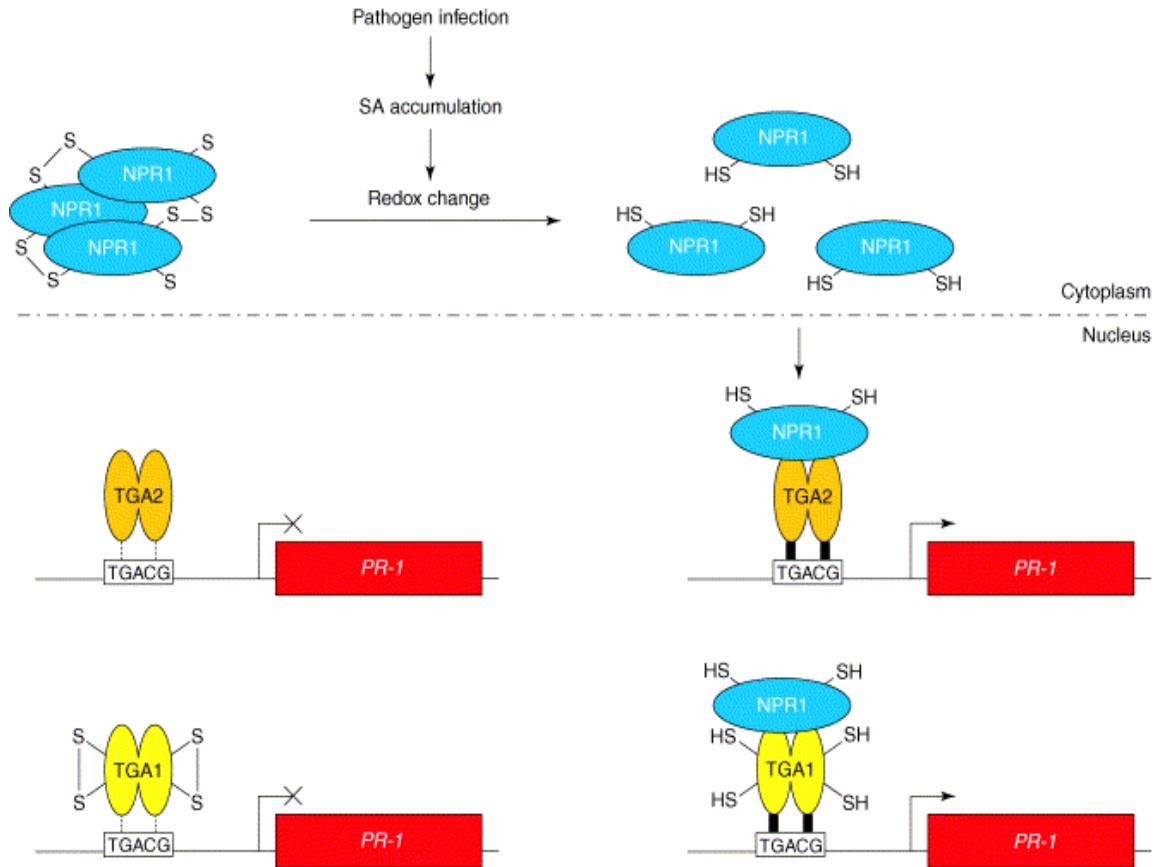
Figure 1.3 Simplified mode of action for *NPR1* defense gene activation (Pieterse and Van Loon, 2004). In a non-induced state, NPR1 proteins cluster in the cytoplasm as oligomers, which are held by disulphide bonds. Upon pathogen invasion, SA and ROS accumulate in the cytoplasm leading to a redox disturbance. This further tends to provoke the dissociation of disulphide bridges within the NPR1 proteins into monomers, which translocates to the nucleus. Monomeric NPR1 proteins bind to TGA transcription factors present in the nucleus resulting in a NPR1-TGA complex (blue-orange and blue-yellow circles on the right hand side). This complex recognizes and binds to TGACG sequences found in the promoter regions of *PR-1* genes leading to the activation of the *PR* genes and hence defense responses. However, in the absence of monomeric NPR1 proteins in the

nucleus, TGA transcription factors are unable to induce *PR* genes and switch on a defense response mechanism (Orange and yellow circles on the left hand side).

In resting cells, *NPR1* is expressed constitutively at very low concentrations (Dong, 2004). Most of the NPR1 proteins are inactive and tend to cluster as oligomers in the cytosol (Kinkema *et al.*, 2000; Mou *et al.*, 2003). Upon activation by pathogens and accumulation of signal molecules (SA, JA and ET) and ROS, the redox-state of the cytosol changes to a more reduced form (Noctor *et al.*, 2002). In the cytosol, disulphide bridges hold NPR1 polymers together and it is well known that an oxidative environment is very essential in keeping disulphide bridges intact (Braden and Tooze, 1999). However, when the cytosolic environment becomes reduced, this leads to the dissociation of the disulphide bridges holding the NPR1 molecules together hence resulting in the formation of monomeric NPR1 (Mou *et al.*, 2003). Further, a change in the cytosolic GSH/GSSG pool also contributes to the cytosolic dissociation of NPR1 polymers (Mou *et al.*, 2003). The exact mechanism how GSH causes reduction is unclear. However, studies carried out by Mou *et al.* (2003) demonstrate that during SA or pathogen induced defense responses there is a change in GSH/GSSG ratio mainly due to the GSH concentration and this is sufficient to reduce NPR1 proteins. Monomeric NPR1 then translocates to the nucleus with this translocation being facilitated by the presence of nucleus localisation signals present in the gene (Kinkema *et al.*, 2000; Mou *et al.*, 2003). In the nucleus, monomeric NPR1 recognizes and binds to transcriptional factors with a core 'TGAC' sequence for the induction of various types of *PR* genes (Zhou *et al.*, 2001).

1.5   **Research aim and objectives**

This MSc project has focused on the detection and expression of the *NPR-like* gene in banana. Currently, the cultivation of banana is hampered by numerous abiotic and biotic stress factors resulting in low productivity and reduced crop performance in many banana varieties. For instance, in the early 1950s, banana production was threatened to extinction by *Fusarium oxysporum* Schlecht f. sp. *cubense* (Smith) Snyd (*Foc*) the causal agent of *Fusarium wilt* (Ploetz and Pegg, 2000). This led to the introduction of more resistant Cavendish varieties. However, evolution of the pathogen has caused some of the Cavendish banana cultivars to become also over time more sensitive to *Foc* causing wilting in plants (Stover and Buddenhagen, 1986). For example, Grand Naine, which was one of the resistant Cavendish banana cultivars that replaced Gros Michel is susceptible to *Mycosphaerella fijiensis* Morelet. This is the causal agent of Black sigatoka (Harelimana *et al.*, 1997; Noupadji and Tomepke, 1999). This cultivar is also susceptible to the burrowing nematode which causes root rot (Stover and Simmonds, 1966). These plants are further susceptible to *Foc*, which causes wilting in plants (Stover and Buddenhagen, 1986). Strategies that have been used to limit the spread and establishment of diseases in most banana plantations include replacement of the susceptible banana varieties with more fungal-resistant varieties, the use of agrochemicals, crop rotation or soil amendment practices and planting of disease-free tissue culture banana plants (Hwang and Ko, 2004; Heslop-Harrison and Schwarzacher, 2007). Tissue culture and somaclonal variation has been successfully applied to obtain a novel banana cultivar designated GCTCV-218 which, unlike the parent line, presently exhibits enhanced

tolerance to race 4 of *F. oxysporum* f. sp *cubense* as well as better horticultural characteristics such as high yield and high fruit quality (Hwang and Ko, 2004). However, a detailed understanding of disease resistance mechanisms and the function of genes involved in this mechanism might ultimately also aid in selection of resistance traits. Genes such as *NPR1*, could be valuable in the improvement of resistance via genetic engineering approaches.

Since the *NPR1* gene has been isolated from different plant species, it was hypothesized at the onset of the project that a *NPR1* gene also exists in banana which might be expressed due to elicitor treatment or pathogen infection. Although the *NPR1* gene has been found in dicot plants and also rice, there is still a need to isolate and characterize the *NPR1* gene from a greater variety of plant species (Fitzgerald *et al*., 2004).

The <u>aim</u> of this project was therefore to isolate and characterize a possible banana *NPR1* gene. To achieve this goal the following three objectives were set:

(1)   To identify via Southern blotting the existence of an *NPR1* gene in banana.

(2)   To use a combination of PCR, genome walking technique and 5',3' RACE technique to subsequently isolate the full length coding region of the identified banana *NPR1* gene.

(3)   To use two banana cultivars (Grand Naine and GCTCV) with different sensitivity to pathogens allowing to quantitatively measure *NPR1* and *PR* gene transcript

levels in banana after treating the plants with either the pathogen *Fusarium oxysporum* f. sp *cubense* or the elicitors salicylic acid and jasmonic acid.

CHAPTER TWO

Materials and methods

2.1 Identification of banana *NPR1*-like genes

2.1.1 DNA isolation

For identification of *NPR1*, templates for PCR amplification were prepared from plasmid and plant genomic DNA. For plasmid DNA isolation, *Escherichia coli* (*E. coli*) cells containing the plasmid pUBINHI3100 (University of California Davis, USA) which harboured the rice *NPR* homologue 1 (*NH1*) were obtained from a glycerol stock stored at -70°C and sub-cultured 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) supplemented with 50 mgL^{-1} ampicillin. Plates were incubated at 37°C overnight and resultant single *E. coli* colonies were cultured in 15 mL Falcon tubes containing 3 mL liquid LB medium (1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), and 1% sodium chloride (w/v), pH 7.5) supplemented with 50 mgL^{-1} ampicillin. The Falcon tubes were incubated at 37°C overnight with constant agitation at 200 rpm on a shaker. Plasmid DNA was then isolated from the overnight liquid culture using the GenElute^{TM} Plasmid Miniprep kit (Sigma-Aldrich, USA) following the manufacturer's instructions.

Plant DNA was extracted from leaves of four available Cavendish banana cultivars (Bluggoe, GCTCV-218, FHIA 25, and Tani) using a modified cetyltrimethylammonium bromide (CTAB) method as described by Gawel and Jarret (1991). DNA was also isolated from leaves of *Arabidopsis thaliana* and *Zea mays* (maize) to serve as genomic

DNA positive controls because they harbour known *NPR1* genes within their genome. For DNA isolation from these plants, 1.5 g of freshly harvested leaf material from each plant was individually ground into a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted in 10 mL of a pre-heated extraction buffer containing: 2% CTAB (w/v), 100 mM Tris-HCl (pH 8); 1.4 mM NaCl; 20 mM EDTA and freshly added 0.1% β mercaptoethanol (v/v). The crude plant extract was thoroughly mixed and then transferred to a 50 mL polypropylene tube (Lasec, South Africa) and incubated for a further 30 min at 65°C.

After incubation, 8 mL of chloroform:isoamyl alcohol (24:1) (Sigma Aldrich, USA) was added to each sample and samples were placed on ice for 15 min. Samples were then centrifuged for 8 min at room temperature at 10,000 rpm and the upper aqueous phase transferred to a clean 50 mL Falcon tube. DNA was recovered after the addition of an equal amount of isopropanol (Sigma Aldrich) and centrifugation at 14,000 rpm for 10 min at 4°C. The pellet was washed in 70% ethanol and spun for 10 min at 14,000 rpm. Pellets obtained were air dried and dissolved in 0.1 mL sterile distilled water (sdH$_2$O) containing 10 mgmL^{-1} of RNase A for the elimination of ribonucleic acid (RNA).

2.1.2   Quantification of DNA

Quantification of DNA in each of the samples was performed with 2 µL of DNA using a NanoDrop® (Fermentas, Canada) and following the manufacturer's instructions. The quality of DNA in each sample was verified visually by agarose gel electrophoresis on a

1% agarose gel. For agarose gel electrophoresis, 0.5 g of low electroendosmosis (LE) agar (Promega, South Africa) was dissolved in 50 mL of 1 X TAE buffer prepared from a 50 X TAE stock solution containing 2 M Tris base (w/v), 5.71% glacial acetic (v/v) acid and 50 mM EDTA. Complete dissolution of the agar was achieved by briefly heating the mixture in a microwave for 1 min at $180^{o}C$. The agar solution was cooled under running tap water and 1 µL ethidium bromide (Sigma Aldrich, USA) was added. The solution was poured in a gel tray containing a comb and allowed to solidify at room temperature. The gel and tray were in a next step placed in a gel tank containing sufficient 1 X TAE buffer (to cover the gel) and the comb removed.

Samples to be loaded were prepared using 50 ng of DNA, 2 µL of a 6 X orange loading dye (for colour visualization and monitoring of the reaction during electrophoresis) and the volume was adjusted to 20 µL with sdH_2O. Samples were loaded in the gel wells and electrophoresis carried out at 100 Volts (V) for 20 min. After electrophoresis, the DNA quality was analysed visually on the gel using an ultraviolet (UV) transilluminator.

2.1.3   Southern blotting

Southern blotting was carried out according to a modified method of Southern (1975) and Maniatis and Sambrook (1982) and using the Random-Prime Labelling kit (Amersham, UK).

2.1.3.1   *DNA digestion and electrophoresis*

Genomic DNA (50 µg) from Bluggoe, GCTCV-218, FHIA 25, Tani, *Arabidopsis* and maize was digested overnight at $37^{o}C$ with restriction enzymes *Eco*RI and *Hind*III (Fermentas, Canada) in 0.1 mL reaction volumes using a suitable buffer recommended by the supplier. The pUBINHIC1300 plasmid DNA was also digested for 1 h using the above mentioned enzymes. Digested DNA was run on a 1% agarose gel at 50 V for 5 h until the coloured dye was 3 mm from the base of the gel.

2.1.3.2   *Treatment of gel containing DNA*

The agarose gel containing the separated DNA fragments was depurinated by immersing the gel in a tray containing 0.125 N HCl solutions. The tray was placed in a bench top shaker and gently agitated for 10 min. After depurination, the HCl solution was discarded and the DNA on the gel was denatured for 15 min in a denaturation buffer (0.5 M NaOH and 1.5 M NaCl) with gentle agitation. This denaturation procedure was repeated twice and the gel was rinsed with sdH$_2$O between each step. After the third denaturation step, the gel was neutralized for 15 min in a neutralization solution (1.5 M NaCl and 0.5 M Trizma® base pH 7.5; Sigma Aldrich) with gentle agitation. The neutralization step was repeated twice.

2.1.3.2 _Transfer of DNA fragments to membrane and pre-hybridization_

For transfer of the nucleic acid from the gel onto a membrane, 10% SSC solution (1.5 M NaCl and 0.15 M sodium citrate-2H_2O) was used as the transfer solution. The treated gel was placed on a wick platform containing a sufficient volume of the SSC solution, in which the lower part of three blotting papers had been submerged. A Hybond™-N+ membrane (Amersham, UK) was placed on top of the gel followed by three sheets of blotting paper and a stack of absorbent paper towels. These were shaped to have the same length and width as the gel and were piled to attain a height of approximately 5 cm. A glass plate and weight (about 750 g) were then placed on top of the paper towels to support the entire system and the DNA transferred over night into the membrane.

After complete transfer, the membrane was marked to indicate the side with the marker for identification purposes. The DNA side of the membrane was UV cross-linked on a UV-transilluminator for 60 s and then placed in a hybridisation tube (with the section containing the DNA facing up). 25 mL of a pre-warmed (60^oC) hybridisation buffer (5 X SSC, 0.1% SDS w/v (Sodium dodecyl sulphate), 5% dextran, 1/20th the volume of a liquid block solution (Amersham) was carefully added and pre-hybridization of the membrane was carried out for 2 h at 60^oC.

2.1.3.4 _Preparation of NPR1 probe_

A _NPRI_ probe (570 bp) was PCR amplified using the plasmid pUBINHIC1300 as a DNA template with primers NH-F1 5'-GAGCTTTTGGATCTCGCACTTGCAGA-3' (forward primer) and NH-R1 5'- CCGAGCTCCACTGTTTTGGAGAGTGCT-3' (reverse primer). The PCR parameters had an initial denaturation phase of 5 min at 95$^{\mathrm{o}}$C, an amplification phase of 30 cycles each consisting of a denaturation step at 95$^{\mathrm{o}}$C for 30 s, annealing at 60$^{\mathrm{o}}$C for 30 s and DNA extension at 72$^{\mathrm{o}}$C for 1 min. This was followed by a final extension step of 7 min at 72$^{\mathrm{o}}$C. The PCR reaction was then separated by agarose gel electrophoresis and the 570 bp PCR product was gel-purified using a gel purification kit (Merck, Gernamy) following the manufacturer's instructions. The concentration of the purified product was determined using the Nanodrop® procedure and DNA quality checked visually via electrophoresis.

2.1.3.5 _Labelling of the NPR1 probe and hybridisation to membrane_

Prior to labelling of the DNA probe, 100 ng of DNA was denatured by incubating at 98$^{\mathrm{o}}$C for 5 min. The reaction was quickly cooled for 5 min on ice. Labelling of the denatured DNA was carried out using the non-radioactive Random-Prime Labelling procedure (Amersham) following the manufacturer's instructions. This was done in a 50 µL reaction volume consisting of 50 ng of the denatured _NPR1_ probe, 5 U Klenow enzyme, dNTP nucleotide mix and DNA primers supplied by the manufacturer (Amersham). The reaction was incubated at 37$^{\mathrm{o}}$C for 1 h and 10 µL of the labelled probe was subsequently

added to the hybridisation buffer in the tube and incubated over night at 60°C. Un-hybridized probe was removed by washing sequentially with 250 mL of three different pre-heated wash solutions at 60°C for 15 min under gentle agitation. The solutions were made up of 2 X SSC and 0.1 % SDS (w/v); 1 x SSC and 0.1 % SDS (w/v); and 0.5 X SSC and 0.1% SDS (w/v), respectively.

2.1.3.6   *Detection of hybridisation signals on membrane*

The hybridisation reaction was stopped using a blocking reagent made up of Buffer A (100 mM Tris HCl, 300 mM NaCl pH 9.5), 0.5 % BSA, 1/10 dilution of a liquid block solution and 1/5000 dilution of anti-fluorescein alkaline phosphate (AP) conjugate (Amersham). This was done by placing the membrane in the solution and agitating at room temperature for 1 h. Unbound conjugates were removed by washing three times at room temperature for 10 min per wash with a solution consisting of 0.3 % Tween 20 in Buffer A (v/v) while shaking gently. The membrane was then placed on a Saran wrap and 4 µL of CDP-Star Detection reagent was added to the membrane and incubated for 5 min at room temperature. After the incubation step, the membrane was then transferred to a new Saran wrap and sealed. The saran wrap was placed in a film cassette (DNA-side facing up) and taken to a dark room. In the dark room, a Hyperfilm-MP of the same dimensions as the sealed membrane was place on top of the membrane. One side of the film was marker (for identification purposes) and the cassette was then closed and the film was exposed for three days in the dark. Development of the film was carried out by submerging the film in a basin containing a sufficient volume of Sognefjord film

developer M (Sognerfjord, SA) and gently agitated for 30 min until exposure spots were visible on the film. Once spots were apparent, the film was transferred and rinsed in a basin containing water to remove the developer. The film was in a last step submerged into a third basin containing a fixation solution and fixed for about 2 min until the film turned transparent. The transparent film was then allowed to dry before analysing.

2.2 Isolation of banana *NPR1*-like gene sequences

2.2.1 Primer design

For the isolation of the *NPR1* gene sequence from banana, PCR primers (forward primer 5'-GAGCTTTTGGATCTCGCACTTGCAGA-3'; reverse primer 5'-CCGAGCTCCACT GTTTTGGAGAGTGCT-3') were designed using Primer 3 software based on sequence information available for the rice *NPR1* gene (GenBank accession no. AY92398).

2.2.2 RNA Extraction and cDNA Preparation

Banana genomic DNA and double-stranded cDNA synthesized from Cavendish banana (Grand Naine) leaves was used as a PCR template. Genomic DNA was isolated as described previously using the CTAB method. For cDNA preparation, total RNA was extracted from banana leaf material following a modified RNA CTAB extraction procedure for Banana RNA extraction by Asif *et al.* (2002) using 1 g of Cavendish banana leaves. The leaves sample was ground into a very fine powder in a mortar using a

pestle and the powder was then dissolved in 10 mL of a pre-heated RNA CTAB extraction buffer [2 % CTAB, 100 mM Tris-HCl (pH 8); 1.4 mM NaCl; 20 mM EDTA (disodium ethylenediaminetetracetate-$2H_2O$)] to which 0.1% mercaptoethanol (v/v) had been added. Samples were transferred to 50 mL polypropylene tubes and incubated for 1 h at $65^{o}C$.

After incubation, an equal volume (10 mL) of chloroform:isoamyl alcohol (24:1) was added to the sample and centrifuged for 15 min at $4^{o}C$ and 12,000 rpm using the Avanti^{TM}J-25I high performance centrifuge, using Rotor JA 25.50 (Beckman Instruments, USA). The upper aqueous phase was then transferred to a clean ice cold tube and the supernatant re-extracted twice as specified above. The final supernatant collected (9 mL) was stored at $-20^{o}C$ overnight after the addition of 2.7 mL of a 10 M lithium chloride (LiCl) solution to obtain a final concentration of 3 M for LiCl. The samples were then centrifuged at $4^{o}C$ for 20 min at 12.000 rpm using the Avanti^{TM}J-25I high performance centrifige, using Rotor JA 25.50 (Beckman Instruments) and the supernatant was then carefully discarded without disturbing the pellets. The pellets were in a next step resuspended in 0.6 mL of sdH_2O the solution was then transferred to a 2 mL Eppendorf tube and the RNA re-extracted sequentially in three centrifugation steps as follows: in the first step, an equal volume (0.6 mL) of RNAse free water-saturated phenol was added to the dissolved pellet in an Eppendorf tube. This was mixed thoroughly and centrifuged at $4^{o}C$ and 12.000 rpm for 10 min. All the above centrifugation steps were done in an Eppendorf bench top centrifuge (Merck, Germany) The upper phase of the centrifuged solution was then carefully transferred to a new Eppendorf tube and an equal

volume of phenol:chloroform (1:1) was added to it. The centrifugation process was repeated as specified above and the upper phase was again transferred to a new Eppendorf tube. To the new supernatant, an equal volume of chloroform was added. This was again centrifuged and the upper phase transferred to a new Eppendorf tube.

To the last supernatant collected, four times the amount of absolute ethanol and 1/3 the volume of 3 M sodium acetate (NaAc-pH 5.2) was then added. Samples were incubated overnight at -70°C to ensure complete precipitation of the RNA in the solution. The pellets were finally collected after centrifugation at maximum speed (14,000 rpm) for 10 min at 4°C. The pellets were washed in 70% ethanol and again spun for 10 min at 14,000 rpm. After discarding the ethanol, the pellets obtained were air dried for 2 min and the RNA recovered after dissolving the pellets in 0.4 mL DEPC treated water. The RNA quality was verified via gel electrophoresis on a 1% denaturing gel and its concentration determined by the Nanodrop® procedure. After confirmation of the RNA's quality and quantity, double-stranded cDNA was synthesized using the cDNA synthesis kit (Sigma Aldrich) following the manufacturer's instructions.

2.2.3 Polymerase chain reaction (PCR)

For amplification of a putative *Musa NPR1*-like fragment in the banana DNA and cDNA templates via PCR, a primer annealing temperature of 55°C was used in a standard PCR reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.25 mM each dNTPs, 5 U of Taq polymerase (Celtic, Germany), 50 ng of either banana genomic DNA or banana

cDNA and 0.02 µM of primer NH-F1 and NH-R1. The PCR cycles were 2 min at 94°C followed by 35 cycles each consisting of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. Final extension occurred at 72°C for 10 min. PCR products were run on a 1% agarose gel at 100 V for 30 min. Bands of the expected size (570 bp) were excised from the gel and DNA recovered using a gel band PCR clean-up kit (Roche Diagnostics, UK) following the manufacturer's instructions.

2.2.4   Cloning of PCR products

Purified DNA fragments were individually cloned using the TOPO TA cloning® cloning kit (Invitrogen, UK). For this, 30 ng of DNA fragments were ligated into 10 ng of PCR®4-TOPO® vector in the presence of 1 µL salt solution (supplied by the manufacturer) for 10 min at room temperature. The ligation mixture (6 µL) was then used to transform DH5ά™-T1^R competent cells (50 µL). This was carried out by incubating the ligate-competent cell reaction for 30 min on ice followed by heat shock at 42°C for 45 s. Samples were quickly cooled on ice for 2 min and 0.2 mL of SOC medium (20 % tryptone, 0.5 % yeast extract, 10 mM NaCL, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose, 10 mM $MgCl_2$) added to it. For maximum growth of cells, samples were incubated while agitating at 200 rpm for 1 h at 37°C after which 0.1 mL of the transformation reaction was spread on LB plates supplemented with 50 mg L^{-1} ampicillin. The plates were then incubated at 37°C overnight. Putative positive transformants (five) in the form of white *E. coli* colonies on the plate were individually cultured in 5 mL liquid broth supplemented with 50 mg L^{-1} ampicillin by agitating overnight at 37°C.

Plasmid DNA was isolated from the liquid cultures using a plasmid DNA isolation kit (Fermentas).

2.2.5 Screening of positive transformants

The presence of the insert was verified by restriction enzyme digestion using 50-100 ng of DNA from each sample. Digestion was carried out for 1 hr at 37°C using 10 U EcoR1 (Fermentas) and a suitable buffer recommended by the supplier following the manufacturer's instructions. The presence of the expected insert size (570 bp) in the digested samples was assessed via agarose gel electrophoresis. The DNA from samples with the expected insert size were further analysed after sequencing for the presence of genes encoding NPR1-like proteins or NPR1-like protein domains.

2.2.6 Amplification of the full length $Musa$ $NPR1$ gene

A combination of both 5' and 3' Rapid Amplification of cDNA Ends (RACE) and genome walking were applied to isolate full-length cDNA clones of $NPR1$ banana sequences. cDNA template for the 5' and 3' RACE reaction was synthesized using reagents that were supplied with the GeneRacer^{TM} kit (Invitrogen, USA) and total RNA extracted from Grand Naine banana leaves using the CTAB RNA extraction method. For synthesis of the RACE template, 5 µg of RNA was dephosphorylated in a 5 µL reaction volume consisting of 1 µL of 10x Calf intestinal phosphatase (CIP) buffer, 10 U of CIP and 40 U of RNaseOut™. The reaction was incubated at 50°C for 60 min, followed by an

incubation step on ice for 5 min. The dephosphorylated RNA was then precipitated by firstly, adjusted the reaction volume to 90 µL and then, adding an equal volume of phenol:chloroform (100 µL). The reaction in the tube was mix vigorously for 30 s and centrifuged at 14,000 rpm for 5 min at room temperature. After centrifugation, the aqueous phase was transferred into a new 1.5 mL eppendorf tube. This was followed by the addition of 200 µg of mussel glycogen, 1/10 the volume of 3 M NaAc (pH 5.2) and two times the volume of absolute EtOH. The reaction in the tube was then briefly vortex and incubated at -80°C for 10 min. The RNA pellet was collected after centrifugation at 4°C for 20 min and 14,000 rpm. The RNA pellet was then washed with 0.5 mL of 70% EtOH and the tube was again centrifuged as mentioned above. The EtOH was carefully removed and the pellet was dried and resuspended in 7 µl of DEPC water.

In a next step, the dephosphorylated RNA was decapped in a 10 µL reaction volume consisting of 1 µL of 10x Tobacco acid pyrophospatase (TAP) buffer, 40 U of RNaseOut, 0.5 U of TAP and 5 µg of dephosphorylated RNA. The reaction was incubated at 37°C for 60 min, followed by incubation for 5 min on ice and then precipitated as described above. The decapped RNA was resuspended in 7 µL DEPC water and used for the ligation reaction with the 5' GeneRacer^{TM} and 5'GeneRacer^{TM} nested primer (5' –

CGACUGGAGCACGAGGACACUGACAUGACAUGGACUGAAGGAGUAGAAA-3') supplied by the manufacturer (Invitrogen, USA). The ligation reaction was carried out by first resuspending the 5' GeneRacer^{TM} primer (0.25 µg) using the decapped RNA mixture. This was followed by an incubation step at 65°C for 5 min and then an

incubation step on ice for 2 min. Thereafter, 10x ligase buffer, one mM ATP, 40 U RNaseOut and 5 U T4 RNA ligase was added to the RNA-primer mix. The volume of the reaction was adjusted to 10 µL with DEPC water and the reaction incubated at 37°C for 60 min. The RNA ligate was in a next step precipitated as described above. After precipitation, the ligated RNA was resuspended in 10 µL DEPC water.

In the final step, the ligated RNA was reverse transcribed as follows: to the ligated RNA, 100 ng of Random hexamer primer and 25 mM dNTP mix was added and incubated at 65 °C for 5 min so as to remove any secondary RNA structure. The reaction was then chilled on ice for 2 min followed by a brief centrifugation for 30 s. This was followed by the addition of 4 µL of 5x Reverse transcriptase buffer, 15 U of Avian Myeloblastosis Virus reverse transcriptase (AMV RT), 40 U of RNaseOut and 2 µL water. The reaction was then mixed and incubated at 25°C for 10 min, 45°C for 60 min and 85°C for 15 min. For synthesizing the 3' cDNA end, the GeneRacer^{TM} 3' primer and nested primer (5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)-3') was used instead of Random hexamer primers in the reverse transcriptase reaction while, decapped RNA was used as the template instead of the ligated RNA.

For isolation of *MNPR1A*, both 5' and 3' cDNA RACE templates were used along with *MNPR1A* gene-specific primers. Two nested gene-specific forward primers: 5'-TGGTGATGACTTGCGGGGAAGATT-3' and 5'-TTGCCATGGACATTGCTCGAGTTG-3'; and two reverse nested primers 5'-AATCTTCCCCGCAAGTCATCACCA-3' and 5'-TGCGG

GTCTCTTCTTTCAGCTTGC-3' were used to amplify the 3'- and 5'-ends, respectively, of the *MNPR1A* gene. Both ends were joined by amplifying with forward primer 5'-CGGCGCGATATGGAAGACAA-3' and reverse primer 5'-GCAGGAGTCAGCAAAA GGAAGC-3' that flank the coding region and a portion of un-translated regions (UTRs) of the *MNPR1A* gene.

Similarly, 3' RACE was performed to isolate the 3' end of *MNPR1B* using two nested gene specific primers 5'-TGATGGCACATCGGAGTTCACC-3' and 5'-GCATCTGGCA CGAATGAGAGCA-3'. The 5' RACE, 5' nested, 3' RACE and 3' nested primers were provided with the GeneRacer™ kit (Invitrogen, USA) that were used together with the gene specific primers. The 5' end of *MNPR1B* was amplified from genomic DNA by genome walking using a series of gene specific and adapter specific primers. The template for the reaction was prepared according to the method described by Siebert *et al.* (1995) and using DNA extracted from Grand Naine leaf material. For preparation of the template, a genomic DNA library was generated using 10 µg of DNA which had been digested overnight with different restriction enzymes (*EcoR*V, *Pvu*II, *Sma*I, *Sca*I and *Stu*I) in a 100 µL reaction volume using buffers that were recommended by the supplier (Fermentas, Life Sciences, Hanover, MD). The digested DNA was in a next step precipitated using the phenol:chloroform method described above and the pellets resuspended in 20 µL of sdH$_2$O. The DNA digests were incubated at 50°C to relax the DNA's structure. The DNA was in a next step ligated to adaptor primers (adaptor1: 5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGG-3', adaptor2: 5'-ACC TGCCC-3') as follows: 150 µM of each adaptor primer was mixed in an Eppendorf tube

and deactivated at 95°C for 5 min; the ligation reaction was carried out in a 30 µL reaction volume consisting of 20 µL of the digested DNA, 150 µM of each adaptor primer, 20 U of T4 DNA ligase and 10x ligation buffer. After overnight ligation at 20°C, the ligase in the reaction was deactivated by incubating at 70°C for 5 min followed by a rapid chilling on ice for 2 min. This was used to generate a library by making a 1:3 dilution of the ligated DNA template. The library was then used as a template for further amplifications using a series of *MNPR1B* gene specific primers. The complete coding region and portions of UTRs of the *MNPR1B* gene were then amplified from a cDNA template using forward 5'-TTGGACGACGGCGGTACACG-3' and reverse 5'-CAGCAT GATCTAGTGGTGTGTCATGG-3' primers. All amplified PCR products were T/A cloned into the PCR®4-TOPO® cloning vector (Invitrogen, USA) and sequenced using M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCT ATGAC-3') primers.

2.2.7   Sequence analysis

Sequencing of the inserts was performed by using the BigDye® Terminator Cycle Sequencing FS Ready Reaction Kit, v 3.1(Perkin Elmer, Applied Biosystems, USA) in an ABI PRISM® 3100 automatic DNA-Sequencer (Applied Biosystems). The BLASTN and BLASTP programs (Altschul *et al.,* 1997) were used for gene sequence similarity searches. Amino acid sequences of selected monocot and dicot *NPR1*-like sequences were aligned using Clustal W (Thompson *et al.*, 1994) and ExPASy (Gasteiger *et al.,*

2003) was utilized for the prediction of amino acid features and identification of conserved domains of *MNPR1A* and *MNPR1B*.

2.2.8   Phylogenetic analysis

To establish a phylogenetic relationship of the *Musa NPR1* sequences with other *NPR1* sequences, the ankyrin domain was used in sequence alignment. *NPR1* sequences from *Oryza sativa* (60308937), *Zea mays* (DN218769), *Musa acuminata* (Monocots) and *Ipomea batatas* (121722583), *Arabidopsis thaliana* (30696995), *Nicotiana tabacum* (111054504), *Lycopersicum esculentum* (49182273), *Capsicum annum* (109693028) *Brassica juncea* (55441973) (Dicots) where retrieved from Genbank and aligned using the Multiple sequence alignment software (MAFFT) (Katoh *et al*., 2002). The sequences were then edited in BioEdit v7.0.0 to obtain the correct reading frame. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 for Neighbour Joining (NJ) and Maximum Parsimony (MP) (Tamura *et al*., 2007) and phylogenetic maximum likelihood version 2.4.4 program was used to obtain the maximum likelihood (ML) (Guindon and Guascuel, 2003). The ML tree was displayed using TreeView (WIN32) version 1.64b (Page, 1996). For each analysis, the bootstrap was set at 1000 replicates.

2.3   *MNPR1* and *PR* genes expression

2.3.1   Growth of banana plants

Expression studies entailed measuring the transcript levels of banana *MNPR1*, *PR-1* and *PR-3* genes in Cavendish banana. For this, two-week old tissue-cultured banana plants (cv Grand Naine and GCTCV-218; Du Roi Laboratory, South Africa) were hydroponically grown in 250 mL cups containing 100 mL of distilled water for three weeks until plants attained a five leaf stage and developed a healthy root system. Plants were kept in a greenhouse under natural light conditions and an 18°C/25 $^{\circ}$C dark/light temperature regime. Plants were fertilized once a week with a nutrient solution [0.6 g/L $Ca(NO_3)_2.H_20$, 0.9 g/L agrasol-0® (Scotts, Scotts-Europe B.V., The Netherlands], 1 mL/L Micromax® stock solution (3 g/L) (Fleuron, Braamfontein 2017, South Africa)

2.3.2   Treatment of banana plants

Fusarium oxysporum-susceptible and tolerant Cavendish banana plants [Grand Naine (Fusarium-sensitive) and GCTCV-218 (Fusarium-tolerant)] were challenged with either an inoculum of *Fusarium oxysporum* f.sp. *cubense*, salicylic acid or methyl jasmonate. Unless stated otherwise, the entire root system was harvested at the beginning of the experiment or at time points 12, 24, and 48 h after starting the treatment, quickly flash frozen in liquid nitrogen and stored at -80°C until needed. Each time point for every

treatment consisted of three plants whose roots were pooled together. The entire experiment was repeated once.

2.3.2.1 *Treatment of banana plants with Fusarium oxysporum f.sp. cubense*

Pathogenic infection of the banana plants was done following the method described by Van den Berg *et al*. (2007). For this, an inoculum was prepared by making use of a cocktail mixture of three highly virulent *Fusurium oxysporum* f. sp c*ubense* (FABI, South Africa) (*Foc*) isolates (CAV 045, 092, 105) belonging to 'subtropical' race 4, vegetative compatibility group (VCG) 0120. These were initially individually cultured for 9 days on 90 cm diameter Petri dishes containing half strength potato dextrose agar (PDA) medium (Biolabs, Merck laboratories, South Africa). Their collective spores were collected by pouring sterile distilled water onto the Petri dishes and loosening the fungal spores with a sterile rod. The spore suspension was filtered through a cheese cloth, combined and the spore concentration adjusted to 2.5×10^5 conidia/mL using a haemocytometer. Each time point ranging from 0 (un-inoculated plants), 12, 24, 48 h post inoculation consisted of three plantlets and one biological replica. The roots of each plant were slightly squeezed and plants were exposed to the inoculum by pipetting 2.5 mL of the inoculum into the cups. Non-infected control samples were treated as mentioned above without addition of a fungal inoculum.

2.3.2.2 *Treatment with salicylic acid and methyl jasmonate*

Treatment with salicylic acid was done following a modified method of Anderson *et al*. (2004). Both the roots and leaves of each plant were sprayed with a 5 mM salicylic acid solution until imminent run-off. Plants were kept in a closed Perspex box until time for collection of samples. Control plants were sprayed in the same way with sterile distilled water. Samples were collected at each time point and stored at -80°C.

Treatment with methyl jasmonate (MeJA) was carried out according to a modified method by Anderson *et al*. (2004). Plants were treated by taping cotton balls containing 400 µL of a 50 µM solution MeJA (Johansson *et al*., 2006; Sigma Aldrich) which had been dissolved in ethanol on the roof of a sealed Perspex box in which banana plants were kept. Samples were collected and stored at -80°C.

2.3.3   RNA extraction and cDNA synthesis

Total RNA was extracted from root material of the two banana cultivars Grand Naine (Fusarium-sensitive) and GCTCV-218 (Fusarium-tolerant) using the Qiagen RNAeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Traces of DNA in the RNA samples were eliminated by treating 5 µg of each RNA sample with DNase 1 (Fermentas Life Sciences, Hanover, MD). The RNA quality was verified by gel electrophoresis and quantified using the Nanodrop® procedure. First strand cDNA was subsequently synthesized from 1.5 µg of the DNA-free RNA samples

by random hexamer priming (Fermentas Life Sciences) using the first strand cDNA synthesis kit according to the manufacturer's instruction (Promega, USA). cDNA synthesis entailed the exposure of the RNA template to 100 ng of random hexamer primers and incubating at 70°C for 5 min followed by cooling on ice. In a further step, 8.4 µL of a mix containing 2.4 µL of 25 mM MgCl, 1 µL of 10 mM of each dNTP, 4 µL Improm-II buffer and 1 U Improm-II reverse transcriptase was added to the primer-template reaction. This was incubated in a PCR machine under conditions of 25°C for 10 min, 42°C for 1 hr and 60°C for 15 min to produce the cDNA.

The quality of cDNA was verified by conducting a standard PCR reaction to amplify a 170 bp actin fragment. Primers used were designed from the gene encoding the banana actin protein and resultant primers consisted of Actin-ff: 5'-ACCGAAGCCCCTCTTAACCC-3', and Actin-RR 5'-GTATGGCTGACACCATCACC-3'. Annealing was done at 55°C and final elongation was for 10 min at 72°C. Presence of the amplified fragment was verified via agarose gel electrophoresis on a 2% agarose gel which was followed by sequence analysis.

2.3.4 <u>Primer design for RT-PCR</u>

Four genes were used for expression studies in Cavendish banana plants. They consisted of genes coding for the MNPR1A, MNPR1B, PR-1 and PR-3 proteins with the *Musa* 25S rRNA as an endogenous control. Primer 3 (Premier Biosoft, Palo Alto, CA, USA) was used to design primers from *MNPR1A* and *MNPR1B* gene sequences while primer

sequences for the amplification of *PR-1*, *PR-3* and *Musa* 25S rRNA PCR products were

obtained from Van den Berg *et al.* (2007). All primers (Table 2.1) were designed to have

a GC content of between 50-55 %, length of 20-22 and which could amplify an amplicon

length of 80-150 bp.

Table 2.1 Quantitative real time PCR primers for amplification in banana

Primer name	Primer sequence (5' – 3')
MNPR1A (forward)	TGAGAGGCAACAACTCAGAGAG
MNPR1A (reverse)	GTCGGCATTGTACCAACACA
MNPR1B (forward)	AGGTTTGCCCGAACAAGAAG
MNPR1B (reverse)	TGAGAGGCAACAACTCAGAGAG
PR-1 (forward)	TCCGGCCTTATTTCACATT
PR-1 (reverse)	GCCATCTTCATCATCTGCAA
PR-3 (forward)	GGCTCTGTGGTTCTGGATGA
PR-3 (reverse)	CCAACCCTCCATTGATGATG
Musa 25s rRNA (forward)	ACATTGTCAGGTGGGGAGTT
Musa 25s rRNA (reverse)	CCTTTTGTTCCACACGAGATT

Primer pairs were assessed by conducting a conventional PCR with cDNA obtained from

banana plants 12 h after SA treatment.

2.3.5   Quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) was performed using the LightCycler® 480, 384-well PCR plates and the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, Germany) following the manufacturer's instructions. All reactions were conducted in triplicate with each PCR reaction consisting of 1 μL of the diluted template (1/10), 1 μM primers, and 5 μL Lightcycler® 480 SYBR-Green I master mix. The reaction volume was adjusted to 10 μL with nuclease-free water. Non-template control reactions contained water instead of cDNA as template. Cycling consisted of an initial denaturation phase of 10 min at 95°C, an amplification phase of 45 cycles each consisting of a denaturation step at 94°C for 5 s, annealing at 63°C for 5 s and extension at 72°C for 10s. Individual PCR products were analysed by melting-point analysis during which samples were heated from 65°C for 10 s to 95°C and the decline in fluorescent signals of each individual sample was assessed.

Five standard curves were made using 5 mM of each primer set using serial dilutions ranging from 1, 1:10, 1:20, 1:50, 1:100 and 1:1000 with cDNA from banana root sample which had been sprayed with distilled water. The standard curve was calculated with the formula $y = mx + b$, where $b = y$ intercept of the standard curve line (crossing point) and m equals to the slope of the standard curve line (this defines the function of the PCR efficiency). Slopes with a value of -3.32 generally indicate that the PCR reaction is 100% efficient. PCR efficiencies other than 100 % can be calculated by replacing the value of the slope(s) into the equation: PCR efficiency = $(10^{(1/-s)})$.

2.3.6   Data analysis

Data were analysed as described in the Applied Biosystems, User Bulletin No. 2 (2000). The relative expression at each time point for all the genes used during the study was calculated making use of the standard curve for the specific target gene and the endogenous control gene (*Musa* 25S rRNA). 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). Values obtained at this stage represent the relative expression values and are unit less. Samples at time point zero were used as the calibrator for each treatment and all other samples are represented in relation to the calibrator by dividing them by the calibrator value (Applied Biosystems, User Bulletin No. 2, 2001).

The standard deviation (SD) and the average input per treatment for each target gene ($SD_{Target\ gene}$) 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$_{\text{control gene}}$ /Average input $_{\text{control gene}}$)^2 + (SD$_{\text{target gene}}$ /Average input $_{\text{Target gene}}$)]* 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. The significance of differences for all treatments and between the two cultivars was analysed by One-way ANOVA and the Tukey Highest Square Difference (HSD) test at $p < 0.05$ using the Statistica software (StatSoft, Inc. 2006).

CHAPTER THREE

Results

3.1   Identification of *NPR1* fragments via Southern blotting

Genomic DNA was digested with *EcoR*I/*Hind*III to identify via DNA hybridization the *NPR1* gene sequence in genomic DNA isolated from Cavendish banana (Bluggoe, GCTCV-218, FHIA 25, and Tani), and also as control in genomic DNA from *Arabidopsis*, and maize. *EcoR*I and *Hind*III were used as restriction enzymes for genomic DNA digestion since they are insensitive to DNA methylation. A smear-like pattern was found for digested DNA after agarose gel electrophoresis which is typical for a total genomic digestion (Fig. 3.1A). When plasmid DNA of pUBINH13100 was digested with *Hind*III, an expected linearized band, which was greater than 5 kb, was obtained due to the presence of a unique *Hind*III site within the plasmid backbone.

Using Southern blot analysis, hybridisation of the DNA fragments with a rice *NPR1 homologue* (*NH1*) probe yielded numerous hybridisation signals (Fig. 3.1B). However, the hybridisation patterns obtained varied for the individual samples. Further, when hybridisation of the rice *NPR1* probe was performed at 50°C, a signal, which was slightly greater than 5 kb, was detected for plasmid pUBINH13100. However, the signal was not very distinct due to smearing around the signal (Fig. 3.1B). Among the banana samples, multiple hybridisation signals were found which were different from each other. However, the visibility of the signal was interfered at 50°C hybridisation temperature by the presence of smears, which were still present in the samples. In *Arabidopsis* about five bands were detected and the banding pattern was different from the banding pattern in banana and very different to two bands only detected in maize. Multiple bands found in

the various plant samples originate either from existing *EcoR*I and *Hind*III restriction sites in *NPR1* gene sequence or from various numbers of copies of the *NPR1* gene in banana, *Arabidopsis* and maize. In addition, gene copy numbers might further be different among the various banana cultivars.

Since smearing, high background noise and appearance of unspecific bands present on the membrane can result from the use of a low hybridization temperature, the membrane was stripped to wash out the existing probe and DNA was re-hybridised at a higher temperature of 60°C. At this temperature, a single band of greater than 5 kb was again detected for plasmid pUBINH13100. However, this band was more distinct than the band obtained at 50°C. Most of the background hybridisation noise was also eliminated in the plant samples. In *Arabidopsis*, a single band of about 2.5 kb was found while in maize a single band of 1 kb was detected (Fig. 3.1C). However, in Bluggoe, GCTCV-218, FHIA 25 and Tani, the multiple hybridisation patterns were again detected. However, these were still very different to each other and very different to maize and *Arabidopsis*. In Bluggoe, six bands with sizes of 4 kb, 2.5 kb, 1.5 kb, 1.2 kb, 900 bp and 500 bp were found. GCTCV-218 had five bands of 4 kb, 2.5 kb, 1.5 kb, 1.2 kb and 500 bp. FHIA 25 had seven bands of 4 kb, 3.5kb, 2.5 kb, 1.5 kb, 1.2 kb, 900 bp and 500 bp. In Tani, five bands were detected ranging in size from 4 kb, 3.5 kb, 2.5 kb, 1.5 kb and 900 bp.
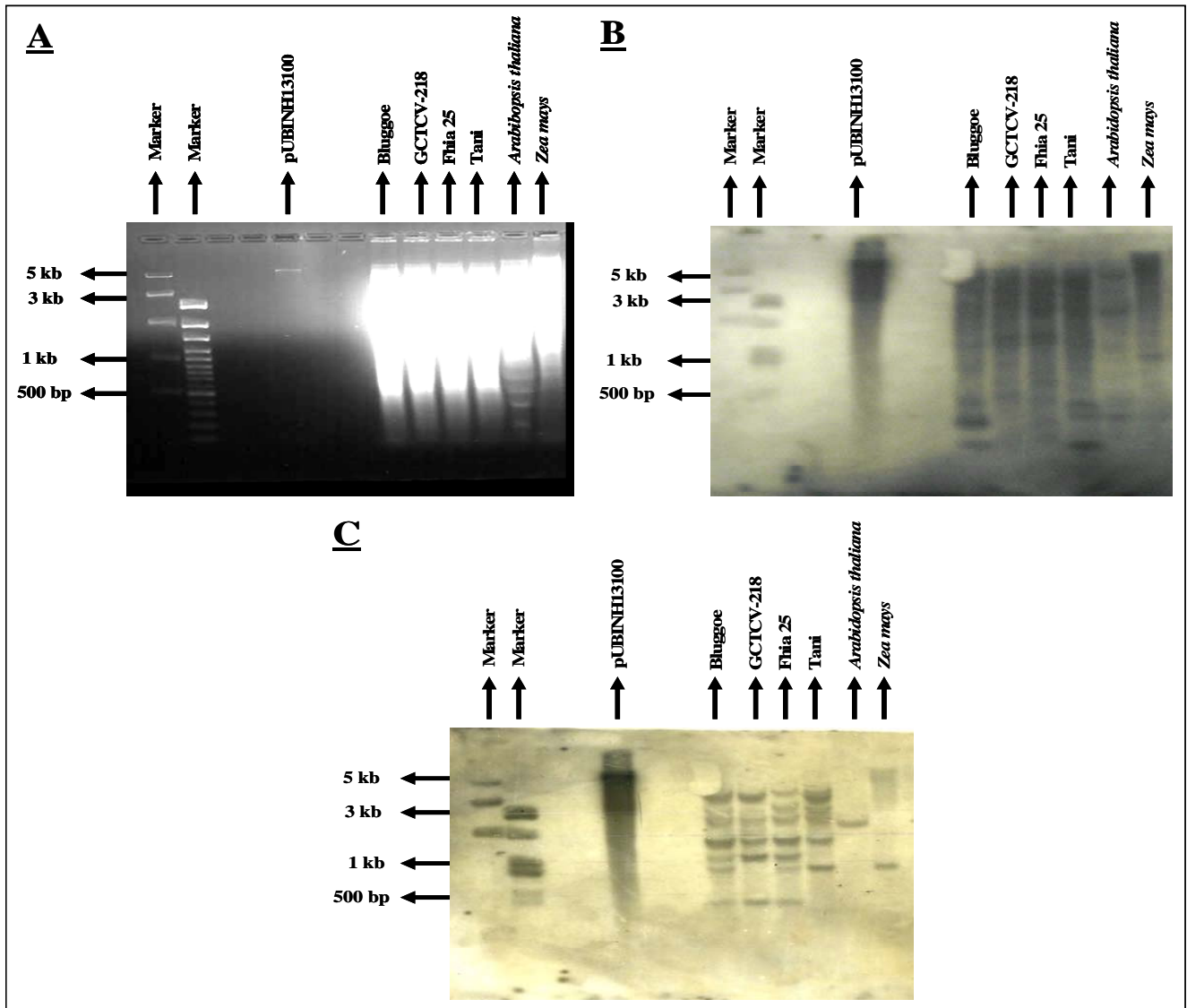
Figure 3.1 Identification of *NPR1* genes in banana using Southern blot hybridisation. (A) Agarose gel electrophoresis of total genomic DNA from different banana cultivars, (Bluggoe, GCTCV-218, FHIA 25, Tani), *Arabidopsis thaliana* and *Zea mays* after digestion with restriction enzymes *EcoR*I/*Hind*III and plasmid pUBINH13100 DNA after digestion with *Hind*III. (B) Hybridisation of the digested DNA at 50°C with a rice *NPR1* homologue (*NH1*) probe. (C) Hybridisation of the digested DNA at 60°C with a rice *NPR1* homologue (*NH1*) probe.

3.2   **Isolation of a partial banana *NPR1* gene sequence from genomic DNA**

Using the accession number AY92398 of the *NH1* gene, known *NPR1* gene sequences from various monocot and dicot plants were retrieved from the National Center for Biotechnology Information database (NCBI). These sequences were used to design degenerate primers (Fig. 3.2). To prove the existence of any *NPR1* gene sequence in banana, genomic DNA was initially used for *NPR1* amplification. For this, banana genomic DNA was isolated from Grand Naine leaves due to the availability of the Grand Naine banana plants at the time and DNA was also isolated from plasmid pUBINH13100. Amplification of the cloned *NPR1* sequence served as a positive control. Using plasmid pUBINH13100, a PCR product with the predicted size (1.4 kb) was obtained after gel electrophoresis (Fig. 3.2A). In Grand Naine, multiple amplification products were found after gel electrophoresis. A 2 kb fragment was detected in Grand Naine which was larger than the expected 1.4 kb fragment amplified from plasmid pUBINH13100. However, the larger size could be due to the possible presence of an intron within the *NPR1* genomic sequence from banana. Both fragments (2 kb and 1.4 kb) were cloned into the vector PCR®4-TOPO®, sequenced and analysed using the program BlastP. Analysis of the 1.4 kb PCR fragment from the plasmid pUBINH13100 yielded *NPR1* similarity to *NPR1* gene sequences from various plants such as O*ryza sativa* (e-value: 3e-67; 99% identity), *Nicotiana tabacum* (e-value: 1e-44; 61% identity)*, Brassica juncea* (e-value: 1e-35; 53% identity) and *Arabidopsis thaliana* (e-value: 1e-30; 48% identity). However, the 2 kb fragment from Grand Naine only showed similarity with expressed sequence tags from

the *Musa acuminata* clone MA4_112I10 and further a retrotransposon (*Musa acuminata* retrotransposon monkey sequence) but not to any *NPR1* gene sequence.
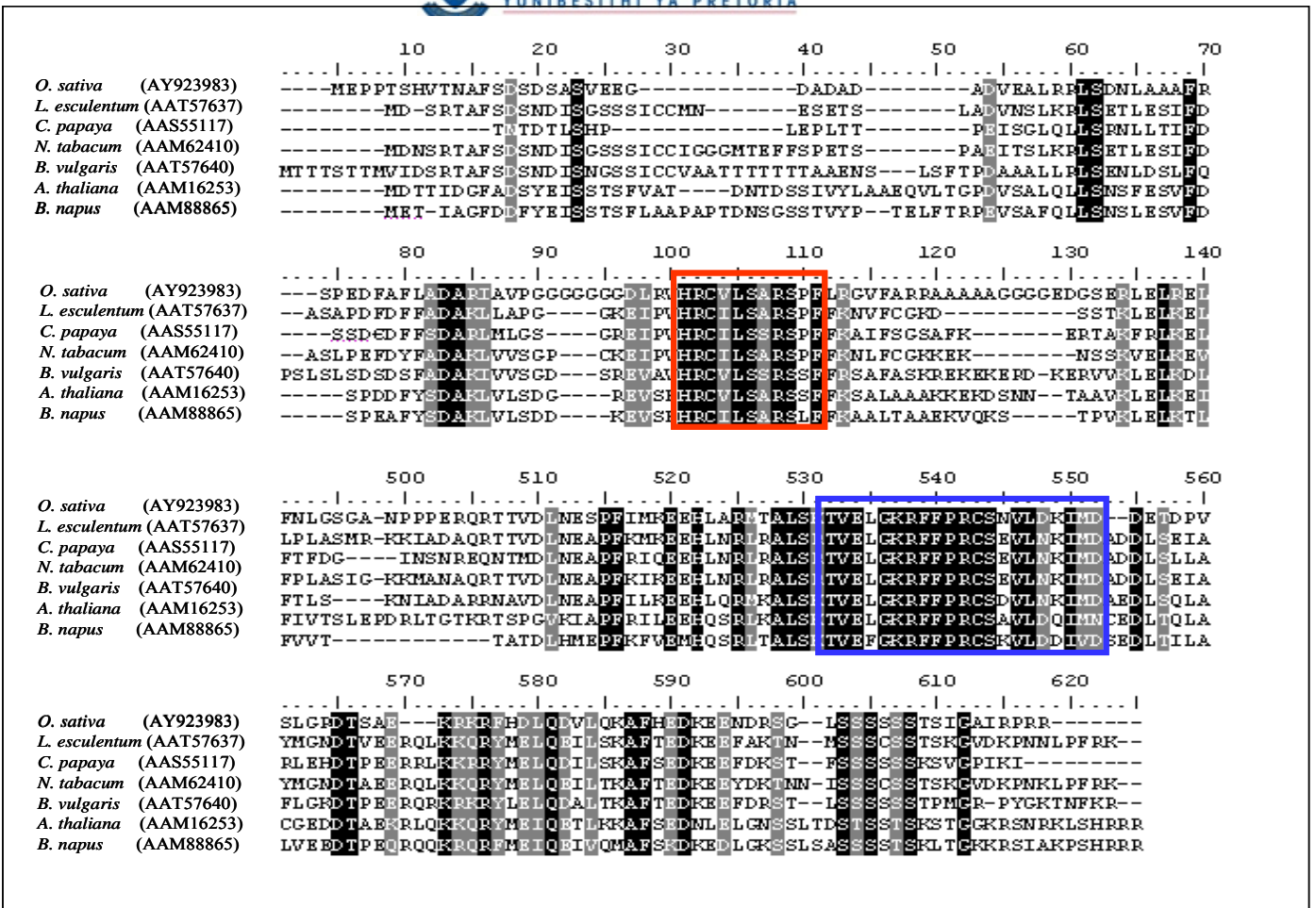
Figure 3.2 Multiple alignment of selected plant NPR1 amino acid sequences showing conserved regions used for designing degenerate primers. NPR1 sequences from *Oryza sativa* (AY923983), *Lycopersicum esculentum* (AAT57637), *Carica papaya* (AAS55117), *Nicotiana tabacum* (AAM62410), *Beta vulgaris* (AAT57640), *Arabidopsis thaliana* (AAM16253) and *Brassica napus* (AAM88865) were retrieved from the National Center for Biotechnology Information database (NCBI) and aligned using the Clustal W multiple alignment software (Thompson *et al.*, 1994). Two conserved blocks (red and blue) where further selected for primer design using the Consensus-degenerate hybrid oligonucleotide primer (CODEHOP) software (Rose *et al.*, 1998).

Due to the presence of multiple PCR products that were obtained from Grand Naine (Fig. 3.3A) and the non-detection of any *NPR1*-related gene sequence after sequence analysis, cDNA was isolated from mRNA isolated from wounded Grand Naine banana leaves. Wounded leaf material was chosen so as to optimise the chances of obtaining a higher copy number for the target NPR1 transcript. The primer design strategy was optimised by designing a primer pair which was very specific to the ankyrin repeat region of the rice *NH1* sequence (Material and methods). The design was based on the assumption that the *NPR1* gene is conserved within monocot plants. PCR amplification using banana cDNA from Grand Naine and the primer pairs designed to amplify the highly conserved ankyrin repeat region of known *NPR1*-like gene sequences yielded a distinct PCR product of approximately 570 bp. (Fig. 3.3B). A single band of about 570 bp was detected in banana, which was similar to the predicted 570 bp fragment amplified from plasmid DNA of pUBINH13100 (Fig 3.3B).
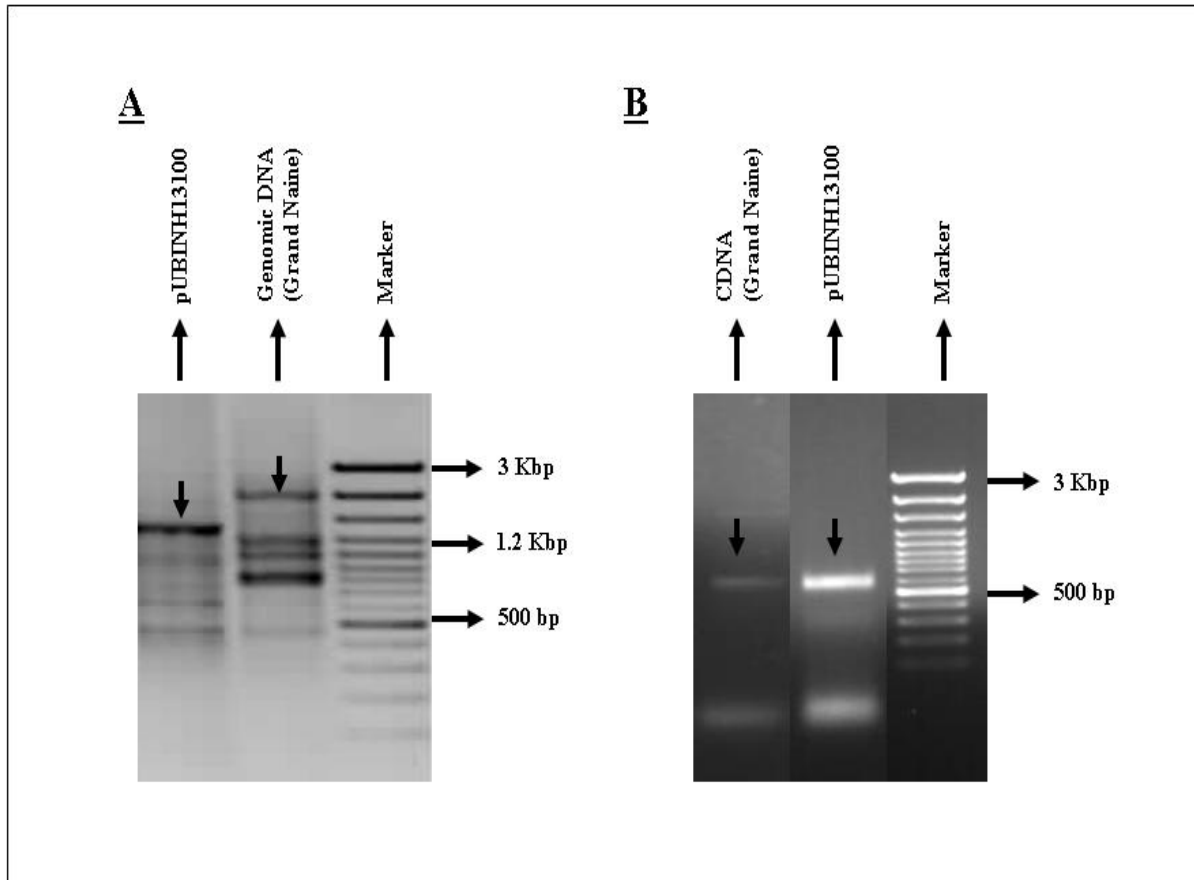
Figure 3.3 PCR amplified products using designed *NPR1* primers and banana leaves from Grand Naine. (A) Amplification products from genomic DNA obtained from leaves of Grand Naine and plasmid pUBINH13100 DNA using degenerate *NPR1* primers. (B) Amplification products obtained after agarose gel electrophoresis from cDNA obtained from leaves of wounded Grand Naine plants and pUBINH13100 plasmid DNA (positive control) using primers designed from the conserved ankyrin repeat region of the rice *NPR1* gene. Internal arrows point to bands that were cloned, sequenced and analysed for the existence of putative *NPR1* information.

When the 570 bp fragment from plasmid pUBINH13100 was cloned, sequenced and analysed using BlastP, a similarity to *NPR1* gene sequences were found with the highest identity to the *NPR1* sequence from *O. sativa* (rice) (e-value: 4e-64; 100% identity). After cloning into the vector pTopo, sequence analysis of different clones revealed the existence of two distinct banana cDNA clones (Fig. 3.4). The two clones, designated *MNPR1A* and *MNPR1B*, had some dissimilarity in their nucleotide sequence (Fig. 3.5) with an 85% identity on both the protein and the nucleotide level (Table 3.1 and Table 3.2). Further, the two partial MNPR1 amino acid sequences had the highest identity with the rice NPR1 sequence (75% identity for MNPR1A and 79% identity for MNPR1B fragments on the protein level) (Table 3.1). At the nucleotide level, the two partial *MNPR1* sequences also had the highest identity with the rice *NPR1* sequence (72% for *MNPR1A* and 74% for *MNPR1B*) and they both had similarities to different extents with other plant *NPR1* sequences (Table 3.2).

>MNPR1A partial nucleotide sequence

GAGCTTTTGGATCTCGCACTTGCAGATATTAACCATAGAAACATCAGAGGCTATACAGTGCTTCACATTGCTGCAATGCGTAAGGAACCCAAGATCA
TTGTGTCGCTGCTAACAAAAGGAGCTAGACCTTCTGATCTAACACTAGATGGAAGGAAAGCAGTTCAGATCTCAAAGCCCAAGATCATTGTGTCGCT
GCTAACAAAAGGAGCTAGACCTTCTGATCTAACACTAGATGGAAGGAAAGCAGTTCAGATCTCAAAGAGACACACCAAGTCCATGGAGTACTACAAG
TCAACTGAAGAAGGACAAGCATCTCCCAAGAGTCGTTTGTGCATCGAGATATTAGAGCAAGCTGAAAGAAGAGACCCGCAAGTAGGAGAAGCTTCTG
CTTTTCTCGCAATAGCTGGTGATGACTTGCGGGGAAGATTACTATACCTTGAGAATCGAGTCACTCTTGCAAGACTATTATTCCCCATGGAGGCAAG
GGTTGCCATGGACATTGCTCGAGTTGATGGCACATNANAATTTACCTTAGGGTCTACTACTANNCATTNTACNGGAAATNAAAGAACTGCAGCGGAT
CTAAATGAAACACCATTCATGATCAAAGAAGAGCATCTAGCACGTATGAGAGCACTCTCCANAACAGTGGANCT

>MNPR1A partial amino acid sequence

MLTIETSEVILCFT*LQCVKNLRSSCHF*QREPDHLILHWMEGKHFRLQRDLPSLWSTSGRLKKEKHLLRVVCALRY*SKLREEIHK
*VKLLYHLQWLVMTCGEDCCILRIELLWQDYCSPWRQELLWTLHKLMAHRSSP*GSTTXHXXGNXRTAADLNETPFMIKEEHLARMR
ALSXTVX

>MNPR1B partial nucleotide sequence

GGCTTTTGGATCTCGCACTTGCAGATGTTAACCATAGAGACTTCAGAGGTTATACTGTGCTTCACATAGCTGCAATGCGTAAAGAACCTAAGATCAT
CGTGTCACTTCTGACAAAGGGAGCCAGACCATCTGATCTTACATTGGATGGAAGGAAAGCACTTCAGATTGCAAAGAGACTTACCAAGTCTGTGGAG
TACCTCAGGTCGATTGAAGAAGGAGAAGCATCTCCTAAGAGTCGTTTGTGCATTGAGATATTAGAGCAAGCTGAGAGAAGAGATCCACAAGTAGGTG
AAGCTTCTGTATCACTTGCAATGGCTGGTGATGACTTGCGGGGAAGATTGTTGTATCTTGAGAATCGAGTTGCTCTGGCAAGACTATTGTTCCCCAT
GGAGGCAAGAGTTGCTATGGACATTGCACAAGTTGATGGCACATCGGAGTTCACCTTAGGGTCTACCAGCAACCGTTCTACTGGAAATCAAAGAACC
GCGATGGATCTAAACGAAGCACCATTCAAGATCAAGGAAGAGCATCTGGCACGAATGAGAGCACTCTCCAAAACAGTGGAGCTCGG

>MNPR1B partial amino acid sequence

MRKEPKIIVSLLTKGARPSDLTLDGRKAVQISKRHTKSMEYYKSTEEGQASPKSRLCIEILEQAERRDPQVGEASAFLAIAGDDLRG
RLLYLENRVTLARLLFPMEARVAMDIARVDGTXXFTLGLPATVLLEIKEPRWI*TKHHSRSRKSIWHE*EHSPKQWSS

Figure 3.4 Partial *MNPR1* nucleotide and amino acid sequences. Cloning, sequencing and analysis of a 570 bp fragment amplified from cDNA of leaves of wounded Grand Naine banana plants yielded two *MNPR1* clones of 569 bp for *MNPR1A* and 571 bp for *MNPR1B*.

```
MNPR1A    GAGCTTTTGGATCTCGCACTTGCAGATATTAACCATAGAAACATCAGAGG  50
MNPR1B    -G.............................G............G...T.......  49

MNPR1A    CTATACAGTGCTTCACATTGCTGCAATGCGTAAGGAACCCAAGATCATTG  100
MNPR1B    T.....T...........A.................A.....T........C.  99

MNPR1A    TGTCGCTGCTAACAAAAGGAGCTAGACCTTCTGATCTAACACTAGATGGA  150
MNPR1B    ....A..T..G.....G.....C.....A.........T...T.G......  149

MNPR1A    AGGAAAGCAGTTCAGATCTCAAAGAGACACACCAAGTCCATGGAGTACTA  200
MNPR1B    .........C........TG.........TT.........TG........CT  199

MNPR1A    CAAGTCAACTGAAGAAGGACAAGCATCTCCCAAGAGTCGTTTGTGCATCG  250
MNPR1B    ..G...G.T..........G...........T..................T.  249

MNPR1A    AGATATTAGAGCAAGCTGAAAGAAGAGACCCGCAAGTAGGAGAAGCTTCT  300
MNPR1B    ....................G........T...A........T.........  299

MNPR1A    GCTTTTCTCGCAATAGCTGGTGATGACTTGCGGGGAAGATTACTATACCT  350
MNPR1B    .TA.CA..T.....G...........................GT.G..T...  349

MNPR1A    TGAGAATCGAGTCACTCTTGCAAGACTATTATTCCCCATGGAGGCAAGGG  400
MNPR1B    ...........TG....G...........G..................A.  399

MNPR1A    TTGCCATGGACATTGCTCGAGTTGATGGCACATNANAATTTACCTTAGGG  450
MNPR1B    ....T...........A.A................G..G..C.........  449

MNPR1A    TCTACTACTANNCATTNTACNGGAAATNAAAGAACTGCAGCGGATCTAAA  500
MNPR1B    .....C.GC....G......................C...GAT.........  499

MNPR1A    TGAAACACCATTCATGATCAAAGAAGAGCATCTAGCACGTATGAGAGCAC  550
MNPR1B    C...G.........A......G...........G.....A..........  549

MNPR1A    TCTCCANAACAGTGGANCT---  569
MNPR1B    ...................CGG  571
```

Figure 3.5 Alignment of partial *MNPR1* nucleotide sequences. The partial 569 bp *MNPR1A* sequence and the 571 bp *MNPR1B* sequence were aligned after sequencing using the Clustal W software. Dots within the alignment represent conserved regions between both sequences with the specific nucleotides shown on the top sequence.

Table 3.1 Percentage identity between partial MNPR1A and MNPR1B amino acid sequences with other plant NPR1 amino acid sequences.

	MNPR1A (partial DNA sequence)	*MNPR1B* (partial DNA sequence)
Musa NPR1A (partial sequence)	100	85
Musa NPR1B (partial sequence)	85	100
Oryza sativa (**60308937**)	72	74
Carica papaya (**45160135**)	68	71
Nicotiana tabacum (**111054504**)	68	71
Arabidopsis thaliana (**1773294**)	60	62
Brassica juncea (**90811463**)	47	58

Table 3.2 Percentage identity between partial *MNPR1A* and *MNPR1B* DNA sequences with other plant *NPR1* sequences.

	MNPR1A (partial protein sequence)	MNPR1B (partial protein sequence)
Musa NPR1A (partial sequence)	100	85
Musa NPR1B (partial sequence)	85	100
Oryza sativa (AAX18700.1)	75	79
Carica papaya (AAS55117.1)	71	74
Nicotiana tabacum (ABH04326.1)	72	77
Arabidopsis thaliana (AAM65726.1)	50	56
Brassica juncea (DQ359129.3)	47	50

3.3 Characterisation of the complete banana *NPR1* gene sequence from cDNA

By applying a combination of the 3' RACE and 5'-end genome walking techniques, full-length *MNPR1* sequences were isolated (Fig.3.6). *MNPR1A* (GenBank accession number DQ925843) had a full-length DNA sequence of 1927 bp (Fig. 3.6) and *MNPR1B* (GenBank accession number EF137717) had a full-length DNA sequence of 2073 bp (Fig. 3.5). These sequences included sequences from the 5' and 3' untranslated regions. The complete amino acid sequences of the two genes (Fig. 3.7) displayed a 78% identity to each other (Table 3.3). Further, these full length sequences had the highest identity with the rice NPR1 amino acid sequence, 63% for MNPR1A and 65% for MNPR1B (Table 3.2) and the least identity with *Helianthus annuus* (40% for MNPR1A and 38% for MNPR1B).

Detailed analyses of the two *MNPR1* sequences revealed that the two sequences harbour a BTB/POZ zinc finger domain and the ankyrin repeat domain (Fig. 3.8). These are typical features of *NPR1* genes, which are highly conserved across many species. However, there is a relative positional change and some amino acid dissimilarities occur in these domains between the two isolated sequences. The BTB/POZ domain of *MNPR1A* was identified at amino acid positions 58 to136 while the ankyrin repeats were identified at amino acid positions 290 to 365 and 324 to 349, respectively (Fig. 3.8). In comparison, the BTB/POZ domain of *MNPR1B* occupies amino acid positions 65 to 148 and the ankyrin repeats are found at positions 302 to 377 and 336 to 361.

>MNPR1A (Coding region = 1728 bp)

ATAACGGCGCGATATGGAAGACAACTATCTCACTGCCGCCCCCGCGTTCTCCGTCTCCGACAACAGCCGCAGCGTCCACTTCGCCGGCGGCGCATCTCCCGACCCCG
CTGCCGATGTGGAGGCCCTCCGCCGCTTATCTGACAACCTTGGTGCCGCATTCGAGTCGCCGGACTTCGAGTTGTTCGCCGACGCCCGCATCGCTGTCGAGGACGGC
GGGGCGCCGGCGCGCGAGGTCGGAGTCCACCGGTGCGTGCTGTCCGCGCGGAGCCCCTTCTTTCGGGAGGTGTTCGCGGAGCGGGAGGGGGCGCTGGCGCCGGTGAG
GCTGGAGCTGTGGAAGCTGGTGAGCGGGTTCGTGGTCGGCTACGACGCCTTGGTGACGGTGCTCGGGTACCTCTACAGAGGGAGGGTGGCGCCGCTGACCAAGGAGG
TGTGCATGTGCGTGGACGAAGAGTGCCGGCACGAGGCGTGCCGGCCGGTGGTCGATTTCATGGTCGAAGTGCTCTACGCCTCCTTCGTCTTCCAAATCTCCGAGCTG
GTCAGCCTCTTCCAGCGACACCTTCTTGATATTCTTGACAAGGTGTCTATAGATGACATTCTGGTAATCCTATCTGTCGCTAACCTGTGTGATAGCTCTTGTGCCAA
ATTATTCAACAAGTGCATAGAGATTGCAGTTAAGTCAGACCTGGACATCATTACGCTTGAGAAGACAATGACTCCTGATATTGTCAAGCAAATCATGGATTCACGCT
TGAATTTGGGAACAGTAGGACCTGAAAGCATCAATTTTTCTGATAAACATGTCAAGAGAATACATGGGGCTCTCGATAATGATGATGTTGATTTAGTAAGAATGCTG
TTGAAGGAGGGGAATACAACACTAGATGATGCATGTGCTTTGCATTATGCAGTGGCATATTGTGACTCAAAGGTTACAACAGAGCTATTAGATCTTGAACTTGCAGA
TATTAACCATAGAAACATCAGAGGCTATACAGTGCTTCACATTGCTGCAATGCGTAAGGAACCCAAGATCATTGTGTCGCTACTAACAAAAGGAGCTAGACCTTCTG
ATCTAACACTAGATGGAAGGAAAGCAGTTCAGATCTCAAAGAGACACACCAAGTCCATGGAGTACTTCAAGTCAACTGAAGAAGGACAAGCATCTCCCAAGAGTCGT
TTGTGCATCGAGATATTAGAGCAAGCTGAAAGAAGAGATCCGCAAGTAGGAGAAGCTTCTGCTTTTCTCGCAATAGCTGGTGATGACTTGCGGGGAAGATTACTATA
CCTTGAGAATCGAGTCACTCTTGCAAGACTATTATTCCCCATGGAGGCAAGGGTTGCCATGGACATTGCTCGAGTTGATGGCACATTAGAATTTACCTTAGGGTCTG
CTACTAGCCATTCTACTGGAAATCAAAGAACTGCAGCAGATCTAAATGAAACACCATTCACGATCAAAGAAGAGCATCTAGCACGTATGAGAGCACTTTCCAGAACA
GTGGAACTCGGGAAGCGCTTTTTTCCCCGGTGCTCAGCGGTTATCAATAAGATCATGGATGATGGCTCGACAGATTTTGCTTATCTTCAGCATGATGCATCAGAAGG
GAAGAGGATGAGATCTTTGGAACTGCAGGATGCCCTGCCGAGAGCATTCAGCGAGGACAAGGAGGAATTCAACAAGTCTGCTTTGTCTTCCTCATCATCATCAACAT
CGGTCGGCATTGTACCAACACAAAGATGATACATTGTGCTGTATCTCCTACATTTCCTATGGCTTCCTTTTGCTGACTCCTGCACTGGGAACATTTTGTTCTTGGCC
CTGCTGTAAATGTATTTTGGTGTGATGGATCTGTTCTGTTTCAAATTTTGTCCTGGAATGCTTCGCACTTCACATTCTATTTAAAAAAAAAAAAAAAAAAAAAAAAA
A

>MNPR1B (Coding region = 1779 bp)

TTGGACGACGGCGGTACACGGCGGGACATGGAACCCAGCTACCTCACGGCCGCCACCGCCTTCTCGGGCTCCGACAACAGCAGCTGCGTGCACTTCTCCGGCGATGC
GGCGGCTGCTGCAGCTCCGGACTCCGCCCCGCCCGCGGCGGAGGTCGAGGGACTCCGTCGCCTGTCGGACCACCTCGGCTCCGCCTTCCAGTCGCCGGACTTCGAGT
TCCTCGCCGACGCCCGCATCGCGGTCGGGCCCCCAGGGGACGGCGGGTCAACGCCCCGCGAGGTCGCGGTACACCGCTGCGTGCTATCCGCCCGGAGCATCGTATTC
CGGGAGGAGTTCGCGAGGCGGGGGAGGGGAACGGCCGCGGCCCCGGTGAGGATGGAGCTGAAGGAGCTGGTAAAGGACTTCGAGGTCGGGTACGACGCCTTGGTGGC
GGTGCTCGGGTACCTCTACACCGGGAGGGTGGCACCGCTGCCCAAGGCGGTGTGCGCCTGCGTCGACGAGGAGTGCCGGCACGAGGCGTGCCGGCCGGCGGTCGATT
TCATGGCCGAAGTGCTCTACGCCTCCTCCGTCTTCCAAATCGCCGAGCTGGTCAGCCTCTTCCAGCGGCACCTCCTTGGTATTCTGGACAAGATGGCAATAGATGAC
ATACCAGTAATTCTCTCTGTTGCTAAACTATGCGATAGCTCATGCGCCAATCTGCTCAGCAAATGCATAGACGTTGTAGTCAAGTCAGACCTAGATACCATCACCCT
AGAGAAGAAGACGCCTCCTGATATTGTTAAGCAAATTATGGATTTACGCTTGAATTTTGGGCTAGTGGGACCTGAAAGCAGCAGCTTTCCTGATAAACACGTCAAGA
GAATACATAGAGCTCTTGACAGTGATGATGTTGACTTAGTAAGAATGCTATTAAAGGAGGGGAATACAACGCTAGATGACGCATGTGCATTGCATTATGCGGTAGCA
TATTGTGATTCAAAAATCACAACAGAGCTGTTAGATCTTGCACTGGCAGATGTTAACCATAGAGACTTCAGAGGTTATACTGTGCTTCACATAGCTGCAATGCGTAA
AGAACCTAAGATCATCGTGTCACTTCTGACAAAGGGAGCCAGACCATCTGATCTTACATTGGATGGAAGGAAAGCACTTCAGATTGCAAAGAGACTTACCAAGTCTG
TGGAGTACCTCAGGTCGATTGAAGAAGGAGAAGCATCTCCTAAGAGTCGTTTGTGCATTGAGATATTAGAGCAGGCTGAAAGAAGAGATCCACAAGTAGGTGAAGCT
TCTGTATCACTTGCAATGGCTGGTGATGACTTGCGGGGAAGATTGTTGTATCTTGAGAATCGAGTTGCTCTGGCAAGACTATTGTTCCCCATGGAGGCAAGAGTTGC
TATGGACATTGCACAAGTTGATGGCACATCGGAGTTCACCTTAGGGTCTACCAGCAACCGTTCTACTGGAAATCAAAGGACTGCGATGGATCTAAACGAAGCACCAT
TCAAGATCAAGGAAGAGCATCTGGCACGAATGAGAGCACTTTCCAGAACAGTGGAACTTGGGAAGCGCTTTTTCCCTCGGTGCTCAGAGGTCATCAACAAGATCATG
GACGACGATCTCACAGAAATCACTGGCCTCGGACACCACACTTCGGAGGAGAAGAGGAGGAGATTTCAGGAGTTGCAGGAAGTCCTGTCAAAAGCATTTAGCCAAGA
CAAGGAGGAATTCGACAGGTCTGCCTTGTCTTCCTCATCCTCATCGTCATCAACATCCATCGACAAGGTTTGCCCGAACAAGAAGATGAGATGATTCCCACCTAGTC
GATTCTATACCTTGTACGCTGATTATGGGTTCTTCGTGCCAAACATGTTTGCTTCGTCTCTCTGAGTTGTTGCCTCTTATAGAACCATGACACACCACTAGATCAT
GCTGTAACATGTGTTATATAACTCTTTGTCTAATATTACTGATTTGTGTTATTTACAAAGGTATTATGAACATCATAATATTAATGCAAAGCAGCTTGGATAGGCTA
CTGTTTCAGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.6 Full-length nucleotide coding sequence of *MNPR1A* and *MNPR1B*. The full-length of *MNPR1A* (1728 bp) and *MNPR1B* (1779 bp) coding region was amplified from both genomic and cDNA obtained from leaves of Grand Naine plants and by applying a combination of genome walking or 5', 3' RACE techniques. Sequences in red represent the start codon (at the beginning of the sequence) and the stop codon (at the end of the sequence).

>MNPR1A full length amino acid sequence

MEDNYLTAAPAFSVSDNSRSVHFAGGASPDPAADVEALRRLSDNLGAAFESPDFELFADARIAVEDGGA
PAREVGVHRCVLSARSPFFREVFAEREGALAPVRLELWKLVSGFVVGYDALVTVLGYLYRGRVAPLTKE
VCMCVDEECRHEACRPVVDFMVEVLYASFVFQISELVSLFQRHLLDILDKVSIDDILVILSVANLCDSS
CAKLFNKCIEIAVKSDLDIITLEKTMTPDIVKQIMDSRLNLGTVGPESINFSDKHVKRIHGALDNDDVD
LVRMLLKEGNTTLDDACALHYAVAYCDSKVTTELLDLELADINHRNIRGYTVLHIAAMRKEPKIIVSLL
TKGARPSDLTLDGRKAVQISKRHTKSMEYFKSTEEGQASPKSRLCIEILEQAERRDPQVGEASAFLAIA
GDDLRGRLLYLENRVTLARLLFPMEARVAMDIARVDGTLEFTLGSATSHSTGNQRTAADLNETPFTIKE
EHLARMRALSRTVELGKRFFPRCSAVINKIMDDGSTDFAYLQHDASEGKRMRSLELQDALPRAFSEDKE
EFNKSALSSSSSSSTSVGIVPTQR

>MNPR1B full length amino acid sequence

MEPSYLTAATAFSGSDNSSCVHFSGDAAAAAAPDSAPPAAEVEGLRRLSDHLGSAFQSPDFEFLADARI
AVGPPGDGGSTPREVAVHRCVLSARSIVFREEFARRGRGTAAAPVRMELKELVKDFEVGYDALVAVLGY
LYTGRVAPLPKAVCACVDEECRHEACRPAVDFMAEVLYASSVFQIAELVSLFQRHLLGILDKMAIDDIP
VILSVAKLCDSSCANLLSKCIDVVVKSDLDTITLEKKTPPDIVKQIMDLRLNFGLVGPESSSFPDKHVK
RIHRALDSDDVDLVRMLLKEGNTTLDDACALHYAVAYCDSKITTELLDLALADVNHRDFRGYTVLHIAA
MRKEPKIIVSLLTKGARPSDLTLDGRKALQIAKRLTKSVEYLRSIEEGEASPKSRLCIEILEQAERRDP
QVGEASVSLAMAGDDLRGRLLYLENRVALARLLFPMEARVAMDIAQVDGTSEFTLGSTSNRSTGNQRTA
MDLNEAPFKIKEEHLARMRALSRTVELGKRFFPRCSEVINKIMDDDLTEITGLGHHTSEEKRRRFQELQ
EVLSKAFSQDKEEFDRSALSSSSSSSSSTSIDKVCPNKKMR

Figure 3.7 Full-length coding sequences of *MNPR1A* and *MNPR1B*. The full-length of *MNPR1A* and *MNPR1B* coding region was amplified from both genomic and cDNA obtained from leaves of Grand Naine plants and by applying a combination of genome walking or 5', 3' RACE techniques. The nucleotide sequences obtained from *MNPR1A* and *MNPR1B* after sequencing was translated into protein sequences in Bioedit.

Table 3.3 Percentage identity between the full-length amino acid coding sequences of MNPR1A and MNPR1B with NPR1 amino acid sequences from other plants.

	MNPR1A	MNPR1B
Musa **NPR1A (MNPR1A)**	100	78
Musa **NPR1B (MNPR1B)**	78	100
Capsicum annum	60	63
Oryza sativa	63	65
Hordeum vulgare	60	62
Nicotiana tabacum	58	62
Lycopersicum esculentum	60	63
Arabidopsis thaliana	47	48
Brassica napus	46	46
Helianthus annuus	40	38

A. thaliana	MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTGPDVSALQLLSNSFESVFDSPD---DFYSDAKLVLSD----	73
MNPR1A	.EDNYLTA.PAFSV.DN.RSVHFAG-----GASPD---PAA..E..RR..DNLGAA.E...--DFELFA..RIAVE---DG	67
MNPR1B	.EPSYLTA.TAFSG.DN.SCVHFSG.-AAAAA.PDSAPPAAE.EG.RR..DHLG.A.Q..--DFE.LA..RIAVGPPGDG	77
C. annum	...-SRTA.S..ND..GS.SICCIGG--MTESFSP.TSP--AEITS.KR..EIL..I...SSPDF..FA.....VP-----	70
L. esculentum	..-SRTA.S..ND..GS.SICCM.E--------S.TS.-A..NS.KR..ETL..I...ASAPDF..FA.....LAP-----	64
N. tabacum	..NSRTA.S..ND..GS.SICCIGGG-MTESFSP.TSP--AEITS.KR..ETL..I...AASPEF.YFA.....IPG----	73
O. sativa	.EPPTSHVTNAFSD.D-.ASVEEGG------AD.D-----A..E..RR..DNLAAA.R..E-DFA.LA..RIAVPGG---	64
H. vulgare	.EAPSSHVTT.FSDCD-.VSME.---------A.PD-----A..E..RR..DNLAAA.R....-DFA.LA..RFAVPG----	61
A. thaliana	---GREVSFHRCVLSARSSFFKSALAAAKKE----KDSNNTAAVKLELKEIAKD----YEVGFDSVVTVLAYVYSSRVRP	142
MNPR1A	GAPA...GV....I......P..REVF.EREG----ALAP------.R...WKLVSG----FV..Y.AL....G.L.RG..A.	134
MNPR1B	GSTP...AV....IV.REEF.RRGRG--TAAAP-----.RM....LV.....F...Y.AL.A..G.L.TG..A.	146
C. annum	--I.K.IPV...I......P...NVFCGKERK----------T.......LM.E------.ASY.AL.N....GK...	132
L. esculentum	--G.K.IPV...I......P...NVFCGKDSS----------T.......LM.E------.S..A..S....L..GK...	126
N. tabacum	--A.K.IPV...I......P...NLFCGK.EKNS---------.S.V....VM.E------.SY.A..S....L..GKI..	137
O. sativa	GGG.GDLLV...I......P.LRGVF.RRAAA--AAGGGGEDGGER...R.LLGGGGEEV...YEALRL..D.L..G..GD	142
H. vulgare	---APDLCV...I......P.LRALFKRRAAA--AGSAGGAEG-DRV..R.LLGG---EV...YEALRL..D.L..G..CD	132
A. thaliana	PPKGVSECADEN-CCHVACRPAVDFMLEVLYLAFIFRIPELVTLYQRHLLDVVDKVVIEDTLVILKLANICGKACMKLLD	221
MNPR1A	LT.E.CM.V..E-.R.E....V....V....AS.V.Q.S...S.F......IL...S.D.I...SV..L.DSS.A..FN	213
MNPR1B	L..A.CA.V..E-.R.E.......A.....ASSV.Q.A...S.F.....GIL...MA.D.IP...SV.KL.DSS.AN..S	225
C. annum	S..D.CV.V.NE-.F.........A.LVQ...AS.T.Q.S...DKF......ILN.AAAD.VMMV.SV.........ER..S	211
L. esculentum	AS.D.CV.V.NE-.L........A..VQ....AS.T.SQ...DKF......IL..A.AD.VMMV.SV.........ER..S	205
N. tabacum	S..D.CV.V.NE-.S........A.LV....IS.T.Q.S...DKF......ILG.AAAD.VMMV.SV.........ER..S	216
O. sativa	L..AACL.V..D-.A..G.H...A..AQ..FA.ST.QVA...TN.F..R....L..EVDNL.L..SV..L.N.S......E	221
H. vulgare	L..TACA.V..GG.A..G.H...S..AQ..FA.ST.QVG..AS.F....LL..EADNLPLV.SV..L.N.S.V..FE	212
A. thaliana	RCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPKVK----KHVSNVHKALDSDDIELVKLILKEDHTNLDDAC	297
MNPR1A	K.I..A...DL.IIT...TMTPDI..Q.M.S.LN..TVG.ESINFSD...KRI.G...N..VD..RM....GN.T.....	293
MNPR1B	K.IDVV...DL.TIT...KT.PDI..Q.M.L.LNF..VG.ESSSFPD...KRI.R......VD..RM....GN.T.....	305
C. annum	S.I.........IIT.D.A..NDI..Q.T.S.T...D.QG.VNHGFPD...KRI.R......V..LRM....G..T.....Y	291
L. esculentum	.ID.........IIT.D...HDI..Q.T.S.A...QG.ESNGFPD...KRI.R......V..LRM....G..T.....Y	285
N. tabacum	S.I.........IIT.D.A..HDI..Q.T.S.A...QG.ESNGFPD...KRI.R......V..LQM..R.G..T.....Y	296
O. sativa	..LDMV.R..L..IT....PDVI.Q....A.LS...IS.EN.GFPN...RRI.R......V...RM..T.GQ......F	301
H. vulgare	..L.RV.R.DL..IT.D.A..LDVI.Q....S.IT...AS.EDNGFPN...RRILS......V...R......GQ......F	292
A. thaliana	ALHFAVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATLEGRTALMIAKQATMAVE	377
MNPR1A	...Y.....DS.VT.E..D.E....I....I........I......KI.V....T...RP.DL..D..K.VQ.S.RH.KSM.	373
MNPR1B	...Y.....DS.IT.E..D.A.......DF......I........KI.V....T...RP.DL..D..K..Q...RL.KS..	385
C. annum	...Y.....DA..TSE..D.A......Q............KI.V....T...RP.DL.SD...RR.RL.D	371
L. esculentum	...Y.....DA..TAE..D.S......Q....H......KI.V....T...RP.DL.SD.KK..Q...RL.RL.D	365
N. tabacum	...Y.....DA..TAE..D.A......Q.S..........KI.V....T...RP.DL.SD..K..Q...RL.RL.D	376
O. sativa	...Y..EH.DS.IT.E..D.A........I...R.R..KI.V....T...RPADV.FD..K.VQ.S.RL.KQGD	381
H. vulgare	...Y..EH.DS.IT.E..DIA.....L.........I...R.RD.KIVV....T...RP.DF.FD..K.VQ...RL.KHGD	372
A. thaliana	CNNIPEQCKHSLKGRLCVEILEQEDKREPIPRDVPPSFAVAADELKMTLLDLENRVALAQRLFPTEAQAAMEIAEMKGTC	457
MNPR1A	YFKST.EGQA.P.S...I.....AER.D.QVGEASAFL.I.G.D.RGR..Y......T..RL...M..RV..D...RVD..L	453
MNPR1B	YLRSI.EGEA.P.S...I.....AER.D.QVGEASV.L.M.G.D.RGR..Y......RL...M..RV..D..QVD..S	465
C. annum	FIKST.EG.SAP.D...I.....AER.D.LLGEASV.L.M.G.D.R.K..Y......G..KL...M..KV..D..QVD..S	451
L. esculentum	FTKST.EG.SAP.D...I.....AER.D.LLGEASL.L.M.G.D.R.K..Y......G..KL...M..KV..D..QVD..S	445
N. tabacum	FSKS..EG.SAS.D...I.....AER.D.LLGEASV.L.M.G.D.R.K..Y......G..KL...M..KV..D..QVD..S	456
O. sativa	YFGVT.EG.P.P.D...I.....AER.D.QLGEASV.L.M.GES.RGR..Y......RIM..M..RV..D..QVD..L	461
H. vulgare	YFGNT.EG.P.PNDK..I....EAER.D.QLGEASV.L.L.G.C.RGK..Y......RIM..I..RV..D..QVD..L	452
A. thaliana	EFIVTSLEPDRLTGTKRTSPGVKIAPFRILEEHQSRLKALSKTVELGKRFFPRCSAVLDQIMNCEDLTQLACGEDDTAEK	537
MNPR1A	..TLG.AT-SHS..NQ..AADLNET..T.K...LA.MR....R................INK..D-DGS.DF.YLQH.AS.G	531
MNPR1B	..TLG.TS-N.S..NQ..AMDLNE...K.K...LA.MR....R................E.INK..D-D...EITGLGHH.S.E	543
C. annum	..PLA.IR-KKMADAQ..TVDLNE...KMK...LN..M...R................E..NK..DAD..SEI.YMGN..P.E	530
L. esculentum	.LPLA.MR-KKIADAQ..TVDLNE...KMK...LN..R...R................E..NK..DAD..SEI.YMGN..V.E	524
N. tabacum	..PLA.IS-KKMVNAQ..TVDLNE....K...LN..R...R................E..NK..DAD..SEI.YMGN....E	535
O. sativa	..NLG.GA-NPPPERQ..TVDLNES..IMK...LA.MT................N...K..D-DE-.DPVSLGR..SAE	538
H. vulgare	..TLG.CT-NPPPE--I.TVDLNDT..KMKD..LA.MR................N...K..D-DE-PE..SLGR.ASSE	527
A. thaliana	RLQKKQRYMEIQETLKKAFSEDNLELGNSS-LTDSTSSTSKSTGGKRSNRKFSHRRR	593
MNPR1A	----.RM.SL.L.DA.PR.....KE.FNK.A-.SS.S....VG---IVPTQR-----	575
MNPR1B	---.RR.FQ.L..V.S.....Q.KE.FDR.A-.SS.S..S.T.IDKVCP.K.MR----	592
C. annum	.QL.......I.T....T..KE.FAKTN-VSS.C.....GVDKPNKLPFRK----	582
L. esculentum	.QL.......L...I.S...T..KE.FAKTN-MSS.C.....GVDKPNNLPFRK----	576
N. tabacum	.QL.......L...I.T....T..KE.FDKTNNISS.C.....GVDKPNKLPFRK----	588
O. sativa	----.RK.FHDL.DV.Q...H..KE.NDR.G-.SS.S....IGAI----RPRR-----	582
H. vulgare	---R.R.FHDLHD..L....KE.FAR.AT.SA.S...PTVARNLTGRPRR-----	576

Figure 3.8 Multiple alignment of *MNPR1A* and *MNPR1B* with selected plant NPR1-like amino acid sequences. Amino acid sequences were aligned by Clustal W multiple

alignment software (Thompson *et al*., 1994). Identical amino acids are represented with dots. Vertical rectangles represent conserved cysteine residues and horizontal rectangles represent BTB/POZ domain (filled) conserved ankyrin repeat domain (not-filled) in both MNPR1A and MNPR1B. Accession numbers used in the alignments are *Capsicum annum* (ABG38308.1), *Lycopersicum esculentum* (AAT57637.1), *Nicotiana tabacum* (ABH04326.1), *Oryza sativa* (NP_001042286.1), *Hordeum vulgare* (CAJ19095.1), *Arabidopsis thaliana* (AAM65726.1).

3.4 **Phylogenetic analysis**

Due to limited information in the gene bank on complete NPR1 sequences especially from monocot plants, a phylogenetic analysis was performed using the conserved ankyrin domain of NPR1 sequences from a limited selection of dicot and monot plants (Fig. 3.9A). Preliminary phylogenetic analysis using Bootstrap consensus for neighbour joining (NJ), Maximum parsimony (MP) and Maximum likelihood (ML) showed that the ankyrin domain of *MNPR1A* and *MNPR1B* grouped closely with other monocot plants like rice and maize (Fig. 3.9B). This suggests a conserved pattern in the *NPR1* sequences among monocot plants. Within the monocots and dicots, there was a relatively close grouping among plants belonging to the same family compared to those belonging to different families. For instance, plants belonging to the Brassicaceae family (*Arabidopsis thaliana* and *Brassica juncea*) formed a separate sub-clade from those of the Solanaceae (*Nicotiana tabacum* and *Lycopersicum esculentum*). In a similar manner, the *Musa NPR1* gene sequences formed a defined sub-clade from *NPR1* sequences of plants belonging to the Poaceae family (*Oryza sativa* and *Zea mays*).

A

```
Musa NPR1A                    ..............GC:...........................AGC...GT 50
Musa NPR1B                    ..............GC:...........................AGC...T  50
Lycopersicum esculentum       GATGATGCGTATGCGCTGCATTATGCGGTGGCGTATTGCGATGCGAAAAC 50
Capsicum annum                ..............................................   50
Nicotiana tabacum             ..............................................   50
Ipomea batatas                ..............................................   50
Oryza sativa                  ..........T:.................AAC:...........AGC:...T  50
zea mays                      ..........................AAC:............AGC:...T  50
Arabidopsis thaliana          ...........A:..GC:..........T:...........A:C:T:.   50
Brassica juncea               ...............AA:..........T:...............AA:...T  50

Musa NPR1A                    G....C...........AA.........A.T.......---:GC:G  97
Musa NPR1B                    T....C.....................A.T.......---:GCG   97
Lycopersicum esculentum       ....CC:...........AGC:......................---:GC:  97
Capsicum annum                CACCAGCGAACTGCTGGATCTGGCGCTGGCGGATGT:GAACCAT:---:AGA  97
Nicotiana tabacum             .....GCG:.........................A.T:......---:GC:  97
Ipomea batatas                .....GTG:..T:..............A:T:.......---:GC:  97
Oryza sativa                  T.....C.........................---:GC:  97
zea mays                      .....C.........................---:GC:  97
Arabidopsis thaliana          G:G:C:..T:........A:A:.....AT:.......---:GC:  97
Brassica juncea               G:.C:.A:C:........A:..GCT:T:........GC:---:GC:  97

Musa NPR1A                    .:ATT:..............................A:T......  147
Musa NPR1B                    :TTTT:..............................A:T......  147
Lycopersicum esculentum       ..............C:.......................  147
Capsicum annum                ACCCGCGCGGCTATACCGTGCTGCATGTGGCGGCGATGCGCAAAGAACCG 147
Nicotiana tabacum             .:AGC:................................  147
Ipomea batatas                .....T:................................  147
Oryza sativa                  .............................A:T......CGC:...CGC:. 147
zea mays                      ...................................CGC:.  147
Arabidopsis thaliana          .............................A:T......  147
Brassica juncea               .............................A:T......  147

Musa NPR1A                    ..............................................  197
Musa NPR1B                    ..............................................  197
Lycopersicum esculentum       AAAATTATTGTGAGCCTGCTGACCAAAGGCGCGCGCCCGAGCGATCTGAC 197
Capsicum annum                ..............................................  197
Nicotiana tabacum             .........G.G:.................................  197
Ipomea batatas                ..............................................  197
Oryza sativa                  ..............................................  197
zea mays                      .........................GCG:...G:....  197
Arabidopsis thaliana          C:GC:G:..C:............GAA:..........A:.G:......AGC:..  197
Brassica juncea               .CCC:G:...C:CTG:.............AA:.G:..CTG:.AA:...G  197

Musa NPR1A                    CTG:..................G:........AGC 228
Musa NPR1B                    CTG:..................G:........AGC 228
Lycopersicum esculentum       ....AAA:.......................  228
Capsicum annum                CAGCGATGGCCGCAAAGCGCTGCAGATTGCG 228
Nicotiana tabacum             ..............................  228
Ipomea batatas                ..............................  228
Oryza sativa                  :TTT:..................G:........AGC 228
zea mays                      :TTT:---------------------------  201
Arabidopsis thaliana          CTG:..A:.......CC:......AT:.......  228
Brassica juncea               CTG:..................CC:.....T:.......  228
```
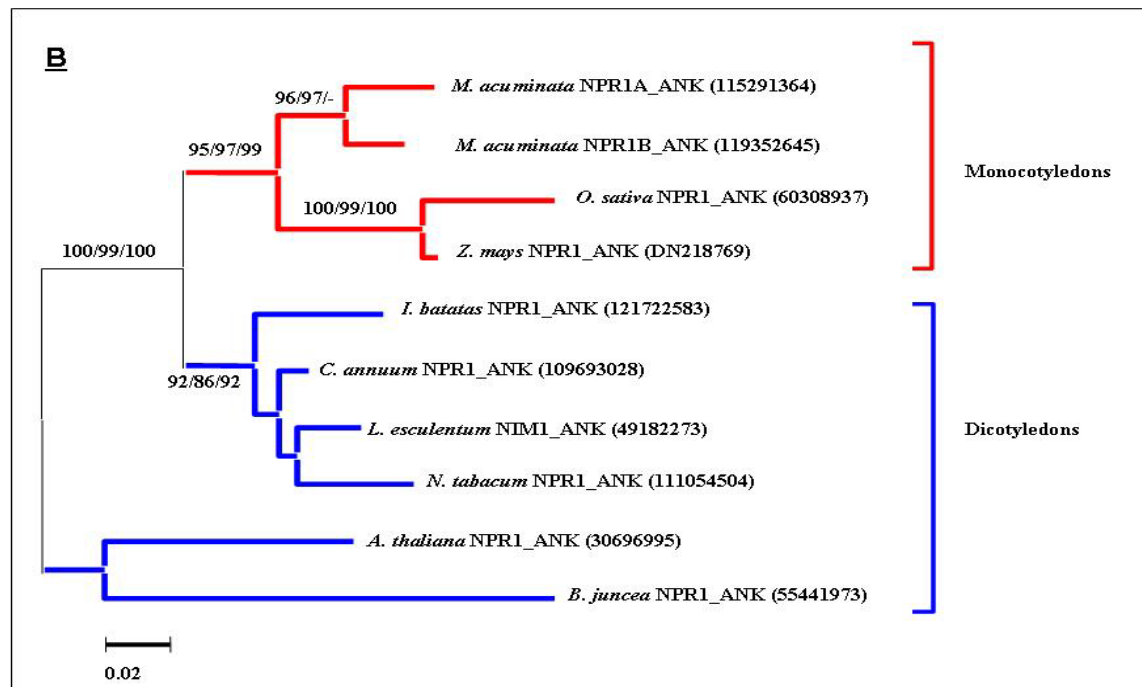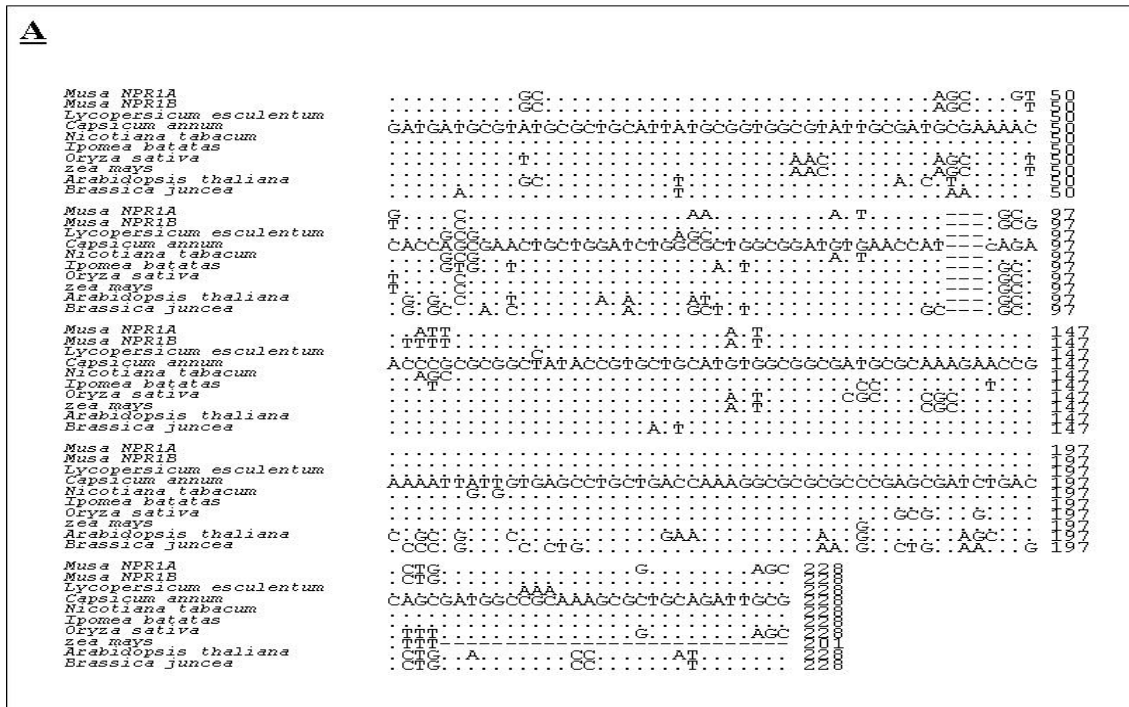
B

Figure 3.9 Multiple alignment and evolutionary relationship of the ankyrin repeats within the *NPR1* gene of various plants. Ankyrin repeat regions of the *NPR1* gene from *Musa*

acuminata (119352645, 115291364), *Arabidopsis thaliana* (30696995), *Brassica juncea* (55441973), *Lycopersicum esculentum* (49182273), *Ipomea batatas* (121722583), *Zea mays* (DN218769), *Oryza sativa* (60308937) were retrieved from Genbank and aligned using the Mafft software program (A). The alignment was then edited in BioEdit to obtain the correct reading frame. The evolutionary history was inferred using the Neighbor-Joining, maximum parsimony (MEGA4) and maximum likelihood (Phyml2.4.4) 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. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (B).

3.5   **_MNPR1 gene expression_**

3.5.1   Elicitor-induced _MNPR1_ gene expression

The expressions of the two _MNPR1_ genes as well as those of two _PR_ genes (_PR-1_ and _PR-3_) were analysed after infection with the economically significant pathogen _Fusarium oxysporum_ f.sp. _cubense_ (_Foc_) and after elicitor (SA and MeJA) treatment to elucidate if either a banana pathogen or elicitors induce _NPR1_ gene transcription and if transcription is directly related to _PR_ gene expression. Further, changes in the steady-state mRNA levels of the _MNPR1_ and _PR_ genes were determined in two banana cultivars, a tolerant _Foc_ cultivar (GCTCV-218) and a susceptible _Foc_ cultivar (Grand Naine).

SA treatment induced _MNPR1A_ and _MNPR1B_ gene expression in both the _Foc_ tolerant GCTCV-218 and the _Foc_ susceptible Grand Naine banana cultivar (Fig. 3.10A and 3.10B). However, _MNPR1A_ expression was not significantly ($P<0.05$) different from basal levels of expression in both banana cultivars over a 48 h period (Fig. 3.10A). _MNPR1B_ expression was significantly increased by SA treatment over a 48 h period in both Grand Naine and GCTCV-218. Induction in _MNPR1B_ expression was further 1.3-fold higher ($P<0.05$) in Grand Naine than in GCTCV-218 24 h after SA treatment. However, _MNPR1B_ expression further increased in GCTCV-218 and was significantly 3.2-fold ($P<0.05$) higher in GCTCV-218 than in Grand Naine 48 h after SA treatment (Fig. 3.10B).

In general, MeJA-induced *MNPR1B* expression was much lower than SA-induced *MNPR1B* expression. When plants were treated with MeJA, a 3.2-fold significant increase ($P<0.05$) in *MNPR1A* expression was observed 12 h after MeJA treatment in GCTCV-218 when compared to expression at the beginning of the treatment. This was followed by a decline in expression (Fig. 3.10C). In Grand Naine, no significant increase of *MNPR1A* expression was found (Fig. 3.10C). In GCTCV-218, a significant increase in *MNPR1B* expression (3.5-fold) ($P<0.05$) at 12 h after MeJA treatment was also observed which was followed by a decline in expression (Fig. 3.10D). In Grand Naine, this increase in *MNPR1B* expression was 2.9-fold ($P<0.05$) but only 48 h after the beginning of the MeJA treatment (Fig. 3.10D).

Figure 3.10 Relative gene expression levels in roots of plants of Cavendish banana cultivars GCTCV-218 (tolerant) and Grand Naine (susceptible). Gene expression was determined for *MNPR1A* and *MNPR1B* after treatment with 5 mM SA (A) and (B), and 50 μM MeJA (C) and (D), respectively. Samples were collected at 0, 12, 24 and 48 h after treatment with the respective elicitor. The experiment was repeated once; the relative expression was determined by quantitative RT-PCR and expressed relative to a 'calibrator', the expression level at 0 h. The relative expression ratios were plotted on the

graph. Results are means ± standard error of the mean (SEM) of six plants. *Significant difference at $P<0.05$ between the two banana cultivars.

SA treatment induced *PR-1* and *PR-3* gene expression in GCTCV-218 (Fig. 3.11A and 3.10B). *PR-1* expression in the tolerant GCTCV-218 was significantly higher ($P<0.05$) at 12 h (1.8-fold), 24 h (5.6-fold) and 48 h (4-fold) after SA treatment than in the susceptible Grand Naine cultivar (Fig. 3.11A). In Grand Naine, no significant increase in *PR-1* expression occurred over 48 h when compared to the beginning of the experiment. However, *PR-3* expression increased after SA treatment in both banana cultivars. Expression in GCTCV-218 was significantly higher ($P<0.05$) at 24 h (1.8-fold) and 48 h (1.5-fold) after SA treatment when compared to Grand Naine (Fig. 3.11B).

When *PR-1* and *PR-3* expression was measured in the two cultivars after MeJA treatment, *PR-1* expression significantly increased (10.9-fold) ($P<0.05$) 12 h post MeJA treatment in GCTCV-218 (Fig. 3.11C) when compared to the expression at the beginning of the experiment. This was followed by a sharp decline in *PR-1* expression. Such an increase in *PR-1* expression was not found for Grand Naine. In Grand Naine, *PR-3* expression significantly increased following MeJA treatment and expression was 13.9-fold ($P<0.05$) higher 48 h after MeJA treatment when compared to *PR*-3 expression at the beginning of the experiment (Fig. 3.11D). In GCTCV-218, no increase in *PR3* expression was found after MeJA treatment over 48 h.
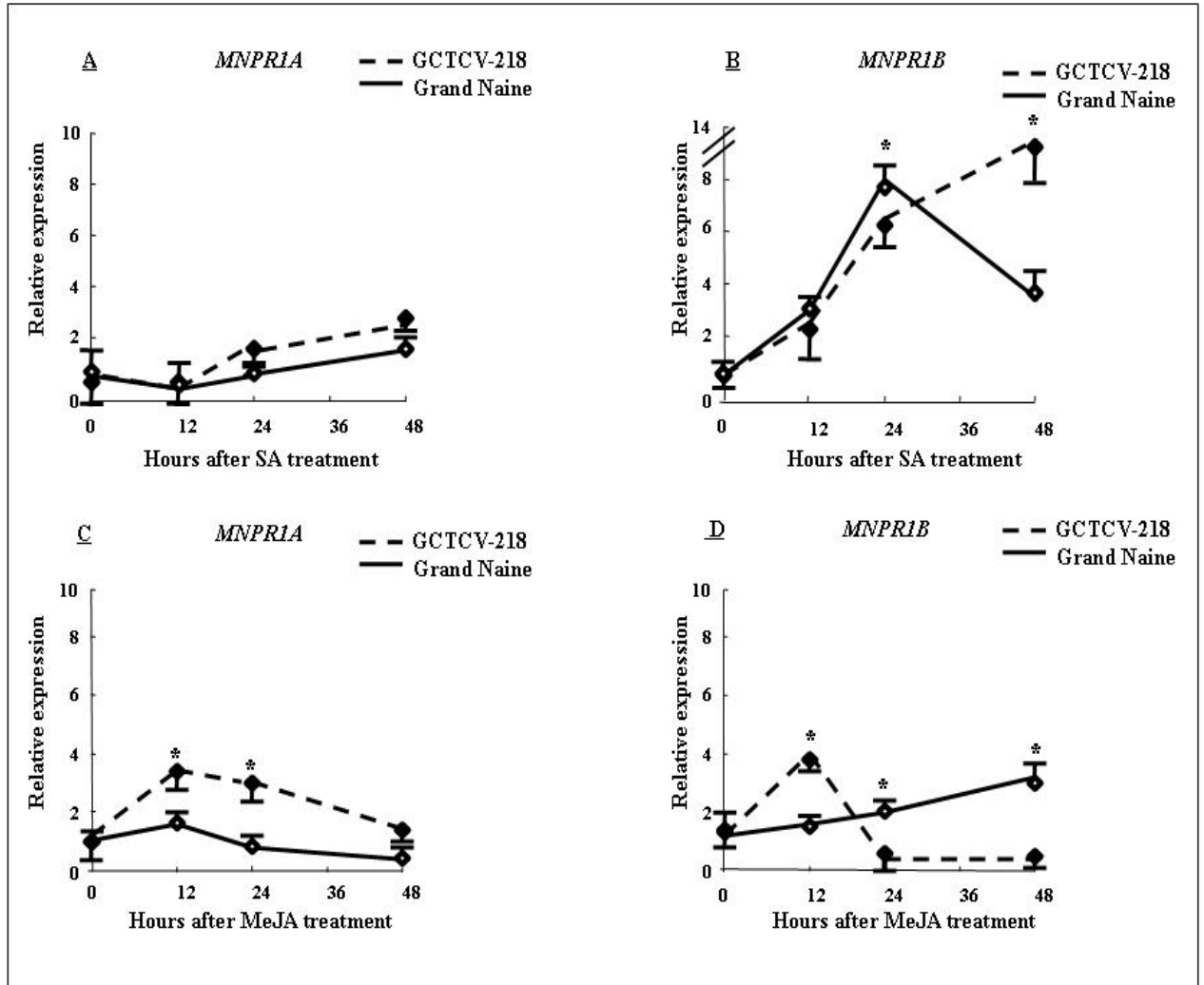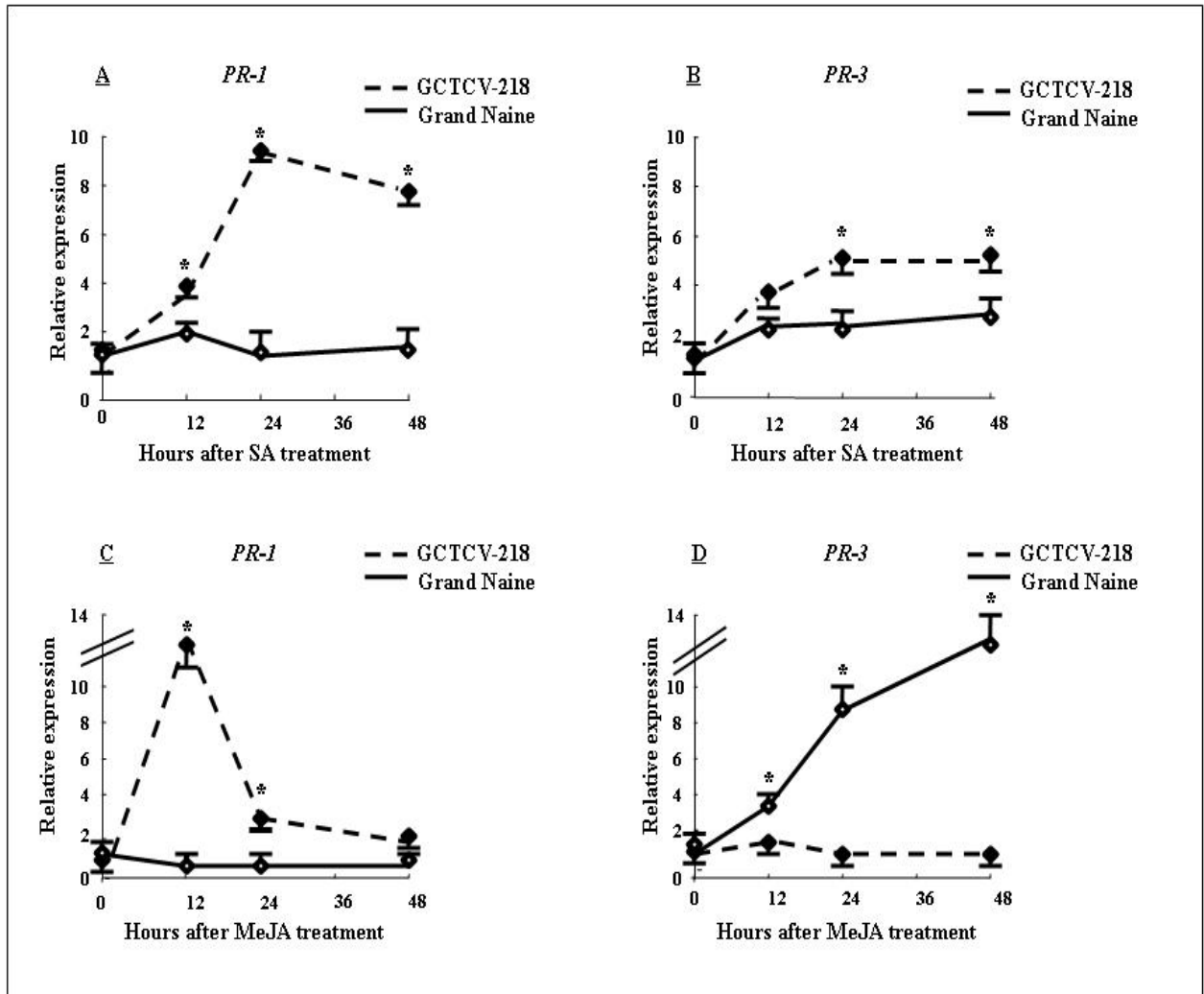
Figure 3.11 Relative gene expression levels in roots of plants of Cavendish banana cultivars GCTCV-218 (tolerant) and Grand Naine (susceptible). Gene expression was determined for *PR-1* and *PR-3* after treatment with 5 mM SA (A) and (B), and 50 μM MeJA (C) and (D), respectively. Samples were collected at 0, 12, 24 and 48 h after treatment with the respective elicitors. The experiment was repeated once; the relative expression was determined by quantitative RT-PCR and expressed relative to a 'calibrator', the expression level at 0 h. The relative expression ratios were plotted on the

graph. Results are means ± standard error of the mean (SEM) of six plants. *Significant

difference at $P<0.05$ between the two banana cultivars.

3.5.2 *Foc*-induced *MNPR1* gene expression

Expression of both *MNPR1* was found in response to *Foc* treatment in both banana cultivars (Fig. 3.12A and 3.11B). In Grand Naine, *MNPR1A* expression at 24 h after infection was significantly 1.9-fold higher ($P<0.05$) than *MNPR1B* expression (Fig. 3.12A). In GCTCV-218, this increase in *MNPR1A* expression was 14.7–fold higher ($P<0.05$) (Fig. 3.12B) when compared to *MNPR1B* expression but much earlier at 12 h after infection.

Both cultivars also expressed *PR-1* and *PR-3* in response to *Foc* treatment (Fig. 3.12C and 3.7D). However, Grand Naine expressed significantly more *PR-3* than *PR-1* with a 1.5-fold difference in expression ($P<0.05$) 24 h after infection (Fig. 3.12C). In GCTCV-218, significantly more *PR-1* than *PR-3* was expressed with a 3.9–fold difference in expression ($P<0.05$) (Fig. 3.12D) 12 h after infection. However, this increase in *PR-1* expression in GCTCV-218 was followed by a sharp decline at 24 h after treatment to near basal levels.
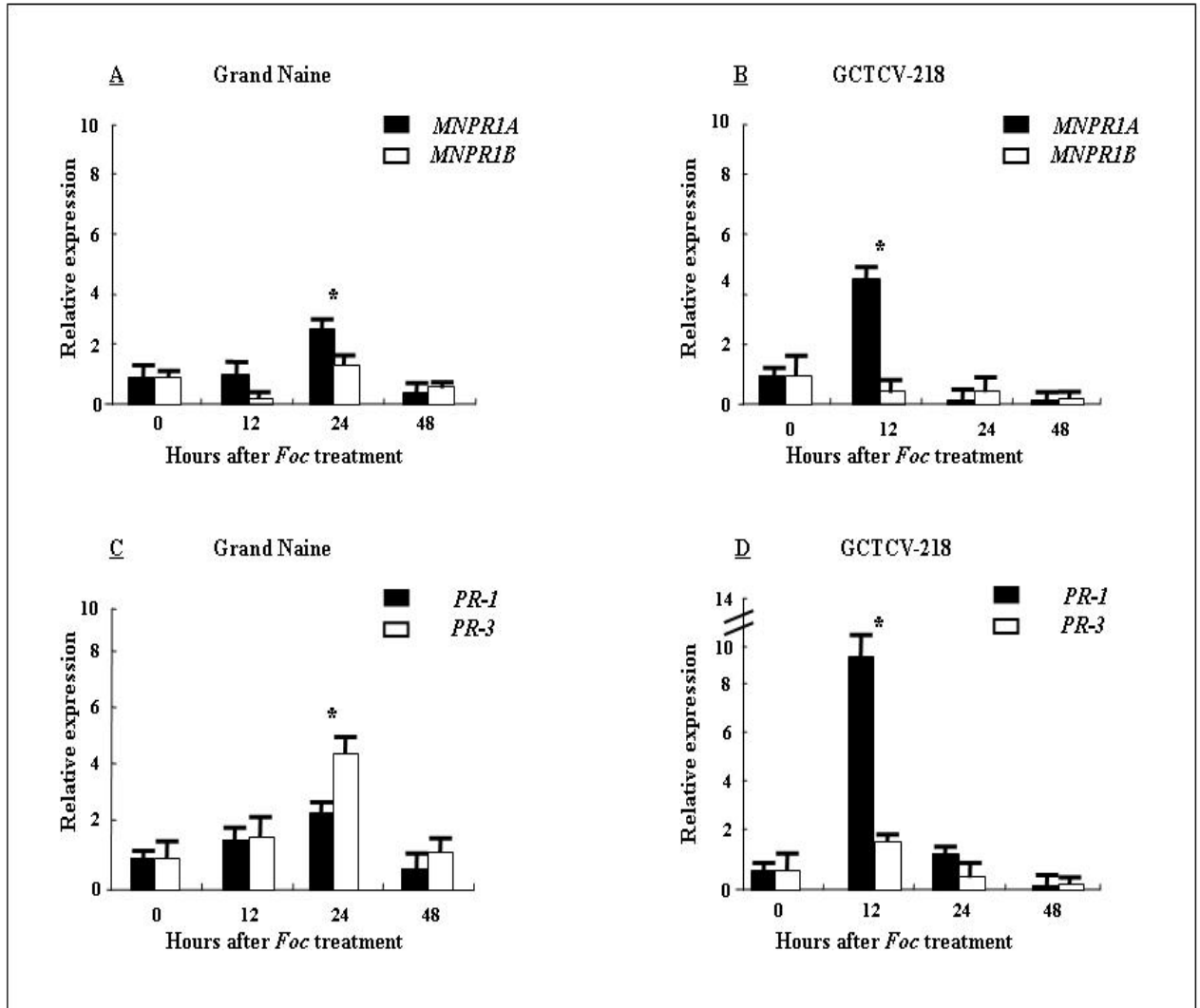
Figure 3.12 Relative gene expression of *MNPR1A* and *PR-1* (dark bars) and *MNPR1B* and *PR-3* (white bars) in *Foc*-infected roots of Cavendish banana cultivar Grand Naine (susceptible) and GCTCV-218 (tolerant). Samples were collected for analyses at 0, 12, 24 and 48 h after treatment. Relative gene expression of *MNPR1A* and *MNPR1B* (A and B) and *PR-1* and *PR-3* (C and D) was determined and compared in each of the cultivars. The experiment was repeated once; the relative expression was determined by quantitative RT-PCR and expressed relative to a 'calibrator', the expression level at 0 h. The relative expression ratios obtained from the only wounded control plants at each time point was

subtracted from those of the infected and wounded samples to obtain the effect due to infection only. The expression ratios due to infection were plotted on the graph. Results are means ± standard error of the mean (SEM) of six plants. *Significant difference at $P<0.05$ between *MNPR1A*/*MNPR1B* and *PR-1*/*PR-3*.

CHAPTER FOUR

Discussion

The cultivation of banana is hampered by numerous abiotic and biotic stress factors resulting in low productivity and reduced crop performance in many banana varieties. For instance, in the early 1950s, the production of Gros Michel banana plants was threatened to extinction by race 4 of the pathogen *Fusarium oxysporum* Schlecht f. sp. *cubense* (Smith) Snyd (*Foc*) the causal agent of *Fusarium wilt* (Ploetz and Pegg, 2000). This led to the introduction of the more resistant Cavendish varieties. However, evolution of the pathogen over time has caused some of the Cavendish banana cultivars to become sensitive to *Foc* causing wilting in plants (Stover and Buddenhagen, 1986). Strategies that are used to limit the spread and establishment of the disease in most banana plantations includes replacement of the susceptible banana varieties with more fungal-resistant varieties, the use of agrochemicals, crop rotation, soil amendment practices and planting of disease free tissue culture generated banana plantlets (Hwang and Ko, 2004; Heslop-Harrison and Schwarzacher, 2007). However, the detailed understanding of disease resistance mechanisms and the function of genes involved in this mechanism might ultimately help to select for resistance traits. Furthermore, such genes could be used for the improvement of resistance via genetic engineering approaches.

This study focused on the isolation and characterization of expression of the *NPR1* gene in banana. There is increasing evidence that the *NPR1* gene sequence might be a crucial component of broad-spectrum resistance in agronomically important crops like rice (Chern *et al*., 2000; Fitzgerald *et al*., 2004) ensuring expression of pathogenesis-related proteins. At the onset of the project there was no evidence for the existence of any *NPR1*

gene sequence in banana as no such sequence was published in the publicly available data bases. Through Southern blot analysis, the existence of *NPR1* gene sequences in the genome of four different banana cultivars belonging to either *Musa acuminata* (A genome) or *Musa balbisiana* (B genome) (Bluggoe (ABB), Tani (BB), FHIA 25 (AAA) and GCTCV-218 (AAA) could be established. Southern blotting is a technique commonly used for the detection of nucleic acid fragments in an agarose gel that are complementary to a specific RNA or DNA sequence (Primrose and Twyman, 2006). This technique is very important in gene identification and has previously been used to identify the presence of the *NPR1* gene in *Brassica juncea* (Meur *et al*., 2006). Detection of more than one hybridization product in banana possibly indicates the existence of more than one copy of the *NPR1* gene sequence within the banana genome irrespective of its genome annotation. However, the two selected DNA restriction enzymes used for genomic DNA digestion might have also contributed to the multiple banding patterns. Characterization of the full-length sequences of the banana *NPR1* genes revealed that both restriction sites (*Hind*III and *EcoR*I) are present in the banana *NPR1* sequences thereby suggesting a role of these enzymes in the production of the multiple banding patterns obtained for the banana samples. Further, the difference in banding patterns found with the banana samples used could have arisen due to sequence variation in banana resulting to a shift in the position of the restriction enzyme sites. Such shifts can account for the production of DNA fragments of different lengths after DNA digestion.

Although the isolation of an *NPR1* gene sequence from genomic DNA was unsuccessful, two novel distinct sequences, *MNPR1A* and *MNPR1B* have ultimately been isolated from

cDNA of wounded Grand Naine plants. Both sequences have been deposited in the GenBank. The failure to amplify the *NPR1* gene from banana genomic DNA could be due to the complex nature of the *Musa* genome and unspecific binding of the primers to areas not related to the banana *NPR1* gene. Compared to the genome size of other species like rice (390 Mb) and *Arabidopsis* (125 Mb), the genome size of banana is relatively large with the haploid genome ranging in size from 560 to 880 Mb (Lysak *et al*., 1999; *Arabidopsis* genome initiative, 2000; Kamate *et al*., 2001; Bartos *et al*., 2005; International rice sequencing project, 2005).

Sequence analysis of *MNPR1A* and *MNPR1B* further revealed typical features shared with other previously described *NPR1*-like gene sequences. This includes two identifiable protein-protein interaction motifs; a zinc finger and ankyrin repeat domains (ARD) (Cao *et al*., 1997; Ryals *et al*., 1997; Després *et al*., 2003). Further, the two sequences share a 78% similarity in their amino acid sequence but vary in their sequence from previously described *NPR1*-like gene sequences (Hepworth *et al*., 2005; Liu *et al*., 2005). Current speculations points to the fact that the two *MNPR1* sequences could be part of a newly discovered *NPR1* gene family in banana and that these genes are possibly involved in a variety of pathogen defense mechanisms like other *NPR1*-like gene sequences in other plants (*Arabidopsis* genome initiative, 2000; Liu *et al*., 2005).

So far, the genomic origin of the two banana sequences is unknown. Cultivated banana plants in the genus *Musa*, such as Grand Naine (AAA), are derived from the wild diploid banana species *M. acuminata* and *M. balbisiana* (Pillay *et al*., 2006) contributing either

the A or B genome, respectively. Cultivars resulting from this hybridisation are diploid (AA, AB, BB), triploid (AAB, AAA, ABB), or tetraploid (AAAB, AABB, ABBB) (Ortiz *et al.,* 1995 and Pillay *et al.,* 2006). In *Brassica juncea*, there is evidence that the two versions of *NPR1* originate from two individual parental genomes (*B. rapa* and *B. nigra*) (Meur *et al.,* 2006). However, since Grand Naine (AAA) only contains the A genome, this genome has very likely contributed both *MNPR1* gene sequences. Alternatively, intra-specific and inter-specific hybridisation of subspecies belonging to the *Musa* genus might have contributed to the overall genome of Cavendish banana resulting in a very complex genome (Bakry *et al.,* 2001; Carreel *et al.,* 2002) in which the A genomes are not identical.

This study also showed that the two banana sequences group more closely with other monocot *NPR1* sequences but less with known dicot sequences. Moreover, *NPR1* sequences from plants within the same family formed distinct sub-clades. These phylogenetic results are supported by previous studies that reveal that there is a closer relatedness between *Musa* and other monocot plants, such as rice, compared to *Musa* and dicot plants such as *Arabidopsis* (Cheung and Town, 2007). Furthermore, the close grouping found between *Arabidopsis* and *Brassica juncea* belonging to the same family (Brassicaceae) has recently been reported by Meur *et al.* (2006), who showed that the *Arabidopsis NPR1* gene is more related to the *NPR1* gene from *B. juncea*. However, these phylogenetic results are preliminary due to the unavailability of *NPR1*-related information in the GenBank.

This study further provided new evidence that the two banana *NPR1* gene sequences were expressed due to elicitor (SA and MeJA) or *Foc* treatment of banana plants. Previous work on the *NPR1* gene expression has revealed that perturbations in the cytoplasm, which either results during pathogen invasion or the accumulation of an elicitor, such as SA and JA, lead to the activation of the NPR1 protein (Cao *et al*., 1998). Monomeric active NPR1 proteins translocate to the nucleus where they bind to specific transcription factors, facilitating the binding ability of these transcription factors to *cis*-acting elements present at the promoter region of *PR* genes hence leading to the induction of these *PR* genes and subsequently defense responses (Bardwell and Treisman 1994; Kinkema *et al*., 2000). Expression of the two banana *NPR1* genes is consistent with findings of other research groups that *NPR1* is expressed when plants sense SA, MeJA or pathogen attack (Cao *et al*., 1997; Cao *et al*., 1998; Pieterse *et al*., 1998, Makandar *et al*., 2006, Yuan *et al*., 2007). However, in comparison to *MNPR1A*, *MNPR1B* was highly responsive to SA-treatment in both banana cultivars and to a much smaller degree to MeJA treatment. In a previous study with *B. juncea* plants, JA was ineffective in both *NPR1* and *PR-1* expression (Meur *et al*., 2006). However, in a recent study expression of the rice *OsNPR1* gene was found after MeJA treatment by Yuan *et al*. (2007). In general, JA pathways have been shown to be activated during herbivore and pathogen attack (Stout *et al*., 1999). Further, there is evidence that *NPR1* is also involved in cross-communication between SA- and JA-dependent defense signalling pathways (Pieterse and van Loon, 2004).

This study also showed that both *MNPR1A* and *MNPR1B* expression was associated with greatly increased *PR* gene expression in the more *Foc*-tolerant cultivar GCTVV-218. This increase was either gradual, as a response to SA treatment, or rapid followed by a sharp decline as a response to MeJA treatment. A similar expression profile was observed in the response of *MNPR1B* to MeJA treatment. In contrast, *PR-3* was highly responsive to MeJA treatment but only in the more *Foc*-sensitive cultivar Grand Naine. Future research has therefore to show if *MNPR1B*, in comparison to *MNPR1A*, is more prominently involved in *PR-3* expression.

In this study, there was also a clear difference in *MNPR1A* and *MNPR1B* expression following *Foc* treatment. *MNPR1A*, but not *MNPR1B*, was more responsive in both cultivars to treatment with *Foc*. Response to *Foc* treatment was also earlier and of a higher magnitude in the more *Foc*-tolerant cultivar GCTCV-218 than in the more *Foc*-sensitive cultivar Grand Naine. Similar observations were also made on the expression of *PR-1* in GCTCV-218 and *PR-3* in Grand Naine. Less sensitivity to *Foc* in GCTCV-218 might be partially attributed to a higher and an earlier expression of both *MNPR1A* and *PR-1* in this cultivar after *Foc* treatment. This response of *PR-1* has also been reported for GCTCV-218 after treatment with *Foc* (Van den Berg *et al*., 2007). There is evidence that necrotrophic pathogens, such as Fusarium, elicit the JA/ethylene-dependent pathway, whereas biotrophic pathogens elicit a SA-dependent pathway (Pieterse and Van Loon 1999; Thaler *et al*., 2004). Recent analysis in *Arabidopsis* further revealed that resistance to *F. oxysporum* requires, besides the ethylene, JA, and SA signalling pathways, also involves the *NPR1* gene (Berrocal-Lobo and Molina, 2004). Since a pronounced response

by *MNPR1A* to SA or MeJA treatment was found in comparison to the high response that was found for *MNPR1B* after SA treatment, it might be speculated that *MNPR1A* might be more responsive to the ethylene dependent pathway when treated with *Foc*.

Future perspective

So far this study did not provide direct evidence that expression of the *MNPR1* genes also confers resistance against banana pathogens. Thus, future work should entail a functional characterization study to investigate the role of the two genes in diseases resistance. This should include transformation of *Arabidopsis*, rice and/or banana itself and challenging the resulting transgenics with a variety of pathogens. An ideal way to determine gene function would be to silence the genes by using for example RNAi induced gene silencing, and then challenging the resulting loss of function transgenics with various classes of pathogens. Further, a more detailed promoter analysis should be carried out. The full length promoters of each of the *MNPR1* genes should be isolated and characterised in order to ascertain the functional domains within the promoter and to understand how this helps in the general activation process of the genes. This might involve isolation of promoter fragments of various lengths for both the *MNPR1A* or *MNPR1B* genes, and to determine their ability to drive expression of the *beta-glucuronidase* (*GUS*) gene or another easily detectable reporter gene system such as the green fluorescence protein (GFP). Gene cassettes comprising the full length and fragments of the *MNPR1* promoters should be transformed into plants, which should subsequently be challenged with various pathogens and exposed to various elicitors, and

the spatial and quantitative expression of *GUS* gene determined. This will shed light on the environmental conditions that affect the expression of the two *Musa* genes.

Results from such work would clearly indicate whether *MNPR1A* or *MNPR1B* is more active during pathogen infection and which of the *MNPR1* genes is directly responsible for the greater induction of *PR* genes. The different defense pathways mediated by the *MNPR1* genes will be characterised to determine if, for example, *MNPR1A* is responsive to nectrophic and *MNPR1B* to biotrophic pathogens (or vice versa) as a result of exposure to *Xanthomonas* or *Fusarium*. Such a study would further contribute to the understanding of the potential of this pathway in conferring broad-spectrum resistance to pathogens and in partiular how this can be used to enhance banana improvement programs.

I   **General solutions and buffers**

I.I   Antibiotics

I.I.I   Ampicillin stock solution (50 mg/mL)

Dissolve 2 g Ampicillin (D(-)-a-Aminobenzylpenicillin sodium salt) (Sigma-Aldrich, Germany) powder in 40 mL of sterile distilled water and filter steralize. Aliquot in 1.5mL Eppendorf tubes and store at -20°C.

I.II   Southern blotting solutions

I.II.I   Neutralization buffer

Combine 121.1 g of Tris base (Sigma-Aldrich, Germany) and 87.7 g NaCl. Add 900 mL distilled water. Adjust pH to 8.0 with concentrated HCl. Adjust volume with distilled water to 1 L and autoclave.

I.II.II   Denaturing buffer

Combine 87.7 g NaCl and 20 g NaOH. Add distilled water to 1 L. Stir to dissolve and autoclave.

I.II.III   20X Sodium Saline-Citrate (SSC)

Combine 174.4 g NaCl, 88.3 g $C_6H_5Na_3O_7.2H_2O$ (Tri Sodium-citrate) in 800 mL distilled water. Stir to dissolve and adjust volume to 1 L. Autoclave.

I.III   Buffers and salt solutions

I.III.I   Agarose gel buffer (TAE) (50X stock)

Combine 968 g Tris base, 228.4 mL glacial acetic acid and 400 mL 0.5 M EDTA, pH 8.0. Add 3 L distilled water and stir until solids dissolve. Adjust volume to 4 L with distilled water. Dilute to 1X before use.

I.III.II   Agarose gel loading buffer (6X)

Combine 0.063 g bromophenol blue (BPB), 0.063 g xylene cyanol FF (XC) and 2.5 mL glycerol. Add distilled water to 25 mL. Aliquot into 1.5 mL tubes and store at -20°C. Add 1 μL to sample before loading onto agarose gel.

I.III.III   3 M Sodium acetate (NaOAc) pH 4.8

Dissolve 40.82 g NaOAc.3H$_2$O in 80 mL distilled water and stir on a hot plate until dissolved. Adjust pH to 4.8 with glacial acetic acid. Adjust volume to 100 mL with distilled water. Autoclave and store at room temperature (RT).

I.III.IV   5 M Sodium chloride

Dissolve 29.22 g NaCl in 80 mL distilled water. Adjust volume to 100 mL, autoclave and store at RT.

I.III.VI   1 M Tris-HCl pH 8.0

Dissolve 12.11 g Tris base in 80 mL of distilled water. Adjust pH to 8.0 using concentrated HCl. Adjust volume to 100 mL with distilled water. Autoclave and store at RT.

I.III.VII   0.5 M EDTA pH 8.0

Dissolve 8 g NaOH pellets in 400 mL distilled water. Add 93.05 g Na$_2$EDTA.2H$_2$O and allow to dissolve. Adjust pH to 8.0 using more NaOH pellets. Adjust volume to 500 mL with distilled water. Autoclave and store at RT.

I.III.VIII   10 N Sodium hydroxide (NaOH)

Dissolve 40 g NaOH pellets in 70 mL distilled water. Adjust volume to 100 mL. Store at RT.

II.   Plasmid maps

II.I   Map of plasmid pCR®4-TOPO® T/A cloning vector (Invitrogen, USA)
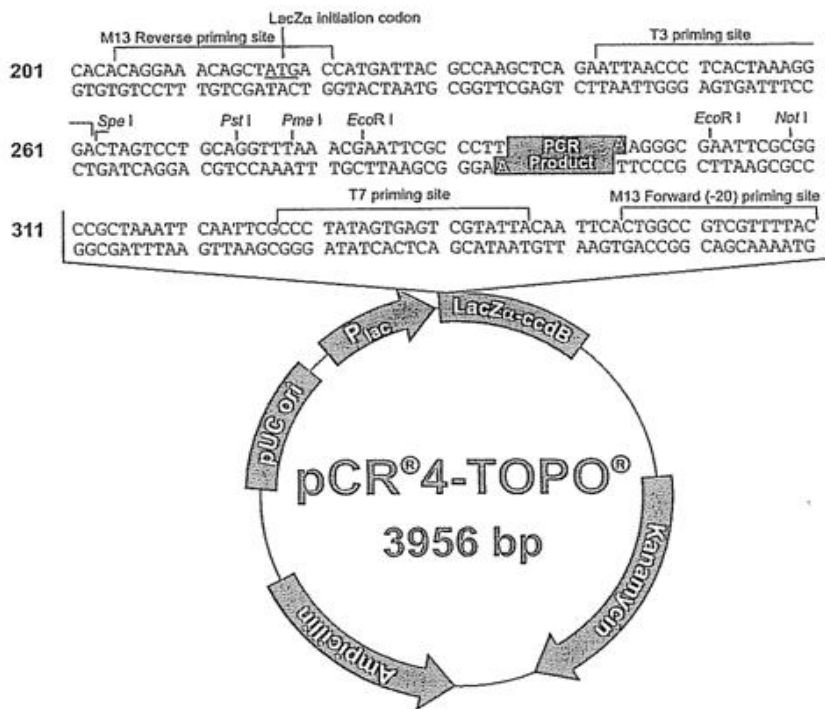
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