# CHAPTER ONE

## INTRODUCTION

## 1.1 Plant defense responses

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

## 1.2 Types of defense responses

### 1.2.1 Basal resistance or innate immunity

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

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

### 1.2.2 Hypersensitive response

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

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

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

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

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

### 1.2.3 Systemic resistance

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

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

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

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

## 1.3 Deciphering metabolic components of the defense network

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

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

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

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

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

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

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

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

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

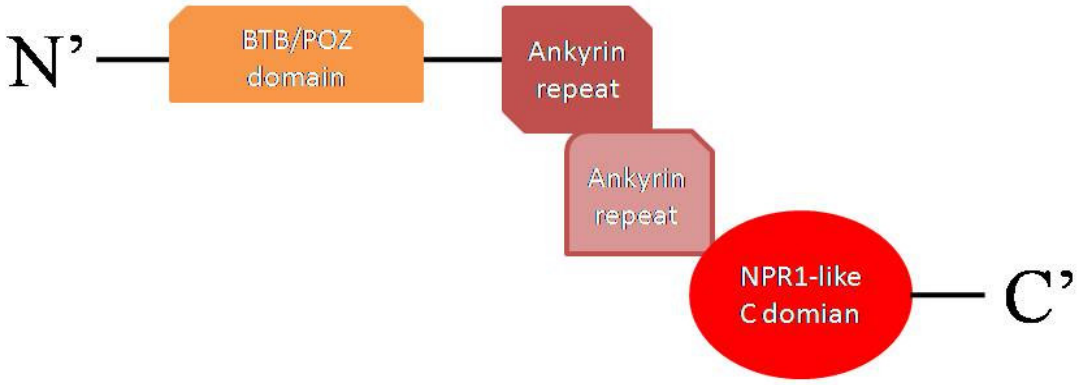


**Figure 1.1** *At*NPR1 protein structure representing three of its functional domains. The *Arabidopsis thialana* NPR1 protein sequence was obtained from genbank (At1g64280) and the conserved regions deduced from the sequence. From the N-terminal (N') to the C-terminal (C') is found the Bric-a-Brack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

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

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

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

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

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

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

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

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

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

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

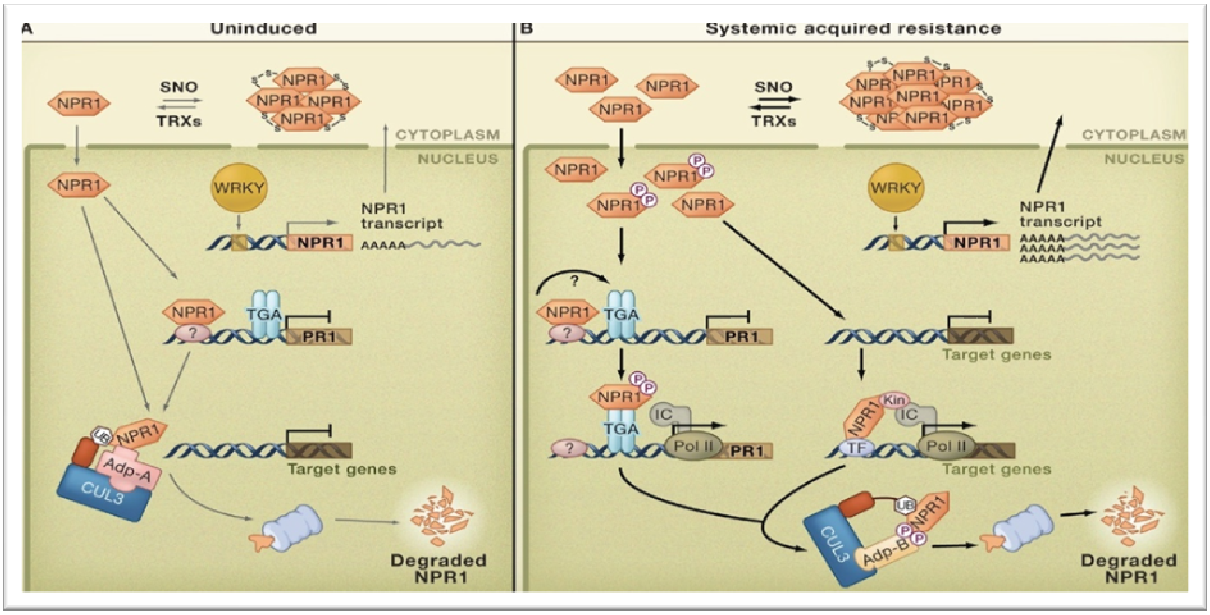


**Figure 1.2** *Arabidopsis thialana* NPR1 pathway (adapted from Mukhtar *et al*., 2009). In a non-induced state (A), *A. thaliana* NPR1 proteins exist predominantly as oligomers held by disulphide bonds in the cytosol. Cytosolic oligomerization is maintained by S-nitrosylation of monomeric NPR1. Few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

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

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

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

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

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

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

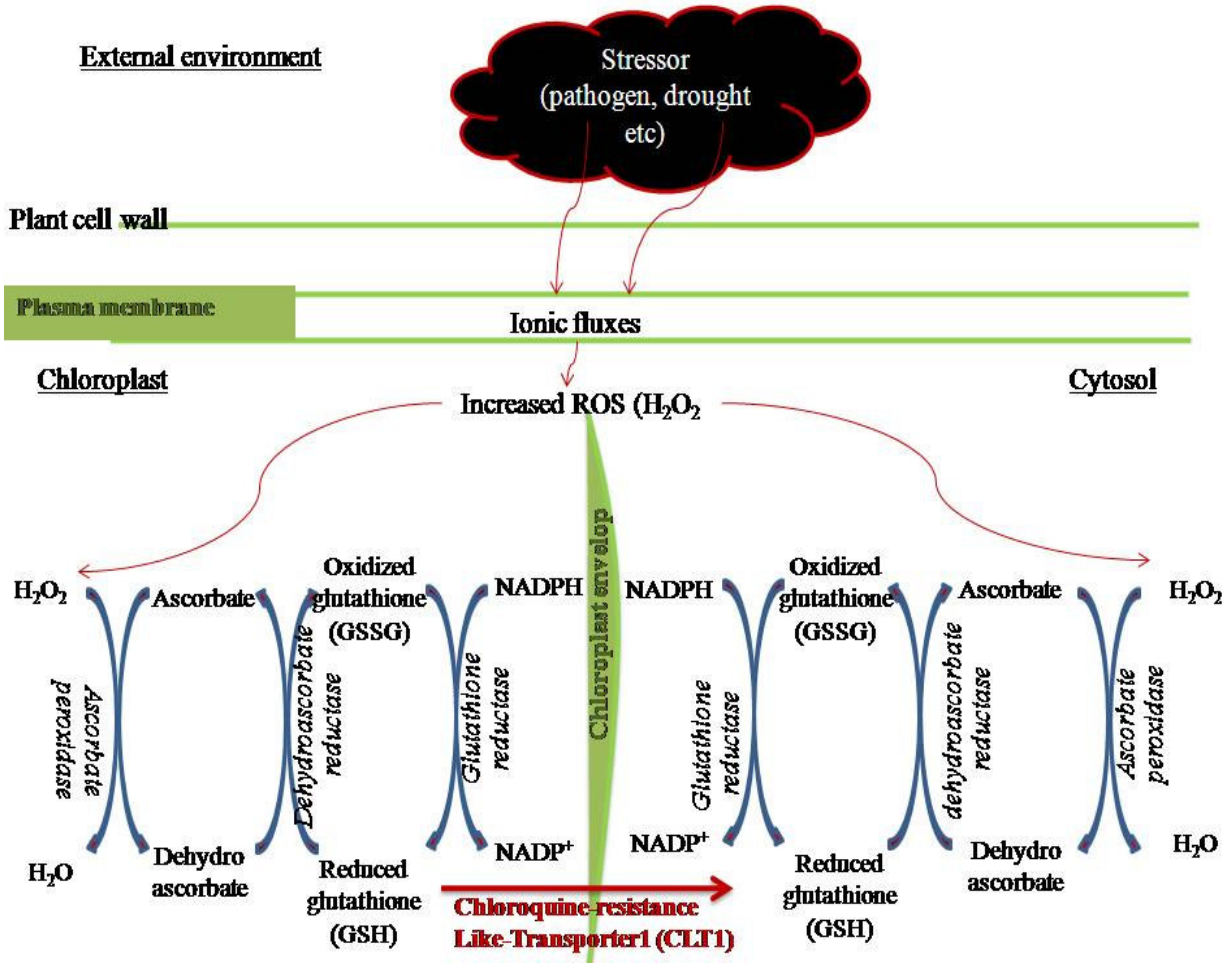


**Figure 1.3** The ascorbate-glutathione cycle (adapted from Smirnoff, 1996; Noctor and Foyer 1998; Asada, 2000). Upon stress perception, changes in ionic fluxes at the plasma membrane triggers an increase in hydrogen peroxide ($H_2O_2$). Detoxification of $H_2O_2$ into water is facilitated by ascorbate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

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

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

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

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

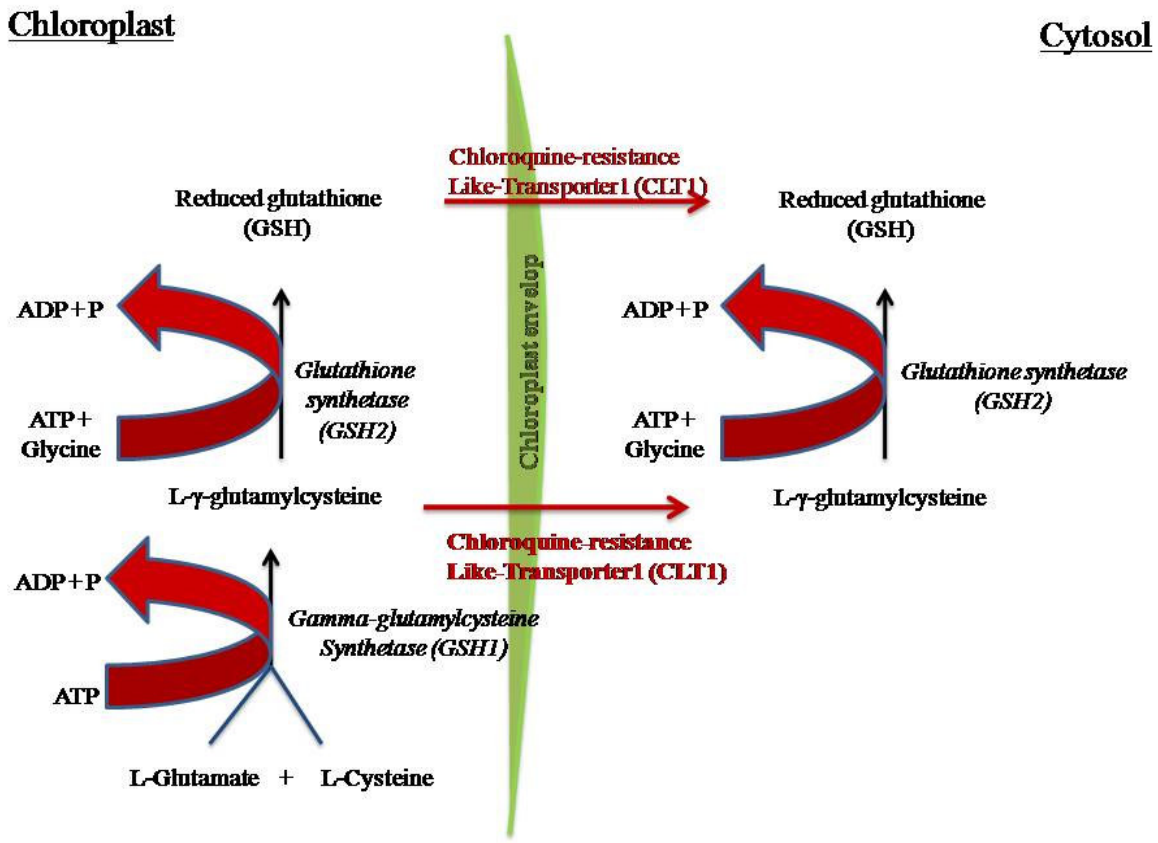


**Figure 1.4** GSH biosynthesis and transportation across the chloroplast. During the synthesis of GSH which begins in the chloroplast, L-glutamine together with L-cysteine react in the presence of gamma-glutamylcysteine synthetase (γ–ECS or GSH1) in a reaction requiring ATP, leading to the formation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

### *1.3.4.2 Phytohormones and NPR1 interaction*

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

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

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

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

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

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

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

#### *1.3.4.3 Transcription factors and NPR1*

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

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

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

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

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

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

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

## 1.4 Working hypothesis and aim of study

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

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

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

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

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