Molecular and bioinformatic analysis of the *Persea americana* (Mill.) NPR1-dependent defence signalling pathway in response to *Phytophthora cinnamomi* infection

by

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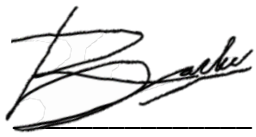
Declaration

I, Robert Backer, declare that the dissertation, which I hereby submit for the degree Ph.D. at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



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Summary

The global avocado industry has experienced significant growth throughout the past two decades, with annual production doubling over that time. However, increased production is accompanied by an ever-increasing threat from a variety of pests and diseases. Phytophthora root rot (PRR) is currently considered the most devastating disease of avocado, causing significant economic losses annually. The causal agent, *Phytophthora cinnamomi*, is a hemibiotrophic oomycete; as such, it utilises both biotrophic and necrotrophic infection strategies to overwhelm its host. Plants use numerous phytohormone regulated defence response pathways, depending on the infection strategy employed by their pathogen. Typically, defence against biotrophic pathogens applies the salicylic acid (SA)-dependent defence response pathway; whereas defence against necrotrophic pathogens is associated with the jasmonic acid/ethylene (JA/ET)-dependent pathway.

Notably, the nonexpressor of pathogenesis-related genes 1 (NPR1) co-transcription factor is crucial to most SA-dependent defence gene expression. Furthermore, it is essential to the establishment of systemic acquired resistance, a plant-wide state of heightened defence readiness. However, the mechanisms required to achieve SAR and effectively defend against different pathogens is exceedingly complex. Therefore, this dissertation aimed to identify and characterise NPR1-like proteins in *Persea americana* (avocado). Furthermore, this study attempted to understand the response of several NPR1 pathway-associated genes, both over-time and comparatively between PRR susceptible and partially resistant avocado rootstocks.

A total of five *NPR1-like* coding genes were described in *P. americana*. Initial *in silico* analyses suggested that three PaNPR1-like proteins could be involved in defence; two of these, PaNPR1 and PaNPR2, were likely associated with positive SAR regulation while PaNPR4 probably served an opposing role. Meanwhile, the remaining two, PaNPR3 and PaNPR5, were most likely involved in tissue development. These suspicions were later confirmed by expression analysis following phytohormone application, *P. cinnamomi* inoculation and tissue-specific sampling. Interestingly, significant differences were observed when comparing the expression

of the several *PaNPR1-like* genes in the PRR susceptible (R0.12) and partially resistant (Dusa®) rootstocks.

Therefore, we identified and annotated 116 orthologs of *Arabidopsis thaliana* NPR1 pathway genes in the *P. americana* genome. Using dual RNA-sequencing data, we characterised the expression of all 116 genes over time in the PRR susceptible rootstock R0.12. Additionally, we compared the expression of the NPR1 pathway-associated genes between R0.12 and the partially PRR resistant rootstock, Dusa®. Our observations suggest that SAR was established in both avocado rootstocks; additionally, expression of the majority of NPR1 pathway-associated genes is regulated to some extent following *P. cinnamomi* challenge. However, significant differences were evident when comparing expression in R0.12 and Dusa®. Primarily, our observations suggest that the SA-defence response pathway is suppressed more effectively in Dusa® following the establishment of SAR. Thus, Dusa® likely responds more appropriately to the pathogen's necrotrophic phase of infection.

The work presented here represents the first step in fully characterising and understanding the NPR1 pathway in *P. americana* and its role in resistance against PRR. Furthermore, we believe that this study will form part of the foundation for further functional characterisation of disease resistance pathways in avocado.

Preface

Chapter 1, entitled "The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and Related Family: Mechanistic Insights in Plant Disease Resistance" attempts to concatenate existing research on distinct aspects of the NPR1 pathway into a palatable mechanistic model. The proposed model includes information on regulation of the *NPR1-like* family during defence responses as well as post-translational modification and the key role players therein. Additionally, these concepts tie into the expression of genes related to systemic acquired resistance and includes less directly-associated topics such as the priming of defence responses, transgenerational immune memory, the role of endoplasmic reticulum resident genes and potential uses in transgenic crops. This work has been published as a review article in Frontiers in Plant Science.

Chapter 2 describes, for the first time, the discovery and partial characterisation of five *NPR1-like* genes in *P. americana*. The observations from this chapter strongly suggest that three *PaNPR1-like* genes might be involved in defence responses; meanwhile, the remaining two are likely to play a role in tissue development. Lastly, comparisons between susceptible and partially resistant rootstocks provides the first evidence that the *P. americana* NPR1 pathway could be a determining factor in resistance against *P. cinnamomi*. This work has been published in Frontiers in Plant Science and is entitled "Identification and analysis of the *NPR1*-like gene family from *Persea americana* (Mill.)"

Therefore, chapter 3 focuses on identifying and characterising NPR1 pathway-associated genes in *P. americana* using genome and dual RNA-sequencing data. This chapter aimed to determine how the *P. americana* NPR1 pathway is regulated over time during *P. cinnamomi* challenge; moreover, whether NPR1 pathway-associated genes are expressed differently in susceptible and partially resistant avocado rootstocks. The majority of NPR1 pathway-associated genes were regulated in agreement with our expectations based on the literature. Additionally, the data strongly suggest that SAR is established, in response to *P. cinnamomi* inoculation, in both susceptible and partially resistant rootstocks. However, suppression of the SA defence response pathway, following the initiation of SAR, seemed to be more

effective in the partially resistant rootstock. Thus, further supporting the notion that regulation of the SA-dependent NPR1 pathway is a deciding aspect in PRR resistance.

The final discussion, study limitations and future work are addressed in chapter 4.

List of Acronyms

ABA	abscisic acid
ADH	alcohol dehydrogenase
BD	binding domain
BiP	luminal-binding protein
BLAST	basic local alignment search tool
BOP	blade-on-petiole
BSMT	benzoic acid/salicylic acid carboxyl methyltransferase
BTB/POZ	Broad Complex, Tramtrack and Bric a brac/Pox virus and Zinc finger
bZIP	basic leucine zipper
CBL	calcineurin B-like
cDNA	complementary DNA
CDS	coding sequence
CIPK	CBL-interacting serine/threonine-protein kinase
CRT	calreticulin
C_t	threshold cycle
CTAB	cetyltrimethylammonium bromide
CUL	cullin
DAD	defender against apoptotic death
DAMP	damage-associated molecular pattern
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dpi	days post-inoculation
E	Efficiency
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ET	ethylene
ETI	effector-triggered immunity

ETS	effector-triggered susceptibility
GSNO	*S*-nitrosoglutathione
GSNOR	*S*-nitrosoglutathione reductase
HDAC	histone deacetylase
hpi	hours post-inoculation
HR	hypersensitive response
hr	hour/s
HSF	heat shock factor
ICS	isochorismate synthase
INA	2,6-dichloroisonicotinic acid
IPL	isochorismate pyruvate lyase
ISR	induced systemic resistance
JA	jasmonic acid
JAZ	jasmonate zim domain
KEGG	Kyoto encyclopaedia of genes and genomes
LDR	linear dynamic range
LHY	late elongated hypocotyl
LSD	least significant difference
MAMP	microbe-associated molecular pattern
MeSA	methyl salicylate
MeSAG	methyl salicylate O-β-glucoside
ML	maximum likelihood
MPK	mitogen-activated protein kinase
NBS-LRR	nucleotide-binding site leucine-rich repeat
NCBI	National Center for Biotechnology Information
NIMIN	NIM(NPR1)-interacting
NJ	neighbour joining
NLS	nuclear localisation signal
NO	nitric oxide
NPR	nonexpressor of pathogenesis-related genes

PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
PAN	perianthia
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogenesis-related
PRR	Phytophthora root rot
PTI	PAMP-triggered immunity
qPCR	quantitative polymerase chain reaction
R^2	coefficient of determination
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
SA	salicylic acid
SAG	SA O-β-glucoside
SAR	systemic acquired resistance
SEM	standard error of the mean
SGE	salicyloyl glucose ester
SNF	sucrose non-fermenting
SnRK	SNF-1 related protein kinase
SUMO	small ubiquitin-like modifier
T_a	annealing temperature
TAE	tris-acetate-EDTA
TF	transcription factor
TGA	TGACG-binding
TOC	timing of cab2 expression
TPM	transcripts per million
TRX	thioredoxin
UV	ultra-violet

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Chapter 1

Review Article

frontiers in Plant Science

REVIEW
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The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and Related Family: Mechanistic Insights in Plant Disease Resistance

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The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and related NPR1-like proteins are a functionally similar, yet surprisingly diverse family of transcription co-factors. Initially, NPR1 in *Arabidopsis* was identified as a positive regulator of systemic acquired resistance (SAR), paralogs NPR3 and NPR4 were later shown to be negative SAR regulators. The mechanisms involved have been the subject of extensive research and debate over the years, during which time a lot has been uncovered. The known roles of this protein family have extended to include influences over a broad range of systems including circadian rhythm, endoplasmic reticulum (ER) resident proteins and the development of lateral organs. Recently, important advances have been made in understanding the regulatory relationship between members of the NPR1-like protein family, providing new insight regarding their interactions, both with each other and other defense-related proteins. Most importantly the influence of salicylic acid (SA) on these interactions has become clearer with NPR1, NPR3, and NPR4 being considered *bone fide* SA receptors. Additionally, post-translational modification of NPR1 has garnered attention during the past years, adding to the growing regulatory complexity of this protein. Furthermore, growing interest in *NPR1* overexpressing crops has provided new insights regarding the role of NPR1 in both biotic and abiotic stresses in several plant species. Given the wealth of information, this review aims to highlight and consolidate the most relevant and influential research in the field to date. In so doing, we attempt to provide insight into the mechanisms and interactions which underly the roles of the NPR1-like proteins in plant disease responses.

Keywords: NPR1, NPR1-like, systemic acquired resistance, salicylic acid, plant disease, *pathogenesis-related*

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INTRODUCTION

The *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES* 1 (*NPR1*), as well as *PATHOGENESIS-RELATED* (*PR*) genes, play a fundamental role in a plant's response to pathogen challenge. NPR1 plays a significant role in the establishment of systemic acquired resistance (SAR) as well as induced systemic resistance (ISR) (Pieterse et al., 1998); it acts as the master key to the plant defense signaling network, mediating cross-talk between the salicylic acid (SA) and jasmonic

acid/ethylene (JA/ET) responses. Constitutive *NPR1* expression within wild-type *Arabidopsis thaliana* ensures a quick response to SA (Cao et al., 1998). NPR1 is then translocated primarily to the nucleus where it indirectly activates *PR* gene expression by recruiting TGA transcription factors (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Kim and Delaney, 2002). The exact mechanisms involved in NPR1 activation, as well as NPR1-dependent/independent *pathogenesis-related* (*PR*) gene expression and the overall role of NPR1 in pathogen defense are important topics of study.

The mechanism by which SA activates NPR1 is not completely understood, yet a lot has been uncovered in recent years. During non-stress conditions, NPR1 can be found as a large cytoplasmic oligomer (Mou et al., 2003). An oxidative burst observed during SA-induced SAR results in a reducing environment as the cell recovers (Mou et al., 2003). This redox state then contributes toward NPR1 monomerization, nuclear localization and *PR* gene expression. Activation of various TGA transcription factors occurs under these conditions (Despres et al., 2000). SA is believed to achieve reducing conditions in two stages: (1) induction of oxidative stress reducing genes (2–3 h after SA treatment) and, (2) NPR1 dependent *PR* gene expression (12–16 h after SA treatment) (Horvath and Chua, 1996; Dong, 2004; Uquillas et al., 2004). Expression of *PR* genes are essential for the development of SAR, *Arabidopsis* mutants deficient in NPR1 show reduced *PR* gene expression and increased susceptibility to pathogens (Cao et al., 1994; Roetschi et al., 2001). Hence NPR1 plays an integral part in the efficacy of plant immune responses.

In studied plant species, two to six *NPR1*-like genes have been found. This family of proteins contain ankyrin repeats and Broad Complex, Tramtrack and Bric a brac/Pox virus and Zinc finger (BTB/POZ) domains, two well documented protein–protein interaction domains (Bardwell and Treisman, 1994; Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999; Hepworth et al., 2005; Li et al., 2006; Spoel et al., 2009). Phylogenetic analysis separates the NPR1-like proteins into three distinct clades, suggesting functional divergence (Hepworth et al., 2005; Zhang et al., 2006). Yet, significant overlap in clade function can be found within the first two clades involved in positive and/or negative SAR regulation (Liu et al., 2005; Le Henanff et al., 2009). The third clade seems to be involved in the development of growing tissues (Hepworth et al., 2005). The varied functional role of NPR1-like proteins suggests a complex functionally important family involved in plant immune responses and development. Thus, this review aims to examine and consolidate these functions, providing mechanistic insights regarding pathogen response.

OVERVIEW OF PLANTS RESPONSES TO PATHOGENS

The interaction between plants and their pathogens has been studied in some detail. Plant–pathogen interactions involve a wide variety of systems on both sides, the balance of which determines the success of either the host or the pathogen (Lodha and Basak, 2012). Compatible interactions occur when a plant is unable to coordinate effective defense responses, enabling the pathogen to colonize and proliferate within the host (Schenk et al., 2000). In contrast, an incompatible interaction occurs when defense responses are sufficient at preventing spread of the pathogen within host tissues (Hammond-Kosack and Jones, 2000). Successful plant immunity relies on both non-specific preformed and inducible defense mechanisms as well as specific induced immune responses. The first line of defense includes physical barriers such as waxy layers, rigid cell walls, antimicrobial compounds and secondary metabolites (Agrios, 2005; Reina-Pinto and Yephremov, 2009). Microbes which overcome these preformed defenses trigger the next line of immune responses. The first of these, pattern-triggered immunity (PTI) is induced by the action of pathogen-recognition receptors (PRRs) which recognize microbe-associated molecular patterns (MAMPs) preventing further invasion of host tissues (Jones and Dangl, 2006; Zipfel, 2009). Additionally, a subset of molecules referred to as damage-associated molecular patterns (DAMPs), are passively released from damaged native plant tissue and capable of activating and perpetuating innate immune responses (Choi and Klessig, 2016).

With the aim of overcoming PTI, pathogens secrete effector molecules which target specific host proteins, manipulating host processes with the purpose of enhancing virulence, a state referred to as effector triggered susceptibility (ETS) (Jones and Dangl, 2006). In response, intracellular resistance (R) proteins, most of which are nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, monitor the status of effector targeted plant proteins or bind directly to them, initiating defense responses in case of attack (Van der Biezen and Jones, 1998; Jia et al., 2000; Dangl and Jones, 2001; Deslandes et al., 2003). These processes initiate effector triggered immunity (ETI), most often characterized by rapid localized programmed cell death (PCD) also known as the hyper-sensitive response (HR), which prevents further spread of the pathogen (Goodman and Novacky, 1994; Van Loon, 1997; Jones and Dangl, 2006). Tissues distal to the initial site of infection experience an increased accumulation of several defense signals, including SA (An and Mou, 2011; Fu and Dong, 2013). Subsequently, systemic production of a collection of pathogen-induced antimicrobial proteins known as PR proteins increase, which enhance resistance to a variety of pathogens (Van Loon and Van Strien, 1999; Durrant and Dong, 2004). This signifies the establishment of SAR, a long lasting systemic broad spectrum resistance which is effective at preventing infection by a wide variety of pathogenic bacteria, fungi, oomycetes, viruses and nematodes (Ryals et al., 1996; Sticher et al., 1997). These basic defenses are intricately interwoven with numerous interactions both within and amongst pathways, which through coordinated signaling events comprise plant immunity.

SALICYLIC ACID AND PHYTOHORMONE CROSS-TALK

Salicylic acid is a phenolic compound produced by various prokaryotes and eukaryotes (An and Mou, 2011). In plants,

its role is as a phytohormone essential to PTI, ETI, and SAR induction (Pieterse et al., 1998; Durrant and Dong, 2004; Tsuda et al., 2009). Whereas the JA/ET signaling pathway is essential for defense against herbivores, insects, and necrotrophic pathogens, the SA signaling pathway is crucial to immune responses against biotrophic and hemibiotrophic pathogens (Shah, 2003; Howe and Jander, 2008). Such pathogen challenge induces the production of endogenous SA which is vital in establishing SAR (Malamy et al., 1990; Metraux et al., 1991; Rasmussen et al., 1991; Gaffney et al., 1993; Delaney et al., 1994). Consequently, mutants deficient in the accumulation of SA such as *SA induction-deficient 2* (*sid2*) or *enhanced disease-susceptibility 5* (*eds5*) and plants expressing the salicylate hydrolase *nahG* gene, display compromised SAR induction (Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002; van Wees and Glazebrook, 2003). Thus, an inability to synthesize or accumulate SA is directly correlated to increased susceptibility to certain pathogens (van Wees and Glazebrook, 2003). Interestingly, SA influences various other hormone signaling pathways including JA and ET as well as auxin (Vlot et al., 2009). In general the balance between these hormones governs the bulk of host defense signaling (Robert-Seilaniantz et al., 2011). This is evident through heightened biotroph resistance resulting in increased susceptibility to necrotrophs and vice versa (Robert-Seilaniantz et al., 2011).

The biosynthesis of SA relies on two pathways, (1) the cinnamic acid pathway which requires PHENYLALANINE AMMONIA LYASE (PAL) and (2) the isochorismate pathway requiring ISOCHORISMATE SYNTHASE (ICS) and ISOCHORISMATE PYRUVATE LYASE (IPL) (Verberne et al., 2000; Wildermuth et al., 2001; Strawn et al., 2007; Chen et al., 2009b; Vlot et al., 2009). The isochorismate pathway is regarded as the predominant biosynthetic pathway during pathogenic threat, evinced by *Arabidopsis ics* mutants which accumulate significantly lower levels of SA following pathogenic stress (Wildermuth et al., 2001; Garcion et al., 2008), a statement also true in *Nicotiana benthamiana* and *Solanum lycopersicum* (Uppalapati et al., 2007; Catinot et al., 2008). Several derivatives of SA exist *in planta* such as SA *O*-β-glucoside (SAG), salicyloyl glucose ester (SGE), methyl salicylate (MeSA), methyl salicylate *O*-β-glucoside (MeSAG) and 2,5 dihydroxybenzoic acid (gentisic acid) (Shulaev et al., 1997; Lee and Raskin, 1998; Seskar et al., 1998; Belles et al., 1999; Song, 2006; Park et al., 2007; Dean and Delaney, 2008). Notably gentisic acid is essential to activating a specific set of *PR* genes (Belles et al., 1999). In fact, many of the aforementioned derivates perform specialized roles in plant immune responses and are required for the complete induction of SA-dependent defense responses, although some are still subject to debate (Nobuta et al., 2007; Vlot et al., 2009; Zheng et al., 2012; Fu and Dong, 2013). Most notably, MeSA has been proven to act as a signal for SAR in tobacco, *Arabidopsis* and potato (Park et al., 2007; Vlot et al., 2008). However, in *Arabidopsis* extended exposure to light following infection can negate the need for MeSA to signal systemic SAR development (Liu et al., 2011). In addition, MeSA might also serve as a volatile cautioning signal to neighboring plants (Koo et al., 2007; Spoel and Dong, 2012). Other compounds such as SAG and SGE ensure an ample supply of SA during pathogen challenge as bioactive free SA is readily hydrolyzed from inactive SAG stored within the vacuole (Dean et al., 2005).

The role of SA in disease resistance is certainly significant in all plant species (Malamy et al., 1990; Gaffney et al., 1993; Delaney et al., 1994; Vernooij et al., 1994; Lawton et al., 1995). Some pathogens even manipulate SA homeostasis to promote host invasion (Feys et al., 1994; Zheng et al., 2012). Several *Pseudomonas syringae* pathovars produce the phytotoxin coronatine, which indirectly represses *ICS1* and activates *BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1* (*BSMT1*) expression, which converts SA into MeSA, to suppress SA accumulation (Feys et al., 1994; Zheng et al., 2012). Although not directly responsible for all signal transduction, SA forms an integral part of a complex network responsible for signal transduction. Initially, global transcriptional profiling discovered extensive crosstalk between SA-, JA-, and ET-pathways in *Arabidopsis* (Glazebrook et al., 2003). Microarray expression profiling in *Arabidopsis* also demonstrated the true extent of cross-talk between various defense signaling pathways (Schenk et al., 2003). This interconnected signaling network serves to fine-tune defense responses through both antagonistic and synergistic interactions (Salzman et al., 2005).

Crosstalk between signaling networks is essential to spatial, temporal and plant–pathogen interaction specificity which informs trade-offs during challenge by multiple biotic and abiotic stresses (Spoel and Dong, 2008). However, phytohormone crosstalk can often be manipulated by pathogens to increase virulence (Spoel and Dong, 2008). Coronatine produced by virulent *P. syringae* for instance, which structurally mimics jasmonyl-L-isoleucine (JA-Ile), stimulates JA responsive pathways thereby suppressing SA signaling (Feys et al., 1994; Bender et al., 1999; Koornneef and Pieterse, 2008). Furthermore, coronatine accumulation is associated with increased abscisic acid (ABA) biosynthesis which in turn leads to reduced SA accumulation, basal defense gene expression and ultimately heightened susceptibility (de Torres-Zabala et al., 2007; Mohr and Cahill, 2007). It has also been clearly demonstrated that auxin, a primary growth hormone, antagonizes SA accumulation (Wang et al., 2007). To this effect, the *P. syringae* effector AvrRpt2 manipulates auxin homeostasis to promote virulence (Chen et al., 2007). These examples highlight the extent of crosstalk between phytohormone pathways which extends beyond SA and JA/ET antagonism, providing insight into the mechanisms plants use to overcome pathogenic threat (Spoel and Dong, 2008; Robert-Seilaniantz et al., 2011). For instance, in wild-type *Arabidopsis* infected with *P. syringae* the accumulation of SA represses the JA mimicking effects of coronatine, while SA indirectly prevents the degradation of the auxin repressor AXR2 thereby avoiding the expression of auxin-responsive genes (Spoel et al., 2003; Wang et al., 2007). Thus it is clear that the fundamental link in phytohormone crosstalk is the

regulation of SA synthesis (Fu and Dong, 2013). For an in-depth review of the topic see (Robert-Seilaniantz et al., 2011; Berens et al., 2017).

NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1

An indispensable player in the SA-defense response pathway is NPR1, a protein involved in fundamental responses to pathogenic challenge. The search for a SA responsive protein led to the discovery of NPR1, a positive regulator of SAR (Glazebrook et al., 1996; Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997). *Arabidopsis npr1* mutants display increased disease susceptibility and a decrease in SAR-triggered *PR* gene expression, specifically *PR1* and *PR5* (Cao et al., 1994; Glazebrook et al., 1996). Whereas complementing *npr1* mutants with wild-type *NPR1* restores resistance and *PR* gene expression (Cao et al., 1997). Various plant species overexpressing *AtNPR1* or its orthologs display enhanced disease resistance to a wide range of pathogens (Cao et al., 1998; Chern et al., 2001, 2005b; Lin et al., 2004; Makandar et al., 2006; Xujing et al., 2006; Malnoy et al., 2007; Potlakayala et al., 2007; Yuan et al., 2007; Wally et al., 2009; Parkhi et al., 2010b; Le Henanff et al., 2011; Kumar et al., 2013; Dutt et al., 2015; Molla et al., 2016). Furthermore, compelling evidence identifies NPR1 as a key element in the crosstalk between the SA and JA/ET responses (Spoel et al., 2003). Hence NPR1 plays a significant role in a broad range of defense responses, acting as the master regulator of plant defense signaling (Dong, 2004).

Specialized domains, specifically the ankyrin repeat and the BTB/POZ domains, facilitate protein–protein interactions (Bardwell and Treisman, 1994; Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999; Hepworth et al., 2005; Li et al., 2006; Spoel et al., 2009). While a bipartite nuclear localization sequence allows for nuclear localization of NPR1 following SA induction (Kinkema et al., 2000). Consequently a subset of the TGA family of basic domain/leucine zipper (bZIP) transcription factors are activated by NPR1 leading to expression of *PR* genes and SAR induction (Zhang et al., 1999; Kinkema et al., 2000; Zhou et al., 2000; Despres et al., 2003). These data suggest that NPR1 is a transcription cofactor responsible for effecting SA-dependent signaling, a concept supported by genome-wide expression analysis of *npr1* mutants (Wang et al., 2006). However, recent advances provide a clearer, malleable and intricate picture of NPR1-dependent defense responses.

NPR1-LIKE FAMILY

Numerous NPR1-like proteins, both putative and confirmed, have been identified in various plant species (**Table 1**). All described NPR1-like proteins contain an ankyrin repeat domain and BTB/POZ domain indicating high levels of functional conservation in the NPR1-like family (Bardwell and Treisman, 1994; Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999; Hepworth et al., 2005; Li et al., 2006; Spoel et al., 2009). However, as the list of NPR1-like proteins in different plant species increases, so does the complexity and variability in function. In *Arabidopsis* alone, five additional *NPR1*-like genes have been described: *AtNPR2, AtNPR3, AtNPR4, AtNPR5/AtBOP1* and *AtNPR6/AtBOP2* (Ha et al., 2004; Hepworth et al., 2005; Liu et al., 2005; Zhang et al., 2006).

Phylogenetic analysis reveals that the NPR1-like family classifies into three clades (**Figure 1**) (Hepworth et al., 2005; Zhang et al., 2006). Each clade seems to fall into a distinct functional niche. Clade 1 (AtNPR1 and AtNPR2) is involved with positive SAR regulation, clade 2 (AtNPR3 and AtNPR4) with negative SAR regulation and clade 3 (AtBOP1 and AtBOP2) with growth and development of leaves and flowers (Cao et al., 1998; Hepworth et al., 2005; Zhang et al., 2006). These clades are not always functionally robust and as such phylogenetic analyses alone are insufficient for functional annotation (Liu et al., 2005; Zhang et al., 2006; Le Henanff et al., 2009). Nonetheless

TABLE 1 | NPR1-like proteins.

Common name	Latin name	Reference
Arabidopsis	*Arabidopsis thaliana*	Cao et al., 1997; Hepworth et al., 2005; Liu et al., 2005
Apple	*Malus pumila*	Malnoy et al., 2007
Apple	*Malus hupehensis*	Zhang et al., 2012
Rice	*Oryza sativa*	Goff et al., 2002
Poplar	*Populus trichocarpa*	Tuskan et al., 2006
Tobacco	*Nicotiana tabacum*	Liu et al., 2002
Tobacco	*Nicotiana glutinosa*	Zhang et al., 2010c
Grapevine	*Vitis vinifera*	Le Henanff et al., 2009
Norton grapevine	*Vitis aestivalis* cv. *Norton*	Zhang et al., 2013
Cotton	*Gossypium hirsutum*	Zhang et al., 2008
Asian pear	*Pyrus pyrifolia*	Faize et al., 2009
Sweet potato	*Ipomoea batatas*	Chen et al., 2009a
Papaya	*Carica papaya*	Zhu et al., 2003; Peraza-Echeverria et al., 2012
Banana	*Musa acuminata*	Endah et al., 2008
Banana	*Musa* spp. ABB	Zhao et al., 2009
Tomato	*Solanum lycopersicum*	The Tomato Genome, 2012
Mustard greens	*Brassica juncea*	Meur et al., 2006
Soybean	*Glycine max*	Sandhu et al., 2009
Cacao tree	*Theobroma cacao*	Shi et al., 2010
Sugar cane	*Saccharum spp.*	Chen et al., 2012a
Coffee	*Coffea arabica*	Barsalobres Cavallari et al., 2013
Orchid	*Phalaenopsis aphrodite*	Chen et al., 2013
Wheat	*Triticum aestivum* L.	Diethelm et al., 2014
Beet	*Beta vulgaris*	Kuykendall et al., 2007
Avocado	*Persea americana*	Backer et al., 2015
Coconut palm	*Cocos nucifera* L.	Nic-Matos et al., 2017
Gladiolus	*Gladiolus hybridus*	Zhong et al., 2015
Canola	*Brassica napus*	Potlakayala et al., 2007
Peanut	*Arachis hypogaea*	Wu et al., 2014
Oriental lily	*Lilium 'Sorbonne'*	Wang et al., 2017
Eucalyptus	*Eucalyptus grandis*	Naidoo et al., 2013

List of plant species which either code for putative or confirmed NPR1-like proteins.

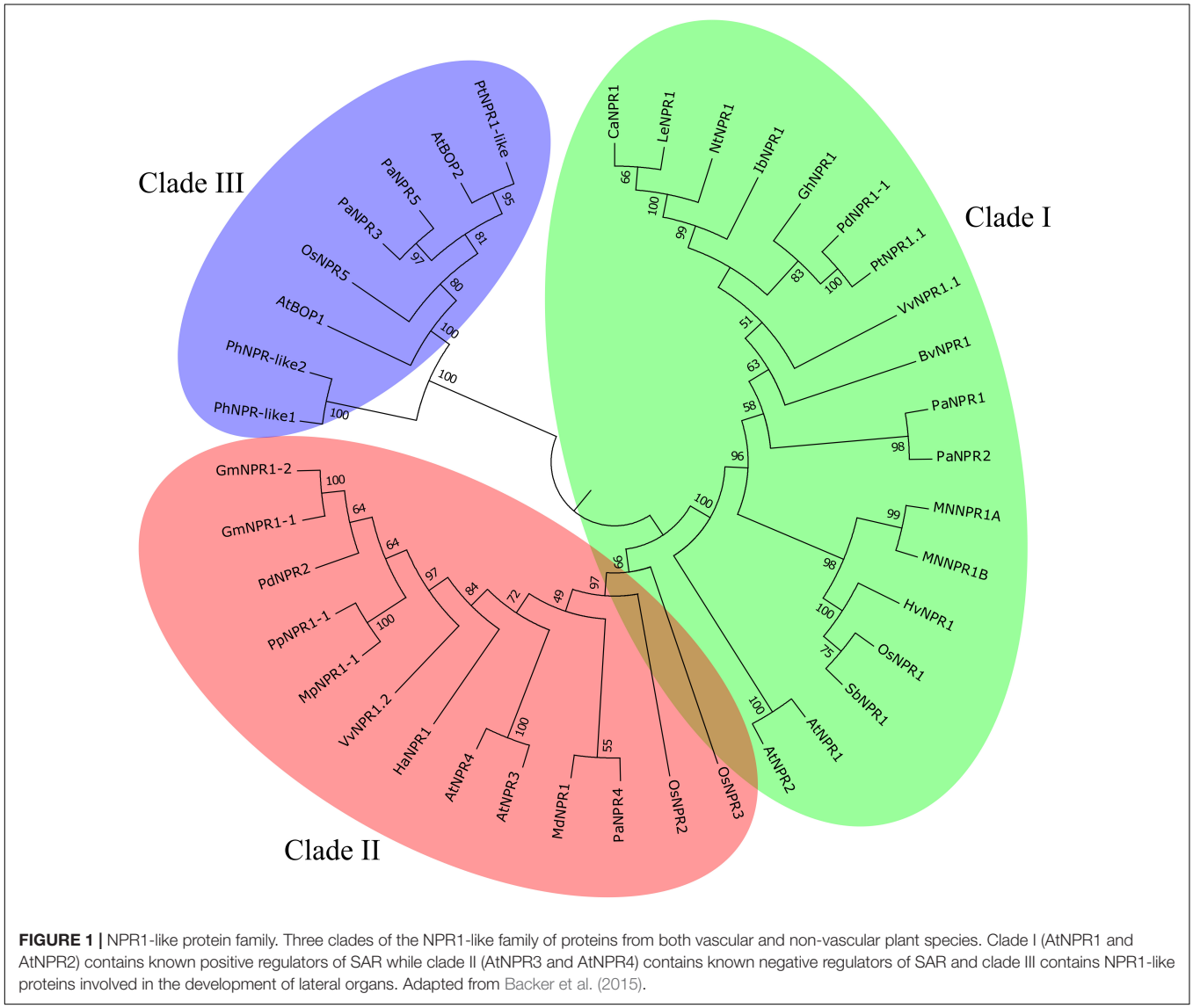

FIGURE 1 | NPR1-like protein family. Three clades of the NPR1-like family of proteins from both vascular and non-vascular plant species. Clade I (AtNPR1 and AtNPR2) contains known positive regulators of SAR while clade II (AtNPR3 and AtNPR4) contains known negative regulators of SAR and clade III contains NPR1-like proteins involved in the development of lateral organs. Adapted from Backer et al. (2015).

phylogenetic grouping provides a foundation for understanding functional variability among NPR1-like proteins.

NPR1 IN CROSSTALK

Effective defense responses rely on correct activation of either the SA- and JA-defense response pathways (Glazebrook, 2005). Although known to interact synergistically, the SA- and JA-defense response pathways are commonly regarded as antagonistic (Felton and Korth, 2000; van Wees et al., 2000; Glazebrook, 2005; El Oirdi et al., 2011). In so doing, plants ensure minimal fitness loss whilst safeguarding disease resistance (Mur et al., 2006; Koornneef and Pieterse, 2008). Research by Spoel et al. (2003) clearly demonstrates antagonistic crosstalk between the SA and JA pathways. In wild-type *Arabidopsis* the combined exogenous application of SA and MeJA favors activation of the SA-defense response pathway, evident through increased *PR1* expression and simultaneous suppression of JA-responsive defense gene expression (Spoel et al., 2003). Furthermore, the simultaneous infection of *Arabidopsis* with both biotrophic and necrotrophic pathogens results in an increased susceptibility to the latter, indicating JA defense suppression via the SA pathway (Spoel et al., 2003, 2007). However, crosstalk seems to be limited to local tissues, thereby preventing necrotrophic pathogens from capitalizing on the suppressed JA response pathway in systemic tissue (Spoel et al., 2007). Suppression also appears to be mediated by cytoplasmic NPR1 which upon SA induction limits JA dependent signaling (Spoel et al., 2003; Ndamukong et al., 2007; Yuan et al., 2007).

Interestingly some pathogens can manipulate this cross talk to promote disease. A necrotrophic fungus, *Botrytis cinerea*, expresses an exopolysaccharide which induces the SA pathway thereby preventing JA dependent gene expression (El Oirdi et al., 2011). El Oirdi et al. (2011) demonstrated the role of NPR1 in *B. cinerea* pathogenesis by infecting tomato plants in which

NPR1 had been silenced. Such NPR1 deficient lines showed significantly reduced disease symptoms. Additionally, transgenic *Arabidopsis* overexpressing NPR1 show enhanced *B. cinerea* susceptibility (El Oirdi et al., 2011). This concept is supported by the observation that NPR1 may play a role in preventing the accumulation of SA during herbivory (Rayapuram and Baldwin, 2007). In NPR1-silenced *Nicotiana attenuata*, SA accumulation was accompanied by an increased susceptibility to herbivores, suggesting that NPR1 might suppress SA production allowing JA-mediated defense responses to dominate (Rayapuram and Baldwin, 2007). Indeed, NPR1 has been shown to prevent the accumulation of SA by negatively regulating *ICS1* upon entry into the nucleus (Zhang et al., 2010a). Additionally, *Arabidopsis npr1* mutants are deficient in mounting ISR (Pieterse et al., 1998). The Cauliflower mosaic virus (CaMV) protein P6 is associated with repressing the SA- and enhancing the JA-defense response pathways (Love et al., 2012). Interestingly, P6 is implicated in the accumulation of inactive NPR1 within the nucleus, an avenue which likely enforces its' effect (Love et al., 2012). Thus, NPR1 is essential in both the SA and JA/ET pathways, regulating the accumulation of SA to activate the appropriate defense signal.

MONOMERIZATION OF NPR1

Although NPR1 is involved in hormone cross talk, its' main purpose is establishing SAR through *PR* gene expression (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). Achieving this requires the nuclear localization of monomeric NPR1 which interacts with TGA transcription factors to form a transcriptional complex (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Kim and Delaney, 2002). This complex then associates with an *activation sequence-1* (*as-1*)-like motif within the *PR* promoter (Lebel et al., 1998; Strompen et al., 1998; Jakoby et al., 2002). Factors such as NIM-INTERACTING2 (NIMIN-2), SNI1, NPR3, and NPR4 seem to be negative regulators which fine-tune NPR1-dependent gene expression (Li et al., 1999; Weigel et al., 2001; Chern et al., 2005a; Zhang et al., 2006; Zwicker et al., 2007).

The nuclear localization of NPR1 is essential for the expression of *PR* genes (Despres et al., 2000; Kinkema et al., 2000; Mou et al., 2003). Preceding cellular oxidative stress NPR1 is primarily found within the cytoplasm in an oligomeric form, though some notable exceptions exist (Kinkema et al., 2000; Mou et al., 2003; Le Henanff et al., 2009; Zhang et al., 2010c; Maier et al., 2011; Peraza-Echeverria et al., 2012; Shao et al., 2013). The production of SA and subsequent oxidative stress decreases cellular reduction potential enforcing an increased production of reducing agents (Mou et al., 2003). Thioredoxins, in particular thioredoxin H-type 3 (TRX-h3) and thioredoxin H-type 5 (TRX-h5), lead to the reduction of Cys156 and disassembly of the NPR1 oligomer (Mou et al., 2003; Tada et al., 2008). Monomeric NPR1 is then translocated to the nucleus via a bipartite nuclear localization signal (NLS) where it induces the expression of *PR1* (Kinkema et al., 2000; Maier et al., 2011). Consequently, inhibiting the formation or nuclear localization of NPR1 monomers decreases *PR1* expression while constitutive monomerization, as in the case of C82A and C216A point mutants, leads to increased *PR1* gene expression (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008). Hence NPR1 is required as a monomer within the nucleus to induce SAR-related defense genes.

Remarkably NPR1 can be constitutively localized within the nucleus of tobacco and grapevine (Le Henanff et al., 2009; Maier et al., 2011). In spite of this, NPR1 within these species still seems to be dependent on SA (Le Henanff et al., 2009; Maier et al., 2011). Moreover, C82A and C216A mutants display even higher expression of *PR* genes following SAR induction (Mou et al., 2003). Confirmation of the SA-dependent nature of NPR1 came from Kinkema et al. (2000), proving that NPR1 nuclear localization was insufficient at inducing SAR in the absence of inducers such as INA (2,6-dichloroisonicotinic acid) or SA. Hence, mechanisms other than simple nuclear localization must play a role in controlling NPR1-dependent transcriptional processes.

TGA TRANSCRIPTION FACTORS AND NPR1

Although the N-terminal half of AtNPR1 exhibits low levels of transcriptional activity it is not likely to induce expression of *PR1* sufficiently (Zhang et al., 1999; Rochon et al., 2006). However, tobacco NPR1 harbors a stronger transactivation domain which is sensitive to SA (Maier et al., 2011). In yeast-1-hybrid screens, a section of NtNPR1 (1–315) exhibits transcriptional activity superior to the VP16 viral transactivation domain (Maier et al., 2011). Yeast cells expressing the Gal4 BD:NtNPR1 fusion protein in SA containing medium had much higher reporter gene activities than cells in media lacking SA (Maier et al., 2011). Hence the extent and way NPR1 is regulated differs between species reflecting their individual evolutionary histories and environments.

Expression of *PR1* in tobacco is highly dependent on *as-1-like* promoter elements known to be responsive to SA (Strompen et al., 1998). Several members of the TGA family of basic leucine zipper protein (bZIP) transcription factors associate with the *as-1-like* promoter element (Strompen et al., 1998; Zhang et al., 1999). Interestingly NPR1 has the ability to strongly interact with several of these transcription factors, namely; TGA1, TGA2, TGA3, TGA4, TGA5, TGA6, and TGA7 (Despres et al., 2000; Zhou et al., 2000; Johnson et al., 2003; Rochon et al., 2006). Interaction occurs predominantly within the nucleus where NPR1 activates TGA transcription factors by increasing their DNA binding affinity, evident through improved TGA2-*as-1-like* complex formation in the presence of wild-type NPR1 (Despres et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002). Two *as-1-like cis* elements can be found within the *PR1* promoter, a positive regulating element, *LS7*, and a negative regulating element, *LS5* (Lebel et al., 1998). Certain TGA transcription factors are able to associate with either of these elements, an association which is significantly enhanced by the presence of NPR1 (Despres et al., 2000). This suggests that NPR1 may not only serve to activate gene transcription but also suppress it in order to establish SAR (Despres et al.,

2000). Further corroboration for the fundamental role of TGA transcription factors in regulation of defense gene expression was found in the promoters for SA-induced genes which showed an overrepresentation of the TGA2 binding sequence TGACTT (Ding et al., 2018).

In rice, *Arabidopsis* NPR1 binds to several bZIP transcription factors: rTGA2.1, rTGA2.2, rTGA2.3, rLG2 (Chern et al., 2001). Correspondingly, rTGA2.1 associates with the *Arabidopsis as-1-like* promoter element as well as the rice *RCH10* proximal promoter element (Chern et al., 2001). Tobacco contains several TGA transcription factors which are also capable of interacting with *Arabidopsis* NPR1, TGA2.1 and TGA2.2 (Niggeweg et al., 2000b). In addition both of these transcription factors are capable of binding to *as-1-like* elements (Niggeweg et al., 2000a,b). Tomato NPR1 was also found to associate with bZIP transcription factors which show high sequence similarity to the *Arabidopsis* TGA family of bZIP transcription factors (Zhang et al., 1999). Thus, a collectively conserved evolutionary role for NPR1 and TGA transcription factors is believed to exist in most if not all plant species.

Several studies have tried to address the *in vivo* role of TGA transcription factors (Pontier et al., 2001; Fan and Dong, 2002; Johnson et al., 2003; Zhang et al., 2003). In tobacco a TGA2 dominant-negative mutant resulted in increased *PR1*, *PR2* and *PR3* induction after SA treatment and enhanced disease resistance (Pontier et al., 2001). Contrastingly, a different dominant-negative TGA2 mutant led to decreased *PR* gene induction and enhanced disease susceptibility in both tobacco and *Arabidopsis* (Niggeweg et al., 2000b; Fan and Dong, 2002). These seemingly contrasting results are most likely due to the unknown interactions of dominant-negative mutants with other TGA transcription factors (Zhang et al., 2003). It seems that TGA2, TGA5, and TGA6 serve redundant roles in NPR1-dependent gene expression (Zhang et al., 2003). Either transcription factor is able to restore wild-type *PR* gene induction or basal expression levels in the *tga6-1tga2-1tga5-1* triple mutant (Zhang et al., 2003).

While *tga6-1tga2-1tga5-1* triple knockout mutants had reduced *PR* gene induction, basal levels of these genes were up to 50-fold higher (Zhang et al., 2003). This would suggest a negative role of TGA factors in basal *PR* expression yet a positive requirement for induction following SA perception. Yet, another study provides evidence that TGA2 is unable to bind to the *PR1* promoter in the absence of SA (Johnson et al., 2003). However, an elegant study by Rochon et al. (2006) clarified the conflicting evidence, showing that TGA2 and NPR1 are able to associate with the *PR1* promoter independently of each other in the absence of SA. Interestingly, NPR1 is capable of associating with TGA2 after SA treatment leading to *PR1* expression. The authors suggest that while TGA2 is a transcriptional repressor, NPR1 becomes a TGA2 transcriptional co-activator after perception of SA (Rochon et al., 2006). Indeed, Boyle et al. (2009) demonstrate that the N-terminal region of TGA2 is a non-autonomous repression domain required for association with *PR1 cis*-elements.

Interaction between TGAs and NPR1 is dependent on a functional ankyrin repeat domain within NPR1 (Zhang et al., 1999; Zhou et al., 2000; Despres et al., 2003). Although not essential to the interaction, the N-terminal domain of NPR1 also appears to be responsible for strengthening the interaction between NPR1 and certain TGAs (Zhou et al., 2000). Several *npr1* mutants deficient in mounting an effective SAR response are unable to interact with *Arabidopsis* TGA2, TGA3 as well as rice TGAs (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Chern et al., 2001). These mutants, specifically *npr1-1, npr1-2, nim1-2* and *npr1-5,* have point mutations in the ankyrin repeat domain (Cao et al., 1994, 1997; Delaney et al., 1995; Glazebrook et al., 1996; Ryals et al., 1997; Shah et al., 1997).

Redox seems to play yet another essential role in the activity of both TGA transcription factors and NPR1. The C-terminal section of interacting TGA transcription factors is required for NPR1-TGA formation *in vitro* and *in vivo* (Zhang et al., 1999; Zhou et al., 2000; Fan and Dong, 2002). While TGA1 and TGA4 were initially considered unable to interact with NPR1, Despres et al. (2003) determined that following SA treatment these transcription factors were able to interact with NPR1 *in planta*. Specifically, residues unique to TGA1 and TGA4, Cys260 and Cys266, mediate the interaction (Despres et al., 2003). During non-induced conditions these residues form an intramolecular disulphide bridge which prevents TGA1 from interacting with NPR1, yet after SA treatment Cys260 and Cys266 are reduced and TGA1-NPR1 interaction occurs (Despres et al., 2003). Similarly, exchanging Cys260 and Cys266 for Asn and Ser, respectively, allows constitutive interaction with NPR1 in the absence of SA (Despres et al., 2003).

NIM INTERACTING PROTEINS

An additional group of NPR1-interacting proteins are NIMINs (NIM INTERACTING) proteins (Weigel et al., 2005; Maier et al., 2011). These proteins are induced by SA or its functional analogs followed by nuclear localization where they interact directly with NPR1 forming a ternary complex with TGA factors (Weigel et al., 2001; Glocova et al., 2005; Weigel et al., 2005). In *Arabidopsis, 35S:NIMIN1* overexpression abolishes the establishment of SAR and reduced *PR* expression (Weigel et al., 2005). However, overexpression of *NIMIN1-2*, which encodes for a mutant protein unable to bind to NPR1, results in near wild-type SAR induction and *PR* expression (Weigel et al., 2005). Moreover, knockout *nimin1-1* mutants showed increased *PR* expression after SA induction (Weigel et al., 2005). Similar results were obtained through overexpression of rice *NRR*, an ortholog of *NIMIN-2* and tobacco *NIMIN-2a* (Chern et al., 2005a). In addition, application of SA/INA to tobacco or *Arabidopsis* substantially reduces the NPR1 interaction potential of NIMIN proteins, specifically NIMIN-1/2 in *Arabidopsis* and NIMIN-2a/2b/2c in tobacco (Maier et al., 2011).

The aforementioned interaction is likely to be affected due to a conformational change which obscures the NIMIN binding motif in the C-terminal end of NPR1 (Maier et al., 2011). A single amino acid change in the C-terminal of the *nim1-4* mutant (Arg432Lys) severely impairs its potential to establish SAR (Ryals et al., 1997). Maier et al. (2011) concluded

that this mutation rendered the interaction between NIMIN-1 and NIMIN-2 to NIM1-4 non-responsive to SA in both *Arabidopsis* and tobacco. Therefore, NIMIN is clearly involved in regulating *PR* expression through modulating NPR1 activity in response to SA.

In *Arabidopsis* NIMIN-1, NIMIN-2, and NIMIN-3 prevent each other from binding to NPR1, this interaction is dependent on the concentration of each protein and supports a theory whereby NIMIN proteins differentially interact with NPR1 (Hermann et al., 2013). In unchallenged *Arabidopsis* plants NIMIN-3 binds to NPR1 to prevent expression of *PR* genes (Hermann et al., 2013). Upon SA detection NIMIN-2 is quickly induced and although it is not involved in suppressing *PR* gene expression it seems to play an unknown role in immediate/early SA responses (Hermann et al., 2013). NIMIN-1 on the other hand delays the expression of *PR* genes, preventing premature activation (Hermann et al., 2013). Similarly, in challenged tobacco plants overexpression of NIMIN-2a does not prevent *PR* expression but rather delays its expression (Zwicker et al., 2007). Correspondingly, down-regulation of NIMIN-2a leads to earlier *PR* gene expression (Zwicker et al., 2007). Additionally these data suggest that NIMIN-2a may be involved in priming tissue distal from the primary site of infection, allowing a quicker response during secondary infection (Zwicker et al., 2007).

STRUCTURE AND FUNCTION OF NPR1

Two domains are essential for the co-activator function of NPR1, the BTB/POZ domain in the N-terminal region as well as a cryptic transactivation domain in the C-terminal region (Rochon et al., 2006; Boyle et al., 2009). Exchanging the α2 and α3 helix residues, which constitute the core of the BTB/POZ domain, with Ala (A-Sub) or removing the first 110 amino acids (Δ110NPR1) of the domain abolishes *PR1* expression (Rochon et al., 2006). However, these mutations do not substantially reduce TGA2-NPR1 binding, providing evidence that the BTB/POZ domain is responsible for co-activation of TGA2 (Rochon et al., 2006). Boyle et al. (2009) were able to confirm this observation by restoring the inducible nature of *PR1* in *Arabidopsis* lines containing A-Sub or Δ110NPR1 in which a truncated TGA2, which lacks the N-terminal repression domain Δ43:TGA2, was coexpressed. The authors were able to demonstrate that the BTB/POZ domain physically interacts with the N-terminal repression domain of TGA2, negating its effect (Boyle et al., 2009).

Furthermore, transcriptional activation via the TGA2-NPR1 complex after treatment with SA requires the C-terminal transactivation domain and two essential cysteine residues, Cys521 and Cys529, in an oxidized state within this domain (Rochon et al., 2006). Surprisingly, while full length NPR1 tethered to a Gal4 DNA-binding domain lacks the ability to activate transcription in the absence of SA, the truncated Δ513:NPR1 C-terminal region containing the transactivation domain can (Rochon et al., 2006). A subsequent study showed that the N-terminal BTB/POZ domain inhibits the C-terminal transactivation domain in SA naïve cells through physical interaction, yet binding of SA to NPR1 disrupts this interaction through a conformational change (Wu et al., 2012b).

It was not until recently that two independent studies provided evidence that NPR1 and its paralogs directly interact with SA, providing invaluable insight into our understanding of SA perception (Fu et al., 2012; Wu et al., 2012b). In the first study, using conventional non-equilibrium ligand binding assays, NPR3 and NPR4 were shown to bind to SA with low and high affinity, respectively (Fu et al., 2012). Subsequently, a different approach utilizing equilibrium dialysis found that NPR1 too can bind to SA with an affinity similar to that of other known hormone-receptor interactions (Wu et al., 2012b). This interaction has been confirmed using three additional methods of detection, irrefutably setting the role of NPR1 as a *bone fide* SA receptor (Manohar et al., 2015). Binding to SA specifically requires Cys521 and Cys529 and the presence of a transition metal, preferably copper, to facilitate it (Wu et al., 2012b). Although orthologs of *Arabidopsis* NPR1 don't harbor the same cysteine residues, the presence of similar residues with electronegative side-chains at comparable positions suggest some likelihood of parallel transition metal associations in other plant species (Wu et al., 2012b). This interaction enforces a conformational change in the C-terminal transactivation domain which reduces its affinity for the N-terminal BTB/POZ domain (Wu et al., 2012b). The authors further demonstrated that reducing conditions alone are not enough for disassembly of the NPR1 oligomer and suggested that the SA-induced conformational change was required for full disassembly (Wu et al., 2012b). Thus, the function of NPR1 is enforced through a conformational changes which rely on direct interaction with SA.

PARALOGS OF NPR1

Paralogs of NPR1, namely NPR3 and NPR4, appear to be negative regulators of *PR* expression (Zhang et al., 2006). Similar structure in these proteins seems to extend to functional similarities, from perception of SA to binding of TGAs (Despres et al., 2000; Kinkema et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002; Mou et al., 2003; Rochon et al., 2006; Zhang et al., 2006; Shi et al., 2013). Initial research suggested that NPR4 could be a positive regulator of disease resistance as *PR* expression priming was compromised in *npr4-2* mutants (Liu et al., 2005). However, *npr3 npr4* double mutants displayed increased *PR* gene expression and increased disease resistance indicating that *npr4-2* contributes to increased *PR* expression in *npr3 npr4* double mutants (Zhang et al., 2006). The redundancy of these proteins was also demonstrated through complementation with NPR3 or NPR4 (Zhang et al., 2006). Interestingly, NPR3 and NPR4 have also been shown to increase JA-dependent gene transcription and *de novo* JA synthesis following the accumulation of SA

likely by promoting the degradation of JA repressing JAZ (JASMONATE ZIM DOMAIN) proteins (Liu et al., 2016). This suggests that NPR3 and NPR4 are essential in preventing disease caused by necrotrophic pathogens on tissues affected by ETI-triggered PCD (Liu et al., 2016). Several studies stimulated substantial debate regarding the exact role of NPR3/NPR4 in defense responses (Fu et al., 2012; Wu et al., 2012b; Kuai et al., 2015). However, their role as co-repressors of SA-inducible defense gene expression is clearly demonstrated by Ding et al. (2018).

Originally, NPR3 and NPR4 were proposed to primarily function as E3 ligases in a model by Fu et al. (2012). The authors demonstrated that NPR3 and NPR4 possess differing affinities for SA, thus allowing them to effectively regulate NPR1-dependent gene expression through CUL3-mediated proteasome degradation of NPR1 (Fu et al., 2012). In naïve cells with low SA concentrations, NPR4 which possess the highest affinity binds to NPR1 preventing ill-timed *PR* expression (Fu et al., 2012). Else, in SAR induced cells higher SA concentrations prevent NPR4-NPR1 association whereas NPR3 gains the ability to interact with NPR1, preventing NPR1-mediated suppression of the HR (Rate and Greenberg, 2001; Fu et al., 2012). Thus, NPR1 turnover rate and ultimately the induction of SAR is determined by the concentration gradient of SA from the initial site of pathogen infiltration to distal tissues. However, the model proposed by Fu et al. (2012) is inconsistent with the ostensible genetic redundancies between *NPR3* and *NPR4* (Kuai et al., 2015). Thus suggesting that NPR3 and NPR4 rather serve redundant roles, in contrast to independently functioning as SA receptors (Kuai et al., 2015). Additionally, no observable interaction occurs between NPR1 and NPR3/NPR4 in yeast-2-hybrid assays or NPR3/NPR4 and Cul3A in co-immunoprecipitation assays (Ding et al., 2018). This suggests, at least, that determining whether NPR3 and NPR4 participate in post-translational modification of NPR1 requires further study.

Instead, Ding et al. (2018) demonstrated that NPR3 and NPR4 are transcriptional co-repressors of SA-induced defense gene expression which function in parallel and independently of NPR1. The authors identified *npr4-4D* (Arg419Gln), a gain-of-function mutation which renders the mutant protein insensitive to SA and constitutively represses SA-inducible defense genes (Ding et al., 2018). Interestingly, mutation of the equivalent amino acid in NPR1 renders it insensitive to SA and is arguably the reason why NIM1-4 does not dissociate from NIMIN1 and NIMIN2 in the presence of SA (Ryals et al., 1997; Maier et al., 2011). An equivalent mutation introduced into NPR3 (Arg428Gln) similarly enables it to suppress defense signaling in the presence of SA, confirming the redundant roles of NPR3/NPR4 (Ding et al., 2018). Transcriptional repression of SA-inducible defense genes was shown to rely on a conserved motif (VDLNETP) within the C-terminal domain of NPR3 and NPR4 with similarity to the ethylene responsive element binding factor associated amphipathic repression motif (EAR; L/FDLNL/F(x)P) (Ohta et al., 2001; Ding et al., 2018). Furthermore, NPR3/NPR4 work together with TGA2/TGA5/TGA6 to suppress the expression of SA-inducible defense genes in SA naïve cells (Ding et al., 2018). The authors also confirmed that NPR3 and NPR4 bind to SA with high affinity. Meanwhile NPR4-4D, while still able to bind to TGA2 and form homodimers, showed a significantly lower (250-fold) binding affinity, demonstrating that R419 is essential in the binding of SA (Ding et al., 2018). Similarly, the NPR1^{R432Q} mutant displays significantly lower SA binding affinity compared to wild-type NPR1 with no apparent effects on interactions with TGA2 or NIMIN1 in yeast-2-hybrid assays (Ding et al., 2018). Thus, both NPR3 and NPR4 are *bone fide* SA receptors with highly similar functionality to that of NPR1, albeit in opposition regarding SA-inducible defense gene expression.

The *BLADE-ON-PETIOLE 1* (*BOP1*) and *BOP2* genes encode proteins with structure similar to other NPR1-like proteins, containing both N-terminal BTB/POZ and C-terminal ankyrin repeat domains (Ha et al., 2003; Ha et al., 2004). However, the C-terminal of several BOP-like proteins in several plant species lack essential features characteristic of defense-related NPR1-like proteins such as a clear bipartite NLS, NIMIN1/2 binding region and the highly conserved NPR1 Arg432 residue (Backer et al., 2015). These differences seemingly translate into functional variation as *bop1 bop2* double mutants display an unaltered wild-type response to *Pseudomonas* infection as well as SA application (Hepworth et al., 2005; Canet et al., 2010a). However, some evidence of defense-related functions exist with BOP1 and BOP2 being implicated in the resistance-inducing activity of MeJA (Canet et al., 2012). Nonetheless, BOP1 and BOP2 are considered transcriptional co-activators which function redundantly and share similar transcriptional patterns, being expressed primarily at the base of lateral organs (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005; Ha et al., 2007; McKim et al., 2008; Jun et al., 2010). Correspondingly, the overwhelming majority of evidence indicates that BOPs are vital to the growth and development of lateral organs (Ha et al., 2004, 2007; Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008). Even though they lack a defined NLS, BOPs can be found within the cytoplasm and nucleus of *Arabidopsis* (Ha et al., 2004; Hepworth et al., 2005). Thus unsurprisingly, BOPs influence transcriptional processes through interaction with TGA transcription factors (Hepworth et al., 2005; Wu et al., 2012a). Of relevance is PERIANTHIA (PAN), a TGA transcription factor with known significance to developmental processes in *Arabidopsis* (Chuang et al., 1999; Hepworth et al., 2005). Interestingly, BOPs have also been implicated in the lignin biosynthesis pathway (Khan et al., 2012). Hence, members of the NPR1-like family together make up one of the most important groups of proteins to study in the field of molecular biology of plant health and development.

WRKY TRANSCRIPTION FACTORS AND NPR1

Microarray of the *Arabidopsis* transcriptome during SAR revealed that not all genes co-regulated with *PR1* contain the expected TGA binding site in their promoters (Maleck

et al., 2000). Instead, W-box *cis*-elements which specifically bind WRKY transcription factors are more common suggesting that WRKY transcription factors might repress a subset of SA-inducible genes, which is alleviated during SAR (Maleck et al., 2000). Indeed, *wrky38* and *wry62* single mutants and to a greater extent *wrky38wrky62* double mutants display enhanced disease resistance and *PR1* expression while overexpression has the opposite outcome (Kim et al., 2008). However, many WRKY transcription factors are positively associated with defense signaling, thus the role of this family in defense is complex (Wang et al., 2006; Zheng et al., 2006; Lai et al., 2008). These transcription factors are involved in defense against a wide range of pathogens, with 43 out of 74 WRKY transcription factors in *Arabidopsis* being linked to pathogenic stress and response to SA (Dong et al., 2003; Ulker and Somssich, 2004; Pandey and Somssich, 2009).

Accordingly, W-box *cis*-elements are found in several indispensable SA-inducible defense response gene promoters including that of *ICS1, TL1-binding transcription factor (TBF1)* and *PR1* (Eulgem et al., 2000; Wildermuth et al., 2001; Turck et al., 2004; Pajerowska-Mukhtar et al., 2012). Furthermore, the presence of multiple W-boxes within the *NPR1* promoter suggest that *NPR1* may be transcriptionally regulated in this manner (Yu et al., 2001). Yu et al. (2001) demonstrated that WRKYs are likely involved in positively regulating the expression of *NPR1*, although the exact WRKY is yet to be identified. Nonetheless, WRKY transcription factors are regulated in both NPR1-dependant and independent manners (Yu et al., 2001; Dong et al., 2003; Wang et al., 2006; Mao et al., 2007; Spoel et al., 2009).

Interestingly, the *CmYLCV* promoter from *Cestrum yellow leaf curling virus* contains both the *as-1* and W-box *cis*-elements in close proximity which associate with both TGA3 and WRKY53 (Sarkar et al., 2018). These elements are essential to the SA-inducibility of the promoter, suggesting that in certain instances both TGA and WRKY transcription factors may work together to regulate transcription (Sarkar et al., 2018). Surprisingly, not only do these transcription factors interact, they require functional NPR1 to induce expression of *Gus* under control of the *CmYLCV* promoter after treatment with SA (Sarkar et al., 2018). Given the complexity of the interactions described here the possibility exists that NPR1 is both positively and negatively regulated by various WRKY transcription factors, although this requires further investigation.

ER RESIDENT PROTEINS AND NPR1

Several genes involved in the secretory pathway are upregulated by NPR1, the most notable are *LUMINAL BINDING PROTEIN 2* (*BiP2*), *Sec61α*, *DEFENDER AGAINST APOPTOTIC DEATH 1* (*DAD1*), and *CALRETICULIN* 3 (*CRT3*) (Wang et al., 2005; Pajerowska-Mukhtar et al., 2012). These secretion-related genes all have a common promoter *cis*-element (*TL1*) which is bound by TBF1 a heat shock factor-like transcription factor instrumental in the growth-defense transition (Wang et al., 2005; Pajerowska-Mukhtar et al., 2012). Increased expression of secretory pathway genes most likely accommodates increased PR protein production during SAR, ensuring proper protein folding (Wang et al., 2005). Support for this conclusion came from mutants of *BiP2*, *Sec61α*, and *DAD1* which all had reduced PR1 secretion after BTH treatment and compromised defense against *P. syringae* (Wang et al., 2005). Similarly, *tbf1* mutants have unaltered *PR1* transcript and protein levels yet significantly less protein is secreted into the apoplast (Pajerowska-Mukhtar et al., 2012). Furthermore, the link between TBF1 and NPR1 is evident from *tbf1* and *npr1-1* mutants which are both similarly compromised in the expression of *BiP2* and *CRT3* (Pajerowska-Mukhtar et al., 2012). Thus, the expression of *NPR1* and *TBF1* are likely co-dependent with the authors suggesting that TBF1 might control *NPR1* directly through *TL1* elements in the promoter, or indirectly through WRKYs, while NPR1 may control *TBF1* through TGAs directly or WRKYs indirectly as both contain the appropriate elements in their promoters, respectively.

NPR1 IN PRIMING

Priming is a process which enhances plant defense responses, enabling earlier and stronger induction of defense genes and enhanced pathogen resistance (Prime et al., 2006). In fact, SAR prepares a plant to defend against future pathogenic stress through priming (Conrath et al., 2002; Prime et al., 2006). Thus unsurprisingly, NPR1 is essential in SA-induced priming in *Arabidopsis* (Kohler et al., 2002; Jung et al., 2009). Of note are pathogen-responsive mitogen-activated protein kinase 3 (MPK3) and MPK6 which are essential to SAR and SA-mediated priming of defense responses (Beckers et al., 2009). Following application of BTH, MPK3/MPK6 mRNA and inactive unphosphorylated proteins accumulate (Beckers et al., 2009). Interestingly, the authors demonstrated that priming of MPK3/MPK6 is NPR1-dependant as *npr1* mutant *Arabidopsis* plants fail to display the same response. Yi and Kwon (2014) and Yi et al. (2015) demonstrated the importance of this finding as NPR1-dependant priming affects early signaling events, such as flg22-triggered MAPK activation.

Furthermore, priming has been shown to affect the progeny of primed *Arabidopsis* as descendants display enhanced resistance against biotic stresses without additional treatment (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012). This transgenerational immune memory requires functional NPR1 (Luna et al., 2012). Transgenerational immune memory relies, at least in part, on increased H3K9 acetylation of the *PR1*, *WRKY6* and *WRKY53* promoters (Luna et al., 2012). Quite surprisingly, the histone deacetylase HDAC19 was shown to be both SA- and NPR1-dependant (Choi et al., 2012). Meanwhile, NPR1 is involved in BTH and *Psm* induced increases in H3K4 trimethylation and subsequent gene activation of the *WRKY6*, *WRKY29* and *WRKY53* promoters (Jaskiewicz et al., 2011). Together these studies suggest a role for NPR1 in histone modification to enforce priming of SA-induced defense genes,

however, understanding the exact part that NPR1 plays in these processes requires further investigation.

POST-TRANSLATIONAL MODIFICATION OF NPR1

An important topic which adds to the complexity of NPR1-dependant transcriptional regulation is that of post-translation modification. The importance of NPR1 post-translational modification is exemplified by regulation of the oligomer-monomer transition in which NPR1 Cys156 is *S*-nitrosylated by *S*-nitrosoglutathione (GSNO) promoting the existence of NPR1 in its oligomeric form, opposing the action of SA-induced thioredoxins (Tada et al., 2008).

Additionally, proteasome-mediated turnover of NPR1 within the nucleus is a requirement for the complete induction of SAR (Spoel et al., 2009). While the antagonistic effects of ABA and SA, which promote and protect against proteasome-mediated degradation, respectively, maintain homeostasis and ensure appropriate defense-related gene expression (Ding et al., 2016). Cullin 3 (CUL3) E3 ligase-facilitated ubiquitinylation and subsequent proteasome degradation is initiated within the N-terminal IκB-like phosphodegron motif of NPR1 (Spoel et al., 2009). Phosphorylation of Ser11/15 present in the phosphodegron motif signals proteasome-mediated degradation (Spoel et al., 2009). Yet, even though degradation of NPR1 is reduced and basal resistance is elevated in npr1$^{S11A/S15A}$, high levels of accumulated NPR1 within the cell prevent HR and the establishment of SAR (Spoel et al., 2009). Compromised induction of SAR is also observed in mutants of NPR1-dependent genes *wrky18* and *wrky38 wrky62*, and similarly in *cul3a cul3b* mutants in which NPR1 is not degraded (Spoel et al., 2009). Hence, turnover seems to be necessary for effective activity of NPR1 (Spoel et al., 2009). This is somewhat expected as inherent instability of transcription factors necessitates turnover in order to preserve peak expression of target genes and is thus conceivably so for co-activators (Salghetti et al., 2000; Collins and Tansey, 2006; Spoel et al., 2009). However, NPR1 does not interact directly with CUL3 and E3 ligases, likely requiring substrate adapters to facilitate degradation, however, attempts to uncover such adapters have not been conclusive (Dieterle et al., 2005; Spoel et al., 2009; Fu et al., 2012).

Interestingly, small ubiquitin-like modifier 3 (SUMO3), which is positively involved in SA-induced defense gene expression, interacts with and sumoylates NPR1 following SA treatment (Wang et al., 2006; van den Burg et al., 2010; Saleh et al., 2015). This interaction requires a SUMO-interaction motif (VIL)-(VIL)-x-(VIL) found within the ankyrin repeat domain of NPR1 (Saleh et al., 2015). Sumoylation alters the association of NPR1 with WRKY and TGA transcription factors, decreasing and increasing association, respectively (Saleh et al., 2015). In addition to the IκB-like phosphodegron motif at Ser11/15, another exists at Ser55/59 and their phosphorylation status influences the ability of SUMO3 to sumoylate NPR1 (Saleh et al., 2015). Phospho-mimic npr1$^{S55D/S59D}$ prevents NPR1 sumoylation while npr1$^{S11D/S15D}$ enhances interaction with SUMO3 and leads to further sumoylation (Saleh et al., 2015). Additionally, SUMO3 is required for phosphorylation of Ser11/15 forming a signal amplification loop which activates more NPR1 increasing defense gene activation and simultaneously targeting NPR1 for ubiquitinylation and degradation by the 26S proteasome (Spoel et al., 2009; Saleh et al., 2015). Together with the results obtained by Spoel et al. (2009), this work emphasizes the importance of NPR1 stability, through post-translational modification, to fine-tune NPR1-dependant defense responses.

Thus unsurprisingly, several kinases have been implicated in the phosphorylation of NPR1 (Xie et al., 2010; Lee et al., 2015). A pathogen-responsive member of the sucrose non-fermenting 1 (SNF1)-related kinase 3 (SnRK3) subgroup, PROTEIN KINASE SOS2-LIKE5 (PKS5) physically interacts with NPR1 (Xie et al., 2010). The authors demonstrate that PKS5 phosphorylates the C-terminal region of NPR1, which contains the Cys-oxidized transactivation domain as well as the bipartite NLS. The *Arabidopsis pks5* mutant as is with the *npr1*$^{S11A/S15A}$ mutant displays reduced expression of *WRKY38* and *WRKY62* (Spoel et al., 2009; Xie et al., 2010). Thus it seems that through phosphorylation of NPR1, PKS5 positively regulates the expression of *WRKY38* and *WRKY62* (Xie et al., 2010).

Similarly, SNF-1 RELATED PROTEIN KINASE 2.8 (SnRK2.8) interacts with and phosphorylates NPR1, specifically Ser589 and likely also Thr373, which are required for nuclear import of NPR1 and subsequent *PR1* gene expression (Lee et al., 2015). Interestingly, SnRK2.8 is produced in response to SA-independent systemic signals and has been implicated in the induction of systemic immunity (Lee et al., 2015). It is possible that, similar to SnRK2.6, nitric oxide (NO) might play a role in SnRK2.8 activation as it plays a proven role in the import of NPR1 monomers into the nucleus of cells in distal tissues during SAR (Lindermayr et al., 2010; Lee et al., 2015). Furthermore, Ser589 resides within the second NLS found in NPR1 (NLS2) (Kinkema et al., 2000). The authors suggest a model by which SA-dependent NPR1 nuclear import, for which NLS1 is required, is predominant close to the site of infection, while distal tissues with only slightly elevated levels of SA, rely on phosphorylation of NLS2 by SnRK2.8 (Kinkema et al., 2000; Lee et al., 2015). Therefore, multisite phosphorylation is clearly a defining feature of NPR1 function and warrants further investigation.

CIRCADIAN RHYTHM AND NPR1

In plants the circadian clock is crucial for synchronizing immune strategies, while redox signaling plays an important role in its implementation (Karapetyan and Dong, 2018). SA levels oscillate throughout the day in a circadian rhythm (Goodspeed et al., 2012). This oscillation is involved in establishing the redox rhythm and influencing the expression of circadian clock genes (Zhou et al., 2015). Captivatingly, the expression of *TIMING OF CAB2 EXPRESSION 1* (*TOC1*), an evening circadian clock gene, is upregulated by the application of SA. However, the timing of its expression does not change irrespective of whether SA is applied at dawn or dusk (Zhou et al., 2015). Due to the

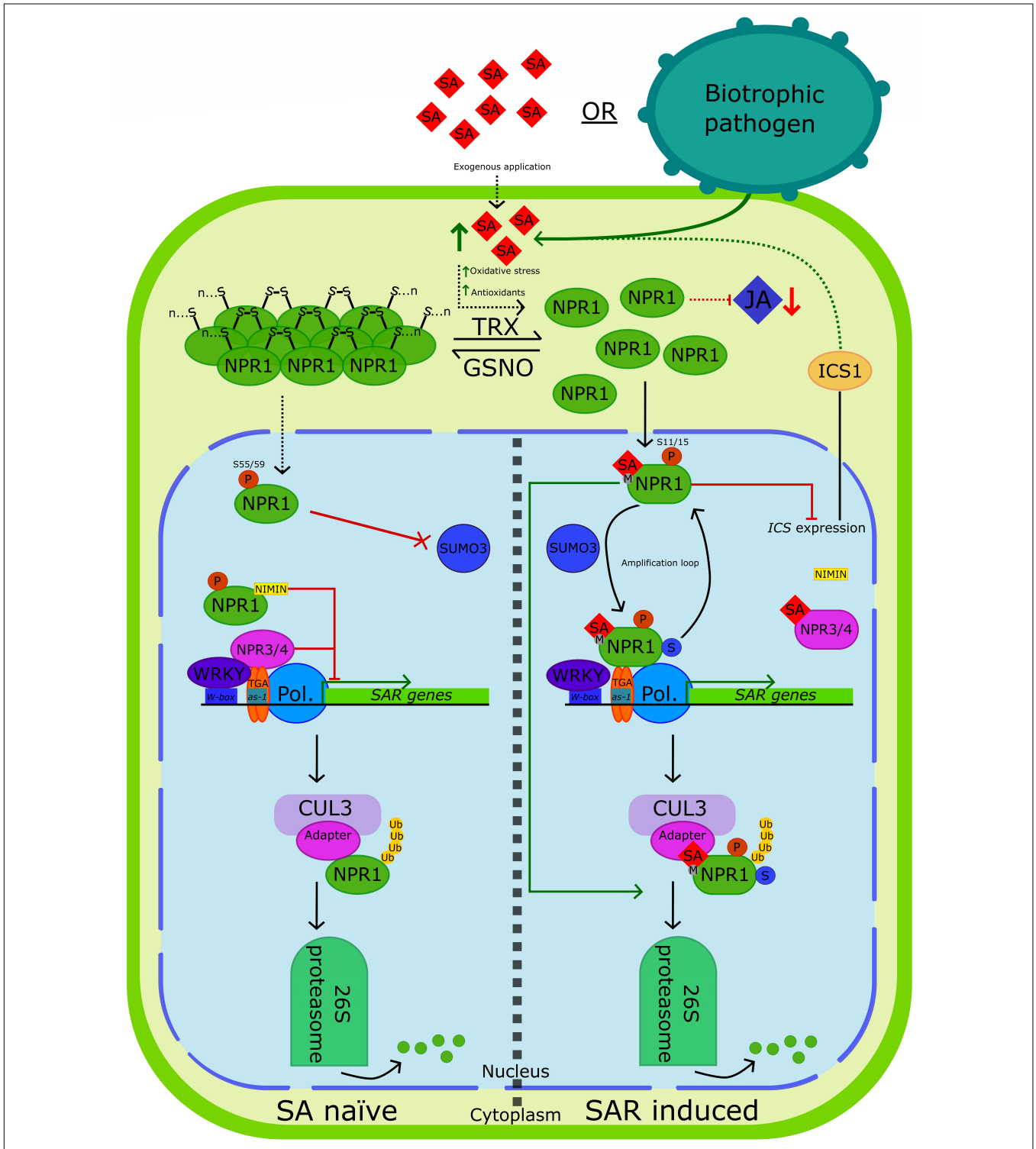

FIGURE 2 | A working model of NPR1, NPR3, and NPR4. The left side of the diagram, partially separated by a dashed line, depicts the regulation of NPR1-dependant defense genes in SA naïve cells. At low SA concentrations, *S*-nitrosylation of NPR1^{Cys156} by GSNO encourages the existence of NPR1 in its oligomeric form. The oligomeric form of NPR1 is predominantly found within the cytoplasm. To prevent the uninduced expression of SAR-related genes, several mechanisms are in place to suppress NPR1-dependant defense gene expression. Phosphorylation of NPR1$^{Ser55/59}$ suppresses defense gene expression and prevents sumoylation of NPR1 by SUMO3, an important aspect of NPR1 activation. The N-terminal BTB/POZ domain of NPR1 interacts with and suppresses the function of the C-terminal transactivation domain of NPR1. Additionally, NIMIN proteins interact with NPR1 to suppress gene expression. Paralogs of NPR1, NPR3, and NPR4, interact with TGA2/TGA5/TGA6 to further suppress transcription. Certain WRKY transcription factors act as transcriptional repressors of a subset of

(Continued)

FIGURE 2 | Continued
SAR-related genes. Finally, NPR1 is degraded by the 26S proteasome following CUL3-mediated ubiquitinylation. However, NPR1 is unable to directly interact with CUL3 and E3 ligases, likely requiring a substrate adapter. The right side of the diagram depicts NPR1 regulation in SAR-induced cells where SA concentration is elevated either due to exogenous application of SA/one of its functional analogs or during biotrophic/hemibiotrophic pathogen challenge. Increased oxidative stress and subsequent increases in antioxidant production leads to the reduction of $NPR1^{Cys156}$, specifically by thioredoxins, leading to the disassembly of the NPR1 oligomer. Within the cytoplasm, NPR1 antagonizes the JA-defense response pathway. Monomeric NPR1 is then translocated to the nucleus via the action of a bipartite nuclear localization signal. Within the nucleus, NPR1 suppresses the expression of *ICS1* which is essential to SA synthesis in response to pathogenic stress, forming a negative feedback loop. Phosphorylation of $NPR1^{Ser11/15}$ within the N-terminal IκB-like phosphodegron motif both enhances interaction with SUMO3 and targets NPR1 for ubiquitinylation and degradation by the 26S proteasome. Sumoylation of NPR1 by SUMO3 also increases and decreases association of NPR1 with TGA and WRKY transcription factors, respectively. SUMO3 is also required for phosphorylation of $NPR1^{Ser11/15}$, creating an amplification loop which leads to the activation of more NPR1, increasing SAR-related gene expression. Interaction of SA and NPR1 requires a transition metal. Following binding of SA to NPR1 a conformational change of C-terminal transactivation domain of NPR1 decreases its affinity for the inhibitory N-terminal BTB/POZ domain. In turn, the BTB/POZ domain of NPR1 interacts with the N-terminal repression domain of TGA transcription factors, thereby activating transcription. Furthermore, binding of SA to NPR1 alters its interaction with NIMINs, relieving repression. Moreover, binding of SA to NPR3/NPR4 diminishes their ability to suppress SAR-related gene expression. Turnover of NPR1 through degradation by the 26S proteasome is essential to preserving peak gene expression and is required for the complete induction of SAR.

redox sensitivity of NPR1, Zhou et al. (2015) hypothesized that NPR1 might play a role in the expression of *TOC1*. Indeed, they demonstrated that basal and SA-induced expression of *TOC1* was reduced and abolished, respectively, in *npr1* mutants (Zhou et al., 2015). This concept was further supported as NPR1 displayed increased association with TGA-binding sites of the *TOC1* promoter in a SA-dependent manner (Zhou et al., 2015). The NPR1 monomer also shows a circadian oscillation, peaking at night (Zhou et al., 2015). The influence that NPR1 has on *TOC1* is reliant on its translocation into the nucleus as *trx-h3 trx-h5* mutants showed decreased basal *TOC1* expression and decreased responsiveness to SA (Zhou et al., 2015). Thus, oscillation in SA and subsequent redox changes drive the nuclear translocation of NPR1, which is required for the regulation of *TOC1* (Zhou et al., 2015). However, these findings do not fully explain why SA-application at dawn showed a delayed induction of *TOC1* until dusk. Through mathematical modeling and *in planta* confirmation it was shown that NPR1 regulates the morning clock gene *LATE ELONGATED HYPOCOTYL* (*LHY*), a known antagonist of *TOC1* (Zhou et al., 2015). This study underpins the importance of NPR1 in defense responses as well as the circadian clock, by interlacing these processes the plant can prioritize growth over increased immunity at night while increasing immunity at dawn when the threat from pathogens is highest (Nozue et al., 2007; Bhardwaj et al., 2011; Korneli et al., 2014; Zhou et al., 2015).

NPR1 IN TRANSGENIC CROPS

Arabidopsis NPR1 has been overexpressed in a multitude of agricultural crops and can enhance resistance to a variety of biotrophic and necrotrophic pathogens (Cao et al., 1998; Chern et al., 2001, 2005b; Lin et al., 2004; Makandar et al., 2006; Xujing et al., 2006; Malnoy et al., 2007; Potlakayala et al., 2007; Yuan et al., 2007; Wally et al., 2009; Parkhi et al., 2010b; Le Henanff et al., 2011; Kumar et al., 2013; Dutt et al., 2015; Molla et al., 2016). This indicates that a high level of functional conservation likely exists in all plant species. However, overexpression studies in rice and strawberry also demonstrated the negative influences constitutive overexpression of *AtNPR1* can have on certain crops (Fitzgerald et al., 2004; Quilis et al., 2008; Silva et al., 2015). Though, specifically expressing *AtNPR1* in only the green tissues of rice, using the $P_{D54O-544}$ promoter, conferred resistance to sheath blight disease caused by the fungus *Rhizoctonia solani* without any detrimental phenotypic effects (Molla et al., 2016). This would suggest that more targeted expression of *AtNPR1* might benefit strawberry as well as any other crops which exhibit sensitivity to global overexpression.

Nevertheless, *AtNPR1* overexpression in crops such as wheat, tomato, carrot, soybean, canola, citrus, tobacco, and cotton display significantly improved disease resistance and even crop yield without any negative phenotypic effects (Lin et al., 2004; Makandar et al., 2006; Potlakayala et al., 2007; Boller and He, 2009; Wally et al., 2009; Parkhi et al., 2010a,b; Gao et al., 2013; Kumar et al., 2013; Matthews et al., 2014). Interestingly overexpression of *AtNPR1* seems to have a negligible effect on basal defense gene expression in many crops, while significantly increasing the response time and strength of defense responses (Wally et al., 2009; Zhang et al., 2010b; Kumar et al., 2013; Boscariol-Camargo et al., 2016). Remarkably, tobacco overexpressing *AtNPR1* also displayed increased resistance to the herbivore *Spodoptera litura* (Meur et al., 2008). Thus, in many cases increasing NPR1 expression not only increases broad spectrum disease resistance but does so without negative impacts on plant growth.

Overexpression of Rice *NPR1* (*OsNPR1/NH1*) in *Arabidopsis* is able to complement the *npr1* mutant, however, several negative consequences are observed, including enhanced herbivore susceptibility (Yuan et al., 2007). This, however, suggests a role for NH1, like that of AtNPR1, in crosstalk between the SA- and JA-defense signaling pathways. Yet, these observations together with the negative phenotypic effects observed during overexpression of *AtNPR1* in rice while such deleterious effects are absent in many other crops, would suggest that some notable differences exist between species regarding the regulation of NPR1. The high basal level of SA in rice which remains unaltered following pathogen infection contrasts with that of *Arabidopsis* and tobacco and supports such a theory (Quilis et al., 2008; Dempsey et al., 2010). Therefore, although NPR1 may serve functionally conserved roles in all plant species, the underlying mechanisms which regulate NPR1-dependant pathways need to be understood for the species under investigation.

Overexpression of *AtNPR1* orthologs from several plant species have also been studied (Malnoy et al., 2007; Potlakayala et al., 2007; Le Henanff et al., 2009, 2011; Shi et al., 2010; Chen et al., 2012b; Yocgo et al., 2012; Zhang et al., 2013; Wang et al., 2017). Apple *AtNPR1* orthologs *MhNPR1* (*Malus hupehensis*) and *MpNPR1* (*Malus pumila*) enhanced resistance to several important pathogens in *Malus x domestica* (Malnoy et al., 2007; Chen et al., 2012b). Likewise, overexpression of *LhSorNPR1* from the oriental hybrid lily 'Sorbonne' in *Arabidopsis* increased wild-type resistance to *P. syringae* (Wang et al., 2017). In tobacco, overexpression of *MhNPR1* increased resistance to *B. cinerea* and interestingly, salt tolerance (Zhang et al., 2012, 2014). Similarly, complementation of *Arabidopsis npr1* using *VaNPR1.1* (*Vitis aestivalis* cv. Norton) increased salt tolerance (Zhang et al., 2013). Additionally, *Arabidopsis npr1* mutants are complemented using *BnNPR1* (*Brassica napus*) while overexpression in *B. napus* enhanced resistance to *P. syringae* without any obvious negative effects, emphasizing similarity between NPR1-dependant defense responses in these two species (Potlakayala et al., 2007). However, *NPR1* incompatibility between species is made apparent as apical dominance is affected in *Arabidopsis* overexpressing *VvNPR1.1* (Le Henanff et al., 2011). These results support the highly versatile and important role of NPR1 in studied plant species. For a thorough review on its potential uses in transgenic crop protection, see Silva et al. (2018).

CONCLUSION AND FUTURE DIRECTIONS

Since its discovery more than 20 years ago, NPR1 has been the focus of countless studies. During this time several NPR1-dependant pathways have been uncovered as have many of the complex mechanisms governing the regulation of NPR1 (**Figure 2**). However, much is still left unanswered, owed to the multifaceted relationships that exist between NPR1, its paralogs and their interacting partners. Truly grasping the extent of such interactions requires an increased effort to discover novel interactions in different species, tissues and during plant–pathogen specific interactions. A topic pursuant to this which is severely underrepresented in the literature is that of tissue specific regulation of NPR1-dependant pathways. Microarray data suggests that significant differences exist regarding the expression of *NPR1*-like genes across various tissues in *Arabidopsis* (Shi et al., 2013). Similarly, the expression of avocado *NPR1*-like genes exhibit unique spatial preferences (Backer et al., 2015). The importance of these observations are highlighted in *Arabidopsis npr3* knockout mutants which display increased resistance to *P. syringae* on developing flowers but not leaves (Shi et al., 2013).

Important aspects of the regulation of NPR1 function and homeostasis which require further attention are that of post-translational modification and proteasome degradation of NPR1. Important questions have been raised regarding these processes, including whether NPR3 and NPR4 act as E3 ligases which lead to the ubiquitinylation of NPR1. Surprisingly, even though Fu et al. (2012) suggested such a role, a more recent report failed to detect interactions between either NPR1 and NPR3/NPR4 or NPR3/NPR4 and CUL3A (Ding et al., 2018). Given the importance of NPR1 turnover to maintain optimal NPR1-dependant gene expression and the role post-translational modifications have in this process, it is imperative to further characterize and understand the process. Discovering how exactly NPR1 is ubiquitinylated by CUL3 given the absence of direct interaction could increase our understanding of post-translational modification as a means of regulating the function of NPR1.

Given that NPR1, NPR3, NPR4, BOP1, and BOP2 all function in the regulation of various transcriptional processes, it is hard to ignore the possibility that NPR2 may serve a similar yet undefined function. This is especially true since *NPR2* is induced by biotic stress and was shown to play a significant role in the perception of SA (Canet et al., 2010b). Phytohormones SA, JA and ET are known to promote leaf senescence and notably, various WRKY and bZIP transcription factors are involved (Zhao et al., 2016). Furthermore, an essential component of SA-induced leaf senescence in *Arabidopsis*, MAPK6, influences the activity and gene expression of *NPR1* (Chai et al., 2014). Therefore it is not surprising that *Arabidopsis npr1-5* null mutants which are impaired in SA biosynthesis suppress precocious leaf senescence characteristic of *pat14* mutants (Zhao et al., 2016). Together, these data clearly suggest that NPR1-dependant signaling is involved in senescence. Interestingly, Shi et al. (2013) noted that NPR2 transcripts were most abundant in senescent tissue, thus it is conceivable that NPR2 may serve a similar or even a more direct role in senescence. Conversely, NPR2 may simply be a redundant or non-functional paralog of NPR1. In either case, it would be worthwhile to investigate and determining which transcription factors interact with NPR2 may be a good place to start.

Given the complex nature of the NPR1-like protein family and uncertainty surrounding the aspects mentioned above, caution should be exercised regarding generalizing statements concerning functions *in planta* and across species. Instead emphasis should be placed on describing temporal, spatial and plant–pathogen interaction specific functions. Nonetheless, continued research on the NPR1-like protein family is warranted and will undoubtedly bring forth novel insights into the molecular pathways involved in plant stress responses and development.

AUTHOR CONTRIBUTIONS

RB conceptualized, drafted, and reviewed the manuscript. SN reviewed and assisted in drafting the manuscript. NvdB conceptualized, reviewed, and assisted in drafting the manuscript. All authors contributed to and approved the final manuscript.

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Chapter 2

Research Article

frontiers in Plant Science

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Phylogenetic and expression analysis of the *NPR1*-like gene family from *Persea americana* (Mill.)

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The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) forms an integral part of the salicylic acid (SA) pathway in plants and is involved in cross-talk between the SA and jasmonic acid/ethylene (JA/ET) pathways. Therefore, NPR1 is essential to the effective response of plants to pathogens. Avocado (*Persea americana*) is a commercially important crop worldwide. Significant losses in production result from Phytophthora root rot, caused by the hemibiotroph, *Phytophthora cinnamomi*. This oomycete infects the feeder roots of avocado trees leading to an overall decline in health and eventual death. The interaction between avocado and *P. cinnamomi* is poorly understood and as such limited control strategies exist. Thus uncovering the role of NPR1 in avocado could provide novel insights into the avocado – *P. cinnamomi* interaction. A total of five *NPR1*-like sequences were identified. These sequences were annotated using FGENESH and a maximum-likelihood tree was constructed using 34 NPR1-like protein sequences from other plant species. The conserved protein domains and functional motifs of these sequences were predicted. Reverse transcription quantitative PCR was used to analyze the expression of the five *NPR1*-like sequences in the roots of avocado after treatment with salicylic and jasmonic acid, *P. cinnamomi* infection, across different tissues and in *P. cinnamomi* infected tolerant and susceptible rootstocks. Of the five *NPR1*-like sequences three have strong support for a defensive role while two are most likely involved in development. Significant differences in the expression profiles of these five *NPR1*-like genes were observed, assisting in functional classification. Understanding the interaction of avocado and *P. cinnamomi* is essential to developing new control strategies. This work enables further classification of these genes by means of functional annotation and is a crucial step in understanding the role of NPR1 during *P. cinnamomi* infection.

Keywords: avocado, *Phytophthora cinnamomi*, NPR1, expression analysis, salicylic acid, jasmonic acid, *pathogenesis-related*

Introduction

Plants recognize and react to external threats much like any other living organism, eliciting a response to combat disease (Robert-Seilaniantz et al., 2011). Defense responses against biotrophic and hemibiotrophic pathogens are mainly dependent on the salicylic acid (SA)

pathway (Glazebrook, 2005). Plants challenged by a biotrophic pathogen show a substantial increase in endogenous SA, a subsequent hypersensitive response (HR) at the site of infection and the onset of systemic acquired resistance (SAR; Malamy et al., 1990; Metraux et al., 1991; Rasmussen et al., 1991; Gaffney et al., 1993; Delaney et al., 1994). SAR is an important part of plant defense, providing long term, broad spectrum resistance which is effective against a wide variety of fungal, viral and bacterial pathogens at tissues distal to the initial site of infection (Frederich et al., 1996; Sticher et al., 1997; An and Mou, 2011; Robert-Seilaniantz et al., 2011). Increases in SA concentration have been conclusively linked to the establishment of SAR, for instance, exogenous application of SA or one of its biologically active analogs, 2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), is able to induce SAR (White, 1979; Ward et al., 1991; Lawton et al., 1995). Conversely, plants that express the transgene *nahG* which encodes for a salicylate hydroxylase, lack functionally active SA and are SAR compromised (Gaffney et al., 1993; Bi et al., 1995; Friedrich et al., 1995; Lawton et al., 1995).

The quest to discover the SA receptor led to the discovery of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), a transcription co-factor protein encoded for by *NPR1* (Cao et al., 1994). The majority of described NPR1 proteins contain ankyrin repeat and Broad Complex, Tramtrack and Bric a brac/Pox virus and Zinc finger (BTB/POZ) domains (Cao et al., 1997; Hepworth et al., 2005; Spoel et al., 2009). These domains are essential for protein–protein interactions and enable NPR1 to function as a co-activator (Cao et al., 1997; Rochon et al., 2006). In *Arabidopsis* NPR1 is found as an oligomer within the cytoplasm of uninduced cells and changes in SA concentration lead to an altered redox environment within the cell, supporting the nuclear localization of NPR1 in its monomeric form (Mou et al., 2003). It is worth noting that NPR1 is constitutively localized within the nucleus of several plant species, yet the perception of a SA signal is still required for the expression of *pathogenesis-related* (*PR*) genes (Kinkema et al., 2000; Le Henanff et al., 2009; Maier et al., 2011).

Multiple NPR1-like proteins seem to be present in most, if not all, plant species. Phylogenetic analysis of this family suggests the existence of three functionally distinct clades (Hepworth et al., 2005; Zhang et al., 2006; Peraza-Echeverria et al., 2012). Members of the first clade, AtNPR1 and AtNPR2, are mostly associated with positive SAR regulation (Cao et al., 1997, 1998). The second clade, AtNPR3, and AtNPR4, is associated with negative SAR regulation, yet is required for effective SAR induction (Liu et al., 2005; Zhang et al., 2006). The third clade, AtBOP1, and AtBOP2, is associated with the development of lateral organs (Hepworth et al., 2005). Phylogenetic analysis has since included NPR1-like proteins from multiple plant species (Le Henanff et al., 2009; Peraza-Echeverria et al., 2012; Shao et al., 2013), and although phylogenetic analysis alone is insufficient for functional annotation it may provide a basis for understanding functional variation (Liu et al., 2005; Zhang et al., 2006).

The most extensively studied member of the *Arabidopsis* NPR1-like family is AtNPR1. Mutants of this protein are more susceptible to virulent pathogens and display compromised expression of *PR* genes when compared to plants expressing wild-type *NPR1* (Glazebrook et al., 1996; Cao et al., 1997). Complementation of these *npr1* mutants using wild-type *NPR1* restores the expression of *PR* genes as well as pathogen resistance and the induction of SAR (Cao et al., 1997). Various plants overexpressing *NPR1* show increased *PR* gene expression and pathogen resistance (Cao et al., 1998; Friedrich et al., 2001; Malnoy et al., 2007; Le Henanff et al., 2011; Chen et al., 2012; Kumar et al., 2012). Overexpressing *OsNPR1*, the ortholog of *AtNPR1* in rice, results in an increased resistance to bacterial blight, yet these transgenic plants show an increased susceptibility to herbivores (Yuan et al., 2007). Interestingly, herbivore hypersensitivity is alleviated when NPR1 is constitutively localized to the nucleus (Yuan et al., 2007). These results suggest that NPR1 is involved in the antagonistic cross-talk between the SA and jasmonic acid/ethylene (JA/ET) pathways, a theory supported by several other studies (Spoel et al., 2003; El Oirdi et al., 2011). Thus, NPR1 is considered the master regulator of defense responses in plants.

Additionally, NPR1 interacts with several members of the TGACG motif-binding factor (TGA) family of basic leucine zipper protein (bZIP) transcriptions factors (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000). These transcription factors associate with the *as-1-like* (TGACG) promoter element within *PR* gene promoters and are responsible, at least in part, for their expression (Fan and Dong, 2002; Zhang et al., 2003). Moreover, the DNA binding affinity of TGA factors is increased when associated with NPR1 (Despres et al., 2000; Fan and Dong, 2002) and NPR1 may also deactivate the repression of *PR* genes by certain TGA factors (Rochon et al., 2006). This interaction describes the basis of NPR1-dependant gene expression, yet the realistically more complex mechanism involves several other factors (Li et al., 1999; Weigel et al., 2001; Chern et al., 2005; Zhang et al., 2006; Zwicker et al., 2007).

Evidence suggests that NPR3 and NPR4 are essential to establishing SAR even though they suppress NPR1-dependant gene expression (Liu et al., 2005; Zhang et al., 2006; Fu et al., 2012). Both NPR3 and NPR4 act as adaptors for Cullin 3 (CUL3) E3 ligase-facilitated ubiquitinylation and subsequent proteasome degradation of NPR1 (Fu et al., 2012). The degradation of NPR1 serves dual roles; turn-over of NPR1 as well as suppression of NPR1-dependant gene expression in SA naïve cells and cells undergoing HR (Spoel et al., 2009). The expression of NPR1-dependant genes during non-stress conditions and HR increases fitness costs and prevents the establishment of HR, respectively (Rate and Greenberg, 2001; Heidel et al., 2004). In SA naïve cells NPR4 strongly interacts with NPR1 thus preventing increases in expression of *PR* genes (Fu et al., 2012). Moreover, increased SA concentrations interrupt this interaction and increase the affinity of NPR3 for NPR1 (Fu et al., 2012). At the site of HR where SA concentrations are the highest NPR1 is rapidly degraded, while at distal tissues with intermediate SA concentrations NPR3 merely facilitates sufficient turn-over of NPR1 (Fu et al., 2012). The turn-over of transcription factors ensures optimal expression of target genes (Salghetti et al., 2000; Collins and Tansey, 2006), as seen for NPR1. Thus by responding to the concentration of SA, NPR3, and NPR4 prevent the untimely expression of *PR* genes,

fine-tuning the defense response. It is therefore clear that understanding the role of the NPR1-like family is an important part of understanding defense responses in plants.

Avocado is an economically important fruit crop with an annual worldwide gross production value of US $ 3835 million[1]. The fruit are highly nutritious and contain high levels of monounsaturated fats making them popular for use in a wide variety of culinary products. The greatest threat to production is Phytophthora root rot (PRR), caused by the hemibiotrophic oomycete pathogen *Phytophthora cinnamomi* Rands (Hardham, 2005). Infection by *P. cinnamomi* results in decreased water and nutrient absorption due to necrosis of the avocado feeder roots, leading to a decline in tree health and eventual death ensuing economic losses (Zentmyer, 1984; Coffey, 1987). With a wide host range of more than 3000 plant species and the ability to persist in the environment (Weste, 1983; Hardham, 2005), effective control of *P. cinnamomi* is limited.

The use of phosphite trunk injections, tolerant rootstocks (e.g., Dusa®) and organic mulching practices are currently utilized by industry as methods for controlling PRR (Coffey, 1987; Giblin et al., 2005). Phosphite trunk injections have been a dependable method for over 30 years (Darvas et al., 1984; Pegg et al., 1985; Coffey, 1987; Kaiser et al., 1997; Giblin et al., 2005), yet evidence suggests that *P. cinnamomi* has the potential to develop decreased sensitivity against this fungicide (Duvenhage, 1994; Dobrowolski et al., 2008). Similar observations occur for metalaxyl, another decidedly effective fungicide (Darvas et al., 1984). Moreover, the lengthy selection process for PRR tolerant rootstocks (Gabor and Coffey, 1991; Menge, 1999; Kremer-Köhne and Mukhumo, 2003) limits the introduction of novel tolerant rootstocks, possibly providing the pathogen with an opportunity to overcome host tolerance.

Although biochemical and histological studies have provided some insight into the avocado – *P. cinnamomi* interaction (Phillips et al., 1987; Botha and Kotze, 1989; Bekker et al., 2006; Sánchez-Pérez et al., 2009; García-Pineda et al., 2010), research on the molecular characteristics of this interaction have only recently gained attention (Mahomed and van den Berg, 2011; Reeksting et al., 2014). Our current understanding of the incompatible *Arabidopsis thaliana* – *P. cinnamomi* interaction provides limited information on compatible interactions. For example, in *Arabidopsis* the JA/ET pathway seems to be essential to *P. cinnamomi* resistance (Rookes et al., 2008), yet in avocado SA inhibits growth and colonization (García-Pineda et al., 2010). Such conspicuous differences between non-host and host interactions highlight the need to elucidate the host specific interaction between avocado and *P. cinnamomi* on a molecular level.

Thus defining the role of NPR1 in avocado could potentially provide novel insights into the avocado – *P. cinnamomi* interaction. This is the first study aimed at discovering and characterizing *NPR1*-like genes in *Persea americana*. We have discovered five *NPR1*-like genes from *P. americana* which harbor the ankyrin repeat and BTB/POZ domains and show sequence similarity to other known *NPR1*-like genes. Phylogenetic analysis reveals that the predicted protein sequences of these genes can be resolved into the three known phylogenetic clades of the NPR1-like family. We describe the expression of these genes in Dusa®, a PRR tolerant avocado rootstock, across five time points during treatment with SA, MeJA, and *P. cinnamomi* using RT–qPCR. Additionally, we measured the basal expression levels for each transcript across six different tissues. The findings of this study provide an invaluable resource for further study and functional characterization of the NPR1-like family in avocado. Future efforts could focus on intracellular interactions and localization as well as overexpression of defense related *PaNPR1*-like genes in wild-type and *npr1* mutant *Arabidopsis*.

Materials and Methods

Sequence Annotation and Phylogenetic Analysis

Five *NPR1*-like gene sequences were obtained from the *P. americana* genome (Unpublished data). Sequences were arbitrarily assigned identifiers as follows: *PaNPR1*, *PaNPR2*, *PaNPR3*, *PaNPR4,* and *PaNPR5*. Open reading frames (ORF's) were annotated using the online prediction tool FGENESH with the *Vitis vinifera* genome-specific parameters selected (Solovyev et al., 2006). Exon/intron positions of predicted CDSs were visualized using GSDS software v2.0 (Guo et al., 2007). Percentage amino acid similarity was calculated using SIAS[2]. Protein domains were predicted using PROSITE (Sigrist et al., 2010). Sequences were submitted to GenBank[3]: *PaNPR1* [GenBank: KR056089], *PaNPR2* [GenBank: KR056090], *PaNPR3* [GenBank: KR056091], *PaNPR4* [GenBank: KR056092], and *PaNPR5* [GenBank: KR056093].

Phylogenetic Analysis

Additional NPR1-like protein sequences from other plant species were attained online at NCBI[4] in order to perform alignments (**Table 1**). Sequences were aligned using Clustal W software v2.1 (Thompson et al., 1994). The best substitution model for the alignment was determined and subsequently used in construction of a maximum likelihood (ML) phylogenetic tree using the tools available in MEGA software v5.2 (Tamura et al., 2011). The initial tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) and bootstrapping (1000 replicates) was used to determine confidence.

Plant Material

One-year-old clonal PRR-tolerant Dusa® rootstock plantlets were provided by Westfalia Technological Services (Tzaneen, South Africa). Two phytohormone treatment groups were assigned and treated with 70 ml sodium salicylate (NaSA) solution (5 mM NaSA (Sigma–Aldrich, St. Louis, MO, USA), 0.1% Tween® 20 (v/v) (Sigma–Aldrich, St. Louis, MO, USA) or 70 ml methyl jasmonate (MeJA) solution (5 mM MeJA (Sigma–Aldrich,

[1]http://faostat3.fao.org/faostat-gateway/go/to/home/E

[2]http://imed.med.ucm.es/Tools/sias.html

[3]http://www.ncbi.nlm.nih.gov/genbank/

[4]http://www.ncbi.nlm.nih.gov/

TABLE 1 | Additional NPR1-like protein sequences from other plant species.

Species	Identifier	Accession number
Arabidopsis thaliana	AtNPR1	[GenBank: NP_176610]
A. thaliana	AtNPR2	[GenBank: NP_194342]
A. thaliana	AtNPR3	[GenBank: NP_199324]
A. thaliana	AtNPR4	[GenBank: NP_193701]
A. thaliana	AtBOP1	[GenBank: NP_001190116]
A. thaliana	AtBOP2	[GenBank: NP_181668]
Populus deltoides	PdNPR1-1	[GenBank: AEY99652]
P. deltoides	PdNPR2	[GenBank: AEE81755]
Beta vulgaris	BvNPR1	[GenBank: AAT57640]
Hordeum vulgare subsp. vulgare	HvNPR1	[GenBank: CAJ19095]
Sorghum bicolor	SbNPR1	[GenBank: XP_002455011]
Helianthus annuus	HaNPR1	[GenBank: AAT57642]
Glycine max	GmNPR1-1	[GenBank: ACJ45013]
G. max	GmNPR1-2	[GenBank: ACJ45015]
Physcomitrella patens	PhNPR-like1	[GenBank: XP_001757508]
P. patens	PhNPR-like2	[GenBank: XP_001759240]
Vitis vinifera	VvNPR1.1	[GenBank: XP_002281475]
V. vinifera	VvNPR1.2	[GenBank: XP_003633057]
Oryza sativa	OsNPR1	[GenBank: AAX18700]
O. sativa	OsNPR2	[GenBank: ABE11616]
O. sativa	OsNPR3	[GenBank: ABE11618]
O. sativa	OsNPR5	[GenBank: ABE11622]
Gossypium hirsutum	GhNPR1	[GenBank: ABC54558]
Ipomoea batatas	IbNPR1	[GenBank: ABM64782]
Solanum lycopersicum	LeNPR1	[GenBank: AAT57637]
Nicotiana tabacum	NtNPR1	[GenBank: AAM62410]
Capsicum annum	CaNPR1	[GenBank: ABG38308]
Musa spp. AAA	MNNPR1A	[GenBank: ABI93182]
Musa spp. AAA	MNNPR1B	[GenBank: ABL63913]
Musa spp. ABB	MdNPR1	[GenBank: ACJ04030]
Malus x domestica	MpNPR1-1	[GenBank: ACC77697]
Pyrus pyrifolia	PpNPR1-1	[GenBank: ABK62792]
Populus trichocarpa	PtNPR1.1	[GenBank: XP_002308281]
P. trichocarpa	PtNPR1-like	[GenBank: XP_002323261]

Accession numbers for several NPR1-like protein sequences. Sequences were used to construct a maximum likelihood (ML) phylogenetic tree along with the five predicted NPR1-like protein sequences from Persea americana.

St. Louis, MO, USA), 0.1% ethanol (99.9%), 0.1% Tween® 20 (v/v)). A third treatment group was inoculated with 20 ml *P. cinnamomi* zoospore suspension (3×10^5 spores/ml) and 70 ml *P. cinnamomi* mycelial suspension. Each treatment contained three biological replicates with two plants per replicate. Control plants were either treated with 70 ml NaSA control solution (0.1% Tween®) or 70 ml MeJA control solution [0.1% Tween®, 0.1% ethanol (99.9%)]. Each control group contained three biological replicates with one plant per replicate. Plants were randomly assigned to either the treatment or control groups. All treatments and controls were applied directly to the soil at the base of the plant. Roots were harvested for all treatment and control groups at 6, 12, 18, 24, and 96 h. Samples were snap frozen in liquid nitrogen and stored at −80°C. Biological replicates were homogenized using the IKA® Tube Mill control (IKA®, Staufen, Germany) until a fine consistency was attained.

Mature grafted trees located at Westfalia (Tzaneen, South Africa) were used for the collection of tissue samples. Six tissue types were selected: feeder roots, mature green stems, mature green leaves, unripe fruit as well as stems and leaves from flush growth (young material). Samples were taken from a single orchard block which contained clonal Hass fruitstocks grafted onto clonal PRR-tolerant Duke 7 rootstocks. Fifteen trees were randomly selected from which two samples of each tissue were taken for each individual tree. Samples were snap frozen in liquid nitrogen and stored at −80°C. Tissue samples were randomly allocated to three groups of five trees, individual tissue samples from each group were then pooled and homogenized using the IKA® Tube Mill control (IKA®).

Phytophthora cinnamomi Infection

Zoospores were produced by placing *P. cinnamomi* colonized blocks of V8 agar (20% V8 juice (v/v), 0.25% $CaCO_3$, agar 17g.l^{-1}) into 90 mm Petri dishes containing 2% V8 broth for 3 days to allow sufficient mycelial growth. Cultures were then rinsed three times with dH_2O and run-off stored for use as mycelial suspension. Filtered stream water was then added and cultures left under UV light for 2–3 days until sufficient sporangia formation was observed. Cultures were then cold-shocked at 4°C for 45 min and placed at room temperature for 1 h to allow zoospore release. Sufficient zoospore release and motility was monitored via microscopy. Inoculation was carried out immediately by pouring both the zoospore and mycelial suspension directly onto the soil at the base of the plants.

Nested PCR

Total genomic DNA was isolated from inoculated root samples. Nested LPV3 PCR was then performed in order to confirm successful infection of plant roots by *P. cinnamomi* as described by Engelbrecht et al. (2013). Results were visualized on 2% TAE agarose gel under non-denaturing conditions.

RNA Extraction

Total RNA was extracted from homogenized plant material using a modified version of the CTAB extraction method described by Chang et al. (1993). The chloroform: isoamyl alcohol step was repeated four to six times until the volume of the interphase diminished and the supernatant was clear. Samples were resuspended in diethylpyrocarbonate (DEPC) treated water containing 30 U/ml RiboLock RNase Inhibitor (Thermo Fisher Scientific Inc., Leicestershire, UK). RNA concentration and purity was assessed using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies Inc., Montchanin, DE, USA). RNA integrity was assessed on 2% TAE agarose gel under non-denaturing conditions.

Total RNA from *P. cinnamomi* infected tolerant (Dusa®) and susceptible (R0.12) avocado rootstocks at 0 h (uninfected control), 6, 12, and 24 h were obtained from Engelbrecht et al. (2013). RNA concentration and purity was assessed using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Montchanin, DE, USA). RNA integrity was assessed on 2% TAE agarose gel under non-denaturing conditions.

cDNA Synthesis

RNA was purified of any contaminants using the RNeasy MinElute Cleanup Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions followed by an on-column RNase-free DNase I (Thermo Fischer Scientific) treatment. cDNA was synthesized using the ImProm-II™ single strand cDNA synthesis kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's guidelines. First strand synthesis was primed using 0.5 μg random hexamers (Thermo Fisher Scientific). cDNA concentration and purity was assessed using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies). Genomic DNA (gDNA) contamination was assessed using the intron-spanning flavone-3-hydroxylase (F3H) primers as described in (Reeksting et al., 2014).

RT-qPCR

Primers for reverse transcription quantitative PCR were designed using CLC Genomics Workbench v5.1 (CLC Bio, Qiagen® Inc., Hilden, Germany) and quality assessed on NetPrimer (Premier Biosoft International, Palo Alto, CA, USA). Primers with annealing temperatures between 55 and 60°C, expected amplicons lengths of <150 bp and quality scores >95.0 (NetPrimer) were synthesized (Inqaba Biotec, Pretoria, South Africa; **Table 2**). Primer specificity was confirmed by performing conventional PCR and sequencing (African Center for Gene Technologies, Pretoria, South Africa) and by the presence of a single melting curve. A 1:3 serial dilution was derived from a comprehensive mix of treated and control cDNA samples. Calibration curves were then performed for each candidate and reference gene across multiple temperatures to ensure that efficiency (E) and correlation (R^2) values were in accordance with MIQE guidelines (Bustin et al., 2009; **Table 3**). All reactions were performed using SensiMix™ SYBR® No-ROX kit (Bioline USA, Inc., Taunton, MA, USA) according to manufacturer's instructions with a final reaction volume of 10 μl. Thermal cycling was performed on the Bio-Rad® CFX 96 instrument (Bio Rad laboratories., Hercules, CA, USA). Thermal cycling conditions were: 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at T_a (**Table 3**) and 10 s at 72°C, a melting dissociation curve was constructed from 60 to 95°C at 0.5°C increments following the final cycle. Three endogenous control genes were used in order to normalize the data for each gene, namely *Actin*, *18S*, and *alpha-1 tubulin* as reported by Reeksting et al. (2014). The stability of the reference genes were analyzed using Bio-Rad® CFX Manager software v1.5 (Bio Rad laboratories; **Table 3**). Normalized relative quantities (fold change) for genes were calculated using the method described by Pfaffl (2001). Expression values for each time-point in a specific treatment were calibrated against a control of the corresponding time-point. Cleanup of RT–qPCR products was performed using Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, Irvin, CA, USA) in preparation for sequencing. Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Thermo Fisher Scientific) according to the manufacturer's guidelines. Reactions were precipitated using 3 M sodium acetate (pH 5.2) and submitted for sequencing (African Center for Gene Technologies, Pretoria, South Africa).

Statistical Analysis

A student's *t*-test was performed to determine significance for quantitative gene expression analysis. SA, MeJA, and *P. cinnamomi* treated samples were compared to respective controls at each time point. Statistical analysis was performed using GraphPad Prism software v6.0.5 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was assessed using a 95% confidence interval. Statistical analysis for various tissue samples was done using JMP 11 (SAS Institute Inc., Cary, NC, USA). Initial analysis was performed using one-way ANOVA followed by Tukey's HSD test, adhoc. Significance was assessed using a 95% confidence interval. A Mann–Whitney unpaired *t*-test was performed to determine significance for quantitative gene expression analysis. Expression in tolerant plants was compared to that of susceptible plants at each respective time point. Statistical analysis was performed with the Statistics Online Computational Resource package[5] Significance was assessed using a 95% confidence interval.

Results

In Silico Identification and Analysis

A total of five *NPR1*-like sequences were obtained from the unpublished *P. americana* genome. *PaNPR1*, *PaNPR2*, *PaNPR3*, *PaNPR4,* and *PaNPR5*, code for putative proteins of 601 aa, 590 aa, 476 aa, 642 aa, and 496 aa, respectively. Amino acid analysis

[5]http://www.socr.ucla.edu

TABLE 2 | Primer sequences used in reverse transcription quantitative PCR analysis.

Gene	Forward 5′–3′	Reverse 5′–3′	Expected product length (bp)
PaNPR1	TGGCTTATCAGTGCTTGCTC	CCTCCTTATCCTCGTTGTATGC	119
PaNPR2	GAACCACTACTAGGAGAAG	TTGCCAGACTAACTCTAC	97
PaNPR3	CTTCCCGACTTATTCTACCTTGAG	CGATCTGCTGTACTCCTTGTC	126
PaNPR4	AGGTGCTGCTGCTGCTAC	TGGATTCGTGGCTTCTCTATGC	94
PaNPR5	GTCGAACAGTTGGCATTG	GAGCACTTTCATCACATCTTC	84
PaPR1	GCGGCTGGAAAGGTTTGT	GGGGCTGTAGTTGCAAGT	102

Primer sequences were designed to amplify fragments no larger than 150bp for each of the five NPR1-like genes identified in P. americana in order to perform RT-qPCR analysis.

TABLE 3 | Reverse transcription quantitative PCR optimization.

Gene	T_a	*E*	R^2	LDR	M1	M2
18S	58°C	108.2%	0.995	1:9–1:729	0.0185	0.0579
α-1 tubulin	58°C	91.8%	0.997	1:3–1:729	0.0064	0.2076
Actin	58°C	101.7%	0.995	1:9–1:729	0.0671	0.0512
PaNPR1	56°C	95.6%	0.996	1:3–1:729		
PaNPR2	59°C	93.2%	0.991	1:3–1:729		
PaNPR3	63°C	96.0%	0.992	1:3–1:729		
PaNPR4	63°C	96.9%	0.996	1:3–1:729		
PaNPR5	62.5°C	93.1%	0.991	1:3–1:729		
PaPR1	58°C	97.6	0.992	1:3–1:729		

Primer sets of the five NPR1-like genes and PR1 from P. americana as well as endogenous control genes were optimized for annealing temperatures (T_a) that yielded sufficient efficiency (E) and coefficient of determination (R^2) values. Linear dynamic range (LDR) indicates the minimum and maximum dilutions used to create a calibration curve. The stability (M-value) of the reference genes is also indicated for SA, MeJA, and Phytophthora cinnamomi treated (M1) and different tissue samples (M2).

revealed that PaNPR1 and PaNPR2 are most similar to AtNPR1 (52.46% identity, 64.08% similarity, and 55.94% identity, 68.35% similarity, respectively) while PaNPR4 is most similar to AtNPR3 (56.74% identity, 66.08% similarity). Furthermore, PaNPR3 and PaNPR5 are highly similar to AtBOP2 (78.90% identity, 83.71% similarity, and 81.57% and identity, 85.18% similarity, respectively). The predicted exon/intron structure further illustrates similarities between the avocado and *Arabidopsis NPR1*-like gene families (**Figure 1A**).

Analysis using PROSITE (Sigrist et al., 2010) reveals that all five PaNPR1-like proteins contain the BTB/POZ and ankyrin repeat domains at similar positions to AtNPR1 (**Figure 1B**). Conversely, only PaNPR1, PaNPR2, and PaNPR4 contain an NPR1-like C-terminal region which has been shown to be an essential component of NPR1 (Cao et al., 1997). The C-terminus contains the nuclear localization signal (NLS), a conserved pentaamino acid motif (LENRV) and a NIM INTERACTING (NIMIN) 1/2 protein binding site (Kinkema et al., 2000; Maier et al., 2011). PaNPR1 contains all five of the conserved basic amino acids that constitute the NLS1, PaNPR2 contains four of the five and PaNPR4 contains several conservative amino acid substitutions (**Figure 1C**). Similarly, the NIMIN1/2 binding region is completely conserved in PaNPR1 with one and three substitutions in PaNPR2 and PaNPR4, respectively, (**Figure 1C**). Furthermore, PaNPR4 contains the putative hinge region (LENRV motif) while PaNPR1 and PaNPR2, respectively, contain a conservative threonine and serine substitution at the third position of this motif (**Figure 1C**). The N-terminal of PaNPR2 contains an IκB-like phosphodegron motif (DSxxxS) which has been shown to be necessary for proteasome-mediated turnover of NPR1 (Spoel et al., 2009) while PaNPR1 contains a similar motif with a serine to lysine substitution at the second position (**Figure 1C**).

The PaNPR1-like protein sequences obtained in this study were subjected to phylogenetic analysis together with 34 full-length NPR1-like protein sequences from vascular and non-vascular plant species (**Figure 2**). This analysis reveals that PaNPR1 and PaNPR2 form a distinct group which is closely related to VvNPR1.1 from grapevine (70.83 and 76.33% similarity, respectively) as well as NPR1 from poplar (PtNPR1.1 and PdNPR1-1) and beet (BvNPR1), clustering within the clade containing AtNPR1 and AtNPR2 (**Figure 2**). On the other hand, PaNPR4 clusters within the clade containing AtNPR3 and AtNPR4 (**Figure 2**). Finally, PaNPR3 and PaNPR5 form a distinct group and are closely related to AtBOP2 from *Arabidopsis* (76.02 and 77.90% similarity, respectively; **Figure 2**).

PaNPR1-like and *PaPR1* Response to SA, MeJA, and *P. cinnamomi*

In order to evaluate all five *PaNPR1*-like genes, it is important to gage their expression in response to hormone treatment and pathogen challenge. It is well-known that SA application increases expression of *AtNPR1* approximately twofold within 24 h, similar results are also obtained when *Arabidopsis* is inoculated with *Hyaloperonospora parasitica* (Ryals et al., 1997). Similarly, MeJA application has been shown to result in increased expression of *NPR1* in rice and banana, although to a lesser extent than treatment with SA (Yuan et al., 2007; Endah et al., 2008). Thus, in order to investigate the response of all five *PaNPR1*-like genes, 1 year-old clonal PRR-tolerant Dusa® rootstock plantlets were treated with either SA, MeJA, or inoculated with *P. cinnamomi* and harvested at 6, 12, 18, 24, and 96 h. Furthermore, an ortholog of *Arabidopsis PR1* from *P. americana*, *PaPR1*, was used as a SAR marker (Reeksting et al., 2014).

The expression of *PaNPR1* was significantly down-regulated during SA treatment at 12 h (0.56-fold), returning to basal levels at 96 h (**Figure 3A**). Treatment with MeJA also decreased the expression of *PaNPR1* but at a later time point, 24 h (0.61-fold), yet expression remained low at 96 h (**Figure 3A**). Infection with *P. cinnamomi* decreased the expression of *PaNPR1* at 12 h (0.68-fold) followed by an increase at later time points, similar to treatment with SA, yet differences between treated and control samples were not significant (**Figure 3A**). However, significant down-regulation was seen at 96 h *P. cinnamomi* infection (0.55-fold), similar to treatment with MeJA (**Figure 3A**).

Treatment with SA significantly up-regulated *PaNPR2* expression at 6 h (2.97-fold) when compared to control samples, returning to baseline levels at 12 h (**Figure 3B**). Plants treated with MeJA showed a progressive decline in the expression of *PaNPR2* with a significant down-regulation at 24 h (0.53-fold) that remained low at 96 h (**Figure 3B**). Similarly, *PaNPR2* was significantly down-regulated at 96 h after infection with *P. cinnamomi* (0.47-fold; **Figure 3B**). Unfortunately the presence of *PaNPR3* could not be reliably detected in either treatment due to low transcript abundance and was therefore omitted from this part of the study.

Significant up-regulation of *PaNPR4* was observed at 6 h (4.63-fold) and 12 h (6.13-fold) with a sharp drop to baseline levels at 18 h (**Figure 3C**). Treatment with MeJA yielded no significant changes in the expression of *PaNPR4*, yet expression seemed to be slightly lower than that of the controls, especially at 96 h (0.44-fold; **Figure 3C**). A significant down-regulation of *PaNPR4* was observed at 18 h *P. cinnamomi* infection (0.53-fold) that remained low until the 96 h time point (**Figure 3C**). Conversely, *PaNPR5* was not significantly altered by any of the hormone

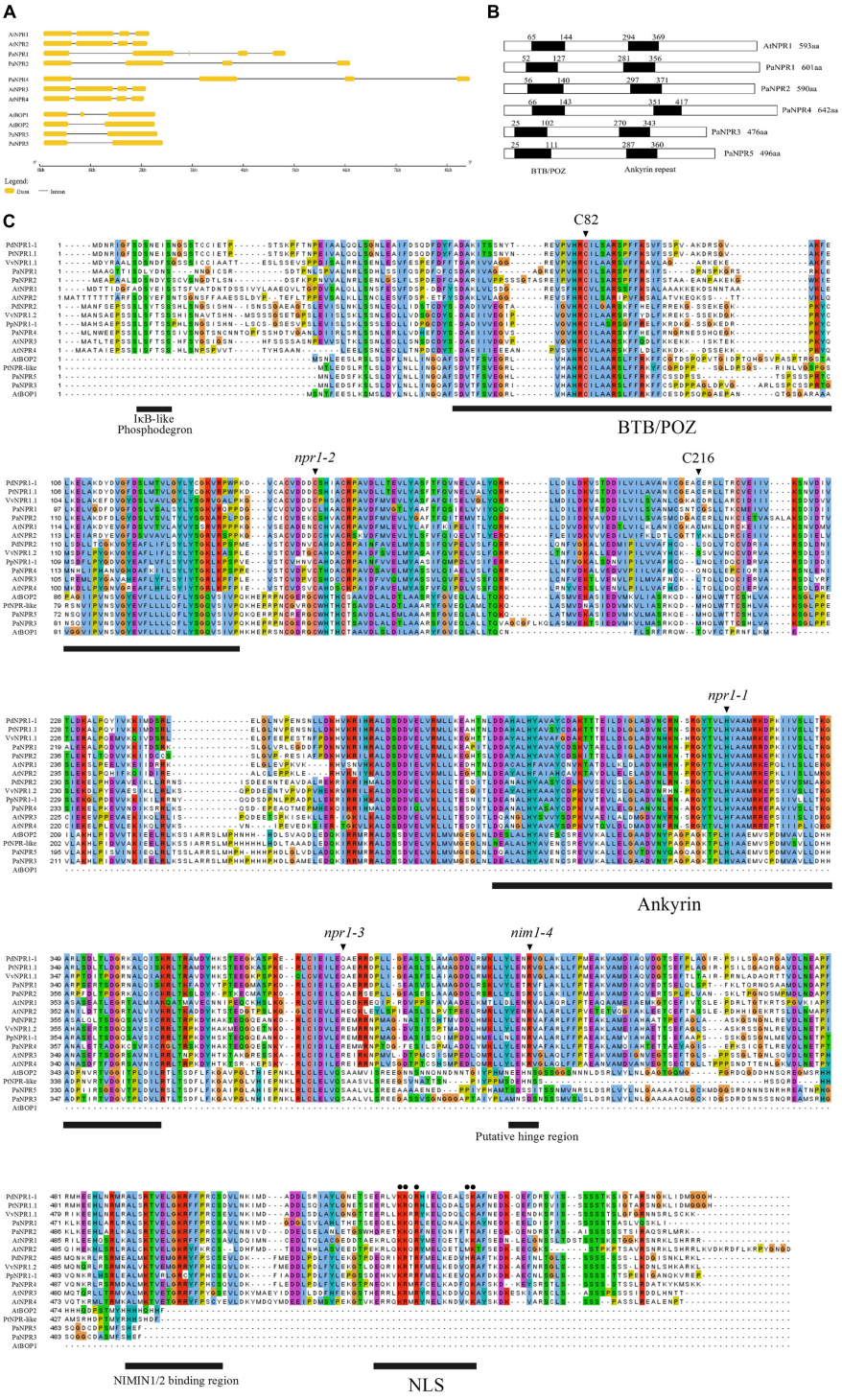

FIGURE 1 | Exon/intron boundary and predicted coding sequence comparison of *PaNPR1*-like genes with other known *NPR1*-like sequences. (A) The predicted exon/intron structure of the *NPR1*-like family from *Arabidopsis thaliana* and *Persea americana*. Exons are denoted by yellow boxes while introns are represented by thin black lines. **(B)** A comparison of the positions of the BTB/POZ and ankyrin repeat domains between the PaNPR1-like and AtNPR1-like family of proteins. **(C)** A multiple alignment of PaNPR1-like proteins and several other known NPR1-like proteins from woody plants and *Arabidopsis*. The positions of amino acid changes causing the npr1-1(H), npr1-2 (C), npr1-3 (*), and nim1-4 (R) mutants as well as the positions of the highly conserved cysteine residues at position 82 and 216 in *Arabidopsis* are indicated by black triangles above the alignment. The BTB/POZ and ankyrin repeat domains are indicated by black bars below the alignment. Several important motifs such as the IκB phosphodegron, LENRV hinge region, NIMIN1/2 binding site, and NLS1, are also indicated by black bars. The positions of important amino acids in the NLS1 of AtNPR1 are indicated by black dots above the alignment.

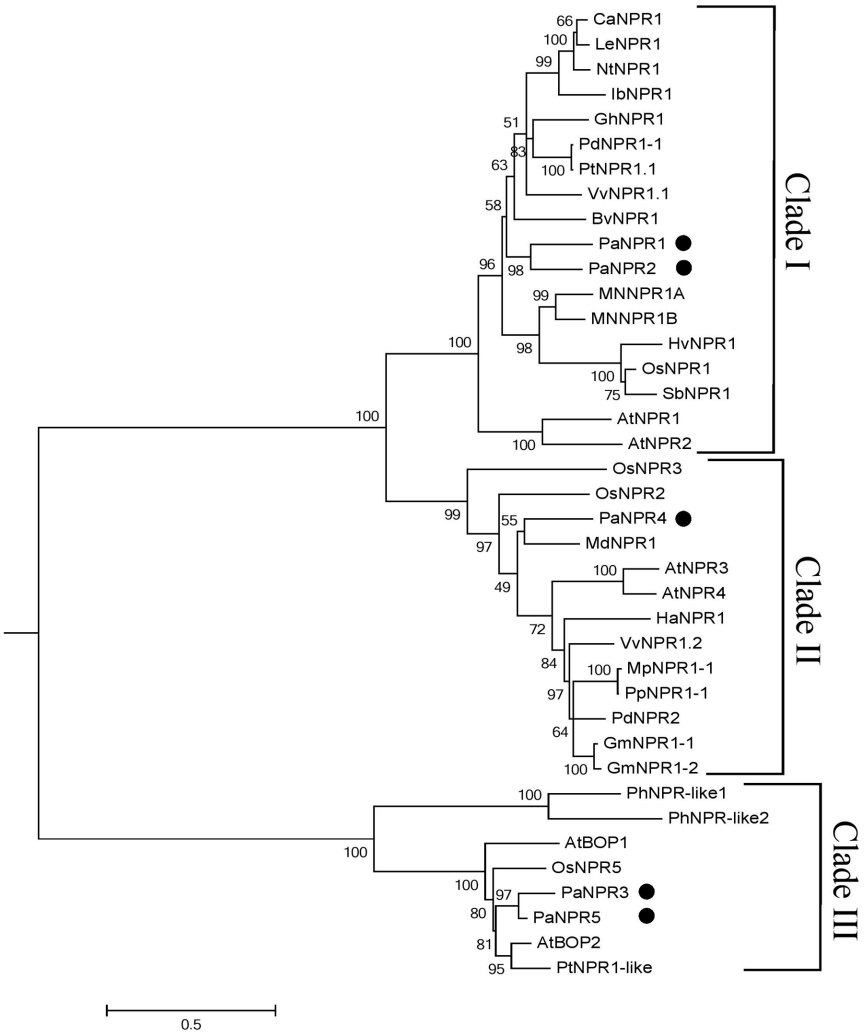

FIGURE 2 | Phylogenetic analysis of the NPR1-like family of proteins from *P. americana* and several other species. A phylogenetic tree of five NPR1-like proteins from *P. americana* as well as NPR1-like proteins from other vascular and non-vascular plant species, including the NPR1-like family from *Arabidopsis*. The tree was generated in MEGA software v5.2 using the maximum likelihood (ML) method. A total of 1000 bootstrap replicates were performed and values are indicated above the branch points. The species of origin, identifiers and accession numbers are summarized in **Table 1**.

treatments, yet was significantly down-regulated by *P. cinnamomi* at 96 h (0.39-fold; **Figure 3D**).

Lastly, expression of *PaPR1* was significantly up-regulated at 6 h (2.71-fold) and 18 h (3.35-fold), with an unexpected slump at 12 h (1.58-fold; **Figure 3E**). This decrease in expression coincided with the highest expression of *PaNPR4* at 12 h and was relieved at 18 h when *PaNPR4* expression returned to basal levels. The expression of *PaPR1* was significantly down-regulated by MeJA at 24 h (0.25-fold) and remained low at 96 h (**Figure 3E**). A significant increase in *PaPR1* expression was seen at 18 h (2.97-fold) followed by a significant decrease at 24 h (0.24-fold) following *P. cinnamomi* inoculation (**Figure 3E**). It was interesting to note that following inoculation with *P. cinnamomi*, the expression of *PaPR1* is similar to that of SA at early time-points and MeJA at later time-points (**Figure 3E**).

PaNPR1-Like Expression in Various Avocado Tissues

AtBOP1 and *AtBOP2* have been shown to be involved in the growth and development of lateral organs and to accumulate extensively in the proximal parts of these tissues (Hepworth et al., 2005). Thus determining whether any of the *PaNPR1*-like genes are overrepresented in specific tissues could assist in identifying possible *AtNPR1* orthologs by eliminating possible ortholog of the *Arabidopsis BOP* genes in avocado. Consequently, feeder roots, mature stems, mature leaves, unripe fruit as well as stems and leaves from flush growth of mature avocado trees were sampled and basal expression of all five *PaNPR1*-like genes was determined.

The expression of *PaNPR1, PaNPR2,* and *PaNPR4* was constitutive in all tissues yet higher basal levels were seen in more mature tissues than in younger tissues. The expression of *PaNPR1*

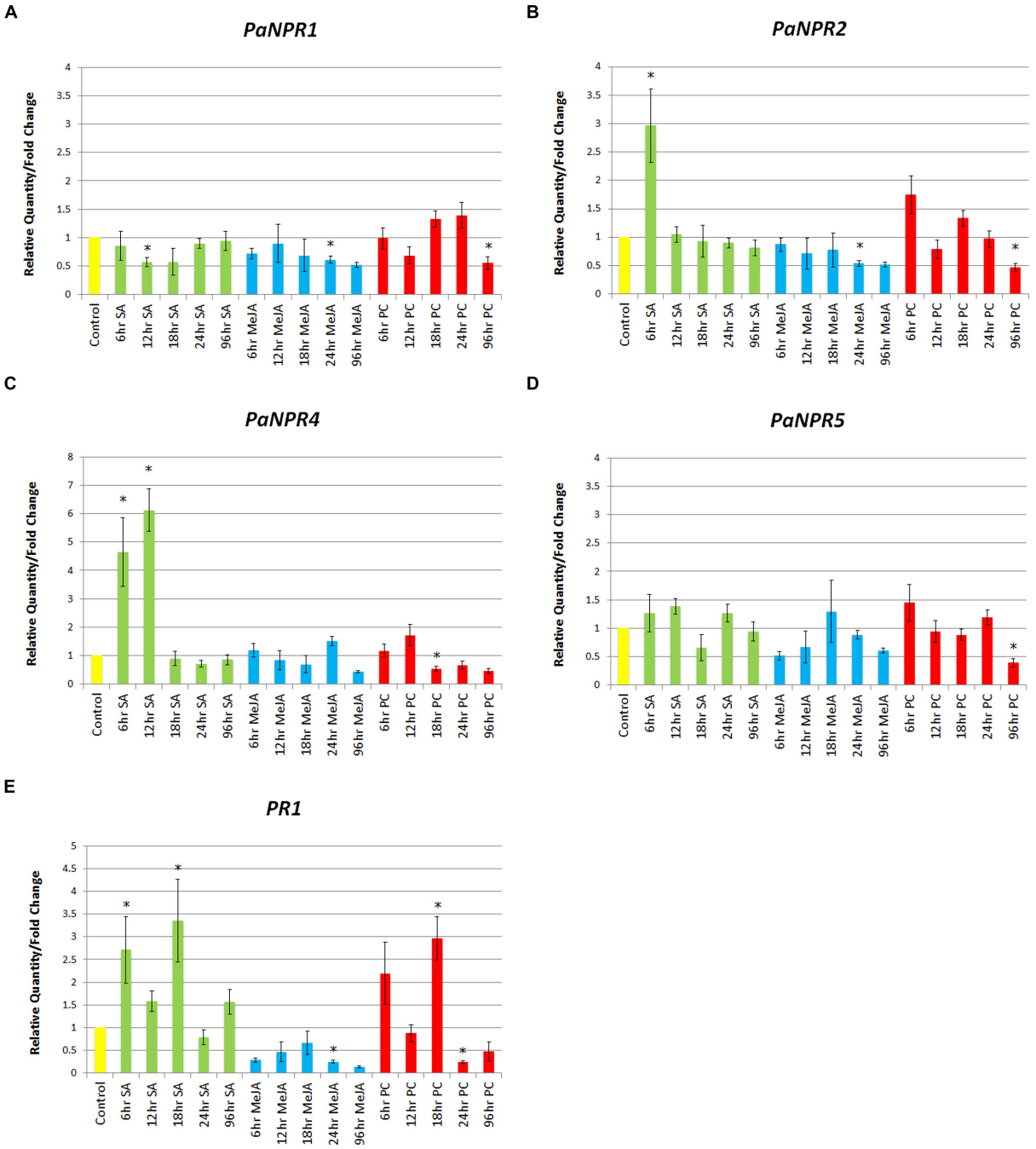

FIGURE 3 | Expression of *PaNPR1*-like genes as well as *PaPR1* in response to SA, JA, and *Phytophthora cinnamomi* treatment. Normalized relative quantities (fold change) for **(A)** *PaNPR1*, **(B)** *PaNPR2*, **(C)** *PaNPR4*, **(D)** *PaNPR5*, and **(E)** *PaPR1* were calculated using the method described by Pfaffl (2001). The response is indicated by vertical bars across five time points for SA (green), MeJA (blue), and *P. cinnamomi* (red) treated samples, and is labeled on the horizontal axis. The samples were compared to untreated samples harvested at each time point, a representative bar for the controls (yellow) is also indicated. SE for each bar is shown. Significant differences ($P < 0.05$) between control and treated samples is denoted by an asterisk (*) above the bar.

was highest in the roots and mature leaves, with the significantly less transcript detected in the young leaves (0.14-fold), young stems (0.11-fold) and unripe fruit (0.28-fold; **Figure 4A**). Similarly, *PaNPR2* had significantly higher expression in roots and mature leaves when compared to young leaves (0.27-fold), young stems (0.24-fold) and unripe fruit (0.22-fold; **Figure 4B**). Expression of *PaNPR4* was significantly higher in mature leaves (2.48-fold) and mature stems (2.61-fold), relative to the young leaves (0.74-fold), young stems (0.70-fold), and unripe fruit (0.44-fold; **Figure 4D**).

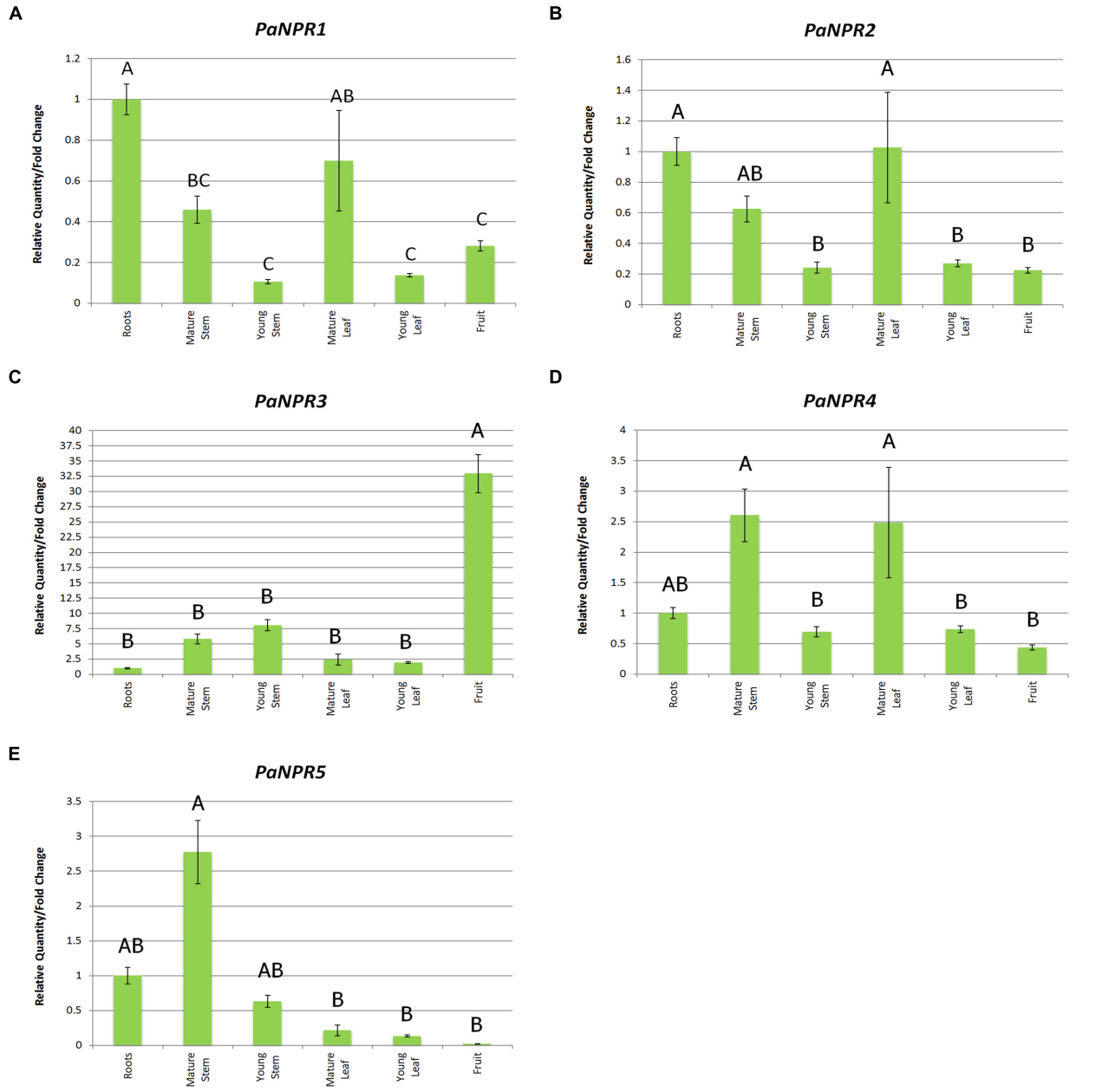

FIGURE 4 | Expression of *PaNPR1*-like genes in various tissues. Normalized relative quantities (fold change) for **(A)** *PaNPR1*, **(B)** *PaNPR2*, **(C)** *PaNPR3*, **(D)** *PaNPR4*, and **(E)** *PaNPR5* were calculated using the method described by Pfaffl (2001). The response is indicated by vertical bars across six tissues; feeder roots, mature green stems, mature green leaves, unripe fruit as well as stems and leaves from flush growth (young material), and is labeled on the horizontal axis. The expression in all tissues was calibrated using expression in the roots. SE for each bar is shown. Bars represented with the same letter are not significantly different at $P < 0.05$.

Conversely, *PaNPR3* and *PaNPR5* displayed patterns of expression unlike those of the aforementioned *PaNPR1*-like transcripts. Extremely high *PaNPR3* transcript levels were found in the unripe fruit (32.94-fold) relative to the roots, mature stems (5.84-fold), young stems (8.08-fold) and young leaves (1.93-fold; **Figure 4C**). Inversely, expression of *PaNPR5* was the lowest in unripe fruit (0.02-fold) and significantly higher in mature stems (2.77-fold; **Figure 4E**). The expression of this gene was also significantly less in mature leaves (0.21-fold) and young leaves (0.13-fold) when compared to mature stems (**Figure 4E**).

PaNPR1-Like Expression in Tolerant and Susceptible Avocado Rootstocks

The expression of *NPR1*-like genes has been shown to differ significantly between susceptible and tolerant banana cultivars challenged with *Fusarium oxysporum* Schlecht f. sp. *cubense*

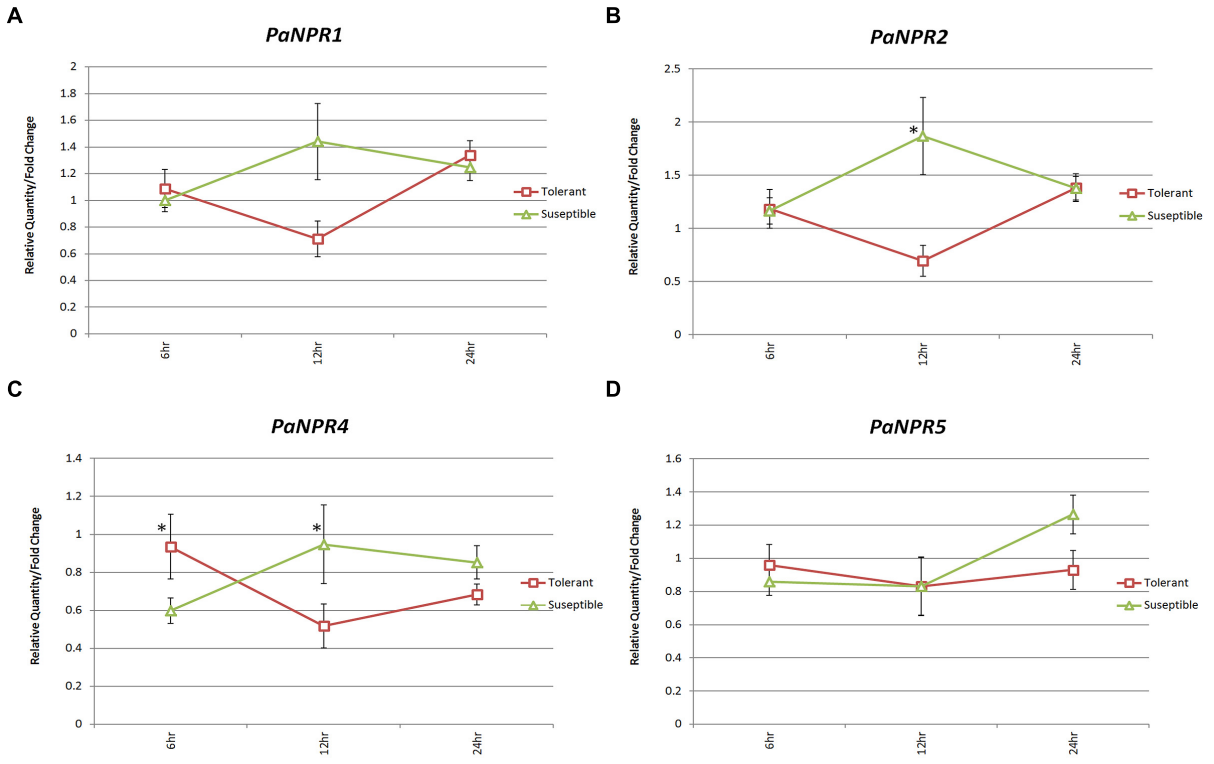

FIGURE 5 | Expression of *PaNPR1*-like genes in tolerant (Dusa®) and susceptible (R0.12) avocado rootstocks infected with *P. cinnamomi*. Normalized relative quantities (fold change) for **(A)** *PaNPR1*, **(B)** *PaNPR2*, **(C)** *PaNPR4,* and **(D)** *PaNPR5* were calculated using the method described by Pfaffl (2001). The response is indicated by horizontal lines (red – tolerant, green – susceptible) across three time points; 3, 6, and 24 h, labeled on the horizontal axis. The expression was calibrated using expression at 0 h (uninfected control) which was set to a normalized relative expression of 1. SE for each bar is shown. Significant differences ($P < 0.05$) between control and treated samples is denoted by an asterisk (*) above the data point.

(Smith) Snyd (*Foc*). Thus determining whether such differences exist between tolerant and susceptible avocado rootstocks could provide insights into molecular differences which may affect *P. cinnamomi* tolerance. Consequently, RNA from both tolerant (Dusa®) and susceptible (R0.12) avocado rootstocks, infected with *P. cinnamomi* and harvested at 0 h (uninfected control), 6, 12, and 24 h, was obtained from Engelbrecht et al. (2013).

The expression of *PaNPR2* was significantly lower in Dusa® (0.70-fold) as compared to R0.12 (1.87-fold) at 12 h (**Figure 5B**). *PaNPR4* was expressed significantly lower in R0.12 (0.60-fold) when compared to Dusa® (0.94-fold) at 6 h (**Figure 5C**). However, *PaNPR4* was expressed significantly lower in Dusa® (0.52-fold) when compared to R0.12 (0.95) at 12 h (**Figure 5C**). Expression of *PaNPR1*and *PaNPR5* was not significantly different when comparing Dusa and R0.12 at any of the time points (**Figures 5A,D**).

Discussion

We identified five *NPR1*-like genes in avocado in an attempt to better understand avocado defense response signaling in general, and with regard to the response to PRR. Studies in numerous plant species have highlighted the fundamental involvement of NPR1 in many defense signaling pathways (Cao et al., 1997; Spoel et al., 2003; Le Henanff et al., 2011). This study is the first investigation of the *NPR1*-like gene family in *P. americana*, and sets the foundation for further functional characterization of the NPR1-like protein family in avocado.

The avocado genome contains five identifiable *NPR1*-like genes; these sequences share similar gene structures and protein sequence identities as well as conserved domains and motifs present in *Arabidopsis NPR1*-like sequences. The *PaNPR1*-like gene sequences have similar exon/intron structures compared to the members of the *Arabidopsis NPR1*-like gene family to which each is most similar. Specifically, the exon/intron structures of *PaNPR1*, *PaNPR2,* and *PaNPR4* are comparable to that of *AtNPR1-4*. Interestingly, *PaNPR1* contains an extra, short, exon (exon 3) which could either be an assembly error or a unique aspect of this gene. Furthermore, the exon/intron structures of *PaNPR3* and *PaNPR5* are similar to that of *AtBOP2*. The predicted coding sequences for all five *PaNPR1*-like genes harbor the BTB/POZ and ankyrin repeat domains, characteristic

of NPR1-like proteins. These domains are crucial components of NPR1 and provide functions relating to NPR1-dependant co-activation of TGA transcription factors and protein–protein binding (Cao et al., 1997; Rochon et al., 2006). Only PaNPR2 contains the complete IκB phosphodegron motif (DSxxxS) found in AtNPR1, while PaNPR1 contains a lysine substitution at the first serine residue. Both serines of this motif are phosphorylated during SA treatment, leading to proteasome mediated turn-over and degradation of NPR1 (Spoel et al., 2009), suggesting that PaNPR2 may be regulated similarly. However, the effect of the serine to lysine substitution in PaNPR1 is unclear, particularly because basic amino acids such as lysine have been shown to undergo phosphorylation (Ciesla et al., 2011). Similar to AtNPR1, NLS1 sequences are also present in PaNPR1, PaNPR2, and PaNPR4 containing five, four, and three of the amino acids known to be essential to nuclear localization of AtNPR1 (Kinkema et al., 2000). Moreover, PaNPR1, PaNPR2, and PaNPR4 contain highly conserved NIMIN1/2 binding regions and LENRV motifs (Maier et al., 2011). Together these data suggest that PaNPR1, PaNPR2, and PaNPR4 are comparable to AtNPR1 and could possibly partake in the perception of SA and regulation of defense responses in avocado. Seemingly distinct, PaNPR3 and PaNPR5 do not contain these motifs and conserved regions, similar to the AtBOP proteins. The substantial difference in protein length and sequence composition of this subset of proteins suggests functionally diverse roles from PaNPR1, PaNPR2, and PaNPR4, and can be suggested that these proteins may be involved in certain aspects of tissue development as seen in AtBOP1 and AtBOP2 (Hepworth et al., 2005).

Phylogenetic analysis reveals that all five PaNPR1-like proteins group with other known NPR1-like sequences, clustering into three distinct clades (Peraza-Echeverria et al., 2012). PaNPR1 and PaNPR2 fall within the same clade as AtNPR1, which is a known positive regulator of SAR (Cao et al., 1997, 1998). PaNPR4 groups with AtNPR3 and AtNPR4 in the second clade. NPR1-like proteins within this group have been shown to negatively regulate SAR (Zhang et al., 2006), yet are able to perceive SA and are vital in mounting SAR (Fu et al., 2012). The last two, PaNPR3 and PaNPR5, fall within the third clade together with AtBOP1 and AtBOP2, which are known for their involvement in development of lateral organs (Hepworth et al., 2005). This phylogenetic analysis provides a second line of evidence suggesting possible functional distinctions between members of the PaNPR1-like protein family.

This study further describes the transcriptional response of the *PaNPR1*-like genes to SA, MeJA, and *P. cinnamomi* treatments as well as their expression levels in different tissues. Surprisingly, *PaNPR1* was down regulated by SA at 12 h, contrasting with *AtNPR1*, which was up-regulated approximately twofold 24 h after SA application in *Arabidopsis* (Cao et al., 1998). This may point to an alternative function of *PaNPR1* during defense responses and warrants further investigation. In banana cultivars which are resistant to *Foc*, NPR1 is up-regulated to a greater extent and at earlier time points after SA treatment than in susceptible cultivars (Endah et al., 2008). Similarly, *PaNPR2* was up-regulated at the earliest time point after SA treatment in the PRR tolerant avocado rootstock Dusa®. Additionally, up-regulation of *PaNPR2* corresponded to an increase in *PaPR1* gene expression. Similarly, *PaNPR4* was up-regulated soon after SA application and reached peak expression at 12 h. Interestingly, the highest expression of *PaNPR4* corresponded to a substantial decrease in *PaPR1* expression, suggesting that PaNPR4 may negatively regulate the expression of *PaPR1*. Treatment with MeJA led to decreased transcript abundance for *PaNPR1*, *PaNPR2,* and *PaNPR4* for extended periods of time, opposite to that seen in rice and banana (Yuan et al., 2007; Endah et al., 2008). The regulation of NPR1 during antagonistic cross-talk between SA and JA mediated defense responses may thus differ between some monocot and dicot plants. Interestingly, the regulation of *PaNPR1*, *PaNPR2,* and *PaNPR4* during *P. cinnamomi* treatment had expression patterns similar to that of SA treatment at earlier time points (6–18 h) and JA treatment at later time points (24–96 h). This could indicate the point at which *P. cinnamomi* switches from a biotrophic to a necrotrophic life stage, thus activating the SA and JA pathways, respectively.

Oddly, the induced expression of *PaNPR2* and *PaNPR4* during SA treatment was not observed during infection with *P. cinnamomi*. In our opinion three possible explanations exist: (1) PaNPR2 and PaNPR4 might be predominantly regulated at the protein level, (2) these proteins might not be involved in defense responses against *P. cinnamomi* or, (3) *P. cinnamomi* suppresses expression of these genes in order to promote successful host invasion. It has been noted that NPR1 is subject to extensive post-translational regulation (Mou et al., 2003; Spoel et al., 2009), thus changes in expression might not reflect the factual role of PaNPR1, PaNPR2, or PaNPR4 during defense response. Furthermore, *Phytophthora* species have been known to alter host gene expression in order to suppress host defense pathways and mediate infection (Oßwald et al., 2014).

A common trend in the expression of *PaNPR1*, *PaNPR2,* and *PaNPR4* in various tissues was seen; transcript levels of these genes were significantly higher in mature tissues than immature tissues, an observation that may be explained by the establishment of SAR in mature tissues. Expression of *PaNPR3* was undetectable in the roots in any of the treatments, yet this gene was expressed at much higher levels in aerial tissues, with the highest levels being detected in fruit. On the other hand, while *PaNPR5* was readily detected in the roots, it was unresponsive to SA or MeJA treatments and significantly down-regulated by *P. cinnamomi* during later time points. These data support our initial hypothesis that PaNPR3 and PaNPR5 are unlikely to be involved in defense responses, and are instead more likely to be involved in development of certain tissues.

Finally, significant differences in the expression of *PaNPR2* and *PaNPR4* were observed when comparing tolerant (Dusa®) and susceptible (R0.12) avocado rootstock cultivars. The expression of *PaNPR2* and *PaNPR4* is significantly lower at 12 h after infection in Dusa® when compared to R0.12. In our opinion these observations could be explained when considering *P. cinnamomi* switching from a biotrophic to a necrotrophic life cycle. In this case, increased expression of *PaNPR1*-like defense related genes would likely suppress the JA/ET pathway and prevent effective

control of *P. cinnamomi*. Thus it is conceivable that *P. cinnamomi* switches to a necrotrophic life cycle somewhere around 12 h after infection and that Dusa® reacts to this change more quickly than R0.12. This would explain, at least to some extent, tolerance in Dusa® and susceptibility in R0.12.

This study provides evidence assisting in the preliminary functional annotation of five newly discovered *NPR1*-like genes from avocado. Sequence structure and homology as well as phylogenetic analyze suggest that three PaNPR1-like proteins may be involved in defense responses, while the remaining two are most likely involved tissue development. Hormone and *P. cinnamomi* treatments, as well as expression in various tissues provide support for this and allow future research to focus on defense related PaNPR1-like proteins. Future efforts would be focused on intracellular interactions and localization of defense related PaNPR1-like proteins as well as the effect of overexpressing defense related *PaNPR1*-like genes in wild-type and *npr1* mutant *Arabidopsis*. Information from this and future studies could aid in understanding PRR tolerance and lead to the development of more tolerant avocado rootstocks.

Author Contributions

RB drafted the manuscript, performed conceptual, and experimental design as well as performed the experimental work. WM assisted in expression analysis and experimental design. JE performed experimental work. BR provided general supervision and assisted in drafting the manuscript. EL sequenced the genome and provided the *PaNPR1*-like sequences. NvdB conceived the study, experimental design and assisted in drafting the manuscript. All authors contributed to and approved the final manuscript.

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Chapter 3

Research Chapter

Holistic investigation of the NPR1-dependent defence response pathway in *Persea americana* (Mill.) following infection with *Phytophthora cinnamomi*

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The following chapter was prepared in the format of a peer-reviewed journal article. Biological material used in dual RNA-sequencing, as well as initial data analyses, were conducted by various members of the Avocado Research Programme. I performed candidate gene selection, manual curation, expression and statistical analysis as well as drafted the manuscript. The study was conceived and supervised by Noëlani van den Berg who also drafted the manuscript. Sanushka Naidoo also supervised the project and drafted the manuscript.

Abstract

A plant's defence against pathogens involves an extensive set of phytohormone regulated, defence signalling pathways. The salicylic acid (SA)-signalling pathway is one of the most well-studied in plant defence. The bulk of SA-related defence gene expression and the subsequent establishment of systemic acquired resistance (SAR) is dependent on the nonexpressor of pathogenesis-related genes 1 (NPR1). Therefore, understanding the NPR1 pathway and all its associations has the potential to provide valuable insights into defence against pathogens. The causal agent of Phytophthora root rot (PRR), *Phytophthora cinnamomi,* is of particular importance to the avocado (*Persea americana*) industry, which sustains massive economic losses on account of this pathogen each year. Furthermore, *P. cinnamomi* is a hemibiotrophic pathogen, suggesting that the SA-signalling pathway might play an essential role in the initial defence response. Therefore, the NPR1 pathway which regulates downstream SA-induced gene expression is likely to be instrumental in defence against *P. cinnamomi*. Thus, we identified 116 NPR1 pathway-associated orthologs from the *P. americana* draft genome and interrogated their expression following *P. cinnamomi* inoculation, using dual RNA-sequencing data. In total, 74 NPR1 pathway-associated genes were temporally regulated in the susceptible *P. americana* rootstock R0.12. Furthermore, 86 NPR1 pathway-associated genes were differentially regulated when comparing R0.12 and the PRR resistant rootstock Dusa®. Although SAR was established successfully in both rootstocks, Dusa® suppressed SA-signalling more effectively following the induction of SAR. This study represents the most comprehensive investigation of the SA-induced, NPR1-dependent pathway in *P. americana* to date. Lastly, this work provides novel insights into the likely mechanisms governing *P. cinnamomi* resistance in *P. americana*.

Keywords: Dual RNA-seq, NPR1, NPR1-like, *Phytophthora cinnamomi*, *Persea americana*, *pathogenesis-related*, systemic acquired resistance, salicylic acid

Introduction

Plants have evolved a complex array of defence mechanisms to combat the virulence strategies employed by various pathogens (LODHA AND BASAK 2012). Ultimately, host-pathogen interactions can be defined, towards either extreme, as compatible/susceptible or incompatible/resistant (HAMMOND-KOSACK AND JONES 2000; SCHENK *et al.* 2000). However, host-pathogen interactions are far from binary, given their complexity, and should instead be described on a spectrum, ranging from entirely susceptible to fully resistant. Understanding, at least some of this complexity may provide insights which could aid in breeding crops for increased pathogen resistance.

The phytohormone salicylic acid (SA) is an essential component of several plant defence responses (PIETERSE *et al.* 1998; DURRANT AND DONG 2004; TSUDA *et al.* 2009). For example, significant accumulation of SA at the initial site of infection is essential to the induction of the hypersensitive response (HR). Subsequently, the accumulation of SA in distal tissues initiates the establishment of systemic acquired resistance (SAR); a long-term, systemic defensive state initiated by SA-dependent gene expression following biotrophic/hemibiotrophic pathogen challenge (RYALS *et al.* 1996; SHAH 2003). Intriguingly, SAR induced plants feature increased resistance to virtually all manner of pathogens, including viruses, bacteria, fungi, oomycetes and nematodes (RYALS *et al.* 1996; STICHER *et al.* 1997). Generally, SAR is defined by a substantial and sustained accumulation of a suite of antimicrobial pathogenesis-related (PR) proteins in tissues distal to the initial site of infection (VAN LOON AND VAN STRIEN 1999; DURRANT AND DONG 2004; AN AND MOU 2011).

The induction of SAR is dependent on the nonexpressor of pathogenesis-related genes 1 (NPR1), a co-transcription factor known as the master regulator of defence responses (CAO *et al.* 1994; GLAZEBROOK *et al.* 1996; CAO *et al.* 1997; RYALS *et al.* 1997; SHAH *et al.* 1997). Not only does SAR not establish in *Arabidopsis thaliana npr1* mutants, the induced expression of *PR-1* and *PR-5* is significantly decreased (CAO *et al.* 1994; GLAZEBROOK *et al.* 1996). Furthermore, the complementation using *NPR1* restores the wild-type *PR* gene expression, as well as the inducibility of SAR (CAO *et al.* 1997). Thus, since its discovery, transgenic overexpression of *NPR1* has proven to increase disease resistance against a variety of pathogens across an

expansive range of crops (BACKER *et al.* 2019). However, the complexity of NPR1-dependent gene expression requires a holistic view of all associated proteins.

Transcription factors are a central aspect of NPR1-dependent gene regulation. The promoters of typical NPR1-dependent genes, such as *PR-1*, contain the SA-responsive *as-1*-like promoter element (STROMPEN *et al.* 1998; DING *et al.* 2018). Importantly, the TGA transcription factor protein family associates with this promoter element and is required for SAR-related gene expression (STROMPEN *et al.* 1998; ZHANG *et al.* 1999; ZHANG *et al.* 2003). Furthermore, NPR1 and various TGA transcriptions factors interact directly, which ultimately increases their DNA-binding affinity (DESPRES *et al.* 2000; ZHOU *et al.* 2000; JOHNSON *et al.* 2003; ROCHON *et al.* 2006). However, TGA transcription factors have also been associated with negative regulatory promoter elements, suggesting that together, NPR1 and TGA transcription factors may also serve to suppress the expression of specific genes (LEBEL *et al.* 1998; DESPRES *et al.* 2000).

Another essential, SA-responsive, transcription factor family are the WRKYs (MALECK *et al.* 2000; KIM *et al.* 2008). Though these transcription factors were initially suggested to suppress the expression of SAR-related genes during non-stress conditions, many have since been implicated in positive regulation of defence signalling (MALECK *et al.* 2000; WANG *et al.* 2006; ZHENG *et al.* 2006; KIM *et al.* 2008; LAI *et al.* 2008). The WRKY transcription factor specific W-box *cis*-elements are common in many SAR-related genes, including *isochorismate synthase 1* (ICS1), *TL1-binding transcription factor (TBF1)*, *PR1* and even *NPR1* itself (EULGEM *et al.* 2000; MALECK *et al.* 2000; WILDERMUTH *et al.* 2001; YU *et al.* 2001; TURCK *et al.* 2004; PAJEROWSKA-MUKHTAR *et al.* 2012). It was also shown that in certain situations, TGAs, WRKYs and NPR1 might all work together to regulate SA-dependent gene expression (SARKAR *et al.* 2018). Thus, various transcription factors serve to extend the influence of NPR1 on SA-dependent gene expression, contributing to its broad regulatory effect.

Although the expression of *NPR1* is itself SA-responsive, post-transcriptional modification of NPR1 seems to be at least as important as increased expression (BACKER *et al.* 2019). The most extensively studied NPR1 post-translational change happens within the cytoplasm; here, during non-stress conditions, NPR1 exists as an oligomer (MOU *et al.* 2003). Following the SA-induced oxidative burst associated with pathogenic stress, and the increased production of reducing agents, NPR1 is monomerised (MOU *et al.* 2003). The action of thioredoxins (TRXs), in particular, are responsible for the reduction of $NPR1^{Cys156}$, resulting in its monomerisation

(MOU *et al.* 2003; TADA *et al.* 2008). By contrast, *S*-nitrosoglutathione (GSNO) promotes the existence of NPR1 as an oligomer (TADA *et al.* 2008). Interestingly, a class III type alcohol dehydrogenase (ADH), *S*-nitrosoglutathione reductase (GSNOR), reduces the amount of available GSNO (LIU *et al.* 2001). Moreover, expression of the gene encoding for GSNOR in *A. thaliana* is induced by SA and essential to the establishment of SAR (FEECHAN *et al.* 2005; XU *et al.* 2013; WANG *et al.* 2014). Thus, taken together, the actions of TRX and GSNOR would reduce the potential for NPR1 to exist as an oligomer and in so doing contribute to the establishment of SAR.

Additionally, several protein kinases are described in the post-translational regulation of NPR1 activity (XIE *et al.* 2010; LEE *et al.* 2015). Interestingly, the NPR1 residue Ser589, which is essential as a part of the nuclear localisation signal (NLS2), is phosphorylated by the sucrose non-fermenting 1 (SNF1)-related protein kinase 2 (SRK2C) protein (KINKEMA *et al.* 2000; LEE *et al.* 2015). Furthermore, *SRK2C* is expressed in response to SA-independent systemic signals and thus, presumably, plays a role in the nuclear import of NPR1 in distal tissues, where SA concentration is lower (XIE *et al.* 2010; LEE *et al.* 2015). Similar to SRK2C, CBL-interacting serine/threonine-protein kinase 11 (CIPK11) interacts with and phosphorylates the C-terminal region of NPR1 (XIE *et al.* 2010). This modification ultimately leads to upregulated expression of *WRKY38* and *WRKY62* in response to *Pseudomonas syringae* pv. *tomato* DC3000 (XIE et al. 2010).

Additionally, phosphorylation of Ser11/15 and Ser55/59 reinforces sumoylation of NPR1 by the small ubiquitin-like modifier 3 (SUMO3), a positive regulator of SA-induced gene expression (WANG *et al.* 2006; VAN DEN BURG *et al.* 2010; SALEH *et al.* 2015). Overall, the sumoylation of NPR1 decreases its interaction with WRKYs, while increasing interaction with TGAs (SALEH *et al.* 2015). Furthermore, sumoylation of NPR1 leads to increased phosphorylation of Ser11/15, reinforcing defence gene expression, followed by ubiquitinylation and subsequent proteasome-mediated turnover of spent NPR1 (SPOEL *et al.* 2009; SALEH *et al.* 2015). Notably, the turnover of NPR1 completes SAR induction, as inherently unstable co-transcription factors likely cannot maintain peak gene expression without being replaced continuously (SALGHETTI *et al.* 2000; COLLINS AND TANSEY 2006; SPOEL *et al.* 2009). However, neither CUL3 or E3-ligases, which ubiquitinylate NPR1, have been shown to interact

with NPR1 directly and therefore likely require a substrate adapter (DIETERLE *et al.* 2005; SPOEL *et al.* 2009; FU *et al.* 2012; DING *et al.* 2018).

Interestingly, NPR1 increases the expression of several protein secretory pathway genes, likely to ensure correct protein processing in response to increased PR protein production (WANG *et al.* 2005; PAJEROWSKA-MUKHTAR *et al.* 2012). These genes all have a common *TL1 cis*-element within their promoters which are bound by the heat stress transcription factor, TBF1 (WANG *et al.* 2005; PAJEROWSKA-MUKHTAR *et al.* 2012). Although *A. thaliana tbf1* mutants do not display decreased *PR-1* transcript or protein levels, the secretion of PR-1 into the apoplast is substantially reduced (PAJEROWSKA-MUKHTAR *et al.* 2012). Interestingly, both *tbf1* and *npr1-1* mutants presented with a decreased expression of *luminal binding protein 2* (*BiP2*) and *calreticulin* 3 (*CRT3*). These observations, together with the presence of the appropriate promoter *cis*-elements, suggest that the expression of *NPR1* and *TBF1* is likely co-regulated (PAJEROWSKA-MUKHTAR *et al.* 2012)

Additionally, SA-responsive negative regulators, such as the NIM(NPR1)-interacting (NIMIN) proteins, are another key component of NPR1-dependent gene expression (WEIGEL *et al.* 2005; MAIER *et al.* 2011). However, the effect of NIMINs is not absolute, and instead, these proteins impact the timing of gene expression (HERMANN *et al.* 2013). Furthermore, proteins such as NPR3 and NPR4, which serve redundant negative regulatory roles, oppose to the function of NPR1 (DING *et al.* 2018). These *bone fide* SA receptors associate with several TGAs and the promoters of SA-inducible genes, preventing expression in the absence of SA (DING *et al.* 2018). Interestingly, the expression of *histone deacetylase 19* (*HDAC19*), a negative regulator of SAR, is NPR1 and SA dependent (CHOI *et al.* 2012). Moreover, repression of *PR-1* and *PR-2* is, at least in part, regulated by HDAC19, which associates with and deacetylates their respective promoters, limiting expression during uninduced conditions (CHOI *et al.* 2012). These studies highlight another critical aspect of SA-inducible, NPR1-dependent gene expression i.e. timing.

The correct timing of defence responses underpins their effectiveness and prevents potential fitness loss due to unnecessary, uninduced defence gene expression. Here, priming forms an integral aspect of SAR, allowing for an earlier, stronger, and thus more effective defence response during subsequent pathogen challenge (CONRATH *et al.* 2002; PRIME *et al.* 2006). In *A. thaliana* the expression of NPR1-dependent, pathogen-responsive *mitogen-activated protein*

kinase 3 (MPK3) and *MPK6* have been implicated in the priming of SA-induced defence responses (BECKERS *et al.* 2009). The accumulation of inactive, unphosphorylated MPK3/6 and their transcripts allows for quicker signal transduction and subsequent responses in reaction to pathogens (BECKERS *et al.* 2009; YI AND KWON 2014; YI *et al.* 2015). Furthermore, expression of the circadian clock genes*, timing of cab2 expression 1* (*TOC1*) and its antagonist *late elongated hypocotyl* (*LHY*), is NPR1-dependent (ZHOU *et al.* 2015). Together, TOC1 and LHY control the balance of growth and defence throughout the day, prioritising defence in the morning when pathogen pressure is at its peak (NOZUE *et al.* 2007; BHARDWAJ *et al.* 2011; KORNELI *et al.* 2014; ZHOU *et al.* 2015).

Previously we described five *NPR1-like* genes in *Persea americana*, three of which are likely to partake in defence responses (BACKER *et al.* 2015). However, attempting to understand the regulation of the NPR1 pathway-associated genes further seems sensible, given the intricacy of NPR1-dependent gene expression. Thus, we believe that regulation of NPR1 pathway-associated genes in *P. americana* will closely resemble expectations based on the literature, following pathogen challenge. Furthermore, we expect to see notable differences in the regulation of several NPR1 pathway-associated genes between susceptible and partially resistant *P. americana* rootstocks in response to *P. cinnamomi* inoculation. Therefore, we endeavoured to identify and partially characterise a wide variety of NPR1 pathway-associated genes from the *P. americana* draft genome. Using dual RNA-sequencing we compared the expression of 116 unique *P. americana* NPR1 pathway-associated genes, of both the *P. cinnamomi* susceptible (R0.12) and partially resistant (Dusa®) rootstocks, following inoculation. We further described the response of the susceptible rootstock to *P. cinnamomi* inoculation across three time-points. Overall, the expression of most NPR1 pathway-associated genes responded as expected based on the literature. However, significant differences were uncovered when comparing the expression of the susceptible and partially resistant *P. americana* rootstocks at 120 hours post-inoculation (hpi). Thus, this study provides the first evidence of significant regulatory differences regarding the expression of NPR1 pathway-associated genes in response to challenge by *P. cinnamomi*.

Materials and Methods

Plant Material

One-year-old clonal Dusa® and R0.12 plantlets, which are partially resistant and susceptible to *P. cinnamomi,* respectively, were provided by Westfalia Technological Services (Tzaneen, ZAF). Plantlets were acclimatised in a temperature-controlled environment at 25°C for 2 weeks. Plantlets were transplanted into a 1:1 perlite vermiculite medium following the removal of the nurse seed and left to acclimatise for an additional 2 weeks.

P. cinnamomi infection trial

P. cinnamomi isolate GKB4 was obtained from the Avocado Research Programme Culture Collection (Pretoria, South Africa). Virulence of the isolate was recovered through apple inoculation followed by single hyphal tip re-isolation (RIBEIRO 1978). Zoospores were produced for the inoculation of *P. americana,* as described below (CHEN AND ZENTMYER 1970). Colonised V8 agar blocks (10% V8 (v/v), 0.1g.l^{-1} $CaCO_3$, agar 17g.l^{-1}) were placed into 90mm Petri dishes containing V8 broth (5% V8 (v/v), 0.1g.l^{-1} $CaC0_3$). Plates were incubated at 25°C for 2 days to allow for enough mycelial growth. Mycelia were rinsed three times using room temperature dH_2O and twice filtered (chromatography paper MN260 (Macherey-Nagel, Düren, DEU) stream water was added to the mycelia. Mycelial blocks were incubated at 25°C for 2 days under fluorescent lighting, at which point ample sporangia formation was observed. Mycelial blocks were cold-shocked by rinsing twice with dH_2O at 4°C and incubated for another 12min. Cultures were then left at room temperature for 1 hour to allow zoospore release and were monitored using light microscopy. Zoospores were collected by pouring the culture through a fine-mesh sieve and counted using a haemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, DEU).

Plantlets were randomly assigned to either the treatment or control group. The treatment group was inoculated by submerging the roots in a zoospore suspension (1.4 x 10^5 zoospore.ml^{-1}), while those of the control group were mock-inoculated by immersion in dH_2O, both treatments were done at midday. Plantlets were replanted in a 1:1 perlite vermiculite medium 2 hours after inoculation. The treatment group consisted of three biological replicates with three plantlets per replicate for both Dusa® and R0.12 which were collected at 6 hr, 12 hr, 24 hr, 48 hr, and 120 hr time-points. The control group consisted of three

biological replicates with three and two plants per replicate for Dusa® and R0.12, respectively, which due to material limitations, were only harvested at the 24 hr time-point. Harvested roots were snap-frozen in liquid nitrogen and stored at -80°C. Biological replicates were homogenised using the IKA® Tube Mill control (IKA®, Staufen, DEU) until a fine consistency was attained.

Dual RNA-sequencing

Total RNA was extracted from homogenised plant material using a modified version of the CTAB extraction method (CHANG *et al.* 1993). The chloroform: isoamyl alcohol step was repeated four to six times until the volume of the interphase diminished, and the supernatant was clear. Samples were treated with DNase I (Fermentas Inc., Vilnius, LTU) and purified using the Qiagen RNeasy clean up kit (Qiagen Inc., Valencia, California, USA). Samples were resuspended in diethylpyrocarbonate (DEPC) treated water containing 30U.ml^{-1} RiboLock RNase Inhibitor (Thermo Fisher Scientific Inc., Leicestershire, GBR). Conventional PCR using LPV3 primers was used to confirm the absence of DNA contamination (KONG *et al.* 2003). RNA concentration and purity were assessed using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies Inc., Montchanin, Delaware, USA). RNA integrity was evaluated on 2% TAE agarose gel under non-denaturing conditions as well as capillary electrophoresis on the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, California, USA). Three inoculated sample time-points, 6 hr, 12 hr and 120 hr from R0.12 and one time-point, 120 hr from Dusa® were selected for further analysis along with all mock inoculated samples from both rootstocks. Paired-end sequencing was performed on these samples, to at least 80x coverage, using the Illumina® HiSeq™ PE150 platform (Novogene Corporation Inc., Chula Vista, California, USA).

Read mapping to the *P. americana* draft genome

Raw data were subjected to quality control and summarised using MultiQC (EWELS *et al.* 2016), and random hexamers priming artefacts were trimmed using Trimmomatic (BOLGER *et al.* 2014). RNA-seq reads were then mapped back to the draft *P. americana* genome (unpublished data), which was obtained from the Avocado Genome Consortium, using HISAT v2.0.6 (KIM *et al.* 2015). Reads that mapped uniquely to the *P. americana* genome were then subjected to further analysis. The entire complement of *P. americana* coding sequences were

obtained from the Avocado Genome Consortium and used as the reference transcriptome. Using the pseudo-alignment technique implemented in Kallisto (Bray *et al.* 2016), and the *P. americana* reference transcriptome, the reads from the RNA-seq experiment were normalized to obtain transcripts per million (TPM) estimates. The TPM data were then used in downstream analyses.

Candidate gene identification and annotation

Proteins that are essential to the establishment of SAR or are NPR1-dependent were identified from the literature (Backer *et al.* 2019). Corresponding *A. thaliana* protein-coding sequences were obtained online from NCBI (http://www.ncbi.nlm.nih.gov/) (Table 1). These sequences were then used to query a local BLAST database generated using the *P. americana* reference transcriptome predicted protein sequences (tBLASTx) in CLC Main Workbench v8.1.3 (CLC Bio, Qiagen® Inc., Hilden, DEU). InterProScan protein domain identifier v5.28-67.0 (Jones *et al.* 2014) was used to identify conserved domains while eggNOG-mapper (Huerta-Cepas *et al.* 2017; Huerta-Cepas *et al.* 2018) was used to complement functional annotation. Four representative plant transcriptomes were obtained from the Ensembl Plants database (https://plants.ensembl.org/index.html) namely; *Cinnamomum micranthum*, *A. thaliana*, *Vitis vinfera*, and *Amborella trichopoda* and used for putative ortholog identification using OrthoFinder (Emms and Kelly 2015). Finally, BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to assign final descriptions to identified proteins through similarity.

Table 1. Candidate sequences identified from the literature used to identify similar sequences from the *Persea americana* reference transcriptome.

Gene	Protein	Accession Number
BiP2	Heat shock protein 70 (Hsp 70) family protein	[Genbank: NM_180788]
CIPK11/PKS5	CBL-interacting serine/threonine-protein kinase 11	[Genbank: NM_128589]
CRT3	Calreticulin	[Genbank: NM_100718]
CUL3A	Cullin-3A	[Genbank: NM_102447]
CUL3B	Cullin-3B	[Genbank: NM_001334418]
DAD1	Phospholipase A(1) DAD1, chloroplastic	[Genbank: NM_001337097]
GSNOR1	*S*-(hydroxymethyl)glutathione dehydrogenase	[Genbank: NM_123761]
HDAC19	Histone deacetylase 19	[Genbank: NM_119974]
HSFB1/TBF1	Heat stress transcription factor B-1	[Genbank: NM_119862]
ICS1	Isochorismate synthase 1, chloroplastic	[Genbank: AY056055]
LHY	Protein LHY	[Genbank: NM_099988]
MPK3	Mitogen-activated protein kinase 3	[Genbank: NM_114433]

MPK6	Mitogen-activated protein kinase 6	[Genbank: NM_129941]
NIMIN-1	Protein NIM1-INTERACTING 1	[Genbank: AJ250184]
NIMIN-2	Protein NIM1-INTERACTING 2	[Genbank: AJ250185]
NIMIN-3	Protein NIM1-INTERACTING 3	[Genbank: AJ250186]
PR1	Pathogenesis-related protein 1	[Genbank: NM_127025]
PR2/BGL2	Beta-1,3-glucanase 2	[Genbank: NM_001339849]
PR5	Pathogenesis-related protein 5	[Genbank: NM_106161]
SARD1	Protein SAR DEFICIENT 1	[Genbank: NM_106040]
Sec61α	Protein transport protein Sec61 subunit alpha	[Genbank: AY093047]
SRK2C/SnRK2.8	Serine/threonine-protein kinase SRK2C	[Genbank: NM_001084370]
SUMO3	Small ubiquitin-related modifier 3	[Genbank: NM_001345118]
TGA2	Transcription factor TGA2	[Genbank: EF470791]
TGA3	Transcription factor TGA3	[Genbank: NM_102057]
TGA4	Transcription factor TGA4	[Genbank: NM_121041]
TGA5	Transcription factor TGA5	[Genbank: NM_203016]
TGA6	Transcription factor TGA6	[Genbank: NM_202564]
TGA7	Transcription factor TGA7	[Genbank: NM_106441]
TOC1	Timing of CAB expression 1 protein	[Genbank: AF272039]
TRX3	Thioredoxin H3	[Genbank: NM_123664]
TRX5	Thioredoxin H5	[Genbank: NM_103588]
WRKY18	WRKY transcription factor 18	[Genbank: NM_119329]
WRKY29	WRKY transcription factor 29	[Genbank: NM_118486]
WRKY38	WRKY transcription factor 38	[Genbank: NM_122163]
WRKY40	WRKY transcription factor 40	[Genbank: NM_106732]
WRKY53	WRKY transcription factor 53	[Genbank: NM_118512]
WRKY6	WRKY transcription factor 6	[Genbank: AF331713]
WRKY60	WRKY transcription factor 60	[Genbank: NM_128058]
WRKY62	WRKY transcription factor 62	[Genbank: NM_120268]
WRKY70	WRKY transcription factor 70	[Genbank: NM_115498]

Expression and statistical analysis

The expression analyses of candidate genes were conducted in Microsoft® Office Excel v16.0.1230 (Microsoft® Corporation Inc., Redmond, Washington, USA) using the TPM data generated in Kallisto. Two different expression analyses were conducted; the first a time-course using R0.12 data from the uninoculated control, 12hpi (hours post-inoculation), 24hpi, and 120hpi and the second being a comparative analyses of R0.12 uninoculated control and 120hpi as well as Dusa® uninoculated control and 120hpi data. All statistical analyses were performed using XLSTAT v2019.3.2 (Addinsoft LLC., Montmartre, Paris, FRA). One-way ANOVA was performed for the time-course analyses followed by a post hoc Fisher's Least Significant Difference (LSD) test. A two-way ANOVA was performed for comparative studies followed by a post hoc Fisher's (LSD) test. Significance was assessed using a 95% confidence interval.

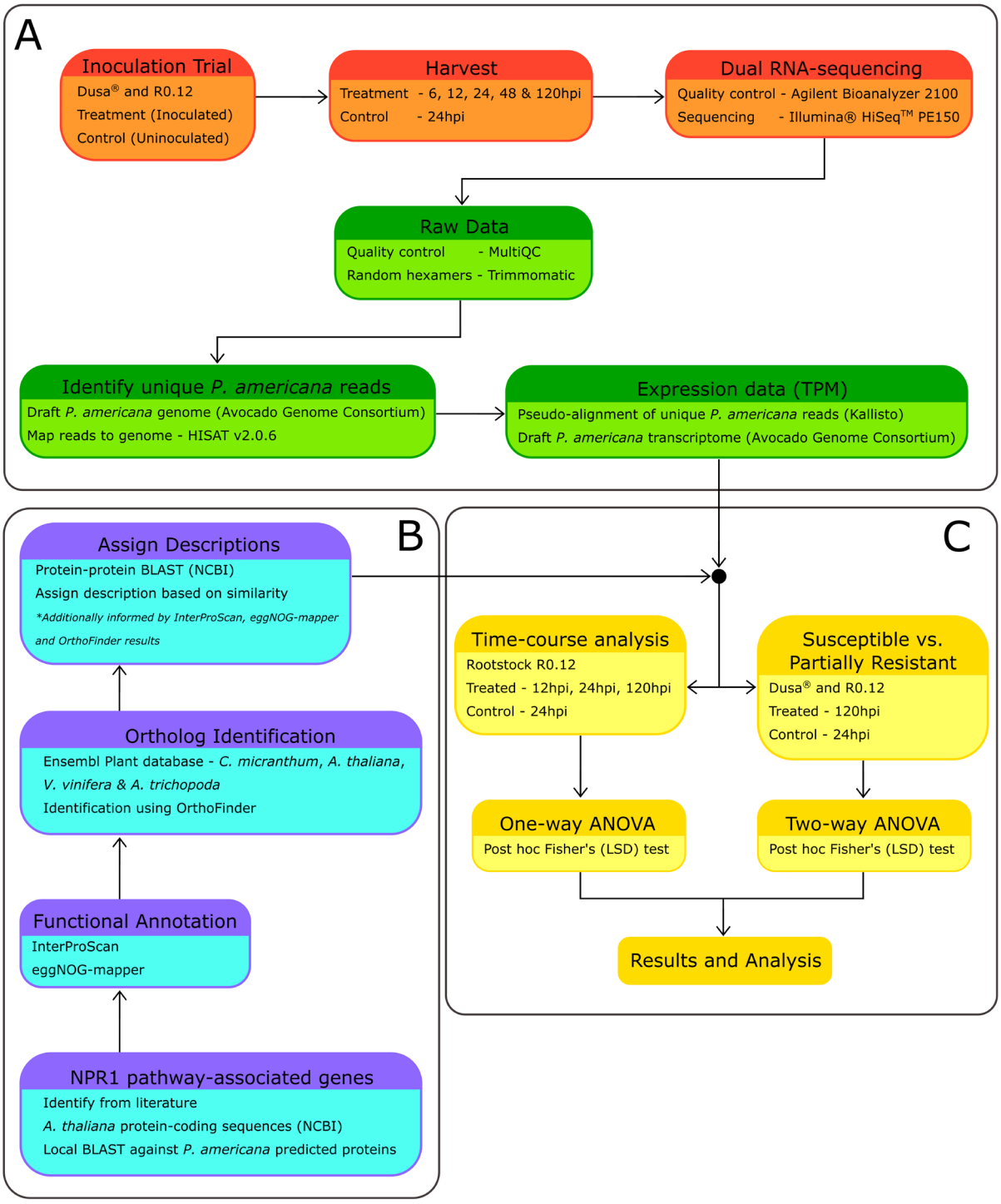


Figure 1. Visual representation of workflow used to ultimately determine differences in the expression of NPR1 pathway-associated genes in *Persea americana*. **A**) Dual RNA-sequencing was performed on libraries derived from both R0.12 and Dusa® rootstock plantlets, either inoculated with *P. cinnamomi* or mock-inoculated with dH_2O. Raw data was then subjected to quality control and aligned to the *P. americana* draft genome to identify *P. americana* transcripts. Pseudo-alignment was performed and used to quantify transcript abundance in the *P. americana* reference transcriptome. **B**) A thorough search of the literature identified

NPR1 pathway-associated genes. A local BLAST search of the reference transcriptome was used to identify possible *P. americana* orthologs. The identified NPR1 pathway-associated orthologs were then described using a multifaceted approach. **C**) The expression data (TPMs) of identified genes were subjected to two analyses. The first included time-course data from the *P. cinnamomi* susceptible rootstock R0.12 and comprised three inoculated time-points as well as an uninoculated control. The second was used to compare the uninoculated control as well as 120hpi of both the susceptible rootstock R0.12 and the partially resistant rootstock Dusa®. Statistically significant results were assessed further.

Results

Dual RNA-seq data analysis

Dual RNA-sequencing produced a total of 1 319 300 784 paired-end reads. On average susceptible (R0.12) sample libraries generated 59 million ± 20 million reads while partially resistant (Dusa®) sample libraries yielded around 68 million ± 33 million reads. About 78% and 82% of R0.12 and Dusa® control sample library reads mapped back to the *P. americana* draft genome, respectively. Whereas 69% and 65% mapped back in the inoculated R0.12 and Dusa® samples. Of the 46 262 coding sequences within the *P. americana* transcriptome, 28 178 expressed at least one TPM.

Identification and annotation of NPR1 pathway-associated genes

A total of 116 unique *P. americana* proteins were identified as significantly similar (data not shown) to the query *A. thaliana* protein-coding sequences (Table 1). These were annotated using a combined approach to arrive at putative functional descriptions (Table S1). InterProScan successfully assigned functional domains to 85.34% of the 116 putative proteins. Meanwhile, eggNOG-mapper could assign functional domains and protein families to 94.83%. Using the four transcriptomes acquired from the Ensembl Plants database, OrthoFinder identified 110 orthologs in *C. micranthum* (94.83%), 93 in *A. thaliana* (80.17%), eight in *V. vinifera* (6.90%) and 90 in *A. trichopoda* (77.59%). Collectively, *C. micranthum* and *A. thaliana* transcriptomes informed OrthoFinder of 99.14% of the 116 identified *P. americana* transcripts while *V. vinifera* and *A. trichopoda* transcriptomes did not add any additional

information. Additionally, online BLASTp analysis revealed that putative *P. americana* NPR1 pathway-associated proteins are most like orthologs in *C. micranthum*, which accounted for 104 (89.65%) of the top-scoring hits. Considering all 116 putative proteins, the average query coverage was 92.85%, percentage identity was 91.75% and E-value averaged 1.54 x10^{-9} (Table S2).

Expression of NPR1 pathway-associated genes over time during a compatible interaction

We examined the normalised RNA-seq expression data (TPMs) of 116 NPR1 pathway-associated genes in the susceptible *P. americana* rootstock, R0.12. The expression data from three inoculated *P. cinnamomi* time-points and one uninoculated control were compared and statistically interrogated. A total of 74 NPR1 pathway-associated genes displayed significantly varied expression over time, or when comparing transcripts from mock-inoculated control samples and *P. cinnamomi* inoculated samples, summarised in figure 2. The mean (TPM), standard error of the mean (SEM), and grouping based on Fisher's (LSD) test for all 116 genes can be found in supplementary table S3.

Significant differences were observed for the expression of three *PaNPR1-like* genes. *PaNPR1* expression increased over time in *P. cinnamomi* inoculated samples, reaching statistical significance at 120hpi. *PaNPR2* expression was significantly higher at all inoculated time points compared to the uninoculated control, reaching its highest level at 120hpi. *PaNPR5* expression was at its highest in the uninoculated control and early inoculated time-points, 12hpi and 24hpi, dropping significantly at 120hpi.

Furthermore, several genes that encode for proteins involved in post-translation modification of NPR1-like proteins showed statistically significant expression differences. Two *AtTRX-like* genes, *PaTRX1-like1* and *PaTRX4-like2,* showed significantly higher expression at 120hpi when compared to the control, an expression pattern like that of *PaNPR1* and *PaNPR2*. In contrast, *PaTRX-like1* decreased over time, and significantly by 120hpi. *PaADH* and *PaADH2*, which encode for proteins that are highly like GSNOR in *A. thaliana,* showed a significant upregulation at 12hpi, which returned to baseline levels by 24hpi. Orthologs of the serine/threonine-protein kinase encoding gene *AtSRK2C*, *PaSAPK2-like1,* and *PaSAPK2-like2*, showed significant upregulation at 12hpi followed by a swift return to baseline levels at 24hpi. However, another likely serine/threonine-protein kinase encoding gene *PaCIPK10-like1* is

significantly downregulated at 24hpi followed by significant upregulation at 120hpi. Curiously, *AtSUMO3* orthologs - *PaSUMO1-like1, PaSUMO1-like2* and *PaSUMO2-like1*, showed stable expression across all time points. *PaNIMIN-2*, the ortholog of *AtNIMIN-2,* which encodes for an NPR1-interacting protein, showed significant downregulation at 120hpi when *PaNPR1* and *PaNPR2* were at their peak expression. Also, the expression of the *PaRHA1B-like* peaked at 12hpi and was downregulated significantly at both 24hpi and 120hpi.

Transcription factors are essential to the organised expression of SAR-related genes (BACKER *et al.* 2019). A total of 19 avocado WRKY-like transcription factor coding genes were found to be significantly differentially regulated in response to inoculation with *P. cinnamomi*, over time. Ten of these were markedly upregulated at 120hpi, while in contrast, only two were downregulated significantly at 120hpi. The expression of five *PaWRKY-like genes* were downregulated at 12hpi, either returning to baseline after that or increasing significantly at later time-points. Two WRKY transcription factor encoding genes, *PaWRKY76-like* and *PaWRKY-like3*, were transiently upregulated at 24hpi and 12hpi, respectively. However, the overall trend of *PaWRKY-like* gene expression indicated a decrease at 12hpi, followed by an often-significant increase at 120hpi.

Although not all were significant, the *PaTGA2-like* and *PaTGA4-like* genes displayed expression patterns which contrasted with most *PaWRKY-like* genes, with expression peaking at 12hpi and dropping after that. Of the 10 *TGA-like genes* identified in avocado, six showed a significant response to *P. cinnamomi* inoculation. *PaTGA2-like1* was significantly upregulated at both 12hpi and 24hpi while *PaTGA4-like1* expression was highest at 12hpi and then significantly downregulated at 24hpi. *PaTGA4-like3* was upregulated considerably at 12hpi, followed by a return to baseline at 24hpi. Additionally, *PaHBP-1b(C38)-like1* expression reduced gradually over time and decreased significantly by 120hpi. Conversely, *PaTGA10-like1* and *PaHBP-1b(C38)-like2* expression showed a gradual rise over time, which reached significant upregulation by 120hpi.

The expression of several PR protein families are dependent on TGA transcription factors and are vital to the establishment of SAR (BACKER *et al.* 2019). We identified eight PR-1-like, twelve PR-2-like, and thirteen PR-5-like coding genes in *P. americana,* of which five *PaPR-1-like*, eight *PaPR-2-like,* and eight *PaPR-5-like* genes were differentially regulated after inoculation with *P. cinnamomi*. The overall expression of both *PaPR-1-like* and *PaPR-2-like* genes tended

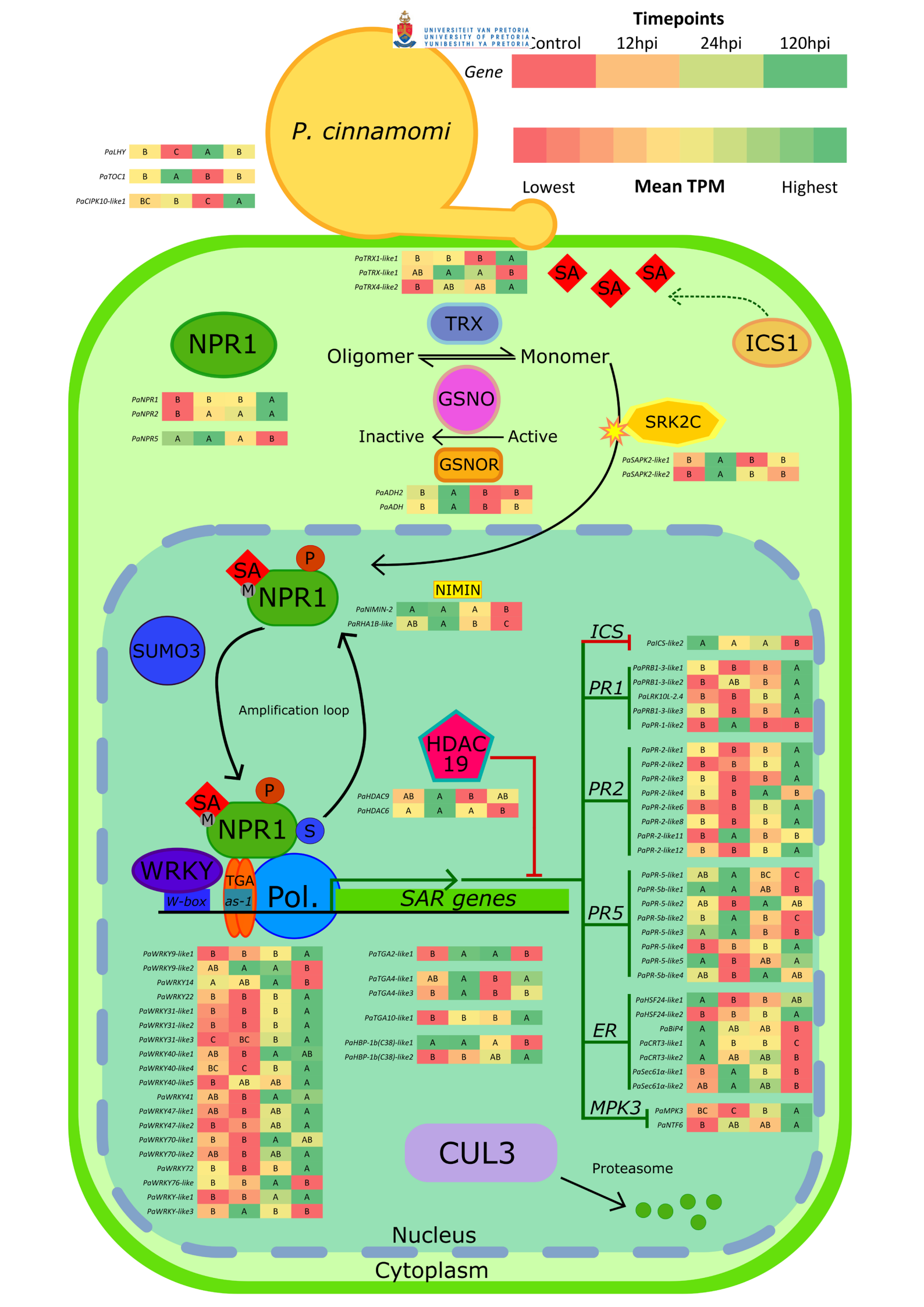
towards an increase over time, reaching their highest expression at 120hpi, although not all were significant. Notable exceptions, *PaPR-1-like2* and *PaPR-2-like4*, were transiently upregulated at 12hpi and *PaPR-2-like11* at 24hpi, followed by a return to baseline levels. Interestingly, unlike *PaPR-1-like* and *PaPR-2-like* genes, *PaPR5-like* genes as a whole had no definitive expression patterns. Expression of three *PaPR-5-like* genes, *PaPR-5-like2*, *PaPR-5-like5,* and *PaPR-5b-like4,* was temporarily downregulated at 12hpi. In contrast, the expression of four *PaPR-5-like* genes was significantly higher at 12hpi when compared to 120hpi. Only the expression of *PaPR-5-like4* increased significantly at 120hpi.

Additionally, the expression of *A. thaliana* histone deacetylase *HDAC19* ortholog *PaHDAC9* was significantly higher at 12hpi and comparatively lower at 24hpi. Furthermore, the expression of *PaHDAC6* seemed to increase, although not significantly, at 12hpi with a significant drop at 120hpi. We also described two *AtICS1-like* genes in avocado, the expression of which tended to decrease following inoculation. Of the two, *PaICS-like2* had the highest expression levels overall, which were significantly downregulated at 120hpi.

Three *AtMPK3/6* orthologs were identified in avocado, namely, *PaMPK3, PaNTF6,* and *PaMMK1*. Although the expression of *PaMMK1* seemed to respond to inoculation by *P. cinnamomi*, increasing slightly by 12hpi and decreasing by 120hpi, it was not significant due to high variability between biological replicates. However, both *PaMPK3* and *PaNTF6* expression was significantly upregulated following inoculation, increasing over time and reaching peak expression at 120hpi.

We further characterised several members of the protein secretory pathway in *P. americana*. The *AtHSFB1-like* orthologs, *PaHSF24-like1* and *PaHSF24-like2* displayed contrasting patterns of expression. *PaHSF24-like2* expression gradually increased over time and was significantly increased compared to the control at 120hpi. Meanwhile, *PaHSF24-like1* expression significantly decreased in response to *P. cinnamomi* inoculation. Similarly, *PaBiP4*, *PaCRT3-like1,* and *PaCRT3-like2* expression reduced substantially over time. Both *AtSec61α-like* orthologs, *PaSec61α-like1* and *PaSec61α-like2*, had significantly elevated transcript levels at 12hpi when compared to 120hpi. Interestingly, both *PaDAD1-like1* and *PaDAD1-like2* had deficient expression levels, which remained virtually unchanged during the study.

Finally, we describe orthologs for the SA-responsive gene, *AtTOC1*, and its antagonist *AtLHY* in *P. americana*. The expression of *PaTOC1* is significantly increased at 12hpi; interestingly expression returns to almost exact control-like levels at both 24hpi and 120hpi. In contrast, *PaLHY* expression is downregulated significantly at 12hpi and upregulated considerably at 24hpi; by 120hpi *PaLHY* expression returns to near baseline levels.


UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Timepoints
Control
12hpi
24hpi
120hpi
Gene
Lowest
Mean TPM
Highest
P. cinnamomi
PaLHY B C A B
PaTOC1 B A B B
PaCIPK10-like1 BC B C A
PaTRX1-like1 B B B A
PaTRX-like1 AB A A B
PaTRX4-like2 B AB AB A
SA
SA
SA
ICS1
NPR1
TRX
Oligomer
Monomer
PaNPR1 B B B A
PaNPR2 B A A A
PaNPR5 A A A B
GSNO
SRK2C
Inactive
Active
GSNOR
PaSAPK2-like1 B A B B
PaSAPK2-like2 B A B B
PaADH2 B A B B
PaADH B A B B
SA
M
NPR1
P
NIMIN
PaNIMIN-2 A A A B
PaRHA1B-like AB A B C
SUMO3
Amplification loop
HDAC 19
PaHDAC9 AB A B AB
PaHDAC6 A A A B
SA
M
NPR1
P
S
WRKY
W-box
TGA
as-1
Pol.
SAR genes
ICS
PaICS-like2 A A A B
PR1
PaPRB1-3-like1 B B B A
PaPRB1-3-like2 B AB B A
PaLRK10L-2.4 B B B A
PaPRB1-3-like3 B B B A
PaPR-1-like2 B A B B
PR2
PaPR-2-like1 B B B A
PaPR-2-like2 B B B A
PaPR-2-like3 B B B A
PaPR-2-like4 B B A B
PaPR-2-like6 B B B A
PaPR-2-like8 B B B A
PaPR-2-like11 B A B B
PaPR-2-like12 B B B A
PR5
PaPR-5-like1 AB A BC C
PaPR-5b-like1 A A AB B
PaPR-5-like2 AB B A AB
PaPR-5b-like2 B A B C
PaPR-5-like3 A A B B
PaPR-5-like4 B B B A
PaPR-5-like5 A B AB A
PaPR-5b-like4 AB B A AB
ER
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PaHSF24-like2 B B B A
PaBiP4 A AB AB B
PaCRT3-like1 A B B C
PaCRT3-like2 A AB AB B
PaSec61α-like1 B A B B
PaSec61α-like2 AB A AB B
MPK3
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PaNTF6 B AB AB A
PaWRKY9-like1 B B B A
PaWRKY9-like2 AB A A B
PaWRKY14 A AB A B
PaWRKY22 B B B A
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PaWRKY76-like B B A B
PaWRKY-like1 B B A A
PaWRKY-like3 B A B B
PaTGA2-like1 B A A B
PaTGA4-like1 AB A B A
PaTGA4-like3 B A B B
PaTGA10-like1 B B B A
PaHBP-1b(C38)-like1 A A A B
PaHBP-1b(C38)-like2 B B AB A
CUL3
Proteasome
Nucleus
Cytoplasm

Figure 2. Visual representation of NPR1 pathway-associated gene expression during *Phytophthora cinnamomi* challenge, over time, in the susceptible *Persea americana* rootstock R0.12. Coloured blocks indicate gene expression (mean TPM); red specifies the lowest observed expression while dark green specifies the highest observed expression, for a given gene across all observations. Four observations were made, one mock-inoculated control (harvested at 24hpi) and three *P. cinnamomi* inoculated (collected at 12hpi, 24hpi and 120hpi) and are indicated, in that order, on the figure. Statistical grouping based on Fisher's Least Significant Difference (LSD) test is indicated by alphabetic indicators (A, B or C) within each respective coloured block. *P. americana* gene names are shown to the left of each respective set of observations. For more detailed information on each gene, including ones not included in this figure, please refer to supplementary table S3. The graphical representation above, which summarises the NPR1 pathway, was adapted from BACKER *et al.* (2019). During challenge from a biotrophic or hemibiotrophic pathogen, in this case, the hemibiotrophic oomycete *P. cinnamomi*, there is an increased accumulation of SA, produced primarily by ICS1. Subsequently, the cell is exposed to increasing levels of oxidative stress, followed by an increase in the production of antioxidants. Specific antioxidants, known as thioredoxins, are involved in promoting the conversion of NPR1 to its monomeric form whereas GSNO encourages NPR1 to exist in its oligomeric form within the cytoplasm. Some specialised members of the alcohol dehydrogenase family, specifically ADH3/GSNOR, reduce the availability of active GSNO and subsequently the oligomerisation of NPR1. Then, the protein kinase SRK2C interacts with and phosphorylates monomeric NPR1^{Ser389}, which is required for the nuclear import of NPR1. Within the nucleus, NIMINs interact with NPR1, either preventing or delaying the expression of target genes. Phosphorylation of NPR1$^{Ser11/15}$ increases sumoylation by SUMO3, which is vital for both NPR1-dependent gene expression and degradation of NPR1. Sumoylation also increases the association of NPR1 and TGA transcription factors, while decreasing association with WRKY transcription factors. In general, WRKY transcription factors are associated with negative and, TGA transcription factors with positive, regulation of SA-dependent genes. Additionally, HDAC19 is associated with repression of several SAR-related genes during uninduced conditions through histone modification. Finally, spent NPR1 is ubiquitinylated by CUL3 and subsequently degraded by the 26S proteasome, an essential aspect of maintaining peak SAR gene expression.

Comparison of NPR1 pathway-associated gene expression during compatible and incompatible interactions

The second set of analyses was used to explore differences in the expression of NPR1 pathway-associated genes from *P. americana* between the susceptible rootstock R0.12 and the partially resistant rootstock Dusa®. These analyses were performed using the normalised dual RNA-seq expression data of all 116 identified genes and included both uninoculated control and *P. cinnamomi* inoculated 120 hr time-point (120hpi) sample libraries from both rootstocks. A total of 86 NPR1 pathway-associated genes were identified as differentially regulated to a statistically significant degree following these analyses (Figure 3). Surprisingly, the regulation of 39 genes were significantly different between the two rootstocks. However, similar responses to *P. cinnamomi* inoculation were noted for 23 genes in both rootstocks. Nonetheless, 24 genes displayed significant up- or downregulation in response to inoculation within a particular rootstock, yet no significant difference was observed when comparing between rootstocks. The mean (TPM), standard error of the mean (SEM), and grouping based on Fisher's (LSD) test for all 116 genes can be found in supplementary table S4.

As in the time-course analyses, the expression of *PaNPR1* and *PaNPR2* in R0.12 are significantly upregulated at 120hpi. However, expression in Dusa® is similar in both the control and 120hpi. Interestingly, expression of *PaNPR1* in the uninoculated control of Dusa® was significantly higher than that of R0.12. The expression of *PaNPR4* in Dusa® was markedly higher in the control as compared to 120hpi while in R0.12, there was no significant difference. However, *PaNPR3* and *PaNPR5* expression was significantly downregulated in response to *P. cinnamomi* inoculation in both R0.12 and Dusa®.

Similarly, the expression of *PaTRX-like1* was significantly downregulated by 120hpi in both Dusa® and R0.12. However, expression of *PaTRX2-like1* and *PaTRX2-like2* in uninoculated Dusa® was substantially higher than in uninoculated R0.12. Furthermore, the abundance of *PaTRX2-like1* decreased significantly in Dusa® at 120hpi while remaining relatively unchanged in R0.12. The expression of *PaTRX4-like1* in Dusa® and not R0.12 was markedly downregulated at 120 hr following *P. cinnamomi* inoculation. Conversely, *PaTDX-like1* expression did not seem to change substantially in response to *P. cinnamomi* inoculation in either rootstock, although considerably lower overall expression was observed in Dusa®. Significant downregulation of *PaADH3* was seen in both rootstocks following *P. cinnamomi* challenge.

Yet, *PaADH-like* was significantly downregulated in Dusa® while remaining unchanged in R0.12, and base-level expression of this gene was significantly higher in Dusa® compared to R0.12.

The expression of *PaCIPK10-like1* increased considerably for both Dusa® and R0.12 following inoculation; transcripts were, however, substantially more abundant in uninoculated R0.12 samples when compared to Dusa®. In contrast, the expression of *PaCIPK10-like2* remained relatively unchanged in Dusa®, while R0.12 transcript levels were significantly reduced at 120hpi. Additionally, the *AtNIMIN-like* ortholog *PaRHA1B-like* was downregulated for both Dusa® and R0.12 at 120hpi. The two remaining *AtNIMIN-like* orthologs, *PaNIMIN-1* and *PaNIMIN-2*, were downregulated in Dusa® and R0.12 respectively while remaining unaffected in the other. Furthermore, while expression for all three *PaSUMO-like* genes remained reasonably constant in R0.12, expression for all three decreased significantly in Dusa® following inoculation. Similarly, the expression of *AtCUL3A* ortholog *PaCUL3A-like1* remained unchanged in R0.12 while decreasing significantly in Dusa®. Additionally, while *PaCUL3A-like2* was not significantly downregulated in either R0.12 or Dusa®, abundance at 120hpi was considerably lower in Dusa®.

As expected, the overall expression for most *PaWRKY-like* genes increased at 120hpi. A total of five *PaWRKY-like* genes were upregulated similarly in both Dusa® and R0.12. Interestingly, four *PaWRKY-like* genes were upregulated in Dusa® only, whereas three were only upregulated in R0.12. Additionally, *PaWRKY9-like1* and *PaWRKY31-like3* were significantly upregulated in both R0.12 and Dusa® although the R0.12 displayed significantly higher upregulation of both compared to Dusa®. Similarly, *PaWRKY-like1* was significantly upregulated in both Dusa® and R0.12 at 120hpi, however, the increase was markedly higher in Dusa® compared to R0.12. In contrast, the expression of *PaWRKY9-like2* and *PaWRKY14* decreased in both rootstocks, although the abundance of *PaWRKY9-like2* was considerably higher in Dusa® for both the control and 120hpi samples. Moreover, although the expression of *PaWRKY40-like1* did not change significantly in either rootstock, transcript abundance in Dusa® was significantly higher in the control sample.

Similarly, Dusa® had significantly higher *PaTGA2-like1* transcript abundance in the control sample compared to R0.12. Furthermore, *PaTGA2-like1* and three additional *PaTGA2-like* genes were significantly downregulated by 120hpi in Dusa® but not in R0.12. In Dusa®, the

expression of both *PaTGA4-like1* and *PaTGA4-like3* was significantly lower in the control and 120hpi samples when compared to R0.12. However, it should be noted that the expression of both *PaTGA4-like* genes remained stable in both rootstocks. In contrast, the expression of both *PaTGA10-like1* and *PaHBP-1b(C38)-like2* was downregulated significantly in R0.12 while remaining stable in Dusa®. Finally, *PaHBP-1b(C38)-like1* was downregulated considerably at 120hpi in both Dusa® and R0.12.

In contrast, the overall expression of both *PaPR-1-like* and *PaPR2-like* genes in Dusa® and R0.12 increased following inoculation. The expression of *AtPR-1-like* orthologs, *PaPRB1-3-like1* and *PaPRB1-3-like2*, was similar in both rootstocks following inoculation with *P. cinnamomi*. However, orthologs *PaLRK10L-2.4* and *PaPRB1-3-like3* were only significantly upregulated in R0.12. Interestingly, Dusa® expressed no detectable *PaPRB1-3-like3* transcripts in the control and virtually none at 120hpi. The upregulation of three *PaPR-2-like* genes was practically identical in both Dusa® and R0.12; however, the remaining *PaPR-2-like* genes showed significantly varied expression. Although *PaPR-2-like1* was upregulated in both rootstocks, induced expression in Dusa® was substantially stronger than that of R0.12. Furthermore, *PaPR-2-like3* and *PaPR-2-like8* were upregulated considerably in R0.12, and while expression did increase somewhat in Dusa®, it was not to a significant degree. Interestingly, the expression of *PaPR-2-like5* decreased significantly in Dusa®, while in R0.12, it increased slightly.

In general, the overall expression of *PaPR-5-like* genes decreased following *P. cinnamomi* challenge at 120hpi. Three of these were downregulated to a similar extent in both Dusa® and R0.12, while another, *PaPR-5b-like1*, was also downregulated in both rootstocks, yet transcript abundance in the control was significantly higher in Dusa®. By contrast, *PaPR-5-like6* was significantly upregulated at 120hpi in Dusa®, and while expression increased somewhat in R0.12, it was not to a significant degree. A similar observation was made regarding the expression of *PaPR-5-like7*; however, the difference in expression at 120hpi in Dusa® was substantially higher. Meanwhile, the expression of *PaPR-5-like4* was significantly upregulated in R0.12 at 120hpi and not in Dusa®. Furthermore, *PaPR-5-like2* and *PaPR-5-like5* were not significantly up- or downregulated at 120hpi in either rootstock; however, there was significantly higher expression overall in Dusa®.

Furthermore, both *PaICS-like* and *PaHDAC-like* gene expression was also generally downregulated following *P. cinnamomi* challenge. Although overall transcript abundance for *PaICS-like1* was low and downregulation was only significant in Dusa®, there was a definite trend indicating downregulation in both rootstocks. The expression of *PaICS-like2* was similar, displaying significant downregulation in both rootstocks following inoculation. Interestingly, the expression of *PaHDAC9* in the Dusa® control was significantly higher than that of R0.12, in which expression remained constant, and was downregulated substantially at 120hpi. Nonetheless, *PaHDAC6* was significantly downregulated in both R0.12 and Dusa®.

By comparison, the expression of *PaMPK3* was upregulated significantly, in both avocado rootstocks, at 120hpi. However, the *PaNTF6* abundance remained constant in Dusa® while being upregulated considerably in R0.12. Conversely, *PaMMK1* was significantly downregulated in Dusa® following inoculation, and while expression in R0.12 tended to decrease, the difference was not significant.

Orthologs of the protein secretory pathway in *P. americana* also displayed some differences in expression between R0.12 and Dusa®. While *PaHSF24-like1* expression was not up- or downregulated significantly in either rootstock following inoculation, abundance was significantly higher in R0.12 prior to inoculation. However, both R0.12 and Dusa® showed significant upregulation of *PaHSF24-like2* following *P. cinnamomi* challenge. By comparison, the expression of both *PaCRT3-like1* and *PaCRT3-like2* was significantly downregulated in both rootstocks, although *PaCRT3-like1* was significantly more abundant in the R0.12 control compared to Dusa®. Lastly, while expression of *PaBiP4* generally trended toward downregulation at 120hpi, only the difference in R0.12 was significant.

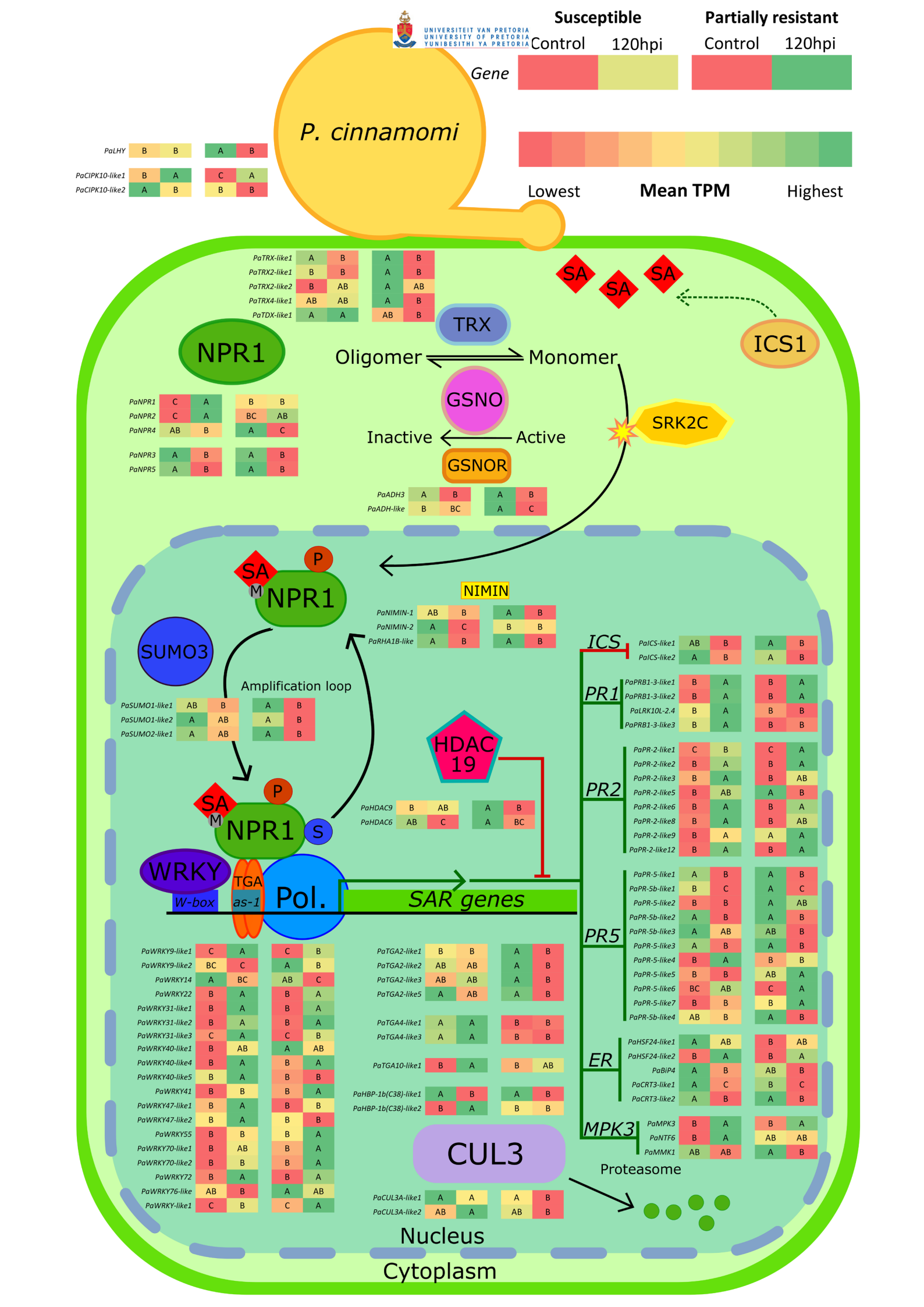
UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Susceptible
Partially resistant
Control
120hpi
Control
120hpi
Gene
P. cinnamomi
PaLHY
PaCIPK10-like1
PaCIPK10-like2
Lowest
Mean TPM
Highest
PaTRX-like1
PaTRX2-like1
PaTRX2-like2
PaTRX4-like1
PaTDX-like1
TRX
SA
SA
SA
ICS1
NPR1
Oligomer
Monomer
PaNPR1
PaNPR2
PaNPR4
PaNPR3
PaNPR5
GSNO
SRK2C
Inactive
Active
GSNOR
PaADH3
PaADH-like
SA
M
P
NPR1
NIMIN
PaNIMIN-1
PaNIMIN-2
PaRHA1B-like
SUMO3
Amplification loop
PaSUMO1-like1
PaSUMO1-like2
PaSUMO2-like1
HDAC 19
PaHDAC9
PaHDAC6
SA
M
P
NPR1
S
WRKY
W-box
TGA
as-1
Pol.
SAR genes
ICS
PaICS-like1
PaICS-like2
PR1
PaPRB1-3-like1
PaPRB1-3-like2
PaLRK10L-2.4
PaPRB1-3-like3
PR2
PaPR-2-like1
PaPR-2-like2
PaPR-2-like3
PaPR-2-like5
PaPR-2-like6
PaPR-2-like8
PaPR-2-like9
PaPR-2-like12
PR5
PaPR-5-like1
PaPR-5b-like1
PaPR-5-like2
PaPR-5b-like2
PaPR-5b-like3
PaPR-5-like3
PaPR-5-like4
PaPR-5-like5
PaPR-5-like6
PaPR-5-like7
PaPR-5b-like4
ER
PaHSF24-like1
PaHSF24-like2
PaBiP4
PaCRT3-like1
PaCRT3-like2
MPK3
PaMPK3
PaNTF6
PaMMK1
PaWRKY9-like1
PaWRKY9-like2
PaWRKY14
PaWRKY22
PaWRKY31-like1
PaWRKY31-like2
PaWRKY31-like3
PaWRKY40-like1
PaWRKY40-like4
PaWRKY40-like5
PaWRKY41
PaWRKY47-like1
PaWRKY47-like2
PaWRKY55
PaWRKY70-like1
PaWRKY70-like2
PaWRKY72
PaWRKY76-like
PaWRKY-like1
PaTGA2-like1
PaTGA2-like2
PaTGA2-like3
PaTGA2-like5
PaTGA4-like1
PaTGA4-like3
PaTGA10-like1
PaHBP-1b(C38)-like1
PaHBP-1b(C38)-like2
CUL3
PaCUL3A-like1
PaCUL3A-like2
Proteasome
Nucleus
Cytoplasm

Figure 3. Visual representation of differences in the expression of NPR1 pathway-associated genes during *Phytophthora cinnamomi* challenge in the susceptible *Persea americana* rootstock R0.12 and partially resistant rootstock Dusa®. Coloured blocks indicate gene expression (mean TPM); red specifies the lowest observed expression while dark green specifies the highest observed expression, for a given gene across all observations. Two observations were made for each rootstock, one mock-inoculated control (harvested at 24hpi) and one *P. cinnamomi* inoculated (collected at 120hpi) and are indicated, in that order, on the figure. Statistical grouping based on Fisher's Least Significant Difference (LSD) test is indicated by alphabetic indicators (A, B or C) within each respective coloured block. *P. americana* gene names are reported to the left of each respective set of observations. For more detailed information on each gene, including ones not included in this figure, please refer to supplementary table S4. The graphical representation above, which summarises the NPR1 pathway, was adapted from BACKER *et al.* (2019) and is briefly described in figure 2.

Discussion

This study explored the expression of NPR1 pathway-associated genes, over time, in a *P. cinnamomi* susceptible *P. americana* rootstock (R0.12), following inoculation. Furthermore, expression at 120hpi was compared between R0.12 and Dusa®, which is known for its partial resistance to *P. cinnamomi*. The observations presented here confirmed the hypotheses set forth for this study. Significant upregulation of several *PR-like* genes indicated the establishment of SAR in both rootstocks. Furthermore, 74 out of 116 identified NPR1 pathway-associated genes were responsive to inoculation with *P. cinnamomi*, in R0.12. These genes conform, mostly, with our expectation based on the literature, representing an intact and responsive SA-induced NPR1-dependent pathway. However, several differences are evident when comparing R0.12 and Dusa®, most notably the upregulation of *PaNPR1* and *PaNPR2* in R0.12, but not Dusa®, at 120hpi. Overall, the evidence suggests that Dusa® might downregulate SA-induced defence responses more efficiently than R0.12 in response to *P. cinnamomi* switching infection strategies to become necrotrophic. Thus, it is likely that the differences in *P. cinnamomi* sensitivity between R0.12 and Dusa®, are primarily dependent on variations in the regulation of the NPR1 pathway.

Challenge with *P. cinnamomi* leads to the establishment of SAR in R0.12

Systemic acquired resistance is initiated in response to biotrophic and hemibiotrophic pathogens, inciting broad-spectrum resistance to succeeding pathogenic stresses (SHAH 2003). The successful establishment of SAR is evidenced by an increased abundance of *PR* transcripts (VAN LOON AND VAN STRIEN 1999; DURRANT AND DONG 2004; AN AND MOU 2011). The expression of four *PaPR-1-like* and six *PaPR-2-like* genes in R0.12 was significantly upregulated by 120hpi. Most importantly, the transcript abundance of *PaPRB1-3-like1* and *PaPRB1-3-like2* was upregulated approximately six- and threefold at 120hpi, respectively. Similarly, *PaPR-2-like1* and *PaPR-2-like2* transcripts were around four times more abundant at 120hpi. Moreover, these genes had the four highest transcript abundances of all 116 investigated genes at 120hpi, underscoring their importance. Thus, by definition, we can confidently confirm that SAR was established in the susceptible *P. americana* rootstock, R0.12, in response to *P. cinnamomi*. Although R0.12 is considered susceptible to *P. cinnamomi*, the likely establishment of SAR explains to some extent why R0.12 is not considered unconditionally susceptible to *P. cinnamomi*.

By contrast, four of the eight significantly regulated *PR-5-like* genes were downregulated at 120hpi. However, three of these were slightly higher at 12hpi than in the control, although not considerably, while *PaPR-5b-like2* was significantly upregulated. It is worth noting that PR-5-like proteins are known to be effective antifungal agents, both *in vitro* and *in vivo* (VIGERS *et al.* 1992; LIU *et al.* 1994; ABAD *et al.* 1996; ZHU *et al.* 1996). Additionally, constitutive overexpression of a tomato *PR-5* in orange plants decreased susceptibility to *Phytophthora citrophthora* (FAGOAGA et al. 2001). Therefore, these observations suggest that some PaPR-5-like proteins may well be involved in the early response against *P. cinnamomi.*

Nonetheless, *P. americana* utilises both the SA and jasmonic acid/ethylene (JA/ET) defence response pathways against *P. cinnamomi* (VAN DEN BERG *et al.* 2018). However, regulation of the SA and JA/ET defence response pathways are most often antagonistic (FELTON AND KORTH 2000; VAN WEES *et al.* 2000; GLAZEBROOK 2005; EL OIRDI *et al.* 2011). Thus unsurprisingly, the SA-pathway is predominantly active during the early stages of infection, when *P. cinnamomi* is in the biotrophic phase (VAN DEN BERG *et al.* 2018). Later, at around 18-24hpi, the JA/ET response pathway dominates as *P. cinnamomi* switches to a necrotrophic infection strategy (VAN DEN BERG *et al.* 2018). Notably, cross-talk between the SA and JA/ET defence pathways is regulated

by the actions of monomeric NPR1 within the cytoplasm (SPOEL *et al.* 2003). Therefore, the upregulation of *PaNPR1* at 120hpi and the continually high expression of *PaNPR2* from 12hpi to 120hpi is somewhat surprising. A previous study comparing *Fusarium oxysporum* Schlecht f. sp. *cubense* (Smith) Snyd (*Foc*) susceptible and resistant banana cultivars noted a delayed response in the upregulation of *MNNPR1A* in the susceptible cultivar compared to the resistant cultivar; still, expression of *MNNPR1A* returned to baseline by 48hpi in the susceptible cultivar (ENDAH *et al.* 2008). By 120hpi, it is highly likely that as *P. cinnamomi* is already well within the necrotrophic phase of infection. Additionally, upregulation of thioredoxin genes *TRX1-like1* and *TRX4-like2* at 120hpi would likely increase monomeric *PaNPR1* and *PaNPR2* within the cytoplasm. Interestingly, we previously reported that *PaNPR1* and *PaNPR2* are not significantly upregulated in Dusa® following *P. cinnamomi* inoculation between 6hpi and 24hpi; instead they were downregulated considerably by 96hpi (BACKER *et al.* 2015). Together, these observations suggest that a likely reason for the success of *P. cinnamomi* in R0.12 could be suppression of the JA/ET pathway by either *PaNPR1*, *PaNPR2*, or both.

Nonetheless, *PaNPR2* seems to be the most likely candidate for the functional ortholog of *AtNPR1*. Not only is *PaNPR2* upregulated much earlier than *PaNPR1*, its transcripts are consistently over three times as abundant. Nevertheless, we also see evidence suggesting potential early post-translational activation of NPR1-like proteins. For instance, the expression of *PaTRX-like1* and *PaTRX2-like2* tends to increase at 12hpi, although not to a significant degree. Additionally, *PaADH2* and *PaADH* were significantly upregulated at 12hpi and given their similarity to AtGSNOR1 it would be reasonable to assume that they too may reduce GSNO. Thus, it is likely that PaTRXs aid in PaNPR1/2 monomerisation at 12hpi, while PaADHs reduce limit re-oligomerisation. Furthermore, the AtSRK2C orthologs *PaSAPK2-like1* and *PaSAPK2-like2* were substantially upregulated at 12hpi, thus increasing the potential for phosphorylation and subsequent activation of PaNPR1/2. Together, these observations suggest that PaNPR1/2 exist primarily as active monomers at 12hpi with the potential to activate SAR.

However, even though PaNPR1/2 likely exist in an active state within the nucleus at 12hpi, we do not see upregulation of *PR-like* genes until 120hpi. This discrepancy is probably due, in part, to several negative SAR regulators. Two *AtNIMIN-like* orthologs, *PaNIMIN-2* and

PaRHA1B-like, are not downregulated until 120hpi and 24hpi, respectively. Collectively, in *A. thaliana* NIMINs do not outright prevent SAR gene expression but rather, through differing sensitivities to SA, delay it (HERMANN *et al.* 2013). Additionally, the orthologs of *AtHDAC19*, *PaHDAC9* and *PaHDAC6*, display a slight increase in abundance at 12hpi, followed by significant downregulation at only 24hpi and 120hpi, respectively. Therefore, we can reasonably assume that although PaNPR1/2 are activated, the actions of PaNIMIN-like and PaHDAC-like proteins delay the expression of SAR-related genes. The inverse relationship observed between the regulation of *PaNIMINs* and *PaHDACs* and that of *PR-1s and PR-2s* further supports these assumptions.

Interestingly, while *PaRHA1B-like* was identified as the ortholog of *AtNIMIN-3*, it is more similar to and likely to encode for an E3 ubiquitin-protein ligase. To date, NPR1 has not been shown to interact directly with either CUL3 or E3 ubiquitin-protein ligases (DIETERLE *et al.* 2005; SPOEL *et al.* 2009; FU *et al.* 2012). Therefore, as NIMIN-like proteins are known to interact with NPR1, it could be worthwhile to determine whether PaRHA1B-like functions as an E3 ubiquitin-protein ligase, and whether it can directly associate with PaNPR1/2.

Besides the influence of negative regulators, further post-translational modification of PaNPR1/2 may still be required to modify SA-dependent gene expression. For example, in *A. thaliana,* CIPK11 has been shown to interact with NPR1 and phosphorylate the C-terminal region (XIE *et al.* 2010). In so doing, CIPK11 positively regulates the expression of *AtWRKY38* and *AtWRKY62*, two negative regulators of the SA-signalling pathway (KIM *et al.* 2008; XIE *et al.* 2010). How exactly the phosphorylation of the NPR1 C-terminal domain leads to increased *WRKY* expression remains to be determined. Nonetheless, the *AtCIPK11* ortholog, *PaCIPK10-like1*, is significantly upregulated at 120hpi, corresponding with the upregulation of several *WRKY-like* genes. Given the data, we surmise that PaCIPK10-like1 leads to upregulation of some *PaWRKY-like* gene expression through phosphorylation of PaNPR1/2. In so doing, *PaCIPK10-like1* indirectly limits SA-signalling, preventing overstimulation of respective defence responses.

Interestingly, the majority of WRKY transcription factors are linked to pathogen responses and SA-signalling (DONG *et al.* 2003; ULKER AND SOMSSICH 2004; PANDEY AND SOMSSICH 2009). However, their collective role is complex, including both negative and positive regulators of the SA-signalling pathway (MALECK *et al.* 2000; WANG *et al.* 2006; ZHENG *et al.* 2006; KIM *et al.*

2008; LAI *et al.* 2008). Even so, negative regulators of SA-signalling are essential for the complete induction of SAR. To that end, SPOEL *et al.* (2009) demonstrated that both WRKY38 and WRKY62 are crucial to the induction of SAR in *A. thaliana* in an NPR1-dependent fashion. Unsurprisingly, the majority of *WRKY-like* orthologs in *P. americana* were significantly upregulated at 120hpi in R0.12. Additionally, the expression of *PaICS1-like2* is downregulated significantly at 120hpi. Together these observations likely indicate that the SA-signalling pathway is being suppressed to some extent by *PaWRKYs* at 120hpi in R0.12. Furthermore, although most *PaWRKYs* are not downregulated significantly at 12hpi, most tend toward decreased expression, likely to prevent suppression of SA-signalling events early on.

Also, three *AtTGA-like* orthologs, *PaTGA2-like1*, *PaTGA4-like1* and *PaTGA4-like3*, are significantly upregulated soon after *P. cinnamomi* inoculation. We can thus safely assume that these three PaTGAs are involved in the expression of SA-induced genes in *P. americana*. However, the upregulation of *PaTGAs* does not coincide with increased *PR* gene expression, and this is likely due to the negative regulators discussed earlier. Interestingly, *PaTGA10-like1* is significantly upregulated a 120hpi. Recently, TGA10 in *A. thaliana* was implicated in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in response to the bacterial PAMP, flg22 (NOSHI *et al.* 2016). However, responses to PAMPs such as flg22 are considered early signalling events and are associated with the oxidative burst seen during the initial stages of infection (FELIX *et al.* 1999).

Thus, it is likely that the upregulation of *PaTGA10-like1* constitutes a primed defence response (CONRATH *et al.* 2002; PRIME *et al.* 2006). We also observed significant upregulation of two *A. thaliana MPK3/MPK6* orthologs, *PaMPK3* and *PaNTF6*, at 120hpi. This upregulation likely corresponds to an increased accumulation of inactive *PaMAPKs*, constituting yet another primed defence response. Together these observations indicate that NPR1-dependent priming occurs in *P. americana*, another hallmark of successful SAR induction.

Finally, we identified two *AtHSFB1* orthologs in *P. americana*, *PaHSF24-like1* and *PaHSF24-like2*. Notably, this transcription factor is instrumental in the transition from growth to defence (PAJEROWSKA-MUKHTAR *et al.* 2012). Interestingly, *PaHSF24-like1* expression is highest in the uninoculated control while *PaHSF24-like2* is significantly upregulated by 120hpi in R0.12. Furthermore, *PaHSF24-like2* displays an expression pattern similar to that of *PaNPR1* and *PaNPR2*. Similarly, the expression of *AtNPR1* and *AtHSFB1* were shown to be co-

dependent (PAJEROWSKA-MUKHTAR *et al.* 2012). Together these observations suggest that *PaHSF24-like2* might be the functional ortholog of *AtHSFB1*. However, the expression of secretory pathway genes, *PaBiP4*, *PaCRT3-like1* and *PaCRT3-like2,* was downregulated significantly at 120hpi. These observations contradict with those made by PAJEROWSKA-MUKHTAR *et al.* (2012). Interestingly, upregulation of the endoplasmic reticulum (ER)-associated protein-coding genes, *PaSec61α-like1* and *PaSec61α-like2*, corresponds to the downregulation of *PaHSF24-like1*. Alternatively, upregulation of ER-associated genes may occur at earlier time-points than those included in this study. Notably, *AtHSFB1, AtCRT3* and *AtBiP2* are upregulated within four hours and return to baseline levels within eight hours of SA treatment (PAJEROWSKA-MUKHTAR *et al.* 2012). Nonetheless, both *PaHSF24-like* and related secretory pathway genes are regulated in response to *P. cinnamomi* inoculation. Thus, further characterisation of their roles in defence against *P. cinnamomi* could prove invaluable.

Differences and similarities between susceptible and partially resistant interactions

Both Dusa® and R0.12 rootstocks show significantly similar upregulation of several of *PR-1-like* and *PaPR-2-like* genes, indicating that SAR is established in both rootstocks. However, some differences are apparent. Two *AtPR-1-like* genes, *PaLRK10L-2.4* and *PaPRB1-3-like3*, remain unchanged in Dusa®, whereas in R0.12 they are significantly upregulated at 120hpi. Moreover, the expression of *PaPRB1-3-like3* in Dusa® is nearly non-existent. Why this discrepancy exists is unclear and warrants further investigation. Furthermore, although *PaPR-2-like1* is significantly upregulated in both rootstocks, expression at 120hpi in Dusa® is markedly higher than in R0.12. Similarly, the expression of *PaPR-2-like2* is approximately 30% higher in Dusa® than in R0.12, although the difference is not statistically significant. Even though most *PaPR-5-like* genes display similar regulation in response to *P. cinnamomi*, the abundance of two *PaPR-5-like* genes is significantly higher in Dusa® before pathogen challenge. Also, the expression of two additional *PaPR-5-like* genes is elevated considerably in Dusa® and not R0.12 following pathogen challenge. Collectively, these data suggest that, at the very least, differences in *PaPR-like* gene expression might contribute to the success of Dusa®.

Arguably, the most apparent difference between Dusa® and R0.12 relates to the expression of defence-related *PaNPR1-like* genes. Following pathogen challenge in R0.12, we see significant upregulation of both *PaNPR1* and *PaNPR2* while the expression of *PaNPR4* remains

relatively unchanged. In contrast, *PaNPR1* and *PaNPR2* abundance remains relatively constant in Dusa®, while *PaNPR4* is significantly downregulated. Therefore, Dusa® likely has far less NPR1-dependent negative regulation of JA/ET defence responses at 120hpi. Meanwhile, decreased expression of *PaNPR4* could account for the higher levels of *PaPR-2-like1* and *PaPR-2-like2* in Dusa®, and possibly other SAR-related genes. Interestingly, the development-related genes, *PaNPR3* and *PaNPR5*, are similarly downregulated at 120hpi in both Dusa® and R0.12. The orthologs of *PaNPR3* and *PaNPR5* in *A. thaliana*, *BOP1* and *BOP2*, are overwhelmingly associated with the growth and development of lateral organs (HA *et al.* 2004; HEPWORTH *et al.* 2005; NORBERG *et al.* 2005; HA *et al.* 2007; MCKIM *et al.* 2008). Together, these observations suggest the prioritisation of defence over growth, following *P. cinnamomi* challenge.

Furthermore, *PaTRXs* and *PaADHs* show both conserved as well as significant differences in their regulation following *P. cinnamomi* challenge. Both *PaTX-like1* and *PaADH3* are significantly downregulated at 120hpi, in Dusa® and R0.12. However, *PaTRX2-like1* and *PaTRX2-like2* are substantially more abundant in uninoculated Dusa® plantlets, while *PaTRX4-like1* also tends to be higher. Similarly, *PaADH-like* is significantly more abundant in uninoculated Dusa®. Therefore, similarities between Dusa® and R0.12 indicate that both rootstocks have a similar potential for NPR1 monomerisation following inoculation with *P. cinnamomi*. However, significantly higher levels of *PaTRXs* and *PaADH-like* in Dusa® could indicate that PaNPR1/2 is more prone to exist as a monomer in Dusa®, or that monomerisation could occur more rapidly following pathogen challenge. Furthermore, although downregulation of *PaTRXs* and *PaADHs* is similar in both rootstocks, a holistic view indicates that downregulation of these genes might be more pronounced in Dusa®. These observations further support the hypothesis that Dusa® might switch to JA/ET-related defence responses more efficiently than R0.12 in response to the hemibiotrophic pathogen.

The protein kinase encoding genes are another example of significant differences between Dusa® and R0.12. In particular, *PaCIPK10-like1* and *PaCIPK10-like2*, the abundance of both was significantly lower in the uninoculated control of Dusa®. In response, negative regulators of SA-signalling such as WRKY38 and WRKY62 would likely be less abundant early on. Otherwise, expression was comparable, with *PaCIPK10-like1* and *PaCIPK10-like2* being up- and downregulated in both rootstocks following inoculation, respectively. Surprisingly,

PaSAPK2-like1 and *PaSAPK-like2* were not significantly different between the two rootstocks. This observation is thus indicating regulatory conservation between Dusa® and R0.12, underscoring the significance of these protein kinases.

Furthermore, three *AtSUMO3-like* orthologs were significantly downregulated in Dusa®, but not R0.12, in response to *P. cinnamomi*. Through the sumoylation of AtNPR1, AtSUMO3 ultimately leads to increased expression of SAR related genes (SALEH *et al.* 2015). Overall, sumoylation of NPR1 leads to decreased association with WRKYs, increased NPR1 turnover, as well as increased association with TGAs (SALEH *et al.* 2015). Therefore, a higher abundance of *SUMO3-like* transcripts early in SA-signalling would probably be required. By contrast, lower abundance would likely limit activation and turnover of NPR1; thus, reducing SA-signalling at later time-points. Therefore, we can reasonably assume that significant downregulation of *PaSUMO-like* genes in Dusa® at 120hpi was inversely associated with NPR1 activity and SAR-related gene expression. Furthermore, *PaCUL3A-like1* was substantially downregulated in Dusa®, but not R0.12 while *PaCUL3A-like2* was significantly lower in Dusa®, compared to R0.12, following *P. cinnamomi* inoculation. Thus, differences are observed between most genes putatively involved in the post-translational modification of PaNPR1/2. Moreover, these observations suggest that post-translational modification may contribute substantially to the increased resistance of Dusa® during *P. cinnamomi* challenge.

Negative regulators of SA-signalling, *PaHDAC9*, *PaNIMIN-1* and *PaNIMIN-2* are also regulated differently in susceptible and partially resistant rootstocks. Whereas *PaHDAC6* and *PaRHA1B-like* are controlled similarly in both rootstocks. Interestingly, while *PaNIMIN-1* is significantly downregulated in Dusa®, *PaNIMIN-2* is downregulated in R0.12. Given that in *A. thaliana,* NIMINs regulate NPR1 differently, it will be interesting to determine which roles different *PaNIMINs* have and how that affects susceptibility or resistance. Nonetheless, we speculate that higher *PaHDAC9* and *PaNIMIN-1* in Dusa® prevents untimely expression of SAR-related genes, in spite of higher uninduced abundance and monomerisation of PaNPR1/2. Therefore, it seems that components of the NPR1 pathway in Dusa® might exist in a primed state, relative to R0.12. In turn, this might lead to earlier and stronger NPR1-dependent defence responses in Dusa® following pathogenic threat.

Generally, in both R0.12 and Dusa® significant upregulation of *PaWRKYs* was observed at 120hpi. However, there were apparent differences, with particular *PaWRKYs* being

upregulated in one rootstock but not the other. Furthermore, the overall trend indicated a stronger global induction of *PaWRKYs* in Dusa®. Therefore, it seems that the SA-signalling pathway is more definitively suppressed at 120hpi in Dusa®, given the general negative regulatory role WRKYs have on SA-signalling.

By contrast, the abundance of *PaTGA-like* transcripts in Dusa® was generally lower at 120hpi than in R0.12. Interestingly, four *PaTGA2-like* genes were significantly downregulated in Dusa®, while expression remained relatively constant in R0.12. Whereas in both rootstocks, *PaTGA4-like* genes were neither up- or downregulated; however, Dusa® had significantly lower transcript abundance overall. These observations further support the idea that the SA-signalling pathway is more efficiently suppressed in Dusa® during the late stages of *P. cinnamomi* infection.

Furthermore, the upregulation of *PaTGA10-like1* was significantly less pronounced in Dusa® than R0.12. Also, *PaNTF6* was not upregulated significantly in Dusa®, contrasting with *PaNTF6* expression in R0.12. Nonetheless, *PaMPK3* was upregulated similarly in both Dusa® and R0.12. These observations are surprising given the putative role of *PaTGA10-like1* and *PaNTF6* in priming defence responses and the initiation of SAR. However, there could be several reasons for these observations. Firstly, it is possible that *PaTGA10* and *PaNTF6* do not have a significant role in the priming of defence responses. Secondly and most likely, upregulation of these genes occurred at an earlier time-point in Dusa®. Lastly, priming may be overstimulated in R0.12 given the late upregulation of *PaNPR1/2*. Thus, further investigations are required to address these issues.

Overall, the regulation of most putative protein secretory pathway genes was similar in both Dusa® and R0.12. However, two notable exceptions were the uninduced expression of *PaHSF24-like1* and *PaCRT3-like1*. The transcripts from both genes were significantly more abundant in R0.12 than Dusa®. However, following inoculation with *P. cinnamomi* the expression in both rootstocks were markedly similar. Thus, another clear difference between Dusa® and R0.12 exists within the putative protein secretory pathway. Nonetheless, as noted before, the differential expression of these genes likely occurs during early SA-signalling events, underscoring the importance of further investigation.

Conclusion

This study represents the first comprehensive investigation into the role of the NPR1 pathway during *P. cinnamomi* challenge in *P. americana*. We demonstrated the establishment of SAR in response to *P. cinnamomi* challenge. We further described the most likely mechanisms employed to achieve SAR, with a focus on NPR1 and associated pathways. Significant differences in the regulation of putative pathway genes were observed when comparing susceptible and partially resistant *P. americana-P. cinnamomi* interactions. Overall, the evidence presented here suggests that the susceptible rootstock, R0.12, may be less efficient at downregulating SA-dependent defence responses following the successful initiation of SAR. Thus, R0.12 would exhibit a less effective JA/ET response during the necrotrophic phase of *P. cinnamomi* infection. By contrast, Dusa® seems to not only initiate SAR to a greater degree but more effectively suppresses components of the SA-signalling pathway by 120hpi; this may be a crucial aspect of effective defence against the necrotrophic phase of *P. cinnamomi* infection. While this study represents a substantial gain in understanding the role of the extended NPR1 pathway in defence against *P. cinnamomi*, it also highlights some important questions. To that effect, it would be worth investigating, to a similar degree as presented here, the regulation of the JA/ET pathway during infection. Additionally, while the evidence presented here might highlight some critical aspects of NPR1-dependent defence responses in avocado, further molecular investigations would be needed to prove any conclusively.

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Supplementary Tables

Supplementary Table S1. Candidate NPR1 pathway-associated genes in *P. americana*. Protein family classification, as well as putative functional descriptions, are the result of a combined annotation approach.

A. thaliana ortholog	*P. americana* ID	Gene name in *P. americana*	Protein family	Putative functional description
BiP2	g13474.t1	*PaBiP5*	Heat shock protein 70 family	luminal-binding protein 5 [*Persea americana* var. *drymifolia*]
BiP2	g16678.t1	*PaBiP4*	Heat shock protein 70 family	luminal-binding protein 4 [*Persea americana* var. *drymifolia*]
CRT3	g39531.t1	*PaCRT3-like1*	Calreticulin-3-like	Calreticulin-3 [*Persea americana* var. *drymifolia*]
CRT3	g7570.t1	*PaCRT3-like2*	Calreticulin-3-like	Calreticulin-3 [*Persea americana* var. *drymifolia*]
CUL3A	g3606.t1	*PaCUL3A-like1*	Cullin family	cullin-3A-like protein [*Persea americana* var. *drymifolia*]
CUL3A	g35218.t1	*PaCUL3A-like2*	Cullin family	cullin-3A-like protein [*Persea americana* var. *drymifolia*]
DAD1	g4709.t1	*PaDAD1-like1*	Phospholipase A(1) DAD1	phospholipase A1 DAD1, chloroplastic [*Persea americana* var. *drymifolia*]
DAD1	g10107.t1	*PaDAD1-like2*	Phospholipase A(1) DAD1	phospholipase A1 DAD1, chloroplastic [*Persea americana* var. *drymifolia*]
HDAC19	g23038.t2	*PaPM1*	Peptidase family M1 domain	Peptidase M1, alanine aminopeptidase/leukotriene A4 hydrolase [*Persea americana* var. *drymifolia*]
HDAC19	g30203.t1	*PaHDAC9*	Histone deacetylase family	histone deacetylase 9 [*Persea americana* var. *drymifolia*]
HDAC19	g30067.t1	*PaHDAC6*	Histone deacetylase family	histone deacetylase 6 [*Persea americana* var. *drymifolia*]

GSNOR1	g13531.t1	*PaADH3*	Alcohol dehydrogenase family	alcohol dehydrogenase 3 [*Persea americana* var. *drymifolia*]
GSNOR1	g13969.t1	*PaADH2*	Alcohol dehydrogenase family	alcohol dehydrogenase 2 [*Persea americana* var. *drymifolia*]
GSNOR1	g13967.t1	*PaADH*	Alcohol dehydrogenase family	alcohol dehydrogenase [*Persea americana* var. *drymifolia*]
GSNOR1	g14178.t1	*PaADH-like*	Alcohol dehydrogenase family	alcohol dehydrogenase-like protein [*Persea americana* var. *drymifolia*]
HSFB1	g24705.t1	*PaHSF24-like1*	Heat shock factor	heat shock factor protein HSF24 [*Persea americana* var. *drymifolia*]
HSFB1	g30822.t1	*PaHSF24-like2*	heat shock factor	heat shock factor protein HSF24-like protein [*Persea americana* var. *drymifolia*]
ICS1	g14724.t1	*PaICS-like1*	Isochorismate synthase	isochorismate synthase, chloroplastic-like protein, isoform X1 [*Persea americana* var. *drymifolia*]
ICS1	g23147.t1	*PaICS-like2*	Isochorismate synthase	isochorismate synthase, chloroplastic-like protein [*Persea americana* var. *drymifolia*]
LHY	g35261.t1	*PaLHY*	Protein LHY-like isoform X1	protein LHY [*Persea americana* var. *drymifolia*]
MPK3	g17292.t1	*PaMPK3*	Mitogen-activated protein kinase	mitogen-activated protein kinase 3-like [*Persea americana* var. *drymifolia*]
MPK3	g42257.t1	*PaNTF6*	Mitogen-activated protein kinase	mitogen-activated protein kinase NTF6-like protein [*Persea americana* var. *drymifolia*]
MPK3	g8979.t1	*PaMMK1*	Mitogen-activated protein kinase	mitogen-activated protein kinase MMK1 [*Persea americana* var. *drymifolia*]
NIMIN-1	g34229.t1	*PaNIMIN-1*	NIMIN family	protein NIM1-INTERACTING 1-like [*Persea americana* var. *drymifolia*]
NIMIN-2	g36175.t1	*PaNIMIN-2*	NIMIN family	protein NEGATIVE REGULATOR OF RESISTANCE-like protein [*Persea americana* var. *drymifolia*]
NIMIN-3	g35159.t1	*PaRHA1B-like*	E3 ubiquitin-protein ligase	E3 ubiquitin-protein ligase RHA1B-like protein [*Persea americana* var. *drymifolia*]

NPR1	g18227.t1	*PaNPR1*	NPR1-like family	nonexpressor of pathogenesis-related protein 1-like 1 protein [*Persea americana* var. *drymifolia*]
NPR1	g33565.t1	*PaNPR2*	NPR1-like family	nonexpressor of pathogenesis-related protein 1-like 2 protein [*Persea americana* var. *drymifolia*]
BOP2	g12308.t1	*PaNPR3*	NPR1-like family	nonexpressor of pathogenesis-related protein 1-like 3 protein [*Persea americana* var. *drymifolia*]
NPR3	g11613.t1	*PaNPR4*	NPR1-like family	nonexpressor of pathogenesis-related protein 1-like 4 protein [*Persea americana* var. *drymifolia*]
BOP2	g6547.t1	*PaNPR5*	NPR1-like family	nonexpressor of pathogenesis-related protein 1-like 5 protein [*Persea americana* var. *drymifolia*]
BGL2	g7475.t1	*PaPR-2-like1*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Persea americana* var. *drymifolia*]
BGL2	g28165.t1	*PaPR-2-like2*	Glycosyl hydrolase 17 family	beta-1,3 glucanase 3 [*Persea americana* var. *drymifolia*]
BGL2	g28166.t1	*PaPR-2-like3*	Glycosyl hydrolase 17 family	beta-1,3 glucanase 3 [*Persea americana* var. *drymifolia*]
BGL2	g7465.t1	*PaPR-2-like4*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Persea americana* var. *drymifolia*]
BGL2	g7469.t1	*PaPR-2-like5*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Persea americana* var. *drymifolia*]
BGL2	g7453.t1	*PaPR-2-like6*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase-like protein [*Persea americana* var. *drymifolia*]
BGL2	g7447.t1	*PaPR-2-like7*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase-like protein [*Persea americana* var. *drymifolia*]
BGL2	g7457.t1	*PaPR-2-like8*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase-like protein [*Persea americana* var. *drymifolia*]
BGL2	g4098.t1	*PaPR-2-like9*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase-like protein [*Persea americana* var. *drymifolia*]
BGL2	g7459.t1	*PaPR-2-like10*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase-like protein [*Persea americana* var. *drymifolia*]

BGL2	g28164.t1	*PaPR-2-like11*	Glycosyl hydrolase 17 family	putative glucan endo-1,3-beta-glucosidase GVI [*Persea americana* var. *drymifolia*]
BGL2	g7473.t1	*PaPR-2-like12*	Glycosyl hydrolase 17 family	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Persea americana* var. *drymifolia*]
BGL2	g13931.t1	*PaPR-2-like13*	Glycosyl hydrolase 17 family	Glucan endo-1,3-beta-glucosidase GII [*Persea americana* var. *drymifolia*]
PR1	g15715.t1	*PaPRB1-3-like1*	Cysteine rich secretory protein family	pathogenesis-related protein PRB1-3-like protein [*Persea americana* var. *drymifolia*]
PR1	g2748.t1	*PaPRB1-3-like2*	Cysteine rich secretory protein family	pathogenesis-related protein PRB1-3-like protein [*Persea americana* var. *drymifolia*]
PR1	g42156.t1	*PaLRK10L-2.4*	Cysteine rich secretory protein family	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like protein 2.4 [*Persea americana* var. *drymifolia*]
PR1	g15714.t1	*PaPRB1-3-like3*	Cysteine rich secretory protein family	pathogenesis-related protein PRB1-3-like protein [*Persea americana* var. *drymifolia*]
PR1	g30797.t1	*PaPR-1-like1*	Cysteine rich secretory protein family	Basic form of pathogenesis-related protein 1 [*Persea americana* var. *drymifolia*]
PR1	g17436.t1	*PaPR-1-like2*	Cysteine rich secretory protein family	pathogenesis-related protein PR-1 [*Persea americana* var. *drymifolia*]
PR1	g17433.t1	*PaPR-1-like3*	Cysteine rich secretory protein family	pathogenesis-related protein PR-1 [*Persea americana* var. *drymifolia*]
PR1	g17427.t1	*PaPR-1-like4*	Cysteine rich secretory protein family	pathogenesis-related protein PR-1 [*Persea americana* var. *drymifolia*]
PR5	g42875.t1	*PaPR-5-like1*	Thaumatin-like protein	pathogenesis-related protein 5-like protein [*Persea americana* var. *drymifolia*]
PR5	g37626.t1	*PaPR-5b-like1*	Thaumatin-like protein	thaumatin-like protein 1b [*Persea americana* var. *drymifolia*]
PR5	g41629.t1	*PaPR-5-like2*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g14064.t1	*PaPR-5b-like2*	Thaumatin-like protein	thaumatin-like protein 1b [*Persea americana* var. *drymifolia*]

PR5	g41635.t1	*PaPR-5b-like3*	Thaumatin-like protein	thaumatin-like protein 1b [*Persea americana* var. *drymifolia*]
PR5	g3865.t2	*PaPR-5-like3*	Thaumatin-like protein	thaumatin-like protein, isoform X2 [*Persea americana* var. *drymifolia*]
PR5	g39310.t1	*PaPR-5-like4*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g39320.t1	*PaPR-5-like5*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g39311.t1	*PaPR-5-like6*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g39315.t1	*PaPR-5-like7*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g33886.t1	*PaPR-5b-like4*	Thaumatin-like protein	thaumatin-like protein 1b, isoform X1[*Persea americana* var. *drymifolia*]
PR5	g39316.t1	*PaPR-5-like9*	Thaumatin-like protein	thaumatin-like protein 1 [*Persea americana* var. *drymifolia*]
PR5	g33373.t1	*PaPR-5-like10*	Thaumatin-like protein	pathogenesis-related protein 5 [*Persea americana* var. *drymifolia*]
SARD1	g36233.t1	*PaSARD1*	Calmodulin binding protein	Calmodulin binding protein-like protein [*Persea americana* var. *drymifolia*]
Sec61α	g34928.t1	*PaSec61α-like1*	SecY SEC61-alpha family	protein transport protein Sec61 subunit alpha-like protein [*Persea americana* var. *drymifolia*]
Sec61α	g22791.t1	*PaSec61α-like2*	SecY SEC61-alpha family	SecY/SEC61-alpha family [*Persea americana* var. *drymifolia*]
CIPK11	g1762.t1	*PaCIPK10-like1*	Non-specific serine threonine protein kinase	CBL-interacting protein kinase 10 [*Persea americana* var. *drymifolia*]
CIPK11	g26871.t1	*PaCIPK10-like2*	Non-specific serine threonine protein kinase	CBL-interacting protein kinase 10 [*Persea americana* var. *drymifolia*]
SRK2C	g32276.t1	*PaSAPK2-like1*	Protein kinase superfamily	serine/threonine-protein kinase SAPK2 [*Persea americana* var. *drymifolia*]

SRK2C	g35660.t1	*PaSAPK2-like2*	Protein kinase superfamily	serine/threonine-protein kinase SAPK2 [*Persea americana* var. *drymifolia*]
SUMO3	g39815.t1	*PaSUMO1-like1*	Small ubiquitin-related modifier family	small ubiquitin-related modifier 1-like protein [*Persea americana* var. *drymifolia*]
SUMO3	g42777.t1	*PaSUMO1-like2*	Small ubiquitin-related modifier family	small ubiquitin-related modifier 1-like protein [*Persea americana* var. *drymifolia*]
SUMO3	g24813.t1	*PaSUMO2-like1*	Small ubiquitin-related modifier family	small ubiquitin-related modifier 2-like protein [*Persea americana* var. *drymifolia*]
SUMO3	g8421.t1	*PaSUMO2-like2*	Small ubiquitin-related modifier family	small ubiquitin-related modifier 2-like protein [*Persea americana* var. *drymifolia*]
TGA6	g5063.t1	*PaTGA2-like1*	TGA transcription factor family	transcription factor TGA2.2-like protein [*Persea americana* var. *drymifolia*]
TGA6	g3842.t1	*PaTGA2-like2*	TGA transcription factor family	transcription factor TGA2.2-like protein [*Persea americana* var. *drymifolia*]
TGA6	g28280.t2	*PaTGA2-like3*	TGA transcription factor family	transcription factor TGA2.2-like protein, isoform X2 [*Persea americana* var. *drymifolia*]
TGA6	g5420.t1	*PaHBP-1b(C38)-like1*	TGA transcription factor family	transcription factor HBP-1bc38-like protein [*Persea americana* var. *drymifolia*]
TGA6	g3464.t1	*PaTGA2-like5*	TGA transcription factor family	transcription factor TGA2 [*Persea americana* var. *drymifolia*]
TGA6	g32178.t1	*PaTGA4-like1*	TGA transcription factor family	transcription factor TGA4, isoform X1 [*Persea americana* var. *drymifolia*]
TGA6	g15923.t2	*PaTGA10-like1*	TGA transcription factor family	bZIP transcription factor TGA10-like protein, isoform X2 [*Persea americana* var. *drymifolia*]
TGA6	g42296.t1	*PaHBP-1b(C38)-like2*	TGA transcription factor family	transcription factor HBP-1bc38-like protein [*Persea americana* var. *drymifolia*]
TGA6	g19423.t1	*PaHBP-1b(C38)-like3*	TGA transcription factor family	transcription factor HBP-1bc38-like protein [*Persea americana* var. *drymifolia*]
TGA6	g2433.t1	*PaTGA4-like3*	TGA transcription factor family	transcription factor TGA4 [*Persea americana* var. *drymifolia*]

TOC1	g36293.t1	*PaTOC1*	Two-component response regulator-like	two-component response regulator-like PRR1 [*Persea americana* var. *drymifolia*]
TRX3	g32937.t1	*PaTRX1-like1*	Thioredoxin family	thioredoxin H-type 1 [*Persea americana* var. *drymifolia*]
TRX3	g15858.t1	*PaTRX-like1*	Thioredoxin family	thioredoxin H-type-like protein [*Persea americana* var. *drymifolia*]
TRX5	g32953.t1	*PaTRX2-like1*	Thioredoxin family	thioredoxin H2-like protein [*Persea americana* var. *drymifolia*]
TRX5	g16081.t1	*PaTRX2-like2*	Thioredoxin family	thioredoxin H2-like protein [*Persea americana* var. *drymifolia*]
TRX5	g41279.t1	*PaTRX4-like1*	Thioredoxin family	thioredoxin H4-1 [*Persea americana* var. *drymifolia*]
TRX5	g21444.t1	*PaTRX4-like2*	Thioredoxin family	thioredoxin H4-1-like protein [*Persea americana* var. *drymifolia*]
TRX5	g28661.t2	*PaTDX-like1*	Thioredoxin family	TPR repeat-containing thioredoxin TDX, isoform X2 [*Persea americana* var. *drymifolia*]
WRKY29	g14581.t1	*PaWRKY22*	WRKY transcription factor family	WRKY transcription factor 22 [*Persea americana* var. *drymifolia*]
WRKY29	g13325.t1	*PaWRKY14*	WRKY transcription factor family	putative WRKY transcription factor 14 [*Persea americana* var. *drymifolia*]
WRKY40	g20708.t1	*PaWRKY40-like1*	WRKY transcription factor family	putative WRKY transcription factor 40 [*Persea americana* var. *drymifolia*]
WRKY40	g30301.t1	*PaWRKY76-like*	WRKY transcription factor family	WRKY transcription factor WRKY76-like protein [*Persea americana* var. *drymifolia*]
WRKY6	g27980.t1	*PaWRKY31-like1*	WRKY transcription factor family	putative WRKY transcription factor 31 [*Persea americana* var. *drymifolia*]
WRKY6	g27775.t1	*PaWRKY31-like2*	WRKY transcription factor family	putative WRKY transcription factor 31 [*Persea americana* var. *drymifolia*]
WRKY6	g14197.t1	*PaWRKY31-like3*	WRKY transcription factor family	putative WRKY transcription factor 31 [*Persea americana* var. *drymifolia*]

WRKY6	g126.t1	*PaWRKY47-like1*	WRKY transcription factor family	putative WRKY transcription factor 47 [*Persea americana* var. *drymifolia*]
WRKY6	g29615.t1	*PaWRKY47-like2*	WRKY transcription factor family	putative WRKY transcription factor 47 [*Persea americana* var. *drymifolia*]
WRKY6	g13934.t1	*PaWRKY9-like1*	WRKY transcription factor family	putative WRKY transcription factor 9 [*Persea americana* var. *drymifolia*]
WRKY6	g1993.t1	*PaWRKY72*	WRKY transcription factor family	putative WRKY transcription factor 72 [*Persea americana* var. *drymifolia*]
WRKY6	g2243.t1	*PaWRKY9-like2*	WRKY transcription factor family	putative WRKY transcription factor 9 [*Persea americana* var. *drymifolia*]
WRKY60	g21100.t1	*PaWRKY40-like2*	WRKY transcription factor family	putative WRKY transcription factor 40 [*Persea americana* var. *drymifolia*]
WRKY60	g9565.t1	*PaWRKY40-like4*	WRKY transcription factor family	putative WRKY transcription factor 40 [*Persea americana* var. *drymifolia*]
WRKY60	g20711.t1	*PaWRKY40-like5*	WRKY transcription factor family	putative WRKY transcription factor 40 [*Persea americana* var. *drymifolia*]
WRKY62	g14855.t1	*PaWRKY70-like1*	WRKY transcription factor family	putative WRKY transcription factor 70 [*Persea americana* var. *drymifolia*]
WRKY62	g41259.t1	*PaWRKY70-like2*	WRKY transcription factor family	putative WRKY transcription factor 70 [*Persea americana* var. *drymifolia*]
WRKY62	g41260.t1	*PaWRKY55*	WRKY transcription factor family	WRKY transcription factor 55 [*Persea americana* var. *drymifolia*]
WRKY70	g9416.t1	*PaWRKY41*	WRKY transcription factor family	putative WRKY transcription factor 41 [*Persea americana* var. *drymifolia*]
WRKY70	g37185.t1	*PaWRKY-like1*	WRKY transcription factor family	DNA-binding WRKY [*Persea americana* var. *drymifolia*]
WRKY70	g25443.t1	*PaWRKY-like2*	WRKY transcription factor family	DNA-binding WRKY [*Persea americana* var. *drymifolia*]
WRKY70	g37189.t1	*PaWRKY-like3*	WRKY transcription factor family	DNA-binding WRKY [*Persea americana* var. *drymifolia*]

Supplementary Table S2. Online BLASTp results of 116 putative *P. americana* NPR1 pathway-associated proteins.

P. americana ID	NCBI Top Hit Description	Max Score	Total Score	Query Cover.	E-value	Per. Ident	NCBI Top Hit Accession
g13474.t1	luminal-binding protein 5 [*Cinnamomum micranthum* f. *kanehirae*]	1335	1335	99%	0	99%	RWR92067.1
g16678.t1	luminal-binding protein 4 [*Cinnamomum micranthum* f. *kanehirae*]	1325	1325	99%	0	98%	RWR72155.1
g39531.t1	Calreticulin-3 [*Cinnamomum micranthum* f. *kanehirae*]	714	714	92%	0	91%	RWR83886.1
g7570.t1	Calreticulin-3 [*Cinnamomum micranthum* f. *kanehirae*]	670	670	96%	0	89%	RWR83886.1
g3606.t1	cullin-3A-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	1498	1498	99%	0	98%	RWR78397.1
g35218.t1	cullin-3A-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	1518	1518	99%	0	99%	RWR89492.1
g4709.t1	phospholipase A1 DAD1, chloroplastic [*Cinnamomum micranthum* f. *kanehirae*]	836	836	99%	0	97%	RWR86719.1
g10107.t1	phospholipase A1 DAD1, chloroplastic [*Cinnamomum micranthum* f. *kanehirae*]	811	811	99%	0	94%	RWR86719.1
g23038.t2	Peptidase M1, alanine aminopeptidase/leukotriene A4 hydrolase [*Cinnamomum micranthum* f. *kanehirae*]	1696	1696	63%	0	93%	RWR72508.1
g30203.t1	histone deacetylase 9 [*Elaeis guineensis*]	841	841	99%	0	93%	XP_010940301.1
g30067.t1	PREDICTED: histone deacetylase 6 isoform X1 [*Nelumbo nucifera*]	590	590	54%	0	88%	XP_010248619.1
g13531.t1	alcohol dehydrogenase 3 [*Persea americana*]	777	777	99%	0	100%	ALR87070.1
g13969.t1	alcohol dehydrogenase 2 [*Persea americana*]	773	773	86%	0	99%	ALR87069.1

g13967.t1	alcohol dehydrogenase [*Cinnamomum micranthum* f. *kanehirae*]	774	774	99%	0	98%	RWR92819.1
g14178.t1	alcohol dehydrogenase-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	770	770	99%	0	95%	RWR96108.1
g24705.t1	heat shock factor protein HSF24 [*Cinnamomum micranthum* f. *kanehirae*]	516	516	99%	0	95%	RWR90087.1
g30822.t1	heat shock factor protein HSF24-like protein [*Cinnamomum micranthum* f. *kanehirae*]	530	530	99%	0	93%	RWR92561.1
g14724.t1	isochorismate synthase, chloroplastic-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	1144	1144	99%	0	96%	RWR80421.1
g23147.t1	isochorismate synthase, chloroplastic-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	1139	1139	99%	0	94%	RWR93149.1
g35261.t1	protein LHY isoform X3 [*Cinnamomum micranthum* f. *kanehirae*]	1343	1343	99%	0	93%	RWR89528.1
g17292.t1	PREDICTED: mitogen-activated protein kinase 3-like [*Nelumbo nucifera*]	669	669	99%	0	81%	XP_010253496.1
g42257.t1	mitogen-activated protein kinase NTF6-like protein [*Cinnamomum micranthum* f. *kanehirae*]	752	752	99%	0	97%	RWR95535.1
g8979.t1	mitogen-activated protein kinase MMK1 isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	726	726	99%	0	94%	RWR89123.1
g34229.t1	protein NIM1-INTERACTING 1-like [*Durio zibethinus*]	56,2	56,2	54%	2E-07	40%	XP_022769782.1
g36175.t1	protein NEGATIVE REGULATOR OF RESISTANCE-like protein [*Cinnamomum micranthum* f. *kanehirae*]	234	234	99%	2E-77	94%	RWR93885.1
g35159.t1	E3 ubiquitin-protein ligase RHA1B-like protein [*Cinnamomum micranthum* f. *kanehirae*]	317	469	91%	6E-107	98%	RWR89445.1
g18227.t1	nonexpressor of pathogenesis-related protein 1-like 1 protein [*Persea americana* var. *drymifolia*]	1143	1143	99%	0	95%	AKH61407.1
g33565.t1	nonexpressor of pathogenesis-related protein 1-like 2 protein [*Persea americana* var. *drymifolia*]	1205	1205	99%	0	100%	AKH61408.1

g12308.t1	nonexpressor of pathogenesis-related protein 1-like 3 protein [*Persea americana* var. *drymifolia*]	867	867	92%	0	99%	AKH61409.1
g11613.t1	nonexpressor of pathogenesis-related protein 1-like 4 protein [*Persea americana* var. *drymifolia*]	1214	1214	99%	0	99%	AKH61410.1
g6547.t1	nonexpressor of pathogenesis-related protein 1-like 5 protein [*Persea americana* var. *drymifolia*]	973	973	98%	0	98%	AKH61411.1
g7475.t1	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Cinnamomum micranthum* f. *kanehirae*]	562	562	97%	0	79%	RWR76103.1
g28165.t1	beta-1,3 glucanase 3 [*Cinnamomum micranthum* f. *kanehirae*]	635	635	99%	0	90%	RWR74719.1
g28166.t1	beta-1,3 glucanase 3 [*Cinnamomum micranthum* f. *kanehirae*]	661	661	99%	0	93%	RWR74721.1
g7465.t1	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Cinnamomum micranthum* f. *kanehirae*]	572	572	85%	0	93%	RWR76104.1
g7469.t1	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Cinnamomum micranthum* f. *kanehirae*]	492	492	99%	1E-172	77%	RWR76103.1
g7453.t1	glucan endo-1,3-beta-glucosidase-like protein [*Cinnamomum micranthum* f. *kanehirae*]	628	628	99%	0	93%	RWR76105.1
g7447.t1	glucan endo-1,3-beta-glucosidase-like protein [*Cinnamomum micranthum* f. *kanehirae*]	621	621	99%	0	93%	RWR76105.1
g7457.t1	glucan endo-1,3-beta-glucosidase-like protein [*Cinnamomum micranthum* f. *kanehirae*]	608	608	99%	0	91%	RWR76105.1
g4098.t1	glucan endo-1,3-beta-glucosidase-like protein [*Cinnamomum micranthum* f. *kanehirae*]	608	608	82%	0	91%	RWR76105.1
g7459.t1	glucan endo-1,3-beta-glucosidase-like protein [*Cinnamomum micranthum* f. *kanehirae*]	608	608	82%	0	91%	RWR76105.1
g28164.t1	putative glucan endo-1,3-beta-glucosidase GVI [*Cinnamomum micranthum* f. *kanehirae*]	657	657	99%	0	95%	RWR74718.1
g7473.t1	glucan endo-1,3-beta-glucosidase, basic vacuolar isoform-like protein [*Cinnamomum micranthum* f. *kanehirae*]	575	575	75%	0	97%	RWR76103.1

g13931.t1	Glucan endo-1,3-beta-glucosidase GII [*Cinnamomum micranthum* f. *kanehirae*]	696	2951	99%	0	64%	RWR92849.1
g15715.t1	pathogenesis-related protein PRB1-3-like protein [*Cinnamomum micranthum* f. *kanehirae*]	318	318	99%	9E-110	93%	RWR73979.1
g2748.t1	pathogenesis-related protein PRB1-3-like protein [*Cinnamomum micranthum* f. *kanehirae*]	317	317	99%	3E-109	96%	RWR77918.1
g42156.t1	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like protein 2.4 [*Cinnamomum micranthum* f. *kanehirae*]	1021	1021	98%	0	87%	RWR76439.1
g15714.t1	pathogenesis-related protein PRB1-3-like protein [*Cinnamomum micranthum* f. *kanehirae*]	314	314	99%	4E-108	95%	RWR73980.1
g30797.t1	Basic form of pathogenesis-related protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	334	546	99%	2E-113	96%	RWR84871.1
g17436.t1	pathogenesis-related protein PR-1 [*Cinnamomum micranthum* f. *kanehirae*]	348	348	99%	2E-121	97%	RWR75340.1
g17433.t1	pathogenesis-related protein PR-1 [*Cinnamomum micranthum* f. *kanehirae*]	348	348	99%	3E-121	96%	RWR75340.1
g17427.t1	pathogenesis-related protein PR-1 [*Cinnamomum micranthum* f. *kanehirae*]	331	331	99%	2E-114	92%	RWR75340.1
g42875.t1	pathogenesis-related protein 5-like protein [*Cinnamomum micranthum* f. *kanehirae*]	464	464	99%	1E-164	97%	RWR74250.1
g37626.t1	thaumatin-like protein 1b [*Cinnamomum micranthum* f. *kanehirae*]	477	477	79%	3E-167	94%	RWR81718.1
g41629.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	588	588	99%	0	94%	RWR92305.1
g14064.t1	thaumatin-like protein 1b [*Cinnamomum micranthum* f. *kanehirae*]	607	607	99%	0	96%	RWR85967.1
g41635.t1	thaumatin-like protein 1b [*Cinnamomum micranthum* f. *kanehirae*]	576	576	99%	0	95%	RWR92307.1
g3865.t2	thaumatin-like protein [*Cinnamomum micranthum* f. *kanehirae*]	484	484	99%	6E-172	97%	RWR74993.1

g39310.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	456	456	99%	8E-162	93%	RWR91969.1
g39320.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	425	425	97%	3E-149	90%	RWR91968.1
g39311.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	457	457	99%	3E-162	94%	RWR91969.1
g39315.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	459	459	99%	6E-163	94%	RWR91969.1
g33886.t1	thaumatin-like protein 1b [*Cinnamomum micranthum* f. *kanehirae*]	542	542	99%	0	90%	RWR79230.1
g39316.t1	thaumatin-like protein 1 [*Cinnamomum micranthum* f. *kanehirae*]	409	409	50%	1E-139	88%	RWR91968.1
g33373.t1	pathogenesis-related protein 5 [*Cinnamomum micranthum* f. *kanehirae*]	433	433	71%	2E-150	87%	RWR85477.1
g36233.t1	Calmodulin binding protein-like protein [*Cinnamomum micranthum* f. *kanehirae*]	743	743	83%	0	90%	RWR93849.1
g34928.t1	protein transport protein Sec61 subunit alpha-like protein [*Cinnamomum micranthum* f. *kanehirae*]	957	957	99%	0	99%	RWR91493.1
g22791.t1	SecY/SEC61-alpha family [*Cinnamomum micranthum* f. *kanehirae*]	857	857	84%	0	99%	RWR84270.1
g1762.t1	CBL-interacting protein kinase 10 [*Cinnamomum micranthum* f. *kanehirae*]	868	868	98%	0	96%	RWR79019.1
g26871.t1	CBL-interacting protein kinase 10 [*Cinnamomum micranthum* f. *kanehirae*]	883	883	95%	0	98%	RWR81223.1
g32276.t1	serine/threonine-protein kinase SAPK2 [*Cinnamomum micranthum* f. *kanehirae*]	652	652	99%	0	94%	RWR89939.1
g35660.t1	serine/threonine-protein kinase SAPK2 [*Cinnamomum micranthum* f. *kanehirae*]	600	600	99%	0	85%	RWR89939.1
g39815.t1	small ubiquitin-related modifier 1-like protein [*Cinnamomum micranthum* f. *kanehirae*]	207	207	99%	1E-67	98%	RWR74569.1

g42777.t1	small ubiquitin-related modifier 1-like protein [*Cinnamomum micranthum* f. *kanehirae*]	216	216	99%	3E-71	100%	RWR87098.1
g24813.t1	small ubiquitin-related modifier 2-like protein [*Cinnamomum micranthum* f. *kanehirae*]	214	214	99%	4E-70	99%	RWR79945.1
g8421.t1	small ubiquitin-related modifier 2-like protein [*Cinnamomum micranthum* f. *kanehirae*]	154	154	98%	5E-47	78%	RWR79945.1
g5063.t1	transcription factor TGA2.2-like protein isoform X2 [*Cinnamomum micranthum* f. *kanehirae*]	667	667	99%	0	98%	RWR79737.1
g3842.t1	transcription factor TGA2.2-like protein isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	607	607	70%	0	95%	RWR74981.1
g28280.t2	transcription factor TGA2.2-like protein isoform X2 [*Cinnamomum micranthum* f. *kanehirae*]	733	733	97%	0	90%	RWR74804.1
g5420.t1	transcription factor HBP-1bc38-like protein isoform X2 [*Cinnamomum micranthum* f. *kanehirae*]	821	821	78%	0	89%	RWR77805.1
g3464.t1	transcription factor TGA2 [*Cinnamomum micranthum* f. *kanehirae*]	993	993	99%	0	98%	RWR83532.1
g32178.t1	transcription factor TGA4 isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	720	720	92%	0	96%	RWR89862.1
g15923.t2	bZIP transcription factor TGA10-like protein isoform X4 [*Cinnamomum micranthum* f. *kanehirae*]	741	741	80%	0	98%	RWR73805.1
g42296.t1	transcription factor HBP-1bc38-like protein [*Cinnamomum micranthum* f. *kanehirae*]	745	745	99%	0	96%	RWR72701.1
g19423.t1	transcription factor HBP-1bc38-like protein [*Cinnamomum micranthum* f. *kanehirae*]	738	738	99%	0	94%	RWR72701.1
g2433.t1	transcription factor TGA4 isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	751	751	99%	0	98%	RWR78443.1
g36293.t1	two-component response regulator-like PRR1 isoform X2 [*Elaeis guineensis*]	555	555	98%	0	56%	XP_029116549.1
g32937.t1	thioredoxin H-type 1 [*Cinnamomum micranthum* f. *kanehirae*]	239	239	99%	3E-79	91%	RWR77614.1

g15858.t1	thioredoxin H-type-like protein [*Cinnamomum micranthum* f. *kanehirae*]	240	240	99%	1E-79	100%	RWR73853.1
g32953.t1	thioredoxin H2-like protein [*Cinnamomum micranthum* f. *kanehirae*]	243	243	99%	5E-81	89%	RWR77629.1
g16081.t1	thioredoxin H2-like protein [*Cinnamomum micranthum* f. *kanehirae*]	225	225	99%	8E-74	91%	RWR73682.1
g41279.t1	thioredoxin H4-1 [*Cinnamomum micranthum* f. *kanehirae*]	281	281	99%	1E-95	97%	RWR79074.1
g21444.t1	thioredoxin H4-1-like protein isoform X2 [*Cinnamomum micranthum* f. *kanehirae*]	240	240	57%	3E-78	95%	RWR80522.1
g28661.t2	TPR repeat-containing thioredoxin TDX [*Cinnamomum micranthum* f. *kanehirae*]	553	553	94%	0	83%	RWR95993.1
g14581.t1	WRKY transcription factor 22 [*Cinnamomum micranthum* f. *kanehirae*]	416	416	89%	1E-144	93%	RWR80315.1
g13325.t1	putative WRKY transcription factor 14 [*Cinnamomum micranthum* f. *kanehirae*]	799	799	99%	0	93%	RWR81887.1
g20708.t1	putative WRKY transcription factor 40 [*Cinnamomum micranthum* f. *kanehirae*]	579	579	99%	0	90%	RWR91137.1
g30301.t1	WRKY transcription factor WRKY76-like protein isoform X2 [*Cinnamomum micranthum* f. *kanehirae*]	583	583	99%	0	91%	RWR82572.1
g27980.t1	putative WRKY transcription factor 31 [*Cinnamomum micranthum* f. *kanehirae*]	1060	1060	94%	0	95%	RWR89042.1
g27775.t1	putative WRKY transcription factor 31 [*Cinnamomum micranthum* f. *kanehirae*]	1068	1068	99%	0	97%	RWR97345.1
g14197.t1	putative WRKY transcription factor 31 [*Cinnamomum micranthum* f. *kanehirae*]	916	916	99%	0	90%	RWR96122.1
g126.t1	putative WRKY transcription factor 47 [*Cinnamomum micranthum* f. *kanehirae*]	914	914	99%	0	93%	RWR94439.1
g29615.t1	putative WRKY transcription factor 47 [*Cinnamomum micranthum* f. *kanehirae*]	885	885	99%	0	92%	RWR95148.1

g13934.t1	putative WRKY transcription factor 9 [*Cinnamomum micranthum* f. *kanehirae*]	884	884	99%	0	94%	RWR92848.1
g1993.t1	putative WRKY transcription factor 72 isoform X1 [*Cinnamomum micranthum* f. *kanehirae*]	1147	1147	99%	0	97%	RWR78811.1
g2243.t1	putative WRKY transcription factor 9 [*Cinnamomum micranthum* f. *kanehirae*]	572	572	85%	0	79%	RWR78594.1
g21100.t1	putative WRKY transcription factor 40 [*Cinnamomum micranthum* f. *kanehirae*]	220	220	87%	1E-67	47%	RWR82957.1
g9565.t1	putative WRKY transcription factor 40 [*Cinnamomum micranthum* f. *kanehirae*]	429	429	99%	3E-150	89%	RWR82957.1
g20711.t1	putative WRKY transcription factor 40 [*Cinnamomum micranthum* f. *kanehirae*]	435	435	99%	7E-153	88%	RWR91132.1
g14855.t1	putative WRKY transcription factor 70 [*Cinnamomum micranthum* f. *kanehirae*]	380	380	63%	5E-131	92%	RWR80504.1
g41259.t1	putative WRKY transcription factor 70 [*Cinnamomum micranthum* f. *kanehirae*]	526	526	99%	0	86%	RWR79059.1
g41260.t1	WRKY transcription factor 55 [*Cinnamomum micranthum* f. *kanehirae*]	671	671	99%	0	91%	RWR79060.1
g9416.t1	putative WRKY transcription factor 41 [*Cinnamomum micranthum* f. *kanehirae*]	618	618	99%	0	90%	RWR93179.1
g37185.t1	DNA-binding WRKY [*Cinnamomum micranthum* f. *kanehirae*]	685	685	99%	0	97%	RWR80234.1
g25443.t1	DNA-binding WRKY [*Cinnamomum micranthum* f. *kanehirae*]	685	685	99%	0	97%	RWR80234.1
g37189.t1	DNA-binding WRKY [*Cinnamomum micranthum* f. *kanehirae*]	620	620	99%	0	92%	RWR80234.1

Supplementary Table S3. Statistical summary of R0.12 time-course analyses.

P. americana ID	Gene name in *P. americana*	Control			12hpi			24hpi			120hpi		
		Mean TPM	SEM	Group	Mean TPM	SEM	Group	Mean TPM	SEM	Group	Mean TPM	SEM	Group
g13474.t1	*PaBiP5*	103.98	8.20	A	105.86	11.63	A	98.35	2.64	A	111.86	5.82	A
g16678.t1	*PaBiP4*	118.50	12.52	A	103.35	7.45	AB	99.25	2.40	AB	82.04	2.45	B
g39531.t1	*PaCRT3-like1*	105.11	1.80	A	90.40	1.51	B	91.34	2.52	B	71.07	0.68	C
g7570.t1	*PaCRT3-like2*	21.57	3.32	A	16.93	1.41	AB	20.00	2.78	AB	10.46	2.38	B
g3606.t1	*PaCUL3A-like1*	18.54	0.57	A	18.09	0.77	A	16.86	0.90	A	16.89	1.00	A
g35218.t1	*PaCUL3A-like2*	14.39	0.37	A	15.41	0.87	A	16.20	0.79	A	16.47	0.84	A
g4709.t1	*PaDAD1-like1*	0.05	0.02	A	0.06	0.05	A	0.04	0.02	A	0.08	0.04	A
g10107.t1	*PaDAD1-like2*	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
g23038.t2	*PaPM1*	27.10	2.31	A	34.35	1.36	A	30.54	2.72	A	30.12	0.44	A
g30203.t1	*PaHD9*	15.79	0.59	AB	19.71	1.31	A	15.13	1.03	B	16.31	1.45	AB
g30067.t1	*PaHD6*	23.13	1.32	A	25.57	0.95	A	23.06	1.00	A	16.73	1.74	B
g13531.t1	*PaADH3*	86.30	10.51	A	75.06	6.53	A	83.58	8.71	A	58.47	5.68	A
g13969.t1	*PaADH2*	181.46	24.40	B	247.23	15.13	A	141.73	11.71	B	143.93	7.83	B
g13967.t1	*PaADH*	44.89	14.84	B	119.23	10.13	A	20.93	6.22	B	40.56	9.40	B
g14178.t1	*PaADH-like*	31.27	1.81	A	29.61	1.06	A	29.81	2.22	A	26.42	2.41	A
g24705.t1	*PaHSF24-like1*	30.51	6.01	A	10.04	1.25	B	15.19	3.04	B	24.02	1.58	AB
g30822.t1	*PaHSF24-like2*	104.93	18.92	B	118.17	11.64	B	136.20	18.38	B	230.43	11.74	A
g14724.t1	*PaICS-like1*	0.85	0.15	A	1.08	0.56	A	0.26	0.13	A	0.20	0.09	A
g23147.t1	*PaICS-like2*	6.18	1.13	A	5.44	0.43	A	5.74	1.04	A	1.57	0.23	B
g35261.t1	*PaLHY*	30.54	2.00	B	10.76	0.48	C	40.56	2.68	A	31.76	1.35	B
g17292.t1	*PaMPK3*	56.19	8.67	BC	45.82	3.84	C	74.96	1.45	B	106.95	4.51	A
g42257.t1	*PaNTF6*	6.64	0.18	B	8.12	0.37	AB	7.41	0.32	AB	9.13	0.71	A
g8979.t1	*PaMMK1*	10.71	2.22	A	13.44	0.98	A	10.47	2.52	A	6.81	1.40	A
g34229.t1	*PaNIMIN-1*	5.11	1.27	A	3.85	1.19	A	5.64	2.34	A	3.87	1.69	A

g36175.t1	*PaNIMIN-2*	198.33	8.46	A	197.89	8.38	A	187.37	8.48	A	90.92	11.67	B
g35159.t1	*PaRHA1B-like*	11.66	1.30	AB	16.85	1.66	A	9.10	0.74	B	2.20	1.40	C
g18227.t1	*PaNPR1*	4.84	0.61	B	6.20	0.59	B	6.21	0.33	B	9.15	0.49	A
g33565.t1	*PaNPR2*	17.44	1.47	B	22.14	1.35	A	22.95	0.26	A	25.05	1.02	A
g12308.t1	*PaNPR3*	0.27	0.03	A	0.29	0.07	A	0.15	0.07	A	0.09	0.04	A
g11613.t1	*PaNPR4*	5.66	0.35	A	5.67	0.60	A	6.51	0.42	A	5.03	0.35	A
g6547.t1	*PaNPR5*	16.46	2.73	A	17.06	1.27	A	14.55	0.95	A	5.11	0.28	B
g7475.t1	*PaPR-2-like1*	780.33	402.80	B	603.34	164.36	B	729.70	212.89	B	3176.65	286.18	A
g28165.t1	*PaPR-2-like2*	434.43	182.60	B	458.93	90.25	B	562.45	99.69	B	1572.10	336.83	A
g28166.t1	*PaPR-2-like3*	108.90	52.33	B	101.04	28.56	B	102.46	14.62	B	506.61	131.08	A
g7465.t1	*PaPR-2-like4*	154.48	30.39	B	127.67	15.68	B	251.77	17.97	A	140.26	7.80	B
g7469.t1	*PaPR-2-like5*	2.46	0.59	A	1.01	0.46	A	9.65	4.55	A	8.27	1.25	A
g7453.t1	*PaPR-2-like6*	0.26	0.14	B	0.23	0.10	B	0.70	0.33	B	1.80	0.27	A
g7447.t1	*PaPR-2-like7*	0.34	0.17	A	0.08	0.06	A	0.44	0.17	A	0.34	0.09	A
g7457.t1	*PaPR-2-like8*	126.55	31.95	B	71.74	19.17	B	122.63	14.31	B	367.30	58.18	A
g4098.t1	*PaPR-2-like9*	0.01	0.01	A	0.03	0.01	A	0.07	0.05	A	0.07	0.01	A
g7459.t1	*PaPR-2-like10*	0.01	0.01	A	0.03	0.01	A	0.07	0.05	A	0.07	0.01	A
g28164.t1	*PaPR-2-like11*	83.46	10.80	B	156.16	4.05	A	85.20	4.71	B	87.04	9.34	B
g7473.t1	*PaPR-2-like12*	18.25	7.17	B	13.98	4.66	B	20.78	6.09	B	75.82	8.09	A
g13931.t1	*PaPR-2-like13*	0.09	0.07	A	0.19	0.13	A	0.06	0.03	A	0.45	0.21	A
g15715.t1	*PaPRB1-3-like1*	632.60	311.67	B	392.47	159.27	B	458.02	128.00	B	3731.54	686.13	A
g2748.t1	*PaPRB1-3-like2*	736.61	286.39	B	1129.10	224.18	AB	902.76	161.97	B	2187.74	434.57	A
g42156.t1	*PaLRK10L-2.4*	3.74	0.44	B	3.60	0.44	B	4.29	0.21	B	6.38	0.79	A
g15714.t1	*PaPRB1-3-like3*	1.88	0.87	B	0.59	0.17	B	1.13	0.25	B	4.58	0.74	A
g30797.t1	*PaPR-1-like1*	0.13	0.11	A	0.05	0.04	A	0.00	0.00	A	0.00	0.00	A
g17436.t1	*PaPR-1-like2*	0.00	0.00	B	0.10	0.04	A	0.00	0.00	B	0.00	0.00	B
g17433.t1	*PaPR-1-like3*	9.09	2.42	A	7.43	2.43	A	10.07	0.93	A	8.36	0.31	A
g17427.t1	*PaPR-1-like4*	2.63	0.97	A	2.31	0.71	A	1.92	0.60	A	2.12	0.49	A
g42875.t1	*PaPR-5-like1*	8.14	1.96	AB	13.02	2.09	A	5.33	0.12	BC	0.35	0.17	C

g37626.t1	*PaPR-5b-like1*	9.42	1.86	A	9.49	1.33	A	5.73	0.51	AB	2.06	0.17	B
g41629.t1	*PaPR-5-like2*	200.73	30.62	AB	144.67	15.88	B	260.76	4.88	A	193.68	24.79	AB
g14064.t1	*PaPR-5b-like2*	60.27	10.39	B	112.29	3.22	A	41.47	2.17	B	12.26	1.39	C
g41635.t1	*PaPR-5b-like3*	17.23	2.73	A	11.34	0.21	A	21.99	4.05	A	12.50	3.58	A
g3865.t2	*PaPR-5-like3*	32.26	5.98	A	36.87	1.57	A	16.20	0.79	B	7.39	0.73	B
g39310.t1	*PaPR-5-like4*	0.11	0.06	B	0.23	0.02	B	0.39	0.15	B	2.01	0.59	A
g39320.t1	*PaPR-5-like5*	2.01	0.41	A	0.26	0.04	B	0.97	0.10	AB	1.80	0.37	A
g39311.t1	*PaPR-5-like6*	170.06	98.41	A	152.59	64.56	A	135.30	77.48	A	914.10	379.06	A
g39315.t1	*PaPR-5-like7*	14.67	7.79	A	6.82	2.32	A	64.58	44.03	A	35.25	10.91	A
g33886.t1	*PaPR-5b-like4*	5.05	0.47	AB	2.76	0.90	B	6.64	0.12	A	4.40	0.95	AB
g39316.t1	*PaPR-5-like9*	0.81	0.09	A	1.19	0.33	A	1.01	0.15	A	0.59	0.10	A
g33373.t1	*PaPR-5-like10*	4.30	1.01	A	4.09	0.55	A	5.02	0.90	A	2.49	1.01	A
g36233.t1	*PaSARD1*	6.23	1.29	A	6.78	0.50	A	10.06	0.20	A	10.44	1.96	A
g34928.t1	*PaSec61α-like1*	37.70	1.88	B	52.58	0.03	A	40.12	0.70	B	36.86	0.70	B
g22791.t1	*PaSec61α-like2*	133.91	5.02	AB	151.39	6.66	A	146.56	6.21	AB	128.78	2.73	B
g1762.t1	*PaCIPK10-like1*	52.42	3.84	BC	56.08	4.00	B	40.84	2.05	C	77.85	3.52	A
g26871.t1	*PaCIPK10-like2*	33.99	2.08	A	33.11	1.99	A	28.07	1.51	A	27.66	0.37	A
g32276.t1	*PaSAPK2-like1*	15.37	1.11	B	34.06	0.74	A	13.90	0.47	B	16.97	3.30	B
g35660.t1	*PaSAPK2-like2*	17.10	1.84	B	31.73	2.46	A	20.21	3.18	B	18.13	0.63	B
g39815.t1	*PaSUMO1-like1*	545.88	52.79	A	633.72	39.50	A	552.65	19.65	A	464.85	50.86	A
g42777.t1	*PaSUMO1-like2*	1039.83	46.27	A	897.91	30.06	A	946.88	14.50	A	877.91	64.45	A
g24813.t1	*PaSUMO2-like1*	424.34	26.03	A	437.66	18.94	A	426.98	24.40	A	364.27	38.51	A
g8421.t1	*PaSUMO2-like2*	0.49	0.40	A	0.13	0.11	A	0.00	0.00	A	0.00	0.00	A
g5063.t1	*PaTGA2-like1*	57.36	0.72	B	68.90	0.16	A	69.47	1.29	A	56.75	0.32	B
g3842.t1	*PaTGA2-like2*	0.26	0.09	A	0.48	0.16	A	0.20	0.08	A	0.19	0.08	A
g28280.t2	*PaTGA2-like3*	12.49	1.17	A	19.77	3.43	A	18.70	1.66	A	17.93	4.81	A
g5420.t1	*PaHBP-1b(C38)-like1*	7.92	0.42	A	7.92	0.21	A	7.47	0.42	A	4.72	0.48	B
g3464.t1	*PaTGA2-like5*	31.96	3.42	A	34.09	2.81	A	32.39	4.05	A	22.54	2.48	A
g32178.t1	*PaTGA4-like1*	14.04	0.94	AB	16.10	0.39	A	11.66	0.93	B	15.68	1.28	A

g15923.t2	*PaTGA10-like1*	2.08	0.29	B	3.31	0.49	B	3.03	1.09	B	7.10	0.61	A
g42296.t1	*PaHBP-1b(C38)-like2*	0.40	0.12	B	0.68	0.11	B	1.02	0.32	AB	1.85	0.36	A
g19423.t1	*PaHBP-1b(C38)-like3*	2.18	0.23	A	1.36	0.31	A	2.17	0.53	A	2.78	0.55	A
g2433.t1	*PaTGA4-like3*	59.05	5.85	B	106.29	5.88	A	53.51	8.34	B	70.75	6.06	B
g36293.t1	*PaTOC1*	7.36	0.23	B	11.81	0.54	A	7.24	0.60	B	7.35	1.15	B
g32937.t1	*PaTRX1-like1*	588.15	13.19	B	593.76	26.40	B	550.66	28.20	B	840.65	100.81	A
g15858.t1	*PaTRX-like1*	123.96	21.86	AB	160.65	21.92	A	145.60	15.37	A	58.77	8.16	B
g32953.t1	*PaTRX2-like1*	142.44	14.43	A	105.34	24.94	A	118.91	14.52	A	88.22	15.03	A
g16081.t1	*PaTRX2-like2*	87.50	6.35	A	110.04	2.39	A	99.43	1.13	A	104.42	10.55	A
g41279.t1	*PaTRX4-like1*	137.48	6.87	A	135.68	6.32	A	157.27	12.10	A	146.83	17.84	A
g21444.t1	*PaTRX4-like2*	1.03	0.11	B	1.67	0.19	AB	1.48	0.27	AB	2.17	0.14	A
g28661.t2	*PaTDX-like1*	25.01	0.89	A	23.75	0.37	A	25.20	2.23	A	25.93	3.09	A
g14581.t1	*PaWRKY22*	13.69	4.04	B	10.21	1.17	B	17.18	1.07	B	44.71	2.09	A
g13325.t1	*PaWRKY14*	7.80	1.25	A	7.45	0.88	AB	8.99	0.92	A	3.95	0.72	B
g20708.t1	*PaWRKY40-like1*	23.73	7.55	AB	15.58	1.66	B	33.27	3.64	A	32.77	1.75	AB
g30301.t1	*PaWRKY76-like*	0.53	0.15	B	0.35	0.08	B	1.93	0.51	A	0.18	0.09	B
g27980.t1	*PaWRKY31-like1*	13.19	4.07	B	12.03	2.33	B	17.81	2.02	B	51.41	6.20	A
g27775.t1	*PaWRKY31-like2*	106.46	23.11	B	100.50	16.66	B	143.39	13.87	B	304.21	30.07	A
g14197.t1	*PaWRKY31-like3*	4.51	0.82	C	4.82	0.72	BC	7.47	0.71	B	11.25	0.57	A
g126.t1	*PaWRKY47-like1*	18.36	3.64	AB	17.21	2.94	B	22.33	4.04	AB	31.13	2.96	A
g29615.t1	*PaWRKY47-like2*	10.50	0.68	B	10.86	0.33	B	12.27	0.84	AB	14.84	0.80	A
g13934.t1	*PaWRKY9-like1*	5.87	1.23	B	6.22	0.22	B	6.90	0.57	B	14.55	1.06	A
g1993.t1	*PaWRKY72*	16.28	1.50	B	13.61	0.69	B	15.68	0.40	B	28.76	1.68	A
g2243.t1	*PaWRKY9-like2*	2.10	0.17	AB	2.58	0.32	A	2.53	0.36	A	1.06	0.29	B
g21100.t1	*PaWRKY40-like2*	1.22	0.50	A	0.43	0.21	A	0.30	0.13	A	1.21	0.31	A
g9565.t1	*PaWRKY40-like4*	6.67	2.20	BC	2.34	0.48	C	10.00	1.13	B	21.06	0.43	A
g20711.t1	*PaWRKY40-like5*	1.37	0.02	B	1.87	0.28	AB	1.72	0.19	AB	3.05	0.65	A
g14855.t1	*PaWRKY70-like1*	4.99	0.72	B	3.34	0.78	B	9.67	1.27	A	6.62	1.07	AB
g41259.t1	*PaWRKY70-like2*	26.91	4.94	AB	20.24	3.16	B	44.68	4.02	AB	54.72	12.44	A

g41260.t1	*PaWRKY55*	0.43	0.08	A	0.69	0.13	A	0.70	0.06	A	0.69	0.04	A
g9416.t1	*PaWRKY41*	14.44	2.09	AB	9.48	1.28	B	19.96	0.07	A	18.91	2.77	A
g37185.t1	*PaWRKY-like1*	4.32	0.63	B	5.02	0.26	B	7.26	0.69	A	8.79	0.13	A
g25443.t1	*PaWRKY-like2*	0.03	0.02	A	0.03	0.03	A	0.00	0.00	A	0.00	0.00	A
g37189.t1	*PaWRKY-like3*	3.25	0.60	B	6.64	0.66	A	3.77	0.98	B	2.76	0.30	B

Supplementary Table S4. Statistical summary of R0.12 and Dusa® comparative analyses.

P. americana ID	Gene name in *P. americana*	R0.12 Control			R0.12 120hpi			Dusa® Control			Dusa® 120hpi		
		Mean TPM	SEM	Group	Mean TPM	SEM	Group	Mean TPM	SEM	Group	Mean TPM	SEM	Group
g13474.t1	*PaBiP5*	103.98	8.20	A	111.86	5.82	A	94.43	4.37	A	109.07	14.06	A
g16678.t1	*PaBiP4*	118.50	12.52	A	82.04	2.45	B	101.79	4.95	AB	74.66	5.53	B
g39531.t1	*PaCRT3-like1*	105.11	1.80	A	71.07	0.68	C	95.82	1.19	B	65.09	2.22	C
g7570.t1	*PaCRT3-like2*	21.57	3.32	A	10.46	2.38	B	23.38	0.59	A	10.81	0.65	B
g3606.t1	*PaCUL3A-like1*	18.54	0.57	A	16.89	1.00	A	16.82	0.92	A	12.71	0.28	B
g35218.t1	*PaCUL3A-like2*	14.39	0.37	AB	16.47	0.84	A	15.05	0.39	AB	13.54	0.54	B
g4709.t1	*PaDAD1-like1*	0.05	0.02	A	0.08	0.04	A	0.03	0.01	A	0.09	0.03	A
g10107.t1	*PaDAD1-like2*	0.00	0.00	A	0.00	0.00	A	0.01	0.00	A	0.00	0.00	A
g23038.t2	*PaPM1*	27.10	2.31	A	30.12	0.44	A	29.84	2.34	A	26.13	1.12	A
g30203.t1	*PaHD9*	15.79	0.59	B	16.31	1.45	AB	19.13	0.52	A	14.16	0.06	B
g30067.t1	*PaHD6*	23.13	1.32	AB	16.73	1.74	C	25.34	1.86	A	17.96	0.68	BC
g13531.t1	*PaADH3*	86.30	10.51	A	58.47	5.68	B	100.62	3.33	A	60.53	1.52	B
g13969.t1	*PaADH2*	181.46	24.40	A	143.93	7.83	A	193.98	20.70	A	140.68	6.31	A
g13967.t1	*PaADH*	44.89	14.84	A	40.56	9.40	A	61.85	17.91	A	43.85	8.10	A
g14178.t1	*PaADH-like*	31.27	1.81	B	26.42	2.41	BC	41.52	2.11	A	19.64	1.41	C
g24705.t1	*PaHSF24-like1*	30.51	6.01	A	24.02	1.58	AB	16.44	2.04	B	22.39	1.19	AB
g30822.t1	*PaHSF24-like2*	104.93	18.92	B	230.43	11.74	A	99.17	6.44	B	190.52	3.86	A
g14724.t1	*PaICS-like1*	0.85	0.15	AB	0.20	0.09	B	0.98	0.28	A	0.25	0.13	B
g23147.t1	*PaICS-like2*	6.18	1.13	A	1.57	0.23	B	4.44	0.28	A	0.87	0.16	B
g35261.t1	*PaLHY*	30.54	2.00	B	31.76	1.35	B	38.81	0.82	A	27.28	1.32	B
g17292.t1	*PaMPK3*	56.19	8.67	B	106.95	4.51	A	63.92	2.61	B	102.94	2.01	A
g42257.t1	*PaNTF6*	6.64	0.18	B	9.13	0.71	A	7.68	0.65	AB	7.41	0.19	AB
g8979.t1	*PaMMK1*	10.71	2.22	AB	6.81	1.40	AB	12.01	0.69	A	6.33	0.32	B
g34229.t1	*PaNIMIN-1*	5.11	1.27	AB	3.87	1.69	B	9.86	1.20	A	2.92	0.65	B

g36175.t1	*PaNIMIN-2*	198.33	8.46	A	90.92	11.67	C	157.77	3.76	B	143.40	11.42	B
g35159.t1	*PaRHA1B-like*	11.66	1.30	A	2.20	1.40	B	13.09	2.05	A	1.69	0.49	B
g18227.t1	*PaNPR1*	4.84	0.61	C	9.15	0.49	A	6.72	0.31	B	7.06	0.31	B
g33565.t1	*PaNPR2*	17.44	1.47	C	25.05	1.02	A	19.63	0.28	BC	23.39	1.31	AB
g12308.t1	*PaNPR3*	0.27	0.03	A	0.09	0.04	B	0.26	0.02	A	0.05	0.01	B
g11613.t1	*PaNPR4*	5.66	0.35	AB	5.03	0.35	B	6.37	0.10	A	3.88	0.17	C
g6547.t1	*PaNPR5*	16.46	2.73	A	5.11	0.28	B	18.53	0.74	A	5.44	0.10	B
g7475.t1	*PaPR-2-like1*	780.33	402.80	C	3176.65	286.18	B	453.22	121.19	C	5347.47	255.37	A
g28165.t1	*PaPR-2-like2*	434.43	182.60	B	1572.10	336.83	A	372.30	74.27	B	1982.72	270.31	A
g28166.t1	*PaPR-2-like3*	108.90	52.33	B	506.61	131.08	A	36.15	4.11	B	228.21	22.66	AB
g7465.t1	*PaPR-2-like4*	154.48	30.39	A	140.26	7.80	A	184.07	14.93	A	114.16	10.25	A
g7469.t1	*PaPR-2-like5*	2.46	0.59	B	8.27	1.25	AB	11.92	3.83	A	2.81	0.35	B
g7453.t1	*PaPR-2-like6*	0.26	0.14	B	1.80	0.27	A	0.19	0.03	B	1.41	0.20	A
g7447.t1	*PaPR-2-like7*	0.34	0.17	A	0.34	0.09	A	0.05	0.03	A	0.30	0.02	A
g7457.t1	*PaPR-2-like8*	126.55	31.95	B	367.30	58.18	A	110.37	2.97	B	250.36	51.71	AB
g4098.t1	*PaPR-2-like9*	0.01	0.01	B	0.07	0.01	A	0.09	0.02	A	0.12	0.01	A
g7459.t1	*PaPR-2-like10*	0.01	0.01	B	0.07	0.01	A	0.09	0.02	A	0.12	0.01	A
g28164.t1	*PaPR-2-like11*	83.46	10.80	A	87.04	9.34	A	76.94	9.79	A	54.42	2.24	A
g7473.t1	*PaPR-2-like12*	18.25	7.17	B	75.82	8.09	A	16.25	4.11	B	81.49	4.68	A
g13931.t1	*PaPR-2-like13*	0.09	0.07	A	0.45	0.21	A	0.04	0.01	A	0.36	0.10	A
g15715.t1	*PaPRB1-3-like1*	632.60	311.67	B	3731.54	686.13	A	310.42	40.97	B	3764.50	361.37	A
g2748.t1	*PaPRB1-3-like2*	736.61	286.39	B	2187.74	434.57	A	674.33	138.64	B	2240.13	96.40	A
g42156.t1	*PaLRK10L-2.4*	3.74	0.44	B	6.38	0.79	A	3.17	0.37	B	2.98	0.33	B
g15714.t1	*PaPRB1-3-like3*	1.88	0.87	B	4.58	0.74	A	0.00	0.00	B	0.24	0.20	B
g30797.t1	*PaPR-1-like1*	0.13	0.11	A	0.00	0.00	A	0.09	0.05	A	0.00	0.00	A
g17436.t1	*PaPR-1-like2*	0.00	0.00	A	0.00	0.00	A	0.03	0.02	A	0.00	0.00	A
g17433.t1	*PaPR-1-like3*	9.09	2.42	A	8.36	0.31	A	11.64	0.50	A	9.09	1.12	A
g17427.t1	*PaPR-1-like4*	2.63	0.97	A	2.12	0.49	A	3.29	0.21	A	3.44	0.25	A
g42875.t1	*PaPR-5-like1*	8.14	1.96	A	0.35	0.17	B	10.77	1.19	A	0.59	0.09	B

g37626.t1	*PaPR-5b-like1*	9.42	1.86	B	2.06	0.17	C	19.70	1.90	A	2.37	0.26	C
g41629.t1	*PaPR-5-like2*	200.73	30.62	B	193.68	24.79	B	294.93	17.96	A	263.47	11.90	AB
g14064.t1	*PaPR-5b-like2*	60.27	10.39	A	12.26	1.39	B	63.36	3.02	A	16.30	0.30	B
g41635.t1	*PaPR-5b-like3*	17.23	2.73	A	12.50	3.58	AB	16.42	1.44	AB	6.88	0.73	B
g3865.t2	*PaPR-5-like3*	32.26	5.98	A	7.39	0.73	B	40.18	2.11	A	5.57	2.09	B
g39310.t1	*PaPR-5-like4*	0.11	0.06	B	2.01	0.59	A	0.33	0.07	B	0.74	0.18	B
g39320.t1	*PaPR-5-like5*	2.01	0.41	B	1.80	0.37	B	2.62	0.27	AB	3.63	0.31	A
g39311.t1	*PaPR-5-like6*	170.06	98.41	BC	914.10	379.06	AB	95.40	15.13	C	1370.77	93.87	A
g39315.t1	*PaPR-5-like7*	14.67	7.79	B	35.25	10.91	B	65.17	8.84	B	821.20	74.08	A
g33886.t1	*PaPR-5b-like4*	5.05	0.47	AB	4.40	0.95	B	7.61	0.67	A	3.12	0.61	B
g39316.t1	*PaPR-5-like9*	0.81	0.09	A	0.59	0.10	A	0.61	0.07	A	0.49	0.06	A
g33373.t1	*PaPR-5-like10*	4.30	1.01	A	2.49	1.01	A	4.22	0.40	A	1.49	0.32	A
g36233.t1	*PaSARD1*	6.23	1.29	B	10.44	1.96	AB	6.93	0.32	AB	12.19	1.38	A
g34928.t1	*PaSec61α-like1*	37.70	1.88	A	36.86	0.70	A	41.79	2.11	A	35.82	0.96	A
g22791.t1	*PaSec61α-like2*	133.91	5.02	A	128.78	2.73	A	128.80	3.75	A	119.62	2.22	A
g1762.t1	*PaCIPK10-like1*	52.42	3.84	B	77.85	3.52	A	32.39	1.27	C	67.29	1.71	A
g26871.t1	*PaCIPK10-like2*	33.99	2.08	A	27.66	0.37	B	27.99	1.16	B	23.91	1.16	B
g32276.t1	*PaSAPK2-like1*	15.37	1.11	A	16.97	3.30	A	14.50	1.40	A	11.05	0.36	A
g35660.t1	*PaSAPK2-like2*	17.10	1.84	A	18.13	0.63	A	17.31	2.52	A	15.48	2.59	A
g39815.t1	*PaSUMO1-like1*	545.88	52.79	AB	464.85	50.86	B	622.94	18.89	A	421.51	18.32	B
g42777.t1	*PaSUMO1-like2*	1039.83	46.27	A	877.91	64.45	AB	964.08	29.26	A	742.98	31.71	B
g24813.t1	*PaSUMO2-like1*	424.34	26.03	A	364.27	38.51	AB	440.96	2.66	A	301.74	21.33	B
g8421.t1	*PaSUMO2-like2*	0.49	0.40	A	0.00	0.00	A	0.07	0.05	A	0.00	0.00	A
g5063.t1	*PaTGA2-like1*	57.36	0.72	B	56.75	0.32	B	61.99	1.61	A	55.43	1.14	B
g3842.t1	*PaTGA2-like2*	0.26	0.09	AB	0.19	0.08	AB	0.37	0.06	A	0.07	0.05	B
g28280.t2	*PaTGA2-like3*	12.49	1.17	AB	17.93	4.81	AB	22.51	4.00	A	8.03	1.18	B
g5420.t1	*PaHBP-1b(C38)-like1*	7.92	0.42	A	4.72	0.48	B	7.89	0.22	A	4.70	0.07	B
g3464.t1	*PaTGA2-like5*	31.96	3.42	A	22.54	2.48	AB	31.25	2.38	A	16.91	0.34	B
g32178.t1	*PaTGA4-like1*	14.04	0.94	A	15.68	1.28	A	9.99	0.88	B	10.25	0.35	B

g15923.t2	*PaTGA10-like1*	2.08	0.29	B	7.10	0.61	A	3.03	0.32	B	4.38	1.29	AB
g42296.t1	*PaHBP-1b(C38)-like2*	0.40	0.12	B	1.85	0.36	A	0.84	0.09	B	0.73	0.24	B
g19423.t1	*PaHBP-1b(C38)-like3*	2.18	0.23	A	2.78	0.55	A	2.39	0.12	A	2.11	0.14	A
g2433.t1	*PaTGA4-like3*	59.05	5.85	A	70.75	6.06	A	39.13	3.40	B	36.72	1.55	B
g36293.t1	*PaTOC1*	7.36	0.23	A	7.35	1.15	A	7.38	0.35	A	5.21	0.49	A
g32937.t1	*PaTRX1-like1*	588.15	13.19	A	840.65	100.81	A	671.17	69.24	A	605.33	59.74	A
g15858.t1	*PaTRX-like1*	123.96	21.86	A	58.77	8.16	B	149.93	11.78	A	39.77	3.50	B
g32953.t1	*PaTRX2-like1*	142.44	14.43	B	88.22	15.03	B	242.61	21.52	A	80.53	11.55	B
g16081.t1	*PaTRX2-like2*	87.50	6.35	B	104.42	10.55	AB	126.86	5.58	A	98.22	6.10	AB
g41279.t1	*PaTRX4-like1*	137.48	6.87	AB	146.83	17.84	AB	168.87	2.49	A	114.07	8.69	B
g21444.t1	*PaTRX4-like2*	1.03	0.11	A	2.17	0.14	A	2.54	0.74	A	1.45	0.43	A
g28661.t2	*PaTDX-like1*	25.01	0.89	A	25.93	3.09	A	19.71	0.89	AB	15.34	0.35	B
g14581.t1	*PaWRKY22*	13.69	4.04	B	44.71	2.09	A	12.32	0.64	B	36.02	1.15	A
g13325.t1	*PaWRKY14*	7.80	1.25	A	3.95	0.72	BC	6.49	0.60	AB	2.23	0.20	C
g20708.t1	*PaWRKY40-like1*	23.73	7.55	B	32.77	1.75	AB	42.42	1.60	A	31.25	1.09	AB
g30301.t1	*PaWRKY76-like*	0.53	0.15	AB	0.18	0.09	B	0.77	0.12	A	0.62	0.14	AB
g27980.t1	*PaWRKY31-like1*	13.19	4.07	B	51.41	6.20	A	12.58	0.64	B	46.03	1.70	A
g27775.t1	*PaWRKY31-like2*	106.46	23.11	B	304.21	30.07	A	114.35	9.85	B	364.64	8.99	A
g14197.t1	*PaWRKY31-like3*	4.51	0.82	C	11.25	0.57	A	3.83	0.29	C	7.29	0.58	B
g126.t1	*PaWRKY47-like1*	18.36	3.64	B	31.13	2.96	A	16.75	1.43	B	20.11	0.69	B
g29615.t1	*PaWRKY47-like2*	10.50	0.68	B	14.84	0.80	A	10.57	0.47	B	8.37	0.21	B
g13934.t1	*PaWRKY9-like1*	5.87	1.23	C	14.55	1.06	A	5.63	0.20	C	10.76	0.61	B
g1993.t1	*PaWRKY72*	16.28	1.50	B	28.76	1.68	A	14.88	1.08	B	25.95	1.50	A
g2243.t1	*PaWRKY9-like2*	2.10	0.17	BC	1.06	0.29	C	3.58	0.37	A	2.42	0.28	B
g21100.t1	*PaWRKY40-like2*	1.22	0.50	A	1.21	0.31	A	0.90	0.28	A	0.90	0.74	A
g9565.t1	*PaWRKY40-like4*	6.67	2.20	B	21.06	0.43	A	11.32	1.92	B	20.89	0.20	A
g20711.t1	*PaWRKY40-like5*	1.37	0.02	B	3.05	0.65	A	0.85	0.10	B	0.70	0.04	B
g14855.t1	*PaWRKY70-like1*	4.99	0.72	B	6.62	1.07	AB	5.62	0.18	B	11.27	2.28	A
g41259.t1	*PaWRKY70-like2*	26.91	4.94	B	54.72	12.44	B	34.59	1.57	B	100.80	14.93	A

g41260.t1	*PaWRKY55*	0.43	0.08	B	0.69	0.04	B	0.72	0.08	B	1.33	0.23	A
g9416.t1	*PaWRKY41*	14.44	2.09	B	18.91	2.77	B	15.87	0.51	B	50.31	2.32	A
g37185.t1	*PaWRKY-like1*	4.32	0.63	C	8.79	0.13	B	6.16	0.74	C	15.71	0.51	A
g25443.t1	*PaWRKY-like2*	0.03	0.02	A	0.00	0.00	A	0.02	0.02	A	0.00	0.00	A
g37189.t1	*PaWRKY-like3*	3.25	0.60	A	2.76	0.30	A	3.00	0.24	A	2.01	0.13	A

Chapter 4

Concluding Remarks

Introduction

The average global production of avocado has more than doubled in the past two decades (http://www.fao.org/faostat/en/#home). Unsurprisingly, avocado consumption boasts several proven health benefits, and thus demand is likely to keep growing (COLQUHOUN *et al.* 1992; LERMAN-GARBER *et al.* 1994; CARRANZA *et al.* 1995; LOPEZ LEDESMA *et al.* 1996; PIETERSE *et al.* 2005; DREHER AND DAVENPORT 2013). However, the avocado industry is ever-increasingly threatened by several noteworthy diseases including Phytophthora root rot (PRR), white root rot and Fusarium dieback (LONSDALE *et al.* 1988; HARDHAM 2005; LÓPEZ *et al.* 2008; FREEMAN *et al.* 2013; ENGELBRECHT *et al.* 2017; PAAP *et al.* 2018; VAN DEN BERG *et al.* 2018b; VAN DEN BERG *et al.* 2019). Of these, PRR is currently regarded as the biggest threat to avocado production worldwide.

The causal agent of PRR, *Phytophthora cinnamomi*, is a hemibiotrophic oomycete with a vast host range exceeding 5000 plant species (HARDHAM AND BLACKMAN 2018). Typical infection by *P. cinnamomi* is characterised by necrosis of the feeder root system, which limits water and nutrient uptake, decreasing productivity and eventually leading to death of the tree (ZENTMYER 1984; COFFEY 1987). Primary control strategies include the use of phosphite trunk injections, strict agricultural hygiene, mulching and irrigation practices as well as development of PRR resistant rootstocks such as Dusa® (COFFEY 1987; GIBLIN *et al.* 2005). However, the development of new rootstocks is a time-consuming endeavour (GABOR AND COFFEY 1991; MENGE *et al.* 2001; KREMER-KÖHNE AND MUKHUMO 2003), one that will easily be overwhelmed by the growing threat from a multitude of pathogens.

Therefore, it has become increasingly important to gain a better understanding of the biological systems involved in resistance against *P. cinnamomi* on a molecular level. The partially resistant rootstock Dusa® has been the focus of several such studies recently (REEKSTING *et al.* 2014a; REEKSTING *et al.* 2014b; REEKSTING *et al.* 2016; VAN DEN BERG *et al.* 2018a; VAN DEN BERG *et al.* 2018c). The early response to *P. cinnamomi* in Dusa® involves elicitation of the salicylic acid (SA) defence response pathway, indicative of the biotrophic phase of the pathogen infection (VAN DEN BERG *et al.* 2018c). By 24 hours post-inoculation, the necrotrophic phase of infection is met overwhelmingly by the jasmonic acid/ethylene (JA/ET) host defence response (VAN DEN BERG *et al.* 2018c). Thus, further characterising these phytohormone

pathways during *P. americana-P. cinnamomi* interactions would undoubtedly provide practical insights into PRR resistance.

Considered a co-transcription cofactor, the nonexpressor of pathogenesis-related genes 1 (NPR1) is required for the majority of SA-induced signalling events, including suppression of the JA/ET defence response pathway (Cao *et al.* 1994; Glazebrook *et al.* 1996; Cao *et al.* 1997; Ryals *et al.* 1997; Shah *et al.* 1997; Spoel *et al.* 2003; Yuan *et al.* 2007; El Oirdi *et al.* 2011). Moreover, to achieve its far-reaching and diverse influence, NPR1 associates with a wide variety of transcription factors including several WRKYs and TGAs (Despres *et al.* 2000; Maleck *et al.* 2000; Zhou *et al.* 2000; Johnson *et al.* 2003; Rochon *et al.* 2006; Wang *et al.* 2006; Zheng *et al.* 2006; Kim *et al.* 2008; Lai *et al.* 2008). Interestingly, NPR1 is also known to influence several endoplasmic reticulum-associated and circadian clock genes, all to better regulate defence responses (Pajerowska-Mukhtar *et al.* 2012; Zhou *et al.* 2015). Furthermore, NPR1 itself is controlled and activated through a complex network of post-translational modifications and protein-protein interactions (Backer *et al.* 2019). Ultimately, NPR1 is essential to the establishment of systemic acquired resistance (SAR), a broad-spectrum systemic resistance effective against a variety of potential pathogens (Ryals *et al.* 1996; Sticher *et al.* 1997; Shah 2003). Knowledge of its integral and complex role in defence responses motivated our interest in the NPR1 pathway for this study.

Summary of findings

This dissertation was designed to address several key questions about the NPR1 pathway in *P. americana.* In chapter 1 (Backer *et al.* 2019), we compiled a comprehensive mechanistic model of the NPR1 pathway based on the literature. The overall purpose was to concatenate existing knowledge in a single meaningful format while removing redundant or outdated observations. In chapter 2 (Backer *et al.* 2015), we described the *in silico* functional classification of the *P. americana NPR1-like* (*PaNPR1-like*) gene family. Subsequently, using RT-qPCR, we investigated the expression of the *PaNPR1-like* genes in response to phytohormone application and *P. cinnamomi* challenge. Additionally, we described expressional differences in PRR susceptible (R0.12) and partially resistant (Dusa®) rootstocks and determined the abundance of *PaNPR1-like* genes in several plant tissues. In chapter 3,

we described 116 unique *P. americana* NPR1 pathway-associated genes. With the aid of dual RNA-sequencing data generated within the Avocado Research Programme (ARP), we interrogated the expression of all 116 genes in the PRR susceptible rootstock R0.12 over-time, following *P. cinnamomi* inoculation. Furthermore, we defined differences between R0.12 and the partially resistant rootstock Dusa® at 5 days post-inoculation (dpi). Together these data provided insight into the most likely *P. cinnamomi* resistance mechanisms employed by Dusa®.

Our initial investigation (Chapter 2) predicted that the *PaNPR1-like* family is comprised of five genes which are highly similar to several members of the *Arabidopsis thaliana NPR1-like* family (BACKER *et al.* 2015). Based on *in silico* identification and analyses, three PaNPR1-like proteins were predicted to play a role in defence, whereas the remaining two were expected to play a role in tissue development (BACKER *et al.* 2015). Moreover, RT-qPCR data supported these *in silico* methods; the expression of defence-related *PaNPR1-like* genes was responsive to phytohormone application and *P. cinnamomi* challenge (BACKER *et al.* 2015). Furthermore, defence-related *PaNPR1-like* genes were significantly more abundant in mature tissues than in young or developing tissues. Meanwhile, development-related *PaNPR1-like* genes were overrepresented in specific tissues and seemed to lack a significant response following phytohormone application (BACKER *et al.* 2015). Finally, we demonstrated that *PaNPR1-like* genes were differentially regulated at 12 hours post-inoculation (hpi) when comparing the PRR susceptible (R0.12) and partially resistant (Dusa®) rootstocks (BACKER *et al.* 2015). Together, these observations classified and differentiated between the defence and development related *PaNPR1-like* genes (BACKER *et al.* 2015). Furthermore, the data suggested that differences in PRR susceptibility between R0.12 and Dusa® likely involves the NPR1 pathway (BACKER *et al.* 2015).

As a result, an expanded investigation (Chapter 3) was initiated to determine the role of the *P. americana* NPR1 pathway in *P. cinnamomi* defence responses. We identified orthologs of 41 *A. thaliana* NPR1 pathway-associated proteins from the *P. americana* draft genome. A total of 116 unique *P. americana* NPR1 pathway-associated coding sequences were identified and annotated. Using dual RNA-sequencing data, we were able to determine that SAR was established in both R0.12 and Dusa® following *P. cinnamomi* challenge. Time-course analysis of NPR1 pathway-associated genes in the PRR susceptible rootstock R0.12 clearly

demonstrated the expected regulation of several genes leading up to the establishment of SAR. These included but were not limited to, genes which encode proteins involved in the post-translational modification of NPR1 and numerous transcription factors essential to SAR. However, in R0.12 the SA-induced signalling pathway was not wholly suppressed following the establishment of SAR and in response to necrotrophy by *P. cinnamomi*. For instance, *PaNPR1* and *PaNPR2* were significantly upregulated by 5dpi, almost certainly leading to suppression of the JA/ET defence response. In contrast, the JA/ET defence response was previously shown to be well established by 24hpi in Dusa® (VAN DEN BERG *et al.* 2018c). Indeed, based on the RNA-sequencing data, we were able to determine that the SA-induced signalling pathway was more efficiently suppressed in Dusa® following the establishment of SAR. Thus, this work investigated the mechanisms utilised by *P. americana* to establish SAR and determined crucial differences between susceptible and partially resistant *P. americana-P. cinnamomi* interactions.

Contribution to current scientific knowledge

Before this study, the molecular information available regarding *P. cinnamomi* defence responses in avocado was relatively limited (MAHOMED AND VAN DEN BERG 2011; ENGELBRECHT AND VAN DEN BERG 2013; REEKSTING *et al.* 2014a; REEKSTING *et al.* 2014b). Additionally, investigations specifically focused on defence against *P. cinnamomi* were limited mainly to *A. thaliana*, generally considered a non-host (ROBINSON AND CAHILL 2003; ROOKES *et al.* 2008; ESHRAGHI *et al.* 2011). Moreover, no information was available on NPR1, arguably the most important regulator of defence responses in plants, and the NPR1-like family in avocado. Thus, this study details the first investigation of the NPR1-like protein family in avocado. Furthermore, our work defines the suspected role of the *P. americana* NPR1 pathway in defence, specifically against *P. cinnamomi*. Moreover, this study provides perceptible advances in the understanding of *P. cinnamomi* resistance in *P. americana*. Lastly, to our knowledge, this study represents the most encompassing investigation of the NPR1-dependent defence response pathway in an agricultural crop.

Regulation of the NPR1 defence response pathway is exceedingly complex, including a multitude of transcription factors, negative regulators, post-translational modifications and

even chromatin remodelling (BACKER *et al.* 2019). Therefore, most studies in previously unstudied plant species focus on either NPR1 or markers of SAR, such as pathogenesis-related (PR)-1, when describing the SA-defence response (ENDAH *et al.* 2008; LE HENANFF *et al.* 2009; PERAZA-ECHEVERRIA *et al.* 2012; SHAO *et al.* 2013). Our initial investigation was very similar, describing and partially characterising the NPR1-like protein family in *P. americana*. Furthermore, by using a combined *in silico* and expression analysis approach, we were able to distinguish between defence and development-related *NPR1-like* paralogs.

However, we utilised a holistic, impartial approach in the second half of this study. This approach has led to a broader understanding of the role the NPR1 pathway plays in the establishment of SAR, and PRR resistance in avocado. Undoubtedly, this design was only possible due to the increased availability and cost-effectiveness of genome and transcriptome sequencing, allowing us to take advantage of the plethora of data. Nonetheless, the observations and opinions presented in this study provide a foundation from which more detailed studies can be informed.

Based on the literature, we expected significant upregulation of several *PR* genes to mark the establishment of SAR (VAN LOON AND VAN STRIEN 1999; DURRANT AND DONG 2004; AN AND MOU 2011). Our observations suggest that SAR was established in both the PRR susceptible (R0.12) and partially resistant (Dusa®) rootstocks by 5dpi, following *P. cinnamomi* inoculation. As such, it is somewhat likely that SAR fulfils an essential role in defence against *P. cinnamomi* in *P. americana*. However, this study further demonstrated that the mere induction of SAR is insufficient during threat from a hemibiotrophic pathogen, as the susceptible rootstock is far less proficient at staving off *P. cinnamomi* infection, despite seemingly inducing SAR. Therefore, it seems that suppression of the SA-signalling pathway in response to the necrotrophic phase of *P. cinnamomi* infection appears to be one of the determining factors governing resistance, at least in the case of Dusa®.

Furthermore, the upregulation of *NPR1-like* genes does not seem to be a prerequisite for the initiation of SAR. Unlike *PaNPR1* and *PaNPR2* expression in R0.12, expression in Dusa® was not upregulated following *P. cinnamomi* inoculation. However, it should be noted that the basal expression of *PaNPR1/2* was higher in Dusa®. Similarly, overexpression of *AtNPR1* in transgenic crops is generally not associated with increased basal defence gene expression, but rather a more effective defence response following pathogenic threat (WALLY *et al.* 2009;

ZHANG *et al.* 2010; KUMAR *et al.* 2013; BOSCARIOL-CAMARGO *et al.* 2016). These observations suggest that post-translational modification of PaNPR1/PaNPR2 may be more important than regulation at the transcript level. In fact, the regulation of several genes related to post-translational modification are comparatively different in Dusa® and R0.12. Notably, the basal expression of several thioredoxins and a putative *S*-nitrosoglutathione reductase was significantly higher in Dusa® when compared to R0.12. Therefore, not only was basal expression of *PaNPR1/2* higher, but PaNPR1/2 might exist primarily as monomers in Dusa®. These observations might suggest that NPR1-dependent gene expression in Dusa® might occur more rapidly than in R0.12. Nonetheless, similarly to NPR1 in tobacco and grapevine, PaNPR1/2-dependent gene expression would likely still be SA-dependent (LE HENANFF *et al.* 2009; MAIER *et al.* 2011).

Priming of defence responses is another crucial component of SAR, aided in part by the increased production of *mitogen-activated protein kinase 3* (*MPK3*) and *MPK6* transcripts and inactive proteins in *A. thaliana* (CONRATH *et al.* 2002; PRIME *et al.* 2006; BECKERS *et al.* 2009; YI AND KWON 2014; YI *et al.* 2015). Therefore, the significant upregulation of *PaMPK3/6-like* genes at 5dpi suggests that *P. americana* might prime defence responses similarly to *A. thaliana*, following the initiation of SAR. Consequently, we found it interesting that the putative transcription factor, *PaTGA10-like1*, was also upregulated at 5dpi. Curiously, *A. thaliana* TGA10 is known to play a role in early, reactive oxygen species (ROS)-mediated pathogen defence responses (FELIX *et al.* 1999; NOSHI *et al.* 2016). As such, we suspect that priming associated with SAR might also include the upregulation of transcription factors which are essential to early defence responses, such as AtTGA10.

In summation, the work presented here demonstrates, for the first time, that the *NPR1-like*, and extended NPR1 pathway-associated genes in *P. americana* likely play a role in determining resistance and susceptibility to *P. cinnamomi*.

Limitations and future work

The work presented here was limited by some factors, which should be addressed in future studies. The first was a lack of molecular work to characterise the PaNPR1-like proteins initially discussed in chapter 2 functionally. Several methods to do so have been detailed in

previous studies and include the complementation of *A. thaliana npr1* mutants, subcellular localisation and constitutive overexpression studies (MALNOY *et al.* 2007; POTLAKAYALA *et al.* 2007; LE HENANFF *et al.* 2009; SHI *et al.* 2010; LE HENANFF *et al.* 2011; CHEN *et al.* 2012; YOCGO *et al.* 2012; ZHANG *et al.* 2012; ZHANG *et al.* 2013; ZHANG *et al.* 2014; WANG *et al.* 2017). However, reliable and tested overexpression vectors, as well as transient and stable transformation protocols, are required to perform these studies. Unfortunately, developing these tools fell outside the scope of this study, albeit not for lack of trying. Alternatively, developing a novel pathosystem which utilises a model species, such as *Nicotiana benthamiana,* which is easily transformed and is naturally susceptible to *P. cinnamomi* would be ideal. Such a pathosystem has recently been developed in the ARP and will undoubtedly be an invaluable tool for addressing future molecular inquiries.

The work detailed in chapter 3 had three evident shortfalls. The first and most crucial was the lack of control samples at each time point of the dual RNA-sequencing experiment. This limitation was reluctantly imposed due to a shortage of sample material in the plant trial. Therefore, although every effort was made to account for all variables, temporal gene regulation, i.e. circadian rhythm, could not be adequately considered. Nonetheless, the impact would mostly be limited to observations at 12hpi in the time-course trial with R0.12.

Another limitation was the lack of comparative data at 12hpi and 24hpi for Dusa®. Unfortunately, this shortfall limits the amount of comparative data available for early defence responses, where SA-induced defence responses are more prevalent. Thus, by including 12hpi and 24hpi dual RNA-sequencing data for Dusa®, we could further expand on the work presented in chapter 3. One particularly informative investigation that could be included would be the modelling of a co-expression network (MUKHTAR *et al.* 2011; GOEL *et al.* 2018). This additional work could potentially identify novel genes and pathways, outside of the NPR1 pathway, involved in *P. cinnamomi* defence.

Moreover, *in silico* and expression analysis do not constitute complete functional characterisation of putative NPR1 pathway associated proteins. Thus, targeted molecular investigations would need to be performed for specific genes of interest within the *P. americana* NPR1 pathway. Again, these investigations would typically include complementation of the appropriate *A. thaliana* mutants, overexpression and subcellular

localisation (ZHANG *et al.* 2003; WANG *et al.* 2005; KIM *et al.* 2008; PAJEROWSKA-MUKHTAR *et al.* 2012).

Additionally, this study is limited by the lack of information regarding gene expression from the perspective of *P. cinnamomi*. However, several members of the ARP are currently focusing on that facet of the dual RNA-sequencing experiment, and therefore such investigations were not form part of this study. Nonetheless, a co-expression network which includes the reads obtained from *P. cinnamomi* could potentially identify effectors with indirect connections to *P. americana* immune receptors (MUKHTAR *et al.* 2011). Furthermore, it would be interesting to compare the pathogen's response to the defence strategies employed by both R0.12 and Dusa®.

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